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Phylogenetic Relationships of Local Durian Species based on Morphological Characteristics and PCR-RFLP Analysis of the Ribosomal Internal Transcribed Spacer (ITS) DNA

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Abstract

In this study, twenty local durian accessions obtained from Central Java in situ collection were characterized using the morphological characteristics and the restriction patterns which generated from the region spanning the internal transcribed spacers ITS LEU and ITS 4. Morphological characteristics of durian leaf, stem, tree, and fruit showed variations for the different accessions, whereas polymerase chain reaction (PCR) products of ribosomal DNA region showed a low length of variation. The size of the PCR products and the restriction analyses with the restriction endonucleases Bsp1431yielded a restriction pattern for each accessions. The results of this study can be utilized by local durian farmers as a preliminary reference for durian propagation. The data obtained need to be supported by further research using the other molecular markers to obtain more accurate data. The clear identity of durian species can help the management of propagation systems by farmers to get superior local durian.

How to Cite

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INTRODUCTION

Durian is the "king of fruit", the South East Asia's popular fruit belonging to the genus *Durio* and the Malvaceae family, specifically the sub-family Bombacaceae. This unique fruit has a strong odor (some people adore it as fragrant; others find the intense and unpleasant aroma), oval shape, large size, and its flesh is covered by thorn husk. Indonesia is one of the centers of origin of durian (*Durio* sp.). The center of ecological diversity for Durians is Borneo Island. Test (2005) found that there were 20 species of the genus *Durio* in there, 18 of them were endemic. The most economically important species is *Durio zibethinus* Murr (Tingal, 1994). According to Nafsi (2007), Indonesia has more than 103 varieties of durian. The morphology characteristics among those 103 durian varieties are different in leaf shape, fruit shape, aroma, taste and seed shape. The diversity of local durian in Indonesia is promising for the improvement of durian quality through plant breeding techniques. Through this technique, it is expected to obtain the superior quality durian cultivar that can be used as the top choice of consumers as great as *monthong* in Thailand and *musangking* in Malaysia. However, there are only a few studies on Indonesian durian diversity. Moreover, the local durian species, especially in Central Java have not been classified and characterized. Also, the management strategies on breeding system of durian in Indonesia especially in Central Java are still rarely done, since the characteristic of all durian species and varieties have not been obviously discovered.

In fact, some cultivars show the similar morphology characteristics which are difficult to distinguish. In the propagation system, the assurance of durian cultivar identity is compulsory. Plant breeding techniques require the certainty of the cultivars identity to avoid confusion. Therefore, the study on durian characteristics, population structure, and genetic diversity are essential for the conservation and management strategies of the durian genetic resources (Karp et al., 1997).

Some findings suggest that the morphology characteristics of durian are affected by the interaction of genotype with its environment (Pandin, 2010; Sobir et al., 2005), even though the morphological characterization is an easy and fast method (Nandariyah, 2007). In Novayandi study (Novayandi, 2004), the results found that there was a discrepancy of morphological characters with the molecular markers using isozymes on the genetic analysis of durian. The local durian cultivars used in this study were the *ex-situ* cultivars collectionwith the average age of trees was 40 years old and an average height of over 20 meters. In addition, the uncertain identities of durian collection werealso yet so difficult for farmers to undertake the efforts to improve its quality and ensure its sustainability.

The determination of the identity of durian is ideally conducted by the integration of the morphological characterization and molecular characterization. Molecular markers can be useful to differentiate and to distinguish the identities among the cultivars (Noveli, 2005; Semagn, 2006; Umar et al., 2013). DNA as the source of analysisis available from any plant organs; therefore, the molecular markers have higher accuracy with the faster timing of characterization to pinpoint an accession genotype. Moreover, the molecular characterization of durian cultivarsgenotype using molecular markers could be done at any stage of plant growth (Retnoningsih et al., 2010). Despite the morphology and molecular characterization, the evolutionary relationship of the local durian is also important to be defined by the phylogenetic analysis. The phylogenetic analysis is a form of germplasm conservation so that the availability of genetic resources for future usage is assured (Bretting & Widrlechner, 1995; Esquinas-Alcazar, 1993).

The polymerase chain reaction (PCR) is a technique to amplify the target of DNA using two designed-primers. The combination of PCR technique with the restriction fragment length polymorphism (RFLP) markers is very useful to detect variations in the level of DNA by mapping and characterization of genes from different species of plants (Tanskley et al., 1989). The PCR-RFLP technique has inherent ability to utilize the small amount of relatively crude genomic DNA and to discriminate between genotypes based upon the presence or absence of restriction sites within the amplified DNA (Karp et al., 1997). PCR-RFLP method utilizes amplification with primers followed by cutting using restriction endonucleases and followed by gel electrophoresis has been widely used for the determination of plant and fungi phylogeny such as *Phaseolus* and banana (Nakamura et al. 1998, Vekemas et al., 1997; Ekasari, 2011). Santoso et al. (2005) studied the genetic variation of 11 accessions of 10 durian species with a PCR-RFLP method using eight restriction enzymes at ndhC-trnV and rbcL region. The advantages of PCR-RFLP approach are in the quality of the data and the information obtained from it. One of the characters that can be used as a molecular identification code

is the internal transcribed spacer region (ITS) of ribosomal DNA. Ribosomal DNA has the nature of homologous on a variety of different organisms, there are many in the cells, and it has a long sequence to allow a statistical test to see the difference. Ribosomal DNA variations can be observed through cutting by specific endonuclease enzyme (restriction enzyme). This enzyme will cut the identified specific sites. This process causes the formation of DNA fragments that differ in size from one organism to another. This polymorphism is then used as an identifier (fingerprint of an organism) (Maras, 2008).

In this study, the morphological and molecular characterization of twenty local durian obtained from *in situ* collection in Central Java were conducted. PCR-RFLP analysis was employed to reveal the molecular identity of local durian accessions.

METHODS

The study was conducted at the Molecular Biology Laboratory, Department of Biology, Semarang State University from March to September 2015. Plant samples were 20 accessions of local durian species collected from the Hortimart Agro Park, Bawen, Kabupaten Semarang, Central Java, Indonesia. The owner of this *ex-situ* collection names the local durian based on the main characters of Javanese traditional puppet as given in Table 1. The scientific names of durian species were unknown.

In this study, 20 local durian accessions were morphologically characterized based on durian descriptors published by Bioversity International (2007). The nature and characteristics of plant organs measured were the trees, leaf, stem, and fruit. Morphological characterization results were then analyzed using the program NTSys-PC 2.0 software

Plant DNA extraction

Leaf samples taken from Hortimart were kept in the clean plastic bag and then it was iceboxed during the transfer into the laboratory. DNA was extracted from 1 g of young leaves of durian accessions using modified CTAB-based protocol of Vanijajiva (2012) followed by brief purification step using illustra Nucleon Phytopure Genomic DNA Extraction Kits (GE Healthcare). Each sample was ground and then was transferred into powder form in liquid nitrogen. Leaf powder was poured into the tube containing with 750 µL 2% CTAB extraction buffer. Samples were incubated at 60 °C for 30 minwith shaking.

Table 1. Twenty accessions of durian species used in this study

Sample code	Local names of *Durio* accession
S1	Wijo
S2	Sadewo
S3	Ngastino
S4	Indrajid
S5	Bolodewo
S6	Arimbi
S7	Ontoseno
S8	Ponconoko
S9	Nogorejo
S10	Matahari
S11	Yodipati
S12	Dewi sinto
S13	Sugriwo
S14	Pergiwo
S15	Romowijoyo
S16	Petruk
S17	Brahmana
S18	Cokro
S19	Bismo
S20	Raja

After incubation, the sample was added by 750 µL phenol: chloroform: isoamyl alcohol (25: 24: 1) and was inverted back until homogeneous. Samples were centrifuged at 10,000 rpm, 4 °C for 15 min to obtain 3 layers. The supernatant (top layer) was transferred to a new tube. Subsequently, the cold isopropanol was added 0.6 × supernatant volumes. The mixture was gently inverted to precipitate the DNA. The DNA precipitates floated to the surfacewas then transferred into a new tube. The DNA pellet was washed using 500 µL 70% ethanol and it was dried at room temperature. After drying, the DNA pellet was dissolved in TE buffer and was allowed to stand at room temperature. Then, 2.5 µL RNase (10 mg/ml) was added to the DNA solution. The purification step was performed by incubation at 37 °C for 30 min. After incubation process was completed, the DNA extraction step was repeated two times starting from the addition of phenol: chloroform: isoamyl alcohol (PCI) until the precipitation process. The DNA solution obtained from leaf samples usually in the form of brown color and slimy texture. This form of DNA cannot be adjusted into PCR reaction. The DNA must be purified using further purification process. In this stu-

dy, the DNA solution was purified using Nucleon Phytopure DNA extraction kit by following the protocol instruction from the kit manufacturer.

DNA amplification

Two pairs of oligonucleotides ITS LEU and ITS 4 primers (Table 2) were used to flank the target regions. Eight restriction enzymes (*Alu*1, *Bsp*1431, *Bsu*R1, *Eco*471, *Ade*1and *Mph*11301) were used to digest the amplicons. The DNA amplification process was performed in a volume of 50 µl containing distilled water, 2x Dream Taq Green PCR master mix (Thermofischer Scientific, USA), 1 mM of each primer (AlphaDNA, Canada), and 60 ng genomic DNA. The PCR mixture was put into the 0.5 ml thin-walled microcentrifuge tube, placed in the thermal cycler machine(Peqlab Biotechnologie GmbH) andthen was amplified for 38 cycles of denaturation process at 94°C for 30 sec, annealing process at 58°C for 50 sec, extension process at 72°C for 45 sec, and final extension at 72°C for 7 min.

Table 2. Targeted DNA regions, primers and theirsequences (Nyffele & Baum, 2001)

DNA region and Primers	Sequences
ITS LEU	5'GTCCACTGAACCTTAT-CATTTAG 3'
ITS 4	5' TCCTCCGCTTATTGATAT-GC3'

First gel electrophoresis was performed in a volume of 12 µl containing 8 µl of distilled water, 2 µl of PCR products, and 2 µl of gel loading buffer. The samples were loaded onto a 2% agarose gel in 1x TAE buffer along with DNA marker. The gel was run in 1x TAE buffer at 80 volts for 1 hour. After that, the gel was dip-stained in the ethidium bromide solution for 15 min and then it was destained in distilled water for 30 min. The image of the stained gel was captured using the photo document system (Alpha Innotech, USA).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Digestion of the PCR products was performed in a volume of 25 µl containing 15 µl distilled water, 2.5 µl 10× TE buffer, 5.0 µl amplicon, and 2.5 µl restriction enzyme. The reaction mixture was placed into the 0.5 µl thin-walled tube. The mixture was mixed by pipetting up and down for several times and incubated at 37 °C for 1.5-3.0 h. The agarose gel electrophoresis and capturing image were then conducted after incubation. Variations of the digested PCR products were scored based on the presence and absence of bands on the images taken from the gels. Based on the presence or absence of band variation of PCR products, the scoring was performed to calculate using the similarity coefficients NTSys-PC 2.0 software. Furthermore, the phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

Plant morphology characterization

Characteristics of the organ leaves that have been observed included 17 properties of the leaf color (the upper and lower surface), leaf shape, leaf density, leaf arrangement, leaf length, leaf width, petioles length, petioles width, leaf texture and degree of leaf slipperiness. Characteristics of tree trunks stature included 9 properties, such as age, tree height, diameter and circumference of tree trunk, tree surface texture, growth direction, board root appearance, branching density, color, and tree trunkstature. Characteristics of fruit included 18 properties, among other forms of fruit; fruit stalk length, fruit weight, fruit diameter, density of spines, thorns shape, skin tone and color intensity on the fruit surface.

Twenty durian accessions showed similarity coefficient ranged from 0.59 to 0.82. The greater the similarity coefficient, the closer relationship among accessions. Based on morphological characters of durian accessions, the relationship between accessions could be analyzed (Figure 1). Based on the similarity coefficient among accessions, all accessions were proved to have different morphological characters. There are four main clusters of durian accessions. The first cluster shows two sub-clusters of durian Cokro and Bismo that have the same similarity coefficient and has a close kinship with Wijo and Pergiwo. In the second cluster, durian Sadewo, Bolodewo, and Romowijoyo are closely related, while the durian Matahari has a close kinship with durian Dewisinto. The second cluster of dendogram, durian Ontoseno and Brahma are proved to be closely related, while durian Arimbi has the similar coefficient close to durian Nogo. Durian Ponconoko with durian Raja has a similarity that is included in the third cluster. While the fourth cluster indicates that durian Ngastino is closely related with Yodipati.

Morphological characters show the characteristics of a living thing. Often, morphological characters of inner and intra-species show

similar character and even the same, or different altogether. Therefore, their identity is difficult to distinguishclearly. Morphological markers have the less precise accuracy of the information because it is influenced by the environment and the interaction between genetic factors and environmental factors (Pandin, 2010; Sobir et al., 2005) Based on the analysis of 20 durian accessions, it proved that all characteristics can distinguish the difference among the accessions. Some durian species have the similar stem and leaf morphological characters, but those have different fruit. In this study, the durian cultivars identity cannot be certainly determined (whether durians are different species, one species, one cultivar, or different cultivars). To determine the precise identity of durian cultivars, it is needed to analyze using molecular techniques to determine the genetic fingerprint of each durian cultivars.

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Plant molecular characterization

Molecular characterization of local durian species was initiated by the DNA extraction. The use of young leaves as DNA sources aimed to get higher DNA quantity. The young leaves have a cell wall structure that is easier to disrupt. The more plant cells that disrupted, the more the amount of DNA that obtained. However, in certain plants, mature leaves are more suitable for DNA extraction process (Small et al., 2004). Durian DNA was extracted and purified by a combination of cetyl trimethyl diamine tetra-acetic acid (CTAB) method and the purification using DNA extraction kit. The purification process using the extraction kit was performed to remove brown color and slimy texture from durian DNA. Durian as a woody plant, in general, contains secondary metabolites such as polyphenol compounds, tannins, and polysaccharides. Polyphenol compounds and polysaccharides when irreversibly interact with proteins and nucleic acids, it forms a brown color and mucus (gelatinous matrix) (Michiels et al., 2003). Polyphenol and tannin compounds causing the browning on the DNA pellets, while the polysaccharide compound causes DNA pellet has a high viscosity, slimy like glue and difficult for the pipetting process. Poor quality of DNA interfere the activity of *Taq* Polymerase enzyme (Michiels et al., 2003). In this study, the combination of CTAB method and kit was helpful to obtain good DNA quality. In the CTAB method, the addition of β-mercaptoethanol concentration serves to reduce oxidation of polyphenol compounds (Fang et al., 1992). The role of kit is used for purification of DNA in order to obtain DNA with far fewer contaminants. The results of DNA electrophoresis are shown in Figure 2. Based on the results of electrophoresis, it indicated that the

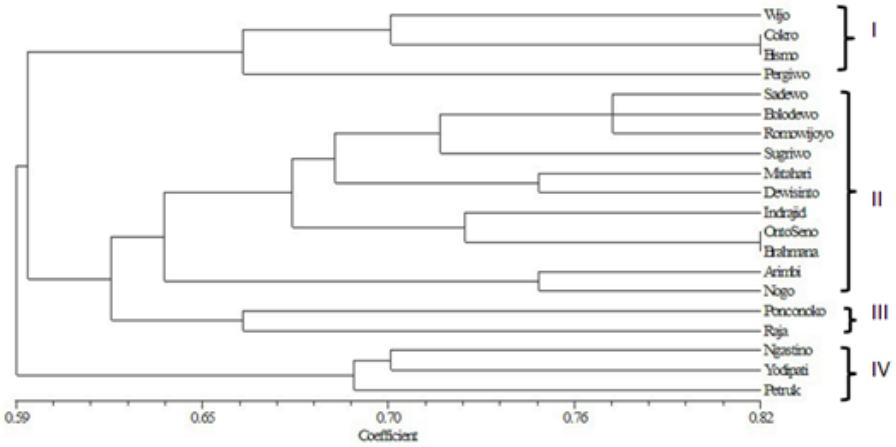

Figure 1. Dendrogram of 20 local durian accessions based on morphological characters.

DNA quality was good enough for the amplification process.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

DNA samples of each cultivar were amplified using primers ITS LEU and ITS 4. Ribosomal ITS DNA can be amplified in all plants using the same primer pairs (Kawata et al., 2003). Research applications of ITS regions of ribosomal DNA amplification in the analysis of genetic diversity have been widely performed. For example, *Adansonia* sp has 787 bp lengths of ITS region (Baldwin et al., 1995). Other studies have shown that *Adansonia killima* and *Adansonia digitata* have the genetic similarity and evolved tetraploidy (Pettigrew et al., 2012). Hikmah (2013) studied the molecular diversity of native durian from Gunungpati, Semarang Indonesia, based on ITS DNA fragment using PCR-RFLP. The study results on Gunungpati native durian showed that each accession shows a typical fingerprint. In this study, the ITS regions of ribosomal DNA of 20 durian accessions produced 850 bp PCR product size (Figure 3). The successful DNA amplification depends on the suitability of the primer pairs as well as the efficiency and optimization of the PCR process. Optimization of annealing temperature becomes the most important part in the process of amplification (Roux, 2009).

In order to determine the molecular characteristics of the twenty local durian accessions, PCR products were digested using 8 restriction enzymes. However, only *Bsp*143I enzyme that showed a success digestion process. The results of DNA digestion are shown in Figure 4. The results showed that each durian accession has a unique DNA fingerprint. PCR-RFLP produced varied restriction fragments with the size of 150 to 850 bp (Figure 4). There are a wide number of fragments produced in each accession ranged from 1 to 8. The PCR-RFLP characterization results indicated that some durian which has different morphological characters proved to have the same molecular character, as indicated by the same characteristic of DNA fingerprints. The phylogenetic tree was generated based on the cutting results. The tree shows the level of relationship between species based on ribosomal DNA, ITS markers. Similarity coefficient was in the range of 22%-100%. Based on phylogenetic tree analysis, it proved that the 20 durian accessions were divided into four clusters. For example, durian Wijo has 1.0 similarity coefficient with durian Ontoseno. Durian Wijo and Ontoseno are closely related to durian Sadewo, Raja, and Ngastino.

Additionally, durian Indrajid, Bolodewo, Arimbi, Dewisinto, and Cokro were proved to

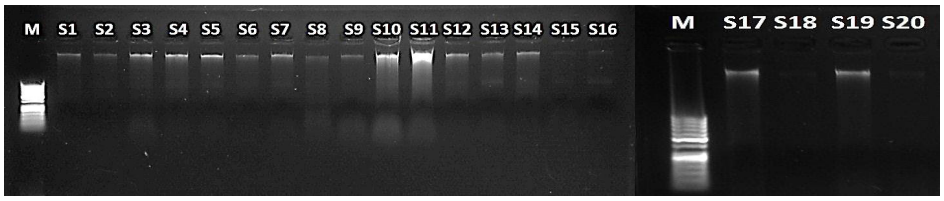

Figure 2. Durian DNA on a 0.8% agarose gel M: DNA ladder 50 bp; S1-S20: genomic DNA sample number 1-20, M: 50 bp DNA ladder.

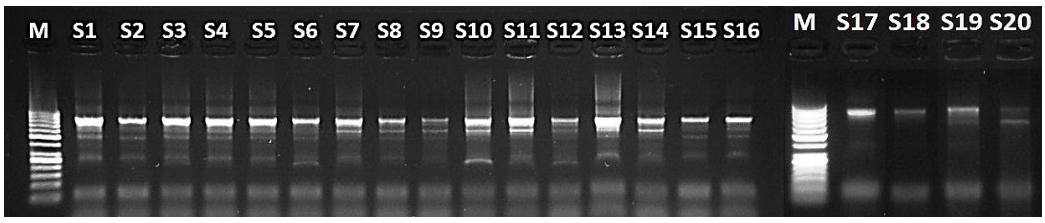

Figure 3. PCR products on a 1.2% agarose gel M: DNA ladder 100 bp; S1-S20: the results of the ITS region PCR samples 1-10

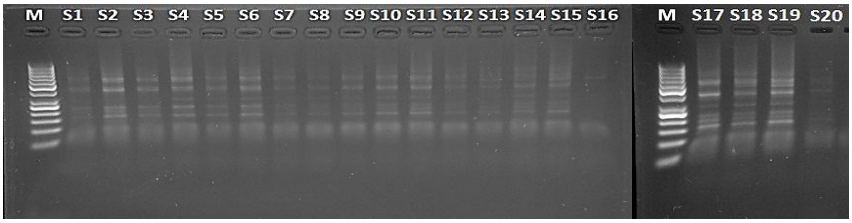

Figure 4. The results of PCR products digestion using Bsp*143I* on 2% agarose gel samples 1-20

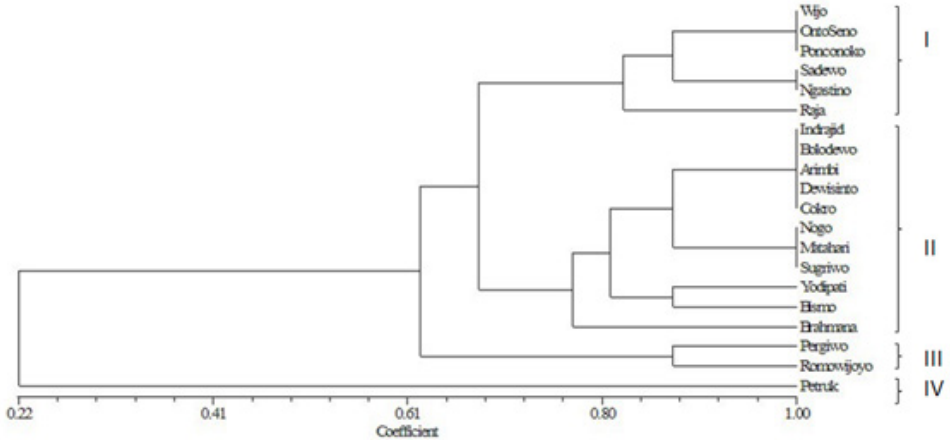

Figure 5. Phylogenetic tree of 20 local durian accessions based on the characteristics of PCR-RFLP on ribosomal ITS DNA.

be the same durian. Based on dendogram, durian Petruk was included in the main cluster durian which was different from the other 19 durian accessionsrevealed that the durian Petruk has a low genetic similarity, suggesting an evolutionary process that occurs in the ITS ribosomal DNA in these species that occur independently. However, based on genetic distance calculation, it can be concluded that genetically, the 20 durian species were not sufficiently varied.

Internal transcribed spacer (ITS) of ribosomal DNA is the region that separates the 5.8S subunit of the 18S and 26S. Variations in the ITS region is very high and the analysis of variations in DNA sequences can be used to access the relationship at a lower taxon-level (Santoso, 2005). Nwakanma et al. (2003) conducted the analysis of PCR-RFLP on ribosomal ITS DNA of *Musa sp*. to determine the variation of the combination of genome A and genome B. Digestion with the enzyme *Rsa*I showed the typical fragment of 530 bp as well 350 bp and 180 bp fragments specific to the banana genomes. In the present study, there were no unique characteristic fragments (polymorphic) that can be used to distinguish the allele and genome markers. However, the DNA fragments formed only shows the fingerprints of the durian genome. Therefore, ITS markers cannot be used as a reference for determining the level of taxa under species. It requires a more accurate analysis using other molecular markers. Utilization of DNA markers can be divided into two types, i.e. based on DNA-DNA hybridization then cut with restriction enzymes, such as AFLP and RFLP and based on DNA amplification such as RAPD and Simple Sequence Repeat (SSR) (Yunus, 2004). The present study was a preliminary study; further analysis is required to determine the species name of each durian accession. In fact, the PCR-RFLP analysis database of durian species name can be used as a reference to determine the durian species name. However, there are only a few data of durian genome found in the gene bank. Further study on DNA sequencing and deeper characterization are necessary to distinguish the clear identity of durian species.

CONCLUSION

Results of the study showed that the genomic DNA of 20 durian accessions were successfully amplified using primers ITS LEU and ITS 4. Data showed that based on the morphological characters, 20 durian accessions are divided into four main clusters. However, based on the PCR-RFLP analysis, DNA fingerprints indicated that the region of the ribosomal ITS DNA does not have a high degree of variation. Therefore, the results cannot be applied as a specific marker for the lower level of taxa. However, this technique has been successfully demonstrated a cluster of 20 accessions division durian, thus, the PCR-RFLP analysis is a useful marker in phylogenetic analysis durian species. This discovery is preliminary data that needs to be supported by further research using other molecular markers to obtain more accurate data. The clear identity of durian species can help the management of breeding systems by farmers to obtain superior durian that can dominate the domestic market and supply the export demands.

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