Review Article

Advances and prospects of orchid research and industrialization

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Abstract

Orchidaceae is one of the largest, most diverse families in angiosperms with significant ecological and economical values. Orchids have long fascinated scientists by their complex life histories, exquisite floral morphology and pollination syndromes that exhibit exclusive specializations, more than any other plants on Earth. These intrinsic factors together with human influences also make it a keystone group in biodiversity conservation. The advent of sequencing technologies and transgenic techniques represents a quantum leap in orchid research, enabling molecular approaches to be employed to resolve the historically interesting puzzles in orchid basic and applied biology. To date, 16 different orchid genomes covering four subfamilies (Apostasioideae, Vanilloideae, Epidendroideae, and Orchidoideae) have been released. These genome projects have given rise to massive data that greatly empowers the studies pertaining to key innovations and evolutionary mechanisms for the breadth of orchid species. The extensive exploration of transcriptomics, comparative genomics, and recent advances in gene engineering have linked important traits of orchids with a multiplicity of gene families and their regulating networks, providing great potential for genetic enhancement and improvement. In this review, we summarize the progress and achievement in fundamental research and industrialized application of orchids with a particular focus on molecular tools, and make future prospects of orchid molecular breeding and post-genomic research, providing a comprehensive assemblage of state of the art knowledge in orchid research and industrialization.

Introduction

With over 750 genera and 28 000 species, Orchidaceae constitutes one of the largest, species-rich families of flowering plants and has successfully colonized all continents except Antarctica [1, 2]. Orchids display extreme specializations particularly in the tropics, presenting several distinctive features like highly diversified flowers driven by orchid-pollinator interactions, dust-like, non-endospermic seeds, obligate association with mycorrhizal fungi, crassulacean acid metabolism (CAM) and unrivaled reproductive strategies contributing to a wide range of adaptation [3, 4]. Although exhibiting enormous diversity, the intrinsic nature of pollinator and mycorrhiza specification makes them particularly vulnerable to environmental change, and unsustainable harvest becomes a major additional risk. As a result, orchids now feature prominently among lists of threatened plant species, including all species in Appendices I and II of CITES [5] and 1601 species on the IUCN Global Red List [6]. Apart from their scientific fascination and significance, orchids are renowned for cut flowers and ornamental potted plants, accounting for a great part of the global floriculture trade [7]. Orchids also have long been commercialized as medicinal products and food [8], whereas breeding and mass production of commercially valuable orchids have long been hindered by a lack of scientific information on the

mechanism involved in orchid growth and flower inducement. Therefore, from the time of Darwin to the present day, there was a great deal of attention to this family in terms of its conservation, evolutionary relationship, and flower diversification. With the scientific tools now available for systematic research, there are emerging studies providing in-depth analysis addressing questions in orchids, with an increasing focus on comparative studies in the area of genomics. By identifying and validating functional genes, reconstructing phylogenetic relationships and performing genome-wide exploration, our understanding of the genetic basis of those unique characteristics of orchids has been fundamentally reshaped. The availability of the wealth of genomic information also stimulates further studies on molecular breeding, new varieties selection and secondary component production that could protect and restore the wild orchids but also develop orchid industrialization in a way that satisfies the demand for commercial uses sustainably.

This review summarizes the prominent strategies and approaches utilized in orchid research including molecular markers, sequencing technologies, transgenic and gene editing techniques, etc. with an enumeration of the studies leading to a major breakthrough in orchid biology such as orchid phylogeny, flower development and evolution of life forms. In addition, recent

advances in biotechnology applied to the orchid industry together with future prospects pertaining to the post-genomic research, conservation, and utilization of orchids are briefly discussed.

Orchid conservation

Over the past 10 years, novel technologies have advanced our understanding of orchids and promoted revolution in orchid research. As modern molecular biology and computer science become more commonplace, the rapid development of genetic techniques has facilitated the unprecedented resolution of uncertainties in orchid biology. These techniques were heavily applied to studies with conservation purposes, including taxonomy, systematics, examination of genetic variation level, effective population size, and mating patterns; namely, population genetics [9]. To visualize the latest knowledge domain and emerging trends of orchid conservation-related literature, CiteSpace [10] was employed to conduct a scientometric investigation (Fig. 1). Figure 1 is generated by time series and depicting the keywords of the research on orchid conservation. It's clear from the timelines that orchid conservation research has gradually shifted from descriptive theories and conventional practices to a high technology-based quantitative science, particularly in a direction toward molecular systematics. Notably, high-frequency keywords like 'phylogenetic analysis', 'biogeography' and 'conservation genetics' have occurred since 2013 (Fig. 1). Indeed, new molecular approaches have emerged in the past decades and will be the most powerful tool in providing new insights and understanding into orchid conservation.

Orchid classification and systematics

Due to frequent hybridization and introgression, delineating taxonomic boundaries of orchids can be very difficult [11, 12]. The resulting uncertainties in the taxonomy and conservation status of orchids can largely hamper effective conservation strategy-making. Nowadays, markers include amplified fragments length polymorphism (AFLP), microsatellite DNA, chloroplast genomes, single nucleotide polymorphisms (SNPs), and restriction-site-associated DNA sequencing (RADseq) have been widely used in systematic and taxonomic studies [13]. These approaches are likely to be most important for delimitating species and intergeneric relationships. More than 10 new recorded genera or new genera with dozens of new species have been published with molecular evidence for their distinctiveness, including new genera *Danxiaorchis* [14], *Hsenhsua* [15], *Shizhenia* [16], and *Yunorchis* [17], the newly recorded genus *Thaia* [18] and genera with phylogenetic replacement, *Cymbilabia* and *Mengzia* [19, 20]. Newly discovered species with support from a phylogenetic tree based on nuclear and plastid DNA markers were found in several genera including *Bulbophyllum*, *Paphiopedilum*, *Gastrodia*, *Dendrobium*, *Liparis*, and *Cymbidium* [21–27]. Although challenges still remain, there have been great strides in the use of molecular tools for the description of new taxa, and the rapid development of new markers can be expected to further expedite the process of orchid systematic studies.

Along with the species-level identification, orchid phylogenetics have now employed next-generation sequencing (mostly Illumina techniques) for understanding higher-level relationships. Given its small size, uniparental inheritance, conservative gene content, and great numbers in cells, plastid genome (plastome) has so far been the most important source of data for plant phylogeny [28]. Since the first plastid genome has been reported in *Phalaenopsis aphrodite* subsp. *formosana* [29], so far there are 642 complete plastid genomes for Orchidaceae in Genbank (accessed 3 May 2022). Indeed, extensive studies have also been done in developing orchid plastid phylogenomics as a well-resolved, strongly supported, time-calibrated phylogeny is fundamental for circumscribing species, tribes and genera. Since the first DNA data-based phylogenetic classification of Orchidaceae was published [30], there has been a great deal of progress in resolving relationships and problematic placements at higher levels. We have seen an exponential growth in plastid phylogenomics to resolve and clarify relationships across orchids, including a supermatrix tree based on 75 chloroplast genes for 39 species covering all orchid subfamilies 16 of 17 tribes [31]; reconstruction of phylogeny and temporal evolution of Orchidaceae based on 76 and 38 coding genes of plastid and mitochondrial genomes, respectively [32]; a first-time phylogenetic placement for Codonorchideae (Orchidoideae), Podochilieae and Collabieae (Epidendroideae) based on 78 plastid coding genes [33]; plastid phylogenomic resolution of subtribes Aeridinae [19] and Goodyerinae [34]; and genera phylogenetics such as *Dendrobium* [35], *Cymbidium* [36], *Holcoglossum* [37] and *Paphiopedilum* [38], suggesting plastome has been a mainstay for addressing finer-scale phylogenetic questions in orchid evolutionary studies.

In parallel with the examination of the plant's organelle genomes, opportunities are rising for using transcriptomic data for orchid phylogenetics. Several orchid phylotranscriptomics have been performed for addressing either broad-scale or shallow-scale orchid relationships including species diversification and genome evolution, as well as key traits like sexual deception [39–43]. These studies also give insights beyond phylogenetic analysis to uncover the morphological evolutionary histories of thousands of unlinked genes. Therefore, repurposing transcriptomic studies provides an additional approach and perspectives for orchid systematic studies. Collectively, drawing on these informative data for orchid classification and systematics has gained important insights into the mechanisms driving the extraordinary species diversity and served as an invaluable resource for conservation implications.

Population genetics

At the population level, identifying species that should be treated as a high priority for conservation is also struggling for conservation planning [44], thus highlighting the need for population genetic analysis. In orchids, unique traits like deceptive pollination and dust-like, wind-dispersed seeds have promoted gene flow between populations [45]. These reproductive strategies have led to significant heterogeneity in the genetic structure among orchid populations [46]. To investigate the population genetics of endangered orchids, marker-assisted approaches have been widely employed in the assessment of genetic diversity, genetic drift, level of inbreeding, and gaining insights into contemporary and historical dispersal for populations identified as being at risk. Genetic diversity studies for the populations of two critically endangered *Amitostigma* species were conducted to identify which entities to preferentially conserve and determine optimal conservation strategy [47, 48]. Studies on *Cypripedium* showed random genetic drift and limited gene flow have a great impact on its genetic diversity and population structure [49, 50], while distinct levels of genetic diversity occur in *Cypripedium* populations with different types of habitats and climates [51, 52]. The accumulation of ancestral variation and genetic admixture from multiple post-glacial colonization routes may explain the high genetic diversity of *Cypripedium* in central Europe, enabling the long-term stability of the species in different biogeographical regions
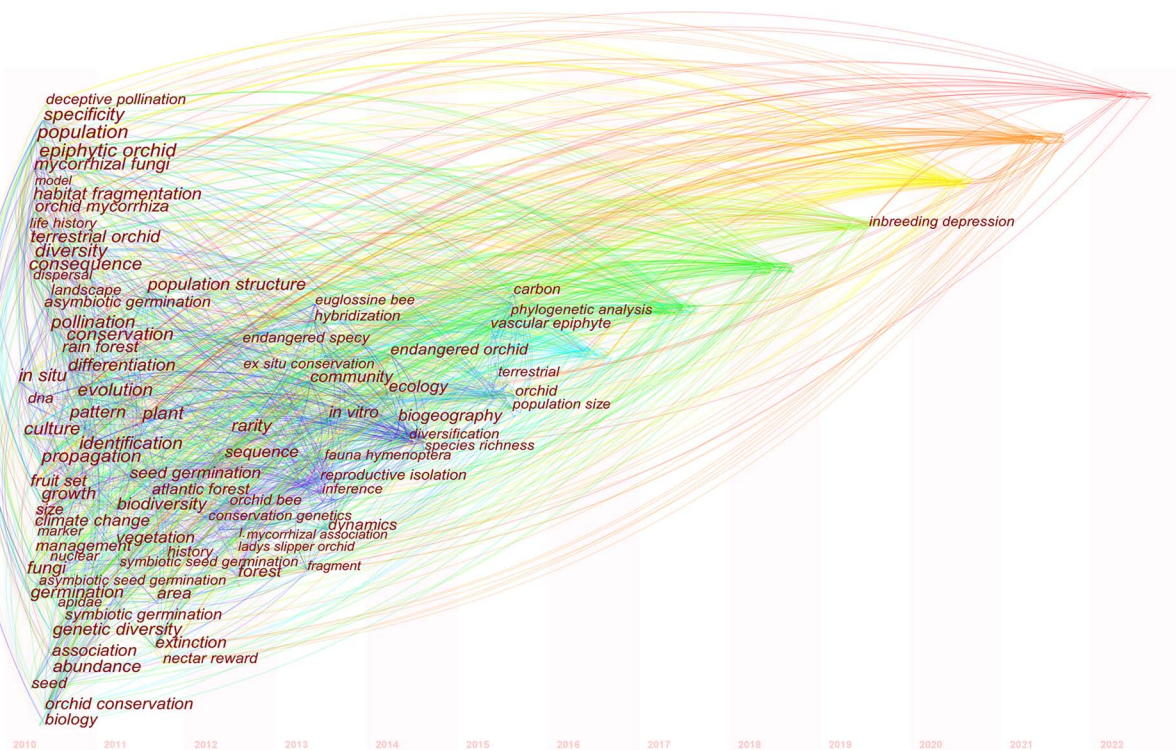
Figure 1. Knowledge mapping of orchid conservation. Visualization of keywords and their frequencies for orchid conservation by time series (2010–2022). Each node represents a keyword that first appears in the analysed data set. The published articles from 2010 to 2022 were retrieved from the Web of Science core collection.

[53, 54]. Anthropogenic disturbance accounts for the major factors driving the orchid habitat fragmentation and deterioration, especially for those thriving as epiphytes [55]. Population genetic structure analysis of an epiphytic orchid, *Bulbophyllum occultum* showed self-pollination and genetic drift have contributed to its high population genetic and phenotypic differentiation, despite it being impacted severely by deforestation [56]. In another case of a dominant clonal *B. bicolor*, low genotypic diversity and lack of spatial genetic structure led by skewed clonal reproduction have contributed to the loss of sex and extinction debt, which demands urgent conservation attention such as *ex situ* collection [57]. Orchids have specific adaptations to pollinators that could promote crossing and avoid inbreeding depression [58]. Several studies have investigated the relative fitness of the population resulting from selfing and outcrossing by using molecular markers. Genetic diversity and structure of *Cattleya* were evaluated by ISSR markers and the results showed that *Cattleya* populations undergo a drastic population decline while deceptive pollination and long-distance seed dispersal may help with higher genetic variability [59, 60]. Blambert *et al.* [61] reported the selfing rates and levels of genetic diversity for two closely related *Jumellea* species with different reproductive systems. The results showed that the selfing rate and magnitude of inbreeding depression are negatively correlated. A study that examined the population genetic patterns of the populations of *Platanthera praeclara* indicated that its small population size could lead to severe inbreeding depression [62]. Genetic diversity and population structure of *Calibrachoa* showed that species presenting inbreeding are more likely to suffer from a loss of alleles, resulting in a low level of population structure and diversity [63]. In a case that used double-digest restriction-site associated DNA (ddRAD) sequencing to evaluate the conservation status of three threatened species of *Corybas*, the gene structure results provided

evidence for hybridization and introgression within the *Corybas* complex, thus leading to blurred taxonomic boundaries [64].

Taken together, the reliability and efficiency of molecular tools have provided more opportunities for examining a wider spectrum of orchid population genetics, facilitating the development of genetic studies on more endangered orchids and the possibility of answering unresolved biological questions, thereafter guiding the conservation practices.

Orchid biology
Deceptive pollination
Orchids have evolved to diverse pollination mechanisms with an overrepresentation of floral mimicry (deceptive pollination) compared to other plants [65]. For a long time, ecological investigations were mostly adopted to understand the driving forces of orchid deception, while the knowledge gap remains to be filled between the underlying mechanisms of the evolution of mimicry and species diversification [66]. Several studies have performed novel and multidisciplinary approaches to provide new insights into this research area. For example, genus phylogeny suggested that the unidirectional transition in nectar presence/absence is associated with food deception, which is an 'evolutionarily stable strategy' in *Epidendrum* [67]. A study incorporated field experiments and chemical findings into phylogenetic analysis concluded that the sexually deceptive orchid *Chiloglottis* undergoes pollinator-driven speciation [68]. A similar attempt was reported in genera *Serapias* and *Iris* to provide evidence for evolutionary transitions between two different pollination strategies [69]. *Stearoyl-acyl carrier protein desaturase* (*SAD*), the first gene involved in the biosynthesis of semiochemicals for sexual deception was found and it's responsible for alkene difference between *Ophrys* species and pollinator-driven 'genic' speciation [70]. Despite the recent advances in our understanding of the orchid deception, it is evident that much

remains to be learnt. In light of the methodological progress, our capability will be greatly enhanced to examine the basis of pollination deception at an unparalleled depth.

Mycorrhizal associations

A common feature of orchids is their obligate mycorrhizal associations, which play crucial roles in orchid life cycle and population dynamics [71, 72]. Identification of orchid mycorrhizal fungi (OMF) has been extensively studied in at least 200 genera [73]. Most OMF belongs to three Basidiomycota lineages, namely Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae [74]. Moreover, orchid distribution is considered associated with OMF diversity [75], which is jointly regulated by environmental factors, spatiotemporal variations, biogeography, and the phylogenetic constraints of host orchids [72]. Multifaceted evidence from radiocarbon (Δ^{14}C), molecular markers and genomic data suggested that both fully mycoheterotrophic and partial mycoheterotrophic orchids obtain carbon from fungi [76, 77]. And the concept that OMF receives nothing in return has been overturned by a study that examined the expression of fungal and plant nitrogen (N) transport and assimilation genes, the results showed mycoheterotrophic orchid and its fungal partner have a mutualistic association [78]. Whereas disentangling the factors determining OMF specificity and molecular mechanisms of this symbiosis still represents a major challenge. Nevertheless, a broader investigation of OMF is needed to translate more orchid-fungi crosstalk, and the availability of fungal reference genomes [79] is expected to enable metagenomic studies to be implemented to address these questions. These studies will be especially important for the regeneration of rare and endangered orchids that link orchids and OMFs in efforts to inform conservation.

Crassulacean acid metabolism

CAM is a key innovation for orchids, representing a specialized drought-adapted photosynthetic pathway that enables plants to grow in the arid environment [80]. Several transcriptomic studies have been done to illuminate the origin and evolution of CAM pathway. PEPC is a key enzyme in CAM pathway, phylogenetic analysis of the PEPC family based on transcriptome data revealed that orchid *PEPC* genes originated from the ancient duplication of monocots, with CAM developed earlier than C4 [81]. Zhang *et al.* [82] reported a comprehensive comparison of carbon fixation pathway genes based on transcriptomic data of 13 orchid species, and revealed that CAM may have evolved primarily by changes in the transcription level of key carbon fixation pathway genes. Differentially expressed genes between circadian day and night were identified in *Pha. equestris*; these genes were mostly enriched in carbon fixation, circadian clock regulation, photosynthesis, and signal transduction pathways [83]. Other studies that employed physiological and biochemical measures to examine the diel dynamics of carbon gain and metabolites of CAM have provided more opportunities to study orchids' adaptation to dry environments, especially in response to projected global warming [84–86].

Whole genome sequencing

With the advent of sequencing techniques, we have witnessed a paradigm shift from microarray-based genotyping studies to whole genome sequencing. The adoption of third-generation sequencing approaches that generate longer reads (e.g. Pacific Biosciences HiFi [87], Pacific Biosciences SMRT [88], Oxford Nanopore [89]) and methods for chromosome conformation capture (e.g. Hi-C) have ushered the revolution of higher quality sequencing and assembly in genome contiguity and accuracy of plants, even for orchids that have large and complex genomes. To date, 16 orchid genomes encompassing four subfamilies (Apostasioideae, Vanilloideae, Epidendroideae, and Orchidoideae) have been published with some of them having updated versions of assemblies and annotations (Fig. 2; Table 1). These genome studies together with high-quality referenced transcriptomic data have given rise to major conceptual or technical breakthroughs of orchid research and overturned many classical views of orchid biology.

Pha. equestris was the first reported orchid genome that contributed to a greater understanding of orchid morphological evolution and physiological adaptation [3]. The highly sophisticated floral structures, epiphytism and CAM were considered pivotal traits that jointly promoted the rapid radiation of orchids. A chromosome level assembly of *Pha. aphrodite* showed that lineage-specific expansion of FRS-like subclade might be an adaptation to the unstable light condition that is associated with epiphytes [90]. The genome sequence of primitive orchid *Apostasia shenzhenica* represents a milestone in research on orchid genomics [91]. By reconstructing an ancestral orchid gene toolkit and evolutionary history of orchids within the angiosperms, together with the exhausted investigation on *Apo. shenzhenica* genome characteristics and transcriptome data covering all orchid subfamilies, this study provided significant insights into key innovations, origins, adaptation and diversification of orchids. Absolute dating of whole-genome duplication (WGD) event in *Apo. shenzhenica* showed a lineage-specific WGD in orchids which occurred shortly before the divergence of five subfamilies. Another interesting feature in orchids is that all orchids showed mycorrhizal fungi association (partial mycoheterotrophy) during seed germination and the early stage of protocorms [92]. Genomes of obligate mycoheterotrophic orchids were reported in two species in *Gastrodia* [93–95] and *Platanthera guangdongensis* [77]. Both studies revealed contracted plastid genomes and substantial loss of genes involved in photosynthesis of fully mycoheterotrophic orchids, while elevated expression of trehalase could be a critical adaptation for mycoheterotrophy, enabling orchids to hijack trehalose from fungi and resynthesize it as sucrose for internal use [77].

For thousands of years, the active compounds in orchids have been used in traditional medicine or as health food supplements. These ancient remedies have now been scientifically scrutinized at the molecular level. *Dendrobium* is the second largest genus in Orchidaceae and is renowned for medicinal use in Asia. Expansion of resistance-related genes and highly expressed polysaccharide synthase gene families in the *Dendrobium* genome suggested a powerful immune system and high tolerance to environmental stress that contributes to its wide distribution [96]. An updated version of the chromosome-level *Dendrobium* genome combined with genome-wide association studies (GWAS) revealed several genes associated with the biosynthesis of active ingredients and stem production in *Dendrobium* species [97]. Another high-quality chromosome level *Dendrobium* genome, *D. chrysotoxum* provided important insights into the interplay among carotenoid, abscisic acid (ABA), and ethylene biosynthesis, demonstrating the regulatory mechanisms of the relatively short flowering period of yellow-flower orchids [98]. The first haplotype-resolved genome assembly using Pacbio HiFi sequencing of orchid is completed in *Bletilla striata*, a traditional medicinal herb in China [99]. The study reconstructed the ancestral karyotype (18 chromosomes) of seven orchids and functionally verified the key transcription factor (MYB) involved in polysaccharide biosynthesis, laying a foundation for molecular-assisted breeding for orchids with high

Table 1. Whole genome sequences of reported orchids species.

| Subfamily	Species	Sequencing technique	Genome size (Gb)	Contig N50 (Mb)	Scaffold N50 (Mb)	Protein-coding genes	Repetitive sequences (%)	Assembly BUSCO (%)	Publications
Apostasioideae	*Apostasia shenzhenica*	Illumina+PacBio	0.35	0.08	3.03	21 841	42.05	92.70	[91]
	Apostasia ramifera	Illumina	0.37	0.03	0.29	22 841	44.99	92.30	[221]
Vanilloideae	*Vanilla planifolia*	Illumina+Nanopore, Hi-C	1.48	0.40	42.00	29 167	44.30	87.60	[100]
	Vanilla planifolia[a]	PacBio HIFI	A: 1.50 B: 1.90	0.92	1.20	59 128	72.00	83.00	[101]
Orchidoideae	*Platanthera zijinensis*	Illumina+PacBio, Hi-C	4.19	1.77	192.35	24 513	77.38	88.66	[77]
	Platanthera guangdongensis		4.20	1.57	193.14	22 559	82.18	72.06	
Epidendroideae	*Gastrodia elata*	Illumina	1.03	0.07	4.90	18 969	66.18	72.40	[94]
	Gastrodia elata	Illumina+PacBio, Hi-C	1.04	21.33		21 115	66.36	62.10	[95]
	Gastrodia elata	PacBio, Hi-C	1.05	9.18	50.60	18 844	74.92	72.60	[222]
	Gastrodia menghaiensis	Illumina+PacBio, Hi-C	0.86	2.37	6.82	14 233	62.57	71.40	[93]
	Phalaenopsis equestris	Illumina	1.09	0.02	0.36	29 431	62.00	89.10	[3]
	Phalaenopsis aphrodite	Illumina	1.20	0.02	0.95	28 902	60.30	91.90	[90]
	Dendrobium catenatum	Illumina	1.01	0.03	0.39	28 910	78.10	89.10	[96]
	Dendrobium huoshanense	Illumina+PacBio, Hi-C	1.29	0.6	71.79	21 070	74.92	87.40	[223]
	Dendrobium chrysotoxum	PacBio, Hi-C	1.37	1.54	67.80	30 044	62.81	90.30	[98]
	Bletilla striata[a]	PacBio HIFI, Hi-C	A: 2.37 B: 2.43	A: 1.65 B: 1.66	A: 146.39 B: 150.22	26 673	/	A: 97.52 B: 97.24	[99]
	Cymbidium sinense	Illumina+Nanopore, Hi-C	3.52	1.11	/	29 638	77.78	87.80	[102]
	Cymbidium goeringii	Illumina+PacBio, Hi-C	4.10	1.04	209.04	29 272	77.65	85.40	[103]
	Cymbidium goeringii	Illumina+PacBio, Hi-C	3.99	0.38	178.20	29 556	88.87	87.80	[224]
	Cymbidium ensifolium	Illumina+PacBio, Hi-C	3.62	1.21	154.88	29 073	80.58	87.00	[104]

[a] A, haplotype-A, B, haplotype-B
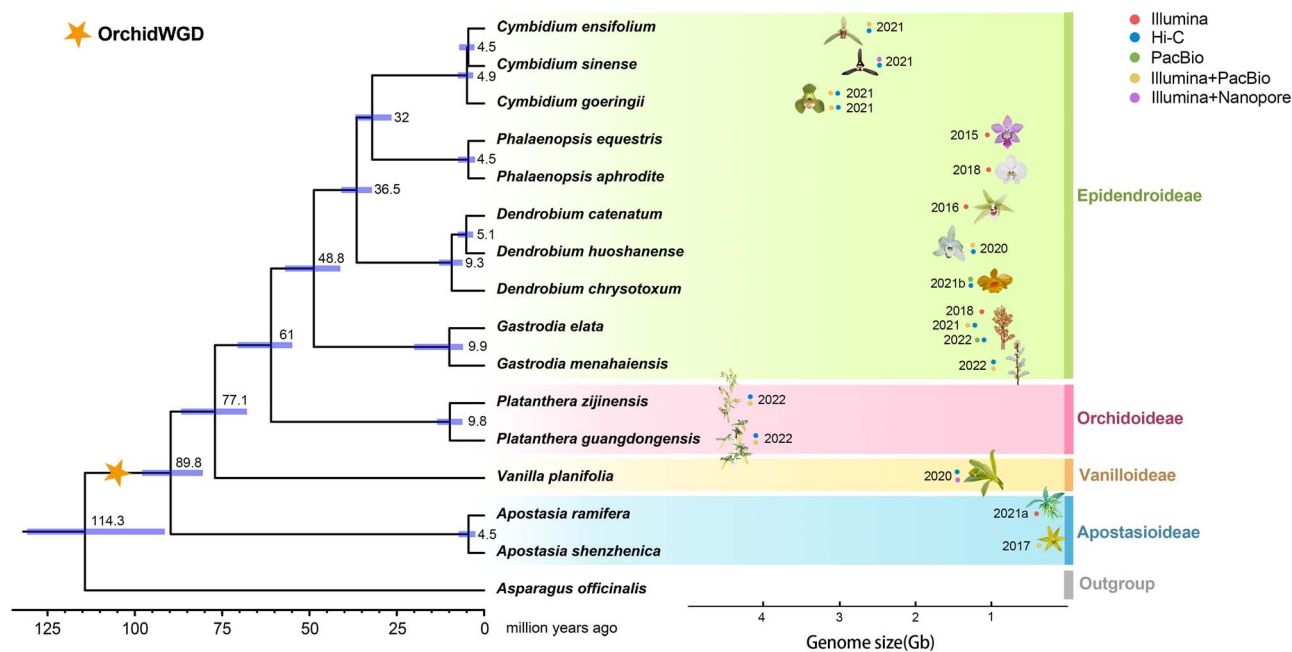
Figure 2. Phylogenetic relationship and divergence time of 15 published orchid genomes. Maximum likelihood phylogenetic tree was constructed using single-copy orthologous sequences from 15 published orchids covering four subfamilies, with *Asparagus officinalis* as an outgroup. Divergence time was calculated by MCMCTree in PAML v.4.9 package. The orange star indicates the lineage-specific WGD (Orchid WGD) event for Orchidaceae. Subfamilies are listed to the right of the tree. Sequencing techniques for each study were marked in dots with different colors.

medicinal values. Vanilla, a stunning spice that is mostly derived from *Vanilla planifolia*'s pods, is now ubiquitous in worldwide food and beverage production. A chromosome-scale genome assembly of *V. planifolia* was reported to provide a better understanding of the genes associated with vanillin pathway, which will enable accelerated breeding of vanilla pods with higher quality and productivity [100]. The other chromosome-level, haplotype-phased *V. planifolia* genome based on Pacbio HiFi sequencing was completed to address the partial endoreplication of *V. planifolia* and take a step forward to further elucidate this complex genome [101].

Orchids exhibit specialized and complex floral structures that led to marvelous species richness; these unique traits play an instrumental role in the course of orchid evolution. A comprehensive inspection of floral shape, color, and scent has been conducted in *Cymbidium*, a genus with vital commercial importance in the world floriculture industry. Three *Cymbidium* genomes (*C. ensifolium*, *C. sinense*, and *C. goeringii*) unveiled the important genetic clues to phenotypic traits such as colorful leaves, diversified flowers, and fragrance, which are primarily regulated by MADS-box, MYB, and TPS gene families, respectively [102–104]. Furthermore, changes in gene number and gene expression can affect flower morphogenesis by altering floral structure and color that foster a variety of mutants, providing great potentials for orchid molecular breeding and floriculture.

In a nutshell, new sequencing technologies are producing genome assemblies of increasing quality, deciphering orchid genomes can provide a comprehensive catalog of genomic information that could empower the studies of important traits and evolutionary mechanisms for the breadth of species with significant ecological and evolutionary importance.

Orchid database

The emergence and availability of massive sequence data have opened new interfaces with computer science, allowing the establishment of multiple orchid databases. OrchidBase was established in 2011 and has now updated to version 5.0, which accommodates whole-genome sequencing and transcriptomic data for *Apo. shenzhenica*, *D. catenatum* and *Pha. equestris*, and floral transcriptomic sequences from 10 orchid species covering all five subfamilies [105] (http://orchidbase.itps.ncku.edu.tw/). Orchidstra 2.0 [106] (http://orchidstra2.abrc.sinica.edu.tw) is another orchid database for transcriptomics resource that includes orchid transcriptome assembly and gene annotations of 18 orchid species belonging to 12 genera across five subfamilies. Other databases like OOGB (http://predictor.nchu.edu.tw/oogb) and PhalDB [107] also provided quantities of sequence information about the genome, transcriptome, and miRNA data of *Oncidium* and *Phalaenopsis*, respectively. These datasets provide the capacity and platform for rapid data mining, generation, and analysis. The aggregation and navigation of different types of orchid data enable the broader biology community to access orchid bioinformatics and perform genome-wide analysis with a lower level of computational skills.

Genome-wide studies

On the basis of large-scale whole-genome data, genome-wide identification and comparative study of a multiplicity of gene families have been conducted in several orchids. Recent publications have reported gene families like YABBY, terpene synthase (TPS), R2R3-MYB, KNOTTED1-like homeobox (KNOX), WRKY, autophagy (ATGs), and MADS-box [108–115] and their expression analysis in *Dendrobium*, *Cymbidium*, and *Phalaenopsis*. Phylogenetics, physicochemical properties, and comparative transcriptomics were performed to identify key genetic basis and molecular mechanisms determining the organogenesis and morphogenesis of orchids. Also, from a perspective of floriculture development, most economically important traits are usually inherited in a quantitative manner such as floral scent and color.
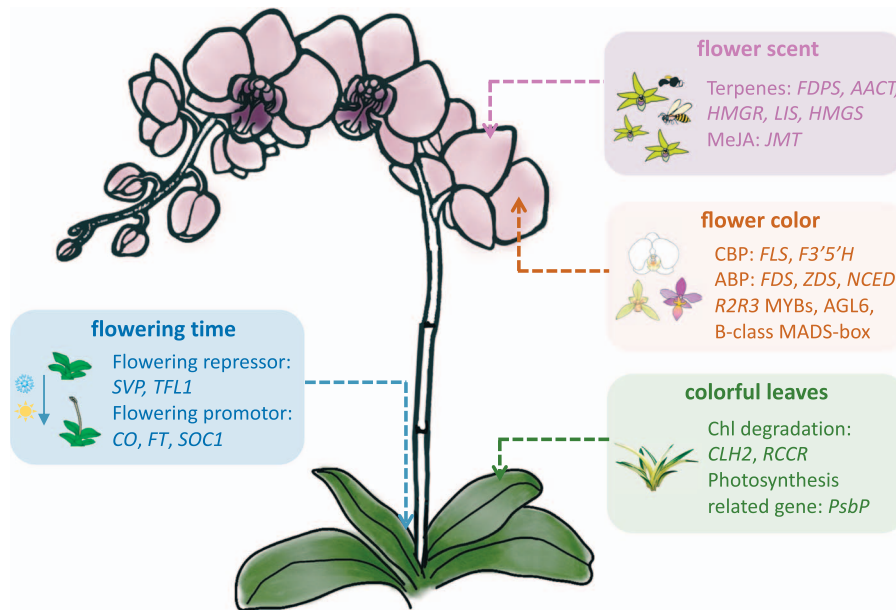
Figure 3. Genes related to key horticulture traits of orchids. Genes involved in floral scent biosynthesis include *FDPS*, *AACT*, *HMGR*, *LIS*, *HMGS*, and *JMT* in methyl jasmonate (MeJA) biosynthesis. Genes regulating chlorophyll degradation such as *CLH2* and *RCCR*, and photosynthesis-related genes *PsbP* are responsible for orchid colorful leaves. Flower color is mainly determined by genes involved in carotenoid biosynthesis (CBP) and anthocyanin biosynthesis pathway (ABP), as well as TFs like R2R3 MYBs, AGL6, and B-class MADS-box. The transition from vegetative development to reproductive growth requires the mediation of multiple flowering repressors (*SVP*-like, *TFL1*-like genes) and promotors (*CO*-like, *FT*-like, *SOC1*-like genes).

Therefore, genome-wide associate studies can contribute to the discovery of candidate genes associated with these important traits. Genotyping-by-sequencing (GBS) approach based on Illumina sequencing has been applied to study four sexually deceptive orchids of *Ophrys*. Highly differentiated polymorphisms were found in genes that are involved in floral scent, including several SNPs linked to pseudo-pheromones related genes [116]. GWAS was performed by combining SNPs and floral aesthetic traits to identify QTL that is associated with color-related traits in *Phalaenopsis* [117]. The results indicated 10 quantitative trait loci and 35 candidate genes that were associated with color-related traits and anthocyanin biosynthesis, respectively, providing important selection markers for *Phalaenopsis* breeding.

Genes associated with important traits

The development of whole genome sequencing, *de novo* transcriptome and molecular toolkit for functional analysis have allowed identification, quantitative comparison and functional characterization of genes related to important traits in orchids. Key genes and transcription factors (TFs) involved in floral pattern, flowering time, coloration, floral scent, colorful leaves, and other important properties were identified and validated (Fig. 3). These studies provide nearly unlimited possibilities in orchid research that could translate complex orchid biology to quantitative information and lay a solid foundation for orchid molecular breeding.

Floral patterning

Floral homeotic genes that encode MADS-box transcription factors play crucial roles in flower development, of which type II MADS-box genes are renowned for their roles in the specification of floral organ identity [118]. Most orchids display a zygomorphic flower with a distinguished lip in the second whorl, this particular floral architecture presents an exciting opportunity to examine the classical ABCDE model of flower development. Since the 'Orchid Code model' [119], 'HOT model' [120] and 'Perianth code'

(P code) [121] have been proposed, a wide-ranging exploration of transcriptome and expression profiles of *Cymbidium* species and their mutants has been conducted to investigate the flower formation of orchids [122–125]. The results from the transcriptomic based analysis were further verified by whole genome data of *C. ensifolium* [104], *C. sinense* [102] and *C. goeringii* [103], which suggested that B- and E- class MADS genes play fundamental and dualistic roles in determining perianth formation while C- and D- class are involved in carpel and gynostemium (column) development (Fig. 4). To better understand the characteristics of ABCDE genes among different lineages, we identified and classified the MADS-box genes for all available orchid genomes (Table 2). Gene duplication in type II MADS genes seems common in orchids [3, 104] and most ABCDE clades look similar in gene numbers between closely related species, while some of them have extremely higher or lower copies (e.g. Bs in *C. ensifolium* and C/D in *G. elata*). In *Apostasia*, fewer B-AP3 and E class genes are considered to form an undifferentiated lip and partially fused column, making it an actinomorphic feature [91], whereas due to the dearth of knowledge pertaining to orchid flower development, the evolutionary buildup of gain and loss of ABCDE genes remains to be investigated. Notwithstanding, an increasing number of functional studies have been done to elucidate the function of these floral organ identity genes. Ectopic expression and virus-induced gene silencing (VIGS) were performed to investigate *SEPALLATA* (*SEP*) genes in determining floral organ identity of *Phalaenopsis* [126]. The model of perianth formation in orchids has been validated by the suppression of L complex activity in lips in *Oncidium* and *Phalaenopsis*, demonstrating *AGAMOUS-LIKE6* (*AGL6*)-like MADS-box genes are exclusively required for lip development, a specialized petal for most orchids [121]. Ectopic expression of *PeMADS28*, a Bs gene of MADS-box gene family in *Arabidopsis* can lead to abnormal ovule development, indicating the conserved function of Bs in ovule integument development [127]. The transgenic *Dendrobium* was generated for elucidating the functions of C- and D-class MADS-Box genes, in which two *AGAMOUS* (*AG*)

genes, *DOAG1* and *DOAG2* were downregulated by microRNA inter-ference [128]. The result showed that both genes exert different roles in specifying reproductive organ identity, *DOAG1* affects floral meristem determinacy and floral organ development, while *DOAG2* regulates perianth and gynostemium (column) develop-ment. A study based on VIGS and transient overexpression of *Pha. equestris* revealed that two *DROOPING LEAF/CRABS CLAW* (*DL/CRC*) genes play an important role in the innovation of orchid reproductive organs [129]. Transcriptome analysis combined with yeast two-hybrid assays of a greenish flower mutant of *Habenaria radiata* showed that this phenotype is caused by loss of function of an E-class MADS-box gene (*HrSEP-1*), which plays an important role in column, lip, and petal development [130]. In addition to MADS-box genes, it was also found that overexpression of miR390, a miRNA involves in diverse processes of plant growth and development from *C. goeringii* to transgenic *Arabidopsis* can alter normal reproductive organ development [131]. Valoroso *et al.* [132] found that *DIV*, *RAD*, and *DRIF* in the MYB gene family might be involved in the establishment of flower bilateral symmetry of *Orchis italica*, an orchid with resupinate flowers. Other regulatory mechanism includes the coordinated signaling of phytohormones such as ethylene, auxin, GA, and ABA also play important roles in orchid floral organ development [133,134]. All these studies specify that the diverse roles of MADS-box genes and other floral identify regulators have contributed to the specialized flower morphogenesis of orchids.

Floral color

Anthocyanin and carotenoid are major pigments that depict flower colors and key TFs such as MYBs play prominent roles in their biosynthesis [135]. In *Phalaenopsis* cultivars, the differential expression of *PeMYB2*, *PeMYB11*, *PeMYB12* was concomitant with complicated floral pigmentation patterning [136]. Transient overexpression of these *R2R3-MYBs* in *Phalaenopsis* verified that they can promote anthocyanin accumulation, and these *PeMYBs* could activate the expression of downstream structural genes. VIGS and transient overexpression of PeMYB4L, a member of R2R3-MYB verified its role in regulating anthocyanin biosynthesis in *Phalaenopsis* [137]. In *Cattleya*, transient overexpression of its two *R2R3-MYBs* (*RcPCP* and *RcPAP*) in a *Phalaenopsis* hybrid showed elevated expression of structural genes involved in carotenoid biosynthesis and anthocyanin biosynthesis, respec-tively, resulting in yellow or red pigmentation in *Phalaenopsis*'s perianth [138]. The insertion of a retrotransposon, *Harlequin Orchid RetroTransposon 1* (*HORT1*) in *PeMYB11* enhanced its expression and resulted in high accumulation of anthocyanins in harlequin flower [139].

It's also widely acknowledged that MYB–basic-helix–loop–helix (bHLH)–WD40 repeat (WDR) (MBW) ternary complex are key reg-ulators determining floral pigmentation and patterning [140]. Comparative transcriptomics of *Pleione* showed a MBW protein complex formed by *PlMYB10*, *PlbHLH20*, or *PlbHLH26* and *PlWD40–1* can repress the expression of *flavonol synthese* (*FLS*), the key structure gene in the anthocyanin biosynthesis pathway that contributes to color formation, resulting in color variations in wild populations [141]. Transient overexpression of a bHLH protein PeMYC4 plus a R2R3 MYB PeMYB4L showed a negative regulation of the anthocyanin accumulation by inhibiting the expression of structure gene *chalcone synthase* (*CHS*) [137]. Characterization of the structure genes involved in the anthocyanin biosynthesis has also been extensively studied in orchids. Metabolite profil-ing and transcriptomic analysis of sexually deceptive *Chiloglottis* showed downregulation and upregulation of *FLS* at young and

mature bud stages, respectively, contributing to the stark color contrast between callus and lip, which enhances the visibility of the mimicry [142]. In *C. kanran*, transient expression of three structure genes *CHS*, *dihydroflavonol reductase* (*DFR*), and *antho-cyanidin synthase* (*ANS*) caused purple-red pigmentation in white *C. kanran* flower [143]. For *Paphiopedilum hirsutissimum*, two structure genes (*F3H* and *CHS*) involved in anthocyanin biosynthesis and major carotenoid biosynthesis genes including *VDE*, *NCED*, and *ABA2* were strongly downregulated in albino phenotype com-pared to normal flowers, suggesting the color variation of *Pap. hirsutissimum* is a result of changes in anthocyanin and carotenoid contents [144]. Anthocyanin biosynthesis is also modulated by several phytohormones including ethylene, cytokinins, and ABA [145–147]. In orchids, two transcriptome-based analysis in *Den-drobium* showed auxin, ABA, and ethylene play various modu-lating roles in flower color formation [148,149]. Whereas the direct association between color formation and phytohormones, in the presence of other regulators that lead to the activation of anthocyanin biosynthesis remains unknown in orchids. Apart from MYB TFs, on the basis of P code in orchids, Hsu *et al.* [121] found that Class B- and *AGL6* MADS-box genes have additional functions than determining floral identifies, in which B and AGL6 proteins form L (OAP3–2/OAGL6–2/OPI) and SP (OAP3–1/OAGL6–1/OPI) complexes that regulate pigmentation of *Phalaenopsis*'s perianth. Also, *AP3/AGL6* genes may act together with *RcPAP1/2*, *RcPCP1* in shaping the spatiotemporal pattern of *Cattleya*'s color formation, further supporting the MADS-box genes' potential role in regulating color differentiation of flower segments [138].

Floral scent

Floral scent is one of the most important signals to attract pollinators, especially for some sexually and food deceptive orchids [150, 151]. On the other hand, fragrant orchids are also coveted commodities in the global orchid market. Studies on orchid floral scents have mainly focused on the characterization of genes encoding the key enzymes responsible for the synthesis of volatile compounds such as terpenes and methyl jasmonate (MeJA) [152]. Volatile terpenoid metabolism genes including *farnesyl diphosphate synthase* (*FDPS*), *acetyl-CoA C-acetyltransferase* (*AACT*), *hydroxymethylglutaryl-CoA reductase* (*HMGR*), *linalool synthase* (*LIS*), and *3-hydroxy-3-methylglutaryl-CoA synthase* (*HMGS*) were found to be responsible for floral scent of *C. goeringii* [103, 153]. In *C. faberi*, *jasmonic acid carboxyl methyltransferase* (*JMT*), an important gene in MeJA biosynthetic pathways was highly upregulated in blooming flowers [154]. Similarly, MeJA biosynthesis-related genes were found exhibited maximal expression levels in opened flowers of *C. ensifolium* [155]. In terms of floral scent regulation, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) TF was reported to regulate diurnal emission of floral scent in *Oncidium* [156] and diurnal scent emission of *Pha. violacea* was positively regulated by circadian clock and light factors which are associated with structural genes and TFs involved in monoterpene biosynthesis [157]. Functional characterization of orchid floral scent has been investigated in *Dendrobium*, *Phalaenopsis*, and *Cymbidium*. In *D. officinale*, genes involved in geraniol synthase (GES) were transiently expressed in *Nicotiana benthamiana*'s leaves, resulting in the accumulation of geraniol *in vivo* [158]. Functional analysis of several TFs (BHLH, Ethylene Response Factor (ERF), NAM, ATAF, and CUC (NAC)) and *TPS* genes involved in monoterpene biosynthesis was performed for *Pha. bellina*, indicating they can induce monoterpene production in scentless orchids [159, 160]. *TPS* genes of *C. faberi* have also been ectopically expressed in *Escherichia coli*, with β-myrcene, geraniol,

Table 2. Numbers and categories of MADS-box genes in published orchid genomes.

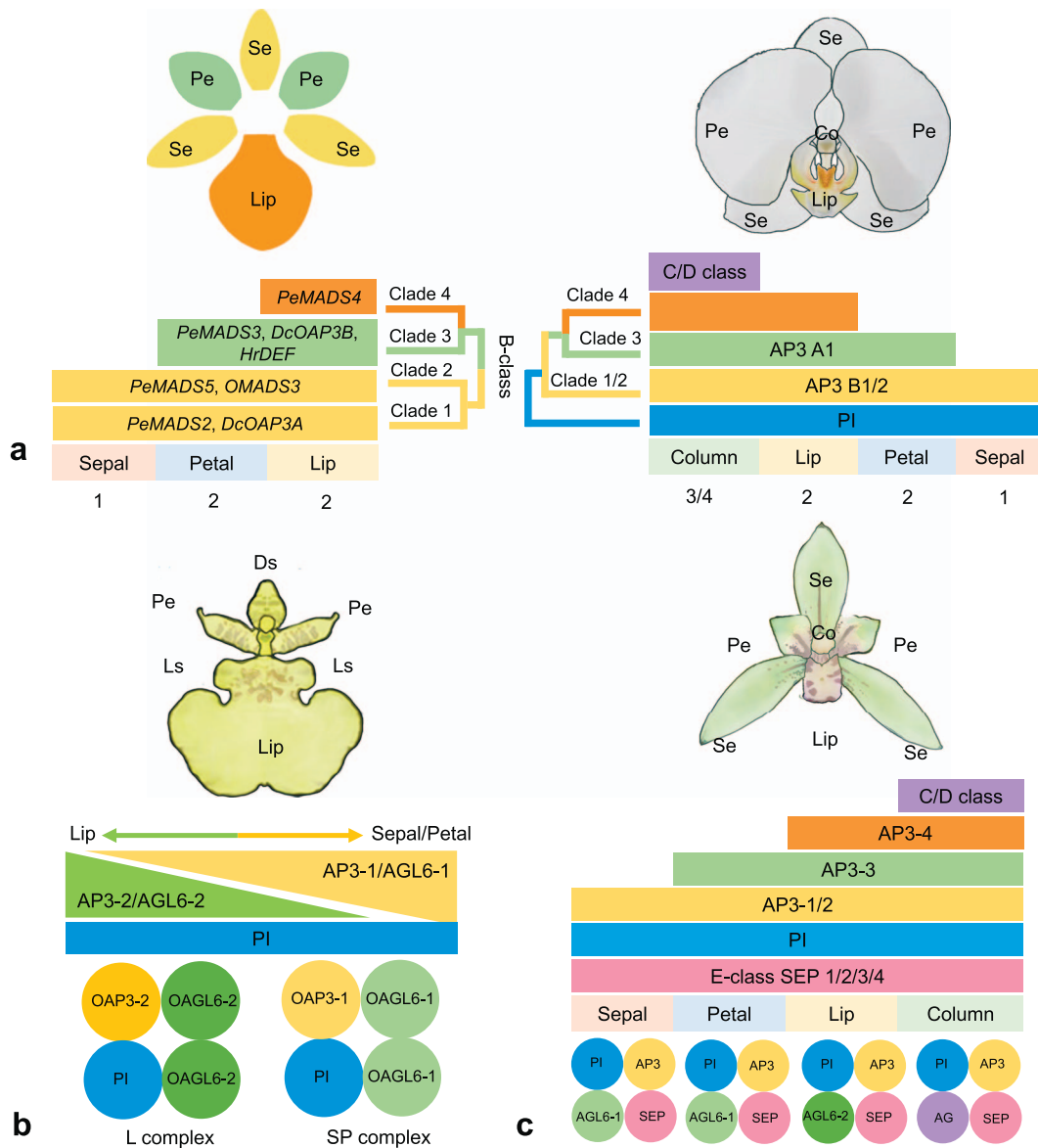| Category	Apostasia shenzhenica	Apostasia ramifera	Vanilla planifolia	Platanthera zijinensis	Platanthera guangdongensis	Gastrodia elata	Phalaenopsis equestris	Phalaenopsis aphrodite	Dendrobium catenatum	Dendrobium chrysotoxum	Cymbidium ensifolium	Cymbidium goeringii	Cymbidium sinense
Type II (Total)	27	23	39	29	27	27	29	45	35	32	38	44	42
MIKCc	25	22	36	27	25	27	28	34	32	28	34	34	41
A	2	2	6	3	3	3	3	3	4	4	4	4	6
Bs	1	1	1	1	1	2	1	1	2	4	7	1	6
B-PI	1	1	1	2	2	3	1	1	1	2	1	1	1
B-AP3	2	2	3	4	4	2	4	4	4	3	4	4	4
C/D	4	3	5	5	5	1	5	7	4	4	4	4	4
E	3	3	2	3	3	3	6	6	5	5	4	6	4
AGL12	1	1	0	1	0	0	0	0	0	1	0	0	0
SOC1	2	0	2	1	1	4	2	2	2	1	3	4	4
SVP	2	3	5	1	1	0	1	2	3	1	2	4	3
ANR1	4	4	4	2	1	4	2	2	3	1	1	1	2
OSMADS32	1	0	3	1	1	0	0	1	1	1	1	1	1
AGL6	2	1	3	3	3	5	3	5	3	1	3	4	6
FLC	0	0	0	0	0	0	0	0	0	0	0	0	0
AGL15	0	1	1	0	0	0	0	0	0	0	0	0	0
MIKC*	2	1	2	2	2	0	1	3	3	4	4	6	1
Type I (Total)	9	7	16	14	16	13	22	9	28	26	33	30	53
Mα	5	5	12	11	14	8	10	3	15	19	27	26	28
Mβ	0	0	0	0	0	0	0	0	0	0	0	0	17
Mγ	4	2	4	3	2	5	12	4	13	7	6	4	8
Total	36	30	55	43	43	40	51	54	63	58	71	74	95

Figure 4. The proposed 'Orchid Code', 'Homeotic Orchid Tepal' (HOT) model, 'Perianth code' (P code) and *Cymbidium ensifolium*'s MADS-box model for flower development of orchids. **a** Orchid code (left) and HOT model (right). In 'orchid code', clades 1–4 of B-class *DEF/AP3*-like genes specify the orchid perianth formation, upon which 'HOT model' proposed that *PI* and *AP3B* clades of B-class MADS-box genes determine all four whorls of flower identity. The joint contribution of *PI* and both *AP3A1* and *AP3Bs* regulate the formation of lateral petals. Both *PI* and *AP3* clade genes are involved in the formation of the lip. C- and D-class MADS-box genes specify column development. **b** Perianth code based on *Oncidium*. For 'P code', *PI* is expressed in lip, sepal and petal and interacts with different AP3-like and AGL-like proteins leading to lip or sepal/petal development. OPI combines with OAP3–2 and two OAGL6–2 to form L complex (Lip program) that determines lip development. SP complex (Sepal/Petal program) is composed of OPI, OAP3–1, and two OAGL6–1, regulating sepal and petal development. **c** MADS-box model of *Cymbidium ensifolium*. In *C. ensifolium*, E-class *SEP*-like and B-class *PI* and *AP3*-like genes are expressed in all flora whorls with C-and D- class genes expressed in column. Co, column; Ds, dorsal sepal; Ls, lateral sepal; Pe, petal; Se, sepal. Numbers 1, 2, 3/4 represent flower whorls.

and *α*-pinene as the main product catalyzed by *CfTPS18* [112]. Still, the type, regulation, and function of volatile compounds biosynthesis require further studies in orchids. Further progress in transcriptomic and functional studies may lead to more solid findings for revealing the molecular basis of orchid floral scent.

Colorful leaf

Colorful leaf is another ornamental character for orchids. Knowledge of the underlying mechanisms that give rise to colorful leaves could help with the effective selection of desirable leaf traits. Leaf color is primarily determined by pigments such as chlorophyll and anthocyanin, mutation or different expression of genes associated with their biosynthesis and metabolism may

lead to leaf color variations [161]. Differential expression of genes involved in the chlorophyll biosynthesis and degradation was found responsible for yellow- or silver-leaf phenotype in *Cymbidium* [104, 162, 163]. Comparative transcriptomic analysis between normal and yellow color-mutant leaves of *C. sinense* 'Dharma' showed that *chlorophyllase (CHL2)* and *red Chl catabolite reductase (RCCR)* which are key genes involved in chlorophyll degradation were highly expressed in the yellow-leaf mutant [164]. In *C. sinense* 'Red Sun', metabolic changes during the red to green leaf color transition were qualitatively and quantitatively analysed [165]. UPLC-MS/MS and PCR results showed that decreasing levels in 15 metabolites associated with anthocyanins biosynthesis, together with the down-regulation of genes encoding anthocyanin

synthesis contribute to the leaf variegation of *C. sinense* 'Red Sun'. Purple leaves mutant of *D. biggibum* leaves is considered associated with the increased expression of MYB2, and the transient expression of *DbMYB2* in *N. benthamiana* showed elevated expression of endogenous anthocyanin genes and thereby the increasing anthocyanin levels [166]. A transcriptomic study based on two *Paphiopedilum* species with green and tessellated leaves revealed that their differentially expressed genes were mostly enriched in processes like chloroplast, cytoplasm, thylakoid membrane, and nucleus [167]. For a *Pha. aphrodite* mutant with leaf variegation, the functional deficiency of PHOTOSYSTEM II SUBUNIT P (PsbP) protein, the extrinsic subunit of the photosystem II, is considered to play an important role in the formation of leaf variegation [168].

Flowering time

Vegetative-to-reproductive transition is a key phase for orchid development and flowering regulation is critical in commercial orchid production. The floral transition of orchids is influenced by environmental cues such as ambient temperature and photoperiod [169], endogenous signals including phytohormones like gibberellic acid (GA) [170] and ABA [171], as well as genetic factors. The regulatory mechanism of orchid flowering varies in different species due to differing growth conditions while several flowering-related genes, either flowering promoters or repressors that have been functionally identified in *Phalaenopsis*, *Dendrobium*, and *Oncidium*, etc. show a similar pattern to affect flower transition [172–176]. It has been reported in *C. goeringii* and *Phalaenopsis* that ectopic overexpression of *FLOWERING LOCUS T* (*FT*) gene can lead to an earlier flowering phenotype in transgenic *Arabidopsis*, with significant upregulation of other flowering time-related genes [177, 178]. The floral meristem identity gene *LEAFY* accumulates high transcript levels in the floral meristem primordia of *Pha. aphrodite*, and its overexpression in rice results in precocious headings [174]. Also, the VIGS-*PhapLFY Pha. aphrodite* showed abnormal flower phenotypes like reduced pigmentation and morphological alterations in cell epidermis. Other orchid flowering promoters include *CONSTANS* (*CO*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*) can also lead to early-flowering phenotypes in different types of transgenic plants examined [179–181]. While overexpression of *SHORT VEGETATIVE PHASE* (*SVP*) in *C. sinense* can significantly inhibit the expression of *FT*, *SOC1* and *APETALA1* (*AP1*) [102]. *Phalaenopsis SVP* also showed negative regulation in floral transition by repressing the expression of *PhaFTs* [182]. Another flowering repressor *TERMINAL FLOWER1* (*TFL1*) plays an antagonistic role with *FT* in repressing flowering, and the overexpression of *TFL* in *Dendrobium* causes delayed flowering and defective floral traits [175, 183].

Moreover, TFs such as MADS-box and zinc finger are thought to play an important role in mediating multiple signaling pathways in orchid flowering [184, 185]. miRNAs are also suggested to be involved in flowering regulation by targeting key genes that function in vegetative to reproductive transition [186–188]. In sum, the current understanding of the regulatory network of orchid flowering is based on the well-characterized genes from model plants, novel regulating factors of floral transition remain to be investigated. Also, the next challenge is to establish reliable protocols to make alterations in the flowering time of orchids to meet the market demand.

Other properties

Genes related to other phenotypic traits or biological processes have been characterized in orchids. For example, secondary metabolites are critical in plant physiology in terms of stress and disease resistance. Functional verification of C-glycosyltransferase (CGT) genes was conducted in *D. catenatum*, which could specifically catalyze O-glycosylation (OGT) that helps drought resistance [189]. *Phalaenopsis* is distinguished by having long-lasting flowers; this feature is determined by the presence of cuticular waxes in perianth [190]. Ectopic overexpression of *R2R3-MYB* genes (*PaMYB9A1* and *PaMYB9A2*) of *Pha. aphrodite* in transgenic tobacco gives rise to a shiny leaf phenotype, indicating these two genes act in regulating the biosynthesis of cuticular wax [191]. WRKY and ARFs gene family have been functionally studied for *C. goeringii*, and their expression in transgenic *Arabidopsis* was highly upregulated after ABA and Indoleacetic acid (IAA) treatment, respectively, suggestive of their roles in response to abiotic stress [192, 193]. These functional genomics studies have elevated the possibilities to verify genes associated with numerous regulating networks and link them with desired traits, thus providing valuable resources for orchid molecular breeding and industrialization.

Biotechnology-aided orchid production

Orchids exhibit several merits by having the elegant appearance, extended longevity, and varieties of cultivars that thrived in floriculture and gained widespread popularity among growers and consumers. Orchid has taken a significant position in the flower market, constituting more than 10% of the international pot plant trade with a 3.0% average annual growth in global import of cut flowers [7]. In addition to ornamental value, orchids such as *Dendrobium*, *Gastrodia*, and *Bletilla* have long been cultivated for medicinal use in Asia and Europe [194], and some *(Vanilla)* have been used for human consumption as flavorings and beverages [8]. Although remaining on a small scale, extracts of orchids have also been gradually applied and traded in the cosmetic, personal care products, and fragrance industries [195]. The significant increase in production and consumption of orchids is largely owing to the utilization of advanced breeding and cultivation techniques. Over the past two decades, tissue culture techniques associated with greenhouse cultivation has largely sped up orchid industrialization. More importantly, advanced biotechnology such as *Agrobacterium*-mediated transformation, particle bombardment and CRISPR/Cas9 genome editing for molecular breeding and large-scale propagation were more likely to be applied to the orchid industry for producing novel varieties with a longer shelf life and shortened production period that contribute to successful commercial orchid farming.

Orchid propagation

Propagation techniques have gained major industrial importance for ornamental plants like orchids in which the commercial demand far exceeds the natural regeneration. Asymbiotic seed germination is a common way for orchid multiplication but it's difficult for some terrestrial orchids (e.g. *Cypripedium*) and less favorable in large-scale production owing to the long juvenile period and heterozygosity in progenies [196]. To obtain propagated orchids with genetic stability and uniformity, micropropagation, usually by diverse forms of tissue culture (e.g. protocorm-like bodies (PLBs)) has been routinely used for mass commercial production and regeneration of endangered orchids [197]. Propagation protocols have been established for many species with commercial values and conservation purposes [198,199]. In spite of the advantages, somaclonal variations induced by prolonged clonal propagation and cryptic genetic effects under microenvironment become the major limitations for maintaining

certain desired traits [200]. Therefore, molecular markers have been applied to examine the genetic variability of *in vitro* raised plants to ensure clonal fidelity [201, 202]. In this context, it has been pinpointed that biotechnological approaches will be a promising tool for orchid micropropagation and simultaneously, commercial production.

Conventionally, it takes three to 13 years to produce a flowering plant from seeds and the transition from the juvenile period to reproductive development is restricted by some environmental conditions such as vernalization [203]. Intriguingly, under a certain combination of growing conditions, orchid protocorms are able to bypass the vegetative phase and directly flower without leaf initiation and root development. It's reported that this process is co-regulated by key TFs like KNOX, R2R3-MYB and Ovate Family Protein (OFP) that can induce a different flowering program [204]. This rapid *in vitro* flowering is of great potential in commercial utilization, especially for species with a long juvenile period. In addition to the reduction of reproduction cycle, enhancing the production of useful secondary metabolites has become an important objective for *in vitro* orchid propagation. Micropropagated orchids with treatment of illumination, abiotic and biotic stress, bioreactors or precursors showed an accumulation of secondary metabolites, indicative of their positive roles in end product production [205–207]. Overall, technical innovations have built the path from laboratory to commercialization for orchid micropropagation, which is expected to gain momentum and give rise to more elite orchid varieties and byproducts.

Transgenic breeding
Genetic transformation
There are mainly two types of gene manipulation techniques, overexpression of exogenous genes (genetic transformation) and silencing of endogenous genes (gene editing/silencing). Genetic transformation is a sought-after technology for introducing agronomically useful genes to modify or recombine already existing traits into target plant species, expanding the gene pool beyond what has been available to conventional breeding means and does not exist in the species of interest. Also, traditional breeding strategies such as crossbreeding with the purpose of improving particular traits are time-consuming for orchids that have prolonged reproductive cycles and multiple backcrossing attempts are required. Therefore, genetic transformation poses a tremendous potential for efficient genetic enhancement of important ornamental plants like orchids. *Agrobacterium*-mediated approach and particle bombardment are two major strategies employed for transgenic orchids [208]. These two methods have been increasingly applied to studies with the aim of transferring desired genes to orchids that could give rise to commercially important traits, such as genes regulating novel flower color, flowering time, plant-pathogen/virus resistance, and cold tolerance (Table 3). These attempts provided efficient, possibly heritable and promising gene transformation to genetically modify specific characteristics of orchids. However, although bioinformatics analyses have provided implications for a wide spectrum of genes associated with orchid characteristics, only a small proportion of these have been functionally verified via gene transformation studies. Besides, it can take many years of painstaking research to develop transformation methods for different species as some orchids (e.g. *Cypripedium*) are recalcitrant for *in vitro* propagation, as mentioned earlier, plus the long reproduction cycle and the limited availability of genomic data. After the transgenic plant with the target gene is successfully generated, it is possible that the desired phenotype and the associated traits could be altered by

the occurrence of somaclonal variation during propagation [208]. So far, transgenic studies have been done exclusively within the Epidendroideae subfamily, with *Cymbidium*, *Dendrobium*, *Oncidium*, *Vanda*, and *Phalaenopsis* as the representative genera for fundamental research and breeding applications. A routine protocol of genetic transformation for other taxa is still considerably lagging behind. Nevertheless, with the help of the ongoing release of high-quality whole genome sequencing in more orchids and the advent of cutting-edge techniques in molecular biology, our understanding of intrinsic mechanisms and modifications of genes of interest for desired traits of orchids will be fundamentally improved.

Genome editing
Genome editing refers to a group of newfangled genetic engineering technologies, in which programmed nucleases composed of sequence-specific DNA-binding domains are employed to induce targeted DNA double-strand breaks (DSBs) in the host that stimulate the cellular DNA repair mechanisms [209]. These site-specific nuclease technologies enable a specific target DNA to be added, removed, or altered at particular locations in the genome, providing great opportunities for plant genome engineering [210]. Before the emergence of sequence-specific nucleases, RNAi-mediated gene knock-down represented an efficient, low-cost, and high-throughput alternative to target gene silencing by homology-directed recombination [211]. There were several flower color/pigmentation-related studies conducted using gene knock-down by RNAi in orchids, including *Dendrobium* Sonia [212], *Oncidium* Gower Ramsey [213] and *Oncidium* Honey Angel [214], whereas this RNAi technology has several drawbacks regarding unpredictable off-target effects and temporary loss-of-function, which could hinder the practical application to a certain extent, due to limited linkage between phenotype and genotype [209]. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system represents a newly developed, efficient tool for introducing site-specific DSBs [215], by which the target gene is completely knocked out and genomic change can be maintained and heritable to the progeny. Although the well-established transformation systems are far from complete in Orchidaceae, this technology has been successfully applied in both natural specimens and their cultivars. Kui *et al.* [216] reported a successful knockout of five target genes (*C3H*, *C4H*, *4CL*, *CCR*, and *IRX*) involved in the lignocellulose biosynthesis pathway in *D. officinale* by CRISPR/Cas9 gene editing. By examining mutation rates of the locus of each target gene, they claimed the established CRISPR/Cas9 system can work efficiently to edit endogenous genes in *D. officinale* and introduce mutations. Three MADS-box genes (*MADS44*, *MADS36*, and *MADS8*) were edited by CRISPR/Cas for producing single-guide RNAs (sgRNAs) and generating different combinations of mutants in *Pha. equestris* [217]. This study illustrated the possibility that several sgRNAs can be constructed into a library and transformed to create mutants with multiple phenotypes. However, whether the editing procedures reported by these two studies can actually produce knock-out phenotypes in orchids remains unknown. Yet only two subsequent studies have provided validated phenotypes correlated with the knockout of target genes using CRISPR/Cas9 technology. In *Pha. amabilis*, phytoene desaturase (*PDS3*) gene which encodes a rate-limiting enzyme in carotenoid synthesis has been edited by CRISPR/Cas9 system and the transformants showed an albino phenotype [218]. Li *et al.* [219] generated a kilobase-scale genomic deletion at the *DOTFL1* locus of *Dendrobium* Chao Praya Smile. The results showed *dotfl1* exhibited earlier occurrence of flowering, pseudobulb

Table 3. Transgenic orchids with commercially applicable traits.

Species/Cultivars	Target genes	Methods	Explant	Aims	References
Phalaenopsis Taisuco Kochdiam × *Phalaenopsis* Taisuco Kaaladian	Cymbidium Mosaic Virus (CymMV) coat protein cDNA	Particle bombardment	Protocorm-like bodies	Improve the resistance of *Phalaenopsis* to CymMV	[225]
Phalaenopsis Amabilis W1–10 × *Phalaenopsis Amabilis* W1–22	CymMV coat protein cDNA	Particle bombardment and *Agr. tumefaciens* strain EHA105- mediated transformation	Protocorm-like bodies	Enable expression of viral and bacterial disease (CymMV) and *Erwinia carotovora* resistant traits	[226]
Phalaenopsis amabilis 'Queen Beer, No.1227'	Lipid transfer protein (LTP) encoding gene	*Agr. tumefaciens* strain LBA4404, vector: pCAMBIA2300	Protocorm-like bodies	Enhance cold resistance in *Phalaenopsis*	[227]
Phalaenopsis Sogo Yukidian 'V3', *Phalaenopsis* OX Honey 'OX1372'	*flavonoid-3',5'-hydroxylase (F3'5'H)*	Transient overexpression, *Agr. tumefaciens* strain EHA105- mediated transformation	Flower	Assessment of F3'5'H 's function in causing violet-blue pigmentation in *Phalaenopsis* flowers	[228]
Phalaenopsis Sogo Yukidian 'SPM313'	Rice GA2-oxidase gene OsGA2ox6	*Agr. tumefaciens* strain EHA105-mediated transformation, vector: Ubi:OsGA2ox6	Protocorm-like bodies	Detect GA2-oxidase gene's role in miniaturizing *Phalaenopsis* species	[229]
Phalaenopsis equestris	Orchid stress-associated protein (SAP) gene, *Pha21*	*Agr. tumefaciens* mediated transformation, vector: pH2GW7	Protocorm	Elucidate the key role of *Pha21* in antiviral immunity enhancement of the RNAi pathway	[230]
Erycina pusilla	Methionine sulfoxide reductase B7 (MSRB7)	*Agr. tumefaciens* strains EHA105-mediated transformation, vector: p35S-AtMSRB7	Protocorm	Verify MSRB gene's function in response to abiotic stress in orchids	[231]
Dendrobium Madame Thong-In	*Dendrobium* Class I KNOX gene DOH1	*Agr. tumefaciens* strain LBA4404 mediated transformation	Protocorm-like bodies	Detect DOH1's function in shaping plant architecture in *Dendrobium*	[232]
Dendrobium Sonia Earsakul	CymMV coat protein cDNA	Particle bombardment	Protocorm-like bodies	Generate resistance to CymMV in naturally CymMV-infected *Dendrobium*	[233]
Dendrobium Chao Praya Smile	*Dendrobium* SOC1	Particle bombardment, vector: pGreen	Callus	Verify SOC1 could promote flowering in *Dendrobium*	[180]
Dendrobium Chao Praya Smile	*Dendrobium* AP1	*Agr. tumefaciens* -mediated transformation	Callus	Verify AP1 could induce early flowering in *Dendrobium*	[173]
Dendrobium Chao Praya Smile	*Dendrobium* FT and FT-INTERACTING PROTEIN 1 (FTIP1)	Particle bombardment *Agr. tumefaciens*-mediated transformation	Callus	Verify that FT interacts with FTIP1 affecting flowering in *Dendrobium*	[234]
Dendrobium Sonia 'Earsakul'	1-aminocyclopropane-1-carboxylic acid oxidase (ACO)	*Agr. tumefaciens* -mediated transformation	Protocorm-like bodies	Demonstrate the effect of reduced antisense ACO activity to vegetative and reproductive development	[235]
Oncidium 'Sweet Sugar' Oncidium 'Sharry Baby' Odontoglossum 'Stirling Tiger' Odontoglossum 'Hansueli Isler'	Ethylene receptor mutant (etr1)-1gene	*Agr. tumefaciens* -mediated transformation	Protocorm-like bodies	Reduce flower's sensitivity to exogenous ethylene and prolong display life	[236]
Oncidium Gower Ramsey	*Oncidium* MADS box gene OSMADS1	*Agr. tumefaciens* - strain LBA4404 mediated transformation	Protocorm-like bodies	Verify OSMADS1 can promote early flowering of *Oncidium*	[237]

formation and termination of inflorescence apices, which are more explicit phenotypes related to floral transition than the previous reported *DOTFL1* knockdown lines by RNAi-mediated silencing [183]. Whereas all these practices have not yet been verified if these edited phenotypes can be retained to F2 and F3 -selfed and -crossed progeny, the gap between research experiment and industry application is still waiting to be filled in. Albeit started late and still in its infancy, these studies showed the great potential of CRISPR/Cas9 systems in efficient and accurate modification of orchid traits and this technique has huge potential to be applied in breeding new and improved orchid varieties without lengthy procedures and unexpected variables led by crossing breeding.

Future prospects

Over the past two decades, the explosion of genomic sequencing technologies combined with recent advances in transgenic techniques have revolutionized the basic and applied biology in orchids. With the wealth of phylogenetic information available, more and more controversial taxonomy of orchid groups has been well solved, framing a reliable evolutionary relationship for this extraordinarily diverse family. Conservation genetics based on molecular markers enable the effective translation of underlying threats into practical conservation measures that are tailored for different orchid communities. The ongoing release of orchid whole genomes has elevated the possibilities of multi-omics studies in orchids in terms of genome mapping, gene expression, comparative genomics, and functional validation. The progress in these studies provides new insights into the origin, evolution, and diversification of Orchidaceae, as well as a molecular vision that uncovers the underlying regulating mechanisms pertaining to exquisite floral morphology, complex life histories, and unrivaled reproductive strategies. As genetic transformation and genome editing have become increasingly routine techniques in plant genome engineering, precise gene characterization and modification are feasible in orchids with complex genomes. Besides, tools like CRISPR-Cas9 system offers new opportunities for gene stacking, in which adding genes-of-interest with desired traits or knocking out genes associated with undesirable traits can be achieved simultaneously in a single practice [217, 220]. Although the current achievement of genome engineering is limited to orchids, these swift and robust approaches pave a way for efficient genetic improvement of important traits in orchids in the foreseeable future.

Apparently, we are now entering a post-genomic era for orchid research and industrialization. Despite the recent advances having unveiled many facets of this intriguing family, its scientific significance has not yet been fully realized. In addition, the deficiency of genome data in most orchid species still hampers the in-depth comparative genomics analysis. Genetic investigation and functional analysis are mainly focusing on well-characterized gene families such as MADS-box, MYB that have been extensively studied in model plants, with fewer efforts put into the inspection of novel regulating factors. Last but not least, genetic transformation protocols available at present are derived from several model orchids like *Dendrobium* and *Phalaenopsis*, which may not be applicable to many other orchid species with specified features, plus the existing transformation efficiency is relatively low in orchids compared to model plants such as *Arabidopsis* and rice; these pending problems will require multi-facet investments to guide future acts. Taken together, the ongoing accomplishments in developing novel genomic tools and techniques have shed new light onto orchid research and production. Our critical next step is to utilize their full potential in the application for orchid conservation, breeding, and industrialization.

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Conflict of interests

The authors declare that they have no conflict of interests.

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