Transcriptome-Wide Identification and Expression Analysis of DIVARICATA- and RADIALIS-Like Genes of the Mediterranean Orchid Orchis italica

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Abstract
Bilateral symmetry of flowers is a relevant novelty that has occurred many times throughout the evolution of flowering plants. In Antirrhinum majus, establishment of flower dorso-ventral asymmetry is mainly due to interaction of TCP (CYC and DICH) and MYB (DIV, RAD, and DRIF) transcription factors. In the present study, we characterized 8 DIV-, 4 RAD-, and 2 DRIF-like genes from the transcriptome of Orchis italica, an orchid species with bilaterally symmetric and resupinate flowers. We found a similar number of DIV- and RAD-like genes within the genomes of Phalaenopsis equestris and Dendrobium catenatum orchids. Orchid DIV- and RAD-like proteins share conserved motifs whose distribution reflects their phylogeny and analysis of the genomic organization revealed a single intron containing many traces of transposable elements. Evolutionary analysis has shown that purifying selection acts on the DIV- and RAD-like coding regions in orchids, with relaxation of selective constraints in a branch of the DIV-like genes. Analysis of the expression patterns of DIV- and RAD-like genes in O. italica revealed possible redundant functions for some of them. In the perianth of O. italica, the ortholog of DIV and DRIF of A. majus are expressed in all tissues, whereas RAD is mainly expressed in the outer tepals and lip. These data allow for proposal of an evolutionary conserved model in which the expression of the orthologs of the DIV, RAD, and DRIF genes might be related to establishment of flower bilateral symmetry in the nonmodel orchid species O. italica.

Key words: Orchidaceae, DIV-like, RAD-like, MYB transcription factors, flower symmetry.

Introduction
Because the origin of angiosperms, bilateral flower symmetry (zygomorphy) has evolved several times in diverse lineages from the ancestral condition of radial symmetry (actinomorphy) (Citerne et al. 2010; Endress 2012). The transition from radial to bilateral symmetry is often related to the coevolution of specialized pollinator insects (Fenster et al. 2009; Ushimaru et al. 2009) and is considered a key innovation associated with angiosperm diversification (Vamosi and Vamosi 2010).

During the last 20 years, the molecular basis of flower symmetry has been studied in Antirrhinum majus (Coen 1996; Luo et al. 1996, 1999; Galego and Almeida 2002). In this species, four genes play a key role in establishment of the asymmetry of the corolla and individual petals: CYCLOIDEA (CYC), DICHOTOMA (DICH), RADIALIS (RAD), and DIVARICATA (DIV). CYC and DICH encode transcription factors of the TCP family that are expressed in the dorsal domains of the snapdragon flower (Luo et al. 1996, 1999; Galego and Almeida 2002; Almeida and Galego 2005). The double mutants cyc:dich have radially symmetric flowers with petals that exhibit ventral identity (Almeida et al. 1997). The DIV and RAD genes encode MYB transcription factors. The DIV protein contains two MYB domains and, although expressed in both the dorsal and ventral parts of the flower, is involved in establishment of the ventral identity (Galego and Almeida 2002). RAD is a small protein containing a single MYB domain that is expressed in the dorsal part of the snapdragon flower (Corley et al. 2005). In A. majus, the DIV and RAD genes work antagonistically to determine the dorso-ventral axis of the flower and their interaction is mediated by another MYB transcription factor, DIV-and-RAD-interacting-factor (DRIF). The RAD protein does not directly interact with DIV but binds the DRIF protein preventing DIV activation in the dorsal domain. DRIF is a co-activator of DIV that is...
expressed in the dorsal and ventral parts of the flower. The DIV-DRIF complex can interact with specific DNA regions to activate other genes involved in ventralization of the flower. In the dorsal domain of the flower, RAD competes with DIV to bind DRIF, inhibiting formation of the DIV-DRIF complex. Thus, DIV cannot activate the genes involved in ventralization in the dorsal domain (Raimundo et al. 2013).

The monocot family Orchidaceae is known for its species richness (more than 20,000) and the beauty of their flowers. Although extremely diversified among species, the orchid flowers share a common basic structure: They are zygomorphic with three outer tepals, two inner lateral tepals and an inner median tepal called labellum or lip. The innermost whorl, the column, is composed of fused male and female reproductive tissues. The pollen grains are at the top of the column and the ovary, whose maturation is triggered by pollination, is at the base (Rudall and Bateman 2002). Many orchids have a resupinate flower in which the pedicel and ovary undergo a 180° rotation resulting in a reversal of the positions of the ventral and dorsal organs in the mature flower (Rudall and Bateman 2002) (fig. 1D).

In orchids, the MADS-box genes involved in formation of the perianth are well characterized (Mondragon-Palomino et al. 2009; Aceto and Gaudio 2011; Cantone et al. 2011; Mondragon-Palomino and Theissen 2011; Pan et al. 2011, 2014; Salemme et al. 2011, 2013a; Aceto et al. 2014; Acri-Nunes-Miranda and Mondragon-Palomino 2014), and recent studies have highlighted the role of the MADS-box gene AGL6 in the formation of the orchid lip (Hsu et al. 2015; Huang et al. 2016). Conversely, few studies are available on orchid TCP genes (Mondragon-Palomino and Trontin 2011; De Paolo et al. 2015; Lin et al. 2016), and the MYB genes that are possibly involved in orchid flower symmetry have not been characterized. The recent release of the genomes of Phalaenopsis equestris and Dendrobium catenatum orchids (Cai et al. 2015; Zhang et al. 2016) and the availability of many orchid transcriptomes represent a valuable resource for analysis of gene families in nonmodel orchid species. The aim of this work was to identify and characterize the DIV- and RAD-like genes expressed in the inflorescence transcriptome of the Mediterranean orchid Orchis italica (sub-family Orchidoideae, tribe Orchidinae) that is characterized by a dense oval inflorescence with numerous pink resupinate flowers (Montieri et al. 2004) (fig. 1A–C). The orthologs of these genes were also identified in the P. equestris and D. catenatum (Epidendroideae) orchid genomes to perform phylogenetic and evolutionary analyses. In addition, the expression patterns of orchid DIV-, RAD-, DRIF-, and AGL6-like genes

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**Fig. 1.**—The Mediterranean orchid Orchis italica. (A) Single floret (early stage), (B) Inflorescence after anthesis; (C) Single floret after anthesis (late stage); (D) diagram showing the positions of the organs of the first (continuous line) and second (dotted line) whorls of the perianth before (left) and after (right) resupination; Te_out, outer tepal; Te_inn, inner tepal.
were examined in different tissues of *O. italica* to evaluate their possible involvement in the bilateral symmetry of the orchid flower.

### Materials and Methods

#### Plant Material

The *Orchis italica* plants used in this study were grown in the greenhouse of the Department of Biology of the University of Naples Federico II (Napoli, Italy). Two developmental stages were examined: Bud stage before anthesis (early) and mature inflorescence after anthesis (late) (fig. 1A–C). Single florets before anthesis were used for *in situ* hybridization experiments. After anthesis, single florets of the same inflorescence were collected, and some were dissected to separate the floral tissues (outer tepals, inner lateral tepals, lip, column, and ovary before pollination). Leaf tissue was also collected. These tissues were stored in RNA-later (Ambion) until nucleic acid extraction.

#### Identification of Orchid DIV- and RAD-Like Genes

The DIV and DVL1 (AAL78741, AAL78742) and RAD and RAD-like (AAK48042, AB14752, AB14753, ABI14755, AJ791699, AJ793240) proteins of *Antirrhinum majus* were used as queries to perform a TBLASTN search (e-value 1e–003) against the inflorescence transcriptomes of *Orchis italica* (Orchidoideae) (De Paolo et al. 2014) and *Ophrys* (Orchidoideae) (Sedeek et al. 2013) and against the genomes of *Phalaenopsis equestris* (Epidendroideae) (Cai et al. 2015) and *Dendrobium catenatum* (Epidendroideae) (Zhang et al. 2016). The same TBLASTN search was conducted on the coding sequences (CDSs) of *P. equestris* downloaded from ftp://ftp.genomics.org.cn/ from BGZS/20130120/. The CDSs with significant hits were used to perform a BLASTN search on the genomic scaffolds of *P. equestris* to verify the eventual presence of introns in the DIV- and RAD-like genes and to identify the exon/intron junctions. Based on the results obtained from *P. equestris*, the scaffolds of *D. catenatum* were scanned to verify the presence and position of introns in regions encoding for DIV- and RAD-like proteins. Virtual translation of the selected nucleotide sequences of *O. italica*, *Ophrys*, *P. equestris*, and *D. catenatum* was performed to check for indels and/or stop codons in the CDSs. Finally, to identify DIV- and RAD-like orthologs in orchids, the best reciprocal hits were obtained from pairwise all-versus-all BLASTP searches conducted between the DIV- and RAD-like predicted amino acid sequences of *O. italica*, *Ophrys*, *P. equestris*, *D. catenatum*, and *A. majus*. In addition, the orthology of these amino acid sequences was predicted through a BLASTP search against the nonredundant protein database and an OrthoMCL analysis (Li et al. 2003). A BLASTCLUST search was conducted for paralog identification within each orchid species using the following parameters: e-value \( \leq 10^{−10} \) and 30% minimum similarity (−S 30) over 75% of the protein (−L 0.75) (Ponting and Russell 2002; Devos et al. 2016).

#### Analysis of the DIV- and RAD-Like Transcripts of *O. italica*

Total RNA was extracted from 10 pooled florets collected from a single inflorescence of *O. italica* after anthesis using Trizol (Ambion) followed by DNase treatment. After spectrophotometric quantification using a Nanodrop 2000 (Thermo Fisher Scientific), RNA (500 ng) was reverse transcribed using the Advantage RT-PCR kit (Clontech) and an oligo dT primer. Using the nucleotide sequences selected from the floral transcriptome of *O. italica*, primer pairs (listed in supplementary table S1, Supplementary Material online) were designed to specifically amplify the entire CDS of the DIV- and RAD-like transcripts, spanning from the 5′ to the 3′ UTR. When the *in silico* assembled DIV- and RAD-like sequence of *O. italica* did not include the 5′ and/or 3′ UTR (OITA_12910, OITA_36312, and OITA_72143), specific primers were designed to perform 5′ and 3′ RACE experiments using the FirstChoice RLM-RACE kit (Thermo Fisher Scientific). PCR amplifications were conducted using 30 ng of first strand cDNA, 0.20 mM dNTPs, 0.5 \( \mu \)M of each primer, 1 × buffer and 2.5 U HotMaster Taq DNA polymerase (5 Prime). The thermal cycle was as follows: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 60 °C for 20 s, 65 °C for a time dependent upon the amplicon size (from 30 s to 3 min), followed by a final extension for 10 min at 65 °C. The amplicons were cloned into the pSC-A-amp/kan vector (Agilent), sequenced using the T3 and T7 plasmid primers and analyzed using an ABI 310 Automated Sequencer (Applied Biosystems).

#### Structural Analysis of the DIV- and RAD-Like Genes of *O. italica*

The total DNA was extracted from leaf tissue of *O. italica* using the ISOLUTE II Plant DNA kit (Bioline) followed by RNase treatment and quantification. Based on the intron position of the DIV- and RAD-like genes of *P. equestris* and *D. catenatum*, specific primer pairs (listed in supplementary table S2, Supplementary Material online) were designed to amplify the introns of these genes in *O. italica*. One hundred-fifty ng of *O. italica* genomic DNA were used as a template in PCR amplifications using the specific primer pair and the Herculase II Fusion DNA polymerase (Agilent) in the amplification conditions suggested by the manufacturer. When the first reaction did not produce a sufficient amount of amplification product, a second reaction was conducted using a 1:10 (v/v) dilution of the first reaction.
with nested primers. dATP tailing was performed on the ends of the amplicons by adding 0.5 U of DreamTaq DNA polymerase (Thermo Fisher Scientific) and 100 μM dATP to the PCR reaction and incubating at 72 °C for 15 min. The amplification products were cloned and sequenced as described above using vector- and intron-specific primers (supplementary table S2, Supplementary Material online). When the intron size exceeded 5,000 bp, direct sequencing reactions were performed on the amplification product. The nucleotide sequences of the DIV- and RAD-like genes of *O. italica* were deposited in GenBank and their accession numbers are listed in table 1.

The intron nucleotide sequences of the DIV- and RAD-like genes of *O. italica*, *P. equestris*, and *D. catenatum* were scanned for transposable/repetitive elements using CENSOR software (Kohany et al. 2006).

### Table 1

The DIV- and RAD-Like Orchid Genes Examined

| Subfamily | Species   | Transcript_Name | cDNA_Size | ORF_Size | Intron_Size | Accession/Scaffold |
|-----------|-----------|-----------------|-----------|----------|-------------|--------------------|
| DIV-like  | *O. italica* | OITA_9548      | 1,361     | 864      | 76          | KY089088           |
|           |           | OITA_23026*    | 737       | 528      | 111         | KY089095           |
|           |           | OITA_3530      | 1,901     | 873      | 201         | KY089091           |
|           |           | OITA_35312     | 1,182     | 894      | 1,615       | KY089094           |
|           |           | OITA_13252*b   | 1,694     | 861      | 3,591       | KY089093           |
|           |           | OITA_13233     | 1,427     | 840      | ~5,000      | KY089092           |
|           |           | OITA_8681      | 1,472     | 855      | ~6,000      | KY089089           |
|           |           | OITA_12910     | 1,101     | 861      | ~10,000     | KY089090           |
|           | Ophrys    | OPH_5397       | 465       | 462      | Unknown     |                    |
|           |           | OPH_23790      | 809       | 708      | Unknown     |                    |
|           |           | OPH_29334      | 1,384     | 840      | Unknown     |                    |
|           |           | OPH_22868      | 1,145     | 675      | Unknown     |                    |
|           | *P. equestris* | PEQU_28155     | 891       | 246      | Unknown     | Scaffold000684_20  |
|           |           | PEQU_02344     | 510       | 79       | Unknown     | Scaffold000002_3686|
|           |           | PEQU_00313     | 846       | 276      | Unknown     | Scaffold000002_542 |
|           |           | PEQU_37054     | 891       | 2,090    | Unknown     | Scaffold000306_1   |
|           |           | PEQU_03826     | 861       | 12,810   | Unknown     | Scaffold000002_5659|
|           |           | PEQU_21184     | 870       | 5,008    | Unknown     | Scaffold000665_25  |
|           |           | PEQU_09358     | 885       | 174      | Unknown     | Scaffold000006_57  |
|           | *D. catenatum* | DCAT_JSDN015056792 | 888       | 124      | Unknown     | JSDN015056792      |
|           |           | DCAT_JSDN015046716 | 525       | 87       | Unknown     | JSDN015046716      |
|           |           | DCAT_JSDN015069654 | 876       | 275      | Unknown     | JSDN015069654      |
|           |           | DCAT_JSDN015055018 | 882       | 1,327    | Unknown     | JSDN015055018      |
|           |           | DCAT_JSDN015002760 | 804       | 8,197    | Unknown     | JSDN015002760      |
|           |           | DCAT_JSDN015031580 | 867       | 2,705    | Unknown     | JSDN015031580      |
|           |           | DCAT_JSDN015018459 | 855       | 3,275    | Unknown     | JSDN015018459      |
|           |           | DCAT_JSDN015042335 | 891       | 231      | Unknown     | JSDN015042335      |
| RAD-like  | *O. italica* | OITA_56510     | 420       | 282      | 1,012       | KY089097           |
|           |           | OITA_103296    | 406       | 285      | 1,056       | KY089098           |
|           |           | OITA_32153     | 369       | 249      | 1,135       | KY089096           |
|           |           | OITA_72143     | 543       | 267      | No          | KY089099           |
|           | Ophrys    | OPH_2593       | 660       | 282      | Unknown     |                    |
|           | *P. equestris* | PEQU_08237     | 288       | 1,775    | Unknown     | Scaffold000054_59  |
|           |           | PEQU_31458     | 294       | 864      | Unknown     | Scaffold000046_4   |
|           |           | PEQU_40575     | 279       | 1,191    | Unknown     | Scaffold198270_12  |
|           |           | PEQU_25415     | 249       | No       | Unknown     | Scaffold000067_1   |
|           |           | PEQU_05110     | 294       | No       | Unknown     | Scaffold000860_202 |
|           | *D. catenatum* | DCAT_JSDN015033743 | 288       | 1,240    | Unknown     | JSDN015033743      |
|           |           | DCAT_JSDN015033707 | 285       | 1,178    | Unknown     | JSDN015033707      |
|           |           | DCAT_JSDN015041675 | 279       | 1,108    | Unknown     | JSDN015041675      |
|           |           | DCAT_JSDN015013347 | 324       | No       | Unknown     | JSDN015013347      |
|           |           | DCAT_JSDN015059067 | 303       | No       | Unknown     | JSDN015059067      |

*NOTE.*—The size is expressed in nucleotides.

*ā* Alternative splicing with intron retention resulting in a premature stop codon.

*ā* Alternative splicing of an additional intron within the 5' UTR (88 bp) that is also conserved in PEQU (86 bp) and DCAT (90 bp).
**Phylogenetic and Evolutionary Analyses**

The predicted amino acid sequences of the DIV- and RAD-like proteins of *O. italica*, *Ophrys*, *P. equestris*, and *D. catenatum* were aligned using MUSCLE (Edgar 2004) and manually adjusted. The corresponding alignments of the DIV- and RAD-like CDSs were obtained using PAL2NAL (Suyama et al. 2006). Maximum likelihood and neighbor joining (NJ) trees of the DIV- and RAD-like amino acid sequences were constructed using MEGA7 software (Tamura et al. 2013) using the JTT + G amino acid substitution model with 1,000 bootstrap replicates.

The presence of shared conserved motifs among the predicted amino acid sequences of the DIV- and RAD-like proteins of *O. italica*, *Ophrys*, *P. equestris*, and *D. catenatum* was verified using the MEME online tool (Bailey et al. 2009), with the search parameters set to any number of repetitions, an optimum width from 6 to 50 and a maximum number of motifs of 10.

The DIV- and RAD-like CDSs of *O. italica*, *Ophrys*, *P. equestris*, and *D. catenatum* were analyzed to test for variation of evolutionary rates at specific codons in the sequences and among branches with the CODEML program from PAML v.4.8 (Yang 1997). Different models (branch, sites, branch-sites, and clades) were compared with measure the ω value (ratio between nonsynonymous and synonymous substitution rates) of the DIV- and RAD-like nucleotide sequences. When ω is 1, neutral selection is acting on the examined sequences whereas purifying or positive selection drive their evolution when it is significantly lower or higher than 1, respectively (Yang and Bielawski 2000). The branch models assume one or different ω values among the branches of the DIV- and RAD-like tree. The sites models verify the presence of positively selected codons among the sequences, and the branch-sites models test for positive selection on individual codons in specific branches of the tree. The clade models check for the presence of different selective constraints between clades after gene duplication. A likelihood ratio test was applied to establish which model best fits the data.

**Identification of the DRIF- and AGL6-Like Transcripts of O. italica**

The sequence of the DRIF proteins of *A. majus* (DRIF1, AGL11918; DRIF2, AGL11919) (Raimundo et al. 2013) and of the AGL6 proteins of the *Phalaenopsis aphrodite* orchid (AGL6-1, PATC154379; AGL6-2 PATC138772) (Su et al. 2013) were used as queries to perform a TBLASTN search against the inflorescence transcriptome of *O. italica*. The total RNA was extracted from floral tissues of *O. italica* after anthesis (fig. 1B and C). Outer tepals (Te_out), inner lateral tepals (Te_inn), labellum (Lip), column (Co), and ovary not pollinated (Ov). The total RNA was also extracted from leaf tissue. After reverse transcription, 30 ng of the first strand cDNA from each tissue were amplified using real-time PCR with SYBR Green PCR Master Mix (Life Technologies) in technical triplicates and biological duplicates and the 5.8S RNA as the endogenous control gene (De Paolo et al. 2015). Specific primer pairs (supplementary table S2, Supplementary Material online) were designed to evaluate the relative expression of the DIV-, RAD-, DRIF- and AGL6-like transcripts of *O. italica*, including two differentially spliced DIV-like transcripts. The relative expression ratio (Rn) was calculated by applying the formula:

$$Rn = \left(\frac{1 + E_{\text{target}}}{1 + E_{5.8S}}\right)^{\frac{\Delta CT_{\text{target}}}{\Delta CT_{5.8S}}}$$

where $E$ is the PCR efficiency and CT the threshold cycle. The mean Rn and standard error (SE) were calculated for each tissue and the statistical significance of the mean Rn differences among and between tissues was assessed using the ANOVA test followed by the Tukey Honestly Significant Difference technique post hoc, and two-tailed t-tests, respectively.

**Results**

**Identification and Structure of Orchid DIV- and RAD-Like Genes**

The analysis of the inflorescence transcriptome of *O. italica* revealed 8 DIV-like transcripts containing two MYB domains. Their lengths range from 737 to 1901 bp and their predicted protein products vary from 175 to 297 amino acids (table 1). The PCR amplification on the inflorescence cDNA of *O. italica* was followed by cloning, sequencing and BLAST analysis validated their expression in the floral tissues. The OITA_23026 and
Phylogenetic and evolutionary analyses because full length transcripts, they were included in the subsequent identification of four DIV DNA of O. italica that belongs to the same sub-family of Orchidoideae. The intron size of the P. equestris and D. catenatum orthologs (table 1). Due to their large size (supplementary fig. S1, Supplementary Material online), the introns of the OITA_13233 (~5,000 bp), OITA_8681 (~6,000 bp), and OITA_12910 (~10,000 bp) genes were only partially sequenced.

Nucleotide sequence analysis performed with CENSOR revealed the presence of traces of transposable/repetitive elements in the introns of OITA_13252 and OITA_13233 and their orthologs in P. equestris and D. catenatum. Table 2 reports the name and class of transposable/repetitive elements found in the introns of orchid DIV-like genes.

There are four RAD-like transcripts containing a single MYB domain in the inflorescence transcriptome of O. italica. Their lengths range from 543 to 420 bp and they encode for 82- to 94-amino acid proteins (table 1). The validation via PCR amplification, cloning, sequencing and BLAST analysis confirmed their expression in the inflorescence of O. italica.

Five RAD-like genes were identified in the genomes of P. equestris and D. catenatum. Three contain a single intron (table 1) with canonical donor and acceptor splicing sites and two do not have any introns. PCR amplification of the genomic DNA of O. italica allowed for amplification of a single intron in three of the four RAD-like genes (table 1). The RAD-like gene OITA_32153 of O. italica shows a premature stop codon compared with its ortholog in P. equestris and D. catenatum, shifting the intron position in the 3′ UTR.

A single RAD-like transcript was identified in the Ophrys transcriptome and was included in the phylogenetic and evolutionary analyses.

Fig. 3A shows the ML tree obtained from amino acid alignment of the DIV- and RAD-like sequences identified in this study. Its topology overlaps with that of the NJ tree (data not shown). Both the DIV- and RAD-like groups have high bootstrap support values (87%). In the DIV-like group, there are three well-supported subgroups (1–3), each with O. italica, P. equestris and D. catenatum sequences, whereas the Ophrys sequences are only in subgroups 1 and 2. There are two well-supported sub-groups within the RAD-like group: The largest includes O. italica, Ophrys, P. equestris and D. catenatum sequences and the other includes only two sequences, one P. equestris and one D. catenatum.

Analysis of the conserved domains (fig. 3B) revealed that nine amino acid motifs are shared among groups of sequences. Motifs 1 to 3 are part of the MYB DNA-binding domain (supplementary fig. 5), Supplementary Material online) and the others have unknown function. Motifs 2 and 3 are shared by all sequences except OPH_5397; motif 1 is in all...
the DIV-like sequences. The distribution of these and the other motifs among the sequences strictly reflects the topology of the ML tree.

The ratio between the mean nonsynonymous and synonymous substitution rates (ω) of orchid DIV- and RAD-like coding regions indicates that purifying selection acts on these genes (ω < 1). Different evolutionary models were compared with determine if the ω ratios differed significantly among the different branches detectable in the phylogenetic tree of the DIV- and RAD-like genes. The results obtained are shown in supplementary table S3, Supplementary Material online; supplementary table S4, Supplementary Material online, reports the statistical significance of each comparison. Within the DIV-like genes, the one-ratio model (that assumes an equal ω for all the branches) can be excluded in favor of the two- and three-ratio models (that consider two and three different ω values, respectively). Excluding the comparison between the three-ratio model and the two-ratio model 1 (that assumes equal ω for DIV-like subgroups 2 and 3), the three-ratio model fits the data better than the two-ratio models. The clade model 2, which assumes different selective pressure on a proportion of sites of the DIV-like subgroup 2

| Sub-family | Species | Gene_Intron | Element_Name | Class |
|------------|---------|-------------|--------------|-------|
| DIV-like   | O. italica | OITA_13252 | hAT-4_STu | DNA/hAT |
| | | | EnSpm2_SB | DNA/EnSpm/CACTA |
| | | | RTE-N18_ATr | NonLTR/RTTE |
| | | | Au | NonLTR/SINE/SINE2 |
| | OITA_8681 | RI_GcSta.3_1-I | LTR/Gypsy |
| | | | MuDR-6_VV | DNA/MuDR |
| | | | MtpHE-E-1a | DNA/Harbinger |
| | PEQU_21184 | Copia-62_BD-LTR | LTR/Copia |
| | PEQU_03826 | hAT-1N1_TC | DNA/hAT |
| | | | LTR-13_Mad | LTR |
| | | | L1-36_NN | NonLTR/L1 |
| | | | Gypsy-20_RC-I | LTR/Gypsy |
| | | | Gypsy-1_RC-I | LTR/Gypsy |
| | | | Gypsy-4_VC-I | LTR/Gypsy |
| | | | Gypsy-4_VC-I | LTR/Gypsy |
| | | | Copia-4_Ba-I | LTR/Copia |
| | | | RTE-1_GM | NonLTR/RTTE |
| | | | EnSpm-18_Aly | DNA/EnSpm/CACTA |
| | | | Gypsy-133_GM-LTR | LTR/Gypsy |
| | DCAT_JSDN01S015018459 | Gypsy-48_Ma-A | LTR/Gypsy |
| | | | Helitron-1_PTr | DNA/Harbinger |
| | DCAT_JSDN01S015031580 | hAT-1_JC | DNA/hAT |
| | | | Gypsy-24_RC-I | LTR/Gypsy |
| | | | RTE-1_NN | NonLTR/RTTE |
| | | | ENSPM1_VV | DNA/EnSpm/CACTA |
| | | | Helitron-1_BDi | DNA/Harbinger |
| | | | Copia-45_PX-I | LTR/Copia |
| | | | Gypsy-27_VV-I | LTR/Gypsy |
| | | | RTE-1_GM | NonLTR/RTTE |
| RAD-like   | O. italica | OITA_32153 | Copia-128_SB-I | LTR/Copia |
| | | | OITA_56510 | LTR/Copia |
| | | | OITA_103296 | LTR/Copia |
| | PEQU_40552 | MuDR-21_VV | DNA/MuDR |
| | | | Helitron-1_GM | DNA/Harbinger |
| | | | HELITRONy1D | DNA/Harbinger |
| | | | WIS2_TM_LTR | LTR/Copia |
| | | | Copia-58_PX-I | LTR/Copia |
| | | | Caulimovirus_1_PTr | IntegratedVirus/Caulimovirus |
| | DCAT_JSDN01S015041675 | HARB-N1_Aly | DNA/Harbinger |
| | DCAT_JSDN01S015033707 | HARB-7_FV | DNA/Harbinger |
relative to the other subgroups, is statistically more supported than its null model, showing that a significant proportion of sites (~34%) of the sequences of subgroup 2 has a different $\omega (0.01754)$ from that of the other subgroups (0.03487). The sites and branch-sites models, that allow for positive selection in specific sites and branches of the DIV-like tree, do not fit the data better than the null model of nearly neutral evolution.

Within the RAD-like genes, the one-ratio model can be excluded in favor of the two-ratio model. The comparison of the sites and branch-sites models does not statistically support the alternative models of positive selection. Moreover, the comparison between the clade model and its null model does not support the presence of sites with different selective pressures.

**Identification of DRIF- and AGL6-Like Transcripts of *O. italica***

There are two putative DRIF-like transcripts in the *O. italica* inflorescence transcriptome: OITA_10599 (1250 nucleotides) and OITA_6376 (2083 nucleotides). The first encodes for a 258-amino acid protein that has 56% identity with the DRIF1 protein of *A. majus*; the second encodes for a 305 amino acid protein that has 39% identity with the DRIF2 protein of}

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**Fig. 3**.—Phylogenetic analysis of orchid DIV- and RAD-like proteins. (A) ML tree showing three major groups in the DIV-like sub-family (1–3) and two in the RAD-like subfamily. The numbers above the nodes indicate the bootstrap percentages greater than 50%; (B) Diagram of the conserved motifs shared by DIV- and RAD-like orchid proteins (see supplementary fig. S2, Supplementary Material online); (C) Structure of orchid DIV- and RAD-like genes. Gray boxes indicate exons, dotted lines indicate introns and black boxes are UTRs. The genes marked with an asterisk are subjected to alternative splicing. The size of the boxes and lines are in scale, except for the large introns (interrupted lines).
A. majus. Higher identity scores (maximum 65% for the first and 56% for the second) after BLASTP searches against the nonredundant protein database correspond to uncharacterized proteins. Both transcripts were successfully PCR amplified from cDNA of the inflorescence of O. italica and their sequence corresponds to that of the transcripts in the assembled transcriptome. DRIF was only characterized in A. majus (Raimundo et al. 2013) and, excluding the presence of two conserved MYB domains, appears to be a poorly conserved protein. BLASTP analysis using the DRIF1 protein of A. majus as the query produces matches with uncharacterized proteins that have 75–78% identity in Lamiales (the same order of Antirrhinum) and the highest percentage of identity in monocots is approximately 60% (e.g., Musa acuminata). Therefore, although not well conserved, we considered OITA_10599 to be the putative ortholog of DRIF1 of A. majus (56% amino acid identity).

There are two AGL6-like transcripts in the inflorescence transcriptome of O. italica, like in Phalaenopsis (Su et al. 2013; Huang et al. 2016) and Oncidium, whereas in Erycina pusilla and Cymbidium goeringii there are three AGL6-like genes (Dirks-Mulder et al. 2017). OITA_1386 (1369 nucleotides) encodes for a 239-amino acid protein that has ~83% identity with AGL6-1 of P. aphrodite and OITA_4335 (1045 nucleotides) encodes for a 243-amino acid protein that has ~86% identity with AGL6-2. After a BLASTP search against the nonredundant protein database using the translated amino acid sequences of OITA_1386 and OITA_4335 as queries, the highest percentages of amino acid identity were obtained for the AGL6 proteins of the Dendrobium hybrid cultivar (87%) and Cymbidium (86%) orchids, respectively. PCR validation confirmed that these transcripts are expressed in the inflorescence tissue of O. italica.

Expression Patterns of DIV-, RAD-, DRIF-, and AGL6-Like Genes in O. italica

RNA in situ hybridization shows the expression of OITA_9548 (putative ortholog of the A. majus DIV gene) and OITA_10599 (putative ortholog of the A. majus DRIF1 gene) in all the early floral tissues of O. italica, whereas the transcript OITA_56510 (putative ortholog of the A. majus RAD gene) is localized in the early lip and outer tepals (fig. 4).

After anthesis, among the DIV-like transcripts some have similar expression patterns and others are very different (fig. 5; supplementary fig. S3, Supplementary Material online).
The OITA_9548 transcript is still expressed at high levels in all the perianth tissues (outer and inner tepals and lip). In addition, it is expressed in the column, ovary and leaf. The OITA_3530 transcript has an expression profile similar to OITA_9548 with lower levels in all tissues and with significant differences between the outer and inner tepals and lip. OITA_8681, OITA_3531, and OITA_13233 are also expressed in all tissues of the perianth; however, their expression profiles are quite different in the column, ovary and leaf, with very low OITA_3531 expression levels in the ovary and leaf and OITA_13233 levels also in the column. OITA_12910 has a very different expression profile from those of the others and is predominantly expressed in the column only. The two differentially spliced transcripts of OITA_23026 differ in their expression level pattern, which is significantly lower in all tissues (excluding leaf) for the isoform that retains the intron (OITA_23026_AS). The expression profile of the other differentially spliced transcript OITA_13252 is also different for the two isoforms. In this case, the isoform that retains the intron in the 5' UTR (OITA_13252_AS) has significantly higher expression levels in all tissues (excluding leaf) than the isoform without the intron (fig. 6).

The RAD-like transcripts have similar expression profiles in the perianth tissues after anthesis, although with different expression levels. They are expressed in the outer tepals and lip with lower expression in the inner tepals. OITA_32153 is also highly expressed in the column and leaf where OITA_56510 is also expressed. All the transcripts are also expressed in the ovary (fig. 5; supplementary fig. S4, Supplementary Material online).

The OITA_10599 transcript is expressed in all floral tissues after anthesis and has a significantly higher expression in the lip whereas the OITA_6376 transcript is expressed at similar levels in all tissues except the ovary and leaf, where it is very weakly expressed (fig. 5; supplementary fig. S5, Supplementary Material online).

The AGL6-like transcript OITA_1386 (AGL6-1) is highly expressed in the lip and, at lower levels, in the outer tepals. OITA_4335 (AGL6-2) is highly expressed in the outer tepals and in the ovary at a much lower level (fig. 5; supplementary fig. S6, Supplementary Material online).

**Discussion**

Structure and Evolution of Orchid DIV- and RAD-Like Genes

Excluding Ophrys, the number of both DIV- and RAD-like genes found in orchids is very similar to that found in other plants; for example, there are up to eight DIV-like genes in
Dipsacales (Howarth and Donoghue 2009) and six RAD-like genes (RAD and five RAD-like) in A. majus and Arabidopsis thaliana (Baxter et al. 2007). This suggests that the number of genes identified in the present study reflects the real DIV- and RAD-like copy number of orchids. The lower number of DIV- and RAD-like transcripts found in the Ophrys transcriptome is probably due to the different assembly strategies used to obtain and assemble the reads for this species. The differences relative to the number of RAD-like genes between O. italica (4) and Phalaenopsis-Dendrobium (5) could be due to the very low expression of the homolog of the fifth RAD-like gene in the O. italica inflorescence, resulting in its absence in the assembled transcriptome. An alternative hypothesis is that there are only four RAD-like genes in the O. italica genome.

The presence of sequences of O. italica, P. equestris, and D. catenatum in almost all the sub-groups of the ML tree (fig. 3A) suggests that the duplications originating the different DIV- and RAD-like orchid genes occurred before the divergence of Orchidoideae and Epidendroideae. The split of the DIV-like genes into three ortholog groups (supplementary table S1, Supplementary Material online) is confirmed by the topology of the ML tree (fig. 3A) and is in agreement with the results obtained in Dipsacales (Howarth and Donoghue 2009), revealing that the origin of these ortholog groups predates the monocot/dicot divergence. The gene organization (intron number and size) and the distribution of the conserved motifs also reflects the division of orchid DIV-like genes into three groups (fig. 3B and C, supplementary fig. S2,
Supplementary Material online). Two ortholog groups are detectable in the RAD-like orchid genes (supplementary table S1, Supplementary Material online). This partition is in general agreement with the ML tree topology (fig. 3A) and with the distribution of the conserved motifs (fig. 3B; supplementary fig. S2, Supplementary Material online). Interestingly, some of the orchid RAD-like genes have a single intron whereas others are intronless (fig. 3C). A unique situation was found in OITA_32153 of *O. italica*, with a premature stop codon that places the intron in the 3' UTR. Although this might appear to be an intermediate situation between intron presence and absence, its orthologs in *Phalaenopsis* and *Dendrobium* have a canonical intron in the coding sequence, suggesting that this intron shift is a derived character of *O. italica*. The intron presence/absence and the presence of an intron in the 3' UTR in the RAD-like genes was also described in *A. majus* and *A. thaliana* (Baxter et al. 2007) and might reflect the evolution of this gene sub-family from an ancestral gene structure that included two exons to a more recent gene organization with a continuous open reading frame. However, it is not possible to exclude that these different gene structures originated independently in the different lineages.

The intron sizes of the orchid DIV- and RAD-like genes are conserved among the orthologs (fig. 3C). Orchids appear to have more large introns than other plant families, with large introns containing numerous traces of transposable elements (Cai et al. 2015; Zhang et al. 2016). Large introns of the DIV- and RAD-like orchid genes contain traces of repetitive/transposable elements (table 2), confirming the results found for other orchid genes (Salemme et al. 2013a, 2013b). In orchids, the abundance of repetitive/transposable elements within large introns might have a functional significance, affecting gene expression. The presence of repetitive/transposable elements within introns can drive antisense transcription and/or promote the formation of heterochromatin, resulting in reduction of transcriptional levels (Feschotte 2008).

The analysis of the evolutionary pressure acting on the orchid DIV- and RAD-like coding sequences shows evidence of strong purifying selection (supplementary table S3, Supplementary Material online). However, different selective constraints act on the three ortholog groups of the DIV-like genes, whereas the evolutionary rates of the RAD-like orchid genes appear more uniform.

**Expression Profiles of DIV-, RAD-, DRIF-, and AGL6-Like Genes in O. italica**

Gene expression patterns are an important tool to infer gene function, in particular in species such as *O. italica* in which it is not possible to perform functional studies based on knock-out techniques.

The transcript OITA_9548 is the putative ortholog of the DIV gene of *A. majus* and its expression profile in the early floral tissues of *O. italica* is similar to that of DIV, expressed in all the parts of the snapdragon flower (Almeida et al. 1997; Galego and Almeida 2002). The OITA_56510 transcript is the putative ortholog of the RAD gene of *A. majus* that is expressed in the dorsal domain of the snapdragon flower. The presence of transcript OITA_56510 in the early lip tissue of *O. italica* resembles the expression profile of the *A. majus* RAD gene. Also the expression profile of the putative ortholog of DRIF I of *A. majus*, OITA_10599, is similar to that described in *Antirrhinum*; it is expressed in all the early floral tissues and in the lip it could bind the RAD-like OITA_56510 and inhibit the formation of the DIV-DRIF complex, thus preventing the activation of ventralization genes. In addition, both the RAD-like OITA_56510 and DRIF-like OITA_10599 are also expressed in the outer tepals, where their interaction could have the same effect as in the lip, inhibiting ventralization. The expression profile of these three transcripts is conserved in the floral tissues after anthesis, where the presence of the transcript of the RAD ortholog is detectable in the ventral part of the flower of *O. italica*. This is because, after resupination, the median inner tepal (dorsal structure) twists and becomes a ventral structure. Taken together, the expression data support a model of interaction among MYB transcription factors to determine the bilateral symmetry of the flower generally conserved between *A. majus* and *O. italica*. However, some differences should be introduced to fit the uniqueness of the orchid flower. This model should be integrated with the expression pattern of the AGL6-like genes whose role in the development of the orchid flower might be prominent in the formation of the lip, as previously hypothesized in *Phalaenopsis* and *Oncidium* orchids (Hsu et al. 2015; Huang et al. 2016), and the outer tepals.

In general, the expression levels of the DIV-like genes in *O. italica* after anthesis are inversely correlated with the intron size, in agreement with the results obtained from the genome-wide analysis of *P. equestris* (Cai et al. 2015). The more efficient transcription of short mRNA molecules than that of longer paralogs is probably due to the lower energetic costs of transcribing and processing shorter transcripts (Castillo-Davis et al. 2002). In addition, the presence of traces of repetitive/transposable elements within the large introns of the DIV-like genes can negatively affect their expression levels (Feschotte 2008).

The DIV-like genes of *O. italica* are mainly expressed in the tissues of the perianth (outer and inner tepals, lip), suggesting a possible redundant role in these tissues. However, some transcripts are also expressed in the column, ovary and leaves, demonstrating the pleiotropic role of these genes, as observed in other species (Howarth and Donoghue 2009). Notably, the expression profile of the alternatively spliced DIV-like isoform OITA_23026_AS (that encodes a short protein with a single MYB domain) resembles that of the RAD-like gene OITA_103296, leading to speculation that this isoform represents an evolutionary step towards the RAD-like genes, possibly evolved from a DIV-like gene after the loss of exon 2...
through alternative splicing. The expression profile of OITA_13252 is similar to that of the alternatively spliced isoform OITA_13252_AS; however, the isoform that retains the intron in the 5′ UTR (OITA_13252_AS) is expressed at higher levels. This difference in expression level suggests a transcriptional regulatory role of the small intron in the 5′ UTR, whose presence might increase the transcription efficiency.

After anthesis, the RAD-like genes of *O. italica* share a similar expression pattern that suggests redundant roles of these transcripts in the organs of the perianth and more specialized roles in reproductive and vegetative tissues.

**Conclusions**

This study is a novel analysis of MYB transcription factors that are potentially involved in the floral symmetry of orchids. The next generation sequencing approach increases the amount of data available at the transcriptomic and genomic levels to analyze gene families of interest in nonmodel species, such as orchids. The present study hypothesizes the existence of an evolutionary conserved program of gene expression between distantly related species, *O. italica* and *A. majus*, to establish bilateral symmetry. Further expression and functional analyses of DIV, RAD and DRIF genes, together with TCP genes, in other orchid species could verify this model.

**Supplementary Material**

Supplementary data are available at Genome Biology and Evolution online.

**Author Contributions**

M.C.V. and S.D.P. performed the transcript and genomic DNA analyses and the expression analysis and participated in the data analysis; M.C.V. and G.I. performed the in situ hybridization experiments; S.A. designed the experiments, performed the in silico analyses, analyzed the data and wrote the manuscript.

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