

## Article

# Combination Breeding and Marker-Assisted Selection to Develop Late Blight Resistant Potato Cultivars

Mariya P. Beketova <sup>1</sup>, Nadezhda A. Chalaya <sup>2</sup>, Nadezhda M. Zoteyeva <sup>2</sup>, Alena A. Gurina <sup>2</sup>, Mariya A. Kuznetsova <sup>3</sup>, Miles Armstrong <sup>4</sup>, Ingo Hein <sup>4,5</sup>, Polina E. Drobyazina <sup>1</sup>, Emil E. Khavkin <sup>1</sup> and Elena V. Rogozina <sup>2,\*</sup>

<sup>1</sup> Institute of Agricultural Biotechnology, Moscow 127550, Russia; m.beketova@gmail.com (M.P.B.); polina.drobyazina@gmail.com (P.E.D.); emil.khavkin@gmail.com (E.E.K.)

<sup>2</sup> Potato Genetic Resources Department/Genetic Department, N.I. Vavilov Institute of Plant Genetic Resources (VIR), St. Petersburg 190000, Russia; spb.chalaya@mail.ru (N.A.C.); zoteyeva@rambler.ru (N.M.Z.); a.gurina@gmail.com (A.A.G.)

<sup>3</sup> Phytopathology Lab., Institute of Phytopathology, Bol'shiye Vyazemy, Moscow 143050, Russia; mari.kuznetsova@gmail.com

<sup>4</sup> School of Life Sciences, Division of Plant Sciences at the JHI, University of Dundee, Dundee DD2 5DA, UK; Miles.Armstrong@hutton.ac.uk (M.A.); Ingo.Hein@hutton.ac.uk (I.H.)

<sup>5</sup> The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

\* Correspondence: rogozinaelena@gmail.com; Tel.: +7-951-656-95-53

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**Abstract:** (1) Background: Although resistance to pathogens and pests has been researched in many potato cultivars and breeding lines with DNA markers, there is scarce evidence as to the efficiency of the marker-assisted selection (MAS) for these traits when applied at the early stages of breeding. A goal of this study was to estimate the potential of affordable DNA markers to track resistance genes that are effective against the pathogen *Phytophthora infestans* (*Rpi* genes), as a practical breeding tool on a progeny of 68 clones derived from a cross between the cultivar Sudarynya and the hybrid 13/11-09. (2) Methods: this population was studied for four years to elucidate the distribution of late blight (LB) resistance and other agronomical desirable or simple to phenotype traits such as tuber and flower pigmentation, yield capacity and structure. LB resistance was phenotypically evaluated following natural and artificial infection and the presence/absence of nine *Rpi* genes was assessed with 11 sequence-characterized amplified region (SCAR) markers. To validate this analysis, the profile of *Rpi* genes in the 13/11-09 parent was established using diagnostic resistance gene enrichment sequencing (dRenSeq) as a gold standard. (3) Results: at the early stages of a breeding program, when screening the segregation of F1 offspring, MAS can halve the workload and selected SCAR markers for *Rpi*-genes provide useful tools.

**Keywords:** *Rpi* genes; parental lines; hybrid progeny; dRenSeq; SCAR markers

## 1. Introduction

A potato cultivar is typically developed through a combination of breeding and selection of progeny clones from a cross of two parents that complement each other in as many valuable traits as possible. The trend towards organic farming makes it important to develop cultivars that are resistant to pathogens and pests. In the Russian breeding centers, interspecific hybrids obtained by sexual hybridization using wild and cultivated potato relatives have been used as donors of resistance traits [1,2]. Resistance to late blight (LB) caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary is among the priority requirements to modern potato cultivars. Cultivated and wild potato accessions from the VIR (N.I. Vavilov Institute of Plant Genetic Resources) were utilized in breeding interspecific hybrids with a pyramid of up to six *Rpi* genes; when tested under the various soil and climate conditions; many of these hybrids maintained high LB resistance for over a decade [3].

Employing DNA markers for target genes in practical breeding makes it possible to accelerate the process of developing cultivars with improved traits and to track individual genes in this process. Marker-assisted selection (MAS) has been established in potato breeding with a focus on resistance to pathogens and pests [4]. Using MAS technologies as compared to phenotypic assessment of resistance to viruses and nematodes by conventional methods proved the former to effectively identify the valuable potato genotypes in the second tuber generation [5]. This trend has been supported by recent studies of the diversity of Russian potato cultivars and breeding lines using DNA markers of disease and pest resistance genes and cytoplasm types [6–9]. However, there is currently insufficient information to inform MAS efficiency at the early stages of breeding for pathogen resistance and on the effect of early selection on the manifestation of economically important traits in breeding material in field trial nurseries. A study using DNA markers linked to the resistance genes *H1*, effective against *Globodera rostochiensis*, *Ry-fsto*, effective against potato virus Y and *Rpi-phu1* effective against LB in a segregating progeny did not reveal any relationship between the presence of various combinations of these markers and agronomical important traits, such as yield, size and shape of tubers [10]. The effectiveness of selecting prospective segregants with higher LB resistance upon the presence of the corresponding DNA markers has been studied but sporadically. Meanwhile, over the past two decades, more than 20 *Rpi* genes have been identified and cloned in potato and related *Solanum* species. The genes *R1*, *R2* and its orthologue *Rpi-abpt*, *R2-like*, and *Rpi-blb3*; *R3a* and *R3b*, *R8*, *R9a*, the orthologues *Rpi-blb1* and *Rpi-sto1*, *Rpi-blb2*, *Rpi-vnt1* and *Rpi-chc1* have been located on chromosomes 4 to 11 and characterized most comprehensively [11]. DNA markers corresponding to the fragments of *Rpi* genes introgressed into cultivated potato from wild relatives are actively deployed for screening breeding material to select promising genotypes combining several *Rpi* genes [12–14]. The dRenSeq method for diagnostic sequencing genome target fragments ensures a high degree of certainty of the identification of full-length sequences of known *Rpi* genes in genetic collections and breeding material [15]. To develop the MAS technology for breeding LB-resistant potato cultivars, it is especially important today to study hybrid populations, to compare the effectiveness of phenotypic and marker selection of segregants, and to evaluate DNA markers of *Rpi* genes as the tools for identifying valuable genotypes at the early stages of breeding process.

In this study, a cross of parents complementary in LB resistance of leaves and tubers together with their F1 progeny were investigated to identify genotypes with high LB resistance and the best combination of favorable traits. Further, this study enabled us to assess the accuracy and efficacy of SCAR (sequence characterized amplified region) markers to track *Rpi* genes as practical breeding tools.

## 2. Materials and Methods

**Plant Material.** The study comprised cv. Sudarynya and the 13/11-09 breeding clone as respective female and male parents and their hybrid progeny (68 individuals). Cv. Sudarynya (released by the Belogorka Research Institute for Agriculture, Leningrad, Russian Federation) was bred from the progeny obtained following the pollination of the 89181/6 clone by a multispecies hybrid 8889/3; the pedigree of the latter lists *Solanum tuberosum*, *S. andigenum*, *S. demissum*, and *S. stoloniferum* [8]. The 13/11-09 clone of an interspecific hybrid was bred at the Institute of Plant Protection, Leningrad, by selecting from the progeny resulting from the pollination of an F2 plant from *S. pinnatisectum* k-17464 × Gitte cross with a pollen mixture from hybrids with high LB resistance [3]. According to the long-term field observations, cv. Sudarynya and clone 13/11-09 noticeably differ in their response to *P. infestans* infection: leaves of the former are more resistant to LB, whereas its tubers are more susceptible than those of the latter. The hybrid seeds were sown in 2017; in subsequent years, F1 progeny individuals were obtained and maintained as clones. In 2018–2020, each F1 clone was planted in the field of the VIR experimental field, located in the North-Western Region of Russia, nearly to St. Petersburg in two replications. Cvs.

Bintje, Elizaveta, Nayada, Sarpo Mira, Alouette, Newskij and Peterburgskij were used as references.

*Methods of Phenotypic and Molecular Analysis.* The color of the skin and base of the tuber eye, the size and intensity of the anthocyanin coloration of the inner side of the corolla, and the manifestation of pigment coloration on the pedicel were assessed, according to the Guidelines for the Conduct of Testing for Distinctness, Uniformity and Stability. Potato (*Solanum tuberosum* L.), RTG/0023/2 form [16].

LB resistance, yield and yield structure were assessed in field experiments in 2017–2020 under the conditions of the VIR experimental field and in laboratory tests at the VIR Department of Genetics (Center International Potato).

To assess LB resistance in the field, plants were examined in the potato stands maintained in the VIR experimental field in 2017–2020 according to the CIP (Center International Potato) Methodological Guidelines [17]. Plant infection under natural infection was followed starting from the day when the susceptible cv. Bintje was affected. Plant damage was recorded weekly following the appearance of the first disease symptoms, using a 9-point scale where 9 stands for high resistance with no symptoms of damage, and 1 means a completely infected and highly susceptible plant. Plants scoring from 7 to 9 points were considered resistant.

The laboratory tests of leaf and tuber resistance were carried out using a highly pathogenic *P. infestans* isolate (1.2.3.4.6.7.10.11) sampled from infected potato plant Lomonosovskij grown in VIR experimental field [18]. The isolate virulence was determined using a set of Black's differential plants (*R1-R11*). To prepare the inoculum, *P. infestans* isolate was grown on tuber slices of the susceptible cv. Dorisa. The cultivars with established LB resistance in the laboratory tests were chosen as the references: Alouette (resistant), Peterburgskij (susceptible) and Newskij (moderately susceptible leaves and resistant tubers). Leaves (three leaf lobes from each plant of a clone), tubers (five from each clone) and tuber slices (three slices cut out from the middle part of each of five tubers) were placed in individual trays and tested in two independent biological replicates. Leaves were tested in mid-July, and tubers in November/December. Incubation proceeded for 8 days at 17 °C and high humidity. Resistance of individual inoculated leaves and tuber slices was assessed using the methods described by M. Brylińska and J. Śliwka [19]. Disease symptoms were scored on days 4 and 8 after the inoculation as the percentage of the lesion area relative to the total leaflet area. The total score of the tuber slices infection was a combination of the infected area percentage and the mycelium growth intensity. When assessing resistance in tubers, the method of decapitated tubers inoculation developed by N. Zoteyeva and E. Zimnoch-Guzowska [20] was used. Disease symptoms in tubers were also scored using a 1–9 scale, where 1 means infection of the entire surface, and 9 means the absence of symptoms. Tubers with scores from 7 to 9 were considered resistant, those with 6 points as moderately resistant, with 5 points as moderately susceptible, and those with 1 to 4 points as susceptible.

*Molecular and Bioinformatics Methods.* Genomic DNA from young leaves was isolated with the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) or DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentration was measured with an UV/Vis NanoPhotometer P300 (IMPLEN, Munich, Germany). In the marker analysis, 11 SCAR markers represented nine *Rpi* genes, namely *Rpi-R1*, *Rpi-R3a*, *Rpi-R3b*, orthologues *Rpi-blb1* and *Rpi-sto1*, orthologues *Rpi-R2* and *Rpi-blb3*, *Rpi-blb2* and *Rpi-vnt1.3* (Rogozina et al., 2021).

DNA amplification was run in a MJ PTC-200 thermocycler (Bio-Rad, Hercules, CA, USA). The PCR mix contained 1 µL of 10 × PCR buffer Mg2+ Plus for *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany), 1 µL of dNTP mix (2.5 mM of each), 1 µL each of forward and reverse primers (1 µM), 5 U of *Taq* DNA polymerase, 30–60 ng/µL of genomic DNA, and sterile deionized water to 10 µL. PCR products were separated by electrophoresis in 1% (*w/v*) agarose in 1 × TAE buffer for 40 min at 6 V/cm and visualized under UV after staining with ethidium bromide using a Gel Logic 100 Imaging System (Eastman

Kodak Company, Rochester, NY, USA). Following electrophoretic separation, PCR-amplified DNA fragments were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

The application of dRenSeq for the identification of functional *Rpi* genes in the 13/11-09 clone was carried out at the James Hutton Institute, Dundee, United Kingdom in accordance with the previously described protocol [15].

Selected amplicons were cloned using pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and sequenced with nucleic acid analyzers ABI PRISM 3130xl (Applied Biosystems, Foster City, CA, USA). Sequenced fragments were assembled using SeqMan package. Lasergene 7.0. BLAST 2.0. and SeqMan, Lasergene 7.0 programs were used to mine genomic databases for *Rpi* genes and their homologues, and their phylogenetic analysis was performed with the MEGA6 package [21].

The data were statistically processed using the parametric and nonparametric statistics methods using the Statistica StatSoft 13 software package (StatSoft Russia; <http://statsoft.ru/products>).

### 3. Results

#### 3.1. Anthocyanin Coloration in Parents and F1 Hybrids

Parental genotypes, cv. Sudarynya and clone13/11-09 and 68 F1 segregants were assessed for the pigment coloration of the skin and base of the tuber eyes. Tubers of cv. Sudarynya, the female parent in the cross, have yellow skin and a red-colored base of the eye. The male parent 13/11-09 has tubers with the red-colored skin and white base of the eye (Figure 1a). Both parents have white tuber flesh. Among F1 clones, we observed individuals, which have maintained the characteristic color patterns of the parents as well as clones with new features, such as yellow tubers and uncolored eyes or anthocyanin pigmentation of the entire tuber surface (Figure 1b). The distribution of color patterns in the segregants was as follows: 17 segregants with red or pink color of the tuber skin and the colored eye base, 10 segregants with red or pink skin and the uncolored eye base, 9 segregants with yellow skin and red eye base, and 32 segregants with yellow tubers and uncolored eyes.



**Figure 1.** (a) Tubers of parental genotypes cv. Sudarynya (right) and 13/11-09 clone (left); (b) tubers of F1 segregants Tubers of cv. Sudarynya (right) and of the 13/11-09 clone (left).

The parents (cv. Sudarynya and 13/11-09 clone) and 56 F1 clones were assessed for the pigment coloration of the corolla and inflorescence. In 12 clones (17%), the plants never bloomed until the end of the study. In cv. Sudarynya, the corolla was colorless, whereas the pedicel ring was pigmented. In the 13/11-09 hybrid, an intense anthocyanin coloration was observed at the greater part of the corolla inner surface (Figure 2a). F1 clones differed in the pigmentation of their corolla and pedicel (Figure 2b). Corolla pigmentation, with varying intensity and area of coloration, was registered in 22 F1 clones. Thirty-four F1 clones had a white corolla. The intensity of the pedicel pigment coloration in F1 clones varied from weak to strong. The pedicel ring was colored in 14 F1 clones.



**Figure 2.** (a) Flowers and leaves of parental genotypes 13/11-09 clone (right) and cv. Sudarynya (left); (b) flowers of F1 segregants.

### 3.2. Resistance of Parents and F1 Progeny to LB of Leaves and Tubers

F1 clones from the cv. Sudarynya × 13/11-09 cross were assessed for resistance to LB of leaves and tubers in field conditions in 2017, 2019 and 2020. In 2018, a hot and dry summer in northwestern Russia prevented early infection of potatoes by LB; the first signs of the disease appeared in late-maturing cultivars at the end of August, when the growing season of F1 plants was over.

In 2017, 2019 and 2020, the first LB symptoms appeared on leaves of the susceptible cultivars Bintje and Peterburgskij in early August, and in the resistant cultivar Sarpo Mira at the end of the second ten-day period of August. However, the development of LB in potato plants during the years of the study occurred at different rates. The fastest epidemic development of the disease was noted in 2017, and a moderate rate of LB development was recorded in 2019 and 2020.

At the end of August 2017, the overall resistance of Sudarynya × 13/11-09 F1 seedlings corresponded to the average resistance of the parents, while the groups of susceptible (1–3 points), weakly resistant (3–5 points) and moderately resistant (5–6 points) progeny individuals were clearly distinguished. About half of the Sudarynya × 13/11-09 progeny (32 individuals) showed resistance from 6 to 8 points, which was at the same level or higher than that of cv. Sudarynya, the best parent (Table 1).

**Table 1.** Distribution of ‘Sudarynya’ × 13/11-09 F1 progeny individuals per classes of leaf and tuber LB resistance assessed by different methods.

Assessment Method *, Date	Number of Individuals	Distribution of Individuals per Classes of Resistance **							Resistance, Average Point	
		1–3	3.1–4.0	4.1–5.0	5.1–6.0	6.1–7.0	7.1–8.0	8.1–9.0	Hybrid Progenies	Parents ***
FLS, 25 August 2017	68	15	5	6	10	14	18	0	5.5 ± 0.3	♀ 6.0 ♂ 5.0
FLC, 14 August 2019	66	0	2	1	4	25	33	1	7.3 ± 0.1	♀ 8.0 ♂ 5.0
FLC, 18 August 2020	67	10	5	16	16	21	0	0	5.3 ± 0.6	♀ 7.0 ♂ 6.0
LLC, 4 dai, 2019	68	0	1	4	31	22	8	2	6.3 ± 0.1	♀ 6.7 ♂ 5.6
LLC, 8 dai, 2019	66	48	5	9	4	0	0	0	2.5 ± 0.1	♀ 3.8 ♂ 2.3
LTC, 2019	66	0	2	15	29	18	1	1	5.6 ± 0.1	♀ 5.9 ♂ 6.7

\* FLS field, leaves of seedlings, FLC field, leaves of clones, LLC laboratory, leaves inoculation, clones, dai days after infection, LTC laboratory, tubers inoculation, clones. \*\* on the 1-9 scale, where 9 is the absence of infection. \*\*\* ♀ ‘Sudarynya’, ♂ 13/11-09.

In mid-August 2019, leaves in half of the F1 progeny (second tuber generation) showed single spots of LB infection, like both parents did. The overall resistance in the progeny (7.3 points) corresponded to the average resistance of the parents (Table 1). One clone (952–49) showed no signs of LB infection of leaves. In the second ten-day period of

August 2019, plants of 18 F1 clones entered the senescence stage, which forced us to harvest the early maturing forms. During the second survey (August 27), plants of F1 clones (48 genotypes), differed in their leaf LB manifestation from weak (affected no more than 25% of the leaf surface) to completely infected plants. High resistance (7 points) was noted in four F1 clones: 952-3, 952-29, 952-51 and 952-62, as well as in cvs. Sarpo Mira and Sudarynya.

In 2020, the average leaf resistance to LB in F1 progeny (third tuber generation) was lower than the average resistance of the parents (Table 1). One third of the progeny (21 genotypes) corresponded to the parents regarding their LB resistance (6–7 points). Among the remaining F1 clones, susceptible, weak and medium resistant individuals (10, 21, and 16 genotypes, respectively) were identified. Spearman's rank correlation coefficient of LB resistance in the field in different years was 0.32–0.54 at  $p < 0.05$ .

In 2019, the detached leaves, whole tubers and tuber slices of F1 clones and parents were artificially infected. Due to the high infectious pressure, strong resistance segregation in the hybrid population was observed already on day 4 after the infection. On day 8, the segregation pattern changed: a large part of resistant and moderately resistant plants shifted towards the class of susceptible ones. The response of reference cultivars to infection corresponded to the characteristics of their resistance (Table 2).

**Table 2.** Leaf and tuber LB resistance of reference cultivars and parents in the Sudarynya × 13/11-09 combination in laboratory tests.

Cultivar	LB Resistance, Average Point					
	Leaf Lobes		Decapitated Tubers		Tuber Slices	
	Day 4	Day 8	Mycelium	Spot	Mycelium	Spot
Alouette	9.0	6.8	1.0	9.0	0.5	9
Peterburgskij	5.3	1.0	2.0	4.0	2.5	3.0
Newskij	7	4	0	6.3	1.0	6.5
Sudarynya	6.7	3.8	0	5.9	0.5	6.0
13/11-09 clone	5.6	2.3	1	6.7	0.2	6.7

In the first survey following leaf inoculation in the laboratory, the average resistance of the F1 progeny corresponded to the average resistance of the parents (Table 1). Two clones, 952-9 and 952-29, as well as the resistant reference cv. Alouette, manifested no signs of LB infection. The infection of the susceptible cv. Peterburgskij equaled 5.3 points. In the second survey, the average resistance of the F1 progeny was slightly lower than the average resistance of the parents. The leaves of more than 70% of F1 clones, as well as the leaves of the susceptible reference cv. Peterburgskij, were completely infected by LB. Four clones, 952-15, 952-59, 952-66 and 952-69, were identified as more resistant (5.5–5.8 points) than the best parent—cv. Sudarynya (3.8 points). In the second survey, resistance in the leaves of clones 952-9 and 952-29 was estimated at 1.3 and 5 points, respectively, the leaves of cv. Alouette scored 6.8 points. A comparison of the data from the first and second surveys of the results of artificial infection using the t-criterion indicated a statistical significance of the difference between mean values ( $t = 35.43$  at  $p < 0.05$ ).

No correlation was found between the results of evaluating F1 progeny for LB resistance in the field and in the case of artificial leaf infection. The isolates collected from potato leaves in the VIR field plots were assayed with several phenotypic and molecular methods; they apparently belong to a single asexual lineage of the pathogen [22]. The laboratory screening has shown a strong decrease in resistance on day 8 after the infection in comparison with the values obtained on day 4. This could be caused by a very strong infection load, i.e., a high inoculum concentration combined with a high aggressiveness of the used *P. infestans* isolate. It should be noted that disease symptoms on individual leaf lobes of cv. Alouette scored 6.3 points, while in our numerous tests using other isolates of the pathogen this cultivar did not show disease symptoms. The data of the survey carried out on day 4 day after the infection made it possible to differentiate the population

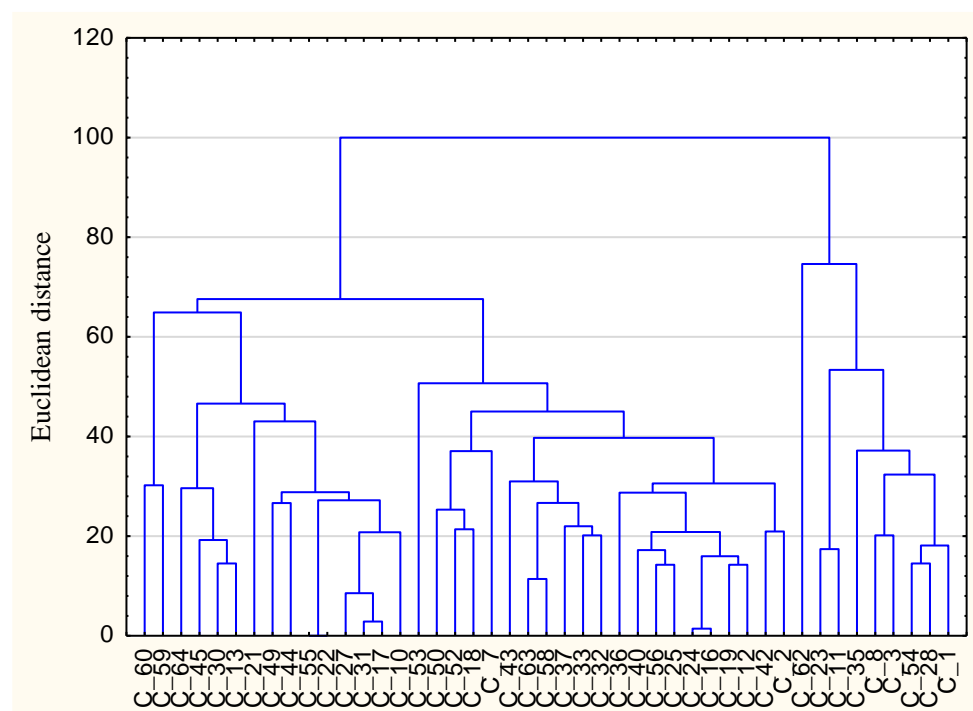


in more detail in terms of resistance and to identify plants with the score of 7–9 points. Such individuals from the Sudarynya × 13/11-09 progeny used in further breeding work were the most promising ones.

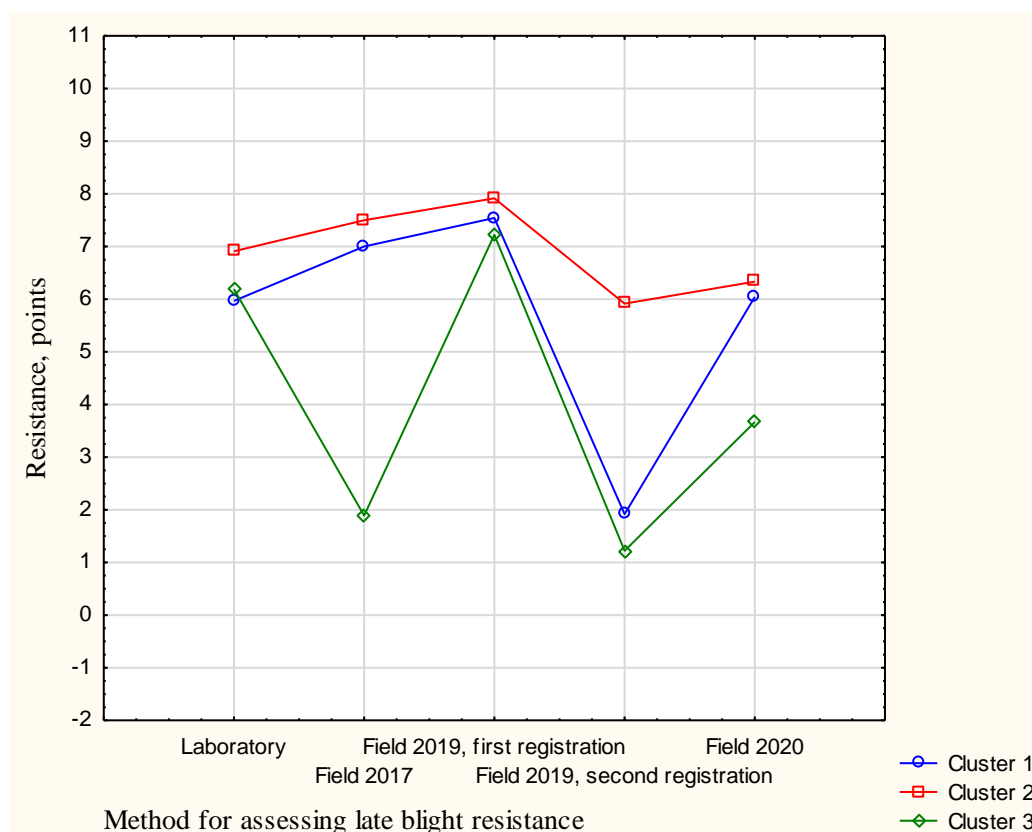
In the case of decapitated tubers inoculation, the average resistance in the F1 progeny was lower than that of the parents (Table 1). When artificially infected, tubers of two clones, 952-7 and 952-11, manifested higher resistance (7–8.2 points) than those of the best parent 13/11-09. The score for the susceptible cv. Peterburgskij was 4.0 points. The tubers of cv. Alouette had no infection spots (9 points), though mycelium growth (1.0 point) was noted in them.

Tuber slices from 17 progeny clones were infected. The size of the infection spot varied from 8 to 4 points, and the development of mycelium, from 0.1 to 3 points. The size of the infection spot in cv. Sudarynya scored 6 points and the formation of mycelium was noted (0.5 points). In 13/11-09, the size of the infection spot was less and scored 6.7 points, and a weak growth of mycelium (0.2 points) was noted. The clone 952-11 was found to have tubers with LB resistance higher than that in the best parent (13/11-09), while tuber resistance of the clone 952-15 corresponded to that of 13/11-09.

Based on the results of the F1 progeny assessment for LB resistance in leaves, the segregants were grouped using two methods: a) the hierarchical classification by constructing a dendrogram on unweighted pairwise average and b) a *k*-means clustering (Figures 3 and 4). According to the diagram shown in Figure 3, F1 progeny is divided into two branches; one of them consists of two clades, which combine 15 and 21 segregants respectively.



**Figure 3.** Hierarchical classification of the F1 progeny regarding LB resistance, genotypes number: C\_1-952-15, C\_2-952-1, C\_3-952-10, C\_7-952-16, C\_8-952-17, C\_10-952-19, C\_11-952-2, C\_12-952-20, C\_13-952-21, C\_16-952-24, C\_17-952-25, C\_18-952-26, C\_19-952-27, C\_21-952-29, C\_22-952-3, C\_23-952-30, C\_24-952-31, C\_25-952-32, C\_27-952-35, C\_28-952-36, C\_30-952-38, C\_31-952-39, C\_32-952-4, C\_33-952-40, C\_35-952-42, C\_36-952-43, C\_37-952-44, C\_40-952-47, C\_42-952-5, C\_43-952-50, C\_44-952-51, C\_45-952-52, C\_49-952-57, C\_50-952-58, C\_52-952-6, C\_53-952-60, C\_54-952-61, C\_55-952-62, C\_56-952-63, C\_58-952-65, C\_59-952-66, C\_60-952-67, C\_62-952-7, C\_63-952-8, C\_64-952-9.



**Figure 4.** F1 progeny clustering by the k-means method regarding LB resistance.

The three clusters resulting from each classification method application were similar in composition. Cluster 2 contains 12 F1 clones that were most resistant to LB in the field experiments and in the laboratory tests; on an average, this group scored 5.9–7.9 points (Figure 4). Cluster 1 comprises 24 F1 clones, with significant leaf damage registered during the second survey in the field experiment of 2019; the average score of this group was 1.9–7.5 points. Cluster 3 includes 9 F1 clones most affected by LB in the field in 2017, 2019 (second survey) and in 2020; the average score of this group was 1.2–7.2 points (Figure 4).

### 3.3. *Rpi* Genes in Parents and F1 Progenies

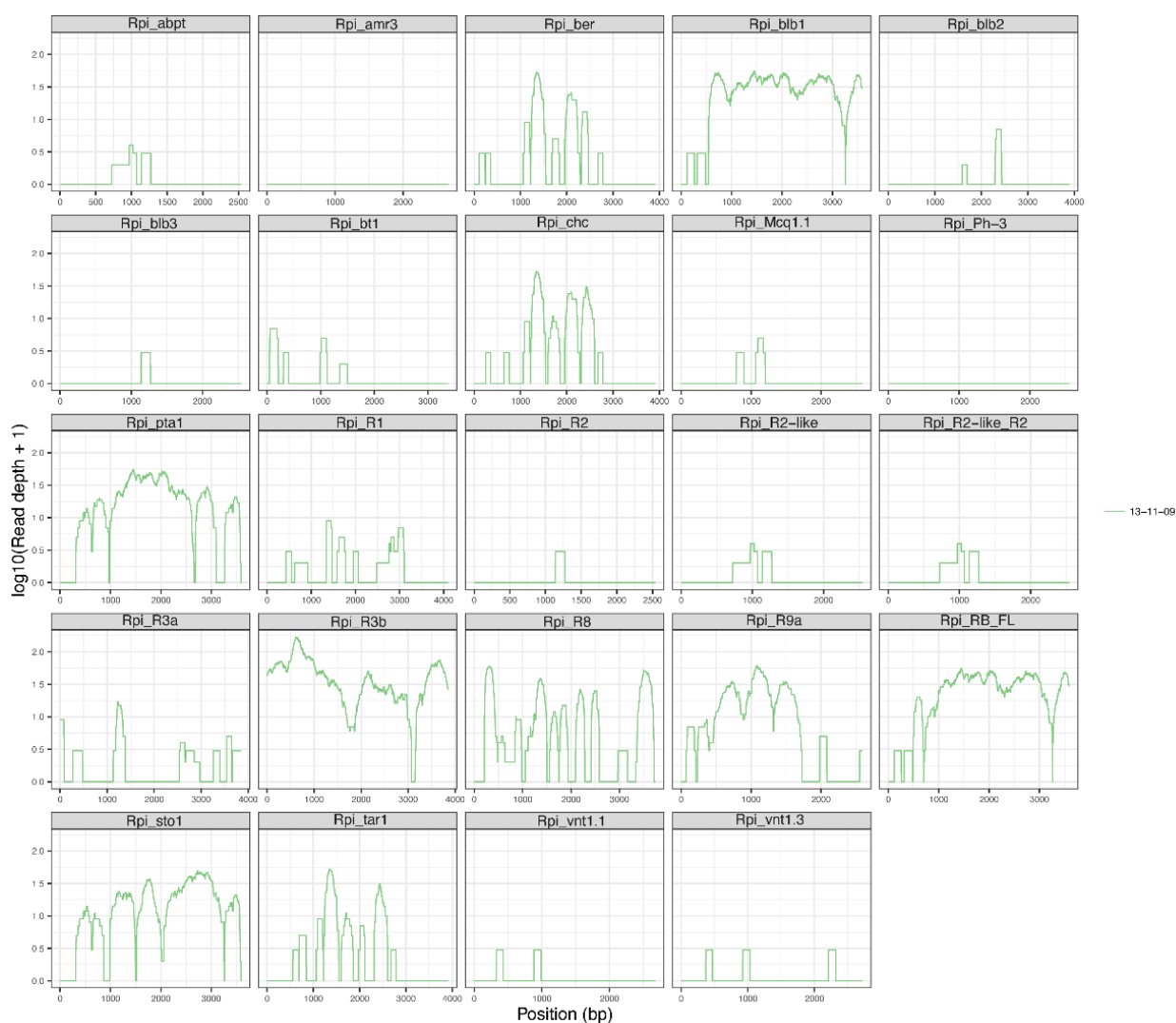
Both parents and 60 F1 progenies were screened with SCAR markers that were developed to track nine *Rpi* genes. Both parents and segregants were devoid of the markers for *Rpi-R1*, *Rpi-blb2* and *Rpi-blb3* genes. Two parents seemed to comprise the *Rpi-R3b*, *Rpi-blb1/Rpi-sto1* genes and differed as regards the *Rpi-R2*, *Rpi-R3a* and *Rpi-vnt1.3* genes. While the markers *Rpi-R2-1137*, *Rpi-R2-686* and *Rpi-blb3-305* correspond to close orthologue and usually manifest perfect agreement, here the latter marker was absent from the genotypes under study, and poor match was observed in the case of two former markers (Table S1. SCAR markers of *Rpi*-genes in F1 progeny and parental lines).

The profile of SCAR markers in cv. Sudarynya is in good agreement with the evidence by Gavrilenko et al. [8] who reported in this genotype the markers of *Rpi-blb1/Rpi-sto1* and *Rpi-R3a* genes.

The pedigree of the hybrid 13/11-09 has not been clearly established; reportedly it lists *S. andigenum*, *S. berthaultii*, *S. demissum*, *S. microdontum*, *S. polytrichum*, *S. pinnatisectum* and *S. xvallis-mexici*. Therefore, it was important that this genotype was also analyzed using the dRenSeq technology, which reliably discerns the full-length gene sequences from non-functional homologs. In this case, only two genes were identified in this hybrid: *Rpi-R3b* and *Rpi-blb1-like* (Figure 5). The *Rpi-R3b* sequence found in these plants was 99.95%



identical to the reference gene *Rpi-R3b* (NCBI Genbank accession JF900492) and completely matched the sequence of *Rpi-R3b*<sup>G1696/G3111</sup> previously described in cv. Innovator [15]. This resulted in 98% of the *Rpi-R3b* sequence being covered by RenSeq reads in the dRenSeq analysis. The sequence of *Rpi-blb1* gene in clone 13/11-09 was 98.9% identical to the reference gene AY336128 differing at only 3 nucleotides in the CDS. Only two of these SNPs result in an amino acid substitution. This resulted in 93% of the *Rpi-blb1* sequence being covered by RenSeq reads in the dRenSeq analysis. Based on the similarity between *Rpi-blb1* and *Rpi-sto1*, the *Rpi-sto1* sequence from clone 13/11-09 was 87% identical to the reference gene EU884421. The presence of the *Rpi-vnt1.3* gene in clone 13/11-09 which was predicted through the corresponding SCAR marker Rpi-vnt1.3-612 was not supported by the dRenSeq analysis suggesting that this SCAR marker yields a false-positive response.



**Figure 5.** dRenSeq analysis in 13/11-09 clone. The sequence representations of known NLRs effective against late blight are shown in each box.

To elucidate this disagreement between the SCAR marker for *Rpi-vnt1* and the dRenSeq analysis, we cloned the marker amplicon Rpi-vnt1.3-612 from the hybrid 13/11-09 (Figure S1. Alignment of *Rpi-vnt1* homologs and predicted amino acid sequences of Rpi-vnt1 protein homologs). The resulting sequence was 97% identical to the functional genes *Rpi-vnt1.1* (NCBI Genbank accession FJ423044) and *Rpi-vnt1.3* (FJ423046) as well as to the *Rpi-vnt1.3* gene in cv. Alouette (MH297492) cloned in the Institute of Agricultural Biotechnology. The predicted amino acid sequence of the amplicon was 95% identical to

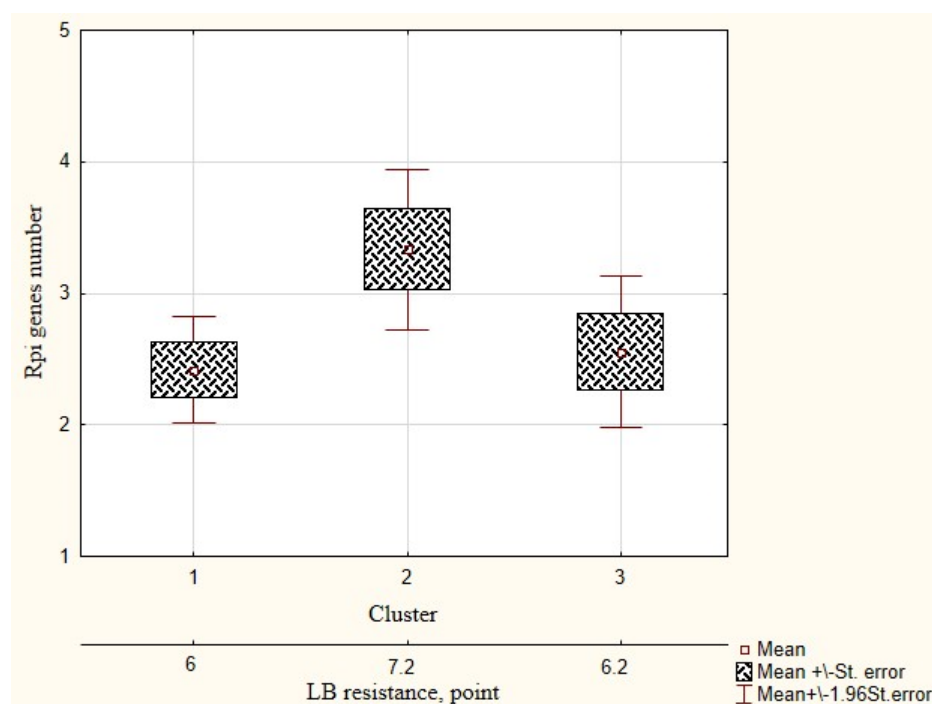
the prototype proteins *Rpi-vnt1.1* (ACJ66594) and *Rpi-vnt1.3* (ACJ66596) as well as to the *Rpi-vnt1.3* proteins in cv. Alouette (QAY29223). However, the marker amplicon *Rpi-vnt1.3-612* was also 99% identical to the pseudogene from *S. microdontum* subsp. *gigantophyllum* (GU338312) and 93% identical to the fragment 59492023-59491423 bp of chromosome 9 of *S. pinnatisectum* cultivar CGN17745 (CP047560). One of these species is the putative source of *Rpi-vnt1.3-612* marker.

This provides evidence that the *Rpi-vnt1* SCAR marker, unlike the *Rpi-R3b* and *Rpi-blb1* SCAR markers, is unable to differentiate between functional and non-functional *Rpi-vnt1* homologs. Indeed, this SCAR marker wrongly predicts the cultivar Bintje, which is susceptible to late blight, to contain *Rpi-vnt1.3* (Table 3).

The segregants manifested diverse combinations of SCAR markers (Table S1); the markers of *Rpi-R3b* and *Rpi-blb1* were most frequent (0.80 and 0.73, respectively). The markers of *Rpi-R2* and *Rpi-R3a* were much less frequent (0.39 and 0.40 respectively). Three progeny genotypes were devoid of any gene markers. The markers *Rpi-blb1-821* and *Rpi-sto1-890* corresponding to widely distant regions of the gene matched perfectly (with a single exception) parents.

Pyramiding several *Rpi* genes in one potato plant can greatly enhance its LB resistance [3]. In the progeny of the Sudarynya × 13/11-09 cross, we find several stacks of markers for *Rpi* genes advantageous for further breeding efforts. We believe that forms combining several *Rpi* genes have the greatest potential for durable LB resistance. Such a combination of genes, according to our data, is quite rare in the starting material for potato breeding and further constrained by the non-specific SCAR marker results for *Rpi-vnt1*. Twelve F1 clones combine the markers of three genes *Rpi-R3a*, *Rpi-R3b* and *Rpi-blb1*, two F1 clones combine the markers for *Rpi-R3b*, *Rpi-blb1/Rpi-sto* and *Rpi-vnt1.3* (the pattern characteristic of the male parent 13/11-09 although the *Rpi-vnt1.3* data apparently indicate the presence of non-functional gene homologue), whereas eight F1 clones each stacked four markers for the genes *Rpi-R2*, *Rpi-R3a*, *Rpi-R3b* and *Rpi-blb1/Rpi-sto* (the profile found in the female parent cv. Sudarynya). Two F1 segregants seem to present another case of *Rpi* gene recombination: *Rpi-R2*, *Rpi-R3b*, *Rpi-blb1/Rpi-sto* (and *Rpi-vnt1.3*), whereas two clones (genotypes number 952-35 and 952-57) revealed a pyramid of as many as five genes from both parents: *Rpi-R2*, *Rpi-R3a*, *Rpi-R3b*, *Rpi-blb1/Rpi-sto* (and *Rpi-vnt1.3*) (Table S1).

A comparison of the results of the marker analysis and classification of F1 clones regarding LB resistance shows that in terms of the *Rpi* genes number, the group of F1 clones from the second cluster (most LB resistant according to the combination of field and laboratory assessments) is superior to the other two clusters (Figure 6). F1 clones showing a consistently high resistance under artificial infection and field trials, were found to contain from 2 to 4 *Rpi* genes. Of the two other clusters, the first one contains F1 clones in which 0 to 3 *Rpi* genes, while the other unites clones with 1 to 3 *Rpi* genes.



**Figure 6.** Predicted *Rpi* genes numbers (based on SCAR markers) in F1 clones from segregant clusters of different LB resistance.

### 3.4. Yield Capacity and Structure in Parents and F1 Progenies

F1 clones significantly differ in productivity, which varies from 32 to 1760 g/plant; the average productivity being 918 g/plant. In accordance with the accepted VIR scale, four clones 952-35, 952-44, 952-57 and 952-69 were characterized as highly productive (100–150% of standard cultivar yield), 21 as medium productive (70–100%) and 17 as low productive (30–70%). The marketability of the yield of F1 clones (the ratio of tubers weighing over 40 g to the weight of all tubers) varies from 0 to 96%, the average marketability being 80%. The marketability of cv. Sudarynya is 84%. The number of tubers (pieces) per plant in F1 clones varies from 1 to 34 pcs. with an average number of 16 tubers. Several F1 clones, such as 952-7, 952-35, 952-42, 952-44, 952-57, 952-59, 952-65 and 952-69 exceeded cv. Sudarynya by their productivity and yield structure elements.

Five F1 clones combined high productivity with LB resistance (Table 3). In two of these clones, 952-35 and 952-62, resistance of naturally and artificially infected leaves was higher than in the parents. By their leaf resistance three other F1 clones corresponded to the best parent cv. Sudarynya. No growth of *P. infestans* mycelium was observed on tubers of all four selected hybrid clones, which means a long incubation period and/or suppression of the pathogen ability to develop. Tubers of parents did not possess such a resistance (Table 3). All clones contained the *Rpi-blb1-like* gene that is the most likely cause of the resistance. As mentioned afore, although the SCAR marker for *Rpi-vnt1.3* predicted the presence of this gene in these clones, we failed to confirm the presence of the functional *Rpi-vnt1.3* in the parents with the dRenSeq analysis. More than that, this SCAR marker was also found in the susceptible cultivar Bintje (LB score of 3) (Table 3).

Among the selected hybrid clones, 952-35 and 952-57 had marker fragments of five *Rpi* genes, three others had marker fragments of four *Rpi* genes (Table 3). According to the morphological characteristics of tubers, clones 952-35 and 952-65 have the phenotype of the female parent, while three other clones have a new combination of color of the skin and eyes of tubers. The productivity of all selected hybrid clones was high, over 1000 g/plant. The marketability was high in three clones, only in 952-62 it was slightly lower

than in cv. Sudarynya. The male parent of the Sudarynya × 13/11-09 combination produced small tubers. Presumably, the insufficient marketability characteristic of clone 952-62 is related to the genes of the low-productive male parent.

Hybrid clones 952-57 and 952-62 apparently inherited a new combination of genes from both parents, and this complementation provided for anthocyanin pigmentation in tubers and eye bases. All five F1 clones selected from the Sudarynya × 13/11-09 progeny by several breeding valuable traits demonstrated LB resistance in leaves and tubers. All selected F1 clones comprised markers of the *Rpi-R2* gene (same as in the female parent) and *Rpi-R3b* + *Rpi-blb1/Rpi-sto* genes, which were apparently inherited from both the female and male parents.

Similar to cvs. Sarpo Mira and Alouette, the best hybrid clones from the Sudarynya × 13/11-09 cross contained markers of the *Rpi-R3a* and *Rpi-R3b* genes, as also the markers of the *Rpi-blb1/Rpi-sto*, which provided resistance to a wide range of LB strains and was not found in cvs. Sarpo Mira and Alouette. However, the best F1 individuals were inferior to some reference cultivars in their leaf LB resistance (Table 3). Additional studies of the progeny of the Sudarynya × 13/11-09 hybrid are necessary to reveal the genetic nature of LB resistance and confirm the functionality of the *Rpi* genes registered with SCAR markers.

**Table 3.** F1 clones from the Sudarynya × 13/11-09 cross with a set of important traits, their parents and potato cultivars.

Clone, Cultivar	LB Resistance, Points		SCAR Markers of <i>Rpi</i> Genes	Productivity, Marketability, %		Tubers (Skin Color, Shape)	Eyes (Depth, Color)
	Leaves <sup>1</sup>	Tubers <sup>2</sup>		g/Plant	%		
952-26	5–8/7	n.d	R2-1137, R2-686, R3b-378, RB-226, Blb-821, Rpi-sto1-890, Rpi-vnt1.3-616 ^	1320	79	yellow	uncolored
952-35	7–8/6.5	5.4	R2-686, R3a-1380, R3b-378, RB-226, Rpi-sto1-890, Rpi-vnt1.3-616 ^	1760	94	yellow	colored
952-57	6–8/6.7	6.3	R2-1137, R2-686, R3a-1380, R3b-378, RB-226, Blb-821, Rpi-sto1-890, Rpi-vnt1.3-616 ^	1490	95	pink, rounded-oval	medium, colored
952-62	7–8/7	5.8	R2-686, R3a-1380, R3b-378, RB-226, Blb-821, Rpi-sto1-890	1040	78	red	colored
952-65	6.5–7/5.8	6.4	R2-1137, R2-686, R3a-1380, R3b-378, RB-226, Blb-821, Rpi-sto1-890	1250	91	yellow	colored
Sudarynya	6–8/6.8	6.0 *	R2-686, R3a-1380, R3b-378, RB-226, Blb-821, Rpi-sto1-890	970	84	yellow, rounded-oval	small, colored
13/11-09	5–6/5.6	6.7 *	R3b-378, RB-226, Blb-821, Rpi-sto1-890, Rpi-vnt1.3-616 ^	900	76	red, rounded-oval	medium, non-colored
Elizaveta	4–5/3	6.7	R1-1205, R3a-1380, R3b-378, RB-226	910	87	yellow, rounded-oval	small, non-colored
Bintje	3/3	3	Rpi-vnt1.3-616 ^	770	78	yellow, rounded-oval	small, non-colored
Sarpo Mira	7–8/7	7	R3a-1380, R3b-378, R8-1276	1250	89	red-pinkish, oval	small, colored
Alouette	9/9	7–9.0 *	R3a-1380, R3b-378, Rpi-vnt1.3-616	1240	87	pink, oval	small, non-colored

<sup>1</sup> under the field \artificial infection. <sup>2</sup> asterisk. \* hybrids and cultivars with tubers with the notable mycelium growth. ^ Rpi-vnt1.3 SCAR markers that do not provide sufficient differentiation between the functional *Rpi-vnt1.3* and its non-functional homologs.

#### 4. Discussion

Individuals in the Sudarynya × 13/11-09 progeny, which differ by a complex of valuable breeding traits manifested the recombination of parental hereditary factors, as evidenced by the results of the analysis of cultivar distinguishing features in F1 clones. The appearance of anthocyanin coloration of different plant organs in potato cultivars is controlled by dominant complementary genes [23]. The basic gene *R* controls the production of red pigment, while the basic gene *P* controls the production of blue and purple pigments. Pigmentation manifests itself in different parts of the plant depending on the presence of *D*, *E* and *F* genes. Tubers with red skin and non-colored eyes (as in the male parent) are produced by plants combining the *R* and *D* genes. Tubers with the non-colored skin and colored eyes appear in plants with a combination of *R* and *E* genes. The proportion of the latter in the Sudarynya × 13/11-09 progeny is 25%. The red-violet color of the corolla is determined by a combination of *R* and *F* genes. Recessive forms and genotypes with individual dominant genes *R*, *D* and *F* have white (yellow) tubers and white corolla. There are about 50% of those in the Sudarynya × 13/11-09 progeny. The appearance of plants with new combinations of genes is also confirmed by the molecular genetic analysis resulting in the identification of two hybrids carrying marker fragments of LB resistance genes of both parents: *Rpi-R2*, *Rpi-R3a*, *Rpi-R3b*, *Rpi-blb1* and *Rpi-vnt1*. The presence of *Rpi-R3b*<sup>G1696/G3111</sup> and a *Rpi-blb1-like* gene as predicted with the SCAR markers was independently confirmed with dRenSeq. Importantly, every progeny clone that was predicted to contain the *Rpi-blb1-like* gene was LB resistant. This is, to our knowledge, the first example where an *Rpi-blb1/Rpi-sto1-like* gene has been successfully established in commercially valuable potato breeding clones by the dRenSeq technology. In contrast, the SCAR marker associated with *Rpi-vnt1.3* proved to be non-specific, it is further confirmed in the fact that susceptible cultivar Bintje was tested positive with this marker.

Depending on the infection load, F1 generation from a cross of two LB-resistant parents demonstrated either an intermediate type of inheritance, or a shift towards a decrease in resistance. The segregation of LB resistance in leaves of the F1 progeny at the seedling stage differed from the segregation in plants of the first and subsequent tuber generations apparently because of different patterns of growth and the annual changes in the composition of the LB pathogen population. The long-term monitoring data evidence significant differences in phytopathological and molecular genetic characteristics of *P. infestans* isolates affecting potato cultivars and interspecific hybrids under the conditions of the VIR field genebank [22]. Therefore, the results of field assessments have poor reproducibility. Obviously, the method of artificial infestation with high infection load, significantly reduced the phenotypic manifestation of the *Rpi* genes, as is evidenced by the prevalence of the affected F1 clones during the second survey of the experiment. However, LB resistance in leaves and tubers in several F1 clones exceeded the resistance of parents or was not inferior by them. Apparently, by combination breeding, we managed to merge the favorable traits of both parents in the progeny.

The application of dRenSeq made it possible to establish the presence of LB resistance genes in one of the parents with incomplete information about its origin.

This method has been successfully used to characterize cultivars and breeding material for the presence of *R* genes that determine potato resistance to pathogens and pests [15,24]. The application of dRenSeq will obviously make the choice of parents with a set of favorable genes more justified and in this way will promote successive breeding program for developing new cultivars with improved properties. Evaluation of the F1 generation confirmed the possibility of using SCAR markers and selecting valuable recombinant genotypes in the progeny when crossing complementary parents with established *Rpi* gene profiles. The presence of marker fragments of target genes requires confirmation of the gene functionality. In addition, the effectiveness of the protective action of *R* genes introduced into hybrid progeny depends on the genetic basis of the second partner in the cross [25]. At the same time, our study showed that the use of MAS selection at the early

stages of a breeding program can significantly reduce the volume of the investigated hybrid population.

Phenotypic assessment of breeding material at the early stages of a breeding program does not identify valuable recombinants regarding their LB resistance. The marker selection of potato forms with 4–5 *Rpi* genes from seedlings or the first tuber generation, when performed at the early stages of the breeding process, will significantly reduce the size of the population to be assessed in subsequent field nurseries and laboratory tests. By using SCAR markers, it is possible to screen a hybrid progeny for the forms combining traits of both parents, and this selection will not entail a decrease in the productivity of the selected material. In our experiment, both F1 clones, each carrying five *Rpi* gene markers, showed very high productivity, almost twice higher than that of cv. Sudarynya.

Our study presents the early results of a comprehensive assessment of the hybrid progeny obtained through combination breeding. The further testing of breeding populations and the work on improving and expanding the set of DNA markers will make it possible to clarify the effect of their application to genetically diverse material, often of insufficiently established origin.

**Supplementary Materials:** The following are available online at [www.mdpi.com/article/10.3390/agronomy11112192/s1](http://www.mdpi.com/article/10.3390/agronomy11112192/s1), Table S1. SCAR markers of *Rpi*-genes in F1 progeny and parental lines Figure S1. Alignment of *Rpi-vnt1* homologs and predicted amino acid sequences of *Rpi-vnt1* protein homologs.

**Author Contributions:** E.K., M.K. and E.R. conceived and designed the research. E.R. bred most hybrids. M.K. and N.C. maintained hybrid collections and evaluated field LB resistance. N.C. and N.Z. evaluated LB resistance in laboratory, M.B., P.D. and E.K. ran the marker and bioinformatics analysis, M.A. and I.H. ran dRenSeq analysis, E.K. and E.R. wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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