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 (*Pi*) 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 no 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**
Dendrobium nobile	KX377961	152018	46.932	93.912
Dendrobium officinale	NC_024019	152221	46.995	94.038
Dendrobium strongylanthum	NC_027691	153059	47.256	94.556
Dendrobium huoshanense	NC_028430	153188	47.294	94.635
Dendrobium chrysotoxum	NC_028549	153953	47.528	95.108
Dendrobium nobile (China)	NC_029456	153660	47.453	94.927
Dendrobium pendulum	NC_029705	153038	47.246	94.542
Dendrobium moniliforme	NC_035154	148778	45.931	91.911
Dendrobium primulinum	NC_035321	150767	46.545	93.14
Dendrobium aphyllum	NC_035322	151524	46.779	93.607
Dendrobium brymerianum	NC_035323	151830	46.873	93.796
Dendrobium denneanum	NC_035324	151565	46.793	93.633
Dendrobium devonianum	NC_035325	151945	46.909	93.867
Dendrobium falconeri	NC_035326	151890	46.891	93.833
Dendrobium gratiosissimum	NC_035327	151829	46.873	93.796
Dendrobium hercoglossum	NC_035328	151939	46.908	93.864
Dendrobium wardianum	NC_035329	151788	46.861	93.77
Dendrobium wilsonii	NC_035330	152080	46.951	93.951
Dendrobium crepidatum	NC_035331	151717	46.837	93.726
Dendrobium salaccense	NC_035332	151104	46.648	93.347
Dendrobium spatella	NC_035333	151829	46.872	93.796
Dendrobium parciflorum	NC_035334	150073	46.331	92.711
Dendrobium henryi	NC_035335	151850	46.88	93.809
Dendrobium chrysanthum	NC_035336	151790	46.861	93.772
Dendrobium jenkinsii	NC_035337	151717	46.839	93.726
Dendrobium lohohense	NC_035338	151812	46.868	93.785
Dendrobium parishii	NC_035339	151689	46.83	93.709
Dendrobium ellipsophyllum	NC_035340	152026	46.935	93.917
Dendrobium xichouense	NC_035341	152052	46.942	93.933
Dendrobium fimbriatum	NC_035342	151673	46.825	93.699
Dendrobium exile	NC_035343	151294	46.707	93.465
Dendrobium fanjingshanense	NC_035344	152108	46.96	93.968
Dendrobium candidum	NC_035745	152094	46.955	93.959
Dendrobium loddigesii	NC_036355	152493	47.077	94.205
Goodyera fumata	NC_026773	155643	48.048	96.151
Goodyera procera	NC_029363	153240	47.306	94.667
Goodyera schlechtendaliana	NC_029364	154348	47.648	95.351
Goodyera velutina	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
Dendrobium nobile	79	22	132	2	2	8	38
Dendrobium officinale	76	0	129	0	0	8	38
Dendrobium strongylanthum	77	0	130	2	2	8	38
Dendrobium huoshanense	76	0	129	2	2	8	38
Dendrobium chrysotoxum	63	0	116	2	2	8	38
Dendrobium nobile (China)	77	0	130	2	2	8	38
Dendrobium pendulum	76	0	129	2	2	8	38
Dendrobium moniliforme	73	0	129	11	2	8	39
Dendrobium primulinum	72	0	132	16	2	8	38
Dendrobium aphyllum	72	0	132	16	2	8	38
Dendrobium brymerianum	72	0	132	16	2	8	38
Dendrobium denneanum	72	0	132	16	2	8	38
Dendrobium devonianum	72	0	132	16	2	8	38
Dendrobium falconeri	72	0	132	16	2	8	38
Dendrobium gratiosissimum	72	0	132	16	2	8	38
Dendrobium hercoglossum	72	0	132	16	2	8	38
Dendrobium wardianum	71	0	131	16	2	8	38
Dendrobium wilsonii	72	0	132	16	2	8	38
Dendrobium crepidatum	72	0	132	16	2	8	38
Dendrobium salaccense	72	0	132	16	2	8	38
Dendrobium spatella	72	0	132	16	2	8	38
Dendrobium parciflorum	72	0	131	16	2	7	38
Dendrobium henryi	72	0	132	16	2	8	38
Dendrobium chrysanthum	72	0	132	16	2	8	38
Dendrobium jenkinsii	72	0	132	16	2	8	38
Dendrobium lohohense	72	0	132	16	2	8	38
Dendrobium parishii	72	0	132	16	2	8	38
Dendrobium ellipsophyllum	72	0	132	16	2	8	38
Dendrobium xichouense	72	0	132	16	2	8	38
Dendrobium fimbriatum	72	0	132	16	2	8	38
Dendrobium exile	72	0	132	16	2	8	38
Dendrobium	72	0	132	16	2	8	38

fanjingshanense							
Dendrobium candidum	75	0	128	0	0	8	38
Dendrobium loddigesii	68	0	120	9	0	8	39
Goodyera fumata	87	0	133	0	0	8	38
Goodyera procera	80	0	127	0	0	8	39
Goodyera schlechtendaliana	81	0	129	0	0	8	40
Goodyera velutina	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
Dendrobium nobile	46576	28853	28039	48381	56892	94957
Dendrobium officinale	46743	28924	28107	48447	57031	95190
Dendrobium strongylanthum	46940	29147	28431	48541	57578	95481
Dendrobium huoshanense	47032	29111	28316	48729	57427	95761
Dendrobium chrysotoxum	47180	29400	28492	48881	57892	96061
Dendrobium nobile (China)	47118	28871	28748	48923	57619	96041
Dendrobium pendulum	46997	29122	28242	48677	57364	95674
Dendrobium moniliforme	45551	28339	27520	47368	55859	92919
Dendrobium primulinum	46191	28750	27909	47917	56659	94108
Dendrobium aphyllum	46417	28917	28057	48133	56974	94550
Dendrobium brymerianum	46509	28968	28123	48230	57091	94739
Dendrobium denneanum	46440	28913	28115	48097	57028	94537
Dendrobium devonianum	46615	28943	28108	48279	57051	94894
Dendrobium falconeri	46591	28911	28040	48348	56951	94939
Dendrobium gratiosissimum	46521	28954	28095	48259	57049	94780
Dendrobium hercoglossum	46592	28941	28131	48275	57072	94867
Dendrobium wardianum	46479	28955	28118	48236	57073	94715
Dendrobium wilsonii	46668	28948	28101	48363	57049	95031
Dendrobium crepidatum	46482	28951	28056	48228	57007	94710
Dendrobium salaccense	46493	28635	27735	48241	56370	94734
Dendrobium spatella	46524	28969	28091	48245	57060	94769
Dendrobium parciflorum	45941	28699	27829	47604	56528	93545
Dendrobium henryi	46550	28936	28093	48271	57029	94821
Dendrobium chrysanthum	46519	28939	28078	48254	57017	94773
Dendrobium jenkinsii	46497	28942	28105	48173	57047	94670
Dendrobium lohohense	46558	28928	28098	48228	57026	94786
Dendrobium parishii	46487	28924	28079	48199	57003	94686
Dendrobium ellipsophyllum	46690	28922	28091	48323	57013	95013
Dendrobium xichouense	46672	28937	28098	48345	57035	95017
Dendrobium fimbriatum	46483	28932	28094	48164	57026	94647
Dendrobium exile	46251	28937	28065	48041	57002	94292
Dendrobium fanjingshanense	46694	28947	28115	48352	57062	95046
Dendrobium candidum	46695	28914	28091	48394	57005	95089
Dendrobium loddigesii	46868	28934	28064	48627	56998	95495
Goodyera fumata	48186	29569	28447	49441	58016	97627
Goodyera procera	47095	29370	28303	48472	57673	95567
Goodyera schlechtendaliana	47822	29206	28146	49174	57352	96996
Goodyera velutina	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**
D. nobile	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. officinale	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. strongylanthum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. huoshanense	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. chrysotoxum	0.3	0.19	0.28	0.22	0.29	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. nobile (China)	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. pendulum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. moniliforme	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.17	0.38
D. primulinum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. aphyllum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. brymerianum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. denneanum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. devonianum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. falconeri	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. gratiosissimum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.17	0.38
D. hercoglossum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. wardianum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. wilsonii	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. crepidatum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. salaccense	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. spatella	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.31	0.14	0.17	0.38
D. parciflorum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.31	0.14	0.17	0.38
D. henryi	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. chrysanthum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. jenkinsii	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. lohohense	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. parishii	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.17	0.38
D. ellipsophyllum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. xichouense	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. fimbriatum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. exile	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.31	0.14	0.16	0.38
D. fanjingshanense	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. candidum	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
D. loddigesii	0.31	0.19	0.27	0.23	0.3	0.2	0.18	0.32	0.32	0.14	0.16	0.38
G. fumata	0.31	0.19	0.26	0.24	0.29	0.2	0.18	0.33	0.32	0.14	0.16	0.38
G. procera	0.31	0.19	0.26	0.24	0.3	0.2	0.17	0.33	0.32	0.14	0.16	0.38
G. schlechtendaliana	0.31	0.19	0.26	0.24	0.29	0.21	0.17	0.33	0.31	0.14	0.16	0.38
G. velutina	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

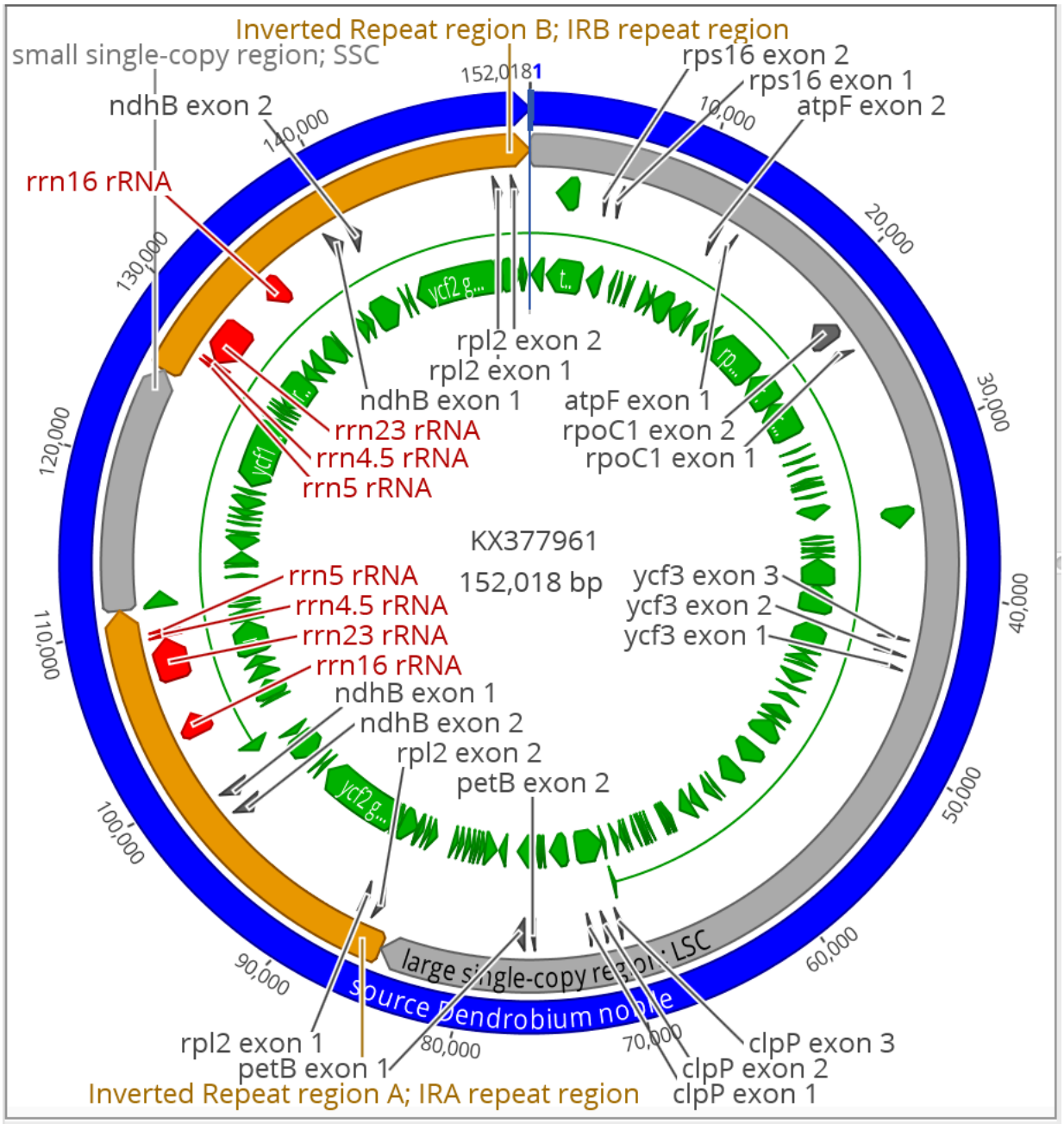
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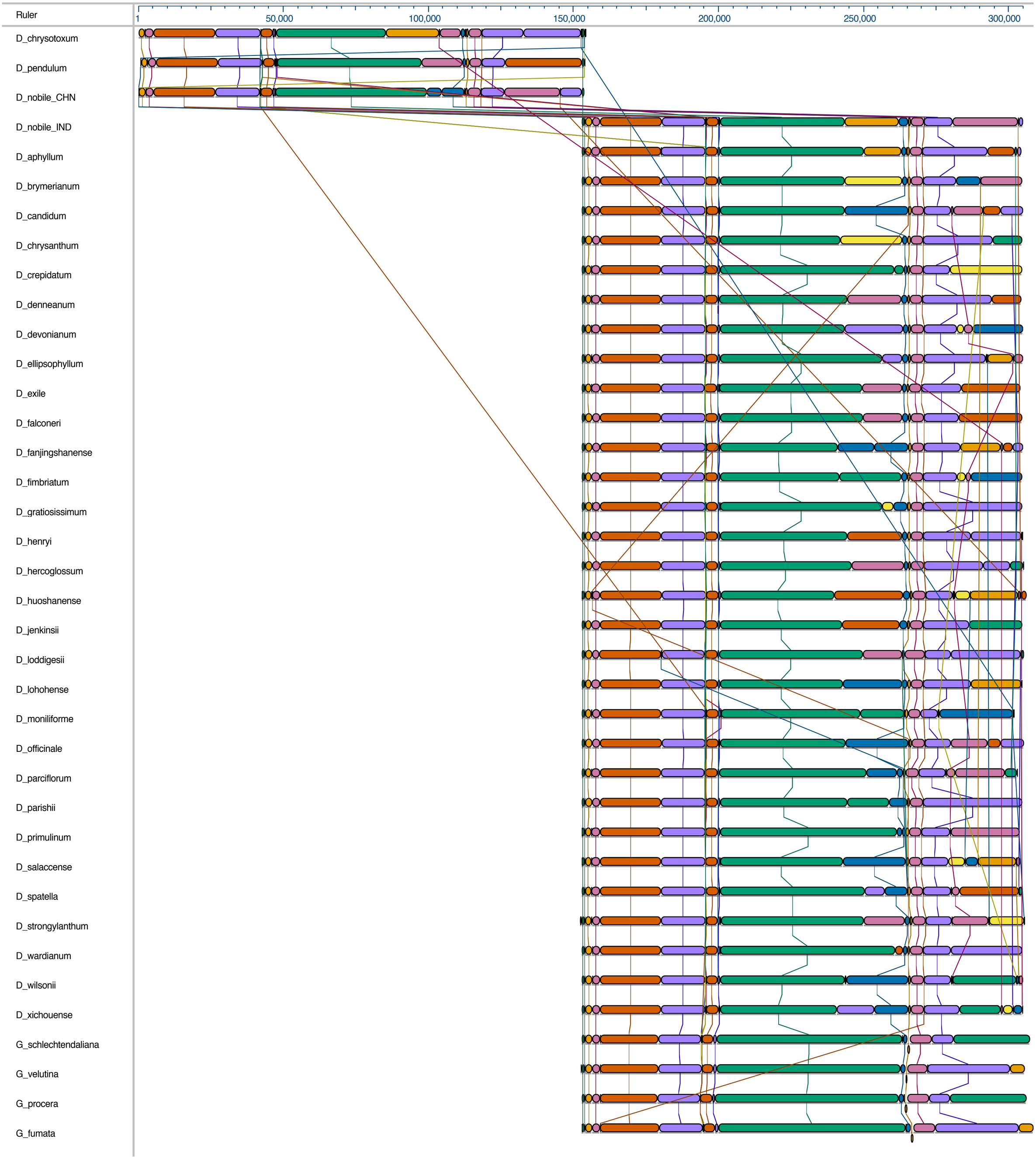
Figure 2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D_gratiosissimum
D_henryi
D_hercoglossum
D_huoshanense
D_jenkinsii
D_loddigesii
D_lohohense
D_moniliforme
D_officinale
D_parciflorum
D_parishii
D_primulinum
D_salaccense
D_spatella
D_strongylanthum
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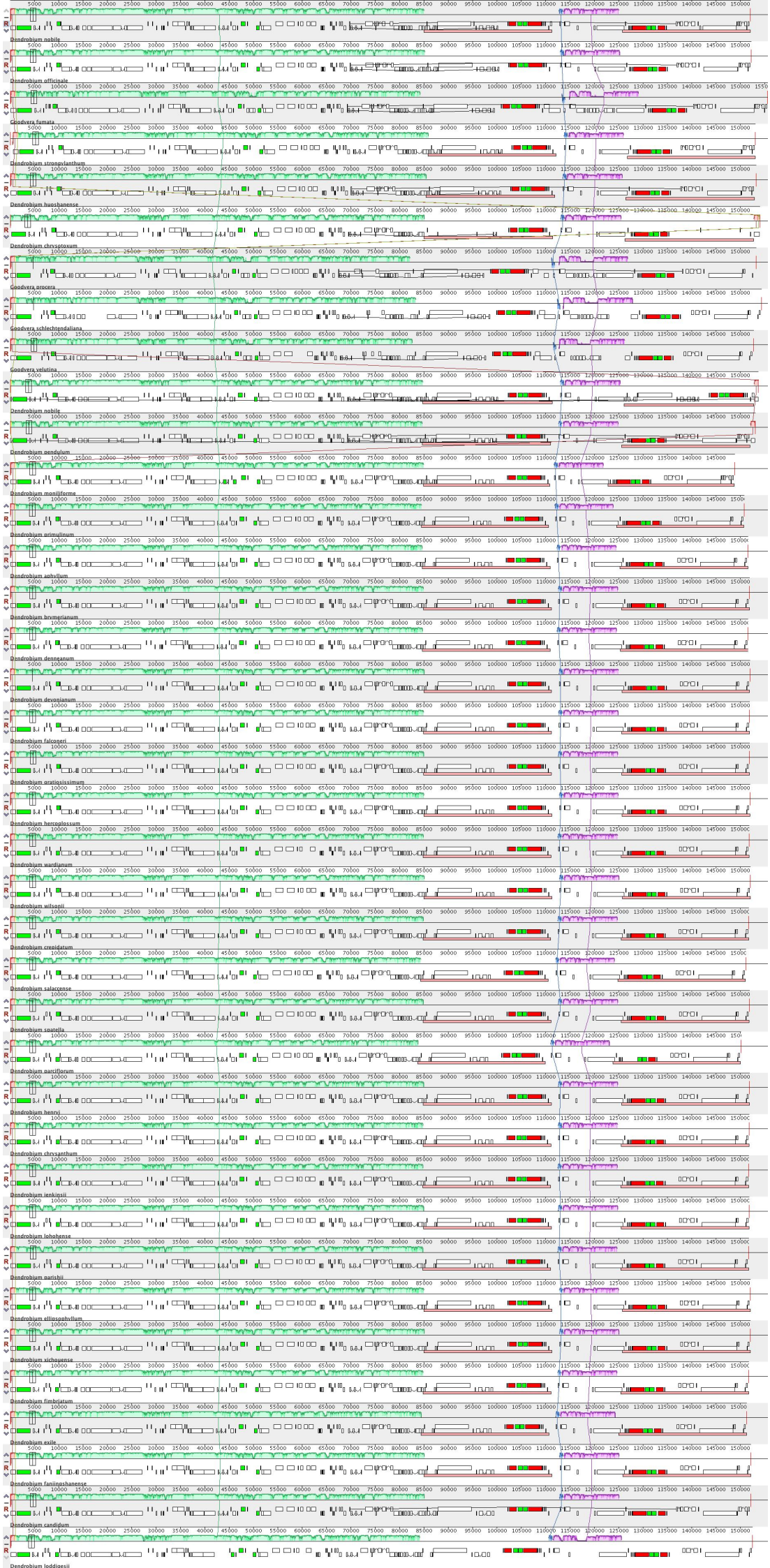
G_velutina
G_procera
G_fumata

Figure 3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Goodyera velutina
Dendrobium nobile
Dendrobium nendulum
Dendrobium monniliforme
Dendrobium primulinum
Dendrobium aphyllum
Dendrobium brymerianum
Dendrobium denneanum
Dendrobium devonianum
Dendrobium falconeri
Dendrobium gratiosissimum
Dendrobium hercoglossum
Dendrobium wardianum
Dendrobium wilsonii
Dendrobium crepidatum
Dendrobium salaccense
Dendrobium spatella
Dendrobium parciflorum
Dendrobium henryi
Dendrobium chrysanthum
Dendrobium jenkinsii
Dendrobium lohohense
Dendrobium parishii
Dendrobium ellipsophyllum
Dendrobium xichouense
Dendrobium fimbriatum
Dendrobium exile
Dendrobium fanlingshanense
Dendrobium candidum
Dendrobium loddigesii

Figure 4

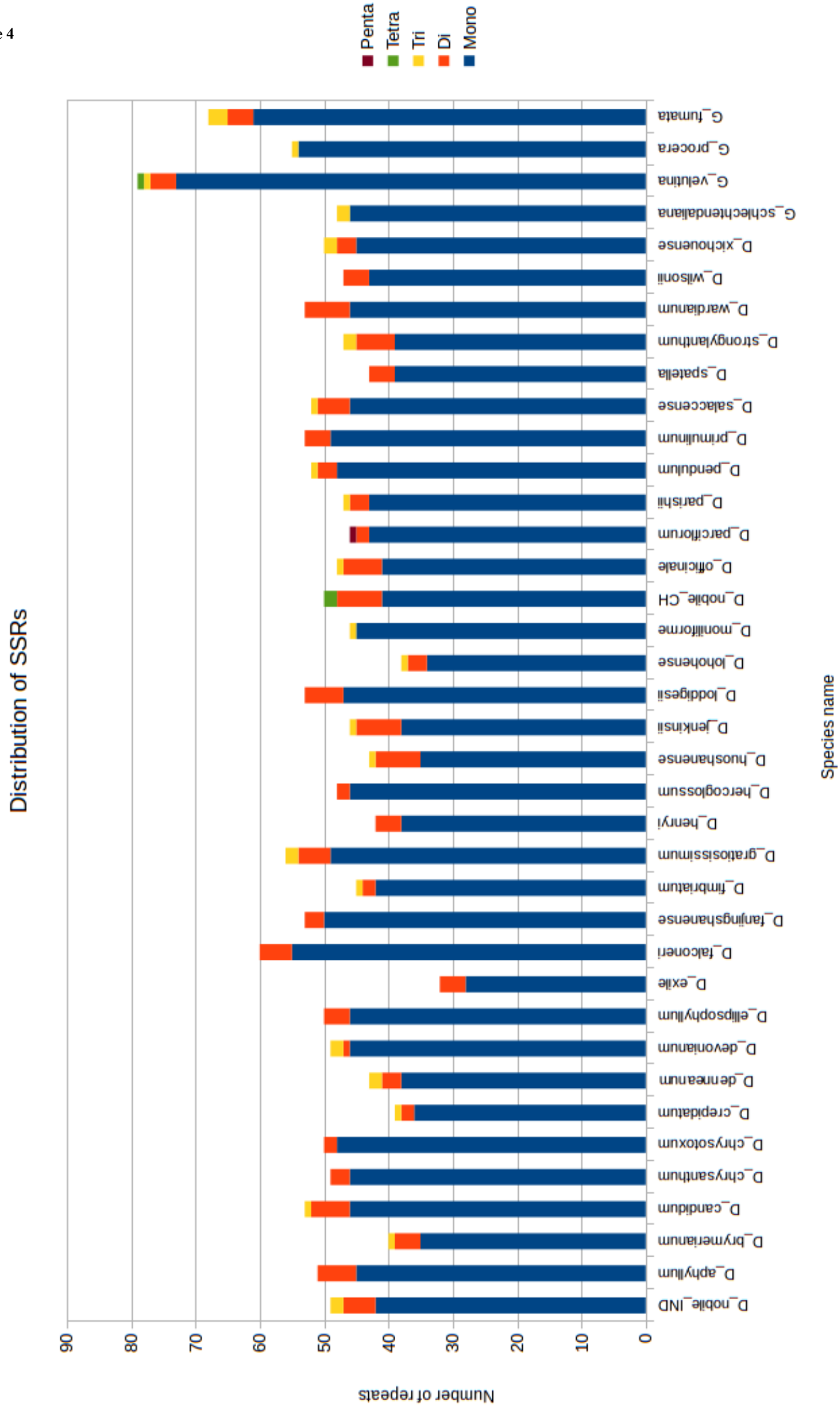

Distribution of SSRs
Penta
Tetra
Tri
Di
Mono
Number of repeats
0
10
20
30
40
50
60
70
80
90
Species name
D_nobile_IND
D_aphyllum
D_brymerianum
D_candidum
D_chrysanthum
D_chrysotoxum
D_crepidatum
D_dennearum
D_devonianum
D_ellipsophyllum
D_exile
D_falconeri
D_fanjingshanense
D_fimbriatum
D_gratiosissimum
D_henryi
D_hercoglossum
D_huoshanense
D_jenkinsii
D_loddigesii
D_lohohense
D_monliforme
D_nobile_CH
D_officinale
D_parcifiorum
D_parishii
D_pendulum
D_primulinum
D_salaccense
D_spatella
D_strongylanthum
D_wardianum
D_wilsonii
D_xichouense
G_schlechtendaliana
G_velutina
G_procera
G_fumata

Figure 5

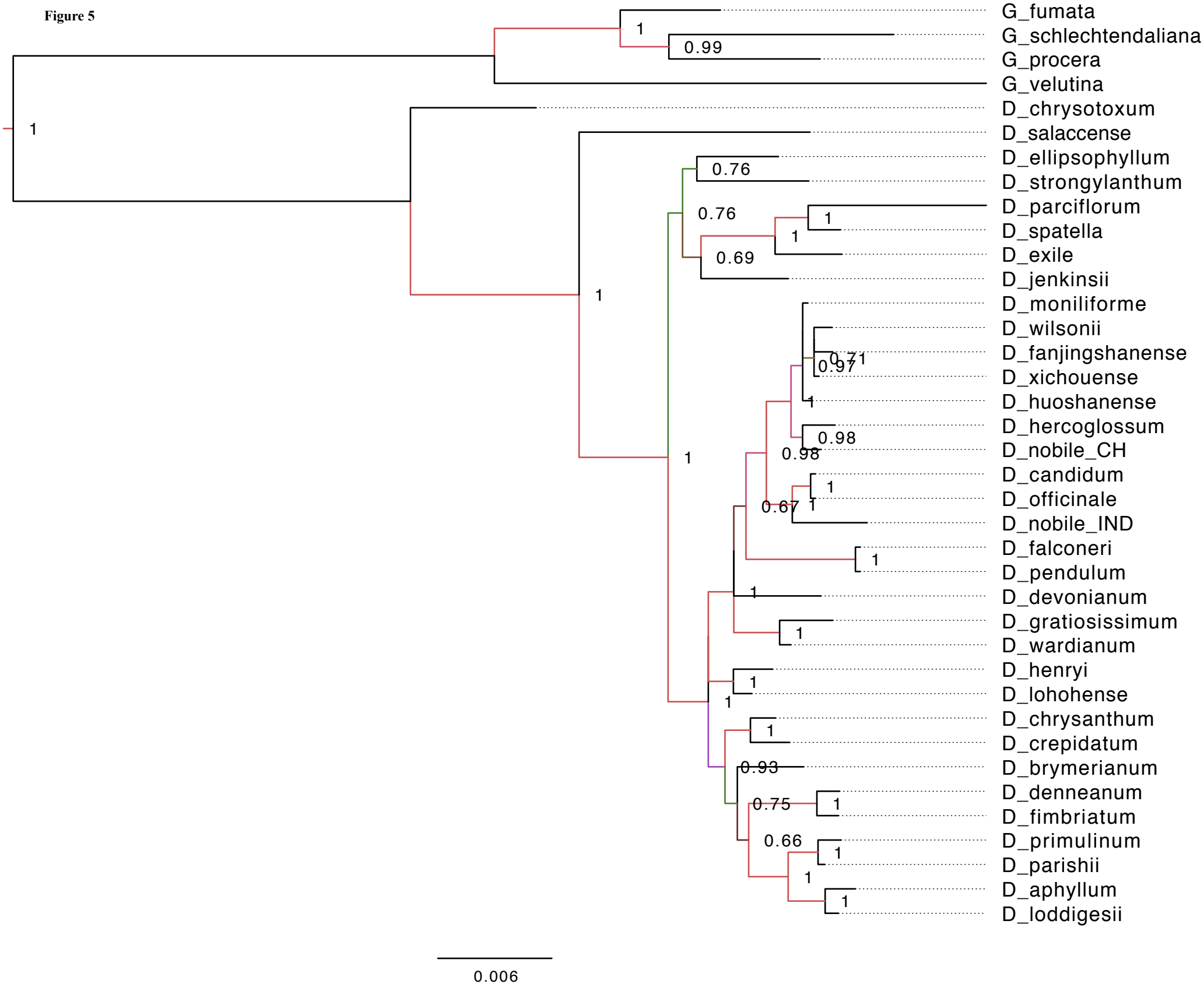
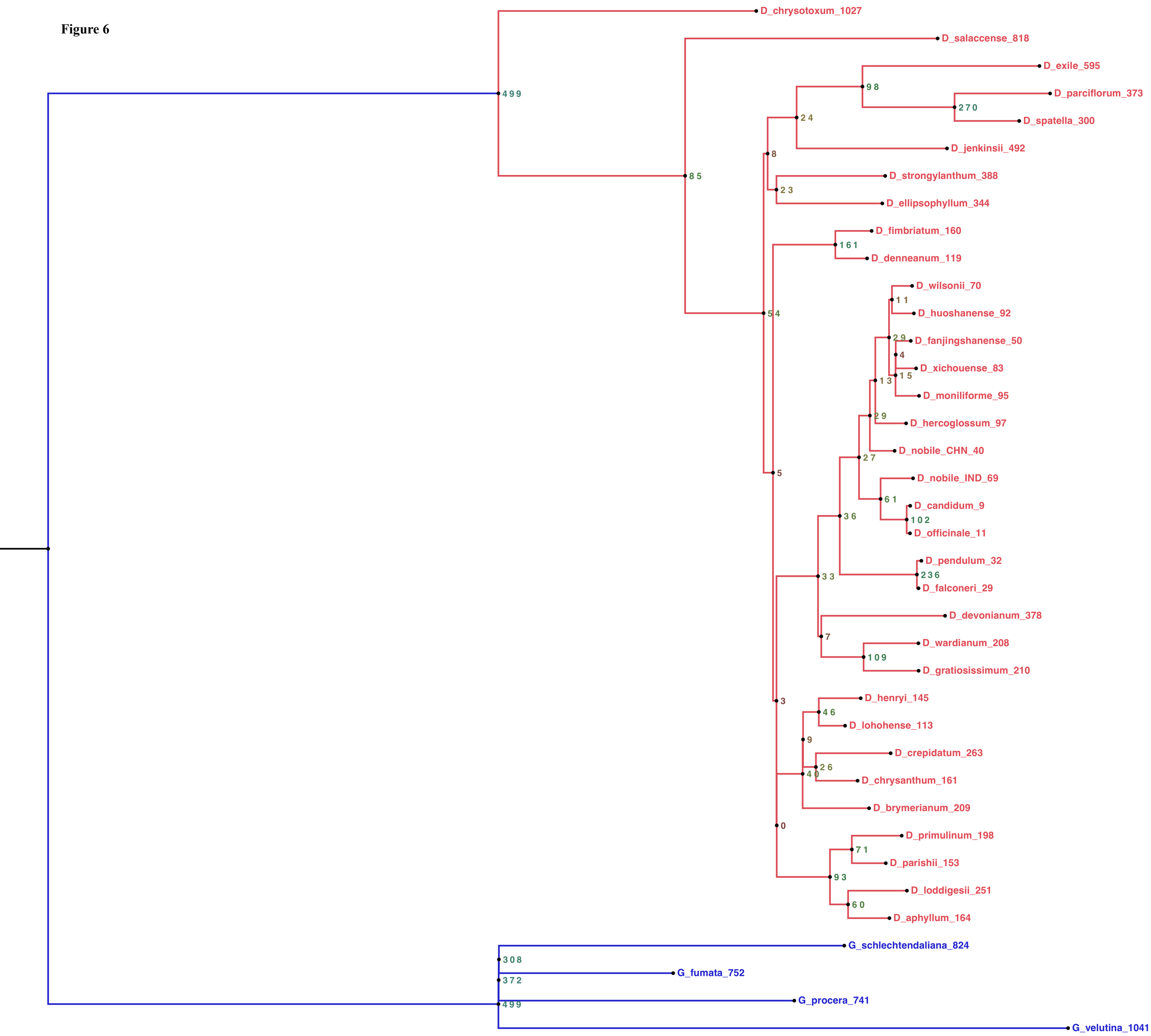

G_fumata
G_schlechtendaliana
G_procera
G_velutina
D_chrysotoxum
D_salaccense
D_ellipsophyllum
D_strongylanthum
D_parciflorum
D_spatella
D_exile
D_jenkinsii
D_moniliforme
D_wilsonii
D_fanjingshanense
D_xichouense
D_huoshanense
D_hercoglossum
D_nobile_CH
D_candidum
D_officinale
D_nobile_IND
D_falconeri
D_pendulum
D_devonianum
D_gratiosissimum
D_wardianum
D_henryi
D_lohohense
D_chrysanthum
D_crepidatum
D_brymerianum
D_denneanum
D_fimbriatum
D_primulinum
D_parishii
D_aphyllum
D_loddigesii
1
0.99
0.76
0.76
0.69
0.71
0.97
0.98
0.98
0.67
0.93
0.75
0.66
0.006

Figure 6


D_chrysotoxum_1027
D_salaccense_818
D_exile_595
D_parciflorum_373
D_spatella_300
D_jenkinsii_492
D_strongylanthum_388
D_ellipsophyllum_344
D_fimbriatum_160
D_denneanum_119
D_wilsonii_70
D_huoshanense_92
D_fanjingshanense_50
D_xichouense_83
D_moniliforme_95
D_hercoglossum_97
D_nobile_CHN_40
D_nobile_IND_69
D_candidum_9
D_officinale_11
D_pendulum_32
D_falconeri_29
D_devonianum_378
D_wardianum_208
D_gratiosissimum_210
D_henryi_145
D_lohohense_113
D_crepidatum_263
D_chrysanthum_161
D_brymerianum_209
D_primulinum_198
D_parishii_153
D_loddigesii_251
D_aphyllum_164
G_schlechtendaliana_824
G_fumata_752
G_procera_741
G_velutina_1041
4 9 9
9 8
2 7 0
2 4
8
8 5
2 3
1 6 1
5 4
1 1
2 9
4
1 5
1 3
2 9
2 7
6 1
1 0 2
3 6
3 3
2 3 6
7
1 0 9
3
4 6
9
2 6
4 0
0
7 1
9 3
6 0
3 0 8
3 7 2
4 9 9