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Comparative analysis of the complete chloroplast genome of two endangered *Dendrobium* species

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Abstract

Dendrobium williamsonii and *Dendrobium cariniferum* (*Orchidaceae*) are endangered perennial herbs, and they are very similar in morphology. Chloroplast genome sequencing technology provides a powerful tool for molecular analysis to get more infomation for phylogenetic analysis and identification of *Dendrobium* species. In this study, the complete chloroplast genomes of *Dendrobium williamsonii* and *Dendrobium cariniferum* were assembled and characterized using *Illumina NovaSeq 6000*. The genome sizes are 159 695 and 159 479 bp, including pairs of inverted repeats (27 055 and 27 024 bp) each separated by small single-copy regions (18 451 and 18 488 bp) and large single-copy regions (87 134 and 86 943 bp). The chloroplast genome overall GC content was 37.11% and 37.13%, respectively. Each chloroplast genome encoded the same number (147) of genes, including 88 protein-coding genes, 51 tRNA genes, and 8 rRNA genes. Comparative analysis of chloroplast genomes revealed the high degree of divergence included *accD-psaL* and *ycf4 -cemA*. The phylogenetic tree showed the two *Dendrobium* species formed only one small clade. A pair of primers that could effectively identify the two *Dendrobium* species were also screened. This study will provide theoretical basis for species identification, genetic breeding, and evolution of *Dendrobium*.

Keywords: chloroplast genome, *Dendrobium*, genetic relationship, sequence divergence, species identification.

Introduction

Dendrobium represents a big genus in family *Orchideaceae*, and there are about 1 500 species worldwide (Feng *et al.* 2015), which are mainly distributed in tropical Asia, subtropical Asia, and Oceania (Zhu *et al.* 2018, Konhar *et al.* 2019). There are at least 80 *Dendrobium* species in China, which occur in the southern regions of the Tsinling Mountains (Tsi *et al.* 1999, Zhu *et al.* 2018). Many species in this genus have been extensively used as herbal medicines for many years in treating diseases, and also the most well known orchids are used in global horticultural trade due to their high ornamental value (Bao *et al.* 2001, Da Silva *et al.* 2016). Nowadays, because of habitat destruction and over-collection, many species of natural *Dendrobium* populations are under severe threat of extinction (Qin *et al.* 2017). Therefore, the protection and effective development and utilization of *Dendrobium* are extremely urgent.

Many species of *Dendrobium* are very similar in appearance and tissue structure, therefore in most cases it is difficult to identify *Dendrobium* species by morphological and anatomic analyses alone (Zhang *et al.* 2005, Niu *et al.* 2018). *Dendrobium williamsonii* and *Dendrobium*

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Abbreviations: cp - chloroplast; IR - inverted repeats; ITS - internal transcribed spacer; LSC - large single-copy; ML - maximum likelihood; SSC - small single-copy.
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cariniferum are ranked as endangered in the Red List of China Higher Plants based on IUCN Red List Categories and Criteria (Qin *et al*. 2017). This two *Dendrobium* species differed only slightly in flower structure. The width of the petal and sepal in *D. cariniferum* is different, the sepal is obviously raised in the middle ribs of the dorsal surface, and the ovary is triangular, but these traits are not in *D. williamsonii* based on Flora of China (Zhu *et al.* 2009) (Fig. 1*E*,*F*). So it is almost impossible to identify the two endangered *Dendrobium* species accurately from their morphology if they are not at flowering stage. *D. cariniferum* is a potential scented *Dendrobium* crossing parent due to its beautiful flowers, which have a pleasant orange fragrance and long-lasting blooms (Zhang and Gao 2021). In addition, *D. cariniferum* contains a variety of medicinal ingredients. *D. cariniferum* is often confused with *D. williamsonii* because of their very similar physical features, thus hindering the normal development and application of the two *Dendrobium* species. Therefore, it is urgent to develop a simple and accurate method for identification of these *Dendrobium* species.

Over the past decades, the promising advances have been achieved in areas of classification, genetic analysis, and selective breeding of *Dendrobium* by molecular markers. A variety of molecular markers like inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and microsatellite (SSR) markers including several other DNA barcode markers from different loci of nucleus have been developed to study *Dendrobium* diversity. However, the *Dendrobium* species are notoriously difficult to identify (Givnish *et al*. 2016). The analysis results are often quite different due to the selection and number of target genes, which is based on DNA sequences in species identification and phylogenetic studies (Luo *et al*. 2014). With the development of sequencing technology, new reference and basis for plant species identification and phylogenetic research are provided by chloroplast DNA (cp DNA) sequencing and comparative analysis.

Chloroplasts are the photosynthetic organelles in plant cells, which provide energy for plant growth and development. The chloroplast genome is an independent genetic element outside the chromosomes, which can replicate itself and usually uniparentally heritable (Sugiura 1992, 1995). Most chloroplast genome sizes range from 120 to 217 kb, which contain 110~130 genes, including 73~86 protein-coding genes, about 30 tRNA and 4 - 8 rRNA (16S, 23S, 4.5S, 5S) (Rodríguez-Ezpeleta *et al*. 2005, Tangphatsornruang *et al*. 2010). Compared with chromosome genome, the chloroplast genome has many advantages such as shorter length, abundant sequence information loci, relatively conservative genome structure and composition (Wakasugi *et al*. 2001, Kahlau *et al*. 2006). Therefore, it is widely used in plant classification, phylogeny, species identification, molecular markers, phylogeny, and transgene studies (Hahn *et al*. 2013).

In this study, complete chloroplast genome sequences of two *Dendrobium* species (*D. williamsonii* and *D.cariniferum*) were first assembled and annotated. Our aim was to determine: *1*) chloroplast genome structure, gene content, and sequence divergence of the two *Dendrobium* species, *2*) the phylogenetic relationship based on the chloroplast genomic data, *3*) primers for effective identification of the two *Dendrobium* species that are very similar in appearance. This study will provide theoretical basis for species identification, breeding, and evolution of *Dendrobium*.

Materials and methods

Plants and DNA extraction: Species were identified and verified by researcher Yunli Jiang, Guizhou Academy of Forestry, Guizhou, China, through morphological identification. The leaf samples of *D. williamsonii* and *D. cariniferum* were collected from Guizhou *Dendrobium* germplasm bank in Guiyang, Guizhou, China (106.73 E, 26.49 N). The specimens were prepared and deposited in the Dendrological Herbarium in Guizhou Academy of Forestry (GZAF, He Li 1043630529@qq.com) with the accession numbers: *D. williamsonii* (202110047) and *D. cariniferum* (202110048). The sample collection was permitted by the Institute of Forestry Biotechnology, Guizhou Academy of Forestry. For extraction of genomic DNA, approximately 100 g healthy fresh leaf tissue per individual were harvested. Total genomic DNA was extracted from healthy fresh leaves with the *Hi-DNAsecure* plant kit *DP350* (*Tiangen Biotech*, Beijing, China), according to the manufacturer's protocols.

Chloroplast genome asembly and annotation: After the genomic DNA isolation, approximately 5 - 10 µg of DNA was sheared, followed by adapter ligation and library amplification. Then, the fragmented DNAs were subjected to *Illumina* sample preparation, and was sequenced by *Shanghai Winnerbio Technology Co.* (Shanghai, China) using *Illumina NovaSeq 6000*. All sequencing depths were over 100×. The subsequent analysis was based on clean reads with high quality. We used the program *MITObim v. 1.4* to perform the reference-guided assemblies using the complete chloroplast genome sequence of *D. officinale* (GenBank number: KJ862886) as reference, and the final circular structure was formed manually.

Annotations of these chloroplast genomes were performed by using *PGA* software (https://github.com/quxiaojian/PGA) (Qu *et al*. 2019) and *GeSeq* (https://chlorobox.mpimp-golm.mpg.de/geseq.html) (Tillich *et al*. 2017) to predict the coding genes, tRNA, and rRNA of the genome, and the boundary regions of start codons, stop codons, and exons/introns in the predicted results were manually corrected. Annotations were performed using the online program *Dual Organellar Genome Annotator* (*DOGMA*) (Wyman *et al*. 2004). All plastome sequences have been uploaded to *NCBI* (Table 1). The circular genome maps were drawn using *CPGview* web server (http://www.1kmpg.cn/cpgview) (Quiñones *et al*. 2023).

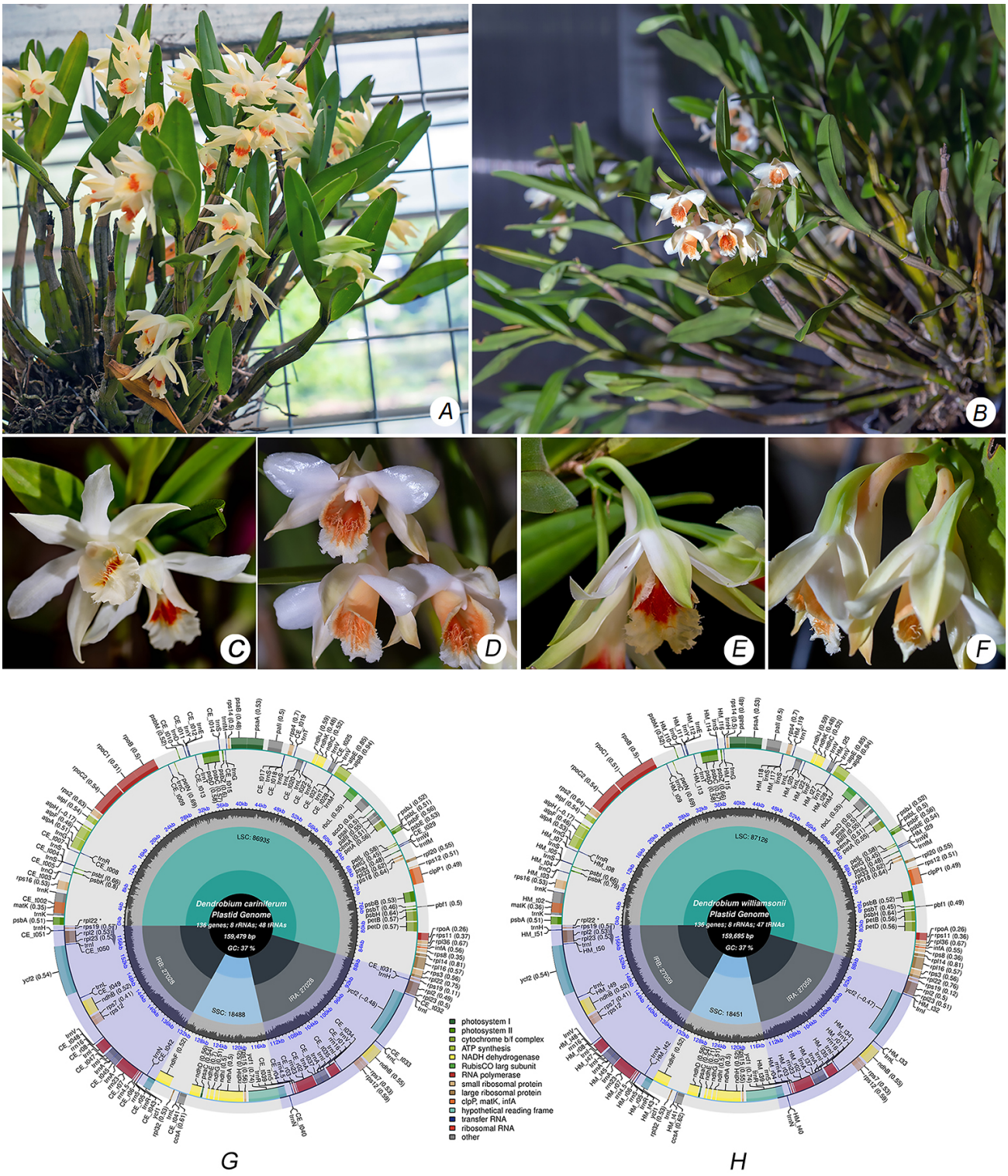

Fig. 1. Images of *Dendrobium* and gene maps of two *Dendrobium* species (*A*, *C*, and *E* are *D. williamsonii*; *B*, *D*, and *F* are *D. cariniferum*. *G* - the chloroplast genome map of *D. cariniferum*; *H* - the chloroplast genome map of *D. williamsonii*.

Chloroplast genome comparative analyses: Four chloroplast genomes of the genus *Dendrobium*, including the two new genomes sequenced in this work, were visualized with the program *mVISTA* (https://genome.lbl.gov/vista/index.shtml) (Mayor *et al*. 2000), and the annotation of *D. officinale* (GenBank number: KJ862886) was used as a reference in the *Shuffle-LAGAN* mode (Frazer *et al*. 2004). The online *IRscope* website

Table 1. Primer name, sequence, and location.

Name	Sequence	Location (*D. cariniferum*)	Location (*D. williamsonii*)	Product variance
Primer 1	F: 5'-GCGTATGCTGTATGTAGTATA-3'	3394..3414	3396..3416	55 bp
	R: 5'-TTCCAAAGTCAAAAGAGTG-3'	4005..3987	4062..4044	
Primer 2	F: 5'-CAACCCAAATCTGATATTGA-3'	48534..48553	48651..48670	66 bp
	R: 5'-TTGAACCCTCACGATTTAA-3'	49076..49058	49259..49241	
Primer 3	F: 5'-CTTCCCTTGAATCAAGATTA-3'	60576..60595	60721..60740	513 bp
	R: 5'-CATATCATATATCCTGCCAT-3'	61581..61562	61213..61194	
Primer 4	F: 5'-AAGAAATCTCAGATAGAATCGACG-3'	62455..62478	62730..62752	34 bp
	R: 5'-CGCAAAAATGGGATATGCAT-3'	63564..63545	63873..63854	
Primer 5	F: 5'-ATGGAAACGTAACAATGGTT-3'	73879..73898	74141..74160	192 bp
	R: 5'-TCTATACCCGATAAGTACCAAT-3'	74479..74458	74549..74528	
Primer 6	F: 5'-GTCTGATACAAAATCCCTTT-3'	76234..76253	76304..76323	90 bp
	R: 5'-AGTTGGAACTTTAGGTGGTT-3'	76922..76903	77082..77063	

(https://irscope.shinyapps.io/irapp/) was used to analyze contraction and expansion of the IR zone boundary (Amiryousefi *et al*. 2018).

Phylogenomic analysis: Phylogenetic trees were constructed using the whole genome, and evolutionary relationships among species were analyzed by clustering of samples. Single-copy orthologous genes were selected from the results analyzed by *OrthoFinder v. 2.5.4* (https://github.com/davidemms/OrthoFinder) (Emms and Kelly 2015, 2019). We used *MAFFT v. 7.505* for multiple sequence alignment (http://mafft.cbrc.jp/alignment/software/) (Zheng *et al*. 2020). Then, *Gblocks v. 0.91b* was used to cut the low quality parts of the results. A phylogenetic tree based on maximum likelihood (ML) method with single copy orthologous genes was built by using *FastTree v. 2.1.11* (http://www.microbesonline.org/fasttree/) (Price *et al*. 2010).

DNA amplification and sequencing: In order to obtain specific primers to identify *D. williamsonii* and *D. cariniferum*, the full-length chloroplast genomes of the two samples were compared globally with the *Align module* in *Vector NTI*. The specific primers were designed separately with *Vector NTI V11.5.1* (https://www.thermofisher.cn/) (Table 1) based on the differences between the two cp genomes, and synthesized by *Shanghai Winnerbio Technology Co*.

Fresh, young leaves of sampled specimens were randomly collected for genomic DNA isolation using the *Hi-DNAsecure* plant kit *DP350*, according to the manufacturer's protocols. PCR was conducted in 20 μL volumes containing 2.0 μL of 10× Taq buffer, 0.2 μL of 5 U Taq, 1.6 μL of 2.5 mM dNTPs mix, 1.0 μL of each primer (the concentration is 0.2 μmol L^{-1}), 1.0 μL of genomic DNA template (the concentration is 20 ng μL^{-1}), and the remaining volume was filled with water (ddH_2O). The amplification was performed in a *MJ Research PTC-100* thermal cycler (Waltham, MA, USA) with a PCR program: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were detected using 2% agarose gel, and sequenced by *Shanghai Winnerbio Technology Co*.

Results

The genome sizes of *D. williamsonii* and *D. cariniferum* were 159 695 bp and 159 479 bp (Table 2). After assembly and annotation, the two cp genomes showed the typical tetrad structure, with a pair of inverted repeats (IRs 27 055 and 27 024 bp) each separated by a small single-copy (SSC) regions (18 451 and 18 488 bp) and a large single-copy (LSC) regions (87 134 and 86 943 bp) (Table 2). The chloroplast genome overall GC content of *D. williamsonii* and *D. cariniferum* were 37.11 and 37.13%, respectively.

When duplicated genes are counted only once, the two *Dendrobium* species' cp genomes contained 145 functional genes including 86 protein-coding genes, 51 tRNA genes, and eight rRNA coding genes (Table 2). The gene numbers and gene orders were identical in chloroplast genomes of two *Dendrobium* species (Table 2). Among the unique genes, 10 genes (*rps16*, *atpF*, *rpoC1*, *petB*, *petD*, *rpl16*, *rpl2*, *ndhB*, *ndhA*, *rpl2*) contained one intron, three genes (*pafI*, *rps12*, *clpP1*) contained two introns in both *D. williamsonii* and *D. cariniferum*. The visualization of two *Dendrobium* species' chloroplast genome structure, size, and gene order were shown in Fig. 1*G*,*H*.

Four cp genomes of species in the genus *Dendrobium*, compared by *mVISTA* with the *Shuffle-LAGAN* method and annotated according to *D. officinale* (KJ862886), was shown in Fig. 2. The variation in *D. williamsonii* and *D. cariniferum* mainly occurred in LSC and SSC regions, which were localized in the intergenic spacers, and intergenic regions with high degrees of divergence included *accD-psaL* and *ycf4-cemA* (Fig. 2). Overall,

Table 2. The features of chloroplast genomes of *Dendrobium* species (LSC - large single-copy region, SSC - small single-copy region, IR - inverted repeat region).

Statistics	*D. williamsonii*	*D. cariniferum*	*D. officinale*	*D. fimbriatum*	*D. longicornu*
Acc. No.	OK173601	OK173600	KJ862886	LC193521	MN227146
Total cp DNA size [bp]	159695	159479	152018	159673	160024
LSC size [bp]	87134	86943	84944	84763	87499
SSC size [bp]	18451	18488	14504	14328	21141
IR size [bp]	27055	27024	26287	30291	25692
Number of genes	145	145	124	137	126
Number of protein-coding genes	86	86	79	91	76
Number of tRNA genes	51	51	37	38	42
Number of rRNA genes	8	8	8	8	8
GC content [%]	37.11	37.13	37.47	37.60	37.15
GC content of LSC [%]	34.79	34.81	35.07	35.15	34.86
GC content of SSC [%]	30.37	30.35	30.32	30.89	31.30
GC content of IR [%]	43.13	43.17	43.37	43.37	43.47

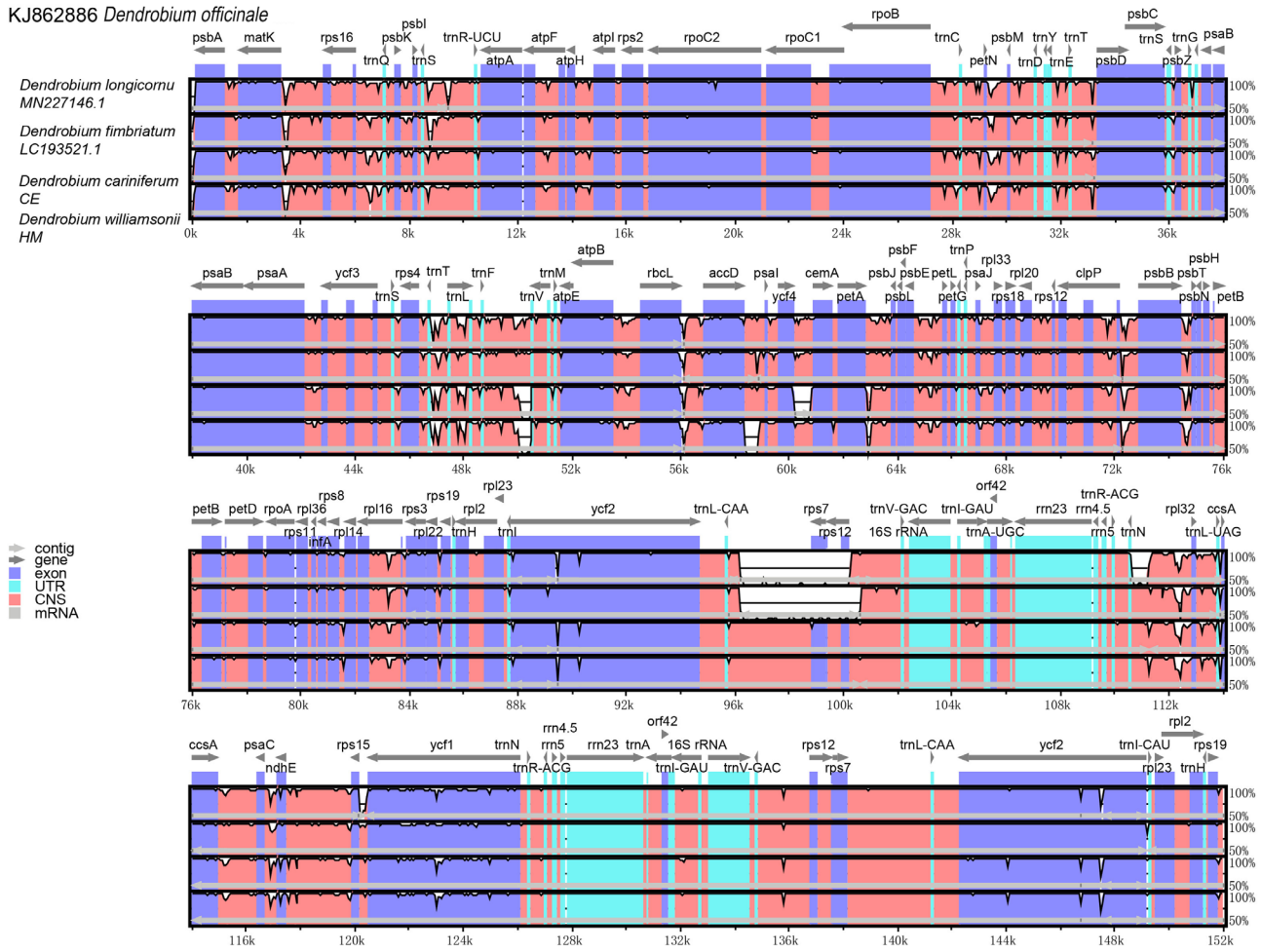

Fig. 2. Visualized alignment of chloroplast (cp) genomes of four *Dendrobium* species with *D. officinale* as a reference. The x-axis represents the base sequence of the alignment and the y-axis represents the pairwise percent identity within 50 - 100%. *The thick gray arrow* at the top of the array indicates gene orientations. *The dark blue regions*, *light blue regions*, *pink regions* and *light gray* represent exon, untranslated region (UTR), conserved coding sequence (CNS), and RNA coding gene, respectively.

the sequence similarity was high among *D. williamsonii* and *D. cariniferum* using *mVISTA*. In the overall four *Dendrobium* species chloroplast genome comparison, the cpDNA coding regions were relatively conserved except for some gene spacer regions (Fig. 2).

The sequences flanking IR/SC junctions were compared between two newly sequenced *Dendrobium* species and three *Dendrobium* species which have been sequenced previously (Fig. 3). The boundaries of IR/LSC rarely changed, but the IR/SSC boundary regions displayed some significant differences in five *Dendrobium* species (Fig. 3). The *ycf1* gene crossed the IRb/SSC boundary region by 5 558 bp in *D. williamsonii* and *D. cariniferum*, and only 309 bp in *D. officinale*, but in *D. fimbriatum*, the *ycf1* gene and *ndhF* gene were overlapped at the IRb/SSC boundary, and in *D. longicornu*, only the *trnN* gene crossed the IRb/SSC boundary region. Meanwhile, the *ndhF* gene crossed the IRa/SSC boundary region by 2 252 bp in *D. williamsonii* and *D. cariniferum*, but the *ycf1* gene crossed the IRa/SSC boundary region by 5 519 bp in *D. fimbriatum* and *D. officinale*, and the *rps15* gene was linked to the *trnN* gene in *D. longicornu*. On the whole, only minor variations were detected at the SC/IR boundaries of *D. williamsonii* and *D. cariniferum* chloroplast genomes.

The phylogenies of *Dendrobium* were examined based on fifty-nine protein-coding genes from the twenty-eight *Dendrobium* cp genomes and six other species (two *Cymbidium* species, two *Paphiopedilum* species, and two *Pleione* species) as the out groups (Fig. 4). Evolutionary trees based on the above data were constructed using maximum likelihood (ML) methods. The closer relationship of *D. williamsonii* and *D. cariniferum* (*versus* other *Dendrobium* species) was clear, and the two *Dendrobium* species formed one small clade alone.

By sequence comparison, six pairs of primers were designed for PCR amplification according to the differential regions of the two *Dendrobium* species, and 2% agarose gel test showed the two *Dendrobium* species could be easily distinguished using the primer 3 and primer 2 (Fig. 5). The PCR products designed by primer 2 were 609 bp and 543 bp, respectively in *D. williamsonii* and *D. cariniferum*, and the size difference was 66 bp (Table 1). The result showed that the PCR product size difference of primer 2 was more than 66 bp in two *Dendrobium* species (Fig. 5). Obviously, primer 2 target region and real amplification were not consistent. The PCR product amplified with primer 3 was sequenced and then compared with chloroplast genome; the result showed that the location of amplification conformed to the design of primers, and the sequences obtained by sequencing were consistent with the expected sequences. These results indicated that the primer 3 (5'-3') CTTCCCTTGAATCAAGATTA and (5'-3') CATATCATATATCCTGCCAT could specifically distinguish *D. williamsonii* and *D. cariniferum*.

Discussion

Structural and sequence comparisons of cp genomes in *Dendrobium*: Many whole cp genomes of *Dendrobium* species were reported in *NCBI*, but no published cp genomes were documented for *D. williamsonii* and *D. cariniferum*. In this study, the chloroplast genomes of *D. williamsonii* and *D. cariniferum* were *de novo* assembled for the first time. The whole chloroplast genome sequence of *D. williamsonii* and *D. cariniferum* was determined to be 159 695 bp and 159 479 bp in length, of which the GC content was 37.11 and 37.13%, respectively. The genome size and GC content in the two *Dendrobium* species were quite similar to other *Dendrobium* cp genomes (Konhar *et al*. 2019). The complete chloroplast genome of the two *Dendrobium* species encoded the same number of genes, including 88 protein-coding genes, 51 tRNA genes, and 8 rRNA genes. In previous studies, the numbers of genes encoded in the cp genome of *Dendrobium* were about 63 - 87 protein-coding genes, 38 - 40 tRNA genes, and 8 rRNA genes (Konhar *et al*. 2019). These results indicated that the chloroplast genome sequences of *Dendrobium* species were diverse. It was worth noting that *D. williamsonii* and *D. cariniferum* not only have similar genome size and genome gene GC content, but

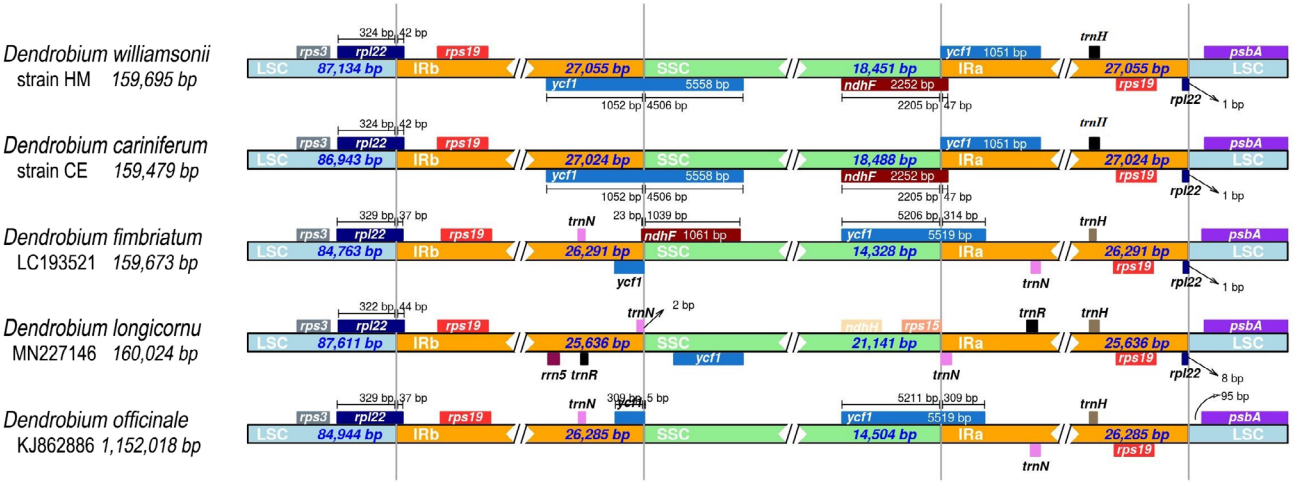

Fig. 3. Comparison of LSC, SSC, and IR borders among five cp genomes of *Dendrobium*.

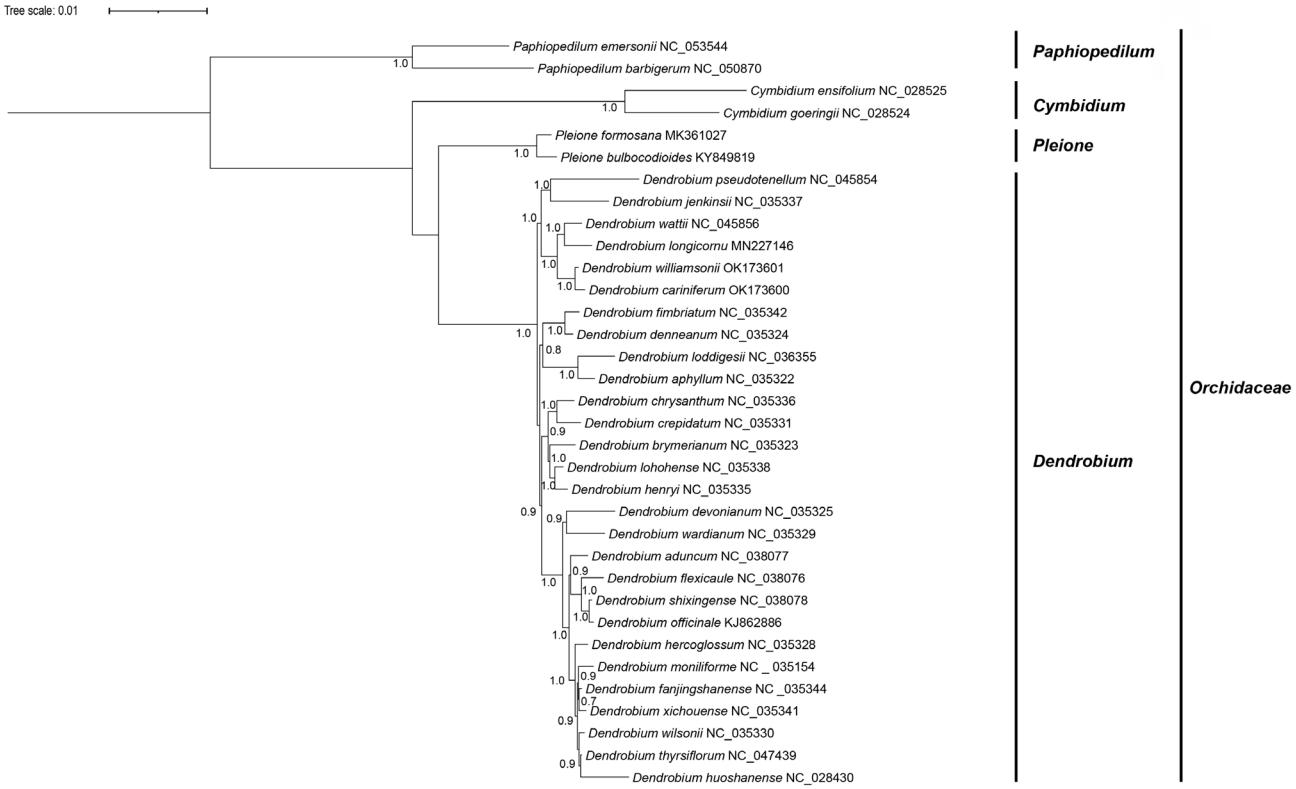

Fig. 4. Phylogenetic tree shows relationship and supported values.

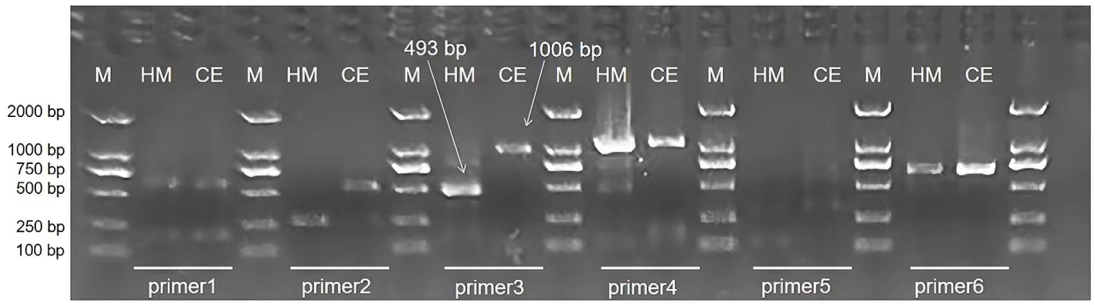

Fig. 5. PCR electrophoresis of six pairs of primers (M - marker; HM - *D. williamsonii*; CE - *D. cariniferum*).

also have the same gene orders. Although cp genomes are highly conserved in terms of genomic structure and size, IR expansion/contraction was usually considered as the reason for size variation in chloroplast genomes (Asaf *et al*. 2016, Dong *et al*. 2016, Yang *et al*. 2016). There was a 216 bp difference in genome size between *D. williamsonii* and *D. cariniferum* chloroplast genomes, probably because of minor variations at the SC/IR boundaries of the two *Dendrobium* species chloroplast genomes. In addition, the sequence similarity was high among *D. williamsonii* and *D. cariniferum* through *mVISTA* analysis. The sequence differences between the two species were mainly the spacer regions of *accD* to *psaL* and *ycf4* to *cemA*.

Phylogenetic analysis: In China, both *D. williamsonii* and *D. cariniferum* belonged to Sect. *Formosae* (Benth. *et* Hook. f.) Hook. F. As we all know, the morphology of *D. williamsonii* and *D. cariniferum* was very similar. So far, there was no molecular evidence for the relationship between *D. williamsonii* and *D. cariniferum*. Phylogenetic analysis using cp genome sequences have resolved numerous lineages within the flowering plants (Jansen *et al*. 2007, Moore *et al*. 2007). Based on the phylogenetic analysis reported here, *D. williamsonii* and *D. cariniferum* had a close relationship with a high correlation, and form one small clade alone. Therefore, we believed that *D. williamsonii* and *D. cariniferum* were treated possibly as sister species according to the morphological and molecular evidence.

DNA barcode development: DNA barcoding has been largely used as a new biological tool to facilitate accurate species identification (Liu *et al*. 2012, Giudicelli *et al*.

2015). Several regions of chloroplast DNA sequences, such as *matK*, *rbcL*, *psbA-trnH*, *atpF-atpH* spacer and the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA, have been advocated as potential plant barcodes (Kress and Erickson 2007, Lahaye *et al*. 2008). Unfortunately, the use of many effective DNA markers (including *rbcL*, *matK* and even the sequences of *ITS* or *ITS2*) could not effectively identify *Dendrobium*, especially for its closely related species because of their close genetic relationships (Zhu *et al*. 2018). Mutational hotspots of plastome have been demonstrated to be conserved among different populations of each species, but polymorphic between various species. Therefore, the mutational hotspots of the plastome are more suitable for the authentication of *Dendrobium* species than those of the ITS region. For example, *D. fimbriatum* was successfully distinguished from other *Dendrobium* species based on the sequence of *psbA-trnH* intergenic spacers (Lu *et al*. 2010). The top ten mutational hotspots *psbB-psbT*, *ndhF-rpl32*, *trnT-trnL*, *rpl32-trnL*, *clpP-psbB*, *trnL* intron, *rpl16-rps3*, *trnE-trnT*, *trnR-atpA*, and *rps16-trnQ* which contain high degree of sequence variability, could be used for the identification of *Dendrobium* species (Zhitao *et al*. 2017). The sequence differences between *D. williamsonii* and *D. cariniferum* were mainly the spacer regions of *accD* to *psaL* and *ycf4* to *cemA*. Therefore, we used these differential sequences to design primers for PCR amplification to effectively distinguish the two *Dendrobium* species. The results showed that this method was feasible to identify the two *Dendrobium* species. Therefore, these mutant "hot spots" could provide sufficient genetic basis for using chloroplast genome as a super-barcode for plant species identification (Hernández-León *et al*. 2013). Further research is necessary to investigate whether these hypervariable regions or complete chloroplast genome sequences could be used as reliable and effective DNA super barcodes for species of *Dendrobium*.

Data availability: The datasets generated during and/or analysed during the current study are available in the [*NCBI*] repository, [*Dendrobium williamsonii* chloroplast, complete genome - nucleotide - *NCBI* (https://www.ncbi.nlm.nih.gov/nuccore/OK173601); *Dendrobium cariniferum* chloroplast, complete genome - nucleotide - *NCBI* (https://www.ncbi.nlm.nih.gov/nuccore/OK173600)]. The associated *BioProject*, *SRA*, and *BioSample* numbers of *Dendrobium williamsonii* are PRJNA910914, SRR22923102, and SAMN32147282 respectively, and the associated *BioProject*, *SRA*, and *BioSample* numbers of *Dendrobium cariniferum* are PRJNA913684, SRR22922860, and SAMN32147278, respectively.

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