RESEARCH ARTICLE

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Determining the genetic diversity of some black cumin genotypes collected in different regions of Türkiye using RAPD markers

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Abstract

Black cumin is one of the important medicinal aromatic plants belonging to the Ranunculaceae family. It is mostly used in the Middle East and for some diseases, especially in the Iranian region. It is important to know the genetic resources of such important medicinal and aromatic plants. Characterization of genetic resources sheds light on both the conservation of genetic resources and the future breeding studies. In this study, a total of 8 black cumin plants were characterized with 17 RAPD primers. Presence (1) and absence (0) scoring of gel images was performed using the Agarose Gel Electrophoresis (AGE) method. In genetic characterization, phylogenetic dendrogram with Bayesian statistics and Principal Coordinate Analysis (PCoA) with Jaccard similarity index were performed. As a result of the findings, the *Nigella damascena*, one of the black cumin plant species, was 100% separated from the *Nigella sativa* species. Additionally, Nigella sativa species differed among Konya, Eskişehir and Çameli genotypes. It gave similar results to Bayesian statistics in PCoA. The analysis indicated that Konya, Eskişehir and Çameli genotypes of *Nigella sativa* species have a higher potential to be used in breeding studies compared to other genotypes.

Keywords: *Nigella sativa*, *Nigella damascena*, RAPD, Plant Breeding, Diversity

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INTRODUCTION

Medicinal and aromatic plants have been used to treat diseases since time immemorial and have a wide range of traditional uses. Even in the Ranunculaceae family, which includes some important medicinal and aromatic plants, more than 2000 species are reported (Luan Nguyen et al. 2023). *Nigella segetalis*, *Nigella sativa*, *Nigella stellaris*, *Nigella koyuncui*, *Nigella gallica*, *Nigella fumariifolia*, and *Nigella damascene* are some of the species belonging to this family. The most studied species among these is *N. sativa*. This plant has different pharmacological effects (for example, it is used in dermatological complications, cancer, and type 2 diabetes) and is also used in traditional treatments (Srinivasan, 2018). In particular, thymoquinone, which is the main component of *N. sativa* oil, has been reported to have antibacterial, anti-cancer, immunostimulant, and antioxidant properties (Havlik et al. 2006). This plant is cultivated in different countries of the world and attracts attention due to its therapeutic properties (Luan Nguyen et al. 2023). In addition, black cumin seeds are used as flavoring in the edible cheese industry (Bourgou et al. 2010). The regions where the plant is most cultivated are Southern Europe, Middle East, and North and East Africa. It is of great importance to know the genetic resources of such an important plant.

Today, with the advancement of technology and molecular biology, the breeding

process is moving even faster. Since morphological markers require expertise and are affected by environmental conditions, researchers have started to use molecular marker methods more frequently (Aydin, 2023). Since molecular markers utilize sequences directly on the genome without being affected by time and environmental conditions, the reliability of the data is higher (Grover and Sharma, 2016). Molecular markers are used in a wide range of fields such as sex determination, species identification, genetic relationships, and determination of parents (Han et al. 2020; Soller, 2020; Song et al. 2023). In addition, it shortens the breeding process by making selection with the help of markers in plant breeding (Hasan et al. 2021). With the help of molecular markers, both population structure and genome structure can be revealed (Song et al. 2023; Yanez et al. 2023). Molecular markers commonly used in such studies are AFLP (Mei-Chao et al. 2020), RFLP (Manjunathagowda, 2021), RAPD (Türkoğlu et al. 2023), SCAR (Xu et al. 2020), ISSR (Venkatesan et al. 2021), SSR (Karaca et al. 2013), and SNP (Meng et al. 2022). Among these markers, RAPD markers were the first PCR-based marker technique and are still very actively used (Bi et al. 2021). RAPD markers are unidirectional universal markers consisting of 10 base pairs developed according to operon technology. The main advantages of this marker technique are that it does not require genomic information, is PCR-based and inexpensive (Amiteye, 2021). Primers form amplicons in PCR and bands in Agarose Gel Electrophoresis (AGE) can be seen when the same primer has reverse binding points on both strands. These markers can produce a large number of amplicons and can be used in genetic studies (Al-Hadeithi and Jasim, 2021). Their major disadvantage is that their primers bind at low temperatures and therefore, if not sensitive, nonspecific bands may appear (Al-Khayri, 2022). Different results may occur in various laboratories. Therefore, conversion of polymorphism bands into SCAR markers shows more permanent results (El-Haggar et al. 2023).

When the web of science database is sought, there are very few molecular marker studies on *Nigella* species. More molecular studies are needed to better understand the genetic structure of such an important plant in the medicinal and aromatic plant group. In this study, 17 RAPD markers were used to reveal the genetic relationships of some *Nigella* genotypes and it was aimed to reveal the genetic relationship in the existing genotypes by analyzing the data in the gel images obtained by agarose gel electrophoresis method.

MATERIALS AND METHODS

Plant Material

A total of 8 *Nigella* sp. genotypes were used in the study. Seven of them belonged to *Nigella sativa* and one genotype belonged to *Nigella damascene*. Six of the genotypes belonging to *Nigella sativa* (Çameli, Eskişehir, Konya, Şanlıurfa, Samsun, and Tokat) were obtained from different regions of Türkiye and one from Syria. The seeds of each plant were planted in small vials and grown under suitable climatic conditions. Leaf samples were collected under sterile conditions after the fourth true leaf was removed and stored at -20 °C for molecular studies.

DNA isolation and Quality-Quantity Determination

After the leaf samples were collected, they were pulverized with the help of liquid nitrogen. DNA isolation was performed according to Karaca et al., (2005) with some modifications. The quantity and quality of the obtained genomic DNAs (gDNA) were determined by Nano-Drop and agarose gel electrophoresis methods.

Primers and Polymerase Chain Reaction (PCR)

Ten nucleotide long unidirectional universal primers developed from operon technology were used in the study (Table 1). In PCR studies, amplicons were generated using Thermo Fisher Scientific (Cat:EP0402) thermal cycling device and Touch-Down PCR method (Karaca et al. 2019). In PCR, the temperature was reduced by 0.5 °C for each cycle from 42 °C to 37 °C and continued with 30 cycles after the first ten cycles. The pre-denaturation phase was continued at 94 °C for 5 min, the denaturation phase at 94 °C for 1 min, the binding temperature at 37 °C for 1 min and the renaturation phase at 72 °C for 2 min. In addition, PCR processes were completed after 10 min at the final renaturation temperature. PCR components and concentrations used were 50 ng gDNA, 2.4 µM of each primer, dNTP 0.28 mM, MgCl2 2.5 mM, 10X buffer 2.5 µL, and 1 Unit of Taq DNA polymerase 25 µL final volume.

Molecular Analysis

Amplicons obtained after PCR were generated by presence (1) and absence (0) scoring. Phylogenetic dendrogram tree was constructed using polymorphic information content (PIC) of primers, principal coordinate analysis (PCoA) and Bayesian statistics. Primer PIC values were calculated according to Smith et al., (1997). Principal coordinate analyses were performed using the Multivariate Statistical Package (MVSP) and Jaccard similarity index. MrBayes program and FigTree version 1.4.4 were used to construct the phylogenetic dendrogram tree.

Table 1. Information on RAPD primers used in the study.

NO	Primer ID	Primer sequence 5'→ 3'
1	OPA-05	AGGGGTCTTG
2	OPA-06	GGTCCCTGAC
3	OPB-12	CCTTGACGCA
4	OPB-13	TTCCCCCGCT
5	OPC-08	TGGACCGGTG
6	OPC-09	CTCACCGTCC
7	OPD-01	ACCGCGAAGG
8	OPD-02	GGACCCAACC
9	OPF-16	GGAGTACTGG
10	OPF-17	AACCCGGGAA
11	OPG-03	GAGCCCTCCA
12	OPG-04	AGCGTGTCTG
13	OPH-07	CTGCATCGTG
14	OPH-10	CCTACGTCAG
15	OPI-11	ACATGCCGTG
16	OPI-14	TGACGGCGGT
17	OPK-15	CTCCTGCCAA
18	OPK-18	CCTAGTCGAG
19	OPN-19	GTCCGTACTG
20	OPN-20	GGTGCTCCGT

RESULTS AND DISCUSSION

Determination of genetic relationships of *Nigella* sp. genotypes in the medicinal and aromatic plant group at molecular level is important for researchers working in this plant group. In this study, genetic characterization of different black cumin genotypes was carried out by using molecular markers. Black cumin seeds available in our unit were germinated and DNA isolation was performed. The quality and quantity of the obtained gDNAs were measured by Nano-Drop (Maestrogen, Hsinchu City, Taiwan, MN-013) and agarose gel electrophoresis methods. The A_{260}/A_{280} reads of around 1.8 should be a quality gDNA, free of proteins (Karaca et al., 2005). Since the ratios of the obtained gDNAs in the specified readings ranged between 1.7-1.9, a quality DNA free of proteins was obtained. In addition, it is not possible to determine whether the gDNA obtained in spectrophotometric readings is broken or not. Therefore, this result can be seen visually by agarose gel electrophoresis method. When agarose gel electrophoresis images were analyzed, it was determined that there was no broken DNA with high molecular weight that would negatively affect the studies.

After PCR, agarose gel electrophoresis method was used to visualize the amplicons. 2% gels were prepared and run for 2, 4 or 6 hours. The images were recorded on computer. Gel images were analyzed with GelAnalyzer (19.1v) and presence (1) and absence (0) bands were recorded. Only 17 of the 20 primers screened produced polymorphic and clearly readable amplicons. The PIC values of these primers varied between 0.218-0.857 for these genotypes. While he lowest PIC value was shown by primer OPD-02, the highest PIC value was shown by primer OPK-15 (Table 2). Only 3 of the primers were below 0.5. The remaining primers were found to be highly polymorphic. In addition, the number of alleles formed by the primers was calculated as 200 in total and the number of alleles per primer was determined as 11.764. The number of alleles for the primers varied between 6-18. The lowest number of alleles was observed in primer OPA-06, while the highest number of alleles was observed in primer OPF-16. The number of patterns formed by the primers in the population was calculated, as well. It was determined that the total number of patterns was 75 and the number of patterns per perimer was 4.411. The lowest number of patterns was 2 in primer OPD-02 and the highest number of patterns was 7 in primer OPK-15. PIC values of the primers are calculated to reveal the efficiency and discrimination power of the molecular markers used in the population and the importance of the primer used (Serrote et al. 2020). It was also determined that the primers used for the black cumin population in this study were effective primers that can be used to distinguish and characterize this population.

Table 2. Polymorphic Information Content of Primers.

NO	Primer ID	Primer sequence 5'→ 3'	Total Alel	Patern Number	PIC
1	OPA-06	GGTCCCTGAC	6	5	0,703
2	OPB-12	CCTTGACGCA	15	6	0,812
3	OPB-13	TTCCCCCGCT	16	5	0,750
4	OPC-08	TGGACCGGTG	12	3	0,593
5	OPC-09	CTCACCGTCC	12	5	0,687
6	OPD-01	ACCGCGAAGG	18	4	0,656
7	OPD-02	GGACCCAACC	7	2	0,218
8	OPF-16	GGAGTACTGG	18	4	0,656
9	OPF-17	AACCCGGGAA	8	4	0,687
10	OPG-03	GAGCCCTCCA	16	6	0,812
11	OPG-04	AGCGTGTCTG	13	3	0,406
12	OPH-10	CCTACGTCAG	8	4	0,562
13	OPI-11	ACATGCCGTG	9	5	0,775
14	OPI-14	TGACGGCGGT	9	5	0,750
15	OPK-15	CTCCTGCCAA	12	7	0,857
16	OPK-18	CCTAGTCGAG	10	3	0,406
17	OPN-19	GTCCGTACTG	11	4	0,612

One of the analyses used in this study is PCoA analysis. In PCoA analysis, principal components are processed and a graph is created with a proximity or distance matrix and reveals the distance between samples (Gower, 2014). In the current study, we revealed the genetic similarity of the existing alleles by using the Jaccard similarity index (Figure 1). As a result of the analysis, *Nigella damascena*, which showed different species characteristics, was completely differentiated from the existing *Nigella sativa* species. Genotypes belonging to *Nigella sativa* species also formed 3 clusters among themselves. One of them was Şanlıurfa, Tokat and Syrian genotypes, another one was Samsun, Konya and Eskişehir and the third one was Çameli variety which was developed as a variety. Here, although the Syrian genotype was obtained from a different country, it was grouped in the same group with Şanlıurfa and Tokat varieties in Türkiye and showed a high rate of similarity. In the PCoA analysis, our population was divided into 4 different clusters and it was determined that it was suitable for use in genetic studies.

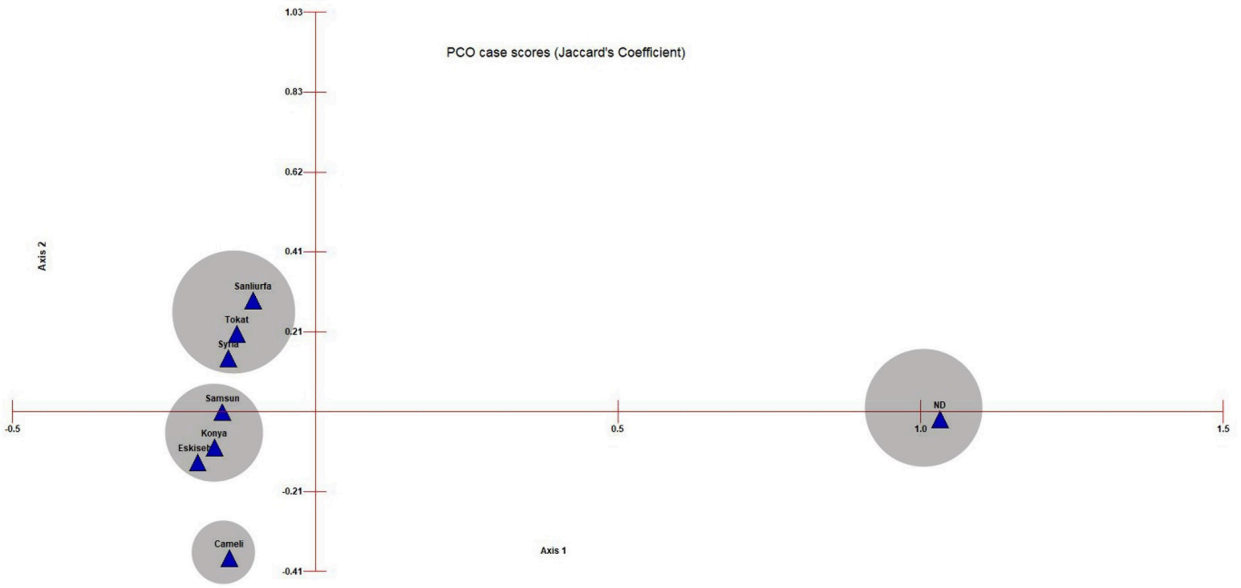

Figure 1. Principal Coordinate Analysis (PCoA).

In addition to these analyses, genetic relationships were analyzed with Bayesian statistics. MrBayes reveals the best relationship between samples or population by including "post probability" estimates in Bayesian statistics in a wide range of phylogenetic and evolutionary models. In this method, the analysis starts with a topology with the highest probability (a prior) and the trees are simulated using the Markov Chain Monte Carlo (MCMC) method and the tree/trees with high post probabilities is/are selected by capturing the best topologies (Karaca et al., 2015). The posterior probability of phylogenetic trees cannot be determined analytically. Instead, MCMC calculates the posterior probability by using the data with the tree created by drawing samples from the "posterior" distribution. As a result of the analyses conducted using MrBayes program, the best tree for revealing the genetic relationship was created with FigTree program. In this study, the analysis was performed with 10 million replications and two main clusters were formed (Figure 2). In one of these clusters, there were genotypes belonging to *Nigella damascena* species and the other cluster included genotypes belonging to *Nigella sativa* species. Among the genotypes of *Nigella sativa* species, Konya, Eskişehir and Çameli genotypes were grouped within themselves.

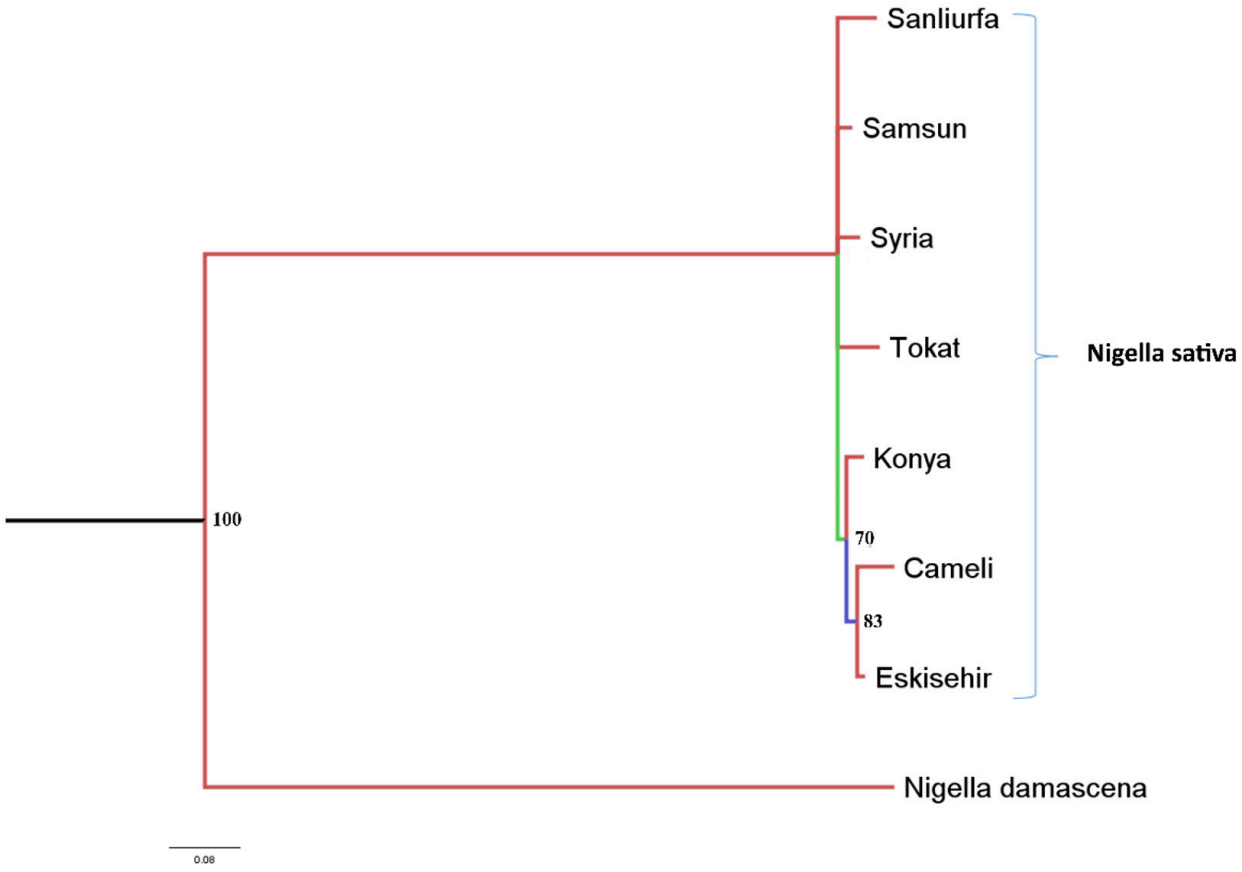

Figure 2. Phylogenetic Dendrogram of Bayesian Statistics.

CONCLUSION

The main objective of the study was to characterize the black cumin plant using molecular markers. In this context, molecular characterization of 7 *Nigella sativa* genotypes and 1 *Nigella damascena* genotype was carried out using 17 RAPD machines. Agarose gel electrophoresis method was used to separate the amplicons generated after PCR. By analyzing the gel images, 17 RAPD primers generated 200 alleles in total and 11.764 alleles were detected per primer. It was determined that 13 of the primers used had high PIC values. Two analysis methods were used for characterization of the genotypes. One of these methods was PCoA analysis with the Jaccard similarity index, and the other was dendrograms using Bayesian statistics. Although there were very few differences between the analyses, they supported each other. While 4 clusters were formed with PCoA analysis, 2 main clusters and one of the clusters formed two clusters in Bayesian statistics.

Consequently, the RAPD primers used in the outputs of this study were found to be effective for this population and can be used in other black cumin populations. Another output was detection of the genetic relationships between

these genotypes. Although the population density was low, the analyses revealed that the variation in the population was high. Especially in PCoA analysis, 4 different clusters were observed. High level of differences was observed in the genotypes of *Nigella sativa* species. These results indicate that *Nigella sativa* genotypes, which is an important medicinal and aromatic plant group, would be used in breeding studies.

Compliance with Ethical Standards

Conflict of interest

There is no conflict of interest regarding the article.

Author contribution

AA: desing, writing and laboratory studies.

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Data availability

Not applicable

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