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GENETIC DIVERSITY OF AN ENDEMIC ORCHID, *COELOGYNE NERVOSA* A. RICH FROM SOUTHERN INDIA USING MORPHOLOGICAL AND MOLECULAR MARKERS

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Abstract

Genetic diversity of *Coelogyne nervosa* A. Rich. was investigated by using SDS-PAGE, RAPD markers, and morphological characters. *Coelogyne nervosa* grows as epiphyte as well as lithophytes in Eastern and Western Ghats of India. Leaf samples collected from these two reference sites were taken for RAPD and protein profile analysis. Vegetative parts such as leaves, pseudobulbs, and roots were fixed in FAA. Free-hand and microtome sections were cut and stained with safranin-fastgreen. The objective of this study was to assess the genetic diversity of an endemic orchid, *C. nervosa* distributed in Southern India. The six populations collected from these two geographical regions exhibited significant variations in their morphological and molecular characters. The stomata were tetracytic and hypostomatic in distribution. The maximum thickness of cuticle and midrib region in leaf and extensive lignification in exodermis and endodermis of root were recorded in populations located in Western Ghats as compared to those of Eastern Ghats; it is interpreted to be associated with the conservation of water. RAPD and protein profile data showed the inter-population diversity between these two reference sites. This can be attributed to the ecological and climatic conditions prevailing in the Eastern and Western Ghats of India.

Introduction

AN ENDEMIC orchid, *Coelogyne nervosa* A. Rich. belongs to the subtribe Coelogyninae of tribe Coelogyneae, family Orchidaceae (Dressler, 1993). The genus *Coelogyne* is distributed in Australasia, Tropical Asia, and China. Western and Eastern Ghats are rich with orchid flora; habitat destruction and illegal collection has jeopardised the size and frequency of orchid natural population. There is an urgent need to evolve conservation strategies for this group of angiosperms before it reaches to extinction. The maintenance of genetic diversity within and among the populations is very important for a long-term conservation programme (Avila-Diaz and Oyama, 2007). Recently genetic polymorphism in many plants has been documented by using various molecular markers including isozymes. The analysis of isozymes and RFLP (Restriction Fragment Length Polymorphism) revealed relatively little polymorphism (Keizo *et al*., 2000). The disadvantages of complex procedures and expensive costs strongly restrict the application of AFLP (Amplified Fragment Length Polymorphism) and SSR (Zha *et al.,* 2009). By contrast, RAPD (Random Amplified Polymorphic DNA), amplified by arbitrary primers could be very useful as low cost genetic markers (Williams *et al.,* 1990). Besse *et al.* (2004) studied the genetic diversity in cultivated *Vanilla* by using RAPD markers. In orchids, the genetic diversity has varied from very low to very high; widespread species in general, have higher levels of variation than the endemic species with a narrow geographical range and usually larger populations have more diversity (Avila-Diaz and Oyama, 2007; Gustafsson, 2000). RAPD is a powerful tool to estimate the range of genetic variability and therefore it is useful to evolve conservation strategies of particular species. The objective of present study was to assess the genetic diversity of *C. nervosa* by using morphological and molecular markers, such as RAPD and SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) protein profiles.

Material and Methods

Study Area

Two major reference sites, *i.e.,* Western and Eastern Ghats of India were selected for the present study (Fig. 1). Populations 2, 4, 5, 6 were situated in Western Ghats whereas remaining two (1 and 3) were in Eastern Ghats (Table 1). Western Ghats harbour a rich variety of plant life in its scrub jungles, moist and dry deciduous forests, tropical wet evergreen, montane grasslands, and sholas. Western Ghats were known for luxuriant growth of orchids. The Eastern Ghats comprises disconnected hill ranges extending along NorthEast-SouthWest direction in the East coast, starts from Tamil Nadu in South and extends up to Orissa through Andhra Pradesh in the North; vegetation was dry deciduous.

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Table 1. *Coelogyne nervosa* populations from Eastern and Western Ghats of India.

Population	Host	Altitude (m)
1. Yercaud (Eastern Ghats)	*Lithophyte*	1500
2. Dodabetta (Western Ghats)	*Terminalia alata*	2623
3. Palani Hills (Eastern Ghats)	*Proteum serratum*	2195
4. Kodaikanal (Western Ghats)	*Pterocarpus marsupium*	2010
5. Waynad (Western Ghats)	*Lithophyte*	1500
6. Munnar (Western Ghats)	*Lithophyte*	1400

Anatomical Studies

Totally six populations (P_1 to P_6) were selected from two major geographical areas. Vegetative parts such as leaves, pseudobulbs, and roots were collected from these six populations growing on different host trees (Table 1). The materials were fixed in formaline-acetic-alcohol. The usual procedure of dehydration and embedding were followed (Berlyn *et al*., 1976; Khasim, 2002). Microtome and free-hand sections were cut at a thickness of 10-15 mm and stained with safranin-fastgreen.

Molecular Studies

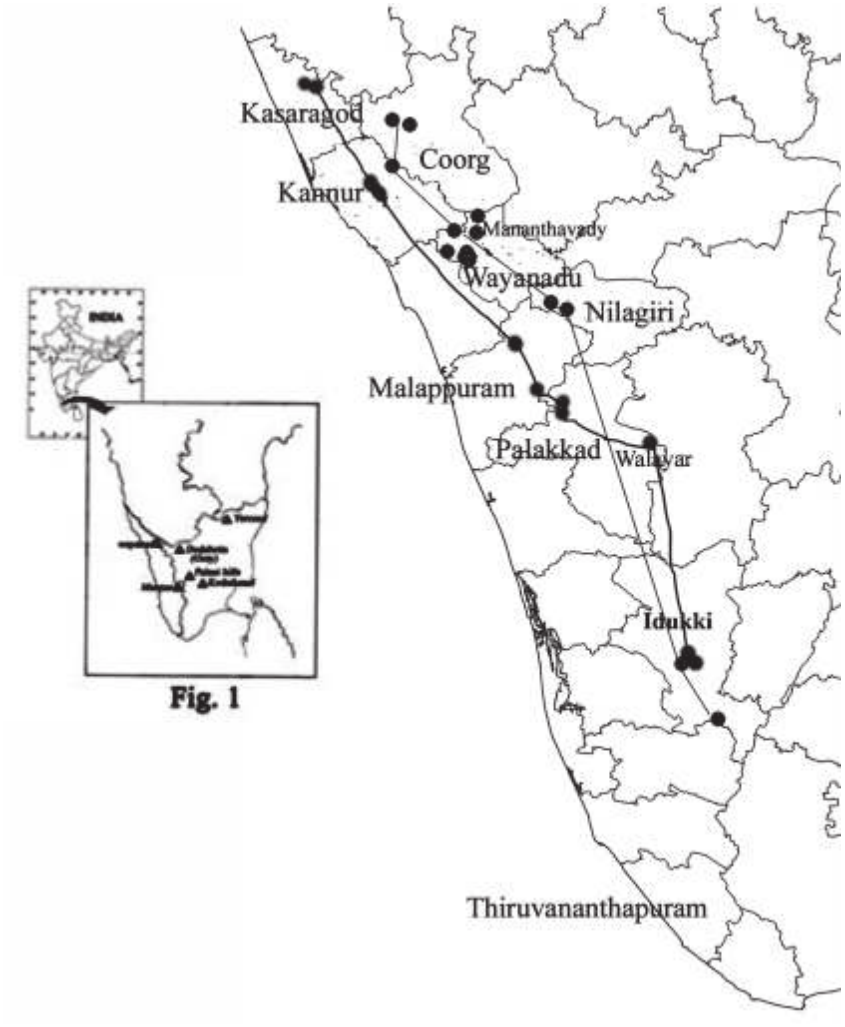

Fig. 1. Study area map showing sampling sites (India).

Leaf material collected from six populations was used for the molecular studies.

SDS-PAGE

Fresh leaves (2 g) were crushed in extraction buffer containing 1.4 M NaCl, 20 mM EDTA (Ethylene Diamine Tetracetic Acid), 100 mM Tris-HCl (pH 8.0), 2% CTAB (N-Cetytl-N, N, N Trimethyl Ammonium Bromide), and 0.2% marcaptoethanol with mortar and pestle, and it was subjected to SDS-PAGE (Shi and Jackowski, 1998). Protein banding pattern was observed and also protein molecular weight marker ranged from 14 kD to 116 kD was used for comparison.

RAPD Analysis

A modified CTAB technique (Doyle and Doyle, 1987) was used for the extraction of genomic DNA and PCR amplification. Only six primers were used in this study (Table 4). PCR was performed in a reaction volume of 25 ml containing 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% triton X-100, 1.5 mM $MgCl_2$, 100 mM each of dNTPs, 25 P mole primer, 100 ng genomic DNA, and 1 unit of Taq DNA polymerase.

Amplified products were resolved electrophoretically on 1.5% agarose gel

Table 2. Morphological characters of *Coelogyne nervosa.*

Morphological characters	P_1	P_2	P_3	P_4	P_5	P_6
Leaf						
Thickness of cuticle (µm)	3, 3-4	4, 4-5	3, 4	3, 4	4, 5-6	4, 6
Thickness of midrib region (µm)	253	298	273	272	307	328
Thickness of laminar region (µm)	203	231	220	205	251	234
Midrib vascular bundle length (µm)	172	190	189	192	182	162
Midrib vascular bundle width (µm)	149	139	132	132	129	143
Guard cell length (µm)	32.3	35	35	31.2	32	32
Guard cell width (µm)	26.2	30	29	24	26	27
Size of the stomatal pore (µm)	14.5	22.2	19.1	20	18	18.2
Number of Phloem cap layers (µm)	5	5	5-6	6-7	6	5
Number of xylem cap layers (µm)	3-4	4	4	4	5	4
Pseudobulb						
Thickness of cuticle (µm)	28	32	35	29.2	32	35
Number of xylem cap layers (µm)	3-4	3	4	3	4	3-4
Number of phloem cap layers (µm)	5	5	5	4	6-7	5-6
Root						
Number of velamen layers (µm)	3-4	4-5	2-4	3-4	4-5	4
Vascular bundle size (µm)	529	512	412	382	332	402
Lignification of exodermis (µm)	20.2	15.2	16.3	15.1	17.2	19.1
Lignification of endodermis (µm)	29	26.9	25	28	26.7	30.2

run at 100 V, visualized by staining with ethidium bromide. RAPD bands are scored as present or absent for each DNA sample and analysed according to Nei and Li (1979) definition of genetic similarity, *i.e.*, SiJ = 2a/(2a+b+c), where SiJ is the similarity coefficient between two individuals (i and J), 'a' is number of bands in both i and J, 'b' is number of bands present in *i* and absent in J and 'c' is the number of bands present in J and absent in J. The matrix of similarity was clustered using UPGMA algorithm and constructed the dendrogram.

Results and Discussion

Morphological and Anatomical Studies

In *Coelogyne nervosa*, leaf was coriaceous and pseudobulbs showed nerve like lines on its surface.

Leaf

Epidermal cells in leaf, relatively larger in the abaxial surface, were rectangular to polygonal in shape. Stomata were confined to abaxial surface (hypostomatic distribution) (Fig. 2a). The leaves were similarly hypostomatic, in most of orchids (Avadhani *et al.,* 1982). Rasmussen (1985) opined that hypostomaty is more frequent in mesophytic orchids and amphistomaty dominates in those of dry and humid habitats. Tetracytic stomata were observed in all six populations (Fig. 2a). The length and width of guard cells is given in Table 2. The maximum and minimum length of guard cells was 35 mm and 31.2 mm in P_2 and P_3, and P_4 respectively.

In transection, leaf was V-shaped at the midrib and flattened at the laminar region (Fig. 2b). Thick cuticle was developed on both surfaces, however, it was more thickened in P_5 and P_6 (both lithophytes from Western Ghats) as compared to other populations. Mesophyll was homogeneous. Highest number of fibre cap layers (6-7) was observed in populations of Western Ghats.

Pseudobulb

In transection, pseudobulb was circular in outline. Highest cuticular thickening was observed in P_3 (Estern Ghats), and also in P_5 and P_6 (both from Western Ghats). Ground tissue consists of large and small parenchymatous cells with abundant mucilage. Large and small vascular bundles were distributed in ground tissue. Air cavities were conspicuous towards phloem cap in all the six populations (Fig. 2c). Such air cavities

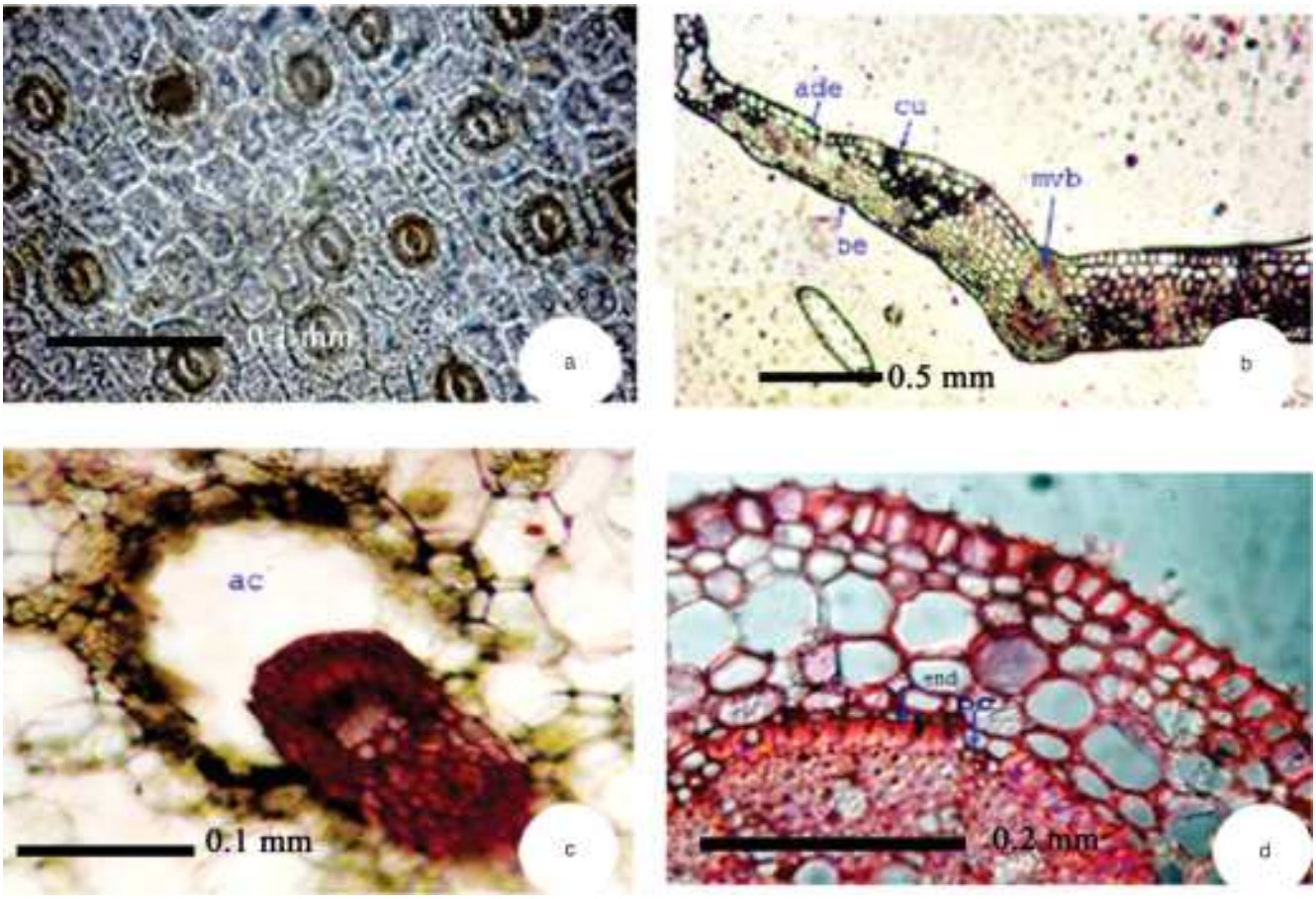

Fig. 2. a-d. Anatomical features of *Coelogyne nervosa*: a, Stomata from abaxial epidermis of leaf; b, Transection of leaf showing midrib vascular bundle; c, Pseudobulb transaction showing air cavity towards phloem cap; d, Root transaction showing 'O' shaped thickened endodermal cells (ade, adaxial epidermis; cu, cuticle; mvb, midrib vascular bundle; ac, air cavity; end, endodermis; pc, passage cell).

were also reported in *Otochilus alba* (Mohana Rao and Khasim, 1987). Presence of air cavities in some members of Coelogyninae enables them to keep weight light (Kaushik, 1983).

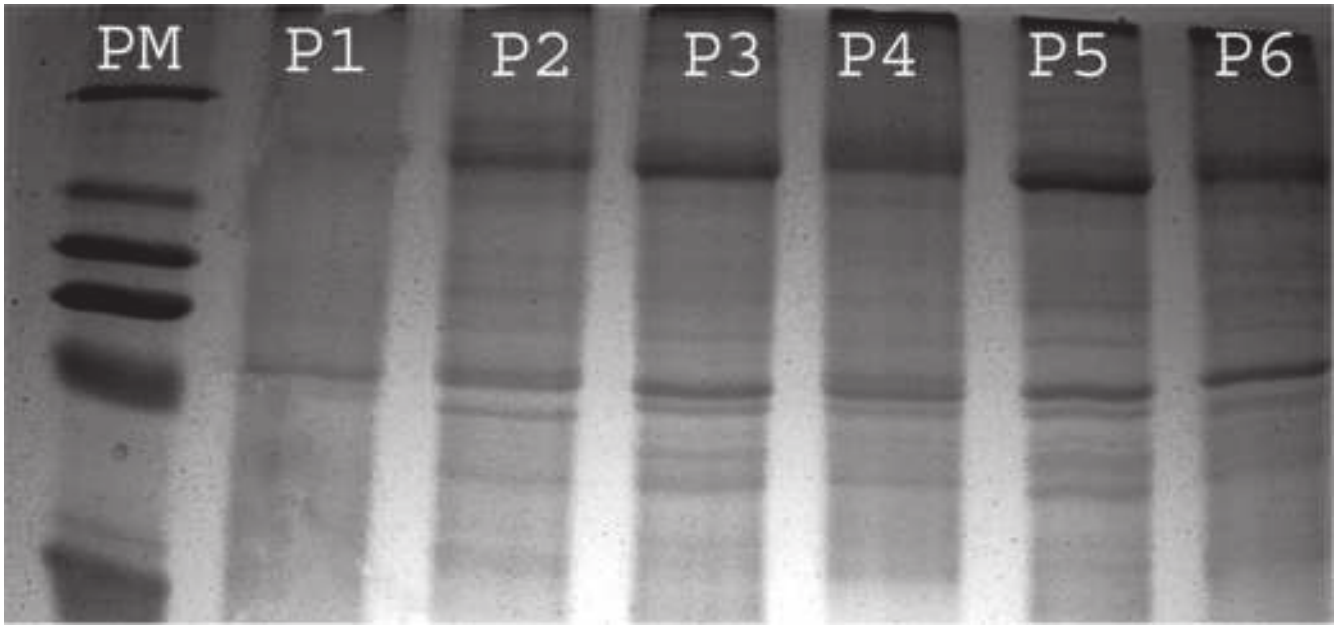

Fig. 3. SDS-PAGE protein banding pattern in six populations of *Coelogyne nervosa* (M-Protein marker, P_1-Yercaud, P_2-Dodabetta, P_3-Palni, P_4-Kodaikanal, P_5-Wayanad, P_6-Munnar).

Table 3. Protein banding pattern and molecular weight in six populations of *Coelogyne nervosa* based on SDS-PAGE.

Protein bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Population 1	81.4	29.3	28.5	27.9	25.2	18.1												
Population 2	110.8	106.3	86.9	76.4	62.3	42.2	36.4	32.0	29.1	27.5	23.0	16.0						
Population 3	109.6	105.9	98.2	91.8	80.6	72.9	41.9	35.4	33.3	31.3	28.5	27.5	24.9	22.9	16.8			
Population 4	110.6	103.5	95.5	76.8	62.8	50.1	43.2	34.7	30.7	29.1	28.6	27.5	25.8	22.6	17.1	14.0		
Population 5	105.9	104.3	94.0	88.0	78.9	70.9	70.7	57.7	48.3	40.6	34.5	30.8	28.6	27.5	25.5	25.8	24.5	22.5
Population 6	102.3	97.0	91.8	72.9	59.3	47.0	41.2	36.3	31.9	29.0	27.8	26.6	24.7	23.8	16.5	14.8		

Root

In all populations of *C. nervosa,* velamentous roots were observed. Exodermis, that lies just below the velamen possessed U-shaped thick-walled cells and also thin-walled passage cells (Fig. 2d). Endodermis was highly lignified with squarish, uniformly thickened cells; it was interrupted by cluster of passage cells lying opposite to passage cells (Fig. 2d). Maximum lignification in endodermal cells was observed in lithophytic population as compared to epiphytic one. Though the Western Ghats were congenial for luxuriant growth of orchids, the lithophytic populations had xerophytic nature. It was also evident from the anatomical data that the host tree plays an important role in supplying nutrients. In this context, Khasim and Ramesh (2010) also opined that the degree of supply of nutrients varied from one host tree to other. The P_2 from Western Ghats showed maximum cuticle thickening and higher number of velamen layers. This attributes that the host tree, *Terminalia alata* on which P_2 grows, would contribute a little amount of nutrient supply. Accordingly, it has undergone structural adaptations so as to conserve the nutrients and utilize them judiciously.

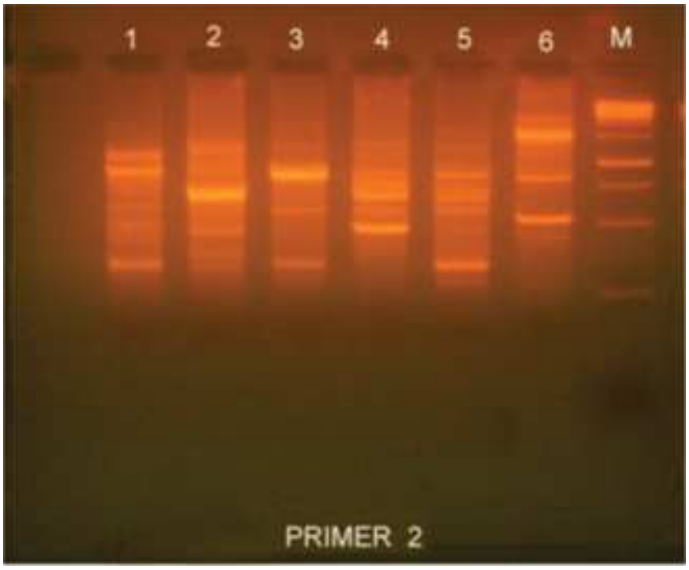

Fig. 4. RAPD amplification profiles of *Coelogyne nervosa* (P_1-Yercaud, P_2-Dodabetta, P_3-Palni, P_4-Kodaikanal, P_5-Wayanad, P_6-Munnar).

Molecular Diversity

SDS-PAGE Protein Profile

In *Coelogyne nervosa*, the SDS-PAGE protein profile showed multiple bands of varied molecular weight ranging from 14 kD to 116 kD in six populations (Fig. 3). Out of eighty three bands, an average of 13 bands per population were observed. There were 36 polymorphic bands observed in all populations. The protein band thickness and staining intensity showed variation amongst six populations.

The SDS-PAGE protein profile also showed that higher molecular weight was represented by P_2 (110.86 kD) and, lowest by P_4 and P_6, all from Western Ghats (14 kD) (Table 3).

RAPD Banding Pattern

The RAPD amplification profile showed variability among six populations of *C. nervosa* (Fig. 4). There were 6

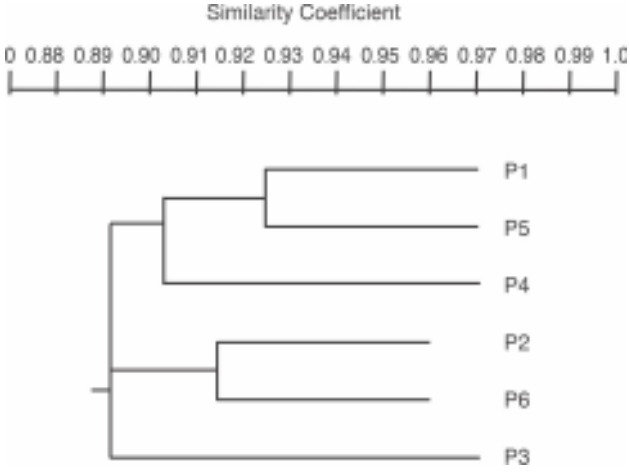

Fig. 5. UPGMA dendrogram of *Coelogyne nervosa* based on RAPD analysis.

Table 4. Primer sequencing, amplified bands, polymorphic bands and percentage polymorphism in RAPD analysis of six populations of *Coelogyne nervosa.*

Primer	Primer Sequence 5'-3'	Amplified bands	Polymorphic bands	Polymorphism (%)
Primer 1	5'GGTGCGGGAA 3'	5	4	80
Primer 2	5'CCCGTCAGCA 3'	5	4	86.6
Primer 3	5'GTTTCGCTCC 3'	4	3	75
Primer 4	5'AAGAGCCCGT 3'	5	4	80
Primer 5	5'GTAGACCCGT 3'	4	2	50
Primer 6	5'AACGCGCAAC 3'	7	5	71.4
	Total	30	22	

primers chosen to generate 31 RAPD fragments, of which 22 bands were polymorphic for all populations (Table 4). Primer 2 was found to produce highest percentage of polymorphism. The percentage of polymorphism ranges from 50.0-86.6%. This data showed that there was considered degree of genetic diversity at interspecific level. The Nei's genetic similarity matrix of all populations was presented in Table 5. The highest value of similarity

Table 5. Nei's genetic similarity matrix of populations of *Coelogyne nervosa* based on RAPD analysis.

Population	P1	P2	P3	P4	P5	P6
P_1	—					
P_2	0.924	—				
P_3	0.901	0.876	—			
P_4	0.905	0.870	0.838	—		
P_5	0.926	0.868	0.879	0.865	—	
P_6	0.910	0.909	0.875	0.862	0.842	—

coefficient (0.926) was found between P_5 and P_1 while the lowest (0.838) in P_4 and P_3. In order to analyse the relationship amongst populations studied, the UPGMA-based dendrogram was constructed using paired matrix values (Fig. 5). From the dendrogram, it is evident that P_1 (Eastern Ghats), P_5 (Western Ghats), and P_4 (Western Ghats) form one cluster and, remaining P_2 (Western Ghats), P_6 (Western Ghats) and P_3 (Eastern Ghats) another cluster. This can be attributed that not only geographical conditions but also species habitat (epiphyte, lithophyte) play vital role in survival of species in the forests.

The present study showed the genetic diversity amongst the populations of same reference site. Besides, there has been a considerable variations found in samples collected from two distinct geographical locations. The gene flow was limited due to the great distance between these two geographical sites. The isolation by distance as well as climatic conditions brought about genetic variations (molecular and morphologically) considerably (Raymond and Rousset, 1995). However, the wide range of molecular weight of protein bands of SDS-PAGE indicate that *C. nervosa* is widely distributed in Western Ghats and there would not be any threat to this species in near future.

According to Misra (1995), as the orchids are highly habitat specific, these suffer a lot due to the destruction of their delicate habitats. Basumatary *et al.* (2008) opined that the epiphytic orchids form a variety of associations in the ecosystem and the knowledge on their community dynamics has much significance in formulating effective conservation measures. Therefore, apart from molecular analysis, the studies on community dynamics and interaction with host trees are equally important before evolving the conservation strategies the orchids (Khasim and Ramesh, 2010).

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