

Evaluation of Genetic Diversity of Rapeseed (*Brassica napus* L.) Cultivars Using SRAP Markers

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ABSTRACT

Rapeseed is cultivated as a valuable oilseed plant throughout the world. The rapeseed oil contains significant amounts of unsaturated fatty acids. A total number of twenty-five cultivars of (winter/spring) rapeseed were grown in greenhouse conditions. In order to investigate genetic diversity, genomic DNA was extracted from the leaves and polymerase chain reaction was performed using 12 pairs of SRAP primers. Based on the results, 96 polymorphic bands were detected, in which the EM10-ME10 primer pairs with 14 bands and the EM1-ME14 and EM10-Me1 primer pairs with 4 bands had produced the maximum and minimum number of polymorphic bands, respectively. The Polymorphic Information Content (PIC) index varied from 0.25 to 0.499 and the genetic diversity based on Nei's index was 0.18 to 0.33. The cluster analysis using UPGMA method, with 95% accuracy of the grouping, was introduced as more appropriate than the other clustering methods. This method divided the studied cultivars into five distinct groups. The most genetic distance was recorded between Hydromel and Elvis, Zarfam, Artist and Okapi cultivars, and the lowest was shown between Artist and Okapi cultivars. Based on the results of principal coordinate analysis, the first and second components explained 83.66% of the variation, which represents the improper distribution of the SRAP markers on the whole genome. Overall, this study demonstrated high genetic diversity among cultivated rapeseed cultivars, which may be attributed to their high genetic background and environmental effects.

Keywords: Cluster analysis, Nei's index, PCoA, PCR, Polymorphic information content.

INTRODUCTION

Rapeseed (*Brassica napus* L.) belongs to brassicaceae family is known as a natural amphidiploid derived from crossing between *Brassica rapa* and wild cabbage (*B. oleracea*) (Iniguez-Luy *et al.*, 2011). Rapeseed is cultivated as a valuable oilseed plant throughout the world and is one the most important crops in the Mediterranean and Middle East. The area under cultivation of rapeseed was 36.7 million hectares with an average yield of 1979 kg/ha and a global production of approximately 72.7 million tons. The maximum production of rapeseed was reported in Canada with 18423000 tons

(FAO, 2017). The rapeseed oil, which is useful in food and industrial applications (Baux *et al.*, 2008), contains the fatty acid compositions that differ from other vegetable oils because of significant amounts of erucic acids (20-45%) and eicosenoic (9-15%) and minor amounts of arachidic, behenic, and lignoeric acids (1-2%) (Downey, 1966). The main fatty acids in oil of grown cultivars of oilseed rape are: Oleic acid- C18:1 (59-68%), Linoleic acid- C18:2 (17-21%) and Linolenic acid- C18:3 (7.8-10%) (Koprna *et al.*, 2006). The oil has improved responsiveness to antioxidants over standard Canola oil, and is useful in food and industrial applications (Baux *et al.*,

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2008). Effective conservation of plant genetic resources is essential for the development of breeding programs, specially to generate commercial hybrids (Abdoli Nasab and Rahimi, 2017) and release much productive cultivars among cultivated species (Maghsoudi Kelardashti *et al.*, 2015). For this purpose, the knowledge of genetic diversity and relationship between accessions is essential (Kresovich *et al.*, 1992). Rapeseed cultivars were classified into winter type, spring type, and semi-winter type groups (Channa *et al.*, 2016). Hybridization between spring, winter and semi-winter cultivars is an important approach to broaden the genetic base of rapeseed germplasm (Kebede *et al.*, 2010). The morphological traits, grain proteins, isozymes and DNA based molecular markers were introduced to evaluate genetic diversity. It is demonstrated that DNA based markers are powerful and reliable tools to investigate diversity and evolutionary relationship in the germplasm (Shengwu *et al.*, 2003). Sequence Related Amplified Polymorphism (SRAP) marker was first introduced by Li and Quiros (2001) and is a novel molecular marker that combines two primers, each containing random sequence with a common motif consisting of CCGG in the forward and AATT in the reverse primer, and generate the polymorphism associated with Open Reading Frames (ORFs) (Farias da Silva *et al.*, 2016). The advantages of this technique are simplicity, high polymorphism, high informative properties, and easy isolation of bands for sequencing (Yousefi *et al.*, 2018). There are several reports about using DNA based markers to evaluate genetic diversity in rapeseed such as SRAP (Lees *et al.*, 2016; Riaz *et al.*, 2014; Tan *et al.*, 2009; Budak *et al.*, 2004; Guo and Luo, 2006; Yancheng *et al.*, 2006), SSR (Karimbeigi *et al.* 2016; Hasan *et al.* 2006; Havlickova *et al.* 2014; Kebede *et al.*, 2010; Jinfeng *et al.*, 2014), ISSR (Havlickova *et al.*, 2014), RAPD (Hala, 2012; Shengwu *et al.*, 2003), RFLP (Girke *et al.*, 2012), AFLP (Qian *et al.*, 2006; Yu *et al.*, 2007), DArT (Raman *et al.*,

2013), and TRAP (Farias da Silva *et al.*, 2016). The method of unweighted average binding among clusters, better known as UPGMA, has been used most frequently in ecology and systematics (James and McCulloch, 1990) and in numerical taxonomy (Sneath and Sokal, 1973). UPGMA is treated as a clustering technique that uses the (unweighted) arithmetic averages of the measures of dissimilarity, thus avoiding characterizing the dissimilarity by extreme values (minimum and maximum) between the considered cultivars. Although SRAP markers are available for rapeseed, a minimum number of this type of marker has not yet been proposed to help in cultivar protection and commercial dispute resolution. The purposes of the present study were to establish the allele patterns and estimate the genetic distances and relationship for 25 rapeseed cultivars that are commercially important in Iran and in many parts of the world using SRAP molecular markers. This information would help the breeders to choose the best parents for crossing and management of the conservative programs.

MATERIALS AND METHODS

Plant Materials

A total of 25 spring and winter rapeseed cultivars (Table 1), which were obtained from Gene Bank of Iran, were grown at research greenhouse of Graduate University of Advanced Technology, Kerman, Iran, during 2017-2018 season. Irrigation was done regularly every three days.

DNA Extraction and PCR Procedure

DNA extraction from young leaves was performed using CTAB method (Murray and Thompson, 1980). The quantity and quality of extracted DNA were evaluated using spectrophotometry and agarose gel

Table 1. Characteristics of the studied rapeseed cultivars.

Row	Cultivar name	Growth Brigade	Origin	Cultivars code
1	Julius	Spring	Germany	P1
2	Hyola 50	Spring	Australia	P2
3	Alonso	Winter	France	P3
4	Delgan	Spring	Iran	P4
5	DK 7170	Spring	Australia	P5
6	Rytmo	Winter	France	P6
7	Agamax	Spring	Germany	P7
8	Danube	Winter	France	P8
9	Hydromel	Winter	France	P9
10	Jerry	Spring	Germany	P10
11	Garou	Winter	Germany	P11
12	Hyola 4815	Spring	Australia	P12
13	Zlanta	Winter	Serbia	P13
14	Elvis	Winter	France	P14
15	Rohan	Winter	Germany	P15
16	Trapper	Spring	Germany	P16
17	Zarfam	Winter	Iran	P17
18	Artist	Winter	France	P18
19	Nataly	Winter	France	P19
20	Ahmadi	Winter	Iran	P20
21	Hyola 61	Spring	Australia	P21
22	Neptune	Winter	France	P22
23	Okapi	Winter	France	P23
24	Zorika	Winter	Serbia	P24
25	DK7130	Spring	Australia	P25

electrophoresis, respectively. Polymerase chain reaction was performed using 12 SRAP primers (Table 2). A 25 μ L reaction mixture containing 50 ng DNA, 10 μ L Master Mix (Ampliqon) and 10 μ M of each of the forward and reverse primers was used. PCR amplification was performed on a thermal cycler with the following parameters: 4 minutes at 94°C for initial denaturation, 35 cycles of amplification at 94°C for 45 seconds, annealing temperature for 45 seconds, and 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The amplified bands were separated on 1.5% agarose gel electrophoresis and staining with ethidium bromide (1 μ g mL⁻¹).

Data Analysis

The distinct and reproducible bands were scored for each SRAP markers as either

present (1) or absent (0). The Polymorphic Information Content (PIC) was calculated to measure the informativeness of the studied molecular markers in differentiating among genotypes according to the formula of Chesnokov and Artemyeva (2015) as follows:

$$\text{PIC} = 1 - [f^2 + (1-f)^2]$$

Where, *f* is the marker frequency in the data set. For the dominant markers, the maximum PIC value is 0.5. Genetic diversity parameters such as number and percentage of polymorphic loci, Number of effective alleles (*N_e*), Shannon information index (*I*) and Nei's gene diversity index (*h*) (Nei, 1972) were analyzed using POPGEN V1.31 software. To provide a better interpretation of the relationship between individuals, and explain genetic variation and show the variation pattern in a multidimensional pattern, Principal Coordinate Analysis (PCoA) was done using

**Table 2.** Sequence and annealing temperature of the SRAP primer pairs used in this study.

Primer combinations	Annealing temperature (Centigrade)	Primer sequence
EM1-Me4	49	5'-GACTGCGTACGAATTAAT-3' 5'-TGAGTCCAAACCGGACC-3'
EM1-Me10	49	5'-GACTGCGTACGAATTAAT-3' 5'-TGAGTCCAAACCGGGAC-3'
EM1-Me14	49	5'-GACTGCGTACGAATTAAT-3' 5'-TGAGTCCAAACCGGCTA-3'
EM6-Me4	47	5'-GACTGCGTACGAATTGCA-3' 5'-TGAGTCCAAACCGGACC-3'
EM6-Me10	53	5'-GACTGCGTACGAATTGCA-3' 5'-TGAGTCCAAACCGGGAC-3'
EM6-Me14	49	5'-GACTGCGTACGAATTGCA-3' 5'-TGAGTCCAAACCGGCTA-3'
EM10-Me4	49	5'-GACTGCGTACGAATTTAG-3' 5'-TGAGTCCAAACCGGACC-3'
EM10-Me10	53	5'-GACTGCGTACGAATTTAG-3' 5'-TGAGTCCAAACCGGGAC-3'
EM10-Me14	49	5'-GACTGCGTACGAATTTAG-3' 5'-TGAGTCCAAACCGGCTA-3'
EM16-Me4	47	5'-GACTGCGTACGAATTCCGG-3' 5'-TGAGTCCAAACCGGACC-3'
EM16-Me10	49	5'-GACTGCGTACGAATTCCGG-3' 5'-TGAGTCCAAACCGGGAC-3'
EM16-Me14	49	5'-GACTGCGTACGAATTCCGG-3' 5'-TGAGTCCAAACCGGCTA-3'

NTSYS V2.02 software. The relative variance of each coordinate indicated the importance of the related coordinate of total variance, which was expressed as a percentage (Nikkhoy and Shiri, 2017). To calculate the Jaccard coefficient of similarity (Sneath and Sokal, 1973) and performing cluster analysis and principal-coordinate analysis, the binary dataset was used in the software NTSYSPC, version 2.2 (Rohlf, 2000). The formula for similarity coefficient calculation was as follows:

$$\text{Jaccard} = \text{NAB} / (\text{NAB} + \text{NA} + \text{NB})$$

Where, NAB is the Number of marker bands shared by two samples (A and B), and NA and NB are the marker bands present only in sample A or B (Nei and Li, 1979). The corresponding cluster analysis was performed using the Unweight Pair Group

Method with Arithmetic Average (UPGMA) and Jaccard similarity Index and its output was used to construct a dendrogram.

RESULTS AND DISCUSSION

The 12 SRAP primer combinations produced sufficient polymorphism in the 25 studied rapeseed cultivars (Figure 1). A total of 116 bands ranging in size from 150 to 4,000 bp with an average of 9.66 bands for individual primer combination were amplified. Among the amplified sites, there were 96 polymorphic loci with an average of eight polymorphic bands per primer combination (Table 3). The percentage of polymorphism was as high as 81.74%.

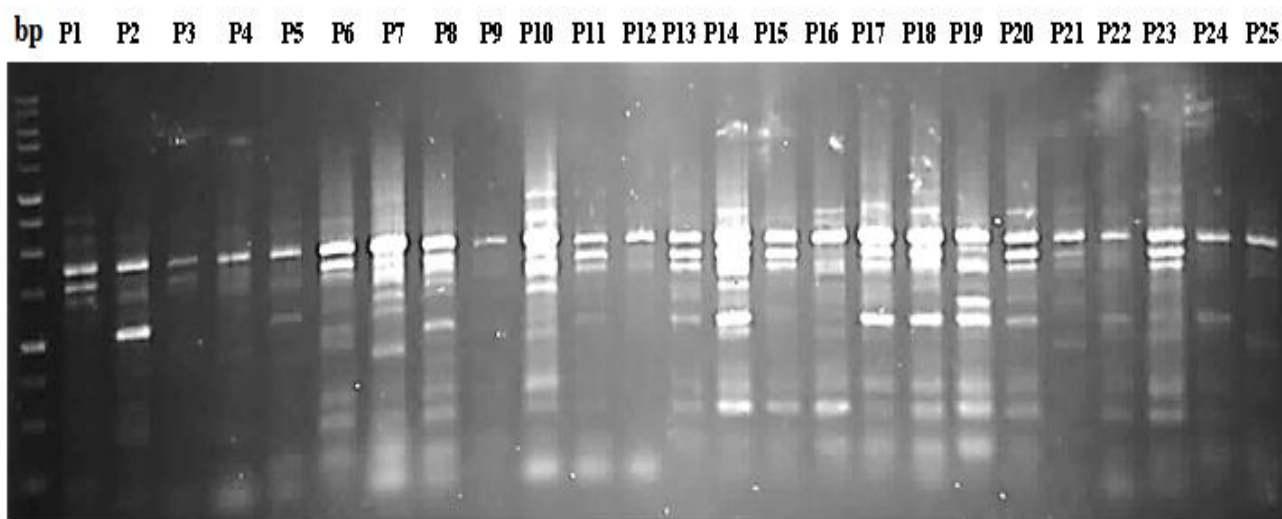


Figure 1. SRAP banding pattern generated by different rapeseed cultivars amplification using EM10-Me10 primer pairs. bp: 1 kb molecular weight marker (Fermentase), P1 to P25: The studied cultivars.

Table 3. The number and frequency of amplified bands, the percent of polymorphism, Polymorphic Information Content (PIC), Nei's gene diversity and Shannon information in the studied rapeseed cultivars.

Primer combinations	No total bands	No polymorphic bands	Polymorphic bands (%)	No effective alleles (Ne)	Nei's index (h)	Shannon' index (I)	PIC
EM1-Me4	10	9	90.00	1.57	0.33	0.49	0.49
EM1-Me10	12	12	100.00	1.52	0.31	0.47	0.49
EM1-Me14	5	4	80.00	1.54	0.30	0.46	0.36
EM6-Me4	8	7	87.50	1.38	0.24	0.38	0.34
EM6-Me10	9	7	77.77	1.46	0.27	0.41	0.34
EM6-Me14	10	9	90.00	1.46	0.28	0.42	0.43
EM10-Me4	9	7	77.77	1.32	0.20	0.33	0.28
EM10-Me10	15	14	93.33	1.50	0.30	0.46	0.46
EM10-Me14	7	4	57.14	1.35	0.20	0.30	0.25
EM16-Me4	12	7	58.33	1.28	0.18	0.28	0.30
EM16-Me10	12	10	83.33	1.27	0.19	0.31	0.44
EM16-Me14	7	6	85.71	1.50	0.29	0.44	0.41
Average	9.66	8	81.74	1.43	0.26	0.40	0.38

The EM10-ME10 primer combination amplified the maximum polymorphic bands (14 bands), while the minimum polymorphic bands (4 bands) were amplified using EM1-Me14 and EM10-Me14 primer combinations. In the studied primer pairs, the highest percentage of polymorphic loci was shown in EM1-Me10 with amount of 100% and the lowest was shown in EM10-Me14 primer combination with the amount

of 57.14%. The number of effective alleles was 1.27 to 1.57, with the average values of 1.43 and the largest was shown in primer combination EM1-Me4, EM1-Me14 and EM1-Me10 (Table 3). The mean PIC value for primer combinations was 0.38, which ranged from 0.25 to 0.499. The highest PIC value was related to EM1-Me10, which introduces it as the most informative primer combination for genetic diversity studies

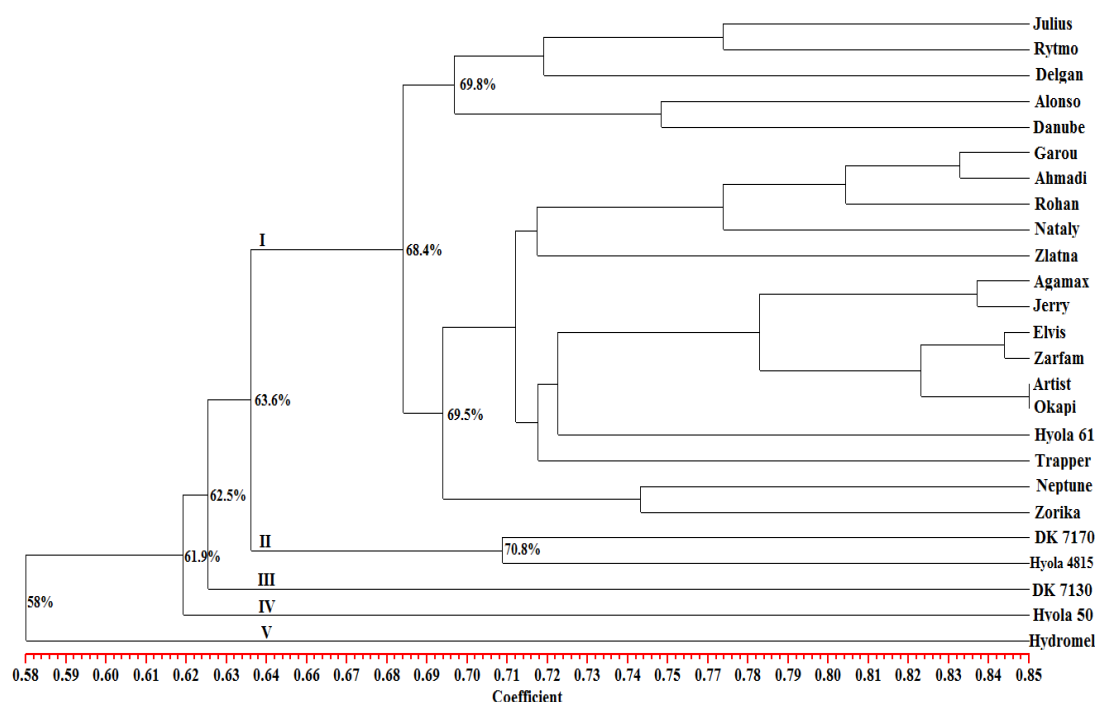


Figure 2. Dendrogram of 25 studied *Brassica napus* cultivars according to UPGMA clustering method and Jaccard similarity index. Dendrogram shows the relationships among cultivars using SRAP molecular markers.

among the studied rapeseed cultivars. The highest Nei's gene diversity index (h) and Shannon polymorphic information index (I) were 0.33 and 0.49, respectively, which were shown in EM1-Me4 primer combination, indicating the high level generic diversity. Based on the SRAP data, the dendrogram grouped the species into five groups (Figure 2).

The first group (I) included Julius, Rytmo, Delgan, Alonso, Danube, Agamax, Jerry, Elvis, Zarfam, Artist, Okapi, Hyola 61, Trapper, Garou, Ahmadi, Rohan, Nataly, Zlatna, Neptune, and Zorika. The second group (II) included DK 7170 and Hyola 4815, the third group (III) DK7130, the fourth group (IV) consisted of Hyola 50, and the fifth group (V) included Hydromel cultivar.

Based on Figure 2 and Table 1, the studied Australian rapeseed genotypes showed the least genetic similarity to Iranian genotypes. On the other hand, Iranian genotypes Delgan and Zarfam showed the highest genetic similarity with German rapeseed genotypes.

Also, the groups II, III and IV included spring Australian genotypes, and group I included the majority of studied genotypes (80 %) from Germany, France, Iran and Serbia. The studied winter genotypes, except Hydromel, were placed in the group I.

The results showed that the genetic distances of 25 rapeseed cultivars varied from 0.15 to 0.42. The largest genetic distance (0.42) was recorded between winter Elvis and Hydromel cultivars; the lowest (0.15) was between winter Artist and Okapi cultivars (Figure 2). Eyni Nargeseh *et al.* (2019) reported that the Hydromel cultivar showed the least grain yield and oil content compared to the other studied cultivars, which could cross with other genotypes in breeding programs. In our study, the Elvis and Zarfam cultivars were classified in one group with high similarity (0.84), in accordance with the finding of Darvish Nia *et al.* (2015) study of genetic diversity in rapeseed, using the SSR and ISJ molecular marker. They reported a high similarity (0.94) between Elvis and Zarfam cultivars.

Also, our results are in line with the findings of Asghari *et al.* (2018) and Mahjoob *et al.* (2014) who classified the Okapi and Zarfam cultivars in the same group with high similarity. Also, Karimbeigi *et al.* (2016) reported a high similarity (0.98) between Elvis and Zarfam cultivars.

The results of PCoA, evidently distinguished that the first two components explain 83.66% of the total variation, which represents the improper distribution of the SRAP markers on the whole genome. Based on PCoA analysis, the studied cultivars were divided into four groups. The first group (I) included Rytmo, Neptune, Zorika and DK 7130 cultivars, the second group (II) included Julius, Alonso, Delgan, and DK7170 cultivars, the third group (III) included Hyola 4815, Nataly, Trapper, Ahmadi, Hyola 61, and Hyola 50 cultivars, and the fourth group (IV) consisted of Agamax, Jerry, Garou, Rohan, Zarfam, Okapi, Elvis, and Artist cultivars. The results of the two methods clustering (shown with black circles) and principal coordinate

analysis (shown with blue circles) were approximately equivalent (Figure 3).

In conclusion, the SRAP molecular markers targeting ORFs as functional regions of the rapeseed genome showed sufficient polymorphism. The results of the present study could be used in breeding programs to obtain rapeseed hybrids; therefore, it is recommended for crossing Hydromel genotypes with other genotypes in breeding programs.

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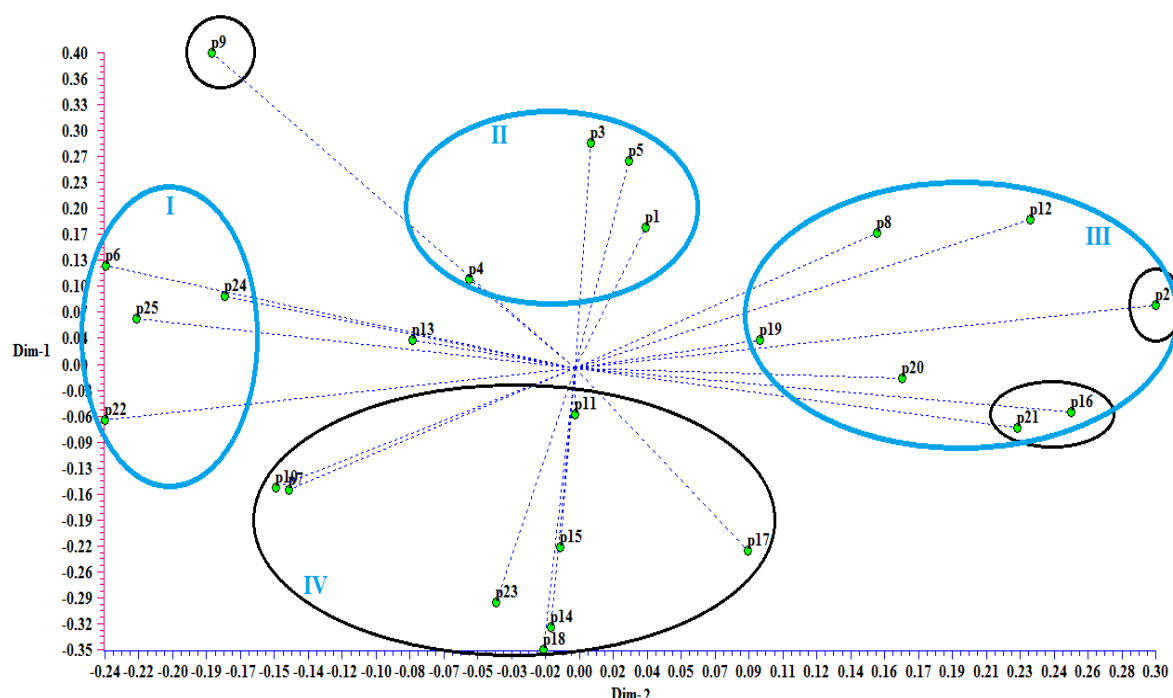


Figure 3. Principle Coordinate Analysis (PCoA) (blue circles) based on the first and second eigenvectors compared with grouping based on clustering analysis (black circles) extracted from SRAP data of 25 rapeseed cultivars used in this study.



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ارزیابی تنوع ژنتیکی ژنوتیپ‌های کلزا (*Brassica napus* L.) با استفاده از نشانگرهای مولکولی SRAP

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چکیده

کلزا به عنوان گیاه روغنی با ارزش در سراسر دنیا مورد توجه می باشد. روغن کلزا دارایی میزان قابل توجهی از اسیدهای چرب غیر اشباع می باشد. تعداد ۲۵ ژنوتیپ کلزا (بهاره - زمستانه) در شرایط گلخانه کشت گردید. به منظور بررسی تنوع ژنتیکی، DNA ژنومی از برگ استخراج و واکنش زنجیره ای پلیمرز با استفاده از ۱۲ جفت آغازگر SRAP انجام گردید. بر اساس نتایج تعداد ۹۶ باند چند شکل به دست آمد، که جفت آغازگر EM10-ME10 با تعداد چهارده باند و جفت آغازگرهای EM1-ME14 و EM10-Me1 با تعداد چهار باند به ترتیب بیشترین و کمترین تعداد باند چند شکل را ایجاد کردند. محتوای اطلاعات چندشکل (PIC) بین ۰/۲۵ تا ۰/۴۹۹ و میزان تنوع ژنی بر اساس شاخص نی از ۰/۱۸ تا ۰/۳۳ متغیر بود. تجزیه خوشه ای به روش UPGMA با و با انجام صحت گروه بندی در حدود ۹۵ درصد معنی داری، مناسب تر از دیگر روش های تجزیه خوشه ای است. تجزیه خوشه ای بر اساس روش جاکارد ژنوتیپ های تحت مطالعه را در پنج گروه مجزا قرار داد. بیشترین فاصله ژنتیکی بین ارقام هیدرومل و الویس و زرفام و کمترین فاصله بین ارقام آرتیست و اکاپی مشاهده گردید. تجزیه به مختصات اصلی نشان می دهد که مولفه اول و دوم ۸۳/۶۶ درصد از تنوع به دست آمده را توجیه می کنند که خود نشان دهنده توزیع مناسب نشانگرهای SRAP در کل ژنوم می باشد. به طور کلی، این مطالعه تنوع ژنتیکی بالایی را ارقام زراعی کلزا نشان داد که می تواند به زمینه ژنتیکی بالای آنها و اثرات محیطی مربوط می باشد.