International Journal of Horticultural Science and Technology
Journal homepage: http://ijhst.ut.ac.ir

Phylogenetic, Genetic Diversity, and Population Structure Analysis of Iranian Black Cumin (*Nigella sativa* L.) Genotypes Using ISSR Molecular Markers

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ARTICLE INFO

Article history:

Received: 14 October 2020,
Received in revised form: 14 August 2021,
Accepted: 15 October 2021

Article type:

Research paper

Keywords:

Breeding,
Germplasm,
Polymorphism information content,
Similarity matrix,
Subpopulation

ABSTRACT

Black cumin (*Nigella sativa* L.) is one of the most important plants in terms of medicine and economics in the world. Breeding of black cumin genotypes by using biotechnology and phytochemistry has always been an important area of different studies. In this study, 24 ISSR molecular markers were used to evaluate the genetic diversity and population structure of Iranian black cumin genotypes. The primers produced a total number of 223 bands, of which 155 were polymorphic bands (indicating 69% polymorphism). By analyzing the similarity matrix based on the simple matching similarity coefficient, the similarity ranged from 0.46 to 0.84. The genotypes were classified into three main groups in the phylogenetic dendrogram, which was based on the similarity matrix and UPGMA algorithm. The average of Polymorphism Information Content, Marker Index, Resolving power, and Observed number of alleles, Effective number of alleles, Nei's gene diversity, and Shannon's information index were 0.26, 1.56, 3.07, 15.79, 13.72, 0.26, and 0.38, respectively. In analyzing the population structure, when the K value was adjusted to range from 2 to 10, two subpopulations were revealed. However, there was a degree of inconsistency when comparing the results of the phylogenetic dendrogram with those of the population structure. The results of this study can expand future inquiries into the assessments of germplasms and provide opportunities for breeding black cumin genotypes.

Introduction

Black cumin (*Nigella sativa*) is an important member of Ranunculaceae family native of Southern Europe, North Africa, and Southeast Asia; it grows in different countries of the world (Islam et al., 2017). The main reason for black cumin cultivation is its highly valuable benefits that have recently increased its breeding potential. As referred to in literatures, the last prophet Hazrat Mohammad (Sm); who told that the "*N. sativa* cure every disease except death" (Islam et al., 2019). Today many studies have proven some of its strong and broad pharmacological applications including antibacterial (Bourgou et al., 2011; Chaieb et al., 2011; Neela et al., 2015; Shaaban et al., 2015; Siddiqui and Chaudhry, 2018), antioxidant (Mariod et al., 2009; Bourgou et al., 2011; Jrah Harzallah et al., 2012; Solati and Baharin, 2014; Abou Khalil et al., 2017), antifungal (Halamova et al., 2010; Rogozhin et al., 2011; Nadaf et al., 2015), anti-schistosomiasis (Mohamed et al., 2005), antidiabetic (Abdelmeguid et al., 2010; Benhaddou-Andaloussi et al., 2011; Mathu et al., 2011), anticancer (Effenberger et al., 2010; Bourgou et al., 2011; Mahmoud and Torchilin,

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DOI: 10.22059/IJHST.2021.311894.402

2013; Majdalawieh et al., 2017; Czajkowska et al., 2017), anti-inflammatory (Chehl et al., 2009; Bourgou et al., 2011; Alemi et al., 2013), antiosteoporotic (Shuid et al., 2012), immunomodulatory activity (Ghonime et al., 2011), nephrotoxicity (Uz et al., 2008; Yildiz et al.,2010), antidepression (Elkhayat et al., 2016), pulmonary protective (Tayman et al., 2013), anti-asthmatic (Boskabady et al., 2010; Barlianto et al., 2017), anticonvulsant (Raza et al., 2008), effects on obesity (Namazi et al., 2018), antimicrobial (Bakal et al., 2017), nutraceutical effects (Ramadan et al., 2007; Srinivasan, 2018), prevention of Alzheimer (Cascella et al., 2018) and anti-pain activity (Mahboubi et al., 2018). Identification and genetic diversity of black cumin genotypes analysis are critical and necessary for different purposes. Genetic resources are the most important, most valuable, and vital reserves in each country and their value is not comparable with others. The essential factor in any breeding program is the existence of high genetic diversity to achieve the desired goals. Therefore, germplasm resources must be collected and evaluated. Determination of genetic diversity in plants is crucial and is the first step in identifying, preserving, and maintaining genetic resources (Nikrouz-Gharamaleki et al., 2019). The selection of genetic diversity analyzing methods depends on the testing objectives, population, and other factors. DNA-based markers are the most appropriate method for estimating genetic diversity. The Inter-Simple Sequence Repeats (ISSR) molecular marker is based on the polymerase chain reaction (PCR) and has favorable characteristics such as simultaneous analysis of a large number of gene amplicon, high precision, and high variation (Reddy et al., 2002).

The main purposes of this study were i) to assess similarity matrix and phylogenetic relationships among genotypes; ii) to evaluate different genetic diversity indices, and iii) to determine the population structure of Iranian black cumin genotypes.

Material and Methods

Plant samples and DNA isolation

In this research, 28 genotypes of black cumin were collected from different parts of Iran (Table 1 and Fig. 1) and cultivated in the experimental farm of Mohaghegh Ardabili University, Ardabil province. Before performing molecular experiments, qualitative traits were investigated for each genotype separately, and the qualitative coefficient of variation was calculated according to the Farmanpour Kalalagh et al. (2016) (Table 2 and Fig. 2). For DNA isolation, unblemished, young, and fresh leaves were taken in June from each genotype and were stored at -80˚C. Total genomic DNA was extracted from 200 mg of dried leaf samples using a CTAB method. Genomic DNA samples were run in 0.8% Agarose gel electrophoresis to check their quality and quantity. The concentration and purity of DNA samples were determined using a Spectrophotometer (6705 UV/Vis model).

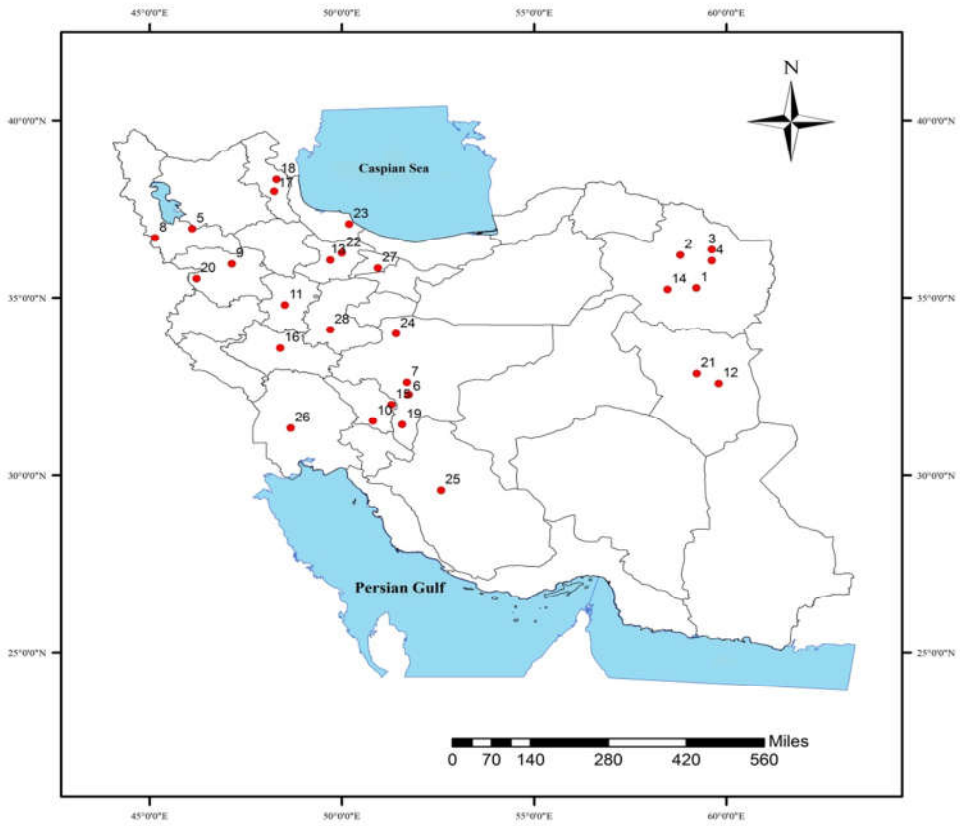

Fig. 1. Collection sites of black cumin (*Nigella sativa* L.) genotypes from different geographical regions of Iran, used in this study

Table 1. Geographical characteristics of collected Iranian black cumin genotypes

No.	Genotype name	Abbreviation	Latitude (N)	Longitude(E)	Altitude(m)
1	Torbat Heydarieh	TH	35°16'47.43"	59°12'58.12"	1363
2	Neyshabour	Ne	36°12'50.71"	58°47'45.93"	1202
3	Mashhad 1	Ma1	36°15'37.66"	59°37'00.32"	1105
4	Mashhad 2	Ma2	36°15'00.02"	59°37'11.28"	989
5	Miandouab	Mi	36°57'34.20"	46°06'19.36"	1296
6	Esfahane 1	Es1	32°32'44.12"	51°44'35.92"	1562
7	Esfahane 2	Es2	32°36'50.70"	51°41'11.74"	1613
8	Piranshahr	Pi	36°41'42.09"	45°08'40.45"	1442
9	Kurdistan	Ku	35°57'19.29"	47°08'10.37"	1874
10	Lordegan	Lo	31°30'59.71"	50°48'51.93"	1574
11	Hamadan	Ha	34°47'55.89"	48°30'54.08"	1838
12	Sarbisheh	Sa	32°34'39.76"	59°47'51.99"	1831
13	Takestan	Ta	36°04'19.55"	49°42'04.85"	1263
14	Kashmar	Ka	35°14'36.32"	58°28'07.35"	1059
15	Boroujen	Bo	31°58'47.25"	51°17'40.58"	2243
16	Lorestan	Lor	33°34'54.63"	48°23'55.75"	1607
17	Ardabi 1	Ar1	38°12'02.85"	48°14'24.10"	1382
18	Ardabi 2	Ar2	38°15'13.45"	48°17'59.96"	1348
19	Semirom	Se	31°24'54.49"	51°34'05.98"	2406
20	Marivan	Ma	35°32'20.31"	46°13'08.33"	1353
21	Birjand	Bi	32°51'53.65"	59°13'34.49"	1468
22	Qazvin	Qa	36°16'25.17"	49°59'53.65"	1307
23	Amlash	Am	37°05'29.88"	50°11'12.98"	39
24	Kashan	Kash	33°59'06.13"	51°24'35.86"	964
25	Shiraz	Sh	29°35'30.36"	52°35'01.31"	1704
26	Ahvaz	Ah	31°19'05.98"	48°40'14.23"	20
27	Karaj	Kar	35°50'24.07"	50°56'20.73"	1329
28	Arak	Ar	34°05'43.28"	49°42'04.85"	1796

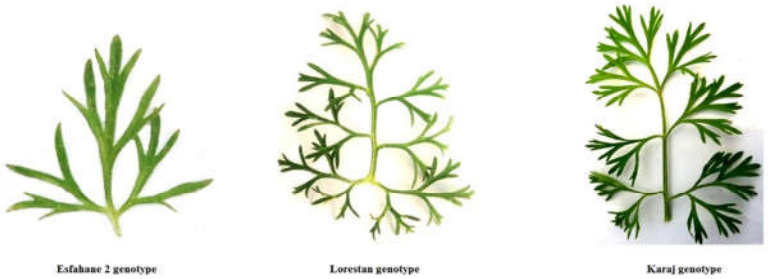

Fig. 2. Qualitative morphological variation in three Iranian black cumin (*Nigella sativa* L.) genotypes

Table 2. Qualitative traits of studied Iranian black cumin genotypes

Genotype \ Trait	SE	FC	SM	FS	FR	FT	LA	BD	GP	STh
Torbat Heydarieh	3	1	2	3	2	2	2	2	3	2
Neyshabour	3	1	2	3	2	2	2	2	4	2
Mashhad 1	4	1	1	2	2	1	1	1	1	2
Mashhad 2	3	1	2	3	2	2	2	2	4	2
Miandouab	3	1	2	3	2	2	2	2	3	2
Esfahane 1	4	1	2	3	1	2	1	1	1	2
Esfahane 2	4	1	1	2	1	1	1	1	1	2
Piranshahr	3	1	2	3	2	2	2	2	3	2
Kurdistan	3	1	2	3	2	2	2	2	3	2
Lordegan	1	2	3	4	3	3	3	3	5	4
Hamadan	2	2	3	4	3	3	3	3	5	3
Sarbisheh	3	1	2	3	2	3	2	2	3	3
Takestan	2	2	3	4	3	3	3	3	5	3
Kashmar	3	1	2	2	1	2	2	2	2	2
Boroujen	4	1	1	1	1	1	1	1	2	1
Lorestan	4	1	2	3	2	2	2	2	3	2
Ardabi 1	2	2	3	4	3	3	3	3	5	3
Ardabi 2	3	1	2	2	2	2	1	2	2	2
Semirom	4	1	1	2	1	1	1	1	1	2
Marivan	2	1	2	3	2	3	2	2	4	3
Birjand	4	1	2	2	2	1	2	1	4	2
Qazvin	4	1	2	3	2	2	1	2	3	2
Amlash	3	1	2	3	2	2	2	2	4	2
Kashan	4	1	1	3	2	2	1	2	1	2
Shiraz	4	1	1	2	1	1	1	1	2	1
Ahvaz	3	1	2	3	1	2	2	2	3	2
Karaj	1	2	3	4	3	3	3	3	5	4
Arak	3	1	1	2	2	1	1	1	1	2
CV(%)	32.55	0	51.85	35.44	51.85	100	54.90	52.83	67.47	44.44

Note: SE (Stem Elasticity: 1=Very low, 2=low, 3=Moderate, 4=High), FC (Flower Color: 1=White, 2=Pale blue), SM (Seed ripening: 1=Early ripening, 2=Middle ripening, 3=Late ripening), FS (Follicle Size: 1=Very small, 2=Small, 3=Moderate, 4=Larg), FR (Flowering Rate: 1=Low, 2= Moderate, 3=High), FT (Flowering Time: 1= Early Flowering, 2=Middle Flowering, 3= Late Flowering), LA (Leaf Area: 1= Narrow, 2= Average width, 3=Broad width), BD (Branch Density: 1=Low, 2=Average, 3=High), GP (Growth Power: 1=Very week, 2=Weak, 3=Moderate, 4=Strong, 5=Very Strong), STh (Stem Thickness:1=Narrow, 2=Medium, 3=Thick, 4=Very Thick), CV(Coefficient of variation for qualitative traits (according to the Farmanpour Kalalagh et al., 2016 formula) = $\frac{Quartile(Q)}{Mean} \times 100 = \frac{Q3-Q1}{Mean} \times 100$).

PCR amplification

Twenty-four Inter-Simple Sequence Repeat (ISSR) primers were used for the classification of genotypes which is used by Farmanpour Kalalagh et al. (2017). Primer names, sequences, annealing temperatures, and other information are given in Table 3. Polymerase chain reaction (PCR) amplification was conducted with a Thermocycler machine (Qantarus model) in 8 µL reaction mixture of 2.05 µL DDW, 1 µL template DNA (10 ng), and 3.75 µL 2 × Master Mix buffer (0.4 mM dNTPs, 0.2 units/µL Amplicon Taq DNA Polymerase, 2 mM MgCl2). The Polymerase Chain Reaction program was conducted using the following thermal protocol: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 40-61.7°C for 30s, elongation at 72°C for 100s and final elongation at 72°C for 7 min. Amplicons were separated on 1.4% agarose gel pre-stained with a GelRed solution (dye) using a 1x TBE (Tris-Borat/ EDTA) buffer. The gels were run for 90 min at 74 volt. Finally, the separated amplicons were visualized by photograph taking (Gel Documentation).

Table 3. Primer names, primer sequences, annealing temperatures, and measured indices in this study.

No.	Primer name	Primer sequence (5′-3′)	Tm (°C)	TA	PA	PPA (%)	PIC	MI	Rp	Na	Ne	h	I
1	AL1	GAGAGAGAGAGACC	45.7	5	4	80	0.35	1.40	1.50	9	7.44	0.29	0.43
2	UBC839	ACACACACACACACACGA	53	8	5	62.5	0.31	1.55	2.55	13	11.42	0.25	0.36
3	UBC811	GAGAGAGAGAGAGAGAT	52	7	4	57.14	0.36	1.44	2.69	11	10.26	0.25	0.36
4	UBC810	GAGAGAGAGAGAGAGAC	43	7	4	57.14	0.34	1.36	0.63	11	8.59	0.15	0.24
5	UBC819	ACACACACACACACACC	52.4	9	7	77.77	0.22	1.54	2.75	16	12.59	0.23	0.35
6	UBC815	CTCTCTCTCTCTCTCTG	44	12	11	91.66	0.16	1.76	6.18	23	20.37	0.37	0.54
7	UBC822	ACACACACACACACACT	49	6	5	83.33	0.29	1.45	1.76	11	8.47	0.36	0.41
8	UBC829	GACAGACAGACAGACA	49	7	6	85.71	0.27	1.62	3.89	13	11.92	0.37	0.54
9	UBC834	GGGTGGGGTGGGGTG	54	9	6	66.66	0.28	1.68	3.34	15	13.94	0.29	0.42
10	ISSR1	CACACACACACACACARG	56	13	8	61.53	0.21	1.68	3.54	21	18.99	0.25	0.36
11	ISSR6	GTGTGTGTGTGTGTGTYC	56.8	8	3	37.5	0.44	1.32	0.98	16	8.15	0.05	0.10
12	ISSR11	BDBACAACAACAACAACA	50	10	9	90	0.19	1.71	3.62	19	16.83	0.37	0.54
13	ISSR12	DDCCACCACCACCACCA	58.8	11	8	72.72	0.21	1.68	3.77	19	17.05	0.30	0.44
14	ISSR16	BDBCACCACCACCACCA	61.7	13	4	30.76	0.36	1.44	2.21	17	16.62	0.12	0.17
15	ISSR19	VVHTTGTTGTTGTTGTTG	50.3	11	9	81.81	0.19	1.71	3.96	14	17.67	0.34	0.55
16	ISSR21	ACTCACTCACTCACTC	51.2	6	4	66.66	0.33	1.32	1.54	10	8.36	0.22	0.34
17	ISSR24	CACCACCACGC	40	9	5	55.55	0.29	1.45	2.12	16	11.15	0.19	0.27
18	ISSR27	CTCTCTCTCTCTCTCTTC	53.7	9	5	55.55	0.32	1.60	2.47	14	11.58	0.17	0.27
19	ISSR28	CTCTCTCTCTCTCTCTAC	53.7	12	12	100	0.15	1.80	5.62	24	21.19	0.42	0.61
20	ISSR29	GACACACACACACACACAC	56.7	10	9	90	0.19	1.71	5.31	19	17.92	0.41	0.58
21	ISSR30	CCACTCTCTCTCTCTCTCT	58.7	12	8	66.66	0.21	1.68	4.76	20	17.33	0.25	0.37
22	ISSR31	ATGATGATGATGATGATG	48.9	11	7	63.63	0.24	1.68	4.04	18	16.05	0.26	0.38
23	ISSR33	GGGTGGGGTGGGGTG	60.8	7	4	57.14	0.32	1.28	1.98	11	8.97	0.17	0.27
24	ISSR35	ACACACACACACACACGT	55.7	11	8	72.72	0.21	1.68	2.7	19	16.65	0.29	0.42
Average	-	-	-	9.29	6.45	69	0.26	1.56	3.07	15.79	13.72	0.26	0.38

Note: Y(C,T), R(G,A), V(A,T), B(A,C), D(T,G), H(C,G), Tm(Annealing temperature), TA(Total Amplicons), PA(Polymorphic Amplicons), PPA(Percentage of Polymorphic Amplicons), PIC(Polymorphism Information Content), M(Marker Index), Rp(Resolving power), Na(Observed number of alleles), Ne(Effective number of alleles), h(Nei's gene diversity), I(Shannon's information index).

Statistical analysis

Clear amplicons were visually scored as a binary matrix for absence (0) and presence (1) of amplicons. The banding patterns were analyzed for genetic relatedness among black cumin genotypes. Indices of genetic diversity such as Nei's gene diversity (h), Shannon's information index (I), the observed number of alleles (Na), and the effective number of alleles (Ne) were obtained using PopGene 32 software. Resolving power (Rp), Marker index (MI), and Polymorphism Information Content (PIC) were calculated using the formulas in Table 4 for each primer. To evaluate the similarity matrix based on the high content of Cophenetic value (r), calculating of r value for Dice, Simple Matching, and Jaccard's coefficients were done based on Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method using NTSYS-pc 2.01 software. The model-based program STRUCTURE 2.3.4 was applied to infer the population structure of Iranian black cumin genotypes. In the first instance, the appropriate population structure analysis was done along with accurate classification of genotypes into subpopulations. Then, the number of K (number of the subpopulations) was calculated by using the Evanno et al. (2005) method. The differences in mean values of the repetitions for adjacent groups were determined by knowing the mean value of the group that was above the mean value and then subtracting it from the group that was below the mean value. The result was named the L'(K). The difference between the L'(K) values of adjacent groups was named L''(K). Using these parameters, the ΔK was calculated by |L''(K)|/Stedv. Finally, by plotting the two-sided diagrams K and ΔK, the peak of the diagram was obtained, which resembles the optimal number of K. Individuals were assigned to subgroups according to the method described by Spataro et al. (2011), and calculation per group gave each individual a membership percentage. According to this method, a genotype can be assigned to a group if the membership percentage of the genotypes is equal to or more than 0.70, whereas genotypes with membership percentages of 0.69 (or less) would be considered mixed genotypes.

Table 4. RP, MI, and PIC formulas used in this study

(1)	$Rp=\sum I_b$ $[I_b= 1-(2\times\lvert 0/5-p\rvert)]$	I_b → represents band informativeness p → fraction of the total genotypes in which the band is present.
(2)	$MI= PIC\times PL$	PL → Polymorphic Loci
(3)	$PIC= 2P_i (1-P_i)$	P_i → frequency of marker fragments that were present. $(1-P_i)$ → *the* frequency of marker fragments that were absent.

Results

In this research, 24 ISSR primers were utilized to identify and assess black cumin genotypes' diversity. As a result, 223 high-resolution amplicons were produced, of which 68 of them were monomorphic and 155 were polymorphic. The size of the amplicons varied between 100 and 1500 bp. AL1 primer, which created five loci obtained the lowest number of amplified loci, and the highest number related to the ISSR1 and ISSR16 primers, which produced 13 loci. The largest polymorphic loci were obtained through the UBC815 primer (Fig. 3), which yielded 11 loci, and the ISSR28, which yielded 12 loci. Three primers including ISSR11, ISSR19 (Fig. 4), and ISSR 29 was also produced 9 polymorphic loci. Each primer's polymorphic percentage varied from 30.76% (ISSR16 primer) to 100% (ISSR28 primer). The average number of the total loci and the average number of polymorphic loci for each primer were 9.29 and 6.45, respectively.

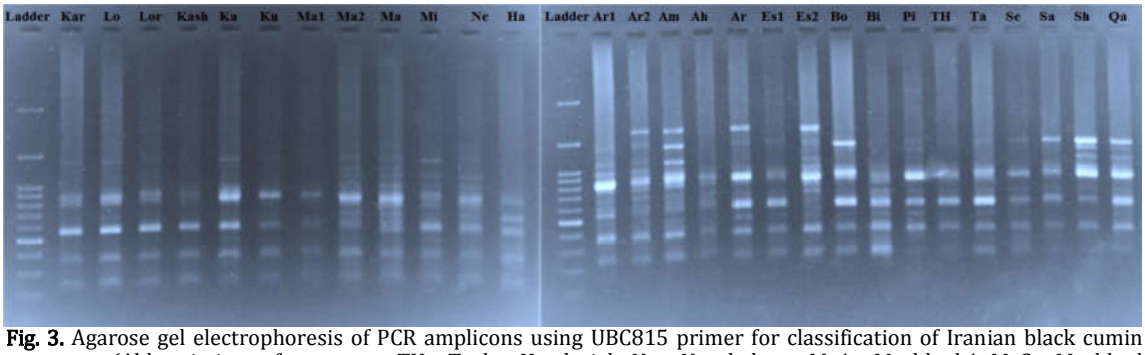

Fig. 3. Agarose gel electrophoresis of PCR amplicons using UBC815 primer for classification of Iranian black cumin genotypes (Abbreviations of genotypes: TH= Torbat Heydarieh; Ne= Neyshabour; Ma1= Mashhad 1; Ma2= Mashhad 2; Mi= Miandouab; Es1= Esfahane 1; Es2= Esfahane 2; Pi= Piranshahr; Ku= Kurdistan; Lo= Lordegan; Ha= Hamadan; Sa= Sarbisheh; Ta= Takestan; Ka= Kashmar; Bo= Boroujen; Lor= Lorestan; Ar1= Ardabi 1; Ar2= Ardabi 2; Se= Semirom; Ma= Marivan; Bi= Birjand; Qa= Qazvin; Am= Amlash; Kash= Kashan; Sh= Shiraz; Ah= Ahvaz; Kar= Karaj; Ar= Arak)

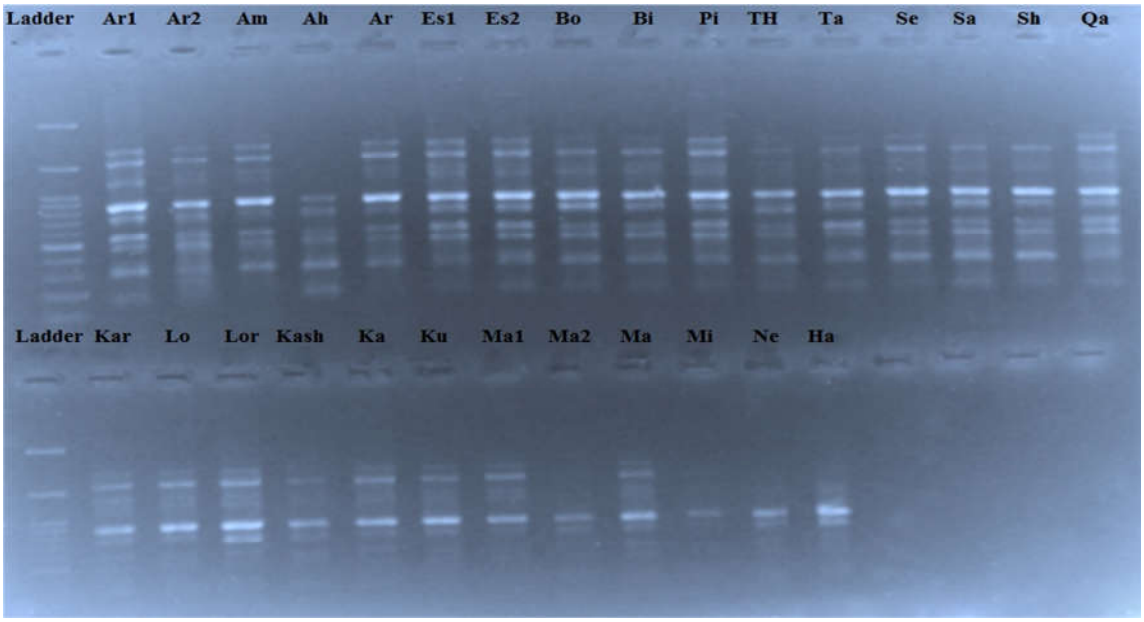

Fig. 4. Agarose gel electrophoresis of PCR amplicons using ISSR 19 primer for classification of Iranian black cumin genotypes (Abbreviations of genotypes: TH= Torbat Heydarieh; Ne= Neyshabour; Ma1= Mashhad 1; Ma2= Mashhad 2; Mi= Miandouab; Es1= Esfahane 1; Es2= Esfahane 2; Pi= Piranshahr; Ku= Kurdistan; Lo= Lordegan; Ha= Hamadan; Sa= Sarbisheh; Ta= Takestan; Ka= Kashmar; Bo= Boroujen; Lor= Lorestan; Ar1= Ardabi 1; Ar2= Ardabi 2; Se= Semirom; Ma= Marivan; Bi= Birjand; Qa= Qazvin; Am= Amlash; Kash= Kashan; Sh= Shiraz; Ah= Ahvaz; Kar= Karaj; Ar= Arak).

The average percentage of polymorphism obtained in this study was 69%, which explains the high genetic diversity among genotypes (Table 3). Also, the values for the average polymorphism information content (PIC), marker index (MI), resolving power (Rp), observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (h), and Shanon's information index (I) were 0.26, 1.56, 3.07, 15.79, 13.72, 0.26 and 0.38, respectively. According to Table 3, the highest values of Rp were 5.31, 5.62, and 6.18, which are attributed to the ISSR29, ISSR28, and UBC815 primers, respectively. The lowest values of Rp were 0.63 and 1.50, which are attributed to the UBC810 and the AL1 primers, respectively. High values of the resolving power obtained for most primers, except the UBC810 and the AL1. The ISSR33 primer exhibited the lowest value of MI (1.28), whereas the ISSR28 exhibited the highest value of MI (1.80). The ISSR6 showed the highest value of PIC (0.44), whereas the ISSR28 showed the lowest value of PIC (0.15). The PIC index determines the differentiation of each primer by the number of alleles in a genetic location and the relative frequency of the amplicons (Muminovic et al., 2004). The maximum PIC value in a double-allele locus is 0.5, and this only happens when the frequencies of the alleles in the population are equal (Mateescu et al., 2005). The marker index (MI) also uses the number of gene loci pertaining to the polymorphic primers in the estimation of their efficiency and resolving power (Powell et al., 1996). Simple Matching (SM) coefficient with high content of cophenetic value ($r = 0.9$) was considered as an appropriate coefficient for the similarity matrix analysis. Therefore, the similarity index was calculated based on the SM coefficient. According to Table 5, the genetic similarity of the genotypes ranged from 0.46 to 0.84 in the black cumin population, and the average of this value was 0.65.

The phylogenetic dendrogram, according to the similarity matrix and the UPGMA algorithm categorized the black cumin genotypes into three groups (Fig. 5). The discriminant analysis confirmed the categorization by 100%. In the analysis of the genetic structure and the segregation of the total population, Table 6 and Fig. 6 show a bilateral graph for determining the optimal K for black cumin genotypes. The peak value of the curve equals 2, thereby indicating that the optimum number of K is equal to 2.

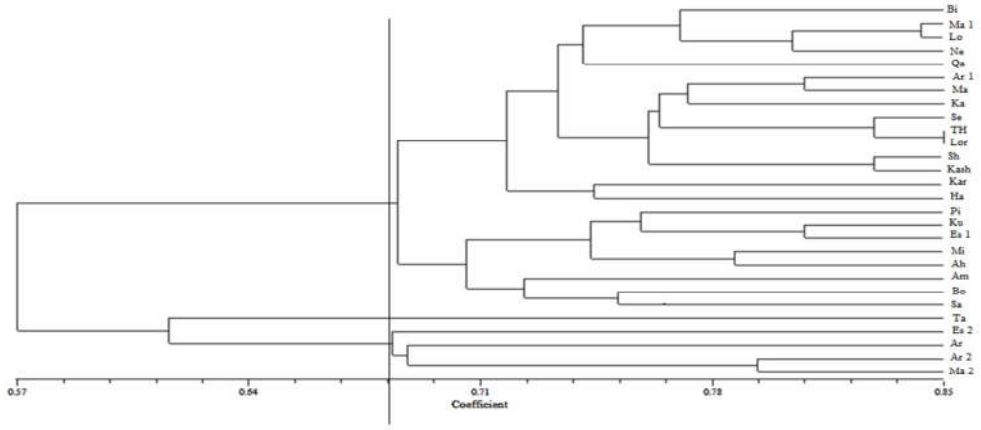

Fig. 5. Phylogenetics dendrogram of Iranian black cumin (*Nigella sativa* L.) genotypes based on the similarity matrix and UPGMA algorithm (Abbreviations of genotypes: TH= Torbat Heydarieh; Ne= Neyshabour; Ma1= Mashhad 1; Ma2= Mashhad 2; Mi= Miandouab; Es1= Esfahane 1; Es2= Esfahane 2; Pi= Piranshahr; Ku= Kurdistan; Lo= Lordegan; Ha= Hamadan; Sa= Sarbisheh; Ta= Takestan; Ka= Kashmar; Bo= Boroujen; Lor= Lorestan; Ar1= Ardabi 1; Ar2= Ardabi 2; Se= Semirom; Ma= Marivan; Bi= Birjand; Qa= Qazvin; Am= Amlash; Kash= Kashan; Sh= Shiraz; Ah= Ahvaz; Kar= Karaj; Ar= Arak).

Table 5. Similarity matrix of Iranian black cumin (*Nigella sativa* L.) genotypes based on Simple Matching (SM) coefficient.

Genotypes	Bi	Kar	Ha	Ta	Ar 1	Ma1	Lo	NE	Qa	Se	Ma	TH	Sh	Kash	Ka	Lor	Pi	Am	Mi	Ah	Bo	Ku	Es1	Es2	Ar	Ar2	Ma2	Sa
Bi	1																											
Kar	0.70	1																										
Ha	0.68	0.74	1																									
Ta	0.60	0.71	0.61	1																								
Ar 1	0.70	0.69	0.75	0.59	1																							
Ma1	0.75	0.72	0.77	0.63	0.77	1																						
Lo	0.75	0.79	0.77	0.62	0.78	0.84	1																					
Ne	0.79	0.67	0.72	0.58	0.77	0.81	0.79	1																				
Qa	0.70	0.66	0.68	0.57	0.76	0.73	0.77	0.74	1																			
Se	0.67	0.67	0.76	0.56	0.75	0.77	0.80	0.79	0.71	1																		
Ma	0.70	0.70	0.71	0.60	0.80	0.73	0.74	0.75	0.76	0.74	1																	
TH	0.69	0.68	0.72	0.54	0.76	0.72	0.72	0.75	0.70	0.82	0.79	1																
Sh	0.66	0.73	0.74	0.64	0.77	0.75	0.74	0.75	0.68	0.75	0.80	0.80	1															
Kash	0.67	0.72	0.70	0.61	0.72	0.72	0.73	0.72	0.65	0.69	0.72	0.71	0.82	1														
Ka	0.72	0.67	0.72	0.61	0.74	0.75	0.73	0.72	0.68	0.72	0.79	0.74	0.81	0.70	1													
Lor	0.72	0.66	0.72	0.59	0.77	0.76	0.72	0.78	0.65	0.82	0.75	0.84	0.81	0.75	0.81	1												
Pi	0.70	0.67	0.75	0.61	0.70	0.72	0.70	0.70	0.67	0.65	0.72	0.68	0.71	0.72	0.75	0.75	1											
Am	0.71	0.68	0.63	0.61	0.68	0.70	0.68	0.73	0.67	0.72	0.72	0.74	0.72	0.69	0.68	0.74	0.73	1										
Mi	0.67	0.63	0.66	0.61	0.68	0.67	0.66	0.73	0.64	0.66	0.70	0.70	0.68	0.65	0.69	0.74	0.75	0.75	1									
Ah	0.66	0.68	0.68	0.59	0.70	0.72	0.72	0.68	0.69	0.67	0.68	0.64	0.69	0.65	0.74	0.73	0.75	0.71	0.78	1								
Bo	0.66	0.65	0.67	0.57	0.70	0.69	0.65	0.67	0.66	0.64	0.66	0.70	0.69	0.67	0.70	0.72	0.71	0.72	0.70	0.75	1							
Ku	0.64	0.70	0.72	0.62	0.72	0.68	0.66	0.66	0.64	0.68	0.70	0.67	0.72	0.65	0.75	0.77	0.76	0.68	0.75	0.77	0.74	1						
Es1	0.59	0.64	0.66	0.55	0.68	0.64	0.63	0.63	0.64	0.66	0.64	0.63	0.70	0.66	0.73	0.70	0.75	0.66	0.66	0.75	0.72	0.80	1					
Es2	0.60	0.53	0.51	0.62	0.53	0.54	0.51	0.61	0.50	0.50	0.54	0.47	0.52	0.52	0.54	0.54	0.58	0.59	0.58	0.56	0.64	0.58	0.62	1				
Ar	0.55	0.54	0.50	0.60	0.51	0.52	0.54	0.57	0.51	0.59	0.59	0.55	0.59	0.60	0.59	0.59	0.57	0.57	0.54	0.54	0.62	0.59	0.61	0.68	1			
Ar2	0.50	0.60	0.54	0.58	0.49	0.50	0.58	0.58	0.53	0.54	0.57	0.52	0.59	0.56	0.59	0.54	0.55	0.54	0.55	0.65	0.60	0.63	0.65	0.62	0.64	1		
Ma2	0.50	0.54	0.50	0.65	0.49	0.49	0.50	0.55	0.46	0.47	0.54	0.50	0.56	0.59	0.58	0.54	0.54	0.56	0.55	0.60	0.61	0.58	0.62	0.73	0.72	0.79	1	
Sa	0.68	0.59	0.61	0.59	0.65	0.65	0.59	0.68	0.63	0.59	0.68	0.63	0.66	0.64	0.67	0.70	0.67	0.71	0.67	0.70	0.75	0.67	0.65	0.65	0.59	0.61	0.65	1

Table 6. Calculated statistic(s) for determination of optimal K using STRUCTURE 2.3.4 software

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-4526.66	1.58	-	-	-
2	**5**	**-3956.54**	**2.38**	**570.12**	**301.74**	**126.74**
3	5	-3688.16	1.80	268.38	108.02	59.89
4	5	-3527.80	12.25	160.36	7.26	0.59
5	5	-3374.70	56.28	153.10	59.14	1.05
6	5	-3280.74	211.45	93.96	101.80	0.48
7	5	-3084.98	103.25	195.76	578.88	5.60
8	5	-3468.10	649.10	-383.12	584.44	0.90
9	5	-3266.78	567.74	201.32	233.34	0.41
10	5	-3298.80	1071.78	-32.02	-	-

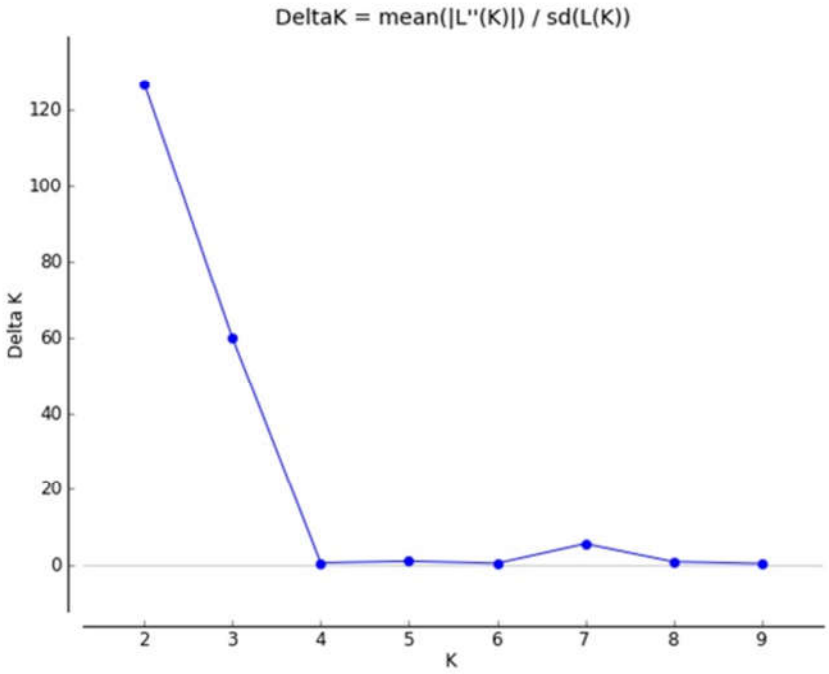

Fig. 6. Estimation of Delta K from calculated K and LnP (K) using the web-based STRUCTURE HARVESTER program in population structure analysis of Iranian black cumin (Nigella sativa L.) genotypes..

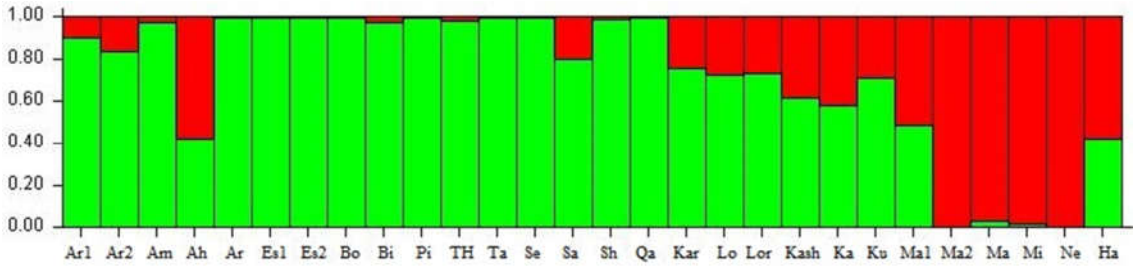

Fig. 7. Inferred population structure of Iranian black cumin (Nigella sativa L.) genotypes based on Inter-Simple Sequence Repeats molecular markers (Abbreviations of genotypes: TH= Torbat Heydarieh; Ne= Neyshabour; Ma1= Mashhad 1; Ma2= Mashhad 2; Mi= Miandouab; Es1= Esfahane 1; Es2= Esfahane 2; Pi= Piranshahr; Ku= Kurdistan; Lo= Lordegan; Ha= Hamadan; Sa= Sarbisheh; Ta= Takestan; Ka= Kashmar; Bo= Boroujen; Lor= Lorestan; Ar1= Ardabi 1; Ar2= Ardabi 2; Se= Semirom; Ma= Marivan; Bi= Birjand; Qa= Qazvin; Am= Amlash; Kash= Kashan; Sh= Shiraz; Ah= Ahvaz; Kar= Karaj; Ar= Arak).

Discussion

Results indicate the black cumin genotypes diversity and the strong ability of ISSR primers to detect differentiation among plant samples. The highest and lowest similarity was observed between the Mashhad 1 with Lordegan, and Qazvin with Mashhad 2 genotypes, respectively. The low similarity shows that the two genotypes have large genetic differences compared to each other, and therefore it is suggested that they can serve as parents in cross-breeding programs. It is assumed that by performing cross-breeding among genotypes that come from the distant group, there is the possibility of producing different progenies in breeding programs. One of the most reliable ways of achieving high levels of heterozygosity in black cumin genotypes is to select parents that are less genetically similar to each other. The identification of crossbreeds with high levels of heterozygosity is an important step in producing hybrid crops, and usually, parents with higher cross-breeding abilities and greater genetic distances can produce hybrids that would be capable of producing higher amounts of yield (Brünjes and Link, 2021).

In our previous genetic diversity evaluation study of Iranian black cumin genotypes based on morphological traits by using multivariate analysis, all genotypes were divided into four main groups without being affected by their geographical origin (Mehri et al., 2018). The results of both studies indicate that the grouping of genotypes based on morphological and molecular traits is not related to each other and also geographical origin has no effect on the classification of genotypes (Mehri et al., 2018). But in present molecular study, genotypes divided into three groups in phylogenetic dendrogram. Whereas, the grouping of genotypes by population structure analysis demonstrated two different groups. Therefore, genotypes can be separated into two groups with different genetic structures, while one group was identified as a mixture that cannot be considered to be in groups 1 or 2 because of their percentage of membership. In molecular diversity study of black cumin (*N. sativa* L.) from Ethiopia as revealed by ISSR markers, neighbor joining and UPGMA results demonstrated the potent classification among accessions collected from the Oromia and Amhara region. The five geographical regions of Ethiopia indicated various levels of genetic diversity (Kapital et al., 2015). Another successful grouping of *N. sativa* landraces by using SCoT markers was reported by Mirzaei and Mirzaghaderi (2015). In this study, most of the landraces of *N. sativa* were grouped together, which were collected from the same regions. On the other hand, it was demonstrated that the landraces of *N. sativa* collected from different regions also can classify into one cluster. In genetic diversity analysis of *N. sativa* from different geographies using RAPD markers, the dendrogram grouped the eight accessions into 4 subgroups based on the UPGMA method. The first subgroup consisted of accessions S1 (India) and S2 (Pakistan). The second subgroup consisted of accessions S4 (Saudi Arabia), S6 (Syria), S8 (Tunisia), and S7 (Turkey) accession. The third subgroup consisted of accession S3 (Egypt). The fourth subgroup consisted of accession S5 (Oman). The dendrogram showed that there is a significant impact of geographic regions on the genetic variation of *N. sativa* accessions (Sudhir et al., 2016). Also, in comparative analysis of RAPD and ISSR markers for assessing genetic diversity in Iranian populations of *N. sativa*, cluster analysis grouped the populations into five subgroups for both molecular markers (Hosseini KorehKhosravi et al., 2018).

In this study, Fig. 5 also displays the inferred structure for the population being studied, using the STRUCTURE software for ISSR primers. Accordingly, genotypes can be separated into two groups with different genetic structures, while one group is identified as a mixture because its percentage of membership does not make its genotypes eligible for either groups 1 or 2. Accordingly, 19 genotypes were assigned to group 1 and 5 genotypes to group 2, while 4 genotypes were described as mixed genotypes. In Golkar and Nourbakhsh (2019) study, population structure analysis assigned *N. sativa* population into 4 and 6 sub-populations for SCoT and SRAP markers, respectively. A population can be divided into subgroups with regard to its genetic structure, and such divisions emanate from the diversity and abundance of alleles. The population being studied in this research was also divided into two main groups. Categorizing the groups based on population structure can lead to different results compared to the results of cluster analysis. Therefore, the identification of genetic structures in populations and in germplasms, as a whole, would strongly be associated with substantial significance (Falush et al., 2003). Thus, the current results can be regarded as a necessary set of information before performing analyses for the identification of relations between linked markers and quantitative trait amplicons in the germplasm of black cumin. This outcome can help prevent researchers from identifying false links, which may ensue because of the differences in population structure.

Conclusion

The ISSR molecular markers used in this study made a correct grouping possible for the Iranian black cumin genotypes. The grouping was successful in two ways: cluster analysis and population structure, both of which were able to separate black cumin genotypes in a manner that

correlated fairly with their geographical distances from each other. The study of genotypes showed high genetic diversity by using qualitative traits and ISSR primers. The twenty-eight genotypes were classified into three groups based on cluster analysis but were grouped in two subpopulations according to the population structure. These results can extend the outlook for breeding programs by offering ways to protect the black cumin germplasm and by introducing the Iranian genotypes to future breeding programs. The study of molecular relationships among the genotypes and the provision of suitable groupings can facilitate the selection of appropriate black cumin genotypes for breeding programs. These steps could ultimately contribute to the production of black cumin genotypes that would be characterized by higher amounts of yield, high quality, and greater adaptability to specific environments.

Acknowledgement
We would like to thank University of Mohaghegh Ardabili (UMA) for supporting this study.

Conflict of Interest
The authors indicate no conflict of interest for this work.

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