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Evaluation of genetic variation in longan (*Dimocarpus longan* Lour) by high annealing temperature random amplified polymorphic DNA (HAT–RAPD)

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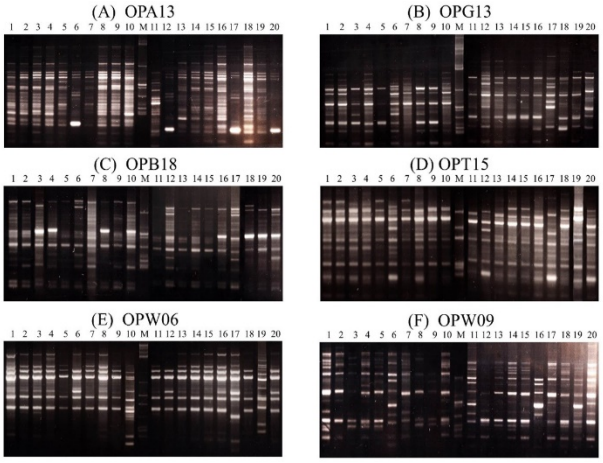
Abstract

Genetic relationships among 20 longan varieties (*Dimocarpus longan* Lour.) collected from Chiang Rai and Lampang province; Thailand, Vietnam and China were investigated using high annealing temperature random amplified polymorphic DNA (HAT–RAPD) technique. Ten primers, OPA 13, OPAK10, OPB 18, OPG 13, OPW 06, OPW 09, OPX 01, OPX 15, OPZ 01 and OPAK 10, produced a total of 305 RAPD markers. All primers generated a high degree of polymorphism, ranging in molecular weight from 100 to 2,500 bp. Hierarchical cluster analysis, based on unweighted neighbor–joining method classified the 20 varieties into 3 major groups. The first two groups could be divided into 2 subgroups and the third group contained only one subgroup. Our data revealed that members in the first group are Daw types. The Narapirom and Petchsakorn varieties exhibited close relatedness, suggesting that they are originated from the same variety. In addition, this technique also detected variation among longan varieties due to selective breeding.



Keywords: *Dimocarpus longan* Lour; High annealing temperature RAPD; Genetic variation; Molecular marker

1. Introduction

Longan (*Dimocarpus longan* Lour, family Sapindaceae) originated in the south of China and has been cultivated in China and Taiwan for over 1,000 years. It has been introduced for cultivation to Hawaii, Central America and throughout Asia [1]. New varieties have been developed and selected to suit various environments. However, original lines of planting stock have been lost and several lines have been mislabeled or renamed. This has

resulted in confusion in the nomenclature of longan varieties and some varieties have been given different names in different locations. In Thailand, longan 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 central and eastern Thailand. To increase productivity, superior varieties / hybrids that are less sensitive to higher temperature should be developed. Any breeding program has to account for the genetic variability encountered in germplasm collections for defined crosses. However, identification of variety by morphology was difficult to distinguish between hybrids or closely related variety. To evaluate genetic variation of germplasm, DNA–based techniques, in particular, random amplified polymorphic DNA (RAPD) is a promising method.

RAPD markers, using arbitrary primers in a polymerase chain reaction (PCR) as described by Williams *et al.* [2] and Welsh and McClelland [3] has been used extensively to analyze the genetic variation of several plant species. It has been used to evaluate population genetic variability [4], for genotype characterization [5, 6] and for hybrid identification [7, 8]. RAPD analysis has also been used to study genetic relationships between species, cultivars and clones and mutants [9 – 14]. Several fruit crops, such as lychee [15], Guava [16], citrus [17] apple [18] and walnut [19] have been investigated by DNA fingerprinting.

Although the RAPD technique is a simple and efficient way to detect DNA polymorphisms, the low reproducibility of results and high sensitivity to PCR reaction conditions have greatly hindered their application [20]. Usually, the technique is performed at low annealing temperatures between 35 and 38 °C [21 – 22]. However, Anuntalabhochai *et al*. [15, 23] and Atienzar *et al.* [24] reported that increasing the annealing temperature above 46 °C in the RAPD reaction results in high resolution and reproduction and a high degree of polymorphism. Moreover, Jiemjuejun *et al.* [25] and Meesangiem *et al.* [26] revealed that genetic relationship of orchids has been analyzed by HAT-RAPD markers. In this work, the high annealing temperature RAPD (HAT–RAPD) method was used to investigate genetic diversity and relationships among longan varieties.

2. Materials and Methods

Plant materials

The twenty longan varieties used in this study have been assembled in germplasm conservation plots at Chiang Rai Horticultural Research Centre, Chiang Rai province, and Hangchat Horticultural Experiment Station, Lampang province, Thailand. The varieties analyzed are listed in Table 1. All, except three varieties in the list (Sukum, Vietnam and Lintchi) are commercially grown. The average age in all varieties was between 5 and 8 years.

DNA extraction

Samples of leaf tissue were ground in liquid nitrogen to a fine powder for DNA extraction following the methods of Doyle and Doyle [27], with minor modifications. About 0.1 g ground leaf tissue was mixed with 700 μL of preheated (60 °C) CTAB extraction buffer (4% (w v^{-1}) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% (w v^{-1}) PVPP (polyvinylpolypyrrolidone), and 0.1% (v v^{-1}) β-mercaptoethanol) in 1.5 mL microcentrifuge tube. The sample was vortexed and incubated at 60 °C in a water bath for 60 min with occasional agitation. The mixture was centrifuged at 12,000 rpm for 5 min, then the supernatant was transferred to a new tube. An equal volume of chloroform: isoamyl alcohol (24 : 1, v v^{-1}) was added. The mixture was spun at 12,000 rpm for 5 min and the supernatant was transferred into a clean tube, to which was added 1.0 vol – 20 °C isopropanol. The solution was mixed by repeated inversion and stored at – 20 °C overnight for precipitation.

After that, it was centrifuged for 5 min at 12,000 rpm. The precipitate was rinsed with 70% (v v^{-1}) cold ethanol, then air dried and dissolved in 100 μL double distilled water. The DNA solution was incubated at 37 °C for at least 3 h, after adding 5 units of RNase ONE™ ribonuclease (Sigma–Aldrich, St. Louis).

An equal volume of phenol was mixed with the DNA solution before centrifuging at 12,000 rpm for 5 min. Then, the upper layer was removed into a 1.5 mL clean tube. An equal volume of chloroform was added, inverted for 5 min and centrifuged at 12,000 rpm for 5 min. Supernatant was collected and mixed well with one volume of precool isopropanol, then left overnight at – 20 °C. The tube was centrifuged at 12,000 rpm for 5 min and drained. The DNA pellet was washed with 70% (v v^{-1}) cold ethanol and air dried, then resuspended in 20 μL TE and stored at – 20 °C. The DNA concentration was measured using a spectrophotometer. The DNA stock solution was kept at a final concentration of 20 ng μL^{-1}.

Table 1 List of longan varieties.

No	Varieties	Source
1	Daw	Hangchat, Lampang
2	Seechompoo	Hangchat, Lampang
3	Biewkhew	Hangchat, Lampang
4	Puangthong	Hangchat, Lampang
5	Daw Lampoon 01	Hangchat, Lampang
6	Narapirom	Hangchat, Lampang
7	Daw Chiang Rai 13	Hangchat, Lampang
8	Lintchi	Hangchat, Lampang
9	Kati	Hangchat, Lampang
10	Puangthong 05	Hangchat, Lampang
11	Haew	Hangchat, Lampang
12	Petsakorn	Hangchat, Lampang
13	Daw Hangchat 46	Hangchat, Lampang
14	Daw Nakornpanom	Hangchat, Lampang
15	Daw Nan	Hangchat, Lampang
16	Daw Luang	Hangchat, Lampang
17	Pingpong	Vietnam
18	Hokkian	China
19	Sukhum	Muang, Chiang Rai
20	Vietnam	Vietnam

HAT–RAPD protocol

Amplification of the DNA was carried out in a 0.2 mL multi ultra PCR tubes, (Sorenson™ Bioscience Inc.) using 20 μL reaction volumes. Decamer primers of arbitrary sequence (Operon Technologies, Almeda, California, USA) were chosen at random for PCR amplification. Each reaction volume contained 1 μL 20 ng DNA

template, 1x buffer (10 mM Tris–HCl pH 8.0, 50 mM KCl, 0.1% (v v^{-1}) TritonX–100, 2.0 mM $MgCl_2$, 200 μM of each dNTP, 0.5 unit *Taq* Polymerase (Promega, Madison, WI) and 2 – 3.5 pmol primers (Table 2). The reaction mixture was placed in a thermocycler (GeneAmp PCR System 2400, Perkin–Elmer, Foster City, CA). The PCR amplification was performed using method described by Anuntalabhochai *et al.* [15], as follows: 2 min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 46 – 48 °C, 45 sec at 72 °C and terminating with 5 min at 72 °C. The amplification products were separated by electrophoresis at 60 V cm^{-1} in 1.4% (w v^{-1}) agarose gels with 1x TBE buffer.

Data analysis

HAT–RAPD amplifications were repeated independently. Only clear and unambiguous bands were considered for analysis. Markers were recorded as 1 to indicate presence and 0 to indicate absence of all those visualized. A dendrogram, depicting the relationship among the taxa, was produced on the basis of the "neighbor–joining" method of the NTSYSpc software program (Exeter Solfware, Setauket, NY, USA).

3. Results and Discussion

The number of monomorphisms and polymorphisms of DNA fingerprints varied according to the combination of arbitrary primers and genomic templates of the samples. Eleven primers were randomly chosen to amplify genomic DNA of the 20 selected longan varieties. Only one primer, OPD20, produced monomorphic bands. Ten primers revealed a high degree of polymorphic banding patterns. Of a total of 305 bands, 178 were polymorphic, ranging in size from 100 to 2,500 bp (Fig. 1). The polymorphisms band occurred in DNA fingerprint may be related to the changes in oligonucleotide priming site due to homolog recombination deletion and mutation [12].

A list and the sequences of the 10 primers are presented in Table 2. The unweighted "neighbor–joining" dendrogram, constructed on the basis of the RAPD markers data, is shown in Fig. 2.

Table 2 List of 10 primers and their sequences used to generate RAPD markers among 20 varieties of longan.

Primers	Sequences (5' – 3')	No. of bands	No.of Polymorphic bands	Molecular weight (bp)
OPA13	CAG CAC CCA C	35	24	100 – 2,400
OPAK10	CTG TCA TGC C	35	14	400 – 2,300
OPB18	CCA CAG CAG T	30	12	200 – 1,200
OPG13	CTC TCC GCC A	30	18	200 – 2,400
OPT15	GGA TGC CAC T	29	18	300 – 1,400
OPW06	AGG CCC GAT G	34	23	200 – 2,400
OPW09	GTG ACC GAG T	36	20	300 – 2,200
OPX01	CTG GGC ACG A	10	9	600 – 1,100
OPX15	CAG ACA AGC C	42	25	170 – 2,500
OPZ 01	TCT GTG CCA C	24	15	200 – 2,500
	Total	305	178	100 – 2,500

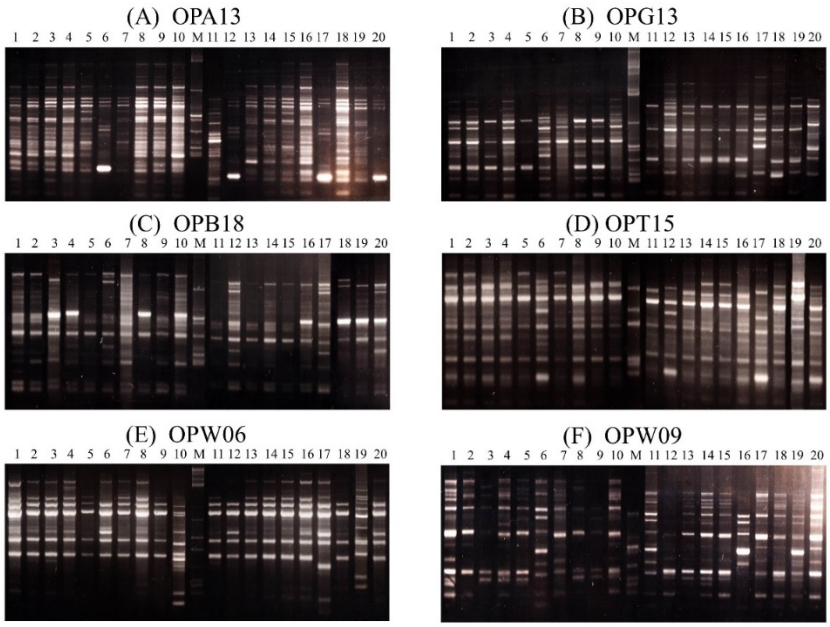


Fig. 1 RAPD amplification products generated from twenty longan cultivars using 6 arbitrary primers : OPA 13 (A), OPG 13 (B), OPB 18 (C), OPT 15 (D), OPW 06 (E) and OPW 09 (F). Lanes : 1) Daw; 2) Seechompoo; 3) Biewkhew; 4) Puangthong; 5) Daw Lampoon 01; 6) Narapirom; 7) Daw Chaing Rai13; 8) Lintchi; 9) Kati; 10) Puangthong 05; 11) Haew; 12) Petsakorn; 13) Daw Hangchat 46; 14) Daw Nakornpanom; 15) Daw Nan; 16) Daw Luang; 17) Pingpong; 18) Hokkian; 19) Sukhum; 20) Vietnam; M) lambda DNA/PstI marker.

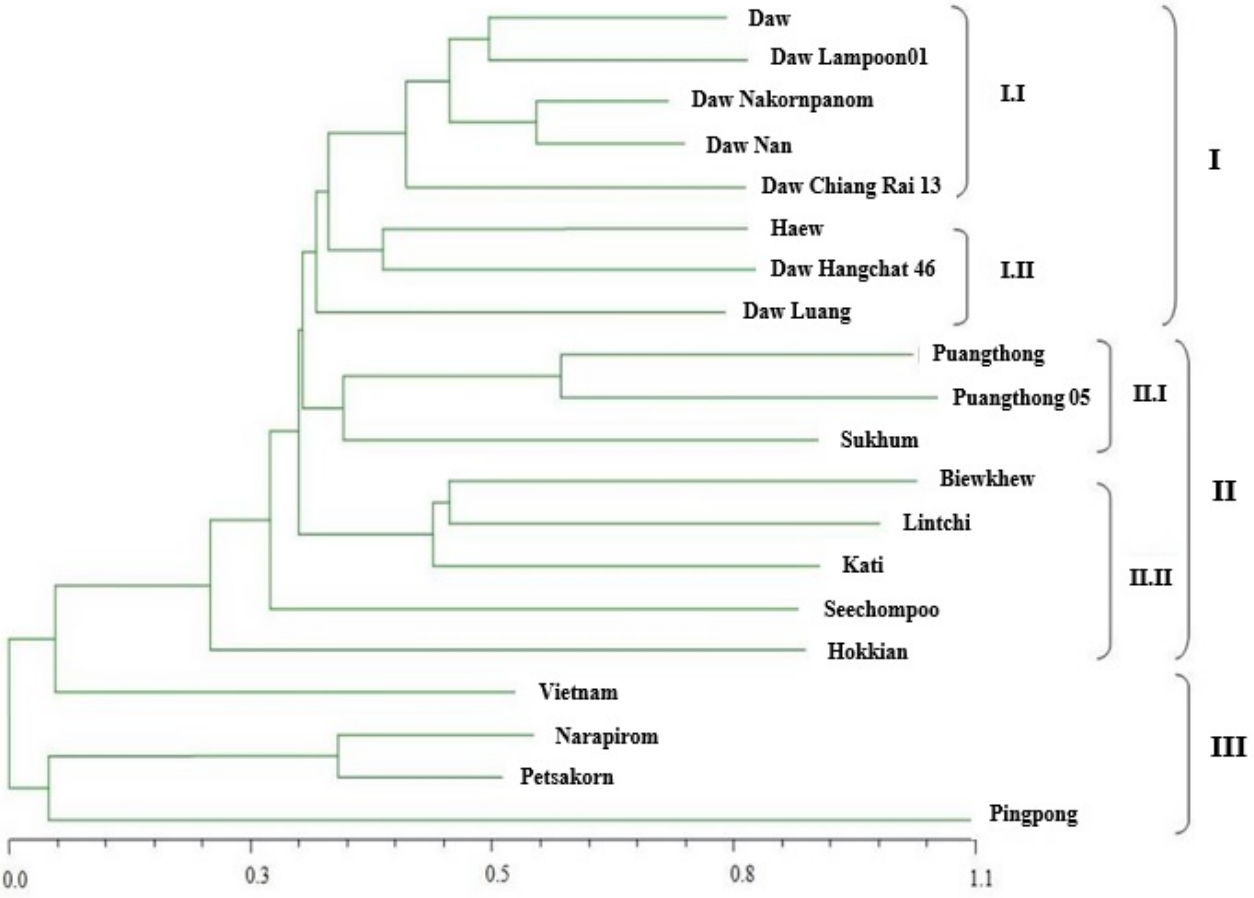


Fig. 2 Dendrogram illustrating genetic relationships among 20 longan varieties, generated by the "neighbor–joining" analysis (NTSYSep) produced by 10 primers. Genetic distance values are indicated at the bottom.

Regarding the historical background, physiological data and phenotypic characters, the dendrogram revealed that the twenty varieties were divided into 3 major groups. The first and the second groups consisted of 2 subgroups, whereas the third group contained one subgroup. Members in the first and the second group required low temperatures for flower bud formation, while members in the third group did not. Moreover, the first and the second groups were distinct due to their harvesting time of production. The first group's members were early harvested cultivars while the second's members were delayed–harvested cultivars.

The dendrogram revealed that all the members in the first groups were the Daw type. The members of each of the subgroups were : subgroup 1.1, Daw, Daw Lampoon 01, Daw Nakornpanom and Daw Nan; subgroups 1.2, Daw Chiang Rai 13, Haew, Daw Hangchat 46 and Daw Luang. In both subgroups, the original Daw type was from Lampoon Province and all members were derived from this Daw type. Previously, several new varieties had been selected from the native varieties for better quality. Consequently, these selected varieties were entered in the longan trade fair festival competition, held by Agricultural Extension Department in Lampoon Province. The best varieties were collected and kept at the Horticultural Research Institute. They were investigated for high yield and better yield components for at least 3 years in different locations. The best varieties in yield and quality were brought to growers. The longan varieties had been named from different locations. The Haew variety was grouped to a member of

subgroup 1.2. Its DNA fingerprint was identical to Daw Yod Dang (data not show). Also, its leaf shape and young leaf color are also similar. In addition, Daw Nakornpanom, Daw Nan and Daw ChiangRai 13 were named after the places where they were first propagated. Whereas Daw Luang was named by its fruit size (Luang is Thai for big.). Therefore, the HAT–RAPD method is able to detect variation in longan varieties caused by selection and propagation.

In the second group, the members of each subgroup were : subgroup 2.1, Puangthong, Puangthong 05 and Sukhum; subgroup 2.2, Biewkiew, Lintchi, Kati, Seechompoo and Hokkian. Our study revealed that DNA fingerprints of the members of the subgroup 2.1, Puangthong and Puangthong 05, were different lines from the same variety propagated from seed. Therefore, a close genetically relationship between the 2 lines was expected. The Sukhum variety was propagate and selected by seed, subsequently, it was named after the owner. Although this variety was classified as belonging to subgroup 2.1 it is only distantly related to the other members of this group. In subgroup 2.2, both the Lintchi and the Kathi varieties had more similar fingerprints than with Biewkiew, because Kathi and Lintchi are derived from the same tree of Biewkiew and had been selected through seed germination for several generations. Both varieties were named according to their typical characters. The flesh color of the Kathi variety is white like coconut meat. Fruits of the Lintchi variety contain a thin membrane under their exocarp, a character also found in lychee fruit. Lintchi is Thai for lychee. The Seechompoo and Hokkian varieties are also delayed- harvesting type as well as the members in group 2. However, their genotypic and phenotypic characters are regarded as distant relatedness to other members in this group.

The third group contained only one subgroup. The members were Vietnam, Narapirom, Petsakorn, and Pingpong. Our data revealed that DNA fingerprinting of both Narapirom and Petsakorn indicated close relatedness. This was confirmed by the similarity of their morphological characteristics. The varieties had been named at different locations. Petsakorn was first planted in Samutsakorn province while Narapirom had been grown at Tambon Narapirom, Nakornpathom province. Vietnam and Pingpong are introduced from Vietnam. The former variety was renamed from the Vinh Chau variety from Soc Trang Province, Vietnam (P. Chueychoom, pers. Commu.). Moreover, members in the third group also shared some common characteristics. Petsakorn and Narapirom have been grown commercially in central of Thailand, where the temperature is not as low as in northern Thailand (over 15 °C) during winter season (November – January). Therefore, low temperatures are not required for flower–bud formation for these two varieties (M. Dhasanondh, person. Commu.) [28]. Additionally, Na Songkhla [29] reported that Pingpong has been cultivated in the south of Vietnam and low temperatures were not required.

Although RAPD method effectively detected variability and have been adopted in a genetical study, plant systematic [30] and plant breeding [31]. However, it has some disadvantages regarding their low sensitivity and reproducible, which caused unstable results [14]. This study showed that increasing the annealing temperature in the RAPD reaction (HAT–RAPD) resulted in high resolution, high degree of polymorphism and reproducible. Thus, HAT–RAPD is used successfully for genetic characterization of numerous plant species and other organisms such as gastrointestinal helminths [32] and snails [33].

4. Conclusion

Our study indicated that the RAPD technique, with high annealing temperature (HAT–RAPD), provides high resolution and clear polymorphism for genetic variation of longan. Ten primers could classify the 20 varieties studied into 3 major groups. It will increase understanding of genetic relationships varieties of this important economic fruit. Furthermore, Genetics diversity data obtained from this study can be applied to genetics conservation in the future.

5. Acknowledgement

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