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STUDY OF GENETIC VARIABILITY IN VEGETABLE CROPS REPRESENTATIVES THROUGH MOLECULAR MARKERS

A B S T R A C T

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The materials of defense are available for free access to those interested in the Central Library of the University of Plovdiv "Paisii Hilendarski" (24, Tsar Asen Str.).

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Title: Study of genetic variability in vegetable crops representatives through molecular markers.

CONTENT

List of abbreviations

COS II	Conserved Ortholog Set II
EMS	Ethyl Methane Sulfonate
ISAP	Inter-SINE Amplified Polymorphism
ISSR	Inter-Simple Sequence Repeat
PIC	Polymorphic Information Content
SINE	Short Interspersed Nuclear Elements
SSR	Simple Sequence Repeat

I. INTRODUCTION

Tomatoes (*Solanum lycopersicum* L.) are of traditional economic importance among vegetable crops in Bulgaria. Potatoes (*Solanum tuberosum* L.) are a vegetable crop that is of great economic importance to mankind. The common bean (*Phaseolus vulgaris* L.) is a promising legume crop for the future nutrition of mankind. Due to their rich protein content, including essential amino acids, legumes have been targeted by all global and European funding organizations in recent years. Despite the observed phenotypic diversity in tomatoes, potatoes, and beans, the genetic variability of these species was determined to be low. The reason is the cultivation of a small number of varieties that bring greater income to farmers. Induced mutagenesis is a reliable technology to overcome the limitations of low genetic variability in plants. The creation of new varieties of tomatoes, potatoes, and beans with high yield potential, resistance to diseases, and enemies important for our country's economy, as well as tolerance to abiotic factors of the environment, is essential. The successful selection of plant species is directly related to the existing genetic diversity, as well as its maintenance and management. The main priority in vegetable crops is the evaluation of genotypes with valuable economic qualities.

The application of various molecular techniques to assess the genetic potential of a given crop species increases and accelerates the efficiency of mutational and traditional breeding.

The introduction of molecular techniques to the study of plant cultivars provides useful information in relation to the protection of copyrights,

the establishment of existing genetic diversity, the identification of hybrid nature, as well as in the discovery of new genetic diversity. The correct selection of appropriate molecular marker systems affecting highly variable regions in the genome is of great importance to establish genetic heterogeneity. Microsatellites, retrotransposons, and restriction sites affect highly variable regions in plant genomes. Techniques that detect single nucleotide substitutions in the genome, particularly in coding regions, are also effective. The present dissertation research, which used a range of molecular marker techniques for genetic variability in the three vegetable crops, reveals perspectives in both fundamental and applied aspects. The establishment of appropriate DNA markers for genotyping makes it possible to screen out phenotypic changes that result from the action of environmental factors so that the selection process is based on genetic variability. The establishment of specific polymorphic profiles and genotyping markers of tomato, potato, and bean samples to be applied at an early stage of plant development would allow accelerating the breeding process. This would be of great importance for the production of the three vegetable crops in our country, based on modern molecular genetic methods, as well as for the restoration of Bulgaria's status as a European leader in the production and export of vegetable crops.

II. REVIEW OF LITERATURE

The literature review includes 6 main points, and in the first 3 of them, the importance of vegetable crops, the application of mutagenesis, and molecular-genetic markers in plant biotechnologies is successively considered. The remaining 3 points emphasize the economic importance of tomatoes, potatoes, and beans worldwide, including in Bulgaria, as well as the application of molecular markers for the study of genetic variability in these crops, the subject of the PhD thesis.

III. OBJECTIVE AND TASKS

➢ **The objective of this PhD thesis was to select molecular marker systems for genotyping the vegetable crops - tomatoes, potatoes, and beans and, based on this, to analyze the genetic variability in selected representatives of them.**

To achieve our goal, we set the following tasks:

1. Selection and testing of the applicability of various molecular marker techniques (SSR, ISSR, ISAP, COS II) for genotyping tomatoes, mutant lines of potatoes, and beans from the collection of Maritsa VCRI.
2. Investigation of the level of polymorphism in varieties and F_1 hybrid lines of tomato from the cultivated species (*Solanum lycopersicum* L.) and varieties obtained with the participation of the wild species *S. pimpinellifolium* L. and *S. chilense* L. by applying SSR, ISSR, ISAP, and COS II molecular techniques.
3. Investigation of the level of polymorphism in potato mutant lines of the species *Solanum tuberosum* L. using the marker systems SSR, ISSR, and ISAP.
4. Investigation of the degree of polymorphism in accessions bean (*Phaseolus vulgaris* L.) by applying microsatellite markers (SSR and ISSR).
5. Comparing the effectiveness of the applied techniques in each of the studied species.

IV. MATERIAL AND METHODS

4.1. Materials

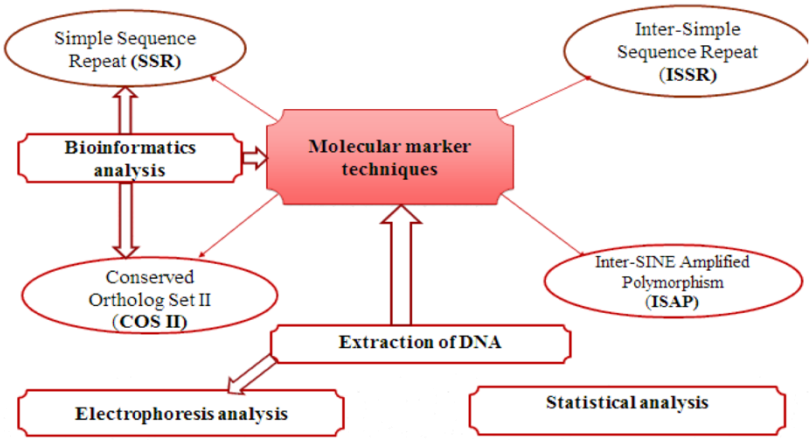
4.1.1. Plant material

1. 4 F_1 hybrids, 3 Bulgarian varieties, and 4 selection lines of tomatoes from the species *S. lycopersicum* L. and two varieties obtained by interspecific hybridization (*S.lycopersicum* L. x *S. pimpinellifolium* L.; *S.lycopersicum* L. x *S.chilense* L.).

2. 16 induced EMS - mutant lines of potato (*S. tuberosum* L.), their initial genotypes and controls.

3. 16 EMS - induced mutant lines of garden bean (*P. vulgaris* L.) by the population of variety "Mastilen 11 b" from the collection of Maritsa VCRI.
4. 20 local accessions and breeding lines of bean (P. *vulgaris* L.).

4.2. Methods



4.2.1. Extraction of DNA

For DNA isolation we used young leaves of tomatoes, potatoes, and beans crushed in mortars with liquid nitrogen. We performed DNA isolation using three protocols - CTAB, Microprep, and Nucleon Phyto Pure Kit.

4.2.2. PCR amplifications

4.2.2.1 PCR amplifications by microsatellite-based SSR reactions

All genomic DNA PCR amplifications were performed in a 20 µL reaction volume containing 20 ng/µL genomic DNA, 1x PCR buffer, 10 µM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 U by Taq DNA polymerase. PCR amplification programme include: *Initial denaturation - 94 °C for 5 min, denaturation - 94 °C for 30 sec, annealing 45 sec, with temperatures of 50 - 55 °C, elongation - 72 °C for 45 sec, 35 cycles, and final elongation 72 °C for 10 min.*

4.2.2.2 PCR amplifications by microsatellite-based ISSR reactions.

Amplification reactions were performed in a total volume of 25 µL. The reaction mixture contained 20 ng/µL of the DNA template, 1x Green PCR, 0.2 mM dNTPs, 10 µM primers of the respective primers, and 0.5 U DreamTaq DNA polymerase. PCR amplification programme include:
Initial denaturation - 94 °C for 5 min, final denaturation - 94 °C for 30 sec, annealing 45 sec, with temperatures of 48 - 56 °C, elongation - 72 °C for 2 min, 35 cycles and final elongation 72 °C for 7 min.

4.2.2.3. PCR amplifications by SINE retrotransposon based - ISAP reactions

Amplification reactions were performed in a total volume of 20 μL. The reaction mixture contained 10 ng/μL of the DNA template, 1x Green PCR buffer (Thermo Scientific, Cat. No B71, Lithuania), 0.2 mM dNTPs, 0.15 μM of the respective primers, 0.1 mg/mL bovine serum albumin and 0.5 U DreamTaq DNA polymerase. PCR amplification programme include: *Initial denaturation - 93 °C for 5 min, final denaturation - 93 °C for 20 sec, annealing for 30 sec at 52 °C, elongation - 72 °C for 2 min, 30 cycles and final elongation at 72 °C for 7 min.*

4.2.2.4. PCR amplifications by COS II reactions in tomato

Amplification reactions were performed in a total volume of 50 μL. The reaction mixture contained 50 ng/μL of the DNA template, 10x Green PCR buffer, 2 mM dNTPs, 10 μM of the respective primers, 25 mM MgCl2 and 0.5 U DreamTaq DNA polymerase. PCR amplification programme include:
Initial denaturation - 94 °C for 5 min, actual denaturation - 94 °C for 1 min, annealing from 50 - 55 °C for 1 min, elongation – 72 °C for 2 min, 35 cycles and final elongation at 72 °C for 10 min.

V. RESULTS AND DISCUSSION

Study of genetic variability in tomatoes, potatoes, bean by applying molecular marker techniques

5.1. SSR analysis in tomato

From the dendrogram, it can be seen that the variety "Kopnezh" F_1 was separated into an independent branch, and the other eight accessions were grouped into one cluster, which was divided into two subclusters (Figure 1).
The first sub-cluster includes the genotypes "Vodoley" F_1, "IZK Olimp" F_1, and "Ideal". The second subcluster splits into two more subclusters. One includes the "Plovdivska karotina" variety, and the other – "IZK Alya", "Aleno sartse", "Rozovo sartse" and "IZK Niki D" F_1.

Table 1. Polymorphic SSR profiles in tomato accessions

Markers	SSR profiles								
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
SSR19	1	1	2	2	3	3	3	1	4
SSR22	1	1	2	1	2	3	4	2	3
SSR38	1	2	3	4	1	3	3	1	1
SSR40	1	2	1	1	1	3	2	4	4
SSR51	1	1	1	2	3	4	5	6	7
SSR61	1	2	2	2	2	3	4	4	2
SSR76	1	2	3	4	5	1	1	1	1
SSR80	1	2	3	2	2	1	2	1	1
SSR96	1	2	3	3	2	3	3	1	1
SSR124	1	1	1	2	1	2	3	3	1
SSR150	1	2	1	3	4	1	1	1	1
SSR223	1	1	1	1	1	2	1	1	1
SSR270	1	1	1	1	1	2	2	2	2
SSR356	1	2	3	4	3	3	5	6	7
SSR586	1	1	1	2	1	1	1	1	1

*Roman numerals correspond to the analyzed cultivars and F_1 hybrids. I – "IZK Niki D" F_1; II – "Rozovo sartse"; III – "Aleno sartse"; IV – "Plovdivska karotina"; V – "IZK Alya"; VI – "Kopnezh" F_1; VII – "Vodoley" F_1; VIII – "IZK Olimp" F_1; IX – "Ideal" F_1

* Arabic numerals corresponding to the different profiles generated.

The results obtained in the thesis show that all cultivars and hybrids created only by representative(s) of the species S. *lycopersicum* L. are grouped in one main cluster. An exception is the F_1 hybrid "Kopnezh", which is grouped in a separate branch and is located closest to two varieties whose origin is associated with wild species – "Plovdivska karotina", obtained by interspecific

hybridization between the cultivated species *S. lycopersicum* L., and the wild species *S. chillense* L., and "IZK Alya", which was created by interspecies hybridization of the cultivated species with the wild species *S. pimpinellifolium* L.

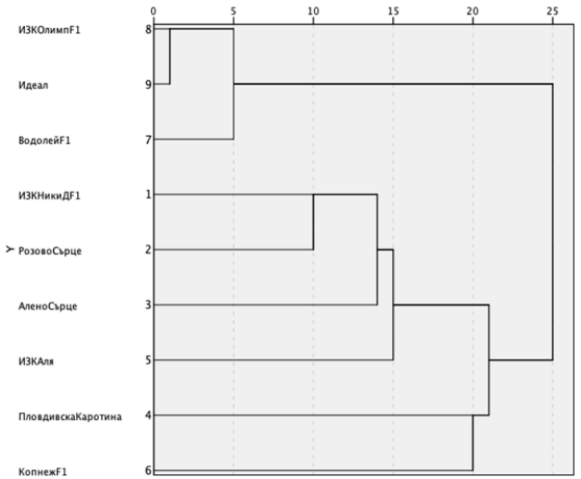


Figure 1. UPGMA dendrogram showing the genetic distance between the investigated tomato accessions based on the generated SSR data.

In the distribution by cluster analysis, both cultivars of interspecific hybrid origin fall into one subgroup (Figure 1).
They also differ from the rest of the studied varieties in terms of their morphological features. The performed cluster analysis, based on SSR microsatellite markers, confirmed the clear differentiation of the cultivars, in the creation of which crosses of the cultivated species with wild species were used, from those of the representatives of the cultivated species. The two cultivars of wild origin were analyzed with SSR markers by Todorovska et al. (2014), who found that "IZK Alya" differs from all other samples and forms its cluster in the UPGMA dendrogram. The data on the amplified monomorphic profile in tomato samples using SSR 66 markers in this study correlates with the results of Gharsallah et al. (2016) who reported relatively low polymorphism found with the same marker. The results reported by the team for other SSR markers studied by us (SSR 19, SSR 22) also match the results obtained in the dissertation work. In a study of seven tomato inbred lines with 30 SSR markers, in 1997 Smulders et al. found an average of 3 alleles per locus.

In a study using 65 polymorphic SSR markers of 17 cultivars and 2 parental tomato lines, He et al. (2003) found an average of 2.7 alleles per locus.
Garcia-Martinez et al. (2006) reported 19 polymorphic loci and found 2 to 10 SSR alleles in a study of 48 tomato accessions.
During the development of the present dissertation, using 19 SSR markers, we identified 63 alleles and genetic similarity between the studied samples in the range of 0.000 to 1.000. The calculated genetic distance between the studied F1 hybrids and the other varieties shows that the F_1 hybrid "Kopnezh" is the most distant, both from the other studied F_1 hybrids and from the other varieties. The reported distance between this variety and "IZK Alya" is 0.707, and between it and "Plovdiv carotene" - 0.791, in contrast to the other studied samples, in relation to which the value is higher.
By examining 9 tomato cultivars using 15 polymorphic SSR markers, we found an average value of the information content of the polymorphism PIC=0.542, and an average heterozygosity H=0.597. Aguirre et al. (2017) reported six alleles identified at the SSR19 marker and a PIC value of 0.782.
In the course of our study of the selected tomato cultivars, we found four alleles with this marker and an average polymorphism index (PIC) of 0.714, which suggests high genetic variability.
The genetic variability found by Popescu et al. (2022) is quite high, which is probably due to the heterogeneous material studied, including determinant and indeterminate cultivars and hybrids.
The plant material studied by us includes 5 indeterminate, 3 determinant, and 1 semi-determinant varieties of tomatoes with the same geographical origin, part of the Bulgarian collection, created and maintained in the Maritsa VCRI, among which we found lower values of polymorphism. Our analysis using 19 SSR reactions showed that the degree of genetic similarity between the studied samples ranged from 0.018 to 0.617, confirming the presence of genetic variability among them, regardless of the fact that they mostly belong to the same species.
We found the highest values of genetic similarity between "IZK Niki D" F_1 and "Rozovo sartse", as well as between "IZK Niki D" F_1 and "Aleno sartse".

5.2. ISSR analysis in tomato

The studied 11 tomato genotypes, created at Maritsa Vegetable Crops Research Institute, were analyzed with 13 ISSR reactions, and with 7 of them we found polymorphic profiles, amplifying a total of 105 fragments with lengths from 250 bp to 3100 bp (Figure 2).

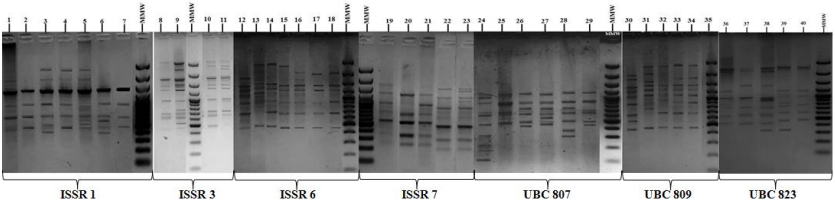


Figure 2. Polymorphic ISSR profiles in tomatoes from the collection of Maritsa VCRI . *MMW – fragment length marker (Gene Ruller 100 bp Plus).

With a greater part of the conducted ISSR reactions in tomatoes, we were able to distinguish five varieties – "IZK Niki D" F_1, "Kopnezh" F_1, "Plovdivska karotina", "Rozovo sartse" and "Aleno sartse". With some of the reactions, the variety "IZK Alya" originating from an interspecific cross of the cultivated species with *S. pimpinellifolium* also amplified an individual profile. The established average value of PIC= 0.353 polymorphism confirms the possibility of applying the ISSR technique in identifying varieties of one species, as well as in species with wild gene plasma to be included in breeding programs. The level of polymorphism detected with the above-mentioned ISSR markers among the selected tomato samples was lower than that detected by the same markers in mutant bean lines (Aziz et al., 2022). In their study, Tomlekova et al. (2012) reported five polymorphic fragments amplified with two ISSR primers that were found in comparative studies of baseline and mutant tomato genotypes. In studies by Vargas et al. (2020) when analyzing 55 tomato samples, by means of 7 ISSR reactions, a total of 63 fragments were amplified and a very high level of polymorphism was found - 90.48%. Ceballos-Aguirre et al. (2017), reported similar results as when analyzing 30 introduced cherry tomatoes with 36 microsatellite markers they found high genetic diversity (He = 0.6946). Sharifova et al. (2017) reported a study of 41 tomato genotypes with 11 ISSR markers, finding a total of 50 amplified fragments, among which 32 were polymorphic. The percentage of polymorphism ranged from 50% to 90%, and the average number of polymorphic fragments was 4.0. The selected 7 ISSR markers reflect significant genetic polymorphism and provide valuable genetic information, indicating their effectiveness in differentiating different tomato samples. The established polymorphism based on the investigated markers in tomato ranged from 36.4 % to 64 %.

5.3. ISAP analysis in tomato

The ISAP marker system has been shown to be very specific and although not generally popular in plant genetics, it is good for using available genomic resources and databases.

In their studies, Seibt et al. (2012, 2016) revealed that the proportion of the genome covered by mobile SINE elements varies between species, being twice as large in potatoes as in other Solanaceae species studied. This statement gives us reason to believe that the selected ISAP method is suitable for application in tomato cultivars, but the SINE elements used in the present study are not intended for the tomato genome. It is necessary to do a further search for more characteristic SINE families that can serve to design primers for amplifications in the tomato genome, as well as their subsequent molecular identification. The design of ISAP primers, however, requires extensive prior genomic information about the SINE elements. The most successful single-family ISAP reactions in tomato were performed with SINE families SolS II and SolS IIIa. The varieties "IZK Niki D" F_1, "IZK Alya", "Rozov blyan" and "Kopnezh" F_1 are distinguished by unique ISAP profiles (Figure 3).

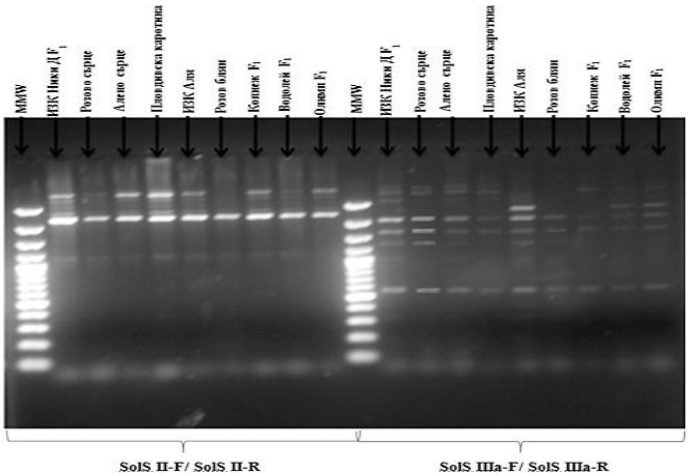


Figure 3. Results of SINE-ISAP reactions in tomato samples; A – SolS II–F/SolS II–R; B – SolS IIIa–F/SolS IIIa–R.

In a study of SINE elements in the family Solanaceae, Seibt et al. (2016) revealed that Capsicum genotypes contained the highest copy number with 21,398 SINEs.

For tomatoes, they found that there were no copies of the SolS VI family, while for all studied Solanaceae species, the abundance was the highest in the SolS II family, varying in the number of copies from 2479 (in tomatoes) to 7044 (in pepper "Zunla-1"). In tomatoes, a large number of copies were also found with SINE – IIIa family (1177), in contrast to pepper (225).

5.4. COS II assay in tomato

After the application of restriction enzyme *Dral* in reaction c2_At2g45620, we established two amplification profiles in Bulgarian tomato varieties. The first profile includes "IZK Niki D" F_1, "Aleno sartse", "Plovdivska karotina" and "Ideal", and the second molecular profile – "IZK Alya", "Kopnezh" F_1, "Vodoley" F_1. After the application of restriction enzyme, in the cultivars "Rozovo sartse" and "Olimp" F_1, we found the absence of amplifications (Figure 4 A).

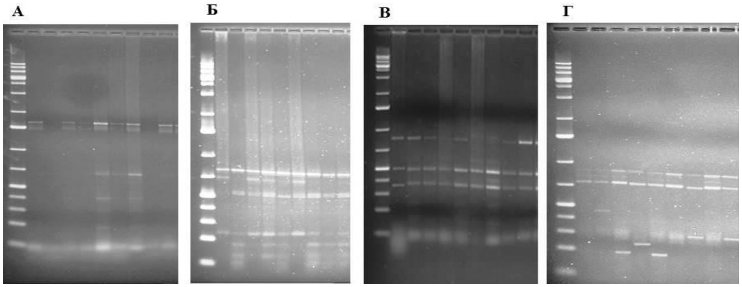


Figure 4. COS II reactions after application of restriction enzymes. A) c2_At2g45620; B) c2_At4g00090; C) c2_At5g64350; D) c2_At5g53000

After the application of restriction enzyme *DpnII* in reaction c2_At4g00090 we identified five amplification profiles. The first belongs "IZK Niki D" F_1, the second profile includes genotypes "Rozovo sartse", "Vodoley" F_1, "Olimp" F_1 and "Ideal". The third molecular profile is characteristic of "Aleno sartse", "Kopnezh" F_1, and the remaining two varieties ("Plovdivska karotina", "IZK Alya") generate individual profiles (Figure 4 B). With reaction c2_At5g64350 we found two profiles after the application of restriction enzyme *Hae III*. The first included two varieties – "IZK Alya" and "Kopnezh" F_1, and the second molecular profile was amplified in all seven other examined tomato samples (Figure 4 B).

In COS II reaction - c2_At5g53000, after the application of restriction enzyme *TaqI*, 9 polymorphic fragments were amplified, with which six molecular profiles were identified. Five of them are individual ("IZK Niki D" F_1, "Rozovo sartse", "Plovdivska karotina", "IZK Alya" and "IZK Olimp" F_1). The remaining four genotypes amplified the same monomorphic profile with this reaction (Figure 4 D).

The Bulgarian tomato varieties "Plovdivska karotina" and "IZK Alya" were identified through six of the eight introduced COS II markers. With most of them, polymorphism was found among representatives of the cultural species ("IZK Niki D" F_1, "Rozovo sartse", "Aleno sartse", "Kopnezh" F_1 "Ideal"). In future studies, knowledge will be expanded to search for and establish genetic diversity in the studied samples, and additional COS II markers located near anthocyanin-related genes will be used. This will also allow the characterization of the quality of the Bulgarian varieties and lines of tomatoes to satisfy the taste needs of the consumer.

5.5. SSR analysis in potato

Through eight SSR reactions, we investigated genetic variability in potato mutant lines, their parent forms, and controls. The number of amplified fragments we detected ranged from 1 to 4 (Table 2) and the number of polymorphic profiles from 3 to 7 (Table 3).

Table 2. The number of amplified fragments and profiles with SSR markers in potato.

SSR markers	**G**	**PF**	**PP**	**PP %**
STM1052	24	2	4	16,67
STM0031	24	3	4	16,67
STM0037	24	3	3	12,5
STM3012	24	2	3	12,5
STI42	24	2	3	12,5
STI53	24	4	7	29,17
STI57	24	3	5	20,84
STI58	24	3	3	12,5
Average		**3,93**	**3,80**	**16,67**

SSR data were statistically processed using NTSYS-2.2j software, with the Ultrametric Dissimilarity option. We constructed a UPGMA-based dendrogram using the Nei and Li coefficients. Furthermore, we applied additional statistical analysis to calculate the matrix correlation coefficient among the potato samples studied.

The summary results of the Mantel test (Mantel, 1967), based on a regression analysis in which the variables are distance dissimilarity (or similarity) matrices, indicated 276 identification points, with a matrix correlation value of r = 0.76267. In the course of our study using 8 SSR markers, we found an average of 4 alleles per polymorphic locus and an average PIC value of 0.502. Ghislain et al. (2004) investigated 156 SSR markers, which allowed the identification of 18 of them as highly informative and easily applicable in the characterization of potato genetic resources. The authors report a PIC value of 0.786 found using SSR marker STM0037 in 931 potato samples. With the same marker in the potato samples from the Bulgarian collection included in the present study, we found a PIC value of 0.456. Ashkenazi et al. (2001) succeeded in distinguishing 12 potato cultivars using 2 SSR markers, finding an average of 5 alleles per polymorphic locus. In the course of our study using the described 8 SSR markers, we found an average of 4 alleles per polymorphic locus. In our study, we identified 3 STM0037-responsive alleles, with amplified fragments ranging in length from 90 to 120 bp. With a unique profile based on this marker, only one of the parental components of the first and fourth mutant groups was identified - PC428, with a fragment length of 90 bp. The polymorphism information content (PIC) value was equal to 0.475. Tiwari et al. (2018) also reported 13 alleles detected with STM0031 reaction and PIC=0.863, indicating a high genetic diversity among the studied samples, while in a study by Tilault and Yevtushenko (2019), the PIC value detected with this marker was 0.674, with 4 alleles generated per locus and amplified fragments ranging in length from 158 to 192 bp. Similar to these results with the same marker, we found four alleles and PIC=0.456 in our studied mutants, their parents, and controls.

5.6. ISSR analysis in potato

The ISSR technique proved to be beneficial in analyzing the Bulgarian collection of potato mutant lines. We would recommend the use of this method to study genetic variability, to identify cultivars and lines, and to speed up the breeding process in potatoes. Of the 12 reactions studied, with 10 we found polymorphic profiles (Table 4). The average value of polymorphism with 10 ISSR reactions in 24 potato genotypes found by us is 44.16%.

Table 3. Results of SSR reactions in potato mutant lines, their initial forms and controls.

Markers	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV
STM1052	1	1	1	2	2	3	4	1	2	1	2	2	2	4	1	1	1	1	1	1	1	1	1	1
STM0031	1	2	2	3	4	3	3	1	2	1	1	3	1	1	1	1	1	1	5	5	1	1	1	1
STM0037	1	2	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
STM3012	1	2	2	2	3	2	2	2	3	2	2	2	2	3	3	2	2	2	2	2	2	2	2	2
STI42	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3
STI53	1	2	3	4	5	3	4	6	3	3	6	3	3	3	5	5	3	3	5	6	7	7	3	3
STI57	1	2	2	1	3	4	1	4	4	4	1	4	4	1	1	1	4	1	1	1	5	1	1	2
STI58	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	2	1	1

** **I** – ПС 428; **II** – ПС 490; **III** – М-I-3; **IV** – М-I-8; **V** – М-I-17; **VI** – М-III-8; **VII** – М-III-9; **VIII** – М-III-25; **IX** –

М-III-30; **X** – М-III-48; **XI** – М-III-50; **XII** – К-III-2; **XIII** – М-IV-14; **XIV** – М-IV-15; **XV** – М-IV-17; **XVI** – К-IV-3; **XVII** – ПС 707; **XVIII** – ПС 538; **XIX** – ПС 757; **XX** – М-VII-7; **XXI** – М-VII-9; **XXII** – М-VII-19; **XXIII** – М-VII-27; **XXIV** – К-VII-4

The STI53 reaction, with which we identified 7 polymorphic profiles, turned out to be the most informative in our research.

Table 4. The amplified polymorphic ISSR markers in potato (*S. tuberosum* L.).

ISSR markers	G	PP	PP %	T	Fragment length (bp)
ISSR 1	24	17	70,8	17	400 - 2100
ISSR 2	24	5	20,8	15	200 - 1400
ISSR 5	24	19	79,2	19	200 – 3000
ISSR 6	24	10	41,7	13	200 – 2000
ISSR 7	24	6	25,0	9	200 – 1000
ISSR 8	24	10	41,7	10	500 – 2800
ISSR 9	24	6	25,0	7	200 –700
ISSR 10	24	5	20,8	12	250 - 2000
UBC807	24	11	45,8	11	480 - 1600
UBC 823	24	17	70,8	13	350 - 2800
Average		**10,6**	**44,16**	**12,6**	

* *G - Number of analyzed genotypes, PP - Polymorphic profiles, PP % - Percentage of polymorphic profiles, T - Total number of fragments.*

5.7. ISAP analysis in potato

The results of the analysis of mutant lines from the collection of Maritsa VCRI with the two most informative SINE families (SolS-IIIa and SolS-IV), conducted as single-family and multiplex reactions, showed a high level of polymorphism among the selected mutant potato lines (Figure 6). Based on the obtained monomorphic and polymorphic ISAP amplification profiles, the starting genotypes from each mutant group were identified with unique profiles (Figure 5).

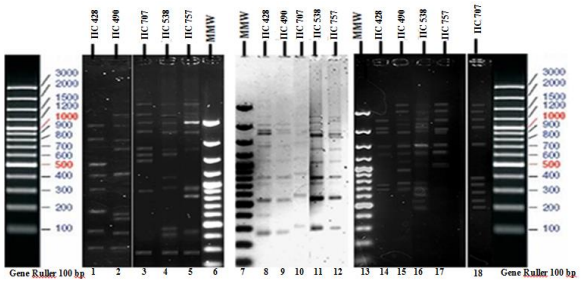


Figure 5. The amplified profiles of the initial potato genotypes by ISAP reactions. Profiles: 1 – 5 (SolS-IIIa-F/SolS-IIIa-R); Profiles 8 – 12 (SolS-IV-F/SolS-IV-R); Profiles 14 – 18 (SolS-IIIa-F/SolS-IV-R); Starts 6, 7, 13 – DNA marker (Gene Ruler 100 bp).

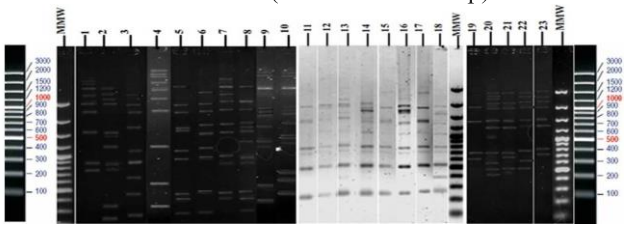


Figure 6. The amplified polymorphic profiles in potato mutant lines by ISAP reactions. SolS-IIIa-F/SolS-IIIa-R (starts 1 – 10); SolS-IV-F/SolS-IV-R (11 – 18); SolS-IIIa-F/SolS-IV-R (19 – 23)

The ISAP genotyping used in the dissertation is based on detecting the presence/absence of SINE elements at a particular locus (Schmit et al., 1998). According to Seibt et al. (2012), the SINEs SolS-IIIa and SolS-IV are the most common in the potato genome, indicating that ISAP reactions with these primers will have the greatest potential for genotyping and identification of potato samples. The study by Tomlekova et al. (2017), conducted with Bulgarian varieties, as well as the results obtained in the dissertation with mutant potato lines, confirm this statement. Based on our obtained results, we can summarize that the primer pairs SolS-IIIa-F/SolS-IIIa-R, SolS-IV-F/SolS-IV-R, and SolS-IIIa-F/SolS-IV-R , used for ISAP amplifications in the present study allowed the generation of highly polymorphic profiles.

The retrotransposon SINE – ISAP markers applied to potato mutant lines showed high sensitivity and managed to distinguish all the initial forms included in the present potato study, as well as some of the mutant lines. Our results demonstrate the potential and accuracy of the ISAP technique to perform precise genotyping in representatives of *S. tuberosum* L., as well as to identify closely related genotypes.

5.8. SSR analysis in local bean accessions

In the course of the studies carried out with local bean samples through 7 SSR reactions, we found that four of them amplified polymorphic profiles, and the remaining three reactions - had a monomorphic profile. Using four microsatellite SSR markers, polymorphism was detected in 20 bean samples, and selection lines were identified – “DG-13-12-22”, “DG-17-38-16”, “DG-17-38-46”, “DG-12-11-20”, as well as the "Skitiya" variety. The calculated mean value of PIC=0.438. According to the generated data, the highest coefficient of genetic distance is observed between genotypes "Skitiya" and "DG-9-11-1". Genotypes "Sadovets", "Belitsa 1", "Belitsa 2", and "Sveta Petka 1" show the highest values of genetic distance compared to selection lines - "DG-17-38-16" and “DG-17-38-38". According to Blair et al. (2012) the expected length of the amplified fragment with the BMd158 reaction was 186 bp. In the course of our study, we identified two amplified fragments with lengths of ~200 bp and 380 bp. In the selection line - "DG-9-11-1", "Sadovets 1", "Belitsa 1" we found the absence of a fragment with a length of ~ 200 bd, while in "DG-12-11-20" a fragment with a length of 380 bd is missing . In the remaining samples, both fragments are amplified, but with different intensity. To study polymorphism in 51 bean samples, Zargar et al. (2016), used SSR markers identified in previous studies by Yu et al. (2000), Gaitan-Solis et al. (2002), Grisi et al. (2007), Hanai et al. (2010), Córdoba et al. (2010). The authors found an average of 11.65 alleles per locus. In our study, the mean number of alleles per locus was 2.43.

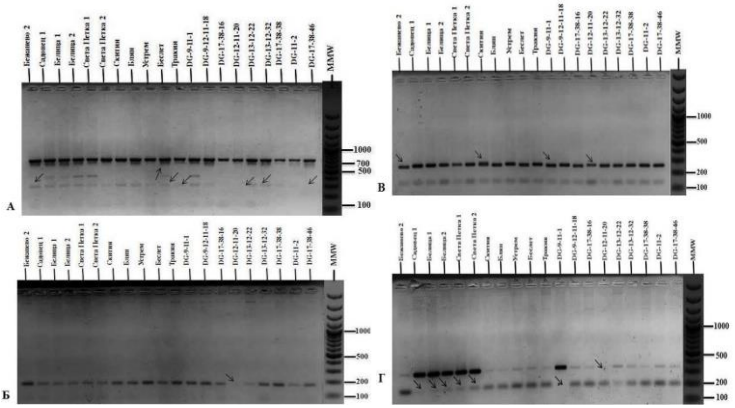


Figure 7. Amplified polymorphic SSR profiles in local bean accessions. * DNA marker (MMW) – Gene Ruller 100 bp Plus; A) BMd128; B) BMd143; C) BMd156; D) BMd158

5.9. ISSR analysis in common bean

Our results demonstrate the applicability of the ISSR technique as an effective method for identifying mutant bean lines and differentiating even closely related genotypes. We found the highest percentage of polymorphic profiles among the studied mutants through ISSR 2T, ISSR 4, ISSR 5 and UBC809 reactions.

Table 5. The amplified polymorphic ISSR markers in bean

(*P. vulgaris* L.).

ISSR markers	G	PP	PP %	T	Fragment length (bp)	PF	P %
ISSR 1	16	8	50,00	17	300-2000	15	88,00
ISSR 2	16	3	18,75	8	200-2500	3	18,75
ISSR 2T	16	12	75,00	15	150-2000	13	87,00
ISSR 3	16	3	18,75	16	350-3100	6	37,50
ISSR 4	16	12	75,00	13	350-2000	13	100,00
ISSR 5	16	14	87,50	20	320-3000	18	90,00
ISSR 7	16	8	50,00	21	150-2000	15	71,40
ISSR 10	16	11	68,75	9	400-2800	6	66,60
ISSR 12	16	9	56,25	13	700-2100	10	76,90
UBC 807	16	5	31,25	17	300-2000	7	41,20
UBC 809	16	14	87,50	14	400-2800	12	85,70

** G = Number of analyzed genotypes, PP = Polymorphic profiles, PP % = Percentage of polymorphic profiles, T = Total number of fragments, PF = Polymorphic fragments, P % = Percentage of polymorphic fragments.*

ISSR markers prove to be an important tool for detecting intraspecies polymorphism and are very promising for practical application. In their study, Tomlekova et al. (2012) reported 5 polymorphic fragments obtained with two ISSR primers generated in the parent and mutant tomato genotypes. Among the bean mutant lines we examined and their parent variety, each of these reactions amplified three profiles.

In studies by Tsonev et al. (2017) reported that there are two nonreproducible primers with trinucleotide repeats (ISSR 8 and ISSR 9) in pepper. In our bean study, these primers amplified monomorphic profiles. Through two other reactions (ISSR7 and ISSR 12), we found a higher polymorphism compared to that reported by Tsonev et al. (2017) in pepper. Our results demonstrate the applicability of the ISSR technique as an effective method for identifying mutant bean lines and differentiating even closely related genotypes. The established and described polymorphic profiles are a good starting point in future research, as they could be used for early identification of mutant genotypes when they are included as initial material in various breeding programs.

In the studies carried out with SSR, ISSR, ISAP, and COS II molecular marker techniques in tomatoes, the microsatellite-based techniques - SSR, ISSR - proved to be the most effective for distinguishing the samples. With the first, we found an average of 4.7 alleles per locus and a PIC value = 0.542, and with the second an average PIC value = 0.353, identifying 7 of the 9 analyzed genotypes. Retrotransposon-based SINE – ISAP and COS II techniques allowed us to distinguish 6 of the tomato cultivars.
The results obtained in the analysis of potato mutant lines, their parents and controls show that the microsatellite-based ISSR technique and the retrotransposon-based ISAP technique are the most effective.
The selected microsatellite ISSR markers also proved their efficiency in the study of the mutant bean collection, created and maintained at Maritsa VCRI.
SSR, ISSR, and ISAP reactions showed relatively high potential for studying genetic diversity in selected vegetable crops. The use of a larger number of informative markers would contribute to the detection of higher genetic variability among accessions.

VII. TO CONCLUDE

The results obtained in the dissertation through the application of various molecular-marker techniques and their discussion give us reason to formulate the following conclusions to conclude:

- In tomatoes, from the 4-technique studies, their effectiveness for detecting polymorphism was in the following order - SSR, ISSR, COS II, ISAP.
- In the case of potatoes, from the conducted research with 3 techniques, the most effective were – ISAP and ISSR.
- In beans from the two technique studies, ISSR was more effective than SSR.

CONCLUSIONS :

1. Fifteen SSR reactions out of nineteen performed in tomato were identified, with amplified polymorphic profiles. Through them, seven of the nine investigated varieties were identified.

2. Seven ISSR reactions out of thirteen conducted in tomatoes were found to amplify polymorphic profiles. Through them, six of the nine studied Bulgarian varieties were identified.

3. Using the ISSR method, representatives of the cultivated species (*S. lycopersicum*) were distinguished from those obtained by interspecific hybridization between cultivated and wild species (*S. pimpinellifolium, S. chilense*).

4. By introducing for the first time in tomato the ISAP molecular method, the potential of 7 of the SINE families has been proven, the most effective being SolS-II-F/SolS-II-R and SolS-IIIa-F/SolS-IIIa-R, with which identified five varieties of tomato.

5. Six of the eight COS II methods were used to identify seven of the nine studied Bulgarian tomato varieties.

6. Through the eight conducted SSR reactions, mostly monomorphic and from three to seven polymorphic profiles were established in the studied potato genotypes. The STI53 reaction is the most informative.

7. Through five of the ten ISSR reactions performed in potato mutant lines, their initial forms, and untreated controls, highly polymorphic profiles were established. Five of the reactions distinguished nine of the mutant lines, both among themselves and relative to the initial forms.

8. The applied two single-family and one multiplex ISAP reactions in potato mutant lines generated highly polymorphic profiles, with all the initial, five of the mutants, and two of the control genotypes amplifying ISAP profiles unique to the studied collection.

9. Through four microsatellite SSR markers, polymorphism was detected in twenty bean samples, and four selection lines and one cultivar were identified.

10. Through eleven of the fourteen ISSR reactions conducted, a high polymorphism was found in the 16 mutant bean lines studied. All mutant lines, such as the parent variety "Mastilen 11b" were identified with different ISSR profiles.

VIII. PhD THESIS CONTRIBUTIONS

7.1. Contributions of an original scientific nature

1. For the first time, the ISAP method was introduced to study the genetic diversity of tomatoes. The used primer pairs SolS-II-F/SolS-II-R and SolS-IIIa-F/SolS-IIIa-R for the ISAP reactions demonstrated a high potential for identifying polymorphic profiles in the culture species.

2. For the first time, the ISAP molecular method was applied to study genetic variability in Bulgarian mutant potato lines, their starting genotypes, and controls. All parental genotypes, five mutants, and three control lines were identified with individual amplification profiles.

3. For the first time, the ISSR molecular method was applied to study genetic variability in the Bulgarian collection (mutant and original forms) of potatoes. The mutant lines from the individual groups were distinguished from each other as well as from the initial genotypes.

4. For the first time, eleven ISSR markers were used to identify polymorphic profiles in Bulgarian mutant bean lines with valuable economic traits originating from the source variety "Mastilen 11b".

5. For the first time, the COS II molecular marker system was used in Bulgarian tomato accessions to study their genetic variability.

7.2. Scientific and applied contributions

1. The applicability of the SSR technique for early identification of tomato accessions has been proven, and the established polymorphism could be used in future breeding programs.

2. The efficiency of ISAP and ISSR techniques for genotyping mutant lines of potatoes, which could be used to accelerate mutational selection in them, has been proven.

3. The established specific SSR profiles for beans are used for the early identification of Bulgarian samples, acceleration of the selection process, copyright protection, and free transfer of genetic material.

4. The genetic diversity established by ISSR markers in advanced (M6) mutant bean lines accelerates the registration of new mutant varieties.

7.3. Confirmatory Contributions

1. The effectiveness of ISAP reactions for the identification of potatoes was confirmed in the study of the Bulgarian collection of mutant lines.

2. The effectiveness of SSR markers for establishing highly polymorphic profiles in tomatoes has been confirmed.

3. The effectiveness of ISSR markers for the study of bean genetic variability has been confirmed.

7.4. Contributions of a methodical nature

1. Three molecular marker techniques based on microsatellites (SSR and ISSR) and retrotransposons (ISAP) were introduced and studied in potato mutant lines created and maintained at Maritsa VCRI.

2. Microsatellite-based molecular techniques (SSR and ISSR) were introduced and studied in selected Bulgarian bean accessions.

3. SSR markers, which are characteristic of the tomato (*S. lycopersicum* L.) genome, were introduced for potato (*S. tuberosum* L.) research.

IX. LIST OF PUBLICATIONS RELATED TO THE PhD THESIS:

1. **Aziz, S**., Kantoglu, Y., Tomlekova, N., Staykova, T., Ganeva, D., Sarsu, F. 2021.Characterization of tomato genotypes by simple sequence repeats (SSR) molecular markers. Biharean Biologist, 15 (2):142-148.Article No:e214501.eISSN:2065-1155; pISSN:1843-5637. **Q4**

2. **Aziz, S.,** Spasova-Apostolova, V., Masheva, V., Tomlekova, N. 2022. Assessing polymorphism within common bean (Phaseolus vulgaris L.) mutant lines originated from variety "Mastilen 11b" using Inter Simple Sequence Repeats markers. Bulgarian Journal of Agricultural Science, 28(4):709–716. eISSN:2534-983X; pISSN: 1310-0351. **Q3**

3. 3.Tomlekova, N., **Aziz, S.,** Nacheva, E., Weber, B., Raina, A., Seibt, K.M.2023. SINE-Markers as a Powerful Tool for Assessing Genetic Diversity to Improve Potato.Advanced Crop Improvement, Volume 2 – Case Studies of Economically Important Crops (eds. Aamir Raina, Mohammad Rafiq Wani, Rafiul Amin Laskar, Nasya Tomlekova, and Samiullah Khan. Springer Nature, Swetzerland AG. ISBN:978-3-031-26668-3 (**in print**).

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NOTES: