Assessing genetic diversity of some banana cultivars using inter simple sequence repeats (ISSR) markers

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ABSTRACT: The purpose of this study was to assess the genetic diversity of 12 banana cultivars using inter simple sequence repeat (ISSR) markers. Twenty ISSR primers were used for screening, seven primers produced 54 total bands and 53 polymorphic bands with an average percentage of polymorphism of 96.43%. The average polymorphism information content (PIC), the average effective multiplex ratio (EMR), the average marker index (MI) and the average resolving power (Rp) were 0.26, 7.46, 2.08 and 4.10 respectively.Genetic similarity matrix from Pearson coefficient between the cultivars ranged from 0.010 to 0.657, with an average of 0.454. The lowest was between Kluai Sao Kratueb Ho and Kluai Hin while the highest was between Kluai Hak Muk Som and Kluai Nam Wa Kom. Cluster analysis using the unweighted pair group method with arithmetic mean analysis (UPGMA) divided 12 cultivars into two major groups. The first group included the main cultivar in this study while the second group comprised of only two cultivars. Although the close relate between different genome type was found, these clustering was acceptable with Cophenetic Correlation Coefficient (CCC) was 0.82.

Keywords: banana, *Musa* spp., genetic diversity, DNA fingerprint, ISSR

Introduction

Banana (*Musa* spp.) is one of the most popular fruits worldwide. Global exports of banana reached an estimated quantity of 18.1 million tons in 2017 (FAO, 2018) demonstrated that the economical and nutritional important food crop in several areas of the world. It also important source of rural income, particularly in some locations where produce them to the home gardens or ornamental varieties of the species. Thailand is the fourth largest banana production in the in ASEAN after Philippines, Indonesia and Myanmar with a total production of 1.1 million tons in 2016 (DITP, 2017). However, the export value of Thai bananas also has the opportunity to growing up, especially in the framework of the JTEPA (Japan-Thailand Economic Partnership Agreement) which specified that Japan will import bananas from Thailand for 8,000 tons per year (DOA, 2016).However, Thailand's export volume is only 1,800 tones (DITP, 2017) because pests, diseases and climate change hamper production of bananas. In order to increase the efficiency of banana production, more knowledge about the genetic diversity, genome structure and phylogenetic relationships of the hybrids, cultivars and wild

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types must be gained. The genomic-based-breeding tools was investigated and used to breed for increased crossbreeding efficiency, disease resistant and high-yielding banana hybrids.

Most of the cultivated bananas are derived from intra- and interspecific hybridizations between two diploid wild species, *Musa acuminate* Colla and *Musa balbisiana* Colla, which contribute the A and B genomes, respectively. These cultivars include diploids, triploids and a few tetraploids in various of genome combinations such as AA, AB, AAB, ABB, BBB and AABB. Taxonomically, banana cultivars and hybrids were classified into different groups according to ploidy analysis and a set of 15 morphological characters (Simmonds and Shepherd, 1955).Edible bananas are widely geographical distributed, accomplished solely by transportation of vegetative planting materials by human agencies, diversified by somatic mutation, and multiplied by vegetative propagation and artificial selection (Simmonds, 1962). At least 140 cultivars in the plantations, 10 wild species and, 4 introduced species were found in Thailand (Rotchanapreeda et al., 2016). Moreover, the occurrence of local names, synonymous and homonymous, plus the high occurrence of somaclonal variation for some cultivars made the genetic relationship of banana cultivars still unclear. Moreover, the genome composition of the banana demonstrates characteristics associated with resistance to pests and diseases. Thus, for further studies on banana improvement, the knowledge on genetic diversity and genome composition of current cultivars was required.

Molecular markers, especially polymerase chain reaction (PCR)-based technique, that provide useful information and new insight into the taxonomy have been available for several years and have been used for evaluate the genetic diversity in *Musa* species, including random amplified polymorphic DNA (RAPD) (Crouch et al. 2000; Pillay et al. 2001), variable number tandem repeats (VNTR) (Garcia et al., 2011), amplified fragment length polymorphism (AFLP) (Opara et al., 2010; Youssef et al., 2011), microsatellites or simple sequence repeat (SSR) (Rotchanapreeda et al., 2016) and sequence-related amplified polymorphism (SRAP) (Phothipan et al. 2005; Valdez-Ojeda et al. 2014).Another PCR-based technique, inter-simple sequence repeats (ISSRs), have been shown to provide a powerful, rapid, simple and reproducible. ISSR technique involves the use of a 5' or 3' anchored or non-anchored nucleotides primer to amplified DNA fragment between two identical microsatellites repeat regions which oriented in opposite direction. The main advantage of ISSR is that no required sequence data for primer construction, make it easier to use than SSR markers which require knowledge of the genomic sequence data to design specific primers. The ISSR primer is randomly distributed throughout the genome that will provide accurate genetic information. Moreover, in terms of expenses, the operational costs, the development costs and labor-intensity are less than AFLP markers (Racharak and Eiadthong, 2007). The ISSR markers have been extensively used for assess genetic diversity and identify the closely related cultivars in many plant species (Srisamoot and Sootsuwan, 2016) such as sugarcane (*Saccharum* spp.) (Mondal et al., 2018), pineapple (*Ananas comosus* (L.) Merr.) (Souza et al., 2017), *Rheum* (Tabin et al., 2016), sorghum (*Sorghum bicolor* (L.) Moench) (Basahi, 2015), *Lilium* (Zhao et al., 2014), wheat

(*Triticum aestivum* L.) (Bararkat et al., 2013; Najaphy et al. 2011) and alfalfa (*Medicago sativa* L.) (Rashidi et al. 2013). For bananas, ISSR markers have been used to characterize and compare the local dessert banana genotypes (Swain et al., 2016), evaluating the genetic diversity among genotypes (Silva et al., 2017), examine genetic integrity and uniformity of different varieties and genetic fidelity testing for micro-propagated plantlets (Surabhi and Pattanayak, 2015). Furthermore, ISSR can be applied in many studies especially in taxonomic studies of closely related species. For these reasons, ISSR marker was selected to investigate the geneticrelationship among banana cultivar in AAA, AAB and ABB groups in Thailand.

Materials and Methods

Sample collection and DNA extraction

Twelve banana cultivars were included in this study. Eleven cultivars are local cultivars viz Kluai Hin, Kluai Phama Haek Kuk, Kluai Hom Khieo, Kluai Nam Wa Kom, Kluai Hak Muk Som, Kluai Sao Kratueb Ho, Kluai Roiwi, Kluai Nak, Kluai Nom Sao, Kluai Nam Wa Tia and Kluai Ngachang. All these cultivars were collected from Kalasin Province and surrounding areas. Only one import cultivar is Kluai Dang Florida. This cultivar was purchased from the ornamental plant market. All samples were planted to preserve germplasm at Phu Sing research and training center, Kalasin University, Kalasin province. The cultivar names and genome types of these cultivars were classified follow to the description by Silayoi (2015).Fresh young banana leaves were collected from the field, packed in plastic bags and kept in iceboxes. The genomic DNA was extracted using the modified cetylmethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Briefly, 100 mg of fresh leaves were ground to fine powder in liquid nitrogen and transferred to a 1.5 mL sterile reaction tube and addition of 1000 µL of CTAB extraction buffer (20 g/L CTAB; 1.4 M NaCl; 0.1 M Tris-HCl; 20 mM Na2EDTA) and 20 µL of Proteinase K (20 mg/mL). The mixture was vortexed and incubated at 65 oC for 60 min. Further, 20 µL of RNase A (10 mg/mL) were added and the sample was homogenized, incubated at 37oC for 30 min. Debris was pelleted by centrifugation and the supernatant was extracted twice with phenol: chloroform: isoamylalcohol (25:24:1) solution and phenol was eliminated by chloroform: isoamyl alcohol (24:1) solution. DNA was precipitated with the equal volume of ice-cold isopropanol and centrifugation. The supernatant was discarded and the pellet was washed twice with 70% ethanol, dried and re-suspended in 50 µL of TE buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8.0). DNA quality was evaluated on a 0.8% agarose gel stained with ethidium bromide. DNA Quantity was measured using a spectrophotometer and the DNA samples were diluted to 20 ng/µL for polymerase chain reaction (PCR) amplification.

ISSR Fingerprinting

A pre-screening of twenty ISSR primers (Srisamoot and Padsri, 2018) were initially screened using two banana cultivars, as a representative sample for A-genome (Kluai Nak) and B-genome (Kluai Hak Muk Som). Only seven primers gave the clearly resolved and polymorphic amplified products within the banana samples (**Table 2**). PCR amplifications were carried out in a final volume of 25 µL, containing 60 ng of DNA template, 0.5 U Taq polymerase, 2.5 µL of 10X reaction buffer, 3.0 mM MgCl2, 0.2 mM of each dNTP and 0.5 µM

ISSR primer. DNA amplifications were performed in the thermo cycler (TGradient 96, Biometra, Germany), under the following conditions: preliminary step of 10 min at 94 ºC, followed by 40 cycles by of 60 sec at 94 ºC, 90 sec to annealing temperature by primer (**Table 2**) and 60 sec extension at 72 ºC with a final 7 min extension at 72 ºC. Amplification products were separated on 1.5% agarose gels electrophoresis at 100 V in TBE 1X buffer. Gels were stained with ethidium bromide for 30 min, visualized under UV light and photographs through gel documentation systems (InGenius, SYNGENE, USA). The obtained bands were compared with the 1kb DNA ladder marker (Invitrogen). In order to evaluate the reproducibility of the DNA profile, PCR reactions were carried out 3 times, and only well-defined and reproducible bands were considered.

Data analysis

DNA banding patterns generated by ISSR-PCR amplification, those with a band on the same migration position on agarose gel were scored as present (1) and those without were scored as absent (0). These binary data were used to analyze the genetic similarity index with Pearson coefficient. The unweighted pair group method with arithmetic averages (UPGMA) was used to construct a phylogenetic dendrogram and cellulate the Cophenetic Correlation Coefficient (CCC) from data matrix via the online dendrogram construction utility DendroUPGMA (Garcia-Vallvé et al., 1999). The 100 bootstrap replicates dendrogram was constructed. The obtained dendrogram was imaged with Tree View 3.0 software (Saldanha, 2004). Because ISSR is dominant markers, so Polymorphism Information Content (PIC) was calculated using the formula PICi = 1-[fi 2 + (1 - fi)2] (De Riek et al., 2001), where PICi is the polymorphic information content of marker i, fi is the frequency of the marker bands present and (1-fi) is frequency of the marker bands absent. Effective multiplex ratio (EMR) is calculated as total number of polymorphic loci (per primer) multiplied by the proportion of polymorphic loci per their total number i.e. EMR = np(np/n), where np is the number of polymorphic loci, and n is the total loci number. The high value of EMR indicated the greater efficient of the primer system (Chesnokov and Artemyeva, 2015). Marker index (MI) is a statistical parameter used to estimate the total utility of the maker system. Marker index is the product of the PIC value and EMR, MI = PIC x EMR (Chesnokov and Artemyeva, 2015). Resolving power (Rp) is a parameter characterizing ability of the primer combination to detect the differences among the large numbers of genotypes (Gilbert et al., 1999; Prevost and Wilkinson, 1999). Rp was calculated using the formula Rp = ΣIB (Prevost and Wilkinson, 1999), where IB (band informativeness) takes the value of: 1 – [2 × (0.5 – p)], p being the proportion of the 12 bananas cultivar containing the bands.

Results and Discussion

DNA detection

Genomic DNA extracted from 12 banana cultivars were visualized by agarose gel electrophoresis, the results showed that the DNA bands for each loading wells were clear and no diffusion. The ratio of A260 nm/A280 nm was between 1.85 - 1.92 (data not show). These results indicated that the extracted genomic DNA of the young banana leaves were purity and high quality.

Primers sequences, number of total bands scored, number of polymorphic bands, percent of polymorphism, polymorphism information content, effective multiplex ratio, marker index and resolving power of primer were showed in **Table 1**.

Table 1 Nucleotide sequence and annealing temperature (Ta) of ISSR primers used in this study and some summary results

Primer	Nucleotide Sequence	Ta (°C)	No. of total bands	No. of polymorphic bands	Polymorphism (%)	PIC	EMR	MI	Rp
ISSR04	CACACACACACAAC	54	7	7	100	0.26	7	1.85	2.50
ISSR05	CACACACACACAGT	55	9	9	100	0.17	9	1.56	2.00
ISSR07	CACACACACACAGG	57	7	7	100	0.29	7	2.03	3.67
ISSR10	GAGAGAGAGAGACC	53	7	7	100	0.36	7	2.54	4.83
ISSR11	GTGTGTGTGTGTCC	57	15	15	100	0.33	15	4.99	8.83
ISSR13	GAGGAGGAGGC	51	4	3	75	0.20	2.25	0.45	4.67
ISSR16	ACTGACTGACTGACTG	58	5	5	100	0.22	5	1.12	2.17
Total			54	53	675	1.85	52.25	14.53	28.67
Mean			7.71	7.57	96.43	0.26	7.46	2.08	4.10

The products of ISSR amplification, a total of 54 strong, clear, and reproducible bands, were amplified from 12 banana cultivars using 7 selected ISSR primes, of which 53 were polymorphic (**Table 1**). The ISSR amplification generated an average of 7.17 DNA bands per primer. The size of the amplified fragments ranged from 300 to 2,000 bp (**Figure 1**). The primer ISSR11 generated a highest number of scoreable bands (15) while the lowest (4) was generated by primer ISSR13. The average percentage of polymorphism was 96.43% indicated the genetic variation of these taxon and does not relied on the number of primers used. Similar to Racharak and Eiadthong (2007) which found 100% polymorphic while examine the genetic variation of subspecies of *M. acuminata* are naturally distributed in Thailand. The results showed that there was significant genetic difference and high genetic diversity among banana cultivars. However, Ruangsuttapha et al. (2007) reported the polymorphism generated by the RAPD primer observed among the Thai banana cultivars was generally low. This may be due to the technique as reported by Salimath et al. (1995) which found the larger number of polymorphisms form ISSR than either RFLP or RAPD analyses.

The PIC value ranged from 0.17 (ISSR05) to 0.36 (ISSR10) with a mean of 0.26. The average PIC in this study is confirmed the good marker system for distinguished between different cultivars as a maximum PIC value of 0.5 for dominant markers (Guo et al., 2014). In general, ISSR primer is used as the forward and reverse primers. PIC can be determined based on both the number and frequency of the amplified fragments. In contrast, the EMR value varied from 2.25 to 15.00 with an average value of 7.46. EMR, was the number of polymorphic

markers generated per assay and indicated the efficiency of the marker system. Low value of EMR in this study seems to low capability of ISSR primer. However, the primers that showed higher polymorphism had higher EMR values which consistent with the report of Najaphy et al. (2011). The MI was lowest with the primer ISSR13 (0.45) and highest with the primer ISSR11 (4.99) with an average of 2.08. MI is a feature of a marker which calculated for all the primers. The higher polymorphism provides higher MI because it is the product of PIC and EMR. In this study, high PIC and low EMR generated a medium value of MI. Although, the higher MI is better (Powell et al., 1996) this medium value also acceptable.

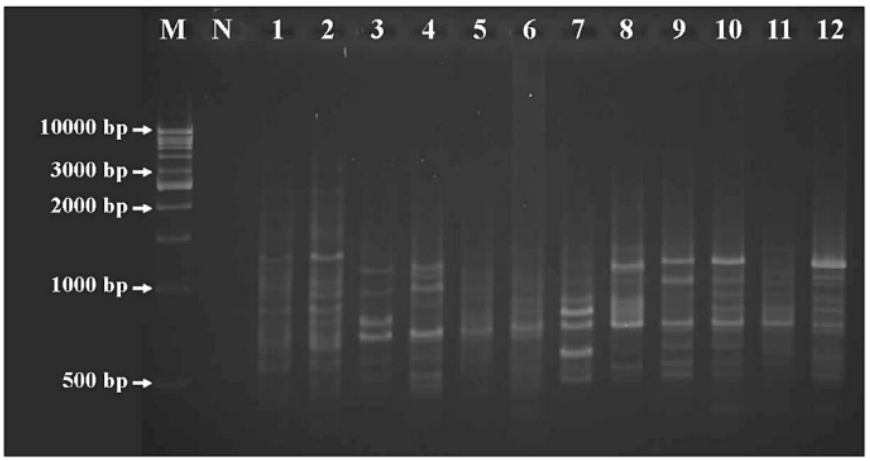

Figure 1 ISSR amplification pattern of 12 banana cultivars obtained with primer ISSR11. M is VC 1kb DNA Ladder marker (Invitrogen), N is negative control. No 1-12 were KluaiHin, Kluai Phama Haek Kuk, Kluai Dang Florida, Kluai Hom Khieo, Kluai Nam Wa Kom, Kluai Hak Muk Som, Kluai Sao Kratueb Ho, Kluai Roiwi, Kluai Nak, Kluai Nom Sao, Kluai Nam Wa Tia and Kluai Ngachang, respectively

The Rp of ISSR primers ranged between 2.00 (ISSR05) and 8.83 (ISSR11) with an average of 4.10. Rp was positively correlated with the number of total amplified bands. Rp value provides a moderately accurate estimate of the number of genotypes identified by a primer (Prevost and Wilkinson, 1999). Only one ISSR primers, ISSR11, occupied the high Rp values and therefore seem to be the most informative primers for distinguishing the banana cultivars. However, Rp had no significant correlation with the other parameters e.g. PIC, EMR and MI in this study. The resolving power provides no information on the ability of a primer to reflect the genetic or taxonomic relationships of a group of genotypes under study (Prevost and Wilkinson, 1999).

Genetic relationships among banana cultivars

Similarity matrix based on ISSR binary data of the 12 banana cultivars were computed with Pearson coefficient are varied from 0.010 (Kluai Sao Kratueb Ho vs. Kluai Hin) to 0.657 (KluaiHakMukSom vs. Kluai Nam Wa Kom) with an average of 0.454 (**Table 2**). These results confirm the close relate cultivar form same genome type e.g. Kluai Phama Haek Kuk vs. Kluai Hin (ABB), Kluai Nam Wa Kom vs. Kluai

Hak Muk Som (ABB) and Kluai Sao Kratueb Ho vs. Kluai Roiwi (AAB) with genetic similarity index of 0.643, 0.657 and 0.573, respectively. However, the high genetic similarity index (0.425) across genome type was found between Kluai Nak (AAA) vs. Kluai Nom Sao (AAB). This may be due to the ISSR primers annealed randomly throughout the target genome and amplified mostly DNA form A genome.

Clustering analysis of 12 banana cultivars

The UPGMA dendrogram divided 12 banana cultivars into two major groups based on present and absent band of ISSR fingerprint on agarose gel.The more identical band indicated the more closely genetic relationship. The first major group can be divided into two subgroups. Group IA included six cultivars: Kluai Sao Kratueb Ho, Kluai Roiwi, Kluai Nam Wa Tia, Kluai Ngachang, Kluai Nak and Kluai Nom Sao. Group IB comprised four cultivars: Kluai Nam Wa Kom, Kluai Hak Muk Som, Kluai Hom Khieo and Kluai Dang Florida. The second major group consist of two cultivars: Kluai Phama Haek Kuk and Kluai Hin (**Figure 2**). Surprisingly, the import cultivar, Kluai Dang Florida, show a close genetic similarity to Thai native cultivars; Kluai Nam Wa Kom, Kluai Hak Muk Som and Kluai Hom Khieo. Moreover, horn plantain type cultivar, Kluai Ngachang have shown close genetic relationship with Kluai Nam Wa Tia (ABB). This indicate that no correlation between the DNA pattern form ISSR primer and the genome type. The reasons might be the polymorphic band amplified by ISSR markers were not located in genomic fragments for genome data, thus that the clustering was not relate with the genome type. Anywise, the position of Kluai Ngachang and Kluai Dang Florida on these cluster based on low bootstrap value (19%). A bootstrap value of 50-70% indicates the low confidence of the genetic relationship. The results showed that the existence of genetic diversity among them.

Table 2 Similarity matrix computed with Pearson coefficient among the 12 banana cultivars as generated by seven ISSR primers

No.	Taxon	1	2	3	4	5	6	7	8	9	10	11	12
1	Kluai Hin	1.000	0.643	0.180	0.058	0.315	0.242	0.010	0.127	0.144	0.162	0.138	0.006
2	Kluai Phama Haek Kuk		1.000	0.131	0.012	0.162	0.201	0.066	0.076	0.003	0.257	0.095	0.064
3	Kluai Dang Florida			1.000	0.373	0.226	0.265	0.236	0.343	0.176	0.197	0.162	0.197
4	Kluai Hom Khieo				1.000	0.426	0.475	0.209	0.311	0.318	0.162	0.138	0.330
5	Kluai Nam Wa Kom					1.000	0.657	0.134	0.182	0.227	0.073	0.400	0.073
6	Kluai Hak Muk Som						1.000	0.289	0.220	0.164	0.115	0.444	0.011
7	Kluai Sao Kratueb Ho							1.000	0.573	0.485	0.324	0.404	0.412
8	Kluai Roiwi								1.000	0.285	0.210	0.417	0.374
9	Kluai Nak									1.000	0.425	0.341	0.348
10	Kluai Nom Sao										1.000	0.185	0.179
11	Kluai Nam Wa Tia											1.000	0.368
12	Kluai Ngachang												1.000
	Average	0.454											
	CCC	0.82											

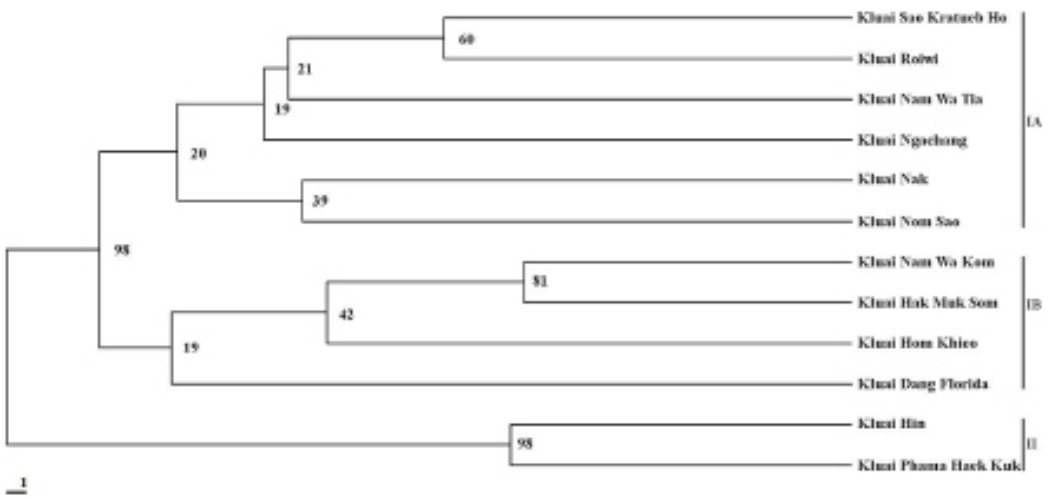

Figure 2 The UPGMA dendrogram of genetic relationships among banana cultivars using ISSR primer

The calculated Cophenetic Correlation Coefficient (CCC) was 0.82. The CCC is the Pearson's linear correlation coefficient between the matrix of distances among cultivars as a result of the original data and the similarity matrix resulting from the dendrogram. According to the maximum value of a CCC is1, the consistency of the clustering pattern is greater (Souza Neto et al., 2014). The calculated CCC from this study implied the good fit between the original data matrix and the matrix resulting from the dendrogram. Therefore, the clustering was acceptably consistent. Consequently, ISSR marker is a very useful and effective method for assessing genetic diversity of some banana cultivars. The present ISSR data generated by seven primers indicated a high genetic diversity in the examined cultivars. However, the generated ISSR fingerprint in this study does not cover all the existent cultivars and low amount number of primers. Further development in the future, these ISSR markers may be an effective tool for genetic diversity assessment and also cultivar identification of all banana.

Conclusions

Twelve banana cultivars generated 54 total bands form using 7 selected ISSR primes. Of these band 53 were polymorphic (96.43%). The average PIC was 0.26 confirmed the good marker system. The average value of EMR was 7.46. The average value of MI was 2.08. The average value of Rp was 4.10. Kluai Hak MukSom and Kluai Nam Wa Kom showed the highest genetic similarity index (0.657). The dendrogram was divided into two main clusters and does not related to genome type.

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