JIPB Journal of Integrative Plant Biology

Research Article
https://doi.org/10.1111/jipb.13462

Comprehensive phylogenetic analyses of Orchidaceae using nuclear genes and evolutionary insights into epiphytism[oo]

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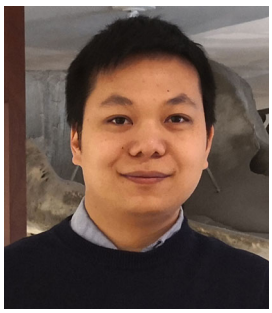

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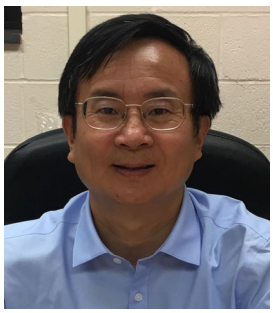

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ABSTRACT

Orchidaceae (with >28,000 orchid species) are one of the two largest plant families, with economically and ecologically important species, and occupy global and diverse niches with primary distribution in rainforests. Among orchids, 70% grow on other plants as epiphytes; epiphytes contribute up to ~50% of the plant diversity in rainforests and provide food and shelter for diverse animals and microbes, thereby contributing to the health of these ecosystems. Orchids account for over two-thirds of vascular epiphytes and provide an excellent model for studying evolution of epiphytism. Extensive phylogenetic studies of Orchidaceae and subgroups have ;been crucial for understanding relationships among many orchid lineages, although some uncertainties remain. For example, in the largest subfamily Epidendroideae with nearly all epiphytic orchids, relationships among some tribes and many subtribes are still controversial, hampering evolutionary analyses of epiphytism. Here we obtained 1,450 low-copy nuclear genes from 610 orchid species, including 431 with newly generated transcriptomes, and used them for the reconstruction of robust Orchidaceae phylogenetic trees with highly supported placements of tribes and subtribes. We also provide generally well-supported phylogenetic placements of 131 genera and 437 species that were not sampled by previous plastid and nuclear phylogenomic studies. Molecular clock analyses estimated the Orchidaceae origin at ~132 million years ago (Ma) and divergences of most subtribes from 52 to 29 Ma. Character reconstruction supports at least 14 parallel origins of epiphytism; one such origin was placed at the most recent common ancestor of ~95% of epiphytic orchids and linked to modern rainforests. Ten occurrences of rapid increase in the diversification rate were detected within Epidendroideae near and after the K-Pg boundary, contributing to ~80% of the Orchidaceae diversity. This study provides a robust and the largest family-wide Orchidaceae nuclear phylogenetic tree thus far and new insights into the evolution of epiphytism in vascular plants.

Keywords: convergence, epiphytes, evolution, orchids, parallel rainforest, phylogenomics, transcriptome

Zhang, G., Hu, Y., Huang, M.-Z., Huang, W.-C., Liu, D.-K., Zhang, D., Hu, H., Downing, J.L., Liu, Z.-J., and Ma, H. (2023). Comprehensive phylogenetic analyses of Orchidaceae using nuclear genes and evolutionary insights into epiphytism. J. Integr. Plant Biol. **65:** 1204–1225.

INTRODUCTION

Orchidaceae (the orchid family) are one of the two largest angiosperm families and contain over 28,000 species, contributing to nearly 10% of angiosperm diversity and providing many economically important plants (Chase et al., 2015; Christenhusz and Byng, 2016). For example, vanilla is a widely used spice in the food industry. In addition, multiple orchids have been used as medicinal herbs in China and other East Asian countries (*Anoectochilus* (marbled jewel orchids), *Blettia* (urn orchids and bai-ji), *Calanthe* (Christmas orchids), *Dactylorhiza* (marsh orchids), *Dendrobium* (shi-hu), and *Gastrodia* (potato orchids and tian-ma)), in Africa (multiple *Eulophia* species (corduroy orchids)), and in North America (*Cypripedium* species (lady's slipper orchids)) (Bulpitt et al., 2007; Gallage and Møller, 2015; Yuan et al., 2018; Teoh, 2019; Hasing et al., 2020). Furthermore, *Cymbidium* has been known as one of the "four most respectable" flowers in numerous traditional Chinese literary works and paintings and widely cultivated in China and other Asian countries since the age of Confucius, who had named it as "the king of fragrant plants" (Hew, 2001; Bulpitt, 2005; Zeng et al., 2020). Today, many orchids are worldwide horticultural plants, such as *Phalaenopsis* (moth orchids), *Cattleya*, and *Cypripedium*.

Rainforests are the most diverse and important terrestrial ecosystems and provide diverse niches, especially the canopy habitats, as the home of most of ~30,000 epiphytic vascular species, which grow on other plants (mainly trees). Vascular epiphytes include ferns and angiosperms and account for ~10% of land plant species (Zotz et al., 2021). Epiphytes mainly grow in rainforests, make up close to 50% of the plant diversity of these ecosystems, and provide food, shelter, and other crucial resources for diverse animals and microbes (Rico-Gray and Thien, 1989; Kelly et al., 1994; Engwald et al., 2000; Stanton et al., 2014; Morales-Linares et al., 2021; Petter et al., 2021; Spicer and Woods, 2022). Epiphytes likely have benefited from available space and sunlight above ground, thus avoiding fierce competition with terrestrial plants, and have diversified during the adaptation to the epiphytic niches through biotic and abiotic interactions (Holbrook and Putz, 1996; Krause et al., 2001). Orchidaceae are crucial ecologically with a global distribution and occupy diverse niches primarily in tropical rainforests, contributing to over one-fifth of the biodiversity in some rainforests (Küper et al., 2004; Huang et al., 2008; Zhang et al., 2015; Givnish et al., 2016; Dewi et al., 2020). Most of the orchids occurring in forests are epiphytes (~20 000 species; ~19 000–21 000 according to different studies) and represent by far the largest group of epiphytic vascular plants (Zotz, 2013; Zotz et al., 2021; Fernández et al., 2023), providing an excellent model for the study of epiphytism.

The evolution of orchids has fascinated many biologists, with some of the earliest studies described in a book by Charles Darwin soon after the publication of his theory of evolution (Darwin, 1877). According to recent systematics, Orchidaceae are divided into five subfamilies, Apostasioideae (~15 species), Vanilloideae (~180 species), Cypripedioideae (~160 species), Orchidoideae (~5,000 species), and Epidendroideae (~22,000 species) (Chase et al., 2015). Three subfamilies (Vanilloideae, Orchidoideae, and Epidendroideae) are further subdivided into a total of 22 tribes and 49–51 subtribes (van den Berg et al., 2005; Chase et al., 2015; Freudenstein and Chase, 2015). Numerous molecular phylogenetic studies in recent decades have made significant progress in the reconstruction of the evolutionary history of orchids, resolving the relationships among all five subfamilies, many tribes, and some subtribes (Cameron et al., 1999; van den Berg et al., 2005; Carlsward et al., 2006; Górniak et al., 2010; Givnish et al., 2015; Zou et al., 2015; Li et al., 2016; Pérez-Escobar et al., 2017, 2021; Y.X. Li et al., 2019; Kim et al., 2020; Eserman et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). Early Orchidaceae phylogenetic studies generally relied on one to a few plastid or nuclear markers (Cameron et al., 1999; van den Berg et al., 2005; Carlsward et al., 2006; Górniak et al., 2010; Zou et al., 2015; Li et al., 2016). More recently, 75–83 plastid genes have been used for Orchidaceae phylogenetic reconstruction (Givnish et al., 2015; Y.X. Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021). The use of organellar DNA sequences (Givnish et al., 2015; Serna-Sánchez et al., 2021) can trace the maternally inherited evolutionary histories, unlike the biparental inheritance of nuclear genes. Thus, nuclear genes from target enrichment (~300 from 75 species and >900 from 28 species) (Eserman et al., 2021; Pérez-Escobar et al., 2021) and transcriptomes (633 genes from 69 data sets) (Wong and Peakall, 2022) have been used for phylogenetic analyses of Orchidaceae. However, the placements of some tribes and subtribes were not well-supported or were inconsistent between some studies (Figures S1, S2). Moreover, many subtribes were not sampled in these studies (as many as 35 sampled out of 51 subtribes; Figures S1, S2), and many sampled subtribes have only one or a few representative species, rendering the placements and monophyly of some subtribes unclear. Furthermore, the taxon sampling of some of these studies lacks some important epiphytic lineages, especially those in relatively small tribes (e.g., Sobralieae) and subtribes (e.g., Adrorhizinae and Chysinae).

The placements of Orchidaceae tribes and subtribes are important for evolutionary studies of important traits, such as epiphytism. For example, Epidendroideae contain a majority of epiphytes, but five tribes in this subfamily, Gastrodieae, Nervilieae, Sobralieae, Triphoreae, and Tropidieae, have inconsistent positions in plastid and nuclear phylogenetic trees (Figures S1, S2). Among these tribes, Sobralieae contain many epiphytes (Zotz et al., 2021), like most of the Epidendroideae tribes, whereas the other four tribes are terrestrial. The uncertain placements of the five tribes have meant that the evolutionary pattern of epiphytism in Epidendroideae remained unclear. Additionally, three of the four largest

Epidendroideae tribes, Cymbidieae, Epidendreae, and Vandeae, together contain >13 000 species, but their relationships were inconsistent among different studies using nuclear and plastid genes (Givnish et al., 2015; Y.X. Li et al., 2019; Eserman et al., 2021; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022) (Figures S1, S2). Furthermore, in Cymbidieae with terrestrial and epiphytic species, the relationships among subtribes were inconsistent among previous plastid and nuclear phylogenetic trees, and the evolutionary pattern of epiphytism in Cymbidieae is still unclear (Górniak et al., 2010; Givnish et al., 2015; Li et al., 2016). Hence, evolutionary analyses of epiphytism and other traits in Orchidaceae can be further improved using a well-resolved phylogenetic topology with sufficient sampling, especially at the tribe and subtribe levels.

To reconstruct Orchidaceae phylogeny and investigate the evolution of epiphytism, here we sampled 610 orchid species with 431 newly generated transcriptomes and 179 publicly available genomic and transcriptomic data sets. The sampling covered all five subfamilies, 19 of 22 tribes, and 44 out of 51 subtribes of Orchidaceae, including 10 Cymbidieae subtribes with epiphytic, terrestrial, or both types of species. We obtained 1 450 low-copy nuclear genes and used various subsets of these genes to reconstruct four coalescent trees. In addition, a supermatrix data set of 299 genes with high taxon coverage was used for phylogenetic reconstruction and for divergence time estimation. The robust phylogenetic trees with well-supported placements of all sampled tribes and subtribes provided a foundation for ancestral state reconstruction of epiphytism.

RESULTS AND DISCUSSION

Multiple nuclear phylogenetic trees of Orchidaceae support monophyly of, and robust relationships among, tribes

We sampled a total of 610 orchid species from 297 genera in this study, covering all five subfamilies (Apostasioideae: two genera with five species; Vanilloideae: five genera with seven species; Cypripedioideae: five genera with 24 species; Orchidoideae: 46 genera with 90 species; Epidendroideae: 239 genera (203 with epiphytes) with 484 species (391 epiphytic)), 19 of 22 tribes, and 44 of 51 subtribes. Our taxon sampling represents the largest phylogenetic sampling of Orchidaceae thus far, greater than the sampling of previous orchid phylogenetic trees reconstructed using several plastid and/or nuclear genes (Cameron et al., 1999; van den Berg et al., 2005; Carlsward et al., 2006; Górniak et al., 2010; Zou et al., 2015; Li et al., 2016). Also, the sampling here is greater than that of recent phylogenomic analyses using plastomic, transcriptomic, and/or genomic sequences; these previous studies sampled from 28 to 264 species and up to 117 genera, which represented up to 28 subtribes and 18 tribes (plastid) or 35 subtribes and 17 tribes (nuclear) (Deng et al., 2015; Givnish et al., 2015; Y.X. Li et al., 2019; Kim et al., 2020; Eserman et al., 2021; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). Among the 297 genera and 610 species sampled here, 131 genera (108 containing epiphytes) and 437 species (334 epiphytic) were not included in any of the previous phylogenomic studies.

We detected 4,450 single- or low-copy orthogroups using 10 public sequenced orchid genomes from three subfamilies (Table S1). From these orthogroups we selected 1 450 as queries (seed genes) according to gene sequence length, species coverage, and number of similar sequences in one or more of the 10 species (see Methods and Materials for details) to search for putative orthologs from other transcriptomes and genomes. To test the robustness and consistency of phylogenetic relationships, four gene-sets were obtained according to the coverage of species, subtribe, and tribe, containing 1,195, 1,016, 834, and 639 genes, respectively. These four gene-sets were used to generate coalescent trees after the removal of putative paralogs and likely non-orchid sequences. These four coalescent trees were used in subsequent discussions of the phylogeny of Orchidaceae. The two larger gene-sets here (1 195 and 1 016) were larger than the previously largest gene-set of 963 genes (from 28 species; Eserman et al., 2021) and all four of our gene-sets were larger than those in two other nuclear phylogenetic analyses (~300 genes from 75 species (Pérez-Escobar et al., 2021) and 633 genes from 69 species (Wong and Peakall, 2022)).

The four coalescent trees here fully and consistently resolved all phylogenetic relationships at or above the subtribal level and consistently resolved the relationships of genera except six nodes (Figures 1–4, S3–S9). Here, the monophyly of Orchidaceae and each of the five subfamilies were maximally supported, and relationships among subfamilies were fully resolved and maximally supported, in agreement with previous studies. Our result also consistently supported the monophyly of all 16 tribes with two or more sampled species with at least 95 bootstrap support (BS) values in all trees (Figures 1–4, S3). Specifically, the monophyly of Neottieae (Epidendroideae) was maximally supported. This is consistent with previous phylogenetic analyses that sampled more than one Neottieae species (Givnish et al., 2015; Y.X. Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021; Wong and Peakall, 2022), although the monophyly of the tribe had <85 BS and <50% quartet support in the phylogenetic analysis using 292 nuclear genes (Pérez-Escobar et al., 2021). Nervilieae contain two subtribes, Epipogiinae and Nerviliinae, but only one of them was sampled in some previous studies (Givnish et al., 2015; Kim et al., 2020; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). The two subtribes were sisters (BS < 85 and quartet support <70%) in orchid phylogenetic trees using either 292 nuclear genes (Pérez-Escobar et al., 2021) or 38 mitochondrial genes. On the other hand, Epipogiinae were sister to Gastrodieae in a plastid phylogenetic tree (Y.X. Li et al., 2019) (Figure S1), possibly due to long branch attraction between fully

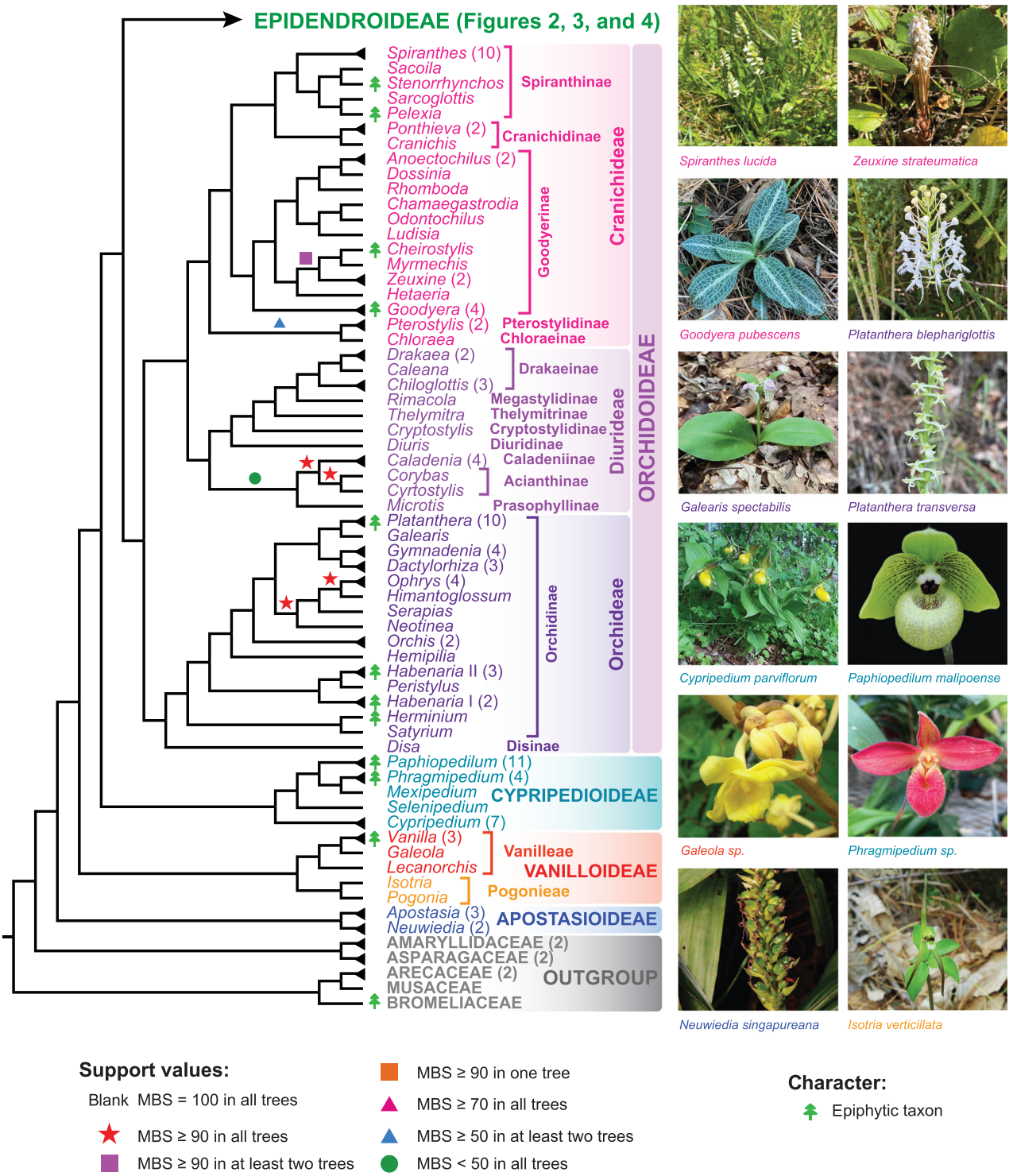

Figure 1. A summary cladogram of Orchidaceae, part 1 (the Apostasioideae, Vanilloideae, Cypripedioideae, and Orchidoideae subfamilies)

A portion of the summary Orchidaceae phylogenetic tree generated from four coalescent trees reconstructed using gene-sets with 1 195, 1 016, 834, and 639 genes, respectively. The relationships among genera of four subfamilies, Apostasioideae, Vanilloideae, Cypripedioideae, and Orchidoideae are shown. The branches have maximal multi-locus bootstrap support values (MBS) in all coalescent trees, unless indicated by colored shapes with corresponding support value shown below the tree. The triangle at the tip of a branch indicates that the corresponding genus contains more than one sampled species, and the number of sampled species of the genus is shown in the parentheses after the genus name. The green tree symbol before a genus name indicates that the genus has epiphytic species. The subfamily, tribe, and subtribe names are shown to the right of genus names. Photographs, with species or genus names below, of some genera/species in the four subfamilies, are presented on the right side. The legends and symbols used here are also the same for Figures 2, 3, and 4.

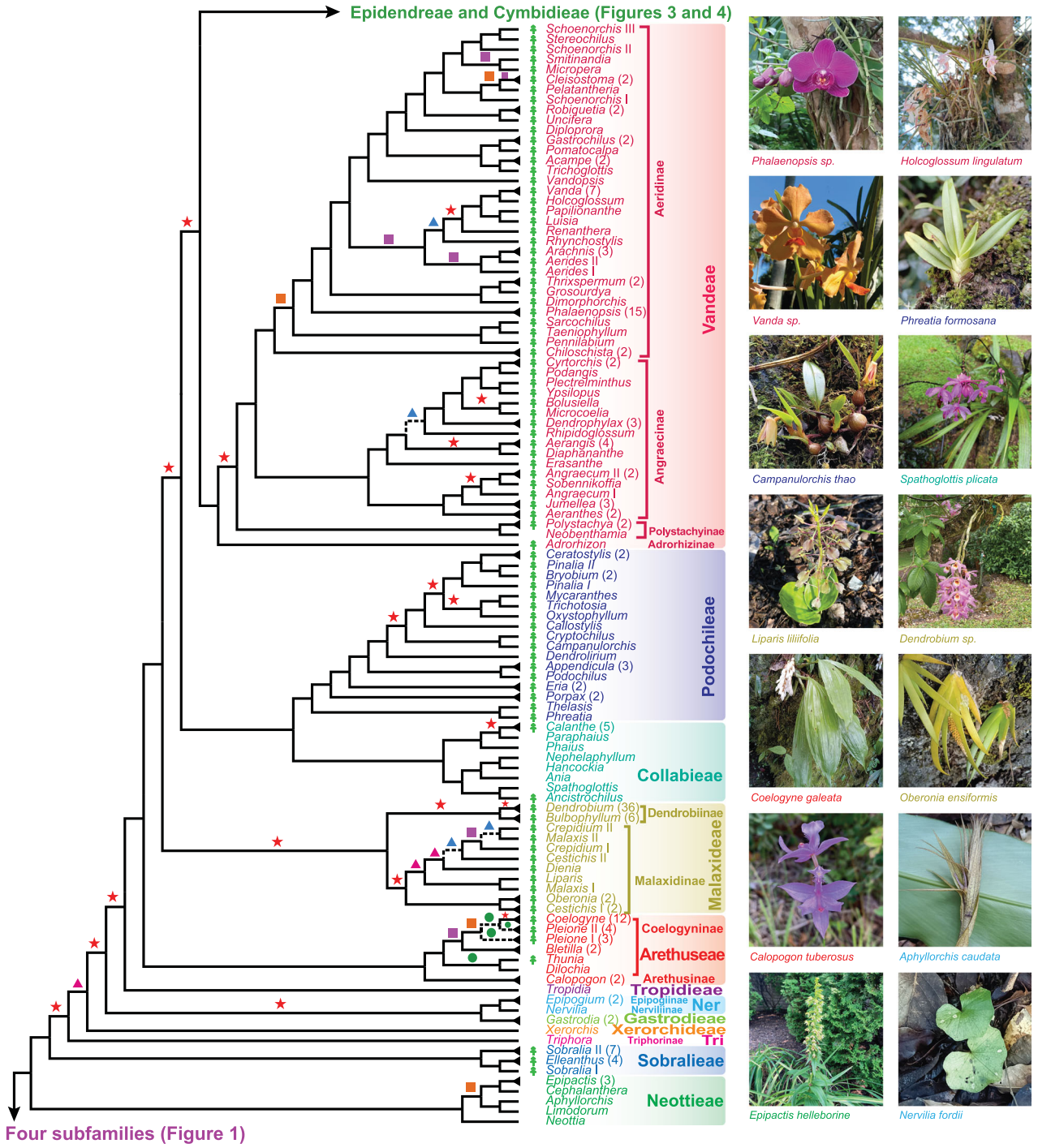

Figure 2. A summary cladogram of Orchidaceae, part 2 (the Epidendroideae subfamily, except the Epidendreae and Cymbidieae tribes)
A portion of the summary Orchidaceae phylogenetic tree generated from four coalescent trees reconstructed using gene-sets with 1,195, 1,016, 834, and 639 genes, respectively, showing relationships of genera of 12 Epidendroideae tribes: Neottieae, Sobralieae, Triphoreae (Tri), Xerorchideae, Gastrodieae, Nervilieae (Ner), Tropidieae, Arethuseae, Malaxideae, Collabieae, Podochileae, and Vandeae. Photographs of representative genera/species of the tribes shown in the tree are presented on the right side. See also legend for Figure 1.

mycoheterotrophic taxa of Epipogiinae and Gastrodieae. The newly obtained phylogenetic trees here with members of both Epipogiinae and Nerviliinae using four gene-sets of up to 1,195 genes, provide consistent and maximal support for the monophyly of Nervilieae, which were sister to Gastrodieae (Figures 2, S3). Additionally, the phylogenetic trees here maximally supported the monophyly of Arethuseae, agreeing with some previous analyses (Givnish et al., 2015; Y.X. Li et al., 2019; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021), whereas other studies only sampled one species for the tribe (Wong and Peakall, 2022) or only one subtribe (Kim et al., 2020).

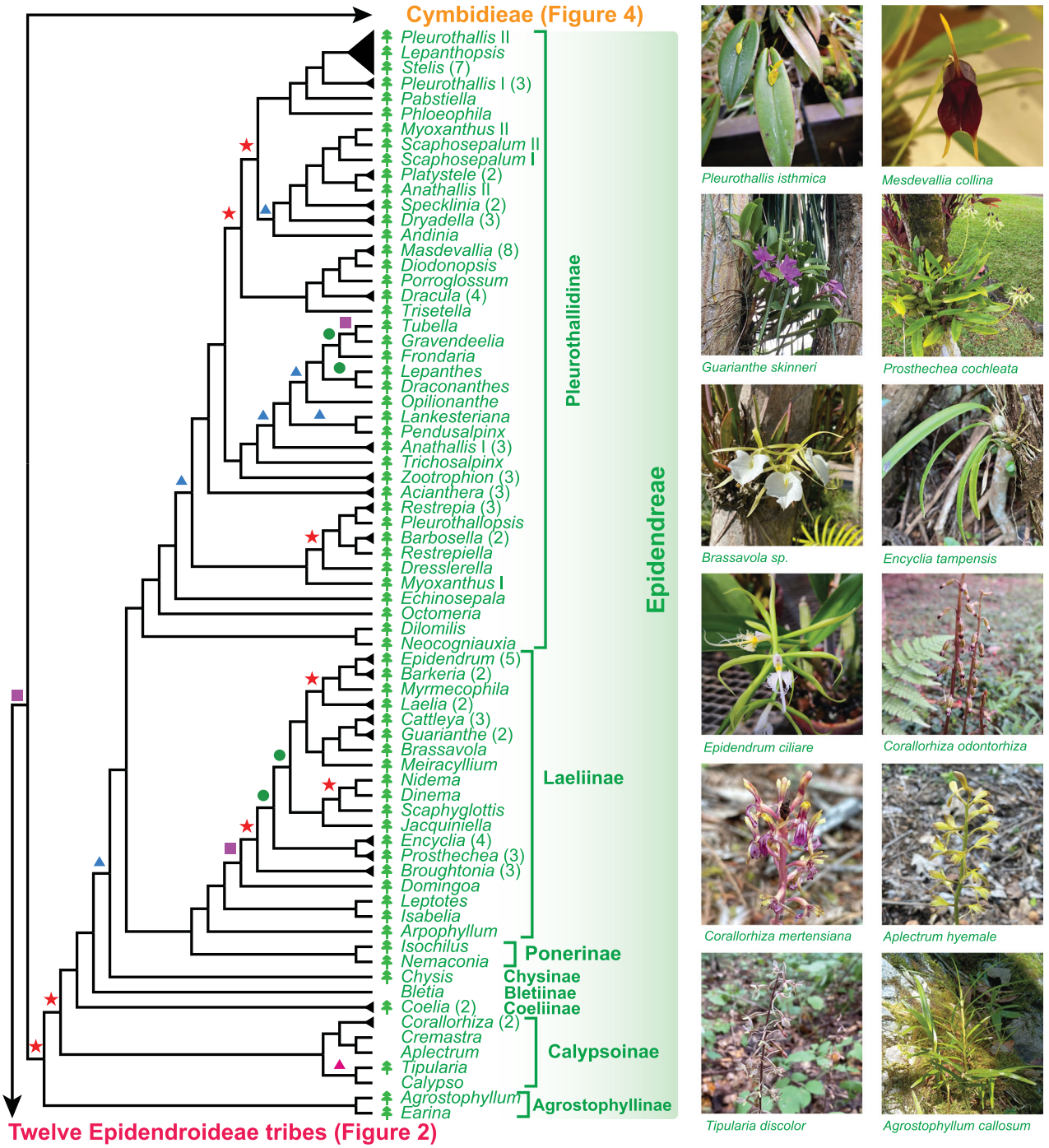

Figure 3. A summary cladogram of Orchidaceae, part 3 (the Epidendreae tribe)
A portion of the summary Orchidaceae phylogenetic tree generated from four coalescent trees reconstructed using gene-sets with 1,195, 1,016, 834, and 639 genes, respectively, showing relationships of genera of Epidendreae. Photographs of representative genera/species of Epidendreae are presented on the right side. See also legend for Figure 1.

The relationships among all 19 sampled tribes were consistently resolved with generally strong supports in all trees, including consistent relationships among tribes of Orchidoideae and Vanilloideae as in previous studies. Several Epidendroideae tribes were previously unresolved or had inconsistent relationships, including those of Sobralieae, Triphoreae, Xerorchideae, Gastrodieae, Nervilieae, and Tropidieae (referred as STXGNT tribes hereafter). They were consistently resolved in our coalescent trees and strongly supported with at least 93 BS values at most nodes (Figures 2, S3). On the other hand, the placement of Xerorchideae had less support (with at least 74 BS values) likely because of the relatively small number (108) of detected genes from the single species of this tribe. Recent phylogenetic trees reconstructed using plastid or nuclear genes

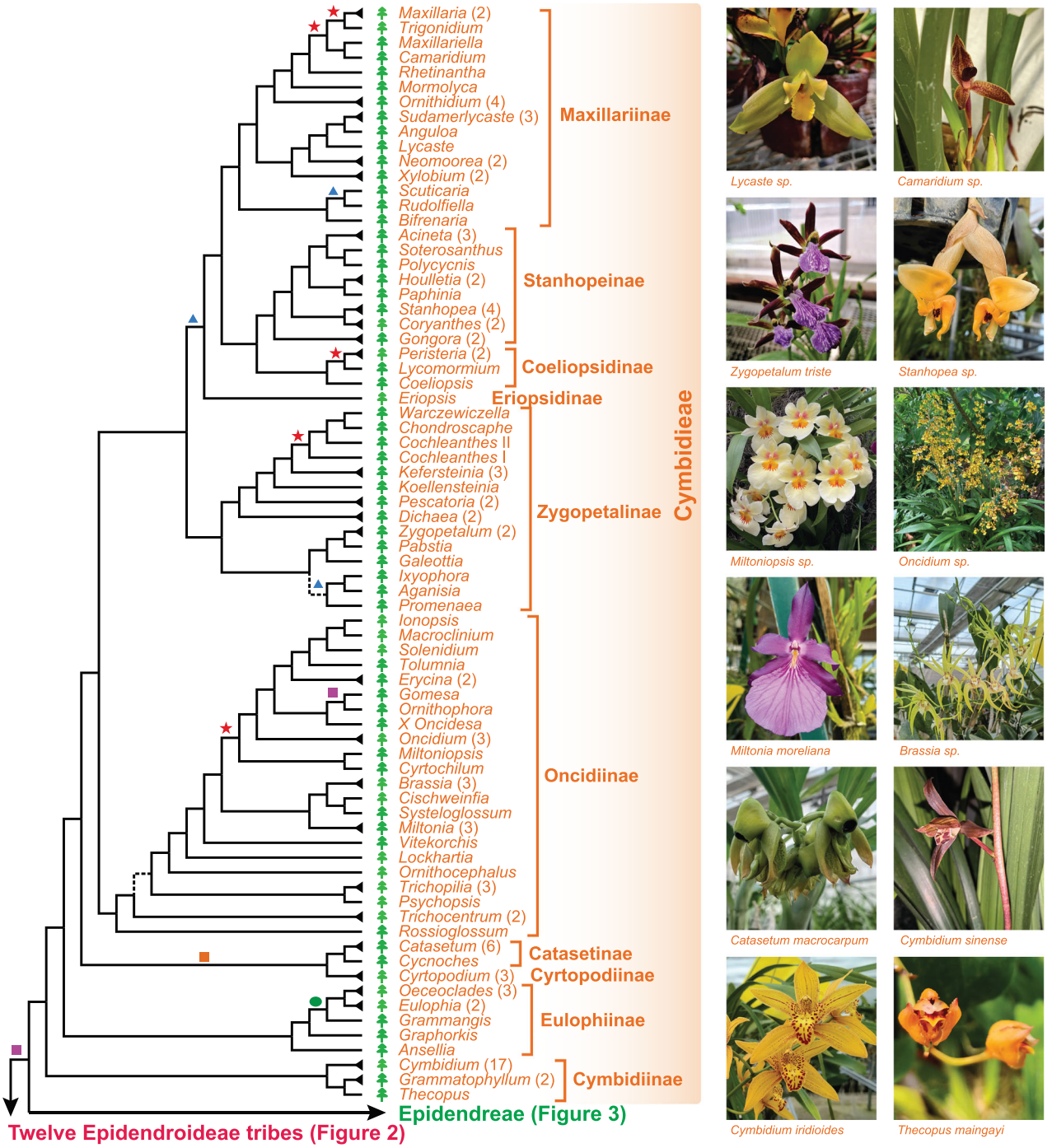

Figure 4. A summary cladogram of Orchidaceae, part 4 (the Cymbidieae tribe)

A portion of the summary Orchidaceae phylogenetic tree generated from four coalescent trees reconstructed using gene-sets with 1 195, 1 016, 834, and 639 genes, respectively, showing relationships of genera of Cymbidieae. Photographs of representative species of Cymbidieae are presented on the right side. See also legend for Figure 1.

lacked one or more of these tribes, and the relationships among the sampled tribes were either not fully resolved or were not strongly supported (Givnish et al., 2015; Y.X. Li et al., 2019; Kim et al., 2020; Eserman et al., 2021; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022) (Figures S1, S2). As mentioned above, our results maximally supported the sister relationship between Gastrodieae and Nervilieae, agreeing with the phylogenetic tree using mitochondrial genes (Y.X. Li et al., 2019), whereas they were either not grouped together (Pérez-Escobar et al., 2021) or Gastrodieae were nested within Nervilieae with BS < 50 in a plastid phylogenetic tree (Y.X. Li et al., 2019).

In addition, the trees here consistently placed Sobralieae and Triphoreae as the second and the third divergent clades

of Epidendroideae, respectively. In previous plastomic analyses, Sobralieae were either placed as the second divergent clade of Epidendroideae with 83–93 BS (Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021) or placed as sister group of Triphoreae with BS of 92 (Givnish et al., 2015). In addition, Triphoreae were either not sampled (Li et al., 2019; Kim et al., 2020) or were sister to Sobralieae (Givnish et al., 2015) or Nervilieae + Tropidieae (BS = 85) (Serna-Sánchez et al., 2021). These two tribes were not sampled in two recent nuclear phylogenetic analyses (Pérez-Escobar et al., 2021; Wong and Peakall, 2022); also only Sobralieae among the STXGNT tribes were sampled in a nuclear phylogenetic analysis with 28 orchids (Eserman et al., 2021) (Figure S2). The unresolved relationships among Sobralieae and five other (TXGNT) tribes affected not only the phylogenetic understanding of Orchidaceae but also the evolutionary studies of growth forms, because Sobralieae have many epiphytic species whereas the TXGNT tribes generally have terrestrial and fully mycoheterotrophic species. Hence, the consistent phylogenetic relationships here among the STXGNT tribes have substantially improved our understanding of evolutionary history of orchid tribes and provided a clear framework for the evolutionary analyses of growth forms and other traits.

Among the remaining seven Epidendroideae tribes, Arethuseae contain many epiphytes, and hence the placement of Arethuseae is important for evolutionary analysis of epiphytism. In the phylogenetic trees here, Arethuseae were placed with maximal support as the sister group of the clade with six tribes (Malaxideae, Cymbidieae, Vandeae, Epidendreae, Collabieae, and Podochileae; the clade named as MCVECP hereafter), which is composed of ~19,000 epiphytic species (Figure 2; see Figures 3, 4 for detailed topologies of Epidendreae and Cymbidieae, respectively). This is consistent with most previous phylogenetic analyses with weak (BS of 51) (Y.X. Li et al., 2019) to strong supports (90–100 BS) (Givnish et al., 2015; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). However, in a previous phylogenetic tree that was used for understanding the evolution of epiphytism in Orchidaceae, Arethuseae were the sister group of a clade containing Collabieae and Epidendreae (Chomicki et al., 2015). Among the MCVECP tribes, four are the largest Epidendroideae tribes (Malaxideae, Cymbidieae, Epidendreae, and Vandeae), with the latter three (CEV) grouped in a strongly supported clade. However, relationships among the CEV tribes were controversial among phylogenetic trees using plastid genes or nuclear genes; for example, Cymbidieae and Epidendreae were sister groups in a nuclear phylogenetic tree (using 292 genes from 75 orchids) (Pérez-Escobar et al., 2021) and a plastid phylogenetic tree (using 76 genes from 74 orchids) (Y.X. Li et al., 2019), whereas Cymbidieae and Vandeae were sister groups in an analysis of 69 orchids using 633 nuclear genes (Wong and Peakall, 2022) and plastid phylogenetic trees using 75 and 78 genes from 39 and 264 orchids, respectively (Givnish et al., 2015; Serna-Sánchez et al., 2021). The phylogenetic trees here consistently resolved the relationships among these three tribes and strongly supported Cymbidieae and Epidendreae as sister tribes and Vandeae as sister to a clade of the other two tribes.

Monophyly of subtribes and resolution of their relationships

In the newly obtained orchid phylogenetic trees, the monophyly of all 31 subtribes with two or more species was consistently and strongly supported (with BS > 94 in all trees). Also, the relationships among all 44 sampled subtribes were consistently resolved with strong supports (with BS > 84 in all trees and >90 in at least one tree) (Figure S3), except for four nodes with moderate or low supports. In two previous nuclear phylogenomic studies, 10 and 11 subtribes of Orchidoideae were sampled, respectively (Pérez-Escobar et al., 2021; Wong and Peakall, 2022). The trees here sampled 15 subtribes (Figure 1), including all subtribes sampled in the two previous studies, providing a comprehensive phylogenetic understanding of Orchidoideae subtribes. The relationships here are consistent with those among sampled subtribes in two previous studies and provide new phylogenetic information on some subtribes (Figures S1, S2). For example in Diurideae, one to three of the subtribes Prasophyllinae, Acianthinae, and Caladeniinae were not sampled in some previous studies (Y.X. Li et al., 2019; Kim et al., 2020; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021). Our results supported Acianthinae and Caladeniinae as sisters (at least 94 BS in all trees), whereas Prasophyllinae were grouped with the other two with weak support (BS < 50), in agreement with a phylogenetic analysis using 633 nuclear genes (Wong and Peakall, 2022). The relationships among other Diurideae subtribes were consistently resolved and maximally supported, consistent with the 633-gene nuclear phylogenetic tree (Wong and Peakall, 2022). In another large Orchidoideae tribe, Cranichideae, our trees showed maximally supported sister relationship for Spiranthinae and Cranichidinae, with Goodyerinae being sister to the clade of Spiranthinae + Cranichidinae, consistent with a previous nuclear phylogenetic analysis (Pérez-Escobar et al., 2021) but different from that in a plastid phylogenetic tree (Serna-Sánchez et al., 2021).

In the Epidendroideae tribe Arethuseae, our phylogenetic trees maximally support the monophyly of two subtribes (Figures 2, S3). Previously, either only one species was sampled for each subtribe (Givnish et al., 2015; Pérez-Escobar et al., 2021; Wong and Peakall, 2022), or the monophyly of one or two subtribes was supported with <80 BS (Y.X. Li et al., 2019; Serna-Sánchez et al., 2021) (Figures S1, S2). In Epidendreae, eight subtribes were represented by 124 sampled species in the newly obtained trees, with maximally supported monophyly for all six subtribes with two or more sampled species (Figures 3, S3). Furthermore, the relationships among subtribes were consistently resolved and are generally in agreement with a previous nuclear phylogenetic analysis that included 16 Epidendreae species from seven subtribes

(Pérez-Escobar et al., 2021). The placements for most subtribes were strongly supported, including that of Agrostophyllinae as the sister of other Epidendreae subtribes. However, previously Agrostophyllinae and Coeliinae were sisters in a plastid phylogenetic analysis (Serna-Sánchez et al., 2021). Our results placed *Coelia* (two species sampled) and Calypsoinae as successive sisters of most subtribes in Epidendreae, in support of the assignment of *Coelia* as a separate subtribe Coeliinae from Calypsoinae. The designation of the subtribe Coeliinae was also supported by several phylogenetic analyses (van den Berg et al., 2005, 2009; Górniak et al., 2010; Pérez-Escobar et al., 2021). However, another analysis placed *Coelia* as sister to other Calypsoinae; this is consistent with either giving *Coelia* its own subtribe or treating it as part of Calypsoinae (Chase et al., 2015; Freudenstein and Chase, 2015). In addition, *Blettia* and *Chysis* occupy non-sister lineages in all four coalescent trees here, consistent with previous analyses (van den Berg et al., 2005, 2009; Górniak et al., 2010; Pérez-Escobar et al., 2021). The phylogenetic positions of *Blettia* and *Chysis* here and in previous studies both support assigning *Blettia* and *Chysis* to respective subtribes Bletiinae and Chysinae.

In Cymbidieae, 72 genera and 133 species representing 10 subtribes were sampled and relationships among them were consistently resolved with generally strong supports. Five of the subtribes form a grade of four lineages (Cymbidiinae, Eulophiinae, Cyrtopodiinae + Catasetinae, and Oncidiinae) outside a large clade of five other subtribes (Figure 4). The relationships among the latter five subtribes, that is, Zygopetalinae, Eriopsidinae, Coeliopsidinae, Stanhopeinae, and Maxillariinae, were consistent among four coalescent trees. Zygopetalinae and Eriopsidinae were placed (with BS from 55–73) as successive sisters of a clade with the remaining three subtribes, among which Maxillariinae were sister to Stanhopeinae + Coeliopsidinae. On the other hand, in a study with one species in each subtribe and 292 genes (Pérez-Escobar et al., 2021), Eriopsidinae, Maxillariinae, and Zygopetalinae formed a grade of three successive sisters of Stanhopeinae + Coeliopsidinae. The consistent subtribe relationships here suggest that this study might have benefited from much greater taxon sampling and up to 1,195 genes used in the phylogenetic analyses.

The orchid phylogenetic analysis here is the largest thus far in taxon sampling and in marker genes with generally highly supported resolution of the relationships among tribes and subtribes and provides a framework for diverse evolutionary studies of this diverse family. For example, the new phylogenetic analysis showed that Sobralieae, a tribe with many epiphytes, are not closely related to other epiphytic tribes, providing new information for understanding evolution of orchid epiphytism (orchids have experienced multiple parallel transitions to epiphytism with one of these events as the ancestor of 2/3 of orchids). The phylogenetic sampling of many genera and species that were not sampled in other nuclear phylogenies (Figure S2) allows their placements with strong supports in an extensive phylogenetic analysis of orchid genera and species, including the large and prominent *Dendrobium* (36 species) and *Cymbidium* (17 species) and 29 other genera with four or more species. The newly reconstructed phylogenetic trees provide a robust evolutionary framework for evolutionary studies involving many genera and species of Orchidaceae.

To investigate the concordance and discordance among gene trees and between coalescent tree and gene trees, quartet supports for the main topology discussed here and alternative topologies were inferred using the gene-set with 1 195 genes. The analysis showed that 437 (~71%) out of 615 internal nodes of the main topology were supported by at least 50% of quartets (Figure S10), and more than 60% of the 437 nodes were supported by at least 80% of quartets, showing a high concordance among gene trees. Among the nodes with <50% quartet support, the vast majority (>90%) was found among closely related species within genera, such as *Dendrobium*, *Vanda*, and *Cymbidium*. In addition, two nodes related to the placement of the tribe Xerorchideae have relatively low quartet supports (36%–41%); these low supports are likely related to the small number of detected genes of the tribe (108 out of 1 195). It agrees with the relatively low BS values of these two nodes and suggests the possibility of other placements of this tribe. Another node with relatively low quartet support (42%) concerns the relationship among three large tribes, that is, Cymbidieae, Epidendreae, and Vandeae. It might partially explain the controversy of relationships among these three tribes in previous studies (Givnish et al., 2015; Y.X. Li et al., 2019; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). The high support values and consistent topology for these three tribes among phylogenetic trees reconstructed using different gene-sets (Figure S3) suggest that the topology provided here is relatively robust. Low quartet supports can be found at several nodes related to the placements of some subtribes, for example, Eriopsidinae (Cymbidieae), Chysinae (Epidendreae), Acianthinae (Diurideae), and Prasophyllinae (Diurideae). Meanwhile, some of them also have relatively low BS support values (Figures 1–4). Although the alternative topologies of some nodes are possible, the consistent phylogenetic topology reconstructed different gene-sets suggests that the topology shown here might be more reliable than the alternative topologies.

Orchids have experienced multiple parallel transitions to epiphytism with one of these events as the ancestor of 2/3 of orchids

To gain an understanding of the evolutionary history of epiphytism and other growth forms in Orchidaceae, we performed ancestral state reconstruction of growth forms. The results showed that the most recent common ancestor (MRCA) of orchids and the ancestor of each subfamily were terrestrial (Figures 5, S11). Epiphytes originated at least 14 times in Orchidaceae (not including re-acquired epiphytism

discussed below), including twice in Cypripedioideae, seven times in Orchidoideae, and at least four (possibly five to seven) times in Epidendroideae, and one origin of the secondary hemi-epiphytes in Vanilloideae (de Lima and Moreira, 2022) (hereafter treated as epiphytes for the convenience of discussion) (Figure S11). One of these transition events from the terrestrial to epiphytic habit occurred at the MRCA of the MCVECP clade with six Epidendroideae tribes, which contain ~20,000 species (~71% of orchids) and ~19,000 epiphytes (~95% of epiphytic orchids) (Zotz et al., 2021). In contrast, the other 13 times affected only a small number of species and generally occurred at genus or species level.

After gaining epiphytism, return to terrestrial growth habits was detected 36 times in the MCVECP clade, showing parallel losses of epiphytism in Orchidaceae (Figures 5, S11). One such loss of epiphytism was at the MRCA of the tribe Collabieae with ~450 species; in addition, the Epidendreae subtribes Calypsoinae (~80 species) and Bletillinae (~50 species) also experienced separate losses of epiphytism. Other losses of epiphytism were shared by closely related genera or within a genus. Moreover, we also detected two and one secondary evolution of the epiphytism in the terrestrial tribe Collabieae and subtribe Calypsoinae, respectively.

In addition, many orchids grow on rocks as lithophytes (Xing et al., 2015; Qin et al., 2020). We detected 46 times parallel origin of lithophytes in Orchidaceae; nearly all of these affected individual genera, except one at the MRCA of three genera in Cypripedioideae (Figure S11). In Cypripedioideae, lithophytes evolved from terrestrial growth forms; on the other hand, most lithophytes in Epidendroideae have evolved from epiphytic ancestors, although several transitions from terrestrial ancestors to lithophytes were detected in Sobralieae, Arethuseae, and Collabieae. In addition, at least 16 transitions to fully mycoheterotrophic habit (lacking photosynthesis) were detected in Orchidaceae. Most fully mycoheterotrophic groups were derived from terrestrial ancestors (e.g., in Neottieae), but they might have originated twice in Cymbidieae from epiphytic ancestors. The history of full mycoheterotrophy involving Gastrodieae and Nervilieae is uncertain. Both Gastrodieae and the subtribe Epipogiinae of Nervilieae are fully mycoheterotrophic, but the subtribe Nerviliinae of Nervilieae is photosynthetic. Thus, one possibility is that the MRCA of Gastrodieae and Nervilieae was fully mycoheterotrophic, whereas the Nerviliinae ancestor regained photosynthetic ability. Alternatively, it is also possible that Gastrodieae and the subtribe Epipogiinae independently gained the fully mycoheterotrophic habit. Additionally, previous studies revealed that the fully mycoheterotrophic species have evolved in parallel among closely related species within the same genus (Barrett et al., 2019; Li et al., 2020), indicating that the gain of the fully mycoheterotrophic habit could happen in different taxonomic ranks and might be frequent in the evolution history of Orchidaceae.

The results here suggested that although the epiphytism evolved parallelly in Orchidaceae, ~95% of epiphytic orchids have inherited epiphytism from a single ancestor of the MCVECP clade. Epiphytism might be one of the factors that promoted the diversification of this extraordinarily large clade containing over two-thirds of the species richness (~20,000 species) of Orchidaceae. A previous study (Givnish et al., 2015) suggested that epiphytes of Arethuseae probably share the same origin of epiphytism with those of the MCVECP clade. However, our analyses using greatly increased taxon sampling, including three terrestrial genera in the two subtribes of Arethuseae, support the idea that the epiphytes in Arethuseae gained epiphytism twice within the subtribe Coelogyninae, separately from that for MCVECP (Figures 5, S11). The phylogenetic tree of another study (Chomicki et al., 2015) proposed that Arethuseae were nested among the tribes of the MCVECP clade; consequently, their character reconstruction using the phylogenetic tree suggested an origin of epiphytism shared by Arethuseae and MCVECP tribes. In addition, the epiphytism of Arethuseae species was shown to be regained after a loss of epiphytism in the common ancestor of the tribe. However, newly obtained phylogenetic trees here highly supported that Arethuseae are sister to MCVECP tribes; thus the character reconstruction here supported likely independent origins of epiphytism for Arethuseae species instead of the re-acquisition of epiphytism. We also detected >30 times loss of epiphytism and cases of re-acquisition of epiphytism in several clades, supporting a highly complex evolutionary pattern of epiphytism. In addition, epiphytism and mycoheterotrophic habit likely originated multiple times within some orchid genera. For example, mycoheterotrophs have originated several times in *Oreorchis* and *Hexalectris* (Barrett et al., 2019; Li et al., 2020b). Hence, the transition times of growth forms in Orchidaceae detected here are probably underestimated because character states were coded at the genus, not species, level in this study. Greater sampling with more species is necessary to further understand the parallel evolution of growth forms of orchids, especially at the species level.

Estimated Orchidaceae origin in the Cretaceous and dramatic divergences in the Cenozoic

To obtain a comprehensive temporal framework of Orchidaceae evolution for comparison with other groups and environments, the divergence history of Orchidaceae was estimated using 14 selected fossil calibrations and two secondary calibrations (Table S2). The stem age and crown age of Orchidaceae were estimated as 131.9 (131.24–138.05) million years ago (Ma) and 101.5 (97.08–102.56) Ma, respectively, suggesting that Orchidaceae had an early Cretaceous origin, and the divergence among extant orchids started near the early to Late Cretaceous boundary with warm climates (Veizer et al., 2000) (Figures 6A, S12). These ages were slightly or substantially older than previous estimations using plastid genomes (99.2–79.91 Ma for crown age; see Figure S13 for 95% highest posterior density (HPD) intervals) (Givnish et al., 2016; Y.X. Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021) or several plastid markers (77–76 Ma for crown age) (Ramírez et al., 2007; Gustafsson et al., 2010) with various fossil calibrations. The origins (stem

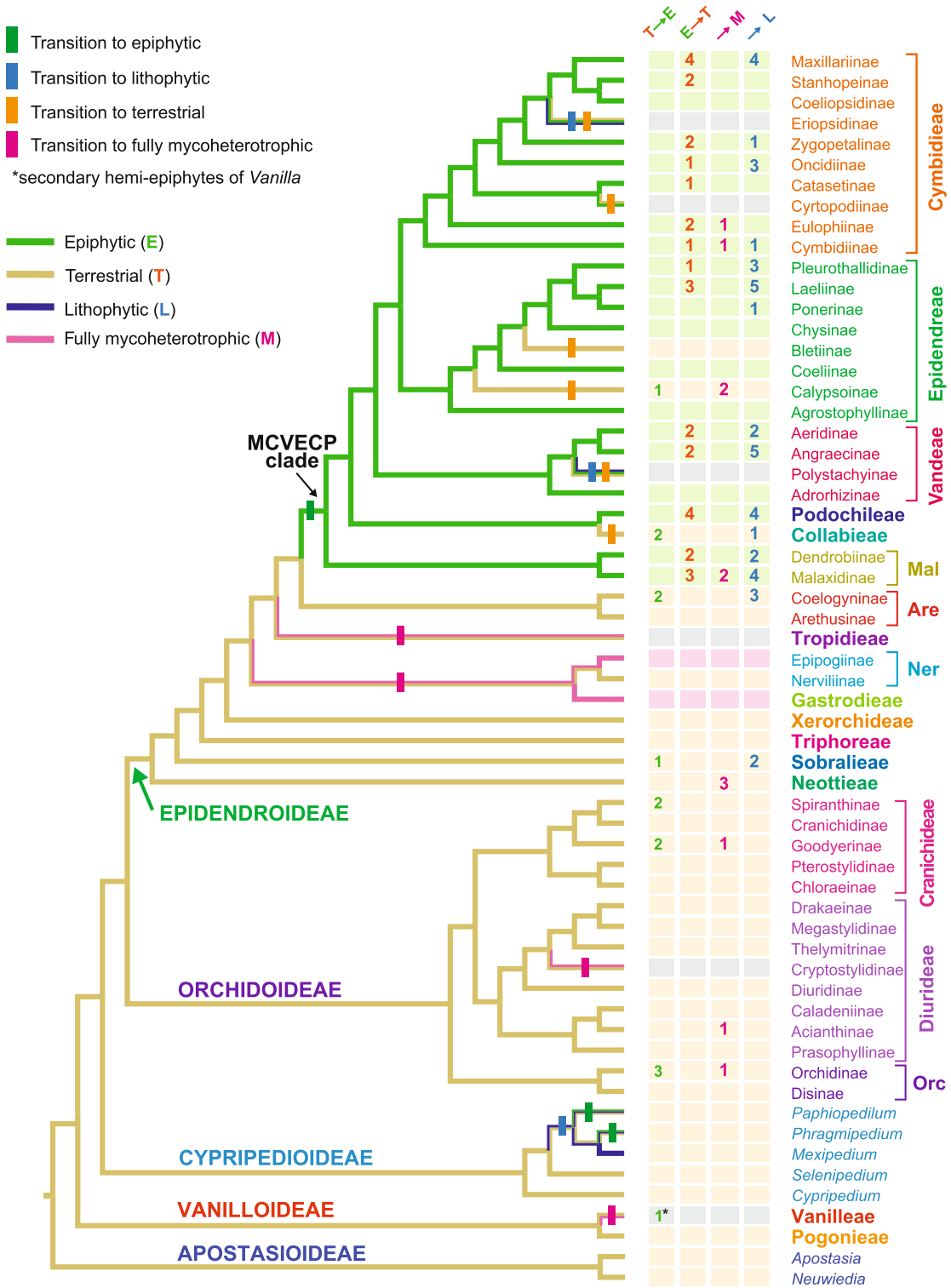

Figure 5. Evolutionary pattern of growth forms in Orchidaceae

A cladogram showing the reconstructed ancestral states of growth forms of Orchidaceae. Corresponding taxon names (genera of Apostasioideae and Cypripedioideae, subtribes of Orchidoideae and Epidendroideae, and tribes of Vanilloideae) are shown on the right side of the tree, and the same color scheme of taxon names in Figures 1–4 is used here. Branch colors indicate the reconstructed growth forms as described on the left side of the tree. The green, purple, blue, and orange vertical bars on branches indicate transitions to epiphytism, full mycoheterotrophy, lithophytism, and terrestrial form, respectively. Numbers in four columns between branch tips and taxon names indicate the times of transitions within the taxon group as indicated at the top: between terrestrial (T) and epiphytic (E), other types to full mycoheterotrophy (M), and other types to lithophytic (L). Orc: Orchideae; Ner: Nervilieae; Are: Arethuseae; Mal: Malaxideae.

ages) of five subfamilies were estimated in the Late Cretaceous from 101.5 to 77.7 Ma, also older than previous results (Ramírez et al., 2007; Gustafsson et al., 2010; Givnish et al., 2016; Y.X. Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021); the crown ages of subfamilies were estimated from 74.3 to 34.4 Ma. Ages of the orchid backbone nodes were generally estimated to be 2–25 million years (Myr) older compared with previous studies (Ramírez et al., 2007; Gustafsson et al., 2010; Givnish et al., 2016; Y.X. Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021). Especially, the stem and crown ages of Epidendroideae were estimated as 77.7 (74.5–79.7) Ma and 72.0 (68.1–74.2) Ma, respectively, as compared with the stem ages <74 Ma and the crown age <60.3 Ma in previous studies; also the crown age of Epidendroideae was estimated within the Cretaceous for the first time.

In Vanilloideae, two tribes diverged at 74.3 (68.4–76.5) Ma and thus are the oldest tribes of Orchidaceae. Tribes of Orchidoideae and Epidendroideae generally originated between 72–50 Ma except that the divergence between Podochileae and Collabieae occurred approximately 41 Ma; the ages here were generally 15–25 Myr older than previous estimations. Specifically, in Epidendroideae, after the divergence of Neottieae in the Late Cretaceous, several tribes quickly diverged within 3 Myr near the K-Pg boundary, providing a possible explanation for the difficulty in resolving these relationships in previous phylogenetic analyses (Givnish et al., 2015; Y.X. Li et al., 2019; Kim et al., 2020; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). The MCVECP clade originated at 54.9 (48.1–55.8) Ma, suggesting that the ancestor of most epiphytic orchids gained epiphytism in the early Eocene near the Paleocene–Eocene Thermal Maximum (PETM) (Figure 6A) (Westerhold et al., 2020). Additionally, the divergence time of Gastrodieae and Nervilieae was estimated as 63.6 (58.9–64.6) Ma, being much older than the 34.9 Ma estimated in Li et al. (2019b). The divergences among three large tribes of Epidendroideae, Cymbidieae, Epidendreae, and Vandeae, occurred near 50–51 (44.2–51.8) Ma, also older than the ~30–40 Ma estimated in previous studies. The divergences of most orchid tribes, especially Epidendroideae tribes, were estimated here to occur in warmer climates in the Paleocene and early Eocene before 50 Ma (Figure 6A), rather than in cooler climates after 50 Ma as suggested by previous studies. Moreover, the crown ages of Epidendroideae tribes are generally 10–20 Myr older than the estimations of previous studies, suggesting that the divergence of Epidendroideae tribes occurred in periods of warmer climates than previous estimates. Especially, the crown age of Arethuseae was estimated as 37.2 (33.5–51) Ma, being much older (and in a warmer period; Figure 6A) than the previous results (~23–15 Ma). Thus the older ages here are consistent with the preference of most extant orchid species for relatively warm climates.

Apostasioideae and Cypripedioideae were not divided into tribes partially because species of each of these subfamilies are morphologically similar compared with those in three other subfamilies. The time estimation here showed that the extant species within these two subfamilies diverged much later compared with the divergences among major lineages in three other subfamilies, agreeing with the morphological evidence. Similarly, the Epidendroideae tribe Sobralieae were estimated to have a much longer gap (lasting ~58.5 Myr) between the origin and divergence compared with other tribes in the same subfamily.

Our results showed that most Orchidaceae subtribes originated from 52 to 29 Ma (Figure 6A, B), but five Cymbidieae subtribes (i.e., Coeliopsidinae, Eriopsidinae, Maxillariinae, Stanhopeinae, and Zygopetalinae) originated more recently in a period from 16 to 10 Ma. The much younger ages of these subtribes compared with those of other Orchidaceae subtribes suggest that the recent diversification of the large tribe Cymbidieae might have been facilitated by some specific factors distinct from those promoting the diversification in other tribes. Divergences within subtribes was estimated from 42 Ma to very recent times (e.g., ~3 Ma for Cyrtopodiinae). In particular, the seven largest subtribes, each containing over 1,000 species, had the starting date of divergences ranging from 41 (42–38; Orchidinae) to 18 (20–14; Laeliinae) Ma, indicating that temporal histories for intra-subtribal divergence are vastly different among Orchidaceae subtribes, with differential impact on the species richness of subtribes. Most genera and species had recent origins that were generally less than 20 Ma (Figures 6B, S12), suggesting that most of diversification in Orchidaceae have occurred recently. Compared with previous studies (Ramírez et al., 2007; Gustafsson et al., 2010; Givnish et al., 2016; Y.X. Li et al., 2019; Kim et al., 2020; Serna-Sánchez et al., 2021), stem and crown ages of subtribes estimated here were generally older. We also newly provided the age estimation for many subtribes that were not included in previous studies. The increased sampling might partially contribute to the older estimations of Orchidaceae groups since the limited taxon representation might underestimate the evolutionary rate of groups. In addition, different gene sequences, fossils, and estimation methods used here and in previous estimations might also contribute to the difference of divergence time.

Multiple increases in diversification rate associated with groups of many epiphytic orchids

To analyze the diversification history of orchids, we estimate the diversification rate shifts using BAMM; since we have much higher (86%) representation at the subtribe level than the genus (<50%) and species (~2.2%) levels, the analysis was initially conducted using subtribe-level sampling fractions. The results showed that the configuration (combination of shifts) that best fit our data sets contained nine shifts of diversification rate (effective sample sizes of the log-likelihood (ESS-L) and of the shift events (ESS-SE) are 541 and 649, respectively) (Figures 6A, S14). Among these nine shifts, eight were each supported by other credible shift

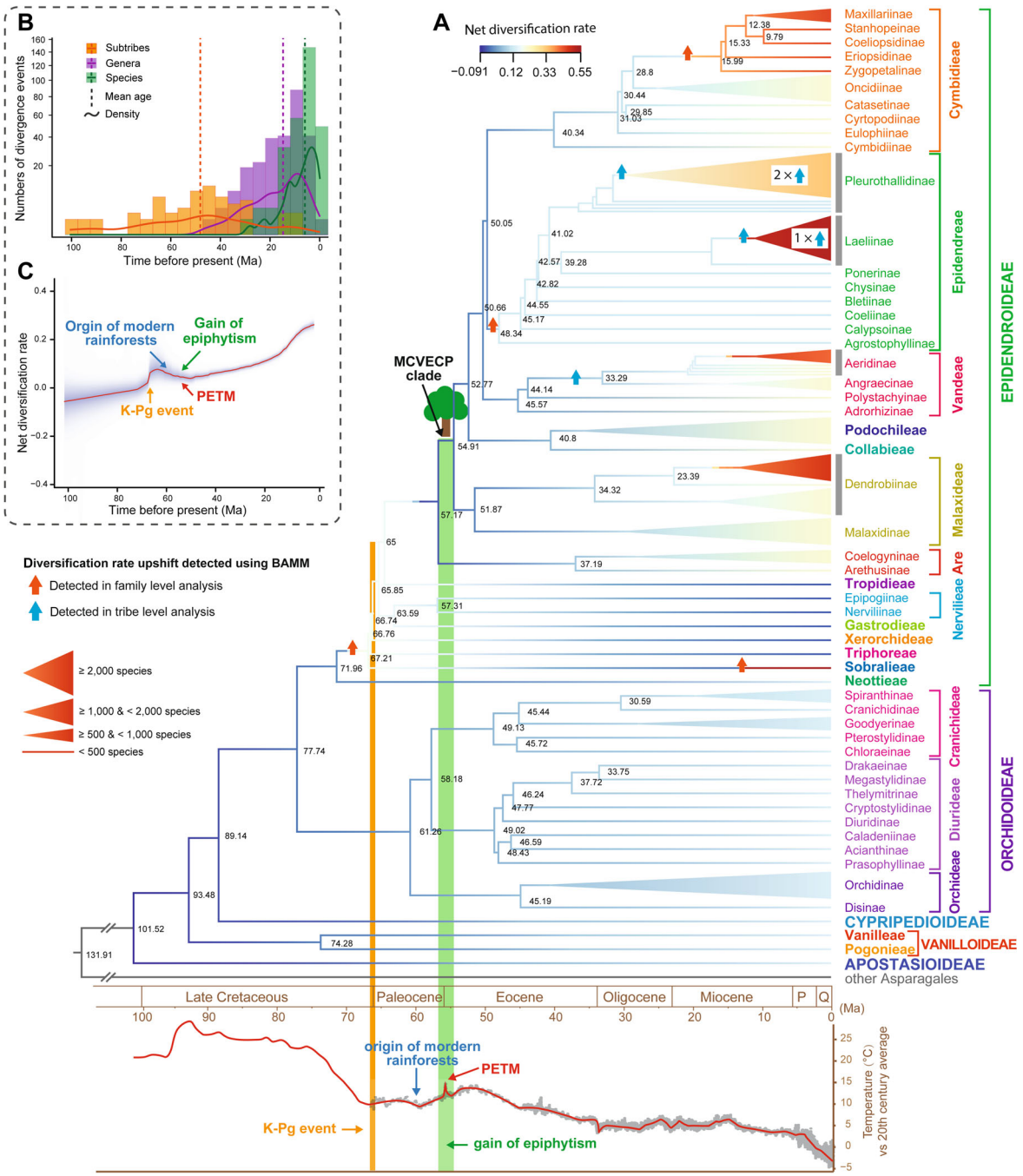

Figure 6. Divergence time and diversification rate upshifts of Orchidaceae

(A) A chronogram inferred using TreePL with the topology of the summary tree from four coalescent trees and with branch length calculated from a concatenated data set containing 299 genes, showing temporal evolutionary patterns of subtribe and higher ranks of Orchidaceae. Numbers near nodes indicate the estimated ages of corresponding nodes. The branch colors indicate the net diversification rate estimated using BAMM, and corresponding rates are shown in the legend at the top of the chronogram. Triangles or lines on terminal branches represent the range of species number of corresponding taxa as indicated to the left side of the chronogram. A geological time scale is shown at the bottom of the chronogram, with an estimated temperature change curve for the Late Cretaceous (Veizer et al., 2000) and the Cenozoic (Westerhold et al., 2020). Also shown are the K-Pg boundary, Paleocene–Eocene Thermal Maximum (PETM), an origin of the modern rainforests (Carvalho et al., 2021), and the origin of epiphytism shared by MCVECP (Malaxideae, Cymbidieae, Vandeae, Epidendreae, Collabieae, and Podochileae) clade inferred in this study. The orange up arrows on branches indicate the net diversification rate upshifts inferred in BAMM using subtribe-level sampling fractions; the blue up arrows on branches and in the triangles indicate the net diversification rate upshifts inferred in Epidendreae and Vandeae using genus-level sampling fractions. The same color scheme of taxon names in Figures 1–4 is used here. **(B)** A histogram plot shows the temporal pattern of divergence events of ancestors of orchid subtribes, genera, and species. The height of each bin indicates the number of divergent events within five million years, and the vertical dashed lines indicate mean ages of divergent events of each rank. **(C)** The temporal change pattern of the Orchidaceae net diversification rate. K-Pg boundary, an origin of the modern rainforests, PETM, and the origin of epiphytism shared by members of the MCVECP clade are indicated.

configurations with high sampling frequency (Figure S15). In this subtribe-level analysis, the diversification shifts detected within subtribes might not be as reliable because the intra-subtribal diversity differences among clades could not be considered. Hence, we conducted further analyses using genus-level sampling fractions for three tribes that had detected shift signals within some subtribes in the initial analysis, that is, Epidendreae, Vandeae, and Malaxideae, which had 67%, 35%, and 42% of representation at the genus level, respectively. In total, we detected five upshifts and one downshift of diversification rate above subtribe level and all of them were in Epidendroideae with ESS-L of 1 927 and ESS-SE of 3,382 (Figures 6A, S14–S17). We also detected five upshifts of diversification rates within subtribes of Epidendreae (Figures 6A, S14–S16); however, we did not detect shifts of diversification rates within subtribes of Vandeae (with ESS-L of 2,185 and ESS-SE of 2,655) and Malaxideae (with ESS-L of 1,314 and ESS-SE of 786) (Figures S17–S18, a shift shared by *Polystachya* of Vandeae with marginal probability of 0.32 is not accounted here), probably due to the lower genus-level representation in these two tribes than that in Epidendreae.

One of the detected upshifts was near the MRCA of all Epidendroideae tribes except Neottieae, shortly before successive divergences of six tribes, suggesting a rapid diversification that contributed diversity of Epidendroideae at the tribe level (Figure 6A). This rapid diversification was close to the K-Pg boundary (Figure 6A), suggesting that the K-Pg mass extinction event might have freed niches allowing the occupation of diverse orchids (Figure 6C). Four of these tribes are fully mycoheterotrophic (Figures 5, S11), a characteristic that can facilitate plant survival with reduced dependence on photosynthesis. Meanwhile, all orchids are associated with symbiotic mycorrhizal fungi for seed germination and hence are mycoheterotrophic at least during some part of their life cycle (Dearnaley et al., 2012; McCormick et al., 2018; D. K. Zhao et al., 2021; Li et al., 2022), suggesting that the ancestor of Orchidaceae had already acquired the capacity for mycoheterotrophic growth. It was hypothesized that after the K-Pg boundary sunlight was blocked for decades; furthermore, saprotrophs, such as fungi, were dominant, whereas floristic diversity was greatly reduced (Vajda and Bercovici, 2014; Kaiho et al., 2016; Berry, 2020; Carvalho et al., 2021). Hence, these orchids that had mycoheterotrophic capabilities beyond the germination stage were probably much less affected by the low level of available sunlight than photosynthetic plants and benefited from the dominant fungal community that appeared after the K-Pg event.

Four of the other nine upshifts were shared by either a tribe or several subtribes. One of these upshifts was near the MRCA of Sobralieae, suggesting a recent rapid diversification of this tribe starting from the late Miocene. Another upshift was shared by the two largest Vandeae subtribes Aeridinae and Angraecinae, together containing ~130 genera and ~2 000 species. A third was shared by five Cymbidieae subtribes that have a combined total of ~1 600 species. These findings suggest that the species richness of Vandeae and Cymbidieae might be related to the rapid diversifications shared by their respective subtribes. We also detected a upshift near the MRCA of Epidendreae, the largest Orchidaceae tribe containing ~7,000 species. Additionally, two upshifts were detected in the large Epidendreae subtribe Laeliinae; one of these two upshifts was at the MRCA of most Laeliinae members except *Arpophyllum*, whereas the other was near the MRCA of *Epidendrum*, a large genus containing ~1,400 species. Furthermore, three upshifts were detected in Pleurothallidinae, the largest subtribe of Orchidaceae having ~4 500 species. These six upshifts all in one single tribe suggest that multiple rapid diversification events occurred in Epidendreae, providing a clear evolutionary pattern of the dramatic (and somewhat uneven) increase in species diversity of the extant orchids. Our time estimation indicated that most upshifts happened after the Oligocene, indicating that the recent diversification is likely the main source of the current diversity of orchids, although both old (~51 Ma) and recent (after 30 Ma) rapid diversifications could have contributed to the current diversity of Epidendreae. Within the MCVECP clade with the vast majority of epiphytic orchids, eight upshifts were detected, suggesting that epiphytism might have accelerated the diversification rate in multiple lineages.

A previous study (Givnish et al., 2015) detected four upshifts of diversification rate in Orchidaceae. One of them, near the MRCA of Epidendreae, was also supported by our results using the new time tree here with more comprehensive sampling. The previously detected upshift near the MRCA of Orchidoideae (Givnish et al., 2015) was not supported by the best configuration here (Figures 6, S14) but was detected in four of the next eight 95% Bayesian credible configurations (Figure S15). However, this shift had relatively low marginal shift probabilities in the four sets (Figure S15) compared with those shifts detected in the best configuration (Figure S14). We did not recover the previously detected upshift shared by Arethuseae plus the MCVECP clade (Givnish et al., 2015). In contrast, we detected a downshift at this node in the first nine 95% credible sets (Figures S14, S15). This difference might be related to different results of divergence time estimation and different sampling sizes. Within Epidendreae, related to the detection of two and three upshifts within subtribes Laeliinae and Pleurothallidinae, respectively, a single upshift was detected previously at the common ancestor of these two subtribes plus Ponerinae (Givnish et al., 2015). It is possible that our sampling of many more taxa, especially more basal lineages of these two subtribes, might have provided a relatively high phylogenetic resolution of the results.

Interactions with rainforests might have promoted the diversification of epiphytic orchids

Extant epiphytic orchids primarily occur in closed-canopy rainforests with mainly angiosperm trees (Zotz, 2016; Fernández et al., 2023; Spicer and Woods, 2022); such angiosperm rainforests were estimated to have formed

approximately 60 Ma (Carvalho et al., 2021) and then dramatically expanded near the PETM (56 Ma) (Jaramillo et al., 2010; Huurdeman et al., 2021). Additionally, previous time estimations showed that some angiosperm groups, for example, Fabaceae and Malvaceae, that contained dominant trees of modern rainforests rapidly diverged approximately at the same time (Cvetkovic et al., 2021; Y. Zhao et al., 2021; Benton et al., 2022). Our study showed that over 95% of epiphytic orchids (~19,000 species from six tribes of the MCVECP clade) were derived from a single ancestor that gained epiphytism approximately 54.9 (48.1–55.8) Ma (Figure 6A), near the PETM event. This estimated time matched that of the expansion of rainforests soon after their origin, suggesting that rainforests might have promoted this transition to epiphytism in Orchidaceae. Modern closed-canopy rainforest ecosystems provide habitats with different levels of sunlight and water along the tree trunks and branches (Shaw, 2004; Wang et al., 2016; Nakamura et al., 2017; Spicer and Woods, 2022), providing vertically diverse niches for epiphytes. Additionally, forest canopies generally hold high concentration of organic matter than the ground soil (Nadkarni et al., 2002), possibly benefiting both the epiphytic orchids and/or mycorrhizal fungi on which the orchids are dependent. Furthermore, these newly appeared niches might have less competition, compared with the diverse terrestrial flora with herbaceous and shrubby plants. Thus the adaptation to epiphytic habits might have allowed orchids to take advantage of these new niches in rainforests and subsequently to diversify.

The MCVECP clade contains ~95% of the epiphytic orchids, but none of the eight upshifts of diversification rate correspond to a node with gain of epiphytism, suggesting that epiphytism alone was not sufficient to cause increased orchid diversification. Besides the abiotic factors, epiphytic orchids have active interactions with other organisms living in forests, such as pollinators (Spicer and Woods, 2022). Orchids have also evolved close interactions with fungi especially for the seed germination, nutrient absorption, and development (Dearnaley et al., 2012; Martos et al., 2012; Gebauer et al., 2016; Alghamdi, 2019). The higher fungal diversity (Cardelus et al., 2009) and microbial biomass (Vance and Nadkarni, 1990) of the rainforest canopies compared with those on the rainforest grounds might have contributed to the diversity of epiphytic orchids.

Moreover, some other characters might have helped orchids in the transition to epiphytic growth and further adaptations. For example, orchids produce thousands of tiny seeds (generally 0.1–1 mm in length) that are easily dispersed by winds, and the light seed weight might help seeds to easily reach suitable epiphytic niches (Madison et al., 1977; Barthlott et al., 2014). Pseudobulbs are thickened stems and are found in many epiphytic orchids but relatively rare in terrestrial orchids. These organs store water, mineral, and carbohydrate and have crucial roles in growth and survival of epiphytic orchids (Ng and Hew, 2000; Yang et al., 2016). In Epidendroideae, pseudobulbs occur in Nervilieae, Arethuseae, and tribes of the MCVECP clade, suggesting that pseudobulbs might have a slightly earlier origin than that of epiphytism at the ancestor of the MCVECP clade and might be one of the factors that promoted the origin of epiphytism in the clade. Orchidaceae have two types of pseudobulbs, heteroblastic and homoblastic, and their shapes and sizes vary. Because of the important storage function of pseudobulbs for orchids in epiphytic habitats with fluctuating environmental condition, the diverse pseudobulbs might have important roles in facilitating the diversification of epiphytes of Orchidaceae by providing different adaptive capacities under selection. Epiphytic orchids also develop a root velamen (spongy outer layer) that has an adaptive function by allowing secure attachment to bark and efficient absorption and retention of water from rain and fog (Zotz and Winkler, 2013; Joca et al., 2017). The connection of environmental factors related to rainforests and epiphytism of orchids suggests that the current diversity of epiphytic orchids might have benefited from their co-evolution and interaction with rainforests.

CONCLUSIONS AND IMPLICATIONS

Greatly increased sampling and gene markers provided the largest and highly resolved nuclear phylogeny of Orchidaceae

The phylogenetic analyses here used up to 1,450 nuclear genes from 610 orchids (representing 297 genera in 44 subtribes), in comparison with recent phylogenomic studies that generally used dozens of plastid genes from up to 264 orchids (up to 28 subtribes and 117 genera) or as many as 633 nuclear genes from dozens of species (Givnish et al., 2015; Y.X. Li et al., 2019; Kim et al., 2020; Eserman et al., 2021; Pérez-Escobar et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). The phylogenetic trees here using four gene-sets with different taxon coverages and gene missing rates consistently resolved previously inconsistent or unresolved relationships among tribes (mostly in Epidendroideae, including the three large tribes, Cymbidieae, Epidendreae, and Vandeae) and many subtribes. Also, these trees provided highly supported placements of many subtribes, genera, and species that were not included in previous phylogenomic studies. Particularly, several basal tribes in Epidendroideae with previously uncertain placements have epiphytes, for example, Sobralieae (Givnish et al., 2015; Y.X. Li et al., 2019; Serna-Sánchez et al., 2021); therefore the highly resolved phylogenetic relationships with greatly increased taxon representation here provide a robust framework for evolutionary studies in Orchidaceae.

An early Orchidaceae transition to epiphytism affecting ~95% of epiphytes

In the ancestral reconstruction of epiphytism using the newly obtained phylogeny, 14 parallel origins of epiphytism were detected, more than the previous estimates of one (Givnish et al., 2015) and 10 times (Chomicki et al., 2015). Specifically, a single gain of epiphytism was shared by six tribes of the MCVECP clade, accounting for around 95% of epiphytic orchids (Figure 5),

whereas Arethuseae appeared to have their own origins, different from the previous suggestion that Arethuseae shared the same origin of epiphytism with the MCVECP clade (Chomicki et al., 2015; Givnish et al., 2015). The parallel origins of epiphytic orchids are similar to multiple origins of epiphytism in other large groups with epiphytes, including ferns, Bromeliaceae, and eudicots (Crayn et al., 2004; Schneider et al., 2004; Schuettpelz and Pryer, 2009; Zotz et al., 2021), suggesting that transition to epiphytism might depend on some morphological or genetic preconditions that are shared by members of these large groups.

Increased diversification of epiphytic vascular plants likely benefited from the rise of modern rainforests

Around 95% of epiphytic orchids, constituting a major portion (~70%) of vascular epiphytes (Zotz et al., 2021), share one origin of epiphytism near PETM at ~55 Ma, nearly coincidental with the expansion of modern rainforests. Multiple subsequent increases of diversification rate of these epiphytic orchids suggested that modern rainforests might have promoted the diversification of epiphytic orchids. Similarly, rapid diversification of epiphytic ferns (accounting for ~10% of vascular epiphytes) also has likely benefited from the formation of modern rainforest (Schneider et al., 2004; Schuettpelz and Pryer, 2009; Du et al., 2021). Three-dimensional structure of rainforests with the relatively closed canopy has provided diverse microhabitats that have a high degree of environmental heterogeneity (including abiotic factors, e.g., water and sunlight, and biotic factors, e.g., mycorrhizal fungi and pollinators) and probably have promoted divergences of organisms in rainforests (Cramer and Willig, 2002; Trevail et al., 2021; Ye et al., 2021). The heterogeneous microhabitats have probably strengthened the isolation among epiphytes and might have also accelerated the diversification of epiphytic vascular plants (Hernández-Pérez et al., 2018). Additionally, during the evolution of modern rainforests following their formation, the host trees (e.g., Fabaceae and Malvaceae), insects, and fungi have rapidly diverged (Cvetkovic et al., 2021; Y. Zhao, et al., 2021; Benton et al., 2022), increasing the complexity of interactions among epiphytes, host trees, and other organisms and benefiting the diversification of epiphytic vascular plants. Diverse rainforest microhabitats might have also promoted the morphological divergence of epiphytes, such as water/nutrient absorption characters, for example root velamen of orchids (Joca et al., 2017), water tanks of bromeliads (Romero et al., 2010; Zotz et al., 2020; Takahashi et al., 2022), and basket-forming morphologies of ferns (Watkins and Cardelús, 2012). These morphological features might have allowed vascular epiphytes to adapt to rainforests and hence further increased the diversity of vascular epiphytes.

MATERIALS AND METHODS

Taxon sampling, RNA isolation, and RNA sequencing

We sampled 610 orchid species from 297 genera in this study, covering 44 out of 51 subtribes, 19 out of 22 tribes, and all five subfamilies; these taxa represent the largest phylogenetic sampling of Orchidaceae thus far. Transcriptomes of 431 orchid species were newly sequenced in this study, and 179 other data sets (including 10 genomes) of Orchidaceae were retrieved from public sources (Table S1). Among taxa that have newly generated transcriptiome data sets for this study, most species were sampled from living collections of the University of California Botanical Garden at Berkeley, the Fairchild Tropical Botanic Garden, and the Shanghai Chenshan Botanical Garden. Other taxa were purchased from commercial suppliers of horticultural orchid species. Some additional species were sampled from field works in accordance with requirements of respective countries and regions, with vouchers deposited at either Department of Biology, the Pennsylvania State University (PAC) or the Chinese National Herbarium (PE). For the species sampled from the living collections, the accession numbers are indicated in Table S1. There was not international material movement of the orchids listed in CITES Appendices I and II for this project. Four species from two other families of Asparagales, two species from Arecales, one species from Poales, and one species from Zingiberales were included as outgroups in the phylogenetic reconstruction (Table S1). To allow the inclusion of more fossil calibrations for greater accuracy of estimated divergence time, we sampled 59 species from basal lineages of angiosperms, eudicots, and additional monocots in the time estimation (Table S1). RNA isolation and library preparation followed methods used in Huang et al. (2022). Paired-end RNA-seq with 150 bp read length was performed using the Illumina HiSeq. 3000 platforms by GENERGY BIO-TECHNOLOGY (Shanghai) or NovaSeq. 6000 platforms by NOVOGENE (California). On average, six gigabases of raw data (with ~40 million reads) were obtained for each species. The raw sequence data generated in this study have been deposited at the National Center for Biotechnology Information Sequence Read Archive (SRA) database under BioProject No. PRJNA923320.

Raw data quality control and transcriptome assembling

The BBduk tool of the BBmap package (Bushnell, 2015) was used for trimming of the adaptors and regions with low sequence quality for each raw data set with default parameters. FastQC (v0.11.8) (Andrews, 2010) was performed to assess the quality of data sets after trimming. De novo assembly of transcriptomes was conducted using Trinity (v2.13.2) (Grabherr et al., 2011) with default parameters. Coding sequences (CDSs) of transcripts were predicted and extracted using TransDecoder (v5.5.0) with minimum length of 50 amino acids for open reading frames, and then redundant predicted CDSs were removed using CD-hit-est (v4.6.8) (Li and Godzik, 2006) with clustering threshold of 0.98. Protein sequences were translated from the de-redundant CDSs using SeqKit (v2.0.0) (Shen et al., 2016). To remove likely contaminant sequences from bacteria, fungi, and other non-plant organisms, BLASTP (Camacho et al., 2009) was performed for the protein sequences of each species against a reference database

generated using 20 monocot genomes (including 10 orchid genomes, Table S1). The sequences without any hit with *e*-value $<1e^{-20}$ were removed, and the retained sequences were analyzed using BUSCO (Simão et al., 2015) to evaluate the quality of assembled transcriptomes. Because Trinity assembles multiple isoforms for some genes, which are not needed for phylogenetic studies and increased the complexity of the analyses, only the longest isoform of each gene was retained for the subsequent ortholog searching process. Whole genome shotgun (WGS) data sets and target enrichment data sets were assembled to contigs following methods used in previous studies (Eserman et al., 2021; Huang et al., 2022).

Orthogroup inference, seed gene selection, and putative ortholog searching

High-quality genomes of 10 Orchidaceae species, including one from Apostasioideae, one from Vanilloideae, and eight from Epidendroideae (Table S1; at the early stages of this study, no sequenced genomes were available for Cypripedioideae or Orchidoideae) were used for the orthogroup inference in OrthoFinder2 (Emms and Kelly, 2019) to obtain phylogenetic hierarchical orthogroups (HOGs). The HOGs shared by the 10 species (the N0 set in our analysis) were used for subsequent seed gene selection. A total of 4 450 HOGs were retained that both (i) were found in at least eight species and (ii) had at most two copies in at most two species. Additionally, genes from multi-copy gene families generally have more complex evolutionary history and might contain phylogenetic noise. Thus to avoid such genes as much as possible, we conducted BLASTP search using protein sequences of the seed genes of each of 10 representative species against the source genome. After the removal of genes which have >4 hits in any of the 10 representative species, 2,415 genes were retained as seed genes. Putative orthologs of these 2,415 seed genes were obtained from transcriptomes using OrthoFinder2 by including four transcriptomes each time together with the above 10 genomes to conduct orthogroup inference; and the inferred orthologs of seed genes of each transcriptome were extracted. Putative orthologs of 2,415 seed genes from WGS and target enrichment contigs were inferred using Hamstr (v13.2.6) (Ebersberger et al., 2009) following methods used in Huang et al. (2022). Because genes with short length and high missing rate tend to have higher uncertainty which may reduce the resolution of phylogeny, 1,450 genes with a median length greater than 300 amino acids and shared by at least half of species (305 species) were retained for subsequent phylogenetic analysis. Moreover, to investigate the robustness of phylogeny, four gene subsets, containing 1,195, 1,016, 834, and 639 genes respectively, were generated from the 1,450 gene-set according to the extent of species and subtribe coverage, that is, species coverage >80% for the 1,195 gene-set, species coverage >80% and subtribe coverage >90% for the 1 016 gene-set, species coverage >85% and subtribe coverage >90% for the 834 gene-set, and species coverage >88% and subtribe coverage >90% for the 639 gene-set. Additionally, the tribe Xerorchideae was represented by a single species (*Xerorchis amazonica*) with a limited data set. To maximize the use of its genes, 108 *Xerorchis* genes were included in each gene-set.

Removal of putative paralogs and phylogeny reconstruction

Sequences of each gene were aligned using MAFFT (v7.487) (Katoh and Standley, 2013), and alignments were trimmed using trimAl (v2.0) (Capella-Gutiérrez et al., 2009) by removing sites with at least 80% missing rate. Phylogenetic trees (hereafter referred as single-gene trees) for orthogroups were reconstructed from the trimmed alignments using RAxML (v8.2.1) (Stamatakis, 2014) with the GTRGAMMA model and 100 rapid bootstrapping replicates, and coalescent trees were inferred using Astral (v5.6.3) (Zhang et al., 2018) with default parameters. The gene selection method used in this study has relatively high accuracy of ortholog inference (Emms and Kelly, 2019); however, our preliminary results showed that some putative paralogs or contaminant sequences were still present. To minimize the effects of the putative paralogs and contaminations, we first reconstructed a coalescent tree of the 1,450 genes without removal of any sequences. According to this tree, the monophyly of Orchidaceae, subfamilies, and multiple tribes was maximally supported (with local posterior probability (LPP) of 1.0) and was selected as the criterion for subsequent identification of putative paralogs or contaminants, which are expected to violate such monophyly (Figure S4). Specifically, we used the criterion to evaluate each single-gene tree of four gene subsets (without deduplication of multi-copies), and those sequences that were inconsistent with the criterion were considered as putative paralogs or contaminations and were removed. Subsequently, single-gene tree of each gene was reconstructed again after the deduplication of multi-copies or isoforms of same species by retaining the longest sequence of each gene. Coalescent trees were reconstructed for four gene subsets, and their summary tree was generated using DendroPy (v4.4.0) (Sukumaran and Holder, 2010) and was used to discuss the phylogeny of Orchidaceae.

Ancestral character reconstruction, time estimation, and diversification rate inference

Information on four growth forms (epiphytic, lithophytic, holomycoheterotrophic, and terrestrial) of orchids and outgroups was obtained from literatures and online sources (Table S1). The ancestral state reconstruction was performed at the generic level using the parsimony method in Mesquite (v3.70) (Maddison and Maddison, 2021). Molecular clock estimation requires a supermatrix data set, whereas such data sets with many hundreds of genes can lead to error in the results (Philippe et al., 2011). In addition, varying degree of missing data can lead to errors in branch length estimation. To reduce errors of the estimation of divergence time, 299 genes with

relatively high taxon coverage (either shared by at least 16 out of 19 Orchidaceae tribes and 40 out of the 59 outgroups that were only used for time estimation or detected in Xerorchideae, which had relatively small number of genes, and found in at least 14 other tribes) were selected from the 639 gene-set for subsequent analyses. A supermatrix data set containing concatenated sequences of these 299 genes was generated for phylogenetic reconstruction with 100 bootstrap replicates in RAxML (v8.2.1) (Stamatakis, 2014) using the summary phylogenetic tree as the constraint tree. Divergence time of Orchidaceae was inferred using treePL (Smith and O'Meara, 2012) and the supermatrix tree with two secondary calibration nodes for MRCAs of angiosperms and eudicots, respectively, and 14 fossil calibrations of monocots (Table S2) selected based on previous studies (Ramírez et al., 2007; Iles et al., 2015; Eguchi and Tamura, 2016; H.T. Li et al., 2019). We performed 10 independent runs of random sampling cross validation analysis to determine the optimal smoothing value by testing values from $1e^{05}$ to $1e^{-20}$. The cross validation results showed that the analysis using smoothing value of $1e^{-08}$ had the lowest χ^2 value; thus this smoothing value was used in the final run to estimate the divergence time of Orchidaceae. Same parameters and options were employed in runs using the 100 trees from bootstrap replicates and the results were used to generate 95% HPD intervals of divergence time in SumTrees 4.4.0 (Sukumaran and Holder, 2010). The estimated time tree (after removal of outgroups) was used to analyze the diversification rate shift using BAMM (v2.5.0) (Rabosky, 2014) with subtribe-level sampling fractions. For three tribes that had detected upshifts within some of their subtribes in the family-wide diversification rate shift analysis, that is, Epidendreae, Malaxideae, and Vandeae, analyses of each tribe were conducted using genus-level sampling fractions and the time tree of the corresponding tribe. Prior parameters were obtained using BAMMtools (Rabosky, Grundler, et al., 2014), and the MCMC simulation with four chains was run for 50 million generations and sampled every 10 000 generations. To assess the convergence of MCMC chains, effective sample sizes (ESS) of each run were calculated to make sure that ESS are greater than 500. The outputs were analyzed in BAMMtools after discarding the first 25% of samples as burn-in to investigate the shift times of diversification rate. Lineage-through-time (LTT) plots were generated using DendroPy and *ggpubr* (v0.4.0) (Kassambara, 2020) to display the temporal pattern of divergence of subtribes, genera, and species.

ACKNOWLEDGEMENTS

We would like to thank Holly Forbes, Corina Rieder, Clare W. Loughran, and Andrew S. Doran at the University of California Botanical Garden at Berkeley, Dr. Chao Hu at Shanghai Chenshan Botanical Garden, Dr. Nichole Tiernan at the Fairchild Tropical Botanic Garden, Dr. Jie Xu, Dr. João Vitor Messeder, Dr. Kun Tan, Mr. Langxing Yuan, and Mr. Xibing Guo for their help with plant materials and photos, and Mr. Leland Burghard for plant care. We thank Drs. Weichen Huang, Taikui Zhang, Lin Zhang, Jun Wang, Xinwei Ma for discussion and technical assistance. This study was supported by funds from the Eberly College of Sciences and the Huck Institutes of the Life Sciences at the Pennsylvania State University and the Forestry Peak Discipline Construction Project of Fujian Agriculture and Forestry University (72202200205).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

H.M. conceived of and designed the study. G.Z., H.M., Y.H., Z.J.L., M.Z.H., W.C.H., J.L.D., H.H., D.K.L., and D.Z. contributed plant materials. G.Z., Y.H., and H.M. performed the experiments and analyses. G.Z. and H.M. wrote the paper with contributions from all other authors. All authors read and approved the manuscript.

Edited by: Hongzhi Kong, Institute of Botany, CAS, China

Received Dec. 18, 2022; **Accepted** Feb. 3, 2023; **Published** Feb. 4, 2023

OO: OnlineOpen

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SUPPORTING INFORMATION

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Figure S1. Comparisons of topologies and the sampling of subtribes and higher ranks of Orchidaceae between previous phylogenetic trees using plastomic data sets and the summary nuclear phylogenetic tree in this study
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