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Efficiency of inter-simple sequence repeat (ISSR) markers in differentiating anise (*Pimpinella anisum* L.) cultivars

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Abstract

The objective of this research was to assess the genetic diversity and similarity of three varieties of anise (Pimpinella anisum L.) by intergenic sequence markers (ISSR) technique. The experiment employed 10 randomly binding primers and yielded the amplification of 112 bands of DNA; out of these, 89 (79.46%) were found to be polymorphic. The results revealed that primer UBC 834 outperformed other primers in identifying genetic differences (92.86% polymorphism). Genetic distance estimation indicated high genetic similarity between varieties 1 and 3 (distance measure 0.16746), whereas variety 2 displayed marked genetic distance (distance measure 0.30927). The UPGMA tree joined varieties 1 and 3 to form a cluster at similarity value 83.25%, whereas each separate cultivar had identity values of 78.73% to be distinct. The experiment had the particularity to note 17 gene bands specific to each cultivar to form its distinct genetic fingerprint. The experiment marked success in identifying genetic differences among the three varieties of anise by employing the ISSR technique; it presented new approaches to preserve and improve genetic material.

Introduction

Anise (Pimpinella anisum L.) represents a very valuable aromatic medicinal plant of family Apiaceae. The characteristics of this plant are its high value from both an economical and medical perspective; from this plant, anethole oil is derived, which is used in pharmaceutical industries as well as in food and cosmetic industries (Arslan et al., 2004). In Iraq, anise is commonly used as medicine in traditional beverages.

The major challenge encountered in anise improvement and breeding is distinguishing among varieties by traditional morphological techniques, which prove inadequate for such characterization (Bayram, 1992). Hence, it became necessary to depend solely on molecular techniques to reveal genetic diversity, which holds the key to any successful improvement scheme (Govindaraj et al., 2015).

The intergenic-sequence marker (ISSR) technique can be considered to be one of the sophisticated tools in molecular biology which not only possesses speed and efficiency but also economical advantages (Bornet & Branchard, 2001). The technique makes use of areas between SSR markers distributed randomly all over the genome to generate a distinct and very common genetic profile (Zietkiewicz et al., 1994).

The efficacy of the ISSR method in assessing genetic diversity among Apiaceae plants has been shown in earlier research; a high rate of polymorphism was reported in cumin (Cuminum cyminum) and dill (Anethum graveolens) species (Akgül et al., 2018; Shayan et al., 2020). The method has also been proved to be efficient in identifying medicinally similar species despite their differences (Talebi et al., 2018).

From these results, this study proposes to: 1. Make an estimate of genetic diversity and relatedness among three varieties of cultivated anise in Iraq. 2. Create a genetic identity for every variety. 3. Assist in rational use of genetic material and conservation of varieties threatened. 4. Assist in their breeding programs to release varieties known by their effectiveness and quality

Materials and Methods

Plant Materials and DNA Extraction:

Samples of anise (Pimpinella anisum) seeds of various geographical origins (1. Iraq, 2. Syria, and 3. Iran) were obtained from local markets in sealed containers containing the variety's documentation. The samples were transferred to sterile containers at the Al-Amin Laboratory of the Holy Shrine of Imam Ali (AS) and stored at -80°C until extraction.

Genomic DNA was extracted from 100 mg of seed powder using a modified CTAB method. DNA purity was assessed using a BioDrop spectrophotometer, with A260/A280 absorbance ratios ranging from 1.8 to 2.0. The measured concentrations were diluted to a standard working concentration of 50 ng/µL using nuclease-free water.

ISSR-PCR Amplification and Electrophoresis:

Ten ISSR primers (Table 1) were selected and have been previously described for the detection of polymorphisms in Apiaceae plants (Prevosti et al., 2004; Gharibi et al., 2011). These primers, originally developed by Zietkiewicz et al. (1994) at the Biotechnology Laboratory at the University of British Columbia, were used in this study. A PCR mixture was prepared in a final volume of 25 µl containing: 50 ng of template DNA, 1× TAC buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, and nuclease-free water for volume adjustment. Appropriate controls were included, including no-template and positive controls.

The amplification program consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, hybridization at the primer-specific temperatures (Table 1) for 45 seconds, and extension at 72°C for 2 minutes, with a final extension step at 72°C for 7 minutes.

The reaction products were purified by gel electrophoresis on a 1.5% agarose gel. The amplified fragments (10 µL per reaction) were separated by size and stained with SYBR Safe. The agarose was visualized using a UV transilluminator and a gel documentation system. A 100-base-pair DNA ladder was used as a molecular size standard to estimate fragment lengths.

Data analysis

Band patterns were recorded as binary data (1 for presence of band, 0 for absence). Data were analyzed using NTSYS-pc (version 2.10) to calculate the total number of bands, percentage polymorphism, and genetic similarity coefficients. Phylogenetic trees were constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm based on pairwise genetic similarity matrices.

Table 1 Details of ISSR primers used and their sequence, annealing temperature used in the study.

Primer Name	**Sequence (5′→3′)**	**Annealing Temperature (°C)**	**References**
UBC 807	$(AG)_8T$	48	(Gajera et al., 2021)
UBC 808	$(AG)_8C$	52	(Gajera et al., 2021)
UBC 809	$(AG)_8G$	53	(Gajera et al., 2021)
UBC 810	$(GA)_8T$	48	(Dhakshinamoorthy et al., 2022)
UBC 811	$(GA)_8C$	50	(Dhakshinamoorthy et al., 2022)
UBC 812	$(GA)_8A$	49	(Dhakshinamoorthy et al., 2022)
UBC 825	$(AC)_8T$	52	(Talebi et al., 2018)
UBC 827	$(AC)_8G$	54	(Talebi et al., 2018)
UBC 834	$(AG)_8YT$	50	(Gajera et al., 2021)
UBC 840	$(GA)_8YT$	50	(Dhakshinamoorthy et al., 2022)

Result and Dissection

Genetic diversity analysis using ten ISSR primers on three anise (Pimpinella anisum L.) cultivars showed high efficiency in discriminating between cultivars. A total of 112 amplified bands, ranging in length from 200 to 2800 base pairs, were obtained. Among these bands, 89 (79.46%) were polymorphic, while 23 (20.54%) were monomorphic. Table (2) shows the performance of each primer in terms of total number of bands, number of polymorphic bands, and percentage of polymorphism.

Table 2 Performance of ISSR primers used to distinguish anise varieties.

Primer name	**Sequence (5′–3′)**	**Total number of bands**	**Number of polymorphic bands**	**Percentage of polymorphism (%)**
UBC 807	$(AG)_8T$	11	8	72.73
UBC 808	$(AG)_8C$	10	7	70.00
UBC 809	$(AG)_8G$	12	10	83.33
UBC 810	$(GA)_8T$	13	11	84.62
UBC 811	$(GA)_8C$	9	7	77.78
UBC 812	$(GA)_8A$	10	8	80.00
UBC 825	$(AC)_8T$	11	9	81.82
UBC 827	$(AC)_8G$	12	10	83.33
UBC 834	$(AG)_8YT$	14	13	92.86
UBC 840	$(GA)_8YT$	10	8	80.00
Total / Average	-	**112**	**89**	**79.46**

Primer UBC 834 showed the highest polymorphism (92.86%), making it the most efficient at detecting genetic variation among the three cultivars, while primer UBC 808 recorded the lowest polymorphism (70.00%).

All primers produced clear and reproducible amplification patterns. 17 unique bands were obtained for each cultivar, allowing for the creation of a unique genetic fingerprint for each cultivar. Primer UBC 810 recorded distinct bands of 1250 base pairs that occurred only in the Iraqi cultivar. Primer UBC 834 recorded bands of 950 base pairs that were unique to the Syrian cultivar. Primer UBC 827 recorded bands of 1550 base pairs that were unique to the Iranian cultivar (Figure 1). These distinct bands can be used as reliable molecular markers to identify and authenticate each cultivar genetically.

The genetic similarity coefficient between cultivars was calculated based on the presence and absence of bands using the Dice similarity coefficient. Then the genetic dimension was calculated as (1 - similarity coefficient). Table (3) shows the genetic dimension matrix between the three varieties.

Table 3 Genetic distance matrix between anise varieties based on ISSR data.

	1	**2**	**3**
1	0.00000		
2	0.30927	0.00000	
3	0.16746	0.21273	0.00000

The results showed the lowest genetic distance was 0.16746 between cultivars 1 and 3, indicating a high genetic kinship between them, while the most distant genetic distance was 0.30927 between the cultivars 1 and 2, showing clear genetic divergence among them.

A dendrogram (phylogenetic tree) was generated from the genetic dimension matrix using the UPGMA algorithm. The results from the genetic dimension matrix were supported by the tree since cultivars 1 and 3 were clustered together at an similarity level of 83.25%, whereas cultivar 2 formed a distinct cluster by combining cluster 1 at an similarity level of 78.73% (Figure 2). The distinct clustering of these cultivars demonstrates genetic order.

The results of this study have shown the high efficiency of the ISSR method in estimating genetic diversity in anise (Pimpinella anisum L.), reaching an overall rate of genetic polymorphism of 79.46%, proving the sensitivity of this method in identifying genetic diversity in the genome level. The results of this study reflect what other researches have shown about estimating the genetic diversity in medicinal and aromatic plants using the ISSR method (Kumar et al., 2023). The results have also confirmed other research (Al-Harrasi et al., 2022), conducted to investigate genetic diversity in cumin. The results of this study showed that the ISSR method reached genetic polymorphism of 82.3% in cumin.

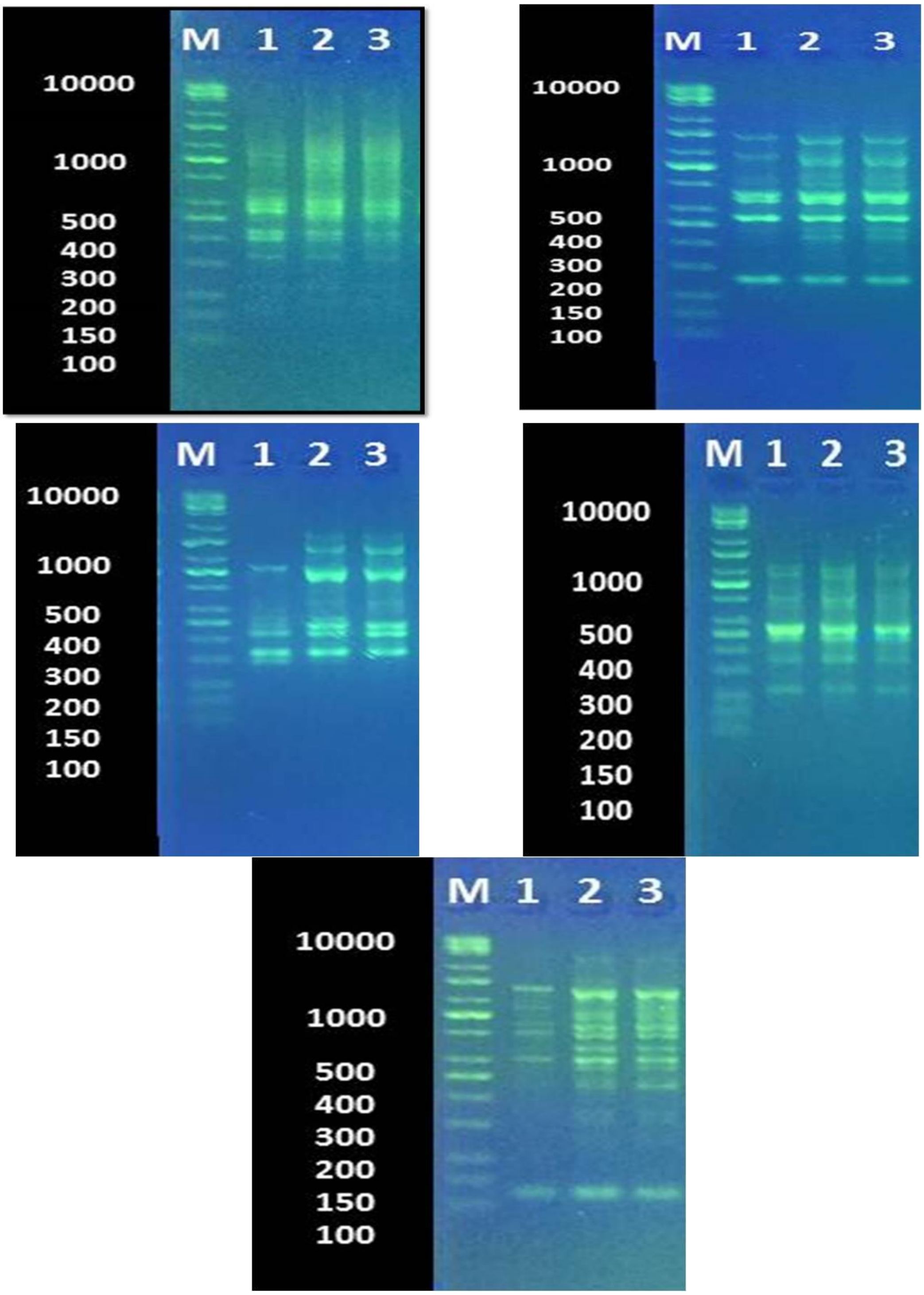

Figure 1 Electrophoretic patterns of the five most polymorphic primers used to distinguish anise cultivars. The image shows the amplification products of the primers (from right to left: UBC 834, UBC 810, UBC 827, UBC 809, UBC 825) separated on a 1.5% agarose gel

Results from analyzing the primers indicated differences in their efficiency in detecting genetic variations; these included the highest value registered by the UBC 834 primer (92.86%) and the lowest registered by the UBC 808 primer (70.00%). The differences were responsible for variations in sequences of nucleotides presented by these primers to regions of genomic variation. Findings presented by Zhang et al., 2024, validate that primers bearing sequences of '(GA) and (AG)' have increased efficiency in detecting genetic variations in plants belonging to species 'Apiaceae'. The reason for this assertion encompasses regions rich in genomic variability presented by these sequences.

The genetic distance test indicated genetic similarity between the Iraqi and Iranian varieties (genetic distance measure of 0.16746), and genetic divergence between varieties from Syria (genetic distance measure of 0 .30927). These findings can be associated to several factors, such as geographical location (Chen et al., 2023), gene flow from adjacent regions (Chen et al., 2023), and isolation (Wang et al., 2024). In agreement with this study (Govindaraj et al., 2015), genetic divergence in the Syrian varieties can be used effectively in genetic improvement for added advantage in hybrid vigor and new traits.

The research unveiled 17 distinct bands that form a special genetic fingerprint for each type of heat treatment and represent an efficient means to safeguard genetic material from deception and plagiarism (Abdullah et al., 2023) as well as ensure sustainable management of genebank (Li et al., 2024). The genetic diversity level reported in this research (79.46%) outperforms what was reported in (Akgül et al., 2018) study about dill (75%), due to the efficiency of the research methodology utilized in this study as well as the special genetic property of the types explored.

The research emphasizes the practical value of genetic fingerprinting in genetic resource conservation. In addition, it provides new perspectives for follow-up research work in terms of increasing sample sources to other regions and combining modern biotechnologies to reach a comprehensive understanding of genetic diversity in anise.

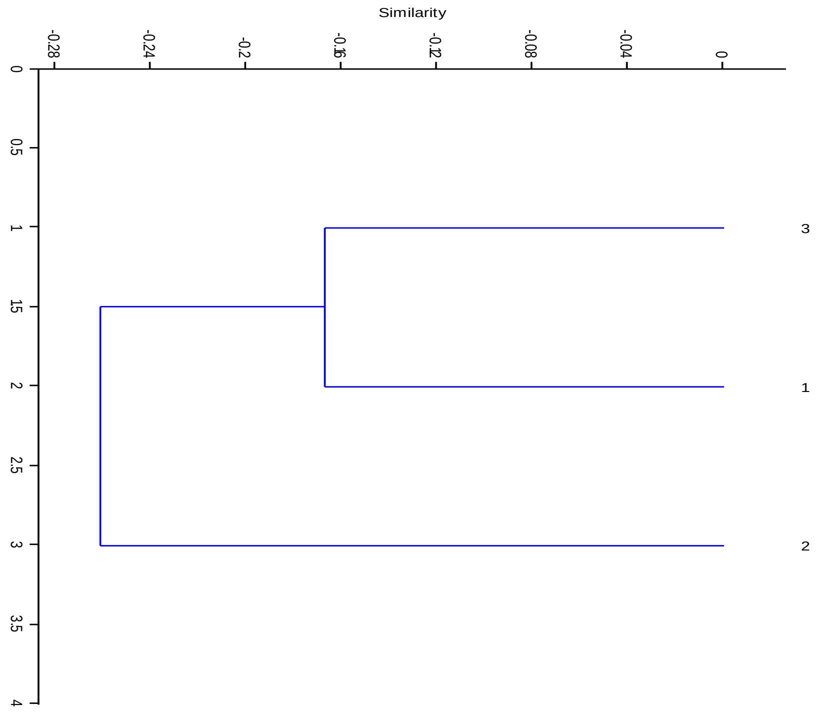

Figure 2 Dendrogram showing the genetic relationships between the three anise varieties (Iraqi, Syrian, Iranian) based on the analysis of ISSR data using the UPGMA algorithm.

Conclusions

In conclusion, this paper provides a comprehensive summary of its chief findings regarding the main role of the Intergenic Sequence Recording (ISSR) marker technique in maximizing genetic diversity in anise cultivars, registering a rate of 79.46% in genetic polymorphism, where the highest rate of genetic polymorphism (92.86%) was registered by primer UBC 834. The study further concluded that there were distinct genetic differences among the cultivated samples, symbolized by the genetic differences between the Syrian cultivated sample and its symbiotic samples from Iraq and Iran. In addition, it presented a distinct genetic fingerprint represented by 17 particular bands enabling precise distinction among varieties and constitutes a critical element in genetic resource conservation efforts as well as fighting business deception. Finally, this research paper provides a strong foundation for genetic science research related to genetic improvement and development models to broaden research to cover other varieties and make use of new genetic techniques to fully harness genetic diversity to improve anise varieties.

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