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Determiation of Genetic Diversity and Relationhips among Thai Litchi Accessions by RAPD and AFLP Markers

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ABSTRACT

Genetic diversity and relationships within Thai litchi cultivars were investigated using RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) markers. Fourteen RAPD primers and seven AFLP primers were chosen, resulting in amplification of 52 and 101 reproducible polymorphic fragment products, respectively. The percentages of polymorphic markers for RAPD and AFLP were 34.6% and 36.3% respectively. Each marker system was able to differentiate all accessions. Even each of the AFLP primers could identify all accessions, while the RAPD markers did not show such efficiency. The polymorphism information content (PIC) scores were calculated for each of 52 RAPD and 101 AFLP polymorphic fragments. It ranged between 0.16 to 0.50 for RAPD markers and 0.22 to 0.50 for AFLP markers. Unweighted pair-group method with arithmetic averages (UPGMA) dendrograms using Jaccards coefficients reflected no clear cut grouping based on neither morphology nor climatic adaptation. However, both dendrograms showed that the 47 litchi accessions could be classified into groups between when the similarity coefficients were as low as 0.37 for RAPD marker and 0.25 for AFLP markers. Two accessions (LH80 and LH109) were found to be genetically very far distant from the other accessions using both markers. RAPD and AFLP marker analyses provided a quick and reliable alternative for identification of litchi accessions and determination of genetic diversity among them.

Key words: litchi, RAPD, AFLP, genetic, diversity

INTRODUCTION

Litchi (*Litchi chinensis*) is an economic fruit of Thailand. Although it has originated in Southern China and Northern Vietnam, many varieties are densely distributed and grown in Thailand. The variability among litchi cultivars is still unknown since breeding for new cultivars done by growers based on a low number of parents. Field collection and preservation in gene banks of Plant Genetic Resources (PGR) has been extensively conducted at the international and national level. To identify genetic materials that may contain useful traits for germplasm enhancement, a systematic evaluation of genetic diversity is required to understand relationship among accessions and their corresponding collecting-site environments (Steiner and Greene, 1996). Understanding the genetic diversity within a germplasm collection facilitates their use, provided that information is available from characterizing germplasm collections (Strauss et al., 1988). Comparison of parents using difference in DNA markers may be one of the method by which breeders can increase the probability of

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selecting those parents with different gene sets. This methods will produce progeny with new and more favorable combinations of genes for quality and yield.

Recent reports have focused on using DNA based markers, particularly random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, to measure genetic diversity in numerous fruit species such as cherry (*Prunus avium*) (Gerlach and Stosser, 1997), lemon (*Citrus lemon* L.) (Machado *et al.*, 1996), mango (*Mangifera indica* L.), peach (*Prunus persica* L.) (Lu *et al*., 1996), grape (*Vitis vinifera*) (Sensi *et al*., 1996), currant (*Ribes grossularia*) (Lanham and Brennan, 1999), and pear (*Pyrus* sp.) (Monte-Corvo *et al*., 2000). Both RAPD and AFLP detected substantial genetic variation within perennial fruit cultivars and generally demonstrate that cultivars can be discriminated on the basis of genetic characteristics (Lopez-Valenzuela *et al*., 1997). Choice of a marker system to use for a particular application depends on its ease of use and the particular objectives of the investigation (Rafalski *et al*., 1996). RAPD and AFLP techniques do not require DNA probes or prior sequence information. These procedures are simple, largely automatable, require only small amounts of DNA, and can be performed without the use of radioactivity (Karp *et al*., 1996). RAPD markers also have limitations such as their dominant character and reproducibility (Williams *et al*., 1990). Reliability may be increased by replicate analyses and PCR performed at different times. Although the AFLP procedure is more labor intensive and expensive than RAPD analysis, but a larger number of loci are detected per reaction in comparison with RAPD-PCR (Powell *et al*., 1996).

The objectives of this study were to : (1) to use RAPD and AFLP analyses to estimate the level of genetic diversity among 47 litchi accessions collected from all over Thailand (2) to determine the relative effectiveness of both markers in revealing variation among closely related cultivars.

MATERIALS AND METHODS

Plant materials

Forty-seven litchi accessions were used in this study (Table 1). They were collected from various locations in Thailand. The fresh young leaves were collected from 5 plants of each accession, and used for DNA extraction by the CTAB method (Doyle and Doyle, 1990).

Random amplified polymorphic DNA (RAPD) analysis

Primers representing 10 random nucleotide sequences, obtained from Operon Technologies (USA) were used in the RAPD assay. PCR reactions were in volumes of 10 μL containing 1 ng/ μL of the extracted genomic DNA, 1 of 10x PCR buffer (100 mM Tris-Cl buffer, 500 mM KCl, 20 mM $MgCl_2$, 0.01% Gelatin), 0.1 mM dNTPs (Promega, USA), 0.2 mM primer, 0.2 unit/μL Taq DNA polymerase (Promega) and 4.8 μL sterile water. Sterile mineral oil (30 μL) was added to each tube to seal the reaction mixture and to prevent evaporation. The PCR was carried out in a DNA Thermal Cycler (Bio Oven III) programmed to run the following temperature profile; 45 cycles of 91.5°C for 1 min, 36°C. for 1 min, 72°C. for 2 min and the final extension for 5 min at 72°C. All PCR products were fractionated by electrophoresis in 1.6% (w/v) agarose gels and visualized by ethidium bromide staining using 0.5 μg/mL in 1x TAE buffer. The fragments of each gel were recorded with Bioprint-version 96,07 system (France). Polymorphism at all loci was confirmed by three repeated tests.

Amplified fragment length polymorphism (AFLP) analysis

The same DNA preparation technique used for RAPD analysis was also employed in AFLP analysis. AFLP analysis was carried out according to Vos *et al*. (1995). Total DNA (500ng) was digested twice with EcoRI(recognition sequence 5'-GAATTC3') and Tru9I (recognition sequence

Table 1 Accessions of litchi used in the variation study.

Number	Accession/local name	Collection site	Origin
1	LH80 Chakra-pad	Tah Ton /Fang/Chiang Mai	
2	LH18 Samphoa Koew	KU station/Pak Chong/ Nakhon Rachasima	
3	LH33 Samphoa Koew	Amphawa-1/Samut Songkhram	
4	LH56 O-Hia	KU station/Pak Chong/ Nakhon Rachasima	
5	LH91 O-Hia	Mae Ngon/Fang/Chiang Mai	
6	LH95 O-Hia (heart shape)	Mae Ngon/Fang/Chiang Mai	
7	LH32 Kra-lok	Amphawa-1/Samut Songkhram	
8	LH13 Hong Huay	KU station/Pak Chong/ Nakhon Rachasima	
9	LH83 Hong Huay	Tah Ton /Fang/Chiang Mai	
10	LH11 Kom (red)	KU station/Pak Chong/ Nakhon Rachasima	
11	LH19 Kom (green)	KU station/Pak Chong/ Nakhon Rachasima	
12	LH22 Kom	KU station/Pak Chong/ Nakhon Rachasima	
13	LH23 Kom	KU station/Pak Chong/ Nakhon Rachasima	
14	LH25 Kom (lamjiak)	Amphawa-1/Samut Songkhram	
15	LH35 Kom (200 years)	Amphawa-2/Samut Songkhram	
16	LH37 Kom	Amphawa-2/Samut Songkhram	
17	LH43 Kom	Amphawa-3/Samut Songkhram	
18	LH69 Kom	Thep Raksa/Pak Chong	
19	LH74 Kom (krathouy)	Thep Raksa/ Pak Chong	
20	LH86 Kom	Mae Ngon/Farng/Chiang Mai	
21	LH29 Jean	Umpawa-1/Samut Sakorn	
22	LH41 Jean	Amphawa-2/Samut Songkhram	
23	LH30 Tai Ohia	Umpawa-1/Samut Sakorn	
24	LH36 Tai Yai	Amphawa-2/Samut Songkhram	
25	LH42 Tai	Amphawa-2/Samut Songkhram	
26	LH34 Kra-thone Thong Phra-rong	Umpawa-1/Samut Sakorn	
27	LH59 Kawaini	KU station/Pak Chong/ Nakhon Rachasima	
28	LH64 Brewster	KU station/Pak Chong/ Nakhon Rachasima	
29	LH67 Golf	KU station/Pak Chong/ Nakhon Rachasima	
30	LH87 Kim-cheng	Mae Ngon/Fang/Chiang Mai	
31	LH100 Jubee-jee	Tah Ton/Fang/Chiang Mai	
32	LH101 Sweet cliff	Horticulture Research Station, Chiang Rai	
33	LH102 Mauritius	Horticulture Research Station, Chiang Rai	
34	LH103 Brewster	Horticulture Research Station, Chiang Rai	
35	LH104 Kom (long leaf)	Horticulture Research Station, Chiang Rai	
36	LH105 Chow Rakam	Horticulture Research Station, Chiang Rai	
37	LH106 Jean -dang	Horticulture Research Station, Chiang Rai	
38	LH107 Nai-Saard	Horticulture Research Station, Chiang Rai	
39	LH108 Tip	Horticulture Research Station, Chiang Rai	
40	LH109 Jean hom	Horticulture Research Station, Chiang Rai	
41	LH112 Jean -lek	Horticulture Research Station, Chiang Rai	
42	LH114 Look-laai	Horticulture Research Station, Chiang Rai	
43	LH116 Jean Kriangsak	Horticulture Research Station, Chiang Rai	
44	LH117 Kra-lok-Bai-Yoh	Horticulture Research Station, Chiang Rai	
45	LH118 Kim Chi	Horticulture Research Station, Chiang Rai	
46	LH119 Sa-laek Tong	Horticulture Research Station, Chiang Rai	
47	LH120 Hak-ip	Horticulture Research Station, Chiang Rai	

5'-GACGATGAGTCCTGAG and TACTCAGGACTCAT-3'). After ligating ER adatptors and MS adaptor to the digested DNA, pre-amplification was conducted with an E R-A- primer (EcoRI adaptor sequence) and Ms-C primer (Ms-C adaptor sequence as a selective nucleotide). The pre-amplification product was used as template DNA for selective amplification. Selective amplification was conducted using an ER-A primer containing two selective nucleotides and Ms-C primer containing three selective nucleotides. 12 combination of the selective primers were used. PCR condition was set for 20 cycles in PCR I and 30 cycles in PCR II. The condition in each cycle was the same as RAPD. The products of selective amplification were denatured at 90 C for 3 min. Electrophoresis was performed on 4.5% polyacrylamide gel in 5xTBE Buffer with a sequencer using 50 watt per gel. AFLP gel was stained by silver staining as described by Bassam *et al*.,1991.

Data analysis

Bands position for each litchi accession and primer combination were scored as present (1) or absent (0) from photographic prints of gels. Only bright, clearly distinguishable polymorphic bands were used in the statistical analysis. The discrimination power of each RAPD and AFLP marker was evaluated by the polymorphism information content (PIC) as described by Anderson *et al*. (1993). Jaccard's (1908) coefficient of similarity was calculated, and the accessions were grouped by cluster analysis using the unweighted pair-group method (UPGMA). NTSYS-pc, Version 2.01d (Rohlf, 1997) program was used for statistical analyses.

RESULTS

RAPD

Fifty primers were screened for their RAPD products generated against DNA samples extracted from litchi accessions. Fourteen primers were selected for the molecular diversity analysis of accessions and evaluation of their relationships based on the total number of bands, the number of polymorphisms, and their reproducibility in three independent applications (Table 2). These selected primers yielded a total of 153 fragments among the 47 litchi accessions of which 52 fragments were polymorphic (34.6%). The size of fragment scored ranged from approximately 500 to 2000 base pairs. The average of two to six polymorphic bands per primer were scored with a mean ± SD of 3.71 ± 1.1 bands per primer. The range of PIC or genetic diversity scores in this study was 0.16 to 0.50 with the mean of 0.31 ± 0.11.

AFLP

The number of bands per primer pair ranged from 31 to 57 with an average of 39.7. A total of 278 AFLP bands were scored with seven primer pair combinations. The total polymorphic bands were 101 (36.3%) with the range from 22.8% to 53.1% (Table 3). The polymorphic bands amplified by any AFLP primer were sufficient to discriminate all litchi accessions. An example of the pattern of amplified products obtained with one AFLP primer pair is presented in Figure 1. The discrimination power of each marker was estimated by the PIC (results not shown), Which found ranging between 0.22 and 0.50 (the expected maximum value for a biallelic locus) with an average of 0.37. A large portion of markers ha a high discrimination power of (≥0.30). Six cultivars, namely Jakrapad, Hong Huay, Kimjeng, O-Hia, O-Hia (heart-shape), and Jubee-jee were tested for intra-varietal variation using primers ACC/CAG, ACC/CAT, and ACC/CAA. A considerable polymorphisms were revealed in this study (Figure 2)

Cluster analysis

RAPD and AFLP cluster analysis is illustrated in the dendrogram of Figure 3 and 4, respectively. The dendrogram constructed by 52

Table 2 List of selected Operon primers, their sequences, number of bands, polymorphism (%) of the RAPD analysis results in 47 litchi accessions.

Sl no.	Operon code	Sequences (5'-3)	RAPD fragment score		Polymorphism%
			Monomorphic	Polymorphic	
1	OPE-04	GTGACATGCC	6	5	45.5
2	OPE-15	ACGCACAACC	8	3	27.3
3	OPE16	GGTGACTGTG	6	4	40
4	OPE-18	GGACTGCAGA	7	3	30
5	OPE-20	AACGGTGACC	7	4	36.4
6	OPF-01	ACGGATCCTG	9	2	18.2
7	OPF-05	CCGAATTCCC	3	4	57.1
8	OPF-10	GGAAGCTTGG	10	4	28.6
9	OPG-04	AGCGTGTCTG	7	2	22.2
10	OPH-04	GGAAGTCGCC	9	4	30.8
11	OPH-15	AATGGCGCAG	6	3	33.3
12	OPI-06	AAGGCGGCAG	11	5	31.3
13	OPK-02	GTCTCCGCAA	6	3	33.3
14	OPL-12	AGAGGGCACA	6	6	50
Total		14	101	52	--
Mean±SD			7.2+1.97	3.71+1.1	

Table 3 List of AFLP primers, their sequences, number of bands, polymorphism (%) of AFLP analysis results in 47 litchi accessions.

Sl. No.	Sequences	AFLP fragment score		Polymorphism%
		Monomorphic	Polymorphic	
1	ACC/CAG	19	12	38.7
2	ACC/CAA	44	13	22.8
3	ACC/CTC	29	15	34.1
4	ACC/CAT	30	12	28.6
5	ACC/CTG	15	17	53.1
6	ACC/CAC	21	19	47.5
7	ACC/CTA	19	13	40.6
Total	7	177	101	--
Mean (±SD)		25.28 ± 9.18	14.43 ± 2.5	36.3

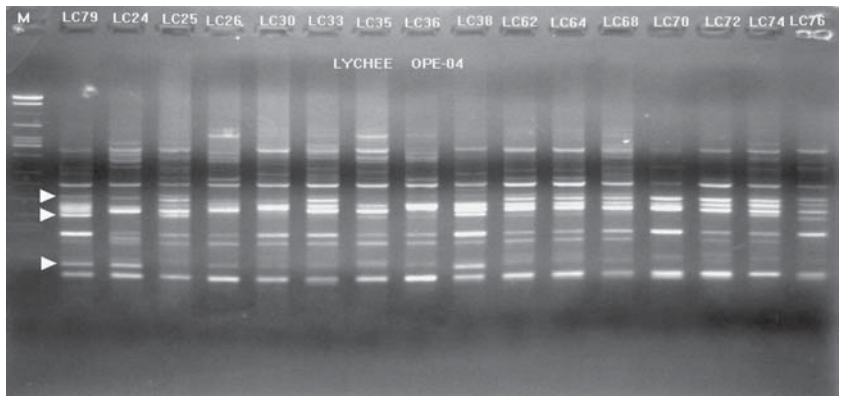

Figure 1 RAPD bands produced by primer OPE-04 with the genomic DNA from each of 16 litchi cultivars. The cultivars are numered as in Table 1.

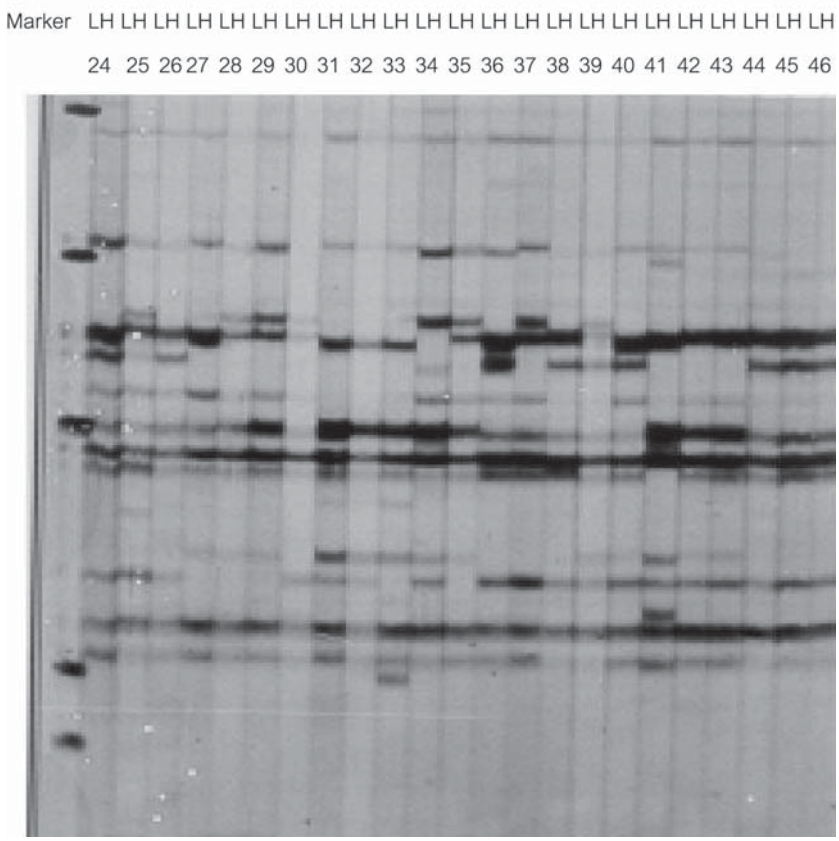

Figure 2 AFLP bands produced by primer ACC/CAG with the genomic DNA from each of 23 litchi cultivars. The cultivars are numered as in Table 1.

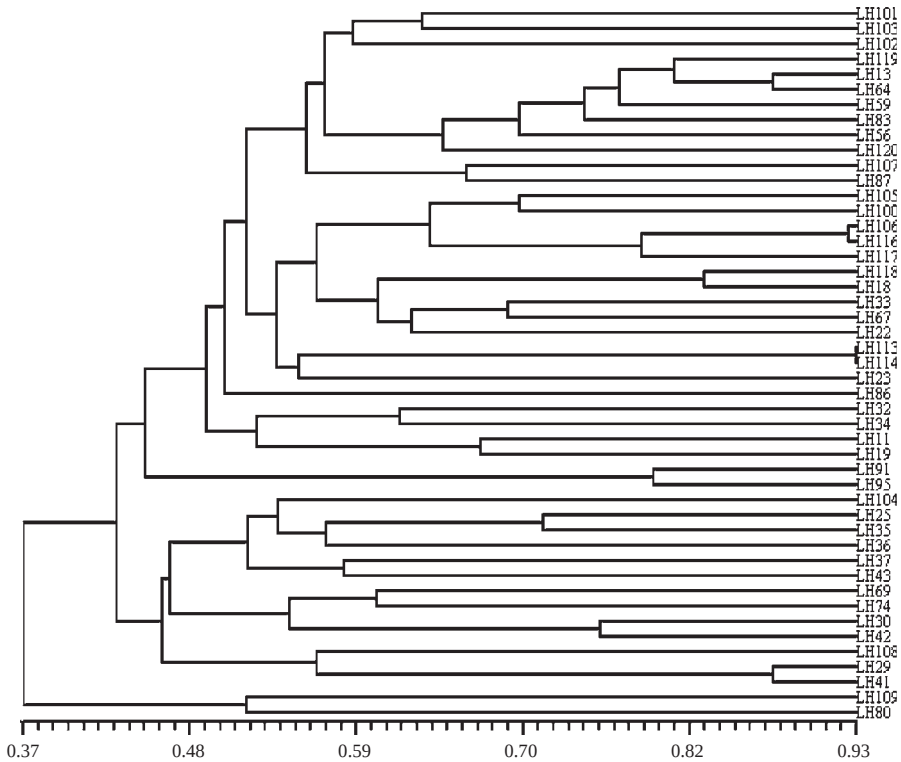

Figure 3 Dendrogram showing genetic relationship among 47 Thai litchi accessions generated by UPGMA cluster analysis calculated from 52 RAPD markers amplified by 14 10-mer primers.

RAPD markers indicated that the Thai litchi accessions were clearly separated into two main groups. One group, which contains the only two red color fruited accessions named Jakra-pad (LH80) and Jean Hom (LH109). The other group could be furtherly divided into six sub-groups at the 0.50 similarity scale, containing 3, 4, 6, 2, 4, and 26 accessions, respectively (Figure 3). Each of these sub-groups could be furtherly divided into several well defined clusters showing a close association among local basis distinct or same accessions. Similarly using the binary data from 101 AFLP markers for UPGMA cluster revealed 47 accessions to be separated into three main groups. It also indicated that the litchi accessions were rather distinctly separated to RAPD dandrogram. Some accessions showed high tendency to be close among themselves the same as RAPD based dendrogram. Range of similarity values in AFLP dendrogram was higher than that of RAPD dendrogram. Accession LH80 and LH109 revealed a high genetic distant in the both marker system analyses.

DISCUSSION

Genetic diversity among 47 selected accessions of Thai litchi was assessed with 52 RAPD and 101 AFLP polymorphic bands. A low number of RAPD polymorphisms per primer was detected among accessions. RAPD marker revealed that its specificity for variety discrimination was comparatively more limited than that of AFLP marker. Similar results also reported by Paran *et al*. (1998) in *Capsicum annuum*. By using RAPD and AFLP primers, there was very low genetic variation within 47 accessions of litchi. For RAPD only

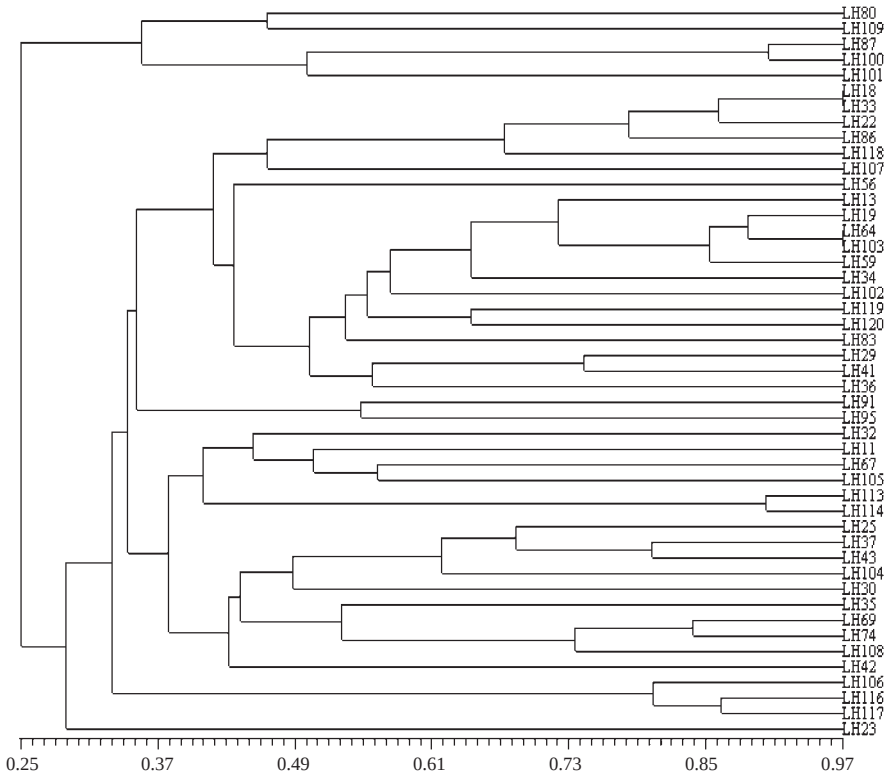

Figure 4 Dendrogram showing genetic relationship among 47 Thai litchi accessions generated by UPGMA cluster analysis calculated from 101 AFLP markers amplified by 7 pair- primers.

34.6% of bands were polymorphic in all accessions. The percentage of polymorphic bands (PPB) in each accession ranged from 18.2% to 50.0%. For AFLP, the PPB was 36.3%, ranging between 22.8% to 53.1% in each accession. These results more or less agreed with RAPD and AFLP analysis in other species (Chowdhury *et al*., 2001; Bellini *et al*.,1998, Paran *et al*., 1998). Both RAPD and AFLP analyses yield similar results and expressed great potential to identify and establish genetic relationship among litchi accessions. Both methods are highly informative and do not require prior knowledge of the litchi genome. The AFLP assay showed some advantages over RAPD, as it is more reproducible and more informative than that of RAPD. Each AFLP primer is sufficient for the identification of all accessions, but it has license restrictions which is limits its use for commercial fingerprinting (Knorr *et al.*, 2001). Different combinations of the banding patterns provided by different primer, is a clear evidence of the high discrimination capacity of these markers. This capacity is particularly useful for management of a germplasm bank, as it provides an inexpensive and reliable method for identification of a large number of cultivars. Moreover, litchi fruit is cross-pollinated and this evidence is easily detected by AFLP markers using two primersACC/CAG, ACC/CAT, and ACC/CAA. Thus the AFLP method is suitable for detecting intravarietal difference.

For similarity coefficients the ranges were 0.25 to 0.97 for AFLP marker, and 0.37 to 0.93 for RAPD data. The mean value was significantly lower in AFLP (0.34) than that of RAPD analysis (0.47), indicating a higher frequency of polymorphic bands scored as presented among the 47 litchi

accessions when using AFLP versus RAPD. Two pairs of accessions (LH18 vs LH33 and LH64 vs LH103) were genetically similar (97% similarity) on the basis of AFLP analysis however, they showed low similarity (65% and 61%) when using RAPD analysis. A possible explanation for the difference in the resolution was the two techniques targeting on different portions of the genome (Karp *et al*., 1996), although, some studies suggested that both RAPD and AFLP markers represented the specific DNA fragments distributing throughout the genome (Becker *et al*., 1995; Williams *et al*., 1990). Higher level of similarity observed among the cultivars originating from the same or nearby geographic origins agreed with both the hypothesis of authoctonal origin as well as the limited diffusion of litchi cultivars from their zones of cultivation (Barranco, 1994). This result suggested that both markers were useful for molecular diversity estimation but had no allelic relationship between the absence or presence of a given band due to the different primer sequences (Baril *et al*.,1997).

Subhadrabandhu (1990) reported that there were two groups of litchi varieties grown in Thailand. One group mainly cultivated in the central part while the other in the northern part of the country. In central part, existing litchi varieties require no or little cool period for inflorescense induction. These varieties are some times classified as low land litchi or tropical litchi, whereas the varieties that require cooler period for flowering are mainly found in the northern part of the country. Both groups also exhibit difference in flowering and harverting time, fruit size and color at maturity. In these respects, both DNA markers did not reveal any clear pattern of grouping based on morphology or putative climatic or geographic origin, as detected in some other crops (Paul *et al*., 1997, Spooner *et al*.,1996). Belaj *et al* (2001) reported similar results in olive germplasm using RAPD markers. Large portions of this litchi germplasm share common ancestry, since Chinese varieties have been highly utilized as parental materials in chance seedling selection by farmers in Thailand. Hence, the gene pool comprising cultivated litchi may be very restricted. It is also possible that some litchi accessions were introduced from Myanmar or Vietnam.

Although both DNA markers were effective in detecting genetic diversity levels in Thai litchi cultivars, AFLP was more efficient than RAPD in number of polymorphic bands detected per primer and the reproducibility involved. Dendrograms constructed based on AFLP and RAPD polymorphisms indicated that these two marker techniques provided no identical phylogenetic information. This observation may be related to larger number of AFLP bands used in the analyses. Estimates of genetic diversity are highly influenced by the genome selected for evaluation and by the number of markers assayed. Since fruit tree cultivars are maintained by vegetative propagation, accurate identification of vegetative materials is crucial for nurserymen and growers, and is required for the plant breeder's rights. Therefore, these DNA marker techniques can be used to identify genetic variation and detect the find relationship between DNA markers and horticultural traits of interest.

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