

Full Length Research Paper

Assessment of genetic diversity in rapeseed cultivars as revealed by RAPD and microsatellite markers

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Microsatellite and RAPD markers were used to detect the genetic variability among 32 rapeseed cultivars. RAPD analysis using 38 primers produced a total of 250 polymorphic bands and microsatellite assay by 10 primer pairs resulted in 143 alleles. A range of 0.60 to 0.91 was observed for polymorphic information content (PIC) of microsatellite markers. A marker related to the RAPD primer 430 was only revealed in the spring rapeseed cultivars. Na12-C01, a microsatellite marker amplifying two different genomic regions in the *Brassica* genome was also observed in the spring cultivars in one of the regions. For each marker, rapeseed cultivars were grouped by cluster analysis using bootstrapping. Combining RAPD and microsatellite markers produced a dendrogram that separated winter and spring cultivars. SWC High Level, SWC3162 and SWC3497 cultivars from Sweden appeared together and distant from other cultivars.

Key words: *Brassica napus*, genetic diversity, markers, microsatellite, RAPD.

INTRODUCTION

The success in breeding programs of a crop species largely relies on the presence of genetic diversity in the germplasm and knowledge about the characteristics of the genotypes and their genetic relationship. Therefore, the methods that evaluate and identify the genotypes more precisely during the growing season, especially at early stages, are preferred by plant breeders (Major, 2002).

Genetic markers are used as a source of genetic information for analysis of genetic diversity in crops and indirect selection of characters for which phenotypic markers are affected by environmental conditions, stage of plant development and lack of precise evaluation methods (Kumar, 1999). The advancements in genetics and cellular biology have resulted in the development of reliable and powerful DNA markers such as techniques based on polymerase chain reaction (PCR) for studying genetic differences in crop species (Mohammadi, 2002).

One of the early PCR-based techniques is random am-

plified polymorphic DNA (RAPD) (Williams et al., 1990). Although this method has low repeatability, but because of its simplicity and speed, it has been used extensively for variety identification, determination of genetic variability, relationship among the crop genotypes and construction of linkage maps (Young, 2000; Jaroslava et al., 2002). Microsatellite or simple sequence repeat (SSR) is another PCR-based marker which is preferred by many geneticists and plant breeders because of higher repeatability, codominant nature, specificity and having multiple alleles (Plieske and Struss, 2001; Halton et al., 2002).

Several researchers have used successfully DNA markers such as RAPD (Ashik Rabbani et al., 1998; Lazaro and Aguinagalde, 1998; Divaret et al., 1999; Vonarx et al., 1999; Xian-Hui et al., 1999; Kimura et al., 2000; Yuan et al., 2004), amplified fragment length polymorphism (AFLP) (Sandip et al., 1999; Lombard et al., 2000; Seyis et al., 2003; Jiang et al., 2007) and SSR (Charters et al., 1996; Szewc et al., 1996; Uzunova and Ecke, 1999; Carolyn et al., 2000; Rudolph et al., 2002; Chao-Zhi et al., 2003) to study genetic diversity in *Brassica* crops.

Kimura et al. (2000) analyzed genetic diversity in a

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collection of 50 *Brassica napus* varieties from China, Japan, Korea, Canada and European countries using RAPD markers. Cluster analysis and principal component analysis based on 181 polymorphic bands resulted from 21 random primers assigned most of the Japanese varieties in one group. The Japanese and Chinese varieties due to similar genetic background were separated from European genotypes. Among the European genotypes, winter types were separated from spring cultivars. According to Yuan et al. (2004) RAPD markers were sensitive enough to reveal the gradual decline of genetic variation with the advance of recurrent selection cycles (C0 to C3) in *B. napus*.

Lombard et al. (2000) studied the genetic differences among 83 spring and winter varieties of rapeseed by AFLP markers. Using 17 primer pairs, 324 markers were identified. Cluster analysis, PCA and analysis of molecular variance distinguished winter from spring types, countries of origin and seed companies. Charters et al. (1996) showed that microsatellite markers are able to discriminate spring from winter types, high from low glucosinolate genotypes and also the origins in oilseed rape. The objective of this investigation was to use SSR and RAPD markers to study genetic variability and relationships among 32 rapeseed cultivars.

MATERIALS AND METHODS

Plant materials

Thirty-two rapeseed cultivars (20 winter and 12 spring type) from Australia, Canada, Germany, Denmark and Sweden were used in this study. A number of seeds from each cultivar were planted in a pot. At the sixth leaf stage, young leaves from each cultivar were harvested for DNA extraction.

DNA extraction

Genomic DNA for each genotype was isolated using the protocol of Saghai-Marooof et al. (1984). The quality and quantity of DNA were determined using agarose gel (0.8 %) electrophoresis and spectrophotometer.

DNA amplification

A final set of 38 RAPD primers and 10 SSR primer pairs selected based on their polymorphism and repeatability were used to evaluate the rapeseed cultivars. RAPD reaction was undertaken by the procedure outlined by Williams et al. (1990) and that of SSR by CIMMYT (2004) protocol. The total reaction volume for DNA amplification was 20 and 10 μ l for RAPD and SSR analyses, respectively.

Data analysis

Banding patterns were scored as binary code of presence (1) and absence (0). For SSR markers, polymorphic information content (PIC), observed heterozygosity and gene diversity were calculated by power marker software (Liu and Muse, 2005). Cluster analysis

was performed based on Neighbor-Joining algorithm using Number of Differences coefficient. The validity of grouping was assessed based on 1000 bootstraps using MEGA 3.0 software (Kumar et al., 2004). The association between marker data and character values measured on the studied genotypes was assessed using stepwise multiple regression analysis.

RESULTS AND DISCUSSION

From 200 RAPD primers only 38 (19%) were polymorphic and showed clear banding pattern. Percentage of polymorphic primers in rapeseed reported in other studies was 76% (Hollden et al., 1994), 14% (Roman et al., 2004) and 6% (Mailer et al., 1997). The 38 RAPD primers produced 250 polymorphic bands in the 32 rapeseed genotypes under study (average of 6.57 bands per reaction). Number of bands ranged from 3 (Primers 407, 427, 431, 486, 515 and 621) to 14 (Primer 516). Size of amplified DNA fragments was in the range of 764 to 3530 bp. Song et al. (2000) stated that larger fragments are more repeatable than smaller fragments.

Average number of bands reported in the literature were 3 (Ishida et al., 2000), 3.36 (Roman et al., 2004), 5.54 (Shiran et al., 2004), 8.6 (Kimura et al., 2000) and 9.6 (Sandip et al., 1999). Higher numbers of bands for each primer indicates the existence of larger genetic diversity among the genotypes under investigation (Agrama and Tuinstra, 2003). Primers with higher polymorphic bands are more efficient in studying genetic diversity and discrimination of the genotypes (Pradhan et al., 2004; Roman et al., 2004).

Among the RAPD primers, primer 430 had a marker that was specific to spring rapeseed cultivars (Figure 1). This marker could be used, therefore, to differentiate spring type genotypes from those of winter types. However, due to low reproducibility of RAPD markers, it is suggested to convert this marker to SCAR (Sequenced characterized amplified region) in order to use it in selection programs.

The characteristics of SSR markers used in this study are shown in Table 1. Among the SSR markers, one had tri-nucleotide repeated sequence and the rest were of di-nucleotide type. Six markers were originated from *B. napus*, two from *B. oleracea*, and two from *B. rapa*. Three markers amplified two loci in the rapeseed genome. This may be attributed to the amphiploidy nature of the rapeseed genome. It is probable that each of the two genomes (A and C) has a primer banding site for these markers (Mitchell et al., 1997; Saal et al., 2001) which may indicate the homology of the A and C genomes (Halton et al., 2002). One of these markers, Na12-EO9 showed similar banding pattern in both regions suggesting the possible duplication of that portion of the genetic material in genomes A and C (Figure 2). Rudolph et al. (2002) also reported this kind of gene duplication in the rapeseed genome. Saal et al. (2001) argued that these similar fragments could be the cause of recombination between large homologous portions in the A and

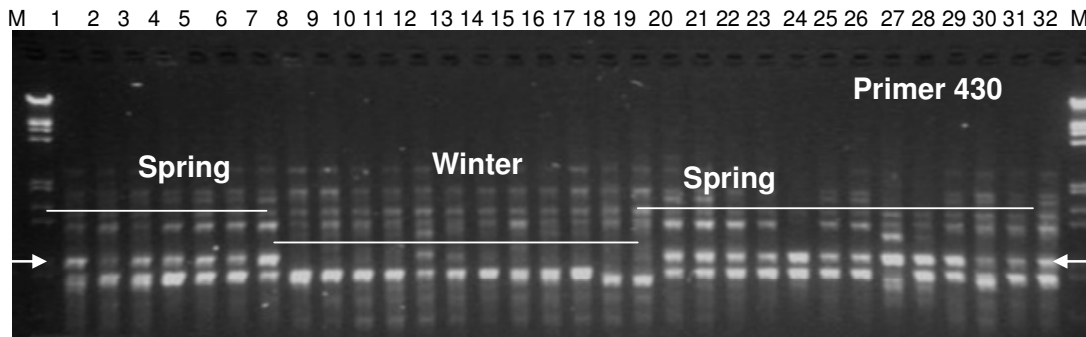


Figure 1. Banding pattern of rapeseed genotypes using RAPD marker 430.

Table 1. Characteristics of SSR markers used in studying the genetic diversity of rapeseed cultivars.

Marker	Origin	Sequence	Allele number	Frequency of predominant allele	Diversity index	Heterozygosity	Polymorphic information content	Allele size range (bp)
Na10 - G05	<i>B.</i>	GA	11	0.38	0.81	0.00	0.79	195 - 242
Na12 - C01	<i>B.</i>	GA	15	0.43	0.78	0.28	0.76	113 - 157
Na12 - C01	<i>B.</i>	GA	10	0.33	0.81	0.39	0.80	45 -- 86
Na12 - E06B	<i>B.</i>	GA	9	0.21	0.85	0.00	0.81	150 - 187
Na12 - E09	<i>B.</i>	GA	16	0.13	0.92	0.40	0.91	320 - 247
Na12 - G11	<i>B.</i>	CT	16	0.20	0.90	0.15	0.89	302 - 228
Na12 - A01	<i>B.</i>	CT	10	0.23	0.86	0.07	0.85	195 - 164
Ol11 - B05	<i>B.</i>	AG	11	0.34	0.82	0.50	0.80	119 - 89
Ol11 - B05	<i>B.</i>	AG	7	0.55	0.64	0.00	0.60	70-94
Ol13 - G05	<i>B.</i>	CCG	8	0.50	0.70	0.035	0.67	111 - 124
Ra2 - G05	<i>B. rapa</i>	GT-GA	15	0.15	0.92	0.20	0.91	150 - 209
Ra2 - G08	<i>B. rapa</i>	GA	15	0.18	0.90	0.13	0.89	297 - 404
Average			11.92	0.30	0.82	0.18	0.81	

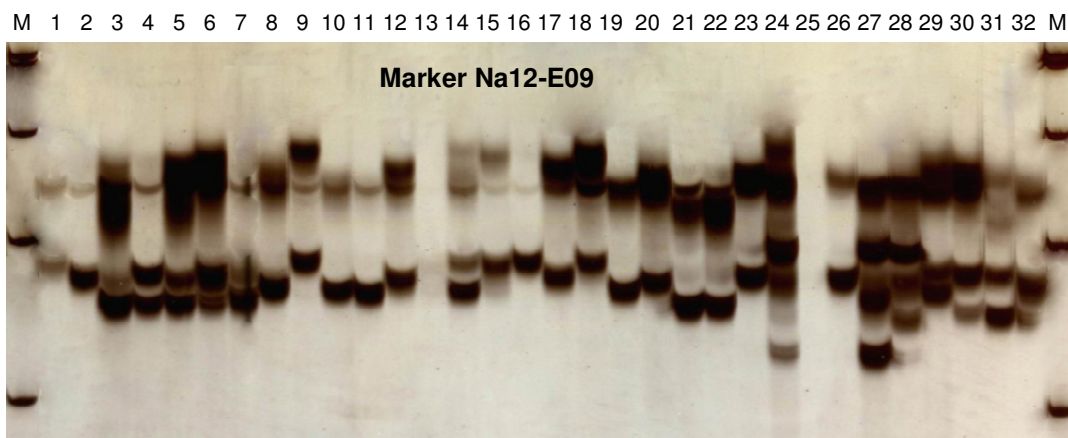


Figure 2. Banding pattern of rapeseed genotypes using SSR marker Na12-E09.

C genomes.

Ten SSR primers produced a total of 143 alleles in 12 loci (average of 11.92 alleles per locus). Other research-

ers have reported the average number of alleles per locus as 2 (Uzunova and Ecke, 1999), 3.9 (Rudolph et al., 2000) and 4.44 (Tonguc and Griffiths, 2004). Large

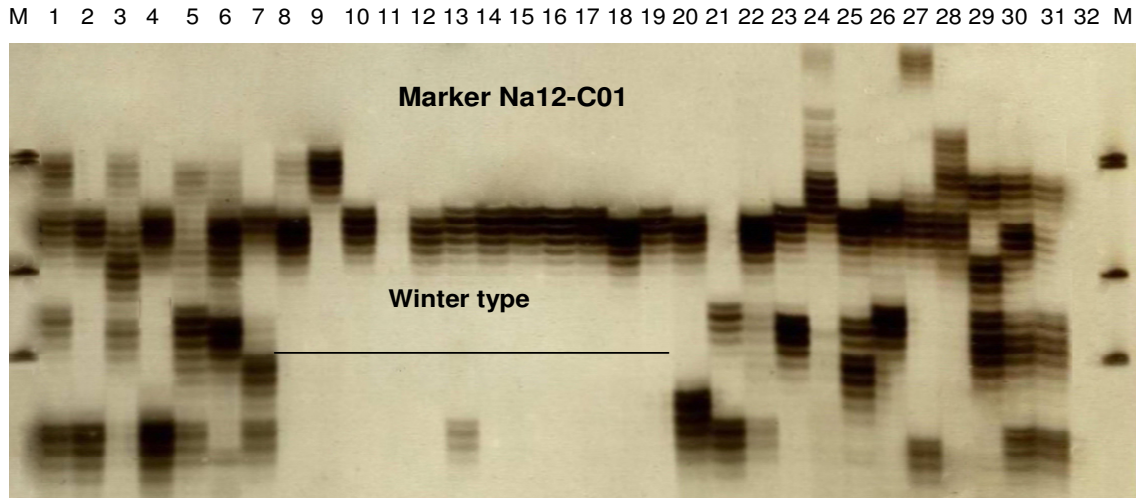


Figure 3. Banding pattern of rapeseed genotypes using SSR marker Na12-C01.

number of alleles per locus observed in our study is the indication of considerable genetic diversity present among the rapeseed cultivars under investigation.

According to Priolli et al. (2002) higher mutation rate in the SSR loci may be the reason for observing larger number of alleles in this marker. Allele size ranged from 45 to 404 base pairs in SSR markers. The second locus of Na12-C01 had the smallest (45 - 86 base pairs) and Ra2-G08 had the largest fragments (297 - 404 base pairs). Thus, markers with different fragment length (for example, Na12-C01 versus Ra2-G08) can be used concurrently in the PCR reaction (Narvel et al., 2000).

Polymorphic information content (PIC) is regarded as one of the important features of the molecular markers and can be used to evaluate the differentiation ability of the markers (Junjian et al., 2002). PIC values ranged from 0.60 (second locus of 0111-B05) to 0.19 (Na12-E09 and Ra2-G05 markers). The tri-nucleotide marker, 0111-G05 with CCG repeated sequence showed lower PIC as compared to other di-nucleotide makers. This may be attributed to the higher mutation rate of di-nucleotide repeated sequence (Vigoruroux et al., 2005).

Similar results have been reported by some researchers (Heckenberger et al., 2002; Agrama and Tuinstra, 2003; Vaz Patto et al., 2004). The amount of PIC is a function of allele number and frequency. Thus, markers with more alleles had larger PIC. For example, Na12-E09 representing largest number of alleles (16) had the highest PIC (0.91). Average PIC of all SSR markers were 0.81 indicating the ability of utilized markers to differentiate the rapeseed genotypes. SSR markers with *B. oleracea* origin showed lower PIC (0.69) as compared to the rest (0.85). Inoue and Nishio (2004) and Tunguc and Griffiths (2004) also obtained lower PIC for markers of *B. oleracea* origin. This is probably, the indication of lower genetic variation exist in the C genome.

Gene diversity ranged from 0.64 to 0.92 with an ave-

rage of 0.80. The second locus of 0111-B05 and the tri-nucleotide marker 0113 - G05, showed the lowest (0.64 and 0.70 respectively), Na12-E09 and Ra2-G05 the highest values (0.92). Hudcovicova and Kraic (2003) recommended the use of markers with the gene diversity of above 0.80 to differentiate the related genotypes.

The correlation coefficient of gene diversity index with PIC was high (0.996) for SSR markers. Senior et al. (1998) stated that if no heterozygosity exists, PIC and diversity index will be identical. Therefore, in homozygote genotypes, there is no need to estimate gene diversity and information from PIC will be sufficient. Average observed heterozygosity of SSR markers was 0.18. The first locus of the marker 0111-B05 showed the highest heterozygosity (0.5). Markers Na10-G05, E06B-Na12 and the second locus of 0111-B05 were not heterozygote. Low heterozygosity observed in this study may be the result of self-sterility nature (Vonarx et al., 1999) and diploid behavior of the rapeseed (Zhang et al., 2000).

The SSR marker 0111 - B05 in the second locus had the highest frequency and Ra2-G05 the lowest frequency of the predominant allele (0.55 and 0.15 respectively). The average frequency of the predominant allele was 0.30. Low frequency of the predominant allele reveals the suitable allelic distribution among the rapeseed genotypes (Priolli et al., 2002). Furthermore, SSR markers with the higher number of alleles per locus, showed the lowest frequency of the predominant allele. Thus, markers with lower frequency of the predominant allele have more differentiation ability than other markers.

The second locus in Na12-C01 SSR marker was only present in the spring genotypes. It may be used therefore, as a marker to differentiate spring from winter cultivars (Figure 3).

Cluster analysis based on RAPD data assigned the genotypes in four main groups (Figure 4). In this grouping winter types were separated from spring genotypes and

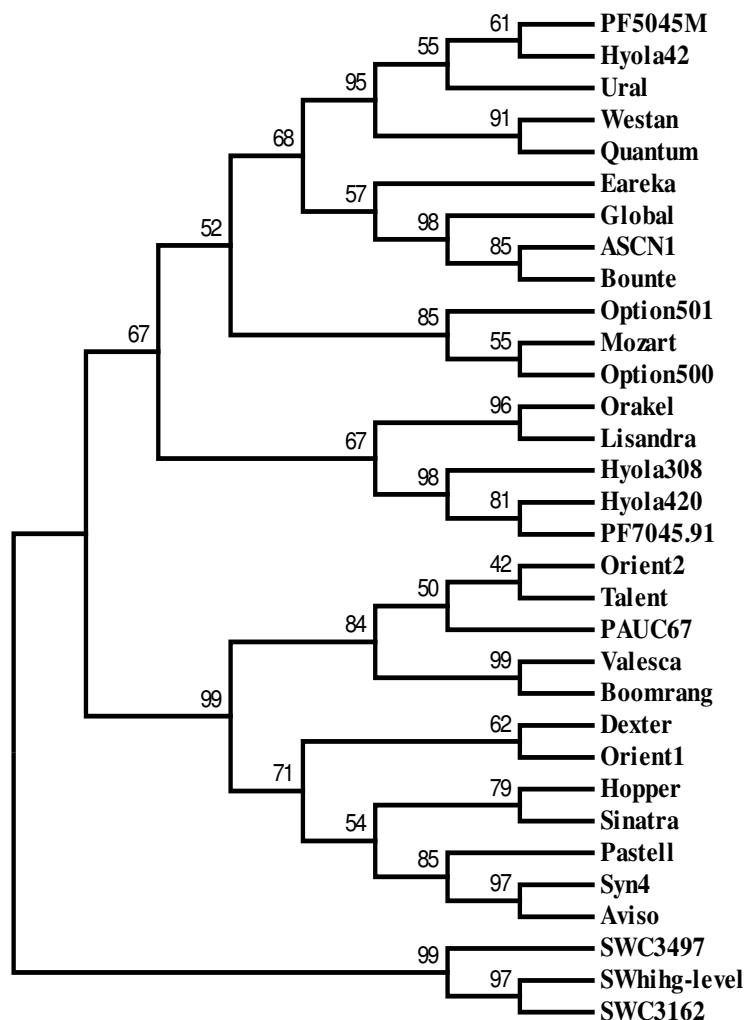


Figure 4. Dendrogram depicting the genetic relationships among rapeseed genotypes using neighbor-joining algorithm and number of differences coefficient using RAPD data.

genotypes with spring growth habit were grouped in three clusters. Chao-Zhi et al. (2003) and Shiran et al. (2004) could differentiate spring and winter rapeseed genotypes by RAPD markers.

However, Hu et al. (2003), in evaluating the *B. napus* germplasm from China and Europe, reported that the relationships between spring and winter accessions from Europe were closer than between Chinese and European accessions. They reasoned that Czech breeders, whose material was mostly evaluated in their study, have used winter \times spring crosses in the breeding programs. Thus, some lines had both winter and spring materials in their pedigrees.

Three Swedish cultivars (SW High Level, SWC3162 and SWC3497) were located in a single cluster and separated from other spring genotypes. These cultivars showed lower 1000 seed weight (2.16) than the average of other genotypes (3.57) and their genetic distances

from the rest of spring cultivars were high. They may be used therefore, as one parent of crosses to maximize variability in the resulted populations.

No association was found between the phenotypic traits and the RAPD markers (except for Swedish varieties with lower 1000 seed weight). Ashiq Rabbani et al. (1998) and Shiran et al. (2004) also reported similar findings. Different environmental conditions may affect the phenotypic characters and thus, identical genotypes may express different phenotypic values (Maric et al., 2004). Furthermore, fewer number of phenotypic variables (Song et al., 2000), measurement error (Maric et al., 2004) and the lack of linkage between phenotypic traits and DNA markers (Agrama and Tuinstra, 2003) may have contributed to this discrepancy.

Grouping based on SSR data showed no distinct separation of winter cultivars from spring types, although most of the spring and also winter genotypes were locat-

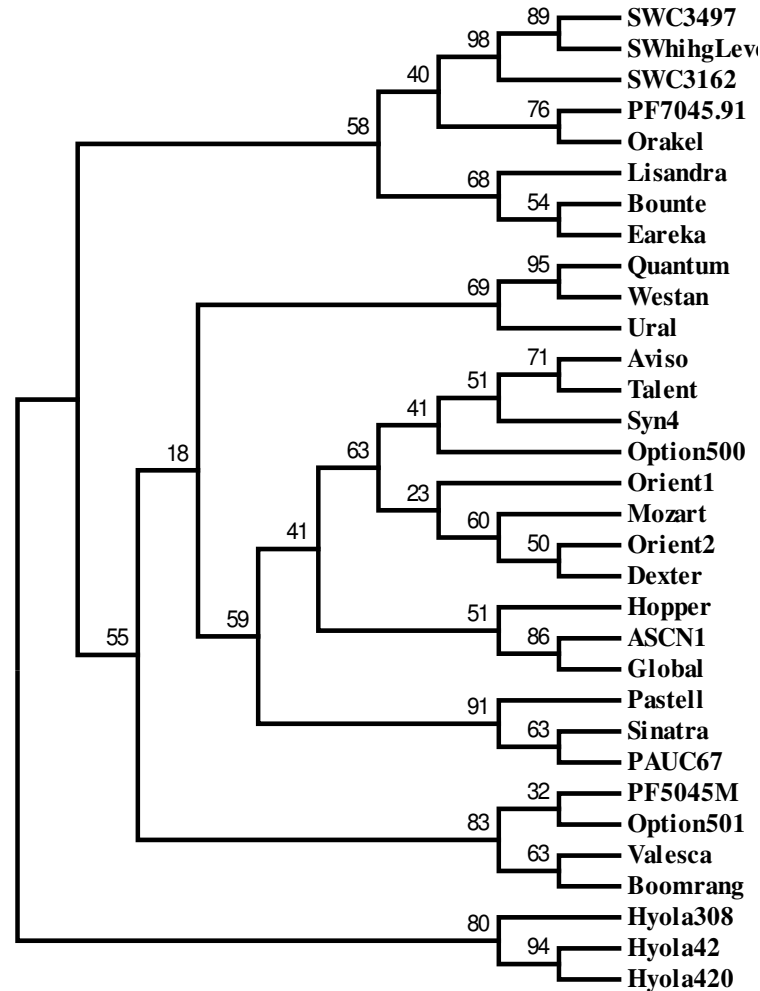


Figure 5. Dendrogram depicting the genetic relationships among rapeseed genotypes using neighbor-joining algorithm and number of differences coefficient using SSR data.

ed close to each other (Figure 5). Low number of SSR markers used in our study could be a possible reason for this result (Fungang et al., 2003; Hodcovicova and Kraic, 2003). Zhang et al. (2002) suggested a minimum of 300 - 400 SSR alleles for efficient grouping of wheat genotypes. They stated that allele number is more important than the marker number for classifying plant materials.

Clustering of the rapeseed cultivars based on the combined RAPD and SSR data revealed similar results with those of RAPD alone and could differentiate winter cultivars from the spring genotypes (Figure 6).

Conclusion

Large genetic variability was observed among 32 oilseed rape cultivars under study. Thirty-eight RAPD primers and 10 SSR primer pairs produced 250 polymorphic bands and 143 alleles respectively. The RAPD primer

430, having the sequence of AGTCGGCACC, had a band that only existed in the spring varieties, thus, capable of use in breeding programs after verifying its repeatability in other experiments and transforming to a codominant SCAR.

The second locus of the SSR marker Na12-C01, also could discriminate the winter and spring types. Cluster analysis based on combined RAPD and SSR data separated winter cultivars from spring cultivars, indicating that the genetic background of spring and winter rapeseed cultivars were different. Results of this study show that, RAPD marker can be used as a suitable tool for genetic assessment of rapeseed germplasm.

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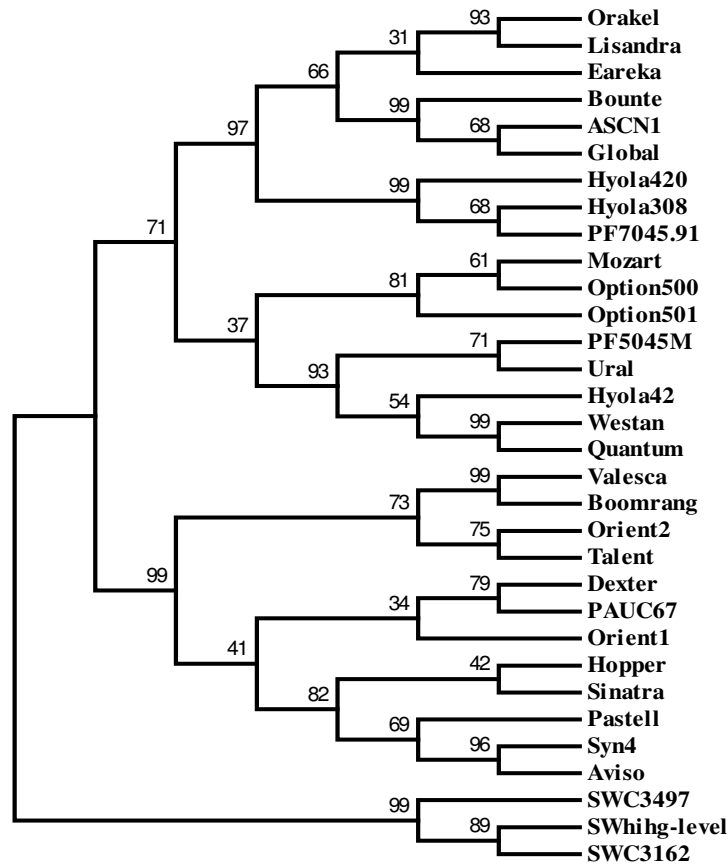


Figure 6. Dendrogram depicting the genetic relationships among rapeseed genotypes using neighbor-joining algorithm and number of differences coefficient using combined RAPD and SSR data.

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