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Assessment of genetic diversity and relationships in pineapple cultivars from Thailand using ISSR marker

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Inter simple sequence repeats (ISSR) markers were applied to assess genetic diversity and genetic relationships among 15 accessions of pineapple (*Ananas comosus* (L.) Merr.) one of the most important agricultural economic fruits in Thailand. Genomic DNA was extracted from fresh leaf samples. Nine ISSR primers were initially screened for analysis and four primers (ISSR1, ISSR3, ISSR 4 and ISSR 5) were chosen for further analysis. A total of 56 DNA fragments, varying from 100-2000 bp, were amplified, of which 27 (48.21%) were polymorphic. A dendrogram showing genetic similarities among pineapple was constructed which based on polymorphic bands using the SPSS program (version 18). Based on the results from the dendrogram, three clusters could be separated with similarity coefficients ranging from 0.316-0.968. The first group included Intrachitdang and Intrachitkhao. The second group included Pattavia, Nanglae and Lakata. The third group included Tadum, Tainan, Pattanie, Phulae, Petburi, Sawee, Phuket, and Tradsithong respectively. Therefore, ISSR analysis is a rapid and suitable method for studying genetic diversity among indigenous Intrachit and others.

Key words: Pineapple, ISSR, genetic diversity, genetic relationships, Thailand.

Introduction

Pineapple (*Ananas comosus* (L.) Merr.) is a member of Bromeliaceae and distinctive for its inflorescence, which is fused into a syncarp, a unique dense rosette of scape-wide leaves and medium to large fruits. They are named for resemblance of the shape of their fruits to the pine cone. Pineapple is normally classified into five groups on the basis of morphology, leaf spination and fruit characteristics, namely: Abacaxi, Cayenne, Maipure or Perolera, Queen and Spanish (Leal and Soule, 1977; Py *et al*., 1987). Pineapple is one of the most popular non-citrus fruits, largely because of its attractive flavour and refreshing sugar-acid balance. Pineapples are found in most tropical and subtropical

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countries. Pineapple was a major tropical fruit cultivated in over 920,000 hectares and 18.2 million tons of pineapple produced in the world (FAO, 2009).

Thailand is one of the major pineapple-growing countries in the world with about 1.9 million tons produced in 2009 (FAO, 2009). On the other hand, Thailand produced about 43% of the world's processed pineapple (Sripaoraya, 2009). Three groups of pineapples have been reported in Thailand (Sripaoraya *et al.,* 2001; Popluechai *et al.,* 2007). The first group is the Queen with small and very spiny leaves. The fruits of the Queen are small and oblong with full yellow shells, small prominent eyes, the flesh are sweet and crispy golden-yellow. The second group is the Cayenne they have leaves with spines confined to the tips. Their fruits are ovoid and medium-sized and pale-yellow, soft and juicy flesh. The last group is the Spanish with varied spines leaves, small to oval or cylindrical-shaped of dark purple fruits with golden-yellow flesh (Bartholomew and Malezieux, 1994; Popluechai *et al.,* 2007).

Like many plants in cultivation, pineapple is highly heterozygous and improvement of many different characters is possible. Breeding programs have made both intraspecific and interspecific crosses and selection has encompassed many aspects of productivity, fruit quality and pest and disease resistance. Over the centuries, numerous pineapple cultivars propagated by vegetative clones have arisen in Thailand. Therefore, homonyms and synonyms may also commonly exist among the names of Thai pineapple cultivars (Sripaoraya *et al.,* 2001).

Understanding the genetic diversity and relationships among pineapple accessions through the use of DNA molecular fingerprinting techniques would assist breeders using pineapple cultivars for breeding programs. Polymerase chain reaction (PCR) based DNA markers provides an opportunity to characterize genotypes and measure genetic relationships more precisely than other markers (Vanijajiva, 2011). Among the various molecular marker techniques, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are easily to be carried out, low cost, and do not require prior genetic information (Williams *et al.,* 1990; Zietkiewicz *et al.,* 1994). The two techniques have been used effectively for identification and assessment of germplasm in pineapple (Prakash *et al.,* 2009). In Thailand the RAPD technique has been used to document the genetic diversity and constructed genetic relationships among Thai pineapple cultivars (Sripaoraya *et al.,* 2001, Popluechai *et al.,* 2007). Although RAPD could amplify the entire information of the genome, but RAPD-PCR has the disadvantage of exhibiting spurious bands due to the low stringency of the technique (Zhao *et al*., 2009; Kumar *et al.,* 2010). ISSR-PCR has been described as an alternative approach for polymorphism studies (Zietkiewicz *et al.,* 1994). The high reproducibility with

ISSR technique is attributable to the use of longer primers allowing for higher annealing temperatures than those of RAPDs (Pradeep *et al.*, 2002). Analysis of genomic DNA using the technique of ISSR is increasingly employed to determine genetic diversity and relationships in plants such as melon and cucumber (Parvathaneni *et al*., 2011), orange (Kumar *et al.*, 2010), mango (Luo *et al.*, 20011), durian (Vanijajiva, 2012), including pineapple (Carlier *et al.*, 2004; Prakash *et al.*, 2009). Assessment of genetic diversity using ISSR techniques in Thai pineapple cultivars may give additional or more information possibly be used in improve breeding of pineapple.

In present study, ISSR polymorphism is used to support the phenotypic identification and to distinguish Thai pineapple cultivars. Genetic diversity and relationships among commercially Thai pineapple varieties were assessed.

Materials and methods

Plant materials

DNA isolation and ISSR analysis were carried out using fresh leaf of samples from 15 accessions collected from pineapple plantation in various provinces from north to south of Thailand (Table 1). Voucher specimens of all accessions are deposited in the Phranakhon Rajabhat University Herbarium.

Genomic DNA isolation

Genomic DNA was extracted from the leaves of 15 accessions using the CTAB method (Vanijajiva, 2011). The leaves (1 g) were ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris–HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol)] 500 µl was added and the solution was incubated at 60 C^o for 30 min. The homogenate was mixed with 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13,000 rpm for 15 min, the upper aqueous layer was transferred to a fresh tube. RNA was removed by treating with 2.5 µl of the RNase (10 mg/ml) for 30 min at 37 C^o. The extraction of DNA with phenol/chloroform/isoamyl alcohol was repeated one more time. DNA in the solution was precipitated with 0.6 volume of ice-cold isopropanol and washed with 70% ethanol. Following this, the DNA was extracted using CTAB DNA extraction protocol without RNase. The process was repeated until the DNA pellet was free of color (two to three times) and the final pellet was dissolved in sterile deionized water. DNA quality and quantity were determined on 0.8% agarose gel. The DNA was stored at -20 C^o, for further use as templates for PCR amplification. The quality of DNA was also evaluated by reading the

absorbance at 260 and 280 nm. The concentration was adjusted to 25 ng/mL used in the PCR analysis.

ISSR-PCR analysis

Nine primers were tested for their ability to amplify ISSR markers. These primers were selected for their ability to give clear, polymorphic, and reproducible patterns of amplification. Each PCR amplification was performed in a final volume of 25 µl of reaction mixture containing 2.5 µl of 10X reaction buffer (100 mM Tris–HCl pH 9, 500 mM KCl, 1% Triton X-100), 0.4 mM of each dNTP, 0.6 mM of primer, 1 unit of Taq polymerase (Promega), 5 mM $MgCl_2$ and 100 ng template DNA. PCR was performed using a Thermohybaid PX2. The first cycle consisted of denaturation of template DNA at 94 C^o for 5 min, primer annealing at 47-55 C^o for 1 min and primer extension at 72 C^o for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was the same as in the first cycle. The last cycle consisted of only primer extension at 72 C^o for 7 min. A negative control reaction in which DNA was omitted and included in every run in order to verify the absence of contamination. The ISSR products were separated by agarose (1.8% w/v) gel electrophoresis at 150 A for 30 minutes in 0.04 M TAE (Tris–acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with ethidium bromide (10 mg/ml), and photographed on a UV transluminator. To determine ISSR profiles, the size of each DNA band was inferred by comparison with a 100 bp DNA ladder (Promega), used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by three repeating tests for each primer at different times.

Gel scoring and data analysis

Only strong and reproducible ISSR bands were scored. Different observed patterns were scored as discrete variables, using 1 to indicate the presence and 0 to indicate the absence of a unique pattern. The SPSS (version 18) data analysis package was used for the statistical analyses. Relationships among individuals were determined by the distance matrix method. Nei and Li's Dice similarity coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix. A dendrogram was constructed from this matrix on the basis of the hierarchical cluster analysis, which is based on the average linkage between groups, i.e. the unweighed pair-group method algorithm (UPGMA). A principal component analysis (PCA) was also conducted using a genetic distance matrix obtained from the binary

data set. It was negated and rescaled (0–1), using the Euclidean distance between pair-wise comparison of individuals (Ludwig and Reynolds, 1988).

Results

DNA isolation

DNA extracted from pineapple leaf using a modified Vanijajiva (2011) gave a good and sufficient quality DNA for PCR reaction, and the amount of DNA extracted from the accessions ranged from 129 to 192 µg/g fresh weight leaf material. The ratios of A260/A280 varied from 1.65 to 1.83. The quality of DNA was also tested by PCR, which confirmed that the DNAs were suitable for PCR reaction.

ISSR polymorphism

In order to obtain primers that can be used effectively in ISSR analysis, nine primers were screened (Table 1) and those primers that generated clear bands were identified. Four primers that could produce reproducible fragments were selected for further investigation (Table 2). For 15 commercial cultivars of pineapple listed in Table 1, the four ISSR primers amplified total of 56 bands and 27 bands of these bands were polymorphic. The percentage of polymorphism of the amplified products was 48.21% (Table 3). The size of amplified bands ranged from approximately 100 to 2000 bp. ISSR5 possessed the lowest polymorphism (41.66%), while ISSR 1 showed the highest polymorphism (53.84 %).

Table 1. Samples of Pineapple used in this study

Pineapple cultivars	Collection site in Thailand	Vouchers	Sample number
Intrachitdang	Chacheosao	OV002-11	P1
Intrachitdang	Petchburi	OV012-11	P2
Intrachitkhao	Chacheosao	OV001-11	P3
Intrachitkhao	Petchburi	OV011-11	P4
Pattavia	Chacheosao	OV003-11	P5
Phuket	Phuket	OV021-11	P6
Tradsithong	Petchburi	OV020-11	P7
Phulae	Chiang rai	OV015-11	P8
Nanglae	Chiang rai	OV016-11	P9
Sawee	Chumporn	OV018-11	P10
Petburi 1	Petchburi	OV022-11	P11
Lakata	Petchburi	OV023-11	P12
Pattanie	Petchburi	OV024-11	P13
Tadum	Petchburi	OV025-11	P14
Tainan	Petchburi	OV026-10	P15

Table 2. Sequences of arbitrary primers, sizes and number of amplified bands, and the percentage of polymorphic bands resulting from the ISSR analysis

Primer No.	Sequence	Size range (bp)	No. of ISSR bands	Polymorphic band%
ISSR1	5'AGGAGGAGGAGGAGGAGG3' $(AGG)_6$	100-2000	13	7 (53.84%)
ISSR2	5'CTCCTCCTCCTCCTCCTC3' $(CTC)_6$	-	-	-
ISSR3	5'AGAGAGAGAGAGAGT3' $(AG)_7T$	100-1,200	19	9 (47.36%)
ISSR4	5'GAGAGAGAGAGAGAGAC3' $(GA)_8C$	200-1,000	12	6 (50%)
ISSR5	5'GAGAGAGAGAGAGAGAT3' $(GA)_8T$	300- 1,500	12	5 (41.66%)
ISSR6	5'TCTCTCTCTCTCTCTCA3'$(TC)_8A$	-	-	-
ISSR7	5'CTCTCTCTCTCTCTCTG3' $(CT)_8G$	-	-	-
ISSR8	5'ACACACACACACACACG3' $(AC)_8G$	-	-	-
ISSR9	5'GGGGTGGGGTGGGGT3' $(GGGGT)_3$	-	-	-

Table 3. Pair-wise genetic similarity of 15 cultivars pineapple according to the index of Nei and Li (1979)

Taxa	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
P1	1.000														
P2	.968	1.000													
P3	.897	.857	1.000												
P4	.857	.897	.968	1.000											
P5	.769	.720	.720	.667	1.000										
P6	.545	.476	.476	.400	.545	1.000									
P7	.545	.609	.316	.400	.769	.769	1.000								
P8	.545	.476	.476	.400	.667	.933	.857	1.000							
P9	.720	.769	.667	.720	.897	.609	.720	.609	1.000						
P10	.476	.400	.400	.316	.720	.897	.897	.897	.545	1.000					
P11	.476	.400	.545	.476	.720	.897	.815	.968	.667	.857	1.000				
P12	.720	.667	.667	.609	.968	.609	.720	.720	.933	.667	.769	1.000			
P13	.609	.545	.667	.609	.720	.815	.815	.897	.545	.857	.857	.667	1.000		
P14	.476	.400	.545	.476	.720	.815	.815	.897	.667	.857	.857	.769	.933	1.000	
P15	.545	.476	.609	.545	.667	.857	.769	.933	.609	.815	.897	.720	.968	.968	1.000

Genetic diversity analysis

In order to estimate genetic diversity among Pineapple cultivars in Thailand, genetic similarity coefficients (GSC) were calculated. The similarity matrix obtained using Nei and Li's coefficient (Nei and Li, 1979) is shown in Table 3. Similarity coefficients ranged from 0.316-0.968 in 15 Pineapple cultivars tested in the present experiment, with the lowest value obtained for

P3-P7 and P4-P10, whereas P1-P2 and P3-P4 show the highest similarity values.

A cluster analysis was performed based on GSC by means of the Nei and Li (1979) and the UPGMA method. The dendrogram grouped the 15 accessions in three main clusters designated as Spanish group Cayenne group and Queen group (Fig. 1). Spanish group included three accessions: Intrachitdang (P1 and P2) and Intrachitkhao (P3 and P4). Cayenne group included three accessions: Pattavia (P5), Nanglae (P9) and Lakata (P12). Queen group included eight accessions: Tadum (P14), Tainan (P15), Pattanie (P13), Phulae (P8), Petburi 1 (P11), Sawee (P10), Phuket (P6), and Tradsithong (P7) respectively. The PCA of the ISSR data supported UPGMA clustering (Fig. 2).

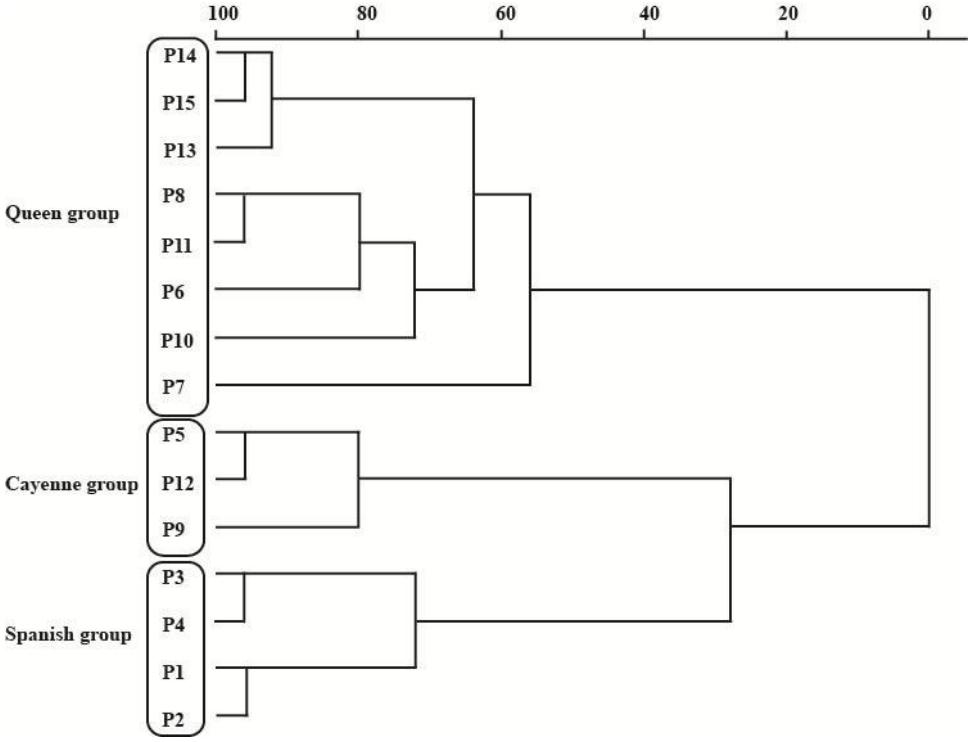

Fig. 1. Dendrogram showing genetic relationship among 15 cultivars of pineapple based on ISSR.

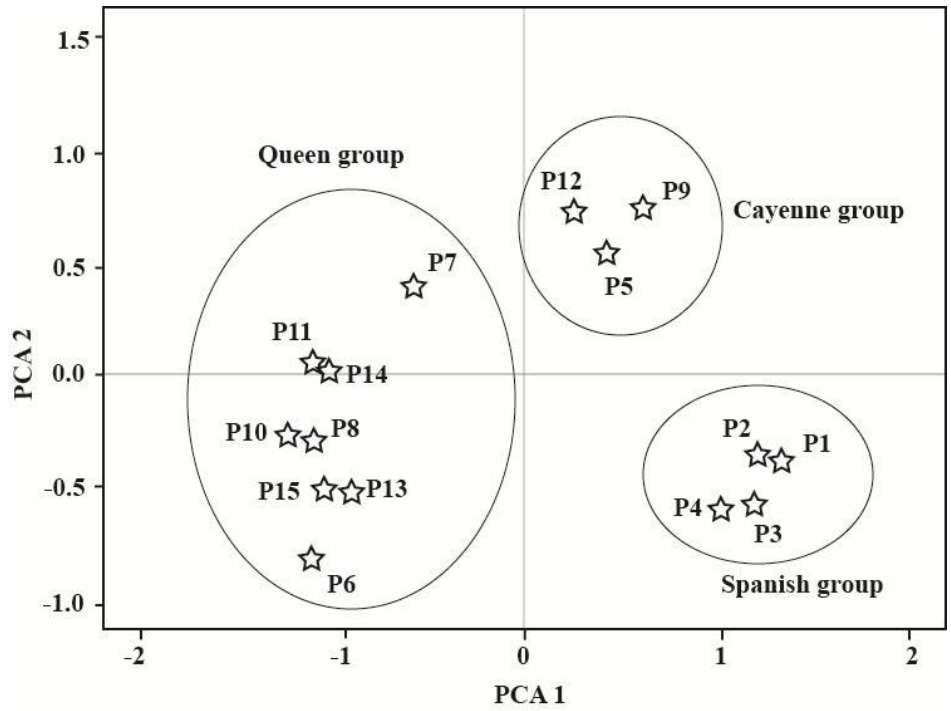

Fig. 2. Plot of PCA analysis of genetic distance of 15 pineapple cultivars from ISSR, showing groups of individual plants

Discussion

This is the first report of using ISSR markers in detecting genetic variations of pineapple cultivars in Thailand. Quite considerable genetic variability does exist among different of Thai pineapple cultivars. In previous studies, Thai pineapple cultivars have been identified by their RAPD profiles (Sripaoraya *et al.*, 2001; Popluechai *et al.*, 2007).

After PCR reaction optimization, the four selected primers gave 27 scorable bands. The average number of amplified bands of each primer was 6.75, and the polymorphism was 48.21%. A high polymorphism indicated that pineapple DNA has a high mutation probability. This high mutation probability could be due to the wide geographic distribution and considerable ecological variations. The results agreed with Aradhya *et al.* (1994), who reported that morphological differentiation among the species of Ananas may be caused by ecological isolation. This could also indicate that random primers have a high potential for detecting polymorphisms at the species level. The ISSR markers are powerful tools for pineapple genetic studies as they can detect very low-level genetic variations. In this study, we found that the number of bands and polymorphic bands produced by each primer was highly varied. The highest

number of bands (19) was produced by primers ISSR3, while primer ISSR4 and ISSR5 generated the lowest amplified bands (12).

ISSR analyses on 15 accessions were clustered into three groups by UPGMA on the basis of 27 polymorphic fragments. The first group included four accessions of two cultivars Intrachitdang, Intrachitkhao with nearly identical amplification profiles, indicating that they possibly have a common ancestry. Low level of molecular polymorphisms between pineapple cultivars of Intrachit cultivars (Spanish group) were also observed using RAPD markers (Popluechai *et al.,* 2007). This low level of the polymorphisms may cause by the narrow genetic background of the cultivated Intrachit pineapple (Popluechai *et al.,* 2007). The second group contains three pineapple cultivars of Cayenne group; Pattavia, Nanglae and Lakata, while the third group included Tadum, Tainan, Pattanie, Phulae, Petburi, Sawee, Phuket, and Tradsithong which belong to the Queen group as previously report using taxonomic characters (Bartholomew and Malezieux, 1994) and RAPD analysis (Sripaoraya *et al.,* 2001; Popluechai *et al.,* 2007). The range of GSC among the 15 accessions was 0.316–0.968. The GSC obtained from our ISSR analysis demonstrated that the level of genetic diversity was relatively high among pineapple accessions. The genetic location of the clusters obtained from PCA also demonstrated wide genetic variability among the clusters. The results of this study illustrated that: (1) the ISSR markers were appropriate for detecting relationships among pineapple accessions, (2) the polymorphism (48.21%) produced by ISSR markers was quite high, and (3) large genetic differentiation existed in pineapple. These results indicated that ISSR markers are useful for the genetic diversity analysis, confirmation of identical genotypes, identification the closest pineapple cultivars, and clones with different names, which is helpful for the management of germplasm and is also useful to improve the current breeding strategies.

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