Comparative analysis of *Dendrobium* plastomes and utility of plastomic mutational hotspots

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*Dendrobium* is one of the largest genera in Orchidaceae, comprising about 800–1500 species mainly distributed in tropical Asia, Australasia, and Australia. There are 74 species and two varieties of this genus in China. Because of their ornamental and commercial value, *Dendrobium* orchids have been studied at low taxonomic levels. However, structural changes and effective mutational hotspots of *Dendrobium* plastomes have rarely been documented. Here, 30 *Dendrobium* plastomes were compared, comprising 25 newly sequenced in this study and five previously published. Except for their differences in NDH genes, these plastomes shared identical gene content and order. Comparative analyses revealed that the variation in size of *Dendrobium* plastomes was associated with dramatically changed length of InDels. Furthermore, ten loci were identified as the top-ten mutational hotspots, whose sequence variability was almost unchanged with more than 10 plastomes sampled, suggesting that they may be powerful markers for *Dendrobium* species. In addition, primer pairs of 47 polymorphic microsatellites were developed. After assessing the mean BS values of all combinations derived from the top-ten hotspots, we recommend that the combination of five hotspots—*trnT-trnL*, *rpl32-trnL*, *clpP-psbB*, *trnL intron*, and *rps16-trnQ*—should be used in the phylogenetic and identification studies of *Dendrobium*.

*Dendrobium*, a genus of the tribe Dendrobieae (Orchidaceae: Epidendroideae), is one of the largest genera in Orchidaceae with approximately 800–1500 species mainly distributed in tropical Asia, Australasia, and Australia. There are 74 species and two varieties of this genus in China, some of which are well known as flowers of Father’s Day in many Asian countries. *Dendrobium* orchids are popular not only for their aesthetic appeal, primarily reflected in their unique flower characteristics, but also for their medicinal value. Owing to their strong health care effects, such as nourishing the kidney, benefiting the stomach, enhancing the body’s immunity, resisting cancer, and prolonging life, many species in this genus have been extensively used as Traditional Chinese Medicine (TCM) for hundreds of years. However, many wild *Dendrobium* species are in extreme danger of extinction (IUCN Redlist of higher plants in China, http://www.zhb.gov.cn/gkml/hbb/bgg/201309/t20130912_260061.htm) due to their low germination rate, slow growing, habitat deterioration, and being over-exploited.

Because of their ornamental and commercial value, *Dendrobium* orchids have attracted intense attention of researchers, leading to numerous taxonomic studies published, particularly in species identification. However, *Dendrobium* species are notoriously difficult to identify. Traditional methods for identifying *Dendrobium* species are based on their morphological characteristics, while many species have overlapping morphological variations due to environmental factors and pollinator selection pressure. Furthermore, after intensively processed, the shoots of *Dendrobium* species become more difficult to distinguish. Therefore, it is urgent to develop a simple and accurate method for identification of *Dendrobium* species.

Recently, a variety of molecular markers have been developed for the studies of *Dendrobium* in terms of species identification, population genetics, and phylogeny. Microsatellite (SSR) markers have been employed to study population genetics of *Dendrobium* species and to investigate the species relationships. Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers are also available for *Dendrobium*. In addition, DNA barcode has been adopted to identify *Dendrobium* species, involving different loci or their combinations, e.g. *ITS14, 15*, *ITS2*, *ITS + matK16*, and *rbcL + matK17*. However, many of these studies resulted in inconsistent conclusions because of using limited number of DNA sequences.

The chloroplast is one of the essential organelles in plant cells, having its own genome called plastome. Plastomes are an ideal resource for selecting mutational hotspots in various lineages because of their maternal inheritance and their lack of recombination among their genes. The plastome sequence is highly conserved in species within a genus and contains many genetic markers. As a result, it is an appropriate tool for phylogenetic studies and evolutionary biology.

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mode of inheritance, dense gene content, and slower evolutionary rates relative to those of nuclear and mitochondrial genomes\(^{17,18}\). A number of hotspots, including \(rbcL\), \(matK\), and \(psbA-trnH\), have been successfully applied to plant species identification and phylogenetic studies\(^{19-21}\). Recently, the comparative plastomic method has been available for mutational hotspot selection, which uses at least two complete plastomes within the study genus to screen for the most informative regions\(^{22,23}\). For instance, the available for mutational hotspot selection, which uses at least two complete plastomes within the study genus to screen for the most informative regions\(^{22,23}\). For instance, the \(psbA-trnH\) and \(trnF-ndhJ\) regions in orchids were demonstrated to be the most useful markers for the phylogenetic analysis of \(Oncidium\)\(^{24}\); and the noncoding loci \(rpl32-trnL\), \(trnE-trnT\), \(trnH-psbA\), \(trnK-rps16\), and \(trnT-trnL\) were shown to be effective in identifying species of \(Cymbidium\)\(^{25}\). However, comprehensive plastome-wide investigation has not been conducted on more powerful loci, which, however, are important for low taxonomic level studies of \(Dendrobium\) species.

In this investigation, we compared 30 plastomes of important \(Dendrobium\) species that contents great medical worth, including the 25 newly sequenced. Our aims were: (1) to evaluate the evolution of \(Dendrobium\) plastomes; (2) to identify more powerful mutational hotspots for low taxonomic level studies of \(Dendrobium\) species on the basis of a wide range of sampling. To achieve these aims, the Maximum likelihood (ML) approach was adopted to evaluate potential hotspot combinations by assessing their mean bootstrap (BS) values.

### Result

**Genome features.** The 25 newly sequenced \(Dendrobium\) plastomes ranged from 150,073 to 152,108 bp in length, with the smallest one belonging to \(D. parciflorum\) while the largest falling into \(D. fianjinghanense\) (Table 1). All plastomes possessed the ancestral angiosperm plastome organization that consisted of a LSC region of 84,273–84,980 bp, a SSC region of 13,821–14,514 bp, and a pair of IR regions of 26,175–26,309 bp each (Table 1, Figure S1a). Similar to other orchid plastomes, \(Dendrobium\) plastomes were also AT-rich (62.27–62.69%). Except for their differences in the total length and composition of retained NDH genes, all plastomes shared identical complements of coding genes, each containing 30 unique tRNA genes, four unique rRNA genes, and 68 unique protein-coding genes. The sequence of eleven NDH genes of \(Dendrobium\) species were compared to \(Cypridipedium formosanum\) (NC_026772), which contains full set of functional NDH genes in orchids (Figure S1b). However, like other Epidendroideae species (e.g. \(Cymbidium\), \(Oncidium\), and \(Phalaenopsis\)), \(Dendrobium\) also experienced the loss of plastid NDH genes. Among them, only \(ndhB\) genes in IR regions were functional with full reading frames, whereas other ten plastid NDH genes were truncated or completely lost.

**InDels coincide with the variation of plastome.** Thirty plastomes of \(Dendrobium\), including our newly sequenced 25, were compiled for comparison. These plastomes experienced different degrees of NDH gene loss, in which the total length of retained NDH genes varied from 3,687–6,336 bp (Table S1). On the other side, the total length of retained NDH genes was uncorrelated with the plastome length (Spearman’s \(r = 0.163, P > 0.05\). In

| Species Name | Plastome length (bp) | LSC region (bp) | SSC region (bp) | IR region (bp) | AT content (%) | Accession | No. vouchers specimen |
|-------------|---------------------|----------------|----------------|----------------|----------------|-----------|----------------------|
| Dendrobium aphyllum | 151524 | 84588 | 14320 | 26308 | 62.40% | LC192953 | NZT2015001 |
| Dendrobium brymerianum | 151830 | 84855 | 14377 | 26299 | 62.40% | LC192954 | NZT2015002 |
| Dendrobium chrysanthaum | 151790 | 84757 | 14441 | 26296 | 62.44% | LC193514 | NZT2015003 |
| Dendrobium chrysotoxum | 151731 | 84785 | 14356 | 26295 | 62.37% | LC193517 | NZT2015004 |
| Dendrobium crepidatum | 151717 | 84811 | 14383 | 26262 | 62.43% | LC193509 | NZT2015005 |
| Dendrobium denneanum | 151565 | 84657 | 14344 | 26282 | 62.37% | LC192955 | NZT2015006 |
| Dendrobium devonianum | 151945 | 84966 | 14435 | 26272 | 62.45% | LC192956 | NZT2015007 |
| Dendrobium ellipsophyllum | 152026 | 84930 | 14488 | 26304 | 62.50% | LC193519 | NZT2015008 |
| Dendrobium exile | 151294 | 84363 | 14315 | 26308 | 62.32% | LC193522 | NZT2015009 |
| Dendrobium falconeri | 151890 | 84862 | 14448 | 26290 | 62.51% | LC192957 | NZT2015010 |
| Dendrobium fianjinghanense | 152108 | 84990 | 14514 | 26302 | 62.49% | LC193523 | NZT2015011 |
| Dendrobium formosanum | 151673 | 84763 | 14328 | 26291 | 62.40% | LC193521 | NZT2015012 |
| Dendrobium gratosissimun | 151829 | 84890 | 14359 | 26290 | 62.43% | LC192958 | NZT2015013 |
| Dendrobium henryi | 151850 | 84878 | 14366 | 26303 | 62.44% | LC193513 | NZT2015014 |
| Dendrobium hercoglossum | 151939 | 84924 | 14397 | 26309 | 62.44% | LC192959 | NZT2015015 |
| Dendrobium jenkinsii | 151717 | 84734 | 14413 | 26285 | 62.40% | LC193515 | NZT2015016 |
| Dendrobium leohense | 151812 | 84876 | 14352 | 26292 | 62.44% | LC193516 | NZT2015017 |
| Dendrobium parciflorum | 150073 | 84442 | 13975 | 26175 | 62.27% | LC192810 | NZT2015020 |
| Dendrobium primulinum | 150767 | 84588 | 14320 | 26308 | 62.69% | LC193510 | NZT2015021 |
| Dendrobium salaccense | 151104 | 84273 | 14315 | 26258 | 62.69% | LC193511 | NZT2015022 |
| Dendrobium spatella | 151829 | 84794 | 14419 | 26308 | 62.42% | LC193511 | NZT2015022 |
| Dendrobium wardianum | 151788 | 84835 | 14359 | 26297 | 62.43% | LC192961 | NZT2015023 |
| Dendrobium waloswii | 152080 | 84988 | 14480 | 26306 | 62.49% | LC193508 | NZT2015024 |
| Dendrobium xichouense | 152052 | 84980 | 14486 | 26293 | 62.49% | LC193520 | NZT2015025 |

Table 1. Characteristics of the 25 newly sequenced \(Dendrobium\) plastomes.
addition, the changed lengths of LSC, SSC, IRs, and whole plastome were compared between each tested species and *D. officinale*. Our analysis indicated that the changed length of LSC, which retains only a few *ndh* residues, was strongly correlated with the changed length of plastome (Spearman’s *r* = 0.908, *P* < 0.01). Meanwhile, the changed lengths of SSC (retaining most of the NDH genes) and IRs (its expansion/contraction having a direct impact on plastome size) were medially correlated with the changed length of *Dendrobium* plastome (Spearman’s *r* = 0.634, 0.721, *P* < 0.05). These results suggested that the changed length of LSC occupied an important position in the changes of plastome sizes.

InDel mutations in plastome were compared between each tested *Dendrobium* species and *D. officinale* (Fig. 1). As a result, a total of 123–352 InDels were identified among these plastomes, with 84–274 in LSC, 18–69 in SSC, and 10–47 in IRs. The InDels located in LSC region accounted for 65–82%; this proportion was significantly greater than those for the InDels situated in SSC and IRs (Mann-Whitney 2-sides, *P* < 0.05), indicating that the locations of InDels in plastome were nonrandom. In order to evaluate the relationship between the variation of *Dendrobium* plastome size and InDel changes, we determined the changed length of InDels based on the differences between insertions and deletions and divided them into two parts: NDH gene-related InDels change and NDH gene-unrelated InDels change (Table S2). The changed length of NDH gene-unrelated InDels was significantly larger than that of NDH gene-related InDels, which was caused by the loss of NDH genes (Mann-Whitney 2-sides, *P* < 0.05). Moreover, the changed length of NDH gene-unrelated InDels was strongly correlated with the variation of plastome size (Spearman’s *r* = 0.867, *P* < 0.01), suggesting that the variation of *Dendrobium* plastome size was largely due to the changed length of InDels.

**Mutational hotspots in *Dendrobium* plastomes.** We identified 92 syntenic intergenic and intronic loci, each longer than 150 bp. Three of them (*matK, rbcL* and *psbA-trnH*) had been widely used as DNA barcode owing to their high variability. Sequence variability (SV) was calculated for each of these loci (Fig. 2 and Table S3). It has been reported that plastomic mutational hotspots are accompanied by biased AT compositions. Consistently, our study showed that the SV of a locus was negatively correlated with its GC content (Spearman’s *r* = −0.809, *P* < 0.01).

Figure 3 shows the SV of the top-ten mutational hotspots from the 25 newly sequenced plastomes. All of these hotspots except *trnL* intron were intergenic spacers. To examine whether the SV of these hotspots changes with increasing number of sampled plastomes, we evaluated their SV rankings among six groups that were randomly composed of different numbers of *Dendrobium* plastomes (Table 2). Only five to six of these mutational spots ranked in the top ten hotspots when sampled plastomes were fewer than ten. However, when more than ten plastomes were sampled, these mutational spots consistently ranked in the top ten. These results indicated that the consistency of the SV of these mutational spots rose with increasing number of sampled plastomes. Therefore, the top ten hotspots (Fig. 2) could be powerful markers for phylogenetic and identification studies of *Dendrobium* species.

**Microsatellites.** We totally retrieved 47 polymorphic SSRs, which are present in at least 15 species, from 92 syntenic intergenic and intronic loci (Table 3). These SSRs consisted of two types: mononucleotide SSRs (44 A/T type and one C/G type), ranging from 8 to 16 nucleotide repetitions; and dinucleotide SSRs (TA), six of them (*trnT-trnL, trnL intron, trnE-trnT*, *tronR-tpA*, and *rps16-trnQ*) were among the top ten hotspots. The SSRs were mainly distributed in LSC, while only one was located in SSC and three in IRs. This signified that the distribution of SSRs was dependent on their locations in plastomes. Our result also revealed that 37 SSRs were located in intergenic spacer regions and 10 SSRs in introns. Primer pairs were developed for all the SSRs (Table 3), which could be used in the amplification of SSRs in *Dendrobium* species for future studies.

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**Figure 1.** InDel distribution among different *Dendrobium* plastomes. The InDel mutations were determined based on the comparison between plastomes of each tested *Dendrobium* species and *D. officinale*. Histograms with different colors indicate the numbers of InDels in LSC, SSC, and IR regions.
Figure 2. The sequence variability (%) and GC content among the 92 syntenic intergenic and intronic loci from Dendrobium plastomes. The red and green lines represent the sequence variability (%) and GC content of each locus, respectively. These syntenic loci are oriented according to their locations in the plastome. The top-ten syntenic intergenic and intronic loci with the highest sequence variability (%) in the tested Dendrobium plastomes were indicated with blue box.

Table 2. SV rankings of the top-ten mutational hotspots in six groups. “—” Means this locus ranked outside the top-ten mutational hotspots. Group A: D. primulinum, D. gratiosissimum. Group B: D. primulinum, D. gratiosissimum, D. hercoglossum, D. falconeri, D. wardianum. Group C: D. primulinum, D. gratiosissimum, D. hercoglossum, D. falconeri, D. wardianum, D. aphyllum, D. devonianum, D. brymerianum, D. denneanum, D. wilsonii. Group D: D. primulinum, D. gratiosissimum, D. hercoglossum, D. falconeri, D. wardianum, D. aphyllum, D. devonianum, D. brymerianum, D. denneanum, D. wilsonii D. spatella, D. crepidatum, D. salaccense, D. ellipsophyllum, D. fangjingshanense. Group E: D. primulinum, D. gratiosissimum, D. hercoglossum, D. falconeri, D. wardianum, D. aphyllum, D. devonianum, D. brymerianum, D. denneanum, D. wilsonii D. spatella, D. crepidatum, D. salaccense, D. ellipsophyllum, D. fangjingshanense, D. chrysotoxum, D. parciflorum, D. exile, D. lohohense, D. fimbriatum. Group F: D. primulinum, D. gratiosissimum, D. hercoglossum, D. falconeri, D. wardianum, D. aphyllum, D. devonianum, D. brymerianum, D. denneanum, D. wilsonii D. spatella, D. crepidatum, D. salaccense, D. ellipsophyllum, D. fangjingshanense, D. chrysotoxum, D. parciflorum, D. exile, D. lohohense, D. fimbriatum, D. chrysanthum, D. henryi, D. xichouense, D. jenkinsii, D. parishii.

Figure 3. Relation between the number of combined hotspots and the mean value of bootstrap (BS). The mean (±SD) BS values were calculated based on different numbers of combined hotspots. Regression analysis: $R^2 = 0.904$, $y = 3.64x + 49.38$. The plateau of the mean BS value reached 71% when five hotspots were combined.
| NO. | Primer name | Position | Region | Location | SSR Type | Primer sequence (5′-3′) | Length | Tm |
|-----|-------------|----------|--------|----------|----------|-------------------------|--------|----|
| 1   | Den ptssr1  | trnT-trnL | LSC    | spacer   | AT × 6   | AGAAAAATCTTACATTTTTCT  | 232    | 51 |
|     |             |          |        |          |          | CATTGATGATGGCCATAT     |        |    |
| 2   | Den ptssr2  | trnT-trnL | LSC    | spacer   | AT × 6   | CTTAAATAGGAATTTCCCT    | 185    | 53 |
|     |             |          |        |          |          | CCAATTACCCCTTCTCTAA    |        |    |
| 3   | Den ptssr3  | trnL intron | LSC  | intron   | T × (8–14) | TGGATTGAGCTTGTGATA    | 231    | 50 |
|     |             |          |        |          |          | TCCCTTCGTCTTTGAGTT     |        |    |
| 4   | Den ptssr4  | trnE-trnT | LSC    | spacer   | A × (8–10) | AATATGACTCTACGACTCC    | 180    | 52 |
|     |             |          |        |          |          | TGACCCGATGACTAGCGAA    |        |    |
| 5   | Den ptssr5  | trnR-2psrA | LSC  | spacer   | A × 9    | TTGGACCGATTTATTTCCT    | 250    | 52 |
|     |             |          |        |          |          | CGAAGAGCTGAACACCTTT    |        |    |
| 6   | Den ptssr6  | rps16-trnQ | LSC  | spacer   | A × (8–13) | AAGTCTCGTGTTAAGTTAT    | 225    | 53 |
|     |             |          |        |          |          | ATGTTGAGGATCAGGAA      |        |    |
| 7   | Den ptssr7  | rpi16 intron | LSC  | intron  | T × (8–11) | CTCCTTGCTATCCCTCCAT    | 225    | 51 |
|     |             |          |        |          |          | CCCACCTGATAACGATGCT    |        |    |
| 8   | Den ptssr8  | petN-pkbM | LSC    | spacer   | A × (8–10) | TCCCTTGCTATGGGGAAG     | 232    | 50 |
|     |             |          |        |          |          | GAGATTAAAGAAGAAGATCT   |        |    |
| 9   | Den ptssr9  | trnL-trnF | LSC    | spacer   | T × (8–15) | TCCCTGCCTTTATTATCC     | 209    | 54 |
|     |             |          |        |          |          | CTTAACGGAGATCACGAGAA   |        |    |
| 10  | Den ptssr10 | rps18-rpl14 | LSC  | spacer   | T × (8–11) | TAGTTATTTGCTCTTCCCT    | 160    | 51 |
|     |             |          |        |          |          | TTACGTAAATACGCGGATTA   |        |    |
| 11  | Den ptssr11 | pkbK-pkbI | LSC    | spacer   | A × (8–10) | AAGGAAAAAGCGATGAA     | 241    | 53 |
|     |             |          |        |          |          | AAAGGAAAAAAGGACAAA      |        |    |
| 12  | Den ptssr12 | pkbK-pkbI | LSC    | spacer   | A × (8–10) | CTTTAAATCAAGTAACCTTT   | 225    | 50 |
|     |             |          |        |          |          | CTATTGAGATGAGCCTCAA    |        |    |
| 13  | Den ptssr13 | pkbK-pkbI | LSC    | spacer   | A × (8–10) | AGATATGAGATGGCGAAGAAA  | 217    | 52 |
|     |             |          |        |          |          | TACAAATCTCCAAGATAAGAT  |        |    |
| 14  | Den ptssr14 | ccsA-nbhd | SSC    | spacer   | T × (8–11) | AATCTGCTGATACGGAAGTC    | 174    | 56 |
|     |             |          |        |          |          | TGGACTCTTTCATTTACGAGA  |        |    |
| 15  | Den ptssr15 | rps16 intron | LSC  | intron  | T × (8–11) | AACTCAAGTTGGAATATTTGT  | 224    | 51 |
|     |             |          |        |          |          | TAAAGGATCAAGGAATAGGAT   |        |    |
| 16  | Den ptssr16 | pkbA-trnK | LSC    | spacer   | T × (8–14) | CTATGCCGAATGTACCAACT   | 246    | 55 |
|     |             |          |        |          |          | CTTCTTTAAACTCTCTCCTCAA |        |    |
| 17  | Den ptssr17 | matS-trnK | LSC    | intron   | A × (8–10) | AATCTGCTTTTTGACCTTGGAA | 214    | 54 |
|     |             |          |        |          |          | AATTTGACAGTTACCCTGAC    |        |    |
| 18  | Den ptssr18 | matS-trnK | LSC    | intron   | A × (8–10) | CTTACCTGGAATGAGCCATA   | 216    | 55 |
|     |             |          |        |          |          | CCGCGACTGATCCTGAAAGGT  |        |    |
| 19  | Den ptssr19 | atpB-rbcL | LSC    | spacer   | T × (8–14) | ATAGCTAAGGTTATGTTTAT    | 224    | 51 |
|     |             |          |        |          |          | CTAGATGATGAAAGGCGGATA  |        |    |
| 20  | Den ptssr20 | atpB-rbcL | LSC    | spacer   | T × (8–14) | TCTTACGTCTATTACCTTTCG  | 262    | 50 |
|     |             |          |        |          |          | GAGATTGAAGAAATAATGATAGT |        |    |
| 21  | Den ptssr21 | atpB-rbcL | LSC    | spacer   | T × (8–14) | CTAATCTTATTACCTTTTCC   | 255    | 50 |
|     |             |          |        |          |          | GAGAATAAATGAAATGATAGTA  |        |    |
| 22  | Den ptssr22 | trnC-petN | LSC    | spacer   | A × (8–12) | ATCTCTTGATCGAATCGTTAC | 216    | 54 |
|     |             |          |        |          |          | CAAATTGCAATACCGCAACC   |        |    |
| 23  | Den ptssr23 | trnC-petN | LSC    | spacer   | A × (8–12) | ACTGATTGATACGCAGAATCA  | 218    | 50 |
|     |             |          |        |          |          | TCTTACTACGGCTTTTATG    |        |    |
| 24  | Den ptssr24 | trnC-petN | LSC    | spacer   | A × (8–12) | ACTAGAGGCCTCTGAGTTGTC  | 235    | 55 |
|     |             |          |        |          |          | TCAATGTTGAGATGATGTTG   |        |    |
| 25  | Den ptssr25 | rps18-rpl20 | LSC   | spacer   | A × 8    | AAACCTCAATAGGAATACAGG  | 213    | 52 |
|     |             |          |        |          |          | ACAAGAGTTGATGGAAACAGGA |        |    |
| 26  | Den ptssr26 | petA-pkbI | LSC    | spacer   | T × (8–14) | AATAAGTGTTGAAAAAGTGC   | 189    | 51 |
|     |             |          |        |          |          | TCCCTTTGTTTTATGTTGTTCC  |        |    |
| 27  | Den ptssr27 | psbK-cemA | LSC    | spacer   | T × (8–14) | AGGAAGAAAAGAGAGGAAATC  | 217    | 54 |
|     |             |          |        |          |          | CCTATACATTACAAAGACA    |        |    |
| 28  | Den ptssr28 | rps2-rpoC2 | LSC    | spacer   | T × (9–13) | CATTATATTGATACGACCCA   | 207    | 55 |
|     |             |          |        |          |          | CTAATACCTAAAGCATTAGTTA |        |    |

Continued
Additionally, we also performed the phylogenetic analyses based on the trnT-trnL combination. The top ten combinations that yielded highest BS values are shown in Table S5, of which only the combination comprising one, two, and three hotspots (Fig. 3). The plateau of mean BS value reached 71% when five hotspots were combined, then rising slightly with further increasing number of hotspots in a combination; the greatest variations existed among the three combinations comprising one, two, and three hotspots (Fig. 3). The variance among combinations declined with increasing number of hotspots in a combination. On the other side, the variance among combinations climbed with increasing number of hotspots in a combination. On the other side, the variance among combinations declined with increasing number of hotspots in a combination; the greatest variations existed among the three combinations comprising one, two, and three hotspots (Fig. 3). The plateau of mean BS value reached 71% when five hotspots were combined, then rising slightly with further increasing number of hotspots in a combination. The top ten combinations that yielded highest BS values are shown in Table S5, of which only the fourth combination consisted of five hotspots (trnT-trnL, rpl32-trnL, clpP-psbB, trnL intron, and rps16-trnQ). Additionally, we also performed the phylogenetic analyses based on rbcL, matK, psbA-trnH and their combinations. Our results showed that the phylogenetic relationships based on the combination of trnT-trnL, rpl32-trnL, clpP-psbB, trnL intron, and rps16-trnQ had a better resolution than other plastid DNA data (Fig. 4).

**Discussion**

In orchids, plastid NDH genes experienced independent loss. The dramatic NDH gene loss/retention has facilitated comparative plastome studies of orchid species. Recently, Kim et al. proposed that the loss of
NDH genes led to the expansion/contraction of IRs. It has been documented that independent loss/retention of NDH genes and expansion/contraction of IRs are largely responsible for the variation of plastomes in different orchid genera and many other species, such as gymnosperms, pines, slender naiad, and saguaro. However, our study demonstrated that neither NDH genes loss/retention nor the expansion/contraction of IRs accounted for the most important role in the variation of Dendrobium plastome sizes (Table S1).

Comparative analyses have shown that InDels commonly occur in plastomes. It is known that InDels are very useful for resolving phylogenetic relationship and can serve as biomarkers. For example, in the Pinaceae plastomes, nine InDels are able to resolve the phylogenetic relationships among different Pinaceae subfamilies. In an investigation of the Fagopyrum plastomes, a number of InDel markers were identified and demonstrated to be effective in differentiating raw or processed buckwheat products. On the other side, few studies have given attention to the relationship between InDels and the variation of plastome size. In the present study, a strong correlation existed between the changed lengths of InDels and plastomes (Fig. 1, Table S2), demonstrating that the variation in size of Dendrobium plastomes was due to dramatical changes in lengths of InDels. However, the changed lengths of InDels is not secure to measure the phylogenetic relationship between the Dendrobium plastomes. In addition, according to Ahmed et al., the distribution of InDel events is dependent on their locations in plastomes, e.g., associated with low GC content, high rate of nucleotide substitutions, or high frequency of SSRs; in line with this, the current research also revealed a nonrandom distribution of InDels (Fig. 1). Nevertheless, the dynamic distribution of the InDels in Dendrobium plastomes is worthy of further investigations.

Recently, the taxonomic study of Dendrobium has become a global concern of biological systematics and been regarded as one of the enormous challenges in Orchidaceae. Numerous studies have focused on searching the most appropriate DNA loci for low taxonomic level studies of Dendrobium species. However, due to limited loci or taxa sampled, some conclusions made in these studies are inconsistent. The mutational hotspots from plastid genome are the most commonly used tool for low-level phylogeographic and phylogenetic studies of plants. Although in many Dendrobium studies, they are supplemented with nrDNA ITS sequences, it is still difficult to obtain sufficient informative sites. Their unavailability makes it very challenging to resolve the phylogenetic relationships among several unplaced species (i.e., D. capillipes, D. trigonopus) and closely related species, and to identify these species. Moreover, the most variable loci in one lineage may not be phylogenetically informative in other lineages. For instance, the loci matK, rbcL, and psbA-trnH are highly variable in angiosperms and have been proposed for DNA barcoding, yet they only showed moderate sequence divergences among all syntenic loci of Dendrobium plastomes tested in this study (Fig. 2, Table S3). Therefore, it is necessary to make a cautious evaluation of specific genetic markers for Dendrobium species.

By comparing 92 syntenic intergenic and intronic loci from 25 Dendrobium plastomes, our analyses revealed that the top ten hotspots listed in Fig. 2 were the fastest evolving loci, which may be used for the phylogenetic study and identification of Dendrobium species. Among these top ten loci, four (psbB-psbT, rpl16-rps3, ...
of these introns contain polymorphic SSRs (Table 3). Considering the functional importance of their secondary structure, we surmise that the polymorphic SSRs might play a role in maintaining the secondary structural features, which are important for a proper splicing. However, eight of these introns contain polymorphic SSRs (Table 3). This finding is in good agreement with the view proposed by Shaw et al. that although the top mutational hotspots are diversified in different lineages, some highly variable loci might remain unchanged in all angiosperm lineages.

Regarding 17 plastid introns, all except trnL-UAA intron belong to self-splicing group II introns; none of them ranked in the top-ten mutational hotspots in our study. Compared to intergenic spacers, the group II introns had lower evolving rates, which could be explained by that their mutations may be constrained by their function in maintaining their secondary structural features, which are important for a proper splicing. However, eight of these introns contain polymorphic SSRs (Table 3). Considering the functional importance of their secondary structural features, we surmise that the polymorphic SSRs might play a role in maintaining the secondary structural features of group II introns.

The subject—"which hotspot and how many hotspots should be used"—has been debated for a long time. Multiple solutions have been put forth in terms of the hotspot region and the number of hotspots in the combination, but no clear consensus result has yet emerged. For example, in Dendrobium, Singh et al. found that the DNA barcode based on three loci, matK, rpoB, and rpoC1 could indentify the maximum number of Dendrobium species. Xu et al. recommended utilizing the combination of ITS + matK as a core DNA barcode; and ITS, rbcL, matK, trnH-psbA, and trnL intron/trnL-trnF were used to resolve the phylogenetic relationship of Dendrobium in the studies of Xiang et al. More recently, Shaw et al. concluded that at least four and up to eight of the most variable hotspots will likely access the majority of the low-level discriminating power of the plastome depending on the lineage of interest. Based on the results of the current research, we recommend that the combination of five hotspots—trnT-trnL, rpl32-trnL, clpP-psbB, trnL intron, and rps16-trnQ—should be used in Dendrobium studies due to three reasons. Firstly, the phylogenetic tree based on this combination showed a strong discriminating ability (nearly all nodes BS value > 75%) for Dendrobium species (Fig. 4). Secondly, five hotspots are necessary to capture the species resolution power of Dendrobium plastome. Empirical data analyses have revealed that greatly increasing the number of hotspots will not improve species-level discrimination because of a "performance plateau." This "performance plateau" was also observed in our study, as manifested by that the mean BS value only slightly increased with more than five hotspots combined. Thirdly, the combination of trnT-trnL, rpl32-trnL, clpP-psbB, trnL intron, and rps16-trnQ contains the lowest number of hotspots while ranking among the top ten combinations that yielded highest BS values (Table S5); hence, it is cost effective to apply this hotspot combination to the phylogenetic and identification studies of Dendrobium.

Methods

Plant materials and DNA extraction. Two grams of fresh leaves were harvested from an individual plant of each tested Dendrobium species (Table 1) grown in the greenhouse of Nanjing Normal University. Total genomic DNA was isolated from the leaves using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. The DNA quality was examined by using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA samples with concentration > 300 ng/μl, A260/A280 = 1.8–2.0, and A260/A230 > 1.7 were used for sequencing.

Plastome sequencing, assembly, and annotation. The total DNA of each tested Dendrobium species was sequenced with an Illumina Hiseq4000 sequencer at 1 Gene, Hangzhou (Hangzhou, China). Approximately 8.75 Gb of 150 bp pair-end reads was yielded for each species; the raw reads were trimmed under the threshold with an error probability < 0.05 and then de-novo assembled on CLC Genomics Workbench 6.0.1 (CLC Bio, Aarhus, Denmark). Contigs > 30 x sequencing depths were collected for reference-based assembly. The plastome of D. officinale (NC_024019) served as a reference sequence. The four junctions between LSC/SSC and IRs were trimmed using specific primers. Plastome annotation was performed using DOGMA and tRNAscan-SE 1.21. The exact boundaries of annotated genes were confirmed by aligning them with the corresponding orthologs from other Dendrobium species.

Identification of InDels. Sequences of large single copy (LSC), small single copy (SSC), inverted repeat (IR) regions, and retained NDH gene residues from each tested Dendrobium plastome were aligned with reference sequences from the plastome of D. officinale according to the MAFFT program. InDel events and lengths were counted and determined with DnaSP v5.

Estimates of sequence variability. To assess sequence variability (SV) among plastomes of Dendrobium species, firstly, we retrieved the sequences of intergenic and intronic loci from 25 newly sequenced plastomes. The loci that are flanked by the same genes/exons were identified as syntenic, while the loci smaller than 150 bp were discarded. Secondly, we compiled 325 pairs of the 25 Dendrobium plastomes, and aligned the sequences of the syntenic loci for each pair by using MUSCLE with the “Refining” option implemented in Mega 5.2. The gaps located at the 5′- and 3′-ends of alignments were excluded. DnaSP v5 was employed to count the numbers of pairwise mutations and InDel events. SV was calculated according to the method of Shaw et al.: SV = (The number of nucleotide mutations + the number of InDel events)/(the number of conserved sites + the number of nucleotide mutations + the number of InDel events) × 100%. Finally, we calculated the average SV of each syntenic locus.
Counts of SSR elements. SSR (simple sequence repeat) elements located in the syntenic loci were detected using GMATo according to the criteria that the "Mini-length" for mono-nucleotide and multi-nucleotide SSRs were set to be 8 and 5 units, respectively.

Phylogenetic analysis. The sequences of top ten hotspots (psbB-psbT, ndhF-rpl32, trnL-trnL, rpl32-trnL, clpP-psbB, trnL intron, rpl16-rps3, trnE-trnT, trnR-tpA, and rps16-trnQ) were retrieved from plastomes of 30 Dendrobium species. Sequence alignments of these loci were separately performed using MUSCLE, and then concatenated into 1023 combinations using SequenceMatrix 1.88 (Table S4). ML trees were constructed using RAxML 8.0.229, with Phalaenopsis aphrodite (NC_007499), Phalaenopsis equestris (NC_017609), and Phalaenopsis (hybrid cultivar) (NC_025593) designated as outgroups. For the maximum likelihood tree analysis, a GTR+GAMMA model was employed, and supporting values of tree nodes were estimated from 1,000 bootstrap replicates.

Statistical analyses. Statistical analyses were performed by using SPSS Statistics 20.0.

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Author Contributions

D.X.Y. designed the study topic. N.Z.T., Z.S.Y. and P.J.J. performed the experiments. N.Z.T., P.J.J., L.L.D. and S.J. analyzed data. N.Z.T. wrote the manuscript.

Additional Information

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