RANDOM AMPLIFIED POLYMORPHIC DNA - POLYMERASE CHAIN REACTION (RAPD - PCR) ANALYSIS OF *Blumea balsamifera* DC

Arthur Almanzo J. Bangguan

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ARTHUR ALMANZO J. BANGGUAN

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Arthur Almanzo J. Bangguan

Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak,
94300 Kota Samarahan
Sarawak

ABSTRACT

*Blumea balsamifera* (Linn.) DC. or ngai camphor is a plant herb that belongs to the family Compositae/Asteraceae. This plant has been frequently prescribed by traditional medical practitioners for remedies of various ailments such as hypertension, diuretic, fever, insomnia and post-parturition aids to mothers. There is a need for establishing genetic markers for future construction of genetic maps of this plant species. The RAPD technique has been chosen for this study as it requires only a simple experimental set-up. The RAPD technique however requires DNA of suitable purity for the enzymatic PCR. Three DNA extraction methods have been tested in this study; (1) the simple extraction method by Thomson and Henry (1995) and Henry (1997), and (2) combination of Thomson and Henry (1995) and Henry (1997) DNA extraction method and Doyle and Doyle (1990) extraction method, and (3) a slightly modified protocol of Doyle and Doyle (1990). The CTAB method by Doyle and Doyle (1990) has consistently yielded DNA that was comparatively better in term of quantity and quality. The DNA recovery with concentration and purity value range between 0.072μg/μl to 0.455μg/μl and 1.663nm to 2.036nm respectively was obtained using this procedure.

In the RAPD-PCR analysis, two 10 base universal primer, namely OPD-OI (5’- ACCGCGAAGG -3’) and OPD-15 (5’- CATCCGTGCT -3’) were tested. However, a negative result is obtained for both of the primers tested, whereby, no bands were observed on the agarose gel. Further studies on both extraction protocol and RAPD-PCR should be taken in order to establish a rapid extraction method and genetic markers for this *B. Balsamifera*.

Key words: *Blumea balsamifera*, DNA extraction, RAPD, genetic markers

ABSTRAK


Kata kunci: *Blumea balsamifera*, pengekstrakan DNA, RAPD, penanda-penanda genetik
1. INTRODUCTION

*Blumea balsamifera* (Linn.) DC. or *ngai* camphor, as it is best known common name by many, is a plant herb that constantly regards by traditional medical practitioners, especially in Asian region as a very useful medicinal plant. Some previous studies have recorded that this plant often prescribed by traditional medical practitioners across Asian region for treating several diseases (Ahmad, 2003; Padua et al., 1999). In Malaysia, it is also known in a variety of names such as Sembong, Sambong, Capa, Telinga kerbau, Sapu, Daun Sembung (Malay); Susuah Mambong, Susuoh, Urok Bung (Sarawak native); Tawawoh and Embong (Sabah native).

This plant was reported to be effective as the remedy for hypertension, diuretic, fever, insomnia and post-parturition aids to mothers, that it has been widely from India, Myanmar, South China, Taiwan to Thailand, Malaysia, Indonesia and the Philippines (Ahmad, 2003; Padua et al., 1999). Apparently, previous researches have revealed that *Blumea balsamifera* DC. do contains some essential oils that are eminent to occur in most of the antiseptic, carminative, spasmolytic and expectorant herb.

DNA extraction is an important matter especially for this *ngai* camphor plant as specific DNA extraction protocol has not been established. Current literature, as indicated by the growing number of DNA isolation protocols for specific plant species, has suggests that extraction of DNA is not always simple or routine, and that published protocols are not necessarily reproducible for all species (Weir et al., 1996;
Khanuja et al., 1999). Weir et al. (1996) has also mentioned that some of the published protocol is not really suitable for extracting DNA from herbaceous plant. Therefore, three DNA extraction methods for this plant have been studied, namely, the simple extraction method (Thomson and Henry, 1995; Henry, 1997), the combination of Thomson and Henry (1995) and Henry (1997) extraction method and Doyle and Doyle (1990) CTAB extraction method, and a slightly modified CTAB extraction method (Doyle and Doyle, 1990).
2. OBJECTIVES

This study is carried out to establish a rapid RAPD-PCR protocol for this plant species and to determine polymorphisms that may exist within this plant species. The finding can be used to facilitate in establishing molecular genetic markers of this plant species in the future. In addition, this study was also done to determine the best possible DNA extraction method that would yield DNA from the ngai champor leaves suitable for randomly amplified polymorphic DNA (RAPD) analysis.
3. LITERATURE REVIEW

3.1. *Blumea balsamifera* (Linn.) DC

This plant (Figure 1), of Compositae/Asteraceae family, is a course, erect, halfwoody, densely yet softly hairy and strongly aromatic herb which could grow between 1.5 to 4 meters in height, and can be found natively from India to South China and South East Asia.

Padua *et al.* (1999), editors of a book entitled "*Medicinal and Poisonous Plant*" had stated that the stem of this plant could grow up to 2.5 centimeters in diameter. Where as, the leaves are simple, alternate, elliptic-lanceolate, 7 to 20 centimeters long, toothed at the margins, pointed blunt at the tip, and narrowed to the short petiole, which is often auricled or appendaged. The flowering heads are stalked, yellow, numerous 6 to 7 millimeters long, and borne on branches of a large terminal, spreading or pyramidal, leafy panicle. The involucral bracts are green, narrow, and hairy. The achenes are 10-ribbed and silky. A brief photo of *Blumea Balsamifera* plant is given in the following page (Figure 1).

This plant, which can be found commonly on the roadsides and open grasslands at low and medium altitudes, contains L-borneol, camphor, cineol, limonene, sesquiterpene, alcohol and phenol phloracetophenon-dimethyl ether (Anonymous, 2004), which are eminent to occur in most of the antiseptic, carminative, spasmylytic and expectorant herb.
Figure 1: *Blumea balsamifera* (L.) DC.

(INSERT) Illustration of *Blumea balsamifera* (L.) DC.
3.2. RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction)

RAPD-PCR is a rapid, sensitive technique that usually uses ten-base synthetic deoxyribonucleotides of random sequence as primers in PCR (William et al., 1990; Welsh et al., 1991). The DNA amplification product will be generated from the region that is flanked by a part of 10-bp priming sites in the appropriate orientation (Chawla, 2002). Genomic DNA from two different individuals often produces different amplification patterns. Williams et al. (1990) showed that the differences as polymorphisms in the pattern of bands amplified from genetically distinct individuals behaved as Mendelian genetic markers. A particular fragment generated for one individual but not for other represents DNA polymorphism and thus can be used as a genetic marker.

The RAPD method can be performed without prior knowledge of that particular species genomic sequence and can be used for constructing linkage maps, tagging genes of interest, determining parentage and detecting genetic variation (Wang et al., 2004). For instance, RAPD-PCR has been used previously to establish genetic linkage maps (William et al., 1990; Chawla, 2002), identify markers linked to a novel powdery mildew resistance gene (ol-2) in tomato (Giovanni et al., 2004), generate molecular markers for Metroxylon sagu (Salleh et al., 2001), study the phylogenetic relationships between species in Allium section Schoenoprasum and for the investigation of the intraspecific differentiation of A. schoenoprasum (Friesen and Blattner, 2000).
RAPD amplification is performed in condition resembling those of PCR, which it typically requires cycling among three temperatures, namely denaturation temperature, annealing temperature and extension temperature. It is usually sufficient to heat the reaction mixture at 94°C for 30 to 60 seconds. However, the initial denaturation step for the plant DNA should be at least 3 minutes for a complete DNA strand separation (Chawla, 2002). Insufficient denaturation is found to be the common problem leading to the failure of the PCR reaction (Chawla, 2002). The temperature for the annealing step will depend to some extent on base composition and the length of primers. A time in the range of 30 seconds to 1 minute is usually sufficient for the annealing step, although this again will depend on a variety of factors such as interference from secondary structure and primary concentration (Chawla, 2002). Whereas, the extension temperature depends on length and concentration of the target sequence (Chawla, 2002), although, in general, the 72 °C is used. The cycle is usually repeated 25 to 45 times. After cycling, an extended extension period of 5 to 10 minutes is included to ensure that all annealed templates are fully polymerized.

3.3. DNA Extraction Protocol

RAPD analysis requires DNA of suitable purity for the enzymatic PCR, and it is often difficult to separate DNA from naturally occurring plant cell contaminants. In particular, polysaccharides (Do and Adams, 1991), phenolic compounds (Newbury and Possingham, 1977) and other secondary metabolites (Khanuja et al., 1999) can form a complex with and become irreversibly bound to
nucleic acids during extraction (Varadarajan and Prakash, 1991), thus interfere with the enzymatic reactions (Khanuja et al., 1999). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of extracted DNA (Khanuja et al., 1999). Successful extraction of PCR-amplifiable DNA can lead to the establishment of a rapid RAPD-PCR procedure for this *ngai* champor plant.

Therefore, for the purpose of this study, three DNA extraction protocols were tested for the determination of the most suitable DNA extraction protocol for this *ngai* camphor plant, namely, the simple extraction method by Thomson and Henry (1995) and Henry (1997), the combination of Thomson and Henry (1995) and Henry (1997) DNA extraction method and Doyle and Doyle (1990) extraction method, and a slightly modified protocol of Doyle and Doyle (1990). The application of both simple extraction method described by Thomson and Henry (Henry, 2001) and Doyle and Doyle CTAB extraction method (Weir et al., 1996) have been reported to be able to consistently yielded PCR-amplifiable DNA. While the simple extraction method offers a reliable and simpler procedure (Henry, 2001), the CTAB extraction method, on the other hand, has been reported to work on various plant species, regardless of plant growing conditions or leaf age (Weir et al., 1996). In addition, Rogers as cited in Weir et al. (1996), has also demonstrated the superiority (reproducibility, purity and PCR-amplificability) of the latter extraction methods over those containing SDS or SDS and CTAB, for a number of plant and fungal species.
3.4. Estimation of DNA Quantity and Purity

DNA quantification can be done by spectrophotometric measurement of UV absorption at the wavelengths of 230, 260 and 280nm. Measures of DNA purity can be determined by the $A_{260}$:$A_{280}$ and $A_{260}$:$A_{230}$ ratios. These ratios provide indications of protein, and polyphenol and carbohydrate contamination, respectively (Weir et al., 1996). A good quality of DNA solution was indicated by the $A_{260}$:$A_{280}$ ratio of 1.8 to 2.0.

According to Sandhya and Arya (n.d.), the $A_{260}$ value provides a measure of concentration, which the value of 1 corresponds to 50μg/ml of double stranded DNA in a 1cm quartz cuvette. The unknown DNA concentration in μg/μl can then be calculated by the formula $A_{260} \times 50 \text{ mg/μl} \times 0.001\text{ μl/ml} \times \text{dilution factor}$. However, relatively large amounts of DNA are required to get accurate readings, for example, 500ng/ml give 0.01 $A_{260}$ units. Thus, this method can many times lead to misleading results if the DNA has RNA or protein contamination. For example, a DNA contaminated with 10% protein or 60% protein can give a $A_{260}$:$A_{280}$ of 1.98 and 1.81 respectively.

In this case, quantification of the DNA can be achieved by running the DNA samples on agarose gel stained with ethidium bromide. A rough estimate of DNA can be obtained by comparing band intensities of the DNA extract and the standards.
3.5. Agarose Gel Electrophoresis

Minigels are run with the purpose of checking the presence of nucleic acids following each extraction protocol or PCR amplification. Ethidium bromide is used to stain the nucleic acids so that the migration of these nucleic acids to different positions based on their molecular weight can be observed. The following table shows the appropriate agarose percentages for different DNA sizes (Weir et al., 1996).

<table>
<thead>
<tr>
<th>Range of Linear DNA (kb)</th>
<th>Agarose Percentages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 – 60</td>
<td>0.3</td>
</tr>
<tr>
<td>1 – 20</td>
<td>0.6</td>
</tr>
<tr>
<td>0.8 – 10</td>
<td>0.7</td>
</tr>
<tr>
<td>0.5 – 7</td>
<td>0.9</td>
</tr>
<tr>
<td>0.4 – 6</td>
<td>1.2</td>
</tr>
<tr>
<td>0.2 – 4</td>
<td>1.5</td>
</tr>
<tr>
<td>0.1 – 3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 1:  The appropriate agarose percentages for different DNA sizes (Weir et al., 1996)
4. MATERIALS AND METHODS

4.1. Plant Material

The plant materials used for the study were sampled from five different locality namely, Semenggok, BDC Stakan Jaya (Kuching), Pusat Latihan Staf UNIMAS, Kg Suba Buan (Bau) and Kg. Meranek (Kota Samarahan), at vicinity such as palm plantation estate, farm, orchard, road side and undeveloped field.

Leaf materials which included the blade and petiole were washed prior to storage and extraction to remove any source of foreign DNA such as fungi, bacteria and insects and insect eggs on the plant materials.

4.2. Plant DNA Extraction and Purification

The leaf materials later subjected to one of the three stated extraction procedures, referred to as the simple extraction method (Thomson and Henry, 1995; Henry, 1997), the combination of Thomson and Henry (1995) and Henry (1997) extraction method and Doyle and Doyle (1990) CTAB extraction method, and a slightly modified CTAB extraction method (Doyle and Doyle, 1990).

The first method was the simple extraction procedure for direct PCR previously described by Thomson and Henry (1995) and Henry (1997). The second method was also the simple extraction procedure by Thomson and Henry
(1995) and Henry (1997) but with the following modifications, which a further DNA purification steps such as the addition of CIA, Isopropanol and wash buffer were implemented to the procedure after the incubation of 10 minutes at 95°C. The third method is based on the protocol previously suggested by Doyle and Doyle (1990), with the following modification, i) The CIA volume ratio to the homogenized mixture was 1:1; ii) The isopropanol incubation was extended to overnight; and iii) All the incubation and centrifugation periods were increased into at least two folds than the previously described Doyle and Doyle CTAB method.

4.2.1. Buffer & Solution (Doyle and Doyle extraction method)

i) CIA (Chloroform Isoamyl Alcohol 24:1)

For the preparation of 250ml CIA, 240ml of chloroform was mixed with 10ml of isoamyl alcohol and stored at room temperature. The prepared CIA reagent must be kept in the dark, preferably using the aluminium foil, for long term storage.

ii) Wash buffer

10mM ammonium acetate (M.W=77.08g)

100% v/v ethanol

For the preparation of 1 liter wash buffer, 240ml of distilled deionized water was mixed with 760ml of pure ethanol. Then, 0.77g of ammonium
acetate was added and dissolved in the mixture. The wash buffer was stored at 4°C prior to use.

iii) Isopropanol

The aliquot is initially stored -20°C.

iv) 1M Tris HCl pH 8.0

Tris base (Biotech Grade) (M.W=121.14g)
Concentrated HCl

For the preparation of 1 liter Tris HCl, 121.1g of tris base was dissolved in 800ml of distilled deionized water. Then, the pH was adjusted to 8.0 with approximately 42ml of concentrated HCl. Later, distilled deionized water was added to make up the solution to 1 liter. The prepared reagent was autoclaved and then stored at room temperature prior to use.

v) 0.5M EDTA pH 8.0

Disodium ethylenediaminetetra-acetate.2H₂O (M.W.=372.2g)
NaOH

For the preparation of 1 liter EDTA, 186.1g of disodium ethylenediaminetetra-acetate.2H₂O was dissolved in 800ml of distilled deionized water. Then, the pH was adjusted to 8.0 by adding approximately 20g of NaOH pellets. Later, the volume was topped up to 1
liter with distilled deionized water, autoclaved and stored at room temperature prior to use.

vi) TE (Tris-EDTA) buffer pH 8.0

10mM Tris HCl pH 8.0
1mM EDTA pH 8.0

For the preparation of 500ml TE buffer, 5ml of 1M Tris HCl pH 8.0 was mixed with 1ml of 0.5M EDTA pH 8.0, and later topped up to 500ml, autoclaved and stored at room temperature.

vii) CTAB (Cetyl trimethyl ammonium bromide) extraction buffer

100mM Tris HCl pH 8.0 (M.W. = 121.14g)
20mM EDTA pH 8.0 (M.W. = 372.2g)
1.4M NaCl (M.W. = 58.44g)
2% CTAB
1% PVP (Polyvinylpyrrolidone)
0.2% β-mercaptoethanol

For the preparation of 1 liter CTAB extraction buffer, 100ml of 1M Tris HCl pH 8.0 was mixed with 40ml of 0.5M EDTA pH 8.0 in 600ml of distilled deionized water. Then, 81.82g of NaCl was added to the mixture. Later, the volume was topped up to 1 liter with distilled deionized water and autoclaved before stored at room temperature.
Immediately before used, 20g of CTAB and 10g of PVP were added to the mixture. Followed by the addition of 40µl (0.2%) of β-mercaptoethanol into 20ml of the extraction buffer.

Firstly, small pieces of plant tissues were ground in liquid nitrogen with a mortar and pestle in order to release the cellular constituents. Then, the ground tissue was homogenized with 1000µl of CTAB extraction buffer (preheat at 65°C) until the slurry is formed. The homogenized mixture was then incubated in water bath at 65°C for at least 30 minutes, followed by the addition of 1 volume of Chloroform-isoamyl alcohol (CIA). The mixture was mixed gently, centrifuged at 13,000 rpm for 10 minutes, and again extracted with 1 volume of CIA and centrifuged at 13,000 rpm for 10 minutes. The aqueous layer was taken and added with 2/3 volume of cold isopropanol (-20°C) and left for overnight incubation in order to precipitate the DNA. The DNA pellet can then be obtained by centrifugation (13,000 rpm for 5 minutes). Next, the DNA pellet was washed with 1ml of wash buffer, incubated at -20°C for at least 30 minutes, and again centrifuged (13,000 rpm for 5 minutes). The DNA pellet was then air-dried and dissolved in TE buffer. The mixture can be either stored at 4°C or used for RAPD-PCR amplification. The plant genomic DNA solution was checked using agarose gel electrophoresis method.
4.3. Estimation of DNA Quantity and Purity

The measurement of DNA quantity and purity was done using the UV spectrophotometer (Ultrospec 1100 Pro UV Visible Spectrophotometer) readily available in the Plant Molecular Biology Laboratory (UNIMAS).

4.4. RAPD-PCR Amplification

4.4.1. Reagent

i) 25mM MgCl₂ (Promega, Madison AV)
ii) *Tag* DNA polymerase buffer (10x) (Promega, Madison AV)
iii) *Tag* DNA polymerase (Promega, Madison AV)
iv) 10mM of dNTPs (Promega, Madison AV)
v) 25 pmol/ul Primer

The universal primers used for the analysis is the OPD (Operon Technologies – 0.5 OD units) set. The respective universal primers sequence is shown in the table below (Table 2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotides Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPD -01</td>
<td>5'- ACCGCGAAGG -3'</td>
</tr>
<tr>
<td>OPD -03</td>
<td>5'- GTCGCCGTCA -3'</td>
</tr>
<tr>
<td>OPD -06</td>
<td>5'- ACCTGAACCGG -3'</td>
</tr>
</tbody>
</table>
vi) Template DNA

vii) Sterile distilled water

Generation of RAPD markers was done essentially according to the procedure of Williams et al. (1990). RAPD reaction was performed in 25μl of reaction mixtures containing 0.5μl of 2.5mM MgCl₂, 2.5μl of PCR buffer 10x, 0.5μl of Taq DNA polymerase, 1μl of 10mM dNTPs, 1μl of universal primer and 2.5μl genomic DNA as the template DNA. Distilled water was added to make up the final volume to 25μl. A negative control containing all components except genomic DNA was included in each set of reactions to check for contamination.