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Assessment of Genetic Diversity between and within Populations of *Coleus* sp using RAPD Marker.

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ABSTRACT

Random Amplification of Polymorphic DNA (RAPD) markers has been extensively used to determine genetic diversity among various medicinal plants. The objectives of the present study was to assess molecular variation and the level of genetic similarity among five strains of *Coleus blumei (ornamental species)* and other *Coleus sps* of medicinal importance, namely *Coleus aromaticus, Coleus rotundifolius*, *Coleus vettiveroides* and *Coleus caninus*. In the present study, six out of the 35 primers screened gave reproducible results. A total of 84 bands were amplified by the six primers with an average of 14 bands per primer and band size ranging from 100 bp - 2000 bp. Nei's gene diversity generated from the data of 84 bands showed that the genetic variation ranged from 0.1975-0.4938. Dendrogram constructed by means of Unweighted Pair Group Method with Arithmetic average (UPGMA) revealed that the five strains of the ornamental species (*Coleus blumei*) stands apart as a major cluster and are far related from the other *Coleus* sps. The genetic distance of two strains of *Coleus blumei,* Co II and Co IV and that between *Coleus vettiveroides* and *Coleus caninus* were 0.1404 and 0.2719 respectively, indicating closer genetic relationship. The study highlighted the clear genetic variation between the ornamental and medicinal species of *Coleus.*

Keywords: *Coleus*, Primers, RAPD, UPGMA.

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INTRODUCTION

Coleus is an aromatic herbaceous perennial plant growing to a height of 1 to 2 feet. It is native to India and Southeast Asia. Flowers are of pale purple or blue colour. The root stock is thick, fibrous, rapidly spreading and typically golden brown. The leaves can be narrow or wide, round or ovate and comes in a wide range of interesting shapes and fancy leaf edges. The coloured foliage for which *Coleus* are so famous can be red, pink, purple, green, yellow, orange, brown, and all shades in between [1]. Besides its medicinal importance, the plant has gained much attention in the floriculture industry because of the development of new varieties with novel foliage colour.

In India, *Coleus* has been popular in traditional medicines because of its curative properties, due to the presence of complex secondary plant metabolites in one or more parts of these plants [2]. *Coleus* finds application in the treatment of eczema and psoriasis. It is also used as anticancer, antidepressant, antidiuretic, bronchodilator, cardiotonic, gastrostimulant, gluconeogenic, immunosuppressant, lipolytic, myorelaxant, neurogenic, thyrotropic and vasodilator [3]. In the present era, herbal medicine is gaining much popularity over the synthetic drugs considering the safety aspect. However if accurate and rapid authentication of plants used in traditional medicine is not achieved it can prove to be potentially toxic and mutagenic. It is here that Random Amplified Polymorphic DNA (RAPD) markers can be used effectively to identify the authenticity of *Coleus sps.*

The detection and documentation of variation existing within and between populations can be achieved by the use of this marker technology which is rapid & inexpensive. It aids in the selection program for the development of *Coleus* varieties with novel foliage colours. Improvement in the yield and quality of secondary plant metabolites of medicinal importance can also be achieved by tracing the specific genes on evaluating the banding patterns obtained in RAPD analysis. The continuous exploitation of these species from the natural flora, is leading to their extinction. Hence, the RAPD marker technology is also an ideal approach for genetic conservation of plant resources. Another merit of RAPD technique is that, it is not affected by environmental and development stage of the plant and can detect polymorphism at DNA level using single random primer [4].

Govarthanan *et al* [5] has reported genetic variability among three *Coleus* sps. (*Coleus amboinicus, Coleus aromaticus* and *Coleus forskohlii)* by RAPD banding pattern analysis. RAPD analysis was also carried out on six varieties of the ornamental *Coleus* (*Coleus blumei*) by Osman [6]. However there has been less or no report on the comparison of genetic variability studies of the ornamental species and *Coleus* sps of medicinal importance grown in Central Kerala, India. The main objective of the present study was to analyse the genetic variation among five strains of *Coleus blumei* (ornamental species different from those reported by Osman) and other *Coleus* species of medicinal importance, namely *Coleus aromaticus, Coleus rotundifolius*, *Coleus vettiveroides* and *Coleus caninus*.

MATERIALS AND METHODS

Sample Collection:

Nine samples of *Coleus* leaves were collected from different regions of Central Kerala, India (Fig 1).

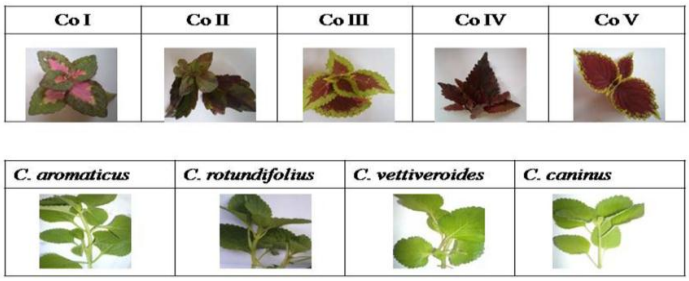

Figure 1: Five strains of *C. blumei*(Co I-V), *C. aromaticus*(Co VI), *C. rotundifolius*(Co VII), *C. vettiveroides*(Co VII), *C.*

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Genetic Diversity Analysis

Isolation of genomic DNA

Isolation of high molecular weight plant genomic DNA is an essential requirement for genetic finger printing by molecular markers, isolation of genes, genetic engineering, genome mapping etc. Due to secondary metabolites it is difficult to isolate DNA from plant tissue compared to animal tissue. For the isolation of DNA from non-ornamental *Coleus sps* modified CTAB (cetyltrimethyl ammonium bromide) method [7] was used. Frozen leaf tissue weighing 1 g was ground to a fine powder in a chilled motor and pestle. To the homogenate 9ml of pre heated CTAB buffer (100mM TrisHCl pH 7.5, 0.7M NaCl, 0.05M EDTA pH 8, 1% CTAB and 0.1% mercaptoethanol)was added and the slurry was transferred into sterile centrifuge tubes. The tubes were incubated at 65^0C for 20 minutes with gentle shaking. After cooling 4.5ml of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 4000 rpm for 10 minutes at room temperature. The top aqueous phase was collected and 6ml of chilled isopropanol was added. The DNA was retrieved with a glass hook, washed with 70% ethanol and transferred to a sterile tube. It was air dried and dissolved in TE (100mM Tris salt, 10mM EDTA) buffer pH 8.0.

The DNA from the ornamental species of *C blumei* were extracted using Sigma Gen Elute^{TM} Plant Genomic DNA Miniprep kit as the quality of DNA obtained by the above mentioned CTAB method did not yield good quality DNA. The coloured pigments present in the ornamental species could have been a hindrance in the DNA extraction process. The concentration and purity of the isolated DNA were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm. The qualities of the DNA samples were analysed using agarose gel electrophoresis stained with ethidium bromide.

RAPD Amplification

Table 1: RAPD primers used in molecular characterization of genotypes

Sl. No	Primer	Sequence (5'-3')
1	OPD05	**TGAGCGGACA**
2	OPB07	**GGTGACGCAG**
3	OPB17	**AGGGAACGAG**
4	OPH20	**GGGAGACATC**
5	OPW06	**ACGCCCGATG**
6	OPW07	**CTGGACGTCA**

A set of 35 decamer primers were employed in RAPD analysis and 6 primers (Table 1) produced reproducible and scorable RAPD profiles. The PCR reaction was carried out in a 40µl reaction mix containing 10X Taq buffer, 2.5mM dNTPs, 10µM primer, 3U/µL Taq DNA polymerase and 50ng of template DNA. Amplification of DNA was performed in a thermal cycler (Eppendorf) using the following conditions: 5 min at 94^oC, followed by 40 cycles of 1 min at 94^oCmin ,1 min at 36^oC and 2 min at 72^oC and finally terminating at 72^oC for 5 min. The RAPD fragments were separated alongside a 100 bp molecular weight marker by 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide and banding pattern was visualized using a Gel Doc System (Vilber Lourmat Gel Documentation Systems, France).

Data Analysis

Scoring of the RAPD-PCR product was done and the results were interpreted using POPGENE version 1.32 software. The datas were scored as ´1´ for the presence of band and ´0´ for absence for each primer DNA combination. A binary data matrix was thus prepared for all the amplified profiles and POPGENE version 1.32 software was used to determine the gene frequency, allele number, polymorphic loci, genetic diversity, genetic distance and genetic variation. Only prominent and reproducible bands obtained for each RAPD primer were considered. By comparing the banding patterns of species for a primer, species-specific bands were identified.

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These data was used to construct a dendrogram for cluster analysis based on the unweighted pair group method with arithmetic average (UPGMA) [8].

RESULTS

In the present study, the DNA from leaves of *Coleus* was isolated using CTAB method (*C. aromaticus, C. rotundifolius C. Vettiveroide s*and *C. caninus*) and kit based method (five varieties of *C. blumei*). Six out of the 35 RAPD primers screened gave reproducible results (Fig. 2, 3, 4). A total of 84 bands were amplified by six primers with an average of 14 bands per primer and band size ranged from 100 bp - 2000 bp. Binary data matrices were prepared in which the presence of a band was coded as '1' and absence as '0'. Fragments of the same molecular weight were considered as the same locus.

***Caninus* (Co IX)**

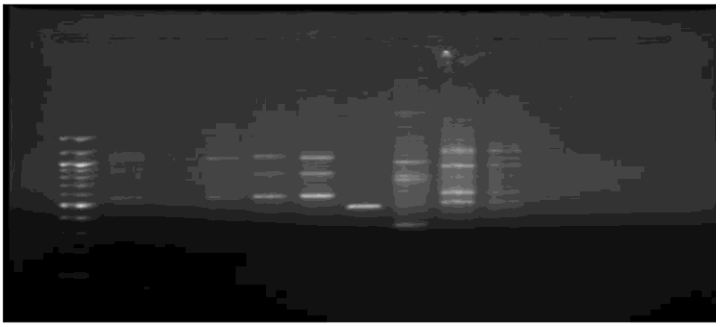

Figure 2: RAPD amplification pattern of different species of *Coleus* sp. using primer OPB07, M-molecular marker (100–1500 bps), five strains of *C blumei*(lanes:2-6),*C. aromaticus*,*C. rotundifolius, C. vettiveroides and C. caninus*

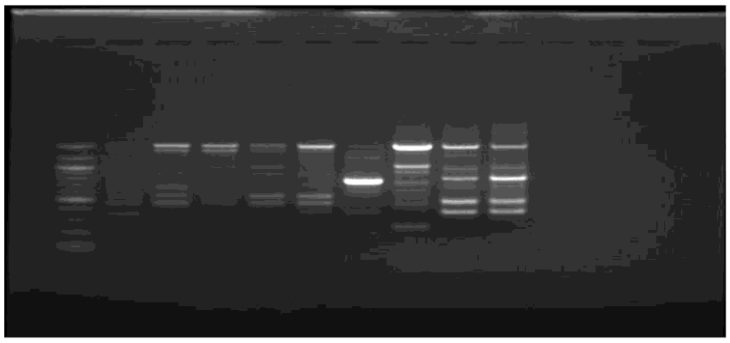

Figure 3: RAPD amplification pattern of different species of *Coleus* sp. using primer OPB17, M-molecular marker (100–1500 bps), five strains of *C blumei*(lanes:2-6),*C. aromaticus*,*C. rotundifolius, C. vettiveroides and C. caninus*

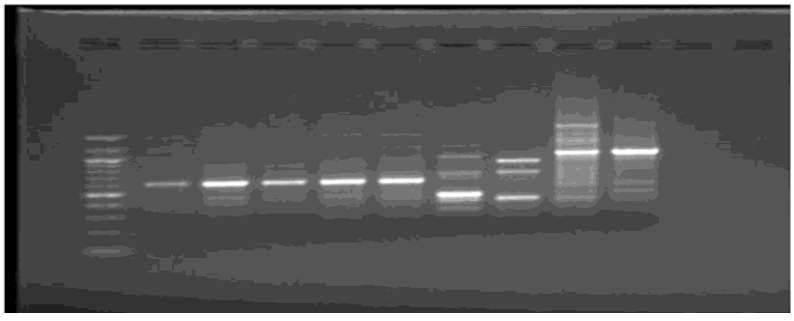

Figure 4: RAPD amplification pattern of different species of *Coleus* sp. using primer OPH20, M-molecular marker (100–1500 bps), five strains of *C blumei*(lanes:2-6),*C. aromaticus*,*C. rotundifolius, C. vettiveroides and C. caninus*

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All bands were scored and gene frequency analysis done by using POPGENE 1.32 software. The results indicated that gene frequency of Allele 0 was ranging from 0.1111 – 0.8889 and Allele 1 was ranging from 0.1111 – 0.8889. The minimum gene frequency value is 0.0000 and maximum value is 1.0000. A value of 1.0000 indicates that the locus is monomorphic and value less than 1.0000 indicates that the locus is polymorphic. From this, we can identify that all the bands formed by different primers are polymorphic in nature. Polymorphism is directly proportional to gene diversity. Polymorphism between bands indicates high level of genetic diversity .Observed number of alleles is 2.0000 and effective number of alleles ranged from 1.2462-1.9756.

Table 2: Nei's genetic distance

Pop ID	1	2	3	4	5	6	7	8	9
1	****								
2	0.3037	****							
3	0.1823	0.1542	****						
4	0.2877	0.1404	0.1967	****					
5	0.2719	0.1823	0.1823	0.1404	****				
6	0.5596	0.5596	0.6466	0.6242	0.6022	****			
7	0.5596	0.5188	0.5188	0.5807	0.5596	0.5596	****		
8	0.6022	0.7932	0.6931	0.8755	0.7932	0.7932	0.9651	****	
9	0.5188	0.7419	0.6022	0.8199	0.6931	0.6931	0.7419	0.2719	****

Even though all organisms exhibited almost similar properties they are genetically diverse. Species specific sites were observed in the populations of *C. blumei* (CoI, CoIV, CoV)*, C. aromaticus, C. rotundifolius, C. vettiveroides* and *C. caninus.* In the present study, Nei's genetic distance ranged from 0.1404-0.9651 (Table 2). Nei's genetic distance [9] revealed that there was a significant genetic diversity among the species. Smaller genetic distance indicates a close genetic relationship. Nei's gene diversity showed that the genetic variation ranged from 0.1975-0.4938. All the six decamer random primers showed molecular polymorphism at amplicon level. The genetic distance of two strains of *Coleus blumei,* Co II and Co IV, Co IV and Co V and Co II and Co V were found to be 0.1404, 0.1404 and 0.1823 respectively. Also the genetic distance between *Coleus vettiveroides* and *Coleus caninus* was 0.2719. Also larger the genetic distance indicates a distant genetic relationship. The genetic distance between *Coleus rotundifolius* and *Coleus vettiveroides* was found to be 0.9651.

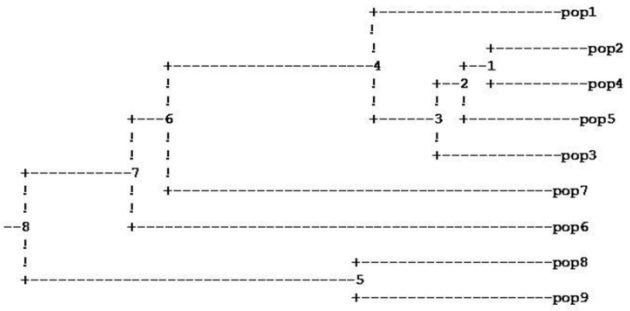

Figure 5: UPGMA Dendrogram analysis based on the genetic distance coefficients of five strains of *Coleus blumei* (pop 1-5), *C aromaticus*(pop 6), *C rotundifolius*(pop7), *C vettiveroides*(pop 8) and *C caninus*(pop9)

From the dendrogram analysis (Fig. 5), CoII and CoIV strains of *Coleus blumei* are closely related. This group is also closely related to CoV strain of *Coleus blumei*. These five strains of *Coleus blumei* together stands apart from the other species of *Coleus* as a major cluster and have a high genetic similarity. Morphologically also they have similarity in shape and colour as shown in Fig.1. *Coleus rotundifolius* and *Coleus aromaticus* are closely related to *Coleus blumei*. The dendrogram also revealed that *Coleus vettiveroides* and *Coleus caninus* are closely related varieties and emerged as another major cluster. They are far related and clustered separately from the rest of the varieties. This also can be noticed from its colour and the shape of the leaves.

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DISCUSSION

Molecular and morphological markers are highly valuable for the identification, conservation and genetic improvement of distinct populations or genotypes [10]. PCR-based RAPD marker is a simple and quick technique that does not require any prior genetic information about the organism. It has been widely used for DNA typing and assessing genetic diversity, phylogenetic relationships in many species, including medicinal plants like *Withania somnifera* [11], *Bacopa monnieri* [12]. Govarthanan *et al* had studied genetic variability among three *Coleus* sps (*Coleus amboinicus, Coleus aromaticus* and *Coleus forskohlii)* by RAPD banding pattern analysis. They had obtained similar clear polymorphic amplification fragments that were and highly reproducible. Very recently, genetic variability studies using RAPD analysis was carried out on six varieties of the ornamental *Coleus* (*Coleus blumei*) by Osman. The results obtained showed variation in genetic similarity between different varieties but a common clustering was observed in the dendrogram analysis similar to our result.

The results obtained suggested that by using RAPD molecular markers, any newly evolved *Coleus sp* can be easily identified. This would be a useful tool in identifying and protecting them from possible infringements in future. Species-specific markers can be identified that would be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred. Genetically distinct cultivars could be identified that could be potentially important sources of germplasm for *Coleus sp* improvement.

CONCLUSION

Molecular tools like DNA marker technology provide valuable data on diversity through their ability to detect variation at the DNA level. The lack of enough data on the assessment of genetic diversity of ornamental as well as medicinal species of *Coleus* grown in Central Kerala has prompted us to carry out the present study. The results of the study highlighted the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of different accessions of an important medicinal plant like *Coleus*. The result further proved that RAPD technique is a reliable method for characterizing variations among species and within a species.

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