2020 • 24 • 7

Print ISSN 2500-0462
Online ISSN 2500-3259

Научный рецензируемый журнал

ВАВИЛОВСКИЙ ЖУРНАЛ ГЕНЕТИКИ И СЕЛЕКЦИИ

Основан в 1997 г.
Периодичность 8 выпусков в год
DOI 10.18699/VJ20.662

Учредители

Федеральное государственное бюджетное научное учреждение «Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук»

Межрегиональная общественная организация Вавиловское общество генетиков и селекционеров

Сибирское отделение Российской академии наук

2020 • 24 • 7

Print ISSN 2500-0462
Online ISSN 2500-3259

Scientific Peer Reviewed Journal

VAVILOV JOURNAL OF GENETICS AND BREEDING

VAVILOVSKII ZHURNAL GENETIKI I SELEKTSII

Founded in 1997
Published 8 times annually
DOI 10.18699/VJ20.662

Founders
Federal State Budget Scientific Institution "The Federal Research Center Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences"
The Vavilov Society of Geneticists and Breeders
Siberian Branch of the Russian Academy of Sciences

ВАВИЛОВСКИЙ ЖУРНАЛ ГЕНЕТИКИ И СЕЛЕКЦИИ

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DOI 10.18699/VJ20.663

Structural and functional features of phytoene synthase isoforms PSY1 and PSY2 in pepper *Capsicum annuum* L. cultivars

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Abstract. The fruits of various pepper cultivars are characterized by a different color, which is determined by the pigment ratio; carotenoids dominate in ripe fruits, while chlorophylls, in immature fruits. A key regulator of carotenoid biosynthesis is the phytoene synthase encoded by the *PSY* gene. The *Capsicum annuum* genome contains two isoforms of this enzyme, localized in leaf (PSY2) and fruit (PSY1) plastids. In this work, the complete *PSY1* and *PSY2* genes were identified in nine *C. annuum* cultivars, which differ in ripe fruit color. *PSY1* and *PSY2* sequence variability was 2.43 % (69 SNPs) and 1.21 % (36 SNPs). The most variable were PSY1 proteins of the cultivars 'Maria' (red-fruited) and 'Sladkij shokolad' (red-brown-fruited). All identified PSY1 and PSY2 homologs contained the phytoene synthase domain HH-IPPS and the transit peptide. In the PSY1 and PSY2 HH-IPPS domains, functionally significant sites were determined. For all accessions studied, the active sites (YAKTF and RAYV), aspartate-rich substrate-Mg^{2+}-binding sites (DELVD and DVGED), and other functional residues were shown to be conserved. Transit peptides were more variable, and their similarity in the PSY1 and PSY2 proteins did not exceed 78.68 %. According to the biochemical data obtained, the largest amounts of chlorophylls and carotenoids across the cultivars studied were detected in immature and ripe fruits of the cv. 'Sladkij shokolad' and 'Shokoladnyj'. Also, ripe fruits of the cv. 'Nesozrevayuschij' (green-fruited) were marked by significant chlorophyll content, but a minimum of carotenoids. The *PSY1* and *PSY2* expression patterns were determined in the fruit pericarp at three ripening stages in 'Zheltyj buket', 'Sladkij shokolad', 'Karmin' and 'Nesozrevayuschij', which have different ripe fruit colors: yellow, red-brown, dark red and green, respectively. In the leaves of the cultivars studied, *PSY1* expression levels varied significantly. All cultivars were characterized by increased *PSY1* transcription as the fruit ripened; the maximum transcription level was found in the ripe fruit of 'Sladkij shokolad', and the lowest, in 'Nesozrevayuschij'. *PSY2* transcripts were detected not only in the leaves and immature fruits, but also in ripe fruits. Assessment of a possible correlation of *PSY1* and *PSY2* transcription with carotenoid and chlorophyll content revealed a direct relationship between *PSY1* expression level and carotenoid pigmentation during fruit ripening. It has been suggested that the absence of a typical pericarp pigmentation pattern in 'Nesozrevayuschij' may be associated with impaired chromoplast formation.

Key words: carotenogenesis; *Capsicum annuum*; pepper fruits; fruit ripening; fruit pigmentation.

For citation: Dyachenko E.A., Filyushin M.A., Efremov G.I., Dzhos E.A., Shchennikova A.V., Kochieva E.Z. Structural and functional features of phytoene synthase isoforms PSY1 and PSY2 in pepper *Capsicum annuum* L. cultivars. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):687-696. DOI 10.18699/VJ20.663

Структурно-функциональные особенности изоформ фитоинсинтазы PSY1 и PSY2 у сортов перца *Capsicum annuum* L.

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Аннотация. Плоды сортов перца характеризуются различной окраской, которая определяется соотношением пигментов, при этом в спелых плодах доминируют каротиноиды, тогда как в неспелых – хлорофиллы (иногда вместе с антоцианами). Ключевым регулятором биосинтеза каротиноидов является фитоинсинтаза, кодируемая геном *PSY*. Геном перца *Capsicum annuum* содержит два гена, кодирующих фитоинсинтазы, одна из которых локализуется преимущественно в пластидах листа (PSY2), другая – в пластидах плода (PSY1). В данной работе были идентифицированы полногеномные последовательности PSY1 и PSY2 у девяти сортов *C. annuum*, различающихся окраской спелого плода. Вариабельность последовательностей составила 2.43 % (69 SNP) и 1.21 % (36 SNP). Наиболее вариабельны белки PSY1 сортов Мария (красный плод) и Сладкий шоколад (красно-коричневый плод). В последовательностях PSY1 и PSY2 определены

фитоинсинтазный домен HH-IPPS и транзитный пептид. Идентифицированы функционально значимые участки в домене HH-IPPS PSY1 и PSY2 анализируемой выборки сортов перца. Для исследуемых образцов области, ограничивающие активные сайты (YAKTF и RAYV), аспартат-богатые субстрат-Mg^{2+}-связывающие сайты (DELVD и DVGED) и другие функциональные сайты, консервативны. Транзитные пептиды были более вариабельны, их сходство у белков PSY1 и PSY2 не превышало 78.68 %. Биохимический анализ показал, что наибольшие количества хлорофиллов и каротиноидов среди исследуемой выборки содержатся в неспелых и спелых плодах сортов Сладкий шоколад и Шоколадный. Значительным содержанием хлорофиллов, но минимальным – каротиноидов отличались спелые плоды сорта Несозревающий (зеленый плод). Профиль экспрессии генов *PSY1* и *PSY2* был определен в перикарпе плода на трех стадиях созревания у сортов Желтый букет, Сладкий шоколад, Кармин и Несозревающий, контрастных по окраске спелого плода – желтой, коричневой, темно-красной и зеленой соответственно. В листьях исследуемых сортов уровни экспрессии *PSY1* значительно варьировали. Для всех сортов был характерен рост транскрипции гена *PSY1* по мере созревания плода, при этом в зрелом плоде максимальный уровень транскрипции выявлен у сорта Сладкий шоколад, а самый низкий – у сорта Несозревающий. Транскрипты *PSY2* были выявлены не только в листе и незрелом плоде, но и в спелых плодах. Оценка возможной корреляции транскрипции *PSY1* и *PSY2* с суммарным содержанием каротиноидов и хлорофиллов показала, что имеется прямая зависимость между уровнем экспрессии гена *PSY1* и каротиноидной пигментацией плода в процессе созревания. Высказано предположение, что у сорта Несозревающий отсутствие типичного для плодов перца паттерна пигментации перикарпа в процессе созревания может быть связано с нарушениями образования хромопластов.
Ключевые слова: каротиногенез; *Capsicum annuum*; плоды перца; созревание плодов; окраска плода.

Introduction

The genus *Capsicum* includes, according to various estimates, 30–35 species, five of which are domesticated: *C. annuum*, *C. chinense*, *C. frutescens*, *C. pubescens*, and *C. baccatum* (Moscone et al., 2007; Dias et al., 2013). Peppers, both sweet and hot (chili), have a high dietary value as they are rich in antioxidants, including vitamin C, flavonoids, and carotenoids (Sun et al., 2007; Cervantes-Paz et al., 2014).

It is known that primates, including humans, do not synthesize carotenoids *de novo*, but are in dire need of them, since, for example, β-carotene and α-carotene are precursors of vitamin A. The antioxidant activity of the carotenoids found in carotenogenic fruits and vegetables helps to reduce the risk of various diseases, such as certain types of cancer, age-related eye pathologies and cardiovascular diseases (Howard et al., 2000; Story et al., 2010; Giuliano, 2017). Among vegetable crops, pepper, which fleshy fruits are enriched with various types of carotenoids, is one of the main sources of antioxidants in the human diet. In this regard, the obtaining of new pepper varieties is an important task of modern breeding (Berry et al., 2019; Sun, Li, 2020).

Pepper species have different antioxidant levels, and many breeding programs use the natural variation to identify the characteristics of "exotic" allelic diversity and donors of specific carotenoid spectra. However, at present, closely related accessions of the same species are predominantly used, which often do not have strong phenotypic differences, usually observed when using wild relatives (Berry et al., 2019).

Carotenoids together with chlorophylls and anthocyanins determine the color of pepper fruits. It should be noted that carotenoids are the dominant pigments in ripe pepper fruits, while chlorophylls (sometimes together with anthocyanins) are in immature, growing fruits. In *C. annuum* cultivars, fruit color depends on the ratio of pigments, as well as on the stage of ripening: from green, yellow, white or purple in unripe fruits (mature fruit stage, MF), to orange, red, dark red, brown and sometimes almost black – in ripe fruits (ripe fruit stage, RF) (Levy et al., 1995; Márkus et al., 1999; Ha et al., 2007). Usually, sweet peppers are harvested at the technical ripeness stage (blanche fruit, intermediate ripe stage, IR), and hot peppers at biological ripeness (RF). The pepper fruit ripening is accompanied by the transition of tissues containing chloroplasts to tissues containing chromoplasts. In chromoplasts, chlorophylls degrade, but the synthesis of carotenoids continues, which, unlike chlorophylls, are able to accumulate in specialized globular structures (Osorio, 2019). This leads to a decrease in the chlorophyll content, the accumulation of carotenoids and, as a consequence, to a change in the ripening fruit color.

Unlike tomato, in ripe fruits of which the main carotenoids are lycopene and β-carotene, in pepper fruits, carotenogenesis goes further – to the formation of xanthophylls; carotenoid spectrum in ripe pepper fruits is represented by major concentrations of red pigments – capsanthin and capsorubin, as well as by various combinations of minor amounts of orange and yellow pigments β-carotene, β-cryptoxanthin, lutein, zeaxanthin, anthraxanthin and violaxanthin (Giuffrida et al., 2013; Mohd Hassan et al., 2019).

Carotenoid pigments are isoprenoid molecules obtained as a result of successive transformations of the universal precursor, isopentenyl pyrophosphate. Several reactions convert this compound into geranylgeranyl pyrophosphate (GGPP), two molecules of which condense head-to-tail by phytoene synthase to form phytoene, the precursor of all carotenoids (Fraser et al., 2000).

Thus, phytoene synthase is a key regulator of carotenoid biosynthesis, supplying the main substrate – phytoene (Fraser et al., 2000). This enzyme is encoded by the *PSY* gene, the expression of which is influenced by intermediate and final products of the pathway (Welsch et al., 2003; Kacha-

novsky et al., 2012; Enfissi et al., 2017). Several types of phytoene synthases have been identified in plants, and phytoene synthase activity depends on the type of enzyme and its intracellular location (Shumskaya et al., 2012). In *Arabidopsis thaliana*, only one *PSY* gene was identified (Zhou et al., 2015), while in tomato *Solanum lycopersicum*, three, and the protein products of these genes have different localization: PSY1 – in fruit plastids, PSY2 – in leaf plastids, PSY3 – in root plastids (Stauder et al., 2018). In pepper *C. annuum*, two genes are currently known that encode phytoene synthases, one of them is mainly localized in the leaf plastids (PSY2), the other – in the fruit plastids (PSY1) (Thorup et al., 2000; Kilcrease et al., 2015). Accordingly, in tomato and pepper, *PSY2* transcripts are mainly present in photosynthetic green tissues, while *PSY1* is found in mature fruits of both crops and in tomato flower petals (Giorio et al., 2008; Kilcrease et al., 2015; Berry et al., 2019; Filyushin et al., 2020). However, both phytoene synthases can be transcribed in all plant organs (Stauder et al., 2018).

This study is focused on identifying the genes of phytoene synthases PSY1 and PSY2 in *C. annuum* cultivars, assessing their intervarietal variability, both structural and functional, as well as possible correlations between the expression of these genes and fruit pigmentation.

Materials and methods

Plant material. Individual plants of nine *C. annuum* cultivars were used in the research: eight cultivars of sweet pepper (Nesozrevayuschij, Karmin, Shokoladnyj, Sladkij shokolad, Ratunda, Maria, Gogoshary, and Zheltyj buket) and one hot pepper cultivar (Mechta hozyayki) (Table 1). The plants were grown in a greenhouse at the Federal Scientific Vegetable Center (FSVC, Moscow Region).

Identification of the whole genome sequences *PSY1* and *PSY2*. Genomic DNA was isolated from freshly collected, ground in liquid nitrogen, leaves of each of the analyzed pepper cultivars, according to (Puchooa, 2004). 100 ng of each obtained preparation was used as a template for *PSY1* and *PSY2* amplification. The amplification primers were previously developed based on the genome sequences of *C. annuum PSY1* (LOC107868281 bifunctional 15-cis-phytoene synthase, chromoplastic, Gene ID: 107868281) and *PSY2* (LOC107859651 phytoene synthase 2, chloroplastic, Gene ID: 107859651) available in the NCBI database. Amplification of the *PSY1* gene was performed with primers CaPSY1F and CaPSY1R (5′-TCAGAATGTCTGTTGCCTTG-3′ and 5′-TCCTG ATTTCATGTTCTTGTAGA-3′), *PSY2* – with primers CaPSY2F and CaPSY2R (5′-AGCATGTCTGTTGCTTT GTTG-3′ and 5′-CTTCATTCATGTCTTTGYTAGTG-3′). High precision LongAmp® Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA), C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the following PCR conditions were used: initial denaturation (94 °C, 10 min); 36 cycles of denaturation (94 °C, 40 s), annealing (56 °C, 40 s) and synthesis (65 °C, 4 min); final completion of the fragments (65 °C, 7 min). Amplified fragments of the expected size were purified from agarose gel using a QIAEX® II Gel Extraction kit (QIAGEN, Hilden, Germany), cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA), and sequenced (2–4 clones for each sample) on ABI Prism 3730 DNA Analyzer (Applied Biosystems, Waltham, MA, USA).

Comparative structural analysis of the *PSY1* and *PSY2*. Alignment and analysis of the obtained nucleotide and amino acid sequences were performed using the MEGA 7.0 (https://www.megasoftware.net/). Known sequences of *C. annuum PSY1* (Gene ID: 107868281) and *PSY2* (Gene ID: 107859651) were used for comparative analysis. Conserved domains in encoded proteins were determined using the NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and UniProtKB (https://www.uniprot.org/). The functional significance of each amino acid (aa) residue substitution was predicted using the PROVEAN (http://provean.jcvi.org/index.php). By radical substitutions is meant those substitutions that can presumably affect the folding of the protein or its functionality.

For cluster phylogenetic analysis of the *PSY1* and *PSY2* genes identified in pepper cultivars, we also used the *PSY* gene sequences in *C. annuum* cv. Zunla 1 (*PSY1*, NC_029980.1:c205334820-205328571; *PSY2*, NC_029978.1: 142877052-142881261) and *C. annuum* cv. Valencia (*PSY1*, GU085273.1). The analysis was performed using the neighbor-joining (NJ) method in the MEGA 7.0 program.

***PSY1* and *PSY2* expression pattern in fruits of the analyzed pepper cultivars during ripening.** Total RNA was isolated (RNeasy Plant Mini Kit, QIAGEN, Germany) from fruit pericarp at three developmental stages (MF, IR, and RF). The resulting preparations were purified from DNA impurities (RNase free DNasy set, QIAGEN, Germany), evaluated qualitatively and quantitatively (spectrophotometrically and by electrophoresis in 1.5 % agarose gel), and used for the cDNA synthesis (GoScript™ Reverse Transcription System, Promega, USA).

To determine the *PSY1* and *PSY2* expression pattern, quantitative real-time PCR (qRT-PCR) was used, which was carried out in three technical replicates with the kit "Reaction mixture for RT-PCR in the presence of SYBR GreenI and ROX" (Syntol LLC, Russia) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Primers for qRT-PCR were developed earlier, based on the *C. annuum PSY1* (X68017) and *PSY2* (XM_016704726.1) mRNA sequences: for *PSY1* – PSY1-F and PSY1-R (5′-GT GAAGAGACAGCTGAGATCG-3′ and 5′-TCTCCGG AGTCATTAGCATCG-3′), and for *PSY2* – PSY2-F and PSY2-R (5′-AAGGAGTCGCAGAACTGAGC-3′ and 5′-GTCGTTCGCTTCAATCTCATCTAA-3′) (Filyushin et al., 2020). To normalize gene transcription level, the reference gene *Actin7* expression and the primers Actin7-F and Actin7-R (5′-CATTGTGCTCAGTGGTGGTTC-3′ and 5′-TCTGCTGGAAGGTGCTAAGTG-3′) were used (Bemer et al., 2012). The qRT-PCR conditions were:

Table 1. Characteristics of fruits of studied *C. annuum* cultivars

C. annuum cultivars	Accession origin (ID, if available)	Fruit color			Sum of chlorophylls, μg/g fresh weight			Sum of carotenoids, μg/g fresh weight		
		Ripening stages								
		MF	IR	RF	MF	IR	RF	MF	IR	RF
Nesozrevayuschij	FSVC	G	G	G	43.8	12.3	29.4	14.5	6.8	55.9
Zheltyj buket	FSVC (9553318)	DG	YG	Y	83.3	14.9	0	27.3	47.4	107.7
Gogoshary	FSVC	G	GR	R, segments of G	18.5	12.6	7.3	6.8	10.2	38.3
Mechta hozyayki (hot)	FSVC (8355299)	DG	GR	R	42.6	11.2	9.0	13.2	25.3	87.6
Maria	FSVC (9809422)	G	R, shade G	R	47.3	7.49	0	18.6	108.7	407.1
Karmin	FSVC (8153509)	G	GR	DR	62.6	14.3	0	26.1	133.0	520.2
Sladkij shokolad	FSVC	G	B	B	119.4	90.77	96.53	61.0	173.7	597.0
Shokoladnyj	FSVC	G	B	B	121.8	–*	130.9	48.5	–*	1009.9
Ratunda**	FSVC	DG	GR	DR	–	–	–	–	–	–

Note. Pepper fruits were analyzed at three stages of ripening: MF – mature green fruit of the final size, IR – intermediate ripe, RF – ripe fruit. Fruit color: G – green, DG – dark green, Y – yellow, YG – yellow green, R – red, DR – dark red, B – brown, GR – green red. Cultivar ID is provided according to the State Register of Breeding Achievements Admitted for Use in RF (https://reestr.gossortrf.ru/).
* At the time of plant material collection, the IR fruits of cv. Shokoladnyj variety were not found; ** at the time of plant material collection, this cultivar had no fruits at the required ripening stages (in different years, the total carotenoids in Ratunda fruits exceeded that of cv. Shokoladnyj up to two times).

95 °C – 5 min; 40 cycles (95 °C – 15 s, 62 °C – 50 s). The obtained data were statistically processed using the GraphPad Prism v. 7.02 (https://www.graphpad.com).

The sum of chlorophylls and the sum of carotenoids in fruit pericarp (together the skin and pulp) were determined spectrophotometrically in chloroform-methanol extracts; the pigment content was calculated using the formulas (Lichtenthaler et al., 1987; Solovchenko et al., 2001), in two biological and three technical replicates.

Results and discussion

Characteristics of the *PSY1* and *PSY2* gene sequences and proteins encoded by them

Previously, it has been shown that the *PSY1* and *PSY2* variability may determine the color of the pepper fruit (Cao et al., 2019; Filyushin et al., 2020). Therefore, for this study, nine *C. annuum* cultivars were selected, which differ in fruit color during ripening: Nesozrevayuschij, Zheltyj buket, Shkoladnyj, Sladkij shokolad, Karmin, Ratunda, Maria, Gogoshary and Mechta hozyayki (see Table 1). Unripe fruit color of all analyzed cultivars was green or dark green, however, the dynamics of color change as they ripen differed among cultivars. In the cv. Nesozrevayuschij, the fruits remained green until biological ripeness, in the cv. Zheltyj buket they were yellow-green at the IR stage and yellow at the RF stage, in cv. Sladkij shokolad and Shkoladnyj, fruits were red-brown at both stages, while the other four cultivars had green-red fruits at the IR stage and red/dark red fruits at the RF stage (see Table 1).

For each of the nine pepper cultivars, the *PSY1* and *PSY2* gene sequences were determined, starting from the ATG codon (Table 2). The length of the *PSY1* gene was 2844 bp in all analyzed cultivars. For comparison, *C. annuum* cv. Zunla 1 *PSY1* available in the NCBI database (Gene ID: 107868281) has the same size, while *S. lycopersicum* cv. Heinz 1706 (Gene ID: 543988) is longer (3302 bp). The length of the *PSY2* gene in the studied cultivars was 2985 bp, with the exception of *PSY2* from cv. Mechta hozyayki (2994 bp, due to the 9-nucleotide insert in the second intron) (see Table 2). The *C. annuum* cv. Zunla 1 *PSY2* (Gene ID: 107859651) is also 2985 bp, whereas *S. lycopersicum* cv. Heinz 1706 *PSY2* (Gene ID: 543964) is 3032 bp. The variability of the *PSY1* and *PSY2* genomic sequences in pepper accessions was 2.43 % (69 SNPs) and 1.21 % (36 SNPs), while 16 and 15 SNPs were localized in exons, respectively. Compared to *S. lycopersicum* cv. Heinz 1706 *PSY1* and *PSY2*, *PSY1* and *PSY2* of the pepper cultivars contained 1072/128 and 818/100 (gene/exons) SNPs.

The coding part of the *PSY1* and *PSY2* genes consisted of six exons and in all studied cultivars was 1260 and 1299 bp, respectively (see Table 2). Found differences in cDNA length were due to the presence of insertions in exons I and VI of *PSY2*. Most of the identified SNPs were concentrated in exon III of *PSY1* (7 SNPs, 43.75 % of all exon substitutions) and in exon VI of *PSY2* (6 SNPs, 40.0 %). Exon II of both genes was invariable and the most conserved with respect to the *S. lycopersicum* cv. Heinz 1706 *PSY* genes. Exon I of both genes turned

Table 2. Characteristics of homologous genes *PSY1* and *PSY2* in *C. annuum* cultivars

C. annuum cultivars	Gene name	NCBI ID	Exon/intron length, bp						Gene, bp	cDNA, bp	Protein, aa
			I	II	III	IV	V	VI			
Zheltyj buket	*PSY1*	MT507241	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507250	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Karmin	*PSY1*	MT507242	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507251	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Maria	*PSY1*	MT507243	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507252	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Sladkij shokolad	*PSY1*	MT507244	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507253	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Shokoladnyj	*PSY1*	MT507245	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507254	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Nesozrevayuschij	*PSY1*	MT507246	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507255	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Gogoshary	*PSY1*	MT507247	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507256	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432
Mechta hozyayki (hot)	*PSY1*	MT507248	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
	PSY2	MT507257	466/101	51/753	173/193	236/219	193/429	180	2994	1299	432
Ratunda	*PSY1*	MT507249	448/96	51/261	173/328	236/250	193/649	159	2844	1260	419
		MT507258	466/101	51/744	173/193	236/219	193/429	180	2985	1299	432

out to be the most polymorphic in comparison with the *S. lycopersicum PSY* genes. Note that since in this work, a limited number of cultivars (nine) were analyzed, the data on gene polymorphism are applicable only to the set of analyzed cultivars.

The *PSY1* and *PSY2* nucleotide sequences have been translated. The putative proteins PSY1 and PSY2 of all analyzed cultivars were 419 and 432 aa, respectively (see Table 2), contained a conserved phytoene synthase domain HH-IPPS (130–412 and 26–310 aa, according to UniProtKB, and 75–405 and 92–430 aa, according to NCBI-CDD) and the N-terminal transit peptide TP (1–129 and 1–25 aa, according to UniProtKB, and 1–74 and 1–91 aa, according to NCBI-CDD). TP cleavage sites in all possible cases were invariant within the analyzed set of cultivars.

Compared to the *C. annuum* cv. Zunla 1 and *S. lycopersicum* cv. Heinz 1706 PSY1 and PSY2, in pepper cultivars, PSY1/PSY2 contained 9/15 and 46/43 aa substitutions, respectively. Out of nine substitutions in PSY1, seven were radical (r) and only two were neutral (n), while all r-substitutions were in the conserved domain, and two n-substitutions were in the transit peptide (Fig. 1). The nC59Y substitution was typical for PSY1 of almost the entire studied set of cultivars, except for the cv. Sladkij shokolad, and all radical substitutions were cultivar-specific. The most variable were PSY1 of cv. Maria and Sladkij shokolad (see Fig. 1).

In the PSY2, of 15 aa substitutions nine were radical. Phytoene synthases PSY2 of cv. Mechta hozyayki, Gogoshary, Ratunda, and Shokoladnyj did not differ from each other or contained nT430A, while each of the other cultivars had one or two r-substitutions in the HH-IPPS domain (see Fig. 1).

The presence of radical aa substitutions in PSY1 and PSY2 of the analyzed cultivars can affect the mature phytoene synthase folding, as well as enzyme ability to interact with protein partners and perform correct catalytic functions. Previously, it was shown that the PSY1 and PSY2 sequences are highly similar (Giorio et al., 2008; Cao et al., 2019). Comparison of the identified *C. annuum* PSY1 and PSY2 confirmed this observation.

Considering the UniProtKB data on the domain localization, HH-IPPS contains 21 variable sites specific for each of the PSY1 and PSY2 protein groups (see Fig. 1). Also, at the PSY1 domain C-terminus, two deletions, P422–S427del and L429del, were identified. The PSY1 and PSY2 domain sequence was highly conserved (92.86 %).

In contrast to the HH-IPPS domain, the TP sequence was found to be highly variable. In comparison with

PSY1	14	59	186	210	214	288	320	321	389	PSY2	71	72	117	178	180	191	236	290	309	383	387	393	394	395	430	C
Zunla 1	V	C	P	A	D	G	G	L	N		T	G	T	T	E	T	V	A	D	K	E	Y	N	N	T	
Maria		Y		D		R	S										D						Y			
Mechta hozyayki		Y																							A	
Gogoshary		Y																								
Karmin		Y																	E		D	N			A	
Ratunda		Y							T																A	
Zheltyj buket		Y			V									N						R			D	Y	A	
Nesozrevayuschij	F	Y									S	R			G	S										
Sladkiy shokolad			T					K					P					S						A	
Shokoladnyj		Y																								
	TP		HH-IPPS domain								TP?		HH-IPPS domain?													*
													HH-IPPS domain?													**

Fig. 1. The PSY1 and PSY2 amino acid polymorphism in the studied *C. annuum* cultivars compared to *C. annuum* cv. Zunla 1.
* According to UniProtKB, ** according to NCBI.

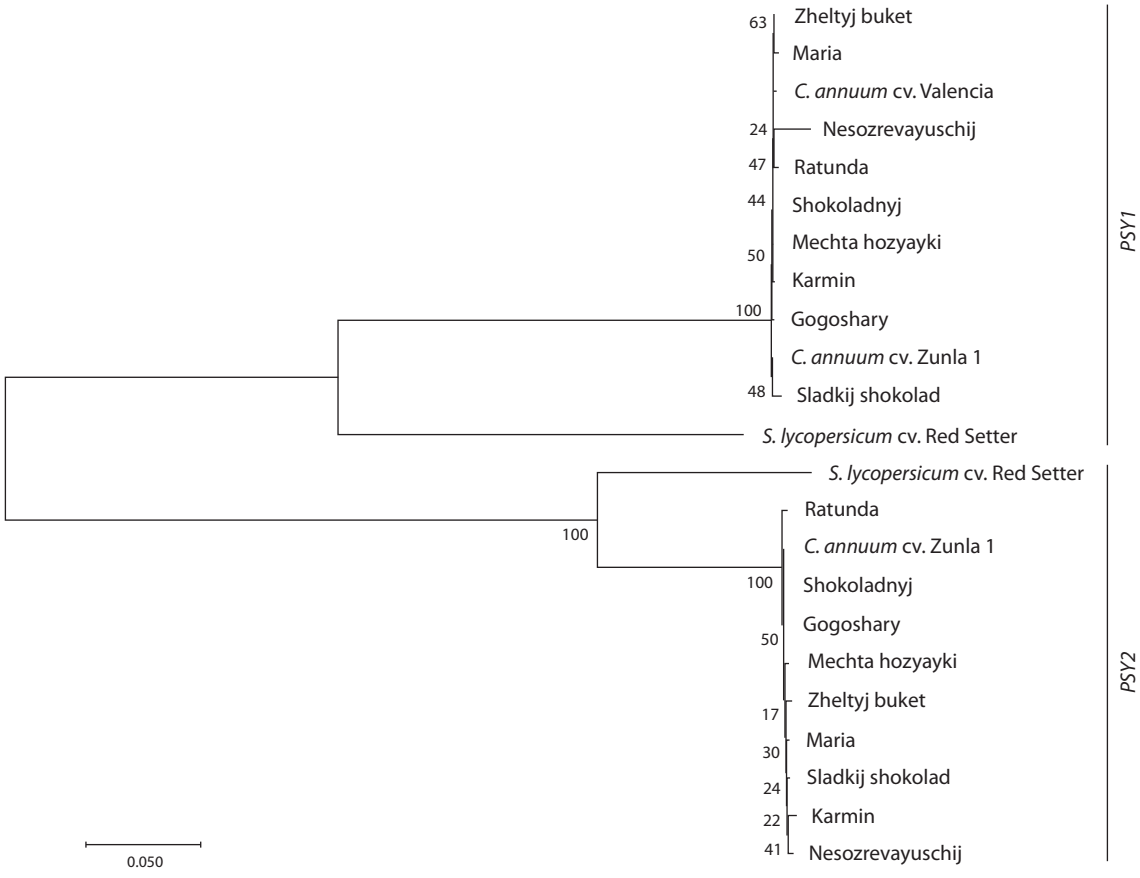

Fig. 2. Phylogenetic analysis of the *PSY1* and *PSY2* full-genome sequences of the studied *C. annuum* cultivars.
The dendrogram was constructed using MEGA 7.0 (NJ method, Tamura–Nei model, bootstrap 1000). For comparison, *Solanum lycopersicum* cv. Red Setter *PSY1* and *PSY2* (EF534740.1, EU021055.1) were used.

PSY2 TP, PSY1 TP contained 29 aa substitutions (21.32 % of aligned length). Thus, the identity of TP in PSY1 and PSY2 in the studied pepper cultivars was 78.68 %; also, two insertions (insF31S33 and insG50) and four deletions (PSY2 numbering: N12del, D35del, L57-R62del, and S64-D65del) were identified in the PSY1 sequence. Apparently, differences in TP sequences may be responsible for the specificity of delivery of each of the phytoene synthases to different types of plastids, as was suggested earlier (Cao et al., 2019).

In the PSY1 and PSY2 of the analyzed pepper cultivars, functionally significant sequences were searched. As a

result, it was shown that the adjoining regions of active sites (143-YAKTF-147/149-YAKTF-153 and 393-RAYV-396/399-RAYV-402), aspartate-rich substrate-Mg^{2+}-binding sites (173-DELVD-177/179-DELVD-183 and 299-DVGED-303/305-DVGED-309), 18 substrate-binding pockets and 15 catalytic residues are conserved for the cultivars under study. The exceptions were the substitutions rE180G (cv. Nesozrevayuschij, PSY2) at the DELVD site, nD309E (cv. Karmin, PSY2) at the DVGED site, and nG288R (cv. Maria, PSY1, substrate-binding pocket). In all cultivars, the sites $F_{147}Y_{148}/F_{153}Y_{154}$ and A_{210}/A_{216} were conserved for both PSY1 and PSY2, with the exception of the rA210D in the cv. Maria PSY1. This composition of functionally important sites determines the similarity of the PSY1 and PSY2 carotenogenic activity level, and, as was shown earlier, the substitution in the A_{210}/A_{216} site is not critical, in contrast to $F_{147}Y_{148}/F_{153}Y_{154}$ (Cao et al., 2019). Therefore, found radical substitution rA210D should not significantly affect the cv. Maria PSY1 activity.

To confirm the structural similarity of the identified *PSY1* and *PSY2*, cluster analysis was performed based on their genome-wide sequences in comparison with the known *S. lycopersicum* cv. Red Setter and *C. annuum* cv. Zunla 1 *PSY1* and *PSY2* (Fig. 2). On the dendrogram, the pepper cultivars were expectedly grouped into two large clusters combining the sequences *PSY1* and *PSY2*, respectively (see Fig. 2). Within each cluster, *C. annuum* accessions formed a single closely related subcluster with insignificant internal bootstrap values (17–50) and the only reliable combination of cv. Maria and Zheltyj buket based on *PSY1*. The *S. lycopersicum* species occupied the base branch in each of the clusters.

Thus, the identified *PSY1* and *PSY2* of nine pepper cultivars, which differ in a ripe fruit color, were highly similar in structure, which suggests that they may preserve the conserved key functions of phytoene synthases in the carotenoid biosynthesis.

The content of chlorophylls and carotenoids in fruit pericarp during ripening

The total content of chlorophylls and carotenoids was measured in fruit pericarp during development in the analyzed pepper cultivars (see Table 1). It was shown that unripe fruit of all cultivars (stage MF) contains comparable amounts of chlorophylls and carotenoids, which characterizes the fruit tissues as photosynthetic. In IR fruits, chlorophyll content decreased by 1.46–5.60 times, depending on the cultivar. In ripe fruits (RF stage), chlorophyll was found in significant quantities in cv. Sladkij shokolad, Shokoladnyj and Nesozrevayuschij, and in small quantities in Gogoshary and cv. Mechta hozyayki. There were no chlorophylls in ripe fruits of cv. Zheltyj buket, Karmin and Maria.

In immature fruits, the carotenoid content was the highest in the cv. Shokoladnyj and Sladkij shokolad (48.5 and 61.0 μg/g), while in the other cultivars it ranged from 6.8 (Gogoshary) to 27.3 μg/g (Zheltyj buket) (see Table 1). In ripe fruits, the primacy remained with the cultivars forming chocolate-colored fruits: the highest carotenoid content was detected in the cv. Shokoladnyj (1009.90 μg/g), while in the cv. Sladkij shokolad, it was reduced by 1.7 times, and in the red-fruited cv. Karmin and Maria – by 1.94 and 2.48 times, respectively (see Table 1). Ripe fruits of the remaining four cultivars accumulated significantly less carotenoids. However, a similar low carotenoid content did not provide the similar ripe fruit color: Zheltyj buket – yellow, Mechta hozyayki – red, Nesozrevayuschij and Gogoshary – green and red-green, respectively. At the same time, cv. Nesozrevayuschij and Gogoshary fruits had on average two times less carotenoids in comparison with the cv. Zheltyj buket and Mechta hozyayki fruits (see Table 1).

In accordance with the obtained biochemical data, it can be assumed that red-fruited cultivars synthesize red pigments typical for peppers – carotenoids capsanthin and capsorubin. In brown-fruited cultivars, the color may be formed by two components – red carotenoids and green chlorophylls. The yellow or green color of fruits at the stage of biological ripeness is most likely determined by the presence of yellow-colored carotenoids (lutein, zeaxanthin) and chlorophylls, respectively.

PSY1 and *PSY2* co-expression pattern in the fruit pericarp during ripening

Carotenoid accumulation in fruits is directly related to the *PSY1* expression level (Meléndez-Martínez et al., 2010); however, although *PSY2* is mainly expressed in photosynthetic tissues, its transcripts have also been found in fruits (Jang et al., 2020). In this study, *PSY1* and *PSY2* expression pattern was characterized in leaves and fruit pericarp (peel and pulp) at three ripening stages (MF, IR, RF) in four pepper cultivars (Fig. 3). The analysis included cv. Zheltyj buket, Sladkij shokolad, Karmin and Nesozrevayuschij, contrasting in the ripe fruit color (RF) – yellow, brown, dark red and green, respectively (see Table 1). Ripe fruits of cv. Karmin and Sladkij shokolad were characterized by a high carotenoid content (520.2 and 597.0 μg/g), while ripe fruits of cv. Nesozrevayuschij, and Zheltyj buket accumulated only 55.9 and 107.7 μg/g, respectively.

In the leaves of the analyzed pepper cultivars, the *PSY1* expression levels varied significantly. For example, the levels were similar in cv. Nesozrevayuschij and Zheltyj buket (0.15 and 0.17, respectively), while three and ten times lower (0.054) in cv. Sladkij shokolad and Karmin (0.012), respectively (see Fig. 3). In the fruits of the analyzed pepper cultivars, a *PSY1* expression pattern was similar – progressive upregulation during fruit ripening. At the final stage of ripening, in the ripe fruit, the maximum *PSY1* expression level was observed in cv. Sladkij shokolad, and the minimum – in cv. Nesozrevayuschij (see Fig. 3).

Phytoene synthase PSY2 is considered to be more specific for photosynthetic tissues (Giorio et al., 2008). In green unripe fruit (stage MF), all analyzed cultivars were characterized by the presence of chlorophylls – the highest in cv. Sladkij shokolad and the lowest in cv. Nesozrevayuschij. Surprisingly, only in these two cultivars, ripe fruits

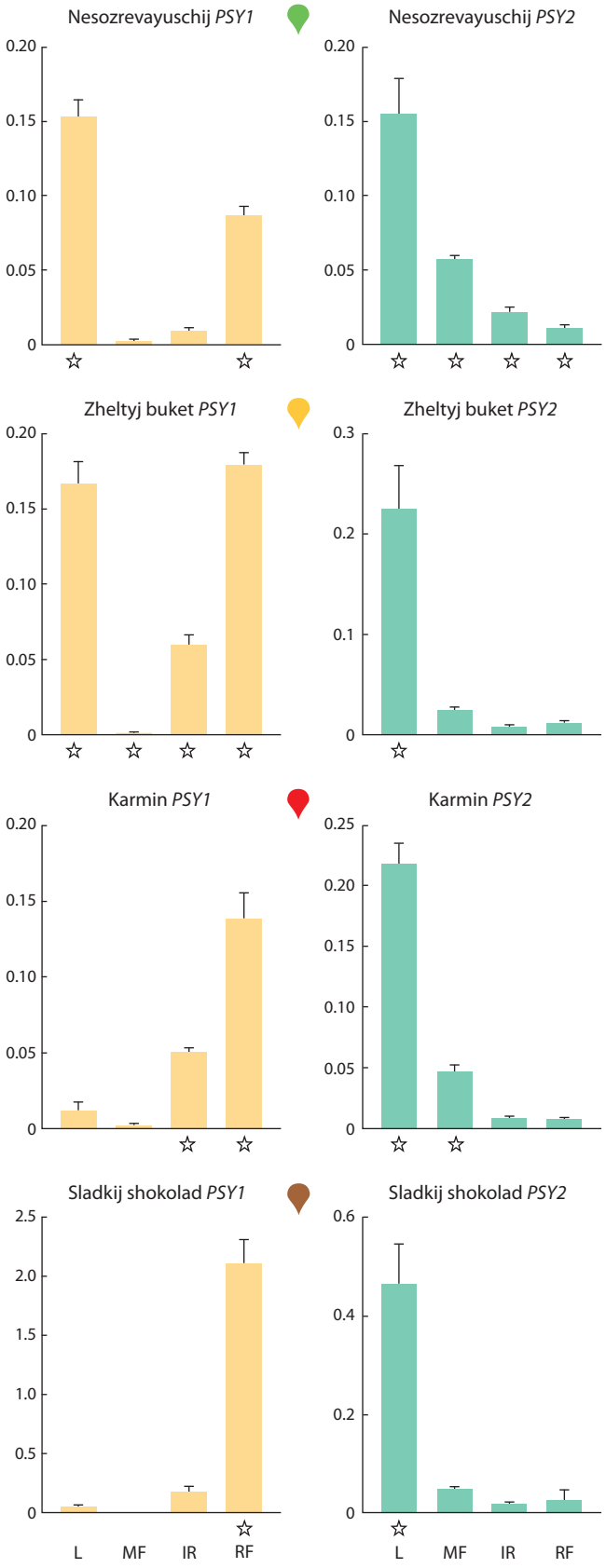

Fig. 3. The *PSY1* and *PSY2* expression pattern in leaves (L) and fruits at three stages of ripening (MF – mature fruit, IR – intermediate ripe fruit, RF – ripe fruit) of *C. annuum* cultivars.

Asterisks indicate gene expression values that differ significantly from its expression in all other tissues of the same accession.

also contained chlorophyll (in the former it was 3 times higher than in the latter).

In the leaves, the *PSY2* expression level in cv. Sladkij shokolad was 2–3 times higher than in the other three cultivars, in which the expression was comparable (see Fig. 3). Besides, *PSY2* transcripts were detected in the fruit pericarp at all ripening stages in all analyzed cultivars. In the immature fruit pericarp (stage MF), *PSY2* expression was approximately at the same level in cv. Nesozrevayuschij (0.057), Sladkij shokolad (0.050), and Karmin (0.046), while in cv. Zheltyj buket, it was twice lower (0.025) (see Fig. 3). In all analyzed cultivars, the *PSY2* expression level decreased as fruits ripen. In ripe fruits (stage RF), the *PSY2* level was similar in cv. Nesozrevayushchij, Zheltyj buket, and Karmin, and 2.3–3.5 times higher in cv. Sladkij shokolad (see Fig. 3).

It can be assumed that the presence of *PSY2* expression in pepper ripe fruits is associated with the chloroplast preservation. This was evidenced by the chlorophyll presence in the ripe fruit pericarp, for example, in cv. Nesozrevayushchij and Sladkij shokolad (see Table 1). However, cv. Zheltyj buket and Karmin also showed *PSY2* expression in ripe fruits, while no chlorophyll was found there. Thus, it can be assumed that *PSY2* can function not only in chloroplasts, but also in chromoplasts. Earlier, using pepper cv. MicroPep Yellow as an example (with lack of *PSY1* gene transcription), it was shown that the synthesis and accumulation of yellow pigments in fruit chromoplasts is associated with *PSY2* expression (Jang et al., 2020).

The regulation of carotenoid biosynthesis and accumulation in pepper fruits is a complex process (Deruère et al., 1994; Kilcrease et al., 2015). In ripe fruits, carotenoids accumulate in chromoplasts in specialized globules, and if their formation is impaired, then carotenoids can be synthesized, but not accumulated (Osorio, 2019). Globule formation is controlled by the Orange protein, which at the same time prevents carotenoid degradation and stabilizes the phytoene synthase PSY activity (Osorio, 2019). Peel of cv. Nesozrevayuschij fruits, as they ripen, retained the green color, while the pulp color changes from light green to yellow-green. In accordance with this and in contrast to the other three analyzed cultivars, there was no significant increase in the carotenoid content (see Table 1), and the *PSY1* transcription level in the ripe fruit of cv. Nesozrevayuschij was 1.6 and 2.1 times lower than that of cv. Karmin and cv. Zheltyj buket, respectively. It can be assumed that in cv. Nesozrevayuschij, a number of processes characteristic of fruit ripening, such as degradation of chlorophyll, transformation of chloroplasts into chromoplasts, and/or *de novo* chromoplast synthesis, are disturbed (Kilcrease et al., 2015; Berry et al., 2019). However, the fruits of cv. Nesozrevayuschij ripen (the seeds are fully formed and viable), although there is no noticeable change in the pericarp color. This confirms the previously shown lack of a relationship between fruit carotenogenesis and ripening (Fraser et al., 2007).

Conclusions

Thus, in the present study, in nine *C. annuum* cultivars, differing in ripe fruit color, *PSY1* and *PSY2* genes encoding phytoene synthases were identified and characterized; the co-expression pattern of these genes in the vegetative and reproductive organs, as well as possible relationships of the expression level with the total carotenoid content were determined. A direct correlation was found between the *PSY1* gene expression level and carotenoid pigmentation of the fruit during ripening. It was shown that in the cv. Nesozrevayuschij, the pericarp pigmentation pattern, typical for pepper fruits during ripening, is disturbed, which may be associated with blocks in the chromoplast formation.

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Acknowledgements. This study was financially supported by the Russian Science Foundation (grant No. 19-16-00016) and, partially, by the Ministry of Science and Higher Education of the Russian Federation (measurement of chlorophyll content in pepper fruits).

Conflict of interest. The authors declare no conflict of interest.

Received May 21, 2020. Revised July 16, 2020. Accepted July 16, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):697-704
DOI 10.18699/VJ20.664

SSR-based evaluation of genetic diversity in populations of *Agriophyllum squarrosum* L. and *Agriophyllum minus* Fisch. & Mey. collected in South-East Kazakhstan

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Abstract. The development of informative polymorphic DNA markers for poorly studied genera is an important step in population analyses of living organisms, including those that play very important ecological roles in harsh environments, such as desert and semi-desert area. Examples of those poorly studied desert species are *Agriophyllum squarrosum* L. and *Agriophyllum minus* Fisch. & Mey. However, a recent RNA-sequencing project in *A. squarrosum* has proposed a large set of hypothetical SSR (simple sequence repeat) markers. In this work, 11 novel polymorphic SSRs were found due to the screening of 24 randomly selected SSRs for three populations of *A. squarrosum* and one population of *A. minus*. The analysis of 11 SSRs revealed 16 polymorphic loci in two *Agriophyllum* species, 8 polymorphic loci within three populations of *A. squarrosum*, and 6 polymorphic loci in the population of *A. minus*. Statistical analyses showed high interspecific, but relatively low intraspecific genetic diversity. The phylogenetic clusterization and population structure analysis have demonstrated a clear segregation of *A. minus* from *A. squarrosum*, as well as the separation of population 1 from populations 2 and 3 of *A. squarrosum*. Thus, we identified the set of novel and informative SSR markers suitable for the study of genetic diversity in *Agriophyllum*.
Key words: sand rice; *Agriophyllum squarrosum*; *Agriophyllum minus*; SSR markers; genetic diversity; population structure.

For citation: Genievskaya Y., Karelova D., Abugalieva S., Zhao P., Chen G., Turuspekov Y. SSR-based evaluation of genetic diversity in populations of *Agriophyllum squarrosum* L. and *Agriophyllum minus* Fisch. & Mey. collected in South-East Kazakhstan. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7): 697-704. DOI 10.18699/VJ20.664

Оценка генетического разнообразия с использованием SSR-маркеров в популяциях *Agriophyllum squarrosum* L. и *Agriophyllum minus* Fisch. & Mey., собранных на юго-востоке Казахстана

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Аннотация. Разработка информативных полиморфных ДНК-маркеров для малоизученных родов – необходимое и важное условие для эффективного популяционного анализа живых организмов, в том числе играющих важную экологическую роль в суровых климатических условиях, в частности в пустынях и полупустынях. Примерами подобных малоизученных пустынных видов являются *Agriophyllum squarrosum* L. и *Agriophyllum minus* Fisch. & Mey. В недавнем проекте по РНК-секвенированию *A. squarrosum* было найдено большое количество гипотетических SSR (простые повторяющиеся последовательности) маркеров. В настоящей работе в результате скрининга 24 случайно отобранных SSR-маркеров для трех популяций *A. squarrosum* и одной популяции *A. minus* обнаружены 11 новых полиморфных SSR-маркеров. Изучение этих 11 SSR-маркеров показало наличие 16 полиморфных локусов для двух видов *Agriophyllum*, 8 полиморфных локусов среди трех популяций *A. squarrosum* и 6 полиморфных локусов для популяции *A. minus*. Статистический анализ выявил высокое межвидовое, но относительно низкое внутривидовое генетическое разнообразие. Филогенетический анализ и анализ структуры популяций позволили обнаружить явное разделение на уровне видов, а

также дивергенцию популяции 1 *A. squarrosum* относительно популяций 2 и 3 данного вида. Таким образом, нами идентифицирован набор новых и информативных SSR-маркеров, подходящих для изучения генетического разнообразия *Agriophyllum*.
Ключевые слова: кумарчик песчаный; кумарчик малый; *Agriophyllum squarrosum*; *Agriophyllum minus*; SSR-маркеры; генетическое разнообразие; структура популяции.

Introduction

Xerophytes and psammophytes (plants with adaptations to survive in water-deficiency environment) become the main plants in the desert region. Despite the harsh environmental conditions, many of these plants have adapted and became a part of complex and diverse desert ecosystems. Due to the wide distribution of desert territories, the study of its wild flora is very important for the ecological prediction and conservation of biodiversity. The Central Asia region, including Kazakhstan, is having numerous large and small sand deserts, such as Gobi, Taklamakan, Karakum, Kyzylkum and Moiynkym. Deserts and semi-deserts occupy more than half of Kazakhstan territory and keep growing (Issanova et al., 2015). The major representatives of wild desert flora here are herbaceous plants, subshrubs, shrubs and subtrees. The list of dominating species includes genera *Artemisia*, *Salsola*, *Ferula*, *Arthrophytum*, *Calligonum*, *Ammodendron*, *Haloxylon*, and *Agriophyllum* (Rachkovskaya et al., 2003). Many of them are endemic for the Central Asian and Kazakhstan deserts.

Agriophyllum squarrosum L. and *Agriophyllum minus* Fisch. & Mey. are important desert species in Kazakhstan. They belong to the tribe Corispermeae within the subfamily Chenopodioideae of the family Chenopodiaceae (Kühn, 1993). The genus *Agriophyllum* includes five species, and four of them, including *A. squarrosum*, grow in Kazakhstan (Ageeva et al., 1960). *A. squarrosum* is also widely spread in all Central Asia territory, Caucasus, and China. Local people in the sandy desert regions of China consume the seed of the species during periods of food shortage, and refer to the plant as 'shami' in Chinese, which translates as 'sand rice' (Chen et al., 2014). Sand rice is an example of psammophyte perfectly adapted for harsh desert environmental conditions. Morphologically it is a shrub-like plant with a height ranging from 20 to 100 cm. The stem of sand rice at the young plant stage is firm, branched, green, and covered with short hairs. Leaves are small, sessile, green, and usually linear. Small flowers are organized in a spike-like inflorescence. *A. squarrosum* blossoms in the late summer and early fall, after that ovoid shape seeds are formed. Seeds of sand rice are very light and covered by a thin husk. After ripening, the husk is cracked into two parts, and seeds are easily dispersed by the wind. The root system is represented by a long taproot penetrating deep into the soil to access stored moisture, and almost equally long lateral roots branching near the soil surface and helping to fix plant in loose sand. Although, there is a lack of information related to the structure of the sand rice's genome, the transcriptomic analysis of *A. squarrosum* ($2n = 18$) had showed presence of 67 741 unigenes and approximately 43 % of them were annotated (Zhao et al., 2014).

Throughout history, sand rice was used for diverse purposes. Aboveground organs (stem and leaves) are eaten by both wild animals and livestock of farmers in arid and semi-arid regions, especially in Western Kazakhstan on camel pasture. Historically, nutritious seeds of sand rice were an alternative to cereals not surviving in hot deserts. In China and Mongolia, the local villagers consume sand rice seed in a variety of dishes. There are many reports about the rich nutrition value of *A. squarrosum* seeds close to its widely-used as food quinoa seeds (Chen et al., 2014). However, sand rice is not a domesticated plant species with several agriculturally unfavorable traits, such as fragile spikes and light seeds. Still, the works on possible domestication of sand rice as a novel crop are reported (Chen et al., 2014). In addition to nutrition purposes, sand rice had found its application in medicine. Back in the days, it was used as antipyretic and analgesic medicine (Gong et al., 2012; Chen et al., 2014). It was reported that the extract of *A. squarrosum* decreases blood glucose levels in type 2 diabetic mice and has the potential for further medical researches (Saqier et al., 2019). Sand rice is useful in combat against shifting sands (Wen-Ming et al., 2004). Climate change and human activities led to the growth of sand desert areas and the migration of sand dunes to agricultural territories. The structure of the sand rice root system and its ability to form seed banks in active sand dunes allow the fixation of the sand surrounding the plant. Thus, *A. squarrosum* has a tremendous potential together with other psammophytes to be used in a large-scale sand fixation (Liu et al., 2007; Ma, Liu, 2008). This species is an interesting model for different studies of morphology and physiology of desert plants. For example, earlier *A. squarrosum* was already used for the study of growth under drought conditions (Mo et al., 1997; Huang et al., 2008) and for the study of fertilizer effect on psammophytes under different rainfall conditions (Yuan et al., 2019).

Endemic species with great economic potential like sand rice are an interesting subject for genetic and molecular researches. One of the most common methods utilized for the studies on biodiversity conservation, population and phylogenetic studies of wild plant species is the usage of molecular DNA markers (Nybom, 2004). Examples of successful application of the most common DNA markers in plants include random amplification of polymorphic DNA (RAPD) (Nybom, Bartish, 2000), amplified fragment-length polymorphisms (AFLP) (Zhang C. et al., 2018), and other nuclear and chloroplast DNA markers (Abugalieva et al., 2017; Almerekova et al., 2018; Turuspekov et al., 2018). Nuclear ribosomal internal transcribed spacer (nrITS) region and five chloroplast DNA (cpDNA) fragments have been used earlier for the study of population dynamics of *A. squarrosum* in China (Qian et al., 2016). The *maturase K* (*matK*) gene of the chloroplast genome and nrITS were used for comparison of *A. squarrosum* and *A. minus* populations in two regions of Kazakhstan (Genievskaya et al., 2017). The literature survey suggests that there is a limited information on the study of *Agriophyllum* species by using SSR markers. However, a recent RNA-sequencing

Table 1. Geographical locations of sampling sites

Sampling site	Species, population	Number of plants	Coordinates	Altitude, m
South-East Kazakhstan, Zhambyl region, Moyynkum desert	*A. squarrosum*, pop1	10	44°34′55.1″ N 76°58′30.3″ E	411
South-East Kazakhstan, Zhambyl region, Moyynkum desert (near Birlik village)	*A. squarrosum*, pop2	25	44°40′56.9″ N 76°41′49.7″ E	407
	A. squarrosum, pop3	19	44°41′36.2″ N 76°44′53.5″ E	407
	A. minus, pop1	20	44°42′08.3″ N 76°44′19.3″ E	403

Fig. 1. *A. squarrosum* (on the left) and *A. minus* (on the right) in Moyynkum desert.

project of *A. squarrosum* populations in China has suggested several thousands of potential SSR markers for this species (Zhang J. et al., 2018).

In this study, we selected 24 SSR markers from this *Agriophyllum* genome resequencing project and used them for the assessment of genetic diversity within and among populations of *A. squarrosum*, and between *A. squarrosum* and *A. minus*.

Materials and methods

Plant material. In total, leaf samples of four wild *Agriophyllum* populations were collected in South-East Kazakhstan and used for the analysis (Table 1). The list included three populations of *A. squarrosum* and one population of *A. minus* sampled in Moyynkum desert of Almaty region in South-East Kazakhstan (Fig. 1). Population 1 of *A. squarrosum* was collected in 2016, while the populations 2 and 3 of *A. squarrosum* and population 1 of *A. minus* were collected in 2019. The distances between populations were at least four kilometers, and plants within the population were sampled in at least 50 meters apart.

DNA extraction, amplification and SSR marker assessment. Five young leaves from each sample were dried in silica gel. The total genomic DNA was extracted from dry leaf tissues using CTAB method (Doyle, 1991). The quality and concentration of extracted DNA were assessed via spectrophotometric test, and 1 % agarose gel electrophoresis.

Twenty-four pairs of SSR markers and their primers (Supplementary Table)[1] were selected from 6150 SSRs in the sequence of *A. squarrosum* genome reported by Zhang J. and co-authors (2018). SSR motives and expected sized of alleles were obtained from the same source.

Annealing temperature (T_a) for primer pairs and concentration of reagents in a PCR reaction mix were determined empirically. All successful PCR reactions were performed in total 16 µl volumes, including 4 mM of each dNTP, 2 mM of $MgCl_2$, 6.4 mM of primer mix (forward+reverse), 1.6 U of Taq polymerase and 50 ng of DNA. The amplification was performed in Veriti Thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation step at 94 °C for 3 minutes, followed by 40 cycles of 94 °C for 30 seconds, optimized T_a °C (see Suppl. Table) for 45 seconds and 72 °C for 1.5 minutes. The final extension step was at 72 °C for 10 minutes. The PCR products were separated on 6 % polyacrylamide gel (PAG). The SSR profile image was

[1] Supplementary Table is available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx10.pdf

captured using the GelDoc gel documentation unit (Bio-Rad Laboratories, Hercules, CA, USA). Allele sizes were estimated visually based on the size of ladder bands and the control sample on each gel.

Statistical analysis. Nei's genetic diversity indices were calculated via POPGENE software ver. 1.32 (Yeh et al., 1997); the polymorphism information content (PIC) (Botstein et al., 1980) was calculated as follows: $PIC = \sum(1-p_i^2)$, where p_i is the frequency of the *i*-th band or percentage of individuals in which the fragment is present. Principal coordinates analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering were performed based on genetic distances among populations and species. PCoA plot was made via GenAlEx (Genetic Analysis in Excel) ver. 6.5 software (Peakall, Smouse, 2012), UPGMA analysis was performed in R statistical software environment (R Core Team, 2018). Bayesian clustering was based on the use of the STRUCTURE software ver. 2.3.4 (Pritchard et al., 2000). The value of *K* was set from 1 to 5 with five iterations for each value of *K*. Both, length of burn-in period, and the number of Markov Chain Monte Carlo (MCMC) repeat after burn-in was set at 100000. Web-tool STRUCTURE HARVESTER (Earl, von Holdt, 2011) based on Evanno's method (Evanno et al., 2005) was used to determine the best fit value of *K*. The molecular variance (AMOVA) test was calculated using GenAlEx software.

Results

Performance of SSR markers in *Agriophyllum* species

Initially, 24 SSR markers were chosen for the analysis (see Suppl. Table), however, successful amplification was performed for 18 markers only. Five of 18 SSRs were multilocus markers, while other 13 SSRs were single-locus. In total, 18 markers allowed identification of 23 loci (Table 2), of which 16 were polymorphic and suitable for the analysis (Table 3).

The screening of sixteen SSR loci has allowed the identification of 43 alleles in the analysis of three populations of *A. squarrosum* and one population of *A. minus*. The molecular sizes of alleles in loci were ranged from 143 to 342 bp. The number of alleles in the study of all four populations varied from 2 to 4.

In total, eight polymorphic loci were observed in *A. squarrosum*, six polymorphic loci were found in *A. minus*, and 16 loci had demonstrated polymorphism between two species (see Table 3). In total, 20 alleles were identified exclusively in *A. squarrosum*, 16 alleles were exclusive for *A. minus*, and 7 alleles were found in both species. The average number of alleles per locus in polymorphic loci was 2.69±0.70 for both species, 2.25±0.16 for *A. squarrosum*, and 2.17±0.17 for *A. minus*, respectively.

Nei's index and PIC value were calculated separately for polymorphic SSR loci of *A. squarrosum*, *A. minus*, and jointly for two *Agriophyllum* species (see Table 3). The genetic distance-based PCoA plot suggested a clear separation of *A. minus* from populations of *A. squarrosum*, as well as distinguishing of the population 1 from populations 2 and 3 in *A. squarrosum* (Fig. 2, *a*). The UPGMA tree had also demonstrated two clusters corresponding to *A. squarrosum* and *A. minus*. The portion of samples in population 1 of *A. squarrosum* was clustered together with populations 2 and 3, while the remaining part formed a separate subcluster (see Fig. 2, *b*). Bayesian distance-based analysis of the structure among two *Agriophyllum* species was congruent with the UPGMA clusterization and also indicated that $K = 3$ is an optimal number of groups in the study (see Fig. 2, *c*).

Table 2. The characteristics of SSR loci successfully amplified in *A. squarrosum* and *A. minus*

No.	Locus	Annealing temperature (T_a), °C	Number of alleles	Range of alleles sizes, bp
1	AGS-02	46	1*	237
2	AGS-03	60	2	266–273
3	AGS-05	42	1*	280
4	AGS-07	46	1*	182
5	AGS-09.1	46	4	178–210
6	AGS-09.2	46	3	264–273
7	AGS-10	46	1*	280
8	AGS-11	54	1*	200
9	AGS-13	46	1*	275
10	AGS-16.1	39	3	159–186
11	AGS-16.2	39	3	284–305
12	AGS-17.1	33	2	228–238
13	AGS-17.2	33	2	287, null allele
14	AGS-18	39	3	176–245
15	AGS-20.1	33	3	298–323
16	AGS-20.2	33	2	359, null allele
17	AGS-23	54	2	170–182
18	AGS-24	46	1*	108
19	AGS-26	54	3	183–199
20	AGS-27.1	46	2	182, null allele
21	AGS-27.2	46	4	229–342
22	AGS-29	42	2	298–328
23	AGS-30	51	3	143–167

* Monomorphic loci excluded from further analysis.

AMOVA results for the set of data containing all *Agriophyllum* samples revealed that the majority (88 %) of the genetic diversity in *Agriophyllum* exists between two species rather than within them (Table 4).

Genetic diversity in *A. squarrosum* populations

Part of this study was focused on the assessment of the genetic diversity within and between populations of *A. squarossum*. In total, 26 alleles were identified in eight SSR loci. The largest number of unique alleles was found in population 1 (8 alleles), while population 2 had only one unique allele (Table 5). The largest values of Nei's index (0.27±0.16) and PIC value (0.25±0.09) were observed in population 1.

Table 3. Assessment of genetic diversity in populations of *Agriophyllum* species based on SSR marker analysis

No.	Locus	*A. squarrosum*				*A. minus*				*A. squarrosum* + *A. minus*			
		na	ne	Nei	PIC	na	ne	Nei	PIC	na	ne	Nei	PIC
1	AGS-03	2	1.85	0.46	0.35	2	1.11	0.10	0.09	2	1.99	0.50	0.38
2	AGS-09.1	2	1.04	0.04	0.04	3	2.06	0.52	0.46	4	1.81	0.45	0.41
3	AGS-09.2	2	1.04	0.04	0.04	2	1.84	0.46	0.35	3	1.80	0.45	0.40
4	AGS-16.1	Monomorphic*				2	1.11	0.10	0.09	3	1.71	0.42	0.34
5	AGS-16.2	3	1.22	0.18	0.17	2	1.11	0.10	0.09	3	1.19	0.16	0.15
6	AGS-17.1	Monomorphic*				Monomorphic*				2	1.63	0.39	0.31
7	AGS-17.2	Monomorphic*				Monomorphic*				2	1.63	0.39	0.31
8	AGS-18	2	1.43	0.30	0.26	Monomorphic*				3	2.22	0.55	0.49
9	AGS-20.1	Monomorphic*				2	1.21	0.18	0.16	3	1.69	0.41	0.34
10	AGS-20.2	Monomorphic*				Monomorphic*				2	1.65	0.39	0.32
11	AGS-23	Monomorphic*				Monomorphic*				2	1.66	0.40	0.32
12	AGS-26	2	1.04	0.04	0.04	Monomorphic*				3	1.69	0.41	0.34
13	AGS-27.1	Monomorphic*				Monomorphic*				2	1.65	0.39	0.32
14	AGS-27.2	3	1.46	0.32	0.29	Monomorphic*				4	2.29	0.56	0.50
15	AGS-29	Monomorphic*				Monomorphic*				2	1.65	0.39	0.32
16	AGS-30	2	1.34	0.25	0.22	Monomorphic*				3	2.13	0.53	0.46
	Mean	2.25	1.30	0.20	0.18	2.17	1.41	0.24	0.21	2.69	1.77	0.42	0.36
	SEM	0.16	0.10	0.06	0.04	0.17	0.17	0.08	0.07	0.18	0.07	0.02	0.02
	Number of plants	54				20				74			
	Number of populations	3				1				4			

Note. na – number of alleles per locus; ne – number of effective alleles; Nei – Nei's genetic diversity index; PIC – polymorphism information content; SEM – standard error of mean. * Monomorphic loci were not considered for the calculation of mean and SEM values.

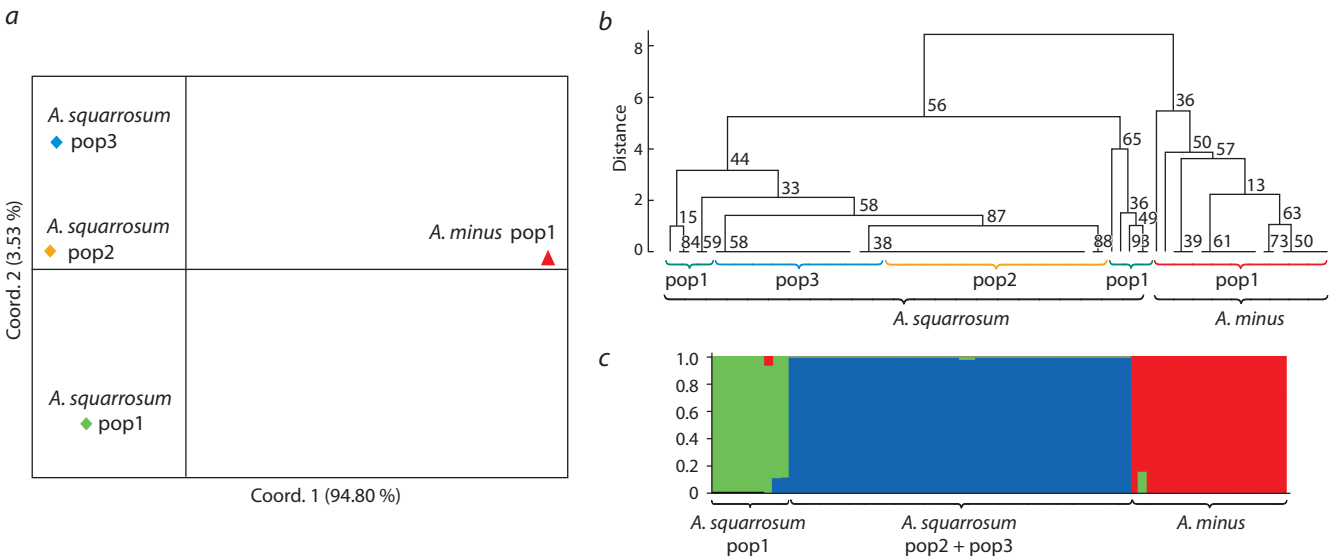

Fig. 2. Population structure among studied *Agriophyllum* species.

a, PCoA of three *A. squarrosum* and one *A. minus* populations; *b*, UPGMA dendrogram showing clusters among 74 samples of both species; *c*, Bayesian clustering of 74 *Agriophyllum* samples at $K = 3$.

Table 4. AMOVA in two species of *Agriophyllum*

Source	df	SS	MS	Est. Var. (%)	*p*
Between species	1	435.289	435.289	14.840 (88)	0.001
Within species	72	151.941	2.111	2.110 (12)	0.047
Total	73	587.230		16.951 (100)	

Note. df – degree of freedom; SS – sum of squares; MS – mean squares; Est. Var. – estimated variance; *p* – significance level.

Table 5. Average values of diversity statistics for eight polymorphic SSR loci across three *A. squarrosum* populations

A. squarrosum	nea	ne	na	Nei	PIC
Population 1	8	1.43 ± 0.32	1.88 ± 0.35	0.27 ± 0.16	0.25 ± 0.09
Population 2	1	1.02 ± 0.06	1.13 ± 0.35	0.02 ± 0.05	0.02 ± 0.05
Population 3	0	1.03 ± 0.09	1.13 ± 0.35	0.03 ± 0.07	0.02 ± 0.06

Note. nea – number of alleles exclusive for population; na – number of alleles per locus; ne – number of effective alleles; Nei – Nei's genetic diversity index; PIC – polymorphism information content.

Table 6. AMOVA among and within three populations of *A. squarrosum*

Source	df	SS	MS	Est. Var. (%)	*p*
Among populations	2	29.185	14.592	0.771 (34)	0.002
Within populations	51	77.556	1.521	1.521 (66)	0.003
Total	53	106.741		2.292 (100)	

Note. df – degree of freedom; SS – sum of squares; MS – mean squares; Est. Var. – estimated variance; *p* – significance level.

The AMOVA for *A. squarrosum* had shown a higher molecular variance within populations than the variance among populations (Table 6).

Discussion

The availability of informative DNA markers is advantageous for the study of rare species, their population structure, and for the development of a proper conservation strategy to prevent their extinction (Adams, Turuspekov, 1998; Kramer, Havens, 2009). SSR markers are one of those types of DNA markers that reliably utilized in population structure analysis due to their hyper-variability, co-dominance, and high reproducibility (Aitken et al., 2005). Successful applications of SSR markers for the identification, classification, and taxonomy of Chenopodiaceae species have been reported previously (Borger et al., 2008; Prinz et al., 2009; Sampson, Byrne, 2012; Nachtigall et al., 2016). However, for the genus *Agriophyllum*, there were no previous studies with the use of SSRs. The situation was changed recently due to the RNA-sequencing project of *A. squarrosum* accessions, and a large number of hypothetical SSR markers were suggested for population studies (Zhang J. et al., 2018). Hence, in this work 24 SSRs were randomly selected for the assessment of three populations of *A. squarrosum* and one population of *A. minus* collected in South-East Kazakhstan. The assessment of selected SSR markers resulted in the identification of 11 novel SSR markers that allowed the identification of 16 polymorphic loci in two *Agriophyllum* species (see Table 3). The large percentage of molecular variance (see Table 4) and a large genetic distance (see Fig. 2) observed between *A. squarrosum* and *A. minus* indicated good discriminative power of studied SSR markers and confirmed a significant genetic difference reported between these species earlier (Genievskaya et al., 2018). Fifteen SSR loci used for both species were in intermediate diversity group with PIC values ranged between 0.32 and 0.50, and Nei's diversity index ranged between 0.39 and 0.56 (see Table 3).

When species were analyzed separately, 8 out of 16 loci were polymorphic for *A. squarrosum*, and 6 loci were polymorphic for *A. minus* (see Table 3). The average diversity in *A. minus* was slightly higher than in *A. squarrosum* and lower than the average interspecies diversity (see Table 3). Thus, *A. squarrosum* and *A. minus* have maintained a low level of genetic diversity. It has been demonstrated that annuals like *Agriophyllum* or short-lived perennials usually demonstrate low levels of genetic diversity compared with long-lived and outcrossing species (Austerlitz et al., 2000). However, the limited amount of samples in studied species (particularly in *A. minus*), their close geographical locations, and a relatively small number of polymorphic SSR markers used for the analysis could influence obtained results.

The study of the genetic diversity within *A. squarrosum* suggested that 66 % of the total variation was within and 34 % between populations (see Table 6), indicating that the difference of population 1 from populations 2 and 3 was rather substantial. The presence of relatively high diversity within populations may be explained by the geographical proximity of *A. squarrosum* populations used in this study (see Table 1),

which may cause a high rate of gene flow in a limited area (Conner, Hartl, 2004). Populations 2 and 3 of *A. squarrosum* had demonstrated an extremely low level of genetic diversity in comparison with population 1 (see Table 5), but the fact that samples from each population clustered together using multiple analyses (PCoA, UPGMA, Bayesian clustering) (see Fig. 2) indicates that there is a high level of heterogeneity in the species. The grouping of 5 samples from population 1 on the UPGMA tree and on the STRUCTURE bar plot into separate subcluster in the entire species cluster may indicate the presence of some barriers between groups of samples within population 1 interfering gene flow. Therefore, even if no physical obstructions were found during the sample collection, probably, two groups in population 1 may be considered as subpopulations (Waples, Gaggiotti, 2006). This assumption is also supported by the relatively higher genetic diversity in population 1 comparing with the other two *A. squarrosum* populations (see Table 5).

Conclusions

In this study, sequences of 24 pairs of oligonucleotides for SSR markers were randomly selected from *A. squarrosum* genome resequencing project, and used for the study of genetic diversity in the genus *Agriophyllum*. Hence, it is the first report exploring the performance of novel SSR markers in the genetic analysis of this genus. The study revealed 16 polymorphic loci in eleven SSR markers using two *Agriophyllum* species, 8 polymorphic loci within three populations of *A. squarrosum*, and 6 polymorphic loci within the population of *A. minus*. Statistical analyses showed high interspecific, but relatively low genetic diversity in populations 2 and 3 of *A. squarrosum*. The phylogenetic clusterization and analysis and population structure analysis demonstrated clear segregation of *A. minus* from *A. squarrosum*, as well as the separation of population 1 of *A. squarrosum* form populations 2 and 3. As a result, we identified the set of novel and informative SSR markers suitable for the study of genetic diversity in *Agriophyllum* species. These results provide an important contribution to the population study and approaches for the development of conservation mechanisms for *Agriophyllum* species.

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Acknowledgements. The research was supported by the project "Key techniques for sand dune stabilization and vegetation recovery in the typical regions of the countries along the Silk Road Economic Belt" (REF: 2016YFE0203400) granted by the Ministry of Science and Technology of China. The study was also supported by the project AP05131621 "Informational system for molecular genetic and botanical documentation of wild flora in Kazakhstan" granted by the Ministry of Education and Science of the Republic of Kazakhstan.

Conflict of interest. The authors declare no conflict of interest.

Received July 29, 2020. Revised September 1, 2020. Accepted September 10, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):705-721
DOI 10.18699/VJ20.665

Потенциально опасные для отечественного картофелеводства карантинные виды и патотипы нематод: изменчивость популяций и генетика устойчивости картофеля

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Аннотация. Обзор посвящен проблеме потенциально опасных для отечественного картофелеводства карантинных видов и патотипов нематод. Картофель поражают более 30 видов паразитических нематод, однако в статье основное внимание уделено самым вредоносным, приносящим большой ущерб картофелеводству представителям родов *Globodera*, *Ditylenchus*, *Nacobbus* и *Meloidogyne*. Проанализированы фитопатологические и молекулярные методы идентификации видов и патотипов и основные достижения в изучении изменчивости популяций паразитических нематод картофеля. Показано, что, благодаря особенностям жизненного цикла нематод и лабильности их геномов, генетическая изменчивость этих организмов очень велика, что создает угрозу образования новых патогенных генотипов паразита. Сведения о внутри- и межпопуляционной изменчивости нематод важны для изучения путей интродукции и распространения отдельных видов, а также поиска корреляций молекулярных маркеров с определенным патотипом. Филогенетические исследования, основанные на современных данных по генетической изменчивости популяций, позволили выявить комплексы видов у *Globodera pallida* (Stone) Behrens и *Nacobbus aberrans* (Thorne) Thorne & Allen (sensu lato), включающие криптические виды. К основным составляющим успешной защиты, предотвращающей массовое распространение паразитических нематод, относятся карантинные мероприятия, агротехнические приемы, биологические способы защиты и возделывание устойчивых сортов. Особое внимание в обзоре уделено вопросам селекции сортов картофеля с длительной устойчивостью к различным видам нематод, поскольку возделывание таких сортов – экологически наиболее безопасный и экономически выгодный способ предотвращения эпифитотий. В настоящее время достигнуты значительные успехи в генетической защите сортов картофеля, особенно в отношении цистообразующих нематод. Приведены сведения об источниках устойчивости картофеля к паразитическим нематодам, выделенных в коллекциях диких и культурных видов. Проанализированы данные об идентифицированных *R*-генах и QTL устойчивости, которые были интрогрессированы в селекционный материал с помощью различных методов и подходов. Представлены результаты изучения структурной и функциональной организации генов устойчивости к цистообразующим нематодам картофеля. Рассмотрены результаты исследований по использованию молекулярных маркеров определенных генов в маркер-опосредованной селекции для создания новых устойчивых сортов, в том числе с групповой устойчивостью.

Ключевые слова: картофель; паразитические нематоды; *Globodera*; *Ditylenchus*; *Nacobbus*; *Meloidogyne*; патотипы; изменчивость популяций; устойчивость сортов; гены устойчивости; QTL.

Для цитирования: Мироненко Н.В., Гавриленко Т.А., Хютти А.В., Афанасенко О.С. Потенциально опасные для отечественного картофелеводства карантинные виды и патотипы нематод: изменчивость популяций и генетика устойчивости картофеля. *Вавиловский журнал генетики и селекции*. 2020;24(7):705-721. DOI 10.18699/VJ20.665

Quarantine nematode species and pathotypes potentially dangerous for domestic potato production: populations diversity and the genetics of potato resistance

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Abstract. The review considers quarantine species and nematode pathotypes potentially dangerous for domestic potato production. Potatoes are affected by more than 30 types of parasitic nematodes, but the review focuses on the most harmful representatives of genera that cause great damage to potato production: *Globodera*, *Ditylenchus*, *Nacob-*

bus and *Meloidogyne*. Phytopathological and molecular methods of identification of species and pathotypes and the main achievements in studying the population variability of parasitic potato nematodes were analyzed. It was shown that due to the peculiarities of the life cycle of nematodes and lability of their genomes, the genetic variability of these organisms is very high, which creates a threat of forming new pathogenic genotypes of the parasites. The information about the intra- and interpopulation variability of nematodes is important for studying the ways of introduction and distribution of separate species, as well as for searching for the correlations of molecular markers with the pathotype. Phylogenetic studies based on modern data on genetic variability of populations have allowed to reveal species complexes in *Globodera pallida* (Stone) Behrens and Nacobbus aberrans (Thorne) Thorne & Allen (sensu lato), including cryptic species. The main components of successful protection preventing a wide distribution of parasitic nematodes are quarantine measures, agricultural techniques, biological methods of protection and cultivation of resistant cultivars. Special attention in the review is paid to the breeding of potato cultivars with durable resistance to various nematode pathotypes, because the cultivation of such varieties is the most ecologically safe and economically advantageous way to prevent epiphytoties. Currently, significant progress has been made in the genetic protection of potato cultivars, especially against cyst-forming nematodes. The review provides data on sources of potato resistance to parasitic nematodes identified in collections of wild and cultivated species. Data on identified R-gens and QTL of resistance that have been introduced into breeding varieties using different methods and approaches are analyzed. The literature data on the study of structural and functional organization of genes for resistance to potato cyst nematodes are given. The results of molecular research on revealing the polymorphisms of loci involved in the control of resistance to cyst and gall nematodes, the development of molecular markers of certain genes and their use in marker-assisted selection for developing of new resistant cultivars, including those with group resistance, are considered.
Key words: potato; parasitic nematodes; *Globodera*; *Ditylenchus*; *Nacobbus*; *Meloidogyne*; pathotypes; population variability; resistance cultivars; resistance genes; QTL.

For citation: Mironenko N.V., Gavrilenko T.A., Khiutti A.V., Afanasenko O.S. Quarantine nematode species and pathotypes potentially dangerous for domestic potato production: populations diversity and the genetics of potato resistance. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):705-721. DOI 10.18699/VJ20.665 (in Russian)

Введение

Картофель (*Solanum tuberosum* L.) является одной из основных сельскохозяйственных культур и возделывается на более чем 19 млн га сельскохозяйственных угодий по всему миру с ежегодным производством более 390 млн тонн картофеля (FAOSTAT, 2019). По валовому сбору картофеля Российская Федерация занимает третье место в мире после Китая и Индии, и его среднегодовое производство в стране составляет примерно 29 млн тонн в год. Основной фактор, снижающий рентабельность возделывания картофеля, – болезни, способом контроля которых продолжают оставаться химические средства защиты. Возделывание сортов картофеля с длительной устойчивостью к болезням – экологически наиболее безопасный и экономически выгодный способ предотвращения эпифитотий.

Паразитические нематоды повсеместно приносят большой ущерб производству картофеля, ежегодные глобальные потери урожая от них составляют 10–15 % и оцениваются в 78 млрд долларов (Fábia et al., 2018). Картофель поражают 34 вида паразитических нематод, 6 из которых, по крайней мере, могут вызывать значительные потери урожая. Среди них распространенными во всех зонах возделывания картофеля и наиболее вредоносными являются цистообразующие картофельные нематоды (ЦКН) – золотистая картофельная нематода (ЗКН) *Globodera rostochiensis* (Woll.) и бледная картофельная нематода (БКН) *Globodera pallida* (Stone). Эти виды считаются объектами внешнего и внутреннего карантина и широко распространены на всех континентах: в Европе, Северной и Южной Америке, Азии, Африке, Океании (EPPO, 2020a, b), ЗКН распространена также и в Австралии (Blacket et al., 2019).

Кроме ЦКН, существенные потери урожая картофеля повсеместно могут вызывать карантинные, широкоспециализированные виды нематод, на настоящий день отсутствующие на территории Российской Федерации. В соответствии с единым перечнем карантинных объектов Евразийского экономического союза (ЕАЭС) от 30 ноября 2016 г. № 158 с изменениями и дополнениями от 8 августа 2019 г. в перечень карантинных вредных организмов, отсутствующих на территории ЕАЭС, входят: ложная галловая нематода *Nacobbus aberrans* (Thorne) (EPPO, 2019), колумбийская корневая галловая нематода *Meloidogyne chitwoodi* Golden et al., корневая галловая нематода *M. enterolobii* Golden et al. и ложная колумбийская галловая нематода *M. fallax* Karssen (https://docs.eaeunion.org/docs/ru-ru/01413200/cncd_06032017_158). Стеблевая нематода картофеля *Ditylenchus destructor* (Thorne) относится к карантинным видам в Европейском союзе (список A2), но из-за широкого распространения в нашей стране входит в список A3 – регулируемых некарантинных вредных организмов на территории Российской Федерации (EPPO, 2019b; EPPO, 2020d; OEPP/EPPO, 2017a).

Вопросы классификации видов паразитических картофельных нематод еще не приведены к единой системе. Согласно классификации, принятой EPPO (EPPO, 2000), виды нематод родов *Globodera*, *Ditylenchus*, *Nacobbus* и *Meloidogyne*, рассмотренных в этом обзоре, относятся к одному классу Chromadorea, и порядку Rhabditida, но к четырем различным семействам: Heteroderidae (виды: *Globodera rostochiensis*, *G. pallida* – цистообразующие нематоды), Anguinidae (*Ditylenchus destructor* – стеблевая нематода картофеля), Pratylenchidae (*Nacobbus aberrans* – ложная галловая нематода) и Meloidogynidae (*Meloidogyne chitwoodi*, *M. fallax* – галловые нематоды). Кроме перечисленных видов рода *Meloidogyne*, рассматриваются также другие виды этого рода, сведения об устойчивости к которым видов рода *Solanum* имеются в литературе:

M. javanica (Treub, 1885), *M. hapla* Chitwood, 1949, *M. arenaria* Chitwood (1949), *M. incognita* Kofoid & White, 1919.

В литературе обсуждается возможность существования криптических видов у ЦКН (Thevenoux et al., 2019) и видовых комплексов, как, например, у *G. pallida* (Subbotin et al., 2011) и *N. aberrans* (Cabrera-Hidalgo et al., 2019). На филогенетическом древе, построенном на основании сходства нуклеотидных последовательностей малой субъединицы рДНК, филум *Nematoda* разделен на 12 клад, из которых 4 включают нематоды, паразитирующие на растениях. Наиболее вредоносные виды нематод, приводящие к значительным экономическим потерям в картофелеводстве, – представители четырех родов: *Meloidogyne* spp., *Globodera* spp., *Ditylenchus* spp. и *Nacobbus* spp., отнесены к кладе *Tylenchomorpha* (Holterman et al., 2006). Развитие исследований по таксономии паразитических нематод необходимо, прежде всего, для понимания эволюционных процессов в популяциях патогенов, связанных с решением проблемы генетической защиты. Например, в работе R. Thevenoux с коллегами (2019) показано, что генетические расстояния между географическими популяциями *G. pallida* на территории Перу могут свидетельствовать о дивергенции популяций с возможным образованием нового вида.

Составляющие успешной защиты, предотвращающей массовое распространение паразитических нематод, – это карантинные мероприятия и возделывание устойчивых сортов. Из существующих биологических способов защиты картофеля от паразитических нематод можно отметить использование растений нехозяев, которые стимулируют выход личинок из яиц, но не являются субстратом для их размножения. Таким видом-ловушкой считается, например, *Solanum sisymbriifolium* (Lam.) (Timmermans et al., 2006). В США (штат Айдахо) использование в качестве ловушки *S. sisymbriifolium* в трех очагах *G. pallida* позволило снизить жизнеспособность яиц на 95–100 % (Dandurand et al., 2019).

Сорта картофеля с устойчивостью к расширенному спектру патогенов более конкурентоспособны на рынке и обеспечивают получение экологически чистой продукции и сохранение окружающей среды. Последние два фактора имеют не только экономическое, но и существенное социальное значение.

Результатом длительной селекции картофеля на устойчивость как в нашей стране, так и за рубежом стала серия сортов, генетически защищенных от наиболее распространенных патотипов цистообразующих картофельных нематод. Например, в списке из 216 сортов картофеля, устойчивых к цистообразующим нематодам, одобренных для использования в ЕС, 213 защищены геном *H1*, детерминирующим устойчивость к наиболее распространенному, в том числе и на территории РФ, патотипу Ro1 (https://lbst.dk/fileadmin/user_upload/NaturErhverv/Sortsliste_til_internettet_marts_2019.pdf). В России в Госреестре селекционных достижений зарегистрировано 455 сортов картофеля, 254 из них устойчивы к ЗКН, т. е. 55.8 % (Государственный реестр селекционных достижений, 2019). При этом необходимо учитывать опыт селекционных программ Великобритании, где во второй половине 20-го века преимущественно возделывались сорта картофеля с геном *H1* устойчивости к ЗКН, что привело к замещению популяций *G. rostochiensis* на *G. pallida* (Evans, 1993).

Основная проблема селекции растений на устойчивость к болезням – высокая адаптивная изменчивость патогенов, приводящая к обязательной потере устойчивости при длительном возделывании сортов, защищенных одними и теми же генами, на больших площадях. В отношении ЦКН, и, в частности ЗКН, широкое использование устойчивых сортов, защищенных геном устойчивости *H1*, эффективным против распространенного патотипа Ro1, создает условия для адаптивной эволюции паразита и опасность появления новых патотипов. Не исключен и занос других патотипов ЦКН, а также вида *G. pallida* с семенным картофелем из Западной Европы. В настоящее время в РФ вид *G. rostochiensis* представлен исключительно одним патотипом, Ro1, а бледная нематода *G. pallida* до сих пор не обнаружена. Такая же ситуация существует в Австралии, где распространен только один вид *G. rostochiensis* и патотип Ro1, а *G. pallida* не обнаружен, что было доказано секвенированием ITS участков рибосомных генов (Faggian et al., 2012).

В наше время активно развиваются молекулярно-генетические методы видовой диагностики паразитических нематод, появились новые данные по механизмам изменчивости популяций цистообразующих и галловых нематод, что позволяет определить происхождение местных популяций патогена и прогнозировать их возможные адаптационные изменения к возделываемым сортам картофеля. Имеются успехи в разработке молекулярных маркеров генов устойчивости и создании сортов картофеля, генетически защищенных против разных патотипов и видов цистообразующих картофельных нематод. Все эти сведения необходимы для разработки стратегии эффективной генетической защиты против ЦКН в Российской Федерации.

В этом обзоре представлены последние достижения в изучении изменчивости популяций паразитических картофельных нематод, молекулярной диагностики, генетики устойчивости картофеля к нематодам, а также результаты селекции на устойчивость к потенциально опасным для России видам и патотипам цистообразующих и галловых нематод. В статье не затрагивались вопросы биологии фитопаразитических нематод, хозяино-паразитных отношений, особенностей патогенеза растений, биохимических факторов устойчивости растений и мер контролирования вредоносности нематод, поскольку эти темы подробно анализировались в ряде иностранных обзоров (Sato et al., 2019; Abd-Elgawad, Askary, 2020; Holbein et al., 2020), а также в монографии по фитопаразитическим нематодам (https://www.rfbr.ru/rffi/ru/books/o_1781721#99; Зиновьева и др., 2012).

Цистообразующие нематоды

Центр происхождения ЦКН – регион Центральных Анд в Южной Америке (Evans et al., 1975; Plantard et al., 2008). Считается, что ЦКН попали в Европу из Южной Америки в середине 19-го века с селекционным материалом картофеля, а оттуда распространились по всему миру. Таким образом, Европа является вторичным центром

происхождения ЦКН (Evans, Rowe, 1998; Hockland et al., 2012; Boucher et al., 2013). На территории Российской Федерации широкое распространение получила ЗКН, которая встречается очагами в 7 федеральных округах 61 субъекта РФ на территории общей площадью около 1.8 млн га (Справочник…, 2017). По данным Европейской и Средиземноморской организации по защите растений (EPPO), в настоящее время ЗКН распространена во всех странах Европы, в том числе и на территории сопредельных стран, таких как Беларусь, Латвия, Эстония, Литва, Украина, Грузия, Армения; на Азиатском континенте в Таджикистане, Японии и Индии (EPPO, 2020a, b). В РФ до настоящего времени БКН не обнаружена (Limantseva et al., 2014; Хютти и др., 2017). В то же время вид *G. pallida* широко встречается в Европе. По данным EPPO, *G. pallida* зарегистрирована во всех странах Европы, кроме Беларуси, Латвии, Литвы, Польши и Словакии (https://gd.eppo.int/taxon/HETDPA/distribution/RU_ru). В Украине первое обнаружение *G. pallida* было отмечено в Ужгороде в 2005 г. (Pylypenko et al., 2005), но после карантинных мероприятий уже в 2014 г. в существующем очаге патоген не был выявлен, EPPO официально зафиксировала отсутствие этого вида для страны. В сопредельных с РФ Европейских государствах *G. pallida* была выявлена в 2018 и в 2019 гг. в Эстонии (NPPO of Estonia (2018-02, 2018-11, 2019-06)), а также в Норвегии и Финляндии (Holgado, Magnusson, 2012). На Азиатском континенте очаги БКН зафиксированы в Индии и Японии. В связи с большими объемами импорта как продовольственного, так и семенного картофеля в Россию в последние десятилетия существует потенциальная возможность заноса БКН на территорию страны.

Известны еще два вида ЦКН из рода *Globodera*, паразитирующие на картофеле. *G. leptonepia* (Cobb & Taylor) была обнаружена только однажды в партии картофеля из Южной Америки, но интенсивные поиски в районе Андского Нагорья, известного как центр происхождения ЦКН, не были успешными, таким образом, *G. leptonepia* – редкий и неизученный вид (Thevenoux et al., 2019). В 2008 г. в США в штатах Айдахо и Орегон обнаружены единичные цисты, которые по морфологической и молекулярной диагностике не соответствовали ни *G. rostochiensis*, ни *G. pallida* (Dandurand et al., 2019). Новый вид был определен как *Globodera ellingtonae* n.sp. (Handoo et al., 2012) и было показано, что картофель и томаты – его растения-хозяева (Skantar et al., 2011). Отсутствие сведений о распространении этого вида в других регионах мира, по-видимому, свидетельствует о наличии локальной эндемичной популяции на Северо-Американском континенте.

Цистообразующие картофельные нематоды – облигатные специализированные седентарные паразиты, для которых характерно наличие в жизненном цикле цист, содержащих яйца и инвазионные личинки. Потери урожая могут достигать более 80–90 % (OEPP/EPPO, 2017b). Основной диагностический признак *G. pallida* – окраска цист: в конце созревания они не приобретают золотистый цвет, как у *G. rostochiensis*, а остаются бледными. Цисты обоих видов могут выживать в почве без растений-хозяев до 10 лет (Sijmons, 1993; Williamson, Hussey, 1996), однако имеются данные о сохранении их жизнеспособности до 30 лет (Winslow, Willis, 1972). Максимальные потери урожая, ассоциированные с поражением ЦКН, определены в 80 % (Brodie, Mai, 1989), но при высокой инфекционной нагрузке ЦКН в почве урожай картофеля может быть полностью уничтожен (Whitehead, Turner, 1998). Четкие симптомы глободероза часто не заметны в период вегетации картофеля (Lilley et al., 2007). Вследствие этого потери урожая могут быть приписаны вторичным болезням, возникшим на уже зараженных нематодой ослабленных растениях картофеля.

Видовая диагностика с применением морфометрических и молекулярных методов широко применяется в практике карантина растений для идентификации видов ЦКН в обнаруженном очаге в посадках картофеля. Морфометрические методы диагностики достаточно подробно описаны в коллективной монографии (Зиновьева и др., 2012), а также в обзорах и методических указаниях (Шестеперов, 2002; Ryss, 2002; Bairwa et al., 2017; Christoforou et al., 2017; OEPP/EPPO, 2017b).

Молекулярные методы идентификации видов ЦКН

Молекулярные методы идентификации видов нематод меняются параллельно с развитием базовых методов молекулярной генетики и зависят от конкретных задач исследователя. В последние годы молекулярную диагностику различных видов нематод проводят методами обычной ПЦР, ПЦР в реальном времени (количественная ПЦР) и обратной ПЦР (ОТ-ПЦР). Во всех случаях обычно используют праймеры, разработанные к последовательностям участков кластера генов рибосомных РНК. Методы идентификации *G. rostochiensis* и *G. pallida*, основанные на рестриктном анализе продуктов амплификации фрагментов ITS1 или ITS1+5.8S+ITS2 (Thiéry, Mugniery, 1996; Fleming et al., 1998), сменились более удобными методами ПЦР с видоспецифичными праймерами (Bulman, Marshall, 1997; Zouhar et al., 2000; Vejl et al., 2002).

В наших работах для молекулярной идентификации видов *G. rostochiensis* и *G. pallida* (Мироненко и др., 2013, 2015; Limantseva et al., 2014) применен метод мультиплексной ПЦР с двумя наборами видоспецифичных праймеров (Bulman, Marshall, 1997). Эти же праймеры используют другие авторы, например, для количественной ПЦР (Nakhla et al., 2010).

Для оценки эффективности методов деконтаминации растительного материала от ЦКН определяют жизнеспособность цист, применяя ОТ-ПЦР как индикатор экспрессии генов «домашнего хозяйства», например гена gpd1, кодирующего глицерол-3-фосфат-дегидрогеназу – одного из основных ферментов гликолиза (Kaemmerer, 2012). В этой работе виды нематод были определены предварительно по методу S.R. Bulman и J.W. Marshall (1997) с помощью стандартной ПЦР, так как ОТ-ПЦР не может быть использована для идентификации видов нематод, пока не будут обнаружены видоспецифичные гены, необходимые для выживания организма, аналогичные генам «домашнего хозяйства». Вероятно, полногеномное секвенирование обоих видов нематод (Cotton et al., 2014; Eves-van den Akker et al., 2016) сделает доступной информацию для выявления таких генов.

Патотипы *G. rostochiensis* и *G. pallida*

Оба вида ЦКН могут быть классифицированы на патотипы на основании их реакций вирулентности/авирулентности на дифференцирующих тест-клонах картофеля с различными генами устойчивости (табл. 1) (Canto Saenz, De Scurrah, 1977; Kort et al., 1977). Для *G. rostochiensis* были определены пять патотипов, Ro1–Ro5; для *G. pallida* – три, Pa1–Pa3 (Kort et al.,1977).

В 1990 г. дифференциация популяций ЦКН была пересмотрена на основании нечетких различий между патотипами, определяемыми на данном наборе, что связано с различной жизнеспособностью инокулюма и наличием количественной частичной устойчивости, уровень которой трудно интерпретировать (Nijboer, Parlevliet, 1990). По мнению авторов, основанном на анализе литературы и собственных экспериментальных данных, возможно идентифицировать только три патотипа: Ro1 (бывшие Ro1 и Ro4), Ro3 (бывшие Ro2 и Ro3) и Ro5 (бывший Ro5). Клоны-дифференциаторы 60.21.19 и 65.346.19 различают только виды нематод, но не их патотипы. В отношении *G. pallida* H. Nijboer и J.E. Parlevliet (1990) считают возможным характеризовать популяцию только как более или менее вирулентную, не выделяя патотипы. В то же время в Нидерландах в 1975 г. было отмечено обильное размножение БКН на сортах, устойчивых к патотипу Pa2. По реакции клона-дифференциатора (VTN)' 62-33-3 изолят был отнесен к патотипу Pa3 (Dellaert, Vinke, 1987). Авторами показан различный уровень размножения патотипа Pa3 на разных генотипах картофеля.

По мнению Европейской и Средиземноморской организации по защите растений, термин «патотип» в отношении ЦКН считается слишком общим, и многие популяции, основываясь на Международной схеме по дифференциации (Kort et al., 1977), не могут быть адекватно идентифицированы и отнесены к тому или иному патотипу (OEPP/EPPO, 2017b). В настоящее время предлагается проводить изучение вирулентности местных популяций *G. rostochiensis* и *G. pallida* на наборах сортов, выращиваемых в той или иной стране, со следующими эталонными популяциями ЦКН: Ro1 Ecosse (INRA, Франция), Ro5 Harmerz (BBA, Германия), Pa1 Scottish (SASA, Великобритания) и Pa3 Chavornay (INRA, Франция). В связи с тем, что в селекции картофеля используются различные генетические источники устойчивости, вирулентность эталонных популяций ЦКН должна регулярно перепроверяться по отношению к этим наборам сортов. Точно так же, если в результате мониторинга будет обнаружено, что характеристики вирулентности популяций ЦКН нематод в Европе изменились, то эталонные популяции ЦКН следует пересмотреть (OEPP/EPPO, 2006, 2017b).

Во всем мире широко распространен патотип ЗКН Ro1, и, соответственно, большинство устойчивых к ЗКН сортов защищены эффективным против этого патотипа геном *H1*. Преодоление этого гена другими патотипами ЗКН зарегистрировано в Польше, где впервые в 2013 г. был выявлен патотип Ro5 (Przetakiewicz, 2013). В США в течение более 50 лет с момента первого обнаружения ЗКН ген *H1* оставался эффективным против распространенного патотипа Ro1. В 1995–1996 гг. на Лонг Айленде и в северной части штата Нью-Йорк на сортах, защищенных геном *H1*, было

Table 1. Set of potato differentials for identifying pathotypes of cyst nematodes (Canto Saenz, De Scurrah, 1977; Kort et al., 1977)

Potato differentials	Pathotypes
Solanum tuberosum ssp. *andigena* hybr. CPC 1673	Ro1,4
S. kurtzianum hybr. KTT/60.21.19	Ro1,2
S. vernei hybr. 58.1642/4	Ro1,2,3
S. vernei hybr. 62.33.3	Ro1,2,3,4; Pa1,2
S. vernei hybr. 65.346/19	Ro1,2,3,4,5
S. multidissectum hybr. P55/7	Pa1
S. vernei hybr. 69.1377/94	Ro1,2,3,4,5; Pa1,2,3

отмечено появление размножающейся популяции ЗКН, которая была определена как патотип Ro2 (Brodie, 1995; 1996). Интересно, что на обширных территориях, например в Российской Федерации и Австралии (Виктория), встречается только один вид, *G. rostochiensis*, и один патотип этого вида, Ro1 (Limantseva et al., 2014; Blacket et al., 2019).

Появление патотипа Pa2/3 *G. pallida* в США в штате Айдахо произошло в результате одной интродукции в 2006 г. Последующие интенсивные исследования показали распространение этого вида на полях общей площадью 369 га, в основном путем механического переноса инокулюма с сельскохозяйственной техникой. В связи с этим немедленно было введено эмбарго на импорт картофеля из этого штата традиционным партнерам в Канаду, Южную Корею и Мексику. Япония прекратила импорт картофеля из США (Dandurand et al., 2019). Таким образом, серьезный экономический ущерб от появления *G. pallida* на небольших площадях одного региона был связан главным образом с необходимыми карантинными ограничениями.

Генетическая изменчивость популяций ЗКН и БКН

Цистообразующие картофельные нематоды группируются в патотипы согласно их способности размножаться на дифференциаторах (Canto Saenz, De Scurrah, 1977; Kort et al., 1977). Идентификация патотипа фитопатологическим методом занимает много времени, поэтому предпринимаются попытки найти молекулярные маркеры, способные идентифицировать патотипы. Однако до сих пор эти попытки были безуспешны.

В работах, посвященных изучению генетической изменчивости ЦКН, употребляется термин «популяция», которую рассматривают как вариант патотипа. Интересно, что разные популяции одного патотипа можно различить с использованием комбинации молекулярных маркеров, например гена-эффектора *rbp-1*, некодирующих участков ядерной (ITS регион) и митохондриальной (scmtDNA IV) ДНК (Hoolahan et al., 2012).

В этом плане представляют интерес результаты изучения нуклеотидного полиморфизма гена-эффектора *pel-2*, который у нематод рода *Globodera* кодирует эффекторную молекулу пектат-лиазу, участвующую в деградации кле-

точной стенки растения-хозяина. Однако наблюдаемый полиморфизм не был связан с определенным патотипом *G. rostochiensis* или *G. pallida*, хотя были выявлены видоспецифичные сайты (Geric Stare et al., 2011).

В качестве генетического маркера патотипов *G. pallida* рассматривают также ген-эффектор *Gp-Rbp-1* (Carpentier et al., 2012), который кодирует секретируемый белок, индуцирующий специфический, или эффектор-активированный иммунитет (effector-triggered immunity), опосредованный геном устойчивости *Gpa2 Solanum tuberosum*. Создана база данных из 158 полиморфных последовательностей гена *Gp-Rbp-1*, найдено 8 сайтов, которые находятся под контролем положительного естественного отбора. Из них только один сайт, P/S 187, подходит для объяснения узнавания *Gp-Rbp-1* геном *Gpa2* (Carpentier et al., 2012).

Данные, полученные при анализе внутри- и межпопуляционной изменчивости ЦКН, важны для оценки путей интродукции и распространения, а также поиска корреляций с патотипом. Для этого используют различные молекулярные маркеры – RAPD (Blok et al., 2006; Мироненко и др., 2015), AFLP (Folkertsma et al., 2001), SSR (Plantard et al., 2008; Boucher et al., 2013), SNP (Davis et al., 2005). Например, с помощью микросателлитных маркеров была оценена изменчивость популяций патотипа Ro1 *G. rostochiensis* в Австралии (штат Виктория). Показано большое сходство между австралийскими популяциями по частотам аллелей микросателлитных локусов и в то же время отличие их от других популяций, собранных по всему миру. Этот факт свидетельствует об одноразовом заносе ЗКН в Австралию, что дает возможность легко контролировать появление новых видов и патотипов нематод (Blacket et al., 2019).

В 2011 г. был разработан новый высокопроизводительный метод генотипирования GBS (genotyping-by-sequencing) (Elshire et al., 2011). Позднее был внедрен метод Pool-Seq, основанный на секвенировании ДНК сложных образцов, пулов, вместо индивидуумов (Futschik, Schlotterer, 2010). Среди ЦКН распространена полиандрия, т. е. множественное оплодотворение самки разными самцами (Turner, Rowe, 2006), поэтому одна циста может содержать несколько сотен генетически различных яиц. Именно пулы цист (по 15 цист) нематод были объектом исследования с помощью комбинации подходов Pool-Seq и GBS, что позволило изучить отношения между 23 популяциями *G. rostochiensis* из девяти стран, представляющими пять патотипов (Mimee et al., 2015). На филогенетическом древе, построенном для 23 популяций на основании частот аллелей для 604 SNP-локусов, популяции сгруппировались по генетическому сходству и патотипному составу в два кластера: Ro1, Ro2 и Ro3, Ro4, Ro5. Отмечено большое генетическое сходство двух популяций ЗКН из США, относящихся к разным патотипам (Ro1 и Ro2). Авторы считают, что из-за длительного использования устойчивых сортов картофеля с геном *H1* развитие вирулентной популяции (Ro2) может быть результатом недавней мутации, а не интродукции нового патотипа ЗКН (Mimee et al., 2015).

Возможно, в ближайшее время будет разработан новый подход к молекулярной диагностике патотипов Ro1 и Ro2, основанный на использовании результатов полногеномного секвенирования множественных линий Ro1 и Ro2, полученных из одной цисты, и выявлении однонуклеотидных полиморфизмов (Dandurand et al., 2019).

Данные литературы по генотипированию популяций нематод с помощью молекулярных маркеров показывают, что один патотип может объединять популяции нематод, различающиеся не только по географическому происхождению, но и генетическим параметрам изменчивости. Например, с использованием комбинации методов Pool-Seq и GBS были получены сведения о генетическом разнообразии популяций, на основании которых рассчитаны индексы фиксации (Fst) при попарном сравнении популяций *G. rostochiensis.* Так, патотип Ro1 был представлен в работе 14 популяциями из девяти стран: минимальное значение, Fst = 0.04, получено при сравнении популяций из Германии и Бельгии, а максимальное, Fst = 0.43, – для популяций из США и Канады. Можно предполагать, что на фоне такой высокой генетической изменчивости высока вероятность адаптивной эволюции, приводящей к образованию новых патотипов (Mimee et al., 2015).

Адаптация паразита к генотипам хозяина, защищенным эффективными генами устойчивости, может быть связана со сниженной фитностью на восприимчивых сортах. «Цена» новой вирулентности состоит в том, что клоны с такой вирулентностью становятся менее агрессивны на сортах с отсутствием комплементарного гена устойчивости, т. е. на восприимчивых сортах. Вследствие этого снижается конкурентоспособность таких особей в природных популяциях паразитов. Такие факты приведены в обзоре (Laine, Barres, 2013) для грибов, оомицетов, вирусов и нематод, в частности *Meloidogyne incognita* (Castagnone-Sereno et al., 2007). На примере *G. pallida* была показана адаптация паразита после 8–10 пассажей на сорте картофеля Iledher с QTL устойчивости $GpaV_{vrn}$, интрогрессированным от *Solanum vernei* и картированным на хромосоме V (Fournet et al., 2013). Авторы пришли к неожиданному выводу об увеличении фитности линий с новой вирулентностью на восприимчивом сорте Desiree, которая выражалась в формировании увеличенных по размеру цист, содержащих большее количество личинок, и в сокращении времени образования личинок из яиц. Поскольку эти данные были получены в лабораторных условиях, то, безусловно, требуется подтверждение такого сценария адаптации *G. pallida* в условиях природных популяций.

Для понимания эволюционной истории *G. pallida* проведены обширные сборы популяций ЦКН на картофельных полях Перу (Picard et al., 2004). Сорок две перуанские популяции G. *pallida* были проанализированы с использованием восьми микросателлитных маркеров. Популяции разделились на южные и северные и образовали пять кластеров. Большее генетическое разнообразие выявлено на юге Перу. В связи с этим была выдвинута идея о том, что процессы горообразования в Андах вызвали множество адаптивных изменений у нематод и стимулировали процесс видообразования у представителей рода *Globodera* (Grenier et al., 2010). Эти исследования были продолжены на более широком материале перуанских популяций *G. pallida*. Были генотипированы 117 популяций с использованием данных секвенирования гена *cathepsin L*, уча-

ствующего в процессе питания нематоды и включающего 12 интронов, и нового набора из 13 микросателлитных локусов (Thevenoux et al., 2019). В результате было выявлено гораздо большее генетическое разнообразие популяций, которые были структурированы в шесть групп: 1a, 1b, 2, 3, 4 и "pallida Chilean type". Оказалось, что генетические расстояния между популяциями *G. pallida* объясняются не только географическим фактором, но и климатическими условиями, а также типом почвы. Показано, что длина продукта амплификации гена *cathepsin L* служит диагностическим видоспецифичным маркером для видов *G. rostochiensis*, *G. pallida* и *G. ellingtonae.* Изоляты групп 1, 2 и 3 имеют один и тот же аллель гена *cathepsin L*, что свидетельствует об их генетической близости и позволяет считать их популяциями одного вида. Популяции группы 4 и группы pallida Chilean type могут считаться криптическими видами внутри видового комплекса *G. pallida*. О генетической отдаленности группы 4 от остальных говорит факт обнаружения у изолятов этой группы уникальной аллели гена *cathepsin L*. О дивергенции группы pallida Chilean type свидетельствуют высокие значения Fst при сравнении с *G. pallida*, которые были выше 0.5, что сравнимо для Fst между перуанскими *G. rostochiensis* и *G. pallida* (=0.58) и *G. pallida* и *G. mexicana* (=0.48) (Thevenoux et al., 2019).

Генетика устойчивости картофеля к цистообразующим нематодам и перспективы селекции

***G. rostochiensis* (ЗКН).** Источниками устойчивости картофеля к различным патотипам ЗКН и БКН служат многие южноамериканские и мексиканские виды картофеля (Castelli et al., 2003; Nunziata et al., 2010; Dalamu et al., 2012). У ряда устойчивых образцов культурных и близкородственных диких видов картофеля было идентифицировано около 25 генетических факторов, детерминирующих устойчивость к ЦКН: *R*-гены, обеспечивающие реакцию сверхчувствительности (*H1*, *Gpa2*) или конститутивно экспрессирующиеся во всех тканях растения (*Gro1-4*), и локусы количественных признаков (QTL), вовлеченные в контроль длительной олиго- и полигенной устойчивости либо в контроль частичной устойчивости к одному или нескольким патотипам одного из видов нематод (табл. 2). Идентифицированы также два QTL, вовлеченные в контроль частичной групповой устойчивости к разным видам ЦКН, оба картированы на хромосоме V в одном и том же кластере: *Grp1*_QTL, определяющий устойчивость картофеля к патотипу Ro5 ЗКН и к патотипам Pa2/3 БКН (Finkers-Tomczak et al., 2009, 2011), и *Ro2_A* QTL устойчивости к патотипу Ro2 ЗКН и к патотипам Pa2/3 БКН (Park et al., 2019).

Гены, контролирующие устойчивость к наиболее распространенным патотипам ЦКН, были клонированы и секвенированы: ген *Gpa2* устойчивости к патотипам Pa2 и Pa3 БКН (Rouppe van der Voort et al., 1997; Van der Vossen et al., 2000), *Gro1-4*, определяющий специфическую устойчивость растений к патотипу Ro1 ЗКН, входящий в сложный кластер генов семейства *Gro1* (Paal et al., 2004), и ген *H1* устойчивости к патотипам Ro1/Ro4 ЗКН (Finkers-Tomczak et al., 2011). Выявлена сложная структура этих локусов, содержащих как полноразмерные, так и дефектные копии гомологов *R*-генов, – RGH (resistance gene homologues). Продукты этих генов относятся к разным классам рецепторных белков, взаимодействующих с эффекторами патогена. Так, ген *Gpa2* кодирует белок семейства LZ-NBS-LRR (Van der Vossen et al., 2000), *H1* – белок семейства CC-NB-LRR (Finkers-Tomczak et al., 2011), а *Gro1-4* – структурно отличный белок, относящийся к семейству TIR-NB-LRR с дополнительным TIR-доменом, гомологичным Toll-подобному рецептору, активирующему IL-1 (Paal et al., 2004).

Для детекции у растений функциональных аллелей *R*-генов устойчивости к ЦКН, а также локусов количественных признаков, контролирующих частичную устойчивость к глободерозам, разработаны многочисленные ДНК-маркеры, которые широко применяются в маркер-опосредованной селекции (MAS) (Dalamu et al., 2012; Хютти и др., 2017), а также мультиплексные системы (Asano et al., 2012), позволяющие существенно повысить эффективность селекционно-генетических программ по созданию устойчивых к ЦКН сортов картофеля.

Как упоминалось выше, наиболее часто нематодоустойчивые сорта защищены доминантным аллелем гена *H1*, сохраняющим уже более 50 лет свою эффективность против наиболее распространенного патотипа ЗКН Ro1 (Ellenby, 1954; Gebhardt et al., 1993). Сорта с генами *Gro1-4*, или *GroV1*, или с QTL-локусами серии *Gro1* также проявляют устойчивость к патотипу Ro1 *G. rostochiensis* (см. табл. 2). В то же время в сортиментах сортов и селекционных клонов разных стран частота встречаемости образцов с этими генами различна. Так, по данным польских коллег, 77 % из 61 протестированных зарубежных сортов обладали маркерами гена *H1* и 28 % – маркером гена *Gro1-4*, причем результаты молекулярного скрининга коррелировали с данными фитопатологических тестов (Milczarek et al., 2011). Из 812 сортов и селекционных клонов японского селекцентра NARO (Хоккайдо) у 33 % образцов были выявлены маркеры гена *H1*, в то же время ни один из протестированных образцов не обладал маркером гена *Gro1-4* (Asano et al., 2012). Аналогично в выборке из 58 селекционных клонов индийской селекции около 60 % генотипов имели маркеры гена *H1*, а генотипов с маркером гена *Gro1-4* не обнаружено (Sudha et al., 2016, 2019). При этом около 20 % из 217 протестированных образцов коллекции UACh Университета Южного Чили оказались MAS-позитивными в скрининге с маркером гена *Gro1-4* (López et al., 2015). Согласно суммарным результатам молекулярного скрининга 225 российских сортов картофеля и сортов ближнего зарубежья (114 из которых входят в Государственный реестр селекционных достижений, допущенных к использованию в РФ), от 2 до 5 маркеров гена *H1* были выявлены у 28 % изученных сортов, а маркеры гена *Gro1-4* – лишь у 2 % (Антонова и др., 2016; Клименко и др., 2017; Гавриленко и др., 2018). Очевидно, что в селекционных программах разных стран использованы разные доноры *R*-генов устойчивости к патотипу Ro1 *G. rostochiensis.* В цитированных выше работах описано различное число маркеров, но в большинстве случаев в наборы входили и наиболее информативные из них: Gro1-4-1 гена *Gro1-4*, а также маркеры TG689 и 57R гена *H1*.

Table 2. Resistance (*R*-) genes/QTLs identified in wild and cultivated *Solanum* species which confer resistance to different pathotypes of potato cyst nematodes and root-knot nematodes

Species of pathogenic nematodes	Pathotypes	Wild and cultivated potato species – source of nematode resistance	*R*-genes/QTLs (chromosomal localization)	References
		Potato Cyst Nematodes		
Golden cyst nematode *Globodera rostochiensis*	Ro1	*S. spegazzinii*	***Gro1-4*** (VII)	Paal et al., 2004; Gebhardt et al., 2006
	Ro1	S. *spegazzinii*	QTL $Gro_{1.2}$ (X) QTL $Gro_{1.3}$ (XI) QTL $Gro_{1.4}$ (III)	Kreike et al., 1993, 1996
		S. vernei	*GroV1* (V)	Jacobs et al., 1996
	Ro2	*S. canasense*, *S. tuberosum* ssp. *andigena*	–	Nunziata et al., 2010
	Ro2	Breeding clones with *S. tuberosum* ssp. *andigena* and/or *S. vernei* in their pedigree	*Ro2_A* QTL (V) *Ro2_B* QTL (V)	Park et al., 2019
	Ro5	Interspecific hybrids *S. tuberosum* with *S. vernei*, *S. oplocense*, ssp. *andigena*	*Grp1*_QTL (V)	Rouppe van der Voort et al., 1998; Finkers-Tomczak et al., 2009
	Ro1, 5	*S. spegazzinii*	*Gro1* (VII)	Barone et al., 1990; Paal et al., 2004
	Ro1, Ro4	*S. tuberosum* ssp. *andigena*	***H1*** (V)	Ellenby, 1952; Ross, 1979; Gebhardt et al., 1993; Bakker et al., 2004; Finkers-Tomczak et al., 2011
White cyst nematode *Globodera pallida*	Pa1	*S. multidissectum*	*H2* (V)	Dunnett, 1960; Strachan et al., 2019
	Pa3	*S. tarijense*	*Gpa3*_QTL (XI)	Wolters et al., 1998
	Pa3	*S. tarijense*	$GpaIV^{I}_{tar}$_QTL (XI)	Tan et al., 2009
	Pa2/Pa3	*S. tuberosum* ssp. *andigena*	*H3* (IV)	Howard et al., 1970; Bradshaw et al., 1998
		S. tuberosum ssp. *andigena*	***Gpa2*** (XII)	Rouppe Van der Voort et al., 1997; Van der Vossen et al., 2000
		S. tuberosum ssp. *andigena*	$GpaIV^{S}_{adg}$ (IV)	Moloney et al., 2010
		S. sparsipilum	$GpaV^{s}_{spl}$_QTL(V)	Caromel et al., 2005
		S. sparsipilum	$GpaXI^{s}_{spl}$_QTL	Caromel et al., 2005
		S. *spegazzinii*	*Gpa*_QTL	Kreike et al., 1994
		S. tuberosum ssp. *tuberosum*	*Gpa* (IV)	Bradshaw et al., 1998
		S. sparsipilum	*Gpa4*_QTL (V)	Wolters et al., 1998
		Interspecific hybrids *S. tuberosum* with *S. vernei*, *S. oplocense*, ssp. *andigena*	*Gpa5*_QTL (V), *Gpa6*_QTL (IX)	Rouppe van der Voort et al., 2000; Bryan et al., 2002
		S. *spegazzinii*	*GpaM1*_QTL (V) *GpaM2*_QTL (VI) *GpaM3*_QTL (XII)	Caromel et al., 2003
		S. vernei	$GpaV_{vrn}$_QTL (V)	Sattarzadeh et al., 2006
		Breeding clones with *S. tuberosum* ssp. *andigena* and/or *S. vernei*	*Ro2_A* QTL (V)	Park et al., 2019
		Interspecific hybrids *S. tuberosum* with *S. vernei*, *S. oplocense*, ssp. *andigena*	*Grp1*_QTL (V)	Rouppe van der Voort et al., 1998; Finkers-Tomczak et al., 2009

Table 2 (end)

Species of pathogenic nematodes	Pathotypes	Wild and cultivated potato species – source of nematode resistance	*R*-genes/QTLs (chromosomal localization)	References
		Root-knot nematodes		
Columbia root-knot nematode *Meloidogyne chitwoodi*	Races 1, 2	*S. bulbocastanum*	$R_{Mc1(blb)}$ (XI) [*RMc1*]	Brown et al., 1996
		S. fendleri	$R_{Mc1(fen)}$ (XI)	Draaistra, 2006
		S. hougasii	$R_{Mc1(hou)}$ (XI)	Brown et al., 1999, 2003
Northern root-knot nematode *Meloidogyne hapla*		*S. tarijense*	$R_{Mh\text{-}tar}$_QTL (VII)	Draaistra, 2006
		S. chacoense	$R_{Mh\text{-}chc}A$_QTL, $R_{Mh\text{-}chc}B$_QTL	
Meloidogyne fallax		*S. chacoense*	$R_{Mf\text{-}chc}$	
		S. fendleri	$R_{Mc1(fen)}$ (XI)	
M. arenaria, *M. incognita*, *M. javanica*		Interspecific hybrids *S. tuberosum* with *S. sparsipilum*	*Mh*	Berthou et al., 2003

Note: Bold type indicates *R*-genes that were mapped, cloned and sequenced.

***G. pallida* (БКН).** Источники устойчивости картофеля к патотипам Ра2/Р3 БКН достаточно широко использованы в селекции. Так, маркеры гена *Gpa2* выявлены у 17.5 % образцов выборки из 812 сортов и селекционных клонов японского селекцентра NARO, (Asano et al., 2012); маркеры гена *Gpa2* и/или *Gpa5*_QTL детектированы у устойчивых к БКН зарубежных сортов и отсутствовали у восприимчивых (Milczarek et al., 2011). Из 66 клонов индийской селекции примерно у половины были детектированы маркеры локусов $GPaV_{vrn}$_QTL и *Gpa5*_QTL устойчивости к Ра2/3 БКН, причем почти все MAS-позитивные генотипы были фенотипически устойчивы к БКН (Sudha et al., 2019). Целенаправленный поиск источников устойчивости к *G. pallida* в нашей стране до последнего времени фактически не проводился, поскольку этот патоген в России не обнаружен. С использованием аллель-специфичного маркера гена *Gpa2* среди 193 отечественных сортов выявлено 24 (12.4 %) MAS-позитивных – потенциальных источников устойчивости к БКН (Гавриленко и др., 2018; Клименко и др., 2019). Особый интерес для последующих селекционных работ, направленных на пирамидирование генов устойчивости к разным вредным организмам, представляют сорта, сочетающие маркеры генов *Gpa2* и *H1* устойчивости к обоим видам цистообразующих нематод – *G. pallida* и *G. rostochiensis*.

Сорта и селекционные клоны картофеля, устойчивые к *G. rostochiensis*, несущие доминантный аллель гена *H1* устойчивости к патотипам Ro1/4, поражаются другими патотипами ЗКН (Brodie, 1995, 1996; Przetakiewicz, 2013), известно также, что растения с маркерами локуса *Gpa5* поражаются ЗКН (Sudha et al., 2019). В связи с этим в последнее десятилетие усилия исследователей разных стран направлены на поиск источников групповой устойчивости к глободерозам. С этой целью проведены фитопатологические тесты, по результатам которых среди образцов диких (*S. kurtzianum*, *S. sparsipilum*, *S. vernei*) и культурных видов (*S. stenotomum*) были отобраны источники устойчивости ко всем известным патотипам *G. rostochiensis* (Ro1–Ro5) и *G. pallida* (Pa1–Pa3) (Dalamu et al., 2012). Такие источники групповой устойчивости были также выделены в различных комбинациях межсортовых скрещиваний (Przetakiewicz et al., 2017). В селекционных программах США удалось отобрать пять селекционных клонов, одновременно устойчивых к трем видам цистообразующих картофельных нематод: *G. rostochiensis* (Ro1 и Ro4), *G. pallida* и *G. ellingtonae* (Whitworth et al., 2018). Интересно отметить, что большая часть генотипов, устойчивых и к *G. rostochiensis* (Ro1 и Ro4), и к *G. ellingtonae*, обладали маркерами локуса *H1*, что позволило авторам предположить наличие у этих клонов нового локуса, тесно сцепленного с *H1*, детерминирующего устойчивость картофеля к *G. ellingtonae* (Whitworth et al., 2018).

В европейской базе данных по сортам картофеля (The European Cultivated Potato Database, ECPD) приведены списки большого количества сортов с устойчивостью к различным патотипам цистообразующих картофельных нематод.

Галловые нематоды

Известно более 90 видов галловых нематод из рода *Meloidogyne* – широко специализированных патогенов, поражающих более 2000 видов растений, в том числе картофель (Hunt et al., 2009; Moens et al., 2009). Наибольший ущерб картофелеводству галловые нематоды наносят в районах тропического и субтропического климата (Lima et al., 2018). Благоприятными для развития большинства видов рода *Meloidogyne* являются условия повышенной температуры, наличие ирригации и легкая песчаная почва (Jones et al., 2017). В таких условиях потери от поражения галловыми нематодами, например такими, как *M. javanica* и *M incognita*, могут составлять 100 %. В регионах с умеренным климатом, таких как Европа и Северная Америка, распространенный и вредоносный на картофеле вид – колумбийская галловая корневая нематода – *M. chitwoodi*. Этот вид может поражать картофель при температуре ниже 6 °C (Jones et al., 2017).

Галловые нематоды – эндопаразиты. Инфекционными являются личинки второй стадии развития, которые, вылупившись из яиц, передвигаются в почве и находят корни растения, а далее с использованием стилета и набора энзимов, проникают в клетки корня и начинают питаться. В результате гипертрофии и гиперплазии соседних клеток образуются гигантские клетки, в которых происходят еще три линьки. Половозрелая самка откладывает в среднем 400–500 яиц между клетками кортикальной паренхимы или на поверхности корней. Первая линька личинок происходит внутри яйца, после чего появляется инфекционная личинка второго возраста. Взрослые самцы не паразитируют на растении, они выходят в почву и погибают (Lima et al., 2018). Пораженные растения картофеля – низкорослые, хлоротичные, с бурыми пятнами на листьях и гнилью клубней.

Для галловых нематод характерны различные типы размножения: от классического амфимиксиса до митотического партеногенеза. Для партеногенетических видов *Meloidogyne*, к которым относятся *M. chitwoodi*, *M. javanica* и *M incognita*, не подходит концепция биологического вида. Идентификация видов построена на определении морфологических признаков и поддержана биохимическими данными, основанными на изоферментном анализе (Dalmasso, Berge, 1978). Для применения этих методов требуются зрелые самки. Последние 30 лет разрабатывали методы молекулярной диагностики галловых нематод на основе ДНК полиморфизмов рибосомной и митохондриальной ДНК и анонимных локусов, таких как RFLP, RAPD и AFLP (Hyman, Powers, 1991; Powers, 2004). Источниками ДНК в этом случае могут быть яйца, личинки или взрослые особи. В настоящее время методы диагностики галловых нематод, как и других, основаны на ПЦР с видоспецифичными праймерами, например для *M. incognita* и *M. javanica* (Donkers-Venne et al., 2000; Dong et al., 2001), *M. chitwoodi* и *M. fallax* (Wishart et al., 2002).

Генетика устойчивости картофеля к галловым нематодам и перспективы селекции

На сегодняшний день устойчивые к галловым нематодам селекционные сорта картофеля еще не выведены, однако источники устойчивости к разным видам рода *Meloidogyne* были выделены среди диких видов, на их основе создаются перспективные селекционные клоны и исследуется генетика устойчивости картофеля к этим видам паразитических нематод. Так, в результате фитопатологического скрининга на устойчивость к галловым нематодам более 5000 индивидуальных растений 64 диких видов картофеля показано, что устойчивые к *M. chitwoodi* и *M. fallax* образцы встречаются в основном среди центрально- и североамериканских видов (Janssen et al., 1996), у которых был установлен моногенный контроль устойчивости (Brown et al., 1989, 1991). Многие диплоидные виды этих регионов принадлежат к третичному генпулу и из-за барьеров презиготической несовместимости не могут скрещиваться с *S. tuberosum*. Интрогрессия гена $R_{Mc1(blb)}$, обеспечивающего резистентность диплоидного мексиканского вида *S. bulbocastanum* к расам 1 и 2 колумбийской галловой корневой нематоды *M. chitwoodi*, была осуществлена с помощью межвидовой соматической гибридизации с *S. tuberosum*. В результате косегрегационного анализа популяций, полученных в возвратных скрещиваниях этих соматических гибридов, проведенного с использованием хромосомспецифичных RFLP-маркеров, ген $R_{Mc1(blb)}$ был картирован на хромосоме XI (Brown et al., 1996).

Ортологичные гены устойчивости к *M. chitwoodi* – $R_{Mc1(fen)}$ и $R_{Mc1(hou)}$ – идентифицированы у аллополиплоидных мексиканских видов *Solanum hougasii* и *S. fendleri* (Brown et al., 1999, 2003, 2009; Draaistra, 2006) (см. табл. 2). В серии работ C.R. Brown с коллегами (1996, 1999, 2003, 2009, 2014; Zhang et al., 2007) получены интрогрессивные формы картофеля поколений BC_5–BC_6 с генами $R_{Mc1(blb)}$, $R_{Mc1(fen)}$ и $R_{Mc1(hou)}$ диких мексиканских видов, а также разработаны сцепленные с ними STS- и CAPS-маркеры для проведения маркер-опосредованной селекции. Устойчивость интрогрессивных форм обусловлена генерацией активных форм кислорода и активацией защитных механизмов, в которых центральную роль играет салициловая кислота. Она запускает реакции сверхчувствительности и каскада физиологических и биохимических реакций, предотвращающих развитие *M. chitwoodi* в корнях растений (Bali et al., 2019). Важно отметить, что клоны с идентифицированным геном $R_{Mc1(fen)}$ устойчивости к *M. chitwoodi* одновременно обладали и устойчивостью к *M. fallax* (Draaistra, 2006).

Среди южноамериканских диких видов картофеля образцов, устойчивых к *M. chitwoodi* и к *M. fallax*, не обнаружено, за исключением единичных образцов у трех видов: *S. berthaultii*, *S. chacoense*, *S. gourlayi*. По результатам AFLP-анализа гибридной популяции *S. tuberosum* × *S. chacoense*, был идентифицирован QTL *RMf-chc* устойчивости к *M. fallax* (Draaistra, 2006).

Многочисленные источники устойчивости к северной галловой нематоде *M. hapla* были отобраны среди как северо-, так и южноамериканских видов (Janssen et al., 1996). Постзиготическая несовместимость, проявляющаяся в большинстве комбинаций межвидовых скрещиваний диких видов Южной Америки с *S. tuberosum*, относительно легко преодолевалась использованием дигаплоидов сортов картофеля или экспериментальных полиплоидов диких диплоидных видов для достижения значений «эффективной плоидности» (EBN), а также подбором совместимых комбинаций скрещиваний (Гавриленко, Ермишин, 2017).

Таким образом, устойчивые к *M. hapla* образцы диких видов Южной Америки относительно легко вовлекались в гибридизацию с *S. tuberosum*; расщепляющиеся гибридные популяции использовали для картирования локусов, обуславливающих частичную устойчивость к *M. hapla* (Draaistra, 2006). В этой работе на хромосоме VII был картирован $R_{Mh\text{-}tar_}$QTL дикого аргентинского вида *S. tarijense*, два других QTL, $R_{Mh\text{-}chc}A$ и $R_{Mh\text{-}chc}B$, так и не удалось картировать из-за нарушений колинеарности хромосом *S. chacoense* и *S. tuberosum*, однако были отобраны AFLP-маркеры, ассоциированные с локусами устойчивости к северной галловой нематоде (Draaistra, 2006) (см. табл. 2).

Важным практическим результатом работ по картированию *R*-генов и QTL устойчивости картофеля к галло-

вым нематодам считается разработка сцепленных с ними ДНК-маркеров (Rouppe van der Voort et al., 1999; Draaistra, 2006), которые применяются в маркер-опосредованной селекции. Следует отметить, что первые результаты исследований по пирамидированию $R_{Mh\text{-}chcA}$_QTL и $R_{Mh\text{-}tar}$_QTL оказались нерезультативными, поскольку среди клонов, отобранных в MAS, не выявлено устойчивых к *M. hapla* (Tan et al., 2009). Исследования, направленные на поиск эффективных комбинаций QTL-локусов и *R*-генов для создания высокоустойчивых к галловым нематодам генотипов, продолжаются.

В работе F. Berthou с коллегами (2003) изучен характер наследования устойчивости к различным видам галловых нематод гибридов ди- и тетраплоидных культурных видов картофеля с близкородственным южноамериканским видом *S. sparsipilum*. Результаты анализа девяти гибридных комбинаций (из F_1, F_2 и возвратных скрещиваний) позволили установить моногенную устойчивость к трем видам галловых нематод, *M. arenaria*, *M. incognita*, *M. javanica*, обусловленную доминантным аллелем гена *Mh* (см. табл. 2). Вместе с тем отобранные устойчивые генотипы поражались *M. mayaguensis*, *M. chitwoodi*, *M. fallax*, *M. hapla*. Важно отметить, что устойчивость гибридов, определяемая геном *Mh*, существенно снижается при повышении температуры от 24 до 29 °C (Berthou et al., 2003), что необходимо учитывать при проведении фитопатологических тестов в регионах с тропическим и субтропическим климатом, где широко распространены галловые нематоды.

Виды паразитических нематод картофеля, генетика устойчивости к которым не изучена

Стеблевая нематода картофеля *Ditylenchus destructor* (*Nematoda*, *Tylenchida*) – широко распространенные виды в регионах с умеренным климатом в Европе, в том числе в России, Северной Америке, Азии, Океании и Южной Африке (Mai et al., 1990; Шестеперов и др., 2006, 2010; Sigareva et al., 2012; OEPP/EPPO, 2017a).

Вредоносность стеблевой нематоды картофеля *D. destructor* состоит в повреждениях клубней, которые покрываются растрескивающимися темными пятнами, при этом симптомы поражения надземной части растения отсутствуют. Весь жизненный цикл нематод длится около шести дней и проходит в клубнях картофеля, в которые они проникают через кожуру около глазков, питаясь крахмальными зернами (Mai et al., 1990).

Стеблевая нематода *D. dipsaci* поражает стебли, столоны и клубни картофеля. На клубнях образуются серовато-бурые пятна, растение отстает в росте. Пораженные стебли изгибаются и становятся вздутыми. Источник инфекции – пораженные клубни (Lima et al., 2018). Многочисленные исследования генетической изменчивости популяций *D. dipsaci* показали, что этот вид фактически является комплексом видов (Subbotin et al., 2005). Для идентификации видов *Ditylenchus* были разработаны видоспецифичные универсальные праймеры к последовательностям генов и спейсерным участкам генов рибосомального кластера, дававшие в ПЦР специфический продукты амплификации: *D. destructor* – 1200 п. о., *D. dipsaci* и *D. myceliophagus* – 900 п. о. (Vrain et al., 1992). Для идентификации нормальной и гигантской рас *D. dipsaci* использовали ПЦР с применением SCAR-праймеров (Esquibet et al., 2003); праймеры на 5.8S рДНК были разработаны для молекулярной диагностики *D. destructor* (Marek et al., 2005). Протоколы по молекулярной диагностике подробно описаны EPPO – European and Mediterranean Plant Protection Organization (OEPP/EPPO, 2017a). Видоспецифичные праймеры на ITS-участки были использованы для идентификации *D. destructor* в Московской области (Mahmoudi et al., 2019). В литературе были сообщения об устойчивых и толерантных к *D. destructor* и *D. dipsaci* сортах картофеля (Mwaura et al., 2015). Генетика устойчивости картофеля к клубневой и стеблевой нематодам не изучена.

Ложная галловая нематода *Nacobbus aberrans* – эндемичный вид для Южно-Американского континента, тем не менее из-за широкого диапазона хозяев, включающего 84 вида растений, и экономического значения для таких культур, как картофель, томат и сахарная свекла, она отнесена к карантинным объектам. Для картофеля потери урожая составили в среднем 65 % в Андском регионе Латинской Америки. Таксономические проблемы рода *Nacobbus* рассмотрены в обзоре (Manzanilla-López, 2010). Современные молекулярные данные позволили присвоить *N. aberrans* статус видового комплекса. Для обнаружения *Nacobbus* spp. в почве и клубнях картофеля используют методы RFLP и секвенирование ITS-rDNA-областей (Reid et al., 2003), ПЦР с видоспецифичными праймерами (Atkins et al., 2005). В литературе сообщалось об источниках устойчивости к *Nacobbus aberrans*, отобранных среди образцов диких южноамериканских видов: *S. microdontum*, *S. acaule*, *S. okadae* (Franco, Main, 2006), генетика устойчивости картофеля к ложной галловой нематоде не изучена.

Заключение

В настоящее время защита картофеля от паразитических нематод сводится к двум основным составляющим: 1) карантинным мероприятиям, которые не всегда эффективны из-за иногда неконтролируемого перевоза посадочного материала и сельскохозяйственной техники между частными хозяйствами, и 2) использованию устойчивых к нематодозам сортов, в том числе с групповой устойчивостью к разным видам нематод. Вследствие этого поиск для селекции новых генов устойчивости, в том числе эффективных против потенциально опасных патотипов и видов паразитических нематод, создадут «подушку безопасности» в случае их заноса на территорию РФ или появления в результате адаптационных процессов. Информация о картированных *R*-генах и QTL устойчивости к нематодам и ассоциированных с ними ДНК-маркерах существенно повышает эффективность селекционно-генетических программ по созданию новых сортов, устойчивых к разным патотипам и видам паразитических нематод.

Перспективным представляется развитие новых молекулярных технологий. Последние 20 лет с момента обнаружения РНК интерференции (RNAi) (Fire et al., 1998), эту технологию пытаются использовать для защиты растений от различных видов нематод (Lilley et al., 2007; Bairwa et al., 2017). Показано, что экспрессия в растении-хозяине двуцепочечной РНК, нацеленной на гены домашнего хозяйства или паразитизма у галловой нематоды, приводит к

устойчивости против этой нематоды (Gheysen, Vanholme, 2007). Дальнейшее развитие молекулярных технологий, а также исследований биологии паразитических нематод картофеля, изменчивости популяций, эволюции, структурной и функциональной геномики как паразитов, так и их хозяев позволит совершенствовать способы контроля заболеваний картофеля, вызываемых паразитическими нематодами.

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Acknowledgements. The work was completed with the support of Russian Science Foundation (grant No. 16-16-04073).

Conflict of interest. The authors declare no conflict of interest.

Received July 13, 2020. Revised October 14, 2020. Accepted October 15, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):722-729
DOI 10.18699/VJ20.666

Pyrenophora tritici-repentis population structure in the Republic of Kazakhstan and identification of wheat germplasm resistant to tan spot

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Abstract. *Pyrenophora tritici-repentis* is a causative agent of tan spot in wheat. In recent years, there has been an increasing spread and harmfulness of wheat tan spot. The aim of the research was to study the racial composition of the *P. tritici-repentis* population in the Republic of Kazakhstan. A collection of 30 common wheat accessions, including promising lines and cultivars from Kazakhstan and CIMMYT–ICARDA, was assessed for resistance to *P. tritici-repentis* in a greenhouse and characterized using the *Xfcp623* molecular marker, diagnostic for the *Tsn1* gene. Monosporic isolates of *P. tritici-repentis* isolated from the southeastern region were assigned to certain races based on the manifestation of symptoms of necrosis/chlorosis on standard differentials (Glenlea, 6B662, 6B365). Five races of *P. tritici-repentis* have been identified, including races 1, 2, 3, 7 and 8. It has been shown that races 1 and 8 of *P. tritici-repentis* are dominant. As a result of the analysis of the frequency of occurrence of the *P. tritici-repentis* races, it was found that race 1 (50 %) producing Ptr ToxA and Ptr ToxB and race 8 (35 %) producing Ptr ToxA, Ptr ToxB and Ptr ToxC turned out to be dominant. From a practical point of view, of greatest interest are 16 wheat samples, which demonstrated resistance to race 1 and confirmed insensitivity to Ptr ToxA in a molecular screening. These include eight Kazakhstani (4_PSI, 10204_2_KSI, 10204_3_KSI, 10205_2_KSI, 10205_3_KSI, 605_SP2, 632_SP2, Dana) and seven foreign lines (KR11-20, KR11-03, KR11-9014, 11KR-13, KR11-9025, KR12-07, GN-68/2003). The results of this study are of interest in wheat breeding programs for tan spot resistance.
Key words: wheat; tan spot; races; molecular markers; *Tsn1*; ToxA.

For citation: Kokhmetova A.M., Kovalenko N.M., Kumarbaeva M.T. *Pyrenophora tritici-repentis* population structure in the Republic of Kazakhstan and identification of wheat germplasm resistant to tan spot. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):722-729. DOI 10.18699/VJ20.666

Структура популяции *Pyrenophora tritici-repentis* в Республике Казахстан и идентификация устойчивой к пиренофорозу гермоплазмы пшеницы

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Аннотация. Возбудитель пиренофороза *Pyrenophora tritici-repentis* – одна из наиболее вредоносных болезней листовых пятнистостей пшеницы. В последние годы отмечаются нарастающее распространение и вредоносность пиренофороза в Казахстане. Расовый состав *P. tritici-repentis* претерпевает изменения из-за климатических и средовых флуктуаций, а также из-за все более усиливающейся тенденции возделывания одних и тех же сортов пшеницы на больших территориях. В настоящее время имеется лишь ограниченная информация о расовой структуре популяции *P. tritici-repentis* в Казахстане. Целью исследований были изучение популяций *P. tritici-repentis* по расовому составу на юго-востоке Республики Казахстан, а также идентификация устойчивых к пиренофорозу образцов пшеницы. Коллекция из 30 образцов мягкой пшеницы, включающая перспективные линии и сорта из Казахстана и из международных центров CIMMYT и ICARDA, была подвергнута оценке устойчивости к возбудителю пиренофороза в теплице и охарактеризована с использованием молекулярного маркера *Xfcp623*, диагностического для гена *Tsn1*. Моноконидиальные изоляты *P. tritici-repentis*, выделенные из популяции патогена юго-восточного регио-

на, были отнесены к определенным расам на основе проявления симптомов некроза/хлороза с использованием стандартных образцов-дифференциаторов (Glenlea, 6B662, 6B365). Идентифицировано пять рас *P. tritici-repentis*, включающих расы 1, 2, 3, 7 и 8. Показано, что доминируют расы 1 и 8 *P. tritici-repentis*. В результате анализа частоты встречаемости рас возбудителя желтой пятнистости *P. tritici-repentis* установлено, что доминирующей оказалась раса 1 (50 %), продуцирующая Ptr ToxA и Ptr ToxB, и раса 8 (35 %), продуцирующая Ptr ToxA, Ptr ToxB и Ptr ToxC. С практической точки зрения наибольший интерес представляют 16 образцов пшеницы, которые демонстрировали устойчивость к расе 1 и подтвердили нечувствительность к токсину Ptr ToxA при молекулярном скрининге. К ним относятся восемь казахстанских линий: 4_PSI, 10204_2_KSI, 10204_3_KSI, 10205_2_KSI, 10205_3_KSI, 605_SP2, 632_SP2, Dana и семь зарубежных линий: KR11-20, KR11-03, KR11-9014, 11KR-13, KR11-9025, KR12-07, GN-68/2003. Результаты этого исследования представляют интерес для программы селекции пшеницы на устойчивость к пиренофорозу.
Ключевые слова: пшеница; пиренофороз; расы; молекулярные маркеры; *Tsn1*; ToxA.

Introduction

One of the main reasons for the reduction in yield of wheat in Kazakhstan are the diseases with airborne infection. Dominant position as a part of the pathogenic complex of wheat in the south and south-east of Kazakhstan took rusts (yellow, stem and leaf rust) (Kokhmetova et al., 2011, 2016b, 2018b; Rsaliyev A.S., Rsaliyev Sh.S., 2018), as well as leaf spot diseases (tan spot and Septoria) (Kokhmetova et al., 2017, 2018a, 2019).

The causative agent of wheat tan spot is the fungus *Pyrenophora tritici-repentis*, which belongs to the class Ascomycetes, the subclass of marsupials, the order Dothidiales, the family Pleorosporaceae. In addition to wheat, *P. tritici-repentis* infects more than 60 species of forage and wild-growing grasses (Koishybaev, 2010; Mironenko, Kovalenko, 2018). The infection is manifested on leaves and leaf sheaths of cereals in the form of small single or multiple spots oval or round shape, yellow or light-brown color, a chlorotic zone is formed around the spot. The source of the primary infection is the ascospores of the fungus, the secondary infection is caused by conidia, which are carried by the wind (Pospekhov, 1989).

The harmfulness of the disease leads to a decrease in the assimilation surface, an increase in transpiration, a decrease in the accumulation of organic matter, the defeat of all aboveground plant organs, a loss of grain quality due to the formation of unfulfilled grain. In conditions favorable for the development of the disease, losses of more than 50 % were noted (Shabeer, Bockus, 1988). *P. tritici-repentis* (PTR) (Died.), the causative agent of tan spot, induces two different symptoms on susceptible varieties – necrosis and chlorosis. Both symptoms are genetically under independent host control. At present, eight PTR races have been identified in the world based on the ability to induce symptoms of necrosis and chlorosis on a set of wheat differentiatial cultivars. Integrated disease control strategies, such as cultivation of resistant cultivars, combined with desired crop rotations and management practices, are the most effective, environmentally friendly and economical means to control wheat tan spot (Singh et al., 2010).

P. tritici-repentis is found in all major wheat-growing regions. The tan spot pathogen is registered in Australia, Canada, the United States of America, South America, Romania, Moldova, England, Kazakhstan, Ukraine, Belarus, Central Asia (Mikhailova et al., 2012). The first information about the distribution of *P. tritici-repentis* in Central Asia was presented by B.A. Khasanov in the early 1980s (Postnikova, Khasanov, 1997). Monitoring of wheat fields in Central Asia and Kazakhstan in 2003 showed that tan spot is most common on winter wheat, while the severity could reach from 50 to 100 % (Koishybaev, 2002; Lamari et al., 2005).

The compatibility reaction between the *P. tritici-repentis* race and the corresponding differential is realized through an intermediary – the host-specific toxin (Host Selective Toxins, HST). To date, four HSTs have been characterized: one toxin inducing necrosis, Ptr ToxA, two toxins inducing chlorosis, Ptr ToxB and Ptr ToxC, and one toxin inducing both necrosis and chlorosis, Ptr ToxD (Balance et al., 1989; Orolaza et al., 1995; Ali et al., 2010).

The population structure investigation of *P. tritici-repentis* in Kazakhstan have received attention since the beginning of the 2000s, and it continued in recent years (Zhanarbekova et al., 2005; Maraite et al., 2006; Kokhmetova et al., 2016b, 2017). The greatest diversity of racial composition in the pathogen population was noted in Azerbaijan, where races 1, 2, 3, 5, 7 and 8 were identified, and in Syria, where races 1, 3, 5, 7 and 8 were observed (Lamari et al., 2005). Race 1 was the most common in Central Asia and Kazakhstan (87 %), while races 2, 3, and 4 were less common (Zhanarbekova et al., 2005; Maraite et al., 2006). Earlier, we carried out a comparative study of the similarities and differences of *P. tritici-repentis* populations in terms of virulence and racial composition in the Republic of Kazakhstan and the North Caucasus region of Russia. It was shown that in recent years race 8 found in high frequency in Kazakhstan (Kokhmetova et al., 2016a, 2017).

The inheritance of resistance to tan spot is both quantitative and qualitative, and genes for resistance to toxins and quantitative trait loci (QTLs) are race-specific and control the process that reduces sensitivity to toxins (Mikhailova et al., 2012). Six major genes for resistance to tan spot *Tsr1–Tsr6,* localized on chromosomes 2BS, 3AS, 3BL, 3DS, and 5BL, have been identified (McIntosh et al., 2013). In the review by P.K. Singh et al. (2016) indicate that numerous genetic studies with the analysis of QTLs have demonstrated that resistance to tan spot is inherited as a polygenic trait, while the main race-specific genes, *Tsr1* to *Tsr6*, often explain the effects of these loci (Singh S. et al., 2008; Singh P.K. et al., 2016). Additional QTLs have been identified and localized on chromosomes 1AL, 2AS, 3AS (Singh S. et al., 2008), 4AL, 5AL, 1BS, 2BL, 3BS, 3BL, 5BL, 2DS, 2DL and 7DS (Singh P. et al., 2016).

To increase the efficiency of breeding for resistance to tan spot, it is necessary to identify promising wheat lines characterized by a diversity of disease resistance genes, and then place them in the territory of the disease spread. Since under the influence of abiotic and biotic factors in nature there are permanent changes in the racial composition of pathogens, it is necessary to regularly analyze the structure of pathogen populations. This makes it possible to assess the dynamics of the variability of the racial composition in the population and to identify isolates with a new spectrum of virulence.

The aim of our research was to study the racial composition of the *P. tritici-repentis* population from the southeastern region of the Republic of Kazakhstan, as well as to search for sources of resistance to tan spot in the collection of wheat samples.

Materials and methods

To determine the distribution area and harmfulness of *Pyrenophora tritici-repentis*, infected wheat leaf samples were randomly collected from winter bread wheat in the southeastern regions in 2018 in the Almaty region of the Republic of Kazakhstan. The analysis of the phytosanitary state of wheat crops was carried out during the period of heading and grain milk stages (June).

The object of the study was a collection of 30 entries of common wheat *Triticum aestivum*, including 17 promising breeding lines and cultivars from Kazakhstan and 13 entries from CIMMYT–ICARDA (see Table 2). The study of the wheat collection is aimed at finding the sources of resistance to PTR based on the assessment of seedling resistance to the dominant races of the fungus, the study of field (adult plant) resistance and molecular screening to *P. tritici-repentis* toxins. Salamouni (Lebanon) cultivar was used as a insensitive control for race 1 of tan spot and Ptr ToxA, Glenlea (Canada) – as a susceptible control for race 1 and Ptr ToxA.

Evaluation of field resistance to tan spot was carried out under conditions of the Kazakh Research Institute of Agriculture and Crop Production (KazNIIZiR), (Almalybak, 43°13′09″ N, 76°36′17″ E, Almaty region) in the 2019–2020 crop season. Experiments were conducted as a completely randomized design with two replicates in 1 m^2. The severity of plants was assessed under conditions of an artificial infectious background on flag leaves in GS 65-69, Zadoks scale (Zadoks et al., 1974). The infectious background was created using infected with tan spot straw stubbles (1 kg/m^2). The level of resistance was assessed according to the scale of 1–100 % for appraising the intensity of disease (Saari, Prescott, 1975). The standard wheat differentials of disease Glenlea (sensitive control) and Salamouni (resistant control) were used as controls.

The differentiation of races was carried out in accordance with the classification proposed by L. Lamari and C.C. Bernier (1998), using a Canadian set of disease differentials (wheat cultivar Glenlea and lines 6B662 and 6B365). The Ptr ToxA toxin induces the formation of necrosis symptoms the Glenlea wheat cultivar, and the Ptr ToxB and Ptr ToxC toxins induce the chlorosis symptoms on the 6B365 and 6B662 lines.

Monoconidial isolates of the fungus were isolated from wheat infectious material collected in the farm and breeding fields of the southeastern region of the Republic of Kazakhstan using the method of L.A. Mikhailova with colleagues (2012). To study the racial composition of the Kazakhstani population of *P. tritici-repentis*, 20 monoconidial isolates were used. The study of the structure of the population by racial composition and virulence was carried out using the method of leaf sections placed in a 0.004 % solution of benzimidazole (Mikhailova et al., 2012). Seedling resistance was also assessed using the benzimidazole method. The degree of development of the disease was assessed on the 7–8th day. The plants were rated for disease based on lesion type; cultivars with a necrotic reaction 1–2 were attributed to resistant (R), and with reaction 3–5 – to susceptible (S) entries (Lamari, Bernier, 1989). The presence or absence of chlorosis was assessed on lines 6B365 and 6B662.

Genomic DNA was extracted from 5-day-old wheat seedlings from plant material using the CTAB method (Riede, Anderson, 1996). To identify the carriers of resistance genes, the method of polymerase chain reaction (PCR) was used with primers flanking diagnostic gene markers and DNA samples from a collection of 30 common wheat samples (*T. aestivum* L.). The cultivars carrying the *Tsn1* gene, which is sensitive to the Ptr ToxA toxin, were identified on the basis of PCR using the SSR marker *Xfcp623* (Zhang et al., 2009; Faris et al., 2010). The marker has two alleles: 380 bp (associated with sensitivity, the dominant allele of the *Tsn1* gene) and the null allele (associated with insensitivity, the recessive allele of the *tsn1* gene) (Zhang et al., 2009). The composition of the reaction mixture and the PCR conditions followed the protocol (Roder et al., 1998). To separate the amplified DNA fragments, electrophoresis was performed in a 2 % agarose gel in TBE buffer (45 mM Tris borate, 1 mM EDTA, pH 8) (Chen et al., 1998). The gels were visualized using a Mega Bio-Print 1100/26M gel documenting system, Vilber Lourmat.

Results

To study the racial composition of the Kazakhstani *P. tritici-repentis* population, 20 monoconidial isolates were analyzed. Using differential lines and cultivars from Canada, 20 isolates were characterized as belonging to certain races of *P. tritici-repentis*, presented in Table 1. In accordance with the generally accepted classification of races (Lamari et al., 1998), the isolates were assigned to race 1 as inducing toxins Ptr ToxA and Ptr ToxC, to race 2 (Ptr ToxA), race 3 (Ptr ToxC), race 4 (non-inducing toxins), race 5 (Ptr ToxB), race 6 (Ptr ToxB and Ptr ToxC), race 7 (Ptr ToxA and Ptr ToxB) and race 8 (Ptr ToxA, Ptr ToxB and Ptr ToxC).

As a result of the analysis of the frequency of occurrence of the races of the pathogen *P. tritici-repentis*, it was found that in isolates from the southeast of Kazakhstan the dominant race was 1 producing Ptr ToxA and Ptr ToxB (50 %), and race 8 producing Ptr ToxA, Ptr ToxB and Ptr ToxC (35 %). Races 4, 5, and 6 were not found in the studied samples of the *P. tritici-repentis* population. Thus, in the southeastern region of Kazakhstan, five races of *P. tritici-repentis* have been identified: 1, 2, 3, 7, and 8.

Inoculation and evaluation of 30 promising lines and varieties of winter soft wheat in laboratory conditions were carried

Table 1. Determination of the races of *P. tritici-repentis* isolates from the south-east of Kazakhstan on the Canadian set of differential cultivars

Isolate catalog number	Reaction of differential cultivars to the *P. tritici-repentis* inoculation			Race number
	Glenlea	6B662	6B365	
1-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
2-Yu-KZ	N (ToxA)	Cl (ToxB)	Cl (ToxC)	8
3-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
4-Yu-KZ	ToxA	ToxB	ToxC	8
5-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
6-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
7-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
8-Yu-KZ	N (ToxA)	Cl (ToxB)	R	7
9-Yu-KZ	N (ToxA)	Cl (ToxB)	Cl (ToxC)	8
10-Yu-KZ	N (ToxA)	Cl (ToxB)	Cl (ToxC)	8
11-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
12-Yu-KZ	N (ToxA)	Cl (ToxB)	Cl (ToxC)	8
13-Yu-KZ	N (ToxA)	Cl (ToxB)	Cl (ToxC)	8
14-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
15-Yu-KZ	R	R	Cl (ToxC)	3
16-Yu-KZ	N (ToxA)	Cl (ToxB)	Cl (ToxC)	8
17-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
18-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
19-Yu-KZ	N (ToxA)	R	Cl (ToxC)	1
20-Yu-KZ	N (ToxA)	R	R	2

Note. Manifestation of symptoms: N – necrosis, Cl – chlorosis; R – resistant response to *P. tritici-repentis* infection.

out (Table 2). Since most of the *P. tritici-repentis* infectious material collected in southeastern Kazakhstan was attributed to isolates of race 1 producing the ToxA toxin (see Table 1), we used an isolate of race 1 to screen wheat material. The analysis of wheat samples was carried out for an isolate of the Kazakhstani population of the fungus from the southern region of the Republic of Kazakhstan (1-Yu-KZ), producing the Ptr ToxA toxin (see Table 2).

The reactions of wheat genotypes to the Ptr 1-Yu-KZ isolate presented in Table 2 indicate that five promising lines are characterized by the highest resistance, which is 16.6 %. This group includes three Kazakhstani (GF_13_CP, GF_16_CP, 10204_3_KSI) and two foreign wheat lines from ICARDA–CIMMYT (KR11-9025 and GN-158/2004). Sixteen samples (53.3 %) showed moderate-resistant (MR) response. Moderately susceptible type of reaction (MS) was identified in three entries. The susceptible type of reaction (S) was observed in six entries, which is 20 % of the total amount of the studied material.

The results of assessing the field resistance to tan spot showed that the development of the disease was observed mainly in the lower and middle tiers of plants, the maximum severity was 40 %. A resistant type of reaction to the disease (10–15 %) was observed in 14 lines, which amounted to 46.6 % of the total amount of the studied material. Four wheat lines were characterized by high field resistance to the disease: GF24_CP, 10204_2_KSI, KR11-20 and 11KR-13.

The search for genotypes-carriers of alleles of the genes of sensitivity, *Tsn1*, and insensitivity, *tsn1*, to the Ptr ToxA *P. tritici-repentis* toxin in promising wheat lines was carried out as a result of molecular analysis of a collection of wheat entries and comparison of these results with screening based on reactions to isolate 1-Yu-KZ, producing Ptr ToxA toxin.

Genotyping of wheat entries using a molecular marker was aimed at identifying carriers of genes that control sensitivity and resistance to the Ptr ToxA toxin. The *Xfcp623* marker amplified a 380 bp fragment associated with the *Tsn1* gene sensitive to the Ptr ToxA toxin in 13 entries (43.3 %) (see Table 2, the Figure). The null allele of the *Xfcp623* marker was found to be linked to toxin insensitivity in 17 wheat entries. Most of the entries (76.4 %) were insensitive to the isolate of race 1 (1-Yu-KZ) producing the Ptr ToxA toxin (see Table 2). The linkage rate of the *Xfcp623* marker to race 1 insensitivity was 76.4 %. An example of an electrophoretogram with the results of PCR, reflecting the presence/absence of the *Tsn1* locus sensitive to Ptr ToxA in the samples under study, is shown in the Figure.

The frequency of the *Xfcp623* marker allele linked to the *tsn1* gene, which controls the insensitivity to the Ptr ToxA toxin, was 56.6 % (17 entries). The frequency of the marker linked to the dominant allele of the *Tsn1* gene linked to the sensitivity to the Ptr ToxA toxin was 43.3 % (13 entries).

The SSR marker *Xfcp623* amplified a 380 bp DNA fragment associated with the dominant ToxA-sensitive *Tsn1* allele in 13 entries and in the control Glenlea. Another allele, found using the *Xfcp623* marker in other wheat entries (17), was a null allele characteristic of ToxA-insensitive genotypes and indicating the recessive state of the *tsn1* allele.

From a practical point of view, of greatest interest are 16 wheat samples, which demonstrated resistance to race 1 and confirmed insensitivity to Ptr ToxA in a molecular screening. These include eight Kazakhstani (4_PSI, 10204_2_KSI, 10204_3_KSI, 10205_2_KSI, 10205_3_KSI, 605_SP2, 632_SP2, Dana) and seven foreign lines (KR11-20, KR11-03, KR11-9014, 11KR-13, KR11-9025, KR12-07, GN-68/2003).

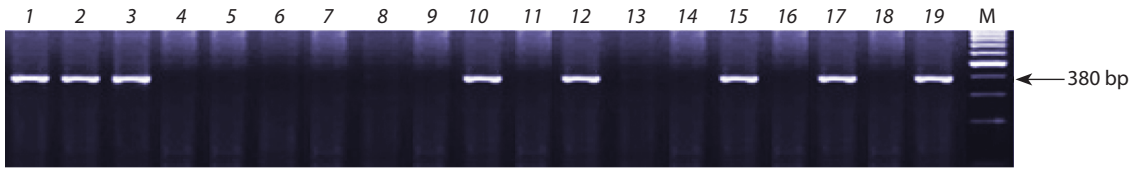
Discussion

The studies presented in this article made it possible to determine the racial composition of *P. tritici-repentis* isolates established in 2018 in the southeast of Kazakhstan. The study of the racial composition of the Kazakhstani southeastern population of *P. tritici-repentis* confirmed the previously obtained results (Kokhmetova et al., 2016a) and revealed the dominance of races 1 and 8 in this territory. An analysis of the frequency of occurrence of the fungus races showed that in the collection

Table 2. Screening of promising lines of winter bread wheat from Kazakhstan and ICARDA–CIMMYT for seedling and field resistance to *P. tritici-repentis*

Name	Origin	Response to isolate Ptr 1-Yu-KZ*	Field resistance to Ptr, %**	*Xfcp623*, *Tsn1*	
GF_13_CP	Kazakhstan	1/0	25	S	380
GF_16_CP		1/1	25	S	380
GF_19_CP		3/3	30	S	380
GF24_CP		3/3	5	R	null
4_PSI		2/2	10	R	null
10204_2_KSI		2/1	5	R	null
10204_3_KSI		1/1	10	R	null
10205_2_KSI		1/2	10	R	null
10205_3_KSI		3/2	10	R	null
602_SP2		2/2	25	S	380
605_SP2		2/2	10	R	null
618_SP2		2/3	30	S	380
632_SP2		2/1	10	R	null
634_SP2		3/2	10	R	null
635_SP2		2/2	30	S	380
Dana		2/2	10	R	null
Dinara		2/1	25	S	380
KR11-20	ICARDA–CIMMYT	2/1	5	R	null
KR11-03		1/2	10	R	null
KR11-9014		2/3	10	R	null
KR11-26		2/2	25	S	380
KR11-29		3/3	10	R	null
11KR-13		2/2	5	R	null
KR11-40		1/2	40	S	380
KR11-9025		1/0	15	R	null
KR12-07		1/2	30	R	null
KR12-9011		2/1	25	S	380
GN-68/2003		2/3	10	R	null
GN-143/2006		2/1	10	S	380
GN-158/2004		1/0	30	S	380
Salamouni – resistant control for race 1 and Ptr ToxA toxin	Lebanon	1/0	5	R	null
Glenlea – susceptible control for race 1 and Ptr ToxA toxin	Canada	3/3	30	S	380

* Isolate 1-Yu-KZ, producing Ptr ToxA toxin; above the line – the score for necrosis; below the line – the score for chlorosis; ** the average percent disease severity for two years are presented (2019–2020); *Xfcp623* – SSR marker of the *Tsn1* locus sensitive to Ptr ToxA amplifies a 380 bp DNA fragment.



DNA amplification products of wheat entries using the diagnostic marker *Xfcp623*, linked to *Tsn1* gene determining susceptibility to Ptr ToxA.

1 – GF_13_CP; *2* – GF_16_CP; *3* – GF_19_CP; *4* – GF24_CP; *5* – 4_PSI; *6* – 10204_2_KSI; *7* – 10204_3_KSI; *8* – 10205_2_KSI; *9* – 10205_3_KSI; *10* – 602_SP2; *11* – 605_SP2; *12* – 618_SP2; *13* – 632_SP2; *14* – 634_SP2; *15* – 635_SP2; *16* – Dana; *17* – Dinara; *18* – Saloumoni – resistant check to Ptr ToxA; *19* – Glenlea – susceptible check to PTR ToxA. M – 100 bp DNA Ladder (Gene-RulerTM, Fermentas). 2 % agarose gel.

of isolates from Kazakhstan in 2018 the races 1 and 8 were dominant. In northern Kazakhstan in the early 2000s, races 1 (Zhanarbekova et al., 2005) and races 2, 3, and 4 (Maraite et al., 2006) were widespread. The indicated differences in the racial composition in Kazakhstan may be due to changes in climatic conditions in different years. The presented differences in the composition of the pathogen indicate the need for an annual analysis of the structure of pathogen populations in order to understand the dynamics of its variability and the distribution areas of *P. tritici-repentis*.

The study of the structure of tan spot population in three different climatic zones of Russia made it possible to determine the racial composition of the *P. tritici-repentis* populations. It was established that races 1 and 2 were dominant. Race 8 was found in all regions. The population from Dagestan lacked races 5, 6, 7, while the population from Western Siberia lacked race 4 (Kremneva et al., 2007; Mikhailova et al., 2010, 2014).

A comparative analysis of Kazakh and Russian samples of pathogen populations, carried out in 2016, showed that isolates from Kazakhstan are the most virulent, but the isolates from the North Caucasus region of Russia are the most phenotypically diverse (Kokhmetova et al., 2016a). The authors revealed a diversity in the virulence of isolates: in Russia, 4 races of *P. tritici-repentis* (1, 2, 4, and 8), and in Kazakhstan – five races (1, 3, 4, 6, and 8) were identified. Study of the population structure of *P. tritici-repentis* in the West Asian regions of Russia and North Kazakhstan showed that a high degree of similarity in the structure of fungi populations from these regions was noted on the basis of toxin formation, which indicates a single epidemiological zone (Gultyaeva et al., 2018).

Studies aimed at assessing the resistance of wheat germplasm to tan spot have received great attention (Chu et al., 2008; Singh P. et al., 2016; Kokhmetova et al., 2017, 2018a, 2019). The present work is due to the need to create genetically heterogeneous sources of resistance, which can be used in breeding wheat varieties resistant to *P. tritici-repentis*. This problem was solved on the basis of the use of DNA technologies and the use of isolates of race 1, which produce the most common toxin in Kazakhstan, Ptr ToxA.

Molecular markers for the diagnosis of insensitivity to Ptr ToxA and Sn ToxA (*Xfcp393* and *Xfcp394*) have previously been developed (Zhang et al., 2009). After the *Tsn1* gene was cloned and sequenced, the dominant SSR marker *Xfcp623* was developed on the inner region of the gene (Faris et al., 2010). The *Xfcp623* marker, proposed as a diagnostic marker for the *Tsn1* gene, is considered more reliable than those previously developed. The reliability of the *Xfcp623* diagnostic marker for detecting wheat genotypes with resistance to the pathogen and insensitivity to Ptr ToxA has been shown in a number of studies (Karelov et al., 2015; Mironenko et al., 2017). Taking into account the higher efficiency of the *Xfcp623* marker, in our study we genotyped wheat germplasm using this marker.

As a result of our research, a collection of 30 common wheat entries was characterized using the *Xfcp623* molecular marker, which is diagnostic for the *Tsn1* gene associated with sensitivity to Ptr ToxA. From a practical point of view, of the greatest interest are 16 wheat samples, which demonstrated resistance to race 1 and confirmed resistance to Ptr ToxA in molecular screening. These include eight Kazakh and seven foreign wheat lines. Susceptibility to Ptr ToxA did not always correlate with susceptibility to race 1 and depended on the genetic background of the host.

The results of genotyping and screening of wheat entries for resistance to the most common isolates of *P. tritici-repentis* in Kazakhstan are of interest in order to increase the efficiency of breeding based on the elimination of carriers of dominant alleles of the *Tsn1* gene sensitive to the aggressive Ptr ToxA toxin from the breeding material. Carriers of the identified *tsn1* gene for resistance to Ptr ToxA can be used in breeding programs for gene pyramiding of genes for resistance to wheat diseases.

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Acknowledgements. This work was supported by the Kazakhstan Ministry of Education and Sciences, project AP05132540.

Conflict of interest. The authors declare no conflict of interest.

Received October 9, 2020. Revised October 23, 2020. Accepted October 23,2020.

Вавиловский журнал генетики и селекции. 2020;24(7):730-737
DOI 10.18699/VJ20.667

Comparative analysis of wild and cultivated *Lathyrus* L. species to assess their content of sugars, polyols, free fatty acids, and phytosterols

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Abstract. Under the condition of climate change, the need for crops resistant to abiotic and biotic stresses is increasing. *Lathyrus* spp. are characterized by a high nutritional value of their green biomass. The grass pea is one of the most resistant to drought, waterlogging, cold, salinity, diseases and pests among cultivated legumes, and it is grown at minimal cost. The creation of new *Lathyrus* L. sorts with an improved nutrient composition of nutrients will allow to obtain high-quality feed in areas with extremely unstable weather conditions. In this connection, we studied the patterns of variability in the parameters of the carbohydrate complex (sugars, their lactone and methyl forms), polyols (including phenol-containing alcohols), phytosterols, free fatty acids (FFA) and acylglycerols in the green mass of 32 samples of *Lathyrus sativus* L., *L. tuberosus* L., *L. sylvestris* L., *L. vernus* (L.) Bernh., *L. latifolius* L., *L. linifolius* (Reichard) Bassler. from the VIR collection, reproduced in the Leningrad region in contrasting conditions 2012, 2013.The content of identified compounds varied depending on the genotype, species, and weather conditions. High temperatures and high level of precipitation in 2013 contributed to the accumulation of monosaccharides, in more colder and drier conditions in 2012 – oligosaccharides, most of polyols and FFA. The cultivated species (*L. sativus*) was distinguished by its high sugar content, and the wild species as follows: *L. latifolius* by FFA; *L. linifolius* by ononitol, myo-inositol, and glycerol 3-phosphate; *L. vernus* by MAG and methylpentofuranoside. The species cultivated in culture (*L. sativus*) was distinguished by a high sugar content, wild species: *L. latifolius* – by FFA, *L. linifolius* – ononitol, myo-inositol and glycerol-3-phosphate, *L. vernus* – MAG and methylpentofuranoside. According to our results, the studied samples are promising for the selection of *Lathyrus* varieties with high nutrition quality and stress-resistant.
Key words: *Lathyrus* L.; wild species; varieties; green mass; gas chromatography; polymorphism of characters.

For citation: Solovyeva A.E., Shelenga T.V., Shavarda A.L., Burlyaeva M.O. Comparative analysis of wild and cultivated *Lathyrus* L. species to assess their content of sugars, polyols, free fatty acids, and phytosterols. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):730-737. DOI 10.18699/VJ20.667

Сравнительный анализ диких и культурных видов чины (*Lathyrus* L.) по содержанию сахаров, многоатомных спиртов, свободных жирных кислот и фитостеролов

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Аннотация. В условиях изменения климата увеличивается потребность в устойчивых к абиотическим и биотическим стрессам сельскохозяйственных культурах. Виды чины характеризуются высокой питательной ценностью зеленой массы. Чина посевная – одна из наиболее устойчивых к засухе, заболачиванию, холоду, засолению, болезням и вредителям бобовых культур, выращивается при минимальных затратах. Создание сортов *Lathyrus* L. с улучшенным составом питательных веществ позволит получать качественные корма в районах с крайне неустойчивыми погодными условиями. В связи с этим нами исследованы закономерности изменчивости показателей углеводного комплекса (сахаров, их лактонных и метильных форм), многоатомных спиртов (в том числе фенолсодержащих), фитостеролов, свободных жирных кислот и ацилглицеролов в зеленой массе 32 образцов *Lathyrus sativus* L., *L. tuberosus* L., *L. sylvestris* L., *L. vernus* (L.) Bernh., *L. latifolius* L., *L. linifolius* (Reichard) Bassler. из коллекции ВИР, репродуцированных в Ленинградской области в контрастных условиях 2012 и 2013 гг. Содержание идентифицированных соединений варьировало в зависимости от ге-

нотипа, вида и метеорологических условий. Высокие температуры и большое количество осадков 2013 г. способствовали накоплению моносахаридов, более холодные и сухие условия 2012 г. – накоплению олигосахаридов, большинства многоатомных спиртов и свободных жирных кислот. Вид, возделываемый в культуре (*L. sativus*), отличался высоким содержанием сахаров, дикие виды – свободных жирных кислот (*L. latifolius*), ононитола, мио-инозитола и глицерол-3-фосфата (*L. linifolius*), МАГ и метилпентофуранозида (*L. vernus*). По результатам изучения большинство исследованных образцов перспективно для селекции новых высокопитательных и устойчивых к стрессам кормовых сортов *Lathyrus*.
Ключевые слова: *Lathyrus* L.; дикие виды; сорта; зеленая масса; газовая хроматография; генетические ресурсы; полиморфизм признаков.

Introduction

The changing climate leads to the expansion of areas with extremely unstable weather conditions, thus enhancing the demand for stress-resistant crops grown for food and feed. Many species in the genus *Lathyrus* L. are used as sources of human food, animal feed, and medications. A majority of wild peavines are exploited as pasture and fodder plants. The best-known species is the grass pea (*Lathyrus sativus* L.) with its millennia-long cultivation history, cultivated on all the continents. This leguminous crop is considered one of the most resistant to drought, waterlogging, and cold (Campbell, 1997). It is adapted to a diversity of soil types, including salinized soils, and would yield harvests in the environments where other crops would die, so it was recognized as 'the food for survival' (Sarkar et al., 2019). The species is resistant to diseases (powdery mildew, rust, etc.) and pests (Sarkar et al., 2019). The yield of grass pea seed reaches 2.9 t/ha, and that of its green biomass is 5.2 t/ha.

Grass pea seed and green biomass are notable for their high nutritional value. The grain contains, depending on growing conditions, from 18 to 34 % of protein (Rizvi et al., 2016; Burlyaeva et al., 2018; Donskoj et al., 2019), and the biomass from 10 to 27 % (Burlyaeva et al., 2015). Seeds of *L. sativus* and *L. cicera* L. are characterized by high concentrations of essential amino acids (63–64 %) and polyunsaturated fatty acids (66.9 and 58.6 %, respectively), predominantly linoleic (Grela et al., 2012). The hay from grass pea matches alfalfa in nutritiousness (Poland et al., 2003). The content of organic acids in the *Lathyrus* green biomass varies from 140.0 to 2140.0 mg/100 g, free amino acids from 11.8 to 610.0 mg/100 g, and secondary metabolites from 4.4 to 224.6 mg/100 g (Solovyeva et al., 2019).

In Russia, compared with other countries, *Lathyrus* spp. occupy a minor niche in the national plant production. Breeding work is conducted in a limited number of institutions, which has a negative effect on the crop's utilization in agriculture.

Our previous investigations of the green biomass of wild and cultivated *Lathyrus* spp. exposed a wide range of compounds in it (organic acids, free amino acids, and secondary metabolites). Accessions from the peavine collection were identified as promising sources for the development of highly nutritious, resistant and officinal cultivars (Solovyeva et al., 2019).

The present study is the next step in the research into metabolomic profiles of peavine green biomass with a focus on the content of saccharides, free fatty acids (FFA), polyols, and acylglycerols. The objective of the study was to assess the interspecific and intraspecific polymorphisms of biochemical characters in *Lathyrus* spp. and the effect of weather conditions on their variability. To solve this task, the technique currently widely known as profiling was used (Steinhauser, Kopka, 2007). This technique is one of the so-called metabolomic methods of analysis, which, in addition to delivering information on individual metabolites in a studied object, makes it possible to evaluate the status of this object (Worley, Powers, 2012; Hong et al., 2016).

Thus, our aim was gaining knowledge about variations in the status of metabolic networks in the accessions of *Lathyrus* spp. in the context of their taxonomic characteristics and weather conditions.

Materials and methods

The experiment encompassed 32 accessions of six *Lathyrus* spp. from the collection of the Vavilov Institute (VIR): grass pea (*L. sativus*), flat pea (*L. sylvestris* L.), spring pea (*L. vernus* (L.) Bernh.), heath pea (*L. linifolius* (Reichard) Bassler), everlasting pea (*L. latifolius* L.), and tuberous pea (*L. tuberosus* L.), grown in the fields of Pushkin experimental laboratories of VIR in 2012 and 2013 according to the guidelines approved by VIR (Vishnyakova et al., 2010). Meteorological conditions during the growing seasons were contrasting. In 2012, the sum of active temperatures was 1885.0 °C, and the precipitation amount 340.7 mm; in 2013, the sum of active temperatures increased to 2474.3 °C, and rainfall to 646.4 mm.

Plants were collected in the phase of first mature pods. Fresh green biomass was analyzed from 5 plants of each accession (leaves, inflorescences, pods in the phase of milk ripeness, and stems). Gas chromatography/mass spectrometry (GC–MS) profiling was performed using the protocol for the analysis of trimethylsilyl derivatives, developed at the Komarov Botanical Institute while working on the program task, theme AAAA-A18-118032390136-5 "Assessment of changes in the correlational structure of metabolite networks in the process of growth and development of fungi and plants from the viewpoint of systemic biology", and adjusted for use on Agilent 6850-MSD 5975 at the Research Park of St. Petersburg State University Center for Molecular and Cell Technologies (Puzanskiy et al., 2018).

The plant material was examined by the GC–MS technique: it was extracted with ethanol, and then evaporated dry on a CentriVap Concentrator (Labconco, USA). The solid residue was dissolved in pyridine containing 1000 ppm of tricosane that served as an internal standard; then, 20 μl of BSTFA (N,O-Bis[trimethylsilyl]trifluoroacetamide) (Supelco, USA) was introduced. To ensure the silylation reaction sufficiency, the vials were kept for 15 min under +100 °C in a special thermal block. The samples were analyzed on an Agilent 6850 chromatographic mass spectrometer with the Agilent 5975 D mass selective detector (USA). Chromato-

graphic separation was done on an Agilent HP-5MS column (USA), length: 30 m, internal diameter: 0.25 mm, stationary phase film thickness: 0.25 μm, linear temperature programming mode: 70 to 325 °C, speed: 6°/min (50 min), carrier gas: helium. The analysis was performed with a constant gas flow velocity through the column (1 mL/min) as follows: evaporator temperature: +300 °C; flow split ratio during sample injection: 1:20; mass spectra scanning range: 50 to 1050 amu; scanning speed: 2 scans/sec. Total ion current (TIC) chromatograms were recorded for the samples.

The results were processed using UniChrom and AMDIS software resources, NIST 2010 mass spectra libraries, and in-house libraries of the Research Park of St. Petersburg State University and the Komarov Botanical Institute.

The amount of trimethylsilyl (TMS) derivatives of the identified compounds was calculated by the internal standardization method for tricosane using UniChrom software. With the semiquantitation approach applied, the detector's sensitivity coefficients for individual compounds need not be taken into account (Worley, Powers, 2012). The data produced by the analysis are presented in conventional units (c. u.) (Sitkin et al., 2013).

Statistical data processing was done with Statistica 7 and Excel 7.0 software for Windows, using the principal factor analysis (PFA) and one-way analysis of variance (ANOVA). Statistical significance of the environmental effect on the expression of biochemical characters was assessed using Fisher's criterion (LSD test), and the factor's effect size (percentage) – η^2 (intraclass correlation coefficient, according to Fisher) – was calculated by the following formula (Ivanter, Korosov, 2003):

$$\eta^2 = \frac{SS_{\text{factor}}}{SS_{\text{total}}} \times 100\ \%,$$

where η^2, % is the effect size percentage of the factor's impact; SS_{factor} is the sum of squared deviations for the factor; SS_{total} is the total sum of squared deviations.

Results

The GC–MS profiling of the green biomass of *Lathyrus* L. spp. identified about 300 components. Organic acids, free amino acids and phenol-containing compounds were discussed in an earlier publication (Solovyeva et al., 2019). This study presents analytical results of comparing the contents of over 60 compounds in the peavine green biomass, including such groups as sugars, polyols, phytosterols, and FFA. The levels of the identified compounds are presented in c. u. (see the Table, Suplementary Material 1)[1].

Carbohydrate composition. Sugars in the peavine green biomass consisted of mono- and oligosaccharides. Monosaccharides were represented by pentoses (ribose, arabinose, lyxose, and xylose) and hexoses (fructose, glucose, sorbose, galactose, mannose, rhamnose, and altrose). Oligosaccharides included disaccharides (sucrose, maltose, and rutinose) and a trisaccharide (raffinose). Fructose, glucose and mannose were the main sugars in the hexose group, xylose in the pentose group, and sucrose in the group of disaccharides (Fig. 1). Besides, metabolically active forms of saccharides were idenified:

Comparative analysis of the content of saccharides, polyols and FFA in the green mass of some *Lathyrus* L. species (c. u.)

Species	Saccharides	Polyols	FFA
L. sativus L.	1808 ± 124*	487 ± 66	162 ± 25
	423–4470**	77–3184	10–732
L. sylvestris L.	825 ± 101	340 ± 73	130 ± 42
	592–1162	244–630	40–288
L. vernus (L.) Bernh.	304 ± 204	1041 ± 22	50 ± 7
	40–569	1019–1062	43–56
L. linifolius (Reichard) Bassler	327 ± 5	2343 ± 247	114 ± 3
	322–331	1895–2791	110–117
L. latifolius L.	488 ± 10	360 ± 31	345 ± 81
	478–4990	329–391	264–427
L. tuberosus L.	144 ± 25	66 ± 17	86 ± 34
	119–169	49–82	52–120

* Arithmetic mean ± standard error of the arithmetic mean; ** variability (min–max).

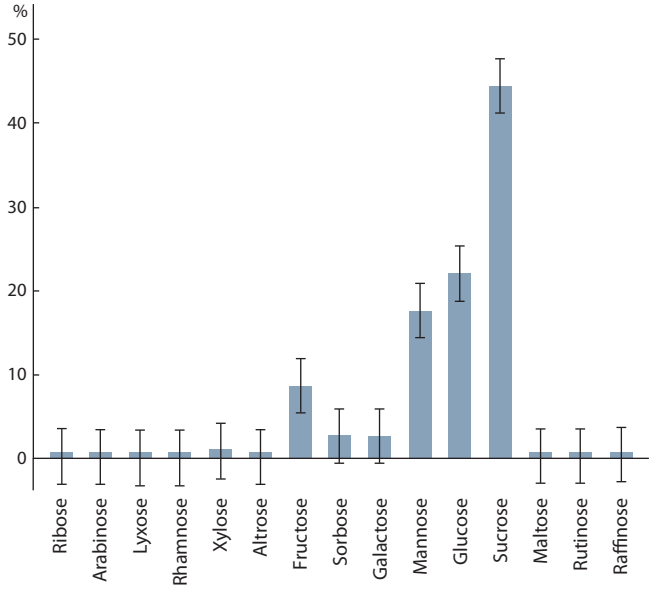

Fig. 1. Component composition profiles of monosaccharides and oligosaccharides: percentage of the total sugar content in the green biomass of *Lathyrus* spp.

lactone (glucose 1,4-lactone), phosphate (fructose 6-phosphate and glucose 1-phosphate), and methyl sugars (methylpentofuranoside and methylglucofuranoside).

The relative content of sugars varied across the years (Fig. 2) and the genotypes. A majority of sugars were monosaccharides (67 % of the total sugars) represented mostly by hexoses (66 % of the total sugars). Pentoses amounted to slightly more than 1 %, and oligosaccharides to 32.6 %; of the latter, disaccharides accounted for 32.3 %, and trisaccharides for 0.3 %. Sucrose had the highest percentage among disaccharides (31.9 %).

[1] Supplementary Materials 1–3 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx11.pdf

The mean value for sugars in 2012 was 1442 c. u. (40 to 4470). The greatest part of sugars was represented by disaccharides, their mean content equal to 903 c. u. (0 to 3983). The mean total amount of monosaccharides was 538 c. u. (40 to 2085). They were represented mostly by hexoses, with the mean level of 530 c. u. (29 to 2082). Pentoses averaged 8 c. u. (0 to 24). Identified among trisaccharides was raffinose whose mean content was 1 c. u. (0 to 14). The mean content of total oligosaccharides was 904 c. u. (0 to 3984).

In 2012, the highest saccharide levels were recorded for the accessions of *L. sativus* (up to 4470 c. u.) and *L. sylvestris* (up to 1162). The remaining peavine species accumulated less than 600 c. u. of sugars. The lowest values were observed in the accessions of *L. tuberosus* (144 c. u.).

In 2013, the total content of sugars, including monosaccharides (hexoses and pentoses), increased on average to 1830 and 1278 c. u., respectively, while the content of sucrose reduced to 551. Compared with 2012, the limits of the variation range changed for total sugars from 144 to 2511 c. u., for monosaccharides from 63 to 1842 c. u. (for hexoses from 63 to 1751 c. u.), and for oligosaccharides from 273 to 1060 c. u. The major part of sugars in 2013 was represented by monosaccharides (hexoses). Mean values for total oligosaccharides in 2013 decreased to 532 c. u., while those for raffinose increased to 11 c. u. The ranges of variability for pentoses and raffinose (0.3–64 and 0–40 c. u., respectively) in 2013 became wider than in the previous year.

High levels of sugars (more than 2831 c. u.) during the entire period of studies were observed in the accessions of *L. sativus*.

Polyols and phytosterols. Most of the identified polyols were hexatomic (sorbitol, dulcitol, mannitol and inositol); their mean total amount was 476 c. u. (see Suppl. Material 1). They were represented mainly by inositol, its isomers and derivatives (myo-inositol, chiro-inositol, methyl-inositol, ononitol and galactinol); their total sum was 413 u. e. In addition to the alcohols mentioned above, glycerol, erythritol, threitol, xylitol, arabinitol and phytol were identified. Besides, phytosterols (campesterol, stigmasterol, β-sitosterol, isofucosterol and taraxasterol) and phenolic alcohols (coniferol, α-tocoferol, kaempferol and pyrogallol) were observed; their total sums were 9 and 3 c. u., respectively. In the phytosterol group, β-sitosterol prevailed (8 c. u.). Phosphate forms of glycerol and inositol were also identified as well as metabolic products of glycerophospholipids (glycerol 3-phosphate and myo-inositol 2-phosphate). Phenol-containing alcohols were discussed earlier (Solovyeva et al., 2019).

The content of polyols significantly varied across the years of the study (see Fig. 2). In 2012, their mean level was 744 c. u., with the limits of variation from 171 to 3184. In 2013, their amount reduced to 317 c. u., and the range of their variation narrowed (77 to 442). This value in *L. sativus* in 2012 (637 c. u.) was lower than the mean (calculated for all accessions), but in 2013 it decreased to 322 c. u. The same tendency was observed in the accessions of *L. sylvestris*: 437 c. u. in 2012, and 275 c. u. in 2013.

The highest content of polyols was found in *L. sativus* (3152 c. u.), a lower one in the accessions *L. linifolius* (2307), *L. vernus* (1050), *L. sylvestris* (619) and *L. latifolius* (348), and the lowest in *L. tuberosus* (66).

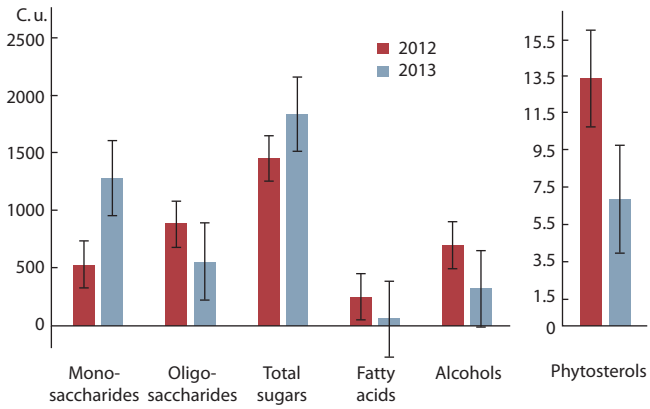

Fig. 2. Mean values for monosaccharides, oligosaccharides, total sugars, fatty acids, polyols, and phytosterols in the *Lathyrus* green biomass in 2012–2013.

The highest amounts of erythritol and phytosterols (1 and 22, respectively) were observed in the accessions of *L. sativus*, and the highest total polyols in *L. sativus* and *L. linifolius* (3115 and 2307 c. u., respectively).

Free fatty acids and acylglycerols. Nineteen FFA were identified, including saturated (capric, undecylic, lauric, palmitic, stearic, arachidic, behenic, lignoceric, cerotic, montanic and melissic) and unsaturated ones (oleic, linoleic and linolenic), hydroxy acids (hydroxyoctadecanoic, hydroxytetracosanoic, hydroxyhexacosanoic, hydroxyoctacosanoic and hydroxytriacontanoic), and monoacylglycerols (MAG-1 C16:0 and MAG-1 C18:0) (see Suppl. Material 1).

In 2012, the total FFA content in the peavine green biomass was 248 c. u. (limits of variation from 37 to 732), with 1 c. u. for MAG (0 to 21). In 2013, the values of FFA and MAG reduced to 52 and 0.5 c. u., and their variation limits narrowed (10 to 138, and 0 to 2, respectively) (see Fig. 2).

Hydroxyhexacosanoic, palmitic, linolenic and stearic acids showed the highest values: their shares in the total content of FFA and hydroxy acids were 32, 22, 14 and 13 %, respectively. The mean percentage of linoleic acids was 7 %, with 5 % of capric, 3 % of oleic, 2 % of hydroxyoctacosanoic, and 1 % of undecylic acid. The percentage of minor FFA was less than 1 %.

The highest mean content of FFA was observed in the accessions of *L. latifolius* (up to 345 c. u.). Other species showed lower levels: 162 c. u. in *L. sativus*, 131 in *L. sylvestris*, 114 in *L. linifolius*, and 86 in *L. tuberosus*. The lowest FFA amounts were found in *L. vernus* (50 c. u.), but this species was distinguished for the contents of MAG-1 C16:0 (up to 11 c. u.) and MAG-1 C18:0 (up to 10). The levels of MAG in the other species were much lower.

Discussion

Our experiment disclosed the presence of significant interspecific and intraspecific variability of the *Lathyrus* accessions both in the quantitative content and qualitative composition of the identified compounds (see Suppl. Material 1).

The analysis of the resulting data showed a strong variation in the values under different weather conditions (see Fig. 2). High temperatures and intense rainfall (2013) contributed

to the accumulation of sugars at the expense of an increase in the percentage of monosaccharides, while the colder and drier conditions (2012) provoked a rise of polyols, FFA and oligosaccharides.

A single factor analysis of variance was applied to ascertain the statistical significance of the effect of weather on the studied characters. Growing conditions were found to produce a statistically significant impact on the variability of the total sugars, total FFA, ribose, arabinose, xylose, altrose, rhamnose, mannose, glucose, galactose, sucrose, maltose, rutinose, raffinose, certain acids (palmitic, stearic, oleic, linoleic, linolenic, lignoceric, hydroxyhexacosanoic and hydroxytriacontanoic), stigmasterol, threitol, glycerol, xylitol, erythritol, ononitol, sorbitol, mannitol, phytol, β-sitosterol, campesterol, phosphate, glucose 1-phosphate, methylphosphate, threono-1,4-lactone, and glucono-1,4-lactone (Fig. 3, Suppl. Material 2). Variations of other compounds were not significantly affected by weather. The strongest effect of growing conditions was observed in the accumulation of mannose (effect size $\eta^2 = 62.9$ %), rhamnose (62.3), glucose (56.7), raffinose (41.1) and altrose (40.9 %) in the peavine biomass. Variability of the total FFA levels was determined by meteorological conditions to the extent of 34.1 %; linolenic (51.1) and palmitic (46.9) acids were the most dependent on them. Among phytosterols, the highest values of η^2 were recorded for campesterol (80.2 %) and stigmasterol (71.6); among polyols, for threitol (53.6 %) and mannitol (37.9). A significant weather impact was produced on the content of phosphoric acid derivatives (60.0 %), glucose 1-phosphate (36.7), and threono-1,4-lactone (31.5). Thus, weather conditions were found to have a significant effect on the accumulation of a considerable number of identified compounds in the green biomass of *Lathyrus* spp.

The principal factor analysis (PFA) was applied to reveal the interplay among biochemical characters and disclose regularities in their variations under the influence of weather conditions, genotypes, and taxonomic (species-specific) attribution. As a result, 10 factors determining 65.8 % of the total variance in plant characters were identified. In the first factor (F1, 20.7 % of the total variance), two large groups of biochemical characters demonstrated negatively correlated concurrent variations. The first group included rhamnose, xylose, altrose, glucose, maltose, rutinose, raffinose, erythritol, threitol, mannitol, campesterol, stigmasterol, phosphate and glucose 1-phosphate; the second one united palmitic, linoleic, linolenic and stearic acids, glycerol, sorbitol, β-sitosterol and threono-1,4-lactone. This factor demonstrates that higher amounts of the compounds from the first group in peavine green biomass are accompanied by lower levels of those from the second group, and vice versa. The dominating characters in F1 (determining variations of the others) are campesterol, stigmasterol, linolenic acid, and palmitic acid. The second factor (F2, 7.5 %) proves the relationship between sorbose and fructose. The third (F3, 6.8 %) ascertains the interactions of methyl-inositol, glyceraldehide, α-methylglucofuranoside, and methylglucoside. The fourth (F4, 6.3 %) shows correlations between capric, oleic and hydroxytetracosanoic acids. The fifth (F5, 5.4 %) incorporates arachidic, behenic, hydroxyhexacosanoic and hydroxyoctacosanoic acids. The sixth factor (F6, 4.8 %) includes melissic and hydroxyoctadecanoic

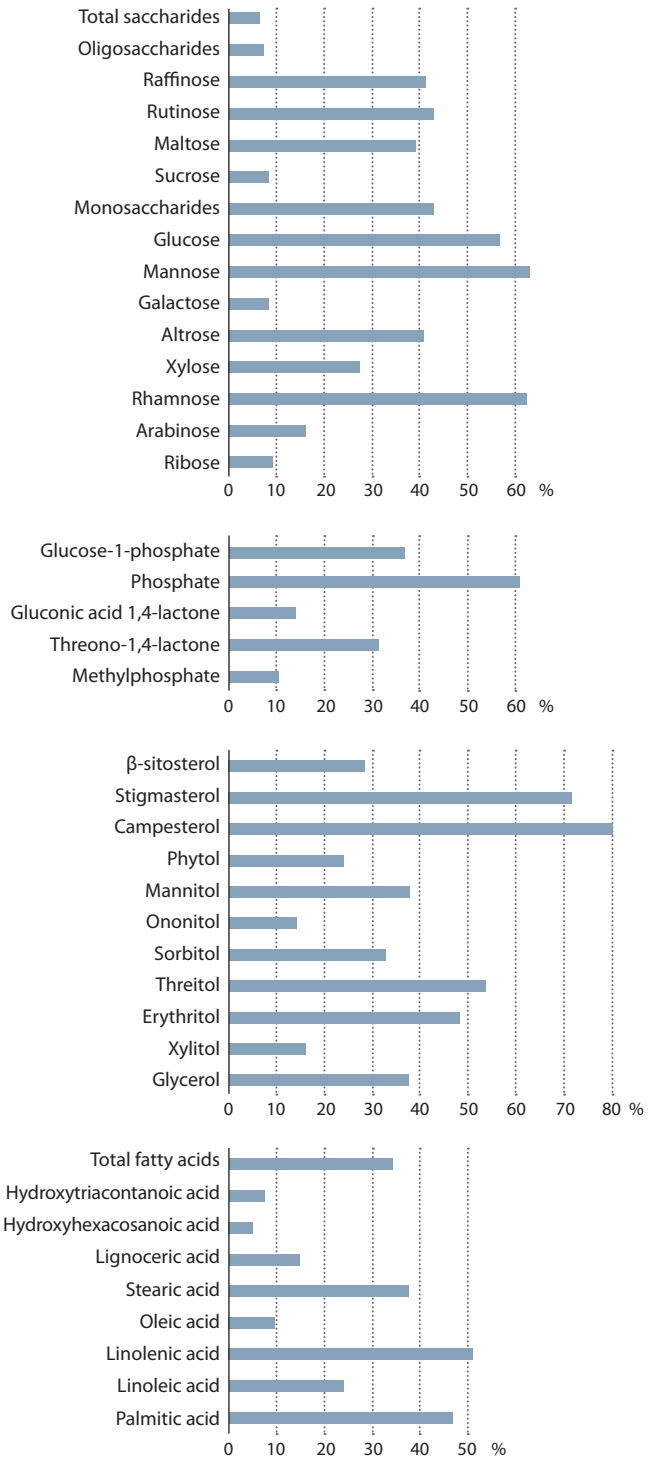

Fig. 3. The effect size percentage (η^2) showing the impact of weather conditions on the variability of biochemical characteristics.

acids, the seventh (F7, 4.1 %) ononitol, the eighth (F8, 3.6 %) glycerol 3-phosphate, the ninth (F9, 3.5 %) lupeol and guanosine, and the tenth (F10, 3.3 %) myo-inositol 2-phosphate and uridine.

While studying the distribution of accessions in the space of the first two factors, it seems obvious that the plants are grouped according to the years of observations (Fig. 4). The right-hand part of the graph contains cultivars with high levels

of saccharides (rhamnose, xylose, altrose, glucose, maltose, rutinose, raffinose, and glucose 1-phosphate), phosphate, erythritol, threitol, mannitol, campesterol and stigmasterol, and low levels of FFA (palmitic, linoleic, linolenic and stearic acids), glycerol, sorbitol, β-sitosterol, threono-1,4-lactone. The left-hand part harbors accessions with the opposite values of the above-listed compounds, the upper part with minimal amounts of sorbose and fructose, and the bottom part with the highest levels of these two sugars. Thus, the accessions reproduced in 2013 clustered in the section with high amounts of most of the sugars, while those of 2012 in the FFA section. Adverse growing condition (2012) provoked the accumulation of glycerol, sorbitol and β-sitosterol, whereas optimal conditions for plant growth and development (2013) favored erythritol, threitol, mannitol, campesterol and stigmasterol. The wild species *L. vernus*, *L. linifolius* and *L. tuberosus* showed intermediate levels of most sugars, polyols and FFA, and low values of fructose and sorbose. The accessions of *L. sylvestris* differed from other wild forms in the higher fructose and sorbose content. Many indicators in *L. latifolius* were close to those in *L. sylvestris*. The cultivated *L. sativus* demonstrated the richest polymorphism of all the studied characters. Most of the cultivars representing *L. sativus* concentrated in the section of intermediate and high levels of fructose and sorbose, but no species-specific groups were observed for the other biochemical characters: the accessions scattered across the graph in accordance with individual features of their genotypes and their normal responses to weather conditions.

Factors F3–F10 highlighted heterogeneity of the accessions in each of the principal components (Suppl. Material 3). Maximums of factor loadings for most of the factors were recorded in the accessions of *L. sativus* (k-34 and k-900), *L. linifolius* (N-597422), and *L. vernus* (N-591179 and N-593953).

According to Chavan (1998), the green biomass of peavines is characterized by a fairy high content of sugars. Within the plant, sugars play the role of osmoprotectants and stabilizers of membranes and proteins, including the same under the impact of low temperatures. Therefore, accessions with high sugar content, especially as far as oligosaccharides and raffinose are concerned, are more resistant to abiotic environmental stressors. Oligosaccharides are accumulated by a plant as a response to the cold stress (Krasenski, Jonak, 2012; Moreno et al., 2018). In our experiment, the growth of the oligosaccharide content was observed in the year with lower temperatures, which confirmed the above-mentioned. The highest levels of raffinose were observed in *L. sativus*, so its accessions may be recommended to breeders to develop stress-adaptable and cold-tolerant cultivars.

Some researchers reported that if the raffinose content in food or feed exceeded 0.4 % dry weight it may cause dyspeptic disorders (Muzquiz et al., 2012). In our experiment, its percentage never exceeded this level, so the green biomass of all studied peavine species may be used as animal feed.

Mannitol, arabinitol, sorbitol, galactinol, dulcitol, erythritol and other polyols improve adaptive properties of a plant against salt, water and temperature stresses. Accessions with a high content of these compounds may be identified as potentially resistant to unfavorable environmental factors (Tibbett et al., 2002; Majumder, Biswas, 2006; Dong et al., 2013; Baudier et al., 2014; Zhou et al., 2014; Patel, Williamson, 2016; Moreno et al., 2018). According to our data, the content of glycerol and sorbitol in peavine green biomass was higher under cold and dry conditions in 2012 (23 and 56 c. u., respectively), which confirmed the results of the authors referred to above. The highest levels of glycerol and sorbitol were observed in *L. sativus*.

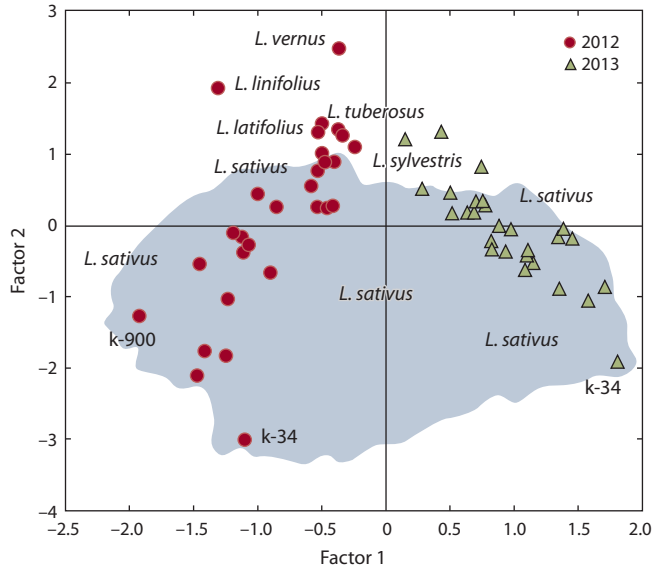

Fig. 4. Distribution of the accessions over the space of the first two factors (F1 and F2).

Inositol, its isomers and derivatives, including phosphate ones (methyl-inositol, chiro-inositol, myo-inositol, ononitol, and myo-inositol 2-phosphate) participate in the cell membrane biosynthesis and plant growth regulation, are incorporated into the composition of the cell's phosphate 'depot', and appear among the antistress factors of plant protection (osmolites) (Dong et al., 2013). In this research, when the plants developed under cold dry conditions, their mean values of ononitol were higher than in an optimal environment (505 and 245 c. u., respectively). The same situation was observed with the levels of mannitol, xylitol and erythritol in the green biomass of *L. sativus*: under unfavorable conditions their content increased. The maximums of methyl-inositol, chiro-inositol, and myo-inositol 2-phosphate were identified in *L. sativus* (200, 40 and 0.5 c. u., respectively), while myo-inositol and ononitol maximums in *L. linifolius* (212 and 2088 c. u., respectively). On the whole, high values in the polyol content were recorded for all *Lathyrus* spp., which explains their resistance to abiotic and biotic stressors.

Phytosterols play an important role in plant growth and development, because they are precursors of phytohormones (Beebe, Turgeon, 1992; Deng et al., 2016; Valitova et al., 2016). According to published data, the main phytosterol in plants is β-sitosterol, which coincides with our results: β-sitosterol accounted for 86.5 % of the total content of identified sterols. In our experiment, the total phytosterol content in the peavine biomass was 39 c. u.

Among FFA on the surface of *L. sativus* seeds, palmitoleic and palmitic acids are prevailing, followed in descending order by stearic, myristic, oleic, arachidic, capric, behenic, linoleic and linolenic acids (Adhikary et al., 2016). In this

study, the major share of identified FFA also went to palmitic, linoleic, linolenic, oleic and stearic acids. The presence of the mentioned FFA in green biomass characterizes peavines as highly nutritional fodder plants promising for cultivation.

Thus, over 300 compounds have been identified in the green biomass of *Lathyrus* spp. (Solovyeva et al., 2019); more than 60 of them are described in this publication. The values of a majority of the analyzed biochemical substances demonstrated a wide range of variability. Their amounts significantly varied across different genotypes, species, and years of testing. The studied accessions showed high intra- and interspecific polymorphisms, both in the quantitative content and qualitative composition of the identified compounds.

Wild species (*L. vernus*, *L. linifolius* and *L. tuberosus*) had medium values in the content of most sugars, polyols and FFA, and low levels of fructose and sorbose. As far as their sugar content is concerned, *L. sylvestris* and *L. latifolius* occupied an intermediate position between the abovementioned wild species and *L. sativus*. The cultivated *L. sativus* stood out for the highest amount of sugars in its green biomass, *L. linifolius* for ononitol, myo-inositol and glycerol 3-phosphate, while *L. vernus* for MAG and methylpentofuranoside.

The comparative analysis helped to identify accessions suitable for further profound investigations into sugars, polyols, FFA, phytosterols, etc. It is especially valuable for future researching, because these compounds are indicators of green biomass quality and resistance to unfavorable environmental factors. The identified accessions can be used to produce both highly nutritional and resistant cultivars of *Lathyrus* spp. Considering the latest achievements in genomics, such accessions may be regarded as quality and resistance sources not only for peavines but for other crops as well.

Conclusion

Considerable polymorphism of biochemical characters has been disclosed in wild and cultivated *Lathyrus* spp. The results obtained attest to the high potential of the studied species for contemporary agricultural production and new breeding trends.

Introducing *L. sativus*, *L. sylvestris*, *L. vernus*, *L. linifolius*, *L. latifolius* and *L. tuberosus* into animal feed production would expand the assortment of the exploited fodder crops. Due to their resistance to abiotic stressors and the disclosed nutritional value, *Lathyrus* spp. could play an important role in food security maintenance in areas with unpredictable weather conditions.

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Acknowledgements. The work was done in the framework of a state task according to the theme plan of VIR, official registration No. 0662-2019-0002.

Conflict of interest. The authors declare no conflict of interest.

Received May 13, 2020. Revised August 04, 2020. Accepted August 17, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):738-746
DOI 10.18699/VJ20.668

Phenotypic diversity of bread wheat lines with introgressions from the diploid cereal *Aegilops speltoides* for technological properties of grain and flour

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Abstract. The creation of varieties adapted to changing environmental conditions, resistant to various pathogens, and satisfying various grain purposes is impossible without using the genetic diversity of wheat. One of the ways to expand the genetic diversity of wheat is to introduce new variants of genes from the genetic pool of congeners and wild relatives into the genotypes of existing varieties. In this study, we used 10 lines from the Arsenal collection created on the genetic basis of the spring variety 'Rodina' and the diploid species *Aegilops speltoides* in the Federal Research Center "Nemchinovka" in 1994. The lines were previously characterized for the presence of translocations and chromosomal rearrangements cytologically and using molecular markers. Technological analyses were performed on grain obtained in Western Siberia and Moscow region. The aim of this study was to establish the possibilities of expanding the phenotypic diversity for technological properties of grain and flour as a result of such hybridization of bread wheat and the diploid cereal *Aegilops speltoides*. The variety 'Rodina' forms a vitreous grain with a high gluten content in Siberia, but has low physical properties of flour and dough. Five derived lines were found to have significantly higher protein and gluten content in grain. The highest values under both growing conditions were found in lines 73/00^{i}, 82/00^{i}, and 84/00^{i}. Two lines (69/00^{i} and 76/00^{i}) showed a high flour strength and dough elasticity, characterizing the lines as strong and valuable in quality. These lines can be used for baking bread. Line 82/00^{i} inherited from *Ae. speltoides* a soft-grain endosperm, which indicates the introgression of the *Ha-Sp* gene, homoeoallelic to the *Ha* gene of bread wheat, into 'Rodina'. Flour of this line is suitable for the manufacture of confectionery without the use of technological additives. The lines generally retained their characteristics in different growing conditions. They can be attracted as donors of new alleles of genes that determine the technological properties of grain and resistance to biotic stresses.
Key words: bread wheat; *Ae. speltoides*; introgression lines; chromosomal rearrangements; grain vitreousness; protein and gluten content in grain; physical properties of dough; soft grain and endosperm grain hardness.

For citation: Shchukina L.V., Lapochkina I.F., Pshenichnikova T.A. Phenotypic diversity of bread wheat lines with introgressions from the diploid cereal *Aegilops speltoides* for technological properties of grain and flour. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):738-746. DOI 10.18699/VJ20.668

Фенотипическое разнообразие линий мягкой пшеницы с интрогрессиями от диплоидного злака *Aegilops speltoides* по технологическим свойствам зерна и муки

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Аннотация. Создание сортов, адаптированных к изменяющимся условиям окружающей среды, устойчивых к различным патогенам и соответствующих различному целевому назначению зерна, невозможно без использования генетического разнообразия пшеницы. Одним из путей его расширения является введение в генотипы существующих сортов новых вариантов генов из генетического пула родственных видов и диких сородичей. В настоящем исследовании использованы 10 линий из коллекции «Арсенал», созданных на основе ярового сорта Родина и вида *Aegilops speltoides* в Научно-исследовательском институте сельского хозяйства центральных районов Нечерноземной зоны (ныне Федеральный исследовательский центр «Немчиновка») в 1994 г. Линии были ранее охарактеризованы цитологическими и цитогенетическими методами, а также с помощью молекулярных маркеров на наличие замещений и перестроек хромосом. Технологические анализы были выполнены на зерне, полученном в Западной Сибири и Московской области. Цель исследова-

ния – установить возможности расширения фенотипического разнообразия пшеницы по технологическим свойствам зерна и муки в результате такой гибридизации. Сорт Родина формирует стекловидное зерно с высоким содержанием клейковины в условиях Сибири, но имеет невысокие физические свойства муки и теста. Обнаружено, что пять производных от него линий имеют достоверно более высокое содержание белка и клейковины в зерне. Наибольшие значения в обоих условиях выращивания установлены у линий 73/00^{i}, 82/00^{i} и 84/00^{i}. Две линии, 69/00^{i} и 76/00^{i}, показали высокую силу муки и упругость теста, характеризующие линии как сильные и ценные по качеству. Зерно этих линий может быть использовано для выпечки хлеба. Линия 82/00^{i} унаследовала от *Ae. speltoides* мягкозерный эндосперм, что говорит об интрогрессии в геном сорта Родина гена *Ha-Sp*, гомеоаллельного гену *Ha* мягкой пшеницы. Мука этой линии пригодна для изготовления кондитерских изделий без применения технологических добавок. Линии в основном сохраняли свои особенности в различных условиях выращивания. Они могут быть привлечены в качестве доноров новых аллелей генов, определяющих технологические свойства зерна и устойчивость к биотическим стрессам.
Ключевые слова: мягкая пшеница; *Ae. speltoides*; интрогрессированные линии; хромосомные перестройки; стекловидность зерна; содержание белка и клейковины в зерне; физические свойства теста; мягкозерность и твердозерность эндосперма.

Inroduction

Climate change on the Earth entails a change in growing conditions for crops. Breeders when faced with the new natural challenges, must have a large arsenal of genetic diversity in order to create varieties with the required properties. In Russia, bread wheat is one of the main cereal crops, which grain is used for food, fodder and for technical purposes. Also, grain is an important export item. In 2020, the area under spring bread wheat in Russia was 12.2 million hectares, while the total area under spring crops was 52 million hectares (Ganenko, Belaya, 2020).

For a long time breeding was focused on the obtaining of high-yielding wheat cultivars; this resulted in a loss of valuable and rare alleles that ensure the development of high-quality cultivars with high gluten content in grain. Changes in the spectrum of pathogens and their racial composition also periodically remove many varieties from the use in production. As a result, the gene pool of wheat cultivars becomes narrower in a practical application.

Currently, breeding is faced with the task of obtaining the cultivars that are adapted to changing environmental conditions, resistant to various pathogens and satisfying various end-use grain purposes (baking yeast bread, making pizza, cookies, pancakes, noodles, etc.) (Peña, 2002). This task requires the expansion of the genetic diversity of wheat in many ways. The classic way to solve this problem is to use ancient varieties and genetic collections of wheat in hybridization (Mitrofanova, 2012; Vikram et al., 2016). The alternative way is hybridization of bread wheat with closely related species and wild relatives that carry gene variants that are absent in the genotype of existing cultivars. This pathway is mainly used to search for genes of resistance to biotic and abiotic stresses (Tsitsin, 1958; Vavilov, 1986; Leonova, Budashkina, 2016; Voronov et al., 2019). Introgression of alien genetic material also affects certain grain quality traits (Krupnova, 2013; Shchukina et al., 2017; Alvarez, Guzmán, 2018).

The main grain components of caryopsis that affect the technological properties of grain are gluten (protein) and starch; their composition and content determine the practical use of grain. The search for the genes that can diversify these parameters in wheat during interspecific and intergeneric hybridization is an urgent area of research. Understanding the relationship between the chromosomal rearrangements and introgressions with the formation of the end-use product of grain enlarges the field of work of breeders. The aim of this study was to establish the possibility of enlarging the phenotypic diversity for technological properties of grain and flour in bread wheat due to chromosomal rearrangements resulting from hybridization with diploid cereal *Aegilops speltoides*.

Materials and methods

Genetic material. We used 10 lines of the spring bread wheat cultivar Rodina from the 'Arsenal' collection (69/00^{i}, 73/00^{i}, 76/00^{i}, 77/00^{i}, 81/00^{i}, 82/00^{i}, 84/00^{i}, 99/00^{i}, 102/00^{i} and 103/00^{i}). They were obtained by selecting individual plants with bivalent meiosis from the progeny F_2M_2–F_4M_4 of asymmetric sex hybrids F_1M_1 ($2n = 49$). The asymmetric sex hybrids were obtained from crossing of the cultivar Rodina ($2n = 42$) with the species *Aegilops speltoides* (sample k-389 from the VIR collection, St. Petersburg) whose pollen was irradiated with gamma rays at a dose of 10 kR (Lapochkina, 1999). The lines were previously characterized for the presence of substitutions and rearrangements of chromosomes by cytological and cytogenetic methods, as well as using molecular markers.

Establishment of the mechanisms of alien material introgression in wheat genome (the presence of substitutions, translocations) was carried out by studying the nature of chromosome pairing in meiosis in specially obtained F_1 hybrids (test line × the original cultivar Rodina). In the lines, the genes for resistance to leaf rust and powdery mildew were identified using microsatellite and STS markers and the test pathotypes of the fungus (Table 1) (Lapochkina et al., 2003, 2005; Gajnullin et al., 2007). The parental cultivar Rodina turned out to be heterogeneous for the chromosomal translocation T1BS/1RS inherited from the cultivar Kavkaz, which was involved in the origin of the cultivar (World Seeds × Kavkaz) (Dorofeev et al., 1987). By analyzing the gliadin storage proteins, the line with the absence of this rearrangement was isolated (hereinafter referred to as cultivar Rodina). This genotype was used as a control in all experiments.

Growing conditions. The lines and the parent cultivar Rodina were grown with spring sowing in the experimental field of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Akademgorodok, Novosibirsk region) in 2003–2005, 2007, and 2013. On

Table 1. Genetic features of the spring bread wheat lines with introgressions from *Aegilops speltoides*

Cultivar and lines	Cytogenetic status	Powdery mildew infection in Moscow region, %	Leaf rust infection in Moscow region, %	Comments
Rodina – control	T 1B/1R (from cultivar Kavkaz)	80	80	The cultivar is susceptible to powdery mildew, leaf and stem rust. Resistant to yellow rust
69/00[i]	Disomic substitution	0–10	$30/_{2}$ –$60/_{4}$	No data
73/00[i]	Disomic substitution 5B/5S	0	0	The line with a group resistance to fungal diseases. Resistant to brown rust the seedling stage as well. Dominant inheritance of resistance
76/00[i]	Disomic substitution 7D/7S, T 1BS/1SS and T 4BL/4SL	5–15	$20/_{2}$ –30–$40/_{3}$	The genes *Pm2*, *Lr10*, *Lr21* (STS) were identified. Based on the results of infection with test pathotypes, the adult plant resistance genes are assumed
77/00[i]	Disomic and telocentric substitutions	15–25	40–60	No data
81/00[i]	Disomic and telocentric substitutions	0–5	0	The genes *Pm2*, *Pm3c*, *Lr21*, *Lr46* were identified
82/00[i]	Disomic substitution	0–5	0	The genes *Lr10*+ and *Lr26*, *Pm3c*, *Pm4в* were identified
84/00[i]	Small translocation	10	$20/_{2}$ –$40/_{2}$ –$60/_{3}$	No data
99/00[i]	Disomic substitution and translocation	5–10	0	The genes *Lr1*, *Lr10*, *Lr21*, *Lr37*, *Lr46*, *Pm2*, *Pm4b* were identified
102/00[i]	Recombination	40	$0–1/_{0;–1}$	The genes *Lr10*, *Lr21*, *Lr35*, *Lr34* (STS) were identified; test pathotypes of fungi *Lr12*, *Lr27*, *Lr 31*
103/00[i]	Disomic substitution and translocation	10–15	40–60	No data

the experimental field of the Federal Research Center "Nemchinovka" (Moscow region), the growing was carried out from 2000 to 2011.

A row seeding scheme was used in tiers with a width of a row 1 m, five rows per sample, 50 grains per row. The soils in Novosibirsk are gray forest, in Nemchinovka – sod-podzolic. Fertilizers were applied to the soil before sowing, in accordance with the agronomic practice adopted for these soils. Harvesting was carried out manually in sheaves, followed by post-harvest ripening for a month, which is necessary for the complete formation of the gluten complex in grain.

According to the agroclimatic zoning of Russia (https://geographyofrussia.com/agroklimaticheskoe-rajonirovanie/), Moscow region and Novosibirsk are situated in the same zone of sufficient moisture for the growing season with droughts in certain years. The meteorological data of the Ogurtsovo station which is the closest in geographical position to the site of the experiments in Novosibirsk, are given in Supplementary Materials 1 and 2[1]. The number of replicates for lines in experimental plots by years in Novosibirsk is indicated in Supplementary Material 3.

Technological analysis of grain included: determination of thousand grains weight, grain vitreousness, flour particles diameter after grinding, protein and gluten content in grain. We also determined the physical properties of dough, water absorption capacity and mixing characteristics of flour obtained from the grain grown in Novosibirsk.

Thousand grains weight was determined by the express method through weighing of 100 grains. Total grain vitreousness was determined visually after cutting 100 caryopses in half. The indicator of total vitreousness is the sum of fully vitreous and half of the amount of partially vitreous grains (State Standard 10987-76). The average flour particles diameter was determined using a PSH-4 device according the previously described method (Shibayev et al., 1974; Egorov, 2000). Wet gluten content in grain in Novosibirsk was determined by hand washing in water from one gram of meal (State Standard R 54478-2011). The amount of wet gluten was expressed as a percentage of the meal weight. At the Federal Research Center "Nemchinovka" the protein and gluten content in grain was determined on a SpectraStar 2400 Infrared analyzer.

The samples were milled on a laboratory roller mill MLV-1, with a 70 % flour yield, for further research on alveograph and farinograph devices.

[1] Supplementary Materials 1–3 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx12.pdf

Physical properties of dough were determined on a Chopin alveograph equipped with a fifty-gram kneader (State Standard R 51415-99 with a modification for research work). Flour strength (W, $J \cdot 10^{-4}$), dough elasticity (P, mm) and dough extensibility (L, mm) were determined. Dough balance was calculated as the ratio of elasticity to extensibility (P/L). Water absorption capacity (WAC, %) and mixing characteristics of flour were determined on a Brabender farinograph with a fifty-gram kneader (State Standard ISO-5530-1-2013 with a modification for scientific research). WAC is amount of water (expressed as a percentage) required to form a dough with a consistency of 500 units of farinograph (u. f.). The mixing characteristics included five characteristics: dough formation time (DF, min), dough stability (DS, min), dough liquefaction (DL, u. f.). valorimetric assessment (comprehensive assessment based on the results of the study of flour on a farinograph) (VA, u. val. – valorimeter units). Electrophoresis of endosperm gliadin proteins in the lines was performed as described earlier (Pshenichnikova, Maystrenko, 1995).

Statistical analysis. The data for each trait for each genotype were averaged over all years of research (see Suppl. Material 3), and the average deviation was calculated. Student's *t*-test was used to determine the significance of differences from the control for each feature. All calculations were performed using Microsoft Office Excel 2013.

Results

The obtained results are grouped in Tables 2 (milling parameters and wet gluten content in grain), 3 (physical properties of dough) and 4 (mixing characteristics of flour). Thousand grains weight of Rodina was 29.2 g in Novosibirsk and 39.1 g in Nemchinovka (see Table 2). In Novosibirsk its grain was vitreous (80.1 %) and medium-hard (20.4 μm). Wet gluten content in grain reached 36.0 % in Novosibirsk, while in Nemchinovka this value was almost 10 % lower. The flour strength was 145 $J \cdot 10^{-4}$, the elasticity was 56 mm, and the dough extensibility was 108 mm. P/L ratio was low (0.55) (see Table 3). Water absorption capacity of flour in the cultivar was 66.6 %. Dough formation took a little over 3 minutes and it retained the stability for 2 minutes. Dough liquefaction and valorimeter number were 58 u. f. and 59 e. val., respectively (see Table 4). In terms of grain quality, the Rodina variety can be classified as a filler variety (Methods of State Variety Testing…, 1988).

According to Table 2 none of the lines surpassed the control in both geographical areas for thousand grains weight. The trait in Novosibirsk was significantly reduced by the lines 73/00^i, 77/00^i, 84/00^i, 99/00^i and 103/00^i. The smallest grain was in the first two lines, which significantly, by 8.9 and 11.6 g, differed from the control. Of these five lines, three (73/00^i, 77/00^i, 99/00^i) also significantly reduced the trait in Nemchinovka (see Table 2). The correlation between the two regions for this trait was highly significant ($r = 0.75$, $p < 0.001$).

Grain vitreousness and flour particles diameter were studied only in Novosibirsk. The lines, in general, did not differ significantly from the Rodina cultivar (see Table 2). The lines 102/00^i and 103/00^i significantly outperformed the control by 8 and 11 %, respectively. The greatest significant decrease in grain vitreousness was observed in the line 82/00^i – 63.3 %, which was accompanied by a twofold decrease in the flour particles diameter (10.0 μm) compared to the original cultivar. The other three lines (69/00^i, 84/00^i and 99/00^i) also significantly reduced the average diameter of flour particles by about 4 μm, as compared with Rodina (see Table 2).

In the lines 73/00^i, 76/00^i, 82/00^i, 84/00^i and 99/00^i, an increase in gluten content was observed in comparison with

Table 2. Average long-term indicators of milling parameters and wet gluten content in grain of introgression lines and the cultivar Rodina

Cultivar and lines	Thousand grains weight, g		Average vitreousness of grain, %	Average size of flour particles, μm	Wet gluten content in grain, %	
	Novosibirsk	Moscow region			Novosibirsk	Nemchinovka
Rodina	29.2 ± 4.5	39.9 ± 0.6	80.1 ± 3.1	20.4 ± 3.5	36.0 ± 3.5	25.8 ± 3.8
69/00^i	25.7 ± 3.6	34.5 ± 7.8	83.7 ± 6.7	15.7 ± 2.4**	37.3 ± 4.0	31.4 ± 0.8
73/00^i	20.3 ± 3.4***	32.5 ± 2.1	81.2 ± 9.163	19.6 ± 3.0	46.1 ± 6.9***	35.8 ± 3.3
76/00^i	27.6 ± 2.4	43.6 ± 0.7**	83.5 ± 7.0	20.3 ± 2.9	39.7 ± 2.8*	28.3 ± 6.6
77/00^i	17.6 ± 1.6***	30.8 ± 0.4**	88.8 ± 5.5	21.3 ± 2.2	38.9 ± 7.3	27.0 ± 3.7
81/00^i	24.1 ± 5.4	41.5 ± 0.7	79.8 ± 8.4	18.3 ± 2.0	37.4 ± 2.0	26.6 ± 0.6
82/00^i	26.1 ± 6.9	39.0 ± 1.4	63.3 ± 5.8***	10.0 ± 0.5***	47.4 ± 2.5***	32.7 ± 1.9
84/00^i	23.1 ± 4.9*	36.7 ± 6.4	77.7 ± 14.2	16.0 ± 3.0*	41.0 ± 4.6*	21.0 ± 0.6
99/00^i	22.6 ± 4.8*	35.4 ± 3.2	80.6 ± 4.7	15.9 ± 2.7*	43.6 ± 7.1*	31.5 ± 0.8
102/00^i	24.6 ± 5.9	43.5 ± 3.5	88.5 ± 5.0*	26.6 ± 6.5	33.0 ± 7.4	26.1 ± 2.6
103/00^i	24.6 ± 2.2*	41.5 ± 0.3	91.2 ± 1.0***	19.3 ± 3.2	37.1 ± 7.8	28.2 ± 0

* $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$.

Table 3. Average values of physical properties of dough in introgression lines and the cultivar Rodina, field, five-year trials (Novosibirsk)

Cultivar and lines	Dough strength, W, J·10^{-4}	Dough tenacity, P, mm	Dough extensibility, L, mm	P/L ratio
Rodina – control	143±53	56±14	108±26	0.55±0.19
69/00^{i}	339±79**	114±20***	95±14	1.25±0.33**
73/00^{i}	121±21	71±1	65±18*	1.18±0.43
76/00^{i}	272±52**	85±18**	116±27	0.79±0.32
77/0^{i}	178±21	78±2**	85±15	0.93±0.13
81/00^{i}	128±28	65±12	80±14	0.84±0.21
82/00^{i}	159±46	79±6**	72±28	1.29±0.63
84/00^{i}	166±19	80±5**	69±8**	1.16±0.11**
99/00^{i}	188±50	75±8	100±5	0.76±0.05
102/00^{i}	129±15	65±9	80±14	0.84±0.28
103/00^{i}	139±7	51±2	114±12	0.45±0.07

* $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$.

Table 4. Mixing properties of dough in introgression lines and cultivar Rodina (Novosibirsk, 2013)

Cultivar and lines	WAC, %	Dough formation, min	Dough stability, min	Dough liquefaction, u.f.	Valorimeter number, u.val.
Rodina	66.6	3′15″	2′00″	58	59
69/00^{i}	68.6	5′00″	4′00″	70	73
73/00^{i}	72.6	3′30″	2′30″	90	58
76/00^{i}	71.5	4′10″	2′20″	48	66
77/00^{i}	68.0	3′30″	1′00″	75	56
81/00^{i}	68.1	3′15″	3′15″	85	63
82/00^{i}	77.5	3′30″	1′00″	145	48
84/00^{i}	70.6	5′00″	2′00″	75	66
102/00^{i}	64.0	3′30″	2′30″	80	59

Note. WAC – water absorbing capacity.

Rodina. The highest value was found in the three lines – 73/00^{i}, 82/00^{i} and 99/00^{i} (see Table 2). The same three lines were superior to the parent variety in Nemchinovka. The average gluten content for all the years of research in Novosibirsk was 10 % higher than in Nemchinovka.

Flour strength of the lines 69/00^{i} and 76/00^{i} significantly exceeded the control, by 196 and 129 J · 10^{-4}, respectively (see Table 3). Dough elasticity of the introgression lines generally increased. In the lines 76/00^{i}, 77/00^{i}, 82/00^{i} and 84/00^{i}, this trait was significantly higher compared to the control, within the limits of 78–80 mm. Dough elasticity of the line 69/00^{i} has almost doubled. Two lines, 73/00^{i} and 84/00^{i}, showed a decreased dough extensibility. P/L ratio of the lines 69/00^{i}, 73/00^{i}, 82/00^{i} and 84/00^{i} has more than 1.0, that is, it has become more balanced. In the lines 69/00^{i} and 84/00^{i}, these changes are significant.

Mixing characteristics of flour in the lines were determined only in one year and in one replication; therefore, it is impossible to draw statistical conclusions about the reliability of the differences between the lines and the control. Nevertheless, some lines are distinguished by a number of parameters (see Table 4). WAC increased in the lines 73/00^{i}, 76/00^{i}, 82/00^{i} and 84/00^{i}. The maximum value of 77.5 % was in the line 82/00^{i} which surpassed the control by more than 10 %. The time of dough formation increased for lines 69/00^{i} and 84/00^{i} compared to the control. Only in the line 69/00^{i} dough stability has doubled. Dough liquefaction mostly increased in the lines. The worst liquefaction value was observed for the line 82/00^{i} – 145 u. f. The valorimetric number in this line was the lowest, only 48 u. val. The line 69/00^{i} had the highest valorimeter number, which outperformed the control by 14 units and consisted 73 e. val. (see Table 4).

Discussion

Currently, the works are underway to transfer the genes from wild relatives to the genome of bread wheat in order to develop a useful genetic diversity for breeding. Several studies were carried out in relation to the technological properties

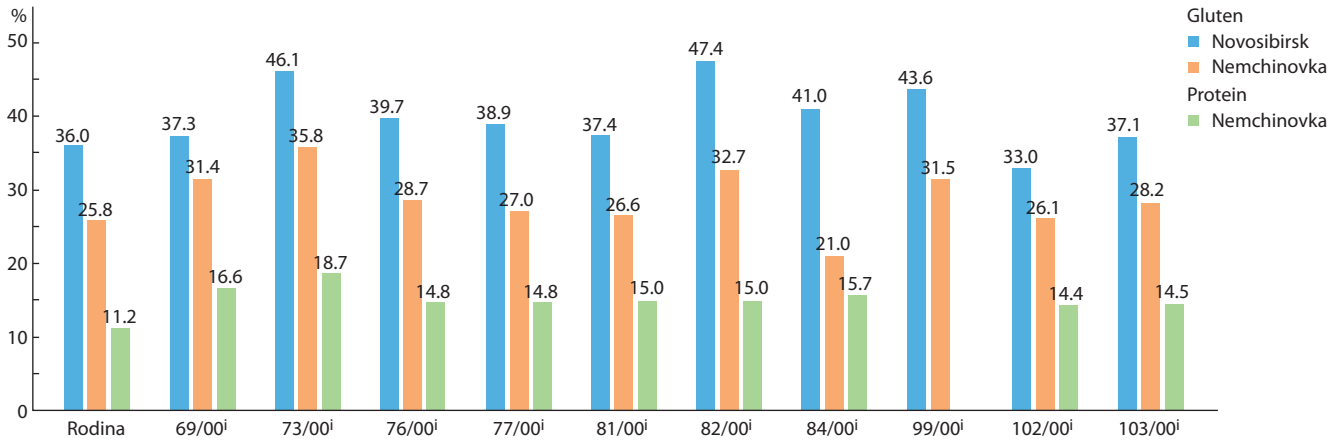

Fig. 1. Wet gluten and protein content in grain of introgression lines and the cultivar Rodina grown in Nemchinovka and wet gluten content in grain of the same lines grown in Novosibirsk.

of grain. In particular, introgression from the wild relative *Aegilops markgrafii* increased the gluten content in grain and improved other technological parameters (Shchukina et al., 2017). Krupnova (2010) showed the effect of translocations which carry *Lr* genes from wild relatives *Agropiron elongatum*, *Triticum dicoccum*, *Agropiron intermedium*, *T. dicoccoides* on the protein content in flour, IDK-1 parameters, sedimentation, falling number and grain test weight.

In our work, we investigated the influence of genetic material from *Ae. speltoides*, introgressed into the genome of bread wheat cultivar Rodina, on technological characteristics of grain and flour. The transfer of alien genetic material was confirmed by genetic, cytogenetic, and molecular methods (Salina et al., 2001; Adonina et al., 2004, 2012; Gajnullin et al., 2007). Long-term studies have shown that the lines carry genes for resistance to fungal diseases; some of them were identified (see Table 1). Milling properties, gluten content in the grain and dough physical properties were studied in the lines. Variability was found in comparison with the original Rodina cultivar for all technological characteristics studied. Some lines showed a high gluten and protein in grain, variability of milling parameters, variability of rheological and mixing properties of the dough. Ten introgression lines were studied in different years of cultivation and in different geographic regions of Russia. At the same time, it is important to note that some of the discovered new properties were stably preserved under various growing conditions.

In accordance with Russian and international trade standards gluten content in grain is the most important indicator when determining the wheat grain grade. Figure 1 shows the comparative values of wet gluten content in the grain grown in the Moscow Federal Research Center "Nemchinovka" and in Siberia. The same figure shows the protein content in grain grown in Nemchinovka. The average values in the lines and the parent cultivar grown in Novosibirsk was higher than in Nemchinovka. The difference in gluten content in Rodina was about 13 %, and in the lines, on average, 10 %. As can be seen from the data obtained, the ratio of wet gluten to protein was approximately 2 : 1. Such ratio is typical for bread wheat grain grown under normal cultivation conditions and is consistent with the data obtained by other researchers (Kozmina, 1969; Kulkarni et al., 1987). The line 73/00^{i} was superior for gluten content to all other lines under both growing conditions and showed the highest protein content in grain (see Fig. 1). The lines 76/00^{i}, 82/00^{i} and 99/00^{i}, in the Moscow region, as well as in Novosibirsk, showed high values of both traits. This suggests that introgressions resulted in the inheritance of the genes that significantly expanded the wheat diversity for protein and gluten content in grain. However, differences were also found in the manifestation of these traits. In the line 69/00^{i}, the protein and gluten content in the Non-Chernozem region was 16.6 and 31.4 %, respectively, which exceeded the control. In Novosibirsk, this line did not differ significantly from Rodina. Significant differences in two growing regions were found for the line 84/00^{i}. Under conditions of Novosibirsk it belonged to the group of lines with the highest gluten content, while in the Non-Chernozem region it did not differ from the parent cultivar (see Table 2, Fig. 1).

In the work of Adonina et al. (2012), using fluorescent hybridization with the Spelt1 and pSc119.2 probes in combination with microsatellite markers it was shown that the line 73/00^{i} carries translocations into the short arm of chromosome 1B and the long arm of chromosomes 5B and 6B. Subsequently, these translocations were transferred to separate lines based on the Rodina cultivar (Adonina et al., 2012). The original line 73/00^{i} possesses a group resistance to a spectrum of fungal diseases (see Table 1). But only the obtained line with translocation into 5B chromosome had the maximum resistance to leaf rust (Adonina et al., 2012). Earlier, in this chromosome the loci were found responsible for the high protein and gluten content in grain (Gonzalez-Hernandez et al., 2004; Pshenichnikova et al., 2012). The line 73/00^{i} was distinguished by a decrease in thousand grains weight and accumulation of a high amount of protein and gluten under various conditions. The water absorption capacity of the dough in the line was increased, which is probably due to the high amount of gluten and protein in grain. However, the flour strength was reduced, and dough liquefaction was increased compared to the control which is undesirable when used for food purposes. At the same time, such wheat genotypes can

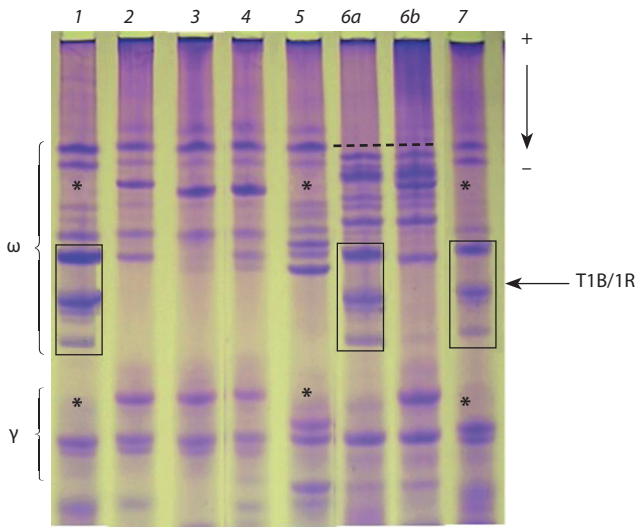

Fig. 2. Electrophoregram of gliadin components in the introgression lines.

The arrow indicates rye secalins introduced by the 1BS/1RS translocation. The asterisks indicate the components of γ- and ω-gliadin that are absent as a result of translocation. The dashed line indicates the rearrangement in locus *Gli-D1*. *1* – 84/00^{i}; *2* – 82/00^{i}; *3* – 76/00^{i}; *4* – cultivar Rodina; *5* – 73/00^{i}; *6a*, *6b* – 69/00^{i}; *7* – 81/00^{i}.

be a valuable source of vegetable protein in the production of feed for livestock and fish farming.

Another line – 82/00^{i}, which is characterized by a consistently high protein and gluten content (see Table 2, Fig. 1), simultaneously demonstrated a significant decrease in the vitreousness of the grain and the average diameter of flour particles in comparison with Rodina. The line showed a very high water absorption capacity and a high dough liquefaction. According to the literature data, the *Ha* locus, located in the subtelomeric region of the short arm of 5D chromosome, is responsible for the hardness and vitreousness of bread wheat (McIntosh et al., 2013). Two closely linked dominant genes in this locus *Pina-D1* and *Pinb-D1*, which encode proteins puroindolines are responsible for the variability of the endosperm structure.

Earlier it was found that the winter line 84/00^{w} from the Arsenal collection with soft-grain endosperm carries the introgression from *Ae. speltoides* in the form of an entire 5S/5A chromosome substitution. It carries the locus *Ha-Sp* homeoallelic to the locus *Ha* (Pshenichnikova et al., 2010). Subsequently, on the basis of the line 84/00^{w}, spring supersoft-grain lines were obtained. They combine in their genotypes the homeoallelic loci *Ha-Sp* of the line 84/98^{w} and *Ha* the latter being obtained from the soft-grain cultivar Chinese Spring (Simonov et al., 2017). These lines are characterized by a supersoft, mealy endosperm of the caryopsis and by a vitreousness of less than 50 % and flour particle size of 10–12 μm. According to cytogenetic data (see Table 1), the line 82/00^{i} carries a disomic substitution as in the line 84/00^{w}. However, the line 82/00^{i} is spring, and it can be assumed that introgression affected only the short arm of chromosome 5A. New spring supersoft grain lines can be obtained using the line 82/00^{i}. Flour of such lines is suitable for the manufacture of confectionery products without the use of technological additives (Peña, 2002).

The same introgression in the proposed region could lead to an increase in wet gluten content in grain. Earlier, in the Weimai × Yannong hybrid wheat population, in the region of chromosome 5A marked with the molecular markers *Xcfa2163.2-Xcwm216* the main locus *QGpc.WY-5A.1* responsible for 53 % of the phenotypic variability for protein accumulation in grain, and the locus *QWgc.WY-5A.2* responsible for 36 % of the phenotypic variability for wet gluten content were co-localized (Li et al., 2012). It should also be noted that the line 82/00^{i}, in contrast to the line 73/00^{i}, had thousand grains weight comparable to the original variety (see Table 2). This indicates the possibility of selection for high gluten and protein content without loss of yield. The line was characterized by a high resistance to fungal diseases carrying the identified resistance genes *Lr10* and *Lr26*, *Pm3c*, *Pm4b* (see Table 1). This level of resistance possibly is also provided by introgression.

The original cultivar Rodina was characterized by low rheological and mixing properties. The cultivar Kavkaz – the carrier of the 1BS/1RS rye translocation is present in its pedigree. The cultivar was heterogeneous for this trait. This translocation is known to significantly impair dough physical properties (Martin, Stewart, 1990) since it affects the composition of high molecular weight glutenins and gliadins. These gluten proteins determine the balance between the elasticity and extensibility of dough. By analyzing the component composition of gliadins (Fig. 2), we selected the line of Rodina which does not contain the translocation. Nevertheless, the physical properties of this line remained low (see Tables 2, 3). According to electrophoretic data, the 1BS/1RS translocation was inherited by the lines 81/00^{i}, 84/00^{i} and 69/00^{i} (see Fig. 2). The last line was heterogeneous for this trait. Basically, these lines were also characterized by a low flour strength like Rodina. Only one line, 69/00^{i}, showed the average value of flour strength allowing to classify the line as strong in quality and use it as an improver for baking purposes. The line 69/00^{i} is characterized by the absence of the most slowly moving components of the ω-fraction of gliadins (see Fig. 2) which are controlled by the *Gli-D1* locus of chromosome 1DS (Pshenichnikova, Maystrenko, 1995). The locus is closely linked to the *Glu-D1* locus, coding high-molecular subunits of glutenins, which largely determine the strength of flour and elasticity. Probably, this region of the chromosome has undergone a recombination as a result of distant crossing. Interestingly, the presence of the 1BS/1RS translocation does not impair the physical properties of this line.

Disomic substitutions were found in the lines 76/00^{i} and 81/00^{i} by cytological methods. Molecular methods identified the complete substitution of 7D chromosome for 7S chromosome from *Ae. speltoides* (Adonina et al., 2004). The line 81/00^{i} additionally carries the 1BS/1RS translocation, and the line 76/00^{i} carries a translocation into the short arm of chromosome 3A. The line 76/00^{i} differs from the line 81/00^{i} in a number of technological parameters for the better. It can be classified as valuable in quality and used as an improver. Gluten content in the line 76/00^{i} was significantly increased; gluten was of better quality. Flour strength in the line reached

272 J · 10^{-4}, and the dough became more elastic (see Table 3). Mixing flour parameters and water absorption capacity have improved (see Table 4). It can be assumed that the substitution of chromosome 7D for 7S has a positive effect on the quality parameters of flour if the rye secalins are absent in gluten composition. In addition, it was shown that the line 76/00[i] carries the 1BS/1SS translocation (see Table 1). Earlier, it has already been noted that the introgression into the short arms of the first homoeologous group chromosomes from species of the genus *Aegilops* improves baking properties (Alvarez, Guzmán, 2018).

Conclusion

Ten spring lines from the 'Arsenal' collection, selected initially for the resistance to powdery mildew or leaf rust, were for the first time studied for a wide range of technological parameters, including milling parameters, gluten and protein content in grain and physical properties of flour and dough. Our research has shown that introgressions from the species *Ae. speltoides* significantly expand the genetic diversity of common wheat for these properties and, as a consequence, the possibilities of the final use of grain and flour. In this work, the lines were identified that combine the new variability for various technological traits with the resistance to various fungal diseases. The lines generally retained their characteristics under different growing conditions in different growing years. They can be involved in breeding work as donors of a complex of agronomically valuable traits.

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Acknowledgements. Technological analysis of grain was fulfilled with the support of Russian Science Foundation project No. 16-16-00011-P. Cultivation in the field was carried out in the conditions of the Center for Collective Use "Breeding and Genetic Laboratory" with the support of the budget project No. 0324-2019-0039-C-01.

Conflict of interest. The authors declare no conflict of interest.

Received July 31, 2020. Revised September 16, 2020. Accepted October 9, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):747-754
DOI 10.18699/VJ20.669

The pattern of genetic diversity of different breeds of pigs based on microsatellite analysis

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Abstract. One of the main tasks of genetics and animal breeding is the assessment of genetic diversity and the study of genetic relationships between different breeds and populations using molecular genetic analysis methods. We analysed the polymorphism of microsatellites and the information on the state of genetic diversity and the population structure of local breeds in Russia: the Kemerovo, the Berkshire, the Liven, the Mangalitsa, and the Civilian; in the Republic of Belarus: the Large White and the Black-and-White; and in Ukraine: the White Steppe, as well as commercial breeds of imported origin of domestic reproduction: the Large White, the Landrace, and the Duroc. The materials used for this study were the tissue and DNA samples extracted from 1,194 pigs and DNA of the UNU "Genetic material bank of domestic and wild animal species and birds" of the L.K. Ernst Federal Research Center for Animal Husbandry. Polymorphisms of 10 microsatellites (S0155, S0355, S0386, SW24, SO005, SW72, SW951, S0101, SW240, and SW857) were determined according to the previously developed technique using DNA analyser ABI3130xl. To estimate the allele pool of each population, the average number of alleles (N_A), the effective number of alleles (N_E) based on the locus, the rarified allelic richness (A_R), the observed (H_O) and expected (H_E) heterozygosity, and the fixation index (F_{IS}) were calculated. The degree of genetic differentiation of the breeds was assessed based on the pairwise values of F_{ST} and *D*. The analysis of the allelic and genetic diversity parameters of the local breeds showed that the maximum and minimum levels of polymorphism were observed in pigs of the Ukrainian White Steppe breed (N_A = 6.500, N_E = 3.709, and A_R = 6.020) and in pigs of the Duroc breed (N_A = 4.875, N_E = 2.119, and A_R = 3.821), respectively. The highest level of genetic diversity was found in the Large White breed of the Republic of Belarus (H_O = 0.707 and N_E = 0.702). The minimum level of genetic diversity was found in pigs of the imported breeds – the Landrace (H_O = 0.459, H_E = 0.400) and the Duroc (H_O = 0.480, H_E = 0.469) – indicating a high selection pressure in these breeds. Based on the results of phylogenetic analysis, the genetic origin of Large White pigs, the breeds, from which the Berkshire pigs originated, and the genetic detachment of the Landrace from the Mangalitsa breeds were revealed. The cluster analysis showed a genetic consolidation of the Black-and-White, the Berkshire, and the Mangalitsa pigs. Additionally, the imported breeds with clustering depending on the origin were characterised by a genetic structure different from that of the other breeds. The information obtained from these studies can serve as a guide for the management and breeding strategies of the pig breeds studied, to allow their better use and conservation.
Key words: pig breeds; microsatellites; genetic diversity.

For citation: Kharzinova V.R., Zinovieva N.A. The pattern of genetic diversity of different breeds of pigs based on microsatellite analysis. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7): 747-754. DOI 10.18699/VJ20.669

Паттерн генетического разнообразия у локальных и коммерческих пород свиней на основе анализа микросателлитов

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Аннотация. Одной из основных задач генетики и селекции животных является оценка генетического разнообразия и исследование генетических взаимоотношений между различными породами и популяциями с помощью методов молекулярно-генетического анализа. Нами проведен анализ полиморфизма микросателлитов и получена информация о состоянии генетического разнообразия и структуры популяций локальных пород свиней, разводимых на территории России (кемеровская, беркширская, ливенская, мангалица, цивильская), Республики Беларусь (крупная белая, черно-пестрая), Украины (степная белая), а также коммерческих пород импортного происхождения отечественной репродукции (крупная белая, ландрас, дюрок). Материалом для исследований служили пробы ткани 1194 образцов свиней из биоресурсной коллекции

«Банк генетического материала животных и птиц» ФГБНУ ФИЦ ВИЖ им. Л.К. Эрнста. Полиморфизм 10 STR-локусов (S0155, S0355, S0386, SW24, SO005, SW72, SW951, S0101, SW240, SW857) определяли по ранее разработанной методике с помощью генетического анализатора ABI3130xl (Applied Biosystems, США). Для оценки аллелофонда каждой породы рассчитывали среднее число аллелей (N_A) и эффективное число аллелей (N_E) на локус, аллельное разнообразие (A_R), вычисленное с применением процедуры рарификации, наблюдаемую (H_O) и ожидаемую (H_E) гетерозиготность, индекс фиксации (F_{IS}). Степень генетической дифференциации пород оценивали на основании попарных значений F_{ST} и *D*. Анализ параметров аллельного и генетического разнообразия локальных пород показал максимальный уровень полиморфности у свиней украинской степной породы (N_A = 6.500, N_E = 3.709, A_R = 6.020), а минимальный – у свиней породы дюрок (4.875, 2.119 и 3.821 соответственно). Наиболее высокий уровень генетического разнообразия выявлен у свиней крупной белой породы Республики Беларусь (H_O = 0.707, H_E = 0.702). Минимальный уровень генетического разнообразия установлен у свиней импортных пород ландрас (H_O = 0.459, H_E = 0.400) и дюрок (H_O = 0.480, H_E = 0.469), что, возможно, указывает на высокое давление отбора в этих породах. По результатам филогенетического анализа выявлена генетическая обособленность пород свиней корня крупной белой породы, в создании которых принимали участие беркширские свиньи, и отдаленность пород ландрас и мангалица. Кластерный анализ показал генетическую консолидированность свиней пород черно-пестрая, беркширская и мангалица. Отличной от других пород генетической структурой характеризовались также импортные породы свиней с кластеризацией в зависимости от происхождения. Информация, полученная в ходе исследований, может служить руководством для стратегий управления и разведения изученных пород свиней с целью лучшего их использования и сохранения.

Ключевые слова: породы свиней; микросателлиты; генетическое разнообразие.

Introduction

Currently, the industrial production of pork is based on the use of a limited number of commercial breeds of imported pigs. These breeds are well adapted for use in intensive production systems, aimed at maximising the genetic potential of productivity (Muñoz et al., 2019). Along with breeds of imported origin, there are local breeds that are carriers of unique forms of variability and constitute the national genetic resources of agricultural animal species. Despite their small size, local breeds have not lost their importance in the modern conditions of the development of animal husbandry. Having a lower productivity compared to commercial ones, such breeds are characterised by a greater individual variability, constitutional strength, stress resistance and good adaptation to local climatic conditions (Kharzinova et al., 2017).

Nowadays, local breeds are considered irreplaceable genetic resources for the creation of geographically oriented systems for organic production of livestock products. According to Stolpovsky (2013), due to the inclusion of transnational livestock industries in the agriculture world, there is a danger of a reduction of the national genetic resources, dependence on food imports, and breeding achievements, and there is a threat of globalisation of the spread of infections and hidden genetic defects. This implies an increasing importance not only to study the gene pool of species of foreign origin of the animals but also the conservation of genetic resources of the local breeds.

According to the guidelines for the development of national plans for the management of farm animal genetic resources (FAO, 1998), FAO proposes an integrated global management of farm animal genetic resources using microsatellite reference markers (short tandem repeats, STR) (Egito et al., 2007). To date, there are many publications that show the applied importance of STR for characterising the genetic diversity and structure of pig breeds for commercial (Zinovieva et al., 2012; Vrtková et al., 2012; Szmatoła et al., 2016) and local breeding (Kaul et al., 2002; Kramarenko et al., 2018). However, comparative studies of the entire variety of local and commercial breeds of pigs bred in Russia have not yet been carried out.

The aim of this study was to characterise the genetic diversity and population structure of eight local and three commercial pig breeds based on the analysis of microsatellites.

Materials and methods

The object of research was biological material obtained from 1,194 pigs, which was stored in the UNU "Genetic material bank of domestic and wild animal species and birds" of the L.K. Ernst Federal Research Centre for Animal Husbandry. Tissue samples (ear pinch) were used as biological material. The presented sample included eight local breeds bred in Russia: the Kemerovo (Kemerovo region, KEM, *n* = 35), the Berkshire (Yaroslavl region, BERK, *n* = 80), the Liven (Orel region, LIV, *n* = 67), the Mangalitsa (Altai Territory, MNG, *n* = 52), the Civilian (Republic of Chuvashia, CVL, *n* = 43); The Republic of Belarus: the Large White (BLW, *n* = 47) and the Black-and-White (BBP, *n* = 98); and Ukraine: the White Steppe (LWUK, *n* = 61), as well as three commercial breeds of imported origin of domestic reproduction, bred in the breeding and genetic centres of Oryol, Voronezh, and Lipetsk regions: the Large White (LW, *n* = 241), the Landrace (LDR, *n* = 250), and the Duroc (DUR, *n* = 223).

DNA isolation was performed using DNA-Extraction kits for genomic DNA isolation (ZAO "Syntol", Russia), in accordance with the manufacturer's protocol. Analysis of polymorphisms of ten microsatellites (S0155, S0355, S0386, SW24, SO005, SW72, SW951, S0101, SW240, and SW857) was carried out according to the previously described method (Kharzinova et al., 2018). The results of the amplified fragments were visualised using fragment analysis by using Gene Mapper v. 4 software (Applied Biosystems, USA).

Analysis of population genetic parameters, the degree of genetic differentiation based on matrices of pairwise values of F_{ST} and *D* and the construction of phylogenetic trees using the Neighbor-Net algorithm were performed in GenAlEx 6.503

(Peakall, Smouse, 2012), SplitsTree 4.14.5 software (Huson, Bryant, 2006) and R package 'diveRsity', with subsequent visualisation using the package 'pophelper' (Keenan et al., 2013).

The genetic structure of the studied pig breeds was assessed using principal component analysis (PCA) in R package 'adegenet' (Jombart, 2008) and was visualised using R package 'ggplot2' (Wickham, 2009) and through clustering using STRUCTURE 2.3.4 software (Pritchard et al., 2000), using a mixed model (the number of assumed clusters, K – from 1 to 20; length of the burn-in period – 100,000; model of Markov chains of Monte Carlo – 100,000). For each value of K, 10 iterations were performed. Structure Harvester (Earl, von Holdt, 2012) was used to determine the optimal number of clusters (ΔK), according to the method proposed by Evanno et al. (2005). Source files were generated in Microsoft Excel format and R 3.5.0 software environment (R Core Team).

Results and discussion

In the analysis of genotypes of ten microsatellites for the entire sample, 69 alleles were detected, which exceeded the number of alleles (48 alleles) detected in the molecular genetic analysis of the Chinese pig breed with a similar number of markers (Yue, Wang, 2003). Locus SW951 had the lowest number of alleles (5 alleles). A similar trend for this locus was revealed in studies of pigs bred in Ukraine (2 alleles) (Kramarenko et al., 2018) and Thailand (7 alleles) (Charoensook et al., 2019). The greatest number of alleles (22) was found at the SO005 locus, which was consistent with the results of the studies by Guastella et al. (2010) and Šalamon et al. (2019), in which this locus exceeded the others in the number of alleles: 19 and 17 alleles, respectively. The minimum mean values of both observed (H_O) and expected (H_E) heterozygosity were noted at the SW951 locus: 0.437 ± 0.067 and 0.482 ± 0.071, respectively. Locus SW857 had the maximum values of indicators: H_O = 0.868 ± 0.018 and H_E = 0.783 ± 0.018.

The analysis of the distribution of genotype frequencies to the Hardy–Weinberg genetic equilibrium for the entire sample (Table 1) showed significant deviations from the state of genetic equilibrium at individual loci in all the breeds studied. In Landrace pigs, deviations from the genetic equilibrium were found at all loci, in Duroc and Large White pigs, at nine and eight loci, respectively. It should be noted that local breeds of pigs were inferior to commercial breeds in terms of the number of loci with significant deviations from the state of genetic equilibrium. The number of such loci varied from three in the Liven breed to seven in the Ukrainian White Steppe breed. These findings can indicate greater selection pressures in commercial pig breeds, compared to local breeds. Of the ten studied loci, highly significant deviations from the genetic equilibrium were established for the SO005 locus, according to Hardy–Weinberg ($p < 0.001$).

An interesting research result published by Kramarenko et al. (2018) showed that in pigs of the Duroc breed bred in the regions of Ukraine, eight out of twelve loci had insignificant deviations from the state of genetic equilibrium.

To assess the degree of genetic diversity of populations and breeds, two main indicators are most often used – the level of polymorphism and the degree of homozygosity (heterozygosity) (Khrabrova et al., 2011), whose results are presented in Table 2. The minimum values of the average number of alleles per locus (N_A = 4.875) were observed in three breeds: CVL, MNG, and DUR, and the maximum values (more than 6.000) were observed in Landrace pigs (LDR, N_A = 6.001) and in the Large White breed bred in the territories of our country (LW, N_A = 6.250) and in Ukraine (LWUK,

Table 1. Results of the test of ten microsatellites in the analysis of the studied breeds of pigs, for compliance with the Hardy–Weinberg genetic equilibrium

Breed	Locus STR									
	SW24	S0155	SO005	SW72	SW951	S0386	S0355	SW240	SW857	S0101
LDR	**	**	***	***	**	**	*	***	***	***
CVL	*	ns	***	ns	ns	***	***	ns	ns	ns
LIV	ns	*	**	ns	ns	ns	ns	**	ns	ns
BLW	*	ns	***	ns	ns	ns	ns	*	ns	*
MNG	ns	ns	***	*	ns	*	***	ns	***	ns
BERK	ns	**	***	ns	***	ns	***	ns	**	ns
KEM	*	ns	***	ns	ns	**	*	ns	**	**
LWUK	ns	**	***	*	*	***	ns	***	ns	*
BBP	ns	ns	***	ns	ns	***	**	ns	*	ns
DUR	**	***	***	***	***	ns	***	***	***	***
LW	***	***	***	ns	***	***	ns	***	***	***

Note. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns – not significant.
Breed hereinafter: KEM – the Kemerovo, BERK – the Berkshire, LIV – the Liven, MNG – the Mangalitsa, CVL – the Civilian; BLW – the Large White and BBP – the Black-and-White of The Republic of Belarus, LWUK – the White Steppe of Ukraine, LW – the Large White, LDR – the Landrace, DUR – the Duroc.

Table 2. Parameters of genetic diversity of the studied breeds of pigs based on the microsatellite analysis

Breed	*n*	N_A	N_E	A_R	H_O	H_E	F_{IS} (F_{IS} 95 %, CI > 0)
CVL	43	4.875 ± 0.398	2.807 ± 0.295	4.810 ± 0.391	0.590 ± 0.074	0.611 ± 0.047	0.059 [–0.100; 0.218]
LIV	67	5.375 ± 0.596	2.979 ± 0.306	5.073 ± 0.514	0.672 ± 0.041	0.639 ± 0.037	–0.060 [–0.159; 0.039]
BLW	47	5.000 ± 0.535	3.672 ± 0.492	4.934 ± 0.527	0.707 ± 0.052	0.702 ± 0.029	–0.002 [–0.088; 0.084]
MNG	52	4.875 ± 0.639	2.723 ± 0.376	4.659 ± 0.613	0.524 ± 0.113	0.545 ± 0.095	0.100 [–0.120; 0.320]
BERK	80	5.125 ± 0.441	2.789 ± 0.206	4.769 ± 0.342	0.575 ± 0.048	0.627 ± 0.028	0.079 [–0.062; 0.220]
KEM	35	5.125 ± 0.611	3.246 ± 0.443	5.444 ± 0.626	0.550 ± 0.055	0.644 ± 0.054	0.139 [0.034; 0.244]
LWUK	61	6.500 ± 0.802	3.709 ± 0.427	6.020 ± 0.657	0.627 ± 0.043	0.709 ± 0.028	0.118 [0.041; 0.195]
BBP	98	5.375 ± 0.595	3.057 ± 0.331	4.828 ± 0.550	0.645 ± 0.062	0.639 ± 0.049	–0.008 [–0.097; 0.081]
DUR	223	4.875 ± 0.295	2.119 ± 0.274	3.821 ± 0.305	0.480 ± 0.088	0.469 ± 0.070	–0.014 [–0.178; 0.150]
LW	241	6.250 ± 0.559	3.349 ± 0.467	5.126 ± 0.518	0.651 ± 0.047	0.672 ± 0.030	0.036 [–0.039; 0.111]
LDR	249	6.001 ± 0.463	2.396 ± 0.492	4.634 ± 0.475	0.459 ± 0.095	0.490 ± 0.073	0.098 [–0.037; 0.233]

Note. *n* – the number of samples; N_A – the average number of alleles per locus; N_E – the number of effective alleles per locus; A_R – allelic diversity; H_O – the observed heterozygosity; H_E – the expected heterozygosity; F_{IS} – inbreeding coefficient with a 95 % confidence interval.

N_A = 6.500). The values of the number of effective alleles per locus (N_E) ranged from 2.119 (DUR) to 3.709 (LWUK).

Another measure that characterises the level of polymorphism is allelic diversity (A_R), which is considered as a strong indicator of the evolutionary potential of a population (Allendorf, 1986; Caballero, García-Dorado, 2013), and it has been suggested that this indicator is of key importance for the conservation and management of the population (Greenbaum et al., 2014). The minimum values of this indicator, which were corrected using the rarefaction method, were detected for DUR – A_R = 3.821, and were maximal in LWUK – A_R = 6.020. According to Greenbaum et al. (2014), a decrease in allelic diversity may lead to a decrease in the population's ability to adapt to future environmental changes. Moreover, according to Wagner (2008), there is evidence that a high allelic diversity, even of simple neutral alleles, increases the evolutionary potential by making less genotypic space available for mutational events.

To date, the most commonly used indicators of the genetic characteristics of populations presented in most studies (Vonholdt et al., 2008; Toro et al., 2009; Andras et al., 2011) are the observed (H_O) and expected (H_E) heterozygosity (Greenbaum et al., 2014). The H_O in the studied breeds of pigs ranged from 0.459 ± 0.095 for LDR to 0.707 ± 0.052 for BLW. According to some authors, a decrease in H_O can lead to a decrease in the average fitness of individuals, and, therefore, this indicator has clear ecological consequences (Reed, Frankham, 2003; Szulkin et al., 2010). Moderate levels of H_E (above 0.5) were observed in nine pig breeds, ranging from 0.545 ± 0.095 for MNG, to 0.709 ± 0.028 for LWUK. Pigs of the Duroc and the Lansrace breeds were an exception, in which this indicator had minimum values: 0.469 ± 0.070 and 0.490 ± 0.073, respectively.

According to the fixation index values, a slight lack of heterozygotes was found in seven breeds of pigs (CVL, MNG, BERK, KEM, LWUK, LW, and LDR) with a variation of positive values of the indicator from 0.036 for LW, to 0.139 for KEM. However, for these breeds, with the exception of KEM and LWUK, the 95 % confidence interval (CI) of the fixation index included the zero value, which indicates non-significant deviations in the number of heterozygotes from the theoretically expected, in these breeds. A slight shift in the genetic balance towards an excess of heterozygotes was noted in four breeds: LIV, BLW, BBP, and DUR, in which the fixation index had negative values, which amounted to 0.060, 0.002, 0.008 and 0.014, respectively.

Among the local breeds, the maximum level of polymorphism was observed in LWUK (N_A = 6.500, N_E = 3.709, A_R = 6.020), and the maximum level of genetic diversity was found in BLW (H_O = 0.707, H_E = 0.702). At the same time,

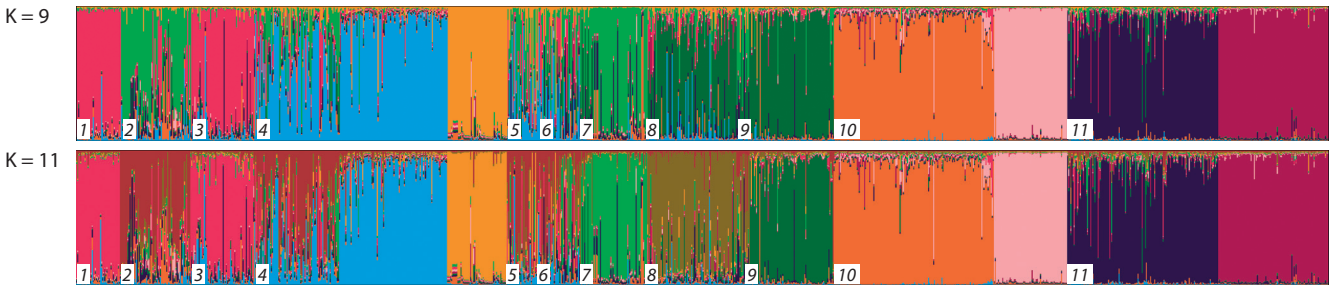

Fig. 1. Results of the cluster analysis of eleven pig breeds based on microsatellites using the STRUCTURE 2.3.4 software.

Breeds: *1* – Civilian; *2* – Liven; *3* – White Steppe of Ukraine; *4* – Large White; *5* – Large White of The Republic of Belarus; *6* – Kemerovo; *7* – Mangalitsa; *8* – Black-and-White of The Republic of Belarus; *9* – Berkshire; *10* – Duroc; *11* – Landrace.

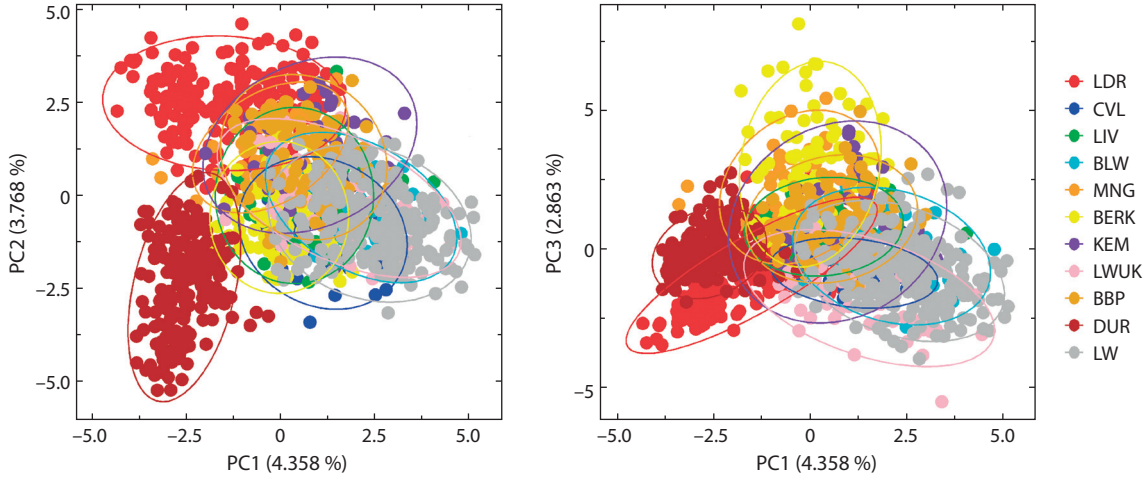

Fig. 2. Projection of the studied samples of the pig breeds on the plane of two coordinates according to PCA analysis.

pigs of the Mangalitsa breed had minimum values for all the analysed parameters: N_A = 4.875, N_E = 2.723, A_R = 0.659, H_O = 0.524, and H_E = 0.545. However, in an study by Druml et al. (2012), the values of genetic parameters characterising the level of genetic diversity of pigs of the Mangalitsa breed of Austria and the National Reserve of Serbia were even lower: N_A = 3.8, H_O = 0.49, and H_E = 0.54 and N_A = 3.94, H_O = 0.58, and H_E = 0.54, respectively. When comparing the animals of imported origin of domestic reproduction, the group of large white pigs exceeded the other two in all aspects: N_A = 6.250, N_E = 3.349, A_R = 5.126, H_O = 0.651, and H_E = 0.672. Of all the studied pig breeds, the minimum level of polymorphism and genetic diversity was found in the Duroc breed: N_A = 4.875, N_E = 2.119, A_R = 3.821, H_O = 0.480, and H_E = 0.469. A similar trend towards a relatively low level of genetic diversity of this breed was noted in the works of other authors. In a comparative analysis of local breeds of Brazil with pigs of specialised breeds (Duroc, Landrace, and Large White), the minimum values of both the average number of alleles per locus and the effective number of alleles were identified in the Duroc breed, which amounted to N_A = 3.65 and N_E = 3.01 (da Silva et al., 2011). In his work, Szmatoła et al. (2016), while studying the genetic diversity of four commercial breeds and one local breed of pigs in Poland using five microsatellites, he revealed the lowest values of the average number of alleles per locus (N_A = 4.6), the number of effective alleles per locus (N_E = 2.78) and allelic diversity (A_R = 4.6). However, the studies by Kim et al. (2005), which focused on the description of the genetic diversity and population structure of four European, two Korean and three Chinese pig breeds, showed that Duroc pigs outnumbered others in these parameters. At the same time, local Korean pigs showed consistently low levels of allelic diversity and heterozygosity, while Chinese pig breeds, except for the Wuzhishan breed, had a relatively high degree of genetic diversity compared to commercial and local Korean pig breeds. The lower values of population genetic parameters detected in our work, both in pigs of the Mangalitsa and the Duroc breeds, possibly indicates a high selection pressure and a minimal or no migration of new genes in the breeds.

To assess the genetic structure of the studied pig breeds, Bayesian cluster analysis was carried out using STRUCTURE (Fig. 1), as well as coordination analysis, using PCA (Fig. 2). Despite the fact that the algorithm based on the values of ΔK (Earl et al., 2012) revealed that the optimal number of clusters for this sample is equal to 9, K = 9 (ΔK = 136.79), the results at K = 11 were also presented.

The breeds LIV, BLW, and LW are characterised by a mixed genetic origin. In addition, a similar genetic pattern was observed in the CVL and LWUK breeds. A clear genetic

Table 3. Genetic distances between the studied breeds of pigs based on the microsatellite analysis

Breed	CVL	LIV	BLW	MNG	BERK	KEM	LWUK	BBP	DUR	LW	LDR
CVL	0	0.101	0.158	0.175	0.271	0.212	0.097	0.207	0.176	0.153	0.250
LIV	0.098	0	0.107	0.147	0.187	0.138	0.195	0.122	0.189	0.168	0.186
BLW	0.098	0.062	0	0.242	0.267	0.113	0.112	0.110	0.285	0.064	0.271
MNG	0.171	0.114	0.132	0	0.265	0.146	0.269	0.185	0.246	0.225	0.150
BERK	0.195	0.120	0.135	0.178	0	0.200	0.277	0.113	0.191	0.221	0.224
KEM	0.152	0.088	0.071	0.099	0.125	0	0.179	0.113	0.270	0.151	0.143
LWUK	0.094	0.125	0.086	0.169	0.168	0.116	0	0.178	0.267	0.077	0.238
BBP	0.150	0.080	0.062	0.118	0.088	0.064	0.120	0	0.249	0.132	0.144
DUR	0.189	0.158	0.199	0.208	0.181	0.208	0.222	0.195	0	0.291	0.186
LW	0.111	0.102	0.037	0.162	0.131	0.107	0.083	0.096	0.190	0	0.224
LDR	0.244	0.171	0.190	0.146	0.189	0.140	0.171	0.115	0.222	0.185	0

Note. *D* values are shown above the diagonal. The F_{ST} values are below the diagonal for pairwise comparison.

structure has been identified in black-and-white, Berkshire, and Mangalitsa pigs. The formation of several clusters of breeds of imported origin is explained by both different origins and different strategies of selection and the breeding work used in the enterprises.

Principal component analysis, the key feature that enables the projection of samples onto orthogonal coordination axes, each of which consisting of a linear combination of allelic or genotypic values (Patterson et al., 2006; Novembre et al., 2008), revealed a genetic mixing and enabled the visualisation of a slight differentiation for most of the studied breeds. Independent cluster formed by representatives of commercial breeds (Duroc, Landrace, and Large White); at the same time, local breeds formed overlapping arrays. According to Jolliffe and Cadima (2016), the lack of clear clustering does not mean the absence of differences but may indicate the similarity of the largest source of variability. In addition, this analysis made it possible to characterise the range of variability in three components. The first component was responsible for most of the genetic variability of the entire data set (4.3 %), while the second and third components reflected 3.7 and 2.8 % of genetic variability, respectively.

To assess the degree of differentiation of populations, two main classes of indicators that determine the quantitative structure of populations are used: fixation indices F_{ST} and Nei's G_{ST}, and indicators of allelic differentiation, such as Jost's *D* and differential entropy (Jost et al., 2018). One of the most commonly used indicators in population genetic studies is the standard method for estimating the F_{ST} fixation index, described by Weir and Cockerham (1984). However, when calculating the genetic distances based on the variability of highly polymorphic markers, the values of the indicator may be shifted (Meirmans, Hedrick, 2011; Hopper et al., 2018). Therefore, we additionally performed calculations of the *D* index proposed by Jost (2008) that takes into account the proportion of allelic variations in populations (Table 3).

The greatest genetic affinity for both indicators was found for pigs of the Russian and Belarusian populations of the Large White breed: LW/BLW F_{ST} = 0.037, D = 0.064. However, regarding the maximum values of the indices, differences were detected: the greatest genetic distance, according to the F_{ST} fixation index, is characteristic of the LDR/CVL = 0.244 group, and the LW/DUR = 0.291 group, according to the *D* index.

To visualise the genetic degree of closeness of the studied pig breeds, the numerical matrices of the pairwise genetic distances, F_{ST} and *D*, were visualised using the Neighbor-Net algorithm and are presented in Figure 3. A separate massif was formed by groups of root pigs of a large white breed (CVL, LWUK, LW, and BLW) and adjacent branches of the Kemerovo and Liven breeds. A separate branch is a cluster of breeds in the creation of which pigs with the blood of the Berkshire breed took part: BBP, BERK, and DUR. The Landrace and the Mangalitsa animals are presented in a separate cluster.

Conclusion

Our studies were aimed at analysing the genetic diversity and studying the relationship between eight local pig breeds and three pig breeds of imported origin of domestic reproduction. In general, the studied local breeds exceeded the groups of imported pigs in both allelic and genetic diversity, which is probably explained by the lack of a practical program of continuous improvement of specific characteristics, to which commercial breeds are subject. On the contrary, the maximum positive values of the fixation index were detected in local breeds (Kemerovo and Ukrainian White Steppe), which can lead to a shift in the genetic equilibrium towards a lack of heterozygotes. The analysis of the main components, carried out on the basis of the allele frequencies of the studied breeds of pigs, made it possible to characterise the range of variability and trace the main patterns of population genetic differentiation of individuals of the studied breeds of pigs.

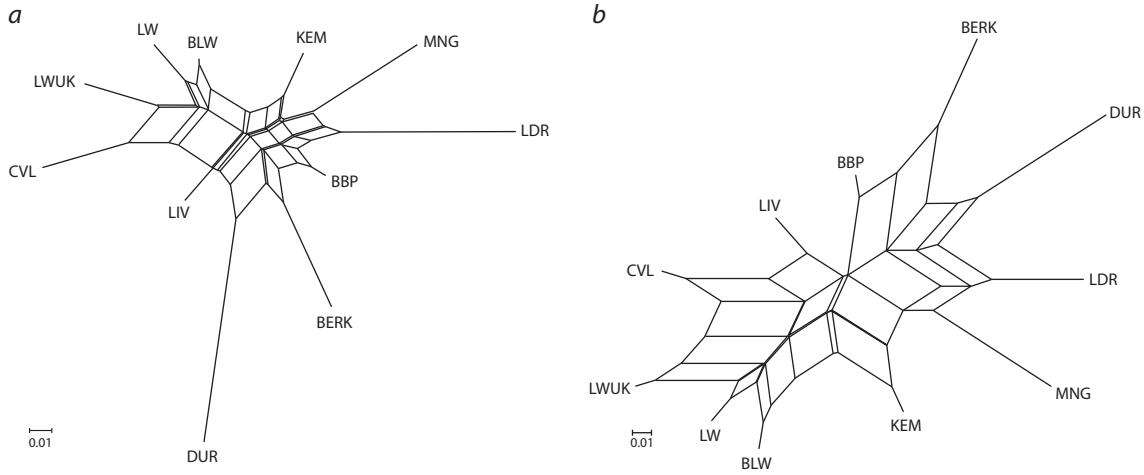

Fig. 3. Phylogenetic dendrogram of the genetic relationships of the studied pig breeds based on the pairwise genetic distances matrix F_{ST} (*a*) and Jost's *D* (*b*) using the Neighbor-Net algorithm.

The information obtained from these studies can guide the management and breeding strategies of the studied breeds in order to better use and conserve them. At the same time, further studies of pigs, both local and specialised breeds, with many microsatellites, using mitochondrial DNA and single nucleotide polymorphism analysis, seem necessary. Future genetic progress will mainly depend on the availability of sufficient genetic variation, and a more holistic understanding of the state of genetic diversity and the structure of pig breeds will bring tremendous benefits for the entire pig industry.

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Acknowledgements. The research was carried out with the support of the Ministry of Science and Higher Education of the Russian Federation AAAA-A18-118021590138-1 on the theme 0445-2019-0026.

Conflict of interest. The authors declare no conflict of interest.

Received April 14, 2020. Revised September 14, 2020. Accepted September 30, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):755-760
DOI 10.18699/VJ20.670

Chickens productivity selection affects immune system genes

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Abstract. The quantitative trait loci associated with the immune properties of chickens are of interest from the point of view of obtaining animals resistant to infectious agents using marker-assisted selection. In the process of selecting markers for genomic selection in broiler-type chickens, a non-standard genotype frequency of the *RACK1* gene allele (SNP Gga_rs15788101) in the B5 line of broiler-type chicken cross Smena 8 was identified and it was suggested that this gene was involved in selection. Therefore, it was decided to investigate the available polymorphisms in the three genes responsible for the IgY titer (*DMA*, *RACK1* and *CD1B*). Molecular typing of single nucleotide polymorphisms of three loci revealed an approach to fixation of the unfavorable allele of the *DMA* gene (SNP Gga_rs15788237), an approach to fixation of the unfavorable allele of the *RACK1* gene and the prevalence of the favorable *CD1B* gene allele (SNP Gga_rs16057130). Analysis of the haplotypes revealed a strong linkage disequilibrium of these genes. This suggests that these genes experience selection pressure. Analysis of the protein-coding sequences of the *CD1B* and *DMA* genes of various breeds of chickens revealed a negative selection of these genes. In order to understand whether the fixation of the studied alleles is the result of artificial selection of the B5 line of the cross Smena 8, an analysis of similar loci in layer chickens Hisex White was carried out. The frequencies of the alleles at the loci of the *CD1B* gene (Gga_rs16057130) and the *RACK1* gene (Gga_rs15788101) in the Hisex White chicken genome differ from the frequencies of the alleles obtained for chickens of the B5 line of the cross Smena 8. It can be assumed that the fixation of the allele in the *DMA* gene (SNP Gga_rs15723) is associated with artificial or natural selection, consistent in broilers and layers. Changes in the loci Gga_rs16057130 and Gga_rs15788101 in the B5 line of the Smena 8 chickens are most likely associated with artificial selection of broiler productivity traits, which can subsequently lead to fixation of alleles at these loci. Artificial breeding of chickens leads to degradation of the variability of genes encoding elements of the immune system, which can cause a decrease in resistance to various diseases. The study of the negative impact of selection of economic traits on immunity should provide means to mitigate negative consequences and help find ways to obtain disease-resistant animals.
Key words: chickens immune system genes; allele fixation; negative selection.

For citation: Borodin A.M., Alekseev Ya.I., Gerasimov K.E., Konovalova N.V., Terentjeva E.V., Efimov D.N., Emanuilova Zh.V., Tuchemskiy L.I., Komarov A.A., Fisinin V.I. Chickens productivity selection affects immune system genes. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):755-760. DOI 10.18699/VJ20.670

Селекция продуктивности кур влияет на гены иммунной системы

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Аннотация. Локусы количественных признаков, связанные с иммунными свойствами кур, представляют интерес с точки зрения получения устойчивых к инфекционным агентам животных при помощи маркер-опосредованной (геномной) селекции. В процессе подбора маркеров для геномной селекции у кур бройлерного типа выявлена нестандартная частота аллеля гена *RACK1* (SNP Gga_rs15788101) у кур линии Б5 мясного кросса селекции СГЦ «Смена» и возникло предположение о том, что этот ген вовлечен в селекцию.

Поэтому было решено исследовать доступные полиморфизмы в трех генах, ответственных за титр IgY (*DMA*, *RACK1* и *CD1B*). Молекулярное типирование однонуклеотидных полиморфизмов трех локусов показало приближение к фиксации неблагоприятных аллелей генов *DMA* (SNP Gga_rs15788237) и *RACK1* и преобладание благоприятного аллеля гена *CD1B* (SNP Gga_rs16057130). При анализе гаплотипов выявлено сильное неравновесное сцепление этих генов. Это свидетельствует о том, что данные гены испытывают селекционное давление. Исследование белок-кодирующих последовательностей генов *CD1B* и *DMA* различных пород кур продемонстрировало негативную селекцию этих генов. Для того чтобы понять, является ли фиксация изученных аллелей результатом направленной селекции кур линии Б5 мясного кросса СГЦ «Смена», проведен анализ аналогичных локусов у кур яичной селекции «Хайсекс белый». Частоты аллелей в локусах гена *CD1B* (Gga_rs16057130) и гена *RACK1* (Gga_rs15788101) в геноме кур «Хайсекс белый» отличаются от частот аллелей, полученных для кур линии Б5 мясного кросса селекции СГЦ «Смена». Можно предположить, что фиксация аллеля в гене *DMA* (SNP Gga_rs15788237) связана с искусственным или естественным отбором, единым для кур мясной и яичной селекции. Изменения в локусах Gga_rs16057130 и Gga_rs15788101 у кур линии Б5 мясного кросса селекции СГЦ «Смена», скорее всего, связаны с искусственной селекцией признаков продуктивности бройлеров, которая в дальнейшем может привести к фиксации аллелей в этих локусах. Искусственная селекция кур ведет к деградации вариабельности генов, кодирующих элементы иммунной системы и, как следствие, уменьшению резистентности к различным заболеваниям. Изучение негативного влияния селекции хозяйственных признаков на иммунитет должно способствовать снижению отрицательных последствий и поиску способов получения резистентных к заболеваниям животных.
Ключевые слова: гены иммунной системы кур; фиксация аллелей; негативная селекция.

Introduction

Selecting production traits in the broiler chicken has a negative effect on the breed's resistance to infectious diseases (Zekarias et al., 2002) and harms their immune competence. On the other hand, resistant chickens are poor producers, e.g. high leucosis resistance in layers reduces egg yield. The only exclusion to this rule has been known so far is resistance to Marek's disease that stimulates egg laying (Zekarias et al., 2002). One of the studies has demonstrated that broilers produce a short-term humoral response, while layers – a long-term humoral and strong cell-mediated response (Koenen et al., 2002). Another study claims genetic selection to improve the broiler's growing trait reduces the humoral and increases the cell-mediated and inflammatory responses (Cheema et al., 2003). There are studies proving intensive selection has no negative effect on poultry immune competence (Emam et al., 2014). At the same time, there is a theory saying the resources necessary for physiologically normal immunity are taken to provide the productivity trait in chicken (Zekarias et al., 2002).

After it was found out that antibody titers are genetically inherited, the certain genes affecting this process were detected (Yonash et al., 2001; Kaiser et al., 2002). The quantitative trait loci associated with the immune properties in *Gallus gallus* belong to different chromosomes (Slawinska, Siwek, 2013; Zhang et al., 2015). A genome-wide search for associations allowed one to detect in chromosomes 1, 3, 5, 12 and 16 nine single-nucleotide polymorphisms (SNPs) in the loci associated with total immunoglobulin Y (IgY) concentration in sera. The most significant five of them are located within a narrow region covering 0.26 Mb of chromosome 16 in the MHC-B locus that determines resistance to viral, bacterial and parasitic infections in chickens. Locus's variability determines a breed's resistance to different pathogens (Iglesias et al., 2019). Keeping in mind that the number of haplotypes in the locus is almost one order lower in broilers than in their wild ancestors, it partially may explain the difference in resistance (Nguyen-Phuc et al., 2016). The genes of this region may play a crucial role in immune response modulation (Zhang et al., 2015), so chickens produce IgY to provide their offsprings with an effective humoral response to the most wide-spread pathogens before their own immune system matures (Dias da Silva, Tambourgi, 2010). While choosing genomic selection markers in the parental lines of the B5 line / Smena 8 cross broilers, a nonstandard allele genotype of the *RACK1* gene was identified and a suggestion was made that it might be involved in the selection process. For that reason, a decision was made to study the polymorphisms of all the three genes responsible for the IgY titer.

Materials and methods

To isolate the DNA, feather samples from the 100 broilers belonging to the 79th generation of the B5 line / Smena 8 cross (Cornish breed) bred at Breeding and Genetic Center "Smena" were collected. To isolate the DNA of layer chickens, the feather samples of 48 the Hisex White layer chickens bred at the Zagorskoe Experimental Farm of Federal Scientific Center "All-Russian Research and Technological Poultry Institute" of the Russian Academy of Sciences were used. The DNA was isolated from the animals' quill of 0.3–0.5 cm in length as it is required by the investigation protocol for the M-sorb kit (No. HG-501, Syntol LLC, Russia). The PCR required 1.5 μl of isolated DNA and was performed in real time using an ANK-M device (Institute for Analytical Instrumentation of the Russian Academy of Sciences, Russia). The SNPs were typed in two different ways: with the primers containing a modified LNA nucleoside on their 3′ end (Latorra et al., 2003); and introducing two different LNA nucleosides into the 5′ end of a probe in a position compliment to an SNP being studied. While the probe/target mismatch destabilized the interaction, the proper positioning facilitated it. This was due to the presence of LNA modification that significantly changed the thermodynamic characteristics of the samples (You et al., 2006). Using two different channels to detect a fluorescent signal enabled us to detect an SNP in a single test tube, increasing the assay's effectiveness and simplifying data interpretation. The primer/probe sequences for SNP detection can be seen in Table 1.

Such dyes as 6-carboxyfluorescein (6FAM) and 5-carboxyrhodamine 6G (5R6G) were used as fluorescent markers, and the BHQ1 dye – as a quencher. The accumulated data

Table 1. Studied SNPs and their positions in chromosome 16, genes responsible for IgY titers, probes and primers used for the analysis

SNP	Gene	Index	Primer/probe sequences (5′→3′)
Gga_rs16057130 G > **A**	*CD1B*	CF	GGATCTGTCCTCCCTTCC
		CR	CTTCCCAAACTACATCCTCA
		CDA	(6FAM)TGCT(A-LNA)CACGAGG(BHQ1)
		CDG	(5R6G)TGCT(G-LNA)CACGAGG(BHQ1)
Gga_rs15788237 T > **C**	*DMA*	DMF	GGGACACATCAGTGAGGA
		DMR	AATGGACATCCCAACTGA
		DMA	(6FAM)CCCC(A-LNA)ACGATGT(BHQ1)
		DMG	(5R6G)CCCC(G-LNA)ACGATGT(BHQ1)
Gga_rs15788101 A > **G**	*RACK1*	RNF	GCAGCAGCCTCAGCTCCAA
		RNR	GAGATAAAGCCCGGTGAGGA
		RT	(6FAM)CTCA(T-LNA)ATTCCCGTC(BHQ1)
		RC	(5R6G)CTCA(C-LNA)ATTCCCGTC(BHQ1)

Note. Here and also in Table 2, 4 the nucleolytic sequence enhancing the IgY titer is marked in bold.

were put through the Ensamble genome browser at https://www.ensembl.org/index.html (Zerbino et al., 2018). The linkage disequilibrium was analyzed using the CubeX web-tool (Gaunt et al., 2007) and the DNASp v.6 software (Rozas et al., 2017). The positive and negative selection was analyzed in the HyPhy software (Kosakovsky Pond, Frost, 2005) from the Datamonkey web server (http://datamonkey.org) using the following sequences from GenBank: AB268588.1, AY849318.1, NM_001024582.1, AB204802.1, AY375530.1 for the *CD1B* gene; AB268588.1, FJ770458.1, NM_001099353.2, HM545127.1, AB426148.1 for the *DMA* gene; and AY393848.1, M24193.1, NM_001004378.2, CR386189.1, AY694127.1 for the *RACK1* gene. Protein secondary structure errors due to mutations were analyzed on the Dim-Pred (Disorder inducing mutation prediction) server at http://www.iitm.ac.in/bioinfo/DIM_Pred/ (Anoosha et al., 2015). The protein 3D structures of corresponding genes were modeled on the SWISS-MODEL web server at http://www.expasy.ch/swissmod/SWISS-MODEL.html (Waterhouse et al., 2018).

Results and discussion

SNP typing of the three loci responsible for enhanced IgY titer in the B5 line / Smena 8 cross broilers was carried out. All the three SNPs were localized within their corresponding genes. Fixation of the allele Gga_rs15788237 determining the lowest IgY titer in the locus was revealed, as well as that of an unfavorable allele Gga_rs15788101 and the predominance of a favorable allele Gga_rs16057130. The results of SNP typing can be seen in Table 2.

***CD1B* gene.** The *CD1* proteins are a family that is similar to MHC class I glycoproteins that expose alien and native antigens, so they can be recognized by T-cells (Barral, Brenner, 2007). The ratio of synonymous (dS) and nonsynonymous (dN) mutations in the 5′ coding sequences of the *CD1B* gene in the chickens of different origin demonstrated a negative selection to present in two regions. The analysis performed using the HyPhy software showed the gene has two codon- alteration regions that result in amino acid replacement (Table 3). However, while replacing nonpolar valine by alanine may not affect the protein's structure and functions, a replacement of polar serine by nonpolar glycine may significantly alter both the structure and the way the protein interacts with ligands. Analyzing the two mutations on the DIM-Pred server demonstrated their destructive effect on the protein's secondary structure in which the coding glycine occurred to bind the favorable SNP Gga_rs16057130 allele. 3D modeling of the protein's structure revealed no visible changes in case of mutual replacements.

DMA gene. The *DMA* gene encodes the alpha-chain of glycoprotein being the receptor to expose alien antigens with specialized T-cells (Chazara et al., 2011). The gene's dN/dS ratio suggests there is one negative selection site in this gene (see Table 3). The Ser27Leu replacement does not disrupt the protein's secondary structure but can probably affect the way it interacts with its surrounding and ligands. However, if the site really presents in the *DMA* gene, a question rises why the unfavorable allele prevails for the IgY titer. A possible explanation can be a balancing selection when a positive selection is substituted by a negative one if the allele frequency becomes high. In this case allele fixation never occurs, so they cannot be regarded either as favorable or unfavorable (Hurst, 2009). In essence, the issue remains open.

RACK1 gene. The gene encodes C1, an activated kinase receptor subunit. Analysis of the gene's dN/dS ratio reveals no signs of the selection determined by the encoding part of the gene, which is hardly a surprise, for this protein is very conservative and 100% match of that in humans. Thus, the hypothetic reason of *RACK1* selection remains unidentified and can be predetermined as by the encoding as by the regulation sites in both encoding and non-encoding domains (Chen, Blanchette, 2007; Koonin, Wolf, 2010). Since structural conservation can not be a strong selection factor of its own (Drake et al., 2006; Katzman et al., 2007), several such factors may probably exist.

The allele fixation effect that produces extended runs of homozygosity as a response to environmental and artificial selection factors has recently been found in chickens (Rubin

Table 2. Genotypes and alleles distribution in the B5 line/Smena 8 cross broilers (n = 100)

SNP	Gene	Genotype	Allele frequency
Gga_rs16057130 G > **A**(1)	*CD1B*	GG = 0.09, AA = 0.47, GA = 0.44	G = 0.31, A = 0.69
Gga_rs15788237 T > **C**	*DMA*	TT = 0.92, CC = 0.00, TC = 0.08	T = 0.96, C = 0.04
Gga_rs15788101 A > **G**	*RACK1*	AA = 0.87, GG = 0.00, AG = 0.13	A = 0.935, G = 0.065

Table 3. Nucleotide changes in the *CD1B* and *DMA* genes, leading to amino acid replacements

Gene	Nucleotide change	Codon	Amino acid replacement
CD1B	T > C	GTG > GCG	Val202Ala
CD1B	G > A	GGC > AGC	Gly283Ser
DMA	CA > TG	TCA > TTG	Ser27Leu

Table 4. Allele and genotype frequencies distribution in the genome region containing immune system genes in the Hisex White chickens (n = 48)

SNP	Gene	Genotype	Allele frequency
Gga_rs16057130 G > **A**	*CD1B*	GG = 0.21, AA = 0.35, GA = 0.44	G = 0.43, A = 0.57
Gga_rs15788237 T > **C**	*DMA*	TT = 0.92, CC = 0.00, TC = 0.08	T = 0.96, C = 0.04
Gga_rs15788101 A > **G**	*RACK1*	AA = 0.25, GG = 0.06, AG = 0.69	A = 0.59, G = 0.41

et al., 2010; Fleming et al., 2016). The runs are 3 million base pairs on average and contain a certain number of linked homozygous SNPs (McQuillan et al., 2008; Keller et al., 2011; Hedrick, GarciaDorado, 2016). Finding such runs allows one to detect the genome regions and genes involved in both natural and artificial selections. In tropical climate, adaptation of chickens to traditional poultry production leads to natural selection of birds with favorable genotypes, so the frequency of corresponding alleles increases in the next generations, affecting the homeostasis and immune system genes (Marchesi et al., 2018). For us to understand if the fixation of studied alleles was the result of artificial selection in the B5 line / Smena 8 cross broilers, analysis of similar loci in the Hisex White layer chickens was carried out (Table 4).

Similar to the results obtained from the B5 line / Smena 8 chickens, the Hisex White layers had an allele Gga_rs15788237 fixation in the locus. However, the allele frequencies Gga_rs16057130 and Gga_rs15788101 in the Hisex White genome are different from the frequencies obtained for the B5 / Smena 8 chickens.

Based on the data obtained, an assumption can be made that the allele fixation in *DMA* gene is related to either artificial or natural selection that is similar for both layer and broiler chickens. The changes in Gga_rs16057130 and Gga_rs15788101 loci observed in the B5 line / Smena 8 cross chickens are most likely related to artificial selection of the productivity traits typical for broilers, which in the future may result in complete allele fixation in these loci. While the fixation, the so-called genetic hitchhiking can take place when an allele changes its frequency not because it is being selected but because it is located next to a gene being selected (Smith, Haigh, 1974; Futuyma, 2013). This phenomenon may involve both favorable and unfavorable alleles of the neighboring gene.

Thus, the presented experiment has confirmed that artificial selection for productivity traits may possibly affect the immune system of chickens, the effect that was first studied by J.J. Li et al. (2017). Typing of the *IFIH1* and *IFIT5* innate immunity genes has demonstrated an interaction between the production traits and the immune system. It can also be assumed that artificial selection of commercial traits may result in passive selection for immune traits. A study to identify artificial selection regions in chickens (Ma et al., 2018) detected two immunity genes: *BCL2L14* (apoptosis mediator) and *CDH13* (encoding protein enhancing immune resistivity to *Campylobacter jejuni*). Resequencing of the cockfighting chicken genome (Guo et al., 2016) detected multiple immunity genes involved in the selection process.

All the authors mentioned above reported about the genes being involved in selection but not directly related to productivity traits. However, they did not mention whether favorable or unfavorable alleles were selected. The fact that unfavorable alleles can be selected as well has long been known since both artificial and natural selection increases the frequency of the rare recessive alleles having a negative effect on viability (Hocking, 2014). This can be exemplified by skeleton and muscle diseases in growing chickens and by multiple ovulations in broilers' adult parents (Hocking, 2014) meaning the corresponding diseased genes are a part of the parent lines. Other negative manifestations of artificial selections include reduced resistance to infectious disease, pulmonary hypertension and osteoporosis that can be promoted by negative pleiotropic effect or genetic hitchhiking (Elferink et al., 2012). A stark example of such unexpected selection results can be a 22% body mass increase in the chickens bred from crossing the Livorno White lines, which were selected for a long term to produce two-yolk eggs (Abplanalp et al., 1977). All these data prove that selection affects multiple genes. In our study, selection of two unfavorable and one favorable alleles of a single trait was observed, which is important because unfavorable alleles can be beneficial for artificial selection. Forecasting of the unfavourability is based on the variants that greatly affect the phenotype but often turn out to be unstable in nature. However, in artificial conditions, these variants may be quite viable (Hedrick, GarciaDorado, 2016; Bosse et al., 2018).

Conclusion

Artificial selection in chickens leads to degradation of the variability of the genes encoding the immune system elements that may worsen the birds' resistance to certain diseases. Studying

the negative effect productive trait selection has on the immunity may give us a tool not only to mitigate the effect but also to breed disease-resistant animals.

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Acknowledgments. The study was performed under state assignment No. 075-01297-20-00 of 18.12.2019 for Breeding and Genetic Center "Smena."

Conflict of interest. The authors declare no conflict of interest.

Received March 02, 2020. Revised August 31, 2020. Accepted September 02, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):761-769
DOI 10.18699/VJ20.671

Метаболический фенотип взрослых потомков мышей, полученных при разных вариантах эмбриональных пересадок

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Аннотация. Вспомогательные репродуктивные технологии (ВРТ) занимают все более заметное место в репродуктологии. Кроме того, в развитых странах ВРТ обеспечивают воспроизводство более 50 % крупного рогатого скота, а в коллекциях генетических линий лабораторных животных являются неотъемлемым компонентом криоархивирования и редеривации. Процедуры ВРТ включают развитие ранних эмбрионов вне материнского организма и высокую вероятность неполной синхронизации физиологического состояния суррогатной матери и пересаживаемых эмбрионов. Поскольку все это происходит на стадии наибольшей восприимчивости зародышей к эпигенетическому перепрограммированию, то полный цикл ВРТ и его отдельные составляющие могут приводить к устойчивым фенотипическим изменениям потомков. Данное влияние подтверждают исследования морфофункциональных характеристик половозрелых потомков мышей аутбредной линии CD1, полученных с использованием разных вариантов трансплантаций ранних эмбрионов. Сравнительные исследования массы и состава тела, базального уровня глюкозы и реакции на глюкозную нагрузку (глюкозотолерантный тест) выполнены на половозрелых самцах и самках, потомках матерей, не подвергавшихся экспериментальным воздействиям в период беременности (группа контроля); двухклеточных эмбрионах, вымытых у беременных самок, после инкубирования до стадии бластоцист и пересаженных суррогатным матерям (группа 2 кл. – бл.); при пересадках двухклеточных эмбрионов (группа 2 кл. – 2 кл.) и бластоцист (группа Бл. – бл.) сразу после вымывания. Во всех экспериментах эмбрионы пересаживали вынашивающим самкам той же линии. Установлено, что половозрелые потомки, полученные при всех вариантах пересадок, характеризуются большим по сравнению с контрольными особями относительным содержанием жира и, соответственно, меньшей тощей массой тела. Этот эффект был выражен сильнее у самок, чем у самцов. В отличие от состава тела пересадки эмбрионов в большей степени влияли на базальную концентрацию глюкозы и показатели глюкозотолерантного теста у самцов, чем у самок. При этом потомки групп 2 кл. – 2 кл. и 2 кл. – бл. характеризовались более высокой толерантностью к нагрузке глюкозой по сравнению с контрольной группой и группой Бл. – бл. Устойчивые отклонения состава тела и показателей гомеостаза глюкозы, выявленные у потомков при разных вариантах эмбриотрансплантаций, свидетельствуют о фенотипической значимости процедур, используемых при вспомогательных репродуктивных технологиях.
Ключевые слова: пересадки эмбрионов; половозрелые потомки; метаболический фенотип; состав тела; глюкозотолерантный тест.

Для цитирования: Анисимова М.В., Гон Я., Юдин Н.С., Мошкин Ю.М., Герлинская Л.А. Метаболический фенотип взрослых потомков мышей, полученных при разных вариантах эмбриональных пересадок. *Вавиловский журнал генетики и селекции*. 2020;24(7):761-769. DOI 10.18699/VJ20.671

Metabolic phenotype of adult mice offspring obtained from different variants of embryo transfer

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Abstract. Assisted reproductive technologies (ART) increasingly occupy the study of human reproduction. In addition, in developed countries they contribute to breeding of more than 50 % of cattle. In the management of collections of genetic lines of laboratory animals, these technologies are obligatory components of cryopreservation and rederivation. ART procedures include the development of early embryos outside the mother's body and the high probability of incomplete synchronization of the physiological state of the surrogate mother and transplanted embryos. Since all this occurs at the stage of the highest susceptibility of embryos to epigenetic reprogramming, the full cycle of ART and its individual components can lead to stable phenotypic changes in the offspring. Their reality is confirmed by studies of the morphological and functional characteristics of sexually mature offspring of CD1 outbred mice, obtained using

different variants of early embryo transplantation. Comparative studies of body mass and body composition, basal glucose level and response to glucose load (glucose-tolerance test – GTT) have been done on sexually mature males and females. Animals were separated in 4 groups according to the variant of embryo transplantation: group (control) – natural mating; group (2cl-bl) – incubation of 2-cell up to blastocysts; group (2cl-2cl) – removal and transplantation of the 2-cell embryo without incubation; group (Bl-bl) removal and transplantation of the blastocysts without incubation. All embryos were transplanted to recipient females of the same line. It was found that sexually mature offspring obtained with all variants of transplantations had a higher relative fat content and, correspondingly, lower lean mass compared to the control. This effect was more pronounced in females than in males. Unlike body compositions, embryo transplantations had a greater effect on basal glucose concentration and GTT in males than in females. In this case, the offspring of the 2cl-2cl and 2cl-bl groups were characterized by a higher tolerance to glucose load (GTT) compared with the control and the Bl-bl group. Stable deviations of body compositions and glucose homeostasis indices detected in experimental groups of progenies indicate the phenotypic significance of the embryo transplantations per se.
Key words: embryo transfer; mature offspring; metabolic phenotype; body composition; glucose tolerance test.

For citation: Anisimova M.V., Gong Ya., Yudin N.S., Moshkin Yu.M., Gerlinskaya L.A. Metabolic phenotype of adult mice offspring obtained from different variants of embryo transfer. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):761-769. DOI 10.18699/VJ20.671 (in Russian)

Введение

В мире более 45 млн брачных пар сталкиваются с бесплодием. Преодолеть эту проблему помогают вспомогательные репродуктивные технологии: экстракорпоральное оплодотворение (ЭКО) и внутриклеточная инъекция сперматозоида (intracytoplasmic sperm injection – ИКСИ). Со времени первого успешного применения ВРТ в клинической практике (1978 г.) число детей, рожденных с помощью ЭКО, возросло до 7 млн во всем мире. Сегодня в развитых странах ~1 % детей рождаются методом ЭКО (International Committee…, 2012). Большинство этих детей относят к категории здоровых (Davies et al., 2012), хотя в некоторых исследованиях отмечаются потенциальные риски патологий беременности и новорожденных (Templeton, 2000; Hansen et al., 2013). При проведении ЭКО повышается риск осложнений беременности (Romundstad et al., 2008; Esh-Broder et al., 2011; Chen et al., 2015), среди которых аномальный рост плаценты, перинатальная смертность, преждевременные роды и низкий вес при рождении (Helmerhorst et al., 2004; Ceelen et al., 2008; Rinaudo, Lamb, 2008; Haavaldsen et al., 2012). Дети, зачатые с помощью ЭКО, в подростковом возрасте демонстрируют статистически значимые различия в динамике роста (Ceelen et al., 2009), отложении жира (Ceelen et al., 2007), уровнях артериального давления и концентрации глюкозы в крови (Ceelen et al., 2008).

При использовании ЭКО у лабораторных животных и крупного рогатого скота наблюдается внутриутробное ограничение роста на ранних сроках беременности, за которым следуют ускоренные темпы развития плода от средней до поздней беременности, что коррелирует с увеличением роста плаценты. Исследования на коровах и овцах дополнительно показывают, что потомки, полученные методом ЭКО, демонстрируют уникальный фенотип, так называемый синдром крупного потомства (Young et al., 1998; Sinclair et al., 2000; Farin et al., 2006).

Успешность преимплантационного развития обеспечивается строго скоординированными физиологическими и эпигенетическими трансформациями в период развития от зиготы до бластоцисты. Поддержка здоровой беременности обеспечивается многочисленными материнскими факторами, контролирующими процессы созревания гамет, оплодотворение, доимплантационное развитие и имплантацию бластоцист. Согласно гипотезе Бакера, неблагоприятные условия материнской среды играют ведущую роль в развитии отклонений в период внутриутробного развития и, как следствие, формировании физиологического и метаболического фенотипа новорожденных, ассоциированного с увеличением риска хронических заболеваний во взрослом возрасте (Barker, 2007). Метаболические потребности развивающегося эмбриона зависят от стадии клеточного деления и удовлетворяются за счет гуморального состава внутриматочной среды, включая питательные вещества и факторы роста, которая меняется по мере того, как эмбрион перемещается из яйцевода в матку (Leese, 2012). Важно отметить, что динамичность гуморального окружения практически отсутствует при культивировании эмбрионов *in vitro*.

Риски долговременных неблагоприятных последствий процедур ЭКО зависят от многих переменных, таких как качество и способ получения ооцитов; метод фертилизации *in vitro* (ЭКО/ИКСИ); состав культуральной среды; физические факторы окружающей среды (CO_2/O_2, температура, влажность); продолжительность развития в условиях *in vitro*. Кроме того, на беременность и онтогенез влияет синхронизация степени развития эмбрионов и морфофункционального состояния организма вынашивающей матери. Плодово-материнская синхронизация во многом зависит от стадии эмбриогенеза и трансплантации зародышей в яйцевод или матку.

В данной работе показаны долговременные последствия инкубирования *in vitro* двухклеточных эмбрионов до стадии бластоцист и пересадок либо двухклеточных эмбрионов в яйцевод, либо бластоцист в матку на метаболический фенотип взрослых потомков. Эти варианты пересадок моделируют ВРТ при: а) редеривации и криоархивировании двухклеточных эмбрионов с последующей пересадкой в яйцевод; б) размножении сельскохозяйственных животных на основе криоархивированных бластоцист; в) выполнении ЭКО или ИКСИ в клинической практике, которые включают оплодотворение *in vitro*, инкубирование до стадии бластоцисты и пересадку суррогатной матери. Результаты показали, что все варианты пересадок влияют на метаболический фенотип половозрелых по-

томков, что проявляется в статистически значимых изменениях состава тела и толерантности к глюкозе. Кроме того, это исследование подтвердило важность использования зачатых *in vivo* эмбрионов с последующей пересадкой суррогатным реципиентам в качестве надлежащих контролей для дальнейшего изучения долговременных фенотипических эффектов экстракорпорального оплодотворения.

Материалы и методы

Условия содержания и животные

Исследование выполнено в ЦКП «Центр генетических ресурсов лабораторных животных» ИЦиГ СО РАН (RFME FI61914X0005 и RFMEFI62114X0010). Мышей аутбредной линии CD1 содержали в контролируемых условиях среды: фотопериоде 14С:10Т, при температуре 22–24 °С и влажности 40–50 %. В качестве подстилочного материала использовали обеспыленные березовые гранулы (ООО «Альбион», Новосибирск). Корм (SNIFF, Германия) и воду давали без ограничений. Корм и подстилку предоставляли животным после автоклавирования (121 °С). Протокол эксперимента одобрен комиссией по биоэтике ИЦиГ СО РАН.

Группы животных

Исследования выполнены на потомках обоего пола линии CD1 в возрасте 3–12 нед., полученных с использованием методов ВРТ. В соответствии с методом получения исследованные животные разделены на четыре группы:

1) потомки матерей, не подвергавшихся экспериментальным воздействиям в период беременности (контроль). Животных исследовали в возрасте 3 (n = 113), 7 (n = 113), 10 (n = 111) и 12 (n = 16) нед.;
2) потомки, полученные после культивирования *in vitro* двухклеточных эмбрионов до стадии бластоцист и пересадки самкам-реципиентам (2 кл. – бл.). Животных исследовали в возрасте 3 (n = 23), 7 (n = 23), 10 (n = 19) и 12 (n = 16) нед.;
3) потомки, полученные путем вымывания эмбрионов на стадии бластоцисты и пересадки самкам-реципиентам (Бл. – бл.). Животных исследовали в возрасте 3 (n = 30), 7 (n = 19), 10 (n = 19) и 12 (n = 16) нед.;
4) потомки, полученные путем вымывания эмбрионов на стадии двух клеток и пересадки самкам-реципиентам (2 кл. – 2 кл.). Животных исследовали в возрасте 3 (n = 27), 7 (n = 27), 10 (n = 27) и 12 (n = 16) нед.

Экспериментальные процедуры

Вазэктомия самцов. Самцам в возрасте 8–10 нед. производили вазэктомию путем пережигания семявыносящих канальцев. Процедура проводилась под общей анестезией (домитор 15 мг/100 г веса мыши, золетил 3 мг/100 г веса мыши).

Подготовка самок-доноров эмбрионов. Для стимуляции овуляции проводили процедуру суперовуляции самок, которую выполняли в два этапа. На первом этапе самкам за 2 ч до выключения света (18:00 по местному времени) вводили внутрибрюшинно по 5 IU гонадотропина сыворотки жеребых кобыл (PMSG) (Intervet International B.V., Нидерланды). На втором этапе, через 48 ч после введения PMSG, этим же самкам внутрибрюшинно вводили по 5 IU человеческого хорионического гонадотропина (hCG) (Intervet International B.V., Нидерланды). Сразу после введения hCG самок по одной подсаживали к фертильным самцам той же линии и утром следующего дня самок проверяли на наличие вагинальных пробок. Через 24 ч после обнаружения вагинальной пробки извлекали яйцеводы и с помощью шприца вымывали двухклеточные эмбрионы. Через 3 сут выделяли матки, из которых вымывали бластоцисты. Вымытые эмбрионы помещали в каплю среды HTF (human tubal fluid). Эмбрионы с нормальной морфологией переносили в заранее подготовленную каплю среды KSOM, покрытую минеральным маслом, и помещали в CO_2-инкубатор (37 °С) до трансплантации эмбрионов псевдобеременным самкам-реципиентам.

Индукция псевдобеременности у самок-реципиентов эмбрионов. Перед выключением света в комнате содержания животных (17:00) в клетку с изолированно содержащимся вазэктомированным самцом подсаживали трех самок. Утром следующего дня самок проверяли на наличие вагинальных пробок. Самок с вагинальными пробками отсаживали в отдельные клетки.

Культивирование эмбрионов. Часть двухклеточных эмбрионов, полученных описанном выше методом, в течение 3 дней инкубировали в культуральной среде KSOM AA при 5 % CO_2 и 37 °С. Бластоцисты без морфологических дефектов пересаживали самкам-реципиентам.

Пересадки эмбрионов. Самок-реципиентов двухклеточных эмбрионов (12 ч после подсадки самок к вазэктомированному самцу) и бластоцист (3–3.5 сут после подсадки самок к вазэктомированному самцу) усыпляли при помощи ингаляционного наркоза – изофлурана (Baxter, США). Наркотизированным самкам подсаживали двухклеточные эмбрионы через воронку в яйцевод, а бластоцисты – в матку через надрез со стороны спины. После подсадки 8–10 эмбрионов самок отсаживали в индивидуальные клетки и содержали одиночно на протяжении беременности и выкармливания.

Исследование потомков

Взвешивание потомков. Потомков взвешивали в возрасте 3 (при отъеме от матерей), 7 и 10 нед.

Определение состава тела. Измерения общего жира и тощей массы проводили половозрелым потомкам в возрасте 7 и 10 нед. при помощи низкопольного магнитно-резонансного томографа (EchoMRI, США).

Глюкозотолерантный тест. Толерантность к глюкозе исследовали у потомков в возрасте 11–12 нед. За 16 ч до инъекций глюкозы из клеток содержания мышей извлекали кормушку. Глюкозу («ПанЭко», Россия) вводили внутрибрюшинно из расчета 10 мкл 20 % глюкозы на 1 г веса мыши. Кровь брали из кончика хвоста в 5 временных точках: 0 – базовый уровень глюкозы до введения и после, 1 – через 15 мин, 2 – через 30 мин, 3 – через 60 мин и 4 – через 120 мин. Измерение уровня глюкозы проводили с помощью глюкометра Contour TS (Bayer, Швейцария). В качестве интегрального показателя глюкозотолерантного теста (ГТТ) рассчитывали площадь под кривой концентрации глюкозы (average under curve – AUC).

Статистический анализ

Проверка на нормальность распределения эмпирических данных показала, что для всех изучаемых параметров можно использовать параметрическую статистику. Межгрупповые сравнения средних проводили с помощью однофакторного дисперсионного анализа, определяя наименьшую достоверную разность (least significant difference – LSD). Статистическую зависимость показателей фенотипа от числа новорожденных и массы тела при отъеме от матерей оценивали на основе линейных корреляций. Влияние вариантов пересадки и пола потомков на массу и состав тела анализировали путем двухфакторного ковариационного анализа (ANCOVA) с факторами «пол» и «вариант пересадки» и ковариатами «число новорожденных» и «масса тела» при отъеме. Для глюкозы и показателей теста толерантности к глюкозе применяли двухфакторный дисперсионный анализ (ANOVA).

Результаты

Плодовитость и масса тела при отъеме от матерей

На размер пометов при отъеме от матерей значимо влиял способ их получения (табл. 1). При пересадках эмбрионов отмечено существенное снижение размера пометов по сравнению с контрольной группой. Вместе с тем разные способы пересадок не влияли на среднее число новорожденных ($p > 0.05$, LSD-тест, см. табл. 1). Соотношение полов при разных вариантах пересадок и в целом по всем экспериментальным группам хотя и было сдвинуто в пользу самцов (45 самцов, 35 самок), но этот сдвиг не был статистически значимым: $\chi^2 = 0.63$, $p = 0.43$ при сравнении с теоретически ожидаемым 1:1. Ковариационный анализ (ANCOVA) изменчивости массы тела трехнедельных потомков с факторами «группа», «пол» и размером помета в качестве ковариаты показал значимые эффекты группы: $F_{3.184} = 11.42$, $p < 0.001$. Фактор пола потомка, а также взаимодействие факторов группы и пола новорожденных не влияли на массу потомков в этом возрасте: $F_{1.184} = 0.671$, $p = 0.414$ и $F_{3.184} = 0.597$, $p = 0.618$ соответственно. При сравнении массы трехнедельных потомков, полученных при разных вариантах пересадок, отмечено, что самцы и самки в группах 2 кл. – бл. были самыми тяжелыми, а в 2 кл. – 2 кл. – самыми легкими (см. табл. 1).

Размер помета и масса тела в возрасте 3 нед. коррелировали с массой тела, относительным содержанием жира и тощей массой потомков в возрасте 7 и 10 нед. (табл. 2), но не коррелировали с концентрацией глюкозы и показателями ГТТ. Исходя из этих результатов, все варианты

Table 1. The size of the litter and body mass when weaned in mice obtained at different variants of embryonic transfers

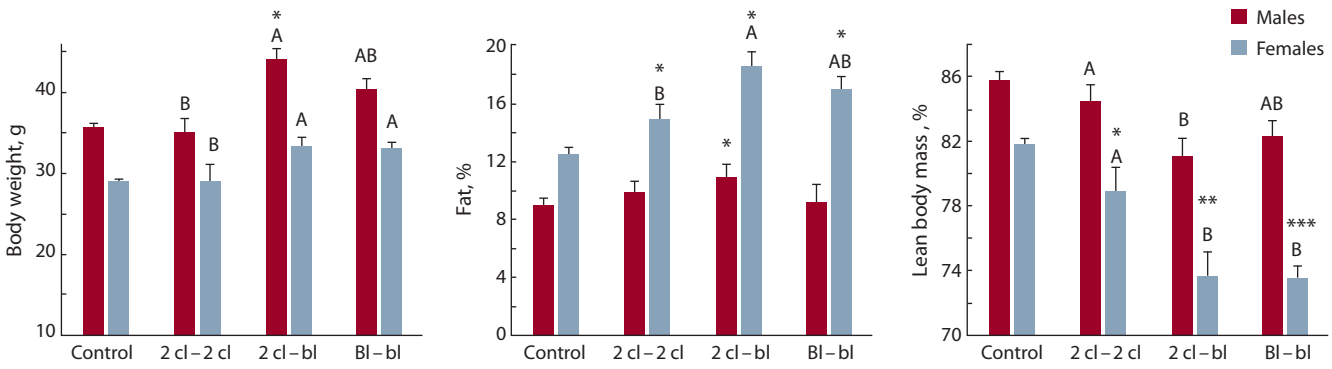
Group	Litter size, Average ± SE (*n*)	Body mass (g), Average ± SE (*n*)	
		Males	Females
Control	12.5 ± 0.58 (9)^{A}	11.7 ± 0.34 (54)^{D}	11.7 ± 0.30 (59)^{D}
2 cl – 2 cl	6.75 ± 1.11 (4)^{B}	15.1 ± 0.77 (17)^{C}	15.0 ± 0.92 (10)^{C}
2 cl – bl	4.43 ± 0.78 (7)^{B}	20.7 ± 0.92 (14)^{A}	20.1 ± 1.00 (9)^{A}
Bl – bl	5.00 ± 0.93 (6)^{B}	18.2 ± 0.77 (14)^{B}	17.8 ± 0.39 (16)^{B}

Note. Group designations here and beyond: Control – descendants derived from natural mating; 2 cl – 2 cl – descendants obtained by washing embryos at the stage of two cells and transplantation to female recipients; 2 cl – bl – descendants obtained after *in vitro* cultivation of two-celled embryos to the stage of blastocysts and transplantation to female recipients; Bl – bl – descendants obtained by washing embryos at the blastocyst stage and transplantation to female recipients.
$^{A, B, C, D}$ – different letters indicate significantly different arithmetic means ($p < 0.05$, LSD-тест); SE – standard error; *n* – number of animals in the group.

Table 2. Correlation of body composition and glucose tolerance test with litter size and body mass of mice when weaned (combined sample data)

Indices	Litter size, *r* ; *p*		Body mass, *r* ; *p*	
	Males	Females	Males	Females
Aged 7 weeks				
Body mass	–0.61; < 0.001	–0.45; < 0.001	0.60; < 0.001	0.39; < 0.001
Fat (%)	–0.26; 0.015	–0.54; < 0.001	0.25; 0.19	0.45; < 0.001
Lean body mass (%)	0.46; < 0.001	0.70; < 0.001	–0.49; < 0.001	–0.64; < 0.001
Aged 10 weeks				
Body mass	–0.60; < 0.001	–0.48; < 0.001	0.53; < 0.001	0.31; 0.003
Fat (%)	–0.43; < 0.001	–0.61; < 0.001	0.35; 0.001	0.45; < 0.001
Lean body mass (%)	0.43; < 0.001	0.60; < 0.001	–0.31; 0.003	–0.41; < 0.001
Glucose concentration and GTT				
Glucose, basal level	0.03; 0.880	0.10; 0.599	–0.10; 0.565	0.03; 0.860
Area under the glucose concentration curve	0.22; 0.218	0.24; 0.195	0.08; 0.664	–0.31; 0.090

Note. *r* – correlation coefficient, *p* – significance level.



Factor	Body weight, g		Fat, %		Lean body mass, %	
Group	$F_{3.171} = 10.92$	$p < 0.001$	$F_{3.171} = 1.60$	$p = 0.192$	$F_{3.171} = 7.55$	$p < 0.001$
Sex	$F_{1.171} = 203.4$	$p < 0.001$	$F_{1.171} = 105.3$	$p < 0.001$	$F_{1.171} = 106.0$	$p < 0.001$
Group × sex	$F_{3.171} = 2.49$	$p = 0.06$	$F_{3.171} = 5.34$	$p < 0.002$	$F_{3.171} = 4.38$	$p < 0.005$

Fig. 1. Body weight and relative values of fat and lean mass as % of body mass in the offspring of mice at the age of 7 weeks.

Hereinafter the table shows the effects of the experimental group and the sex. ANCOVA with covariates (effects not shown) by the litter size and body weight at the age of 3 weeks. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control group. Different letters indicate significantly different arithmetic means ($p < 0.05$, LSD test). ANCOVA calculated for males and females of each experimental group.

межгрупповых сравнений массы и состава тела выполняли с помощью ковариационного анализа (ANCOVA), в который наряду с анализируемыми факторами включали две ковариаты – «размер помета» и «вес потомков» при отъеме от матерей.

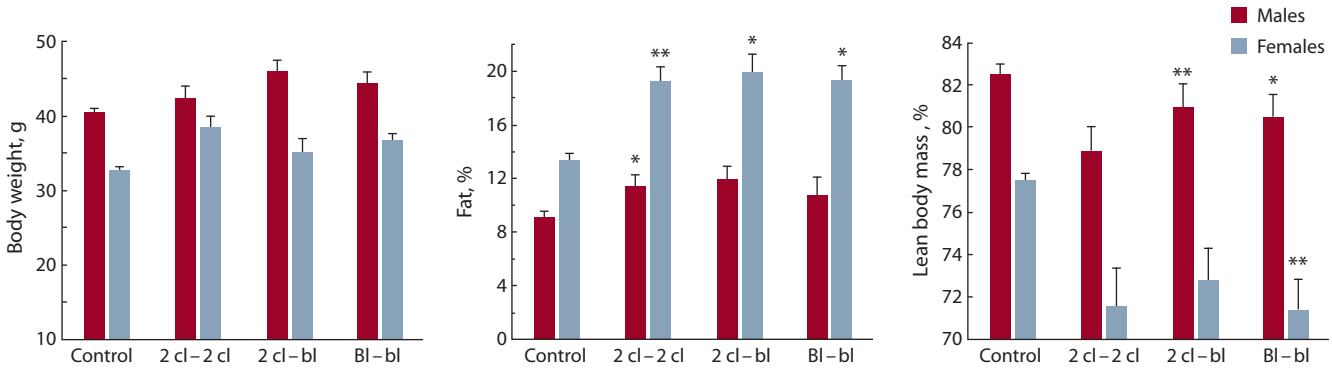
Масса и состав тела половозрелых потомков

Ковариационный анализ (ANCOVA) показал значимое влияние факторов группы, пола и их взаимодействия на массу и состав тела потомков в возрасте 7 нед. (рис. 1). Статистическую значимость отличий самцов и самок каждой экспериментальной группы от контрольной группы также оценивали с помощью ANCOVA. У самцов в возрасте 7 нед. установлено достоверное превышение контрольных значений массы тела и относительного содержания жира в группе 2 кл. – бл. Масса тела самок в этом возрасте в контрольной и экспериментальной группах значимо не различалась. Вместе с тем содержание жира у самок, полученных при разных вариантах пересадок, достоверно превосходило значения, наблюдаемые у контрольных особей. Соответственно, тощая масса самок экспериментальных групп была ниже таковой в группе контроля. Различная фенотипическая реакция на пересадки самцов и самок подтверждена статистически значимыми эффектами взаимодействия факторов «группа» и «пол» (см. рис. 1). Объединение в общую группу данных по всем пересаженным потомкам показало, что содержание жира и тощей массы статистически значимо отличалось от группы контроля только у самок: жир – 16.8±0.54 против 12.5±0.39 % ($p < 0.05$, ANCOVA); тощая масса – 75.4±0.60 против 81.9±0.43 % ($p < 0.05$, ANCOVA).

Поскольку размер помета достоверно не различался между экспериментальными группами, для выявления эффектов, обусловленных разными вариантами пересадок, статистические сравнения между этими группами проводили отдельно для самцов и самок с помощью однофакторного дисперсионного анализа. Наибольшие значения массы тела установлены у особей обоего пола в группе 2 кл. – бл., наименьшие – в группе 2 кл. – 2 кл. (см. рис. 1). Относительное содержание жира, как и масса тела, были наибольшими у самцов и самок группы 2 кл. – бл., а значения тощей массы, соответственно, наименьшими.

Статистический анализ межгрупповых различий в возрасте 10 нед., выполненный по изложенной выше схеме, показал значимое влияние экспериментальной группы, пола и взаимодействия факторов (группы и пола) на общую и тощую массу потомков (рис. 2). На содержание жира фактор «группа» не оказывал значимого влияния, но достоверно влиял фактор пола, а также взаимодействие факторов – «пол» и «группа». При сравнении экспериментальных групп с контрольной установлено, что у самцов доля жира превышала контрольные значения только в группе 2 кл. – 2 кл., а тощая масса была ниже, чем в контроле, в группах 2 кл. – бл. и Бл. – бл. У самок всех экспериментальных групп относительное содержание жира превышало контрольные значения. Соответственно, тощая масса была ниже, чем в контроле, но статистически значимым это различие было только в группе Бл. – бл. Как и в возрасте 7 нед., изменение состава тела, обусловленное пересадкой эмбрионов, было более выраженным у самок, чем у самцов. При объединении данных групп потомков разных вариантов эмбриональных пересадок статистически значимые отличия от контрольной группы были выявлены только у самок: жир – 19.5±0.65 против 13.4±0.46 % ($p < 0.05$, ANCOVA); тощая масса – 71.8±0.65 против 77.5±0.46 % ($p < 0.05$, ANCOVA).

Экспериментальные группы обоего пола между собой достоверно не различались. Эти результаты показывают, что если влияние способа получения потомков на массу тела нивелируется в возрасте 10 нед., то различия в жире и тощей массе по сравнению с группой контроля сохраняются и в этом возрасте.



Factor	Body weight, g		Fat, %		Lean body mass, %	
Group	$F_{3.165} = 6.67$	$p < 0.0012$	$F_{3.165} = 0.61$	$p = 0.610$	$F_{3.165} = 3.81$	$p = 0.011$
Sex	$F_{1.165} = 189.4$	$p < 0.001$	$F_{1.165} = 112.6$	$p < 0.001$	$F_{1.165} = 136.5$	$p < 0.001$
Group × sex	$F_{3.165} = 3.28$	$p = 0.022$	$F_{3.165} = 4.35$	$p = 0.006$	$F_{3.165} = 3.08$	$p < 0.028$

Fig. 2. Body weight and relative values of fat and lean mass as % of body mass in the offspring of mice at the age of 10 weeks.
* $p < 0.05$; ** $p < 0.01$ compared with control group. ANCOVA with covariates by the litter size and body weight at the age of 3 weeks.

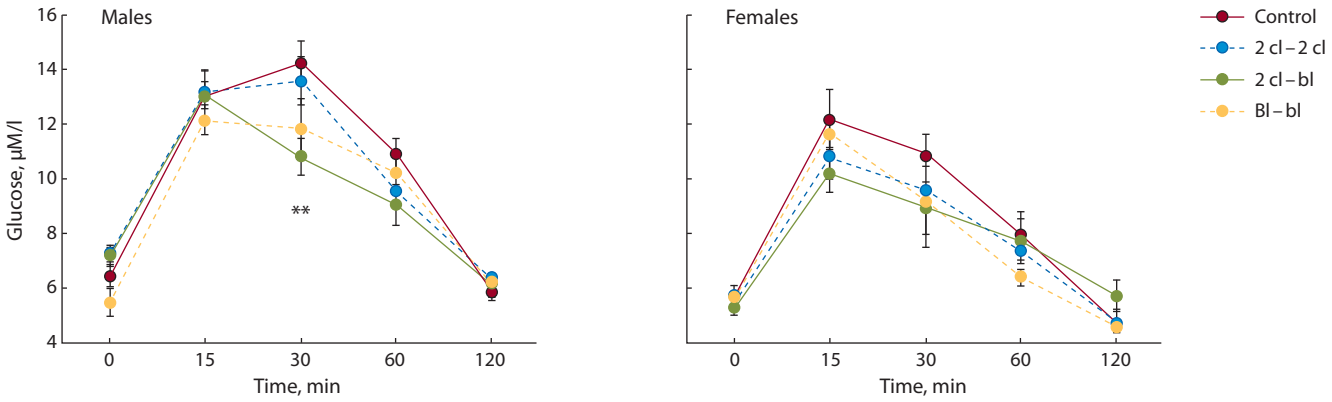
Глюкозотолерантный тест

Уровень глюкозы до и во время нагрузочной пробы (ГТТ) не коррелировал с числом потомков в пометах и массой тела при отъеме от матерей. Это обстоятельство позволило проанализировать влияние пересадок и пола, не прибегая к использованию ковариат. Двухфакторный дисперсионный анализ показал, что базальный уровень глюкозы существенно зависел от пола потомков ($F_{1.56} = 19.74$, $p < 0.001$) и взаимодействия факторов «пол» и «группа» ($F_{3.56} = 3.69$, $p = 0.017$) (табл. 3). Собственный эффект экспериментальной группы был статистически незначимым ($F_{3.56} = 1.90$, $p = 0.14$). Концентрация глюкозы в разные сроки нагрузочной пробы (ГТТ) была достоверно ниже у самок разных групп, чем у самцов (рис. 3). Варианты

Table 3. Basal glucose levels
in mature male and female mice
obtained at different variants of embryonic transfer

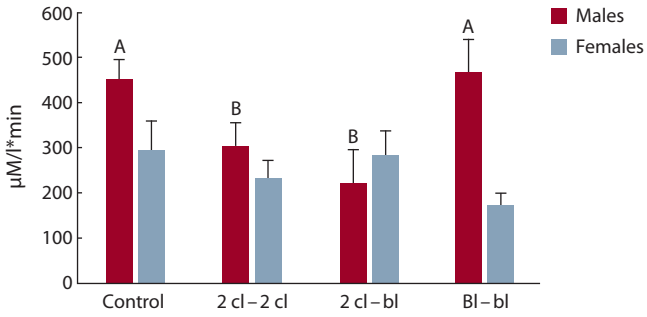
Group	Males, Average ± SE (*n*)	Females, Average ± SE (*n*)
Control	6.46 ± 0.39 (8)$^{A, B}$	5.65 ± 0.46 (8)
2 cl – 2 cl	7.23 ± 0.35 (9)^{A}	5.46 ± 0.18 (7)
2 cl – bl	7.17 ± 0.34 (9)^{A}	5.24 ± 0.21 (7)
Bl – bl	5.50 ± 0.50 (7)^{B}	5.62 ± 0.18 (9)

$^{A, B}$ – different letters indicate significantly different arithmetic means ($p < 0.05$, LSD test); SE – standard error, *n* – number of animals in the group.



Factor	*df*	0 min		15 min		30 min		60 min		120 min	
Group	3.56	$F = 1.90$	$p = 0.139$	$F = 0.58$	$p = 0.631$	$F = 4.20$	$p = 0.009$	$F = 1.40$	$p = 0.251$	$F = 1.22$	$p = 0.311$
Sex	1.56	$F = 19.74$	$p < 0.001$	$F = 9.22$	$p = 0.004$	$F = 25.6$	$p < 0.001$	$F = 31.37$	$p < 0.001$	$F = 17.42$	$p < 0.001$
Group × sex	3.56	$F = 3.69$	$p = 0.017$	$F = 1.09$	$p = 0.361$	$F = 0.63$	$p = 0.600$	$F = 1.33$	$p = 0.272$	$F = 1.03$	$p = 0.386$

Fig. 3. Glucose concentration after carbohydrate load.
The table below shows the effect of the experimental group and sex on glucose concentration at different times after carbohydrate load. ** $p < 0.01$ compared with control group (Student's *t*-test).



Factor	*df*	Area under curve (AUC)	
Group	3.56	$F = 1.90$	$p = 0.139$
Sex	1.56	$F = 8.46$	$p = 0.005$
Group × sex	3.51	$F = 3.69$	$p = 0.021$

Fig. 4. Areas under the glucose increase curve in the descendants of experimental groups.

The table below shows the effect of the experimental group and sex on AUC values. Differing means ($p < 0.05$, LSD test) are marked by different letters.

пересадок статистически значимо влияли на уровень глюкозы, измеренный на 30-й мин ГТТ. При этом наибольшие значения отмечены у контрольных особей, а наименьшие – у потомков группы 2 кл. – бл.

Площадь под кривой прироста концентрации глюкозы (AUC) варьировала в зависимости от пола животных и взаимодействия факторов пола и группы (рис. 4). Последнее обстоятельство выражалось в том, что варианты пересадок влияли на AUC только у самцов. При этом наибольшие значения AUC отмечены у контрольных животных и потомков, полученных при вымывании и пересадке бластоцист (группа Бл. – бл.). У самцов групп 2 кл. – 2 кл. и 2 кл – бл. значения AUC были достоверно ниже.

Обсуждение

Результаты наших исследований показали, что пересадка эмбрионов суррогатной матери даже без предварительной инкубации оказывает долгосрочное влияние на метаболический фенотип взрослых потомков. Эти данные подтверждают положение о высокой вероятности эпигенетического перепрограммирования на доимплантационной стадии развития (Fleming et al., 2004; Rehfeldt, Kuhn, 2006; Burdge et al., 2007; Nelson, Lawlor, 2011; Fleming et al., 2012; Mulligan et al., 2012). Следует отметить, что в подавляющем большинстве исследований долговременных последствий ЭКО в качестве главного фактора рассматривают состав культуральной среды (Fernández-Gonzalez et al., 2004; Dumoulin et al., 2010; Bouillon et al., 2016). Наше исследование показывает, что влияние культивирования эмбрионов *in vitro* от двух клеток до бластоцисты проявляется главным образом в большей массе тела и содержании жира у самцов в возрасте 7 нед. по сравнению с самцами, полученными естественным способом. В отличие от самцов масса тела самок, полученных при разных вариантах пересадок, не отличалась от группы контроля в возрасте 7 и 10 нед. Однако самки экспериментальных групп имели большее, чем в группе контроля, относительное содержание жира и меньшие значения тощей массы. Поскольку эти же отличия от контрольных особей были статистически достоверными как в экспериментах с культивированием эмбрионов от двух клеток до бластоцист, так и при пересадках двух клеточных эмбрионов и бластоцист без культивирования, можно заключить, что сама процедура эмбриотрансплантации вносит вклад в метаболические изменения потомков женского пола.

Кроме того, условия преимплантационного этапа и стадия развития в момент пересадки влияли на толерантность к глюкозе потомков мужского пола, полученных после пересадок двух клеточных эмбрионов и бластоцист и после культивирования *in vitro*. При этом следует отметить, что самцы, рожденные после пересадок интактных бластоцист, не отличались по этому признаку от контрольных особей. Полученные в нашем исследовании данные массы тела и толерантности к глюкозе демонстрируют некоторую схожесть долговременных последствий культивирования на преимплантационной стадии развития с метаболическими изменениями у взрослых потомков (Donjacour et al., 2014; López-Cardona et al., 2015). Анализ данных литературы о влиянии ВРТ на пре- и постимплантационное развитие, а также постнатальный фенотип показывает, что воздействие ВРТ зависит от вида млекопитающих, пола, используемой культуральной среды, а также возраста модельных видов (Duranthon, Chavatte-Palmer, 2018). При этом авторы подчеркивают, что очень трудно сделать общие выводы, за исключением того факта, что процедуры ВРТ влияют на липидный обмен и метаболизм глюкозы. Предложено несколько связанных между собой гипотез, объясняющих, каким образом неблагоприятные факторы на ранних этапах развития могут оказывать долгосрочное влияние на здоровье потомков (Feuer, Rinaudo, 2012, 2017).

Гипотеза начального триггера предполагает, что в основе относительно сходных метаболических фенотипов (метаболизм глюкозы, липидный обмен и изменения артериального давления) лежат эпигенетические механизмы программирования генома. Период развития от стадии зиготы до бластоцисты является наиболее эпигенетически уязвимым, поэтому считается, что реакция эмбриона на искусственную среду приводит к изменениям экспрессии генов, которые включены в программу формирования обмена веществ. Согласно следующей гипотезе, влияние манипуляций *in vitro* на экспрессию ключевых генов, выполняющих функции регулятора транскрипции в предимплантационных эмбрионах, может по-разному влиять на типы клеток и объяснить специфичность профилей экспрессии генов в разных тканях, наблюдаемых при использовании ВРТ. Наконец, предполагается влияние условий преимплантационного развития на глобальную экспрессию генов, обусловленное транскрипционными изменениями, которые эпигенетически поддерживаются в нескольких локусах, в том числе при дифференцировке клеток (Feuer et al., 2014).

Следует отметить, что значительно меньше внимания уделяется долговременным последствиям ВРТ, обусловленным реакцией суррогатной матери на эмбриональные антигены, формирующей гуморальное обеспечение для нормального развития плодов. Сегодня уже нет никаких сомнений, что материнские Т- и В-клетки распознают

антигены плода и реагируют на его присутствие. Это явление подтверждено на экспериментальных моделях, в которых ответы Т- и В-клеток на природные или модельные антигены прослеживались *in vivo* и *in vitro* (Moldenhauer et al., 2010; Taglauer et al., 2010). Кроме того, наличие антиген-реактивных Т-лимфоцитов и антител к генам гистосовместимости хорошо известно и задокументировано у беременных женщин (James et al., 2003; Kahn, Baltimore, 2010). Результаты наших ранее опубликованных исследований показывают долговременные эффекты иммуногенетических различий между суррогатной матерью и эмбрионом на стадиях двух клеток и бластоцисты на метаболический и поведенческий фенотип взрослых потомков (Gerlinskaya, Evsikov, 2001; Gerlinskaya et al., 2019). Влияние факторов иммуногенетического диалога матери и плода на фенотип потомков может отличаться при пересадках, выполняемых на аутбредных (в нашем случае линия CD1) и инбредных линиях мышей.

Заключение

Таким образом, развитие доимплантационных эмбрионов *in vitro* и даже их пересадки без предварительного культивирования влияют на метаболический фенотип потомков. Эти ключевые процедуры ВРТ могут существенно влиять на воспроизводимость результатов при использовании технологий криоархивирования для воспроизводства генетических линий лабораторных животных, хозяйственно значимые признаки и репродукцию ценных пород сельскохозяйственных животных, а также вероятность метаболических отклонений у новорожденных, зачатых с помощью экстракорпорального оплодотворения.

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Acknowledgements. The research was conducted with the support of Russian Science Foundation, grant No. 20-14-00055. In the work were used the resources of the Center for Genetic Resources of Laboratory Animals of the Federal Research Center Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences with the support of budget projects (AAAA-A17-17072710029-7 and 0259-2019-0004).

Conflict of interest. The authors declare no conflict of interest.

Received June 1, 2020. Revised July 20, 2020. Accepted July 21, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):770-776
DOI 10.18699/VJ20.672

The composition of peripheral immunocompetent cell subpopulations and cytokine content in the brain structures of mutant *Disc1*-Q31L mice

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Abstract. The *DISC1* (*disrupted in schizophrenia 1*) gene is associated with brain dysfunctions, which are involved in a variety of mental disorders, such as schizophrenia, depression and bipolar disorder. This is the first study to examine the immune parameters in *Disc1*-Q31L mice with a point mutation in the second exon of the *DISC1* gene compared to mice of the C57BL/6NCrl strain (WT, wild type). A flow cytometry assay has shown that intact *Disc1*-Q31L mice differ from the WT strain by an increase in the percentage of $CD3^+$ T cells, $CD3^+CD4^+$ T helper cells and $CD3^+CD4^+CD25^+$ T regulatory cells and a decrease in $CD3^+CD8^+$ T cytotoxic/suppressor cells in the peripheral blood. A multiplex analysis revealed differences in the content of cytokines in the brain structures of *Disc1*-Q31L mice compared to WT mice. The content of pro-inflammatory cytokines was increased in the frontal cortex (IL-6, IL-17 and IFNγ) and striatum (IFNγ), and decreased in the hippocampus and hypothalamus. At the same time, the levels of IL-1β were decreased in all structures being examined. In addition, the content of anti-inflammatory cytokines IL-4 was increased in the frontal cortex, while IL-10 amount was decreased in the hippocampus. Immune response to sheep red blood cells analyzed by the number of antibody-forming cells in the spleen was higher in *Disc1*-Q31L mice at the peak of the reaction than in WT mice. Thus, *Disc1*-Q31L mice are characterized by changes in the pattern of cytokines in the brain structures, an amplification of the peripheral T-cell link with an increase in the content of the subpopulations of $CD3^+CD4^+$ T helpers and $CD3^+CD4^+CD25^+$ T regulatory cells, as well as elevated immune reactivity to antigen in the spleen.

Key words: *Disc1*-Q31L mice; cytokines; T cells; B cells; antibody-forming cells; brain; peripheral blood; spleen.

For citation: Gevorgyan M.M., Zhanaeva S.Ya., Alperina E.L., Lipina T.V., Idova G.V. The composition of peripheral immunocompetent cell subpopulations and cytokine content in the brain structures of mutant *Disc1*-Q31L mice. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):770-776. DOI 10.18699/VJ20.672

Субпопуляционный состав периферических иммунокомпетентных клеток и содержание цитокинов в структурах мозга у мутантных мышей линии *Disk1*-Q31L

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Аннотация. Нарушения в гене *DISC1* (*disrupted in schizophrenia 1*) ассоциированы с дисфункциями мозга, характерными для ряда психических заболеваний (шизофрения, депрессия, биполярное расстройство и др.). В данной работе впервые изучены иммунологические параметры у мышей линии *Disc1*-Q31L с точечной мутацией во втором экзоне гена *DISC1* (замена глутамина на лейцин в 31-м положении) по сравнению с мышами линии C57BL/6NCrl (дикий тип). Методом проточной цитофлуориметрии показано, что по сравнению с мышами дикого типа у интактных *Disc1*-Q31L мышей в периферической крови увеличено процентное содержание $CD3^+$ Т-лимфоцитов, $CD3^+CD4^+$ Т-хелперов и $CD3^+CD4^+CD25^+$ Т-регуляторных клеток при снижении $CD3^+CD8^+$ Т-цитотоксических/супрессорных клеток. С помощью мультиплексного анализа выявлены различия в содержании цитокинов в структурах мозга *Disc1*-Q31L мышей по сравнению с мышами дикого типа. Содержание провоспалительных цитокинов повышалось во фронтальной коре (IL-6, IL-17 и IFNγ) и стриатуме (IFNγ), а в гиппокампе и гипоталамусе, напротив, уменьшалось. При этом IL-1β снижался во всех исследованных структурах. Наряду с этим обнаружено увеличение количества противовоспалительного цитокина IL-4 во фронтальной коре и снижение IL-10 в гиппокампе. Иммунная реактивность на введение антигена эритроцитов барана, анализируемая по числу антителообразующих клеток в селезенке, на пике иммунного ответа

у *Disc1*-Q31L мышей была выше, чем у мышей дикого типа. Таким образом, мыши линии *Disc1*-Q31L характеризуются изменением паттерна цитокинов в структурах мозга, усилением периферического Т-клеточного звена с повышением субпопуляций $CD3^+CD4^+$ Т-хелперов и $CD3^+CD4^+CD25^+$ Т-регуляторных клеток, а также увеличением иммунной реактивности на антиген в селезенке.
Ключевые слова: *Disc1*-Q31L мыши; цитокины; Т-клетки; В-клетки; антителообразующие клетки; мозг; периферическая кровь; селезенка.

Introduction

It is now well established that a variety of social, environmental and genetic factors may cause inflammatory responses that, over time, may result in development of multiple diseases, including neuropsychiatric disorders (Haroon et al., 2012; Felger, Lotrich, 2013; Dantzer, 2018). The inflammatory processes are closely associated with alterations in the production of cytokines (IL-6, IL-2, IL-1β, TNFα, etc.), the composition of T-cell subsets with different functional activities ($CD4^+$ T-helper cells, $CD8^+$ cytotoxic/suppressor T-cells, T-regulatory cells), both in the peripheral immune system and in the central nervous system (Haroon et al., 2012; Felger, Lotrich, 2013; Dantzer, 2018). Animal models have provided valuable opportunities to study the impact of immune dysfunctions and related alterations in neurotransmitter and hormonal systems in the pathogenesis of neuropsychiatric disorders caused by multiple risk factors, including genetic background. As shown previously, animals with genetic predisposition to depressive or aggressive behavior are characterized by changes in the distribution and ratio of the main subpopulations of T-cells in the blood and spleen, immune responsiveness to T-dependent antigen, as well as cytokine variations in the periphery and brain structures (Alperina et al., 2007, 2019; Idova et al., 2013, 2015, 2019; Takahashi et al., 2018).

Disrupted-in-Schizophrenia-1 (*DISC1*) gene has been functionally linked to brain dysfunctions associated with impaired neurodevelopment processes and intracellular signaling pathways that predispose to schizophrenia, major depression, and bipolar disorder (Lipina et al., 2010; Hikida et al., 2012; Mathieson et al., 2012; Lipina, Roder, 2014; Serykh et al., 2020). Several mouse models based on *DISC1* dysfunction have been generated to date, including a homozygous *Disc1*-Q31L$^{-/-}$ mouse line with a point mutation in exon 2 of chromosome 8, leading to glutamine to leucine substitution at amino acid 31 in the DISC1 protein (Q31L). Analysis of emotional, social and cognitive behaviors of this mice line showed a range of behavioral abnormalities that may be considered as a depression-like endophenotype (Lipina et al., 2013; Lipina, Roder, 2014; Dubrovina et al., 2018; Serykh et al., 2020). The Q31L mutation in *DISC1* gene is also known to be associated with changes in the dopaminergic (DA) activity (Lipina et al., 2013) and other neuromediator systems, which are involved in the neurobiological mechanisms of psychiatric disorders and in the control of immune function (Saurer et al., 2006; Devoino et al., 2009; Al'perina, 2014).

However, peculiar changes in immunological variables in the peripheral immune system and in the brain characteristic of *Disc1*-Q31L$^{-/-}$ mice remain to be elucidated. Given a role of the immune system both in the development of different psychoemotional states and in neuroimmunomodulation (Devoino et al., 2009; Idova et al., 2018, 2019; Alperina et al., 2019), the aim of this study was to analyze the basal content of T- and B-cells in the peripheral blood and spleen, as well as the level of pro- and anti-inflammatory cytokines in the brain structures of *Disc1*-Q31L$^{-/-}$ mice. Immune reactivity to the antigen by the number of antibody-forming cells (AFC) was also determined.

Materials and methods

Animals. The experiments were performed in adult (3.0–3.5 months old) homozygous male mice of the *Disc1*-Q31L$^{-/-}$ strain (n = 23) and there wild type (WT) littermates of the C57BL/6NCrl strain (n = 23) weighing 27–30 g. Mice were bred in the animals facility of the Scientific Research Institute of Physiology and Basic Medicine ("Biological collection – genetic biomodels of neuropsychic diseases", No. 493387). Mice were kept in standard cages (OptiMice Biotech A.S.; 40 × 25 × 15 cm) in groups for 5 animals per cage under standard vivarium conditions and free access to food and water. All experimental procedures were performed in accordance with the requirements of the European Community Directive (86/609/EC) and approved by Local Ethical Committee of the Scientific Research Institute of Physiology and Basic Medicine, protocol No. 10 (17.12.2015).

Design of experiments. The levels of T- and B-lymphocytes and their subpopulations in the peripheral blood and spleen, as well as the content of proinflammatory (IL-1β, IL-2, IL-6, IL-17, TNFα, IFNγ) and anti-inflammatory (IL-4 and IL-10) cytokines in the brain structures (prefrontal cortex, striatum, hippocampus, hypothalamus) were assessed in intact mice of the *Disc1*-Q31L and WT strains (10 animals of each strain). The immune reactivity to sheep red blood cells (SRBC) was analyzed by measuring the number of antibody-forming cells (AFC) in the spleen of mice of both strains (n = 13 of each strain). SRBC were suspended in saline and injected once, intravenously into the tail vein at a dose of $5 \cdot 10^8$.

Blood was immediately collected after the animals were decapitated into tubes containing K3EDTA (Becton Dickinson, USA). Spleens were removed on ice on day 4 after SRBC injection and placed in tubes with cooled RPMI-1640 medium (Sigma-Aldrich, USA). Brain structures were dissected on ice; brain samples were frozen in liquid nitrogen and stored at –70 °C until analysis.

Determination of cell subpopulation. To analyze cell subsets, 25 μl of blood was incubated for 30 minutes in a dark place with 1.5 μl (0.2–0.5 μg/μl) labeled rat anti-mouse monoclonal antibodies (MoAB) against surface markers: CD3 (allophycocyanin, APC), CD4 (peridinin-chlorophyll protein, perCP), CD8 (phycoerythrin, PE), CD25 (Brilliant Violet 421), CD19 (fluorescein isothiocynate, FITC) (all obtained from BD Pharmingen™, USA). Erythrocytes of the blood were lysed with Lysing Solution BD FASC (Becton

Dickinson, USA). After a 10-minute incubation, the cells were washed once with phosphate buffered saline (PBS), the cell pellet was resuspended in 100 µl of PBS.

The spleen was cut into several pieces, and then disaggregated mechanically into single-cell suspension, which was passed through a 50 µm cell strainer. The suspension was washed twice with RPMI-1640 medium at 200 g for 5 minutes. The cell pellet was resuspended in RPMI-1640 medium, adjusted to $1 \cdot 10^6$/100 µl of the suspension and placed into plates in a volume of 100 µl in each well. The cell suspension was incubated with the same MoAB as the blood cells for 20 minutes, and fixed by adding 1 % paraformaldehyde to each tube. Isotypic antibodies were used as a control.

The study of cell populations was performed on a FACS CANTO™ II flow cytometer (Becton Dickinson, USA) using multi-stage gating. At least 50000 cells were analyzed in each sample. Data analysis was performed using the FACSDiva software. The contents of $CD3^+$ T-lymphocytes, $CD3^+CD4^+$ T-helpers, $CD3^+CD8^+$ cytotoxic/suppressor T-lymphocytes, $CD3^+CD4^+CD25^+$ T-regulatory cells, $CD19^+$ B-lymphocytes as a percentage of the total number of cells were determined. Immunoregulatory index was measured as a ratio of the content of $CD4^+$ to $CD8^+$ T-cells.

Determination of cytokines in the brain structures. For the analysis of cytokines, detergent-soluble fractions of brain tissues were prepared. The samples were thawed on ice, homogenized in lysis buffer cooled to +4 °C containing PBS (pH 7.4), 0.1 % Triton X-100, 1 mM EDTA, and 1 mM PMSF using plastic pestles. The homogenates were incubated on ice for 30–40 minutes. The tissue extracts were centrifuged (Centrifuge 5415 R) at 4500 rpm for 20 minutes at +4 °C. Cytokine concentrations were determined in the supernatants. The concentration was normalized to tissue weight (pg/g tissue).

The content of cytokines in brain homogenates was determined according to the manufacturer's protocol by multiplex immunoassay on a multiplex protein and nucleic acid analyzer (Milliplex Luminex 200, Merk Millipore) using a kit (Milliplex MAP Mouse Cytokine/Chemokine, Millipore). The results were analyzed using the xPONENT and Analist software.

Determination of antibody-forming cells. The immune response was assessed by the relative (per 10^6 spleen cells) and absolute (per total number of cells in the spleen) number of IgM-AFC using the standard method (Ladics, 2007).

Statistical analysis. The data were analyzed using Statistica 10.0 software. To verify whether data were normally distributed, the Kolmogorov–Smirnov and Shapiro–Wilk tests were used. Normally distributed data (the content of T-cells and their subpopulations, and B-cells) were assessed by one-way ANOVA. The Mann–Whitney test was used for abnormally distributed data (cytokine content and AFC number). Data are presented as mean and mean error (M ± m) with significance set at a level of $p < 0.05$.

Results

Content of T-cells, their subpopulations, and B-cells in the peripheral blood and spleen of *Disc1*-Q31L mice. There were differences in the content of all analyzed immunocompetent blood cells between nonimmunized *Disc1*-Q31L and WT mice. The percentage of $CD3^+$ T-lymphocytes in mice of the *Disc1*-Q31L strain was significantly higher than in WT mice ($F(1.18) = 45.2$, $p < 0.001$). Analysis of T-lymphocyte subpopulations showed an increase in the content of $CD3^+CD4^+$ T-helpers ($F(1.17) = 15.5$, $p < 0.01$) in *Disc1*-Q31L mice compared to WT strain, while the number of $CD3^+CD8^+$ T-cytotoxic/suppressor cells was decreased ($F(1.17) = 12.6$, $p < 0.01$). As a result, the immunoregulatory index, determined as the ratio of the content of $CD4^+$ to $CD8^+$ T-lymphocytes, in mutant mice was 1.3 times higher ($F(1.18) = 27.5$, $p < 0.01$) than in WT mice. The content of T-regulatory cells with the $CD3^+CD4^+CD25^+$ phenotype in *Disc1*-Q31L mice was also higher than in WT mice ($F(1.17) = 5.3$, $p < 0.05$). The number of $CD19^+$ B-lymphocytes was decreased in the peripheral blood of mutant mice compared to WT mice ($F(1.17) = 5.7$, $p < 0.05$) (see the Table).

In contrast to the observed increase in the number of $CD3^+$ T-lymphocytes in the blood, their percentage in the spleen decreased ($F(1.18) = 10.58$, $p < 0.01$). The levels of the subpopulations of $CD3^+CD4^+$ T-helpers ($F(1.18) = 0.68$, $p > 0.05$), $CD3^+CD4^+CD25^+$ T-regulatory cells ($F(1.18) = 0.23$, $p > 0.05$), $CD3^+CD8^+$ T-cytotoxic/suppressor cells ($F(1.18) = 1.66$, $p > 0.05$), the ratio of $CD4^+/CD8^+$ T-cells ($F(1.18) = 1.36$, $p > 0.05$), and $CD19^+$ B-cells ($F(1.18) = 0.23$, $p > 0.05$) in the spleen of *Disc1*-Q31L mice were similar to those of WT mice (see the Table).

Cytokines in the brain structures in mice of the *Disc1*-Q31L strain. Analysis of the cytokine profile in brain structures of intact *Disc1*-Q31L mice revealed regional differences in the content of cytokines between mutant and WT mice (Fig. 1).

In the frontal cortex, levels of the three pro-inflammatory cytokines IL-6 ($p < 0.01$), IL-17 ($p < 0.01$) and IFNγ ($p < 0.01$) were higher in *Disc1*-Q31L mice than in WT mice, while the level of IL-1β ($p < 0.05$) decreased. IL-2 and TNFα levels were similar between the mutant and WT strains ($p < 0.05$). As to the content of anti-inflammatory cytokines, the level of IL-4 in *Disc1*-Q31L mice was higher than in WT mice ($p < 0.01$), while the level of IL-10 did not change ($p > 0.05$) (see Fig. 1, *a*).

In the striatum, the content of IFNγ ($p < 0.01$) was found to be increased in *Disc1*-Q31L mice compared to WT animals. The levels of other pro-inflammatory cytokines – IL-1β ($p < 0.01$), IL-2 ($p < 0.001$) in the mutant mice were lower than in WT mice, while the levels of IL-6, IL-17 and TNFα remained unchanged ($p > 0.05$). Similarly, there were no significant strain differences in the levels of anti-inflammatory cytokines IL-4 and IL-10 ($p > 0.05$) (see Fig. 1, *b*).

When compared to WT mice, *Disc1*-Q31L mice showed lower levels of IL-1β ($p < 0.01$), IL-2 ($p < 0.01$) and IL-17 ($p < 0.05$) in the hypothalamus, while the levels of the rest cytokines were unchanged (IL-4, IL-6, IL-10, IFNγ, TNFα) ($p > 0.05$) (see Fig. 1, *c*).

The levels of proinflammatory cytokines IL-1β, IL-2, IL-17, TNFα ($p < 0.05$) were significantly lower in the hippocampus of *Disc1*-Q31L mice than in WT mice, with more pronounced decrease in IFNγ content ($p < 0.001$). The levels of IL-6 were equivalent between *Disc1*-Q31 and WT mice ($p > 0.05$). The content of anti-inflammatory cytokine IL-10 in the hippocampus of *Disc1*-Q31 mice was also decreased

Content of the subpopulations of T- and B-lymphocytes (%) in the peripheral blood and spleen of *Disc1*-Q31L mice (M ± m)

CD cell markers	Blood		Spleen	
	WT	*Disc1*-Q31L	WT	*Disc1*-Q31L
$CD3^+$	30.8 ± 0.8	37.8 ± 0.7***	36.8 ± 1.1	26.4 ± 1.0**
$CD3^+CD4^+$	62.6 ± 0.8	68.3 ± 1.4**	59.9 ± 0.6	59.0 ± 9.7
$CD3^+CD4^+CD25^+$	6.3 ± 0.5	8.3 ± 0.7*	10.7 ± 0.4	10.5 ± 0.3
$CD3^+CD8^+$	35.5 ± 0.6	30.1 ± 1.5**	35.9 ± 0.7	36.9 ± 0.5
$CD4^+/CD8^+$	1.8 ± 0.05	2.3 ± 0.09**	1.67 ± 0.05	1.61 ± 0.04
$CD19^+$	64.4 ± 1.4	60.6 ± 1.1*	59.9 ± 1.8	59.0 ± 0.7

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with WT mice (ANOVA test). Number of animals in each group = 9–10.

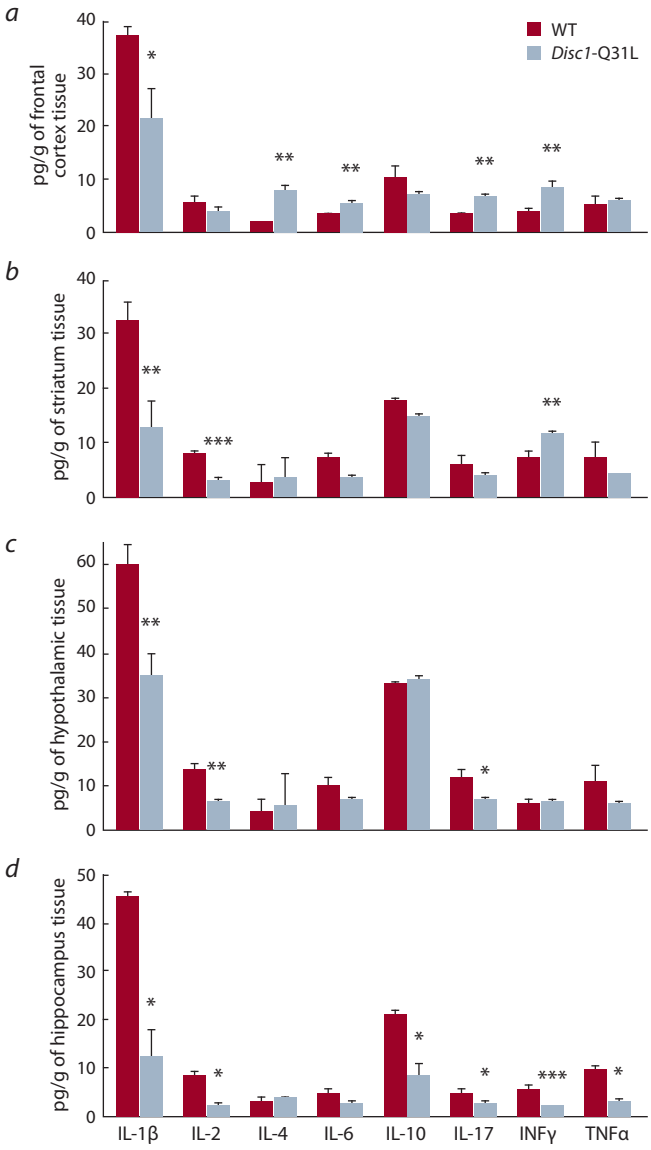

Fig. 1. Content of cytokines in the brain structures: frontal cortex (*a*), striatum (*b*), hypothalamus (*c*), hippocampus (*d*) in WT mice and *Disc1*-Q31L mice.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT mice (Mann–Whitney U-test). Number of animals in the groups = 9–10.

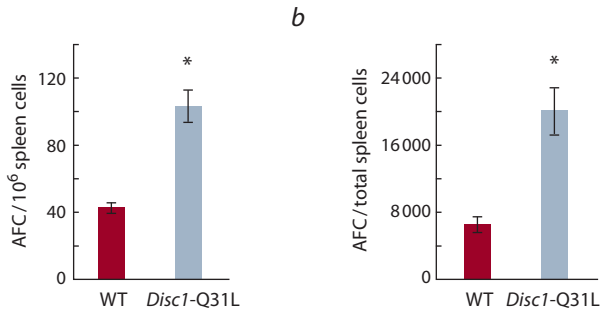

Fig. 2. Relative (*a*) and absolute (*b*) numbers of AFC in the spleen of WT and *Disc1*-Q31L mice on the 4th day following immunization with SRBC ($5 \cdot 10^8$).

* $p < 0.001$ compared to WT mice (Mann–Whitney U-test). Number of animals in the groups = 13.

($p < 0.05$), while the level of IL-4 did not differ from that of WT mice ($p > 0.05$) (see Fig. 1, *d*).

Immune reaction of *Disc1*-Q31L mice to the antigen. Immunization of *Disc1*-Q31L mice with SRBC produced an increase of the immune response at the peak of its development in the spleen of WT mice. The relative ($p < 0.001$) and absolute ($p < 0.001$) numbers of AFC in *Disc1*-Q31L mice were significantly higher than in WT mice (Fig. 2).

Discussion

Changes in DISC1 protein activities caused by mutations in the *DISC1* gene are known to be involved in multiple mental disorders, such as schizophrenia, depression, bipolar disorder (Lipina et al., 2010, 2013, 2014; Hikida et al., 2012; Mathieson et al., 2012). Alterations in immune variables associated with these disorders may differentially contribute to disease development. Schizophrenia has been found to be accompanied by elevated serum numbers of B-cells, along with a decrease in the content of T-cells, $CD4^+$ T-helpers, and the ratio of $CD4^+$ to $CD8^+$ T-cells (Steiner et al., 2010). Aggressive behavior, as observed in a variety of animal models, is also associated with an increase in T-helpers and the $CD4^+/CD8^+$ ratio, as well as a higher immune response generated by an antigen (Devoino et al., 2009; Idova et al., 2015; Takahashi et al., 2018).

On the other hand, depression is characterized by increasing numbers of $CD3^+CD8^+$ T-suppressor/cytotoxic cells, a decrease in the immunoregulatory index and the immune response suppression (Alperina et al., 2007; Devoino et al., 2009; Haroon et al., 2012; Felger, Lotrich, 2013; Idova et al., 2013).

The present study demonstrates that, compared to WT mice, intact mice of the *Disc1*-Q31L strain have raised blood levels of $CD3^+$ T-lymphocytes, and their subpopulations, such as $CD3^+CD4^+$ T-helpers and $CD3^+CD4^+CD25^+$ T-regulatory cells, with a consequent increase of the immunoregulatory index. At the same time, the mutant mice showed lower percentage of $CD3^+$ T-lymphocytes in the spleen, that might lead to a predominance of $CD19^+$ B-cells, thereby suggesting a redistribution of these cell subsets within the immune system.

Redistribution of T- and B-lymphocytes, which are known to produce specific sets of cytokines, and the ratio of these cells in the immunocompetent organs may significantly affect inflammatory and immune processes characteristic of genetically determined behaviors and psychopathology (Ottaway, Husband, 1994; Devoino et al., 2009). It seems, thus, possible that higher ability of *Disc1*-Q31L mice to respond to antigen challenge, as measured by the numbers of AFC in the spleen, may be related to changes in immune cell distribution among different compartments of the immune system.

Our results have also shown that the pattern of cytokine variations over brain structures differ in *Disc1*-Q31L and WT mice and depends on the brain area, in which these cytokines are localized. The levels of IL-6, IL-17 and IFNγ were found to be simultaneously increased only in the frontal cortex of *Disc1*-Q31L mice compared to WT animals. These pro-inflammatory cytokines has long been known as very potent signaling molecules of neuroinflammation implicated in the pathophysiology of depression, bipolar disorder, and schizophrenia (Grigor'ian et al., 2014; Lesh et al., 2018). Moreover, the frontal cortex has also been associated with the development of various psychiatric diseases (Clapcote et al., 2007).

Only the IFNγ level was increased in the striatum of *Disc1*-Q31L mice compared to WT mice, whereas the concentrations of other cytokines decreased. Levels of pro-inflammatory cytokines were also lower in the hippocampus and hypothalamus of mutant mice than in WT mice. Changes in the distribution of brain cytokines found in *Disc1*-Q31L mice suggest that this mutation may contribute to neuroinflammation, which is an important etiological factor for affective disorders. The observed increase in the level of anti-inflammatory cytokine IL-4 in the frontal cortex of *Disc1*-Q31L mice could reflect a compensatory response to the elevations of pro-inflammatory cytokines that occurred in this brain area. These findings are consistent with previous reports, showing that various forms of depression-like behavior or aggression are associated with impaired balance between pro- and anti-inflammatory cytokines in a number of brain regions including the frontal cortex and hippocampus (Takahashi et al., 2018; Alperina et al., 2019; Idova et al., 2019).

DISC1 has been found to form a complex with other intranuclear transcription factors, which mediate the expression of several genes implicated in behavioral changes resembling human psychiatric disorders (Lipina, Roder, 2014). A wide range of studies on behavioral phenotype of *Disc1*-Q31L produced conflicting results. Some data indicate that mice with Q31L mutation in *Disc1* have a depressive-like enodophenotype (Lipina et al., 2013; Dubrovina et al., 2018; Serykh et al., 2020), while others did not show significant behavioral differences in this strain compared with the WT control in any of the tests (Shoji et al., 2012). Recent evidence suggests that *Disc1*-Q31L mice may also display aggressive behavior (Serykh et al., 2020). In contrast to the data obtained in other models, in which animals developing depression-like responses showed immunosuppression (Alperina et al., 2007; Devoino et al., 2009; Idova et al., 2013), our results revealed higher immune reactivity in *Disc1*-Q31L mice compared to WT control. At the same time, immune parameters characteristic of *Disc1*-Q31L mice are more relevant to those observed in aggressive animals. It may be due to the diverse behavioral phenotype of these mice displaying not only depression, but also aggressive behavior (Serykh et al., 2020) that is associated with increased immune function and specific pattern of cytokines.

However, the mechanisms underlying alterations in peripheral immune parameters and the profile of brain cytokines are unknown. There is growing evidence that immune mediators such as cytokines are involved in the interactions between the immune and neuroendocrine systems and can change the activity of central neuromediator systems that contribute to cognitive, behavioral, and brain structure abnormalities seen in affective disorders (Grigor'ian et al., 2014; Lesh et al., 2018). It is possible that the immune status of *Disc1*-Q31L mice could be related to the neurochemical pattern of the brain characteristic of this strain. *Disc1*-Q31L mice have been shown to have decreased levels of DA combined with DOPAC increase in the nucleus accumbens (Lipina et al., 2013), known to implicate in neuroimmunomodulation (Saurer et al., 2006; Devoino et al., 2009; Al'perina, 2014). There is also data that the DOPAC/DA ratio, which may reflect the metabolic rate of DA and synaptic activity, increases under immunostimulation observed in animals experienced excessive aggression associated with elevated activity of the DA system (Devoino et al., 2009; Al'perina, 2014). Taking into account changing DAergic activity in the brain structures of *Disc1*-Q31 mice (Lipina et al., 2013) and the critical role of this system in the control of aggression and neuroimmunomodulation (Saurer et al., 2006; Devoino et al., 2009; Al'perina, 2014), it is likely that DA may contribute to the enhancement of immune function found in the *Disc1*-Q31 strain.

However, it remains unclear whether variations of central cytokines are related to brain alterations of monoamines specific for *Disc1*-Q31L mice or the Q31L mutation determines their profile. Moreover, it has been found that not only neurotransmitters can affect the production of cytokines (Kawano et al., 2018), but also cytokines can modulate mediator neurotransmission and promote changes in the neurochemical pattern of the brain (Dunn, 2006; Felger, Lotrich, 2013).

Conclusions

Our data indicate that the Q31L point mutation in the *DISC1* gene leading to the substitution of glutamine to leucine at amino acid 31 has a significant influence on immunity

and may result in an amplification of peripheral T-cell link with an increase in the content of $CD3^+CD4^+$ T-helpers and $CD3^+CD4^+CD25^+$ T-regulatory cell subpopulations, as well as elevated immune reactivity in the spleen induced by the antigen. Alterations in the peripheral immune variables are accompanied with changes in the distribution of pro- and anti-inflammatory cytokines within brain structures, which are involved both in the control of different forms of behavior and in immune function. The *Disc1*-Q31L mouse strain is a promising model for further study of the relationships between genetic factors and neuroimmunological mechanisms and their implication in the development of psychoemotional disorders.

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Acknowledgements. This work was supported by budget from the Project No. AAAA-A16-116021010228-0 of the SRIPhBM. The authors are grateful to Dr. E.V. Melnikova for technical assistance and advice in setting up the flow cytofluorometry method.

Conflict of interest. The authors declare no conflict of interest.

Received February 21, 2020. Revised August 19, 2020. Accepted August 19, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):777-784
DOI 10.18699/VJ20.673

The cell cycle regulatory gene polymorphisms *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) in lung cancer: a meta-analysis

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Abstract. Lung cancer is one of the most common types of cancer in the world. Although the mechanism of lung cancer is still unknown, a large number of studies have found a link between gene polymorphisms and the risk of lung cancer. The tumor suppressor p53 plays a crucial role in maintaining genomic stability and tumor prevention. MDM2 is a critical regulator of the p53 protein. Despite the importance of p53 pathway in cancer, data on the contribution of SNPs of *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) to the development of lung cancer are very contradictory. A meta-analysis that collects quantitative data from individual studies and combines their results has the advantage of improving accuracy, providing reliable estimates, and resolving those issues in which studies on individual associations are not effective enough. The aim of this study was to determine whether the *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) polymorphisms confer susceptibility to lung cancer. A meta-analysis was conducted on the associations between the *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) polymorphisms and lung cancer. A total of 51 comparison studies including 25,366 patients and 25,239 controls were considered in this meta-analysis. The meta-analysis showed no association between lung cancer and *MDM2* (*rs2279744*) under any model. A noteworthy association of *TP53* (*rs1042522*) with susceptibility to lung cancer in overall pooled subjects was observed under three different models (allele contrast, homozygote contrast (additive) and dominant). Stratification by ethnicity indicated an association between the *TP53* (*rs1042522*) and lung cancer in Asians and Caucasians. This meta-analysis demonstrates that the *TP53* (*rs1042522*), but not *MDM2* (*rs2279744*) polymorphism may confer susceptibility to lung cancer.
Key words: *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) gene polymorphism; lung cancer; meta-analysis.

For citation: Bulgakova O., Kussainova A., Bersimbaev R. The cell cycle regulatory gene polymorphisms *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) in lung cancer: a meta-analysis. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):777-784. DOI 10.18699/VJ20.673

Полиморфизмы генов *TP53* (*rs1042522*) и *MDM2* (*rs2279744*) в раке легкого: метаанализ

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Аннотация. Рак легкого – один из наиболее распространенных видов рака в мире. Хотя механизм возникновения заболевания по-прежнему остается в значительной степени неизвестным, благодаря многочисленным исследованиям была выявлена связь между полиморфизмами генов и риском развития рака легкого. Решающую роль в поддержании стабильности генома и профилактике опухолей играет онкосупрессор p53. Ключевым регулятором белка p53 является MDM2. Несмотря на важность p53 сигнального пути в канцерогенезе, данные о вкладе SNP *TP53* (*rs1042522*) и *MDM2* (*rs2279744*) в развитие рака легкого очень противоречивы. Метаанализ, собирающий количественные данные из отдельных исследований и объединяющий их результаты, имеет преимущество, которое заключается в повышении точности, предоставлении надежных оценок и решении тех вопросов, когда исследование отдельных ассоциаций недостаточно эффективно. Целью нашей работы было изучение роли полиморфизмов *TP53* (*rs1042522*) и *MDM2* (*rs2279744*) в формировании предрасположенности к раку легкого. Проведен метаанализ ассоциации полиморфизмов *TP53* (*rs1042522*) и *MDM2* (*rs2279744*) и рака легкого. В общей сложности рассмотрено 51 исследование типа «случай–контроль», включающее 25 366 пациентов с раком легкого и 25 239 здоровых индивидуумов. Результаты метаанализа показали отсутствие связи между раком легкого и *MDM2* (*rs2279744*) во всех моделях. Примечательно, что ассоциация *TP53* (*rs1042522*) с предрасположенностью к раку легкого наблюдалась в трех разных моделях (мультипликативная, аддитивная и доминантная). Стратификация по этническому признаку также указывает на связь между *TP53* (*rs1042522*) и риском развития рака легкого как в азиатской, так и в европейской популяции. Проведенный метаанализ позволяет сделать вывод, что полиморфизм *TP53* (*rs1042522*), но не *MDM2* (*rs2279744*), может обусловливать предрасположенность к раку легкого.
Ключевые слова: полиморфизм генов *TP53* (*rs1042522*) и *MDM2* (*rs2279744*); рак легкого; метаанализ.

Introduction

Lung cancer remains one of the most common forms of cancer in the world. Every year World Health Organization (WHO) includes lung cancer in the lists of the leading cause of death worldwide. Thus, there were 2.1 million cases of lung cancer and 1.8 million deaths in 2018 (https://www.who.int/ru/news-room/fact-sheets/detail/the-top-10-causes-of-death). Cancer incidence rate varies in different regions of our planet, so the highest incidence of lung cancer is observed in Eastern Europe and Central and East Asia (Bray et al., 2018).

A large number of researches have been conducted to study the molecular base of lung cancer. One of the risk factors for the development of pulmonary neoplasms is genes polymorphisms. The main cause of carcinogenesis is disorders in the regulation of cell cycle control. The tumor suppressor gene *TP53* plays an important role in regulating the cell cycle. p53 protein is known as the "guardian of the genome". p53 regulates many genes expression in response to cellular stress induced by various adverse environmental factors (Haronikova et al., 2019). This protein plays a key role in processes such as DNA repair, cell cycle arrest, apoptosis and senescence (Nicolai et al., 2015). *MDM2* is a key regulator of p53 protein activity and degradation. Polymorphic variants of the *TP53* and *MDM2* genes have been found in various types of cancer, including lung cancer. Analysis of the literature data showed that polymorphisms of the *TP53 Arg72Pro* (*rs1042522*) and *MDM2 SNP309* (*rs2279744*) genes cause an increased predisposition to tumor development. The *TP53 (rs1042522*) gene polymorphism is localized on chromosome 17 position 7676154 Genotype frequency in the Caucasian population GG: 0.074, CC: 0.503, CG: 0.423. In the East Asian population, GG: 0.173, CC: 0.345, CG: 0.482 (http://www.ensembl.org/).

Meta-analyses have shown that the *TP53 Arg72Pro* polymorphic allele is associated with the development of stomach cancer (Xiang et al., 2012), bladder (Xu et al., 2012), colorectal cancer (Tian et al., 2017) and acute lymphocytic leukemia (Tian et al., 2016). However, no association was found between *TP53 Arg72Pro* and the risk of acute myeloid leukemia (Tian et al., 2016), oral squamous cell carcinoma (Sun et al., 2018), and esophagus cancer (Jiang et al., 2011).

The polymorphic allele of the *MDM2* gene *rs2279744* is located at 68808800 position on chromosome 12 Genotype frequency in the Caucasian population TT: 0.404, GG: 0.113, GT: 0.483. In the East Asian population TT: 0.200, GG: 0.276, GT: 0.524 (http://www.ensembl.org/).The *MDM2 SNP309* polymorphism was also found to increase the risk of colorectal cancer (Qin et al., 2013), breast cancer (Cheng et al., 2012) and liver cancer (Tang et al., 2014). But there was no association with prostate, urinary tract (Ding et al., 2016) and stomach (Ma et al., 2013).

Many population studies have been conducted on the influence of the mutant alleles *TP53 Arg72Pro* (*rs1042522*) and *MDM2 SNP309* (*rs2279744*) on the predisposition to the development of pulmonary neoplasia. It was shown that the polymorphism of the *TP53 Arg72Pro* gene is associated with a high risk of small cell lung cancer among Spaniards (Fernández-Rubio et al., 2008). Similar data were found for non-small cell lung cancer in Norwegians (Lind et al., 2007) and Poles (Szymanowska et al., 2006), squamous cell lung cancer in German residents (Popanda et al., 2007), and lung adenocarcinoma in the Chinese population (Zhang X. et al., 2006; Ren et al., 2013).

Data on the contribution of *MDM2 SNP309* to the development of lung cancer are very contradictory. Most studies have shown an association of the *MDM2* (*rs2279744*) mutant allele with a high risk of lung tissue carcinogenesis (Enokida et al., 2014; Wang X. et al., 2015; Li, 2017). However, Pine et al. (2006) did not find that *MDM2 SNP309* is associated with lung neoplasia in the European population.

The data on the association of polymorphisms of the *TP53* genes *Arg72Pro* (*rs1042522*) and *MDM2 SNP309* (*rs2279744*) with the development of tumors as a whole are very contradictory. Therefore, it would be interesting to perform a meta-analysis on the association of *TP53 Arg72Pro* (*rs1042522*) and *MDM2 SNP309* (*rs2279744*) with a risk of developing lung cancer in Asian and European populations.

Materials and methods

Search strategy. Search for relevant studies was conducted using online databases, such as Scopus, PubMed and Web of Science. The search strategy was performed using a combination of the following keywords: "*TP53*", "Murine double minute 2" or "*MDM2*", "polymorphism", "SNP", "*rs1042522*", "*rs2279744*", "*Arg72Pro*", "*codon 72 Arg*", "c.215C > G", "SNP309", "c.291 T > G" "lung cancer", "non-small cell lung cancer", "association".

Inclusion and exclusion criteria. The eligible inclusion criteria for the meta-analysis were (i) case-control study, (ii) identification of different histological types of lung cancer which was confirmed histologically or pathologically, (iii) having an available genotype for estimating an odds ratio (OR) with 95 % confidence interval (95 % CI), (iv) genotype frequencies in controls were consistent with those expected from Hardy–Weinberg equilibrium ($p > 0.05$).

The studies were excluded when (i) they were not case-control studies, (ii) with duplicated data from previous articles, (iii) they were not original articles, e. g. review, (iv) inadequate genotype data were available.

Data extraction and quality assessment. Two researchers (O.B. and A.K.) evaluated the eligibility of all retrieved studies and extracted the pertinent data from the specified publications in standardized tables. The extracted data included: (i) the first author name, (ii) publication year, (iii) ethnicity, (iv) lung cancer patients and healthy controls sample size for each studied polymorphism. Disagreement was resolved by consulting with a third investigator (R.B.). The study quality was assessed in accordance with the Newcastle–Ottawa Scale (NOS) (Wells et al., 2009).

Statistical analysis. Hardy–Weinberg equilibrium (HWE) in control population was assessed utilizing the "Calculation of Chi-square test for deviation from Hardy–Weinberg equilibrium" online software (http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/kitest.htm). The statistical analysis was performed using Comprehensive Meta Analysis version 2.2.064 (Biosta, Englewood, NJ, USA). Estimates were summarized as ORs with 95 % CIs for each study. The heterogeneity was evaluated by using the I^2 index. An I^2 value of > 50 % was considered to indicate high heterogeneity (Lee, 2015). The random effects model for analysis was used in case

Table 1. Characteristics of the studies of *TP53* (*rs1042522*) polymorphism included in the meta-analysis

First author, year	Lung cancer			Control			Ethnicity	*p* (HWE) Control
	GG	GC	CC	GG	GC	CC		
Kawajiri, 1993	148	127	53	144	165	38	Asian	0.65
Murata, 1996	80	89	22	53	76	23		0.59
Wang Y., 1999	68	74	52	47	75	30		0.56
Pierce, 2000	41	51	19	82	65	23		0.67
Hiraki, 2003	68	99	24	90	106	43		0.60
Sakiyama, 2004	398	460	144	302	310	73		0.67
Zhang X., 2006	321	506	279	425	731	264		0.56
Jung, 2008	108	130	42	120	136	37		0.64
Sreeja, 2009	70	84	57	98	76	37		0.65
Sobti, 2009	37	73	41	42	73	36		0.54
Chua, 2010	28	69	26	42	88	31		0.53
Kiyohara, 2010	206	194	62	162	175	42		0.66
Piao, 2011	1458	1821	657	734	776	190		0.66
Liu D., 2013	144	137	79	126	115	119		0.51
Li Y., 2013	118	146	99	161	196	89		0.58
Mostaid, 2014	27	40	39	62	35	19		0.69
Saikia, 2014	95	125	52	225	260	59		0.65
Zhang F., 2014	124	311	205	133	330	222		0.44
Chowdhury, 2015	19	19	12	11	18	21		0.40
Bulgakova, 2019	12	62	11	30	6	6		0.78
Birgander, 1995	148	127	53	144	165	38	Caucasian	0.65
To-Figueras, 1996	52	32	6	92	47	8		0.79
Pierce, 2000	84	46	8	87	76	10		0.72
Fan, 2000	212	204	66	237	212	61		0.67
Liu G., 2001	604	465	99	728	441	87		0.76
Papadakis, 2002	27	27	0	24	64	11		0.56
Miller, 2002	367	299	101	496	339	92		0.72
Wu, 2002	299	177	40	352	156	32		0.80
Su, 2003	28	31	4	9	9	3		0.64
Szymanowska, 2006	110	120	10	311	225	40		0.74
Popanda, 2007	209	156	40	244	131	29		0.75
Nadji, 2007	55	64	10	41	29	19		0.62
Mechanic, 2007	166	125	16	193	122	20		0.76
Giuliani, 2007	32	26	5	30	15	5		0.75
Fernández-Rubio, 2008	308	249	32	338	209	35		0.76
Buyru, 2008	32	25	8	24	49	14		0.56
Souto-García, 2012	341	267	43	318	198	32		0.76

high heterogeneity (Lee, 2015). Otherwise, the fixed-effects model was used. Publication bias was measured via "Begg's funnel plot" and "Egger's linear regression" method (Egger et al., 1997). A two-tailed p-value < 0.05 implied a statistically significant publication bias.

Results

Studies included in the meta-analysis

A total of 531 potential articles were identified from the databases search. After 236 duplicate records were removed, a total of 295 potential articles were reviewed. Amongst these articles, 216 were excluded after titles and abstracts review. Afterwards, we excluded 28 studies for no case-control design. Finally, 51 studies with a total of 25,239 controls and 25,366 cases that met the inclusion criteria were included in this meta-analysis (Suppl. Fig. 1)[1].

Characteristics of studies included in this meta-analysis

A total of 37 articles that examined *TP53* (*rs1042522*) association with lung cancer risk were determined. Two of these articles included data of two different sets (*TP53* (*rs1042522*) and *MDM2* (*rs2279744*)) (Zhang X. et al., 2006; Chua et al., 2010) and these sets were examined autonomously. Thus, the identified 37 articles encompassed case-controls studies involving 16,229 lung cancer patients and 14,897 controls (Table 1). Among 37 articles, 20 studies were established in Asian populations and 17 in Caucasian populations. The

[1] Supplementary Figures 1– 5 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx13.pdf

Table 2. Characteristics of the studies of *MDM2* (*rs2279744*) polymorphism included in the meta-analysis

First author, year	Lung cancer			Control			Ethnicity	*p* (HWE) Control
	GG	GC	CC	GG	GC	CC		
Hu, 2006	166	373	178	274	538	271	Asian	0.5
Zhang X., 2006	249	561	296	418	711	291		0.54
Jun, 2007	113	280	189	122	299	161		0.47
Chua, 2010	29	65	29	51	83	25		0.58
Kohno, 2011	68	183	126	79	151	95		0.48
Enokida, 2014	153	379	230	152	335	213		0.46
Li, 2017	58	96	32	44	101	51		0.48
Li G., 2006	419	472	135	408	573	164	Caucasian	0.60
Lind, 2006	130	156	55	161	207	44		0.64
Pine, 2006	150	167	54	182	187	52		0.65
Liu G., 2008	702	802	283	530	631	199		0.62
Mittelstrass, 2008	270	293	70	547	598	149		0.65
Zhuo, 2012	419	472	135	408	573	164		0.61
Javid, 2015	20	56	24	40	50	10		0.65

genotype frequencies in controls of all studies were consistent with those expected from HWE ($p > 0.05$).

Another 14 articles identified *MDM2* (*rs2279744*) association with increased lung cancer risk were retrieved (Table 2). These 14 articles encompassed case-controls studies involving 9,137 lung cancer patients and 10,342 controls. Among 14 articles, 7 studies were established in Asian populations and 7 in Caucasian populations. The genotype frequencies in controls of all studies were consistent with those expected from HWE ($p > 0.05$).

All estimated published articles were executed under accredited genotyping methods.

Meta-analysis of the relationship between the *TP53* (*rs1042522*) polymorphism and lung cancer risk

Meta-analysis of *TP53* (*rs1042522*) polymorphism was associated with lung cancer (G versus C: OR = 0.82, 95 % CI 0.71–0.94, $p = 0.005$; GG versus CC: OR = 0.86, 95 % CI 0.74–0.99, $p = 0.039$; GG+GC versus CC: OR = 0.86, 95 % CI 0.76–0.98, $p = 0.02$; GG versus GC+CC: OR = 1.12, 95 % CI 0.89–1.42, $p = 0.336$). And the association was statistically significant under allele model (G versus C), homozygote model (GG versus CC) (Suppl. Fig. 2) and dominant model (GG+GC vs. CC) (Suppl. Fig. 3) ($p < 0.05$). A summary of meta-analysis findings concerning associations between the *TP53* (*rs1042522*) polymorphism and lung cancer risk is shown in Table 3.

Further subgroup analysis was conducted on the association between *TP53* (*rs1042522*) polymorphism and the risk of lung cancer (see Table 3). After stratifying by ethnicity, this meta-analysis indicated an obvious association of *TP53* (*rs1042522*) and lung cancer risk among Caucasians (G versus C: OR = = 0.83, 95 % CI 0.73–0.95, $p = 0.005$; GG versus CC: OR = 0.83, 95 % CI 0.73–0.95, $p = 0.005$; GG+GC versus CC: OR = 0.88, 95 % CI 0.77–1.00, $p = 0.045$) and among Asians (G versus C: OR = 0.76, 95 % CI 0.63–0.92, $p = 0.005$; GG versus CC: OR = 0.84, 95 % CI 0.67–1.06, $p = 0.136$; GG+GC versus CC: OR = 0.81, 95 % CI 0.69–0.96, $p = 0.012$).

Meta-analysis of the relationship between the *MDM2* (*rs2279744*) polymorphism and lung cancer risk

In this meta-analysis was shown no association *MDM2* (*rs2279744*) polymorphism with lung cancer (G versus T: OR = 0.86, 95 % CI 0.71–1.03, $p = 0.1$; GG versus TT: OR = 0.86, 95 % CI 0.71–1.03, $p = 0.1$; GG+GT versus TT: OR = 0.90, 95 % CI 0.79–1.02, $p = 0.5$; GG versus GT+TT: OR = 1.10, 95 % CI 0.94–1.22, $p = 0.276$). A summary of meta-analysis findings concerning associations between the *MDM2* (*rs2279744*) polymorphism and lung cancer risk is shown in Table 4. Subgroup analysis detected no association *MDM2* (*rs2279744*) polymorphism with lung cancer.

Heterogeneity and publication bias

Between-study heterogeneities were found in all subjects for both polymorphisms *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) (see Table 3, 4). Because of this the meta-analysis was designed using "a random effect model" to establish pooled OR and corresponding 95 % CI for all models. We performed the meta-regression to explore the potential source of between-study. A big problem for meta-analysis is the disproportionate number of positive studies that leads to a bias in the publication. The funnel plot indicated some evidence of publication bias for Caucasians, but not for Asians in analysis of *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) gene polymorphisms (Suppl. Fig. 4, 5). The publication bias was observed from Egger's test ($p \leq 0.05$) also for Caucasian population (see Table 3, 4).

Discussion

The tumor suppressor gene *TP53* (previously named *p53*), is key regulator of a cell cycle network, apoptosis and DNA repair pathway. *TP53* is one of the most carcinogenesis-associated genes. There were several studies assessing the effects of *TP53* polymorphisms on the risk of lung cancer, but the results are very contradictory. For example, no associations of the *TP53* (*rs1042522*) polymorphism with lung cancer were found in Jung et al.'s (2008) article. But, increased risk

Table 3. Meta-analysis of the association between *TP53* (*rs1042522*) polymorphism and lung cancer risk

Polymorphism	Test of association			Test of heterogeneity		
	OR	95 % CI	*p*	Model	I^2	*p*
G versus C						
Overall	0.82	0.71–0.94	0.005	Random effects model	64	0.02
Asian	0.76	0.63–0.92	0.005	Random effects model	73	0.5
Caucasian	0.83	0.73–0.95	0.005	Fix effects model	26	0.001
GG versus CC						
Overall	0.859	0.744–0.993	0.039	Random effects model	66	0.001
Asian	0.84	0.67–1.06	0.136	Random effects model	82	0.001
Caucasian	0.83	0.73–0.95	0.005	Fix effects model	34	0.06
Dominant model (GG+GC vs. CC)						
Overall	0.86	0.76–0.98	0.02	Random effects model	62	0.01
Asian	0.81	0.69–0.96	0.012	Random effects model	71	0.29
Caucasian	0.88	0.77–1.00	0.045	Fix effects model	39	0.002
Recessive model (GG vs. GC+CC)						
Overall	1.08	0.89–1.42	0.336	Random effects model	86	0.624
Asian	1.12	0.89–1.42	0.336	Random effects model	91	0.749
Caucasian	1.12	0.98–1.27	0.086	Random effects model	61	0.01

Table 4. Meta-analysis of the association between *MDM2* (*rs2279744*) polymorphism and lung cancer risk

Polymorphism	Test of association			Test of heterogeneity		
	OR	95 % CI	*p*	Model	I^2	*p*
G versus T						
Overall	0.86	0.71–1.03	0.10	Random effects model	75	0.38
Asian	0.81	0.63–1.10	0.122	Fix effects model	73	0.49
Caucasian	0.82	0.63–1.10	0.122	Random effects model	26	0.49
GG versus TT						
Overall	0.86	0.71–1.03	0.10	Random effects model	75	0.001
Asian	0.82	0.63–1.06	0.122	Random effects model	73	0.001
Caucasian	0.90	0.71–1.06	0.435	Random effects model	72	0.04
Dominant model (GG+GT vs. TT)						
Overall	0.90	0.79–1.02	0.50	Random effects model	60	0.09
Asian	0.89	0.74–1.10	0.212	Random effects model	66	0.58
Caucasian	0.91	0.76–1.10	0.303	Random effects model	56	0.05
Recessive model (GG vs. GT+TT)						
Overall	1.10	0.94–1.22	0.276	Random effects model	74	0.14
Asian	1.18	0.99–1.40	0.059	Random effects model	57	0.43
Caucasian	0.98	0.84–1.14	0.812	Random effects model	69	0.05

to develop lung cancer was observed in association with the *Pro/Pro* genotype variant in Chowdhury et al.'s (2015) research. Mostaid et al. (2014) found that *TP53 Arg72Pro* and *Pro72Pro* genotype significantly associated with increased relative risk of lung cancer. Our previous study also demonstrated the association of genotype *Arg72Pro* of *TP53* gene with lung cancer risk (Bulgakova et al., 2019). Papadakis et al. (2002) demonstrated that subjects with *Arg72Arg* genotype of *rs1042522* had significantly increased lung cancer risk. We comprehensively searched the up-to-date electronic databases to reveal the associations between *TP53* genetic polymorphisms (*rs1042522*) and risk of lung cancer. The genome-wide association study (GWAS) is very popular method to detect a variation in SNPs with variation in common diseases. In 2017, data from a study of new loci of susceptibility to lung cancer were published. The study identified *RNASET2*, *SECISBP2L*, *NRG1*, *CHRNA2*, *OFBC1* and *RTEL1* as candidate genes associated with lung cancer (McKay et al., 2017). The polymorphisms of *TP53* (*rs1042522*) and *MDM2* (*rs2279744*) weren't detected in this GWAS (McKay et al., 2017).

A total of 37 case-control comparisons for *TP53* (*rs1042522*) (16,229 lung cancer patients and 14,897 healthy controls) were

investigated in this meta-analysis. A noteworthy association of *TP53* (*rs1042522*) with susceptibility to lung cancer in overall pooled subjects was observed under three different models: the allele contrast, homozygote contrast (additive) and dominant model. Also, stratification analysis explained a strong evidence of this variant with risk of lung cancer among Asians and Caucasian under allelic, homozygote (only for Caucasian) and dominant models. Moreover, the *Arg72Arg* genotype was associated with the obvious protective effect (OR = 0.82, 95 % CI 0.71–0.94, $p = 0.005$).

Compared to *TP53*, whose role has been widely discussed in lung cancer developing, its main negative modifier – *MDM2*, has not been sufficiently studied. The data on the association of polymorphism of *MDM2* (*rs2279744* or *309T > G*) with the risk of developing lung cancer as well as in the case of *TP53* (*rs1042522*) are contradictory. Thus, Enokida et al. (2014) did not found any association between polymorphism of *MDM2* (*rs2279744*) and lung cancer risk. Chua et al. (2010) demonstrated that the *MDM2 (rs2279744*) TT rather than the GG genotype is associated with increased risk of lung cancer in Asian. But, the *MDM2* TT genotype was associated with a decreased risk of developing NSCLC compared with that of the *MDM2* GG genotypes in Li G. et al.'s (2006) research. A total of 14 case-control comparisons for *MDM2* (*rs2279744*) (9,137 lung cancer patients and 10,342 healthy controls) were investigated in this meta-analysis. There were no significant associations between *MDM2* (*rs2279744*) polymorphisms and lung cancer with regard to G allele vs. T allele: OR = 0.86, 95 % CI 0.71–1.03, $p = 0.1$; homozygote model: OR = 0.86, 95 % CI 0.71–1.03, $p = 0.1$; dominant model: OR = 0.90, 95 % CI 0.79–1.02, $p = 0.5$ and recessive model: OR = 1.10, 95 % CI 0.94–1.22, $p = 0.276$. The stratification analysis also did not demonstrate the association of this polymorphism with risk of lung cancer among Asians and Caucasian under all models. Thus, *MDM2* (*rs2279744*) polymorphism does not affect the risk of developing lung cancer.

This meta-analysis has some limitations. First, heterogeneity level was high. But we tried to eliminate this effect using a random effects model rather than a fixed effects model. Publication bias could also have biased the results, as studies that produced negative results may not have been published. Despite our use of Egger's regression test, we cannot eliminate the possibility of bias. Second, the relative importance of the *MDM2* (*rs2279744*) polymorphism during the development of lung cancer may vary between ethnic groups, but we were only able to perform ethnic-specific meta-analysis in Asians and Europeans. Thus, our results are applicable to only these ethnic groups. Therefore, additional studies with other ethnic populations are warranted to assess the association between *MDM2* (*rs2279744*) polymorphism and the risk of lung cancer.

But, the present meta-analysis has also several strengths. We used a strong comprehensive search strategy, and had a well-defined inclusion and exclusion criteria. Reviewers performed the study selection and extracted data independently. Moreover, we assessed the quality of the included studies by predefined criteria and the score of included studies was high. Finally, all genotype data extracted from the studies were reported in the study. The advantage of this study over other meta-analyzes is a more complete review of literature and the inclusion of recent data.

Conclusion

In summary, this meta-analysis study indicated evidence of association for *TP53* (*rs1042522*), but not *MDM2* (*rs2279744*) variants with lung cancer based on 51 case-control published studies. Additionally, stratified analysis based on ethnicity observed an obvious association of *TP53* (*rs1042522*) both among Asian and European subjects under allelic, homozygote and dominant models. However, polymorphism *MDM2* (*rs2279744*) may not impart susceptibility to lung cancer in either Asians or Europeans.

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Acknowledgements. This study was partially supported by the Ministry of Science and Education of the Republic of Kazakhstan (grant No. AP05135213).

Conflict of interest. The authors declare no conflict of interest.

Received June 16, 2020. Revised September 8, 2020. Accepted September 10, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):785-793
DOI 10.18699/VJ20.674

Кандидатные SNP-маркеры, изменяющие сродство TBP к промоторам Y-связанных генов *CDY2A*, *SHOX*, *ZFY*, снижают ряд показателей репродуктивного потенциала мужчин

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Аннотация. Репродуктивный потенциал – уровень физического и психического состояния организма, позволяющий при достижении социальной зрелости воспроизводить здоровое потомство. В узком биомедицинском смысле определение включает комплекс функциональных показателей репродуктивной системы, но в более широком смысле его можно рассматривать как совокупность физиологических, поведенческих, адаптивных, ментальных, антропометрических и генетических характеристик особи, способствующих размножению. Целью настоящей работы было расширить область применимости созданного ранее Web-сервиса SNP_TATA_Z-tester для поиска кандидатных маркеров однонуклеотидного полиморфизма (SNP) на хромосоме Y человека, связанных с мужским репродуктивным потенциалом (МРП). В поиске кандидатных SNP-маркеров для МРП мы сосредоточились на генах хромосомы Y человека. Изучены 35 SNP в промоторах генов *CDY2A*, *SHOX* и *ZFY*, представляющих все три типа генов хромосомы Y человека: уникальный, псевдоаутосомный и паралог гена хромосомы X человека соответственно. Предсказаны 11 кандидатных SNP-маркеров ослабления МРП из-за изменения сродства TATA-связывающего белка (TBP) к этим промоторам. Выборочно верифицированы *in vitro* величины сродства «TBP-промотор», предсказанные в этой работе. Установлена достоверная корреляция ($r = 0.94$, $p < 0.005$) между ними и результатами измерения *in vitro* сродства TBP человека к олигонуклеотидам, идентичным сайтам TBP-связывания исследуемых промоторов. Проведя поиск в базе данных PubMed по ключевым словам, мы нашли клиническое описание патологических состояний человека, соответствующих изменению экспрессии генов, несущих предсказанные нами кандидатные SNP-маркеры. Среди них оказались такие патологии, как нарушение сперматогенеза (*ZFY*: rs1388535808 и rs996955491), задержка полового созревания (*CDY2A*: rs200670724), нарушения эмбриогенеза (*SHOX*: rs28378830) и непропорционально низкий рост с деформациями Маделунга (*SHOX*: rs1452787381). Они свидетельствуют, что в случае SNP-промоторов генов хромосомы Y человека следует ожидать изменений широкого круга показателей МРП, выходящих далеко за рамки генетического контроля собственно мужской репродуктивной функции.
Ключевые слова: человек; репродуктивный потенциал; хромосома Y; ген; промотор; TATA-связывающий белок (TBP); TBP-связывающий сайт (TATA-бокс); однонуклеотидный полиморфизм (SNP); SNP-маркер; верификация *in vitro*.

Для цитирования: Пономаренко М.П., Шарыпова Е.Б., Драчкова И.А., Савинкова Л.К., Чадаева И.В., Рассказов Д.А., Пономаренко П.М., Осадчук Л.В., Осадчук А.В. Кандидатные SNP-маркеры, изменяющие сродство TBP к промоторам Y-связанных генов *CDY2A*, *SHOX*, *ZFY*, снижают ряд показателей репродуктивного потенциала мужчин. *Вавиловский журнал генетики и селекции*. 2020;24(7):785-793. DOI 10.18699/VJ20.674

Candidate SNP-markers altering TBP binding affinity for promoters of the Y-linked genes *CDY2A*, *SHOX*, and *ZFY* are lowering many indexes of reproductive potential in men

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Abstract. Reproductive potential is the most important conditional indicator reflecting the ability of individuals in a population to reproduce, survive and develop under optimal environmental conditions. As for humans, the concept of reproductive potential can include the level of the individual's mental and physical state, which allows them to reproduce healthy offspring when they reach social and physical maturity. Female reproductive potential has been investigated in great detail, whereas the male reproductive potential (MRP) has not received the equal amount of attention as yet. Therefore, here we focused on the human Y chromosome and found candidate single-nucleotide polymorphism (SNP) markers of MRP. With our development named Web-service SNP_TATA_Z-tester, we examined *in silico* all 35 unannotated SNPs within 70-bp

proximal promoters of the three Y-linked genes, *CDY2A*, *SHOX* and *ZFY*, which represent all types of human Y-chromosome genes, namely: unique, pseudo-autosomal, and human X-chromosome gene paralogs, respectively. As a result, we found 11 candidate SNP markers for MRP, which can significantly alter the TATA-binding protein (TBP) binding affinity for promoters of these genes. First of all, we selectively verified *in vitro* the values of the TBP-promoter affinity under this study, Pearson's linear correlation between predicted and measured values of which were $r = 0.94$ (significance $p < 0.005$). Next, as a discussion, using keyword search tools of the PubMed database, we found clinically proven physiological markers of human pathologies, which correspond to a change in the expression of the genes carrying the candidate SNP markers predicted here. These were markers for spermatogenesis disorders (*ZFY*: rs1388535808 and rs996955491), for male maturation arrest (*CDY2A*: rs200670724) as well as for disproportionate short stature at Madelung deformity (e. g., *SHOX*: rs1452787381) and even for embryogenesis disorders (e. g., *SHOX*: rs28378830). This indicates a wide range of MRI indicators, alterations in which should be expected in the case of SNPs in the promoters of the human Y-chromosome genes and which can go far beyond changes in male fertility.

Key words: human; reproductive potential; chromosome Y; gene; promoter; TATA-binding protein (TBP); TBP-binding site (TATA-box); single nucleotide polymorphism (SNP); candidate SNP marker; verification *in vitro*.

For citation: Ponomarenko M.P., Sharypova E.B., Drachkova I.A., Savinkova L.K., Chadaeva I.V., Rasskazov D.A., Ponomarenko P.M., Osadchuk L.V., Osadchuk A.V. Candidate SNP-markers altering TBP binding affinity for promoters of the Y-linked genes *CDY2A*, *SHOX*, and *ZFY* are lowering many indexes of reproductive potential in men. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):785-793. DOI 10.18699/VJ20.674 (in Russian)

Введение

Следуя идеям R.N. Chapman (1931) и E.R. Pianka (1976), популяционные экологи используют концепцию репродуктивного потенциала в качестве самого важного показателя шансов воспроизвести потомков и довести их до репродуктивного возраста при наилучших условиях как на уровне индивида, так и на уровне популяции (Axelsson et al., 2010). Теория ожидаемой продолжительности жизни (Bowles, 1998) связывает репродуктивный потенциал, качество и продолжительность жизни человека с предрасположенностью к болезням и стрессам, закодированной в его геноме. Наконец, прогресс медицины, достижения науки, развитие технологий и улучшение образования позволяют увеличить репродуктивный потенциал суперпопуляции людей, хотя рост народонаселения, урбанизация, загрязнение окружающей среды и эпидемии могут его уменьшить.

Базовая концепция предиктивно-превентивной персонализированной медицины (Trovato, 2014), предполагающая достоверные различия между больными и здоровыми добровольцами по встречаемости у них клинических маркеров однонуклеотидного полиморфизма (SNP) (Varzari et al., 2016), указывает на возможность по расшифрованным индивидуальным геномам людей улучшить качество и продолжительность жизни как их, так и потомков. По SNP-маркерам генов, контролирующих показатели репродуктивного потенциала в геноме пациента, врач может прогнозировать способность к зачатию, предрасположенность к репродуктивным нарушениям, стрессорную реактивность и хронические болезни, способные ухудшить его репродуктивное здоровье, качество жизни и долголетие, а также рекомендовать адекватный образ жизни, профилактические мероприятия, диету и форму взаимодействия пациента с врачом во избежание развития нежелательного жизненного сценария.

Крупнейший научный проект XXI в., «1000 геномов» (Telenti et al., 2016), как основа предиктивно-превентивной персонализированной медицины, уже выявил сотни миллионов SNP, собранных в базе данных dbSNP (Sherry et al., 2001), между десятками тысяч расшифрованных индивидуальных геномов людей и эталонным (референсным) геномом человека в базе данных Ensembl (Cunningham et al., 2019). Наконец, база данных dbWGFP (Wu et al., 2016) собирает, систематизирует и приоритизирует сведения о каждом из всех 10 млрд потенциально возможных SNP человека в качестве первоосновы анализа индивидуального генома пациента в предиктивно-превентивной персонализированной медицине (Trovato, 2014).

Поскольку врачебное решение на основе наличия или отсутствия тех или иных SNP-маркеров в геноме пациента нацелено на его здоровье, продолжительность и качество жизни, то приемлемы только биомедицинские SNP-маркеры, клинически доказанные сравнением больных и условно доровых людей (Varzari et al., 2016). Учет необходимых затрат времени, ручного труда и финансов для каждого такого теста исключает возможность оценить на практике проявление каждого из 10 млрд SNP человека (Wu et al., 2016) в патогенезе каждой из 55 тыс. болезней человека, согласно Международной классификации болезней и сопутствующих проблем со здоровьем людей, МКБ-11 (Pocai, 2019). Однако остается неясным, необходимо ли клинически проверять все SNP человека при условии, что дилемма J.B.S. Haldane (1957) и теория нейтральной эволюции (Kimura, 1968) предсказывают нейтральность абсолютного большинства SNP человека. Сейчас для будущего клинического теста чаще всего вручную эвристически выбирают кандидатный SNP-маркер заданной патологии среди всех SNP генов, уже связанных с этой болезнью (Varzari et al., 2016). Выбор таких кандидатных SNP-маркеров можно сделать объективнее, быстрее и прицельнее за счет малозатратного биоинформатического выявления абсолютного большинства нейтральных SNP во всем референсном геноме человека (Ponomarenko M. et al., 2017). Хотя точность биоинформатических прогнозов для SNP все еще остается ниже порога их применимости в клинической практике (Yoo et al., 2015), она непрерывно увеличивается с каждым годом (Putlyaeva et al., 2018).

Сейчас наилучшую точность биоинформатических прогнозов достигли для SNP белок-кодирующих частей генов (Amberger et al., 2015), чье негативное влияние неустранимо ни сменой образа жизни, ни терапией (Mitsuyasu et al., 1998). В то же время значительно меньше

исследованы SNP регуляторных районов генов (Zerbino et al., 2015), лишь модулирующих уровень экспрессии, что корректируемо как сменой образа жизни, так и лекарствами (Ponomarenko M. et al., 2013)). Поэтому регуляторные SNP сайта связывания TATA-связывающего белка (TBP, каноническая форма сайта – TATA-бокс, ≈15 % TATA-содержащих генов у человека), который необходим перед стартом любого транскрипта (Martianov et al., 2002; Rhee, Pugh, 2012) и который модулирует транскрипцию пропорционально сродству TBP-промотор (Mogno et al., 2010), выглядят многообещающими в силу их предсказуемости и пользы для медицины (Ponomarenko M. et al., 2013, 2017).

Ранее мы создали Web-сервисы SNP_TATA_Comparator (Ponomarenko M. et al., 2015) и SNP_TATA_Z-tester (Sharypova et al., 2018) для оценки статистической значимости влияния SNP на сродство TBP к промоторам генов человека и других эукариот с использованием библиотеки BioPerl (Stajich et al., 2002), доступа к базе данных Ensembl (Cunningham et al., 2019) и с помощью других источников данных соответственно. На этой методической основе мы предсказали кандидатные SNP-маркеры ожирения (Arkova et al., 2015), агрессивности (Chadaeva et al., 2016), нарушений циркадного ритма (Ponomarenko P. et al., 2016), атеросклероза (Ponomarenko M. et al., 2019), аутоиммунных заболеваний (Ponomarenko M. et al., 2016), болезни Альцгеймера (Ponomarenko P. et al., 2017), резистентности к противоопухолевой химиотерапии (Turnaev et al., 2016), предрасположенности к социальному доминированию и подчинению у людей (Chadaeva et al., 2019).

Целью настоящей работы было расширить область применимости наших Web-сервисов (Ponomarenko M. et al., 2015; Sharypova et al., 2018) для оценки SNP сайтов TBP-связывания посредством поиска кандидатных SNP-маркеров на хромосоме Y человека, связанных с показателями мужского репродуктивного потенциала (МРП). Эта методология была использована при изучении женского репродуктивного потенциала (Chadaeva et al., 2018), тогда как МРП еще не получил должного внимания.

Материалы и методы

Биоинформатический анализ данных. Мы исследовали последовательности ДНК $S = \{s_{-70}…s_i…s_{-1}\}$ длиной 70 п. о. перед стартами транскрипции ($TSS \equiv s_0$; где $s_i \in \{a, c, g, t\}$) промоторов белок-кодирующих генов хромосомы Y человека, как показано на рис. 1 и описано ранее, например в недавней работе (Ponomarenko M. et al., 2019). Стрелками на рис. 1 указано, как с помощью (*а*) Web-сервиса UCSC Genome Browser (Haeussler et al., 2015) и (*б*) базы данных dbSNP (Sherry et al., 2001) мы вводили исходные данные в наш (*в*) Web-сервис SNP_TATA_Z-tester (Sharypova et al., 2018), где окно “Result” содержит результат обработки этих исходных данных в рамках нашей биоинформатической модели трехшагового связывания TBP/промотор (Ponomarenko P. et al., 2008).

Каждый SNP анализировали независимо от остальных таким образом, что если было предсказано недостоверное изменение сродства TBP к минорному варианту промотора с этим SNP в сравнении с нормой, то он исключался из дальнейшего анализа (данные не показаны). Для оставшихся SNP с помощью стандартного поиска в базе данных PubMed (Lu, 2011) по ключевым словам находили сообщения о клинически значимых показателях МРП, которые соответствовали значимым изменениям экспрессии генов, несущих предсказанные кандидатные SNP, вследствие достоверного изменения сродства TBP к промоторам этих генов.

Эксперимент *in vitro*. Рекомбинантный TBP человека (hTBP) экспрессировали в клетках *Escherichia coli* BL21(DE3) с плазмиды pAR3038-hTBP (любезно предоставлена проф. B. Puhg, The Pennsylvania State University, Pennsilvania, США) согласно (Peterson et al., 1990; Pugh, 1995), за исключением концентрации ИПТГ (1 мМ вместо 0.1 мМ) и времени индукции (3 ч вместо 1.5 ч).

В работе использовали олигодезоксирибонуклеотиды (ODN) длиной 26 п. о., синтезированные и дополнительно очищенные электрофорезом в ПААГ («Биоссет», Новосибирск). Для получения меченых двуцепочечных ODN обе цепи метили ^{32}P-ATP («Биосан», Новосибирск) с помощью Т4-полинуклеотидкиназы («СибЭнзим», Новосибирск), отжигали при 95 °C (в эквимолярном соотношении) и медленно (не менее 3 ч) охлаждали до комнатной температуры. Отожженные дуплексы очищали и анализировали электрофорезом в 15 % ПААГ в неденатурирующих условиях с последующей радиоавтографией на фосфоримиджере Molecular Imager PharosFX Plus (Bio-Rad) (Drachkova et al., 2005). Немеченые дуплексы получали так же и использовали без дополнительной очистки в ПААГ.

Для определения равновесной константы диссоциации (K_D), а также времени полураспада ($t_{1/2}$) констант скорости образования (k_a) и распада (k_d) комплексов TBP-ODN проводили не менее трех экспериментов по связыванию TB-ODN при 25 °C в буфере, содержащем 20 мМ HEPES-KOH (pH 7.6), 5 мМ $MgCl_2$, 70 мМ KCl, 1 мМ DTT, 100 мкг/мл BSA, 0.01 % NP-40, и 5 % глицерина с фиксированной концентрацией 2 нМ активного TBP. Каждый эксперимент включал 32 реакции связывания (8 временных точек, каждая с четырьмя концентрациями ODN). Четверка реакций связывания (1 временная точка) запускалась одновременно добавлением TBP к реакционной смеси, содержащей ODN, и немедленным переносом в термостат на 25 °C. По окончании реакции связывания все реакционные смеси наносили на ПААГ одновременно при напряженности поля 10 В/см. Комплексы TBP-ODN отделяли от несвязанного ODN с помощью количественного метода электрофоретической задержки (EMSA). Электрофорез проводили в нативном 5 % ПААГ на Tris-глициновом буфере (pH 8.3) в течение 40 мин при температуре 10 °C и напряженности поля 25 В/см.

Затем гели высушивали и экспонировали с экраном Imaging Screen-K (Kodak) для фосфоимиджера Molecular Imager PharosFX Plus (Bio-Rad). Экран сканировали на фосфоимиджере и с помощью программы Quantity One – 4.5.0 (Bio-Rad) проводили количественный анализ радиоавтографов. Полученные зависимости концентрации комплексов TBP-ODN от концентраций ODN вводили как исходные данные в общедоступный Web-сервис GraphPad Prism 5 (http://graphpad-prism.software.informer.com/5.01), результатом которого были величины K_D, $t_{1/2}$, k_a и k_d анализируемых комплексов TBP-ODN, а также оценки стандартных ошибок средних (± SEM) для этих величин.

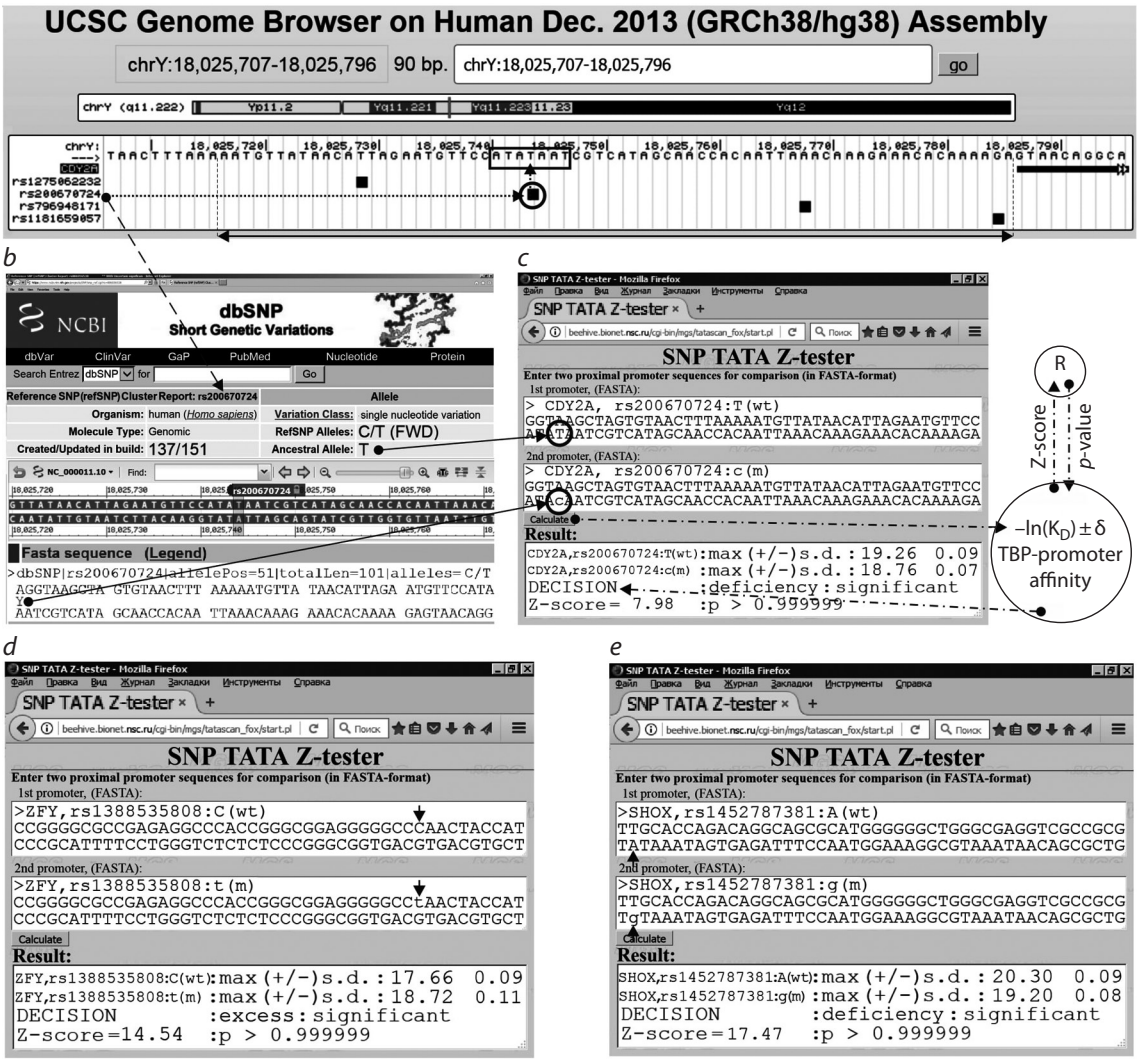

Fig. 1. Analysis of the 70 base long proximal promoter (↔) upstream of the transcription start of the human *CDY2A* gene with four unannotated SNPs (■). One of them (rs200670724; dashed arrow) has been proposed as a candidate marker for human male maturation delay and poorer reproduction potential caused by poorer expression of the gene (Stahl et al., 2012) associated with the presence of the SNP in the TBP binding site (boxed).

(*a*) Presentation of the human *CDY2A* promoter with the UCSC Genome Browser (Haeussler et al., 2015). (*b*) Annotation of rs200670724 SNP in dbSNP (Sherry et al., 2001). (*c*) The SNP_TATA_Z-tester application formerly developed by us. Line "DECISION" in its "Result" window present the prediction of CDY2A deficiency with the minor allele of the promoter as compared to the ancestral SNP variant, (R) means the R software (Waardenberg et al., 2015). (*d*, *e*) Corresponding predictions of lower male reproductive potentials done by SNP_TATA_Z-tester for candidate SNP markers rs1388535808 and rs1452787381 in the *ZFY* and *SHO* genes, respectively.

Статистический анализ. Биоинформатические оценки сродства TBP к вариантам SNP-промоторов генов хромосомы Y человека сравнивали с экспериментальными величинами сродства TBP к ODN, идентичным этим промоторам, с помощью STATISTICA (Statsoft™).

Результаты и обсуждение

Проанализированы все 35 неаннотированных SNP, доступных в выпуске № 151 базы данных dbSNP (Sherry et al., 2001), для проксимальных промоторов длиной 70 п. о. генов *CDY2A*, *SHOX* и *ZFY*, представляющих все три типа генов на хромосоме Y человека: уникальный, псевдоаутосомный и паралог гена хромосомы X человека соответственно (табл. 1). В результате было выявлено 11 кандидатных SNP-маркеров снижения МРП из-за достоверных изменений сродства TBP к этим промоторам (см. табл. 1). Рассмотрим сначала наиболее подробно единственный кандидатный SNP-маркер rs200670724 в гене *CDY2A*, связанный с ослаблением МРП, чтобы затем, благодаря аналогиям с этим иллюстративным примером, кратко описать 10 остальных кандидатных SNP-маркеров в генах *ZFY* и *SHOX*.

Уникальный **ген *CDY2A*** на хромосоме Y человека кодирует хромодомен 2A и несет четыре неаннотированных SNP в своем проксимальном промоторе длиной 70 п. о. (см. рис. 1, *a*), лишь один среди которых, rs2276109 (см.

Table 1. Candidate SNP markers of male reproductive potential (MRP) capable of altering the affinity of TBP for the promoters of the human Y chromosome genes (according to our predictions made in this work)

Gene	dbSNP (Sherry et al., 2001)	5′-flank	wt / mut	3′-flank	K_D, nM wt / mut	Δ	Z	α	Candidate SNP marker	♂	References
CDY2A (unique male-specific protein-coding gene)	rs200670724	atgttccata	**t** / c	aatcgtcata	**4** / 7	<	7	10^{-6}	Increased risks of male maturation arrest	↓	Stahl et al., 2012
ZFY (paralogous to X-linked genes)	rs1388535808	ggaggggcc	**c** / t	aactaccatc	**21** / 7	>	15	10^{-6}	Increased risks of azoospermia and infertility	↓	Jan et al., 2018
	rs996955491	gggggcccaa	**c** / a	taccatcccg	**21** / 13	>	8	10^{-6}		↓	
SHOX (pseudo-autosomal gene)	rs1452787381	gtcgccgcgt	**a** / g	taaatagtga	**2** / 5	<	17	10^{-6}	Increased risks of disproportionate short stature and Madelung deformity	↓	Ramachandrappa et al., 2018
	rs1405831103	gtcgccgcgt	**ataa** / t	atagtgagat	**2** / 8	<	22	10^{-6}		↓	
	rs1273755135	tcgccgcgta	**t** / c	aaatagtgag	**2** / 4	<	17	10^{-6}		↓	
	rs375938368	aggtcgccgc	**g** / t	tataaatagt	**1.5** / 1.9	<	3	10^{-3}		↓	
	rs771395540	caggagccaa	**t** / c	aggggtcttc	**62** / 70	<	2	0.05		↓	
	rs28378830	aggggtcttc	**g** / a*	agtcaccctg	**62** / 30	>	13	10^{-6}	Increased risks of pathoembryogenesis	↓	Brosens et al., 2014
	rs894540003	caataggggt	**c** / a	ttcgagtcac	**62** / 23	>	15	10^{-6}		↓	
	rs970127768	cgaggtcgcc	**g** / a*	cgtataaata	**1.5** / 1.3	>	2	0.05		↓	

Note. wt – norm (reference human genome); mut – minor allele; K_D – equilibrium dissociation constant of TBP-promoter complex; Δ – alteration in the affinity of TBP for promoter, namely: increase (<) and decrease (>); Z – Z-value of Fisher's Z-test; α – statistical significance estimate (α = 1 – *p*, where: *p*, probability estimate for random reasons shown in Fig. 1, *d*); ♂ – alteration in MRP, namely: increase (↑) and decrease (↓). Genes: *CDY2A* – chromodomain Y-linked 2A; *ZFY* – zinc finger protein Y-linked; *SHOX* – short stature homeobox.

* In addition to the indicated minor allele, other alleles are present, which insignificantly alter the affinity of TBP to the corresponding human promoter.

рис. 1, *б*), достоверно снижает экспрессию этого гена, согласно нашему прогнозу (см. рис. 1, *в*). Как представлено в первой строке табл. 1, этот дефицит экспрессии гена *CDY2A* вызван снижением сродства «TBP-промотор» с 4 нМ в норме до 7 нМ для минорного аллеля rs2276109.

Прежде всего мы экспериментально проверили этот прогноз методом задержки электрофоретической подвижности (EMSA). Электрофореграммам для нормального и минорного аллелей rs2276109 соответствуют рис. 2, *а* и рис. 2, *б*, тогда как зависимость концентрации комплексов TBP-ODN от концентраций ODN, которые были построены на основе этих электрофореграмм, демонстрируют рис. 2, *в* и 2, *г*.

На этих рисунках можно видеть снижение сродства "TBP-ODN" с 160 нМ в норме до 500 нМ для минорного аллеля rs2276109. Это свидетельствует об адекватности оценок Web-сервиса SNP_TATA_Z-tester (Sharypova et al., 2018) для оценки сродства TBP к промоторам генов на хромосоме Y человека.

Наконец, с помощью поиска по ключевым словам в базе данных PubMed (Lu, 2011) мы нашли клинические данные (Stahl et al., 2012) о дефиците экспрессии гена *CDY2A* при задержке полового созревания. Это позволило нам предложить кандидатный SNP-маркер rs200670724 как генетическую причину снижения МРП вследствие задержки полового созревания мужского организма.

Ген *ZFY* (Y-связанный белок «цинковый палец») является паралогом гена *ZFX* на хромосоме X человека. В его промоторе мы нашли два неаннотированных SNP, rs1388535808 и rs996955491, способных вызвать избыток *ZFY*, как представлено на рис. 1, *г*.

S.Z. Jan с коллегами (2018) сообщили о суперэкспрессии *ZFY* в сперматоцитах как клиническом маркере остановки их мейоза, азооспермии и бесплодия у мужчин. В границах применимости этих клинических наблюдений без гипотез о причинно-следственных связях мы предсказали rs1388535808 и rs996955491 как кандидатные SNP-маркеры, повышающие риск остановки сперматогенеза на стадии мейоза с последующей азооспермией и бесплодием, что безусловно, значительно снижает мужской репродуктивный потенциал (см. табл. 1).

Ген *SHOX* (гомеобокс низкорослости) локализован в псевдо-аутосомном районе 1 (PAR1) хромосомы Y человека. В проксимальных промоторах длиной 70 п. о. перед его стартами транскрипции мы нашли пять кандидатных SNP-маркеров для дефицита этого транскрипционного фактора (см. рис. 1, *е*: rs1452787381) и три кандидатных SNP-маркера для его избытка (см. табл. 1: rs28378830). Согласно табл. 1, они снижают МРП посредством непропорционально низкого роста с деформациями Маделунга (Ramachandrappa et al., 2018) либо нарушений эмбриогенеза (Brosens et al., 2014) соответственно.

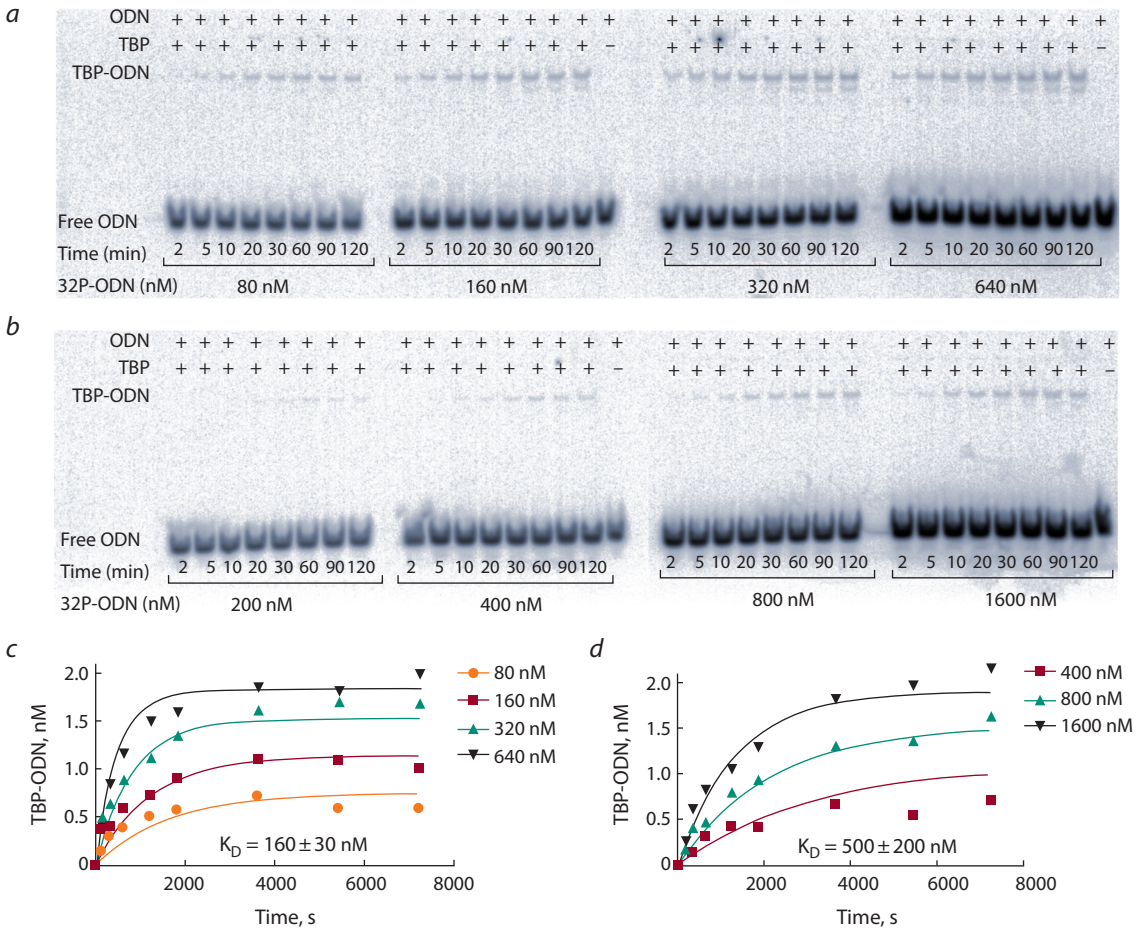

Fig. 2. Measuring the kinetics of TBP binding to two TATA-containing ODNs identical to the human *CDY2A* gene promoter.

Legend: (*a* and *b*) Electropherograms of the wild-typed ancestral and minor alleles of the unannotated SNP rs200670724 under this study, respectively; the concentration of TBP was 2 nM in all the experiments; the concentrations of an ODN containing a tested SNP allele that we used are indicated; (*c* и *d*) dependences of reaction rates on ODN concentrations in the cases of the ancestral and minor alleles of the SNP rs200670724, respectively; the K_D value of the equilibrium dissociation constant was inferred from the dependences of reaction rates on ODN concentrations according to the Web-service GraphPad Prism 5 (http://graphpad-prism.software.informer.com/5.01).

Экспериментальные данные *in vitro* для выборочной проверки трех кандидатных SNP-маркеров снижения МРП, rs20067072 (*CDY2A*), rs1452787381 (*ZFY*) и rs1452787381 (*SHOX*), представлены в табл. 2. С использованием метода EMSA были измерены, прежде всего, равновесная константа диссоциации (K_D), а также время полураспада ($t_{1/2}$), константы скорости сборки (k_a) и распада (k_d) комплексов TBP-ODN, как это было показано на иллюстративном примере rs200670724 (см. рис. 2).

Достоверные робастные корреляции – линейная Пирсона (*r*), обобщенная Гудмана–Крускала (γ), ранговые Спирмена (R) и Кендалла (τ), – между нашими предсказанными (см. табл. 1) и экспериментальными (см. табл. 2) значениями равновесной константы диссоциации (K_D), выраженными в натуральных логарифмических единицах, показаны на рис. 3. Они доказывают адекватность наших прогнозов для величин сродства TBP к промоторам генов Y-хромосомы человека. В порядке обсуждения можно коротко отметить, что несовпадение между шкалами вертикальной (эксперимент) и горизонтальной (прогноз) осей на рис. 3 соответствует различию в концентрациях TBP (т. е. в неконтролируемой доле димеров TBP, которые имеют K_D = 4 ± 1.5 нМ (Colemanet al., 1995) и не связывают ДНК) в этой работе (2 нМ) и ранее (Ponomarenko P. et al., 2008) при оптимизации трехшаговой модели связывания «TBP-промотор» (0.3 нМ), использованной Web-сервисом SNP_TATA_Z-tester (Sharypova et al., 2018) для прогнозов в этой работе.

Самая близкая к канонической последовательность TATA-бокса наблюдается в промоторе гена *SHOX* с K_D = 39 ± 7 нм (см. табл. 2). Минорный вариант SNP rs1452787381 (замена «A» на «G» в TATA-боксе) приводит к снижению сродства почти в 7 раз, что обусловлено снижением скорости образования комплексов (k_a) в 22 раза. Скорость распада комплексов (k_d) снижается в 3 раза, а также увеличивается время жизни комплексов ($t_{1/2}$). Все эти характеристики могут привести к повышенному риску формирования непропорционально низкого роста с деформациями Маделунга.

Наихудшее сродство "TBP-OND", K_D = 1000 ± 400 нм, было определено для комплексов TBP с промотором гена *ZFY*, имеющего последовательность (CCCAACTAC),

Table 2. EMSA-based *in vitro* analysis of a complex of TBP and synthetic 26 bp ODNs identical to natural promoters near the SNPs being tested

Gene dbSNP ID (Sherry et al., 2001)	Allele	ODN, sequence, 26 bp	Prediction	Experiment			
			$-\ln(K_D)$	K_D, nM	$t_{1/2}$, min	k_a, $M^{-1}s^{-1}$	k_d, $s^{-1}\times10^{-4}$
CDY2A rs200670724	*WT* T-43C	agaatgttccataTaatcgtcatagc agaatgttccataCaatcgtcatagc	19.27 18.76	160 ± 30 500 ± 200	28.2 ± 5.4 64.2 ± 18.2	2500 ± 300 350 ± 50	4.1 ± 0.7 1.8 ± 0.6
ZFY rs1388535808	*WT* C-56T	ggcggaggggccCaactaccatccc ggcggaggggccTaactaccatccc	17.67 18.18	1000 ± 400 2000 ± 1000	50.2 ± 15.8 57.8 ± 17.8	230 ± 40 90 ± 50	2.3 ± 0.6 2.0 ± 0.7
SHOX rs1452787381	*WT* A-45G	gaggtcgccgcgtAtaaatagtgaga gaggtcgccgcgtGtaaatagtgaga	20.31 19.21	39 ± 7 260 ± 70	19.3 ± 3.1 57.8 ± 14.5	17000 ± 2000 770 ± 80	6.0 ± 1.0 2.0 ± 0.5

Note: $t_{1/2}$, half-life; K_D, dissociation constant; k_a and k_d, assembly and dissociation rate constants, respectively.

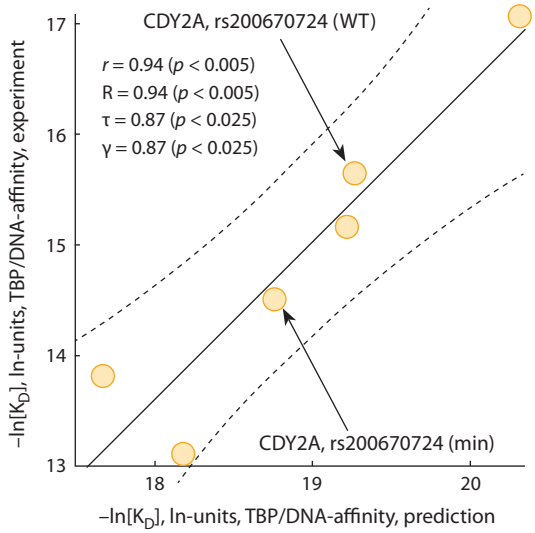

Fig. 3. The significant robust correlations between the predicted *in silico* and experimentally measured *in vitro* values of the TBP–DNA affinity (in natural logarithmic units).

Legend: solid and dashed lines denote the linear regression and boundaries of its 95 % confidence interval, calculated by means of standard software package STATISTICA (Statsoft™, Tulsa, USA); arrows pinpoint the ancestral (WT) and minor (min) alleles of the SNP being studied (rs200670724 of CDY2A), an analysis of which is depicted in Figures 1 and 2 as an example of the application of our Web service SNP_TATA_Z-tester (Sharypova et al., 2018) in this work and its *in vitro* selective verification here; *r*, γ, R, τ , and *p* are coefficients of linear correlation, Goodman–Kruskal generalized correlation, Spearman's and Kendall's rank correlations, and their *p* values, respectively.

лишь весьма отдаленно напоминающую консенсус TATA бокса (TATAAAAG). Замена «C» на «T» не спасает положение – скорость образования комплексов (k_a) снижается в 2.5 раза, и незначительно изменяется скорость их распада (k_d) и время жизни ($t_{1/2}$). Можно предположить, что предсказанная нами *in silico* суперэкспрессия минорного аллеля SNP rs1388535808 в сперматоцитах, приводящая к остановке сперматогенеза на стадии мейоза, азоospермии и бесплодию, в условиях *in vivo* обеспечивается участием TBP-ассоциированных факторов (TAFs) и других регуляторных белков в образовании транскрипционного комплекса в случае TATA-несодержащего промотора гена *ZFY* человека.

Полученные данные о снижении сродства TBP к промотору гена *CDY2A* более чем в 3 раза для аллеля *C* соответствуют клиническим данным о дефиците продукта этого гена как генетической причине задержки полового созревания.

Заключение

В настоящей работе исследованы все 35 SNP в промоторах генов *CDY2A*, *SHOX* и *ZFY*, представлявших все три типа генов хромосомы Y человека: уникальный, псевдоаутосомный и паралог гена хромосомы X человека соответственно. Из них отобраны 11 кандидатных SNP-маркеров, способных изменить сродство TBP к этим промоторам и таким образом способствовать ослаблению МРП. Прежде всего, мы выборочно верифицировали *in vitro* величины сродства «TBP-промотор», предсказанные в этой работе, и установили статистически достоверную линейную корреляцию между ними и экспериментально измеренными величинами ($r = 0.94$, $p < 0.005$). Используя поиск по ключевым словам в базе данных PubMed, мы нашли клинические данные о физиологических признаках патологий человека, которые соответствовали изменению экспрессии генов, несущих предсказанные кандидатные SNP-маркеры. Среди них были показатели процесса сперматогенеза (*ZFY*: rs1388535808 и rs996955491), задержки полового созревания (*CDY2A*: rs200670724), непропорционально низкого роста с деформациями Маделунга (например, *SHOX*: rs1452787381) и нарушения процесса эмбриогенеза (например, *SHOX*: rs28378830). В совокупности полученные результаты свидетельствуют, что в случае SNP-промоторов генов хромосомы Y человека следует ожидать изменений широкого круга показателей МРП, выходящих далеко за рамки генетического контроля собственно мужской репродуктивной функции.

Это заключение согласуется с выводом авторов реконструкции коэволюции хромосом X и Y у разных видов (опоссум, бык, крыса, мышь, мармозетка, макака-резус, шимпанзе и человек) на основе данных высокопроизводительного секвенирования (Bellott et al., 2014), что, кроме мужской репродуктивной функции, хромосома Y определяет также гендерный диморфизм в предрасположенности к заболеваниям и жизнеспособность мужчин. Предсказанные и экспериментально подтвержденные здесь изменения сродства TBP к минорным вариантам SNP генов *CDY2A*, *SHOX* и *ZFY* человека могут снизить МРП лишь при аномальных изменениях уровней белков, кодируемых этими генами, в соответствующих тканях на

определенных стадиях онтогенеза. Это с необходимостью требует клинической проверки, на снижение затрат которой благодаря улучшению ее адресности нацелено наше экспериментально-биоинформатическое исследование.

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Acknowledgements. The concept, supervision of the work, computerized predictions, and their biological interpretation (LVO and AVO) were supported by the Russian Science Foundation, project 19-15-00075. The manuscript was written by MPP and IVCh, support by the Russian Federation Ministry of Science and Education, Program for Improvement of the Competitive Ability of Russian Universities among the Global Research and Educational Centers (project 5-100). The work with the Web service (PMP and DAR) and the *in vitro* experiment (EBSh, IAD, and LKS) were supported by State Budgeted Projects 0324-2019-0040-S-01 and No. 0324-2019-0042-S-01, respectively.

Conflict of interest. The authors declare no conflict of interest.

Received December 09, 2019. Revised July 13, 2020. Accepted July 15, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):794-801
DOI 10.18699/VJ20.675

Эколого-генетическая оценка последствий влияния радиации на загрязненных территориях

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Аннотация. Объектами исследования являются районы Западно-Казахстанской области Республики Казахстан, прилегающие к полигону Капустин Яр: Бокейординский, Жангалинский, Жанибекский, Казталовский, Акжаикский и Сырымский. Цель работы – радиоэкологическое обследование загрязненных территорий и исследование содержания загрязнителей в объектах среды, биоте (грызунах, рыбах и биосубстратах домашних животных) физико-химическими методами для оценки воздействия полигона на биоту и человека. Представлены результаты экспедиционных и лабораторных исследований объектов окружающей среды прилегающих к полигону районов. Приведены показатели гамма-съемки уровней радиационного фона обследуемых территорий, данные о загрязнении почвы, поверхностных и подземных вод, доминантных форм растений, биосубстратов (шерсти домашних животных: верблюда, лошади, коровы). Использованы стандартные способы отбора проб, общепринятые методы исследования: радиологический и цитогенетический (микроядерный), атомно-абсорбционная спектрофотометрия. Проведено рекогносцировочное и радиоэкологическое обследование объектов окружающей среды с использованием аналитических методик, что позволило определить количественное содержание токсичных компонентов, приоритетных загрязнителей и радиоактивных изотопов. Установлено, что значения объемной активности природных и техногенных радионуклидов в пробах почвы, питьевой воды и биосубстратах (шерсти домашних животных, образцах периферической крови человека) из населенных пунктов соответствуют величине контрольного уровня для данного региона. Измерения гамма-излучений показали, что по периметру территории полигона и в близлежащих населенных пунктах уровень радиации находится в пределах 0.06–0.14 мкЗв/ч. Незначительное превышение уровня радиоактивности сохраняется вблизи падения ракет в Бокейординском районе. Исследованные районы характеризуются незначительным уровнем радиационного фона: среднее значение МЭД составляет 0.014 мкЗв/ч. Абсолютный максимум, 0.73 мкЗв/ч, зарегистрирован в пунктах падения ракет в Казталовском районе.
Ключевые слова: радиация; радионуклиды; радиоактивность; биосубстрат; полигон; мутация; цитогенетика; мутагены; экология.

Для цитирования: Бигалиев А.Б., Шалабаева К.З., Шимшиков Б.Е., Кобегенова С.С., Адилова Л.М., Кожахметова А.Н., Шарахметов С., Бурханова М.Н. Эколого-генетическая оценка последствий влияния радиации на загрязненных территориях. *Вавиловский журнал генетики и селекции*. 2020;24(7):794-801. DOI 10.18699/VJ20.675

Ecological and genetic assessment of the consequences of radiation influence on contaminated areas

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Abstract. The objects of study are the territory of the districts of the West Kazakhstan region of the Republic of Kazakhstan adjacent to the Kapustin Yar test site: Bokeyordinsky, Zhangalinsky, Zhanibeksky, Kaztalovsky, Akzhayiksky and Syrymsky. The purpose of the work is to conduct a radioecological survey of contaminated areas and to study the content of pollutants in environmental objects, biota (rodents, fish and biosubstrates of domestic animals) by physicochemical methods to assess the risk of the landfill's impact on biota and humans. This paper presents the results of expeditionary and laboratory research on the pollution of environmental objects in the areas adjacent to the landfill. Indicators of the gamma-survey of the levels of radiation background of the surveyed territories as well as data on pollution of soil, surface and ground waters, dominant forms of plants, biosubstrates (hair of domestic animals: camel, horse, cow) are given. The generally accepted research methods were used: standard sampling me-

thods, radiological, atomic adsorption spectrophotometry, cytogenetic (micronucleus) method. A reconnaissance and radioecological examination of environmental objects was carried out using analytical methods, which made it possible to determine the quantitative content of toxic components, the content of priority pollutants and radioactive isotopes. It has been established that the values of the volumetric activity of natural and man-made radionuclides in soil samples, drinking water and biosubstrates (pet hair, human peripheral blood samples) from settlements correspond to the value of the control level for this region. The measurements of radiation activity by gamma radiation showed that along the perimeter of the surveyed territory of the test site and in nearby settlements the radiation level is in the range of 0.06–0.014 μSv/h. A slight excess of the level of radioactivity persists in the area near the fall of missiles in the Bokeyordin region. The investigated regions are characterized by an insignificant level of background radiation, the average DER value for the regions as a whole is 0.14 μSv/h. The absolute maximum, 0.73 μSv/h, was recorded at the points of missile impact in the Kaztal region.
Key words: radiation; radionuclides; radioactivity; biosubstrate; polygon; mutation; cytogenetics; mutagens; ecology.

For citation: Bigaliev A.B., Shalabaeva K.Z., Shimshikov B.E., Kobegenova S.S., Adilova L.M., Kozhakhmetova A.N., Sharakhmetov S., Burkhanova M.N. Ecological and genetic assessment of the consequences of radiation influence on contaminated areas. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7): 794-801. DOI 10.18699/VJ20.675 (in Russian)

Введение

Вокруг ракетно-ядерных полигонов в Атырауской и Западно-Казахстанской областях Республики Казахстан сложилась сложная экологическая обстановка, вызванная радиационным загрязнением природной среды. В 2004–2008 гг. на территории военных полигонов Капустин Яр и Азгир и в прилегающих районах проведен ряд экологических работ с применением полевых и аналитических методов исследования. Тем не менее вопрос о степени влияния деятельности полигонов на окружающую среду и здоровье населения остается нерешенным[1] (Мухаметжанова, 2017). На протяжении нескольких десятков лет ученые Казахского национального университета имени аль-Фараби проводят исследования, направленные на поиск чувствительных биологических маркеров, специфичных для оценки радиационного воздействия и информативных как в раннем, так и отдаленном периоде облучения. В настоящее время одними из немногих биологических показателей (наряду с ЭПР-спектроскопией эмали зубов), в полной мере отвечающих этим требованиям, являются хромосомные аберрации в лимфоцитах периферической крови. Принципы цитогенетического метода индикации радиационного воздействия достаточно убедительно обоснованы во многих отечественных и зарубежных исследованиях, результаты которых послужили основой для рекомендаций ВОЗ, МАГАТЭ и НКДАР ООН по использованию анализа хромосомных аберраций в лимфоцитах крови в качестве тест-системы для количественной оценки действия мутагенных факторов радиационной природы (WHO…, 2010). Данные о «биологической» дозе, полученные с помощью цитогенетических методов шире, чем ее физическое значение, так как отражают не только результат радиационного воздействия на организм человека, но и его индивидуальную радиочувствительность, что позволяет более корректно прогнозировать ранние и отдаленные последствия облучения. В настоящее время в большинстве случаев, при которых люди подвергаются воздействию радиации как от естественных, так и техногенных источников, речь идет об облучении в небольших дозах (Zhumadilov et al., 2013). Поэтому основную проблему составляют последствия радиационного воздействия в малых дозах, особенности биологических эффектов которого до сих пор являются предметом активных дискуссий (Hoshi, Saimova, 2017).

Актуальность предлагаемой работы определена необходимостью изучения современного состояния природных популяций растений, животных и человека в условиях различного фона ионизирующей радиации при длительном хроническом облучении в местах ядерных испытаний на территории полигона Капустин Яр. В свою очередь, оценка влияния деятельности полигона требует разработки природоохранных мероприятий по уменьшению техногенного воздействия, ранней диагностики устойчивости генома природных популяций, подвергающихся давлению антропогенного пресса. При этом требуются учет и прогнозирование текущих и отдаленных последствий влияния факторов среды обитания на биоту и здоровье населения, что представляет чрезвычайную актуальность для данного региона и имеет научно-практическую значимость (Bigaliev, 2016; Markabayeva et al., 2018).

Материалы и методы

Использованы общепринятые методы отбора проб почвы, воды, образцов растений, животных и человека. Отбор проб почвы производили согласно стандартной методике № 5.05.008-99 г. (Островская и др., 2014). Пробы почвы плотно запечатывали в кюветах и перед спектрометрическими измерениями выдерживали в течение 2–3 нед. для установления подвижного равновесия между изотопами ^{226}Ra, ^{224}Ra и продуктами их распада (^{222}Ra, ^{220}Ra и др.). Исследования проводили согласно утвержденному и внесенному в государственный реестр Республики Казахстан документу «Методика измерения активности радионуклидов с использованием сцинтилляционного гамма-спектрометра с программным обеспечением «Прогресс» (рег. № KZ.07.00.00304-2014)[2]. Из населенных пунктов, расположенных на прилегающей к полигону территории, отобраны биосубстраты домашних животных, образцы доминантных видов растений, взяты образцы периферической крови жителей. Все они являются зве-

[1] Национальный доклад о состоянии окружающей среды и использовании природных ресурсов Республики Казахстан за 2011–2014 годы. Под ред. РГП на ПХВ «Информационно-аналитический центр охраны окружающей среды». Алматы, 2015;214.

[2] Инструкция по отбору проб почвы при радиационном обследовании загрязненной территории, 1987.

ньями трофической цепи питания и участвуют в миграции радионуклидов и тяжелых металлов (Постановление Правительства РК «Об утверждении Правил объявления чрезвычайной экологической ситуации» № 431 от 5 мая 2005 г.). Определение активности радионуклидов проводили спектрометрическим методом с использованием гамма-спектрометров «МКС-01А Мультирад» (ООО «НТЦ «Амплитуда», Россия), Canberra CR-4018 (США). Для выявления содержания тяжелых металлов применена атомно-абсорбционная спектрофотометрия. Исследования проведены в физико-химической лаборатории факультета биологии и биотехнологии Казахского национального университета имени аль-Фараби в соответствии с общепринятыми стандартами (ГОСТ 26929-86, ГОСТ 30178-96, ИСО 8288-1986) на спектрофотометре «МГА-915МД» (ГК «Люмэкс», Россия) (Техногенные потоки…, 2001).

Для цитогенетического исследования использовали микроядерный тест. Цитологические препараты для микроядерного анализа готовили согласно общепринятым методикам (Руководство…, 2002), фотографировали в световых микроскопах Axioskop 40 (Carl Zeiss, Германия) и Micro Optic (Австрия). Забор образцов крови для микроядерного анализа проводили из фаланги пальцев в условиях сельских поликлиник, соблюдая принципы антисептики. Объектом исследования служила популяция коренных жителей, включающая 107 человек, проживающих на территории, прилегающей к полигону Капустин Яр.

Результаты и обсуждение

Планомерные работы по оценке влияния деятельности полигонов Капустин Яр и Азгир на окружающую среду начались в 2001 г., став первым этапом программы исследований в регионе, охватывающем шесть южных районов Западно-Казахстанской области и два района Атырауской области. Работы первого этапа завершены в 2002 г. (Loomis et al., 1990) – представлены результаты полевых и лабораторных исследований в Бокейординском и южной части Жангалинского районов. Выполнены инструментальные измерения радиационных параметров территории данных районов, используемой для падения отделяющихся частей ракет. Однако изучение состояния здоровья населения проведено только с использованием медицинской и демографической статистики. Данные медицинских исследований получены разными составами врачей, без сравнения с контрольным районом и проведения необходимых генетических исследований (популяционных и цитогенетических), должной статистической обработки полученных результатов.

В 2018–2019 гг. мы впервые взяли пробы, образцы и биосубстраты в этих же районах Западно-Казахстанской области для лабораторных исследований. Для рекогносцировочного изучения загрязненных участков, выявленных по результатам предыдущих исследований, проведено экспедиционное обследование экологического состояния компонентов окружающей среды: произведен отбор проб почвы, воды (подземных и поверхностных), растений, биосубстратов (шерсти домашних животных, органов и тканей рыб, грызунов, ящериц, образцов периферической крови жителей). В 2018 г. в Жангалинском районе взято 78 проб и образцов, Бокейординском районе – 89, Жанибекском районе – 75, Казталовском районе – 91. Всего по четырем районам – 333. В 2019 г. в Акжаикском районе отобрано 78 проб и образцов, бывшем Тайпакском районе – 89, Сырымском районе – 46. Всего по трем районам – 213. Проведены измерения радиометрических параметров среды в точках отбора проб в соответствии с методикой гамма-съемки[3].

Полевые исследования

Результаты радиологического исследования активности радионуклидов техногенного происхождения в пробах и образцах тест-объектов, взятых с территорий зоны полигона, представлены в табл. 1.

Проведенные радиоэкологические исследования объектов окружающей среды с использованием аналитических методик позволили определить количественное содержание приоритетных загрязнителей и радиоактивных изотопов. Установлено, что значения объемной активности природных и техногенных радионуклидов в пробах почвы, питьевой воды и биосубстратах (шерсти домашних животных) из исследуемых населенных пунктов соответствуют показателям контрольного уровня. Измерения радиационной активности методом гамма-излучения показали, что по периметру обследованной территории полигона и в близлежащих населенных пунктах уровень радиации находится в пределах 0.06–0.014 мкЗв/ч, что соответствует уровню радиации для данного региона. Незначительное превышение уровня радиоактивности сохраняется на территории вблизи падения ракет в Бокейординском районе. Таким образом, исследованные районы характеризуются незначительным уровнем радиационного фона, среднее значение МЭД составляет 0.14 мкЗв/ч (Постановление Правительства РК «Об утверждении Правил объявления чрезвычайной экологической ситуации» № 431 от 5 мая 2005 г.). Абсолютный максимум, 0.73 мкЗв/ч, зарегистрирован в пунктах падения ракет в Казталовском районе.

Определено содержание тяжелых металлов в подготовленных пробах (почвы, растений и воды) методом атомно-абсорбционной спектрометрии согласно ГОСТ СТ РК ИСО 11047-2008 на приборах «МГА-915» и ASS-1 (табл. 2).

Наибольшая концентрация тяжелых металлов выявлена в подземных водах обследованных районов. Так, максимальные показатели содержания Zn составили 0.05–0.71 мг/л, Mn – 0.838 (с. Базаршолан), Cr – 0.052, Fe – 0.06–0.88 мг/л (с. Базартобе).

Незначительно увеличенное содержание тяжелых металлов определено на территориях с относительно повышенным радиационным фоном (с. Харкен, Базаршолан, Базартобе) – как в образцах почвы, так и растений. Значительное накопление тяжелых металлов Ni, Zn, Co, Cr установлено в почвах обследованных территорий, прилегающих к полигону. Полученные результаты позволяют констатировать, что загрязнение окружающей среды представляет определенную опасность для биоты и человека. К стойким химическим загрязнителям кумулятивного действия со специфическими токсическими свойствами

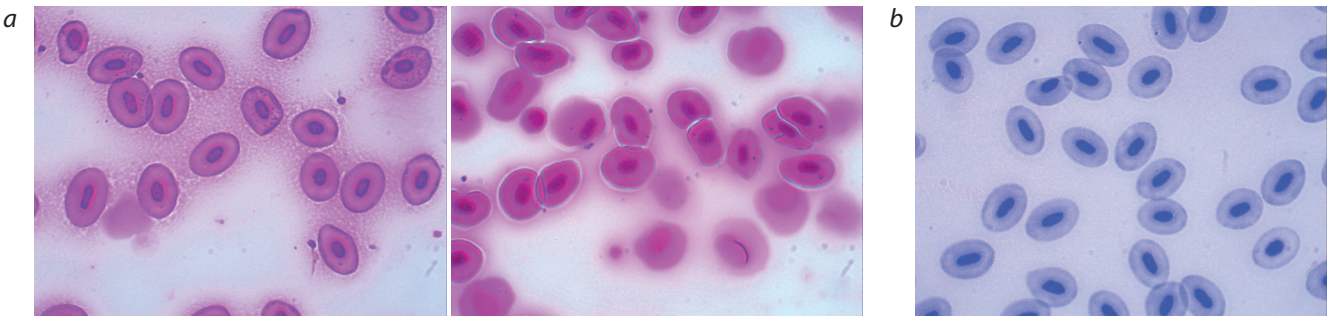
[3] Коллектив авторов ИРБЭ НЯЦ РК – отчет по теме: «Оценка влияния деятельности военных полигонов на окружающую среду и определение мер по ее реабилитации», 2002.

Table 1. Activity of technogenic radionuclides in samples and samples of test objects

Location name	Object type	Mass (g)	Cs-137	Ra-226	Th-232	K-40	r-90
Bokeyordinsky district							
Bisen	Plants	89	< 3.0	38.5 ± 18.3	16.7 ± 9.0	220.0 ± 32.0	< 3.0
	Soil	942		36.8 ± 15.2	18.2 ± 7.0	324.0 ± 42.0	
Khan Ordasy (rocket crash site)	Soil	108		30.6 ± 11.6	19.2 ± 6.0	251.0 ± 37.0	
	Plants	249		30.7 ± 14.0	20.0 ± 10.0	100.0 ± 27.0	
	Salt film on the surface of groundwater	40		40.9 ± 16.0	27.0 ± 13.0	110.0 ± 24.0	
Zhanibeksky district							
Mashteksay village (point cemetery)	Soil	494	< 3.0	55.8 ± 11.7	7.7 ± 5.0	98.0 ± 27.0	< 3.0
	Plants	55		57.9 ± 13.2	9.4 ± 4.0	105.0 ± 23.0	
Onege village (point 3 km to the north)	Plants	90		59.3 ± 15.4	7.6 ± 5.0	109.0 ± 25.0	
	Soil	542		56.6 ± 11.0	11.3 ± 6.0	104.0 ± 20.0	
Zhangalinsky district							
Zhana Kazan village (Bolshoy Ozen river bank)	Soil	95	< 3.0	44.7 ± 11.0	18.7 ± 7.0	90.8 ± 28.0	< 3.0
	Plants	97		40.5 ± 14.0	23.3 ± 11.0	97.2 ± 26.0	
Kazstalovsky district							
Kaztalovka (Seksenbay-1)							
point 3 km to the west	Plants	54	< 3.0	62.6 ± 10.0	21.1 ± 10.0	218.5 ± 13.0	< 3.0
point 3 km to the south	Soil	355		73.5 ± 9.0	22.6 ± 12.0	226.2 ± 14.0	
Kaztalovka (Seksenbay-2)							
point 3 km to the north	Plants	50		59.5 ± 10.0	24.4 ± 10.0	195.9 ± 18.0	
point 3 km to the south-west	Soil	297		54.9 ± 12.0	17.9 ± 13.0	97.7 ± 20.0	
Bozoy village							
3 points along the perimeter	Plants	240		49.7 ± 15.0	25.4 ± 9.0	223.3 ± 10.0	
point 3 km to the north	Soil	1002		58.6 ± 13.0	18.2 ± 9.0	228.2 ± 11.0	
Konys village							
3 points along the perimeter	Plants	24		56.8 ± 11.0	17.6 ± 10.0	193.6 ± 19.0	
point 3 km to the north	Soil	257		55.8 ± 13.0	25.9 ± 8.0	219.4 ± 12.0	
Novokazanka village (litter along the road)	Plants	137		59.4 ± 10.0	21.8 ± 9.0	195.5 ± 20.0	
Combined samples of 5 individuals of each species (biosubstrates)							
Bokeyordinsky district (village Saigyn point)	Wool (cow)	104	< 3.0	13.8 ± 8.05	< 3.0	15.0 ± 10.0	< 3.0
	Wool (horse)	102		11.6 ± 6.03		13.2 ± 8.0	
	Wool (camel)	107		12.5 ± 7.11		13.43 ± 7.1	
Zhangalinsky district (points Novokazanka, Zhangala)	Wool (cow)	100	< 3.0	11.6 ± 6.15	< 3.0	12.2 ± 7.31 11.2 ± 6.30	< 3.0
	Wool (horse)	102		10.4 ± 5.83		13.11 ± 8.2	
	Wool (camel)	107		11.5 ± 6.22		12.2 ± 7.31	
Zhanibeksky district (point Bozoy, aul Konys)	Wool (cow)	120	< 3.0	12.4 ± 7.51	< 3.0	13.3 ± 6.52	< 3.0
	Wool (horse)	125		13.6 ± 8.14		13.7 ± 6.82	
	Wool (camel)	135		14.7 ± 7.66		15.2 ± 9.33	
Kaztalovsky district (item drop location)	Wool (cow)	110	< 3.0	11.4 ± 6.42	< 3.0	10.7 ± 5.81	< 3.0
	Wool (horse)	125		13.6 ± 8.14		13.7 ± 6.73	
	Wool (camel)	105		10.8 ± 5.54		11.2 ± 7.12	

Table 2. The content of heavy metals in soil, plant and water (surface and underground) samples in the Kapustin Yar landfill area

Location name	Cu	Zn	Cd	Pb	Ni	Co	Mn	Cr	Fe
In soil samples in a layer of 0–10 cm, mg/kg									
Lbischensky (at the akimat)	18.67	118.71	0.77	15.00	91.43	17.08	766.66	71.43	52100.80
Aksuat (Baturino)	23.11	121.29	0.56	27.50	377.14	17.50	883.33	65.71	47058.82
Shandarzhap (Harken)	16.00	83.87	0.21	17.50	331.43	13.33	608.33	45.71	26890.75
Tompak	14.89	87.74	0.28	27.50	3371.42	15.42	666.66	42.86	31932.77
Tinali (at the well)	22.44	127.74	0.70	27.50	480.00	16.25	733.33	54.28	33613.44
Bazarsholan	18.44	105.80	0.49	35.00	245.71	13.33	733.33	45.71	35294.41
Karaultobe	21.11	116.13	0.63	62.50	411.43	19.58	950.00	68.57	48739.49
Bazartobe	17.11	149.68	0.35	32.50	251.43	4.58	633.33	37.14	16806.72
Tolepkol	26.22	121.29	0.56	40.00	137.14	13.75	416.66	37.14	48739.50
Saykuduk	18.22	110.97	0.63	57.50	297.14	14.17	725.00	48.57	18487.39
J. Moldagaliev	15.11	103.22	0.70	37.50	131.43	10.00	725.00	37.44	33613.44
Zhympity	20.33	117.42	0.77	47.50	262.86	14.17	900.00	51.43	21848.74
In plant samples, mg/kg									
Lbischensky (near the akimat), gray wormwood	15.83	90.11	0.80	3.81	10.90	3.00	7.14	16.18	694.44
Aksuat (Baturino), gray wormwood	11.33	67.03	0.76	5.71	8.00	2.33	26.19	25.43	944.44
Chapaev, white wormwood	14.33	60.44	0.53	7.62	8.73	2.33	3.81	34.68	333.33
Tinali (at the well), wormwood	6.67	49.45	0.49	9.52	5.09	2.00	17.62	32.37	777.77
Tompac, gray wormwood	8.00	27.47	0.53	5.71	5.82	1.67	12.38	39.31	750.00
Shandarzhap (Harken), itsigek	6.83	40.66	0.98	11.43	5.82	3.00	10.95	34.68	694.44
Tolepkol, green wormwood	9.67	65.93	0.49	7.62	7.27	4.00	3.33	18.50	916.66
Bazarsholan, kochia	10.33	39.56	0.71	9.52	10.18	2.67	20.00	32.37	1916.66
Bazartobe, wormwood	9.50	59.34	0.53	7.62	9.45	2.67	11.91	34.68	1527.77
Karaultobe, wormwood	8.16	26.37	0.58	93.52	5.09	1.67	12.38	23.12	694.44
Saykuduk, wormwood	9.16	42.86	0.53	13.33	10.90	2.67	14.29	32.37	1666.66
J. Moldagaliev, wormwood	12.16	60.44	0.71	11.43	8.73	2.67	13.33	34.68	1472.22
Zhympity, wormwood	9.16	52.75	0.89	13.33	5.09	3.67	9.05	23.12	1027.77
In water samples, mg/l									
Aksuat (Baturino), water tower	0.00322	0.05358	0.001145	0.02365	0.0095	0.0084	0.008571	0.02446	0.1277
Bazarsholan, drinking water	0.00458	0.04956	0.001955	0.02514	0.0183	0.01503	0.8380	0.034335	0.024
Tinali, well	0.00967	0.02677	0.000773	0.01546	0.01353	0.05993	0.001933	0.033526	0.0177
Lake near Aksuat	0.0048	0.03428	0.000773	0.009143	0.0136	0.0050	0.2743	0.01387	0.0053
Lbischenski, underground water	0.003513	0.05859	0.000772	0.01771	0.005787	0.005373	0.01476	0.03583	0.0689
Karaultobe, drinking water	0.00447	0.02279	0.00057	0.00813	0.006913	0.004473	0.002614	0.02538	0.01525
J. Moldagaliev, drinking water	0.000353	0.00058	0.00024	0.00505	0.003180	0.002297	0.00025	0.02696	0.0029
Saykuduk	0.00171	0.0089	0.00074	0.01131	0.00612	0.00540	0.0108	0.02122	0.0120
Zhympity, drinking water	0.00219	0.01704	0.00074	0.01131	0.004387	0.004543	0.00201	0.02065	0.0235
Budarinsky Canal	0.00324	0.02314	0.00122	0.02057	0.00544	0.00918	0.002571	0.0250	0.0195
Bazartobe, water from a well	0.00544	0.01266	0.00448	0.07863	0.03456	0.0528	0.021486	0.05216	0.8800
Tolepkol, a well for sheep	0.0050	0.05220	0.00096	0.01905	0.007393	0.006667	0.1357	0.03237	0.0250
Shandarzhap (Harken), water supply	0.00309	0.7011	0.000772	0.01105	0.005027	0.00522	0.00193	0.02145	0.02094



Pathologies of erythrocytes of crucian carp: *a* – micronuclei, displacement of the nucleus to the periphery, anisacytosis; *b* – micronuclei, invagination of the nucleus (×1000, coloration with basic fuchsin according to Pfeffer).

относятся прежде всего тяжелые металлы. Тройку наиболее экологически опасных тяжелых металлов составляют свинец, ртуть и кадмий. Данные металлы являются потенциально опасными токсикантами, способными вызывать нарушения жизнедеятельности водной и наземной биоты, и, следовательно, могут быть дестабилизирующим фактором в экологической системе сложившегося биоценоза (Фомин Г.С., Фомин А.Г., 2001). Также общеизвестно, что тяжелые металлы, накапливаясь в тканях организма, изменяют транскрипционную активность хромосом (Теплая, 2013; Chaizhunusova et al., 2017; Serzhanova et al., 2018) и приводят к нарушениям устойчивости генома. Обнаруженный относительно высокий уровень активности радионуклидов в локальных точках среди исследуемых тест-объектов отмечается в почвах, подземных водах, растениях и биосубстратах в пределах контрольных величин.

Цитогенетические исследования

В популяциях человека и животных имеются индивидуумы и особи с различной устойчивостью к мутагенным факторам. Особое внимание в этой связи следует обратить на виды с выраженной нестабильностью генома (Bigalyev et al., 2014).

Микроядерный анализ образцов рыб. Из образцов отловленных на исследуемой территории видов рыб были приготовлены гематологические препараты для микроядерного теста. У обследованных рыб эритроциты были представлены молодыми бластными формами и зрелыми клетками (см. рисунок). Молодые клетки в зависимости от степени развития представляли собой круглые или слегка вытянутые клетки, размеры ядер которых варьировали от крупных, занимавших большую часть клетки, до мелких. В большинстве случаев зрелые эритроциты имели эллипсовидную форму, вытянутое ядро красно-фиолетового цвета, прозрачную цитоплазму серо-розового цвета. Наряду со здоровыми клетками были зарегистрированы и патологические.

Вобла. Клетки крови воблы в большинстве случаев характеризовались неправильной формой эритроцитов. Патологии эритроцитов включали микроядра, смещение ядра к периферии, анизоцитоз, инвагинацию ядра, что несколько затрудняло проведение микроядерного анализа.

Судак. У особей судака умеренно часто встречались патологии двух групп: смещение ядра к периферии, вызываемое набуханием клетки, микроядра, возникающие при нарушениях клеточного деления, и инвагинация ядра, являющаяся маркером дегенерации эритроцитов. Отмечены единичные очаги ядерных теней, возникающих при разрушении эритроцитов.

Жерех. У особей жереха были наиболее выражены патологии первой группы. Отмечено большое количество эритроцитов неправильной формы – грушевидной, серповидной, пятиугольной (пойкилоцитоз). Незначительно представлены ядерные патологии в виде инвагинации ядра и микроядер.

Карась. В эритроцитах изученных карасей присутствовали как относительно крупные, так и мелкие микроядра. При этом в одной клетке в некоторых случаях наблюдалось по одной-две и более микроядер. Однако наиболее часто встречались клетки с одним микроядром помимо основного ядра. Кроме того, наблюдались смещение ядра к периферии клетке и инвагинация ядра (см. рисунок).

Нарушения эритроцитов, выявленные у исследуемых видов, свидетельствовали о дестабилизации физиологических процессов в организме обследованных рыб, приводящей к развитию патологии митоза. Так, анизо- и пойкилоцитоз показывают функциональную недостаточность кроветворных органов, а также наблюдаются при анемии. Наряду с вышеуказанными патологиями обнаружено смещение ядер к периферии (возникающее при набухании). К дегенеративным изменениям также можно отнести инвагинацию ядра, свидетельствующую о деградации самого эритроцита. Последующий микроядерный анализ мазков показал увеличение спонтанной частоты клеток с микроядрами в 1.5–2 раза в загрязненных районах. Вероятность встречаемости эритроцитов с микроядрами в периферической крови при спонтанном мутагенезе составляет 0.5–1.0 % (Fenech, 2011).

Полученные результаты в дальнейшем будут сопоставлены с результатами гистопатологического анализа внутренних органов (жабр, печени, кишечника, мышц и гонад) для оценки токсикологического состояния среды обитания рыб.

Микроядерный анализ клеток человека. Объектом исследования служила свежеполученная кровь из пальца смешанной популяции, включающей 107 человек, проживающих в районах с неблагополучной экологической обстановкой из-за близости полигона Капустин Яр. Сводные группы для микроядерного анализа составили 23 человека с наследственными дегенеративными заболеваниями

Table 3. The number of erythrocytes with micronuclei in the blood of individuals from the study area

Experimental group	Number of patients, *n*	The number of analyzed eritrocytes in thousands	Erythrocytes with micronuclei	
			absolute	at %
Control group	50	482.4		0.427 ± 0.01
Patients:				
with perinatal pathology	25	212.6	148	1.411 ± 0.07
children with congenital malformation	24	258.8	300	2.99 ± 0.11
with hepatocerebral dystrophy	27	259.6	774	3.82 ± 0.12
with other inherited degenerative diseases	23	245.1	991	3.68 ± 0.13
with Down syndrome	8	98.1	902	4.3 ± 0.11

нервной системы, 27 – с гепатоцеребральной дистрофией, 24 – с врожденными пороками развития, 25 – с перинатальной патологией, 8 – с синдромом Дауна. Средний возраст больных – 33.9 года. В контрольную группу вошли 50 практически здоровых людей в возрасте 20–37 лет. Результаты микроядерного теста представлены в табл. 3.

Количество выявленных микроядер достоверно выше у пациентов с наследственно-дегенеративными заболеваниями ($t = 21.68$, $p < 0.01$), гепатоцеребральной дистрофией ($t = 33.93$, $p < 0.01$), врожденными пороками развития ($t = 25.63$, $p < 0.01$), синдромом Дауна ($t = 38.73$, $p < 0.01$) и перинатальной патологией ($t = 14.05$, $p < 0.01$) в сравнении с контрольной группой – 0.427 ± 0.01.

По данным различных авторов, спонтанный уровень микроядер у здоровых людей колеблется от 0.24 ± 0.01 до 0.34 ± 0.1 %. Больные с высоким показателем микроядер обследованы повторно через месяц, у них обнаружена тенденция повышения количества микроядер в эритроцитах периферической крови.

В зависимости от размера микроядра распределены на две группы: крупные и мелкие. Эритроциты с мелкими микроядрами составляли 88 %, а с крупными микроядрами – 12 %. Типы нарушений ядер соматических клеток различаются по количеству и форме в зависимости от видовой, тканевой принадлежности. По данным авторов, высокий уровень эритроцитов с микроядрами зарегистрирован у больных с различными формами миопатии. Дальнейшие исследования позволили прийти к выводу, что нестабильность генома подтверждается и другими тестами (Ильинских и др., 1992). Проведен корреляционный анализ, который свидетельствует, что образование крупных микроядер тесно связано с геномными нарушениями хромосомного аппарата ($r = 0.70$, $p < 0.05$), тогда как уровень клеток с мелкими микроядрами коррелирует с частотой нарушений в структуре хромосом ($r = 0.60$, $p < 0.05$). Установлено, что частота эритроцитов с мелкими микроядрами не зависит от уровня патологии митоза, многогрупповых мета- и анафаз с мостами (во всех случаях $p > 0.05$). Уровень клеток с крупными микроядрами тесно связан с патологией митоза – отставанием отдельных хромосом в мета- и анафазах – и свидетельствует о том, что крупные микроядра, по-видимому, образованы отставшими хромосомами, в то время как мелкие – в основном структурными аберрациями хромосом. Авторы утверждают, что приведенные данные показывают тесную связь между цитогенетическими нарушениями и образованием микроядер (Djokovic-Davidovic et al., 2016).

Заключение

Значения объемной активности природных и техногенных радионуклидов в пробах почвы, питьевой воды, биосубстратах (шерсти домашних животных и образцах периферической крови человека) из населенных пунктов, расположенных рядом с полигоном Капустин Яр, соответствуют величине контрольного для Западно-Казахстанской области уровня (радиационный фон в пределах 0.06–0.014 мкЗв/ч). Незначительное превышение уровня радиоактивности сохраняется на территории вблизи падения ракет в Бокейординском районе. Цитогенетические исследования с использованием микроядерного теста в соматических клетках рыб показали увеличение спонтанной частоты клеток с микроядрами в 1.5–2 раза в загрязненных районах. Вероятность встречаемости эритроцитов с микроядрами в периферической крови при спонтанном мутагенезе составляет 0.5–1.0 %. Анализ мазков крови человека на микроядерный тест также показал увеличение спонтанной частоты клеток с микроядрами в 1.5–2 раза в загрязненных районах по сравнению со спонтанной частотой 0.5–1.0 %.

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Conflict of interest. The authors declare no conflict of interest.

Received September 10, 2020. Revised October 16, 2020. Accepted October 16, 2020.

Вавиловский журнал генетики и селекции. 2020;24(7):802-807
DOI 10.18699/VJ20.676

mRNA technology as one of the promising platforms for the SARS-CoV-2 vaccine development

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Abstract. After the genome sequence of SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus 2) was published and the number of infected people began to increase rapidly, many global companies began to develop a vaccine. Almost all known approaches to vaccine design were applied for this purpose, including inactivated viruses, mRNA and DNA-vaccines, vaccines based on various viral vectors, synthetically generated peptides and recombinant proteins produced in cells of insects and mammals. This review considers one of the promising vaccine platforms based on messenger RNA. Until recent years, mRNA-vaccination was out of practical implementation due to high sensitivity to nuclease degradation and consequent instability of drugs based on mRNA. Latest technological advances significantly mitigated the problems of low immunogenicity, instability, and difficulties in RNA-vaccine delivery. It is worth noting that mRNA-vaccines can efficiently activate both components of the immune system, i. e. T-cell and humoral responses. The essential advantage of mRNA-vaccines includes fast, inexpensive, scalable and uniform production providing a large output of desirable products *in vitro*. Synthesis and purification processes significantly simplify the process technology of mRNA drugs with injectable purity. Thus, mRNA production via *in vitro* transcription is more advantageous as compared with DNA-vaccines since it is a chemical process without the use of cells. mRNA techniques make it possible to pass all the phases of vaccine development much faster in comparison with the production of vaccines based on inactivated viruses or recombinant proteins. This property is critically important when designing vaccines against viral pathogens as the main problem of disease control includes a time gap between an epidemic and vaccine development. This paper discusses studies on the development of vaccines against coronaviruses including SARS-CoV-2 with special attention to the mRNA technique.
Key words: coronavirus; SARS-CoV-2; COVID-19; mRNA-vaccines.

For citation: Ilyichev A.A., Orlova L.A., Sharabrin S.V., Karpenko L.I. mRNA technology as one of the promising platforms for the SARS-CoV-2 vaccine development. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2020;24(7):802-807. DOI 10.18699/VJ20.676

Технология мРНК как одна из перспективных платформ для разработки вакцины против SARS-CoV-2

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Аннотация. После того как была опубликована последовательность генома SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus 2), а количество заболевших стало стремительно возрастать, многие глобальные компании начали разработку вакцины от данного вируса. Для создания вакцины задействованы практически все известные на данный момент способы – это вакцины на основе инактивированного вируса, мРНК и ДНК, вирусных векторов, синтетических пептидов и рекомбинантных белков, произведенных в клетках насекомых и млекопитающих. В обзоре рассматривается одна из перспективных вакцинных платформ, созданная на основе матричной РНК (мРНК). До недавнего времени мРНК-вакцинация не рассматривалась с практической точки зрения в силу высокой чувствительности к нуклеазной деградации и, как следствие, нестабильности препаратов на основе мРНК. Последние технологические достижения в значительной степени преодолели проблемы низкой иммуногенности, нестабильности и трудности доставки РНК-вакцин. Важно отметить, что мРНК-вакцины способны эффективно активизировать оба звена иммунитета – как Т-клеточный, так и гуморальный ответы. Существенным преимуществом мРНК-вакцин является быстрое недорогое масштабируемое и однотипное производство, обеспечивающее высокие выходы желаемого продукта в условиях *in vitro*. После синтеза и процедуры очистки технологически значительно проще добиться получения препарата мРНК инъекционной чистоты. Таким образом, производство мРНК путем транскрипции *in vitro* предпочтительнее в сравнении с производством ДНК-вакцин, так

как в действительности является химическим процессом без использования клеток. По сравнению с производством вакцин на основе инактивированного вируса или рекомбинантного белка мРНК-технологии позволяют гораздо быстрее пройти все этапы разработки. Этот параметр имеет первостепенное значение для создания препаратов против вирусных патогенов, основной проблемой борьбы с которыми является временной разрыв между эпидемией и разработкой вакцины. В данном обзоре мы обсуждаем работы, связанные с разработкой вакцины против коронавирусов, включая SARS-CoV-2, с акцентом на технологии мРНК.
Ключевые слова: коронавирус; SARS-CoV-2; COVID-19; мРНК-вакцины.

After the first sequence of the severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) genome was published by Chinese researchers in January 2020, many scientific organisations and pharmaceutical companies began developing a vaccine against SARS-CoV-2 (Zhou P. et al., 2020). For this purpose, almost all known approaches for vaccine design were applied, including inactivated viruses, mRNA and DNA vaccines, vaccines based on various viral vectors, recombinant proteins produced in cells of insects and mammals, and synthetic peptide-based vaccines.

In this article we will consider the advantages of mRNA vaccines.

SARS-CoV-2 belongs to the family of *Coronaviridae*, which also includes dangerous viruses such as severe acute respiratory syndrome-related coronavirus (SARS-CoV) and Middle East respiratory syndrome-related coronavirus (MERS-CoV)[1]. These viruses have a single-stranded RNA genome about 30 kb in size, which is the largest known RNA virus genome. The complete genomes of SARS-CoV-2 and SARS-CoV have very high homology, suggesting that the mechanisms of entry of these viruses into human cells are similar (Zhou P. et al., 2020). The viral envelope consists of a lipid bilayer in which the structural proteins of the membrane (M), envelope (E), and spike (S) are fixed. The nucleocapsid (N) protein, together with the viral RNA genome, form a helical core located within the viral envelope. The ratio of S:E:M:N proteins corresponds to 20:1:300:100. The globular part of the S protein contains many dominant antigenic epitopes involved in the mechanisms of the humoral and cellular immune response (Zhou Y. et al., 2018). The S protein plays a crucial role in the attachment of coronaviruses to a cell surface receptor and, consequently, entry of the virus into the cell. For SARS-CoV-2 and SARS-CoV, the S protein receptor is angiotensin converting enzyme 2 (ACE2). Therefore, the S protein is considered the most suitable target for vaccine development (He et al., 2006).

Research on the development of vaccines against the SARS-CoV (2002) and MERS-CoV (2012) viruses were carried out but were never completed. This is partly due to the fact that the SARS-CoV epidemic lasted for a relatively short time, about 15 months, and the last case was recorded in June 2003. In total, more than 8,000 people were infected (Kim et al., 2019). Since 2004, no cases of SARS-CoV infection have been reported. The MERS-CoV virus has caused sporadic outbreaks in various countries, with the most recent case detected in February 2020 in Qatar (de Wit et al., 2020). However, this existing research on the development of a vaccine against these viruses has provided an important understanding of the mechanisms that mediate the induction of a protective immune responses against SARS-CoV and MERS-CoV, and these findings are being taken into account by the designers of SARS-CoV-2 vaccines.

A number of studies have demonstrated that antibodies generated against the SARS-CoV S protein can protect laboratory animals from virus infection (Yang et al., 2004). However, the humoral response was short-lived in people who had SARS-CoV infection (Tang et al., 2011). At the same time, a virus-specific T cell response was recorded up to 11 years after infection (Ng et al., 2016). These data highlight the importance of the T cell response, which should be taken into account when developing effective immunogens that can stimulate cytotoxic and helper responses against SARS-CoV-2.

Different approaches were used to develop vaccines against SARS-CoV and MERS-CoV, including vaccines based on inactivated virus, viral vectors, recombinant proteins, peptides, and DNA and RNA vaccines. The same approaches are being used to create a vaccine against SARS-CoV-2 now. According to the website of the World Health Organization (WHO), on August 13, 2020, more than 100 SARS-CoV-2 vaccine prototypes were being developed (Draft landscape of COVID-19 candidate vaccines, 2020). Such a variety of prototypes in the first stages is understandable as there is no universal solution to the problem at the moment.

It should be noted that more than 10 vaccines from this list have been developed on the basis of mRNA, a rapidly developing technology in recent years (Table).

Among the developers of mRNA vaccines are such research centres and companies as Moderna Inc. (USA), CureVac and BioNTech (Germany), Oxford University (UK), CanSino Biologics Inc. (China), VIDO-InterVac (Canada), and BIOCAD (Russia).

Until recently, the development of preventive and therapeutic RNA-based vaccines has been fraught with problems due to mRNA instability and inefficient delivery. Progress in this area can be attributed to advances in mRNA synthesis technology, optimisation of the secondary structure of mRNA and the cap structure, increasing resistance to RNA degradation by nucleases by the inclusion of modified nucleosides such as pseudouridine and 5-methylcytidine, and improvements in methods for RNA purification and delivery (Pardi et al., 2018). The necessary enzymes and ingredients are currently commercially available, which allows the production of mRNA in the necessary quantities for mass vaccination of the population. In recent years, a number of mRNA vaccines have been developed and tested against a variety of infectious diseases (influenza, rabies virus, Zika virus, HCV, HMV, etc.) and several types of cancer. These vaccines have shown promising results in both animal and human models (Pardi et al., 2018). It is important to note that mRNA vaccines can

[1] WHO, https://www.who.int

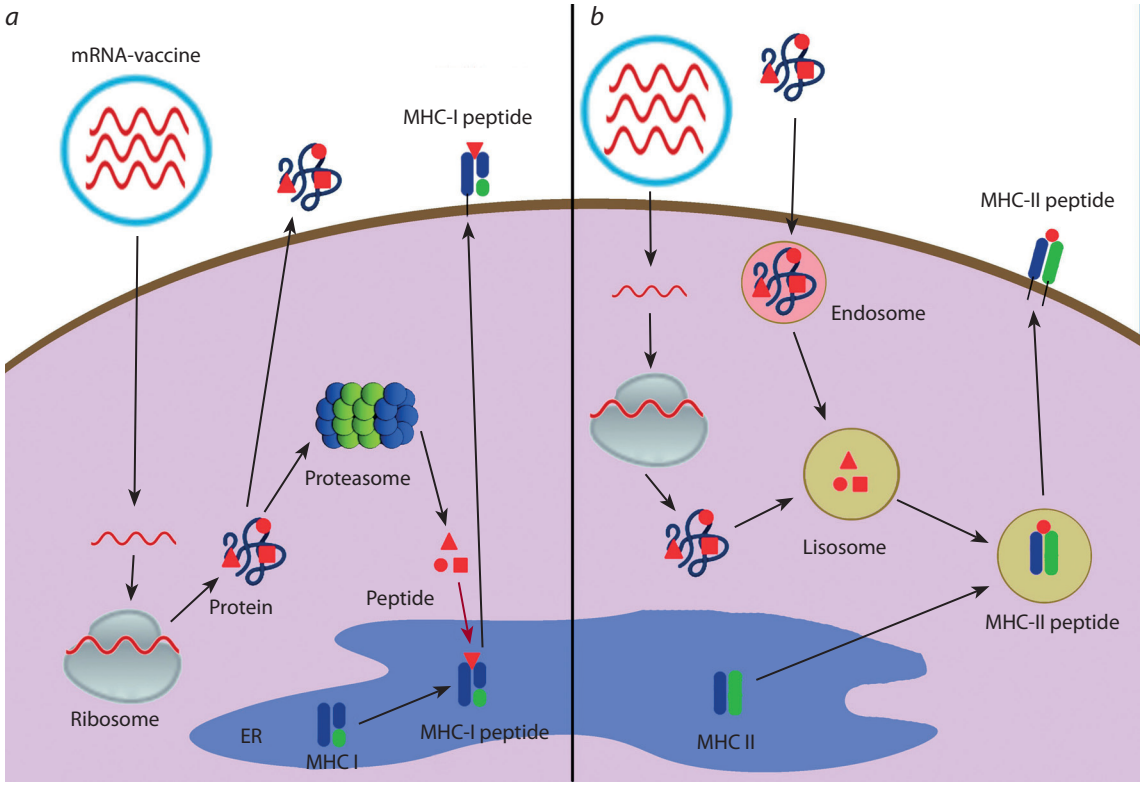
List of mRNA-based vaccines under development against COVID-19 registered by WHO as of October 2, 2020

A candidate vaccines in clinical evaluation	
Type of candidate vaccine	Developer, clinical stage, clinical trial registration number
LNP-encapsulated mRNA	Moderna / National Institute of Allergy and Infectious Diseases (NIAID): phase 1 – NCT04283461, phase 2 – NCT04405076, phase 3 – NCT04470427
3 LNP-mRNAs	BioNTech / Fosun Pharma / Pfizer: phase ½ – 2020-001038-36 ChiCTR2000034825, phase 3 – NCT04368728
mRNA	Arcturus / Duke-NUS: phase ½ – NCT04480957
	Curevac: phase 1 – NCT04449276, phase 2 – NCT04515147
	People's Liberation Army Academy of Military Sciences / Walvax Biotech: phase 1 – ChiCTR2000034112
LNP-nCoVsaRNA	Imperial College London: phase 1 – ISRCTN17072692
A candidate vaccines in preclinical evaluation	
Type of candidate vaccine	Developer
mRNA	Selcuk University
LNP-mRNA	Translate Bio / Sanofi Pasteur
	CanSino Biologics / Precision NanoSystems
LNP-encapsulated mRNA cocktail encoding VLP	Fudan University / Shanghai Jiao Tong University / RNACure BioPharma
LNP-encapsulated mRNA encoding RBD	
Replicating defective SARS-CoV-2 derived RNAs	The National Centre for Biotechnology, Spain
LNP-encapsulated mRNA	University of Tokyo / Daiichi-Sankyo
Liposome-encapsulated mRNA	BIOCAD, Russia
Several mRNA candidates	RNA immune Inc.
mRNA	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo, Russia
	China CDC / Tongji University / Stermina
LNP-mRNA	Chula Vaccine Research Center / University of Pennsylvania
mRNA in an intranasal delivery system	eTheRNA
mRNA	Greenlight Biosciences
	IDIBAPS-Hospital Clinic, Spain
Self-amplifying RNA	Gennova

effectively activate both parts of the immune response – both T cells and humoral responses (Zhang et al., 2019).

RNA-based vaccines can be divided into two types: non-replicating mRNAs and self-amplifying RNAs (Iavarone et al., 2017). Non-replicating RNA vaccines are composed of the mRNA that encodes the amino acid sequence of the target protein (the immunogen) together with all necessary elements for the translation process. Self-amplifying RNA vaccines are replicons constructed from positive single-stranded RNA viruses, such as alpha viruses and flaviviruses. Such replicons usually consist of two parts: one of them encodes non-structural proteins that carry out viral RNA replication, while the other encodes the target protein (immunogen) (Iavarone et al., 2017). Self-amplifying RNA vaccines are characterised by higher and longer expression of the target gene compared to non-replicating analogues. However, these RNA replicons are very sensitive to the size of the embedded target. In addition, a large vector size (about 10 kb) may limit the efficiency of cell internalisation (Schwendener, 2014).

A schematic diagram of the mRNA-based vaccine and the mechanism of antigen presentation are presented in Figure. After the mRNA enters the cell, it is translated through the



Messenger RNA vaccine and antigen presentation.
a – muscle cells; *b* – specialised antigen-presenting cells.

cellular mechanism of protein synthesis. Translation can occur both on ribosomes located in the cytoplasm in free form and on ribosomes associated with the membranes of the endoplasmic reticulum. There are some variants of antigen presentation pathway (see Figure).

1. The protein enters the proteasome where it undergoes processing and is cleaved into small peptides (epitopes). These peptides are then transferred to the lumen of the endoplasmic reticulum by transporter associated with antigen processing proteins, where they bind to MHC class I molecules. The resulting complex in the vesicle is directed to the plasma membrane of the cell and exposed on the cell surface, where it is recognised by the $CD8^+$ receptors of cytotoxic lymphocytes, stimulating a specific cytotoxic cellular response.
2. The protein is enclosed in vesicles called lysosomes, where the antigen is cleaved into peptide fragments by lysosome-associated enzymes (acid proteases). The lysosome merges with the vesicle that carries the MHC class II molecule. Within this structure, an epitope complex with MHC II is formed. The complex is transported to the cell membrane and brought to the surface, where it is recognised by $CD4^+$ lymphocyte receptors. As a result, both the T-helper response and humoral immunity (activation of B lymphocytes) are activated.
3. The protein can be secreted by the cell, activate B lymphocytes, and induce the humoral immune response.

mRNA-based vaccines have a range of useful features in comparison to other types of vaccines, such as classic vaccines (based on a live attenuated or inactivated virus), and protein and DNA vaccines. Firstly, mRNA vaccines are known for their safety. mRNAs are non-infectious, unlike classical viral vaccines, and they have low reactogenicity. An important point of difference from classical vaccines is the lack of strict temperature control required for storage of drugs based on mRNA. Currently, most vaccines need to be transported and stored under cold chain conditions, which causes serious challenges for their delivery to remote regions. The lyophilised mRNA vaccine can be stable at 5–25 °C for 36 months, which makes it possible to eliminate this disadvantage. Unlike a DNA vaccine, mRNA cannot integrate into the cell genome and cause mutations. Thus, there is no risk of insertion of foreign genetic information into the patient's genome. mRNA, being a minimal genetic vector, does not lead to the antivector immune response observed when using viral carriers. Thus, it can be used for immunisation multiple times.

Messenger RNA is subject to physiological destruction as a result of processes occurring in the cell. Its half-life can be regulated by modifications of the RNA sequence and its delivery method (Kauffman et al., 2016; Guan, Rosenecker, 2017).

A significant advantage of mRNA vaccines is its fast, inexpensive, scalable, and uniform production, which provides high yields of the desired product *in vitro.* It does not require the cultivation of bacteria or the use of cell cultures or chicken embryos, which are necessary for most types of antiviral vaccines. All that is required for the production of an mRNA vaccine is a DNA matrix that carries the target gene under the control of the T7 phage promoter, in addition to a set of enzymes for matrix synthesis. After the synthesis and purification procedure, it is technologically much easier to

obtain an injectable mRNA preparation than a DNA vaccine. Thus, the production of mRNA by *in vitro* transcription is more attractive than the production of DNA vaccines because it is essentially a chemical process that does not require the use of cells (Liu et al., 2019).

An important but non-optimised part of mRNA vaccine technology is its delivery. To perform its task, the mRNA must enter the cell's cytoplasm where the protein encoded by it can be translated. A range of mRNA delivery methods have been described, including administration of the vaccine by electroporation, injection into muscles, lymph nodes, or directly into organs, or administration intranasally, rectally, or orally (Gómez-Aguado et al., 2020; Wadhwa et al., 2020). Messenger RNA vaccination is also hindered by its degradation by various extracellular ribonucleases, which are abundant in tissues and in the intercellular space (Houseley, Tollervey, 2009).

A variety of approaches have been used to deliver and protect mRNA from degradation by nucleases. Lipid nanoparticles are currently one of the most commonly used means of delivering mRNA. Standard lipid nanoparticles consist of four components: cationic lipid, cholesterol, auxiliary phospholipids, and polyethylene glycol. Cationic polymer materials such as dendrimers and polyethylenimine, among others, are promising materials for facilitating the delivery of nucleic acids. Gene gun and electroporation techniques can also be used (Capasso et al., 2018; Kowalski et al., 2019). It is possible to increase the stability of the mRNA molecule by including nucleotide analogues such as pseudouridine, methylpseudouridine, and methylcytodine. However, sometimes the use of such modifications leads to a decrease in the efficiency of translation.

An important advantage of mRNA vaccine technology compared to the production of vaccines based on inactivated virus or recombinant protein is the ability to quickly pass all stages of its development. This quality is very important for the development of vaccines against viral pathogens, the main problem of which is the time gap between the start of the epidemic and the development of the vaccine. To prevent outbreaks of newly emerging and rapidly evolving pathogens, the speed of response to the pandemic with the creation of a preventive vaccine is of paramount importance. It has recently been shown that by using a synthetic biology approach including bioinformatics, a prototype vaccine against a target viral pathogen in mRNA format can be developed in a week (Rauch et al., 2018).

The developers of the mRNA vaccine against SARS-CoV-2 in the United States (Moderna Inc. together with the National Institute of Allergy and Infectious Diseases, NIAID) created a prototype of the mRNA-1273 vaccine in an unprecedentedly short time[2]. It took just 63 days from the selection of the viral sequence to the development of the vaccine for the first phase of clinical testing, in which 45 volunteers were given three different doses over 6 weeks to obtain initial safety data and demonstrate the desired immune response.

The most interesting thing is that the mRNA-1273 vaccine did not pass all of the preclinical tests before it was used in the first phase of clinical trials after proving its specific activity. What prompted the researchers from the United States to choose this approach? Firstly, the development of this vaccine was based on previous projects by the developers to create vaccines against other types of coronavirus, such as SARS and MERS, which were unfortunately never completed. There are also dozens of studies on the use of mRNAs as therapeutic vaccines for the treatment of cancer, with no significant adverse reactions to the vaccine observed (Sebastian et al., 2014; Pardi et al., 2020).

In conclusion, we can say with some confidence that RNA-based vaccines can be effective against pandemics caused by viruses, including SARS-CoV-2, as this approach offers a relatively simple and fast solution for newly emerging and returning viral pathogens.

[2] Moderna press-release: https://investors.modernatx.com/news-releases/news-release-details/moderna-ships-mrna-vaccine-against-novel-coronavirus-mrna-1273

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Acknowledgements. The study was conducted under the state assignment of State Research Center of Virology and Biotechnology "Vector."

Conflicts of interest. The authors declare no conflict of interest.

Received May 25, 2020. Revised October 12, 2020. Accepted October 13, 2020.