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VARIABILITY OF GENOMIC RGA-LOCI OF MODERN RUSSIAN POTATO CULTIVARS: NBS-PROFILING DATA

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Abstract

The plant immunity is aimed at protecting against biotic and abiotic stresses and, therefore, at adapting to adverse environmental conditions. At the first protection step, a wide range of phytopathogen receptors encoded by resistance *R*-genes is employed. The presence of a conserved NBS-domain in the receptors makes it possible to profile the plant genome by amplification of *R*-gene analogs. The multilocus NBS-profiling method makes it possible to efficiently characterize the plant genome in terms of the representativeness and variability of the NBS-domain containing *R*-genes. This method is used to study the diversity of *R*-gene loci in crops and related wild species, as well as introgressive hybridization and the *R*-gene evolution in plant species with varying degrees of pathogen resistance. NBS-profiling is also applied for genotyping GenBank collections, developing markers and saturating genetic maps. The requirement for cultivar genotype certification and profiling, along with a limited number of similar studies in Russia, makes research on the molecular profiling of domestic and foreign cultivars farmed in the Russian Federation relevant. In the present work, NBS-profiling was used for genotyping 65 potato *Solanum tuberosum* cultivars of mainly modern domestic breeding, as well as the related species *Solanum stoloniferum* (as an outgroup). Using two primer/enzyme combinations (NBS7/MseI and NBS9/MseI), 204 NBS fragments were generated, of which 144 (70.6%) were polymorphic and one fragment was unique to cv. Gala. For each cultivar, a specific spectrum of NBS fragments was determined. Analysis of genetic distance matrix revealed a high level of polymorphism (GD = 0.18-0.45 with an average value of 0.33) among the studied cultivars. Genetic distances within the analyzed cultivars varied more than between the cultivars and *S. stoloniferum* accession (GD = 0.27-0.40). The most related cultivars were Solnechny/Pamyati Rogacheva (GD = 0.18) and Velikan/Vympel (GD = 0.19) originated from Lorch Potato Research Institute, and the most distant cultivars were Charoit/Red Scarlett (GD = 0.45). Statistical analysis of NBS-profiling data clustered studied potato cultivars in accordance with different traits and resistance to phytopathogens. On the dendrogram and graphs generated using the PAST and Structure 2.3.4 software, a pronounced tendency to group cultivars by traits of resistance to the Potato virus Y (*Potyvirus*, *Potyviridae*) and the Potato leafroll virus (*Polerovirus*, *Luteoviridae*) was shown. The primer/enzyme systems used in this study for NBS-profiling can be applied to study the mechanisms of potato resistance to biotic stresses.

Keywords: *Solanum tuberosum*, Russian cultivars, foreign cultivars, genomic polymorphism, NBS-LRR-profiling, RGA-analysis

Plant immunity is designed to protect against biotic threats and provide adaptation; it contains a broad range of *R*-genes (resistance genes) that encode phytopathogen receptors of several classes, NLR (nucleotide-binding leucine-rich repeat proteins) being the most common class [1, 2]. As the products of *R*-genes

contain conservative domains, plant genome can be analyzed by amplifying RGAs, or resistance gene analogs. Plant genome can contain hundreds of these genes due to tandem and ectopic duplications followed by local gene r rearrangements and conversion [2]. NBS-LRR receptors contain two main conservative domains: the central domain NBS (nucleotide-binding site) and C-terminal domain, or LRRs (Leucine-Rich Repeats) [1, 3]. Gene families encoding NBS-LRR receptors have been identified by whole genome sequencing in several plant species: 57 genes in *Cucumis sativus*, 149 in *Arabidopsis thaliana,* and 653 in *Oryza sativa*. The genome of *Solanum tuberosum* L. was found to have 435 genes and 179 pseudogenes of NBS-LRR family [1].

Whilst the LRR domain sequence is relatively polymorphic, the NBS domain consists of several highly conservative and strictly ordered motifs: P-loop, Kinase-2, Gly-Leu-Pro-Leu (GLPL); mutations affecting them can disrupt receptor functioning [1]. Multilocus NBS profiling helps effectively describe plant genome in terms of the representation and variability of *R*-genes, the product of which contains an NBS domain. The method is based on amplifying genomic DNA sequences flanking the region that encodes the NBS domain of *R*-genes; it uses degenerate primers complementary to the highly conservative regions of the NBS domain [4].

NBS profiling is commonly used in research of the RGA locus diversity in crops such as potatoes, tomatoes, wheat, lettuce, etc. [5-7], as well as in related wild species [2, 4, 8]. NBS profiling also makes it possible to study introgressive hybridization between cultivated and wild species [6]. Besides, NBS profiling is used to study the evolution of *R*-genes in species that vary in their pathogen resistance; the method is employed to genotype gene bank collections, develop codominant markers, and saturate genetic maps [4, 6, 7].

This paper presents NBS profiling of potato cultivars and promising breeding clones; most have been bred in Russia.

The goal was to evaluate the NBS-LRR R-genes polymorphism and to find a possible correlation between clustering based on NBS analysis and resistance traits to various potato-affecting phytopathogens.

Materials and methods. For NBS-LRR profiling, 60 *Solanum tuberosum* cultivars and five promising breeding clones bred in Russia and abroad were used; for control, we used a related species, *Solanum stoloniferum*, which, among its other uses, is often utilized by breeders as a source of resistance to various phytopathogens. These cultivars were picked as they were (and are) part of research efforts covered by Russia's Federal Potato Breeding and Seed Farming Research Program. Most of these cultivars (59 out of 65; 90.77%) are in the Russian Public Register of Selected Breeds and Cultivars Allowed for Use in 2020 (http://reestr.gossortrf.ru/reestr/culture/159.html). Tubers were provided by the Russian Potato Research Center (Lorch Institute) and grown in a standard greenhouse environment (23 °C/25 °C, 16 h/8 h day/night).

Genomic DNA was extracted from tissues of 5-day sprouts by the CTAB protocol [9, 10] in two analytical replicates.

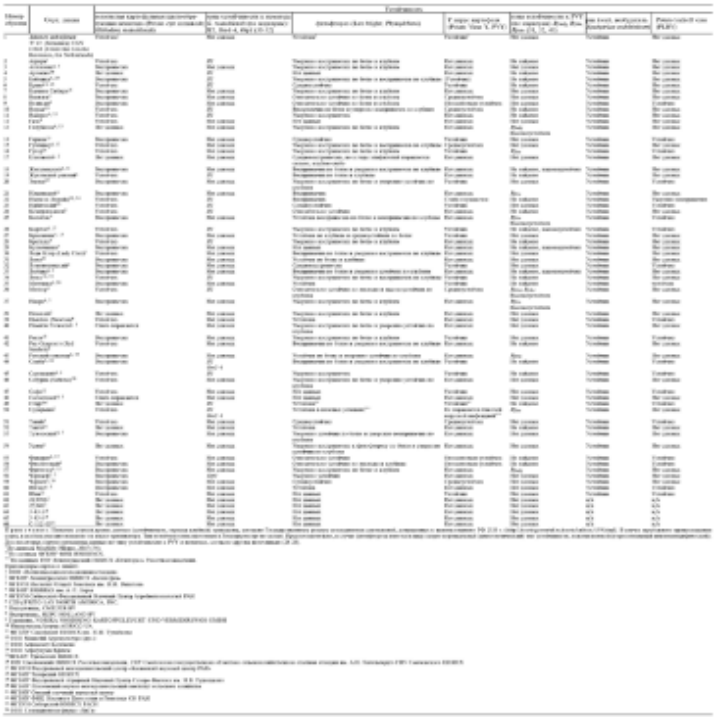
NBS profiling followed the standard protocol [4]. For analysis, 350 ng of genomic DNA from the accessions was digested with the MseI endonuclease (Thermo Fisher Scientific, USA). The resulting fragments were ligated to appropriate adapters and then used in two-round amplification at a T100™ amplifier, ThermoCycler, Bio-Rad, USA. The first polymerase chain reaction (PCR) round had 30 cycles (denaturation 30 s at 95 °C, primer annealing 1 minute at 55-60 °C, elongation 2 minutes at 72 °C) using NBS7, NBS9 primers [4, 11]. An aliquot of the PCR mix (0.5 μl) was used in the second PCR round (same parameters) with an IRD 700/800-labeled NBS primer and a Msel-adapter [4] primer. Amplification products were separated in a 6% denaturing polyacrylamide gel and visualized

with a LI-COR 4300 gel analyzer (LI-COR operator manual; LI-COR, USA).

For statistical analysis of the NBS profiling results, the obtained NBS spectra were documented as binary matrices in Microsoft Excel. The matrices were used to identify variety-specific DNA markers, to find pairwise genetic similarity coefficients (GS) and genetic distances (GD = 1 – GS), to run neighbor-joining (NJ) and principal coordinates analysis (PCA), and to group genetically similar accessions (PAST software, http://www.nhm.uio.no/english/research/infrastructure/past/) [12]. The genomic structure of the accessions was analyzed in Structure v. 2.3.4 (https://web.stan-ford.edu/group/pritchardlab/structure_software/release_versions/v2.3.4/html/structure.html), a package that can find shared genetic blocks and calculate their ratios in each accession [13, 14].

Results. Table 1 shows the specifications of the cultivars and their accessions (see http://www.agrobiology.ru for more info)

1. Specifications of potato cultivars and accessions studied by NBS analysis (for details, see http://www.agrobiology.ru)



NBS profiling. This study involved analysis of the adaptively significant *R*-gene family in 60 cultivars and five promising breeding clones of *S. tuberosum* as well as *S. stoloniferum* accession.

Amplification utilized degenerate primers NBS7 and NBS9 complementary to two main conservative motifs of the NBS domain, P-loop and GLPL, which enabled to tag both the 5′-, and the 3′-regions of NBS-LRR genes (Fig. 1).

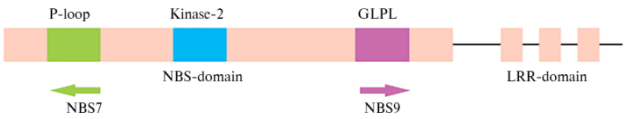


Fig. 1. Layout of NBS7 and NBS9 primers for NBS profiling:: NBS — Nucleotide Binding Site, LRR — Leucine-Rich Repeats, GLPL — Gly-Leu-Pro-Leu.

By using two NBS primer/enzyme combinations, NBS7/MseI and NBS9/MseI, we obtained individual spectra of the NBS fragments sized 70 to

550 bps, which could presumably be associated with the specific pathogen resistance in the analyzed cultivars.

A total of 204 NBS fragments were sampled, including 144 polymorphic fragments (70.6%) and a single unique fragment for cv. Gala, see Table 2. Potato accessions that have the most divergent *R*-genes spectra could be considered potential donors of resistance genes for breeding programs.

2. Polymorphism revealed by NBS profiling in 65 potato cultivars, breeding clones, and *Solanum stoloniferum*

Primer/enzyme combination	Number of NBS fragments			
	total	polymorphic		unique
		total	%	
NBS7/MseI	108	70	64.8	1
NBS9/MseI	96	74	77.1	0
Total	204	144	70.6	1

Statistical analysis of NBS spectra. NBS-LRR profiling data was used to obtain binary matrices for cluster analysis.

GD values of the tested cultivars varied within 0.18 to 0.45 (GD_{mean} = 0.33). Profiling found Lorch Institute-bred cultivars to be the closest: Solnechny and Pamyati Rogacheva (GD = 0.18), Velikan and Vympel (GD = 0.19). Maximum genetic distances were found between Charoit and Red Scarlett (GD = 0.45). Notably, GD between potato cultivars and *S. stoloniferum* accession varied insignificantly (GD = 0.27-0.40). This might be due to the fact that *S. stoloniferum* is commonly used in potato breeding as a donor of valuable resistance genes alleles that provide resistance foremost to viral pathogens [20]. Most of the tested cultivars were modern varieties, thus likely using in breedin programs interspecies hybridization involving several wild species as shown in [21].

There is only limited evidence on the *R*-genes variability in *Solanum* (including potato cultivars), which is why it seems interesting to compare the obtained data with the other plant species. Thus, NBS profiling of 32 Russian and non-Russian apple (*Malus domestica* Borkh.) cultivars showed their *R*-gene polymorphism (49% polymorphic fragments, GD_{mean} = 0.14) [22] being far below that of the tested potato cultivars (GD_{mean} = 0.33). NBS genotyping of wheat cultivars from Turkey, Kazakhstan, and Europe detected a comparable level of *R*-gene polymorphism (GD_{mean} = 0.30) [23]. Analysis of pepper cultivars (*Capsicum annuum* L.) results in an inter-variety polymorphism (GD of up to 0.26) at half the value detected in wild accessions of the same species (GD of up to 0.58) [8]. So it may be assumed that the resistance genes polymorphism in cultivars depends on both the variability of *R*-genes in the species genome and on the involvement of wild material in breeding.

Today, cultivar- and species-specific NBS profiling sees increasing use not only in genotyping, but also for the purpose of identifying new resistance genes to various diseases [2, 24]. The above-described primer systems for NBS analysis further could find use in studying biotic stress resistance mechanisms in potato cultivars.

A dendrogram was plotted on the basis of the obtained NBS spectra; the tested accessions form a high-polymorphism cluster split into groups with low bootstrap support (Fig. 2).

It seemed interesting to assess how accession clustering correlated with specific traits and with the breeding center (Table 1). No clear clustering by affiliation with the breeding center was found, that may be due to the intensive exchange of breeding material. Six non-Russian cultivars clustered into pairs (Red Impala and Saturna, Newton/Lady Clair, Gala/Impala); however, these pairs were not breeding center-specific (Fig. 2). Nevertheless, the dendrogram shows four clusters that mainly contain Lorch Institute-selected cultivars (see Fig. 2).

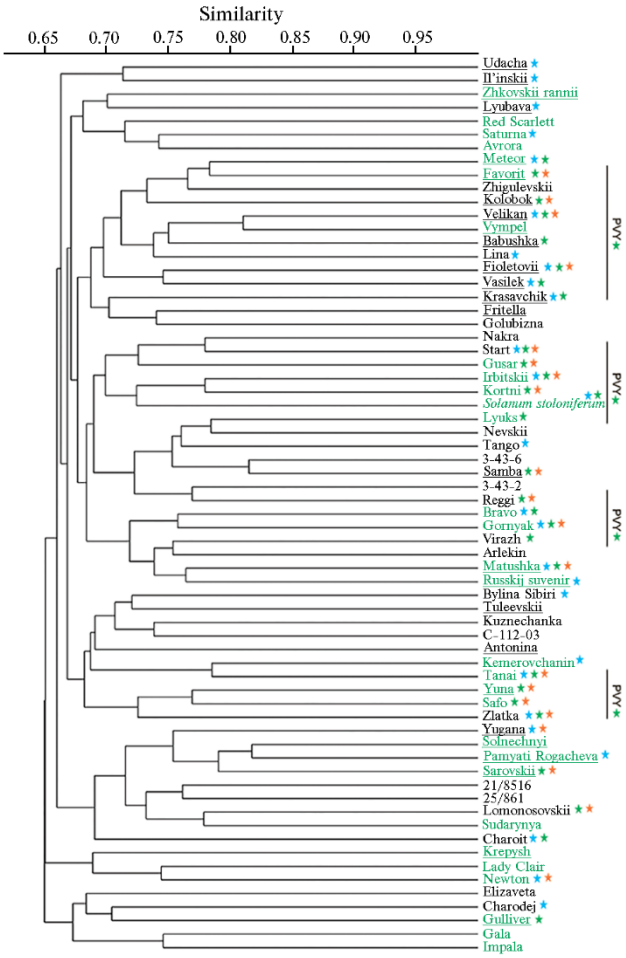


Fig. 2. Dendrogram of genetic variabitity in 65 tested cultivars and breeding clones of potatoes and *Solanum stoloniferum*, plotted by neighbor-joining (NJ, PAST software) **on NBS profiling data.** The highlighted cultivars are resistant to nematode (green font), Phytophthora (blue asterisk next to name), PVY (Potato virus Y, green asterisk), and PLRV (Potato leafroll virus, orange asterisk). Cultivars of Lorch Institute breedind are bold and underscored. For cultivar discription, see Table 1.

Possible clustering of samples by resistance to phytopathogens was analyzed. Several small clusters were found that featured resistance to *Globodera rostochiensis* (Fig. 2); this could be due to the involvement of various donors including several wild species such as *Solanum acaule*, *S. spegazzinii*, or *S. vernei* [25]. Of all the five known *G. rostochiensis* pathotypes [26], only one is common in Russia: Ro1 [27]. Resistance to this pathotype is determined by the dominant alleles of the following genes: *H1* (Chromosome 5) and *Gro1* (Chromosome 7) [17, 28-32]; their loci contain numerous RGA copies [33, 34]. *H1* has been introduced by introgression from singular nematode-resistant *S. tuberosum* subsp. *andigenum*, *S. vernei* accessions [26, 35], whereas *Gro1-4* originated from *S. spegazzinii* [28].

Solanum demissum accessions are the core donors of resistance to phytophthorosis [25]. As far as this trait is concerned, the tested cultivars are scattered all over the dendrogram without apparent clustering (see Fig. 2). This might be due to the fact that BAK1/SERK3 proteins, which are involved in the protective response to phytophthorosis pathogens in plants, are leucine-rich repeat receptor-like kinases (LRR-RLK) that lack an NBS domain [36, 37].

PVY (Potato virus Y, *Potyvirus*, *Potyviridae)* is one of the most economically significant pathogens [38]. Upon infection, NBS-LRR gene Ny-1 (Chromosome

9) recognizes the viral effector [38, 39]. In the dendrogram, PVY-resistant cultivars fall into 4 groups (Fig. 2).

PLRV (Potato leafroll virus, *Polerovirus*, *Luteoviridae*) is considered the second most critical pathogen. Three PLRV resistance-associated quantitative trait loci are localized on Chromosomes 11 and 5 [40, 41]. Interestingly, clustering by PLRV resistance was found to overlap with clustering by PVY resistance(Fig. 2). Main donors of resistance to both viruses are wild species *S. demissum*, *S. acaule*, *S. chacoense*, and *S. stoloniferum* [25 This may be the reason that *S. stoloniferum* accession grouped together with both PVY-resistant and PLRV-resistant cultivars in the dendrogram (Fig. 2).

Two cultivars, Fioletovy and Vasilek, had very peculiar clustering. Those have higher anthocyanin concentrations, making them the only cultivars in the tested population to have bluish-violet skin (Fioletovy also has similarly colored flesh). Both cultivars are Phytophthora and PVY-resistant. However, they have different breeding records. Although the intensity of anthocyanin accumulation has already been shown to correlate with the strength of response to phytophthorosis [42], this grouping indicates rather similarity in *R*-gene patterns and not with coloration. Interestingly, cultivar resistance description that the originators published in the Public Register do not always match molecular profiling data [30-32, 43, 44], which might be due to the difficulty of determining infection response symptoms by visual cues.

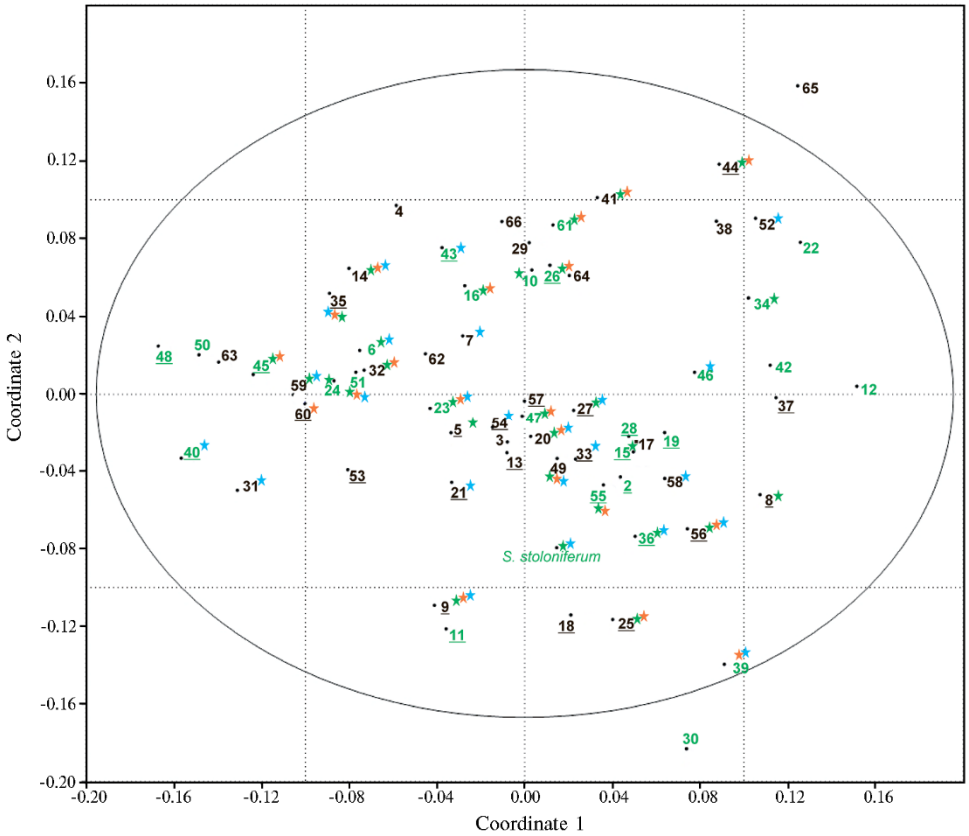


Fig. 3. PCA analysis of NBS profiling data for 65 studied potato cultivars, breeding clones, and *Solanum stoloniferum*. Cultivars resistant to nematode (green numbers), Phytophthora (blue asterisk next to number), PVY (Potato virus Y, green asterisk next to number), and PLRV (Potato leafroll virus, orange asterisk next to number) are indicated. Cultivars of Lorch Institute breedind are underscored. For cultivar discription, see Table 1.

Cluster analysis was complemented with PCA that confirmed there was a single polymorphic group of cultivars and lines selected in Russia and other countries (Fig. 3). The PCA diagram shows a slightly different grouping from the dendrogram. Accessions tended to converge when they shared nematode and PVY/PLRV resistance or were from the same breeding center, although this tendency was weak. Non-Russian cultivars mainly occupied the right side of the diagram, whilst *S. stoloniferum* was in the center.

Also, according to the NBS-marking data, the analysis of the genomic structure of 65 analyzed potato accessions was carried out using the Structure 2.3.4 program. Variants of division into subgroups from k = 2 to k = 15 were tested, and the best result was obtained for k = 10 (LnLike = -75339.2). In Figure 4, the NBS labeling data of the analyzed samples are presented in the form of different ratios of blocks with sets of resistance genes.

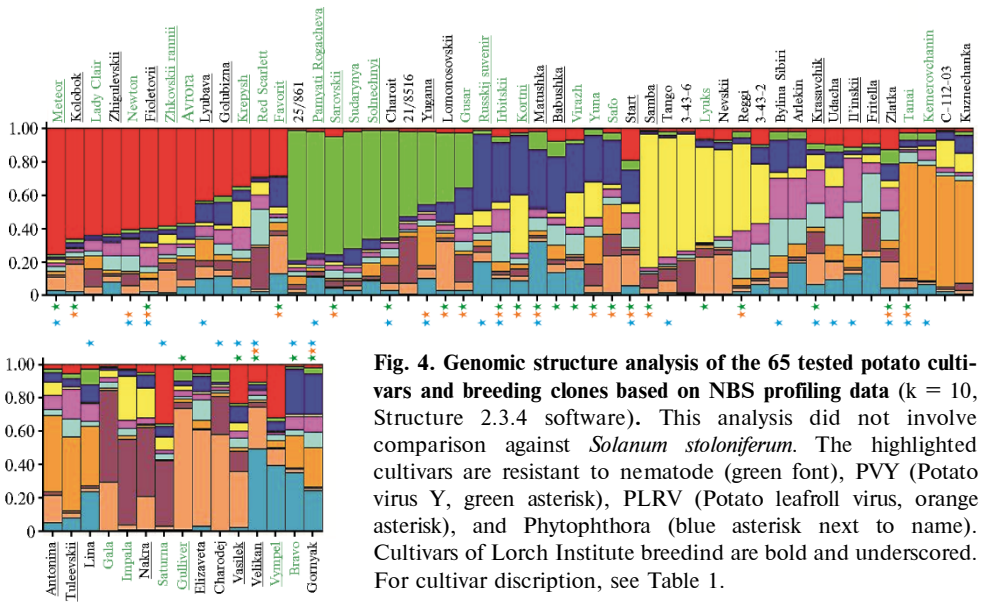


Fig. 4. Genomic structure analysis of the 65 tested potato cultivars and breeding clones based on NBS profiling data (k = 10, Structure 2.3.4 software)**.** This analysis did not involve comparison against *Solanum stoloniferum*. The highlighted cultivars are resistant to nematode (green font), PVY (Potato virus Y, green asterisk), PLRV (Potato leafroll virus, orange asterisk), and Phytophthora (blue asterisk next to name). Cultivars of Lorch Institute breedind are bold and underscored. For cultivar discription, see Table 1.

The analysis made it possible to identify 10 clusters of accessions, in five of which one of the blocks in the genome prevailed. Also one group was identified where there were no major blocks, and the samples of this group showed the greatest polymorphism. The analysis performed did not reveal a single potato accession that would be represented by one or two blocks of resistance gene sets (Fig. 4).

Thus, as a result of multilocus analysis using the NBS-marking method, a collection of 60 cultivars and five breeding clones (*Solanum tuberosum*) of Russian and foreign breeding was characterized for the first time. In general, a high level of NBS-LRR gene polymorphism was revealed in Russian potato cultivars. We identified groups of accessions with the most similar and different patterns of NBS-LRR genes. Accessions tended to converge when they shared nematode and PVY (Potato virus Y)/PLRV (Potato leafroll virus) resistance or were from the same breeding center, although this tendency was weak. Primer/enzyme systems used in this study can be further used for NBS genotyping as part of primary screening for resistance to various biotic stresses, or in research of biotic stress resistance mechanisms.

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