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Original scientific paper

EVALUATION OF RAPD MARKERS AS A MARKER-ASSISTED SELECTION TOOL FOR VARIETY TYPE AND ERUCIC ACID CONTENT IN RAPESEED

Dragana MILADINOVIĆ^{1*}, Marko MILER², Ana MARJANOVIĆ JEROMELA¹, Ivana IMEROVSKI¹, Aleksandra DIMITRIJEVIĆ¹, Branislav KOVAČEVIĆ³, Siniša JOCIĆ¹, Sandra CVEJIĆ¹, Nada HLADNI¹, Dragana OBREHT-VIDAKOVIĆ⁴

¹Institute of Field and Vegetable Crops, Novi Sad, Serbia

²Department of Cytology, Institute for Biological Research “Siniša Stanković“, University of Belgrade, Belgrade, Serbia

³Institute for Lowland Forestry and Environment, University of Novi Sad, Novi Sad, Serbia

⁴Department of Forest and Conservation Sciences, Faculty of Forestry, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

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Random amplification of polymorphic DNA (RAPD) analysis was performed on twelve rapeseed genotypes from Institute of Field and Vegetable Crops, Novi Sad, Serbia, genepool in order to identify markers that could be used in marker assisted selection (MAS) for different growing type and selection of the varieties with low or zero level of erucic acid. Out of fifteen RAPD markers, three were monomorphic, whereas twelve had polymorphic profiles. Three primers amplified specific fragments in spring varieties. UBC 25 and UBC 191 amplified the fragments of 450 and 750 bp, respectively, in all tested spring varieties, except in JR-NS-36. Primer UBC 72 generated a fragment of 700 bp that was present in all spring varieties. These fragments were not present in any of winter varieties. None of the tested RAPD primers amplified fragment(s) uniquely present either in varieties with or without (0%) erucic acid or with different erucic acid content. Cluster analysis showed a concordance between the position of varieties in the cluster and their pedigree information, but also enabled separation of spring and winter varieties. Contingency analysis confirmed that fragment

Corresponding author: Dragana Miladinović, Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad; E-mail: dragana.miladinovic@ifvcns.ns.ac.rs; Phone: 0214898421

UBC 72_700 is specific for spring varieties, while for erucic acid content, only moderate association was found with UBC 137_750.

Keywords: *Brassica napus*, cluster analysis, contingency analysis, molecular markers, erucic acid.

INTRODUCTION

Rapeseed (*Brassica napus* L.) is today the world's third-leading source of both vegetable oil and oil extraction meal (FRIEDT and SNOWDON, 2009). The diversity of forms and varieties enables its growing in virtually all climatic conditions, mostly for oil which is extracted from seeds (MARJANOVIĆ JEROMELA *et al.*, 2016). The difference between winter and spring rapeseed varieties is in the genetic mechanism that controls vernalization and the beginning of flowering (HASAN *et al.*, 2006). The spring varieties do not require vernalization and are not resistant to cold, so they are sown in the spring and development of the stem begins immediately after the germination. Winter varieties, on the other hand, are sown in the fall and survive winter conditions as leafy rosettes on the soil surface (SNOWDON *et al.*, 2007). Winter varieties usually have higher yields than spring ones, but they can be grown profitably only in the areas where they can survive in winter conditions.

Significant improvement of quality and production of rapeseed has been achieved with the creation and cultivation of varieties with low erucic acid and glucosinolates in seed oil (MARJANOVIĆ JEROMELA *et al.*, 2007). Earlier varieties of rapeseed contained up to 50% erucic acid, which is harmful to health, and has no nutritional value. Today's varieties obtained by selection have significantly lower or minimal amounts of erucic acid (MARJANOVIĆ JEROMELA *et al.*, 2016).

Up to date, different markers were used for analysing rapeseed genome, including restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR), sequence-related amplified polymorphism (SRAP), cleaved amplified polymorphic sequence (CAPS) and simple sequence repeats (SSR) (MILADINOVIĆ *et al.*, 2014 a). The usefulness of RAPD markers in the evaluation of genetic diversity in the genus *Brassica* (YU *et al.*, 2005; MARJANOVIĆ JEROMELA *et al.*, 2009), as well for discrimination between winter and spring varieties (SHIRAN *et al.*, 2006; MOGHADDAM *et al.*, 2009) has been confirmed in several studies. They have also been used for constructing the genetic linkage map in *B. napus* (FOISSET *et al.*, 1996; LOMBARD and DELOURME, 2001) and for locating QTLs for erucic acid content (XUE-PING *et al.*, 2005). JOURDEN *et al.* (1996) and RAJCAN *et al.* (1999) used RAPD analysis for detection of molecular markers associated with erucic acid levels in rapeseed.

The aim of our study was to detect differences between winter and spring rapeseed varieties from Institute of Field and Vegetable Crops, Novi Sad, Serbia germplasm using RAPD markers and to identify markers that could be used in marker assisted selection (MAS) oriented towards production of rapeseed varieties with low or zero level of erucic acid.

MATERIALS AND METHODS

Plant material

To test the value of RAPD markers in the detection of different growth type's two sets of varieties were used: six winter and six spring rapeseed varieties (Table 1). The winter varieties

differed in erucic acid content and were used for identification of markers for this trait. The winter varieties had different erucic acid content.

Table 1. Types and erucic acid content of tested varieties

Variety	Type	Erucic acid content (%)
Mira	Spring	-
Jovana	Spring	-
JR-NS-6	Spring	-
JR-NS-26	Spring	-
JR-NS-28	Spring	-
JR-NS-36	Spring	-
NS-L-31	Winter	0
NS-L-128	Winter	0.25
NS-L-134	Winter	9.05
NS-L-136	Winter	0.94
NS-L-137	Winter	0
Banaćanka	Winter	0.16

DNA extraction and polymerase chain reaction (PCR) assay

Bulk samples of leaves harvested from ten plants of each variety were collected. DNA extraction was performed according to modified cetyltrimethyl ammonium bromide (CTAB) method (PERMINGEAT *et al.*, 1998). DNA polymorphism was analyzed by 15 randomly-chosen RAPD primers (Table 2).

Table 2. Sequences of primers

Primer	Sequence
UBC 5	5'-CCT GGG TTC C-3'
UBC 13	5'-CCT GGG TGG A-3'
UBC 25	5'-ACA GGG CTC A-3'
UBC 33	5'-CCG GCT GGA A-3'
UBC 72	5'-GAG CAC GGG A-3'
UBC 126	5'-CTT TCG TGC T-3'
UBC 137	5'-GGT CTC TCC C-3'
UBC 146	5'-ATG TGT TGC G-3'
UBC 159	5'-GAG CCC GTA G-3'
UBC 168	5'-CTA GAT GTG C-3'
UBC 191	5'-CGA TGG CTT T-3'
UBC 219	5'-GTG ACC TCA G-3'
UBC 225	5'-CGA CTC ACA G-3'
UBC 268	5'-AGG CCG CTT A-3'
UBC 513	5'-ACG GCA GTG G-3'

PCR was performed in the Mastercycler Gradient (Eppendorf, Germany), in 15 µl of reaction mixture that contained 1xPCR buffer, 1.5 mM MgCl₂, 0.4 mM of dNTPs, 0.5 µM of primer, 1 U of DNA polymerase, 2.5 µg BSA and 30 ng of genomic DNA. Thermocycling conditions were as follows: an initial denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 2 min, then 1 min at 36°C and 2 min at 72°C. The final extension was carried out for 10 min at 72°C. All PCR products were evaluated by electrophoresis on 2% metaphor agarose gels using 1 × TBE buffer. The gels were stained with ethidium bromide 10 mg ml⁻¹ and visualized with the BIO-Print system (Vilber Lourmat, France). Fragment size was evaluated with program BIO-CAPT V.97 (Vilber Lourmat, France).

Data analysis

Each fragment that was amplified using RAPD primers was treated as binary unit character and scored “0” for absence and “1” for presence. An un-weighted pair group arithmetic means method (UPGMA) cluster analysis was done to generate a dendrogram, using complete linkage method. Polymorphic information content (PIC) value was calculated following KUMAR *et al.* (2014). Association between RAPD markers and traits was measured by contingency coefficient. The statistical significance of the association was evaluated by independence test. Statistical analysis was carried out using Statistica 10 (StatSoft 2010).

RESULTS AND DISCUSSION

Out of 15 RAPD markers, three were monomorphic (UBC 33, UBC 168 and UBC 513), whereas twelve had polymorphic profiles. The primers produced DNA fragments that ranged from 100 to 2500 bp (Table 3).

Table 3. Characteristics of amplified DNA profiles and polymorphic information content (PIC) values

Primer	Number of bands	Band size range (bp)	PIC*
UBC 5	5	750-2000	0.095
UBC 13	5	600-2000	0.375
UBC 25	8	450-2020	0.413
UBC 33	5	330-1500	monomorphic
UBC 72	3	650-1050	0.375
UBC 126	9	600-2500	0.467
UBC 137	7	500-1500	0.200
UBC 146	7	550-2000	0.408
UBC 159	7	375-1200	0.362
UBC 168	5	300-1000	monomorphic
UBC 191	4	400-1000	0.478
UBC 219	6	380-1700	0.314
UBC 225	5	625-1250	0.255
UBC 268	13	250-2500	0.355
UBC 513	5	375-1500	monomorphic

*Polymorphic information content

For each polymorphic RAPD marker PIC value was calculated (Table 3). PIC values ranged from 0.095 to 0.478, with an average of 0.341. Four out of twelve markers were highly informative (PIC >0.40), whereas all others, except UBC 5, were moderately informative (0.20 < PIC < 0.40).

RAPD profiles of spring and winter varieties

Three primers made clear and visible bands and amplified specific fragments in spring varieties. UBC 25 amplified a fragment of 450 bp in all tested spring varieties, except in JR-NS-36. This fragment was not present in any of winter varieties. The same stands for 750-bp fragment obtained with UBC 191. This is in agreement with the results of SHIRAN *et al.* (2006) and ASGHARI *et al.* (2011), who also found that RAPD markers were able to separate spring and winter type rapeseed cultivars.

Similar to results of MOGHADDAM *et al.* (2009), who found a RAPD marker that was only present in the spring rapeseed cultivars, in our work primer UBC 72 generated a fragment of 700 bp that was uniquely present in spring varieties (Figure 1). The next step in the validation of the marker UBC 72_700 as a candidate for MAS would be the examination of its amplification stability and repeatability on a larger sample of winter and spring cultivars and further conversion into SCAR marker with improved specificity and stability (YANG *et al.*, 2013; CHENG *et al.*, 2015). Besides that, this procedure enables conversion of a dominant marker (RAPD) into co-dominant marker (SCAR), making it even more breeder friendly (SPOONER *et al.*, 2005).

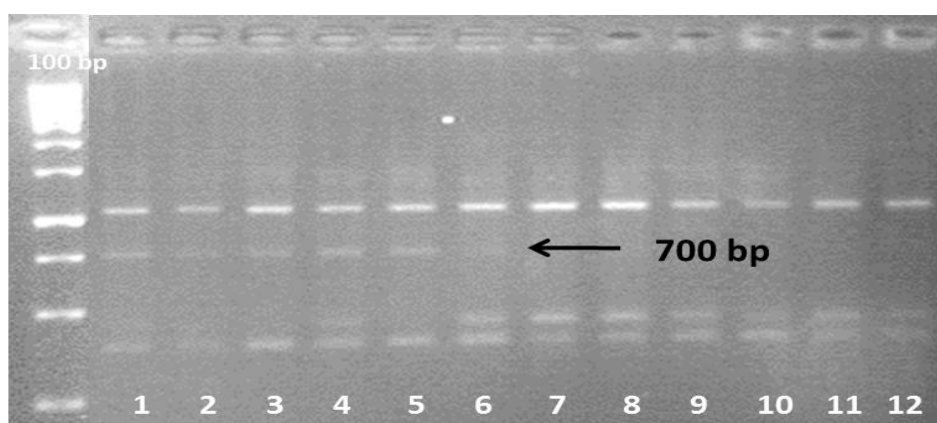


Figure 1. RAPD profiles of the primer UBC 72 (1–6 – spring varieties as in Table 1; 7–12 – winter varieties as in Table 1)

RAPD profiles of varieties with different erucic acid content

JOURDREN *et al.* (1997) and RAJCAN *et al.* (1999) identified RAPD markers associated with erucic acid level that could be used for selecting low or high erucic acid lines. In our work, none of the tested RAPD primers amplified fragment(s) uniquely present either in varieties with or without (0%) erucic acid or with different erucic acid content. According to AMAR *et al.* (2008) two major QTLs that are involved in biosynthesis of erucic acid and that are responsible for the acid content in winter oilseed rape cultivars are located on the linkage groups N8 and

N13. According to DEY *et al.* (2006), the main reason for the discrepancy between the grouping based on the RAPD markers and QTLs is that QTLs are usually controlled by several genes (polygenic). That is why, it is necessary to test more RAPD markers to identify those which are able to detect the variations in erucic acid content (XUE-PING *et al.*, 2005).

Cluster analysis

RAPD analysis combined with cluster analysis was found to be an adequate method for genetic characterization and grouping of many plant species, including rapeseed (FAHMI *et al.*, 2012) and these markers can also be useful in taxonomic studies (DIMITRIJEVIĆ *et al.*, 2014). Our results of cluster analysis showed a concordance between the position of varieties in the cluster and their pedigree information, but also enabled separation of spring and winter varieties (Figure 2). All tested varieties except JR-NS-6, have been grouped into one cluster (cluster A), while the NS-JR-6 formed a separate cluster (cluster B). Cluster A branched into two groups (C and D). Inside the group C, spring varieties with a common parent Mira and Jovana formed a separate cluster (C1), while JR-NS-26 and NS-JR-28, which also share one parent, grouped together (C2). Within the group D, spring variety JR-NS-36 formed a separate cluster (D1). As observed by other authors (SHIRAN *et al.*, 2006; MOGHADDAM *et al.*, 2009), RAPD markers produced a dendrogram that separated winter and spring varieties. Winter varieties grouped together (D2), with NS-L-31, NS-L-136, NS-L-128 and NS-L-134 forming one sub-cluster (D2.1) and NS-L-137 and Banačanka, the varieties without erucic acid and with a common parent, forming other one (D2.2).

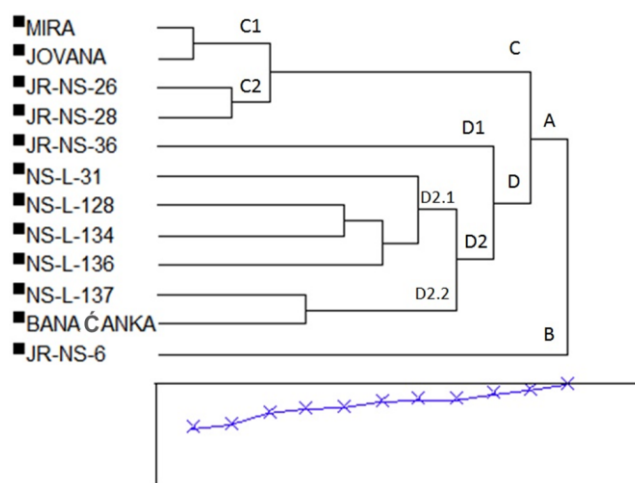


Figure 2. UPGMA dendrogram based on RAPD data of the tested varieties

Contingency analysis

In order to check and statistically confirm if there was significant association of specific fragments with variety type and/or erucic acid content, contingency coefficient test was done. This test was also used by HASSAN *et al.* (2011) and IMEROVSKI *et al.* (2013) for identifying genetic markers for *Orobanche* resistance in sunflower, as well as by MILADINOVIĆ *et al.* (2014 b) for association of tolerance to *Sclerotinia sclerotiorum* (Lib. de Bary) in wild sunflower populations. Contingency test was also used for genomic analysis of regions involved in rapeseed resistance to stem canker (FOPA FOMEJU *et al.*, 2015). As in the work of MOGHADDAM *et al.* (2009), a significant marker-trait association was established for one RAPD marker and variety type. Fragment UBC 72_700 was found to be specific for spring varieties (Table 4). For erucic acid content, only moderate association was found with UBC 137_750. This is in agreement with the results of other authors who found no association between the phenotypic and morphological traits and the RAPD markers (BURLACU *et al.*, 2011; FAHMI *et al.*, 2012; PAULAUSKAS *et al.*, 2013).

Table 4. Association of fragments with the variety type and/or presence or absence of erucic acid in all tested varieties expressed by contingency coefficients

Primer	Trait		
	Fragments*	Spring / winter	Erucic acid (0% / >0%)
UBC 5	UBC 5_1800	0.500	-
UBC 25	UBC 25_450	0.645	-
UBC 72	UBC 72_650	0.577	-
	UBC 72_700	0.707	-
UBC 137	UBC 137_500	0.500	-
	UBC 137_750	-	0.577
UBC 146	UBC 146_600	0.500	-
UBC 159	UBC 159_1200	0.577	-
UBC 191	UBC 191_750	0.645	-
UBC 225	UBC 225_780	0.577	-
	UBC 225_1000	0.577	-

* – only alleles that showed significant association with variety type and/or presence or absence of erucic acid are presented in the table

CONCLUSIONS

Random amplification of polymorphic DNA (RAPD) analysis has detected differences between winter and spring rapeseed varieties. From the aspect of breeding, the most significant is the fragment UBC 72_700, which was found to be specific for spring varieties. We did not identify markers that could be used for screening the presence of erucic acid. Selected marker UBC 72_700 will be further assessed on a larger number of spring and winter varieties. Subsequent studies will be devoted to the development of sequence characterized amplified region (SCAR) markers specific for this trait.

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OCENJIVANJE MOGUĆNOSTI KORIŠĆENJA RAPD MARKERA ZA DETEKCIJU OZIMIH I JARIH SORTI I SADRŽAJA ERUKA KISELINE KOD ULJANE REPICE

Dragana MILADINOVIĆ^{1*}, Marko MILER², Ana MARJANOVIĆ JEROMELA¹,
Ivana IMEROVSKI¹, Aleksandra DIMITRIJEVIĆ¹, Branislav KOVAČEVIĆ³, Siniša JOCIC¹,
Sandra CVEJIĆ¹, Nada HLADNI¹, Dragana OBREHT-VIDAKOVIĆ⁴

¹Institut za ratarstvo i povrtarstvo, Novi Sad, Srbija

²Department za citologiju, Institut za biološka istraživanja "Siniša Stanković", Univerzitet u Beogradu, , Beograd, Srbija

³Institut za nizijsko šumarstvo i životnu sredinu, Univerzitet u Novom Sadu, Novi Sad, Srbija

⁴Department of Forest and Conservation Sciences, Faculty of Forestry, University of British Columbia, Vancouver, BC V6T 1Z4, Kanada

Izvod

Urađena je RAPD analiza dvanaest genotipova uljane repice stvorenih u Institutu za ratarstvo i povrtarstvo, Novi Sad, Srbija, kako bi se identifikovali markeri koji bi mogli biti korišćeni za marker asistiranu selekciju ozimih i jarih genotipova, kao i sorti sa niskim ili nultim nivoom eruka kiseline. Od petnaest ispitivanih RAPD markera, tri su bila monomorfna, dok je dvanaest dalo polimorfne profile. Tri prajmera su umnožila fragmente specifične za jare sorte. UBC 25 i UBC 191 su dali fragmente od 450, odnosno 750 bp, u svim testiranim jarim sortama osim u JR-NS-36. Prajmer UBC 72 je generisao fragment od 700 bp koji je bio prisutan u svim jarim sortama. Ovi fragmenti nisu bili prisutni ni u jednoj od ozimih sorti. Nijedan od testiranih RAPD prajmera nije umnožio fragmente karakteristične za sorte sa ili bez (0%) eruka kiseline ili sa različitim sadržajem eruka kiseline. Klaster analiza pokazala je postojanje veze između položaja sorti u klasteru i njihovog porekla, ali i omogućila razdvajanje ozimih i jarih sorti. Analiza kontingencije je potvrdila da je fragment UBC 72_700 specifičan za jare sorte, dok je pronađena samo umerena veza UBC 137_750 i sadržaja eruka kiseline.

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