TROPICAL AGRICULTURAL SCIENCE

Bali Bananas (*Musa* spp. L.) Genetic Relationship Based on Internal Transcribed Spacer 2 (ITS-2)

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ABSTRACT

Banana is one of the most essential commodities in Bali island. It is not only for nutrition sources but also for cultural and religious aspects. However, Bali banana genetic diversity has not been explored; therefore, in this study, we focused on its genetic relationship using a molecular approach. This research aimed to determine the genetic relationship of Bali banana cultivars using the internal transcribed spacer 2 (ITS-2) region as a molecular marker. A total of 39 banana samples (*Musa* spp. L.) were collected from Bali island. The ITS-2 DNA regions were then amplified and sequenced from both ends. ITS-2 sequences were predicted using the ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/). The multiple sequences alignment was performed using ClustalX for nucleotide-based tree and LocARNA to

Fenny Martha Dwivany, Muhammad Rifki Ramadhan, Carolin Lim, Agus Sutanto, Husna Nugrahapraja, Ketut Wikantika, Sigit Nur Pratama, Karlia Meitha and Aksarani Sa Pratiwi

provide the secondary structure information. Phylogenetic trees were constructed using neighbor-joining (Kimura-2-parameter model, 1,000 bootstrap). The result showed that two clades were formed, one clade was abundant in A genome (AA and AAA), and the other rich in the B genome (BB and ABB). This result suggested that cultivars that had similar genomic compositions tended to be grouped within the same clade and separated with different genomic compositions. This study gives perspectives that ITS-2 sequences in bananas are quite similar and differ much compared to other families. Secondary structure has been described to provide more robust resolving power in phylogenetic analysis.

Keywords: Genetic diversity, ITS-2, phylogenetic tree, secondary structure

INTRODUCTION

Banana is one of the most important crops, with 100 million metric tons being produced annually in 130 tropical and subtropical countries (Pillay & Tenkouano, 2011). It was reported that bananas were initially derived from naturally crossing of bananas in Papua New Guinea and Eastern Indonesia, then differentiated and spread to Southeast Asia and finally throughout the world (Nelson et al., 2006). In Indonesia, banana is a valuable commodity with the highest biodiversity throughout the country (Hapsari et al., 2017). Particularly on the island of Bali, in which most of its inhabitants are Hindus, bananas have pivotal roles in the procession

aspects of the customs, culture, and religious ceremonies. Not only in quantity, but the diversity of bananas is also crucial for the people of Bali, especially for custom processions and offerings (Rai et al., 2018).

DNA barcoding is a method used in species identification using standard areas of DNA in an organism. This method uses short molecular markers in organism DNA and is considered promising to identify species of an organism and determine their relationship with one another. The molecular markers used should be variable enough and enable closest species identification but need to be conservative to simplify PCR amplification and alignment of sequences among distant species. One type of molecular marker used universally is the internal transcribed spacer (ITS) (Zhang et al., 2015).

Most of the banana accessions are polyploid (diploid or triploid) and believed that various cultivars originated from intra- and inter-specific hybridizations between wild bananas of *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) (De Langhe et al., 2009). To date, the immense diversity of banana cultivars on the island of Bali has not been fully explored, especially in the aspect of genetics and molecular biology. To the best of our knowledge, there are some molecular studies on banana in Indonesia (Hapsari et al., 2018; Meitha et al., 2020). However, there was no report on banana molecular relationships in Bali. As one of the most popular markers in phylogenetics, ITS-2 sequences are considered accurate owing to their conserved nucleotides. As a means of

identification by using the DNA barcoding method, ITS-2 can supplement the use of morphological characteristics analysis (Guzow-Krzemińska & Węgrzyn, 2000).

ITS is a region located between the ribosomal rDNA locus. This region is often used by researchers to determine biodiversity and molecular relationship, especially in plants. The molecular marker of the ITS region is chosen because it has several advantages. The ITS area is relatively short (at around 200 bp) and has a high-copy-number in the genome, making it easier to be isolated, amplified, and analyzed (Poczai & Hyvönen, 2010). ITS-2 region, the primary source of variations in the ITS sequence, is shorter and easier to be sequenced. Besides, the ITS-2 region exhibits significant sequence variability at the species level or lower (Yao et al., 2010). Therefore, the ITS-2 region has been considered as an excellent phylogenetic

marker and a promising standard region for barcode DNA (Zhang et al., 2015). The purpose of this research is to determine the genetic variability of banana cultivars in Bali Island using the ITS-2 region as a molecular marker.

METHODS

Tissue Collection and Preservation

We collected thirty-nine cultivars of bananas from nine regencies in Bali, as presented in Table 1 and Figure 1 was visualized according to area coordinates, as shown in Table 2. Cultivars were sampled one replicate by cutting approximately 10 cm of the cigar leaves with a sterile blade and stored in a 50 mL conical tube filled with 2 g silica gel (SiO_2) to reduce the humidity. Once arrived in Bandung, liquid nitrogen was used to preserve the cigar leaves and leaves were stored in -80°C before used.

Table 1

Banana cultivars collected from Bali island

No.	Cultivar	Locality	No.	Cultivar	Locality
1.	Kayu	Gianyar	11.	Sobo	Klungkung
2.	Sabe Macan	Klungkung	12.	Buah	Karangasem
3.	Mas Bali	Gianyar	13.	Ketip Kerta	Gianyar
4.	Sangket	Klungkung	14.	Poh	Gianyar
5.	Keladi	Klungkung	15.	Bali	Gianyar
6.	Tulang	Gianyar	16.	Lumut	Gianyar
7.	Kepok Tanjung	Klungkung	17.	Ambon Kuning	Gianyar
8.	Rojo Molo	Klungkung	18.	Mas Marlin	Gianyar
9.	Tembaga	Gianyar	19.	Nangka	Gianyar
10.	Gancan	Klungkung	20.	Ketip Tulang	Gianyar

Table 1 *(Continued)*

No	Cultivar	Locality	No	No Cultivar	Locality
21.	Klutuk	Denpasar	31.	Bile	Buleleng
22.	Padi	Gianyar	32.	Ketip Sari	Jembrana
23.	Muli Kuning	Gianyar	33.	Raja Bulu	Gianyar
24.	Kaiki	Karangasem	34.	Susu Hijau	Denpasar
25.	Siam	Gianyar	35.	Panjang	Gianyar
26.	Kapal	Gianyar	36.	Tanduk	Jembrana
27.	Bunga	Karangasem	37.	Seribu	Klungkung
28.	Cavendish	Gianyar	38.	*Musa velutina*	Gianyar
29.	Kepok	Gianyar	39.	*Musa ornata*	Gianyar
30.	Kole	Gianyar			

Table 2

Locations of sample collection with the village of each regency and city

| Regency/City	Village	Coordinate of Global Positioning System
Denpasar	Penatih	8°36'28.79"S, 115°14'14.87"E
Gianyar	Guwang	8°36'39.23"S, 115°17'23.82"E
	Celuk	8°36'5.59"S, 115°15'53.85"E
	Kerta	8°19'49.64"S, 115°17'32.69"E
Klungkung	Besan	8°31'5.73"S, 115°27'5.85"E
	Pesinggahan	8°32'58.88"S, 115°27'27.02"E
Karangasem	Rendang	8°25'41.02"S, 115°25'7.79"E
Bangli	Jehem	8°24'54.90"S, 115°22'15.16"E
Buleleng	Anturan	8°9'12.42"S, 115°3'4.93"E
Badung	Abiansemal	8°32'15.60"S, 115°12'51.66"E
Tabanan	Marga	8°25'51.67"S, 115°10'34.55"E
Jembrana	Bading Kayu	8°25'24.13"S, 114°54'59.59"E
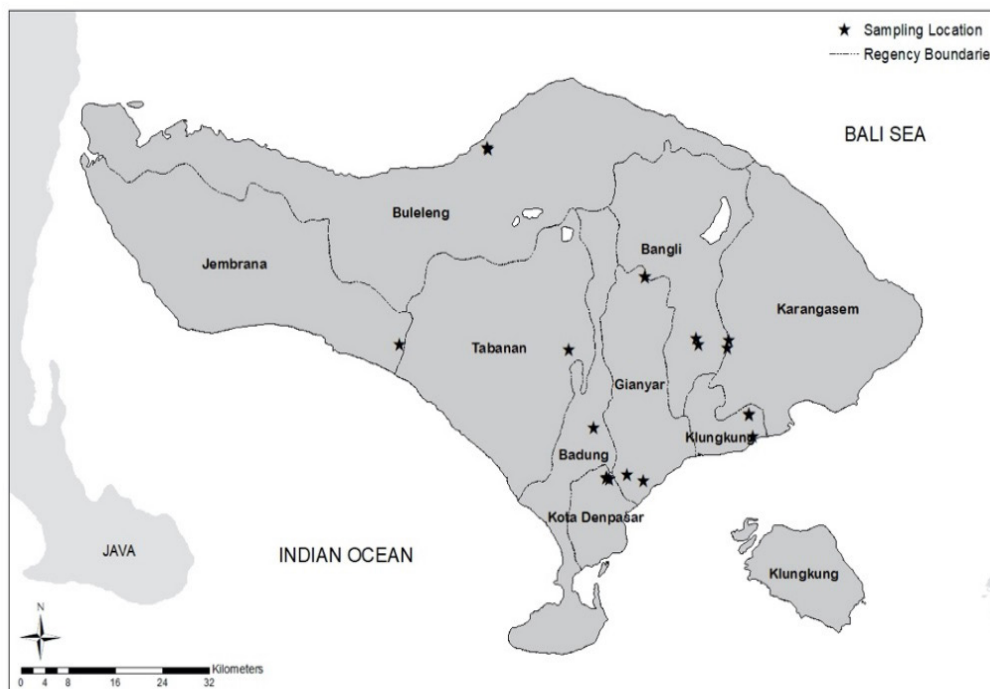
Figure 1. Sampling locations of banana cultivars in Bali island with stars showing the geographical position of Penatih, Guwang, Celuk, Kerta, Besan, Pesinggahan, Rendang, Jehem, Anturan, Abiansemal, Marga, and Bading Kayu area

DNA Isolation and Amplification of ITS-2 Region

Thirty-nine (39) samples had been collected and each sample had one replicate. All samples were ground using mortar and pestle with the help of liquid nitrogen, which was poured periodically until the sample became powdery fine. The DNA was isolated using the modified Doyle and Doyle method, by multiple washing and cleaning process using Isopropanol (Doyle & Doyle, 1991). The quality and quantity of DNA isolates were measured using NanoDrop™ spectrophotometer. The 260/280 ratio was measured to check the quality and quantity of the DNA in which TE buffer was used as a solvent. Gene amplification was performed by polymerase chain reaction (PCR) method (initial denaturation temperature 94°C for 5 minutes, annealing temperature of 55°C for 30 seconds, and elongation temperature of 72°C for 10 minutes; with 40 denaturation cycle) (Meitha et al., 2020). The 50 µL of PCR reaction comprised 5 µL of DNA samples and mixed with 25 µL of GoTaq® Green Master Mix (serial number: AF9PIM712 1016M712, Promega Co., USA), which consisted of DNA Polymerase, Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400

Fenny Martha Dwivany, Muhammad Rifki Ramadhan, Carolin Lim, Agus Sutanto, Husna Nugrahapraja, Ketut Wikantika, Sigit Nur Pratama, Karlia Meitha and Aksarani Sa Pratiwi

µM dCTP, 400 µM dTTP, and 3mM $MgCl_2$ (Promega Co., USA); 15 µL of nuclease-free water; and 2.5 µL of each of the primary ITS-S2F and ITS-S3R. The primer sequences used in the reaction were ITS-S2F: 5'-ATGCGATACTTGGTGTGAAT-3' and ITS-S3R: 5'-GACGCTTCTCCAGACTACAAT-3' (Gu et al., 2013). The PCR results were confirmed using electrophoresis (agarose 1%, TAE 1x buffer, 70V-30 minutes). The PCR amplified products were sequenced using Sanger sequencing platform via Macrogen Inc., South Korea. Samples that have been successfully sequenced then used to make contig using the CodonCode Aligner application (http://www.codoncode.com/aligner/).

Prediction of ITS-2 Secondary Structure and Phylogram Construction

The secondary structure ITS-2 region was predicted using ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) with E-value cut off less than 1e-16 and model possessing the highest transfer helices while as for motif prediction, E-value less than 0.01 and Viridiplantae model were opted (Keller et al., 2009; Koetschan et al., 2009). Multiple ITS2 sequences were aligned with ClustalX algorithm (Sievers & Higgins, 2014), within an integrated program, namely SeaView 4 (Gouy et al., 2010). Consensus representation of multiple sequence alignment was illustrated using WebLogo 3 (http://weblogo.threeplusone.com/), and the probability of the bases are shown in bits unit (Crooks et al., 2004).

Logo consensus was manually juxtaposed to its motif prediction to depict the conserved nucleotides better.

We added other closely related plants of *Musa* (*Ensete ventricosum*, *Ensete glaucum*, and *Musella lasiocarpa*) to inquire further whether the ITS-2 sequences among them are conserved but adequately robust to different group species within Musaceae family. To provide a broader illustration of plant evolution and systematics through phylogeny, different ITS-2 sequences of various plants from 4 families were designated as operational taxonomical units (OTUs). Those group of plants collected from ITS2 Database were Strelitziaceae (*Strelitzia reginae* and *Strelitzia alba*), Poaceae (*Hordeum vulgare*, *Zea mays*, and *Orzya sativa*), Brassicaceae (*Brassica rapa*, *Arabidopsis thaliana*, and *Arabidopsis lyrata*), and Fabaceae (*Medicago cretace* and *Medicago polyceratea*) (Koetschan et al., 2009). ITS-2 rRNA sequences of Bali bananas have been uploaded to GenBank and can be freely accessed, whose accession numbers are MN718989-MN719027.

Phylogram construction comprised multiple sequence alignment, tree inference, and visualization. Firstly, ITS-2 (51 OTUs) sequences were aligned with ClustalX software (Sievers & Higgins, 2014). The tree was inferred using neighbor joining (NJ) with Kimura-2-parameter (K2P) model and 1,000 bootstrap disturbances using SeaView program (Gouy et al., 2010). To compare, we managed to construct a phylogram using the same tree inference method enriched with secondary structure

information of ITS-2 sequence using Clustal algorithm coupled with LocARNA program (Will et al., 2012). Last, trees were visualized and annotated using FigTree 1.4.4 (Rambaut, 2018).

RESULTS AND DISCUSSION

Amplification of ITS-2 Area

The ITS-2 region amplification produced fragments with a size of ~500 bp. The amplified products did not only consist of the ITS-2 region, which based on the literature had a length of 205 bp to 227 bp (Hřibová et al., 2011), but also the 5.8S rDNA region and the 28S rDNA region which were the flanking sequences of the ITS-2 region (Yao et al., 2010). This was not a problem in constructing the phylogeny of ITS-2 due to a trimming process that was conducted following ITS-2 annotation. The visualization of the amplification results can be seen in Figure 2.

ITS-2 Secondary Structure

We evaluated the secondary structure of ITS of *M. acuminata* (A genome donor) and *M. balbisiana* (B genome donor), as shown in Figure 3. A variety of molecular and evolution mechanisms have resulted in ITS-2 that can be distinguished based on their secondary structure. The most striking differences observed were the internal loop size and the extra loop in helix I in B genome (Figure 3B) compared to the A genome (Figure 3A). We also found that from the alignment of Bali bananas represented in logo consensus, the first motif of U-U mismatch (II left) was the least conserved motif among the other three motifs.
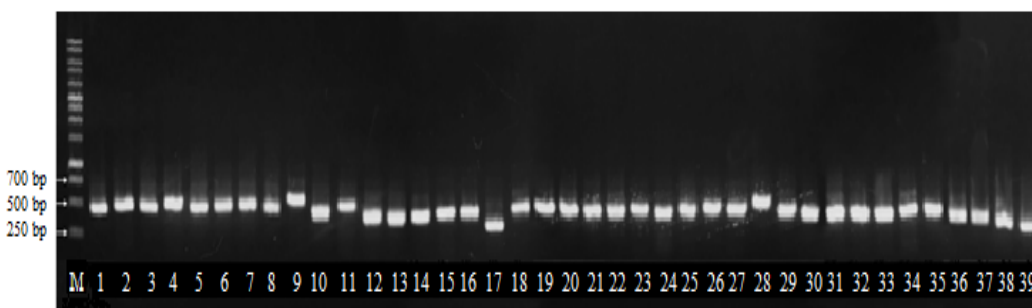

Figure 2. Electrophoregram of DNA amplicons of 5.8S rDNA, ITS-2, and 28S rDNA of 37 banana cultivated varieties, *Musa ornata*, and *Musa velutina* with no band shearing: M = 1 kb DNA marker ladder, 1 = Kayu, 2 = Sabe Macan, 3 = Mas, 4 = Sangket, 5 = Keladi, 6 = Tulang, 7 = Raja Molo, 8 = Tembaga, 9 = Gancan, 10 = Sobo, 11 = Buah, 12 = Ketip Kerta, 13 = Poh, 14 = Bali, 15 = Lumut, 16 = Ambon Kuning, 17 = Mas Marlin, 18 = Nangka, 19 = Ketip Tulang, 20 = Klutuk, 21 = Padi, 22 = Muli Kuning, 23 = Kaiki, 24 = Siam, 25 = Kapal, 26 = Bunga, 27 = Cavendish, 28 = Kepok, 39 = Kole, 30 = Bile, 31 = Ketip Sari, 32 = Raja Bulu, 33 = Susu Hijau, 34 = Panjang, 35 = Tanduk, 36 = Seribu, 37 = Kepok Tanjung, 38 = *Musa ornata*, and 39 = *Musa velutina*. The bands are shown to have lengths of 500 bp, hence the flanking sequences were trimmed to produce the ITS-2 sequence only
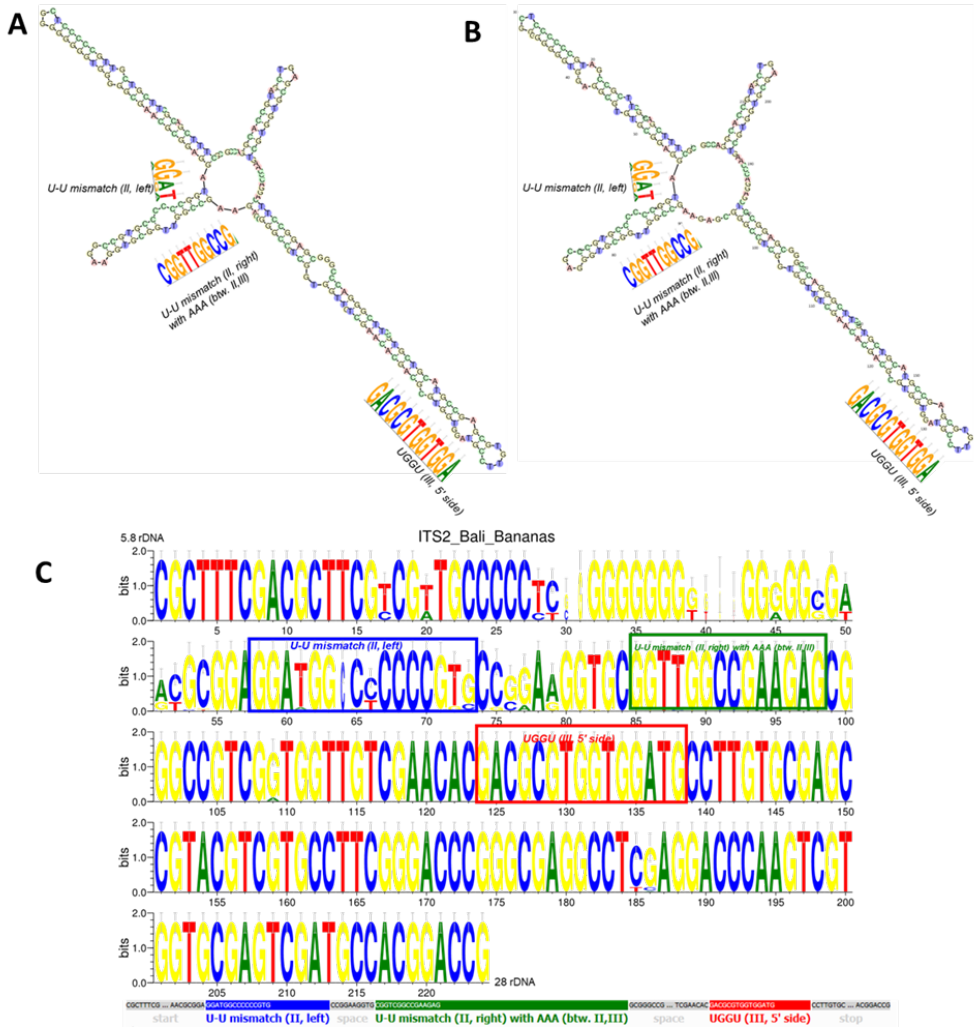
Pertanika J. Trop. Agric. Sci. 43 (4): 583 - 597 (2020)

589

Figure 3. Secondary structure and motif prediction of ITS-2: A) secondary structure of ITS-2 predicted from A genome banana, B) secondary structure of ITS-2 predicted from B genome banana, and C) consensus of ITS-2 sequences of 32 banana cultivars represented in nucleotides logo (C = blue, G = yellow, T = red, A = green). The probability of each nucleotide is represented in bits unit. The 224 bp ITS-2 is paired with the motif prediction comprises start, two spacers, three motifs (represented in boxes outlined with blue, green, and red colors), and stop sequence

ITS-2 functional secondary structure is proven to be conserved in plants DNA (Hershkovitz & Zimmer, 1996; Jobes & Thien, 1997). ITS-2 spacer had four unique helices structure, as shown in Figure 4, and the analysis of the two-dimensional structure proved that the structure was highly conserved throughout the eukaryotes (Schultz et al., 2005). Helix II is characterized by the content of pyrimidine-pyrimidine nodules. Helix III characterized as the longest and containing the most conserved primer on 5' includes TGGT (UGGU sequences for RNA) sequence (Schultz et al., 2005). As for helix, I and IV are more diverse, and only helix II and III that could be identified in general to all eukaryote (Coleman, 2007). The functions of ITS-2 as spacer are largely unknown, and ITS-2 sequences are not incorporated into the mature rRNA. Nevertheless, they encode signals for the rRNA transcripts processing properly (Hillis & Dixon, 1991). This inferred function is itself dependent on secondary structure of the ITS RNA.

Phylogenetic Tree Analysis

Standard multiple sequence alignment programs, such as MAFFT (Katoh & Standley, 2013) or T-Coffee (Notredame et al., 2000), cannot be used to construct a phylogenetic tree by including the secondary structure information. Rather, these tools use virtually similar approaches based on nucleotides information such as similarity. Despite significant variations in nucleotides, the ITS-2 secondary structure has been described to be highly conserved, possessing four helices and motifs (Schultz et al., 2005). As an alternative, combining multiple loci of gene markers with the ITS-2 will provide a better substitute to infer a phylogenetic tree. The demerit of using additional loci is that it is time-consuming and can be costly; furthermore, adding nucleotides information does not guarantee the truest evolutionary history (Huelsenbeck et al., 1996).

To provide a comparison, we inferred trees using two alignments: nucleotides information-based and one with additional secondary structure information, as shown in Figure 4.

To analyze the genetic variability among *Musa* species and the family of Musaceae, we also studied other families of Strelitziaceae, Poaceae, Brassicaceae, and Fabaceae. Coupled with additional OTUs of *Ensete* and *Musella* species, this strategy hopefully can shed light in terms of ITS-2 usefulness. Particularly, it may provide a depiction of major and minor classification in plants. Based on the same taxa of families, both phylograms, as shown in Figure 4, showed a consistent clade clustering. Our analyses showed the monophyletic origin of Musaceae comprised *Musa*, *Ensete*, and *Musella* species, this result is concordant to previous analysis (Hřibová et al., 2011). Musaceae is shown to closely relate to Strelitziaceae as they are the group in order of Zingiberales. Given two types of strategies in constructing the phylogenetic tree, the secondary structure is far more conserved in comparison with the nucleotide sequences. The genera of *Ensete* and *Musella* are not shown as outgroups
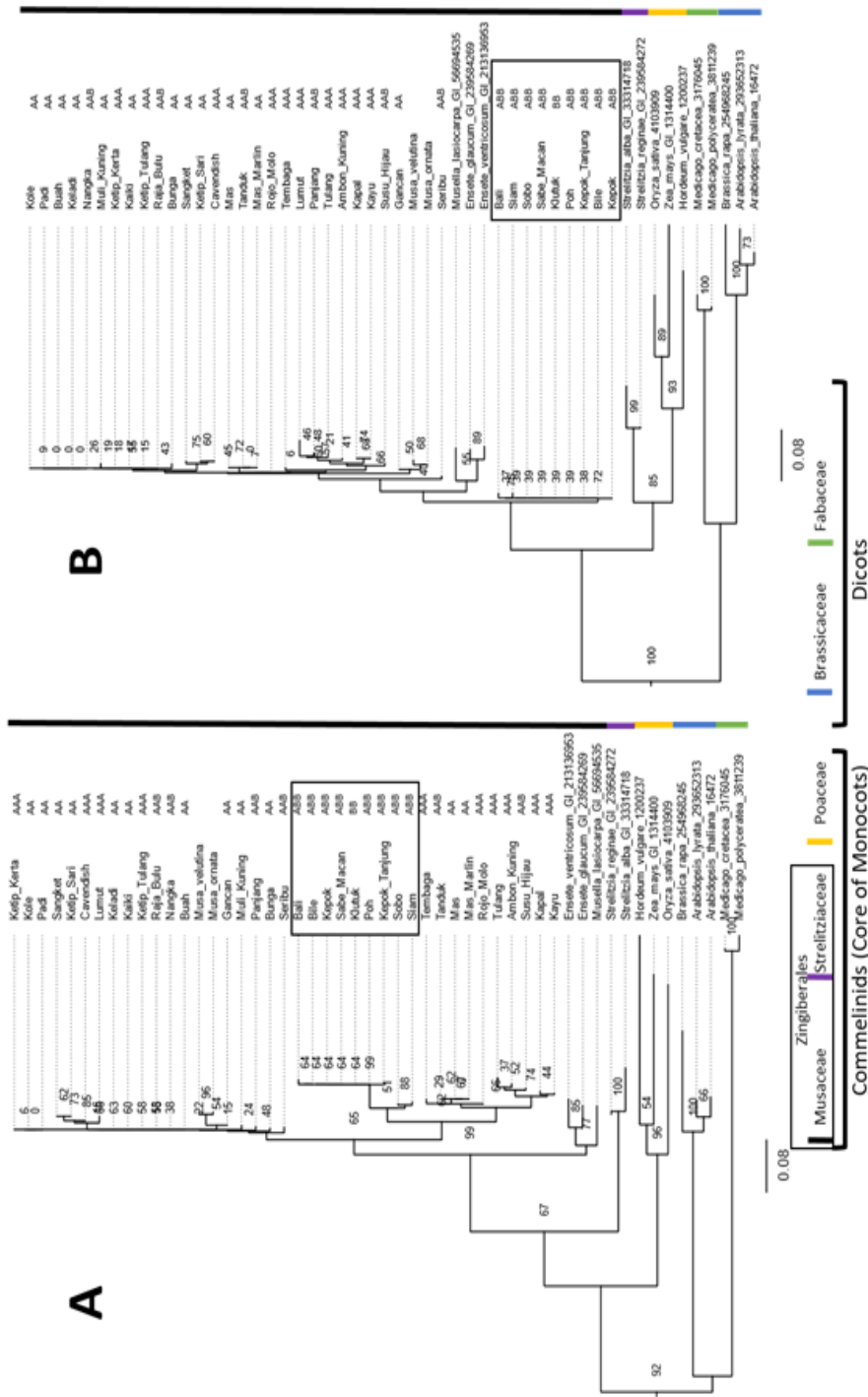
Fenny Martha Dwivany, Muhammad Rifki Ramadhan, Carolin Lim, Agus Sutanto, Husna Nugrahapraja, Ketut Wikantika,
Sigit Nur Pratama, Karlia Meitha and Aksarani Sa Pratiwi

Figure 4. Phylograms constructed from the ITS-2 region from banana cultivars and other plants from other families (Strelitziaceae, Poaceae, Brassicaceae, and Fabaceae): A) NJ tree inferred using NJ (neighbor-joining) with no information of secondary structure, B) NJ tree constructed from the information of nucleotide sequences and secondary structure. Numbers on the branches show bootstrap value, and the terminal nodes represent species name in which genome composition for banana cultivars are shown. The numbers following non-*Musa* spp. are the GI accession of ITS-2 that were retrieved from the database

within *Musa* clade in the tree constructed from nucleotide and secondary structure information (Figure 3B).

The monophyletic group of sub-class of commelinids, known as the core of monocots, has been described as a closely related group based on molecular analysis (Barrett et al., 2013). The group of comelinids, such as corn (*Zea mays*), barley (*Hordeum vulgare*), and paddy (*Oryza sativa*) are shown topologically close to bananas. Not only they have extant similar phenotypic characteristics, but they also have a close molecular relationship based on ITS-2 rDNA analysis. Liliopsida (monocots) groups are shown as distant relatives meaning a greater variance above taxa of families was observed.

The drawback of using the ITS-2 rDNA marker in phylogenetic analysis is that ITS-2 may be heterogeneous. This characteristic can be understood by its presence in plant species and polyploid ones as multiple divergent ITS sequences in several loci. ITS-2 sequences have been used in a wide range of taxonomy analysis ranging from order to subspecies while other barcoding markers such as *rbcL* (gene encoding ribulose biphosphate carboxylase large chain) and (mitochondrial DNA) mtDNA have narrower ranges (Coleman, 2003). Although their versatility of usefulness, ITS-2 sequences appeared to be less conserved (differ too much) in major groups or higher taxa (Baldwin et al., 1995). We found that this claim could be well-conceived as we evaluated the alignment of different families, particularly in the subdivision of dicots.

Thus, the strategy used in choosing an alignment approach remained a challenging task.

One of the intricate comprehensions in phylogenetics is polytomy, a clade of OTUs that are not formed a series of two way-splits. Commonly, this phenomenon appears as a result of unresolved phylogeny owing to the limitedness of phylogenetic software (Slowinski, 2001). This is not the case when it comes to several ITS-2 region clusters. Our data showed that some banana cultivars have 100% identity, such as in the B genome group, and appeared as a polytomy. Subsequently, we reconstructed the trees by excluding non-banana plants as well as ornamental bananas (*M. velutina* and *M. ornata*) (Figure 5).

The phylogenetic trees in Figure 5 show that the OTUs based on ITS-2 were clustered according to their families in which banana cultivars were grouped into two clades indicating their dominant genome composition: A genome and B genome. This clade described the genome composition of the banana cultivar. The first clade contained banana cultivars that had the genome AA, AAA, and AAB. In the second clade, banana cultivars had the genomic composition of ABB and BB. In conclusion, ITS-2 region can be used to classify banana species rather than banana cultivars. However, the closely related *M. velutina* and *M. ornata* made the clustering analysis seemed complicated (Figure 4). Constructed trees of ingroup edible bananas (nucleotide information only and one added with secondary structure information) seem to have similar topology
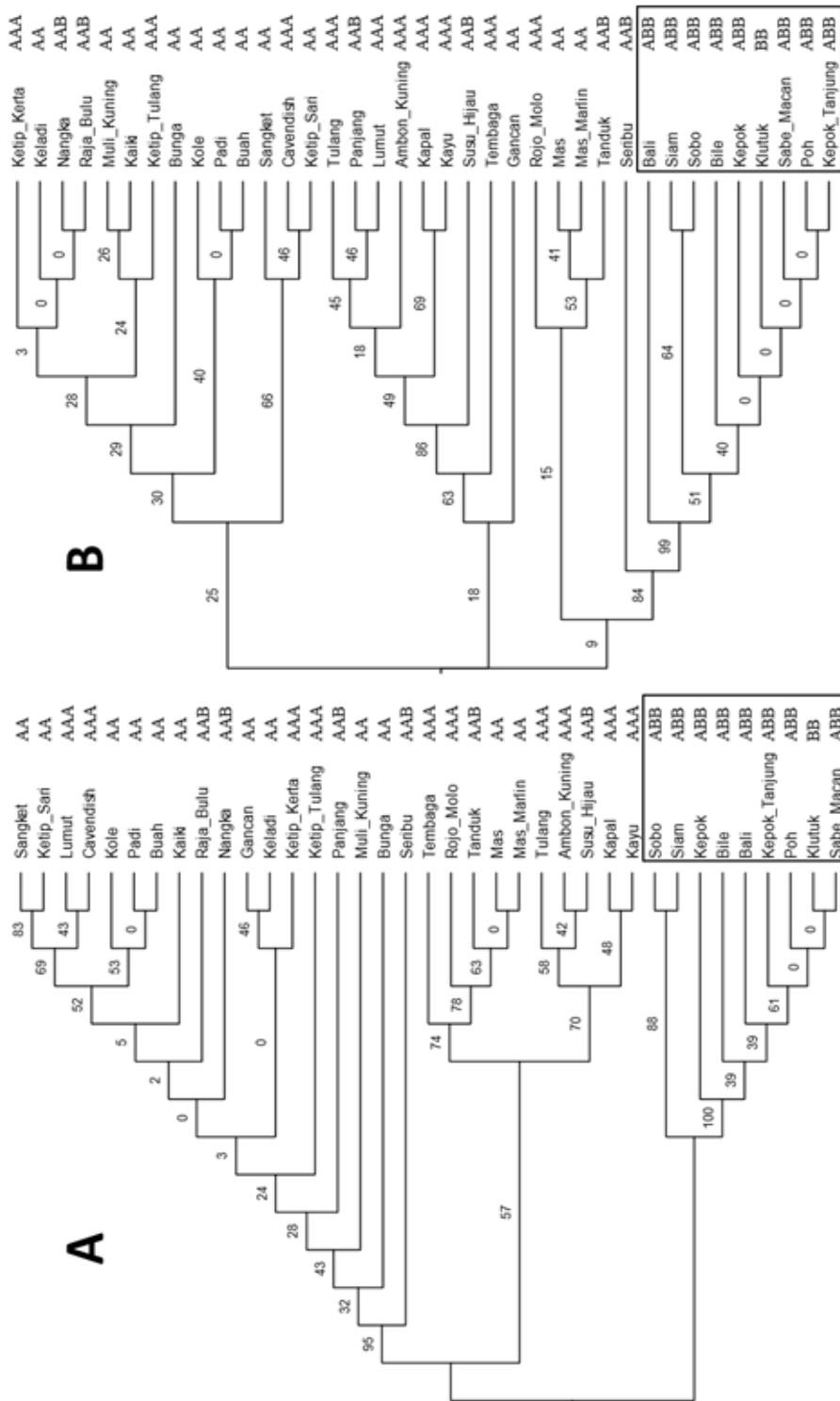
Fenny Martha Dwivany, Muhammad Rifki Ramadhan, Carolin Lim, Agus Sutanto, Husna Nugrahapraja, Ketut Wikantika,
Sigit Nur Pratama, Karlia Meitha and Aksarani Sa Pratiwi

Figure 5. Dendograms of edible banana cultivars inferred from ITS-2 sequences: A) tree constructed using NJ and B) tree constructed using NJ with secondary structure information attained from LocARNA software

594

Pertanika J. Trop. Agric. Sci. 43 (4): 583 - 597 (2020)

and cluster (Figure 5). Both strategies were able to group bananas based on their genome composition even though the B genome group was clearly separated in the former strategy.

In terms of Bali bananas diversity, it is also imperative to be concerned about the cultivation and conservation of useful cultivars such as 'Kayu AAA' and 'Bile ABB' cultivar (also known as 'Haji' cultivar in Nusa Tenggara). 'Kayu' cultivar possesses distinctive value since the variety is only cultivated in Bali island, generally harnessed for offering in ceremonies (Rai et al., 2018).

Being one of the most diverse cultivars resulting from several wild species crossing, bananas have more than thousand of cultivars. Molecular barcoding such as this is beneficial to evaluate the diversity of bananas regarding their chromosomal genome character. Further analysis of multiple ITS-2 sequence types origin in terms of evolutionary mechanisms, and their utilization can shed some light on genome relationship and polyploid ancestry (Bailey et al., 2003; Dadejová et al., 2007). Banana cultivars that have a relative proportion of similar genome compositions are placed in the same clade. Thus the molecular relationship of them is closer than that of a banana cultivar with a different relative proportion of genomic compositions. By juxtaposing two phylograms, we observed that additional information from a secondary structure could provide a stronger resolving power in phylogenetic analysis rather than just solely relying on nucleotide information.

CONCLUSION

This study showed that from thirty-nine banana samples from Bali island, two clades were formed, one clade was abundant in A genome (AA and AAA), and the other rich in the B genome (BB and ABB). This ITS-2 study also suggested that cultivars with similar genomic compositions would tend to be grouped within the same clade.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Fenny Martha Dwivany, Muhammad Rifki Ramadhan, Carolin Lim, Agus Sutanto, Husna Nugrahapraja, Ketut Wikantika, Sigit Nur Pratama, Karlia Meitha and Aksarani Sa Pratiwi

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597