Journal of Tropical Biodiversity and Biotechnology
Volume 09, Issue 03 (2024): jtbb91511
DOI: 10.22146/jtbb.91511

Research Article

# Characterization of Flower's Color based on *CHS* Gene Structure in *Phalaenopsis* 'OX Queen' and *Dendrobium* 'Cheddi Jagan' Orchids

**Yumna Rahmadias Hanifa[1], Elke Gildantia[1], Pauline Destinugrainy Kasi[2], Aziz Purwantoro[3], Endang Semiarti[4]***

1)Study Program of Biotechnology, Postgraduate School, Universitas Gadjah Mada, Daerah Istimewa Yogyakarta, 55281, Indonesia
2)Study Program of Doctoral Biology, Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Daerah Istimewa Yogyakarta, 55281, Indonesia
3)Faculty of Agriculture, Gadjah Mada University, Sekip Selatan, Yogyakarta 55281, Indonesia
4)Laboratory of Biotechnology, Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Daerah Istimewa Yogyakarta, 55281, Indonesia
* Corresponding author, email: endsemi@ugm.ac.id

**Keywords:**
*CHS* gene
*Dendrobium* 'Cheddi Jagan'
flower's colour
*Phalaenopsis* 'OX Queen'
phenotypic
variegated flower
**Submitted:**
06 December 2023
**Accepted:**
02 Mei 2024
**Published:**
02 September 2024
**Editor:**
Ardaning Nuriliani

## ABSTRACT

Orchids (Orchidaceae) are ornamental plants known for their high aesthetic value attributed to the shapes, colours, and fragrances of their flowers. Two types of hybrid orchids with attractive flowers, namely the *Phalaenopsis* 'OX Queen' orchid and the *Dendrobium* 'Cheddi Jagan' boast attractive flowers were used in this research, because of the beauty of its flower colour. The objective of this research is to characterise the morphology of flower colour and *CHS* (*Chalcone Synthase*) gene content that induces flower colour. The method used in this research analyzing the flower's colour by using the RHS (*Royal Horticultural Society*) colour chart and molecular analysis by DNA genomic isolation and PCR amplification of gDNA for *CHS* gene specific primers. The results showed that purple colour is observed through the RHS, with *P.* 'OX Queen' coded as Deep Purple Pink (N73A) and *D.* 'Cheddi Jagan' coded as Strong Reddish Purple (N72C). The *CHS* gene can be amplified in *P.* 'OX Queen' 1,287 bp and *D.* 'Cheddi jagan' 3,731 bp. In both orchids, the results of amplification showed CHS motifs with conserved domains PLN03172 and PLN03170. The research results show that there is a significant difference in the morphology of the flowers of orchids. Purple colour is observed through the RHS, with *P.* 'OX Queen' coded as N73A and *D.* 'Cheddi Jagan' coded as N73C. The results showed that gDNA can be isolated by using CTAB method according to Murray and Thomson, and the *CHS* gene can be amplified by using CHS primers, resulting 1200 bp of *P.* 'OX Queen' and 2500 bp for *D.* 'Cheddi Jagan'. Through this study, preliminary data is expected to be obtained for future research, which is the formation of variegated flowers through editing the CRISPR/Cas9 genome in the *CHS* gene. This research is intended to support further studies on the formation of variegated flower patterns in *P.* 'OX Queen' and *D.* 'Cheddi Jagan', focusing on the *CHS* gene using CRISPR/Cas9 technique.



## INTRODUCTION

Orchids are ornamental plants that have high diversity. In Indonesia, orchid diversity it's about 23% of the total of 30,000 orchid species in the

world and it is estimated around 5,000 species (Karoy et al. 2022). *Phalaenopsis* and *Dendrobium* orchids are two types of orchids that captivate attention with the beauty of their flower morphology (Semiarti et al. 2020) and widely used as parents of hybrid orchids (Li et al. 2021). The uniqueness of the orchid is the shape, colour and aroma of their flowers. *P.* 'OX Queen' is an orchid hybrid, resulting from a cross between *P.* 'Brother Success' as the female parent and *P.* 'Plum Rose' as the male parent (Royal Horticultural Society 2024a). Based on the Orchid Roots recognized by the Royal Horticultural Society, *D.* 'Cheddi Jagan' is also an orchid hybrid, resulting from a cross between *D.* 'Cheong Chee Yon' as the female parent and *D.* 'Genting Rose' as the male parent (Royal Horticultural Society 2024b). Hybrid orchids are popular because they have beautiful colours and are commonly used as decorative flowers both indoors and outdoors. *P.* 'OX Queen' and *D.* 'Cheddi Jagan' orchids were used in this study because both of them have plain purple flowers which will then be carried out *CHS* gene analysis on both orchid flowers.

Flower's colour is one of the things that causes plants to be attractive and in demand, the colour formed in this is produced through the biosynthesis of pigments which include anthocyanins, carotenoids and betalains. The *Chalcone Synthase* (*CHS*) gene encoded an enzyme responsible for initiating the flavonoid biosynthesis pathway in plants. CHS enzyme is a key in the flavonoid biosynthetic pathway that determines colour which contributes to flower petal colours, against UV radiations, attract pollinators, as well as plant growth and development (Kong et al. 2020; Jia et al. 2023). Flavonoids are responsible for pigmentation in flowers and fruits from orange to pink, red, violet and blue. The biosynthesis of flavonoids starts with the condensation of one molecule of p-coumaroyl-CoA and three malonyl-CoA molecules, which is catalyzed by CHS to produce naringenin chalcone (Sun et al. 2015). In sepals, petals, and flower labellums, anthocyanin pigments are the most commonly found pigments, anthocyanin pigments are divided into three colour groups such as pelargonidin which produces orange or red, cyanidin which produces pink, and delphinidin which produces purple or blue (Grotewold 2006; Pratama et al. 2023). Several studies related to the *CHS* gene such as on *Malus* Crabapple (Tai et al. 2014), *Paeonia suffruticosa* (Zhou et al. 2011), *Dahlia variabilis* (Ohno et al. 2018), *Phalaenopsis* orchids (Han et al. 2006) (Kuo et al. 2019), and many more. Research on the *CHS* gene in orchids was previously carried out by Linggabuwana et al (2024) was the inspiration for this research. The beautiful flower's colour of this hybrid orchid has sparked interest among researchers in characterization of the *CHS* gene which codes for flower coloration and can construct sgRNA that will be used in genome editing. In future research it is hoped that researchers can change the colour of orchid flowers from plain flower colours such as pink or purple to become variegated flowers on the marginal part, then the uniqueness of this flower can be developed on an industrial scale, so that it can increase its economic and aesthetic value.

Regarding identification, digital image processing techniques (Putra 2021) can be employed to accurately identify various orchid types based on the texture of their flowers. Molecular analysis serves as a complementary tool to morphological analysis, aiding in the development of new orchid varieties within plant breeding programs (Arif & Ratnawati 2018). Genetic research on orchids holds the potential to unveil valuable information concerning genetic diversity, floral development, and the species adaptations to their environment. The isolation of DNA from orchid flowers has become essential in understanding the genetic and bio-

logical aspects of the abundant orchid species in Indonesia. Furthermore, this molecular analysis using plant DNA supports increasingly important genetic conservation efforts, particularly in light of several orchid species being threatened by environmental changes and human activities. Currently, various DNA isolation techniques have been developed, tailored to the unique characteristics of orchids, such as their robust cell walls and specific chemical components within floral tissues (Arif & Ratnawati 2018). Research on the isolation of orchid flower DNA is crucial for comprehending the most efficient and reliable methods for obtaining high-quality DNA samples from various orchid species. The two main stages of flowering are called flower initiation and flower development. The onset of flowers is thought to be regulated by a number of biochemical and physiological processes. The growth of flowers involves the establishment of the different floral elements, which are represented in changed and carefully controlled expression of genes resulting in the development of many flower-specific mixtures (Herdenberger et al. 1990).

This research will study the experimental procedures carried out about characterisation of flower phenotypes and genotypes from *P.* 'OX Queen' and *D.* 'Cheddi Jagan'. Through this study, preliminary data is expected to be obtained for future research, which is the formation of variegated flowers through editing the CRISPR/Cas9 genome in the *CHS* gene.

## MATERIALS AND METHODS

### Materials

#### Plant Materials

Orchid plant that were used in this research were *P.* 'OX Queen' orchid flower and *D.* 'Cheddi Jagan' orchid Nambangan Orchid nursery owned by Hasan Sulaiman, S.P., located at 43 Telaga Warna Street, Rejowinangun, Nambangan, North Rejowinangun, Central Magelang Subdistrict, Magelang City, Central Java.

#### Morphological Analysis

Writing ruler (smallest scale is 1 mm), Canon SX430 IS camera (Japan), and identifying flower color based on the RHS Color Chart.

#### Genomic DNA Extraction

Chemical materials for isolation of plant genome DNA were 3% CTAB (containing 1 M Tris HCl, 0.5 M EDTA, 3% CTAB powder, isopropanol, isoamyl alcohol, NaCl, and pure water), PVP 1%, chloroform, absolute ethanol, 70% ethanol, TE buffer pH 8. The ingredients for the PCR reaction are 2x MyTaqTM HS Red Mix (Bioline), KOD FX Neo kit (Toyobo), pure water, specific primers for *Actin* and *CHS* genes for forward and reverse, presented in Table 1. below, and genome DNA of *Phalaenopsis* 'OX Queen' and *Dendrobium* 'Cheddi Jagan'.

#### Gene Amplification

The amplification of the orchid *P.* 'OX Queen' genome DNA was conducted using the *PaCHS2* primers. Meanwhile, the genome DNA of *D.* 'Cheddi Jagan' was amplified using *DcCHSII1* primers. The chemical materials for electrophoresis are agarose gel powder, TAE 1X, 6X loading dye (Geneaid), 100 bp DNA ladder (Geneaid), 1 kb DNA ladder (Geneaid), ddH2O, RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology), and visualised with tools UV transilluminator (Extragene UV3CL, Taiwan).

**Table 1.** List of primer used in this research

| Primer | Sequence |
|---|---|
| *PaCHS2* F | 5'-AGATCTTTGCAATAATTTTAAAAAAAAATTC-3' |
| *PaCHS2* R | 5'-GTTTTATTCGAGGCTGAGTTTG-3' |
| *DcCHSII1 F* | 5'-AAATAGCTGCCCACGCTCTTG-3' |
| *DcCHSII1 R* | 5'-GCAAAAAAGATTCATAAGACTTCTTTATTA-3' |
| *Actin* F | 5'-GTATTCCCTAGGATTGTTGGT-3' (accession number AY134752) (Semiarti et al. 2007) |
| *Actin* R | 5'-CAGAGTGAGAATACCTCGTTTG-3' (accession number AY134752) (Semiarti et al. 2007) |

## Methods

### Flower Morphological Observation

Three samples of *P.* 'OX Queen' and three samples of *D.* 'Cheddi Jagan' which has purple colours were used in this study. To observe orchid morphology, a writing ruler (smallest scale was 1 mm) is used to measure orchid habitus, size of roots, leaves, and flowers. An RHS Colour chart is used to determine flower colour codes, and a Canon SX430 IS camera (Japan) is used for documentation.

### Genome DNA Extraction

The isolation of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' flower genomic DNA was performed using a modified version of the Murray and Thompson method (Murray & Thompson 1980) using isopropanol. The DNA isolation results of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' were visualized using 1% gel electrophoresis using the Mupid-exU Submarine electrophoresis system (Japan).

### Amplification of *CHS* Gene

The results of DNA isolation were used as a template for amplifying the *CHS* and *Actin* gene sequences using the Polymerase Chain Reaction (PCR) method. The *CHS* gene was used to detect anthocyanin pigments (Linggabuwana et al. 2024), and the *Actin* gene was used as an internal positive control (Zhao et al. 2012). The composition of the reagents in the PCR process is presented in Table 2.

**Table 2.** Reagent Components for PCR Reaction of *CHS* and *Actin* gene.

| Reagent Component | Volume (µL) |
|---|---|
| Genome DNA (ng/µL) | 2 |
| *2x PCR Buffer* (mM) | 12.5 |
| *Forward* primer (10 pmol/µL) | 0. 75 |
| *Reverse* primer (10 pmol/µL) | 0. 75 |
| *ddH₂O* (µL) | 3.5 |
| *dNTPs* (mM) | 5 |
| *KOD Fx Neo* (units/µL) | 0.5 |
| Total | 25 |

The PCR process for the *PaCHS2* and *DcCHSII1* gene was carried out with a protocol of 30 cycles, pre-denaturation at 94°C for 2 minutes, denaturation at 98°C for 30 seconds, annealing temperature based on optimization results *P.* 'OX Queen' at 59°C, *D.* 'Cheddi Jagan' at 57°C and *Actin* at 51°C for 30 seconds, extension at 68°C time based on optimiza-

tion results *P.* 'OX Queen' for 40 seconds, *D.* 'Cheddi Jagan' for 2 minutes and *Actin* for 5 seconds and hold at 4°C.

## Data Analysis

Phenotypic analysis was carried out by observing plant morphology including the flower's colour of the orchids. Genotype analysis was carried out on the *CHS* gene sequencing results of the orchids using bioinformatic tools that are the NCBI BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov)) is used to align genome DNA obtained with the database, NCBI Conserved Domain Search (NCBI Conserved Domain Search (nih.gov)) is used to view conserved domains in both orchid sequences. The *CHS* gene motif can determine using the website Prosite Expasy (Expasy - PROSITE), Prosite Translate (Expasy - Translate tool) and Biomodel Sequence Massager (Sequence Massager (uah.es)) as well as the MultAlin (http://multalin.toulouse.inra.fr/multalin/) was used to align orchids sequences, ApE and UGENE applications are used to align orchids sequences with reference sequences and mRNA to determine the location of exons and introns in both orchids.

## RESULTS AND DISCUSSION

### Morphological Character of *Phalaenopsis* 'OX Queen'

Morphological observations of *P.* 'OX Queen' as displayed in Figure 1, there are 10 orchid flowers in a single cluster, predominantly displaying purple hues and accentuated by white edges along their margin. This is in accordance with van Tongerlo et al. (2021), that the *Phalaenopsis* orchid is famous for its enchanting beauty and abundant flowers. Each stalk has the potential to produce between 5 and 10 flowers simultaneously, with each flower lasting up to 3 months. This figure indicate that this orchid plant exhibits monopodial stem growth with a plant size of approximately 15-17 cm. The leaves of *P.* 'OX Queen' are dark green and lance-shaped, with a length of approximately 7-25 cm and a width of about 4-10 cm. The flowers are round with slightly overlapping petals and sepals. Fully bloomed flowers measure around 10-15 cm in length and 7-8 cm in width, displaying a plain purple color.

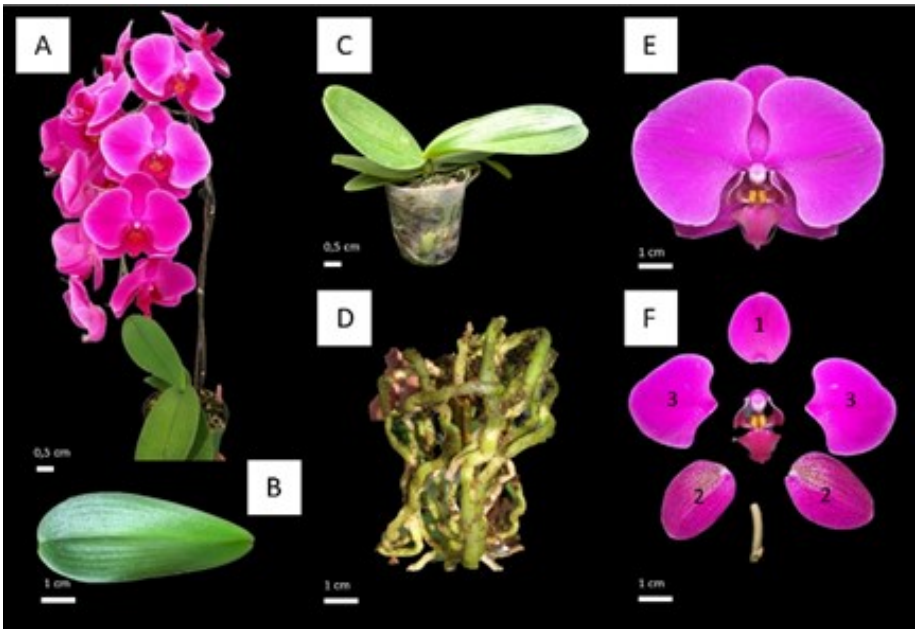


**Figure 1.** Habitus of *P.* 'OX Queen' hybrid. A= Habitus of Reproductive stage of *P.* 'OX Queen'; B= Leave; C= Vegetative Stage; D) Roots; E= Flowers from the front view; F= Flower parts consists of dorsal sepals (1), lateral sepals (2) and petals (3).

## Morphological Character of *Dendrobium* 'Cheddi Jagan'

Morphological observations of *D.* 'Cheddi Jagan,' as presented in Figure 2, reveals that this orchid plant exhibits sympodial stem growth with a plant size of approximately 20-25 cm. The leaves of *D.* 'Cheddi Jagan' are dark green and lance-shaped, with a length of approximately 7-15 cm and a width of about 4-5 cm. Typically, each *D.* 'Cheddi Jagan' plant has four pseudobulbs, each measuring around 10-13 cm in height. The flowers are oval-shaped with slightly overlapping petals and sepals, and their texture resembles velvet or velvety material. Fully bloomed flowers measure approximately 7-8 cm in length and 5-6 cm in width, and they are of a plain purple color. The pollen is yellow with a white operculum. De et al. (2015) states that the *Dendrobium* genera have sympodial characteristics and have pseudobulbs, these pseudobulbs can be long, short or swollen. The inflorescences are terminal or subterminal with various sizes and colours, this is what makes the *Dendrobium* orchid a popular orchid used as a decorative flower indoors, as a plant in a pot, or as a cut flower.

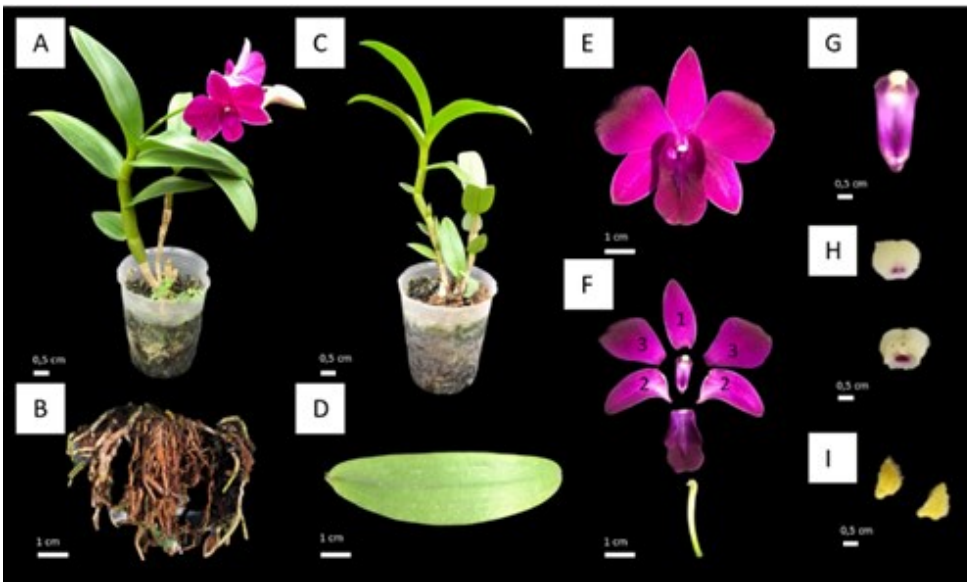


**Figure 2.** Habitus of Reproductive stage of *D. 'Cheddi Jagan'*. A= Habitus *D.* 'Cheddi Jagan'; B= Roots; C= Vegetative Phase; D= Leave; E= Flowers from the front view; F= Flower parts consists of dorsal sepals (1), lateral sepals (2) and petals (3); G= Columna; H= Operculum; I= Pollinia.

## Flower Morphology of *P.* 'OX Queen'

**Table 3.** Colour identification of *P.* 'OX Queen' hybrid flower based on RHS Colour Chart.

| Flower Sample | Colour Code | Colour Identification Based on RHS Colour Chart |
|---|---|---|
|  | Red purple Group N73 A | |

## Morphology Flower of *Dendrobium* 'Cheddi Jagan'

**Table 4.** Colour identification of *Dendrobium* 'Cheddi Jagan' hybrid flower based on RHS Colour Chart.

| Flower Sample | Colour Code | Colour Identification Based on RHS Colour Chart |
|---|---|---|
|  | Red purple Group N72 C | |

Table 3 displays the results of flower colour identification based on the RHS colour chart for *P.* 'OX Queen,' which is characterized by a purple colour with code N73A colour card group or Deep Purplish Pink colour (sRGB: R207, G116, and B171),. while the, *D.* 'Cheddi Jagan' that displayed in Table 4 is identified with a purple colour denoted by code N72C colour card group which means Strong Reddish purple (sRGB: R193, G104, and B160). Codes A to D or the first, second, third, and fourth colour chips are respectively lighter in colour but have the same colour hue (Voss 2001). *P.* 'OX Queen' and *D.* 'Cheddi Jagan' both exhibit purple colours, but differ in the nuances of the flower colour. *D.* 'Cheddi Jagan' appears to have a deeper shade of purple compared to *P.* 'OX Queen'.

## Genotypic Analysis

### Analyze of DNA and PCR

Based on the qualitative DNA (Figure 3) tested using gel electrophoresis, it was determined that the DNA sample sizes of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' were both greater than 10,000 bp.

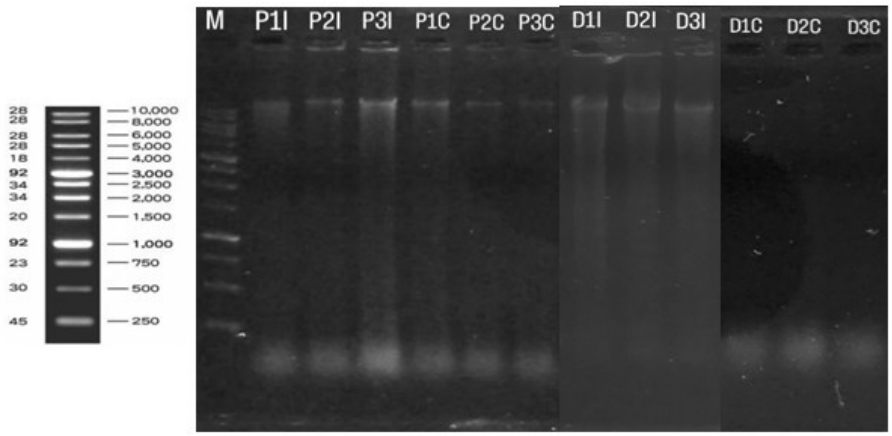


**Figure 3.** DNA genome visualization from *P.* 'OX Queen' (P1-P3) and *D.* 'Cheddi Jagan' (D1-D3).

Based on Table 5, the purity of the DNA produced in the *P.* 'OX Queen' orchid is included in the good category, whereas in the *D.* 'Cheddi Jagan' orchid the DNA purity produced is still not good due to the presence of contaminants in the DNA sample. DNA isolation methods in different plants can produce different purities and concentrations due to various factors, including the chemical content in the plant, the efficiency

of DNA separation or purification, and the DNA precipitation process (Rizko et al. 2020). According to Green and Sambrook (2019), the purity ratio value of A280/A260 should be in the range of 1.8–2.0. A purity ratio of A260/A280 below this range indicates that there is protein contamination.

**Table 5.** Quantity analysis of *P.* 'OX Queen' (P1-P3) and *D.* 'Cheddi Jagan' (D1 -D3) using NanoDrop Spectrophotometers.

| Sample ID | Conc. | Units | A260/A280 | A260/A230 |
|---|---|---|---|---|
| P1 | 113.016 | ng/µL | 1.92 | 1.085 |
| P2 | 132.076 | ng/ µL | 1.848 | 1.079 |
| P3 | 120.8 | ng/ µL | 1.996 | 0.998 |
| D1 | 73.407 | ng/ µL | 1.517 | 0.564 |
| D2 | 138.732 | ng/ µL | 1.309 | 0.438 |
| D3 | 134.57 | ng/ µL | 1.398 | 0.400 |

Meanwhile, based on the A260/230 purity ratio value, a sample has good DNA purity if it has an A260/230 purity ratio value ranging from 1.8-2.0 for DNA (Dwiyani et al. 2016). Based on orchid DNA electrophoresis, electropherogram results were obtained with a size of more than 10 kb. According to Waluyo et al. (2013), a good DNA band in an electropherogram is if the DNA band is clearly visible and has uniform length of base pairs without any smearing. The quantity of orchid DNA was determined using the UV spectrophotometric method at wavelengths of 260 and 280 nm (Semiarti et al. 2020).

## Amplification *of CHS* gene Fragmen in *P.* 'OX Queen' and *D.* 'Cheddi Jagan'

The results of DNA isolation were used as a template for amplifying the *CHS* and *Actin* gene sequences using the PCR method. The *CHS* gene was used to detect anthocyanin pigments (Linggabuwana et al. 2024) and this was used to determine the specifications of the two orchid genera and the *Actin* gene was used as an internal positive control (Zhao et al. 2012).

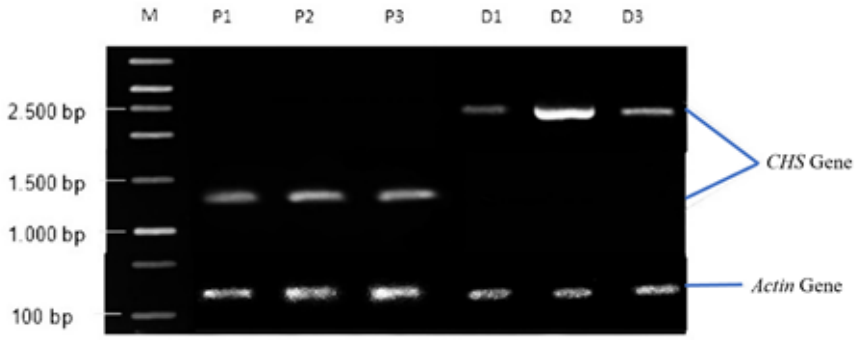


**Figure 4.** Detection of *CHS* and *Actin* gene in *P.* 'OX Queen' and *D.* 'Cheddi Jagan' purple zone using the PCR method.

Figure 4 shows the PCR results of the *CHS* gene in *P.* 'OX Queen' of 1,287 bp and in *D.* 'Cheddi Jagan' of 3,731 bp. These results were validated by primary *Actin* as a housekeeping gene and positive control. The *actin* primer has a size of 114 bp, showing the successful amplification of the expected DNA fragment. The *actin* primer shows strong and clear band intensity, conforming the presence of target DNA in the sample. These results strengthen confidence in validity and reliability of the data resulting from PCR analysis, and ensure that the amplification of target

and control DNA has proceeded as expected.

Based on the qualitative DNA test using gel electrophoresis, it was determined that the DNA sample sizes of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' were both bigger than 10,000 base pairs. Modification of the Murray and Thompson method with the addition of isopropanol aims to precipitate DNA. Heikrujam et al. (2020) stated that apart from precipitating DNA, isopropanol can also dissolve non-polar solvents such as chloroform so that it can remove impurities in DNA and produce good quality DNA. This is in accordance with research by Sari and Restanto (2022) namely that PVP is used to bind phenolic components and avoid oxidation. The genome DNA isolated by Murray and Thompson method (1980) were used for amplification of *CHS* and *Actin* genes.

The sequence results obtained showed that the number of base pairs was different from the target size of the primers used, possibly because the primers were prepared based on reference sequences from different orchid species contained in the database. As a next step, mapping and characterization of the *CHS* sequence can be carried out in both the *P.* 'OX Queen' orchid and the *D.* 'Cheddi Jagan' orchid to see differences in the size of the DNA sequence. Several factors that need to be considered to get PCR results that match expectations are the quantity and quality of DNA isolation results. Good quality DNA has a purity between 1.8 – 2.0 and a concentration above 100 ng/µL based on measurements with a spectrophotometer. *Actin* serves as a housekeeping gene used as an internal positive control (Yonindi et al. 2022). Based on the visualized DNA results via gel electrophoresis, a 114 bp DNA band from the Actin gene amplification can be observed.

## *CHS* gene Motifs Analysis

The DNA results obtained from Oxford Nanopore Technology sequencing were 1.287 bp for *P.* 'OX Queen' and 3.731 bp for *D.* 'Chedi Jaggan'. It can be seen in Figure 5 which shows a schematic of the *CHS* gene in the DNA sequence of orchid flowers. The sequence of the *CHS* gene in *P.* 'OX Queen' is at bases 1.171 to 1.300, while in *D.* 'Chedi Jaggan' is at bases 2.731 to 2.860. In this base there is a *CHS* motif encoded in both orchid flowers.

Figure 5 shows the results of aligning the sample sequence with the database sequence. The *P.* 'OX Queen' consensus was aligned with the '*Phalaenopsis* hybrid cultivar *Chalcone Synthase* gene' with accession number AY825502.1, while the *D.* 'Cheddi Jagan' consensus was aligned with the '*Dendrobium catenatum* unplaced genomic scaffold' with accession number NW_021318618.1. In *P.* 'OX Queen' and *D.* 'Cheddi Jagan', the presence and conservation of the CHS domain suggest their capability to produce flavonoids. The CHS conserved domain in different orchid species can contribute to the identification of genetic variations that may influence the flavonoid biosynthesis and consequently, the phenotypic traits such as flower's colour.

In Table 6, it can be seen that for *P.* 'OX Queen', two sequences were found that were related to the *CHS* gene. The first sequence is registered in super families PLN03172 and accession number cl30448, while the second sequence is registered in super families PLN03170 and accession number cl30450. These two sequences had very low significance values (1.20e-19 and 4.69e-163), indicating that the *CHS* gene is very conservative and important in flavonoid synthesis in *P.* 'OX Queen'. Meanwhile, for *D.* 'Cheddi Jagan', there are three sequences related to the *CHS* gene. The first two sequences are registered in super families PLN03172 and accession number cl30448, while the third sequence is registered in

**Table 6.** The CHS Conserved Domain of *P.*'OX Queen' and *D.* 'Cheddi Jagan'.

| Sample | Name | Accession | Description | Interval | E-value |
|---|---|---|---|---|---|
| *P.* 'OX Queen' | PLN03172 super family | cl30448 | chalcone synthase family protein; Provisional | 5-205 | 1.20e-19 |
| | PLN03170 super family | cl30450 | chalcone synthase; Provisional | 294-1283 | 4.69e-163 |
| *D.* 'Cheddi Jagan' | PLN03172 super family | cl30448 | chalcone synthase family protein; Provisional | 103-222 | 1.35e-07 |
| | PLN03170 super family | cl30450 | chalcone synthase; Provisional | 1186-1272 | 6.12e-04 |
| | PLN03172 super family | cl30448 | chalcone synthase family protein; Provisional | 281-1207 | 0,00E+00 |
| | PLN03170 super family | cl30450 | chalcone synthase; Provisional | 198-278 | 4.92e-10 |

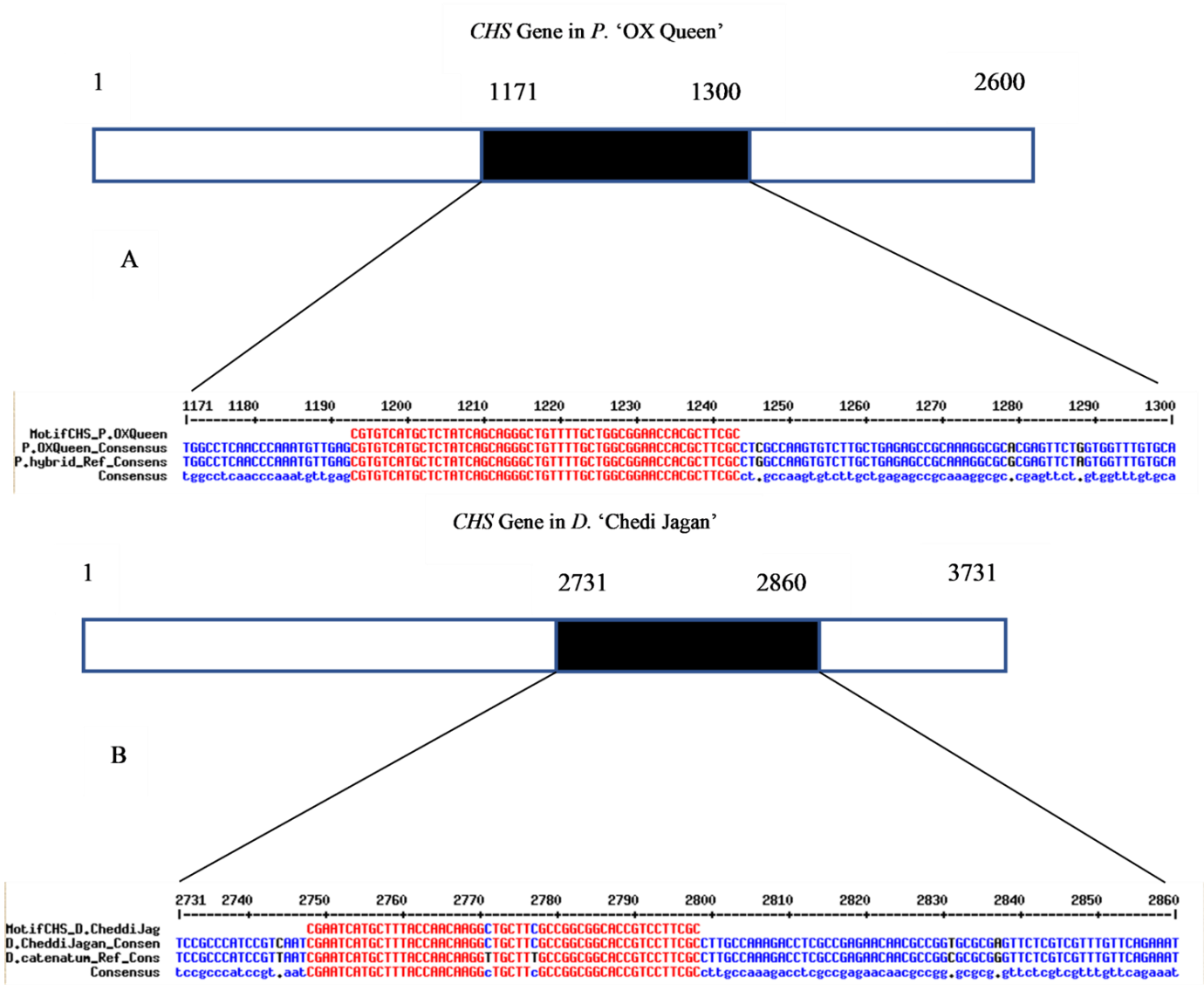


**Figure 5.** MultAlin alignment of the *CHS* gene in (A) *P.* 'OX Queen' and (B) *D.* 'Cheddi Jagan'.

super families PLN03170 and accession number cl30450. The first sequence has a fairly low significance value (1.35e-07), while the second and third sequences have a higher significance value (6.12e-04 and 0.00E+00). This suggests a variation in *CHS* gene sequences between *P.* 'OX Queen' and *D.* 'Cheddi Jagan'. So, these results indicate that the *CHS* gene plays a role in flavonoid biosynthesis in both orchid species, but the level of conservation and significance may vary between different species. This is in accordance with the statement from Hartono et al. (2021) that the smaller the E value (large negative exponent value), the higher the level of significance. Thus, the results indicate that the sequence match to the protein family or domain determined in the analysis is highly significant.

Based on the analysis of amino acid motifs shown in Figure 6., it was found that in *P.* 'OX Queen' there is a CHS protein motif in amino acid sequences 538 to 554 while in *D.* 'Cheddi Jagan' has a CHS protein motif in amino acid sequences 658 to 674. Apart from the CHS protein, in *P.* 'OX Queen' there is the EGF-1 (EGF-like domain signature 1) protein which is located at sequence 149 to 160, it is also shown in Figure 5A. with a red box. The *CHS* gene encodes the enzyme CHS which is an enzyme that is important in flavonoid biosynthesis. This protein accumulates anthocyanins in plant organs such as roots, stems, leaves and flowers (Linggabuwana et al. 2024). These pigments accumulate in vacuoles and their stability depends on intravacuolar conditions which include pH and pigment concentration (Luo et al. 2017). Anthocyanin accumulation is formed through phenylpropanoid pathways and catalysed by a number of enzymes including CHS, chalcone-flavanone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose: flavonoid 3-O glycosyltransferase (UFGT) (Liu et al. 2022).

The morphological and molecular analysis of *P.* 'OX Queen' and *D* 'Cheddi Jagan' orchids aim to lay the groundwork for CRISPR/Cas9-mediated genome editing, particularly in sgRNA determination. This research utilizes CRISPR/Cas9 in floricultural crops, focusing on enhancing flowering traits like colour modification, prolonging shelf life, initiation and development of flowers, and altering ornamental foliage colour through genome editing. Researchers aim to comprehensively under-

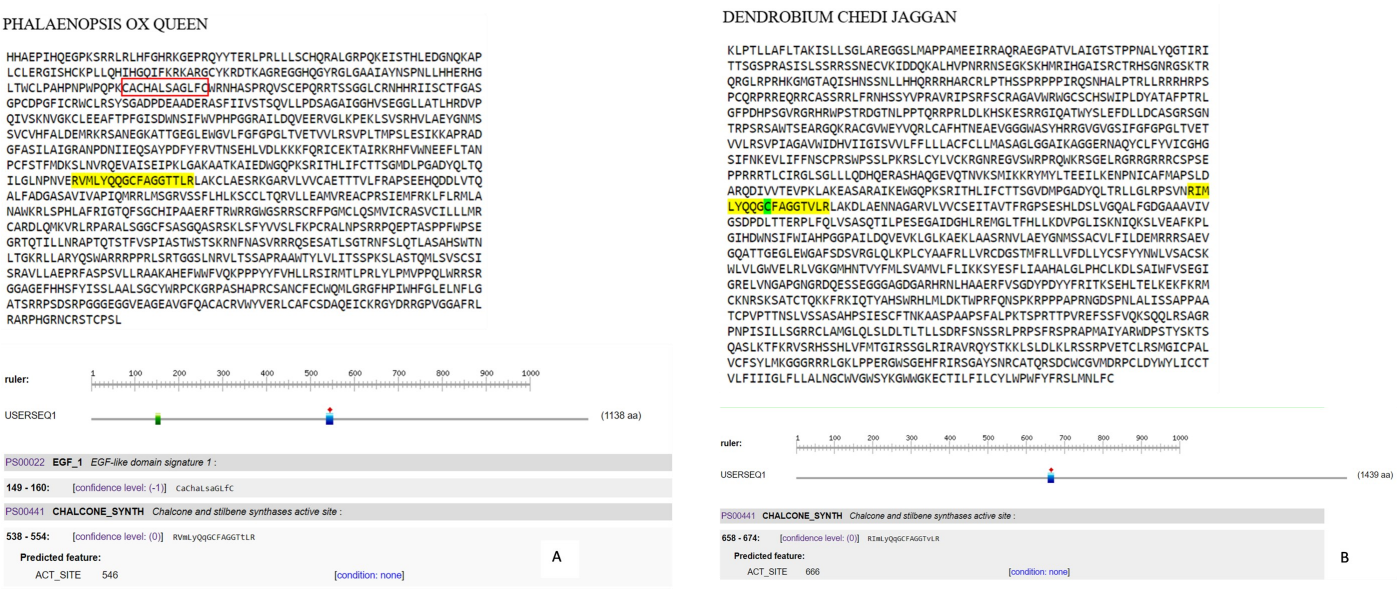


**Figure 6.** Amino acid motifs in CHS protein of *P.* 'OX Queen' (A) and *D.* 'Cheddi Jagan' (B) from PROSITE Analysis.

stand the morphological and molecular aspects of both orchid species. Morphological analysis identifies targetable unique traits, while molecular analysis delves into genetic details and gene expression (Arif & Ratnawat 2018).

This experiment provides crucial insights for price genome editing, emphasizing effective sgRNA identification and design, results are expected to deepen understanding of the genetic basis of *P.* 'OX Queen' and *D.* 'Cheddi Jagan', facilitating more targeted genome editing for variegated flower creation, primarily targeting the CHS protein function essential for flower coloration.

## CONCLUSION

This research found that the *CHS* gene fragment was successfully isolated from the genomes of two hybrid orchids *Phalaenopsis* 'OX Queen' and *Dendrobium* 'Cheddi Jagan' which have plain pink and purple flowers, apparently in both orchids there are CHS protein with the CHS family domain motif that function for flavonoid synthesis, but they have slight differences in the DNA structure of the *CHS* gene. This indicates the possibility that the results of the phenotypic analysis of flower colour and *CHS* gene structure can be used to determine target sequences for sgRNA to create *Phalaenopsis* and *Dendrobium* orchids with variegata patterned of flower's color using the CRISPR/Cas9 Genome editing system.

## AUTHOR CONTRIBUTION

E.S. designed the research and supervised all the process, Y.R.H. and E.G. responsible for laboratory activities, phenotypic and molecular data analysis and writing the manuscript, P.D.K assisted in the wet lab and dry lab activities and manuscript proofreading and A.P. conducted data and manuscript proofreading.

## ACKNOWLEDGMENTS

This research was supported by a Research Thesis Magister (PTM), Universitas Gadjah Mada 2023 with Contract No: 3201/UN1/DITLIT/Dit-Lit/PT.01.03/2023 that ES as the PI. The authors would like to thank Nambangan Orchid for supplying orchids and have very well cooperation with authors for this research.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this research.

## REFERENCES

Arif, A. & Ratnawati., 2018. The Relativity Relation of Dendrobium Based on Morphological And Anatomical Leaf Characters. *Jurnal Prodi Biologi*, 7(4), 213.

De, L.C. et al., 2015. Morphological Characterization in Dendrobium Species. *Journal of Global Biosciences*, 4(1), 1198–1215.

Dwiyani, R. et al., 2016. Transformasi Gen Pembungaan melalui Agrobacterium tumefaciens Secara In-Vitro pada Tanaman Anggrek Vanda tricolor. *AGROTROP*, 6(1), pp.83–89.

Green, M.R. & Sambrook, J., 2019. Analysis of DNA by Agarose Gel Electrophoresis. *Cold Spring Harbor Protocols.*, 2019(1). doi: 10.1101/pdbtop100388. PMID: 30602561.

Grotewold, E., 2006. The Genetics and Biochemistry of Floral Pigments. *Annual Review of Plant Biology*, 57(1), pp.761–780. doi: 10.1146/annurev.arplant.57.032905.105248.

Han, Y.Y. et al., 2006. Cloning and characterization of a novel chalcone synthase gene from *Phalaenopsis* hybrida orchid flowers. *Russian Journal of Plant Physiology*, 53(2), pp.223–230. doi: 10.1134/S1021443706020129.

Hartono, D.A., Setyorini, S. & Karimah, S.A., 2021. Model Komputasi Blast Pada Lingkungan Hadoop. *eProceedings of Engineering*, 8(1), pp.908-917.

Heikrujam, J., Kishor, R. & Mazumder, P.B., 2020. The Chemistry Behind Plant Dna Isolation Protocols. In *Biochemical Analysis Tools - Methods For Bio-Molecules Studies*. IntechOpen. doi: 10.5772/intechopen.92206.

Herdenberger, F. et al., 1990. Isolation of flower-specific cDNA clones from sunflower. *Plant Science*, 69(1), pp.111–122. doi: 10.1016/0168-9452(90)90109-2.

Jia, Y. et al., 2023. Molecular characterization of a chalcone synthase gene RhCHS from Rhododendron × hybridum Hort. *Gene*, *857*. doi: 10.1016/j.gene.2023.147176.

Karoy, R.E., Kainde, R.P. & Pangemanan, E.F., 2022. Inventarisasi Anggrek Hutan Di Tahura Gunung Tumpa. *COCOS*, 14(4), pp.1-13. doi: 10.35791/cocos.v1i1.38918

Kong, X. et al., 2020. Identification of chalcone synthase genes and their expression patterns reveal pollen abortion in cotton. *Saudi Journal of Biological Sciences*, 27(12), pp.3691–3699. doi: 10.1016/j.sjbs.2020.08.013.

Kuo, Y.T. et al., 2019. Segmental and tandem chromosome duplications led to divergent evolution of the chalcone synthase gene family in phalaenopsis orchids. *Annals of Botany*, 123(1), pp.69–77. doi: 10.1093/aob/mcy136.

Li, C. et al., 2021. A review for the breeding of orchids: Current achievements and prospects. *Horticultural Plant Journal*, 7(5), pp.380–392. doi: 10.1016/j.hpj.2021.02.006.

Linggabuwana, A. et al., 2024. Isolation and Characterization of Chalcone Synthase (CHS) Gene in Variegated-Flower of Dendrobium "Enobi"and Phalaenopsis Hybrid Orchids. *HAYATI Journal of Biosciences*, 31(2), pp.382–391. doi: 10.4308/hjb.31.2.382-391.

Liu, J. et al., 2022. Color fading in lotus (Nelumbo nucifera) petals is manipulated both by anthocyanin biosynthesis reduction and active degradation. *Plant Physiology and Biochemistry*, 179, pp.100–107. doi: 10.1016/j.plaphy.2022.03.021.

Luo, H. et al., 2017. Characterization of active anthocyanin degradation in the petals of *Rosa sinensis* and *Brunfelsia calycina* reveals the effect of gallated catechins on pigment maintenance. *International Journal of Molecular Sciences*, 18(4), 699. doi: 10.3390/ijms18040699.

Murray, M.G. & Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, 8(19), pp.4321–4326. doi: 10.1093/nar/8.19.4321.

Ohno, S. et al., 2018. Post-transcriptional silencing of chalcone synthase is involved in phenotypic lability in petals and leaves of bicolor dahlia (Dahlia variabilis) 'Yuino.' *Planta*, 247(2), pp.413–428. doi: 10.1007/s00425-017-2796-3.

Pratama, A.N. et al., 2023. Low-light intensity reprogramed flower pigmentation in Dendrobium Sonia via downregulation of dihydroflavonol 4-reductase and anthocyanidin synthase genes. *Scientia Horticulturae*, 312, 111853. doi: 10.1016/j.scienta.2023.111853.

Putra, R.P., 2021. Identifikasi jenis tanaman anggrek melalui tekstur bunga dengan tapis Gabor dan M-SVM. *JOINTECS (Journal of Information Technology and Computer Science)*, 6(1), pp.29-34.

Rizko, N. et al., 2020. Isolasi DNA Daun Jeruk Bali Merah (Citrus maxima Merr.) dengan Modifikasi Metode Doyle and Doyle. *Berkala Bioteknologi*, 3(2).

Royal Horticultural Society., 2024a, '*Phalaenopsis* OX Queen, OX Orch. 2008' in *Royal Horticultural Society*, viewed February 6, 2024, from https://www.rhs.org.uk/The International Orchid Register / RHS Gardening.

Royal Horticultural Society., 2024b, '*Dendrobium* Cheddi Jagan, Singapore Bot.Gdns 1994' in *Royal Horticultural Society*, viewed February 6, 2024, from https://www.rhs.org.uk/The International Orchid Register / RHS Gardening.

Sari, V.K. & Restanto, D.P., 2022. Review Artikel: Metode Ekstraksi Dna Genom Untuk Tanaman Tinggi Kandungan Polisakarida Dan Metabolit Sekunder. *Agroteknika*, 5(2), pp.118–129. doi: 10.55043/Agroteknika.V5i2.155

Semiarti, E. et al., 2007. Agrobacterium-mediated transformation of the wild orchid species Phalaenopsis amabilis. *Plant Biotechnology*, 24, pp.265–272. doi; 10.5511/plantbiotechnology.24.265

Semiarti, E. et al., 2020. Innovation of Natural Orchid Cultivation Technology For Tourism Development In Banyunganti Hamlet, Jatimulyo Village, Girimulyo Sub- District, Kulon Progo District, Yogyakarta. *Journal of Tropical Biodiversity And Biotechnology*, 5(3), pp.178–182. doi: 10.22146/jtbb.46283.

Sun, W. et al., 2015. Molecular and biochemical analysis of chalcone Synthase from freesia hybrid in flavonoid biosynthetic pathway. *PLoS ONE*, 10(3), e0119054. doi: 10.1371/journal.pone.0119054.

Tai, D. et al., 2014. A malus crabapple chalcone synthase gene, McCHS, regulates Red petal color and flavonoid biosynthesis. *PLoS ONE*, 9 (10), e0110570. doi: 10.1371/journal.pone.0110570.

van Tongerlo, E. et al., 2021. Vegetative traits can predict flowering quality in Phalaenopsis orchids despite large genotypic variation in response to light and temperature. *PLoS ONE*, 16(5), e0251405. doi: 10.1371/journal.pone.0251405.

Voss, D.H., 2001. Relating Colorimeter Measurement of Plant Color to the Royal Horticultural Society Colour Chart. *HortScience*, 27(12), pp.1256-1260. doi: 10.21273/HORTSCI.27.12.1256.

Waluyo, Sustiprijatno & Suharson., 2013. Transformasi Genetik Tembakau dengan Gen Cold Shock Protein melalui Perantara Agrobacterium tumefaciens. *Jurnal AgroBiogen*, 9(2), pp.58-65.

Yonindi, N.B. et al., 2022. Stabilitas Genetik Klon Tanaman Tebu Transgenik Berbasis Rnai Dan Ketahanannya Terhadap Infeksi Scmv. *Agriprima : Journal of Applied Agricultural Sciences*, 6(1), pp.35–44. doi: 10.25047/Agriprima.V6i1.452

Zhao, D. et al., 2012. An actin gene as the internal control for gene expression analysis in herbaceous peony (*Paeonia lactiflora* Pall.). *African Journal of Agricultural Research*, 7(14), pp.2153–2159. doi: 10.5897/AJAR11.1613.

Zhou, L. et al., 2011. Molecular characterization and expression analysis of chalcone synthase gene during flower development in tree peony (*Paeonia suffruticosa*). *African Journal of Biotechnology*, 10(8), pp.1275 -1284. doi: 10.5897/AJB10.599.