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Genetic Diversity and Relationships among Sugarcane (*Saccharum* sp.) from Thailand Revealed by RAPD and AFLP Markers

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Abstract

Objective: The objective of the present research was to evaluate the genetic diversity and relationships of sugarcane (*Saccharum* sp.) that collected from the sugarcane germplasm collection, Thailand. **Methods:** Genetic diversity of sugarcane was detected by DNA sequencing, Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) markers. **Findings:** The *trn*L- *trn*F region of the chloroplast genome was amplified for 26 cultivars with C and F primers that the fragment was approximately 950 base pairs (bp). The taxonomic status and relationships of these sequences are not resolved. RAPD marker was used to study genetic variation and relationships among 56 sugarcane varieties and their hybrids. Ninety-six RAPD primers were screened and nine primers of them produced reproducible and recordable bands were used in analysis. The eighty-eight polymorphic bands (96.70%) were detected and genetic similarity was 0.657. The best five AFLP primer combinations were selected and generated 187 polymorphic bands (80.26%). Comparing the results from the RAPD and AFLP markers in 19 sugarcane cultivars, the genetic similarity ranging from 0.582 to 0.927, with the average of 0.756 for AFLP and ranging from 0.449 to 0.865, with the average of 0.657 for RAPD that demonstrating a low level of genetic diversity. For dendrogram analysis, AFLP marker showed itself to be more efficient at discriminating sugarcane cultivars. However, the genetic relationship of the samples slightly correlated with sucrose content. **Application:** RAPD and AFLP markers can be used to help plant breeder to select cultivars for future sugarcane improvement programs.

Keywords: AFLP, Genetic Diversity, RAPD, *Saccharum* sp., Sugarcane

1. Introduction

Sugarcane (*Saccharum* spp.) is a member of the grass family (Poaceae) in tribe Andropogoneae[1] which is an important crop to produce sugar and ethanol. Typically, this genus is composed of six different species: *Saccharum spontaneum*, *S. robustum*, *S. officinarum*, *S. barberi*, *S. edule*, and *S. sinense*[2]. Sugarcane can be used as a raw material for industry to produce not only the world's sugar, but also cellulosic ethanol. Therefore, better understanding in genetic background of sugarcane cultivars and improved sugarcane varieties are important strategies to increase the cane yield. In recent years, improved varieties or modern commercial varieties are inter-specific hybrids of *Saccharum* sp. which are usually hybrid between *S. spontaneum* and *S. officinarum*[3]. *S. officinarum* has very high sugar content, low fibre content and poor disease resistance, while *S. spontaneum* is a highly polymorphic species that has lower sugar content, high fibre content, higher levels of disease resistance, drought tolerance and disease and pest resistance[4].

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In *Saccharum* species, morphological features are highly influenced by environment factors that make it very difficult to identify and distinguish the genotypes. Molecular markers are powerful tools to estimate the complex genetic variation of sugarcane without environmental impact[5]. In *Saccharum* complex, several molecular techniques have been investigated for genetic diversity such as random amplified polymorphic DNA: RAPD[1,5], restriction fragment length polymorphism: RFLP[6], amplified fragment length polymorphism: AFLP[7], inter simple sequence repeats: ISSR[8] and microsatellite or simple sequence repeats: SSR[9] markers.

In Thailand, the RAPD technique has been used to determine the genetic variation and relationship among some sugarcane cultivars and germplasm[10]. The genetic diversity is essential for germplasm groups and cultivars identification. Including, selection of cultivars for breeding that will be useful for sugarcane breeding program. As well as, both RAPD and AFLP markers does not require knowledge of the genome sequence. Therefore, the purpose of this research to evaluate genetic diversity among *Saccharum* species and hybrid using both RAPD and AFLP markers.

2. Materials and Methods

2.1 Sugarcane Samples

This study was carried out using fifty-six sugarcane cultivars leaves that collected from the sugarcane germplasm collection: Khon Kaen Field Crops Research Center, Muang, Khon Kaen province and Suphan Buri Field Crops Research Center, U Thong, Suphan Buri province, Thailand. A detailed description of 56 sugarcane cultivars are given in table 1. The leaves were transported to our laboratory and kept at -80°C until DNA extraction.

Table 1. Description 56 sugarcane cultivars for use in genetic diversity study

Cultivars	Species/hybrid	Cultivars	Species/hybrid	Cultivars	Species/hybrid
Samples from *Suphan Buri Field Crops Research Center,* U Thong, Suphan Buri province					
SP80***	Hybrid	CO82-32**	Hybrid	UT1	Hybrid
KK80**	Hybrid	85-2-352**	Hybrid	UT2	Hybrid
UT10***	Hybrid	ROC1	Hybrid	UT3	Hybrid
UT11**	Hybrid	K84-200**	Hybrid	UT5	Hybrid
UT8**	Hybrid	Mauritius	Hybrid	SP50**	Hybrid
LK92-11	Hybrid	CO-290***	Hybrid	Trojan	Hybrid
Jeandang*	Hybrid	Egypt*	Hybrid	Badilla	*S.officinarum*
Samples from Khon Kaen Field Crops Research Center, Muang, Khon Kaen province					
B37-161**	Hybrid	ThS97-42	*S. spontaneum*	Mahasarakham*	Hybrid
B43-62**	Hybrid	KKJ03-425	Hybrid	TP07-124-1*	Hybrid
KU60-5***	Hybrid	ThS97-41*	*S. spontaneum*	B47-419*	Hybrid
B41-227*	Hybrid	ThS97-51*	*S. spontaneum*	Thai127*	Hybrid
Songkonphureau*	Hybrid	ThS97-10*	*S. spontaneum*	SO10-06***	Hybrid
TP07-424*	Hybrid	Chumporn2	Hybrid	TP07-395	Hybrid
TP07-426	Hybrid	TP07-428	Hybrid	Asatoa	Hybrid
E-heaw	Hybrid	KK3***	Hybrid	Asawa**	Hybrid
ThS97-48*	*S. spontaneum*	SO10-08*	Hybrid	Aiwa	Hybrid
KK1***	Hybrid	TP07-305***	Hybrid	B34-164	Hybrid
K88-92*	Hybrid	KU60-3*	Hybrid	B40-98**	Hybrid
ThS97-45*	*S. spontaneum*	B37-16*	Hybrid		

* used for DNA sequencing analysis, ** used for AFLP analysis

*** used in both DNA sequencing and AFLP analysis

2.2 DNA Extraction

Total genomic DNA was extracted from leaf tissues using a modification of CTAB (Cetyltrimethylammonium bromide) protocol of Doyle and Doyle[11]. First step, the leaf sample (1 to 2 g) was ground into a fine powder in liquid nitrogen and suspended in 700 µl of 2 X CTAB extraction buffer having 1% PVP. Then, transfer homogeneous solution into a new microcentrifuge tube and adding β-mercaptoethanol. Mixed gently and incubated at 65°C for 4 hours. The homogenate was mixed in equal volume of chloroform: isoamyl alcohol (24: 1, v/v) by gentle inversion and centrifuged at 14,000 rpm for 5 min. The chloroform: isoamyl alcohol step was repeated at least three times. After that, transfer the upper aqueous layer to a new microcentrifuge tube. RNA was removed by treating with 2 µl of the RNase (20 mg/ml) and incubate at 37°C for 1 hour, add 50 µl of 10% CTAB in 0.7 M NaCl and centrifuged at 14,000 rpm for 5 min. Then, upper aqueous phase was transferred to new tube and adding two-third volume of cold isopropanol for DNA precipitation. The final step, the DNA pellet was washed with ethanol and dissolved in 100 µl of TE buffer. Concentration and purity of DNA was measured and kept at -20 °C until further used.

2.3 PCR Amplification and Sequencing

For DNA sequencing, the *trn*L- *trn*F region of the chloroplast genome was amplified for 26 samples using C primer (5'-CGAAATCGGTAGACGCTACG-3') and F primer (5'-ATTTGAACTGGTGACACGAG-3')[12]. PCR amplification was carried out in 25 µl total volume containing 50 ng of DNA template, 0.8 pM of each primer, 200 µM dNTP mixed, 1 unit of Taq DNA polymerase and 1X PCR buffer. The PCR condition was programmed as follows: initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C or 52°C for 1 min and extension at 72°C for 2 min and the final extension at 72°C for 5 min. PCR fragments were visualized using 1.5% agarose gel running in 1X TBE buffer comparing with 100 base pairs DNA ladder (Vivantis). The purify PCR products were sequenced by First Base Laboratories, Malaysia. The sequences were aligned and analyzed using BioEdit and MEGA6 software.

2.4 RAPD Analysis

RAPD marker was carried out with decamer primers[13]. The 20 µl reaction volume consisting of 1.5X standard *Taq* reaction buffer (New England BioLabs, USA), 2-2.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase, 200 µM of dNTPs, 2 mM of each RAPD primer and 250 ng genomic DNA. The following PCR program was used: initial denaturing at 94°C for 5 min, 40 cycles of 1 min of denaturation at 94°C, 1.30 min of primer annealing at 36°C and 2 min of extension at 72°C and final extension at 72°C for 2 min. The amplified RAPD fragments were separated and visualized using 1.5% agarose gel running in 1X TBE buffer along with 100 bp of DNA marker (Vivantis). The gel was photographed using gel documentation system (InGenius: Syngene, UK).

2.5 AFLP Analysis

The AFLP protocol was performed as described by Vos et al[14] and Bess et al[15] with slight modification. DNA sample was double digested with *Eco*RI and *Mse*I enzyme at 37°C overnight. After completed digestion, digested DNA was ligated to *Eco*RI and *Mse*I adapter in T4 ligase buffer at 37°C for 3 hours. Preselected amplification step was carried out using primers having complementary to the adapters contained one extra nucleotide (*Eco*RI+A and *Mse*I+C). Then, selective PCR amplification step was performed using three selective nucleotide (*Eco*RI+ANN, *Mse*I+CNN) in table 2. Forty-nine *Eco*RI/*Mse*I primers were used to screen for four samples of sugarcane cultivars. PCR product was added an equal volume of AFLP loading dye (98% formamide, 10 mM EDTA, pH=8.0, 0.1% bromo-phenol blue and 0.1% xylene cyanol). For denaturation, the samples were heated at 90°C for 5 min and chilled on ice. The amplified DNA fragments of each sample and primer were separated by denaturing 6% polyacrylamide gel. Banding patterns were determined using the silver nitrate staining method.

Table 2. Primers combinations employed for AFLP analyses

Eco**R1 + ANN**	Mse**1 + CNN**
AAC	CTC, CAA,CAC
AAG	CTA, CAA, CAG, CAT, CAC, CTC, CTT
ACA	CTG, CAG, CTA, CAT, CTC
ACC	CAT, CAA, CAC, CTC, CTG, CTT
ACG	CTC, CAT, CAG, CAA, CTA, CTT, CAC, CGA, CGG
ACT	CTG, CAC, CAG, CAT, CTA, CTT
AGA	CAC, CAA
AGC	CTA, CAA, CAG, CAT, CTC, CTT
AGG	CTA, CTC, CTG, CAA, CAT

2.6 Data Scoring and Analysis

Both data scoring and analysis of RAPD and AFLP markers, Banding patterns were compared and scored as 1 for presence and 0 for absence in a binary character. The data were used to evaluate genetic similarity using similarity index. The simple matching coefficients among the sugarcane cultivars were calculated. The dendrogram was constructed by genetic similarity matrices using UPGMA by the NTSys-pc software package.

3. Results

3.1 DNA Sequencing

The *trn*L- *trn*F region of the chloroplast genome from 26 cultivars (21 hybrid and 5 *S. spontaneum* samples) were amplified using the C and F primers. The sizes of DNA fragments are 950 base pairs (bp) long in all cultivars. The sequences were queried against GenBank using BLAST, all cultivars are *S. officinarum*. Including, we analyzed the genetic diversity among 26 sugarcane cultivars, two *S. officinarum* (AY116253 and EU434103), two *S. spontaneum* (JN642308 and AY116259) and four related *Saccharum* spp. (*Erianthus arundinaceus* (GQ870009), *Saccharum giganteum* or *Erianthus giganteus* (DQ004979), *Miscanthus sinensis* (DQ005095) and *Saccharum narenga* or *Narenga porphyrocoma* (JN642307). When constructed the homology tree (data not shown), those sequences have 100% homology, except *E. arundinaceus* and *M. sinensis*. Thus, the *trn*L- *trn*F region is not suitable in addressing the questions of relationships among sugarcane cultivars. The report by Zhu et al[16] in which the thirteen sugarcane cultivars and two reference cultivars were clustered into two groups at a 58% homology level based on the multiple sequence of chloroplast DNA. The four sugarcane cultivars were placed into one group. Whereas, the eight clones of *S. spontaneum* and the three of F1 progeny (*S. spontaneum* X *Saccharum* spp.) were divided into the other group which demonstrate that the chloroplast DNA of *S. spontaneum* was maternally inherited. However, these data indicate that the *trn*L- *trn*F region alone did not resolve among intraspecific relationships. In this experiment, we demonstrate that the sugarcane cultivars in Thailand are the complex hybrids cultivars. In addition, no strong evidence was found to support the *Erianthus* segregation. The *trn*L- *trn*F region is adequate for classifying such as *S. giganteum* and *S. narenga* which coincided with the report by Welker et al[17]. Furthermore, the closely related genera such as *Erianthus* and *Miscanthus* could be made available for use in directed breeding for genetic improvement of sugarcane.

3.2 RAPD Analysis

Initially, 91 decamer primers were screened in three cultivars (SP80, UT84-10 and UT84-11), only 15 primers were produced DNA patterns with recordable bands. However, only 10 primers showed easily recordable bands among 56 cultivars which were used to construct the dendrogram. The number of recordable loci for each primer ranging from 7 (OPB17) to 13 (OPC19) and ranging in size from 350 (OPC02 and OPD08) to 1300 bp (OPB17, OPC19, OPD02 and OPD08). A summary of primers, sequences of primer and amplified products from this study is shown in table 3. From total 89 bands were scored, 88 bands (96.70%) were found to be polymorphic. This result is consistent with the result of previous research using RAPD as genetic markers. Nawaz et al[1] investigated genetic diversity in 40 accessions of sugarcane in Pakistan with 92.05% polymorphism. Hapsoro et al[18] compared the genetic diversity among 38 genotypes of sugarcane (*S. officinarum*) consisted of 8 genotypes from Australia, 7 from Africa, 10 from America, and 13 from Asia with 78.45% polymorphism.

Genetic distance and genetic similarity are commonly used to measures of genetic diversity. In this study, the genetic similarity of sugarcane samples were recorded between 0.427 and 0.888 with an average value of 0.657 (data not shown). We found that the highest genetic similarity was 0.888 between Asatoa and Asawa, KK88-92 and SO10-08 as well as the lowest genetic similarity was 0.427 between UT11 and UT3, UT11 and UT5. These data results suggest that using RAPD marker revealed the existence of low level of genetic diversity that are consistent with the finding of Pan et al[19]. However, Hapsoro et al. studied the assessment of genetic variation in sugarcane germplasm showed moderate level of genetic diversity[18].

The simple matching coefficient among 56 sugarcane cultivars was used to calculate and create a UPGMA dendrogram as shown in figure 1. The population was clustered into three major groups. There were 2 subgroups in group 1 and 3, three subgroups in group 2. The first group (I) consisted of 12 sugarcane genotypes namely SP80, UT11, Mauritius, UT10, 85-2-352, Jeandang, CO82-32, ROC1, UT8, LK92-11, K84-200 and CO-290 that were collected

Table 3. Primer codes and sequences of the RAPD primers used and fragment sizes of the generated RAPD markers

Primer codes	Sequence (5'-3')	Fragment size (bp)
OPB17	AGGGAACGAG	400,550,600,800,1000,1100,1300
OPC02	GTGAGGCGTC	350,450,550,600,700,800,900,1000,1200
OPC18	TGAGTGGGTG	600,700,800,900,1000,1050,1100,1150,1200,1250
OPC19	GTTGCCAGCC	400,450,550,600,650,700,800,900,1000,1050,1100,1150,1300
OPD02	TCGGACGTGA	450,500,600,700,800,900,1000,1100,1200,1300
OPD08	GTGTGCCCCA	350,450,500,600,700,800,900,1000,1100,1200,1300
OPH05	AGTCGTCCCC	550,600,700,800,900,1000,1100,1150,1200
OPU03	CTATGCCGAC	500,600,700,900,1000,1100,1150,1200
OPZ04	AGGCTGTGCT	450,500,600,700,800,900,1000,1100,1150,1200

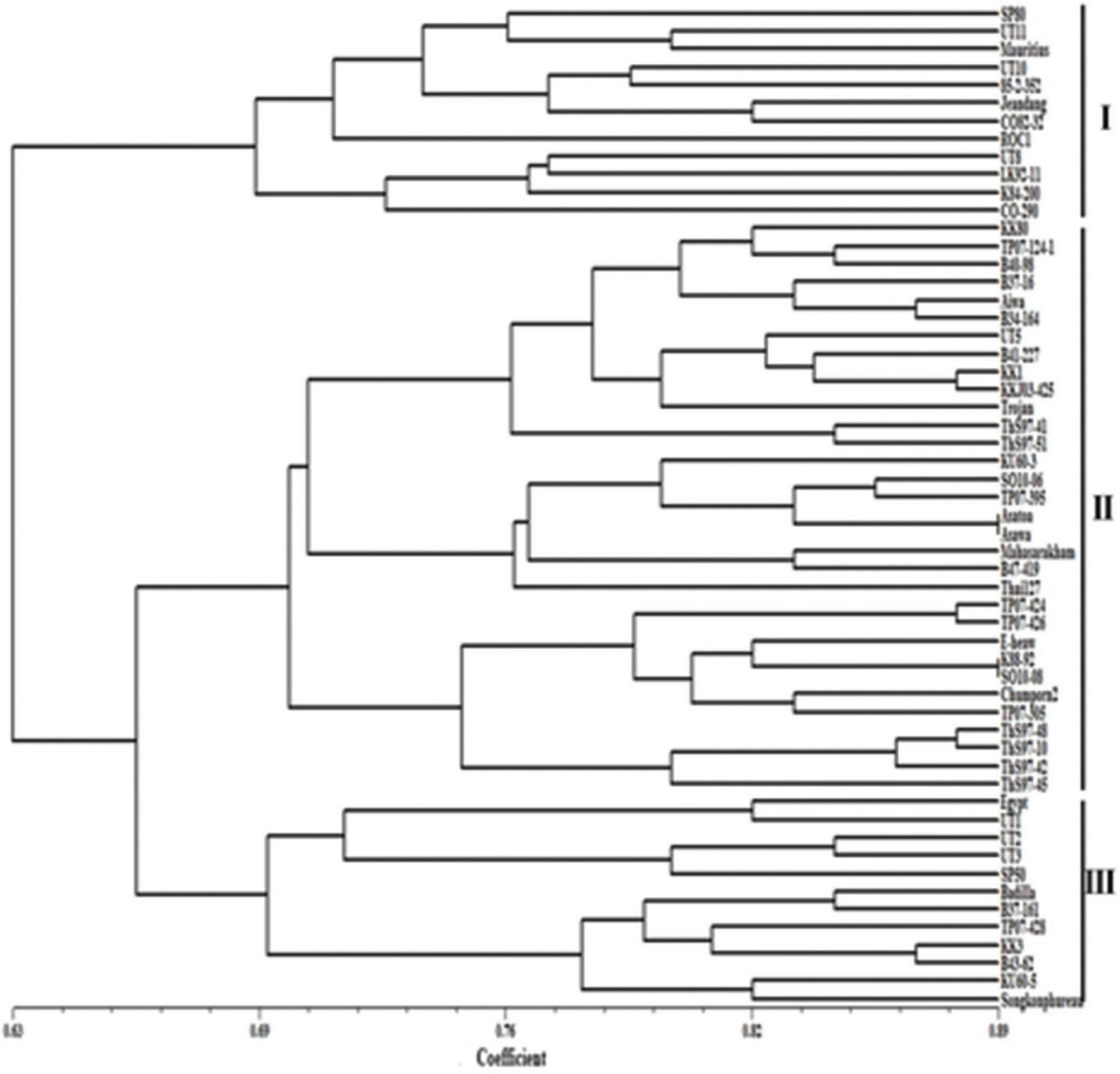

Figure 1. A dendrogram of 56 sugarcane cultivars as shown by UPGMA cluster analysis based on RAPD markers using nine primers.

from Suphanburi field crops research center. The second group (II), most of samples pick up from Khonkhane field crops research center. The third group (III), Egypt, UT1, UT2, UT3, SP50, Badilla, B37-161, TP07-428, KK3, B43-62, KU60-5 and Songkonphureau that consisted of cultivars from Suphanburi and Khonkhane field crops research center. *S. spontaneum* are grouped together in second group, whereas *S. officinarum* is in the third group. SP50 is the cultivar which higher sugar values contains and grown commercially for sugar production. So, the genetic relationship was slightly correlated with sucrose content.

3.3 AFLP Analysis

The 19 sugarcane cultivars (SP80, KK80, UT10, UT11, UT8, CO82-32, 85-2-352, K84-200, SP50, B37-161, KK3, B43-62, KU60-5, KK1, TP07-305, SO10-06, Asawa and B40-98) were examined by AFLP markers. Forty-nine AFLP primers were tested on four sugarcane cultivars (SP80, UT10, K84-200 and CO-290) which selected from RAPD profile. Five AFLP primers (ACG-CTA, AAG-CAA, AGG-CTG, AAG-CAG and AGG-CTT) were adequate and selected to study genetic relationships within 19 sugarcane cultivars. These five primer pairs produced up to a total of 233 bands of which 187 (80.26%) were polymorphic as shown in table 4. The total number of bands between 38 (AGG-CTT) and 57 (AAG-CAG) with an average of 46.60 bands per primer combination. This result was related to AFLP markers across 21 varieties of the sugarcane (*S. officinarum*) were grown at Mitrphol Research Center, Chaiyahpume, Thailand that found 75.76% polymorphism using five primer combinations. However, in other countries showed moderated polymorphism like Lima et al[20] revealed an average of 50% polymorphism within Brazilian sugarcane samples using 21 AFLP primers. Whereas, Rodríguez et al[6] reported an average of 46.70% of polymorphism on fifteen commercial varieties were obtained from the germplasm bank in Cuba as well as 54% of polymorphism on fifteen cultivated in Mexico[21]. Moreover, Selvi et al[22] published an average of 52% polymorphism within samples from India. The similarity values based on AFLP marker ranged from 0.582 to 0.927 with an average of 0.756 (data not shown). We found that genetic similarity between CO-290 and K84-200 and between CO82-32 and SO10-06 are the highest and lowest similarity, respectively. UPGMA cluster analysis was used to construct a dendrogram which grouped 19 sugarcane cultivars into six groups (A-F) (Figure 2A). While, B37-161, SP50 and B40-98 formed independent groups in group C, E and F, respectively.

3.4 RAPD and AFLP Analysis

Comparison of AFLP and RAPD molecular markers for assessment of genetic diversity among 19 sugarcane cultivars. Generally, when comparing the results from RAPD and AFLP data, there were many more similarities than major differences between them (Figure 2A-B). The AFLP dendrogram (Figure 2A) grouped into three main groups (A, B and D) and three independent groups (C, E and F). The RAPD dendrogram (Figure 2B) grouped into two main groups (I, II). Group I comprised of 8 cultivars (SP80, UT10, CO82-32, 85-2-352, UT11, UT8, K84-200 and CO-290) were split into 2 groups (A and D) that analyzed by AFLP marker. Group II contained 11 cultivars, most of them were grouping in group B based on AFLP marker analysis, except KK80, B37-161, SP50 and B40-98. While, TP07-305 cultivar was formed independent in group II. Normally, AFLP markers simultaneously amplification of large numbers of loci throughout the genome. In this study, genetic similarity was somewhat higher for AFLP (0.582-0.927) than for RAPD (0.449-0.865). The results showed low level of genetic diversity in the

Table 4. Six AFLP primer combinations and percent polymorphism were used in nineteen sugarcane cultivars

Primers (Eco**RI**/ Mse**I**)	**Number of amplified fragments**	**Number of polymorphic fragments**	**Percent polymorphism**
ACG/CTA	41	33	80
AAG/CAA	42	35	83
AGG/CTG	55	43	78
AAG/CAG	57	38	66
AGG/CTT	38	38	100
Total	233	187	80.26

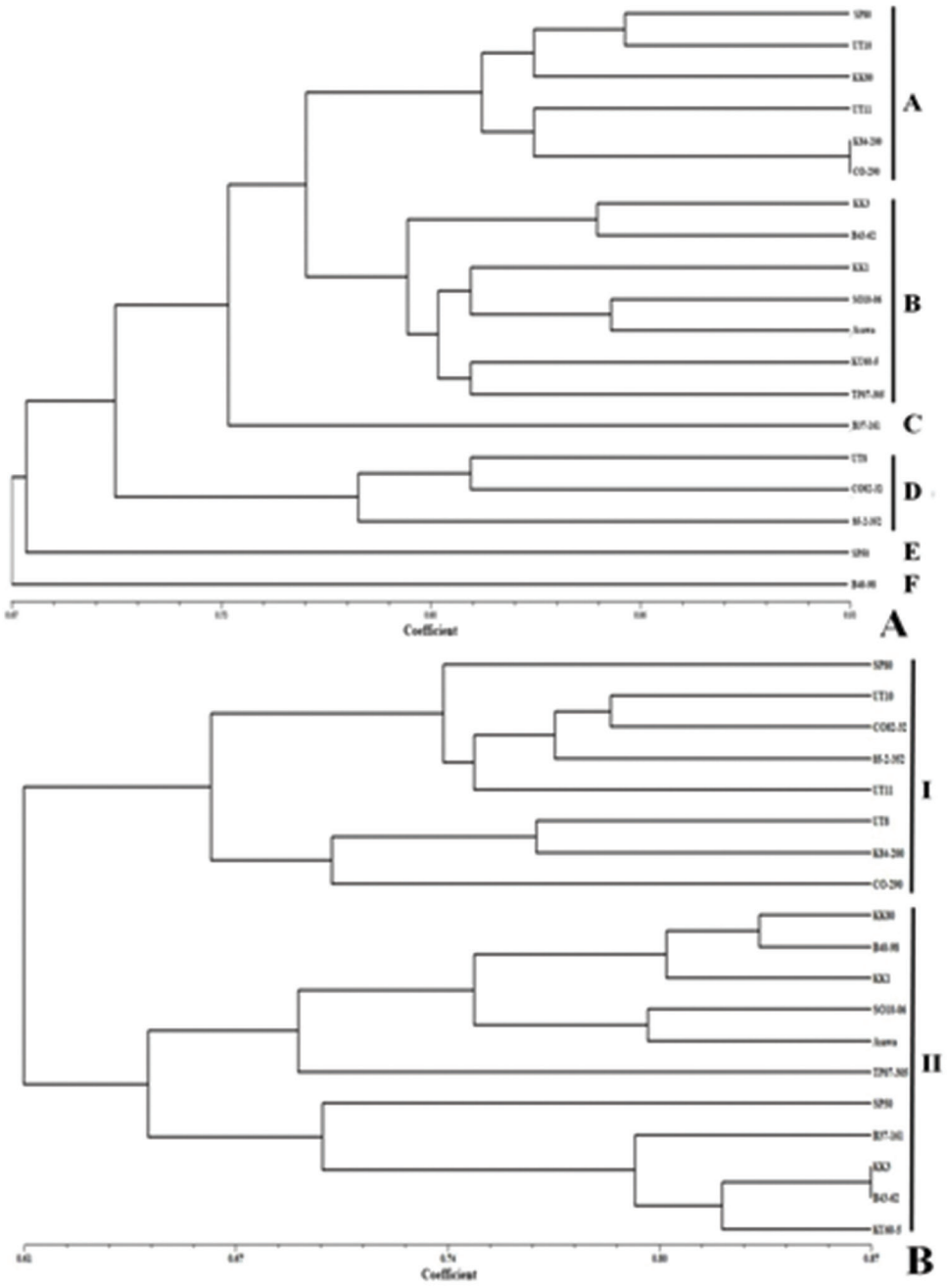

Figure 2. Dendrograms of 19 sugarcane cultivars as shown by UPGMA cluster analysis based on AFLP marker using five primer combinations (A) and RAPD marker using nine primers (B).

germplasm bank of sugarcane in Thailand. Furthermore, the usefulness of the AFLP approach was sufficient to show very high accuracy in providing information for grouping based on UPGMA dendrogram which were able to better discriminate closely related cultivars. The TP07-305, B37-161 and B40-98 cultivars are imported from overseas formed independent groups of RAPD and AFLP markers. For breeding purpose, knowing genetic similarity of the sugarcane cultivars was very important to design an effective breeding program that takes advantage of diverse progeny. For example, two cultivars were selected first one SP50 which carrying high sugar content and second cultivars belonging to either group A or D were suggested to be selected as parents to be crossed.

4. Conclusion

This present study was conducted to reveal genetic diversity among sugarcane cultivars in Thailand using sequencing, RAPD and AFLP markers. The most of them are hybrids cultivars, only one *S. officinarum* and five *S. spontaneum*. For sequencing, the *trn*L- *trn*F region of the chloroplast DNA is not suitable in addressing the questions of relationships. For RAPD marker, only ninety-six RAPD primers were screened and nine primers were analyzed among 56 sugarcane cultivars. The eighty-eight polymorphic bands (96.70%) were detected and genetic similarity was ranging from 0.427 to 0.888, with the average of 0.657 that demonstrated a low level of genetic diversity. When comparing the results from the RAPD and AFLP markers for the genetic similarity in 19 sugarcane cultivars which ranging from 0.582 to 0.927, with the average of 0.756 for AFLP and ranging from 0.449 to 0.865, with the average of 0.657 for RAPD. In this study was showed the genetic base of the population was narrow. Normally for develop a new sugarcane variety, in hybridization process will be selected the samples haveing low level of genetic similarity. The RAPD and AFLP dendrograms represented a similar layout, closely approximating the known pedigree data. However, AFLP marker showed itself to be more efficient at discriminating sugarcane cultivars that are genetically closely related. From this result, RAPD and AFLP markers can be used to help and facilitate the plant breeder to identify and select cultivars for future sugarcane breeding programs.

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