Australian Journal of Crop Science AJCS
AJCS 7(3):361-367 (2013) ISSN:1835-2707

Comparison of morpho-agronomic traits versus RAPD and ISSR markers in order to evaluate genetic diversity among *Cuminum cyminum* L. accessions

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Abstract

Cuminum cyminum is a valuable medicinal plant belongs to family Apiaceae. In this study genetic variation among 42 cumin accessions were collected from different regions of Iran plus two accessions from Syria and Afghanistan were assessed based on three marker systems namely, ISSR, RAPD and morpho-agronomic traits. In overall, banding patterns of 22 ISSR primers and 13 RAPD primers revealed 202 (67.32%) and 85 (54.90%) polymorphic bands, respectively. The range of similarity coefficient in ISSR and RAPD markers were 0.48-0.92 and 0.25-0.94, respectively. Using primers as pairwise combination in this study did not offer higher polymorphism but provided different band pattern. Specific grouping were carried out by each cluster analysis including ISSR, RAPD, ISSR+RAPD and morpho-agronomic markers based on their similarity matrix making 8,7,6 and 3 groups respectively. The results showed that grouping based on molecular markers and morpho-agronomic traits are different so these two systems could not discriminate accessions as a same way. All of Mantel tests between extracted similarity matrices from each marker system were significant except between ISSR marker and morpho-agronomic traits. It could be concluded that among three different molecular data sets, the RAPD and RAPD+ISSR data have a significant and closer relationship to morpho-agronomic data.

Keywords: *Cuminum cyminum*, ISSR, Mantel test, primer pairwise combination, RAPD
Abbreviations: ISSR: Inter Simple Sequence Repeat, PIC: Polymorphic Information Content, RAPD: Random Amplified Polymorphic DNA

Introduction

Cumin (*Cuminum cyminum)*, from the family Apiaceae is an annual plant native of Mediterranean regions. Cumin as a valuable medicinal plant had been used with the people of India and Egypt since ancient time. Nowadays, this plant is cultivated in huge scale in other different countries such as, China, Pakistan, Iran, Iraq, Turkey, Syria and Morocco (Omidbaigi, 2007). In Iran, attempts to cumin breeding especially concerning compatibility to various climates and resistance to biotic and abiotic stresses are restricted (Bagheri and Mahmoudi, 2003). A plant-breeding program, to be success, needs a system to conservation of genetic reservoirs (Sakti and Khadag, 1995). Both variation and selection are requisites of each plant breeding program as we can say, having diversity and wide amplitude of genetic pool is necessary to breeders (Ehdaei, 1988). In addition, progressions in genomics have given means to raise value of breeding programs (Yunbi and Jonath, 2008). From another perspective, since we are not able to sequence all plants, analysis and identity of genetic diversity among them will be worthwhile (Agarwal et al., 2008). Applying DNA molecular markers, for assessment of genetic variation in plants has shown advantages over other markers based on the phenotype; they are neutral, not related to age and tissue type, not influenced by the environmental conditions, feasibility, lower costs and more informative than morphological markers (Da Mata et al., 2009). Among the all kinds of DNA markers, RAPD and ISSR are two of the most popular markers based on polymerase chain reaction (PCR). This types of molecular markers have been used widely to analyze genetic diversity among different species of plants (Herrera et al., 2002; Talhineas et al., 2003; Chen et al., 2005; Salhi et al., 2005; Hadian et al., 2007; Isshiki et al., 2007; Zamani et al., 2009; Verma et al., 2009; Pezhmanmehr et al., 2009) and beside of morphological markers. For example, Dey et al. (2006) used RAPD markers and agronomic traits to assess genetic diversity of bitter gourd (*Momordica charantia*) genotypes and revealed that there was a low fitness between grouping and genotype place of collection. Sensoy et al. (2007) carried out same study on Turkish melon (*Cucumis melo* L.) but found different results that were locating genotypes with close regions in same groups based on cluster analyses. ISSR and morphological markers together have been used to study greek tomato (*Solanum lycopersicum* L.) by Terzopoulos and Bebeli (2008) and they did not found correlation between two data sets. The aim of current investigation was to evaluate genetic diversity between 40 cumin accessions, collected from various regions of Iran plus two accessions from Syria and Afghanistan, based on morpho-agronomic traits and DNA molecular markers (RAPD and ISSR).

Result and discussion

Morpho-agronomical analysis

In the field experiment of this study, results showed significant differences among accessions based on measured traits (Table 1). This table provides an overview of mean, maximum,

minimum and LSD value for each trait and coefficient of variation (CV) values across all 31 accessions. Except two traits

Table 1. The morphological characters and basic statistical data of 31 accessions of *Cuminum cyminum*

Trait	Mean	Max	Min	LSD	CV (%)	P*(F-test)
Yield (kg)	623.6	1215.3	140.3	8.53	8. 38	<0.0001
Number of umbel Per plant	38.96	69.07	18.35	7.91	12. 36	<0.0001
Biological yield	1181.2	2347.6	306.8	15.31	7. 98	<0.0001
Harvest Index	52.93	63.02	46.54	4.18	4. 82	<0.0001
Plant Height (cm)	24.42	20.57	15.29	0.96	2. 42	<0.0001
Thousand Seed Weight (gr)	3.42	4.25	2.92	0.35	4. 67	0.0003
Number of miniumpel in umbe	3.39	4.39	2.85	0.23	4. 35	0.0273
Number of seed in umbel	11.23	14.27	7.21	1.02	5. 60	<0.0001
Number of branch	5.64	8.25	4	0.4	4. 34	<0.0001
Percent disease incidence	10.96	48.2	0	0.85	58. 36	<0.0001
Days to germination	16.73	19.66	15	1.16	4. 25	<0.0001
Days to flowering	65.66	71.33	62.67	3.62	3. 38	<0.0001
Days to Physiological Maturity	83.61	87.67	80.33	3.07	2. 24	0.1077
Days to maturity	95.65	99.33	93.33	3.36	2. 15	0.0039

* $p < 0.01$= highly significant

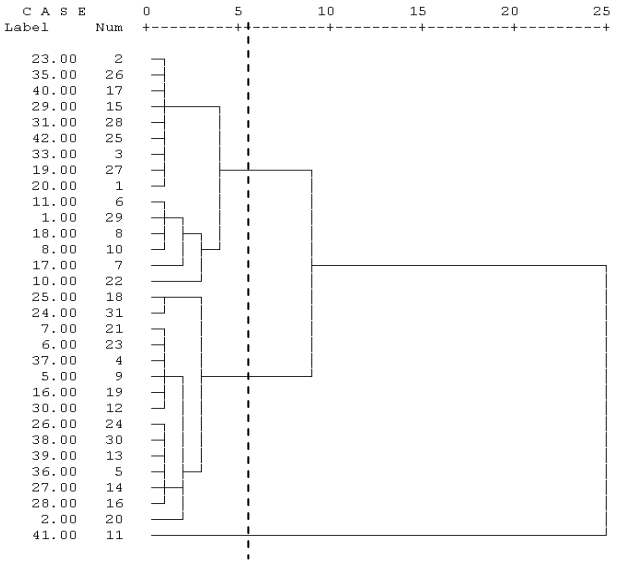

Fig 1. Dendrogram derived from cluster analysis based on morpho-agronomic traits.

including number of umbel per plant (12.36) and percent disease incidence (58.36) others had low CV, generally fewer than 10. In a similar study, Bahraminejad et al. (2011) indicated a significant diversity among and within populations derived from nine provinces of Iran for all the measured phenotypic traits. They introduced Kerman and Esfahan genotypes as high yielding ones. Cluster analysis based on measured traits classified accessions into three groups not completely match to geographic regions (Fig 1). In contrast, Hashemi et al. (2008) showed that cluster analysis of similarity data, grouping of the Persian cumin (*Bunium persicum*) ecotypes were according to their geographic origin. Accessions of 6, 5, 27, 4, 10, 22, 18, 28, 29, 26, 19, 20 and 2 were located in second cluster, 31 in third group and other ones occupied first cluster. The first group of accessions in term of some traits such as seed yield, biological yield and percent disease incidence were more similar than other traits. The single accession in the third cluster, showed a significant excellence concerning almost economically traits including seed yield, biological yield, plant height and number of umbel per plant. The other accessions in the second group were almost similar according to other traits.

DNA molecular marker analyses

Among the 31 ISSR primers in molecular section, only 22 of them successfully amplified polymorphism bands. The all ISSR primers amplified 136 polymorph bands out of 202 (67.32%). The number of bands varied from three (primer UBC-873) to 15 (primer UBC-864). The highest and lowest percentages of polymorphism belonged to UBC-864 primer (93%) and UBC-857 primer (13%), respectively. There is no record of ISSR data in literature on cumin germplasm to compare with their values.

Although using primers as pairwise combination did not offer higher polymorphism but it provided different band pattern. Primer pairwise combination for assess genetic diversity in various researches related to molecular markers has been used (Correa et al., 1997; Debener and Mattiesch, 1998; Cekic et al., 2001) but restricted in assessment of genetic diversity. The differences among produced band patterns by application of two primers and their combination among accessions were completely explicit. The 13 RAPD primers that used in this study, produced 153 bands which 84 of them were found to be polymorphic (54.90%), varied from 4 (primer E10) to 23 (primer AB1). Several marker systems have been reported for analyze genetic diversity in cumin and other species belongs to the family of Apiaceae. Pezhmanmehr et al. (2009) observed 86% polymorphism after using 38 RAPD primers among 20 Iranian populations of *Bunium persicum*. In another research, Domblides et al. (2010) found 11 and 10.2 polymorph bands in average among parsley (*Petroselinum crispum* (Mill.) Nym.) samples per RAPD and ISSR primers, respectively. The value of polymorphism created by ISSR primers was higher than

Table 2. Mantel test for comparisons of similarity matrices derived from different data sets in Cumin accessions.

	RAPD	ISSR	RAPD+ISSR
ISSR	*r = 0.541 **p-value <0.0001		
Agronomical traits	r = 0.108 p-value=0.02	r = 0.047 p-value=0.325	r = 0.098 p-value= 0.036

*r = correlation value, **P < 0.05 and P < 0.01 are considered as significant and highly significant respectively.

RAPD ones. Yang et al. (2007) stated that the ISSR marker could detect more genetic variation when compared to RAPD. This situation has been observed in some other studies including, Ye et al., (2008); Praveen et al., (2009); Sarwat et al., (2008). PIC index for ISSR primes ranged from 0.09 to 0.50 and for RAPD were from 0.13 from 0.50. The PIC value has been used for evaluate genetic variation in many studies (Najaphy et al., 2011).

Genetic relationships among accessions and cluster analysis

Cluster analysis based on Jaccard similarity matrix via Centroid method were performed for ISSR, RAPD and ISSR+RAPD binary data In order to understand genetic relationships among accessions. To have least "chaining effect" which represent individual groups and complicated the interpretation of results (Mohammadi and Prasana, 2003), different methods for cluster analysis were tested and finally Centroid method was selected.

Molecular markers analyses

Molecular markers are a useful complementary tool to morphological and physiological characterization of plants because they have many advantages for example they are plentiful, independent of environmental effects, and cultivar identification early in plant development (Manifesto et al., 2001). According to the Jaccard`s similarity matrix for ISSR data, the amount of similarity varied from 0.48 (between accession 18 and 40) to 0.92 (among accession 30 and 31) with 0.73 in average (data not shown). The dendrogram is illustrated based on Centroid analysis of the ISSR data in Figure 2A. Cluster analysis resulted in a classification of 42 accessions into six groups in 0.14 distance unit. Totally, the accessions in this research were almost belong to Khorasan (45%) and Kerman provinces (26%) that were well discriminated by this method of classification. As groups 3, 4 and 5, which contained 47% of accessions almost, were belonged to Khorasan province and there was no accession related to Kerman province that occupied other clusters. Nonetheless, there was some kind of aversion between genetic divergence and place of collection. For example, cluster analysis did not separate two abroad accessions (Syria and Afghanistan) from Iranian accessions, which could be because of close geographical relatedness. Based on Jaccard similarity matrix for RAPD data, the most similar accessions (0.94 similarity) observed between 34 (Mashhad) and 35 (Gorgan) while, the least of it (0.25) observed among accessions 22 (Gonbad-e-Kavous) and 39 (Daregaz). The average of similarity (0.74) was just a little more than what found in ISSR primers (0.73). The genetic similarity ranged from 0.37 to 0.95 among Persian cumin (*Bunium persicum*) accessions in a research conducted by Hashemi et al. (2008). Cluster analysis based on RAPD data, according to Centroid method classified accessions into seven groups in 0.15 distance unit (Fig. 2B). Discrimination between two major groups of accessions (Kerman and Khorasan province) observed here too. All accessions belong to Kerman province, were placed in the last three clusters and none of them were present in other clusters. ISSR and RAPD markers demarcated 42

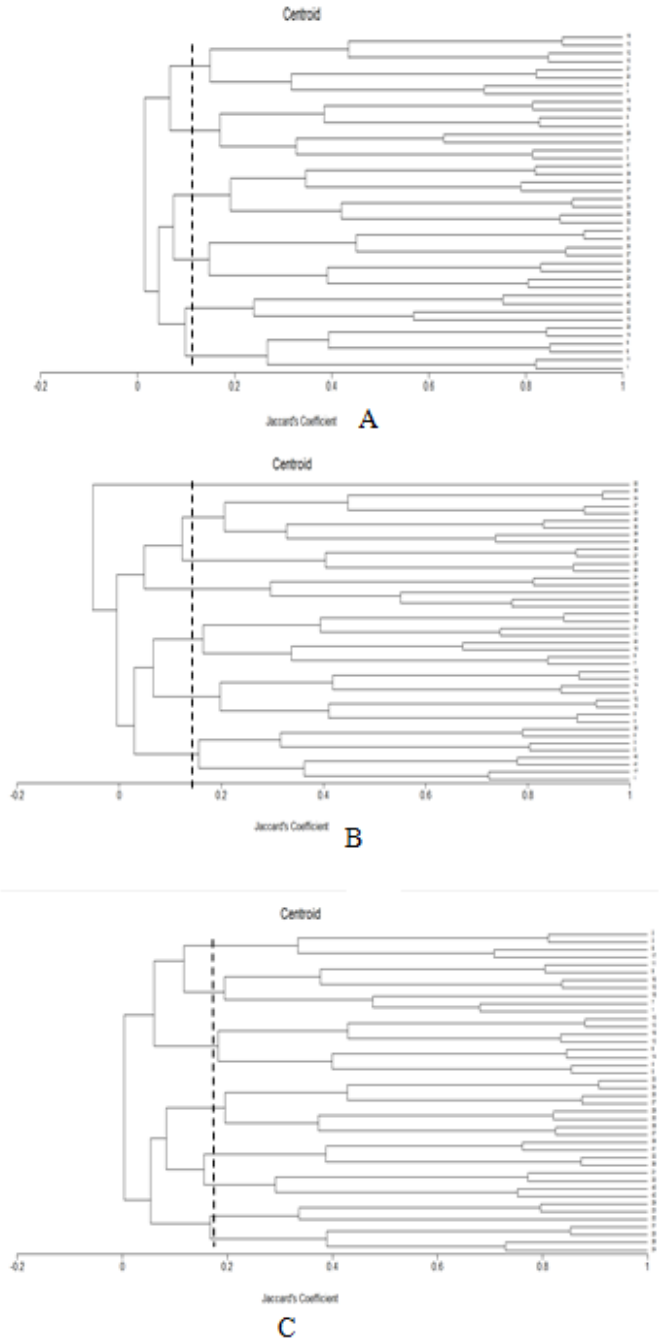

Fig 2. Dendrogram derived from a Centroid cluster analysis of (A) ISSR, (B) RAPD and (C) RAPD+ ISSR markers using Jaccard' similarity coefficient of cumin accessions (Dividing them into six, seven and eight groups respectively).

Table 3. List of 42 Cumin accessions and their place of collection.

Code	Place of collection	code	Place of collection
1	Mahan	22	Gonbad-e-kavous*
2	Honak	23	Shahroud
3	Sirach*	24	Nahbandan
4	Jopar*	25	Tabriz
5	Koohbanan	26	Sabzevar2
6	Sabzevar1	27	Ghochan
7	Koohpaye	28	Beshrooyeh
8	Shiraz	29	Dastgerdan
9	Baft*	30	Boushehr
10	Shahdad	31	Gonabd
11	Badrood	32	Ferdos-e- Sivand*
12	Ghanaghestan*	33	Kashmar
13	Birjand*	34	Mashhad*
14	Ravar*	35	Gorgan
15	Tabas*	36	Turbat-e-haeidarye
16	Kerman 1	37	Syria
17	Bojnoord	38	Chenaraan
18	Khomain	39	Daregaz
19	Kerman2	40	Esfahan
20	Ashkhaneh	41	Afghanistan
21	Shirvan*	42	Khaf

*= these accessions were eliminated in field section because of very low germination and not having appropriate plant density.

Table 4. Location and condition of the field in this experiment

Longitude	E47 04
Latitude	N39 34
Altitude	1319
The average rainfall	455 mm
Soil texture	Clay loam
Weather and natural conditions	Moderate cold

germplasms into six and seven groups respectively; clustering of accessions within groups was not completely similar. The main reason for the difference in resolution of RAPD and ISSR is that the two marker techniques targeted different parts of the genome (Souframanien and Gopalakrishna, 2004). When all binary data from two molecular marker systems were gathered, the Jaccard similarity matrix showed a range from 0.45 (between accessions 22 and 26) to 0.90 (between 33 and 34). The related dendrograms represented in Figure 2C. In this state, unlike to two mentioned separate analyses, cluster analysis made eight groups in dendrogram in 0.17 distance unit (number of groups in all three dendrograms were chosen according to results from analysis of molecular variance (AMOVA). The dendrogram was more similar to ISSR cluster rather than RAPD one, which might be due to higher number of ISSR primers in comparison to RAPD primers. As we found about ISSR marker, other dendrograms also did not provide any clear pattern of clustering according to their locations that were collected indicating little location specificity among cumin germplasm. Similar observations were reported in groundnut (Dwivedi et al., 2001) and Shisham (Mohd Arif et al., 2009).

Principle coordinate analysis

The principle coordinate analysis was performed with ISSR, RAPD and ISSR+RAPD data in order to establish the relationship among samples and comparison to cluster analysis (Fig 3). Distribution pattern of accessions in this aspect was mainly similar to the result extracted from cluster analysis.

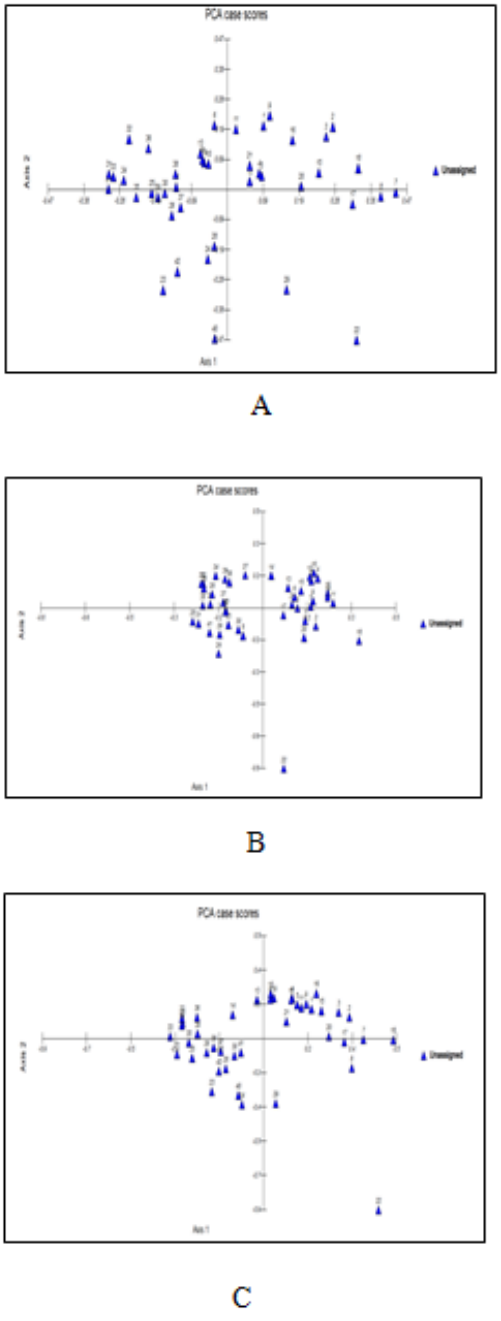

Fig 3. Plot of cumin accessions by principal coordinate analysis using the Jaccard's similarity coefficients (A) ISSR, (B) RAPD and (C) RAPD+ISSR markers (Each plot shows relationships of 42 Cumin accessions based on the marker used).

Mantel test

Finally**,** Mantel test was performed to provide a comparison between extracted similarity matrices from three kinds of markers. The results offered a significant correlation between RAPD similarity matrix and the same extracted matrix from morpho-agronomic traits, while the comparison of ISSR and morpho-agronomic traits similarity matrices was not significant. Tatineni et al. (1996) observed high value for this correlation (r = 0.63) in their study in order to evaluate cotton genetic diversity using RAPD and morphological markers which might be due to inclusion of highly heritable and stable characters. On

Table 5. ISSR and RAPD primers and their amplification results in Cumin genome.

No.	primer	Primer's sequence	PB	TNB	PIC	No.	primer	Primer's sequence	PB	TNB	PIC
ISSR		(3`-5`)	(%)			ISSR		(3`-5`)	(%)		
1	UBC-854	(TC)8RG	86	7	0.44	20	UBC-809&UBC-811	-	42	12	0.40
2	UBC-112	(GACA)4	50	10	0.37	21	UBC-864& UBC-820	-	83	6	0.50
3	UBC-856	(ACAC)4YG	70	10	0.30	22	UBC-827& UBC-864	-	73	11	0.22
4	UBC-811	(GA)8C	50	6	0.47	mean	-	-	66.27	8.86	0.39
5	UBC-820	(ACTG)4	64	14	0.49	RAPD					
6	UBC-855	(AC)8YT	67	9	0.15	1	OPC07	GTCCCGACGA	75	9	0.43
7	UBC-808	(AG)8C	75	12	0.49	2	E17	CTACTGCCGT	82	14	0.48
8	UBC-818	(CA)8G	63	8	0.39	3	T19	GTCCGTATGG	33	3	0.45
9	UBC-872	(GATA)4	86	7	0.48	4	E10	GGTGACTGTG	25	1	0.13
10	UBC-873	(ATG)6	67	3	0.24	5	OPC08	TGGACCGGTG	50	4	0.26
11	UBC-841	(GACAC)4	75	8	0.50	6	OPC15	GACGGATCAG	67	4	0.41
12	A	(GACA)3RT	86	7	0.50	7	T9	CACCCCTGAG	47	7	0.36
13	B	YR(GACA)3	20	5	0.09	8	U11	AGACCCAGAG	79	11	0.17
14	C	(GACAC)2	75	8	0.45	9	A7	GAAACGGGTG	43	3	0.45
15	UBC-864	(ATG)4	93	15	0.39	10	AB1	CCGTCGGTAG	57	13	0.50
16	UBC-809	(AG)8G	40	10	0.45	11	OPC13	AAGCCTCGTC	8	1	0.04
17	UBC-857	(AC)8T	13	8	0.50	12	C16	CACACTCCAG	50	7	0.49
18	UBC-827	(GACT)4	90	10	0.44	13	T18	GATGCCAGAC	64	7	0.41
19	C&A	-	89	9	0.42	mean	-	-	52.3	6.46	0.35

PB= Polymorphic Bonds; TNB= Total Number of Bonds; PIC= Polymorphic Information Content.

the other hand, the low correlation between ISSR marker system and morphological traits that could be caused by marker sampling error and biased representation of genome differences revealed by ISSRs (Schut et al., 1997). The Mantel test also could represent a significant correlation between RAPD+ISSR markers and morpho-agronomic traits based on their similarity matrices in the level of 0.05. All values for each comparison are given in Table 2.

Materials and methods:

Plant materials

In the middle of April, seeds of forty-two cumin accessions (Table 3) were sown in growing season 2010-2011 at the field described in Table 4. In the field experiment, 11 accessions were removed because of very low germination rate and do not having appropriate plant density so 31 accessions in field experiment and 42 accessions in molecular experiment were tested (when morpho-agronomical and molecular data were compared with each other, the additional accessions in molecular experiment were not considered). The laboratory experiments were performed in the Biotechnology Laboratory of Campus of Agriculture and Natural Resources, Razi University of Kermanshah, Iran.

Morphological and phenological characterization

In the current research, the accessions were planted in plots with 1.5 × 1.5 m long. The row spacing and distance between plants were 30 and 5 cm, respectively. Agricultural operations performed under dry conditions and all plants were treated in a uniform manner. After elimination of border effects, plants were harvested and selected eight of them randomly for measuring of all traits.

DNA purification

In the laboratory experiment, cumin young fresh leaves for DNA isolation were harvested from all accessions. DNA extraction carried out based on CTAB method described by Murray and Thompson, (1980). Finally, DNA samples were stored in 20°C before ISSR and RAPD analyses.

RAPD and ISSR amplification

Twenty two ISSR (18 single primers + 4 pairwise combination i.e. used equal amount of two well-done primers instead of one in the PCR mix) and 13 RAPD markers used for screening and exhibiting genetic variation among all accession (Table 5). PCR reaction was performed in a total volume of 25 µl in a FLEXCYCLER thermocycler. The reaction mixture including 2.5 µl PCR buffer (10 mMTris- HCl, 50 mM KCl), 1.6 µl $MgCl_2$ (10 mM), 2.5 µl primer (10µM), 0.4 dNTP mix (0.1 mM), 2.5 µl template DNA (5 ng/µl), 0.2 µl *Taq*-polymerase (5U) and 15.3 µl DDW. Other steps conducted according to Williams et al. (1990). After PCR operation, amplified products were run in 1.2% agarose gel with 0.5×TBE and 1 Kbp DNA ladder. After that, gels were stained with ethidium bromide and visualized via ultra violet.

Statistical analysis

Quantitative analyses of morpho-agronomic traits carried out using SAS (2003) software (analysis of variance and comparison of means with LSD test) and SPSS 16.0 (cluster analysis based on Euclidean distance square). In order to molecular analysis, all amplified bands for each marker among all accessions were scored for the absence (0) or presence (1). MVSP software version 3-13r and NTSYS-pc software version 2.02 were used for cluster analysis, performed via Centroid method and Principle coordinate analysis (PCoA), respectively. Finally, the Mantel`s test (Mantel, 1967) was performed via XLSTAT software.

Conclusion

In conclusion, the present molecular analyses have the potential to be a complementary tool for agro-morphological markers in studying the genetic diversity in cumin germplasm. What could

be concluded from this research is the high ability of molecular makers along with agro-morphological markers to determine the genetic variation of cumin.

Acknowledgment

The authors would like to acknowledge Dr Leila Zarei for her helpful comments and suggestions in the development of this research and all who help us gathering cumin accessions from different regions. Razi University of Kermanshah financially supported this research.

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