Towards conservation and breeding of Ajowan (*Trachyspermum ammi*) by assessing ISSR, morphological traits and germination variability

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Abstract

Ajowan is one of the most important members of medicinal plants of the Apiaceae, consumed by humans. The aim of this study was to explore genetic variability of 20 ajowan populations by ISSR fingerprinting and evaluation of traits at germination. Out of the 30 ISSR primers, ten generated reproducible and polymorphic bands. A total of 93 bands were detected out of which 65 were polymorphic. Highest values for resolving power (Rp) were detected for UBC818 and ISSR5 primers (7.2 and 4.6, respectively) which their sequences had $(AC)_n$ or $(CA)_n$ motifs. All genotypes were separated in dendrogram drawn by the ISSR data. For all traits measured at the germination stage, there were highly significant differences among genotypes (P≤0.01). A low germination percentage (42.1%) was observed in average. Qom was the best population considering germination properties such as germination percentage (95%) and shoot length (3.8 cm). The highest number of branches and number of umbels were observed for Sabzevar (with 83 and 91, respectively). High broad sense heritability values were obtained for germination and morphological traits (ranging from 0.68 to 0.99). In conclusion, two populations of Sabzevar and Qom populations were the best for several traits and are suggested for further analysis.

Key words: Cluster analysis, Domestication, Genetic diversity, Heritability, Molecular markers.

INTRODUCTION

Ajowan (*Trachyspermum ammi*) (2n=18) (Syn. *Carumc opticum*) is one of the first members of aromatic and medicinal plants of the Apiaceae family consumed by human. It was originated from India and is cultivated in India, Iran, Afghanistan, Pakistan and Egypt. It is an annual cross-pollinating plant with white flowers and small brownish fruits (Boskabady and Shaikhi, 2000). Ajowan seed is used for various purposes as food additives and for making medicine and cosmetics. Modern experiments with crude extract of *T. ammi* seeds have demonstrated its antispasmodic, bronchodilator, antihypertensive and hepatoprotective activities (Gilani *et al*., 2005). There is evidence that this plant has relaxant and anti-cholinergic effects. Thymol is the most important component in ajowan essential oil (Boskabady and Shaikhi, 2000).

After the appearance of side effects of chemical medicines, in recent years, there has been a renewed interest in using medicinal plants for curing diseases. There is a growing need for medicinal plants around the world and plant-based medicines are fast becom-

ing the main part of modern medicine market. Currently, more than 80 percent of world population use plant derived medicine because they do not have side effects in comparison to synthetic medicines (Verma and Singh, 2008). Wild harvesting of medicinal plants by local sellers will increase the risk of their extinction (Canter *et al.,* 2005). There is a growing concern about the loss of genetic diversity and degradation of natural habitats of medicinal plants. High commercial value of these plants will encourage sellers to harvest more plants from the wild life. For this reason many habitats near big cities where in past were rich in medicinal plants, nowadays are empty. Overgrazing and overharvesting are the leading causes of medicinal plants disappearance in areas around cities along with drought stress caused by climate change (Adnan *et al*., 2015). Therefore, there is a concern that medicinal plants will be extinct in the near future and there is an urgent need to address the medicinal plant extinction in the country. Thus, it is well accepted that collecting medicinal plants for their conservation and domestication is essential to meet the society need. This has to be done in two steps. Firstly, estimating the plant genetic diversity by classic and modern methods and secondly domestication for cultivation in modern farms for commercial production. These activities will help classic and modern breeding procedures to improve yield and medicinal characteristics of the plants. The knowledge of genetic diversity is fundamental in domestic cultivation of this plant as a crop. Through domestication and cultivation, the diversity of a plant will be maintained and also the need of the society for medicinal plants will be satisfied (Potvin *et al*., 2003).

Germination is a challenge for wild plants as sometimes it needs special ecological requirements such as chilling and soaking (Canter *et al*., 2005). One of the main obstacles of medicinal plant domestication is seed germination under farm conditions. To start any breeding program, the germination barriers need to be removed. Uniform and rapid germination in farm is an inevitable property of a commercial cultivar as it is essential for promising productive growth during the life cycle.

Little is known about ajowan genetic diversity and its population structure despite its importance as an aromatic and medicinal plant. In plant breeding, the existence of genetic diversity has a pivotal role in successful selection. Therefore, genetic diversity estimation will be necessary for establishing a starting population for breeding programs. Also estimation of genetic diversity can play an important role in addressing the issue of genetic drift in a plant germplasm. Estimation of genetic diversity based on morphological traits can help management of breeding program and domestication activities. Several studies have analyzed the essential oil of ajowan (Boskabady and Shaikhi, 2000; Khajeh *et al*., 2004) and there are a few reports about genetic diversity of this plant (Modareskia *et al*., 2012; Fadaei Heidari *et al*., 2016).

Although morphological diversity estimation reveals the variability of some important traits such as yield components, but it cannot reveal exactly the genetic diversity at molecular level. During the last few years, molecular techniques have been used widely for characterization, evaluation of genetic diversity and inter- or intra- species relationships. Several studies have documented the usefulness of molecular markers to estimate genetic diversity. Molecular markers are especially helpful in differentiating genotypes which are similar to each other phenotypically. Inter simple sequence repeat (ISSR) markers (Zietkiewicz *et al*., 1994) have been used with success to identify and determine relationships between species, population and cultivar levels in many plant species, including several aromatic and medicinal plants such as *Achillea millefolium* (Farajpour *et al*., 2012), *Artemisia capillaries* (Shafie *et al*., 2009), *Zingiber officinale* Rosc .(Kizhakkayil and Sasikumar, 2010), *Salvia miltiorrhiza* Bunge (Peng *et al*., 2014), *Changiums myrnioides* (Qiu *et al*., 2004) and *Curcuma longa* (Singh *et al*., 2012). Although there are many studies about ajowan essential oil components and its therapeutic effects (Khajeh *et al*., 2004; Mohagheghzadeh *et al*., 2007) there are a few studies regarding genetic diversity of this plant (Modareskia *et al*., 2012; Fadaei Heidari *et al*., 2016). ISSR marker system is widely applicable because it is an inexpensive and rapid method, it needs small amount of DNA template and does not require prior knowledge of DNA sequences (Godwin *et al*., 1997).

While some research studies have been carried out on molecular diversity of ajowan, no studies are found dealing with germination, morphological and molecular diversity of this plant in a same experiment. The aims of the present work were (1) to determine genetic diversity based on morphological traits measured at maturity to help the establishment of starting material required for breeding purposes, (2) evaluate germination traits to help for better germination and (3) molecular fingerprinting of genotypes by ISSR for better management of germplasm. Introduction of a new cultivated variety could be a contributing factor in conserving this plant. The findings will help to plan the best strategy for genetic improvement of this plant.

Table 1. The name and collection sites of 20 populations used for this study.

Code	Population	Province	Latitude	Longitude	Elevation (m)
1	Falavarjan	Isfahan	32º 36' N	51º 26' E	1610
2	Samian	Ardebil	38º 20' N	48º 15' E	1298
3	Marvdasht	Fars	29º 52' N	52º 48' E	1602
4	Alamut	Ghazvin	36º 29' N	50º 25' E	2046
5	Khanaman	Kerman	30º 25' N	56º 34' E	1560
6	Qom	Qom	34º 39' N	50º 57' E	934
7	Arak	Markazi	34º 05' N	49º 42' E	1748
8	Shazand	Markazi	33º 54' N	49º 24' E	2002
9	Khomein	Markazi	33º 38' N	50º 04' E	1811
10	Shiraz	Fars	30º 06' N	52º 45' E	1616
11	Sadough1	Yazd	30º 51' N	54º 54' E	1426
12	Sabzevar	Khorasan Razavi	36º 13' N	57º 40' E	994
13	Isfahan	Isfahan	32º 38' N	51º 39' E	1576
14	Tehran	Tehran	35º 41' N	51º 24' E	1181
15	Gorgan	Golestan	36º 50' N	54º 25' E	129
16	Karaj1	Alborz	35º 48' N	50º 30' E	1839
17	Karaj2	Alborz	36º 12' N	50º 54' E	1556
18	Zahedan	Sistan Balochestan	29º 32' N	60º 54' E	1381
19	Parsabad	Ardebil	39º 45' N	47º 50' E	46
20	Yazd	Yazd	32º 07' N	56º 37' E	1144

MATERIALS AND METHODS

Plant materials

The plant material consisted of 20 ajowan populations (Table 1). Eighteen populations were provided by the gene bank of Forests, Range and Watershed Management Organization, Tehran, Iran. The next two populations, Sabzevar and Gorgan, were provided by local farmers.

Germination experiment

In order to determine germination response of genotypes, an experiment with three replications each consisting of 25 seeds was conducted in completely randomized design (CRD) at 25 . Seeds were surface sterilized with 0.5% sodium hypochlorite for 30 seconds, were monitored daily and considered germinated when the radicle length was approximately ≥2 mm. Estimations of the time taken for cumulative germination to reach 50% in each replicate (D50) were interpolated from the germination progress curve versus time. Germination test followed for ten days, when no differences were observed in the number of germinated seeds. Germination rate (GR) was calculated as below (Roundy and Biedenbender, 1996):

$$GR = 1/D50 \quad (1)$$

Mean germination time (MGT) was calculated according to the following formula (Patane and Gresta, 2006).

$$\text{MGT (days)} = \frac{\sum T_i N_i}{S} \quad (2)$$

Where Ti is the number of days after beginning of experiment, Ni is the number of seeds germinated on day i and S is the total number of germinated seeds.

After 10 days, shoot length (SL) and root length (RL) were measured per each plant. Shoot and root samples were oven dried at 72 ºC for 48 hours and shoot dry matter (SDM) and root dry matter (RDM) were measured.

Morphological variability assessment

To assess morphological diversity among ajowan genotypes, an experiment was conducted using a completely randomized design with three replications under controlled conditions in a greenhouse. In each pot 15 seeds were sown; after emergence seedlings in each pot were thinned to five. Pots were 25 cm in diameter and 30 cm in height. They were filled with soil and manure in 3:1 volume ratio, respectively. Soil texture was sandy loam (50% sand, 25% silt and 25% clay) prepared from a local research field. Total nitrogen of the field soil was 0.19% and available phosphorus and potassium was 28 and 588 ppm, respectively. The maximum and minimum temperature of greenhouse was 32 and 25 °C, respectively. Relative humidity was 60% and photoperiod was14-h. Main stem diameter (SD), inflorescence diameter (ID), plant height (PH), number

of branches (NB), leaf width (LW), leaf length (LL), petiole length (PL), number of umbels (NU), number of umbellets in an umbel (NUI), number of flowers per umbellet (NFU), internode length (IL), shoot dry weight (SDW) and number of days to flowering (DF) were recorded at the flowering stage. Data recorded from five plants in each pot were averaged to have replications.

ISSR experiment

Genomic DNA extraction

For DNA extraction, healthy fresh leaves were sampled from 10 independent plants per population and were bulked and immediately ground to a powder in liquid nitrogen and then stored at -80 °C. DNA was extracted based on the method described by Murray and Thompson (1980). Prior to polymerase chain reaction (PCR) analysis, extracted DNA was quantified by visual comparison with a molecular size marker (1 kb ladder) on an ethidium bromide stained agarose gel. High quality DNA samples were then diluted by distilled water to 50 ng/µl.

PCR amplification

Thirty ISSR primers (Sinaclon company, Tehran, Iran) were used for the PCR amplification. DNA amplification reactions and electrophoresis were performed according to Thomas and Bebeli (2010). PCR reactions were carried out in 25 µl volume containing 1× PCR buffer, 0.4 mM dNTPs, 2mM $MgCl_2$, 0.5 µM ISSR primer, 1.0 U Taq DNA polymerase and 50 ng genomic DNA. Temperature profile for amplification included one cycle of 5 min at 94 ˚C, followed by 45 cycles of amplification. Each cycle of amplification had a denaturing step at 94 ˚C for 30 s, an annealing step at 56-59 ˚C (depending on primer sequence) for 45 s and an extension step at 72 ˚C for 2 min. After the final cycle, samples were held for an extra 5 min at 72 ˚C. To rule out the possibility of contamination in the PCR reaction, always a negative control (a tube without DNA) was included in each assay. Amplification products were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide in 1× TAE buffer. ISSR bands were detected and photographed by a Gel Doc system (UVP, Bio Doc, Upland, USA). The molecular sizes of bands were assigned based upon their positions relative to the 1kb DNA ladder.

Data analysis

The data obtained from germination and morphological diversity experiments were analyzed by SAS software (Version 9.00). Variance analysis were performed and heritability estimates were calculated with the following formula (Schwartz *et al*. 2009)

$$H^2 = \frac{\sigma_g^2}{\sigma_p^2} = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{R}} \quad (3)$$

Where σ_g^2 equals the variance of genotypes, σ_g^2 equals the phenotypic variance, σ_g^2 equals the error variance, and R equals the number of replications. Genetic coefficient of variability was calculated according to Johnson *et al*. (1955) as given below:

$$GCV = \frac{\sqrt{\sigma_g^2}}{\bar{x}} \times 100 \quad (4)$$

where σ_g^2 equals the variance of genotypes and $\bar{x}$ is the mean of trait.

Hierarchical cluster analysis for germination experiment and morphological diversity experiments were carried out using UPGMA method by NTSYS. Mean scores of measured morphological traits were subjected to PCA analysis, using R software (R2.14.1).

ISSR amplicons were scored for presence or absence in each genotype, and the data were entered into a binary matrix as discrete variables (‘1’ presence and ‘0’ absence). The ability of the most informative primers to differentiate between the genotypes was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (1999), using the following formula:

$$Rp = \sum Ib \quad (5)$$

Where I_b is band information and is calculated as $I_b = 1 - [2 \times |0.5 - p|]$ where p is the population proportion containing the band. The polymorphism information content (PIC) of each marker was expressed using this formula:

$$PIC = 1 - [f^2 + (1-f)^2] \quad (6)$$

Where ‘f’ is the frequency of the marker in the data set (De Riek *et al*., 2001). To obtain a measure of the utility of the marker system, marker index (MI) was also calculated as

$$MI = PIC \times \eta\beta \quad (7)$$

Where PIC is the mean PIC value, η the number of bands, and β is the proportion of polymorphic bands (Powell *et al*., 1996).

For all pairs of genotypes, genetic similarity values were calculated using the Jaccard’s coefficient (Sneath and Sokal, 1973). The similarity matrices were used for the construction of dendrograms using UPGMA (Mo-

Table 2. Mean squares of ANOVA for traits measured in the germination experiment along with descriptive statistics, genetic variance estimations, broad sense heritability *(H2)* and genotypic coefficient of variation (GCV).

Source of variations	df	Shoot length (cm)	Root length (cm)	Shoot dry matter (g)	Root dry matter (g)	Mean germination time (day)	Germination percentage (%)	Germination rate
genotype	19	1.64^{**}	0.115^{**}	7.8^{-7**}	8.8^{-8**}	0.07^{**}	1683^{**}	7.3^{-6**}
error	40	0.02	0.002	1.7^{-8}	4.5^{-9}	0.014	35	1.2^{-6}
mean		2.62	1.43	0.001	0.0003	2.27	42.1	0.714
min.		1.15	1.02	0.0003	0.0001	1.98	11.1	0.711
max.		3.83	1.81	0.0017	0.0005	2.48	95.5	0.718
genetic variance		0.54	0.037	2.5^{-7}	2.7^{-8}	0.018	549.3	2^{-6}
H^2		0.98	0.98	0.97	0.94	0.8	0.97	0.83
GCV		28	13	45	53	6	56	0.2

**significant at P≤0.01 probability level.

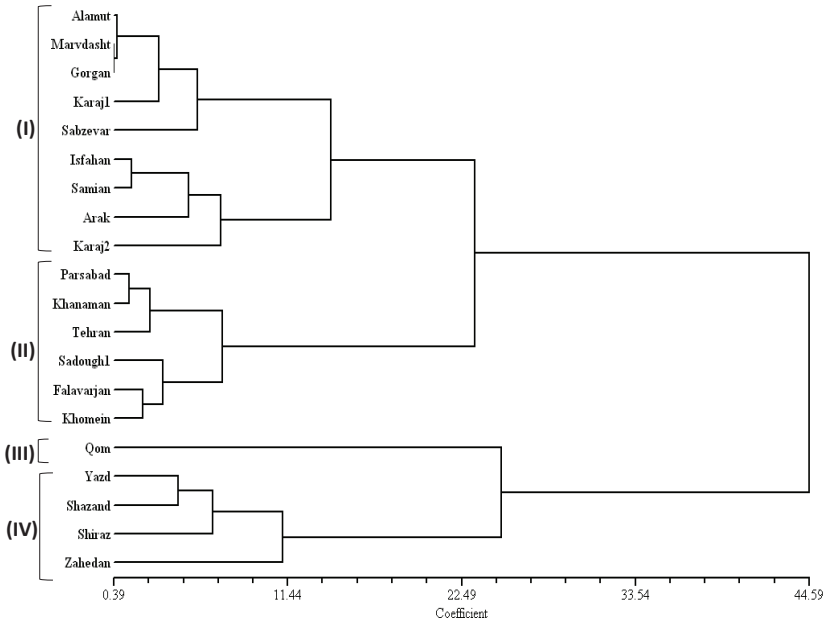

Figure 1. Dendrogram based on UPGMA generated from germination data. Genotypes were grouped in four classes.

hammadi and Prasanna, 2003). To test the goodness of fit of the UPGMA cluster analysis, the co-phenetic correlation coefficient was calculated. Data management and analyses were performed using NTSYS-pc version 2.02 software (Rohlf, 2005).

RESULTS

Germination experiment

There was a high genotypic coefficient of variation among genotypes for some traits such as shoot dry weight, root dry weight and germination percentage (Table 2). The highest variable trait among genotypes was root dry matter as its genotypic coefficient of variation (GCV) was 53%. These results show that there is a high genetic variation among ajowan genotypes across the country which can be exploited in breeding programs for the improvement of germination-related traits. Heritability estimates ranged from 0.8 for mean germination time to 0.98 for shoot length. Considering the shoot length, genotypes Qom and Yazd were the best (with 3.8 and 3.7 cm, respectively) and the lowest amount was observed for Tehran and Falavarjan (with 1.1 and 1.4 cm, respectively). The highest root length was measured for Shazand and Shiraz (with 1.7 and 1.8 cm, respectively) while the lowest value was recorded for Sadough1 and Karaj2 (with 1 and 1.15 cm, respectively). Qom had the best values for germination parameters (with 95% of germination) and the worst one was Falavarjan (with 13% of germination).

Figure 1 shows an overview of genotypes relationships considering germination data. In cluster analysis,

Table 3. Mean of measured traits in the germination experiment in center of clusters developed by Kmeans cluster analysis.

Cluster	Shoot length (cm)	Root length (cm)	Shoot dry matter (g)	Root dry matter (g)	Mean germination time (day)	Germination percentage (%)	Germination rate
1	2.75	1.36	0.0012	0.0003	2.25	40	0.715
2	1.89	1.38	0.0005	0.0001	2.31	17	0.713
3	3.83	1.62	0.0017	0.0006	2.10	96	0.717
4	3.15	1.65	0.0016	0.0005	2.31	71	0.715

genotypes were clustered in four main groups (I-IV). Table 3 shows the means of traits in each cluster. In group (I) genotypes with moderate performance gathered. In group (II) all genotypes with weak performances considering all germination traits were clustered. Interestingly, in group (III) there was only one case, Qom, which showed a remarkable performance for all germination related traits. Genotypes with high values for shoot and root characteristics were clustered in group (IV). There was a large difference among genotypes in germination factors. In Table 2 the minimum and maximum values for each trait is presented. For example, the germination percentage ranged from 11.1% (Sadough1) to 95.5% (Qom), germination rate ranged from 0.711 (Khanaman) to 0.718 (Alamut) and mean germination time ranged from 1.98 days (Alamut) to 2.48 days (Samian). No acceptable germination percentage was observed among genotypes as its average was not greater than 42.1 percent. The range of this trait was between 11.1 and 95.5 percent (Table 2)

Morphological variability

Differences among genotypes were significant at P≤0.01 probability level for all measured morphological traits (Table 4). Strong evidence for genetic variation was found when broad sense heritability was estimated. Heritability ranged from 0.82 from internode length to 0.99 for days to flowering. Means of traits are presented in Table 5. The minimum time needed for flowering was 61 days (for Sabzevar) while the maximum time was 81 days (for Khanaman). Plant height is an important factor for commercialization probability of a cultivar as it is critical for mechanical harvesting. Interestingly, the range of this trait was between 45 to 78 cm for Arak and Falavarjan, respectively. The best genotype for the number of branches was Sabzevar (83) while Karaj2 had the lowest number (18). Alamut was one of the genotypes with the highest value for petiole length, leaf length, leaf width and stem diameter. Inflorescence diameter, number of umbels in plant, number of umbelletes in umbel and number

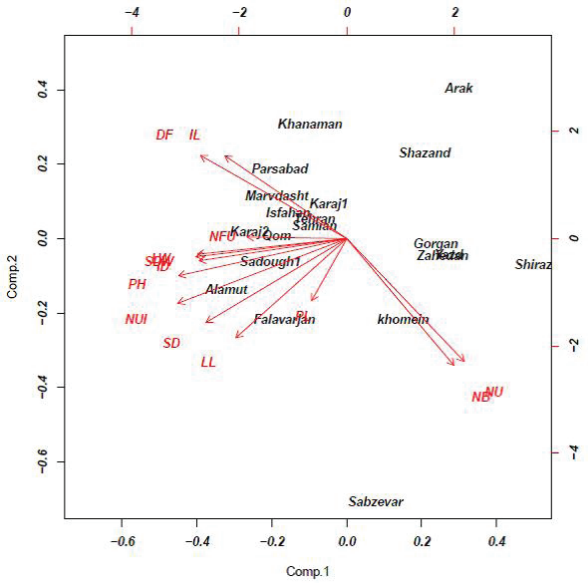

Figure 2. Biplot of first across second PCA extracted from PCA analysis of different morphological traits. Arrows show the projection of different traits. Main stem diameter (SD), inflorescence diameter (ID), plant height (PH), number of branches (NB), leaf width (LW), leaf length (LL), petiole length (PL), number of umbels (NU), number of umbellets in an umbel (NUI), number of flowers per umbellet (NFU), internode length (IL), shoot dry weight (SDW) and days to flowering (DF).

of flowers in umbellete are important components in determining final seed yield. Regarding the inflorescence diameter the best genotype was Qom with 39 mm width. In Sabzever, the number of umbels was the maximum (91) while the number of umbelletes in umbel was the maximum in Karaj2 with 17.8. Number of flowers in umbellete was maximum for Qom and Alamut both with 20. The number of total flowers in a plant is the product of umbels in plant, number of umbelletes in umbel and the number of flowers in umbellete. Regarding the number of total flowers, the best genotypes were Sabzevar, Falavarjan and Sadough1. Qom, Alamut and Isfahan had the highest dry biomass with 10.3, 10 and 9 g plant^{-1}, respectively. In Figure 2, biplot of first two components of PCA analysis is shown where

Table 4. Mean squares of ANOVA for traits measured in pot experiment along with descriptive statistics, genetic variance estimate and broad sense heritability (H^2).

Source of variations	df	Days to flowering	Plant height (cm)	Number of branches	Petiole length (cm)	Leaf length (cm)	Leaf width (cm)	Stem diameter (mm)	Inflorescence diameter (mm)	No. of umbles in plant	No. of umbellets in umbel	No. of flowers In umbellets	Internode length (cm)	Dry biomass (g)
genotype	19	101**	283**	856**	4**	7.8**	3.36**	3.51**	59.2**	1184**	24**	7.4**	3.4**	6.2**
error	40	0.66	24	18	0.58	1	1.08	0.34	6	30	1.9	0.85	0.62	0.4
mean		72.17	65.64	43.56	2.75	9.10	6.31	5.94	32.86	45.57	14.49	17.85	5.16	7.46
min.		61.67	45.72	18.67	1.07	6.71	4.46	3.71	24.99	18.33	9.89	15.22	3.12	3.38
max.		81.00	78.89	83.17	5.11	11.90	7.83	7.37	39.74	91.50	17.83	20.56	8.18	10.30
genetic variance		33.45	86.33	279.33	1.14	2.27	0.76	1.06	17.73	384.67	7.37	2.18	0.93	1.93
H^2		0.99	0.92	0.98	0.86	0.87	0.68	0.90	0.90	0.97	0.92	0.89	0.82	0.94

**significant at P ≤ 0.01 probability level.

Table 5. Mean values for different morphological traits measured. Means were compared using Duncan multiple test at p≤0.01.

Genotype	Days to flowering*	Plant height (cm)	Number of branches	Petiole length (cm)	Leaf length (cm)	Leaf width (cm)	Stem diameter (mm)	Inflorescence diameter (mm)	No. of umbles in plant	No. of umbellets in umbel	No. of flowers in umbellets	Internode length (cm)	Dry biomass (g)
Alamut	75.0 cd	75.4 ab	34.7 fg	4.6 ab	11.8 a	7.6 a	7.4 a	32.5 bcd	18.3 h	17.6 a	20.1 ab	5.5 bc	10.0 ab
Arak	72.7 e	45.7 f	45.4 cde	1.1 g	6.8 e	5.3 abc	3.7 h	27.5 de	35.1 def	9.9 g	17.4 cdefg	4.8 bcd	6.6 ghi
Isfahan	75.7 cd	71.5 ab	35.0 efg	2.2 defg	9.3 bcde	6.4 abc	6.0 abcdef	33.7 abc	37.4 def	17.2 ab	19.2 abc	5.7 bc	9.1 bc
Yazd	68.7 g	56.9 cdef	76.7 a	1.9 efg	9.1 cde	5.1 abc	5.7 bcdefg	25.6 e	45.9 d	11.4 efg	15.9 efg	5.0 bcd	5.9 i
Falavarjan	75.0 cd	78.9 a	52.2 c	3.2 abcdef	9.3 bcde	7.4 a	6.6 abcd	37.0 ab	63.7 c	16.7 abc	18.7 abcd	5.3 bc	8.7 cd
Gorgan	64.7 j	57.6 cdef	53.1 c	2.2 defg	8.2 de	6.6 abc	4.6 fgh	29.9 cde	71.0 bc	10.9 efg	16.3 defg	5.0 bcd	7.0 fghi
Karaj1	70.7 f	67.8 abc	32.7 fg	1.2 g	7.9 de	7.2 ab	7.1 a	36.3 ab	39.7 def	13.8 bcde	17.7 cdef	5.2 bc	6.9 fghi
Karaj2	79.7 ab	74.6 ab	18.7 h	4.0 abcd	11. 9a	7.4 ab	7.2 a	32.3 bcd	20.8 gh	17.8 a	15.5 fg	5.8 bc	8.0 cdef
Khanaman	81.0 a	65.5 bcd	22.0 h	4.3 abc	9.2 cde	6.9 abc	4.3 gh	36.3 ab	27.3 fgh	13.4 cdef	17.3 cdefg	6.0 b	7.6 defgh
khomein	67.7 gh	56.8 cdef	52.2 c	2.8 bcdefg	11. 4abc	6.6 abc	5.5 cdefg	30.0 cde	62.7 c	12.9 defg	16.2 efg	3.9 cd	7.0 fghi
Marvdasht	74.3 de	78.4 a	27.7 gh	1.4 fg	9.5 abcd	6.9 abc	6.9 abc	32.6 bcd	32.3 defg	16.6 abc	16.7 defg	6.5 ab	8.6 cde
Parsabad	76.7 c	74.4 ab	36.5 efg	1.5 fg	9.7 abcd	7.6 a	6.1 abcde	36.4 ab	43.3 de	15.5 abcd	17.8 cdef	8.2 a	6.3 hi
Qom	75.7 cd	65.6 bcd	36.8 defg	3.6 abcde	8.5 de	6.4 abc	6.0 abcde	39.1 a	30.3 efgh	17.0 ab	20.6 a	4.6 bcd	10.3 a
Sabzevar	61.7 k	67.3 abc	83.2 a	3.4 abcdef	11.7 ab	6.0 abc	6.7 abcd	36.3 ab	91.5 a	16.8 abc	18.0 bcde	3.9 cd	7.5 efgh
Sadough1	78.7 b	71.5 ab	47.2 cd	2.2 defg	9.5 abcd	7.8 a	7.0 ab	39.7 a	44.2 de	16.2 abcd	19.3 abc	5.3 bc	7.5 efgh
Samian	75.7 cd	64.9 bcde	46.1 cd	3.3 abcdef	8.7 de	5.3 abc	6.7 abcd	31.9 bcd	27.9 fgh	17.8 a	19.4 abc	5.6 bc	7.7 defg
Shazand	65.0 ij	54.9 def	28.7 gh	2.5 cdefg	7.8 de	4.7 bc	4.8 efgh	31.1 bcde	40.2 def	9.9 g	19.6 abc	4.7 bcd	6.8 fghi
Shiraz	66.7 hi	54.9 def	65.6 b	2.5 cdefg	6.7 e	4.5 c	4.8 efgh	25.0 e	79.7 ab	10.2 fg	15.2 g	3.1 d	3.4 j
Tehran	75.7 cd	76.9 ab	34.3 fg	2.1 defg	8.3 de	5.4 abc	6.6 abcd	37.4 ab	38.3 def	15.3 abcd	18.7 abcd	4.6 bcd	7.4 fgh
Zahedan	62.7 k	53.2 ef	42.6 cdef	5.1 a	6.7 e	5.3 abc	5.3 defg	26.7 de	61.7 c	12.9 defg	17.3 cdefg	4.5 bcd	7.0 fghi

* means with the same alphabetic letter are not significant at p≤0.01.

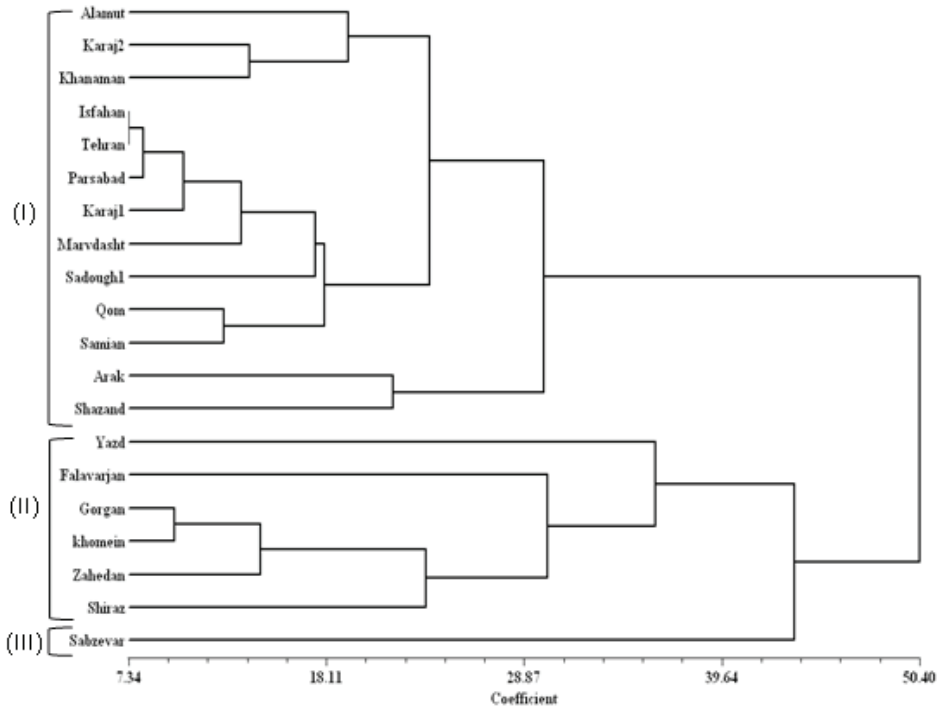

Figure 3. Dendrogram based on UPGMA generated from morphological data shows the relationship among 20 ajowan genotypes.

the relative value for each trait in each genotype can be deduced. The biplot is quite revealing in several ways. First cosine of angle between eigenvectors shows the correlation between traits. For example, there was a significant high positive correlation (r=0.77) between the number of umbles (NU) and the number of branches (NB) and their angle in the plot were very small. Furthermore, the projection of each genotype on each vector shows its relative value for the given trait. For example, the genotype Sabzevar had the highest value for NB and NU with 83 and 91, respectively (Table 5). In biplot, the highest projection on these two vectors was observed with Sabzevar. Thus, the plot provides profound information about the genotypes. However, the drawback of this plot is that it includes 62 percent of the variation and gives a general view of genotypes instead of detailed description.

UPGMA method was used for clustering genotypes based on the morphological data. Genotypes were clustered in three groups (Figure 3). Mean days to flowering for group (I) was 75 days while for group (II) and (III) was 67 and 61 days, respectively. Number of branches in group (I), (II) and (III) was 34, 57 and 83, respectively. There was a large difference among three groups considering the number of umbels in plant. The number of umbels in plant was 33 for group (I) while it was 64 for group (II). Sabzevar population in group (III) had the extraordinary value for this trait with 91 umbels per plant.

ISSR analysis

The primer sequence, number of amplified bands and polymorphism parameters of ISSR experiment are shown in Table 6. Out of the 30 ISSR primers tested, ten produced reproducible bands with at least one polymorphic band. The repeatability of results was confirmed by comparing the banding pattern of two separate assays for each primer pair. The size of the amplified products varied between 250 and 1500 bp. The most polymorphic primer was UBC818 which produced 14 bands, and it was followed by the ISSR5 and UBC857 which generated 12 polymorphic bands, each. Altogether, 66 percent of the amplified bands were polymorphic. The amplification pattern of primer ISSR5 is shown in Figure 4. In Table 6, PIC reflects the polymorphic information content of each primer. Based on the average polymorphic information content of each primer, the best primers were UBC818 and ISSR7 both with PICs of 0.33. Nevertheless, the Rp values of these two primers were completely different. Primer UBC818 had the highest value (7.2) for Rp; im-

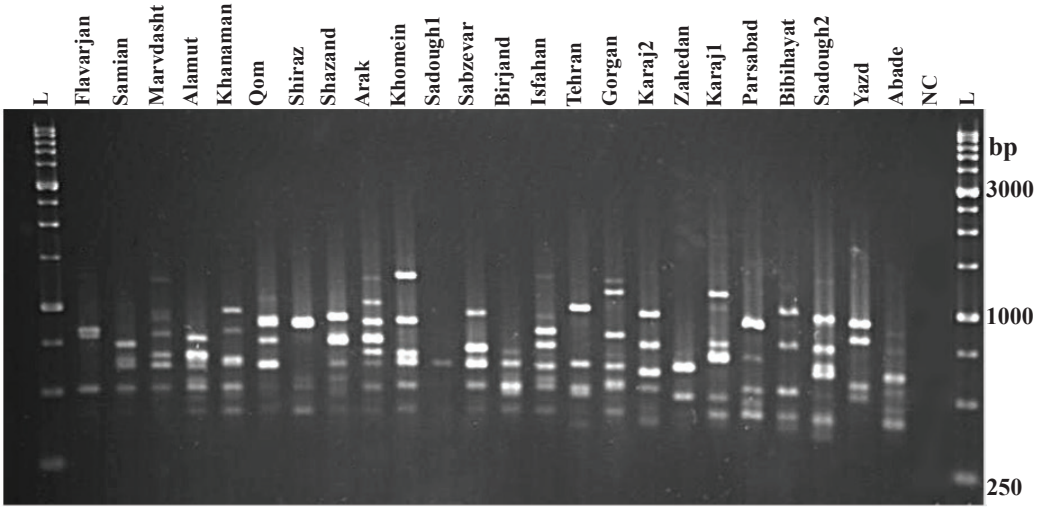

Figure 4. ISSR amplification pattern of ajowan genotypes using primer ISSR5. L: DNA ladder, NC: negative control.

Table 6. The nucleotide sequence, polymorphism, PIC, MI and Rp values for 10 ISSR primers used for analyzing genetic diversity.

Primer	Sequence 5'-3'	Number of amplified bands		Polymorphism (%)	PIC	MI	Rp
		Total	Polymorphic				
UBC810	$(GA)_8T$	6	2	33	0.085	0.17	0.6
UBC812	$(GA)_8A$	6	2	33	0.14	0.28	1.3
UBC817	$(CA)_8A$	10	5	50	0.13	0.66	1.8
UBC818	$(CA)_8G$	14	14	100	0.33	4.6	7.2
UBC827	$(AC)_8G$	7	4	57	0.15	0.58	1.6
UBC857	$(AC)_8Y^*G$	12	11	92	0.26	2.9	4.3
ISSR01	$(ACTG)_4$	13	4	30	0.12	0.47	2.3
ISSR5	$(AC)_8TG$	12	12	100	0.28	3.4	4.6
ISSR7	$(GT)_8C$	7	7	100	0.33	2.4	3.3
ISSR8	$(AC)_8CT$	6	4	66	0.3	1.2	3.1
Sum		93	65	-	-	-	-
Mean		9.3	6.5	66	0.21	1.66	3

*Y = C, T.

plying its power in differentiating genotypes from each other. Using data of this primer for cluster analysis with Jaccard's similarity equation and UMGMA clustering algorithm, all 20 genotypes were discriminated from each other, clearly. According to the similarity matrix of this primer, the highest value was 0.9 which means the identity of genotypes can be detected without ambiguity. The primers based on $(AC)_n$ and $(CA)_n$ produced the maximum number of polymorphic bands while the minimum number of bands were produced by $(GA)_n$ primers (Table 6).

The dendrogram of cluster analysis with ISSR data is shown in Figure 5. The co-phenetic correlation between similarity matrix and co-phenetic matrix was 0.85. No agreement existed among three clusters generated from germination, morphological and molecular experiments. In germination experiment, the closest genotypes to each other were found to be Alamut, Marvdasht and Gorgan. In the classification based on morphological traits, Isfahan, Tehran and Parsabad

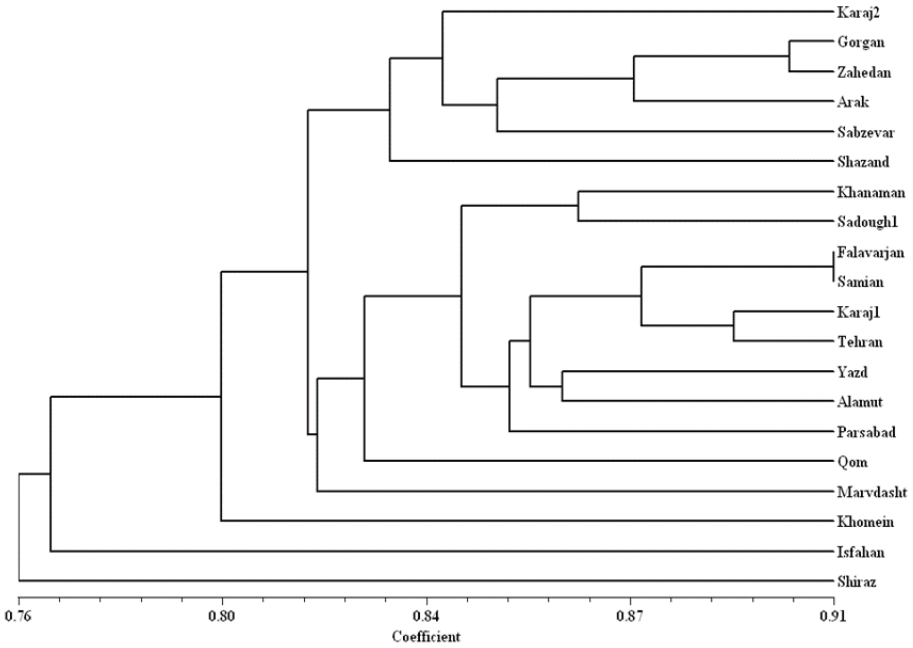

Figure 5. Dendrogram of genotypes based on UPGMA algorithm. Similarity matrix was created using Jacard function.

were located close to each other. In the molecular experiment, Falavarjan and Samian were located very close to each other. Marvdasht and Gorgan which had nearly the same behavior in germination experiment were located in different groups in the molecular based dendrogram. In morphological dendrogram, Isfahan and Tehran were not separated from each other while in the molecular based dendrogram they were in different groups. Even the closest genotypes in ISSR dendrogram had a similarity near 0.91 (Falavarjan and Samian) which could easily be discriminated from each other. Further analysis showed that the correlation between ISSR similarities and morphological distance was low (r= -0.22) however it was significant, statistically. In Figure 6, the morphological distance versus ISSR similarity is plotted.

DISCUSSION

Germination experiment

It is believed that the first essential step for domestication of a plant is to understand enough about its seed germination (Potvin *et al*., 2003). Acceptable germination is a key factor in preserving endangered plants artificially or naturally (Butola and Badola, 2004). The range of germination percentage (11.1 to 95.5%) was very diverse (Table 2). What is surprising is that the difference among genotypes were highly significant for all traits ($P \leq 0.01$). These results are in accordance with previous studies regarding the genetic diversity of some Iranian medicinal plants (Farajpour *et al*., 2012; Izadi-Darbandi *et al*., 2013).

For low germination populations further analysis should be carried out to understand the reason. One possibility is seed dormancy. If seed dormancy is governed genetically, then genotypes with no dormancy are expected to arise via selection. High germination percentage helps farmer for to use fewer seeds for sowing. A more important factor than germination percentage is homogeneity of germination. Covering the soil surface with crop in a short period of time will not allow weeds to invade the farm. Also, homogeneity of germination will lead to homogeneity of maturity which is important for harvesting. Germination rate and mean germination time reflect this homogeneity. Stronger seedling will help the plant to have better growth in its whole life cycle. Regarding seedling characteristics, the best genotype was Qom which had the best shoot and root length. So far very little attention has been paid to developing new cultivars in medicinal plants. Producing inbred lines in this plant is possible by inbreeding, however, development of a hybrid cultivar is technically challenging as in ajowan the size of flowers is very small and the source of male sterility has not been discovered yet. Therefore, the best way for producing an acceptable cultivar is planning for an open pollinating or synthetic cultivar. In both approaches

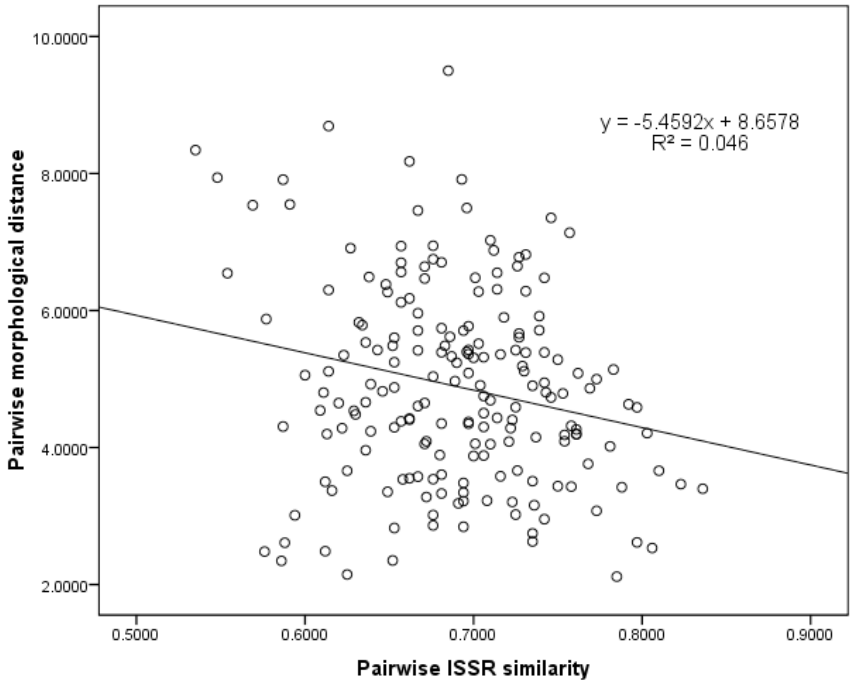

Figure 6. Correlation of ISSR similarities calculated with Jacard function with morphological distance matrix.

germination percentage and its homogeneity should be taken into consideration.

Morphological variability

Heritability was very high for flowering date (H^2=0.99) which further supports the idea that this trait is controlled by more than one gene. High heritability for this trait was reported previously in a different plant species (Izadi-Darbandi *et al*., 2013). For the Mediterranean climate, future cultivars should have early maturity. In most parts of the country the precipitation after the end of April is not enough for proper plant growth. Another important characteristic especially important for developing a new cultivar is stem diameter as it has a high correlation with tolerance to lodging. Altogether, morphological data showed that none of the genotypes had an acceptable value for all traits, although Sabzevar was the best genotype considering several measured traits. Therefore, a new commercial cultivar should be developed from these genotypes by crossing and selecting among progenies. Shiraz with unacceptable values for most traits would be better to be discarded from starting population for breeding. Considering open pollinating cultivars, a starting population could include Sabzevar (for showing the best values of days to flowering, total numbers of flowers, number of branches and leaf length), Falavarjan (for showing the best plant height), Alamut (for showing the best stem diameter and petiole length) and Qom (for showing the best shoot dry weight). A simple strategy for improving the population for developing a new cultivar is genotypic mass selection. There were significant negative correlation between days to flowering and the number of umbels. Thus, early flowering is a better criterion for selection. For a synthetic cultivar development, the starting material has to be grouped in different maturity classes as the range of maturity was from 61 to 81. The first 10 populations with the longest time for flowering (Table 5) can be used for poly cross analysis to developa late maturity cultivar and the second 10 genotypes with the shortest time to flowering can be used for developing an early maturity cultivar. Overall, the results of the cluster analysis indicated that the relative position of genotypes in dendrograms does not match their collection site. Other researchers have also reported this disagreement, as well (Roy *et al*., 2006; Kumar *et al*., 2007; Fadaei Heidari *et al*., 2016). The range of plant height was between 46 to 79 cm. Some genotypes are too short for mechanical harvesting.

ISSR experiment

The main purpose of ISSR experiment was to fingerprint the genotypes, manage their conservation in the gene bank and recognize the genotype identity during cultivar development. In this experiment, 66 percent

of total 93 bands were polymorphic. Modareskia *et al*. (2012) obtained 61% polymorphism out of 153 bands while trying to characterize 15 Ajowan ecotypes by ISSR. Most polymorphic bands were produced from primers with $(AC)_n$ and $(CA)_n$ motifs which are consistent with the results obtained from previous studies about ISSR diversity in Apiacea family (Qiu *et al*., 2004; Modareskia *et al*., 2012). The closest genotypes in the ISSR dendrogran (Falavarjan and Samian) were obtained from geographically distant regions. Gorgan and Zahedan populations are also from distant areas in Iran while they are very close to each other based on the ISSR dendrogram. Similar to morphological analysis, these results of cluster analysis indicated that the relative position of genotypes in dendrogram does not match their collection site. Other researchers have also reported this type of disagreement, as well (Roy *et al*., 2006; Kumar *et al*., 2007; Fadaei Heidari *et al*., 2016). The co-phenetic correlation between similarity and co-phenetic matrices was 0.85 implying good fitness of cluster to similarity matrix. Primers UBC818, UBC857 and ISSR5 were the best as they showed the highest Rp values and are suggested for future studies. Thenotworthy finding was that the results of germination, morphological and molecular parts were not consistent with each other implying that they may be independent to some extent. These results are consistent with previous studies in which both morphological and molecular diversities were assessed simultaneously in different plant species (Bruschi *et al*., 2003; Reed and Frankham, 2001). This inconsistency may be due to the fact that ISSRs do not amplify the functional parts of the genome. The results of ISSR may help us to preserve the seed samples in the gene bank for better conservation of this plant as they can differentiate different populations from each other. The present results of morphological, germination and ISSR diversity are significant in at least two major respects, first, conservation of this plant and second for developing new commercial cultivars. Howeve, further work is required to establish a breeding program for genetic improvement and domestication of this plant.

CONCLUSION

The aim of this investigation was to assess genetic diversity of ajowan considering germination, morphological traits along with ISSR fingerprinting. This experiment confirmed that there was a considerable genetic diversity among ajowan genotypes. Germination properties of genotypes were characterized and the best genotype for each morphological trait was detected. According to ISSR results all genotypes were separated from each other. Taken together, these results suggest that there is enough diversity in the germplasm for the development of new promising cultivars. This research will serve as a base line for future studies dealing with genetic improvement and domestication of this plant. Since there is a high genetic diversity among accessions of this plant, their conservation would be very helpful for the future breeding programs. More investigation is needed to estimate narrow sense heritability which is more practical in plant breeding. Future experiments also should assess the general combining ability of genotypes for developing a synthetic cultivar. The findings of this research have a number of practical implications for introducing the starting plant material. As the germination of most genotypes was not acceptable, it is recommended that further research be performed on understanding the obstacles of germination of these genotypes.

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Compliance with Ethical Standards

This research did not involve any human participants or animals.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Herewith I confirm, on behalf of all authors, that the information provided is accurate.

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