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CERTIFICATION OF RUSSIAN RED CLOVER (*Trifolium pratense* L.) VARIETIES BASED ON SSR AND SRAP MARKERS

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Abstract

Molecular-genetic certification is a powerful strategies and efficient addition to the traditional methods of variety testing and agricultural crops identification. Russia, as well as a world in a whole, introduces the current DNA technologies in the breeding programs, in a variety registration process and in a system of seed production. However, the traditional approaches, based on observation and recording the morphological characters, are the prevalent now for the forage crops. It influences negatively on efficiency of selection, increases the terms and coasts of the new varieties development, registration and breeders rights protection. In this paper, the results of creation a system for identification and genetic certification of Russian red clover cultivars on the base of SSR and SRAP-markers are submitted for the first time. The seeds of 15 domestic varieties from gene pool collection of Federal Williams Research Center of Forage Production and Agroecology and 6 accessions of foreign breeding from Vavilov All-Russian Institute of Plant Genetic Resources were used for investigations. The genome DNA was extracted from 7-day seedlings' tissue. Bulk DNA samples were formed from 30 individual genotypes per each variety. We used basic SDS-method in own modifications. Quantity and quality of extracted DNA was analyzed by agarose gel electrophoresis and measurement of concentration and purity. The final concentration of DNA samples was 30 ng/μl. PCR amplification was performed using 35 SSR from the Red Clover Marker Database (http://marker.kazusa.or.jp/Red_clover), and 40 SRAP markers. A total of 476 PCR products were generated with SSR markers for 12 red clover varieties. A set of eight microsatellite loci was selected for identification the tested samples. With application of 40 SRAP markers, we selected 18 informative combinations for analysis of the red clover collection of 16 varieties. Total 812 PCR products were revealed and 85 (10.5 %) among them were determined as polymorphic. The set of 7 informative markers were identified for samples differentiation on the base of SRAP analysis. Unique varieties-specific DNA fragments were sequenced (Evrogen Lab company, Russia) for validation the results of analysis. Nucleotide sequences, identifying Russian red clover varieties Trifon, Mars, Topas, Atlant, Tetraploidniy VIK, Meteor, VIK 77, were included in the GenBank NCBI (https://www.ncbi.nlm.nih.gov/). The data of DNA fingerprinting we used for development the molecular-genetic formulas representing microsatellite loci allele composition and polymorphism in exon and intron regions of genome. As a result of this study, 10 etalon genetic certificates were designed for Russian red clover varieties.

Keywords: forage crops, genetic diversity, SSR markers, SRAP markers, DNA polymorphism, genetic certification

Among genomic biotechnologies in agriculture of the Russian Federation, special attention is focused on DNA identification and genetic certification of breeding achievements. Widespread use of these approaches will increase the efficiency of registration of new varieties and protect the copyright of breeders, and

will help in the fight against counterfeit in the seed market. Currently, the assessment of varieties for compliance with the the DUS (distinctiveness, uniformity and stability) test is based on the description of morphological characteristics. However, this is a labor-intensive and time-consuming protocol that requires appropriate professional scale and, in some cases, special conditions for testing (for example, vernalization of barley and wheat) [1]. The task is also complicated by the limited number of evaluation descriptors, which are also subject to the influence of environmental conditions. These are, in particular, traits of disease resistance that must be assessed when certifying some vegetable varieties (e.g., tomatoes) or flowering time for many outbreeding species (e.g., ryegrass) [2-4]. For a number of crops, identifying the characteristics that determine the originality of a variety is complicated by a high degree of intrapopulation variation or, on the contrary, interspecific morphological similarity (twin species) [5, 6). As the number of varieties increases, the genetic base shrinks because breeding tends to focus on a few of the most important agronomic traits, making it difficult to distinguish differences on a morphological basis [7]. Moreover, in perennial species, the manifestation of a number of traits requires a long time and an appropriate stage of development.

The efficiency of assessment can be significantly increased by integrating methods based on the use of molecular DNA markers into the variety testing system. An almost unlimited number of such markers and a high degree of detectable polymorphism (regardless of the plant part being studied and environmental conditions) allow differentiation of even difficult-to-distinguish varieties. DNA markers can reveal hidden variability which improves the accuracy of results and the resolution of analysis [8-10].

Genetic identification is based on determining the combination of alleles of a particular gene that are characteristic of the organism being studied. If you have informative and convenient methods, the results can be easily documented and a molecular genetic passport of the variety can be developed indicating the length of DNA fragments in specific chromosome regions. The passport allows you to determine the uniqueness of the sample and the level of genetic variability of the species, assess the degree of relationship with known varieties and compliance with the standard, and analyze the uniformity of seeds.

The methods underlying the creation of genetic passports are successfully used not only in variety testing, but also in breeding. The ability to select valuable genotypes at the initial stage of plant development reduces the duration of testing and increases the efficiency of the breeding process, especially in cases where selection by phenotype turns out to be lengthy and not reliable enough [11]. Analysis of DNA polymorphism provides breeders with information that can be used to control the results of hybridization, when selecting parental forms for crosses, and to identify the sources of genes that affect economically valuable traits.

Molecular markers used to assess genetic variability and identify lines, varieties and forms must meet certain requirements, i.e., to be highly polymorphic, reproducible, and evenly distributed throughout the genome, covering its different regions. Most often for this purpose, RFLP- (restriction fragment length polymorphism), RAPD- (random amplified polymorphic DNA), AFLP- (amplified fragment length polymorphism), SSR- (simple sequence repeats) markers, which are anonymous DNA fragments reflecting polymorphism in randomly selected regions of the genome [14, 15].

However, the results of polymorphism assessment do not always correlate with known morphological characters. For the purposes of applied genetics and breeding, analysis of functional molecular markers that identify changes in transcribed coding DNA sequences, i.e., the genes is more rational [16-18]. The main

resource for the development of such markers are EST (Expressed Sequence Tags) libraries which contain sequenced DNA fragments 500-700 bp long. For many crops, databases have now been created with information on EST-SSR markers developed based on complementary DNA (cDNA) [19, 20]. SSR markers provide the detection of polymorphisms of simple repeated DNA sequences (1-10 bp) which vary in length in some plant accessions [21]. The identification is also based on the variability of these genome regions, suggesting that genotypes of the same variety (if it is sufficiently aligned) should contain alleles of the same size.

In recent years, to assess the genetic polymorphism of varieties and species, the system of SRAP markers (sequence related amplified polymorphism) designed to amplify DNA fragments in the intron-exon regions of a gene has been successfully used [22]. Their advantages are ease of use and statistical processing, high information content and stability of results, much higher than with RAPD marking. With the SRAP method, it is easy to detect polymorphism even in closely related breeding material, so it is successfully used to distinguish varieties of different crops [23-25].

In Russia, as throughout the world, the DNA analysis is involved in breeding programs, registration of new varieties and commercial distribution of seeds. Genetic passports have been developed for a number of agricultural crops, mainly those for food purposes (rice, wheat, beets, potatoes, etc.) [26-29]. Forage grasses remain less studied, with phenotypic assessment predominant. This significantly reduces the efficiency of the selection of parental and breeding materials, increases the time for the creation of new varieties and the costs of their testing and registration. The solution will be a methodology for analyzing the genetic variability of varieties and forms at the DNA level, which should be high-performance, relatively inexpensive and independent of morphological parameters.

Of the forage perennial grasses in the regions of the Non-Chernozem Zone of Russia, meadow clover is the most common. It is used not only as a valuable high-protein feed for animals, but also as an excellent precursor for other crops and plays an important role in the biologization of agriculture [30]. To date, through the efforts of Russian breeders, more than 100 varieties of this species have been created. The use of modern methods of molecular analysis expands the possibilities of their reliable identification and legal protection, and also helps to accelerate the process of creating new forms that are high-yielding and disease-resistant.

In the presented work, we for the first time assessed the genetic polymorphism of a collection of Russian and foreign varieties of red clover using SSR and SRAP markers, identified variety-specific DNA fragments for differentiating the studied material, the uniqueness of which was confirmed by sequencing. Based on the results obtained, reference genetic passports have been compiled for a number of Russian varieties.

Our goal was to develop a system for determining varietal identity and reference genetic passports based on SSR and SRAP markers for Russian meadow clover varieties.

Materials and methods. The research was carried out in 2019-2021 at the Williams Federal Scientific Center for Forage Production and Agroecology. The material seeds of 15 Russian varieties of meadow clover (*Trifolium pratense* L.), from the Center for Shared Use Biological Collections of Forage Plants and 6 accessions of foreign origin from the Collection of Genetic Resources VIR ((FSC Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg) were used.

Genomic DNA was isolated from a bulk sample of the 7-day-old seed-

lings, 30 genotypes from each variety. The common SDS method with modifications was used [31]. DNA preparations were evaluated using electrophoresis in agarose gel and spectrometry (a UV-vis Nabi spectrophotometer, MicroDigital Co., Ltd., Korea) to measure the concentration and purity. The final concentration of samples was adjusted to 30 ng/μl before use in PCR. In genotyping, we used 35 microsatellite markers developed by S. Sato et al. [32] for the genome structure of red clover and placed in the Red Clover Marker Database (http://marker.kazusa.or.jp/Red_clover) and 40 combinations of primers for SRAP markers [22, 33].

A reaction mixture for SSR analysis of 15 μl per sample contained 3 μl of 10× PCR buffer (Taq Turbo Buffer); 0.2 μl of polymerase (Tag DNA Polymerase, 5 units/μl); 0.1 μl of each primer (100 μM); 1 μl 50× dNTP (dNTP mix 10 mM each) and 1 μl DNA sample (30 ng/μl) (all reagents from Evrogen Lab LLC, Russia). Amplification (a T100 thermal cycler, Bio-Rad, USA) by a modified Touchdown PCR program includes an initial denaturation for 3 min at 94 °C followed by 3 stages of sequential reduction in the primer annealing temperature by 2 °C every 3 cycles, the 30 s at 94 °C, 30 s at 68 °C (3 cycles), 30 s at 94 °C, 30 s at 66 °C (3 cycles), and 30 s at 94 °C, 30 s at 64 °C (3 cycles). A chain elongation was run for 30 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C (3 cycles); 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C (3 cycles); 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C (3 cycles). Then, the hybridization temperature reduced to optimal value of 55 °C was maintained for the remaining 30 reaction cycles: 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C. The final stage of chain elongation took 10 min at 72 °C [32, 34]. The reproducibility of the results was checked by repeating the experiments three times, including using bulk DNA preparation from different seedling samples as a matrix.

The resulting PCR products were preliminarily analyzed after electrophoresis in a 1.6% agarose gel (LE2, Lonza, USA) and detection using a GelDoc™ XR+ device (Bio-Rad, USA). The size of the fragments was determined with the molecular marker 100 bp GeneRuler DNA Ladder (Thermo Fisher Scientific, USA) in the ImageLab program (Bio-Rad Lab., Inc., USA). The results of the analysis were summarized in a general table and the presence of variety-specific alleles for each marker was revealed.

To validate alleles unique to the variety, the PCR products were cloned in the pAL2-T vector (ZAO Evrogen, Russia) and sequenced (an ABI PRISM 3130XL genetic analyzer, Applied Biosystems, Inc., USA) using a Big Dye terminator v.3.1 cycle sequencing kit (Applied Biosystems, Inc., USA). Sequencing data were analyzed in the Ugene program [35] and then aligned using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). EST/genomic sequences from the Red Clover Marker Database (http://marker.kazusa.or.jp/Red_clover) were used as a reference.

To increase the accuracy of determining the length of all fragments identified in the DNA profile of candidate varieties for certification, control markers were developed. Based on the electrophoresis in 10% acrylamide gel (BIO-RAD Tetra Cell chamber, USA), the allele sizes were determined vs. the control marker.

Components of the reaction mixtures for most of the SRAP markers corresponded to that proposed by H.B. Rhouma et al. [23], however, for successful amplification of some primer combinations, the reagent content and PCR program had to be optimized. The modified mixture, 20 μl per sample, was 3 μl 10× PCR buffer (Taq Turbo Buffer); 0.5 μl 50× dNTP; 0.1 μl 100 μM primer; 0.4 μl polymerase (Tag DNA Polymerase, 5 units/μl); 1.0 μl DNA sample (30 ng/μl).

Amplification (a T-100 Thermal Cycler, Bio-Rad, USA) program was as follows: 4 min at 94 °C (initial denaturation); 1 min at 94 °C, 1 min at 35 °C,

1 min at 72 °C (10 cycles); 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C (30 cycles); 5 min at 72 °C (final elongation). The size of amplified DNA fragments was measured in horizontal electrophoresis vs. a 1 kb molecular marker (Evrogen Lab LLC, Russia). Target (variety-specific) PCR products were excised from agarose gel and purified on columns with a Cleanup Standard kit (Evrogen Lab LLC, Russia), and then the purified amplicons were cloned in the pAL2-T vector (ZAO Evrogen, Russia) as per the manufacturer's instructions and sequenced. The results of sequence analysis performed in the Ugene and BLAST programs [35] were used to select SRAP markers identifying individual varieties.

Genetic relationships between the studied samples were visualized by Principal Coordinate Analysis (PCoA) and the GenAlEx software package (version 6.2) [36].

Results. The red clover varieties used in this study are listed in Table 1.

1. List of red clover (*Trifolium pratense* L.) varieties involved in the study and the labeling system used

Variety	Originator (country of origin/catalog number in the VIR collection)	Year of entry into the State Register	Marking system
Rannii 2	Williams FRC VIK	1995	SSR, SRAP
Trifon	Rudnitsky FASC North-East	2014	SSR, SRAP
Pamyayi Lisitsyna	Federal Scientific Center for Leguminous and Cereal Crops; Williams FRC VIK; Siberian FSC of Agrobiotechnologies RAS	2005	SSR, SRAP
Pelikan	Federal Scientific Center for Bast Crops	1992	SSR, SRAP
Trio	Williams FRC VIK	1995	SSR, SRAP
Veteran	Williams FRC VIK	2011	SSR, SRAP
Tetraploid VIK	Williams FRC VIK	1973	SSR, SRAP
Mars	Williams FRC VIK	1993	SSR
VIK 771	Williams FRC VIK	2006	SSR
Mateor	Siberian FSC of Agrobiotechnologies RAS; Williams FRC VIK	2007	SSR
Topaz	Williams FRC VIK OOO Agrokompleks-N	2000	SSR
Atlant	FRC Tyumen Scientific Center RAS; Siberian FSC of Agrobiotechnologies RAS	2007	SSR
Altyn	Williams FRC VIK; ONO Morshansk breeding station	1999	SRAP
VIK 84	Williams FRC VIK	1991	SRAP
Vorontzhskii	Williams FRC VIK	2015	SRAP
Marathon	K-48013 (France)	–	SRAP
Freedom	K-51532 (USA)	–	SRAP
Ganymed	K-53648 (Czech Republic)	–	SRAP
Metis	K-53792 (Denmark)	–	SRAP
Nemaro	K-50958 (Germany)	–	SRAP
Norlac	K-51526 (Canada)	–	SRAP

N o t e. Dashes mean that the variety is not included in the State Register of the Russian Federation.

To assess the genetic polymorphism of red clover varieties, a representative sample of genotypes is required (at least 30-50 per sample) in order to accumulate the maximum number of markers characteristic of cross-pollinated populations with a high level of heterogeneity. In such cases, the use of individual genotypes for analysis of the total DNA sample (bulk strategy) can significantly reduce the costs of labor, time and financial resources [37, 38]. Previously, our modifications of the SDS method for DNA extraction [39] ensured good quality of preparations suitable for use in PCR with different types of markers from a common sample formed from highly watered tissue of 30 seedlings of each variety [31].

The main our criteria for selecting microsatellite loci were the number of detected alleles, coverage of all linkage groups by location on chromosomes, and the short length of the resulting PCR fragments (100-300 bp). All SSR markers were di- and tri-nucleotide repeats. To determine the degree of homogeneity, random samples of seedlings of the varieties Ranniy 2, Mars, Tetraploid VIK, Trifon (10 genotypes of each) were analyzed for microsatellite loci RCS1307,

RCS5600 and RCS5208. A fairly high genetic evenness of the material was revealed, which is not typical for cross-pollinated crops with a high level of gametophytic self-incompatibility. Other studies have also reported a significant degree of DNA polymorphism identified within red clover populations with markers of different types (RAPD, AFLP, SSR), from 67.5 to 83.6% [17, 38, 40]. Apparently, our results were influenced by the limited genetic material used in the breeding schemes (the samples included in the analysis had a common originator, the Williams Federal Scientific Center WIK).

Figure 1 shows the general scheme of assessing intervarietal genetic polymorphism to develop genetic passports of red clover.

To study intervarietal genetic variability using 35 pairs of SSR primers and subsequent certification, 476 amplification products were generated for 12 varieties of meadow clover, with an average of 13.6 alleles per locus. These data are comparable to those of other researchers. I. Radinovic et al. [41] in a study of 46 red clover genotypes of different ecological and geographical origins revealed an average of 13.36 alleles per locus for 14 SSR markers. Researchers in Brazil [42] genotyped 57 red clover accessions from North America and Europe and determined that there was an average of 9 alleles for each of the 7 SSR loci tested.

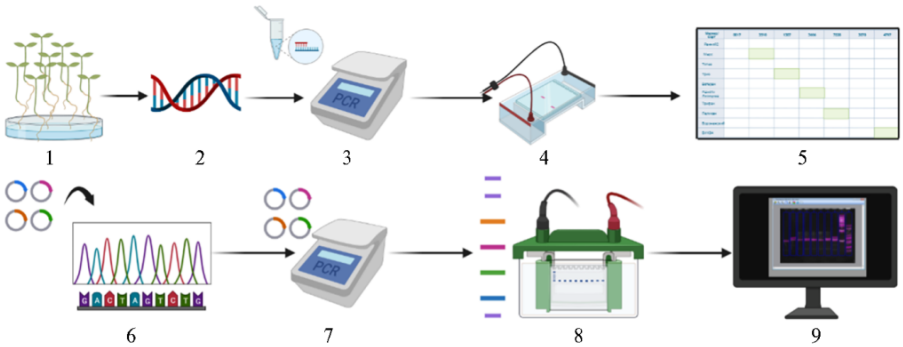

Fig. 1. Scheme of red clover (*Trifolium pratense* L.) DNA fingerprinting for genetic certification: 1-9 — procedures using SSR markers, 1-6 — procedures using SRAP markers; 1 — formation of a total sample of seedlings of 30 genotypes of each variety, 2 — DNA extraction, 3 — amplification in a thermal cycler (Bio-Rad, USA), 4 — preliminary electrophoresis in agarose gel, 5 — selection of variety-specific markers, 6 — Sanger sequencing and analysis of results, 7 — PCR with plasmid DNA, 8 — development of a specific (control) marker and vertical PAGE electrophoresis, 9 — determination of product sizes in comparison with a control marker to compile a molecular genetic formula of the variety (scheme created in the Biorender program (https://app.biorender.com/).

In our work, the size of the amplicons varied from 91 bp (with primer pair RCS5305) to 359 bp (RCS1535). We identified a set of 8 informative microsatellite loci that generated reproducible polymorphic products and were suitable for distinguishing varieties in the sample. Thus, with primers to the RCS1307 marker, unique alleles were discovered that distinguished the varieties Mars, Topaz and Trifon, and the RCS0017 locus distinguished Meteor and Topaz.

In general, based on SSR analysis, a low level of both intravarietal and intervarietal DNA polymorphism was revealed, which is likely due to the origin and selection history of the material. The average value of genetic variability between varieties was 5.3%. This turned out to be significantly lower than the indicators known from the works of other researchers [43, 44].

The use of additional marker systems based on sequence polymorphism in various structural elements of the genome can significantly increase the resolution of the analysis. Using a set of 40 combinations of SRAP primers, we genotyped an expanded collection of red clover samples composed of the Russian varieties that could not be certified using a panel of SSR markers and the foreign

ones involved to compare genetic polymorphism.

The suggested PCR program [23] was ineffective for the SRAP marker combinations F10-R9, Me4-R9, ME1-EM1. By optimizing the components of the reaction mixture, the temperature, time parameters and using 18 combinations of primers, we obtained distinct and reproducible products (812 in total). The largest number of amplified fragments was detected with F13-R9 (62 amplicons), while with ME1-EM2 this figure was minimal (22 amplicons) with an average value of 45.1 for each combination. A total of 85 amplicons turned out to be polymorphic, which amounted to 10.5% of the total value. DNA fragments unique to individual varieties (up to 10) were found using F9-R9, F10-R8 and F10-R9 and only one variety-specific fragment was revealed for F9-R14 and for Me4-EM1.

Based on this information, we assessed the genetic relationships between the studied samples and visualized variations in their distribution on the coordinate plane using PCoA analysis (Fig. 2).

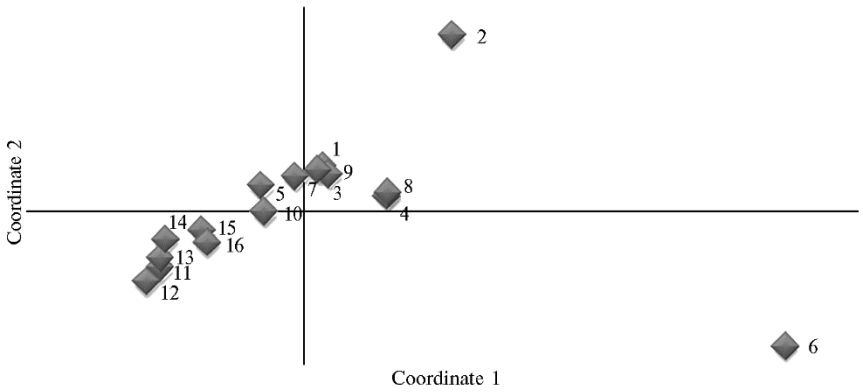

Рис. 2. Clustering of Russian and foreign varieties of meadow clover (Trifolium pratense L.) using the principal coordinate method (PCoA analysis) according to SRAP analysis: 1 — Ranny 2, 2 — Trifon, 3 — Pamyati Lisitsyn, 4 — Pelican, 5 — Trio, 6 — Veteran, 7 — Altyn, 8 — VIK 84, 9 — Tetraploid VIK, 10 — Voronezhsky, 11 — Norlac, 12 — Freedom, 13 — Ganymed, 14 — Metis, 15 — Nemaro, 16 — Marathon.

The clustering showed two relatively compact groups. One group is the varieties of foreign origin, and the other group is ultra-early ripening diploid varieties Trio, Altyn, Voronezhsky, Ranniy 2, tetraploids Pamyati Lisitsyna and Tetraploid VIK originated from Williams Federal Scientific Center VIK. The samples in the first group overlapped significantly, indicating high genetic similarity between them, possibly due to intense gene flow. The most genetically close were Norlac (Canada) and Freedom (USA), as well as the European varieties Ganymed and Metis. At a small genetic distance from them are varieties from Western Europe Nemaro (Germany) and Marathon (France).

The revealed similarity between the varieties of the second cluster was probably a consequence of their genetic relatedness and common breeding history. The tetraploid varieties VIK 84 and Veteran (the Williams Federal Scientific Center VIK) were located on the coordinate plane separately from the other varieties of their group. The Veteran variety stood out among other samples due to its high photosynthetic and symbiotic activity and longevity in agrophytocenoses. The VIK 84 variety has an increased resistance to damage by clover cancer, anthracnose and fusarium, high winter hardiness and drought resistance. It was clustered together with the Pelican variety (Penza Research Institute of Agriculture), bred by the method of a complex hybrid population from several varieties of early ripening clover, including that selected at the Williams Federal Scientific Center VIK. The diploid single-cut variety Trifon (Rudnitsky Research Institute of

North-East Agriculture) generated through the multiple recurrent selection based on the variety Krano (Denmark) is at a considerable distance from the others. The meadow clover variety Trifon is winter-hardy and resistant to sclerotinia and fusarium, since biotypic selection was carried out under an artificial increased load to these pathogens [45]. A high genetic similarity was evidenced by the close location of the Voronezhsky and Trio samples on the graph. The Voronezh variety was created within an ecological selection program at the Voronezh Experimental Station by selection of the best varieties of the Williams All-Russian Research Institute of Feeds under different conditions. The genotyping results suggest that plants of the Trio variety also participated in the formation of the new variety. Currently, the Voronezh variety is considered a source of high winter hardiness, drought resistance and seed productivity. Due to its good adaptive potential, it is widely in demand in the seed market.

To validate the analysis results, Sanger sequencing of the identified unique amplification fragments was performed (21 using SSR markers, 5 using SRAP). Analysis of the obtained data in the BLAST search engine revealed a high degree of sequence homology in comparison with nucleotide sequences from the reference database (Table 2).

2. Alignment of red clover nucleotide sequences obtained by SSR analysis vs. the reference from the database Red Clover Kazusa (http://marker.kazusa.or.jp/Red_clover)

Variety	Primer	Clone number/product length, bp.	Match with reference	
			identity	indels
Mars	RCS1307	1.3/155	149/155 (96 %)	1/155 (0 %)
	RCS3666	1.4/216	210/216 (97 %)	4/216 (1 %)
		1.5/226	28/28 (100 %)[a]	0/28 (0 %)[a]
			179/182 (98 %)[a]	0/182 (0 %)[a]
Topas	RCS1307	2.2/151	140/161 (87 %)	16/161 (9 %)
	RCS0017	9.1/150	100/101 (99 %)[a]	0/101 (0 %)[a]
			24/24 (100 %)[a]	0/24 (0 %)[a]
		9.2/144	100/101 (99 %)[a]	0/101 (0 %)[a]
			44/44(100 %)[a]	0/44 (0 %)[a]
Trifon	RCS1307	3.3/152	149/155 (96 %)	3/155 (1 %)
		3.5/157	152/157 (97 %)	2/157 (1 %)
Pamyati Lisitsyna	RCS1307	4.5/169	154/169 (91 %)	14/169 (8 %)
Atlant	RCS1307	5.5/141	136/155 (88 %)	15/155 (9 %)
		5.6/137	135/155 (87 %)	19/155(12 %)
	RCS2199	7.4/198	190/198 (96 %)	8/198 (4 %)
		7.6/200	190/200 (95 %)	10/200 (5 %)
	RCS4797	8.2/175	36/36 (100 %)[a]	0/36 (0 %)[a]
			141/143 (99 %)[a]	1/143 (0 %)[a]
		8.3/158	116/116 (100 %)[a]	0/116 (0 %)[a]
			44/44 (100 %)[a]	0/44 (0 %)[a]
Tetraploid VIK	RCS1307	6.2/158	152/157 (97 %)	2/157 (1 %)
		6.6/155	151/155 (97 %)	1/155 (0 %)
	RCS5781	9.4/205	187/208 (90 %)	12/208 (5 %)
		9.6/201	184/203 (91 %)	6/203 (2 %)
	RCS4532	10.2/235	234/237 (99 %)	2/237 (0 %)
Meteor	RCS0017	8.4/166	164/166 (99 %)	2/166 (1 %)
		8.6/172	164/172 (95 %)	8/172 (4 %)
	RCS3070	4.1/333	136/137 (99 %)[a]	1/137 (0 %)[a]
			123/125 (98 %)[a]	0/125 (0 %)[a]
		4.2/323	136/137 (99 %)[a]	1/137 (0 %)[a]
			125/125 (100 %)[a]	0/125 (0 %)[a]
	RCS3510	5.5/254	252/271 (93 %)	17/271 (6 %)
		5.6/257	255/271 (94 %)	14/271 (5 %)
Trio	RCS0017	10.5/164	164/164 (100 %)	0/164 (0 %)
Veteran	RCS0017	11.2/164	149/164 (91 %)	0/164 (0 %)
	RCS4797	6.2/180	180/190 (95 %)	10/190 (5 %)
VIK 77	RCS3510	2.1/249	243/271 (90 %)	22/271 (8 %)
	RCS7228	3.5/182	181/185 (98 %)	3/185 (1 %)
		3.6/181	180/185 (97 %)	4/185 (2 %)

N o t e. [a] — nucleotide sequences that have a large insertion or deletion compared to the database reference.

Figure 3 shows, as an example, the results of alignment of the nucleotide

sequence of a PCR fragment obtained with a combination of SRAP markers ME1-EM1.

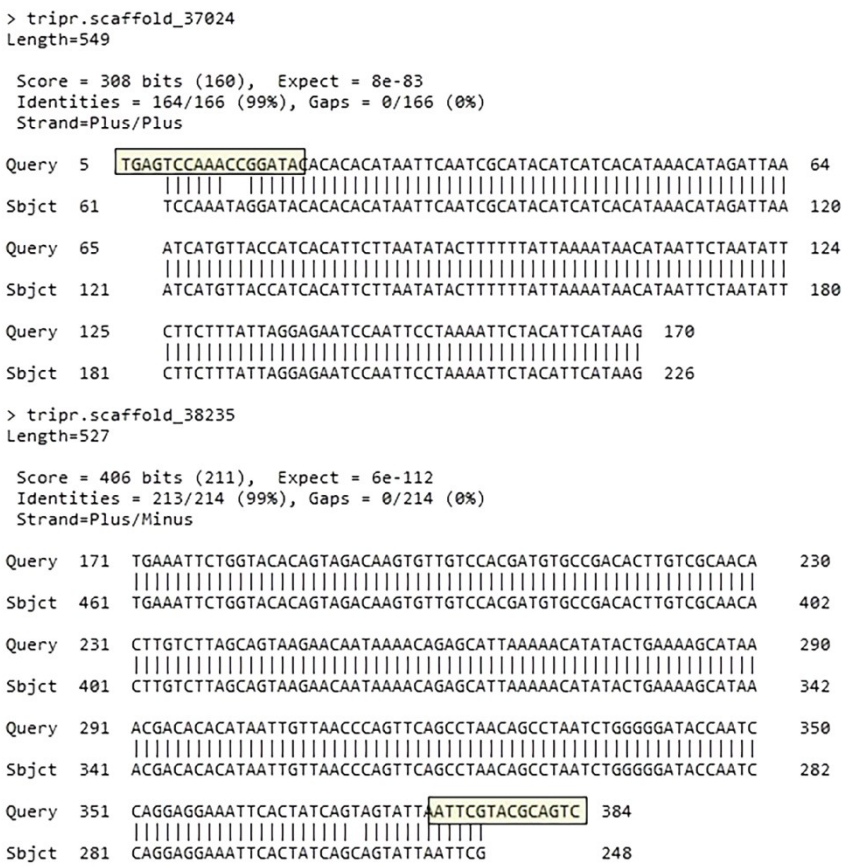

FIg. 3. Sequence alignment of a unique PCR fragment for red clover (*Trifolium pratense* L.) variety Trio amplified with the SRAP combination ME1-EM1 (query) vs. the *T. pratense* genome (sbjct). Primer sequences are marked.

Annotated nucleotide sequences identifying the varieties Trifon, Mars, Topaz, Atlant, Tetraploid VIK, Meteor, VIK 77 are included in the international database of genetic resources GenBank NCBI (https://www.ncbi.nlm.nih.gov/) under individual numbers MW520170; OP493602 and OP493613; OP493603 and OP493612; OP493606 and OP493607; OP493608 and OP493609; OP493610 and OP493611; OP546652 and OP546653. This data can be used for basic and applied research on red clover, including development of novel technologies. To increase the accuracy of determining the sizes of alleles that are not unique to the variety in the sample under study, but were identified as part of the DNA profile (8 loci), we created specific control markers which are PCR products obtained using plasmids.

Commercial standard markers were not suitable, since the large range in the size of divisions of the marker ladder did not allow the establishment of insignificant differences of several nucleotides between amplicons. In our study, we compared the size of alleles in the DNA profiles of the studied samples with the control marker after electrophoresis in 10% PAGE (Fig. 4).

For the studied collection, identification DNA markers were determined: eight pairs of SSR loci and seven SRAP combinations, which revealed the uniqueness of the allelic composition of at least two Russian varieties (Table 3).

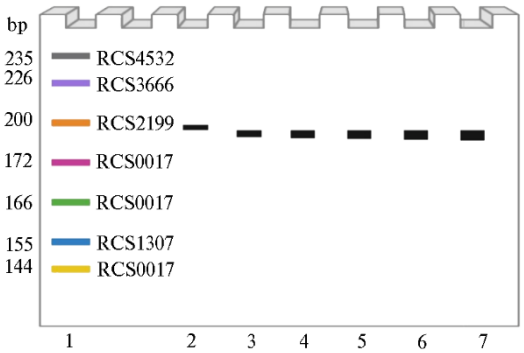

Fig. 4. Electrophoresis with a control marker to verify the allele sizes in DNA profiles of red clover (*Trifolium pratense* L.) varieties certified based on SSR markers: 1 — control marker composed of amplicons produced from clones 9.2 (144 bp), 1.3 (155 bp), 8.4 (166 bp), 8.6 (172 bp), 7.6 (200 bp), 1.5 (226 bp), 10.2 (235 bp), 2 — amplicon from clone 7.4 of the certified Atlant variety (SSR marker RCS 2199, 198 bp), 3-7 — PCR products with genomic DNA of the varieties Mars, VIK 77, Meteor, Topaz, Tetraploid VIK (SSR marker RCS 2199).

3. SSR- and SRAP markers for DNA identification of red clover (*Trifolium pratense* L.) Russian varieties

Marker (reference)	Nucleotide sequence 5′-3′	PCR fragment size, bp	Marker code
	SSR markers		
RCS3666 (32)	CATGGCTGCCTGAGGTTAAT/ TCTGTTTCTTGTCTCGGCCT	216-230	A
RCS3510 (32)	TTCACAAGTTTTTCGGGTGA/ GCCAAAGGGAAGGTTCAATC	249-257	B
RCS7228 (32)	TCAACAATGTGGCTTCTCCT/ AAGGTTCCCAACCCAATTTC	179-187	C
RCS4797 (32)	GCCCGTCTACCTTTTGTTCA/ GCGCCATAAGCAACTGTGTA	155-180	D
RCS2199 (32)	AAAAAGAAAGCGTTAAAGGGG/ GCATTGCCTTTTGCTTCTTC	178-200	E
RCS5781 (32)	GATCGATCCGAAAACCAAAA/ TGCCATCGAGAGAGAAGGTT	165-210	F
RCS1307 (32)	CCCTTCTAGCCTAGCAACCA/ GCGGAAAAGATTCAGCCTAA	137-158	G
RCS0017 (32)	GCGGAAAAGATTCAGCCTAA/ GGACTTCTCTGATATTGAACTGAATG	144-177	H
	SRAP markers		
F10-R9 (23)	GTAGCACAAGCCGGAAG/ GACTGCGTACGAATTTCA	116-1442	A
Me4-R9 (22)	CGAATCTTAGCCGGAAT/ GACTGCGTACGAATTTCA	165-519	B
ME1-EM1 (22)	TGAGTCCAAACCGGATA/ GACTGCGTACGAATTAAT	125-700	C
F13-Em2 (23)	CGAATCTTAGCCGGCAC/ GACTGCGTACGAATTCGG	234-763	D
F13-R9 (23)	CGAATCTTAGCCGGCAC/ GACTGCGTACGAATTTCA	115-1315	E
F11-R7 (23)	CGAATCTTAGCCGGATA/ GACTGCGTACGAATTGAG	232-1762	F
Me4-EM5 (22, 23)	CGAATCTTAGCCGGAAT/ GACTGCGTACGAATTAAC	139-748	G

The information obtained by sequencing DNA fragments unique to the variety and verifying the sizes of the remaining alleles in its DNA profile (compared to the control marker) was used to compile an individual molecular genetic formula. Capital letters of the Latin alphabet in the formulas denote the studied loci (marker code, see Table 3), and the lower digital index indicates the size of the identified alleles in nucleotide pairs (Table 4).

Molecular formulas served as the basis for creating genetic passports of red clover using two marking systems (six with SSR loci, four with SRAP markers). Additionally, the passport included information on the origin of the variety, regions of cultivation, main morphobiological characteristics and economically valuable

properties (Fig. 5).

4. Molecular genetic formulas of the studied red clover (*Trifolium pratense* L.) Russian varieties based on SSR and SRAP markers

Variety	Formula
	SSR marking
MArs	**A216/226**B252/252C180/180D155/169E189/194F192/208**G155/155**H165/177
VIK 77	A219/219**B249/249C182/182**D165/174E180/180F193/207G156/156H144/144
Meteor	A219/219B**254/257**C187/187D170/179E178/189F165/209G142/142**H166/172**
Topas	A220/230B252/252C179/179 D157/164E181/190F194/209**G151/151**H144/**150**
Atlant	A221/221B252/252C179/184**D158/175E198/200**F195/210G137/141H145/145
Tetraploid VIK	A218/230B252/252C179/179D170/180E181/193**F201/205**G155/**158**H144/144
	SRAP marking
VIK 84	A331B286**C446**,356D700,447,290,234E1070,565,180,115F1055,685,534,313,232G748,638,523,359
Trio	A400,305B286**C393**D700,234E1070, 300,115F685,313,232G609,359,139
Voronezhslky	A900,800,585,400,317,168**B129**D700,270,234E1070,300,115F800,685,534,313,232G748,638,204,139
Pelikan	**A338**,175B286C356,234D700,234E1070,115F800,685,313,232G748,638,359

N o t e. Letters of the Latin alphabet are marker code, digital index means size of amplification fragments (bp); Unique products confirmed by sequencing are highlighted in bold.

Fig. 5. Molecular genetic passports of red clover (*Trifolium pratense* L.) variety Mars based on the SSR marker system (left) **and variety Trio based on the SRAP marker system** (right)**.**

Thus, here we assessed the genetic polymorphism of meadow clover varieties for SSR and SRAP markers. The genotyping technologies have been optimized with regard of the properties of the crop studied, including modified DNA extraction from seedlings and amplification conditions for SRAP markers. For each marking system, unique DNA profiles and markers for genetic identification were obtained. The sizes of variety-specific DNA fragments identifying seven commercially important Russian varieties of red clover were confirmed by sequencing. Annotated nucleotide sequences are deposited in the GeneBank NCBI. Molecular genetic formulas of varieties have been compiled that reflect the allelic composition of microsatellite loci and polymorphism of intron-exon regions of the genome. These data formed the basis for the development of 10 reference genetic passports (six for SSR loci, four for SRAP markers) for Russian red clover varieties. Using a reference passport, you can identify a variety, analyze its homogeneity and genetic purity, and establish the compliance of an anonymous sample with a known standard. The proposed method for certification of red clover went through a triple control system, including 3-fold repetition of experiments, cloning and sequencing of the main fragments, and the use of a control marker to increase the accuracy of determining the lengths of amplified products. These techniques provide accurate allelic profiles of varieties, reduce the likelihood of genotyping errors, serve as confirmation of the reliability of the results obtained, and can be used in the

development of genetic passports of various forage crops.

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