*Full Length Research Paper*

# Development of SSR markers of mangosteen (*Garcinia mangostana* L.)

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Assessment and utilization of diversity in plant genetic resources is very important to improve of plant species. A mangosteen (*Garcinia mangostana* L.) seed was formed obligate apomicts process. Genetic diversity of mangosteen was developed by using SSR markers. An inter-simple sequence repeat (ISSR)-suppression - PCR technique established to SSR marker of plant spesies. DNA library construction was used restriction enzyme blunt end *Rsa* I and adaptor (consist of 48-mer: 5'-GTAATACGACTCACTATAGG GCACGCGTGGTCGACGGCCCGGGCTGGT-3' and 8-mer with the 3-end capped by an amino residue: 5'-ACCAGCCC-NH2-3). Primer designed by using compound SSR (AC)10; (TC)6(AC)5 or (AC)6(AG)5 and an adaptor primer AP2 and nested PCR using AP1 primers. The PCR product integrated into the plasmid PGEMT Easy Vector System and competence cell *Escherichia coli* (strain DH5α) and sequence. Eight sequence from sample #G17 with SSR compound (AC)10, (TC)6(AG)5 and (AC)6(AG)5 produced two primer pairs. The primer pairs from sequence positive clone 4 of SSR compound (TC)6(AC)5 were forward primer: 5'-GGCCGTT AAAGTAGCTCAAGAA-3' and reverse primer:5'-CCGCATAGCATCAGTATCTG TC-3', while primer pairs from sequence positive clone 10 of SSR compound (AC)6(AG)5 were forward primer: 5'-GTGTTTCCATTTGTTACGCGCT-3' and reverse primer: 5'TAATGCCGTTGGGCAGTGA-3'.

**Keywords**: *Garcinia mangostana*, SSR compound. SSR primer

## INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is a tropical fruit tree species known for its delicate exotic appeals, hence the tree is referred to as '*Queen of tropical fruit*' (Wieble, 1993). Mangosteen fruit has a high economic value, thus it has good prospects to be developed into an excellent export commodity. Recently, the Indonesian government has place high priority to develop mangosteen for export. Available statistical data showed that in 2004, the production of mangosteen fruit was 62 117 metric tons and this rose to 78 674 metric tons in 2008, an increase of almost 17%. In 2008, the volume of mangosteen fruits exported was 9 465.665 metric tons (Directorate General of Horticulture, 2009).

Mangosteen fruit can be consumed fresh or as processed food. Besides its being used as food,

mangosteen also has medicinal properties. In South-east Asia, the pericarp of mangosteen fruit has been used traditionally as medicine to treat inflammation, diarrhea, dysentery, wounds and skin infection (Obolskiy *et al.*, 2009). The pericarp of mangosteen fruit contain the secondary metabolite xanthones and more than 80 xanthones have been isolated and characterized from the various parts of *G. mangostana* plant. The major constituents of xanthones isolated from mangosteen are α-mangostin and γ-mangostin (Jung *et al.*, 2006). According to Han *et al.* (2009), the biological effects of mangosteen xanthones are diverse and include antioxidant, antibacterial, antifungal, antimalarial, anti inflammatory, cytotoxic, and HIV-1 inhibitory activities.

Mangosteen is an erect trees and slow growing. It is caused by bed rooting system, low of intake water and mineral, low of photosynthesis rate, a long of dormancy phase. Characteristic of mangosteen trees are: (1) slow growth rate of seedling, this is due to the lack of root

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system in which both formation root hairs were few and low capacity of $CO_2$ capture leaves (Lim, 1984),  (2) long juvenile phase, the primary mangosteen fruit reached 10-15 years after planting (Wieble, 1993).

The mangosteen seeds are formed obligate apomicts and in the group seed recalcitrant. The seed is not from the results of pollination and fertilization (Richard, 1990), but it's comes from nucellus cells. Embryo that appear derived from somatic embryos, so it can be said that mangosteen is propagated vegetatively. All mangosteen trees are believed to be genetically identical or homogenous (Richards, 1990) and genetic variability of mangosteen is very limited. The lack of genetic variability mangosteen make conventional breeding hybridization technique and selection is impossible also, because of lack of fertile pollen and rudimentary (Wieble, 1993). In plant breeding, information of genetic diversity is very important to decided improving stage for future mangosteen.

The mangosteen cultivated mainly in South-east Asia. Almeyda and Martin (1976) proposed that mangosteen is native of Indonesia, because of it's distributed almost throughout the archipelago in Indonesia with the main populations in Sumatra, Kalimantan and Java Island. However, the production centers of mangosteen are in Province of West Sumatra, West Java, Central Java, East Java, and Bali (Sobir and Purwanto, 2007). Identification germplasm can be done by distinguishing morphological characteristics of plant phenotypic, biochemical content analysis of plants and molecular marker (Gallego and Martinez, 1996).

Simple sequence repeats (SSR) or microsatellite is becoming the marker of choice in both animal and plant species. SSR is short stretches of DNA, consisting of tandem repeated nucleotide units (1-5 nucleotides long). SSR marker is usually very polymorphic due to the high level of variation in the number of repeats (Gianfranceschi et al., 1998). Polymorphisms associated with a specific locus are due to the variation in length of SSR, which in turn depends on the number of repetitions of the basic motif (Rallo et al., 2000). SSR marker is highly popular genetic markers as possess: co-dominant inheritance, high abundance, enormous extent of allelic diversity, ease of assessing SSR size variation through PCR with pairs of flanking primers and high reproducibility. However, the development of SSR marker requires extensive knowledge of DNA sequences, and sometimes they underestimate genetic structure measurements, hence they have been developed primarily for agricultural species, rather than wild species (Mondini et al., 2001).

Development of SSR marker requires the identification and sequencing of SSR loci and the construction of primers that can be used to amplify the alleles. Polymorphism at SSR loci can be efficiently assessed by PCR. To improve the efficiency of SSR marker development, it was developed a method, named the ISSR-suppression-PCR technique, which did not require enrichment and screening procedures (Lian et al., 2001).

Gene Research Center, University of Tsukuba was collected 39 germplasms of Garcinia from several locations in Indonesia, Thailand and Myanmar such as G. mangostana, G. mallacensis, G. cowa, G. atroviridis and G. schomurgkinae. But identification germplasm of Garcinia by using SSR marker not reported yet by researchers. The objectives of this work were: i) to construct a SSR marker-rich DNA library of G. mangostana; ii) to identify, select and sequence SSR marker DNA clones G. mangostana of ISSR-suppression-PCR technique.

## MATERIAL AND METHODS

### Plant material and DNA isolation

Mangosteen leaves samples were used genotype #G12 (Tasikmalaya); #G15 (Purwakarta); #G17 (Jambi); #G111 (Malinu East Kalimantan) from Indonesia, while genotype #G23 (Mon State) from Myanmar. Total genomic DNA of mangosteen leave was extracted using modified CTAB procedure describes by Doyle and Doyle (1990). Dried leave with gel silica and liquid nitrogen will be ground in a mortal. The product is transferred to eppendrof  tubes 700 µl extract buffer CTAB, 0.2 % mercaptoethanol and 0.1 % PVP. Furthermore, it was vortexes and incubated at 60 $^o$C for 30 minute in water bath, and then spin down at 10 000 rpm for 10 minute. The supernatant was been transferred to new eppendorf tube added 700 µl an equal volume buffer extract  of chloroform: isoamylalcohol (24:1) and was vortexes  and spin down at 10 000 rpm for 10 minute. The supernatant transferred to new eppendorf tube added 400 µl cool isopropanol and spin down at 10 000 rpm for 10 minute. The DNA pellet washed 100 µl with wash buffer and spin down at 10 000 rpm for 5 minute.  Pellet DNA added 100 TE buffer are stored at 4$^o$C (freezer). DNA concentration was measured using a spectrophotometer (Beckman Coulter DU 640) and DNA quality was used 1 % gel agarose in electrophoresis and soaking ethidium bromide and documentation under UV.

### DNA library construction

DNA genomic (~400 ng/µl) digested by restriction enzymes Eco RV; Eco105; Rsa I and Dra I (Toyobo Co.) and then incubated 37 $^o$C for overnight. Check digested DNA fragment by gel electrophoresis 1.5 % gel agarose. The solution added with ammonium acetate (x1/3 volume) and 100 % ethanol and then spin down at 10 000 rpm for 10 minute. The pellets are washed by 70 % ethanol and spin down at 10 000 rpm for 10 minute.  The pellet dried air for 60 minute and dissolved in TE buffer (100 µl).
 The restricted fragment has been legated by a specific blunt Adaptor (consist of 48-mer: 5′-GTAATACGACTCACTATAGGGCACG CGTGGTCGACGGCCCGGGCTGGT-3′ and 8-mer with the 3′-end capped by anamino residue: 5′-ACCAGCCC-NH2-3′) by use of a DNA ligation kit(Takara Shuzo Co.). The restricted fragment (~50 ng/µl) 8 µl;  dDW 8 µl; 20 µl Takara ligation kit and 4 µl annealed adaptor (100pmol/µl) and then incubated 16 oC for 1 hour. The solution added equal amount of chloroform: isoamylalcohol (24:1) and spin down 14 000 rpm for 10 minute. Take supernatant and move to other tube and added 96 % ethanol and spin down 14 000 rpm for 10 minute. The pellet washed by 70 % ethanol spin down 14 000 rpm for 5 minute and dissolved in TE buffer (50 µl).
 Primer designing of a compound SSR marker fragments were

amplified from the *Rsa I* DNA library using compound SSR primer $(AC)_{10}$; $(TC)_6(AC)_5$ or $(AC)_6(AG)_5$ and an adaptor primer AP2 (5′-CTATAGGGCACGCGTGGT-3'). The primary PCR cocktail solution one reaction as bellow: 10x *ex taq buffer* (5 µl); dNTP mixture (4 µl); AP2 (2 µl) adaptor DNA (1 µl); *ex taq* DNA (0.25 µl) and dDW 35.75 µl. The amplification DNA by using PCR machine (Gene Amp PCR System 9700) consists of 5 cycles an initial denaturation for 4 min at 94 °C followed by 30 second at 94 °C annealing for 30 second at 62 °C and elongation for 1 minute at 72 °C and then 38 cycles of 30 second at 94 °C, 30 second at the annealing temperature 60 °C, 30 second elongation at 72 °C, and a final extension step of 5 min at 72 °C. PCR product checked with 1.5 % gel agarose.

The secondary PCR used an adaptor AP1 primers (5′-CCATCCTAATACG ACTCACTATAGGGC-3') nested in the primary PCR product. The secondary PCR cocktail solution one reaction as bellow: 10X *ex taq* buffer (5 µl); dNTP mixture (4 µl); AP1 (2 µl) adaptor DNA (1 µl); *ex taq* DNA (0.25 µl) and dDW 35.75 µl. The amplification consist of 5 cycles an initial denaturation for 4 min at 94 °C followed by 30 second at 94 °C annealing for 30 second at 62 °C and elongation for 1 minute at 72 °C and then 38 cycles of 30 second at 94 °C, 30 second at the annealing temperature 60 °C, 30 second elongation at 72 °C, and a final extension step of 5 min at 72 °C. PCR product checked with 1.5 % gel agarose.

The DNA fragment (200-700 bp) required of secondary PCR product and cutting by using clean razor blade. Chop the trimmed gel slice and place the pieces into the filter cup of the Quantum prep ᵀᴹ Freeze N Squaeze DNA gel extraction spin columns (Bio-rad Co). Place the filter cup into dolphin tube. Place the Quantum prep ᵀᴹ Freeze N Squaeze DNA gel extraction spin columns (filter cup nested within dolphin tube) in a -20 °C freezer for 5 minute. The sample was spin down at 14 000 rpm for 5 minute at room temperature. Collect the purified DNA from collection tube and added chloroform: isoamylalcohol (24:1) equal volume and then spin down 14.000 rpm 10 minute. Take supernatant and move to other tube and added 96 % ethanol and spin down 14 000 rpm for 10 minute. The pellet washed by 70 % ethanol spin down 14 000 rpm for 5 minute and it was dissolved in TE buffer (20 µl).

### Cloning

The PCR product was integrated into the plasmids PGEMT Easy Vector System (Promega Co.). The composition were dDW (2.0 µl); 2X buffer (4.0 µl); PCR product (0.5 µl); PGEMT Easy Vector (0.5 µl); T4 DNA Ligase (1.0 µl) and incubate 37 oC for overnight (16 hours). Take competence cell *Escherichia coli* (strain DH5α) and put on cruise ice for 30 minute. The DNA ligation product (8.0 µl) and competence cell *E. coli* (42.0 µl) and put in ice for 60 minute. Furthermore, put incubate block heater at 42 oC for 45 second and transfer on ice for 2 minute. Added SOC solution (450 µl) in hood and shaking incubator at 37oC for 2 hours and then spin down 2 500 rpm for 5 minute. Discard the 350 µl by pipette and 50 µl spread on the LB plate (containing of amphicillin antibiotic; IPTG; Xgal) and then incubate LB plate at 37 oC overnight (16 hours).

The evaluation LB plates between white or blue colony. Pick a white colony (positive colony) by using sterilized toothpick and rinse the tip in the PCR cocktail. The PCR cocktail solution one reaction as bellow: 10x *ex taq* buffer (2 µl); dNTP (1.6 µl); forward primer (M13 F) (1 µl); reverse primer (M13 F) (1 µl); ex Taq (1 µl) and dDw (4.3 µl). The amplification consist of 25 cycles of 30 second at 94 °C, 30 second at the annealing temperature 55 °C, 30 second elongation at 72 °C, and a final extension step of 7 min at 72 °C. The PCR colony checked with 1.5 % gel agarose. The colony PCR can be purificated by using exo-sap. The PCR product (8 µl) mixed with exo-sap (3 µl) is ready for cycles PCR as bellow: 30 minute for 37 °C and 15 minute for 80 °C.

### Sequencing

The PCR cocktail for sequencing have been prepared with composition as bellow: Big Dye (0.5 µl); Big Dye sequencing 5X buffer (1.5 µl); forward primer (1.6 pmol) (1µl); PCR product (16 ng) (0.5µl) and dDW (6.5 µl). The amplification consist of 27 cycles of 30 second at 94 °C, 20 second at the annealing temperature 50 °C, 4 minute elongation at 60 °C. Purification PCR product with 75 % isopropanol (40 µl) and incubate at room temperature for 15 minute. Furthermore, spin down 15 000 rpm for 20 minute at room temperature and discard isopropanol. Washing with 75 % isopropanol 125 µl and spin down 15 000 rpm for 20 minute at room temperature and discard isopropanol. Dry the pellet at room temperature for 30 minute.  Resuspend the sample in injection buffer with Hi-Di formamide and denature of the sample 95 °C for 5 minute and chill on ice for at least 5 minute. Transfer samples to sequencer machines (Genetic Analyzer 3130, Applied Biosystem, Hitachi). The sequence data was analyzed by using software Websat (Martin *et al.*, 2009).

## RESULT

### Plant material and DNA isolation

DNA isolation of mangosteen process is quite difficult, because the leaf of mangosteen contains a high polyphenol compounds. In order that buffer extraction CTAB can be added PVP. The qualities of DNA have been visualized by 1 % gel agarose in electrophoresis and documentation under UV (*unpublished data*). The quantities of DNA are calculated by using a spectrophotometer and measurement was done three times. The DNA concentration of genotype #G12; #G15; #G17; #G111; and #G23 were 322.4 ng/µl; 305.2 ng/µl; 417.3 ng/µl; 382.1 ng/µl; 379.6 ng/µl respectively. DNA concentration has been required in the process of DNA library construction were 400 ng/µl.

### DNA library construction

The DNA library construction was used two sample #G12 and #G111.  The both samples have been digested by a blunt end restriction enzyme such as *Dra I, Eco RV, Eco 105* and *Rsa I*. The result showed that restriction enzyme *Rsa I* can digested fragment DNA of two samples with a uniform smeared, whereas the restriction enzyme *Dra I* and *Eco RV* cut only a part DNA fragment. The restriction enzyme *Eco105* only cut partially for sample #G111, while sample #G17 can not be cutting (*unpublished data*). Furthermore, the experiment used four samples, namely #G15, #G17, #G111 and #G23 have been digested by only restriction enzyme of *Rsa I*. Results showed all samples were cut off, except for sample #G15 did not cut. As showed in Figure 1.

The DNA concentration of genotypes #G17; #G111; and #G23 were 35.3 ng/µl; 43.9 ng/µl; 13.9 ng/µl respectively. The highest concentration of DNA contained is at the sample #G17 and the lowest is at sample #G23. The DNA concentration of sample need for ligation were 50 ng/µl. The DNA concentration is very important to
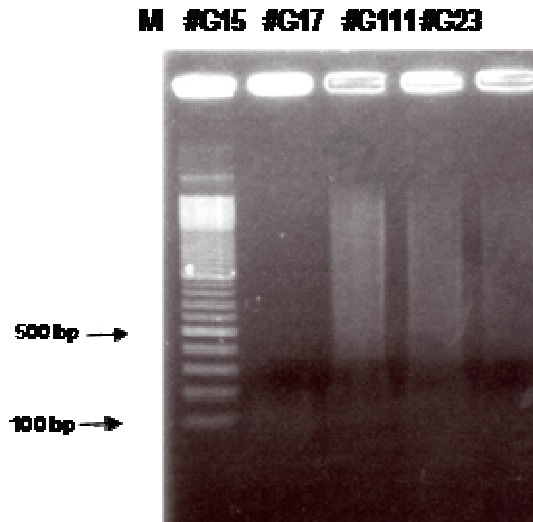
**Figure** 1. DNA fragment sample #G15; #G17; #G111; #G23 digested by restriction enzyme of *Rsa I*; M=100 bp ladder

process ligation of DNA fragments. Ligation of DNA fragments by using an adaptor 48 mer: 5′-GTAATACGACTCACTATAGGGCA CGCGTGGTCGA-3' and an adaptor 8-mer with the 3′-end capped by an amino residue: 5′-ACCAGCCC-NH$_2$-3′ by use of a DNA ligation kit. Because of digested by using restriction enzyme blunt end, so to ligation process needed adaptor. DNA fragments from ligation process arrange on PCR machine to produced PCR product. A primary PCR using SSR primers compound (AC)$_{10}$; (TC)$_6$(AC)$_5$ or (AC)$_6$(AG)$_5$ and an adaptor primer AP2 (5'-CTATAGGGCACGC GTGGT-3 ') and three DNA fragment samples. PCR fragment were amplified from *G. mangostana* genome using different SSR compound. The results showed that the SSR primers compound (AC)$_{10}$ of three samples (#G17; #G111 and #G23) are smeared, while the compound SSR (TC)$_6$(AC)$_5$ and (AC)$_6$(AG)$_5$ have size fragments were 1200 bp. The primary PCR product will be continued to the secondary PCR by using primers AP1 (5'-CCATCCTAATACGACTCACTA TAGGGC-3'). The secondary PCR product nested in the primary PCR product. After checking secondary PCR product by using 1.5% gel agarose DNA fragment/band pattern of the three samples showed that almost the same (Figure 2). The product amplified by (AC)$_{10}$; (TC)$_6$(AC)$_5$ and (AC)$_6$(AG)$_5$ were used for cloning and sequencing.

The visualization of secondary PCR product on electrophoresis in 1.5 % gel agarose and subsequently the band required cut into 200-700 bp. The process of making pellets made in accordance with the procedures. The process of cloning is done by using pGEMT easy vector and competent cells of *E. coli* strain DH5a. The experimental results showed that *E. coli* able to carry

DNA fragment through pGEMT easy vector. As evidence, in the plates there are many white colony, while the blue colony showed that the vector does not successfully carried DNA fragments (Figure 3).

To select the optimum size of positive clones, we choose sixteen colonies each SSR compounds. The colonies in the plate have been harvested by using toothpick and put a cocktail colony PCR by using M13 forward primer.

Fifteen of 48 colonies amplified fragment on 1.5 % gel agarose in electrophoresis (Figure 4). The DNA fragments with sizes ranging from 300 bp to 700 bp were selected and sequenced. Eight of 15 fragments can be sequenced by using Genetic Analyzer 3130 (Applied Biosystem, Hitachi).

The result showed that sample of #G17 with SSR compound of (AC)$_{10}$ only positive clone 4; 6; 14; 15 and 16 were amplified on 1.5 % gel agarose in electrophoresis. SSR compound of (TC)$_6$(AC)$_5$ only positive clone 1; 2; 4, 6; 14 and 16 were amplified. SSR compound of (AC)$_6$(AG)$_5$ only positive clone 4, 6, 10 and 11 were amplified. The fragment size (300-700 bp) have been choose and then sequence. We have eight fragments to sequence and analyze primer pairs to flanking region such as SSR compound (AC)$_{10}$ positive clone 4;15;16; SSR compound (TC)$_6$(AC)$_5$ positive clone 1; 2; 4; 14 and SSR compound (AC)$_6$(AG)$_5$ only positive clone 10. The eight sequences fragment as showed in Table 1.

The sequence of sample #G17 has two primer pairs amplified single band. The two primer pairs from sequence positive clone 4 of SSR compound (TC)$_6$(AC)$_5$ and sequence positive clone 10 of SSR compound
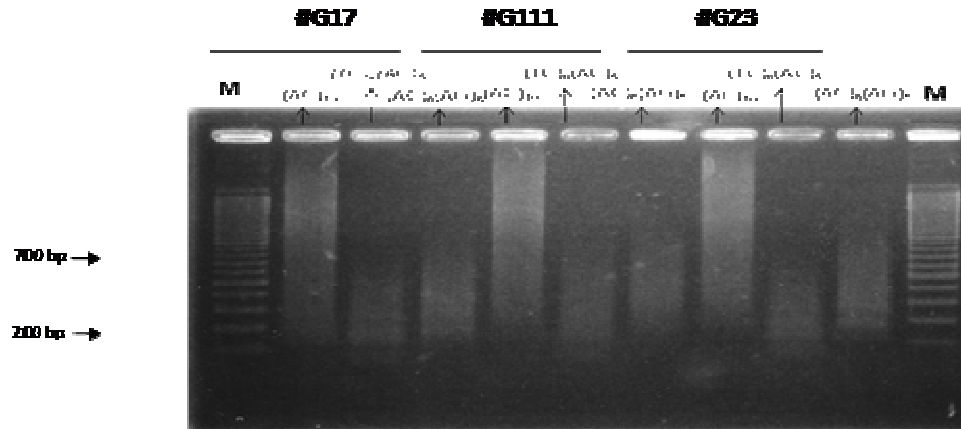
**Figure** 2. Secondary PCR product using primers AP1 of samples #G17; # G111; # G23 by using SSR compound $(AC)_{10}$; $(TC)_6(AC)_5$; $(AC)_6(AG)_5$; M= 100 bp ladder
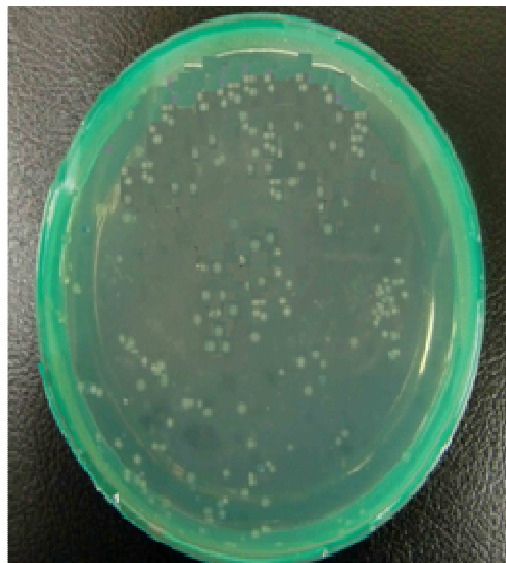


**Figure** 3. Colony of positive clones in plate sample of #G17

$(AC)_6(AG)_5$. The primer pairs from sequence positive clone 4 of SSR compound $(TC)_6(AC)_5$ were forward primer: 5'-GGCCGTTAAAGTAGCTCAAGAA-3' and reverse primer: 5'-CCGCATAGCATCAGTATCTGTC-3' with repeat motif $(TC)_6$ and product size 293 bp. Annealing temperature of primer (Tm) was 59.9 $^o$C. While primer pairs from sequence positive clone 10 of SSR compound $(AC)_6(AG)_5$ were forward primer: 5'-GTGTTTCCATTTGTTACGCGCT-3' and reverse primer: 5'TAATGCC GTTGGGCAGTGA-3' with repeat motif $(G)_{12}$ and product size 125 bp. Annealing temperature of primer (Tm) was 63 $^o$C.

## DISCUSSION

Isolation SSR marker was performed by ISSR suppression-PCR as outlined by Lian (2001, 2003). To optimize of activity restriction enzyme were used genotype #G12 and #G111 with *Eco RV, Eco105; Dra I* and *Rsa I*. Genotype #G17, #G111 and #G23 were used for isolation SSR. DNA genomic was separately digested with *Rsa I* restriction enzyme. Three amplified product exhibited smeared banding pattern in gel agarose electrophoresis. The fragments were then legated to a specific adaptor consist of 48-mer: 5′-
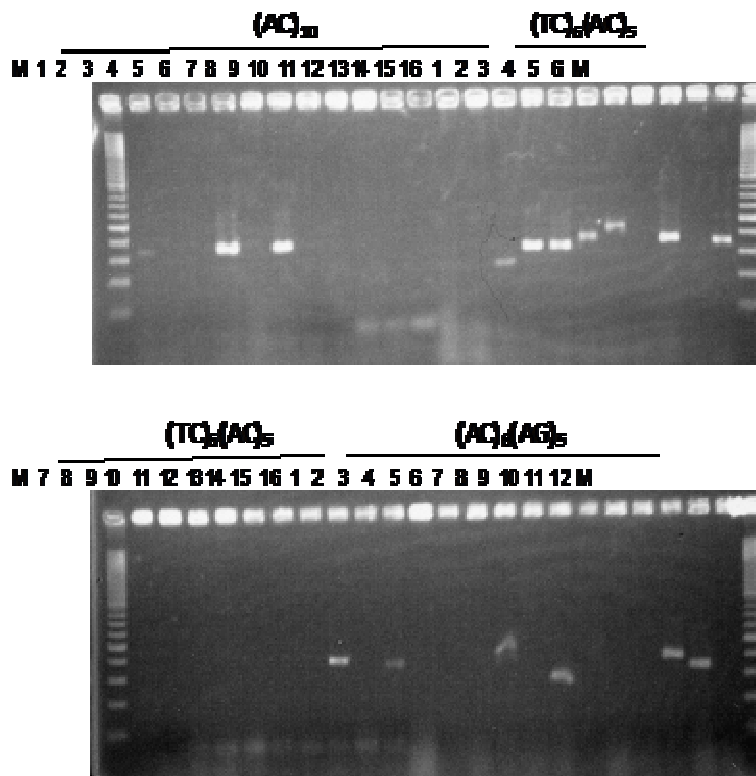
**Figure 4**. DNA fragment colony PCR from sample #G17 by using compounds SSR  $(AC)_{10}$; $(TC)_6(AC)_5$ and $(AC)_6(AG)_5$

GTAATACGACTCACTATAGGGCACGCGTGGTCGACG GCCCGGGCTGGT-3′ and 8-mer with the 3′-end capped by an amino residue: 5′-ACCAGCCC-NH$_2$-3′) by use of a DNA ligation kit (Takara Shuzo Co.). Adaptor used to replace one type of protruding terminus with another. During ligation, the protruding 5' end of the adaptor becomes joined to complementary terminus of the target DNA (Sambrook and Russel, 2001).

As a primary step, fragment flanked by SSR compound $(AC)_{10}$, $(TC)_6(AC)_5$ and $(AC)_6$ $(AG)_5$ at one end were amplified from each of the adaptor legated and adaptor primer AP2 designed from the longer strand of the adaptor. Figure 2 showed that SSR compound and adaptor primer AP2 Amplified products of these three samples exhibited smeared banding patterns on agarose electrophoresis. The fragment of #G17 integrated into the plasmids PGEMT Easy vector System (Promega Co.) and were transformed into *E. coli* strain of DH5α and then sequence. The secondary step was performed to determine the sequence of the other flanking region of each SSR. Eight of 15 sequences have been chosen to design for the determination of the unknown flanking region. The ISSR sequences in the other fragments were different from each other. These results suggested that we could quickly determine sequences of one of both flanking regions of many microsatellites with one ISSR

amplification. If several kinds of SSR primers were used together to amplify the fragments of inter-simple sequence repeats, more diverse ISSR sequences might be obtained.

Hayden *et al.* (2004) recently prepared primers flanking a compound SSR sequence from the bread wheat genomic sequence database, and reported that PCR amplification with a prepared primer and a compound SSR primer resulted in co-dominant electrophoretic bands and successfully showed high polymorphism of wheat lineages. Lian *et al.* (2006) showed that designed primer in combination with acompound SSR primer $(TC)_6(AC)_5$ or $(AC)_6(AG)_5$ to amplify each compound SSR region of 22 *D. trifidus* individual trees.

In *Acanthus ilicifolius*, two compound SSR primers $(AC)_6(TC)_5$ and $(TC)_6(AC)_5$ were used. Fifteen fragments that contained $(AC)_6(TC)n$ (6) or $(TC)_6(AC)_n$ (9) repeats at one end were obtained and used to design the IP1 primer. While In *Lumnitzera racemosa*, four compound SSR primers $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ and $(TC)_6(AC)_5$ were used. Thirty fragments that contained a compound microsatellite region at one end were obtained and used to design the IP1 primers, of which four, four and 22 fragments were amplified by $(AC)_6(AG)_5$, $(AC)_6(TC)_5$ and $(TC)_6(AC)_5$ primers, respectively (Geng *et al.*, 2008).

**Table 1.** Positive clone sequences sample of #G17 of SSR compound

| clone name | Sequences (5' – 3') |
| --- | --- |
| clone 4 of SSR compound $(AC)_{10}$ | GGGAAGGTCGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGC ATGCTCCCGGCCGCTAAAGGGGCCGTGGGAATTCGATTACACACACACACAGAGA TATAGCCATGTACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTGAGTCGTA TTAGGATGAAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGA GCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTT GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAAAGACTCTGCTATGCTCGTTTT TGTGTCTCCAAATGGTGACCTGCTGCCCCTCGCCTCAGGGGACCCACTAAACCCTA TATAGGGCAGCTGGGCAAGCCCGCGGGCCGGCCCCCACGCGGCGGTCAAGAACT CCCTGCTTT TTGTGGGGGGAGGGCCCCCCGCCATTCCTTCTGGACA GCTTCACAA CGCGG GCTTA GG |
| clone 15 of SSR compound $(AC)_{10}$ | GGTTGGGCGGCAGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGT CGCATGCTCCCGGCCGTTGATGGGGGTCTTGGAATTCGATTACACACACACACAGA GAGATTGTCTTGTACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTGAGTCG TATTAGGATGGAATCACTAGTGAAATCGCGGCCGCCTGCAGGTCGACCATATGGGA GAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGC TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA |
| clone 16 of SSR compound $(AC)_{10}$ | GGGTCCGGGCAGTCATGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCG CATGCTCCCGGCCGTTGAGGTGGTCTTTGGAATTCGATTACACACACACACAGAGA GAGAGAGAGAGAGAGTACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTGA GTCGTATTAGGATGGAATCTCCCCTGAATTTCAAGGCCGCCTGCAGGTCGACCATA TGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTA AATAGCTTGGCGTAATCATGGTCATAGCTGGTCCCTGGAGAGAA |
| clone 1 of SSR compound $(TC)_6(AC)_5$ | TACGCATGATGTTCGATACTAGCGATGCGACGTGATGTCGAGTATGACTCGATCAT CTATCGACCATAGCGCCTTTTGATGTGATCAAATAGGCGTGAGGCCGGGGGAAAAA TTTATTGGGCCCCGCGCAGGCCCCAAGTTGATGTTTTATACTACCCCTAAGTTGGTA ATTGGTAGGTCCGGGAAC |
| clone 2 of SSR compound $(TC)_6(AC)_5$ | AAGTGAAGGCGGGGAGCTTCGGATTCGATCCATCCTAATACGAAAAAGGGGGTCG GCTGTGGTCGACTTGCCCGGGCTGGTACACATGTGTGGAAACATGTGTTCTATCTG TTGCTGTGTGAGTAAAGAGAAAGAGAGAGAGAGAGAGAGAGAGAGAGAGTGCG CGTGTGTGCGTGTGTTTGTGTGTTTTGTGTGTGTGTTTTGTGTTTTAGAAGAGATAA TTTCTTTTTTTTCGCCCCCCTACGCCACTTTTG |
| clone 4 of SSR compound $(TC)_6(AC)_5$ | GGTTCGCGGCGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCG CATGCTCCCGGCCGTTAAAGTAGCTCAAGAATTCGATTTCTCTCTCTCTCACACCAA AGCTCAAATAGAACACATGTTTCCACACATGTGTACCAGCCCGGGCCGTCGACCAC GCGTGCCCTATAGTGAGTCGTATTAGGATGGAATCACTAGTGAATTCGCGGCCGCC TGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATT CTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGA CAGATACTGATGCTATGCGGAGGTAGGGACTCTCAAATCGTCACAAGCACGGGCCT CTTATAGGGAGAGCCCGCGCAACGAAAGGGGAGCGCGGCAAGACGGGCGGTACA GATTGGGATGGGGGGGGGTATTCCTCCCCTCTAGTAAAATACACCACCCAATTCGCG GCTCATAGCACGAAGCAGCAAACCGCCATTCCCATTAGGATCAAGACCTTAATTGC CTCTGTTTAAAATAAACCCGGAAACCTTGGGGAAA |
| clone 14 of SSR compound $(TC)_6(AC)_5$ | GGTGGAAGGCGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCG CATGCTCCCGGCCGCTAGGGGGTCGCGGGAATTCGATTACCACACACACACGCAC CAAATGCAGAAGCTTGTGTTGGAGGAGAGGAGGAAATACAGGGAGAAGGGGAGAC AGAGCGTACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTGAGTCGTATTAG GATGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCT CCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGC GTAATCATGGTCATAGCTGTTTCCTGTGTGAAGCGCGAGGCTCTTTGGTCGGAGGG GTGTTTCCATTTGTTACGCGCTGCCACCCCCCCCCGAAAGAGGACAACCTAACAAC CACTATTGGGGCCCGTGTTTTGGGGGGGGGGGGGCTCCTCCCCCCCCCGTTTCACT GCCCAACGGCATTAATCACGCTCACCA |
| clone 10 of SSR compound $(AC)_6(AG)_5$ | GGCCCCTCCCGCCCATTCTGTGTGACCGACTCACTATAGGGCGATTGGGCCCGAC GTCGCATGCTCCCGGCGTGGTGGGTCTCTGGAATTCGATTCCATCCTAATACGACT TATCGGGTCGCGTGGTCGACGGCCCGGGCTGGTACCATACACATGTGTGGAAACA TGTGTTCTATCTTTTTTTCCTAACAAAAGAAAAAGAGAGAGAGAAATCACTAGTGAATT CGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATA GCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGT TTCCTGTGTGAA |

Lian *et al.* (2006) stated that SSR marker was codominant markers with high polymorphism such as *Dendropanax trifidus* were easily developed by this technique. Because of requires sequencing only once in comparison with our previous technique. This approach substantially reduces time and cost in the development of co-dominant polymorphic markers. Another merit is that because a common fluorescent compound SSR primer can be used in polymorphism analyses for different loci and the fluorescent primer is rather expensive, this may further save investigation costs (Hayden *et al.*, 2004). Finally, when co-dominant polymorphic markers must be developed simultaneously for several species, a common fluorescent compound SSR primer could also be used in polymorphism analyses for these several different species. This technique is, in principle, applicable to other species and successful isolation of co-dominant compound SSR markers for several other plant species, by use of this method, is currently in progress.

The results indicated that the ISSR suppressor PCR methods enable to develop easily SSR marker from *G. mangostana* genome. The ISSR suppressor-PCR methods successful for *Salix reinii, Pinus densiflora* and *Robinia pseudoacacia* (Lian *at al.*, 2001), *Tricholoma matsutake* (Lian *at al.*, 2003), *Brassica rapa* (Tamura *et al.*, 2005), *Karenia mikimotoi* (Nishitani *et al.,* 2009). The major advantage of this method is that library construction and screening can avoided.

## CONCLUSION

DNA library construction used restriction enzyme blunt end Rsa I and primer designed by using SSR compound $(AC)_{10}$; $(TC)_6(AC)_5$ or $(AC)_6(AG)_5$ and an adaptor primer AP2 and nested PCR using AP1 primers. Eight sequence from sample # G17 with SSR compound $(AC)_{10}$, $(TC)_6(AG)_5$ and $(AC)_6(AG)_5$ produced two primer pairs. An inter-simple sequence repeat (ISSR) suppressor-PCR methods to develop SSR markers of *G. mangostana* L. The primer pairs from sequence positive clone 4 of SSR compound $(TC)_6(AC)_5$ were forward primer: 5'-GGCCGTTAAAGTAGCTCAAGAA-3' and reverse primer:5'-CCGCAT AGCATCAGTATCTGTC-3', while primer pairs from sequence positive clone 10 of SSR compound $(AC)_6(AG)_5$ were forward primer: 5'-GTGTTTCCATTTG TTACGCGCT-3' and reverse primer: 5'TAATGCCGTTGGGCAGTGA-3'. This primer pairs could contribute to analyze genetic diversity of *G. mangostana* L. or other species Garcinia.
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## ACKNOWLEDGEMENTS

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