The Complete Chloroplast Genome of *Dendrobium nobile*, an endangered medicinal orchid from Northeast India and its comparison with related chloroplast genomes of *Dendrobium* species.

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Abstract

The medicinal orchid genus *Dendrobium* belonging to the Orchidaceae family is the largest genus comprising about 800-1500 species. To better illustrate the species status in the genus *Dendrobium*, a comparative analysis of 33 newly sequenced chloroplast genomes retrieved from NCBI Refseq database was compared with that of the first complete chloroplast genome of *D. nobile* from north-east India based on next-generation sequencing methods (Illumina HiSeq 2500-PE150). Our results provide comparative chloroplast genomic information for taxonomical identification, alignment-free phylogenomic inference and other statistical features of *Dendrobium* plastomes, which can also provide valuable information on their mutational events and sequence divergence.

Keywords: *Dendrobium*, Next generation sequencing, Chloroplast, RNA Editing, Codon usage, SNP

Introduction

Dendrobium is a huge genus of the tribe Dendrobieae (Orchidaceae: Epidendroideae) that was established by Olof Swartz in 1799. The present-day *Dendrobium* is the largest genus with approximately 800-1500 species and occurs in diverse habitats throughout much of Southeast Asia, including China, Japan, India, and the Philippines, Indonesia, New Guinea, Vietnam, Australia and many of the islands in the Pacific. [1].

Many species and cultivars of this genus are well-known floral motifs and have featured in artwork. *Dendrobium* orchids are popular not only for their visual appeal in cut flower market, but also for their herbal medicinal history of about 2000 years in east and south Asian countries [2]. Due to their diverse medicinal values namely for treating kidney and lung ailments, as a potent tonic for treating gastrointestinal problems and strengthening body's immunity, improving sexual potency, anti-cancerous properties, treatment for lumbago and arthralgia etc., many species in this genus have been extensively used as herbal medicines for several hundreds of years. However, many *Dendrobium* species in the wild face an extreme threat of extinction due to their low germination and slow growth rate, habitat decline and over exploitation arising out of anthropogenic activities [3].

Dendrobium orchids have overwhelmed researchers because of their high economic importance in global horticultural trade and in Asian traditional medicine leading to extensive plant systemic studies particularly in species identification, novel marker development, breeding and conservation. In the past two decades, promising advances have been made in areas of molecular taxonomy and systematics and selective breeding of *Dendrobium* species by intensive use of molecular markers. Recently, a variety of molecular markers like microsatellite (SSR), Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers including several other DNA barcode markers from different loci of nuclear and chloroplast (cp) regions have been developed to study *Dendrobium* diversity. However, these species are notoriously difficult to identify [4].

The complete cp genome usually contains a uniparentally inherited DNA, a feature which makes it an obvious choice for plant taxonomical analyses, phylogenomics and phylogeographic inferences at different taxonomic levels. Studies pertaining to plastome genome sequences are useful in investigating the maternal inheritance in plants, especially those with polyploid species, owing to their high gene content and conserved genome structure [5,6 & 7]. The advent of High-throughput sequencing technologies has enabled a rapid increase in the rate of completion of cp genomes with faster and cheaper methods to

sequence organellar genomes [8, 9]. At the time of writing this manuscript, chloroplast genomes from 33 *Dendrobium* species have been reported as per NCBI Organellar genome records (<https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/dendrobium>).

Of the many highly prized medicinal plants in the genus *Dendrobium*, *D. nobile* Lindl. is one such endangered medicinal orchid listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II that demands immediate attention for its protection and propagation. Here, we report the first complete chloroplast genome of *D. nobile* from north-east India based on next-generation sequencing methods (Illumina HiSeq 2500-PE150) and further compare its structure, gene arrangement and microsatellite repeats with related species of 33 other newly sequenced *Dendrobium* chloroplast genomes. Our results provide comparative chloroplast genomic information for taxonomical identification, phylogenomic inference and other statistical features of *Dendrobium* plastomes, which can also provide valuable information on their mutational events and sequence divergence. The availability of complete cp genome sequences of these species in the genus *Dendrobium* will benefit future phylogenetic analyses and aid in germplasm utilization of these plants.

Materials and Methods

Sample collection, DNA extraction and sequencing

Fresh leaves of *D. nobile* were collected from plants growing in greenhouse of National Research Centre for Orchids, Sikkim, India and voucher specimen was deposited with Botanical Survey of India as well as with the Department of Botany, North Eastern Hill University, Shillong. The high molecular weight DNA was extracted using a modified CTAB buffer, and treated according to a standard procedure for next generation sequencing on Illumina HiSeq 2500-PE150. The quality and quantity of the genomic DNA was assessed through agarose gel electrophoresis, Nanodrop and Qubit detection method. The experiments included both paired-end and mate-pair libraries. Tagmentation was carried out with ~4µg of Qubit quantified DNA and the tagmented sample was washed using AMPURE XP beads (Beckman Coulter #A63881) and further exposed to strand displacement. The strand-displaced sample of 2-5kb and 8-13kb gel was size selected and taken for overnight circularization. The linear DNA was digested using DNA Exonuclease. Subsequently the circularized DNA molecules were sheared using Covaris microTUBE, S220 system (Covaris, Inc., Woburn, MA, USA) for obtaining fragments in the range 300 to 1000bp. M280

Streptavidin beads (ThermoFisher Scientific, Waltham, MA) was used to cleanse the sheared DNA fragments with biotinylated junction adapters. Further the bead-DNA complex was subjected to End repair, A-Tailing and Adapter ligations.

Data processing

The data quality assessment for Illumina WGS raw reads was carried out using FastQC tool. Perl scripts were written for adapter clipping and low quality filtering. Cp genomes of *D. officinale*, *D. huoshanense* and *D. strongylanthum* retrieved from NCBI-Refseq database was used as reference for the assembly. BWA-MEM algorithm with default parameter settings was used for aligning the adapter clipped and low quality trimmed processed reads with the *Dendrobium* cp genomes [10]. SPAdes-3.6.0 program was used for k-mer based (k-mer used 21, 33, 55 and 77) de-novo assembly with the aligned reads and the quality of the assembled genome was gauged using Samtools and Bcftools (read alignment and genome coverage calculation) [11]. (<https://samtools.github.io/bcftools/bcftools.html>).

Genome annotation and codon usage

Basic Local Alignment Search Tool (BLAST; BLASTN, PHI-BLAST and BLASTX) [12], chloroplast genome analysis platform (CGAP) [13] and Dual Organellar GenoMe Annotator (DOGMA) [14] was used to annotate protein-coding and ribosomal RNA genes. The boundaries of each annotated gene with putative start, stop, and intron positions were manually determined by comparison with homologous genes from other orchid chloroplast genomes. Further tRNA genes were predicted using tRNAscan-SE [15] and ARAGORN [16]. RNA editing sites in the protein-coding genes of *D. nobile* were predicted using Plant RNA Editing Prediction & Analysis Computer Tool (PREPACT) (<http://www.prepact.de>). For this analysis, *D. nobile* cp genome was BLAST aligned against *Nicotiana tabacum*, *Oryza sativa*, *Japonica* Group, *Phalaenopsis aphrodite* subsp. *Formosana*, *Physcomitrella patens* subsp. *Patens* and *Zea mays* with a cutoff E-value set to 0.08. The circular genome maps were drawn in CLC Genomics Workbench followed by manual modification. The sequencing data and gene annotation were submitted to GenBank with accession number KX377961. MEGA 7 was used to analyze and calculate GC content, codon usage, nucleotide sequence statistics and relative synonymous codon usage (RSCU) [17].

Simple sequence repeats analysis

MISA (<http://pgrc.ipk-gatersleben.de/misa/misa.html>), a tool for the identification and location of perfect microsatellites and compound microsatellites was used to search for potential simple sequence repeats (SSRs) loci in the cp genomes of all the *Dendrobium* species. The threshold point for SSRs identification was set to 10, 5, 4, 3, and 3 for mono-, di-, tri-, tetra-, and penta-nucleotides SSRs, respectively. All the repeats found were manually curated and the redundant ones were removed.

Phylogenetic reconstruction with whole genome alignment and rearrangement analysis

For phylogenetic reconstruction we included *D. nobile* cp genomes from India and China along with 32 other *Dendrobium* cp genomes retrieved from GenBank. Four *Goodyera* species were taken as outgroup. The cp genome sequences were aligned with MAFFT v7.0.0 [18] and manually curated by visual inspection. The complete cp genome sequences and protein coding genes (PCGs) were used for the Bayesian phylogenetic reconstruction using MRBAYES 3.2.6 [19]. To further validate our results we employed "K-mer Based Tree Construction" in CLC Genomics Workbench that uses single sequences or sequence lists as input and creates a distance-based phylogenetic tree. For visualization and testing the presence of genome rearrangement and inversions, gene synteny was performed using MAUVE as implemented in DNASTAR 12.3 with default settings. Comparative analysis of nucleotide diversity (P_i) among the complete cp genomes of *Dendrobium* was performed using MEGA 7.

Single Nucleotide Polymorphism identification and phylogenetic analysis without genome alignment

Phylogenetic tree was constructed based on the Single Nucleotide Polymorphisms (SNPs) identified in the whole chloroplast genomes using kSNP3.0 with default settings except for k-mer size [20]. SNPs were identified with k-mer size set to 23 based on which approximately 79% of the k-mers generated from median-length genome were unique.

Results and Discussion

Genome organization and features

The complete cp genome of *D. nobile* was determined from the data generated out of a whole genome project initiative of the same species by Paired-end and Mate pair data from Illumina HighSeq with 150*2 and Illumina NextSeq500 with 75*2 respectively. Further the aligned

Illumina reads were separated and assembled using CLC Main Workbench Version 7.7.1 into the single longest scaffold. The *D. nobile* cp genome is a typical circular double-stranded DNA with a quadripartite structure; it is 152,018 bp in size and consists of Large Single Copy (LSC) (1..84,944; 84,944 bp), Small Single Copy (SSC) (111,230..125,733; 14504 bp), and two Inverted Repeat (IR) regions of 26,285 bp: IRA (84,945..111,229) and IRB (125,734..152018). In total 134 unique genes (79 PCGs, 8 rRNA genes, 7 pseudogenes and 38 tRNA genes) were successfully annotated, of which 12 genes {rps16, atpF, rpoC1, ycf3, rps12 (2), clpP, petB, rpl2 (2), ndhB (2)} are reported with introns (Fig. 1). We could identify a total of 20, 81 and 11 genes duplicated in the IR, LSC and SSC regions respectively in the *D. nobile* cp genome.

Potential RNA editing sites

All 49 RNA editing sites (Table 1) were congruently predicted in 23 genes of *D. nobile* from at least 75% of the reference organisms (*Nicotiana tabacum*, *Oryza sativa Japonica* Group, *Phalaenopsis aphrodite* subsp. *Formosana*, *Physcomitrella patens* subsp. *Patens* and *Zea mays*) and resulted in amino acid substitutions.. All the RNA-editing sites were non-silent and edited C to U. Of the 49 RNA editing sites 89.8% (44) editing sites appeared in the second position of triplet codon, 10.2% (5) editing sites appeared in the first position of triplet codon whereas no editing sites appeared in the third base of triplet codon. The genes ndhD, rpoB, rpoC1 had 8, 6 and 4 RNA editing sites respectively. All the 49 RNA editing sites led to changes in the amino acid. The most frequent amino acid conversion was hydrophilic to hydrophobic (S to L, 22 occurrences and S to F, 8 occurrences), followed by hydrophobic to hydrophobic conversions (P to L, 12 occurrences). Seven conversions were found to be hydrophilic to hydrophilic (H to Y, 5 occurrences and T to M, 2 occurrences).

Comparison with other chloroplast genomes within the genus *Dendrobium*

We compared thirty-four chloroplast genomes representing different species within the genus *Dendrobium* (Table 2). The length of the *Dendrobium* species cp genomes ranged from 148,778 to 153,953 bp, with *D. chrysotoxum* being the largest cp genome and *D. moniliforme* the smallest. The cp genomes have acquired the familial angiosperm plastome organization comprising of a LSC, an SSC and a pair of IR regions each. *Dendrobium* cp genomes are also AT-rich (62.26–62.39%) quite similar to other orchid cp genomes. Differences in the cp genome size of these species are primarily due to the variations in the length of LSC, SSC and IR regions. Synteny comparison revealed a lack of genome rearrangement and

inversions, thereby, substantiating for the highly conserved nature in the genomic structure, including gene number and gene order in these cp genomes. However, structural variation was predominant in the LSC/IR/SSC boundaries (Fig. 2), which could be harnessed for predicting potential biomarkers for species identification.

IR regions are generally considered to be highly conserved regions in the chloroplast genome. In the evolutionary ladder, SSC and IR border regions experience expansion and contraction that overall contribute to the variation in chloroplast genome length [21, 22]. Thus, the positions of LSC/IRA/SSC/IRB borders were examined in the overall alignment of *Dendrobium* whole cp genomes and all of them were found to have similar structures at the IR/LSC junction (Fig. 3).

A comparative nucleotide sequence statistics (counts of annotations, AT/GC counts, nucleotide frequency in codon positions etc.) for all the *Dendrobium* species including representatives from outgroup are outlined in Tables 3, 4 and 5. The relative synonymous codon usage is given in parentheses following the codon frequency (averages over all taxa) involved (Table 6). Maximum Likelihood analysis of natural selection codon-by-codon was carried out. For each codon, estimates of the numbers of inferred synonymous (s) and nonsynonymous (n) substitutions are presented along with the number of sites that are estimated to be synonymous (S) and nonsynonymous (N) (Table S1). These estimates were calculated using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model [23] of codon substitution and Felsenstein 1981 model [24] of nucleotide substitution. For estimating ML values, a tree topology was automatically computed. The test statistic dN - dS was used for detecting codons that have undergone positive selection, where dS is the number of synonymous substitutions per site (s/S) and dN is the number of nonsynonymous substitutions per site (n/N). A positive value for the test statistic indicates an overabundance of nonsynonymous substitutions. In this case, the probability of rejecting the null hypothesis of neutral evolution (P-value) was calculated [25, 26]. Values of P less than 0.05 are considered significant at a 5% level and are highlighted [Table S2]. Normalized dN - dS for the test statistic is obtained using the total number of substitutions in the tree (measured in expected substitutions per site). The analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding and all positions containing gaps and missing data were eliminated. There were a total of 108,594 positions in the final dataset.

Characterization of simple sequence repeats

SSRs were identified in MISA perl scripts with a minimum of 10 bp repeats among all the *Dendrobium* species. Of all the SSRs, the mononucleotide A/T repeat units occupied the highest proportion. A higher proportion of di-, tri- repeats are reported rather than tetra- and penta-nucleotide repeats across *Dendrobium* cp genomes (Fig. 4). The SSRs could be further investigated for identifying potential markers that can aid in barcoding analysis.

Phylogenetic analyses

In the present study, we employed two different approaches for phylogeny reconstruction. First we aligned the whole cp genomes and exported the alignment matrices for creating a Bayesian tree (Fig. 5). Two independent MCMC chains were run with first 25% of the cycles removed as burn-in, coalescence of substitution rate and rate model parameters were also examined and average standard deviation of split frequencies was carried out and generations added until the standard deviation value was lowered to 0.01. Secondly we performed a phylogenetic tree construction using an alignment free approach. In this case we identified the SNPs from the cp genomes and utilised them in constructing the phylogenetic tree (Fig. 6). A total of 13,839 SNPs were identified in the 38 genomes analyzed, of which 2,203 were homoplastic SNPs i.e. SNPs that do not correspond to any node in the parsimony tree. The fraction of k-mers present in all genomes is 0.482. The numbers at the nodes in the phylogenetic tree indicate the number of SNPs that are present in all of the descendants of that node and absent in others (Fig. 6). The numbers at the tips indicate the number of SNPs unique to each particular species.

The two different methods that employed both alignment and alignment-free approach resulted in highly reliable identical phylogenetic trees within each data set. Different analyses based on the two datasets generated largely congruent topologies (Figs. 5 and 6) with *Dendrobium* species forming one clade and *Goodyera* species forming another clade as an outgroup.

Conclusions

This study provides the first comparative account on the complete chloroplast genome of *D. nobile* from north-east India with 33 other species from the genus *Dendrobium* that revealed higher sequence variation in SSC and LSC regions compared with IR regions in both coding and non-coding regions. The gene order, gene content and genomic structure were highly conserved with those of other sequenced *Dendrobium* species. However, IR contraction is

observed within the genus and several SNPs identified from these cp genomes were quite instrumental in generating alignment-free robust phylogenetic trees that congrued with trees generated from aligned matrices of whole cp genomes. This gives an indication that the SNPs, insertions and deletions, LSC and SSC regions in the cp genomes of this medicinal orchid genus can be utilized for barcoding and biodiversity studies. Further this would augment more and more plastome sequencing of *Dendrobium* species that are not reported in this study.

Data Availability

The following information was supplied regarding the GenBank accession, BioSample, SRA and BioProject pertaining to this study.

NCBI GenBank accession number: KX377961.

BioSample: SAMN05190527; Sample name: SO_5373; SRA: SRS1473719

BioProject Accession: PRJNA323854; ID: 323854

Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

Ruchishree Konhar performed the experiments, analyzed the data, contributed analysis tools, prepared figures and/or tables, authored and reviewed drafts of the paper. Manish Debnath performed the in silico analysis and prepared tables and figures. Atanu Bhattacharjee and Durai Sundar analyzed the data, contributed analysis tools, authored or reviewed drafts of the paper, and approved the final draft. Debasis Dash provided physical resources, contributed analysis tools, authored or reviewed drafts of the paper, and approved the final draft. Devendra K Biswal and Pramod Tandon conceived and guided on the overall design of experiments, provided physical resources, contributed analysis tools, authored and reviewed drafts of the paper, approved the final draft.

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Figure Legends

Figure 1. Gene map of *Dendrobium nobile* chloroplast genome from Northeast India.

Genes shown inside the circle are transcribed clockwise, and those outside are transcribed anticlockwise. Color coding indicates genes of different functional groups. A pair of inverted repeats (IRA and IRB) separate the genome into LSC and SSC regions.

Figure 2. Whole chloroplast genome alignment of 38 orchid species.

The whole chloroplast genome alignment includes 34 *Dendrobium* species and 4 species from the genus *Goodyera* as outgroup. Each genome's panel contains its name, sequence coordinates and a black coloured horizontal centre line with coloured block outlines appearing above and below it. Each block represents homology with the genes, internally free from genomic rearrangement, connected by lines to similarly coloured blocks depicting comparative homology across genomes.

Figure 3. Comparison of the borders of LSC, SSC and IR regions across *Dendrobium* chloroplast genomes.

Figure 4. SSR distribution among different *Dendrobium* plastomes. The SSR were determined in MISA per scripts based on the comparison between plastomes of each tested *Dendrobium* species and *D. nobile*. Histograms with different color codes indicate the numbers of SSRs. The minimum number (thresholds) of SSRs was set as 10, 5, 4, 3, and 3 for mono-, di-, tri-, tetra-, and penta-nucleotides SSRs, respectively.

Figure 5. Phylogenetic tree based on bayesian inference from the whole genome alignment matrix of *Dendrobium* chloroplast genomes. The tree yielded monophyletic groupings of the genus *Dendrobium* and *Goodyera* species emerged as outgroup with a separate clade. Posterior probability/bootstrap values are indicated on the internal nodes, which are highly supportive of the overall tree topology.

Figure 6. Alignment free phylogenetic tree reconstruction based on SNP identification.

The optimum kmer size for the dataset is determined that calculates FCK, a measure of diversity of sequences in the dataset (Kchooser) and a consensus of the equally most parsimonious trees are reported. The numbers at the nodes indicate the number of SNPs that

are present in all of the descendants of that node and absent in others. The numbers within parentheses at the tips indicate the number of SNPs unique to each particular species.

Supplementary Files

Table S1. Maximum Likelihood analysis of natural selection codon-by-codon

Table S2. Results from the Fisher's Exact Test of Neutrality Selection across the chloroplast genome sequences in the genus *Dendrobium*

Table 1. RNA editing sites predicted in *Dendrobium nobile* chloroplast genome

Gene	Nucleotide Position	Amino Acid Position	Triplet position within codon	Bases	Codon change	Amino Acid Conversion	Count	Percentage
matK	1258	420	1	C→U	CAC→UAC	H→Y	4/5	80%
	913	305	1	C→U	CAU→UAU	H→Y	4/6	80%
rps16	143	48	2	C→U	UCA→UUA	S→L	4/7	100%
atpA	773	258	2	C→U	UCA→UUA	S→L	4/8	100%
atpF	92	31	2	C→U	CCA→CUA	P→L	4/9	100%
atpI	629	210	2	C→U	UCA→UUA	S→L	4/10	100%
	428	143	2	C→U	CCU→CUU	P→L	4/11	100%
rpoC1	617	206	2	C→U	UCG→UUG	S→L	4/12	100%
	488	163	2	C→U	UCA→UUA	S→L	4/13	100%
	182	61	2	C→U	UCU→UUU	S→F	4/14	100%
	41	14	2	C→U	CCA→CUA	P→L	4/15	100%
rpoB	2426	809	2	C→U	UCA→UUA	S→L	4/16	80%
	623	208	2	C→U	CCG→CUG	P→L	4/17	80%
	566	189	2	C→U	UCG→UUG	S→L	4/18	100%
	551	184	2	C→U	UCA→UUA	S→L	4/19	100%
	473	158	2	C→U	UCG→UUG	S→L	4/20	100%
	338	113	2	C→U	UCU→UUU	S→F	4/21	100%
rps14	149	50	2	C→U	CCA→CUA	P→L	4/22	100%
ycf3	191	64	2	C→U	CCA→CUA	P→L	4/23	100%
	185	62	2	C→U	ACG→AUG	T→M	4/24	100%
	44	15	2	C→U	UCU→UUU	S→F	4/25	100%
atpB	1184	395	2	C→U	UCA→UUA	S→L	4/26	100%
accD	1184	395	2	C→U	UCA→UUA	S→L	4/27	100%
	1412	471	2	C→U	CCA→CUA	P→L	4/28	100%
	1430	477	2	C→U	CCU→CUU	P→L	4/29	100%
psaI	80	27	2	C→U	UCU→UUU	S→F	4/30	100%
psbF	77	26	2	C→U	UCU→UUU	S→F	4/31	100%
petL	5	2	2	C→U	CCU→CUU	P→L	4/32	100%
rpl20	308	103	2	C→U	UCA→UUA	S→L	4/33	80%
clpP	559	187	1	C→U	CAU→UAU	H→Y	4/34	100%
	82	28	1	C→U	CAU→UAU	H→Y	4/35	100%
petB	611	204	2	C→U	UCA→UUA	S→L	4/36	100%
rpoA	830	277	2	C→U	UCA→UUA	S→L	4/37	100%
	368	123	2	C→U	UCA→UUA	S→L	4/38	100%
	200	67	2	C→U	UCU→UUU	S→F	4/39	75%
rpl2	2	1	2	C→U	ACG→AUG	T→M	4/40	100%
ndhD	878	293	2	C→U	UCA→UUA	S→L	4/41	100%
	674	225	2	C→U	UCG→UUG	S→L	4/42	100%
	383	128	2	C→U	UCA→UUA	S→L	4/43	100%
ndhA	473	158	2	C→U	UCA→UUA	S→L	4/44	100%

ndhB	149	50	2	C→U	UCA→UUA	S→L	4/45	100%
	467	156	2	C→U	CCA→CUA	P→L	4/46	100%
	586	196	1	C→U	CAU→UAU	H→Y	4/47	100%
	704	235	2	C→U	UCC→UUC	S→F	4/48	100%
	737	246	2	C→U	CCA→CUA	P→L	4/49	100%
	830	277	2	C→U	UCA→UUA	S→L	4/50	80%
	836	279	2	C→U	UCA→UUA	S→L	4/51	80%
	1481	494	2	C→U	CCA→CUA	P→L	4/52	100%
rpl23	71	24	2	C→U	UCU→UUU	S→F	4/53	80%

Table 2. Summary of characteristics in chloroplast genome sequences of thirty-four *Dendrobium* species and four *Goodyera* species (taken as outgroup).

Organism	Accession Number	Length	Weight (single-stranded) Mda	Weight (double-stranded) Mda
<i>Dendrobium nobile</i>	KX377961	152018	46.932	93.912
<i>Dendrobium officinale</i>	NC_024019	152221	46.995	94.038
<i>Dendrobium strongylanthum</i>	NC_027691	153059	47.256	94.556
<i>Dendrobium huoshanense</i>	NC_028430	153188	47.294	94.635
<i>Dendrobium chrysotoxum</i>	NC_028549	153953	47.528	95.108
<i>Dendrobium nobile (China)</i>	NC_029456	153660	47.453	94.927
<i>Dendrobium pendulum</i>	NC_029705	153038	47.246	94.542
<i>Dendrobium moniliforme</i>	NC_035154	148778	45.931	91.911
<i>Dendrobium primulinum</i>	NC_035321	150767	46.545	93.14
<i>Dendrobium aphyllum</i>	NC_035322	151524	46.779	93.607
<i>Dendrobium brymerianum</i>	NC_035323	151830	46.873	93.796
<i>Dendrobium denneanum</i>	NC_035324	151565	46.793	93.633
<i>Dendrobium devonianum</i>	NC_035325	151945	46.909	93.867
<i>Dendrobium falconeri</i>	NC_035326	151890	46.891	93.833
<i>Dendrobium gratiosissimum</i>	NC_035327	151829	46.873	93.796
<i>Dendrobium hercoglossum</i>	NC_035328	151939	46.908	93.864
<i>Dendrobium wardianum</i>	NC_035329	151788	46.861	93.77
<i>Dendrobium wilsonii</i>	NC_035330	152080	46.951	93.951
<i>Dendrobium crepidatum</i>	NC_035331	151717	46.837	93.726
<i>Dendrobium salaccense</i>	NC_035332	151104	46.648	93.347
<i>Dendrobium spatella</i>	NC_035333	151829	46.872	93.796
<i>Dendrobium parviflorum</i>	NC_035334	150073	46.331	92.711
<i>Dendrobium henryi</i>	NC_035335	151850	46.88	93.809
<i>Dendrobium chrysanthum</i>	NC_035336	151790	46.861	93.772
<i>Dendrobium jenkinsii</i>	NC_035337	151717	46.839	93.726
<i>Dendrobium lohohense</i>	NC_035338	151812	46.868	93.785
<i>Dendrobium parishii</i>	NC_035339	151689	46.83	93.709
<i>Dendrobium ellipsophyllum</i>	NC_035340	152026	46.935	93.917
<i>Dendrobium xichouense</i>	NC_035341	152052	46.942	93.933
<i>Dendrobium fimbriatum</i>	NC_035342	151673	46.825	93.699
<i>Dendrobium exile</i>	NC_035343	151294	46.707	93.465
<i>Dendrobium fanjingshanense</i>	NC_035344	152108	46.96	93.968
<i>Dendrobium candidum</i>	NC_035745	152094	46.955	93.959
<i>Dendrobium loddigesii</i>	NC_036355	152493	47.077	94.205
<i>Goodyera fumata</i>	NC_026773	155643	48.048	96.151
<i>Goodyera procera</i>	NC_029363	153240	47.306	94.667
<i>Goodyera schlechtendaliana</i>	NC_029364	154348	47.648	95.351
<i>Goodyera velutina</i>	NC_029365	152692	47.138	94.328

Table 3. Summary features of chloroplast genome sequences of thirty-four *Dendrobium* species and four *Goodyera* species

Organism	CDS	Exon	Gene	Misc. feature	Repeat region	rRNA	tRNA
<i>Dendrobium nobile</i>	79	22	132	2	2	8	38
<i>Dendrobium officinale</i>	76	0	129	0	0	8	38
<i>Dendrobium strongylanthum</i>	77	0	130	2	2	8	38
<i>Dendrobium huoshanense</i>	76	0	129	2	2	8	38
<i>Dendrobium chrysotoxum</i>	63	0	116	2	2	8	38
<i>Dendrobium nobile (China)</i>	77	0	130	2	2	8	38
<i>Dendrobium pendulum</i>	76	0	129	2	2	8	38
<i>Dendrobium moniliforme</i>	73	0	129	11	2	8	39
<i>Dendrobium primulinum</i>	72	0	132	16	2	8	38
<i>Dendrobium aphyllum</i>	72	0	132	16	2	8	38
<i>Dendrobium brymerianum</i>	72	0	132	16	2	8	38
<i>Dendrobium denneanum</i>	72	0	132	16	2	8	38
<i>Dendrobium devonianum</i>	72	0	132	16	2	8	38
<i>Dendrobium falconeri</i>	72	0	132	16	2	8	38
<i>Dendrobium gratiosissimum</i>	72	0	132	16	2	8	38
<i>Dendrobium hercoglossum</i>	72	0	132	16	2	8	38
<i>Dendrobium wardianum</i>	71	0	131	16	2	8	38
<i>Dendrobium wilsonii</i>	72	0	132	16	2	8	38
<i>Dendrobium crepidatum</i>	72	0	132	16	2	8	38
<i>Dendrobium salaccense</i>	72	0	132	16	2	8	38
<i>Dendrobium spatella</i>	72	0	132	16	2	8	38
<i>Dendrobium parciflorum</i>	72	0	131	16	2	7	38
<i>Dendrobium henryi</i>	72	0	132	16	2	8	38
<i>Dendrobium chrysanthum</i>	72	0	132	16	2	8	38
<i>Dendrobium jenkinsii</i>	72	0	132	16	2	8	38
<i>Dendrobium lohohense</i>	72	0	132	16	2	8	38
<i>Dendrobium parishii</i>	72	0	132	16	2	8	38
<i>Dendrobium ellipsophyllum</i>	72	0	132	16	2	8	38
<i>Dendrobium xichouense</i>	72	0	132	16	2	8	38
<i>Dendrobium fimbriatum</i>	72	0	132	16	2	8	38
<i>Dendrobium exile</i>	72	0	132	16	2	8	38
<i>Dendrobium</i>	72	0	132	16	2	8	38

<i>fanjingshanense</i>							
<i>Dendrobium candidum</i>	75	0	128	0	0	8	38
<i>Dendrobium loddigesii</i>	68	0	120	9	0	8	39
<i>Goodyera fumata</i>	87	0	133	0	0	8	38
<i>Goodyera procera</i>	80	0	127	0	0	8	39
<i>Goodyera schlechtendalana</i>	81	0	129	0	0	8	40
<i>Goodyera velutina</i>	79	0	126	0	0	8	39

Table 4. Counts of nucleotides in the chloroplast genomes.

Nucleotide	Adenine (A)	Cytosine (C)	Guanine (G)	Thymine (T)	C + G	A + T
<i>Dendrobium nobile</i>	46576	28853	28039	48381	56892	94957
<i>Dendrobium officinale</i>	46743	28924	28107	48447	57031	95190
<i>Dendrobium strongylanthum</i>	46940	29147	28431	48541	57578	95481
<i>Dendrobium huoshanense</i>	47032	29111	28316	48729	57427	95761
<i>Dendrobium chrysotoxum</i>	47180	29400	28492	48881	57892	96061
<i>Dendrobium nobile (China)</i>	47118	28871	28748	48923	57619	96041
<i>Dendrobium pendulum</i>	46997	29122	28242	48677	57364	95674
<i>Dendrobium moniliforme</i>	45551	28339	27520	47368	55859	92919
<i>Dendrobium primulinum</i>	46191	28750	27909	47917	56659	94108
<i>Dendrobium aphyllum</i>	46417	28917	28057	48133	56974	94550
<i>Dendrobium brymerianum</i>	46509	28968	28123	48230	57091	94739
<i>Dendrobium denneanum</i>	46440	28913	28115	48097	57028	94537
<i>Dendrobium devonianum</i>	46615	28943	28108	48279	57051	94894
<i>Dendrobium falconeri</i>	46591	28911	28040	48348	56951	94939
<i>Dendrobium gratiosissimum</i>	46521	28954	28095	48259	57049	94780
<i>Dendrobium hercoglossum</i>	46592	28941	28131	48275	57072	94867
<i>Dendrobium wardianum</i>	46479	28955	28118	48236	57073	94715
<i>Dendrobium wilsonii</i>	46668	28948	28101	48363	57049	95031
<i>Dendrobium crepidatum</i>	46482	28951	28056	48228	57007	94710
<i>Dendrobium salaccense</i>	46493	28635	27735	48241	56370	94734
<i>Dendrobium spatella</i>	46524	28969	28091	48245	57060	94769
<i>Dendrobium parviflorum</i>	45941	28699	27829	47604	56528	93545
<i>Dendrobium henryi</i>	46550	28936	28093	48271	57029	94821
<i>Dendrobium chrysanthum</i>	46519	28939	28078	48254	57017	94773
<i>Dendrobium jenkinsii</i>	46497	28942	28105	48173	57047	94670
<i>Dendrobium lohohense</i>	46558	28928	28098	48228	57026	94786
<i>Dendrobium parishii</i>	46487	28924	28079	48199	57003	94686
<i>Dendrobium ellipsophyllum</i>	46690	28922	28091	48323	57013	95013
<i>Dendrobium xichouense</i>	46672	28937	28098	48345	57035	95017
<i>Dendrobium fimbriatum</i>	46483	28932	28094	48164	57026	94647
<i>Dendrobium exile</i>	46251	28937	28065	48041	57002	94292
<i>Dendrobium fanjingshanense</i>	46694	28947	28115	48352	57062	95046
<i>Dendrobium candidum</i>	46695	28914	28091	48394	57005	95089
<i>Dendrobium loddigesii</i>	46868	28934	28064	48627	56998	95495
<i>Goodyera fumata</i>	48186	29569	28447	49441	58016	97627
<i>Goodyera procera</i>	47095	29370	28303	48472	57673	95567
<i>Goodyera schlechtendaliana</i>	47822	29206	28146	49174	57352	96996
<i>Goodyera velutina</i>	47554	28694	27658	48786	56352	96340

Table 5. Counts of nucleotide frequency in codon positions across the chloroplast genomes.

Nucleotide per position	1 A	1 C	1 G	1 T	2 A	2 C	2 G	2 T	3 A	3 C	3 G	3 T
<i>D. nobile</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. officinale</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. strongylanthum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. huoshanense</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. chrysotoxum</i>	0.3	0.19	0.28	0.22	0.29	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. nobile (China)</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. pendulum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. moniliforme</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.17	0.38
<i>D. primulinum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. aphyllum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. brymerianum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. denneanum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. devonianum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. falconeri</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. gratiosissimum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.17	0.38
<i>D. hercoglossum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. wardianum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. wilsonii</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. crepidatum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. salaccense</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. spatella</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.31	0.14	0.17	0.38
<i>D. parciflorum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.31	0.14	0.17	0.38
<i>D. henryi</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. chrysanthum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. jenkinsii</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. lohohense</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. parishii</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.17	0.38
<i>D. ellipsophyllum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. xichouense</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. fimbriatum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. exile</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.31	0.14	0.16	0.38
<i>D. fanjingshanense</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. candidum</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>D. loddigesii</i>	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
<i>G. fumata</i>	0.31	0.19	0.26	0.24	0.29	0.2	0.18	0.33	0.32	0.14	0.16	0.38
<i>G. procera</i>	0.31	0.19	0.26	0.24	0.3	0.2	0.17	0.33	0.32	0.14	0.16	0.38
<i>G. schlechtendalia</i>	0.31	0.19	0.26	0.24	0.29	0.21	0.17	0.33	0.31	0.14	0.16	0.38
<i>G. velutina</i>	0.31	0.19	0.27	0.24	0.29	0.21	0.18	0.33	0.32	0.14	0.16	0.38

Table 6. Relative synonymous codon usage (in parentheses) following the codon frequency across the chloroplast genomes in the genus *Dendrobium*.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	2018.1	1.16	UCU(S)	1330	1.63	UAU(Y)	1371	1.38	UGU(C)	706.9	1.24
UUC(F)	1459.2	0.84	UCC(S)	882.8	1.08	UAC(Y)	621.4	0.62	UGC(C)	437	0.76
UUA(L)	918.4	1.14	UCA(S)	999.4	1.23	UAA(*)	970.5	1.05	UGA(*)	1065	1.15
UUG(L)	970.9	1.21	UCG(S)	576.9	0.71	UAG(*)	732.2	0.79	UGG(W)	691.4	1
CUU(L)	1068.9	1.33	CCU(P)	638	1.13	CAU(H)	919.7	1.43	CGU(R)	336.1	0.63
CUC(L)	629.2	0.78	CCC(P)	547.8	0.97	CAC(H)	369.3	0.57	CGC(R)	220.7	0.41
CUA(L)	762.8	0.95	CCA(P)	689.4	1.23	CAA(Q)	952.8	1.38	CGA(R)	545.2	1.02
CUG(L)	473.7	0.59	CCG(P)	375.4	0.67	CAG(Q)	423.2	0.62	CGG(R)	343	0.64
AUU(I)	1635.7	1.21	ACU(T)	646	1.21	AAU(N)	1580	1.39	AGU(S)	659.9	0.81
AUC(I)	1072.9	0.8	ACC(T)	530.8	1	AAC(N)	695	0.61	AGC(S)	435.8	0.54
AUA(I)	1337.4	0.99	ACA(T)	610.3	1.15	AAA(K)	1914	1.31	AGA(R)	1171	2.2
AUG(M)	891.4	1	ACG(T)	343.2	0.64	AAG(K)	1009	0.69	AGG(R)	576	1.08
GUU(V)	709.4	1.36	GCU(A)	467.5	1.29	GAU(D)	1038	1.43	GGU(G)	523.7	0.99
GUC(V)	366.7	0.7	GCC(A)	326.4	0.9	GAC(D)	413.9	0.57	GGC(G)	314.4	0.59
GUA(V)	647.8	1.24	GCA(A)	438.7	1.21	GAA(E)	1335	1.37	GGA(G)	754.1	1.43
GUG(V)	366.9	0.7	GCG(A)	221.5	0.61	GAG(E)	618.3	0.63	GGG(G)	521.8	0.99

Figure 1

Figure 2

Figure 4

Figure 5

Figure 6

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