Genetic Characterization of Selected Medicinal Dendrobium (Orchidaceae) Species Using Molecular Markers

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Abstract

Molecular characterization of orchids is imperative for the prevention of elite germplasm erosion in breeding programs mainly during development of synthetic hybrids. The Indian Dendrobium species comprise the third largest genus in the family Orchidaceae and have their remarkable value in horticultural, agricultural and medicinal realms. The present study is focused on the latter characterizing five selected medicinal Dendrobium species from North-eastern India, using Random Amplified Length Polymorphism (RAPD) as well as Simple Sequence Repeat (SSR) markers. The RAPD genotypes were determined using the Polymerase Chain Reaction (PCR) followed by electrophoresis in agarose gel and the genetic relationship among studied species was estimated using Nei’s genetic distance. Five successful random primer-pairs generated a total of 124 RAPD fragments containing 25 species-specific fragments. On an average each primer-pair produced 28 amplified DNA fragments and the amplicon size varied between 250 to 800 base pairs (bp). Using multiple nucleotide repeat loci, 30 individuals from different species were studied using as biological replicates. The size of amplified DNA varied between 152-230 bp. A total of 27 highly reproducible DNA fragments showed polymorphism with average frequency of 5.4 allele per locus. The number of SSR alleles detected per locus ranged from 4 to 7. The highest number of 7 alleles were scored with DO-03 primer and observed to be the most polymorphic marker (100%). The expected heterozygosity varied between 0.0788 (with primer set DO-12) to 0.7209 (with primer set DO-07) with an average expected heterozygosity of 2.805. The cluster analysis based on both type of molecular markers grouped all species into two different clusters, however, genetic relationships at inter-specific level comparable to their morphological sections could be established with SSR marker. Thus the preliminary study using UPGMA method showed that SSR markers are superior in the detection of polymorphism among Dendrobium species segregating them into their respective pre-defined morphological sections, than RAPD markers. Further studies of other species using more microsatellite loci with larger sample sizes can reveal the intricate genetic relationships of Indian orchids.

Keywords: Dendrobium, Phylogeny, RAPD, SSR, Molecular Markers

1. Introduction

Orchids belong to the largest and most diverse group among the angiosperms. The genus Dendrobium is the third largest in the family Orchidaceae comprising of about 1184 species worldwide (Leitch et al, 2009). Till date, more than 103 species of the genus Dendrobium have been reported from India mainly from North-East region (Singh et al, 2001) (Figure 1). The Dendrobium species are broadly categorised into horticultural, agricultural, medicinal or dual purpose species depending upon their utility per se, however, the latter got limited exploration so far. The medicinally important species have specific qualities of tonic, disease resistance and anti-depressant.
For example, the species *Dendrobium macraei* is important for ayurvedic treatment as being source of *Jivanti* that contains an alkaloid ‘Jebantine’ and used as tonic (Khasin & Rao, 1999).

From *Dendrobium moschatum*, two phenanthrenes named Rotundatin and Moscatin have been reported to be useful in inhibition of the aggregation of platelets induced by arachidonic acid and collagen. Similarly, two bibenzyl (Gigantol and Moscatilin) and one alkaloid (Dendrobinae) have been reported as leading compound in *Dendrobium nobile* (Miyazawa et al, 1997). Both compounds showed strong antimutagenic potential and were found to be anti carcinogenic against lung carcinoma, ovary adenocarcinoma and promyelocytic leukemia (Lee et al, 1995). Of the different medicinal *Dendrobium* species reported from Chinese Pharmacopoeia, (2000), namely *D. aphyllum, D. bellatulum, D. densiflorum, D. fimbriatum* and *D. nobile* are the native species to India (Singh et al, 2001). These species are adapted to varied climates in North-East India and could be elite germplasm for current breeding programs. However, because of extensive demand of commercial hybrids of *Dendrobium* having the least concern on their medicinal aspects, a number of these species are now being outbred. As a result, some of the native medicinal species are at the verge of extinction. Hence, there is an urgent need to conserve these species and breeds. The species characterization is the primary step towards the germplasm conservation programme. The systematics of the genus *Dendrobium* has been extensively studied on the basis of morphological key characters (Dressler, 1993). However, the accuracy of phenotypic characterization is often affected by the influence of the environment and underlying genetic complexity. Hence, it becomes tremendously difficult to distinguish closely related species due to limited information available on chemical components in *Dendrobium* (Wood, 2006).

A number of investigations have shown genetic polymorphism in many plant taxa by the use of various fingerprinting techniques including the analysis of isozymes and RAPD, RFLP, AFLP, SSRs markers (Xu et al, 2001 and Begum et al, 2009). RAPD markers have been identified for revealing high levels of polymorphism in many medicinal plants due to their low investment and requirement of minute amounts of starting plant materials, though with certain limitations. Instead, microsatellite markers, by virtue of their codominant and multiallelic nature prove to be efficient in genetic diversity studies as compared to RAPD (Nagaraju et al, 2001). Thus microsatellites have become markers of choice in characterization of orchid species. In spite of the evolutionary significance of the Indian *Dendrobium* species, the available data on characterization of these species using reproducible and reliable markers is scanty. Previously, 12 set of SSR markers have been proposed in *Dendrobium officinale* based on highly reproducible microsatellites with di-nucleotide repeats (Gu et al, 2007). In the present study the genetic relationship among five medicinally important species of *Dendrobium* from North-East India has been established by combination of RAPD and SSR markers.

![Figure 1. Geographical regions of North-East India Showing the Site of Collection of *Dendrobium* Species i.e. Kalimpong and Shillong](image)

**2. Materials and Methods**

**2.1. Collection of Plant Material**

For the present study, biological samples from five different species of the genus *Dendrobium* were collected from different parts of the Northern-East India (Figure 1). These species are *D. bellatulum, D. densiflorum, D. fimbriatum, D. nobile* and *D. aphyllum*, which belong to different morphological sections as defined earlier (Table 1, Figure 2). All authenticated plant materials were collected either under the supervision of Dr. U.C. Pradhan, an eminent Orchidologist from Kalimpong, West Bengal, India; or from Botanical Survey of India, Shillong. All collected individual plants were maintained in the orchidarium in replicates of two to six individuals per species as per the availability.

**2.2. DNA Extraction from Leaf Tissues**

Young leaves were harvested from different species in at least three biological replicates and stored at -20 °C for DNA isolation. The genomic DNA was extracted using the standard molecular biology protocol (Bhattacharyya & Mandal, 1999). The isolated DNA was dissolved in 50 µl
TE (Tris EDTA) buffer. The quality and quantity of extracted DNA samples were assessed by electrophoresis on agarose gel (1.2%) and by UV spectrophotometric analysis, respectively. The concentration of extracted DNA was adjusted to 10-20 ng/µl and samples were stored at -20 °C.

3.3. The RAPD Markers

A total of 16 primers (Bangalore Genei, India) were taken initially and utilized for the amplification from all studied species. Five RAPD markers (RD-03, RD-04, RD-05, RD-11 and RD-14) were analysed by PCR using GeneAMP PCR System 9700 (Applied Biosystems, USA). The sequences of these primers used are given in Table 2.

3.4. PCR Amplification and Electrophoresis of RAPD Markers

25 µl of PCR reaction was comprised of 6 µl (60 ng) template DNA, 2.5 µl 10X PCR buffer, 4 µl 2.5 mM dNTPs, 4 µl 1U Taq DNA polymerase and 10.5 µl double distilled sterile water. Reaction condition was programmed as initial hold at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing of primer at 36 °C for 45 seconds, extension at 72 °C for 1 minute and 30 seconds and the final hold at 4 °C for 5 minutes. The amplified DNA products were resolved on 1.2% agarose gel, visualized by ethidium bromide staining, and photographed under UV light. A DNA marker (λDNA/EcoRI + HindIII) was used to estimate DNA fragments generated by RAPD.

### Table 1. Source of Five Medicinally Important Dendrobium Species from North-East India

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Common name</th>
<th>Section</th>
<th>Locality</th>
<th>Collected from</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>D. bellatulum</em></td>
<td>Formosae</td>
<td>Kalimpong, W.B.</td>
<td>U C Pradhan</td>
</tr>
<tr>
<td>2.</td>
<td><em>D. densiflorum</em></td>
<td>Densiflora</td>
<td>Shilong, Meghaloya</td>
<td>Botanical Survey of India</td>
</tr>
<tr>
<td>3.</td>
<td><em>D. fimbriatum</em></td>
<td>Holochrysa</td>
<td>Shilong, Meghaloya</td>
<td>Botanical Survey of India</td>
</tr>
<tr>
<td>4.</td>
<td><em>D. nobile</em></td>
<td>Dendrobium</td>
<td>Kalimpong, W.B.</td>
<td>U C Pradhan</td>
</tr>
<tr>
<td>5.</td>
<td><em>D. aphyllum</em></td>
<td>Dendrobium</td>
<td>Kalimpong, W.B.</td>
<td>U C Pradhan</td>
</tr>
</tbody>
</table>

### Figure 2. Five Representative Medicinal Dendrobium Species Used in this Study: (a) *D. bellatulum*; (b) *D. densiflorum* (c) *D. fimbriatum* (d) *D. nobile* and (e) *D. aphyllum*

### Table 2. The Sequences Oligonucleotide Primers used for RAPD Analysis and Banding Patterns Obtained from the Five Species of Dendrobium

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>T</th>
<th>V</th>
<th>P (%)</th>
<th>Distinctive Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD-03</td>
<td>GGAGGAGGAG</td>
<td>26</td>
<td>25</td>
<td>096.15</td>
<td>A 06</td>
</tr>
<tr>
<td>RD-04</td>
<td>CCGCCTAGTC</td>
<td>23</td>
<td>23</td>
<td>100.00</td>
<td>05</td>
</tr>
<tr>
<td>RD-05</td>
<td>AAGGGCGCAC</td>
<td>25</td>
<td>25</td>
<td>100.00</td>
<td>04</td>
</tr>
<tr>
<td>RD-11</td>
<td>AGAGGGCAC</td>
<td>29</td>
<td>29</td>
<td>100.00</td>
<td>09</td>
</tr>
<tr>
<td>RD-14</td>
<td>GGTGGGTGATG</td>
<td>21</td>
<td>20</td>
<td>095.23</td>
<td>04</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>124</td>
<td>122</td>
<td>098.39</td>
<td>28</td>
</tr>
</tbody>
</table>

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3.5. The SSR markers

SSR markers were selected from the list of SSR markers developed earlier by Gu et al (2007). Of the 12 sets of developed markers, 5 highly polymorphic markers were considered suitable for this analysis.

3.6. Genotyping with SSR Markers

Dilute DNA samples were subjected to PCR amplification using selected SSR markers in GeneAmp PCR System 9700 (Applied Biosystems, USA). The PCR mix was prepared in 25 µL buffer II (Applied Biosystems) using 200 µM of each dNTP, 0.2 µM of each primer (forward and reverse), 5 ng genomic DNA and 0.5 U of Taq Gold (Applied Biosystems). Reaction condition was programmed as initial hold at 94 °C for 5 minutes, and then 10 cycles for denaturation at 94 °C for 20 seconds, annealing of primer at 45.5 °C for 45 seconds, extension at 72 °C for 1 minute and 30 seconds followed by final hold at 4 °C for 5 minutes. The amplified DNA products were resolved on 1.2% agarose gel, visualized by ethidium bromide staining and photographed under UV light. A 25-bp DNA ladder (Promega) was used to estimate alleles.

3.7. Data Analysis

All the genotypes were scored for the presence and absence of the amplified products from both types of molecular markers. The data were entered into a binary matrix as desired variables; 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. The genetic relationship among the species was estimated using the Euclidean distance matrix. The similarity matrix was processed using XLSTAT 7.5.2 (http://www.xlstat.com). The RAPD and SSR data were also evaluated by Principal Coordinate Analysis (PCA) as an average distance, using XLSTAT 7.5.2. Genetic distance estimates based on pair-wise comparisons served as elements in the proximity matrix in a cluster analysis by UPGMA and plotted using the XLSTAT 7.5.2. The resulting clusters were represented in the form of a dendrogram.

4. Results

4.1. Analysis of Genetic Variability using RAPD Markers

Five medicinally important species of Dendrobium were procured from North-Eastern India and authenticated with scientific experts and established in the orchidarium for material collection. The leaf samples from each species were used in replicates for DNA isolations having similar morphology. The RAPD markers were used to assess the genetic variability among five species of Dendrobium i.e. D. bellatulum, D. densiflorum, D. fimbriatum, D. nobile and D. aphyllum which belong to different morphological sections and genetic relationships were established (Table 1) (Wood, 2006). To identify the RAPD markers with confidence, the individual PCR reactions were repeated three times for each of the five sample species with atleast three biological replicates every time resulting into the highly reproducible DNA fragments. Five RAPD markers (RD-03, RD-04, RD-05, RD-11 and RD-14) could be identified distinguishing the studied material. The size of amplified DNA fragments produced by these primers varied between 250 to 800 bp. The random primers generated a total of 124 RAPD fragments containing 25 species-specific fragments and 122 variable bands from the five species (Table 2). Each primer produced approximately 21 to 29 fragments in any particular reaction with the average number of 28 fragments. Among all DNA amplified fragments, certain polymorphic fragments could be generated using all five primers in all studied species. The number of such polymorphic fragments varied as 6 for D. bellatulum, 5 for D. densiflorum, 5 for D. fimbriatum, 2 for D. nobile and 7 for D. aphyllum. These polymorphic RAPD markers had high diagnostic value since they were highly distinctive for each species and absent from other species, with no exceptions. Representative fingerprints generated using RD-03 and RD-11 primers are shown in Figure 3.

To establish the genetic relationship among studied species based on successful RAPD markers, a dendrogram was constructed according to UPGMA cluster analysis using the Neighbour-Joining method (NJ) and Euclidean distance matrix (Figure 4). Such moderate to high genetic similarity was well reflected on the dendrogram also, where the studied species could be grouped into two distinct clusters. In the first cluster D. bellatulum and D. densiflorum were grouped with D. fimbriatum, and in second cluster D. nobile and D. aphyllum were grouped together (Figure 4). In parallel, two distinct clusters were observed following UPGMA method analysis, with three species in cluster I and two species in cluster II, respectively.

4.2. Analysis of Genetic Variability Using SSR Markers

In the present study, genetic polymorphism in the five medicinal Dendrobium species was analyzed by using 12 microsatellite markers, which were known to be polymorphic in D. officinale populations (Gu et al, 2007). Five microsatellite markers showed polymorphism with clear and reproducible fragments while others were monomorphic in all studied species (Table 3). Allele sizes were determined using commercial size standards. A total of 27 well repeatable DNA fragments were scored from all tested species, with average frequency of 5.4 allele per locus (Table 3). The highest number of allele (7 alleles) was detected from DO-03 primer with the alleles varying
in size from 152 to 230 bp. The expected heterozygosity ranged from 0.0788 to 0.7209 (mean value = 0.56116) and the observed heterozygosity ranged from 0.0612 to 0.8125 with an average of 0.6202. In all 5 loci, the observed heterozygosities were higher than the expected heterozygosities. The effective numbers of alleles ($n_e$) were observed ranging from 1.0855 to 3.5842 (mean value = 2.75778). Representative fingerprints from DO-02 and DO-09 were shown in Figure 5. In all 5 polymorphic loci, the observed heterozygosities were higher than the expected heterozygosities. No significant ($p<0.05$) departures from the Hardy-Weinberg equilibrium (HWE) expectations were observed. A fingerprinting dataset of 5 SSR loci with 27 alleles were selected for cluster analysis based on SM coefficient of DNA banding pattern similarity. In order to study the correlation at inter-specific level, a dendrogram was constructed to infer phylogenetic relationship among five medicinally important *Dendrobium* species using UPGMA algorithm. These species were grouped into two main clusters with a similarity level of 0.637 (Figure 6). Cluster I consisted of only *D. bellatulum* (from section Formosae) and got diverged from other species concluding that this species is more diversified. Cluster II was comparatively more complex having sub-clusters where *D. nobile* and *D. aphyllum* (section Dendrobium) grouped

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence (5’-3’)</th>
<th>$T_a$ (°C)</th>
<th>Repeat Motif</th>
<th>Size (bp)</th>
<th>$N_a$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$n_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO-02</td>
<td>F:CTCCACGCATGAACATTAG R:TTTGACCATACTGTGGGCT</td>
<td>47</td>
<td>(GA)$_{11}$</td>
<td>203-224</td>
<td>5</td>
<td>0.7045</td>
<td>0.7030</td>
<td>3.3664</td>
</tr>
<tr>
<td>DO-03</td>
<td>F:GCCCGACTACATCCAAAC R:GGTGTTGCTTCCGATCTAA</td>
<td>50</td>
<td>(GA)$_{10}$</td>
<td>219-230</td>
<td>7</td>
<td>0.7143</td>
<td>0.6680</td>
<td>3.0120</td>
</tr>
<tr>
<td>DO-07</td>
<td>F:AGGGCTTTCTTGGGTTCG R:TCGCTGCTGTTGGAAGTTG</td>
<td>54</td>
<td>(CT)$_{28}$</td>
<td>190-200</td>
<td>6</td>
<td>0.8085</td>
<td>0.7209</td>
<td>3.5842</td>
</tr>
<tr>
<td>DO-09</td>
<td>F:GGGAAGGTGGGTGCATGTC R:GTCAGCGGCACGTGCACAA</td>
<td>55</td>
<td>(TG)$_1$(A(GT)$_1$(T(TG)$_5$ T(TG)$_2$T(TG)$_8$</td>
<td>171-183</td>
<td>4</td>
<td>0.8125</td>
<td>0.6351</td>
<td>2.7408</td>
</tr>
<tr>
<td>DO-12</td>
<td>F:CGGATGATGTGACAAAAA R:GCTCAAGATGGGTAGACT</td>
<td>51</td>
<td>(GT)$_7$</td>
<td>152-172</td>
<td>5</td>
<td>0.00612</td>
<td>0.0788</td>
<td>1.0855</td>
</tr>
<tr>
<td>Total Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27</td>
<td>5.4</td>
<td>3.04592</td>
<td>3.04592</td>
</tr>
</tbody>
</table>

$T_a$: Annealing Temperature; $H_E$: Expected Heterozygosity; $H_O$: Observed Heterozygosity; $n_e$: Effective Number Of Allele (Locus Name and Primer Sequences are from Gu et al, 2007)

Figure 3. RAPD Fingerprint of the Five *Dendrobium* Species Obtained with RD-03 and RD-11. Lane Mr: Marker; Lane A: *D. bellatulum*; Lane B: *D. densiflorum*; Lane C: *D. fimbriatum*; Lane D: *D. nobile*; and Lane E: *D. aphyllum*

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with *D. densiflorum* (section Densiflora) sharing a common root of origin, with more diverged species *D. fimbriatum* (section Holochrysa) (Figure 6). In the dendrogram based on Nei’s genetic distance obtained (Figure 6), the populations were highly differentiated by their own genetic distances. The genetic diversity at interspecific level was expressed in terms of average heterozygosity.

![Figure 4. Dendrogram Constructed According to UPGMA Cluster Analysis, Based on the Euclidean Distance from RAPD Profile, Showing the Genetic Relationships Among the Five Species of *Dendrobium*](image)

5. Discussion

DNA-based molecular markers could be utilized for the comparison of genetic architecture of different plant species even when morphological descriptions are most similar. Among all, the PCR-amplification based markers have been proved to be advantageous even in the absence of any prior genetic/sequence information (Levinson & Gutmen, 1987 and Williams et al, 1990). RAPD markers are dominant markers and used for the simplicity of the technique. On the other hand, SSR markers are co-dominant in nature and have been used for better reproducibility. In our view, simultaneous use of dominant and co-dominant markers may result into better comprehensiveness of the diversity data. Therefore, in the present study, the RAPD and SSR markers were employed to estimate genetic relatedness and diversity among five medicinally important species of *Dendrobium*. We mainly focused on five selected medicinal species from all other species of North-East India, on the basis of their extensive history for use in flock medicine by different ethnic communities. Among the studied species, high proportion of genetically diverse loci suggested the significant genomic heterogeneity available naturally. This would indeed exclude any possibility of unequivocal identification of medicinal plants species required for the assurance of quality, efficacy, and safety in the herbal medicine industry (Lau et al, 2001; Zhang et al, 2005 and Sucher & Carles, 2008). Also, the characterization of the genetic diversity and examination of the genetic relationship among *Dendrobium* species are important for sustainable conservation and increased use of plant genetic resources.

The present work indicates that the RAPD markers may be considered as an effective and convenient method to differentiate *Dendrobium* species. Out of 16 RAPD
primers, only those 5 primers were selected which exhibited polymorphism in three independent experiments among three replicates of all studied species. The genetic variation and dendrogram constructed based on RAPD data also demonstrated the genetic relationships among five species of *Dendrobium*. Earlier, genetic characterization of nine other medicinal *Dendrobium* species using RAPD has been shown where 16 random primers had generated a total of 340 RAPD fragments containing 17 species-specific bands (Zha et al, 2009). Therefore, the RAPD approach for genetic characterization is highly sensitive and reproducible, and even scarce amount of DNA template are sufficient for analysis. Currently, other medicinally important plants belonging to Orchidaceae are being defined using RAPD in contemporary studies (Salim et al, 2010 and El-Domyati et al, 2011).

The SSR technique is particularly powerful to reveal the genetic diversity of the medicinally important species of the genus *Dendrobium* because it requires little information available for genetic characterization of species genomes. Earlier, Gu et al (2007) have identified and characterized 12 SSR markers in *D. officinale* revealing high degree of polymorphism. Since the development of SSR markers is very time-consuming and high cost process, for the present study, these 12 SSR markers were tested in five other *Dendrobium* species for their authentication, identification and to explore their genetic relation. However, in result study, only 5 markers were observed to be polymorphic with observed heterozygosities higher than the expected heterozygosities. This may be due to small samples size and presence of null alleles. This data may probably reflect the consequences of overexploitation and habitat fragmentation on the species’ population genetic structure. Therefore, the SSR-targeted genotyping is particularly robust technique for the identification of germplasm and evaluation of genetic diversity and phylogeny among plant species and populations of the same species (Jarne & Lagoda, 1996; Boonsrangsom et al, 2008 and Fan et al, 2009). While deducing phylogeny it was also observed that SSR markers produce more convincing result in terms of the level of polymorphism than that of RAPD markers, though the reproducibility was comparable with both types of markers. In the dendrogram derived from RAPD fingerprinting data, two clusters were observed, while in SSR, there are also two different clusters but the second cluster is further divided into three different subclusters. Therefore, in dendrogram derived from SSR fingerprinting data, species are distributed in accordance with the presently known sections (Wood, 2006). Moreover, the previous studies from our laboratory have established the evolutionary trends among different *Dendrobium* species based on ITS (Internal Transcribed Spacer), seed traits and pollinia micro-morphology (Chattopadhyay et al, 2010 and Chaudhary et al, 2012). The cluster dendograms derived particularly from SSR markers in the present study showed high congruence with ITS based phylogeny in *Dendrobium* validating the present observations on species relatedness (Chaudhary et al, 2012).

6. Conclusion

The present study highlights the importance of molecular markers mainly RAPD and SSR for the characterization of genetic diversity among medicinally important *Dendrobium* species complex. The reported RAPD and SSR markers are very sensitive and highly reproducible. However, in deducing phylogeny SSR markers are observed to have more confidence level than that of RAPD markers. Moreover, the SSR markers are investigated for the first time to distinguish among medicinally important *Dendrobium* species from North-East India and 5 SSR markers were characterized that to be used for the study of genetic diversity of *Dendrobium* species and showed the consequences of overexploitation and habitat fragmentation on the species’ population genetic structure.

Acknowledgements

This Work Was Financially Supported By Council of Scientific and Industrial Research, Government of India. We Also Thank Dr. A Lokho of Department of Botany, Dr. S.J. Pukhan, Dr. A. Mao, and Dr. T.M. Hennuita of Botanical Survey of India, Dr. U.C. Pradhan, Orchid Research Group, India for their Support in Identification and Collection of Plant Material.
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