Biosaintifika 10 (2) (2018) 252-259

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Characterization of Rambutan Cultivars (*Nephelium lappaceum*) Based on Leaf Morphological and Genetic Markers

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DOI: http://dx.doi.org/10.15294/biosaintifika.v10i1.122218

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History Article

Received 30 November 2017
Approved 3 March 2018
Published 30 August 2018

Keywords

Dendrogram; ISSR; UPGMA

Abstract

Rambutan (*Nephelium lappaceum*) is an economically important plant which is native to Indonesia and Malaysia. The diversity of rambutan in Indonesia is abundance especially in Kalimantan where the wild relatives still grow naturally. Rambutan cultivars are usually differed from each other based on fruit morphological characters. However, rambutan tree begins to fruiting for the first time in 3-4 years. Therefore, another character is needed to characterize each cultivar in a short period. The objectives of this study were to distinguish rambutan cultivars using leaf morphological and Inter-Simple Sequence Repeat (ISSR). As many as 30 rambutan cultivars collected from Cipaku Orchard and Mekar Sari Park were observed for their morphological and ISSR characters. Six characters of were surveyed for leaf morphological character. For the genetic character, 6 out of 31 ISSR primers were assessed which resulted in 58 polymorphic bands (87%). As a result, leaf morphological characters overlapped among cultivars causing difficulties distinguishing each cultivar. ISSR marker, three major clusters have been identified according to UPGMA method. Index similarity among rambutan accessions from ISSR data ranged from 48-93%. As a conclusion, ISSR marker could be potentially applied rambutan cultivars characterization.

How to Cite

Manggabarani, A. M., Chikmawati, T., & Hartana, A. (2018). Characterization of Rambutan Cultivars (*Nephelium lappaceum* L.) Based on Leaf Morphological and Genetic Markers. *Biosaintifika: Journal of Biology & Biology Education*, 10(2), 252-259.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Rambutan (*Nephelium lappaceum* L.) is one of tropical fruit that has a high potential value. It belongs to *Sapindaceae* which originated from South East Asia primarily in Indonesia and Malaysia (Leenhouts, 1986). Indonesia, as one of rambutan origin, preserves many rambutan cultivars as well as its wild relatives which can be found in many regions especially in Kalimantan. The species is most commonly cross-pollinated plant allowing its high genetic variation in nature (Tindall *et al*. 1994).

The cultivation of rambutan as one of the fruit-producing plant has been widely undergone to generate superior rambutan plant with the desirable trait. Based on its value, Rambutan variation was divided into two types, the popular type and the local type. There are many rambutan cultivars found in Indonesia which is promising and interesting for the consumer such as Rapiah, Binjai, and Garuda. Other local cultivars are most commonly used for rootstocks such as Sinyonya and Sitangkue. Generally, every cultivar has specifically fruit characters. Its fruit is varied in many characters such as its skin color of ripe fruit, flesh texture, flesh flavor, spintern density (Kuswandi *et al*. 2014). Nevertheless, the plant takes more than a year to produce fruits allowing the needs to find another desirable character in differentiating every cultivar.

Several studies had been done to determine character for differentiating among rambutan. Compared to fruit characters, leaves of rambutan are highly varied which are potential to be studied as a cultivar marker. Several leaf characters of rambutan had been assessed as a specific marker, yet the specific character has not been obtained (Barreto *et al*. 2015). In contrast to morphological character, the molecular character has been used immensely as a marker in differing cultivars as well as detecting genetic variability in many intraspecies levels (Boczkowska & Tarczyk 2013). Wild relatives of rambutan found in Kalimantan had been examined using Inter-Simple Sequence Repeat (ISSR) marker which showed high similarity between the relatives (Napitu et al. 2016). In addition, genetic variability among rambutan accessions from Malaysia had also been studied using Random Amplified Polymorphic DNA (RAPD) technique (Chew *et al*. 2005). However, RAPD technique produce lacks DNA fragment reproducibility due to its low annealing temperature. Meanwhile, Inter-Simple Sequence Repeat (ISSR) is a molecular marker amplifying DNA sequence region between two identical microsatellites which generates a lot of DNA loci. It gives a high reproducibility DNA result which applied to detect genetic variation in lower taxa (Zietkiewicz *et al.* 1994).

The purpose of the present study was to characterize rambutan cultivars using leaf morphological and genetic character using ISSR marker. This study may be useful as the first step in increasing economic value of Rambutan which is still lower than that of other popular tropical fruit such as banana and mango as well as identifying potential genotype from local races for future development.

METHODS

Plant materials used in this study were 30 rambutan cultivars collecting from Mekar Sari Park (MSP) and Cipaku Nursery (CN) Bogor (Table 1). The observation was conducted in Plant Genetic and Physiology and also Ecology and Plant Resource Laboratoriums, Biology Department, Faculty of Mathematics and Natural Science, Bogor Agricultural University.

Leaf Morphological Observation

Twenty leaves of each rambutan cultivar were collected and observed for their leaf morphological characters. The leaves were collected from the front, back, left and right shoots. Rambutan leave is a pinnately compound leaf composed of 4-7 leaflets. The leaf morphological character observed was whole leaf shape, leaflets arrangement, leaflet number, leaflet size, leaflet tip shape, and leaflet base shape. Six characters were selected to be analyzed in differing each cultivar. The observed characters were based on rambutan descriptor (IPGRI 2003) and *Manual of Leaf Architecture* (Ash *et al*. 1999).

DNA Isolation, Amplification and Visualization

DNA from dried rambutan leaves were extracted according to *Cetyl Trimethyl Ammonium Bromide* (CTAB) protocol (Doyle 1991) with several modifications by Hariri (2017), Souza *et al*. (2012) and Riupassa (2016). The modification included the addition of Sorbitol buffer before CTAB buffer extraction to minimize the high mucus content of the leaves, and also the addition of repeated DNA purification process to separate polysaccharide and other secondary metabolite residues from the DNA samples. Six out of 31 ISSR primers (Degani *et al*. 2003, Hariri 2017, Riupassa 2016) were selected to produce the clearest and showed the highest polymorphic

Table 1. List of rambutan accessions collected from Mekar Sari Park and Cipaku Nursery

Accession code	Accession name	Collected from	Acces-sion code	Accession name	Collected from
A6B	Aceh 6B	CN	KMS	Kering Manis	CN
ADL	Aceh Gundul	CN	KR	Kerikil	MSP
AGDG	Aceh gendong	CN	NM	Narmada	MSP
AGLG	Aceh Gelong	MSP	PB	Pirba	CN
AGT	Aceh gendut	CN	PBN	Padang Bulan	CN
AKG	Aceh Kuning	CN	RP	Rapiah	MSP
AL	Aceh Lebak	MSP	SB	Sibulan	CN
AP	Aceh Plat	MSP	SC	Simacan	MSP
ASM	Asam Manis	MSP	SDK	Sindang Langka	MSP
ATG	Antalagi	CN	SKA	Sikoneng asam	MSP
ATM	Aceh tombong	CN	SKM	Sikoneng manis	MSP
BJ	Binjai	MSP	SKWL	SKWL	CN
GLB	Gula Batu	MSP	STG	Sitangkwe	CN
GR	Garuda	MSP	SY	Sinyonya	CN
KM	Kalimantan	CN	TG	Tangkue	CN

*CN = Cipaku Nursery; MSP = Mekar Sari Park

Table 2. ISSR primers for primer selection

Primer	DNA sequence	Primer	DNA sequence
Primer 1	$(GA)_9$ T	ISSR 4	$(GAG)_5$ AC
Primer 2	T $(GA)_9$	ISSR 5	$(GAG)_5$ AT
UBC 819	$(GT)_8$ A	PIET 7	$(GA)_9$ A
UBC 825	$(AC)_8$ T	PIET 8	$(GA)_9$ C
UBC 836	$(AG)_8$ YA	PIET 10	$(GT)_9$ T
UBC 841	$(GA)_8$ YC	ISSR 10	$(GA)_6$ CC
UBC 847	$(CA)_8$ RC	ISSR 15	$(GTG)_3$ GC
UBC 856	$(AC)_8$ YA	ISSR 18	$(GATA)_3$ GG
UBC 857	$(AC)_8$ YG	ISSR 23	$(GACA)_3$ CC
UBC 858	$(TG)_8$ RT	UBC 807	$(AG)_8$ T
N/ISSR 1	$(CA)_6$ AT	UBC 813	$(CT)_8$ T
N/ISSR 2	$(AG)_8$ TC	UBC 817	$(CA)_8$ A
N/ISSR 4	$(CT)_8$ GG	UBC 818	$(CA)_8$ G
N/ISSR 5	$(GA)_8$ TT	UBC 820	$(GT)_8$ C
ISSR 8	$(AC)_8$ TA	UBC 821	$(GT)_8$ T
ISSR 1	$(AGG)_5$		

Y = pyrimidine (C,T); R = purine (A,G)

DNA bands (Table 2). DNA amplification was performed using a total of 25 µL reaction mixture containing 2 µM of template DNA (20 ng/ µl), 1 µM of primers (10 ng/ µl), 12.5 µl of 2x PCR mix solution (*Gotag Green Mix*) and 9.5 µl of nuclease-free water (Promega, USA). PCR amplification was performed in a thermal cycler (ESCO Swift™ Maxi model SWT-MY-BLC-7, USA) following several steps which are an initial denaturation at 94°C for 3 minutes; 30 cycles of denaturing at 94°C for 1 minute, annealing at 45.0-51.6°C for 50 seconds, elongation at 72°C for 2 minutes; and a final elongation at 72°C for 10 minutes. PCR amplification products along with 100 bp and 1

kb DNA ladder were separated by electrophoresis in 1% agarose gel (1 x TBE) (stained with ethidium bromide) for 1 hour and 30 minutes at 80 volts. Electrophoresis results were visualized on UV *transilluminator* and documented using *Wise Capture* 1.0.0.1 application.

Data Analysis

The leaf morphological characters were not included for further analysis due to the number of characters used was too small. DNA band resulted from ISSR primer at the same location was scored as 1 and 0 coded for the presence and absence band respectively. Genetic similarity among accessions was evaluated based on *Simple Matching* (SM) similarity coefficient. The genetic relationship among rambutan cultivars was analyzed using *Unweighted Paired Group Method using Arithmetic Mean* algorithm (UPGMA). Data analysis was performed using *Numerical Taxonomy and Multivariate Analysis System for* PC (NTSys-PC) 2.1.1a software (Exeter Software, New York) (Rohlf 1998).

RESULT AND DISCUSSION

Variation of Leaf Morphology among Rambutan Accessions

Rambutan trees were varied in shape and size of their leaves (Figure 1). Rambutan leave is a pinnately compound leaf containing 4-7 leaflets. The shape of the observed leaflets was oval or obovate, the leaf tip and base were acute, obtuse, or rounded while the size ranged from 12-16 cm in length and 5-9 cm in width. The leaf variations may be used as informative characters for determination of rambutan cultivars. Meanwhile, whole leaf shape and leaflets arrangement characters were not included due to no significant difference found among cultivars.

The morphological character has been successfully used to identify genotypes (Krisnawati & Adie 2017), as well as cultivars (Suo *et al*. 2005). Based on leaf characters, several cultivars showed a specific group of leaf characters which can be used for rambutan cultivar marker. Instead of one specific character, there was a group of leaf characters for eight cultivars. Those cultivars (Table 3) included three popular cultivars such as Aceh Lebak, Rapiah, and Simacan. Other rambutan cultivars could not be distinguished using leaf morphological character. In general, the leaf morphological characters used in this method are not helping very much in discriminating the observed rambutan cultivars.

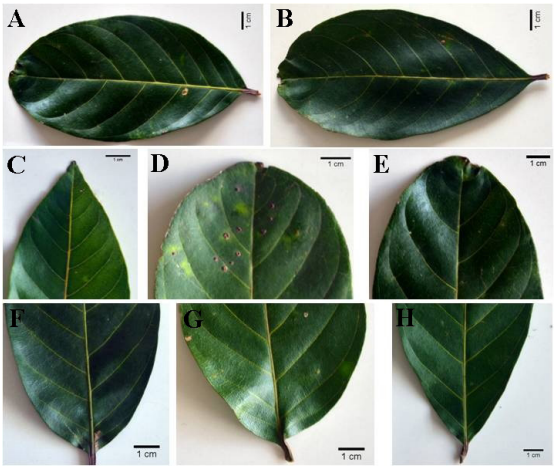

Figure 1. Rambutan leaflets variation. Leaflet shape (A) oval, (B) obovate; leaflet tip (C) acute, (D) rounded, (E) obtuse; leaflet base (F) obtuse, (G) rounded, (H) acute.

Genetic Variation among Rambutan Accessions based on ISSR Marker

As many as 31 primers were tested to amplify rambutan DNA. 6 primers were selected from those 31 primers which successfully showed scorable and clear multiband patterns in each cultivar (Figure 3). A total of 67 bands were amplified from 30 accessions using the six selected ISSR

Table 3. Accessions with specific leaf characters

Accession	Location	Specific leaf characters
Aceh Gelong	MSP	Oval, obtuse base, 12-14 cm length, 5-6 cm width, 4-5 leaflets
Aceh gendut	CN	Oval, rounded base, 15-16 cm length, 7-9 cm width, 6-7 leaflets
Aceh Kuning	MSP	Oval, rounded tip and base
Aceh Lebak	MSP	Oval, base acute, 15-16 cm length, 5-6 cm width, 6-7 leaflets
Asam Manis	MSP	Oval, acute tip, 12-14 cm length
Kalimantan	CN	Obovate, obtuse tip, 6-7 leaflets
Rapiah	MSP	Obovate, obtuse tip, 5-5 leaflets
Simacan	MSP	Obovate, acute tip

*CN = Cipaku Nursery; MSP = Mekar Sari Park

primers, of which 58 bands (87%) were polymorphic. The average band number per primer was 9. The highest polymorphic band was resulted from primer ISSR 23 while the lowest one was from primer UBC 807. Band sizes ranged from 200-2000 base pair (bp) (Table 4).

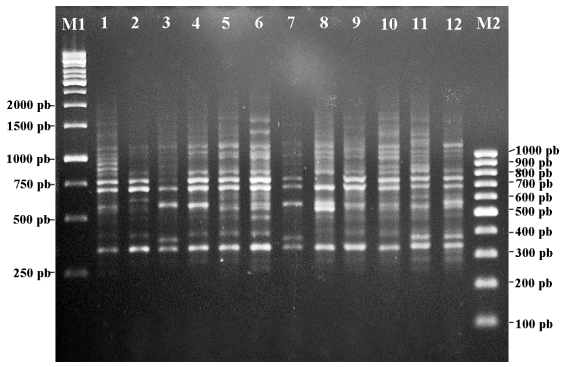

Figure 3. Polymorphic banding pattern resulted from ISSR 15 primer. M1=Marker 1 Kb, 1=Kerikil, 2=SKWL, 3=Aceh Tombong, 4=Aceh gendut, 5=Sinyonya, 6=Simacan, 7=Pirba, 8=Padang Bulan, 9=Binjai, 10=Aceh Gundul, 11=Gula Batu, 12=Narmada, M2=Marker 100 pb.

A dendrogram showing relationships among rambutan cultivars based on ISSR data was constructed using Simple Matching coefficients and UPGMA method. The genetic variation analysis using ISSR marker has been applied as an initial attempt on crop improvement such as on *Cucumis* spp. (Chaudhary *et al*. 2016), *Cicer arietinum* (Gautam *et al*. 2016), dan *Sorghum bicolor* (El-Amin & Hamza 2016). According to ISSR data, 30 cultivars were grouped into three major clusters (Figure 4). The index similarity among rambutan ranged from 48 to 93%. On 65% of similarity index, cluster I included 22 cultivars. Cluster II was comprised of 7 cultivars, while cluster III consisted of only one cultivar which is Pirba. The cluster III was separated from the other clusters on 48% of similarity index or 52% of genetic variability.

It can be shown that cluster I contained the most rambutan cultivars. Aside from sweet flavor of the flesh, it is preferable to consume rambutan fruit which the flesh is easily peeled off from its seed. Among 30 analyzed cultivars, 23 accessions are having the character, and 19 of them were grouped into cluster I.

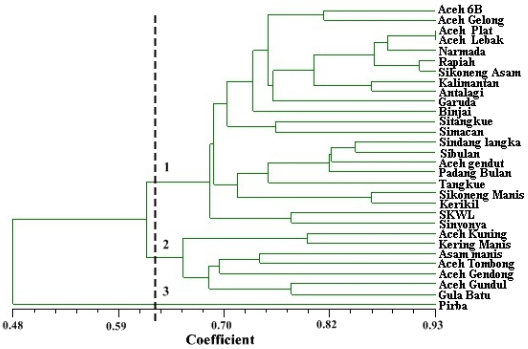

Figure 4. Dendrogram of 30 rambutan cultivars based on molecular character using UPGMA method

Among the rambutan cultivars, highly similarity index was found on several rambutan cultivars. For example, Aceh Plat and Aceh Lebak cultivars had the highest similarity index of 93%. Both cultivars shared similar morphological character of fruits easily detached from its seed. In addition, both of their leaflet were also oval, the tip of leaflet were obovate, while the base were acute. Beside its high similarity with Aceh Lebak, Aceh Plat also had a high similarity with Rapiah by having similarity index of 87%. By the morphological character, including the taste, and the hair density of Aceh Plat and Rapiah resemble to one another. Rapiah accession and Sikoneng Asam also showed similarity index of 90% which was high.

The highest dissimilarity among rambutan cultivars observed was shown by Pirba. Pirba was separated from other cultivars by 48% of simila-

Table 4. ISSR primers and DNA band profile from amplification result

Primer name	Primer DNA sequences (5'-3')	DNA fragment length (bp)	Polymorphic bands quantity	Total bands
ISSR 1	$(AGG)_5$	450-1800	8	11
ISSR 5	$(GAG)_5$ AT	450-1400	9	9
ISSR 10	$(GA)_6$ CC	270-2000	12	13
ISSR 15	$(GTG)_3$ GC	330-1200	9	11
ISSR 23	$(GACA)_3$ CC	280-1400	13	13
UBC 807	$(CA)_8$ A	200-1200	7	10
Total			58	67

rity index, or 52% of the genetic distance. In agreement with the clustering result, Pirba has the shortest panicle length compare to other rambutan cultivars (Ishaq *et al*. 2015). Pirba accessions have a superior fruit character such as sweet flesh flavor, hard and dry flesh texture, as well as the flesh easily detached from its seed. Therefore, this cultivar is potential to be improved in the future. As a result, only one

There are specific DNA banding patterns observed from several accessions using ISSR primers. Specific DNA banding patterns found in Pirba using ISSR 1, and UBC 807 primer (Figure 5) while Asam Manis and Aceh Gendong showed specific DNA banding pattern using ISSR 10 primer (Figure 6). Those specific patterns might be applied as a specific marker for rambutan cultivar determination. However, more primers as well as more technique are required to reveal more specific DNA banding pattern for every rambutan cultivar.

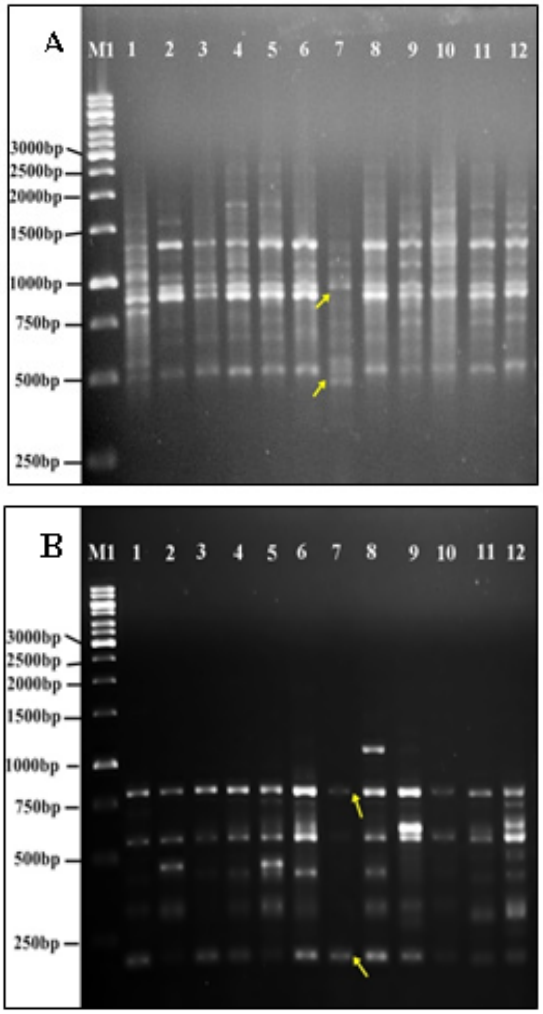

Figure 5. DNA genome amplification results using A) ISSR 1, and B) UBC 807 primers. M1=Marker 1 Kb, 1=Kerikil, 2=SKWL, 3=Aceh Tombong, 4=Aceh gendut, 5=Sinyonya, 6=Simacan, 7=Pirba, 8=Padang Bulan, 9=Binjai, 10=Aceh Gundul, 11=Gula Batu, 12=Narmada. The arrows in A and B show specific band for Pirba.

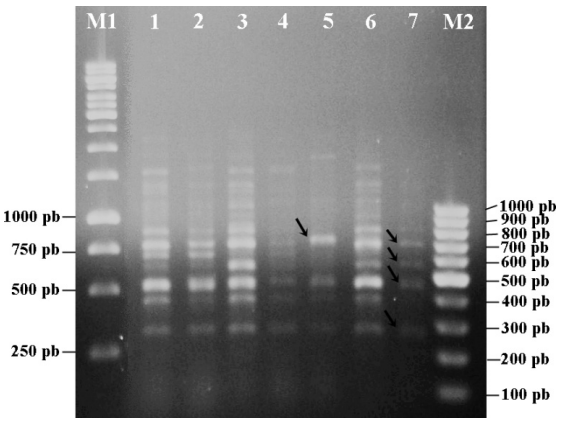

Figure 6. DNA genome amplification results using ISSR 10 primer. M1=Marker 1 Kb, 1=Sibulan, 2=Tangkue, 3=Sitangkue, 4=Kering Manis, 5=Asam Manis, 6=Antalagi, 7=Aceh Gendong. The arrows in the 5th and 7th column show specific band for Asam Manis and Aceh Gendong respectively.

The genetic variability among rambutan accession was successfully detected using ISSR primers. Rambutan is a cross-pollinated plant causing high genetic variability. Consequently, the propagation is rarely done by seed which will result in new undesirable character. Generally, popular rambutan cultivar is originated from local rambutan which is lack of the information about their parents. This information might reveal the relationship among rambutan accessions for the further breeding program. ISSR application for detecting genetic variability has been widely used for the initial of the breeding program on several species including *Cucumis* spp. (Chaudhari *et al*. 2016), *Cicer arietinum* (Gautam *et al*. 2016), and *Sorghum bicolor* (El-Amin & Hamza 2016).The rambutan cultivar characterization according to the result of leaves character was difficult to be distinguished clearly. Although several characters of leaves differed from the other, there was possibility to find those characters in other cultivar due to its high overlapped. In this research, molecular approach to support the morphological character has been analyzed to clearly distinguish each cultivar. However, the result also could not reveal the relationship between rambutan cultivar obviously. The overlapping character among cultivars along with the highly plasticity of leaf

morphological character resulting on the difficulties to make obvious border among cultivars. As stated by Leenhouts (1986), rambutan had a high variation and overlapped morphological characters. Similar case were also found in the characterization of mango cultivar (Fitmawati & Hartana 2010). The result of classification of mango cultivar based on morphological character were different from molecular classification by using RAPD. Moreover, the overlapping character and some of transition form were found in mango cultivar which causing it more difficult to be distinguished to one another.

CONCLUSIONS

Leaf morphology among rambutan cultivars was varied in leaflet number per petiole, the shape of leaflet lamina, tip, base, as well as the leaflet size. As many as eight cultivars showed a specific group of leaflet morphological character. Polymorphic DNA fragment was revealed by 6 ISSR primers, ISSR 1, ISSR 5, ISSR 10, ISSR 15, ISSR 23, and UBC 807. Specific DNA banding patterns were found in Pirba using ISSR 1, and UBC 807; Asam Manis using ISSR 10; and Aceh Gendong using ISSR 10.

ACKNOWLEDGEMENTS

The authors thank to Cipaku Nursery Bogor and Mekar Sari Park Bogor for supplying leaf material of rambutan accessions, and Directorate General of Higher Education 2014–2015 for the financial support.

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