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Original Article

Isolation and characterization of *LEAFY* homologous gene partial sequences from *Dendrobium capra* J.J. Smith Endemic Orchid of Java Island

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Abstract

Dendrobium capra is an endemic orchid species on the island of Java, Indonesia, which blooms only once a year, usually in February. In the flowering process, *LEAFY* (*LFY*) gene and its homologs are considered as key regulators in flowering of higher plants, including orchids. To understand floral regulatory mechanisms in orchids, information on the flowering gene structure needs to be explored. Partial sequences of *LFY* homologue of *D. capra* has been isolated and consists of 220 bp encoding 72 amino acid sequences. Bioinformatic analysis using Blastp search showed high similarity between the *LFY* genes of *D. capra* with other orchid *LFY* genes and has adjacent evolutionary relationship among orchid'sLFY. Amino acid sequence alignment result showed similar characteristic with other orchid's LFY with conserved motif leucine-rich repeat and DDPRRRLLLLSPDQHN amino acid motif.

Keywords: *Dendrobium capra*, *LFY* gene, orchid *LFY*

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Introduction

Indonesia is home to around 5.000 orchid species of the world and some of them are endemics. *Dendrobium capra* is one of those endemic orchids, native to Java especially in middle to east Java. *D. capra* lives as an epiphyte, mainly on teak tree (*Tectona grandis*) as its host and classified under Appendix II category by CITES (Yulia & Ruseani, 2008; UNEP-WCMC, 2014). It has small green flowers that appear in cluster of 2-4 flowers and have good scents (Fig. 1). Those characteristics make *D. capra* highly valuable in both conservation and commercial perspective.

Figure 1. Flower morphology of *Dendrobium capra* from Gunung kidul, Java Island. Bar: 1 cm

LEAFY (*LFY*) gene is considered one of key regulators of flowering in a model plant *Arabidopsis thaliana*. It is because flowering pathways, such as photoperiod, vernalization, autonomous and gibberellin pathway came converged in *LFY*. *LFY* upregulation will induce expression of *APETALA3* (*AP3*), *AGAMOUS* (*AG*) and *PISTILLATA* (*PI*) as floral organ identity genes (Busch et al., 1999; Parcy et al., 1998; Weigel & Meyerowitz, 1993) with *UNUSUAL FLORAL ORGAN* (*UFO*) and *WUSCHEL* (*WUS*) as coactivators (Honma & Goto, 2000; Lenhard et al., 2001).

Until now there are various *LFY* homologs that have been identified in many higher plants. The *LFY* homologs have a similar characteristic that has two positions of conserved introns. In orchids, *LFY* homolog is strongly predicted to play significant role to induce flowering as it is located directly upstream of floral organ identity genes (Wang et al., 2017). Recent researches on identification of orchid *LFY* are able to identify homologues on various orchid plants such as *PhalLFY* from *Phalaenopsis* hybrida (cv. wedding promenade) (Zhang et al., 2010), *ChLFY* from *Cymbidium ensifolium* (Yang & Zhu, 2015) and *PhapLFY* from *Phalaenopsis aphrodite* (Jang, 2015).

In this work, it has been isolated part of the homologous *LFY* gene from *D. capra* ((hereinafter referred to as *DcLFY*) to find out the protein motif that governs the flowering process/ mechanism in *D. capra*. Knowing that the main structure of the *DcLFY* gene is expected to be used as a basis for inducing/accelerating flowering on *D. capra*, it is even possible to induce flowering more than once a year. This will be very beneficial to conserve this *D. capra* orchid in its natural habitat.

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Methods

Plant Materials

Three years old plants of *Dendrobium capra* orchid from Gunung kidul were obtained from local market of horticulture (PASTY). Plants were maintained in greenhouse at 30-34 ℃ (day) and 26-29 ℃ (night). Plants

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were watered and fertilized using 2 g.L^{-1} of Hyponex® fertilizer (N: P: K, 20:20:20) once a week.

Genomic DNA Isolation

Genomic DNA of *D. capra* was extracted using CTAB method described by Semiarti et al. (2007) with minor modification. 100 mg leaf materials were grinded using ice-cold pestle with 500 ul CTAB buffer and transfered into 1.5 mL microtube. Leaf lysate was incubated at 55-65 ℃ for 30 mins, then 500 ul chloroform was added into lysate, then it shaked at 120 rpm for 30 mins. After shaking, microtubes were opened to throw away the gas, then it centrifuged at 5,000 rpm for 5 mins. Supernatants were transfered into new microtube, then added equal volume of isopropanol (1:1, v/v), then incubated at room temperature for 10 mins. The samples were centrifuged at 5.000 rpm for 5 mins and removed the supernatant. DNA pellets were washed using 70% ethanol, then centrifuged at 5.000 rpm for 5 mins. DNA pellet was dried for 15 mins and resuspended by using 50 ul TE buffer with addition of 1 ul of Rnase A (10 mg/mL).

LFY homologs were amplified from genomic DNA of *D. capra* using forward primer (5'-TCT TGT CGG CGA GCG ATA TG-3') and reverse primer (5'-GTT GTG TTG GTC GGG TGA GA-3') designed by Primer-BLAST (Ye *et al.*, 2012) based on *DnLFY* sequence (KC568360.1). PCR reactions were performed using GoTaq Green master mix (Promega) under following conditions: pre-denaturation 95 ℃ for 5 mins; 30 cycles of denaturation 95 ℃ for 30 s, annealing 55 ℃ for 30 s, extension 72 ℃ for 30 s; final extention 72 ℃ for 15 mins. Amplified DNA (amplicon) was resolved on 1% agarose gel with SYBR safe staining and visualized under uv transilluminator. The positive result sent to 1st Base DNA sequencing.

Bioinformatics Analysis

Sequencing results were used to create contigs using DNA Baser Assembler then converted to amino acid sequence and analyzed for similarity comparison using blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) then some of them were picked for alignment against *D. capra* LFY sequence using MUSCLE algorithm on MEGA7 (Edgar, 2004; Kumar et al., 2016) and arranged using BoxShade version 3.21. Multiple Sequence Alignment result used to generate phylogenetic tree by Neighbor Joining method using 1000 Bootstrap replicates on MEGA7 (Kumar et al., 2016). Conserved region was determined by Gblocks version 0.91b (Castresana, 2000).

Results

Isolated partial DNA resulting in 220 bp clean reads that showed predicted conserved region of orchid's'LFY gene in *DcLFY*, based on the sequence of *PhapLFY*. This region showed high homology between *DcLFY* and *PhapLFY* with 70% similarities that encodes 72 amino sequences. Blastp search for that sequence confirmed that the sequence has high similarity on LFY especially in orchids. Multiple sequence alignment using MUSCLE algorithm showed that *D. capra* LFY has high similarity with LFY from other orchids (Fig. 2).

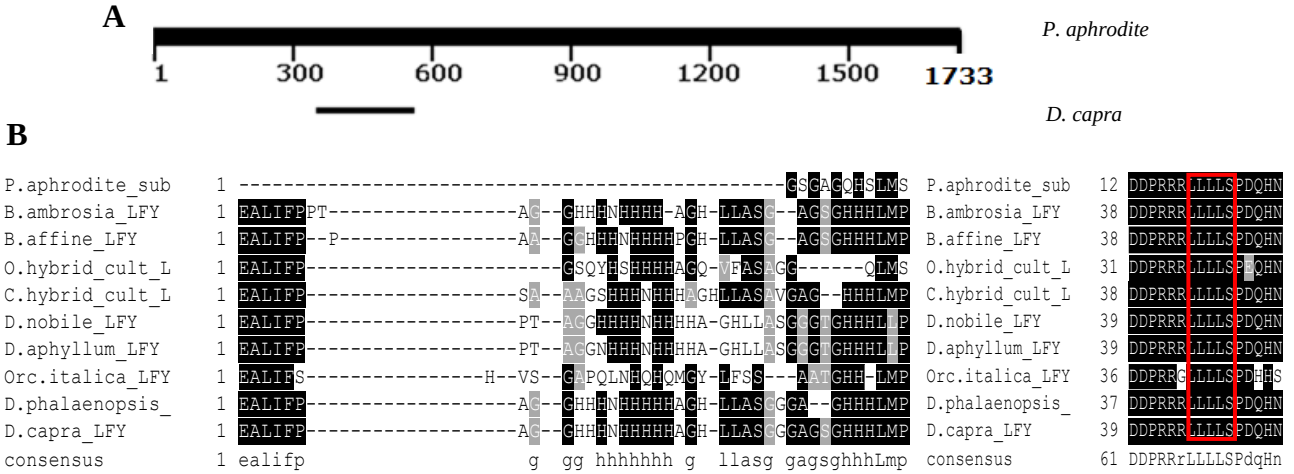

Figure 2. Multiple sequence alignment result between *DcLFY* and other orchid *LFY* using MUSCLE algorithm found a conserved region in DcLFY amino acid sequence. (A) Comparison between 1733 bp full length of *PhapLFY* sequence (ALM87388.1; Jang, 2015) and partial *DcLFY* sequence. *DcLFY* sequence showed 70% similarities with *PhapLFY* and covered nucleotide 351 to 561 (B). Protein allignment showed that within the conserved region, there is a leucine-rich repeat shown in the red box as one of common characteristic of LFY protein, as described in some references (Coen et al., 1990; Weigel et al., 1992; Zhang et al., 2010). Sequences used to align with *D. capra LFY* were *Bulbophyllum affine* (AHC05839.1), *D. phalaenopsis* (AHC05821.1), *Orchis italica* (BAC54955.1), *B. ambrosia* (BAC05840.1), *Cymbidium* hybrid cult. (AGE45851.1), *Phalaenopsis aphrodite* subsp. formosana (ALM87388.1), *Oncidium* hybrid cult. (ADL57240.1), *D. nobile* (AHC05836.1), *D. aphyllum* (AHC05806.1).

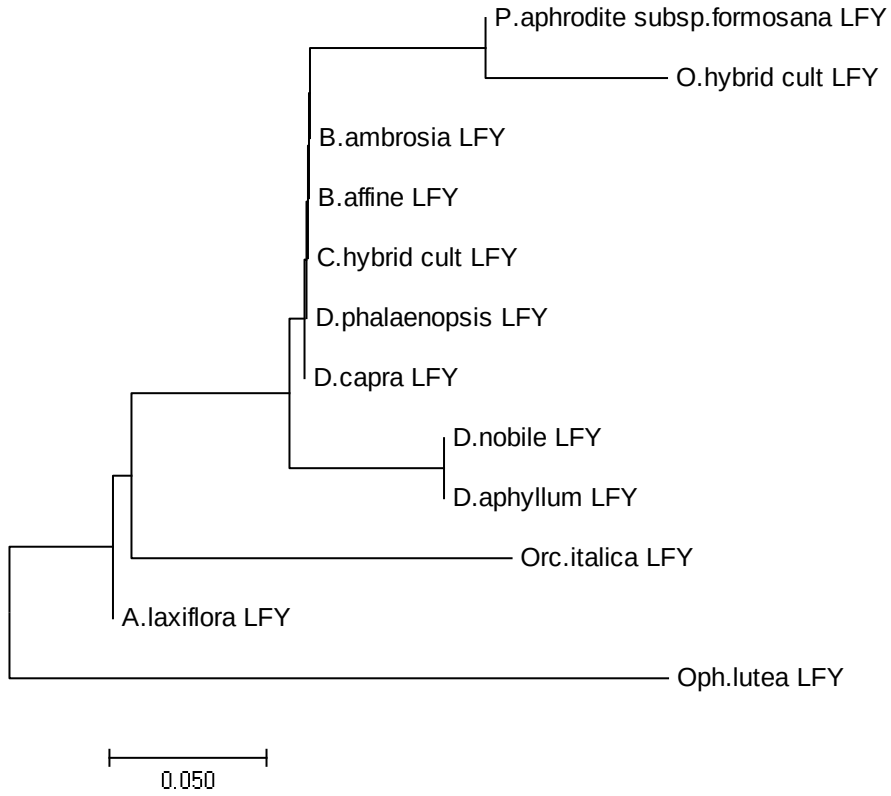

Figure 3. Phylogenetic tree of *D. capra LFY* and *LFY* from other orchids species. Twelve orchids such as, *Anacamptis laxiflora* (BAC55080.1), *Bulbophyllum affine* (AHC05839.1), *D. phalaenopsis* (AHC05821.1), *Orchis italica* (BAC54955.1), *B. ambrosia* (BAC05840.1), *Cymbidium* hybrid cult. (AGE45851.1), *Phalaenopsis aphrodite* subsp. formosana (ALM87388.1), *Oncidium* hybrid cult. (ADL57240.1), *D. nobile* (AHC05836.1), *D. aphyllum* (AHC05806.1), *Ophrys lutea* (CCB84677.1) were used to predict evolutionary relationship of *D. capra* LFY with other orchid LFY using Neighbor Joining method and 1000 Bootstrap replicates.

Discussions

As the main regulator of genetic regulation in flowering plants, the role of the *LFY* gene is very important, and this is determined by the structure of DNA sequences that will determine the amino acid motif of the *LFY* functional protein. *LFY* upregulation will induce the expression of floral organs identity genes *AP3, AG* and *PI* (Busch et al. 1999, Parcy et al., 1998; Weigel & Meyerowitz, 1993) with UFO and WUS as coactivators (Honma & Goto , 2000; Lenhard et al., 2001). Thus, the isolation and characterization of the *DcLFY* gene structure will help to understand flowering mechanisms in *D. capra.*

To look further into their evolutionary relationship, phylogenetic tree using Neighbor Joining method was constructed. The tree showed that *D. capra* LFY, has adjacent evolutionary relationship among *LFY* in orchids (Fig. 3). Orchid LFY is separated into 2 large groups with DcLFY has nearest relationship with *D. phalaenopsis* LFY with highly conserved DDPRRRLLLLSPDQHN amino acid motif. Those two share 95% similarity and in the motif has a leucine-rich repeat which is one of the common *LFY* characteristic (Coen et al., 1990; Weigel et al., 1992; Zhang et al., 2010). DcLFY also share high similarity with *Cymbidium* LFY (84%) and *Bulbophyllum* LFY (86 – 88%). The high similarity between those LFY and DcLFY are relevant to its phenotype as a group member of plants with a sympodial growth system and the timing of the appearance of flowers. This is different from *Phalaenopsis* which has a monopodial growth system and can be flowered many times a year. Other parts or positions of this gene and its contigs need to be more explored to fully understand the structure and function of this gene related to flower initiation. Orchids *LFY* homologues were also identified on various orchid plants such as *PhalLFY* from *Phalaenopsis* hybrida (cv. wedding promenade) (Zhang et al., 2010), *ChLFY* from *Cymbidium ensifolium* (Yang & Zhu, 2015) and *PhapLFY* from *Phalaenopsis aphrodite* (Jang, 2015). In accordance with Wang et al. (2017), it is helpful to understand the mechanism of flowering that *LFY* homolog is strongly predicted to play significant role to induce flowering as it is located directly upstream of floral organ identity genes.

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