

# Assessment of genetic divergence of *Costus speciosus* genotypes using RAPD marker

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**Abstract-** The present work assessed the genetic divergence among the genotypes of *Costus speciosus* collected from five blocks of Balaghat district of M.P., India by RAPD (Randomly Amplified Polymorphic DNA) using Seventeen random declaimer primers as the plant was found to adored its therapeutic efficacy in Ayurveda and traditional system of medicines and is most important medicinal plants used in Arthritis. A deprogram was constructed for cluster analysis using an un-weighted pair group method with arithmetic means (UPGMA) grouped the genotypes into 2 major clusters based on win boot. Out of the seventeen random primers used for studying genetic divergence, twelve primers were found to be polymorphic. Out of 17 primers, 1 was found to be 100% polymorphic generating a total of 21 alleles with an average of 4.2 products per polymorphic primer. The total numbers of alleles were amplified 393 and percent polymorphic was 43.36 for tall seventeen markers. Genetic similarity/dissimilarity among genotypes was evaluated by generating a similarity matrix based on Jaccard's co-efficient ranging from 56 to 0.70. Results showed that both environmental and genetic factors were constrained in observing variations. The degree of genetic variations detected among the accessions of *Costus speciosus* suggested that RAPD marker advent seemed to be best suited for assessing with high accuracy the genetic relationships among distinct *Costus speciosus* accessions.

**Index Terms-** RAPD, Genetic diversity, *Costus speciosus*, variation, Polymorphism, Primer.

## I. INTRODUCTION

Medicinal plants have been of great importance in human culture to meet the primary health care needs. Many people in developing countries use medicinal plants as traditional drugs. According to World Health Organization, up to 80% of the world's population relies on traditional medicinal system for some aspect of primary health care [1].

Medicinal plants produce a variety of compounds having known therapeutic properties. [2]. India is a varietal emporium of medicinal plants and is one of the richest countries in the world in regard to genetic resources of medicinal plants. It exhibits a wide range of topography and climate, which has bearing on its vegetation and floristic composition. Moreover, the agro-climatic conditions are conducive for introducing and domesticating new exotic plant varieties [3].

*Costus speciosus* (Koen ex. Retz.) Sm. belongs to the family Zingiberaceae comprises 175 species in all over the

world. It is commonly called Creep ginger. It can grow up to 5ft. tall in frost-free areas, but typically grow to about 6 ft. tall in cooler regions. In India *Costus speciosus* alone widely distributed at Western Ghats of Tamilnadu and some other moist places of India. The plant is mainly used for healing in burning sensation, constipation, leprosy, worm infection, skin diseases, fever, asthma, and bronchitis. The leaf infusion is used by the patients while bathing with high Febrifuge [4]. It is an erect plant up to 2.7 m high with tuberous root stalk, a sub-woody stem at the base flowers are larger, white, in thick, cone like terminal spikes, with bright red bracts. *Costus speciosus* is native to the Malay peninsula of the south-east Asia. In India, the plant naturalizes in sub-Himalayan tract of central India and parts of Western Ghats of Maharashtra, Karnataka and Kerala [5].

Madhya Pradesh is a second largest state of India. The state is the home of many tribal's such as Baigas, Gonds, Korku tribes[6]. Madhya Pradesh is the heart of Indian Peninsula and has largest concentration for tribal population, about 28% of the total state's population. Madhya Pradesh has different types of growing healing herb, which is used in arthritic medicine like Ayurveda, Siddha and Unani. Medicinal and aromatic plants found in forest areas throughout the Madhya Pradesh form the plain to the hills.

Molecular markers are DNA sequences that are randomly present throughout the DNA and shows specific location in the genome. Molecular markers are of many types but we use RAPD marker. Markers in combination with morphological characterization can be used to evaluate genetic variation among different species of plants. RAPD is a reliable and specific marker in which two orbitatory primers are used which randomly selects and gives results in the form of polymorphism. Due to advancement in plant biology our researchers focuses on the study of molecular characterization of genetic analysis of medicinal plant used in the treatment of arthritis. Molecular characterization in plant biology is used to study to molecular genetic data, molecular biology experimentation, and system analysis. Molecular markers are DNA sequences that are randomly present throughout the DNA and shows specific location in the genome. Molecular markers are of many types but we use RAPD marker. Markers in combination with morphological characterization can be used to evaluate genetic variation among different species of plants. RAPD is a reliable and specific marker in which two orbitatory primers are used which randomly selects and gives results in the form of polymorphism.

## II. MATERIALS AND METHODS

### Plant material

A total of five genotypes were collected from genotypes of *Costus speciosus* collected from five blocks of Balaghat district of M.P., India. The Genotype name their collection sites are given in Table 1.

**Table 1: Five genotypes of *Costus speciosus* and their collection sites**

S. No	Place of collection	Latitude	Longitude	Altitude (m)
1.	Block Birsa (Kaniya)	21 <sup>o</sup> 50` N	080 <sup>o</sup> 50` E	606 m
2.	Block Baihar (Pathri)	21 <sup>o</sup> 20` N	080 <sup>o</sup> 42` E	497 m
3.	Block Parswada (Geegali ghoghara)	22 <sup>o</sup> 09` N	080 <sup>o</sup> 14` E	524 m
4.	Block Balaghat (Gangulpara)	21 <sup>o</sup> 53` N	080 <sup>o</sup> 18` E	374 m
5.	Block Lanji (Devarbeli)	21 <sup>o</sup> 37` N	080 <sup>o</sup> 39` E	357 m

### DNA isolation, amplification and data analysis

The leaf tissue (500 mg) was collected and total genomic DNA was isolated from this leaf tissue sample by following the protocol of Doyle and Doyle (1987)[7] with some minor modifications Fig.1. The leaf tissue was powdered in liquid nitrogen and immediately transferred to 50 ml polypropylene tubes containing 15 ml of preheated (65°C) extraction buffer. DNA quantity and quality was evaluated spectrophotometrically using a Nano-Drop spectrophotometer Table 2. DNA amplification was carried out in an Eppendorf Master-cycler gradient using 10-mer RAPD primers obtained from Integrated DNA Technologies, USA. 50 ng of genomic DNA was used for each PCR amplification reaction. Each PCR reaction of 20 µl reaction volumes contained 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTP mixes, 10 pmol of primer (Integrated DNA Technologies, USA) and 1U of Taq polymerase. All the chemicals used in the PCR reaction were procured from (Bangalore Genei, India) unless otherwise stated. Amplification conditions were: initial denaturation of 5 min at 95°C followed by 45 cycles each of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C and finally a 8 min extension at 72°C. All the experiments were repeated twice to ensure reproducibility. Amplified DNA fragments were separated by electrophoresis at 100 V in 1 X TAE for 3 hrs on 1.2% agarose gel. Gels were stained with ethidium bromide and photographed by the gel documentation system (Bio-Rad).



**Fig. 1 Genomic DNA of Five genotypes of *Costus speciosus***

**Table 2: Genomic DNA quantification**

S. No.	Place of collection	Ratio 260/280	DNA Con. (ng/ul)
01	Birsa (L <sub>1</sub> )	1.75	500
02	Baihar (L <sub>2</sub> )	1.78	800
03	Parswada (L <sub>3</sub> )	1.69	1000
04	Balaghat (L <sub>4</sub> )	1.88	500
05	Lanji (L <sub>5</sub> )	1.89	1200

### Analysis of RAPD data

Amplified fragments were scored for the presence (1) and absence of (0) bands. The data matrices were analyzed by the SIMQUAL program of NTSYS-pc (ver. 2.02e)[8] and the similarities between genotypes were estimated using Jaccard's coefficient. A dendrogram was constructed from the resultant similarities matrices using the unweighted pair group mean averages (UPGMA) method [9]. Statistical analysis was performed in the program PAST3 [10].

## III. RESULTS AND DISCUSSION

Genetic variability in *Costus speciosus* in five blocks of Balaghat district has been carried out using RAPD markers. Seventeen primers generated reproducible, informative and easily scorable RAPD profiles (Table 3 & Fig. 2 to Fig. 11).

The number of alleles was amplified ranged from 3 to 9. A total 393 alleles were amplified with average 23.17 alleles per primer. Out of 393, 170 polymorphic bands were observed. The same type of bands occurred at different frequencies in all populations. There were many additional bands neglected which were not reproducible. Seventeen different RAPD primers (primer-01, primer-02, primer-03, primer-04, primer-05, primer-06, primer-07, primer-08, OPA-12, OPY-11, OPB-04, OPD-01, OPE-01, OPG-4, OPM-16, OPB-11 and OPT-01) were used to evaluate the level of genetic diversity amongst the different samples of *Costus speciosus*. The amplified product was scored on the basis of presence and absence of bands. The scoring of

bands was done independently and only the distinct well separated bands were used to generate the input 1, 0 matrixes that were used for all further computations. A total of 392 bands were amplified in five *Costus speciosus* samples using 17 RAPD primers. Out of these 392 bands, 170 bands were polymorphic and 223 bands were monomorphic (Table 2). Maximum

numbers of bands were generated from the primer OPT-01 giving 34 bands out of which 09 were polymorphic, followed by primer OPG-04 generating 31 bands with 06 polymorphic bands. A 100% polymorphism was obtained with the primers OPE-01 and followed by primer OPY-11 (90.47).

**Table 3: List of the RAPD markers used for this study**

So.No	Primer code	Nucleotides sequence (5`-3`)	Annealing temperature (T <sub>m</sub> )	Size of fragment (bps)	TNB	PB	MB	PP	PIC
1	Primer 1	TCTGTGCCAC	34.9 <sup>0</sup> C	75-1kp	18	8	10	44.44	0.5
2	Primer 2	GTGTGCCCAA	35.9 <sup>0</sup> C	75 -400bp	17	12	5	70.59	0.47
3	Primer 3	GACTAAGCCC	30.6 <sup>0</sup> C	50 -500bp	19	14	5	73.68	0.52
4	Primer 4	TCCCCATCAC	32.8 <sup>0</sup> C	50 -500bp	14	9	4	64.29	0.38
5	Primer 5	AATCGGGCTG	35.1 <sup>0</sup> C	200-900bp	24	9	15	37.50	0.66
6	Primer 6	CAAACGTCGG	34.2 <sup>0</sup> C	200-900bp	21	6	16	28.57	0.52
7	Primer 7	GTTGCGATCC	33.5 <sup>0</sup> C	100-900bp	26	6	20	23.08	0.72
8	Primer 8	GTAGACCCGT	32.6 <sup>0</sup> C	200-800bp	16	11	5	68.75	0.44
9	OPA-12	TCGGCGATAG	36.7	200-550bp	16	11	5	68.75	0.44
10	OPY-11	AGCGTGTCTG	32.8	200-500bp	21	19	2	90.48	0.58
11	OPB-4	GGACTGGATG	24.6	200-800bp	32	2	30	6.25	0.88
12	OPD-1	ACCGCGAAGG	44.1	200-800bp	30	10	20	33.33	0.83
13	OPE-1	CCCAAGGTCC	37.3	200-500bp	21	21	0	100.00	0.58
14	OPG-4	AGCGTGTCTG	29.2	200-900bp	31	6	26	19.35	0.1
15	OPM-16	GTAACCAGCC	32	200-900bp	30	15	15	50.00	0.88
16	OPB-11	GTAGACCCGT	32	200-500bp	22	2	20	9.09	0.61

17	OPT-1	GGGCCACTCA	32	100-900bp	34	9	25	26.47	0.8
<b>Total</b>					<b>393</b>	<b>170</b>	<b>223</b>	<b>43.36</b>	

The clear bands were scored from the gel and “0” and “1” were standardized as the least and maximum of dissimilarity respectively. The dissimilarity coefficients used for cluster analysis was based on the unweighted neighbor- joining method and a dendrogram was generated to study the relationship among *Costus speciosus* samples collected from different regions of Balaghat district (M.P.). The genetic dissimilarity index calculated varied from 0.07 to 0.60 for all the five costus samples (Table 3). The maximum genetic diversity i.e. 0.60 was

calculated for samples **collected from Baihar (L2) and Lanji (L5)**. This shows that the *Costus speciosus* samples collected from these two places are highly genetically diverse. The sample L2 and L4 collected from **Baihar (L2) and Balaghat (L4)** respectively do not show much diversity (0.07). This clearly indicated that these two samples may have same genetic makeup with no or very little genetic difference and these may have spread to different areas because of human intervention.

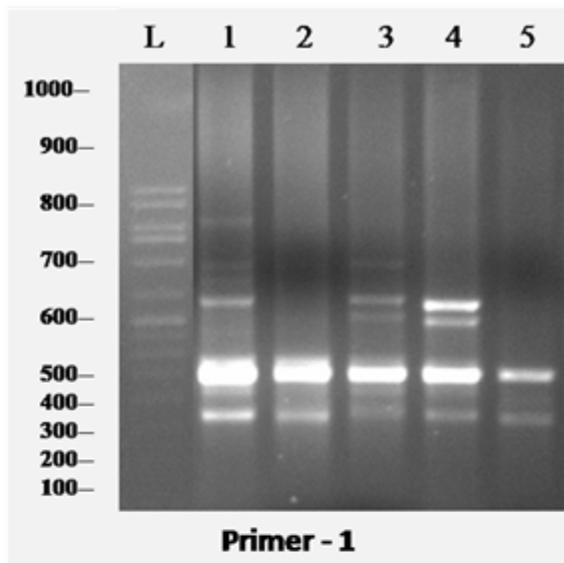


Figure 2: Gel picture showing the RAPD amplification patterns generated by Primer 1 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

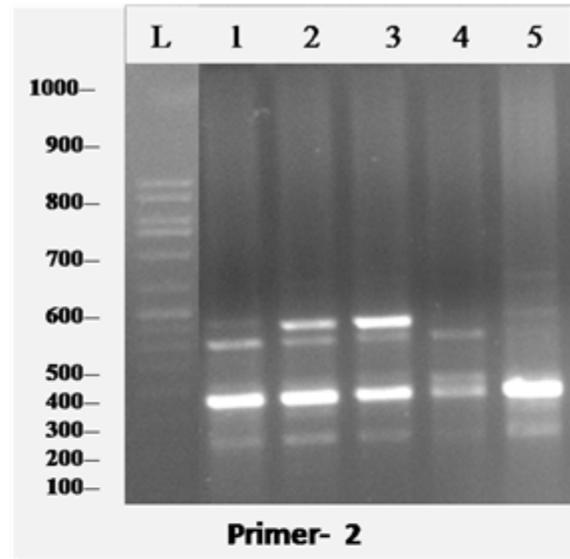


Figure 3: Gel picture showing the RAPD amplification patterns generated by Primer 2 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

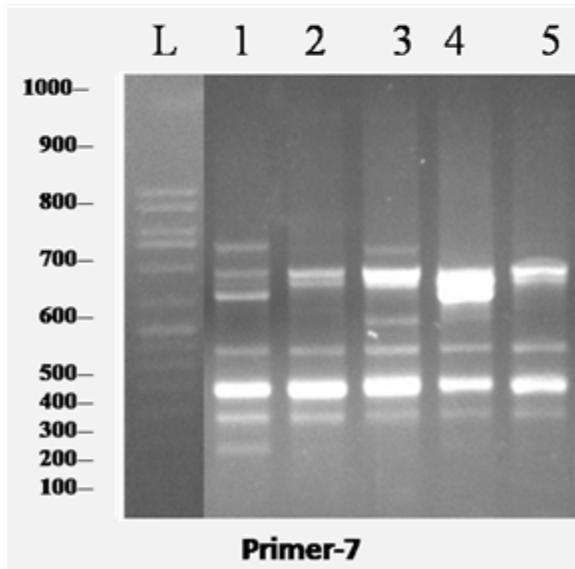


Figure 4: Gel picture showing the RAPD amplification patterns generated by Primer 7 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

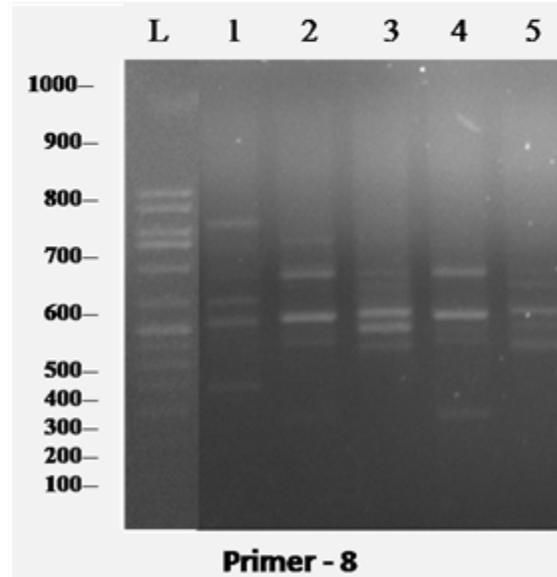


Figure 5: Gel picture showing the RAPD amplification patterns generated by Primer 8 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

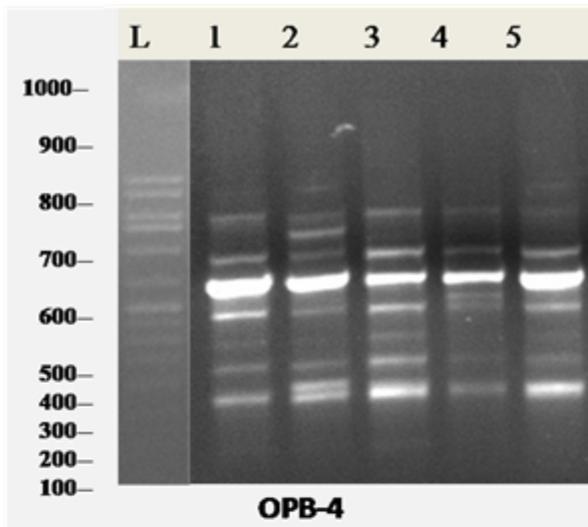


Figure 6: Gel picture showing the RAPD amplification patterns generated by Primer OPB-4 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

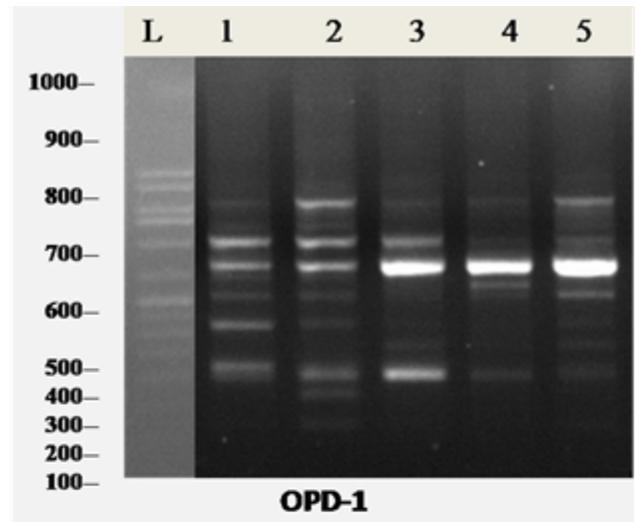


Figure 7: Gel picture showing the RAPD amplification patterns generated by Primer OPD-1 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

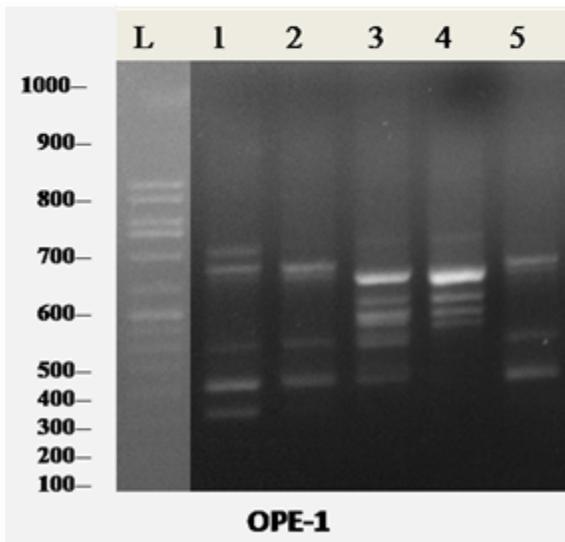


Figure 8: Gel picture showing the RAPD amplification patterns generated by Primer OPE-1 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

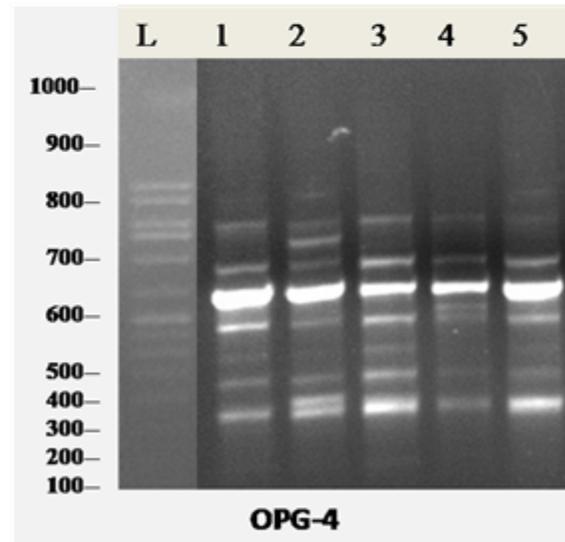


Figure 9: Gel picture showing the RAPD amplification patterns generated by Primer I [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

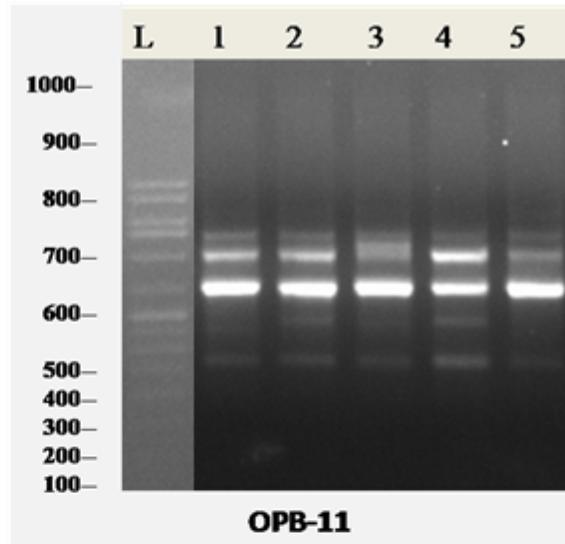
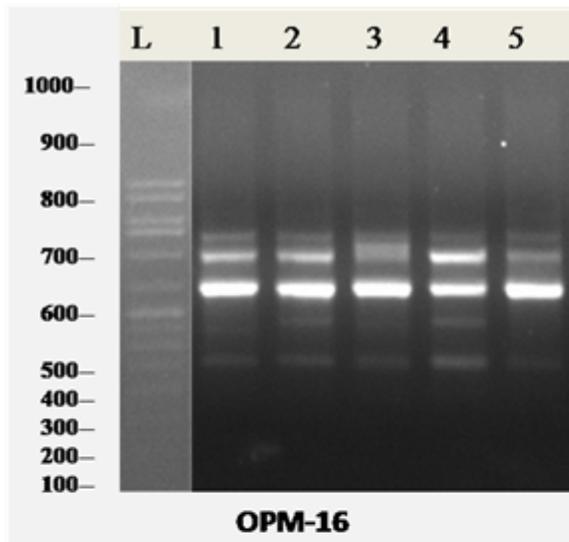


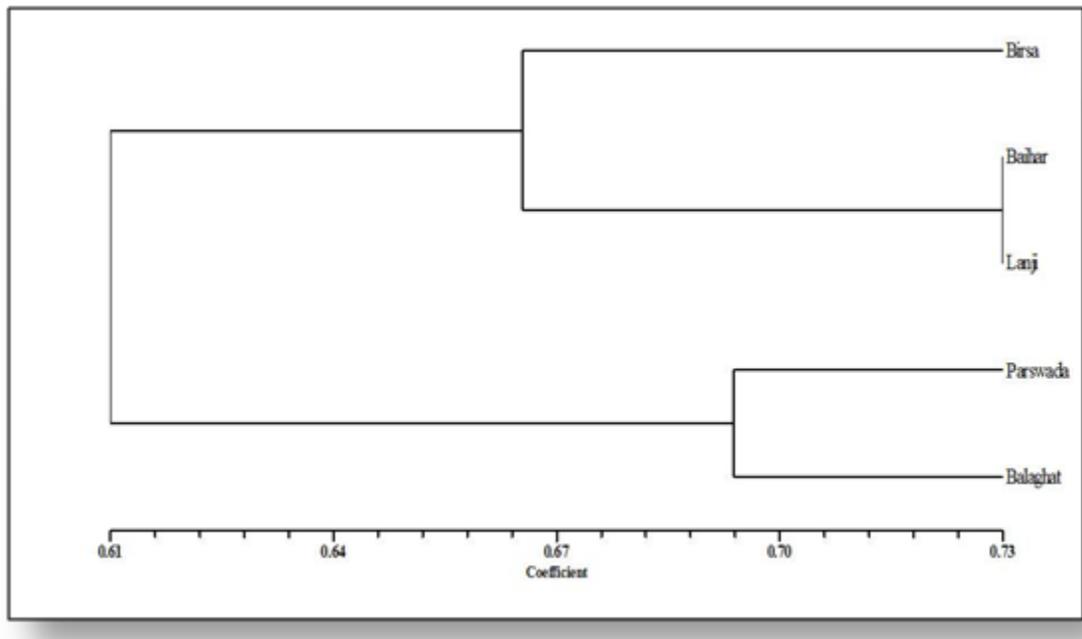
Figure 10: Gel picture showing the RAPD amplification patterns generated by Primer OPM-16 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

Figure 11: Gel picture showing the RAPD amplification patterns generated by Primer OPB-11 [L-Ladder, Names of Samples: 1-Birsa; 2- Baihar; 3- Parswada; 4- Balaghat; 5- Lanji.]

Table Analysis of genetic dissimilarity:

	L1	L2	L3	L4	L5
L1	1.00				
L2	0.28	1.00			
L3	0.27	0.17	1.00		
L4	0.07	0.20	0.44	1.00	
L5	0.51	0.60	0.51	0.38	1.00

L1-Birsa, L 2 - Baihar, L 3 - Parswada, L 4 - Balaghat, L5 - Lanji



**Figure 1: UPGMA based dendrogram of five *Costus speciosus* samples**

The generated dendrogram based on neighbourhood joining method approach of the UPGMA method showed three distinct clusters (Fig 1). All five genotypes were classified into two cluster; cluster 1 and cluster 2. The genetic similarity between all five genotypes ranged from 61% to 73%. Cluster 1 has three genotypes which were collected from Birsa, Baihar and Lanji and showed around 66% similarity between each other. The highest similarity was observed between Baihar and Lanji and that was 73%. Cluster 2 and only two genotypes collected from Parswada and Balaghat and showed around 69% similarity to each other.

Nowadays DNA analysis has become routine technique to estimate genetic diversity in many plant species [11, 12 and 13, 14] including medicinal plants [15 and 16]. A large number of medicinal plant species produce different secondary metabolites which can hamper the DNA isolation procedures. Because of this fact optimization of DNA isolation protocols is often necessary as a primary step in molecular analysis of medicinal plant [17]. The first step of presented study concerned on identification of effective method of DNA isolation from fresh tissue. DNA extracted with CTAB method characterized bad purity and quality. DNA pellets were sticky and have brown or green colour. After electrophoresis in UV light many pollutants around of DNA were observed. After PCR with selected RAPD primers no amplification products were observed. In the second experiment isolated DNA characterized better purity and acceptable quality than obtained using CTAB method, but after PCR no amplification product were observed too. Because of fact that pollutants migrated in agarose gels to purify obtained DNA long and slow electrophoresis and isolation DNA from gels were used. After that procedure DNA characterized small amount but purity and quality were sufficient to conduct PCR. Second part of the experiment was estimating genetic diversity among *A. Montana* genotypes using RAPD method. Randomly amplified

polymorphic DNA is a technique, which identified relatively high number of polymorphic products [18 and 19].

#### IV. CONCLUSIONS

Presented experiment showed that isolation of pure DNA from *A. montana* plants is very difficult. The good way to obtain good quality of DNA is using long and slow electrophoresis and isolation of DNA from agarose gels. Present study demonstrated that RAPD markers provide a useful and effective method to estimate the genetic diversity among *A. montana* genotypes. Analyzed *A. montana* genotypes characterized quite high genetic similarity. The highest genetic similarity was estimated among GA17 and GA18 genotypes, which were closely located on the obtained dendrogram.

**Conflict of interest:** Hari Shankar Yadav and P.K. Saluja declare that they have no conflict of interest.

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