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Morphology and molecular characterization of *Vanda tricolor* × *Vanda limbata* orchid hybrid based on *VOH1* gene characters

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ABSTRACT *Vanda* is a monopodial epiphyte orchid that spreads throughout Asia and Southeast Asia reaching 70 species. Indonesia itself has its own endemic *Vanda* orchid such as *Vanda tricolor* and *Vanda limbata*. A hybrid of *V. tricolor* and *V. limbata* is predicted to form a new specific character in the flower and leaf. The purpose of this study was to determine the morphological and molecular differences between *V. tricolor*, *V. limbata*, and *Vanda* hybrids resulting from crosses between that two orchids, by analysing the morphological characteristics of the roots, leaves, flowers and the structure of the *Vanda Orchid Homeobox1* (*VOH1*) shoot-forming gene isolated from *V. tricolor*, *V. limbata*, and their hybrids. The morphological analysis was conducted using RHS colour chart, size measurement of plants, and the transversal preparation of the leaf. Molecular analysis was performed by PCR using *Dendrobium Orchid Homeobox 1* (*DOH1*) primers, followed by sequencing and bioinformatic analysis. Morphologically, the flower's colour of the hybrid is most similar to *V. limbata* but the flower's patterns are more similar to *V. tricolor* meanwhile the leaf colour of the hybrid is brighter than the both parents. The slides illustrate the sclerenchyma tissue is made up of strongly thickened walls containing lignin indicates the presence of homeobox *DOH1* gene homolog, namely *VOH1*. The molecular result displayed by the phylogenetic tree of the *VOH1* indicates that the hybrid has more similarities with *V. limbata*.

KEYWORDS *Vanda* hybrid; *Vanda limbata*; *Vanda tricolor*; *VOH1* gene

1. Introduction

The Orchidaceae is the most diverse family of flowering plants (Chase et al. 2015). These plants exhibit a wide variety of floral forms, sizes, colors, scents, and properties (Zhang et al. 2018). The genus *Vanda* Jones ex R.Br. contains between 75–80 species composed of epiphytes and lithophytes. The morphological characteristics of *Vanda* species include upright growth of shoots with monopodial type and leaves that are slim, dense, and lateral (Risdiana et al. 2023).

Among the species, *V. tricolor* is one of the most well-known, with white flowers adorned with red purple-brown dots (Kusumastianto et al. 2015). This orchid is found in Sleman Regency, Yogyakarta, on the slopes of Mount Merapi. This orchid has white blooms with specks of reddish-purple (Rineksane et al. 2021). *V. limbata* which can be found in Nusa Tenggara Barat, is characterized by yellow spots on its petals and a maroon base (Dwiyanto et al. 2017). The most beneficial traits of both parents species can be combined to create hybrid offspring when two individuals with opposing traits are crossed (Li et al. 2021). One hybrid orchid being produced is a cross between *V. limbata* as the male and *V. tricolor* as a female which contribute the ovule (Mellissa 2019). It is anticipated that orchid hybrids with a variety of traits will be produced, as both orchids have remarkable traits as parent crossings (Tuwo and Indrianto 2016).

Morphological observations are easy to conduct since the dominant characteristic are present in many different parts of the plant (Indraloka et al. 2019). Determining the variations in morphological characteristics between vegetative and generative organs is important for this characterization. To assess morphological traits, plant components such as stems, flowers, leaves, roots, seeds, and fruit were recognized and recorded (Hartati et al. 2021).

Along with the morphological traits that have been used for plant identification, molecular characterization method, which measures genetic variety directly at the scale of the DNA molecule also has great importance (Iroka et al. 2015) and (Andrade-Rodriguez et al. 2019).

KNOTTED1-LIKE HOMEOBOX (*KNOX*) genes are one of the five classes of plant homeobox genes that are categorised according to sequence similarities in the homeodomain and surrounding areas (Yu et al. 2000). *C*lass-1 *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) genes were transcriptionally active in the preservation of the

SAM, which functions in the growth and maturation of shoots, regulator of lignin biosynthesis in plant tissues, and participate in the formation of lobes and leaflets (Wang et al. 2022) and (Townsley et al. 2013). Yu et al. (2000) reported their findings in which they isolated and characterised *DOH1*, an orchid homeobox gene that codes for a member of the class 1 *KNOX* gene family.

Previously *VOH1* gene in *V. tricolor* has been found by Ruben et al. (2022). It was found that the *VOH1* gene functions similarly to *DOH1*, which is involved in *Dendrobium* shoot development. Though *Dendrobium* and *Vanda* have different types of stem growth, in which *Vanda* is a monopodial plant, while *Dendrobium* is a sympodial plant, their homeobox genes are quite similar. Therefore, this study was carried out to continue the characterization of *VOH1* in the *V. limbata* and *Vanda* hybrid.

2. Materials and Methods

2.1. Materials

The materials applied in this study include three plants with eight year old of age each, a *V. tricolor* from orchid nursery in Balelawang, a *V. limbata* from Griya Anggrek Yogyakarta, and a *Vanda* hybrid from Karanggayam Greenhouse in which 2 leaves of each plants are taken for DNA isolation. To make transversal sections of leaves, alcohol series of 70–100%, FAA (Formaldehyde Acetic Acid), xylol, orange G, safranin staining, paraffin, and Canada balsam were used. Chemical materials for DNA isolation such as alcohol 70 percent, Cetyltrimethylammonium bromide (CTAB), chloroform, isopropanol, sodium acetate, iso-amyl alcohol. Meanwhile for PCR amplification, MyTaq HS Red Mix (Bioline), ddH_2O, primers forward and reverse of *DOH1* and *ACTIN* as the positive control. Lastly, for the electrophoresis process, Agarose, TAE, and 1st Base florosafe DNA stain was used.

2.2. Methods

2.2.1 Morphology observation

Habitus of *V. tricolor*, *V. limbata*, and *Vanda* hybrid were observed and photographed. The length and width of each part were then measured using a ruler and written down in a table to be compared. The colours of flowers, leaves, pedicel, and root were compared using the physical Sixth Edition of Royal Horticultural Society (RHS) Color Chart for accuracy in colour measurement.

The preparations of leaf transversal sections were made following the method according to Dionne and Spicer, 1958 Ruzen (1951), first is fixation of leaves. Leaf samples of *V. tricolor*, *V. limbata*, and *Vanda* hybrid were cut transversely. The samples were fixed into a fixative solution of FAA (Formaldehyde Alcohol Acetic Acid) for 24 hours. Dehydration samples where samples were put into alcohol with different percentages and duration. Dealcoholization, where the samples were then put into solution alcohol: xylol 1 (3:1), alcohol: xylol 2 (1:1), alcohol: xylol 3 (1:3) until the samples became translucent. Embedding where the samples are put into a xylol paraffin put into the mold for 24 h at room temperature. Cutting the samples was done using a microtome rotary and put on the glass object with glycerin albumin and distilled water and heated. Staining was done using safranin 1%, Orange G 2% solution and Tanic acid. Samples then washed and put into Iron Alum 1%. Lastly the steps of dehydration and dealcoholization were repeated, and mounting was done using Canada balsam.

2.2.2 DNA isolation

The method for DNA isolation to be used in this research is Doyle (1990). As the first step three different mature leaves of *V. tricolor*, *V. limbata*, and *Vanda* hybrid were cut from their plants. The leaves were then cleaned and measured for 100 mg per sample to be preserved in a 1.5 microcentrifuge tube. The samples are then grinded using micropestle. Once the samples were crushed, 200 µL of cetyl trimethyl ammonium bromide (CTAB) were transferred into each sample and mixed. An additional 300 µL of CTAB were added, and the samples were incubated in a waterbath at 65 °C for 60 min, with the inversion every 10 min. After being taken out of the waterbath, the samples were let to remain at room temperature for two minutes. Following that, they were combined for five minutes with 480 µL of chloroform and 20 µL of isoamyl alcohol in a gyratory shaker. The samples were then centrifuged at 12,000 rpm for 15 min. The supernatant were weighed and transferred into a 1.5 microcentrifuge tube. Next, 3M sodium acetate was added to half of the supernatant volume, and isopropanol was added to the remaining two thirds. After that, the samples are incubated at -20 °C for 24 h. After a day, the samples were centrifuged for 10 min at 12,000 rpm. The particle should be visible at the tube's bottom after the supernatant was eliminated. Subsequently, 500 microliters of 70% ethanol were used to cleanse the DNA. The samples were then centrifuged at 12,000 rpm for 5 min. After another discard, a few minutes were given for the supernatant to air dry. After that, 50 microliters of TE buffer were mixed in with the pellet. The samples were then incubated in a waterbath at 37 °C for an hour. The DNA isolation findings were then stored at a temperature of -20 °C in a freezer. DNA amplification was carried out using the *DOH1* primer following the method used by Ruben et al. (2022).

PCR Mixture was made following MyTaqTM HS Red Mix (Bioline) protocol. To make a PCR mix, 12.5 µL of MyTaqTM HS Red Mix, 9.5 µL ddH_2O, 1 µL of *DOH1* forward primer, and 1 µL of *DOH1* reverse primer was added into a 0.2 mL microtube. Then 1 µL of DNA template was added into the tube. Afterward, 24 µL of the PCR mix were added into each DNA template and mixed. One cycle of DNA amplifications of *DOH1* primer composed of, predenaturation in 95 °C for 1 min, denaturation in 95 °C for 15 s, annealing in 52.7 °C for 15 s, extension in 72°C for 30 s, and final extension in 72 °C for 5 min.

Electrophoresis was done to visualise the DNA ampli-

fication result. One percent of agarose gel was used therefore 50 mL of 1× TAE was mixed with 0.4 gr of Agarose. The mixture was then heated using a microwave for 30 s, shaken, and added 1.5 µL of 1st Base florosafe DNA stain. Then the mixture was poured into the mold with the comb that has been placed. After several minutes, the gel was taken and put into the electrophoresis chamber. 2 µL of DNA amplifications result was added into the well along with 1 µL of 100 bp DNA ladder. The electrophoresis was then turned on for 50 min at 50 V. Afterward, the result was viewed using a UV transilluminator and photographed. The results were sent to 1st Base (Apical Scientific Sdn Bhd, Malaysia) for sequencing.

2.2.3 Data analysis

Data of the morphological character were analyzed and observed using Microsoft excel and the photographs are edited using Adobe Photoshop 2022 to make the black background cover of each parts therefore it can be viewed clearly and ImageJ to add the scale bar to the preparations figures. Meanwhile, molecular character data in the form of DNA sequencing results were analyzed using NCBI BLAST to find the homologue gene to *DOH1*. The sequences are aligned using MULTALIN webpage. The phylogenetic tree was made using MEGA11 application to find the similarity between the species of *V. tricolor*, *V. limbata*, and *Vanda* hybrid.

3. Results and Discussion

3.1. Morphological characters observed

All of the observed samples of *V. tricolor*, *V. limbata*, and *Vanda* hybrid have 8 years of age, all have monopodial stem growth orientation, meaning that the flowers emerge from the axils of their leaves and grow with a single stem. All three have the same sort of inflorescence, a raceme, with a single peduncle supporting many pedicels on each flower. Since each leaf apex is pointed it is called acute and the tips are varied in length, one is shorter than the other, all three have strap leaf types. Their leaf vein is straight therefore it is linear Their whole leaf border and leaf apex are obtuse, with a linear leaf vein. Their leaf margin is entire. All their root forms are rounded with blunt tip. For all three flowers, the sepal and petal have the same obovate form which means that the base is smaller and wavy edges (Figure 1–3).

The labellum of *V. limbata* flower has mentum which is a pocket from the lateral sepal that binds with the labellum (Figure 2), while *V. tricolor* and *Vanda* hybrid do not have it. The labellum of *V. limbata* has spur which is a pocket from lateral sepal that bind with the labellum. In addition, the shapes differ the hypochilium of *V. limbata* is broad, while the hypochilium of *Vanda* hybrid is thinner. *V. tricolor* also have wings that fold to each side and the mesochilium is erect with lobes in the epichilium. The epichilium of *V. limbata* lacks lobes, and the mesochilium is narrow. Lastly, the epichilium of *Vanda* hybrid has lobes, and the mesochilium is narrow. The columns are identical, with the pollen being sealed with an anther cap but varying in size (Figure 1–3).

The colour comparison using RHS colour chart of *V. tricolor*, *V. limbata*, and *Vanda* hybrid indicates that whereas the roots of *V. tricolor* and *V. limbata* are have the same colour which is 144 Strong Yellow Green B, the roots of *Vanda* hybrid are brighter (Figure 1–3). When it comes to leaf colour, *V. tricolor* has 145 Strong Yellow Green A, *Vanda* hybrid has 140 Brilliant Yellowish B, and *V. limbata* is brighter which is 144 Strong Yellow Green B. The petals and sepals colour of Vanda hybrid is brighter than that of *V. limbata*. While *Vanda* hybrid has a foundation colour of 179 Moderate Red A and spots with a similar pattern to *V. tricolor* of 10 Brilliant Yellow A, *V. limbata* has a colour base of 169 Strong Reddish Orange A with yellow dots of 9 Vivid Yellow A. On the other hand, *V. tricolor* differs in that its base colour is NN155 White B, just like the pedicel, and the spots on its petals and sepals are 183 Moderate Red C. The size of the labellum of *Vanda* hybrid is the same as that of *V. limbata*, with the exception that the base of the labellum is lighter with colour N81 Light Purple D, whereas the tip of *V. tricolor* is lighter with colour N74 Vivid Reddish Purple, which is similar to *V. tricolor*'s colour of NN74 Strong Reddish Purple (Figure 1–3).

Table 1 shows that *Vanda* hybrids have roots and stem lengths that are more similar to *V. limbata*, while *V. tricolor* is larger overall. The flowers are comparable in

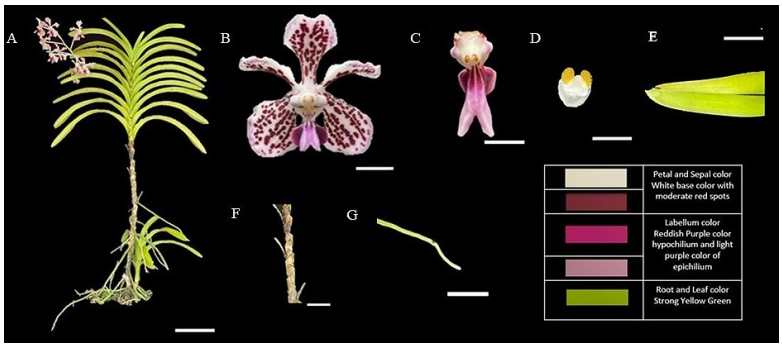

FIGURE 1 Morphology of *Vanda tricolor*. (A) Habitus, (B) Flower, (C) Flower parts and the pedicel, (D) Pollen, (E) Anther cap, (F) Column and Lip fornt view, (G) Column and Lip side view, (H) Leaf, (J) Root (Bars = 1.5 cm).

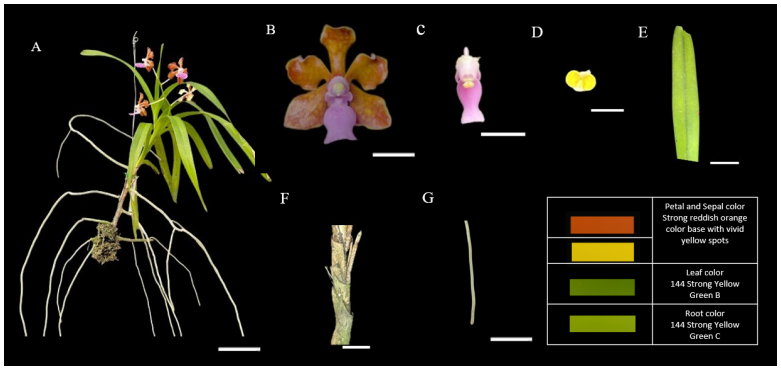

FIGURE 2 Morphology of *Vanda limbata*. (A) Habitus, (B) Flower, (C) Flower part and the pedicel, (D) Coumn and Lips fornt view, (E) Column and Lip side view, (F) Pollen, (G) Root, (F) Leaf, (I) Stem (Bars = 1.5 cm).

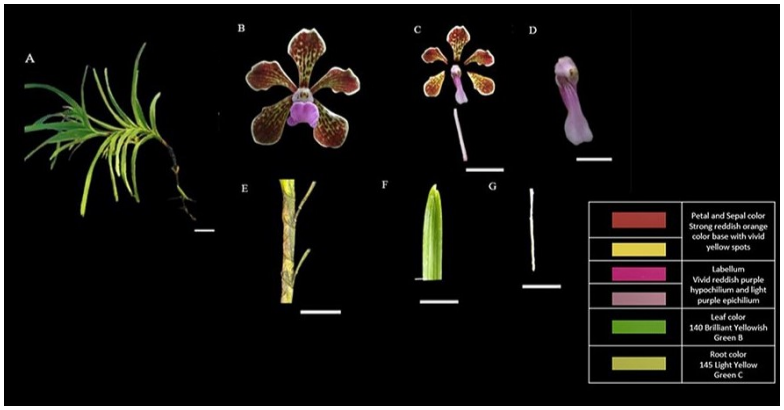

FIGURE 3 Morphology of *Vanda tricolor* × *Vanda limbata*. (A) Habitus, (B) Flower, (C) Flower including sepals, petals, columns, labellum and stem, (D) Labellum, (E) Stem, (F) Leaf, (G) Root (Bars = 1.5 cm).

TABLE 1 Morphological comparison on the size of root, leaves and flowers among *Vanda tricolor*, *Vanda limbata* and *Vanda hybrid*

Orchid / Characters	*V. tricolor* (cm)	*V. limbata* (cm)	*V. tricolor* × *V. limbata* (cm)
Root length	56 ±1.5	52 ± 2.5	54.3 ± 2.5
Root diameter	0.5 ± 2.5	0.5 ± 2.9	0.5 ± 1.81
Leaf length	30.7 ±3.9	32 ± 1.7	33.7 ± 2.36
Leaf width	5 ± 0.2	4 ± 0	4 ± 0.14
Pedicel length	7.4 ± 0	5 ± 0	4 ± 0.12
Flower diameter	6 ± 0.2	4.5 ± 0.5	5 ± 0,25
Sepal dorsal length	3 ± 0	2.4 ± 0.16	2.5 ± 0,12
Sepal lateral length	3.1 ± 0.08	2.2 ± 0.1	3 ± 0,12
Petal lateral length	3 ± 0	2.2 ± 0.16	3 ± 0
Column length	1.3 ± 0	1 ± 0	1 ± 0.1
Labellum length	2.6 ± 0.06	1.6 ± 0.17	2 ± 0.2
Labellum width	2.4 ± 0	1.3 ± 0.05	1.5 ± 0
Pollen length	0.5 ± 0.08	0.4 ± 0	0.4 ± 0

size, with *V. tricolor* being larger than both *V. limbata* and *Vanda* hybrids. This can be observed in the length and width of the labellum, as well as in the flower diameter, petal, column, spur, and sepal dorsal and lateral in Figure 1–3. Regarding their root width, leaf width, flower diameter, sepal dorsal length, sepal lateral length, pollen and viscidium length, and other dimensions, *V. limbata* and *Vanda* hybrid are quite comparable in size. On the other hand, *V. limbata* and *Vanda* hybrid have a similar-sized spur.

Plants may respond to changes in their surroundings. Environmental factors can affect plant growth directly through physical factors that alter basic growth processes, or indirectly through developmental adaptation. Numer-

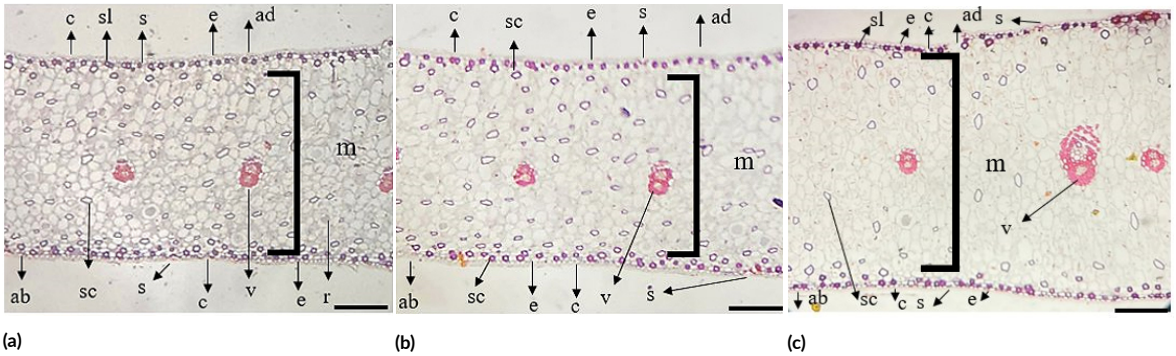

FIGURE 4 Transversal leaf anatomy of *Vanda tricolor*, *Vanda limbata* and *Vanda* hybrid. (A) *V. tricolor*, (B) *V. limbata*, and (C) *Vanda* hybrid; ad : adaxial (upper leaf); ab : abaxial (lower leaf); e : epidermis; r : raphid cystal: c : cuticle; v : vascular bundle; m : mesophyll tissue; sl : sclerenchyma; sc : sclereid (Bars = 15 µm).

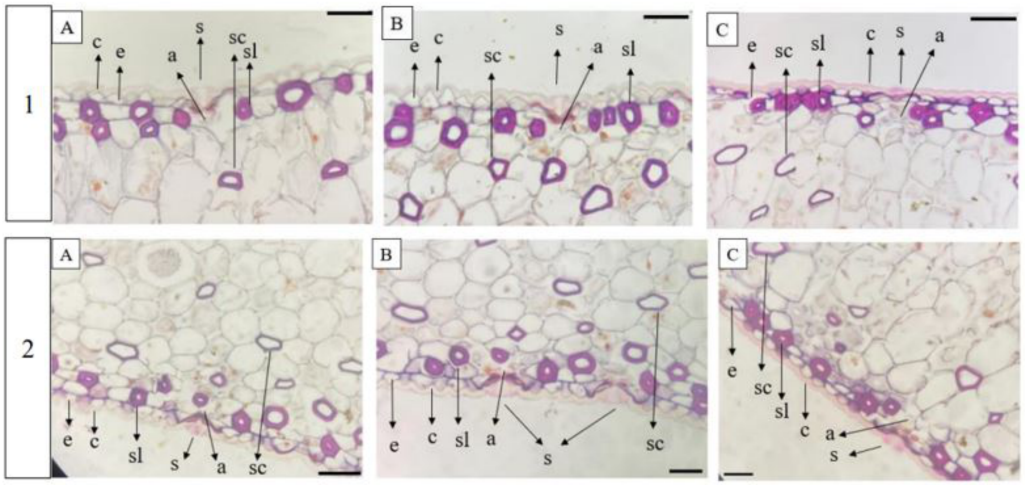

FIGURE 5 Adaxial and abaxial leaf anatomy of *Vanda tricolor*, *Vanda limbata* and *Vanda* hybrid. (1) Adaxial, (2) Abaxial, (A) *V. tricolor*, (B) *V. limbata*, (C) *Vanda* hybrid; Sc : sclereid; SI : sclerenchyma; e : epidermis; c : cuticle; a : air cavity; s : stomata (Bars = 15 µm).

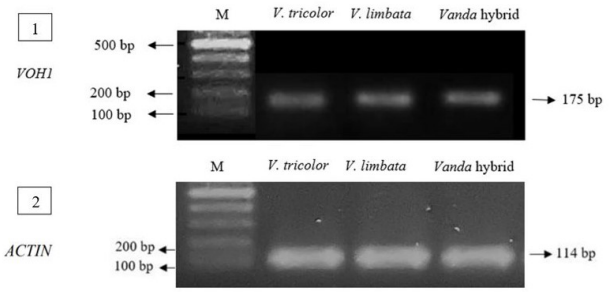

FIGURE 6 Electrophoregraph of amplified *VOH1* gene of *Vanda tricolor*, *Vanda limbata* and *Vanda* hybrid. (1) *VOH1* gene (2) *ACTIN* gene as internal/positive control (M = 100bp ladder (Geneaid)).

ous aspects of the environment, such as temperature, light, nutrient availability, and water availability and limited resources, have an impact on plant development. Many plant characteristics are climate-dependent (Li et al. 2015). Since all three of the plant's samples were obtained in a similar environment in warm and humid conditions, this may help to explain the similarities in morphological characteristics of *Vanda* hybrid influencing the qualities that both species share in their hybrid, *Vanda* hybrid.

The species of *V. tricolor* used in this study is forma Merapi, which is distinguished from *V. tricolor* from Bali or Java by having purple dots and a white petal base (Semiarti et al. 2020). Contrarily, *V. limbata* has a wide range of colours. In Java, they often have net patterns and pale brown petals, whereas in Flores Island, most people observe them with red petals (Metusala 2011).

The transversal leaf anatomic of *V. tricolor*, *V. limbata*, and Vanda hybrid shows in Figure 5 and 6. There are differences that can be viewed such as the epidermis shapes, in *V. tricolor* and *V. limbata* they have triangular shapes while in *Vanda* hybrid, the shape is rectangular. Similarly, *V. tricolor*, *V. limbata*, and *Vanda* hybrid have cuticles covering the epidermis.

There are also sclerenchyma spread throughout the tissue especially in the epidermis and vascular bundles area. When mature, sclerenchyma tissue is made up of dead cells with strongly thickened walls that contain lignin and a high percentage of cellulose (60–80%). Primary and secondary walls are the two types of cell walls seen in sclerenchyma cells. The secondary wall gives the cell and tissue a great deal of stiffness and hardness. It is very thick and heavily lignified (15–35%) (Carrillo-López and Yahia

2019). Given their function in controlling the formation of lignin, as demonstrated by their participation in lignification, it is highly likely that *KNOX* genes have the potential to be useful regulators of lignification in plant tissues (Townsley et al. 2013) (Figure 5–6).

3.2. Molecular characterization

Due to the presence of lignin in sclerenchyma walls, the molecular characterization aims to identify the *KNOX1* homologous gene in the leaf, in *Phalaenopsis* it is called *POH1*, in *Vanda* it is called *VOH1*, and in *Dendrobium* it is called *DOH1*. Figure 7 illustrates that the DNA was amplified using the *DOH1* primer and the *VOH1* gene was found with a length of 175 bp in all three species, *V. tricolor, V. limbata*, and *Vanda* hybrid. Figure 8 display the nucleotide sequences of *V. limbata*, and *Vanda* hybrid are aligned with *V. tricolor*, *Phalaenopsis equestris POH1*, *Dactylorhiza fuchsii KN4*, *Dendrobium grex* Madame Thong-IN *DOH1*, *Dendrobium nobile DOH1*, also the model plant *Arabidopsis thaliana KNAT1* to find the similarities and differences. These sequences are amplified from 781–1027. The red and blue coloured letters symbolized the conserved gene in *VOH1*, while the black symbolized the non conserved gene. There are seven most conserved gene found with red letters.

A collection of species' most recent common ancestor relationships can be concluded in phylogenetic trees. Measuring the degree of discordance or resemblance between two suggested trees is frequently necessary (Weyenberg and Yoshida 2016). By using DNA sequence data, the

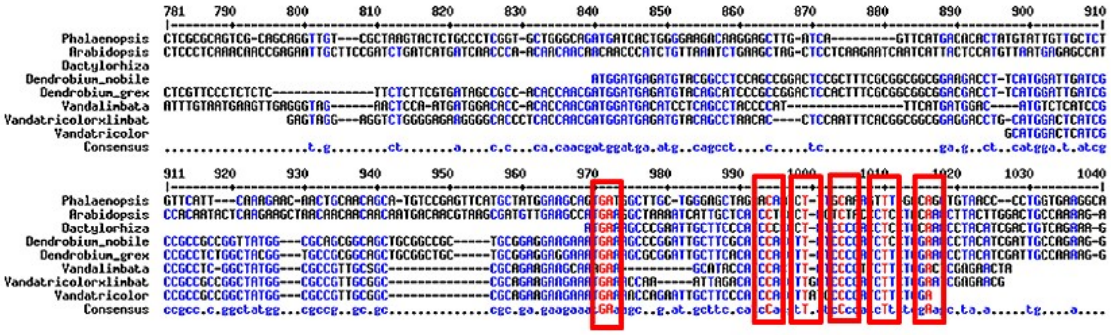

FIGURE 7 DNA alignment of the *VOH1* gene from *Vanda tricolor*, *Vanda limbata* and *Vanda* hybrid with *VOH1* homologous gene from other plant species.

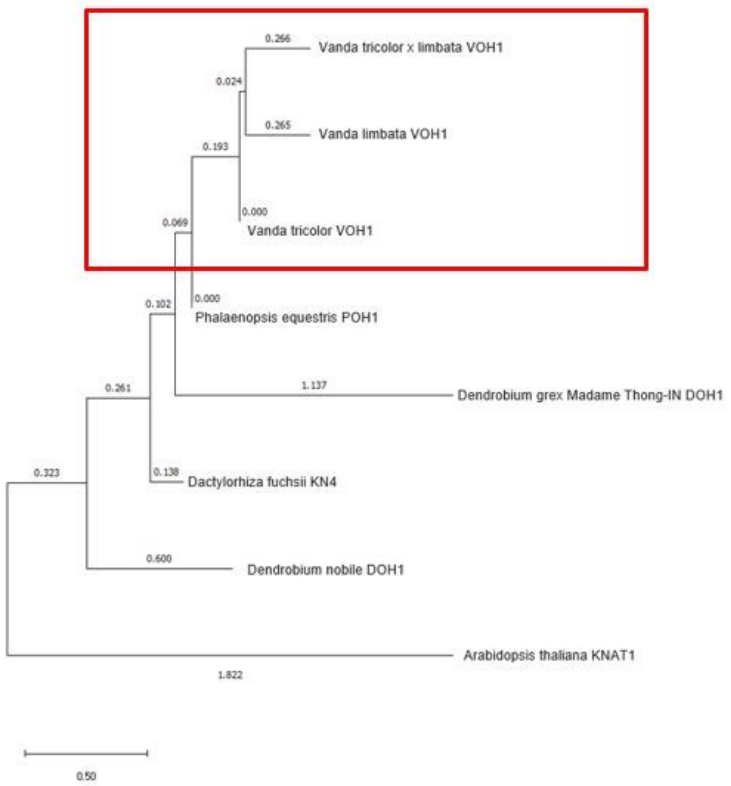

FIGURE 8 Phylogenetic tree of *VOH1* gene of *Vanda tricolor*, *Vanda limbata* and *Vanda* hybrid with *VOH1* homologous gene from other plant species.

understanding of phylogenetic relationships across all taxonomic groups of terrestrial plants has greatly increased as a result of the latest developments in analytical tools, DNA extraction, and sequencing (Liu et al. 2022). Using Gamma Distribution 5 and the JTT (Jonas-Taylor-Thornton) Model, phylogenetic analysis was performed using MEGA11 software. *Arabidopsis thaliana KNAT1*, the model plant, was used as the outgroup in the formation of a clade. One subclade can be seen in the picture, with *Vanda* hybrid being the most similar to *V. limbata aH1*, and *D. nobile DOH1*, which are also orchids. Hence, it may be concluded that the discovered *VOH1* is homologous to the other *KNOX1* homologous gene of *D. nobile*, *D. fuchsii*, *P. equestris*, and *A. thaliana* (Fig. nd *V. tricolor*. Subsequently, there was *P. equestris POH1*, *D. grex* Madame Thong-IN DO8).

4. Conclusions

Based on the research that has been conducted, there are several conclusion that can be drawn, the morphological differences between *V. tricolor*, *V. limbata*, and *Vanda* hybrid are in the shoot growth development that *V. tricolor* grow uniformly upright, *V. limbata* grow disorganize upright, and *Vanda* hybrid grow intermediate as the mix. The sepals and petals colour are also distinct, with *Vanda* hybrid is more similar to *V. limbata* as the male parent but its size and the dot patterns on *Vanda* hybrid is similar to *V. tricolor* as the female parent. The transversal leaf sections between *V. tricolor*, *V. limbata*, and *Vanda* hybrid have similar structures of thick sclerenchyma walls which were regulated by *KNOX1* gene. Molecularly, the *VOH1* of *V. tricolor*, *V. limbata*, and *Vanda* hybrid are managed to be isolated and found to be similar with one another with 36 conserved genes in the *VOH1* alignment and found to be homologue with *KNOX1* of other plants species indicating that they have similar functions in SAM maintenance for shoot growth, lignin synthesis, and compound leaf formation. Therefore this could serve as a recommendation for future breeding programs and given that the differentiation in the *VOH1* gene is predominantly observable through length variations, it is imperative to extend the investigation to the *CHS* gene and identify the colour differences that are morphologically observed in the gene of *V. tricolor*, *V. limbata*, and *Vanda* hybrid.

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Authors' contributions

HA and CAM carried out the morphological observation and DNA isolation. Writing, data analysis, and paper preparation were completed by HA. ES was in charge of supervising the implementation of the research and facilitating the discussion of its conclusions. All writers have examined and approved the final content.

Competing interests

The authors declare that there are no conflicts of interest related to this publication.

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