DNA Barcoding of *Vanda tricolor* Lindl. Based on *matK*, *rbcL* and ITS2 Sequences

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Abstract. *Vanda tricolor* Lindl. is one of the important ornamental plants favored by many people in the world. However, this plant required many years for vegetative growth before the first flowers emerge. Since the flower is one of the most important organs in morphological identification, an alternative approach through DNA barcoding is one of the best breaking approaches for fast and accurate identification regardless of floral organs availabilty. In this study, three different primer sets (*matK*, *rbcL*, ITS2) were used for molecular identification. DNA isolation was carried out using plant genomic isolation kit and was applied as template in PCR reaction. After confirmation using agarose gel electrophoresis and visualized under UV transilluminator, the PCR products were then purified and subjected for sequencing analysis. The sequences length obtained were 408 bp, 317 bp and 461 bp for *matK*, *rbcL*, and ITS2, respectively. Further analysis using BLAST and phylogenetic tree construction revealed that all three primer sets are applicable for identification of *Vanda* orchids. Notably, the ITS2 sequence showed specificity and able to discriminate up to the species level, thus recommended to be used in molecular identification of *Vanda* orchids.

Keywords: Molecular identification · Specific marker · Vanda orchids

1 Introduction

Indonesia as one of the world's mega biodiversity is estimated to have 25% of the world's flowering plant species, including orchids. Orchid (Orchidaceae) is a flowering plant family with the most species members up to 4,000 species. Orchids have economic value as ornamental plants with high aesthetics [1]. One of the most popular orchids possessing beautiful flowers is the *Vanda* species [2]. *Vanda* is orchids type which is estimated to have ±40 species. *Vanda tricolor* Lindl. is one of the orchids species with attractive flower and possess medicinal properties such as anti-cancer and aromatherapy. Most of these orchids are taken from the forest and traded under the local name "pandan orchid" [3]. Generally, *V. tricolor* can be identified based on characteristics such as leaf shape, flower color, texture and flower petals [4]. The leaves shape of *V. tricolor* has similarities with *Vanda limbata* Blume i.e., linear with flat leaf edges (entire) [5]. The first flowering time of *V. tricolor* orchids is relatively long, about five years since seed

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sowing, therefore, it is difficult to find these orchids in a flowering state in nature [6]. Hence, an alternative identification method is needed other than based on morphological characters, namely DNA barcoding [7].

DNA barcodes are standardized short DNA sequences with special characters that can be used to speed up and facilitate species identification. DNA barcodes can be obtained from the chloroplast, nuclei, and mitochondria [8]. The Consortium for the Barcode of Life (CBOL) suggested the use of chloroplast-resident *matK* and *rbcL* genes as barcodes for plant DNA [9]. The *matK* gene provides high resolution in comparing plant species, but the gene is difficult to amplify. In contrast, the *rbcL* gene is easy to amplify but has a low resolution so that it is difficult to distinguish species in close relatives [7]. The Internal Transcribed Spacer (ITS) region is a potential barcode originating from the nuclei. This region has a small size (± 700 bp) and has many copies in the genome so that it is easy to isolate, amplify and analyze [10].

With above-mentioned background and consideration, this research aimed to identify potential barcodes using three molecular markers i.e., *matK*, *rbcL* and ITS2 for *V. tricolor* orchids.

2 Materials and Method

2.1 Sample Collection and Genomic DNA Isolation

The sample of *Vanda tricolor* orchid used in this study was obtained from the Mount Gumitir area, Jember Regency, East Java, Indonesia. Genomic DNA isolation was carried out using GenEx kit (Korea). The leaves (0.1 g) were crushed into finely powder using mortar and pestle, then subjected to proceed with several buffer provided and following the genomic extraction procedures as stated in manufacturer's instruction. The resulted homogenate then subjected for precipitation using isopropanol and washed with 70% ethanol. In the final stage, the genomic DNA (pellet) was dissolved with resuspension (RE) buffer and stored for further use.

2.2 PCR Analysis and Sequencing

Three different primer sets were used for DNA amplification i.e., maturase-k (*matK*), riboluse-1.5-bisphosphate carboxylase (*rbcL*) and Internal Transcribbed Spacer 2 (ITS2) [11]. Total volume for PCR was 20 μL, consisting of ddH_2O 16 μL, DNA template 2 μL, primer set 2 μL, and PCR premix (AccuPower® PCR PreMix Bioneer, Korea). The amplification reaction was performed in three steps include pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min 30 s, and the final extension at 72 °C for 5 min. The PCR product was separated in 1.25% agarose gel electrophoresis containing EtBr and visualized under UV transilluminator. The resulted DNA bands were then compared with marker 100 bp Plus DNA Ladder (Bioneer). The PCR product were sent to 1st BASE (Singapore) for purified and sequencing.

2.3 Data Analysis

Data analysis were performed using Bioedit, ClustalX 2.1 and MEGA applications. The sequencing results were edited using Bioedit software [12], then subjected into The Basic Local Alignment Search Information (www.ncbi.nlm.gov). The phylogenetic analysis was carried out by processing the sequences in each region using ClustalX 2.1 [13] and then reconstructing the phylogenetic tree using MEGA X software [14].

3 Result and Discussion

3.1 Morfological Characteristics

Several reliable references were used for exploring morphological characteristics of *Vanda tricolor* Lindl. Based on our observation, the *V. tricolor* is a monopodial orchid. The shape of the stem is straight, slender with a stem length of ± 45 cm. The leaves of the *V. tricolor* orchid are green, ligulate, the leaf margins are flat (integer) with a ragged (truncatus) tip (Fig. 1a). The flower stalks emerge from the sides of the stem alternately.

The flowers size of *V. tricolor* are ± 7 cm, white in color with brownish red spots. The flower arrangement consists of three sepals and two petals displaying a spoon-like shape. The sepals and petals of *V. tricolor* are ± 2 cm length. These flowers have a gynostegium, a side lobe and a middle lobe. Labellum of *V. tricolor* is white with a side lobe of ± 6 x 6 mm in size. The middle lobe has a large size with three ridges located in the middle (Fig. 1b). These observations are consistent with previous study which stated that the flower arrangement of *V. tricolor* consists of sepals, petals, gynostegium, side lobes and middle lobes [15]. In spite of its attractive appearance, this flower also releases a fragrant aroma.

Fig. 1. Morphology of *Vanda tricolor* Lindl. Orchid. (a) Habitus, (b) flowers: sepals (S), P (petal), Sl (side lobe), Ml (middle lobe), G (gynostegium).

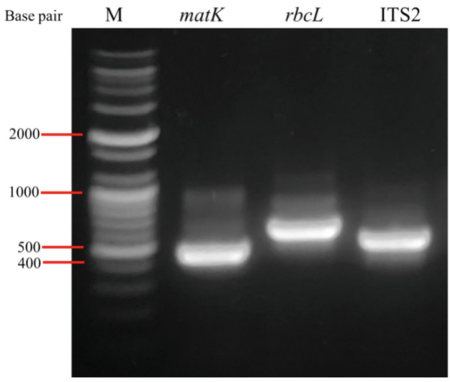

Fig. 2. Amplification of DNA target using three different primers set (*matK*, *rbcL*, ITS2) through PCR and visualized under UV-transilluminator.

3.2 Barcodes Characteristics

Amplification of target DNA was performed using three specific primer sets (*matK*, *rbcL*, and ITS2) with PCR condition as described in method. The results showed that DNA bands obtained from PCR process are clear and well-visualized (Fig. 2).

The PCR product using *matK* primer was clearly visible and showed the predicted size (± 495 bp), in accordance to the previously reported by Kumar et al. [14]. The same result for *rbcL*, the DNA bands amplified using *rbcL* primers were clearly visible with 550 bp in size, in accordance with the statement [16] that the size of *rbcL* ranged from 550–600 bp. The resulting ITS2 is clearly visible with size of ± 500 bp, in accordance with previous study that stated the resulted amplicon of ITS2 are ± 400–500 bp [17].

In the next, all of the PCR products were purified, subjected to sequencing and further analyzed (BLAST, alignment, phylogenetic). The query lenght of *matK*, *rbcL*, and ITS2 were 408 bp, 317 bp, and 461 bp, respectively. BLAST analysis for *matK* sequence showed that *V. tricolor* orchid sample had 100% homology with *Vanda tricolor* species (MT518856.1) from the USA (Table 1). These results indicate that the molecular marker *matK* can distinguish to the species level in the genus *Vanda.*

The *rbcL* sequences for *V. tricolor* are not yet available on NCBI, hence, our exploration for *rbcL* from these orchids is the first to report. The results of BLAST analysis of the *rbcL* sequence of *V. tricolor* showed the highest similarity with *Vanda coerulea* (KX344633.1) from India with percentage identity of 98.91% (Table 1). This value indicates that these two sequences have high similarity but not identical. With this high value, the *rbcL* sequence is still not the best barcode candidate yet for identification of *V. tricolor*.

The results of BLAST analysis of ITS2 sequences from *V. tricolor* showed high homology with three accession of *V. tricolor* originating from Switzerland (EF670373.1, EF670375.1, EF670374.1) with percentage identity more than 93% (Table 1). This value indicates the congruence of the *V. tricolor* sequence originated from sample with the sequence deposited in the database. High identity percentage indicates that the two species are closely related or come from a common ancestor [18]. In contrast to *rbcL*, the overall BLAST analysis of *matK* and ITS2 sequence revealed *Vanda tricolor* as

Table 1. BLAST analysis of *matK*, *rbcL* and ITS2 sequences from *Vanda tricolor* Lindl

Sequences	Name	Accession number	Per. Ident (%)	Sources
matK	*Vanda tricolor*	MT518856.1	100	USA
	Vanda tricolor	MT518855.1	100	USA
	Vanda ustii	KC823026.1	100	Philippines
rbcL	*Vanda coerulea*	KX344633.1	98.91	India
	Vanda coerulea	KX344587.1	98.53	India
	Vanda coerulea	KX344582.1	98.34	India
ITS2	*Vanda tricolor*	EF670373.1	93.71	Switzerland
	Vanda tricolor	EF670375.1	93.49	Switzerland
	Vanda tricolor	EF670374.1	93.49	Switzerland

Table 2. Alignment analysis of *matK*, *rbcL* and ITS2 sequences from *Vanda tricolor* Lindl

Primer	Sequence Number	Substitution
matK	298	C → T
rbcL	250 350	G → T C → A
ITS2	25, 37, 42, 71, 75, 167, 189, 291, 321 11, 58, 109, 166, 210, 314 49, 84	C → T C → G C → A

the highest percentage. These results bring *matK* and ITS2 as a strong candidate and recommended as potential barcode for molecular identification of *V. tricolor*.

The alignment analysis aims to compare and determine the level of homology between sequences [19]. As shown in Table 2, there were 1 and 2 bases differences observed in the *matK* and *rbcL* sequences, and 17 different bases found in ITS2 sequence. Together with BLAST results, alignment data indicate that *matK* and *rbcL* has low intraspecific variation and can not distinguish intraspecies variation in some *Vanda* species, while ITS2 showing much variation in bases different. These bases different is due to the fact of ITS2 is chromosomal DNA which are much more affected by recombination, this led to more sequence variation.

Phylogenetic analysis used the Neighbor-Joining (NJ) method and the bootstrap with a number of replications of 1000. The phylogenetic construction was carried out by taking several sequences data in the database with highest similarity to the sample used. The results of the *matK* phylogenetic analysis showed that the *V. tricolor* species closely related to *V. ustii* from the Philippines with a high bootstrap value i.e., 100 (Fig. 3). The similarity of genetic patterns and biochemical properties possessed by an organism means that these organisms originated from a common ancestor (monophyletic) [20]. *Vanda tricolor* and *V. ustii* belong to one section i.e., Deltoglossa which is classified

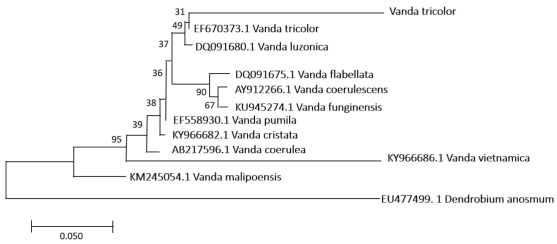

Fig. 3. Phylogenetic tree of *matK* sequences of *Vanda tricolor* and its relatives.

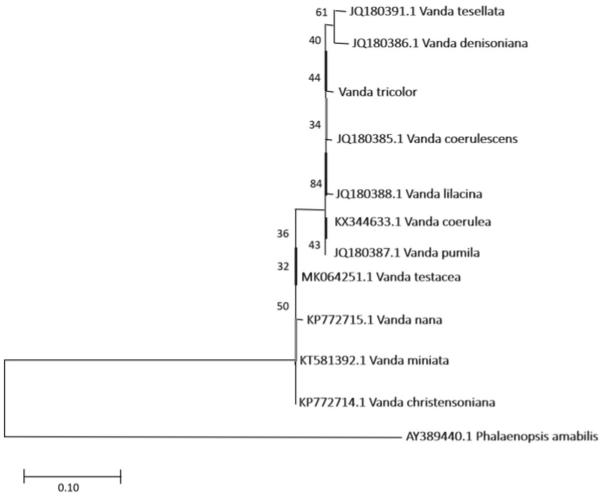

Fig. 4. Phylogenetic tree of *rbcL* sequences of *Vanda tricolor* and its relatives.

based on the similarity of cylindrical column shape with thick bases, ribbon-shaped and thick flesh leaves, and spoon-shaped petals. The difference between the two species lies in the flower pattern in which *V. tricolor* has white flowers with brownish red spots while *V. ustii* has yellowish light cream flowers colors [21].

The results of phylogenetic tree construction based on *rbcL* sequences showed that *V. tricolor* formed a cluster with *V. coerulea* (from India) with a moderate bootstrap value of 72 (Fig. 4). The morphological characteristics of *V. coerulea* include stems are ± 50 cm in height with a diameter of ± 1–1.5 cm, leaves are oval in shape, stiff with have segments. Flowers are between 7.5–10 cm, pale blue in color and round [22].

The results of phylogenetic tree construction based on the ITS2 sequence showed that *V. tricolor* sampel formed a cluster with *V. tricolor* originating from Switzerland (Fig. 5) with bootstrap value of 31. The emergence of three *V. tricolor* in BLAST analysis and contructed phylogenetic tree shows closely related and supporting the statement that ITS2 can be recommended as a molecular marker for *V. tricolor* orchids. In addition, the results showed that the ITS2 barcode had a better discriminating ability in identifying the samples in this study. The speed of ITS2 evolution affects the intraspecific variation of a species [20].

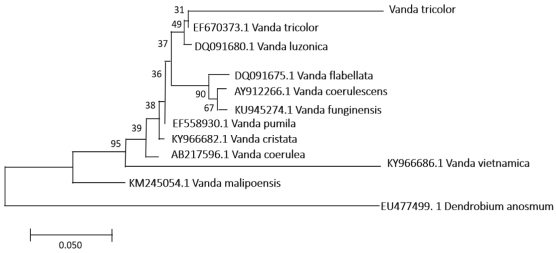

Fig. 5. Phylogenetic tree of ITS2 sequences of *Vanda tricolor* and its relatives.

Vanda tricolor Lindl. Samples were successfully amplified using three sets of primers *matK*, *rbcL* and ITS2. The query lenght sequences obtainded from *matK*, *rbcL* and ITS2 were 408 bp, 317 bp and 461 bp, respectively. The ITS2 sequence used in *V. tricolor* study is specific and can discriminate up to the species level. Based on this, the ITS2 sequence can be used as a molecular marker in determining barcodes for identification of *V. tricolor* orchids.

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