

Discordance Down Under: Combining Phylogenomics and Fungal Symbioses to Detangle Difficult Nodes in a Diverse Tribe of Australian Terrestrial Orchids

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Abstract.—Orchid mycorrhizal fungi (OMF) associations in the Orchidaceae are thought to have been a major driver of diversification in the family. In the terrestrial orchid tribe Diurideae, it has long been hypothesized that OMF symbiont associations may reflect evolutionary relationships among orchid hosts. Given that recent phylogenomic efforts have been unable to fully resolve relationships among subtribes in the Diurideae, we sought to ascertain whether orchid OMF preferences may lend support to certain phylogenetic hypotheses. First, we used phylogenomic methods and Bayesian divergence time estimation to produce a genus-level tree for the Diurideae. Next, we synthesized decades of published fungal sequences and morphological/germination data to identify dominant fungal partners at the genus scale and perform ancestral state reconstruction to estimate the evolutionary trajectory of fungal symbiont shifts. Across the tribe, we found phylogenomic discordance stemming from incomplete lineage sorting. However, our results also revealed unprecedented phylogenetic niche conservatism of fungal symbionts within the tribe: entire genera, subtribes, and even groups of related subtribes associate with only a single fungal family, suggesting that fungal symbiont preferences in the Diurideae do indeed reflect phylogenetic relationships among orchid hosts. Moreover, we show that these relationships have evolved directionally from generalist associations with multiple fungal families towards more specific partnerships with only one fungal family. Orchid symbiont preferences here provide new insights into the placement of several groups with longstanding phylogenetic uncertainty. In spite of complex evolutionary histories, host-symbiont relationships can be used to help detangle alternative phylogenetic hypotheses. [Key words: Ancestral state reconstruction; Diurideae; incomplete lineage sorting; mycorrhizae; Orchidaceae; phylogenomics; phylogenomic discordance; symbiosis.]

The evolutionary histories of plants and fungi are inextricably linked, and the development of mycorrhizal symbiosis is considered a key innovation that may have facilitated the successful colonization of terrestrial environments by early land plants (Field et al. 2015; Feijen et al. 2018; Jacquemyn and Merckx 2019; Wang et al. 2021b). Approximately 90% of terrestrial plants form mycorrhizal associations, 74% of which associate with c. 300–1600 taxa of arbuscular mycorrhizal fungi (van der Heijden et al. 2015). In contrast, members of the angiosperm family Orchidaceae predominantly partner with three orchid mycorrhizal fungal (OMF) families broadly labeled as “rhizoctonias”: Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae (Yukawa et al. 2009; Dearnaley et al. 2012). Not only can these associations be remarkably specific, with some orchid species partnering with just one or few putative fungal species (Jacquemyn et al. 2015; van der Heijden et al. 2015; Phillips et al. 2020), but all orchids are also obligately dependent on their mycorrhizal partners to germinate (Rasmussen and Rasmussen 2009; Wang et al. 2021a). With approximately 28,000 described species, the Orchidaceae is one of the most speciose families of flowering plants (Christenhusz and

Byng 2016). The evolution of OMF associations—particularly the recruitment of ubiquitous, saprotrophic fungi as mycorrhizal partners—may have facilitated niche expansions and radiations in Orchidaceae by enabling access to a wider suite of potential habitats and nutritional niches (Yukawa et al. 2009; Nurfadilah et al. 2013; Selosse et al. 2022). The orchid-mycorrhizal relationship is therefore hypothesized to have been a major driver of diversification in the family (Waterman and Bidartondo 2008; Waterman et al. 2011; Wang et al. 2021a).

A striking degree of orchid-mycorrhizal specificity is evident in the predominantly Australian terrestrial orchid tribe Diurideae (Orchidoideae). In contrast to European orchids, which on average partner with c. 13 fungal operational taxonomic units (OTUs) per species, intensive sampling combined with germination trials has revealed that many Australian orchid species associate with just one or two fungal OTUs (Phillips et al. 2020). Furthermore, any one genus of Diurideae will typically associate with an average of only three to four fungal OTUs from a single fungal family (Warcup 1981; Pridgeon and Chase 1995; Linde et al. 2014; Jacquemyn et al. 2017; Phillips et al. 2020; Freestone et al. 2021,

2022; Li et al. 2021a; Oktalira et al. 2021; Arifin et al. 2022, 2023). With estimates of diversity ranging from 900 to over 1,000 species, the Diurideae encompasses approximately 75% of Australia's terrestrial orchid species (Weston et al. 2014; Chase et al. 2015; Jones 2021; Peakall et al. 2021). Among these are some of the world's most charismatic terrestrial orchids, including *Rhizanthella*, the rare underground orchids, and an unusually high incidence of species pollinated by sexual deception of male insects, documented in over 150 species across at least nine genera (Peakall 1990, 2023; Schiestl 2005; Peakall et al. 2010; Gaskett 2011; Phillips et al. 2013; Weston et al. 2014; Ackerman et al. 2023). While Australia represents the center of diversity for the Diurideae, the tribe also extends to New Zealand, New Caledonia, Malesia, and East Asia (Kores et al. 2001). As to *why* such a staggering degree of ecological specificity has evolved both above and below ground in the Australian terrestrial orchid flora remains unknown.

Our ability to interrogate such evolutionary questions within the Diurideae has so far been hampered by a lack of a robust phylogeny of the tribe (Kores et al. 2001). While Diurideae itself is a well-supported tribe, some subtribal relationships continue to be uncertain, with molecular

phylogenetic reconstructions of the tribe to date either lacking resolution or exhibiting incongruent topologies (Kores et al. 2001; Cameron 2006; Weston et al. 2014; Chase et al. 2015; Givnish et al. 2015; Nauheimer et al. 2018; Peakall et al. 2021; Wong and Peakall 2022; Zhang et al. 2023; Pérez-Escobar et al. 2024). The placement of subtribes such as Prasophyllinae and Acianthinae has been particularly variable (Fig. 1).

Over the past two decades, several molecular studies have independently investigated individual subtribes of the Diurideae (Indsto et al. 2009; Peakall et al. 2010; Lyon 2014; Miller and Clements 2014; Nargar et al. 2018; Nauheimer et al. 2018; Wong et al. 2022). However, there has been a more limited investigation of the relationships among these subtribes. Peakall et al. (2021) recovered the most recent tribal-scale phylogeny with near-complete genus representation, employing a custom-designed exon capture bait set focused on the tribe. While a tribal-scale phylogeny was published, this publication was primarily concerned with the target-capture methodology and subsequent bioinformatic pipelines. Nevertheless, despite the addition of thousands of phylogenetically informative sites, the trees recovered in that study exhibited short branch

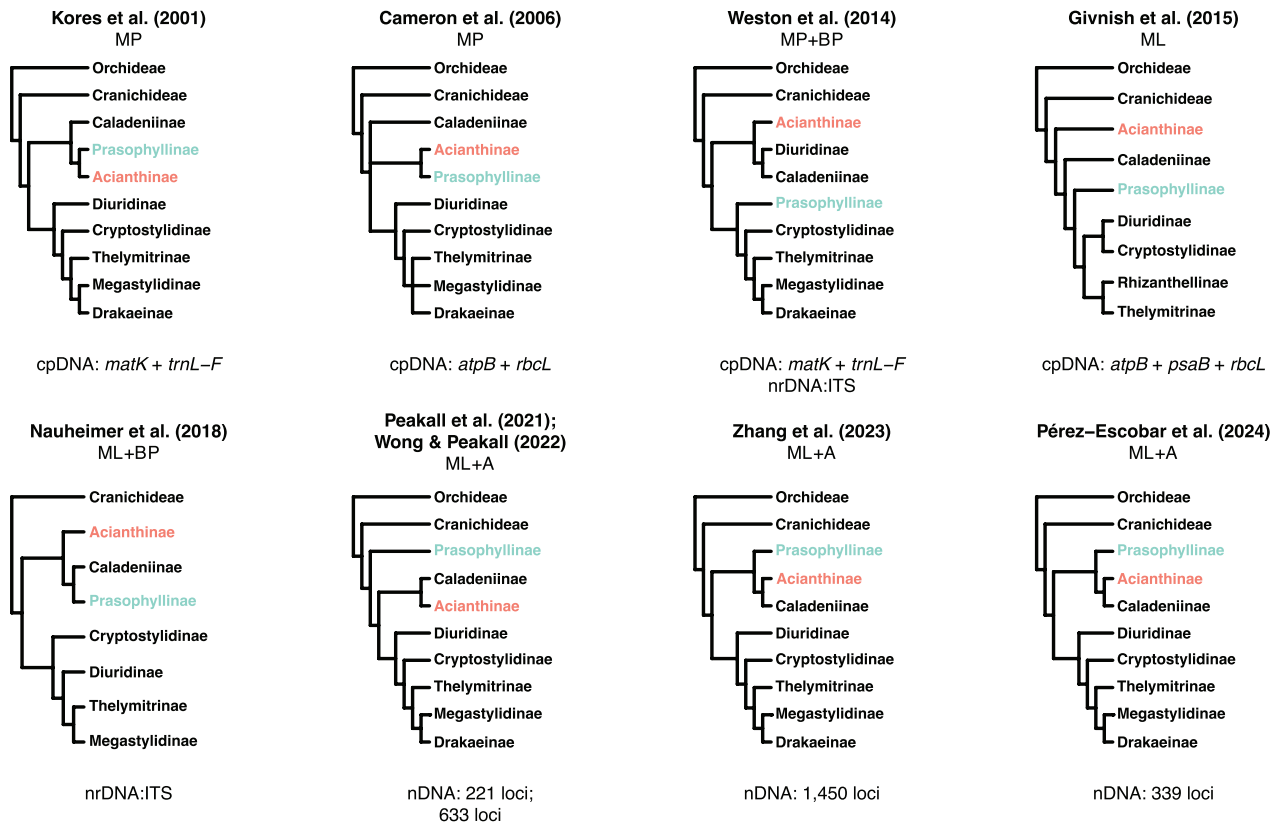


FIGURE 1. Subtribal topologies recovered for the Diurideae adapted from previous publications arranged chronologically with tree inference methods outlined above, and chloroplast (cpDNA), nuclear ribosomal (nrDNA), and nuclear (nDNA) gene regions used in each study outlined below each tree. The problematic subtribes Prasophyllinae and Acianthinae are highlighted. Serna-Sánchez et al. (2021) and Pérez-Escobar et al. (2021) are not shown here due to insufficient subtribal representation of the Diurideae. Note: Rhizanthellinae is nested within Prasophyllinae in Weston et al. (2014). Unless explicitly mentioned, Rhizanthellinae is otherwise unrepresented in the studies shown here. MP = Maximum Parsimony; BP = Bayesian Probability; ML = Maximum Likelihood; and A = ASTRAL Summary.

lengths and poor gene concordance along the backbone of the tree, suggesting the presence of either incomplete lineage sorting (ILS) or deep introgression, possibly resulting from rapid radiation. This pattern was echoed in a recent phylogenomic study of the Orchidaceae with the most genomic coverage to date (Zhang et al. 2023). Even with Zhang et al. (2023)'s inclusion of 1450 low-copy nuclear genes, subtribal relationships in the Diurideae were still poorly resolved, particularly with respect to the Prasophyllinae and Acianthinae.

Phylogenomic studies across the Tree of Life incorporating increasingly large amounts of data have repeatedly uncovered widespread phylogenetic discordance, highlighting that the systematist's goal of complete phylogenetic resolution may ultimately be limited by the inherent complexity of biology (Guo et al. 2023; Stull et al. 2023). To that end, it is clear that we must find alternate means of distinguishing among alternative evolutionary hypotheses in spite of confounding biological processes. Given the possibility that patterns of fungal symbioses may reflect evolutionary relationships among host orchids in the Diurideae, we explore here whether host-symbiont relationships may offer clues that can assist in the resolution of phylogenetically recalcitrant clades. Warcup (1981) observed that genera within the Diurideae that shared symbionts from the same fungal family appeared to be closely related, and hypothesized that these relationships may reflect the orchid phylogeny. Kores et al. (2001) also suggested that fungal associations may indeed bear phylogenetic significance and recommended further investigation against a robust phylogeny. More recently, phylogenetic niche conservatism of fungal symbionts (i.e., considering the fungal symbiont as a component of the orchid's niche) has been demonstrated at the subtribal scale (Arifin et al. 2023), raising the question of whether this phenomenon extends throughout the Diurideae more broadly. Thus, our goals in this study were to: 1) determine the dominant fungal partners for genera within the Diurideae; 2) ascertain whether patterns of fungal symbioses are phylogenetically structured in the tribe; 3) reconstruct the evolutionary history of these interactions; and 4) assess whether these relationships can be used to discriminate between alternate phylogenetic hypotheses.

MATERIALS AND METHODS

Taxon Sampling

To recover a genus-level phylogeny of the Diurideae, three data sets were curated: 1) a nuclear transcriptome set (tsDNA) consisting of 11 transcriptomes with eight accessions representing all subtribes of Diurideae and one accession of *Pterostylis curta* (Orchidoideae; Cranichideae) generated by Peakall et al. (2021), one newly sequenced transcriptome for *Rhizanthella slateri* (Diurideae; Prasophyllinae) generated using similar methods outlined in Wong et al. (2024) (raw RNA-seq data deposited in the NCBI Sequence Read Archive (SRA) under the BioProject

and SRA accession PRJNA661963 and SRR31372432, respectively), and one sample of *Gymnadenia densiflora* (Orchidoideae; Orchideae) sourced from the GenBank (Clark et al. 2016) Transcriptome Shotgun Assembly database (SRR8175725); 2) a chloroplast data set (cpDNA) extracted from the same transcriptomes used for the tsDNA set, to assess topological differences between nuclear and chloroplast trees; and 3) an extended set (esDNA) that combined nuclear data from 54 target-capture accessions and the tsDNA transcriptomes recovered by Peakall et al. (2021), the newly sequenced *Rhizanthella slateri* transcriptome, and 116 other publicly available accessions sourced from the NCBI SRA (Supplementary Table S1; <https://doi.org/10.5061/dryad.b2rbnzsqp>). Taxa were selected to cover the breadth of Diurideae subtribes, along with representatives from subtribes across the Orchidaceae. To provide adequate calibration points for divergence time estimation, additional outgroup taxa were included in the esDNA set from the Asparagales, Liliales, Zingiberales, Arecales, Poales, Dioscoreales, and Alismatales. A total of 32 of 35 accepted genera (POWO 2024) of the Diurideae were included for analysis. Genome-scale sequence data were not available for three monotypic genera; *Aporostylis*, *Coilochilus*, and *Waireia*.

Use of Generic Names in Diurideae

Accepted generic names here follow treatments outlined in Kew's Plants of the World Online (POWO 2024). Of particular importance is: 1) the treatment of the genera *Cyanicula*, *Elythranthera*, *Ericksonella*, and *Glossodia* under an expanded *Caladenia*; 2) the treatment of *Phoringopsis* and *Thynninorchis* under an expanded *Arthrochilus*; and 3) the treatment of *Paracaleana* as a synonym of *Caleana*. These treatments deviate from those provided by the Australian Plant Name Index (APNI 2024), along with several Australian herbaria. As we have included many non-Australian genera as outgroups in this study, rather than adopt several conflicting classifications from different herbaria for different components of our data set, we have instead elected to adopt Kew's POWO treatment. However, we note that our choice to adopt POWO's taxonomic treatment of these genera does not necessarily reflect the taxonomic opinions of the authors.

Read Pre-Processing and Target-Capture Assembly

To remove adapter sequences, trim, and filter raw paired-end reads, Trimmomatic v0.39 (Bolger et al. 2014) was used using the following settings: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:2:True LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:50. HybPiper v2.1.6 (Johnson et al. 2016) was used to perform reference-based assembly, extract coding sequences, and identify putative paralogs from trimmed reads using the default settings with BWA (Li and Durbin 2009) selected as the read aligner. A reference file of target loci for HybPiper was sourced from "Set

2" in Peakall et al. (2021). "Set 2" is a set of 245 nuclear loci identified to be common across the tribe Diurideae, along with the outgroup *Pterostylis* (Orchidaceae; Cranichideae). As this set was targeted at Diurideae at the tribal scale, it was considered most appropriate for this study. To supplement the "Set 2" references, nuclear Angiosperms353 (Johnson et al. 2019) references were added to the target file to allow for the inclusion of several publicly available target-capture sequences.

Paralogy Resolution

To reduce the confounding effects of paralogous loci in downstream analyses, sequences recovered by *HybPiper* were processed using orthology inference methods described by Yang and Smith (2014) and adapted for use with target-capture data sets by Morales-Briones et al. (2022) as the Python package *ParaGone* v0.0.14rc (available at github.com/chrisjackson-pellicle/ParaGone). *ParaGone* takes the output from the *HybPiper* function *paralog_retriever* as input. The *paralog_retriever* function outputs two folders that can be used for further analysis: the first with all sequences recovered by *HybPiper* with putative paralogs; and the second with the same sequences, but with sequences flagged as putative chimeric sequences by *HybPiper* removed. To reduce the possible impact of chimeric sequences on further downstream analyses, only the second folder output by *paralog_retriever* with putative chimeric sequences removed was used for further analyses. *ParaGone* was run using the *full_pipeline* function with default settings and *G. densiflora* set as the outgroup for the tsDNA set, and *Sagittaria latifolia* (Alismataceae; Alismatales) for the esDNA set using the *--internal_outgroups* flag. Paralogs were resolved using the Rooted subTrees/Rooted Ingroups (RT) algorithm option as this is the method considered most appropriate by Yang and Smith (2014) for data sets with known high-quality outgroup representatives and probable genome duplication events (i.e., most plant families). This method uses user-designated outgroups to iteratively identify a subtree with the largest number of ingroup taxa. Outgroup taxa are subsequently pruned from this subtree, and the subtree is then used to infer gene duplications from root to tip. The full *ParaGone* pipeline outputs resolved and aligned sequences with trimmed terminal ends. As designated outgroups are pruned by the RT algorithm, final alignments were stripped of sequences from *G. densiflora* and *S. latifolia*. Alignments output by *ParaGone* <500 bp in length were excluded from further analysis. For the tsDNA set, any alignments below 100% taxon coverage were also removed, whereas filtering was relaxed to 50% taxon coverage for the esDNA set.

Chloroplast Assembly and Alignment

To recover chloroplast sequences from the transcriptome reads, reference-guided assembly was also performed using *HybPiper* with the same settings outlined above. A target file of chloroplast regions was

built using whole plastome sequences available on GenBank sampled from the Diurideae (*Corybas diene-mus*, MK867775; *Chiloglottis cornuta*, MK848867; *Microtis unifolia*, MK796239; *Rhizanthella gardneri*, GQ413967) and Cranichideae (*Goodyera fumata*, KJ501999; *Hetaeria oblongifolia*, MW589525). To standardize annotations between plastomes retrieved from GenBank, all reference plastomes were re-annotated using the organellar annotation web application *GeSeq* (Tillich et al. 2017) (available at chlorobox.mpimp-golm.mpg.de/geseq.html) with ARAGORN v1.2.38 and *Chloë* v0.1.0 enabled. Annotated regions were then extracted from the re-annotated .gb files output by *GeSeq* using the Python script *get_annotated_regions_from_gb.py* (available at github.com/Kinggerm/PersonalUtilities/blob/master/get_annotated_regions_from_gb.py) developed by Zhang et al. (2020). Target regions extracted from reference plastomes were included in subsequent cpDNA alignment and tree inference steps. Each region recovered by *HybPiper* was aligned using MAFFT v7.453 (Katoh and Standley 2013) with the following arguments: *--globalpair --maxiterate 1000 --leavegappyregion*. Subsequent alignments were trimmed using *trimAl* v1.4.rev22 (Capella-Gutiérrez et al. 2009) using the same arguments used by *ParaGone* in its *trimAl* trimming step (*-gapthreshold 0.12 -terminalonly -gw 1*).

Nuclear Phylogenetic Inference

Gene trees for each locus recovered for the tsDNA and esDNA sets were estimated using IQ-TREE v2.2.2.7 (Minh et al. 2020b). Best-fitting substitution models for each alignment were determined by *ModelFinder Plus* (Kalyaanamoorthy et al. 2017) as implemented in IQ-TREE by calling *-m MFP*. To assess branch support, 1,000 bootstrap replicates were calculated for each alignment using the SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010). Clades in bootstrapped unrooted gene trees output by IQ-TREE with 0% SH-aLRT support were collapsed using the *Newick Utilities* v1.6.0 function *nw_ed* (Junier and Zdobnov 2010) as recommended by Simmons and Gatesy (2021) for downstream summary tree analyses.

Chloroplast Phylogenetic Inference

Final trimmed and aligned chloroplast regions were concatenated and analyzed as a single locus using IQ-TREE with the argument *-p* which concatenates all alignments within an input directory into a supermatrix for further tree inference. Substitution model selection was then performed using *ModelFinder Plus* using the argument *-m MFP+MERGE* which first determines the optimum number of partitions by iteratively merging alignments until model fit does not improve further and subsequently estimates substitution models for each resulting partition. Branch support was estimated with 1,000 SH-aLRT bootstrap replicates as above, with the addition of 1,000 ultrafast bootstrap approximation (UFBoot) replicates (Hoang et al. 2018).

Discordance Analysis

To recover a phylogeny that accommodates confounding evolutionary processes such as ILS and introgression, summary species trees for the tsDNA and esDNA sets were inferred from unrooted gene trees using *ASTRAL*-III v5.7.8 (Zhang et al. 2018) with the *-t* 32 flag used to calculate alternate quartet support values. *ASTRAL* node support is quantified with a local posterior probability score, with values of 1.0 considered strong support, 0.90–0.99 moderate support, 0.80–0.89 weak support, and values below considered poor support. To quantify phylogenetic discordance, the *ASTRAL* tree was used as an input species tree to calculate gene concordance factors (gCF) using *IQ-TREE* (Minh et al. 2020a). Site concordance factors (sCF) were calculated using the updated likelihood calculation option (*-scf*) introduced by Mo et al. (2023). Coalescent theory dictates that under the assumption of ILS, gene trees or sites supporting alternative topologies should occur with approximate equal frequency (Huson et al. 2005; Green et al. 2010; Martin et al. 2015). Therefore, alternative explanations other than ILS (i.e., introgression) are supported when alternate topology frequencies are not equal. To test whether discordant topological frequencies at problematic nodes were significantly different, an exact binomial test was conducted on alternate topology discordance factors output by *IQ-TREE* (Supplementary Methods S1) and on alternate topology normalized quartet scores output by *ASTRAL* (Supplementary Methods S2). Gene concordance factors were visualized as a heatmap color gradient on the *ASTRAL* species tree using the *Interactive Tree of Life* (iTOL) v.6.7 platform (Letunic and Bork 2021).

Four-Cluster Likelihood Mapping

Four-cluster likelihood mapping (FcLM) (Strimmer and von Haeseler 1997) was completed using *IQ-TREE* to assess support for alternative topologies present within our alignments. Analyses were conducted on the concatenated supermatrix using the *-p* flag. Automatic model selection for each partition was performed using the *-m* MFP flag for the tsDNA and esDNA sets, and *-m* MFP+MERGE for the cpDNA set. All unique quartets were drawn for each analysis. Two separate cluster analyses were run to assess support for: 1) relationships among Prasophyllinae, Acianthinae, Caladeniinae, and the remainder of the Diurideae and 2) relationships among Prasophyllinae, a Caladeniinae+Acianthinae cluster, a Diuridinae+Cryptostylidinae+Thelymitrinae+Megastylidinae+Drakaeinae cluster, and outgroup taxa.

Divergence Time Estimation

To gain an understanding of the time scale of the evolution of the Diurideae, divergence time estimations were carried out using the *ASTRAL* species tree from analysis of the esDNA set as the main input topology. A

total of 14 fossil calibration points within the monocots were incorporated in our analyses (Supplementary Table S2). This calibration set includes three Orchidaceae fossils that have been widely used in previous divergence time estimates within the Orchidaceae (Ramírez et al. 2007; Conran et al. 2009; Gustafsson et al. 2010), along with three more recently published Orchidaceae fossils (Poinar 2016a, 2016b; Poinar and Rasmussen 2017). With a total of six Orchidaceae fossil calibrations, this study incorporates the highest number of Orchidaceae fossils utilized to date in the literature. The remaining fossil calibrations include six internal calibrations within the monocots, and one root calibration based on the approximate age of the monocots (113–125 Ma) utilized by Zhang et al. (2023) with dates sourced from Iles et al. (2015) and Eguchi and Tamura (2016).

Bayesian divergence time estimation using an approximate likelihood method was conducted using *MCMCtree* v4.10.6 (Rannala and Yang 2007) as implemented in the software package *PAML* (Yang 1997). The R script *genesortR* (Mongiardino Koch 2021) was used to calculate several metrics of phylogenetic informativeness to assess the suitability of recovered loci in the esDNA set for divergence time estimation analyses. Output from *genesortR* (Supplementary Methods S3) showed that phylogenetic signal decreased beyond the top 200 sorted loci, therefore only these loci were retained for further analysis. The 200 selected loci were then concatenated into a single alignment and then split into three separate partitions by the first, second, and third base-pair positions of the alignment using *AMAS* (Borowiec 2016). The three partitions were then input into the *PAML* program *BASEML* which was used to calculate maximum likelihood branch length estimates, along with the gradient and Hessian matrix necessary for subsequent divergence time estimation using *MCMCtree*. Output from *BASEML* (out.BV) were then used as input parameters for the *MCMCtree* divergence time calculation step. Burn-in was set to 10,000 with a sample frequency of 120, with 20,000 samples required, resulting in a total of 2,410,000 iterations. Two independent runs were completed to check for convergence by comparing mean estimates between runs (Supplementary Fig. S1).

Orchid Mycorrhizal Fungi Taxonomic Notes

As noted already, orchid mycorrhizal fungi (OMF) are predominantly drawn from the families Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae. Unfortunately, there is considerable confusion surrounding the use of generic names within these families due to ongoing nomenclatural issues stemming from the use of dual anamorphic/teleomorphic typified names. Despite attempts to unify fungal taxonomy under a “one fungus, one name” principle (Hawksworth 2011; Stalpers et al. 2021), the use of superseded fungal generic names is still commonplace within the orchid literature. This issue is further confounded by the fact that a great deal of orchid mycorrhizal literature predates

molecular taxonomy and the adoption of the “one fungus, one name” principle. Thus, we provide a summary of these nomenclatural issues below to provide clarity and consistency regarding how we addressed the taxonomy of these families in the remainder of this study.

The Ceratobasidiaceae encompasses putatively orchid mycorrhizal genera including *Ceratobasidium*, *Ceratorhiza*, *Rhizoctonia*, and *Thanatephorus*. All genera within the Ceratobasidiaceae are here considered synonymous under the unified generic name *Rhizoctonia*. Refer to the 2024 Outline of Fungi (Hyde et al. 2024) and Oberwinkler et al. (2013) for a detailed discussion of this treatment. Orchid mycorrhizal species of Tulasnellaceae are identified in the literature under the generic names *Tulasnella* and *Epulorhiza*. As per Stalpers et al. (2021), *Tulasnella* and *Epulorhiza* are treated as synonymous here under the name *Tulasnella*. The order Sebaciniales was split by Weiß et al. (2016) into two families; Sebacinaceae (“Sebaciniales Group A”); and the monogeneric family Serendipitaceae (“Sebaciniales Group B”), which contains the genus *Serendipita* (= *Piriformospora*). The majority of green orchid mycorrhizal fungi in Sebaciniales are considered to be species of *Serendipita*, although there are some species of *Sebacina* that partner with fully or partially mycoheterotrophic orchid species (Weiß et al. 2016). Older orchid mycorrhizal literature primarily refers to orchid mycorrhizal fungal species as *Sebacina*, but where possible in this study, we have attempted to corroborate these data with molecular data and split Sebaciniales sequences into their respective families following Weiß et al. (2016).

Identification of Orchid Mycorrhizal Fungal Partners

To identify the dominant fungal partners for each of the orchid genera included in this study, we performed a literature search and mined publicly available fungal sequences (last assessed: 23 May 2024). Our literature search spanned all known mycorrhizal and germination studies for all genera of the Diurideae, and the most closely related Cranichideae outgroup subtribes Pterostylidinae and Chloraeinae (Supplementary Table S3). GenBank was then mined for orchid-associated fungal sequences by searching for each orchid genus and filtering to display only sequences from fungi. This was repeated for orchid nomenclatural synonyms to ensure synonymous genera were captured. Retrieved sequences were then cross-referenced with studies identified in the literature search to ensure all published sequences were included. Sequences from published studies that were not retrieved in the preliminary searches were subsequently retrieved manually. Unpublished fungal sequences isolated from members of the Acianthinae generated by Lyon (2014) were shared with us by the author. To ensure only fungal Internal Transcribed Spacer (ITS) sequences were retained for further analysis, fungal sequences retrieved from GenBank were then processed using *ITSx* v1.1.3 (Bengtsson-Palme et al. 2013) which extracts the ITS region from input sequences. Sequences with no ITS

region recovered by *ITSx* were subsequently discarded from further analyses.

To accommodate mis/unidentified accessions, retained fungal sequences were re-classified using *BLASTn* v2.12.0, searching against the RefSeq Fungal ITS database (O’Leary et al. 2016), which contains representative fungal ex-type sequences. Initial *BLAST* searches against the RefSeq Fungal ITS database failed to correctly identify sequences confidently known to represent species (i.e., ex-type sequences from recently described species) of the dominant OMF families; Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae. These initial *BLAST* searches also failed to accurately resolve sequences split across the Sebacinaceae and Serendipitaceae (e.g., type sequences of species of *Serendipita* were not identified as *Serendipita*, despite being confidently placed within Serendipitaceae in their source publications). As searches against the default RefSeq Fungal ITS Database could not accurately classify sequences within these families, a custom database (Supplementary Data S1) was created by adding additional sequences known to represent ex-type sequences for as many described species as possible of *Rhizoctonia*, *Sebacina*, *Serendipita*, and *Tulasnella*. This resulted in the addition of 54 ex-type sequences which were then able to be referenced in *BLAST* analyses. Family-level taxonomic assignments for each *BLASTed* sequence were retrieved by matching the species name of the top *BLAST* match against the Global Biodiversity Information Facility (GBIF 2024) Backbone Taxonomy using the R package *rgbif* v3.8.0 (Chamberlain and Boettiger 2017). Sequences not belonging to putatively orchid mycorrhizal fungal families—Ceratobasidiaceae, Tulasnellaceae, Serendipitaceae, Sebacinaceae, Thelephoraceae, Russulaceae, Tubercellaceae, Clavulinaceae, Hymenochaetaceae, Marasmiaceae, Psathyrellaceae, Mycenaceae, and Physalacriaceae (Dearnaley et al. 2012; Wang et al. 2021a)—were filtered out and excluded from subsequent analyses.

The dominant fungal partner for each orchid genus included in this study was coded as the fungal family that was identified in over 90% of sequences. For genera that did not associate with a clear dominant fungal family, fungal families recovered in over 10% of isolated sequences were coded as polymorphic, with each family over 10% included as a character state. Fungal sequences were coded as Ceratobasidiaceae, Tulasnellaceae, or Serendipitaceae if they were identified within these three major orchid mycorrhizal fungal families. Fungal sequences identified as Sebacinaceae, Thelephoraceae, Russulaceae, Tubercellaceae, Clavulinaceae, Hymenogastraceae, or Inocybaceae were coded as ECM (ectomycorrhiza), and sequences identified as Pezizaceae, Pyronemataceae, Hymenochaetaceae, Marasmiaceae, Psathyrellaceae, Mycenaceae, or Physalacriaceae were coded as SAP/ECM (Saprotrophic/ECM) following Wang et al. (2021a). If sequence data were unavailable for a taxon, the dominant fungal partner was inferred from available literature based on putative identifications based

on morphology, and the ability of these fungi to induce germination and protocorm formation. Orchid genera lacking sequence data or fungal data in the literature were excluded from further analyses. Following sequence classification, no genera in our data set were associated with enough sequences classified as SAP/ECM for it to be coded as a possible character state and it was consequently excluded as a character state from further analyses.

Ancestral State Reconstruction

To reconstruct the evolutionary history of mycorrhizal associations within the Diurideae, we performed marginal ancestral state reconstruction (Supplementary Methods S4), with the two most closely related Cranichideae subtribes—Pterostylidinae and Chloraeinae—used as outgroups. To determine whether conclusions garnered from ancestral state reconstructions were consistent across alternate phylogenetic hypotheses, ancestral state reconstruction was carried out on the final species tree recovered from analysis of the esDNA data (henceforth referred to as Topology 1), along with constrained topologies matching the two alternate topologies tested in previous analyses: Topology 2—(Prasophyllinae, (Clade A, Clade B)); and Topology 3—(Clade A, (Prasophyllinae, Clade B)). To create the constrained alternate topologies, the function *tree.merger()* from the R package *RRphylo* v.2.8.0 (Castiglione et al. 2018) was used to re-bind the Prasophyllinae. Genera with multiple species representatives in these trees were collapsed to a single branch per genus using the *keep.tips()* function as implemented in *ape* v.5.7.1 (Paradis and Schliep 2019).

To determine the most appropriate trait evolution model for our data set, several models were fitted and compared using the *phytools* v.1.5.13 function *fitpolyMk()* (Revell 2012). This method fits a continuous-time Markov chain model (i.e., an extended Mk model *sensu* Lewis (2001) and Harmon (2019)) to estimate ancestral states for discrete polymorphic characters. The best model was determined by comparing the AIC scores for each model fit. Character models were fit using four different parameterizations; 1) an equal rates model which assumes all character transitions happen at an equal rate; 2) a symmetric model, which assumes that each backward and forward transition pair can have different rates; 3) an all-rates-different model, which assumes that every character transition may have a different rate; and 4) a transient model, which assumes that polymorphism is acquired at a constant rate and lost at a faster rate based on the proposition that polymorphism is inherently less stable than a monomorphic state (Harmon 2019). Each of these trait models was fitted considering character states as either unordered (where any state may transition to any other state), or ordered (where certain transitions are impossible without first moving through an intermediate state) (Harmon 2019). Ordered evolutionary models were fit for the ordered set {C, T, S, ECM}, representing the categories “Ceratobasidiaceae,” “Tulasnellaceae,”

“Serendipitaceae,” and “Ectomycorrhizal Fungi,” respectively, based on patterns of ordered evolution observed by Yukawa et al. (2009), Waterman et al. (2011), and Shefferson et al. (2010). Ordered models implemented in *phytools* assume that state changes are bidirectional between character states (i.e., $A \leftrightarrow B$ vs. $A \rightarrow B$). At the time of writing, the *phytools* function *fitpolyMk()* does not support uni-directional (i.e., $A \rightarrow B$) models. Thus, in order to model uni-directional transitions, polymorphic states observed in our data set were re-coded as ‘monomorphic’ character states (e.g., “C” = 1, “C+T” = 2, etc.) and a custom transition matrix was built which allowed character transitions only in one direction. These data were then used as input to fit unidirectional models using the *fitMk()* function. Across all topologies and models tested, the ordered-transient bidirectional model was the best-supported model based on AIC scores (Supplementary Table S4). Consequently, only ordered-transient models were retained for further analyses. Marginal ancestral state reconstruction was then performed using the *phytools* function *ancr()* which takes a fitted model as input and calculates empirical Bayes posterior probabilities (marginal ancestral states) for each node in the input tree.

RESULTS

Sequence Recovery and Alignments

Following filtering and quality control of assembled and aligned loci, 271 loci for the 10-taxon tsDNA set with 100% taxon coverage were retained for further analysis. For the 179-taxon extended set (esDNA), 259 loci were retained at the 50% coverage threshold. Final alignments for each set included 78,676 sites for the 16-taxon cpDNA set (6,081 parsimony informative), 330,369 sites for the tsDNA set (38,245 parsimony informative), and 276,243 sites for the esDNA set (153,763 parsimony-informative).

Subtribal Topologies Within the Diurideae

Subtribal topologies for the Diurideae recovered in this study were largely congruent with previous studies of the group at this scale (Kores et al. 2001; Weston et al. 2014; Peakall et al. 2021; Zhang et al. 2023; Pérez-Escobar et al. 2024). All analyses consistently recovered two main clades within the Diurideae: the first containing Diuridinae, Cryptostylidinae, Thelymitrinae, Megastylidinae, and Drakaeinae, branching in that order (henceforth referred to as Clade A), and the second containing Caladeniinae and Acianthinae (henceforth referred to as Clade B). Rhizanthellinae clustered with the Prasophyllinae across all analyses. However, the placement of the Prasophyllinae+Rhizanthellinae clade was variable. Trees estimated from the tsDNA and cpDNA sets (Fig. 2) placed Prasophyllinae+Rhizanthellinae as sister to Clade B, whereas analysis of the esDNA set

Nuclear (tsDNA) ML + A tree

271 loci; 330,369 sites

Chloroplast (cpDNA) ML tree

78,676 sites

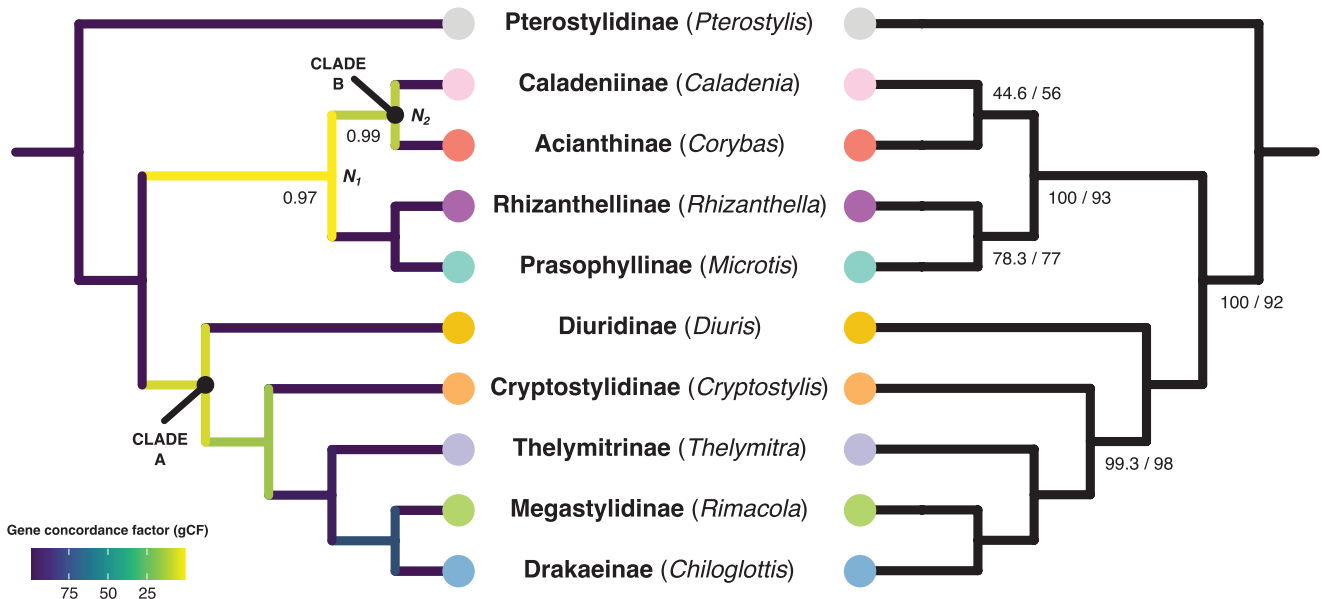


FIGURE 2. Species tree cladograms recovered for the transcriptome (tsDNA) and chloroplast (cpDNA) sets with tsDNA branches coloured by gene concordance factor (gCF). Nodes on the tsDNA tree with *ASTRAL* local posterior probabilities (LPP) <1.0 and nodes on the chloroplast tree with SH-aLRT/UFBoot <100 are indicated with printed probability scores. All other unlabeled nodes have an LPP = 1.0, or SH-aLRT/UFBoot = 100. For the unpruned version of the cpDNA tree with reference plastome tips displayed, refer to [Supplementary Data S2](#). Nodes with *ASTRAL* normalized quartet scores visualized in [Fig. 4](#) are highlighted here as N_1 and N_2 . ML = Maximum Likelihood; A = *ASTRAL* Summary.

placed Prasophyllinae+Rhizanthellinae at the base of Clade A (Fig. 3).

Nodes at the base of Clades A and B were generally poorly supported across all measures and analyses. All subtribes within the Diuridae were recovered as monophyletic with the exception of the Megastylidinae, which was recovered as a series of species-poor clades sister to the Drakaeinae. This finding is consistent with the results of Kores et al. (2001), Cameron (2006), Weston et al. (2014), Peakall et al. (2021), and Pérez-Escobar et al. (2024), all of which similarly recovered the Megastylidinae as non-monophyletic. As Megastylidinae has consistently been shown to be non-monophyletic, revision of this subtribe is necessary. Similarly, Rhizanthellinae was confidently recovered as nested within the Prasophyllinae, echoing the results of Weston et al. (2014). Here, *Rhizanthella* was recovered as sister to a clade containing *Prasophyllum* and *Genoplesium*, whereas Weston et al. (2014) placed *Rhizanthella* within a clade containing genera now considered synonymous with *Microtis*. As Rhizanthellinae is nested within Prasophyllinae, the two subtribes should be considered synonymous. Thus, for the remainder of this manuscript, Rhizanthellinae will be considered as part of the Prasophyllinae. Our results also recovered *Genoplesium* as nested within the clade containing all accessions of *Prasophyllum*, suggesting that the two genera are most likely congeneric.

A binomial test of discordant topology frequencies (Fig. 4a) for the (Prasophyllinae, (Caladeniinae, Acianthinae)) node in the tsDNA tree found that the number of gene

trees and sites supporting alternate topologies did not differ significantly ($P = 0.26$; 1.0, respectively). The (Caladeniinae, Acianthinae) node in the tsDNA tree similarly showed gene and site alternate topology frequencies that were not significantly different ($P = 0.25$; 0.34). Binomial tests of *ASTRAL* normalized quartet scores for the same nodes in the tsDNA tree similarly failed to find any significant difference ($P = 1.0$; 0.24). A binomial test of the (Prasophyllinae, (Clade A)) node in the tsDNA tree similarly found no significant difference in alternate gene tree and site topology frequencies ($P = 0.51$; 0.91). However, a binomial test of the (Caladeniinae, Acianthinae) node in the tsDNA tree found alternate gene tree frequencies that were not significantly different ($P = 1.0$), but alternate site frequencies that were significantly different ($P = 0.04$). Binomial tests of *ASTRAL* normalized quartet scores for all nodes mentioned here also failed to find any significant difference in alternative quartet support scores (Methods S2). As the majority of these tests found no significant differences between alternate topology frequencies or normalized quartet scores, ILS is the most probable driver of phylogenetic discordance observed at these nodes.

Four-Cluster Likelihood Mapping of Diuridae Topologies

When testing arrangements of Prasophyllinae with respect to Clade A, Clade B, and remaining outgroup taxa, four-cluster likelihood mapping found that the majority of quartets across all analyzed sets (tsDNA = 80.0%;

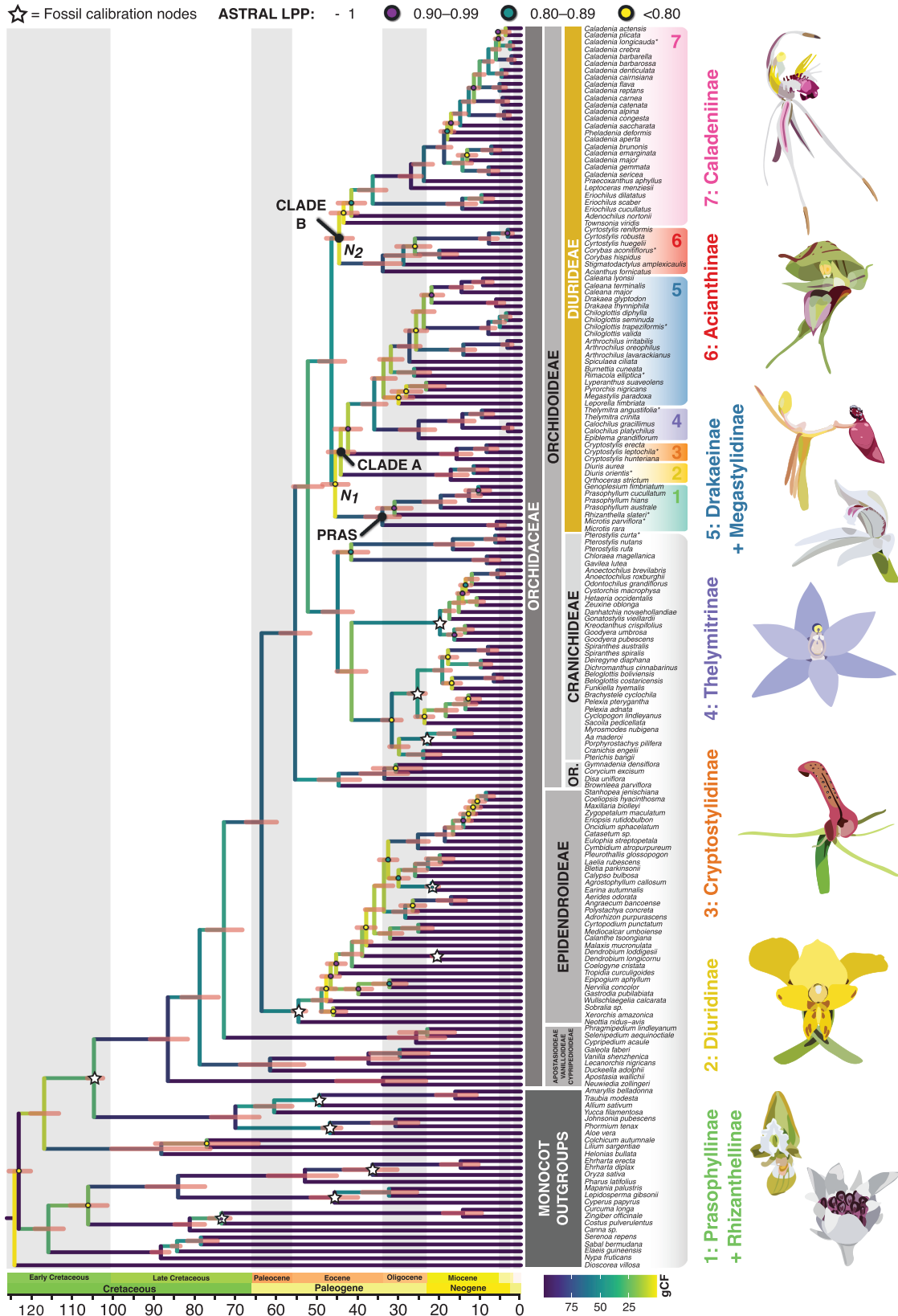


FIGURE 3. Chronogram of the Diurideae and outgroup taxa recovered from analyses of the esDNA dataset with branches coloured by gCF values. Nodes with ASTRAL LPP <0.80 are highlighted with coloured points. Fossil calibration nodes are indicated with stars. Nodes with ASTRAL normalized quartet scores visualized in Fig. 4 are highlighted here as N_1 and N_2 . * = samples used in the tsDNA and cpDNA sets. For the raw version of the time tree with mean node ages and 95% Highest Posterior Density (HPD) intervals, refer to Supplementary Data S3. Genus names follow POWO (2024)—see methods for explanation.

esDNA = 38.1%; cpDNA = 46.8%) supported a Prasophyllinae+Clade B cluster and a Clade A+Outgroup cluster (Fig. 4b). When testing topological arrangements of Prasophyllinae, Acianthinae and Caladeniinae with respect to Clade A, the majority of quartets across nuclear sets (tsDNA = 66.7%; esDNA = 35.6%) support a Prasophyllinae+Clade A cluster and a Caladeniinae+Acianthinae cluster, whereas the majority of quartets inferred from the chloroplast set support a Prasophyllinae+Caladeniinae cluster and an Acianthinae+Clade A and outgroup cluster (Fig. 4b).

Divergence Time Estimation

Bayesian divergence time estimates placed the crown diversification of the Orchidaceae in the Late Cretaceous at c. 86.4 Ma (95% HPD 81.59–90.77 Ma), consistent with other recent divergence time estimates of the family (Pérez-Escobar et al. 2024). The Cranichideae–Diurideae split was inferred to have occurred in the Eocene c. 52.14 Ma (95% HPD 48.60–55.68 Ma), with the Diurideae crown age inferred at c. 46.35 Ma (95% HPD 43.07–49.35 Ma). The timing of the Cranichideae–Diurideae split is consistent with the separation of Australia from Antarctica and South America (van den Ende et al. 2017). Our results push the stem age of the Pterostylidinae back by over 10 million years compared with estimates obtained by Nargar et al. (2022), which used only secondary calibrations, suggesting that the Pterostylidinae had already begun to split from the remainder of Cranichideae by the time Australia, Antarctica, and South America separated. All subtribes within the Diurideae were inferred to have stem ages between 46 and 36 Ma, with all subtribes having split by the Eocene–Oligocene Boundary.

Ancestral State Reconstruction of Orchid–Fungal Associations

Following cleaning and curation of 3,271 sequences retrieved from GenBank and from unpublished studies, 3,097 fungal ITS sequences for 27 orchid genera were retained for taxonomic assignment and subsequent fungal character coding (Supplementary Data S4). Fungal characters for six orchid genera (*Genoplesium*, *Orthoceras*, *Burnettia*, *Adenochilus*, *Leptoceras*, and *Praecoxanthus*), which had no fungal sequences available, were inferred from the literature using morphology/germination data. Two genera from the Diurideae (*Epiblema* and *Townsonia*) were excluded from further character state analyses due to an absence of morphology/germination or sequence data. Fungal data was also unavailable for the three monotypic genera lacking orchid sequence data; *Aporostylis*, *Coilochilus*, and *Waireia*. In total, 33 orchid genera were included in character state analyses (Fig. 5). A table outlining the number of species for each genus with molecular or morphological data available, along with an estimated sampling fraction (i.e., the total number of species per genus with fungal data divided by the number of accepted species per genus) can be found in Supplementary Table S5.

Ceratobasidiaceae associates predominantly with the tribe Cranichideae (subtribes Pterostylidinae and Chloraeinae) and the Diurideae subtribe Prasophyllinae (*Prasophyllum*, *Genoplesium*, and *Rhizanthella*), with the exception of *Microtis*, which predominantly associates with Tulasnellaceae, with occasional observations of Serendipitaceae (Fig. 5). Clade A was found to associate almost entirely with Tulasnellaceae, except for the monotypic genus *Leporella*, which was found to associate with Ceratobasidiaceae. Tulasnellaceae was also

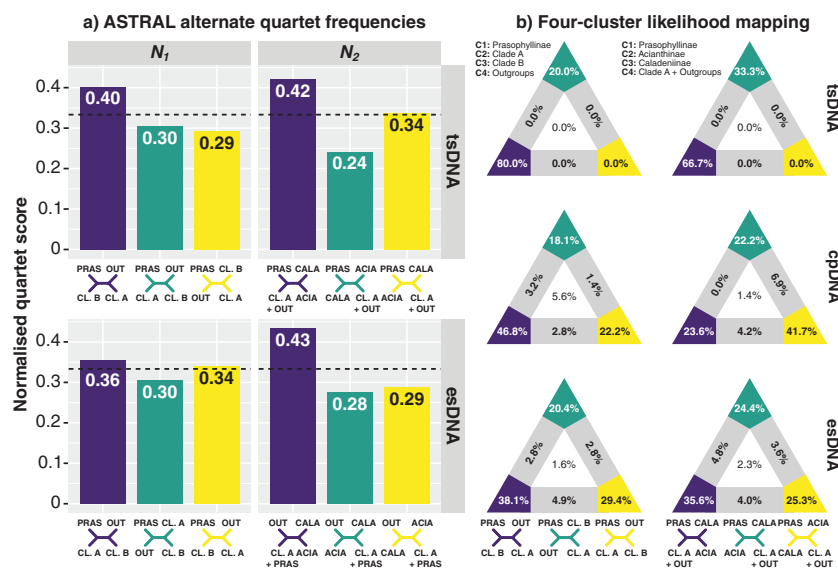


FIGURE 4. a) Alternate topologies and normalized quartet scores for nodes of interest identified in Figs. 2 and 3 from ASTRAL species trees for the tsDNA and esDNA sets; b) Four-cluster likelihood mapping for the tsDNA, esDNA, and cpDNA sets. CL. A = Diuridinae + Cryptostylidinae + Thelymitrinae + Megastylidinae + Drakaeinae; CL. B = Caladeniinae + Acianthinae; PRAS = Prasophyllinae (inc. Rhizanthellinae); CALA = Caladeniinae; ACIA = Acianthinae; OUT = Outgroups.

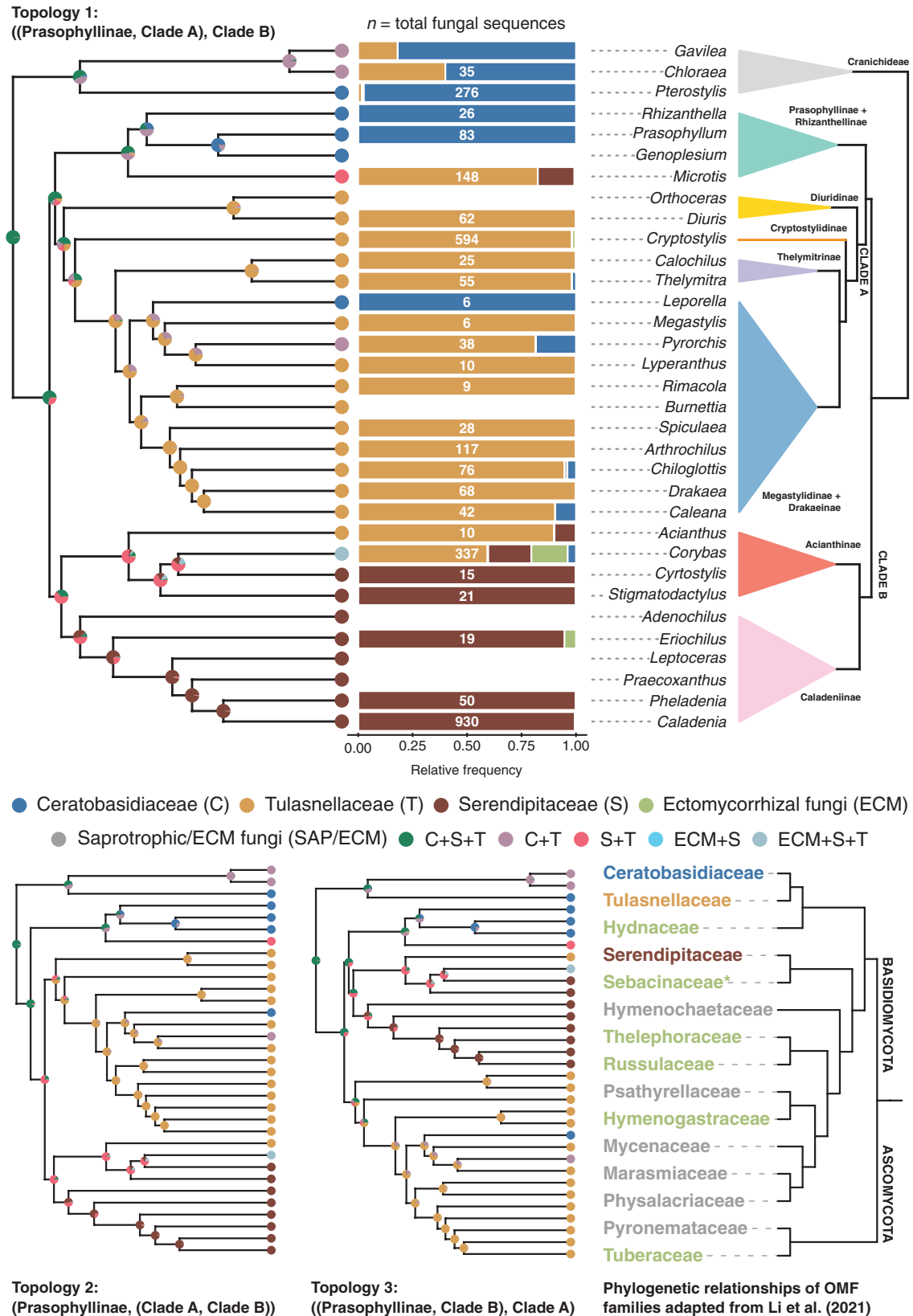


FIGURE 5. Alternative topologies of the Diurideae with Pterostylidinae and Chloraeinae (Cranichideae) used as outgroups with marginal ancestral state estimates of dominant fungal symbionts at the nodes. Topology 1 is the same topology outlined in Fig. 4. A guide tree with subtribes highlighted as collapsed clades is shown on the right. The central bar plot illustrates the relative frequency of fungal family sequences

found to be the dominant symbiont for one genus in Acianthinae; *Acianthus*. The genus *Corybas* was predominantly associated with Tulasnellaceae; however, there were also substantial numbers of sequences of Serendipitaceae and other ECM fungi. Associations with Serendipitaceae were also observed in other Acianthinae genera, namely *Cyrtostylis* and *Stigmatodactylus*, which exclusively partnered with Serendipitaceae, while one sequence was identified from the genus *Acianthus*. Associations with the Serendipitaceae were entirely absent in Clade A, and were primarily observed within Caladeniinae, wherein all genera partnered exclusively with Serendipitaceae.

Regardless of which topology was used for ancestral state reconstruction, the ancestral Diurid was inferred to have been either capable of partnering with Serendipitaceae and Tulasnellaceae, or a mycorrhizal generalist capable of partnering with all three major OMF families. The ancestor of Clade A was inferred to have been a Tulasnellaceae specialist, whereas the ancestor of Clade B was most likely capable of partnering with Tulasnellaceae and Serendipitaceae. A shift to exclusive partnerships with Serendipitaceae was inferred to have occurred at the most basal node of the Caladeniinae. Nodes with substantial probabilities of a Ceratobasidiaceae+Tulasnellaceae state occurred at the base of the Prasophyllinae and Cranichideae; however, there was considerable uncertainty as to the ancestral symbiont of the Prasophyllinae. Nonetheless, reconstructions across all topologies show a pattern of moving from generalist associations towards more specialized associations through time. Posterior probabilities for all states and topologies can be found in [Supplementary Data S4](#).

DISCUSSION

Summary

While previous work on the Diurideae has demonstrated that mycorrhizal specificity predominates at the species, genus, and individual subtribe scales, we show that this specificity extends even further than previously recognized. Not only do most genera in the Diurideae predominantly associate with a single fungal family, but so too do most subtribes and clades of related subtribes. Ancestral state reconstructions suggest that fungal relationships within the Diurideae evolved away from broader associations with multiple fungal genera to become increasingly specialized over time. Our results support the hypothesis that closely

related orchid genera share similar fungal partners, and confirm that these associations reflect phylogenetic relationships among host orchids. Moreover, patterns of host-symbiont preference here provide supporting evidence for the phylogenetic placement of problematic subtribes and genera.

This study represents the synthesis of decades of work towards understanding the mycorrhizal relationships of the Australian terrestrial orchid flora. Previous large-scale studies of mycorrhizal relationships across the Orchidaceae primarily focused on the phylogenetic structure of orchid trophic mode shifts with respect to the functional guilds of their fungal partners ([Wang et al. 2021a](#)). However, this study is, to the best of our knowledge, the first that attempts to quantify orchid-fungal relationships at the tribal scale using dominant fungal families as opposed to functional guilds. Additionally, this is also one of the first studies to attempt to use host-symbiont preferences to support evolutionary hypotheses where genetic data alone has previously proved insufficient.

Incomplete Lineage Sorting is a Significant Driver of Phylogenetic Discordance in the Diurideae

Matching recent studies ([Peakall et al. 2021](#); [Wong and Peakall 2022](#); [Zhang et al. 2023](#)), we found widespread phylogenetic discordance within the Diurideae. Despite the implementation of paralogy resolution methods not used by [Peakall et al. \(2021\)](#), our present study produced comparable results with little improvement in resolution. It is thus unlikely that hidden paralogy is a major contributor to the discordance observed. Based on the consistent lack of significant difference between alternative topology frequencies, ILS remains the most likely driver of phylogenetic discordance within the Diurideae.

Rapid radiation is a known cause of ILS and phylogenetic discordance ([Stull et al. 2023](#)), and rapid diversification was suggested by [Peakall et al. \(2021\)](#) as a possible cause of the discordance within the Diurideae. Interestingly, family-scale diversification rate estimation across the Orchidaceae has not indicated elevated diversification rates within the Diurideae ([Givnish et al. 2015](#); [Zhang et al. 2023](#)). However, these previous results may under-represent diversification rate shifts at the subfamily scale due to the relatively rapid diversification rates inferred for the large subfamily Epidendroideae. When investigating diversification rate shifts at the subfamilial scale, [Thompson et al. \(2023\)](#) recently found significantly higher speciation rates within the Australian Diurideae and Cranichideae relative to the rest of the subfamily Orchidoideae, thus

supporting the hypothesis that rapid diversification has played a role in shaping this lineage of Australian orchids.

Our divergence time estimates lend further support for rapid radiation at the base of the Diurideae, considering that all subtribes are estimated to have originated within a period of only 10 million years. Curiously, the most problematic nodes in the Diurideae phylogeny appear to coincide with core fungal symbiont shifts within the tribe. Indeed, transitions to near-exclusive Tulasnellaceae partnerships in Clade A, and exclusive Serendipitaceae partnerships in Caladeniinae, are inferred to have occurred in a small window of approximately five million years between 46–41 Ma. This raises the question of whether fungal niche differentiation early in the tribe's evolutionary history may represent a key innovation, wherein specializing on different fungal groups opened up a wider range of nutritional niches that subsequently facilitated rapid diversification, resulting in the patterns of ILS observed here. Experimental studies on several Australian orchids, including members of the Diurideae, have shown that OMF are capable of accessing different environmental nutrients with varying abilities and preferences (Nurfadilah et al. 2013; Davis et al. 2022). Additionally, several Australian plant groups have developed specialized root adaptations to cope with the continent's ancient, nutrient-poor soils (Orians and Milewski 2007; Flores-Moreno et al. 2023). It is possible, then, that the evolutionary trend towards increasingly specialized symbiont associations in the Australian terrestrial orchid flora, which is dominated by the Diurideae and Pterostylidinae, is another example of an Australian plant group adapting to depauperate soils. Given this possibility, future studies should test whether these fungal symbiont shifts are indeed associated with diversification rate shifts within the Diurideae and Pterostylidinae.

Fungal Symbiont Specificity in the Diurideae Extends Beyond the Subtribe Scale; Symbiont Preferences Reflect the Orchid Phylogeny

Based on the observation that fungal symbionts were generally congeneric, Warcup (1971, 1973, 1981) predicted that mycorrhizal specificity occurred from the species to subtribal level within the Diurideae. He further noted that orchid genera associated with Tulasnellaceae or Serendipitaceae generally did not associate with other fungal genera, and argued on the basis of this observation that the systematic placement of *Lyperanthus* at that time, was likely incorrect. Indeed, Warcup's hypothesis that *Lyperanthus* was not a member of Caladeniinae (cf. Dressler (1981)), was later supported by molecular phylogenies that placed *Lyperanthus* within Clade A (Kores et al. 2001; Weston et al. 2014; Peakall et al. 2021; this study).

The narrow range of fungal associations within Clade A is now well-established, and members of this clade associate near-exclusively with Tulasnellaceae

(Bonnardeaux et al. 2007; Roche et al. 2010; Smith et al. 2010; Linde et al. 2014; Reiter et al. 2018, 2023; Nguyen et al. 2020; Arifin et al. 2022, 2023). Caladeniinae is similarly well-studied with respect to its fungal associations, with overwhelming evidence that all genera in the subtribe associate exclusively with species of the Serendipitaceae (Warcup 1971, 1981, 1988; Ramsay et al. 1986; Huynh et al. 2004, 2009; Bonnardeaux et al. 2007; Swarts et al. 2010; Sommer et al. 2012; Davis et al. 2015; Phillips et al. 2016; Whitehead et al. 2017; Oktalira et al. 2019, 2021; Reiter et al. 2020).

Our synthesis here of several decades worth of collective germination, morphological, and fungal sequence data covering over 400 orchid species demonstrates that mycorrhizal relationships within the Diurideae are highly specific, with this specificity extending from the genus level to beyond the subtribe scale, congruent with Warcup's prediction. We demonstrate that genera, subtribes, and even groups of related subtribes associate with only a single fungal family, suggesting that fungal symbiont preferences in the Diurideae do indeed reflect phylogenetic relationships among orchid hosts.

Fungal Symbiont Shifts in the Diurideae Became Increasingly Specialized Over Time

Given its association with the earliest-diverging orchids (subfamily Apostasioideae), Yukawa et al. (2009) proposed that Ceratobasidiaceae is likely the ancestral symbiont for the Orchidaceae. Within the Orchidoideae (containing the Orchideae, Cranichideae, and Diurideae), fungal specialization and directional fungal transitions may be the norm. For example, Waterman et al. (2011) found mycorrhizal relationships at the subtribal scale to be highly conserved, with early-diverging members of the Coryciinae (Orchideae) specializing on Ceratobasidiaceae, with subsequently diverging clades specializing on *Tulasnella*, and *Serendipita* (as Sebacinaleae "Clade B") in that order. Shefferson et al. (2010) reported a similar pattern at the genus scale in *Goodyera* (Cranichideae). However, while Yukawa et al. (2009), Waterman et al. (2011), and Shefferson et al. (2010) hypothesized the order in which these fungal symbiont transitions occur, they did not include ancestral state reconstructions.

Our results here suggest that the ancestor of the Diurideae was most likely capable of partnering with multiple OMF families. Over time, subtribes appear to have evolved to be increasingly more specific with respect to their fungal familial associations. However, as our analyses incorporated multiple alternative phylogenetic hypotheses, it was not possible to determine whether the ordered progression Ceratobasidiaceae→Tulasnellaceae→Serendipitaceae→ECM is a consistent phenomenon within the Diurideae. It also remains to be tested if a similar pattern of mycorrhizal generalism moving towards more specific associations would be revealed with the testing of alternative phylogenetic hypotheses in other groups. Nevertheless, ordered transition models outperformed unordered models in all analyses

here, suggesting that there is an element of directionality contributing to the patterns we have observed.

The strong phylogenetic structure of fungal symbiont preferences within the Diurideae suggests that there is a genetic basis to this interaction. Indeed, transcriptomic and proteomic studies coupled with histological analyses have revealed several putative molecular mechanisms mediating orchid-mycorrhizal symbiosis. They include the involvement of plant cell wall degrading, biosynthesis, and remodeling enzymes (Li et al. 2018; Adamo et al. 2020; Miyauchi et al. 2020; Chen et al. 2022), plant-microorganism response pathways (Valadares et al. 2021), nodulin-like genes (Perotto et al. 2014), plant immune response genes (Valadares et al. 2014; Chen et al. 2017), common symbiosis genes (Miura et al. 2018), and sugar transporter genes (Zhao et al. 2024). Recent studies have also established the crucial roles of the plant hypoxia-responsive pathway (Xu et al. 2023), and a unique gibberellin signaling mechanism (Miura et al. 2023) in enabling symbiosis between orchids and their fungal symbionts. We predict that these molecular mechanisms may hold the key to unlocking the basis of these phylogenetic patterns of symbiont association. It follows, given its strong pattern of symbiont specificity that the Diurideae offers a model system for future studies of the molecular physiology and evolution of OMF interactions (and host-symbiont interactions more broadly).

Can Orchid Symbiont Preferences Be Used to Discriminate Among Alternative Phylogenetic Hypotheses?

As previously noted, closely related genera and subtribes within Diurideae appear to associate with similar fungal partners. Can we use such general patterns of fungal association to discriminate among alternative phylogenetic hypotheses? Below, we consider fungal symbiont preferences as a supporting line of evidence in relation to three problematic phylogenetic relationships: 1) the sister relationship between Acianthinae and Caladeniinae; 2) the placement of Diuridinae within Clade A; and 3) the placement of *Rhizanthella* within the Prasophyllinae, sister to *Prasophyllum* s.l.

In genomic-scale studies, Acianthinae is consistently recovered as sister to Caladeniinae (Peakall et al. 2021; Wong and Peakall 2022; Zhang et al. 2023; Pérez-Escobar et al. 2024; this study). However, in pre-genomic studies which relied on only one to three nuclear or plastid regions, Acianthinae was recovered as either sister to Prasophyllinae (Kores et al. 2001; Cameron 2006), sister to a Caladeniinae+Diuridinae clade (Weston et al. 2014), sister to a Caladeniinae+Prasophyllinae clade (Nauheimer et al. 2018), or as sister to the rest of the Diurideae (Givnish et al. 2015). The subtribe contains genera observed to partner with Tulasnellaceae (*Acianthus* and *Corybas*) and Serendipitaceae (*Acianthus*, *Corybas*, *Cyrtostylis*, and *Stigmatodactylus*). Crucially, while members of the Acianthinae have been observed to partner with Serendipitaceae, members of Clade A have not. As Acianthinae contains genera capable of

partnering with both Tulasnellaceae and Serendipitaceae, its placement alongside the Serendipitaceae-associating Caladeniinae is sound. This placement is supported further by morphology based on trees inferred using 102 morphological characters by Weston et al. (2014), which similarly recovered the Acianthinae and Caladeniinae as sister clades. The combination of molecular, morphological, and fungal symbiont data thus support the Acianthinae and Caladeniinae as sister subtribes.

A second subtribe exhibiting variable phylogenetic placement is the Diuridinae. Topologies recovered by Kores et al. (2001), Cameron (2006), Peakall et al. (2021), Wong and Peakall (2022), and Zhang et al. (2023) suggest the Diuridinae precede Cryptostylidinae in branching order, which is echoed here. However, molecular trees recovered by Weston et al. (2014) placed Diuridinae as a sister to Caladeniinae. This placement has not been recovered in any other study of the Diurideae or Orchidaceae, and contrasts with the majority of reconstructions, which cluster Diuridinae with Clade A. As Diuridinae appears to associate exclusively with Tulasnellaceae (Smith et al. 2010), with no evidence of association with Serendipitaceae, its placement alongside other Tulasnellaceae-associated genera in Clade A is parsimonious.

A final phylogenetic conundrum resolved in this study is the underground mycoheterotrophic genus *Rhizanthella*. Previous phylogenetic studies utilizing chloroplast markers have failed to resolve the position of *Rhizanthella* due to its highly degraded plastome resulting from its parasitic lifestyle (Delannoy et al. 2011; Givnish et al. 2015). *Rhizanthella* has previously been recovered as nested within genera now considered synonymous with *Microtis* (Weston et al. 2014) or nested within Clade A, sister to Thelymitrinae (Givnish et al. 2015). For the first time, using genome-scale nuclear transcriptomic data, our results now place *Rhizanthella* within the Prasophyllinae as sister to the clade containing *Prasophyllum* and *Genoplesium*. Furthermore, as these orchid genera associate exclusively with Ceratobasidiaceae (Warcup 1981, 1985; Burns-Balogh 1984; Mursidawati 2004; Bougoure et al. 2009, 2010; Freestone et al. 2021, 2022), their systematic placement together is consistent with their shared fungal associations.

The placement of the subtribe Prasophyllinae remains uncertain, and the genus *Microtis* confounds attempts to use fungal symbiont preferences to evidence the placement of this subtribe. While a substantial number of sequences sourced from *Microtis* were of Serendipitaceae, the collective literature on *Microtis* (Prasophyllinae) encompassing morphological, germination, and molecular data has consistently found Tulasnellaceae to be more effective in inducing germination and subsequent protocorm formation (Milligan and Williams 1988; Warcup 1988; Perkins et al. 1995; Bonnardeaux et al. 2007; De Long et al. 2013; Lim 2015). Although research to date has been mostly restricted to the clade containing *Microtis media*, Tulasnellaceae

is likely to be the dominant mycorrhizal partner of *Microtis* at large. Consequently, topologies placing Prasophyllinae as a sister to either Clade A or Clade B are equally plausible. However, if Ceratobasidiaceae is indeed the ancestral symbiont of the Orchidaceae as suggested by Yukawa et al. (2009), Waterman et al. (2011), and Shefferson et al. (2010), then topologies placing Prasophyllinae as the earliest-diverging Diurideae lineage (Peakall et al. 2021; Wong and Peakall 2022) would be favored by this prediction.

CONCLUSIONS

In this study, we sought to understand the evolution of mycorrhizal specificity within the Diurideae, and determine whether host-symbiont preferences may provide clues to aid the resolution of phylogenetic relationships in one of the most systematically recalcitrant tribes of the entire Orchidaceae. We inferred and dated a phylogeny for the Diurideae, and identified phylogenetic discordance stemming from ILS, indicating that problems in the Diurideae may not be resolved simply with the addition of more genetic data. However, we also found that fungal symbioses in the tribe are highly specialized, reflect the phylogeny of the orchid host, and have evolved away from broad associations with multiple fungal families towards more narrow associations with only a single fungal family. Leveraging the patterns of these associations has provided clarity as to the systematic placement of several problematic orchid subtribes and genera, where genetic data alone has proved insufficient. Thus, we demonstrate that it is possible to use host-symbiont relationships to help arbitrate between alternate phylogenetic hypotheses in spite of confounding evolutionary processes.

SUPPLEMENTARY MATERIAL

Supplementary material including data files, code, and online-only appendices used in this study are available from the Dryad Digital Repository: <https://dx.doi.org/10.5061/dryad.b2rbnzsqq>.

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AUTHOR CONTRIBUTIONS

RPO, DCJW, RP, and CCL designed the research. RPO, DCJW, and RP acquired the data. RPO analyzed the data. RPO wrote the manuscript. All authors edited and revised the manuscript.

CONFLICT OF INTEREST STATEMENT

None declared.

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