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Molecular characterization of Iranian black cumin (*Nigella sativa* L.) accessions using RAPD marker

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Abstract

Nigella sativa L. ($2n$ = 12) is an annual flowering plant belonging to the family *Ranunculaceae* known for medicinal properties demonstrating valuable components that are widely used in food and pharmaceutical industries. Nine Random Amplified Polymorphic DNA markers (RAPD) were used in this study, in order to characterize the genetic variation of 16 black cumin varieties collected from Iran's different regions. The amplification reaction produced 95 bands with sizes ranging from about 100 to 500 bp. The average frequency of bands was 20 while the averages of polymorphism were about 8.11 per primer. The Jaccard similarity coefficient and the Unweighted Pair-Group Method Analysis (UPGMA) clustering algorithm were applied to the RAPD data sets in order to understand the genetic relationships among the tested accessions. The accessions were categorized into three groups using cluster analysis. The results were supported by the Principal Coordinate Analysis (PCoA) cluster analysis. The results showed that the tested black cumin genotypes had a high genetic diversity and could be used in black cumin germplasm conservation programs. Moreover, the RAPD is a versatile approach to the diversity analysis of native accessions of black cumin.

Key words: genetic diversity, *Nigella sativa*, RAPD, PCoA

Introduction

Black cumin (*Nigella sativa* L.) is an annual flowering plant belonging to the family *Ranunculaceae*. This plant has more than 100 valuable components such as thymoquinone, monoterpenes like p-cymene and pinene, nigellidine, nigellimine and saponin. *Nigella* is used to flavor traditional foods, especially bakery products (Iqbal et al., 2011). Recently, more attention has been given to high-quality oil and to medicinally important bioactive compounds present in black cumin (Ramadan and Morsel 2003; Iqbal et al., 2011). Extracts obtained from seeds of *Nigella* are usually used in the field of medicine in treating diseases such as respiratory infections, abdominal pains, gastrointestinal diseases, and as an antitumor agent (Hajhashemi et al., 2004; Mashhadian and Rakhshandeh, 2005). Furthermore, extracts of *Nigella* have insect repellent features and may be used for the production of organic pesticides (Khan et al., 2003). The seeds of Nigella, rich in essential polyunsaturated fatty acids, can also become a more economical alternative for the use of traditional crops in edible oil production.

The main fatty acids reported to be present in *N. sativa* L. oil were palmitic (C16:0), oleic (C18:1), and linoleic acids (C18:2) (Iqbal et al., 2005). Edible oils containing oleic acid are more desirable for cooking whereas high concentrations of linolenic acids are undesirable because of their low stability. They also oxidize rapidly. For the growth and health of humans, polyunsaturated fatty acids are essential. Furthermore, polyunsaturated fatty acids play an important role in reducing cholesterol and diminishing the risk of heart disease (Mohamed et al., 1995).

Characterization of different accessions of *Nigella* can help in breeding programs especially to obtain plants with high quality and quantity oil. Despite the published research, there are few reports of analysis in genetic

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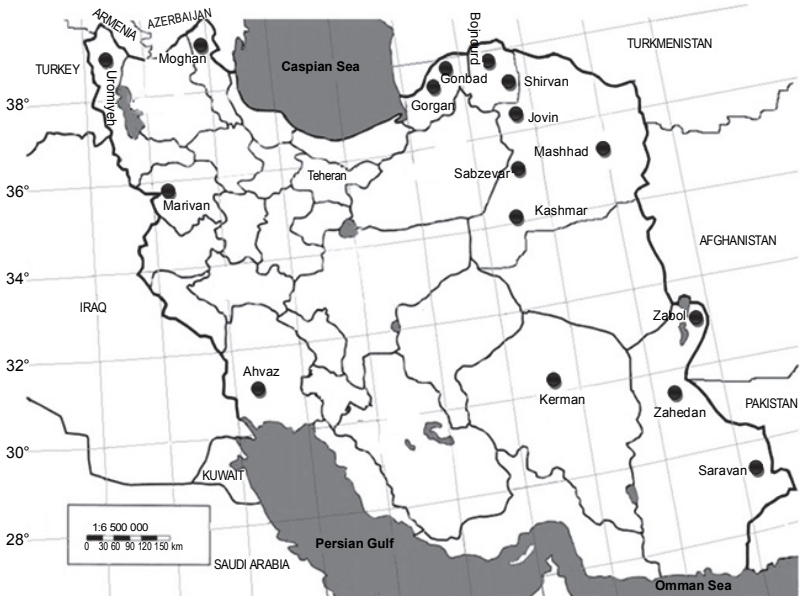

Fig. 1. Collection sites of *N. sativa* accessions in different regions of Iran (black dots)

Table 1. List of *N. sativa* accessions used in the study

Sample code	Samples region	Province of Iran	260/280 nm ratio
IR 1	Gorgan	Golestan	1.87
IR 2	Gonbad	Golestan	1.89
IR 3	Uromia	West Azerbaijan	1.83
IR 4	Ahvaz	Khozestan	1.9
IR 5	Bojnourd	North Khorasan	1.91
IR 6	Joveyn	Razavi Khorasan	1.87
IR 7	Mughan	Ardabil	1.87
IR 8	Zabol	Sistan and Baluchestan	1.87
IR 9	Zahedan	Sistan and Baluchestan	1.85
IR 10	Sabzevar	Razavi Khorasan	1.85
IR 11	Saravan	Sistan and Baluchestan	1.9
IR 12	Shirvan	North Khorasan Province	1.8
IR 13	Kashmar	Kashmar	1.8
IR 14	Kerman	Kerman	1.87
IR 15	Marivan	Kurdistan	1.81
IR 16	Mashhad	Razavi Khorasan	1.87

diversity, especially in the domestication centers such as Iran.

Genetic diversity may be assessed in populations (between or within them) at molecular levels including allozymes and DNA (Panahi et al., 2013a). During the past decades, molecular markers have been widely used for revealing polymorphisms at DNA level. In order to study genetic diversity, different DNA markers such as Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), and Random Amplified Polymorphic DNA (RAPD) are available; all of them have been used to determine genetic diversity in various populations (Panahi et al., 2013a; Panahi et al., 2013b; Mahmoudi et al., 2014).

Table 2. Sequences of the 9 selected primers used for the RAPD analysis of 16 accessions of *Nigella sativa* L.

Primer	Sequence 5′-3′	Total of bands	Polymorphic bands	Polymorphic %
OPH-04	GGAAGTCGCC	16	11	68.7
OPH-20	GGGAGACATC	17	10	58.8
OPH-15	AATGGCGCAG	10	7	70
OPA-04	AATCGGGCTG	13	8	61.5
OPA-10	CTGCTGGGAC	12	11	91.6
UB12	CCTGGGTCCA	10	5	50
UB25	ACAGGGCTCA	11	7	63.6
UB30	CCGGCCTTAG	9	8	88.8
UB76	GAGCACCAGT	12	9	75
Mean		12.2	8.11	69.8

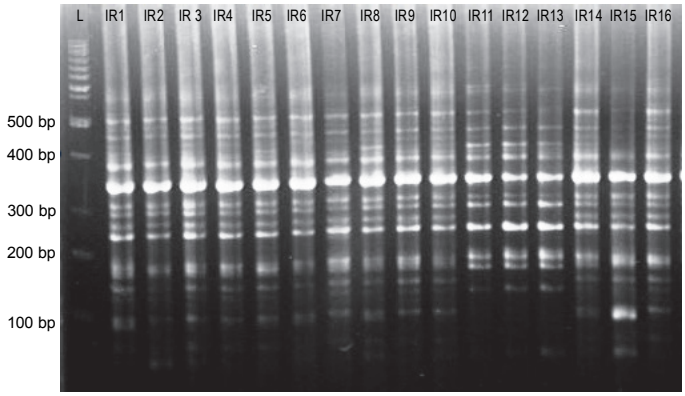

Fig. 2. Amplification patterns of OPH20 primer. The electrophoresis was performed in a 3% agarose gel. L: 100 bp ladder (Quick-Load)

RAPD is an inexpensive and effective technique for surveying the genetic diversity in plants, and as it involves using short (10 bp) arbitrary primers for amplifying unknown sequences (Khan et al., 2003), it is suited for plants with poor genomic sequence information such as *N. sativa* (Panahi et al., 2013b).

In *N. sativa* L., the information regarding DNA polymorphism has not been sufficient to date. For this reason, the aim of this research was to analyze the diversity and efficiency of the RAPD system in the clustering of different *N. sativa* L. accessions cultivated in different regions of Iran.

Materials and methods

Plant material

The 16 accessions of *Nigella* were collected from different regions of Iran (Fig. 1) and were sown in April 2014 in the experimental field at the College of Agriculture in Shirvan Higher Education Complex, Shirvan, Iran. The experiment was done in a randomized complete block design (RCBD) with three repeats. In each block, there were three rows, and in each row, 50 seeds were sown. The names and origins of accessions are shown in Table 1.

Isolation of genomic DNA

In this study, for genomic DNA extraction, the Cetyl Trimethylammonium Bromide (CTAB) method was used with modifications (Doyle and Doyle 1990). Leaves of each *N. sativa* accession (2 g) were used for DNA extraction. First, they were triturated to fine powder with polyvinylpyrrolidone in liquid nitrogen. Next, 10 ml of 1% CTAB buffer (100 mM Tris–HCl buffer pH 8.0, 1.4 M NaCl, 20 mM, EDTA, 1% mercaptoethanol) was added to homogenates, mixed, and centrifuged at 10 000 rpm for 15 min. In the following step, two volumes of 2% CTAB buffer were added to the collected aqueous phases.

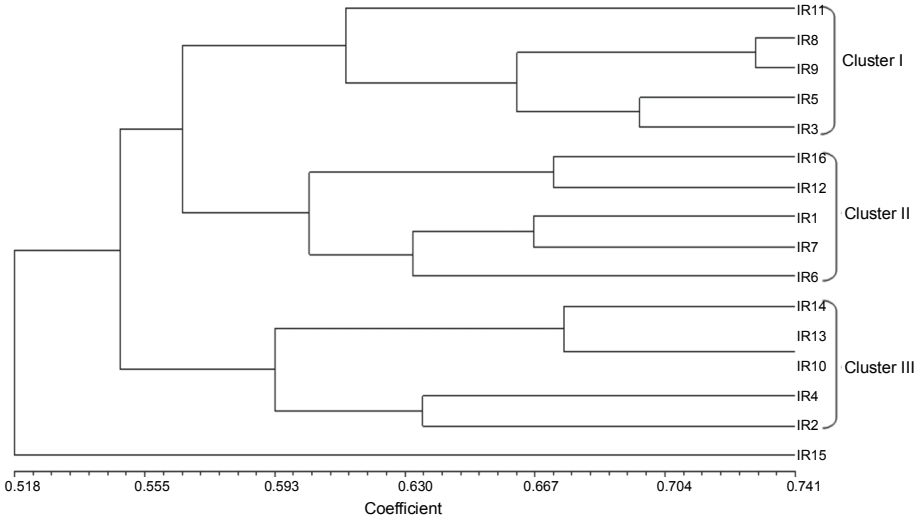

Fig. 3. Phylogeny relation of black cumin accessions, accession names presented according to Table 1

The mixtures were incubated at 65 °C for 60 min. Then, the suspensions were cooled at room temperature and equal volumes of chloroform and isoamyl alcohol (24 : 1) were added to the solutions and mixed. Then, the mixtures were centrifuged at 10 000 rpm for 15 min. In the next step, 0.6 volume of cold isopropanol and 1/30 volume of sodium acetate (3 M, pH 5.2) were added to the collected aqueous phases and were incubated at –20 °C for 1 h. The samples were centrifuged at 10 000 rpm for 15 min to obtain DNA pellets. The pellets were washed twice with 80% ethanol, air-dried, and dissolved in TE buffer. The isolated DNAs were treated with RNase I (Qiagen) at 37 °C for 30 min. DNA quality and quantity were analyzed by 1.4% agarose gel electrophoresis and NanoDrop Spectrophotometer (Thermo Scientific, Germany).

Optimization of RAPD

For the consistent, strong amplification of products and the rate of polymorphism, totally 30 arbitrary RAPD primers were used for the initial analysis. Based on our analysis, 21 primers were monomorphic among all the accessions, and therefore they were excluded from the experiment. According to a method developed by Williams et al. (1990), the remaining 9 RAPD primers (Table 2) were selected to perform a random amplification. The PCR thermal cycler was programmed for the first denaturation step at 94 °C for 1 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. The RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1 × TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator using a digital camera.

Data analysis

The amplified products were scored for the presence (1) or absence (0) of bands and were assembled into a binary matrix for total RAPD bands. Using NTSYS-pc software version 2.1, the binary matrices were subjected to statistical analyses (Rohlf, 2000). The Jaccard similarity coefficient was used to compute pairwise genetic similarities. Using the Unweighted Pair-Group Method Analysis (UPGMA), a similarity matrix was used for the cluster analysis and the construction of a dendrogram (Sneath and Sokal, 1973), while for the principal coordinate analysis (PCA), the NTSYSpc version 2.1 (Rohlf, 2000) was used.

Results and discussion

In *N. sativa,* genetic diversity analyses collected from Iran's different regions have been carried out using RAPD markers. In this regard, the purity of samples was determined from the 260/280 ratio by the NanoDrop Spectrophotometer analysis in order to select the high-quality DNA samples. Results showed that 260/280 ratio in DNA samples ranged from 1.8 to 1.91 indicating high purity (Panahi et al., 2013c; Shahriari Ahmadi et al., 2013).

Among the accessions, 21 primers were monomorphic, and therefore they were rejected from further

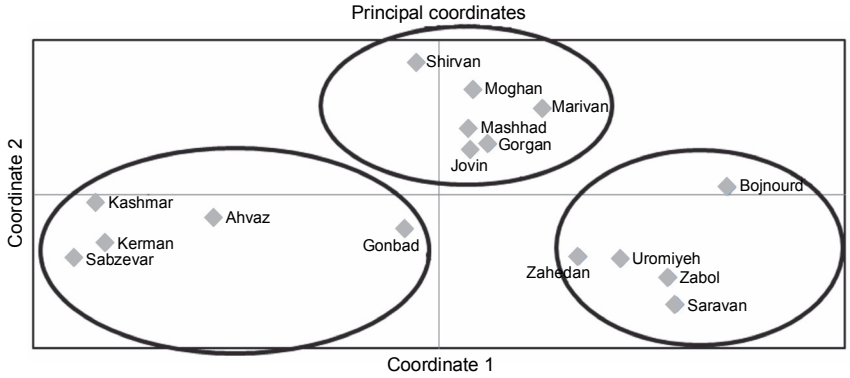

Fig. 4. Principal coordinate analyses for 9 RAPD primers applied on *16 N. sativa* accessions

analyses. The remaining 9 RAPD primers were used for genetic diversity assessment. Nine RAPD primers produced a total of 95 bands across 16 accessions of *N. sativa* ranging from 17 (OPH-20) to 9 products (UB-30). The products ranged from 100 to 500 bp. For each primer, the average frequency of bands was 20. The number of polymorphic products (P) ranged from 3 to 11 with the average 8.11 per primer (Table 2). Figure 2 shows the pattern of RAPD bands produced by OPH-20 primer.

Sixteen accessions were grouped in three main clusters based on the dendrogram generated by UPGMA algorithm (Fig. 3). Cluster I included IR11 (Saravan), IR8 (Zabol), IR9 (Zahedan), IR5 (Bojnourd), and IR3 (Urmia). In this cluster, IR8 (Zabol) and IR9 (Zahedan) created a subcluster while IR5 (Bojnourd) and IR3 (Urmia) formed another subcluster (Fig. 3). Cluster II included IR16 (Mashhad), IR12 (Shirvan), IR1 (Gorgan), IR7 (Mughan), and IR6 (Joveyn). This cluster also comprised 2 subclusters. Subcluster I included IR16 (Mashhad) and IR12 (Shirvan) whereas the second subcluster comprised IR1 (Gorgan), IR7 (Mughan), and IR6 (Joveyn) (Figure 3). In cluster III, IR3 (Urmia), IR10 (Sabzevar), IR4 (Ahvaz), and IR2 (Gonbad) accessions were included. In this cluster, IR3 with IR10 and IR4 with IR2 formed two subclusters.

The genetic relationship among all 16 accessions was also visualized by performing the Principal Coordinate Analysis (PCoA) based on RAPD data (Fig. 4). The PCoA is a method to explore and visualize similarities or dissimilarities of data. It starts with a similarity matrix or dissimilarity matrix (distance matrix) and assigns for each item a location in a low-dimensional space. PCoA tries to find the main axes through a matrix. It is a kind of the Eigen analysis, and it calculates a series of eigenvalues and eigenvectors. Each eigenvalue has an eigenvector, and there are as many eigenvectors and eigenvalues as there are rows in the initial matrix (Anderson et al., 2003).

The first two Eigen values accounted for 63.25% of variation observed in the accessions of Iran's different regions. The two-dimensional plot generated from the PCoA also supported the clustering pattern of the UPGMA dendrogram.

Neither the cluster analysis nor the PCA revealed a clear relationship between the diversity pattern and the geographical origin of accessions that were also randomly distributed over the whole dendrogram. This is consistent with Kapital et al.'s (2015) results. Using inter-simple sequence repeat (ISSR) markers, the Authors investigated the genetic diversity of black cumin in Ethiopia and reported that different geographical regions showed different levels of genetic variation.

RAPD markers have been found to be efficient methods that may be used for grouping closely related species and for identifying their different varieties (Palai and Rout 2007). Islam and coworkers (Islam et al., 2009) reported that RAPD can be efficiently used to distinguish the diversity among individuals in population. It is important to maintain high genetic diversity within populations and local accessions in plants, in order to conserve and utilize alternative breeding programs (Hamrick and Loveless, 1989).

In conclusion, our results showed that there is a high genetic diversity among accessions of *N. sativa* in Iran, and RAPD can be used to cluster these accessions based on genetic similarities. Because there have been no other studies reported on the diversity of *N. sativa*, especially no analysis of their diversity in native popula-

tions, our findings can be used for the basal conservation programs of this species.

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