

Cytoplasmic genetic diversity of potato varieties bred in Russia and FSU countries

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Male sterility in potato is little studied since traditional breeding is based on the vegetative reproduction of highly heterozygous tetraploid varieties. The rapid development of hybrid diploid breeding contributes to growing interest in studying the male sterility of this important crop. In this work, a set of 6 cytoplasmic markers was employed to describe cytoplasmic genetic diversity of 185 potato cultivars bred in Russia and FSU countries. Three cytoplasm types were identified, T (40.0 %), D (50.8 %) and W/γ (8.7 %), which according to literature are associated with male sterility. With a single exception (0.5 %), cytoplasm types characteristic of male fertile forms (A, P) were not found in the subset of 185 cultivars. A comparison of these results with previously published data suggested expanding the subset to up to 277 cultivars, all developed in Russia or FSU countries; however, the resulting differentiation into three cytoplasm types (T, D and W/γ) was nearly the same. Fertility phenotyping helped identify both male-sterile and male-fertile genotypes within the three groups of varieties with T-, D- and W/γ-type cytoplasm. Fifteen genotypes differing in cytoplasm type and male sterility/fertility traits were selected for direct sequencing of 8 mtDNA loci. Fragments of the *nad2*, *nad7*, *cox2*, *atp6* and *CcmFc* genes were identical in all 15 selected genotypes. The polymorphism, detected in the *rps3*, *atp9* and *CcmFc* loci, was not associated with male sterility. Two SNPs in the *nad1/atp6* and *nad2* loci differentiated 7 genotypes with W/γ-type cytoplasm into five genotypes with tetrad sterility, and two with fertile pollen. The results of an NGS analysis confirmed the association of these SNPs with tetrad sterility in a larger set of 28 genotypes of different origin, all with W/γ-type cytoplasm. A heteroplasmy state was observed both in male-sterile and in male-fertile genotypes.

Key words: potato; *Solanum*; male sterility; cytoplasmic types; DNA markers.

For citation: Gavrilenko T.A., Klimenko N.S., Alpatieva N.V., Kostina L.I., Lebedeva V.A., Evdokimova Z.Z., Apalikova O.V., Novikova L.Y., Antonova O.Yu. Cytoplasmic genetic diversity of potato varieties bred in Russia and FSU countries. Vavilovskii Zhurnal Genetiki i Seleksii = Vavilov Journal of Genetics and Breeding. 2019;23(6):753-764. DOI 10.18699/VJ19.534

Генетическое разнообразие сортов картофеля российской селекции и стран ближнего зарубежья по типам цитоплазм

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Мужская стерильность у картофеля малоизучена, поскольку традиционное картофелеводство основывается на вегетативном размножении высокогетерозиготных тетраплоидных сортов. Быстрое развитие диплоидной гибридной селекции картофеля обуславливает возрастание интереса к изучению мужской стерильности у этой важной культуры. В настоящей работе охарактеризовано генетическое разнообразие по типам цитоплазм 185 сортов картофеля селекции России и стран ближнего зарубежья с использованием набора из шести цитоплазматических маркеров. Сорта выборки были дифференцированы на три группы с T (40.0 %), D (50.8 %) и W/γ (8.7 %) типами цитоплазм, которые, согласно литературным данным, ассоциированы с мужской стерильностью. За единичным исключением (0.5 %), типы цитоплазм, характерные для мужскофертильных форм (A, P), в изученной выборке не найдены. Сопоставление полученных результатов с ранее опубликованными данными позволило расширить выборку до 277 отечественных сортов, однако дифференциация на три типа цитоплазм (T, D, W/γ) сохранилась. На основании результатов фенотипизации среди сортов с T-, D- и W/γ-типами цитоплазм были выявлены не только мужскостерильные, но и фертильные генотипы.

У 15 отобранных генотипов, различающихся по типу цитоплазм и признаку мужской стерильности/фертильности, методом прямого секвенирования были проанализированы восемь локусов мт-генома. Фрагменты мт-генов *nad2*, *nad7*, *cox2*, *atp6*, *CcmFc* были идентичны независимо от типа цитоплазмы и мужской стерильности/фертильности сортов. Индели/замены нуклеотидов в локусах *rps3*, *atp9*, *CcmFc* не были ассоциированы с признаком мужской стерильности. На ограниченной выборке из семи образцов с W/γ-типом цитоплазмы были выявлены различия генотипов с тетрадной мужской стерильностью и с фертильной пыльцой по двум однонуклеотидным заменам в локусах *nad1/atp6* и *nad2*. Результаты NGS-анализа подтвердили ассоциацию этих SNP-вариантов с тетрадной мужской стерильностью на большей выборке из 28 образцов с W/γ-типом цитоплазмы различного происхождения. Показано, что гетероплазматическое состояние характерно как для генотипов с тетрадной мужской стерильностью, так и для генотипов с мужской фертильностью.

Ключевые слова: картофель; *Solanum*; мужская стерильность; типы цитоплазм; молекулярные маркеры.

Introduction

In more than 300 years, covering the breeding history of potato (*Solanum tuberosum*) outside South America, where it was domesticated, thousands of cultivars have been developed by breeders; they are presently cultivated in nearly 150 countries. This 300-year period may be divided into three stages: (1) the breeding process of the 18th–19th centuries, based on a restricted genetic diversity of relatively not numerous South America's indigenous cultivars introduced to Europe; (2) beginning from the early 20th century, targeted crossing of cultivated potato with wild species in order to introgress *R* genes, first of all, conferring resistance to fungal and viral pathogens; and (3) the third phase that we are now standing witness to is marked by the emergence of essentially novel trends in potato breeding: cygenesis, genome editing, and diploid hybrid breeding. The latter trend, based on crossing inbred diploid lines in order to obtain in plenty hybrid seeds (true potato seeds, TPS) of heterotic F_1 hybrids, manifests a radical change of the paradigm in the breeding new potato varieties and in their reproduction (Lindhout et al., 2011; Jansky et al., 2016).

The rapid development of diploid hybrid breeding is coupled with the prospects to cardinaly shorten the duration of the breeding process and reduce the costs of producing and multiplying healthy clones (Lindhout et al., 2011, 2018; De Vries et al., 2016; Jansky et al., 2016), because most of potato pathogens are not transmitted with pollen or TPS. The onset of diploid hybrid breeding called for a need to study genetic bases of self-incompatibility (Phumichai et al., 2005) and inbreeding depression in potato (Zhang et al., 2019) in order to obtain inbred diploid lines, as well as to promote research on male sterility and CMS-*Rf* genetic systems, aimed at the development of effective interline hybridization techniques (Anisimova, Gavrilenko, 2017). Previously, those challenges have had not been so relevant, so far as the conventional potato production is based on vegetative reproduction of highly heterozygous tetraploid cultivars.

Some few publications have addressed the problems of male sterility in potato, despite the fact that this trait is quite frequent in improved cultivars. There are several known types of male sterility in potato:

(1) Genetic-cytoplasmic male sterility (Grun et al., 1962), expressed as various abnormalities in the development of reproductive organs and as the plant's inability to set berries,

which is caused by interactions between dominant alleles of nuclear genes responsible for male sterility (for example, *Ms* gene) and genetic factors of the T (Tuberosum) type of cytoplasm (Grun et al., 1977; Iwanaga et al., 1991);

(2) Functional male sterility, when plants produce morphologically normal, well stainable, but non-functional pollen grains. This type of sterility has been described for hybrids, breeding clones and cultivars with the cytoplasm of wild Mexican species, *S. demissum* (Dionne, 1961; Hosaka, Sanetomo, 2012);

(3) Cytoplasmic male sterility (CMS), expressed in certain interspecies combinations. For example, virtually all studied cultivars and breeding clones with the cytoplasm of another wild Mexican species *S. stoloniferum* can participate in crosses only as female parents (Ross, 1986; Lössl et al., 2000; Song, Schwarzfischer, 2008), because they manifest tetrad sterility (tetrads do not disintegrate in the end of microsporogenesis, and over the entire process of subsequent microgametogenesis microspores remain united into 'permanent tetrads'). Using the method of metabolic profiling on anthers, it has been shown that tetrad sterility is associated with an abrupt disorder of the carbonic exchange in anthers, mainly as far as the polysaccharide spectrum is concerned, and the metabolism of amino and fatty acids (Shishova et al., 2019).

Among the carriers of the above-mentioned cytoplasm types, genotypes with male fertility occur with varied frequency. For example, H. Ross (1986) reported that a third of the varieties with the most widespread T-type cytoplasm were unable to develop berries. Studying the genetic control of fertility restoration resulted in identifying the nuclear *Rt* gene (a male fertility restorer gene) that can result in the recovery of male fertility in the T-type cytoplasm holders (Iwanaga et al., 1991; Ortiz et al., 1993). These authors reported a wide distribution of functional *Rt* gene alleles in the breeding gene pool. At the same time, the *Rt* gene, same as the nuclear *Ms* gene, has not been relevantly studied at the molecular level. Japanese researchers have screened hybrids and breeding clones carrying the *S. demissum* cytoplasm and selected genotypes that function in crosses as pollinators, which may be associated with effects of nuclear-cytoplasmic interactions (Sanetomo et al., 2011). At the same time, R. Ortiz et al. (1993, 2009) failed to detect the *Rt* or *Ms* genes among the samples with D-type cytoplasm. Recently, using the methods of comparative genomics, homologs of *RFL-PPR* genes were

identified in potato and their structural polymorphism was studied (Anisimova et al., 2019).

The data on structural rearrangements analysis of the mtDNA loci associated with the sterilizing cytoplasm of the wild Mexican species *S. stoloniferum* (Lössl et al., 1999) and the *S. demissum* (Sanetomo, Hosaka, 2011, 2013) are quite sporadic. At present, the potato cytoplasm types are identified with marker-assisted selection techniques, using organelle DNA markers (mitochondrial (mtDNA) and chloroplast (cpDNA)).

Plastid DNA markers. The first data concerning cpDNA polymorphism in potato species were obtained using restriction and RFLP analyses. As a result, the T-type of cpDNA was identified, with the 241 bp specific deletion in the *ndhC/trnV* locus, and 4 more types, differing in the restriction sites BamHI, HindIII and PvuII (Hosaka, 1986, 2003). The initial classification was later revised, so five main cpDNA types are now recognized in potato: A, C, S, T and W. Their identification requires employment of the entire package of SSR, STS and CAPS markers from the set of K. Hosaka and R. Sanetomo (2012) (Fig. 1).

Mitochondrial DNA markers. The mtDNA polymorphism was first reported for potato species using the data of RFLP analysis, which resulted in identifying there major (α , β , γ) and two minor (δ , ϵ) types of mtDNA (Lössl et al., 1999). Later, PCR primers were developed, with specificity to the regions of mt-genes *atp6*, *cob* and *rps10*, which detect α , β and γ types of mtDNA (Lössl et al., 2000). In time, it became clear that only one pair of primers, ALM_4/ALM_5 (specific to *rps10* mtDNA locus) was enough to identify major mtDNA types from the marker set developed by A. Lössl et al. (2000) (Song, Schwarzfischer, 2008; Hosaka, Sanetomo, 2012; Antonova et al., 2018); these primers are also included in the set of Hosaka, Sanetomo (2012). Afterwards, markers amplifying different regions (Regions 1–3) of the chimeric fragment *Band1*, which incorporates a region of the *rps19* gene of mtDNA were developed (see Fig. 1). Chimeric fragment *Band1* is typical for the hybrids and cultivars having *S. demissum* in their maternal ancestry (Sanetomo, Hosaka, 2011, 2013).

Markers for cytoplasm types. The first set of PCR markers for potato cytoplasm identification, developed by A. Lössl et al. (2000), made it possible to identify three major combinations of cpDNA and mtDNA markers in improved cultivars: T/ β for T-type cytoplasm, W/ α for the cytoplasm of various wild species, including *S. demissum*, and W/ γ for the cytoplasm introgressed from *S. stoloniferum*. Cultivars with cytoplasm of the W/ γ -type were shown to display tetrad sterility in their pollen (Lössl et al., 2000; Song, Schwarzfischer, 2008).

Another set, offered by the Japanese researchers K. Hosaka and R. Sanetomo (2012), contains a larger number of markers for cpDNA, includes also a pair of ALM_4/ALM_5 primers from the set reported by A. Lössl et al. (2000) and, additionally, a D marker (Region 1) for identification of the D-type cytoplasm of *S. demissum*. With the help of this set, the modern classification of potato cytoplasm types has been developed; it includes 8 main types: A, M, P, W (W/ α , W/ β , W/ γ), T (T/ β) and D (see Fig. 1).

It should be kept in mind, however, that the cytoplasm types identified with the marker sets described by Lössl et al. (2000) and Hosaka, Sanetomo (2012) are not identical. A set

of markers from the Japanese researchers' diagnoses 'the true W-type' (wild). When the set developed by Lössl et al. (2000) is used, the W-type, identified by the absence of the 241 bp deletion in the *ndhC/trnV* locus, combines the A, M, P and W types of cytoplasm (see Fig. 1) which can be found both in cultivated and in wild potato species. Thus, the W-type of cytoplasm is interpreted in publications twofold: as 'the true W-type' (Hosaka, Sanetomo, 2012; Sanetomo, Gebhardt, 2015; Gavrilenko et al., 2018) and as 'a contraposition to the T-type' (Lössl et al., 2000; Song, Schwarzfischer, 2008), which has been responsible for discrepancies in the interpretations of molecular screening results. Therefore, after 2012, the primers ALM_4/ALM_5 from the set described by Lössl et al. (2000) have been used to detect the mtDNA types, while the types of cytoplasm are commonly identified using the molecular marker set of Hosaka, Sanetomo (2012) (see Fig. 1).

None of the 8 cytoplasm types is species-specific, although the T-type is found in up to 90 % of Chilean indigenous varieties (Hosaka, 2003; Spooner et al., 2007; Gavrilenko et al., 2013) and in a majority of old European cultivars (Provan et al., 1999; Gavrilenko et al., 2007; Sanetomo, Gebhardt, 2015). In view of this, the T-type is often referred to as the 'cultivated' or Chilean. The A- and P-types of cytoplasm are characteristic of male-fertile diploid and tetraploid indigenous Andean cultivars, while the W/ α and W/ β types of many wild potato species (Hosaka, Sanetomo, 2012; Mihovilovich et al., 2015). The D-type of cytoplasm is typical for *S. demissum* and the cultivars developed with its involvement (as a maternal progenitor), and has been found among the accessions of wild allohexaploid and allotetraploid Mexican potato species (Hosaka, Sanetomo, 2012, 2014). W/ γ cytoplasm type (and γ -type of mtDNA) has been detected in all hybrid clones and cultivars with the cytoplasm of *S. stoloniferum*, manifesting tetrad sterility (Song, Schwarzfischer, 2008; Antonova et al., 2018). At the same time, Hosaka and Sanetomo (2012) reported detection of W/ γ -type cytoplasm in potato accessions of *S. chacoense*, *S. pampasense*, *S. pinnatisectum* and *S. vernei*.

Large subsets of foreign varieties, analyzed with the marker set of Hosaka, Sanetomo (2012), showed a low level of cytoplasm genome diversity: more than 90 % had three cytoplasm types, T, D and W/ γ , associated with male sterility, while about 10 % possessed cytoplasm of rare types: W/ β , A, M or W/ α (without the D marker) (Sanetomo, Gebhardt, 2015). Varieties with P-type cytoplasm were not found in the European gene pool; however, the P-type was present in ~6 % of Japanese varieties (Hosaka, Sanetomo, 2012) (see Supplement 1)¹.

Studying agronomic traits in groups with different cytoplasm types revealed significantly higher starch content in hybrids and breeding clones with W/ γ - and W/ α -type cytoplasm than in those carrying the other cytoplasm types (Lössl et al., 2000). Higher starch content and later plant maturity were reported by R. Sanetomo and K. Gebhardt (2015) for the holders of W/ γ -type cytoplasm, compared with those carrying the other types. The same authors also observed that the level of field resistance to late blight in potato accessions with D-type cytoplasm was higher than in those with the T-type.

The data on the distribution of different cytoplasm types in domestic varieties (bred in the USSR, Russia, or adjacent

¹ Supplementary Materials 1–10 are available in the online version of the paper: <https://vavilov.elpub.ru/jour/manager/files/application/SupplGavrilenkoEngl.pdf>

Identification of cytoplasm types in potato using marker set of Hosaka, Sanetomo (2012)		Identification of cpDNA types using marker set of Hosaka, Sanetomo (2012):						
		SAC-marker (locus <i>cemA</i> of cpDNA)						
		Presence of the restriction in the BamHI site in the PCR-product of SAC primers				Absence of the BamHI-restriction		
		H1-marker (locus <i>ndhC/trnV</i> of cpDNA)						
		241 bp deletion in the PCR-product of H1 primers		241 bp deletion is absent				
		S-marker (locus <i>rps16/trnQ</i> of cpDNA)						
		48 bp deletion is absent					48 bp deletion in the PCR-product of NTCP6 primers	
		A-marker (locus <i>rpl32/ccsA</i> of cpDNA)						
		Absence of the BamHI-restriction				Restriction of the BamHI site in the PCR-product of A primers		Absence of the BamHI-restriction
D-marker: amplification of chimeric fragment <i>Band1</i> – PCR-product of primers D (Region 1)		Identification of mtDNA types:		Types of cpDNA:				
		PCR with primers ALM_4/ALM_5 (Lössl et al., 2000), locus <i>rps10</i> of mtDNA		T	W	C	A	S
		CYTOPLASM TYPES:						
D	527 bp	α	2400 bp		D			
Other types	Absence of PCR product	β	1600 bp	T	W/α			
		γ	Absence of PCR product		W/γ	M	A	P

Fig. 1. Identification of cytoplasm types using the marker set of Hosaka, Sanetomo (2012).

countries) are not numerous. The set of PCR markers developed by A. Lössl et al. (2000) was used to study 98 domestic cultivars (Gavrilenko et al., 2007). In the context of previous considerations, only 40 of them, with the T-type, match to the modern classification. The marker set described by Hosaka, Sanetomo (2012) was employed to determine cytoplasm types in 25 Russian cultivars preserved in foreign genebanks (Sanetomo, Gebhardt, 2015) and in 28 cultivars developed by Russian breeders (Gavrilenko et al., 2018) (see Supplement 1). The present research continues studying cytoplasm type genetic diversity of domestic potato varieties preserved in the VIR collection.

Materials and methods

The research material included 185 potato cultivars released in Russia and adjacent countries and preserved in the VIR collection (see the Table). For this cultivars the data on cytoplasm types were determined for the first time with the aid of the markers A, D, S, SAC and H1 from the marker set

of Hosaka, Sanetomo (2012). For 158 cultivars of this set, mtDNA types were earlier identified using the pair of primers ALM_4/ALM_5, specific to the *rps10* locus of mtDNA (Gavrilenko et al., 2007; Antonova et al., 2018). Male sterility phenotyping and molecular screening with the *R1* and *R3a* markers involved additional varieties from an extended subset ($N=277$), for which the types of cytoplasm had been identified earlier (Gavrilenko et al., 2007, 2018; Sanetomo, Gebhardt, 2015) (see Supplement 1).

DNA isolation was performed on plant leaves, using the CTAB extraction technique modified by Gavrilenko et al. (2013).

Molecular screening. Six STS/CAPS/SSR markers from the set of Hosaka, Sanetomo (2012) were used to identify cytoplasm types (see Supplement 2). Molecular screening for the presence of the *R1* and *R3a* gene markers controlling race-specific late blight resistance was conducted using the primers mentioned in Supplement 3.

The cytoplasm types of domestic potato varieties ($N = 185$) bred in Russia, USSR and adjacent countries which were identified in present research using marker set of Hosaka, Sanetomo (2012)

Cytoplasm types	N (%)	Variety name
T (T/β)	N = 74 (40.0 %)	Alisa, Ametist, Antonina, Avrora, Belorusskiy ranni, Bezhitskiy, Brat-2, Bryanskiy delikates, Bryanskiy nadezhny, Bryanskiy priusadebny, Bryanskiy ranni, Chayka, Druzhny, Falenskiy, Filatovskiy, Fioletovy, Gart, Gornouralskiy, Granat, Gulliver, Iskra, Kalinka, Katyusha (bred at the Polar station of VIR), Kemerovskiy, Khibinskiy ranni, Kivi, Kolpashevskiy, Korenevskiy, Krasavchik, Krasnaya gorka, Krasnaya zarya, Krasnoufimskiy, Kristall, Kustarevskiy, Lakomka, Laymdota, Lekar, Lider, Lina, Lyuks, Musinskiy, Nadezhda, Nart 1, Narymka, Nauka, Ognivo, Pamyati Rogacheva, Pribrezhny, Prigozhny 2, Primorskiy (=Pri-12), Rossiyanka, Rusalka, Ryabinushka, Safo, Sentyabr, Severyanin, Shaman, Shurminskiy 2, Sineva, Smena, Solnechny, Svetlyachok, Varsna, Vasilek, Virazh, Volzhanin, Volzhskiy, Vyatka, Yavar, Yubileyny Osetii, Yupiter, Zagadka, Zauralskiy, Zolskiy
D (W/α)	N = 94 (50.8 %)	Alena, Alpinist, Amur, Antoshka, Arkhideya, Babushka, Barin, Baron, Bashkirskiy, Belosnezhka, Belukha, Bolshevik, Bolvinskiy, Borodyanskiy rozovy, Bryanskiy yubileyny, Buket, Bylina Sibiri, Chaya, Delfin, Dina, Divo, Dontsovskiy, Effekt, Fermer, Garant, Gornyak, Goryanka, Gubernator, Irbitskiy, Kamenskiy, Kemerovchanin, Kormilets, Kortni, Krasavitsa, Krasnaya roza, Kuznechanka, Ladozhskiy, Lasunak, Lazar, Lazurit, Lyubava, Malinovka, Manifest, Mars, Matushka, Maugli, Nalchikskiy, Nesterovskiy, Nikulinskiy, Parus, Pransa, Prestizh, Prizer, Prolisok, Ramzay, Rapso-diya, Rassvet, Reggi, Rezerv, Romashka, Rosinka, Rummyanka, Rusich, Sambo, Saprykinskiy, Sarovskiy, Sintez, Skarb, Skoroplodny, Solnyshko, Start, Svenskiy, Tanay, Tango, Teshcha, Tomich, Tuleevskiy, Udacha, Ukrainskiy rozovy, Uspek, Utenok, Veselovskiy 2-4, Veteran, Viza, Vympel, Vyotok, Yugana, Yuna, Zhavoronok, Zhigulevskiy, Zhivitsa, Zlatka, Zov, Zvezdochka
W/γ	N = 16 (8.7 %)	Bryanskiy krasny, Barmaley, Fokinskiy, Ilinskiy, Kolobok, Korona, Meteor, Moskvoretskiy, Nakra, Olimp, Pogarskiy, Resurs, Sokolskiy, Vektar, Yubiley Zhukova, Zdyabytak
A	N = 1 (0.5 %)	Katyusha (bred in Ukraine)

PCR was performed using a 20 μL reaction mixture, containing 10 ng of the total DNA of potato varieties, a 1× reaction buffer (Dialat Ltd, Moscow), 2.5 mM MgCl₂, 0.4 mM of each dNTP, 0.2 μM of the forward and reverse primer, and 1 unit of Taq polymerase (Dialat Ltd, Moscow).

Amplification. The conditions of PCR complied with the recommendations of the primer developers (Supplements 2 and 3). In a number of cases, we optimized the programs using the TOUCHDOWN function. All reactions that involved the use of SCAR markers had no less than three replications.

Restriction. When CAPS markers were used, the treatment of PCR products with the BamHI restriction enzyme was performed in a 30 μL of reaction mixture according to the protocols of enzyme producers (SibEnzyme, <http://russia.sibenzyme.com/>).

Electrophoretic separation of fragments. PCR products were separated using horizontal 2 % agarose gel electrophoresis in a TBE buffer, followed by staining with ethidium bromide and UV visualization.

Sequencing. We developed primers for amplification of mtDNA loci (Supplement 4) on the basis of the complete sequence of the cultivated potato mt genome (GenBank number of the sequence: JF772172.1), using the Primer3Plus software. Amplified fragments were sequenced by the Sanger method on an ABI 3500x analyzer. High-throughput sequencing (NGS) was made according to the Illumina Inc. technology on an Illumina MiSeq (USA), using MiSeq® ReagentKit v3 (600 cycle) with twofold read coverage (2*300 nt).

Evaluation of male fertility. Pollen fertility (PF) was assessed using acetocarmine staining; no less than 300 pol-

len grains of each genotype were scanned in two replications. Preparations of stained pollen grains were analyzed under a light microscope Axio Scope.A1 (Zeiss, Germany), ×200 zoom. In addition, controlled crosses were made in 28 combinations, where 15 cultivars with D-type cytoplasm served as pollinators, and varieties with different cytoplasm types as female parents; immediately after the removal of yet unripened anthers, the emasculated flower buds were covered with isolators.

Statistic analysis. Data on the starch content and plant maturity score as well as foliage and tuber late blight resistance were taken from the State Register of Breeding Achievements (2010–2018) and the catalogues of potato cultivars for various years. Differences in these agronomic traits among the three groups of cultivars with the T-, D- and W/γ-types of cytoplasm were estimated by nonparametric statistic methods with the StatSoft Statistica 13.3 software tools (Khalafyan, 2010), using the Kruskal–Wallis test by ranks (one-way ANOVA on ranks) and the Mann–Whitney U test, at the significance level of 5 %. The same methods were used to assess correlation between type of cytoplasm and the presence/absence of molecular markers for the race-specific late blight resistance genes (*R1* and *R3a*).

Results

Identification of cytoplasm types in potato varieties

Types of cytoplasm were identified in 185 improved potato cultivars, and the resulting data are presented in the Table and Fig. 2. In this whole subset, only one cultivar, ‘Katyusha’ (bred

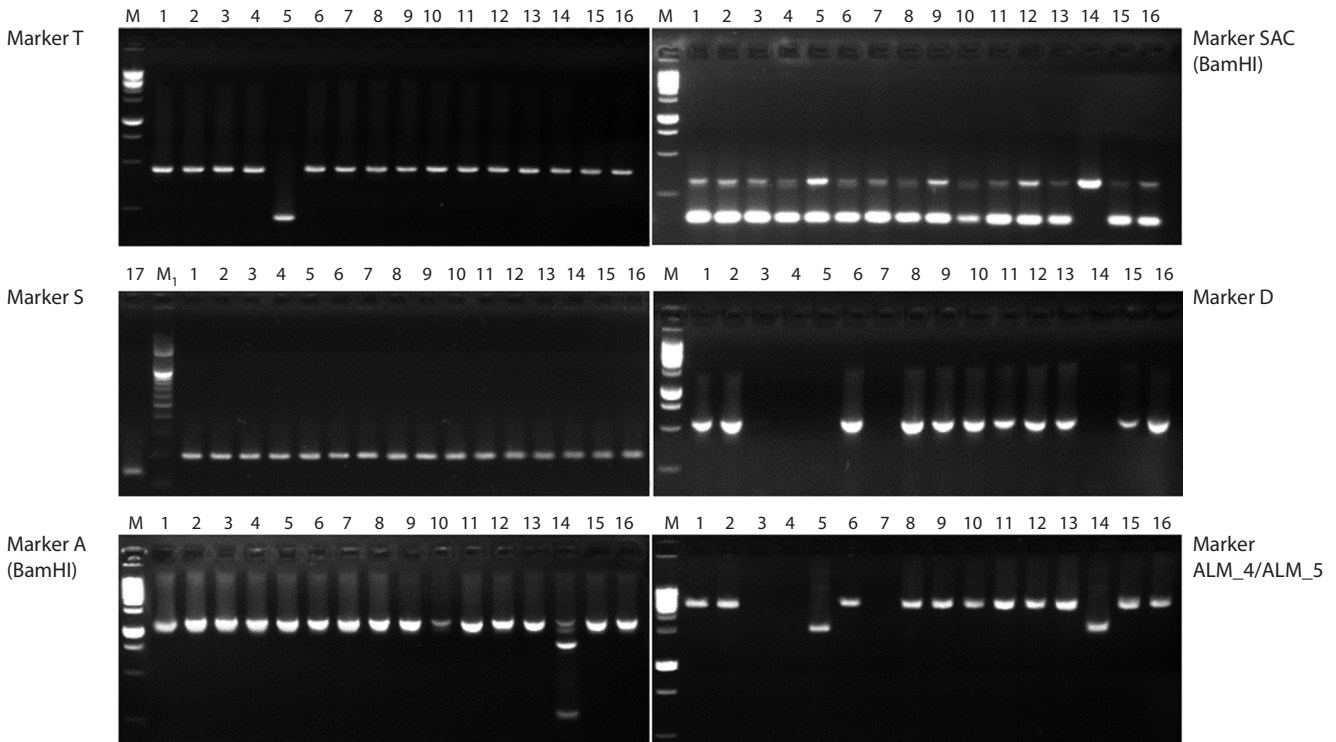


Fig. 2. Identification of cytoplasm types in potato varieties using markers set of Hosaka, Sanetomo (2012). Varieties: 1, Manifest (D); 2, Prestizh (D); 3, Kolobok (W/γ), 4, Nakra (W/γ), 5, Avrora (T); 6, Sintez (D); 7, Olimp (W/γ), 8, Tanay (D); 9, Zlatka (D); 10, Romashka (D); 11, Sambo (D); 12, Tomich (D); 13, Uspekh (D); 14, Katyusha (A); 15, Fermer (D); 16, Alpinist (D). Cytoplasm type is indicated in parentheses after the variety name. 17, control sample – *S. phureja* k-9386 with P-type cytoplasm having 48 bp deletion in the NTCP6 locus of cpDNA. M – DNA Ladder “Sibenzyme 1 kb”; M₁ – DNA Ladder “Sibenzyme 100 bp 1500”.

in Ukraine), demonstrated the absence of the BamHI site in the PCR product of SAC primers (locus *cemA* of cpDNA); further restriction analysis resulted in detecting the fertile A-type cytoplasm in it (see Fig. 1, 2 and the Table). It should be kept in mind that among Russian cultivars there is one with the same name, ‘Katyusha’ bred at the Polar Experiment Station of VIR, which carries T-type cytoplasm. In the remaining 184 cultivars, the BamHI restriction site was detected in the PCR product of SAC primers (see Fig. 1), therefore they may be counted among the varieties with T-, D- and W/γ- types of cytoplasm, which are associated with the male sterility traits according to Hosaka and Sanetomo (2012). The Table shows the results of their subsequent differentiations. Seventy-four cultivars had a 241 bp specific deletion in the *ndhC/trnV* intergenic spacer of cpDNA (T-type cpDNA) and the β-type mtDNA (see the Table and Fig. 2).

Two pairs of primers developed for amplification of two different regions (Region 1 and Region 2) of the chimeric *Band1* fragment were used to identify the D-type cytoplasm in PCR analysis. The screening results appeared fully identical for both primer pairs. Approximately a half of the studied subset, 94 out of 185 cultivars, had cytoplasm of the D type (see the Table), which attested to the presence of *S. demissum* in their maternal ancestry. For all 94 cultivars with D-type cytoplasm, the ALM_4/ALM_5 primer pair detected the α-type mtDNA. All cultivars with D-type cytoplasm simultaneously possessed the ‘true W type’ of cpDNA – they had the BamHI restriction site in the PCR product of SAC primers, but did not have the

BamHI restriction site in the PCR product of A primers and 241 bp deletion in the *ndhC/trnV* intergenic spacer of cpDNA (see Fig. 1, 2).

In the remaining 16 cultivars with ‘the true W-type’ of cpDNA, PCR products were not identified for the *Band1* fragment, hence they did not possess D-type cytoplasm (see Fig. 1, 2 and the Table). Since the same cultivars were earlier reported to have γ-type mtDNA due to the absence of amplification with the ALM_4/ALM_5 primers (Antonova et al., 2018), they may be recognized as having the W/γ-type of cytoplasm.

Thus, there were no cultivars with fertile cytoplasm types in the studied subset, except for the single (0.5 %) cv. ‘Katyusha’ (A-type) released in Ukraine, which confirmed the result earlier obtained by Hosaka, Sanetomo (2012) and Sanetomo, Gebhardt (2015), who had analyzed this cultivar. The remaining 99.5 % of the sampled cultivars possess three cytoplasm types: T, D and W/γ (see the Table), which are associated, according to Hosaka, Sanetomo (2012), with male sterility. With this in view, we carried out phenotyping of male sterility in the plants that took part in molecular screening.

Studying male sterility in plants

Pollen fertility (PF) was assessed in 121 cultivars from the extended subset (see Supplement 1): 45 with T-type, 60 with D-type, and 16 cultivars with W/γ-type cytoplasm. Of those, 26.7 % of cultivars with T-type cytoplasm developed colorless

and morphologically abnormal pollen grains; among them were 'Bryanskiy ranniy', 'Golubizna', 'Zolskiy', 'Lorkh', 'Pribrezhnyy', 'Rusalka' and 'Severyanin'. In 28.9 % of cultivars, the PF exceeded 60 %; in the rest it varied from 15 to 60 %. It should be mentioned that a considerable variability of pollen fertility was recorded in different years.

Out of the 16 cultivars with the sterilizing W/γ-type cytoplasm, tetrad sterility was observed in 14 (87.5 %). This trait was variable in different cultivars: for example, in cvs. 'Moskvoretskiy', 'Nakra', 'Olimp', 'Sokolskiy' and 'Yubiley Zhukova' up to 100 % of pollen grains remained clustered in 'permanent tetrads' at stage of opening flowers (Fig. 3, a).

Cvs. 'Bryanskiy krasnyy', 'Pogarskiy' and 'Korona' were observed to have about 50 % of 'permanent tetrads' against a background of sterile monads. Cvs. 'Kolobok' and 'Resurs' showed up to 20 % of 'permanent tetrads' with a background of sterile monads. Cvs. 'Barmaley' and 'Fokinskiy' with unknown pedigrees were two exceptions, as they developed only monads, 70 % of which were stainable with acetocarmine and had normal morphology (see Fig. 3, b).

In the cultivars with D-type cytoplasm, the pollen fertility varied from 20 to 80 %. Since Hosaka, Sanetomo (2012) reported functional pollen sterility for the holders of this cytoplasm type, we made 28 combinations of controlled crosses, where cultivars with D-type cytoplasm served as pollinators. Practically all crosses (except the combinations with cv. 'Skazka') resulted in berries with seeds, which is an evidence of functional pollen fertility in 14 out of 15 pollinators having the D-type cytoplasm of *S. demissum* (see Supplement 5).

Molecular screening with markers for the *R1* and *R3a* genes, introgressed into the breeding gene pool from *S. demissum*. Molecular screening of extended subset was performed to verify the assumption that, in the case of functional male sterility of D-type cytoplasm holders which took part in cultivar' pedigrees, markers of the *R1* and *R3a* genes can be detected only in the varieties with the cytoplasm of *S. demissum*; and when such forms possess male fertility – in varieties with various cytoplasm types. The results of molecular screening showed that markers of the *R1* and/or *R3a* genes had been identified in cultivars carrying various cytoplasm types in almost equal ratios (see Supplement 6).

Thus, the tree groups of cultivars with the T, D and W/γ cytoplasm types were shown to contain both male-sterile and male-fertile genotypes, which may be caused by the specificity of nuclear-cytoplasmic interactions and the polymorphism of mtDNA loci associated with the CMS trait.

Studying polymorphism of mtDNA loci. Based on the results of male sterility studies, 15 accessions were selected to form three groups with the T, D and W/γ cytoplasm types, so that each of them contained both male-sterile and male-fertile genotypes. They underwent direct sequencing to analyze 8 loci of the mt genome (fragments of the genes *atp6*, *atp9*, *cox2*, *CcmFc*, *nad2*, *nad7*, *rps3*, and the *nad1/atp6* intergenic spacer), whose polymorphism is often associated with CMS in various plant species (Ducos et al., 2001; Kim D.H., Kim B.D., 2006; Das et al., 2010; Liu et al., 2011). To amplify these 8 loci, we developed 8 pairs of primers, whose sequences are presented in Supplement 4. All the studied 15 genotypes, re-

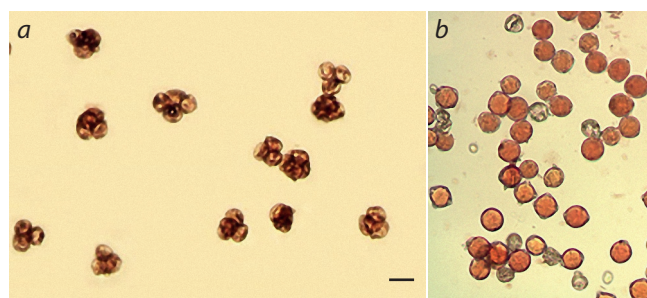


Fig. 3. Tetrad sterility in cv. 'Moskvoretskiy' (a) and pollen grains of cv. 'Fokinskiy' (b), both cvs. with W/γ-type cytoplasm.

gardless of their cytoplasm type or male sterility/fertility traits, demonstrated identical sequences of the *nad2*, *nad7*, *cox2*, *atp6*, *CcmFc* loci, which matched with the corresponding regions on the complete mt genome sequence (JF772172.1) of cultivated potato. Nucleotide InDels/substitutions were identified in the *rps3*, *atp9* and *CcmFc* loci; however, they were not associated with the male sterility feature. For the *nad1/atp6* and *nad2* (the second intron) loci, single-nucleotide substitutions were identified, which differentiated 7 holders of the W/γ cytoplasm into two groups: five genotypes with tetrad male sterility, and two with fertile pollen. In the second intron of the *nad2* gene (position 216310 in the complementary strand of the sequence JF772172.1), an A→C transversion was found: SNP variant 'A' was present in male sterile genotypes, and 'C' – in male fertile ones (Fig. 4, a). As for the *nad1/atp6* intergenic spacer, SNP variant 'T' in position 48923 was identified for sterile genotypes, and 'G' for male fertile ones (see Fig. 4, b). In the accessions with cytoplasm of the T- and D-types, regardless of their male sterility/fertility traits, the sequences of these two loci were similar.

These two SNP variants were identified while analyzing a limited number (7) of genotypes with W/γ-type cytoplasm. To confirm their association with tetrad sterility, we made an NGS analysis of the *nad2* and *nad1/atp6* loci on a larger subset of accessions of various origin, all having W/γ-type cytoplasm. The analysis covered 12 additional cultivars with W/γ-type cytoplasm, developed both in Russia and Europe having *S. stoloniferum* in their maternal ancestry, and 10 breeding clones with W/γ-type cytoplasm, developed on the basis of the Mexican species *S. guerreroense* (Zoteeva et al., 2017). These accessions were used to make three bulk samples, each uniting genotypes with W/γ-type cytoplasm of similar origin and with similar appearance of the male sterility or fertility trait (see Supplement 7). The results of the NGS analysis confirmed that SNP variants 'A' in the *nad2* locus (position 216310) and 'T' in the *nad1/atp6* spacer (position 48923) were associated with tetrad male sterility in all studied genotypes. In addition, minor mitotypes were identified in the polymorphic site for each bulk sample, which may be explained by either heterogeneity of a bulk sample or the heteroplasmic state of the analyzed genotypes – when substoichiometric quantities of different mtDNA variants are present in plant cells.

To find the cause of the observed heterogeneity, we developed a CAPS marker, Int2NAD2/Sse9I (F: GGGCTTCTTGCTACGCTACA; R: TTAAGCCTGGGCGAAGATGG,

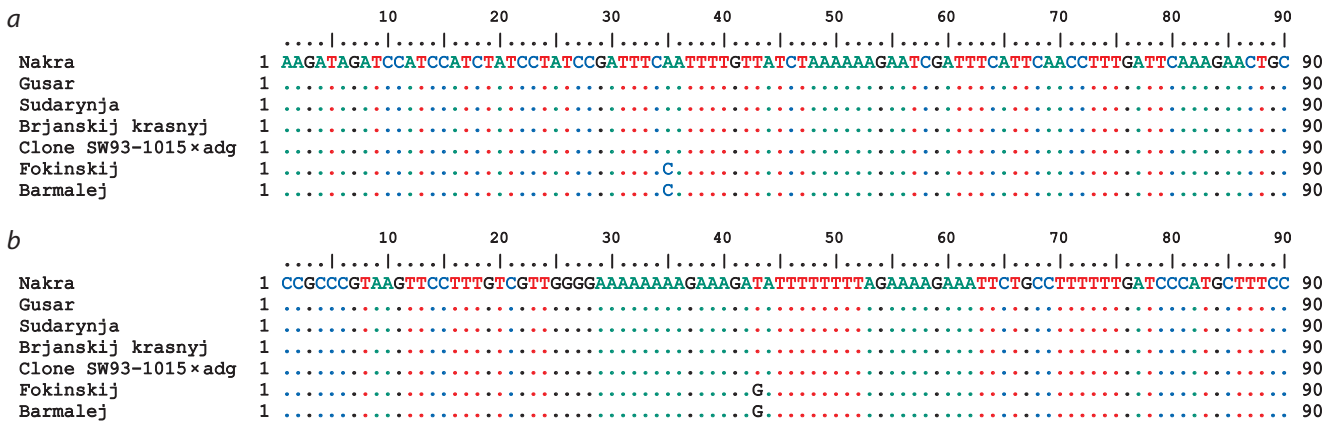


Fig. 4. Alignment of fragments (a) of the second intron of the *nad2* gene and (b) of the *nad1/atp6* intergenic spacer in 7 accessions having W/γ-type cytoplasm. From top to bottom – 5 genotypes with tetrad sterility: varieties Nakra, Gusar, Sudarynja, Bryanskiy krasny and breeding clone SW93-1015 × adg (Zoteyeva et al., 2017) and the last two: Fokinskiy, Barmaley – with fertile pollen grains forming only monads.

with subsequent restriction Sse9I), for the polymorphic SNP site A/C in the intron of the *nad2* gene. This marker makes it possible to distinguish the site AATT (genotypes with tetrad sterility) from the site CATT (genotypes with fertile pollen and W/γ cytoplasm). Each of the 28 selected accessions was analyzed individually with this marker, and all bulk samples appeared uniform – the accessions therein had a restriction site corresponding to the main nucleotide for the given group (see Supplement 7). Thus, the polymorphism of SNP variants in the second intron of the *nad2* gene may be explained by the presence of various mitotypes in substoichiometric amounts.

Differences between cultivars with different cytoplasm types in their agronomic traits. Pairwise comparison among the cultivar groups with different cytoplasm types, using the Kruskal–Wallis test by ranks and the Mann–Whitney U test, showed the absence of significant differences in starch content, field resistance of foliage to late blight, and presence of the markers of the *R1* and *R3a* genes, as well as the presence of significant differences in tuber late blight resistance between three cultivar groups ($p = 0.044$), with higher resistance in the varieties with W/γ-type cytoplasm. When the Mann–Whitney test was applied, the differences in earliness between the cultivars with D-type (mean = 6.4) and with W/γ-type (mean = 5.5) cytoplasm were significant ($p = 0.037$), but with the Kruskal–Wallis criterion the differences were not essential.

Discussion

According to the published data, more than 90 % of foreign potato cultivars (released in East Europe, North America and Japan) manifest three types of cytoplasm: T, D and W/γ, associated with variously expressed male sterility (see Supplement 1). The occurrence rate for cultivars with the A, M, W/β and W/α (without the D marker) cytoplasm types in total does not exceed 10 % (Hosaka, Sanetomo, 2012; Sanetomo, Gebhardt, 2015). Similar results were obtained in this research for potato cultivars developed in Russia and adjacent countries: 99.5 % of 185 cultivars were found to have three cytoplasm types: T (40.0 %), D (50.8 %), and W/γ (8.7 %). Except for cv. ‘Katyusha’ (A type, 0.5 %) released in Ukraine, no cultivars were observed to have fertile cytoplasm types.

Comparing the obtained results with our previous results (Gavrilenko et al., 2007, 2018) and published data (Sanetomo, Gebhardt, 2015) suggested expansion of the sampled subset of domestic cultivars to 277, but their variability in cytoplasm types remained similar as it had been: T (48.4 %), D (44.4 %), W/γ (6.8 %), and A (0.4 %). Having screened the extended subset, we found neither any cultivars with A-type or P-type cytoplasm typical for male fertile tetraploid and diploid Andean landraces, nor any varieties with M, W/β or W/α (without the D marker) cytoplasm types characteristic of the related South American wild potato species: *S. acaule*, *S. spegazzinii* and *S. sparsipilum* (= *S. brevicaule*), frequently used in breeding programs. At the same time, many varieties from the studied extended subset had earlier proved to possess molecular markers of nuclear *R* genes conferring resistance to harmful organisms: *H1*, *Gpa2*, *Gro1-4*, *Rx1*, *Rx2*, and *Ry_{adg}* introgressed into the breeding gene pool from the above-listed species (Gavrilenko et al., 2009, 2018; Biryukova et al., 2015; Antonova et al., 2016; Klimenko et al., 2017, 2019). It may be explained by male fertility of their hybrids, which participated in crosses, like the parental accessions of cultivated and wild South American species, as pollinators (Ochoa, 2004).

Wide distribution of the T-type cytoplasm, especially among old improved cultivars, may be due, on the one hand, to its positive effect on the yield (Plaisted, 1972) and, on the other, to the use of carriers of this cytoplasm type in breeding as female parents, because of the male sterility feature expressed in them (Grun et al., 1977). By the end of the 20th century, when interspecific hybridization had become the main tool of broadening the genetic diversity of breeding materials, the frequency of cultivars with T-type cytoplasm was abruptly decreased, whereas the occurrence rate of cultivars with the cytoplasm of wild Mexican species (D and W/γ) went up (see Supplement 8). It should be also mentioned that in the domestic gene pool the occurrence of cultivars with the D-type cytoplasm from *S. demissum* is two to three times higher than in West European or Japanese cultivars (see Supplement 1).

The spreading of the cytoplasm type inherent in wild Mexican species over the breeding gene pool is explained on the one hand by interspecific incompatibility (as a rule,

S. demissum and *S. stoloniferum* participate in interspecific crosses as maternal parents) and on the other hand – male sterility of the obtained hybrids (Dionne, 1961; Irikura, 1968; Song, Schwarzfischer, 2008; Hosaka, Sanetomo, 2012; Gavrilenko, Yermishin, 2017). Because of that, the introgression of nuclear genes responsible for race-specific late blight resistance (*R1–R3*, *R4*, *R8*, *R10*) from *S. demissum* and the *Ry_{sto}*/*Ryf_{sto}* genes of resistance to PVY from *S. stoloniferum* was simultaneously and unintentionally accompanied by a transfer of cytoplasmic determinants from wild Mexican species into the breeding gene pool.

It is reported in publications that the *W/γ*-, D- and T-types of cytoplasm have a specific effect on the male fertility/sterility traits, respectively inducing tetrad sterility, functional sterility of pollen, and abnormalities in the development of reproductive organs (see Hosaka, Sanetomo, 2012). According to the results of phenotyping performed in the framework of this research, each of the three groups of domestic cultivars with the T, D and *W/γ* cytoplasm types contained not only male-sterile, but also highly fertile genotypes. Male fertility in the T-type cytoplasm holders is explained in publications by the presence of a dominant allele of the *Rt* gene (a male fertility restorer gene) and, for a number of interspecies combinations, by the homozygosity (*ms/ms*) for the recessive allele of the *Ms* gene (male sterility gene) (Iwanaga et al., 1991; Ortiz et al., 1993; Mihovilovich et al., 2015). Among the varieties with T-type cytoplasm there are many efficient pollinators that have served as male parents in the development of many domestic cultivars, such as ‘Avrora’, ‘Dina’, ‘Zarevo’, ‘Kameraz’, ‘Priyekulskiy ranniy’, ‘Smena’ and ‘Shurminskiy 2’ (see the Table and Supplement 1).

The present results of successful crosses with pollinator varieties carrying D-type cytoplasm did not confirm the information about the functional pollen sterility in the *S. demissum* cytoplasm holders. An indirect evidence of male fertility in cultivars with D-type cytoplasm is the results of molecular screening with markers of the *R1* and *R3a* genes of race-specific late blight resistance, introgressed into the breeding gene pool from *S. demissum*. Markers of these genes were detected in cultivars with D-type, T-type and *W/γ*-type cytoplasm (see Supplement 9), which may be due to the involvement into the breeding process of donors of the *R1* and *R3a* genes (D-type cytoplasm holders) as pollinators.

Contrariwise, markers for the *Ry_{sto}* and *Ryf_{sto}* genes conferring extreme resistance to PVY, occur only in the cultivars having the *W/γ* cytoplasm transferred from *S. stoloniferum* (Flis et al., 2005; Song, Schwarzfischer, 2008; Antonova et al., 2018; Gavrilenko et al., 2018) (see Supplement 10), that confirms the sterilizing effect of this cytoplasm type. The analysis of the obtained results and published data (Song, Schwarzfischer, 2008) makes it possible to conclude that all genotypes with tetrad pollen sterility have *W/γ* cytoplasm type. On the other hand, the holders of this cytoplasm type may be sporadically interspersed with a few genotypes with fertile pollen, carrying cytoplasm of other wild species, for example, *S. vernei*, as shown by K. Hosaka and R. Sanetomo (2012). In our study, two (12.5 %) of the 16 cultivars with *W/γ*-type cytoplasm developed fertile pollen; it may well be that their maternal pedigree also included accessions of the South American species *S. vernei* or *S. chacoense*, whose *W/γ* cy-

toplasm type was ascertained by Hosaka, Sanetomo (2012).

The present research succeeded in differentiating sterile and fertile accessions both with *W/γ*-type cytoplasm according to single-nucleotide substitutions in two mtDNA loci (*nad1/atp6* and *nad2*). The CAPS marker Int2NAD2/Sse9I, developed by us, can be used in future for additional differentiation of the *W/γ*-type cytoplasm and for selection of male-sterile/fertile genotypes. The NGS analysis technique was for the first time applied to study the heteroplasmic state of mtDNA in potato genotypes with the sterilizing *W/γ* cytoplasm type. It has been shown that a heteroplasmic state is equally characteristic for the genotypes with tetrad sterility (where a minor mtDNA variant typical for genotypes with fertile pollen has been identified) and for male fertile cultivars (where a minor mitotype typical for cultivars with tetrad sterility has been found). The significance of stoichiometric quantities in the CMS phenotype expression has been demonstrated for various crop species: *Pennisetum glaucum* (Feng et al., 2009), *Beta vulgaris* (Bragin et al., 2011), *Brassica napus* (Chen et al., 2011), *Oryza sativa* (Bentolila, Stefanov, 2012), etc.

Conclusion

It may be summarized in the conclusion that Russian potato cultivars and those of the adjacent countries, along with the breeding gene pools in foreign countries, are represented mostly by plants with the T, D and *W/γ* cytoplasm types, while genotypes with fertile types of cytoplasm are practically absent. It seems obvious that breeders have not previously paid much attention to this trait, since the cultivated potato follows a vegetative reproduction pattern. In present research pairwise comparison of the cultivar groups with different cytoplasm types failed to find statistically significant differences in a number of economically useful traits, except for higher tuber late blight resistance in cultivars with *W/γ*-type cytoplasm.

The analysis of published data and results of the presented research makes it possible to conclude that the presently known cytoplasm markers for potato are not informative enough to be used in the selection of male sterile and male fertile genotypes. It is safe to assume that after the identification of the *Rt* and *Ms* genes in potato the efficiency of this research trend will be enhanced. Besides, further development of molecular markers is required to accomplish more detailed typing of mitochondrial DNA loci involved in male sterility control. Studying the CMS-*Rf* genetic systems and working out molecular marker techniques for sterilizing types of cytoplasm and fertility restorer genes are promising for the development of a new trend – heterosis-based potato hybrid breeding. Developing molecular markers for the CMS-*Rf* genetic systems are also promising for conventional breeding in the context of facilitating the selection of parental lines for crosses and, *inter alia*, pyramiding genes conferring resistance and other agronomical traits in one genotype.

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Acknowledgements. The research was supported by a grant from the Russian Scientific Foundation (Project No. 16-16-04125). The authors are grateful to Y.I. Karabitsina for her assistance in making pollen grain preparations and we acknowledge Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' in ARRIAM.

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

Received April 22, 2019. Revised June 7, 2019. Accepted June 10, 2019.