Assessment of Genetic Diversity among *Musa* Cultivars based on Sequence-Related Amplified Polymorphism Technique

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Abstract

Bananas are one of the world's most eatable fruits. Edible bananas belonging to the *Eumusa* section are believed to contain genomes from two wild diploid species, *Musa acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome). Almost cultivated bananas are diploid, triploid or tetraploid and classified into different genome groups according to several morphological characteristics. 'Kluai Namwa Mali-Ong' is the most suitable use as a raw material for the sun-dried banana production in Bangkratum District, Phitsanulok Province, Thailand. The varietal identification among hybrid bananas is difficult due to there is a small difference in morphological characters and local names of bananas based on fruit traits results in abundant synonyms of banana cultivars. Therefore, it is essential for choosing the correct banana cultivars for marketing and gaining understanding of banana breeding using biotechnology approaches. The study aimed to assess the genetic diversity among *Musa* (ABB group) 'Kluai Namwa' cultivars and two outgroup taxa (AA/AAA and BB groups) with DNA markers. Based on ten sequence-related amplified polymorphism (SRAP) markers, a total of 80 DNA bandings were scored, of which 74 were polymorphic (92.50%). Polymorphic information content (PIC) values ranged from 0.16 to 0.30 with an average 0.25. The bands size ranged from 100 to 1200 base pairs. An unweighted pair group method arithmetic average (UPGMA) analysis based on Dice coefficients demonstrated that the accessions had a similarity range from 0.42 to 1.00 with a mean of 0.86 which suggested extreme genetic diversity among *Musa* samples. However, the low genetic diversity was found among 12 *Musa* (ABB group) 'Kluai Namwa' samples. In the present study, dendrogram derived from ten SRAP markers showed that all samples could be categorized into two major groups (ABB-BB and AA/AAA genome groups). The results based on SRAP markers in this study could be useful for germplasm collection and banana breeding in the future.

Keywords: genetic diversity, banana, *Musa*, DNA marker, SRAP

Introduction

Bananas (*Musa* spp.) are one of the world's most eatable fruits. It originated from Southeast Asia, India to Polynesia (Simmonds, 1962). Edible bananas belonging to the family of Musaceae, *Eumusa* section, are believed to contain genomes from two wild diploid species, *Musa acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome). Almost cultivated bananas are diploid (AA, BB and AB), triploid (AAA, AAB, ABB and BBB) or tetraploid (ABBB, AABB and AAAB) and are classified into different genome groups based on 15 morphological characteristics according to Simmonds and Shepherd's genome-based system (Simmonds & Shepherd, 1955). The total scores of 15 important banana characters that show totally differences between *M. acuminata* and *M. balbisiana* range from 15 (AA or AAA genomes) to 75 (BB or BBB genomes) in genome categorization and determine the relative contribution of these two wild species to the genome composition of the banana cultivars. Among economically important bananas cultivated in Thailand, *Musa* (ABB group) 'Kluai Namwa' which is rich in nutrients, is widely grown in every provinces of

Thailand. Various types of Thai food are made from raw 'Kluai Namwa' varieties. One of several varieties, 'Kluai Namwa Mali-Ong', is the most suitable use as a raw material for the sun-dried banana production in Bangkratum District, Phitsanulok Province, Thailand. By using the 'Kluai Namwa Mali-Ong' variety as raw material in sun-dried banana process, its color and taste is optimal. However, the varietal identification among hybrid bananas is difficult due to there is a small difference in morphological characters. Also, local names of bananas based on fruit traits results in abundant synonyms of banana cultivars. Therefore, it is essential for choosing the correct banana cultivars for marketing and gaining understanding of banana breeding using biotechnology approaches like genetic markers.

In the past, genetic diversity in plants can be estimated mainly using morphological characteristics. Recently, the development of molecular markers provided access to the genetic variance reported in many plant species, which was benefic for genetic improvement and characterizing the germplasm (Mohan et al., 1997; Carvalho, Vitorino, de Souza, & Bessa, 2019). There are many types of molecular markers using in the genetic diversity studies of crop plants. Sequence-related amplified polymorphism (SRAP) technique is a tool for genetic diversity detection in the open reading frames (ORFs) of plant genomes and associated organisms (Li & Quiros, 2001). Based on two primer combination, the forward and reverse primers are 17 and 18 nucleotides long, respectively and contain three major elements: (1) filter sequences with no specific nucleotides at the 5' end, (2) core motif with four nucleotides long as CCGG for the forward primer and AATT for the reverse primer, and (3) three particular nucleotides long at the 3' end (Li & Quiros, 2001). Earlier studies, the SRAP technique has been reported to be an effective tool for the evaluation of genetic diversity in many studies of other crop plants including orchardgrass (Zeng, Zhang, Lan, & Yang, 2008), grape (Liu et al., 2012; Fan et al., 2015), pistachio (Guenni, Aouadi, Chatti, & Salhi-Hannachi, 2016), Chinese wild bermudagrass (Zheng, Xu, Liu, Zhao, & Liu, 2017), jojoba (*Simmondsia chinensis*) (Kumar, Heikrujam, Sharma, & Agrawal, 2019). In this study, SRAP technique was used to assess the genetic diversity among *Musa* (ABB Group) 'Kluai Namwa' cultivars and two outgroup taxa (AA/AAA and BB groups).

Methods and Materials

Plant materials and genomic DNA extraction

To assess the genetic diversity among *Musa* cultivars with DNA markers, fresh young leaves of 12 *Musa* cultivars including three different genome groups (AA/AAA, BB and ABB) were collected from banana plants at Plant Propagation Center No. 6, Muang Phitsanulok District, Phitsanulok Province (Table 1). Moreover, other four 'Kluai Namwa Mali-Ong' samples were collected from different provinces of Thailand including Chon Buri, Phetchaburi, Samut Songkhram and Suphan Buri Province. Then, the genomic DNA of 16 *Musa* samples was extracted from the young leaves using the BioFACT^{TM} Genomic DNA Prep Kit for Plant (BIOFACT Co., Ltd., Korea) according to the manufacturers guide. The quality and concentration of DNA were determined by a UV spectrophotometer (Microplate reader, synergy H1 BioTech, USA) at A_{260} nm, the A_{260}/A_{280} ratio, and by electrophoresis using 0.8% agarose gel with 1X TAE buffer, and stained with Safe DNA Dye (Hydragreen^{TM}, USA). The resuspended DNA was then diluted in sterile distilled water to 50 ng/μl concentration for use in amplification reactions.

Table 1 List of 16 *Musa* samples, scientific name/ hybrid name, genome group and place of sample collection used in this study.

No.	Cultivar name	Scientific name/ Hybrid name	Genome group (Suvittawat et al., 2014)	Place of sample collection (province)
1	'Kluai Hom Champa'	*Musa acuminata*	AA/AAA	Phitsanulok
2	'Kluai Hom Eisan'	*Musa acuminata*	AA/AAA	Phitsanulok
3	'Kluai Tani Dam'	*Musa balbisiana*	BB	Phitsanulok
4	'Kluai Tani 167 Buriram'	*Musa balbisiana*	BB	Phitsanulok
5	'Kluai Namwa Dam'	*Musa x paradisiaca*	ABB	Phitsanulok
6	'Kluai Namwa Tha Yang'	*Musa x paradisiaca*	ABB	Phitsanulok
7	'Kluai Namwa Phrarachthan'	*Musa x paradisiaca*	ABB	Phitsanulok
8	'Kluai Namwa Nuanchan'	*Musa x paradisiaca*	ABB	Phitsanulok
9	'Kluai Namwa Ubon'	*Musa x paradisiaca*	ABB	Phitsanulok
10	'Kluai Namwa Khieo'	*Musa x paradisiaca*	ABB	Phitsanulok
11	'Kluai Namwa Mali-Ong' (1)	*Musa x paradisiaca*	ABB	Phitsanulok
12	'Kluai Namwa Pakchong 50'	*Musa x paradisiaca*	ABB	Phitsanulok
13	'Kluai Namwa Mali-Ong' (2)	*Musa x paradisiaca*	ABB	Chon Buri
14	'Kluai Namwa Mali-Ong' (3)	*Musa x paradisiaca*	ABB	Phetchaburi
15	'Kluai Namwa Mali-Ong' (4)	*Musa x paradisiaca*	ABB	Samut Songkhram
16	'Kluai Namwa Mali-Ong' (5)	*Musa x paradisiaca*	ABB	Suphan Buri

SRAP-PCR amplification

SRAP primer combinations (Li & Quiros, 2001) were obtained from 4 forward and 7 reverse primers (Table 2). PCR reactions were performed in a total volume of 25 µl consisted of 2X MyTaq™ HS Mix (Bioline, USA) including *Taq* DNA polymerase, dNTP mix, gel loading dye, 5 µM SRAP primer each, and 100 ng of genomic DNA. Amplifications were carried out in a thermal cycler (Bio-Rad T100™, USA) with an initial denaturation at 94 °C for 3 min; 5 cycles of 3 steps: 1 min for denaturation at 94 °C, 1 min for annealing at 35 °C and 2 min for extension at 72 °C; followed by 35 cycles with an annealing temperature at 50 °C, and a final prolongation step of 5 min at 72 °C. PCR products were separated in a 1.5% agarose gel using 1X TAE buffer, and stained with Safe DNA Dye (Hydragreen™, USA).

Table 2 Forward and reverse SRAP primers used in this study (Li & Quiros, 2001).

Forward primer	Sequence (5' → 3')	Reverse primer	Sequence (5' → 3')
Me1	TGAGTCCAAACCGG**ATA**	Em1	GACTGCGTACGAATT**AAT**
Me4	TGAGTCCAAACCGG**ACC**	Em2	GACTGCGTACGAATT**TGC**
Me5	TGAGTCCAAACCGG**AAG**	Em3	GACTGCGTACGAATT**GAC**
Me6	TGAGTCCAAACCGG**TAA**	Em4	GACTGCGTACGAATT**TGA**
		Em6	GACTGCGTACGAATT**GCA**
		Em7	GACTGCGTACGAATT**CAA**
		Em8	GACTGCGTACGAATT**CTG**

Data analysis

Amplified SRAP amplicons were scored in a binary matrix as the presence (1) or absence (0) of each band for each cultivar. DNA bands that could not be easily detected were not scored. The total number of amplified bands, polymorphic bands, percentage of polymorphism (%) and average number of bands per primer were calculated. Polymorphic information content (PIC) valuation was calculated using the formula PIC = $1 - \sum p_i^2$, where p_i is the i^{th} allele frequency for individual p (Ott, 1991). An unweighted pair group method with arithmetic average (UPGMA) analysis based on the Dice similarity coefficients was constructed and bootstrap of phylogenetic trees was analyzed using a freeware program; FreeTree and TreeView (Hampl, Pavlíček, & Flegr, 2001).

Results

SRAP-PCR analysis

In this study, ten SRAP primer combinations were used to assess the genetic diversity among *Musa* (ABB group) 'Kluai Namwa' cultivars and two outgroups. A total number of generated DNA bands were 80, of which 74 bands (92.50%) were polymorphic among *Musa* samples (Table 3). Allele size ranged from 100 to 1200 base pairs (bp). A number of amplified bands per SRAP primer combinations varied from six to 13 with a mean of eight bands per marker. The PIC values varied from 0.16 (Me4/Em7 primer combination) to 0.30 (Me6/Em8 primer combination) with an average of 0.25. The SRAP profiles of 16 *Musa* samples generated by Me4/Em8 and Me6/Em7 primer combinations are shown in Figure 1.

Table 3 Polymorphism obtained from ten SRAP primer combinations among 16 *Musa* samples.

No.	SRAP Primer combination	Allele size range (bp)	Amplified bands			PIC value
			Total	Polymorphic	Polymorphism (%)	
1	Me1/Em6	250 - 800	9	9	100	0.25
2	Me4/Em7	250 - 1000	7	5	71	0.16
3	Me4/Em8	100 - 500	7	6	86	0.21
4	Me5/Em1	100 - 1200	13	12	92	0.28
5	Me5/Em8	100 - 1000	7	6	86	0.26
6	Me6/Em2	150 -1000	6	6	100	0.27
7	Me6/Em3	100 - 1200	10	9	90	0.27
8	Me6/Em4	100 - 1000	7	7	100	0.24
9	Me6/Em7	150 - 1000	7	7	100	0.24
10	Me6/Em8	200 - 1000	7	7	100	0.30
	Total		80	74	-	-
	Average		8.0	7.4	92.5	0.25

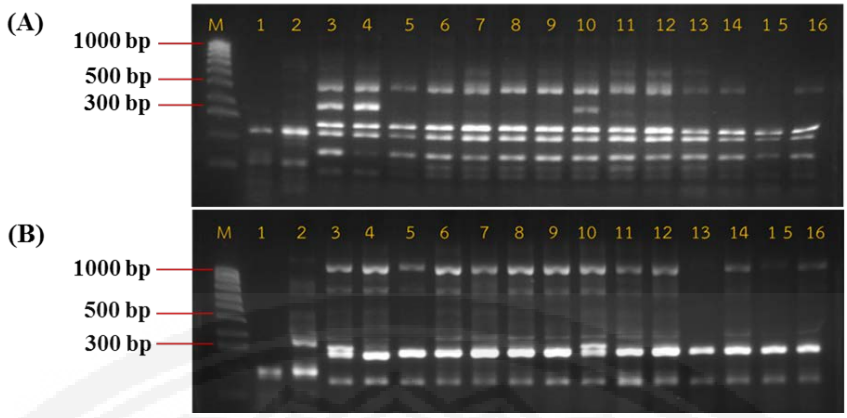

Figure 1 SRAP profiles of 16 *Musa* samples (1-16) generated by (A) Me4/Em8 and (B) Me6/Em7 primer combinations. Lane M is 100 bp DNA ladder (HyperLadder™ 100 bp, Bioline, USA).

Cluster analysis

The range of the similarity coefficients varied from 0.42 to 1.00, with an average of 0.86 (Table 4). The lowest similarity coefficient (0.42) was observed between 'Kluai Hom Champa' (AA/AAA group) and 'Kluai Tani Dam' (BB group). Among 12 *Musa* (ABB group) 'Kluai Namwa' cultivars, the similarity coefficients ranged from 0.89 to 1.00, with an average of 0.96. The lowest similarity coefficient among hybrid bananas was observed between 'Kluai Namwa Dam' and 'Kluai Namwa Khieo' (0.89) while the highest similarity coefficient among hybrid bananas was observed between 'Kluai Namwa Nuanchan' and 'Kluai Namwa Ubon' (1.00). Among five 'Kluai Namwa Mali-Ong' samples collected from different provinces of Thailand, 'Kluai Namwa Mali-Ong' (1) which collected from Phitsanulok Province was the most distinct genotype from the others. The UPGMA analysis based on Dice coefficients categorized the 16 *Musa* samples into two different groups based on 74 polymorphic SRAP bands (Figure 2). The first main group was separated into two subgroups: I-A with all 12 hybrid banana cultivars (5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16) and I-B with two wild banana cultivars (3 and 4), while the second group had another two wild banana cultivars (1 and 2).

Table 4 Similarity coefficient matrix of 16 *Musa* samples based on ten SRAP primer combinations.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.00															
2	0.79	1.00														
3	0.42	0.50	1.00													
4	0.45	0.53	0.97	1.00												
5	0.48	0.59	0.82	0.83	1.00											
6	0.54	0.61	0.86	0.87	0.93	1.00										
7	0.51	0.60	0.84	0.85	0.95	0.98	1.00									
8	0.53	0.60	0.84	0.85	0.93	0.98	0.98	1.00								
9	0.53	0.60	0.84	0.85	0.93	0.98	0.98	1.00	1.00							
10	0.49	0.57	0.88	0.87	0.89	0.97	0.95	0.95	0.95	1.00						
11	0.55	0.62	0.86	0.89	0.93	0.98	0.97	0.97	0.97	0.95	1.00					
12	0.54	0.62	0.85	0.86	0.92	0.99	0.97	0.99	0.99	0.96	0.97	1.00				

Table 4 (Cont.)

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
13	0.52	0.61	0.80	0.83	0.93	0.95	0.96	0.96	0.96	0.91	0.95	0.96	1.00			
14	0.52	0.61	0.82	0.83	0.93	0.96	0.96	0.96	0.96	0.93	0.95	0.97	0.98	1.00		
15	0.49	0.59	0.81	0.81	0.91	0.94	0.95	0.95	0.95	0.90	0.92	0.95	0.97	0.97	1.00	
16	0.50	0.60	0.81	0.82	0.93	0.96	0.97	0.97	0.97	0.92	0.94	0.96	0.99	0.99	0.98	1.00

Note: Name of *Musa* samples (1-16) was shown in Table 1.

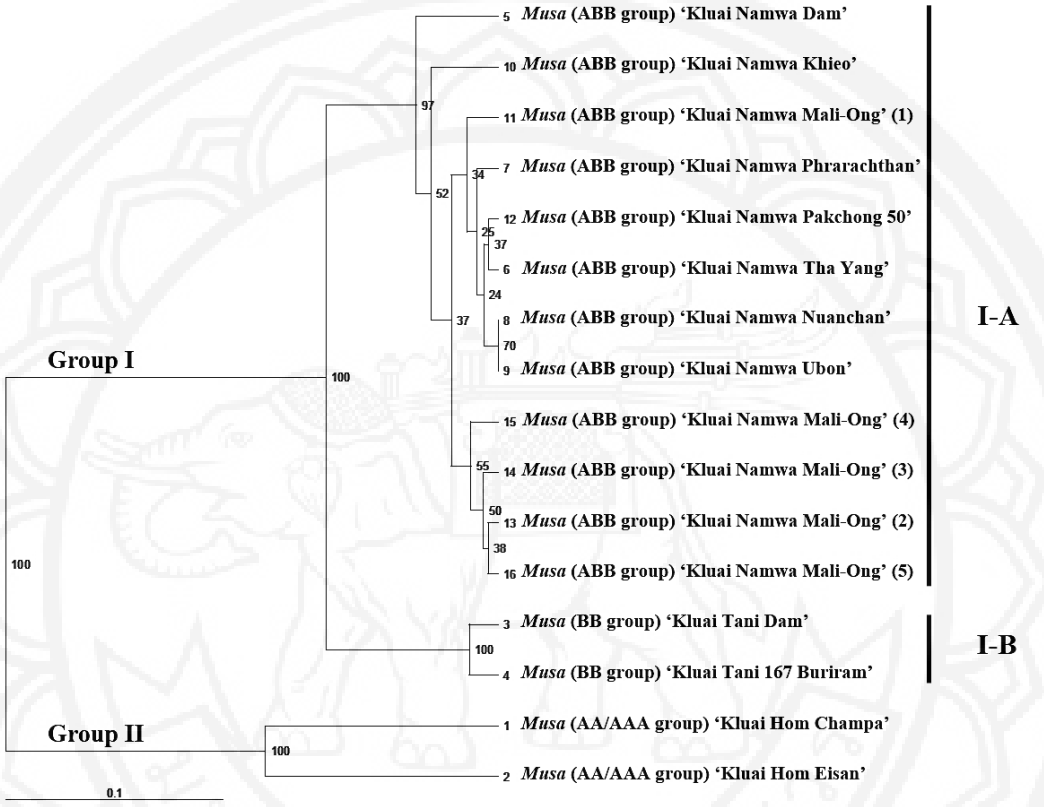

Figure 2 Dendrogram constructed by UPGMA analysis based on SRAP data of the 16 *Musa* samples. The numbers represent values from the bootstrap analysis.

Discussion

Study on plant genetic diversity is important due to it provides breeder an opportunity to develop new varieties with desirable traits. The assessment of genetic diversity within and between plants is commonly achieved based on various techniques such as (1) morphological marker, (2) biochemical marker and (3) DNA marker (Govindaraj, Vetriventhan, & Srinivasan, 2015). Since DNA markers are largely unaffected by the environmental factors and developmental stages, they are widely used as a biotechnology tool for genetic diversity assessment. Previously, many types of DNA markers have been used to assess genetic diversity in the genus *Musa* such as random amplified polymorphic DNA (RAPD) (Phothipan, Silayoi, Wanichkul, & Apisitwanich, 2005; Brown, Venkatasamy, Khittoo, Bahorun, & Jawaheer, 2009; Zozimo, Ratanasut, Boonsrangsom, & Sujipuli, 2018), high annealing temperature random amplified polymorphic DNA (HAT-RAPD) (Suvittawat, Teinseree, Saradhuldhat, Karintanyakit, & Phengchang, 2014), inter simple sequence

repeats (ISSR) (Wanvisait, Pinthong, Tankrathok, Sukkapan, & Srisamoot, 2019), simple sequence repeat (SSR) (Cyrille, Désiré, da Sylvère, & Fulgence 2019), amplified fragment length polymorphism (AFLP) (Opara, Jacobson, & Al-Saady, 2010), start codon targeted marker (SCoT) (Siritheptawee, Maneenet, Kunpuksri, Thanananta, & Thanananta, 2017) and sequence-related amplified polymorphism (SRAP) (Phothipan et al., 2005; Youssef, James, Rivera-Madrid, Ortiz, & Escobedo-GraciaMedrano, 2011).

Since SRAP technique has many advantages such as simplicity, reliability, high degree of reproducibility and high polymorphism rate (Li & Quiros, 2001). In this study, ten SRAP markers were used to assess genetic diversity among *Musa* (ABB group) 'Kluai Namwa' cultivars and two outgroups. 74 polymorphic bands were achieved with an average of 7.4 bands per marker which is greater than 4.5 polymorphic bands per marker detected in Turkey bananas by SRAP marker (Pinar et al., 2015) and 6.2 fragments per marker detected in 38 Indian banana cultivars by RAPD marker (Motilal, Rajiv, & Asha, 2016). The PIC values varied from 0.16 to 0.30 with an average of 0.25, suggesting that this SRAP technique could be used to generate median polymorphism which is useful for genetic difference of banana plants.

The genetic similarities of 16 *Musa* accessions varied from 0.42 to 1.00, with an average of 0.86. The results implied an extreme level of genetic diversity between *Musa* accessions. The lowest similarity coefficient (0.42) was observed between 'Kluai Hom Champa' (AA/AAA group) and 'Kluai Tani Dam' (BB group), indicating that they are not closely related because of the differences in their genome groups as they have AA/AAA and BB genome, respectively. High genetic diversity has also been reported in the South Indian banana cultivars (Motilal et al., 2016). Among twelve *Musa* (ABB group) 'Kluai Namwa' cultivars, the similarity coefficients ranged from 0.89 to 1.00, with an average of 0.96, suggesting that they are the most closely related among the 12 *Musa* hybrid cultivars evaluated. The lowest similarity coefficient among *Musa* 'Kluai Namwa' cultivars (0.89) was observed between 'Kluai Namwa Dam' and 'Kluai Namwa Khieo' while the highest similarity coefficient among hybrid bananas (1.00) was observed between 'Kluai Namwa Nuanchan' and 'Kluai Namwa Ubon'. Similarly, a small range of Dice similarity coefficients from 0.82 to 0.89 with a mean of 0.86 was observed among five *Musa* (ABB group) when analyzed with RAPD technique (Motilal et al., 2016). Although most of *Musa* (ABB group) 'Kluai Namwa' cultivars have a slightly difference in morphology, at the DNA level, most of them showed low genetic variation, it possibly caused by genetic mutation as bananas were vegetative propagated crop (Pinar et al., 2015). Also, the low genetic diversity was found among 'Kluai Namwa Mali-Ong' samples collected from different provinces of Thailand.

In the present study, the UPGMA analysis based on Dice coefficients categorized the 16 *Musa* samples into two different groups (I and II) which were relative to the banana genome groups. The first main group was separated into two subgroups including twelve hybrid banana genotypes (ABB genome) (I-A) and two wild banana genotypes (BB group) (I-B). The AA or AAA *Musa* genotypes were placed into the second group. Although there was unclear separation among the hybrid bananas, most of ABB *Musa* genotypes were closely related. Suvittawat et al. (2014), classified eight Thai banana cultivars (ABB group) with standard morphological identification and HAT-RAPD technique, reported that hybrid bananas (ABB group) were morphologically classified by (1) pseudo-stem height and (2) color of fruit peel and fruit core. The results based on SRAP markers in this study could be useful for germplasm collection and banana breeding in the future.

Conclusion and Suggestions

This study aimed to assess the genetic diversity among *Musa* (ABB group) 'Kluai Namwa' cultivars and two outgroup taxa (AA/AAA and BB groups) with SRAP markers. Based on ten SRAP markers, a total of 80 DNA bandings were scored, of which 74 were polymorphic (92.50%). Polymorphic information content (PIC) values ranged from 0.16 to 0.30 with an average 0.25. The bands size ranged from 100 to 1200 base pairs. The UPGMA analysis based on Dice coefficients demonstrated that the accessions had a similarity range from 0.42 to 1.00 with a mean of 0.86 which suggested extreme genetic diversity among *Musa* samples. The low genetic diversity was found among 'Kluai Namwa' samples. In the present study, dendrogram derived from ten SRAP markers showed that all samples could be categorized into two major groups (ABB-BB and AA/AAA genome groups). In the future, a number of *Musa* samples and genetic markers could be added to study the genetic diversity and varietal identification. The results based on SRAP markers in this study could be useful for germplasm collection and banana breeding in the future.

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