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Genetic Analysis of Guava Germplasm using AFLP Markers

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Abstract Forty-nine guava accessions consisting of 20 pink, 20 white, five creamy and four maroon-fleshed clones from 15 countries were analyzed using amplified fragment length polymorphism (AFLP) markers to evaluate genetic diversity among accessions and flesh types. Seven informative AFLP primer combinations generated a total of 113 scorable AFLP fragments, of which 61 (54%) were polymorphic. Genetic analysis using Nei and Li similarity coefficient showed very closely related samples with the similarity coefficients ranged from 0.80 to 1.0. Cluster analysis with UPGMA showed that at similarity index of $\geq$ 0.92, the accessions were divided into three groups and two outsiders. Group I includes eight accessions consisting of six pink, one white and one creamy-fleshed clones. Group II has 35 accessions, which comprised 12 pink, all of the other white and creamy-fleshed type. Within this group, they are further divided into three sub-groups that most white-fleshed accessions are grouped separately from pink-fleshed accessions. All four accessions of maroon-fleshed type were clustered in group III. Two outsiders joined the cluster at similarity index of 0.91 and 0.86. Commercial clones of Thailand were closely related and high level of similarity among cultivated guavas originated from different regions suggested that further breeding program needs to incorporate wider germplasm.

Keywords: genetic diversity, guava breeding, *Psidium guajava* L., cluster analysis

Introduction

More than 80 genera and 3,000 species are members of Myrtaceae. Among these genera, *Psidium* is one of the most important for cultivation and consists of more than 150 species (Cobley, 1956). Guava (*Psidium guajava* L.) is the highest valuable cultivated species native to tropical America such as Mexico, Brazil (Cobley, 1956; Morton, 1987). Most commercial cultivars are diploid (2n=2X=22) (Hirano and Nakasone, 1969); although there are a few triploid (2n=3X=33) commercial clones reported in Thailand (Srisuwan, 2003).

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Guava is an excellent tropical fruit because of its high productivity, wide adaptability (Nakasone and Paull, 1998), and a high level of nutrition values such as vitamin C, dietary fiber and antioxidants (Jimenez-Escrig *et al*., 2001; Thaipong *et al*., 2006).

Guava cultivation is presently found distributed throughout tropical and subtropical regions such as India, South Africa, Florida, Hawaii, Egypt, Brazil, Colombia, Cuba, Venezuela, New Zealand, the Philippines (Yadava, 1996), Vietnam (Le *et al*., 1998), and Thailand (Tate, 2000). Commercially, guava is propagated asexually; therefore, genetic diversity among cultivated genotypes may not be much. However, their phenotype varies greatly in both vegetative and reproductive characters (Nakasone and Paull, 1998).

In Thailand, guava production areas of nearly 6,593 ha (OAE, 2017) are located in the Central and Western parts of the country, especially Samut Sakhon, Ratchaburi and Nakhon Pathom provinces; however, a guava plant can grow and produce fruits well in most regions in Thailand throughout the year. Major commercial cultivars are 'Paen Seethong' and 'Kim Joo'. These white flesh cultivars account for more than 95% of fresh guava consumption. New cultivars with high nutritional value, excellent flavor, tolerant to biotic and abiotic stresses are increasingly important.

For developing new guava cultivars with high fruit quantity and quality, tolerant to biotic and abiotic stresses, and for future consumer preferences, availability of wide genetic resources is of primary importance. Accurate information of the genetic relationship of germplasm would assist in the best selection of parents to making diverse genetic crosses in the breeding program. In addition, it would assist to eliminate duplicates in the germplasm collection. Amplified fragment length polymorphism (AFLP) markers developed by Vos *et al*. (1995) was widely used for genetic analysis and cultivar identification in many crops such as persimmon (Kanzaki *et al*., 2001), plum (Goulao *et al*., 2001), jackfruit (Schnell *et al*., 2001), sweet cherry (Zhou *et al*., 2002), peach (Aranzana *et al*., 2003), grape (Upadhyay *et al*., 2007) because it shows higher reproducibility compared to random amplified polymorphic DNA (RAPD) markers and lower costs compared to microsatellites (Powell *et al*., 1996).

The objective of the present research was to analyze genetic relationship among guava germplasm originated or developed in tropical areas using AFLP markers.

Materials and methods

Plant materials

Table 1. The guava materials used for genetic relationship analysis.

No.	Cultivar	Origin	Source	Flesh color	Note[1/]
1	Diminutive	Hawaii	HILO, Hawaii	Pink	GDL, SD
2	Keynok Crae	Thailand	Central Thai	Pink	GDL, SD
3	Keynok Daeng	Thailand	Central Thai	Pink	GNL, SD
4	Keynok Karn	Thailand	Western Thai	Creamy	GNL, SD
5	Pelock	Thailand	Western Thai	Pink	GNL, SD
6	Keynok Rayong	Thailand	Eastern Thai	Pink	GNL, SD
7	Local Phethabun	Thailand	Northern Thai	Pink	GNL, SD
8	Philippines	Philippines	Philippines	Maroon	MNL, SD
9	Thai Maroon	Thailand	HILO, Hawaii	Maroon	MNL, SD
10	Daeng Angkhang	Thailand	Northern Thai	Maroon	MNL, SD
11	Daeng Siam	Thailand	Central Thai	Maroon	MNL, SD
12	Samseekrob	Thailand	Central Thai	White	GNL, SD
13	Paen Seethong	Thailand	Central Thai	White	GNL, SD
14	Yensong	Thailand	Central Thai	White	GNL, SD
15	Kao-amporn	Thailand	Central Thai	White	GNL, SD
16	Klom Salee	Thailand	Central Thai	White	GNL, SD
17	XaLi	Vietnam	Vietnam	White	GNL, SD
18	Vitnam	Vietnam	HILO, Hawaii	White	GNL, SD
19	Bangkok Apple	Thailand	Central Thai	White	GNL, SL
20	Sampran Seedless	Thailand	Central Thai	White	GNL, SL
21	Saleethong	Malaysia	Central Thai	White	GNL, SL
22	Indonesian Seedless	Indonesia	HILO, Hawaii	White	GNL, SL
23	KUHP 12	Myanmar	Myanmar	Creamy	GNL, SD
24	KUHP 13	Myanmar	Myanmar	Creamy	GNL, SD
25	Kim Joo	Taiwan	Central Thai	White	GNL, less seed
26	KUHP 14	Taiwan	Taiwan	White	GNL, SD
27	Pear	Taiwan	HILO, Hawaii	White	GNL, SD
28	Pearl	Taiwan	Taiwan	White	GNL, SD
29	KUHP 38	Japan	Okinawa, Japan	Pink	GNL, SD
30	KUHP 16	Brazil	Brazil	White	GNL, SD
31	KUHP 84	Ethiopia	Ethiopia	White	GNL, SD
32	KUHP 85	Ethiopia	Ethiopia	Pink	GNL, SD
33	KUHP 81	Bhutan	Bhutan	Creamy	GNL, SD
34	KUHP 91	Bhutan	Bhutan	Pink	GNL, SD
35	KUHP 90	Bhutan	Bhutan	Creamy	GNL, SD
36	PuertoRico2	Puerto Rico	HILO, Hawaii	Pink	GNL, SD
37	Hongkong Pink	Hong Kong	HILO, Hawaii	Pink	GNL, SD
38	Hongkong White	Hong Kong	HILO, Hawaii	White	GNL, SD
39	Red Indian	Florida	HILO, Hawaii	Pink	GNL, SD
40	Gushiken Sweet	Hawaii	HILO, Hawaii	Pink	GNL, SD
41	PC12-102	Thailand	Northern Thai	Pink	GNL, SD
42	PC13-10	Thailand	Northern Thai	Pink	GNL, SD
43	Kelknoi	Thailand	Northern Thai	Pink	GNL, SD
44	Beaumont	Hawaii	HILO, Hawaii	Pink	GNL, SD
45	Waiakea	Hawaii	HILO, Hawaii	Pink	GNL, SD
46	Holmberg	Florida	HILO, Hawaii	Pink	GNL, SD
47	Poamoho Pink	Hawaii	HILO, Hawaii	Pink	GNL, SD
48	Panyak Seethong	Thailand	Central Thai	White	GNL, SD
49	Allahabad Safeda	India	HILO, Hawaii	White	GNL, SD

[1/] GDL = green dwarf leaf; GNL = green normal leaf; MNL = Maroon normal leaf; SD = seedy; SL = seedless.

Forty-nine guava accessions consisted of 20 pink, 20 white, five creamy and four maroon-fleshed types were collected or introduced from 15 countries (Table 1). All accessions are maintained at the Department of Horticulture, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, Thailand. These materials were initial introduction for the breeding program which began in 1999.

DNA extraction

Total genomic DNA was extracted using the CTAB procedure (Doyle and Doyle, 1990) with some modifications. PVP, β-mercaptoethanol and phenol were not used for the extraction. Approximately five grams of very young fresh leaf tissue from each accession was ground into fine powder in a mortar with liquid nitrogen. The powder was mixed with 20 ml of pre-warmed (at 60°C for 30 min) extraction buffer (3% CTAB, 100 mM Tris-HCl pH 8, 20 mM EDTA pH 8.0, 1.4 M NaCl) and incubated at 60°C for 60 min with an intermittent gentle shaking in a water bath. The mixture was allowed to cool at room temperature and 10 ml of chloroform : isoamyl alcohol (CIA) (24:1 v/v) was added and mixed well by inversion for 20 min to form an emulsion. The mixture was then centrifuged at 2,000 rpm at 4°C for 15 min to separate the phase and the supernatant was recovered. This step was repeated until clear supernatant was obtained by adding an equal volume of CIA. Then, DNA was precipitated by adding an equal volume of cold isopropanol. The mixture was gentle mixed well by reversion and kept at -20°C for 30 min to accentuate the precipitation of DNA. Subsequently, the cold mixture was centrifuged at 2,000 rpm to pellet the DNA. The DNA pellet was washed twice with 75% ethanol (ETOH) and was then centrifuged at 2,000 rpm at 4°C for 15 min. The DNA pellet was dried at room temperature prior to dissolve in 10 mM Tris-HCl and 1 mM EDTA (TE) by incubating at 60°C to accentuate the dissolve of DNA.

The DNA solution was purified by a RNase (10 mg/ml) treatment at 37°C for 1 h. The mixture was then added with 400 µL of 3 M NaOCA and 10 mL of cold 100% ETOH and kept in -20°C for 10 min to accentuate the precipitation of DNA. The DNA fragment was then hooked and moved to a new micro-centrifuge tube containing 500 µL of 75% ETOH and then centrifuged at 13,000 rpm at 4°C for 5 min and the supernatant was removed. The DNA pellet was washed with 75% ETOH for 2 to 3 times and dried at room temperature. The dried DNA pellet was re-dissolved in TE as a stock solution. Concentration and purity of the extracted DNA was determined by visual comparison with EZload Precision Molecular Mass Standard (Bio-Rad, USA) in a 0.8% (w/v) agarose gel (Promega, USA) stained with ethidium

bromide and by spectrophotometically. The concentration of the DNA samples was adjusted to 50 ng/µL with sterile reverse osmosis water as working DNA solution. The DNA stock and working solutions were stored at -20°C until used.

AFLP reaction

AFLP analysis was performed as described by Vos *et al*. (1995) with some modifications using the AFLP kit from Invitrogen, USA. Total genomic DNA (250 ng) was digested with 2.5 unit *Eco*RI (BioLabs, England) and 2.5 unit *Mse*I (BioLabs, England) in 25 µl reaction mixtures. The mixtures were then incubated at 37°C for 16 h and were inactivated at 70°C for 15 min. Then, 25 µl of *Eco*RI and *Mse*I adapter mixtures contained of 1 µl of 5 pmole *Eco*RI adaptor (Bio Basic, Canada), 2 µl of 50 pmole *Mse*I adapter (Bio Basic, Canada), 5 µl of 10X ligase buffer [50 mM Tris-HCl (pH 7.5 at 25°C), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 25 µg/ml BSA], and 0.6 µl of 5 U/µl T4 DNA ligase (SibEnzyme, USA) were ligated to the digested DNA fragments. The ligation was incubated at 20°C for 3 h. The ligated DNA was diluted 10 times before pre-amplification analysis.

Pre-amplification was performed with the *Eco*RI + A and *Mse*I + C primer pairs (Bio Basic, Canada) in 50 µl reaction consisted of 2 µl of 5 pmole *Eco*RI + A primer, 2 µl of 5 pmole *Mse*I + C primer, 5 µl of each dNTP, 5 µl of 10X PCR buffer plus 1.5 mM $MgCl_2$, 0.5 µL of 2 U/µl Taq polymerase and 5 µl of diluted DNA. The reaction was carried out in a PTC-0225 Peltier Thermal Cycler (MJ Research Inc., USA). The pre-amplification reactions were subjected to 20 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 60 s. The pre-amplification reactions were diluted 50 folds and used as template for the selective amplification.

Selective amplification was performed with *Eco*RI + ANN primers (Bio Basic, Canada) combined with *Mse*I + CNN primers (Bio Basic, Canada) in 20 µl polymerase chain reaction (PCR) in 0.2 ml PCR tubes on the PCR machine. PCR mixtures for selective amplification contained 5 pmole *Eco*RI + ANN primer, *Mse*I + CNN primer, 1X PCR buffer, 2 mM of each dNTP, 1 U Taq polymerase, and 5 µl diluted template DNA. The selective amplification temperature profile was programmed as: one cycle of 60 s at 94°C, 60 s at 65°C, 90 s at 72°C followed by 10 cycles with 1°C lower annealing temperature in each cycle and 22 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The amplification products were stored at -20°C until used.

Electrophoresis analysis

The PCR products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA pH 8.0, 0.05% bromophenol blue, and 0.05% xylene cyanol). Then, the mixtures were denatured at 94 °C for 3 min and cooled immediately in ice before loading onto pre-warmed 6% polyacrylamide gel [(acrylamide:bisacrylamide,19:1), 7.5 M urea, 1X TBE buffer]. Thereafter, aliquots (4 µl) of the mixture were loaded onto the gel and the AFLP fragments were separated electrophoretically in 1X TBE buffer at a constant voltage (295 V) in a Dual Slab Gel Unit (DSG-200g, C.B.S. Scientific Co., USA) until formamide dye had advanced on ¾ of the gel. Then, the gels were silver stained as described in Bassam *et al*. (1991). The gels were dried at room temperature for 48 h prior to visually scoring band.

Data analysis

Unambiguous and reproducible AFLP bands from two independent replications of each accession and primer combinations were scored as present (1) or absent (0) to create the binary data set. The genetic similarity between pairs of accessions was estimated according to Nei and Li (1979). The similarity coefficient [$S_{ij} = 2a/(2a+b+c)$, where S_{ij} is the similarity between the two individuals i and j, a is the number of bands present in both individuals, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i] in the SIMQUAL procedure. Cluster analysis was performed from the similarity data matrices to construct a dendrogram using the unweighted pair-group method with arithmetic averages (UPGMA) in the SAHN procedure. The SIMQUAL and SAHN procedures are the packaging of the Numerical Taxonomy and Multivariate Analysis System program package for personal computer (NTSYS-pc version 2.00).

Results and Disscussion

DNA extraction

Genomic DNA extraction method using very young fresh leaf tissue was useful for isolation of DNA as it was low in fibrous, phenols and polysaccharides that make easy to grind leaves in powder and purify their DNA. In addition, some highly toxic chemicals such as PVP, β-mercaptoethanol and phenol were absent for purifying DNA. The DNA extraction protocol used in this experiment resulted in high amount of white

DNA pellet and not degraded. This extraction method yielded a range DNA content of 0.5 to 5 µg/g of fresh leaf tissue, and purified DNA revealed A260/A280 = 1.3 to 2.5 (data not shown). However, very young leaves were not useful for making dry tissue and isolation of DNA from dry powder as they were burnt on drying (Prakash *et al*., 2002).

AFLP analysis

A total of ten AFLP primer combinations recommended by the AFLP Instruction Manual (Invitrogen, USA) were evaluated and seven were selected for the analysis. The seven informative AFLP primer combinations (Table 2) generated a total of 113 reproducible amplification bands across all guava accessions, of which 61 (54%) bands were polymorphic. The highest level of polymorphism (75%) was observed using the primer combination *Eco*RI-ACT/*Mse*I-CTC. The number of amplified AFLP bands per primer combination ranged from 10 to 20 with an average of 16.1 bands. The range of polymorphism bands per primer recombination varied from 4 (40%) to 15 (75%) with an average of 8.7 (54%) bands (Table 2). This level of polymorphism is lower than reported for Mexican guava AFLP study that was 71.9% (Sanchez-Teyer *et al*., 2010). This may be due to the fact that the Mexican accessions may be land races as Mexico is believed to be the center of origin of guava (Cobley, 1956; Morton, 1987), while guava materials used in this study are mainly cultivated clones or developed in breeding programs. However, lower levels of polymorphism have been reported in some AFLP fruit crop studies such as 45.1% in jackfruit germplasm (Schell *et a*l., 2001), fewer than 19% in sweet cherry (Zhou *et al*, 2002), and 15.8% in peach cultivars (Aranzana *et al*., 2003).

Table 2. The primer combinations used for amplified fragment length polymorphism analysis and the respective number of DNA fragment generated.

Selective amplification primer pairs	Amplified bands	Number of polymorphic bands	Polymorphism (%)
*Eco*RI-AAC/*Mse*I-CTA	18	10	55.6
*Eco*RI-ACT/*Mse*I-CTC	20	15	75.0
*Eco*RI-AAC/*Mse*I-CAT	10	4	40.0
*Eco*RI-ACA/*Mse*I-CAC	15	8	53.3
*Eco*RI-AAG/*Mse*I-CAC	20	8	40.0
*Eco*RI-AGC/*Mse*I-CTG	17	10	58.8
*Eco*RI-ACC/*Mse*I-CAG	13	6	46.1
Total	113	61	
Average	16.1	8.7	54.0

Genetic relationships among accessions

The 49 accessions evaluated in this research include 20 from Thailand, four from Taiwan, three from Bhutan, five from Hawaii, two each from Florida, Hong Kong, Myanmar, Vietnam and Ethiopia, and one each from Philippines, Brazil, Japan, Malaysia, India, Puerto Rico and Indonesia (Table 1). Guava has been under cultivation in tropical and subtropical regions for centuries. The true origin of material may not be related to the source from which it was obtained. Under central Thailand conditions, some accessions differ visibly in tree vigor, leaf color, size, shape, flower color, fruit shape, skin color, flesh texture, flesh color (data not shown), fruit size, percent edible flesh, seed weight and number, titratable acidity, pH, and ascorbic acid (Thaipong and Boonprakob, 2006).

Genetic analysis using Nei and Li similarity coefficient showed very closely related samples. The similarity coefficients ranged from 0.80 between 'Daeng Angkhang' and KUHP85 to 1.0 between the two white flesh commercial cultivars of Thailand 'Klom Salee' and 'Yensong'. Therefore, new accessions would be required to widen the genetic base for breeding program. The acquirement of new accessions should be concentrated in the center of diversity of the species such as Mexico. The levels of genetic similarity in this study are lower than those reported for 68 Mexican guava done by AFLP analysis with the similarity coefficients varied from 0.46 to 0.90 (Sanchez-Teyer *et al*., 2010). Based on RAPD markers, Prakash *et al*. (2002) reported the levels of genetic similarity between 41 Indian guava accessions of five *Psidium* species were moderate (0.46) to high (0.89).

Cluster analysis with UPGMA showed that the guavas were divided into three groups at threshold 0.915 of similarity index (Fig. 1). Group I includes eight accessions of six pink, one white and one creamy-fleshed type. Two dwarf accessions, 'Diminutive' and 'Keynok Crae', were included in this group. 'Diminutive' is a seedling introduced from the Tropical Plant Genetic Resource Management Unit (HILO), Hawaii; while 'Keynok Crae' was collected from a local market in Thailand. Both accessions have light pink-fleshed.

Group II has 35 accessions, which comprised of 12 pink, all of the other white and creamy-fleshed type. Within this group, they were further divided into three sub-groups in which most white-fleshed accessions were clustered separately from pink-fleshed accessions. The pink-fleshed accessions are all high acid flavor used mainly for processing juice and paste. In Thailand, there are a few leading white-fleshed commercial cultivars such as 'Paen Seethong', Klom Salee' and 'Yensong'. 'Klom Salee' and 'Yensong' showed genetic identical which is in agreement with previous phenotypic evaluation for quantitative traits such as fruit weight, titratable acidity, pH, and ascorbic acid

contents (Thaipong and Boonprakob, 2006). 'Klom Salee' has been cultivated over 30 years, while 'Yensong' was recently found in the same production region. Via air-layering which is the main propagation method, 'Yensong' could have been originated as bud sport of 'Klom Salee'.

All four accessions of maroon-fleshed and leafed type were clustered in group III and separated from other green leafed accessions. This indicated their close similarity. At threshold 0.915 of similarity index, there were two accessions, KUHP85 and KUHP91, that could not be grouped to any cluster. KUHP85 is a light pink-fleshed cultivar introduced from Ethiopia, while KUHP91 is a light pink-fleshed cultivar introduced from Bhutan.

Genetic relationships among fleshed types

There are four types of flesh color among *P. guajava* species that were white, pink, creamy and maroon in which most cultivars have white and pink-fleshed. In Thailand, all commercial cultivars are white-fleshed type. The genetic similarity between flesh type guava accessions were between 0.89 and 0.92 and similarity within a type was slightly greater than those between types (Table 3). Within the same type of flesh color, the similarities were 0.94 among white, 0.92 among pink, 0.94 among creamy and also 0.94 among maroon (Table 3). Cluster analysis based on 113 AFLP markers could only distinguish four maroon-fleshed accessions from the other types of flesh color (Fig. 1).

Table 3. Average and standard error of Nei and Li similarity coefficients of guava within and between flesh color types.

Flesh color	White	Pink	Creamy	Maroon
White	0.94 ± 0.02			
Pink	0.91 ± 0.02	0.92 ± 0.02		
Creamy	0.92 ± 0.02	0.91 ± 0.02	0.94 ± 0.04	
Maroon	0.92 ± 0.02	0.89 ± 0.03	0.89 ± 0.01	0.94 ± 0.03

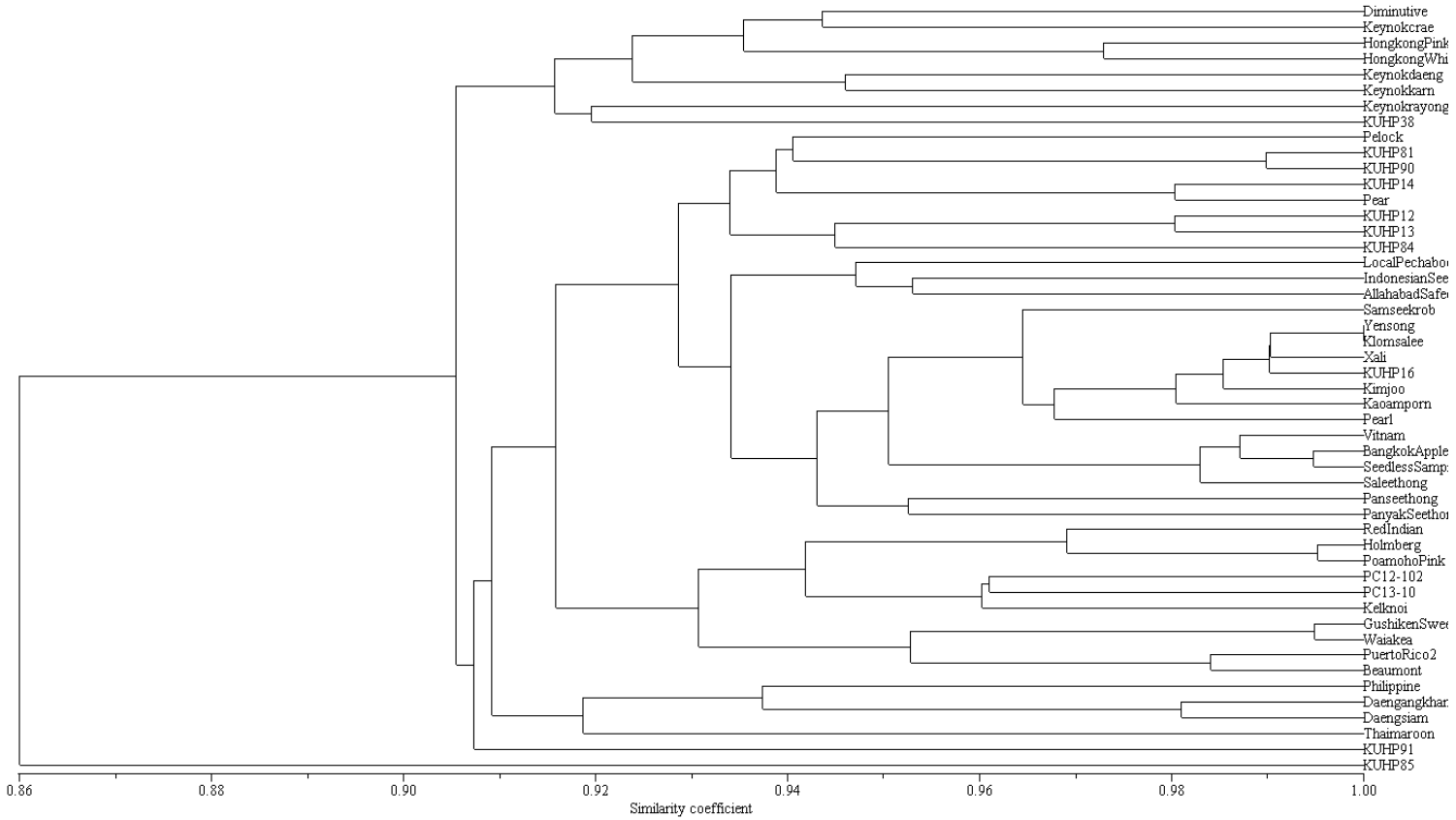

Figure 1. Dendrogram of 49 guava accessions using UPGMA method on Nei and Li similarity of 113 AFLP markers.

Conclusion

Genetic relationships among 49 guava accessions originated from several countries based on 113 AFLP markers generated from seven primer pairs were very closely related despite their high phenotypic variation. Commercial guavas in Thailand have narrow genetic base. Therefore, new accessions would be required and should be introduced from the center of diversity of the species to widen the genetic base for further breeding program. Two white-fleshed leading commercial cultivars of Thailand 'Klom Salee' and 'Yen Song' showed genetic identical. Based on the 113 AFLP markers, cluster analysis could not distinguish between phenotypes of white, pink and creamy-fleshed, except maroon flesh color.

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