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Pan-Genomic Evolution of R2R3-MYB and bHLH Transcription Factors in *Dendrobium*

Tiancai Wang [1,†], Mengke Qin [1,†], Danni Luo [1], Runjie Guo [1], Linxia Bai [1], Haotian Zhou [1], Yang Wang [1], Yufei Liu [1], Jinpo Su [1], Yingjie Luo [1] and Xiaokai Ma [1,2,*]

[1] Fujian Provincial Key Laboratory of Haixia Plant Systems Biology, Center for Genomics and Biotechnology, Haixia Institute of Science and Technology, College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China; 52362043013@fafu.edu.cn (T.W.); 12462110007@fafu.edu.cn (M.Q.); 12462110064@fafu.edu.cn (D.L.); 52462043082@fafu.edu.cn (R.G.); 12562110027@fafu.edu.cn (L.B.); 12562110077@fafu.edu.cn (H.Z.); 52562043003@fafu.edu.cn (Y.W.); 12562110033@fafu.edu.cn (Y.L.); 12405014029@fafu.edu.cn (J.S.); 52462043086@fafu.edu.cn (Y.L.)

[2] Key Laboratory of Orchid Conservation and Utilization of National Forestry and Grassland Administration, Fujian Agriculture and Forestry University, Fuzhou 350002, China

* Correspondence: maxk@fafu.edu.cn

† These authors contributed equally to this work.

Abstract

R2R3-MYB and bHLH transcription factors (TFs) are key regulators of floral secondary metabolism and epidermal development in flowering plants. Orchids exhibit remarkable floral diversity, which is critical for pollination success and ornamental value, yet the evolutionary and functional diversification of these TF families within the genus remains largely unexplored. Here, we conducted a comprehensive pan-genome dissection of R2R3-MYB and bHLH TF families across 18 *Dendrobium* species, integrating orthologs assignment, phylogenetics, duplication profiling, cis-regulatory annotation, and tissue-specific expression analysis. We identified 3074 R2R3-MYB and 2282 bHLH genes, classified into 69 and 61 orthologous gene groups (OGGs), respectively. Core OGGs accounted for two-thirds of both families, indicating strong evolutionary conservation, whereas variable OGGs reflected lineage-specific diversification. Phylogenetic analyses resolved R2R3-MYBs into 24 canonical subfamilies and revealed conserved heterogeneous expansion patterns in bHLH subfamilies. Promoter architectures of R2R3-MYB genes were enriched in hormone-, stress-, and light-responsive elements, whereas bHLH promoters were dominated by development-related motifs. Tissue-specific expression profiling in *Dendrobium* 'Chao Praya Smile' showed that floral bud-enriched genes were associated with flavonoid/anthocyanin biosynthesis, whereas root-enriched genes were linked to stress and hormone responses. Integration of pan-genomics and transcriptomics highlighted evolutionary trajectory and functional divergence between core and variable gene sets within *Dendrobium*. Our study establishes a comprehensive, genus-wide framework for understanding the evolutionary and functional characteristics of MYB–bHLH regulatory networks in *Dendrobium*. These findings provide valuable genetic resources and key candidate targets for functional characterization and molecular breeding, with important implications for genetic improvement of reproductive traits, floral quality, stress resistance, and ornamental and agronomic value in orchids.

Keywords: pan-genome; gene family; reproduction; R2R3-MYB; bHLH; transcription factors; *Dendrobium*; orchid

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Academic Editor: Matthew Hegarty

Received: 29 January 2026
Revised: 24 February 2026
Accepted: 26 February 2026
Published: 27 February 2026

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1. Introduction

The MYB and bHLH transcription factor (TF) families constitute core regulatory components of gene expression networks that govern floral development, sexual reproduction, and adaptive trait formation in higher plants [1,2]. These two TF families play central roles in controlling the differentiation and development of floral organs, and the associated biosynthesis of secondary metabolites that are essential for flower coloration, scent production, and reproductive success [3]. By integrating developmental programs with hormonal and environmental signals, MYB and bHLH TFs enable plants to coordinate reproductive development with ecological adaptation, thereby contributing to phenotypic diversity and evolutionary fitness [4]. Among plant transcription factors, the MYB family represents one of the largest and most functionally diverse groups. MYB proteins regulate a wide range of biological processes by binding to specific cis-acting elements in the promoter regions of target genes, particularly those involved in flavonoid and anthocyanin biosynthesis, epidermal cell differentiation, and stress-responsive pathways [5,6]. In parallel, bHLH transcription factors, characterized by a conserved basic helix–loop–helix domain, are key regulators of floral organ development, pollen viability, and secondary metabolism, and frequently act in concert with MYB proteins to fine-tune gene expression during reproductive development [7].

Within the MYB family, R2R3-MYB proteins constitute the most abundant and functionally well-characterized subfamily. They are typically characterized by two highly conserved MYB DNA-binding domains (R2 and R3 repeats), which mediate specific interactions with target gene promoters and enable precise transcriptional regulation [8]. Extensive studies have demonstrated that R2R3-MYB transcription factors play central roles in regulating plant secondary metabolic pathways, including anthocyanin, flavonoid, terpenoid, and phenylpropanoid biosynthesis, as well as epidermal cell differentiation, organ morphogenesis, and floral and fruit development [9–11].

In terms of flower color regulation, R2R3-MYB transcription factors are key determinants of anthocyanin accumulation. In *Cymbidium ensifolium*, the R2R3-MYB genes *CeMYB52* and *CeMYB104* have been identified as key regulators associated with anthocyanin accumulation and flower color variation [12]. Consistently, MYB–bHLH cooperative regulation has been demonstrated in other species; in *Chrysanthemum morifolium*, the bHLH transcription factor *CmbHLH2* acts as an essential partner of *CmMYB6* to regulate anthocyanin biosynthesis and flower color formation [13].

In floral scent biosynthesis, MYB transcription factors have been shown to directly regulate the production of volatile compounds. In *Hedychium coronarium*, multiple MYB transcription factors can bind to the promoters of terpenoid- and benzenoid-related floral scent biosynthetic genes, and their silencing leads to a significant reduction in volatile emission [14]. Similarly, in the *Cymbidium* cultivar 'Sael Bit', *CsMYB1* functions as a core regulator of phenylpropanoid- and ester-derived floral scent biosynthesis [15].

Beyond secondary metabolism, specific R2R3-MYB subgroups are directly involved in reproductive development. In cotton, *GhMYB35* has been identified as a key regulator of anther development and pollen viability, and its complete loss results in pollen abortion, anther indehiscence, and male sterility [16]. In addition, members of the R2R3-MYB subgroup 19 (SG19) have been reported to participate in floral development, flower maturation, and floral scent emission in various angiosperms [17]. Collectively, these findings indicate that although R2R3-MYB transcription factors are functionally conserved, their specific regulatory roles exhibit pronounced species specificity and functional divergence.

In contrast to MYB TFs, bHLH TFs are defined by a conserved basic helix–loop–helix domain. The basic region mediates binding to E-box or G-box elements in DNA, while the HLH region facilitates homo- or hetero-dimerization of proteins, thereby enabling fine-

tuned regulation of target gene transcription [7]. The bHLH family is also one of the most abundant transcription factor families in plants and is widely involved in the regulation of anther development, pollen viability, secondary metabolism, and stress responses in crops, and horticultural plants [18]. In multiple plant species, bHLH transcription factors have been demonstrated to play crucial roles in reproductive development. For instance, in maize, a regulatory cascade consisting of Ms23–Ms32–bHLH122–bHLH51 precisely controls anther development [18]. In tomato, *Solyc01g102300.3* has been identified as a key bHLH regulator required for maintaining pollen viability, and its mutation significantly reduces pollen viability without affecting vegetative growth [19].

Notably, R2R3-MYB and bHLH transcription factors often function cooperatively by forming MYB–bHLH or MYB–bHLH–WD40 (MBW) complexes to regulate secondary metabolism and developmental processes. This regulatory mode is particularly well characterized in anthocyanin biosynthesis and flower coloration [20]. For example, in Gentian, *GtMYB3* interacts with *GtbHLH1* to activate late anthocyanin biosynthetic genes, thereby determining the accumulation of gentiodelphin in petals [21]. In kiwifruit, *AcMYBF110* interacts with *AcbHLH1/4/5* and *AcWDR1* to form multiple MBW complexes with distinct functions, establishing a positive feedback amplification regulatory network for anthocyanin biosynthesis [20].

As one of the most species-rich angiosperm families, the Orchidaceae is distinguished by its remarkable diversity in floral structure, color, and fragrance [22,23]. Within Orchidaceae, the genus *Dendrobium* comprises more than 1500 species and possesses significant ornamental, medicinal, and economic value. The diverse flower colors, specialized labellum structures, and complex secondary metabolite profiles make *Dendrobium* an ideal system for investigating transcriptional regulation and trait evolution [11]. In recent years, multiple studies have revealed the critical roles of MYB and bHLH transcription factors in trait formation in *Dendrobium*. In *Dendrobium catenatum*, *DcTT8*, *DoMYB5*, and *DobHLH24* have been identified as core regulators of anthocyanin biosynthesis and stem color differentiation [24,25]. In *Dendrobium* cultivars with Phalaenopsis-like floral morphology, *DhMYB2* and *DhbHLH1* cooperatively activate multiple anthocyanin biosynthetic genes, thereby determining color differences among floral organs [26]. Moreover, in *Dendrobium nobile* and *Dendrobium chrysotoxum*, MYB and bHLH transcription factors jointly constitute core regulatory networks controlling anthocyanin and flavonoid biosynthesis, driving flower and stem coloration [27,28].

Despite these advances, current studies have largely focused on individual *Dendrobium* species or specific traits, and the systematic genus-level comparisons of the genetic variations, evolutionary relationships, and functional divergence of the R2R3-MYB and bHLH families remain limited [11]. Furthermore, transcriptomic studies in *Dendrobium* are often restricted to a limited number of tissues or developmental stages, which constrains a comprehensive understanding of cross-organ regulatory mechanisms mediated by these transcription factors [29]. In the present study, we systematically identified the members of R2R3-MYB and bHLH TF families from 18 *Dendrobium* genomes and employed a pan-genome strategy to investigate their genomic distribution patterns, gene duplication modes, selective pressures, gene structures, conserved motifs, and phylogenetic relationships. This study was carried out at the Center for Genomics and Biotechnology, Fujian Provincial Key Laboratory of Haixia Plant Systems Biology, Haixia Institute of Science and Technology, Fujian Agriculture and Forestry University, which has long been dedicated to research on the molecular basis of floral diversity and agronomic trait improvement in orchids. We established a comprehensive framework covering the entire *Dendrobium* genus, providing a foundation for dissecting the evolution and functional characteristics of the MYB–bHLH regulatory network; these results not only enrich our understanding

of transcription factor evolution and regulatory mechanisms in orchids, but also have important impacts at both agronomic and commercial levels. Agronomically, the candidate regulatory factors and genetic resources identified in this study provide key targets for molecular breeding, facilitating the genetic improvement of important traits such as floral quality, stress tolerance, and reproductive efficiency in Dendrobium and other economically valuable orchids. Commercially, the findings lay a foundation for breeding new orchid varieties with higher ornamental value, longer flowering duration, and stronger cultivation adaptability, which will effectively promote the sustainable development and economic benefits of the global orchid industry. Meanwhile, this genus-level regulatory framework provides a valuable reference for accelerating functional characterization and reducing the cycle and cost of molecular breeding, further strengthening the practical application value of this study in agricultural production and commercial horticulture.

2. Materials and Methods

2.1. Data Sources of Genome Assemblies and Annotations

The 18 Dendrobium genome sequences and annotations were collected from public databases. The genome assemblies of *Dendrobium* Chao Praya Smile, *Dendrobium discolor*, *Dendrobium crocatum*, *Dendrobium smilliae*, *Dendrobium leonis*, *Dendrobium crumenatum*, *Dendrobium formosum*, *Dendrobium secundum*, *Dendrobium ellipsophyllum*, *Dendrobium tetragonum*, *Dendrobium hercoglossum*, *Dendrobium lindleyi*, *Dendrobium aphylium*, and *Dendrobium jenkinsii* were retrieved from Figshare (https://doi.org/10.6084/m9.figshare.26342338.v1) [30]. The genome assemblies for *Dendrobium chrysotoxum* (PRJNA161865), *Dendrobium nobile* (PRJNA725550), *Dendrobium officinale* (PRJNA662181), and *Dendrobium catenatum* (PRJNA262478) were retrieved from NCBI database [31–34].

The genome assemblies and annotations for other species, such as *Apostasia shenzhenica* (PRJNA310678), *Vanilla planifolia* (PRJNA633886), *Phalaenopsis equestris* (PRJNA192198) and *Platanthera guangdongensis* and *Platanthera zijinensis* (PRJNA739531) were retrieved from the NCBI database [34–37]. The datasets for *Gastrodia menghaiensis* were downloaded from Figshare (https://figshare.com/s/f759784e78e86ba71c7c) (accessed on 28 September 2025 [38], while those of *Angraecum sesquipedale* were obtained from the National Genomics Data Center (NGDC; assembly identifier: 88029) [39], and the datasets for *Cremastra appendiculata* were retrieved from the China National GeneBank (CNGB) Data Resources (project identifier: CNP0001656) [40].

2.2. Identification of R2R3-MYB and bHLH Genes

Hidden Markov Model (HMM) profiles for the MYB_DNA-binding domain (PF00249) and the bHLH domain (PF00010) were downloaded from the Pfam protein family database (http://pfam.xfam.org/) [41]. Using BLASTP (2.16.0) (E-value < 1×10^{-10}), protein sets from the 18 *Dendrobium* genomes were queried with 126 *A. thaliana* R2R3-MYB sequences and 172 *A. thaliana* bHLH sequences obtained from The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/) [42,43]. Resulting hits were further screened with HMMER (E-value < 1×10^{-10}; http://hmmer.org/download.html) [44] to confirm the presence of the MYB_DNA-binding (PF00249) or bHLH (PF00010) domains, and only sequences with complete domains were retained as candidates. To ensure reliability, all candidate R2R3-MYB and bHLH proteins were validated using the NCBI Conserved Domain Database (CDD; E-value threshold = 0.01; maximum hits = 500; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (http://smart.embl-heidelberg.de). For R2R3-MYBs, only proteins containing complete R2 and R3 repeats confirmed by both tools were retained; for bHLHs, only proteins with an intact basic helix–loop–helix domain—

including the N-terminal basic DNA-binding region and the C-terminal HLH dimerization region—validated by both resources were kept for downstream analyses.

2.3. Physicochemical Properties of R2R3-MYB and bHLH Proteins

The physicochemical characteristics of *Dendrobium* R2R3-MYB and bHLH proteins, including amino acid composition, molecular weight (MW), isoelectric point (pI), and grand average hydropathicity (GRAVY), were examined using the ProtParam tool on the ExPASy website (https://web.expasy.org/protparam/) [45]. The subcellular localization of these proteins was predicted using the WoLF PSORT online program (https://wolfpsort.hgc.jp/) [45].

2.4. Identification of Orthologous Gene Groups for R2R3-MYB and bHLH

Orthologous gene groups (OGGs) for R2R3-MYB and bHLH genes among the 18 *Dendrobium* genomes were identified using OrthoFinder (v2.4.0) [46] with default parameters. The identified OGGs were categorized into four groups: core (present in all 18 accessions), softcore (present in 15–17 accessions), dispensable (present in 2–14 accessions), and private (present in only one accession). For each OGG, the longest protein sequence was selected as the representative sequence.

2.5. Identification of Cis-Elements in R2R3-MYB and bHLH Genes

To investigate cis-elements in the promoter regions, the 2000 bp genomic DNA sequences upstream of all R2R3-MYB and bHLH genes in *Dendrobium* were analyzed using the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The main parameters for motif identification were set as follows: the background model Bm (default: pre-compiled Arabidopsis thaliana model, adjustable for different species), motif length W (default: 8 bp, range: 5–15 bp), number of motifs N (default: 6, with identified positions masked in subsequent searches), maximum copy number C (default: 1, adjusted to avoid noise), and maximum overlap O (default: 2, to reduce redundancy) [47].

2.6. Chromosomal Distribution, Duplication, and Selection Pressure Analysis

The chromosomal distribution of R2R3-MYB and bHLH genes in *Dendrobium* was analyzed with the R package "ideogram" (v1.5.2), using *D.CPS* genome as a representative. Gene duplication events were identified using the DupGen_finder [48]. To calculate Ka/Ks of each orthologous gene pair, the amino acid sequences were aligned using MUSCLE (5.3) [49], transformed into CDS alignments with PAL2NAL (v14) [50], and then fed into the KaKs_Calculator (v2.0) [51].

2.7. Analysis of the Structure and Conserved Sequences

The MEME program (https://meme-suite.org/meme/tools/meme) [52] was used to identify motifs in R2R3-MYB and bHLH proteins in *Dendrobium*. For the R2/R3 repeats of R2R3-MYBs and the HLH domains of bHLH proteins across *Dendrobium* and *A. thaliana*, we performed multiple sequence alignments with ClustalX (v2.1) [53] and generated sequence logos using TBtools (v2.0) [54].

2.8. Multiple Sequence Alignment and Phylogenetic Analysis

The amino acid sequences of R2R3-MYB and bHLH genes in *Dendrobium* were aligned using the MAFFT program [55]. A maximum likelihood phylogenetic tree was constructed using IQ-TREE, with the best-fit substitution model selected by ModelFinder (v2.1.10). Branch support was assessed with 1000 bootstrap replications. The phylogenetic tree was visualized using the iTOL website (https://itol.embl.de/) [56].

2.9. RNA-Seq Data and Differential Expression Analysis

Transcriptome (RNA-seq) data for *Dendrobium* 'Chao Praya Smile' (*D. CPS*) were retrieved from the NCBI BioProject PRJNA1088629. Raw counts were generated using HTSeq 2.0 [57] and were analyzed in DESeq2 [58] to identify differentially expressed genes ($|\log2(\text{fold change})| \geq 1$; FDR-adjusted $p < 0.05$). DEGs with elevated expression in one or more tissues were designated tissue-preferential to explore organ-specific roles of R2R3-MYB and bHLH genes.

3. Results

3.1. Distribution of R2R3-MYB and bHLH TFs in Dendrobium and Related Orchidaceae Species

Considering the limitation of a single reference genome to capture the genetic information of a species [59–61], we analyzed R2R3-MYB and bHLH genes at the pan-genome level to better characterize their genetic diversity. Across 18 published *Dendrobium* species with eight related Orchidaceae species, we identified 3074 R2R3-MYB genes and 2282 bHLH genes (Supplementary Tables S1 and S2; Figure 1A).

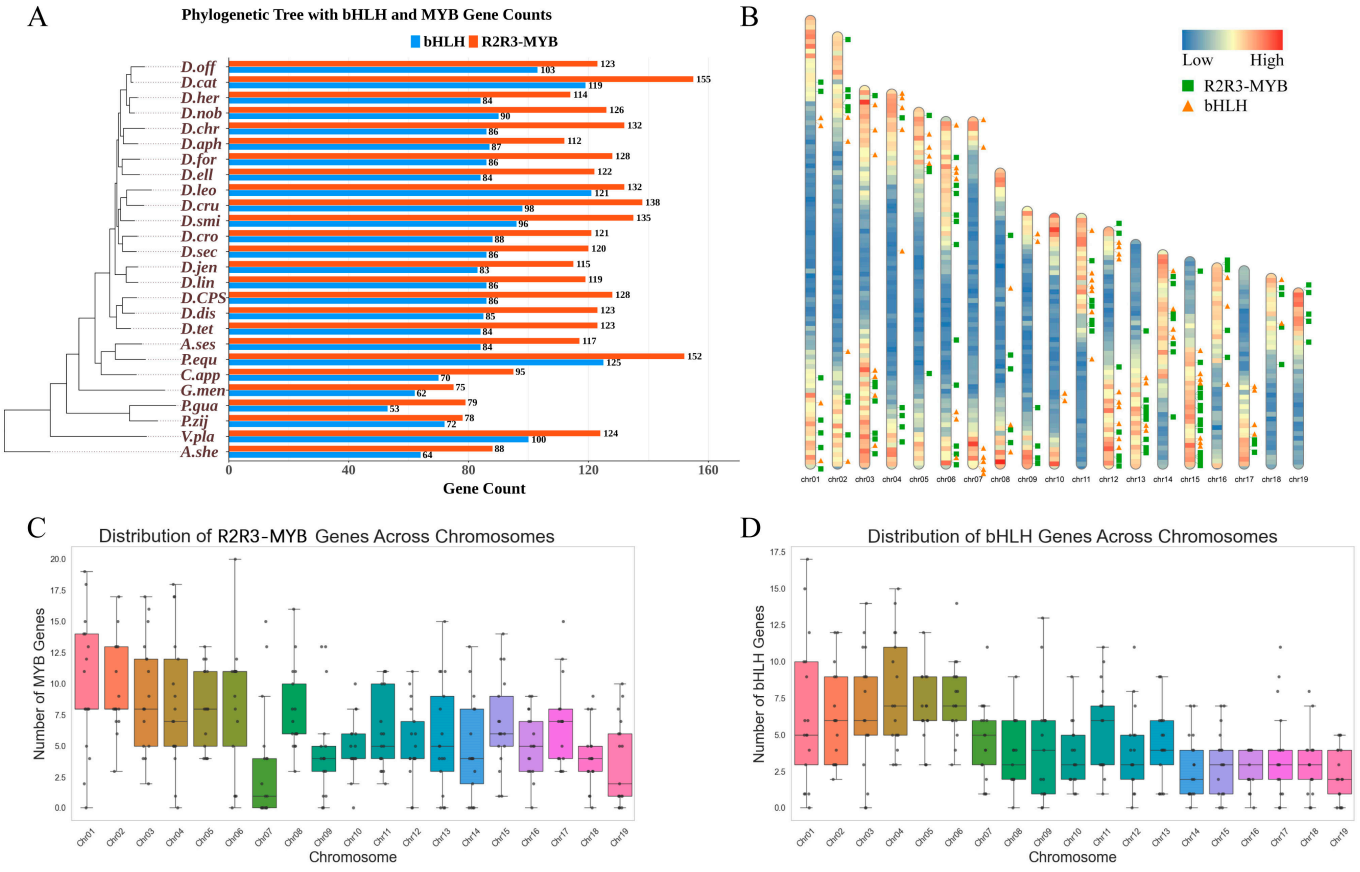


Figure 1. Distribution and chromosomal organization of R2R3-MYB and bHLH gene families in *Dendrobium*. (**A**) Phylogenetic relationship of 18 *Dendrobium* species and representative Orchidaceae species with corresponding numbers of R2R3-MYB (red bars) and bHLH (blue bars) genes. Species names are indicated on the left, and gene copy numbers are shown at the end of each bar. (**B**) Chromosomal distribution and density of R2R3-MYB and bHLH genes in *Dendrobium* 'Chao Praya Smile'. Chromosomes (Chr01–Chr19) are displayed as vertical bars colored according to gene density (low to high). Green squares indicate R2R3-MYB genes, and orange triangles indicate bHLH genes. The distribution of R2R3-MYB (**C**) and bHLH (**D**) genes across chromosomes in *Dendrobium* 'Chao Praya Smile'. Each dot represents the gene count on an individual chromosome.

Within *Dendrobium*, R2R3-MYB copy numbers ranged from 87 in *D. aphylium* to 119 in *D. catenatum*, whereas bHLH counts ranged from 83 in *D. jenkinsii* to 121 in *D. leonis*, indicating a moderate expansion of both families. By comparison, non-*Dendrobium* orchids such as *Gastrodia menghaiensis*, *Phalaenopsis equestris*, and *Vanilla planifolia* harbored 62, 125, and 100 bHLH genes, and 75, 152, and 124 R2R3-MYB genes, respectively. Notably, the mycoheterotroph *G. menghaiensis* showed marked reductions in both families, consistent with its highly degenerated photosynthetic system, whereas *P. equestris* and *V. planifolia*, the species with active floral development and secondary metabolism, retained relatively high copy numbers.

Chromosomal distribution analysis in *Dendrobium* 'Chao Praya Smile' (*D. CPS*) revealed that R2R3-MYB and bHLH genes were distributed across all 19 chromosomes (Figure 1B,D). However, the number of genes varied markedly among chromosomes, with some chromosomes showing relatively high gene densities and others containing only a few members. This uneven chromosomal distribution suggests non-random genomic organization of both IF families.

Although the sizes of the R2R3-MYB and bHLH gene families are relatively conserved within the *Dendrobium* genus, the numbers of R2R3-MYB and bHLH genes were unevenly distributed across the 19 chromosomes (Figure 1C,D), exhibiting a clear pattern of higher gene numbers on the first chromosomes and lower numbers on the later ones.

3.2. Orthologous Group of R2R3-MYB and bHLH Genes in Dendrobium

Clustering by sequence similarity across 18 *Dendrobium* genomes identified 69 orthologous gene groups (OGGs) for the R2R3-MYB family and 61 OGGs for the bHLH family. The cumulative number of OGGs approached a plateau as additional genomes were included (Figure 2A,B), indicating that the current dataset adequately captures the pan-R2R3-MYBs and pan-bHLHs TF repertoires in *Dendrobium*. Based on the presence and absence of genes from the two TFs across the *Dendrobium* genomes analyzed in this study, orthologous gene groups (OGGs) were classified into core genes (present in all accessions), softcore genes (present in most accessions), dispensable genes (present in a subset of accessions), and private genes (present in only one accession) (Figure 2E,F).

For R2R3-MYBs, core OGGs (present in all 18 genomes) constituted ~59.42% (41/69), followed by softcore (~11.59%, 8/69), dispensable (~7.25%, 5/69), and private (~21.74%, 15/69) OGGs (Figure 2C; Supplementary Table S4). For bHLHs, core OGGs likewise predominated (~70.49%, 43/61), with softcore (~22.95%, 14/61), dispensable (~0%), and private (~6.56%, 4/61) OGGs comprising the remainder (Figure 2D; Supplementary Table S5). These patterns point to a large, highly conserved core set for both families across *Dendrobium*, consistent with essential roles in development and secondary metabolism.

Ka/Ks analyses showed significantly lower Ka/Ks ratios in core OGGs than in variable groups (softcore + dispensable + private) in both MYB and bHLH families (Figure 2G,H), implying stronger purifying selection on core members to maintain key regulatory functions. By contrast, variable OGGs displayed greater sequence divergence and duplication activity (Figure 2I,J), suggesting relaxed constraints and possible neo-functionalization associated with lineage-specific adaptation under positive selection.

Notably, several private OGGs (e.g., R2R3-MYB *D.chr.MYB62*, *D.off.MYB106*, *D.smi.MYB7* and bHLH *D.leo.bHLH64*, *D.smi.bHLH49*) were restricted to single species (Supplementary Tables S4 and S5). Functional annotations linked these genes to pigment biosynthesis, organ development, and stress responses. Given the established roles of MYB/bHLH TFs in anthocyanin–flavonoid metabolism and floral morphogenesis, these lineage-specific members likely contribute to species-specific coloration, organ specialization, or ecological adaptation within *Dendrobium* [25].

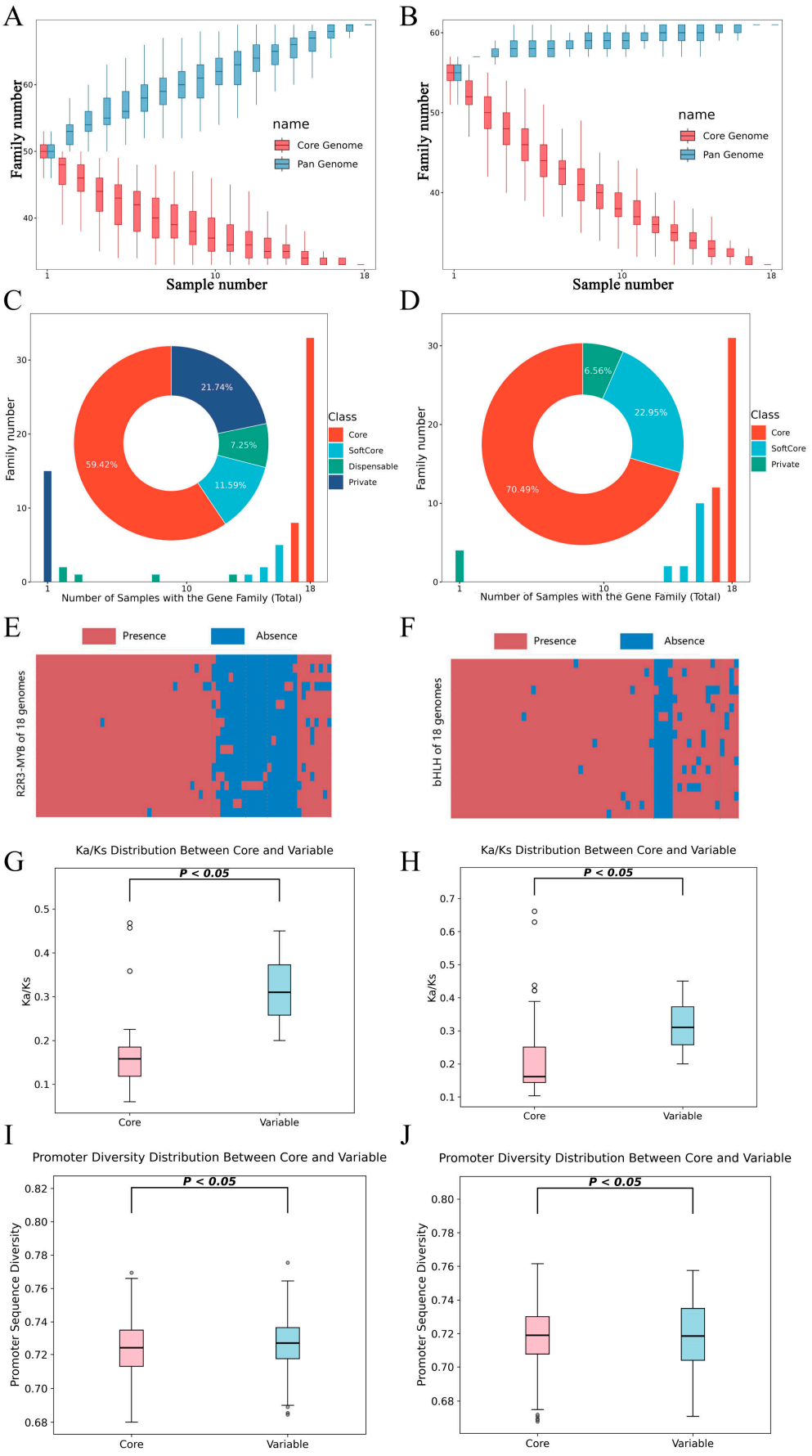


Figure 2. Pan-genome architecture and evolutionary constraints of R2R3-MYB and bHLH gene families in *Dendrobium*. (**A**,**B**) Core and pan-genome accumulation curves of the R2R3-MYB (**A**) and

bHLH (**B**) gene families across 18 Dendrobium genomes. Red boxplots represent the number of core gene families, while blue boxplots represent the number of pan-gene families as genomes are sequentially added. (**C**,**D**) Classification and distribution of R2R3-MYB (**C**) and bHLH (**D**) gene families in the Dendrobium pan-genome. Donut charts indicate the proportion of gene families belonging to different categories (core, softcore, dispensable, and private for MYBs; core, softcore, and private for bHLHs). Bar plots show the number of genomes in which each gene family is present. (**E**,**F**) Presence/absence variation (PAV) heatmaps of R2R3-MYB (**E**) and bHLH (**F**) gene families across 18 Dendrobium genomes. Red indicates presence and blue indicates absence of a gene family in each genome. (**G**,**H**) Comparison of Ka/Ks ratios between core and variable gene families for R2R3-MYB (**G**) and bHLH (**H**). Variable genes include softcore, dispensable, and private categories. Statistical significance was assessed using the Wilcoxon rank-sum test ($p < 0.05$). (**I**,**J**) Comparison of promoter sequence diversity between core and variable gene families for R2R3-MYB (**I**) and bHLH (**J**). Variable gene families exhibit significantly higher promoter sequence diversity than core gene families ($p < 0.05$).

3.3. Protein Structural Features of R2R3-MYBs and bHLHs in Dendrobium

We further analyzed the physicochemical properties, structural characteristics, and subcellular localization of R2R3-MYB and bHLH transcription factors identified in *Dendrobium*. A total of 2154 R2R3-MYB and 1512 bHLH proteins were characterized in terms of amino acid length, molecular weight, theoretical isoelectric point (pI), and predicted localization.

For the R2R3-MYB family, protein lengths ranged from 68 to 4669 amino acids, with an average of 371 amino acids. The majority (73.0%) contained 200–400 amino acids, suggesting that most MYBs are of moderate size. Molecular weights ranged from 7.90 to 525.37 kDa (average 41.88 kDa). The theoretical pI values varied from 4.37 to 11.20 (average 7.15). Among them, 55.0% of MYBs were acidic (pI < 7), while 44.0% were basic (pI $\geq$ 7) (Supplementary Table S9). For the bHLH family, protein lengths ranged from 82 to 3925 amino acids, with an average of 422 amino acids. More than half (53.0%) were between 200 and 400 amino acids. The molecular weights ranged from 9.20 to 444.90 kDa (average 46.81 kDa). The theoretical pI ranged from 4.19 to 10.56 (average 6.63), with 70.0% being acidic proteins (Supplementary Table S10). These results indicate that *Dendrobium* R2R3-MYB and bHLH proteins are generally small to medium-sized acidic transcription factors, consistent with the conserved physicochemical traits of these families in plants.

Subcellular localization prediction revealed that both families are primarily nuclear proteins, consistent with their roles as transcriptional regulators. Among bHLHs, 82.5% were localized to the nucleus, followed by the chloroplast (7.8%), cytoplasm (5.0%), peroxisome (1.3%), plasma membrane (1.2%), Golgi apparatus (1.0%), and other minor compartments (<1%). Similarly, 96.2% of R2R3-MYBs were localized in the nucleus, 1.7% in the cytoplasm, and fewer than 1% in other organelles including the chloroplast, mitochondria, plasma membrane, peroxisome, and Golgi apparatus. These results demonstrate that both families predominantly function in the nucleus, although a small subset may exert regulatory roles in other cellular compartments. Collectively, the conserved physicochemical characteristics and nuclear localization of R2R3-MYB and bHLH TFs highlight their shared functional features as key regulators of gene expression in *Dendrobium*.

Sequence logo analysis further revealed that the R2 and R3 repeats of R2R3-MYB proteins exhibit highly conserved amino acid residues that are critical for maintaining the helix–turn–helix structure and DNA-binding activity (Figure 3A,B). In the R2 repeat, three regularly spaced and highly conserved tryptophan residues (Trp, W) were clearly identified, corresponding to the three α-helical regions (Helix 1, Helix 2, and Helix 3). These conserved Trp residues are known to play essential roles in stabilizing the three-helix bundle and ensuring the structural integrity of the MYB DNA-binding domain. Notably,

this Trp conservation pattern was consistently observed in both *A. thaliana* and *Dendrobium*, indicating strong evolutionary conservation of the R2 repeat.

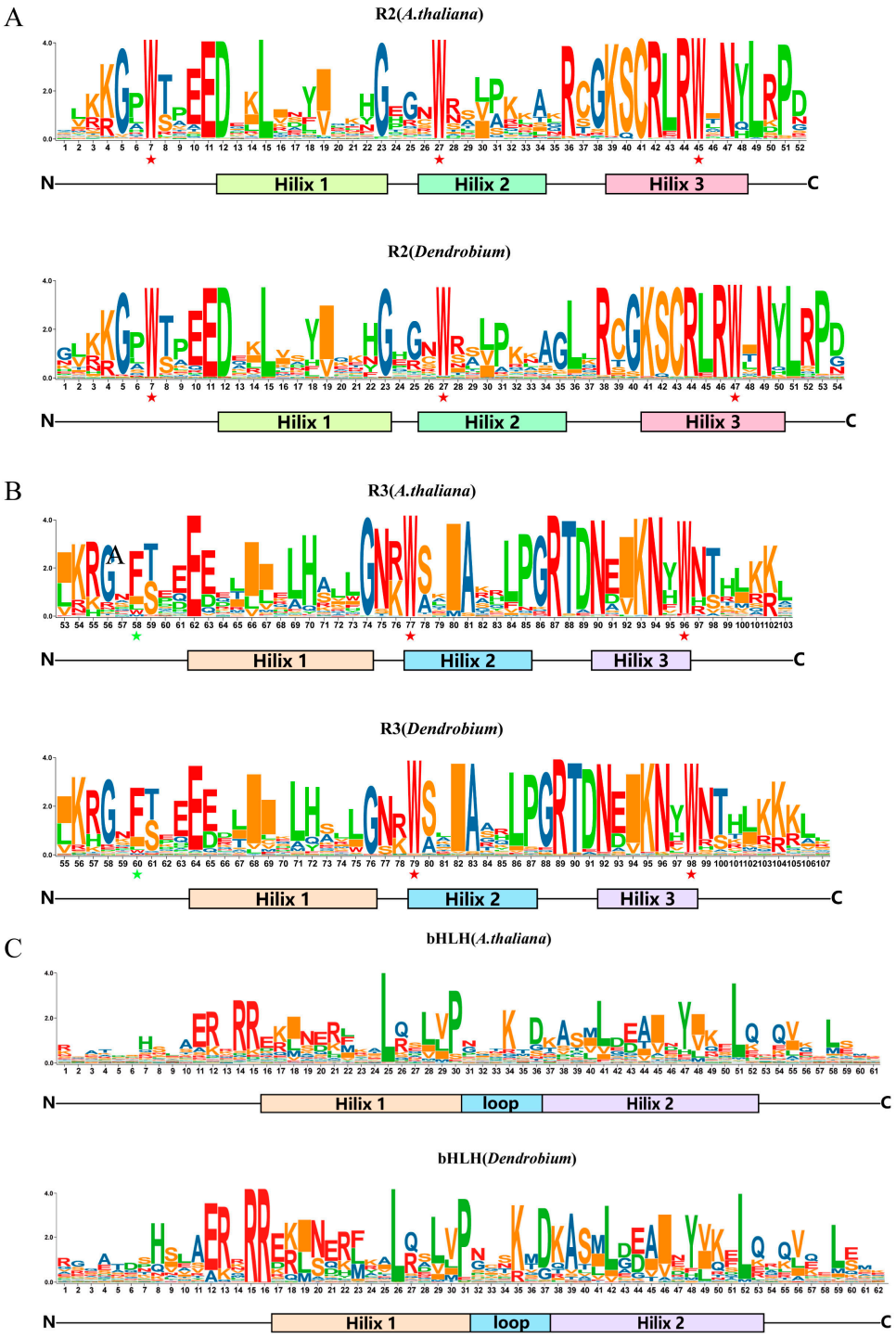


Figure 3. Sequence characteristics of the R2R3-MYB and bHLH domains in *A. thaliana* and *Dendrobium*. (**A**) Sequence characteristics of the R2 repeats in R2R3-MYB proteins from *A. thaliana* and *Dendrobium*. (**B**) Sequence characteristics of the R3 repeats in R2R3-MYB proteins from *A. thaliana* and *Dendrobium*. The locations of the three α-helices (helices 1–3) are indicated. Conserved tryptophan residues (Trp, W) within the MYB domain are highlighted with red asterisks, while substituted residues in the R3 repeat are denoted by green asterisks. The bit score represents the information content of each amino acid at specific positions within the sequence. (**C**) Sequence logo of the bHLH domain from *A. thaliana* and *Dendrobium*, showing conserved and variable residues within the basic helix–loop–helix structure. The loop region is indicated in the middle, and Helix 1 and Helix 2 are marked below.

In contrast, the R3 repeat contained only two conserved Trp residues, which correspond to the second and third Trp positions of the R2 repeat. The first Trp residue in the R3 repeat was frequently replaced by phenylalanine (Phe, F), and occasionally by isoleucine (Ile, I) or leucine (Leu, L), a substitution pattern that has been widely reported in plant R2R3-MYB proteins. This conserved replacement suggests that aromatic or hydrophobic residues at this position may compensate for the loss of Trp while maintaining the overall structural stability of the R3 repeat.

Moreover, differential conservation was observed among the three helices. Helix 1 and Helix 2 showed relatively lower sequence conservation, whereas Helix 3 was markedly more conserved and enriched in basic and hydrophobic amino acids, consistent with its direct involvement in DNA recognition and binding. Several positions within Helix 3 exhibited strong residue preference across species, further supporting the functional constraint imposed on this region. These results demonstrated that while the overall R2R3-MYB DNA-binding domain is highly conserved, limited sequence divergence at specific positions may contribute to functional diversification among MYB members in *Dendrobium*.

For bHLH transcription factors, the sequence logo of the bHLH domain revealed a highly conserved basic region at the N-terminus, characterized by the enrichment of positively charged residues such as arginine (Arg, R) and lysine (Lys, K), which are essential for DNA binding (Figure 3C). The subsequent Helix 1 and Helix 2 regions contained several conserved hydrophobic residues, facilitating homo- or hetero-dimer formation, whereas the intervening loop region displayed higher sequence variability. Importantly, the overall conservation pattern of the bHLH domain was highly similar between *A. thaliana* and *Dendrobium*, indicating that the fundamental DNA-binding and dimerization mechanisms of bHLH proteins are well preserved across plants.

3.4. Phylogenetics, Cis-Elements, and Duplication Patterns of R2R3-MYBs and bHLHs in Dendrobium

To elucidate the evolutionary relationships and potential functional diversification of the R2R3-MYB and bHLH transcription factor families, we reconstructed the phylogenetics relationships, predicted the cis-element compositions, and investigated the subfamily-level duplication patterns for members in R2R3-MYB and bHLH families in *Dendrobium*.

3.4.1. Phylogenetic Relationships Among Members in R2R3-MYB and bHLH Gene Families

Neighbor-joining (NJ) phylogenetic trees were reconstructed for R2R3-MYB and bHLH gene families in *Dendrobium*. Protein sequences from the model species *Arabidopsis thaliana* and *Oryza sativa* were included to stabilize subfamily boundaries. For both gene families, one representative sequence (the longest protein) was selected from each orthologous gene group (OGG) for reconstructing the phylogenetic trees (Supplementary Tables S3 and S6). Based on 69 representative OGG sequences from *Dendrobium*, together with 126 *A. thaliana* and 117 *O. sativa* R2R3-MYB proteins, the phylogenetic tree resolved 24 subfamilies that were largely consistent with the canonical R2R3-MYB classification in *Arabidopsis* (Supplementary Tables S11 and S12). Most subfamilies contained multiple *Dendrobium* members, indicating a high degree of conservation at the subfamily level. In addition, several subfamilies showed relatively concentrated distributions of *Dendrobium* genes, suggesting subfamily-level expansion or diversification. Subgroups previously associated with flavonoid and anthocyanin metabolism (e.g., S6, S7, and S12), epidermal cell fate determination (e.g., S1 and S15), and developmental regulation (e.g., S4, S9, and S22) were clearly recovered.

Phylogenetic reconstruction based on 61 *Dendrobium* bHLH OGG representatives, together with 172 *A. thaliana* and 167 *O. sativa* bHLH proteins, yielded subfamily classifications consistent with established bHLH frameworks in plants (Supplementary Tables S13 and S14).

Members within the same subfamily clustered closely, reflecting conserved evolutionary relationships, while several subfamilies exhibited distinct clustering patterns indicative of diversification at the subfamily scale.

3.4.2. Cis-Element Compositions of R2R3-MYB and bHLH Promoters

Cis-acting regulatory elements were annotated within the 2 kb upstream promoter regions of 3074 R2R3-MYB genes from 25 orchid species, including 18 *Dendrobium* species. In *Dendrobium*, hormone-responsive elements were the most abundant (48.6%), followed by stress-responsive (22.0%), development-related (15.9%), and light-responsive elements (13.4%) (Supplementary Table S7). Representative motifs included ABRE (abscisic acid response), MBS (drought response), WUN-motif (wounding response), G-box/Box4 (light response), and ARE (hypoxia response), indicating that R2R3-MYB genes may integrate hormonal, environmental, and developmental regulatory signals.

Analysis of the 2 kb promoter regions of 2282 bHLH genes from the same set of species showed that development-related elements predominated in *Dendrobium* (89.9%), whereas light-responsive (5.5%), stress-responsive (2.4%), and hormone-responsive elements (2.3%) were less frequent (Supplementary Table S8). Common cis-elements including TATA-box and CAAT-box (core/developmental elements), G-box/Box4 (light response), CGTCA/TGACG motifs (jasmonic acid response), ABRE, MBS, WUN-motif, LTR (low-temperature response), and ARE suggested relatively conserved regulatory architectures for bHLH genes.

3.4.3. Subfamily-Level Duplication Patterns

To further characterize gene family expansion at a finer evolutionary scale, duplication modes of R2R3-MYB and bHLH genes were examined at the subfamily level (Figure 4B,D). Based on phylogenetic classification, duplication origins of genes within each subfamily were categorized as singleton, whole-genome duplication (WGD), tandem duplication (TD), proximal duplication (PD), transposed duplication (TRD), or dispersed duplication (DSD). The compositions of duplication modes among subfamilies indicated heterogeneous expansion patterns. Overall, the relative contributions of different duplication types varied across subfamilies, suggesting that R2R3-MYB and bHLH genes experienced divergent evolutionary trajectories with respect to duplication and retention following gene family expansion.

Together, phylogenetic relationships, promoter cis-element composition, and subfamily-level duplication patterns indicate that the R2R3-MYB and bHLH TF families are evolutionarily conserved in *Dendrobium*, while also exhibiting diversification at the subfamily level. Specifically, promoters of R2R3-MYB genes were enriched in hormone-, light-, and stress-responsive cis-elements, whereas bHLH gene promoters were predominantly associated with development-related regulatory elements.

3.5. Tissue-Specific Expression and Functional Divergence of R2R3-MYBs and bHLHs in Dendrobium

To assess the tissue-specific expression and potential functional divergence of R2R3-MYB and bHLH TFs, we constructed the transcriptome expression profiling across eight tissues of *Dendrobium* 'Chao Praya Smile' (e.g., *D. CPS.*). Global expression patterns indicated clear tissue-dependent differences for both R2R3-MYB and bHLH genes: a subset of members showed higher expression levels or stronger tissue preference in floral buds and roots, whereas overall expression changes were relatively weaker in leaves and photosynthetic stems (PSs) (Figure 5A,B). We further identified significantly differentially expressed genes (DEGs) in four representative tissues (root, PS, leaf, and floral bud) and summarized their subfamily distributions (Figure 5D; Supplementary Tables S15 and S16), thereby charac-

terizing tissue partitioning of these TF families from three perspectives—expression variation, DEG abundance, and subfamily composition.

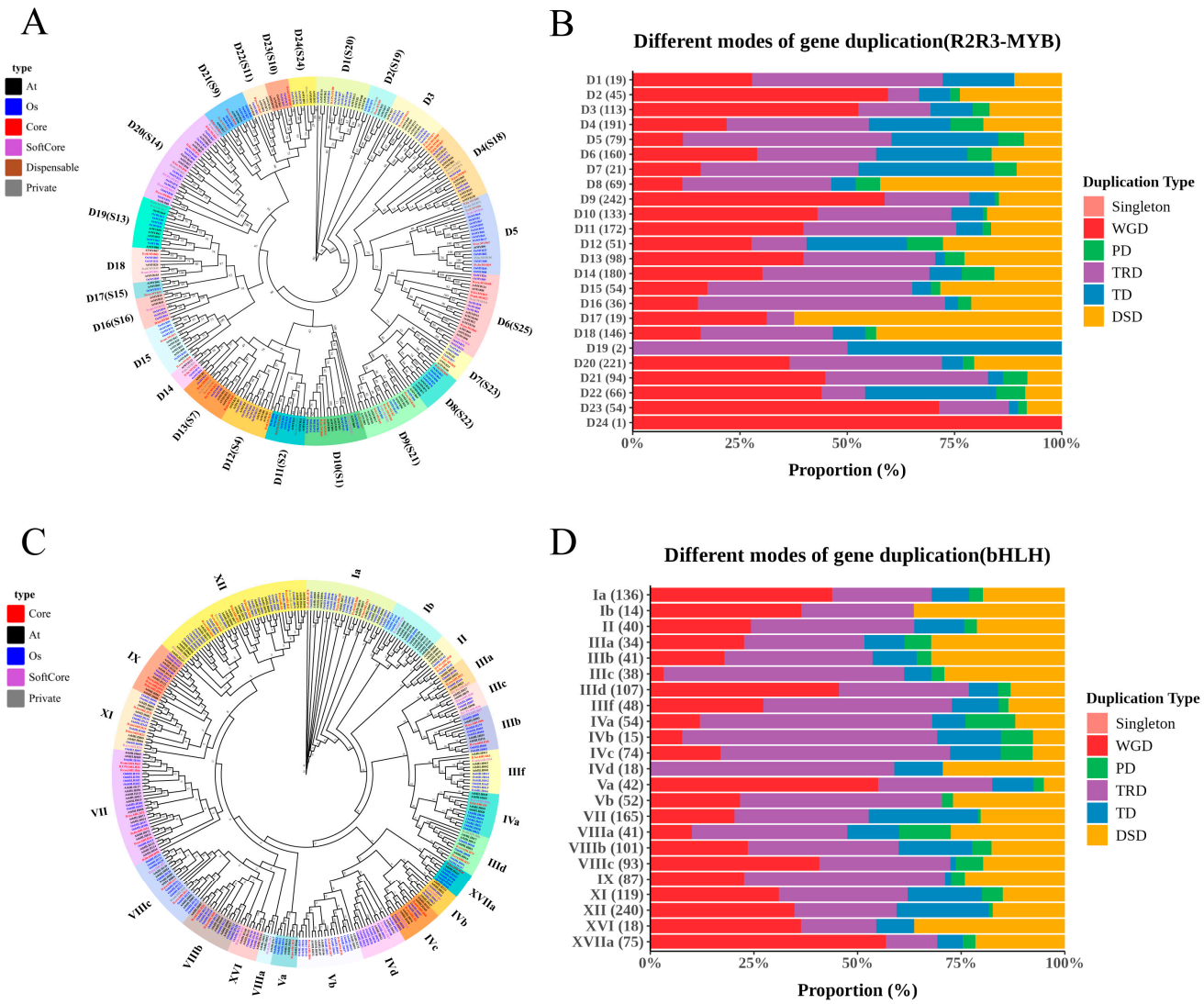


Figure 4. Phylogenetic relationships and duplication patterns of R2R3-MYB and bHLH TF families in *Dendrobium*. (**A**) Maximum likelihood (ML) phylogenetic tree of R2R3-MYB proteins from *Dendrobium*, *Arabidopsis thaliana* (At), and *Oryza sativa* (Os). The outer rings indicate the pan-genome categories of *Dendrobium* genes (core, softcore, dispensable, and private). Subfamily classifications are labeled according to established R2R3-MYB groupings. (**B**) Distribution of different gene duplication modes across R2R3-MYB subfamilies in *Dendrobium*. Stacked bar charts show the relative proportions of single-copy genes (Singleton), whole-genome duplication (WGD), proximal duplication (PD), tandem duplication (TD), transposed duplication (TRD), and dispersed duplication (DSD) within each subfamily. (**C**) Maximum-likelihood phylogenetic tree of bHLH proteins from *Dendrobium*, *Arabidopsis thaliana* (At), and *Oryza sativa* (Os), with subfamily classifications indicated. (**D**) Distribution of different gene duplication modes across bHLH subfamilies in *Dendrobium*. Stacked bar charts represent the relative contributions of various duplication types within each bHLH subfamily.

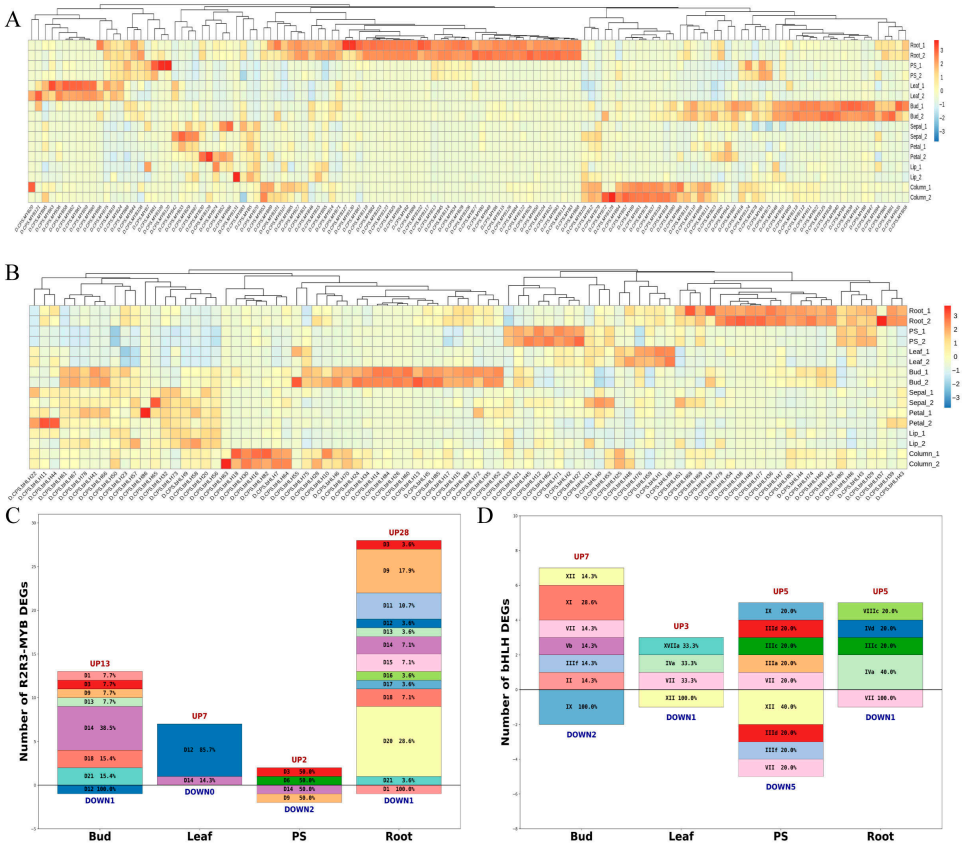


Figure 5. Expression profiles of R2R3-MYB and bHLH genes in different tissues of *Dendrobium*. (**A**,**B**) Heatmaps of gene expression: (**A**) R2R3-MYB genes; (**B**) bHLH genes. Expression levels are shown as $\log_2$-transformed FPKM values and were row-normalized prior to hierarchical clustering. Each row represents one tissue (with two biological replicates), and each column represents a single gene. (**C**,**D**) Stacked bar plots of differentially expressed genes (DEGs): The number of DEGs for R2R3-MYB (**C**) and bHLH (**D**) genes in four tissues (floral bud, leaf, photosynthetic stem (PS), and root) of *Dendrobium* 'Chao Praya Smile' are shown. Different colors correspond to different subfamilies within each gene family. "UP" modules indicate upregulated genes, while "DOWN" modules indicate downregulated genes.

4. Discussion

Dendrobium has key ornamental traits, such as flower color, scent, and form, which directly affect pollinator attraction and reproductive success, making them the primary targets in horticultural breeding [62]. Functional studies in *Dendrobium* have identified several R2R3-MYB and bHLH genes implicated in floral scent, pigmentation, and secondary metabolic regulation [15,40]. However, the functional mechanisms of many R2R3-MYB and bHLH genes in this genus remain largely unresolved [11,63]. In recent years, multiple high-quality genomes have been assembled for *Dendrobium*, representing diverse lineages within the genus, providing an effective pan-genome framework to investigate the evolution of transcription factor families in genus [30–33].

Orthologous clustering reveals a stable, conserved core of OGGs in both R2R3-MYB and bHLH families in *Dendrobium*, alongside a dynamic accessory non-core subset subject to expansion, loss, and specialization (Figure 2C,D), providing a genetic substrate for phenotypic diversification in *Dendrobium* genus. The predominance of core lineages indicates that the MYB–bHLH regulatory backbone is largely conserved across *Dendrobium*, consistent with their fundamental roles in development, secondary metabolism, and environmental responses [64]. Previous phylogenetic and comparative genomic studies on *Dendrobium* have illustrated that the genus has undergone extensive adaptive radi-

ation and lineage-specific diversification since its origin, driven by geological changes (e.g., compression of the Indian Plate), sea-level fluctuations, and shifts in precipitation patterns [65]. Specifically, the diversification of *Dendrobium* species was accelerated during the middle Miocene, a period characterized by global climatic cooling and aridity, which also triggered the independent evolution of crassulacean acid metabolism (CAM) photosynthesis in at least eight lineages of *Dendrobium*, providing novel ecological adaptations for niche expansion [66]. During this evolutionary process, genome duplication events (including whole-genome duplication shared with *Phalaenopsis*) have been identified as key drivers of genetic innovation, facilitating the expansion of gene families related to floral development, environmental adaptation, and medicinal polysaccharide synthesis—consistent with the dynamic changes observed in the non-core OGGs of R2R3-MYB and bHLH families in our study [34]. Additionally, studies focusing on *Dendrobium* species diversification have shown that pollinator-mediated selection (e.g., adaptation to specific insect pollinators) and habitat differentiation (e.g., adaptation to epiphytic, lithophytic, or terrestrial habitats) have further promoted lineage-specific specialization, which may explain the functional divergence of non-core OGGs in regulating floral traits and stress responses [67]. *Dendrobium* also retains appreciable proportions of softcore and variable OGGs (Figure 4A,C), reflecting lineage-specific expansion and diversification and implying that non-core components may constitute an important genetic substrate for the evolutionary diversification of floral and reproductive traits [68]. Selection pressure analysis based on Ka/Ks ratios showed that most duplicated gene pairs in both R2R3-MYB and bHLH families had Ka/Ks < 1, indicating that these genes have mainly undergone purifying selection during evolution (Supplementary Tables S17 and S18). In contrast, only a small number of gene pairs showed Ka/Ks > 1, suggesting that positive selection has acted on a limited subset of genes. Meanwhile, the Ka/Ks ratios and promoter sequence variation in core genes were significantly lower than those of softcore, dispensable, and private genes, supporting that core transcription factors are under stronger functional constraint. In addition, the widespread presence of duplicated gene pairs with Ka/Ks < 1 also suggests that neutral evolution and purifying selection following gene duplication have contributed greatly to the expansion and functional retention of the R2R3-MYB and bHLH families in *Dendrobium* [14]. In these two TFs, the Ka/Ks ratios of core genes and the sequence variation in their promoter regions were significantly lower than those of variable genes (softcore, dispensable, and private), indicating that core genes have experienced stronger purifying selection during evolution [69].

Secondary metabolites play important roles in floral pigmentation, scent releasing and reproductive success, of which biosynthesis is primarily controlled by the MYB–bHLH–WD40 (MBW) regulatory complex which integrates light cues, abiotic stresses, and phytohormones via the synergy between environmental and hormonal signals [70]. Consistent with this, studies on related orchid species such as *Phalaenopsis equestris* have demonstrated that the MBW complex directly regulates anthocyanin and flavonoid biosynthesis in floral tissues, with MYB and bHLH proteins acting as core regulators to fine-tune pigment accumulation and floral coloration—traits closely associated with ornamental value and pollination success [71]. Beyond orchids, similar regulatory mechanisms have been reported in other flowering plants, such as *Arabidopsis thaliana* and *Oryza sativa*, where MYB–bHLH interactions mediate the integration of environmental signals (e.g., light intensity) and hormonal cues (e.g., jasmonate) to regulate secondary metabolism, highlighting the evolutionary conservation of this regulatory module [72]. Practically, manipulating the MBW complex in orchids has been shown to alter floral color and pigment content, providing a feasible strategy for molecular breeding of new ornamental varieties with enhanced commercial value [25]. Our cis-regulatory analysis showed that the DNA-binding domains

of *Dendrobium* R2R3-MYB and bHLH proteins are highly conserved, whereas their promoter architectures diverge. It should be noted that computational prediction of cis-elements is inevitably affected by potential false-positive signals—short nucleotide sequences similar to known cis-elements may be identified by chance, though we took preliminary measures to reduce false positives by excluding low-abundance motifs and requiring high similarity to core motifs. Additionally, the choice of background sequence model is critical for reliable motif enrichment analysis; we adopted the pre-compiled *Arabidopsis thaliana* background model, a common and reasonable choice for non-model plants like *Dendrobium* due to the lack of a species-specific background, which effectively controlled for general nucleotide composition bias while providing a robust reference for motif enrichment evaluation. Our motif enrichment analysis further revealed distinct regulatory preferences between the two TF families: promoters of R2R3-MYB genes are enriched for hormone-responsive motifs and substantial fractions of stress- and light-responsive elements (Supplementary Table S7). In contrast, bHLH promoters are dominated by core and development-related elements but consistently include light-responsive motifs, jasmonate-responsive motifs, and abscisic acid-responsive motifs (Supplementary Table S8). These architectures suggest that both TFs participate not only in fundamental developmental regulation but may also participate in integrating endogenous hormonal and environmental cues to fine-tune reproductive organ development, pigment biosynthesis, and broader secondary metabolic pathways. This is consistent with prior evidence that MYB/bHLH TFs play pivotal roles in floral anthocyanin–flavonoid metabolism, morphogenesis, and scent-related metabolism [42]. The high conservation of the R2R3-MYB and bHLH functional domains further supports their conserved and essential roles in transcriptional regulation. In the R2R3-MYB DNA-binding domain, the characteristic substitution of the first tryptophan (Trp) in the R3 repeat by phenylalanine, isoleucine, or leucine represents a conserved adaptive change that maintains structural stability while reducing excessive hydrophobicity [73]. This substitution is biologically meaningful because it preserves the overall helix–helix packing required for proper folding, but introduces subtle structural flexibility that may facilitate functional diversification among different MYB subgroups. Meanwhile, the significantly higher conservation of Helix 3 reflects strong functional constraints, as this region directly mediates DNA recognition and target specificity.

For the bHLH domain, the highly conserved basic region enriched in arginine and lysine underscores its essential function in DNA binding, whereas the conserved hydrophobic residues in the two helices ensure reliable homo- and hetero-dimerization [74]. The relatively higher variability in the loop region provides the necessary structural plasticity for regulatory versatility, as demonstrated in previous studies showing that the loop region's sequence flexibility enables bHLH proteins to form diverse homo- and hetero-dimers with distinct regulatory partners, thereby expanding their functional scope [75]. Together, the conserved domain architecture and key amino acid substitution patterns observed in both transcription factor families indicate that the core DNA-binding and dimerization mechanisms are evolutionarily conserved across plant species, while minor but critical variations at key sites provide the structural basis for functional divergence and lineage-specific regulatory innovation in *Dendrobium* [25]. Our findings support the possibility that lineage-specific MYB and bHLH members contribute to species-specific floral pigmentation, organ differentiation, scent regulation, and ecological adaptation, thereby promoting floral trait diversification in *Dendrobium*.

In *Dendrobium* 'Chao Praya Smile', differential expression analysis revealed pronounced tissue-dependent differences in the magnitude of transcriptional reprogramming for the two TF families. In floral buds of this species, R2R3-MYB DEGs were predominantly upregulated (UP 13, DOWN 1), mainly assigned to subfamilies D14 and D18, indicating

strong activation of these MYB lineages during the bud stage in this species. In parallel, multiple bHLH genes were also upregulated in buds of this species, spanning subfamilies III, VII, and XI, forming a multi-subfamily induction pattern. The synchronous upregulation of MYB and bHLH genes in floral buds of this species suggests a coordinated transcriptional regulatory tendency during early floral organ differentiation in this species. In contrast, leaves of this species displayed relatively few DEGs for both families, largely restricted to a small number of subfamilies (e.g., D12 for MYB and IVa/VII for bHLH), indicating a limited scope and magnitude of differential expression in leaves under our sampling conditions. In PSs of this species, DEG numbers were likewise low, with a more balanced pattern of up- and downregulation, yet involving a relatively diverse set of subfamilies, suggesting an overall stable transcriptional landscape supported by multiple TF lineages in this tissue of the species. Roots of this species exhibited the most pronounced differential expression: R2R3-MYB DEGs were markedly more abundant than in other tissues of this species (UP 28, DOWN 1), involving multiple subfamilies (D20, D9, D14, D15, and D18), while upregulated bHLH DEGs were primarily enriched in subfamilies IVa and III, reflecting robust MYB/bHLH-associated regulatory activity in roots of this species. Overall, in this species, buds and roots showed stronger differential expression and subfamily enrichment for both R2R3-MYB and bHLH genes than leaves and PS, consistent with tissue-dependent regulatory demands in this specific species (Figure 5C,D; Supplementary Tables S15 and S16). Transcriptomic analysis observed pronounced tissue-specific expression and functional partitioning across the R2R3-MYB and bHLH families in *Dendrobium*. In floral buds, *D.CPS.MYB91* and *D.CPS.bHLH60* belong to the D13 and IIIf subfamilies, respectively, both of which are mainly enriched in flavonoid and anthocyanin biosynthesis pathways.

In *Dendrobium* 'Chao Praya Smile', these genes may exhibit coordinated expression and function through the MYB–bHLH–WD40 (MBW) regulatory axis and related networks, thereby promoting pigment accumulation and potentially further coordinating floral morphogenesis [70]. The specific functions of these candidate members of both transcription factors in floral regulation and reproductive development remain to be further experimentally validated in *Dendrobium* 'Chao Praya Smile'.

5. Conclusions

Our study presents a comprehensive pan-genomic analysis of R2R3-MYB and bHLH transcription factors across 18 *Dendrobium* genomes. We identified 3074 R2R3-MYB and 2282 bHLH genes, categorized into 69 and 61 orthologous groups, respectively. Phylogenetic analysis indicated a conserved evolutionary core in both families alongside lineage-specific diversification in variable gene sets. Examination of protein motifs, gene structures, and cis-regulatory elements further elucidated the regulatory potential of these transcription factors. Tissue-specific expression profiling in *Dendrobium* 'Chao Praya Smile' implicated these TFs in reproductive development, including floral pigmentation, secondary metabolism, and stress adaptation. Integrating pan-genomic and transcriptomic data clarified the evolutionary patterns and functional partitioning between conserved and variable gene sets, offering targets for future functional studies. Taken together, our findings advance our understanding of R2R3-MYB and bHLH TFs families in *Dendrobium* and provide a foundation for molecular breeding to improve floral traits, stress tolerance, and other important horticultural characteristics. Furthermore, our work contributes to the broader field of pan-genomics by illustrating patterns of gene family evolution and functional diversification in orchids.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy16050521/s1.

Author Contributions: Conceptualization, X.M.; methodology, X.M., T.W., M.Q., D.L. and R.G.; formal analysis, T.W., M.Q., D.L., R.G., L.B. and H.Z.; investigation, X.M., T.W., M.Q., D.L., R.G., L.B., H.Z., Y.W., Y.L. (Yufei Liu), J.S. and Y.L. (Yingjie Luo); data curation, T.W., M.Q., L.B., Y.W. and Y.L. (Yufei Liu); writing—original draft preparation, T.W., M.Q.; writing—review and editing, X.M., T.W., M.Q., D.L., R.G., L.B., H.Z., Y.W. and Y.L. (Yingjie Luo); visualization, T.W., M.Q., D.L., R.G., L.B., H.Z., J.S. and Y.L. (Yingjie Luo); supervision, X.M.; project administration, X.M.; funding acquisition, X.M. All authors have read and agreed to the published version of the manuscript.

Funding: The National Natural Science Foundation of China (Project Nos. 32470246; 31700199 to X.M.), the Natural Science Foundation of Fujian Province, China (Project No. 2021J01142 to X.M.), and the Foundation from Fujian Agriculture and Forestry University (Project No. 11899001001; KFb22112XA to X.M.).

Data Availability Statement: Genome assemblies and annotation files were obtained from Figshare (https://doi.org/10.6084/m9.figshare.26342338.v1; https://figshare.com/s/f759784e78e86ba71c7c (accessed on 28 September 2025)), the NCBI database under BioProject numbers PRJNA161865, PRJNA725550, PRJNA662181, PRJNA262478, PRJNA310678, PRJNA633886, PRJNA192198, and PRJNA739531, the National Genomics Data Center (NGDC; assembly identifier 88029), and the China National GeneBank (CNGB) Data Resources (project identifier CNP0001656). Transcriptome data were downloaded from the NCBI database under BioProject PRJNA1088629.

Conflicts of Interest: The authors declare no competing interests.

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