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Full Length Research Paper

Characterization of specific random amplified polymorphic (RAPD) DNA fragments related to catechin content for early detection methods in gambier plant (*Uncaria gambir* (Hunter) Roxb.)

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The existence of a target DNA fragments associated with the potential high level of catechins in gambier plant was carried out preliminary using RAPD markers. Target fragment was ligated and transformed into *Escherichia coli* DH5α strains using pGem-T Easy vector. The purpose of this study was to obtain information of specific DNA fragments and to generate specific primer combinations associated with potentially high content of catechins. DNA sequence was successfully generated with 206 nucleotides in length. The combination of specific primers, designated as Udtg3 (5'- CAGATCCTTTGCCACACTGA-3') and Udtg4 (5'- CGTACCTGCCAA CATA ACAG - 3') were successfully designed and could be applied as a diagnostic marker in detection of catechin content production of gambier plant.

Key words: Gambier, RAPD, specific primers, MAS, catechin.

INTRODUCTION

Gambier plants (*Uncaria gambier* (Hunter) Roxb.) is one of the export of plantation commodities possesing high economic value and this is based on their commercial role. The gambier resin contains mainly catechins that is necessary in the pharmaceutical industries, cosmetics, paint, and others. Gambier plant in Indonesia is largely dispersed and cultivated in West Sumatra; so-called specific plants of West Sumatera.

Fauza (2009) described four genotypes of gambier mainly found in West Sumatera, namely: Udang, Cubadak, Riau Gadang dan Riau Mancik. Among this genotypes, Udang is the most productive genotype than the other three based on their sap and catechin content yield (Hasan et al., 2000). This was further proved by Ferita et al. (2009) that they showed range productivity of 14-45%; 3-33%; 9-27% and 9-17% for each Udang, Riau Mancik, Ria Gadang and Cubadak respectively.

Developing a superior cultivars containing high level of catechin , is one of gambir breeding program goals. But such breeding goal was hindered by the unavailability of method that can be used for early detection of catechin contents in the seedlings stage. For that reason, it is necessary to identify the genetic characters associated with high catechin level and use them as a marker. The success of the identification and characterization of such marker could speed up the breeding program of this crop.

Molecular techniques such as random amplified polymorphic DNA (RAPD) can be used to support plant breeding activities, such as identification of specific characters through fingerprinting on gambier. RAPD is one of a DNA fingerprinting technique based on polymerase chain reaction (PCR) technology that can multiply the amount of DNA millions of times from its original amount. The technique has several advantages over hybridization methods, one of which is not necessary to use radioactive materials as used in the process of hybridization, rapid analysis systems, high

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sensitivity, and can identify the organism in small quantities (Babaloka, 2003; Jamsari et al, 2004; Lee et al., 1997; Lopez et al., 2003; Pastrik and Rainey, 1999). Unfortunately, RAPD technique has several drawbacks, for instance this technique was believed to be very sensitive to reaction conditions bringing the consequencies to less consistent results (Yu and Pauls, 1992). Many factors affect the volatility and sensitivity of RAPD markers for example ratio of template DNA and primers, concentration of Mg ions and Taq-polymerase type and even the type of used PCR machine, as well as the low temperature and short RAPD primer that commonly iis used in the procedure (Yang et al., 1996). Based on those arguments, many RAPD based molecular markers were converted into other marker system, for instance sequence tagged sites (STS). STS marker system is a marker system based on DNA sequence information of target region. Primers can be developed from specific region associated with some important agronomical traits.

The purpose of this study was to identifiy DNA specific fragments genetically associated with the potency of catechins production level and further develop specific primer combinations that can be used for early detection system during positive selection of gambier plant containing high level of catechin production.

MATERIALS AND METHODS

Genotype of Udang was used in this study. They were arranged into two groups, which were bulked into high and low catechin content. Each group consisted of five individual plant. The high group was characterized by ≥ 15% of catechin content, while the low group was characterized by < 15% of catechin content.

Molecular experiments such as: DNA isolation, PCR amplification, and cloning of DNA fragments were performed at the Laboratory of Biotechnology and Plant Breeding Faculty of Agriculture, University of Andalas Padang while the sequencing of specific DNA fragments was conducted at the Institute of Biology Molecular Eijkmann Jakarta. Plant materials used in this research was Udang type based on their morphological cahracter. Sampels were collected from gambier collection facility at Faculty of Agriculture-Andalas University.

Plant material and RAPD-based differential fingerprinting

Differential fingerprinting experiment was performed to visualize any polymorphism character between low and high catechin content of gambir collection. DNA isolation based on CTAB protocol as described by Doyle and Doyle (1990) was applied prior to differential screening. RAPD based marker system was applied in pool of low and high catechin content gambir fingerprinting. Each pool contained DNA from five individual plants. Pool was composed based on their catechin content for instance the low pool content of plants having of ≤ 15% of catechin content, and the high pool content plants having of > 15% of catechin content. 98 RAPD-primers were used in the differential screening (Table 1). All PCR reactions were performed using a T-Professional termocycler (Biometra-Germany), using standard PCR condition cycled for 45 times of 5 min, 95°C denaturation, 2 min of 38°C annealing and 5 min of 72°C extension. PCR product was checked by electrophoresis technique on 1.5% agarose. Primers successfully showing polymorphism between low and high pools were used for individual fingerprinting analysis. Similar principle was performed during individual differential screening of DNA composing DNA pool. Only fragments showing stabile performance were choosen for further analysis.

Cloning and sequencing of specific RAPD fragments linked to catechin level

Specific RAPD fragments from individual screening were further used for the cloning step. Fragments were cut from the gel using sterile scalpel and further purified using Wizard-Gel Purification Kit (Promega-USA). Purified fragments were subsequently proceeded for cloning step. Ligation condition and components were applied as recommended by the kit producer. Genetic transformation of recombinant plasmid into *Escherichia coli* strain DH5α were done using heat-shock technique as described by Sambrook and Russell (2001). Transformation suspension were plated on Luria Bertani selective medium containing IPTG and X-Gal to facilitate blue-white selection. Transformant were checked via mini preparation of plasmid DNA as described by Birnboim and Doly, (1979). Successful isolated DNA was further analyzed using PCR technique by applying T7 and SP6 primers. RTG-PCR bead (LG-healt care-UK) was used as PCR cocktail. Amplification reaction was done using the condition as follow: 5 µl of amplification product was controlled by electrophoresis technique on 1% agarose gel. Positive product was subjected directly for sequencing using both primers. Sequencing was done at the Institute of Molecular Biology Eijkmann Jakarta, following polymerase chain termination technique as described by Sanger et al. (1977). Sequence data was edited and subjected for BLAST (http://www.ncbi.nlm.nih. gov / BLAST) analysis for homology search.

Primer design and marker evaluation

Primer design was done by using the Primer3 software accessible on line at http://frodo.wi.mit.edu/primer3.2010. Synthesis of the primer was done at one-base (first-base-Singapore). The designed primers were checked for their optimum temperature using T-Gradient professional PCR machine (Biometra-Germany). Successful primer were tested against their original genome. Only primer pairs capable of producing clear and single fragment were taken for further evaluation. Primer evaluation was aimed for their accuracy among different genetic background of genotypes. Accuracy level was measuerd using the formula:

$$\text{Accuracy (\%)} = \frac{\text{Number of samples with expected product}}{\text{Number of tested samples}} \times 100\%$$

RESULTS

Isolation of DNA

Prior to differential screening of polymorphism between low and high catechin content in the pool step, genomic DNA isolation was performed. DNA generated from fresh youg leaf of each sample showed good condition in quantity as well as in quality (Figure 1).

RAPD-based differential screening

In order to speed up selection step, RAPD-based

Table 1. RAPD primers and sequences used in selection bulk (high and low pool) contents of catechin Udang type.

Number	Primer name	Sequence	Fragment of high catechin	Fragment of low catechin	Polymorphism
1	OPA-02	TGC CGA CCT G	6	6	0
2	OPA-04	AAT CGG GCT G	0	0	0
3	OPA-05	AGG GGT CTT G	0	0	0
4	OPA-06	GGT CCC TGA C	0	0	0
5	OPA-09	GGG TAACGC C	6	6	0
6	OPA-11	CAA TCG CCG T	4	4	0
7	OPA-13	CAG CAC CCA C	6	6	0
8	OPA-15	TTC CGA ACC C	2	2	0
9	OPA-19	CAA ACG TCG G	5	5	0
10	OPB-01	GTT TCG CTC C	6	6	0
11	OPB-06	TGC TCT GCC C	4	4	0
12	OPB-09	TGG GGG ACT C	2	2	0
13	**OPB-11**	**GTA GAC CCG T**	**4**	**3**	**1**
14	OPB-14	TCC GCT CTG G	1	1	0
15	OPB-15	GGA GGG TGT T	0	0	0
16	OPB-17	AGG GAA CGA G	5	5	0
17	OPC-02	GTG AGG CGT C	8	8	0
18	OPC-08	TGG ACC CGT C	3	3	0
19	OPC-14	TGC GTG CTT G	0	0	0
20	OPC-15	GAC GGA TCA G	1	1	0
21	OPC-16	CAC ACT CCA G	0	0	0
22	OPD-04	TCT GGT GAG G	0	0	0
23	OPD-05	TGA GCG GAC A	1	1	0
24	OPD-06	ACC TGA ACG G	0	0	0
25	OPE-03	CCA GAT GCA C	2	2	0
26	OPE-04	GTG ACA TGC C	0	0	0
27	OPE-05	TCA GGG AGG T	3	3	0
28	OPE-08	TCA CCA CGG T	3	3	0
29	OPE-14	TGC GGC TGA G	6	6	0
30	OPE-18	GGA CTG CAG A	5	5	0
31	OPF-05	CCG AAT TCC C	0	0	0
32	OPF-08	GGG ATA TCG G	5	5	0
33	OPF-12	ACG GTA CCA G	0	0	0
34	OPF-13	GGC TGC AGA A	0	4	4
35	OPF-14	TGC TGC AGG T	0	0	0
36	OPG-04	AGC GTG TCT G	0	0	0
37	OPG-05	CTG AGA CGG A	4	4	0
38	OPG-06	GTG CCT AAC C	1	1	0
39	OPH-03	AGA CGT CCA T	2	2	0
40	OPH-04	GGA AGT CGC C	1	1	0
41	OPH-05	AGT CGT CCC C	2	2	0
42	**OPI-01**	**ACC TGG ACA C**	**2**	**1**	**1**
43	OPI-02	GGA GGA GAG G	0	0	0
44	OPI-03	CAG AAG CCC A	0	0	0
45	OPJ-04	CCG AAC ACG G	0	0	0
46	OPJ-05	CTC CAT GGG G	0	0	0
47	OPJ-06	TCG TTC CGC A	3	3	0
48	OPK-04	CCG CCC AAA C	5	5	0
49	OPK-06	CAC CTT TCC C	5	5	0
50	OPK-07	AGC GAG CAA G	3	3	0
51	OPK-12	TGG CCC TCA C	4	4	0
52	OPK-13	GGT TGT ACC C	5	5	0
53	**OPK-15**	**CTC CTG CCA A**	**6**	**5**	**1**
54	OPL-09	TGC GAC AGT C	0	0	0
55	OPL-10	TGG GAG ATG G	5	5	0
56	OPL-11	ACG ATG AGC C	5	5	0
57	OPL-13	ACC GCC TGC T	5	5	0
58	OPM-04	GGC GGT TGT C	0	0	0
59	OPM-05	GGG AAC GTG T	0	0	0
60	OPM-06	CTG GGC AAC T	3	3	0

Table 1. Contd.

61	OPN-06	GAG ACG CAC A	5	5	0
62	OPN-10	ACA ACT GGG G	0	0	0
63	OPN-11	TCG CCG CAA A	1	2	1
64	OPN-12	CAC AGA CAC C	3	3	0
65	OPN-14	TCG TGC GGG T	3	3	0
66	OPN-15	CAG CGA CTG T	3	3	0
67	**OPN-16**	**AAG CGA CCT G**	**8**	**4**	**4**
68	**OPN-19**	**GTC CGT ACT G**	**7**	**4**	**3**
69	OPO-04	AAG TCC GCT C	5	5	0
70	OPO-05	CCC AGT CAC T	1	1	0
71	OPO-06	CCA CGG GAA G	2	2	0
72	OPP-04	GTG TCT CAG G	0	0	0
73	OPP-06	GTG GGC TGA C	4	4	0
74	OPQ-11	TCT CCG CAA C	0	0	0
75	OPQ-12	AGT AGG GCA C	1	1	0
76	OPR-11	GTA GCC GTC T	3	3	0
77	OPS-03	CAG AGG TCC C	4	4	0
78	OPS-18	CTG GCG AAC T	8	8	0
79	OPU-08	GGC GAA GGT T	4	4	0
80	OPW-01	CTC AGT GTC G	7	7	0
81	OPW-02	CAT CGC CGC A	5	5	0
82	OPW-03	GTC CGG AGT G	6	6	0
83	OPW-11	CTG ATG CGT G	6	6	0
84	OPW-14	GGT CGA TCT C	5	6	1
85	OPW-19	CAA AGC GCT C	4	6	2
86	OPX-01	CTG GGC ACG A	6	6	0
87	OPX-04	CTG GGC ACG A	6	6	0
88	OPX-07	GAG CGA GGC T	7	7	0
89	**OPX-09**	**GGT CTG GTT G**	**4**	**0**	**4**
90	OPX-15	CAG ACA AGC C	7	7	0
91	OPX-19	TGG CAA GGC A	5	5	0
92	OPY-04	GGC TGC AAT G	7	7	0
93	OPY-08	AGG CAG AGC A	6	6	0
94	OPY-09	AGC AGC GCA C	5	8	0
95	OPY-10	TCG CAT CCC T	4	4	0
96	OPY-16	GGG CCA CTG T	8	8	0
97	OPY-20	AGC CGT GGA A	7	7	0
98	OPZ-13	GAC TAA GCC C	0	0	0

fingerprinting differentiation was combined with bulked segregant analysis (Michelmore, et al. 1991). A total of 98 operon RAPD primers (Almaeda, USA) were used in the first step differential screening in the pool level. Results of the screening is shown in Table 1. There were 629 total fragments generated and six fragments on average could be generated from each primer. However, some primer even could not produce any fragment. Six out of 98 primers, OPB-11, OPI-02, OPK-15, OPN-16, OPN-19, and OPX-09 produced polymorphism fragment between low and high catechin content pool (Figure 2A). Figure 2B is an example of polymorphism shown by primer OPK-15. Theses primers were further proceeded to individual differential screening level using individual plant DNA composing the pool. However, only one primer OPK-15 could consistently differentiate between high and low catechin producing plants in both level screening. This was indicated by a single 700 bp fragment (Figure 3). The other five primers were then discharged from further analysis.

Sequence characteristic of RAPD specific fragment linked to catechin production

Specific RAPD fragmentt putatively linked to the cateching production was subjected to the subsequent analysis. Cloning procedures of specific fragments into pGemT-Easy vector (Promega-USA) was performed mainly as described by manufacturers recommendation. Transformation suspension was plated onto LB selective medium containing IPTG, X-Gal and supplemented with ampicilin. Selection of transformants were facilitated by expression of Lac-Z gene allowing blue-white selection procedure.

In further step, recombinant colonies were subjected in

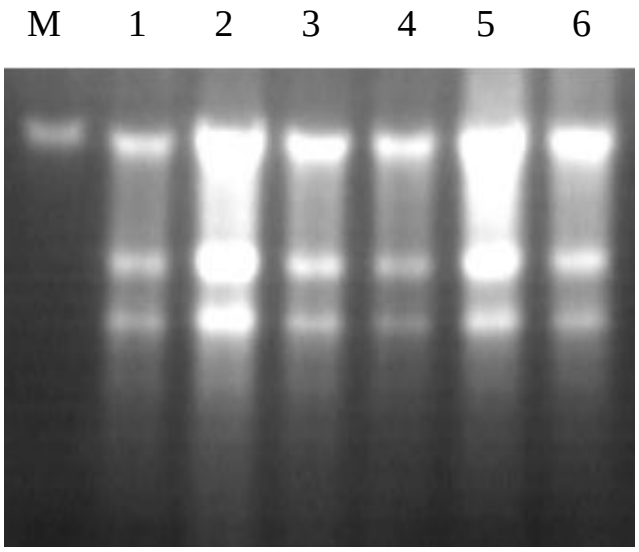

Figure 1. Electrophoresis performance of DNA from 6 *Uncaria gambir* plants. M = λ DNA (50 ng/µl), U1-U6 are samples.

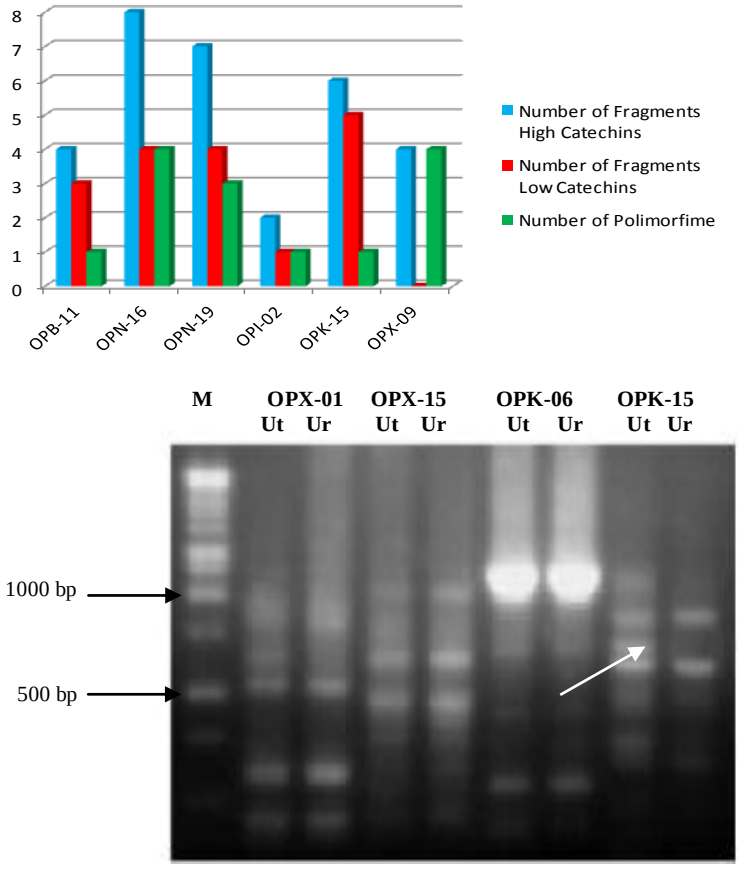

Figure 2. A. Diagram appearance of fragments amplified by RAPD primers showing polymorphism between DNA pool with high catechin and low catechin DNA gambier. **B.** The results of PCR amplification with primer OPX-01, OPX-15, OPK-06 and OPK-15 on a sample of Udang high catechin (Ut) and Udang low catechin (Ur). M = 1kb ladder. The arrow is showing polymorphism.

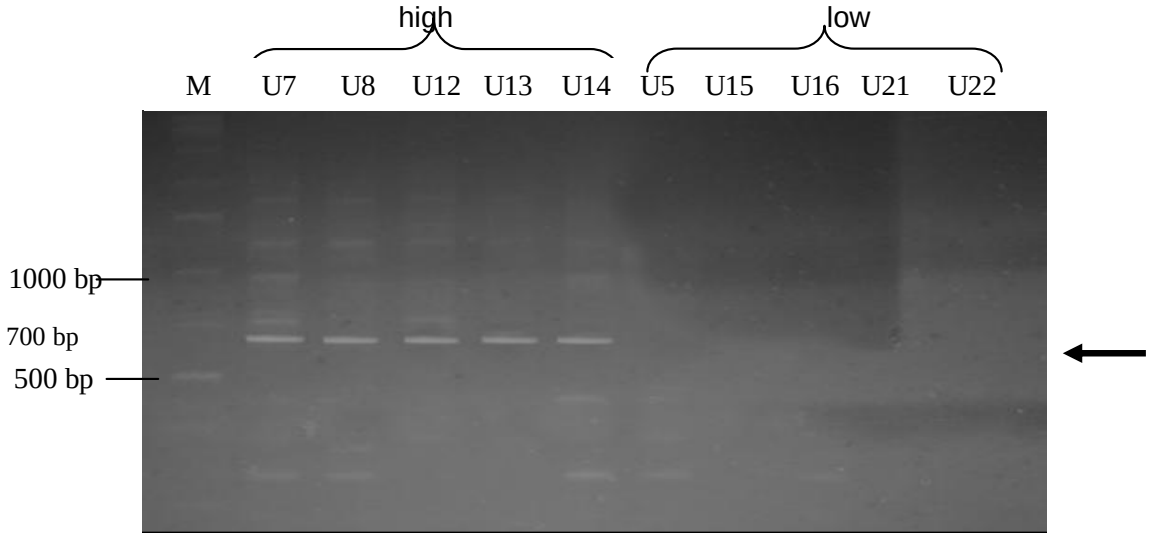

Figure 3. The results of PCR amplification with OPK-15 primer, with sample: U7, U8, U12, U13, U14, (Udang high catechins), and U5, U15, U16, U21, U22 (Udang low catechin). M = 1 kb ladder (Fermentas, USA). The arrows show the position of the fragment polymorphisms.

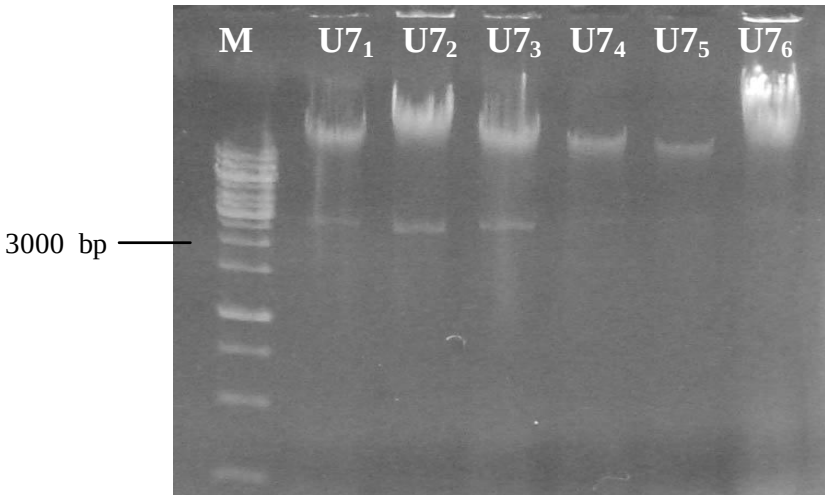

Figure 4. The results of plasmid DNA isolation. M = 1 kb ladder marker (Fermentas, USA); U7$_1$ - U7$_6$, sample of Udang 7.

plasmid DNA isolation. Six white colonies were used as sample for DNA isolation, and all of the samples seemed contained insert (Figure 4). Proving insert availability, PCR based analysis was performed. This was done by using T7 and SP6 primer combination (Figure 5).

Sequencing of specific RAPD fragments linked to catechin content

In order to unravel nucleotide composition of specific RAPD fragments, five samples U7, U8, U12, U13, and U14 were subjected for sequencing. After trimming and editing umbiguous nucleotide of five sequence data, 349 bp in average of nucleotide sequence was produced. They ranged from 314 to 445 bp. Plasmid sequence was eliminated by means of Vecscreen software available at NCBI website (http://www.ncbi.nlm.nih.gov/ VecScreen/). After performing this step, we could determine the inserted sequence ranging from 206 to 294 bp in length.

Marker development linked to catechin level content

Designing of specific primer was performed using Primer3 software available at http://frodo.wi.mit.edu/primer3/. Based on availability of sequence data, five primer pairs were designed.

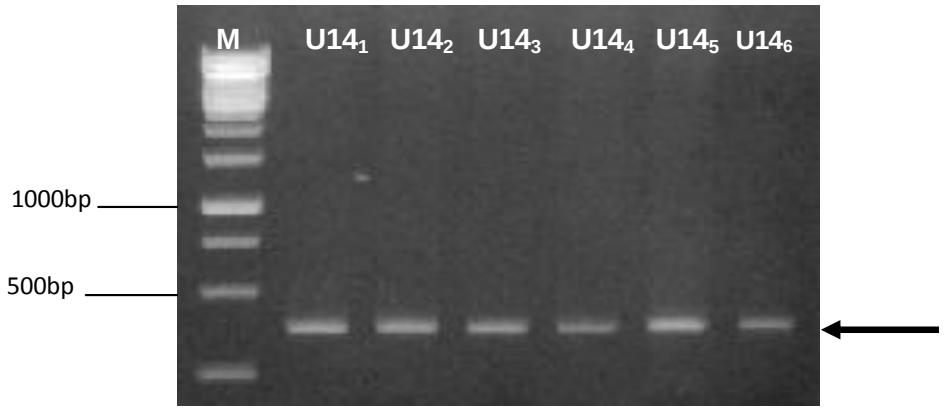

Figure 5. The results of PCR amplification with T7 and SP6 primer. M = 1 kb ladder marker-Fermentas, USA); U14$_1$ -U14$_6$, sample of Udang 14. Arrows are fragments of the insert.

Table 2. Primer sequences designed with the Primer3 program.

Name	Sequence	Size prediction
Udtg3	CAGATCCTTTGCCACACTGA	178 bp
Udtg4	CCTGCCAACATAACAGCGTA	

Synthesis of the primer sequence was ordered via first base (Firstbase-Singapore). However, after testing all the designed primer, only one primer pair which was named Udtg3 and Udtg4 (Table 2). This primer pair was generated from U14. After testing these primer pair via PCR-based technique, we could observe a single fragment of about 178 bp in lenght from all five samples identified as high-content catechin producing plant group (Figure 6).

In order to investigate the accuracy of the primer, we further tested the primer in more wide genetic background. For this purpose, we used 24 plants after previously identifying their catechin level production. There were ten samples positive fragments as expected product, seven samples from high contents of catechins, three samples from low contents of catechins, and 14 samples of negative fragments.

$$\text{Accuracy (\%)} = \frac{\text{Number of samples with expected product}}{\text{Number of tested samples}} \times 100\%$$

$$\text{Accuracy (\%)} = \frac{[7\ (\text{high catechin}) + 3\ (\text{low catechin})]}{24} \times 100\%$$

Accuracy (%) = 41.7%

DISCUSSION

Gambier extract contains several important components which are useful for health and pharmaceutical process. In general, the extract of gambier contain catechin (1 to 33%), Tannates catecu acid (20 to 55%), pirocatecol (20 to 30%), gambier flaoresenci (1 to 2%), red catecu (3 to 5%), quercetin (2 to 4%), fixed oil (1 to 2%), wax (1 to 2%), and small amounts of alcohol (Nazir, 2000). Among those, catechin is one of the most important substances, since it is more commonly used for many industrial activity. Based on this reason, more study on catechin and its production aspect will be more interesting in the future. However, study on the molecular aspect as we performed in this study is scarce. That is why this our preliminary results will be very valuable for Gambier role for industrial side.

At least microgram of DNA could be produced from each sample. CTAB method in this case seemed to be suitable for genomic DNA isolation of *Uncaria gambir*. This result shows the suitability of CTAB method in *Uncaria gambir* species DNA isolation. This is surprising, since Uncaria gambir contain polyphenolic compounds in high amount. Normally, DNA isolation from tissue containing polyphenol compunds in high amount is tedious and needs more effort to dissolve the protein (Smalla et al., 1993). However, only CTAB-based protocol could produce genomic DNA in high quantity and quality. One of the reason in such case, could be lying on the type of tissue used for DNA isolation. Fresh young leaf tissue had soft and releasable cells, making lysis procedure more effective. Combined with high incubation temperature (65°C), lysis activity could be effectively improved (Bruce et al., 1992). Average percentage of white colony was 88.1% and blue colony was 0.7%.

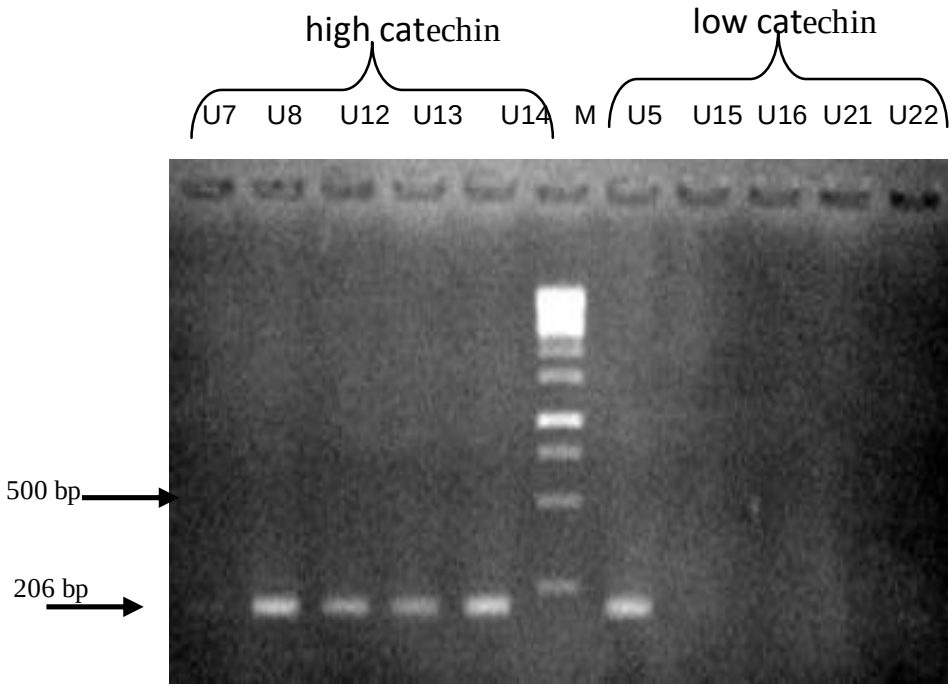

Figure 6. The result of PCR product in testing of primer pair Udtg3 and Udtg4. U7, U8, U12, U13, U14 are samples from high catechin content, while U5, U15, U16, U21, U22 belong to low level catechin content group. M = 1 kb marker (Fermentas-USA).

Transformation eficiency which reached 88.1% observed in this experiment is relatively high compared to the results published by other authors elsewhere (Jamsari, 2008).

The presence of white colonies indicated that the Lac-Z gene expression which change the X-Gal compounds became blue and was no longer able to perform its function, since specific RAPD fragment has interrupted the integrity of Lac-Z gene. Lac-Z gene encodesa β-galactosidase enzyme that catalyzes the breakdown of lactose (Xgal) into glucose and galactose.

After PCR step by T7 and SP6 primer, it could be concluded that 59.6% of the colonies contained positive insert, and only 40.4% did not contain insert. This result shows, that transformation eficiency in this experiment was higher than what was obtained by Jamsari (2008) for similar work with *Colletotrichum capsici* and *Colletotrichum gleosporides*. They got tranformation eficiency of 45 and 47.6% for both Colletotrichum species respectively.

All six samples used for PCR analysis produced approximately a 400 bp single fragment. However, in fact not all six samples were truly white, some of the colonies showed light blue colour, indicating that *Lac-Z* gene expression could still take place. This is actually commonly observed, when the inserted fragment is relatively short. Counting of the 400 bp of PCR product generated from T7 and SP6 primer, showed that the RAPD specific fragment that was successfully inserted to the pGem T Easy vector could be only about 250 bp. This is calculated by reducing PCR product with plasmid fragment flanked by T7 and SP6 primer.

However, comparison between cloned RAPD fragments with PCR product generated from T7 and SP6 primer pair showed inconsistencies. The cloned RAPD fragments was about 700 bp, meanwhile PCR product obtained was only about 400 bp. This results indicate that RAPD fragments cloned in this experiments were not single product. By comparison, short fragment in this case (about 250 bp) is more easier than the longer fragment (about 700 bp). The inconsistencies results is regarded as one of the drawback of the RAPD marker system as described by Yu and Pauls (1992). Furthermore, its sensitivity was affected by many factors for instance ratio of template DNA and primers, concentration of Mg^{2+} ions and type of Taq-polymerase used and also the type of PCR maschine used in the experiment (Meunier and Grimont, 1993; Rajput, et al., 2006; Jamsari, 2008). For this reason, RAPD marker system was preferentially converted to other marker system such as STS, CAPS or SCARs (Jamsari, 2004).

BLAST analysis of the five sequence data exhibited no sigificant hit, indicating no sequence of *Uncaria sp*. was available currently. Sequence allignment of those five nucleotide sequence showed differences in the sequence. Substitution, and insertion-deletion (indel) events could be observed among them. Indels events were observed in 16 position, while substitution was

observed involving 1 to 3 bases (data not shown).

The length of PCR product was in accordance with our expectation. However, applying the primer pair againts five low-content catechin producing plant group still produced an unexpected result since one among them (U15) which belonged to thelow level group produced similar fragment (Figure 6).

The percentage of Udtg3/Udtg4 primer accuracy was still low at 41.7% in detected gambier plant for high contents of catechins potential. The low accuracy of the primer, which is determined by high proportion of recombinants indicated that genetically primer loci of Udtg3/Udtg4 is located far enough from the gene of interest. Besides, low accuracy of primer was developed in this study, probably due that catechins content is quantitatively controlled by many genes. In this manner, every single gene contributes only small part in the catechin production. It could be possible that among the genes involved in the catechin production, some of them is/are major gene(s) located anywhere in the chromosome. However, until now, genomic study of *Uncaria* species is very rare.

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