UDC 575:633.63
DOI: 10.2298/GENSR1103575N
Original scientific paper

ESTIMATION OF GENETIC VARIATION AMONG RELATED SUGAR BEET GENOTYPES BY USING RAPD

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Nagl N., K.Taški-Ajduković, A. Popović, Ž. Ćurćić, D.Danojević and L. Kovačev (2011): *Estimation of genetic variation among related sugar beet genotypes by using RAPD.* - Genetika, Vol 43, No. 3,575 -582
In marker assisted breeding programs, determination of genome polymorphism and development of suitable molecular markers is of the greatest importance. The aim of this research was development of RAPD markers, which will enable quick and cost efficient DNA polymorphism analysis among closely related sugar beet genotypes. The research was conducted on twelve sugar beet genotypes from population of closely related genotypes. Reactions with eight RAPD primers and five primer mixtures resulted in stable and reproducible bands in all samples, with 44 polymorphic and 14 monomorphic loci, and average of 6.13 bands per primer. In two-primer reactions nine new polymorphic bands were detected.

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Polymorphism information content (PIC) for each primer was calculated, while genetic variation was estimated by calculation of the number of polymorphic loci and their percentage, observed number of alleles, effective number of alleles, and Nei's gene diversity. An unweighted pair group arithmetic mean method (UPGMA) cluster analysis showed that samples were divided in two groups with relatively high coefficient of similarity. The presented results showed that RAPD markers can be suitable for genetic diversity analysis in breeding material with high levels of homology and homozygosity.

Key words: *Beta vulgaris* L., genetic variation, RAPD

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is a crop of significant economic importance and it accounts for about 25% of worldwide sugar production (DRAYCOTT, 2006). The basic requirement of every improvement program is presence of genetic variation in the investigated germplasm. Therefore, the assessment of genetic diversity is essential for the efficient organization and use of breeding material (FERNIE *et al*., 2006). Sugar beet breeding is increasingly supported by use of DNA markers, who reflect the actual level of genetic variation existing among genotypes and therefore provide much more accurate estimate than phenotypic and pedigree information.

The random amplified polymorphic DNA (RAPD) (WILLIAMS *et al*., 1990) marker system has been used in many different applications in sugar beet breeding and genetic research, such as: detection of DNA sequence polymorphism (LORENZ *et al.*, 1994, NAGL *et al.*, 2008), analysis of genetic relationships (SHEN *et al*., 1998), construction of linkage maps (UPHOFF and WRICKE, 1995) and identification of markers linked to genes of interest (PELSY and MERDINOGLU, 1996, HALLDEN *et al.*, 1997, HAGIHARA *et al.*, 2005). Despite a few drawbacks, such as their dominant nature and poor reproducibility, RAPD markers allow an inexpensive and rapid analysis of the polymorphisms in many individuals with good coverage of the entire genome (NAGL *et al.*, 2011).

RAPD analysis normally employs single-primer PCR to amplify a set of random DNA fragments. In principle, however, there is no limit to the number of primers that can be used simultaneously in a given RAPD reaction. If two primers are used, it is expected that some DNA fragments will be generated by one primer only, while others will be generated by combination of two different primers. The major advantage of using two-primer mixtures is, of course, development of many markers at very low cost in very short time. Theoretically, half of the amplification products can be expected to have the same primer at the both ends, while the other half should have different primers. In practice, however, the increase in number of PCR products is lower (SAAL *et al*., 2000, MILADINOVIC *et al*., 2011), mostly due to competition for priming sites in the genome (WILLIAMS *et al.*, 1993), which depends on complementarity between primers and target sites. Amplification is probably initiated in many sites, but only a subset of all possible products is detected as visible

bands after amplification. The degree of competition in part determines the copy number of amplified fragments in the final products (HALLDEN *et al*., 1996).

The aim of this research was development of fast and inexpensive RAPD marker protocols with one-primer and two-primer mixtures, as method of choice for use in preliminary testing of genetic variability in sugar beet germplasm.

MATERIALS AND METHODS

From F2 population made by crossing two closely related monogerm sugar beet genotypes, twelve genotypes were taken for DNA polymorphism analysis. Total genomic DNA was isolated from leaves according to the protocol of SOMMA (2004).

In RAPD analysis were used eight previously used decamer primers and five primer mixtures (NAGL *et al.*, 2008), who were selected for their polymorphism and stabile expression of amplification products (Table 1). PCR was carried out in a 25-µl reaction volume containing 2.5 µl buffer; 0.2 mM of each dNTP; 0.5 µM of primer; 2 units of *Taq* polymerase (Fermentas) and 30 ng of DNA. In two-primer reactions 0.25 µM of each primer was used. Reactions were performed in Tpersonal PCR (Biometra) termocycler with the amplification profile: denaturation at 94ºC for 4 min, followed by 40 cycles with 94ºC for 2 min, 36ºC for 1 min and 72ºC for 2 min, with final elongation on 72ºC for 10 min. PCR products were separated on 1.2% or 1.7% agarose gels containing 0.005% ethidium bromide and visualized under UV light.

Each amplified fragment was treated as binary unit character and scored “0” for absence and “1” for presence. In order to measure informativeness of the markers, the polymorphism information content (PIC) for each primer was calculated (SMITH *et al*., 1997). PIC provides an estimate of discriminatory power of a marker by taking into account not only the number of alleles at a locus, but also their relative frequencies. Estimation of genetic variation was carried out by using the POPGENE software package version 1.32 (YEH *et al*., 1997) for calculation of the following parameters: number of polymorphic loci and their percentage, observed number of alleles (number of alleles with nonzero frequency), effective number of alleles (estimates the reciprocal of homozygosity) and Nei’s gene diversity (based on allelic frequencies).

An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed, based on Jaccard's coefficient of similarity, as available in NTSYSpc software package version 2.11a (ROHLF, 2000). Dendrogram was drawn using SAHN clustering method and generated by using TREE display option.

RESULTS AND DISCUSSION

All tested RAPD primers and primer mixtures generated 44 polymorhic, stable and reproducible bands in all samples (example Fig. 1), ranging from 250 to 2000 bp, with average number of bands per primer of 6.13 (Tab. 1). The highest number of bands was achieved with primer A08 (12 bands), while the amplifications with primer A09 resulted in only one band. The most informative primer was A04 with PIC value of 0.391. Although primer A09 had only one monomorphic band, and

PIC value 0, it was used for further analysis due to its previously shown low competitiveness and ability to generate bands in reactions with primer mixtures. In two-primer reactions nine new polymorphic bands were amplified, their size varying in size from 100bp to 1100bp. Since only selected primer combinations were investigated their informativness was higher than that of most single primers, with an average number of amplification products 62% higher than in single-primer RAPD. The obtained results are in agreement with our previous investigations (NAGL *et al.*, 2008), and results of HU *et al*. (1995) and SAAL *et al*. (2000), where two-primer reactions amplified approximately 40% more bands than single-primer did. It should also be mentioned that these primers had an excellent performance in our previous investigation (NAGL *et al.*, 2007) on different sugar beet breeding material. This confirms the idea of HANSEN *et al.* (1998) that if there is a defined set of primers associated with high numbers of polymorphisms, their use in RAPD reactions will result with high number of products regardless of plant DNA source and without any prior screening or reaction optimization.

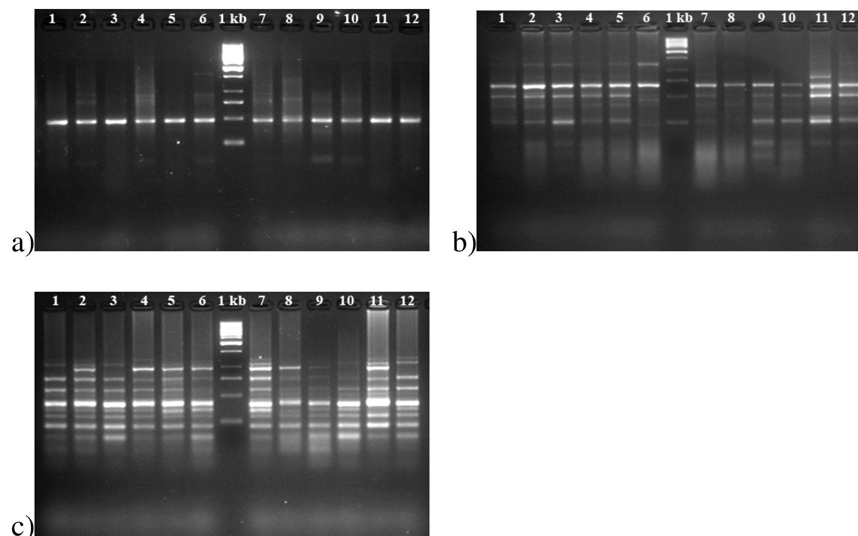

Figure 1. RAPD profile obtained after reaction with primer A09 (a), A20 (b) and primer mixture A09/20 (c). 1kb – GeneRuler 1kb DNA Ladder (Fermentas)

The multiplication products were scored and used for estimation of genetic variation among selected sugar beet genotypes, which is presented in Table 2. The number of polymorphic loci was 44, i.e. 75.86% of all scored loci. The value of Nei's gene diversity index was 0.252, which agrees with gene diversity values estimated by dominant markers in monogerm sugar beet germplasm (MCGRATH *et al.*, 1999), or within beet accessions (POULSEN *et al.*, 2007) . These values are lower than reported by KRAFT *et al.* (1997) and LI *et al.* (2010), but the difference can be explained by the fact that in both cited investigations were used codominant markers.

Table 1. Primers used for RAPD analysis and reaction products

Primer	Sequence (5'-3')	Max. no. of bands	Band size range (bp)	PIC
A04	AATCGGGCTG	4	300-2000	0.391
A05	AGGGGTCTTG	6	300-950	0.212
A07	GAAACGGGTG	7	500	0.372
A08	GTGACGTAGG	12	200-1500	0.249
A09	GGGTAACGCC	1	200-1200	0.0
A14	TCTGTGCTGG	5	350-1000	0.133
A19	CAAACGTCGG	5	250-1300	0.198
A20	GTTGCGATGC	8	450-1800	0.270
Primer mixture		No. of new bands	Band size (bp)	
	A07/A08	1	1100	0.219
	A07/A09	2	270, 300	0.302
	A08/A20	1	100	0.264
	A09/A19	1	240	0.300
	A09/A20	4	150, 240, 250, 270	0.317

Table 2. Estimates of genetic variation among sugar beet genotypes using RAPD markers

	P (no.)	P (%)	Na	Ne	He
Mean	44	75.86	1.759	1.428	0.252
St. deviation			0.432	0.367	0.192

P (no) – number of polymorphic loci, P (%) – percentage of polymorphic loci, Na - observed number of alleles, Ne - effective number of alleles, He – Nei's gene diversity

UPGMA dendrogram was drawn to visualize relationships among sugar beet genotypes (Fig.2). Two major groups were formed, first with eight genotypes and second with four. The first group comprised of two sub-groups, one with six genotypes (1, 3, 8, 6, 4, 5) and the other with two genotypes (11, 12), with coefficients of similarity within over 0.70. The second group included sub-group with three genotypes (2, 7 10) to which genotype 9 was attached.

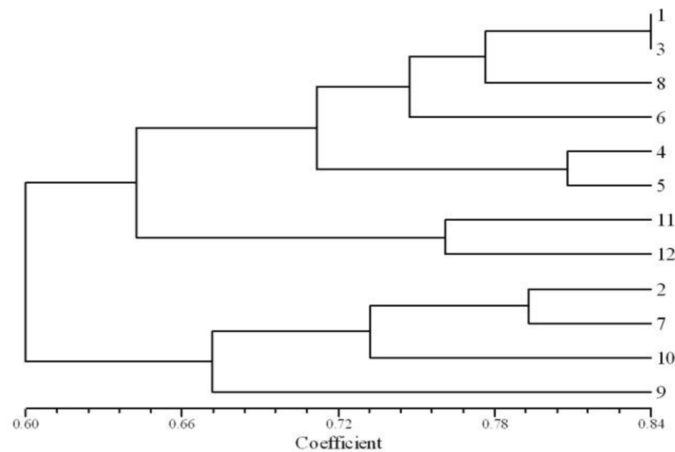

Figure 2. Dendrogram for cluster analysis of sugar beet genotypes based on Jaccard's similarity coefficient

CONCLUSION

RAPD markers were able to detect polymorphism between all tested genotypes which showed them to be marker system suitable for genetic diversity analysis in breeding material with high levels of homology and homozygosity.

Increase in informativness and average number of amplification products in most two-primer reactions, presents simple improvement of RAPD analysis, generating more markers at low cost. It enables development of two-primer reaction protocols, where selected primer combinations could be used in genetic variation analysis giving both, PCR products obtained with single primers as well as heteroduplex bands.

ACKNOWLEDGEMENTS

This study was funded by Ministry of science and technological development, Serbia, under the project TR31015.

Received, July25rd2 011

Accepted, November 17th 2011

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OCENA GENETSKE VARIJABILNOSTI IZMEĐU SRODNIH GENOTIPOVA ŠEĆERNE REPE POMOĆU RAPD MARKERA

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I z v o d

U programima oplemenjivanja uz pomoć molekularnih markera, određivanje polimorfizma genoma i razvoj odgovarajućih markerskih sistema je od najveće važnosti. Cilj ovog rada je bio razvoj RAPD marker sitema sa ciljem njegove primene u brzoj i jeftinog analizi DNK polimorfizma u populacijama srodnih genotipova šećerne repe. Predstavljena istraživanja su sprovedena na dvanaest srodnih genotipova šećerne repe. Kao rezultat reakcija sa osam RAPD prajmera i pet mešavina prajmera dobijeni su stabilni i ponovljivi produkti reakcija kod svih genotipova, sa 44 polimorfna i 14 monomorfnih lokusa, odnosno prosečno 6.13 traka po prajmeru. U reakcijama sa dva prajmera detektovano je devet novih polimorfnih traka. Informativnost prajmera i prajmerskih mešavina je određena pomoću PIC (polymorphism information content) vrednosti, dok je genetska varijabilnosti procenjena određivanjem broja polimorfnih lokusa i njihovim procentom, uočenim brojem alela, efektivnim brojem alela, i genetskog diverziteta po Nei –u. UPGMA (unweighted pair group arithmetic mean method) klaster analiza je pokazala da su uzorci bili grupisani u dve glaven podgrupe sa značajnim koeficijentom sličnosti unutar njih. Predstavljeni rezultati pokazuju da RAPD markeri mogu omogućiti analizu genetske divergentnosti u oplemenjivakom materijalu kod koga se očekuje povećana homologija i stepen homozigotnosti. '

Primljeno 27. VII. 2011.
Odobreno 17. XI. 2011.