Life Science and Biotechnology
Volume 2, Number 1, May 2024
Pages: 33 – 40

DNA Barcode Characteristic of Dendrobium crumenatum based on ITS2

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Abstract

Dendrobium crumenatum, known as the pigeon orchid, is a type of orchid that is used as medicine by local people. Morphological identification to the *D. crumenatum* has an obstacle due to the similar reproductive stucture to the same genus of *D. heterocarpum.* DNA barcoding is an alternative technique for identifying *D. crumenatum* which provides a more objective measure based on genetic data. One of the molecular markers that is reliable and widely used in DNA barcoding is ITS2. The purpose of this research is to identify the ITS2 sequence as a molecular barcode effective for *D.crumenatum.* Genomic DNA isolation of *Dendrobium crumenatum* was carried out with the CTAB method (*Cetyl Trimethyl Ammonium* Bromide) and DNA amplification using PCR. The results showed that DNA barcoding research using the ITS2 sequence in *D. crumenatum* provides specific results to differentiate *D. crumenatum* from other Dendrobium species. The similarity of flower morphology in *D. crumenatum* and *D. formosum* proposed as the cause of close proximity shown in phylogenetic tree. Based on these results, it is highly recommended to use the ITS2 sequence as a molecular marker for barcoding orchids, especially *D. crumenatum* since its capability for differentiating between species with morphological similarities.

Keywords: *Dendrobium crumenatum*, DNA Barcoding, ITS2

Introduction

Indonesia is a country of rich in flowering plants, especially Orchids. The family Orchidacea was estimated covered around 5,000 species spread around the country (Rukmana, 2000). Common utilization of orchids is as ornamental plant, this plant can be used also as natural material for medicinal purposes (Silalahi et al., 2015). Orchids are also sold as cut flower commodities with a great demand (Suradinata et al., 2016). Genus Dendrobium with an estimated 275 species is a kind of the popular orchids in Indonesia (Widiastoety et al., 2010).

Dendrobium is a large genus of epiphytic orchid species. Several species of this genus are also known to be used as traditional medicine in China (Xing et al., 2013). Dendrobium is extensively dispersed from Japan to Indo-Malayan and Indonesia to Australia, the Pacific islands and New Zealand (Yukawa et al., 1992). The taxonomy and systematics of the genus Dendrobium will be easier to identified through a combination of morphological and molecular identification. This is important for considering the problems in differentiating numerous species of Dendrobium, such as *D. crumenatum* and *D. heterocarpum* (Sandamali et al., 2020). *Dendrobium crumenatum*, known as the pigeon orchid, is a type of orchid that is used as medicine by local people. This orchid is often used as a medicine for earache by dripping water from its pseudobulb (Teoh, 2016). One of proven identification techniques capable of identifying orchid species including the genus Dendrobium is DNA barcoding (Silva et al., 2015).

DNA barcoding is an alternative technique for identifying a species using molecular markers (Herbert, 2003). The basic principle of DNA barcoding is the identification using short DNA sequences (barcodes) of standard parts of the genome of the organism being studied. Species identification using DNA is a fast and consistent method since DNA characters are more constant than morphological characters. DNA sequences resulting from DNA barcoding can be used to determine the relationships of a species using phylogenetic trees (Rohimah et al., 2018). This technique has provided a breakthrough in modern taxonomy for the study of species identification and biodiversity. One of the molecular markers that is reliable and widely used in DNA barcoding is ITS2 (Putranto, 2016). ITS2 in plants, especially

monocots, gymnosperms and ferns, has been identified as having a bp length of 100-480 bp. ITS2 shows significant sequence variability at the species level or below (Yao et al., 2010; Su'udi et al. 2022). In addition, the specificity of ITS2 has also been reported previously in several orchids such as four Thrixspermum species (Rohimah et al. 2020; Rohimah et al. 2021), *Phalaenopsis deliciosa* (Maulidya et al. 2020), Dendrobium *linearifolium* (Su'udi et al. 2022), *Vanda tricolor* (Su'udi et al. 2022), and *Bulbophyllum lobbii* (Su'udi et al. 2024). Therefore, DNA barcoding research using ITS2 marker on *Dendrobium crumenatum* is a necessity to enrich the database for future use.

Materials and Methods

Plant Materials and Morphological Confirmation

The orchid sample used in this research was purchased from Wawan Wibowo Nursery, Malang, Indonesia. The orchids were maintained in the greenhouse at Botany Laboratorium until used. For morphological confirmation, various identification books, such as Orchids of Papua New Guinea (Millar, 1978), Lowland Orchids of Papua New Guinea (O'Byrne, 1994), Key to the genera of Orchidaceae of New Guinea (Schuiteman, 1995), Flora Malesiana: Orchids of New Guinea (Schuiteman et al., 2010), and recommended website (https://powo.science.kew.org/) were used.

DNA Isolation, Amplification, and Sequencing

Genomic DNA isolation of *Dendrobium crumenatum* was carried out with CTAB method (*Cetyl Trimethyl Ammonium Bromide*) (Doyle, 1991). The process was initiated by grinding 0.5 gram of the orchid leaf sample in a CTAB buffer with mortar and pestle. The 1 ml solution containing the leaf samples was transferred into a microtube and incubated at 65°C for 1 hour. Subsequently, the 0.5 ml of chloroform was added and incubated for 5 minutes at room temperature and then centrifuged at 10,000 rpm, at the temperature of 25°C, for 15 minutes. The supernatant (0.6 ml) resulting from the centrifugation process was transferred into a new tube and 5 µl of RNase was added. The mixture was then incubated for 30 minutes at 37°C. In the next, a 0.6 ml of cold isopropanol was added to the supernatant and homogenized by inverting. The solution centrifuged at 10,000 rpm, for 11 minutes with two repetitions, at the temperature 4°C. The supernatant was discarded and 0.5 ml of 70% ethanol was added and centrifuged at 10,000 rpm, at the temperature 4°C, for 10 minutes. The ethanol was discarded and the DNA pellet was dried using a desiccator for 10 minutes. The final stage was resuspension using 50 µl of TE buffer.

Amplification of ITS2 fragment using PCR began with making a total of 20 µl cocktail containing PCR master mix, 2 µl of DNA template, 1 µl of forward primer DR2F, 5'-GGCTCTCGCATCGATGAAGA-3', 1 µl of reverse primer ITS_26SE, 5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3', and 16 µl distilled water. DNA amplification was carried out using following conditions: 1 cycle of pre-denaturation at 95°C for 5 seconds, 35 cycles of (denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension 72°C for 1 minute and 15 seconds), 1 cycle of final extension at 72°C for 5 minutes, and hold at 16°C.

DNA amplification result was confirmed using electrophoresis in a 1.25% agarose gel containing ethidium bromide (EtBr), followed by visualization using a UV-Transilluminator. The observed DNA bands were then compared with 100 bp DNA ladder marker for ensuring the congruence of PCR product size. The sequencing analysis was carried out using the service of Bioneer, Republic of Korea.

Data Analysis

Data analysis was carried out using computational methods with several software, including BLAST for sequence alignment in GenBank database, GeneDoc for aligning selected sequences, and MEGA XI for constructing the phylogenetic tree and evolutionary study.

Results

The research was carried out sequentially consisting of morphological observations, determination of molecular marker (barcode) characteristics and genetic distance studies through phylogenetic tree construction. The results of morphological observations show that the *Dendrobium crumenatum* orchid has a round stem, with brownish pseudobulbs at its base (Figure 1a). The leaves have an oblong-lanceolate shape with a curve at the tip. The leaves are yellowish green with a width of ± 2 cm and a length of ± 5.5 cm and a smooth surface with flat edges (Figure 1b).

Dendrobium crumenatum orchid flowers are white with yellow in the middle of the labellum. The length of the sepals and petals is ± 3 cm (Figure 1c). The morphological identification results above are under previous research which stated that there are pseudobulbs on this orchid, the length of the sepals and petals is 3 cm (Teoh, 2016), the leaves are yellowish green (Purnamasari et al., 2016) with a width of 2-2.5 cm and flat leaf edges and smooth surfaces (Sofiyanti, 2014). The flowers are white with yellow in the middle of the labellum (Teoh, 2016).

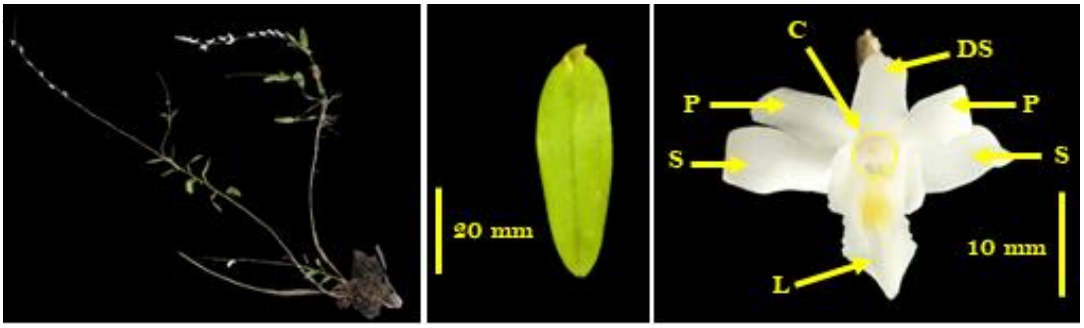

Figure 1. Morphology of *Dendrobium crumenatum*. (a) Habitus; (b) Leaves; (c) Flower. L (labellum), S (sepal), P (petal), C (columna), DS (dorsal sepal)

The characteristics of ITS2 in *D. crumenatum* were explored by analyzing the sequence and comparing it with the ITS2 sequence available in Genbank (NCBI). For this purpose, several stages such as DNA isolation, amplification using PCR, and reading the sequence through sequencing were conducted. The DNA isolation stage was carried out utilizing the CTAB technique and was directly used as a template in PCR analysis. The results of PCR amplification showed a bright single band with the correct size. The ITS2 of *D. crumenatum* was successfully amplified at position of ± 480 bp (below the 500 bp marker) (Figure 2). This result is in accordance with Yao et al. (2010) who stated that ITS2 in plants, especially monocots, gymnosperms and ferns, were identified with the length of 100-480 bp.

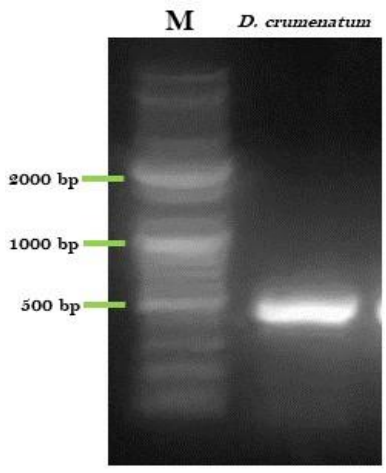

Figure 2. Visualization of ITS2 amplification from *Dendrobium crumenatum* using DR2F and ITS_26SE primers loaded on agarose gel at concentartion of 1.25%. M: DNA marker

The amplified PCR product were further processed for sequencing. The sequencing results (ITS2 nucleotide sequence) from *D. crumenatum* were analyzed for the level of homology with the *D. crumenatum* orchid as well as other orchids available in NCBI GenBank using BLAST. The results of BLAST analysis revealed that the ITS2 sequence of *D. crumenatum* has a high level of homology with ITS1-ITS2 of *D. crumenatum* (Acc. MH763846.1) originating from Sri Lanka at 99.79% followed by ITS1-ITS2 of *D. formosum* (Acc. AB972337.1) from Thailand at 99.58% (Table 1). This high homology possibly is due to the similarity of the flower morphology, i.e. having yellow appearance in the middle of the labellum (Baishnab and Datta, 2019). The BLAST results also show an E-Value of 0.0, which indicates that the BLAST results have no other possibility in the alignment carried out (Sabbathini et al., 2017). A lower E-Value value indicates a higher homology value (Asnani et al., 2015).

To provide a detailed illustration, alignment analysis was also carried out using Genedoc software (Nichlolas et al., 1997). Alignment was carried out by aligning the sequence results with sequences from NCBI, both of the same species and different species. The alignment results of *D. crumenatum* from this study with several *D. crumenatum* literature (from NCBI) shows several base differences. The *D. crumenatum* sequence has different nucleotide bases from the literature *D. crumenatum* sequence (from NCBI) with accession numbers HM054625.1, JF713095.1 and JF713096.1 in the order of 28th, namely guanin (G)

but has the same nucleotide with others (accession numbers AB972336.1 and AB593537.1) (Figure 3). Meanwhile, the alignment results of *D. crumenatum* with other Dendrobium species show differences in several nucleotide bases (Figure 4). This proves that the ITS2 sequence is specific for *D. crumenatum* and is able to distinguish up to species level (Yao et al., 2010).

Table 1. BLAST results of the ITS2 sequence from the *Dendrobium crumenatum* sample with its homologs

Spesies Name	Accession No.	Query Cover	E Value	Per. Ident	Origin
Dendrobium crumenatum	MH763846.1	100%	0.0	99.79%	Sri Lanka
Dendrobium formosum	AB972337.1	100%	0.0	99.58%	Thailand
Dendrobium crumenatum	AB972336.1	100%	0.0	99.58%	Thailand
Dendrobium crumenatum	AB593537.1	95%	0.0	99.78%	Jepang
Dendrobium calceolum	AB593513.1	95%	0.0	94.74%	Jepang
Dendrobium parciflorum	AB593628.1	95%	0.0	94.29%	Jepang
Dendrobium crumenatum	HM054625.1	84%	0.0	97.98%	India
Dendrobium acinaciforme	AB847640.1	91%	0.0	95.17%	Jepang

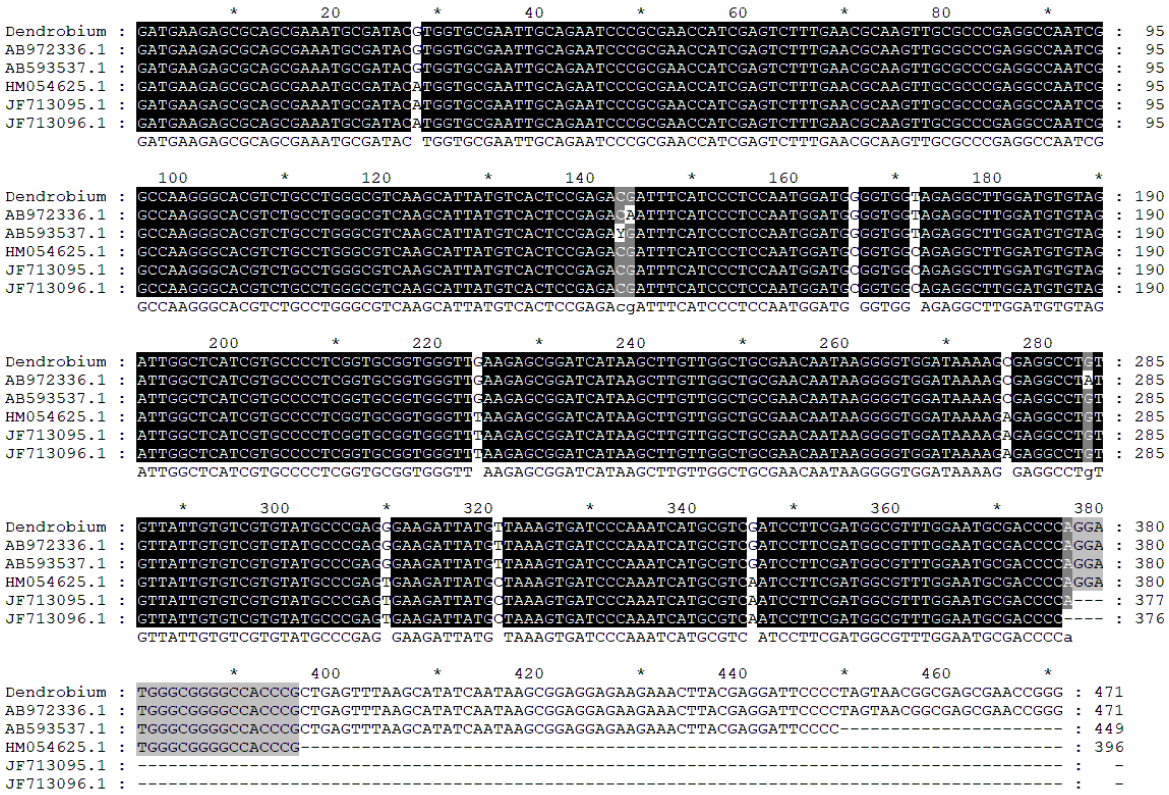

Figure 3. Alignment of the ITS2 sequence from *Dendrobium crumenatum* samples with those in the NCBI database

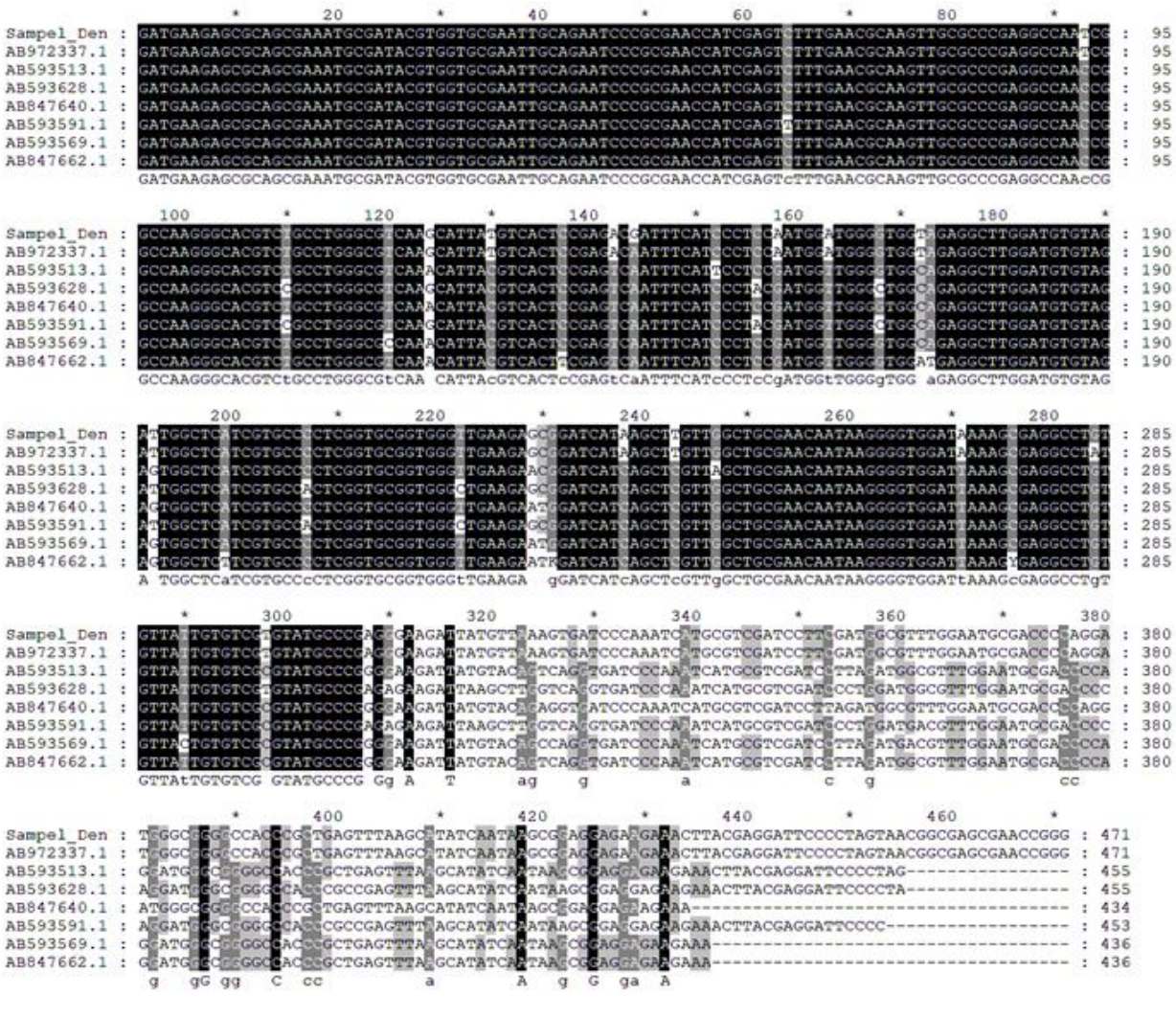

Figure 4. Alignment of the ITS2 sequence from *Dendrobium crumenatum* samples with those of other Dendrobium species available in the NCBI database

The aligned sequences were then phylogenetically constructed using MEGA X. The results of the phylogenetic tree construction reveal that *D. crumenatum* is closely related to *D. formosum* and *D. calceolum* (Figure 5).

Dendrobium crumenatum which belongs to the Crumenata section has similar flowers to *D. formosum* of Formosae section (Takamiya et al., 2014). The flowers of both species are white with a yellow center labellum (Baishnab and Datta, 2019). Hence, it is a plausible reason for the close relationship between the two species illustrated in a phylogenetic tree constructed. Meanwhile, *D. calceolum* which belongs to the Aporum section also has a close relationship with *D. crumenatum* based on a phylogenetic tree construct. Following the research by Takamiya et al. (2014) Section Crumenata and Section Aporum are included in the Clade E group which includes *D. crumenatum* and *D. calceolum*. This supports the results of the phylogenetic construction of this research which is characterized by the close proximity of the two species.

In sum, DNA barcoding research using the ITS2 sequence in *D. crumenatum* provides specific results to differentiate *D. crumenatum* from other Dendrobium species. The similarity of flower morphology in *D. crumenatum* and *D. formosum* is proposed as the cause of close proximity shown in phylogenetic tree. Based on these results, it is highly recommended to use the ITS2 sequence as a molecular marker for barcoding orchids, especially *D. crumenatum* since its capability for differentiating between species with morphological similarities.

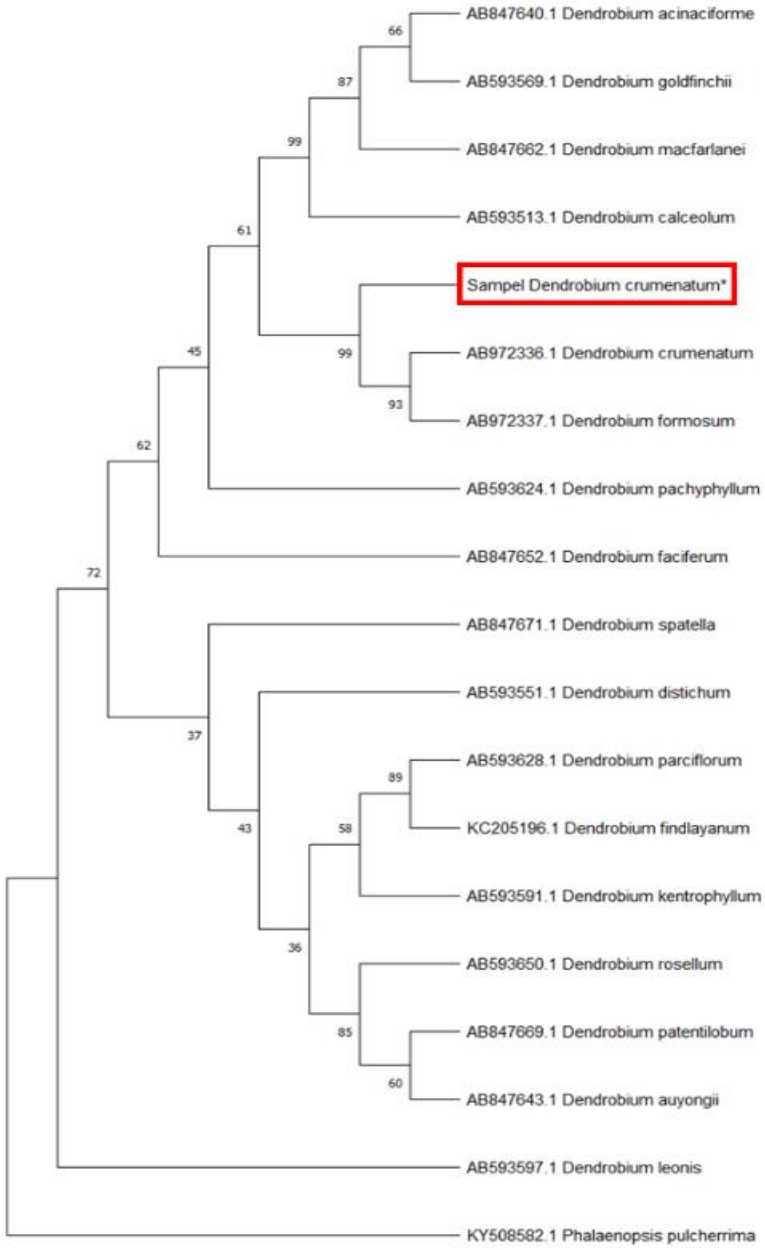

Figure 5. Phylogenetic tree of *Dendrobium crumenatum* (red boxed) with other Dendrobium species using ITS2 sequences. *Phalaenopsis pulcherrima* was used as outgroup

Acknowledgments

Authors thanks to the University of Jember's Institute of Research and Community Services for funding this research through the Hibah KeRis year of 2020.

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