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DNA Barcode Characteristics of *Paphiopedilum supardii* Braem & Loeb

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ABSTRACT

***Paphiopedilum* is a protected orchid (Orchidaceae) genus with the most endangered species due to its unique aesthetic qualities, totaling 15 species. The excessive exploitation of orchids has led to a decline in their natural populations. This has prompted comprehensive conservation efforts to preserve these species. However, it is quite challenging to identify orchids that have not yet bloomed because of the similar morphology of their vegetation among closely related species, necessitating alternative identification approaches that are both rapid and accurate, especially for endangered orchids. DNA barcoding is a rapid and accurate molecular method for species identification. In this study, the selected rare orchid was *Paphiopedilum supardii* Braem & Loeb. The research procedure included DNA sample isolation using a Kit, DNA amplification using *rbcL* and ITS primers, PCR product purification, and sequencing. Based on the research results, DNA samples were amplified using *rbcL* and ITS primers. The DNA band resulting from *rbcL* primer amplification was 600 bp in size, whereas the ITS amplification result was 900 bp. The PCR products were then purified directly using a NEXprep™ Plant DNA Mini Kit (NEX™ Diagnostics, Korea). The PCR and purification results were separated on a 1.25% agarose gel. Based on data analysis, BLAST results from NCBI and sequence alignment using MEGA11, Clustal X2, and GeneDoc showed that the *rbcL* sequence had a higher homology level than ITS. Therefore, the ITS sequence is more specific for use as a molecular marker in DNA barcoding research on *P. supardii*.**

Keywords: DNA barcode, *ITS, P. supardii,* rbcL

INTRODUCTION

Orchids (Orchidaceae) comprise approximately 20,000 species across 899 genera, representing around 7% of all flowering plant species worldwide (Perwitasari *et al.* 2020). Indonesia has approximately 6,000 orchid species (Fandani *et al.* 2018). Orchids are ornamental plants with high beauty and aesthetic potential (Su'udi *et al.* 2024). Due to the high demand for wild orchids, their removal from natural habitats has become uncontrolled. This excessive exploitation has led to a decline in wild orchid populations. The scarcity of orchid species is driven by habitat fragmentation, deforestation, forest fires, and land conversion (Sindiya *et al.* 2018). Paphiopedilum, an endangered orchid in Indonesia, is threatened with extinction in its natural habitat due to environmental degradation. All of its species are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), including 15 *Paphiopedilum* species that are endangered and protected based on Permen P.20, 2018, indicating that this orchid is classified as an endangered species and its trade is strictly restricted (CITES, 2023; Kartikaningrum *et al.* 2021).

Paphiopedilum supardii Braem & Loeb is an endemic orchid of Kalimantan with a critically endangered status according to the IUCN (International Union for Conservation of Nature and Natural Resources) (Handini *et al.* 2016). *Paphiopedilum supardii* is a lithophytic and monopodial orchid species. The population of these orchids is only found in limited locations on the island of Kalimantan (endemic), and in recent decades, its population density has been decreasing due to over collection, human disturbance, as well as deforestation, logging and the expansion of agricultural land, which is cause habitat degradation (Rankou & Sullivan 2015). This has encouraged the development of comprehensive conservation strategies to preserve them, including *in situ* through habitat management and protection, *ex situ* through seed bank and *in vitro* culture, DNA barcoding for species identification using moleculer analysis, and policy to support the sustainability of the species and its habitat (Sindiya *et al.* 2018; Seaton *et al.* 2010; Sarmah *et al.* 2025; Phillips *et al.* 2020; Gostel & Kress 2022).

Paphiopedilum supardii is a monopodial orchid with elongated, ribbon-shaped leaves that have pointed tips (acutus), smooth edges (integer), and a smooth surface (laevis). *Paphiopedilum supardii* has a brown synsepalum and dorsal petals with brown vertical stripes. The leaves of this orchid are thick and fleshy

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(Figure 1), so only a small piece is needed for DNA extraction. DNA extraction from fresh samples should use young leaves, as their tissue tends to produce higher DNA yields compared to older leaves due to the higher cellular activity and lower levels of interfering compounds such as polyphenols and secondary metabolites. Therefore, the need for chemicals to reduce these compounds during the isolation process is less when using newer plant tissue (Buchori *et al.* 2023).

DNA barcoding is a molecular-based species identification technique that uses DNA sequences from small genome fragments and is known for its speed, accuracy, and consistency (Rohimah *et al.* 2020). This process involves amplification, sequencing, and matching DNA sequences with a reference database containing genetic information from various species (Letsiou *et al.* 2024). The use of molecular markers (barcodes) offers several advantages, including consistent data, recognition across various tissue types and developmental stages, and resistance to environmental factors (Sindiya *et al.* 2018). However, there are also several disadvantages, such as the absence of a single plastid marker comparable to CO1 in animals, thus requiring a combination of several moleculer markers; hybridization and recent speciation can cause bias because new species have not yet genetically diverged; relatively high technical cost; and the analysis results require a complete database reference (Hollingsworth *et al.* 2011; Jones *et al.* 2020; Kartzinel *et al.* 2025). Some commonly used marker genes in plants include *matK, rbcL*, and ITS (Gostel *et al.* 2020). Although there are already several database for *Paphiopedilum,* there is still no database for *Paphiopedilum* native to Indonesia. Therefore the objective of this research is to analyze DNA barcoding of *Paphiopedilum supardii* orchids from Indonesia using the molecular markers *rbcL* and ITS as the basis for molecular identification.

METHODS

This study was conducted from November 2018 to April 2019 at the Biotechnology Laboratory, Faculty of Mathematics and Natural Sciences, University of Jember. The instruments used in this research included a mortar, micropipette, electrophoresis, PCR, and UV illuminator. The materials used were *P. supardii* purchased from from the Kebon Agung Orchid Nursery, Jl. Sri Gunting, Krajan, Jumerto, Patrang, Jember subdistrict, *rbcL* primers (forward sequence/F: 5'-ATGTCACCACAAACAGAGACTAAAGC-3', reverse/R: 5'-CGGGGTGGACTTGATTTTAC-3'), and ITS primers (forward sequence/F/ITS_17SE: 5'-ACGAATTCATGGTCCGGTGAAGTGTTCG -3', reverse/F/ITS_26SE: 5'-CGAACACTTCACCGGACCATGAATTCGT-3'). PCR master mix, ddH_2O, NEXprep™ Plant DNA Mini Kit (NEX™ Diagnostics, Korea), agarose, TAE buffer, isopropanol, TE buffer, 70% alcohol, sterile distilled water, ethidium bromide (EtBr), chloroform, and RNAse.

Sampling and DNA Isolation

This study used a DNA barcoding approach with *rbcL* and ITS markers to obtain more accurate and molecularly standardized species identification. The leaf samples, weighed 0.5 grams were ground and then *P. supardii* genomic DNA was isolated using NEXprep™ Plant DNA Mini Kit according to the manufacture's procedure. The isolated DNA was then amplified using PCR with *rbcL* and ITS primers. Amplification was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 1 min 15 s, and a final extension at 72°C for 5 min. The PCR products were purified and sent to 1st BASE (Singapore) for sequencing (Perwitasari *et al.* 2020).

(a) (b)

Figure 1 Vegetative (a) and generative (b) morphology of *P. supardii* Braem & Loeb. Scale: 10 cm.

Data Analysis

Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) to determine the degree of homology with sequences in GenBank (NCBI) which can be accessed online at https://www.ncbi.nlm.nih.gov/, *rbcL* and ITS sequences from top five species with highest similarity to the samples were collected in FASTA format. Phylogenic analysis was performed using MEGA11 software (Tamura *et al.* 2021). The aligned sequences were then transferred to Clustal X software to be converted to .msf format (Larkin *et al.* 2007), and then visualized with Genedoc software (Nicholas *et al.* 1997). Phylogenetic trees were constructed using MEGA11 with the Neighbor Joining method (Tamura *et al.* 2021).

RESULTS AND DISCUSSION

Amplification and DNA sequencing

Based on the research results, DNA isolation of *P. supardii* samples should be carried out using the Kit method, the sequencing process was successful. Isolation using the CTAB method produces short DNA sequences that are only readable up to 100 bp, with unclear re-sequencing results. In contrast, the use of produces. In general, the CTAB method for plant DNA isolation tends to produce genomic DNA with low purity levels and is still prone to contamination from polysaccharides, polyphenols, and other secondary metabolites. In contrast, In contrast, the use of NEXprep™ Plant DNA Mini Kit is able to produce clearly readable sequences. In general, the CTAB method for plant DNA isolation tends to produce genomic DNA with low purity levels as the method is still prone to contamination from polysaccharides, polyphenols, and other secondary metabolites. On the other hand, the Kit method produces genomic DNA with higher purity (Jabeen *et al.* 2022).

Paphiopedilum supardii leaves have thick and hard textures. After grinding with CTAB buffer, the sample showed a brownish color, indicating a high polyphenol content in the plant tissue. Therefore, the isolation method using the Kit is a better alternative because it can produce genomic DNA with higher purity. The genomic DNA was then separated using 1.25% agarose gel electrophoresis to determine its size, which was found to be greater than 10,000 bp (Figure 2 (a)).

Genome electrophoresis results showed thick DNA bands in the tested samples, indicating that the extracted DNA has a high concentration. Although smears (rocket tails) are still visible, this suggests the possibility of DNA degradation (Figure 2(a)). The quality and purity of the resulting genomic DNA depend on the isolation method used (Buchori *et al.* 2023). Additionally, high levels of polyphenols, polysaccharides, and other secondary metabolites can interfere with the DNA isolation process, thereby reducing the purity of the obtained genomic DNA (Masoomi-Aladizgeh *et al.* 2023). Visualization of PCR products on a 1.25% agarose gel showed that amplification using *rbcL* and ITS primers produced thick and clear DNA bands (Figure 2 (b)).

The DNA band of *P. supardii* was approximately 600 bp in size in the *rbcL* gene, while in ITS, it was approximately 900 bp in size. According to Sunaryo (2015), PCR products for barcoding purposes are usually short, approximately 600-800 bp in size. A thick and clear DNA band is a requirement that must be met for successful DNA purification (Figure 2(b)). The concentration of the amplified DNA affects the success of purification and sequencing.

Purification was performed using a Purification Kit (Jena Bioscience) directly from the PCR product (not from an agarose gel), as the separated PCR product showed a single DNA band of the target size. The purification results are shown in a pure DNA sample with a clear band (Figure 2(c)). A thick DNA band is required for sequencing. Based on the figure, the *rbcL* gene DNA band was thicker than the ITS band. This indicates that the DNA concentration from amplification with the *rbcL* primer was higher than that from amplification with the ITS primer. In addition to the DNA concentration, the purity of the purification results also affects the quality of the sequenced DNA.

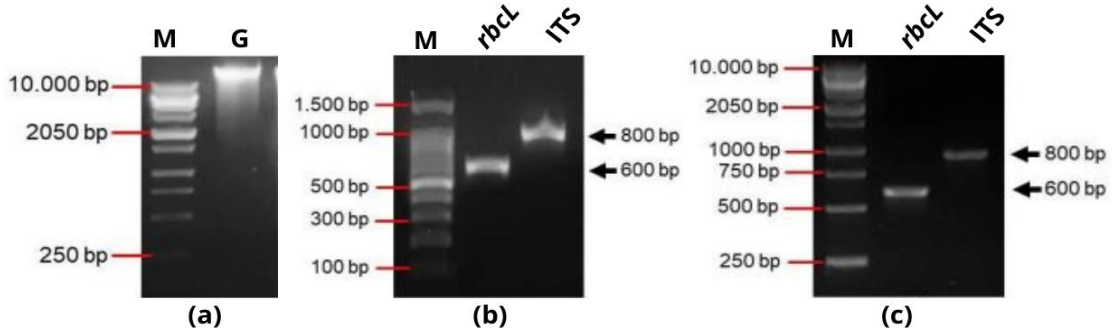

Figure 2 (a) Genomic DNA of *Paphiopedilum supardii* orchid (G), M: 1 Kb DNA ladder. (b) Visualization of gel electrophoresis separation of amplified (PCR) samples of *P. supardii* using *rbcL* and ITS primers, (c) Visualization of gel electrophoresis separation of purified PCR products of *P. supardii* samples using *rbcL* and ITS primers.

The disadvantage of the CTAB method is the poor quality of the DNA, resulting in PCR products that cannot be read after sequencing, and even DNA degradation during shipping, as occurred with the *P. supardii* sample. To obtain purer *P. supardii* DNA, DNA extraction was performed using a kit. This was done to prevent DNA degradation during shipping and ensure that the sequencing results could be read properly. However, the disadvantage of using a kit is its high cost.

DNA Barcode Characteristics of Paphiopedilum supardii

The BLAST results of the *P. supardii* sequence showed high homology with *P. supardii* (from the USA), *P. spicerianum* (from India), *P. fairrieanum* (from India), *P. charlesworthii* (from Thailand), and *P. wardii* (from China), with an identity value of 100% and an E-value of 0.0 (Table 1). In this study, the *rbcL* primer was only able to distinguish samples at the genus level. High homology indicates low intraspecific genetic variation. Therefore, *rbcL* cannot be used as a molecular marker (barcode) for *P. supardii*.

The ITS sequence of *P. supardii* had the highest homology with two *P. supardii* species originating from the USA and Taiwan, with an identity percentage of 100%. BLAST results also showed homology with *P. stonei* (from the UK), *P. rothschildianum* (from Malaysia), and *P. ooii* (from Poland), with an identity percentage above 99%. The identity values in the ITS BLAST analysis results varied, indicating differences in the number and location of sequences between *P. supardii* and other sequences in the NCBI GenBank, yet still allowing for specific identification at the species level (Table 2).

The alignment results of the *P. supardii rbcL* gene sample showed high homology with other species (Figure 3). However, in the ITS sequence of *P. supardii*, one sequence differed from the sequences of the compared species, specifically at the 138th base pair between G and T. Another difference was also observed in the 189 bp sequence between T and C, as well as in the 507 bp sequence between A and T, but in both nucleotide base positions, the *P. stonei* sequence (JQ929348) showed no differences from the *P. supardii* sequence (Figure 4). The sample sequence was aligned with five *Paphiopedilum* sequences based on the BLAST results from. Based on the alignment results, the *rbcL* gene exhibited lower genetic variation than ITS.

The *rbcL* gene sequence exhibits low genetic variation. The *P. supardii* sample sequence formed a cluster with *P. spicerianum* and *P. fairrieanum*. However, the *P. supardii* sequence from GenBank formed a separate cluster outside this group. Species within the in-group exhibit identical DNA sequences, as determined by BLAST analysis, when compared to other species. Although the *rbcL* gene sequences of *Paphiopedilum* are identical to those of *P. supardii*, the two species are not grouped together in a single phylogenetic cluster (Figure 5). This high level of homology is due to the *rbcL* gene originating from chloroplasts, having a low mutation rate, and being widely distributed across nearly all the plant species. Therefore, this gene is believed to retain genetic information from the plant ancestors (Rohimah et al. 2018).

The ITS sequence phylogenetic tree reconstruction indicate that the sample sequence forms a single cluster with *P. supardii* NCBI was closely related to *P. stonei*, *P. rothschildianum*, and *P. ooii* (Figure 6). The ITS sequence can distinguish identification at the species level and is more variable than the *rbcL* gene sequence, making the ITS marker gene more specific and effective as a DNA barcode for *P. supardii* than *rbcL*. Phylogenetic analysis revealed the relationship between the samples and other *Paphiopedilum* species. These findings represent an initial step in developing a DNA barcoding database to support species identification and conservation strategies for protected, the conservation of endangered orchids.

Table 1 Results of BLAST analysis of *P. supardii rbcL* gene

Name	Accession	Query Cover (%)	E-Value	Per. Ident (%)	Origin
P. supardii	MT519332	100	0.0	100	USA
P. spicerianum	HQ998581	100	0.0	100	India
P. fairrieanum	HQ998560	100	0.0	100	India
P. charlesworthii	KX755540	100	0.0	100	Thailand
P. wardii	JN181467	100	0.0	100	China

Table 2 Results of BLAST analysis of *P. supardii* ITS sequence

Name	Accession	Query Cover (%)	E-Value	Per. Ident (%)	Origin
P. supardii	AY643454	85	0.0	100	USA
P. supardii	GQ505309	77	0.0	100	Taiwan
P. stonei	JQ929348	84	0.0	99.72	UK
P. rothschildianum	EF459731	98	0.0	99.52	Malaysia
P. ooii	KX931035	93	0.0	99.50	Poland

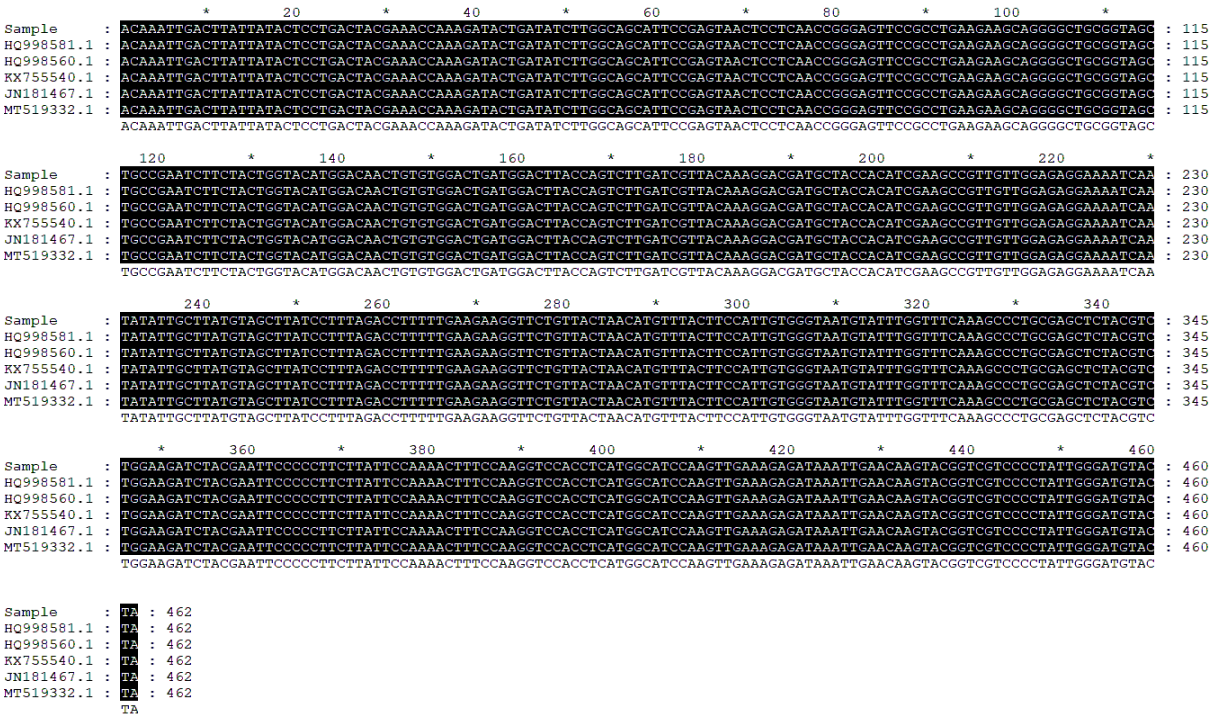

Figure 3 Alignment results of *rbcL* sequences of orchid *P. supardii*.

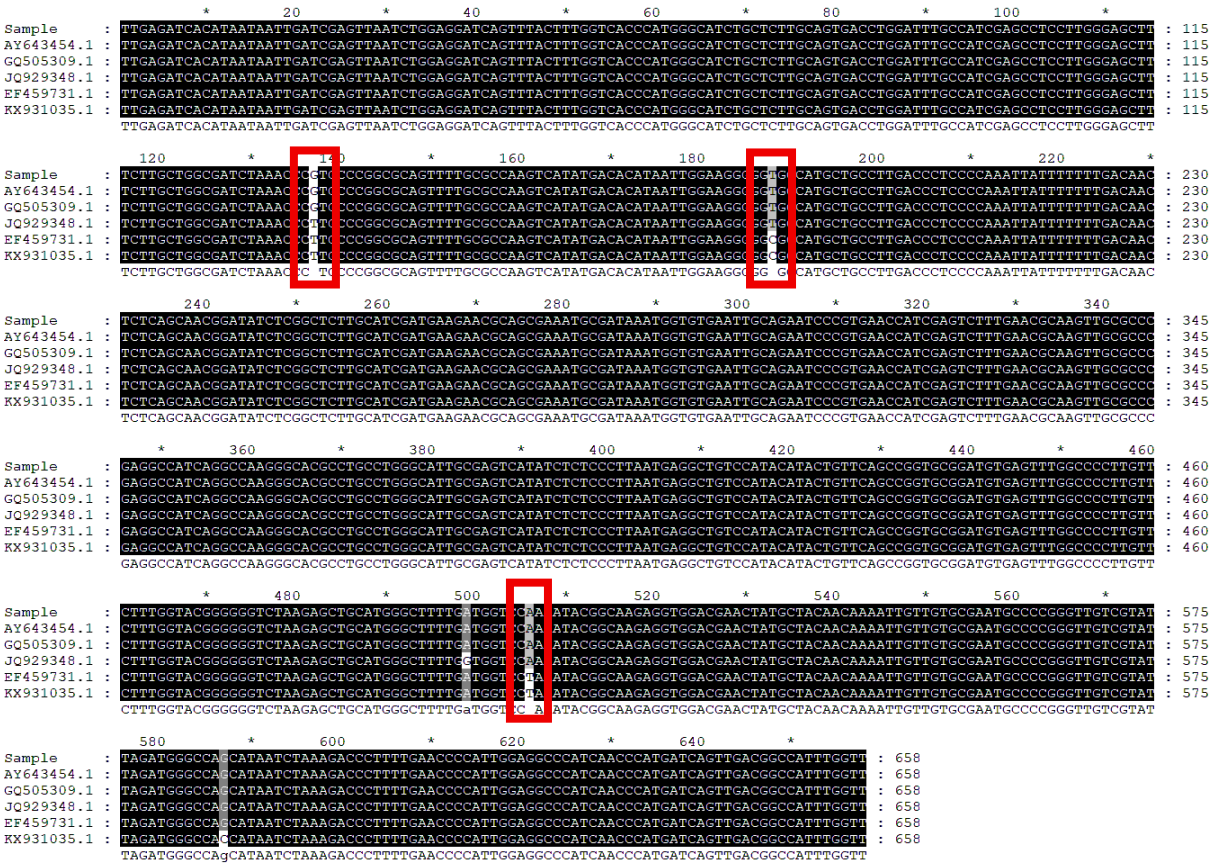

Figure 4 Alignment results of ITS sequences of orchid *P. supardii*.

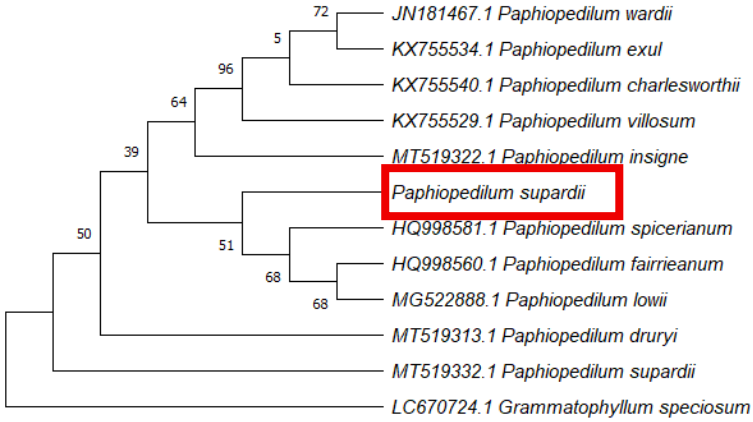

Figure 5 Phylogenetic tree of *P. supardii rbcL* gene sequences with closest relatives in NCBI.

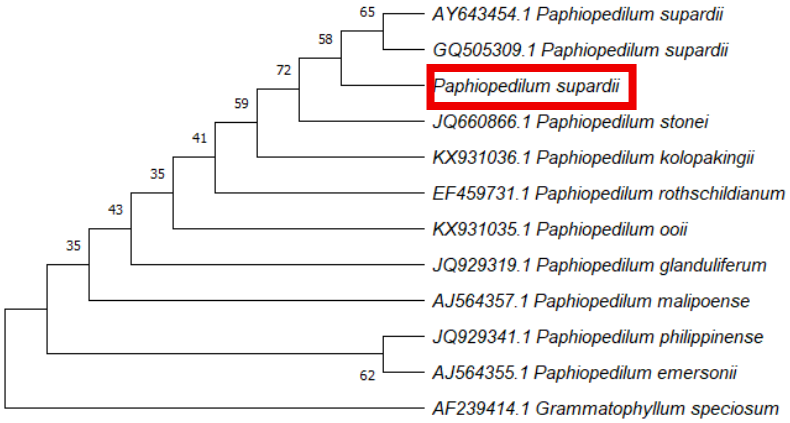

Figure 6 Phylogenetic tree of *P. supardii* ITS sequences with closest relatives in NCBI.

CONCLUSION

The rbcL and ITS primers produced good sequencing data from the amplified DNA templates. The ITS sequence can provide more specific results for DNA barcoding and can identify species up to the species level compared to the rcbL sequence, making it more suitable as a barcode for P. supardii. A kit method was used for genomic DNA isolation to ensure successful sequencing.

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