**Research**

# ISSR-based molecular variation of some fennel (*Foeniculum vulgare* Mill.) populations

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ABSTRACT

This research was conducted to determine the molecular variation between different fennel populations. In this research, six fennel populations, of which two were collected from natural flora, were used as a material. The molecular characterization of the populations was performed using six ISSR (Inter simple sequence repeat) primers. A total of 57 bands were obtained, of which 48 were evaluated as polymorphic. The average polymorphism percentage was 85%. The PIC values changed from 0.33 (ISSR-16) to 0.43 (ISSR-11) with an average of 0.37. The average RP (Resolving Power) value of the primers was found as 4.17, and the highest value was obtained from the ISSR-4 (6.33) primer. The mean EMR value was 6.78, and the highest value was obtained from the ISSR-4 (10.29) primer. The mean MI (Marker Index) value was estimated to be 2.47, with the highest value similarly observed in the ISSR-4 (3.67) primer. ISSR-4 was the most prominent primer in terms of these three parameters and the maximum total and polymorphic bands. The similarity values of the fennel populations were found to be between 0.30 and 0.95. The Mental test conducted between Jaccard's similarity matrices and the cluster result revealed the r value as 0.99, showing a very good correlation between the ISSR-based similarity matrix and the cluster. The populations of Burdur, Denizli, Isparta and Izmir, which were cultivated and traded, were genetically similar in terms of the ISSR primers examined, whereas Manisa Yunt Mountain and Odemis populations collected from natural flora were in separate groups.

## Bazı rezene popülasyonlarının (*Foeniculum vulgare* Mill.) ISSR markörlerine dayalı moleküler varyasyonu

ÖZET

Bu araştırma, farklı rezene popülasyonları arasındaki moleküler varyasyonunu belirlemek amacıyla yürütülmüştür. Araştırmada materyal olarak farklı kaynaklardan sağlanan ve ikisi doğal floradan toplanmış olan 6 adet rezene popülasyonu kullanılmıştır. Popülasyonların moleküler karakterizasyonu 6 adet ISSR primeri ile belirlenmiştir. Toplam 57 bant elde edilmiş, bunlardan 48 adedi polimorfik olarak değerlendirilmiştir. Ortalama polimorfizm oranı %85 olarak bulunmuştur. PIC değerleri 0.33 (ISSR-16) ile 0.43 (ISSR-11) arasında değişim göstermiş, ortalama ise 0.37 olarak hesaplanmıştır. Primerlerin ortalama RP değeri 4.17 olarak hesaplanmış, en yüksek değer ISSR-4 (6.33) primerinden elde edilmiştir. Ortalama EMR değeri 6.78 bulunurken en yüksek değer ISSR-4 (10.29) primerinden elde edilmiştir. Ortalama MI değeri 2.47 olarak tahmin edildi, en yüksek değer yine ISSR-4 (3.67) primerinde gözlenmiştir. ISSR-4 primeri bu üç parametre ve en yüksek toplam ve polimorfik bant sayısı bakımından öne çıkan primer olmuştur. Rezene popülasyonları arasındaki benzerlik değerleri 0.30 ile 0.95 arasında bulunmuştur. Jaccard'ın benzerlik matrisleri ile küme sonucu arasındaki zihinsel test r = 0.99 (Kofenetik korelasyon katsayısı) olarak bulunmuştur ve ISSR tabanlı benzerlik matrisi ile küme arasında çok iyi bir korelasyon olduğunu göstermektedir. İncelenen ISSR primerleri bakımından ekimi ve satışı yapılan Burdur, Denizli, Isparta ve Izmir popülasyonları birbirlerine genetik bakımdan oldukça

benzer bulunurken doğal floradan toplanan Manisa Yuntdağı ve Ödemis popülasyonları ayrı gruplarda yer almışlardır.

## 1. Introduction

Fennel (*Foeniculum vulgare* Mill.), belonging to the Umbelliferae (Apiaceae) family, is an annual or perennial plant that can reach 60-200 cm in height and has yellow flowers and leaves with leaflets fibrous (Baytop, 1999; Ceylan, 1997). It has two significant varieties, which are *Foeniculum vulgare* var. *vulgare* (bitter fennel) and *Foeniculum vulgare* var. *dulce* (sweet fennel). Fennel fruit contains 10-20% fatty oil (60-75% petroselinic, oleic, linoleic fatty acids), volatile oil (3-6% in bitter fennel, 2-4% in sweet fennel) and 15-20% protein. In addition, it contains flavonoids, sterol, sugar, and apiol (Akgul, 1993). The major constituents of fennel essential oil are *trans*-anethole, estragole, fenchone, and limonene (Telci et al., 2009, Avci, 2013, Rather et al., 2016, Coban et al., 2018). The major components of the wild fennel populations from Middle Black Sea Region flora of Turkey, has been characterized as the estragole and fenchone (Telci et al., 2019).

Fennel is used for various purposes in food, cosmetic and pharmaceutical industries. The vegetative parts of the plant are used in green salads, its fruit in spices, and the essential oil in its seeds in the production of perfume, soap, medicine, and cosmetics. Recent studies have shown that the essential oil extracted from this plant is a valuable antioxidant with antibacterial, anticancer and antifungal properties (El-Awadi and Esmat, 2010, Moura et al., 2005, Bahmani et al., 2013). The increasing commercial value of fennel requires the identification, recognition and preservation of the existing diversity of this plant.

Various molecular markers techniques based on PCR (polymerase chain reaction) have been used to reveal genetic diversity in many plant species. Inter simple sequence repeat (ISSR) markers have great potential in studies at population and species levels. In addition to being economically important species, it is also possible to use these markers in natural populations. They have been utilized in the investigation of genetic variation between closely related individuals and identification in various species (Zietkiewicz et al., 1994). ISSR markers have been also successfully employed to determine relationships at the population and species levels and a variety of plant species, including various aromatic and medicinal plants; e.g., *Pimpinella anisum* (Akcali Giachino, 2019), *Achillea millefolium* (Farajpour et al., 2012), *Artemisia capillaries* (Shafie et al., 2009), *Thymus daenensis* (Rahimmalek et al., 2009), safflower (Yang et al. 2007), and *Helichrysum* Mill. species (Azizi et al., 2019). In fennel, Yadav and Malik (2018), Choudhary et al. (2018), Salami et al. (2017), Grover and Malik (2017), Gehan and Alhamd (2015), and Bahmani et al., (2012) conducted studies using ISSR markers. This method is widely applicable since it only requires small amounts of DNA, is fast, inexpensive, and easy to apply, exhibits high polymorphism, and does not require prior knowledge for the primer design (Godwin et al., 1997) in contrast to simple sequence repeat markers.

This study aimed to determine the effectiveness of ISSR markers in identifying the genetic diversity of different fennel populations.

## 2. Materials and Methods

### 2.1 Plant materials

DNA samples from six fennel populations; cultivated and traded in Turkey (1: Burdur, 2: Denizli, 3: Isparta, and 4: Izmir), and collected from the natural flora (5: Manisa Yunt mountain and 6: Odemis) were sampled from the seeds. Molecular analyses were carried out at the central laboratory of Ege University Faculty of Agriculture (EGE AGROLAB).

### 2.2 DNA isolation

DNA was isolated using the GenEluteTM Plant Genomic DNA Miniprep isolation kit in directly ground seeds (Sigma-Aldrich). Genomic DNA concentration was determined by a spectrophotometer and 0.8% agarose gel. The DNA samples were diluted to 25 ng per µl for the polymerase chain reaction (PCR) analysis.

### 2.3 ISSR-PCR Analysis

The PCR reaction volume in ISSR amplification was 10 µl, which contained 10xTaq DNA polymerase buffer, 2.5 mM MgCl2, 0.5µM primer (Sigma), 200 µM of each dNTP (dATP, dTTP, dCTP, and dGTP), 50 ng genomic DNA, and 0.5 unit of Taq DNA polymerase enzyme (Sigma).

The PCR procedure was performed on a thermal cycler (Thermo Scientific Arktik with Gradient) at 94 ºC for 1.30 min for one cycle, followed by 94 ºC for 45 sec, and the annealing phase of the primer was undertaken at 45-60.8 ºC (depending on the primer) for 45 sec., followed by 72 ºC for 1.30 min for 45 cycles, and finally 72 ºC for seven minutes.

### 2.4 Data analysis

A data matrix was created using 1 and 0 for the presence and absence of the ISSR bands, respectively. Using this data matrix, the genetic distance values according to Jacard's similarity coefficients were

obtained from NTSYS-pc 2.20j (Numerical Taxonomy and Multivariate Analysis System, Rohlf, 2000) statistical package program (Jaccard, 1908). The same software was utilized to obtain dendrograms of the genotypes grouped according to the unweighted pair-group method with arithmetic average (UPGMA). The polymorphism information content (PIC) values of each primer were calculated as follows (Anderson et al., 1993):

$$PIC = 1 - \Sigma\, fi^2$$

where fi indicates the frequency of the ith allele.

The RP (Resolving Power) of each primer was calculated in accordance with Prevost and Wilkinson (1999) as follows:

$$RP = \Sigma\, Ib$$

where Ib is the band informativeness with

$$Ib = 1 - [2 \times (0.5 - p)]$$

and $p$ is the proportion of six populations containing the amplified products. MI was obtained by multiplying the PIC values with the effective multiplex ratio (EMR). The effective multiplex ratio (EMR) is the number of polymorphic bands detected per test. (Powell et al. 1996; Milbourne et al. 1997):

$$MI = EMR \times PIC$$

To analyse fennel populations was performed the multivariate analyses including cluster analysis and principal component by using of the genetic similarity coefficient matrix. Statistical analyse was made Mantel statistics Z test (Mantel, 1967) and NTSYS-pc software (Rohlf, 2000).

## 3. Results and Discussion

Six of the 11 ISSR primers used in the study yielded assessable bands. Table 1 presents the names, base sequences, annealing temperature, total number of bands, number of polymorphic bands, % polymorphic band ratios, PIC, resolving power (RP), EMR and marker index (MI) values of the six primers evaluated. As a result of the ISSR analysis, the six primers evaluated produced a total of 57 bands, of which 48 were polymorphic. The average polymorphism percentage was found to be 85% with high molecular diversity at the population level. Similarly, Salami et al. (2017) found the average polymorphic band percentage in outcross and self-pollinated fennel populations to be 88.3% and 82.3%, respectively. Again, Bahmani et al. (2012), who used ISSR markers to evaluate the genetic diversity between 25 ecotypes of Iranian fennel, reported 89% polymorphism. However, Grover and Malik (2017) obtained a relatively low polymorphism of

39.1% in their studies using ten ISSR markers to assess genetic diversity between seven fennel genotype lines. This may be due to the variations in specific genotypes and the differences between the primers used. In the current study, the ISSR-16 primer provided the least number of bands (n = 6) while the highest number of bands was obtained from the ISSR-4 primer (n = 14). ISSR-16 primer (100%) showed the highest polymorphism. The mean PIC value obtained from the ISSR bands was calculated as 0.37. The highest and lowest PIC values were observed in ISSR-11 (0.43) and ISSR-16 (0.33), respectively.

Only the ISSR-11 primer (0.43) exceeded the mean PIC, thus exhibiting the highest polymorphism. Similar results are reported by Choudhary et al. (2018) (0.35) and Jadidi and Kalantar (2016) (0.36). In their study using 12 IS SR primers in ten different fennel genotypes, Poudineh et al. (2018) found that the PIC values ranged from 0 to 0.47. The RP of the primers ranged from 2.67 to 6.33, with the highest value being obtained from the ISSR-4 primer and the lowest from ISSR-16. The average RP was calculated as 4.17. The highest EMR (10.29) was obtained from ISSR-4 and the lowest (4.50) from ISSR-19, with an average of 6.78 per primer. The average MI was estimated as 2.47, with the maximum value found in ISSR-4 (3.67) and lowest in ISSR-19 (1.63). ISSR-4 was the primer providing the highest values in terms of RP, EMR, MI, and the total number of bands and polymorphic bands. The ISSR-11 primer achieved the highest PIC value (0.43), while ISSR-16 had the highest polymorphism percentage (100%). The band profiles of these primers are shown in Figure 1. According to the results of their genetic analysis, Poudineh et al. (2018) reported that the ISSR-8 primer had the highest number of alleles, polymorphism, Shannon index, and heterozygosity and PIC values.

The genetic similarity values obtained according to Jaccard's similarity coefficient of the fennel populations are shown in Table 2. The similarity matrix was calculated using 57 ISSR fragments and used to perform the UPGMA cluster analysis.

The cophenetic correlation between the cluster results and the genetic similarity matrix was found to be r = 0.99 (Mantel, 1967), indicating that the clustering dendrogram was in high agreement with the similarity matrix. Bahmani et al. (2012), who evaluated 25 fennel ecotypes collected from different regions of Iran using ISSR markers, reported that the similarity matrix was moderately consistent with the dendrogram with a cophenetic correlation value of 0.79.

The genetic similarity values of the populations changed between 0.30 and 0.95. The mean similarity ratio of the populations was 0.59. Table 2 shows that the fennel populations cultivated and marketed in Turkey were genetically very similar with 0.90-0.95 genetic similarity values according to the ISSR primers examined. Burdur and Denizli populations had the

highest genetic similarity with a value of 0.95. Two wild populations (Manisa Yunt Mountain and Odemis), which were collected from natural flora but were also cultivated and traded in Turkey, were observed to be the most distant populations with values of 0.30-0.45.

Table 1.  ISSR primers and measured marker parameter results

| Primer | Sequence 5′–3′ | Temp (°C) | NTB | NPB | PBR% | PIC | RP | EMR | MI |
|---|---|---|---|---|---|---|---|---|---|
| ISSR 4 | 5'-HVH(TCC)$_5$-3' | 60.8 | **14** | **12** | 86 | 0.362 | **6.33** | **10.29** | **3.67** |
| ISSR11 | 5'-(GA)$_8$YG-3' | 50 | 8 | 7 | 88 | **0.43** | 4.67 | 6.13 | 2.63 |
| ISSR 13 | 5'-(AC)$_8$YG-3' | 57.1 | 10 | 8 | 80 | 0.36 | 4.00 | 6.40 | 2.31 |
| ISSR 16 | 5'-(AG)$_8$C-3' | 46.8 | 6 | 6 | **100** | 0.33 | 2.67 | 6.00 | 2.00 |
| ISSR 19 | 5'-(CT)$_8$G-3' | 45 | 8 | 6 | 75 | 0.36 | 3.00 | 4.50 | 1.63 |
| ISSR 25 | 5'-(AC)$_8$G-3' | 54.9 | 11 | 9 | 82 | 0.35 | 4.33 | 7.36 | 2.59 |
| **Average** | | | **9.5** | **8** | **85**[a] | **0.37**[a] | **4.17**[a] | **6.78**[a] | **2.47**[a] |
| **Minimum** | | | **6** | **6** | **75** | **0.33** | **2.67** | **4.50** | **1.63** |
| **Maximum** | | | **14** | **12** | **100** | **0.43** | **6.33** | **10.29** | **3.67** |
| **Total** | | | **57** | **48** | **-** | **-** | **-** | **-** | **-** |

Temp °C: Annealing temperature, NTB: Number of total bands, NPB: Number of polymorphic bands, PBR: Polymorphic bands ratios, PIC: Polymorphism information content, RP: Resolving power, EMR: Effective multiplex ratio, MI: Marker Index, Y = G/C, H = A/T/C, V = 3A/G/C
[a] Average of the column
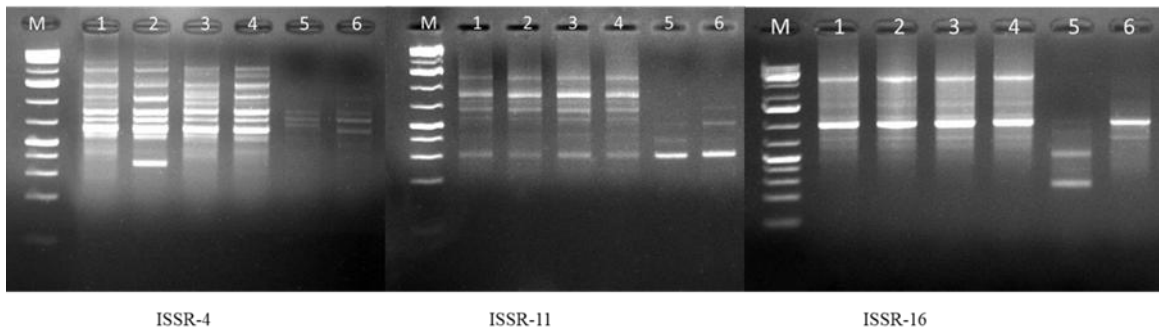


M: marker DNA, 1: Burdur, 2: Denizli, 3: Isparta, 4: Izmir (cultivated populations), 5: Manisa Yunt Mountain, 6: Odemis (populations collected from natural flora).

Figure 1.   Band profiles of ISSR-4, ISSR-11 and ISSR- 16 primers in fennel populations.

Table 2. The genetic similarity values among six fennel populations

| Populations | Burdur | Denizli | Isparta | İzmir | Manisa | Odemis |
|---|---|---|---|---|---|---|
| Burdur | 1.00 | | | | | |
| Denizli | **0.95** | 1.00 | | | | |
| Isparta | 0.90 | 0.90 | 1.00 | | | |
| İzmir | 0.91 | 0.91 | 0.90 | 1.00 | | |
| Manisa | **0.30** | **0.30** | **0.30** | **0.30** | 1.00 | |
| Odemis | 0.45 | 0.45 | 0.45 | 0.45 | **0.30** | 1.00 |

Highest and lowest values demonstrated in bold

In their study of 55 fennel accessions using 12 SRAP (Sequence-Related Amplified Polymorphism) primers, Maghsoudi Kelardashti et al. (2015) found that the genetic similarity values between these accessions ranged from 0.71 to 0.91. Kameli et al. (2013) evaluated 10 Satureja populations with 10 ISSR primers and reported genetic similarity values varying between 0.57 and 0.99.

Figure 2 presents the UPGMA dendrogram of the fennel populations obtained according to the Jaccard coefficient and shows that Burdur, Denizli, Isparta and Izmir populations, which are cultivated, form one group.
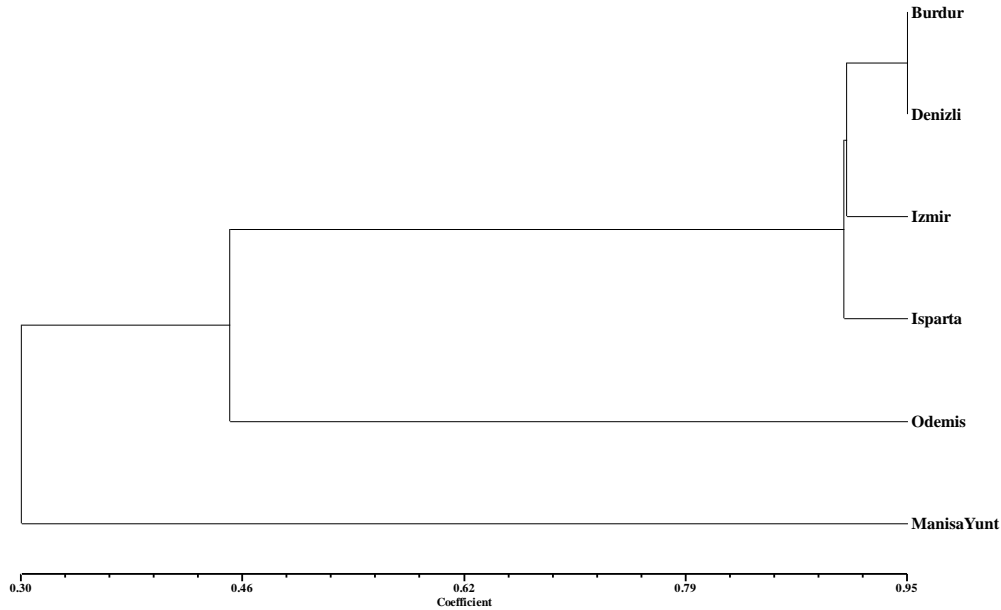


Figure 2: Phylogenetic tree derived from genetic similarity between fennel populations.
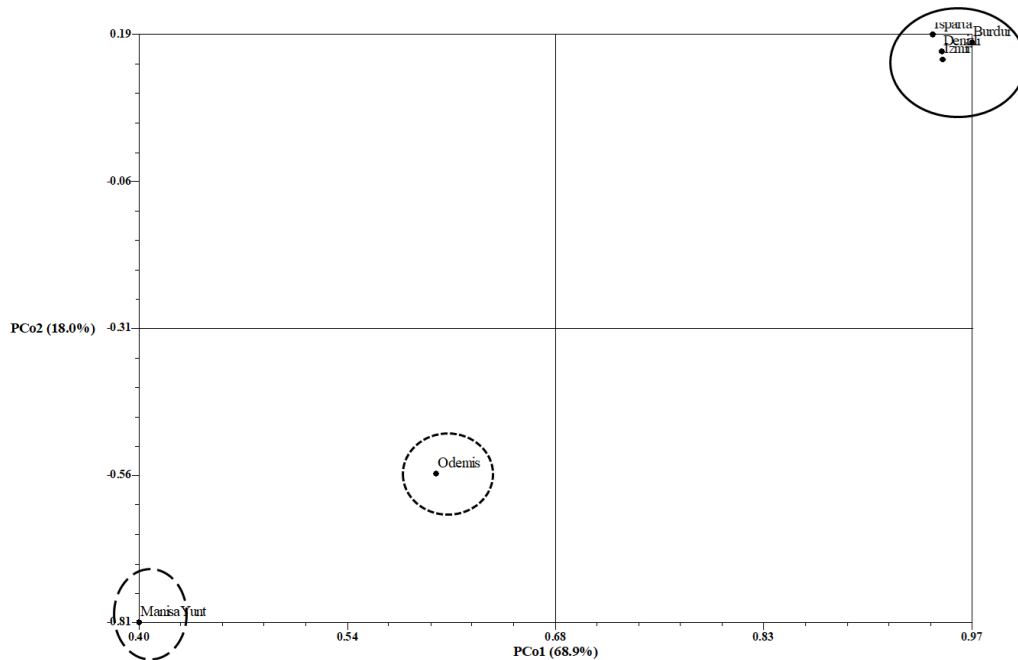


Figure 3: Principal coordinate plot produced from the Jacards similarity matrix of six fennel populations.

Burdur and Denizli populations are the closest with a value of 0.95. Manisa and Odemis populations collected from natural flora are included in the dendrogram separately. Manisa Yunt Mountain wild population with a value of 0.30 is the most genetically remote in relation to all other populations. In their study conducted with eight ISSR primers, Shojaiefar et al. (2015) constructed a dendrogram that divided 20 fennel accessions into four clusters in 0.71 distance units.

The results of PCoA for the ISSR data are shown in Figure 3. The results of the PCoA analysis were in good accord with the cluster analysis. The fennel populations were grouped similarly in the dendrogram and the 2D-dimensional plot of PCoA (Principal Coordinate Analysis). The first two principal coordinates accounted for 68.9 and 18.0% of the total molecular variation, respectively. This explained 86.9% of the total variation. While the fennel populations cultivated and traded in Turkey (Burdur, Denizli, Isparta, and Izmir) were clustered in one group, Manisa Yunt Mountain and Odemis populations were included in separate groups.

## 4. Conclusion

Characterization and identification of plant species play an important role for biodiversity, conservation and sustainable use of genetic resources. DNA-based techniques are used extensively in the characterization of medicinally important plant species. These methods provide precise and reliable results, especially in plant species or varieties that are often mixed with other species, as well as those that cannot be morphologically and/or phytochemically differentiated.

In this study, the genetic variation between different fennel populations was evaluated and their genetic relationships were determined using ISSR markers. The results of the cluster analysis revealed high variability between the investigated fennel populations. The relationship between the fennel populations was confirmed by similar grouping on the UPGMA dendrogram and on the PCoA. Based on the examined ISSR primers, the fennel populations that are cultivated and traded in Turkey; i.e., Burdur, Denizli, Isparta and Izmir populations, were found to be genetically very similar while Manisa Yunt Mountain and Odemis populations were included in separate clusters. According to the various parameters examined, ISSR-4 was the most prominent primer in terms of the total number of bands, the number of polymorphic bands, RP, EMR and MI, while the ISSR-11 primer had the highest PIC value and ISSR-16 exhibited the highest percentage of polymorphism. The information revealed by this research will help breeders acquire knowledge of the genetic variation of fennel populations and broaden the genetic base in fennel breeding. It can also contribute to the protection and improvement of medicinal and aromatic species, which are becoming recognized and increasing their importance. The results showed that ISSR markers were successfully applied in the evaluation of the fennel plant. In addition, ISSR markers present as a favourable system with faster results and easier application than the other DNA markers and more repeatability and less cost compared to RAPD (Random Amplified Polymorphic DNA) markers.

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