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Research paper

# Plastid RNA editing reduction accompanied with genetic variations in *Cymbidium*, a genus with diverse lifestyle modes

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ABSTRACT

Recent sequencing efforts have broadly uncovered the evolutionary trajectory of plastid genomes (plastomes) of flowering plants in diverse habitats, yet our knowledge of the evolution of plastid post-transcriptional modifications is limited. In this study, we generated 11 complete plastomes and performed ultra-deep transcriptome sequencing to investigate the co-evolution of plastid RNA editing and genetic variation in *Cymbidium*, a genus with diverse trophic lifestyles. Genome size and gene content is reduced in terrestrial and green mycoheterotrophic orchids relative to their epiphytic relatives. This could be partly due to extensive losses and pseudogenization of *ndh* genes for the plastid NADH dehydrogenase-like complex, but independent pseudogenization of *ndh* genes has also occurred in the epiphyte *C. mannii*, which was reported to use strong crassulacean acid metabolism photosynthesis. RNA editing sites are abundant but variable in number among *Cymbidium* plastomes. The nearly twofold variation in editing abundance is mainly due to extensive reduction of ancestral editing sites in *ndh* transcripts of terrestrial, mycoheterotrophic, and *C. mannii* plastomes. The co-occurrence of editing reduction and pseudogenization in *ndh* genes suggests functional constraints on editing machinery may be relaxed, leading to nonrandom loss of ancestral edited sites via reduced editing efficiency. This study represents the first systematic examination of RNA editing evolution linked to plastid genome variation in a single genus. We also propose an explanation for how genomic and posttranscriptional variations might be affected by lifestyle-associated ecological adaptation strategies in *Cymbidium*.



## 1. Introduction

In photosynthetic plants, the plastid genome (plastome) is typically conserved in size, structure, and gene content (Wicke et al., 2011; Mower and Vickrey, 2018). However, in response to altered habitats and changes in external nutrient sources, the plastome may experience mild to severe functional disruption by progressive loss of genes in different functional categories due to altered selective regimes (Barrett and Davis, 2012; Graham et al., 2017; Kim et al., 2020). The early stage of genome degradation often includes the pseudogenization and/or loss of NADH dehydrogenase-like (*ndh*) genes, which are proposed to function as protection against photo-oxidative stress by balancing cellular redox levels (Martín and Sabater, 2010). The *ndh* genes act to relieve the effects of frequent episodes of environmental stresses but could be dispensable under favorable environments (Ruhlman et al., 2015).

Compared to the evolution of plastid genome architecture, relatively little is known about the evolutionary dynamics of posttranscriptional modifications. Plastid RNA editing, which mostly converts cytidine to uridine (C-to-U) at the post-transcriptional level, is widespread in land plants (Freyer et al., 1997; Small et al., 2020). Plastid transcripts in early land plants are generally heavily edited, yet most ancestral edits tend to be lost in higher plants (Tillich et al., 2006; Small et al., 2020). In angiosperms, RNA editing can affect dozens to >180 sites in the plastome

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(Hein et al., 2016; Ishibashi et al., 2019; Smith, 2020). Comparisons of homologous editing sites have indicated that many sites are conserved in different angiosperm clades (Tsudzuki et al., 2001; Tillich et al., 2006). Slight differences in editing positions have also been observed in *Arabidopsis* ecotypes (Tillich et al., 2005). However, the evolutionary rates of plastid RNA editing sites may also be rapid. For example, plastid RNA editing is extremely high (up to ~ 3500 sites) and extraordinarily variable (>6-fold change) in a lycophyte genus, *Selaginella* (Oldenkott et al., 2014; Smith, 2020).

How is the frequency of plastid RNA editing influenced by external and internal stimuli? Do sites of posttranscriptional modifications evolve in tight association with genetic variation? We answered these questions by examining the evolution of plastid genome architecture and RNA editing profiles for 11 orchids from *Cymbidium*, which comprises approximately 52 recognized species that are mainly distributed in Asian tropical and subtropical regions and northern Australia (Du et al., 2007; Yang et al., 2013). *Cymbidium* species have diverse life forms; ~70% are epiphytic, whereas a minority are terrestrial or lithophytic, and there is one partial mycoheterotroph, *Cymbidium macrorhizon* Lindl (Motomura et al., 2008; Kim et al., 2018; Suetsugu et al., 2018). These differences in life forms probably reflect changes in ecological adaptations to photosynthetic fluctuations and altered nutrient sources. Epiphytic plants are better able to compete for sufficient light when living on trees but need to balance transpirational water loss and photosynthetic rates. Terrestrial and mycoheterotrophic orchids compensate for their limited photosynthesis via increased fungal dependence. It has been reported that mycoheterotrophic orchids obtain 30–50% more carbon than terrestrial orchids to make up part of the nutrients needed for growth (Motomura et al., 2010). Thus, the diversity of *Cymbidium* plants provides an excellent opportunity to explore the genomic and posttranscriptional variation that may be associated with lifestyle-associated ecological adaptations.

## 2. Materials and methods

### 2.1. Taxon sampling

We sampled 11 *Cymbidium* species (*Cymbidium dayanum* Rchb. f., *Cymbidium ensifolium* (L.) Sw., *Cymbidium faberi* Rolfe, *Cymbidium floribundum* Lindl., *Cymbidium lancifolium* Hook., *Cymbidium lowianum* Rchb. f., *C. macrorhizon*, *Cymbidium mannii* Rchb. f., *Cymbidium sinense* (Jackson ex Andr.) Willd, *Cymbidium tracyanum* L. Castle, *Cymbidium tortisepalum* Fukuyama) with a diversity of lifestyle strategies (epiphytic, terrestrial and mycoheterotrophic). Stems and flower tissues were collected for the leafless mycoheterotroph *C. macrorhizon*, and fresh leaves were collected for other orchids. Sampling and sequencing details are included in Table S1.

### 2.2. Assembly and annotation of plastomes

Genome sequencing, assembly and annotation were performed as described previously (Fan et al., 2019; Liu et al., 2020). Briefly, total cellular DNA was isolated using the cetyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1987), and sequenced on an Illumina HiSeq X Ten machine, generating 1.2–68.6 Gb of 150 bp paired-end reads per sample. Plastomes were assembled with NOVOPlasty v.3.2 (Dierckxsens et al., 2017) and annotated using the Geneious v.9.1.3 trial version (Kearse et al., 2012) with manual adjustment. The assembled plastomes are deposited in GenBank under accession numbers: MW582681–MW582691.

### 2.3. Strand-specific RNA-Seq library construction and transcriptome sequencing

To efficiently capture plant organelle-derived transcripts that generally lack poly(A) tails, we adopted the Ribo-minus RNA sequencing strategy. Briefly, total RNA was isolated using TRIzol reagent (Invitrogen) and treated with Ribo-Zero™ rRNA Removal Kit (Epicentre, Madison, WI, USA) for rRNA depletion and then with DNase I (TaKaRa Bio Inc., Japan) to ensure the removal of trace amounts of DNA. Strand-specific RNAseq libraries were constructed from purified mRNA using random hexamers, generating approximately 12 Gb of 150 bp paired-end reads for each species (Table S1). All raw sequencing data have been deposited in Sequence Read Archive under accession numbers SRR13674023–SRR13674033 and SRR13734467–SRR13734477.

### 2.4. Identification of editing sties

Raw RNAseq reads were filtered using Fastp v.0.20.1 (Chen et al., 2018) and then aligned to the respective plastomes with bowtie2 v.2.1.0 (Langmead and Salzberg, 2012). Expression signals were estimated via read coverages and calculated using Bedtools (Quinlan and Hall, 2010). RNA editing sites were detected using VarScan v.2.4.4 (Koboldt et al., 2012) and visually examined with Tablet v.1.19.09.03 (Milne et al., 2013) as described previously (Fan et al., 2019).

### 2.5. Phylogenetic construction and ancestral states

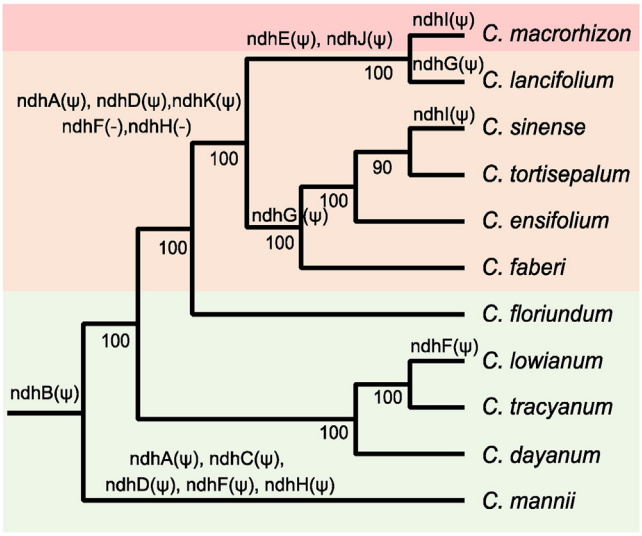
A 104.4-kb alignment of plastome sequences (including sequences of single copy regions and one copy of the inverted repeat) was performed using MAFFT v.7.475 (Katoh and Standley, 2013) that included all 11 *Cymbidium* species, along with *Erycina pusilla* (L.) N.H. Williams & M.W. Chase (NC_018114) and *Oncidium sphacelatum* Lindl. (NC_028148) as outgroups. A maximum likelihood-based plastid phylogeny was constructed using RAxML v.8.2.12 (Stamatakis, 2014) with a GTRGAMMAI substitution model and 100 bootstrap replicates. Ancestral states of genes and RNA editing were estimated using Dollo parsimony implemented in Count (Csűös, 2010).

## 3. Results

### 3.1. The trajectory of Cymbidium plastome evolution

To assess the evolutionary dynamics of plastid genome features, which may be associated with variation in lifestyles and other photosynthetically-related traits among *Cymbidium* orchids (Table S1), 11 plastomes were sequenced and assembled. All sequenced plastomes are largely syntenic in structure (Fig. S1). In general, plastomes are slightly larger in epiphytes compared with terrestrial and mycoheterotrophic orchids (Fig. 1), with genome sizes ranging from 149,728 bp in *C. macrorhizon* (a mycoheterotrophyte) to 156,616 bp in *Cymbidium tacyanum* (an epiphyte). *Cymbidium* plastomes encode 70–78 functional protein-coding genes. The differences in total gene numbers are due to different retention, loss and pseudogenization of the 11 *ndh* genes. All other typical plastid protein-coding genes are intact in *Cymbidium*.

The extent of *ndh* gene retention and loss, including pseudogenization via point mutations, fragmentation and gene deletions, varies among *Cymbidium* species (Fig. 1; Table S2). A previously documented 1-bp insertion at the 5′-end of *ndhB* (Kim et al., 2018) is shared with all 11 sampled *Cymbidium* species, and this frameshift might represent the initial degradation of the NDH complex in




| | Genome Size (bp) | Protein Genes | Edited Genes* | Editing Sites |
|---|---|---|---|---|
| *C. macrorhizon* | 149,727 | 70 | 25/4 | 57 |
| *C. lancifolium* | 149,797 | 70 | 31/5 | 65 |
| *C. sinense* | 150,168 | 71 | 33/4 | 77 |
| *C. tortisepalum* | 150,162 | 72 | 34/4 | 76 |
| *C. ensifolium* | 149,883 | 72 | 30/4 | 67 |
| *C. faberi* | 150,069 | 72 | 31/4 | 67 |
| *C. floriundum* | 155,382 | 78 | 38/1 | 91 |
| *C. lowianum* | 155,447 | 77 | 36/2 | 100 |
| *C. tracyanum* | 156,616 | 78 | 38/1 | 99 |
| *C. dayanum* | 155,312 | 78 | 35/1 | 90 |
| *C. mannii* | 153,024 | 73 | 30/3 | 69 |

**Fig. 1. Phylogenetic comparisons of genomic features of 11 *Cymbidium* plastomes.** Left: The tree was inferred on the concatenated data set of a copy of inverted repeats and single-copy regions as described in Methods. Epiphytic, terrestrial, and mycoheterotrophic taxa are colored with light green, orange, and pink, respectively. The relative timing of pseudogenization (with "Ψ") or complete loss (with "-") of *ndh* genes are highlighted on each branch. Bootstrap supports are depicted below each branch. Right: The genome size, functional protein-coding genes, edited genes, and total editing sites are summarized for each species. *: The total number of edited functional genes and pseudogenized protein-coding genes were summarized before and after the separator.

these genes. Other than the frameshifted *ndhB*, epiphytic *Cymbidium* orchids have generally retained nearly all plastid *ndh* genes, while most *ndh* genes have become progressively pseudogenized or lost in terrestrial and mycoheterotrophic taxa. Moreover, consistent with previous studies (Luo et al., 2014; Kim et al., 2018), *ndh* genes were also extensively pseudogenized in *C. mannii*, an epiphyte with strong crassulacean acid metabolism (CAM photosynthesis) (Zhang et al., 2016). Notably, the pseudogenized *ndh* genes are dispersed along the plastome (Fig. S1), indicating multiple mutational hotspots.

### 3.2. *Extensive loss of plastid C-to-U RNA editing in* Cymbidium *plastomes*

To further investigate plastome evolutionary dynamics at the posttranscriptional level, we performed non-polyA enriched transcriptome sequencing to efficiently capture nascent and mature organellar RNAs. Interestingly, RNAseq read mapping revealed that nearly all of the plastome is transcribed (mean depth = 2.0–5.9 × $10^4$), with the regions closest to *ycf1* and *ycf2* showing the lowest expression signals relative to others (Fig. 2). Moreover, intergenic regions and pseudogenes have relatively high expression levels, presumably due to co-transcription with neighboring genes.

The deep levels of expression obtained for most of the plastome provides ample data for the systematic identification of plastid RNA editing events. A total of 57–100 genomic sites are C-to-U edited in *Cymbidium* plastome transcripts. *C. macrorhizon* comprises the smallest set (57) of edits while epiphytic *Cymbidium* orchids (excluding *C. mannii*) generally have ≥90 edited sites. As many as 25–39 functional protein-coding genes are edited at one or more sites (Fig. 1; Table S3), mostly at their 1st and 2nd codon positions (Fig. S2). Interestingly, many pseudogenized *ndh* genes are still edited to some extent (Fig. 1). For example, of the seven pseudogenized *ndh* genes in *C. macrorhizon*, four were edited. A small number of intergenic and intronic edits (12.3–21.0%) were also identified (Table S3).

Comparisons of homologous editing sites revealed that the nearly two-fold differences in editing numbers were mainly due to progressive loss of ancestral edits, and to a much lesser extent, lineage-specific gains (Figs. 3 and S3). Not surprisingly, the *ndh* genes, both intact and pseudogenized, exhibit the most variation in their editing status in *Cymbidium*, requiring editing at 2–11 sites in *ndhB*, 0–3 sites in *ndhC*, 1–4 sites in *ndhD*, and 0–4 sites in *ndhF* (Table S3). Reconstruction of ancestral states indicates that editing of *ndh* transcripts is dramatically reduced in terrestrial and mycoheterotrophic orchids. Nearly 57–80% of the 30 edits that were probably present ancestrally in coding regions of *ndh* transcripts are lost in these orchids (Fig. S2). The epiphyte *C. mannii*, which also exhibits independent loss and pseudogenization of its *ndh* genes (Fig. 1), has only retained 9 ancestral editing sites in *ndh* transcripts. In contrast, the epiphytic orchids that have retained mostly functional *ndh* genes show only mild variation in *ndh* editing frequency.

Editing in non-*ndh* genes, such as other photosynthetic genes (*pet*, *psa* and *psb*) and ribosomal protein genes (*rpl* and *rps*), generally fluctuate in their editing profiles in a species-specific pattern (Fig. S2). Genes in other functional groups, such as those encoding *atp*, *rpo* and *ycf* proteins, are relatively conserved in RNA editing in all 11 *Cymbidium* species.

### 3.3. *The extent of plastid RNA editing reduction varies among* Cymbidium *lineages and phylogenetic depths*

Using the inferred ancestral editing status for each internal node, we compared the percentage of RNA editing reduction at different phylogenetic depths (Fig. 3A). The rampant RNA editing reduction of terrestrial and mycoheterotrophic orchids begins prior to the divergence of terrestrial orchids and the epiphytic *Cymbidium floriundum*, in which nearly 21.4% *ndh* and 2% non-*ndh* ancestral edits were lost (Fig. 3A). An additional 36.4% reduction of *ndh* edits occurred in the common ancestor of all terrestrial and mycoheterotrophic *Cymbidium* orchids. Subsequent RNA editing reduction is extensive for the mycoheterotrophic *C. macrorhizon*,

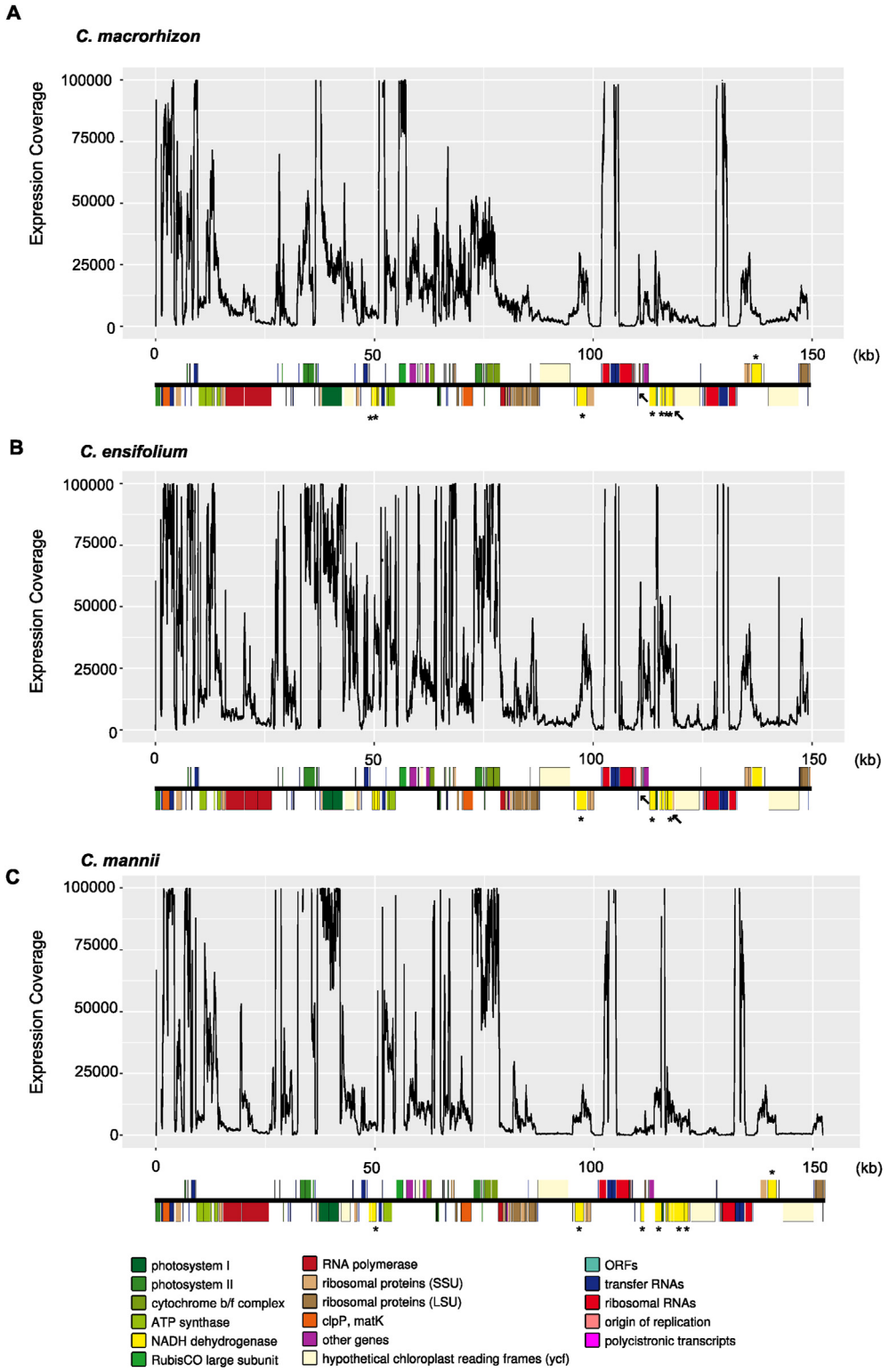



**Fig. 2. Plastome expression profiles of three representative *Cymbidium* species.** The expression levels of one mycoheterotrophic (*C. macrorhizon*), one terrestrial (*C. ensifolium*), and one epiphytic (*C. mannii*) orchid plastome are shown. All of these representative species exhibited extensive *ndh* gene pseudogenization and/or loss events and with similar high-level expression profiles. Read coverages (with a cutoff of 100000) for each genomic site are plotted along each chromosome. Pseudogenized *ndh* genes are highlighted with asterisks and the ancestral locations of lost *ndh* genes are marked with arrows.

while terrestrial orchids generally demonstrate lineage-specific editing reduction.

Among epiphytic orchids, the evolution of RNA editing was found to contrast between *C. mannii* and other taxa. Nearly 68% of RNA editing in *ndh* genes in *C. mannii* were lost, considerably higher than that in non-*ndh* genes. In contrast, only a small number of editing sites were affected in other epiphytic orchids, and these affected both *ndh* genes and non-*ndh* genes.

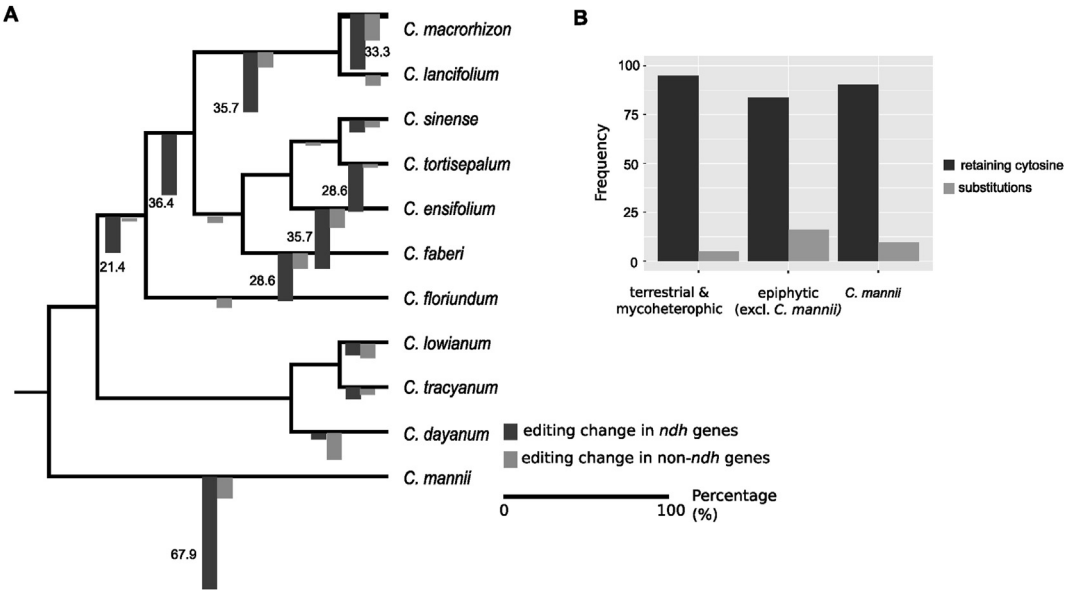



**Fig. 3. The relative timing and extent of loss of ancestral editing sites and the nucleotide status.** A: The percentages of loss of ancestral editing sites (relative to the editing status of the former nodes) are depicted at each branch. Changes over 20% are highlighted. Loss of editing in *ndh* genes are shown in black and that in non-*ndh* genes are in grey. B: Shown as the frequency of the nucleotide status for cytosines retained (black) and sites with substitutions (grey) in terrestrial and mycoheterotrophic, epiphytic (excluding *C. mannii*) and *C. mannii* orchids.

### *3.4. Loss of plastid editing is mainly due to reduced editing efficiency*

The reduction of editing sites could not be simply explained by gene deletion. In fact, sequence deletion only eliminates the editing of six genomic sites (one in *ndhA*, one in *ndhB* and four in *ndhF*) (Fig. S2). To examine the cause of editing loss, we examined the nucleotide states at each homologous editing site (Fig. 3B). Nucleotide substitutions (i.e., converting an edited cytosine (C) to another nucleotide) make up only a small fraction (<15%) of the editing loss at ancestral sites. Instead, the majority of ancestral edit sites that have lost editing have become unedited cytosines. These unedited cytosines account for 95% of loss events for terrestrial and mycoheterotrophic plants, 90% for *C. mannii,* and 85% for the other epiphytic orchids (Fig. 3B). This pattern indicates that losses of plastid editing sites in terrestrial and mycoheterotrophic *Cymbidium* orchids and *C. mannii* plastomes are mainly due to reduced editing efficiency rather than removal of the edited cytosine by deletion or nucleotide substitution. This is further supported by the extent of RNA editing in the transcript pool; *ndh*, *petL*, *rpl* genes tend to have low editing efficiency and reduced editing, whereas other genes (such as ATP synthase genes) have higher editing efficiency and less editing loss (Fig. S3).

## 4. Discussion

Collectively, these data suggest that throughout the diversification of *Cymbidium* species, both gene content and RNA editing profiles have changed to a certain extent, particularly in terrestrial and mycoheterotrophic species, which also have broader variation in genome architecture than epiphytic orchids (Fig. 1). This might be explained by increased opportunity for nutrient recovery from mycorrhizal partners when moving from trees to the ground, which ultimately may provide adaptive benefit to the switch to partial or full mycoheterotrophy (Motomura et al., 2010). One hallmark of orchids is their seeds generally lack endosperm and depend on fungi for nutrients during germination and the early stages of seedling growth (Du et al., 2007). *Cymbidium* plants with different lifestyle forms may have variable nutrient dependence on fungi. While epiphytic plants can absorb sufficient light, the terrestrial and mycoheterotrophic *Cymbidium* orchids generally grow in shade under the tropical forest all year round, which increases both their chances and their requirements for nutrients from fungal partners. This ecological adaptation could relax the functional constraints on photosynthetic components, typically on *ndh* genes, which function to protect against photo-oxidative stresses (Martín and Sabater, 2010).

However, we also found independent pseudogenization of most *ndh* genes in the plastome of the epiphytic *C. mannii* (Fig. 1). This may provide a good example of different ecological adaptation strategies leading to the convergent reduction of *ndh* genes. However, the underlying reasons for this reduction of *ndh* genes in *C. mannii* are still not well understood. One possibility is that *C. mannii* has evolved strong CAM photosynthesis, which could reduce transpirational water loss and improve photosynthetic rates by monitoring the opening and closing of stomatal aperture in a day–night fashion (Motomura et al., 2010; Zhang et al., 2016). This circadian regulation of photosynthesis could also relax selection on *ndh* genes by optimizing cellular oxidative redox. If this is true, we would expect such reduction of *ndh* genes would be repeatedly observed when more plastomes are sequenced in other strong CAM lineages.

How does the evolution of plastid RNA editing profiles associate with genetic variation in *Cymbidium* plastomes? To answer this question, we performed transcriptomic sequencing of all sampled *Cymbidium* taxa with a non-polyA enriched strategy, which efficiently captures early editing status in nascent RNAs (Germain et al., 2013). To our knowledge, our transcriptomic sequencing efforts represent the largest scale comparison of plastid RNA editing in a genus. Our findings provide strong evidence that non-random loss of ancestral edits in *ndh* genes in terrestrial and mycoheterotrophic species as well as *C. mannii* mainly accounts for the variation in total RNA editing sites (Figs. 1 and 3), although several other sites may fluctuate in editing levels. Because of this correlated

reduction of RNA editing sites in *ndh* genes accompanied with *ndh* pseudogenization, it seems that plastid RNA editing provides little or no advantage to counteract genetic variation at the DNA level.

How are RNA editing profiles in terrestrial and mycoheterotrophic orchids and *C. mannii* affected by relaxed selection? Among plastid genes, RNA editing is relatively abundant in *ndh* genes (Martín and Sabater, 2010). In our study, it appears that *ndh* genes continuously accumulated mutations in these lineages, suggesting increased mutation burden for the site recognition of editing machinery. If so, one would expect editing efficiency could be reduced at ancestral editing sites in the *ndh* genes for these particular lineages. By analyzing the proportion of edited reads within the total read coverage, we found relatively lower editing efficiency in *ndh*, *petL*, *rpl* genes compared with other genes (Fig. S3). Moreover, the contrasting evolution of *ndh* and non-*ndh* derived editing sites among *Cymbidium* plastomes further support this hypothesis (Fig. 3A). In summary, we conclude that the plastid RNA editing system could be fragile and the evolution of RNA editing profiles could provide additional evolutionary footprints left by altered external and internal environments.

## Author contributions

A.Z. and J.Y. designed and conceived this study; M.Z., L. Z., F.L, Y.H and W.F performed this experiments and analyses; M.Z and A.Z. wrote the manuscript; funding acquisition, J.Y.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2021.07.002.

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