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Molecular genetic bases of seed resistance to oxidative stress during storage

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Abstract. Conservation of plant genetic diversity, including economically important crops, is the foundation for food safety. About 90 % of the world's crop genetic diversity is stored as seeds in genebanks. During storage seeds suffer physiological stress consequences, one of which is the accumulation of free radicals, primarily reactive oxygen species (ROS). An increase in ROS leads to oxidative stress, which negatively affects the quality of seeds and can lead to a complete loss of their viability. The review summarizes data on biochemical processes that affect seed longevity. The data on the destructive effect of free radicals towards plant cell macromolecules are analyzed, and the ways to eliminate excessive ROS in plants, the most important of which is the glutathione-ascorbate pathway, are discussed. The relationship between seed dormancy and seed longevity is examined. Studying seeds of different plant species revealed a negative correlation between seed dormancy and longevity, while various authors who researched *Arabidopsis* seeds reported both positive and negative correlations between dormancy and seed longevity. A negative correlation between seed dormancy and viability probably means that seeds are able to adapt to changing environmental conditions. This review provides a summary of *Arabidopsis* genes associated with seed viability. By now, a significant number of loci and genes affecting seed longevity have been identified. This review contains a synopsis of modern studies on the viability of barley seeds. QTLs associated with barley seed longevity were identified on chromosomes 2H, 5H and 7H. In the QTL regions studied, the *Zeo1*, *Ale*, *nud*, *nadp-me*, and *HvGR* genes were identified. However, there is still no definite answer as to which genes would serve as markers of seed viability in a certain plant species.
Key words: seeds; barley; QTL; seed longevity genes; genetic markers; biochemical markers.

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Молекулярно-генетические основы устойчивости семян к окислительному стрессу при хранении

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Аннотация. Сохранение генетического разнообразия растений, в том числе хозяйственно значимых культур, является основой продовольственной безопасности. В мире около 90 % генетического разнообразия культурных растений сохраняется в виде семян в генных банках. В процессе хранения в семенах накапливаются свободные радикалы, в первую очередь активные формы кислорода (АФК). Повышение уровня АФК вызывает окислительный стресс, который негативно влияет на качество семян и может привести к полной потере их жизнеспособности. В обзоре обобщены сведения о биохимических процессах, влияющих на продолжительность жизни семян. Проанализированы данные о деструктивном действии свободных радикалов по отношению к макромолекулам клетки растения и пути устранения избыточного количества АФК в растениях, наиболее важным из которых является аскорбат-глутатионовый путь. Рассматривается вопрос взаимосвязи периода покоя и длительности сохранения семян. В исследованиях на семенах разных видов растений была выявлена отрицательная корреляция между периодом покоя и долголетием семян, тогда как в работах с семенами *Arabidopsis* различные авторы выявили как положительную корреляцию между периодом покоя и длительностью сохранения семян, так и отрицательную. Отрицательная корреляция между периодом покоя и жизнеспособностью, вероятно, свидетельствует о способности семян адаптироваться к изменяющимся условиям окружающей среды. Нами проанализирована информация по генам *Arabidopsis*, связанным с жизнеспособностью семян. В настоящее время выделено значительное количество локусов и генов, влияющих на долголетие семян. Статья знакомит

с современными исследованиями жизнеспособности семян ячменя. Локусы количественных признаков (QTL), связанные с долголетием семян ячменя, были определены на хромосомах 2H, 5H и 7H. В изученных областях QTL выявлены гены *Zeo1*, *Ale*, *nud*, *nadp-me* и *HvGR*. Однако вопрос о том, какие гены являются маркерами жизнеспособности семян растений определенного вида, остается открытым.
Ключевые слова: семена; ячмень; QTL; гены долголетия семян; генетические маркеры; биохимические маркеры.

Introduction

Conservation of plant genetic diversity, including economically important crops, is a task of topmost priority. Since the end of the previous century plant genebanks have been sprouting up all over the world. Currently, about 90 % of crop accessions are stored worldwide in genebanks as seeds (Li, Pritchard, 2009; http://www.fao.org). The N.I. Vavilov Institute of Plant Genetic Resources (VIR) holds over 320,000 viable accessions of cultivated plants and their wild relatives, including more than 250,000 seed accessions in its Kuban Seed Genebank, founded in 1976 (Loskutov, 2009; Silaeva, 2012).

Seeds represent a stage in the life cycle when plants suffer particularly high levels of genotoxic stress, which can lead to the genome's instability (Waterworth et al., 2011). Seed ageing is regarded as the accumulation of structural and metabolic injuries leading to functional failures and reduced resistance to unfavorable environments, which may result in loss of viability (McDonald, 1999; Smolikova, 2014). According to their behavior under storage, seeds are classified into orthodox and recalcitrant (Walters, 2015). In the end of their ripening period, orthodox seeds, as a rule, lose water and dry out down to a moisture content of 10 %; they can be preserved in such state for many years without any loss of germination rate. This property of orthodox seeds was called 'desiccation tolerance' (Dekkers et al., 2015). Contrariwise, dehydration in recalcitrant seeds entails loss of their germination ability and death. Therefore, they are considered desiccation-sensitive. Usually recalcitrant seeds are not dried prior to their placement in a genebank for conservation. Orthodox seeds are dried down to a moisture content of 5 % or less, or frozen before storage. As a rule, the duration of storage periods for orthodox seeds in genebanks may be extended if they are kept under lower moisture and temperature conditions (Bewley et al., 2013). For example, barley seeds retain their viability from six months to 7–9 years at the air temperature of +20 °C and relative humidity (RH) of 50 % (Priestley et al., 1985; Nagel, Börner, 2010), whereas under negative temperatures (–18 °C) and 4–8 % RH, according to calculated estimates, seed viability may remain intact up to more than 80 years (Walters et al., 2005).

In addition to environmental factors, such as humidity, temperature, light, and absence of pathogens (Schmidt, 2000), seed ageing rates are affected by genetic factors determining seed color and weight and influencing the activity of protein non-enzymatic glycosylation processes, lipid peroxidation, cell membrane structure, generation and neutralization of reactive oxygen species (ROS) and free radicals, and other processes (Wettlaufer, Leopold, 1991; Ponquett et al., 1992; Khan et al., 1996; Wojtyla et al., 2016; Frolov et al., 2018; Antonova et al., 2019).

The causes of seed viability losses and deaths are not quite clear, because a lot of processes are involved, including damage to macromolecules (such as DNA, lipids and proteins) resulted from ROS-induced responses. This issue was studied by numerous researchers. Using various plant species as examples, they showed that seed ageing rates depended on protection mechanisms against stress and the ability of seeds to withstand ROS-induced changes. In different plant species antioxidants are differently involved in removing excessive ROS. In oil crops, for example, a more active role is played by lipophilic antioxidants (Bailly, 2004; Bahin et al., 2011; Waterworth et al., 2011; Jeevan Kumar et al., 2015; Kong et al., 2015). The research efforts employing germplasm materials preserved in the IPK (Germany) and the USDA (United States) genebanks showed that seed longevity varied not only across different plant species, but also among different genotypes within a single species, although interspecific differences in seed shelf-life exceeded intraspecific ones. Similar research was performed on different plant species: barley, wheat, rapeseed, etc. (Walters et al., 2005; Nagel et al., 2009; Nagel, Börner, 2010; Rehman Arif et al., 2017; Rehman Arif, Börner, 2019). The fact that genotypes within the same species demonstrated different seed life spans may serve as a basis for genetic analysis of seed longevity.

Seed dormancy and longevity

Physiological dormancy may be described as a preprogrammed state limiting the set of environmental conditions under which the seed germinates (Nikolaeva, 1982, 1999; Baskin J., Baskin C., 2007). Dormancy is an adaptive trait that enables seeds to survive lengthy periods of unfavorable conditions (Bewley et al., 2013; Sliwinska, Bewley, 2014). To optimize germination and maintain viability during long unfavorable periods, seeds enter into the state of dormancy (Rajjou, Debeaujon, 2008). Seed dormancy is regulated through abscisic acid (ABA) and other bioactive compounds, such as plant hormones: gibberellins, cytokinins, and ethylene (Nonogaki, 2017). Entering the state of seed dormancy is accompanied by a considerable increase in ABA content, whereas releasing from dormancy, on the contrary, is associated with a decrease in ABA.

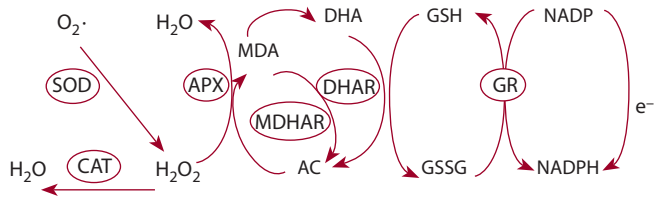
The issue of the interplay between seed dormancy and seed longevity has been analyzed by numerous researchers

for decades, although the conclusions made on the results of those studies are contradictory. Clerkx et al. (2004) studied this problem on *Arabidopsis* plants with mutations in certain developmental and biochemical pathways. They reported reduced seed longevity for such mutations as *abscisic acid insensitive3* (*abi3*) and *abscisic acid deficient1* (*aba1*). The same mutations also provoked reduction of seed dormancy duration for *abi3* and *aba1*. Thus, the authors supposed that a positive correlation existed between seed longevity and dormancy. Miura et al. (2002), while studying interrelations between dormancy and seed viability in rice, came to an opposite conclusion – that these two physiological states are controlled by different genetic factors. The QTL analysis showed that the seed longevity loci were on chromosomes 2, 4 and 9, while the QTLs associated with seed dormancy were identified on chromosomes 1, 3, 5, 7 and 11. Nguyen et al. (2012) also observed a negative correlation between seed longevity and dormancy in *Arabidopsis* plants. Having analyzed six populations of recombinant inbred lines (RILs), the authors identified 5 loci linked with seed germination ability after storage (*GAAS1–GAAS5*). They reported that the *GAAS* (*germination ability after storage*) loci correlated with those responsible for seed dormancy, i. e. *DOG* (*delay of germination*) loci. Their correlation was negative. A detailed analysis of *GAAS5* and *DOG1* QTLs revealed that the *DOG1* locus reduces seed longevity and at the same time increases seed dormancy period (Nguyen et al., 2012). A homolog of the same gene was annotated by Nagel et al. (2019) on the barley chromosome 3H. The authors reported that the *DOG1* locus plays a role in extending the period of barley seed dormancy, although a less significant role than in the case of *Arabidopsis*.

Thus, the interaction between seed dormancy and seed longevity is interpreted ambiguously, so further research would be required to clarify this issue. The presence of loci that either extend the life span of seeds or prolong their dormancy period within an individual plant accession may provide it with adaptive plasticity, which will lead to the manifestation of an optimal phenotype under various environmental conditions.

Biochemical processes affecting seed longevity

In the process of storage, seeds suffer physiological stress consequences, one of which is the accumulation of free radicals, primarily reactive oxygen species (ROS). An increase in the ROS level results in oxidative stress. In most cases under oxidative stress a superoxide anion radical (superoxide radical, $O_2\cdot$) is formed; it quickly converts into other ROS forms: hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$). Damage caused by free radicals leads to a rupture in the genome integrity within the nucleus, which in its turn may entail a complete loss of seed viability (Bailly, 2004; Kranner et al., 2006; Bailly, Kranner, 2011). The effect of free radicals and lipid peroxidation induces



Ascorbate-glutathione pathway: CAT – catalase; SOD – superoxide dismutase; APX – ascorbate peroxidase; MDHAR – monodehydroascorbate reductase; DHAR – dehydroascorbate reductase; GR – glutathione reductase; AC – ascorbate; MDA – monodehydroascorbate; DHA – dehydroascorbate; GSSG – oxidized glutathione; GSH – reduced glutathione.

membrane structure degradation and DNA disintegration, accompanied by a decrease in the activity of a majority of enzymes in the cell. One of the causes of such decline in activity may be the deterioration of enzymes or malfunctions in the protein-synthesizing complex under the aggregate impact of free radicals. There is a universal mechanism of oxidative stress prevention, usable against excessive ROS in plants. An important role in the antioxidant protection system of the cell is played by the enzymes of superoxide dismutase (SOD). The main function of these enzymes is converting the superoxide radical into H_2O_2 and oxygen (Jajic et al., 2015). Three SOD isoforms differing in metals within the active center have been identified in plant cells: Fe-SOD (chloroplast), Mn-SOD (mitochondria and peroxisomes) and Cu-Zn-SOD (cytoplasm, chloroplast and peroxisomes). Hydrogen peroxide is in its turn decomposed by catalase (CAT), which is found in glyoxysomes and peroxisomes (Willekens et al., 1995), except the isoform which is mitochondrial (Scandalios et al., 1997). Glutathione peroxidase (GPX) also removes excessive H_2O_2 and organic peroxides (Eshdat et al., 1997).

The ascorbate-glutathione or the Foyer–Halliwell–Asada pathway also plays a significant role in the cell's antioxidant protection (Bailly, 2004; Foyer, Noctor, 2005) (see the Figure). In this pathway, H_2O_2 reduction to water is catalyzed by ascorbate peroxidase. It is accompanied by ascorbate oxidation to monodehydroascorbate (MDA), which can be reduced back to ascorbate by MDA reductase, employing NADPH. As a result of a spontaneous redox reaction, MDA can produce dehydroascorbate (DHA), which is converted back to ascorbate via glutathione oxidation. The cycle is completed with the NADPH-assisted reduction of the oxidized glutathione form (Medvedev, 2013).

Redox potential of the water-soluble antioxidant glutathione (GSH) is of particular importance as a regulator of the cell's redox potential in the case of orthodox seeds (Schafer, Buettner, 2001; Noctor et al., 2011). Since orthodox seeds contain insignificant amounts of ascorbate (Kranner et al., 2006), Kranner and her coauthors hypothesized that glutathione is the main and possibly the oldest redox buffer, and a change in the GSSG/GSH reduction potential can serve as a universal seed viability marker

(Mittler, 2002; Kranner et al., 2006). In addition to water-soluble antioxidants, plant seeds also contain fat-soluble (hydrophobic) antioxidants: α-, β-, γ-, σ-tocopherols and carotenoids (Sharova, 2016); their contribution to the cell's antioxidant protection depends on the plant species. For example, fat-soluble antioxidants are more significant for oil crops whose seeds are rich in fatty acids.

Seed longevity is also affected by some other compounds with an antioxidant function, such as polyphenols, flavonoids and peroxiredoxins (Landry et al., 1995; Sattler et al., 2004; Sharova, 2016). It should be mentioned that the cell's antioxidant mechanisms control the content of ROS, but do not eliminate them completely. It happens because small amounts of ROS are important signaling molecules, participating in plant growth, development, and stress response. In seeds, ROS play an important role, associated with viability and release from dormancy. An excess in ROS leads to a loss of seed viability, but ROS production is required for seed dormancy breakage and facilitates seed germination (Bailly, 2004; Oracz et al., 2009).

Thus, ROS play a dual role in seed physiology. On the one hand, ROS possess an exceptionally high reactivity. They are capable to induce chain reactions and oxidize practically all organic compounds, causing irreversible oxidative damage to most important biomolecules, such as proteins, lipids or DNA. On the other hand, ROS participate in cell growth regulation, protection against pathogens, and redox status control in cells. Besides, ROS function as a positive signal in seed dormancy alleviation and germination (Bahin et al., 2011; Jeevan Kumar et al., 2015).

Candidate genes for seed longevity identified in *Arabidopsis thaliana* (L.)

The role of important factors affecting seed longevity was analyzed in mutants of *Arabidopsis thaliana* (L.) Heynh. and transgenic lines. Seed maturation is known to be genetically controlled by four main regulators: *ABI3* (*ABSCISIC ACID-INSENSITIVE3*), *LEC1*, *LEC2* (*LEAFY COTYLEDON1* and *LEAFY COTYLEDON2*) and *FUS3* (*FUSCA3*) (Raz et al., 2001). Mutations in these key regulators lead to a quick loss of seed viability under storage. For example, *abi3*, *lec1* and *fus3* mutants demonstrated reduced seed longevity (Ooms et al., 1993; Clerkx et al., 2004). Significant reduction of seed longevity is also caused by seed coat mutations. Seed coat acts as a structural barrier against biotic and abiotic stresses. For example, *tt* (*transparent testa*), *ttg* (*transparent testa glabra*) and *ats* (*aberrant testa shape*) mutants were reduced germination percentage than the control plants (Debeaujon et al., 2000; Clerkx et al., 2004). A decrease of seed longevity was observed in *vte1* and *vte2* mutants involved in vitamin E biosynthesis (lipophilic antioxidant) and in completely tocopherol-free ones (Sattler et al., 2004).

After UV exposure, *Arabidopsis* mutants deficient in flavonoid biosynthesis showed increased lipid peroxidation by 60 %, which correlated with a decrease in seed viability (Landry et al., 1995). The content of some oligosaccharides, such as galactinol, correlated with seed longevity (Obendorf, 1997). The research conducted on *Arabidopsis*, cabbage and tomato revealed a positive correlation between galactinol content and seed longevity in these crops (de Souza Vidigal et al., 2016). Reduced seed viability, compared with the wild-type plants, was observed in *Arabidopsis* mutants that lacked the functional malate hydrogenase enzyme (Yazdanpanah et al., 2019). The researchers who made such observation assume that the NADP-ME1 enzyme activity is required to protect seeds from oxidation during long-term seed storage (Yazdanpanah et al., 2019). Oxidative stress negatively affects seed quality; it is confirmed by a reduction in germination percentage of the *frostbite1* (*fro1*) mutant which constitutively accumulated ROS (Lee et al., 2002; Clerkx et al., 2004). At the same time, Clerkx et al. failed to find a reliable reduction of germination rate for two mutants with ROS-scavenging mechanisms: *vitamin C deficient1-1* (*vtc1-1*) and *cadmium sensitive2-1* (*cad2-1*), the latter being glutathione-deficient.

By now, a considerable number of loci and genes associated with seed longevity have been identified in *Arabidopsis* plants. Such genes may be classified into several groups according to the mechanisms of their effect on seed viability; the most significant among them are the groups of genes whose effect is connected with plant hormones, such as abscisic and gibberellic acids, and redox processes.

Candidate genes for seed longevity identified in barley

Studying seed longevity of various plant species, such as *Arabidopsis* (Clerkx et al., 2004; Bentsink et al., 2006), barley (Nagel et al., 2009), wheat (Landjeva et al., 2010; Rehman Arif, Börner, 2019), rice (Miura et al., 2002), *Aegilops* (Landjeva et al., 2010), maize (Revilla et al., 2009), lettuce (Schwember, Bradford, 2010), etc., has shown that seed longevity is controlled by several genetic factors, which facilitates the identification of quantitative trait loci (QTLs). In a number of published works QTLs associated with seed longevity were identified for different plant species using natural and artificial ageing techniques. Artificial ageing methods are employed to predict eligibility of seed samples for long-term storage (Safina, Filipenko, 2013; Smolikova, 2014). They are essentially based on artificial acceleration of the senescence process by briefly exposing seeds to higher temperatures and humidity close to critical levels for a given crop. There are two generally accepted seed ageing methods: AA (accelerated ageing) and CD (controlled deterioration). In the AA test, seeds are exposed for short period of time to high temperature and high relative humidity (100 %). CD treatment differs from AA in that seeds are shortly stored under higher moisture content (MC) (18–20 %) and temperatures in aluminum foil bags (Nagel et al., 2009, 2015). The ISTA (International

Seed Testing Association) standards prescribe a definite MC value to the CD test according to the following formula:

$$m_{H_2O} = \left(\frac{100 - smc_1\,(\%)}{100 - smc_t\,(\%)}\right) \times m_1,$$

where m_{H_2O} is the amount of added water (g); smc_1 is the initial seed moisture content (%); smc_t is the taget seed moisture content (%); and m_1 is the initial seed weight (g).

It should be mentioned that under accelerated ageing seeds are experiencing high water contents and high temperatures. In such a situation, the main negative processes within the seeds are lipid peroxidation and loss of membrane phospholipids. Meanwhile, during long-term storage of dry seeds, mostly non-enzymatic reactions would take place, and they do not require much water (Walters, 1998; Murthy et al., 2003; Veselovsky, Veselova, 2012; Frolov et al., 2018; Antonova et al., 2019). Therefore, the question on the extent to which the processes within seeds under artificial ageing would mimic those undergoing naturally during long-term storage remains unanswered (Agacka-Mołdoch et al., 2016; Bankin et al., 2018). Nonetheless, artificial ageing techniques are widely used by various researchers in their efforts to study seed viability.

The development of new sequencing methods, achievements in a wide range of analytical technologies, and emergence of bioinformatics procedures contributed to higher quality of research not only on model species but also on agricultural crops. Several significant specific features of *Hordeum vulgare* (L.), such as the diploid nature of the cultivated barley genome with a high degree of self-pollination, small number of sufficiently large chromosomes ($2n$ = 14), easy crossability, and simplicity of cultivation in very diverse climates, promote the wide use of this crop in genetic studies. Modern DNA technologies applied to various barley accessions helped to identify candidate genes potentially responsible for extending the life span of seeds. Nagel et al. in 2009 used two induced ageing methods (AA and CD) to detect QTLs correlating with seed longevity in the following barley plant populations: OWB, S × M ('Steptoe' × 'Morex') and W766 (Nagel et al., 2009). The authors identified the greatest number of QTLs associated with seed longevity on chromosomes 2H, 5H and 7H. One significant QTL was detected within a distal region of chromosome 2H associated with the gene *Zeo1* (*Zeocriton 1*). The *Zeo1* gene determines plant height and spike compactness. Such plants are known to have low fertility. The *Ale* (*Aleurain*) gene encoding thiol protease was identified in the QTL on the long arm of chromosome 5H. The expression of *Ale* is regulated by gibberellic and abscisic acids which play important roles in seed germination (Nagel et al., 2009). The *nud* gene determining hulled/naked caryopsis was identified as a candidate for QTL on chromosome 7H. In 2016, Nagel et al. conducted additional research on seed longevity. Most of the QTLs associated with seed longevity, like in the 2009 tests, were detected in two areas: on chromosome 2H between 110 and 172 cM, the locality of the *Zeo1* gene, and in the centromeric region of 7H from 73 to 95 cM, also incorporating the *nud* gene.

Further annotation of gene functions in the QTL areas by the authors revealed the presence of the glutathione reductase enzyme in the same area, which suggests a relationship with oxidative stress (Meyer, Hell, 2005; Rouhier et al., 2008; Nagel et al., 2016). Bahin et al. studied sunflower and barley seed longevity in 2011 and found out that in the process of storage barley seeds, unlike those of sunflower, did not accumulate ROS. The authors supposed that an excess in ROS neutralized the antioxidant glutathione (GSH) (Bahin et al., 2011), which could serve as a marker in barley seed longevity studies. Wozny et al. in 2018 proposed the NADP-dependent malic enzyme (NADP-ME) as a candidate gene to be used in future seed longevity studies on barley (Wozny et al., 2018). The enzyme was localized on chromosome 2H within the area of the QTLs correlating with seed longevity in the studied barley accessions (Wozny et al., 2018).

Thus, most of the QTLs associated with barley seed longevity have been identified on chromosomes 2H, 5H and 7H. The following genes have been detected in the studied QTL areas: *Zeo1*, *Ale*, *nud*, *nadp-me*, and *HvGR*. Different researchers offer different candidate genes for further research on barley seed longevity. By now, however, no one has found a reliable universal marker to be associated with loss of viability in barley seeds. Presently, there are no readily available genetic markers for seed longevity that could prove handy to search for an optimum frequency of seed reproduction in the process of barley germplasm conservation.

Conclusion

Mechanisms of seed viability reduction and death during long-term storage may differ depending on plant species. For barley, they have been identified only partially. The data presently available on the identification of genes associated with barley seed longevity have been obtained using artificial seed ageing methods, but they cannot simulate natural ageing processes accurately enough. Genetic markers associated with seed longevity that could appear suitable to optimize reproduction of seed accessions have not yet been conclusively identified. Eventually, glutathione reductase may be suggested as a candidate gene for further studies of barley seed longevity. This enzyme is directly involved in neutralization of excessive ROS, antioxidant defense, and cell signaling in plants.

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The consensus rye microsatellite map with EST-SSRs transferred from wheat

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Abstract. Microsatellite (SSR) markers with known precise intrachromosomal locations are widely used for mapping genes in rye and for the investigation of wheat-rye translocation lines and triticale highly demanded for mapping economically important genes and QTL-analysis. One of the sources of novel SSR markers in rye are microsatellites transferable from the wheat genome. Broadening the list of available SSRs in rye mapped to chromosomes is still needed, since some rye chromosome maps still have just a few microsatellite loci mapped. The goal of the current study was to integrate wheat EST-SSRs into the existing rye genetic maps and to construct a consensus rye microsatellite map. Four rye mapping populations (P87/P105, N6/N2, N7/N2 and N7/N6) were tested with CFE (EST-SSRs) primers. A total of 23 *Xcfe* loci were mapped on rye chromosomes: *Xcfe023*, *-136* and *-266* on chromosome 1R, *Xcfe006*, *-067*, *-175* and *-187* on 2R, *Xcfe029* and *-282* on 3R, *Xcfe004*, *-100*, *-152*, *-224* and *-260* on 4R, *Xcfe037*, *-208* and *-270* on 5R, *Xcfe124*, *-159* and *-277* on 6R, *Xcfe010*, *-143* and *-228* on 7R. With the exception of *Xcfe159* and *Xcfe224*, all the *Xcfe* loci mapped were found in orthologous positions considering multiple evolutionary translocations in the rye genome relative to those of common wheat. The consensus map was constructed using mapping data from the four bi-parental populations. It contains a total of 123 microsatellites, 12 SNPs, 118 RFLPs and 2 isozyme loci.
Key words: *Secale cereale*; SSR; *Triticum aestivum*; microsatellite markers; genetic mapping.

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Консенсусная микросателлитная карта ржи с интегрированными EST-SSR маркерами пшеницы

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Аннотация. Микросателлитные (SSR) маркеры широко используют для картирования генов ржи и анализа транслокационных линий пшеницы и тритикале. SSR-маркеры с известной внутрихромосомной локализацией очень востребованы для картирования экономически значимых генов и QTL-анализа. Одним из источников новых SSR-маркеров у ржи являются микросателлитные маркеры пшеницы. Несмотря на несколько наборов микросателлитных маркеров, доступных у ржи, по-прежнему необходимо расширение списка SSR, сопоставленных с хромосомами ржи, поскольку на некоторых генетических картах количество SSR-маркеров невелико. Цель настоящего исследования состояла в том, чтобы интегрировать EST-SSR пшеницы в существующие генетические карты ржи и построить консенсусную микросателлитную карту ржи. Четыре картирующих популяции ржи (P87/P105, N6/N2, N7/N2 и N7/N6) тестировали с использованием праймеров CFE (EST-SSR). В результате в молекулярно-генетические карты ржи было интегрировано 23 микросателлитных локуса *Xcfe*: *Xcfe023*, *-136* и *-266* на хромосоме 1R, *Xcfe006*, *-067*, *-175* и *-187* на 2R, *Xcfe029* и *-282* на 3R, *Xcfe004*, *-100*, *-152*, *-224* и *-260* на 4R, *Xcfe037*, *-208* и *-270* на 5R, *Xcfe124*, *-159* и *-277* на 6R, *Xcfe010*, *-143* и *-228* на 7R. За исключением *Xcfe159* и *Xcfe224*, все картированные локусы *Xcfe* были обнару-

жены в ортологичных позициях с учетом множественных транслокаций в ходе эволюции генома ржи по сравнению с пшеницей. Консенсусная карта построена с использованием данных по четырем картирующим популяциям ржи. Она содержит в общей сложности 123 микросателлитных маркера, 12 SNP, 118 RFLP и 2 изоферментных локуса.
Ключевые слова: *Secale cereale*; SSR; *Triticum aestivum*; микросателлитные маркеры; генетические карты.

Introduction

Several linkage maps of rye carrying RFLP, AFLP, SSR, DArT and SNP markers are available to date (Devos et al., 1993; Philipp et al., 1994; Senft, Wricke, 1996; Korzun et al., 2001; Bednarek et al., 2003; Hackauf, Wehling, 2003; Khlestkina et al., 2004; Varshney et al., 2007; Bolibok-Brągoszewska et al., 2009; Gustafson et al., 2009; Milczarski et al., 2011, 2016; Xu et al., 2012; Bauer et al., 2017).

SSRs (microsatellites) are among the most widely used DNA-markers in rye genetics. For example, SSR markers were used for mapping the *sy1*, *sy9*, *sy18* and *sy19* asynaptic genes (Malyshev et al., 2009; Dolmatovich et al., 2013a, b), the gene *mo1* for supernumerary spikelets (Dobrovolskaya et al., 2009), several anthocyanin biosynthesis genes (Khlestkina et al., 2009, 2011, 2013), *Ddw1* (Tenhola-Roininen, Tanhuanpää, 2010) and *Ddw3* (Yang et al., 2018) dwarfing genes, the powdery mildew resistance locus (Wang et al., 2010), the *Elm-R1* gene related with embryo lethality in wheat-rye hybrids as well as the hybrid dwarfness gene *Hdw-R1* (Tikhenko et al., 2011; Tsvetkova et al., 2018), aluminum tolerance loci in rye and triticale (Fontecha et al., 2007; Benito et al., 2010; Niedziela et al., 2014) and several QTL for agronomic traits including grain yield (Hackauf et al., 2017).

SSRs can be suitable for marker-assisted breeding (Lapitan et al., 2007), detection of the genetic variability in rye and triticale (Bolibok et al., 2005; Vyhnánek et al., 2009) as well as for marker-assisted identification of rye genetic material in wheat cultivars and lines (Silkova et al., 2006; Schlegel, Korzun, 2008; Schneider, Molnár-Láng, 2009; Adonina et al., 2011; Silkova et al., 2011; Schlegel, 2015).

In spite of several sets of microsatellite markers available in rye, broadening a list of SSRs mapped to rye chromosomes is still needed, since some rye chromosome maps still have just a few microsatellite loci mapped (Khlestkina et al., 2004). The goal of the current study was to integrate wheat EST-SSRs, expressed sequence tag SSR (from map of L.Y. Zhang et al. (2005)) into the existing rye microsatellite map and construct the consensus microsatellite map of rye genome.

Materials and methods

Four rye F_2 mapping populations (P87/P105; N6/N2, N7/N2 and N7/N6; see detailes in (Khlestkina et al., 2004)) were used in PCR assays with CFE primers available at GrainGenes database (http://wheat.pw.usda.gov). DNA was available from previous studies (Korzun et al., 2001; Khlestkina et al., 2004). PCR and analysis of the amplified fragments length was performed as described in L.Y. Zhang et al. (2005). Chromosome arm location of homologous sequences carrying the CFEs (http://wheat.pw.usda.gov) was performed using BLAST analysis (Altschul et al., 1990) of the corresponding wheat ESTs given at http://wheat.pw.usda.gov against wheat chromosome survey sequences available at https://urgi.versailles.inra.fr/blast/blast.php. Linkage maps were constructed with MAPMAKER 2.0 (Lander et al., 1987) using Kosambi function (Kosambi, 1944), based on genotyping data obtained in the current study and previously (Korzun et al., 2001; Khlestkina et al., 2004; Varshney et al., 2007). The consensus map was constructed using JoinMap 2.0 program (Stam, 1993).

Results and discussion

Despite the possibility of a high-throughput marker analysis using SNPs (Bauer et al., 2017), microsatellites remain convenient and low-cost markers for mapping genes and marker assisted selection in rye and triticale. For these purposes microsatellite markers with known precise intrachromosomal location are needed. The sources for mapping novel SSR loci in rye were rye EST-SSRs (Hackauf, Wehling, 2003; Khlestkina et al., 2004), or wheat genomic microsatellites (Khlestkina et al., 2004). In the current study, we used wheat EST-SSRs for genotyping rye mapping populations.

The parents of the four rye mapping populations (P87/P105, N6/N2, N7/N2 and N7/N6) were tested with 301 CFE primer pairs. Thirty-two pairs revealed polymorphism between the parents of one or more mapping populations: 10 between P87 and P105, 13 between N6 and N2, 11 between N7 and N2 and 15 between N7 and N6. The portion of polymorphic CFE markers (10.6 %) is comparable with that described for genomic wheat SSRs GWM transferred to the same set of mapping populations parents (9.2 %) (Khlestkina et al., 2004).

Twenty-three of the 32 markers were segregating in the mapping populations, while nine pairs produced monomorphic PCR-products, that can be explained by rye heterogeneity. Twenty-three *Xcfe* loci were genetically mapped on rye chromosomes (see the Table and Supplementary Materials)[1].

A consensus map was constructed using mapping data for the four populations. The consensus map contains 11 microsatellite (*Xcfe*..., *Xrems*... or *Xgwm*...) markers on chromosome 1R, 23 on 2R, 10 on 3R, 15 on 4R, 29 on 5R, 17 on 6R, 18 on 7R (see the Figure). In addition to these 123 SSR markers the consensus map contains 12 SNPs (*Xgbs*...), 118 RFLP markers (other *X*... names), and two isozyme loci. The former rye consensus map constructed in 2009 contained 10 microsatellite markers only (Gustafson et al., 2009).

Most of the microsatellites mapped in the current study consist of 3 bp repeats (15 loci), 5 of the mapped SSRs were dinucleotide, 2 sequences carried tetra- and 1 hexanucleotide repeat (see the Table).

Twenty-one of the 23 *Xcfe* loci mapped in orthologous positions (see the Table) considering multiple evolutionary translocations in the rye genome relative to those of common wheat, as described in detail by K.M. Devos et al. (1993). Two loci *Xcfe159-6R* and *Xcfe224-4R* have no orthology with wheat *Xcfe159* (5A, 5D) and *Xcfe224* (5B). The portion of the *Xcfe* loci showing orthology between wheat and

[1] Supplementary Materials are available in the online version of the paper: http:/www.bionet.nsc.ru/vogis/download/pict-2020-24/appx5.pdf

Characterization of CFE markers mapped in the current study

CFE No.	Motif	bp in wheat	bp in rye*	Chromosome location in wheat**	Chromosome location in rye*	Orthologous between wheat and rye
004	$(GAG)_6$	217	124, 130	3B, 4BS, 4DS	4RS	Yes
006	$(CGT)_7$	239	304, 307	2AL, 2BL, 2DL	2RL	Yes
010	$(AGG)_8$	324	311, 314	7AS, 7BS, 7DS	7RS	Yes
023	$(CGA)_5$	231	198, 207, 213	1AS, 1BS, 1DS	1RS	Yes
029	$(GA)_7$	201	194, 196	3AL, 3B, 3DL	3RL	Yes
037	$(TACG)_3$	150	159, 171	5AL, 5BL, 5DL	5RL	Yes
67	$(AG)_{11}$	175	180, 182, 194	2AS, 2BS, 2D	2RS	Yes
100	$(TG)_6$	243	237, 241, 255	7AS, 7BS, 7DS	4R(cent)	Yes
124	$(GAACCC)_3$	263	250, 272	6AL, 6BL, 6DL	6RL	Yes
136	$(AG)_7$	160	194, 200	1AL, 1BL, 1DL	1RL	Yes
143	$(CAGG)_4$	150	168, 172	5BL, 5DL	7RS	Yes
152	$(CGA)_5$	150	226, 232, 235, 238	6AS, 6BS, 6DS, 7AS	4RL	Yes
159	$(CAG)_6$	163	164, 167, 170, 173, 176	5BL, 5DL	6R (cent)	No
175	$(GGC)_6$	216	216, 219, 222	2AL, 2BL, 2DL	2RL	Yes
183	$(AG)_{14}$	215	210, 212	2AL, 2BL, 2DL	2RL	Yes
208	$(AGG)_4$	249	268, 274	5A, 5BS, 5DS	5RS	Yes
224	$(GCC)_{7+4}$	275	212, 215	5BL	4R(cent)	No
228	$(CTG)_{13}$	207	324, 333, 348	4AL, 5BL	7RS	Yes
260	$(CCT)_7$	149	136, 139	4AL, 4BS, 4DS	4RS	Yes
266	$(ACC)_4$	251	239, 244, 247	1AL, 1BL, 1DL	1RL	Yes
270	$(GTG)_7$	126	129, 132	4DL, 5DL, 4BL, 5AL	5RL	Yes
277	$(ACA)_4$	202	197, 203	6D	6RL	Yes
282	$(GAC)_7$	149	129, 138	3AS, 3B, 3DS, 4DL	3RS	Yes

* Data obtained in the current study (different length of the PCR products correspond to different parents of the rye mapping populations used; each microsatellite studied was monolocus and homozygous in all parents of the mapping populations, amplifying one fragment in each parental genotype).

** Chromosome location of homologous sequences carrying the CFEs (http://wheat.pw.usda.gov) was performed using BLAST analysis of the corresponding wheat ESTs given at http://wheat.pw.usda.gov against wheat chromosome survey sequences available at https://urgi.versailles.inra.fr/blast/blast.php. Further information is given according to http://wheat.pw.usda.gov.

rye (91 %) is higher than that found previously for genomic SSR loci *Xgwm* (73 %) (Khlestkina et al., 2004). This may reflect conservatism of the coding portion of plant genome, in particular that of the regions complementary to the primers, flanking microsatellites.

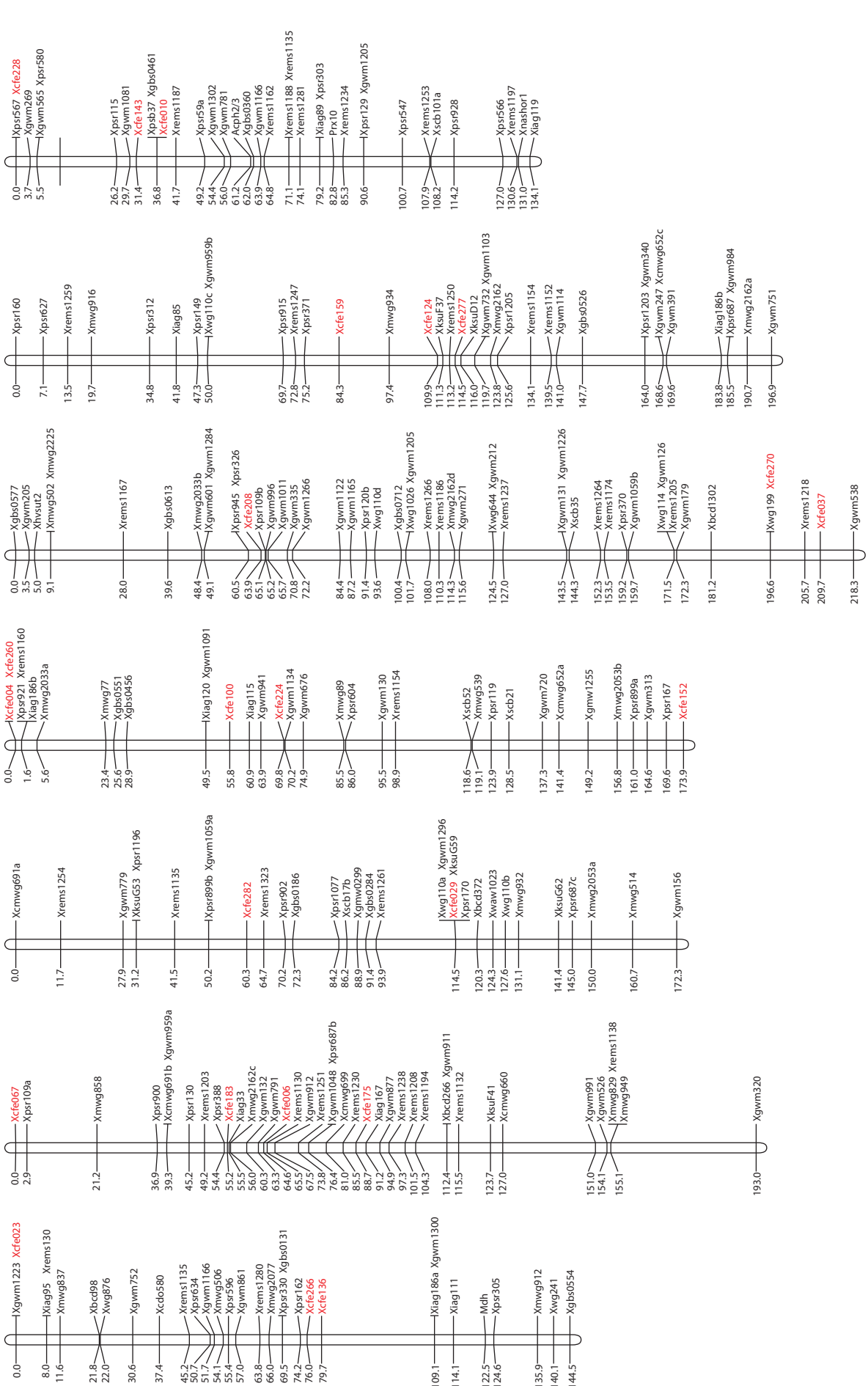
Usually the markers mapped to 7RS are found in a comprehensive region of the chromosome 7R corresponding to ancient translocation, while just a few markers are available for the small proximal region not involved in this translocation (Devos et al., 1993; Korzun et al., 2001; Khlestkina et al., 2004). The *Xcfe010-7R* locus mapped in the current study is located in this region (see the Figure) and can be used for tagging the part of chromosome 7RS, which is orthologous to the short arm of chromosome 7 of Triticeae (Devos et al., 1993).

The *Xcfe* loci mapped can be recommended for various applications in rye genetics and breeding. Some of them locate in the regions carrying known rye genes and therefore have a potential for marker-assisted selection. For example, comparison of the consensus map (see the Figure) with data available from previous gene mapping studies suggests *Xcfe270-5R* to be close to the dwarfing gene *Ddw1* (mapped by (Tenhola-Roininen, Tanhuanpää, 2010)), while the *Xcfe006-2R* locus (see the Figure) is mapped in the region highly comparable with location of the asynaptic genes *sy9* and *sy18* on chromosome 2R (Malyshev et al., 2009; Dolmatovich et al., 2013a).

Overall, the consensus map of rye contains 123 microsatellites. The list of mapped SSRs can be broaden in the future based on 856 SSRs recently found in rye genome shotgun survey sequences (Li et al., 2018).

Conclusion

The consensus map constructed in the current study contains a total of 123 microsatellites (including 23 SSRs transferred in our study from wheat to rye map), 12 SNPs, 118 RFLPs



Rye consensus microsatellite map constructed in the current study based on the map data from our study (for wheat EST-SSRs) and previous studies (for SNPs, Varshney et al., 2007; rye SSRs and wheat gSSRs, Khlestkina et al., 2004; RFLPs, Korzun et al., 2001) using JoinMap 2.0 program (Stam, 1993).

and 2 isozyme loci. Co-linearity between rye and wheat chromosome regions carrying these microsatellite loci was shown using 21 from 23 SSRs. These markers can be useful for both comparative mapping between wheat, rye and triticale as well as for marker-assisted breeding.

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Genomic regions of *Solanum tuberosum* L. associated with the tuber eye depth

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Abstract. Potato (*Solanum tuberosum* L.) is one of the most important food crops in the world. The genome of this potato species is autotetraploid and has a high level of heterozygosity, also this potato species is a cross-pollinated plant. These characteristics complicate the genetic analysis and breeding process. The tuber's eye depth is an important trait that affects the suitability of potato varieties for processing. Potato breeding for this trait is based on phenotypic assessment. Identification of the loci that control tuber eye depth would allow diagnostic markers for the marker-assisted selection to be created. The aim of this study is to search for loci associated with the eye depth by analyzing *Solanum tuberosum* varieties from the GenAgro collection of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, genotyped using the Illumina 22K SNP potato array DNA chip. The 24 significant markers associated with the "eye depth" trait were identified using 15,214 SNP markers genotyped with the Illumina 22K SNP potato array chip and the general linear model (GLM) taking into account the population structure. Data obtained showed the presence of SNPs in four genomic regions: on chromosome 4 (1 marker in the 3.92 Mb area), 5 (1 marker in the 4.67 Mb area) and 10 (1 marker in the 4.87 Mb area and 21 markers in the region between 48.1–48.9 Mb). The results of localization in the region 48.1–48.9 Mb of chromosome 10 correspond to previously published studies, the remaining three regions were detected for the first time. DNA sections containing SNPs linked to the tuber's eye depth were studied in the SolTub_3.0 potato genome assembly (https://plants.ensembl.org/). KASP markers were developed based on the data obtained. It will be possible to screen the breeding material and to breed the varieties more effectively using current markers associated with a shallow tuber's eye depth.

Key words: GWAS; SNP; potato; eye depth.

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Геномные районы *Solanum tuberosum* L., ассоциированные с глубиной залегания глазков клубней

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Аннотация. Картофель (*Solanum tuberosum* L.) – одна из важнейших в мире продовольственных культур. Геном вида автотетраплоидный, отличается высоким уровнем гетерозиготности, этот вид является также перекрестноопыляемым. Все это затрудняет генетический анализ и селекционный процесс. Глубина залегания глазков клубня картофеля продовольственного назначения – важный признак, влияющий на пригодность сортов картофеля для переработки. Селекция по этому признаку ведется на основе фенотипической оценки. Идентификация локусов, контролирующих данный признак, позволила бы проводить маркер-контролируемый отбор гибридов, отбраковывая формы с глубоким залеганием глазков на ранних этапах селекции. Целью настоящего исследования было выявление геномных районов, ассоциированных с глубиной залегания глазков, путем анализа сортообразцов картофеля *S. tuberosum* L. из коллекции ГенАгро Института цитологии и генетики СО РАН. При использовании 15214 SNP-маркеров, генотипированных с помощью чипа

Illumina 22K SNP potato array, и обобщенной линейной модели (General Linear Model, GLM) с учетом популяционной структуры найдены 24 значимых маркера, ассоциированных с признаком «глубина залегания глазков». Полученные данные показали наличие SNP в четырех геномных районах: в хромосомах 4 (1 маркер в районе 3.92 Мб), 5 (1 маркер в районе 4.67 Мб) и 10 (1 маркер, относящийся к району 4.87 Мб, и 21 маркер в районе 48.1–48.9 Мб). Сопоставление выявленных геномных районов в нашем исследовании с более ранними работами подтвердило, что локус между 48.1–48.9 Мб был известен ранее, остальные три района обнаружены впервые. Участки ДНК, содержащие SNP, сцепленные с глубиной залегания глазков, были изучены в сборке генома картофеля SolTub_3.0 (https://plants.ensembl.org/), и на основе полученных данных были разработаны KASP-маркеры, при применении которых можно будет более эффективно вести скрининг селекционного материала и селекцию сортов с мелким залеганием глазков.
Ключевые слова: GWAS; SNP; картофель; глубина залегания глазков.

Introduction

Potato *Solanum tuberosum* L. is the important food crop. The genetic analysis and breeding process of this crop is complicated by the autotetraploid nature and a high level of heterozygosity of this species. In addition, *S. tuberosum* is a cross-pollinated species, that also makes genetic research and breeding more difficult. (Prashar et al., 2014). Potato varieties are reproduced vegetatively in view of the low fertility and the impossibility of ripening fruits in the climatic conditions of many countries. This type of reproduction allows this crop to maintain the identity of variety genome in different reproduction, despite the heterozygosity.

Mapping of quantitative trait loci (QTL) and genes of autotetraploid potato using biparental populations is a difficult task. The biparental populations method for autotetraploids requires obtaining and analysis of numerous progeny. However, the low fertility of most potato varieties does not allow obtaining big mapping populations. Researches often overcome these limitations of mapping studies by transition to diploid level, at which in addition interspecific hybridization is possible. However this approach still requires high level of fertility. All these features limit the use of *S. tuberosum* varieties in genetic mapping and QTL studies. However, the possibility of applying a genome-wide association studies (GWAS) with the development of high performance (HP) sequencing and other HP-genotyping methods (including application of SNP-arrays) made it possible to intensify research aimed on identification of genomic loci related with quantitative traits, avoiding all difficulties mentioned above for biparental mapping and QTL analysis. The vegetative reproduction way of cultivated forms of potato facilitates GWAS method compared to other cross-pollinated plants, however some adaptation of the method is still needed taking into account the autotherapoid nature and heterozygosity (Prashar et al., 2014; Khlestkin et al., 2019).

The depth of the tuber eyes is an important trait for the suitability of potato varieties for processing. The volume of losses during peeling, which should not be higher than 15 % (Zemtcova, Timofeeva, 2011), depends on the eye depth. Accordingly, this trait affects the cost of peeling during processing. Based on the conditions for obtaining minimal waste during mechanized abrasive peeling, the tuber eye depth should be no more than 1.5 mm (Pshechenkov, Mal'cev, 2011). Evaluation of potato tuber eye depth is carried out using various scales. A number of studies used scales divided into three to nine grades (Li et al., 2005; Prashar et al., 2014; Hara-Skrzypiec et al., 2018).

The first genetic studies of tuber eye depth were performed at the beginning of the twentieth century. R.N. Salaman (1911) showed that deep eyes dominate shallow ones. Later W. Black (1930) also suggested that the eye depth is controlled by genetic factors, but he hypothesized that this trait has an intermediate type of inheritance or incomplete dominance when extreme phenotypes (very deep and very shallow eyes) are probably homozygous and intermediate phenotypes (medium depth of the eye) are heterozygous. B. Maris (1966) suggested that the eye depth is controlled by one major gene with a additive effect. Some authors have reported that shallow eyes are dominant (Howard, 1974). H. Kukimura (1972) and H.W. Howard (1974) indicated that when crossing two samples with shallow eyes, in segregation can appear samples with deep and medium eyes. Studies that using genetic markers have shown existence of the major locus that controls the eye depth trait (Li et al., 2005). These studies have also shown that the deep eye (Eyd) dominates the shallow eye (eyd). The Eyd/eyd locus, which is responsible for the depth of the eye, is located on chromosome 10 (Li et al., 2005).

W. Black (1930) was first who suggested eye depth to be quite dependent on environmental conditions. Later, B. Maris (1966) demonstrated a high susceptibility of this trait to changes under the influence of environmental factors. However, H.W. Howard (1974) argued that the eye depth is determined mainly by the genotype. Later, a number of studies also showed a high heritability of the eye depth (Gopal et al., 1992; Love et al., 1997; da Silva et al., 2014; Ney et al., 2016) and an insignificant environmental influence (Love et al., 1997). Other authors show that the genotype most strongly affects the trait, but the influence of environmental factors is also quite high (Hara-Skrzypiec et al., 2018). A number of studies have shown the ability of the eye depth to change under the influence of somaclonal variation (Evans et al., 1986; Thieme, Griess, 2005).

In traditional breeding, the depth of the eyes is estimated in the first generation after crossing. However, the tubers of hybrids grown from botanical seeds are too small in size and in this case it is difficult to adequately estimate the depth

of the eyes. To solve this problem, DNA markers can be used that will allow effective selection at the first generation stage and reduce the volume of subsequent studies.

The aim of this study was to search for loci associated with the eye depth by analyzing *S. tuberosum* potato varieties from the GenAgro collection of the Institute of Cytology and Genetics SB RAS, genotyped using the Illumina 22K SNP potato array.

Materials and methods

The plant material. A total of 88 potato varieties from the collection of the GenAgro Institute of Cytology and Genetics SB RAS (Table 1) were phenotyped. Most of the potato collection was represented by varieties and hybrids of domestic selection, some varieties studied were from abroad (Ukraine, Germany, China, etc.).

Plants were grown in the field in two field plots on the territory of the Michurinsky village, Novosibirsk region from May to August 2017.

Field tests were carried out according to the following scheme: the number of rows for each genotype was 2; number of plants in a row – 10; row length – 3 m; the distance between the rows – 0.75 m; the distance between the plants in the rows – 0.30 m; planting method – manually (by hand) on furrows, filling furrows with harrows; landing date is the third decade of May.

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

Meteorological conditions of the growing season:

May. Air temperature: long-term average 10.90 °C; average monthly 12.60 °C; effective temperature sum 197.60 °C. Precipitation: long-term average 37.00 mm; the amount for the month is 33.90 mm.

June. Air temperature: long-term average 16.90 °C; average monthly 19.30 °C; effective temperature sum 576.00 °C. Precipitation: long-term average 55.00 mm; the amount for the month is 71.90 mm.

July. Air temperature: long-term average 19.40 °C; average monthly 18.50 °C; effective temperature sum 1004.00 °C. Precipitation: long-term average 61.00 mm; the amount for the month is 99.50 mm.

August. Air temperature: long-term average 16.20 °C; average monthly 16.80 °C; effective temperature sum 1408.00 °C. Precipitation: long-term average 67.00 mm; the amount for the month is 65.60 mm.

Evaluation of the eye depth was carried out in 2017 in accordance with the VIR methodology (Kiru et al., 2010). The eye depth was determined on a scale of 1 to 3: 1 – shallow (less than 1–1.3 mm), 2 – medium (1.4–1.6 mm), 3 – deep (more than 1.7 mm). Five typical eyes of a potato tuber were measured. These data were compared with the data presented in the database of the State Register of breeding achievements approved for use (http://gossortrf.ru/gosreestr.html) and data from the European cultivated potato database (https://www.europotato.org/).

For genotyping, DNA was isolated from the skin of potato tubers using the DNeasy Plant Mini kit (Qiagen, CA, USA) according to the manufacturer's protocol. The concentration and purity of the test samples was determined using gel electrophoresis and using a Nanodrop 2000 apparatus.

All 88 varieties were genotyped using the Illumina 22K SNP potato array (GGP Potato V3) DNA chip at Traitgenetics GmbH (Gatersleben, Germany).

The 21,226 SNP dataset was filtered using Excel software. Low quality data was deleted, all monomorphic markers and markers containing more than 95 % of one allele. For further analysis, 15,214 (71.7 %) SNPs were taken. The chromosomal position for SNP has been determined using data of (Vos et al., 2015).

An analysis of the associations between the eye depth and the genomic regions was carried out using the Tassel 5.2.59 software package (Bradbury, 2007). A generalized linear model (GLM) was used taking into account the population structure (Q). The population structure of the collection was analyzed in previous works (Khlestkin et al., 2019) using the STRUCTURE v 2.3.4 program (Pritchard et al., 2000), based on data from all 15,214 markers taken for further analysis.

Since the TASSEL software package was developed for the analysis of the genomes of diploid species, for its application to the tetraploid genome, the data were transcoded into a digital format taking into account the representation of the effector allele (Khlestkin et al., 2019).

Two criteria were used to determine the significance of associations: (1) the Bonferroni correction, which was defined as dividing the statistical significance level (0.05) by the total number of trials, in our case, by the number of markers (15,214) and amounted to $3.28 \cdot 10^{-6}$, and (2) Benjamini–Hochberg test (Benjamini, Hochberg, 1995) (false discovery rate, FDR). In this case, only those SNPs whose *p*-value (FDR) did not exceed the threshold value of 0.05 were considered significant, taking into account the amendments to the Benjamini–Hochberg method.

The development of KASP markers and genotyping of 86 varieties using them was carried out by LGC Genomics LLC (Teddington, UK). Sections of 100 bp DNA containing SNP associated with the eye depth trait were converted to KASP markers (Supplementary 1)[1]. Data about the nucleotide composition of these sites are presented in the SolTub_3.0 potato genome assembly (https://plants.ensembl.org/). In the future, it will be possible more effectively carry out of screening breeding material and selection varieties with a shallow eye depth using these developed KASP markers.

[1] Supplementary Materials are available in https://vavilov.elpub.ru/jour/manager/files/SupplTotsky_engl.pdf

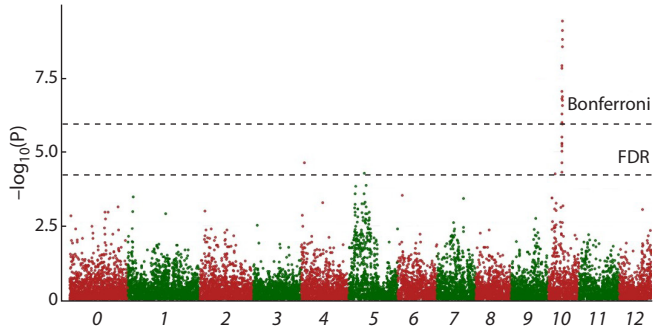
Table1. The results of phenotypic estimation of the potato tubers eye depth

No.	Sample name	Eye depth	No.	Sample name	Eye depth
1	785/8-5	Medium	45	Lyubava	Medium
2	Agata	Shallow	46	Lyuks	Shallow
3	Alyona	Medium	47	Maret	Medium
4	Antonina		48	Matushka	Shallow
5	Arlekin		49	Meteor	Medium
6	Babushka	Shallow	50	Milavica	
7	Bozhedar	Deep	51	Monaliza	Shallow
8	Bravo	Medium	52	Nakra	Medium
9	Vasilyok	Shallow	53	Nayada	Shallow
10	Velikan	Medium	54	Nevskij	
11	Virazh		55	Nikulinskij	
12	Vympel		56	Novosibirskij	Deep
13	G 06-08-2015		57	Pamyati Osipovoj	
14	G 3-43-2	Shallow	58	Pamyati Rogachyova	Shallow
15	G 3-43-6	Medium	59	Pikasso	
16	Gala	Shallow	60	Radonezhskij	Medium
17	Golubizna		61	Reggi	Shallow
18	Gornyak		62	Red Skarlet	
19	Granat	Medium	63	Redstar	
20	Granola	Shallow	64	Ruslan	
21	Gulliver		65	Russkij souvenir	
22	Gusar		66	S-112-03 (Dochka)	
23	Debryanskij	Medium	67	Samba	
24	Di Dzhon 12	Deep	68	Sante	
25	Diamant	Shallow	69	Sarovskij	Medium
26	Zhavoronok	Medium	70	Safo	Shallow
27	Zhigulyovskij	Shallow	71	Svitanok kievskij	
28	Zhukovskij rannij		72	Sel'ma	
29	Zagadka	Medium	73	Skala	
30	Zekura	Shallow	74	Solnechnyj	Medium
31	Zizella	Medium	75	Start	Shallow
32	Zlatka		76	Stigniya	
33	Il'inskij		77	Sudarynya	Medium
34	Impala	Shallow	78	Tanaj	Shallow
35	Irbitskij		79	Tango	Medium
36	Kemerovchanin	Medium	80	Tuleevskij	Shallow
37	Klada	Shallow	81	Udacha	
38	Kolobok	Deep	82	Favorit	
39	Kortni		83	Fioletovyj	Medium
40	Krepysh	Medium	84	Fritella	Shallow
41	Kuznechanka		85	Hozyayushka	
42	Ladozhskij		86	Charoit	
43	Lina		87	Yugana	
44	Lomonosovskij	Shallow	88	Yuna	

Results

Phenotyping conducted on 88 samples of the collection showed that most of the collection, 49 samples, had a shallow eye depth. Also, a large part of the collection was represented by samples having a medium eye depth – these were 33 genotypes. Samples with deep eyes represented a small part of the sample and were represented by only 6 samples (see Table 1).

Association studies using GLM and taking into account the population structure revealed 24 SNPs significantly associated with the tuber's eye depth (Table 2, see the Figure). After applying the Bonferroni multiple test correction at 5 % ($p < 3.28 \cdot 10^{-6}$), only 15 of 24 SNPs remained significant for the tuber's eye depth, the remaining nine SNPs remained significant only using the FDR criterion ($p < 0.05$).



Manhattan plot showing significant SNP, associated with eye depth when using GLM analysis taking into account the population structure.

1–12 – chromosome designation; *0* – SNPs unassigned to certain chromosomes.

Table 2. SNPs associated with the eye depth, which identified using GLM analysis

No.	Marker	Chromosome	Physical map position (bp)	*p*	FDR	Alleles
1	PotVar0111687*	10	48721966	3.99E–10	6.06917E–06	T/C
2	solcap_snp_c2_25485*	10	48737840	8.22E–10	6.25189E–06	A/G
3	solcap_snp_c1_8019*	10	48863165	1.62E–09	8.21252E–06	A/G
4	solcap_snp_c2_25471*	10	48808404	2.90E–09	1.10302E–05	A/G
5	solcap_snp_c2_25526*	10	48617149	1.27E–08	3.85736E–05	A/G
6	solcap_snp_c2_25522*	10	48617457	1.53E–08	3.87577E–05	T/C
7	solcap_snp_c2_25532*	10	48591792	9.29E–08	0.000201844	C/G
8	solcap_snp_c1_8021*	10	48862950	1.41E–07	0.00026906	A/G
9	solcap_snp_c2_25529*	10	48593621	1.62E–07	0.000273091	T/G
10	solcap_snp_c1_16351*	10	48761642	1.86E–07	0.000282357	T/C
11	solcap_snp_c2_55948*	10	48982352	2.80E–07	0.000387016	A/G
12	solcap_snp_c2_25528*	10	48616808	5.26E–07	0.000667235	T/C
13	solcap_snp_c2_25527*	10	48616993	1.05E–06	0.001228589	A/G
14	solcap_snp_c2_25469*	10	48808653	1.21E–06	0.001315794	A/G
15	solcap_snp_c2_45611*	10	48203431	3.15E–06	0.003191491	A/G
16	solcap_snp_c2_25530	10	48593576	5.13E–06	0.004875707	T/C
17	solcap_snp_c1_13531	10	48149384	5.48E–06	0.004901056	A/G
18	solcap_snp_c2_45606	10	48218826	6.41E–06	0.005414409	A/G
19	solcap_snp_c2_25523	10	48617343	9.66E–06	0.007735919	T/A
20	PotVar0106879	4	3916249	2.40E–05	0.01823474	T/C
21	solcap_snp_c2_49532	10	48443334	2.40E–05	0.017394673	A/C
22	solcap_snp_c2_45612	10	48203384	4.93E–05	0.034120853	T/C
23	solcap_snp_c2_23017	5	4670373	5.39E–05	0.035637141	T/C
24	PotVar0107893	10	4870467	5.45E–05	0.034526271	A/G

Note. 1–15*– SNPs, which are significant according Bonferroni multiple test correction at 5 % ($p < 3.28 \cdot 10^{-6}$); 16–24 – SNPs, which are significant according FDR.

Table 3. Correspondence of the allele of marker and phenotypic condition of the eye depth

No.	Marker	Alleles	Eyes		
			shallow	medium	deep
1	**PotVar0111687**	4 C	0	0	1
		C+T	38	29	5
		4 T	**10**	**2**	**0**
2	**solcap_snp_c2_25526**	4 C	0	0	0
		C+T	37	28	6
		4 T	**11**	**4**	**0**
3	**solcap_snp_c1_16351**	4 C	0	0	1
		C+T	35	29	4
		4 T	**13**	**3**	**0**
4	**solcap_snp_c2_55948**	4 C	0	0	1
		C+T	34	28	5
		4 T	**14**	**3**	**0**
5	**solcap_snp_c1_8019**	4 G	0	0	1
		G+A	36	30	5
		4 A	**10**	**1**	**0**
6	**solcap_snp_c2_25471**	4 G	0	0	1
		G+A	38	31	5
		4 A	**10**	**1**	**0**
7	**solcap_snp_c2_25522**	4 A	0	0	1
		G+A	38	29	5
		4 G	**10**	**3**	**0**
8	**solcap_snp_c2_25528**	4 A	0	0	1
		G+A	34	27	5
		4 G	**14**	**5**	**0**
9	solcap_snp_c2_25469	4 A	0	0	1
		G+A	33	28	4
		4 G	15	4	1
10	solcap_snp_c2_25529	4 A	0	0	1
		C+A	33	27	4
		4 C	15	5	1
11	solcap_snp_c2_25527	4 T	0	0	1
		C+T	33	28	4
		4 C	14	4	1

Note. Haplotypes predicting small eyes depth are highlighted in bold.

SNPs associated with the eye depth are found in four genomic regions, two of which are located on chromosome 10. SNPs that are significant when using Bonferroni's multiple test corrections ($p < 3.28 \cdot 10^{-6}$) were located on one of the two loci of chromosome 10. SNPs that are significant when using FDR ($p < 0.05$) are found at all four loci on chromosomes 10, 4, and 5. On chromosomes 4 and 5, only one significant SNP was located. Twenty-one SNPs were located on chromosome 10 in a relatively small area between 48.1 and 48.9 Mb. Another SNP was located

in another region of chromosome 10 at position 4.87 Mb (this SNP is significant when using FDR).

The most significant SNP PotVar0111687 refers to a non-coding sequence. In total, among 18 significant SNPs, 6 are located in non-coding regions of the genome, and 11 are located in protein coding sequences. Annotation of genes containing significant SNPs is given in Supplementary 2. Due to the lack of accurate information on the genetic control of the eye depth, it turned out to be difficult to definitely relate the identified genes to the formation of the eye depth.

KASP genotyping analysis results

KASP genotyping was carried out using 11 KASP markers developed on the basis of SNP that significant when using Bonferroni multiple test corrections, which were linked to the eye depth and located on the 10th chromosome between 48.1 and 48.9 Mb (Table 3). Eight markers correlated with the eye depth, or rather, the homozygous state of one of the alleles of these markers correlates with a shallow eye.

Haplotypes that are 100 % predictive for shallow eyes are: PotVar0111687 (homozygote for T), solcap_snp_c1_16351 (homozygote for T), solcap_snp_c2_55948 (homozygote_s2, 55848), solcap_snp_c2_25522 (homozygous for G), solcap_snp_c2_25528 (homozygous for G). The carriers of this haplotype are the Vasilyok, Gulliver, Lyuks, Matushka, Nayada, Dochka, Tuleevskij, Favorit, Charoit varieties.

Discussion

X.Q. Li et al. (2005) conducted genetic mapping of the locus responsible for the eye depth. As a result, the Eyd locus was mapped on chromosome 10 proximal to the CT240 marker (12 cM) and distal to the STM0051 marker (13 cM). BLAST analysis of the sequences of these markers indicates the position of CT240 in the region of 51 Mb, and STM0051 in the region of 23.4 Mb. It can be assumed that the locus that we identified on chromosome 10, which is located between 48.1 and 48.9 Mb, corresponds to the Eyd gene. In a number of works (Śliwka et al., 2008; Prashar et al., 2014; Rosyara et al., 2016 and others), a locus linked to eye depth was regularly detected in this region.

A. Prashar et al. (2014) using Infinium 8303 Potato Array compiled a genetic map of diploid potatoes. After selecting the most valuable SNPs, the map contained 1355 different loci and 2157 SNPs. In this work, it was found that the main locus for the tuber's eye depth is located on chromosome 10 (SNP: c1_8020) and linked to the tuber-shaped locus. This SNP is located on chromosome 10 in the region of 48.8 Mb, as well as locus 4 detected in the current study. Also A. Prashar et al. (2014) found two SNPs linked to the eye depth on chromosomes 2 (c2_7422) and 3 (c2_37119), which were not so significant and most likely could have an auxiliary effect.

U.R. Rosyara et al. (2016), also used GWAS to search for SNPs linked to the eye depth. In their study, the DNA chip had 3.5 thousand markers. A highly significant SNP for the eye depth was located at the 48.9 Mb position on chromosome 10 (coinciding with the locus detected by A. Prashar et al. (2010) and locus 4, identified in our work). The less significant SNP c2_11685, which was also linked to the tuber's eye depth, was located on chromosome 5 at 2.3 Mb position. In our work, we also detected SNP associated with the eye depth, located on chromosome 5, but in a distinct region – 4.7 Mb.

H. Lindqvist-Kreuze et al. (2015) also found potato tubers eye depth QTL on chromosome 10 at position 49.4 Mb, as well as another locus on chromosome 12. Researchers have identified a number of candidate genes that underlie significant QTL and are linked to the eye depth. One of them is the BEL-1-like homeobox gene, found at a distance of 1.37 Mb from the QTL marker toPt-437059 on chromosome 10. The second is the α-expansion gene, which was found in the region of significant QTL on chromosome 10 at a distance of 1.78 Mb from the QTL marker toPt-437059. Also, some genes associated with the production and modification of pectins were found in the close proximity to toPt-437059 marker on chromosome 10.

A. Hara-Skrzypiec et al. (2018) also performed mapping for a number of potato traits, including the eye depth. Seven QTLs that were linked to the eye depth were found: one per each chromosome 1, 4, and 11, and two per each chromosomes 3 and 5. However, unlike other studies, the major QTL was located on chromosome 4 (at position 68.8 Mb) and accounted for 22.6 % of the dispersion in the average data set. We found in our study a significant SNP PotVar0106879 located on chromosome 4 at position 3.9 Mb.

Thus, the key locus responsible for the eye depth is located on chromosome 10, while loci with a minor effect are located on chromosomes 1, 2, 3, 4, 5, 11, and 12, as well as in other parts of chromosome 10.

Conclusion

Despite a wide range of genetic studies of the potato tubers eye depth trait and the identification of genes and QTL associated with eye depth variability, DNA markers are still not used in the breeding of potatoes to this trait. Meanwhile, the use of diagnostic DNA markers allows to carry out more efficient pre-breeding research (screening of potato genetic resources to identify donors of valuable allelic variants) and marker-assisted selection in breeding programs (Gebhardt et al., 2006; Chen et al., 2017; Klimenko et al., 2017, 2019). We were able to develop a number of new PCR markers that can be convenient for screening the genetic resources and breeding material of potato. After additional verification of these proposed markers on an extended sample, they can be used to select shallow-eyed plants by analysis at the DNA level. We selected PCR markers located on chromosome 10 at positions between 48.62 and 48.98 Mb. These data coincide with the data of other authors on the location of the supposed gene, which is responsible for the eye depth. However, due to the larger number of markers that we found, these data allow us to clarify the localization of the

gene of interest to us and suggest that it is located at positions between 48.0 and 49.0 Mb. According to our data, selection according to the haplotype which includes 8 SNPs located on chromosome 10 is optimal (PotVar0111687 (homozygote of T), solcap_snp_c2_25526 (homozygote of T), solcap_snp_c1_16351 (homozygote of T), solcap_snp_c2_55948 (homozygote of T), solcap_snp_c1_8019 (homozygote of A), solcap_snp_c2_25471 (homozygote of A), solcap_snp_c2_25522 (homozygote of G), solcap_snp_c2_25528 (homozygous for G)). The carriers of this haplotype are the varieties Vasilyok, Gulliver, Lyuks, Matushka, Nayada, Dochka, Tuleevskij, Favorit, Charoit, which are characterized by a shallow eye depth. The use of these varieties as donors of this trait (which are at the same time donors a number of other valuable properties) in combination with PCR analysis of the offspring to select carriers of the corresponding haplotype will provide a more economical and accelerated method of creating potatoes with a shallow tuber eye depth.

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Analysis of genetic relationships of genotypes of the genus *Rosa* L. from the collection of Nikita Botanical Gardens using ISSR and IRAP DNA markers

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Abstract. In connection with the development of breeding and the creation of new plant varieties, the problem of their genotyping and identification is becoming increasingly important, therefore the use of molecular methods to identify genetic originality and assess plant genetic diversity appears to be relevant. As part of the work performed, informative ISSR and IRAP DNA markers promising for the study of genetic diversity of the *Rosa* L. genus were sought and applied to analysis of genetic relationships among 26 accessions of the genus *Rosa* L. from the gene pool collection of Nikita Botanical Gardens. They included 18 cultivated varieties and 8 accessions of wild species. The species sample included representatives of two subgenera, *Rosa* and *Platyrhodon*. The subgenus *Platyrhodon* was represented by one accession of the species *R. roxburghii* Tratt. Cultivated roses were represented by varieties of garden groups hybrid tea, floribunda, and grandiflora. The tested markers included 32 ISSRs and 13 IRAPs. Five ISSR markers (UBC 824, ASSR29, 3A21, UBC 864, and UBC 843) and three IRAPs (TDK 2R, Cass1, and Cass2) were chosen as the most promising. They were used for genotyping the studied sample of genotypes. In general, they appeared to be suitable for further use in studying the genetic diversity of the genus *Rosa* L. The numbers of polymorphic fragments ranged from 12 to 31, averaging 19.25 fragments per marker. For markers UBC 864 and UBC 843, unique fingerprints were identified in each accession studied. The genetic relationships of the studied species and varieties of roses analyzed by the UPGMA, PCoA, and Bayesian methods performed on the basis of IRAP and ISSR genotyping are consistent with their taxonomic positions. The genotype of the species *R. roxburghii* of the subgenus *Platyrhodon* was determined genetically as the most distant. According to clustering methods, the representative of the species *R. bengalensis* did not stand out from the group of cultivated varieties. When assessing the level of genetic similarity among the cultivated varieties of garden roses, the most genetically isolated varieties were 'Flamingo', 'Queen Elizabeth', and 'Kordes Sondermeldung'; for most of the other varieties, groups of the greatest genetic similarity were identified. This assessment reflects general trends in phylogenetic relationships, both among the studied species of the genus and among cultivated varieties.
Key words: *Rosa* L.; rose; genetic resources; DNA-markers; ISSR; IRAP; genetic diversity.

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Анализ генетических взаимосвязей генотипов рода *Rosa* L. из коллекции Никитского ботанического сада с использованием ISSR- и IRAP- ДНК-маркеров

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Аннотация. В связи с развитием селекции и появлением новых сортов растений все более важным становится вопрос паспортизации и структуризации генофонда, поэтому использование молекулярно-генетических методов для выявления генетической оригинальности и оценки генетического разнообразия растений представляется актуальным. В рамках настоящей работы осуществлены поиск информативных ISSR- и IRAP- ДНК-маркеров, перспективных для изучения генетического разнообразия рода *Rosa* L., и анализ с их помощью генетических взаимосвязей образцов из генофондовой коллекции роз Никитского ботанического сада. Материалом для генотипирования послужили 26 образцов, 18 из которых являются культурными сортами, а 8 образцов относятся к дикорастущим видам. В выборку видов включены представители двух подродов *Rosa* и *Platyrhodon*. Подрод *Platyrhodon* представлен одним образцом вида *R. roxburghii* Tratt. Среди культурных роз присутствовали сорта

садовых групп: чайно-гибридной, флорибунда и грандифлора. В исследовании было задействовано 32 ISSR- и 13 IRAP-маркеров. После апробации были отобраны как наиболее перспективные пять ISSR-маркеров (UBC 824, ASSR29, 3A21, UBC 864, UBC 843) и три IRAP-маркера (TDK 2R, Cass1, Cass2). Они использовались для генотипирования исследуемой выборки генотипов и в целом перспективны для дальнейшего изучения генетического разнообразия рода *Rosa* L. Количество полиморфных фрагментов варьировало в диапазоне от 12 до 31, в среднем 19.25 фрагмента на маркер. По маркерам UBC 864 и UBC 843 выявлены уникальные фингерпринты для каждого изученного образца. Оценка генетических взаимосвязей изученных видов и сортов роз с использованием методов UPGMA, PCoA и байесовского анализа, выполненная на основе данных IRAP- и ISSR-генотипирования, согласуется с таксономическим положением образцов. Генотип вида *R. roxburghii* подрода *Platyrhodon* определен как генетически наиболее отдаленный образец. Представитель вида *R. bengalensis* методами кластеризации не выделился из группы культурных сортов. При оценке уровня генетического сходства среди культурных сортов садовой розы наиболее генетически обособленными сортами оказались 'Flamingo', 'Queen Elizabeth', 'Kordes Sondermeldung', для остальных сортов были определены группы наибольшего генетического сходства. Данная оценка отражает общие тенденции в филогенетических отношениях как между изученными видами рода, так и между культурными сортами.

Ключевые слова: *Rosa* L.; розы; генофонд; генотипирование; ДНК-маркеры; IRAP; ISSR; генетическое разнообразие.

Introduction

According to the Plant List database (www.theplantlist.org), the genus *Rosa* L. includes 373 recognized species. Sixteen of them occur in the natural flora of the Crimea (Ena, 2012). Currently, the world range of garden roses includes more than 30 thousand varieties. The international classification divides all this diversity into 36 garden groups according to its decorative and biological characteristics (McFarland, 2007). Rose breeding efforts have increased in recent years in Japan, China, India, Canada, and New Zealand (Plugatar et al., 2017). In Russia, breeding work with roses has been successfully carried out in the Nikita Botanical Gardens (NBG) since 1824 (Plugatar, 2016). Roses from the garden groups floribunda, grandiflora, miniature, and hybrid tea have flowering periods from 180 to 200 days a year, depending on the variety, and are the most promising and popular in gardening (Plugatar et al., 2017).

The mobilization and preservation of genetic resources of the entire diversity of rose cultivars and species that participated in their creation is one of the main directions in the creation of new varieties that would meet the requirements of modern decorative floriculture for specific regions of cultivation of this crop (Schanzer, Vagina, 2007; Korkmaz, Dogan, 2018).

The current development of DNA marking methods and their introduction into scientific practice contributes to the improvement of the efficiency of research aimed at clarifying the genetic relationships of varieties at the intra- and interspecies level; study of the genetic structure of collections of gene pools; creation of collections; certification and registration of the existing gene pool. In addition, DNA marking methods can be effectively used to seek donors of genes for breeding-valuable traits, identify duplicate accessions, and resolve disputes when classifying newly received specimens. The use of data at the level of genetic similarity in combination with phenotypic characteristics of varieties in the formation of parent pairs in breeding programs can be promising.

The earliest phylogenetic studies of the genus *Rosa* L. using molecular genetic markers include the work by Millan et al. (1996). The study used RAPD markers (Random Amplified Polymorphic DNA) to assess polymorphism at the intraspecific and interspecific levels in representatives of various sections of *Rosa*. Three clusters were established by the UPGMA method: the first cluster included accessions of sections *Pimpinellifoliae* and *Synstylae*, the second cluster was formed by sections *Chinenses* (*Indicae*) and *Gallicanae*, and the third was represented by species of sections *Cassiorhodon* (*Cinnamomeae*) and *Caninae*. However, a later work with a significantly broader sample of 109 specimens belonging to 39 species (Atienza et al., 2005) failed to obtain an unambiguous distribution of samples by taxa. These results were not completely consistent with a slightly earlier study by American authors (Jan et al., 1999), who also used RAPD markers to analyze another sample of 119 accessions of 36 *Rosa* species.

Koopman et al. (2008) used AFLP DNA markers for phylogenetic analysis. By analysis of a series of 92 samples belonging to 46 species of the genus *Rosa* L., the phylogeny of species within the genus *Rosa* was reconstructed. Multilocus DNA-markers have been widely used to elucidate genetic relationships, both in gene pool collections and in the study of natural populations of various species of the genus *Rosa*. Thus, using complex data obtained by ISSR and RAPD analysis, a group of scientists from Turkey analyzed genetic relationships among 27 *Rosa* species growing in Turkey (Korkmaz, Dogan, 2018). RAPD, ISSR, and SSR markers were used to study the genetic relationship between Taif rose accessions and those collected in Syria and Egypt, including the Damascus rose. The analysis of the degree of genetic similarity based on the results of genotyping revealed the greatest degree of genetic affinity of Taif roses to Damascus roses of Gory variety growing in Syria (El-Assal et al., 2014). Molecular analysis methods, including ISSR markers, were also successfully used in a number of works aimed at studying the genetic relationships in the genus *Rosa* and clarifying issues related to phylogeny performed by Russian scientists (Schanzer, 2013, 2015).

The objective of this work is to study the genetic relationships among *Rosa* accessions of various origins from the NBG's collection using IRAP and ISSR multilocus markers.

Materials and methods

As plant material for genotyping, 26 accessions of the genus *Rosa* L. were selected in the study, 18 of which were cultivated varieties, and 8 belonged to wild species (Table 1). The sample of species included representatives of two subgenera *Rosa* and *Platyrhodon*. The subgenus *Platyrhodon* was represented by a single specimen of the species *R. roxburghii* Tratt. Cultivated roses were represented by hybrid tea, floribunda, and gran-

Table 1. Varieties of the genus *Rosa* selected for genotyping

Sample #	Species, variety	Origin
1	'Korallovyi Suyrpriz'	'Kordes Sondermeldung' × 'Queen Elizabeth'
2	'Miskhor'	'Angelique' × 'Zvezda Oktyabrya'
3	*R. multiflora* Thunb.	Wild-growing species
4	'Gloria Dei'	Seeds: ('George Dickson' × 'Souvenir de Claudius Pernet') × ('Joanna Hill' × 'Charles P. Kilham'); pollen: 'Margaret McGredy'
5	'Ayu-Dag'	'Chrysler Imperial' × 'Kordes Sondermeldung'
6	'Kronenbourg'	Clone of 'Peace' variety
7	*R. foetida* Herrm.	Wild-growing species
8	'Flamingo'	Seedling × 'Lady Like'
9	*R. hugonis* Hemsl.	Wild-growing species
10	*R. indica* Linn.	Wild-growing species
11	'Klimentina'	'Kordes Sondermeldung' × 'Gloria Dei'
12	'Prekrasnaya Tavrida'	'Gloria Dei' × pollen mix of 'Crimson Glory' + 'Poinsettia'
13	*R. bracteata* J.C. Wendl	Wild-growing species
14	'Yves Piaget'	('Pharaoh' × 'Peace') × ('Chrysler Imperial' × 'Charles Mallerin')
15	'Chatyr-Dag'	'Charles Mallerin' × 'Chrysler Imperial'
16	'Chrysler Imperial'	'Charlotte Armstrong' × 'Mirandy'
17	'Prince de Monaco'	'Tamango' × 'Matangi'
18	'Mechta'	'Karl Herbst' × 'Spek's Yellow'
19	*R. roxburghii* Tratt.	Wild-growing species
20	'Queen Elizabeth'	'Charlotte Armstrong' × 'Floradora'
21	'Rouletii' (*R. rouletii* Correvon, *R. chinensis* Jacq. f. *minima*)	Wild-growing species
22	'La France'	'Madame Victor Verdier' × 'Madame Bravi'
23	*R. bengalensis* Pers.	Wild-growing species
24	'Traviata'	'Porta Nigra' × 'Paola' × 'William Shakespeare'
25	'Alisa'	'Jubile du Prince de Monaco' × 'Flamingo'
26	'Kordes Sondermeldung'	'Baby Chateau' × 'Crimson Glori'

diflora groups of varieties. DNA was extracted from young leaves by the CTAB method (Murray, Thompson, 1980).

ISSR and IRAP markers from various literature sources were chosen for DNA genotyping (Arzate-Fernandez et al., 2005; Jawdat et al., 2010; Krishna Parvathaneni et al., 2011; Yuying et al., 2011; Senkova et al., 2013; Suprun et al., 2014). A total of 32 ISSR markers and 13 IRAP markers were used. The markers were tested for the applicability to genotyping samples of the genus *Rosa*. The PCR schedule was as follows: predenaturation at 95 °C for 3 min; 35 cycles: denaturation at 95 °C for 35 s, annealing of primers at 50 °C (55 °C in case of IRAP markers) for 1 min, elongation at 72 °C for 1.5 min; postextension at 72 °C for 5 min. Concentrations of reagents in the PCR mixture: 2.5 µl of 10-fold buffer for Taq DNA polymerase (Sibenzyme, Russia), 0.5 or 2.5 µl of dNTP (2.5 mM), 1 unit of Taq DNA polymerase, 2 µl of primer (3.75 mM) and 40–50 ng of total DNA in the total volume of 25 µl. Electrophoresis of PCR products was performed at 100 V in 2.5 % agarose gel stained with ethidium bromide (2 % agarose gel was used for testing markers at 120 V). DNA was visualized under ultraviolet illumination.

On the base of the genotyping results, a binary matrix was constructed for further use of data in statistical processing programs. For statistical processing of the results of ISSR and IRAP genotyping and analysis of genetic relationships of the studied gene pool, the program PAST version 2.17c (UPGMA and PCoA analysis) was used. Structure 2.3.4 (Bayesian analysis) was used to evaluate the genetic structure of the series. Various values of hypothetical populations from K = 2 to K = 7 (burn-in period = 200,000; 500,000 iterations) were used in the calculation.

Results

The main criterion for choosing DNA markers was the quality of fingerprints of the tested markers on the rose genotypes (for testing markers, DNA of the varieties 'La France' and

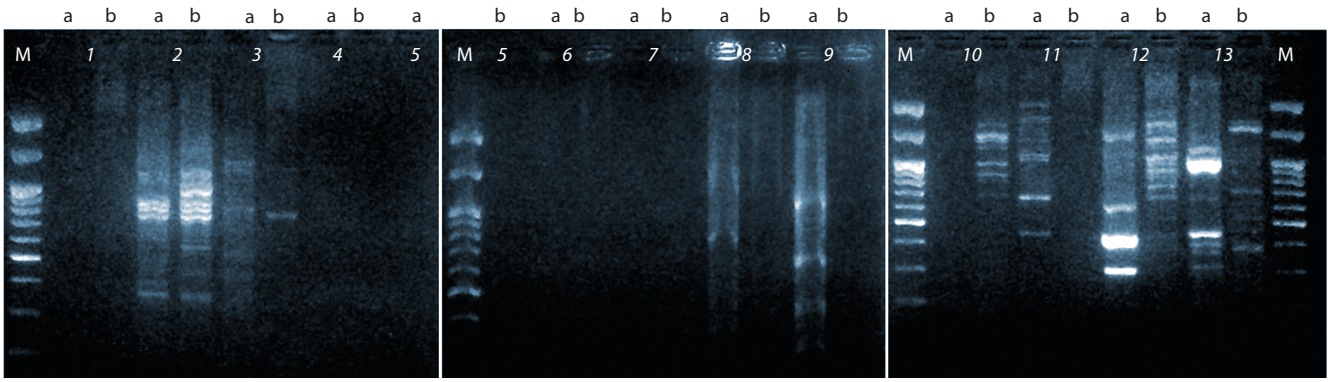

Fig. 1. DNA fingerprints of varieties (a) 'La France' and (b) 'Kronenbourg' tested with IRAP markers.
Lane pairs: *1*, TDK 1F; *2*, TDK 2R; *3*, TDK 2F; *4*, TDK 12F; *5*, TDK 12R; *6*, TDK 13F; *7*, MET 2F; *8*, MET 2R; *9*, BARE 1; *10*, LTR 3; *11*, LTR 15; *12*, Cass1; *13*, Cass2; M, DNA ladder.

'Kronenbourg' was used). The spectra of amplified fragments from the studied markers are shown in Fig. 1.

Markers with the best fingerprints were selected for further work. The quality criteria for fingerprints included the number of DNA fragments, their clarity, and brightness in the electrophoretic image. This is necessary for reliable evaluation of genotyping results. Five ISSR (UBC 824, ASSR29, 3A21, UBC 864, and UBC 843), and three IRAP (TDK 2R, Cass1, and Cass2) markers were chosen for genotyping.

A series of 26 genotypes of representatives of the *Rosa* genus was analyzed with 5 ISSR and 3 IRAP markers (Table 2). For various markers, the ranges of polymorphic alleles varied from 12 to 31 fragments, 19.25 fragments per marker on the average. The UBC 864 marker had the largest number of polymorphic fragments (31), and significant numbers of polymorphic fragments were also found with TDK 2R and ASSR29. Two markers from the set (UBC 864 and UBC 843) gave unique fingerprints for each accession. The UBC 864 marker also had the largest number of unique fragments identified in a single instance in one of the accessions of the sample. Based on the results of genotyping, a binary matrix was constructed for further use of data in statistical processing programs.

Eight markers used allowed us to obtain 153 polymorphic DNA fragments from a series of 26 accessions. This number is sufficient for phylogenetic analysis. Application of the method of principal coordinates (PCoA) to all accessions identified a separate group, including cultivated varieties of roses (Fig. 2). It should be noted that the species *R. bengalensis* was considered varietal. Among the rose species, the farthest position is occupied by the genotype of *R. roxburghii*. The species *R. hugonis* and *R. foetida* are located at a distance from the bulk of genotypes in the series. The species most closely related to domestic varieties are *R. bracteata*, *R. multiflora*, and *R. indica*. However, *R. indica* is located separately with regard to the two above-listed species, and its position is closer to domestic forms.

The Bayesian analysis of the results of genotyping accessions with K values ranging from 2 to 7 was performed using Structure 2.3.4 program. At K = 2, accessions of *R. multiflora*, *R. foetida*, *R. hugonis*, *R. indica*, *R. bracteata*, and *R. roxburghii* were allocated to a separate group. The second group

Table 2. Characteristics of the chosen markers

Marker	Polymorphous fragments	F_{ed}*	Number of unique genotypes
UBC 824	12	3	21
ASSR29	21	3	25
3A21	17	4	18
UBC 864	31	9	26
UBC 843	18	0	26
TDK 2R	21	0	25
Cass1	17	6	22
Cass2	16	5	22

* F_{ed} – the number of unique fragments detected in only one genotype.

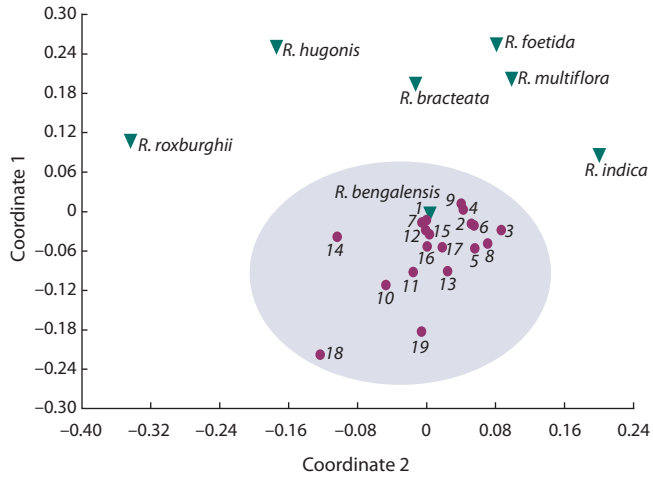

Fig. 2. The results of PCoA analysis for studied rose accessions.
Dots represent rose cultivars, inverted triangles indicate species accessions. *1*, 'Korallovyi Syurpriz'; *2*, 'Miskhor'; *3*, 'Gloria Dei'; *4*, 'Ayu-Dag'; *5*, 'Kronenbourg'; *6*, 'Flamingo'; *7*, 'Klimentina'; *8*, 'Prekrasnaya Tavrida'; *9*, 'Yves Piaget'; *10*, 'Chatyr-Dag'; *11*, 'Chrysler Imperial'; *12*, 'Prince de Monaco'; *13*, 'Mechta'; *14*, 'Queen Elizabeth'; *15*, 'Rouletii'; *16*, 'La France'; *17*, 'Traviata'; *18*, 'Alisa'; *19*, 'Kordes Sondermeldung'.

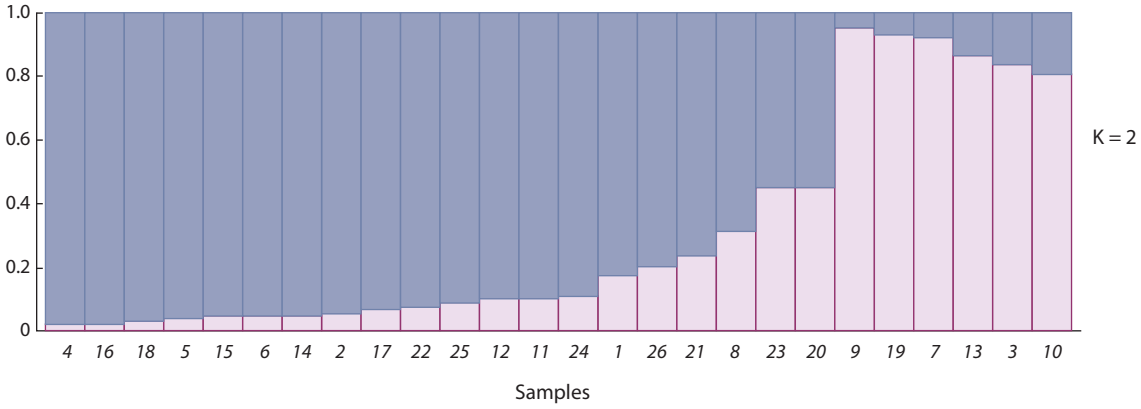

Fig. 3. Bar plots of Bayesian analysis in Structure 2.3.4.

1, 'Korallovyi Syurpriz'; *2*, 'Miskhor'; *3*, *R. multiflora*; *4*, 'Gloria Dei'; *5*, 'Ayu-Dag'; *6*, 'Kronenbourg'; *7*, *R. foetida*; *8*, 'Flamingo'; *9*, *R. hugonis*; *10*, *R. indica*; *11*, 'Klimentina'; *12*, 'Prekrasnaya Tavrida'; *13*, *R. bracteata*; *14*, 'Yves Piaget'; *15*, 'Chatyr-Dag'; *16*, 'Chrysler Imperial'; *17*, 'Prince de Monaco'; *18*, 'Mechta'; *19*, *R. roxburghii*; *20*, 'Queen Elizabeth'; *21*, 'Rouletii'; *22*, 'La France'; *23*, *R. bengalensis*; *24*, 'Traviata'; *25*, 'Alisa'; *26*, 'Kordes Sondermeldung'.

was formed by domestic varieties (Fig. 3). The accessions of *R. bengalensis*, 'Queen Elizabeth', and 'Flamingo' occupied an intermediate position between these groups. At further increase of K, the trend towards this distribution persists. Within these groups, differentiation by this method is poorly visible. Thus, the method allowed us to reliably divide the series into two major groups: wild rose species and domestic forms.

The results of clustering by the UPGMA method revealed patterns in the distribution of the studied genotypes, which were also noted when using the PCoA analysis (Fig. 4). In general, the clades of the constructed dendrogram show low bootstrap values. The most remote genotype is *R. roxburghii*. Two genotypes representing the species *R. hugonis* and *R. foetida* form a separate cluster. The next cluster is represented by *R. bracteata* and *R. multiflora*. The species *R. indica* occupies a separate position relative to other genotypes. All species accessions except for *R. bengalensis* occupy an external position relative to the cluster that includes cultivated forms of roses. Among the rose varieties, the most remote from the total mass of genotypes are 'Queen Elizabeth' and 'Flamingo'. Also, a remote position is occupied by the group represented by varieties 'Kordes Sondermeldung' and 'Alisa'. Other varieties can be divided into 4 clusters: (1) 'Rouletii', 'La France', 'Traviata', and *R. bengalensis*; (2) 'Korallovyi Syurpriz', 'Ayu-Dag', 'Gloria Dei', 'Kronenbourg', and 'Miskhor'; (3) 'Chrysler Imperial', 'Chatyr-Dag', 'Prince de Monaco', 'Yves Piaget', and 'Mechta'; and (4) 'Klimentina' and 'Prekrasnaya Tavrida'.

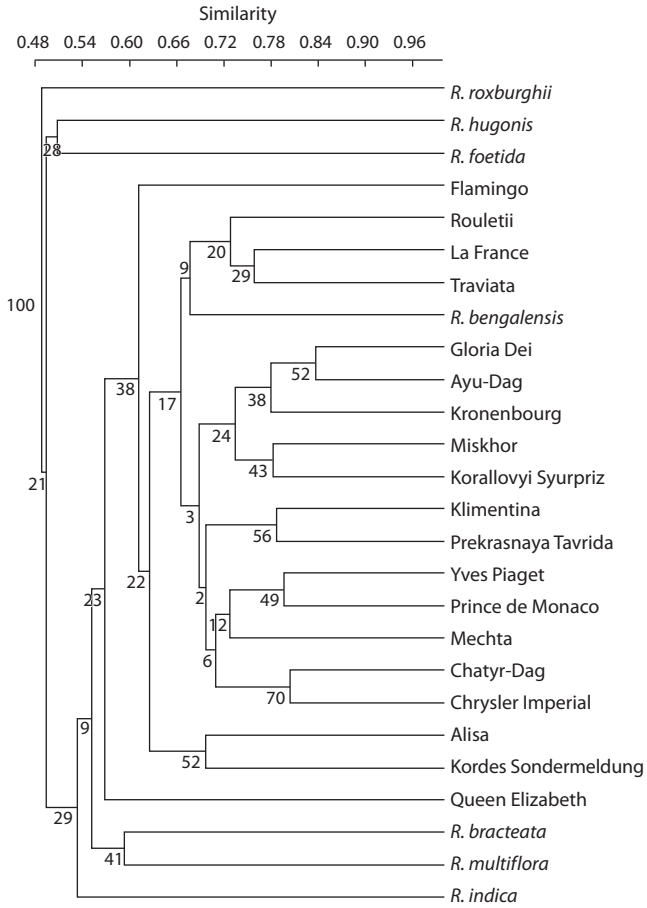

Fig. 4. UPGMA clustering results.

Discussion

In this work, we evaluated the genetic relationship of varieties from the gene pool collection of roses of the Nikita Botanical Gardens. The interpretation of the sample distribution in clustering using various methods is described below.

The Bayesian analysis allows us to determine the genetic contribution of ancestral forms for each genotype studied. Since most rose varieties have a hybrid origin, the analysis of the genetic relationship of samples found that diploid species are clearly separated from cultivated varieties, without making a significant contribution to the gene pool of the studied varieties. However, two varieties have a minor contribution of wild species 'Flamingo' and 'Queen Elizabeth', in its turn, the genotype of *R. bengalensis* occupies an intermediate position between the groups of wild species and cultivated varieties. This distribution stems from the origin of cultivated roses from few wild rose species, with one of these species being *R. bengalensis*.

The isolation of domestic rose forms in clustering can be clearly shown by other methods, such as PCoA and UPGMA. However, in contrast to Bayesian analysis, the other two methods provide a more detailed picture. The PCoA method differentiates wild species by their distance from domestic forms. Similar data on the distribution of samples were obtained by clustering using the UPGMA method. In turn, the low confidence values of the dendrogram clade may be due to the complex hybrid origin of both species and varietal accessions (Bruneau et al., 2007). Therefore, the UPGMA results can be further interpreted when compared with similar data obtained by other methods.

Summarizing the data on the distribution of species samples and cultivated varieties of roses, we can make certain inferences. The phylogenetic data obtained from the results of IRAP and ISSR genotyping are consistent with the information about the systematic position of the studied accessions. The species most distant from the cultivated forms is *R. roxburghii*, representing the subgenus *Platyrhodon* of the genus *Rosa*, and the other species and domestic varieties belong to the subgenus *Rosa* of the same genus. This distribution agrees with taxonomy data. However, the results of a number of molecular studies on the phylogeny of the genus *Rosa* do not distinguish the species *R. roxburghii* from the group of species of the subgenus *Rosa* (Wissemann, Ritz, 2005; Koopman et al., 2008; Fougère-Danezan et al., 2015). Within the *Rosa* subgenus, two species of the section *Pimpinellifoliae* occupy a separate position. Studies conducted on chloroplast DNA markers confirm the proximity of these species (Wissemann, Ritz, 2005). This section of the *Rosa* subgenus is probably the least close to the cultivated varieties in the series. The types of *R. multiflora*, *R. bracteata*, *R. indica* form the closest clusters with varietal accessions, and their contribution to the formation of the domestic gene pool of roses is not ruled out. *Rosa bengalensis* forms a common genetic group with domestic varieties. The contribution of this species to the formation of cultivars is beyond question. It should also be noted that the genetic unity of cultivars indicates the generality of their gene pool.

The analysis of genetic relationships among rose varieties from the results of IRAP and ISSR genotyping was based on PCoA and UPGMA clustering data. Comparing the data of these two methods, one can identify the most reliable groups of varieties that are consistently detected by both methods. The first group is 'Rouletii' and 'La France'. Both varieties are of Western European origin, and their relationship is not obvious. The second group is 'Prince De Monaco', 'Yves Piaget', 'Chatyr-Dag', and 'Chrysler Imperial'. Two varieties from this group have 'Chrysler Imperial' among their ancestors: 'Yves Piaget' and 'Chatyr-Dag'. Whereas the relationship of the three varieties 'Chrysler Imperial', 'Yves Piaget', and 'Chatyr-Dag' can be explained by the origin of the last two from the first, their clustering into one group with 'Prince De Monaco' is difficult to explain. The third group includes 'Gloria Dei', 'Kronenbourg', 'Ayu-Dag', and 'Korallovyi Syurpriz'. There are two varieties in this group whose parents have 'Kordes Sondermeldung', namely, one variety 'Gloria Dei' and its sport (clonal mutant) 'Kronenbourg'.

Varieties such as 'Flamingo', 'Queen Elizabeth', 'Kordes Sondermeldung' and 'Alisa' are the most genetically contrasting. However, a number of varieties in the series are derived from these three, such as 'Ayu-Dag', 'Klimentina', 'Korallovyi Syurpriz' ('Kordes Sondermeldung'), 'Korallovyi Syurpriz' ('Queen Elizabeth') and 'Alisa' ('Flamingo').

Conclusions

The distribution of species accessions of roses in clusters based on their genetic relationships is consistent with the generally recognized phylogeny. It is worth to note that the accession of the species *R. bengalensis* is included in the cluster formed by cultivated varieties. This fact may point to a contribution of wild roses of Indian origin to the formation of the gene pool of modern rose varieties. In turn, the analysis of the relationship of cultivated varieties of garden roses reveals that the varieties 'Flamingo', 'Queen Elizabeth', 'Kordes Sondermeldung', and 'Alisa' stand out from the total mass of the studied varieties. The remaining rose varieties were divided into groups with the greatest genetic similarity. Most of the results of cultivar clustering were explained based on information about the pedigree of varieties, but the position of some varieties was difficult to interpret. Further research is required to determine their relationships. Thus, the markers used in this work have shown their effectiveness in the study of the genus *Rosa*.

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Identification of species in the genus *Nitraria* L. (Nitrariaceae) based on nucleotide variability of nuclear ribosomal DNA

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Abstract. Intragenomic polymorphism of ITS1 and ITS2 of nuclear ribosomal DNA sequences was analysed in 33 samples belonging to the *Nitraria* species *N. schoberi*, *N. sibirica*, and *N. komarovii*. The nucleotide variability of the ITS region was detected in the *Nitraria* species as single-nucleotide substitutions (mainly transitions) and single-nucleotide deletion. Information about the nucleotide variability of fragments is given for the first time by us. The ITS1-*5.8S*-ITS2 region contained 17 phylogenetically informative single-nucleotide polymorphisms. Eleven single-nucleotide substitutions (transitions, C/T) were detected in ITS1. The ITS2 spacer contained 273–274 bp and was more conservative. A total of 5 phylogenetically informative single-nucleotide polymorphisms (4 transitions: C/T, G/A, one transversion: G/C), one single-nucleotide deletion (T/–) were detected in ITS2. The average GC content was 61.5 %. The GC content was lower in *N. sibirica* (59.2 %) than in *N. schoberi* and *N. komarovii* (62.7 %). It has been shown that the shorter ITS2 is a suitable molecular marker separating these species, due to the low interspecific variability and simultaneous available intraspecific variability. Phylogenetic ML and BI trees constructed separately for the ITS1 and ITS2 spacers, as well as separately for the full-size ITS region and the ITS2 spacer, were congruent. The results obtained on the intraspecific differentiation of *N. sibirica* revealed two main ribotypes among the samples of this species: the main Siberian *sibirica*-ribotype and the main Kazakh *sibirica*-ribotype. Geographical features of the distribution of *N. sibirica* ribotypes, as well as the presence of significant differences between the main Siberian and Kazakh *sibirica*-ribotypes (3 single-nucleotide substitutions) indicated significant inter-population differences and taxonomic heterogeneity of *N. sibirica*. Most likely, the processes of homogenization of nuclear ribosomal DNA of *N. sibirica* samples, the origin of which is associated with hybridization and speciation, are currently continuing.

Key words: *Nitraria*; *N. schoberi*; *N. sibirica*; *N. komarovii*; genetic variability; taxonomy; molecular identification; ITS; transition.

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Идентификация видов рода *Nitraria* L. (Nitrariaceae) на основе нуклеотидной изменчивости ядерной рибосомной ДНК

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Аннотация. Проведен сравнительный анализ внутригеномного полиморфизма последовательностей внутренних транскрибируемых спейсеров ITS1 и ITS2 ядерной рибосомной ДНК у 33 образцов, принадлежащих трем видам *Nitraria* – *N. schoberi*, *N. sibirica*, *N. komarovii*. Выявлена нуклеотидная изменчивость региона ITS у изученных видов *Nitraria* в виде однонуклеотидных замен (преимущественно транзиции) и однонуклеотидной делеции. Сведения о нуклеотидной изменчивости фрагментов приводятся впервые нами. Регион ITS1-*5.8S*-ITS2 у изученных видов *Nitraria* содержит 17 филогенетически информативных однонуклеотидных замен. В межгенном спейсере ITS1 выявлено 11 однонуклеотидных замен – транзиций (C/T). Спейсер ITS2 содержит 273–274 п.н. и отличается большей консервативностью. Всего в ITS2 у изученных образцов выявлено пять филогенетически информативных однонуклеотидных замен (четыре транзиции: C/T, G/A, одна трансверсия: G/C), одна однонуклеотидная делеция (T/–). Среднее значение содержания G+C составляет 61.5 %. Величина содержания GC-состава ниже у *N. sibirica* (59.2 %), чем у *N. schoberi* и *N. komarovii* (62.7 %). В сравнении с полноразмерным фрагментом ITS, более короткий ITS2 является подходящим молекулярным маркером, дискриминирующим виды, из-за низкой межвидовой изменчивости и одновременно выраженной внутривидовой вариабельности. Филогенетические ML и BI деревья, построенные как отдельно по

спейсерам ITS1 и ITS2, так и отдельно по полноразмерному ITS-региону и спейсеру ITS2, оказались конгруэнтны. Полученные результаты по внутривидовой дифференциации *N. sibirica* позволяют выделить среди образцов этого вида два основных риботипа: основной сибирский *sibirica*-риботип и основной казахстанский *sibirica*-риботип. Географические особенности распространения риботипов *N. sibirica*, а также наличие существенных различий между основными сибирским и казахстанским *sibirica*-риботипами (три однонуклеотидные замены) свидетельствуют о существенных межпопуляционных различиях и таксономической неоднородности *N. sibirica*. Вероятнее всего, в настоящее время продолжаются процессы гомогенизации рибосомной ДНК образцов *N. sibirica*, происхождение которых связано с гибридизацией и видообразованием.
Ключевые слова: *Nitraria*; *N. schoberi*; *N. sibirica*; *N. komarovii*; генетическая изменчивость; таксономия; молекулярная идентификация; ITS; транзиция.

Introduction

The molecular approach is now becoming a common aspect of plant research at the various taxonomic levels. Non-encoded regions of internal transcribed spacers (ITS) nuclear ribosomal DNA genes are the most promising molecular markers for plant taxa identification (CBOL, 2009; Shneyer, Rodionov, 2018). Along with other DNA fragments, ITS1 and ITS2 spacers were recognized as standard DNA barcodes (Hollingsworth, 2011; Li et al., 2011; Shneyer, Rodionov, 2018). Correct identification of plant species is established in 80 % of cases using only ITS marker, which is significantly higher than the commonly used loci in plant DNA barcoding (Bolson et al., 2015). Despite the limitations of ITS region, which consist in the presence of several thousand copies of sequences at the same time, including those located on different chromosomes (Song et al., 2012; Rodionov et al., 2016), the ITS locus was recognized as the most significant in the molecular taxonomy research of closely related taxa. The high importance of the entire ITS region in plant species identification was shown, for example, for genus *Spiraea* (Polyakova et al., 2015), *Uncaria* (Zhang et al., 2015), *Artemisia* (Wang et al., 2016). The high significance of ITS2 spacer in plant species identification was also revealed (Gao et al., 2010; Ren et al., 2010; Zhang et al., 2015; Feng et al., 2016). First of all, the success of using ITS spacers is related to efficient amplification, optimal size of amplicons for sequencing, and the level of divergence acceptable for interspecies comparisons (Shneyer, 2009; Rodionov et al., 2016). The divergence of the ITS region is usually correlated with the direction and rate of morphological speciation (Shneyer, 2009; Song et al., 2012; Rodionov et al., 2016).

Species of the genus *Nitraria* L. (Nitrariaceae) are a good object for studying the mechanisms of divergence, due not only to the variability of morphological features, but also to their ancient origin. Most of these species are morphologically poorly differentiated. Phenotypically different variants are often accepted as separate species, intraspecific forms, or ecological races (Banaev et al., 2015; Kovtonyuk et al., 2019; Tomoshevich et al., 2019). Widespread and polymorphic species (*N. schoberi* L. and *N. sibirica* Pall.), which are most interesting to researchers, are often difficult to distinguish from each other, especially for herbarium specimens (Peshkova, 1996; Koropachinskii, 2016).

The taxonomy of siberian *Nitraria* species has already been tried using karyological (Muratova et al., 2013; Banaev et al., 2018), phytochemical (Banaev et al., 2015) and morphological (Banaev et al., 2017) methods, however, molecular markers have a number of undeniable advantages over them, demonstrating significant differences at the genetic level without the involvement of environmental factors. Although sequencing of DNA fragments is still an expensive method of analysis, it can be provided accurate and highly informative data on the variability of genomes.

The purpose of this study was to conduct a comparative analysis of the nucleotide variability of the ITS region and identify its significance in the taxonomy of *Nitraria*.

Materials and methods

Taxon sampling. Research specimens were collected from various locations (19 locations *N. sibirica*, 12 – *N. schoberi*, and 2 – *N. komarovii* Iljin & Lava ex Bobrov) in Russia (Altay region, Novosibirsk region, Crimea, Khakassia, Tuva), Kazakhstan, Tajikistan in 2011–2017 (Table 1). Herbarium specimens are stored in the Central Siberian Botanical Garden of the Siberian Branch of the Russian Academy of Sciences (Herbarium of the laboratory of dendrology, NSK Collection, Digital Herbarium CSBG SB RAS (http://herb.csbg.nsc.ru:8081).

DNA extraction, PCR amplification, and sequencing. Total genomic DNA was extracted from silica-dried leaf tissue using standard methods (CTAB) (Doyle J.J., Doyle J.L., 1990). The concentration and amount of DNA were evaluated in 0.8 % agarose gel, as well as on a spectrophotometer (NanoPhotometer P-Class, P-360, Implen).

The ITS sequences were amplified with primers ITS6 (5′-tcgtaacaaggtttccgtaggtga-3′) and ITS9 (5′-ccgcttatt gatatgcttaaac-3′), designed for East Asian species of the tribe Spiraeeae (Potter et al., 2007) and made in company Eurogen (Moscow). A ready-made set of reagents was used for PCR (GenePak® PCR Core, Laboratory Izogen, Moscow). The PCR cycle consisted of 5 min at 95 °C, 30 cycles of 1 min at 94 °C, 50 s at 58 °C, 1 min at 72 °C, and 5 min at 72 °C. PCR products were examined by electrophoresis on 1.5 % agarose gel, and the DNA fragments were subsequently extracted from the ethidium bromide-stained gel and purified using Diatom DNA Elution Kit (Laboratory Izogen, Moscow). ITS fragments were sequenced in the forward and reverse directions (Eurogen, Moscow).

Nucleotide sequence and phylogenetic analyses. The nucleotide sequences of the ITS region of all *Nitraria* specimens were aligned pairwise with BioEdit v.7.1.9

Table 1. Single-nucleotide polymorphisms in ITS2 in *Nitraria* species

Species, specimen	Origin	Ribotype	Position with variable nucleotide					
			33	81	140	158	207	217
N. schoberi Krim	Crimea	H1	G	C	A	G	C	–
N. schoberi Kaspii	Kazakhstan	H1	G	C	A	G	C	–
N. schoberi Kulunda	Altai	H1	G	C	A	G	C	–
N. schoberi Malinovoe		H1	G	C	A	G	C	–
N. schoberi Bagan	Novosibirsk region	H1	G	C	A	G	C	–
N. schoberi Koktal	Kazakhstan	H1	G	C	A	G	C	–
N. schoberi Balhash		H1	G	C	A	G	C	–
N. schoberi Aidarli		H1	G	C	A	G	C	–
N. schoberi Raz'ezd 47		H1	G	C	A	G	C	–
N. schoberi Pyandzh	Tajikistan	H1	G	C	A	G	C	–
N. schoberi Sariozek	Kazakhstan	H1	G	C	A	G	C	–
N. schoberi Lepsi*		H6	G	C	A	G	C	–
N. komarovii Balhash 1		H1	G	C	A	G	C	–
N. komarovii Balhash 2		H1	G	C	A	G	C	–
N. sibirica Bele	Khakassia	H2	A	C	A	G	T	T
N. sibirica Kulunda	Altai	H2	A	C	A	G	T	T
N. sibirica Rubtsovsk		H2	A	C	A	G	T	T
N. sibirica Noven'koe		H2	A	C	A	G	T	T
N. sibirica Veseloyarsk		H2	A	C	A	G	T	T
N. sibirica Balansor		H2	A	C	A	G	T	T
N. sibirica Kuchuk		H2	A	C	A	G	T	T
N. sibirica Shara-Nur	Tuva	H2	A	C	A	G	T	T
N. sibirica Koktal	Kazakhstan	H2	A	C	A	G	T	T
N. sibirica Karatal		H3	A	T	G	C	T	T
N. sibirica Balhash		H3	A	T	G	C	T	T
N. sibirica Kurgan		H3	A	T	G	C	T	T
N. sibirica Kainar		H3	A	T	G	C	T	T
N. sibirica Basshi		H3	A	T	G	C	T	T
N. sibirica Dzhira	Altai	H4	A	C	G	G	T	T
N. sibirica Gornyak		H4	A	C	G	G	T	T
N. sibirica Bahar	Kazakhstan	H4	A	C	G	G	T	T
N. sibirica Raz'ezd 47		H5	A	T	G	G	T	T
N. sibirica Kurti**		H7	A	T	G	C	T	T

Note. * The sample has a singleton at position 71; ** the sample has a singleton at position 201. A dash (–) is a single-nucleotide deletion.

(Hall, 1999). Multiple alignments were performed in the ClustalW2 program with subsequent verification of ambiguous positions on chromatograms and manual editing. The nucleotide composition in the ITS region, the analysis of aligned sequences, selection of the nucleotide substitutions model, and evolutionary constructions were generated using MEGA X software (Kumar et al., 2018) based on the Bayesian information criterion BIC by jModelTest v.2.1.7 (Guindon, Gascuel, 2003; Darriba et al., 2012). The evolutionary distances were obtained by the Maximum Likelihood analytical method (ML) using the 3-parameter Tamura model (Tamura, 1992). Branch support was

Table 2. The GC content (%) of the ITS region in *Nitraria* species

Species	ITS1		ITS2		ITS		G+C average value
	G	C	G	C	G	C	
N. schoberi	24.8	39.3	31.7	31.7	27.8	32.9	62.7
N. komarovii	24.8	39.3	31.7	31.7	27.8	32.9	62.7
N. sibirica	24.8	31.7	31.4	31.4	27.6	30.7	59.2

estimated with 1000 bootstrap replicates in ML analyses (Felsenstein, 1985). Evolutionary constructions are also performed using MrBayes (Bayesian inference, BI) version 3.2.6 (Ronquist, Huelsenbeck, 2003; Ronquist et al., 2012) based on the substitution model – GTR (General Time Reversible) with a gamma distribution to approximate the rate of nucleotide replacement. The Markov chain Monte Carlo (MCMC) algorithm was set to run four chains simultaneously for ten million generations with a sampling of trees every 1000 generations. BI trees were visualized in FigTree version 1.4.3. *Peganum harmala* L. was used as the outgroup (GenBank NCBI: KX282320), closely related to the genus *Nitraria*. The boundaries of the ITS2 spacer are determined by comparing the ITS sequences obtained with the same fragments deposited in GenBank NCBI (*N. schoberi*: KP087771.1; *N. sibirica*: DQ267178.1).

Results and discussion

The dataset used in this study included 33 specimens, belonging to 3 species *Nitraria* – *N. schoberi*, *N. sibirica*, *N. komarovii*. The ITS region of *Nitraria* was studied to solve phylogenetic problems (Temirbayeva, Zhang, 2015); however, information about the nucleotide variability of these fragments is given for the first time by us. The total of 577 bp of the rDNA ITS region (ITS1-*5.8S*-ITS2) was composed of 558 conservative sites, 17 – potentially parsimony informative sites (all of them are single-nucleotide substitutions/polymorphisms) and 2 singletons.

Eleven single-nucleotide substitutions, which are transitions (C/T), were detected in the intergenic spacer ITS1. The gene *5.8S* consisted of 157 bp and was conservative, as expected. The intergenic spacer ITS2 contained 273–274 bp and was more conservative than ITS1. The ITS2 dataset comprised 5 parsimony informative sites (4 transitions: 2 – C/T, 2 – G/A; one transversion: G/C), one single-nucleotide deletion/insertion (T/–), 2 singletons (see Table 1). The GC content of the ITS region was 61.5 % and ranged from 59.2 to 62.7 % (Table 2). The GC content was lower in *N. sibirica* (59.2 %), than in *N. schoberi* and *N. komarovii* (62.7 %).

All the transitions in ITS1 clearly separated *N. sibirica* from the species *N. schoberi* and *N. komarovii*, while no differences were found between *N. schoberi* and *N. komarovii*, and no intraspecific polymorphism was observed in this part of the studied samples genome. In the ITS2 spacer, both species-specific polymorphisms that distinguish *N. sibirica* from the other two species were identified, as well as intraspecific variability of *N. sibirica* specimens.

It is known that the ITS2 spacer is offered as a DNA barcode for plant identification (Feng et al., 2016). Compared to the full-size region of ITS, the shorter fragment of ITS2 is a suitable molecular marker that distinguished the studied species, due to low interspecific variability and at the same time expressed intraspecific variability. The results showed that the intergenic spacer ITS2 was different between *N. sibirica* and *N. schoberi*, as well as between *N. sibirica* and *N. komarovii* by 6 positions (5 single-nucleotide polymorphisms and one single-nucleotide deletion/insertion). For the ITS sequence data set, *p*-distance value was 0.092 between *N. schoberi* и *N. sibirica*, what is comparable to well-distinguished species. For example, the average *p*-distance value calculated for ITS data set *Dendrobium* species and sections ranged from 0.069 to 0.112 (Srikulnath et al., 2015). Interspecific differences in the complex of phenolic compounds were also identified for *N. sibirica* and *N. schoberi* (Banaev et al., 2015) and species specificity of metric and qualitative morphological features was shown (Banaev et al., 2017).

Phylogenetic trees constructed separately for the ITS1 and ITS2 spacers, as well as separately for the full-size ITS region and the ITS2 spacer, were congruent. The ML and BI phylogenetic trees have branches with high bootstraps and are consistent with the morphology and taxonomy of the *Nitraria* genus. At the same time, during the study of the phylogeny of the *Nitraria* based on the analysis of combined data of ITS sequences and fragments of chloroplast DNA (6 genes) (Temirbayeva, Zhang, 2015) the species *N. schoberi*, *N. sibirica* and *N. komarovii* were grouped in one clade together with the Australian species *N. billardieri* DC., while *N. komarovii*, *N. billardieri* and *N. sibirica* were more closely located.

A comparison of the topologies of ML and BI trees (Fig. 1, 2) showed the similarity of *N. schoberi* and *N. komarovii* and the complex intraspecific differentiation of *N. sibirica*.

The species *N. schoberi* and *N. komarovii* with the same ITS sequences formed one separate clade and, accordingly, one ribotype – H1 (see Table 1). The exception was a sample of *N. schoberi* Lepsi from Kazakhstan, characterized by the presence of a singleton in position 71 of the spacer ITS2 (see Table 1).

The specimens of *N. sibirica* were grouped into two subclades on the ML phylogenetic tree (see Fig. 1), and

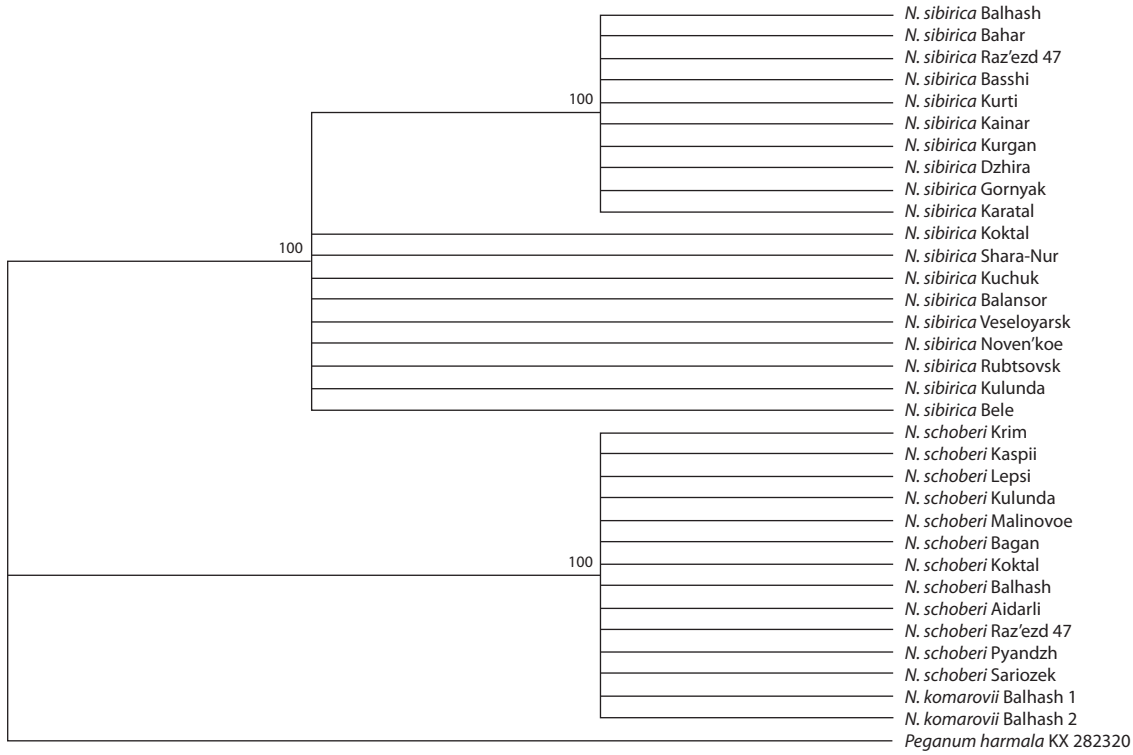

Fig. 1. Phylogenetic tree based on a comparison of the sequences of the ITS2 spacer for *Nitraria* samples using the maximum likelihood method.

The branches indicate the name of the species and the place of collection of the investigated sample.

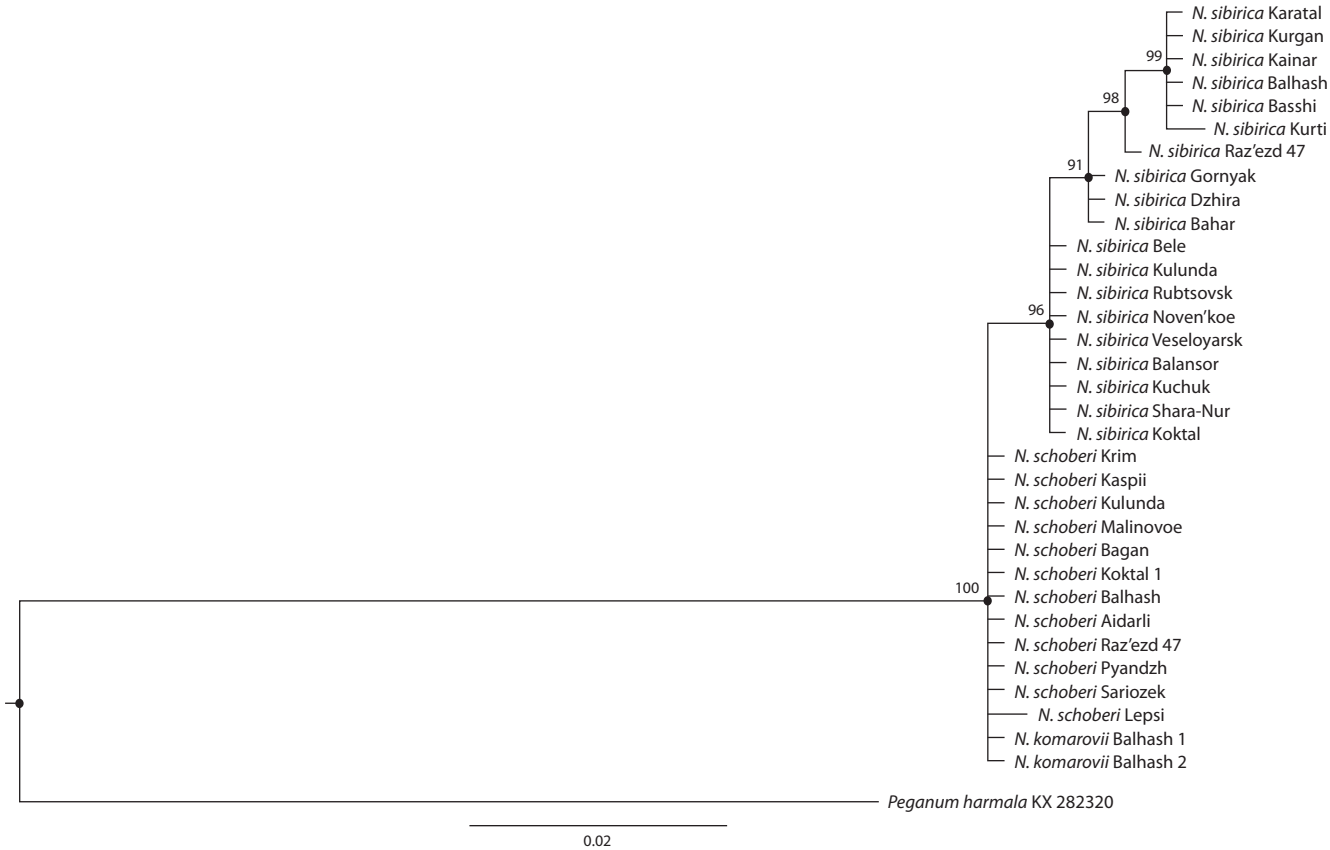

Fig. 2. Phylogenetic tree based on a comparison of the sequences of the ITS2 spacer for the *Nitraria* samples using the Bayesian (BI) method.

The branches indicate the name of the species and the place of collection of the investigated sample.

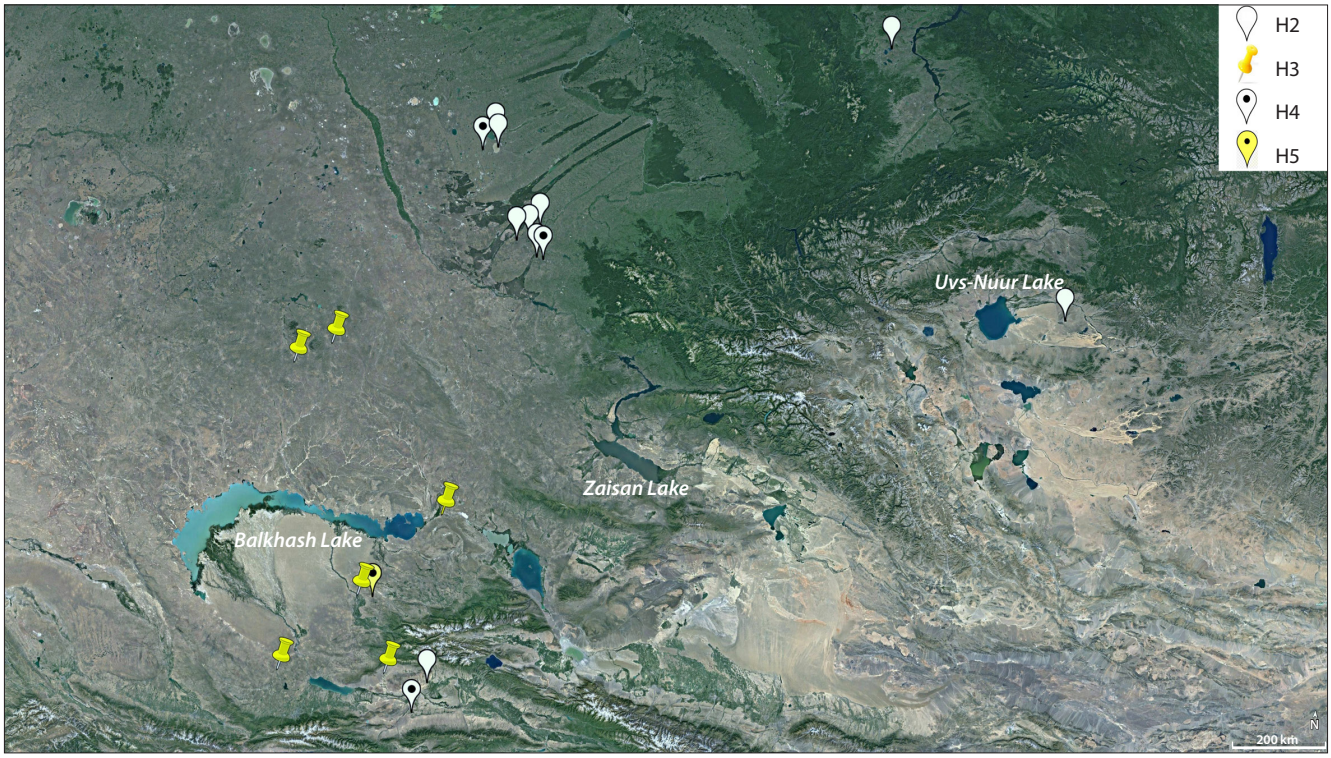

Fig. 3. Spatial distribution of ITS ribotypes of *N. sibirica* (H2, H3, H4, H5).

three subclades – on the BI tree (see Fig. 2). One of the ribotypes (H3) of *N. sibirica* differed in six single-nucleotide substitutions from the *N. schoberi* and *N. komarovii*, which indicates an independent taxonomic rank of these populations. The average intergroup genetic distance, which was 0.024 and was the same for both the H1/H2 and H1/H4 groups, confirmed the same. Ribotypes H2, H3, H4 belonging to *N. sibirica* differed by 1–3 single-nucleotide substitutions. Each of the H5, H6, and H7 ribotypes had one-point mutation (substitution).

As a result of our research on the intraspecific differentiation of *N. sibirica* the samples were divided into two main ribotypes: the main Siberian *sibirica*-ribotype (H2) and the main Kazakh *sibirica*-ribotype (H3) (Fig. 3).

The H2 ribotype was common in the Siberian populations of *N. sibirica* – the Altai territory (Kulundin steppe), Khakassia, and Tuva. The H4 ribotype, which differed in one single-nucleotide substitution from the main Siberian *sibirica*-ribotype, was also common in populations growing mainly in Kulunda, excluding two populations of *N. sibirica* from South-Eastern Kazakhstan on the border with China – Koktal and Bahar, where the Siberian *sibirica*-ribotype (H2) and the H4 ribotype close to it were found.

The main Kazakh *sibirica*-ribotype (H3) was distributed in the Ili-Balkhash region (Ili, Karatal, Ayaguz river basins) and the Kazakh shallow-water area. Ribotypes H5 and H7, close to the H3 ribotype, were also found in the distribution region of the main Kazakh *sibirica*-ribotype.

We noted significant inter-population differences and taxonomic heterogeneity of *N. sibirica* due to geographical distribution of *N. sibirica* ribotypes, as well as significant differences between the main Siberian and main Kazakh *sibirica*-ribotypes (3 single-nucleotide substitutions). Most likely, the processes of homogenization of nuclear ribosomal DNA of *N. sibirica* samples, whose origin is associated with hybridization and speciation (Rauscher et al., 2003; Xu et al., 2017; Efimova et al., 2019), are currently continuing. Previously, it was shown that the populations of *N. sibirica* were heterogeneous and differentiated into separate groups according to ecological and geographical features and the gradient of height above sea level by a complex of phenolic compounds (Banaev et al., 2015).

Conclusion

The obtained results of comparative analysis of the nucleotide variability of the ITS region demonstrated the reliability of the ITS2 spacer as a molecular genetic marker in the identification of *Nitraria* species. In the case of complex morphological identification of *Nitraria* samples, a genetic analysis of the variability of the short ITS2 spacer could be sufficient. However, it should be noted that the ITS region may not always fully resolve all taxonomic issues. Thus, in our study, the species *N. schoberi* and *N. komarovii* had identical its sequences. In addition, difficulties in interpreting the obtained sequence data set could be related to multiple copies of ITS, which are paralogs or orthologs. Answers to further questions related to the taxonomy and evolution of *Nitraria* species can be obtained by identifying these homologues, cloning its fragments, and using additional genetic markers of the chloroplast genome. In

addition, the identified species-specific genetic polymorphisms in the ITS region in the studied *Nitraria* species will allow further selection of restriction enzymes and thus simplify and reduce the cost of obtaining patterns of genetic variability of closely related taxa *Nitraria*.

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Priority trends and prospects of blackberry breeding in conditions of Central Russia

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Abstract. This overview substantiates the possibility and expediency of blackberry breeding in Central Russia, where it is in demand, but not widespread in horticulture. Significant achievements of world breeding, which gave modern cultivars a large set of economically important qualities and growing interest in it all over the world, including Russian gardeners, make it relevant to work with blackberries as an object of selection, and as a promising garden plant. However, insufficient frost and winter hardiness of the bulk of the cultivars of this culture cause certain difficulties when growing it in the areas with cold winters to which the Central zone of Russia belongs. The expansion of the market of berry products also imposes increasingly high requirements on the complex of economic indicators of new cultivars, primarily the quality of blackberry fruit. In this regard, improving the existing range of varieties of the culture, increasing its adaptive properties and commodity qualities of berries are urgent tasks for breeders when creating new cultivars. The relevance of blackberry breeding is also dictated by the fact that in Russia its domestic range of varieties is represented by only one modern cultivar obtained in the southern region and adapted, first of all, to it. For the Central zone of the country, the cultivars of this plant have not been developed (except for the limited experiments of I.V. Michurin conducted almost 100 years ago). Therefore, the breeding of adapted cultivars of the culture in the climatic conditions of this region may be promising. It is also possible to grow here (with shelter for the winter) the cultivars already created abroad that can give with the right agricultural technology a good industrial harvest, which is confirmed by the practice of amateur and farm gardening, as well as scientific research. The purpose of this work is to designate the leading directions of blackberry breeding, the most important in the conditions of Central Russia and to show prospects of the development of new cultivars of this valuable culture in the specified climatic zone. The analysis of world trends and experience in the blackberry breeding and variety study, as well as the results of our own research of the culture conducted in the Orel region, allow us to consider it promising and relevant to work on improving the range of varieties of this plant in Central Russia. All priority areas of blackberry breeding, indicated in foreign and domestic breeding programs (winter hardiness, high quality of fresh and processed fruit, the correct shape of berries, their large size, the necessary values of biochemical composition, high productivity of plants, thornless shoots and high resistance to diseases and pests), are relevant for this region of our country, while high winter hardiness is currently the most important of them.

Key words: blackberries; breeding, trends and priorities of breeding; Central Russia.

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Приоритетные направления и перспективы селекции ежевики в условиях средней полосы России

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Аннотация. В обзоре обосновывается возможность и целесообразность селекции ежевики в центральной части России, где она является востребованной, но малораспространенной в садоводстве ягодной культурой. Значительные достижения мировой селекции, давшие современным сортам большой набор хозяйственно важных качеств, растущий интерес к культуре во всем мире, в том числе у российских садоводов, делают актуальной работу с ежевикой и как с объектом селекции, и как с перспективным садовым растением. Однако недостаточные морозо- и зимостойкость основной массы сортов этой культуры создают определенные трудности при выращивании ее в зонах с холодными зимами, к которым относится средняя полоса России. Расширение рынка ягодной продукции тоже предъявляет все более высокие требования к комплексу хозяйственных показателей новых сортов, в первую очередь к качеству плодов ежевики. В связи с этим улучшение имеющегося сортимента культуры, повышение его адаптивных свойств и товарных

качеств ягод – насущные задачи для селекционеров при создании новых сортов. Актуальность селекции диктуется также тем, что в России отечественный сортимент ежевики представлен всего одним сортом – Агатовая, полученным в южном регионе и адаптированным прежде всего к нему. Для центральной же зоны страны сорта не создавались (за исключением ограниченных опытов И.В. Мичурина, проведенных около ста лет назад). Поэтому выведение адаптированных сортов ежевики в климатических условиях этого региона может оказаться перспективным. Не исключается также возможность выращивания здесь (при укрытии на зиму) уже созданных за рубежом сортов, которые могут давать при правильной агротехнике хороший промышленный урожай, что подтверждает практика любительского и фермерского садоводства, а также выполненные научные исследования. Цель данной работы – на основании анализа результатов зарубежных и отечественных исследований обозначить ведущие направления селекции ежевики, важнейшие в условиях средней полосы России, показать перспективность создания новых сортов этой ценной культуры в указанной климатической зоне. Анализ мировых тенденций и опыта в селекции и сортоизучении ежевики, а также результаты собственных исследований культуры, проведенных в условиях Орловской области, позволяют считать перспективной и целесообразной работу по совершенствованию сортимента этого растения в средней полосе России. Все приоритетные направления селекции ежевики, обозначенные в зарубежных и отечественных селекционных программах (зимостойкость, высокое качество плодов в свежем и переработанном виде, правильная форма ягод, крупный их размер, необходимые значения биохимического состава, высокая продуктивность растений, бесшипность побегов, высокая устойчивость к болезням и вредителям), актуальны и для данного региона нашей страны; при этом важнейшим направлением является в настоящее время создание сортов с высокой зимостойкостью.
Ключевые слова: ежевика; селекция; направления и приоритеты селекции; средняя полоса России.

Introduction

In recent years, more and more attention has been paid to berry crops that are not widely distributed in Russia, such as actinidia, blueberries, blackberries, honeysuckle, viburnum, sea buckthorn and others, which can significantly expand the range of berry products and enrich the diet of the population with useful substances, while representing considerable commercial interest. Their fruits are a rich source of valuable substances for the human body (micro- and macronutrients, vitamins), including antioxidant action. The content of these substances in them is often higher than that of traditionally grown in most regions – strawberries, raspberries, black currants, etc., or is at the same level (Sedov, Gruner, 2014), which confirms the importance of actively involving cultivars of such plants in the practice of gardening.

Among the crops of this group, blackberries are of considerable interest at present, which may eventually occupy a worthy niche in Russian berry growing, as has happened in a number of countries around the world (Strik et al., 2008; Strik, Finn, 2012), where it is among the leading berry plants (Clark, Finn, 2011, 2014; Finn, Clark, 2012).

Blackberries bear fruit after most other berry crops, significantly extending the pipeline of vitamin products in the growing regions. Its fruits contain a significant amount of important biologically active substances of the antioxidant complex (Gruner, Anikeyenko, 1995; Connor et al., 2005; Kolbas et al., 2012; Milošević et al., 2012; Lee, 2017), which are involved in many processes of human metabolism (Kolbas et al., 2012). Their number is: from 500 to 900 mg/100 g of P-active substances-flavonoids (including 17 to 30 mg/100 g of ellagic acid and 85–390 mg/100 g of ellagotanins), from 10 to 50 mg/100 g of ascorbic acid, about 0.6 mg/100 g of carotenoids. In addition, blackberries contain 5 to 14 % sugars (mainly glucose and fructose), 1.3 % organic acids, as well as a significant number of important mineral macro- and microelements (in terms of dry weight) – phosphorus (up to 254 mg/100 g), calcium (about 283 mg/100 g), magnesium (up to 315 mg/100 g), iron (up to 11 mg/100 g), etc. Pleasant taste and delicate aroma of the large black fruit in combination with the specified components of the chemical composition make this culture attractive to gardeners and numerous berry consumers in different regions of our country. The demand for and at the same time the shortage of blackberries in the market of garden products in Russia, including the middle zone, is indirectly evidenced by the high prices for its fruits and planting material of cultivars.

Improved over the years of breeding productivity indicators (up to 20 t/ha) (Clark et al., 2019), high self-fertility (Gruner, 2019) and resistance of many modern cultivars to the most dangerous diseases and pests (Clark, Finn, 2011; Finn, Clark, 2012), good recovery ability after various injuries (Gruner, 2019), relative ease of vegetative reproduction (Podorozhny, Romanova, 2010; Knyazev et al., 2012), responsiveness to bush formation (Clark, Finn, 2011; Takeda et al., 2013), new thorn-free cultivars allow growing blackberries at present without any problems. A certain vulnerability of this plastic garden crop is associated with reduced winter hardiness of almost all its cultivars in the regions with cold and long winters, especially with unstable snow cover, as in the middle zone of Russia (Yakimov, 2010; Evdokimenko, Kulagina, 2015; Gruner, 2019). Therefore, the introduction of blackberries in these climatic conditions is associated with corresponding difficulties, which can be overcome in two ways: selection and technological, as well as their combination.

Blackberry breeding in the world has passed a long historical path (Darrow, 1937; Shoemaker, 1958; Ourecky, 1981; Vitkovskiy, 2003; Clark, Finn, 2011; Finn, Clark,

2012), which resulted in numerous (several hundred) highly productive cultivars (Ourecky, 1981; Yakimov, 2010; Clark et al., 2012; Finn, Clark, 2012). Modern blackberry assortment with a complex of valuable properties allows using it as a source material for further selection, improving individual qualities, or adding additional ones without losing the rest. This makes plant breeding promising, including in the conditions of the center of our country, where there are not as many competing crops that produce high-quality fruits as in the South. Encouraging in terms of promoting blackberries to the North are the positive results already obtained on its selection for winter hardiness in the United States and some European countries (Danek, Kolodziejczak, 1993; Stanisavljevic, 1999; Danek, Orzeł, 2004; Clark et al., 2012; Clark, 2013; Orzeł et al., 2016).

Orel region, where this work is carried out, has a combination of climatic factors of the growing season (Agroclimatic Reference Book…, 1960), in most cases favorable for the growth, development and fruiting of blackberries (Gruner, 2019). The success of breeding blackberries, as well as other fruit and berry crops, in any climate zone is determined, first of all, by the availability of good source material and the correct choice of priority areas, which should be based on the already achieved world results and correspond to them.

1 Taxonomic affiliation, the most important species for breeding and some morphobiological features of blackberries

Blackberries are included in the genus *Rubus* L., subgenus *Eubatus* Focke (= *Rubus* Watson), which, according to various authors, has 132 species in the world's flora (Focke, 1910) to 200 or more species (Rozanova, 1937; Vitkovskiy, 2003) and a significant number of interspecies forms. The complexity of the systematics of wild blackberries was mentioned by the famous researcher of this plant, S.V. Yuzepchuk (1941). Within this subgenus, natural polyploidy is widespread, represented by a polyploid series of forms that have sets of chromosomes from $2x$ ($2n = 14$) to $12x$ ($2n = 84$) with the main number $x = 7$, including aneuploids of different ploidy levels. At the same time, such series can also be within some species (Rozanova, 1937; Ourecky, 1981; Clark, Finn, 2011). The size of blackberry chromosomes is 1–4 μm (Clark, Finn, 2011). In its cultivated cultivars, polyploid rows also occur, with a predominance of tetraploids (Thompson, 1995; Clark, Finn, 2011).

Wild species of North America and Europe became the originators of most cultivars of the *Eubatus* subgenus (Rozanova, 1937; Vitkovskiy, 2003). The most significant in the creation of blackberry cultivars were such North American species as: *R. allegheniensis* Porter, *R. argutus* Link. and *R. canadensis* L. (which gave rise to the first erected and most hardy cultivars, such as Agawam, Jumbo, Lawton, Snyder, Erie, etc.), *R. ursinus* Cham. & Schlecht., *R. macropetalus* Dougl., and *R. loganobaccus* Bailey (the most important species from which the best trailing cultivars of blackberries and raspberry-blackberry hybrids have been obtained: 'Logan', 'Young', 'Boysen', etc.), *R. laciniatus* Willd. (well-known 'Thornless Evergreen' was obtained from its thorn-free chimera), *R. trivialis* L. (one of the parent forms of the erected drought-resistant cultivar Brazos, which was actively used in creating modern cultivars), *R. ulmifolius* Schott. (a tetraploid source of the recessive thornless gene, 'Merton Thornless' was derived from it), (Darrow, 1937; Clark, Finn, 2011), and others. It is usually impossible or very difficult to determine the species identity of modern cultivars, since their genomes usually contain the genoplasm of several species and their hybrid descendants, as well as cultivars of different origin, which in turn is complicated by polyploidy and related heterozygosity of the culture (Rozanova, 1937; Ourecky, 1981; Clark, Finn, 2011).

Blackberry flowers are usually bisexual, but there are also dioecious species (for example, *R. ursinus* in the United States). Self-pollination is good, but cross-pollination is also successful. The color of the corolla varies from pure white to bright pink. The stamens and pistils are numerous. The fruit is a compound drupe, with a color from dark cherry or blue to almost black, sometimes with a glaucous coating, when maturing, separating from the calyx along with the edible fruit. The number of drupes in it can vary from a few pieces in wild species and forms (for example, *R. caesius* L.) to one and a half hundred in modern cultivars (as in 'Natchez'). Set of drupes is usually high, often provided by facultative apomixis (pseudogamy), characteristic of polyploid forms of the *Eubatus* subgenus in various types of pollination (Ourecky, 1981). Flowers and fruits are collected in racemes containing them from several pieces to several dozen.

In Russia, the largest number of wild species is distributed in the Caucasus (about 40), but in the conditions of the middle zone of our country, only two species prevail: *R. caesius* L. with trailing shoots and *R. nessensis* W. Hall with erected canes (Yuzepchuk, 1941; Grossgeim, 1952).

The blackberry plant is a semi-shrub with a long-term underground part (branched rhizome with branching roots and buds from which shoots grow) and above-ground, consisting of two types of stems: generative biennial and vegetative shoots of the current year (annual). After the crop matures, the stems of the second year of life die off. There are also primocane forms that lay generative organs and bear fruit on the shoots of the current year.

There are 3–4 main morphological groups of blackberries that differ in the nature of growth and way of natural vegetative reproduction (Finn, Strik, 2014; Gruner, 2014). The group of erected blackberries includes cultivars and forms that have vertically growing shoots and restrained early ending growth (until the beginning of August in the middle zone of Russia). The natural way of vegetative reproduction in them is with root offshoots (as in red raspberries *R. idaeus* L.). Trailing blackberry plants have a protracted

growth, long (up to 3–8 m) shoots of various thickness, which by the end of the growing season slow down the growth, but do not stop, and in the upper zone form a section of rhizogenesis with reduced leaves, rooting under favorable conditions. Representatives of semi-erect and semi-trailing blackberries complete or significantly slow down the growth after erect, but earlier trailing forms (in the middle zone of Russia, it is the beginning of September). Given that the last two groups are similar to each other, in foreign literature they are usually combined, calling them "semierect" i. e. semi-straight growing (Finn, Strik, 2014).

2 State of blackberry breeding in Russia

In our country, blackberry breeding is now at the initial stage of its path, despite the abundance of wild species and forms, especially in the southern regions, where they could have long served as a source material for selection, but aroused interest mainly as botanical resources (Yuzepchuk, 1941; Grossgeim, 1952). At the beginning of the last century, this plant, as an object of selection, seriously drew the attention of a well-known breeder and experienced gardener – I.V. Michurin (1949), who, as a result of sowing seeds from free pollination of American cultivars, selected several promising seedlings, which later became cultivars and received recognition in amateur gardening. But in the future, these cultivars were preserved mainly in the collections of scientific institutions, as the plants had thorned shoots, low winter hardiness and were affected by diseases ('Izobilnaya', Tekhas).

Major domestic works on blackberries and their cultivars, created by that time in the world, were published in 1930s (Bologovskaya, 1934; Rozanova, 1937). Later, translated foreign publications with detailed information about this culture were published (Shoemaker, 1958; Ourecky, 1981; etc.). Collections of wild species and some foreign cultivars in the 1950s–70s and subsequent years were collected at Maikop (main collection) and Pavlovskaya (single samples) experimental stations of VIR (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) in order to study and select the most promising of them for breeding. N.L. Neronova (1973), a participant of several scientific expeditions to the Caucasus in order to replenish the species collection of blackberries of MOS VIR (Maikop Experimental Station of the N.I. Vavilov All-Russian Institute of Plant Genetic Resources), wrote about the great potential value of wild Caucasian blackberry species, including for introduction into culture in this zone.

Several works performed in the southern zone of horticulture at the end of the last and beginning of this century (Gruner, 1992; Semyonova, Dobrenkov, 2001; Zakharova, 2002) using the gene pool of the Maikop OS VIR culture were devoted to the study of the biological characteristics and economic value of blackberries and the selection of genetic sources for breeding. By this time, a collection of blackberries has been also formed on the Crimean OSS VIR (Crimean Experimental Selection Station of the N.I. Vavilov All-Russian Institute of Plant Genetic Resources), where targeted selection of this culture (the collection gene pool of which numbered 25 introduced cultivars during the study period) has been carried out since 2003 (Podorozhny, 2016), culminating in 2016 with the inclusion of the first and only modern domestic cultivar – 'Agatovaya' in the State Register of the Russian Federation (State Register…, 2019). The priority areas of blackberry breeding in this institution are both improving the indicators of adaptability to climate factors in the southern region (including drought and heat resistance), and improving other characteristics identified as leading in domestic and foreign breeding programs, such as berry quality, productivity, thornlessness, etc. (Kichina et al., 1995; Clark, Finn, 2011; Podorozhny, 2016).

The most obvious reasons for the long absence of breeding research on blackberries in Russia were, first, the abundance of other valuable berry crops distributed in different regions and adapted to them, second, a large variety of wild-growing species of this plant in the southern climate zone, which gave the local population a sufficient number of berries, and third, the strong thornness of intensively growing shoots, both in wild-growing species and in old foreign cultivars, which made blackberries perceived more as a weed and inconvenient for cultivation, and, in-fourth – low winter hardiness of its aboveground part, which did not allow to count on stable berry yields.

With the introduction of a whole series of modern foreign blackberry cultivars to our country over the past decade and a half, the situation in crop breeding may change significantly, since the level of most economic indicators is very high and makes it possible to choose the best genotypes for crosses. Collections of the latest generations of foreign blackberry cultivars are already available and are being studied in a number of Research Institutes, including in the Central part of Russia: VNIISPK (Russian Research Institute of Fruit Crop Breeding, Orel region) – 28 cultivars, Bryansk SAU – about 10 cultivars (private message of Evdokimenko S.N.), I.V. Michurin Federal Research Center (Tambov region) – about 30 cultivars (private message of Gurieva I.V.).

3 Blackberry breeding abroad, priorities and achievements

For more than 170 years, blackberries have been selected in the United States (Darrow, 1937; Shoemaker, 1958; Ourecky, 1981; Vitkovskiy, 2003; Clark, Finn, 2011; Finn, Clark, 2012), where a large variety of wild species of this plant, which gave rise to many cultivars, is also concentrated. Some of the first cultivars appeared spontaneously and were accidentally discovered in private gardens and wild populations, serving as a base and incentive for subsequent selection search (Darrow, 1937; Clark, Finn, 2011). A big breakthrough in blackberry breeding was the development of the thornless 'Merton Thornless' cultivar in England (John Innes Horticultural Institute program), from which

the recessive gene of thornlessness was transmitted to many modern tetraploid ($2n = 28$) cultivars (erect and semi-erect). Two other donors of genes of dominant thornlessness, S_f and S_{fl}, 'Austin Thornless' ($2n = 56$) and 'Lincoln Logan' ($2n = 42$) were used in the creation of trailing thornless cultivars (for more information, see section 5.4).

To date, US scientists are recognized as world leaders in creating and improving the assortment of blackberries. Their research gave an impetus to the development of culture breeding in other countries. The main contribution to the creation of new cultivars of this plant was made by scientists of the world's leading breeding institutions, such as: USDA-ARS Beltsville St. Maryland (Dr. D. Scott, Dr. D.P. Ink) – USA; Arkansas University (Dr. J. Moore, Dr. J. Clark) – USA; United States Department of Agriculture ARS St. Oregon (Dr. Ch. Finn, Dr. B. Strik) – USA; Scottish Crops Research Institute (Dr. D. Jennings) – Great Britain; Fruit Experiment Station of the Research Institute of Pomology and Floriculture (Dr. J. Danek, Dr. A. Orzel) – Poland; New Zealand Institute for Plant and Food Research (Dr. H. Hall) – New Zealand; Fruit Research Institute, Čačak (Dr. M. Stanisavljevič) – Serbia and others.

All modern breeding programs are aimed at further improving the assortment of blackberries by leading economic characteristics. The analysis of major reviews on blackberry breeding (Ourecky, 1981; Clark, Finn, 2008, 2011; Finn, Clark, 2012) shows that the priority directions in the world breeding programs are currently: the quality and size of fruits, productivity, plant adaptability to climate factors, thornlessness, optimal architecture of shoots, fruiting on canes of the current year, resistance to diseases and pests. In other words, the range of problems to be solved remains quite wide, since the requirements for cultivars are constantly increasing, while the genetic potential of the crop identified by many years of research (Clark, Finn, 2011; Finn, Clark, 2012) allows to count on the success of their solution.

It should be noted that the first place is given to the quality of fruits associated with increased market requirements. The emphasis in the selection of adaptability to low winter temperatures in the United States, where for a long time there was active selection for this trait in combination with the straightness of shoots, partly shifted to another valuable direction, also in the future able to largely solve the problem of winter hardiness – the creation of primocane cultivars of blackberries (Clark, Finn, 2011; Clark et al., 2012; Finn, Clark, 2012; Clark, 2013, 2014) (for more information, see section 5.2).

The main results of the long-term and tedious work on the blackberry breeding were high-yielding cultivars with a complex of valuable characteristics. New breeding achievements have aroused great interest in the culture around the world, the level of production of its berries has grown significantly over the past decades and continues to grow (Strike et al., 2008; Strike, Finn, 2012; Clark, Finn, 2014).

The breeders managed to increase the winter hardiness in combination with the plant thornlessness only to a certain level (Stanisavljevic, 1999; Clark, Finn, 2011; Orzeł, 2016; Telepenko, 2018), which is still insufficient for regions with long frosty periods in winter, such as those in the middle zone of Russia (Evdokimenko, Kulagina, 2015; Gruner, 2019). Therefore, along with the appearance of industrial cultivars, the best climatic zones for growing blackberries were determined, and technologies for its cultivation began to be worked out, including those that provide winter shelter for shoots (Takeda, Handley, 2006; Takeda et al., 2013; Mettler, Hatterman-Valenti, 2018). As a result, the leading producers of blackberries in the world today are the United States, Mexico, China, Serbia, Hungary, New Zealand, and in general – about 30 countries (Strik et al., 2008), where climate conditions allow it.

4 Factors limiting blackberry cultivation in the Central part of Russia (on the example of the Orel region)

Orel region, where our blackberry research is conducted, is located in a zone of moderate continental climate, generally favorable for gardening (Agroclimatic Reference Book…, 1960). The temperature of the coldest month (January) is –9…–10 °C. The absolute minimum air temperature for the long-term period is –39.9 °C in the region (2012, according to the VNIISPK weather station). According to the average annual data, frosts stop in the region in the second five days of May (possible fluctuations in the timing of frosts – from the first decade of April to the first decade of June). The average dates of autumn frosts are at the end of September (the earliest start of frosts was observed in the first decade of September, and the latest was in the third decade of October). The snow cover reaches maximum height from mid-February to mid-March. Its average height is 20–25 cm.

During our research, according to VNIISPK meteorological observations, significant decreases in winter temperatures (corresponding to regional minimums) were observed in late November 2014 (–20 °C), in mid-December 2016 (–20.6 °C), in the first decade of January 2015 (–24.5 °C), in the second decade of January 2016 (–29.3 °C), in early February 2017 (–31.5 °C), in late February (–26.0 °C) and at the end of the third decade of March 2018 (–21.6 °C). At the same time, in January, February and March in some years there were long (up to two or more weeks) periods of round-the-clock negative temperatures (with lows at –10…–15 °C) (Gruner, 2019).

The limit of frost resistance of the main mass of blackberry cultivars are considered –10…–20 °C, and only for some of them –25…–30 °C (Gruner, 1986; Takeda, Handley, 2006; Wojcik-Seliga, Wojcik-Gront, 2013; Telepenko, 2018, etc.). It is established both experimentally, using artificial freezing of cuttings, and by field observations, which are the most accepted when evaluating breeding seedlings (Kichina et al., 1995; Kazakov et al., 1999; Clark, Finn,

2011). In any case, the specified limits of blackberry resistance to frost are not sufficient for the region of our research, and this is the main obstacle to the introduction of the crop in this climate zone. Nevertheless, there is already a positive experience of fairly large blackberry plantations (4–7 ha) in the South of the Central zone of Russia – in the Voronezh region: farm "Sladunika" (URL: https://sladunika.ru) and "Divny Sad" (URL: https://divnyi-sad.ru). However, even there they usually require winter shelter, with the exception of certain cultivars that are not frozen even in the harshest winters, such as the Polish cultivar 'Orkan' and the old American cultivar 'Agawam' (private message of the head of the farm "Divny Sad" – A.N. Prodan).

In addition to the lack of winter hardiness of the main assortment, the factors that hinder the cultivation of blackberries in the middle zone of the country and other regions with cold winters (–20 °C and below), can be attributed to the related need for shelter of plants (a labor-intensive and costly event), as well as the diversity of cultivar habit, requiring an individual approach to each of them when growing; mandatory use of trellis due to high productivity and long shoots; and sometimes insufficient knowledge of the biology of the plant, resulting in certain errors in agrotechnics.

5 Results of blackberry cultivar study in the center of Russia, current breeding trends, genetic sources of valuable traits

Purposeful selection evaluation of cultivated blackberries in the climatic conditions of Central Russia has not been carried out since the creation of the first cultivars by I.V. Michurin (Michurin, 1949). In connection with the increased interest of the population in cultivars of this plant, their biology and cultivation techniques, the appearance of new selection products at the market, we conducted research on the variety study of blackberries in experimental plantings of the Department of berry breeding of VNIISPK in 2014–2018 (Orel, Russia). To evaluate the collection and selection material, we used generally accepted research methods (Kichina et al., 1995; Kazakov et al., 1999).

The cycle of works performed in these years to study the adaptive and other economic qualities of representatives of the main morphological groups of blackberries (erect, trailing and intermediate between them) has shown that this valuable berry crop can be successfully grown here under certain conditions (first of all using winter shelter) and give high yields of berries. This requires trellis and variety-specific preparation of plants for winter (Gruner et al., 2018). It is important to select cultivars according to the maturation period, since adverse weather conditions during the growing season of individual years can lead to non-maturation of part of the crop of the most late cultivars, prolonged growth of shoots of the current year and, as a result, their significant freezing in winter (Gruner, 2019). It has been found that, as a rule, all blackberry phenophases occur at a favorable time for the culture and fit into the growing season of the region (Gruner, Kuleshova, 2014). It is revealed that among the studied and involved in the collection new blackberry genotypes (for the latest information prior) there are cultivars ('Agawam', 'Erie', 'Thornfree', 'Brzezina', 'Ouachita', 'Loch Tay', 'Natchez', 'Chester', etc.) and selected forms (seedlings of 'Cheyenne', 'Black Satin', 'Loch Ness', and hybrid form 'Thornfree' × *R. caucasicus* I, etc.) that allow to count on successful selection for improvement as indicators of adaptability, i. e. hardiness and drought resistance (Gruner, 2019) and other economically important traits, including weight, taste and other qualities of the berries (Clark, Moore, 2005, 2008; Yakimov, 2010).

Practical tips and positive forecasts for growing blackberries in the more severe conditions of the Samara region, information about the numerous cultivars of the world assortment of this culture were reflected in the book written by V.V. Yakimov, a connoisseur of this culture (Yakimov, 2010), based on his own experience and a review of foreign literature sources.

The authors of the study of blackberry winter hardiness without winter shelter in the Bryansk region rightly believe that it is impractical to grow blackberries in this zone without such shelter due to the freezing of most cultivars to the level of snow, but some of the cultivars studied by them stand out ('Thornfree',' Black Satin', 'Gazda', 'Orkan', 'Marion' and 'Smoothstem') as promising for household and industrial use in the region (Evdokimenko, Kulagina, 2015). Current trends in blackberry breeding in Russia were formulated earlier (Kichina et al., 1995) and supplemented with respect to the Central zone in this article. Of these, in the first place is breeding for winter hardiness and necessary qualities of blackberry plants associated with it. Let's focus on this direction and some others in more detail.

5.1 Breeding for winter hardiness

The creation of hardy blackberry cultivars was important for the United States due to the significant freezing of most *Eubatus* representatives in almost all regions of this country (Finn, Clark, 2012). The work of North American scientists to create erect genotypes originally obtained from wild species with such a habit – *R. allegheniensis*, *R. argutus* and others was valuable in the breeding of relatively frost- and winter-hardy blackberry cultivars. The first cultivars with the genoplasm of these species were thorny, characterizing by increased frost resistance and restrained growth: 'Agawam', 'Lawton', 'Snyder', 'Erie', 'Darrow', and others (Darrow, 1937; Clark, Finn, 2011). Winter hardiness of such cultivars is associated with the early completion of growth processes; they are also characterized by the depth and duration of organic dormancy. As a result of the long-term breeding, thorny erect 'Illini Hardy' and thornless semi-trailing 'Chester Thornless' are among the most winter-hardy cultivars (Yakimov, 2010; Finn, Clark, 2012). However, over time, US breeders have recognized that climatic conditions with regular winter temperature

drops to certain critical values are necessary for effective selection of genotypes with higher winter hardiness (Clark, Finn, 2011; Finn, Clark, 2012).

The latest generations of erect blackberries ('Navaho', 'Arapaho', 'Natchez', 'Ouachita', 'Caddo', and others, received at Arkansas University, USA) (Andersen, 2001; Clark, Moore, 2005, 2008; Clark, Finn, 2011; Clark et al., 2019), have thornless canes and improved winter hardiness, however, they complete growth (our preliminary observations) later than thorny ancestors, which is probably connected with the involvement of thornless, but long-term vegetative genotypes and therefore moderately winter-hardy genotypes (in particular, 'Thornfree'). Therefore, in the Central zone of Russia, the winter hardiness of these cultivars is likely to be insufficient and closer to this indicator in semi-trailing forms. The morphological proximity of new erect cultivars to this intermediate group is also confirmed by their creators (Finn, Clark, 2012). At the same time, crossing such cultivars among themselves can probably lead to a certain increase in the hardiness of hybrids, due to the saturation with the genoplasm of the hardy ancestors of their parents, in particular, 'Darrow', which was used in the creation of most of them (Clark, Moore, 2005, 2008; Clark et al., 2019). Among the blackberry cultivars, checked by us in the Central zone of Russia in winter, without shelter, only the old American erect tetraploid ($2n = 28$) cultivar 'Agawam' can be grown here, however, when using it in breeding, one should take into account the dominant thornness of shoots and the formation of a large number of root offshoots, which are absent in modern thornfree cultivars with erect habit.

Breeding for increased winter hardiness of *Eubatus* representatives is successfully carried out in Poland (Fruit Experiment Station of the Research Institute of Pomology and Floriculture), where a number of cultivars were obtained that do not require shelter in the climatic conditions of this country (Danek, Kolodziejczak, 1993; Orzeł et al., 2016) and which, as already mentioned, in the South of Central Russia show good resistance to frost. This is, first of all, the mentioned cultivar 'Orkan', which confirmed the specified quality also in the Ukraine along with the American cultivar 'Ouachita' (Telepenko, 2018). These cultivars can be promising in the course of further breeding improvement of blackberry winter hardiness indicators, combined with the thornlessness of shoots.

For cover cultivars in the Central zone of Russia, increased winter hardiness and elasticity (flexibility) of shoots are also relevant, necessary for significant snow cover, garter to the trellis, shelter for the winter; trailing and semi-trailing habit of the bush and early ripeness. According to our research (Gruner, 2019) and preliminary observations, 'Loch Tay' (from the VNIISPK collection) and the hybrid form 'Thornfree' × *R. caucasicus* I, as well as seedlings LN-4, 13, 14 obtained from seeds from open pollination of 'Loch Ness' can be potential sources of these traits' genes. In the conditions of the region, it may be useful to study also cultivars with a typically trailing habit (at least for the purposes of amateur gardening), which are laid on the ground for winter shelter easier than others, giving preference to thorn-free genotypes (with genes S_f and S_{fl}).

5.2 Breeding of primocanes fruiting

One of the current trends that can solve the problem of winter hardiness of blackberries in the future is the creation of new primocane cultivars. Fruiting on shoots of the current year is a relatively new direction in blackberry breeding, but it is very important for expanding the production of berries both to the South and to the North (Clark, Finn, 2011). In the southern regions with warm winters, such cultivars fully bear fruit (and can even produce two crops per season) without the impact of negative temperatures, which ordinary (non-primocane) cultivars need, in particular, for normal differentiation of generative buds. When moving blackberries to the North, shoots of primocane cultivars, in the case of early (before the onset of negative temperatures) ripening of berries on an annual wood, can be cut (or mowed) after harvest, thus solving the problem of winter hardiness and resistance to diseases and pests.

The original source of the gene for this trait was discovered in 1949 in a natural population of erect diploid blackberry form ($2n = 2x = 14$), called 'Hillquist' after the name of the person who found it (L.G. Hillquist from Ashland) in the United States. From it, by breeding (crossing with the tetraploid cultivar 'Brazos' at the University of Arkansas), tetraploids were obtained (including the form Ark. 593, used in further selection), bearing the primocane trait. The recessive nature of the gene for this trait is assumed (Clark, 2008; Clark, Finn, 2011). 'Prime-Jan' and 'Prime Jim' (2003 and 2004) were the first breeding achievements of this type, Prime-Ark-45 (2009) and subsequent cultivars have improved fruit quality. The most important results in this direction were obtained at the University of Arkansas in the USA (where work on the creation of such cultivars continues). Breeding for primocanes is also conducted in Poland (Orzeł et al., 2016). In the currently created representatives of cultivars of this type of fruiting, berries can fully ripen on the shoots of the current year only in the southern zones of horticulture. Therefore, the goal of breeding in this direction, including in the center of Russia, is to provide earlier full ripening on one-year shoots and greater winter hardiness of the underground parts of plants. All other parameters of cultivars and breeding directions in the Central zone of our country correspond to the world priorities identified by leading breeders based on the long-term experience with blackberries (Clark, Finn, 2008, 2011; Finn, Clark, 2012). We give them below.

5.3 Breeding for improved fruit quality

Most modern blackberry cultivars have a set of indicators of berries that ensure their demand in the market and were formed by long-term breeding. This also applies to representatives of the group of erect and semi-erect culti-

vars, which have received a lot of attention to improving the quality of berries (Clark, Moore, 2005, 2008; Clark, Finn, 2011).

The sweet taste of fruit (provided by the amount of soluble solids, SS = 10–15 %) is considered key to expanding the market for fresh blackberry products (Clark, Finn, 2011). In addition to this indicator, acidity, aroma, and tartness are also important. Other traditional high-quality components are the density of the berry, the strength of the skin, small seeds, bright color, easy separation during harvesting, and good post-harvest safety.

When creating new cultivars, it should be taken into account that the ripening of berries is a special period when growing blackberries. On the one hand, the fruits acquire a cultivar-specific taste, gain the maximum amount of sugars, acquire juiciness and aroma, on the other hand, their transportability decreases, and a beautiful gloss is often lost. For this reason, berries are often removed at the stage of technical maturity, and they are not suitable for dessert consumption. The task of breeders is to combine high taste qualities in mature fruits (first of all, sweet taste) with the brilliance of black drupelets and the strength of the skin for successful transportation.

Post-harvest characteristics of blackberry berries include: attractiveness of appearance (preservation of black color, gloss), strength of the fruit (evaluated before and after storage), good taste, lack of the development of fungal and bacterial infections. The quality of berries after harvesting strongly depends on weather conditions during harvest, so it is practiced to grow blackberries, as well as other berry crops, in high tunnels (Finn, Clark, 2011; http://www.korolevagro.ru/teplichnye-tekhnologii/vysokie_tunneli/).

For processing, blackberries must have an intense aromatic taste and color, a high level of soluble solids and titrated acidity, low pH and low drupeletness. These characteristics must be preserved when the berries are frozen. There are significant differences between the cultivars of this crop in the content of phenolic compounds of the antioxidant complex (Connor et al., 2005), which allows for selection to improve this indicator in the offspring.

The shape of blackberry fruit can vary significantly (Gruner, 2014), which means that there are prospects for its selective improvement. It is important to equalize the shape, the fruit are not acceptable cloven or invalid. Desirable berries are rounded, conical or barrel-shaped (cylindrical). It is believed that long berries can provide the most attractive appearance of fresh fruit (Clark, Finn, 2011).

Large size of berries is one of the main tasks of blackberry breeding, and is a component of productivity in addition to the marketability indicator. However, it is considered that an excessive mass of berries (more than 10–15 g) is undesirable for processing and placement in packages, as well as for berry mixes, where such fruits will prevail. Large fruits are varieties of ‘Natchez’, ‘Triple Crown’, ‘Kiowa’, ‘Tupi’ and a number of other cultivars have large size of fruit (Yakimov, 2010), which are potential sources of genes for this trait. The weight of the berry may vary slightly within the cultivar depending on the conditions and methods of cultivation.

The productivity (yield per bush) of blackberries is usually high, but varies greatly both between cultivars and within a single cultivar, depending on climatic conditions and growing technology, and can reach 20 or more kilograms. However, an excessive crop can lead to a decrease in shoot-forming ability (Clark, Finn, 2011) and even in the frequency of fruiting. Therefore, it is necessary to manage the productivity of high-yielding cultivars with the help of timely formation of shoots, their normalization. Sources of high productivity genes are ‘Thornfree’, ‘Chester Thornless’, ‘Black Satin’, ‘Loch Ness’, ‘Triple Crown’, and many others (Yakimov, 2010).

5.4 Breeding for thornlessness

Creating new thorn-free cultivars is a popular direction of blackberry breeding. To date, at least 40 such cultivars have been created in the world, and these are valuable sources of genes for this trait for further selection. ‘Thornfree’, ‘Smoothstem’, ‘Black Satin’, ‘Loch Ness’, ‘Loch Tay’, ‘Chester Thornless’, ‘Triple Crown’, ‘Cacanska Bestrna’, ‘Orkan’, ‘Apache’, ‘Ouachita’ and many other cultivars are descendants of the tetraploid ($2n = 4x = 28$) source of the recessive gene of this trait – ‘Merton Thornless’ and can be used in breeding for thornlessness. Given that this gene is introduced into the genoplasm of relatively hardy tetraploid cultivars, they are probably the ones that should be used for breeding in the Central zone of Russia. At the early stages of ontogenesis, its carriers are identified by the absence of glandules on the germ leaves (Clark, Finn, 2011), so selection for this trait can be conducted at the very beginning of the selection process.

Two other sources of thornlessness i. e. ‘Austin Thornless’ ($2n = 8x = 56$) with the dominant S_f gene, created in England, and ‘Lincoln Logan’ ($2n = 6x = 42$) with the dominant S_{fl} gene, created in New Zealand, are used for breeding cultivars of the trailing type, usually with a higher level of ploidy than tetraploids. Plants with the S_f gene can have thorns on the basal part of the stem up to a height of 30 cm, in the zone of fruiting; the stems are devoid of spines. Therefore, the identification of thorn-free offspring in this case is possible only after the seedlings reach a height of more than 0.3 m (Clark, Finn, 2011). Descendants of ‘Austin Thornless’ are the thorn-free cultivars ‘Waldo’, ‘Black Perl’, ‘Nightfall’, and ‘Black Diamond’, created at the University of Oregon in the United States. Among the latest achievements of this institution is thorn-free ‘Wild Treasure’ created by crossing the ‘Waldo’ cultivar and the wild species *R. ursinus* (Finn et al., 2010). New thornless cultivars with the S_{fl} gene are ‘Columbia Star’, ’Columbia Sunrise’, and ‘Columbia Giant’ (Finn et al., 2014, 2018), which may be sources of this gene of thornlessness in further breeding. Such cultivars are more suitable for growing in the southern regions of horticulture in Russia,

but with a reliable shelter for the winter, they can probably be cultivated in amateur gardens in the center of Russia.

5.5 Breeding for resistance to pests and diseases

Currently, the majority of blackberry cultivars grown have a high level of resistance to harmful organisms. However, the more blackberries are grown, the more often there are reports of an increased number of cases of fungal diseases and pests, with a much higher frequency than bacteria or viruses (Clark, Finn, 2011; Finn, Clark, 2012). The main blackberry breeding for resistance to diseases and pests was carried out by simple selection of healthier or uninfected plants in the seedling population (Clark, Finn, 2011). In order to understand the degree of potential danger of various pathogens, below we provide information about the most harmful of them, which are represented to varying degrees in the regions of cultivation of the discussed crop (Clark, Finn, 2011; Finn, Clark, 2012).

The most common in the world fungal diseases of blackberries are: anthracnose (*Gloeosporium venetum* Speg.), gray rot (*Botrytis cinerea* Pers.: Fr). and leptosphaeria (*Leptosphaeria coniothyrium* (Fuckel) Sacc.). Infection with these pathogens can vary greatly depending on the type of berries, growing locations, weather, and plant care. Orange rust (*Gymnoconia peckiana* (Howe) Trott.) is sometimes seen in plantings, and removal of infected plants is required. Resistance to orange rust is found in almost all eastern (erect) cultivars of the United States, with the exception of the 'Navaho' cultivar. For many years in the southern States of the United States, the most dangerous disease was the doubling of the rosette – cercosporellosis (*Cercosporella rubi* (G. Winter) Plakidas) in the production of blackberries. However, the resistance to this disease was identified, which turned out to be associated with the presence of a recessive gene of thornlessness in the blackberry genoplasm. Therefore, cultivars derived from the source of this gene – 'Merton Thornless' cultivars, are resistant to this disease (Clark, Finn, 2011). However, with the intense infection in the US States of Mississippi and Louisiana, the resistance was not always maintained.

In some years, *Botrytis cinerea* Pers.: Fr., *Septoria rubi* Westend, *Septocyta ruborum* (Lib.) Petr. and *Didymella applanata* (Niessl) Sacc. were observed in parts of Chile, Mexico, New Zealand, and the Western United States. Also in these areas and in areas of the UK and Europe, *Peronospora sparsa* Berk. can create a serious problem. Raspberry and blackberry hybrids, such as Boysenberry and Loganberry, can also be significantly affected by this disease. Powdery mildew (*Podosphaera macularis* Wallr.) is observed in some areas of the world, especially in Mexico. With the increase in plantings in Mexico and other areas, these diseases are becoming more economically important, and it is necessary to find sources of resistance and use them in breeding.

From bacterial diseases, root cancer (*Agrobacterium tumefaciens* [E.V. Smith & Townsend] Conn.) is the most common and can lead to weakening of blackberry plants and significant loss of yield. One of the main viral diseases of the *Rubus* genus for many years was bush dwarfism caused by *Raspberry bushy dwarf virus* (*RBDV*), but it was most pronounced in raspberry species (i. e. *R. idaeus*, *R. parviflorus*, *R. spectabilis*). *Tomato ringspot virus* (*ToRSV*) and *Tobacco ringspot virus* (*TRSV*) have been identified in most regions where blackberries are produced.

All production regions have problems with blackberry pest control. The most common pests are: raspberry glassworm – *Pennisetia hylaeiformis* (Harris), goldenrod – *Agrilus ruficollis* (Fabricius), blackberry mite – *Acalitus essigi* Hassan., etc. Some production regions are heavily affected by pests that require active protection programs. For example, in New Zealand, the cultivars 'Boysenberry', 'Marion' and all other *Rubus* representatives are strongly attacked by bud moth (*Heterocrossa rubophaga* Dugdale and *Eutorna phaulacosma* Meyrick). Green vegetable beetle *Nezara viridula* L. and leafhopper species including *Epiphyas postivittana* Walker, *Planotortrix exessana* Walker etc. can also be a serious problem in New Zealand. In 2008, California first reported a spotted winged Drosophila (*Drosophila suzukii* Matsumura) native to Japan, and then it spread widely across the Western States and in China. It was recently identified in Europe. The depth of this problem has not yet been studied, but there is a danger that this pest will become the main one for blackberries. Much of the breeding effort is focused on field assessments of seedlings for pest resistance and the use of the most resistant ones in crosses (Clark, Finn, 2011; Finn, Clark, 2012).

As we can see, the main danger of diseases and pests of blackberries are for regions with a warm climate, so Central Russia may be safer for the cultivation of this crop. However, breeding for resistance to biotic environmental factors is certainly more effective with a high natural infectious background or with the use of artificial infection.

According to the observations of the authors of this article, in recent years in the Orel region, where this study is conducted, the flower-eating beetle – olenka shaggy (*Epicometis hirta* Poda) has spread, which begins to cause noticeable damage to early-flowering cultivars of blackberries (especially the most winter-hardy 'Agawam'), which makes us pay special attention to this pest and cultivars that are not affected by it, for use them in breeding.

6 Prospects of blackberry breeding in Central Russia

The great achievements of blackberry breeding in the world, the availability of new cultivars with a complex of valuable characteristics for Russian researchers, and the work carried out on the territory of Russia to study it, allow us to consider it promising to improve the assortment of this plant not only in the South, but also in the Central part of our country. The climate of this region can contribute to the effective formation of genotypes with the necessary signs of adaptability, first of all, high frost and winter hardiness,

since negative temperatures critical for the culture occur here almost every winter. At the same time, the selection process itself can be conducted in the field, without the use of artificial freezing of plants.

It is known that the majority of representatives of the subgenus *Eubatus* Focke belong to mesophytic plants by ecological affiliation. In the natural populations of the South of Russia, this is manifested in the fact that they are confined to fairly humid and often protected from the active sun habitats (Yuzepchuk, 1941; Grossgeim, 1952; Neronova, 1973; Ourecki, 1981). For most cultivars of this crop, similar conditions are also preferred. At the same time, in the southern zone, hot dry periods during the growing season of blackberries occur almost annually, often causing the dark berries to bake with a corresponding loss of commodity qualities and even the drying of generative organs (Semenova, Dobrenkov, 2001). In the conditions of the Orel region, where it is planned to conduct breeding research on blackberries, such periods occur much less often (2–4 times in 10 years). At the same time, they allow us to objectively assess the degree of drought tolerance of the crop in this region and, if necessary, to conduct selective selection for this indicator.

Among the other "advantages" of the Central zone of Russia, which are necessary for the cultivation of blackberries, we can mention a much lower potential danger of diseases and pests than in the South due to climate barriers that prevent their spread. Given the already high potential for resistance to these harmful factors, the crop may be very promising in this zone for organic farming, the relevance and necessity of which are recognized in many regions of the world (Doroshenko et al., 2012) and in Russia in particular (https://soz.bio/o-soyuze/). At the same time, certain vegetation periods in the middle zone are favorable for the development of fungal infections, so the identification of resistant blackberry genotypes in breeding research is also possible here.

Selection in other priority areas of blackberry breeding can be carried out in the region without any climatic or other restrictions.

Conclusion

By 2020, VNIISPK has studied a collection of blackberries in the amount of 28 cultivars (3 cultivars were obtained from MOS VIR, 25 – from the nurseries "Divny Sad" of the Voronezh region and "Zeleny Shum" of the Orel region) of the world assortment and 18 selected and elite breeding forms obtained by the authors of the article earlier. Breeding forms were grown from seeds from open pollination of a number of cultivars sent to VNIISPK from the US University of Arkansas and the Swedish Institute of Agricultural Sciences in 1995. Elements of the technology of cultivating the varietal and selection gene pool with the use of winter shelter have been worked out. The first crosses aimed at implementing the priorities of crop breeding in the region were carried out. An annual replenishment of the blackberry collection is planned with new genetic sources of economic and useful traits, first of all, the most winter-hardy.

The analysis of world trends and experience in blackberry breeding and cultivar study, as well as the results of own researches of the crop, carried out in conditions of the Orel region, allow us to consider a promising and feasible work on improving the assortment of this plant in Central Russia.

All the priority areas and tasks of blackberry breeding identified in foreign and domestic breeding programs are relevant for this region of our country, and the most important of them is currently the creation of blackberry cultivars with high winter hardiness.

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Содержание желтых пигментов в зерне твердой пшеницы (*Triticum durum* Desf.): биосинтез, генетический контроль, маркерная селекция

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Аннотация. Зерно с высоким содержанием каротиноидных пигментов ценится за ярко-желтый цвет пасты, производимой из него, и провитаминную (витамин А) и антиоксидантную активность пигментов. Цель настоящего обзора – обобщение современных знаний о биосинтезе и генетическом контроле накопления пигментов в зерне твердой пшеницы и оценка основных результатов исследований и селекции за последние двадцать лет за рубежом и в России. Признак «концентрация каротиноидных пигментов в зерне» (Ypc) относится к разряду количественных. Тем не менее превалирование сильных аддитивных эффектов генов и высокая наследуемость способствовали значительному прогрессу в селекции по этому признаку. Методами молекулярного маркирования локусов количественных признаков (QTL), контролирующих синтез каротиноидных пигментов и значения индекса желтизны (IY), установлено их распределение по всем хромосомам генома твердой пшеницы. Основные генетические локусы, определяющие более 60 % варьирования признака, были картированы в хромосомах 7AL и 7BL. Вклад этих локусов связан с аллельными вариациями, влияющими на активность фермента фитоенсинтетазы (PSY). В других хромосомах были локализованы минорные генетические факторы, из которых наиболее значимы QTL, расположенные в хромосомах 3AS (ассоциирован с геном LCYE-ликопин-ε-циклаза) и 4BS (аллель Lpx-B1.1c). При этом показано, что аллель Lpx-B1.1c вносит вклад в снижение активности липоксигеназы, окисляющей каротиноиды в процессе изготовления конечных продуктов. Рассмотрены и обсуждены проблемы использования молекулярных маркеров в селекционных программах, нацеленных на увеличение концентрации пигментов в зерне и улучшение цветовых характеристик пасты.
Ключевые слова: твердая пшеница; каротиноиды; концентрация пигментов; индекс желтизны; маркерная селекция.

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The content of yellow pigments in durum wheat (*Titicum durum* Desf.) grains: biosynthesis, genetic control, marker selection

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Abstract. Grain with high contents of yellow pigments will add the natural bright-yellow colour to the paste, which unlike a paste with a high level of whiteness, are preferred by consumers. The provitamin activity (vitamin A) and antioxidant activity of the carotenoid pigment increase the biological and nutritional value of the grain with high contents of these pigments. The purpose of this review is to summarize modern knowledge about the biosynthesis and genetic control of pigment accumulation in durum wheat and to assess the main results of research and selection over the past 20 years abroad and in Russia. The trait "concentration carotenoid pigment in grain" (Ypc) is quantitative. However, the prevalence of strong additive gene effects and high heritability have contributed to significant progress in breeding for this trait. Molecular labeling of quantitative trait loci (QTL) that control the synthesis of the carotenoid pigment and the yellowness index (YI) found that they are distributed across all chromosomes of the durum wheat genome. The main QTLs, which determine 60 % of the variation of the trait, were mapped to 7AL and 7BL chromosome. The contribution of these QTLs is associated with allelic variations that control the activity of phytoene synthase (PSY). QTLs with minor effects found on the remaining chromosomes are also reliably mapped using molecular markers. As confirmed in a number of experiments, most of them are QTLs located on 3AS (linked to the LCYE (lycopene ε-cyclase) allele and on 4BS (the LpxB1.1c gene). It has been shown that the LpxB1.1c allele

contributes to a decrease in the activity of lipoxygenase, which oxidases carotenoids during the production of end products. This review considered and discusses the problems of molecular markers in breeding programs to increase the concentration of pigments in the grain and improve the color characteristics of the paste.
Key words: durum wheat; carotenoids; concentration pigment; yellow index; marker-assisted selection.

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Введение

Твердая пшеница (*Triticum durum* Desf.) ежегодно выращивается во всем мире на площади около 17.0 млн га. Производство зерна колеблется по годам от 32 до 42 млн тонн. Продукты из твердой пшеницы используются почти во всех странах, но основными регионами потребления являются страны Средиземноморского бассейна, где зерно твердой пшеницы применяется в качестве сырья для различных продуктов, в основном пасты и круп кус-кус и булгур (Kabbaj et al., 2017). Содержание и структура белка (качество клейковины), а также цвет изделий (макароны, крупа) – наиболее ценные для пищевой, технологической промышленности и на рынке конечных продуктов в этих регионах (Sisson, 2008; Mazzeo et al., 2017). Желтый цвет определяется накоплением каротиноидов в эндосперме, обладающих провитаминными и антиоксидантными свойствами. В процессе размола зерна, замеса теста и изготовления макарон происходит окисление кислородом воздуха полиненасыщенных жирных кислот, каротиноидов, фенолов, что приводит к накоплению бурого (коричневого) пигмента. Окисление катализируется ферментами: липоксигеназой, полифенолоксидазой, пероксидазой, альдегидоксидазой (N'Diaye et al., 2017). Наличие спексов (темных вкраплений) также может снижать уровень желтизны семолины и макаронных изделий (Васильчук и др., 2009).

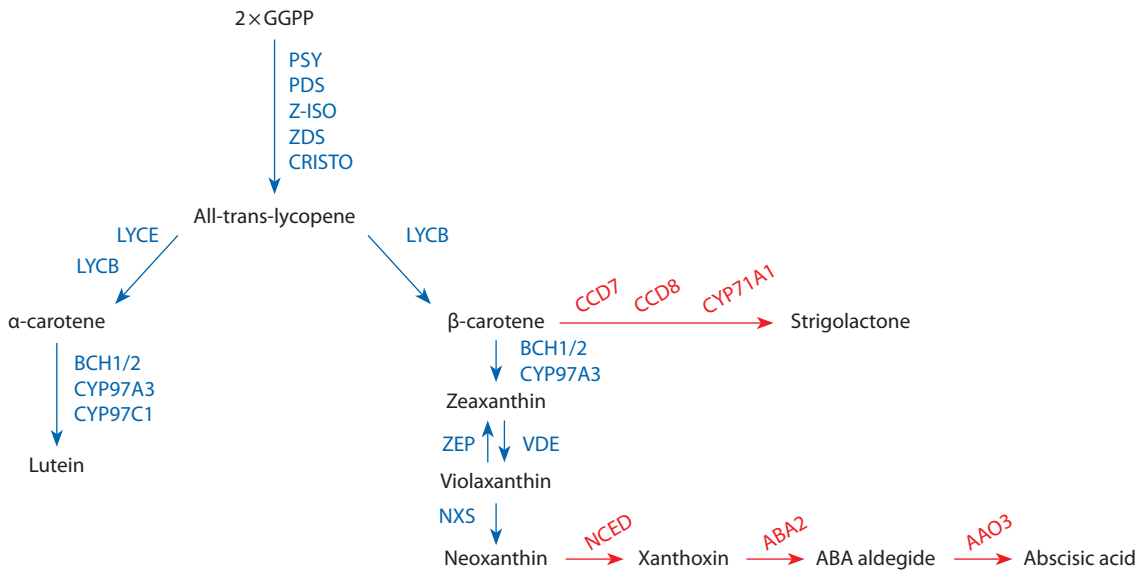
Результаты изучения наследования всего комплекса желтых пигментов в зерне твердой пшеницы показали, что оно носит количественный характер с высокими значениями коэффициента наследуемости и преобладанием аддитивных эффектов генов (Clarke et al.,1998; Borelli et al., 1999; Digesù et al., 2009; Blanco et al., 2011; Roncallo et al., 2012; Schulthess et al., 2013). В связи с этим правомерно предположение о том, что этот признак удобен для молекулярного маркирования соответствующих QTL и их картирования на хромосомах.

Биосинтез, содержание и распределение каротиноидов в зерновке твердой пшеницы

Средняя концентрация каротиноидов в зерне твердой пшеницы составляет 6.2 ± 0.13 мг/кг в сухом весе (Brandolini et al., 2015) с варьированием, в зависимости от сорта и условий среды (год, пункт), от 2.8 до 12.3 мг/кг (Colasuonno et al., 2017a). По мнению Н.С. Васильчука с коллегами (2009), в Поволжье при низкой активности окислительных ферментов достаточно иметь зерно с концентрацией 4.5 мг/кг для получения макарон золотисто-желтого цвета. Каротиноиды – не единственные желтые пигменты в зерне пшеницы и других злаков. Сравнивая общую концентрацию каротиноидов, определенную методом высокоэффективной жидкостной хромотографии (ВЭЖХ), с общим содержанием пигментов, A.M. Digesù с коллегами (2009) показали, что доля каротиноидов у культивируемых и диких тетраплоидных видов пшеницы составила 33.2 % от общего количества желтых пигментов. По результатам своих исследований A. Blanco с колегами (2011) также сообщили о доле каротиноидов, составившей 37 % в общем объеме желтых пигментов, что означает наличие в экстрактах твердой пшеницы неизвестных желтых пигментов, поглощающих свет при 435 нм. По данным (Fu et al., 2017), полученным при изучении канадских сортов твердой пшеницы Navigator и Strongfield, фенольные соединения могут вносить существенный вклад в степень желтизны при экстракции пигментов. Brandolini с коллегами (2008) пришли к выводу, что, хотя каротиноиды являются наиболее важными пигментами при определении желтого цвета пшеничной муки и макарон, их точное измерение может быть достигнуто только с помощью ВЭЖХ анализа. Тем не менее среди желтых пигментов именно по каротиноидам имеется более подробная научная информация.

К каротиноидам относятся пигменты – каротины и ксантофиллы. Химически они представляют собой изопреноидные углеводороды, содержащие 40 углеродных атомов (Кретович, 1986). Биосинтез каротиноидов тщательно исследован на различных растениях – арабидопсисе, рисе, кукурузе, перце, томатах, апельсине и других культурах (Colasuonno et al., 2017a; Rodrigues-Concepcion et al., 2018; Sun et al., 2018).

В зерне твердой пшеницы представлен широкий набор каротиноидных пигментов: лютеин, β-каротин, зеаксантин, β-криптоксантин, β-апокаротенал, антраксантин, тараксантин (лютеин-5,6-эпоксид), авоксантин и тритикоксантин. Каротины α и β в основном находятся в зародыше, превалирующий в зерне среди каротиноидов лютеин (86–94 %) одинаково распределен по слоям и частям зерновки (Digesù et al., 2009). Его доля в семолине при размоле зерна твердой пшеницы составляет 83 %, во фракциях отрубей – 75 % (Fu et al., 2017). Установлено, что характер распределения желтых пигментов по фракциям, выделяемым в процессе размола, варьирует, в зависимости от генотипа. Концентрация лютеина в эндосперме сорта Navigator была выше, чем в отрубях, в то же время у сорта Strongfield наблюдалась обратная закономерность (Fu et al., 2017). В процессе помола и получения крупки (семолины) концентрация большинства каротиноидных пигментов уменьшается, что связано с увеличением контакта измельченных частиц зерна с кислородом воздуха и активностью ферментов. Лютеин и зеаксантин имеют более высокую стабильность при помоле и изготовлении конечных продуктов, по сравнению с другими каротиноидами (Kean et al., 2011).



Biochemical reactions of the carotenoid pathway (Colasuonno et al., 2019).

The main components of the biosynthetic pathway are shown in black; all enzymes involved in carotenoid synthesis, in blue; enzymes of the dioxygenase group involved in carotenoid metabolism in growing plants under stress and accumulating hormone-like ingredients – abscisic acid and strigolactones – in red.

Накопление каротинов в зерне, особенно β-каротина, обуславливает изменение интенсивности окраски семолины от желто-оранжевого до красноватого оттенка. Ксантофиллы обеспечивают желто-оранжевую окраску крупки и макарон. Схема биосинтеза каротиноидов показана на рисунке. Исходным веществом для биохимического синтеза каротинов служит 5-углеродный (C-5) изопреноид – изопентилпирофосфат. Конденсация этого изопреноида представляет собой основу для образования геранилгеранилпирофосфата (C-20). В результате соединения двух молекул геранилгеранилпирофосфата при участии фермента фитоенсинтетазы образуется фитоен (C-40) – первое промежуточное вещество в биосинтезе каротинов. Этот этап является ключевым – скорость биосинтеза и накопления фитоена влияет на весь пул каротиноидов (Cazzonelli, Pogson, 2010; Ke et al., 2019). Фитоен в результате десатурации под действием ферментов фитоендесатуразы (PDS), z-каротиндесатуразы (ZDS), каротинизомеразы (CRTISO) и последовательного удаления четырех атомов водорода превращается в ликопин. Ликопин – каротиноид, определяющий красную и оранжевую окраску плодов, – исходное вещество для синтеза α-каротина/лютеина (класс ксантофиллов) – главного каротиноида зерна твердой пшеницы и β-каротина/зеаксантина (класс ксантофиллов) – главного компонента каротиноидных пигментов в зерне кукурузы (Zhang, Dubcovsky, 2008). Дальнейшее гидроксилирование α-каротина приводит к образованию желтого зейоксантина и лютеина. Трансформация β-каротина продуцирует образование β-криптоксантина, зеаксантина, антраксантина, виолаксантина и неоксантина. Эти реакции катализируются двумя негемовыми β-каротингидроксилазами (BCH1 и BCH2) и двумя гемгидроксилазами (CYP97A и CYP97C) соответственно (Sun et al., 2018). Последняя фаза биосинтеза каротиноидов, катализируемая неоксантиноксидазой (NXS), заключается в превращении виолаксантина в неоксантин. Окисление виолаксантина и неоксантина приводит к образованию ксантоксина, превращаемого в растительный гормон – абсцизовую кислоту (ABA), которая способствует регулярному и сбалансированному накоплению пигментов в растениях и формированию устойчивости к абиотическим стрессам (Al-Babilli, Bowmeester, 2015; Nisar et al., 2015). Еще одна ветвь трансформации β-каротина представляет превращение его под действием ферментов диоксигеназной группы (CCD7, CCD8, CYP711A1) в стриголактоны – ингредиенты гормональной природы, регулирующие развитие и ростовые процессы растений (Colasuonno et al., 2019).

Методы определения общего и компонентного состава каротиноидных пигментов в зерне

Содержание пигментов в зерне, крупке и макаронах относится к сложным признакам. Для общего и компонентного определения их содержания существует несколько мотодов. Эталонные методы для определения общего содержания каротиноидов – стандартный метод 152 (ICC Method 152, 1990) Международной ассоциации науки и техники о зерне (ICC) и международный официальный метод (AACC 14-50.01 (AACC International, 2013). Эти две процедуры основаны на экстракции всех пигментов в водонасыщенном н-бутаноле с последующим спектрофотометрическим количественным определением оптической плотности спиртового экстракта при 435.5 нм (длина волны максимального поглощения лютеина, доминирующего каротиноида в твердой пшенице). В качестве альтернативы концентрации для характеристики насыщенности пигментами зерна применяется индекс желтизны (YI) на основе количественного определения коэффициента отражения света. Хроматограф Minolta CR-300 (Konica

Minolta Pty Ltd, Macquarie Park, NSW), оснащенный импульсной ксеноновой дуговой лампой, – наиболее часто используемый инструмент для анализа индекса желтизны. Этот прибор позволяет определять коэффициенты индекса желтизны и коричневатости (100-L) семолины и макарон. В России в некоторых селекционных центрах для определения индекса желтизны применяется прибор Specol 10 по методологии, предложенной Н.С. Васильчуком (2001). Методы быстродействующей жидкостной хромотографии высокого давления (HPLC) с применением инфракрасной спектроскопии позволяют определить химический состав каротиноидных пигментов и измерить количество каждого компонента в крупке и пасте (Brandolini et al., 2008; Fu et al., 2017).

Картирование локусов, контролирующих синтез пигментов

Концентрация пигментов в зерне твердой пшеницы контролируется различными генами с аддитивными эффектами и зависит от условий внешней среды (Васильчук и др., 2009; Мальчиков, 2009; Schulthess, Schwember, 2013; Гапонов и др., 2018; Мясникова и др., 2019). Систематическое сортоизучение в различных экологических условиях дает необходимую информацию о свойствах сортов и наличии у них соответствующих QTL. Достоверные, значительные и стабильные различия между генотипами по величинам YPC (Yellow Pigment Concentration) и YI (Yellowness Index) свидетельствуют о функционировании локусов количественного признака. Для маркирования и локализации QTL используют рекомбинантно-инбредные линии (RIL), созданные от двуродительских скрещиваний контрастных по величине признака сортов и отобранные в поколениях (от одного парного скрещивания) из беккроссных или дигаплоидных популяций (Elouafi et al., 2001; Pozniak et al., 2007; Singh et al., 2009; Colasuonno et al., 2014). В настоящее время для этих целей используется метод полногеномного генотипирования для поиска корреляций между генотипами и фенотипами в наборах селекционных линий, образцов генетических коллекций и выявления аллельных вариантов молекулярных маркеров и функциональных генов (Vargas et al., 2016; Colasuonno et al., 2017a; Fiedler et al., 2017). В последнее десятилетие этот подход получил распространение благодаря наличию большого количества ДНК-маркеров, равномерно распределенных в геноме, полученных на основе однонуклеотиного полиморфизма (SNP) и совершенствования статистических инструментов (Чесноков, Артемьева, 2011; Maccaferri et al., 2011; Wang et al., 2014; Sehgal, Dreisigacker, 2019). Картирование QTL предполагает хромосомную локализацию локуса в геноме по результатам оценки признаков, полученных в нескольких экспериментах.

Воспроизводимость результатов может зависеть от влияния на признак в исследуемой картирующей популяции многочисленных генов с аддитивными эффектами, родительских компонентов, взаимодействия генотип–среда, числа используемых маркеров и способа измерения каротиноидов. В настоящее время для YPC и YI идентифицирован 81 QTL, включая синглетоны и кластеры QTL, которые распределены по всем хромосомам (Colasuonno et al., 2019). Локализация некоторых QTL была подтверждена с использованием нескольких картирующих популяций, что указывает на присутствие стабильных аллелей, влияющих на улучшение цвета и пищевой ценности зерна твердой пшеницы. Стабильные QTL были обнаружены в хромосомах 1A, 1B, 2A, 2B, 3B, 4A, 6A, 6B, 7A, 7B (Parker et al., 1998; Hessler et al., 2002; Patil et al., 2008; Zhang et al., 2009; Pozniak et al., 2012).

По эффектам на фенотипическую вариабельность признаков QTL распределяются на группы с сильным (40 %), средним (10–40 %) и незначительным (<10 %) влиянием (Colasuonno et al., 2019). Главные локусы с сильным влиянием на YPC и YI были картированы в хромосоме 7AL и дистальной области 7BL (Elouafi et al., 2001; Pozniak et al., 2007; Patil et al., 2008; Zhang, Dubcovsky, 2008). В частности, в длинном плече хромосомы 7A были идентифицированы два QTL с противоположными эффектами. Локус с негативным эффектом по отношению к признакам YPC и YI был ассоциирован с аллельными вариациями гена альдегидоксидазы (AO) – фермента, катализирующего деградацию каротиноидов в результате их окисления (Colasuonno et al., 2014, 2017b). Второй (QTL-73), расположенный в области локализации гена Psy-1, оказывал положительный эффект (до 60 % вариации) на признак YPC. Аналогичные ассоциации QTL и YPC установлены в хромосоме 7BL – негативный эффект одного локуса на фенотипическое проявление YPC составлял 29 %, позитивное влияние второго локуса – 52 % (He et al., 2008, 2009; Zhang, Dubcovsky, 2008). В публикации (N'Diaye et al., 2017) сообщается о картировании YPC и Yi методом конструирования гаплотипных блоков. Гаплотипные блоки формируются с применением насыщенных молекулярных SNP карт. При этом SNP, расположенные в хромосоме в пределах 5.3 cM, группируют в один гаплотипный блок, определяющий один локус, который обозначается префиксом "hap" с указанием номера хромосомы и порядкового номера локуса на хромосоме. Этот подход подтвердил существование высокозначимого QTL (hap_7A_32, маркер Tdurum_conting 54832_139) в хромосоме 7AL, объясняющего 35.6 % фенотипической дисперсии общего пигмента и индекса желтизны в семолине твердой пшеницы и связанного с локусом Psy-A1. Использование подобного метода подтвердило достоверную значимость QTL в хромосомах 2A (hap_2A_18), 7B (hap_7B_36) и 4B (hap_4B_6).

Незначительные по эффектам QTL обнаружены в хромосомах 3A (Parker et al., 1998), 4A и 5A (Hessler et al., 2002), 2A, 4B и 6B (Pozniak et al., 2007), 4B и 6B (Zhang, Dubcovsky, 2008), 1A, 3B и 5B (Patil et al., 2008), 3B и 5B (Howitt et al., 2009), 1A, 1B, 3B и 4A (Zhang et al., 2009).

Гены, участвующие в биосинтезе каротиноидов и их окислении в процессе изготовления конечных продуктов

В настоящее время получена информация о генах-кандидатах, расположенных в регионах локализации локусов со значимым влиянием на признаки YPC и YI. В данном случае к ним относятся гены, контролирующие активность основных ферментов биосинтеза каротиноидов пшеницы, и катаболические гены, вызывающие деградацию каротиноидов. Количественные различия генотипов по на-

Table 1. Genes and their markers associated with the concentration of yellow pigments in grains and processed products located in wheat genomes A and B

Gene	Locus	Allele, haplotype	Marker	Chromosome	Reference
PSY-1	Psy-A1	hap_7A_32	T.durum_conting54832_139	7AL	N'Diaye et al., 2017
		PsyA1a, PsyA1b	YP7A	7AL	He et al., 2008; Ravel et al., 2013
		PsyA1a, PsyA1b, PsyA1c	YP7A-2	7AL	He et al., 2009; Crawford, Francki, 2013; Ravel et al., 2013;
		Psy-A1o, Psy-A1e, **Psy-A1p**, Psy-A1a Psy-A1c, **Psy-A1t**	Psy-A1_STS	7AL	Singh et al., 2009; Crawford, Francki, 2013
		PSY1-AI	YP7A-2, Psy-1SSR Qyp.macs-7A	7AL	Campos et al., 2016; Vargas et al., 2016; Patil et al., 2018
		Psy-A1a, Psy-A1k, Psy-A1ka, Psy-A1kb, PsyA1e, Psy-A1ca, Psy-A1cb, Psy-A1p, PsyA1ra, Psy-A1rb, Psy-A1j, Psy-A1t	Psy-A1_R_49	7AL	Ravel et al., 2013
	Psy-B1	hap_7B_36		7BL	N'Diaye et al., 2017
		Psy-B1a, Psy-B1b	YP7B-1		He et al., 2009; Ravel et al., 2013
		Psy-B1c	YP7B-2		He et al., 2009
		Psy-B1d	YP7B-3		He et al., 2009
		Psy-B1e, Psy-B1a, Psy-B1aa, Psy-B1b, Psy-B1ca, PsyB1m, Psy-B1d	YP7B-4		Ravel et al., 2013
PSY-2	PSY-B2		Xwmc73 – Xgwm274 -wPT-3661	5BS	Colasuonno et al., 2014
ZDS	Zds-A1	Zds-A1a, Zds-A1b	YP2A-1		Dong et al., 2012
LCYE	e-LCY (lutein)	e-LYC3Aa, e-LYC3Ab3A		3A	Crawford, Francki, 2013
			Lipoxygenase activity (LOX)		
Lpx-B1	Lpx-B1.1	hap_4B_6 Lpx-B1.1c	T.durum_conting51688_681	4BS	Verlotta et al., 2010
			Polyphenol oxidase activity (PPO)		
PPO	Tc1		Xgwm312@2A	2AL	Watanabe et al., 2006
	Tc2			2BL	Watanabe et al., 2006
PPO-1	PPO-A1	PPO-A1b, PPO-A1f, PPO-A1e, PPO-A1g, hap_2A-18	PPO18, MG18, BobWhite_c41527_201	2AL	He et al., 2009; Taranto et al., 2012; N'Diaye et al., 2017

коплению пигментов связаны с аллельным разнообразием генов PSY – фитоенсинтетазы (Pozniak et al., 2007; He et al., 2008; Dibari et al., 2012; Colasuonno et al., 2014; Campos et al., 2016), LCYE – ликопин-ε-циклазы (Howitt et al., 2009; Crawford, Francki, 2013), LCYB – ликопин-β-циклазы (Zeng et al., 2015), HYD – β-гидроксилазы (Qin et al., 2012), PDS (фитоендесатуразы) и ZDS – каротиндесатуразы (Cong et al., 2010). Катаболические гены контролируют активность альдегидоксидазы – AO (Colasuonno et al., 2017b), полифенолоксидазы – PPO (Watanabe et al., 2004, 2006; Si et al., 2012), липоксигеназы – LOX или Lpx (DeSimone et al., 2010; Randhawa et al., 2013) и пероксидазы – PER (Ficco et al., 2014), снижающих концентрацию пигментов и потребительские качества конечных продуктов. Наиболее значимые гены, их аллельные варианты и маркеры обсуждаются в тексте и представлены в табл. 1.

Как уже отмечено, ключевым ферментом в сложной цепочке биосинтеза каротиноидов является PSY. Известны три различных гена, кодирующих активность этого фермента: PSY 1, PSY 2, PSY 3, картированные в гомеологичных хромосомах 7-, 5- и 3-й групп соответственно (Dibari et al., 2012). Ген PSY-1 был локализован в хромосомах 7A и 7B твердой пшеницы. Обнаружено, что ген PSY-B1, расположенный в хромосоме 7B, сегрегирует совместно с QTL, ассоциированным с каротиноидным пулом, с изменчивостью YI и YPC от низкого уровня (10 %) до среднего (10–30 %). Ген PSY-A1, расположенный в хромосоме 7A, ведет себя как кодоминантный маркер, объясняет фенотипическую изменчивость, в зависимости от генофона, в диапазоне от среднего (10–30 %) до высокого (30–50 %) и очень высокого (>50 %) уровня (Colasuonno et al., 2014). В целом влияние альтернативных аллелей PSY-A1, по-видимому, является наиболее важным в изменчивости концентрации пигментов и индекса желтизны крупки. Этот вывод основан на результатах изучения различных популяций твердой пшеницы (Campos et al., 2016; Vargas et al., 2016; Patil et al., 2018). Ген PSY-B2, локализованный в 5BS, не оказывал существенного влияния на концентрацию каротиноидов в зерне и крупке (Colasuonno et al., 2014). Локализованный в длинном плече хромосомы 5B ген PSY-B3 повышал экспрессию в листьях и корнях в условиях абиотического стресса (засуха, засоление)

Table 2. Distribution of the Lpx-B1 genes and alleles among cultivars of different breeding periods (Verlotta et al., 2010)

Varieties	Country	Haplotypes		
		I (Lpx-B1.1b +Lpx-B1.3)	II (Lpx-B1.1a +Lpx-B1.2)	III (Lpx-B1.c +Lpx-B1.2)
Varieties created before 1971 of the year				
Matarese, Trinakria, Timilia, Grifoni, Capeiti 8	Italy	+		
Aziziah	Libya	+		
Kiperounda	Cyprus	+		
Polesine	Italy		+	
Russello	Italy		+	
Taganrog	USSR		+	
Cannizzara	Italy			+
Varieties created in the period 1971–1990 years				
Primadur, Tresor, Neodur	France		+	
Karel, Latino, Duilio	Italy		+	
Valnova, Valgerardo, Creso, Grazia, Ofanto, Valforte, Simeto	Italy			+
Produra	USA			+
Varieties created in the period 1991–2005 years				
Marco, Gianni, Bronte, Rusticano, Tiziana, Svevo, Giotto, Iride, Dupri, Italo, Radioso, Claudio, Torrebianca	Italy		+	
Brindur, Nefer	France		+	
Messapia, Gargano, Carpio, Cirillo, Adamello, Colosseo, Quadrato, Ciccio, Vesuvio, Platani, Giusto, Solex, S. Carlo, Bradano, Preco, Fortore, Varano, Lesina, Zenit	Italy			+
Colorado	Italy/USA			+
Navigator	Canada			+
Strongfield	Canada		+	

Note. The "+" sign indicates the belonging to the haplotype.

и был связан с увеличением пула абсцизовой кислоты (Dibari et al., 2012). Гены, контролирующие активность других ферментов, также имеют значимое влияние на концентрацию пигментов в зерне и конечных продуктах. Так, ген ликопин-ε-циклазы (LCYE), ассоциированный с QTL на хромосоме 3A, играет определяющую роль в процессах накопления лютеина – главного каротиноида зерна твердой пшеницы (Howitt et al., 2009). Ген z-каротиндесатуразы (ZDS), маркируемый кодоминантным функциональным маркером YP2A-1 на основе полиморфизма двух аллелей, расположенный на хромосоме 2A, объяснял 11.3 % фенотипической дисперсии YPC и IY в популяции дигаплоидных линий (Dong et al., 2012).

Наиболее четко выражены гены-кандидаты, участвующие в катаболическом пути окисления каротиноидов. Липоксигеназа (Lpx) у растений продуцирует активные формы кислорода, приводящие к деградации каротиноидов и обесцвечиванию конечных продуктов, получаемых из зерна твердой пшеницы (Borrelli et al., 2003). У твердой пшеницы существуют разные гены Lpx (Borrelli, Trono, 2016). На стадии зрелого зерна у сортов с контрастной активностью (окислительной способностью) была установлена различная степень транскрипции генов Lpx-1 и Lpx-3, в то время как транскрипты Lpx-2 на стадии зрелого зерна отсутствовали (De Simone et al., 2010). Локус Lpx-B1 расположен на коротком плече хромосомы 4B, в котором обнаружены три тесносцепленных гена: Lpx-B1.1, Lpx-B1.2 и Lpx-B1.3. Ген Lpx-B1.1 представлен тремя аллелями – Lpx-B1.1a, Lpx-B1.1b, Lpx-B1.1c (Hessler et al., 2002; Carrera et al., 2007; Verlotta et al., 2010). Анализ QTL у твердой пшеницы показал, что 35–54.0 % вариации активности Lpx объясняется Lpx-B1. Аллель Lpx-B1.1c отличается делецией в нуклеотидной последовательности от второго интрона до последнего экзона (Carrera et al., 2007). Этот аллель коррелирует с высоким уровнем желтизны и относительно слабой деградацией пигментов в макаронных изделиях (Carrera et al., 2007; Verlotta et al., 2010). Изучение коллекции твердой пшеницы с включением в нее ландрасов и современных сортов позволило идентифицировать три гаплотипа: первый включал гены и аллели – Lpx-B1.3+Lpx-B1.b, второй – LpxB1.2+LpxB1.1a и третий – LpxB1.2+LpxB1.1c. Эти гаплотипы демонстрировали, соответственно, высокий, средний и низкий уровни функциональных транскриптов Lpx-B1 и ферментативной активности в созревшем зерне.

Известны коммерческие сорта-носители разных генов, Lpx-B1.1, Lpx-B1.2, Lpx-B1.3, и аллелей в локусе Lpx-B1.1. В частности, сорта Kofa и Aureo содержат аллель Lpx-B1.1c. Описаны возможности накопления каротиноидов и генетика липоксигеназы у сортов: Prima-

dur (имеет высокое содержание каротиноидов и высокую активность липоксигеназы), Cosmodur (высокая концентрация каротиноидов, низкая активность липоксигеназы – Lpx-B1.1c), Trinakria (низкая концентрация каротиноидов, высокая активность липоксигеназы), Creso (низкое содержание каротиноидов и низкая активность липоксигеназы – Lpx-B1.1c) (De Simone et al., 2010). По данным A. Verlotta с коллегами (2010) в табл. 2 показано распределение сортов разных периодов селекции (до 1971 г. и в 1971–2005 гг.) по их принадлежности к различным гаплотипам. Вызывает внимание увеличение частоты встречаемости второго и третьего гаплотипов в сортах последних периодов селекции. Необходимо подчеркнуть, что этот результат достигнут селекционерами без понимания и учета в селекционных процедурах генетики окислительных процессов в зерне.

Второй по значимости воздействия на цвет конечных продуктов катаболический фермент – полифенолоксидаза (PPO). Активность фермента контролируют два гена, идентифицированных на гомеологичных хромосомах второй группы, 2A и 2B (Jimenez, Dubcovsky, 1999; Simeone et al., 2002; Watanabe et al., 2004, 2006). В частности, R. Simeone с коллегами (2002) сообщили о значительном генетическом эффекте на активность PPO локуса на длинном плече 2A хромосомы. Гены Tc1 и Tc2 в дистальных частях 2AL и 2BL на расстоянии 46.08 и 40.7 cM от центромеры картированы N. Watanabe с коллегами (2004). Изучение RIL, полученной от скрещивания сортов Jennah Khetifa и Cham 1, показало, что локус в хромосоме 2A обеспечивал 49.1 % активности PPO, низкая активность сегрегировала с молекулярным маркером Xgwm312@2A (Watanabe et al., 2006).

Два гомологичных семейства PPO были картированы по второй гомеологичной группе хромосом и названы PPO-1 (PPO-A1 и PPO-B1) и PPO-2 (PPO-A2 и PPO-B2) (Beecher et al., 2012). Использование маркера, специфичного для мягкой пшеницы (PPO18), для анализа твердой пшеницы позволило обнаружить четыре аллеля PPO-A1: PPO-A1b, PPO-A1f, PPO-A1e, PPO-A1g (He et al., 2009). Используя 111 образцов твердой пшеницы, F. Taranto с коллегами (2012) определили связь различных аллелей PPO-A1 с уровнем активности фермента. Аллель PPO-A1f был связан с высокой, тогда как PPO-A1b и PPO-A1g – с низкой активностью фермента. Эти ученые разработали также новый маркер (MG18), способный обнаружить те же аллели, что и при помощи маркера PPO18, но более эффективно и с более низкой вариабельностью активности PPO внутри каждой группы сортов, несущих один и тот же аллель (Taranto et al., 2012). В маркер-ассоциированной селекции с целью снижения активности PPO целесообразно использовать и паралогичные гены PPO-B1 и PPO-B2 с применением соответствующих маркеров MG08 и MG33, предложенных F. Taranto с коллегами (2015). Гены PPO-B1 и PPO-B2 были расположены на расстоянии 11.4 cM от центромеры на хромосоме 2BL. Скрининг коллекции твердой пшеницы с помощью маркеров MG08 и MG33 позволил идентифицировать четыре и два аллеля соответственно, включая три новых аллеля гена PPO-B1: PPO-B1b, PPO-B1c и PPO-B1d, и один новый аллель PPO-B2 – PPO-B2d. Маркер MG33 способен распознавать два аллеля PPO-B2, связанных с высокой (PPO-B2d) и низкой (PPO-B2a) активностью фермента (Taranto et al., 2015).

Значительное влияние на признаки YI, YPC и цвет макаронных изделий оказывает альдегидоксидаза (AO; EC 1.2.3.1) (Colasuonno et al., 2017b). Три изоформы фермента пшеницы, AO1, AO2, AO3, были локализованы на 2-, 5-, 7-хромосомных группах соответственно. Третий ген альдегидоксидазы, AO-A3, расположенный на хромосоме 7AL, связан с QTL, влияющим на признаки YI и YPC (Colasuonno et al., 2017b). Эксперименты с двумя сортами, Cicco (низкое содержание каротиноидов в зерне) и Svevo (высокое содержание каротиноидов), с применением метода qRT-PCR выявили высокий уровень экспрессии гена AO-A3 у первого сорта и низкий у второго, что подтверждает отрицательный эффект продуктов гена в период накопления каротиноидов. На основе SNP данных, соответствующих изоформе фермента, на хромосоме 7AL картирован маркер IWB59875, который предложен для процедур маркерной селекции для повышения содержания каротиноидов в зерне и цвета макаронных изделий (Colasuonno et al., 2017b).

Пероксидазы – ферменты, катализирующие общую реакцию: $ROOH + H_2O_2 = ROH + H_2O + 1/2O_2$ (Feillet et al., 2000). Паста, произведенная из зерна сортов с высоким уровнем активности пероксидазы, имеет буро-коричневый цвет и низкие потребительские качества (Sisson, 2008). В то же время в процессе изготовления макарон пероксидаза не проявляется в связи с недоступностью перекиси водорода (Ficco et al., 2014). Активность ферментов этой группы в зерне твердой пшеницы значительно меньше, чем в зерне мягкой пшеницы. В связи с этим большинство исследований по их изучению проведено на мягкой пшенице. В литературе отсутствуют сведения о специфических маркерах в геноме твердой пшеницы, связанных с QTL или генами низкой активности. В ряде публикаций отмечено, что гены пероксидазы расположены в гомеологичных хромосомах групп 1, 2, 3, 4 и 7 (Liu et al., 1990; Wei et al., 2015). В зерне твердой пшеницы установлено функционирование 12 изоформ пероксидазы, различающихся по активности в период налива, созревания и прорастания зерна. Некоторые изоформы имеют специфическую локализацию в зерне – перикарп, эндосперм, зародыш. Наиболее важная изоформа, P-5, расположена в эндосперме и оказывает значимое влияние на потемнение (коричневатость) макаронных изделий (Feillet et al., 2000). При использовании полногеномного секвенирования и нулли-тетрасомных линий сорта Чайниз Спринг обнаружены и локализованы два гена, TaPod-A2 и TaPod-D1, на хромосомах 7AS и 7DS. Анализ SNP выявил для двух аллелей локуса TaPod-D1 два функциональных маркера, POD-7D1 и POD-7D6, с высокой и низкой активностью пероксидазы соответственно (Geng et al., 2019). Эти данные, полученные на мягкой пшенице, с учетом ортологичности геномов A и B в перспективе можно адаптировать к проблемам маркерной селекции твердой пшеницы. В отечественной литературе известна публикация А.А. Вьюшкова (2004), в которой приведены значительные сортовые различия твердой пшеницы по активности пероксидазы в крупке (эндосперме).

Результаты и перспективы применения MAS в селекции твердой пшеницы

Несмотря на большое количество работ по локализации и маркированию QTL, связанных с высокой концентрацией каротиноидов в зерне и крупке, результаты прямой проверки применения MAS (селекция с помощью маркеров) представлены ограниченно. Patil с коллегами (2018) сообщили о высокой эффективности использования маркера Psy-A1SSRe, сцепленного с QTL и локусом Psy-A1, на хромосоме 7AL. Маркер Psy-1SSR разработан на основе вариаций в промоторной области PSY-1, он позволяет идентифицировать восемь аллелей Psy-A1 и семь аллелей Psy-B1 одновременно. Маркер Psy-A1SSRe, расположенный в 7AL в большом QTL для YPC, вместе с ранее установленным маркером Qyp.macs-7A был идентифицирован в популяции RIL-PDW233/Bhalegaon 4. Родительский сорт PDW 233 в этой популяции является носителем QTL высокой концентрации пигментов. Эти маркеры были применены для улучшения индийских сортов MACS 3125 и HI8498 с низкой концентрацией пигментов (3.57 и 3.26 ppm соответственно), которые были взяты в качестве рекуррентных родителей в скрещиваниях с PDW 233 – донором QTL для YPC с высокой концентрацией пигментов (8.36 ppm). Селекционные линии, полученные с применением методов MAS на основе MACS 3125 и HI 8498, показали значительное увеличение YPC: 6.16–7.7 и 5.0–7.46 ppm соответственно. В настоящее время MAS используется в CIMMYT и в Канаде для отбора селекционных линий с низкой активностью липоксигеназы при помощи маркера LOXA, нацеленного на аллель Lpx-B1.1c (Randhawa et al., 2013; Dreisigacker et al., 2016; N'Diaye et al., 2017, 2018).

В России во всех лабораториях, осуществляющих селекцию на увеличение концентрации каротиноидов в зерне, индекса желтизны семолины и конечных продуктов, применяются методы традиционной селекции. За период научной селекции и особенно за последние 30 лет эти признаки были улучшены. Сорта, созданные на первых этапах, – Мелянопус 69, Гордеиформе 432, Мелянопус 26, Гордеиформе 179, Гордеиформе 675, накапливают в зерне 3.6–5.0 ppm каротиноидных пигментов. Сорта, широко возделывавшиеся в 60-80-х годах XX в., – Безенчукская 105, Харьковская 46, Безенчукская 139, – превышают этот уровень незначительно (~5 %) (Мясникова и др., 2019). Положительные изменения наблюдались у сортов Светлана (1987 – год включения в реестр) и Саратовская золотистая (1993), которые накапливали 6.0–7.0 ppm пигментов в зерне (Васильчук, 2001). Среди современных сортов яровой твердой пшеницы заметно выделяются по содержанию каротиноидов в зерне Безенчукская золотистая (8.5–9.0 ppm) и Безенчукская крепость (7.5–8.5 ppm). Весь набор современных сортов образует непрерывный ряд изменчивости с шагом в 10–15 % и разницей между крайними вариантами в 200 % (Безенчукская золотистая – Алтайская нива). Изучение отечественных сортов разных этапов селекции в одиннадцати экологических средах позволило установить, что фенотипическое варьирование признака определяется генотипической и средовой вариансами с незначительными эффектами генотип-средовых взаимодействий. По результатам кластерного анализа параметров адаптивности, стабильности и отзывчивости, сорта отчетливо распределялись на кластеры. В группу с оптимальным сочетанием величины, стабильности и отзывчивости признака вошли Безенчукская золотистая, Безенчукская крепость, Безенчукская 210, Саратовская золотистая. Эти генотипы рекомендуется использовать для создания рекомбинантных инбредных линий с целью маркирования QTL, контролирующих синтез каротиноидов в зерне твердой пшеницы, и организации на этой основе маркер-опосредованной технологии селекции (Мясникова и др., 2019).

Заключение

Результаты исследований, рассмотренные в этой статье, показывают реализованные за последние двадцать лет цели в понимании биохимических, генетических механизмов регулирования метаболизма каротиноидных пигментов в твердой пшенице. Данные, полученные на разнообразном растительном материале с применением современных классов ДНК-маркеров, и согласованные в разнообразных экспериментах генетические карты позволили выявить наиболее важные гены, участвующие в контроле биосинтеза, накопления и катаболизма каротиноидов. Наиболее изученными и подтвержденными в ряде экспериментов QTL являются те, которые расположены на хромосомах 3AS (связаны с геном LCYE), 7AL и 7BL (оба тесно связаны с аллелями PSY 1). Перспективно для MAS применение маркера LOXA, нацеленного на аллель Lpx-B1.1c для снижения активности липоксигеназы. Технология MAS с использованием известных для этих QTL маркеров отличается от методов традиционной фенотипической селекции высокой производительностью и эффективностью. В то же время в ряде исследований были представлены данные о том, что большинство QTL не могут быть широко использованы в процедурах MAS, что связано с уровнем их валидации в фенотипических соотношениях в локально адаптированных группах селекционного материала (Pozniak et al., 2012).

Другие QTL, представленные и охарактеризованные в обзоре, требуют дополнительных исследований для идентификации генов-кандидатов, участвующих в накоплении/деградации каротиноидов. В перспективе можно ожидать широкое внедрение методов геномной селекции в программы улучшения цвета конечных продуктов из твердой пшеницы. С учетом аддитивных эффектов генов, контролирующих уровень пигментов и превалирование генотипа признака над средой, применение методов геномной селекции позволит ускорить селекционный процесс на основе не только молекулярной идентификации функционирующих в популяции необходимых QTL, но и целенаправленного получения трансгрессий в результате пирамидирования различных генов. В связи с этим дальнейший акцент в селекционно-генетических мероприятиях будет сделан на анализе генетической изменчивости в коллекциях зародышевой плазмы твердой пшеницы и мутантных популяциях. Перспективны методы редактирования генома с использованием CRIS-Cas9, если их применять, исходя из понимания функций гомеологичных генов с аддитивным эффектом (Patil et al., 2018). Характеристика каждого гена позволит вырабатывать стратегии

диверсификации генетической системы каротиноидных пигментов и расширить существующие вариации, доступные селекционерам.

Непосредственное применение маркеров QTL, апробированных на растительном материале зарубежных стран в России в процедурах MAS и геномной селекции, возможно, если в качестве доноров использовать сорта зарубежной селекции, несущие соответствующие QTL и маркеры. При этом маркеры должны быть эффективными в экологических зонах российских селекционных центров. Однако и в этом случае могут быть проблемы преодоления недостаточной адаптивности привлекаемых в качестве исходного материала генотипов. Селекция на увеличение концентрации пигментов в зерне и продуктах его переработки в России, безусловно, может использовать генетический материал и маркеры, разработанные в иностранных центрах, но базироваться она должна на отечественном исходном материале и адаптированных к нему технологиях маркер-опосредованной селекции.

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Study of the effect of the introduction of mitochondrial import determinants into the gRNA structure on the activity of the gRNA/SpCas9 complex *in vitro*

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Abstract. It has long been known that defects in the structure of the mitochondrial genome can cause various neuromuscular and neurodegenerative diseases. Nevertheless, at present there is no effective method for treating mitochondrial diseases. The major problem with the treatment of such diseases is associated with mitochondrial DNA (mtDNA) heteroplasmy. It means that due to a high copy number of the mitochondrial genome, mutant copies of mtDNA coexist with wild-type molecules in the same organelle. The clinical symptoms of mitochondrial diseases and the degree of their manifestation directly depend on the number of mutant mtDNA molecules in the cell. The possible way to reduce adverse effects of the mutation is by shifting the level of heteroplasmy towards the wild-type mtDNA molecules. Using this idea, several gene therapeutic approaches based on TALE and ZF nucleases have been developed for this purpose. However, the construction of protein domains of such systems is rather long and laborious process. Meanwhile, the CRISPR/Cas9 system is fundamentally different from protein systems in that it is easy to use, highly efficiency and has a different mechanism of action. All the characteristics and capabilities of the CRISPR/Cas9 system make it a promising tool in mitochondrial genetic engineering. In this article, we demonstrate for the first time that the modification of gRNA by integration of specific mitochondrial import determinants in the gRNA scaffold does not affect the activity of the gRNA/Cas9 complex *in vitro*.
Key words: mitochondrial DNA; CRISPR/Cas9; the mitochondrial import determinants; heteroplasmy.

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Изучение влияния детерминант митохондриального импорта в структуре нРНК на активность комплекса нРНК/SpCas9 *in vitro*

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Аннотация. О том, что нарушения структуры митохондриального генома приводят к широкому спектру нейромышечных и нейродегенеративных заболеваний, известно уже давно, но до сих пор не найдено эффективного метода лечения болезней митохондриального происхождения. В основном проблемы с терапией подобных заболеваний обусловлены состоянием гетероплазмии митохондриальной ДНК (мтДНК). Ввиду многокопийности митохондриального генома мутантные копии мтДНК часто сосуществуют с молекулами дикого типа в одной органелле. Клинические симптомы митохондриальных заболеваний и степень их манифестации напрямую зависят от количества мутантных молекул мтДНК в клетке. Смещая уровень гетероплазмии в сторону молекул дикого типа мтДНК, возможно добиться снижения негативного влияния мутации. Для этой цели разработано несколько генно-терапевтических подходов на основе TALE-нуклеаз и нуклеаз типа «цинковые пальцы», однако конструирование белковых доменов таких систем является долгим и трудоемким процессом. Система CRISPR/Cas9 принципиально отличается от данных систем простотой использования, высокой эффективностью и ме-

ханизмом действия. Все присущие характеристики и возможности системы делают ее перспективным инструментом в области генетической инженерии митохондрий. В настоящей статье мы впервые демонстрируем, что модификации направляющей РНК за счет встройки последовательностей, способствующих импорту нРНК в митохондрии, не влияют на функциональную активность комплекса нРНК/SpCas9 в условиях *in vitro*. Полученные результаты указывают на возможность модификации системы с сохранением ее функциональности и использования в перспективе для редактирования митохондриального генома.
Ключевые слова: митохондриальная ДНК; CRISPR/Cas9; детерминанты импорта в митохондрии; гетероплазмия.

Introduction

CRISPR/Cas9 methodology is based on bacterial and archaean defense systems against viruses, transposable genetic elements, and other exogenous DNA species. Lately it has been widely utilized as an efficient multifunctional instrument for genome editing across taxonomy. Its mechanism of action differs from the one for zinc-finger nucleases (ZFNs) and TALE-nucleases (TALENs) and is based on the recognition of a target genome sequence by 20 nucleotide spacer guide RNA (gRNA) and further introduction of a double-stranded break (DSB) via recruitment of Cas9 nuclease (Jinek et al., 2012).

The first necessary step for site-specific DNA recognition and cleavage is the formation of a functional effector complex (Jinek et al., 2014; Jiang, Doudna, 2017). The identification of a target DNA sequence and the consequent nuclease conformational change proceeds via binding of hairpin loops at the 3′ end of gRNA to aminoacids of Cas9 nuclease domain, which in turn leads to the induction of nuclease activity (Wright et al., 2015). The level of complementarity between specific gRNA and Cas9 enzyme determines the thermodynamic stability of a complex and as a result the effectiveness of a target DNA cleavage (Anders et al., 2014). It was shown previously by using crystallography that four base pairs (bps) in the hairpin loops ‘tetraloop’ and ‘stem loop 2’ of a guide RNA extend beyond the ribonucleoprotein complex gRNA/Cas9 while not participating in the interaction with the side amino acid chains of Cas9 (Nishimasu et al., 2014; Konermann et al., 2015). We hypothesize that a substitution of these ‘loose’ gRNA loops with analogous hairpin structures derived from other RNA species does not affect the activity of the complex. Similar RNA modifications have been tested in studies on epigenetic regulation of nuclear gene expression (Mali et al., 2013; Konermann et al., 2015; Komor et al., 2017). It becomes obvious that gRNA molecules could be adjusted for mitochondrial genome editing as well.

A multitude of RNA species with diverse functions are expressed in eukaryotic cells. At the same time, only a minor fraction of them could be transported into mitochondria (Jeandard et al., 2019). The transport of nucleic acids into mitochondria has been a major point of a disagreement in the scientific field, therefore the application of CRISPR/Cas9 system for the suppression of mitochondrial DNA (mtDNA) mutations is generally considered questionable. However, some studies suggest the existence of specific pathways of a targeted import of cytosolic RNAs into mitochondria. Partial mitochondrial localization of synthetic RNAs modified with F- and D-domains of yeast $tRNA^{Lys}$ (CUU) has been demonstrated for yeast mitochondria undergoing stress (Martin et al., 1979; Kamenski et al., 2007). By adding similar hairpin structures others have built recombinant RNA molecules for effective import into mammalian mitochondria and consequent specific inhibition of mtDNA replication (Comte et al., 2013; Tonin et al., 2014). Similar studies utilizing RNA components of RP (Doersen et al., 1985; Holzmann et al., 2008) and MRP (Chang, Clayton, 1987) mitochondrial ribonucleases suggest that their domains can participate in targeted nucleic acid transport into these organelles (Wang et al., 2012). All the described cytosolic RNA species transported into mitochondria are short, non-coding, and contain palindromic sequences for hairpin formation, which are necessary for this type of RNA transport. Artificial introduction of such secondary structures into a gRNA could potentially facilitate RNA transition into mitochondrial matrix.

In this study for the first time, we modify RNA component of CRISPR/Cas9 complex – gRNA for its specific transport inside mitochondria. Knowing that protein component of CRISPR/Cas9 has been already adapted for mitochondrial import (Orishchenko et al., 2016), we hypothesize that reprograming of gRNAs will enable to regulate mammalian mtDNA heteroplasmy level.

Materials and methods

Plasmids and constructs. A fragment of human mitochondrial DNA (DNA substrate), including protospacer in mtND1 gene, was amplified by PCR with L2797 5′-GTCCTAAACTAC CAAACCTGC-3′ and H3733 5′-ATGATGGCTAGGGTG ACTTC-3′ primers and Q5 polymerase (NEB). Guide RNAs (gRNAs) were designed for the target mtDNA sequence by online cloud-based informatics platform Benchling (https://benchling.com/). gRNA with the least number of off-target sites was chosen using the online service http://crispr.mit.edu/. Maps of the gRNA plasmids were designed in SnapGene software (https://www.snapgene.com/). All sequences of the modified gRNAs were analyzed *in silico* to predict secondary structure by RNAfold software from the ViennaRNA Package (Lorenz et al., 2011). To assemble plasmids for gRNA expression, oligonucleotides with overlapping ends were hybridized and inserted into the gRNA_Cloning Vector, kindly provided by Dr George Church (Addgene plasmid # 41824; http://n2t.net/addgene:41824; RRID: Addgene_41824), by Gibson assembly (NEB, USA) according to the manufacturer’s instructions. Plasmid sequences were confirmed by Sanger sequencing.

***In vitro* cleavage assay.** Guide RNA was *in vitro* transcribed using HiScribe™T7 Quick High Yield RNA Synthesis Kit (NEB E2050) and the DNA template generated by PCR from the gRNA plasmids with primers T7_wtgRNA 5′-TAATAC GACTCACTATAGGGAGTTTTATGGtGTCAGCG-3′ and R_T7_gRNA_Cas9 5′-AAAAAAAGCACCGACTCGGT GCC-3′. RNAs were purified by phenol-chloroform extraction and ethanol precipitated. The concentration of RNA was measured using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific) and diluted to 300 nM. Cleavage reactions were carried out in a total volume of 30 μl and contained 1 μl

1 μM nuclease Cas9 *Streptococcus pyogenes* (NEB M0386L) (~30 nM), 3 μl 10× reaction buffer (NEB B0386A), 1 μl gRNA (300 nM). The reaction volume was adjusted with nuclease-free water. After preliminary incubation for 10 min at 25 °C, 1 μl of 30 nM DNA substrate was added to the reaction mixture and incubated at 37 °C for 45 min. The reactions were stopped by the addition of 1 μl Proteinase K (20 mg/ml) and incubated at room temperature for 10 min. Cleavage products were analyzed by electrophoresis on a 1.5 % agarose gel. The presence of 678 and 298 bp fragments is indicative of a specific cleavage of the DNA substrate. *In vitro* cleavage reactions were performed in three independent repeats. The cutting efficiency of the DNA substrate was determined by quantitative assessment of DNA in the bands by gel densitometry in Image Lab software (Bio-Rad, USA). Significant differences were calculated by Student's *t*-test complex. The differences were considered significant at a significance level of $p \leq 0.05$.

Results

Design of modified guide RNAs. To study the effect of modifications in the nucleotide sequence of the guide RNA on the activity of the CRISPR/Cas9 system *in vitro*, their primary structure was designed. Since the tetraloop and the stem loop 2 of the constitutive part of gRNA partially extend beyond the ribonucleoprotein complex (Nishimasu et al., 2014) (Fig. 1, *a*), the introduction of modifications to these loci most likely should not affect the binding of the gRNA/Cas9 complex to a target DNA sequence, as well as its functional activity. Therefore, the GAAA nucleotides of the corresponding loops (tetraloop or stem loop 2) of the gRNA were replaced by a nucleotide sequence of one of the four mitochondrial RNA import determinants (HD, HF, RP, MRP hairpins) (see Fig. 1, *b*) in all possible conformations (direct, reverse, complement, reverse-complement). We designed 32 variants of gRNA with insertion of mitochondrial RNA import determinants in different conformations into the tetraloop or the stem loop 2.

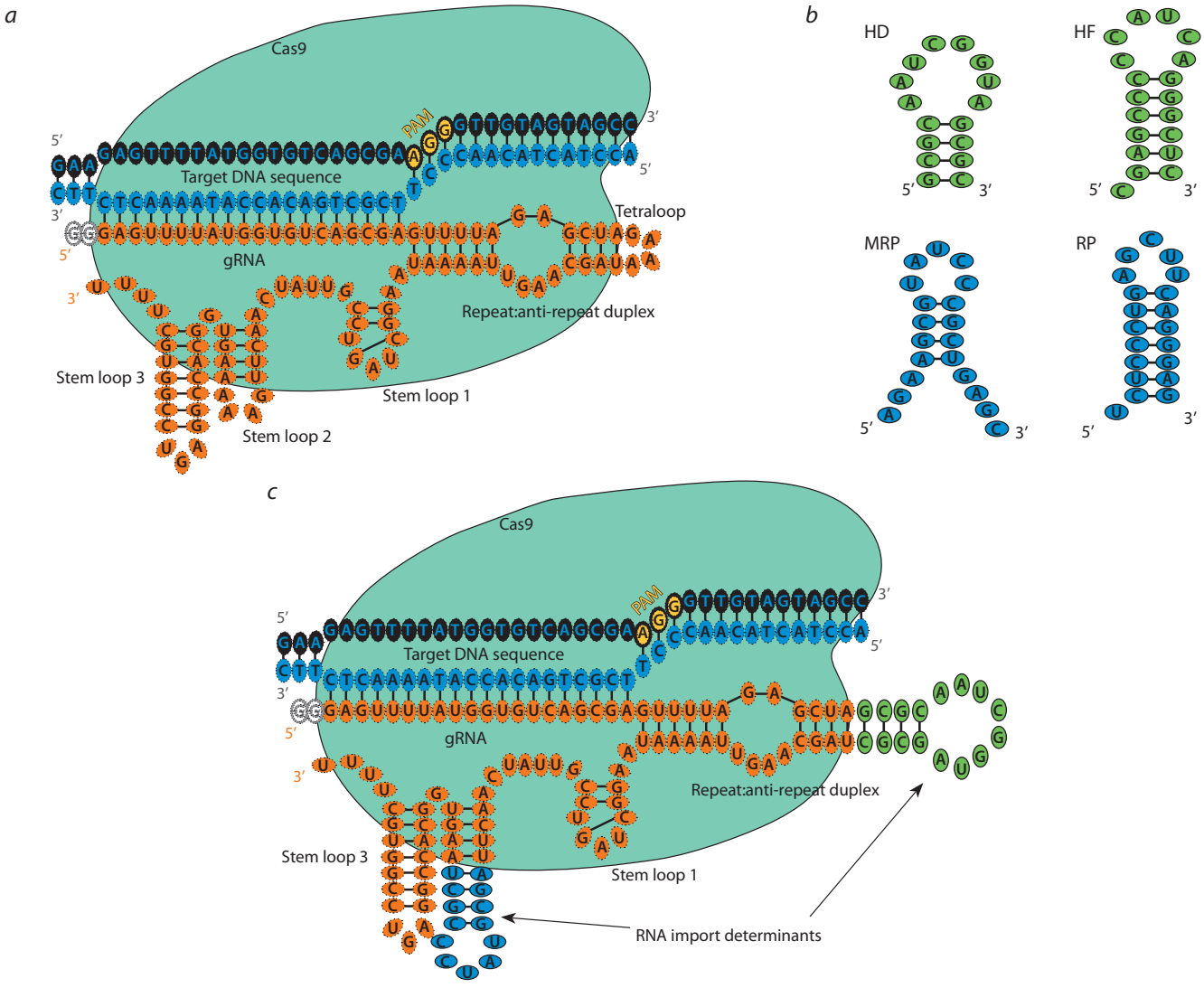

Fig. 1. Design of recombinant gRNA.

a – organization of the target DNA-gRNA-Cas9 complex, where the tetraloop and stem loop 2 of gRNA are free from the interaction with the nuclease; *b* – the hairpin structures are proposed to act as mitochondrial RNA import determinants; *c* – the substitution of a part of the gRNA scaffold loops with import determinants.

Characteristics of modified gRNAs

gRNA modification	The import determinants position	The import determinants conformation	5′–3′ secondary structure
NEG	Without modifications	–	(((((((.((((....)))).)))))))..... .((((....)))).((((((...)))))) Tetraloop Stem loop 2
MRP-TLR	Tetraloop	Reverse	(((((((.((((....(((((.....)))).)))))...)))))))..... .((((....)))).((((((...))))))
RP-TLO	Tetraloop	Original	(((((((.((((.((((((((...))))))))))))...)))))))..... .((((....)))).((((((...))))))
HD-TLO	Tetraloop	Original	(((((((.((((((((.....)))))))))...)))))))..... .((((....)))).((((((...))))))
HF-TLR	Tetraloop	Reverse	(((((((.((((((((.....)))))).)))).)...)))))))..... .((((....)))).((((((...))))))
MRP-SLO	Stem loop 2	Original	(((((((.((((....)))).)))))))..... .(((.....)))..... .((((((...))))))
RP-SLO	Stem loop 2	Original	(((((((.((((....)))).)))))))..... .((..(((((((((...)))))))))..)).((((((...))))))
HD-SLO	Stem loop 2	Original	(((((((.((((....)))).)))))))..... .(((((((((.....))))))))).((((((...))))))
HF-SLO	Stem loop 2	Original	(((((((.((((....)))).)))))))..... .((((.(((((.....))))))))).((((((...))))))

Note. The secondary structure of gRNAs is represented as dot-bracket notation. Each symbol corresponds to a base in the gRNA. The bracket denotes a paired base pair located in the sequence. The dots denote unpaired bases which correspond to loops in the hairpin structure. Unmodified tetraloop and stem loop 2 highlighted in gray.

For each variant of the modified gRNA, a secondary structure was predicted using RNAfold web server from the ViennaRNA software package (Lorenz et al., 2011). The *in silico* predicted structures of the modified gRNAs were compared with the theoretical one for non-modified gRNA. Eventually for each of the import determinants inserted in the tetraloop or the stem loop 2, one of the most optimal conformation was selected. A total of eight variants of modified gRNA were obtained; secondary structures of which had minimal differences from unmodified gRNA (see the Table). All variants of modified gRNA were cloned into a gRNA-cloning vector. The HF-SL variant has not be cloned due to technical difficulties, which are most likely associated with the secondary structure in the nucleotide sequence.

Analysis of the effect of gRNA modifications on the functional activity of the gRNA/Cas9 complex *in vitro*. The activity of the Cas9 nuclease in a complex with modified gRNA was assessed using *in vitro* cleavage reactions. The reactions used gRNA synthesized by *in vitro* transcription with T7 RNA polymerase and recombinant Cas9 nuclease from *S. pyogenes*. A double-stranded DNA fragment of 976 bp amplified by PCR was used as a substrate for *in vitro* cleavage reactions. The protospacer was selected to form two fragments of 298 and 678 bp long, if a double-stranded break in the DNA substrate was successfully introduced by the gRNA/Cas9 complex. Control *in vitro* cleavage reactions were performed with unmodified gRNA (NEG) and without the addition of any gRNA. All reactions were carried out in three independent repeats. The results of agarose gel electrophoresis of the products of *in vitro* cleavage reactions are presented in Fig. 2, *a*. As shown in Fig. 2, *a*, using all variants of modified gRNAs, a specific cleavage of the DNA substrate occurs and the fragments of the expected size are formed. Thus, despite the modifications in the structure of the gRNA, the gRNA/Cas9 complex retains its activity.

A quantitative analysis of the efficiency of the DNA substrate cutting was carried out using densitometry. The cleavage efficiency was determined by the ratio of the pixel density in the bands corresponding to the cleaved DNA substrate to the original uncut DNA fragment (see Fig. 2, *b*). The efficiency of cutting the DNA substrate with Cas9 nuclease in complex with unmodified gRNA (NEG) is 67 %. Modification of gRNA by inserting the HD hairpin in direct conformation into the stem loop 2 of gRNA (HD-SLO variant) significantly ($p \leq 0.05$) reduced the efficiency of DNA substrate cleavage to 32 %, i. e. more than twice compared to unmodified gRNA (NEG). Other variants of gRNA modifications did not lead to any statistically significant changes in the efficiency of DNA substrate cleavage.

Discussion

CRISPR/Cas9 methodology is a revolutionary approach for nuclear genome editing. It has broadened our capabilities for the basic studies of biological processes as well as in the development of human disease therapies. CRISPR/Cas9 adaptation for mtDNA editing is an exciting topic for many laboratories worldwide (Verechshagina et al., 2019). However, unambiguous demonstration of its effective functioning in mitochondria remained unresolved supporting the current opinion about impracticality of using CRISPR/Cas9-derived systems for mitochondrial genome manipulation (Gammage et al., 2018).

Various complications with adaptation of this system for mitochondria are associated with inaccessibility of mitochondrial matrix for the system components due to the presence of outer and inner mitochondrial membranes. A mitochondrion consists of approximately 1500 different proteins with diverse functions, and only 13 of them are encoded by mtDNA and synthetized in the organelle itself (Calvo, Mootha, 2010). Unsurprisingly, there are many known mechanisms of protein transport into different mitochondrial subcompartments (Pfanner et al., 2019). We (Orishchenko et al., 2016) and others (Jo et al., 2015; Loutre et al., 2018; Bian et al., 2019) have demonstrated that introduction of a mitochondrial localization signal at the N-terminus of Cas9 leads to an effective Cas9 import into mitochondrial matrix. Therefore, protein component of CRISPR/Cas9 system could be imported inside mitochondria.

The second task in CRISPR/Cas9 adaptation for mtDNA modification is gRNA mitochondrial import. Unfortunately,

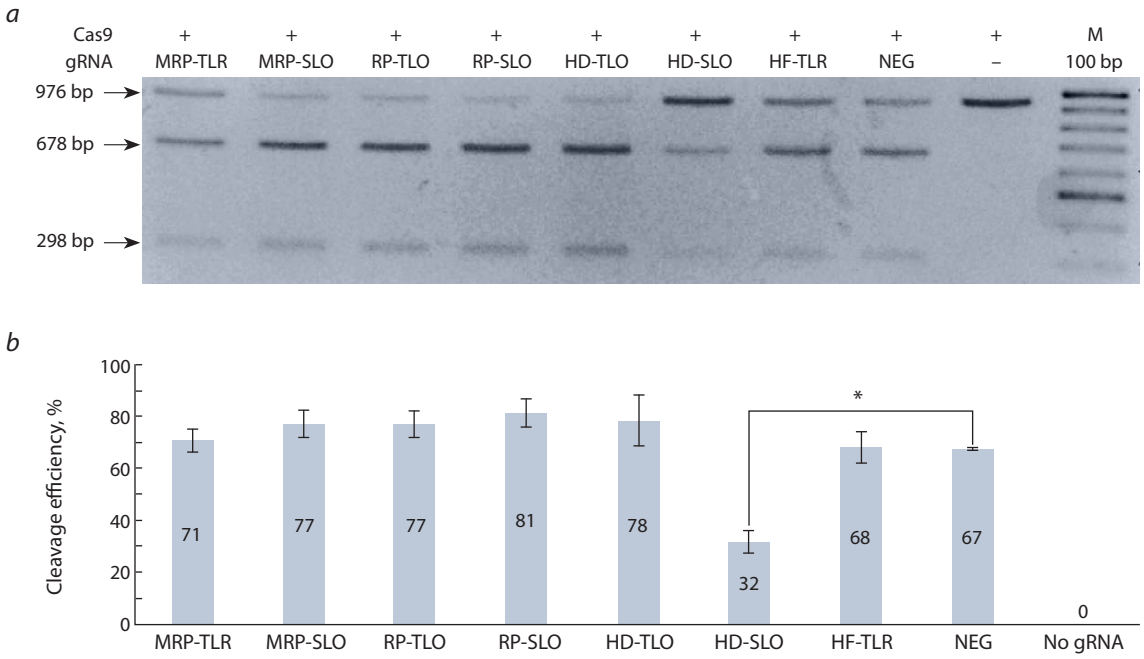

Fig. 2. *In vitro* DNA cleavage by the gRNA/SpCas9 complex with modified gRNA.

Cleavage efficiency was assessed by agarose gel electrophoresis (*a*) and measured using densitometry (*b*). Standard deviation from the mean is shown as error bars (+/–). M – 100 bp DNA ladder. Statistical assessment is made by Student's *t*-test; * indicate significant differences between NEG and modified gRNA, $p \leq 0.05$.

the molecular mechanisms of RNA transport across mitochondrial membranes have not been unambiguously described yet. Moreover, there is no consensus on RNA species imported, their function in mitochondria, and intramembrane channels through which they get transferred. Therefore, development and optimization of gRNA mitochondrial import represents a bottleneck in the overall adaptation of the system.

On the contrary, there have been many recent publications demonstrating successful import of diverse RNA species into mitochondria (Rubio et al., 2008; Wang et al., 2010; Fan et al., 2019; Jeandard et al., 2019). Generally, import of these RNAs is mediated by a stem-loop type hairpin structures. It has been shown that HF and HD hairpins in yeast *Saccharomyces cerevisiae* tRNA are responsible for intramitochondrial transport of $tRNA^{Lys}$ CUU (tRK1). Introduction of these hairpins to other RNAs leads to their *in vivo* mitochondrial import with a consequent restoration of functions initially disturbed by mtDNA mutations (Kazakova et al., 1999; Kamenski et al., 2010; Gowher et al., 2013; Tonin et al., 2014). Additionally, it was demonstrated that RP and MRP hairpins mediate mitochondrial import of H1 and 7-2 RNAs respectively (Wang et al., 2010; Noh et al., 2016; Markantone et al., 2018) thus suggesting that the addition of such components into gRNA structure enables their effective transfer into mitochondrial matrix.

In earlier studies both components of CRISPR/Cas9 system have been extensively modified to achieve high effectiveness and specificity, and to increase its potential functional repertoire. There are several main structural elements of a gRNA: a spacer – a sequence approximately 20 nt in length at the 5′ end of gRNA which is complementary to a target genome sequence, and four hairpins – secondary RNA structures of a stem-loop type (tetraloop, stem loop 1/nexus, stem loop 2, and stem loop 3). The tetraloop contains a lower stem, an overhang, and an upper stem (Briner et al., 2014; Nishimasu et al., 2014). By using site-directed mutagenesis it was shown that the overhang and the stem loop 1/nexus are the key elements of a gRNA necessary for the action of the CRISPR/Cas9 complex. At the same time, the upper stem in the tetraloop and the stem loop 2 could be substantially modified or even eliminated from the gRNA while not compromising gRNA/Cas9 complex activity (Briner et al., 2014; Konermann et al., 2015). Moreover, lengthening of the tetraloop and the stem loop 2 increases the stability of the gRNA and the effectiveness of an assembly of the complex gRNA/dCas9 (Ma et al., 2016; Shao et al., 2016). Therefore, we hypothesized that introducing mitochondrial localization signals into these hairpin structures will facilitate intramitochondrial transfer of gRNAs without affecting the functional performance of gRNA/Cas9 complex.

In the current study, we add HD, HF, RP, and MRP hairpins in different conformations into the tetraloop or the stem loop 2 gRNA structures. Subsequently, using *in vitro* cleavage assay we assess the effects of gRNA modifications on the activity of gRNA/Cas9 complex. We detect specific cleavage of DNA substrates by the combinations of Cas9 with every of our modified gRNA variants (see Fig. 2, *a*) which suggests that introduced modifications do not affect the formation of gRNA/Cas9 complex, as well as the specificity of DNA binding. Importantly, some of gRNA modifications lead to both the increase and the decrease of DNA substrate cleavage rate which could be associated with the influence of the modifications on the gRNA stability, the effectiveness of gRNA/Cas9 complex formation, and Cas9 nuclease activity (Nowak et al., 2016).

An analogous approach for gRNA transport into mitochondrial matrix was taken by R. Loutre and colleagues even though the import determinants were added at either the 5′ or the 3′ end of the gRNA (Loutre et al., 2018). In the case of the 3′ end modification, the activity of gRNA/Cas9 complex *in vitro* matches its activity while using unmodified gRNA. On the contrary, the 5′ end modification significantly diminishes the complex activity. Most likely, this effect is associated with processing and degradation of the 5′ end of modified gRNA. This has been demonstrated for gRNAs with an increased spacer region as well as with an insertion of MS2 and PP7 hairpins at the 5′ end (Ran et al., 2013; Zalatan et al., 2015; Nowak et al., 2016). The 3′ end modifications could decrease the expression and the stability of a gRNA which affects the activity level of gRNA/Cas9 complex (Zalatan et al., 2015).

Conclusion

Therefore, the tetraloop and the stem loop 2 are potentially the optimal regions for the insertion of mitochondrial localization sequences. However, these variants should be tested not only *in vitro* but *in vivo* in cell cultures to analyze both gRNA/Cas9 complex activity and the effectiveness of intramitochondrial transport of both components.

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Generation of microdissected DNA probes from metaphase chromosomes when chromosome identification by routine staining is impossible

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Abstract. Application of microdissected DNA libraries and DNA probes in numerous and various modern molecular cytogenetic studies showed them as an efficient and reliable tool in the analysis of chromosome reorganization during karyotypic evolution and in the diagnosis of human chromosome pathology. An important advantage of DNA probe generation by metaphase chromosome microdissection followed by sequence-independent polymerase chain reaction in comparison with the method of DNA probe generation using chromosome sorting is the possibility of DNA probe preparation from chromosomes of an individual sample without cell line establishment for the production of a large number of metaphase chromosomes. One of the main requirements for successful application of this technique is a possibility for identification of the chromosome of interest during its dissection and collection of its material from metaphase plates spread on the coverslip. In the present study, we developed and applied a technique for generation of microdissected DNA probes in the case when chromosome identification during microdissection appeared to be impossible. The technique was used for generation of two sets of Whole Chromosome Paints (WCPs) from all chromosomes of two species of free-living flatworms in the genus *Macrostomum*, *M. mirumnovem* and *M. cliftonensis*. The single-copy chromosome technique including separate collection of all chromosomes from one metaphase plate allowed us to generate WCPs that painted specifically the original chromosome by Chromosome *In Situ* Suppression Hybridization (CISS-Hybridization). CISS-Hybridization allowed identifying the original chromosome(s) used for DNA probe generation. Pooled WCPs derived from homologous chromosomes increased the intensity and specificity of chromosome painting provided by CISS-Hybridization. In the result, the obtained DNA probes appeared to be good enough for application in our studies devoted to analysis of karyotypic evolution in the genus *Macrostomum* and for analysis of chromosome rearrangements among the worms of laboratory cultures of *M. mirumnovem*.
Key words: metaphase chromosome microdissection; Whole Chromosome Paints; FISH; sequence-independent polymerase chain reaction.

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Получение микродиссекционных ДНК-проб из метафазных хромосом в случае невозможности идентификации целевой хромосомы методами рутинного окрашивания

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Аннотация. При проведении многочисленных и разнообразных молекулярно-цитогенетических исследований микродиссекционные ДНК-библиотеки и ДНК-пробы зарекомендовали себя как надежный и эффективный инструмент как в диагностике и анализе хромосомных патологий человека, так и в работах, посвященных изучению реорганизации хромосом в ходе кариотипической эволюции. Важным преимуществом микродиссекционных ДНК-проб перед хромосомоспецифичными ДНК-пробами, полученными с помощью хромосомного сортинга, является возможность их приготовления из хромосомного материала индивидуальных животных без дополнительного этапа создания клеточных культур, предназначенных для производства большого числа метафазных хромосом. Одно из основных условий успешного использования микродиссекционной техники – идентификация целевой хромосомы на препаратах метафазных хромосом, что позволяет, используя микроманипуляционную технику, осуществлять сбор непосредственно ее материала с цитологических препаратов. В настоящей работе предложена технология создания ДНК-проб для индиви-

дуальных хромосом даже в том случае, когда рутинное окрашивание не дает провести их надежную идентификацию. Представленный подход апробирован при получении наборов хромосомоспецифичных ДНК-проб для хромосом двух видов свободноживущих плоских червей рода *Macrostomum – M. mirumnovem* и *M. cliftonensis*. Кариотипы этих видов содержат три пары мелких, близких по размеру метацентрических хромосом, надежная идентификация которых после окрашивания красителем Гимза оказалась невозможной. Раздельный сбор всех метафазных хромосом из одной метафазной пластинки с последующей амплификацией их ДНК позволил создать ДНК-пробы, специфически окрашивающие исходные хромосомы при проведении даже частичной супрессионной гибридизации *in situ*. При анализе результатов такой супрессионной гибридизации *in situ* идентифицированы хромосомы, из которых были получены ДНК-пробы. Последующее пулирование ДНК-проб, созданных из гомологичных хромосом, способствовало увеличению интенсивности их специфического окрашивания при проведении их супрессионной гибридизации *in situ*. Это, в свою очередь, обеспечило возможность успешного применения предлагаемого подхода в экспериментах, посвященных изучению кариотипической эволюции в роде *Macrostomum*, а также при анализе хромосомных перестроек, имеющих место в лабораторных культурах *M. mirumnovem*.
Ключевые слова: микродиссекция метафазных хромосом; микродиссекционные ДНК-пробы; флуоресцентная *in situ* гибридизация; сиквенс-независимая полимеразная цепная реакция.

Introduction

Comparative cytogenetics as a special area in the modern biology arose after the development of methods for high-quality metaphase chromosome preparation. Its progress is associated mostly with the development of techniques for chromosomes and chromosome regions identification. Since the 1970s, researchers successfully used the GTG-banding method for comparative cytogenetic analysis of chromosomes of different species of mammals and birds (Graphodatsky et al., 2000). The next step in development of comparative cytogenetics was the homeologous gene assignment to chromosomes or chromosome regions in different species of mammals that served as markers of their homeology. In the first studies devoted to gene assignments to the chromosomes, the panels of interspecific hybrids of somatic cells were used (Rubtsov et al., 1981). The obtained data were combined with a comparison of GTG-banding patterns of chromosomes containing homeologous genes (Rubtsov et al., 1988).

Significant progress in comparative cytogenetics has been associated with the development of fluorescent *in situ* hybridization (FISH) technique for nucleic acids in the early 1980s (Bauman et al., 1980). This technique made it possible to localize cloned DNA fragments precisely to small chromosomal regions, and then specifically paint the whole chromosomes or extended chromosome regions (Nesterova et al., 1991). One of the pivotal moments in the development of comparative cytogenetics appeared to be the development of physical isolation of chromosomal material. Two different techniques, namely metaphase chromosome microdissection and chromosome flow sorting, are used for generation of the whole chromosome or partial chromosome paints (WCPs and PCPs, respectively). These paints are generated from isolated chromosomal material through sequence-independent DNA amplification in a polymerase chain reaction with partially degenerated MW6 primer, or by using the special WGA-kits (whole genome amplification). Chromosome *in situ* suppression hybridization (CISS-hybridization) painted specifically the original chromosome or chromosome region and also homeologous chromosomes and correspondent region in related species (Ferguson-Smith, Trifonov, 2007). The quality of such WCPs depends on the efficiency of DNA amplification of the collected chromosomal material, the number of isolated chromosome copies used on the start of DNA amplification, and the quality of DNA of the collected chromosomal material. The high quality of whole chromosome paints can be achieved by collecting many hundreds of chromosome copies using flow sorting. In the case of microdissection, the number of obtained chromosome copies is limited due to the high complexity of the microdissection procedure. The problems of identification chromosome of interest could make the application of chromosome microdissection technology even more complicated task.

In the molecular cytogenetic analysis of chromosomes of free-living worms of the genus *Macrostomum*, we encountered had to solve the problem of whole chromosome paints generation from chromosomes that avoid reliable identification after chromosome staining. The karyotypes of species belonging to the genus *Macrostomum* can be divided into three groups based on their chromosome number and morphology. The karyotypes of species from two groups ($2n = 6$ and $2n = 12$) consist of small metacentric chromosomes suggesting that a recent whole genome duplication (WGD) could take place in the evolution providing species with chromosome number $2n = 12$. This hypothesis is in a good agreement with the results of molecular cytogenetic analysis of asymmetric karyotypes of *M. lignano* and *M. janickei* species. In the karyotypes of these species, there are clear traces of a recent WGD event (Zadesenets et al., 2017a, b). In addition to a WGD in their evolution, there was a fusion of one haploid set of ancestral chromosomes into one large metacentric chromosome (Zadesenets et al., 2017a, b).

The hypothesis of chromosome number doubling in a result of WGD can be verified by generation of WCPs from individual chromosomes of the *Macrostomum* species having the $2n = 12$ karyotype and further CISS-hybridization on metaphase chromosomes of the species. The specific painting of two pairs of paralogous chromosomes with the WCP derived from individual chromosome would indicate to a recent duplication of this one in karyotype evolution of studied species. The same results obtained for all chromosomes will confirm the hypothesis of the WGD that recently took place in the genome evolution of analyzed species. The high level of similarity of all chromosomes have complicated the generation of specific WCPs that could be applied for such a study. Such

similarity of the morphology and size of all chromosomes was revealed in all currently karyotyped species of the genus *Macrostomum* with the chromosome set $2n = 12$ (Zadesenets et al., 2020).

We investigated the karyotypes of new *Macrostomum* species that potentially could be involved in these studies. Additionally, we developed the method for the generation of WCPs that painted original chromosomes in species with morphologically indistinguishable chromosomes.

Material and methods

Laboratory cultures of the free-living *Macrostomum* worms. Laboratory cultures of *M. cliftonensis* and *M. mirumnovem* were kindly provided by Dr. Lukas Schärer (Zoological Institute, University of Basel, Switzerland). The outbred cultures of *M. cliftonensis* and *M. mirumnovem* were maintained in the laboratory of Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences. The karyotype of *M. cliftonensis* ($2n = 6$) consists of three pairs of small metacentric chromosomes of similar size and morphology (Zadesenets et al., 2020). Karyotyping of *M. mirumnovem* revealed a high karyotypic diversity, with the most common chromosome number $2n = 9$ (Zadesenets et al., 2020). In this study, we used only the worms with the $2n = 9$ karyotype.

Metaphase chromosome slide preparation. Chromosome slide preparation was carried out according to the previously published protocol for single-worm karyotyping (Zadesenets et al., 2016). To describe the karyotype, we analyzed at least ten metaphase plates per each specimen. For microdissection, chromosome slides were prepared from chromosome suspension, as described earlier (Zadesenets et al., 2016).

Metaphase chromosome staining. For routine karyotyping, chromosomes were stained with DAPI (4', 6-diamidino-2-phenylindole) (Vector Laboratories, USA) under the standard protocol. For microdissection, chromosomes were stained with 0.1 % Giemsa solution for 3 min at room temperature (RT). After staining, they were rinsed in distilled water and air-dried. After drying, the chromosomes should remain soft enough for effectively cutting with an extended glass needle.

Microscopy. Microimages of metaphase chromosomes after DAPI-staining and FISH were captured using a CCD-camera installed on an Axioplan 2 Plus microscope (ZEISS, Germany) equipped with a fluorescence filter cube set, #49, #10 and #15 (ZEISS, Germany). AxioVision (ZEISS, Germany) or ISIS4 (METASystems GmbH, Germany) software was applied for caption and analysis of chromosome microimages. Microscopy was performed at the Center for Microscopic Analysis of Biological Objects of SB RAS (Novosibirsk, Russia).

Metaphase chromosome microdissection and whole chromosome paint generation. In general, metaphase chromosome microdissection in species of the genus *Macrostomum* and DNA sequence-independent amplification of DNA of dissected material for WCP generation were mainly described previously (Zadesenets et al., 2016, 2017a, b). Briefly, only complete metaphase plates were used in microdissection experiments. The material in the metaphase plate had to be well spread without chromosome contacts and overlapping. Such quality of chromosome spreading allowed us to carefully isolate all chromosomes from the metaphase plate and transfer the material of each chromosome into a separate tube. For microdissection of the *M. mirumnovem* chromosomes, we used only individuals with the $2n = 9$ karyotype. Their chromosome set included the unpaired largest metaphase chromosome MMI1, the pair of large metacentrics MMI2, and three pairs of small metacentric chromosomes MMI3–MMI5. Under microscopic control, the material of the isolated chromosome was transferred to 40 nl of the reaction mixture (Zadesenets et al., 2016), placed in the extended siliconized tip of the Pasteur pipette. Microscopic control guaranteed reliable and complete transfer of isolated chromosome. Then, its material was treated with proteinase K and transferred to 10 µl of the reaction mixture within a 0.5 ml Eppendorf Safe-Lock microcentrifuge tube. Further, DNA preparation for amplification and the amplification itself was performed according to the previously described protocol (Zadesenets et al., 2016, 2017a, b). After polymerase chain reaction (PCR), the resulting DNA product was labeled in 20 additional PCR cycles in the presence of Flu-12-dUTP [fluorescein-5(6)-carboxamidocaproyl-[5(3-aminoallyl)2′-deoxyuridine-5′-Triphosphate] (Biosan, Novosibirsk, Russia) or TAMRA-5-dUTP (5-tetramethylrhodamine-dUTP) (Biosan) using the Whole Genome Amplification 3 Kit (WGA3, Sigma-Aldrich, USA) (Zadesenets et al., 2016, 2017a, b). The WCPs were tested by CISS-hybridization with metaphase chromosomes of the original species.

CISS-hybridization with metaphase chromosomes of *M. cliftonensis* and *M. mirumnovem*. Due to the small body size of *M. cliftonensis* and *M. mirumnovem* (the mean body length of adult worms does not exceed 1.22 and 1.17 mm, respectively) (Schärer et al., 2020), it was impossible to obtain a sufficient amount of Cot1/Cot2 DNA (fraction of highly repetitive DNA) for routine CISS-hybridization. Previously we developed a modification of the CISS-hybridization with WCPs generated from microdissected chromosomes of some *Macrostomum* species (Zadesenets et al., 2017a, b, 2020). This CISS-hybridization gave different painting patterns in different chromosomes regions, depending on their enrichments with DNA repeats. The euchromatic regions at the original chromosome showed specific painting patterns. Less intense fluorescence was observed at other chromosome euchromatic regions containing dispersed DNA repeats. In contrast, more intense signals were found in the heterochromatic regions enriched for DNA repeats homologous to those in the WCPs.

Results and discussion

Chromosomes of *M. cliftonensis*. Before microdissection, we repeatedly checked the karyotypes of randomly chosen 100 specimens of *M. cliftonensis*. All metaphase plates in analyzed samples contained the standard for *M. cliftonensis* chromosome set, $2n = 6$, consisting of three pairs of small metacentric chromosomes (Fig. 1).

Generation and testing of WCPs derived from metaphase chromosomes of *M. cliftonensis*. For obtaining of metaphase plates of *M. cliftonensis*, suspension of mitotic cells was dropped on a cold, wet glass coverslip (60 mm × 24 mm × 0.17 mm), and chromosome slide was immediately put horizontally into warm water vapors (65–70 °C). After air-

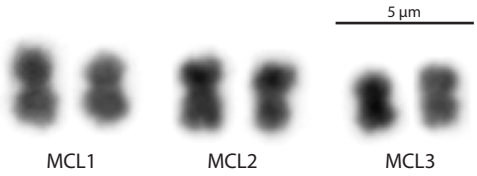

Fig. 1. The karyotype of *M. cliftonensis* (2*n* = 6) consists of three pairs of small metacentric chromosomes showing similar size and morphology.

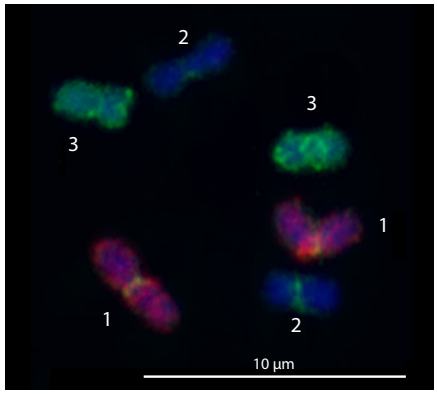

Fig. 2. CISS-hybridization with the WCPs derived from chromosome 1 (red signal) and chromosome 3 (green signal) of *M. cliftonensis* on the metaphase chromosomes of *M. cliftonensis*.

Chromosome numbers are indicated.

Fig. 3. The karyotype *M. mirumnovem* (2*n* = 9) consists of three large chromosomes and three pairs of small metacentric chromosomes showing similar size and morphology.

drying slide was rinsed in phosphate buffer (1xPBS, pH = 7.2) for 1 min at RT and immediately transferred in 0.1 % Giemsa solution for 3–4 min at RT. After staining, the slide was rinsed in distilled water and slightly dried. The chromosomal material should remain wet and soft for easy and careful its collection without breaking into fragments with extended siliconized glass needle. This procedure of chromosome preparation for microdissection reduced DNA degradation and allowed the quantitative collection of chromosome material.

Microdissection was carried out on an AxioVert10 inverted microscope (ZEISS, Germany) equipped with two micromanipulators, one of which controlled an extended glass needle. At the same time, the other served to fix the Pasteur pipette with an extended tip during the transfer of dissected material. For more efficient microdissection, a special rotating sliding stage was installed on AxioVert10 inverted microscope.

Material of all chromosomes from the selected metaphase plate was collected and transferred under microscopic control into reaction mixture solution in the extended siliconized tips of the Pasteur pipettes (the diameter of the tip was about 40 µm). Then it was transferred into the separate PCR tubes contained 10 µl of the reaction mixture (Zadesenets et al., 2016). To ensure that the material was transferred completely, we broke off, the extended tip of the Pasteur pipette in the PCR tube. Further, the preparation of DNA for amplification (proteinase K treatment, DNA fragmentation, DNA library preparation) and DNA amplification itself were carried out according to the standard protocol (Zadesenets et al., 2016, 2017a, b, 2020). The resulting PCR products were labeled, and two-color CISS-hybridization was performed for testing the quality of the obtained WCPs and to determine WCPs generated from homologous chromosomes.

The CISS-hybridization with obtained WCPs painted entirely one pair of chromosomes and gave a signal in the pericentromeric regions of other chromosomes (Fig. 2). The last could be provided by insufficient suppression of repetitive DNA hybridization. The DNA libraries generated from homologous chromosomes were pooled together. After CISS-hybridization, the WCPs based on the combined DNA libraries gave more intense and more specific signal on the original chromosome. They also provided more intense FISH signal at the pericentromeric regions of all chromosomes. At the same time, the painting intensity at the euchromatic regions of other chromosomes did not increase. Two-color CISS-hybridization with obtained WCPs did not reveal chromosome translocation in the *M. cliftonensis* karyotype. These results confirmed our previous suggestion that the *M. cliftonensis* karyotype is highly stable.

Generation and testing of WCPs derived from metaphase chromosomes of *M. mirumnovem*. Since we uncovered high karyotype instability in the laboratory culture of *M. mirumnovem* (Zadesenets et al., 2020), the worms with the 2*n* = 9 karyotype were chosen for the WCP generation. At the beginning of the cultivation of *M. mirumnovem* worms under the laboratory conditions, the most common karyotype revealed among the specimens was 2*n* = 9 (Fig. 3). The same protocol of metaphase chromosome microdissection and sequence-independent DNA amplification described for *M. cliftonensis* was applied for generation of the WCPs from the *M. mirumnovem* chromosomes. The material of all nine chromosomes was isolated separately from one metaphase plate, and the WCPs were obtained by DNA amplification of the collected material. The following CISS-hybridization was performed on metaphase chromosomes of *M. mirumnovem*, and pairs of WCPs derived from homologous chromosomes were determined. Microdissected DNA libraries obtained from homologous chromosomes were pooled together and were further used for the production of the WCPs. As a result, we received the set of WCPs that includes four WCPs derived from the MMI2-MMI5 chromosome pairs and one WCP derived from one copy of unpaired chromosome MMI1.

CISS-hybridization of WCPs obtained from small chromosomes MMI3–MMI5 gave intensive and specific fluorescent signals on the original chromosome, less intensive signal at the pericentromeric regions of the remaining chromosomes, and weak non-specific signals at the euchromatic regions of other small metacentrics. However, the painting pattern of

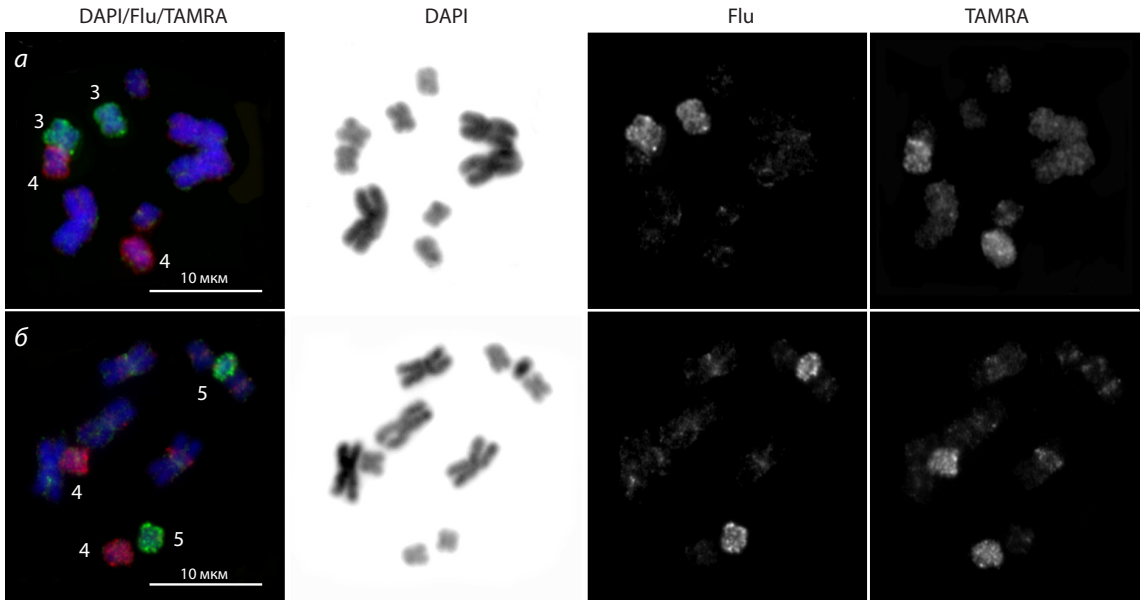

Fig. 4. CISS-hybridization with the WCPs on metaphase chromosomes of *M. mirumnovem*.

a – the whole chromosome paints derived from small chromosomes MMI3 (green signal) and MMI4 (red signal); *b* – the whole chromosome paints derived from small chromosomes MMI4 (red signal) and MMI5 (green signal). Chromosome numbers are indicated.

large chromosomes appeared to be more complicated for interpretation. The WCPs obtained from chromosomes MMI3–MMI5 painted specifically but less intensively and unevenly different regions of the MMI2 chromosome, and they painted even less intensively and less evenly the MMI1 chromosome (Fig. 4).

CISS-hybridization with the WCPs derived from the large chromosome MMI1 and two copies of the MMI2 painted intensively the original chromosomes. However, the pattern of chromosome painting was uneven. On low condensed chromosomes, areas of intense fluorescence alternated with less intensely painted regions. We should note that CISS-hybridization with the WCPs derived from large metacentrics MMI1 and MMI2 gave weak specific fluorescent signals on small metacentric chromosomes MMI3–MMI5. We believe that the obtained painting patterns of the *M. mirumnovem* chromosomes indicate to the WGD event in the evolutionary scenario of this species. However, it has been accompanied by intensive genome and karyotype reorganization, possibly leading to the rediploidization of the modern *M. mirumnovem* genome. A similar scenario of genome and karyotype evolution was previously described for the other *Macrostomum* species, *M. lignano* and *M. janickei*, belonging to another phylogenetic lineage (Schärer et al., 2020; Zadesenets et al., 2020).

Conclusion

The proposed and tested approach for the preparation of DNA probes from individual whole chromosomes allowed us to obtain the WCPs for chromosomes of *M. cliftonensis* and *M. mirumnovem*. The generated WCPs efficiently identified the material of their original chromosomes in both species. Moreover, in the *M. mirumnovem* chromosomes, the WCPs revealed the paralogous regions, resulting from the recent WGD followed by subsequent reorganization of ancestral chromosomes.

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Migration of primordial germline cells is negatively regulated by surrounding somatic cells during early embryogenesis in *Drosophila melanogaster*

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Abstract. Cell migration is an important morphogenetic process necessary at different stages of individual development and body functioning. The initiation and maintenance of the cell movement state requires the activation of many factors involved in the regulation of transcription, signal transduction, adhesive interactions, modulation of membranes and the cytoskeleton. However, cell movement depends on the status of both migrating and surrounding cells, interacting with each other during movement. The surrounding cells or cell matrix not only form a substrate for movement, but can also participate in the spatio-temporal regulation of the migration. At present, there is no exact understanding of the genetic mechanisms of this regulation. To determine the role of the cell environment in the regulation of individual cell migration, we studied the migration of primordial germline cells (PGC) during early embryogenesis in *Drosophila melanogaster*. Normally, PGC are formed at the 3rd stage of embryogenesis at the posterior pole of the embryo. During gastrulation (stages 6–7), PGC as a consolidated cell group passively transfers into the midgut primordium. Further, PGC are individualized, acquire an amoeboid form, and actively move through the midgut epithelium and migrate to the 5–6 abdominal segment of the embryo, where they form paired embryonic gonads. We screened for genes expressed in the epithelium surrounding PGC during early embryogenesis and affecting their migration. We identified the *myc*, *Hph*, *stat92E*, *Tre-1*, and *hop* genes, whose RNA interference leads to premature active PGC migration at stages 4–7 of embryogenesis. These genes can be divided into two groups: 1) modulators of JAK/STAT pathway activity inducing PGC migration (*stat92E*, *Tre-1*, *hop*), and 2) *myc* and *Hph* involved in epithelial morphogenesis and polarization, i.e. modifying the permeability of the epithelial barrier. Since a depletion of each of these gene products resulted in premature PGC migration, we can conclude that, normally, the somatic environment negatively regulates PGC migration during early *Drosophila* embryogenesis.

Key words: *Drosophila melanogaster*; embryogenesis; germline cells; cell migration; embryonic gonad development.

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Миграцию клеток зародышевой линии в раннем эмбриогенезе *Drosophila melanogaster* негативно регулируют окружающие соматические клетки

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Аннотация. Клеточная миграция – важный морфогенетический процесс, необходимый на разных этапах индивидуального развития и функционирования организма. Инициация и поддержание состояния движения клеток требуют активации множества факторов, участвующих в регуляции транскрипции, преобразованиях

сигналов, адгезивных взаимодействиях, модуляциях мембран и цитоскелета. Однако клеточная миграция зависит не только от статуса клеток, способных к активному движению, но и от состояния окружающих клеток, с которыми взаимодействуют движущиеся клетки. Окружающие клетки или матрикс не просто формируют субстрат для перемещения, но могут также участвовать в пространственно-временной регуляции миграции. В настоящее время нет точных представлений о генетических механизмах этой регуляции. Чтобы определить роль клеточного окружения в регуляции индивидуальной клеточной миграции, в настоящей работе мы изучали миграцию клеток зародышевой линии (КЗЛ) в раннем эмбриогенезе *Drosophila melanogaster*. В норме КЗЛ обособляются на 3-й стадии эмбриогенеза на заднем полюсе эмбриона. Во время гаструляции (6–7-я стадии) КЗЛ в виде консолидированной группы пассивно перемещаются внутрь эмбриона и оказываются в кармане первичной кишки. Далее КЗЛ индивидуализируются, приобретают амебоидную форму, активно перемещаются сквозь эпителий кишки и мигрируют в 5–6-й брюшные сегменты эмбриона, где формируют парные первичные гонады. Мы провели скрининг генов, экспрессирующихся в окружающем КЗЛ эпителии в раннем эмбриогенезе и влияющих на их миграцию. Выявили гены *myc*, *Hph*, *stat92E*, *Tre-1* и *hop*, РНК-интерференция которых приводит к преждевременной активной миграции КЗЛ на 4–7-й стадиях эмбриогенеза. Эти гены можно разделить на две группы: модуляторы активности JAK/STAT сигнального каскада, индуцирующего миграцию в КЗЛ, – гены *stat92E*, *Tre-1*, *hop*, и гены, вовлеченные в морфогенез и поляризацию эпителия, т. е. модифицирующие проницаемость эпителиального барьера, – *myc*, *Hph*. Так как снижение количества продуктов каждого из этих генов приводило к преждевременной миграции КЗЛ, то можно сделать вывод, что в норме на ранних стадиях эмбриогенеза соматическое окружение негативно регулирует миграцию клеток зародышевой линии.
Ключевые слова: *Drosophila melanogaster*; эмбриогенез; клетки зародышевой линии; миграция клеток; формирование эмбриональных гонад.

Introduction

Cell migration is an important morphogenetic process necessary at different stages of development and organism functioning. Large-scale migration of cells occurs during the formation of germ layers, then at the stage of differentiation of organs and tissues (Aman, Piotrowski, 2010; Schumacher, 2019). Also, some differentiated cells retain the ability to migrate when performing their specialized functions (Ratheesh et al., 2015; Barros-Becker et al., 2017; Shapouri-Moghaddam et al., 2018). The initiation and maintenance of cell movement state requires the activation of many factors involved in the regulation of transcription, signal transduction, adhesive interactions, modulation of membranes and the cytoskeleton (Devreotes, Horwitz, 2015). Molecular bases of these processes are evolutionary conserved with high homology in different cell types and different species. Therefore, various aspects of cell migration and the mechanisms of its regulation have been successfully studied in model organisms, both *in vivo* and *in vitro*. *Drosophila melanogaster* embryo represents an excellent model to study these processes (Reig et al., 2014).

Early *Drosophila* embryo develops as a syncytium. During first 15 min (first stage of embryogenesis), male and female pronuclei fuse and undergo 13 rounds of mitoses. At the third stage of embryogenesis, the first cells are formed. These are primordial germ cells (PGCs) that bud at the posterior pole of the syncytial embryo in the pole plasm region. The rest of the nuclei continues mitoses and acquires cell membranes only at the fifth stage of embryo development during the process of cellularization. During gastrulation, PGCs as a consolidated group are passively internalized from posterior pole to the midgut pocket by the invagination of the embryonic surface. At the tenth stage of embryogenesis, PGCs in the midgut pocket loose cell-cell contacts, individualize and acquire an amoeboid form. At the same time, the process of epithelial to mesenchymal transition (EMT) is activated in midgut primordium cells, which results in partial loss of apical-basal polarity accompanied by diminished intercellular contacts. This allows PGCs to actively move through midgut epithelium and migrate to the region of gonad formation. During active migration, PGCs split up into two groups and coalesce with mesoderm cells in fifth abdominal segment to form paired embryonic gonads (Dansereau, Lasko, 2008; Richardson, Lehmann, 2010).

Earlier, we showed that transcription factor GAGA (GAF), encoded by *Trl* gene in *Drosophila*, participates in the regulation of PGC migration during early embryogenesis (Dorogova et al., 2016). Primordial germ cells of *Trl* mutants, instead of passive translocation as a consolidated group of cells, actively migrate from posterior to the interior of the early embryo. Furthermore, PGCs loose round shape, acquire cytoplasmic protrusions reminiscent of lamellipodia, move chaotically and as a result do not participate in gonad formation. We showed that Trl protein was absent in PGCs, but the effect of their premature migration during early embryogenesis depended on the expression of *Trl* in somatic cells surrounding PGCs (Dorogova et al., 2016). Current study focuses on the identification of the transcription factor GAF target genes, participating in the regulation of PGC migration by surrounding somatic epithelial cells.

Materials and methods

D. melanogaster strain *Hikone AW* – laboratory stock of the Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia) – was used as a wild type. All other fly strains were obtained from National Institute of Genetics (NIG), Japan, and Bloomington Stocks Center, USA. Stock numbers and corresponding genotypes are represented in Table 1. All strains listed in the Table 1 carry genetic constructs for

Table 1. *Drosophila* stocks used in the project

Gene	Stock center	Stock number	Genotype
+/+	Bloomington	4	Wild type (control 1)
tub-GAL4	Bloomington	30029	y[1] w[1118]; P{w[+mC]=tubP-GAL4}LL7 P{ry[+t7.2] = neoFRT}82B/TM6B, Tb[1]
myc	NIG	51454	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03189}attP40
myc	Bloomington	25783	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01761}attP2
stat92E	Bloomington	33637	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00035}attP2
Tre-1	NIG	HMS00433	y[1] sc v[1]; P{TRiP}attP2
Hph	Bloomington	34717	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01196}attP2
Hph	NIG	1114R-2	y[1] sc v[1]; P{TRiP}attP2/TM3,Sb
hop	NIG	2720R-1	y[1] sc v[1]; P{TRiP}attP2
hop	NIG	HMS00779	y[1] sc v[1]; P{TRiP}attP2
for	NIG	10033R-1	y[1] sc v[1]; P{TRiP}attP2
for	NIG	GL00026	y[1] sc v[1]; P{TRiP}attP40
mbc	NIG	10379R-1	y[1] v[1]; P{TRiP}attP40/CyO
mbc	NIG	HMC03172	y[1] sc v[1]; P{TRiP}attP2
pod1	Bloomington	41705	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02270}attP2
pod1	Bloomington	31219	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01774}attP2
rib	NIG	HMC03083	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03083}attP2
rib	NIG	7230R-2	y[1] sc v[1]; P{TRiP}attP2
ptp4E	Bloomington	38369	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01838}attP2
ptp4E	Bloomington	60008	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS05000}attP40
tll	NIG	HMS01316	y[1] sc v[1]; P{TRiP}attP2
tll	Bloomington	27242	y[1] v[1]; P{TRiP.JF02545}attP2
tao	NIG	HMS01226	y[1] sc v[1]; P{TRiP}attP2
tao	Bloomington	31226	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01803}attP2
shg	Bloomington	27698	y[1] v[1]; P{TRiP.JF02769}attP2/TM3, Sb1
shg	NIG	HMS00693	y[1] sc v[1]; P{TRiP}attP2
Sdc	NIG	10497Rb-2	y[1] sc v[1]; P{TRiP}attP2
bbg	NIG	HMJ23903	y[1] v[1]; P{TRiP.JF02769}attP2/TM3, Sb1
unc-115a	NIG	31352R-3	y[1] sc v[1]; P{TRiP}attP2
Crk	NIG	HMJ22995	y[1] sc v[1]; P{TRiP}attP2

ectopic RNA interference (RNAi) of corresponding gene. To induce RNAi, flies carrying RNAi construct under the UAS promoter were crossed to flies carrying *tub*-GAL4, ubiquitously expressing GAL4 transcription factor that specifically binds to UAS sequence and induces expression. Flies were maintained on a standard agar/corn media at 25 °C.

Dissection, fixation and immunostaining of embryos were described earlier (Dorogova et al., 2014). Mated females were let to lay eggs overnight, after which embryos were collected, fixed and stored in methanol at –20 °C. For the analysis, embryos were rehydrated and immunostained. Following antibodies were used: primary rabbit anti-Vasa (1:30, SC-30210, Santa Cruz Biotechnology), secondary anti-rabbit Alexa 568 (1:300, Molecular probe, A-11011). After incubation with antibodies embryos were stained with DAPI (2 mg/ml in 1 × PBS, pH 7.4) for 5 min and mounted in Mowiol containing 10 % DABCO. For each combination (RNAi of gene induced by ubiquitous *tub-GAL4*) 400–500 embryos of different stages were analyzed. Wild type flies *Hikone AW*, *tub-GAL4* flies and fly strains listed in Table 1 in the absence of RNAi induction were used as controls. 100–200 embryos at different stages were analyzed for each control group. Slides were analyzed using AxioImager Z1

equipped with ApoTome (Zeiss, Germany) and AxioCam MR (Zeiss).

Bioinformatic analysis was performed using following databases: FlyExpress, Fly-FISH, Berkeley Drosophila Genome Project and DRoID. By comparing results from the above databases, we obtained a list of genes expressed at the desired stage of embryogenesis and potentially regulated by GAGA transcription factor. The analysis of non-canonical binding sites was performed using SITECON (Omelina et al., 2011). Regulatory elements 500 base pairs upstream of the transcription start site were used for the analysis.

Results

In our previous work we showed that mutants for *Trl* gene, which encodes transcription factor GAGA (GAF), had defects in PGC migration during embryogenesis (Dorogova et al., 2016). The absence of GAF resulted in premature transepithelial movement of PGCs from the posterior pole to the interior part of the embryo. We found that the effect of early PGC migration depended on zygotic expression of *Trl* in somatic cells of posterior embryo pole. Since GAF is a transcription regulator, we reasoned that its effect on PGC migration is mediated via its target genes. To date, experimental data exist on binding of GAF with promoters of about 300 genes (van Steensel et al., 2003; Omelina et al., 2011) participating in a wide range of fundamental cell processes.

In this work, to determine the role of cell environment in regulating the individual cell migration during early embryo development, we conducted a screen for genes that expressed in somatic cells, surrounding PGCs during 4–6 stages of embryogenesis. This pattern is characteristic of *Trl* expression at the indicated stages, based on published data and Berkley Drosophila Genome Project database (https://insitu.fruitfly.org/cgi-bin/ex/insitu.pl). The set of 81 genes was identified that included *stat92E*, *hop*, *Trl* and *Tre-1* – well known regulators of cell migration (Kunwar et al., 2003, 2008; Li et al., 2003; Sheng et al., 2009; Dorogova et al., 2016). According to database DroID, 67 % of the selected genes represent potential targets of transcription factor GAF. Therefore, we analyzed these genes for the presence of GAF binding sites using SITECON (Omelina et al., 2011). Regulatory elements 500 base pairs upstream of the transcription start site were used for the analysis. As a result, we found 39 potential GAF binding sites of the GAGnGAG type, and 68 of the GAGnnnGAG type in the region –500…+1. As an initial verification of identified binding sites functionality we analyzed the presence of GAF binding peaks using ModEncode database (Embryo_0_12h_GAF_ChIP_chip; http://www.modencode.org/). For the majority of genes (32 from 39 for GAGnGAG sites and 62 from 68 for GAGnnnGAG sited) GAF binding was observed during embryogenesis (for two genes, the data were absent).

Interestingly, the density of GAF binding sites distribution in –500…+1 region of genes expressing in early embryogenesis in somatic cells surrounding PGCs, was not

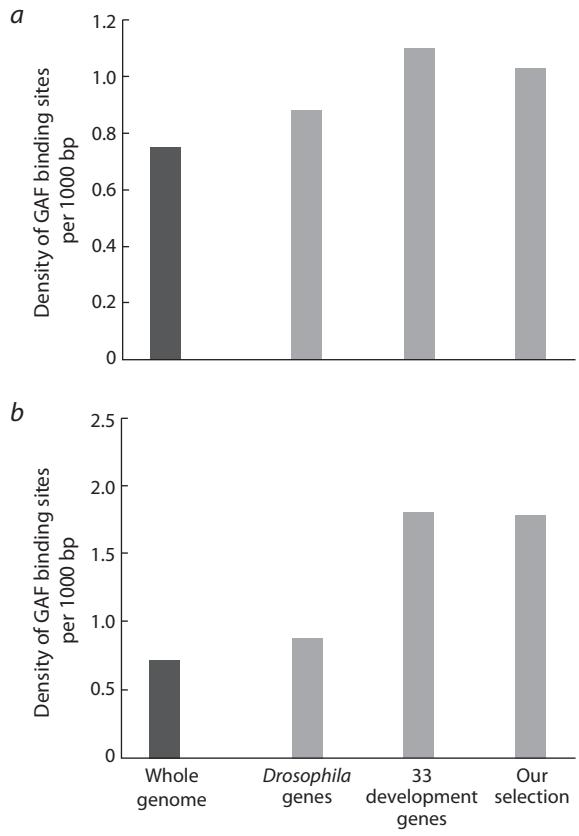

Fig. 1. The distribution density of potential GAF binding sites (per 1000 nucleotides) of the GAGnGAG (*a*) and GAGnnnGAG (*b*) types in various gene sets.

The black column is the density of GAF binding sites throughout whole *Drosophila* genome; the light gray columns are the density of GAF sites in the promoter regions (–500…+1) of various gene sets: 6947 random *Drosophila* genes, 33 *Drosophila* development genes (Omelina et al., 2011), and our set of genes which expressed in the epithelial cells at the posterior pole of 4–6 stage embryos.

random (Fig. 1) and exceeded the average density of such sites throughout whole *Drosophila* genome. For example, density of GAGnnnGAG sites for our gene set was more than twice higher compared to the genome-wide distribution density (see Fig. 1, *b*). What is more intriguing is that the density of GAF binding sites in our set of genes was similar to that of *Drosophila* development genes. Our gene set consisted of genes expressed in early embryogenesis, however, most of them were not early development genes.

Next we analyzed the migration of PGCs during RNAi of genes from our gene set. To induce RNAi we used the GAL4/UAS system with ubiquitous *tub-GAL4* driver (Table 2, Fig. 2). For many genes, transgenic fly stocks with RNAi constructs against different parts of the gene were available. In such cases we used all available stocks in independent experiments. *Hikone AW* wild type stock, *tub-GAL4* stock and stocks carrying *UAS-RNAi* (in the absence of GAL4 induction) were used as controls. In all controls premature PGC migration was not observed.

Premature PGC migration in early embryogenesis was observed during RNAi of genes *myc* (~90 %, n^* = 60;

Table 2. PGC migration phenotypes during RNA interference of the corresponding gene

Gene, stock number	Expression pattern at 1–5 stages of embryogenesis	RNA-interference phenotype
myc 51454	1–4 stages – ubiquitously*, stage 5 – epithelial cells at embryo poles	Premature PGC migration at the stage 5
myc 25783		Premature PGC migration at the stage 5
mbc 51446	1–4 stages – ubiquitously*, stage 5 – all blastoderm cells, except PGCs	No defects in early migration. However, PGCs loose orientation after the exit from the midgut primordium at the stage 11
mbc 10379R-1		No defects in early migration. Small defects in migration direction at the stage 11
hop 32979	1–4 stages – strong ubiquitous expression*, stage 5 – weak ubiquitous expression, including PGCs	Defects in PGCs exit from the midgut primordium at the stage 10
hop 00779		Defects in PGCs exit from the midgut primordium at the stage 10
stat92E 33637	1–5 stages – ubiquitously*	Premature migration at early stages and during the middle embryogenesis. Some cells remain in the midgut pocket at 10–11 stages
Hph 1114R	1–4 stages – ubiquitously*, vanished to the stage 5	High level of embryonic lethality, many embryos do not survive to gastrulation stage. Survived embryos have defects in epithelium formation; PGCs randomly spread over the embryo surface
Hph 34717		High level of embryonic lethality during middle embryogenesis. Survived embryos show defects at early and late stages; PGCs spread over the embryo surface
Tre-1 3171R	1–5 stages – posterior pole of the embryo, pole plasm region	One third of the embryos die. Abnormalities in gastrulation; PGC migration is defective at both early and middle stages
Tre-1 HMS00433		One third of the embryos die. Abnormalities in gastrulation; PGC migration is defective at both early and middle stages
ptp4E 60008	1–5 stages – ubiquitously*, with gradual decrease	Defects in PGC migration at 10–11 stages, some cells remain in the midgut pocket
ptp4E 38369		Some cells remain in the midgut pocket at 10–11 stages
pod1 31219	1–5 stages – ubiquitously*	No defects
pod1 41705		No defects
rib 7230R-2	1–4 stages – no expression, stage 5 – epithelial cells at both embryo poles	No defects
rib 50682		No defects
for 10033R-1	1–3 stages – ubiquitously*, stage 4 – ubiquitously, except PGCs, stage 5 – epithelial cells at the posterior embryo pole	No defects
for GL00026		No defects
tll HMS01316	1–4 stages – no expression, stage 5 – pole plasm	No defects
tll 27242		No defects
tao HMS01226	1–4 stages – ubiquitously*, stage 5 – PGCs	No defects
tao 31226		No defects
shg 27698	1–5 stages – ubiquitously*	No defects
shg HMS00693		No defects
bbg HMJ23903	1–4 stages – no expression, 5–6 stages – epithelial cells at the posterior embryo pole	No defects
unc-115a	1–4 stages – ubiquitously*, degrades by the stage 5	No defects
Sdc 10497	1–6 stages – ubiquitously*, except PGCs	The embryo degrades, and PGCs die at early stages, no migration defects
Crk 22995	1–6 stages – ubiquitously*	Defects at the stage 10 during migration through midgut epithelium. Some PGCs remain in the midgut pocket

* Ubiquitously – means the localization of mRNA of the corresponding gene throughout the whole volume of the embryo including pole plasm region at 1–3 stages and the formed PGCs at 4–6 stages (unless otherwise indicated).

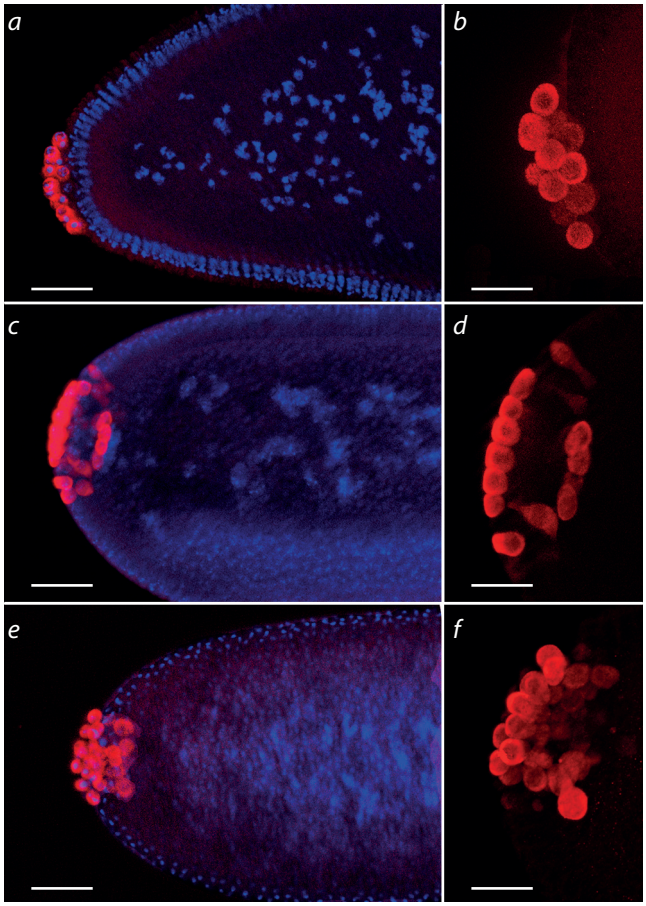

Fig. 2. Primordial germ cells at 4–5 stage of embryonic development in control (*a*, *b*), in *tub-GAL4/UAS-myc-RNAi* (*c*, *d*) and *tub-GAL4/UAS-Hph-RNAi* (*e*, *f*).

a, *b* – primordial cells are located at the posterior pole of the embryo and have a spherical shape typical for this stage; *c*, *d* – premature germ cells migration in *tub-GAL4/UAS-myc-RNAi embryos*; *e*, *f* – premature germ cells migration in *tub-GAL4/UAS-Hph–RNAi* embryos. The nuclei are stained with DAPI, Germ cells – antibodies to the VASA protein. Scale: *a*, *c*, *e* – 30 μm; *b*, *d*, *f* – 50 μm.

* hereinafter *n* – the number of viewed embryos of genotype *tub-GAL4/UAS–RNAi* at 4–6 stage of embryogenesis), *Hph* (~90 %, *n* = 45), *stat92E* (~90 %, *n* = 50), *Tre-1* (~70 %, *n* = 40) and *hop* (~40 %, *n* = 40). In addition to defects in migration at early stages, RNAi of *stat92E*, *Tre-1* and *hop* genes also resulted in PGC migration defects during middle embryogenesis, at stages 10–11. Several genes, *shg*, *hop*, *mbc*, *Hph*, *ptp4E* and *Crk*, were found to be involved in PGC migration through primordium midgut epithelium at stages 10–11. We did not reveal any effect of RNAi of *tll*, *tao*, *for*, *bbg*, *unc-115a*, *Sdc*, *pod1* and *rib* genes on PGC migration in embryogenesis.

Discussion

We discovered the premature PGC migration phenotype that depended on the transcription factor GAF (encoded by *Trl* gene in *Drosophila*) expression in somatic cells surrounding PGCs (Dorogova et al., 2016). This phenomenon prompted us to search for other genes regulating PGC migration in early embryogenesis. Interestingly, 67 % of genes that expressed in epithelial cells surrounding PGCs at 4–6 stages of embryo development are potential targets of transcription factor GAF, which suggests its active role in PGC migration regulation. We compared our data with data on transcriptional regulation of other types of migrating cells in *Drosophila* embryo (Bae et al., 2017): caudal visceral mesoderm (precursors of longitudinal muscles of the gut, they migrate collectively), and hemocytes (the *Drosophila* equivalent of blood cells, they migrate individually). Among 73 genes common for somatic migrating cells, the expression of 64 genes (88 %) can be regulated by GAF. Also, it is worth mentioning that the distribution density of GAF binding sites in the promoters of genes from our gene set was two times higher than the random density, and corresponded to the density in early development genes (Omelina et al., 2011). Therefore, we propose that GAF regulates not only embryonic cell migration, but also early *Drosophila* development in general.

Since genes participating in the control of embryonic cell migration were enriched with targets of GAF transcription factor, we further selected genes involved in PGC migration regulation from surrounding somatic cells. We analyzed 17 genes from obtained gene set for their effect on PGC migration, and revealed a number of genes that negatively regulated this process. We showed that RNA interference of *myc*, *Hph*, *stat92E*, *Tre-1* and *hop* genes resulted in premature transepithelial migration of PGCs in early embryogenesis. The involvement of last three genes was known before: *hop* (*hopscotch*) and *stat92E* encode JAK kinase and transcription factor STAT92E (Signal Transducer and Activator of Transcription), respectively. The products of both of these genes activate evolutionary conserved JAK/STAT signaling pathway that induces cell migration in *Drosophila*, mice and humans (Li et al., 2003; Silver et al., 2005). Premature PGC migration that we observed during RNAi of *hop* is in agreement with J. Li and colleagues (2003) who showed that *hop* expressed only in somatic cells of the embryo, and its mutations resulted in hyperactivation of STAT92E and premature PGC migration. It should be noted that to initiate migration, JAK/STAT pathway needs to be activated in PGCs themselves. During early embryogenesis, JAK/STAT signaling cascade in PGCs is activated by Tor kinase (Li et al., 2003). In somatic cells surrounding PGCs, this cascade is activated by JAK kinase and seems to control the reorganization of cytoskeleton and polarization of epithelial cells.

The role of JAK/STAT in epithelium morphogenesis and polarization has been shown for many tissues including *Drosophila* embryonic intestine (Josten et al., 2004). *Tre-1* is also a part of JAK/STAT pathway and it encodes G protein-coupled receptor (GPCR) – transmembrane chemoattractant receptor (Kunwar et al., 2003, 2008; Sheng et al., 2009). The activation of *Tre-1* initiates significant intracellular rearrangements – the cell changes its polarization and cytoskeleton regulation, which altogether creates the conditions for active cell movement. It has been shown that *Tre-1* is also important for cell polarization at early stages

of embryo development (Richardson, Lehmann, 2010). We hypothesize that the RNA interference of *Tre-1* leads to the weaker polarization of epithelial cells and, as a result, to increased permeability of epithelium for migrating cells.

The most interesting for us is the result on the role of *myc* and *Hph* in the regulation of PGC migration. *myc* (or *dm – diminutive*) encodes the well-known transcription factor homologous to vertebrate Myc protooncogene, which is important for cell proliferation and growth. Surprisingly, recent genetic screen for modulators of tumor invasion identified Myc as a negative regulator that blocked tumor invasion and metastasis (Ma et al., 2017). Ectopic expression of human cMyc potently suppressed JNK-dependent cell invasion and migration in both *Drosophila* and lung adenocarcinoma cell lines (Ma et al., 2017). The authors showed that Myc, together with its transcriptional partner, upregulated the expression of tyrosine kinase *puc* (*puckered*), which, on the one hand, is involved in polarization and morphogenesis of epithelial cells, and, on the other hand, inhibits JNK signalling pathway critical for tumor invasion and cell migration (Ma et al., 2017). Therefore, the decrease of Myc in somatic cells might promote PGC migration due to the increase in the permeability of surrounding epithelium.

Hph (*HIF prolyl hydroxylase*) encodes HIF prolyl-4-hydroxylase which acts as an oxygen sensor. Mutations in *Hph* result in defects in embryonic tracheal development. In *Drosophila* oogenesis, *Hph* regulates border cell migration speed in a dose-dependent manner: overexpression of *Hph* increases border cell migration, whereas *Hph* depletion has opposite effect (Doronkin et al., 2010). In addition, *Hph*-mutant mosaic clones show diminished expression levels of *slbo* (*slow border cells*) – key regulator of border cell migration and *shg* (*shotgun*) – gene encoding cell adhesion protein DE-cadherin (Doronkin et al., 2010).

To sum up, we revealed negative regulators of PGC migration in early embryogenesis that falls into two categories: modifiers of JAK/STAT signaling pathway activity that induces cell migration in many organisms, and genes involved in epithelium polarization and morphogenesis. Genes in the first group normally reduce the invasiveness of migrating primordial germ cells, whereas genes in the second group are responsible for the impermeability of the epithelial layer.

Conclusion

In this work we performed screen for genes involved in primordial germ cell migration during early embryo development. A key feature of our screen was that we searched for the regulators not in migrating PGCs themselves but in surrounding somatic cells. We identified five genes, *myc*, *Hph*, *stat92E*, *Tre-1* and *hop*, whose RNA interference resulted in a premature PGC migration in early embryogenesis. The premature migration phenotype demonstrates that surrounding cells, somatic cells in particular, not only form a substrate for PGCs movement, but can also actively regulate the migration itself. This aspect of migration regulation is poorly studied and far from being well-understood. Nevertheless, it requires further detailed studies because of homology with other types of individual cell migration, including cell migration during metastasis.

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Влияние диеты с повышенным содержанием жира на липидный профиль ооцитов мышей

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Аннотация. Существуют предпосылки того, что у женщин с ожирением возможно снижение качества ооцитов. При этом остается неясным, как связано это изменение с ожирением: опосредованно или напрямую, через изменение содержания и/или состава липидов в ооцитах. Целью настоящей работы было изучение на мышах влияния богатой жирами диеты, применяемой к самкам-донорам, на качественный состав и общее количество липидов в незрелых и созревших *in vivo* ооцитах. Установлено, что диета, богатая липидами, приводит к увеличению массы тела самок мышей по сравнению с контролем ($p < 0.001$; 44.77 ± 1.46 и 35.22 ± 1.57 соответственно), а также уровня холестерина ($p < 0.05$; 2.06 ± 0.10 и 1.78 ± 0.10 соответственно) и триглицеридов ($p < 0.05$; 2.13 ± 0.23 и 1.49 ± 0.21 соответственно) в крови этих животных. Эта диета не повлияла на степень ненасыщенности внутриклеточных липидов незрелых (0.207 ± 0.004 в эксперименте и 0.206 ± 0.002 в контроле) и зрелых ооцитов (0.212 ± 0.005 в эксперименте и 0.211 ± 0.003 в контроле). При созревании ооцитов *in vivo* наблюдалось возрастание содержания внутриклеточных липидов. В зрелых ооцитах количество липидов было больше в экспериментальной группе по сравнению с контролем ($p < 0.01$; 8.15 ± 0.37 и 5.83 ± 0.14 соответственно). Выявлено увеличение количества внутриклеточных липидов при созревании ооцитов как после стандартной диеты ($p < 0.05$; 4.72 ± 0.48 и 5.83 ± 0.14 соответственно), так и после диеты, богатой жирами ($p < 0.001$; 3.45 ± 0.62 и 8.15 ± 0.37 соответственно). Таким образом, при созревании ооцитов мышей *in vivo* возрастает содержание внутриклеточных липидов, богатая жирами диета приводит к повышенному содержанию липидов в зрелых ооцитах.

Ключевые слова: мыши; диета; ооциты; внутриклеточные липиды; нильский красный; конфокальная лазерная сканирующая микроскопия; спектроскопия комбинационного рассеяния света.

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Effects of a high-fat diet on the lipid profile of oocytes in mice

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Abstract. There are evidences that obese women exhibit a detrimental oocyte quality. However, it remains unclear how this change is associated with obesity, indirectly – or directly through a change in the content and/or composition of lipids in oocytes. The aim of this work was to study effects of a high-fat diet applied to female donor mice on the amount and qualitative composition of lipids of immature and *in vivo* matured oocytes. A high-fat diet caused larger body weight in female mice compared with the control ($p < 0.001$; 44.77 ± 1.46 and 35.22 ± 1.57, respectively), and increased the blood levels of cholesterol ($p < 0.05$; 2.06 ± 0.10 and 1.78 ± 0.10, respectively) and triglycerides ($p < 0.05$; 2.13 ± 0.23 and 1.49 ± 0.21, respectively). At the same time, this diet does not affect the level of unsaturation of lipids in immature (0.207 ± 0.004 in the experiment and 0.206 ± 0.002 in the control) and matured oocytes (0.212 ± 0.005 in the experiment and 0.211 ± 0.003 in the control). Total lipid content increased during *in vivo* maturation of mouse oocytes. The amount of lipids was greater in mature oocytes in the experimental group compared to the control ($p < 0.01$; 8.15 ± 0.37 and 5.83 ± 0.14, respectively). An increase in intracellular lipid amount during oocyte maturation was revealed both after a standard diet ($p < 0.05$; 4.72 ± 0.48 and 5.83 ± 0.14, respectively) and after a

fat-rich diet ($p < 0.001$; 3.45 ± 0.62 and 8.15 ± 0.37, respectively). Thus, during *in vivo* oocyte maturation in mice the content of intracellular lipids enhanced, the high-fat diet aggravated this dynamics of lipid increase during *in vivo* maturation of oocytes.
Key words: mice; diet; oocytes; intracellular lipids; Nile Red; confocal laser scanning microscopy; Raman spectroscopy.

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Введение

Ооцит и клетки кумулюса, составляющие кумулюс-ооцитный комплекс (КОК), содержат липидные гранулы (ЛГ), которые связаны с другими органеллами, участвующими в клеточном метаболизме (Kruip et al., 1983; Dunning et al., 2014; Ellenrieder et al., 2016). Предыдущие исследования, проведенные при помощи световой и электронной микроскопии, выявили связь между эндоплазматическим ретикулумом, митохондриями и ЛГ в ооцитах крупного рогатого скота; эти кластеры назвали метаболическими единицами (Kruip et al., 1983). Тесная связь между этими органеллами способствует клеточному метаболизму, в частности β-окислению липидов (Ellenrieder et al., 2016). Цитоплазматические ЛГ являются хранилищами жиров, которые могут быть использованы в качестве энергетического субстрата (Thiam et al., 2013). Гидрофобное содержимое этих гранул, состоящее в основном из триацилглицеридов и сложных эфиров стеролов, таких как холестерин, окружено монослоем фосфолипидов (Walther, Farese, 2009). В настоящее время ЛГ считают активными внутриклеточными структурами, играющими важную роль в клеточном гомеостазе (Walther, Farese, 2009; Welte, Gould, 2017). Кроме того, недавние исследования показали их протекторную, а также регуляторную функцию, в частности их участие в белковом метаболизме и работе ядра (Welte, Gould, 2017).

Влияние ожирения на качество ооцитов – важный медицинский аспект. Избыточная масса тела отрицательно сказывается на репродуктивном здоровье людей, о чем свидетельствуют клинические данные, полученные при применении вспомогательных репродуктивных технологий – ВРТ (Robker, 2008; Souter et al., 2011; Dickey et al., 2012). Установлено, что у женщин с ожирением наблюдается снижение качества ооцитов в циклах ВРТ по сравнению с имевшими нормальный вес тела (Robker, 2008). Между тем не ясно, связано ли это с избыточной массой тела пациенток либо с изменением содержания и состава липидов в ооцитах (Pantasri et al., 2015).

Эффект обогащенной липидами диеты на развитие ооцитов подтверждается и в экспериментах на различных видах животных (Zeron et al., 2002; Minge et al., 2008; Wu et al., 2010; Dunning et al., 2014). Так, в работе на мышах, в которой самок держали на диете с повышенным содержанием жиров, продемонстрировано, что у особей с вызванным таким питанием ожирением было низкое качество ооцитов, а эмбрионы из них хуже развивались в культуре *in vitro* (Minge et al., 2008). Однако до сих пор остается неизвестным, что именно вызывает эти изменения. Цель нашей работы – изучение на мышах влияния богатой жирами диеты, применяемой к самкам-донорам, на качественный состав и общее количество внутриклеточных липидов незрелых и зрелых ооцитов мышей.

Материалы и методы

Экспериментальные животные. В исследовании для получения незрелых и зрелых ооцитов использовано 56 самок-доноров мышей линии CD1 в возрасте 2.5 мес. (16 в контроле и 40 в экспериментальной группе) и 6 стерильных самцов этой же линии. Животных содержали в клетках с подстилкой из опилок в стандартных условиях конвенционального вивария Института цитологии и генетики СО РАН (Новосибирск, Россия): при комфортной температуре 22–24 °С, свободном доступе к полнорационному сухому гранулированному корму для лабораторных грызунов «Чара» (ЗАО «Ассортимент-Агро», Россия) и очищенной воде, 12:12-часовом цикле дня : ночи. Все эксперименты на животных одобрены Комиссией по биоэтике Института цитологии и генетики СО РАН (протокол № 5 от 13.05.2011) и соответствуют Европейской конвенции о защите позвоночных животных, используемых для экспериментальных и других научных целей.

Оценка эффективности диеты. Самок-доноров содержали как на стандартной диете (контроль), так и на специализированной, когда дополнительно к обычному корму животным добавляли свиное сало и семена подсолнечника (экспериментальная группа). Пищевые добавки начинали давать с возраста пяти недель. Откормочный эксперимент длился в течение восьми недель. Для подтверждения эффективности диеты мышей обеих групп взвешивали перед эвтаназией, а также у самок натощак собирали кровь после декапитации, центрифугировали при 3 250 об/мин (1000 g) в течение 5 мин, после чего собирали плазму и оценивали уровень холестерина и триглицеридов с использованием наборов Холестерин-Ново (АО «Вектор-Бест», Россия) и Триглицериды-Ново (АО «Вектор-Бест»), как рекомендовано производителем.

Незрелые ооциты получали от 10 самок в контрольной и 30 – в экспериментальной группе. Животных на стадии проэструса подвергали эвтаназии при помощи декапитации. Яичники извлекали и измельчали в среде Flushing Solution (FertiPro, Бельгия). Выделенные КОК оценивали под стереомикроскопом Leica S8 APO с увеличением ×80 (Leica Microsystems, Германия). Для исследования брали только те КОК, в которых было не менее пяти слоев кумулюсных клеток, плотно прилегающих к прозрачной оболочке ооцита (Hillier et al., 1985). Если КОК имели существенные дефекты, то их отбраковывали.

Получение стерильных самцов. Стерилизацию самцов проводили путем вазэктомии не менее чем за две недели до начала эксперимента, как было описано ранее (Hogan et al., 1994). Самцов мышей линии CD1 в возрасте шести недель наркотизировали при помощи внутрибрюшинного введения 0.25 мг/кг препарата медетомедина гидрохлорида (Domitor 1 mg/mL, Orion-Corporation, Фин-

ляндия) и через 10 мин – 50 мг/кг препарата золетила (Zoletil, SA, Virbac Sante Animale, Франция). После наркотизации подкожно вводили антибиотик: 0.01 мл амоксициллина тригидрата 150 мг/мл (ОАО «Синтез», Россия). Затем животных помещали на подогреваемый столик, шерсть в зоне операционного поля сбривали, а кожу обрабатывали 70 % этиловым спиртом. При помощи хирургических ножниц делали горизонтальный надрез кожных покровов мошонки длиной ~5 мм. Подтягивали эпидидимисы к краю хирургической раны и разворачивали их так, чтобы были видны семенные канатики. Семявыносящие каналы отделяли от сопряженных тканей и пережигали раскаленным пинцетом в двух местах, удаляя участок канала между ними. Эпидидимисы возвращали в первоначальное положение. Затем в рану засыпали 2 мг амоксициллина тригидрата (ОАО «Синтез»). Зашивали надрезы наложением двух швов и обрабатывали их Раносаном (ООО «Апи-Сан», Россия).

Получение зрелых ооцитов. Для выделения зрелых ооцитов проводили стерильное спаривание с использованием шести вазэктомированных самцов. В эксперименте участвовало 16 самок (6 в контроле и 10 в экспериментальной группе). Зрелые ооциты выделяли через 20–22 ч после стерильного спаривания. С этой целью выполняли эвтаназию самок-доноров при помощи декапитации, извлекали яичники с прилегающими к ним яйцеводами. Органы переносили в питательную среду M2 (Merck, Германия), разрезали ампулярную часть яйцевода и извлекали зрелые ооциты. Для удаления кумулюсных клеток использовали гиалуронидазу (Merck) в концентрации 80 МЕ/мл (Brinster, 1971).

Определение степени ненасыщенности липидов. Методом рамановской спектроскопии (комбинационного рассеяния света – КРС) было выполнено сравнение ненасыщенности липидов в незрелых и зрелых ооцитах. Для исследования изменений в степени ненасыщенности липидов измеряли спектры КРС в диапазоне от 1000 до 3000 $см^{-1}$. Для каждого ооцита измеряли от 30 до 60 спектров КРС от разных локальных областей клетки. Латеральный и продольный размеры областей, от которых измеряли комбинационное рассеяние света, составляли ~1 и 10 мкм соответственно. Для каждого отдельного набора данных с использованием метода главных компонент выделяли вклад липидов, аналогично подходу, применявшемуся ранее на эмбрионах мыши (Okotrub et al., 2017). Для характеризации ненасыщенности углеводородных цепочек липидов исследовали соотношение интенсивностей пиков КРС, относящихся к валентным колебаниям двойных C=C связей (~1660 $см^{-1}$) и симметричным валентным колебаниям метиленовых групп (2850 $см^{-1}$). Ооциты переносили в стеклянный контейнер с лункой, глубиной 300 мкм в капле среды KSOM (Merck), покрывали тонким листом слюды и герметизировали. Измерения осуществляли с использованием лабораторной экспериментальной установки, состоящей из модифицированного микроскопа (Orthoplan, Leitz, Германия) и решеточного монохроматора SP2500i (Princeton Instruments, Trenton, NJ, США), оснащенного многоканальным детектором Spec-10:256E/LN (Princeton Instruments). Точность определения абсолютной частоты КРС была более чем 1 $см^{-1}$; спектральное разрешение составляло 2.5 $см^{-1}$. Для возбуждения КРС использовали излучение твердотельного лазера (Excelsior, Spectra Physics, США) с длиной волны 532.1 нм.

Оценка общего количества липидов ооцитов. Оценка изменения внутриклеточного состава липидов в ооцитах мышей после содержания самок-доноров на двух диетах проведена при помощи окрашивания флуорохромом нильским красным – Nile Red Staining Kit (Merck) с последующей конфокальной лазерной сканирующей микроскопией (КЛСМ). Метод подробно описан ранее (Romek et al., 2011), при этом нами внесены изменения. Ооциты перед исследованием были зафиксированы в 4 % параформальдегиде (Merck) на фосфатном буфере – PBS (Merck) в течение двух часов, затем трижды отмыты в 50 мкл PBS с содержанием одного мг/мл поливинилпирролидона (Merck) по 5 мин каждая. Стоковый раствор флуорохрома Nile Red (1 мг/мл) был приготовлен посредством разведения красителя в диметилсульфоксиде. Перед окрашиванием стоковый раствор разводили до рабочей концентрации 10 мкг/мл. Ооциты инкубировали в рабочем растворе в течение трех часов при 37 °C, чтобы добиться максимальной интенсивности окрашивания внутриклеточных липидов, как описано ранее (Genicot et al., 2005). Материал в капле PBS монтировали на предметные стекла.

Изображения образцов получали с помощью инвертированного конфокального лазерного сканирующего микроскопа LSM 780 NLO Axio Observer Z1 (Zeiss, Германия) с применением программного обеспечения Zen 2012 (Black Edition) (Zeiss). Все образцы фотографировали объективом Plan-Apochromat ×20 (0.8 NA), возбуждение флюорохрома проводили на длине волны 488 нм аргоновым газовым лазером на мощности 0.1 % × 30 мВт ≈ 30 мкВт, главное дихроичное зеркало было выбрано на 488 нм. Разрешение детектора 512 × 512 пикселей, с задержкой на каждый пиксель в 3.15 мкс. Для детекции сигнала использовали GaAsP детектор (Gallium Arsenic Phosphorus). Полная трехмерная визуализация ооцитов была выполнена с помощью опции Z-stack, установленной на толщину оптического среза в 2.5 мкм. Спектры производили на длинах волн 494–687 нм, с шагом в 9 нм. Число оптических срезов – 40, общая толщина всех оптических срезов – 100 мкм. Все изображения были созданы детектированием в режиме счета фотонов. Итоговое изображение представляет из себя трехмерную матрицу, где для каждого элемента имеется информация о числе детектированных фотонов (абсолютное число фотонов – а. ч. ф.). Для вычитания фона делали три изображения на стекле, где отсутствует материал, при тех же условиях, что были описаны выше, с последующим вычислением среднего. Все оптические срезы (как образца, так и фона) суммировали с применением скрипта для ImageJ, чтобы сформировать итоговое изображение. Для вычитания фоновой флуоресценции использовали Python 3.8 с библиотекой OpenCV.

Статистический анализ. Данные анализировали посредством языка программирования R 3.6.2 и с графической оболочкой RStudio Desktop 1.1.463. Анализ на нормальность распределения данных проводили критерием согласия Андерсона–Дарлинга, с учетом коэффициентов эксцесса и асимметрии. Корреляцию между массой тела

и уровнем холестерина в крови самок-доноров ооцитов, а также между массой тела самок-доноров ооцитов и абсолютным числом фотонов оценивали с использованием ранговой корреляции Стьюдента. Значения представлены как среднее ± SEM. Различия средних между группами оценивали *t*-критерием Стьюдента, а для неравных дисперсий – критерием Кохрана–Кокса. Уровень значимости принимали при $p < 0.05$.

Результаты

Данные по влиянию диеты на массу животных, уровень холестерина и триглицеридов в крови представлены в табл. 1. Установлено, что диета с высоким содержанием липидов статистически достоверно ($p < 0.001$) приводит к увеличению веса тела животных по сравнению с контролем. Диета с повышенным содержанием липидов достоверно ($p < 0.05$) вызывала повышение уровня холестерина и триглицеридов в крови по сравнению с контрольной группой. Имелась достоверная корреляция ($r = 0.68$; $p < 0.01$) между массой тела самок-доноров и уровнем холестерина в их крови.

Результаты по влиянию диеты на степень ненасыщенности липидов в незрелых и зрелых ооцитах приведены в табл. 2. В ходе исследований не выявлено достоверных различий по степени ненасыщенности липидов в незрелых и зрелых ооцитах после содержания самок-доноров на двух разных диетах.

Влияние диеты на содержание липидов в незрелых и зрелых ооцитах продемонстрировано на рис. 1 и 2. В результате проведенной работы не выявлено различий по количеству липидов в незрелых ооцитах после содержания самок-доноров на двух разных диетах. Но в зрелых ооцитах количество липидов было больше ($p < 0.01$) в группе мышей, бывших на диете, богатой жирами, по сравнению с контролем (8.15 ± 0.37 и 5.83 ± 0.14 млн фотонов соответственно). Кроме того, установлено возрастание количества внутриклеточных липидов у зрелых ооцитов, по сравнению с незрелыми, как у самок, находящихся на стандартной диете (5.83 ± 0.14 и 4.72 ± 0.48 млн фотонов соответственно, $p < 0.05$), так и у самок, содержавшихся на диете, богатой жирами (8.15 ± 0.37 и 3.45 ± 0.62 млн фотонов соответственно, $p < 0.001$). Имелась достоверная корреляция ($r = 0.91$, $p < 0.001$) между массой тела самок-доноров и количеством внутриклеточных липидов в ооцитах.

Table 1. Effects of diet on body weight and concentrations of cholesterol and triglycerides in blood of CD1 mice

Parameters	Group (number of animals)	
	Control ($n = 16$)	High-fat diet ($n = 40$)
Body weight, g	35.22 ± 1.57	44.77 ± 1.46***
Cholesterol concentration, mM	1.78 ± 0.10	2.06 ± 0.10*
Triglyceride concentration, mM	1.49 ± 0.21	2.13 ± 0.23*

Differences from the control group are significant at * $p < 0.05$; *** $p < 0.001$.

Table 2. The degree of unsaturation of intracellular lipids in immature and mature oocytes of CD1 mice depending on diet

Oocytes	Group (number of oocytes)	
	Control	High-fat diet
Immature (*n*)	0.206 ± 0.002 ($n = 3$)	0.207 ± 0.004 ($n = 3$)
Mature (*n*)	0.211 ± 0.003 ($n = 6$)	0.212 ± 0.005 ($n = 10$)

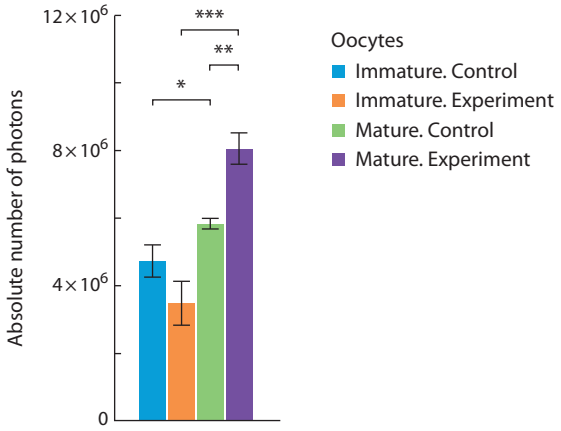

Fig. 1. Total fluorescence intensity of lipids in immature and mature oocytes in CD1 mice.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

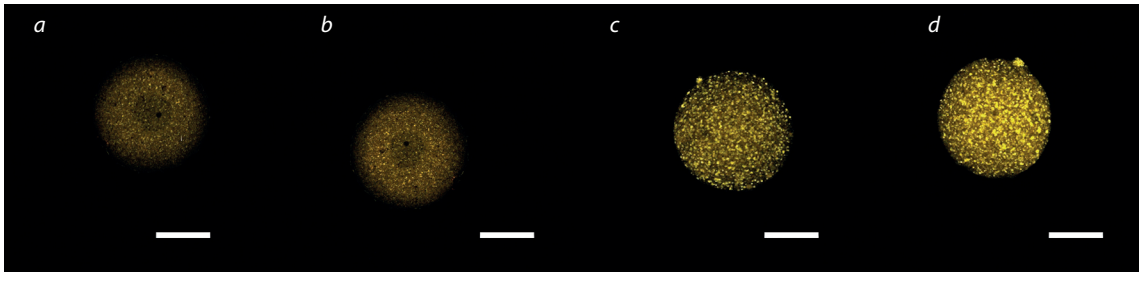

Fig. 2. Confocal laser scanning microscopy of immature and mature oocytes of CD1 mice after staining with Nile Red.

(*a*) Immature oocytes, control group; (*b*) immature oocytes, experimental group; (*c*) mature oocytes, control group; (*d*) mature oocytes, experimental group.
Color corresponds to the wavelength of intracellular lipids. Scale bar = 30 μm.

Обсуждение

В ходе проведенных экспериментов установлено, что диета с повышенным содержанием липидов приводит к увеличению массы тела мышей, а также повышению уровня холестерина и триглицеридов в их крови, что соответствует данным других исследователей (Ma et al., 2012; Li et al., 2018). В настоящее время есть несколько работ, в которых ученые пытаются оценить липидный профиль ооцитов *in vivo* после влияния диеты (Zeron et al., 2002; Wu et al., 2010; Li et al., 2018). Результаты этих работ достаточно противоречивы.

Используемая нами диета не повлияла на качественный состав липидов в незрелых и зрелых ооцитах мышей от самок-доноров с ожирением. Эти данные могут быть обусловлены тем, что диета была сбалансирована по насыщенным и ненасыщенным жирным кислотам, поэтому никаких изменений качественного состава липидов в ооцитах не происходило, как описано ранее в экспериментах со сходным дизайном (Dunning et al., 2014; Amstislavsky et al., 2019). Более того, в работе, проведенной на овцах (Zeron et al., 2002), отмечено, что диета, богатая полиненасыщенными жирными кислотами (с добавлением рыбьего жира), влияла на изменение состава внутриклеточных липидов в клетках кумулюса, но не в незрелых ооцитах, что может быть связано с высоким содержанием в добавке ненасыщенных жирных кислот.

В нашем исследовании обнаружено, что при содержании самок-доноров на диете, богатой жирами, липидов в зрелых ооцитах становилось больше, чем в контроле, т. е. при применении стандартной диеты. Между тем в незрелых ооцитах не было различий по общему количеству липидов при содержании мышей на разных диетах. Некоторые исследования на мышах отмечают возрастание количества липидов в незрелых ооцитах при содержании мышей на жирной диете (Wu et al., 2010). Однако в недавно опубликованной работе (Li et al., 2018) продемонстрировано возрастание числа крупных липидных гранул в клетках кумулюса, но не в самих незрелых ооцитах. Возможно, наблюдаемое в нашем эксперименте отсутствие повышения общего уровня липидов в незрелых ооцитах после специальной обогащенной жирами диеты связано с тем, что эти липиды уходят в другие клетки, в частности кумулюса, в то время как на последующей стадии развития происходит накопление липидов непосредственно в ооцитах.

Противоречия в выводах описанных выше работ, выполненных разными группами исследователей, могут быть связаны с различиями между линиями, а также с тем, что употреблялись корма, отличающиеся по своему составу. В целом можно сказать, что, в отличие от многочисленных работ, в которых используется модель изменения качественного и количественного состава липидов в репродуктивных клетках, культивируемых *in vitro* (Брусенцев и др., 2019; Amstislavsky et al., 2019), аналогичные эксперименты в условиях *in vivo* сопряжены с воздействием большого числа различных факторов, что, по-видимому, и приводит к разнородности результатов.

Обнаружена динамика возрастания количества липидов от незрелых ооцитов к зрелым, наблюдавшаяся при обеих диетах. Но более выраженной эта зависимость была при диете с повышенным содержанием жира. Действительно, жирные кислоты, имеющиеся в крови матери, ответственны за липидный профиль фолликулярной жидкости, из которой они поступают в ооцит через окружающие его клетки кумулюса (Valckx et al., 2014). Ранее с помощью микро- и спектроскопических методов были исследованы распределение, локализация и размер липидных гранул в процессе созревания ооцитов мышей (Dunning et al., 2014; Bradley et al., 2016). В частности, при использовании когерентного антистоксового комбинационного рассеяния выявлено возрастание размера и числа агрегированных ЛГ при созревании ооцитов мышей как *in vivo*, так и *in vitro* (Bradley et al., 2016). Сходная закономерность отмечена в работе на крупном рогатом скоте: полуколичественным методом с применением КЛСМ и флуорохрома BODIPY продемонстрировано достоверное возрастание внутриклеточных липидов при созревании ооцитов *in vitro*, хотя при созревании ооцитов *in vivo* наблюдалась лишь тенденция к такому возрастанию (Collado et al., 2017).

Однако в работе на свиньях показано снижение количества внутриклеточных липидов по мере развития ооцитов *in vivo*: на стадии герминального везикула содержание липидов было на 21 % выше, чем у созревших до стадии метафазы второго деления мейоза (Romek et al., 2011). Таким образом, имеются и видовые отличия в изменении содержания внутриклеточных липидов в ходе созревания ооцитов (Romek et al., 2011; Dunning et al., 2014; Bradley et al., 2016; Collado et al., 2017). В частности, у свиней снижение количества жиров в ходе созревания ооцитов, вероятней всего, связано с их активным расщеплением, так как у этого вида животных липиды могут выступать в качестве основного энергетического субстрата (Bradley, Swann, 2019).

Заключение

Результаты нашего исследования показывают, что увеличение содержания липидов по мере созревания *in vivo* ооцитов мышей может быть усилено при содержании самок-доноров на диете, богатой жиром. Есть вероятность того, что липиды в большом количестве поступают в ооцит мыши из клеток кумулюса при его созревании (Li et al., 2018). Большее количество липидов в зрелых ооцитах, полученных от самок-доноров, содержавшихся на диете, богатой жирами, по сравнению с контролем, по всей видимости, может быть связано с накоплением энергетического субстрата и особенностями метаболизма ожиревших особей, например неспособностью клетки быстро утилизировать поступающие жиры. Имеются примеры того, что фактический состав жирных кислот в ЛГ ооцитов зависит от рациона матери, который определяет жирные кислоты, доступные для ооцита во время развития яичников (Bradley, Swann, 2019).

Таким образом, диета, богатая липидами, приводит к увеличению массы тела самок мышей, а также повышает уровень холестерина и триглицеридов в крови этих животных. Содержание самок-доноров на такой диете не влияет на качественный состав липидов незрелых и зрелых ооцитов. При этом наблюдается повышение общего содержания липидов при созревании ооцитов мышей *in vivo*. Диета, обогащенная жирами, приводит к большему накоплению липидов в зрелых ооцитах.

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Генетические маркеры о распространении древних морских охотников в Приохотье

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Аннотация. Представлен обзор сведений о генетическом полиморфизме современного и древнего населения Севера Азии и Америки с целью реконструкции истории миграций древних морских охотников в Охотоморском регионе. Проанализированы данные о полиморфизме митохондриальной ДНК и распространенности «арктической» мутации – варианта rs80356779-A гена *CPT1A*. Известно, что «арктический» вариант гена *CPT1A* с высокой частотой распространен в современных популяциях эскимосов, чукчей, коряков и других народов Охотоморского региона, хозяйственный уклад которых связан с морским зверобойным промыслом. Согласно палеогеномным данным, самые ранние находки «арктического» варианта гена *CPT1A* обнаружены у гренландских и канадских палеоэскимосов (4 тыс. лет назад), представителей токаревской культуры Северного Приохотья (3 тыс. лет назад) и носителей культуры позднего дзёмона острова Хоккайдо (3.5–3.8 тыс. лет назад). Результаты анализа позволили выявить несколько миграционных событий, связанных с распространением морских охотников в Охотоморском регионе. Самая поздняя миграция, оставившая следы у носителей культуры эпи-дзёмон (2.0–2.5 тыс. лет назад), привнесла с севера Приохотья на Хоккайдо и соседние территории Приамурья митохондриальную гаплогруппу G1b и «арктический» вариант гена *CPT1A*. Следы более ранней миграции, также привнесшей «арктическую» мутацию, зарегистрированы у населения позднего дзёмона Хоккайдо (3.5–3.8 тыс. лет назад). Проведен филогенетический анализ митохондриальных геномов, относящихся к редкой гаплогруппе C1a, встречающейся у населения Дальнего Востока и Японии, но в филогенетическом отношении родственной C1-гаплогруппам американских индейцев. Результаты показали, что дивергенция митохондриальных линий в пределах гаплогруппы C1a происходила в диапазоне от 7.9 до 6.6 тыс. лет назад, а возраст японской ветви гаплогруппы C1a составляет ~5.2 тыс. лет. Пока неизвестно, связана ли эта миграция с распространением «арктического» варианта гена *CPT1A* или же присутствие C1a-гаплотипов у населения островов Японии маркирует собой еще один, более ранний, эпизод миграционной истории, связывающей население северо-западной Пацифики и Северной Америки.
Ключевые слова: митохондриальная ДНК; ген *CPT1A*; популяции человека; палеогеномика; культура морских охотников; Охотоморский регион.

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Genetic markers on the distribution of ancient marine hunters in Priokhotye

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Abstract. This is a review of studies on the genetic polymorphism of modern and ancient populations of the north of Asia and America, with the aim of reconstructing the history of migrations of ancient marine hunters in the Okhotsk Sea region. The data on mitochondrial DNA polymorphism and the "Arctic" mutation distribution – the rs80356779-A variant of the *CPT1A* gene – were analyzed. It is known that the "Arctic" variant of the *CPT1A* gene is widely distributed in modern populations of the Eskimos, Chukchis, Koryaks, and other peoples of the Okhotsk Sea region, whose economic structure is associated with marine hunting. According to paleogenomic data, the earliest cases of the "Arctic" variant of the *CPT1A* gene were found in the Greenland and Canadian Paleoeskimos (4 thousand years ago), among representatives of the Tokarev culture of the Northern Priokhotye (3 thousand years ago), and among the bearers of the culture of the late Jomon of Hokkaido (3.5–3.8 thousand years ago). The results of the analysis revealed several migration events associated with the spread of marine hunters in the Okhotsk Sea region. The latest migration, which left traces on bearers of the Epi-Jomon culture (2.0–2.5 thousand years ago), introduced the mitochondrial haplogroup G1b and the "Arctic" variant of the *CPT1A* gene from the north of Priokhotye to Hokkaido and neighboring territories of the Amur Region. Traces of earlier migration, which also brought the "Arctic" mutation, were recorded in the Hokkaido population of the late Jomon period (3.5–3.8 thousand years ago). A phylogenetic analysis of mitochondrial genomes belonging to the rare haplogroup C1a, found in populations of the Far East and Japan, but phylogenetically related to the C1-haplogroups of the Amerindians, was carried out. The results of the analysis showed that the divergence of

mitochondrial lineages within the C1a haplogroup occurred in the range from 7.9 to 6.6 thousand years ago, and the age of the Japanese branch of the C1a haplogroup is approximately 5.2 thousand years. It is not yet known whether this migration is associated with the spread of the "Arctic" variant of the *CPT1A* gene or the presence of C1a haplotypes in the population of the Japanese islands marks another, earlier, episode of the migration history linking the populations of Northwest Pacific and North America.

Key words: mitochondrial DNA; *CPT1A* gene; human populations; paleogenomics; culture of marine hunters; Okhotsk Sea region.

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Введение

Согласно результатам археологических и палеогеномных исследований, 4.5–2.8 тысячелетия назад в обширном регионе – от Чукотки и Аляски и до Гренландии – существовала общность племен (палеоэскимосов), объединявшая искусных охотников на морских млекопитающих (Rasmussen et al., 2010; Flegontov et al., 2019). Предполагается, что формирование высокоспециализированной культуры морских зверобоев проходило главным образом на американском континенте и связано с развитием «арктической традиции малых орудий», а на Чукотке палеоэскимосские памятники возрастом от 3.5 до 2.9 тыс. лет обнаружены в ограниченном числе мест – на острове Врангеля и поселении Уненен (Гребенюк и др., 2019). По палеогеномным данным, первая миграция предков палеоэскимосов из Сибири в Америку произошла ~5.5 тыс. лет назад (Rasmussen et al., 2010). В генетическом отношении палеоэскимосы ближе всего к современным чукотско-камчатским народам (корякам, чукчам, ительменам), а не американским индейцам (Flegontov et al., 2019). Результатом другой миграции из Сибири в Америку (~4 тыс. лет назад) стало появление собственно современных эскимосов (неоэскимосов) севера Америки, Гренландии и Чукотки (Achilli et al., 2013). Неоэскимосы формировались на палеоэскимосской основе, но, несмотря на объединяющую носителей этих культур традицию морского зверобойного промысла, в генетическом отношении они различались (Rasmussen et al., 2010; Raghavan et al., 2014; Flegontov et al., 2019; Sikora et al., 2019).

Установлены также две волны обратных миграций из Америки на крайний Северо-Восток Азии. Одна из них связана с миграцией палеоэско-алеутских групп ~3.5 тыс. лет назад, а другая – с миграциями неоэскимосов ~2.5 тыс. лет назад (Гребенюк и др., 2019; Flegontov et al., 2019; Grugni et al., 2019; Sikora et al., 2019). В результате первой из указанных обратных миграций на Чукотке возникла палеоэскимосская традиция, а в Северном Приохотье – токаревская культура (Гребенюк и др., 2019). Результатом второй обратной миграции стало развитие неоэскимосских культур в Беринговморье (Raghavan et al., 2014; Flegontov et al., 2019).

Генетические маркеры древних морских охотников

Генетическая реконструкция событий, произошедших на крайнем Севере Азии и Америки, стала возможной благодаря исследованиям распределения вариантов митохондриальной ДНК (мтДНК) и Y-хромосомы, наследуемых по материнской и отцовской линиям соответственно, у современного и древнего населения этого региона. Исследования полиморфизма мтДНК показали, что только несколько митохондриальных гаплогрупп объединяют генетически популяции крайнего Севера Америки (эскимосов) и Северо-Востока Азии (эскимосов, чукчей и коряков) – это гаплогруппы A2a, A2b, D2a и D4b1a2a1a (Derenko et al., 2007; Tamm et al., 2007; Dryomov et al., 2015). Гаплогруппа D2a маркирует собой предков палеоэскимосов, поскольку она характерна для представителей палеоэскимосских культур Саккак и Дорсет, а также токаревской культуры Северного Приохотья, а остальные гаплогруппы связаны уже с относительно недавней неоэскимосской экспансией (Raghavan et al., 2014; Flegontov et al., 2019; Sikora et al., 2019). Аналогично по распределению вариантов Y-хромосомы только гаплогруппа Q-B143 маркирует древний палеоэско-алеутский компонент, отмеченный также у современных коряков, чукчей и юкагиров (Rasmussen et al., 2010; Malyarchuk et al., 2011; Karmin et al., 2015; Grugni et al., 2019). Между тем присутствие мужской гаплогруппы Q-B34 у азиатских эскимосов и коряков связано с обратной миграцией, приведшей к появлению неоэскимосских культур на Чукотке (Grugni et al., 2019).

Из аутосомных генетических вариантов наиболее интересна «арктическая» мутация гена *CPT1A*, кодирующего карнитин пальмитоилтрансферазу типа IA – один из ключевых ферментов транспорта жирных кислот в митохондрии. В результате нуклеотидной замены G→A в локусе rs80356779 гена *CPT1A* образуется аминокислотная замена пролина на лейцин в позиции 479 фермента CPT1A (замена P479L), которая относится к числу патологических, поскольку приводит к понижению ферментативной активности CPT1A (Greenberg et al., 2009). Проведенные исследования установили, что «арктическая» мутация rs80356779-A с высокой частотой распространена только на крайнем Севере Азии и Америки (Rajakumar et al., 2009; Lemas et al., 2012; Clemente et al., 2014; Малярчук и др., 2016). Ее частота составила более 70 % у американских и гренландских эскимосов, 66 – у коряков, 56 – у чукчей и 30 % – у охотских эвенов (Малярчук и др., 2016). Результаты филогенетического анализа протяженных участков гена *CPT1A* продемонстрировали однократное возникновение мутации rs80356779-A у эскимосов, чукчей и коряков (Clemente et al., 2014), а ее появление у эвенов и эвенков связано не с независимым происхождением этого варианта полиморфизма, а с межэтническими контактами (Малярчук и др., 2016).

Обнаружено, что поддержанию высокой частоты «арктического» варианта гена *CPT1A* у эскимосов, чукчей и

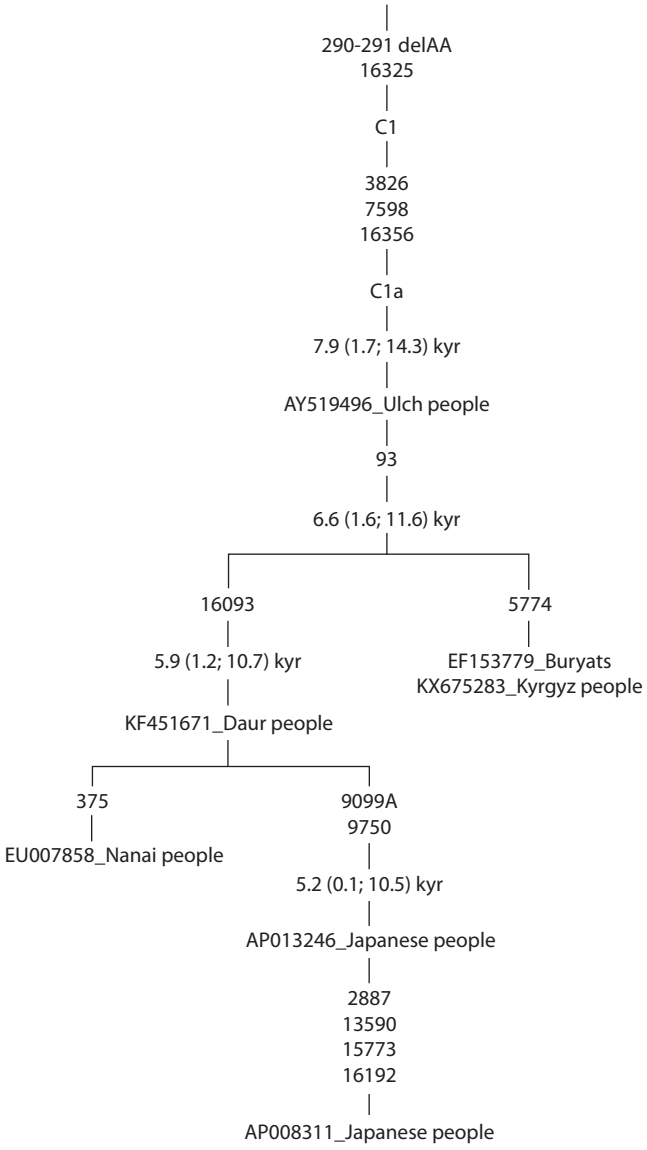
коряков способствовал отбор, связанный, в первую очередь, с адаптацией к традиционной диете морских зверобоев, основанной на потреблении жира и мяса ластоногих и китов (Clemente et al., 2014). По всей видимости, в ответ на высокий уровень кетогенеза, возникающий при постоянном потреблении жирной пищи, у морских охотников произошли некоторые изменения метаболизма, например у них отпала необходимость в высокой активности ферментов метаболизма полиненасыщенных жирных кислот, которыми богаты продукты морского зверобойного промысла. Аминокислотная замена P479L как раз приводит к снижению каталитической активности фермента CPT1A (Greenberg et al., 2009). В результате носители «арктического» варианта в большей степени защищены от кетогенеза, что вполне оправданно при соблюдении традиционной диеты, но в современных условиях (в случае отхода от традиционной диеты) эта аминокислотная замена стала вредной. Так, у эскимосов Северной Америки и Гренландии – носителей «арктической» мутации в гомозиготном состоянии (их частота составляет 40–70 % в различных популяциях) – дефицит фермента CPT1A сопровождается гипокетонной гипогликемией, синдромом внезапной детской смерти, бóльшей предрасположенностью к ожирению, диабету 2-го типа, жировой болезни печени и др. (Greenberg et al., 2009). Вызывает тревогу, таким образом, высокая частота гомозиготного носительства «арктического» варианта» (генотип rs80356779-AA) у коренного населения крайнего Северо-Востока Азии (47 % у коряков, 33 у чукчей и 8 % у охотских эвенов), тем более в условиях отсутствия неонатального скрининга «арктической» мутации у новорожденных детей коренного населения.

Распространенность «арктического» варианта гена *CPT1A* в современных и древних популяциях

Предполагается, что распространению «арктического» варианта гена *CPT1A* в популяциях коренного населения Северо-Восточной Азии способствовали миграции морских охотников вдоль побережий северных морей (Малярчук, 2018). Кроме эскимосов, чукчей и коряков, случаи этой мутации мозаично были зарегистрированы у эвенков Якутии (с частотой 1 %) (Малярчук и др., 2016), долгано-нганасанского населения Таймыра (3 %) (Smolnikova et al., 2015), нанайцев северокитайской провинции Хэйлунцзян (10.3 %) (Li et al., 2018). Поскольку в соседних по отношению к указанным этническим группам популяциях «арктический» вариант гена *CPT1A* отсутствовал, то наиболее вероятным представляется, что случаи нахождения варианта rs80356779-A вдали от Чукотки и Северного Приохотья могут объясняться миграциями древних морских охотников. Согласно археологическим данным, группы морских охотников из Берингоморья проникали на Таймыр ~3 тыс. лет назад (Гурвич, Симченко, 1980). Неоднократно также археологами отмечалась экспансия морских охотников с севера на юг вдоль побережья Охотского моря (Лебединцев, 1990; Befu, Chard, 2017).

Результаты молекулярного датирования показали, что эволюционный возраст «арктического» варианта гена *CPT1A* составляет от 6 до 23 тыс. лет (Clemente et al., 2014). Между тем сведения палеогеномных исследований позволяют считать, что «арктическая» мутация появилась у коренного населения Арктического и Охотоморского регионов на протяжении последних четырех тысячелетий. Согласно палеогеномным данным, «арктическая» мутация отсутствовала у представителей позднего палеолита арктической Восточной Сибири (стоянка Яна, 32.5 тыс. лет назад) (Sikora et al., 2019) и Юго-Восточной Сибири (стоянка Мальта, 24 тыс. лет назад) (Raghavan et al., 2014), а также в Северной Америке у представителя культуры Кловис (12.6 тыс. лет назад) (Rasmussen et al., 2014) – девочки из погребения Upward Sun River (Аляска, 11.5 тыс. лет назад) (Moreno-Mayar et al., 2018) и Кенневикского человека (8.3–9.2 тыс. лет назад) (Rasmussen et al., 2015). В мезолите и неолите «арктическая» мутация также не была выявлена по результатам исследований древнего индивидуума из Дуванного Яра (Чукотка, ~9.8 тыс. лет назад), а также древних жителей пещеры Чертовы ворота (Приморье, ~7.5 тыс. лет назад) (Sikora et al., 2019). «Арктический» вариант отсутствовал также у индивидуумов со стоянки Усть-Белая (Прибайкалье, 4.5–6.6 тыс. лет назад) (Sikora et al., 2019).

Впервые «арктический» вариант гена *CPT1A* был обнаружен в гетерозиготном состоянии у палеоэскимоса, представляющего культуру Саккак из Гренландии (4 тыс. лет назад), а также с частотой ~50 % у канадских и гренландских представителей палеоэскимосской культуры Дорсет (1.4–1.6 тыс. лет назад) (Rasmussen et al., 2010; Clemente et al., 2014). «Арктический» вариант зарегистрирован также у двух представителей токаревской культуры (Северное Приохотье, 3 тыс. лет назад) и у древних жителей эскимосского поселения Эквен (Чукотка, 1.9–2.1 тыс. лет назад) (Sikora et al., 2019). Недавние исследования продемонстрировали, что и для представителей Южного Приохотья – носителей культуры позднего дзёмона (Хоккайдо, 3.5–3.8 тыс. лет назад) также был характерен «арктический» вариант гена *CPT1A* (Kanzawa-Kiriyama et al., 2019). Авторы предположили, что частота варианта rs80356779-A в популяции позднего дзёмона Хоккайдо была высокой, а ее закреплению способствовал тот факт, что древние жители активно охотились на морских животных (морских котиков, сивучей, морских львов, дельфинов). Возможно, что присутствие «арктического» варианта у представителей позднего дзёмона Хоккайдо связано с воздействием древних северо-восточноазиатских популяций, повлиявших также и на предков других народов Приамурья и Сахалина – например нивхов, у которых также обнаружен «арктический» вариант гена *CPT1A* (Zhou et al., 2019). Между тем результаты исследования полиморфизма мтДНК показали, что для древнего и современного населения Хоккайдо преемственность от древности до современности сохраняется для митохондриальных гаплогрупп N9b, D4h2 и M7a, которые отсутствуют у народов Северо-Востока Азии (коряков, ительменов, чукчей) (Adachi et al., 2018). Общим же звеном для всех указанных популяций служит присутствие гаплогруппы G1b. Эта гаплогруппа мтДНК появилась у представителей эпи-дзёмона на Хоккайдо 2.0–2.5 тыс. лет назад и распространена у современных айнов с частотой около 16 % (Adachi et al., 2011). Ее появление явно связано



The phylogenetic tree of whole mitochondrial genomes belonging to haplogroup C1a.

Transitions are indicated on tree branches; for transversions, the results of nucleotide replacements are shown; deletions are marked as del. The evolutionary ages of mtDNA clusters (in thousands of years, kyr) are given in accordance with the mutation rate in the whole mitogenome, equaling 1.665×10^{-8} substitutions per site per year (Soares et al., 2009). For mitogenomes, GenBank numbers and ethnicities are indicated. The phylogenetic tree is built with the program mtPhyl 4.015 (https://sites.google.com/site/mtphyl/home).

с миграциями с северо-востока, поскольку гаплогруппа G1b установлена как у мезолитического индивидуума из Дуванного Яра (Чукотка, 9.8 тыс. лет назад), так и у токаревцев (3 тыс. лет назад) (Sikora et al., 2019). По всей видимости, на Хоккайдо эта гаплогруппа мтДНК попала в связи с распространением токаревской культуры на юг Приохотья.

Экспансия носителей митохондриальной гаплогруппы G1b на рубеже нашей эры в популяциях Охотоморского региона, вероятно, повлекла за собой и распространение «арктического» варианта гена *CPT1A*. Однако поскольку гаплогруппа G1b не обнаружена на Хоккайдо в более раннее время (т. е. 3.5–3.8 тыс. лет назад), то вполне возможно, что источник «арктического» варианта гена *CPT1A* у них мог быть связан с другой, более ранней, миграцией. Анализ данных о полиморфизме мтДНК у населения северной части Восточной Азии показывает, что в литературе имеются сведения о присутствии в восточноазиатских популяциях очень редкой митохондриальной гаплогруппы C1a, которая в филогенетическом смысле является сестринской ветвью по отношению к распространенным у индейцев Америки гаплогруппам C1b, C1c и C1d. Гаплогруппа C1a обнаружена у японцев (0.5 %) (Maruyama et al., 2003), ульчей (0.6 %) (Starikovskaya et al., 2005), ороков Сахалина (11.5 %) (Бермишева и др., 2005), нанайцев (1.2 %) (Tamm et al., 2007), дауров (2.2 %) (Kong et al., 2003), монголов (1.3 %) (Kolman et al., 1996; Derenko et al., 2007), алтайцев (0.7 %) (Dulik et al., 2012), бурят (0.7 %) (Derenko et al., 2007), киргизов (0.5 %) (Tamm et al., 2007), казахов (0.8 %) (Tamm et al., 2007). Предполагается, что присутствие гаплогруппы C1a на северо-востоке Азии связано с обратной миграцией из Америки (Tamm et al., 2007), произошедшей ~8.6 тыс. лет назад, судя по эволюционному возрасту этой гаплогруппы мтДНК (Derenko et al., 2010). Конечно, по результатам филогенетического анализа и возрасту гаплогруппы трудно определить, когда именно была предполагаемая миграция. Неизвестно, появились ли мутации, определяющие гаплогруппу C1a (рисунок), в Азии или же произошел перенос в Азию уже сформировавшейся гаплогруппы C1a, возникшей очень локально в Америке.

Филогенетический анализ всех известных к настоящему времени C1a-митогеномов показывает, что эволюционный возраст гаплогруппы составляет ~8 тыс. лет, а дивергенция основных гаплотипов от корневой последовательности мтДНК произошла ~6.6 тыс. лет назад, при этом японская ветвь гаплогруппы C1a имеет возраст ~5.2 тыс. лет (см. рисунок). Таким образом, учитывая достаточно широкий диапазон времени формирования «арктического» варианта гена *CPT1A* (от 6 до 23 тыс. лет назад), можно предположить, что C1a-миграция из американской части Берингии могла быть осуществлена носителями «арктического» варианта гена *CPT1A*. В таком случае находит объяснение присутствие как C1a-гаплотипов мтДНК у современных японцев и народов Сахалина и Приамурья, так и «арктического» варианта гена *CPT1A* у представителей позднего дзёмона Хоккайдо и современных народов Сахалина и Приамурья. В настоящее время ареал C1-гаплогрупп у народов Америки смещен к югу, однако палеогеномные исследования установили присутствие C1b-гаплотипа на Аляске 11.5 тыс. лет назад (Tackney et al., 2015). Поэтому вполне вероятно, что митохондриальные генофонды берингийцев включали в свой состав C1-линии, одна из которых могла стать родоначальницей гаплогруппы C1a.

В отношении происхождения «арктического» варианта гена *CPT1A* установлено, что для варианта rs80356779-A предковым является гаплотип, характерный для населения Восточной Азии (Clemente et al., 2014). Однако этапы дальнейших изменений этого гаплотипа, приведших к возникновению «арктической» мутации, равно как и место ее происхождения (Америка или Азия) пока неизвестны.

Заключение

Полученные результаты позволили выявить несколько миграционных событий, связанных с распространением морских зверобоев в Охотоморском регионе. Самая поздняя миграция, оставившая следы у носителей культуры эпи-дзёмон, привнесла с севера Приохотья на Хоккайдо и соседние территории Приамурья митохондриальную гаплогруппу G1b и «арктический» вариант гена *CPT1A*. Следы более ранней миграции, также привнесшей «арктическую» мутацию, зарегистрированы у населения позднего дзёмона Хоккайдо. Пока неизвестно, связана ли эта миграция с распространением митохондриальной линии C1a или же присутствие C1a у населения островов Японии маркирует собой еще один, более ранний, эпизод миграционной истории, связывающей население Берингии и всего Охотоморского региона. Следует отметить, что в этнологической литературе уже давно существует предположение об этногенетическом родстве предков нивхов, чукчей, коряков, эскимосов и американских индейцев (Jochelson, 1926). Предполагается также существование в прошлом циркумохотской культурной общности, имевшей глубокие связи с сопредельными культурами азиатского побережья и островов северо-западной Пацифики и Северной Америки (Лебединцев, 2003).

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