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Morphology and Molecular Identification of Local Cultivars of Pisang Raja (*Musa* spp.) from Yogyakarta, Central Java and East Java, Indonesia

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ABSTRACT

Pisang Raja is well-known banana cultivars in Indonesia. Particularly in Java, there are not less than 40 local cultivars of Pisang Raja were recorded which needs to be clearly classified and identified. Genomic group identifications were conducted both morphology and molecular to thirteen local cultivars of Pisang Raja originated from Yogyakarta, Central Java and East Java collection of Purwodadi Botanic Garden – Indonesian Institute of Sciences. Morphological identification was using taxonomic scoring of fifteen dianogstic characters between *M. acuminata* and *M. balbisiana* wild species as their ancestral parents, whereas molecular identification was using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism/PCR-RFLP of Internal Transcribed Spacer/ITS method. Result showed that genomic group of 13 Pisang Raja local cultivars based on morphology consists of 8 AAB, 3 ABB, and 2 AAA; whereas molecular result consists of 4 AAB, 3 ABB, and 6 AAA. About 4 out of 8 banana cultivars which identified as AAB morphologically were revealed as AAA molecularly. Molecular approach using PCR-RFLP of ITS to identify genomic group of Pisang Raja local cultivars provide more objective, consistent and reliable result, also less time-consuming than morphology. Nonetheles morphological description of bananas is still necessary to conduct as part of a longer-term systematic evaluation and selection basis for further utilization and development. Genomic group result of Pisang Raja local cultivars examined are as follows: Raja Bandung (ABB), Raja Siem (ABB), Raja Prentel (ABB), Raja Gintung (AAB), Raja Temen (AAB), Raja Lingi (AAB), Raja Marto (AAB), Raja Kenanga (AAA), Raja Ketan (AAA), Raja Nangka (AAA), Raja Pendek (AAA), Raja Talun (AAA) and Raja Warangan (AAA).

Key words: genomic group, identification, ITS, morphology, molecular, PCR-RFLP, Pisang Raja

INTRODUCTION

Pisang Raja is indigenous and well-known banana cultivar in Indonesia and Malaysia. It is also present in the Philippines, Thailand and Papua New Guinea with their own local names (Espino *et al.* 1992; Lim 2012). It is characterized by its large fruit, thick coarse skin, orange; flesh creamy-orange, coarse texture, sweet taste; bunches bear 6-8 hands (Espino *et al.* 1992). Particularly in Java; there are many local cultivars of Pisang Raja, not less than 40 local cultivars of Pisang Raja were recorded from some previous inventory and diversity studies of bananas in Java by Jumari & Pudjoarinto (2000); Ekasari *et al.*, 2012; Ayuningtyas (2013); Indraswari (2014); Firdausi *et al.* (2015); Hapsari *et al.* (2017).

Pisang Raja plays important roles in Javanese cultural life, therefore become highly prized in the market. It is one among the obligatory components of offerings and decorations in the ritual ceremonial activities. Raja means king, symbolising hopes for the happy, successful prosperity of kings and to become a wise and righteous person. Pisang Raja used for rituals are limited to certain local cultivars *i.e.* Pisang Raja Sajen, Raja Temen and Raja Pulut (Hapsari *et al.* 2017). Fruit of Pisang Raja cultivars can be consumed both as dessert and processed, or considered as dual purpose banana. Banana fruits are nutritious food recommended for people at all ages, especially for baby, also diet food for adults but consumption must be limited for diabetic and kidney problem patients (Hapsari & Lestari, 2016). The carbohydrate levels of some Pisang Raja cultivars are following; Pisang Raja 28.95 %, Raja Siem 23.66 %, Raja Sere 23.97 %, Raja Pakuwan 20.49 %, Raja Nangka 27.94 % (Heyne, 1987) and Raja Bandung 31.13 % (Hapsari & Lestari, 2016)

In taxonomic view, Pisang Raja is a hybrid species and should correctly be written as *Musa* x *paradisiaca* L. or as *Musa acuminata* x *Musa balbisiana* (AAB group) since it is a triploid hybrid with two

genomic sets contributed by *M. acuminata* Colla (A genome) and one by *M. balbisiana* Colla (B genome). According to morphological characteristic using taxonomic scoring, Pisang Raja supposed to have intermediate characters of both ancestral parents (using 1-5 degree diagnostic) with score 26-46 (Simmonds & Shepherd, 1955; Simmonds 1959; Espino *et al.* 1992). However, morphological characteristics of the high diversity of Pisang Raja local cultivars in Java doesn't always resemble *Musa* AAB group. Therefore, it needs to be clearly classified and identified.

Identification and characterization using morphological attributes (whole-plant including floral and fruit) are found difficult, subjective and time consuming; however, it still important to perform. As a more reliable and fast alternative, various molecular techniques also have been used to study the genetic diversity and taxonomy of cultivated bananas to confirm the previous morphological identification. Some molecular techniques have been extensively used in identification and banana genetic analysis such as Amplified Fragment Length Polymorphism/AFLP (Wong *et al.*, 2002), Polymerase Chain Reaction-Restriction Fragment Length Polymorphism/PCR-RFLP (Nwakanma *et al.*, 2003; Ekasari *et al.*, 2012; Hapsari *et al.*, 2015), Random Amplified Polymorphic DNA/RAPD (Pillay *et al.* 2000; Sukartini, 2008, Poerba & Ahmad, 2010), DNA barcoding using some markers (Liu *et al.*, 2010; Hrˇibova´ *et al.*, 2011; Li *et al.*, 2013; Hapsari, 2015), microsatellites (Retnoningsih 2009; Windarti *et al.*, 2009; Lu *et al.*, 2011; de Jesus *et al.*, 2013; Poerba & Ahmad, 2013), etc.

The objective of this study is to identify the genomic group of 13 different local cultivars of Pisang Raja originated from Yogyakarta, Central Java and East Java using morphology and molecular assessed by PCR-RFLP of the Internal Transcribed Spacer/ITS; also study its phenetic and genetic relationship. PCR-RFLP of ITS is effective and more economically molecular technique to identify the genomic group on bananas cultivars than other techniques (Nwakanma *et al.*, 2003; Ekasari *et al.*, 2012); although it was not able to distinguish individual's homozygous diploid and triploid (Hapsari *et al.*, 2015). A proper and correct classification and characterization of local banana cultivars is important as basis information for further utilization and development, evaluation and conservation management, also for further banana breeding programmes.

MATERIALS AND METHODS

Plant materials

Plant materials examined in this study were thirteen local cultivars of Pisang Raja originated from Yogyakarta, Central Java and East Java collection of Purwodadi Botanic Garden-Indonesian Institute of Sciences (Table 1). Genomic group identifications were conducted both morphology and molecular. Plant part used for molecular analysis was fresh young cigar leaf (furled leaf) tissues of each cultivar.

Table 1. Plant materials Pisang Raja local cultivars examined in this study

No	Cultivar name	Vak location	Registration number	Locality
1	Raja Lingi	XXIV.E. 14-abc	P19760188	Yogyakarta
2	Raja Warangan	XXIV.D.48	P1994058	Yogyakarta
3	Raja Bandung	XXIV.D. 69-ab	P19940525	Yogyakarta
4	Raja Gintung	XXIV.D.88a	P19940533	Yogyakarta
5	Raja Siem	XXIV.D. 23-ab	P1975062	Prembun, Kebumen, Central Java
6	Raja Marto	XXIV.D. 57-a	Not available	Temanggung, Central Java
7	Raja Ketan	XXIV.D. 22-ab	P19760732	Siman, Ponorogo, East Java
8	Raja Nangka	XXIV.E. 7-bc	P19810567	Purwodadi, Pasuruan, East Java
9	Raja Kenanga	XXIV.E. 24-a	P19810568	Purwodadi, Pasuruan, East Java

No	Cultivar name	Vak location	Registration number	Locality
10	Raja Prentel	XXIV.E. 2-bc	P19940520	Nongkojajar, Pasuruan, East Java
11	Raja Pendek	XXIV.E.70	P2012030021	Pajarakan, Probolinggo, East Java
12	Raja Temen	At Nursery	P2012030017	Pasrepan, Pasuruan, East Java
13	Raja Talun	XXIV.D. 20b	P1972054	Purwodadi, Pasuruan, East Java

Morphological method

Taxonomic scoring for bananas method designed by Simmonds & Shepherd (1955) were conducted to identify the genomic group of Pisang Raja local cultivars. By using scoring method of fifteen characters, each of characters were scored depends on the diagnostic differences between *M. acuminata* and *M. balbisiana* wild species as their ancestral parents (Table 2). For each character in which the cultivar agreed with wild *M. acuminata* the score of 1 was given; for each character in which the cultivar agreed with *M. balbisiana* the score of 5 was given; and intermediate expressions of the character were assigned score of 2, 3 or 4 according to its intensity.

Identification of genomic group was then determined by its respective total score range. The scoring technique provides for a range of 15 (15 x 1) for wild *M. acuminata* and 75 (15 x 5) for wild *M. balbisiana* species. *M. acuminata* cultivars should have total scores of 15 to 25 while *M. balbisiana* cultivars ranged 70 to 75. The hybrid cultivars (*M. acuminata x M. balbisiana*) are expected to score between 26 to 69 points; it comprises of AAB (26-46), AB/AABB (47-49), ABB (59-63), and ABBB (67-69) (Simmonds & Shepherd, 1955; Silayoi & Chomchalow, 1987).

Molecular method

Molecular analysis was conducted at Laboratory of Plant Physiology, Tissue Culture & Microtechnique, Biology Department, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia. Whole genome DNA from leaf tissues was isolated using Promega Wizard® Genomic DNA Purification Kit, Wisconsin, USA. Protocol of DNA isolation was conducted following its manufacturer's instructions for plants. Amplifications of ITS region were using primer pairs of ITSL (5'-TCG TAA CAA GGT AGG CGT TTC TG-3 ') as a forward primer and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') as a reverse primer (White *et al.*, 1990; Hsiao *et al.*, 1994; Nwakanma *et al.*, 2003). The amplifications were performed at a volume of 15 µl consisting of 1,5 µl 25 ng DNA sample, 1,5 µl of 10 pmol forward and reverse primers, 1,5 µl of nuclease-free water and 7,5 µl DreamTaq Green PCR Master Mix (2x) from Thermo Scientific, California, USA. PCR thermal cycle protocol consisted of 1x initial denaturation (95 °C for 3 minutes); followed by 25x of denaturation (95 °C for 30 seconds), annealing (53 ºC for30 seconds), and extension (72 ºC for 30 seconds); and final extension (72 ºC for 7 minutes). Successful amplifications were confirmed by electrophoresis separation on 1,5% agarose gels, stained with 1 µg/ml of *Ethidium bromide* in TBE buffer, then visualised under UV light. DNA ladder Gene-ruler 100 bp (Thermo Scientific, California, USA) were used to estimate the sizes of amplified DNA fragments.

PCR products were then digested using *RsaI* restriction endonuclease enzyme (Thermo Scientific, California, USA). The digestion protocol consists of 10 µl PCR product, 18 µl nuclease-free water, 2 µl 10x Buffer Tango and 2 µl *Rsa*I. Incubation was conducted at 37 °C water bath for overnight (± 12 to 16 hours). The digested DNA fragments were confirmed using electrophoresis separation on 2% agarose gels, stained with 1 µg/ml of Etbr in TBE buffer, then visualised under UV light. DNA ladder Gene-ruler 50 bp (Thermo Scientific, California, USA) were used to estimate the sizes of digested DNA fragments.

Table 2. Characters used in banana classification through taxonomic scorecard Simmonds & Shepherd (1955)

No.	Character	*Musa acuminata* (score 1)	*Musa balbisiana* (score 5)
1	Pseudostem color	More or less heavily marked with brown or black blotches	Blotches slight or absent
2	Petiolar canal	Margin erect or spreading, with scarious wings below, not clasping pseudostem	Margin inclosed, not winged below, clasping pseudostem
3	Peduncle	Usually downy or hairy	Glabrous
4	Pedicels	Short	Long
5	Ovules	Two regular rows in each loculus	Four irregular rows in each loculus
6	Bract shoulder	Usually high (ratio < 0.28)	Usually low (ratio > 0.30)
7	Bract curling	Bract reflex and roll back after opening	Bracts lift but do not roll
8	Bract shape	Lanceolate or narrowly ovate, tapering sharply from the shoulder	Broadly ovate, not tapering sharply
9	Bract apex	Acute	Obtuse
10	Bract color	Red, dull purple or yellow outside; pink, dull purple or yellow inside	Distinctive brownish-purple outside; bright crimson inside
11	Color fading	Inside bract color fades to yellow towards the base	Inside bract color continuous to base
12	Bract scars	Prominent	Scarcely prominent
13	Free tepal of male flower	Variably corrugated below tip	Rarely corrugated
14	Male flower color	Creamy white	Variably flushed with pink
15	Stigma color	Orange or rich yellow	Cream, pale yellow or pale pink

Phenetic and genetic relationship reconstruction

Clustering analyses were performed to reconstruct relationship pattern of Pisang Raja local cultivars both morphology and molecular. Prior to analysis, the scoring data of morphology characters (1-5) was transformed to 0-1 matrices. Whilst for molecular data, the band pattern of digested DNA fragments was quantified into numeric data score (absent=0; present=1). Clustering analyses both phenetic and genetic were conducted using Paleontological Statistics (PAST) software version 1.94b; with unweighted, paired group algorithm and Jaccard similarity measure (Real & Vargas, 1996).

RESULTS AND DISCUSSIONS

Morphological characteristics

Taxonomic scoring result showed that morphological characteristics of thirteen Pisang Raja local cultivars examined were varying. It comprises of three genomic groups, comprises of 8 AAB, 3 ABB, and 2 AAA (Table 3).

Pisang Raja local cultivars which identified as *Musa* AAB group including Raja Gintung, Raja Ketan, Raja Lingi, Raja Marto, Raja Nangka, Raja Talun, Raja Temen and Raja Warangan (Table 3). General characteristic of *Musa* AAB group is having intermediate characters, mostly scored 2-4 of their both ancestral parents, with two rows in each loculus of ovules. Key character of each cultivar are following: Pisang Raja Gintung has 1-5 seeds per fruit (Fig. 1-A) while Pisang Raja Ketan has persistent flower relicts on the fruit (Fig. 1-B). Pisang Raja Lingi has persistent neutral/male flowers and presence of withered bracts on the rachis (Fig. 1-C). Pisang Raja Marto has degenerating early or absent male axis (Fig. 1-D), Pisang Raja Nangka is included as plantain type (Valmayor *et al*., 2000); the fruit is starchy so that requires some cooking to become palatable (Fig. 1-E). Pisang Raja Talun has spiral bunch shape; all fruit is attached to a unique crown coiled around the stalk (Fig. 1-F). Pisang Raja Temen (temen=true) means the true Pisang Raja; it bears bunches with 6-10 hands with flesh creamy-orange and sweet taste (Fig. 1-G). It is popular cultivar mostly used for obligatory requirement in traditional rituals of Javanese culture (Hapsari *et al.* 2017). Pisang Raja Warangan has sharp curved and slightly ridged fruit shape (Fig. 1-H).

Pisang Raja Bandung, Raja Prentel and Raja Siem were identified as *Musa* ABB group (Table 3). General characteristic of *Musa* ABB group is close related to *M. balbisiana*, mostly scored 3-5, with four rows in each loculus of ovules and the fruits are mostly seeded 3-10 seeds per fruit. Key character of each cultivar are following: Pisang Raja Bandung has compact bunch and very waxy fruits (Fig. 2-A), Pisang Raja Sri has pseudostem height less than 2 m, small and slightly ridged fingers (Fig. 2-B), and Pisang Raja Siem has fruit with pointed shape and persistent style (Fig. 2-C).

Raja Kenanga and Raja Pendek were identified as *Musa* AAA group (Table 3). General characteristic of *Musa* AAA group is having characters close related to *M. acuminata*, mostly scored 1-2, with two rows in each loculus of ovules, curved fruit shape, sweet taste and aromatic fruits. Key character of each cultivar are following: Pisang Raja Kenanga has pseudostem height up to 3 m with green-yellow fruit at ripening (Fig. 2-D), whilst Pisang Raja Pendek has dwarf type plant appearance with yellow-green fruit at ripening (Fig. 2-E).

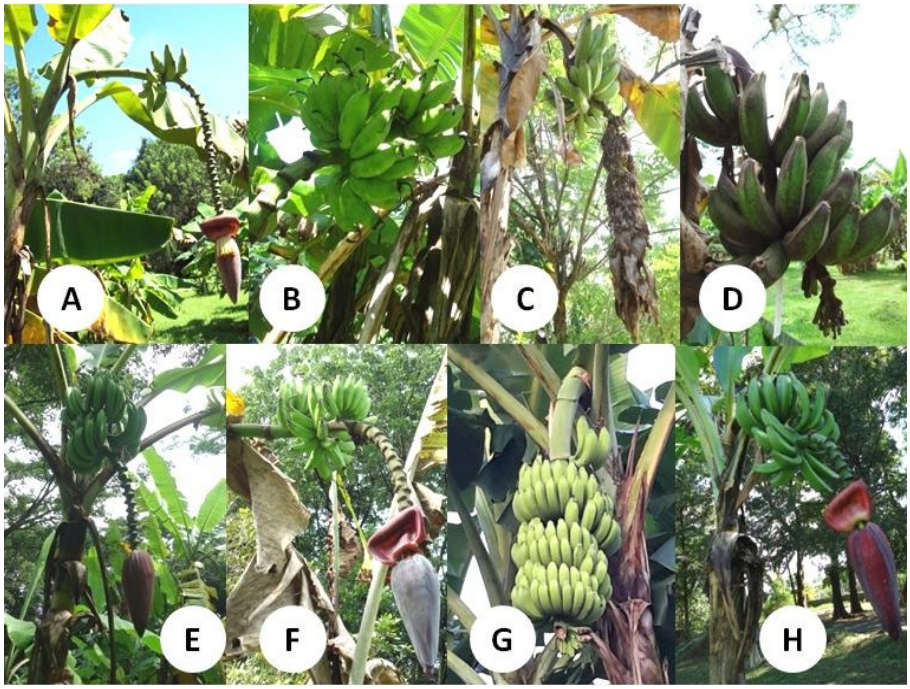

Figure 1. Plants and bunches appearance: A. Raja Gintung, B. Raja Ketan, C. Raja Lingi, D. Raja Marto, E. Raja Nangka, F. Raja Talun, G. Raja Temen and H. Raja Warangan

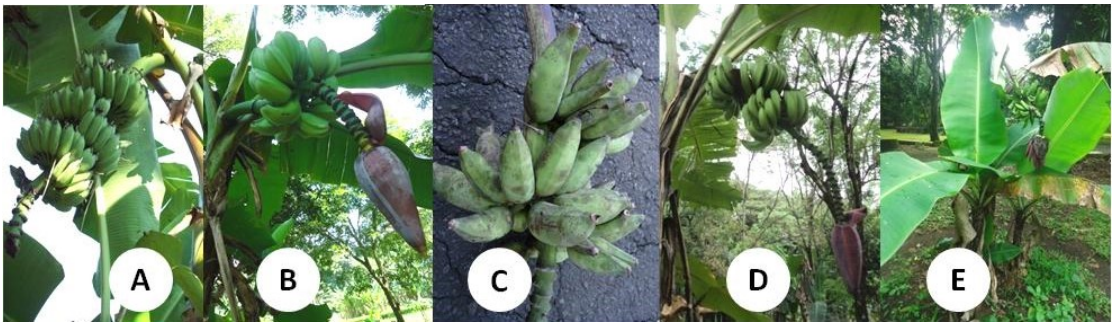

Figure 2. Plants and bunches appearance: A. Raja Bandung, B. Raja Prentel, C. Raja Siem, D. Raja Kenanga and E. Raja Pendek.

Table 3. Morphological characteristic scoring result of 13 Pisang Raja local cultivars

No	Character	Raja Bandung	Raja Prentel	Raja Siem	Raja Gintung	Raja Ketan	Raja Lingi	Raja Marto	Raja Nangka	Raja Talun	Raja Temen	Raja Warangan	Raja Kenanga	Raja Pendek
1	Pseudo-stem colour	4	4	4	3	1	2	2	1	2	2	2	1	1
2	Petiolar canal	5	4	5	2	4	4	3	2	3	3	2	2	2
3	Peduncle	4	4	2	3	3	2	3	2	3	3	2	1	1
4	Pedicel	5	4	5	4	2	3	3	2	3	4	3	1	1
5	Ovules	5	5	5	1	1	1	1	1	1	1	1	1	1
6	Bract shoulder	4	4	3	2	2	3	1	3	2	3	1	1	2
7	Bract curling	3	4	3	2	2	4	2	1	2	3	1	1	1
8	Bract shape	3	3	3	2	2	3	1	3	2	3	3	2	1
9	Bract apex	3	3	3	2	2	3	1	3	2	3	3	2	1
10	Bract colour	5	5	4	3	3	4	4	2	3	3	3	1	2
11	Bract colour fading	5	5	5	3	4	3	5	2	4	3	2	1	1
12	Bract scars	4	4	3	3	3	3	5	3	2	2	2	1	1
13	Free tepal of male flower	4	4	5	2	4	3	3	3	3	3	3	2	2
14	Male flower colour	5	3	5	3	2	2	3	3	3	3	3	2	1
15	Stigma colour	5	3	5	2	3	4	5	3	3	2	3	1	1
Total score		**64**	**59**	**60**	**37**	**38**	**44**	**42**	**34**	**38**	**41**	**34**	**20**	**19**
Genomic group		**ABB**	**ABB**	**ABB**	**AAB**	**AAB**	**AAB**	**AAB**	**AAB**	**AAB**	**AAB**	**AAB**	**AAA**	**AAA**

Molecular characteristic

Amplifications of ITS region to thirteen Pisang Raja local cultivars produced ±700 bp (Fig. 3-A) fragment that is equivalent in size to the ITS of most Angiosperms (Baldwin et al., 1995). The digestion of PCR products using *Rsa*I showed 530 bp and 120 bp fragment unique to the A genome and two fragments of 350 bp and 180 bp that were specific to the B genome. The band expression is quantitative based on its genomes contribution, banana cultivars which possessed two sets of 'B' genomes showed stronger band intensity at 350 bp and 180 bp than those with single 'B' genome (Fig. 3-B). This study results are in accordance to some previous studies by (Nwakanma *et al.*, 2003; Ekasari *et al.*, 2012; Hapsari *et al.*, 2015).

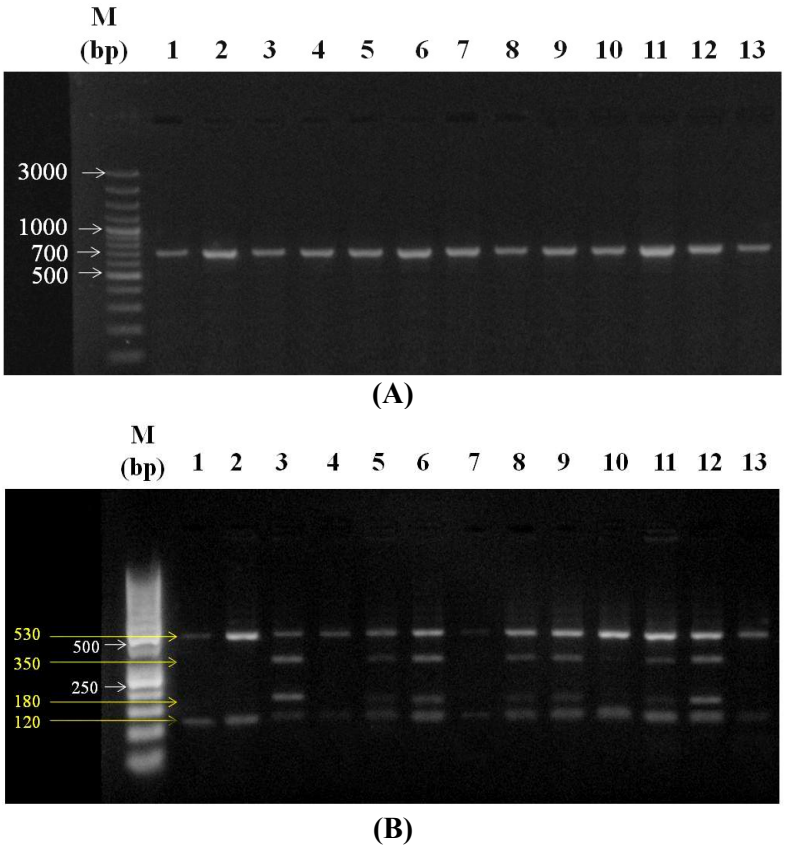

Figure 3. Visualization result on agarose gel: A. Amplification of ITS and B. Digestion of ITS PCR products using *Rsa*I. Remarks: 1. Raja Warangan, 2. Raja Kenanga, 3. Raja Prentel, 4. Raja Ketan, 5. Raja Marto, 6. Raja Siem, 7. Raja Pendek, 8. Raja Lingi, 9. Raja Temen, 10. Raja Nangka, 11. Raja Gintung, 12. Raja Bandung, and 13. Raja Talun.

Genomic group identification using PCR-RFLP of ITS to thirteen Pisang Raja local cultivars showed that it comprises of 3 ABB, 4 AAB, and 6 AAA. The finding of this study is about 4 out of 8 banana cultivars which identified as AAB morphologically were revealed as AAA molecularly *i.e.* Raja Ketan, Raja Nangka, Raja Talun, and Raja Warangan (Fig. 3-B). The final genomic group identification result were shown in Table 4.

Molecular approach using PCR-RFLP of ITS to identify genomic group of Pisang Raja local cultivars provide more objective result, consistent and reliable, also less time-consuming than morphology. PCR-RFLP method is simple and considerable less expensive with consistent compared to other molecular method. Nonetheles morphological description of bananas is still needed due to it is related with customer preference attributes of banana cultivars. Molecular method is needed to confirm the genomic constitution of banana cultivars for correct identification and classification.

Table 4. Differences of genomic group identification result using morphplogy and molecular

No.	Cultivar name	Genomic group		Species name (final result)
		Morphology	PCR-RFLP ITS	
1	Raja Bandung	ABB	ABB	*M. acuminata* x *M. balbisiana* (ABB)
2	Raja Prentel	ABB	ABB	*M. acuminata* x *M. balbisiana* (ABB)
3	Raja Siem	ABB	ABB	*M. acuminata* x *M. balbisiana* (ABB)
4	Raja Gintung	AAB	AAB	*M. acuminata* x *M. balbisiana* (AAB)

No.	Cultivar name	Genomic group		Species name (final result)
		Morphology	PCR-RFLP ITS	
5	Raja Ketan	AAB	AAA	*M. acuminata* (AAA)
6	Raja Lingi	AAB	AAB	*M. acuminata* x *M. balbisiana* (AAB)
7	Raja Marto	AAB	AAB	*M. acuminata* x *M. balbisiana* (AAB)
8	Raja Nangka	AAB	AAA	*M. acuminata* (AAA)
9	Raja Talun	AAB	AAA	*M. acuminata* (AAA)
10	Raja Temen	AAB	AAB	*M. acuminata* x *M. balbisiana* (AAB)
11	Raja Warangan	AAB	AAA	*M. acuminata* (AAA)
12	Raja Kenanga	AAA	AAA	*M. acuminata* (AAA)
13	Raja Pendek	AAA	AAA	*M. acuminata* (AAA)

Phenetic and genetic relationship of 13 Pisang Raja local cultivars

Dendogram relationships based on morphology (phenetic) and molecular (genetic) of thirteen Pisang Raja local cultivars were clustered in three groups following its genomic group but in different pattern (Fig. 4). Phenetic relationship showed that AAA group were closer related to AAB group (similarity 12%) than to ABB group (similarity 8%). The morphological characteristics of AAB and AAA groups are quite similar so that become problems in taxonomic scoring, therefore their phenetic relationship were become sister group. Among AAB group, it was separated into two sub-groups i.e. sub-group I (Raja Marto, Raja Gintung, Raja Talun and Raja Ketan), and sub-group II (Raja Lingi, Raja Temen, Raja Nangka and Raja Warangan). Whilst, among ABB group, Raja Bandung were closer related to Raja Siem with similarity of 42% (Fig. 4-A). Meanwhile, genetic relationship showed that ABB and AAB groups were closer related (similarity 50%) than AAA group (similarity 34%). In addition, genetic relationship showed 100% similarity within group, due to the band patterns which are very specific to each genomic group (Fig. 4-B).

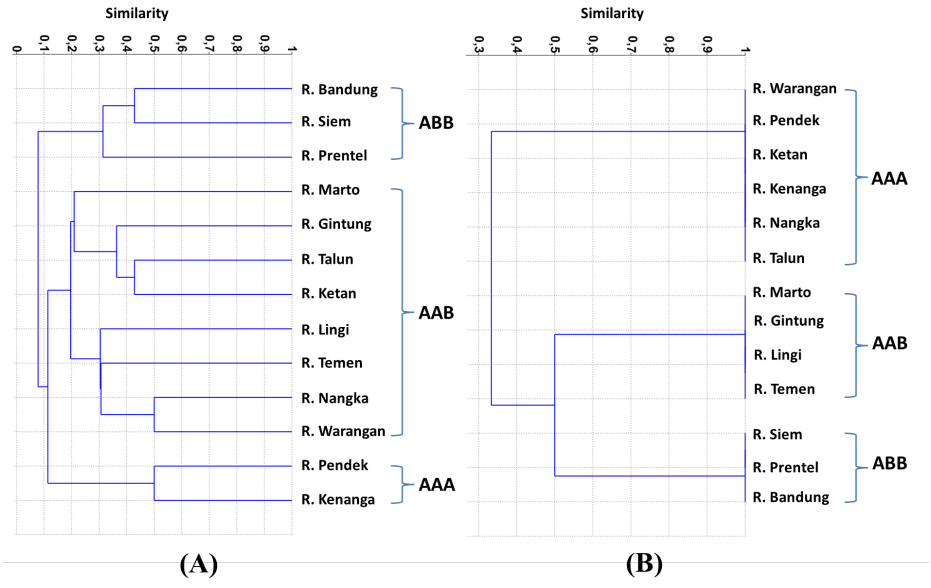

Figure 4. Dendogram phenetic (A) and genetic (B) relationship of thirteen Pisang Raja local cultivars

CONCLUSION

This study was clearly identified and classified the genomic group of thirteen Pisang Raja local cultivars. Genomic group result based on morphology was slightly different to molecular method, in which

about 4 out of 8 banana cultivars which identified as AAB morphologically were revealed as AAA molecularly *i.e.* Raja Ketan, Raja Nangka, Raja Talun, and Raja Warangan. Phenetic and genetic relationships were clustered in three groups following its genomic group. Dendogram of phenetic relationship showed that *Musa* AAA group was closer related to AAB group than to ABB group. Whilst, genetic relationship showed that *Musa* ABB were closer relatd to AAB group than AAA group.

Molecular approach using PCR-RFLP of ITS to identify genomic group of Pisang Raja local cultivars provide more objective, consistent and reliable result, also less time-consuming than morphology. However, both morphological description and molecular confirmation of genomic group of the rich local genetic variety of Indonesian banana cultivars is necessary to conduct as part of a longer-term systematic evaluation and selection basis for further utilization and development.

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