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Fast Assessment of Phylogenetic Diversity among Five *Hyacinthus orientalis* Cultivars using Molecular Markers

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ABSTRACT

Hyacinthus is one of the most popular bulbous flowers in the world, the hyacinth is a favorite spring fragrant flower for outdoor landscapes and as a cut flower. Although there are numerous horticultural variants and cultivars of hyacinth, it only has one origin. Understanding the genetic relationships between *Hyacinthus* cultivars is necessary to enhance agricultural productivity and gain economic benefits. This research aimed to study the relationships between five different *Hyacinthus* cultivars using Random Amplified Polymorphic DNA markers (RAPD). Six universal primers were used to screen the five cultivars for polymorphisms. For the RAPD–PCR investigation, the number of amplified fragments varied with the primer, a total of 49 amplified bands of which 16 were polymorphic. With the unweighted pair-group approach of arithmetic averages (UPGMA), cluster analyses and phylogenetic tree were carried out, the cultivars clustered according to color, with the colorful cultivars forming the first cluster and the white cultivar forming the second. The genetic distance between the five cultivars ranged from 0.142 to 0.834.

INTRODUCTION

Hyacinth, or *Hyacinthus orientalis* L., is a monocotyledonous plant that belongs to the Hyacinthaceae family Angiosperm phylogeny group (2009). One of the most widely grown plants in the world, Hyacinthus varieties are known for their fragrant and colored blossoms. The hyacinth features a huge, oval bulb that resembles a modified stem and leaves. The modified leaves eventually turn into scale leaves, while the stem decreases and flattens into the disc. They are a well-liked option among ornamentals because they come in a wide range of beautiful blossom colors, such as orange, yellow, pink, purple, blue, red, and white. The raceme-shaped hyacinth inflorescence has two to forty flowers on a single spike with different quantities and densities (Wong *et al*., 2022). Hyacinths have been used as cut flowers, as well as potted plants (Hu *et al*., 2011).

Due to the variety of chromosomal ploidy levels and the increased possibility of getting hybrid offspring from parents with higher ploidy, it is challenging to evaluate the legitimacy of hybrid offspring in hyacinth cultivars. Since hyacinth begins to bloom from seeds in the second or third year, early recognition and selection can greatly lower the cost of generating a new variety. (Wong *et al*., 2022).

Morphological markers were traditionally used to distinguish and identify varieties in agricultural and horticultural crops. Even so, molecular markers have many advantages over morphological and biochemical markers due to their higher level of polymorphism, independence from environmental factors, and exceptional automation capability. RAPD (Random Amplified Polymorphic DNA) markers are examples of molecular markers (Shinsaku *et al*., 1991). A common and useful technique for the diversity of genes based on the marker methodology is the (RAPD), which is used to explore genetic variation at the population level and among different genotypes, the RAPD method offers a better level of polymorphism and a quick, informative approach (Epe *et al*., 2021).

Molecular markers such as RAPD, ISSR (Inter Simple Sequence Repeats), and SSR (Simple Sequence Repeats) may be used to study evolution and population genetics (Sharma *et al*., 2022). SSRs are DNA sequences that include a variable number of tandem repeats located across the genome (Litt and Luty, 1989). RAPD is one of the fingerprint methods developed over the past few years that do not require prior investments in sequence analysis, primer synthesis, or DNA probe. RAPD, the most commonly used technique in genetic diversity, relies on the amplification of random DNA fragments using arbitrarily chosen PCR primers. This technique detects differences at the DNA level using small amounts of genomic DNA without prior sequence knowledge, any DNA fragment pattern can be generated. The patterns produced are determined by the PCR primer sequence and the nature of the template DNA (Vos *et al*., 1995). RAPD marker has been recently one of the best methods for analyzing genetic relationships in many plants because RAPD targets both coding and non-

coding areas of the genome, it may quickly and cheaply differentiate taxa below the species level without the need for preexisting DNA sequencing (Nagaraju *et al*., 2023).

Our study aimed to determine the phylogenetic relationships of the five *Hyacinthus* cultivars using RAPD molecular markers for future use in breeding programs.

MATERIALS AND METHODS

This study was conducted in the Faculty of Agriculture, Floriculture Department, Tissue Culture and Biotechnology Laboratory, Alexandria University, Egypt, Between 2022 and 2023.

1. Plant material

Hyacinthus orientalis flowering bulbs, five different varieties, were purchased from a commercial nursery in the Alexandria governorate, Egypt. All bulbs were almost the same size, with an average height of 5.3 cm and 7.4 cm in diameter, and weighing an average of 100 g. The bulbs were grown in 8 by 8 cm pots and kept at the Department of Floriculture Nursery, Faculty of Agriculture, Alexandria University, Egypt. Later, the bulbs were utilized as a source of DNA.

The five cultivars of flowering bulbs from the *H. orientalis* species were (A) Pink Pearl: 20 to 30 cm in height with fragrant erect spikes carrying pink flowers with a pale pink color edge, leaves are broad with rich green color. (B) Jan Bos: is an erect, highly scented cultivar that reaches a height of 25 cm with brownish green leaves and a brownish red spike carrying dark reddish-pink florets (C) Blue Pearl: about 25 cm in height with tightly clustered bell-shaped blue fragrant floret heads, and dark green foliage, (D) Serene Blue: 20 to 30 cm in height with bright leaves and, thickly flowered spikes with a light blue color and lighter edges, and (E) White Pearl: about 30 cm in height, with three to four light green leaves and sweet fragrant white spikes (Figure 1).

***2.* Extraction of DNA and purification used in RAPD analysis**

DNA was obtained from the leaves using the GeneJET Plant Genomic DNA Purification Kit # K0791. From each cultivar, 100 mg of healthy green leaves were properly cleaned using water and ethyl alcohol to eliminate dirt and other pollutants before being ground under liquid nitrogen to a fine powder. Using the Thermo Scientific plant tissue DNA purification kit, the DNA was extracted, according to the manufacturer's manual. The presence of DNA was detected by electrophoresis on agarose gels 1.2%. DNA was kept at -20°C for future use. After the extraction of total DNA and purification, the hyacinth, the concentration of the DNA concentration was calculated using the GeneQuant II RNA/DNA Calculator, Model 80–2105–98, and the absorbance was measured at 260 and 280 ηm.

Master Mix (Dream Taq TM green PCR Master Mix (2x) containing (DreamTaq DNA polymerase + DreamTaq buffer + MgCl2 and dNTPs) from Thermo-Scientific was used according to the manufacturer's instructions. For the RAPD analysis, the amplification reactions of PCR were carried out in a final volume of 50 µl, containing 25 µl Green PCR Master Mix, 2 µM single primer (Table 1), 1 µg DNA template, and completed to 50 µl by nuclease-free water. Amplification was programmed with an initial denaturation temperature at 94°C for 3 min, 35 denaturation cycles at 94°C for 30 sec, 42°C annealing temperature for 30 sec, 72°C extension for 1 min, 72°C final extension for 5 min, and 4°C storage temperature using Techne TC 3000 Thermal Cycler.

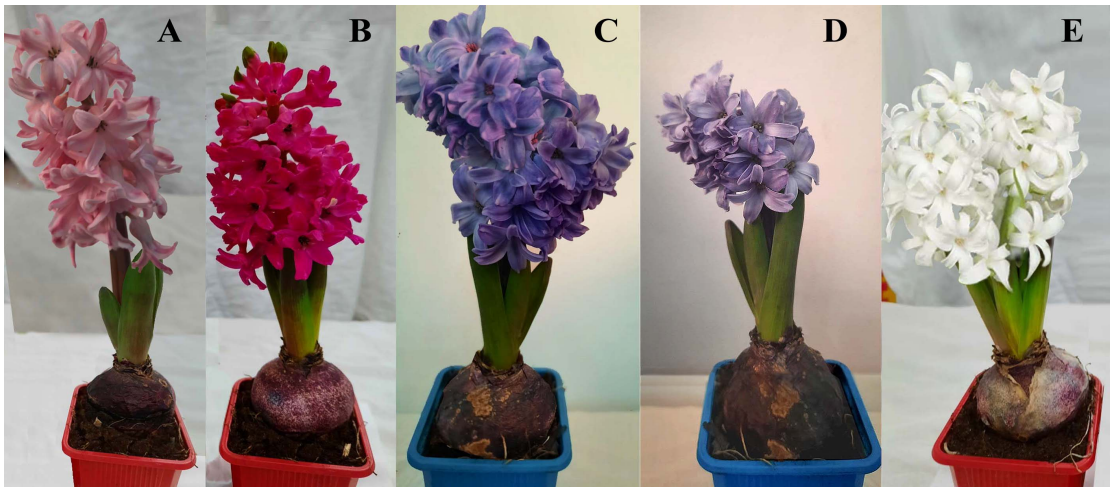

Figure 1: The different five cultivars of *H. orientalis* flowering bulbs where (A) represents Pink Pearl, (B) Jan Bos, (C) Blue Pearl, (D) Serene Blue, and (E) White Pearl cultivars respectively planted in 8×8 cm pots.

Table 1: Primers for RAPD-PCR reactions for screening the five *H. orientalis* cultivars, annealing temperature, and guanine–cytosine percentage.

S/N	Primers code	Sequence (5'-3')	Annealing temperature (°C)	GC%
P1	OPC–12	TGTCATCCCC	32	60
P2	OPD–05	TGAGCGGACA	32	60
P3	OPH–20	GGGAGACATC	32	60
P4	UBC–231	AGGGAGTTCC	32	60
P5	UBC–245	CGCGTGCCAG	36	80
P6	UBC–261	CTGGCGTGAC	34	70

3. Electrophoresis of DNA

The PCR amplified products were electrophoresed at 100 V for 40 min on a 1.5% agarose gel stained with ethidium bromide in (1x) TBE buffer using a Cleaver horizontal gel electrophoresis unit. Fermentas' Gene ladder 100 bp was used as a standard to determine the size of amplified DNA bands. The UV transilluminator light documentation unit (Vilber Lourmat ECX-15M) was used for gel imaging.

4. Data analysis

Six primers were used to screen the samples for polymorphisms. The total number of bands, polymorphic bands, and percentage of polymorphism for each primer was calculated. PyElph, a software for the phylogenetics and analysis of gel images, was used to perform the polymorphic tree and cluster analysis (version 2.6.5). The unweighted pair group method (UPGMA) was used to create a dendrogram.

RESULTS

1. The RAPD and phylogenetic analysis

For the RAPD–PCR investigation, highly pure DNA extracts from the five cultivars of *H. orientalis* were utilized as templates. DNA isolated from the samples of leaves using the specified methodology was amplified successfully in all cultivars tested. RAPD–PCR reaction shows bands size ranging from 200 to 1300 bps depending on the primer, the data in (**Table 2)** and (**Figure 2, Figure S1;** Appendix A**)** reveal that the number of amplified fragments depended on the primer with a total of 49 bands with an average of 8.16 bands for each primer, 16 bands were polymorphic producing 32.65 polymorphism percentage. Additionally, primer OPC–12 produced the most bands (16), whereas primer UBC–245 produced the lowest number of bands (4) (Figure S2; Appendix B). The primers generated fragments ranging in size from 200 to 1300 bps. The phylogenetic tree (Figure 3) of the five cultivars revealed that the colorful cultivars were in the first cluster, while the White Pearl cultivar was in the second. The first cluster was further divided into three clusters: the pink and red inflorescences were in one cluster, the blue inflorescences were in two clusters, and the white one was in a separate cluster. The genetic distance between the five cultivars of hyacinth ranged from 0.142 to 0.834.

DISCUSSION

Molecular markers are frequently employed in biological samples to determine genetic connections and variation (Ganie *et al*., 2015). Currently, a variety of molecular markers are accessible to analyze genetic diversity. The research's objective is the primary factor in determining the best markers and methodology to use (Scariot *et al*., 2007). For genetic analysis in several plant species, RAPD markers are as effective as AFLP (amplified fragment length polymorphism) markers, ISSR (inter-simple sequence repeat), and SSR (simple sequence repeat) markers. RAPD is quick and inexpensive, and it is appropriate for creating a genetic profile for different horticultural crops (Behrooz *et al*., 2010). In the current study, the RAPD technique was used to investigate the genetic relationship between *Hyacinthus* cultivars. Hu *et al*. (2015) used twelve ISSR molecular markers to investigate the phylogenetic relations of 29 *Hyacinthus* cultivars and mentioned that *H. orientalis* is widely used as an attractive ornamental plant, so understanding the genetic diversity of the cultivar is essential for commercial benefits. They constructed a UPGMA tree, UPGMA is regarded as a clustering technique that employs (unweighted) arithmetic averages of dissimilarity measures, thereby not using extreme values (minimum and maximum) to describe how different the genotypes are from one another under consideration (Cosme *et al*., 2014), in this UPGMA tree, the same colored cultivars are generally clustered together; they concluded that among hyacinth cultivars the genetic relationships correlated with the inflorescence colors. Similar results were found in our study using the unweighted pair group method with arithmetic mean UPGMA, as we found that the pink and red colored inflorescences were in the same cluster, and those with blue inflorescences also clustered together, whereas the white colored one was in a separate cluster. He *et al*. (2022) working on eight diploid hyacinth cultivars found that the cultivars were divided into three groups by cluster analysis, Pink Pearl and Red Pearl (red cultivars) were clustered together in group I while Purple Sensation and Blue Pearl (blue cultivars) were in another group Ⅱ,

Table 2: Shows the number of amplified fragments for RAPD analysis using various primers, polymorphic bands, and percentage of polymorphism.

Primers code		Number of fragments				
		Pink Pearl	Jan Bos	Blue Pearl	Serene Blue	White Pearl
P1 OPC–12	P2	4	3	3	4	2
OPD–05		1	2	1	1	1
P3 OPH–20		2	2	2	1	1
P4 UBC–231	P5	3	3	1	0	1
UBC–245		1	1	1	1	0
P6 UBC–261		3	2	1	1	0
Total number of bands		14	13	9	8	5
Polymorphic bands		4	3	3	4	2
polymorphism %		28.57	23.07	33.33	50.0	40.0

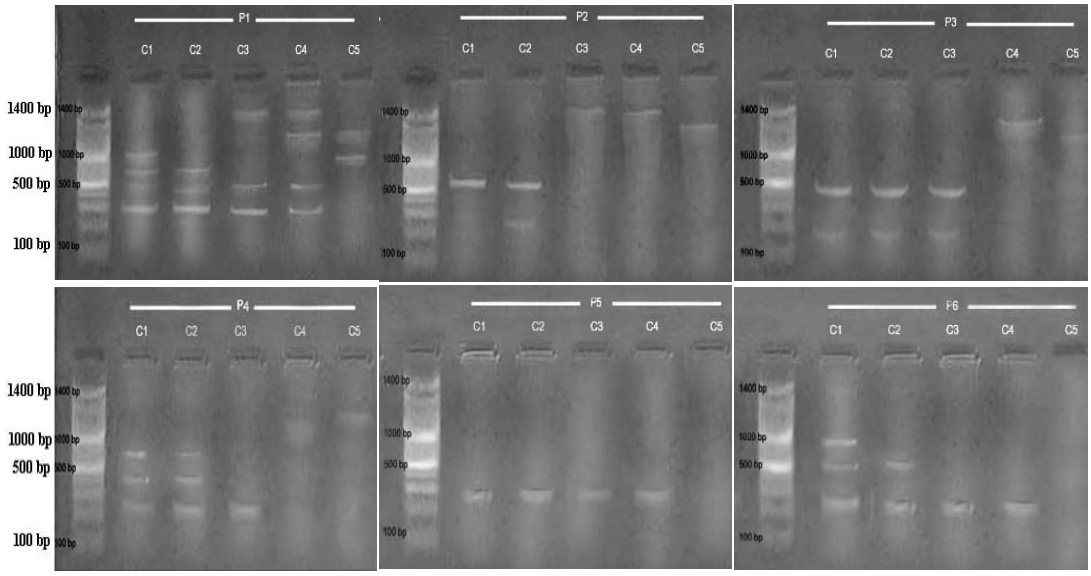

Figure 2: Gel electrophoresis of the RAPD–PCR reaction shows band sizes ranging from 200 to 1300 bps. Lanes C1 to C5 represent Pink Pearl, Jan Bos, Blue Pearl, Serene Blue, and White Pearl cultivars, respectively. P1 to P6 are the six universal primers OPC–12, OPD–05, OPH–20, UBC–231, UBC–245, and UBC–261 respectively.

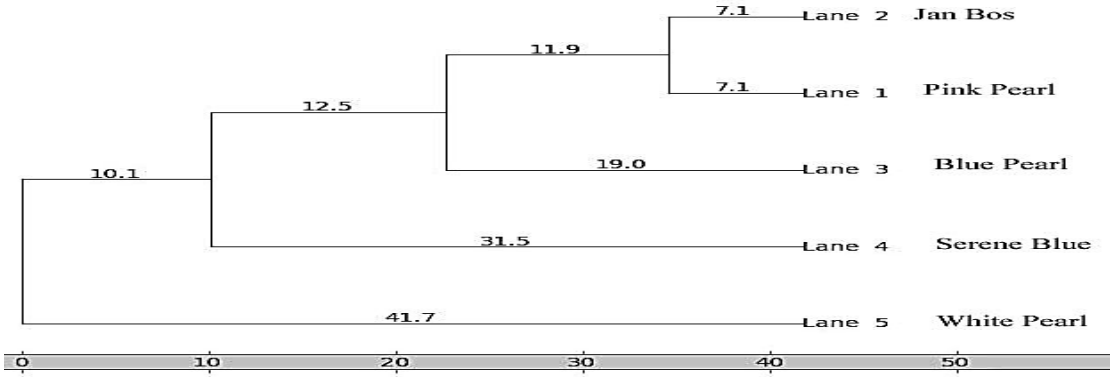

Figure 3: The polymorphic tree of the five cultivars of *H. orientalis* using the unweighted pair group method (UPGMA) PyElph a software system for phylogenetics and analysis of gel images, lanes from 1 to 5 represent Pink Pearl, Jan Bos, Blue Pearl, Serene Blue, and White Pearl cultivars respectively.

and the yellow cultivar Gypsy Princess was clustered alone in group Ⅲ. Hosokawa (1999) reported that blue-flowered *Hyacinthus* had a higher content of anthocyanin pigments than red and pink-flowered bulbs. El-Naggar *et al*. (2023) working on *in vitro* produced *Hyacinthus* reported that the pink and red cultivars (Jan Bos and Pink Pearl), followed by the blue flowering cultivars (Blue Pearl and Serene Blue), exhibited the maximum chlorophyll a and b concentrations while the White Pearl cultivar had the lowest chlorophyll level. Similar findings were also reported for *Moringa* genotypes, where a strong correlation between genetic diversity and phenotypic features was discovered (Hassanein and Al-Soqeer 2018; Kordrostami *et al*., 2016). This was the case in our phylogenetic tree (Figure 3) of the five cultivars, revealing that the colorful cultivars were genetically clustered together, whereas the white cultivar was genetically clustered alone.

In our study, a total of 49 bands were magnified with a total of 16 polymorphic bands representing 32.65% of all bands, revealing a moderate level of genetic diversity between cultivars of hyacinths. The geographical distribution of populations will most likely develop genetic differences as a result of adaptation to different environments (Mekbib *et al*., 2020).

CONCLUSION

The genetic diversity in the *H. orientalis* accessions under this study is moderate, according to the data, despite the fact that the accessions were of various colors. This could be because only a few markers or accessions were used to study the genetic diversity between cultivars.

For the six RAPD primers investigation using the unweighted pair group approach (UPGMA), it was found that among hyacinth cultivars the genetic affinity had a correlation with inflorescence colors where two clusters were formed, one for the colored cultivars, red and blue, whereas the white-colored one was in a separate cluster. The data obtained in our study could be useful for the commercial production and identification of hyacinth cultivars. Further investigation utilizing more samples, cultivars, and markers is required to further comprehension of the genetic structure of the population of *H. orientalis*.

Declarations

Disclosure statement

All the authors declare that they have no conflicts of interest.

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Ethics Approval

Not applicable.

Competing Interests

The authors declare no competing interests.

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الملخص العربي

تقييم سريع للتنوع الجينى بين خمسة أصناف هياسينت باستخدام الواسمات الجزيئية

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الهياسينت هي واحدة من الأبصال المزهره الأكثر شعبية في العالم، الهياسينت هى زهرة عطرية ربيعية مفضلة للتنسيقات الطبيعية خارجيا وكزهرة قطف. على الرغم من وجود العديد من المتغيرات البستانية وأصناف الهياسينت، إلا أنها لها أصل واحد فقط. يعد فهم العلاقات الوراثية بين أصناف Hyacinthus ضروريا لتعزيز الإنتاجية الزراعية والحصول على فوائد اقتصادية. هذا البحث يهدف إلى دراسة العلاقات الوراثية بين خمسة أصناف مختلفة من زهور الهياسينت باستخدام البصمة الوراثية العشوائية (RAPD). تم استخدام ست بادئات (برايمرات) لفحص الأصناف الخمسة بحثا عن التنوع الجيني. بالنسبة لفحص RAPD-PCR، اختلف عدد الحزم المضخمة مع البرايمر، بإجمالي ٤٩ حزمة منها ١٦ حزمة بها تباين جيني. باستخدام نهج المجموعات الزوجية غير المرجحة للمتوسطات الحسابية (UPGMA)، تم إجراء التحليلات العنقودية وشجرة التباين الجيني، وتجمعت الأصناف وفقا للون، حيث شكلت الأصناف الملونة المجموعة الأولى والصنف الأبيض الذي يشكل المجموعة الثانية. تراوحت المسافة الوراثية بين الأصناف الخمسة من ٠,١٤٢ إلى ٠,٨٣٤.