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MOLECULAR AND MORPHOLOGICAL STUDIES IN *COELOGYNE NERVOSA* A. RICH, AN ENDEMIC ORCHID FROM SOUTHERN INDIA

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Abstract

An analysis of genetic diversity in six populations of *Coelogyne nervosa* from Eastern and Western Ghats revealed significant variations in their morphological and molecular characters. Western Ghat populations supported thicker foliar cuticle and highly lignified exodermal and endodermal cells in roots than those in Eastern Ghat populations. RAPD and protein profiles also showed inter population diversity between the reference sites. The diversity seems to be related to the climatic conditions along the distribution range.

Introduction

THE ORCHIDS represent a group of highly diverse and evolved plants that depends on a variety of factors for continued reproduction in nature. They are well represented in India; the Himalayan, the NorthEastern, and the peninsular regions are the major orchid habitats in the country. Several species are highly polymorphic and these include many that are endemic and threatened of survival. The Western and Eastern Ghats, in the peninsular India, are rich in orchid diversity. Several species are polymorphic. Habitat destruction and unregulated commercial collections have, however, jeopardized the size and frequency of natural populations in many species. *Coelogyne nervosa* A. Rich., an epiphytic taxon endemic to Southern India, is one such species. There is a need to conserve them for posterity.

Genetic polymorphism has been documented in many plant species, using a variety of molecular markers including isozymes. In orchids, the genetic diversity varies from very low to very high; widespread species in general have higher levels of variation than the endemic ones with a narrow geographical range, the larger populations are often more diverse (Gustafsson, 2000). The maintenance of genetic diversity within and between the populations is very important for a long-term conservation program (Avila-Diaz and Oyama, 2007). Random amplified polymorphic DNA (RAPD), a low cost genetic marker, is a powerful tool to estimate the range of genetic variability besides formulating conservation strategies (Williams *et al.*, 1990). Besse *et al.*, (2004) assessed the genetic diversity in cultivated *Vanilla* with the help of RAPD markers, whereas zha *et al.* (2009) made similar studies in *Dendrobium.*

This paper aims to assess its genetic diversity using both the morphological and molecular (DNA and Protein profiles) markers.

Material and Methods

Two major reference sites, *i.e.*, Western Ghats and Eastern Ghats in Southern India were selected for sourcing the plant material (Fig. 1). Western Ghats harbour a rich and luxuriant growth of plant diversity including orchids in scrub, moist & dry deciduous, and tropical wet & evergreen forests, besides in montane grasslands and sholas. The Eastern Ghats comprising disconnected hill ranges extending along NorthEast and SouthWest direction in the East coast, harbours dry deciduous vegetation. *Coelogyne nervosa* is an

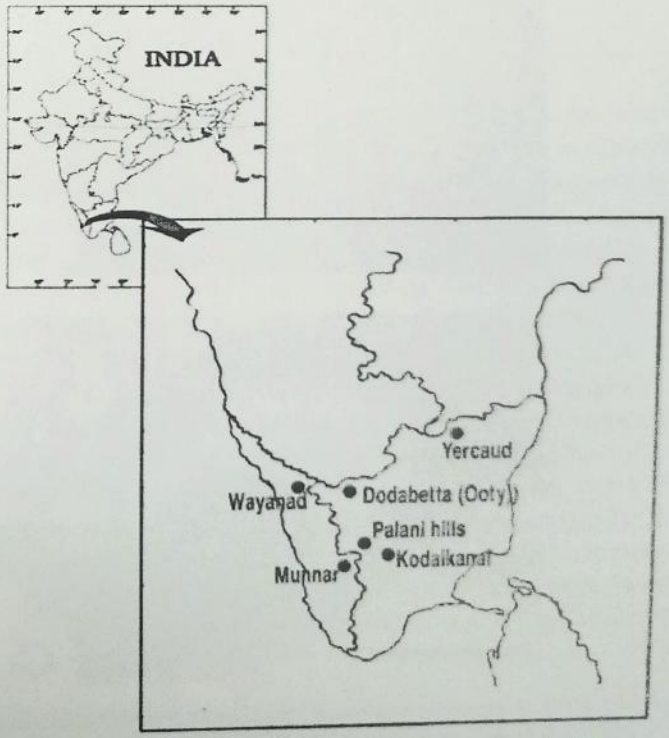

Fig. 1. Study area map showing sampling sites (India).

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epiphytic species growing on a variety of broad-leaved phorophytes. It also dwells as a lithophyte on humus covered rocks. A total of six populations (P-1 to P-6) of *Coelogyne nervosa* were selected; four (P-2, P-4, P-5, P-6) from Western Ghats, and two (P-1, P-3) from Eastern Ghats. Table (1) lists their source..

Anatomical Studies

Vegetative anatomy was studied in the leaves, pseudobulbs and roots for which purpose these were fixed in FAA (formaline-acetic acid-alcohol) solution. The usual procedure of dehydration and embedding were followed (Berlyn and Mikshe, 1976; Khasim, 2002). Microtome and free-hand sections were cut at a thickness of 10-15 m and stained with safranin - fastgreen combination.

Molecular Studies

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

SDS-PAGE

Fresh leaves (2 g) were crushed using mortar and pestle, and an extraction buffer [1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% CTAB, and 0.2% marcaptoethanol]. The extract was subjected to SDS-PAGE following Shi and Jackowski (1998).

Table 1. *Coelogyne nervosa populations:* Source and habit.

Population	Source with altitude	Habit
1.	Yercaud (E. Ghats) 1500 m	Lithophyte
2.	Dodabetta (W. Ghats) 2623 m	Epiphyte *(Terminalia alata)*
3.	Palani Hills (E. Ghats) 2195 m	Epiphyte *(Proteum serratum)*
4.	Kodaikanal (W. Ghats) 2010 m	Epiphyte *(Pterocarpus marsupium)*
5.	Waynad (W. Ghats) 1500 m	Lithophyte
6.	Munnar (W. Ghats) 1400 m	Lithophyte

The pattern of protein bands was observed and analyzed through a comparison with molecular weight (14 kD to 116 kD) marker proteins.

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 l containing 50 mM KCl 10 mM Tris HCl (pH 9.0), 0.1% triton X-100, 1.5 mM MgCl2, 100 M each of dNTPs, 25 P mole primer 100 ng genomic DNA and 1 unit of Taq DNA

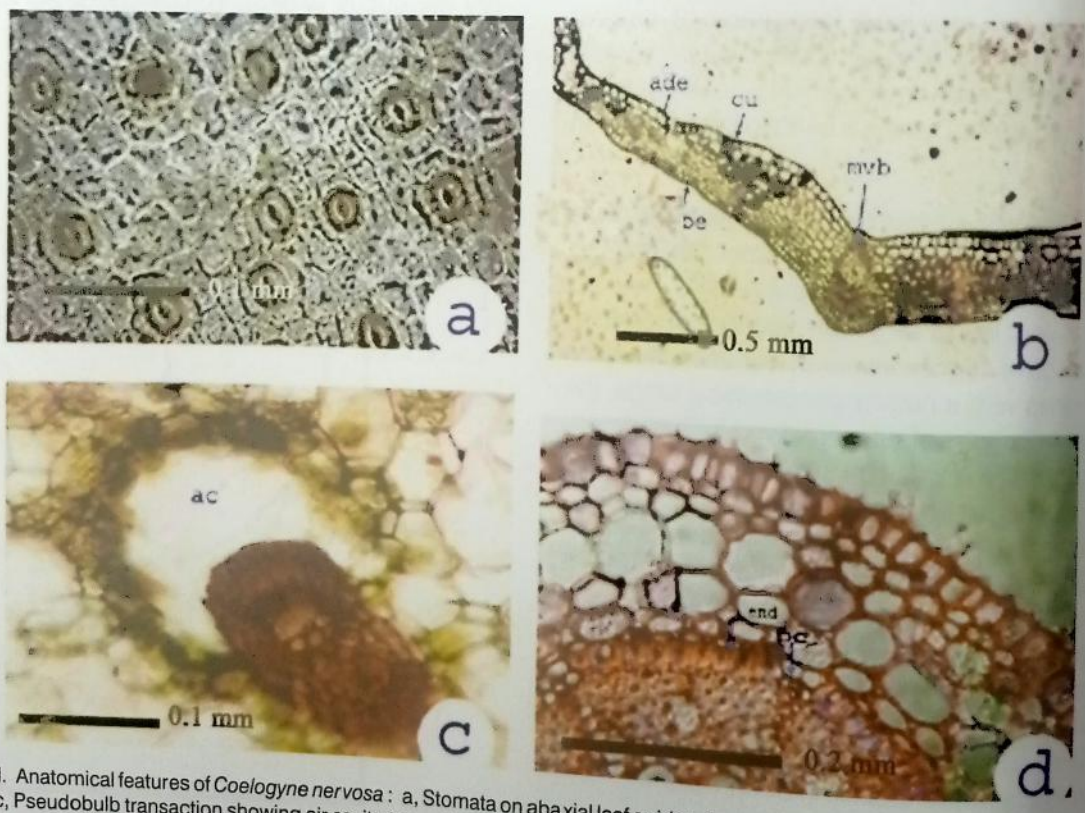

Fig. 2 a-d. Anatomical features of *Coelogyne nervosa* : a, Stomata on abaxial leaf epidermis; b, Transection of leaf showing midrib vascular bundle; c, Pseudobulb transaction showing air cavity towards phloem cap; d, Root transaction showing 'O' shaped thickened endodermal cells. (ade=adaxial epidermis,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

Table 2. Morphological characters of *Coelogyne nervosa*.

Morphological characters	Populations					
	P1	P2	P3	P4	P5	P6
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
Phloem cap layers (Number)	5	5	5-6	6-7	6	5
Xylem cap layers (Number)	3-4	4	4	4	5	4
Pseudobulb						
Thickness of cuticle (μm)	28	32	35	29.2	32	35
Xylem cap layers (Number)	3-4	3	4	3	4	3-4
Phloem cap layers (Number)	5	5	5	4	6-7	5-6
Root						
Velamen layers (Number)	3-4	4-5	2-4	3-4	4-5	4
Vascular bundle size (μm)	529	512	412	382	332	402
Exodermis thickness (μm)	20.2	15.2	16.3	15.1	17.2	19.1
Endodermis thickness (μm)	29	26.9	25	28	26.7	30.2

polymerase. The amplified products were resolved electrophoretically on 1.5% agarose gel run at 100 V, visualized by staining with ethidium bromide. RAPD bands were 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.

Result and Discussion

Morphological and Anatomical Studies

Leaf

In all the *C. nervosa* populations, the leaves were uniformly coriaceous and pseudobulb invariably showed nerve like lines on its surface. The epidermal cells were rectangular to polygonal in shape, and relatively larger on the abaxial surface. The leaves were hypostomatic in accord with similar findings in most of the orchids (Avadhani *et al.*, 1982). Incidentally, hypostomaty is more frequent in mesophytic orchids as compared to amphistomaty which dominates in orchids of dry and humid habitats. Tetracytic stomata were observed in all six populations (Fig. 2a). The measurements of guard cells are given in Table 2. Longest guard cells were discerned in the populations P2 and P3. In a transection, the leaf appeared V-shaped in the midrib and flattened at the laminar region (Fig. 2b). Thick cuticle was observed on both the leaf surfaces; the thickenings were more pronounced in the lithophytic populations (P-5, P-6), both from Western Ghats. Mesophyll was homogeneous. Highest number of fibre cap layers (6-7) was observed in populations from Western Ghats.

Pseudobulb

In a transection, the pseudobulb was circular in outline. Highest cuticular thickening was observed in P3

Table 3. SDS-PAGE based protein bands and their molecular weight in *C. nervosa* populations.

Population \ Protein band	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	81.4	29.3	28.5	27.9	25.2	18.1												
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						
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			
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		
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
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		

(Eastern Ghats), and also in P-5 and P-6 (both from Western Ghats). Ground tissue consists of parenchymatous cells with abundant mucilage. Large and small vascular bundles were distributed in ground tissue. Air cavities were conspicuous towards phloem cap in all six populations (Fig. 2c). Such air cavities were also reported in Otochilus alba (Mohana Rao and Khasim, 1987). Presence of air cavities in some members of Coelogyninae enables them to keep light in weight (Kaushik 1983).

Root

In all populations of *C. nervosa* the roots were velamenous. The exodermis comprising U-shaped thick-walled cells was dotted with some thin-walled passage cells (Fig. 2d). The endodermal cells were highly lignified and uniformly thickened; the lignification was more pronounced in the lithophytic populations. Highly thickened cuticle and mid-rib in the leaves, and higher number of velamen layers and more pronounced lignification of exo- and endodermal cells in the accessions P5 and P6 (Table 2) seem to be related to water deficient conditions on their lithophytic abodes. A relatively thicker cuticle and multiplicity of velamen layers in population growing on *Terminalia alata* trees (P2) is interesting and may be attributed to xeric conditions on their phorophytic abodes. In this context, it may not be out of place to mention that the host tree leachates play an important role in satisfying the water and nutrient requirement of the epiphytic vegetation. Earlier, Khasim and Ramesh (2010) suggested that the degree of nutrient supply varies with the host tree.

Molecular Diversity

Protein Profile

In *C. 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 among six populations.The SDS-PAGE protein profile (Table 3) 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).

RAPD Banding Pattern

The RAPD amplification profile showed variability among six populations of *C. nervosa* (Fig. 4). There were 6 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 50-86.6%. These data show that there was considered degree of genetic diversity at interspecific level. The Nei's genetic similarity matrix of all populations is presented in Table 5. The highest

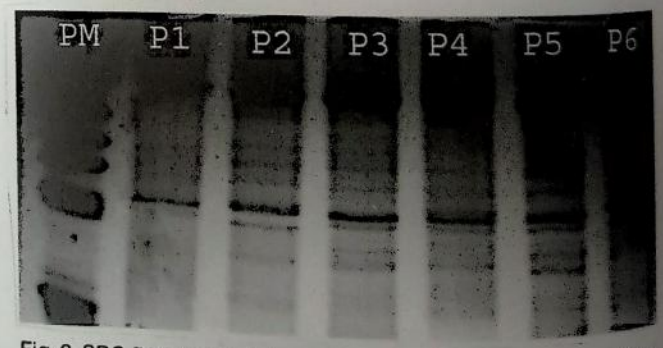

Fig. 3. SDS-PAGE protein banding pattern in *C. nervosa* populations (P1 to P6). (PM-Protein marker, P1-Yercaud, P2-Dodabetta, P3-Palni, P4-Kodaikanal, P5-Wayanad, P6-Munnar)

value of similarity 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 among 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

2012)

Table 4. RAPD Analysis : Primer sequences, amplified and polymorphic bands, and frequency of polymorphicity in *C. nervosa* populations.

S.No.	Primer	Primer Sequence 5'-3'	Amplified bands	Polymorphic bands	Polymorphicity (%)
1	Primer 1	5'GGTGCGGGAA 3'	5	4	80.0
2	Primer 2	5'CCCGTCAGCA 3'	5	4	86.6
3	Primer 3	5'GTTTCGCTCC 3'	4	3	75.0
4	Primer 4	5'AAGAGCCCGT 3'	5	4	80.0
5	Primer 5	5'GTAGACCCGT 3'	4	2	50.0
6	Primer 6	5'AACGCGCAAC 3'	7	5	71.4
		Total	**31**	**22**	

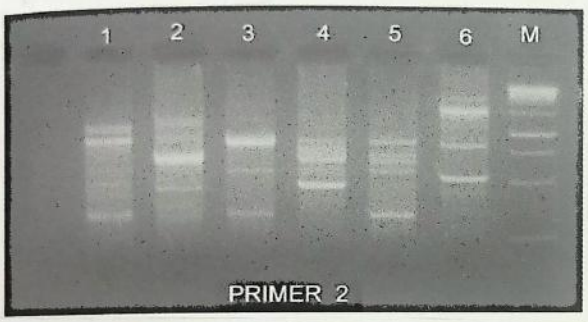

Fig. 4. RAPD amplification profiles of *C. nervosa* populations (P1-P6). (P1-Yercaud, P2-Dodabetta, P3-Palni, P4-Kodaikanal, P5-Wayanad, P6-Munnar)

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 habitat (epiphyte, lithophyte) play vital role in survival of species in the forests.

The present study shows genetic diversity even among the populations of same reference site. Besides, there has been a considerable variation 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 genetic variations, according to Raymond and Rousset (1995), are induced by isolation through distance as well as climatic conditions. 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.

The orchids are habitat specific and they suffer much due to the destruction of their delicate habitats (Misra, 1995). According to Basumatary *et al.*, (2008), the epiphytic orchids exihibit a variety of associations in the ecosystem and a knowledge on their community dynamics is highly significant in formulating effective conservation strategies. Therefore, apart from molecular analysis, the studies on community dynamics and interaction with host tree are equally important before evolving the conservation strategies of orchids (Khasim and Ramesh, 2010).

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

Populations	P1	P2	P3	P4	P5	P6
P1	–					
P2	0.924	–				
P3	0.901	0.876	–			
P4	0.905	0.870	0.838	–		
P5	0.926	0.868	0.879	0.865	–	
P6	0.910	0.909	0.875	0.862	0.842	–

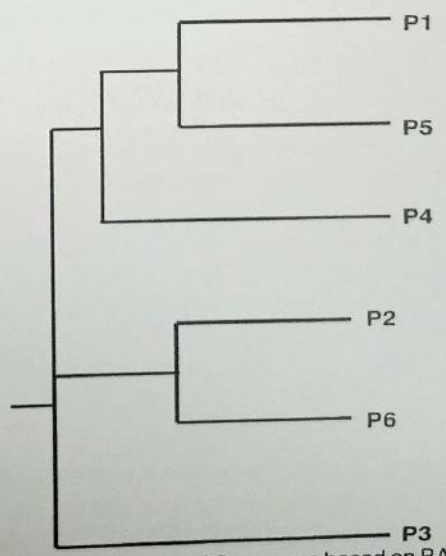

Fig. 5. UPGMA dendrogram of *C. nervosa* based on RAPD analysis

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