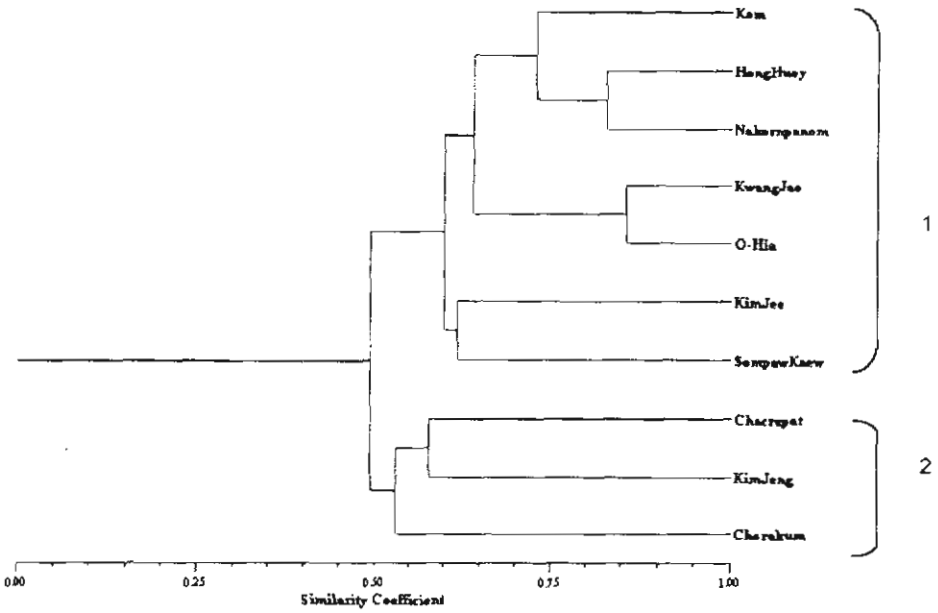

Figure 18 Dendrogram of 10 lychee cultivars using the SSR marker. The scale 0 to 1 indicates genetic similarity calculated using the Dice coefficient in the NTSYS program

4.4 HAT-RAPD analysis

In the analysis of 10 lychee cultivars, all 10 tested primers revealed polymorphisms. A total Usually, products below 200 bp or above 3 kb gave faint and non-reproducible bands, hence most of the scored products are in the range of 0.3–2 kb. The HAT-RAPD primers amplified between 5 and 15 bands per genotype; however only 5–13 of them were scorable, with a molecular size range of 200–4507 bp (Table 16). Using ten RAPD primers, a total of 215 bands were scored. Figure 19, 20 and 21 shows an example of such a typical HAT-RAPD pattern using the OPH-03, OPG-13 and OPH-11 primer, respectively.

Table 16 . List of HAT-RAPD primers, their sequence, number of fragments and size range.

Primers	Sequence (5' ⟶ 3')	Total number of amplified fragments	Number of polymorphic fragments	Fragment size range (bp)	Primers
OPH03	AGACGTCCAC	26	13	200-4507	OPH03
OPD08	GTGTGCCCCA	17	11	250-2850	OPD08
OPU15	ACGGGCCAGT	26	10	250-2500	OPU15
OPAK10	CAAGCGTCAC	16	8	350-2560	OPAK10
OPH13	GACGCCACAC	17	11	200-4000	OPH13
OPH11	CTTCCGCAGT	23	12	200-2110	OPH11
OPG13	CTCTCCGCCA	25	13	200-4507	OPG13
OPD20	ACCCGGTCAC	25	12	200-2500	OPD20
OPAS10	CCCGTCTACC	19	10	200-4507	OPAS10
OPB18	CCACAGCAGT	21	10	200-4507	OPB18
Total		215	110		Total

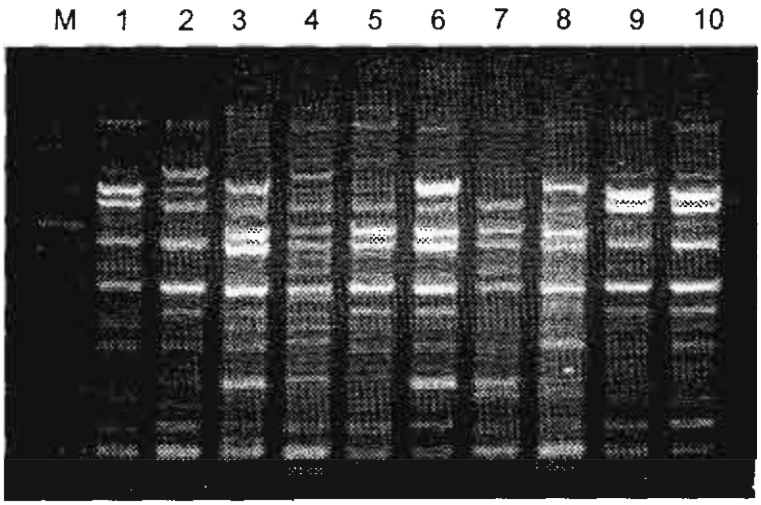

Figure 19. HAT-RAPD profiles generatred using OPH-03 primer. Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, 100 bp marker

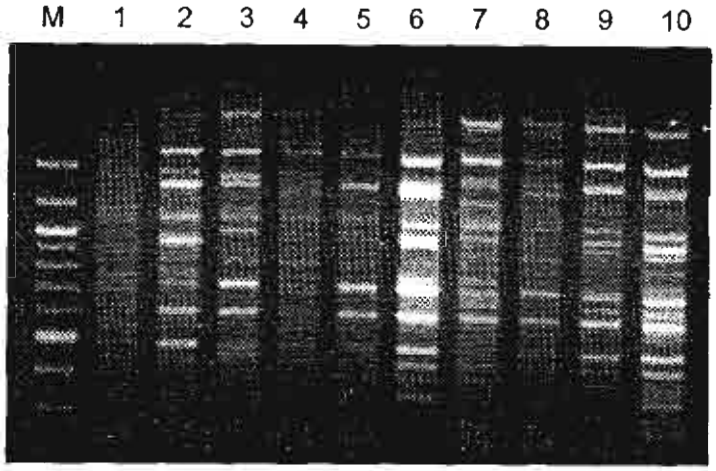

Figure 20 HAT-RAPD profiles generatred using OPG-13 primer . Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, 100 bp marker.

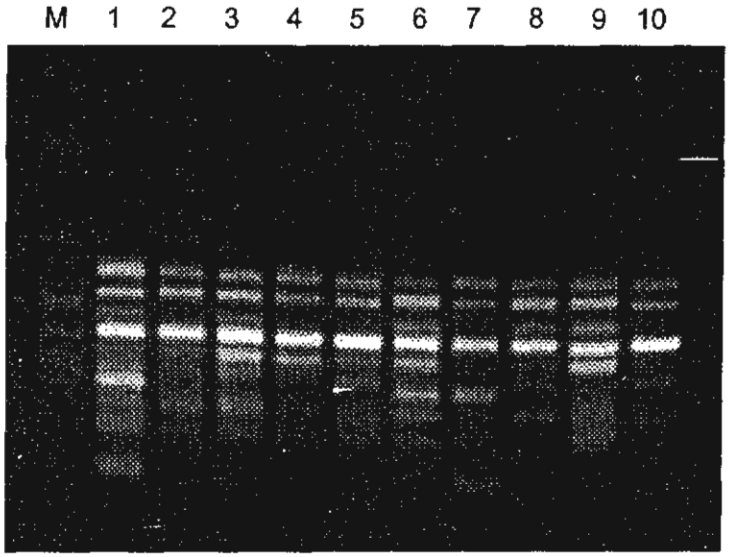

Figure 21 HAT-RAPD profiles generatred using OPH-11 primer. Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, lamda/PstI marker.

Table 17. Dice similarity coefficients showing the relationships between 10 lychee cultivars. Results are based on 110 HAT-RAPD fragments generated by 10 primers

Cultivar	1	2	3	4	5	6	7	8	9	10
Kom	1.00									
Chakrapat	0.41	1.00								
HongHuey	0.47	0.65	1.00							
KimJeng	0.44	0.69	0.62	1.00						
KwangJao	0.36	0.74	0.63	0.67	1.00					
O-Hia 0.46	0.72	0.76	0.71	0.82	1.00					
KimJee0.51	0.56	0.65	0.55	0.52	0.64	1.00				
Nakornpanom	0.47	0.45	0.54	0.43	0.38	0.53	0.56	1.00		
SampawKaew	0.72	0.56	0.61	0.51	0.48	0.56	0.57	0.52	1.00	
Chorakum	0.67	0.55	0.48	0.48	0.45	0.54	0.58	0.51	0.78	1.00

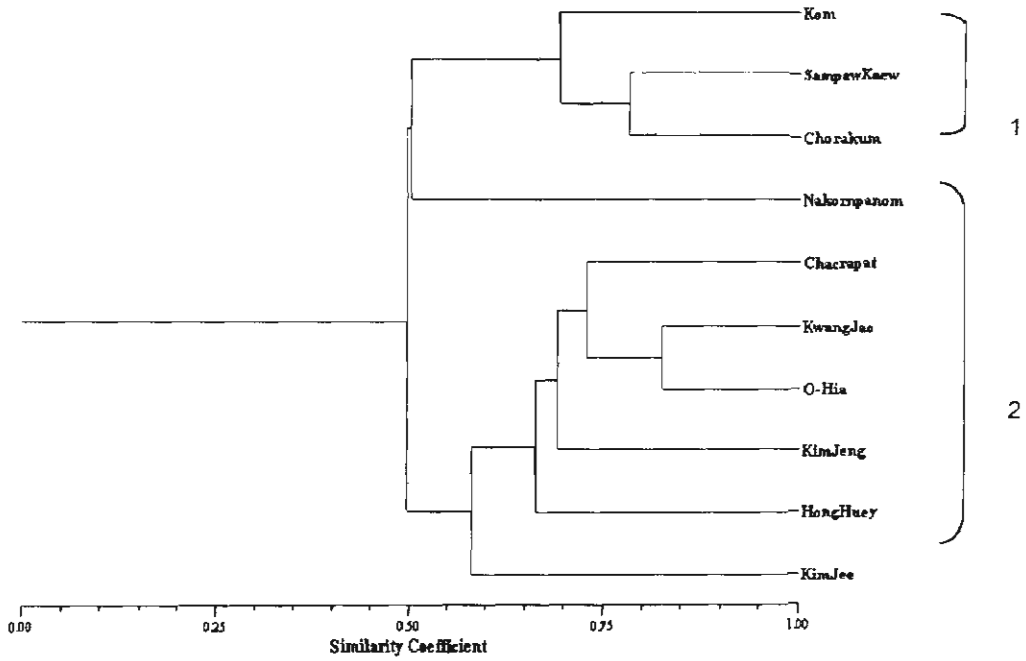

Figure 22 Dendrogram of 10 lychee cultivars obtained HAT-RAPD marker. The scale 0 to 1 indicates genetic similarity calculated using the Dice coefficient in the NTSYS program.

Pair-wise estimates of similarity ranged from 0.39 to 0.79. O-Hia and KwangJao were the closest genotypes with the highest similarity index of 79%, while KimJee and Chakrapat were the least similar cultivars (39%).

The UPGMA dendrogram produced using the Dice coefficient (Table 19.) between ten cultivars is showed in Figure 25. The phylogeny tree obtained indicated that the ten lychee cultivars were clearly separated into two major groups. The first formed by Kom, SampawKaew, Chorakum and Nakornpanom, the second formed by HongHuey, Chakrapat, O-Hia, KimJeng, KimJee and KwangJao

Table 18. List of 10 primer combinations used in AFLP analysis, their sequence, number of fragments and size range.

Primer name	**Combinations**	**Number of amplified fragments**	**Number of polymorphic fragments**	**Fragment size range (bp)**
A	E+ACA/M+CTA	38	21	200-4507
B	E+ACT/M+CAC	56	32	250-2850
C	E+ACT/M+CTA	45	30	250-2500
D	E+ACA/M+CAC	60	41	350-2560
E	E+ACT/M+CTT	44	27	200-4000
F	E+ACC/M+GCC	54	33	200-2110
G	E+ACA/M+CTT	51	30	200-4507
H	E+GCC/M+CGC	52	32	200-2500
I	E+AAC/M+CTT	58	27	200-4507
J	E+ACG/M+ACC	55	28	200-4507
Total		513	301	

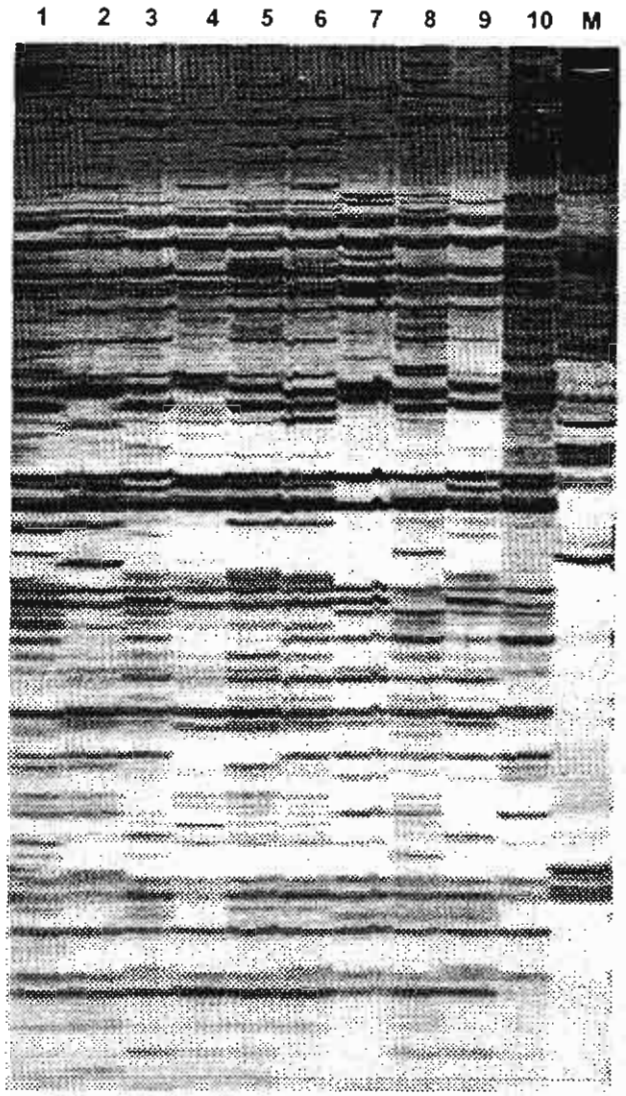

Figure 23 . AFLP profiles generated by using primer pair of E+ACC/M+GCC. Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, lamda/PstI marker.

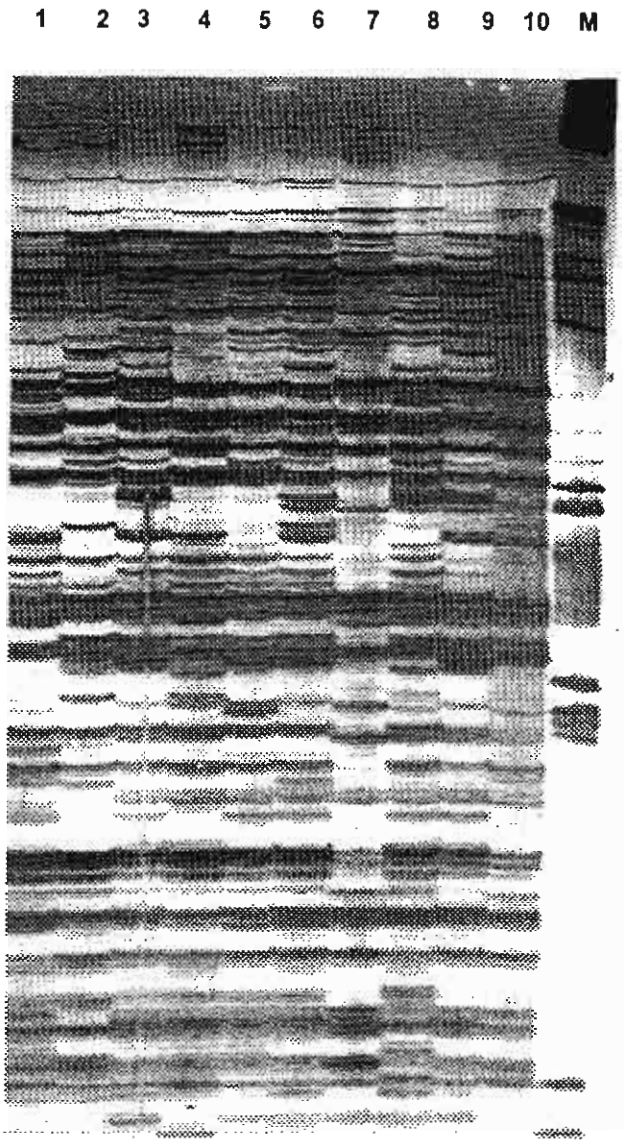

Figure 24. AFLP profiles generatred using primer pair of E+GCC/M+CGC. Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, lamda/*PstI* marker.

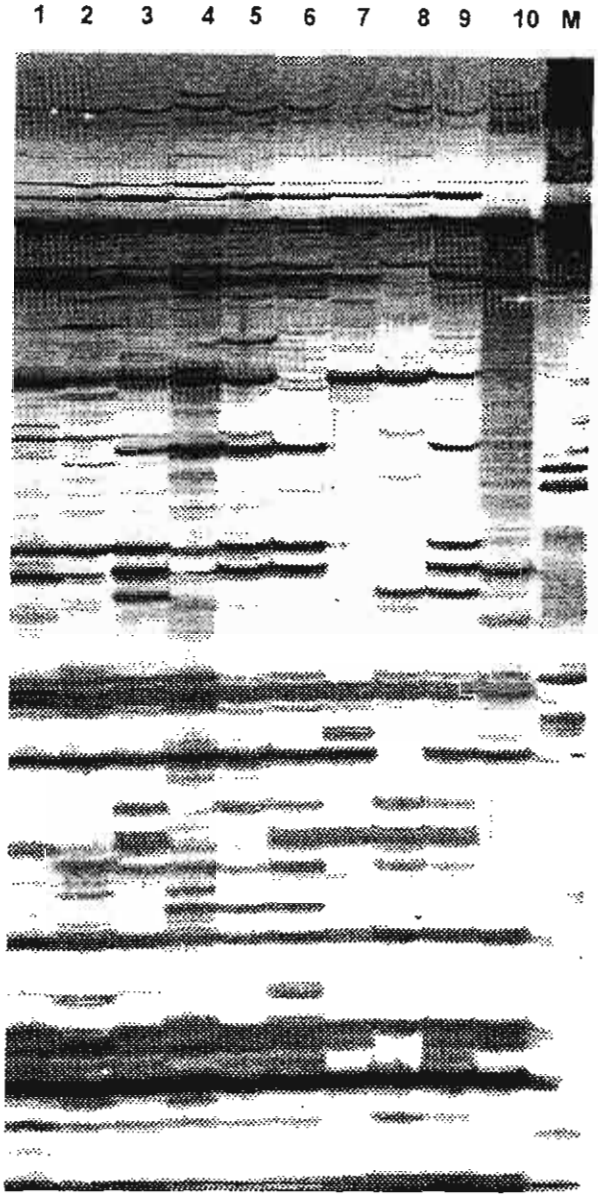

Figure 25 . AFLP profiles generatred using primer pair of E+ACG/M+ACC. Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, lamda/*PstI* marker.

Table 19 Dice similarity coefficients showing the relationships between 10 lychee cultivars. Results are based on 513 AFLP fragments generated by 10 primer combinations

Cultivar	1	2	3	4	5	6	7	8	9	10
Kom	1.00									
Chakrapat	0.54	1.00								
HongHuey	0.61	0.57	1.00							
KimJeng	0.53	0.64	0.57	1.00						
KwangJao	0.54	0.63	0.72	0.67	1.00					
O-Hia 0.57	0.59	0.79	0.67	0.79	1.00					
KimJee0.46	0.39	0.55	0.46	0.49	0.56	1.00				
Nakornpanom	0.59	0.48	0.71	0.44	0.57	0.60	0.41	1.00		
SampawKaew	0.64	0.43	0.74	0.46	0.59	0.67	0.57	0.58	1.00	
Chorakum	0.66	0.45	0.58	0.44	0.47	0.55	0.51	0.53	0.73	1.00

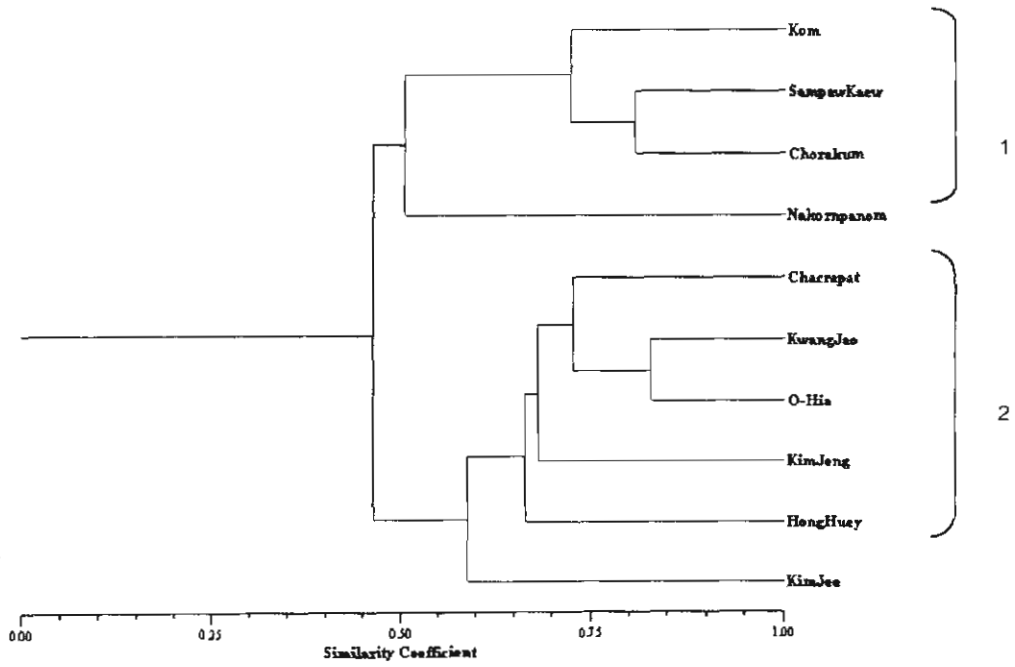

Figure 26 Dendrogram of 10 lychee cultivars using AFLP marker. The scale 0 to 1 indicates genetic similarity calculated using the Dice coefficient in the NTSYS program

4.6 Comparison of RAPD, PCR-RFLP, SSR, HAT-RAPD and AFLP markers

All of molecular approches used in this study were able to uniquely fringerprint each of the 10 lychee cultivars. The total number of assay units varied for each marker system from only 10 primer combinations for AFLP to 65 primers for RAPD (Table 20). Similarity, the number of bands scored ranged from 103 (from SSR) to 513 (from HAT-RAPD).

Table 20. Analysis of the RAPD, PCR-RFLP, SSR, HAT-RAPD and AFLP-generated banding patterns

Markers	Number of assay units	Total number of bands	Number of polymorphic bands (%)	Number of bands per assay unit
RAPD	65 (primers)	168	78 (46.4%)	1.2
PCR-RFLP	30 (10 primers, 3 enzymes)	143	71 (49.7%)	2.37
SSR	10 (primers)	103	42 (40.8%)	4.2
HAT-RAPD	10 (primers)	215	110 51.2%)	11
AFLP	10 (primer combinations)	513	301 (58.7%)	30.1

The percentage of polymorphic bands for each assay did not correlate to the total number of bands. Only 103 bands were scored for SSR, which was the lowest numbers, 143 bands for PCR-RFLP, 168 bands for RAPD, 215 bands for HAT-RAPD and the highest numbers was 513 bands were scored for AFLP. There was wide variation in the average number of genotypes revealed by each marker system. With genetic similarity matrices were obtained based on each marker type using Dice algorithm. The correlation between marker-based genetic similarity matrices were tested using Mantel test (Mantel, 1967). This test compares the elements of two matrices and estimates the degree of correlation between the matrices by means of a test criterion, Z, and a product-moment

correlation, *r*. The correlation coefficient among the five similarity matrices shows in table 21. All the estimates of correlation coefficients among genetic similarity data were highly significant ($P<0.01$). Correlation coefficients of SSR marker data (r = 0.26600, r = 0.38012, r = 0.46932 and r = 0.47655 with RAPD, HAT-RAPD, PCR-RFLP and AFLP, respectively) with those obtained using other marker systems were lower than those among similarity estimates based on RAPD, PCR-RFLP, HAT-RAPD and AFLP (r = 0.59158, r = 0.79704, r = 0.59691, r = 0.50162, r = 0.53966 and r = 0.97945, respectively between RAPD and PCR-RFLP, RAPD and HAT-RAPD, RAPD and AFLP, PCR-RFLP and HAT-RAPD, PCR-RFLP and AFLP and HAT-RAPD and AFLP).

Table 21. Mantel test correlation among 5 markers

Marker	RAPD	PCR-RFLP	SSR	HAT-RAPD	AFLP
RAPD	1.00000				
PCR-RFLP	0.59158	1.00000			
SSR	0.26600	0.46932	1.00000		
HAT-RAPD	0.79704	0.50162	0.38012	1.00000	
AFLP	0.59691	0.53966	0.47655	0.97945	1.00000

The values of the Mantel test correlation showed a good fit of the data using HAT-RAPD, AFLP and RAPD. The r correlation value was 0.97945 between HAT-RAPD and AFLP, 0.79704 between HAT-RAPD and RAPD and 0.59691 between RAPD and AFLP. These data indicate that the genetic similarity index obtained with HAT-RAPD and AFLP shared a good correlation and thus the results are comparable.

All methods for detecting polymorphic markers have strengths and weaknesses. Microsatellites or SSR are generally highly polymorphic and are widely distributed in the genome. Microsatellite markers can readily reveal mixed infections in uncloned samples and allelic interpretation is straightforward, but to detect them requires prior knowledge of sequence. While the recent growth in sequence information in public databases is quite phenomenal, the number of parasites for which such information is unavailable. Microsatellite analysis can often be conducted on crude lysates rather than purified DNA but even if conducting multiple PCRs per tube it can be problematic to screen more than three or four markers simultaneously. AFLP, in contrast, detects less polymorphism and requires purified DNA to ensure that complete restriction enzyme digestion is achieved;

however, it does not require prior sequence information. The number of polymorphisms detected from AFLP technique show much higher than that revealed

Like AFLP, RAPD analysis is applicable to DNA of any origin and complexity without prior sequence information. Both RAPD and AFLP techniques are poor at detecting mixed infections and care is required in allelic interpretation. However, AFLP uses high-stringency PCR, which improves reproducibility. Furthermore, AFLP markers are more suitable for genetic mapping, whereas RAPD markers have less frequently been found useful for segregation analysis.

We focus on the result of AFLP and HAT-RAPD. Both techniques surveys on the 10 lychee cultivars revealed 58.7% and 51.2% of polymorphic bands respectively, which are higher than RAPD, PCR-RFLP and SSR. If reproducible ability in amplification is considered, AFLP and HAT-RAPD are more reliable than the other as our experiment showed. Molecular markers based on PCR reaction and using unspecific primer is highly sensitive to the reaction condition, including the concentration of Mg^{2+}, *Taq* polymerase, dNTP, primers, DNA purity, etc (Skroch and Nienhuis 1995; Fritsch and Rieseberg 1996). The length of primer for RAPD is alway 10 bp, and thus the annealing temperature is always under 42°C. As a consequence, it is possible to generate non-specific products because of primer mismatching in PCR amplification, so that the standard procedure should be established and carried out with more caution to obtain good-quality and reproducible bands. AFLP and HAT-RAPD is robust, however, because they are less sensitive to the reaction conditions than RAPD.

In this study we have assessed the patterns of genetic diversity among ten lychee cultivars based on ten decamer primers for HAT-RAPD, ten AFLP primer combinations and ten selected SSR primers. All of the markers were able to uniquely fingerprint each of cultivar. Each technique not only differs in principal, but also in the type and amount of polymorphism detected. The level of polymorphism detected with each marker system varied widely and the index comparing their informativeness are reported in Table 2. In this study the correlation coefficients between HAT-RAPD and AFLP were higher than those between SSR and AFLP. The genetic similarity values obtained with HAT-RAPD and AFLP were equivalent and the dendrogram obtained were analogous whereas SSR gave a difference. The same results was reported by Bohn *et al.* (1999) that low correlations between AFLP and SSR data among winter wheat cultivars and speculated

that this may be due to a low linkage between marker loci from different marker systems resulting in sampling of different genome parts. In contrast to this, Russel *et al.* (1997) and Peglia and Morgante (1998) found overall low correlation coefficients comparing RAPD data with SSR, AFLP and RFLP data, while data based on SSRs and AFLPs were more closely correlated. Pejic *et al.* (1998) assumed that the high rate of mismatch annealing of RAPDs may be the main reason for this.

With HAT-RAPD and AFLP marker systems two major clusters were detected. The first cluster mainly consists of the favorite cultivars to grow in the north part of Thailand (Kwang Jao, O-Hia, Chakrapat, Kim Jeng, Hong Huey and Kim Jae) (Subhadrabandhu,1990). Kwang Jao and O-Hia had similar fingerprints because they were imported from the same city from China at different times (Subhadrabandhu,1990). Chakrapat and Kim Jeng were clustered in the equal group because they have the same flowering time (February) as well as Kim Jae and Hong Huey (December – January) (Montri, personal communication). In the second group, all they have been grown in the central part of Thailand. Most of them (Kom, Sampawkaew, Saraketong, and Nakornpanom) have been cultivated through seed germination and have adapted to grow for certain environmental conditions. Only Nakornpanom that required the low temperature for induced the flowering. So it showed the distant relationship from the others. From these results indicated that the HAT-RAPD and AFLP displayed the agreeability with the historical information. Therefore, HAT-RAPD was chosen to detect genetic variation among 33 lychee cultivars because they are being cheaper, faster and reproducible (Anuntalabhochai *et al.*, 2001).

4.7. HAT-RAPD analysis for 33 lychee cultivars

From the previous section, HAT-RAPD showed the powerful for the detection of genetic diversity among 10 lychee cultivars. So this technique was chosen to assessment of genetic variation within 33 lychee cultivars by using 5 decamer primers (OPH11, OPX04, OPAS10, OPC09 and OPD20). The totals of 316 reliable HAT-RAPD markers were visually scored. Of these fragments, 272 bands displayed polymorphic. The size of these polymorphic markers ranged approximately from 200 bp to 4500 bp (Table 22).

Table 22. List of HAT-RAPD primer, their sequence, number of fragments and size range

Primer	Sequence (5' ⟶ 3')	Number of amplifie fragments	Number of polymorphic fragments	Fragment size range (bp)
OPH11	CTTCCGCAGT	68	61	200-4000
OPX04	CCGCTACCGA	63	55	250-4000
OPAS10	CCCGTCTACC	67	59	300-5200
OPC09	CTCACCGTCC	55	46	200-1500
OPD20	ACCCGGTCAC	63	51	350-4000
Total		316	272	

Based on similarity coefficients using HAT-RAPD data, cluster analysis was performed by UPGMA in order to determine the relationships among different cultivars. The cluster analysis suggested the existance of two major groups (Fig. 32). Eighteen of the 33 cultivars formed a largest group 1. It could be further sub-divided into two sub-groups. The sub-group 1A comprised the 13 cultivars (KimJeng, which needed the long period of low temperature for induce the flowering. Five cultivars fell into a closely related subgroup 1B which contained only the abroad cultivars (HaakYip from China, SweetCliff from, Kaimana from Hawaii , Maritius from Israel and Brewster from Australia), which needed the same condition for flower induction.

Fifteen cultivars formed a separate group 2 showing their distinctness from the another group. Almost of them are the lowland cultivar, which needed the shorter period of low temperature for flower inducing.

The genetic variability in lychee has previously been characterized using isozyme (Degani et al., 1995) and the level of phenolic compound (Spranger *et al.*, 1998). In this study, molecular markers were subjected to measure the relationships among the genotypes. The amplification of total genomic DNA with random primers produced fragments with differnt staining intensities between genotypes. Intensity differences may be associated with the degree of homology between the primer and a given priming site on the template DNA or with the amplification of other fragments in the sample (Thormann et al., 1994).

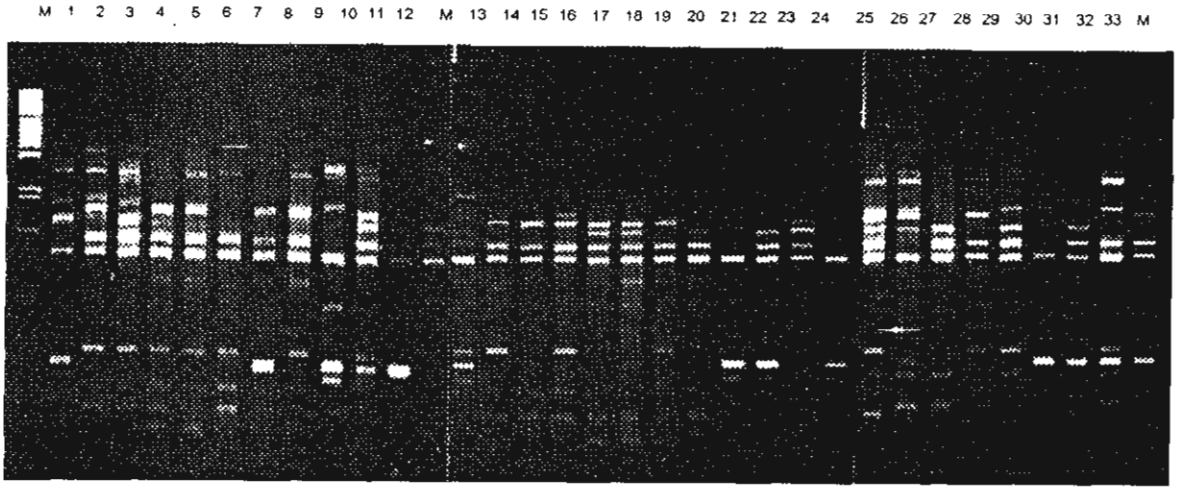

Figure 27. HAT-RAPD profiles generated by OPH-11 primer. Lane 1, Kim Jeng; lane 2, Chacrapat; lane 3, Kim Jae; lane 4, O-Hia; lane 5, Hong Huey; lane 6, Kwang Jao; lane 7, Brewster 1; lane 8, Phang 11; lane 9, Phang 80; lane 10, Phang 50; lane 11, Phang 46; lane 12, Phang 13; lane 13, Kom; lane 14, Thip; lane 15, Sarake Tong; lane 16, Sampaw Kaew; lane 17, Luk Lai; lane 18, Chorakum; lane 19, Nakornpanom; lane 20, Nai Sa-ard; lane 21, Jean Yai; lane 22, Jean Lek; lane 23, Kaloke Bai Oor; lane 24, Kaloke Bai Yor; lane 25, Kwai May Pink; lane 26, Jean dang; lane 27, Jean kreng suk; lane 28, Haak Yip; lane 29, Sweet cliff; lane 30, Salatheil; lane 31, Mauritius; lane 32, Brewster 2; lane 33, Kaimana and M, lamda/*Pst*I marker

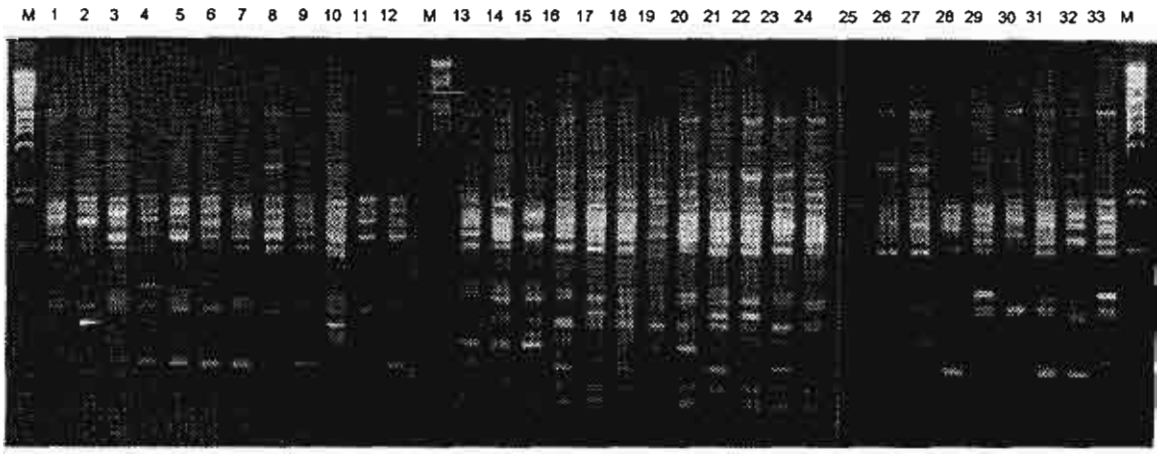

Figure 28 . HAT-RAPD profiles generated by OPD-20 primer. Lane 1, Kim Jeng; lane 2, Chacrapat; lane 3, Kim Jae; lane 4, O-Hia; lane 5, Hong Huey; lane 6, Kwang Jao; lane 7, Brewster 1; lane 8, Phang 11; lane 9, Phang 80; lane 10, Phang 50; lane 11, Phang 46; lane 12, Phang 13; lane 13, Kom; lane 14, Thip; lane 15, Sarake Tong; lane 16, Sampaw Kaew; lane 17, Luk Lai; lane 18, Chorakum; lane 19, Nakornpanom; lane 20, Nai Sa-ard; lane 21, Jean Yai; lane 22, Jean Lek; lane 23, Kaloke Bai Oor; lane 24, Kaloke Bai Yor; lane 25, Kwai May Pink; lane 26, Jean dang; lane 27, Jean kreng suk; lane 28, Haak Yip; lane 29, Sweet cliff; lane 30, Salatheil; lane 31, Mauritius; lane 32, Brewster 2; lane 33, Kaimana and M, lamda/*Pst*I marker.

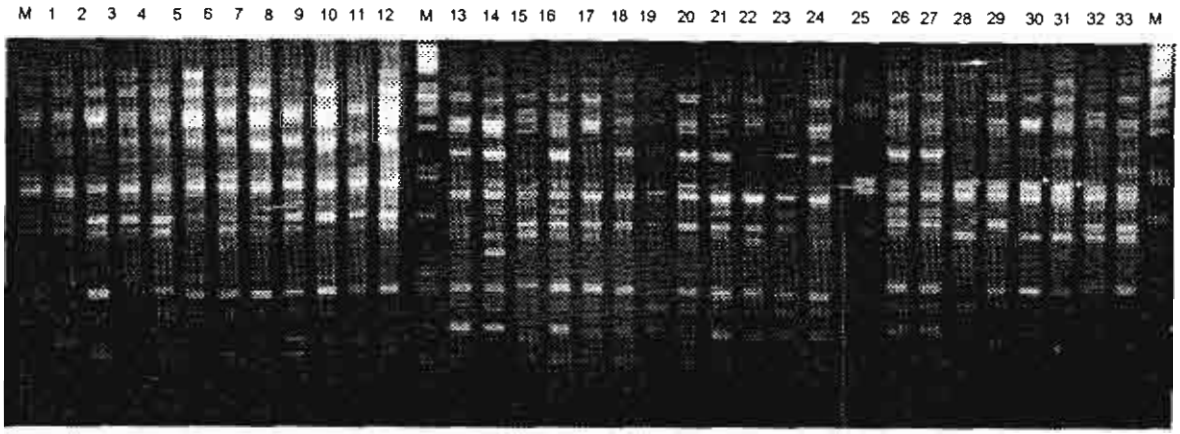

Figure 29 . HAT-RAPD profiles generated by using primer OPS-10. Lane 1, Kim Jeng; lane 2, Chacrapat; lane 3, Kim Jae; lane 4, O-Hia; lane 5, Hong Huey; lane 6, Kwang Jao; lane 7, Brewster 1; lane 8, Phang 11; lane 9, Phang 80; lane 10, Phang 50; lane 11, Phang 46; lane 12, Phang 13; lane 13, Kom; lane 14, Thip; lane 15, Sarake Tong; lane 16, Sampaw Kaew; lane 17, Luk Lai; lane 18, Chorakum; lane 19, Nakornpanom; lane 20, Nai Sa-ard; lane 21, Jean Yai; lane 22, Jean Lek; lane 23, Kaloke Bai Oor; lane 24, Kaloke Bai Yor; lane 25, Kwai May Pink; lane 26, Jean dang; lane 27, Jean kreng suk; lane 28, Haak Yip; lane 29, Sweet cliff; lane 30, Salatheil; lane 31, Mauritius; lane 32, Brewster 2; lane 33, Kaimana and M, lamda/*Pst*I marker.

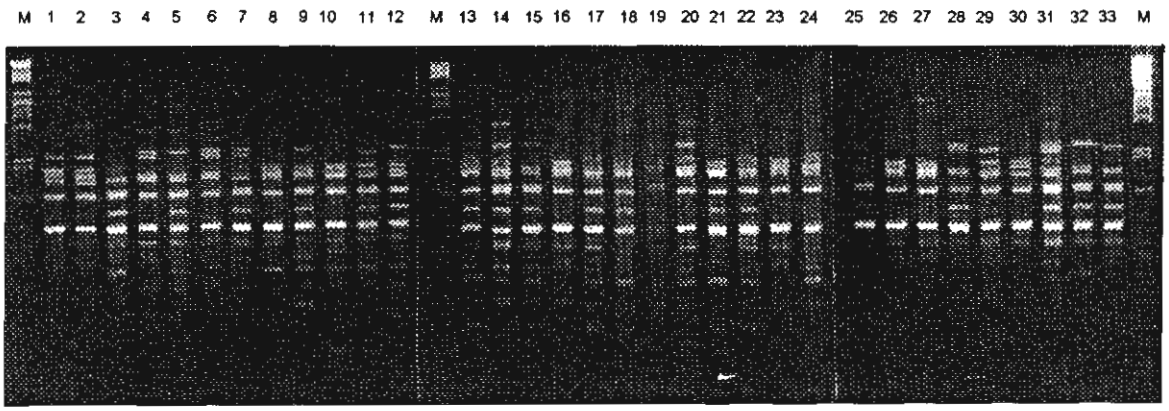

Figure 30 . HAT-RAPD profiles generated by OPC-09 primer. Lane 1, Kim Jeng; lane 2, Chacrapat; lane 3, Kim Jae; lane 4, O-Hia; lane 5, Hong Huey; lane 6, Kwang Jao; lane 7, Brewster 1; lane 8, Phang 11; lane 9, Phang 80; lane 10, Phang 50; lane 11, Phang 46; lane 12, Phang 13; lane 13, Kom; lane 14, Thip; lane 15, Sarake Tong; lane 16, Sampaw Kaew; lane 17, Luk Lai; lane 18, Chorakum; lane 19, Nakornpanom; lane 20, Nai Sa-ard; lane 21, Jean Yai; lane 22, Jean Lek; lane 23, Kaloke Bai Oor; lane 24, Kaloke Bai Yor; lane 25, Kwai May Pink; lane 26, Jean dang; lane 27, Jean kreng suk; lane 28, Haak Yip; lane 29, Sweet cliff; lane 30, Salatheil; lane 31, Mauritius; lane 32, Brewster 2; lane 33, Kaimana and M, lamda/*Pst*I marker

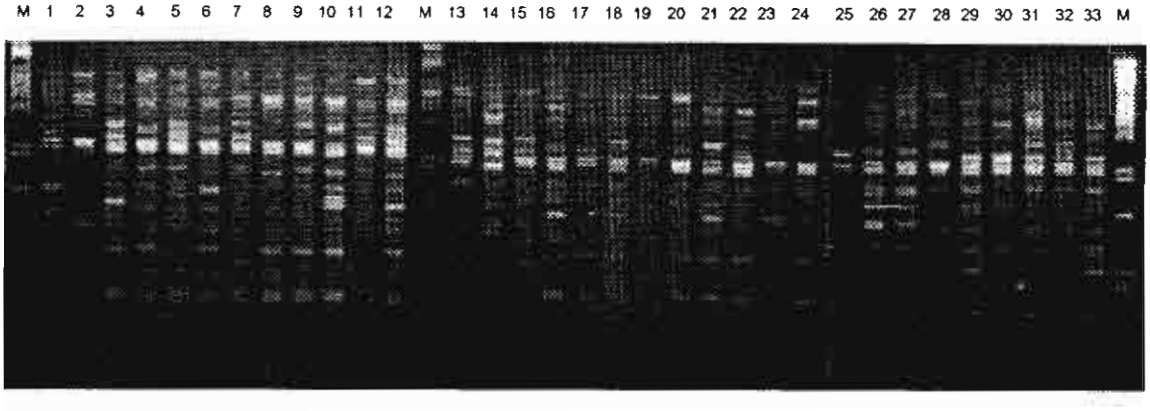

Figure 31. HAT-RAPD profiles generated by OPX-04 primer. Lane 1, Kim Jeng; lane 2, Chacrapat; lane 3, Kim Jae; lane 4, O-Hia; lane 5, Hong Huey; lane 6, Kwang Jao; lane 7, Brewster 1; lane 8, Phang 11; lane 9, Phang 80; lane 10, Phang 50; lane 11, Phang 46; lane 12, Phang 13; lane 13, Kom; lane 14, Thip; lane 15, Sarake Tong; lane 16, Sampaw Kaew; lane 17, Luk Lai; lane 18, Chorakum; lane 19, Nakornpanom; lane 20, Nai Sa-ard; lane 21, Jean Yai; lane 22, Jean Lek; lane 23, Kaloke Bai Oor; lane 24, Kaloke Bai Yor; lane 25, Kwai May Pink; lane 26, Jean dang; lane 27, Jean kreng suk; lane 28, Haak Yip; lane 29, Sweet cliff; lane 30, Salatheil; lane 31, Mauritius; lane 32, Brewster 2; lane 33, Kaimana and M, lamda/*Pst*I marker.

HAT-RAPD fingerprinting of 33 genotypes with five primers revealed a total number of 272 polymorphic amplified DNA fragments. On average, 54 polymorphic loci were scored per primer. The genetic similarity coefficients for the 33 clones ranged from 0.30 to 0.98. The UPGMA analysis made it possible to discriminate all of the genotypes of this study.

The dendrogram (Figure. 32) showed a clear distinction into a major and minor cluster. Thirty-three cultivars were separated into two major groups. The first group consists of 19 cultivars, and another contains 14 cultivars.

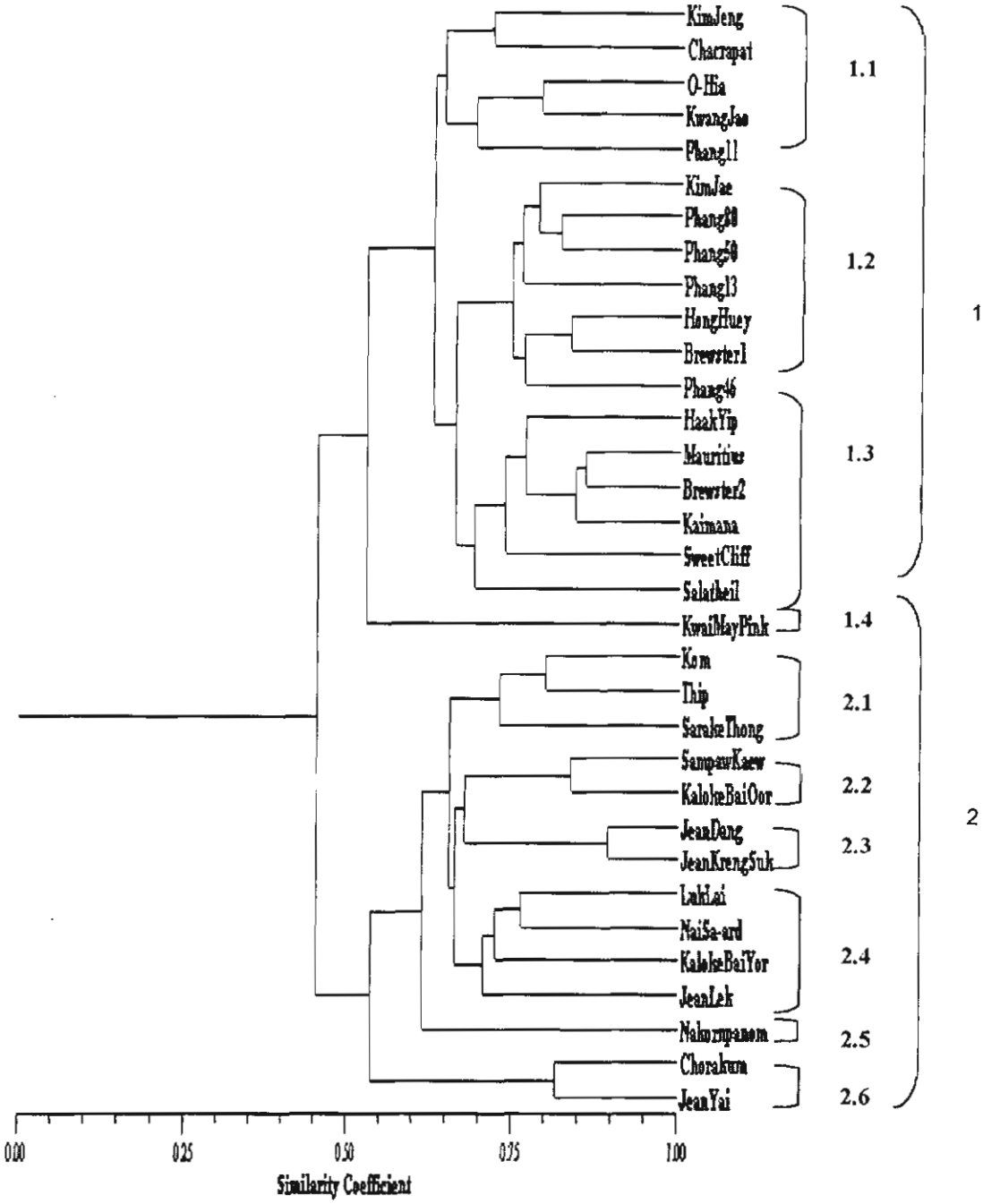

Figure 32 . Dendrogram of 33 lychee cultivars obtained by HAT-RAPD marker. The scale 0 to 1 indicates genetic similarity calculated using the Dice coefficient in the NTSYS program **1:** upland lychee group; **2 :** Lowland lychee group

For the first group, all of them are the Upland cultivars, which were planted in the north part of Thailand such as Chiangrai, Chiangmai and Lumphun. The sharable character of these group is needed the long period of low temperature for flower induction. Nineteen cultivars were clustered into 4 subgroups:

Subgroup1.1: O-Hia, KwangJao, Chacrapat, KimJeng, Phang 11

Subgroup1.2: KimJae, Phang 80, Phang 50, Phang 13, HongHuey, Brewster1, Phang 46

Subgroup1.3: HaakYip, Mouritius, Brewster2, Kaimana, SweetCliff, Salattheil

Subgroup1.4: Kwai May Pink

O-Hia, KwangJao, Chacrapat, KimJeng, (subgroup1.1) were grouped together because they were imported from the same province of China. They have the same flowering time (mid Febuary – March) and fruiting time (May – July). These four cultivars are the most popular cultivars in both Thailand and abroad. For Phang 11, it was clustered into this group because it is probable the hybrid of KwangJao and O-Hia.

Subgroup 1.2 consists the four hybrids (Phang 80, Phang 50, Phang 13 and Phang 46) and parents (KimJae, HongHuey and Brewster1). Unfortunately, the information of the hybrids are confusion because they were selected by the local farmers. So, our results could be used to define them. HongHuey, Brewster1 and Phang 46 showed the close relationship as Brewster1 was not imported from USA but it was selected by the Chiangrai Horticultural center as well as Phang 46 and gave the name to them.

Subgroup 1.3, most of them are the imported cultivars. Brewster2, HaakYip, Mauritius and SweetCliff were introduced from USA while Salatheil from Australia and Kaimana from Israel. These six cultivars need the lower temperature than the other cultivars in this group for activation of flower bud.

Kwai May Pink (subgroup 1.4) was separated from the other. It is posible that this cultivar originated from the hybridization of different cultivar for this experiment.

In the second group, most of them are the Lowland cultivars. The main character of these cultivars is no need the low temperature or need a short period of low temperature for flower induction. Fourteen cultivars were clustered into 6 subgroups:

Subgroup 2.1: Kom, Thip, SarakeThong
Subgroup 2.2: SampawKaew, KalokeBaiOor
Subgroup 2.3: JeanDang, JeanKrengSuk
Subgroup 2.4: LukLai, NaiSa-ard, KalokeBaiYor, JeanLek
Subgroup 2.5: Nakornpanom
Subgroup 2.6: Chorakum, JeanYai

JeanDang and Jean KrengSuk (subgroup 2.3) showed the very closely relationship because both of them are the same cultivar which was imported to Thailand in different times.

Nakornpanom (subgroup 2.5) was separated from the other because it was selected from the seed and planted at Nakornpanom province. The plant that has the good character or can adapt to the climate were collected and rename.

Lowland lychee cultivars have the closely relationship because almost cultivars originated as chance seedlings from unknown parents. The plant that carried good characters, such as high-yield, good flavor, good taste ,were selected. So some misnaming of cultivars have occurred. Identical cultivars or synonymies have the different names in different regions, and the homonyms or distinct cultivars can be found with identical name. For example, JeanLek and KalokeBaiYor originated from the seed propagation in China and directly introduced to the central part of Thailand

This study was conducted to determine the extent of genetic diversity in a sample of the Chiangrai germplasm of Thailand, based on the molecular marker system . Until recently, morphology was used extensively to determine relationships among plants. This was also the case in Lychee in which morphological and agronomic characters were used to assess genetic relationships (Ortiz 1997; Ortiz et al. 1998; Karamura 1998). Morphological markers are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith and Smith 1989). In contrast, DNA markers are found in abundance and are not influenced by the environment or developmental

stage of a plant, making them ideal for genetic relationship studies. This is the first study that has made use of molecular markers to examine genetic diversity of lychee cultivar in Thailand.

Low levels of genetic diversity in a group of closely related organisms are generally ascribed to factors such as domestication, a genetic bottleneck (temporary reduction in population size) or founder effects (establishment of a population by a single or few individuals). There appears to be no documented evidence to support the latter two effects, except Champion's (1970) hypothesis on a single ancestor. Consequently, we suggest that domestication could also be a major factor responsible for the reduced variability in the lowland cultivars. Lychee were introduced into Thailand long time ago (Simmonds 1966) and, therefore, have a long and intense history of cultivation (De Langhe 1961). Limited genetic diversity has also been recorded for many other domesticated species including beets (Jung et al. 1993), barley (Saghai-Maroof et al. 1994) and lentils (Ferguson et al., 1998).

The high level of DNA diversity contrasts with the low level of morphological variability present in these plants. Probable reasons for this discordance are the following. The HAT-RAPD primers can anneal in both areas of the genome responsible for the morphological variation and non responsible for the morphological variation, resulting in random sampling of the genome and an sufficient number of polymorphisms. The morphological variation is the result of genotype × environmental interaction. There are many report of environmental effects on lychee characters.

One of the interesting aspects of this study was the clustering of the two different climate , Upland and Lowland cultivars. Field data show that the two groups are similar in morphology. Upland is considered to be a recent introduction directly or indirectly from south China (Joubert, 1986; Menzell and Simpson, 1986; Taylor, 1993; Tindall, 1994) via the norther of Thailand while the low land cultivars were introduced directly from China to the central. Morphological descriptions were available for all 33 cultivars. The datas showed that all cultivars have the very closely characters. So it is very difficult to compare the clustering based on HAT-RAPD profiles with the morphological characteristics of lychee. DNA clustering patterns were in general agreement with climatic respondent characteristics of the cultivars. The abroad cultivars, which were grouped together with the Upland cultivars because most of them needed the long period of low temperature for

flower induction while the Lowland cultivars don't need the same condition for induce the flowering.

Cultivar identification allows agriculturists and processors to be assured that varieties offered for sale are of the correct genotype and specified provenance. The fundamental importance to good husbandary of recognizing specific distinct varieties is shown by practices of indigenous people of the Amazon basin in Peru who describe varieties of manioc in great detail and require new varities to be distinct (Boster 1985). Morphological characters have traditionally provided signatures of varietal genotype and purity. However, molecular characters that more quickly and accurately reveal genetic differences without the obscurance of environment, provide significant advantages in discrimination, reliability, timeliness and reduced cost.

Molecular genetic markers have the potential to be a powerful tool in managing plant germplasm collections, both in *situ* or ex *situ*. As molecular technique advance, the efficiency, amount of information generated and ease to use increase while the cost and time involved decrease. Conversely, cost associated with field space, repository space (including seed testing, regeneration and seed dissemination) are incresing. Therefore, every tool available which make the collection less expensive to store and analyse and more useful to plant improvement, must be exploited to its fullest potential.

The measure of genetic diversity and the fingerprinting of genotype using molecular markers are two distinc, but related, techniques. Genetic diversity is a relative measure of the genetic distances between genotype in a defined set or study using a pre-selected marker. These distances depend on the composition of genotype in the set, and the markers used for measurement. Fingerprinting is an absolute measure of the genetic makeup of an individual or a line, and must be unique to that individual or line in order to distinguish it from all others (not only in one study, but from all others in existance, except for twins, clone and completely identcal inbreds or dubble hapliod).

Genetic markers have been proposed to aid in plant genetic resource management in resource acquisition (sampling strategies and determining which groups may be under-represented in a collection), maintenance (maintaining trueness to type and monitoring changes in allele frequencies in population), characterization (of new acquired accessions when few or no data are available) and utillization (aiding in pre-breeding and introgression and marker-assisted selection). Specifically, fingerprinting and genetic diversity measures can be of use to germplasm bank curators and breeders in the following capacities: (1) to search for correlations of traits and markers (without mapping) in related individuals using a common database to store multiple types of Data), (2) to

narrow the search for new alleles in loci of interest, (3) to verify pedigrees and fill in the gaps in incomplete pedigree or selection history (4) to assign lines and population to heterotic groups (5) to choose parents for mapping, marker-assisted selection and backcrossing shemes (6) to mornitor changes in allele frequencies in populations (7) to study the evolutionary history of wild relatives.

Molecular markers are proving useful in the maintenance of and access to the diversity present in germplasm collections. In order to screen even a fraction of the total number of lines in a large collection, high throughput marker techniques and efficient data managment are essential components of a system that must be optimized before undertaking such a monumental task. The importance of data storage and analysis cannot be understated; access to marker data in an integrated database will allow future analysis and experiments in silico which were not imagined at the time the lines were fingerprinted but which will be able to take advantage of the existing data without the need for further lab work.

In large germplasm collection, it is not possible-or even desirable-to adapt all accessions for use by breeders. However, using markers, we can quickly screen accessions for the presence of novel alleles of useful genes. In the future, as the genomics revolution provides sequences of genesof interest to breeders, markers in gene itself will allow us to maximize the true potential of germplasm collections by screening for new alleles of these loci which can be transfered by introgression or transformation to elite germplasm. This represents a more directed search for new alleles of know loci, which may provide increased levels of adaptation to poor environments, resistance to pests, yield and new metabolites than currently available to breeders and genetictists.

In many situations, the most easily obtained assessment of genetic variation is that of measuring morphological or phenotypic variation. The sharing of phenotypic characters is interpreted as an indication of relatedness. Morphological traits are, however, often influenced by environmental conditions (Jasienski, 1997) which in turn may influence the estimation of genetic variation and relatedness. Consequently, to be really useful, morphological measurements should be accomplished on plant material that is grown in comparative trials. This may be both expensive and time consuming, and moreover, almost impossible to accomplish for some species that are very difficult to grow. However, if morphological characters are shown to be heritable, they will nevertheless reflect the genetic structure within the plant material.

When studying a plant species, there are several sets of phenotypic characters that may be used for discrimination and relatedness. Horticulturally important traits are

valuable for a species in cultivation, and form the basis for the breeders' selection of promising plant material. Other morphological traits are used mainly for identification of genotypes and cultivars, e.g. the UPOV (International Union for the Protection of New Varieties of Plants) guide-lines for evaluation of distinctness, homogeneity and stability. A third type of traits are used for evaluation of the genetic variation within a species, e.g leaf shape analysis. The use of automated image analysis of shape has several advantages over the scoring of morphological characters by hand. It allows rapid and cost-effective scoring of variation, in that a large number of plants can be screened easily. In addition, the process is separated into two phases (image aquisition and shape description): once the outlines have been stored, different approaches to shape description can be used with the possibility to choose the descriptor system that is best suited to a particular problem (White *et al.* 1988). Different descriptor suites are moment invariants (White *et al.* 1988) and elliptic Fourier coefficients (McLellan & Endler 1998). The moment invariants descriptor system describes leaf shape by quantities that measure te distribution of the Cartesian coordinates (x and y) of image points along the outline of the leaf. In contrast, the elliptic Fourier coefficients approach approximates the coordinates of points around the outline of the leaf to a trigonometric function. Moment invariants tend to be more efficient in describing shape differences at the between-plant level, whereas elliptic Fourier coefficients yield a better separation at the population or regional levels (Lonn & Prentice 1990)

The use of different methods to evaluate genetic diversity may reveal different patterns of variation. Phenotypic differences are not necessarily correlated with the number of underlying gene mutations, and differences in phenotypic characters are not necessarily reflections of different genetic events (Bachmann, 1992). RAPDs can potentially cover the entire genome (coding as well as non-coding regions), and since most of the genome is composed of non-coding DNA, it is plausible that the majority of the amplified fragments are from these regions. Mutations in non-coding DNA are selectively neutral and therefore, the RAPD analysis is able to detect even small differences in DNA which are not associated with phenotypic variation. Morphological traits are prone to selection since they often are related to fitness. However, RAPD markers have also been shown to be under selective pressure. In their analysis of *Triticuin dicoccoides,* Li *et al.* (1999) found a substantial amount of plant differentiation at the DNA level, which was associated with microclimatic stress. Moreover, quantitatively inherited characters like leaf morphology are often influenced by phenotypic plasticity but see McLellan, (2000). Consequently, a combination of morphological and molecular

analyses may be the most useful alternative when trying to understand all aspects of genetic variation within a species (Olsson, 1999).

Once the morphological traits or the generated molecular marker profiles have been evaluated, there are different strategies how to estimate the similarity or dissimilarity between the analysed individuals. Similarity indices measure the amount of closeness between two individuals, the larger the value the more similar are the two individuals. There is a variety of alternative measures for expressing similarity, like Jaccard's coefficient of similarity which can be used for binary data and often is applied in RAPD-based studies. This coefficient is based on number of positive matches between two individuals whereas joint absences are excluded. Dissimilarity coefficients instead estimate the distance or unlikeness of two individuals, the larger the value the more different are the two individuals. The Euclidean distance and the squared Euclidean distance are two commonly used measures of dissimilarity in both morphological and molecular analyses.

The amount of genetic diversity in a population depends on many factors. A few factors include: inbreeding, plant reproductive system, environment, diversity in founding population, time that population has been in existence, and gene immigration. Within a hybrid parent, less diversity means a more uniform hybrid; however, the more genetic diversity between the hybrid parents, the greater the heterosis seen in the resultant hybrid. The amount of diversity present in a wild population determines the number of individuals that need to be sampled in each population and the total number of populations that ought to be sampled to adequately represent the genetic diversity contained in those populations. It also indicates the population size that needs to be grown out to maintain the total genetic diversity originally present in a population. The relationship between two inbreds, lines, or populations can be defined in terms of genetic distance. Knowledge of genetic distances among genotypes within germplasm sources is critical for the continued success of plant breeding programs. Conservation methods for crop germplasm may also be affected by genetic relationships among commercial and breeding lines. Although pedigree information and taxonomic studies provide useful information for plant breeders interested in questions of genetic diversity, the ability to utilize DNA technology, such as molecular markers, provides a more accurate and reliable glimpse into these genetic relationships.

Distance measures with molecular markers generally are based on a presence/minus basis. With two inbreds, four types of matches between the inbreds are

possible: presence of the band in both inbreds (1-1), presence of a band in one inbred but not in the other (1-0 and 0-1), and absence of the band in both inbreds (0-0). Matrices developed from these data may be analyzed with a number of different distance measures, including those summarized by Dudley: the simple matching coefficient, Nei and Li's coefficient, Gower's coefficient, and Rogers' distance. Each of these methods has been used successfully to measure genetic distance among cultivated accession of the crop plants.

In recent years, molecular markers have been widely used as a tool to provide an estimate of germplasm similarity (or diversity) and the data derived from banding patterns were judged as more informative than those coming from pedigree analyses (Stiles *et al.*, 1993). Our results demonstrate that HAT-RAPD markers are useful to identify lychee genotypes and showed the good fit with the historical information and the dominant character of each cultivars. In the next chapter, we used the different marker, Sequence characterized amplified region (SCAR) for identification of some cultivars.

Chapter V

5.1 Sequence-Characterized Amplified Regions (SCARs)

Since the polymerase chain raction (PCR) technique was published, many workers have recognized its potential utility in the genetic characterization of organisms as compared with the restriction fragment length polymorphism (RFLP) technique. Later, other derived PCR-based techniques were developed which either need a previous knowladge of genomic DNA sequence to design specific primers such as SSRs, RAPDs and AFLPs. Most of these techniques have been successfully applied to plants for different purposes (Weising *et al.*, 1995).

PCR based techniques are generally quick and straight forward to perform and the fact that PCR requires only small amounts of DNA makes it useful even with *in vitro* plantlets. It is quick , easy and requires no prior sequence information. Marker assisted selection with RAPDs is not always possible because RAPDs designed for one population are not always polymorphic for another, or since they lack reliability among laboratories (Jones *et al.*, 1997; McGregor *et al.*, 2000). The use of RAPD results for the characterisation and evaluation of germplasm and genetic resources and for the identification of markers for use in breeding programmes at wide-scale – implies a standardisation to yield reproducible results since the error intrinsic to the procedure greatly compromises the value of the technique for marker-assisted selection and virtually precludes its use in seed quality control applications. Some attempts have been made to achieve such standardisation (Jones *et al.*, 1997; Claros *et al.*, 2000), but the best option to overcome these problems is the use of specific molecular markers.

These several techniques were adapted to resolve many viticulture problems when ampelographic method either could not be used or were too time consuming. The choice of using the fingerprint technique depends on it applications. For identification purposes, RAPD and AFLP techniques generate relatively complex patterns, making the analysis of gels tedious and time consuming. The AFLP method typically produce in each reaction between 50 to 100 bands, whereas the RAPD method often detects between 5 to 10, but can produce some false positives and negatives (Lamboy 1994) due to its competitive reactions (Heun and Helentjaris 1993). Alternatively, SSRs are highly specific but are sparsely distributed in plant genomes (Weber, 1991). Therefore, efficient

methods are still required for single and accurate identification of plant cultivars in routine procedures.

Specific molecular markers can be deduced from unique, single copy segments of the genome and can be considered co-dominant and can be used in closely related species (Shah *et al.*, 2000), while non-specific markers like RAPD are dominant markers (the homozygote is not distinguishable from the heterozygote) and non-locus specific. There are three ways to obtain specific molecular markers: one type is deduced from microsatellite sequences that consists of tandemly repeated multicopies of small oligonucleotide motifs. Many microsatellite alleles are too large to be amplified, and the microsatellites identified so far are species specific and costly to develop. The second are markers deduced from gene sequences. The third way is to convert polymorphisms to a RFLP probe or a SCAR (Paran and Michelmore 1993). SCAR markers are based on the sequencing of RAPD or AFLP fragments and furture definition of more specific primers (Naqvi and Chatoo, 1996; Adam-Blondon *et al.*, 1998; Negi *et al.*, 2000). Hence, the PCR products allow reproducibility of the technique on a relatively wide range of reactive conditions and in different laboratories (Lawson *et al.*, 1998). The use of restriction enzymes to detect polymorphic nucleotides by the loss or gain of a restriction enzyme recognition site is called CAPS (Konieczny and Ausubel 1993) and used to increase the number of polymorphisms detected by a single marker.

SCARs will allow for rapid marker development, even though they are not highly polymorphic. SCARs are advantageous over RAPD markers as

(i) they detect only single, genetically defined loci,

(ii) their amplification is less sensitive to reaction conditions,

(iii) they can potentially be converted into codominant markers that will increase the available information in a marker-assisted selection program,

(iv) they are not aware of the presence of introns that could eliminate the priming sites,

(v) scoring results obtained by SCARs are more straightforward than other PCR-based markers.

The ideal case is the one where SCARs can be used as an ASAP (presence/absence assay tc detect the product (Gu *et al.*, 1995), eliminating the need for electrophoresis to resolve the amplifications as well as decresing the cost and incresing the speed of the analysis. Consequently, SCAR markers offer the most practical method

for screening numerous samples in a time and labour-saving manners, being accurate, feasible to use and cost efficient (Kasai et al., 2000).

The development of sequence characterized amplified regions (SCARs) (Paran and Michelmore 1993) and allele-specific associated primers (ASAPs) (Gu et al., 1995), derived from RAPD fragments, introduced the possibility of obtaining reliable techniques and single DNA markers linked to genes in lettuce and beans, respectively. In a similar way, Mondon et al. (1997) defined the sequence specific DNA primer (SSDP) from RAPD markers for the molecular typing of *Aspergillus fumigatus* strains. SCAR marker have been recently developed for many plant species such as grape (Xu et al., 1995; Lahogue et al., 1998), barley (Deng et al.,1997; Hernandez et al., 1999; Eckstein et al., 2000).

In previous chapter, we have identified, characterised and established relationships of 33 lychee cultivars in Thailand. This approach was conducted with HAT-RAPD markers. Since the reproducibility of polymorphisms described is essential for a guarantee certificate of accuracy, we have focus on the development of a minimal number of specific molecular markers (SCAR) that can identify the 33 cultivars in a reproducible and easy way. Indeed, the specific markers described here could be used in furture assays of breeding and quality control.

5.2 Materials and methods

5.2 Plant material

The thirty-three different lychee-tree cultivars defined in previous chapter are used.

5.3. Cloning the selected HAT-RAPD fragments

5. 3.1 DNA extraction and HAT-RAPD analyses are performed as previous chapter described.

5. 3.2 The amplified products of adequate HAT-RAPD are excised from agarose gels. The gels slice is melted in TE buffer and the equal volume of phenol.

5. 3.3 Incubate at 50°C for 10 min or until the gel slice has completely dissolved.

5. 3.4 After the gel slice has dissolved completely, centrifuge the tube for 5 min at 13,000 rpm.

5. 3.5 Remove the supernatant to the new tube and add 1 volume of chloroform to the solution. Mix the solution by inverting the tube 5 min.

5. 3.6 Centrifuge the tube at 13,000 rpm for 5 min.

5. 3.7 Remove the supernatant to the new tube and add 1 volume of isopropanol.

5. 3.8 To precipitate the DNA fragment, keep the solution in -20°C freezer for over night.

5. 3.9 After the precipitation, centrifuge the tube at 13,000 rpm for 15 min.

5. 3.10 Remove the supernatant and add 70% cold ethanol for washing the DNA.

5. 3.11 Dry the DNA at room temperature for 10 min and dissolve with TE buffer.

5. 3.12 The DNA fragment is then ligated into pGEM-T-easy vector

5. 3.13 Set up ligation reactions as described below:

2X Rapid Ligation Buffer	5µl
pGEM-T Easy Vector (50ng)	1µl
PCR product	25 ng
T4 DNA ligase (3 Weiss units/µl)	1 µl
Deionized water to a final volume of	10 µl

5. 3.14 Mix the reaction by pipetting and incubate the reaction tube 1 hour at 16°C.

5. 3.15 Clone the pGEM-T-easy vector with insert into a competent *Escherichia coli* strain DH5°.by using Ion-beam transformation method (Anuntalabhochai *et al.*, 2001).

5.4. Ion Beam Transformation

5.4.1 To prepare the bacterial cells for transformation, spread the bacterial cells on fresh LB agar plate and incubate at 37°C for 6-8 hours.

5.4.2 Place the sterile adhesive tapes at the appropriate position on the petri dish.

5.4.3 Smear the bacterial cells from 15.1 on sterile adhesive tapes and keep it in sterile condition.

5.4.4 Place the petri dish in the sample holder and take it into the target chamber.

5.4.5 Inside the target chamber the operating pressure is about 10^{-3} Pa and the temperature of the target chamber is about $0^{\circ}C$.

5.4.6 Bacterial cells are bombarded with Argon ions at 26 keV to fluence of $5x10^{14}$, $1x10^{15}$, $2x10^{15}$ and $4x10^{15}$ ions/cm^2.

5.4.7 The ion-bombarded bacterial cells are removed from the vacuum chamber and promptly, within about 10-15 minutes, suspended in 1.5-ml microfuge tubes containing 150 µl LB medium for 2-5 minutes.

5.4.8 10 µl of the cell suspension are incubated separately with 10 µg of recombinant-plasmid DNA on ice for 5 minutes.

5.4.9 Transfer the cell suspension into 3-ml LB medium and incubate at $37^{\circ}C$ for 2 hours with gently shaking.

5.4.10 Transfer 100 µl of cell suspension onto LB agar medium containing 100 µg/ml ampicillin antibiotic.

5.4.11 Store the plate at room temperature until the liqid has been absorbed

5.4.12 Invert the plates and incubate at $37^{\circ}C$. Transformed colonies should appear in 12-16 hours.

5.5. Plasmid preparation by alkaline lysis with SDS: Minipreparation

5.5.1 Inoculate 2 ml of LB medium containing ampicillin (100µg/ml) with a single colony of transformed bacteria. Incubate the culture overnight at $37^{\circ}C$ with vigorous shaking.

5.5.2 Pour 1.5 ml of culture into a microcentrifuge tube. Centrifuge at maximum speed for 30 second in microfuge. Store the unused portion of the original culture in $4^{\circ}C$.

5.5.3 Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

5.5.4 Resuspend the bacterial pellet in 100 µl of ice-cold alkaline lysis solution I by vigorous vortexing.

5.5.5 Add 200 µl of freshly prepared alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. Store the tube on ice for 5 minutes.

5.5.6 Add 150 µl of alkaline lysis solution III (Appendix). Close the tube and disperse alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 5-10 minutes.

5.5.7 Centrifuge the bacterial lysate at maximum speed for 10 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.

5.5.8 Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture for 2 hours at -20°C.

5.5.9 Collect the precipitated nucleic acids by centrifugation at maximum speed for 10 minutes.

5.5.10 Remove the supernatant by gentle aspiration. Stand the tube in an invert position on a paper towel to allow all of the fluid to drain away.

5.5.11 Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes.

5.512 Again remove all of the supernatant by gently aspiration.

5.5.13 Remove all beads of ethanol that on the side of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube.

5.5.14 Dissolve the nucleic acids in 50 µl of TE buffer (Appendix). Store the DNA solution at -20°C.

5.6. DNA Sequencing

5.6.1 For each set of sequencing reactions, label four the 0.5-ml microfuge tubes (G, A, T, C). Add 2 µl of the appropriate d/ddNTP Mix to each tube. Add 1 drop of mineral oil to each tube. Cap the tubes and store on ice until needed.

5.6.2 For each set of sequencing reactions, mix the following reagents in a microfuge tube:

plasmid template DNA	50 ng
DNA sequencing 5x buffer	5 µl

SP6 or T7 primer	100 ng
Distilled water to final volume	16 µl

5.6.2 Add 1µl of Sequencing Grade *Taq* DNA polymerase (5u/µl) to the primer/template mix. Mix briefly by pipetting.

5.6.3 Add 4 µl of the enzyme/primer/template mix from 16.3 to each labeled tube containing d/ddNTP and mix briefly.

5.6.4 Briefly centrifuge in a microcentrifuge.

5.6.5 Place the reaction tubes in a thermal cycler that has been preheated to 95°C and using the following cycling profiles:

95°C for 2 minutes, then	
95°C for 30 seconds	(denaturation)
42°C for 30 seconds	(annealing)
70°C for 1 minutes	(extension)
For 55 cycles total, then 4°C o/n	

5.6.6 After the program has been completed, add 3 µl of DNA Sequencing Stop Solution to each tube. Briefly centrifuge in microcentrifuge to terminate the reactions.

5.6.7 Heat the reaction at 70°C for 2 minutes immediately before loading 3.5 µl of each reaction on a 6% polyacrylamide sequencing gel with 0.4 mm spacers.

5.7 SCAR primer designed

For each cloned HAT-RAPD amplification product, two oligonucleotides (one from each end) are designed to be used as SCAR primers. Each primer contained the original 10 bases of the HAT-RAPD primer plus the next 8, 9 or 10 internal bases from the end.

5.8 SCAR assay

Amplification of genomic DNA (10 ng/µl) with SCAR primers is carried out in a GeneAmp PCR system 2400 (Perkin-Elmer) in standard PCR reaction that consisted in 30 cycles of 30 sec at 94°C, 30 sec at the annealing temperature, and 45 sec at 72°C. Amplification products are resolved electrophoretically in 1.4% agarose gel. The polymerase used is HotStartTaq (QIAGEN) at 1 unit/ µl. The annealing temperature is first calculated as 4-fold the number of GC plus 2-fold the number of AT and decreasing the result by 4°C.

5.9 Result and Discussion

5.9.1 SCAR marker for Kom variety

5.9.1.2 HAT-RAPD amplification and cloning

From HAT-RAPD fingerprint of 33 lychee cultivars obtained by using primer OPH11 (Figure 33), one cultivar-specific HAT-RAPD marker was selected for transformation into SCAR marker. The $OPH11_{250}$ band from Kom was selected because it produced an intense band from Kom DNA compared to the faint bands revealed from the other lowland cultivars. The fragments were separated on 1% agarose, the gel slice containing the fragments of interest were cut out and the DNAs were purified as . The cultivar-specific band was successfully cloned into plasmid pGEM-T-Easy vector (Promega) and transformed into *E.coli* competent cells by three methods; chemical transformation, electrotransformation and ion-beam transformation. This is the first report that could be transformed the foreign DNA into bacterial cells by using the low energy ion beam (Anuntalabhochai et al, 2001).

The selection of positive clones was carried out by PCR analysis. DNA templates from white colonies were amplified with T7 (TAATACGACTCACTATAGGG) and SP6 (TATTTAGGTGACACTATAG) primers. Positive colonies were grown overnight in 2 ml of LB medium containing 100 µg/ml amplicillin. For each cloned marker, purification of plasmid DNA was carried out from five independent transformed clones using alkaline lysis procedure (Sambrook and Russel, 2001). The size of DNA inserts were checked by *Eco*RI enzyme digestion (Promega) followed by separation in 1.4% agarose gel.

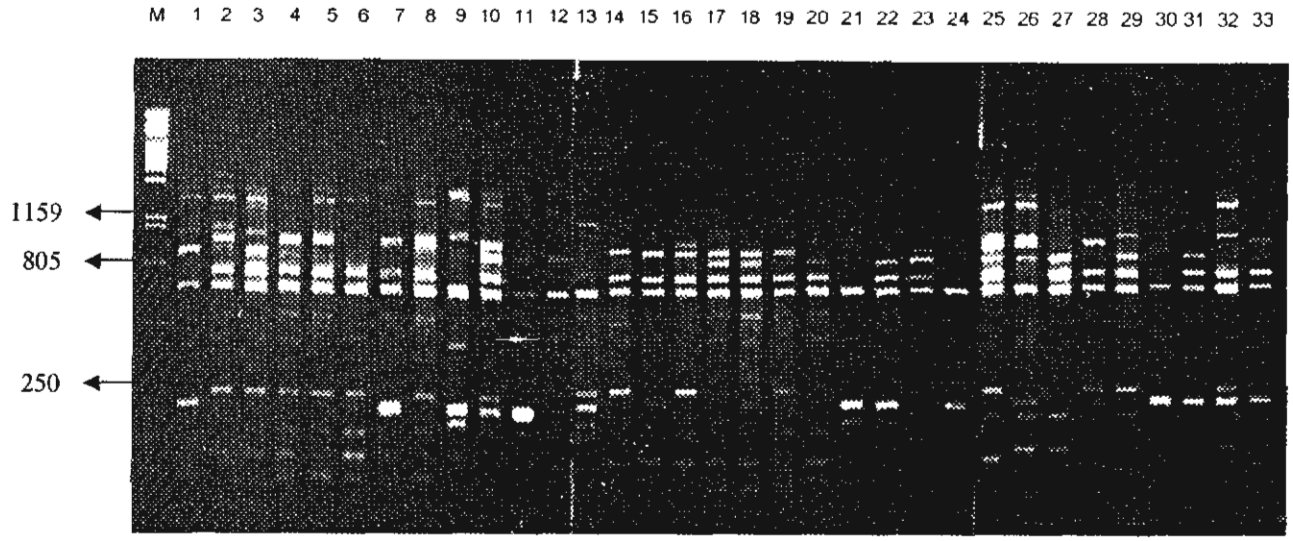

Figure 33. HAT-RAPD profiles generated by OPH-11 primer. Lane 1, Kom; lane 2, HongHuey; lane 3, Chakrapat; lane 4, O-Hia; lane 5, Sampawkaew; lane 6, Choragum; lane 7, Nakornpanom; lane 8, KimJeng; lane 9, KimJee; land 10, KwangJao and M, lamda/*Pst*I marker. Numer (left panel) indicates molecular weight in base pairs.

5.9.1.2 Sequencing of HAT-RAPD fragment and specific primer design

The selected fragment (250 bp) was sequenced by using the SILVER SEQUENCE™ DNA Sequencing System (Promega), then three pairs of oligonucleotides were designed to be tested as SCAR primers (Table 23). Each primer contained the original 10-mer RAPD primer as described by Paran and Michekmore (1993); only the next eight, nine or ten internal bases from the end were added. The initial test of PCR conditions was performed using the genomic DNA of ten lychee cultivars with each primer pair from the set.

Table 23. Sequence-specific oligonucleotide primers derived from HAT-RAPD fragment of Kom cultivar. The primer pair, annealing temperature and unexpected bands for initial specific amplification are note.

SCAR primer[a]	5' to 3' Sequence[b]	Annealing temperature	unexpected bands
$OPH11F1_{250}$	CTTCCGCAGTTGGTCTAGG	65^0C	Yes
$OPH11R1_{250}$	CTTCCGCAGTAACAGAATT		
$OPH11F2_{250}$	CTTCCGCAGTTGGTCTAGGA	65^0C	Yes
$OPH11R2_{250}$	CTTCCGCAGTAACAGAATTT		
$OPH11F3_{250}$	CTTCCGCAGTTGGTCTAG	65^0C	No
$OPH11R3_{250}$	CTTCCGCAGTAACAGAATT		

[a] The letters and numbers preceding the R (reverse) and F (forward), refer to the progenitor primer used (OP:Operon Technologies). The subscript indicates the size of marker in bp.

[b] The underlined sequences represent the original sequence of the progenitor RAPD primer

The preliminary screening determined the optimal primer pairs and the annealing temperature for specific amplification. Only one primer pair, $OPH11F3_{250}$/$OPH11R3_{250}$ produced a single and specific marker on the expected size of Kom cultivar. For the other primer pairs, they produced the unexpected fragments in at least 2 cultivars. So $OPH11F3_{250}$/$OPH11R3_{250}$ were chosen to amplified the genomic DNA of 33 lychee cultivars. The result showed that primer $OPH11F3_{250}$/$OPH11R3_{250}$ generated the specific marker for Kom cultivar identification (Figure. 37).

For the previous study (Anuntalabhochai *et al.*, 2001), RAPD was able to identify some lychee cultivars by specific markers but these markers were amplified and visualized into patterns with other RAPD bands. Moreover, the RAPD technique has been questioned due to its lack of total reproducibility (Buscher *et al.*, 1993; Lamboy 1994) which is an important pitfall both to cultivar identification in routine procedures and to data exchange among laboratories. In this study, our objective was to clone one specific RAPD marker and to transform it into single SCAR marker. The reliability of

SCAR markers linked to a unique locus has been reported in some important crops (Naqvi and Chaltoo 1996; Barret *et al* 1998) as well as fruit (Lahogue *et al.*, 1998; Deputy *et al.*, 2002).

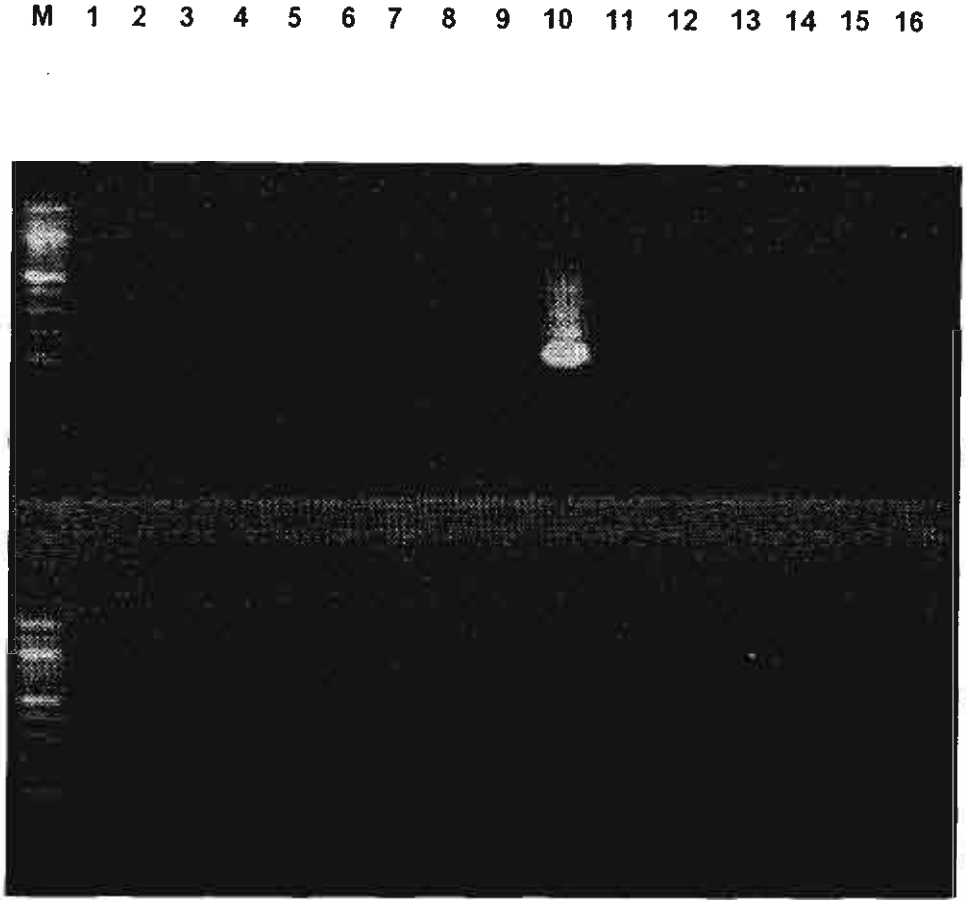

Figure 34. Lychee cultivar identification with sequence-specific primers $OPH11F3_{250}$ and $OPH11R3_{250}$ (SCAR marker). The expected amplification product presented in lane 10, which is Kom cultivar.

The SCAR markers described here have some advantages over RAPD markers used to distinguish lychee tree. The use of RAPD markers allowed us to identify molecular markers within a few months, but due to their dominant nature, the amplification of multiple loci, and their sensitivity to reaction conditions, restricted their further use. SCARs are more independent to reaction conditions, and are usually codominant markers that amplify a single locus, making them robust and more reproducible assay than could be obtained with the short primers used for RAPD analysis. This is the reason why we have used primers a little shorter than the 24-mer

described by Paran and Michelmore, since they can produce fragments in most genotype (Gu *et al.*, 1995)

In conclusion, we have been able to describe a cheap, reliable procedure to identify 33 lychee cultivars. The proposed methodology represents a simple and reliable approach for the generation of effective and specific molecular markers to assist in lychee paternity testing, variety identification and mapping.

5.9.2 Identification of lowland lychee cultivars

In section 5.9.1 a specific identification for Kom variety by SCAR marker (Figure 34) was reported, hereby a specific primer for lowland lychee cultivars is described.

HAT-RAPD fingerprints of 33 lychee cultivars amplified by OPH-11 was demonstrated previously (Figure 34). The fragment at 250 bp. Product from lane 2 (Hong Huey cultivar) was excised from agarose gel, consequently subcloned into P-Gem-T-easy vector (Promeca) then sequenced. Based on the sequence 4 oligonucleotide primers, named OPH11F1, OPH11R1, OPH11F2 AND OPH11R2 (Table 24), were designed and synthsized (BSU, Bioservice). For the forward primers, OPH11F1 AND OPH11F2, both primers were designed by extendtion 3 (TAA) and 5 (TAAGA) nucleotides following sequence of OPH11 pirmer (CTTCCGCAGT) respectively. Whereas the reverse primers, OPH11R1 and OPH11R2, were extended with 3 (GCC) and 5 (GCCAA) nucleotides after OPH11 sequence as well.

Genomic DNA of 22 cultivars including upland and lowland cultivars including Kim Jeng, Chacrapat, Kim Jae, O-Hia, Hong Huey, Kwang Jao, Phang 11, Phang 80, Phang 50, Phang 46, Phang 13, Kom, Thip, Sarake Tong, Sampaw Kaew, Luk Lai, Chorakum, Nakornpanom, Nai Sa-ard, Jean Yai, Jean Lek, and Kaloke Bai Oor, were amplified using combination of these primers under PCR condition (as mentioned in section 5.9.1.1). Only a combination of OPH11F2 and OPH11R2 revealed a single intended band at expecting size of 250 bp in all 11 lowland cultivars. (Figure 36). However number of faint-unspecific bands were also observed. Interestingly, none of the bands was amplified in all 11 upland cultivars.

Table 24. The designed oligonucleotide primers derived from HAT-RAPD fragment of Honh Huey cultivar.

Primers	Sequences
OPH11F1	CTTCCGCAGTTAA
OPH11R1	CTTCCGCAGTGCC
OPH11F2	CTTCCGCAGTTAAGA
OPH11R2	CTTCCGCAGTGCCAA

F: forward primer and R: reverse primer. The underline indicate additional nucleotides following OPH11 sequence (CTTCCGCAGT)

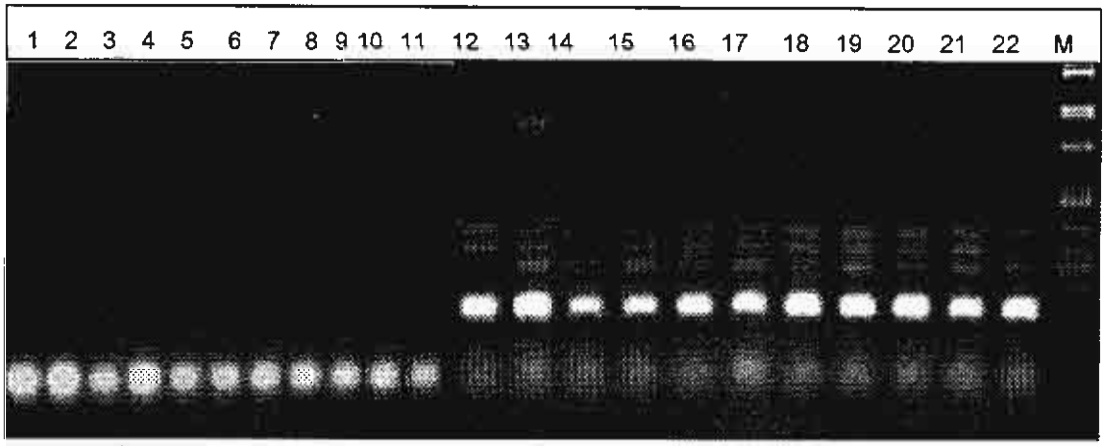

Figure 35 Lychee cultivars amplifiled with specific primers OPH11F2 and OPH11R2. The expected amplification product (250 bp) presents in lane 12 – 22. None of the expexted product (250 bp) is detected in lane 1-11. In panel A represents the upland lychee cultivars. In panel B representis the lowland cultivar group. Lane 1, Kim Jeng; lane 2, Chacrapat; lane 3, im Jae; lane 4, O-Hia; lane 5, Hong Huey; lane 6, Kwang Jao; lane 7, Phang 11, lane 8, Phang 80; lane 9, Phang 50; lane 10, Phang 46; lane 11, Phang 13; Panel B represents for low land cultivars; lane 12, Kom; lane 13, Thip; lane 14, Sarake Tong; lane 15, Sampaw Kaew; lane 16, Luk Lai; lane 17, Chorakum; lane 18, Nakornpanom; lane 19, Nai Sa-ard; lane 20, Jean Yai; lane 21, Jean Lek; lane 22, Kaloke Bai Oor . M, lamda/*Pst* I marker.

As mentioned previously, the lowland cultivars usually do not required low temperature for floral induction. Whereas the upland cultivars need low temperature (10-15 $^{\circ}$C) at certain period of time for flowering. The presence of the certain DNA fragment in genomic DNA of lowland cultivars may imply that the insertion of the DNA fragment might inactivate function of genes involving in responsibility for flowering induced by the low temperature. Therefore for further work, characterization and function analysis of the DNA fragment in the lowland cultivars will be carries out.

Chapter VI

6.1 Hybrid detection in lychee cultivars by HAT-RAPD markers

Genotypic identification of breeding lines, hybrids and clones are necessary in many plants but commonly these are based on morphological and anatomical features (Crawford, 1990). Phenotypic traits seldom serve as unambiguous descriptors, are vulnerable to environmental and physiological conditions of the plant, and therefore have limited use for clear genetic identification. As an alternative, laboratory methods have been developed such as karyological measurements, chromatography, protein electrophoresis, and isozyme analysis (Brown *et al.*, 1993), where only isozyme analysis has had some level of acceptance and has proven a useful comparable method for a number of taxa (Torres *et al.*, 1993). But even isozyme analyses have drawbacks in that they detect only very limited amounts of polymorphism amongst closely related genotypes. These limitations were discussed by Naton *et al.*, (1992) and they came to the conclusion that for some somatic hybrids of tobacco produced through protoplast fusion, isozyme separation did not correlate well with the morphological characteristics of some hybrids.

Molecular markers, which detect variation at the DNA level overcome most of the limitations of morphological and biochemical markers. As demonstrated by their use in various plant species, molecular markers are best suited for estimation of genetic diversity and varietal identification. Besides their unlimited numbers, molecular markers are not affected by environmental and developmental stage. Use of molecular markers gains further importance for perennial crops like lychee where progress in crop improvement is often hampered by its long generation period. Molecular markers have been applied for several purposes for instance identification in number of plant cultivars and species (Prevost and Wilkinson,1999; Pasakinskiene *et al.*, 2000), phylogenetic evaluation (Wang *et al.*, 1998; Blair *et al.*, 1999), tagging and marker aided selection of ergonomically important genes (Akagi *et al.*, 1996; Ratnaparkhe *et al.*, 1998; Hittalmani *et al.*, 2000), linkage map construction and the mapping of quantitative traits loci (QTL) (Wang *et al.*, 1998; Debener and Mattiesch, 1999; Dunemann *et al.*, 1999; Garcia *et al.*, 2000; Takeuchi *et al.*, 2001). Many of the complications of a phenotype based assay can be mitigated through direct identification of genotypes with DNA based diagnostic

assays (Tingey and del Tufo, 1993). DNA changes (mutations) are usually stably inherited at every cell division cycle, and assays can be carried out on single cells through to complete individuals. Two such methods include DNA fingerprinting using predominantly mini- and micro-satellite DNA sequences, and RFLP analysis (Rafalski and Tingey, 1993). Although these methods are able to potentially disclose unlimited polymorphic markers, they require some knowledge of the plant genome to target, they require large amounts of DNA, they are lengthy, costly, labour intensive and also commonly use radioactivity.

Welsh and McClelland (1990) and Williams *et al.*(1990) reported an identification technique based on the amplification of random genomic DNA sequences by PCR using single, short arbitrary primers, commonly called RAPD-PCR. Amplified RAPD products usually segregate in a Mendelian fashion and these can be effectively used as genetic markers. RAPD technology therefore surveys (scans) numerous loci in the genome, which makes this method particularly attractive for analysis of genetic distance and similarity between species (Clark and Lanigan, 1993). Some other advantages of this technique include no knowledge of the plant DNA is needed, its ability to scan and detect extensive polymorphism, simplicity, rapidity, use no radioactivity and requires very small amounts of genomic DNA. Since 1990, RAPD has been used to distinguish species, cultivars, hybrids and clones in a variety of different plants (Baird *et al.*, 1992). However, the number of DNA markers is still insufficient because when 10-base primers were used for RAPD analysis in many plant species and hybrids only 10-16% of them amplified polymorphic bands (Yamagishi, 1995). Therefore, it is important to develop new DNA markers for genetic evaluation of horticultural important traits in plant species.

Usually, the technique is performed at low annealing temperatures between 35 and 38°C resulting in low reproducibility DNA polymorphism in number of plant species. Currently, Anuntalabhochai *et al.*, (2000) reported that increasing the annealing temperature above 46 °C in the RAPD reaction provides high resolution, reproduction and a high degree of polymorphism.

In this chapter, we used the HAT-RAPD marker to genetically identify lychee hybrids cross-pollinated from different pairs of lychee parents.

6.1.2 Materials and methods

6.1.2.1 Plant material

The plant materials for this study comprised of 4 commercial cultivars including Chacrapat, KimJeng, Hong Huey and O-Hia. Among these cultivars they were selected for cross pollination to produce hybrids (Table 25).

Table 25 List of lychee parents and their hybrids.

Female parent (♀) x Male parent(♂)	Hybrid
Chacrapat x KimJeng	No.11 No.14 No.16
KimJeng x Chacrapat	No.25 No.31 No.32
KimJeng x Hong Huey	No.1 No.7 No.8
Hong Huey x KimJeng	No.17 No.18 No.19
Chacrapat x O-Hia	No.29 No.30

6.1.2.2 DNA extraction

Isolation of total genomic DNA for molecular marker analysis was carried out utilizing the cationic hexadecyl trimethyl ammonium bromide (CTAB) method of Weising *et al.* (1991) with small modifications.

1 Young leaf pieces (0.5 – 1g) were washed twice in sterilized distilled water and ground to a fine powder with liquid nitrogen in a sterile mortar and pestle.

2 The powder obtained (300 mg) was transferred to a 1.5 microcentrifuge tube and resuspended in 700 µl preheat CTAB extraction buffer.

3 Samples were incubated at 60°C in the water bath for 60 minutes.

4 Proteinase K (10mg/ml) were added and carefully inverted several times then left for 60 minutes at 37°C.

5 The tubes were centrifuged at 12,000 rpm for 5 minutes.

6 The supernatants were transferred to the new tubes and added with the equal volume of chloroform.

7 The tubes were inverted for 5 minutes and centrifuged at 12,000 rpm for 3 minutes.

8 The supernatants were removed to the new tubes, then added the equal volume of cold isopropanol and incubated at -20°C overnight.

9 The tubes were spun at 12,000 rpm for 15 minutes and the supernatants were removed.

10 The pellets were washed with 500 µl cold 70% ethanol.

11 The tubes were spun at 12,000 rpm for 5 minutes and the supernatants were removed.

12 The nucleic acid (DNA, RNA) pellets were dried at a room temperature and resuspended in 50 µl of TE buffer.

13 For the DNA purification, RNase A (10mg/ml)(Sigma) were added in the solutions and placed at 65°C for 15 minutes.

14 After that, 300 µl of TE buffer and 300 µl of phenol were added. The tubes were inverted for 5 minutes and spun at 10,000 rpm for 5 minutes.

15 The supernatants were transferred to the new tubes and added with the equal volume of chloroform:isoamyl alcohol (24:1).

16 The tubes were inverted for 5 minutes and spun at 10,000 rpm for 5 minutes.

17 Repeated following step 2.8 to 2.12 again.

18 DNA were quantified and qualified by UV absorbance of DNA with a spectrophotometer (Beckman, DU-7500) at 260 nm and by 0.7% agarose gel electrophoresis.

6.1.2.3. HAT-RAPD analysis

1 Genomic DNA of each lychee cultivar was diluted into 10 ng and stored at -20°C

2 The amplifications followed the protocol of Anuntalabhochai *et al.* (2001). PCR was performed in a total volume of 20 µl containing;

- 10xPCR buffer (Tris-HCl 100 mM, pH8.3, KCl 500 mM) (QIAGEN)
- 100 µM each dNTP (dATP, dTTP, dGTP, dCTP) (Promega)

- 1.5 mM $MgCl_2$ (QIAGEN)

- 0.3 μM 10-base primers (obtained from Operon Technologies Inc., Alameda, California)

- 0.5 unit of *Taq* DNA polymerase (QIAGEN)

- 10 ng of DNA template

3 Placed the reaction tubes in Perkin-Elemer thermal cycler (Gene Amp PCR system 2400) and used the following cycling profile:

95°C 2 min., 95°C 30 sec., 46-55°C 30 sec., and 72°C 45 sec., for 35 cycles , then the final cycle 72°C 5 min.

4 After the thermal cycling program has been completed, the amplification samples were stored at 4°C prior for electrophoresis.

6.1.3 Results and discussion

6.1.3.1 DNA isolation

The yield of DNA via CTAB extraction method was greatest for both parents and their hybrids. The DNA extracted was always contaminated with RNA, but this was easily removed by treatment with RNase irrespective of species or hybrids. The $A_{260/280}$ ratio (1.65-1.98) also suggest that the DNA isolated from lychee was not heavily contaminated with proteins and polysaccharides and that the PCR reaction was not inhibited by these compounds. Small inconsistencies between replicates were apparent during some RAPD-PCR amplification reactions, but this problem was easily eliminated by diluting any PCR reaction inhibitors in the DNA preparation by the TE buffer. This is a common approach to diluting out possible inhibitors in sample preparation for PCR. A concentration of 1.5 mM $MgCl_2$ was found to be optimum for primers used for 30 cycles, but each primer had its own optimum temperature for primer annealing.

6.1.3.2 DNA fingerprinting

Ten random primers (Table 26) were selected to detect the genetic variation in five hybrid combinations of lychee by using high-annealing temperature random amplified polymorphic DNA (HAT-RAPD). Figure 36, 37 and 38 show the HAT-RAPD patterns obtained from Among five hybrid combinations of lychee Chacrapat x

KimJeng, KimJeng x Chacrapat, KimJeng x Hong Huey, Hong Huey x KimJeng and Chacrapat x O-Hia, respectively.

Table 26. Primer lists for the genetic analysis of lychee hybrids

Primer name	Sequence (5 ⟶ 3)
OPL-11	TGGTGGACCA
OPC-09	CTCACCGTCC
OPH-13	GACGCCACAC
OPU-03	CTATGCCGAC
OPD-18	GAGAGCCAAC
OPG-03	GAGCCCTCCA
OPG-13	CTCTCCGCCA
OPD-20	ACCCGGTCAC
OPD-20	ACCCGGTCAC
OPW-19	CAAAGCGCTC

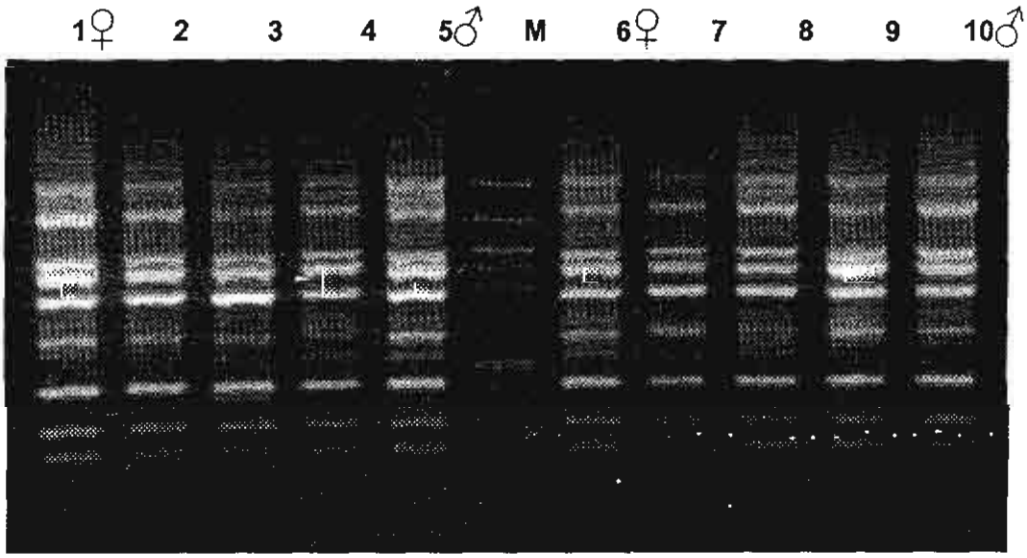

Figure 36. HAT-RAPD analysis of DNA from Lychee cultivars and hybrids using primer OPL11, separated in 1.4% agarose and stained with ethidium bromide. Lane 1, Chacraput; lane 2, hybrid no. 11; lane 3, hybrid no. 14; lane 4, hybrid no.16; lane 5, KimJeng; lane 6, KimJeng; lane 7, hybrid no. 25; lane 8, hybrid no. 31; lane 9, hybrid no. 32; lane 10, Chacraput and lane M, molecular weight marker 100 bp.

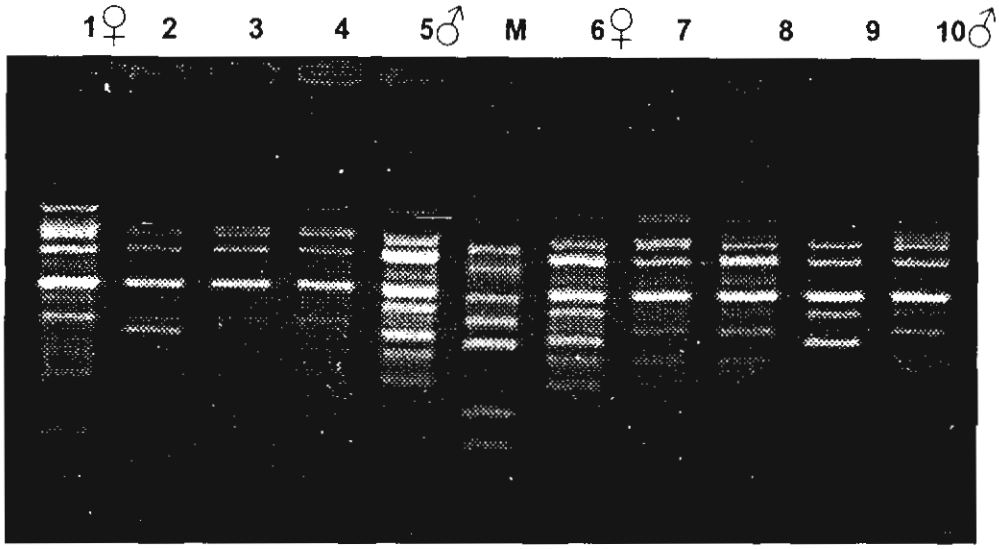

Figure 37. HAT-RAPD analysis of DNA from Lychee cultivars and hybrids using primer OPC-09, separated in 1.4% agarose and stained with ethidium bromide. Lane 1, KimJeng; lane 2, hybrid no. 1; lane 3, hybrid no. 7; lane 4, hybrid no.8; lane 5, HongHuey; lane 6, HongHuey; lane 7, hybrid no. 17; lane 8, hybrid no. 18; lane 9, hybrid no. 19; lane 10, KimJeng and lane M, molecular weight marker 100 bp.

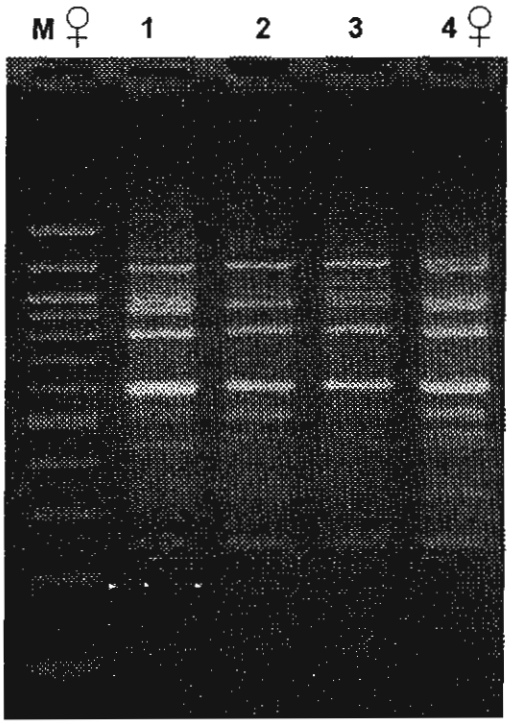

Figure 38. HAT-RAPD analysis of DNA from Lychee cultivars and hybrids using primer OPC-09, separated in 1.4% agarose and stained with ethidium bromide. Lane 1, Chacraput; lane 2, hybrid no. 29; lane 3, hybrid no. 30; lane 4, O-Hia and lane M, molecular weight marker 100 bp.

6.1.3.3 Hybrid analysis

All 9 primers (Table 26) yielded polymorphic bands for RAPD anlysis. Regarding to band sharing between hybrid and its parents, seven hybrid types of *Louisiana irises* (Arnold *et al.*, 1991) and Chrysanthemum (Huang *et al.*, 2000) have been proposed. These types were as following:

Type I: markers shared bands in both parents, and offspring;

Type II: markers shared bands in male and female parents;

Type III markers shared bands in male parent and offspring;

Type IV markers shared bands in female parent and offspring;

Type V markers were presented in the male parent only;

Type VI markers were present in the female parent only;

Type VII markers were present in offspring only.

Therefore due to this criteria our result revealed that hese HAT-RAPD marker could also be classify into seven types (Table 27). Among HAT-RAPD markers, the band patterns in the hybrids were found to be not completely additive. A similar phenomenon also appeared in the interspecific hybridization in *Cyrtandra* (Smith *et al.*, 1996), and intraspecific crosses of sugarcane varieties (Huchett and Botha, 1995). In the hybrid combinations of Chacrapat x KimJeng, markers of offspring revealed 80.8% shared markers with parents, including Type I, III, and IV. The hybrid combinations of KimJeng x Chacrapat, KimJeng x Hong Huey, Hong Huey x KimJeng and Chacrapat x O-Hia revealed 83.6%, 76.5%, 73% and 80.7% bands shared with parents, respectively.

Arnold *et al.* (1991) identified the natural hybrids of Louisiana irises by bands shared with both species. Therefore, Type I, III, and IV markers are good markers to identify the new hybrid from parents to ensure effective selection by plant breeders. In addition, Type III markers are especially important markers to identify the true male parent.

Sources of polymorphisms in HAT-RAPD assay may include base change within priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a DNA fragment without preventing its amplification (Williams *et al.*, 1990). In addition, the polymorphisms of HAT-RAPD markers were observed as different-sized DNA fragments from amplification. Therefore, differences in markers from parents to offspring may be the result of DNA recombination, mutation, or random segregation of chromosome in meiosis processing during hybridization (Huchett and Botha, 1995; Darnell *et al.*, 1990).

In this study, 17.8%, 16%, 22.9%, 25.6% and 19.2% markers from parents, including type II, V, and VI markers were not found in hybrid combinations of Chacrapat x KimJeng, KimJeng x Chacrapat, KimJeng x Hong Huey, Hong Huey x KimJeng and Chacrapat x O-Hia, respectively. In lychee, the strict outcrossing results in higher levels of heterozygosity (Wolff *et al.*, 1994). The low number of bands not shared with parents in offspring of lychee is probably due to segregation of heterozygous chromosomes during meiosis. Chromosomal crossing-over during meiosis may result in the loss of priming sites and thus markers are present in parents but not in offspring (Smith *et al.*, 1996).

Furthermore, the phenomenon of non-Mendelian inheritance could be detected because of the existence of competition in RAPD analysis (Lu *et al.*, 1995; Hallden *et al.*, 1996). The aforementioned problem is less serious in the investigation of haploids or completely homozygous material, whereas heterozygous material is more problematic (Hallden *et al.*, 1996). Therefore, it is not surprising to find only a portion of the bands from each parent in the hybrid of lychee..

The identification of cultivars or breeding lines is very important in all horticultural and agricultural species in order to protect the rights of plant breeders (Wolff *et al.*, 1995). In lychee, cultivars are identified in flowering trials, and breeders' rights are presented by cultivar characteristics including flower, leaf and growth morphology (Wolff *et al.*, 1995). The present study revealed relatively narrow diversity in Indian coconut populations. This information is important for ongoing efforts to develop high yielding hybrids and varieties. Inclusion of more exotic collections with desirable traits will be a useful step towards achieving lychee breeding objectives.

Table 27. The seven types of HAT-RAPD markers were identified for five hybrid populations of lychee cultivars.

Type of markers	Property of markers			HAT-RAPD markers for hybrid combinations									
	Male □	Female □	Offspring	Chacrapat x KimJeng		KimJeng x Chacrapat		KimJeng x Hong Huey		Hong Huey x KimJeng		Chacrapat x O-Hia	
				No.	%	No.	%	No.	%	No.	%	No.	%
I	+	+	+	72	51	70	47	72	50	68	46	77	52.7
II	+	+	-	8	5.7	12	8	15	10.4	13	8.8	11	7.5
III	+	-	+	22	15.6	23	15.3	20	14	18	12	16	11
IV	-	+	+	20	14.2	32	21.3	18	12.5	22	15	25	17
V	+	-	-	11	7.8	8	5.3	12	8.3	17	11.4	9	6.2
VI	-	+	-	6	4.3	4	2.7	6	4.2	8	5.4	8	5.5
VII	-	-	+	2	1.4	1	1	1	1	2	1.4	0	0
Total				141		150		144		148		146	

6.2.1 Detection genetic variation in lychee accession within Hong Huey and in Chacrapat cultivars from different locations

Lychee (*Litchi chinensis* Sonn,) originated in south-eastern China and has been cultivated for more than 2000 years. It has been introduced for cultivation widely in tropical and subtropical regions (Cambell and Knight, 1990). In Thailand, lychee is commercially grown in the north, where temperatures are lower than 15° C for 30-45 days continuously. However, some varieties have been grown in the warmer climates of the central and eastern parts of Thailand. New varieties have been developed and selected to suit its environments of certain regions by seedling and cross pollination to obtain new hybrids. However, original lines of planting stock have been lost and several have been mislabeled or renamed. This had resulted in confusion in the nomenclature of lychee varieties and some varieties have been given different names in different locations.

Since phenotypic traits depend on environmental and physiological condition of the plant (Neilsen et al., 1985) they provide ambiguous descriptions. In addition, lychee classification is based on their growth habits, breeding behavior and other morphological characters. Such the parameters are of limited use for clear identification (De Filipps *et al.*, 1996). Therefore, several techniques mainly in DNA-based procedures have been developed currently for support classification and identification in many plant taxa (Tingey and Tufo, 1993).

Since molecular markers, in particular HAT-RAPD, have been proven for a methods for choice for distinguish genetic variation in species, varieties, clones, and hybrid in number of plant taxa as mentioned above. Therefore , this method was chosen to assess variation in lychee varieties, in particular Chacrapat and Hong Hueng varieties, from different provinces in Thailand.

6.2.3.1 Materials and methods

6.2.3.1.1 Plant material

Two lychee varieties, Hong Huey and Chacrapat, from three provinces including Leoy, Petchaboon and Chiangrai, were chosen to study. Both are the commercial varieties which preferential cultivation in northern and north-east of Thailand. Hong Huey variety is represented as an early flowering variety whereas Chacrapat variety is

for late flowering variety. Young leaves from different braches of lychee trees were collected for genomic preparation.

6.2.3.1.2 DNA extraction and HAT-RAPD analysis

Both methods have been described previously (6.1.1.2 and 6.1.1.3). For HAT-RAPD reaction, five arbitrary primers named OPH-11, OPD-20, OPS-10, OPC-09, OPX-04 (Table 28) were used for this investigation.

Table 28 List of primers and their sequences for analysis accession of lychee

Primer	Sequence (5' ⟶ 3')
OPH11	CTTCCGCAGT
OPX04	CCGCTACCGA
OPAS10	CCCGTCTACC
OPC09	CTCACCGTCC
OPD20	ACCCGGTCAC

6.2.3.2 Results and discussion

6.2.3.2.1 Analysis of genetic variation within lychee intervariety

Of all 5 primers (Table 28), only OPH-11 provided DNA polymorphism in both Hong Huey and Chacrapat varieties (Figure 39). The others generated monomorphic DNA bands. Figure 39 showed the DNA polymorphic DNA bands (difference in molecular weights at 1,500 1,000, 900, 400, 350, 290 and 150 bp.) in Hong Huey cultivars. While the polymorphic bands (2000, 1200, 990, 900, 860, 820, 600, 460, 320, 155 bp.) were observed in Chacrapat cultivar.

Regarding to lack of appropriate tools for identification in lychee, our result revealed that lychee samples of the same varieties, both Chacrapat and Hong Huey from different provinces in Thailand, showed unidentical genotypes. The present study revealed relatively narrow diversity within intraspecific cultivars, Hong Huey and

Chacrapat. This information is important for developing a specific marker to identify particular lyuchee accession for patenting and/or approval of new variety with DNA marker.

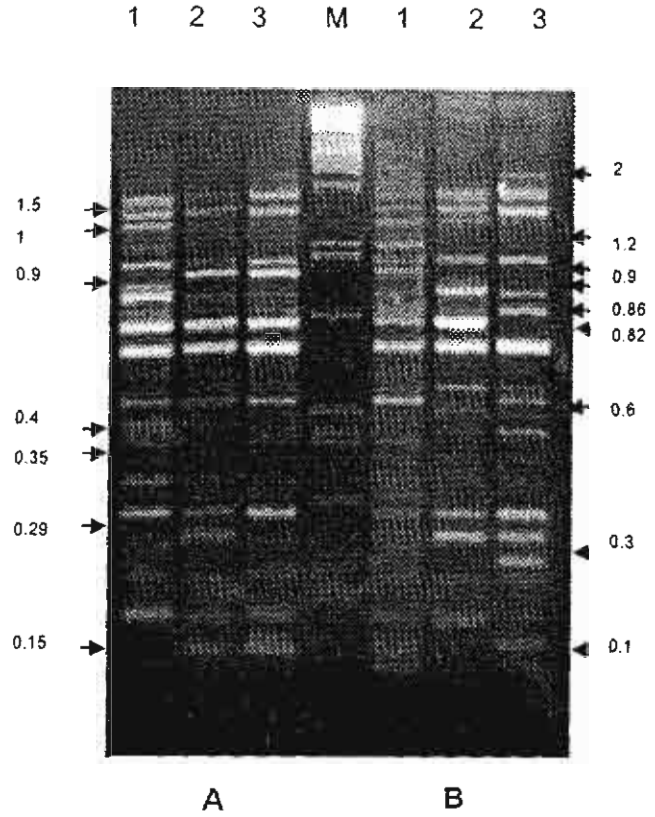

Figure 39. HAT-RAPD analysis of lychee DNA in two cultivars; Hong Huey (panel A) and Chacrapat (panel B). Lane 1-3: lychee accessions from Leoy, Petchboon and Chiangrai province repectively. M: Lamda DNA/ *Pst*I markers. Arrows indicated DNA polymorphic bands in each culktivars. Numbers in right and left both panels represented molecular weight in kb.

. This study also demonstrated the efficacy of HAT-RAPD markers to analyze genetic intervariety and relationship in the germplasm. HAT-RAPD markers revealed a genetic relationship pattern that was in agreement with the results obtained with AFLP markers (Chapter IV). In view of this, HAT-RAPD markers, which require less cost and infrastructure compared to other presently available markers, can be effectively used for the estimation of genetic distances among other plant-germplasm collections in major lychee growing developing countries.

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Output of the project

International publications

1. Anuntalabhochai, S., Chandej, R., Phanchisiri, B., Yu., L.D., Vilaithong, T., and Brown, I.G. (2001) Ion-Beam-Induced deoxyribose nucleic acid transfer. Applied Physics Letters. 78(16):1-3.
2. Anuntalabhochai, S., Chandej, R., Chiangda, J., and Apavatjrut, P. (2000). Genetic Diversity within Lychee (Litchi chinensis Sonn.) based on RAPD analysis. Acta Horticulturae. 575: 253-259.
3. Chandej, R., Anuntalabhochai, S. et al., (2003). Compariosn of molecular markers in analysis of genetic diversity in lychee (Litchi chinensis Sonn.) cultivars. Plant Science (Manuscript in preparation)
4. Chandej, R., Anuntalabhochai, S. et al., (2003) Development of species specific SCARs marker in lychee in Thailand. Crop Science. . (Manuscript in preparation)

Presentation in International Conferences

1. Proceedings of International Symposium on Molecular Markers for Characterizing Genotypes and Identifying Cultivars in Horticulture. Montpellier, France. 6-8 March 2000
2. International Symposium on Tropical and subtropical Fruits, Nov 26th – +Dec 1st . Canne, Australia.

National Conferences/symposiums

1. การเสนอผลงานนักศึกษาปริญญาเอกกาญจนาภิเษก ครั้งที่ 1 ตุลาคม 2542 โรงแรมจอมเทียน ปาล์มบีช รีสอร์ท พัทยา
2. การเสนอผลงานนักศึกษาปริญญาเอกกาญจนาภิเษกสัญจร ด้านเทคโนโลยีชีวภาพ ครั้งที่ 2 ตุลาคม 2543 อาคารบัณฑิตวิทยาลัย มหาวิทยาลัยเชียงใหม่

การสร้างนักวิจัยระดับปริญญา ตรี โท เอก ดังนี้

1 นาย รัฐพร จันทร์เดช (สำเร็จการศึกษาระดับปริญญาเอกแล้ว)
2 น ส สุปราณี สิทธิพรหม (สำเร็จการศึกษาระดับปริญญาเอกแล้ว)
3 นส สุภัค มหัทนพรรค (กำลังศึกษาต่อระดับปริญญาเอก)
4 นส สุกัลญา พิทักษ์รัตนนุกูล (สำเร็จการศึกษาระดับปริญญาโทแล้ว)
5 นส ต่อนภา ผุสดี (สำเร็จการศึกษาระดับปริญญาโทแล้ว)
6 นาย วีรชัย ตีรอรุณศิริ (สำเร็จการศึกษาระดับปริญญาโทแล้ว)
7 นส ณัฏฐิณี บัวพงษ์ (กำลังศึกษาต่อระดับปริญญาโท)
8 นส ศิริรัตน์ จงแสง (กำลังศึกษาต่อระดับปริญญาโท)
9 นส สุพรรณี พรมเทพ (กำลังศึกษาต่อระดับปริญญาโท)
10 นาย ศาตรา ลาดปะละ (กำลังศึกษาต่อระดับปริญญาโท)
11 นส น้ำฝน พันธารัตน์ (สำเร็จการศึกษาระดับปริญญาตรีแล้ว)
12 นส สุปราณี บุญเนินแต้ (สำเร็จการศึกษาระดับปริญญาตรีแล้ว)

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Ion-beam-induced deoxyribose nucleic acid transfer

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We report our observations of the interaction of energetic ions with bacterial cells, inducing direct deoxyribose nucleic acid (DNA) transfer into *Escherichia coli (E. coli)*. Argon- and nitrogen-ion beams were used to bombard the bacteria *E. coli* in a vacuum with energy of 26 keV and fluence in the range $0.5-4\times10^{15}$ ions/cm^2. Three DNA plasmids, pGEM2, pGEM-T easy, and pGFP, carrying different marker genes, were subsequently transferred (separately) into the appropriately ion-bombarded bacteria and successfully expressed. The results of this study indicate that ion beams with an energy such that the ion range is approximately equal to the cell envelope thickness, at a certain range of fluence, are able to generate pathways for macromolecule transfer through the envelope without irreversible damage. © *2001 American Institute of Physics.*
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Ion bombardment of material results in radiation damage to the near-surface target structure and introduction of foreign atoms and electric charge to the target.[1] Energetic heavy-ion beams have been used to bombard biological materials for genetic modification purposes, particularly for the mutagenesis of living organisms including both plants and bacteria.[2–5] In this application, the deoxyribose nucleic acid (DNA) structure inside the cell nucleus is modified randomly by ion-beam irradiation and positive mutations are subsequently selected for propagation. More recently, ion-beam bombardment has been used for the direct transfer of exogenous macromolecules such as vital dye and DNA into plant cells. *GUS* gene transfer into rice cell suspension[6] and tobacco pollen,[7] and transfer of Trypan blue (a vital dye) into *Curcuma* embryo[8] induced by heavy-ion-beam bombardment have been described. In ion-beam-induced DNA transfer only the cell envelope is bombarded so as to allow a subsequent orderly transfer of whole DNA into the internal cell region. We point out that the cell envelope can be a complex structure consisting of a number of distinguishable regions such as outer membrane, cell wall, and plasma membrane,[9] and the ion bombardment must provide a path for the external DNA or other macromolecules through the thickness of the entire envelope. Although ion-beam-induced DNA transfer into plant cells, and ion-beam-induced mutation of the *LacZ* gene in the bacteria *Escherichia coli (E. coli)* (Ref. 10) have been demonstrated, ion-beam-induced DNA transfer into bacterial cells has not been reported up to now. Here, we describe our work showing that ion-beam bombardment can lead to direct DNA transfer into living bacterial (*E. coli*) cells.

To prepare the *E. coli* strain DH5α for ion bombardment, the bacteria were smeared as a thin layer on sterile adhesive tape in a petri dish that was then placed inside a sample holder. The holder was capable of sequentially exposing a number of samples to the ion beam, as well as housing an unbombarded control sample. Ion bombardment was carried out using the mass-analyzed heavy-ion-implantation facility at Chiang Mai University.[11] Argon or nitrogen ions at 26 keV bombarded the *E. coli* to fluences of 5×10^{14}, 1×10^{15}, 2×10^{15}, and 4×10^{15} ions/cm^2. Inside the target chamber the operating pressure was about 10^{-3} Pa and the temperature of the target was about 0 °C. The samples were maintained under these conditions for about 1.5–2 h, allowing for system pump-down and ion bombardment.

Our procedure was to carry out the ion bombardment as described above, followed by DNA transfer by incubating a mixture of the DNA and the ion-bombarded bacteria, followed finally by biological testing procedures to confirm that the DNA was indeed transferred. Testing for DNA transfer was done sequentially by two independent methods: detection of marker genes, and measurement of the transferred DNA molecular size. In our work, three marker genes were selected: *amp*$^+$, *LacZ*, and *GFP* genes. The *amp*$^+$ gene product is resistant to ampicillin. The *LacZ* gene product is an enzyme called β-galactosidase, which can hydrolyze an artificial chemical called X-gal to release a blue pigment.[12] The *GFP* gene product is a protein called green fluorescent protein, which can be visualized in green under ultraviolet

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TABLE I. DNA plasmids used in the study.

DNA plasmid	Molecular size (kb)	Marker gene carried
pGEM2	2.8	*amp*$^+$
pGEM-T easy	3.0	*amp*$^+$, *LacZ*
pGFP	3.3	*amp*$^+$, *GFP*

(UV) light. The gel electrophoresis technique is used to measure the molecular size.[13]

We used three different DNA plasmids—pGEM2, pGEM-T easy, and pGFP—with different molecular sizes and carrying different marker genes, as shown in Table I. The ion-bombarded bacterial cell samples were removed from the vacuum chamber and promptly, within about 10–15 min, suspended in 150 μl LB$^-$ medium for 2–5 min (LB medium, "Luria broth," is a bacterial culture medium, and LB$^-$ is LB medium without ampicillin supplement). 10–15 μl of the cell suspension were incubated separately with 10 μg of each plasmid on ice. The incubation period was 30 min and a 15 min period was also used for the pGFP case. Then, the mixtures were cultured in 3 ml LB$^-$ at 37 °C for 2 h with vigorous shaking. For testing the property of ampicillin resistance, the cultured mixtures were added with ampicillin antibiotic to a final concentration of 100 μg/ml (the medium called LB$^+$) and cultured further overnight under the same conditions as above. Vacuum-treated but nonbombarded control samples were cultured in both LB$^-$ and LB$^+$ media. Since pGEM-T easy and pGFP plasmids contain not only the *amp*$^+$ gene but also others, the properties of *LacZ* and *GFP* genes were subsequently tested for in the *E. coli* that had positively shown the presence of the *amp*$^+$ gene. For detecting the *LacZ* gene, the *E. coli* samples bombarded at a fluence of 2×10^{15} ions/cm^2, incubated with pGEM-T easy and cultured in LB$^+$, were then cultured overnight on agar-solidified LB$^+$ together with X-gal and a chemical inducer IPTG (for activating the gene). For detecting the existence of the *GFP* gene, the *E. coli* incubated with pGFP was further cultured overnight on solid LB$^+$ at 30 °C and then viewed under UV light. For the molecular-size measurements, following conventional preparation,[14] the transferred plasmids were individually digested by various restriction enzymes (*Pst*I, *Eco*R1, and *Xba*1) and then analyzed by gel electrophoresis. All the experiments were repeated at least twice.

The observed results were as follows. The control samples that were exposed to the vacuum and low-temperature environment but not ion bombarded grew well in LB$^-$ but not in LB$^+$, thus indicating that the harsh conditions did not suppress cell viability. The *E. coli* bombarded with fluences of 5×10^{14}, 1×10^{15}, and 2×10^{15} ions/cm^2 grew well in LB$^-$ but not in LB$^+$, whereas those bombarded with 4×10^{15} ions/cm^2 did not grow in the media, implying that the experiment on the lower-fluence ion-bombarded bacteria was meaningful. Growth of the *E. coli* into which pGEM2 was transferred was found to be dependent on the ion species and bombardment fluence. For Ar-ion bombardment with fluences of 1×10^{15} and 2×10^{15} ions/cm^2, the bacteria grew successfully in the LB$^+$ medium, whereas survival growth of the bacteria bombarded with a fluence of 5×10^{14} was not observed. None of the cells bombarded with any fluence of N ions and subsequently incubated with the plasmids was able to grow in the LB$^+$ medium, nor the control sample. The *E. coli* containing the other two transferred plasmids behaved similarly, in particular showing good growth for the bacteria treated with an Ar fluence of 2×10^{15} ions/cm^2 even though incubated for different time periods. Blue colonies were exhibited for the *E. coli* detected with the transferred *LacZ* gene, as shown in Fig. 1(a). Green colonies were visible under UV light for the *E. coli* detected with the transferred *GFP* gene, as shown in Fig. 1(b). This behavior of the color colonies demonstrates a completely successful transfer of the corresponding genes into the bacteria without loss of genes originally carried by the DNA plasmids. Successful DNA transfer was further confirmed by the measured molecular sizes. As shown in Figs. 1(c) and 1(d), the gel electrophoresis displacement locations of the pGEM-T easy and pGFP transferred plasmids correspond to standard positions of molecular sizes 3 and 3.3 kb, respectively. These results indicate that indeed the original exogenous plasmids were transferred into the ion-bombarded *E. coli*.

Physical mechanisms that offer an explanation for ion-beam-induced macromolecule transfer into cells have been discussed previously for the case of plant cells.[3,6] Two possibilities have been considered: electroporation,[15] in which pores in the plasma membrane may be opened by ion-beam-induced electric-charge transfer across the cell envelope, and etching of the cell by ion-beam sputtering leading to perforation of the cell envelope (including outer membrane, cell wall, and plasma membrane). Electroporation is a transient effect, however, occurring promptly at the time of cell charging and of gate width small compared to a second. In the present case where the DNA transfer occurs not simultaneously with ion bombardment but instead at a time of about 10 min or so after the bombardment, electroporation would seem to be ruled out.

Collisional effects of the beam in the cell envelope can be due to the energetic primary ions and/or to the lower-energy secondary particles associated with the collision cascade. Perforation of the cell envelope can be brought about by direct sputter removal (etching) of material at relatively high collision energy and also by breaking of chemical bonds resulting in damage to the cell envelope at considerably lower energy. Molecular-bond breaking can be effected not only by energetic primary ions but also by secondary particles in the collision cascade, and in this case the number of atoms displaced can be several hundreds for each primary ion. The ion range (distance below the cell surface to the peak of the implanted ion distribution) is critical for optimal perforation of the cell envelope—cell damage should extend through the thickness of the cell envelope but should be minimal in the interior cell region (cytoplasm). Estimates of ion range contain considerable inherent uncertainty because of a lack of precise knowledge of the cell envelope composition, mass density, and physical state under our experimental conditions (low pressure and temperature within the vacuum chamber). Nevertheless, Monte Carlo and analytical calculations using the TRIM code[16,17] and the PROFILE code[18] and with our best knowledge of the cell envelope[9,19] indicate

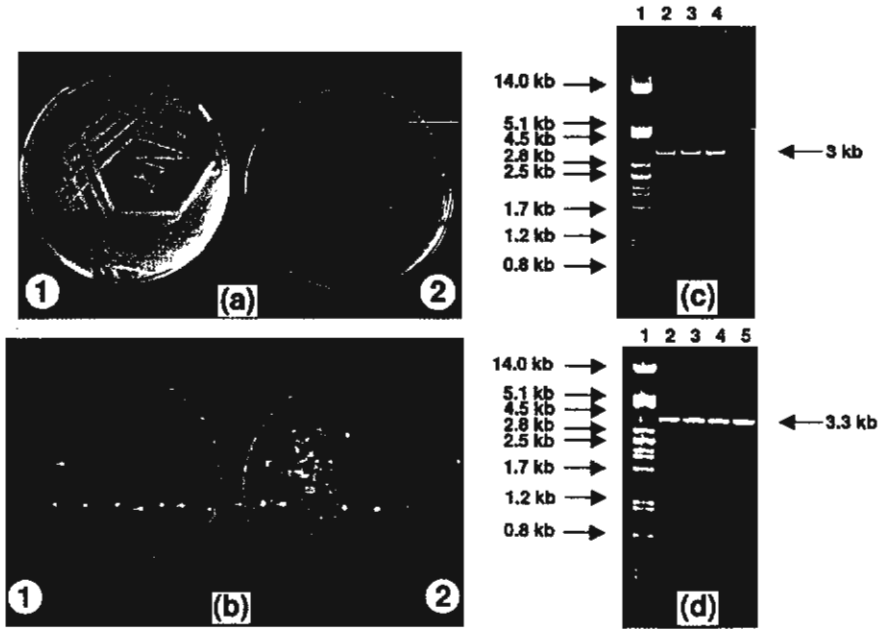

FIG. 1. (Color) Analysis of transferred pGEM-T easy and pGFP plasmids in ion-bombarded *E. coli* strain DH5α. (a) β-galactosidase activity in LB media added with X-gal and IPTG. The *E. coli* without pGEM-T easy (1), and with the transferred pGEM-T easy (2), cultured in LB^- and LB^+, respectively. (b) Expression of green fluorescent protein in *E. coli*. The *E. coli* without pGFP (1), and with the transferred pGFP (2), plated on LB^- and LB^+, respectively, visualized under UV light. (c) Measurement of molecular size of pGEM-T easy in gel electrophoresis. The transferred pGEM-T easy was digested with restriction enzymes *Pst*1 (lane 2), and *Eco*R1 (lane 3). The original pGEM-T easy digested with *Pst*1 is in lane 4. A standard molecular-size marker is shown in lane 1. (d) Measurement of molecular size of pGFP in gel electrophoresis. The transferred pGFP was digested with restriction enzymes *Pst*1 (lane 2), *Eco*R1 (lane 3), and *Xba*1 (lane 4). The original pGFP digested with *Pst*1 is in lane 5. A standard molecular-size marker is shown in lane 1.

that a range of 500–600 Å can be expected for 26 keV Ar ions, with damage due to the secondary cascade extending yet deeper. This is in good agreement with our estimates of the cell envelope thickness.[9,19] The *E. coli* cell has a 30 Å cell wall, a periplasmic space of about 150 Å, and a 75 Å plasma membrane, all surrounded by a complex outer membrane of thickness 300–700 Å as indicated by our transmission electron microscopy measurements; the entire cell envelope is thus of thickness some 500–900 Å. The range of nitrogen ions is about twice that of Ar ions of the same energy due to the smaller stopping power for the lighter ion species.[20] DNA transfer into the cells was not observed subsequent to N-ion bombardment.

The model that emerges is thus of perforation of the cell envelope by ion-beam bombardment in the appropriate regime of energy and fluence, allowing subsequent transport of the large DNA molecule into the interior cell region. When the ion range is approximately equal to the cell envelope thickness and the bombardment fluence is adequate, a pathway for macromolecule transfer through the cell envelope is formed. So long as the ion-bombardment damage is not too severe (bombardment fluence not too great) the cell envelope can recover, and the cell will live and propagate. If the ion fluence is too low, the cell envelope is not perforated and DNA transfer into the cell does not occur, and if the ion fluence is too high then the cells are damaged irreversibly. Further investigation is needed to provide a detailed explanation of the mechanisms involved in creating passages or channels through the cell envelope and enhancing the permeability through the cell envelope.

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Characteristics of heavy ion beam-bombarded bacteria *E. coli* and induced direct DNA transfer

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Abstract

The goal of the work described here was to study ion beam interactions with bacteria and thus develop an understanding of the mechanisms involved in ion bombardment-induced direct gene transfer into bacterial cells. Ar ion beams at an energy of 26 keV and fluences ranging from 5×10^{14} to 4×10^{15} ions/cm^2 were used to bombard bacterial cells of *Escherichia coli* strain DH5α. The bacteria were able to survive the low-temperature and low-pressure treatment conditions for at least a few hours. The ion bombardment created novel crater-like structures on the surface of the bacterial cell envelope, as observed by scanning electron microscopy. Four variously sized DNA plasmids carrying the ampicillin resistance gene were transferred and expressed in *E. coli* cells bombarded with ion fluences of 1×10^{15} and 2×10^{15} ions/cm^2. The dependence of the DNA transfer on the plasmid DNA size, ion fluence and incubation time all suggests that the ion beam-induced surface crater-like structures provide the pathway for the mechanism that is responsible for the ion beam-induced DNA transfer.

Keywords: Ion beam bombardment; Bacteria; *Escherichia. coli*; Plasmid DNA transfer; Cell envelope

1. Introduction

Low-energy ion beam bioengineering has recently been developed for genetic modification (e.g. [1]). Not only has mutation been induced by the technique, but also direct gene transfer. Ion beam-induced DNA transfer has been successfully accomplished in plants, such as rice cell suspension [2] and tobacco pollen [3]. The first evidence of DNA transfer into bacterial cells by ion beam techniques was recently reported by our group [4]. The aim of the work reported here was to further study the effects of ion beam bombardment as related to DNA transfer into bacterial cells, and to develop an understanding of the mechanisms involved. The structure of the bacterial cell envelope, consisting of several different structural layers, is more complicated than that of the plant cell, which is mainly the cell wall [5], and thus successful DNA transfer into bacterial cells calls for a higher degree of precision of the technical parameters and procedure. Moreover, due to the vastly more rapid regeneration rate of bacterial compared to plant cells, it is more significant and meaningful to examine the transferred genes maintained in the regenerated cells. Here we describe the characteristics of plasmid DNA transfer into bacteria *Escherichia coli* induced by low-energy Ar ion bombardment, and provide some preliminary evidence for the transfer mechanisms involved.

2. Experiment

Two or three separate single colonies of competent cells of *E. coli* strain DH5α, which is ampicillin sensitive (i.e. without the ampicillin resistance gene), cultured on Luria Bertani (LB) solid medium, were smeared as a thin layer on sterile adhesive tape and placed on each of five different locations on a Petri dish. The dish was then affixed to a specially designed

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Table 1
DNA plasmids used in the experiment

	Plasmid			
	pUC18	pGEM2	p35s	pBI221
Molecular size (kbp)	2.7	2.8	4.5	5.7

bio-sample holder, by means of which four locations were sequentially exposed to the ion beam, while a fifth location was unexposed and used as a control. The experiment was carried out under standard microbiological safety conditions.

Ion bombardment was carried out using the 150-kV mass-analyzed heavy-ion implantation facility at Chiang Mai University [6,7]. Ar ions at 26 keV of energy, magnetically scanned across the target, bombarded the exposed bacterial samples to ion fluences of 0.5, 1, 2 and 4×10^{15} ions/cm^2. The beam current density was approximately 5–10 μA/cm^2. The fluence of each scanned pulse irradiating the target was approximately $3–5\times10^{12}$ ions/cm^2. During ion bombardment, the pressure in the target chamber was kept at approximately 10^{-4} Pa, and the temperature of the target in this environment was estimated to be approximately 0 °C. The time duration for the whole treatment process, including vacuum pump-down, ion bombardment and normal pressure recovery, was approximately 1.5–2 h.

After ion bombardment, the control, the *E. coli* colonies grown on LB solid medium, and each treated sample, including both the ion-bombarded samples and the samples that were exposed to vacuum only, were separately suspended in 150 μl of LB liquid medium in sterile Eppendorf tubes for a few minutes.

To investigate the ion fluence dependence of the bacterial viability, 12 μl of each suspension was incubated overnight in 3 ml of LB liquid medium at 37 °C on a rotating shaker.

To investigate the DNA transfer, 12 μl of each cell suspension, including the control and the vacuum-exposed and ion-bombarded samples, was separately mixed with 10 μg of various DNA plasmids (as shown in Table 1) in sterile Eppendorf tubes, and incubated in an ice box for times of 5, 10, 15 and 30 min. The plasmid DNA carried the gene for ampicillin resistance. Then 12 μl of each sample was transferred into 3 ml of LB liquid medium and incubated at 37 °C on a rotating shaker for 2 h. To test for DNA transfer, carbenicillin was added to each tube to a final concentration of 100 μg/ml (50 μg/ml for pBI221 plasmid DNA); the tubes were further incubated overnight under the same conditions. To confirm the transfer of DNA into the bacterial samples, the samples in the tubes that were found to express antibiotic resistance were processed for plasmid DNA purification. The resulting plasmid DNA was separated by 1.5% agarose gel electrophoresis and measured for molecular size [4,8].

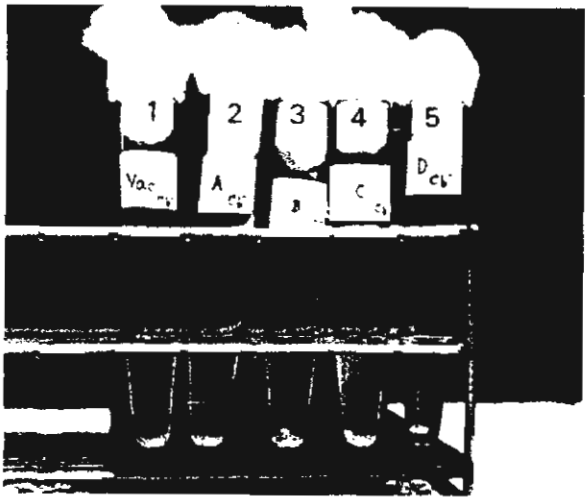

Fig. 1. Effect of vacuum and ion bombardment on *E. coli* viability. Treatment time was 2 h. Post-treatment conditions: 12 μl of each *E. coli* cell suspension was incubated overnight in 3 ml of LB liquid medium at 37 °C on a rotating shaker. Conditions of the tubes: Tube 1, vacuum-treated only; tubes 2, 3, 4 and 5, Ar ion beam-treated with fluences of 0.5, 1, 2 and 4×10^{15} ions/cm^2, respectively. The turbidity of the medium is the indicator of *E. coli* growth. (The fresh control refers to Fig. 4a.)

Transmission (TEM) and scanning electron microscopy (SEM) were used to observe the bacterial cell envelope microstructure after ion bombardment. For TEM, the bacterial cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.3 at 4 °C for 1 h immediately after ion bombardment was completed. The cells were then centrifuged to a pellet. The cell pellet was rinsed several times with the same buffer and post-fixed with 1% OsO_4 in 0.1 M PBS for 1 h. The fixed specimens were washed with the same buffer and dehydrated in increasing concentrations of alcohol and placed in 100% acetone. The specimens were dehydrated and dried at the critical point, then infiltrated with increasing concentrations of Spurr's 1969 resin in acetone, embedded in a box. Polymerization was carried out at 70 °C. Thin sections were prepared and sequentially stained with uranyl acetate and lead citrate and examined under a JEOL-JEM 1200 JEX II transmission electron microscope. For SEM, the bacterial samples were directly gold-coated within 15 min of ion bombardment and then examined under a JEOL-JSM 840A scanning electron microscope.

3. Results and discussion

After 2 h under the low-pressure, low-temperature conditions in which ion bombardment was carried out, the vacuum-treated and most of the ion beam-treated

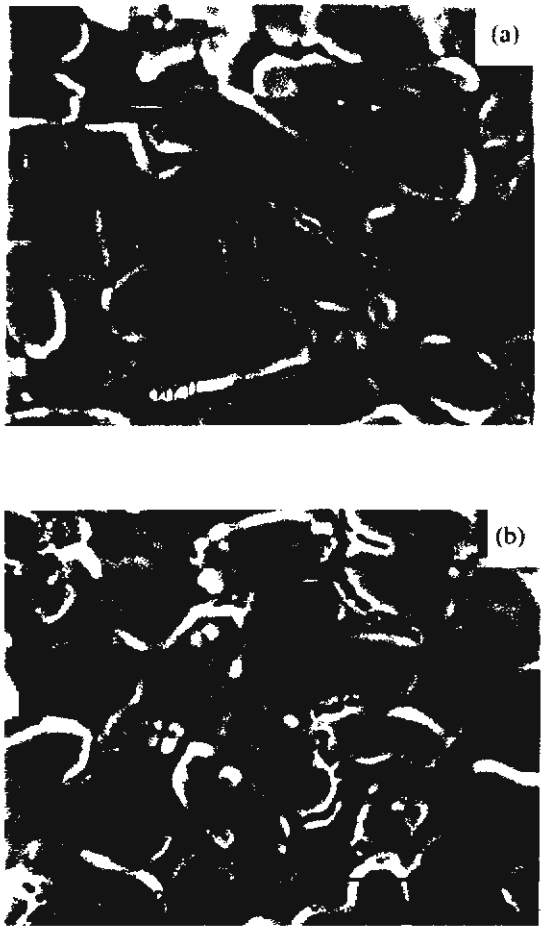

Fig. 2. SEM photographs of *E. coli* cell surface: (a) the vacuum-treated control; and (b) bombarded with 26-keV Ar ions to a fluence of 1×10^{15} ions/cm^2. Scale: the width of the micrograph is 6 μm.

bacteria, except the sample treated at the highest dose, survived and were able to grow, as indicated by the turbidity of bacterial growth in the liquid medium shown in Fig. 1. This indicates that cell residence for some hours in vacuum at low temperature does not affect the bacterial viability; similarly, the lower-dose ion beam bombardment does not affect cell viability either.

Ion beam-induced damage and physical changes to the surface of the bacterial cell envelope were observed, as shown in Figs. 2 and 3. The surface of the complex outer membrane of the cell envelope of the control is fairly smooth (Fig. 2a and Fig. 3a), and the underside of the cell wall is also very clear (Fig. 3a). In contrast, the ion-bombarded outer surface of the cell envelope is markedly undulatory and the cell wall has become somewhat indistinct (Fig. 3b). Some 'pimple-like' structures, or craters, with a diameter of approximately 100 nm can be observed (Fig. 2b; the side view in Fig. 3b shows approximately the same size), obviously piercing the cell through the entire envelope, including the cell wall. This suggests that ion beam etching first causes the cell envelope undulations, and subsequently, at certain weak locations (the cell envelope thickness is naturally inhomogeneous), the envelope may be pierced as a result of the ion beam-induced atomic displacement cascades, forming a micro-hole. Possibly, due to higher pressure inside the cell or to biological reaction of the cell, the substance inside may then erupt through the hole to form the crater or pimple. We further hypothesize that lower ion bombardment fluence creates less and non-fatal damage to the cell, since the radiation damage

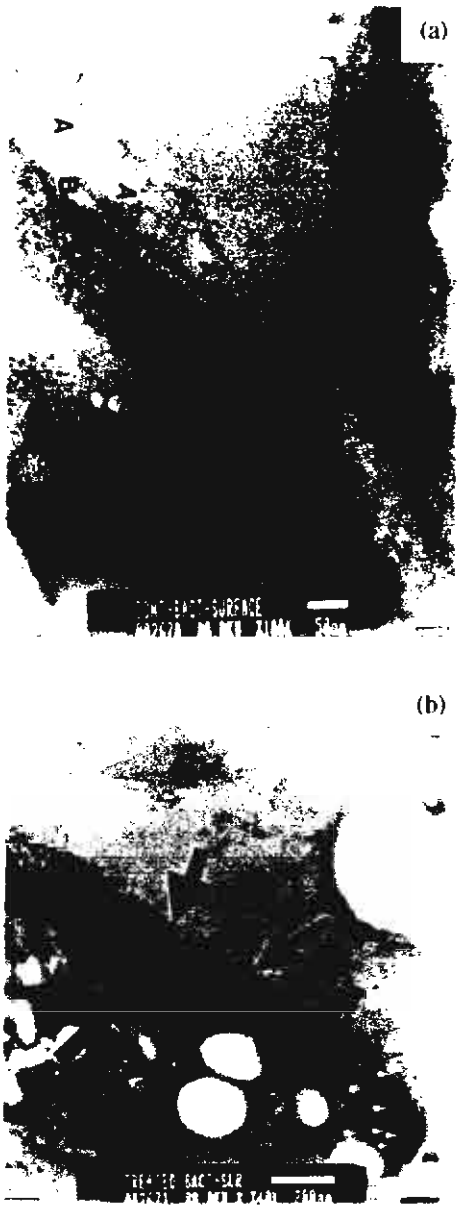

Fig. 3. TEM photographs of the *E. coli* cells. (a) The vacuum-treated control: A indicates the complex outer membrane, and B the cell wall. (b) The ion-bombarded cell: two smaller arrows point to the external side of the cell envelope, and the larger arrow indicates a deep pit piercing the cell through the cell envelope.

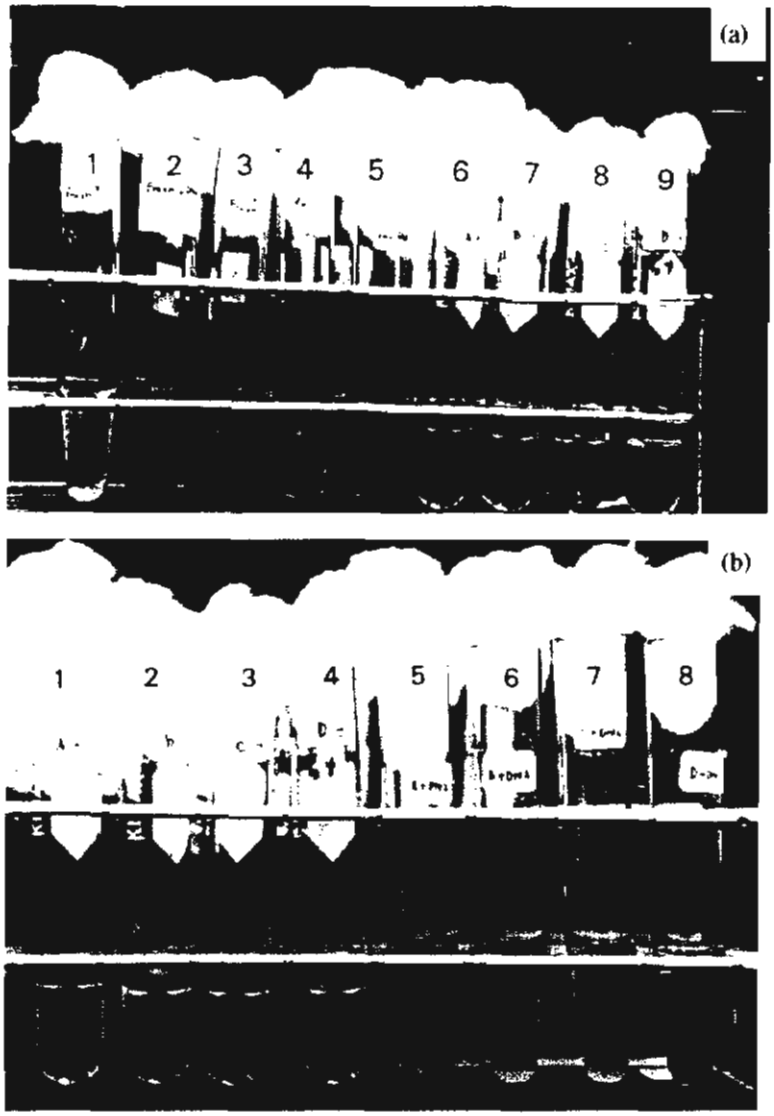

Fig. 4. Effect of ion beam bombardment on plasmid DNA pGEM2 transfer into *E. coli* incubated in antibiotic medium. Incubation conditions: 12 μl of each sample suspension were incubated overnight in 3 ml of LB liquid medium containing carbenicillin (LB^+) at 37 °C on a rotating shaker. (a) Control of ion-bombarded *E. coli* without DNA transfer. Tube no 1: control of fresh *E. coli* cultured in LB medium without the antibiotic (LB^-); no 2: fresh *E. coli* with DNA transfer treatment, 12 μl of fresh *E. coli* cell suspension + 10 μg of pGEM2 for 30 min, incubated in LB^- for 2 h and then added with antibiotic to a final concentration of 100 μg/ml and incubated overnight at 37 °C; no 3: fresh *E. coli* cultured in LB^+; no 4: vacuum-treated *E. coli* cultured in LB^+; no 5: vacuum-treated *E. coli* with DNA transfer treatment, 12 μl of vacuum-treated *E. coli* cell suspension + 10 μg of pGEM2 for 30 min, incubated in LB^- for 2 h and then added with antibiotic to a final concentration of 100 μg/ml and incubated overnight at 37 °C; nos 6, 7, 8 and 9: ion-bombarded *E. coli* with fluence of 5×10^{14}, 1×10^{15}, 2×10^{15} and 4×10^{15} ions/cm^2, respectively, without DNA transfer. (b) Ion-bombarded *E. coli* with DNA transfer, compared with those without DNA transfer. Tube nos 1, 2, 3 and 4: ion beam-bombarded with fluence of 5×10^{14}, 1×10^{15}, 2×10^{15} and 4×10^{15} ions/cm^2, respectively, without transferred DNA; and tube nos 5, 6, 7 and 8: ion beam-bombarded with fluence of 5×10^{14}, 1×10^{15}, 2×10^{15} and 4×10^{15} ions/cm^2, respectively, with transferred DNA. The turbidity of the medium is the indicator of *E. coli* growth.

is proportional to ion bombardment fluence, and hence is compatible with bacterial survival and growth. On the other hand, the highest fluence of 4×10^{15} ions/cm^2 may lead to lethal damage to the bacterial cells.

The effect of ion beam bombardment on DNA transfer into bacteria is shown in Fig. 4. Fig. 4a shows the controls. Without any plasmid DNA pGEM2 transfer operation, *E. coli* cells, whether they were ion bombarded or not, were not able to survive in the LB medium containing carbenicillin. With DNA transfer treatments but without ion bombardment, *E. coli* could not survive either. However, after the DNA transfer, the *E. coli* cells that were bombarded with fluences of 1×10^{15} and 2×10^{15} ions/cm^2 were able to grow in antibiotic medi-

Table 2
Dependence of Ar ion beam-induced DNA transfer into *E. coli* on the plasmid DNA size, ion fluence and incubation time

Incubation time (min)	pUC18		p35s		pBI221	
	Fluence (ions/cm^2)		Fluence (ions/cm^2)		Fluence (ions/cm^2)	
	1×10^{15}	1×10^{15}	1×10^{15}	2×10^{15}	1×10^{15}	2×10^{15}
5	−	+	−	−	−	−
10	+	+	−	−	−	−
15	+	+	−	+	−	+
30	+	+	−	+	−	+

'+', succeeded; '−', failed.

um, as indicated by the turbidity shown in Fig. 4b, thus demonstrating that the bacteria possessed the antibiotic resistance gene (*amp*+: ampicillin resistance gene), which could only come from the plasmid DNA, pGEM2. The dependence of successful ion beam-induced DNA transfer on the DNA size, ion fluence and incubation time is shown in Table 2. In order to confirm that the transferred plasmid DNA indeed came from the original DNA, the electrophoresis-measured molecular size of the transferred plasmid DNA was compared with that of the original. The result shows that the original pure plasmid DNA, pGEM2, and the DNA extracted from the transferred *E. coli* are identical in molecular size and shape (data not shown).

Mechanisms that can lead to ion beam-induced DNA transfer have been discussed in the literature (e.g. [2]). A very likely mechanism has to do with the effect of ion beam sputtering of the surface and radiation damage-formed pathways in the cell envelope. We have pointed out in previous publications [4,9] that ion beams with energy such that the ion range is approximately equal to the effective cell-envelope thickness, and at a certain range of fluence, are able to generate pathways for exogenous macromolecule transfer through the envelope without irreversible damage. The holes or craters observed in the cell envelope after ion bombardment could provide such pathways. The dependence of successful ion beam-induced DNA transfer on DNA size, ion fluence and incubation time has been analyzed, as shown in Table 2. First, successful transfer depends on the DNA size: the smaller the DNA, the easier the transfer. It is reasonable that smaller DNA can more readily be transported through pathways of limited size. Second, transfer depends on the ion fluence, and success can be related to a certain optimal fluence: a certain higher fluence of ions can create more pathways for the DNA to pass through. Third, the transfer depends on the incubation time: the longer the time, the greater the degree of transfer. This suggests that since the DNA transfer needs time, longer incubation provides the DNA with greater access to the pathways and with sufficient time to interact with the pathways and be transported.

Energetic ion bombardment deposits energy to the target that is converted into displacement of target atoms to form structural damage, which generally distributes in agreement with the ion distribution [10,11]; this applies to any material. The range of Ar ions in the plant cell-wall material has been calculated in our previous studies [9]. Although the cell envelope of the gram-negative *E. coli*—consisting of a 3-nm cell wall, a 15-nm periplasmic space, a 7.5-nm plasma membrane and a thick, complex outer membrane [5,12], approximately 30–70 nm, as measured from the TEM photograph (Fig. 3b)—is somewhat different from the plant cell envelope structure, we can apply the plant cell-wall data in our calculation, provided the deviation is small. The depth on the deeper side of the full width at half-maximum (FWHM) of the Gaussian-like depth profiles for all four fluences used is approximately 50 nm, which is the characteristic penetration depth of the ions. Surface sputtering losses [10,11] are 3, 6, 11 and 22 nm for fluences of 0.5, 1, 2 and 4×10^{15} ions/cm^2, respectively. Thus, the total characteristic penetration depths are 53, 56, 61 and 72 nm. Comparing the results calculated with the entire thickness of the *E. coli* cell envelope, approximately 50–90 nm, leads to the following interpretation. For the highest fluence, 4×10^{15} ions/cm^2, some ions completely penetrate through the cell envelope and enter the inner organelles of the cell, due to the characteristic penetration depth of 72 nm, which is greater than many parts of the bacterial cell-envelope thickness; this results in lethal damage to the cell. For lower fluences, the characteristic penetration depth is not much greater than the average thickness of the cell envelope, and thus less damage is expected to the envelope and cells can survive. However, the damage produced by the lowest fluence, 5×10^{14} ions/cm^2, may be too small to form adequate pathways for the exogenous DNA to pass through, and thus for this fluence no DNA transfer was observed. For the two intermediate fluences of 1 and 2×10^{15} ions/cm^2, the damage created by ion bombardment is adequate for the formation of some pathways for DNA transfer without killing the cells. Our argument for a mechanism based on ion beam-created pathways is supported by the fact that DNA transfer always succeeds when it is carried out promptly after the specimens are hydrated (suspended in 150 μl of LB liquid medium), but never after a long time delay. We speculate that a long time delay to incubation of the ion beam-bombarded cells in the DNA solution could allow the bacteria to self-recover the damage created, and thus close the pathways.

We point out that the DNA transfer observed should be a regeneration effect, since the fast bacterial regeneration rate leads to a specific regeneration time definitely within the transfer operation time, some several 10s of minutes. However, in the case of DNA transfer into plant cells, the effect might be evidenced for several

days or even weeks [2] because of the low regeneration rate of plants.

4. Conclusion

For appropriately moderate ion fluences, low-energy (26 keV) Ar ion-beam bombardment can induce successful plasmid DNA transfer into bacterial cells. The mechanism responsible is discussed in terms of ion bombardment-induced certain forms of damage in the cell envelope, such as micro-holes or craters, which may play a role in acting as channels for exogenous DNA molecules to be transferred.

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Genetic Diversity within Lychee (*Litchi chinensis* Sonn.) based on RAPD Analysis

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Keywords: PCR, RAPD, *Litchi chinensis*, DNA fingerprinting, cultivars

Abstract

An analysis of genetic variation within 20 lychee cultivars (*Litchi chinensis* Sonn.) was investigated using random amplified polymorphic DNA (RAPD) technique. Out of 69 arbitrary primers, five primers named OPB18, OPC09, OPAK10, OPAQ12 and OPAS10 produced reliable DNA polymorphism ranging in molecular weight from 200 to 2000 bp. DNA patterns from RAPD data were analyzed by cluster analysis and UPGMA to present a dendrogram depicting the degree of genetic relationship among the 20 cultivars. These cultivars were classified into 2 major groups with each containing three sub-groups. Our analysis revealed that some cultivars known as O-Hia and Haak Yip displayed identical fingerprint patterns indicating that it is the same cultivar, known under two different names. Others such as, Kwang Jao and Jean Hom, and Haew and Luk Lai exhibited a high similarity in their patterns, indicating a close genetic relationship.

INTRODUCTION

Lychee or litchi (*Litchi chinensis* Sonn.) originated in south-eastern China and has been cultivated for at least 2,000 years. It has been introduced widely as a cultivated plant in the tropical and subtropical world (Cambell and Knight, 1990). Generally, horticulturists have developed cultivars suited to the environment of certain regions. Plantings derived from the origins have been lost and several lychee cultivars have been renamed or mislabeled, resulting in confusion in the nomenclature of lychee cultivars (Aradhya et al., 1995; Degani et al., 1995). Apparently, the same cultivar has been described under different names, and in some regions different cultivars have been given the same name (Menzel and Simpson, 1990).

Since phenotypic traits depend on environmental and physiological conditions of the plants (Nielsen, 1985) they provide ambiguous descriptions. Such traits are of limited use for clear identification (De Filippis et al., 1996). In addition, related hybrids also exhibit complexity of their characteristics resulting in confusion for discrimination.

Currently, several techniques have been developed for support classification and identification purposes, for instance karyological investigations, thin layer chromatography and isozyme analysis (Brown et al., 1993). Only isozyme analysis has been proven a reliable tool. (Degani et al., 1995; Baes and van Cutsem, 1993; Apavatjrut et al., 1999). However, polymorphism among closely related genotypes, such as some somatic hybrids of plant species can limit isozyme separation (Naton et al., 1992). Finally, DNA-based procedures have been described as methods of choice to identify many plant taxa (Tingey and Tufo, 1993).

Random amplified polymorphic DNA using single arbitrary primers in PCR was described by Williams et al. (1990) and Welsh and McClelland (1990). RAPD-PCR is simple and rapid to scan extensive DNA polymorphism. Moreover, small amounts of genomic DNA and no radioactivity are required in the reaction. RAPD-PCR has been used to distinguish cultivars of different plant species (Yu and Nguyen, 1994; Cho et al., 1995), interspecific hybrids (Inai et al., 1993), clonal variation (Munthali et al., 1996, Piola et al., 1999) and it has also been used to detect plant genetic stability (Gavidia et al., 1996).

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The objective of this study is to genetically characterize a wide range of lychee cultivars in Thailand using RAPD markers and also to analyze their genetic relationships.

MATERIALS AND METHODS

Plant Material

Twenty lychee cultivars including the Chinese and American cultivars (Table 1) used in this experiment were kindly provided by the Chiangrai Horticultural Research Center, Chiangrai province and Phang Experimental Station, Chiangmai province, Thailand.

DNA Isolation

A minipreparation protocol utilizing the cationic hexadecyl trimethyl ammonium bromide (CTAB) method of Weising et al., (1991) was used with small modifications. Young leaves (0.5-1g) were ground to a fine power in liquid nitrogen. The powder was mixed with CTAB extraction buffer (4% w/v CTAB, 1% w/v PVPP, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 0.1% v/v 2-mercaptoethanol), incubated at 60°C in a water bath for 60 min. Proteinase K (10 mg/ml) (Sigma) was added and the mixture was incubated for another 60 min. at 37°C. The mixture was extracted twice with 24 chloroform: 1 isoamyl alcohol (v/v), then the nucleic acid was precipitated in isopropanol with 0.1 M NaCl and kept at-20°C for overnight. After the pellet was washed by chilled 70% ethanol, it was treated once with RNase A (10mg/ml) (Sigma) for 60 min. at 37°C. Consequently, the DNA was extracted once with 25 phenol: 24 chloroform: 1 isoamyl alcohol and was precipitated in cold isopropanol (-20°C). Finally the DNA was resuspened in TE buffer. Quantitative and qualitative determination of DNA was accomplished by UV absorbance of DNA with a spectrophotometer (Beckman, DU-7500) at 260 nm. and by electrophoresis on 0.8% agarose gel .

RAPD Amplification

Decamer arbitrary primers from Operon Technologies Inc.(Alameda, California., USA) were chosen at random for PCR amplification. A list of the primers is in Table 2. The PCR was performed in a 25 µl reaction volume consisting of: 40 ng of primer; 0.1 unit of *Taq* DNA polymerase (Promega); 10 ng of genomic DNA; 1x reaction buffer providing from manufacture and added with 2mM $MgCl_2$ and 150 µM of each dNTP. Generally, the annealing temperature of RAPD reaction was carried out at 35-38°C due to Tm values of their primer sequences. In order to increase annealing stringency of the primers and DNA templates, in this experiment, the annealing temperature was performed at 46 °C. This modified RAPD is so-called HAT-RAPD (high annealing temperature Random amplified polymorphic DNA). The amplification was carried out in a DNA Thermal Cycler PCR system (Perkin Elmer; Gene Amp PCR System 2400) programmed for an initiation denaturation step of 3 minutes at 94 °C, followed by 40 cycles each consisting of 30 sec. at 94°, 46° and 72 °C respectively. The final cycle was 5 minute step at 72°C. After amplification, the sample was separated in 2% agarose gels in a presence of a molecular marker (Promega) at 60 $V.cm^{-1}$ on 1x TAE buffer (40mM Tris-acetate, (pH8.0); 1 mM EDTA). The PCR products were stained with ethidium bromide (0.5µg/ml) and the gels were photographed under UV light. RAPD-PCR reactions were repeated independently at least twice.

Data Analysis

The banding patterns obtained by the chosen primers were compared among the different taxa. The bands were recorded as discrete variables, using 1 to indicate presence and 0 to indicate absence of visual bands. A dendrogram depicting the relationships among the taxa was produced on the basis of an hierarchical cluster analysis performed by SPSS software using the UPGMA (unweighted pair-group arithmetical mean) method.

RESULTS AND DISCUSSION

Sixty-nine primers of arbitrary nucleotide sequences were randomly chosen to amplify genomic DNA of 20 lychee genotypes. Four primers (6%) were not able to amplify PCR products, whereas monomorphic and few polymorphic bands were produced by sixty primers (87%). Only these primers (7%) named OPB 18, OPC 09, OPAK 10, OPAQ 12, OPAS 10 revealed a high degree of polymorphism among of the twenty lychee cultivars (Fig 1). A list of these five primers and their sequences is presented in Table 2.

The 66 polymorphic RAPD bands generated by these five primers varied in size ranging from 200 to 2000 bp. Of these primers, the primers OPS10 and OPC09 produced the highest number of polymorphic bands (Fig 1c and 1d). The differences in RAPD profiles are determined by a competition between potential priming sites in genomic DNA rather than by the total number of priming sites available (Rafalski et al., 1991).

Cluster analysis using RAPD-PCR data produced reliable and consistent results. The fragments were scored as characters for presence and absence. The data were arranged in a matrix and then subjected to UPGMA cluster analysis to generate a dendrogram (Fig. 2). Cluster analysis has been reported to have an advantage over other grouping methods (Wilkie et al., 1993). Two individuals or cultivars were grouped together at a particular level and the most similar cultivars were combined step by step.

In all RAPD-PCR amplification reactions, genomic DNA of longan (*Dimocarpus longan* Lour.), Puang Thong cutivar, was included for intergeneric comparison. According to the dendrogram, longan was the furtherest removed compared to all the lychee taxa. The 20 varieties were divided into two major clusters and each was subdivided into three subclusters. For the first cluster, the members of each subclusters were: subcluster 1, Sarake Tong, Hong Huey and Sampow Kaow; subcluster 2, Kwang Jao, Jean-Hom, O-Hia, Haak-Yip, Jean-Lek and Kaloke Bai Yaow; subcluster 3, Brewster and Kim Cheng. For the second cluster, members of each subclusters were: subcluster 1, Kom, Jean-Yai and Kratone Tongprarong; subcluster 2, Haew, Luk-Lai and Chacrapat; subcluster 3, Kaloke Bai Oor, Kim Jee and Nakornpanom.

Because of incomplete historical information and lack of appropriate tools for identification, some early cultivars introduced from China were misidentified or mislabeled, resulting in confusion in the nomenclature of lychee cultivars (Aradhya et al., 1995). Our results showed identical DNA fingerprints for Sarake Tong (from central Thailand) and Hong Huey (from northern Thailand), leading to conclude that Sarake Tong was misidentified (director of Chiangrai Horticultural Research Center, personal communication). Moreover, identical fingerprints were observed for O-Hai and Haak-Yip. The two cultivars have been reported as the same cultivar but are known under different names in different countries (Menzel and Simpson, 1990; Subhadrabandhu, 1990). Both Kwang Jao and Jean-Hom had similar fingerprints because they were imported from China at different times, so are suggested to be of a similar genetic origin. Two members in subcluster 2 of the second cluster, Haew and Luk-Lai, had many similar bands, suggesting a close relationship between the two cultivars. Both cultivars have been cultivated through seed germination from the same origin for several generations.

These evidences indicate that RAPD technique is sensitive and powerful enough to detect genetic variation and similarity of DNA among lychee cultivars. Further study of RAPD-PCR-based procedures should reduce any confusion in the identification of lychee varieties, especially when combined with other tools such as SCAR (sequence characterized amplified region). Parent and Page (1998) have proposed that the SCAR technique provides relevant markers for the identification of raspberry cultivars. In combination with other techniques such as RFLP and AFLP will be possible to clarify genetic variations of lychee cultivars in more detail.

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Tables

Table 1. List of lychee cultivars.

Cultivar	Source	Location in Thailand
Kaloke Bai Oor	Samutsongkram	Central
Sampow Kaow	Samutsongkram	Central
Sarake Tong	Samutsongkram	Central
Kom	Samutsongkram	Central
Chacrapat	Chiangmai	North
Brewster	Chiangmai	North
Kwang Jao	China	North
O-Hia	China	North
Hong Huey	Chiangrai	North
Kim-Cheng	China	North
Jean-Lek	China	Central
Jean-Yai	China	Central
Luk-Lai	China	Central
Jean-Hom	China	North
Nakornpanom	Samutsongkram	Central
Kratone Tongprarong	Samutsongkram	Central
Kaloke Bai Yaow	Samutsongkram	Central
Haew	Samutsongkram	Central
Haak-Yip	USA	North
Kim-Jee	China	North

Table 2. Nucleotide sequences of primers used for generating RAPD markers among 20 lychee varieties in Thailand.

Code	Sequence (5'-3')	Number of polymorphic Markers
OPB 18	CCA CAG CAG T	12
OPC 09	CTC ACC GTC C	14
OPAK 10	CAA GCG TCA C	13
OPAQ 12	CAG CTC CTG T	8
OPAS 10	CCC GTC TAC C	19

Figures

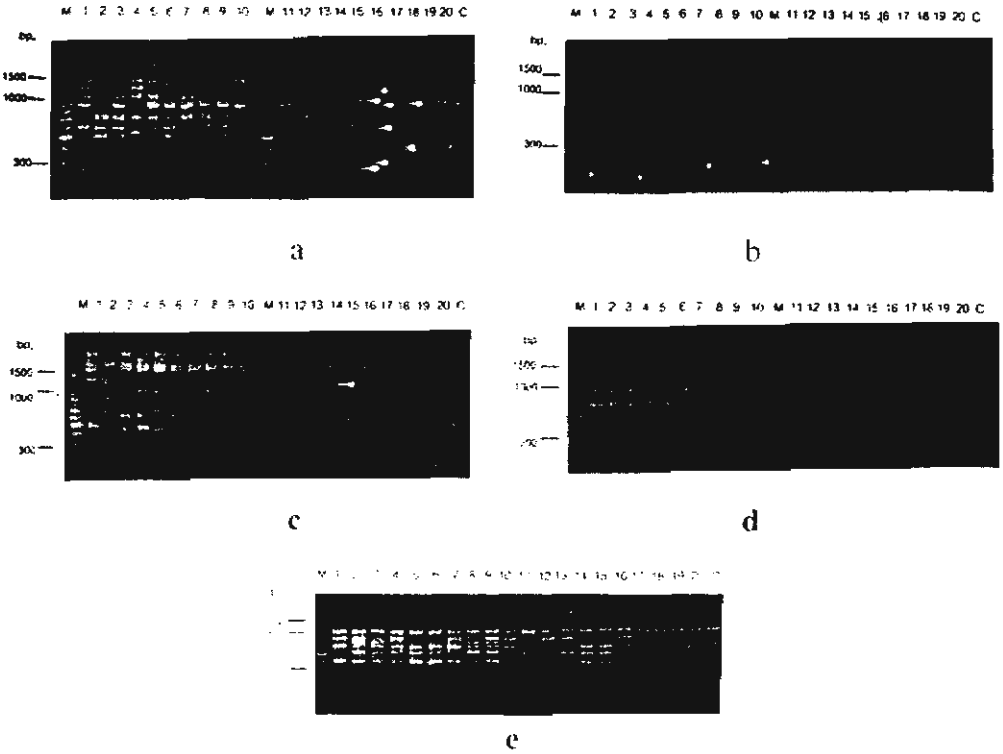

Fig. 1. Amplification products of 20 lychee cultivars generated using primers OPAK 10 (a), OPB 18 (b), OPAS 10 (c), OPC 09 (d) and OPAQ 12 (e). Number above each lane corresponded to the following cultivars: (1) Kaloke Bai Oor, (2) Sampow Kaow, (3) Salake Tong, (4) Kom, (5) Chacrapat, (6) Brewster, (7) Kwang Jao, (8) O-Hia, (9) Hong Huey, (10) Kim-Jeng, (11) Jean-Lek, (12) Jean-Yai, (13) Luk-Lai, (14) Jean-Hom, (15) Nakornpanom, (16) Kratone Tongpralong, (17) Kaloke Bai Yaow, (18) Haew, (19) Haak-Yip and (20) Kim-Jee. Lane C represents RAPD products of longan (*Dimocarpus longan* Lour.) cv. Puang Tong used as the intergeneric control. Lane M is molecular weight marker of 100- bp ladder (Promega).

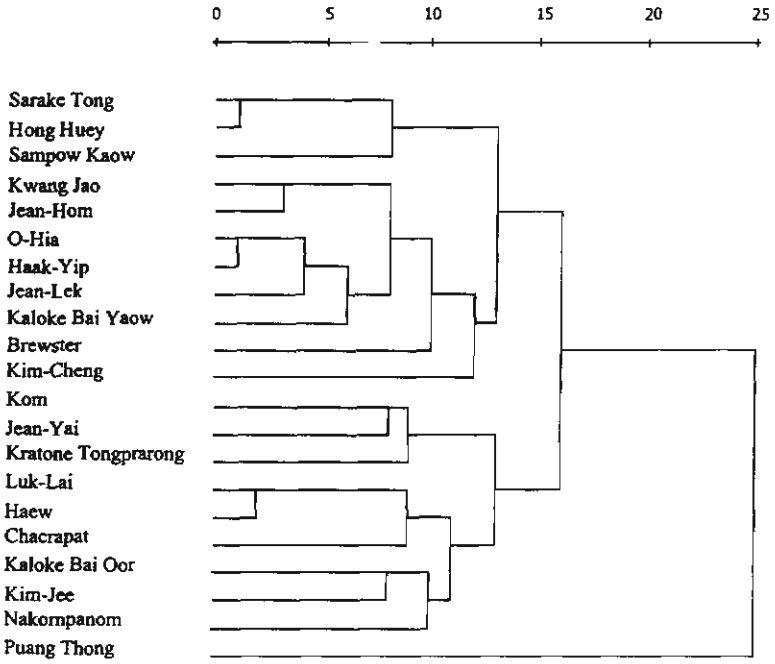

Fig. 2. Dendrogram depicting the genetic relationships among lychee cultivars. The scale indicated dissimilarity index.