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Corrigendum to: Retention of an apparently functional plastome in an apparently mycoheterotrophic orchid, *Dipodium roseum* D.L.Jones & M.A.Clem. (Orchidaceae)

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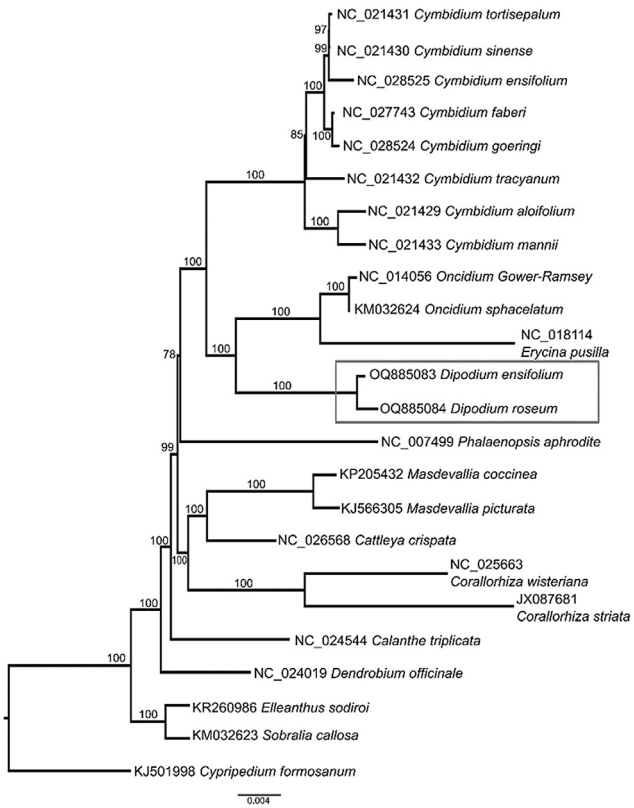
The authors advise of an error to Fig. 4 of the published paper, which is regretted. The GenBank accession numbers for *Dipodium ensifolium* and *Dipodium roseum* were missing in the figure. The corrected figure is provided below.



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Australian Journal of Botany

Retention of an apparently functional plastome in an apparently mycoheterotrophic orchid, *Dipodium roseum* D.L.Jones & M.A.Clem. (Orchidaceae)

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ABSTRACT

Context. Giving up photosynthetic function is a bold evolutionary step for a plant, yet the evolutionary transition from autotrophy to mycoheterotrophy has occurred dozens of times. Comparing plastome sequences of mycoheterotrophs and autotrophs has identified recurring patterns of gene loss throughout a range of land plants, though more comparisons are required to see how broadly these patterns apply across the diversity of terrestrial plants. Mycoheterotrophy is especially common in Orchidaceae, with more than 40 transitions from autotrophy to mycoheterotrophy in the family. **Aims.** We sought to test generalised patterns of plastome degradation seen in other mycoheterotrophs by comparing two species in the genus *Dipodium* (Orchidaceae); one species is photosynthetic and the other appears to be a facultative mycoheterotroph species. **Methods.** We sequenced and assembled the plastomes of two *Dipodium* species and compared the two genomes to identify gene degradation or loss. **Results.** The two plastomes were nearly identical, with no degradation of photosynthesis genes in the putative mycoheterotroph, and both species have undergone loss or pseudogenisation of all plastid *ndh* (nicotinamide adenine dinucleotide + hydrogen specific dehydrogenase) genes. **Conclusions.** These results contrast with most other comparisons between photosynthetic and likely mycoheterotrophic relatives, where rapid degradation in mycoheterotroph plastome genes is common, and may suggest the leafless *Dipodium* species are capable of photosynthesis and may be in the early stages of transitioning to a fully heterotrophic lifestyle. **Implications.** Further investigation of trophic transitions in *Dipodium*, including sequencing more plastomes and measuring photosynthetic capability of the putative heterotrophs, will yield insights into the evolution of plant lineages that lose the ability to photosynthesise.

Keywords: *Dipodium*, gene loss/degradation, mixotrophy, mycoheterotrophy, *ndh* genes, Orchidaceae, plastome, symbiosis.

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Introduction

Mycorrhizal symbiosis operates over a continuum of ecological syndromes. True mutualism lies at one extreme. In the middle are mixed strategies including both obligate and facultative mixotrophy, while obligate parasitism (mycoheterotrophy) lies on the other extreme (Selosse *et al.* 2006). Mycoheterotrophic plants act as obligate parasites of fungi, and having lost the capacity for photosynthesis, rely on mycorrhizal associations to obtain carbon. These plants feature similar evolutionary morphological trends as holoparasites: they are frequently not green, leaves are typically scale-like or entirely absent, vascularisation of the stems is often reduced, stomata are mostly absent from above-ground/vegetative parts, and the below-ground organs of mycoheterotrophic plants are typically highly modified (Merckx and Freudenstein 2010). Although losing the ability to photosynthesise seems like a significant evolutionary risk for plants, mycoheterotrophy has evolved approximately 47 times in land plants (Merckx 2013).

Photosynthetic ability in land plants is conferred by the chloroplast, a specialised plastid organelle obtained from a cyanobacteria in an ancient endosymbiosis event

(Timmis *et al.* 2004). In plants that have evolved a degree of mixotrophy and no longer rely directly on autotrophic photosynthesis, the plastid genome (plastome) is typically reduced, featuring gene pseudogenisation, deletions or rearrangements (dePamphilis and Palmer 1990; Bungard 2004). This includes holoparasites (*Epifagus* Nutt., dePamphilis and Palmer 1990; *Cytinus* L., Roquet *et al.* 2016; *Hydnora* Thunb., Naumann *et al.* 2016; *Cuscuta* L., Braukmann *et al.* 2013; *Cistanche* Hoffmans. & Link, Li *et al.* 2013; *Pilostyles* Guill., Bellot and Renner 2016; *Rhopalocnemis* Jungh., Schelkunov *et al.* 2019; *Parasitaxus* de Laub., Qu *et al.* 2019), hemiparasites (*Viscum* L., Petersen *et al.* 2015), and mycoheterotrophs (*Corrallorhiza* Gagnebin, Barrett *et al.* 2019; *Rhizanthella* R.S.Rogers, Delannoy *et al.* 2011; *Neottia* Jacq., Logacheva *et al.* 2011; *Epipogium* R.Br., Schelkunov *et al.* 2015; *Corsia dispar* D.L.Jones & B.Gray, Bodin *et al.* 2016; *Sciaphila* Blume, Lam *et al.* 2015; Orobanchaceae Vent., Frailey *et al.* 2018; *Burmannia* L., Li *et al.* 2019; *Epirixanthes* Blume, Petersen *et al.* 2019; *Monotropa* L., Gruzdev *et al.* 2016). The ultimate state of degradation of plastome function in heterotrophic plants is exemplified by an apparent complete loss of the plastome in several holoparasitic taxa (*Rafflesia* R.Br., Molina *et al.* 2014; *Schoepfia* Schreb., Su and Hu 2016). Non-parasites have also been identified as having reduced plastomes and gene loss (i.e. *Tahina spectabilis* J.Dransf. & Rakotoarin, Barrett *et al.* 2016; *Carnegiea gigantea* Britton & Rose, Sanderson *et al.* 2015; *Saniculiphyllum guangxiense* C.Y.Wu & T.C.Ku, Folk *et al.* 2020). Sequencing heterotroph plastomes (including partial heterotrophs and mixotrophs) has led to inferences of general evolutionary trends of gene pseudogenisation or loss, including an increase in AT%, indels and gene rearrangements, and the degradation of photosynthetic ability (Graham *et al.* 2017).

Lineages that include both photosynthetic and fully mycoheterotrophic species have been studied to test whether there are common or repeated pathways of plastome degradation. The current consensus holds that losing genes in the NADH (nicotinamide adenine dinucleotide) dehydrogenase complex (*ndh* genes) can begin a heterotrophy cascade (Lam *et al.* 2016; Barrett *et al.* 2018), which leads to the degradation of a set of photosynthesis genes (including *psa, psb*, *pet* and *rpo* genes, and *rbcL*). These are lost soon after the transition to heterotrophy and lead to a loss of photosynthetic ability, though this does not always inhibit the plant from producing chlorophyll (Wickett *et al.* 2011; Barrett and Davis 2012; Cusimano and Wicke 2016; Feng *et al.* 2016). However, in many heterotrophic species the plastome is retained and patterns of reduction converge on a conserved minimal gene set (Delannoy *et al.* 2011; Logacheva *et al.* 2011). This core set includes ribosomal and transfer RNAs (rRNAs, tRNAs), ribosomal protein genes, *clp*P, *acc*D, *ycf*1, and *ycf*2. For example, even the very reduced plastomes of *Epipogeum* (~11 kb) are thought to be functional in some way as they contain a high proportion of coding DNA, an absence of non-synonymous mutations in protein-coding genes, and apparently functional tRNAs (Schelkunov *et al.* 2015). Studies with dense sampling of mycoheterotrophs and related autotrophs have found significant plastome sequence variation at even very fine taxonomic scales (including within species variation) and rapid degradation of plastid genomes associated with a transition to heterotrophy (Barrett *et al.* 2018, 2019; Schneider *et al.* 2018). Conversely, examples where a photosynthetic species and an apparent (facultative) heterotroph have similar plastomes are rare in the literature (but see Kim *et al.* 2020). The extent of plastome gene degradation therefore appears to vary widely among transitions to heterotrophy. Identifying whether this variation is due to lineages being at different places on the same path, or whether different paths of degradation are taken by different lineages is key to better understanding the evolution of heterotrophy. Ideally, we need to accumulate more studies of phylogenetically independent autotrophic–heterotrophic contrasts.

Approximately half of all facultative mycoheterotrophs occur in the Orchidaceae. This represents at least 30 transitions to full mycoheterotrophy (Barrett *et al.* 2018) and many of the sequenced plastomes of mycoheterotrophs have been orchids. However, the plastomes of photosynthetic orchid species also commonly feature loss and degradation of *ndh* genes (Neyland and Urbatsch 1996; Kim *et al.* 2015). Sequencing across Orchidaceae has revealed that *ndh* gene loss is complex; within subfamilies it appears that loss of full-length copies of the gene family occurs multiple times (Kim and Chase 2017). *Ndh* genes have also been lost in several non-orchid mycoheterotrophs (*Petrosavia* Becc, Petrosaviaceae, Logacheva *et al.* 2014), parasitic plants (*Cuscuta*, Convolvulaceae, Haberhausen and Zetsche 1994), carnivorous plants (*Utricularia* L., Utricularieaceae, Silva *et al.* 2016; *Genlisea* Rchb., Lentibulariaceae, Silva *et al.* 2018), aquatic plants (*Najas* L., Hydrocharitaceae, Peredo *et al.* 2013), as well as other photosynthetic lineages (Gnetales and Pinaceae, Braukmann *et al.* 2009; *Erodium* L'Hér. (Chris Blazier *et al.* 2011) and *Melianthus* L. (Weng *et al.* 2014), Geraniaceae; *Capparis* L., Capparaceae, Maurya *et al.* 2020). Under optimal growth conditions these genes have been found to be dispensable (Burrows *et al.* 1998) as the NADH dehydrogenase complex functions to fine tune photosynthesis that may not be required in terrestrial habits (Ruhlman *et al.* 2015) or under low stress conditions (Wicke *et al.* 2011). In orchids, the complex patterns of *ndh* loss or retention irrespective of the mode of carbon acquisition may confuse generalisations about the loss of this gene family and mycoheterotrophy.

The orchid genus *Dipodium* R.Br. (Orchidaceae, subfamily Epidendroideae) provides a useful study system for understanding the transition from photosynthetic-capable (autotrophic) to non-photosynthetic (likely facultative mycoheterotrophs) as the genus includes both leafy, photosynthetic and leafless, likely mycoheterotrophic species. The photosynthetic species are typically epiphytic or climbers, have large leaves (up to 400 mm long), and occur in tropical regions of Australia and south-east Asia. The apparent mycoheterotrophic species

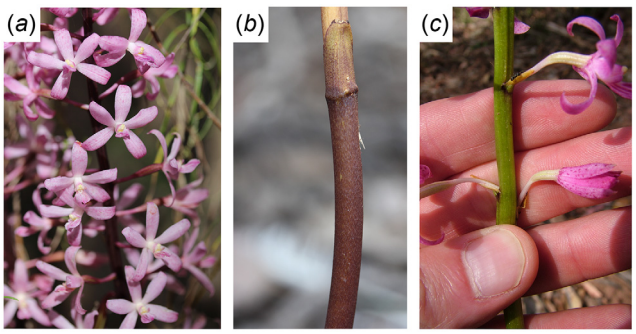

Fig. 1. *Dipodium roseum*, showing flowering inflorescence with reddish-brown stem (*a*; plant from Gumtree Reserve, Eltham, Victoria), scale leaf (*b*; plant from Gumtree Reserve, Eltham, Victoria), and green inflorescence stem (*c*; plant from Cathedral Range State Park, Victoria). Photos: M. Bayly.

are terrestrial and entirely subterranean when not flowering. The inflorescence stem is typically brown (Fig. 1*a*, *b*) with leaves reduced to scales (up to 30 mm long), and they occur in south-eastern Australia, New Caledonia and New Guinea (O'byrne 2014). One species, *D. ensifolium* is terrestrial but has leafy stems. The literature on whether the leafless terrestrial *Dipodium* species are fully or partially mycoheterotrophic is mixed (Dearnaley and Le Brocque 2006*a*; O'byrne 2014), and some populations of apparently mycoheterotrophic species can be found with green stems (Fig. 1*c*; M. Bayly, personal observation; Jeanes and Backhouse 2006; see images on *Dipodium* profile at VicFlora 2022). Mycorrhizal associations of the Australian terrestrial *Dipodium* are dominated by the genus *Russula* Pers. which is typically ectomycorrhizal with trees (Dearnaley and Le Brocque 2006*a*). This reflects associations of other mycoheterotrophic orchids with *Russula* (*Cymbidium* Swartz, Ogura-Tsujita *et al.* 2012; *Limodorum* Boehm., Girlanda *et al.* 2006; *Goodyera* R.Br., Suetsugu *et al.* 2019) and stands in contrast to the major diversity of Australian terrestrial orchids, which associate with saprophytic lineages *Tulasnella* J.Schröt., *Serendipita* P.Roberts, and *Ceratobasidium* D.P. Rogers (Warcup 1981; Dearnaley and Le Brocque 2006*b*; Whitehead *et al.* 2017; Freestone *et al.* 2021; Arifin *et al.* 2022). Here, we compare the plastomes of *D. ensifolium*, a likely photosynthetic species with green leaves, and *D. roseum*, an apparent facultative mycoheterotrophic species of *Dipodium*. This comparison aims to test generalised patterns of plastome degradation seen in other mycoheterotrophs.

Methods

Sampling, DNA extraction, library preparation and sequencing

To compare the plastome sequence of a mycoheterotroph and a photosynthetic species, we obtained a fresh sample of the leafless and putatively heterotrophic *Dipodium roseum* F.Muell. (MELUM155142a) and an herbarium specimen of the photosynthetic, green and leafy *D. ensifolium* F.Muell. (BRI AQ0839318). Genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) protocol (Shepherd and McLay 2011). DNA extraction for each sample was performed at separate times to avoid contamination between samples. Libraries were prepared for Illumina sequencing following Schuster *et al.* (2018). The *Dipodium* samples were pooled equally and then pooled with two other libraries to be 45% of the final concentration. The final library was sequenced using a MiSeq V3 300PE kit, at the Walter and Eliza Hall Institute, Melbourne, Victoria, Australia.

Plastome assembly

Raw read data of total genomic DNA was *de novo* assembled using CLC genomics workbench ver. 8.5.1 (https://www.qiagenbioinformatics.com/) with default settings, minimum length 1000 bp. Resulting contigs were then mapped to *Cymbidium aloifolium* (NC021429, Yang *et al.* 2013) in Geneious ver. 9.1.8 (http://www.geneious.com, Kearse *et al.* 2012), using custom sensitivity settings; gaps allowed set to a maximum of 20% per read, maximum gap size of 3000 and two to three iterations. Four contigs spanned the entire reference genome in *D. roseum* and five contigs in *D. ensifolium*. The only gaps present between contigs represented the second inverted repeat (IR) region, otherwise contigs overlapped at each end. The IR region was duplicated and remapped to the genome allowing for draft assembly of a complete circular plastome. Raw reads were mapped back over the draft genome to check for any deletions and SNP (single nucleotide polymorphism) differences between *de novo* assembled contigs used to make the draft and the raw reads. Mean raw read coverage of the *D. roseum* plastome was 202× bp, and 74× bp for the *D. ensifolium* plastome. A consensus sequence was then made for each sample. Plastid genomes were aligned using the MAFFT pairwise alignment plug-in (MAFFT, ver. 7.222; Katoh and Standley 2013) in Geneious Prime ver. 2019.2.1 with default settings. Gene annotations were transferred from GenBank published reference genomes and manually checked for changes to length and reading frame.

Testing for relaxed selection in *D. roseum* plastome genes

To compare the ratio of substitution rates at non-synonymous and synonymous sites, we performed dN/dS tests in MEGA7 (Kumar *et al.* 2016) using the Nei-Gojobori method (Nei and Gojobori 1986); the variance of the difference was computed using the bootstrap method (1000 replicates). We compared 63 genes from the two *Dipodium* genomes to *Cymbidium aloifolium*. Ribosomal RNAs, translational RNAs, and *ndh*

genes were not tested. We also tested for relaxed selection pressure on *D. roseum* genes using RELAX v.2.1 (Wertheim *et al.* 2015) in HyPhy (Pond *et al.* 2005) using the Datamonkey webserver (Pond and Frost 2005). RELAX extends dN/dS calculations by estimating an intensity parameter (k), and tests whether selection is relaxed or intensified along a branch. *D. roseum* was set as the test branch and *D. ensifolium* was set as the reference branch. Model acceptance or rejection was determined using a likelihood ratio test.

Phylogenetic position of *Dipodium*

Twenty-four complete plastid genomes from species in subfamily Epidendroideae were downloaded from GenBank. Gene regions were extracted from each genome (58 genes, excluding the *ndh* genes, ribosomal RNAs, translational RNAs), gene reading frames and annotations were checked by eye, and were aligned using the MUSCLE plug-in (Edgar 2004) in Geneious with default settings. All genes were concatenated together to produce an alignment 52 466 bp long. The alignment was analysed using RAxML (ver. 8, Stamatakis 2014) with five maximum likelihood searches under both the GTRCAT and GTRGAMMA model, with *Cypripedium formosanum* as an outgroup. Support for the RAxML topology was tested by performing 1000 'rapid' bootstrap repetitions, the 10 independent runs were checked for topological convergence, and bootstrap values were mapped on to the best tree as defined by likelihood scores.

NADH dehydrogenase (*ndh*) gene evolution

The loss of the *ndh* complex in the plastome was characterised using the parameters outlined in Kim *et al.* (2015); genes are either present (1, i.e. full length and in frame), pseudogenised (2, i.e. stop codons reduce length of transcript), truncated (3, >10% gene length deleted), or completely absent (4). The loss of genes in the *ndh* complex was reconstructed onto a cladogram using a directional step matrix following Kim *et al.* (2015), where any transition towards loss (e.g. 1–>2, 3–>4, or 1–>4) required one step, and any reversal required five steps. The characters were mapped onto a cladogram featuring *Phaelenopsis, Cymbidium, Dipodium*, *Erycina*, *Oncidium*, with *Calanthe* as an outgroup as all *ndh* genes are fully functional in that genus. Character state reconstructions were performed in Mesquite v3.7 (Maddison and Maddison 2021) and only non-equivocal states were recorded.

Results

Comparative analyses of two *Dipodium* plastomes

The results of DNA sequencing and plastome assembly, including reads mapped to plastome, coverage, plastome size and GC (guanine-cytosine) content are shown in Table 1. Comparing the plastome structure and gene content between the two species shows that there is no loss in genes or change in structure in the apparent facultative mycoheterotrophic *D. roseum* compared to the photosynthetic *D. ensifolium*. Both species have the typical quadripartite structure and organisation of orchid plastomes (Fig. 2). The pairwise genetic similarity between the two species across the aligned plastomes was 97.77% and across protein-coding genes it was 99.25%.

Structurally, the number and order of genes in both species of *Dipodium* remain consistent. However, relative to other members of Orchidaceae, there has been a small rearrangement in the placement/location and direction of *petN* and *psbM* genes compared to *Cymbidium* species. Both species of *Dipodium* exhibit an alternative initiation codon for *matK* relative to other closely related orchids with sequenced whole plastomes. Such alternative out-of-frame initiation codons are well documented, particularly in the Orchidaceae (Barthet *et al.* 2015).

No significant changes in selection between the *Dipodium* species

The Nei-Gojobori test indicated that three genes *rps*14 (300 bp), *rpl*23 (279 bp), and *psbF* (117 bp) had significantly different dN/dS ratios between the two species, indicating reduced selection in *D. roseum* in those genes (Fig. 3). There was no significant difference between the mycoheterotroph and the autotroph in the remaining 60 genes analysed; most genes in the *D. roseum* plastid genome are evolving under purifying selection (dN/dS < 1). For both species, the gene *psbH* (309 bp) appears to be under

Table 1. Sequencing and assembly characteristics of the two sequenced *Dipodium* plastomes.

Species	Total reads	Reads mapped	Plastome length (bp)	LSC length (bp)	SSC length (bp)	IR length (bp)	Mean coverage	GC (%)	GenBank accession number	Voucher
Dipodium roseum (001)	7 000 412	177 302	141 239	81 836	10 446	24 534	201	37.7	OQ885084	MELUM155142a
Dipodium ensifolium (004)	2 085 448	44 603	142 016	82 176	10 998	24 430	73	36.9	OQ885083	BRI AQ0839318

LSC, large single copy; SSC, small single copy; IR, inverted repeat.

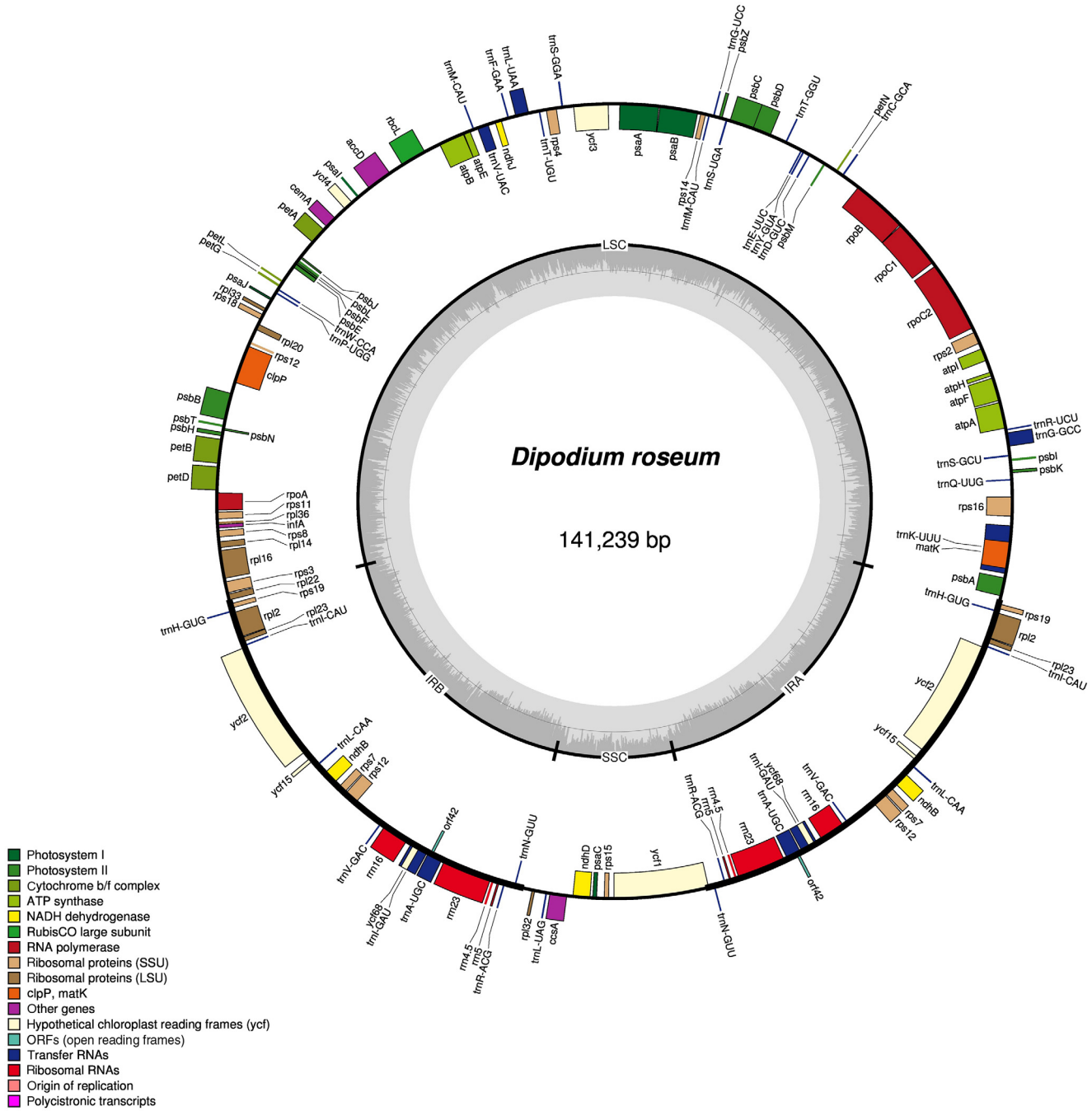

Fig. 2. Plastome map of *Dipodium roseum*, made with OGDraw (using the chlorobox toolkit – https://chlorobox.mpimp-golm.mpg.de/OGDraw.html, accessed March 2020).

relaxed selection compared to *Cymbidium*. RELAX analyses of the same genes revealed no significant evidence for relaxation or intensification of selection on any genes between the two species.

Phylogenetic position of *Dipodium*

Phylogenetic analysis of 58 plastome genes resolved *Dipodium* in Orchidaceae subfamily Epidendroideae, sister to *Oncidium* and *Erycina* (Fig. 4). The 10 independent tree searches between the two models (GTRGAMMA and GTRCAT) produced identical topologies. Limited taxonomic sampling restricts the usefulness of this dataset to fully resolve the currently uncertain position of the *Dipodium* in the tribe Cymbidieae (Freudenstein and Chase 2015), or determine whether it belongs in its own subtribe, Dipodiinae (Li *et al.* 2016).

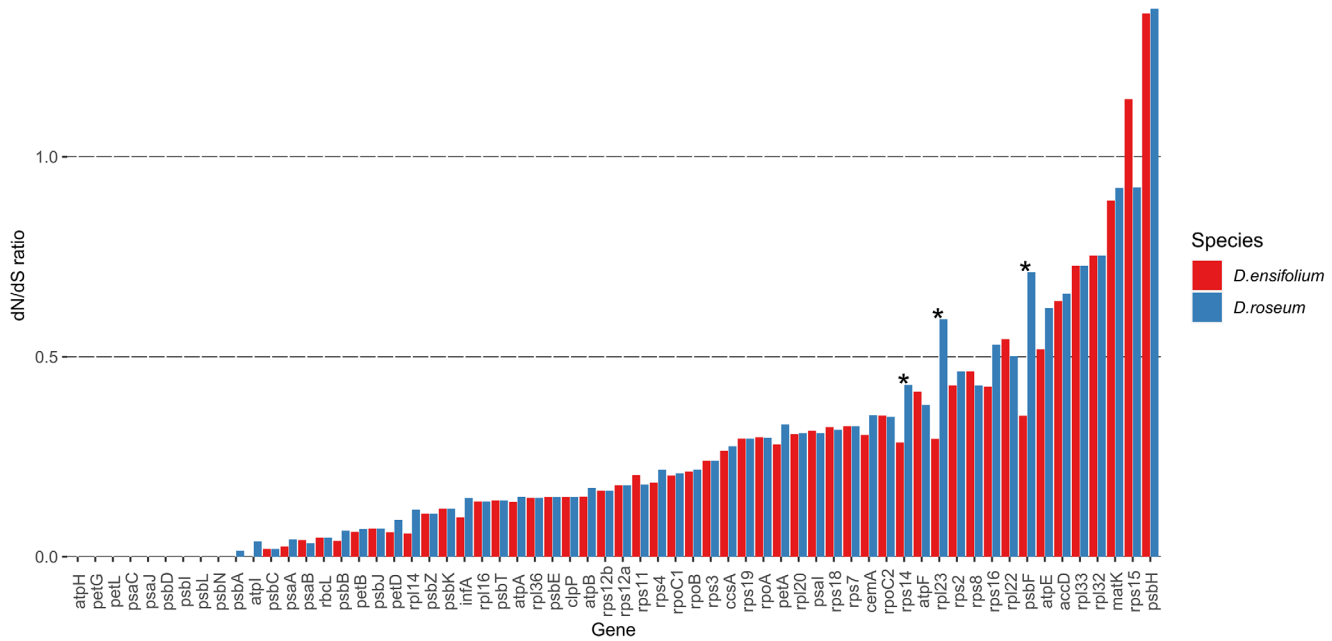

Fig. 3. dN/dS calculations of *D. roseum* (blue) and *D. ensifolium* (red) compared to *Cymbidium aloifolium*. Values below 1 represent purifying selection, values of 1 represent stabilising selection, and values above 1 represent relaxed selection.

NADH dehydrogenase (*ndh*) gene evolution

We found that all 11 *ndh* genes typically present in plant plastomes display identical changes in the plastomes of both *Dipodium* species. Using the parameters of *ndh* loss outlined by Kim *et al.* (2015), eight genes have been deleted (*ndh*A, *ndh*C, *ndh*E, *ndh*F, *ndh*G, *ndh*H, *ndh*I, *ndh*K) and three genes (*ndh*B, *ndh*D, *ndh*J) have been truncated by greater than 10% of the original length (Table 2). Character state reconstruction of the *ndh* gene family on a simplified phylogeny shows that most of these losses or truncations have occurred within the *Dipodium* lineage, and that *ndh* gene degradation has independently occurred repeatedly within the genera compared here (Fig. 5, Supplementary material S1).

Discussion

We sequenced the plastomes of two *Dipodium* species; the photosynthetically capable *D. ensifolium*, and the putative mycoheterotroph, *D. roseum*. Although these species lie on opposite sides of a substantial evolutionary transition in metabolism, the genomic architecture of plastomes in both species was very similar. We found no evidence of genome size reduction, gene deletions or pseudogenisation in *D. roseum* relative to the autotrophic *D. ensifolium*. Three plastid genes showed a significant signal for reduced selection in *D. roseum*, but their sequences indicate they remain functional. These results contrast with the majority of other sequenced facultative mycoheterotroph plastomes, where genome size reduction and gene loss or pseudogenisation is common in the transition to mycoheterotrophy (Merckx and Freudenstein 2010; Barrett *et al.* 2018).

Our discovery that the likely-mycoheterotroph *D. roseum* has a near-identical plastome to a photosynthetic congener is unusual amongst studies of plastome evolution in mycoheterotrophs. The only other study to identify an apparent mycoheteroph and photosynthetic congener with a near-identical plastome was that of Kim and Chase (2017) in the leafless *Cymbidium macrorhizon* and relatives. That species lacks leaves and roots, but when flowering the inflorescence rachis is pale green. A complementary study found that species photosynthesises during flowering and fruiting, with the additional carbon resources likely contributing to seed production (Suetsugu *et al.* 2019). The more typical finding in similar studies is that substantial genomic changes are associated with the transition from autotrophy to mycoheterotrophy (Graham *et al.* 2017; Barrett *et al.* 2018).

Our finding of no substantial differences in plastome architecture is surprising in context with other contrasts at the sub-generic level. Perhaps the most well-studied transition to full mycoheterotrophy and loss of photosynthetic capability in orchids is in *Corallorhiza*. Extensive studies in the genus have found that fully mycoheterotrophic species have degradation in a suite of key photosynthetic genes (including *pet*, *psa*, *rpo* complexes and *ccsA*, *cemA* and *rbcL*, as well as *ndh* complex), and phylogenetic reconstruction of gene loss in the genus identified several independent losses of photosynthetic genes (Barrett *et al.* 2019). However, mycoheterotrophs did not necessarily have reduced plastome sizes (Barrett *et al.* 2018), leaflessness was not correlated with plastome degradation, and some mycoheterotrophs have detectable levels of

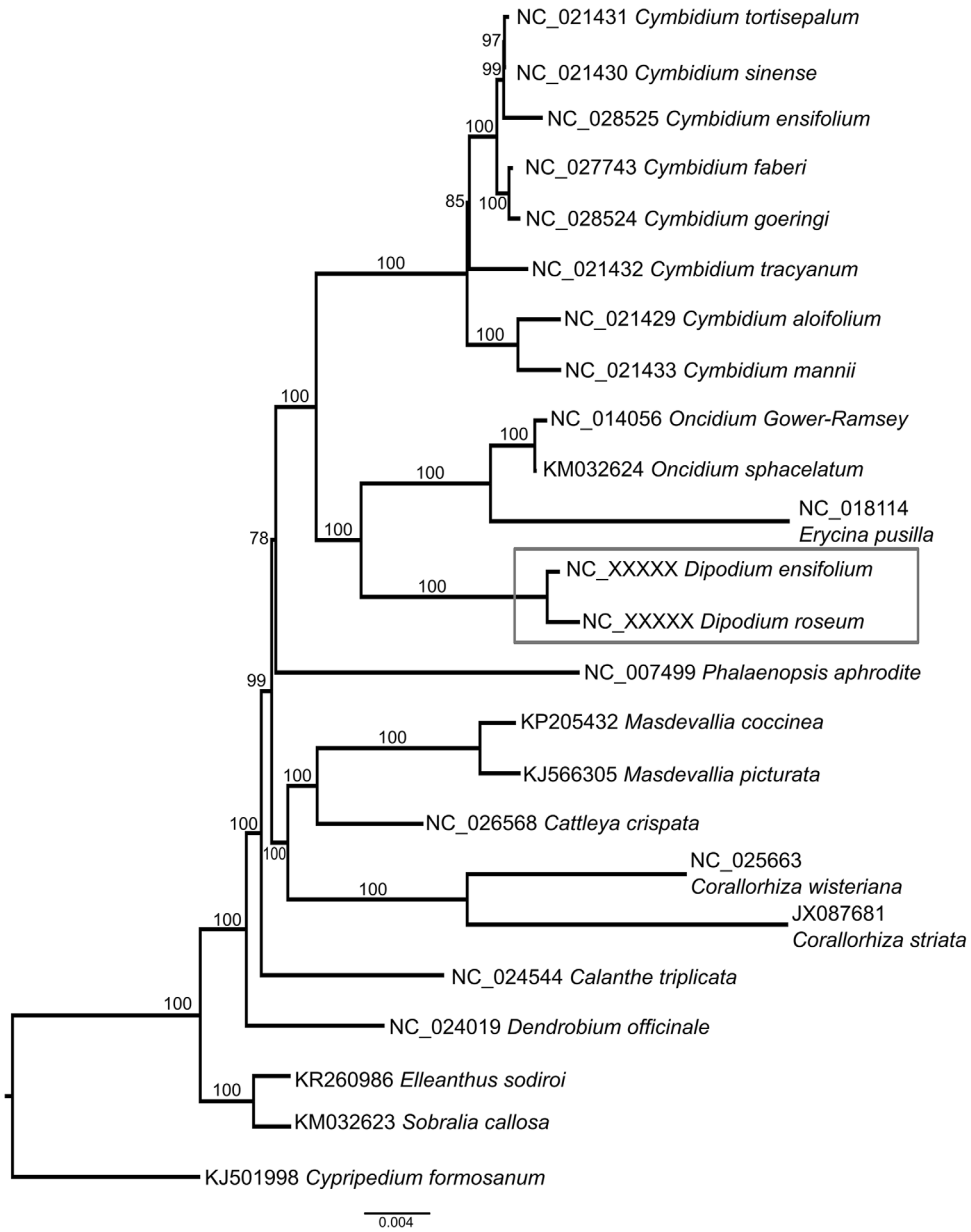

Fig. 4. Maximum likelihood phylogeny of Epidendroideae including two samples of *Dipodium*, based on 58 plastid genes. Rapid bootstrap support values from RAxML are shown.

chlorophyll (10-fold less than autotrophs; Barrett *et al.* 2014). Other examples include *Hexalectris*, an orchid genus of nine species with six independent losses of photosynthetic capability (Barrett and Kennedy 2018), and the tribe Neottieae (Feng *et al.* 2016; Zhou and Jin 2018), which has had three to four independent transitions to full mycoheterotrophy accompanied by a loss of important photosynthetic genes (Lallemand *et al.* 2019). These comparative analyses have shown that degradation can be rapid and frequently there are multiple transitions to full mycoheterotrophy amongst closely related species.

We found *ndh* gene loss in both species of *Dipodium*, which is consistent with generalisations that orchids appear to lose *ndh* genes readily, even in autotrophic species (Kim *et al.* 2020). The *ndh* gene family is important in environments with highly variable light intensities but functional *ndh* genes in the plastome are clearly dispensable within the Orchidaceae; loss of *ndh* genes is common and widespread in Orchidaceae and has been found in four of five subfamilies (Kim *et al.* 2020). *Ndh* gene loss appears to be randomly distributed throughout the family and within subfamilies (Kim *et al.* 2015), which makes any implication of *ndh* gene loss beginning a transition away from autotrophy difficult to tease out. Our data support this, with evidence of *ndh* loss in *Dipodium* further complicating the pattern in the

Table 2. Summary of differences in genes between the two *Dipodium* plastomes and *Cymbidium aloifolium*, with a particular focus on the *ndh* gene family.

Gene	*Dipodium ensifolium* OQ885083 (bp)	*Dipodium roseum* OQ885084 (bp)	*Cymbidium aloifolium* NC021429 (bp)
*ndh*A	Deleted (0)	Deleted (0)	Pseudogenised (2199)
*ndh*B	Truncated (811)	Truncated (800)	Pseudogenised (2224)
*ndh*C	Deleted (0)	Deleted (0)	Pseudogenised (363)
*ndh*D	Truncated (1179)	Truncated (1006)	Pseudogenised (1481)
*ndh*E	Deleted (0)	Deleted (0)	Functional (306)
*ndh*F	Deleted (0)	Deleted (0)	Pseudogenised (2211)
*ndh*G	Deleted (0)	Deleted (0)	Pseudogenised (531)
*ndh*H	Deleted (0)	Deleted (0)	Pseudogenised (1210)
*ndh*I	Deleted (0)	Deleted (0)	Pseudogenised (504)
*ndh*J	Truncated (387)	Truncated (387)	Functional (477)
*ndh*K	Deleted (0)	Deleted (0)	Pseudogenised (886)
*mat*K	Functional (1545)	Functional (1539)	Functional (1554)
*psb*G	Deleted (0)	Deleted (0)	Pseudogenised (746)
*ycf*15	Functional (243)	Functional (243)	Pseudogenised (231)

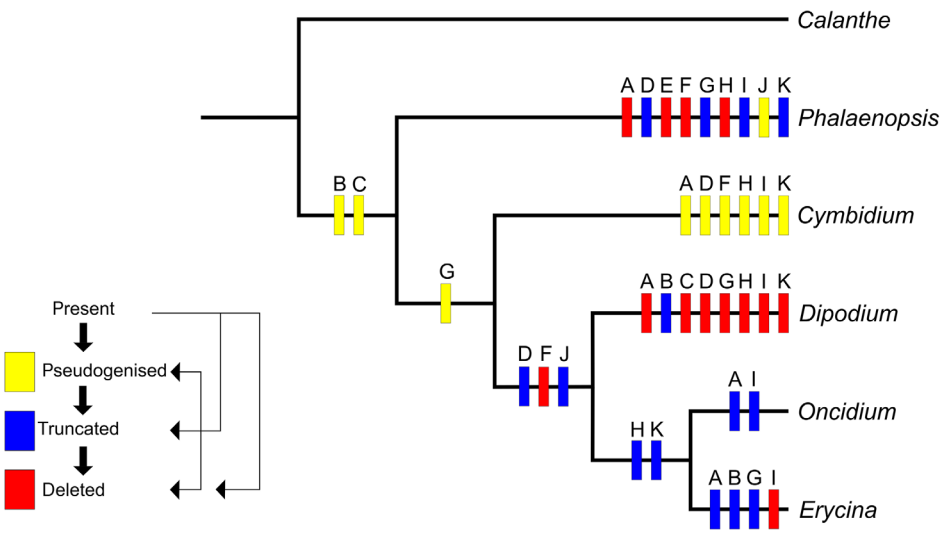

Fig. 5. Inferred evolution of *ndh* genes in Epidendroideae, incorporating data and the approach to character state optimisation used by Kim *et al.* (2015). Letters A to K refer to the 11 genes, *ndh*A to *ndh*K. The data matrix used for this character mapping is presented in Supplementary material S1.

Epidendroideae (Fig. 5) compared to the findings of Kim *et al.* 2015. There is some evidence that *ndh* subunits from the nuclear genome are lost alongside those from the plastome (Lin *et al.* 2017), but an alternative hypothesis of transferral of *ndh* genes to the nuclear or mitochondrial genome(s) is also plausible (Timmis *et al.* 2004). Further sequencing of both nuclear and mitochondrial genomes of orchids will allow testing of these hypotheses and determine the destination of *ndh* genes as they leave the plastome.

Relative to the currently understood general case of transition to mycoheterotrophy, *Dipodium* appears to be somewhat of an anomaly. *D. roseum* has many of the morphological characteristics of a mycoheterotroph but does not have any plastome degradation compared to the photosynthetic *Dipodium ensifolium*, illustrating that significant morphological change can be decoupled from functional loss of photosynthesis. Alternatively, *D. roseum* might represent a very recent transition away from autotrophy, and we happen to be observing that transition before the ensuing rapid gene loss. *D. roseum* is known to have fungal associations (Dearnaley and Le Brocque 2006*a*) with mycorrhizae commonly associated with mycoheterotrophy, and fungal

peletons can be observed in the body of the plant (Gross 1991; M. Whitehead, personal observation). Based on the contents of its plastome this species is possibly still capable of photosynthesis. Some populations of *D. roseum* have green stems (Fig. 1*c*; M. Bayly, personal observation), which indicates the plants are still capable of producing chlorophyll, although the presence of chlorophyll does not necessarily confer photosynthetic capability (Julou *et al.* 2005). It is possible that *D. roseum* can still photosynthesise, but only does so during flowering, and is on an evolutionary pathway towards full mycoheterotrophy. Were this the case, *D. roseum* may be better described as a mixotrophic species, capable of obtaining carbon both through photosynthesis and fungal mutualism (Dearnaley and Le Brocque 2006*a*; Girlanda *et al.* 2006; Yagame *et al.* 2012; Bellino *et al.* 2014).

Further research is required to confirm whether other terrestrial *Dipodium* species are facultative mycoheterotrophs, how frequently mycoheterotrophy may have evolved in the genus, what the pattern of *ndh* loss is within autotrophic species, and an assessment of the associated fungal diversity. Ideally, this would include a dated genus-level phylogeny, sequencing plastomes of all species in the genus (ideally multiple samples within a species), measures of photosynthetic capability of populations, and characterisation of the fungal associations of leafy and terrestrial species. If the terrestrial species of *Dipodium* are in the early stages of a transition to mycoheterotrophy, the genus may prove to be important in understanding the bold evolutionary step of abandoning photosynthesis in favour of fungal symbioses.

Supplementary material

Supplementary material is available online.

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