Genetic relationship assessment of pineapple germplasm in Thailand revealed by AFLP markers

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ABSTRACT

The study is aimed to depict phylogenetic relationship among 37 pineapple accessions collected from government research center, private company germplasm repository and commercial growing areas in Thailand using AFLP technique. Twenty–one primer combinations were used and 675 AFLP markers were produced. Of these, 468 markers (69.33%) were found to be polymorphic. Jaccard similarity coefficient among the samples was 0.62-1.0. Cluster analysis was performed using UPGMA method. The phylogenetic tree could separate all pineapple accessions into 9 groups at genetic similarity of 0.83. The first cluster, consisted of 14 accessions, which represented morphological characteristics of Cayenne group. The second cluster, consisting of 9 accessions, was in Queen group. The third cluster was White jewel, in Perolera group. Clusters 4-9 were hybrid, Spanish, Pernambuco groups and exotic varieties. Principle component analysis (PCA) was also performed and the results were in agreement with phylogenetic analysis. PCA1, 2 and 3 were counted for 60.19% of total variation indicated moderate genetic diversity of the samples. The present study also showed that AFLP technique clearly identified 4 commercial hybrid varieties close to Queen or Spanish group. These data will be useful for hybrid selection in breeding program and germplasm evaluation in the future.

Keywords: AFLPs; *Ananas comosus*; genetic relationship; pineapple

INTRODUCTION

Pineapple (*Ananas comosus* L., Merr., 2n=50), is an economically important perennial monocot of the family Bromeliaceae. There are about 30 commercial pineapple cultivars worldwide, which were classified into 5 groups, including Cayenne, Queen, Spanish, Pernambuco (or Abacaxi) and Perolera (or Maipure). Morphological classification showed clear differences in length and shape of leaf, fruit size, fruit shape or spine characteristics among these groups. (Py *et al.*, 1987; Coppens d'Eeckenbrugge *et al.*, 2011).

Thailand is one of five pineapple producing countries in the world. The export products of pineapple include canned, sliced, prepared or preserved products generated about 510.72 millions of US dollars in 2013 (FAOSTAT, 2016). There are many plantation areas distributed in the country, which main areas located in Prachuap Khiri Khan, Phetchaburi, Rayong, Chachoengsao, Chanthaburi and Trat provinces. There are only 3 groups of commercial pineapples in Thailand. The first group is Cayenne, which is best for fresh fruits and canned, such as Pattavia, Sriracha and Nanglae cultivars. The second group is Queen consisting of Phuket, Pulae, Trad Sithong and Sawee cultivars. The third group is Spanish including Intra Chit Dang and Intra Chit Khaw cultivars. Generally, pineapple is mostly self–incompatible species which required genetic differences between parents to produce hybrids (Zhang *et al*., 2014). However, some cultivars were selected from spontaneous mutation or breeding program with unknown of pedigree. No information

of the genetic relationships of all varieties has been reported (Sripaoraya *et al.*, 2001)

Recently, DNA fingerprint analysis has been used to access genetic makeup of pineapples such as ISSR markers which was used for genetic diversity study in pineapples (Vanijajiva, 2012; Sousa *et al*., 2013) or screening of segregation populations between *Ananas bracteatus* and *A. comosus* (Carlier *et al*., 2004). Simple Sequence Repeat (SSR) markers, one of the most powerful markers for quantifying and comparing levels of inter-species genetic variations, could not clearly identify cultivars into correct pineapple groups. DNA profiling from SSR analysis should be utilized for cultivar protection systems (Shoda *et al*., 2012; Feng *et al*., 2013), or analysis of different pineapple germplasm collections and elucidation of synonymies and homonymies present in the different collections (Rodríguez *et al*., 2013). Cluster analysis of pineapple cultivars in Thailand was also performed by RAPD technique (Popluechai *et al*., 2007)

The genetic diversity of germ plasm collection can be assessed through pedigree analysis and DNA fingerprints. Amplified fragment length polymorphism (AFLP) is a rapid and efficient method for producing DNA fingerprints without sequence information. The technique is stable and reproducible (Vos *et al*., 1995; Kladmook *et al*., 2010). AFLP markers have been used to estimate genetic diversity in several plant species, such as gladiolus (Ranjan *et al*., 2010), lotus (Hu *et al.*, 2012), curcuma (Das *et al*., 2011), cassumunar ginger (Kladmook *et al*., 2010), mandarin (Dorji and Yapwattannaphun, 2015) and pea (Dyachenko *et al*., 2014). AFLP markers are used in pineapple for analysis of genetic diversity and germplasm evaluation (Kato *et al.*, 2004; Tapia *et al*., 2005, Pérez *et al*., 2009; Paz *et al*., 2012), *in vitro* somaclonal variation detection (Pérez *et al*., 2011), genetic mapping (Carlier *et al*., 2004; Carlier *et al*., 2012, Sousa *et al*., 2013) and genetic fidelity or homogeneity (Scherer *et al*., 2015).

In this study, AFLP markers were used for evaluation of genetic relationship among 37 pineapple accessions collected in Thailand. The genetic information data combined with morphological characters could be used for breeding selection program and screening or managing of germplasm collection.

MATERIALS AND METHODS

Plant materials

Fourteen pineapple accessions from Petchaburi Agricultural Research and Development Center (PB ARDC), Department of Agriculture, Ministry of Agriculture and Cooperative; fifteen accessions from pineapple germplasm collection of Tipco Biotech Limited Company (Tipco) Prachuap Khiri Khan and 8 accessions from other growing areas in Thailand were collected (Table 1).

DNA extraction

The genomic DNA was extracted from young leaves using CTAB method modified from Doyle and Doyle (1990). Quality of the DNA was observed on a 0.8% agarose gel and the concentration was evaluated using a NanoDrop ND–1000 spectrophotometer (NanaDrop Technologies, Wilmington, USA).

AFLP analysis

AFLP analysis was performed according to Vos *et al.* (1995) method. DNA (500 ng) was digested with 2 restriction enzymes (*Eco*RI and *Mse*I). The digested DNA was ligated into adapters which have specific sites for the two restriction enzymes: *Eco*RI adapter 5'–CTCGTAGACTGCGTACC–3' /3'– CATC TGACGCATGGTTAA–5' and *Mse*I adapter5'–GACG ATGAGTCCTGAG–3' /3'–TACTCAGGACTCAT–5'. The *Eco*RI+A (5'–AGACTGCGTACCAATTCA–3') and *Mse*I+C (5'–GACGATGAGTCCTGAGTAAC–3') oligonucleotide primers were used for the pre–selective amplification step. The second selective amplification step was conducted using variouscombination between *EcoR*I+ACA/AAG/ACA/ACC/ACG/ACT/AGC/AGG /ATG and *Mse*I+CAC/CTA/CAA/CAT/CAG/CTC/ CTT/CTG. List of AFLP primer combinations used in this study is shown in Table 2. The PCR products were separated on 6% denaturing polyacrylamide gel and stained with silver nitrate as described by Caetano–Anolles (1997)

Data analysis

Total number of bands were scored and analyzed by NTSYSpc version 2.1m program (Rohlf, 2005). The Jaccard's similarity coefficient was used in estimation of genetic similarity among samples. Phylogenetic tree was generated by Unweighted pair group method with arithmetic analysis (UPGMA). The test for the goodness of fit for clustering in UPGMA algorithm, cophenetic value and Mantel's test (Mantel, 1967) were evaluated. Principal component analysis (PCA) was calculated for determining genetic relationships of each sample. FreeTree program (www.natur.cuni.cz/flegr/programs/ freetree.htm) was also performed by NTSYSpc software for approval of the degree of confidence at the nodes of the phylogenetic tree by bootstrap

analysis. Polymorphic information content (PIC) was calculated from randomized selection of the frequency of polymorphism between two samples by the formula: $PIC = 1–\sum p_i^2$ where p_i is the frequency of the i^{th} band (Ott, 1991).

Table 1 Pineapple accessions (*Ananas* sp.) used in this study.

Acc.no.	Cultivar	Scientific name	Group	Source
1	Pattavia	*A. comosus*	Cayenne	Tipco
2	Sriracha	*A. comosus*	Cayenne	Tipco
3	Raimuang	*A. comosus*	Cayenne	Loei
4	Nanglae	*A. comosus*	Cayenne	Tipco
5	Huai Mun	*A. comosus*	Cayenne	Tipco
6	Lampang	*A. comosus*	Cayenne	Tipco
7	Tadam	*A. comosus*	Cayenne	Chanthaburi
8	Clone 10	*A. comosus*	Cayenne	Tipco
9	Clone 30	*A. comosus*	Cayenne	PB ARDC
10	Wansingkorn	*A. comosus*	Cayenne	Prachuap Khiri Khan
11	Phuket	*A. comosus*	Queen	Tipco
12	Sawee	*A. comosus*	Queen	Tipco
13	Phulae	*A. comosus*	Queen	Tipco
14	Trad Sithong	*A. comosus*	Queen	Tipco
15	Singapore	*A. comosus*	Queen	PB ARDC
16	Singapore-Pattavia (spiny)	*A. comosus*	Queen	PB ARDC
17	Singapore-Pattavia	*A. comosus*	Queen	PB ARDC
18	Phetburi	*A. comosus* var.Tainan 41	Queen	PB ARDC
19	Homsuwan	*A. comosus*	Cayenne	Tipco
20	MD-2 (spiny)	*A. comosus*	Cayenne	Rayong
21	MD-2	*A. comosus*	Cayenne	Rayong
22	MD-2 (tissue culture)	*A. comosus*	Cayenne	Rayong
23	Leuang Sam Roi Yod	*A. comosus*	Cayenne	Prachuap Khiri Khan
24	Tropical Gold	*A. comosus*	Cayenne	PB ARDC
25	Seiko	*A. comosus*	Cayenne	PB ARDC
26	HANA 17	*A. comosus* var. comosus ‘ Natal’	Queen	PB ARDC
27	HANA 25	*A. comosus* var. comosus ‘ Macgregor’	Queen	PB ARDC
28	HANA 58	A. hybrid , Wild Brazil X Lot520	Intra-specific hybrid	Tipco
29	HANA 63	*A. erectifolius*	*Ananas* sp.	Tipco
30	HANA 64	*A. bracteatus*	*Ananas* sp.	PB ARDC
31	HANA 100	*A. comosus* var. comosus, Cayenne # 59 4N	Cayenne	PB ARDC
32	HANA 114	*A. comosus* var. comosus, Cayenne Bottleneck	Cayenne	PB ARDC
33	HANA 133	*A. comosus* var. comosus, ‘Kew’	Cayenne	PB ARDC
34	White Jewel	*A. comosus*	Perolera	Tipco
35	Brazil	*A. lucidus*	Pernumbuco	Tipco
36	Intra Chit Dang	*A.* comosus	Spanish	Chachoengsao
37	Intra Chit Khaw	*A.* comosus	Spanish	Chachoengsao

PB ARDC = Petchaburi Agricultural Research and Development Center; Tipco = Tipco Biotech Limited Company

Table 2 List of AFLP primer combinations, number of amplified bands, percentage of polymorphism and polymorphic information content obtained from the analysis of 37 pineapple accessions.

No.	Primer pair	Total bands/Polymorphic bands	%Polymorphism	PIC
1	E+ACA/M+CAC	33/21	63.64	0.147
2	E+ACA/M+CAT	25/11	44.00	0.109
3	E+ACA/M+CTA	25/18	72.00	0.179
4	E+AAG/M+CAC	26/12	46.15	0.080
5	E+AAG/M+CAT	32/20	62.50	0.119
6	E+AAG/M+CTA	40/32	80.00	0.153
7	E+ACA/M+CAA	37/17	45.95	0.099
8	E+ACA/M+CAC	23/16	69.57	0.186
9	E+ACC/M+CAG	29/23	79.31	0.165
10	E+ACC/M+CTC	28/21	75.00	0.164
11	E+ACG/M+CAT	18/15	83.33	0.182
12	E+ACG/M+CTA	17/13	76.47	0.138
13	E+ACT/M+CAA	52/38	73.08	0.173
14	E+ACT/M+CAG	44/35	79.55	0.147
15	E+ACT/M+CAT	37/29	78.38	0.195
16	E+ACT/M+CTA	38/28	73.68	0.187
17	E+ACT/M+CTC	43/31	72.09	0.165
18	E+ACT/M+CTT	67/45	67.16	0.131
19	E+AGC/M+CTG	18/14	77.78	0.087
20	E+AGG/M+CAA	21/13	61.90	0.157
21	E+AGG/M+CTT	22/16	72.73	0.183
	Total	**675/468**	**-**	**3.146**
	Average	**32.14/22.29**	**69.33**	**0.150**

RESULTS AND DISCUSSION

AFLP polymorphisms

Genetic variation among 37 pineapple accessions was determined using AFLP technique. Twenty-one from 72 primer combinations, of *Mse*I/*Eco*RI clearly showed that they could be utilized to differentiate the pineapple groups (Cayenne, Queen, Spanish, Perolera and *Ananas* sp.). Totally, 675 AFLP markers were scored and 468 markers were found to be polymorphism (69.33%) while 207 markers (30.67%) were monomorphism. PIC scores of all AFLP markers ranged from 0 to 0.50 with an average PIC at 0.15. Out of the total 675 markers, 211 markers (31.26%) had PIC scores between 0.00 to 0.05, following by 133 markers (19.70%) had PIC scores between 0.05 to 0.10 and only 71 markers (10.52%) were in between 0.45 to 0.50. The rest of 260 markers (38.52%) had PIC scores distributed in wide ranges, between 0.10 to 0.45 (Figure 1, Table 2). PIC value is generally used as an index of genetic diversity. The maximum PIC score for dominant markers is 0.50. The average PIC value (0.15) in this study indicated low genetic variation among pineapple accessions. PIC value was correlated with the average polymorphism which was 69.33% in this study. This polymorphism level was consistent with the results of Popluechai *et al.* (2007), who reported 70.4% of the average polymorphism among 9 Thai pineapple cultivars based on RAPD analysis and Paz *et al.* (2012), who reported 64.3% of the polymorphism among 55 genotypes of *Ananas comosus* collection in Cuba using AFLP markers.

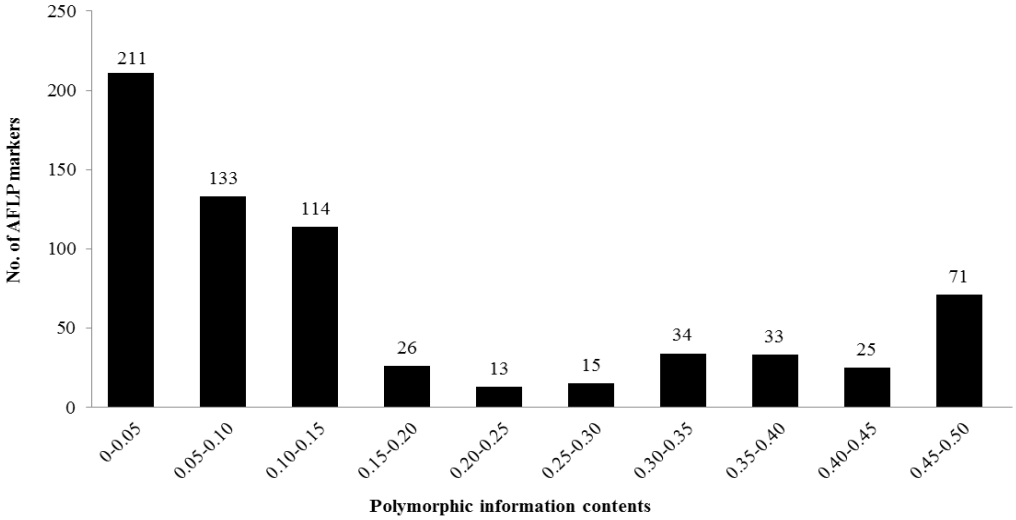


Figure 1 Polymorphic Information Contents (PICs) of 675 AFLP markers from 37 pineapple accessions.

Cluster analysis

The genetic similarity was employed to evaluate genetic relationship among commercial cultivars and within *Ananas* species using Jaccard coefficient (Jaccard, 1908). The genetic similarity among the samples ranged from 0.62 to 1.00 and at genetic similarity of 0.83, the 37 pineapple accessions could be separated into 9 major clusters (Figure 2). Cluster 1 consisted of all varieties in Cayenne group which were Pattavia, Sriracha, Lampang, Clone 10, Raimuang, Clone 30, Nanglae, Huai mun, Tadam, Wansingkorn, HANA 100, HANA114, HANA 133 and Seiko. The results were inconsistent with Vanijajiva (2012) who reported the classification of Tadum cultivar into Queen group based on ISSR marker analysis. However, based on the morphology characteristic classification such as the spineless, Tadum cultivar should be in Cayenne group. All pineapple cultivars in cluster 1 were showed very close relationships among themselves with high genetic similarity (0.95) and could be divided into 5 subclusters (1.1–1.5) as shown in Figure 2. Pineapples in Cayenne group had similar morphological characteristics, which were either absence of spine or presence of a few spines near the leaf tip. However, some cultivars in cluster 1 showed differences in fruit size, flesh color and thickness of fruit eyes. The variations of these characteristics in pineapple have been reported as climate and ecological adaptations (Ruas *et al.*, 2001; Coppens d'Eeckenbrugge *et al.*, 2011). Our results indicated these cultivars were found to be separated by few AFLP DNA bands. Similarity between Pattavia and Sriracha was 100%, whereas these two cultivars were 99.7% similar to Lampang cultivar. However, Pattavia cultivar could not be differentiated from Sriracha cultivar in that they both exhibited similar morphological characteristics of plant, fruit shape and also DNA band patterns. The results suggested that they were the same cultivar, but Pattavia was grown in the south, whereas Sriracha cultivar was grown in the Eastern region of Thailand.

Cluster 2 included seven pineapple cultivars and two varieties in Queen group which were Phuket, Phulae, Sawee, Trad Sithong, Singapore, Phetburi, Singapore–Pattavia, HANA 17 and HANA 25. These pineapple cultivars could be divided into 4 subclusters (2.1–2.4) as shown in Figure 2. The Queen group could be distinguished from other groups with clear morphological characteristics based on the presence of spines along the edge of leaves (Adikaram and Abayasekara, 2012). In the Queen group, two Singapore–Pattavia showed the most distinct accessions with the genetic similarity around 0.84 compared with the rest of accessions. Genetic similarity between Pluket and Plulae was 100%, whereas these two cultivars were 99.7% similar to Sawee. The AFLP analysis suggested that Phuket and Phulae could be the same cultivar, similar to the results from RAPD reported by Popluechai *et al.* (2007). In fact, it is known that Phuket and Sawee have originated in the south of Thailand, whereas Phulae is Phuket cultivar that cultivated in the Northern part of Thailand in Chiang Rai province. Phulae cultivar has smaller plant and fruit size with yellow flesh, crispier, more fragrant and sweeter than Phuket cultivar. These different characteristics could be caused by different agricultural practices, geographical area and climatic zone. However, other types of markers with high detection efficiency might be able to distinguish these two cultivars.

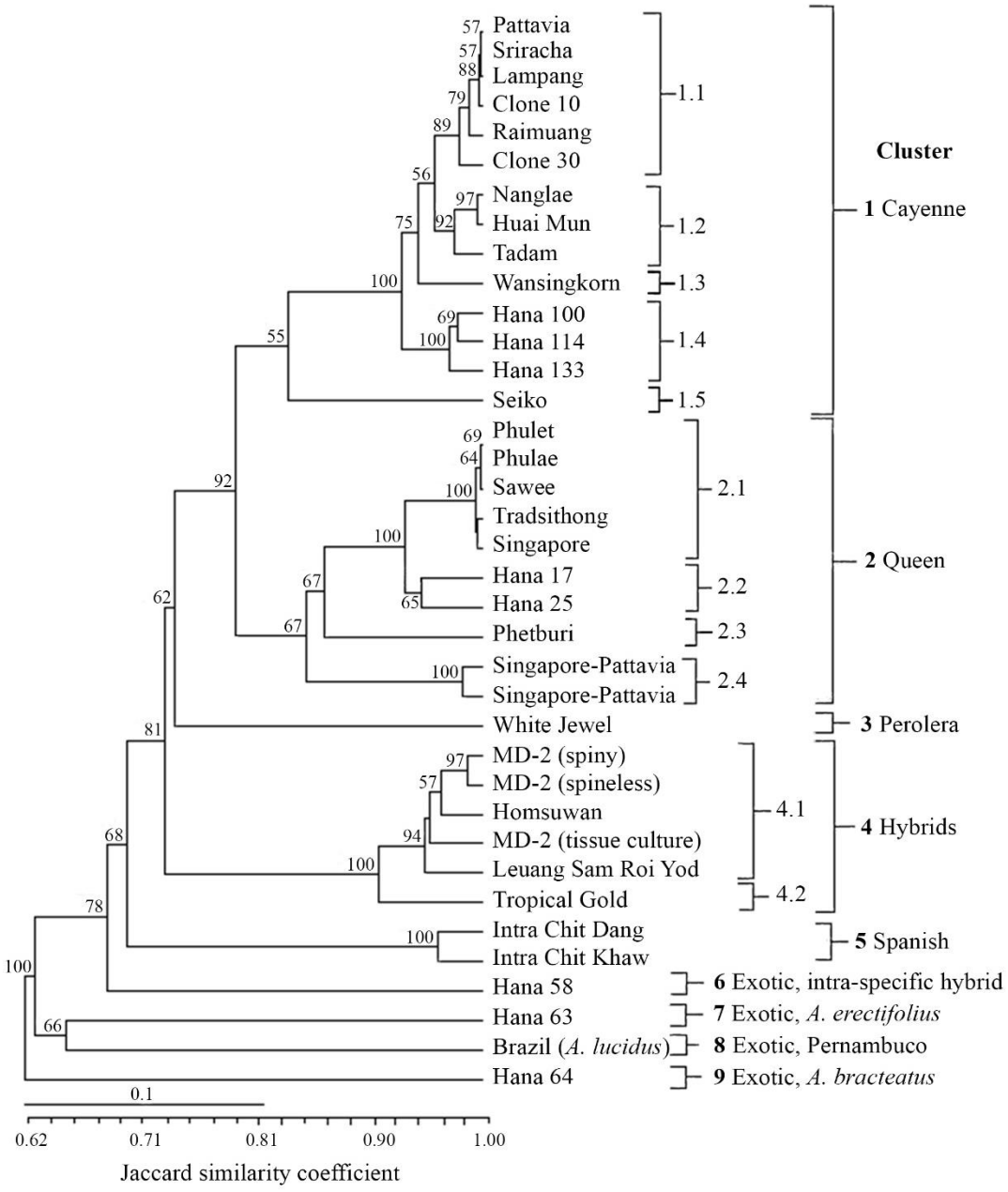


Figure 2 Cluster analysis of 37 pineapple accessions obtained through UPGMA method by NTSYS-pc version 2.10m software using DNA patterns from AFLP technique. Numbers indicate bootstrap values greater than 50% for 500 replications.

Cluster 3 was the White Jewel, a variety that was classified into Perolera group. The White Jewel is an exotic variety, originally imported from Brazil. With no spine present, it has piping leaf, sweet fruit with strong fragrance and pearly white pulp. It was a selected clone from multiplication by micropropagation done by Petchaburi Agricultural Research and Development Center. This variety was registered in Thailand as Phetburi No.2. Cluster 4 included four hybrid cultivars which were Homsuwan, MD-2, Leuang Sam Roi Yod and Tropical Gold. The hybrid cultivars were classified into two subclusters (4.1 and 4.2). The phylogenetic tree indicated that MD-2, one of the hybrid cultivars developed in Hawaii at the Pineapple Research Institute, was genetically close to Queen and Spanish group similar to the study using ITS sequences by Hidayat *et al.* (2013).

Furthermore, genetic variations among spiny and spineless accessions from the same cultivars were very low. The genetic similarity among MD-2 (spineless),

MD-2 (spiny) and MD-2 (tissue culture) in cluster 4 were 0.995, whereas genetic similarity between Singapore-Pattavia (spineless) and Singapore-Pattavia (spiny) from cluster 2 was 0.859. The results suggested that the presence or absence of spine in pineapple possibly was not determined primarily by genetic differences, although some genetic variations could be transmitted to the progeny (Cabral *et al.*, 1997).

Cluster 5 was Spanish group including Intra Chit Dang and Intra Chit Khaw. Intra Chit Dang has a long history of more than 300 years. It was imported to Thailand by Portuguese during the period of 1670–1700 (Collins, 1960). This cultivar is still grown in Bang Khla district, Chachoengsao province. Some morphological characteristics, such as shoot size is bigger than Pattavia cultivar. It has red-brown color at the leaf edge with regularly curved spine. The fruit is small, protruding eyes with high fiber content, (Adikaram and Abayasekara, 2012). Incontrast, Intra Chit Khaw, which has green color on leaf, is reported to be a mutant of Intra Chit Dang (Popluechai *et al.*, 2007).

The varieties belonging in clusters 6 to 9 were exotic varieties. Pineapple variety in cluster 6, Hana 58 (*Ananas* hybrid, Wild Brazil X Lot520), was the intra-specific hybrid. Cluster 7, Hana 63 (*A. erectifolius*), and cluster 8, Brazil (*A. lucidus*), were classified as members of Pernambuco group. Lastly, cluster 9, Hana 64, was found to be *A. bracteatus.* This variety is an exotic from Hawaii and is collected for the purpose of breeding program at Petchaburi Agricultural Research and Development Center.

Cophenetic value and Mantel's test were evaluated from AFLP markers in order to test for the goodness of fit for clustering in UPGMA algorithm. The cophenetic correlation value from this study was r=0.98, meaning good representative of the data matrix in the dendrogram of cluster analysis. Whereas, the PCA was calculated using genetic similarity from 675 markers of 37 pineapple accessions. The distribution pattern from PCA was correlated with the UPGMA clustering (Figure 3) and related with morphological characteristic classification of each pineapple group. Examples of some pineapple cultivars in each group were demonstrated in Figure 4. The PCA 1, 2 and 3 were accounted for 41.2, 10.5, and 8.47% of the total variations, respectively.

In summary, AFLP technique can be used for study of pineapple genetic relationship in Thailand. Results obtained from this study revealed that the AFLP marker could be used to classify 37 pineapple accessions into 9 clusters, which were consistent with the morphological characteristic classification. The AFLP profile and cluster analysis revealed moderate genetic diversity among some pineapple cultivars in Thailand. Some of AFLP markers suggested close relationships between some well-known cultivars. The results from the present study will be useful for parent selection in breeding program and germplasm management/maintenance in the future.

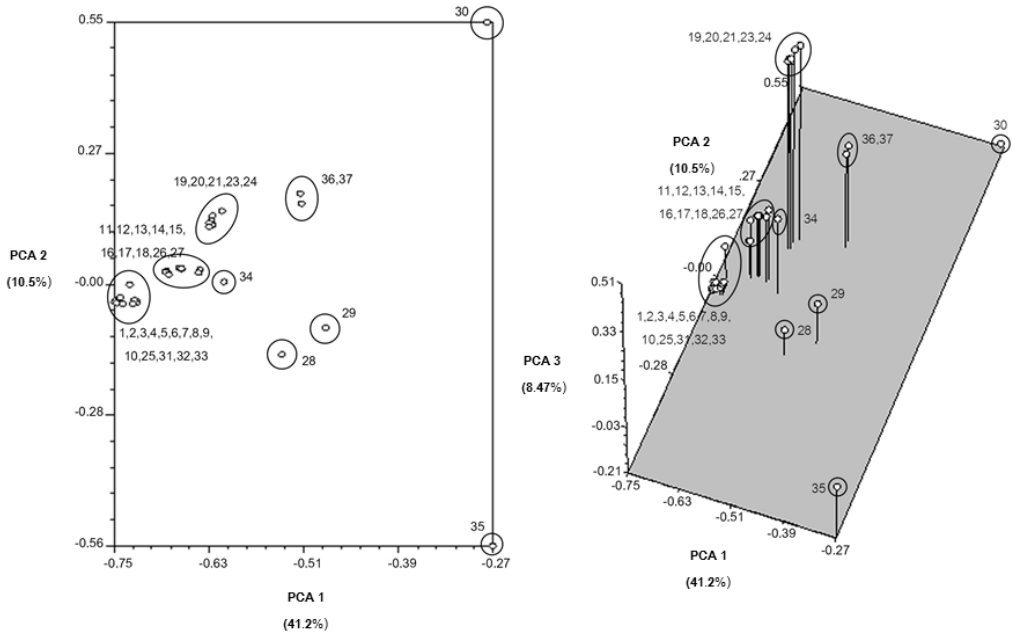


Figure 3 Two and Three-dimensions of a principal component analysis (PCA) based on AFLP binary matrix showed the genetic relationship among 37 accessions of pineapples. Numbers of accessions are described in Table 1. PCA 1, 2 and 3 were accounted for 41.2%, 10.5% and 8.47% of variation, respectively.



Figure 4 Some of pineapple cultivars in different morphological groups and *Ananas* sp. used in AFLP study. a, Nanglae (Cayenne); b, Trad Sithong (Queen); c, White Jewel (Perolera); d, MD-2 (Cayenne, Hybrid); e, Intra Chit Dang (Spanish); f, Hana 58 (*Ananas* hybrid); g, Hana 63 (*Ananas erectifolius*); h, Brazil (*Ananas lucidus*) and i, Hana 64 (*Ananas* .*bracteatus*).

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