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Molecular Diversity Analysis in Potato (*Solanum tuberosum* L.) Genotypes Through RAPD Markers

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ABSTRACT

This study aimed to analyze genetic diversity and similarities between 144 potato accessions, composed by hybrid populations 3 created starting from sexual hybridization at the National Institute of Research and Development for Potato and Sugar Beet Brasov (NIRDPSB Brasov) and their genitors, based on the PCR technique and polymorphism generated between potato genotypes, using RAPD primers. 12 RAPD primers were tested, of which only four were chosen for analyses (OPAB 11, OPA 04, OPA 03, OPC 02), based on the polymorphism generated between the studied potato genotypes. These molecular markers were able to identify and amplify DNA fragments specific to each studied genotype. All primers generated a total of 64 DNA amplification products of which 57 where polymorphic ranging between 398 bp and 2124 bp. The highest informative primer based on PIC value was OPC 02. Genetic similarity presented by phylogenetic tree, resulted in six clusters and the value ranges between 15% and 95%, indicating that all genotypes had a high level of similarity, according to their geographical origin and morphological characteristics of the genitors. Also, the screen plot corresponded with PCA is demonstrating this goal.

Keywords: *Solanum tuberosum* L., breeding, PCR, RAPD molecular markers, identification.

INTRODUCTION

Worldwide, potato crop has a high share in the economic balance, also in Romania, where is known as “second bread” and is considered as a strategical food (Stefan et al., 2022).

The introduction and development of new potato cultivars (*Solanum tuberosum* L.) has been an important strategy to increase crop productivity of this important staple food, fourth after rice, wheat and corn (Rocha et al., 2010; Chiru and Olteanu, 2013).

DNA fingerprinting has evolved as a result of major achievements in the field of obtaining new potato varieties through breeding programs (Hoque et al., 2013; Onamu et al., 2016). The analysis of genetic diversity and inter- or intraspecific, population or individual relationships, led to the use of molecular methods with the aim of detecting hereditary differences between individuals. The simple highlighting of a genetic polymorphism is a goal in itself.

The use of molecular markers, through the analysis of genomes, determined a strong rise in the efficiency of potato breeding activities (Kazuo, 2015), with practical applicability in the construction of genetic maps (Perez et al., 1999), in determinate the genetic relationships between cultivars or elite line which help breeders to identify and use unrelated and complementary parents in their traits (Orona-Castro et al., 2006); in germplasm diversity of potato (Yasmin et al., 2006); in genetic diversity (Gauchan et al., 2012) and conducting taxonomy and phylogenetic studies in potato (Vos et al., 1995; Sun et al., 2003).

Variety identification is essential to certify the identity and purity of genotypes hence might be useful in the analysis of genetic variability among cultivars, determining distinct parental combinations to produce segregating progenies that capture maximum genetic variability to enhance selection options among progeny (Alaklabi, 2017; Wang et al., 2019).

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Molecular markers highlight differences between nucleotide sequences in DNA extracted from different individuals, differences that are not necessarily visible in the phenotype (Hadi and Nurchasanah, 2020). A single different nucleotide, in a gene or even in repetitive DNA, can determine the formation or disappearance of a molecular marker (Jones et al., 1997; Balazs, 2011). Unlike morphological markers, such as tuber shape, leaf type and color, as well as flower color (Rosa et al., 2010; Lopez-Vizcón and Ortega, 2012) which are time consuming, difficult to assess and are influenced by environmental factors. The molecular markers have the great advantage that they are numerous and do not disturb the organism`s physiology.

They present many important characteristics, are independent of the phenotypic expression of the gene, do not present epistasis and pleiotropy, are transmitted Mendelian dominant or codominant and are indifferent to selection pressure (Botez et al., 2013).

For a correct management of genetic resources like the exact identification of plant cultivars is an important requirement both for the practical purposes of breeding and for fields close to it, such as the protection of property rights over newly created varieties (Novakova et al., 2009).

The specific molecular methods (e.g., techniques based on DNA sequencing and analysis) allow obtaining a "genetic fingerprint" characteristic of each genotype, with obtaining detailed data and information and thorough knowledge of the existing genetic resource pool and its use value (Naznin et al., 2020).

The PCR (Polymerase Chain Reaction) technique, discovered in 1988 by Kary Mullis and his team (Saiki et al., 1985), is based on the enzymatic amplification of DNA, starting from a very small amount of DNA template (Islam et al., 2022).

One of the techniques that does not require sequence information is Random Amplified DNA Polymorphism (RAPD) analysis (Williams et al., 1990; Karp et al., 1997; Tabkhkar et al., 2012). Unknown DNA fragments are amplified with primers having a random nucleotide sequence (Taoutaou et al., 2022). These markers are widely and most commonly used marker because they are dominant, technically simple, quick, non-radioactive, largely automatable, relatively inexpensive and require small amounts of DNA, that is why polymerase chain reaction (PCR) generating RAPD fragments is more understood (Selaocoe et al., 2019, cited by Singh et al., 2021). In some cases, patterns characteristic of a certain genotype result, which are a true genetic fingerprint, represented like a barcode.

Amplicons (amplified DNA fragments) result by fixing the primers to partially or totally complementary sites of each DNA strand. While nothing is known about the identity and context of the sequence from which a particular amplification product originates, its presence or absence in different organisms is important information for assessing genetic diversity and relatedness.

Although widely used for different purposes, there is a lack of knowledge about the nucleotide sequence of RAPD markers and the binding sites of primers in the target genome (Mori and Fani, 2013). The same authors agree that certain RAPD bands could be generated by self-priming events when hairpin loops form at the 3' terminus of the initially amplified products.

However, one of the shortcomings of RAPD markers is the low reproducibility of this type of analysis. This is because the primers are not specific to a particular locus, and thus some of the amplification products are the result of defective primer attachment. The analysis is thus sensitive to a series of factors such as: primer attachment temperature, the quality and concentrations of the extraction buffer components, the DNA polymerase used, etc. (Hadrys et al., 1992).

The aim of study was to estimate the genetic variability of potato genotypes created at NIRDPSB Brasov and to confirm the phylogenetic relationship between them, using RAPD molecular markers. DNA fingerprint results for each cultivar will contribute to the morpho-physiological

description existent in the database of NIRDPSB Brasov.

MATERIAL AND METHODS

The biological material analyzed consisted of 144 samples, which are vegetative hybrid populations (P3) obtained by sexual hybridization and their genitors (Table 1). The hybrid combinations were obtained in the breeding process done at the Genetic Breeding and Plant Selection Laboratory of NIRDSPB Brasov. The potato breeding process, in addition to careful study of the genitors has a well-established procedure, aiming at a rigorous schema, lasting 10-12 years: Year I - Seed field (first generative hybrid population); Years II-IV - Selection field - vegetative hybrid populations (P1-P3); Years V-VII - Selection field - vegetative descendants (D1 and D2); Years VIII-IX - Comparative cultures of orientation years I and II; Years X-XII - Comparative culture of concurrence (Stefan et al., 2016).

Table 1. The biological material analyzed

Vegetative hybrid populations P3			
Genitors ♀ x ♂		Hybrid combination	Number of genotypes/ combination
Brasovia	Saviola	1957	12
	Tivoli	1971	10
	Aladin	1979	9
Caruso	Tivoli	1982	8
	Svenja	1977	8
	Aladin	1976	9
Cosiana	Aladin	1947	9
Patricia		1980	11
Christian	Saviola	1965	15
Leandra		1960	4
Brasovia	Blue Congo	1963	3
Robusta		1970	6
Marvis	Orchestra	1962	7
Europrima		1966	4
Sarmis		1958	3
Marvis	Patricia	1949	3
Opal		1956	6
Total: 17 genitors			127 genotypes
Total: 144 samples			

DNA isolation, PCR-RAPD and polymorphism analyses. The molecular analyzes were performed at the Genetics Laboratory of the Faculty of Agriculture from University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. For total DNA isolation, young leaves were harvested from the upper third of the plant. They stored in a freezer at -80°C until the analysis. DNA extraction was performed using the ISOLATE II Plant Kit (Bioline). The purity and quantity of DNA was carried out spectrophotometrically using NanoDrop 1000 device. The PCR amplification was performed in a volume of 15 µl containing: 30 ng DNA, 2x PCR master-mix (ready-to-use, Bioline), 0.2 µM/primer and free water (Fermentas). The reaction mixture was overlaid with 15 µl mineral oil. Amplification was performed using a Techne TC-4000 thermal cycler, with the following profile of temperature: initial denaturation step of 95°C for 3` follows by 45 cycles of 1` at 93°C, 1` at 34°C, 1` at 72°C and 10` at 72°C - final extension.

For genetic diversity and DNA fingerprinting, 12 RAPD primers produced by Operon Technologies, Inc. USA, were used (Table 2).

Agarose gel electrophoresis. The amplification products were separated into

1% agarose gel. To stain the DNA fragments, 5 µl of the Red Safe dye (Bioline) was added to the agarose solution. Gels were run for 1.5 h at 80 V and visualized under UV light (UVP BioImaging Systems). The standard 100 bp DNA Step Ladder molecular marker (Fermentas) it was used to calculate the bands sizes.

Image analysis. Only bands with a high light intensity were included in the statistical analysis. The presence of the band was marked with 1 and the absence was marked with 0. The gels were analyzed using the TotalLab TL 100 program.

Value of PIC (Polymorphism Information Content) it was determined to show how many monomorphic and polymorphic amplification products were generated by each primer. The Polymorphic Information Content (PIC) was calculated according to the formula Roland-Ruiz et al., 2001:

$$PIC = 2 * fi * (1-fi)$$

where:

fi - is the frequency of the amplified allele (band present);

(1-fi) - is the frequency of the null allele (band absent).

The genetic distance between genotypes was calculated using Jaccard's (1908) similarity coefficient. Cluster analysis was obtained using the PAST software version 2.1 (Harper, 2001) and a dendrogram was generated based on genetic distance obtained from the same coefficient. Genotype data of marker was correlate with the genotypes using PAST software version 2.1, resulting the PCoA (Principal Coordinates Analysis). This is a multivariate statistical technique used for visualizing the similarities and differences between samples based on a distance matrix.

Table 2. RAPD primers used to generate the genetic variability and its sequences

Crt. no.	Primer name	The nucleotide sequence of the primers (5'- 3')
1	OPB 08	GTCCACACGG
2	OPE 14	TGCGGCTGAG
3	OPC 02	CAGGCCCTTC
4	AB 11	CAATCGCCGT
5	OPA 18	AGGTGACCGT
6	OPB 17	AGGGAACGAG
7	OPAB 11	GTGCGCAATG
8	OPA 20	GTTGCGATCC
9	OPX 03	TGGCGCAGTG
10	OPA 04	AATCGGGCTG
11	OPA 03	AGTCAGCCAC
12	OPC 02	GTGAGGCGTC

RESULTS AND DISCUSSION

Reproducible results with the RAPD method are guaranteed only when utilizing a DNA template of high purity. The quantity of DNA varied between 32.1 and 856 ng/µl, with a purity between 1.67 and 2.05.

Out of the 12 RAPD primers used, only four were able to generate polymorphism for each genotype at molecular level, this was OPA 03, OPA 04, OPC 02 and OPAB 11. Primer selection was based on three criteria: reproducibility, levels of polymorphism detected in a specific group of genotypes and number of polymorphic loci per assay. The rest of primers were either monomorphic or did not amplify any molecular fragments. Table 3 shows the results obtained following amplification with RAPD primers used. All primers had a characteristic RAPD pattern consisting of 12 to 18 fragments, with their size ranging from 398 pb to 2124 pb. All primers generated a total of 64 DNA bands (amplification products) of which 57 were polymorphic. Only OPA 03 generate 12 polymorphic bands and the rest of primers had the same number of polymorphic bands (15 bands). The percentage of polymorphism

it was 89.3%. Polymorphism percentage obtain by Simon et al. (2023) was between 20 and 75%.

Das et al. (2010) used OPA 03 which generated 10 amplified band (8 polymorphic bands) with the size range between 300-2000 pb. But in the studies made by Naznin et al. (2020), OPA 03 did not show any band in gel electrophoresis.

Table 3. Fragment size, total band amplified and value of PIC (Polymorphism Information Content), results using RAPD primers

Primer name	Total DNA bands	Polymorphic bands	Polymorphism (%)	Fragment size min-max (pb)	Total bands amplified	PIC
OPA 03	13	12	92.3	633 - 1505	387	0.191
OPA 04	16	15	93.7	559 - 2124	712	0.020
OPC 02	18	15	83.3	398 - 1439	549	0.196
OPAB11	17	15	88.2	425 - 1241	392	0.148
Total	64	57	357.5	-	2040	-
Mean	16	14.25	89.3	-	-	-

The few electrophoretic profiles obtained after amplification with the primer OPA 04 are showed in Figures 1 - 3.

The difference between individuals observed in samples 33 and 39, which present only one amplified fragment, and in samples 8, 21, 22, 26, 42, 43, which present four fragments, the rest have much more amplified fragments each. Also, OPA 04 did not amplify any fragment in sample 17.

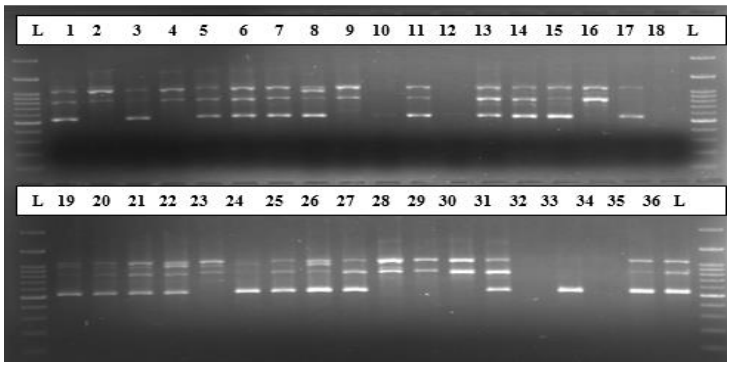

Figure 1. The electrophoretic profile obtained with the OPA 04 primer for the first 36 potato samples (1-36). *The upper side*: Arizona-1, Tivoli-2, Robusta-3, Saviola-4, Patricia-5, Orchestra-6, Bettina-7, Cosiana-8, Blue Congo-9, Caruso-10, Brasovia-11, Svenja-12, Europrima-13, Christian-14, Opal-15, Leandra-16, Aladin-17, Tresor-18. *The bottom side*: 1947/1-19, 1947/2-20, 1947/3-21, 1947/4-22, 1947/5-23, 1947/6-24, 1947/8-25, 1947/9-26, Sarmis-27, Marvis-28, 1979/1-29, 1979/2-30, 1979/3-31, 1979/4-32, 1979/5-33, 1979/6-34, 1979/7-35, 1979/10 -36, L: Ladder DNA-100 bp (Bioline).

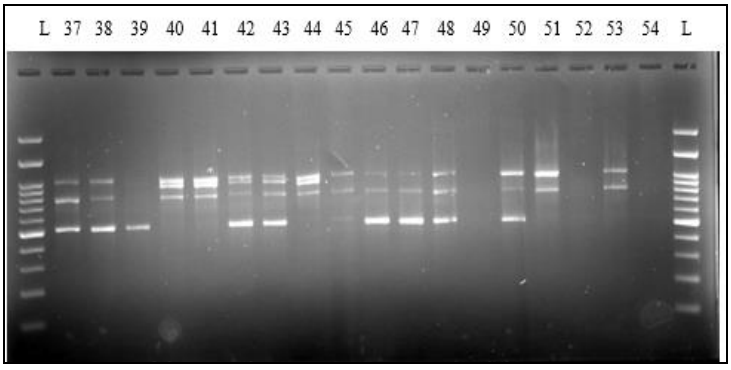

Figure 2. Electrophoretic profile obtained with OPA 04 primer in potato samples 37-54 (37-1979/11, 38-1982/1, 39-1982/2, 40-1982/2A, 41-1982/3, 42-1982/4, 43-1982/4.1, 44-1982/5, 45-1982/6, 46-1958/2, 47-1958/3, 48-1958/4, 49-1963/2, 50- 1982/2A 50-1963/3, 51-1963/4, 52-1965/1, 53-1965/2, 54-1965/3), L: Ladder DNA-100 bp (Bioline).

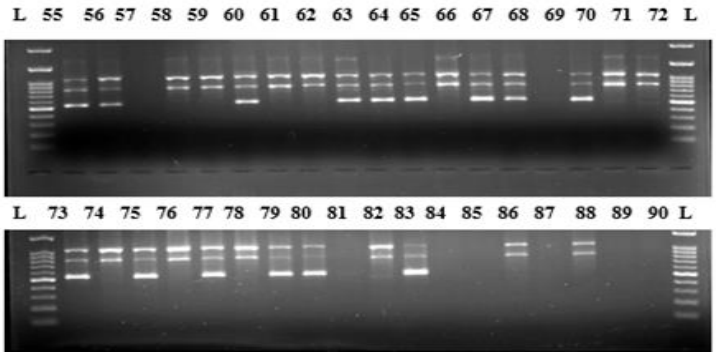

Figure 3. Electrophoretic profile obtained with OPA 04 primer in potato samples 55-90.
The upper side: 55-1965/6, 56-1965/7, 57-1965/8, 58-1965/9, 59-1965/10, 60- 1965/11, 61-1965/12, 62-1965/13, 63-1965/14, 64-1965/15, 65- 1965/16, 66-1965/17, 67-1966/2, 68-1966/3, 69-1966/4, 70-1966/6, 71-1962/1, 72-1962/2.
The bottom side: 73-1962/3, 74-1962/4, 75-1962/5, 76-1962/7, 77-1962/8, 78-1970/1, 79-1970/2, 80-1970/3, 81-1970/5, 82-1970/6, 83-1970/7, 84-1957/1, 85-1957/2, 86-1957/4, 87-1957/5, 88-1957/8, 89-1957/9, 90-1957/11, L: Ladder DNA-100 bp (Bioline).

In Figure 3 it can observe the amplified bands with sizes between approximately 500 and 2100 bp, but this primer did not amplify part of the samples between 81 and 90 (81-1970/5, 84-1957/1, 85-1957/2, 87-1957/5, 89-1957/9, 90-1957/11) and samples 57-1965/8 and 69-1966/4. Saimon et al. (2023) used six RAPD primers and the patterns of the amplified bands was ranges between 500 to 3000 bp for all primers.

The PIC value represents the discriminatory power of a DNA marker produced by a specific PCR primer (Roland-Ruiz et al., 2001). Details about the primers used in these studies are shown by PIC values. The coefficient had a value between 0 and 1. A PIC value of 0.00 indicates monomorphism, but if is > 0.00 are considered polymorphic. When the value of PIC ≥ 0.5 are the best primers that can be used as molecular markers and is the expected threshold for DNA fingerprint identification.

Our results range the PIC value between 0.020 and 0.196, with the highest value attributed to the OPC 02 primer. Based on this, the RAPD markers were classified as informative OPC 02, followed by OPA 03, OPAB 11 and less informative it was OPA 04. The low PIC values in the experiment seems reasonable given the close relationship between the genotypes analyzed. Rocha et al. (2010) evaluate a molecular profile to study genetic diversity in 16 potato cultivars and the PIC value for RAPD markers was between 0.12-0.94. While Hadi and Nurchasanah (2020) used seven RAPD markers to study the genetic diversity of 13 accessions of potato and the value of PIC was between 0.31 and 0.78.

The analysis of genetic distances between the potato varieties studied, calculated based on the Jaccard coefficient, highlighted that the only four RAPD markers used, divided the potato varieties into six major clusters based on their genetic origin (Figure 4).

In the first cluster, we found all 11 genotypes from the 1980 hybrid combination and one part of the genotypes (six samples) from the 1976 hybrid combination. Here we found four genotypes from 1960 hybrid combination and three genotypes from 1957. The hybrid combinations found in cluster I have the Saviola variety as their common genitor. Here, the highest similarity was recorded between 1980/11 and 1980/12 (90%), between 1976/9 and 1976/10 (60%), 1976/8 and 1980/5 with 49%, because Aladin is their common genitor (Figure 4). The genetic similarity among all genotypes in the first cluster ranges from 22% to 90%. Onamu et al. (2016) reported an average genetic similarity of 0.61 ± 0.02 between pairs of 35 potato accessions, with a range of 0.46-0.87.

In the second cluster, all genotypes belonging to the 1977 hybrid combination were included, along with the six genotypes from 1971 and 1976, as all these combinations share Tivoli and Aladin as their genitors. The average genetic similarity

among all genotypes here is between 26 and 85%. The highest similarity was recorded between two genotypes from 1971 hybrid combinations.

The third cluster included the cultivars which were used as genitors for all hybrid combinations. As we know, the molecular markers are used to determinate the genetic relationships between cultivars (Orona-Castro et al., 2006), to confirm the phylogenetic studies or to study the genetic origine between genitors and their descendants. Furthermore, it includes all genotypes belonging to the 1979 and 1947 hybrid combinations, where one of the genitors is the Aladin variety. Additionally, next to these two combinations, we found all six genotypes from 1956 hybrid combination, because Patricia is a common genitor with the 1947 hybrid combination. Genetic similarity in this cluster ranges from 18 to 95%. Saimon et al. (2023) used Jaccard coefficient to establish the genetic similarity of each variety, and this ranged from 0.63 to 0.90.

The fourth cluster comprises genotypes from the 1970 hybrid combination (with six genotypes) and the 1957 hybrid combination (with six genotypes; the remaining four belong to the last cluster). These two have the same genitor, Brasovia genotype. Also, it can find genotypes from 1982 hybrid combination that share the same genitor with genotypes from 1957 hybrid combination, the Tivoli cultivar. Our results confirmed that RAPD markers can be used to establish pedigree-based genetic diversity estimates in cultivated potato hybrids. The results are in accordance with research of Sun et al. (2003).

In the next cluster (the fifth), is included all 15 genotypes from 1965 hybrid combination, four genotypes from 1966 hybrid combination and one part the genotypes from 1962. The last two have the Orchestra cultivar as a genitor.

In this cluster, we found the smallest genetic similarity between all genotypes, which ranges from 15% to 67%.

In the last cluster (sixth cluster) are clustered a few genotypes from 1957 hybrid combination, near two genotypes from 1965 combination 1965/2 and 1965/3, which have the highest genetic similarity from this cluster.

Similarity coefficients ranged from 0.29 to 0.93 was obtained by Das et al. (2010), and in their opinion "cluster analysis reflected the expected trends in relationships of the full and half-sib potato genotypes".

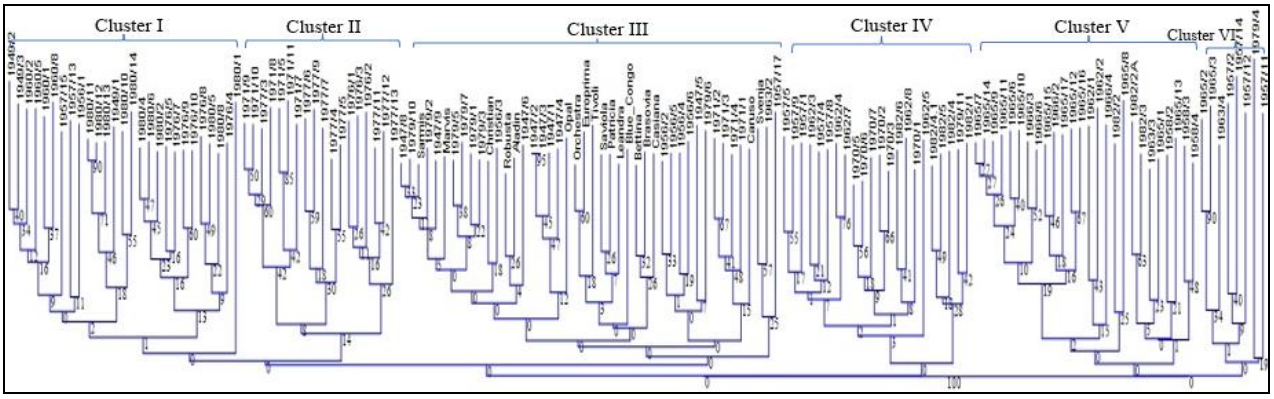

Figure 4. Dendrogram generated based on the genetic distribution of the vegetative populations 3 and their genitors using Jaccard's (1908) similarity coefficient

The data obtained by us align with those in the specialized literature, indicating that RAPD markers can differentiate the studied potato varieties and, at the same time, highlight the degree of genetic similarity among them (Rocha et al., 2010; Hadi and Nurchasanah, 2020; Naznin et al., 2020).

All the results are confirmed by Principal Component Analysis (PCoA) which were largely in agreement with those obtained by

the cluster analysis, further supporting the genetic similarity of the genotypes, as shown in Figure 5. To explain the total variance exhibited on the PC axis, the first step in PCA analysis is to evaluate eigenvalues. In our analysis, the value of the eigenvalue was between 0.020 and 4.792 and the percentage of variance (% variance) was between 0.031 and 7.488.

The screen plot from figure 5 shows the relationship between all 144 potato genotypes using genetic similarity. The area with the most potato varieties is on the left side, where is included all the potato genotypes, like in the third cluster of the dendrogram.

PCA based on correlation distributes the genotypes into the two-dimensional plane, that explains the correlation and variability among the genotypes (Raza et al., 2019).

In the right and upper area of the plot, the genotypes included correspond to those found in the second cluster of the dendrogram, while in the right and down area we found the genotypes from the fifth and sixth cluster from dendrogram. The results are in accordance with research of Sun et al. (2003).

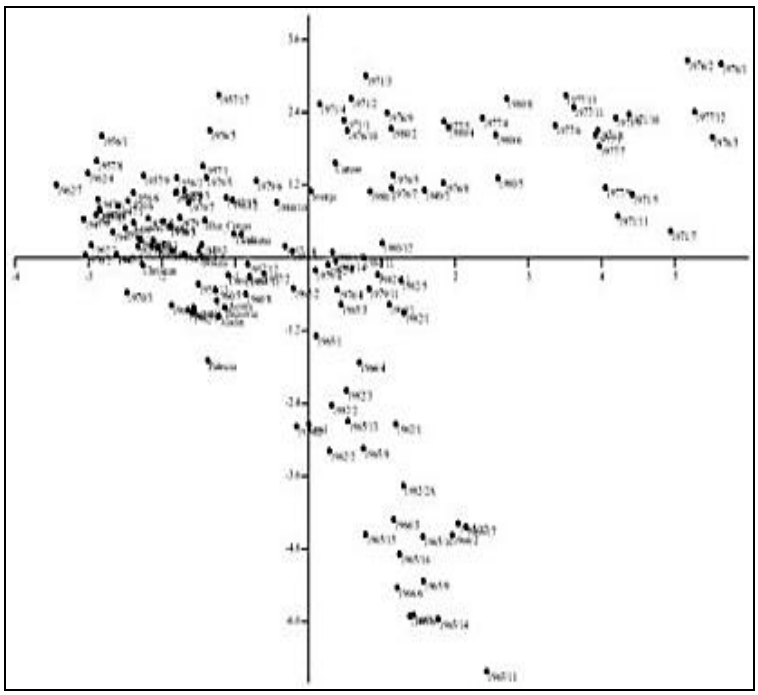

Figure 5. Screen plot analysis of principal component analysis (PCoA) contributing to genotype variability of all potato genotypes studied based on RAPD molecular markers

CONCLUSIONS

12 RAPD primers were chosen from the specialized literature, of which only four generated polymorphism and were used in the analyses, to highlight the differences and similarities between the potato genotypes studied and to confirm the establish pedigree-based genetic diversity estimates in cultivated. These primers are: OPA 03, OPA 04, OPC 02, OPAB 11.

Regarding the number of polymorphic bands all primers generated a total of 64 DNA bands (amplification products), of which 57 were polymorphic. Only OPA 03 generated 12 polymorphic bands, while the remaining primers each had 15 polymorphic bands.

The PIC value for all primers used ranges between 0.020 and 0.196, with the highest value attributed to the OPC 02 primer. The best informative primers were OPC 02, followed by OPA 03, OPAB 11 and less informative were OPA 04. Based on the results of the phylogenetic tree constructed using RAPD amplified bands, all 144 potato genotypes were divided into six clusters according to their genetic origin. The third cluster is the largest, including all 17 parents and three hybrid combinations along with their genotype combinations.

The genetic similarity among the genotypes analyzed in this research ranges between 15% and 95%, indicating that all genotypes had a high level of genetic similarity.

PCA based on correlation arranges the genotypes on a four-dimensional plane, elucidating the correlation and variability among them.

In conclusion, the study showed that genetic diversity among 144 potato genotypes was easy detected by four RAPD markers. But for a more conclusive and exactly results are needed to use a higher number of RAPD primers, or a more precise technique for improved accuracy.

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