The application of ISSR markers in genetic variance detection among Durian (*Durio zibethinus* Murr.) cultivars in the Nonthaburi province, Thailand

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Abstract

In this study, inter-simple sequence repeats (ISSR) markers were applied to assess genetic diversity and genetic relationships of 14 cultivars of Durian (*Durio zibethinus* Murr.), one of the most important agricultural economic fruits in Thailand. Genomic DNA was extracted from fresh leaf samples of 14 accessions collected from the Nonthaburi province. Nine ISSR primers were initially screened for analysis and five primers (ISSR1, ISSR3, ISSR4, ISSR5 and ISSR9) were chosen for further analysis. A total of 50 DNA fragments, varying from 50-2000 bp, were amplified, of which 19 (38%) were polymorphic. A dendrogram showing genetic similarities among Durian was constructed based on polymorphic bands using the SPSS program (version 18). Based on the results from the dendrogram, two clusters could be separated with similarity coefficients ranging from 0.632-1.00.

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1. Introduction

Durian (*Durio zibethinus* Murr.) is an economically fruits in Thailand. The country is the world’s largest producer and exporter of Durian, followed by Malaysia and Indonesia [1]. Durian belongs to the family Malvaceae [2] and distinctive for its large size, unique odour, and thorn-covered husk. Over the centuries, numerous Durian cultivars propagated by vegetative clones have arisen in Thailand. At present, there are over 200 durian names have been cultivated in Thailand [3]. However, the difference between its cultivars is practically not studied. There is not much information available on the genetic variance

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between cultivated Durian varieties in Thailand, particularly in the Nonthaburi province, where it has been cultivated for hundreds of years. Earlier classification and evaluations of Durian were done primarily based on phenotypic expression of the plants such as shape of fruit, size of thorns on the skin and other morphological characters. Unfortunately, morphological variation has limited ability to distinguish genetically similar individuals. For this reason, the use of molecular markers has become a standard method to study variability among closely related taxa [4]. Genetic markers, such as isozymes [5] and polymerase chain reaction (PCR) based methods, are more reliable for identification of genetic diversity than morphological markers, although each technique has advantages and limitations. Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. This method is similar to RAPDs, since both require no prior knowledge of the genome, cloning or specific primer design, yet it has higher reproducibility than RAPDs because of high annealing temperatures, and the cost of the analyses is lower than the cost of AFLPs. Therefore, ISSRs have been broadly and successfully used in studies on genetic diversity, phylogenetics, genetic mapping and evolutionary biology in a wide range of plant species [6].

The objective of this study was to use the ISSR technique to evaluate the genetic diversity and relatedness of 14 cultivars of Durian in the province of Nonthaburi using nine ISSR primers. To date, there is no published literature on the assessment of genetic diversity in Durian cultivars using ISSR techniques in this province of Thailand.

2. Materials and methods

2.1 Plant materials

DNA isolation and ISSR analysis were carried out using fresh leaf samples from 14 accessions collected from the Nonthaburi province. All cultivars are cultivated in a greenhouse at the Faculty of Science and Technology, Phranakhon Rajabhat University. Voucher specimens of all accessions are deposited in the Phranakhon Rajabhat University Herbarium.

| Durian Cultivars | Collection site in Nonthaburi | Vouchers | Sample number |
|------------------|-------------------------------|----------|---------------|
| Kop Chainam      | Bang Kruai                    | OV012-10 D1 |
| Kop Saonoi       | Bang Kruai                    | OV015-10 D2 |
| Kop Watklaul     | Bang Kruai                    | OV011-10 D3 |
| Kop Maethao      | Bang Kruai                    | OV007-10 D4 |
| Kop Tatao        | Bang Kruai                    | OV002-10 D5 |
| Kop Takum        | Bang Kruai                    | OV019-10 D6 |
| Kanyao           | Bang Bua Thong                | OV020-10 D7 |
| Yummawad         | Bang Kruai                    | OV004-10 D8 |
| Luang            | Bang Kruai                    | OV022-10 D9 |
| Thongyoichat     | Bang Kruai                    | OV026-10 D10 |
| Kampan           | Bang Kruai                    | OV027-10 D11 |
| Chatsrithong     | Bang Kruai                    | OV032-10 D12 |
| Monthong         | Pak Kret                      | OV003-10 D13 |
| Chanee           | Pak Kret                      | OV005-10 D14 |
2.2 Genomic DNA isolation

Genomic DNA was extracted from the leaves of 14 accessions using the CTAB method [7]. The leaves (1 g) were ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris–HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol)] 500 ml was added and the solution was incubated at 60 °C for 30 min. The homogenate was mixed with 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13,000 rpm for 15 min, the upper aqueous layer was transferred to a fresh tube. RNA was removed by treating with 2.5 ml of the RNase (10 mg/ml) for 30 min at 37 °C. The extraction of DNA with phenol/chloroform/isoamyl alcohol was repeated one more time. DNA in the solution was precipitated with 0.6 volume of ice-cold isopropanol and washed with 70% ethanol. Following this, the DNA was extracted using CTAB DNA extraction protocol without RNase. The process was repeated until the DNA pellet was free of color (two to three times) and the final pellet was dissolved in sterile deionized water. DNA quality and quantity were determined on 0.8% agarose gel. The DNA was stored at -20 °C, for further use as templates for PCR amplification.

2.3 ISSR-PCR analysis

To optimize the PCR amplification condition, experiments were carried out with varying concentrations of MgCl₂ and DNA template. Three different concentrations of MgCl₂ (3, 4, 5 mM) and three different concentrations of DNA template (25, 50, 100 ng) were used. PCR mixture (25 μl) contained: 10 Promega reaction buffer (100 mM Tris–HCl pH 9, 500 mM KCl, 1% Triton X-100), 0.4 mM of each dNTP, 0.6 mM of primer, 0.5 unit of Taq polymerase (Promega), 5 mM MgCl₂ and 100 ng template DNA. PCR was performed using a Thermohybaid PX2, programmed for an initial melting step at 94 °C for 4 min, followed by 45 cycles, each cycle consisting of three steps of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. A final extension step at 72 °C for 4 min was performed after the 45 cycles. A negative control reaction in which DNA was omitted was included in every run in order to verify the absence of contamination. The ISSR products were separated by agarose (1.8% w/v) gel electrophoresis at 100 A for 30 minutes in 0.04 M TAE (Tris–acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with ethidium bromide (10 mg/ml), and photographed on a UV transluminator. To determine ISSR profiles, the size of each DNA band was inferred by comparison with a 100 bp DNA ladder (Promega), used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by three repeating tests for each primer at different times.

2.4 Gel scoring and data analysis

Only strong and reproducible ISSR bands were scored. Different patterns observed were scored as discrete variables, using 1 to indicate the presence and 0 to indicate the absence of a unique pattern. The SPSS (version 18) data analysis package was used for the statistical analyses. Relationships among individuals were determined by the distance matrix method. Nei and Li’s Dice similarity coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix. A dendrogram was constructed from this matrix on the basis of the hierarchical cluster analysis, which is based on the average linkage between group, i.e. the unweighed pair-group method algorithm (UPGMA) [7].
3. Results and Discussion

3.1 ISSR profiles

The number of fragments produced by a primer ranged from 5 to 17 (Table 2). Examples of the ISSR polymorphisms produced by the random primer ISSR1 and ISSR3 are shown in Figure 1. The maximum numbers of bands were observed in ISSR3 primer (17), while minimum number of bands was recorded with ISSR5 primer (5) (Table 2). Quite considerable genetic variability does exist among different varieties of Durian cultivated in Nonthaburi province.

![Figure 1](image-url)  
**Fig. 1.** Examples of the RAPD polymorphisms from 14 Durian cultivars revealed by decanucleotide primers (A) ISSR1 (B) ISSR3 (left to right: lane M, molecular weight marker 1 kb ladder DNA; lanes D1–D14 stand for individual of plants)

| Primer number | Sequence                         | Size range (bp) | No. of ISSR bands | Polymorphic band% |
|---------------|----------------------------------|-----------------|-------------------|-------------------|
| ISSR 1        | 5'-AGGAGGAGGAGGAGG-3'            | 150-450         | 6                 | 2 (33%)           |
| ISSR 3        | 5'-AGAAGAAGAAGAAGT-3'            | 50-2000         | 17                | 7 (41%)           |
| ISSR 4        | 5'-GAGGAGGAGGAGGAGAC-3'          | 150-900         | 10                | 3 (30%)           |
| ISSR 5        | 5'-GAGGAGGAGGAGGAGAT-3'          | 200-750         | 5                 | 2 (40%)           |
| ISSR 9        | 5'-GGGGTGGGGTGGGGT-3'            | 150-950         | 12                | 5 (42%)           |

3.3 Relationships between cultivars

In order to estimate genetic variability among Durian cultivars in Nonthaburi, genetic similarity coefficients were calculated. The similarity matrix obtained using Nei and Li’s coefficient \([x]\) is ranged from 0.632-1.00 in 14 Durian cultivars tested in the present experiment. Based on ISSR bands amplified by five ISSR primers, genetic distances among the 14 accessions were calculated and a dendrogram was constructed by UPGMA method (Fig. 2). The dendrogram consisted of two major clusters. The first cluster contained a group of the eight Durian cultivars of Kob varieties (D1-D6), Thongyoichat (D10) and Kampan and was divided into three sub-groups. The first subgroup is made up of three cultivars: D1, D6 and D5. Inside the second subgroup of the first main cluster are three cultivars: D2, D4 and D3, related to third subgroup D10 and D11. The remaining cultivars were divided into two sub-groups. Cultivars belonging to the second main cluster are grouped in two sub-clusters. The first subgroup is made up of two cultivars: D9 and D14. D8, D12 and D13 form the second sub-cluster with a distantly related D7. The detail grouping between cultivars Durian in Nonthaburi based on ISSR data, differs slightly from that of the
RAPD marker [8] and the taxonomic characters taxonomic characters, for example, shape of fruit, size of thorns on the skin and leaf characters [1].

In conclusion, ISSR markers provide basic genetic knowledge among the cultivars of Durian. This research supports the current varietal classification of Durian. This is a preliminary study, and that a more detailed molecular study such as SSR and AFLP could help solve this: the existence of homonyms and synonyms, particularly with regard to varieties that have been cultivated for centuries and are widely distributed in Nonthaburi.

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