RESEARCH ARTICLE

Analysis of Fennel Breeding Populations Based on Distinctness, Uniformityand stability (DUS) testing via Morphological Descriptors and DNA Molecular Markers

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Abstract A bred plant variety is a valuable tool for enhancing crop production and total yield. Recently, our breeding team developed three high seed yielding synthetic cultivars of bitter fennel. To characterize these newly breeding populations and differentiate them from each other and their parental populations, we conducted a Distinctness, Uniformity, and Stability (DUS) assessment using morphological traits evaluated over two consecutive years, as well as 20 Start Codon Targeted (SCoT) markers. The evaluation of morphological traits revealed that, five of qualitative traits exhibited monomorphism, while two other qualitative traits displayed polymorphism. Additionally, the investigation of eight quantitative traits showcased significant diversity. The Combined Over Years Distinctiveness analysis at a 1% significance level, based on eight quantitative characteristics, indicated distinctiveness for two breeding populations, namely Early and Medium Maturity Synthetic cultivars, compared to other populations. The Combined Over Years Uniformity analysis identified that all of the breeding populations and their parental populations were uniform in terms of the measured traits. The differences between Phenotypic Coefficients of Variation and Genotypic Coefficients of Variation were not particularly noticeable in the experimental material used currently. The majority of the measured traits showed high heritability, highlighting their stability over time. PCA utilized for quantitative morphological features, the first two principal components collectively accounted for more than 86.8% of the phenotypic variation. Subsequently, a dendrogram was constructed, which clustered breeding and parental populations into three distinct groups. The 15 SCoT markers produced a total of 164 extended fragments, with 139 (85%) of them showed polymorphic.The genetic similarity between the populations exhibited a range of 0.08–0.81, and cluster analysis utilizing Jaccard similarity coefficients and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) technique revealed clear distinctions among the three breeding populations and their parental populations. Altogether, the findings indicate that morphological DUS descriptors effectively distinguish some the populations, while others with overlapping expressions cannot be discriminated. Notably, SCoT markers demonstrate a fine ability to detect distinctions among fennel populations, suggesting the possibility of utilizing them as a complement to morphological traits in the DUS test. As a conclusion, these three

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newly breeding populations of fennel are distinct from each other and from their parental populations, and they can advance for registration and seed purity testing.

Keywords DUS test · Fennel · Morphology · SCoT marker · Distinctiveness · Synthetic cultivars

Introduction

Discoveries in Kurdistan's Shanidar Cave suggest that the history of human use of medicinal plants dates back as far as 60,000 years ago (Lietava 1992). Bitter fennel (*Foeniculum vulgare Mill. var. vulgare*), as the source subspecies of fennel derived products, is one of the earliest medicinal plants, with numerous applications in food, beverage, and pharmaceutical industries. Bitter fennel, hereafter referred to simply as fennel, originated from the Mediterranean regionbut nowadays it is cultivated almost worldwide. The most economically valuable product of fennel plants is the seeds, which are mostly used for essential oil extraction (Rather et al. 2016; Barros et al. 2010). Numerous research indicated that fennel herbal medicines possess valuable curative properties such as antifungal, anti-cancer, antibacterial, anti-inflammatory, and antioxidant effects (Hamdy Roby et al. 2013; Ouariachi et al. 2014; Oliveira et al. 2015; Salami et al. 2016; Afifi et al. 2021; Akbari et al. 2023). Additionally, various pharmaceutical products in the market are formulated based on the phytochemical substances found in fennel such as trans anethol and methyl chavicole (Kooti et al. 2015; Edoardo et al. 2010; Elagayyar et al. 2001).

The global fennel seed production is estimated at nearly 800 thousand tons, with Iran emerging as a major contributor (FAO 2021). Iranian fennels exhibit remarkable diversity in habitats, and their adaptation to local environments over an extended period suggests the presence of distinct fennel landraces in each region. This has been proved through morphological research (Bahmani et al. 2012a, b, 2015, 2024; Shojaiefar et al. 2015; Akbari et al. 2015), phytochemical combinations (Akbari et al. 2023, 2024; Izadi Darbandi et al. 2023; Bahmani et al. 2016), genetic properties (Sheidai et al. 2007; Bahmani et al. 2012a, b, 2013; Shojaiefar et al. 2015).

To meet the rising global demand for fennel seeds, it is imperative to increase fennel seed yield. To achieve this, our options are applying optimized agronomic management and along with developing high yielding fennel cultivars. To improve agronomic management in fennel farms, some researchers reported different kinds of chemical and organic fertilizers, irrigation plans, sowing date, and depth, and plant density to get more seeds and essential oil per area (Ehsanipour et al. 2012; Khorshidi et al. 2010; Mohamed and Abdu 2004). All of these factors can increase fennel yields but not to the level that can fulfill the world's need, while plant breeding has almost endless prospects in developing high yielding cultivars.

Intentional exploitation of heterosis through breeding efforts has led to significant yield increases in numerous crops with hybrid breeding playing a pivotal role. Successful hybrid breeding relies on effective pollination control, and for this, male sterility systems are highly useful. In the absence of such system for hybrid cultivars, synthetic cultivar arises as an alternative approach to utalize hybrid vigor (Liu et al. 2020; Maydell et al. 2021). Heterosis exhibits notable efficacy in outcrossing crops such as fennel with cross-pollination rates ranging from 82.2 to 91.4% (Ramanujam et al. 1964; Sawargaonkar et al. 2018). Population improvement through methods such as synthetic cultivar development is highly recommended for fennel breeding programs (Poehlman and Sleper 1995).

Any newly developed cultivars must be distinct (D) from previously released cultivars, uniform (U) withing the population, and stable (S) in terms of morphological and other examined features (Pourabed et al. 2015). The International Union for the Protection of New Varieties of Plants (UPOV) criteria require the fundamental Distinctness, Uniformity, and Stability (DUS) test for identifying and registering newly developed cultivars. The establishment of distinctiveness is the most important aspect of DUS testing. If the differences in traits are constant and obvious, a variety might be deemed distinctive (UPOV 2002). The DUS tests are mostly founded on morpho-physiological features recorded from plants grown in the same environment in a close vicinity (Cooke 1995). There are 27 morphological DUS descriptors that are suggested for performing DUS test for fennel (UPOV 2018). Because there

are so many existing cultivars, the traditional method of DUS experiments is time-consuming and costly, necessitating high expenses and specialized workers, as well as often subjective decisions (Cooke 1995). Furthermore, most of those characters are quantitative, and their expression is influenced by environment, so replicated assessment is highly necessary. Compared to morphological traits, incorporating molecular markers into DUS testing has been demonstrated to be faster and more cost-effective (Mailer et al. 1994). Molecular markers, consisting of nucleotide sequences displaying polymorphism among individuals due to variations like insertion, deletion, point mutations, duplication, and translocation, and they do not necessarily impact their corresponding gene's activity. An optimal DNA marker should showcase co-dominance, uniform genomic distribution, high reproducibility, and the ability to detect significant levels of polymorphism (Nadeem et al. 2018). Various types of molecular markers, including microsatellites (Wang et al. 2015; Sousa et al. 2017; Tiwari et al. 2018; Gupta et al. 2022), have been assessed for their efficacy in DUS tests. Restriction Fragment Length Polymorphism (RFLP) (Gebhardt et al. 1989), Amplified Fragment Length Polymorphism (AFLP) (De Riek et al. 2001), Sequence tagged Microsatellite Site (STMS) (Singh et al. 2004), Random Amplified Polymorphic DNA (RAPD) (Yadav and Sciences 2010), Inter Simple Sequence Repeats (ISSR) (Prevost and Wilkinson 1999), RAPD and ISSR (Shukla et al. 2011; Lu et al. 2009) are reliable and effective methods of cultivar identification.

The Start Codon Targeted (SCoT) polymorphism is widely used in PCR-based molecular markers due to its rapidity, cost-effectiveness, minimal DNA quantity needed, and the absence of a prerequisite for DNA sequence knowledge. SCoT markers produce more reliable and uniform bands, establishing them as influential polymorphic markers that effectively highlight distinctions among individuals. Hence, this method can find applied in diverse fields, encompassing genetic mapping and population research in the realm of plants (Collard and Mackill 2009).

Our research group has developed innovative fennel breeding populations, each consisting of three synthetic cultivars strategically designed to improve yield performance. For any newly developed population to be considered a new variety, distinctness, uniformity, and stability are crucial factors. This study aimed to evaluate the second-generation breeding populations against their parental populations over two years, utilizing the DUS test through morphological traits. Additionally, 20 SCoT markers were employed to enhance the discrimination and identification of fennel breeding populations.

Materials and methods

Plant material

Our breeding team developed breeding populations comprising three synthetic cultivars by crossing various populations possessing enhanced general combining ability to improve yield performance (Bahmani et al. 2023). The breeding populations, alongside selected elite parents were utilized in this research. Specifically, Early Maturity Synthetic involving two of its parental populations (Hashtgerd and Fasa), Medium Maturity Synthetic involving four of its parental populations (Moghan, Fozve, Meshkinshahr, Khash), and Late Maturity Synthetic involving two of its parental populations (Chahestan and Hajiabad) were employed in this research. The study included DUS testing based on morphological traits recorded in 2018 and 2019, as well as the analysis of 20 populations using SCoT markers (Table 1).

Morphological characterization

Field experiment

The investigation took place in the 2018 and 2019 growing seasons, where seeds of the populations were sown in an open field testing operation under a randomized complete block design (RCBD) with three replications. The field had sandy loam soil and was located in Pakdasht (Tehran province, Iran), with a semi-arid climate (Tables 2). Each population was planted in a 3 m^2 plot, with an interrow distance of 25 cm to achieve a plant density of 10 plants per m^2 in both years (Khorshidi et al. 2010). No diseases or pests were detected throughout the growing seasons.

Table 1 Geography profile of origination regions of fennel populations

No	Ecotype	Altitude (m)	Latitude	Longitude	No	Ecotype	Altitude (m)	Latitude	Longitude
1	Meshkin shahr	1568	38 23 N	47 40 E	11	Nairiz	1632	29 12 N	54 20 E
2	Fozveh	1612	32 36 N	51 26 E	12	Sardasht	1670	36 9 N	45 30 E
3	Chahestan	27	27 13 N	56 22 E	13	Givi	1682	37 41 N	48 28 E
4	Khash	1394	28 13 N	61 12 E	14	Ardabil	1332	38 15 N	48 17 E
5	Moqhan	31	39 39 N	47 55 E	15	Fasa	1288	28 58 N	53 41 E
6	Sarpol zahab	548	34 27 N	45 54 E	16	Arak	1708	34 6 N	49 46 E
7	Haji abad	931	28 19 N	55 55 E	17	Rafsanjan	1580	30 25 N	55 54 E
8	Hasht gerd	1426	35 65 N	50 43 E	18	Early maturity synthetic (Early-Syn)			
9	Divandare	2142	36 4 N	46 55E	19	Medium maturity synthetic (Medium-Syn)			
10	Razan	1870	35 21 N	49 4 E	20	Late maturity synthetic (Late-Syn)			

Table 2 The climatic conditions of the experimental site

Months	Monthly average temperature (°C)		Monthly relative humidity (%)		Monthly total rainfall amount (mm)	
	2018	2019	2018	2019	2018	2019
January	5.8	6	46.1	50	31.75	49.29
February	8.4	7.2	42	43	27.43	18.53
March	17	10.5	25.6	41.4	5.32	62.48
April	16.7	15.5	36.2	35.7	54.35	65.02
May	21.4	24	34.9	24	24.88	6.09
June	29.1	30.5	20	17.1	5.58	1.02
July	34	33	12.9	17	0	0
August	31.7	30.5	18.6	19.4	0	0
September	26.9	26.6	20.7	23	0.25	0
October	19.2	19.6	38.9	38.5	14.23	66.82
November	10.7	9.4	59.2	55.3	72.39	21.84
December	8.9	7.8	49.9	55.9	26.92	24.13
Average	19.4	18.4	34.1	35	–	–
Total	–	–	–	–	263.10	315.22

Data collection

For the two-year data collection, we randomly selected twenty plants in each plot and assessed a range of morphological traits. These traits comprised eight quantitative and seven qualitative characteristics recommended by the DUS test guidelines (Table 3).

Statistical analysis for the morphological data

To establish distinctiveness based on visually assessed traits, we considered distinctiveness between two populations evident if the expression of one or more traits distinctly fell into two states, as outlined in the test guidelines. The analysis of the eight quantitative DUS traits was performed using DUSNT software (Watson 2004). Following UPOV guidelines (2008a and 2008b), we conducted analyses for Combined Over Years Distinctiveness (COYD) and Combined Over Years Uniformity (COYU). Comparing the genotypic and phenotypic coefficients of variation (GCV and PCV), as well as heritability using pooled data of quantifiable variables collected over two years, allowed for the assessment of stability. To provide more information, Principal Component Analysis and Clusters Analysis were executed utilizing R statistical software (version 4.2.3).

Table 3 Characters used in Distinctness, Uniformity, and Stability (DUS) testing of fennel populations

No	Character	State	Types of assessment	
1	Only cultivars without grumolo: Young plant: length of cotyledons (LC)	Short	3	MS/VG
		Medium	5	
		Long	7	
2	Foliage: intensity of green color (IGC)	Very ligh	1	VG
		Light	3	
		Medium	5	
		Dark	7	
		Very dark	9	
3	Only cultivars without grumolo: Young plant: length of petiole of first leaf (LPPFL)	Short	3	MS/VG
		Medium	5	
		Long	7	
4	Leaf: curvature of tip (LCT)	Absent	3	VG
		Weakly expressed	5	
		Strongly expressed	7	
5	Foliage: color (FC)	Green	1	VG
		Brownish	9	
6	Grumolo formation (GF)	Absent	1	VG
		Present	9	
7	Foliage: attitude (FA)	Erect	3	VG
		Semi-erect	5	
		Horizontal	7	
8	Foliage: density (FD)	Sparse	3	VG
		Medium	5	
		Dense	7	
9	Time of appearance of main umbel (TAMU)	Early	3	VG
		Medium	5	
		Late	7	
10	Time of beginning of flowering (TBF)	Early	3	VG
		Medium	5	
		Late	7	
11	Main stem: height at flowering (HF)	Short	3	MS/VG
		Medium	5	
		Long	7	
12	Main umbel: diameter (UD)	Small	3	MS/VG
		Medium	5	
		Large	7	
13	Fruit: thousand fruit weight (TFW)	Low	3	MG
		Medium	5	
		High	7	
14	Leaf: length (LL)	Small	3	MS/VG
		Medium	5	
		Large	7	
15	Male sterility (MS)	Absent	1	VS
		Present	9	

PCR amplification through SCoT markers

DNA extraction

Genomic DNA was extracted from young leaves of the 20 populations using the CTAB method as described by Doyle and Doyle (1987) with specific modifications, as outlined by Kafkas et al. (2006). The quality of the DNA samples was verified using a Qubit Fluorometer (Invitrogen) and electrophoresis. Subsequently, the concentration of DNA samples were adjusted to 5–10 ng/μl using Tris–EDTA buffer, and the samples were then stored at −20 °C for further analysis.

Genetic assessment

As indicated in Table 4, 20 SCoT primers were used for amplification to genetically evaluate the 20 populations (Collard and Mackill 2009; Yan et al. 2016). Polymerase chain reaction (PCR) was carried out in a final volume of 20 μl using 10 μl of master mix (2X amplicon), 2 μl of primer (10 M), 2 μl of genomic DNA, and 6 μl of deionized water after adjusting the amplification conditions. The following temperature condition was used to amplify PCR: initial denaturation at 94 °C for 4 min, followed by 35 cycles including DNA denaturation at 94 °C for 1 min and DNA annealing at 49–62 °C for 1 min, then extension at 72 °C for 2 min and a final extension at 72 °C for five minutes. The PCR product was separated using electrophoresis for 1 h at 80 V in a %1.5 agarose gel with TAE buffer. The separated bands were observed using ethidium bromide and UV light.

Statistical analysis for the SCoT markers

After electrophoresis of the PCR products, the clear and sharp bands were scored so that each allele amplified with the primers was scored as 1 for presence, while absence was indicated by 0. To analyze the genetic data, the number of total bands (TB), the number of polymorphic bands (PB), and the percentage of polymorphism (% PL) were determined. Marker performance measures such as polymorphic information content (PIC), marker index (MI), and resolving power (RP) were

Table 4 Primers codes and sequence used in Start Codon Targeted (SCoT) analyses

	Primer code	Sequence (5′–3′)	GC (%)
1	SCoT-2	CAA CAA TGG CTA CCA CCC	55.6
2	SCoT-5	CAACAATGGCTACCACGA	50
3	SCoT-9	CAACAATGGCTACCAGCA	50
4	SCoT-11	AAGCAATGGCTACCACCA	50
5	SCoT-14	ACG ACA TGG CGA CCA CGC	66.7
6	SCoT-16	ACCATGGCTACCACCGAC	61.1
7	SCoT-17	ACC ATG GCT ACC ACC GAG	61.1
8	SCoT-19	ACCATGGCTACCACCGGC	67
9	SCoT-24	CAC CAT GGC TAC CAC CAT	55.6
10	SCoT-26	ACC ATG GCT ACC ACC GTC	61.1
11	SCoT29	CCATGGCTACCACCGGCC	72.2
12	SCoT-30	CCA TGG CTA CCA CCG GCG	72.2
13	SCoT-31	CCA TGG CTA CCA CCG CCT	66.7
14	SCoT-34	ACCATGGCTACCACCGCA	61.1
15	SCoT-35	CATGGCTACCACCGGCCC	72
16	SCoT-36	GCAACAATGGCTACCACC	55
17	SCoT-37	CAA TGG CTA CCA CCG GCC	72.2
18	SCoT-40	CAATGGCTACCACTACAG	50
19	SCoT-44	CAATGGCTACCATTAGCC	50
20	SCoT-59	ACAATGGCTACCACCATC	50

calculated. The PIC value for each primer was calculated as $PIC_i = 2 f_i (1-f_i)$, where PIC_i represents the PIC of the ith marker, f_i denotes the frequency of the fragments that are present, and $1-f_i$ denotes the frequency of absent fragments (Roldán-Ruiz et al. 2000). Prevost and Wilkinson (1999) determined the marker index (MI) employing the following equation: $MI = PIC \times$ number of polymorphism bands. According to Gilbert et al. (1999), the equation $Rp = \Sigma Ib$ represents the Resolving power (Rp), where the formula for Ib is $1 - 2 \times |0.5 - p|$. In this equation, p denotes the frequency of the presence of a specific band (or allele) in the population. To determine similarity level among the populations, Jaccard's coefficient of similarity was estimated (Jaccard 1908). Cluster analysis based on the Unweighted Pair Group Method with Arithmetic

Mean (UPGMA) technique was conducted using R statistical software, version 4.2.3.

Results

Morphological characterization for DUS test

All eleven fennel populations exhibited consistent scores for seven qualitative traits measured over two years of study. Among these traits, foliage attitude, curvature tip of the leaf, foliage color, plant grumolo formation, and male sterility showed no variation, while the remaining two traits displayed only minor variation (Table 5, Fig. 1).

On the other hand, the eight quantitative traits revealed significant variability among the populations over the two years. For instance, leaf length ranged from 26.62 cm (Early Maturity Synthetic) to 29.963 cm (Chahestan). Cotyledon length varied from a maximum of 6.829 cm (Fasa) to a minimum of 4.981 cm (Hajiabad). Fasa displayed the earliest onset of flowering at 75.14 days, while Chahestan took the longest at 120.48 days. The tallest height at flowering time was recorded in Hajiabad (148.858 cm), while the shortest was from Hashtgerd (91.75 cm). The primary umbel diameter ranged from 6.50 cm in Hajiabad to 12.79 cm in Hashtgerd. Moghan exhibited the highest thousand-seed weight at 4.086 g, whereas Chahestan recorded the lowest weight at 2.527 g. Petiole length of the first leaf varied from 8.671 cm in Hashtgerd to 11.238 cm in Meshkinshahr. Additionally, the main umbel appeared earliest in Early Maturity Synthetic at 74.4 days and latest in Late Maturity Synthetic at 119.01 days. Tables 5 and 6 present the observed qualitative and quantitative morphological characters.

COY-D and COY-U analysis

The data collected from the eight quantitative traits underwent COY-D statistical analysis at a 1% significance level. Each population was treated as a candidate variety and compared to the remaining 10 populations, serving as reference populations, to generate a pairwise distinctiveness matrix through COY-D analysis (Table 7). Results from comparing the Early Maturity Synthetic with other populations revealed distinctiveness across all examined quantitative traits. Similarly, the Medium Maturity Synthetic, when compared with other populations, demonstrated distinctiveness across all examined quantitative traits. Furthermore, the comparison of the Late-Maturity Synthetic with other populations indicated distinctiveness across most traits compared to the other populations. However, non-distinctiveness was observed from its parental population, Hajiabad (Table 7). Additionally, a COY-D analysis utilizing the MJRA model was performed on the eight quantitative traits (Table 8). For all traits, the F1 ratio (Ratio of Genotype MS to Genotype×Year MS) was significant, suggesting that the environment had less

Table 5 Qualitative characters of synthetic cultivars and parental populations of fennel

Genotype	Foliage attitude	Leaf colour	Leaf: curvature of tip	Foliage: color	Plant:grumolo formation	Foliage: density	Male sterility
Fasa	Horizontal	Light	Absent	Green	Absent	Open	Absent
Moghan	Horizontal	Medium	Absent	Green	Absent	Medium	Absent
Meshkinshahr	Horizontal	Medium	Absent	Green	Absent	Dense	Absent
Fozveh	Horizontal	Medium	Absent	Green	Absent	Medium	Absent
Hajiabad	Horizontal	Dark	Absent	Green	Absent	Dense	Absent
Hashtgerd	Horizontal	Light	Absent	Green	Absent	Open	Absent
Early maturity synthetic	Horizontal	Light	Absent	Green	Absent	Open	Absent
Medium maturity synthetic	Horizontal	Medium	Absent	Green	Absent	Medium	Absent
Late maturity synthetic	Horizontal	Dark	Absent	Green	Absent	Dense	Absent
Khash	Horizontal	Medium	Absent	Green	Absent	Medium	Absent
Chahestan	Horizontal	Dark	Absent	Green	Absent	Dense	Absent

Table 6 Statistical and Genetic parameters for measurable traits in fennel populations

Traits	Minimum	Maximum	Mean ± SE	Genotypic variance	Pheno-typic variance	GCV	PCV	Heritability
Leaf: length (cm)	26 (syn 1)	29.963 (Chahestan)	29.66 ± 1.05	0.47	2.011	2.38	4.96	23.10
length of cotyledons (cm)	4.981 (Hajiabad)	6.829 (Fasa)	5.82 ± 0.096	0.44	0.47	11.44	11.78	94.18
Time of beginning of flowering	75.114 (Fasa)	120.481 (Chahestan)	96.84 ± 1.38	268.42	271.16	17.72	17.81	98.99
Time of appearance of main umbel	74.400 (Syn 1)	119.008 (Syn 3)	95.7 ± 0.96	270.18	274.16	16.40	16.52	98.55
Height at flowering (cm)	91.75 (Hashtgerd)	148.858 (Hajiabad)	117.08 ± 10.67	68.23	87.62	7.25	7.21	77.78
Main umbel: diameter (cm)	6.50 (Hajiabad)	12.79 (Hashtgerd)	8.63 ± 1.21	0.85	1.04	11.24	12.49	80.90
Thousand seed weight (g)	2.527 (Chahestan)	4.086 (Moghan)	3.38 ± 0.97	0.10	0.11	9.68	10.16	90.73
Length of petiole of first leaf (cm)	8.67 (Hashtgerd)	11.23 (Meshkin-shahr)	10.30 ± 0.261	0.48	0.68	6.61	7.85	70.90

Syn 1: Early maturity synthetic; Syn 3: Late maturity synthetic
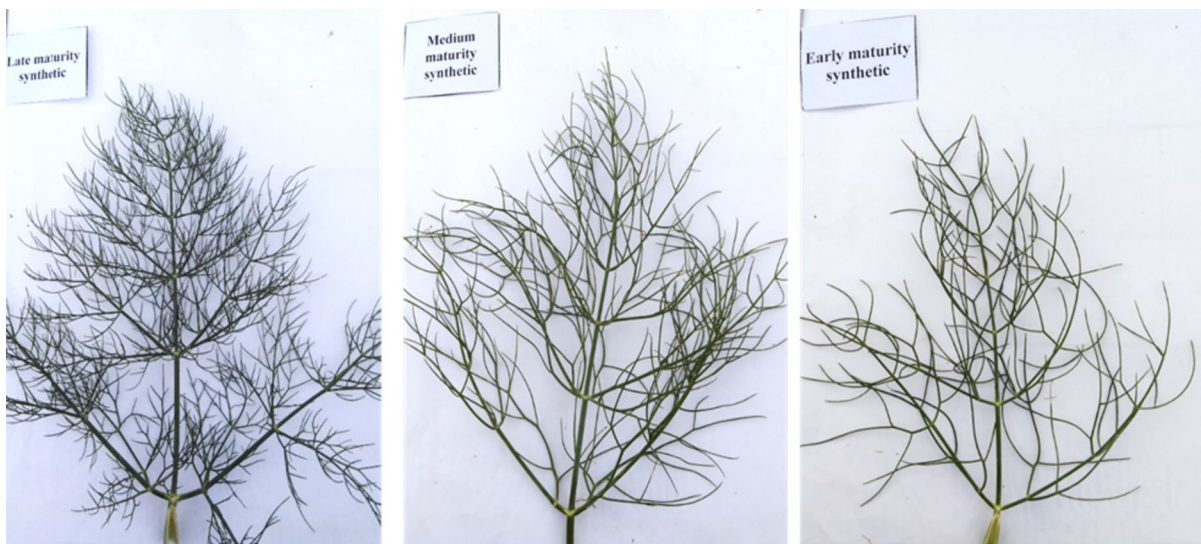
Fig. 1 Foliage density in synthetic cultivars of fennel

of an impact on the expression of the trait. The following traits: time of main umbel appearance, length of petiole, length of cotyledons, time of beginning of flowering, and umbel diameter showed a significant F2 ratio (Ratio of Genotype × Year MS to Genotype × Rep MS). This indicates that the consistency in distinctiveness patterns for these traits varied across the two years of the experiment. In summary, the environment significantly influenced the distinctiveness patterns among the populations for these traits, limiting their ability to establish varietal distinctiveness within the current set of populations.

COY-U analysis was conducted for eight quantitative traits using pairwise comparisons of the standard deviations (SDs) for each trait (Table 9). A thorough examination revealed that among the 11

Table 7 Pairwise distinctiveness matrix of synthetic cultivars and parental populations obtained from COYD analysis

	Genotypes	1	2	3	4	5	6	7	8	9	10	11
1	Chahestan	–	D	D	D	D	D	D	D	D	D	D
2	Khash	D	–	D	D	ND	D	D	ND	D	D	D
3	Fasa	D	D	–	D	D	D	D	D	D	D	D
4	Hashtgerd	D	D	D	–	D	D	D	D	D	D	D
5	Meshkinshahr	D	ND	D	D	–	D	D	ND	D	D	D
6	Hajiabad	D	D	D	D	D	–	D	D	D	D	ND
7	Fozveh	D	D	D	D	D	D	–	D	D	D	D
8	Moghan	D	ND	D	D	ND	D	D	–	D	D	D
9	Syn1	D	D	D	D	D	D	D	D	–	D	D
10	Syn2	D	D	D	D	D	D	D	D	D	–	D
11	Syn3	D	D	D	D	D	ND	D	D	D	D	–

Table 8 Combined over years distinctiveness assessment of eight measurable traits utilizing modified joint regression analysis (MJRA)

	Leaf length	Height at flowering	Main umbel diameter	Time of beginning of flowering	Time of appearance of main umbel	Length of petiole	length of cotyledons	Thousand seed weight
Year MS	0.71	6.686	0.467	187.51	53.46	0.303	0.332	0.008
Genotype MS	8.579	525.622	5.083	1728.351	1684.205	3.250	2.715	0.642
Genotype.year MS	3.304	18.93	3.023	28.522	13.746	0.75	0.235	0.025
F1 ratio	2.596	27.766	1.681	60.597	122.523	4.336	11.553	25.323
Gen. rep MS	3.627	18.698	0.589	4.704	1.475	0.27	0.059	0.02
F2 ratio	0.911	1.012	5.132	6.063	9.322	2.774	4.008	1.3
Between SE	0.742	1.776	0.71	2.18	1.514	0.353	0.198	0.065
Within SE	0.777	1.765	0.313	0.885	0.496	0.212	0.099	0.057

fennel populations, Khash and Late Maturity Synthetic exhibited uniformity for all eight characters, followed by Chahestan, Fozveh, and Medium Maturity Synthetic, which showed uniformity for seven characters. Moreover, there was a lack of uniformity observed for most of the traits, albeit staying within the permissible limits recommended by UPOV criteria.

The pooled analysis of the quantitative traits showed a relatively minor difference between PCV and GCV which is a reflection of the low environmental effects on the traits expressions underscoring consistency and stability of the traits (Table 6). Phenotypic and genotypic coefficients of variation were classified into low (< 10%), moderate (10–20%), and high (> 20%) categories. The time of the beginning of flowering (17.81 and 17.72) and the time of appearance of the main umbel (16.52 and 16.40)

displayed moderate values of PCV and GCV. Conversely, lower PCV and GCV values were noted for leaf length (4.96 and 2.38). These results suggested a significant impact of genetic components on the expression of most of the traits, while environmental factors had minimal influences. This result revealed high heritability for the time of beginning of flowering (98.99%), time of appearance of the main umbel diameter (98.55%), and thousand seed weight (80.90%), suggesting that both additive and non-additive gene effects have an equal role in their heritability. In contrast, low heritability (23.10%) was observed for leaf length, signifying a prevalence of non-additive gene effects and greater environmental influence on its expression,

thereby limiting its performance stability over time. Overall, the analysis of various genetic parameters for eight quantitative DUS descriptors revealed

Table 9 Combined over years uniformity analysis using the standard deviation of synthetic cultivars and parental populations of fennel

Genotype	Leaf: length	Height at flowering	umbel: diameter	Time of beginning of flowering	Time of appearance of main umbel	Length of petiole	length of cotyledons	Thousand seed weight
khash	90	104	107	114	85	92	83	72
Fasa	95	100 [1]	105	97	110	116 [1]	89	105
Hashtgerd	104	88	108 [1]	77	99	93	104 [1]	61
Meshkinshahr	99	108 [1]	89	112	100	84	125 [2]	105
Fozveh	98	106	81	95	93	96	100	126 [1]
Moghan	106	105	106	80	103	92	87	151 [2]
Chahestan	98	88	91	92:1	78	106	100	115
Hajiabad	98	109 [1]	102	91:1	125 [1]	106	88	67
Early maturity synthetic	109 [1]	107	105	107	101	116 [1]	105:1	133 [2]
Medium maturity synthetic	98	96	106	125:1	99	97	107	78
Late maturity synthetic	104	89	100	109	106	102	112	86

Symbols:1, 2 indicate the frequency of instances where the standard deviation within a year surpasses the UPOV criterion

substantial variability and heritability within the populations. However, leaf length stood out due to its lower heritability, emphasizing the importance of these parameters in accurately characterizing fennel populations.

Principal component analysis (PCA)

PCA stands out as a highly precise tool for grouping germplasm, effectively subdividing them into subgroups based on the extent of variations in their characteristics. In this study, PCA was implemented on the quantitative morphological traits to group the 11 fennel populations. The first two principal components together represented 86.8% of the phenotypic variation, as indicated by PCA eigenvectors. PC1 accounting for 73.4 percent of the total variation, was directly linked to the time of appearance of the main umbel, time of the beginning of flowering, height at flowering, and, leaf length. Meanwhile, PC2, contributing to 13.4 percent of the overall variance, primarily reflected the traits of main umbel diameter, length of cotyledons, thousand seed weight, and petiole length. Visualization of the first two PCs in a biplot (Fig. 2) effectively demonstrated the population relations. PCA successfully categorized the cultivars based on

their quantitative traits, grouping each synthetic cultivar with its respective parental populations in one cluster. Notably, in the biplot, the Chahestan population exhibited distinct separation from all the others populations, even the other late maturity populations, potentially reflecting its unique origins and extensive diversity in morphological traits.

Clustering analysis

The dendrogram constructed based on eight quantitative traits, put breeding populations and parental populations, in three major clusters (Fig. 3) which was similar to the results from the PCA. The first cluster of the dendrogram consisted of Late Maturity Synthetic with its parental populations Hajiabad and Chahestan, the second cluster consisted of Medium Maturity Synthetic with its parental populations Fozve, Moghan, Khash, and Meshkinshahr, and the third cluster consisted of Early Maturity Synthetic with its parental populations Hashtgerd and Fasa. This approach was able to perfectly distinguish the three synthetic cultivars from their relevant parental populations.
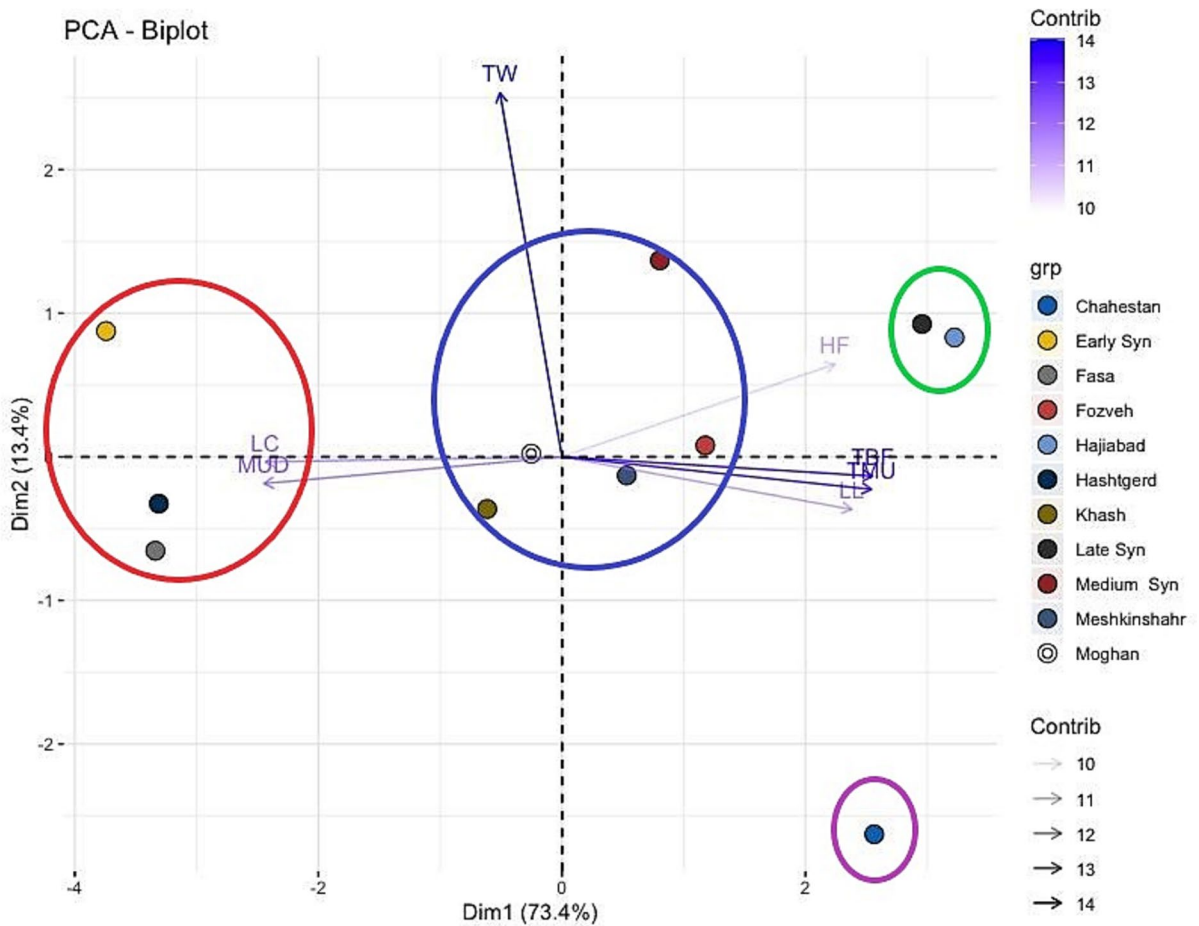
Fig. 2 PCA biplot analysis of fennel populations based on quantitative traits

Molecular markers characterization

Assessment of polymorphism using SCoT marker

Out of the 20 SCoT primers, 15 amplified clear, sharp, and reproducible bands in the 20 fennel populations. The SCoT primers generated a total of 164 amplified bands, of which 139 (85%) were polymorphic with an average of 10.93 bands for each primer, SCOT-9 with 18 bands had the most bands, whereas SCoT-35 primers with 3 bands had the least. Among the populations, the Late Maturity Synthetic had the most bands (129) while the Sarpolzahab had the least (64). Table 10 shows the findings for the primers that were utilized in this study. The percentages of polymorphisms in the tested primers varied from 54 to 100%. The two primers, SCoT-29 and SCoT-31,

showed 100% polymorphism, indicating their significant potential to distinguish different populations from each other.

The polymorphism bands ranged from 2 (SCoT-2) to 17 (SCoT-9) with an average of 9.13. The amount of polymorphism of a marker is represented by the polymorphic information content (PIC), which can range from zero to half, and it is calculated using the frequency and number of amplified fragments (Roldàn-Ruiz et al. 2000). Markers with greater PIC values have a stronger ability to distinguish populations. The PIC values for fifteen primers ranged from 0.10 (SCoT-31) to 0.40 (SCoT-11), with an average of 0.25. Moreover, the resolving power (RP), a key indicator of a primer's ability to distinguish between various populations (Powell et al. 1996), ranged from 1.20
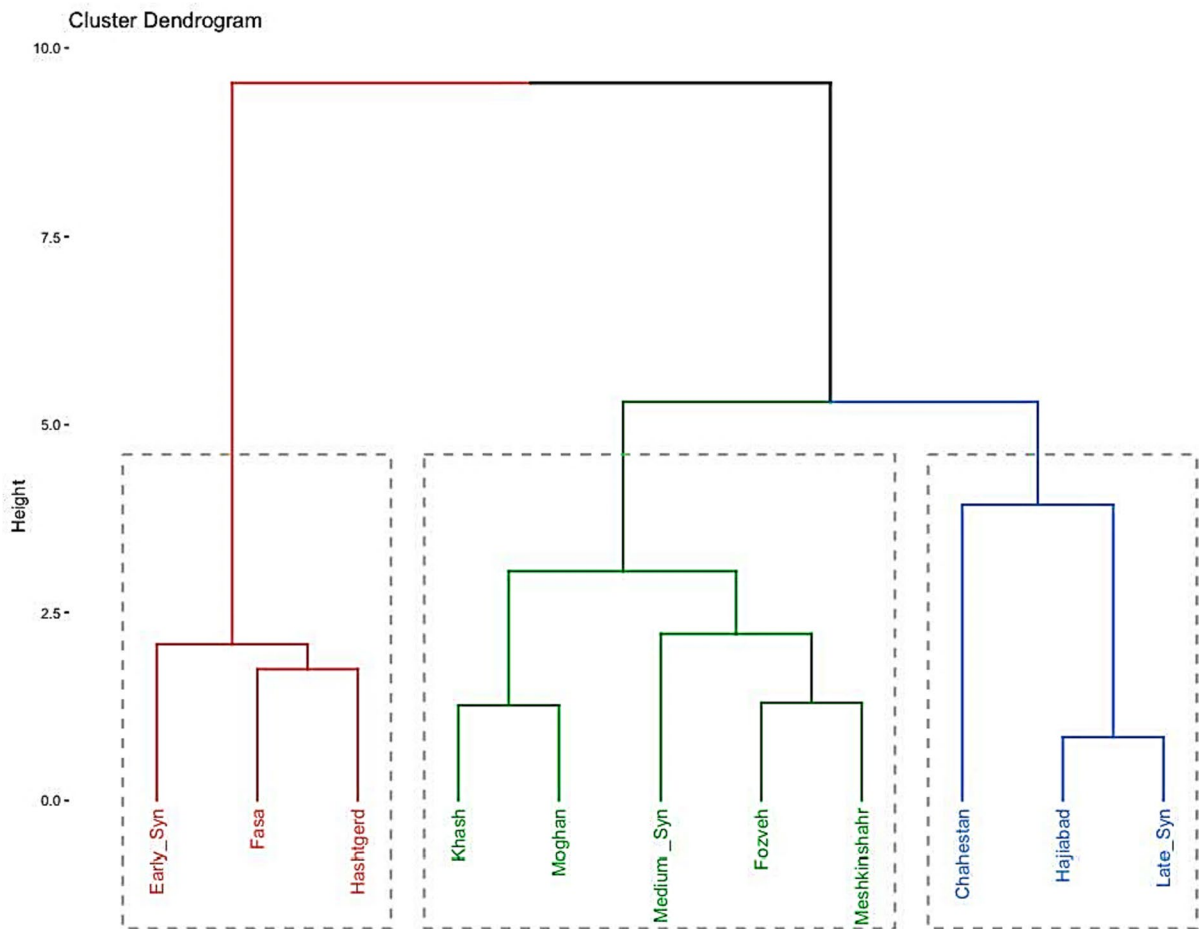
Fig. 3 Dendrogram of 11 fennel populations based on quantitative traits

(SCoT-35) to 7.50 (SCoT-9) in our study, with an average of 4.03. Due to their high RP, SCoT-9 and SCoT-11 were able to differentiate most of the populations. Marker Index (MI), as an indicator for the total effectiveness of a primer, in the study ranged from 0.38 (SCoT-2) to 4.80 (SCoT-11) with the average of 2.50. Considering the PIC, RP, and MI measures, revealed that primer SCoT-11 was the most efficient in examining the variation among the populations (PIC 0.40, RP 7.20, and MI 4.80). Other primers such as SCoT-9 and SCoT-30 were also performed well.

Analysis of genetic relationships

The 20 fennel populations were discriminated by the polymorphic SCoT markers employed in this investigation. The Jaccard coefficient was used to calculate the genetic similarity matrix for the fennel populations (Fig. 4). The genetic similarity of the fennel populations varied from 0.08 to 0.81; this indicated that there is a sufficient variation among the fennel populations. The similarity matrix showed that among all the 20 populations, Sardasht and Nayriz were the most similar, whereas Early Maturity Synthetic and Rafsanjan were the least similar populations.

Cluster analysis based on marker data, a useful way to evaluate genetic relationships among individuals (Randi and Lucchini 2002). Cluster analysis was performed using Jaccard similarity coefficients and the UPGMA technique, which effectively distinguished all the 20 populations. The fennel populations were classified into seven distinct groups, highlighting a

Table 10 Amplification results of Start Codon Targeted (SCoT) primers used for fennel populations

Primer	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)	PIC	RP	MI
SCoT-2	10	6	2	40	0.10	1.50	0.38
SCoT-9	18	1	17	95	0.28	7.50	4.77
SCoT-11	12	0	12	100	0.40	7.20	4.80
SCoT-14	13	6	7	54	0.19	3.50	1.30
SCoT-17	9	0	9	100	0.25	3.20	2.30
SCoT-19	10	1	9	90	0.24	3.00	2.16
SCoT-24	9	2	7	78	0.24	3.00	1.65
SCoT-26	10	0	10	100	0.255	3.60	2.55
SCoT29	13	0	13	100	0.27	4.4	3.52
SCoT-30	14	0	14	100	0.31	6.5	4.35
SCoT-31	9	3	6	67	0.18	2.3	1.06
SCoT-34	9	1	8	88	.0.27	3.4	2.16
SCoT-35	3	1	2	67	0.25	1.2	0.5
SCoT-36	11	1	10	91	0.31	4.8	3.11
SCoT-37	14	3	11	79	0.26	5.4	2.83

Fig. 4 Heatmap indicating the genetic similarity among 20 different fennel populations

Cluster Dendrogram
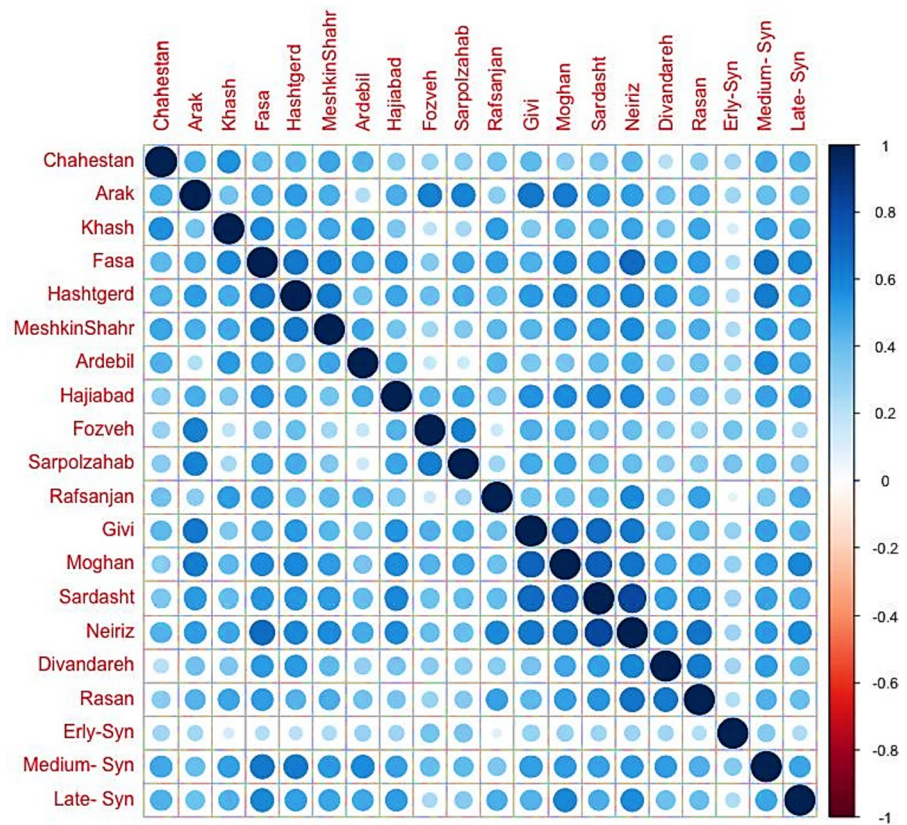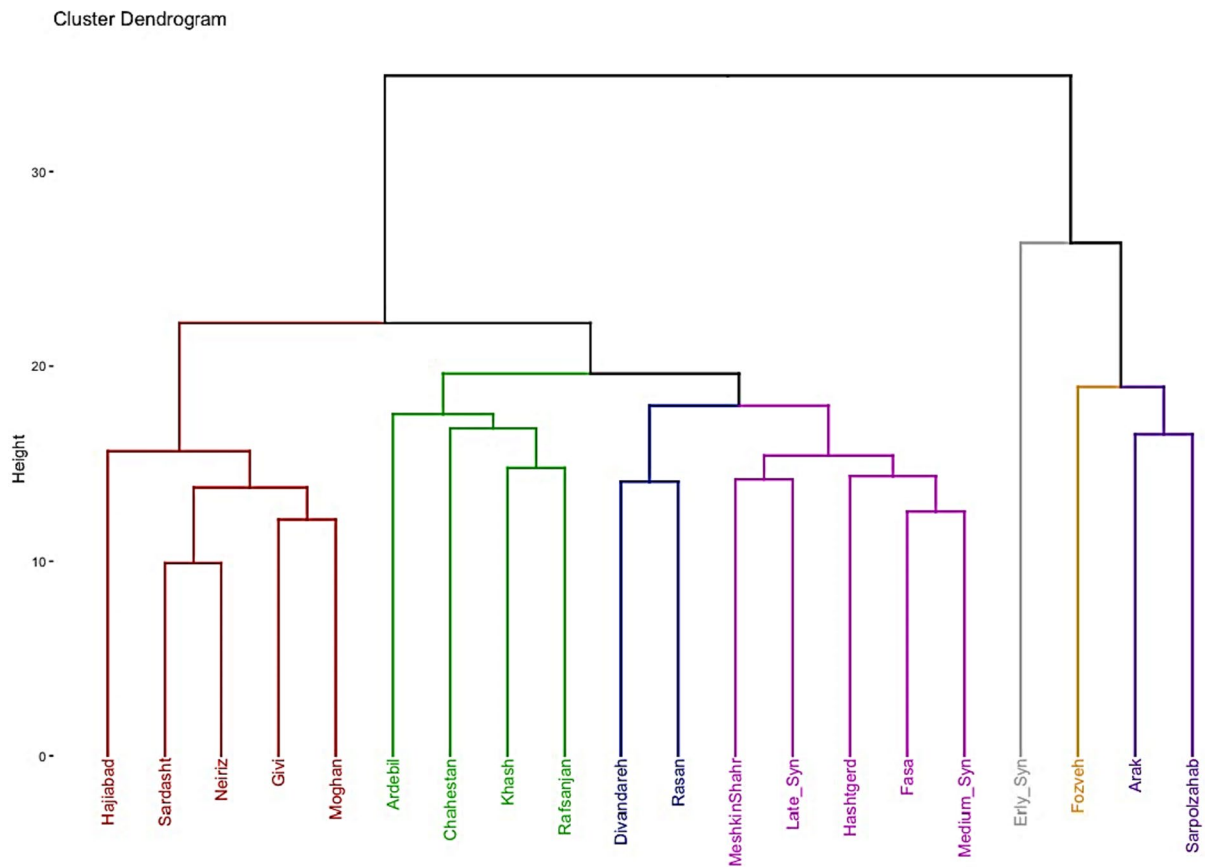
Fig. 5 Cluster analysis of all populations with molecular data based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method using Jaccard's genetic similarity

wide range of genetic diversity (Fig. 5). Groups V and VI each comprised a single fennel population, namely Early Maturity Synthetic and Fozveh, respectively. Within Group IV, which comprised multiple populations, including Medium Maturity Synthetic, Late Maturity Synthetic, Meshkinshahr, Fasa, and Hashtgerd, were clustered together. This classification successfully differentiated Early and Late Maturity Synthetic cultivars from their respective parent populations and discriminated among three synthetic fennel cultivars and their parental populations.

Discussion

Accurate identification and evaluation of plant materials is necessary for variety creation, the registration process, and breeders' rights protection

(Hale et al. 2006). In this study, the distinctness, uniformity and stability of three breeding populations of fennel and their elite parental populations was evaluated using morphological traits and SCoT markers.

The findings indicate that certain morphological DUS descriptors effectively can distinguish some of the populations. Therefore, additional markers or descriptors may be necessary to complement the morphological DUS descriptors for distinctiveness. Morphological traits are commonly employed to identify plant materials, however, this approach is debatable since environmental factors can have a significant impact on morphological traits. Furthermore, due to the time and expense needed, this method is inefficient (Singh et al. 2004). Moreover, morphological traits on theirown are

incapable of detecting discrimination between certain cultivars that are morphologically similar but exhibit diverse agronomical characteristics. DNA analysis can help to overcome all of these constraints because it is quicker, more cost-effective, and more precise (Korir et al. 2013). In recent years, various novel marker approaches have emerged, and among them, Start Codon Targeted (SCoT) markers are becoming increasingly popular owing to their high polymorphism and resolvability (Guo et al. 2012; Bhattacharyya et al. 2013). The SCoT marker system is characterized by its simplicity, cost-effectiveness, polymorphic nature, repeatability, and reliability (Gorji et al. 2011; Zhang et al. 2015). To use molecular approaches for cultivar identification, first must understand the level of polymorphism shown by the different methods in the species under investigation. In their study, Farshadfar et al. (2017) found that employing ten SCoT primers to amplify DNAs from 16 fennel accessions resulted in 55 bands, of which 51 were polymorphic, indicating a polymorphism rate of 93%. Similarly, Bahmani et al. (2013) reported obtaining 1043 polymorphic DNA fragments in their research involving 25 fennel accessions from various regions in Iran, utilizing 10 RAPD markers to assess genetic diversity. Furthermore, Bahmani et al. (2012a, b) and Poudineh et al. (2018) examined genetic diversity among fennel cultivars using ISSR markers. Both investigations disclosed a notable level of polymorphism. Our results demonstrated a substantial percentage of polymorphic primers (85 percent), indicating that the diversity of SCoT markers utilized in this study serves as an effective tool for distinguishing and identifying cultivars. Out of the 20 SCoT markerse used in our study, 15 of threm were able to discriminateall the populations, showcasing their potential utility in DUS testing as well. Numerous research recorded the utilization of molecular markers in crop registration testing. In rice (Singh et al. 2004), cucumber (Bernet et al. 2003), and rapeseed (Tommasini et al. 2003), fingerprinting has extensively employed RAPD, AFLP, and SSR markers. When comparing molecular and morphological features, various scientists have revealed that molecular markers provide for more exact discrimination than morphological features, as demonstrated in maize

(Dillmann et al. 1997). According to previous findings molecular markers could be employed for pre-screening or classification of existing and emerging cultivars. Furthermore, the outcomes of clustering using SCoT markers and morphological features in the present investigation did not seem comparable, revealing differences in exploring genetic diversity. This could be due to the fact that different molecular markers represent genetic variety in different sections of the genome. The current study highlighted the efficacy of SCoT markers in demonstrating the distinctness of the fennel populations for DUS test.

Conclusion

In summary, this DUS test using both SCoT markers and morphological traits was able successfully show the distinctiveness, uniformity, and stability of our newly developed fennel cultivars. According to the results, it is easier to properly identify and classify cultivars when molecular markers and morphological characteristic analysis are combined. These analyses revealed that the newly developed populations of native fennel are generally distinct from the parent populations and can be utilized for seed purity registration and testing. Beside our previous open field testing operations to assess yiled performance of the three fennel synthetic cultivars, the DUS test here in this current study also showed that synthetic cultivar can be a great breeding approach to develop high yielding cultivars in crops such as fennel.

Declarations

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