ORIGINAL ARTICLE

Comparison of cultivar identification methods of longkong, langsat and duku: *Lansium* spp.

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Abstract

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DNA amplification (RAPD), sequencing (RAPD-based PCR) and quantification (flow cytometry) were tested for their ability to identify *Lansium* spp.; duku, langsat and longkong. The results revealed that all the techniques tested can be used successfully. It was clearly seen that DNA amplification and sequencing are cumbersome, expensive and time consuming. Flow cytometry is simple and quick to use, less labour intensive and accurate/repeatable and hence can be considered a practical alternative to the others. The data for measurement of DNA content showed that duku has the lowest DNA content while longkong has the highest DNA content and langsat was intermediate.

Key words : cultivar identification, *Lansium* spp., PCR, RAPD, DNA sequencing, flow cytometry, phylogenetic relationship

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การเปรียบเทียบวิธีการตรวจสอบพันธุ์ลองกอง สางสาด และดูกู: *Lansium* spp.

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การตรวจสอบดีเอ็นเอโดยเทคนิค RAPD การตรวจหาลำดับเบสของยีนบางยีน และโฟลไซโตเมทรี เป็นเทคนิคที่ใช้ในการศึกษานี้เพื่อตรวจสอบพันธุ์พืชในสกุล *Lansium* คือ ดูกู ลางสาดและลองกอง ผลการศึกษาพบว่า RAPD based PCR มีความยุ่งยาก ประกอบด้วยขั้นตอนจำนวนมาก ต้องใช้ความชำนาญสูง และมีค่าใช้จ่ายแพง ในขณะที่เทคนิคโฟลไซโตเมทรีง่ายขั้นตอนน้อยไม่ยุ่งยากซับซ้อน และใช้เวลาสั้นเพียง 10-15 นาที สามารถบอกความแตกต่างของปริมาณดีเอ็นเอในพืชทั้งสามพันธุ์ได้ดังนี้คือ ดูกูเป็นพืชที่มีปริมาณดีเอ็นเอที่เป็นองค์ประกอบน้อยที่สุด ในขณะที่ลองกองมีปริมาณดีเอ็นเอที่เป็นองค์ประกอบมากที่สุด ส่วนลางสาดมีปริมาณดีเอ็นเอที่เป็นองค์ประกอบอยู่กลางระหว่างสองพันธุ์

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Lansium spp. includes three main important cultivars; longkong, langsat and duku. Among these cultivars, longkong is very popular to the growers. The fruit of this cultivar is reported to have the best quality. It has no latex, few seeds, sweet berry and a pleasant aroma. As the morphological appearance of all cultivars is nearly the same, identification of the cultivars is very difficult for growers. In the case of duku, it can be identified by a bitter leaf taste at seedling stage while the other two cultivars are able to be identified after bearing fruit, which requires 5-7 years. This time period causes a great economic loss when seedlings or grafted plants of undesirable cultivars are used. Early identification of the cultivars before planting would be an economic advantage.

The technologies of polymerase chain reaction (PCR) and RAPD techniques hold promise as useful tools for identification of plant cultivars (Willium *et al*., 1990). Recently, DNA polymorphisms amplified by using oligonucleotide primer, 9 or 10 nucleotides in length or longer, were used as genetic marker (Gustavo *et al*., 1991; Hu and Quiros, 1991; Nybom, 1994 and Willium *et al*., 1990) for fingerprinting or genomic mapping (Dirlewanger and Bodo,1994; Gardiner *et al*.,1994; Hu and Quiros, 1991; King, 1994 and Weeden *et al*., 1994) and identification of a resistant gene (Yang and Kruger, 1994). For flow-cytometry, there is no need to extract DNA. Leaf samples were chopped in a mixture of two different buffer solutions, nucleus isolating and staining buffers. The solutions containing nuclei were passed through a flow cytometer under the detection of ultraviolet system. Most of flow cytometry works are concerned with ploidy level screening (Mishiba and Mii, 2000; Kato and Mii, 2000; Mizuhiro *et al*., 2001; Kato *et al*., 2001; Te-chato *et al*., 2000). In this present study, to identify cultivar of *Lansium* spp. efficiently, PCR-based techniques, i.e. RAPD, DNA sequencing and flow cytometry were compared.

Materials and Methods

Plant material preparation

In this experiment, four cultivars of *Lansium* (longkong, duku, langsat and duku pramare) were used as plant materials in RAPD analysis. The fruits of all cultivars were collected from an orchard in Narathiwas. The seeds were excised, the aril removed and soaked in 10% sodium carbonate solution for 15 minutes. The seed surface was then sterilized in 1% sodium hypochlorite for 20 minutes

followed by five successive washings with sterile distilled water. The seeds were then sown *in vitro* on 1/2 strength MS medium without phytohormone. The cultures were maintained under dim illumination for two weeks. After germination was observed, the cultures were transferred to continuous light of 3,000 lux illumination and cultured for two weeks. Then fully expanded leaves of each cultivar were collected for DNA extraction and analysed.

Different methods such as RAPD, DNA sequencing and flow cytometry were used to identify the cultivars. While plants grown *in vitro* conditions were used for RAPD and DNA sequencing, plants *in situ* were used for flow cytometry

1. RAPD-based PCR

1.1 DNA extraction

Leaf samples (20 mg) of each cultivar were collected in Eppendorf tubes. A 150 µL extraction buffer (TE buffer) which consisted of 20 mM Tris-HCl (pH 8.0) and 0.1mM EDTA in the presence of 20 µL 10% SDS was added to each tube. The leaf tissue was crushed in the tube by a glass rod until a homogeneous suspension was obtained. The suspension was incubated at 70ºC for 15 min and 110 µL of 5M ammonium acetate was added and mixed with a vortex mixture. The solution was put on ice for 30 minutes and then centrifuged at 15,000 rpm for 10 min. After centrifugation, the supernatant was collected. Isopropanol (500 µL) was added twice to the suppernatant and left at room temperature for 10 minutes after which a white crystal of DNA could be seen. The solution was again centrifuged at 15,000 rpm for 10 minutes, the supernatant was discarded and the pellet of DNA was rinsed with 70% ethanol. The sample was again cetrifuged at 15,000rpm for 5 minutes. The pellet of DNA was dried and dissolved in 20 µL of TE buffer and the DNA solution kept in a freezer for amplification.

1.2 DNA amplification and size separation

A series of 10-mer primers from Operon Technology (Alamenda, California) was selected. The details of each primer are shown in Table 1. PCR reaction mixture (25 µL final volume) contained 1X reaction buffer, 500 µM dNTPs, 5 µM primer, 0.5 unit of Taq polymerase and 25 ng of genomic DNA template. Amplification was carried out in a PCR machine (PTC-10, MJ Research, Inc.) programmed for 3 cycles of 2 min denaturation at 94ºC, 2 min annealing at 35ºC and

Table 1. Primers used in amplification of *Lansium* spp.

Series of primer	Sequence (5'———>3')
A-06	GGTCCCTGAC
A-07	GAAACGGGTG
A-08	GTGACGTAGG
A-09	GGGTAACGCC
A-10	GTGATCGCAG
A-13	CAGCACCCAC
B-01	GTTTCGCTCC
B-02	TGATCCCTGG
B-03	CATCCCCCTG
B-04	GGACTGGAGT
K-01	CATTCGAGCC
K-02	GTGTCCGCAA
K-03	CCAGCTTAGG
K-04	CCGCCCAAAC
K-05	TCTGTCGAGG
K-06	CACCTTTCCC
T-01	GGGCCACTCA
T-02	GGAGAGACTC
T-03	TCCACTCCTG
T-04	CACAGAGGGA
T-05	GGGTTTGGCA
T-06	CAAGGGCAGA
T-07	GGCAGGCTGT
T-08	AACGGCGACA
T-09	CACCCCTGAG
T-10	CCTTCGGAAG
T-11	TTCCCCGCGA
T-12	GGGTGTGTAG
T-13	AGGACTGCCA
T-14	AATGCCGCAG
T-15	GGATGCCACT
T-16	GGTGAACGCT
T-17	CCAACGTCGT
T-18	GATGCCAGAC
T-19	GTCCGTATGG
T-20	GACCAATGCC

2 min extension at 72ºC, followed by 40 cycles of 0.3 min at 94ºC, 1 min at 35ºC and 2 min at 72ºC.

Fragments generated by amplification were separated according to size on 3% agarose gel (Seaplaque) run in 1xTE buffer at a constant voltage of 100 V for 5 h. The gel was stained with ethidium bromide and photographed under a UV transilluminator. To determine an optimum primer by RAPD-based PCR, the size of each band was inferred by comparison with a 1 kb ladder.

2. DNA sequencing

The DNA isolated from the leaf samples of three cultivars, langsat, longkong and duku was ampified by PCR technique. To study the homology of DNA among cultivars in this experiment, specific primers localized in the chloroplast genome, *rbc*L were used. The primer consists of forward (FWD) and reverse (RVS) directions. Sequences of bases in the primers are shown in Table 2. This primer was designed to amplify a 1118 bp fragment, encompassing 78% of the Rubisco large subunit (*rbc*L) sequence of the tobacco (Shinozaki *et al*., 1986);

The polymerase chain reactions were carried out in volumes of 20 µL containing 20 ng of genomic DNA, 1 µL of each primers, 2 µL dNTP, 50mM $CaCl_2$ and 1 Unit of *Taq* polymerase (Gold Taq) with the following thermal cycler profile: 94ºC for 10 min one cycle; 94ºC for 30 s, 56ºC for 30 s, 72ºC for 60 s for 40 cycles; 94ºC for 30 s, 72ºC for 120 s. for one cycle. The amplified DNA fragments were checked electrophoretically before passing through purification with the NUCLEO TRAP® extraction kit. Purified DNA was then sequenced. A 4 µL DNA sample was sequenced on a long distance polyacrylamide gel electrophoresis. The data were computerized by ABI PRISM Model version 2.1.1. Similarity among the species was interpreted by nucleotide private homology data of GENETYX.

Table 2. Specific primers used for gene specific PCR amplification of DNA of *Lansium* spp.

Primer	Sequence (5'——>3')
rbcL-FWD	TTGGCAGCATTCCGAGTAA
rbcL-RVS	TGTCCTAAAGTTCCTCCAC

3. Flow cytometry analysis

Leaf samples from five trees of three different cultivated *Lansium*; duku, langsat and longkong were collected from field grown mature trees at the Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University. The leaves were cleaned by rinsing under running tap water. A small leaf sample of about 1 cm^2 was excised from each cultivar and chopped with razor blade in 1.5 ml of DAPI (4', 6-Diamidino-2-penyinidole dihydrochloride) or Partec solution under cool conditions (put on ice). The former buffer consists of a mixture of 2mg/l DAPI, 12 g/l tris, 1 ml/l triton-X and 2 mM $MgCl_2$. The latter commercial buffer, a 1:5 mixture of Partec type-P solutions A and B (Partec Company, Germany) was used for isolation and staining of nuclei. Solution A isolates the nuclei and solution B is used for staining nuclei as DAPI staining.

Results

1. RAPD analysis

A large amount of DNA (10-30 ng) from each cultivar could be extracted from the leaf samples (25 mg). Polymorphisms among total genomic DNA of four cultivars of *Lansium domesticum* were readily obtained by OPA, OPT and OPK but without OPE. Some of the operon series could randomly amplify a large number of DNA fragments while the others could amplify only few fragments (Figure 1). However, all operon series clearly produced the variation among *Lansium* cultivars. The number of amplified DNA fragments or polymorphisms produced by the 25 primers using four cultivars of *Lansium domesticum* Correa. ranged from 1 by primer OPT6 to 23 produced by OPT8 (Figure 2). DNA fragments produced by primer OPT8 and OPT9 ranged from 194 to 1353 base pairs (bp). The fragment size produced by primer OPT6 and OPT7 ranged from 234 to 1078 bp.

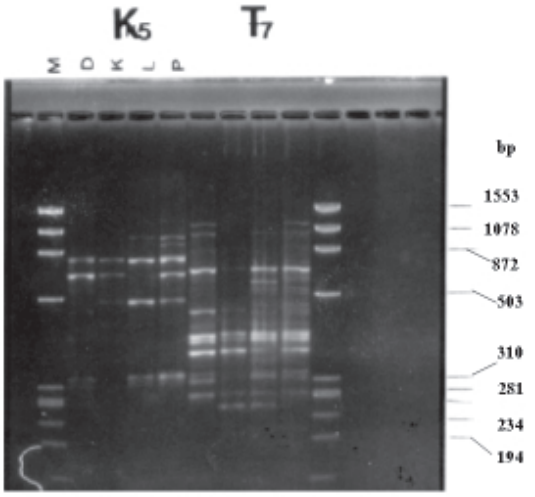

Figure 1. Comparison of DNA Polymorphisms amplified by OPK (K5) with OPT (T7).
M: molecular weight markers
D: duku
K: longkong
L: langsat
P: pramare (duku)

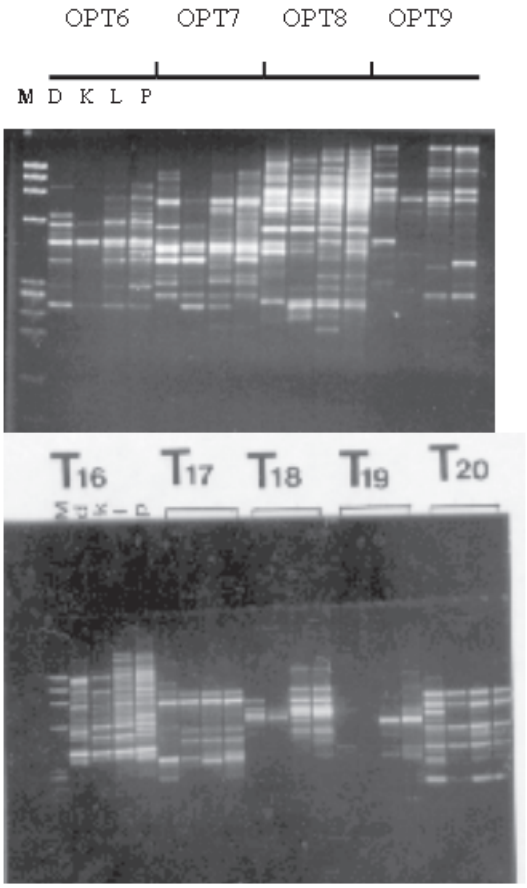

Figure 2. Patterns of DNA amplified by various series of primer T (OPT). M: molecular weight markers, D: duku, K: longkong, L: langsat, P: pramare (duku)

The profiles of the RAPD indicated that each primer could generate a major band (strong band) which could be used as RAPD markers for detecting the differences among the four cultivars. The differences in polymorphism may be due to the differences in amount of genetic variation that exist among the different cultivars. The primers, and conditions for DNA amplification, chosen in this study produced reasonably consistent results. RAPD markers illustrated in this study can be used to identify the cultivars.

2. DNA sequencing

The genetic relationship among the three cultivars of *Lansium* was revealed by comparison of the sequence of a 400 base pair of the *rbc*L gene fragment in the chloroplast genome. The duku cultivar was mid-way between langsat and longkong cultivars (Figure 3). This result was obtained using *rbc* FWD as a sequencing primer. The other primers and DNA from the other related cultivars were not ready to examine. Therefore, a conclusion about the genetic relationship cannot be made at this point. However, the result obtained suggests that longkong has a greater genetic distance from langsat and duku.

3. Flow cytometry analysis

The results from flow cytometry clearly revealed the ploidy level of each species in *Lansium*. Ploidy level of various clones in the cultivars was consistent. A histogram produced from each clone contained the same amount of DNA content. Among the three cultivars tested, duku contained the smallest amount of DNA, followed by langsat while longkong had the greatest DNA content (Figure 4). From the above

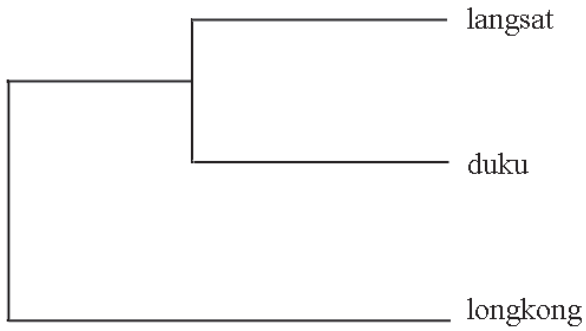

Figure 3. Phylogenetic relationship among three cultivated *Lansium*.

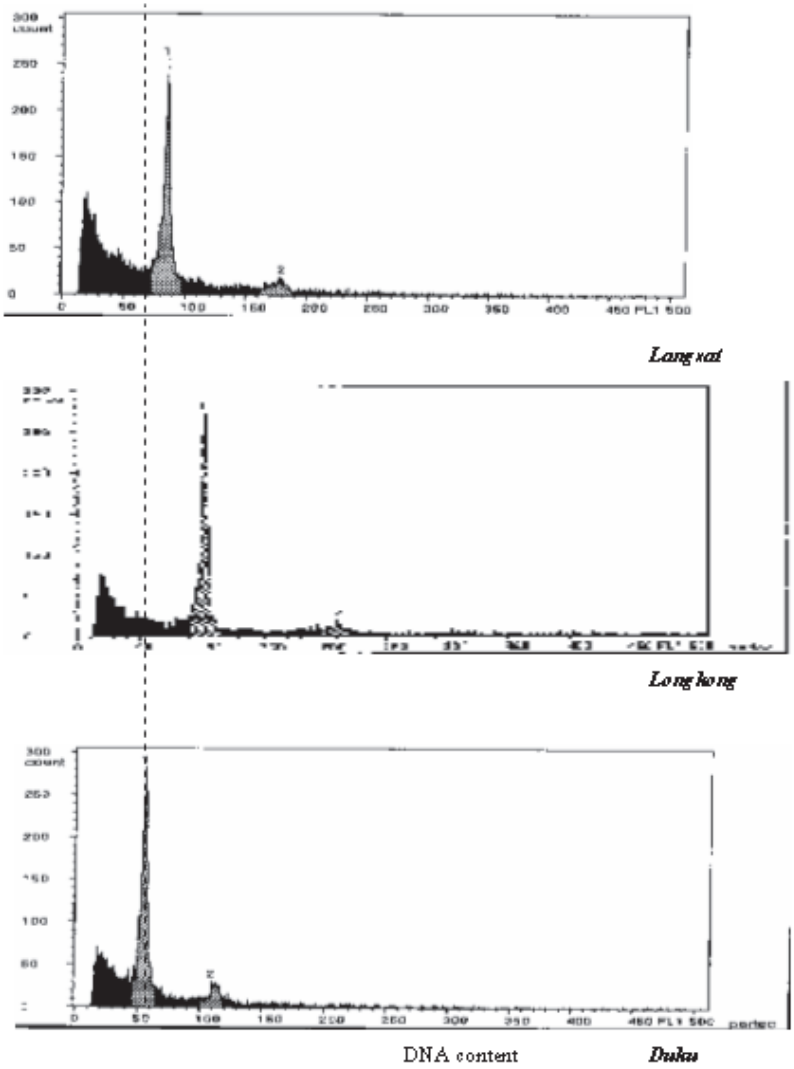

Figure 4. Ploidy level of some important cultivated *Lansium* spp.

result of DNA contents the ploidy level can be determined and clearly distinguished among the three cultivars. Duku has the lowest DNA content while longkong has the largest amount of DNA content and langsat was intermediate. Staining the nucleus by two different staining buffers provided different results. DAPI gave inferior peak on the histogram due to a low efficiency in staining. Identification of the species could not be made by DAPI whereas Partec buffer could stain the DNA of the species very well leading to a clear resolution peak of the histogram.

Discussion

Until a few decades ago, the *Lansium* spp. were not well known or as popular as other fruit crops. Growing these fruit trees was limited to only small or backyard orchards. More recently, the fruit trees of this genus has become economically important. New technological advances for production of the trees need to be developed. However, before focusing on other fields of development, a reliable methods of cultivar identification must be developed. A good or true-to-type cultivar must be screened and distributed to growers. As growing of this fruit tree is carried out by using seedlings, many constraints are encountered from this technique. Firstly, the time consumed from planting to blooming is too long, ranging from 7-10 years. Secondly, there is no reliable way to distinguish this cultivar seedlings being planted due to similar morphological characteristics of the seedlings. Many approaches of morphological identification have been attempted but an efficient result was not obtained (Autchanakul, 1990). Biochemical markers by means of isozyme technique was carried out (Te-chato and Nawarangsan, 1995). Again, the resolution of the results was still ambiguous. By this method, physiological ages and planting area play a significant role in resolution of isozyme patterns. So far, there is only one report about identification of the mixed species of *Lansium* by RAPD-based PCR (Konlasuk *et al*., 2001). In practice, only conventional technique was routinely performed by growers recently. Among those techniques tasting the leaves of seedlings was the most potent and practical way to screen before planting in the field. By this technique, it is very difficult to identify langsat from longkong. There still has not been an appropriate technique developed for solving this problem of growing longkong. DNA analysis has proved to be the best way for cultivar classification and identification in broad range of fruit crops and perennial trees. RAPD technique could not be used due to there being no specific banding or fingerprint of longkong DNA. Similar results were also reported by Konlasuk *et al.* (2001). Many testing steps and skillful labour are required for DNA works, especially DNA sequencing. At least three procedures must be done. Time consumed from starting procedure to final step is generally from one to three days. Moreover, it is very expensive. In this investigation, it is quite clear that flow cytometry is potent and a short cut for identification of longkong cultivar. The procedure was simple with a short time requirement (within a few munites) for obtaining a good result. Leaf sample of the species was only chopped in an appropriate nuclear staining buffer solution. Then the solution containing stained nucleus was passed through flow cytometry and the ploidy level or content of DNA was detected. All procedures could be performed within 10 to 15 minutes. In the future, more research is needed to establish a more broadly applicable DNA fingerprint method for identifying *Lansium* seedlings or adult trees.

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