UDC 575.630
https://doi.org/10.2298/GENSR2002765V
Original scientific paper

GENETIC DIVERSITY AND RELATIONSHIP OF TUNISIAN CASTOR (*Ricinus communis* L.) GENOTYPES REVEALED BY SSR MARKERS

Martin VIVODÍK*[1], Ezzeddine SAADAOUI[2], Želmíra BALÁŽOVÁ[1], Zdenka GÁLOVÁ[1]

[1]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Nitra, Slovak Republic
[2]National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia

Vivodík M., E. Saadaoui, Ž. Balážová, Z. Gálová (2020). *Genetic diversity and relationship of Tunisian castor (Ricinus communis L.) genotypes revealed by SSR markers*-.Genetika, Vol 52, No.2, 765-776.

The aim of this study was to assess genetic diversity within the set of 55 Tunisian castor genotypes using 20 SSR primers. PCR amplification of DNA using 20 primers for SSR analysis produced 141 DNA fragments that could be scored in all 55 genotypes of castor. The number of amplified fragments varied from 4 (Rco 15) to 10 (Rco 29 and Rco 33). Of the 141 amplified bands, all 141 were polymorphic, with an average of 7.05 polymorphic bands per primer. The polymorphism information content (PIC) value varied from 0.719 (Rco 15) to 0.879 (Rco 29), with an average of 0.825 and index diversity (DI) value varied from 0.745 (Rco 15) to 0.881 (Rco 29) with an average of 0.832. 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis. Probability of identity (PI) was low ranged from 0.002 (Rco 29 and Rco 41) to 0.018 (Rco 15) with an average of 0.007. A dendrogram based on UPGMA analysis separated 55 Tunisian castor genotypes into 4 clusters (1, 2, 3, 4). Knowledge on the genetic diversity of castor can be used for future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production.

Keywords: ricin, microsatellites markers, polymorphism, dendrogram**,** PIC

INTRODUCTION

Castor (*Ricinus communis* L., 2n = 2x = 20, *Euphorbiaceae*), is industrially important non-edible oilseed crop widely cultivated in the arid and semi-arid regions of the world (GOVAERTS *et al*., 2000). The seed of castor contain more than 45% oil and this oil is rich (80–90%) in an unusual hydroxyl fatty acid, ricinoleic acid (JEONG and PARK, 2009). Castor oil is the only vegetable oil soluble in alcohol, presenting high viscosity, and requiring less heating than

Corresponding authors*:* Martin Vivodík, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, Nitra, phone: +421376414269, e-mail: martin.vivodik@uniag.sk

others oils during the production of biodiesel (JEONG and PARK, 2009). Castor is a cross pollinated crop and is usually cultivated as a hybrid in India, as hybrids give significantly greater yields than pure lines or varieties (BIRCHLER *et al*., 2003 and REIF *et al*., 2007). A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner (SANTALLA *et al*., 1998; PERVAIZ *et al*., 2010). DNA-based molecular analysis tools are ideal for germplasm characterization and phylogenetic studies (SHINWARI *et al*., 2011). Among the various DNA-based markers, microsatellites (SSR), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have been used to study genetic diversity (RABBANI *et al*., 2010). These markers elucidate the phylogenetic relationships among various lines, for their efficient use in breeding and genetic resource management. These methods, however, involve the use of expensive enzymes, radioactive labeling, and are cumbersome and hence, appear unsuitable (TURI *et al*., 2012). Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers on the other hand, require only small amounts of DNA sample without involving radioactive labels and are simpler as well as faster (KAPTEYN and SIMON, 2002; JAN *et al*., 2011). SSR has proven to be quite efficient in detecting genetic variations and used for diversity assessment and for identifying germplasm in a number of species (MIKIĆ *et al.*, 2018; NIKOLIĆ *et al.*, 2018; SALEHI *et al*., 2018; RAJA *et al*., 2018; DARESTANI *et al*., 2019; ROGIĆ *et al.*, 2019; SAVIĆ *et al*., 2019). The polymerase chain reaction (PCR), which is the basic technique of SSR markers, has been used by many authors, such as LABAJOVÁ *et al.*, (2011); ŽIAROVSKÁ *et al.*, (2013); ŠTEFÚNOVÁ *et al.*, (2014); ŽIAROVSKÁ *et al.*, (2014); KALLAMADIA *et al.*, (2015); SHARAWY *et al.*, (2015); VYHNÁNEK *et. al*., (2015); RAŽNÁ *et. al*., (2016); ŽIAROVSKÁ *et. al.*, (2017); ANSARI *et al*., (2018); EL-FIKI *et al.*, (2019); ŠTIASNA *et al*., (2019). The present study is focused on estimation of genetic distance between 56 Tunisian castor genotypes, based on 20 SSR markers.

MATERIALS AND METHODS

Plant material and SSR amplification

Fifty-five castor (*Ricinus communis* L.) genotypes were used in the present study. Seeds of castor were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia (Figure 1). The ricin genotypes were obtained from 12 regions of Tunisia: S- Souassi, BT- Bouthay, GH- Ghomrassen, BA- Sidi Bou ali, MT- Matmata, AG- Mateur, N- Nefza, MD- Mednine, M- Mornag, G- Gabes, K- Kebili, KJ- Ksar jedid (Table 3.). Genomic DNA of castor cultivars was extracted from leaves of 14-day old plantlets with GeneJET Plant Genomic DNA Purification Mini Kit according to the manufacturer's instructions. DNA concentrations were estimated by UV-Vis spectrophotometer Q5000, Quawell.

Amplification of SSR fragments was performed according to BAJAY *et al.*, (2009, 2011) (Table 1). Polymerase chain reaction (PCR) were performed in 25 µl of a mixture containing 10.5 µl H_2O, 12.0 µl Master Mix (Genei, Bangalore, India), 0.75 µl of each primer (10 pmol) and 1 µl DNA (100 ng). Amplification was performed in a programmed thermocycler (Biometra, Germany) and amplification program consisted of an initial denaturing step at 94°C for 1 min, followed by 35 cycles of amplification [94°C (1 min), 1 min at the specific annealing temperature of each primer pair (Table 1), 72°C (1 min)] and a final elongation step at 72°C for

10 min. Amplification products were confirmed by electrophoresis in 7% denaturing polyacrylamide gels and silver stained and documented using gel documentation system Grab-It 1D for Windows.

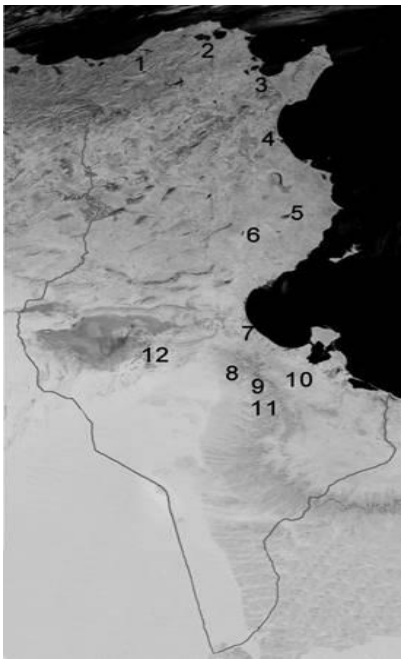

Figure 1. Localization in the map of Tunisia of the populations that are at the source of the seeds used in the present study. (1) Nefza, (2) Mateur, (3) Mornag, (4) Sidi Bou Ali, (5) Souassi, (6) Bouthady, (7) Gabes, (8) Matmata, (9) Ksar Jedid, (10) Medenine, (11) Ghomrassen, (12) Kebili.

*Table 1 List of SSR primers (*BAJAY *et al., 2009, 2011)*

Marker name	*Ta* (°C)	Repeat motif	Sequence of the primer (5′ - 3′)
Rco02	60	$(AC)_{12}$	**F: CTAGCTTTGGGGCACAGTC** **R: GGAAAATAGGTGCGTATGAAAC**
Rco05	60	$(TG)_6(GA)_{22}(GAA)_4$	**F: AGCCCAGAAATTGGAAAAGA** **R: CAAACCCAAGCAAACCTCA**
Rco06	60	$(TG)_{11}$	**F: GGGTGAAAATGAAGAGATTGG** **R: ATAACCCGTGAAGCATGGAC**
Rco08	60	$(TG)_{10}$	**F: CGTGTGTCTGTGTGCATGTC** **R: CCTCAACCCTTTGCTGTTTC**
Rco09	60	$(AC)_{11}$	**F: CCAACTCCCTTGTCTGCAA** **R: GTGAATGGCAAGCAGCAAT**
Rco13	62	$(GA)_{23}$	**F: GGTGCTTCCAGAAATTCAGTT** **R: GGAGGGGAAAGACAGGATTC**

Rco15	60	$(AG)_{18}$	**F: CACGCACGTTAAAGCAAACT** **R: GCGAAGAAACCAAAATGGAG**
Rco18	60	$(CA)_{17}$	**F: AGGGGGATAAGCGTGATATG** **R: CCGTTATGAAAAGGAAAGCA**
Rco20	60	$(TC)_{23}$	**F: CCAAAAGGAATGTGGGACTC** **R: TGTGGAGAGGATGAAGAGGAA**
Rco22	62	$(AAAC)_3(AC)_9(TC)_5$	**F: ATCCGCCGACAATAGCAG** **R: GCAACACTCTCTTCCCTGAA**
Rco23	62	$(GA)_{15}(AG)_8$	**F: CATGGATGTAGAGGGTCGAT** **R: CAGCCAAGCCAAAGATTTTC**
Rco26	62	$(CT)_{19}$	**F: TTGCTTGTCAAAGGGGAGTT** **R: TCATTTTGAGGGAGAAACCA**
Rco29	60	$(GA)_7$	**F: GGAGAAAAGAAAGGGAGAAGG** **R: GCCAAAAGCACACTTAATTTGA**
Rco30	60	$(AG)_{19}$	**F: TGAAACTTTGGAGCTTGGAGA** **R: GGTCCCACACATTCATACACA**
Rco31	60	$(TC)_{12}(TCTA)_4(AC)_{10}$	**F: ACAATGCGTGTGTCTGTGTG** **R: CCTCAACCCTTTGCTGTTTC**
Rco33	60	$(TCT)_{11}$	**F: ACATACATGCAGGGAGACCA** **R: TCTGCTTTAATGGCTGATCG**
Rco34	60	$(GT)_{11}$	**F: TCGGTTAAGGGTATGGGTTG** **R: CACACTTCATTTCGCAGACC**
Rco35	60	$(AG)_{16}$	**F: GGAAGAATTGGGTTGGAAGT** **R: AACAAACACAGGTGCATCAT**
Rco40	60	$(TC)_5(CT)_7$	**F: AACTGGATAAAGGGGTATTTGG** **R: GCTTTTTGGTAGCAGGTTTGA**
Rco41	60	$(CT)_{17}(CA)_{11}$	**F: CATGTTGTTTTTGGCAGCTC** **R: CGTTCACACTCATCAATCCA**

Ta- annealing temperature

Data analysis

Data obtained from SSR analysis were scored as presence (1) or absence (0) of fragments for each castor genotype and entered into a matrix. Based on the similarity matrix, a dendogram showing the genetic relationships between genotypes was constructed using unweighted pair group method with arithmetic mean (UPGMA) by using the SPSS professional statistics version 17 software package. For the assessment of the polymorphism between castor genotypes and usability of SSR markers in their differentiation diversity index (DI) (WEIR, 1990), the probability of identity (PI) (PAETKAU *et al.*, 1995) and polymorphic information content (PIC) (WEBER, 1990) were used.

Diversity index (DI)

$$DI = 1 - \sum p_i^2$$

Probability of identity (PI)

$$PI = \sum p_i^4 + \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{n} \left(2 p_i p_j\right)^2$$

Polymorphic information content (PIC)

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2 \right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 \cdot p_j^2$$

where p_i and p_j are frequencies of i^{th} and j^{th} fragment of given genotype.

RESULTS

PCR amplification of DNA using 20 primers (Table 1) for SSR analysis produced 141 DNA fragments that could be scored in all 55 genotypes of castor (Figure 2). The number of amplified fragments varied from 4 (Rco 15) to 10 (Rco 29 and Rco 33). Of the 141 amplified bands, all 141 were polymorphic, with an average of 7.05 polymorphic bands per primer. Results indicated the presence of wide genetic variability among different genotypes of castor. Variations in DNA sequences lead to polymorphism. Greater polymorphism is indicative of greater genetic diversity. Using of parents with greater genetic diversity results in broad genetic base of the hybrids.

The polymorphism information content (PIC) value varied from 0.719 (Rco 15) to 0.879 (Rco 29), with an average of 0,825 and index diversity (DI) value varied from 0,745 (Rco 15) to 0,881 (Rco 29) with an average of 0,832. 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis.

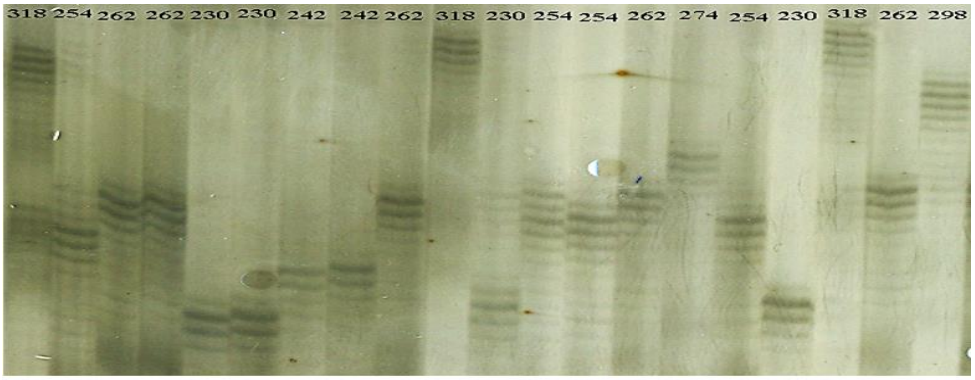

Figure 2. PCR amplification products of 20 genotypes of castor produced with SSR marker Rco 29. Lanes 1- 20 are castor genotypes

Probability of identity (PI) was low ranged from 0.002 (Rco 29 and Rco 41) to 0.018 (Rco 15) with an average of 0.007 (Table 2). For better differentiation of analysed ricin genotypes, it is necessary to use a higher number of SSR markers.

Table 2. List of SSR primers, total number of bands and the statistical characteristics of the SSR markers used in castor

Marker name	Number of alleles	DI	PIC	PI
Rco02	7	0.834	0.827	**0.007**
Rco05	9	0.862	0.860	**0.004**
Rco06	5	0.792	0.778	**0.010**
Rco08	7	0.834	0.830	**0.006**
Rco09	6	0.814	0.806	**0.007**
Rco13	8	0.839	0.835	**0.006**
Rco15	4	0.745	0.719	**0.018**
Rco18	8	0.841	0.838	**0.006**
Rco20	6	0.818	0.809	**0.008**
Rco22	7	0.832	0.826	**0.006**
Rco23	8	0.861	0.856	**0.003**
Rco26	6	0.822	0.816	**0.006**
Rco29	10	0.881	0.879	**0.002**
Rco30	5	0.774	0.758	**0.015**
Rco31	7	0.843	0.837	**0.004**
Rco33	10	0.876	0.872	**0.004**
Rco34	7	0.842	0.835	**0.005**
Rco35	8	0.871	0.867	**0.002**
Rco40	5	0.786	0.776	**0.012**
Rco41	8	0.870	0.866	**0.002**
Average	**7.05**	**0.832**	**0. 825**	**0.007**

DI- diversity index, PIC- polymorphic information content, PI- probability of identity

Table 3. Coordinates, altitudes and bioclimates of studied sites

Population	Number of plants	Latitude (N)	Longitude (E)	Altitude (m)	Bioclimate
Nefza	4	36° 58’ 36.53”	9° 03’ 51.01”	34	Humid and
Mateur	5	37° 01’ 51.80”	9° 52’ 51.67”	23	Sub-humid
Mornag	5	36° 41’ 40.78”	10° 18’ 21.19”	52	Upper and inferior
Sidi Bou Ali	5	35° 57’ 05.03”	10° 28’ 17.92”	20	semi-arid
Souassi	5	35° 20’ 20.77”	10° 32’ 24.30”	55	Upper and inferior
Bouthady	4	35° 04’ 10.43”	10° 15’ 56.32”	124	arid
Gabes	4	33° 52’ 23.25”	10° 07’ 40 04”	46	
Matmata	4	33° 32’ 25.81”	09° 57’ 95.03”	375	
Ksar Jedid	5	33° 18’ 03.93”	10° 17’ 37.84”	208	
Medenine	4	33° 21’ 40.50”	10° 28’ 59.44”	100	
Ghomrassen	5	33° 03’ 32.33”	10° 19’ 35.01”	93	
Kebili	5	33° 38’ 44.94”	8° 59’ 38.66”	45	Saharan

A dendrogram based on UPGMA analysis separated 55 Tunisian castor genotypes into 4 clusters (1, 2, 3, 4) (Figure 3). Cluster 1 contained 2 unique genotype K-4 and BA-5. Cluster 2 was divided into 2 subclustes (2a and 2b). Subcluster 2a contained 7 genotypes of castor and subluster 2b contained 4 genotypes of castor. Cluster 3 contained 6 genotypes of castor. Cluster 4 with 36 genotypes was divided into three subclusters (4a, 4b, 4c). Subcluster 4a contained 2 genotypes (GH-2 and GH-5) and in the subcluster 4b was 6 genotypes of ricinus. Subclaster 4c was the largest of all subclusters and contained 28 Tunisian castor genotypes.

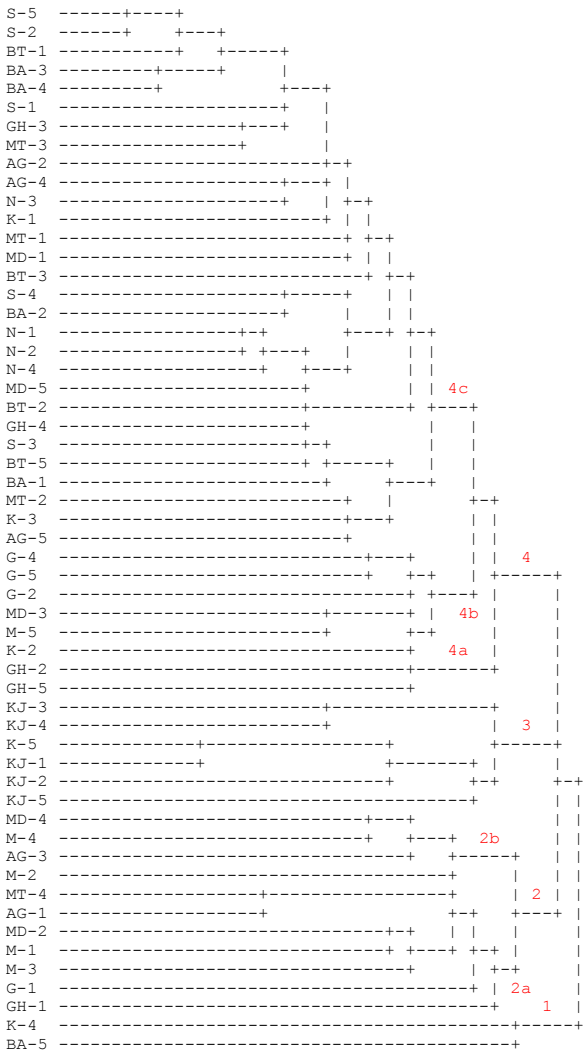

Figure 3. Dendrogram of 55 Tunisian castor genotypes based on data from 20 SSR markers

DISCUSSION

PECINA-QUINTERO *et al.* (2013) assess the diversity and genetic relationships among accessions of *R. communis* from the state of Chiapas, México using AFLP and SSR markers. In this research 53% of the AFLP markers were polymorphic. Additionally, 100% of the SSR primers were polymorphic, with an average of 5.5 alleles per locus. The SSR primer Rco23 generated a PIC value of 0.812, an SI of 1.919 and four uncommon alleles (0.05, 0.06, 0.07, 0.07), indicating that this SSR primer was particularly informative for this study. A dendrogram was generated with the markers obtained from these studies, allowing for the identification of two main groups of accessions. GÁLOVÁ *et al.* (2015) study the genetic diversity within the set of 60 ricin genotypes using 10 SSR primers. Ten SSR primers revealed a total of 67 alleles ranging from 4 to 9 alleles per locus with a mean value of 6.70 alleles per locus. The PIC values ranged from 0.719 to 0.860 with an average value of 0.813 and the DI value ranged from 0.745 to 0.862 with an average value of 0.821. Probability of identity (PI) was low ranged from 0.004 to 0.018 with an average of 0.008. A dendrogram was constructed from a genetic distance matrix based on profiles of the 10 SSR loci using the unweighted pair-group method with the arithmetic average (UPGMA). The objectives of study MACHADO *et al.* (2016) were to identify the genetic variability and estimate the level of homozygosity in a castor bean F4 population using microsatellite markers (SSR). To this end, it was performed the genotyping of the population through 53 pairs of SSR primers. Allele frequencies were estimated by number of alleles per locus, expected heterozygosity (He), observed heterozygosity (Ho) and polymorphic information content (PIC). Polymorphism was detected in a total of eight loci (15.09%) of the 53 evaluated, with the presence of two alleles per locus. Allele frequencies varied between 0.71 and 0.53, and the PIC, between 0.32 and 0.37. The average observed heterozygosity Ho (0.30) was lower than the expected heterozygosity He (0.47). In study of KIRAN *et al.* (2017), 110 simple sequence repeats (SSR) markers were employed for fingerprinting 2 popular castor varieties, 2 hybrids and their parental lines. 15 unique alleles were identified for 6 parental lines and a total of 132 alleles was observed with 56 polymorphic markers. The analysis of plant-to plant variation within the parental lines of the hybrid DCH177 and DCH519, using informative markers indicated residual heterozygosity at two marker loci. RUKHSAR *et al.* (2017) was conducted with 27 diverse castor inbreds to evaluate diversity using 13 morphological characters and 14 simple sequence repeat (SSR) markers. A set of 14 SSR primers detected 44 alleles with a mean of 3.14 alleles/locus. The polymorphic information content ranged from 0.16 (GB-RC-3) to 0.68 (GB-RC-2) witha mean of 0.43. The molecular marker based clustering revealed four distinct groups. Similar results achieved also CHAUDHARY *et al.* (2019) which study 15 castor genotypes to evaluate diversity using 14 microsatellite (SSRs) markers. In SSR analysis, 14 primers generated a total of 31 amplicons with mean number of 2.21 polymorphic amplicons per primer. The range of molecular weight of these amplicond was from 120 to 300 bp.The polymorphic information content (PIC) ranged from 0.231 (SSR-8) to 0.684 (SSR-10) with an average of 0.413. The primers SSR-7 and SSR-10 possess higher PIC value above 0.6. The highest genetic similarity was noticed between the genotypes, GEETA and 48-1 whereas; least genetic similarity was reported between the genotypes namely, JI-96 and SH-72. Clustering pattern of dendrogram generated by pooled SSR data showed two main clusters. The cluster A was the largest with eight genotypes and cluster B contained seven genotypes.

CONCLUSION

The analysis showed that the SSR markers are very effective molecular markers for the assessment of the genetic diversity in castor bean. A dendrogram based on UPGMA analysis separated 55 Tunisian castor genotypes into 4 clusters (1, 2, 3, 4). Cluster 1 contained 2 unique genotype K-4 and BA-5. Cluster 2 was divided into 2 subclustes (2a and 2b). Subcluster 2a contained 7 genotypes of castor and subluster 2b contained 4 genotypes of castor. Cluster 3 contained 6 genotypes of castor. Cluster 4 with 36 genotypes was divided into three subclusters (4a, 4b, 4c). Subcluster 4a contained 2 genotypes (GH-2 and GH-5) and in the subcluster 4b was 6 genotypes of ricinus. Subclaster 4c was the largest of all subclusters and contained 28 Tunisian castor genotypes. Our analysis proved utilization of SSR markers for differentiation of used set of castor genotypes. SSR markers are useful in the assessment of castor bean diversity, the detection of duplicate sample in genotype collection, and the selection of a core collection to enhance the efficiency of genotype management for use in castor bean breeding and conservation.

ACKNOWLEDGMENTS

This work was supported by project APVV-15-0543, VEGA project No. 1/0246/18 and KEGA project No. 025SPU-4/2018.

Received, November 11th, 2019
Accepted March 18th, 2020

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GENETIČKI DIVERZITET I ODNOSI GENOTIPOVA TUNISKOG RICINUSA (*Ricinus communis* L.) NA OSNOVU SSR MARKERA

Martin VIVODÍK*[1], Ezzeddine SAADAOUI[2], Želmíra BALÁŽOVÁ[1], Zdenka GÁLOVÁ[1]

[1]Slovački univeryitet za poljoprivredu u Nitri, Fakultet za biotehnologiju i hranu, Departman za biohemiju I biotehnologiju, Nitra, Slovačka Republika.
[2]Nacionalni Institut za istraživanje i ruralni inžinjering, vode i šumarstvo (INRGREF), Regionalna Stanica Gabès, BP 67, Gabès Manara, 6011, Tunis.

Izvod

Cilj ove studije bio je procena genetske raznolikosti unutar skupa od 55 tuniskih genotipova ricinusa koristeći 20 SSR prajmera. PCR amplifikacija DNK korišćenjem 20 prajmera za SSR analizu proizvela je 141 fragment DNK koji su se mogli oceniti u svih 55 genotipova ricinusa. Broj amplifikovanih fragmenata varirao je od 4 (Rco 15) do 10 (Rco 29 i Rco 33). Od 141 trake, sve su bile polimorfne, sa prosekom 7,05 polimorfnih traka po prajmeru. Vriednost sadržaja polimorfizma (PIC) varirala je od 0,719 (Rco 15) do 0,879 (Rco 29), sa prosekom 0,825, a vriednost indeksa diverziteta (DI) varirala je od 0,745 (Rco 15) do 0,881 (Rco 29) sa prosekom od 0.832. 100% korišćenih SSR markera imalo je vrednosti PIC i DI veće od 0,7 što znači visoki polimorfizam izabranih markera korišćenih za analizu. Verovatnoća identiteta (PI) je bila niska u rasponu od 0,002 (Rco 29 i Rco 41) do 0,018 (Rco 15), sa prosekom 0,007. Dendrogram na osnovu UPGMA analize razdvojio je 55 tuniskih genotipova ricinusa u 4 grupe (1, 2, 3, 4). Znanje o genetskoj raznolikosti ricinusa može se koristiti za buduće programe oplemenjivanja za povećanu proizvodnju ulja kako bi se zadovoljila sve veća potražnja ricinusovog ulja za industrijsku upotrebu kao i za proizvodnju biodizela.

Primljeno 11.XI.2019.
Odobreno 18. III. 2020

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