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Unraveling Genetic Diversity Through Molecular Insights: RAPD Profiling of *Hybanthus enneaspermus* (L.) F. Muell.

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Abstract

Hybanthus enneaspermus (L.) F. Muell, commonly known as the Indian Violets or Purple Balsam, is a flowering plant belonging to the family Violaceae. It is widely distributed in tropical and subtropical regions. It holds cultural and medicinal importance in various traditional systems of medicine. Extracts from different parts of the plant have been traditionally employed to address ailments ranging from respiratory conditions to skin disorders. RAPD (Random Amplified Polymorphic DNA) analysis is a molecular biology technique used to generate fingerprint-like patterns of genomic DNA. In this study, we employ RAPD analysis to investigate the genetic diversity and relationships within species/Population/group of interest. Through the amplification of random genomic regions, we aim to generate informative banding patterns that will serve as molecular fingerprints for the individuals under investigation. This study clearly demonstrates the efficacy of this technique as a robust tool for molecular characterization, underscoring its relevance in contemporary genetic research.

Keywords: *Hybanthus enneaspermus*, Electrophoresis, Genetic diversity, RAPD.

INTRODUCTION:

Genetic diversity is a fundamental aspect of biological systems, playing a critical role in adaptation, evolution, and speciation. The ability to assess and characterize genetic variations within populations is essential for understanding their dynamics and implementing effective conservation and breeding strategies (Joshi *et al,* 1999). Molecular markers have emerged as powerful tools for the elucidation of genetic diversity, providing insights into the genomic landscapes of organisms.

Among the diverse array of molecular markers, Random Amplified Polymorphic DNA (RAPD) analysis has gained prominence for its simplicity, cost-effectiveness, and ability to generate high-resolution genetic profiles. RAPD relies on the amplification of random genomic regions using short, arbitrary primers. The resulting banding patterns reveal polymorphic markers that can be utilized for various genetic studies, including population genetics, phylogenetics, and marker-assisted breeding (Welsh and McClelland, 1999).
Random Amplified Polymorphic DNA (RAPD) is a PCR (Polymerase Chain Reaction) based technique used for identifying genetic variation. RAPD involves the use of single 'arbitrary' primer in a PCR and results in the amplification of several discrete DNA products (Joshi *et al*, 1999). The technique was developed independently by two different laboratories (Williams *et al*, 1990., Welsh and McClelland, 1999) and called as RAPD and AP-PCR (Arbitrary Primed PCR) respectively. Using just one primer with any nucleotide sequence, this method finds nucleotide sequence polymorphisms in a DNA amplification-based experiment.
Hybanthus enneaspermus (L.) F. Muell, commonly known as the Indian Violets or Purple Balsam, is a flowering plant belonging to the family Violaceae. Widely distributed in tropical and subtropical regions, this species is recognized for its ecological significance, traditional medicinal uses, and ornamental appeal. As an integral part of local ecosystems, *H. enneaspermus* has drawn attention from researchers, conservationists, and traditional healers alike. The distinctive botanical features of *H. enneaspermus* include its small, vibrant violet flowers, characteristic heart-shaped leaves, and a prostrate or ascending growth habit. The plant's adaptability to diverse habitats, ranging from open grasslands to forest margins, reflects its ecological versatility.

MATERIALS AND METHODS:

Isolation of DNA from Plant sample- *Hybanthus enneaspermus*

Grind 200 mg of -20°C stored plant leaf tissue to a fine paste in 2 ml CTAB buffer by using pre chilled mortar and pestle. Transfer CTAB/plant extract mixture to a micro centrifuge tube and add 0.2% β-Mercaptoethanol (v/v) and incubate the mixture in a water bath for 1 h at 60˚C with frequent inversion. After incubation, spin the CTAB/plant extract mixture at 12,000 rpm for 10 min at room temperature (20°C). To each tube add equal volumes of Chloroform: Isoamylalchol (24:1) and mix the solution by inversion for 5 min and centrifuge at 12,000 rpm for 10 min. Transfer the aqueous phase only to a clean micro centrifuge tube and repeat the step again (until solution is transparent). The upper aqueous phase contains DNA. Add equal volume of chilled isoproponol to precipitate the DNA. Mixed the solution for 5 min by inversion and incubate at - 60˚C for 10-30 min (depending on the precipitation). Centrifugation at 12,000 rpm for 15 min and the supernatant is discarded. Wash pellet in 70% chilled ethanol by inversion and centrifuge at 12,000 rpm for 5 min (Repeat the same step 2-3 times). Discard the supernatant and the pellet is air dried at room temperature (ethanol residue should be remove but it should not be over dried as it would be hard to re-suspend the DNA). Add 100-200 µl of high salt TE buffer to re-suspend the DNA pellet. RNaseA (10 mg/ml) treatment can be done to remove RNA from the sample (3-5 µl RNase is added and maintain at 37°C for 45 min). DNA concentration (quality and yield) was measured by running aliquots on 1% TAE agarose gel, stained with ethidium bromide and bands were observed in gel documentation system. The DNA samples were stored at -20°C until further use. The DNA thus obtained is transferred to PCR tubes along with forward and reverse primers to perform PCR.

PCR Master mix preparation

The PCR reaction was carried out in 20µl reaction mixture containing 10µl Takara mix, 1µl of 10µM forward and reverse primer, 100-200ng of template DNA and made up with sterile distilled water.

PCR Components	**Stock**	**Volume to be taken**
Distilled water	-	Made up to 20 µl
Forward Primer	10 µM	1 µl
Reverse primer	10 µM	1µl
Takara master mix	2X	10µl
Template DNA	-	100-200ng
Total Volume		**20 µl**

- The amplification reaction was performed in a thermal cycler (SimpliAmp, Thermo Fisher) using the PCR conditions with the steps of initial denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, annealing temperature based on Tm of primers for 30 sec, and 72°C for 1 minute 30 secs. Final extension was done at 72°C for 10 minutes and cooling at 4°C for ∞.

GEL ELECTROPHORESIS OF PCR PRODUCTS

The assessment of PCR product is required when working with DNA. This can be done using Agarose gel electrophoresis. This is relatively inexpensive and easy to use method, originally introduced nearly 50 years ago independently for the separation of topoisomers of mtDNA and the separation of restriction enzyme DNA fragments (Borst, P. 2005). This technique will allow the visualization of the PCR amplification reaction. Agarose gel electrophoresis separates DNA molecules based on their size.

Board of agarose gel is assembled with wells on top. The gel is submerged in electrophoresis buffer inside the electrophoresis tank. The PCR products are then deposited on the wells and an electrical current is passed through the gel, conducted by the salts in the electrophoresis buffer. Before applying DNA into the wells of the gel, the DNA is mixed with loading buffer which is composed of glycerol and bromophenol blue dye. The loading buffer does not interfere with electrophoresis. To know the approximate size of PCR product, a DNA molecular weight marker is added to one of the wells. By comparing the bands from the unknown PCR products with the lanes of molecular weight marker, we can estimate the size of our PCR product.

RESULTS :

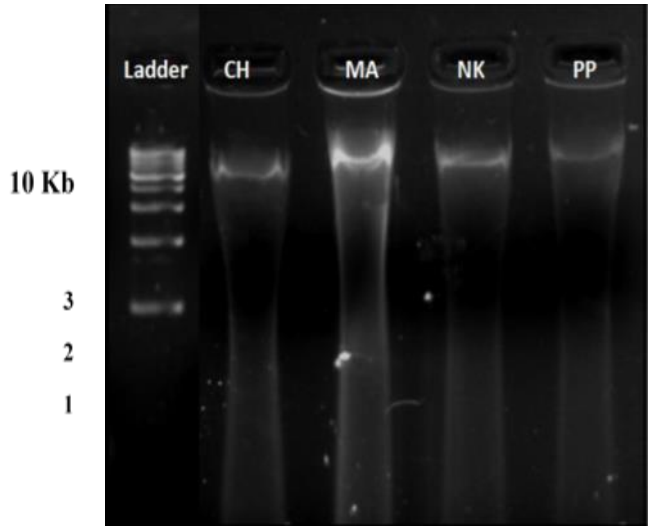
DNA Quantification:

The amount of DNA obtained is tabulated below.

Sample code	Concentration (µg/ml)	Purity
CH	440	1.81
MA	712	1.79
NK	354	1.85
PP	745	1.84

(CH- Chothavilai, MA- Marthandam, NK-Nagercoil, PP- Petchiparai)

From the table, it is obtained that the purity of DNA is 1.85 in the sample obtained from Nagercoil region followed by 1.84 in Petchiparai region, 1.81 in Chothavilai region and 1.79 in Marthandam region. The amount of DNA thus obtained is now subjected to agarose gel electrophoresis to obtain the gel picture of isolated DNA. The below Figure shows the gel picture of isolated DNA.

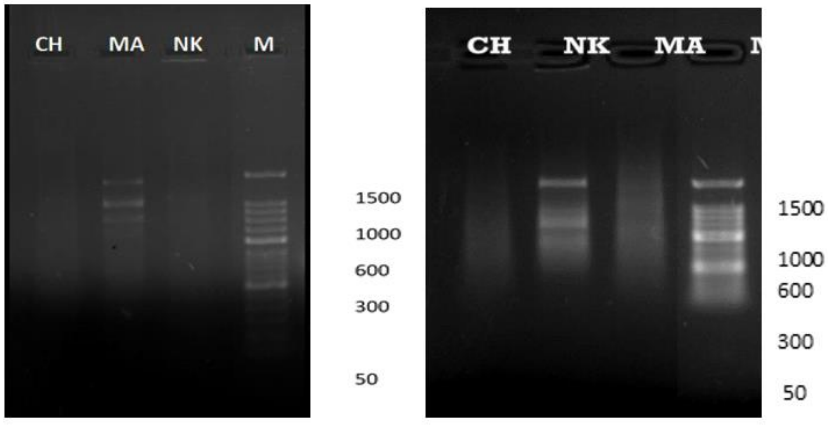


PCR

After the process of PCR, the sequence obtained is tabulated below

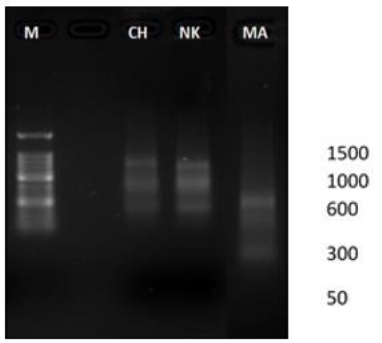
PCR Primer	Sequence	Tm
OPA01	CAGGCCCTTC	33
OPA02	TGCCGAGCTG	33
OPA03	AGTCAGCCAC	28.9
OPA09	GGGTAACGCC	33
OPA10	GTGATCGCAG	28.9

OPA01, OPA02, OPA03, OPA09, OPA10 were the PCR primers used. Among the primers used from the OPA kit, OPA01, OPA02, OPA03, OPA09 and OPA10 shows polymorphic DNA. Tm value difference is corresponding to primers used. In OPA01, OPA02, OPA03, OPA09 andOPA10, the bands were found. In OPA01, two bands were obtained in Marthandam region. The band is 1500Kb and 1000Kb. In OPA02, one prominent band of 1500 Kb were seen in Nagercoil region and one of 1500 Kb in Petchiparai region. In OPA03, a distinguishable bands of 1000 Kb and 600 Kb in Nagercoil and Chothavilai region. In OPA09, a solid band of 600 Kb is found in Petchiparai region. In OPA10, a distinguishable band of 600 Kb is seen in Nagercoil and Petchiparai region. Hence it can be said that Nagercoil and Petchiparai plant samples shows some resemblances corresponding to DNA sequence.

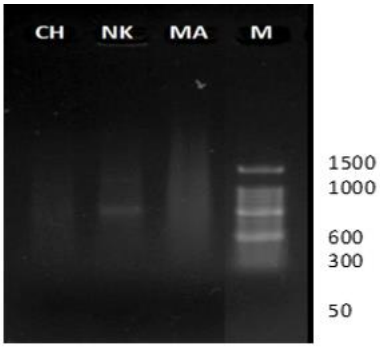


OPA01

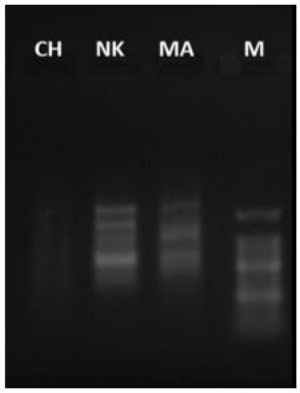
OPA02



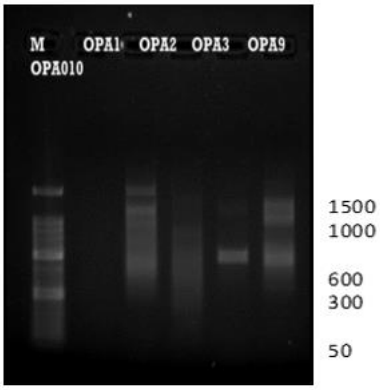
OPA03



OPA09



OPA10



Petchiparai region

(CH- Chothavilai, MA- Marthandam, NK-Nagercoil)

DISCUSSION

For information on the level of polymorphism and diversity, RAPD analysis has been demonstrated to be a valuable approach. In order to conserve genetic resources and use breeding effectively, fingerprinting and assessing genetic diversity among accessions are important. They do not allow the quantification of the

genotypic similarity between accessions as do genetic distances from DNA polymorphism. The simplicity of laboratory assay for RAPD markers makes them an attractive method for obtaining intraspecific distinctions (Nayak *et al*, 2013). In numerous underutilised fruit crops, including loquat (Badenes *et al*., 2004), tamarind (Diallo *et al*., 2007), and for the protection of plant patents (Baird *et al*., 1996). This method is already employed for cultivar identification and genetic variability study. More polymorphic bands were found in the current study, which demonstrated that RAPD fragments are fairly polymorphic and particularly useful for estimating the genetic relationships between *Hybanthus* genotypes. Similar discussion was found in Bael (Nayak *et al*, 2013). Numerous studies on DNA-based molecular markers, including as RAPD, ISSR, AFLP, and microsatellites, are now being conducted in research institutes all over the world. The RAPD method has advantages over other DNA-based genetic markers in that it is relatively quick, simple to perform, relatively inexpensive, highly informative, requires no information to template the DNA sequence, and synthesises specific markers more amenable to automation than traditional techniques, although nanogram quantities of DNA are needed (Das B K *et al*., 2009).

SUMMARY AND CONCLUSION

RAPD analysis was employed in this study to assess the genetic diversity and relationships among the samples under investigation. A set of arbitrary short primers was used to amplify random regions of genomic DNA, resulting in distinct banding patterns. The generated profiles provided a molecular snapshot of the genetic variations within the studied population. The banding patterns reflected the presence or absence of specific DNA sequences, contributing valuable information about the genetic makeup of the samples. RAPD analysis has provided valuable insights into the genetic diversity of the studied samples, laying the groundwork for further investigations and applications in the field of molecular biology and genetics.

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