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Assessment of Genetic Diversity and Correlation in Fennel (*Foeniculum vulgare* Mill.) Varieties by RAPD and ISSR Markers

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ABSTRACT

Fennel (*Foeniculum vulgare* Mill) belongs to the family: *Apiaceae*, well recognized for its nutritional and pharmacological purposes. In this study, three varieties of fennel were evaluated by molecular characterization using both Random amplified polymorphic DNA (RAPD), Inter-simple sequence repeats (ISSRs) assays to identify their advantages as marker-assisted selection (MAS) in breeding programs. Ten RAPD and five ISSR primers amplified a total of 62 and 24 fragments with a 58.06 and 70.83% polymorphism, respectively. In addition, RAPD markers gave the greatest number of molecular markers 33 with 53.23%, whereas the ISSR loci scored seven markers with 29.17%. The highest genetic distance (GD) for RAPD (0.67) was identified between *Anethum foeniculum* Clairv and *Anethum rupestre* Salisb and ISSR (0.92) and combined (0.73) were recognized between *Anethum foeniculum* Clairv, *Anethum foeniculum* L. In contrast, the lowest GD was (0.48) differentiated between *Anethum foeniculum* L, and *Anethum rupestre* Salisb for RAPD and *Anethum foeniculum* Clairv, and *Anethum rupestre* Salisb for ISSR (0.71) and *Anethum foeniculum* L, and *Anethum rupestre* Salisb for combined (0.56). We found that the ISSR gave the highest number of polymorphism, compared with RAPD. Therefore, a combination of ISSR and RAPD markers is the best method for the genetic variability analyses of different fennel varieties in breeding programs.

Keywords: Cluster analysis, DNA markers, Genetic variability, Heat map analysis, similarity index.

1. Introduction

Hundred years ago, the common fennel (*Foeniculum vulgare* Mill) (2n = 22) was utilized as a pioneering source of medicine. There are various synonyms of *F. vulgare*, such as, *Foeniculum azoricum* Mill, *Anethum foeniculum* clairv, *A. rupestre* Salisb, *A. foeniculum* L, *Feniculum commune* Bubani, *F. foeniculum* (L.) H. Karst, *F. officinale*, *F. capillaceum* Gilib, and *F. dulce* DC, (Hakim *et al.,* 2019). It belongs to the family *Apiaceae* (*Umbelliferaceae*). The fennel is very important for chemical, pharmaceutical, and medical purposes. It cultivates in mild climates and needs to low quantity of water (Omidbaigi, 2007). *Foeniculum vulgare* Mill, one of the important medicinal plants in this family, is a perennial that cultivates naturally in most of the countries, e.g. Egypt, India, and China…etc (Akgul, 1986). Fennel is well recognized and used since antiquity for its taste and for medical purposes (He and Huang 2011; Lucotte *et al.,* 2018; Syed *et al.,* 2019). Besides, the ancient Egyptians and Greeks utilized it as food and in the medical purposes, in China it was considered a snake bite remedy, and Romans and Indians cultivated it for its aromatic fruits (Dellaporta *et al.,* 2003; Aboelsoud, 2010). *Foeniculum vulgare* Mill is applied in medicine for the treatment of several diseases. At present, its herbal remedial and essential oils are widely applied for fever, diarrhea, liver pain, colics in children, flatulence, constipation, stomachache, conjunctivitis, abdominal pains, gastritis, insomnia, arthritis, irritable colon, mouth ulcer, and other conditions (Koppula and Kumar, 2013; Badgujar *et al.,* 2014). Furthermore, many studies have reported not only its anti-microbial, anti-fungal, antioxidant, and anti-cancer characterization, but also its hepatoprotective, hypoglycemic,

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and estrogenic purposes (El-Soud *et al.,* 2011). *Foeniculum vulgare* Mill contains different minerals, trace elements, vitamins, amino acids, fat- and water-soluble, and essential oils (Xue *et al.,* 2006; Rezayat *et al.,* 2018). Despite its interesting pharmaceutical and agronomic properties, *Foeniculum vulgare* Mill still a genetically understudied species. In general, given the lack of genomic data available in the genbanks, DNA-based markers for this species were widely applied to determine the genetic variability of genotypes and to examine the genotoxicity and uniformity of plants micropropagated by organogenesis and embryogenesis (Abou El-Nasr *et al.,* 2013). These assays are widely used because they will present fast results and do not need a design of primer sequences (Godwin *et al.,* 1997). Nowadays, Kelardashti *et al*., (2015) applied Sequence-related amplified polymorphism (SRAP), Random amplified polymorphic DNA (RAPD), and Inter-simple sequence repeats (ISSRs) to assess the genetic variability in 11 *Foeniculum vulgare* Mill individuals.

In this study, three varieties of fennel were evaluated by molecular characterization using both RAPD and ISSR assays to identify their advantages for marker-assisted selection (MAS) in breeding programs.

2. Materials and Methods

2.1. Plant materials

Three different varieties of fennel (*Anethum foeniculum* Clairv, *Anethum foeniculum* L, and *Anethum rupestre* Salisb) were obtained from Al-Sharqia governorate during May 2022. The plants were identified by Mrs Teriza Labib, Al-Orman herbarium. A voucher specimen kept in herbarium of National Research Centre, Dokki, Giza, Egypt.

2.2. Isolation of DNA

Total DNA was isolated from *Foeniculum vulgare* according to the described method by Dellaporta *et al.,* (1983).

2.3. RAPD and ISSR assays

The ten RAPD and five ISSR primers were used to generate reproducible polymorphic DNA products. **Tables (1 and 2)** show the base sequences of these DNA primers for both RAPD and ISSR markers that produced informative polymorphic bands. The amplification reaction was carried out in a DNA thermocycler (Biometra, Germany) according to the protocol proposed by Williams *et al*., (1990). The PCR amplification was performed in a 25 µl reaction volume containing the following: 2.5 µl of dNTPs (2.5 mM), 1.5 µl of $MgCl_2$ (25 mM), 2.5 µl of 10x buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50 ng/µl), 0.3 µl of *Taq* polymerase (5 U/µl) and 14.7 µl of sterile ddH_2O. The reaction of RAPD was subjected to one cycle at 95°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 37°C for 30 seconds, and 72°C for 30 seconds, then a final cycle of 72°C for 12 min. For ISSR, reactions were performed with the following temperature profiles: an initial denaturation step of 94°C/4 min followed by 40 cycles consisting of a denaturation step of 94°C/30 s, a primer annealing step of 52°C/45 s, and an extension step of 72°C/2 min. The last cycle was followed by 72°C/7 min for final extension.

2.4. Gel electrophoresis

The amplified DNA (15 µl) for all samples was electrophoresed on 1% agarose containing ethidium bromide. (0.5 µg/ml) in 1X TBE buffer at 75 constant volt, and determine with a UV transilluminator. The size of each fragment was estimated with reference to a size marker of 100 bp DNA ladder (BioRoN, Germany).

2.5. Data analysis

A matrix for RAPD, ISSR, and combined were generated by scoring reproducible bands as 1 for their presence and as 0 for their absence across three fennel varieties. Genetic similarity coefficients were computed following Nei and Li, (1979). The data was subsequently used to construct a dendrogram using the un-weighted pair group method of arithmetic averages (UPGMA) (Sneath and Sokal, 1973) employing sequential, agglomerative hierarchic, and non-overlapping clustering (SAHN). All the computations were carried out using the software NTSYS-PC (Numerical Taxonomy

and Multivariate Analysis System), version 2.1 (Rohlf, 2000). Correlation coefficients were calculated using similarity coefficients obtained from RAPD, ISSR, and combined analyses.

Table 1: List of the RAPD primer names and their nucleotide sequences used in the study.

NO.	Name	Sequence	No.	Name	Sequence
1	**OP-A02**	5` TGCCGAGCTG 3`	**6**	**OP-A01**	5′ TCGGGGATAG 3`
2	**OP-C09**	5` CTCACCGTCC 3`	**7**	**OP-A07**	5′ GAA AGG GGTG 3`
3	**OP-C12**	5` TGTCATCCCC 3`	**8**	**OP-A18**	5` AGGTGA CCGT 3`
4	**OP-D05**	5` TGAGCGGACA 3`	**9**	**OP-B02**	5′ CCCTGTCGCA 3`
5	**OP-D09**	5` CTCTGGAGACG 3`	**10**	**OP-C03**	5′ GAT GAC CGC C 3`

Table 2: List of the ISSR primer names and their nucleotide sequences used in the study

No	Name	Sequence	No	Name	Sequence
1	**HB-11**	5` GTGTGTGTGTGTTGTCC3`	**4**	**HB- 15**	5′ TGGTGGTGGC 3`
2	**HB- 12**	5′CACCACCACGC 3`	**5**	**HB-14**	5′ CTCCTCCTCGC 3`
3	**HB-13**	5` AGGAGGAGGC 3`			

2.6. Principal component analysis (PCA)

PCA was also carried out to show multiple dimensions of the distribution of the three fennel varieties in a scatter-plot by PAST software version 1.62 (Hammer *et al.,* 2001).

3. Results

3.1. RAPD-PCR fingerprinting

The genetic diversity among three fennel varieties (*Anethum foeniculum* Clairv, *Anethum foeniculum* L, and *Anethum rupestre* Salisb) was estimated by using ten RAPD-PCR primers to identify the DNA fingerprints among tested varieties (Table 3 and Fig. 1). The ten RAPD primers gave 62 amplicons ranged from 180 to 1230 bp, involving 36 polymorphic bands (58.06%) and 26 fragments were monomorphic (41.94%). The number of produced amplicons per primer varied from three for RAPD primers OP-C09 and OP-C12 to ten for RAPD primer OP-A18. The number of polymorphic bands also ranged from one for primers RAPD OP-C12 and OP-D09 to seven for RAPD primer OP-C03. Moreover, the RAPD primer OP-C09 recoded the greatest value of polymorphism of 100%. In contrast, RAPD primer OP-D09 gave the smallest value of polymorphism of 25%. In addition, ten RAPD primers gave 23 molecular markers with different molecular sizes, such as, *Anethum foeniculum* Clairv, which scored two negative markers of (-320 and -920 bp), using primer RAPD OP-D05. Besides, *Anethum foeniculum* L showed 12 markers with different molecular sizes of (-800), (-560 and -840), (+380), (-601), (+610 and +690), and (-280, -405, -500, -560, and -700) bp, using primers RAPD OP-A02, OP-C12, OP-A07, OP-A01, OP-B02, and OP-C03, respectively. Finally, *Anethum rupestre* Salisb exhibited 16 specific bands of -400 bp (primer OP-D09), -190, +350, +400, +645, and +650 (primer OP-A18), -410, -500, -605, and -900 bp (primer OP-A07), +700 and -300 bp (primer OP-A01), +310 and +860 bp (primer OP-B02), and +200 and +900 bp (primer OP-C03) (Table 3).

Table 3: The RAPD and ISSR marker analyses of the three fennel varieties.

Molecular type	Primer code	Range of amplified (bp)	Total bands	Monomorphic bands	Polymorphic bands	Polymorphic Percentage %	Specific marker
RAPD	OP-A02	220 -1230	5	2	3	60	+220,-800
	OP-C09	370-560	3	0	3	100	0
	OP-C12	480-840	3	2	1	33.3	-560,-840
	OP-D05	320-920	5	2	3	60	-500,-920
	OP-D09	400 -780	4	3	1	25	+500,+700,-400
	OP-A01	205-700	7	4	3	42.86	-300,-601
	OP-A07	180-900	8	3	5	62.5	+380,-410,-500,-605,-900
	OP-A18	190-700	10	5	5	50	+350,+400, +645,+650,-190
	OP-B02	300-860	9	4	5	55.56	+310, +370,+610, +690, +860
	OP-C03	200-900	8	1	7	87.5	+200,+900, -280,-405,-500,-560,-700
Total		**180-1230**	**62**	**26(41.94%)**	**36**	**58.06**	**33 (53.23%)**
ISSR	14A	300 - 520	3	0	3	100	0
	44B	240 - 1230	5	2	3	60	-240,-1230
	HB-11	320 - 1290	5	2	3	60	+430,+1290
	HB-12	200 - 600	4	2	2	50	-200,-400
	HB-13	330 - 400	2	0	2	100	0
	HB-15	280 - 1360	5	1	4	80	-1360
Total		**200-1360**	**24**	**7 (29.17%)**	**17**	**70.83**	**7 (29.17%)**

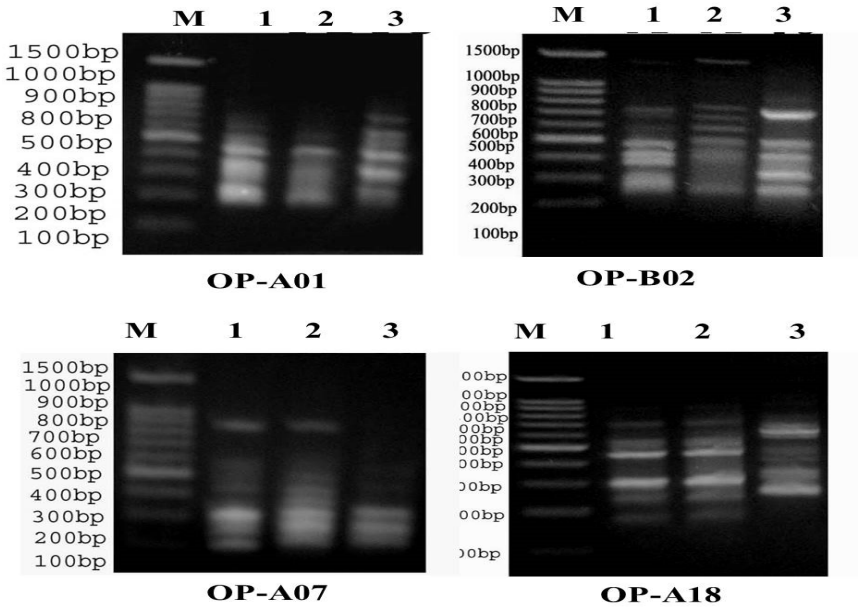

Fig. 1: RAPD profiles of three different varieties of fennel using primers OP-A18, OP-A07, OP-A01, and OP-B02. Lane M: 100 bp DNA ladder. Lane 1: *Anethum foeniculum* Clairv; lane 2: *Anethum foeniculum* L., and lane 3: *Anethum rupestre* Salisb.

3.2. Cluster analysis depending on RAPD-PCR

The cluster analysis among the three different fennel varieties of RAPD-PCR was assessed based on the UPGMA. The genetic closeness was determined between 0.67 and 0.48, revealing a high level of similitude. The maximum genetic distance (GD) was (0.67) identified between *Anethum foeniculum* Clairv, and *Anethum rupestre* Salisb, while the minimum value was (0.48) differentiated between *Anethum foeniculum* L. and *Anethum rupestre* Salisb. The dendrogram revealed two clusters (I and II); the first cluster (I) involved *Anethum foeniculum* Clairv and *Anethum rupestre* Salisb. However, the second cluster (II) consisted of var. *Anethum foeniculum* L. (Figs. 3 and 4).

3.3. ISSR-PCR fingerprinting

The genetic variability among three fennel varieties was determined by using five ISSR markers to identify the DNA fingerprints (Table 3 and Fig. 2). The five ISSR-PCR primers produced 24 amplified fragments ranging from 200 to 1360 bp, involving 17 polymorphic amplicons (70.83%), and seven bands were monomorphic (29.17%). The number of produced bands per primer varied from two for ISSR HB-13 to five for primers HB-13. The number of polymorphic bands also ranged from two for primer HB-13 to four for primers 44B, HB-11, and HB-15. Furthermore, primer 14A and primer-HB-13 scored the highest value of polymorphism of 100%. On the contrary, ISSR primer-HB-12 gave the lowest value of polymorphism of 50%. On the other hand, five ISSR primers recorded seven markers with different molecular sizes, e.g., *Anethum foeniculum* Clairv displayed two positive markers of (+430 and +1290 bp), using primer ISSR-HB-11. Furthermore, *Anethum rupestre* Salisb displayed five negative markers with different molecular sizes of (-240 and -1230), (-200 and -400), and (-1360) bp, using primers 44B, HB-12, and HB-15, respectively (Table 3).

3.4. Cluster analysis depending on ISSR-PCR

The cluster analysis among the three different fennel varieties of ISSR markers was determined depending on the UPGMA. The genetic closeness was determined between 0.71 and 0.92, revealing a high level of similitude (Fig. 3). The most elevated genetic similarity was (0.92) identified between *Anethum foeniculum* Clairv and *Anethum foeniculum* L., while the least value was (0.71) discriminated between *Anethum foeniculum* Clairv, and *Anethum rupestre* Salisb. The dendrogram revealed two clusters (I and II); the primary cluster (I) has *Anethum foeniculum* Clairv and *Anethum foeniculum* L. However, the second cluster (II) consisted of var. *Anethum rupestre* Salisb (Figs. 3 and 4).

3.5. Cluster analysis depending on RAPD and ISSR combined

The hereditary likeness and clustering structure among the three fennel varieties of RAPD and ISSR combined depended on the heat map. The hereditary likeness was determined between 0.56 and 0.73 (Figs. 3). The highest similarity (0.73) was recognized between *Anethum foeniculum* Clairv and *Anethum foeniculum* L, while the lowest identity was (0.56) differentiated between *Anethum foeniculum* L, and *Anethum rupestre* Salisb. The dendrogram revealed two major clusters (I and II): The 1st cluster (I) contained *Anethum foeniculum* Clairv and *Anethum foeniculum* L., and the 2nd cluster (II) consisted of *Anethum rupestre* Salisb (Figs. 3 and 4).

3.6. A principal component analysis

A principal component analysis (PCA) plot was applied for the three fennel varieties using ISSR, RAPD markers, and combined. The PCA classified three varieties into three groups A, B, and C (Fig. 5). The results of the PCA plot correlate with the cluster analysis produced by the UPGMA dendrogram and show results that match those of the dendrogram.

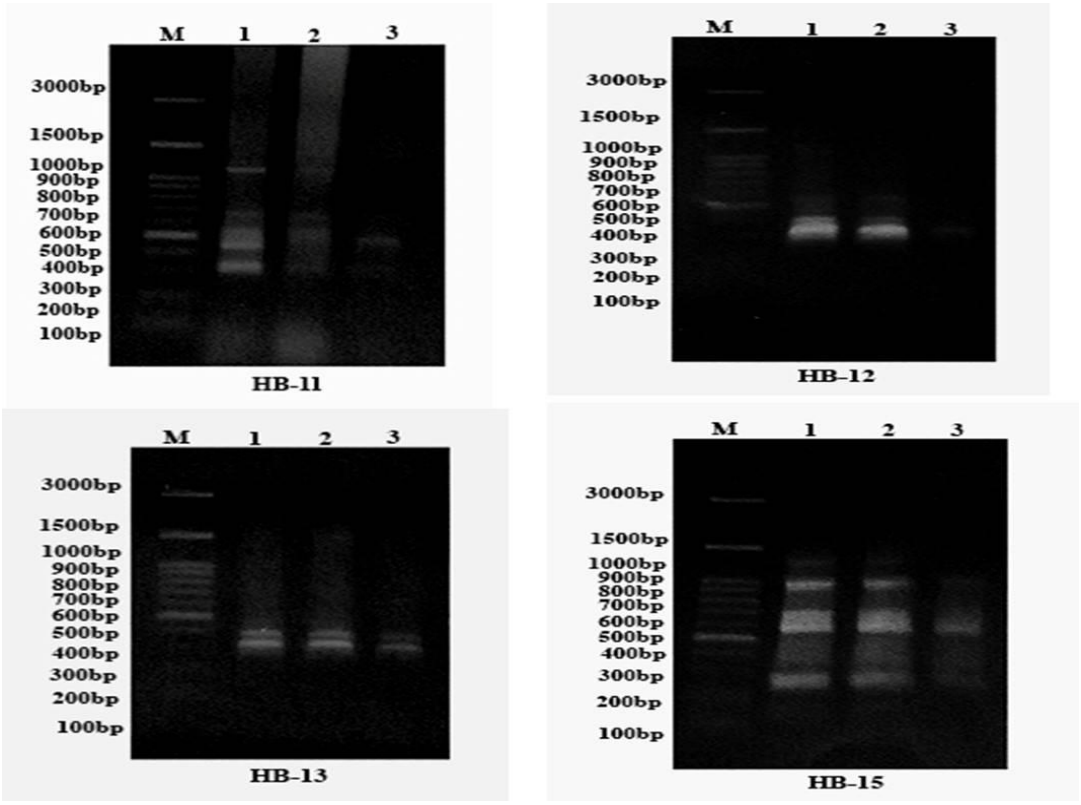

Fig. 2: ISSR profiles of three different varieties of fennel using primers OP-A18, OP-A07, OP-A01, and OP-B02. Lane M: 100 bp DNA ladder. Lane 1: *Anethum foeniculum* Clairv; lane 2: *Anethum foeniculum* L., and lane 3: *Anethum rupestre* Salisb.

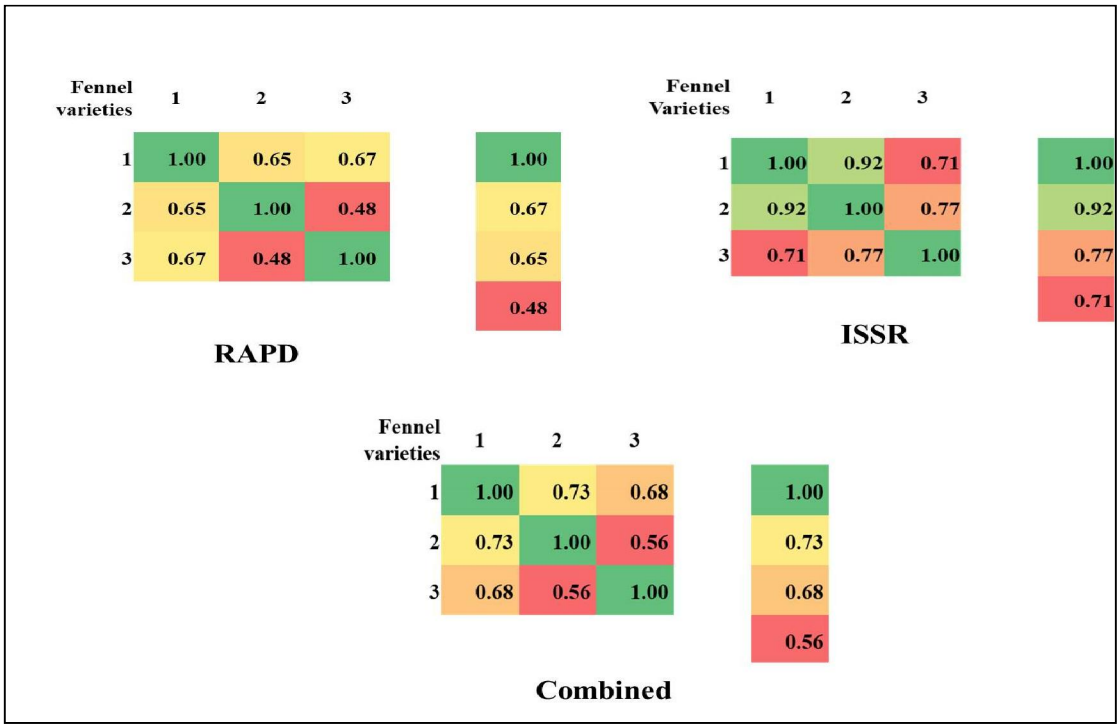

Fig. 3: Heat map of three fennel varieties depending on RAPD, ISSR, and combined by UPGMA algorithm using Jaccard's similarity coefficient. Number refers to variety code.1: *Anethum foeniculum* Clairv; 2: *Anethum foeniculum* L., and 3: *Anethum rupestre* Salisb.

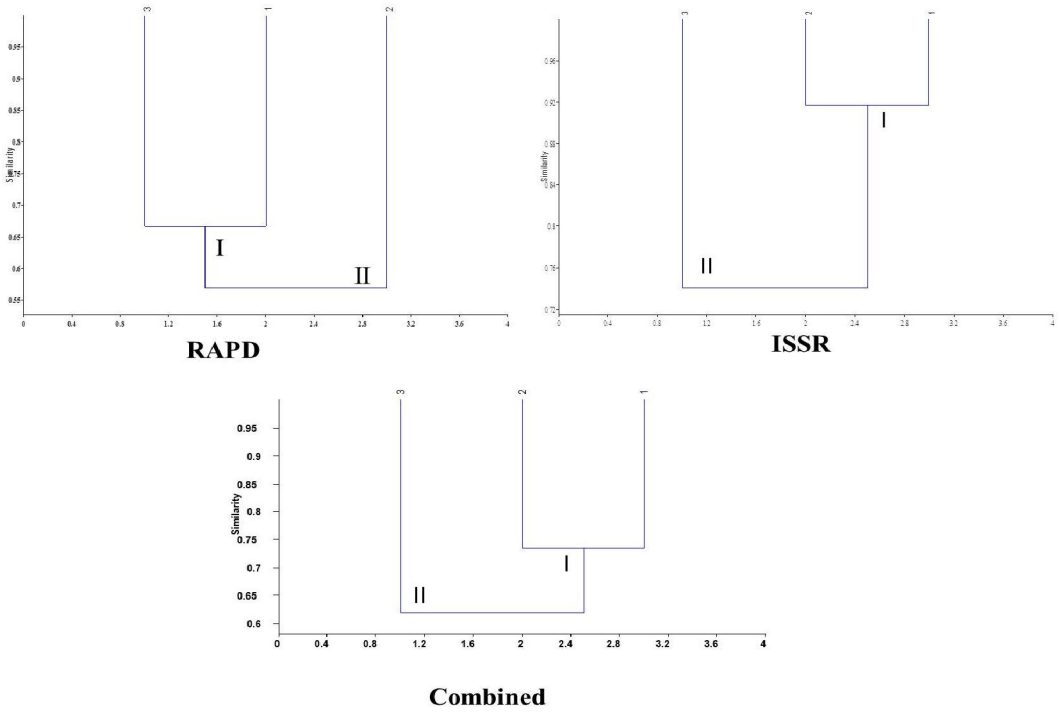

Fig. 4: The cluster tree of three fennel varieties based on RAPD, ISSR, and combined polymorphism. Number refers to variety code. 1: *Anethum foeniculum* Clairv; 2: *Anethum foeniculum* L., and 3: *Anethum rupestre* Salisb.

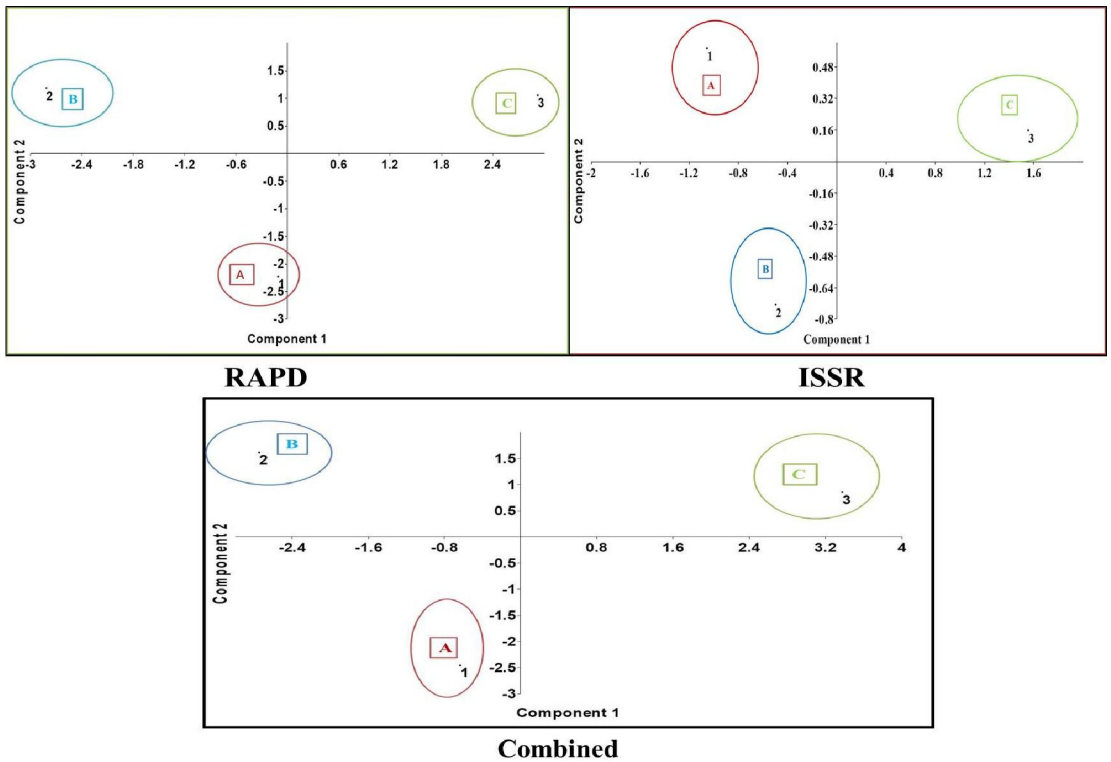

Fig. 5: The principal component analysis (PCA) of three fennel varieties based on RAPD, ISSR, and combined polymorphism. Number refers to variety code. 1: *Anethum foeniculum* Clairv; 2: *Anethum foeniculum* L., and 3: *Anethum rupestre* Salisb.

4. Discussion

The determination of genetic diversity is the basis for the identification of germplasm to discriminate plant species. DNA markers e.g., RAPD or ISSR assays are important methods for the analysis of the genetic variability, and screening of favorite germplasm for crosses as well as for genotype preservation in gene banks. In addition, the polymorphism that was estimated by these molecular markers is one of the useful criteria for study of individuals and understanding of their genetic variability (Zietkiewicz and Labuda, 1994; Alvarez *et al.,* 2007). In this study, the enforcement of RAPDs and ISSRs as genetic markers to describe the *Foeniculum vulgare* Mill varieties was evaluated. The results showed that a total number of magnified bands were higher for RAPD (62 bands), compared with ISSR (24 fragments). In addition, ISSR gave the highest percentage of polymorphic bands of 70.83% compared with RAPD of 58.06. Therefore, ISSR loci were more polymorphic compared with RAPD markers because the ISSR markers include microsatellite sequences, distributed throughout the genome. Besides, ISSR technique has a high rate of mutation and may link to functionally important sites (Penner, 1996). However, RAPD techniques are placed in non-coding regions. Additionally, the ISSR markers are more polymorphic (Goulao and Oliveira, 2001). Lal *et al.,* (2010) reported that the greatest polymorphism (95%) was recorded in ISSR loci, compared with RAPD (87%) markers. They tested the efficiency of all these DNA markers in *Cicer arietinum* L. and *Cajanus cajan* L.

Ramadan *et al.*, (2019) studied the genetic diversity among the six varieties of fennel plants by six ISSR markers and indicated that the percentage of the polymorphism was 60.3%. Previous studies reported that the ISSR markers are more effective compared with RAPD technique and differentiate closely related varieties in different plant species (Singh *et al*., 2012). In contrast, other reports showed that the RAPD markers are more polymorphic than the ISSR technique because it includes the coding and non-coding sequences into the genome (Patel *et al*., 2016).

Andrea *et al.*, (2004) studied the genetic stability and uniformity of *Foeniculum vulgare* regenerated plants through organogenesis and somatic embryogenesis. They observed that the use of RAPD markers is more serviceable than RFLP markers because large numbers of samples can be analyzed economically and fast, the specific DNA fingerprints gained are independent of ontogenic expression, and most of the genome can be sampled with a potentially unlimited number of markers.

In the current investigation, the cluster analysis among the three different fennel varieties of RAPD, ISSR, combined was assessed based on the UPGMA. The results indicated that the highest genetic distance (GD) for RAPD (0.67) was identified between *Anethum foeniculum* Clairv, and *Anethum rupestre* Salisb. However, ISSR (0.92) and combined (0.73) were recognized between *Anethum foeniculum* Clairv and *Anethum foeniculum* L. In contrast, the lowest GD was (0.48) differentiated between *Anethum foeniculum* L., and *Anethum rupestre* Salisb for RAPD, and *Anethum foeniculum* Clairv and *Anethum rupestre* Salisb for ISSR (0.71) and *Anethum foeniculum* L., and *Anethum rupestre* Salisb for combined (0.56). Therefore, the cluster analysis among the three fennel varieties depended on RAPD and ISSR combined matched with ISSR. So, the molecular characterization by may prove to be useful for the genetic improvement, breeding programs, and keeping of DNA variability of fennel species. Cluster analysis is an efficient method for grouping populations or grmplasms (Morphy *et al*., 1992). Lamare and Rao, (2015) mentioned that a combination of RAPDs and ISSR assays is considered the best methods for studying of genetic diversity among population.

In this regard, PCA plot was applied for the three fennel varieties using ISSR, RAPD markers, and combined. The PCA classified three varieties into three groups A, B, and C. The results of the PCA plot correlate with the cluster analysis generated by the UPGMA dendrogram. These results were in agreement with Perkins, (1972) who mentioned that PCA analysis is widely used in the expression of genetic diversity. This analysis can be applied to estimate variability patterns as multi-dimensional. PCA displays populations in more than one dimension compared with the cluster analysis, which decreased variability of data in a single dimension (number of principle components). This action presents the possibility of further interpretation with reference to the relationship between tested cultivars.

This study showed new data at the molecular level for the genetic variability of *Foeniculum vulgare* for a public phylogenetic relationship between the three different fennel varieties of this plant. The polymorphism information generated can be applied in plant breeding programs, crop

amelioration programs and might be helpful in future planning for the development of new fennel genotypes.

5. Conclusions

In the current work, RAPD-PCR, ISSR-PCR, and combined were used to analyze the genetic diversity among three different fennel varieties. The results showed that RAPD-PCR and ISSR-PCR combined is considered the best method for the analysis of genetic diversity among tested fennel varieties. The cluster analysis among the three varieties depended on RAPD and ISSR combined was determined. The results showed that the highest hereditary similarity was recognized between *Anethum foeniculum* Clairv and *Anethum foeniculum* L., while the lowest identity was identified between *Anethum foeniculum* L., and *Anethum rupestre* Salisb. So, the molecular characterization may prove to be useful for the genetic improvement, breeding programs, and keeping of DNA variability of fennel species.

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