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INTERNAL TRANSCRIBED SPACER 2 (ITS2) VARIATIONS IN SEVERAL CULTIVARS OF 'HAJI' BANANA (MUSA X PARADISIACA L.) FROM LOMBOK ISLAND, INDONESIA

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SUMMARY

The banana cultivar 'Haji' (*Musa x paradisiaca* L.) is native to the Lombok Island, Indonesia. The said cultivar has an excellent feature of lengthy shelf life and the potential to significantly improve the banana through breeding. However, cultivar 'Haji' has various local names and morphological variations, and therefore, its characterization by molecular markers is necessary to confirm the observed genetic variations. The presented study comprised the examination of the Internal Transcribed Spacer 2 (*ITS2*)'s capability as a marker to identify the banana cultivar 'Haji.' Genetic divergence analysis using *ITS2* sequences revealed the banana cultivar Haji's 10 accessions were closely related, with a divergence coefficient of 0.000 to 0.023. Phylogenetic analysis based on the *ITS2* sequence showed all the banana accessions were in the same clade, separating from the out-group accession. The results authenticated all 10 banana accessions with different local names and morphological characters belonged to the cultivar 'Haji' (*Musa x paradisiaca* L.). These findings are vital in developing *ITS2* as a DNA barcode for the banana cultivar 'Haji' (*Musa x paradisiaca* L.).

Keywords: Banana cultivar 'Haji,' DNA barcode, genetic divergence, *ITS2*, molecular marker, morphological characters, phylogenetic analysis, shelf life

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Key findings: The size of the *ITS2* sequence of the banana cultivar 'Haji' (*Musa x paradisiaca* L.) accessions was 220 bp, with a 10 bp base difference. The genetic divergence of the cultivar 'Haji' accessions based on *ITS2* ranged from 0.000 to 0.023, indicating a close kinship. Phylogenetic tree construction showed the banana cultivar 'Haji' accessions belonged in the same clade and were far from the out-group accession.

INTRODUCTION

Identifying plants with superior characteristics has several benefits, especially in agriculture, conservation, and medical applications. Such approaches considerably contribute to our understanding of plant diversity and the development of improved cultivars with good yields. One of the superior characteristics of fruit plants is a long shelf life, which also play a vital role in reducing post-harvest losses in various types of bananas. The identification of diverse banana cultivars with a long shelf life has progressed, including *Musa balbisiana* Colla (Dwivany *et al*., 2021), Talas banana (*Musa x paradisiaca*) (Sunaryo *et al*., 2017), and the cultivar 'Haji' (Gusmiati *et al*., 2018).

Banana cultivar 'Haji' (*Musa x paradisiaca* L.) is an indigenous genotype found in Lombok Island, Indonesia. Gusmiati *et al.* (2018) stated the 'Haji' banana has several distinctive autapomorphic characteristics. These are a pink inner pseudo stem, the horizontal position of the fruit bunch, a vast layer of wax on the leaves, and a yellow-pink shade on the midrib ventral surface. Gusmiati *et al.* (2018) also declared the 'Haji' cultivar has the ABB genome. The 'Haji' fruit has a shelf life of around 20 days at room temperature. However, the said banana cultivar on Lombok Island has different local names and morphological characteristics, and therefore, a speculation exists where each banana accession may resemble different ecotypes. Kurnianingsih *et al.* (2018) revealed the 'Haji' cultivar in East Lombok has the local names, Kelak and Sambelia. One of their distinct traits is in the pigmentation of the tepal compound of the male flower.

The morphological characteristics of crop plants have served to identify and map the plant relationships and taxonomy (Long *et al.*, 2023); however, morphological characteristics are lacking in their expression because of the environment's influence. In addition, the identification using morphological features is limited to mature plants, as many species require flowering and fruiting for accurate classification. The aforementioned process is often lengthy and requires the anticipation of particular growth stages (Sravani *et al*., 2024). Therefore, molecular identification is essential to overcome the weaknesses of detection using morphological characters. Molecular identification methods employed have succeeded to identify the locally cultivated banana cultivars (Sravani *et al.*, 2024).

Molecular plant identification often uses the DNA barcoding (Letsiou *et al.*, 2024). DNA barcodes are short DNA sequences used for species identification and discrimination (Wang *et al*., 2023). The DNA barcoding allows rapid, precise species documentation, overcoming the problems associated with conventional taxonomic identification based on morphological attributes (Wäldchen *et al*., 2018). The DNA barcoding is a technique complementing conventional taxonomy by providing the precise data on previously unknown taxa (Gostel and Kress, 2022; Rohit and Solanki, 2022; Stuart *et al*., 2023). Species identification and classification can also continue with combining the results of DNA barcode sequences and the morphological characters (Kowalska *et al*., 2019).

The Internal Transcribed Spacer-2 (*ITS2*) is one of the DNA barcodes certified to identify and differentiate the banana cultivars, as well as, determine their genetic links (Dhivya *et al*., 2020). *ITS2* is shorter and more straightforward to sequence than other possible barcoding regions, making it more useful for routine identifying tasks (Rahmah *et al*., 2023). *ITS2* can group banana cultivars in phylogenetic analysis based on their genome composition (Dwivany *et al*., 2020a). The *ITS2* region can identify endemic bananas, such as,

Loka Pere bananas. Thus, *ITS2* can be a new and reliable DNA barcode marker (Rahmah *et al*., 2023). *ITS2* can offer durable cultivar-specific markers from generation to generation, which makes it helpful in distinguishing banana cultivars (Hou *et al*., 2022). Unlike traditional morphological character-based identification methods, *ITS2* provides a more reliable and unambiguous identification methodology (Dhivya *et al*., 2020), which is extremely useful for practical plant breeding and its enhancement programs.

Conducting banana cultivars identification based on molecular characteristics needs to support the breeding and improvement programs for banana cultivars with potential superior characteristics, including those with a long shelf life. Therefore, the promising study aimed to confirm the molecular characteristics of several accessions of banana cultivar 'Haji' with different local names on the Lombok Island, Indonesia.

MATERIALS AND METHODS

Plant material and preparation

A sample collection of the banana cultivar 'Haji' (*Musa x paradisiaca* L.) 10 accessions started in the five Districts of Lombok Island Region, West Nusa Tenggara, Indonesia. These districts are Mataram (one accession), West Lombok (one accession), Central Lombok (one accession), North Lombok (one accession), and East Lombok (six accessions) (Table 1). The selection of five districts represents the area and genetic diversity of 'Haji' cultivars on Lombok Island. The five districts spread less than 400 meters above sea level (masl) and in between 400–700 masl.

The identification of 'Haji' cultivar plants relied on the method of Gusmiati *et al.* (2018). The 'Haji' has the banana cultivars characterized with the ABB group. It has several distinctive characteristics, i.e., a pink inner pseudo stem, the horizontal position of the fruit bunch, a vast layer of wax on leaves, and a yellow-pink shade on the midrib ventral of the leaf. Sampling for the out-group accession (*M. balbisiana* Colla, Klutuk Sukun) commenced in West Lombok, Indonesia. *M. balbisiana* Colla is one of the two main progenitor species of many banana cultivars (Martens *et al*., 2022).

Molecular analysis proceeded in the laboratory using fresh curled leaves (cigar leaves). The cigar leaves' trimming reached ±4 cm segments. The leaves bore wrapping in tissue, placed in a plastic clip containing silica gel. In the laboratory, the cigar leaves' grinding with liquid nitrogen continued until becoming powder in form, and remained stored at -80 °C until applying genomic DNA extraction.

Table 1. Location and local names of 10 accessions of 'Haji' cultivar on Lombok Island and Out-group (*M. balbisiana* Colla, Klutuk Sukun accession).

Location (Village)	Local Name	GPS Coordinate
Accessions of the cultivar 'Haji'		
Monjok Barat (M1)	Haji	08°57'66.9"S, 116°10'75.8"E
Lebah Sempaga (LS)	Sembalun, Sambelia	08°55'02.8"S, 116°25'62.2"E
Aik Bukak (AB)	Sembalun	08°56'94.7"S, 116°32'25.6"E
Pesanggrahan (Te1)	Bile	08°58'99.6"S, 116°40'80.4"E
Kembang Sari (Se)	Kelak, Bile	08°65'83.6"S, 116°53'25.8"E
Sapit (Sa)	Kelak, Sembalun	08°48'58.2"S, 116°55'46.0"E
Belanting (KN)	Sambelia, Bile	08°30'85.4"S, 116°65'61.0"E
Belanting (Pa)	Sambelia, Bile	08°28'21.5"S, 116°61'29.2"E
Pengadangan (Pe)	Saba sembalun	08°51'96.9"S, 116°47'11.0"E
Bayan (DT)	Kelak	08°30'29.2"S, 116°42'43.5"E
Out-group: *M. balbisiana* Colla, Klutuk Sukun accession		
Meninting (KS)	Batu	08°55'03.7"S, 116°07'33.0"E

Amplification of *ITS2* region and sequence analysis

The genomic DNA extraction from 100 mg of ground banana leaf used the CTAB method, with slight modifications (Aboul-Maaty and Oraby, 2019). In the presented research, the modifications included using 2% CTAB, 2% β-mercaptoethanol, and 1% PVP 40. Measuring the genomic DNA concentration utilized the Nanodrop Spectrophotometers (Thermo Scientific). Genomic DNA amplification applied the Internal Transcribed Spacer 2 (*ITS2*) primers, consisting of *ITS2*-S2F (ATGCGATACTTGGTGTGAAT) and *ITS2*-S3R (GACGCTTCTCCAGACTACAAT) (Dwivany *et al*., 2020a, b; Meitha *et al*., 2020). The PCR reaction comprised initial denaturation (94 °C for 3 min), denaturation (94 °C for 30 s), annealing (58 °C for 90 s), extension (72 °C for 1 min), and the final extension (72 °C for 10 min). The PCR process engaged 20 µl of a total volume, comprising 165 ng DNA template (2 µl), 10 µmol forward and reverse primers (1 µl each), 10 µl Go Taq® Green Master Mix (Promega, United States), and 4 µl nuclease-free water. The PCR product (DNA target) attained visualization with the UV transilluminator, following electrophoresis on the 2% agarose gel. Then, sequencing the amplified DNA employed the Sanger method at 1st Base Ltd., Malaysia.

The *ITS2* sequences' reconstruction and analysis ran the Molecular Evolutionary Genetics Analysis software, version 11.0 (MEGA 11). The *ITS2* database was available online (http://*ITS2*.bioapps.biozentrum.uni-wuerzburg.de (accessed on July 18, 2023), which aided the extraction of *ITS2* sequences. The obtained sequences sustained subsequent multiple alignments using the ClustalW on MEGA 11 software.

Genetic divergence and polymorphic sites

Genetic divergence analysis engaged the MEGA 11 software. The assessment of DNA polymorphism and polymorphic sites used the DnaSP version 6.0.

Phylogenetic analysis

Producing the phylogenetic tree construction employed the MEGA 11 software using an Unweighted Pair-Group Method with Arithmetic mean (UPGMA). For the phylogeny test, the bootstrap method with 1000 bootstrap replications was applicable. For the substitution type, start by selecting nucleotide, followed by selecting the Kimura 2-parameter model.

RESULTS AND DISCUSSION

Molecular identification of the banana cultivar 'Haji' (*Musa x paradisiaca* L.) materialized to complement the morphological data already recorded. The 'Haji' cultivar has five local names: Kelak, Bile, Sambelia, Sembalun, and Saba Sembalun. Moreover, they have differences in morphological characters. These are in leaf habitus, pseudostem color, pseudostem pigmentation, leaf color and size, bracteal shape and color, and compound tepals on male flowers. In the latest research, the *ITS2* marker employed for molecular identification of the cultivar 'Haji' ensued, given the *ITS2* marker is species-specific (Zhao *et al*., 2016) and could also distinguish the different plant cultivars (Dhivya *et al*., 2020; Dwivany *et al*., 2020a, b; Meitha *et al*., 2020; Adriyana *et al*., 2023). The *ITS2* marker has several Important criteria, including the availability of conserved regions for universal primer design, simplicity in amplification, and providing enough variations to distinguish the closely related plant species (Mursyidin and Setiawan, 2023).

Amplification of *ITS2* region and sequence analysis

In the presented research, the amplification of genomic DNA using *ITS2* primers resulted in a band of ~500 bp (Figure 1). The amplified product contained *ITS2* region. Dwivany *et al*. (2020a) stated the ~500 bp fragment not only consists of the *ITS2* region, but also includes the flanking sequences of the *ITS2* region,

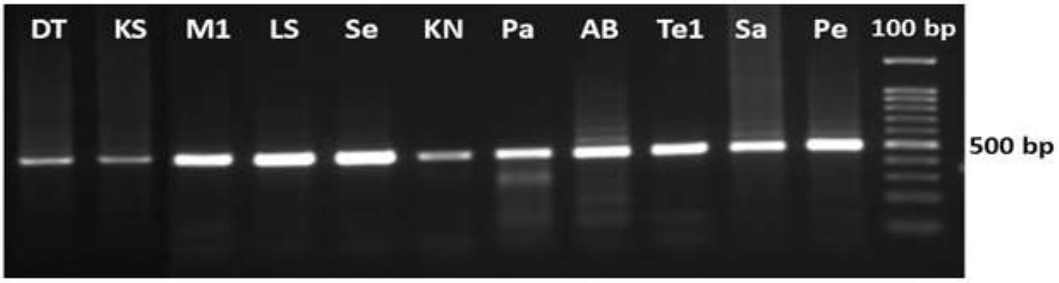

Figure 1. *ITS2* amplification of the banana cultivar 'Haji' accessions and the out-group accession (*M. balbisiana* Colla, Klutuk Sukun).

Table 2. Genetic information of *ITS2* sequences of the banana cultivar 'Haji' accessions obtained from Lombok Island, Indonesia, and the out-group accession.

Parameters	*ITS2*
Number of sites (bp)	220
Pairwise identity of 10 Haji cultivar sequence (%)	99.1
Identical Site of 10 Haji cultivar sequence (%)	97.3
Number of polymorphic (segregating) sites (bp)	10
Substitution (transition-transversion) sites (bp)	10
Indel (insertion-deletion) sites (bp)	0
Singleton variable sites (bp)	6 (bases 17, 28, 47, 48, 49, and 75)
Parsimony informative sites (bp)	4 (bases 15, 30, 42, and 72)

5.8S rDNA, and 28S rDNA. After the 5.8S and 28S sections' elimination from the sequence, the length of the amplified *ITS2* fragments ranged from 218 to 222 (Meitha *et al*., 2020).

Genetic characteristics of *ITS2* sequences from 10 accessions of the cultivar 'Haji' and the out-group had a pairwise identity percentage of 99.1%, and with identical bases of 97.3%. The total mutation and polymorphic (segregating) site of the *ITS2* sequence of the cultivar 'Haji' and the out-group was 10, and all of them were substitution mutations (Table 2).

In this study, the *ITS2* length of the banana cultivar 'Haji' 10 accessions was 220 bp (Table 2). The lengths of *ITS1* and *ITS2* spacers in all studied banana accessions ranged from 213 to 223 bp and 205 to 219 bp, respectively, and the total length of the ITS1-5.8S-*ITS2* sequence region ranged from 566 to 593 bp in the banana accessions (Cao *et al*., 2022). However, other research showed the length of the *ITS2* in *Musa* accessions was 208 to 222 bp (Dwivany *et al*., 2020a, b; Meitha *et al*., 2020). The *ITS2* length of the cultivar 'Haji' obtained in this research was the same as the *ITS2* in other banana cultivars belonging to the genomes ABB, AA, AB, and *Musa balbisiana* Colla (BB group).

The banana cultivars from the ABB genome group, with an *ITS2* length of 220 bp, were the cultivar Kepok in Indonesia (found in West Java, Bali, East Nusa Tenggara, and Papua) and Poh (*Musa* spp.). With the *ITS2* length of 220 bp, other banana cultivars were the Mas (*Musa acuminata* with AA subgroup 'Sucrier'), Bile (*Musa* spp with group AB), Mas marlin (*Musa acuminata* with AA subgroup 'Berlin'), and *Musa balbisiana* Colla (BB group) (Meitha *et al*., 2020). The *ITS2* length of banana *Musa acuminata* with genome AAA varied from 118 to 222 bp, while the *ITS2* of *Musa troglodytarum* L. (TT genome) gave a report of 208 bp (Dwivany *et al*., 2020a, b). The banana cultivar Bile has the ABB genome and was typically the cultivar 'Haji' in Nusa Tenggara, Indonesia. However, in this research, all accessions of cultivar 'Haji' have a different base of *ITS2* sequences from the banana cultivar 'Bile' found in Bali, Indonesia.

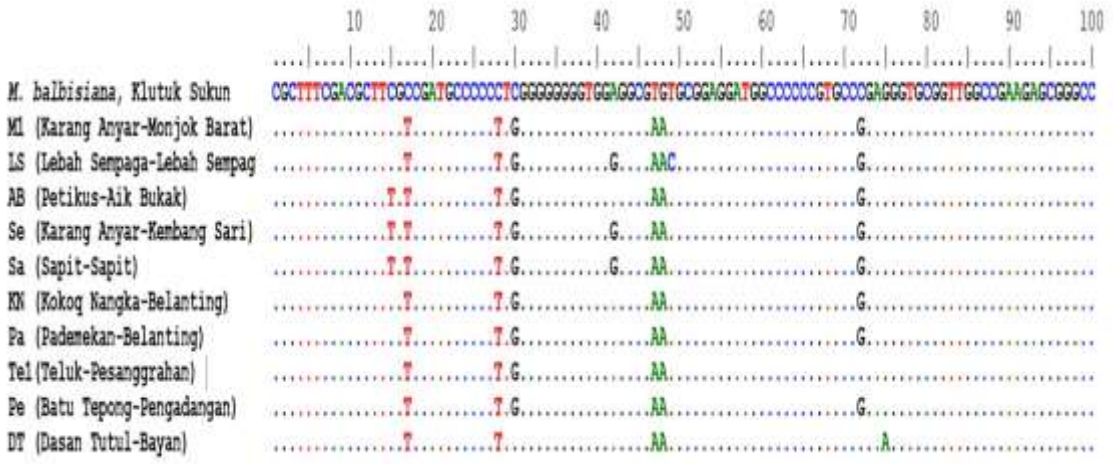

Figure 2. Differences in *ITS2* sequent bases of the banana cultivar 'Haji' accessions and the out-group accession (*M. balbisiana* Colla, Klutuk Sukun).

Genetic divergence and polymorphic sites

In the relevant study, the cultivar 'Haji' revealed the substitution mutations. The mutation occurred in bases 15, 17, 28, 30, 42, 47, 48, 49, 72, and 75 (Figure 2). Substitution mutations in the *ITS2* sequence of the 'Haji' cultivar accessions consist of transition mutations (bases 15, 17, 28, 42, 48, 49, and 75) and transversion mutations (bases 30, 47, and 72) (Figure 2). The multiple alignments of the *ITS2* sequence of the cultivar 'Haji' accessions also showed the presence of substitution mutations. The *ITS2* belongs to the ITS region, a DNA spacer connecting the small and large subunit ribosomal RNA genes. The ITS has a high nucleotide substitution rate (Chen and Shiau, 2015; Mursyidin and Setiawan, 2023). A larger nucleotide substitution rate in ITS is ideal for studying the genetic and germplasm diversity (Chen and Shiau, 2015). In the *ITS2* region, compensatory base changes (CBC) substitution can also occur. The CBC in *ITS2* can restore base pairs and maintain the secondary structure of *ITS2* (Cao *et al*., 2022). The secondary and tertiary structures of *ITS2* are essential for the ribosome function (Acharya *et al*., 2022). The secondary structure of *ITS2* can influence the structure and function of ribosomes, which can affect the expression of morphological characters (Dhivya *et al*., 2020).

Genetic divergence analysis based on *ITS2* sequences revealed the 10 accessions of the cultivar 'Haji' have a close relationship, with a divergence coefficient (0.000–0.023). The accessions KN and M1, accessions Pa and M1, accessions Pe and M1, accessions Pa and KN, accessions Pa and Pe, and accessions Sa and Se exhibited a divergence coefficient of 0.000. Divergence coefficient of 0.023 was notable between the accession DT and the accessions LS, Se, and Sa of the banana cultivar 'Haji' (Figure 3). The lower the divergence coefficient value, the closer the genetic relationship between the compared accessions. The 10 accessions of the cultivar 'Haji' have a close genetic relationship based on the *ITS2* sequence divergence coefficient, even though there were mutations in the sequence. Low genetic divergence based on the *ITS2* region in banana cultivars indicates a close evolutionary relationship among those cultivars (Dhivya *et al*., 2020; Fendiyanto *et al*., 2023). Pere *et al.* (2023) stated based on the *ITS2* region, a low divergence in intraspecies exists. *ITS2* is an ideal DNA barcode for distinguishing species and genetic diversity studies.

Mutations in the *ITS2* sequence of the cultivar 'Haji' accessions do not cause morphological variations. Since *ITS2* is a non-coding section of ribosomal DNA (rDNA), mutations in the *ITS2* sequence usually do not

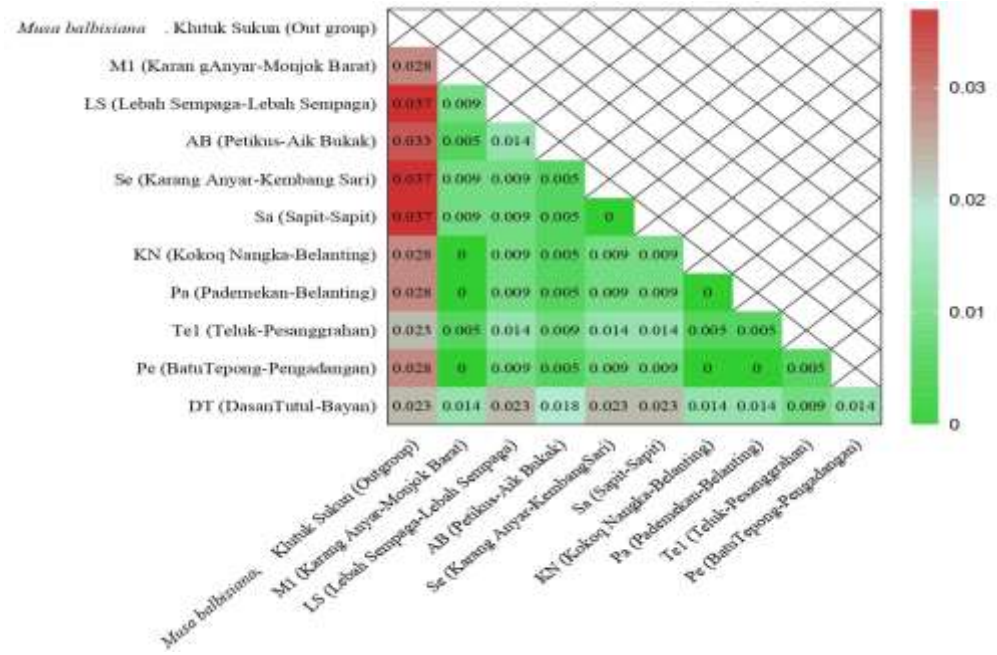

Figure 3. Heatmap of the relationship between the banana cultivar 'Haji' accessions and the out-group accession based on the *ITS2* sequence genetic divergence coefficient.

result in morphological variations. As the *ITS2* region primarily participates in the development of the ribosomal RNA, mutations affect the rRNA's functionality than the organism's appearance. Specific secondary structures give rise to the rRNA functionality, which is essential for the healthy operation of the ribosomes, as maintained by the *ITS2* sequence under evolutionary constraints (Mishra *et al*., 2021; Karuwal *et al*., 2024). The phylogenetic analysis results also support these findings.

Phylogenetic analysis

Construction of a phylogenetic tree, using genetic distances from 10 *ITS2* sequences of the cultivar 'Haji' accessions and the out-group, divides the banana accessions into two large groups. These groups are clade I, having 10 accessions of the cultivar 'Haji', and clade II, containing only the out-group accession. Clade I comprises six subclades. The sub-clade A consists of accessions Pa, Pe, KN, and M1, sub-clade B with the accession Te1, while sub-clade C has the accession AB. The sub-clade D consists of accessions Se and Sa, sub-clade E with accession LS, and sub-clade F having the accession DT (Figure 4). A scale of 0.01 indicates the genetic distance between sequences.

Phylogenetic construction of the cultivar 'Haji' accessions showed all the accessions appeared in the same clade and separated from the out-group accession (*M. balbisiana* Colla, Klutuk Sukun). Phylogenetic analysis helps identify the inter-specific relationship of Musaceae, which is necessary for more effective management and conservation of the species. Phylogenetic analysis using molecular data, such as, ribosome (*ITS*) and chloroplast (*trnL-F*) sequences, has revealed Musaceae generally have well-conserved genome sizes and genes (Fu *et al*., 2022; Gardoce *et al*., 2024). The results of this study can support molecular systematic studies, phylogenetics, and the development of banana germplasm with superior characteristics, such as, long shelf life in the 'Haji' cultivar.

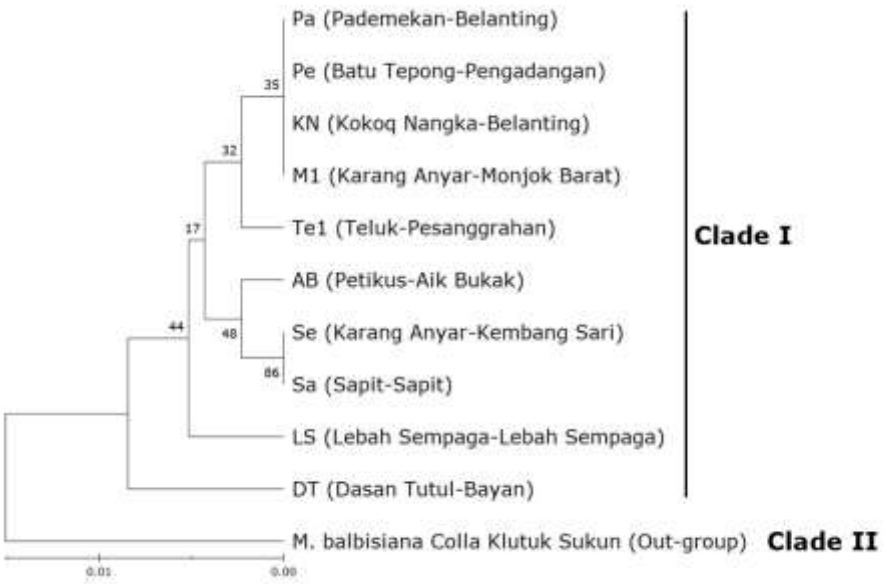

Figure 4. Construction of a phylogenetic tree based on the *ITS2* sequence of the banana cultivar 'Haji' accessions and the out-group accession (*M. balbisiana* Colla, Klutuk Sukun).

CONCLUSIONS

The *ITS2* was able to differentiate the accessions of banana cultivar 'Haji' (*Musa x paradisiaca* L.) from other *Musa* sp. The *ITS2* size was 220 bp in the cultivar 'Haji'. The substitution mutations were also evident in the *ITS2* sequence of the cultivar 'Haji' accessions at the 10 bp. The divergence coefficient of the cultivar 'Haji' accessions was low, indicating the close kinship among the accessions. The phylogenetic analysis based on the *ITS2* sequence showed all the accessions of cultivar 'Haji' positioned in the same clade and separated from the out-group accession (*M. balbisiana* Colla Klutuk Sukun). The results indicated all obtained accessions of the cultivar 'Haji' found in Lombok Island, Indonesia were genetically identical.

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