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Molecular Characterization of *Dendrobium* Orchid Species from Western Ghat Region of Karnataka using RAPD and SSR Markers

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A B S T R A C T

Orchids are the most beautiful flowering, highly evolved ornamental plants and also have medicinal importance. In the present study, genetic diversity among six *Dendrobium* species was analysed using morphological characters and molecular markers including RAPD and SSR markers. Morphological characters analysed were leaf shoot and flower characters of six *Dendrobium* species. The variability in morphology was found among the species. Further, the genetic diversity among the species was determined by using RAPD and SSR markers. Out of 52 RAPD primers, 34 were selected for diversity analysis, producing 222 amplified bands. Of which 216 bands depicted 97.29 per cent polymorphism with Jaccard's similarity coefficient varied from 0.19 to 0.35. The dendrogram was constructed by Unweighted Pair Group Method Using Arithmetic Average (UPGMA), separated six Dendrobium species into two main clusters, one with four species and the other with two species. Out of 11 SSR primers, eight were selected for diversity analysis, producing 12 amplified bands. Of which eight bands depicted 66.66 per cent polymorphism with Jaccard's similarity coefficient varied from 0.37 to 0.60. The dendrogram was constructed by UPGMA, separated six Dendrobium species into two main clusters, one with five species and the other with one species. RAPD markers were more reliable than the SSR markers. Species specific SSR markers are to be developed in future.

Introduction

Orchids are most beautiful flowering and highly evolved plants. They are the most unique plants distributed in wide range of habitats (Kull *et al.,* 2006; Singh *et al.,* 2007). There are about 17,000 wild orchid species in 750 genera reported in the world (Rao 1998). Till the year 2016, 29199 accepted orchid species were reported (Govaerts *et al.,* 2016). The orchids in India are found mostly in North Eastern region, Eastern and Western

Ghats (Bhanwra *et al.*, 2006). Rao (1998) has described 65 species belonging to Kodugu region of Karnataka, an important part of Western Ghats. The genus *Dendrobium* found to be an important genus in the Orchidaceae family with 1190 species as per the Royal Botanic Gardens, Kew. *Dendrobium* plants exhibit distinctive ecological diversification. They can be found in terrestrial, epiphytic, and lithophytic life forms. In addition to ornamental values, orchids like *Dendrobium* have therapeutic effects, viz., significant hepatoprotective activity against CCl4 induced hepatotoxicity (*Dendrobium ovatum)* (Ganpathy *et al.*, 2013), anti-oxidant and anti-glycation (*Dendrobium aqueum)*(Mukharjee *et al.*, 2012), inhibitory activity on phytopathogenic fungi (*Dendrobium herbaceum)* (Akarsh *et al.*,2016), *Dendrobium* substances have been used in traditional medicine in many Asian countries (Bulpitt *et al*., 2007).

Genetic diversity studies and determination of the genetic relationship among *Dendrobium* species are important for the conservation and potential use of plant genetic resources to produce hybrid *Dendrobium* orchids.The development of molecular marker techniques for genetic variation analysis in the past decade has led to advancement analysis of orchid genetic diversity. Molecular markers have been used to study DNA sequence variation in and among orchid species (Wang *et al*., 2009). Related knowledge about more plant species is prerequisite for efficiently managing and exploiting *Dendrobium* genetic resources. In the present study, genetic diversity among six *Dendrobium* species from Western Ghat regions of Karnataka was established using RAPD and SSR markers.

Materials and Methods

Orchid plant samples for present study were collected mainly from Kodugu district, Western Ghats region of Karnataka. *D. aqueum, D. crepidatum, D. herbaceum, D. jerdonianum, D. macrostachyum and D. Ovatum* was the six species collected and established in orchidarium at College of Agriculture, Bengaluru (Table 1). Morphological characters were recorded based on descriptor for *Dendrobium* prescribed by Nation Research Centre on Orchids, Sikkim.

Plant Genomic DNA isolation

Tender leaves were selected for genomic DNA isolation. Leaf samples were harvested and stored at -20˚C. Genomic DNA was isolated from leaves using modified Cetyltrimethyl ammonium bromide (CTAB) method (Khan *et al*., 2007). The isolated DNA was dissolved in 20µL Tris EDTA (TE) buffer. Quantity and quality of genomic DNA were determined by using NanoDrop spectrophotometer and agarose gel electrophoresis respectively.

RAPD analysis

PCR reaction for RAPD primers was carried out in 10µL reaction volume containing 50ng of genomic DNA, 1U *Taq* polymerase (Thermo scientific fisher), 2mM dNTPs, 10x PCR reaction buffer with 1.5mM $MgCl_2$and 5 picomole of RAPD primer. Standardised amplification conditions consisted of Initial denaturation at 94˚C for 5minutes, denaturation at 94˚C for 2 minutes, annealing at 37˚C for 1 minute 30 seconds, extension at 72˚C for 2 minutes and final extension at 72˚C for 8 minutes with 40 cycles.The amplified DNA products were resolved on 1.5 per cent agarose gel, visualized by Ethidium bromide staining and photographed under UV light gel documentation system.

SSR analysis

PCR reaction for SSR primers was carried out in 10µL reaction volume containing 50ng of

genomic DNA, 1U *Taq* polymerase (Thermo scientific fisher), 2mM dNTPs, 10x PCR reaction buffer with 1.5mM $MgCl_2$and 2.5 picomole of each primer (forward and reverse). Standardised amplification conditions consisted of Initial denaturation at 94˚C for 4minutes, denaturation at 94˚C for 2 minutes, annealing from 48˚C to 56˚C (standardized for each primer pair) for 45 seconds, extension at 72˚C for 1minute, 30 seconds and final extension at 72˚C for 8 minutes with 35 cycles.The amplified DNA products were resolved on 3 per cent agarose gel, visualized by Ethidium bromide staining and photographed under UV light gel documentation system.

The bands were scored based on the gel images with the presence of band scored as one '1' and absence as zero '0'. Scored data were entered into binary matrix and subjected to further analysis. Similarity index was calculated using the simple clustering (SM) coefficient (Sneath and Sokal 1973). Similarity matrices were obtained using the subprogram SIMQUAL to generate pair-wise Jaccard's similarity coefficient (NTSYS-pc.Version 2.0). Further, using similarity indices, the clusters were built by Unweighted Pair Group Method Using Arithmetic Average (UPGMA) procedure. A dendrogram was constructed using NTSYS-pc Version 2.0.

Results and Discussion

The morphological study of the six species of *Dendrobium* was undertaken with various descriptive traits and quantitative characters. All the six *Dendrobium* species collected had lanceolate leaf shape with entire leaf margin (Plate 1). All the species studied are epiphytic in nature as listed (Table 2). Similarly, *Dendrobium monileforme* was found to have lanceolate type of leaves (Xiaohua *et al.*, 2009).

Dendrobium aqueum and *Dendrobium crepidatum* were having cane clavate fleshy type of shoots, *Dendrobium herbaceum* and *Dendrobium jerdonianum* were having cane cylindrical type of shoots whereas, *Dendrobium macrostachyum* and *Dendrobium ovatum* were having cane woody type of shoots (Plate2). Similar type of results regarding leaf shape, leaf length and width were reported for *Dendrobium macrostachyum.* Leaves were found to have Linear-lanceolate to ovate-lanceolate shape with 4 to 10 cm of length and 1 to 2.4 cm of breadth (Reddy *et al.,* 2002).

Dendrobium aqueum, Dendrobium crepidatum, Dendrobium macrostachyum and *Dendrobium ovatum* were having acute leaf apex. *Dendrobium herbaceum and Dendrobium jerdonianum* were found to have retuse type of leaf apex (Plate 1).

Shapes of apex of sepals and apex of petals of the species such as *Dendrobium crepidatum, Dendrobium herbaceum, Dendrobium jerdonianum* and *Dendrobium ovatum* were found to be obtuse type. *Dendrobium macrostachyum* was found to have acute type of apex of sepals and petals (Table 3). Four species namely, *Dendrobium crepidatum, Dendrobium herbaceum, Dendrobium ovatum* and *Dendrobium macrostachyum* had uniformly white coloured sepals and petals while, *Dendrobium jerdonianum* had uniformly orange coloured sepals and petals (Plate 3).

Out of 52 RAPD primers, 34 primers were amplified with high resolution for all the six *Dendrobium* species. The six *Dendrobium* species produced a total of 222 amplified bands, out of which 6 were monomorphic bands and 216 were polymorphic bands with an average of 6.35 bands per primer.

Number of bands amplified ranged from 2

(Primer: OPE 12) to 12 (Primer: OPBA 3 and OPA 09) with mean of 6.5 bands per primer (Plate 4). Out of 34 amplified markers Primer OPE 12 showed lowest number of polymorphic bands of one (1) whereas, the OPA 09 showed highest polymorphic bands of twelve (12). The primers were 97.30 per cent polymorphic across the species, with lowest polymorphism of 50 per cent produced by OPE 12 primer and highest polymorphism of 100 % produced by OPA 18, OPM 15, RD 03, RD 04, RD 11, RD 14, OPC 08, OPB 01, OPC 13, OPMA 18, OPB 04, OPA 03, OPC 01, OPE 07, OPA 15, OPA 10, OPA 02, OPA 12, OPC 11, OPA 09, OPB 12, OPA 19, OPA 08, OPA 11, OPA 04, OPA 20, OPB 08 and OPF 9 primers (Table 4).

The Jaccard's genetic similarity coefficient ranged from 0.19 to 0.35 (Table 1). The *Dendrobium aqueum* showed lowest similarity index (0.19) with *Dendrobium herbaceum* and the highest similarity index (0.35) was found between Dendrobium *macrostachyum* and *Dendrobium ovatum.* The Polymorphic Information Content (PIC) value, a reflection of allele diversity and frequency among the species, were uniformly high for all the RAPD loci tested. The PIC of studied species ranged from 0.23 for OPE 12 primer to 0.36 for OPA 10 primer with an average PIC of 0.29 (Table 5).

The dendrogram constructed separated six species into two main clusters, one with 4 species (*Dendrobium aqueum, Dendrobium crepidatum, Dendrobium macrostachyum* and *Dendrobium ovatum)* and other with two species *Dendrobium herbaveum* and *Dendrobium jerdonianum* (Fig. 1). Clusters were formed were mainly divided based on descriptive characters of the species which were measured. The four species (*Dendrobium aqueum, Dendrobium crepidatum, Dendrobium macrostachyum* and *Dendrobium ovatum)* in cluster I have acute type of leaf apex, remaining two species (*Dendrobium herbaceum* and *Dendrobium jerdonianum)* in Cluster II have retuse type of leaf apex and shortleaves. The species present in sub cluster I of first main cluster have same shoot character *viz.,* Cane clavate (fleshy) and medium leaf length. The species present in sub cluster II of first main cluster have cane woody type of shoot nature.

Similarly, RAPD primers were used to establish phylogenetic relationship between nine *Dendrobium species.* Nine *Dendrobium* species were grouped into four main clusters using dendrogram (Zha *et al.,* 2009). Ten RAPD primers were used by Niknejad *et al.* (2009) to analyse the genetic relationship between 20 *Phalaenopsis* species. They could group twenty *Phalaenopsis* species into three major groups based on similarity index between species. RAPD primers were used for cluster analysis in *Vanda* species by Lim *et al.* (1999) and in *Dendrobium* species by Khosravi *et al.* (2008).

Out of 11 SSR primer pairs, 7 primer pairs were amplified with high resolution. The six *Dendrobium* species produced a total of 12 amplified bands, out of which 4 were monomorphic bands and 8 were polymorphic bands with an average of 1.5 bands per primer.

Number of bands amplified ranged from one (OA 25, OA 12, OA 08 and OA) to two (DO 12, DO 03, OA 07 and OA 23) with mean of 1.5 bands per primer (Table 6). The primers were 66.66 per cent polymorphic across the species, with lowest polymorphism of 0 per cent produced by DO 03 primer and highest polymorphism of 100 per cent produced by OA 25, OA 12, OA 08, OA 07 and OA primers (Plate 5).

The Jaccard's genetic similarity coefficient ranged from 0.37 to 0.60 (Table 7). The *Dendrobium crepidatum* showed lowest

similarity index (0.37) with *Dendrobium herbacium* and *Dendrobium aqueum.* Highest similarity index (0.60) was found between Dendrobium *aqueum* and *Dendrobium herbaceum, Dendrobium macrostachyum, Dendrobium ovatum.* 0.60 similarity index was also found between *Dendrobium herbaceum* and *Dendrobium macrostachyum, Dendrobium ovatum.*

Table.1 Orchid species collected and conserved in College of Agriculture, Bengaluru

Sl.No.	Species	Location	Coordinates
1	*D. aqueum*	Ponnampet	12°08'41.8"N 75°56'17.4"E
2	*D. crepidatum*	Halligattu	12º7'47.6"N 75º55'18.4"E
3	*D. herbaceum*	B Shettigeri	12°08'22.7"N 75°55'18.7"E
4	*D. jerdonianum*	Kumaralli	12°39'39.8"N 75°42'17.2"E
5	*D. macrostachyum*	Halligattu	12°07'47.5"N 75°55'18.3"E
6	*D. ovatum*	Kanur	12°04'41.9"N 76°02'48.6"E

Table.2 Descriptive traits (vegetative plant parts) recorded for the six *Dendrobium* species

Sl.No.	Species name	Nature of shoot	Shape of leaf	Apex of leaf	Leaf margin
1	*D. aqueum*	Cane clavate fleshy	Lanceolate	Acute	Entire
2	*D. crepidatum*	Cane clavate fleshy	Lanceolate	Acute	Entire
3	*D. herbaceum*	Cane cylindric(fleshy)	Lanceolate	Retuse	Entire
4	*D. jerdonianum*	Cane cylindric(fleshy)	Lanceolate	Retuse	Entire
5	*D. macrostachyum*	Cane woody	Lanceolate	Acute	Entire
6	*D. ovatum*	Cane woody	Lanceolate	Acute	Entire

Table.3 Sepal and petal morphological characters of flowers of *Dendrobium* species

Species	Apex of sepal	Shape of sepal	Sepal dominant colour	Apex of petal	Shape of petal	Petal dominant colour
D. crepidatum	Obtuse	Oblong	White	Obtuse	Oblong	White
D. herbaceum	Obtuse	Linear	White	Obtuse	Linear	White
D. jerdonianum	Obtuse	Linear	Orange	Obtuse	Linear	Orange
D. macrostachyum	Acute	Oblong	White	Acute	Oblong	White
D. ovatum	Obtuse	Oblong	White	Obtuse	Oblong	White

Table.4 Description of the RAPD products obtained from six *Dendrobium* species

Sl. No	Primer name	No. of bands	No. of polymorphic bands	Polymorphism (%)	PIC
1.	OPA 05	7	6	85.71	0.28
2.	OPA 18	7	7	100	0.29
3.	OPBA 03	12	10	83.33	0.27
4.	OPE 12	2	1	50	0.23
5.	OPM 15	9	9	100	0.30
6.	RD-03	10	10	100	0.30
7.	RD-04	7	7	100	0.30
8.	RD-05	11	9	81.81	0.26
9.	RD-11	7	7	100	0.32
10.	RD-14	6	6	100	0.35
11.	OPC 08	6	6	100	0.31
12.	OPB 01	3	3	100	0.32
13.	OPC 13	10	10	100	0.32
14.	OPAM 18	6	6	100	0.31
15.	OPB 09	6	6	100	0.30
16.	OPA 03	9	9	100	0.30
17.	OPC 02	4	3	75	0.24
18.	OPC 01	3	3	100	0.27
19.	OPE 7	6	6	100	0.30
20.	OPA 15	4	4	100	0.27
21.	OPA 10	3	3	100	0.36
22.	OPA 02	6	6	100	0.32
23.	OPA 12	8	8	100	0.28
24.	OPC 11	5	5	100	0.30
25.	OPA 09	12	12	100	0.30
26.	OPB 12	6	6	100	0.29
27.	OPA 07	6	5	83.33	0.21
28.	OPA 19	5	5	100	0.28
29.	OPA 08	4	4	100	0.35
30.	OPA 11	5	5	100	0.34
31.	OPA 04	10	10	100	0.31
32.	OPA 20	6	6	100	0.29
33.	OPB 08	4	4	100	0.35
34.	OPF 9	7	7	100	0.30
	Mean	**6.53**	**6.35**	**95.85**	**0.29**

Table.5 Similarity indices of six *Dendrobium* species using RAPD markers

	Dendrobium aqueum	***Dendrobium crepidatum***	***Dendrobium herbaceum***	***Dendrobium jerdonianum***	***Dendrobium macrostachyum***	***Dendrobium ovatum***
Dendrobium aqueum	1.00					
Dendrobium crepidatum	0.32	1.00				
Dendrobium herbaceum	0.19	0.22	1.00			
Dendrobium jerdonianum	0.29	0.25	0.34	1.00		
Dendrobium macrostachyum	0.27	0.24	0.22	0.28	1.00	
Dendrobium ovatum	0.26	0.26	0.24	0.24	0.35	1.00

Table.6 Description of the SSR products obtained from six *Dendrobium* species

Sl. No.	**Primer name**	**No. of bands**	**No. of polymorphic bands**	**Polymorphism (%)**	**PIC**
1	DO 12	2	1	50	0.11
2	OA 25	1	1	100	0.34
3	OA 12	1	1	100	0.34
4	DO 03	2	0	0	0.00
5	OA 08	1	1	100	0.24
6	OA 07	2	2	100	0.24
7	OA	1	1	100	0.24
8	0A 23	2	1	50	0.17
	MEAN	**1.5**	**1**	**75**	**0.21**

Table.7 Similarity indices of six *Dendrobium* species using SSR markers

	Dendrobium aqueum	***Dendrobium crepidatum***	***Dendrobium herbaceum***	***Dendrobium jerdonianum***	***Dendrobium macrostachyum***	***Dendrobium ovatum***
Dendrobium aqueum	1.00					
Dendrobium crepidatum	0.37	1.00				
Dendrobium herbaceum	0.60	0.37	1.00			
Dendrobium jerdonianum	0.43	0.44	0.42	1.00		
Dendrobium macrostachyum	0.60	0.57	0.60	0.42	1.00	
Dendrobium ovatum	0.60	0.57	0.60	0.42	0.60	1.00

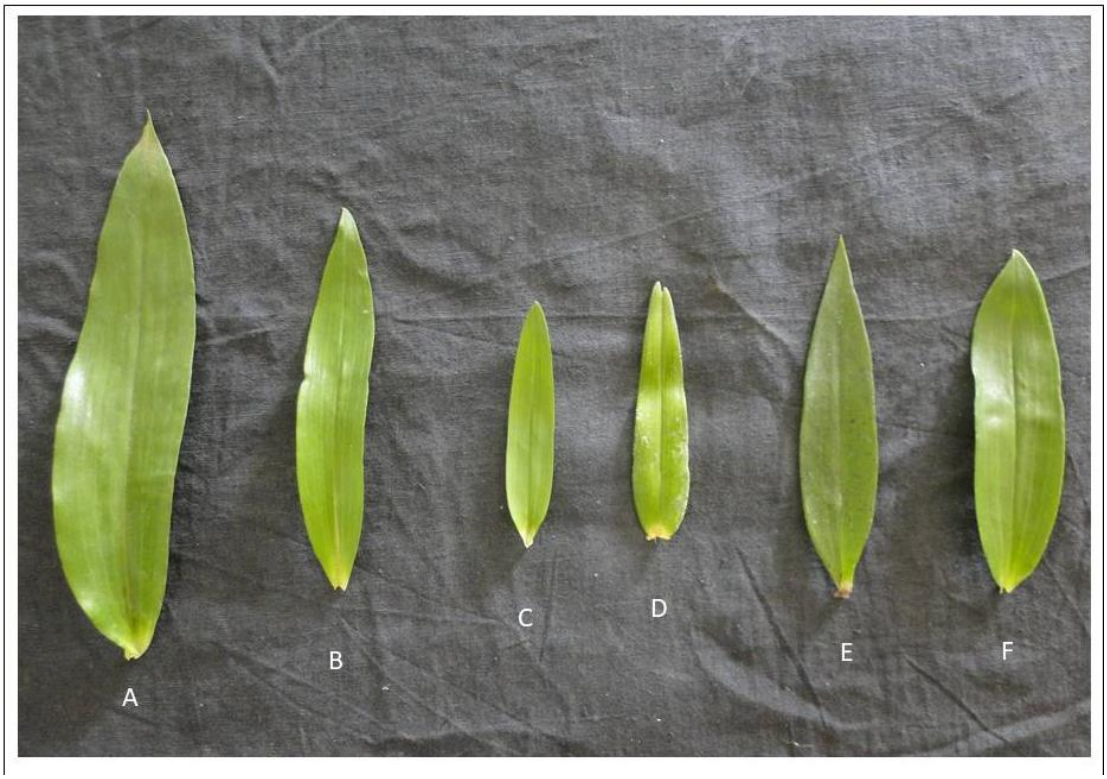

Plate.1 Shape of leaf apex of six *Dendrobium* species

A: ***Dendrobium aqueum*****; B:** ***Dendrobium macrostachyum*****; C:** ***Dendrobium ovatum;*** **D:** ***Dendrobium herbceum*****; E:** ***Dendrobium jerdonianum*****; F:** ***Dendrobium crepidatum.***

Plate.2 Nature of shoots of six *Dendrobium* species
A: *Dendrobium aqueum*; B: *Dendrobium crepidatum*; C: *Dendrobium herbaceum*; D: *Dendrobium jerdonianum*; E: *Dendrobium macrostachyum*; F: *Dendrobium ovatum.*

Plate.3 Flower morphology of *Dendrobium* species
A: *Dendrobium crepidatum;* B: *Dendrobium herbaceum*; C: *Dendrobium jerdonianum*; D: *Dendrobium macrostachyum;* E: *Dendrobium ovatum*

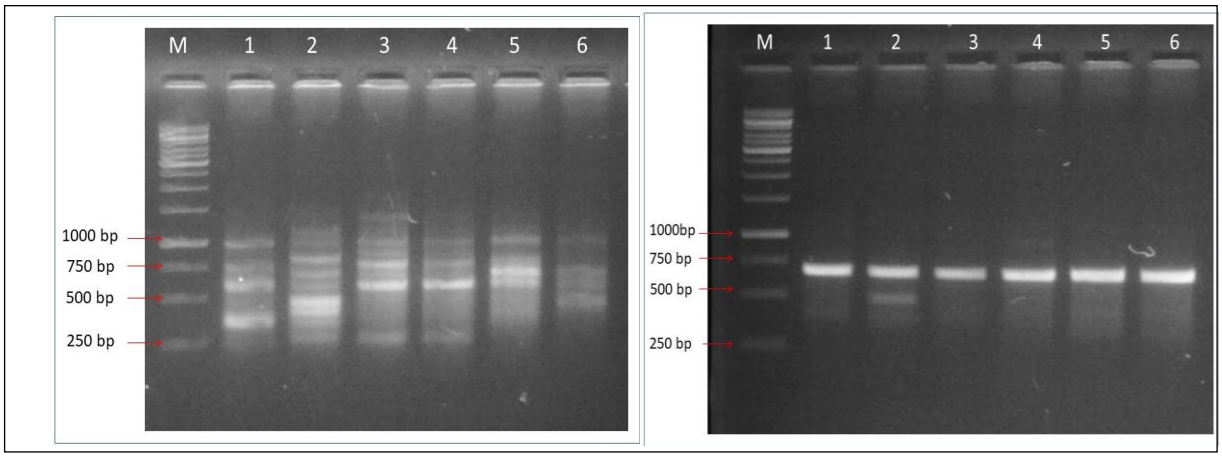

Plate.4 RAPD banding pattern of six Dendrobium species obtained with OPBA 3 and OPE 12. M: ladder (1kb); Lane 1: *D. Aqueum*; Lane 2: *D. Crepidatum*; Lane 3: *D. herbaceum*; Lane 4: *D. Jerdonianum*; Lane 5: *D. Macrostachyum*; Lane 6: *D. ovatum*

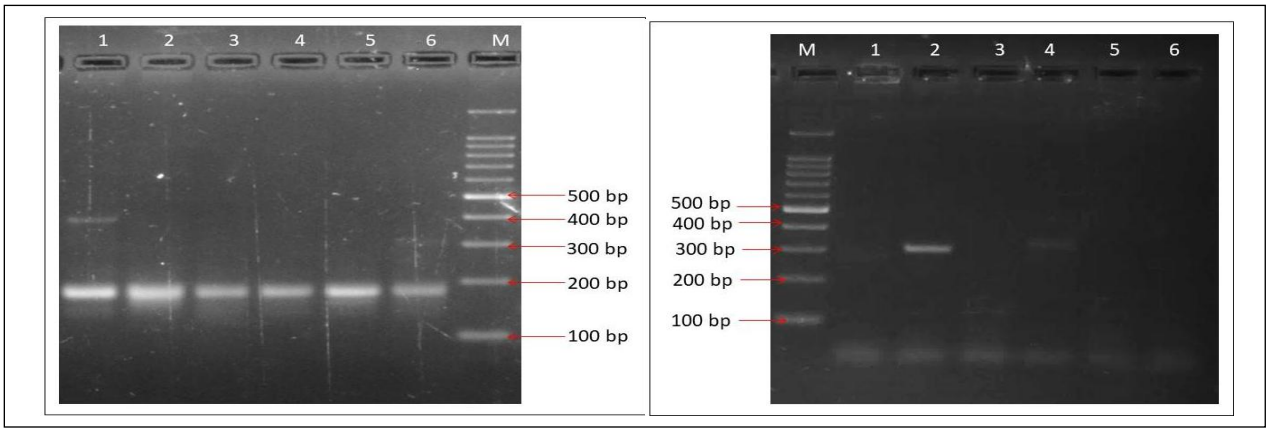

Plate.5 SSR banding pattern of six Dendrobium species obtained with DO 12 and OA 25. M:Ladder(1kb); Lane 1: *D. Aqueum*; Lane 2: *D. Crepidatum;* Lane 3: *D .herbaceum*; Lane 4: *D. Jerdonianum*; Lane 5: *D. Macrostachyum*; Lane 6: *D. ovatum*

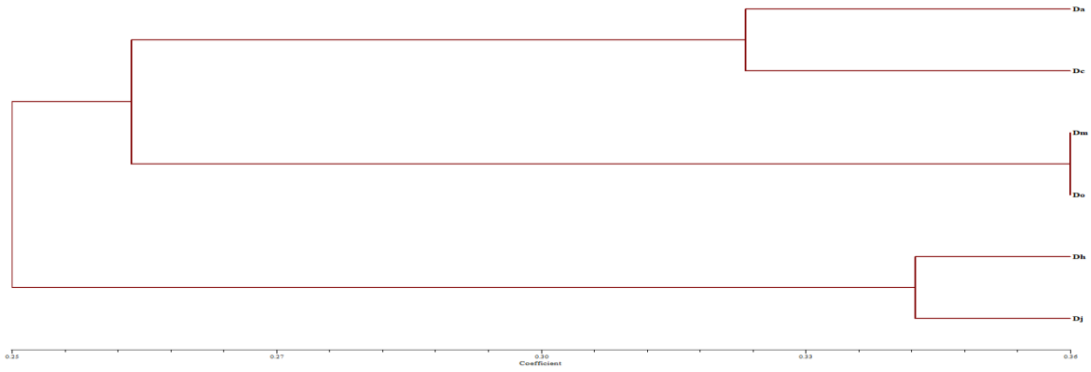

Fig.1 UPGMA dendrogram representing relationship among six *Dendrobium* species using RAPD markers. Da: *Dendrobium aqueum*; Dc: *Dendrobium crepidatum*; Dh: *Dendrobium herbaceum*; Dj: *Dendrobium jerdonianum* and Dm: *Dendrobium macrostachyum*; Do: *Dendrobium ovatum*

Fig.2 UPGMA dendrogram representing relationship among six *Dendrobium* species using SSR markers. Da: *Dendrobium aqueum*; Dc: *Dendrobium crepidatum*; Dh: *Dendrobium* herbaceum; *Dj*: *Dendrobium jerdonianum* and Dm: *Dendrobium macrostachyum*; Do: *Dendrobium ovatum*

The dendrogram constructed separated the six species into two main clusters, one with five species (*Dendrobium aqueum, Dendrobium crepidatum, Dendrobium macrostachyum, Dendrobium herbaceum* and *Dendrobium ovatum)* and other with one species (*Dendrobium jerdonianum*) (Fig. 2).

Clusters were formed based on descriptive and quantitative characters. The five species in cluster I have whitish coloured flowers; *Dendrobium jerdonianum* in Cluster II has orange coloured flowers. Similarly, SSR markers were used to determine the genetic variability among 12 *Dendrobium* Species and species specific SSR markers were identified by Liu *et al.* (2014). The individuals of *Dendrobium officinale* collected from different places were differentiated by using 13 SSR markers (Lu *et al.,* 2012).

The phylogenetic relationship among the species obtained using RAPD markers was found to be more relevant, because the species which were grouped into same main and sub-clusters were found to have similar morphologically descriptive characters. The phylogenetic relationship among the species obtained using SSR markers was able to differentiate the species based on the flower colour morphology.

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