Faculty of Resource Science and Technology

PCR - RFLP (POLYMERASE CHAIN REACTION - RESTRICTION FRAGMENTS LENGTH POLYMORPHISM) ANALYSIS IN *BLUMEA BALSAMIFERA* DC.

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Polymerase Chain Reaction – Restriction Fragments Length Polymorphisms (PCR-RFLP) Analysis in *Blumea balsamifera* DC.

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ABSTRACT

Blumea balsamifera DC is one of the medicinal plants in the *Compositae* family. It is used as traditional medicine for local people especially for afterbirth mother and fever. Scientific studies reported that the plant contains essential oils such as camphor, l-borneol, limonene and sesquiterpene. The study is to analyze the level of genetic variation that may occur within the species of *Blumea balsamifera* from different locations in Sarawak using PCR-RFLP analysis. The locations include Kuching, Bau and Kota Samarahan using the genetic molecular marker, PCR-RFLP. Furthermore, the study is also to select the best method for DNA extraction. Four DNA extraction methods have been used to extract DNA from *Blumea balsamifera*. Amongst the methods, Doyle & Doyle (1987) has the best yield with the purity in the range of 1.4 to 2.0 and amount of 50 to 500µg/ml. The PCR analysis was done using a set of 13 random oligonucleotides primers shows a negative result. Further investigation using different combination of primers need to be carried out in the future.

Key words: Blumea balsamifera, camphor, PCR-RFLP

ABSTRAK

Blumea balsamifera DC adalah merupakan salah satu tumbuhan ubatan yang tergolong dalam famili *Compositae*. Tumbuhan ini digunakan sebagai ubatan traditional bagi masyarakat tempatan terutamanya bagi rawatan untuk ibu selepas melahirkan anak dan demam. Kajian saintifik menunjukkan bahawa tumbuhan ini mengandungi bahan seperti camphor, l-borneol, limonene dan sesquiterpene. Kajian ini adalah bertujuan untuk menganalisis tahap variasi genetik *B. balsamifera* yang mungkin berlaku menggunakan kaedah PCR-RFLP. Sampel diperoleh dari daerah berbeza di Sarawak iaitu Kuching, Bau dan Kota Samarahan. Di samping itu, kajian ini bertujuan untuk menentukan kaedah pengekstrakan DNA yang terbaik. Empat kaedah mengekstrak DNA telah digunakan untuk mengekstrak tumbuhan ini. Antara kaedah-kaedah ini, kaedah Doyle & Doyle telah berjaya mendapatkan nilai ketulenan DNA dalam lingkungan 1.4 hingga 2.0 dan kepekatan DNA dalam lingkungan 50 hingga 500µg/ml. Analisis PCR yang dilakukan menggunakan 13 primer oligonukleotid secara rawak telah mendapat keputusan yang negatif. Kajian ini perlu diteruskan pada masa depan menggunakan gabungan primer berlainan.

Kata kunci: *Blumea balsamifera*, camphor, PCR-RFLP

CHAPTER 1

INTRODUCTION

Blumea balsamifera DC or locally known as capa, sembong, telinga kerbau or daun sembang is one of the plants from the family of *Compositae*. It is also known internationally as ngai camphor or Blumea camphor plant (Retrieved from www.akitalherbalmedicine.com, 2004). This plant commonly grows at low and medium altitudes and open places such as open grassland, the roadside or by the riverside. It reported to be found in various countries that include Philippines, India, Southern China, Malaysia and Moluccas (Retrieved from www.akitalherbalmedicine.com, 2004).

Figure 1: *Blumea balsamifera DC* (Retrieved from Philippine Medicinal Plants website, n.d)

Blumea balsamifera is a course, tall, erect and half-woody and strongly aromatic herb that is densely and softly hairy (Figure 1). The plant possesses strong camphoraceous odour and a pungent taste. A mature *B. balsamifera* can grow in the range of 1.8 metres to 3.6 metres. The

stems can grow up to 2.5 centimetres in diameter. The leaves are simple, exstipulate and alternate. The shapes of the leaf are elliptic to oblong-lanceolate, 7 to 20 centimetres long and toothed at the margins. The leaves also pointed blunt at the tip (Figure 2). The flowering heads are stalked, numerous 6 to 7 millimetres long, and borne on branches of a large terminal, spreading or pyramidal, leafy panicle (Retrieved from www.akitalherbalmedicine.com, 2004).

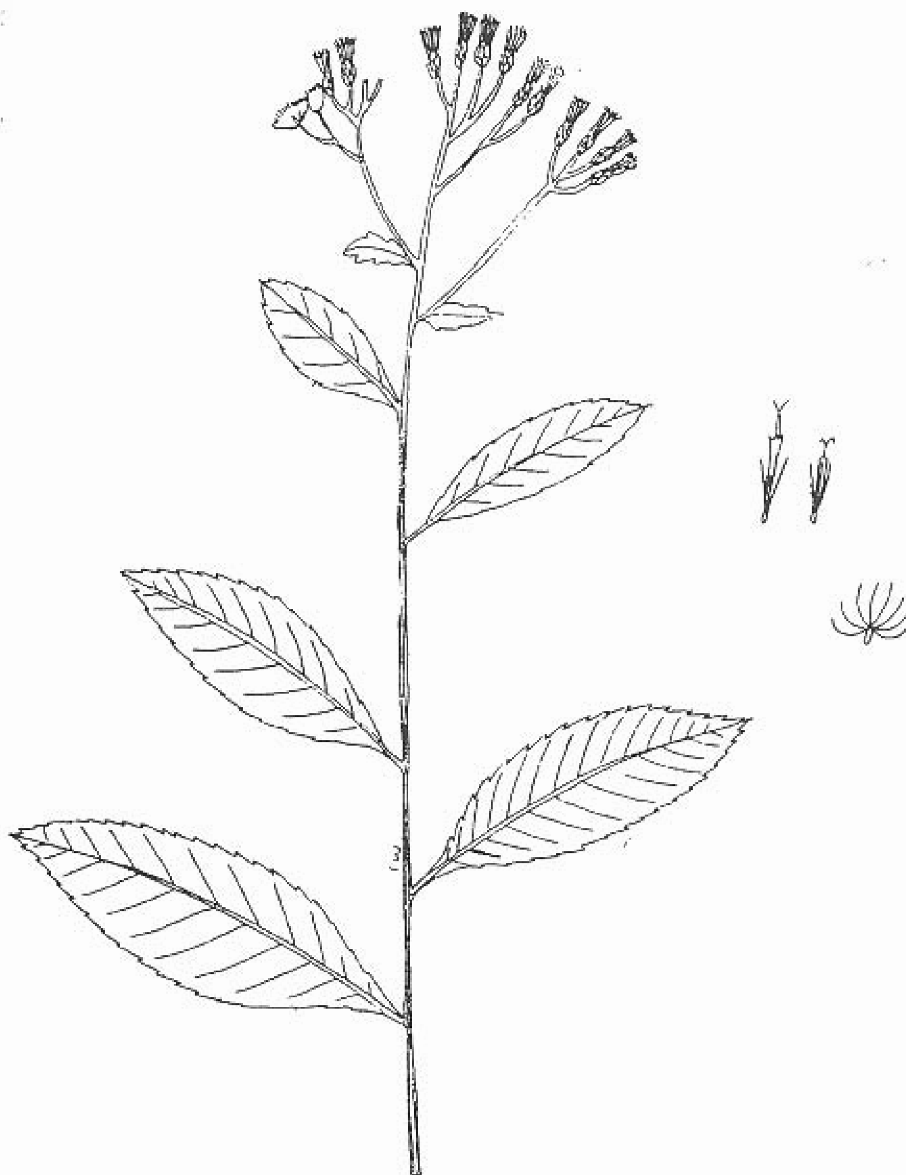

Figure 2: *Blumea balsamifera DC* illustration (Wiart, 2000)

B. balsamifera is one of the many medicinal plants that can be found in Malaysia. Local people have used the *B. balsamifera* for traditional medicine purposes. Some finding has proven that a lot of local people have used this plant for purposes such as treating women especially after birth and fever (Ahmad and Raji, 2004). Some use the plant for relieving the body pain. Most people use the leaves and roots by boiling them with water and either drinking the water or use it for external purposes such as relieving body pain The leaves are also used for treating beriberi (Retrieved from www.akitalherbalmedicine.com, 2004). The leaves are crushed and applied externally as a styptic on the wounds. Lotion also can be made from the boiled leaves used for lumbago, rheumatism and for soothing the skin of children (Ahmad and Raji, 2004).

Other countries besides Malaysia also have been reported to use this plant especially for medicinal purposes. In Cambodia, the plants are used externally in scabies. The fresh juice of the leaves is dropped into the eyes for chronic and purulent discharges. Internally, the decoction is both astringent and anthelmintic. It is also given for worms, dysentery and chronic uterine discharges. The powder of the leaves is used as snuff. Besides these, the infusion of the leaves is also made as an alternative for tea in Philippines. It is believed that the tea could suppress stomach pains (Retrieved from www.akitalherbalmedicine.com, 2004).

The Department of Health of Philippines have approved *B. balsamifera* as one of ten herbs that being effective in treating certain disorders. The Department of Science and Technology also has conducted extensive tests on *B. balsamifera*. Through clinical studies, they have shown *B. balsamifera* to be both safe and effective in the cases of kidney stones and hypertension. There are even *B. balsamifera* based products in the Philippines market that have

been approved by the Bureau of Food and Drugs and are routinely prescribed by doctors (Retrieved from phillipinesherb.com, n.d). According to the Asian Herbal Product (2004) website, through some extensive studies *B. balsamifera* has been proven to be effective as a diuretic. Recent studies have shown that it is capable to reduce or eliminating the kidney stone without surgery (Asian Herbal Product, 2004).

The scientific studies have reported that the plant leaves and stems contain essential oils. This includes the L-borneol (0.1 to 0.4 %), camphor (25%), limonene (75%), cineol, sesquiterpene, sesquiterpene alcohol, palmitin, myristic acid and phenol phloracetophenon-dimethyl ether (Retrieved from www.akitalherbal.com, 2004; Wiart, 2000; Philippines Medicinal Plant website, n.d). These components are well known to occur in most of the antiseptic, carminative, spasmolytic and expectorant essential oils (Wiart, 2000). Camphor is an essential oil that is commonly used in Chinese medicine. Another essential oil, borneol is used by many Asian cultures. It is one of a very important ingredient in a lot of Japanese incense formula (Oller, 2004). Studies found that the leaves contain 0.1 to 0.4 percent of yellow oil with camphor like odour is the almost pure form of L-borneol. The l-borneol is easily oxidized to camphor (Retrieved from www.akitaherbalmedicine.com, 2004).

CHAPTER 2

OBJECTIVE

The objective of this study is to look at the banding pattern of *Blumea balsamifera* generated by using PCR-RFLP technique. The study carried out is also to determine the best genomic DNA extraction method for yielding high quality and quantity DNA from this plant species.

CHAPTER 3

LITERATURE REVIEW

3.1 PLANT TOTAL GENOMIC DNA EXTRACTION

According to Henry (2001), DNA extraction is the basis of any genotype analysis. The approach of the preparation of DNA from plants is determined by species, the type of tissue or sample available and the analysis required on the DNA. There are three commonly used techniques that include the CTAB (hexadecyltrimethylammonium bromide) method, protein precipitation method and caesium chloride method (Milligan, 1992).

These methods differ in the detergent used to solubilize the cellular membrane, the equipments requirements, quality of DNA isolated and their yield. Most of the tissue used for population surveys will be probably collected and stored on ice until it can be frozen in liquid nitrogen, pulverized and stored at –70 °C. These three techniques successfully extract DNA from tissue prepared via this way as well as from fresh tissues (Milligan, 1992).

The cationic detergent, CTAB, is one of the most widely used techniques for isolating plant DNA. This detergent is used to solubilize the plant membranes and form a complex with the DNA. One of the advantages of the CTAB based DNA extraction method is that extensive preparation of the plant tissue is not required and it is adaptable to various types of tissues. This will include the tissues such as the leaves, roots, seeds, embryos, endosperm, pollen and suspension culture. Furthermore, this method can easily accommodate a wide range of sample

sizes from milligram of quantities of herbarium, mummified, or fossil tissue to many grams of freshly harvested tissues (Milligan, 1992).

Total genomic DNA extraction generally involves steps such as the isolation of specific tissues, tissue disruption, extraction to solution, solvent purification and precipitation. Based on Henry (2001), the tissue of the plant that one wishes to isolate, the DNA will be isolated and undergo the initial step that is the disruption of the selected tissue. The purpose of disrupting the tissue is to allow the DNA to be released into the extraction solution.

Some of the plant tissues are difficult target for DNA extraction because the presence of materials such as polysaccharides and secondary metabolites that react with the DNA or co-purify with the DNA during extraction. According to Kim *et al.* (1997), problem arises in extracting DNA from plants containing high content of polyphenolics. Secondary plant products such as phenolic terpenoids that may bind to DNA after cell lysis mediate the DNA degradation. Therefore, it is difficult to isolate high quality DNA from plants containing high contents of polyphenolics compounds. Several rapids methods exist for genomic DNA extraction, however there are no simple methods for obtaining large quantities of DNA from plant containing high contents of polyphenolics compounds.

3.2 PCR – RFLP ANALYSIS

PCR-RFLP is a molecular marker technique that involves both polymerase chain reaction (PCR) and the amplified DNA products digestion using specific restriction enzyme. The PCR-RFLP is different compared to conventional RFLP (Southern RFLP) whereby the PCR based RFLP offer a simple, fast, highly repeatable, cost effective and non-radioisotopic approach in detection of the DNA polymorphism. If more probes are sequenced informative primers from known sequences are made available, one may expect to accumulate sufficient number of polymorphic markers to use PCR-RFLP for classification purposes. Besides, PCR-RFLP also can be applied in marker-aided selection and gene mapping projects (Ghareyazie *et al.*, 2001). The technique includes PCR amplification and restriction enzyme digestion analysis.

3.3 POLYMERASE CHAIN REACTION (PCR)

According to Vicente and Fulton (2000) PCR is a rapid, inexpensive and simple way of copying specific DNA fragments from minute quantities of DNA material even when the source DNA is of poor quality. Moreover, this method does not involve the use of radioisotopes or toxic chemicals. PCR is invented by Kary Mullis of Cetus in the year 1985. From single copy of DNA, millions copies of the DNA can be make using this technique. This is an ultimately sensitive detection system since it able to detect one molecule in a reaction because of its specificity and accuracy (Bains, 1993).

The main components for PCR include the Taq polymerase. This polymerase enzyme is an enzyme that makes new DNA. This polymerase is isolated from the bacteria *Thermus aquaticus*. Other components other than Taq polymerase are such as the primers (forward and reverse), short DNA molecules or dNTPs (dATPs, dGTPs, dCTPs and dTTPs). These dNTPs are complementary to two sites either side of the piece of DNA to be amplify. The primers are usually oligonucleotides, which have been synthesized (Bains, 1993).

There are three main steps in the PCR process. The first step is the denaturation. In this step the DNA fragments are typically heated at high temperature that reduces the DNA double helix to single strands. These single strands are accessible to the primers. The second step is the annealing process. In this process, the reaction mixture is cooled down and the primers is annealing to the complementary regions in the DNA template strands. The primers and the complementary strands will form the double strands product. The third step is the extension process. The DNA polymerase will synthesize the complementary strands by reading the

opposing strands sequence and extends the primer by adding nucleotides in the order in which they can pair. The whole process will repeat over and over (Vicente and Fulton, 2000).

The repeated cycle of heating and cooling will stimulate the primers to bind to the original sequences and to newly synthesised sequences. The enzyme will again extend primer sequences. This cycling of temperatures results in repeated copying process thus leading to an exponential increase in the number of copies of specific sequences. Since the amount of DNA placed in the tube at the beginning is very small, almost all the DNA at the end of the reaction cycles is the copied sequences (Vicente and Fulton, 2000).

3.4 RESTRICTION FRAGMENTS LENGTH POLYMORPHISM (RFLP)

RFLP was firstly described and used in 1978 by Kan and Dozy (Walker and Rapley, 1992). Their research showed that there is a difference in the pattern of digestion with the restriction from normal individuals and patients with sickle cell anaemia. RFLP can be defined as the DNA fragments that isolated using particular restriction endonuclease and existing in various lengths (Berg and Singer, 1992; Kreuzer and Massey, 1996). These different sizes of fragments are generated from the same region of the genome of the different individuals. The words 'poly' means many and 'morph' means form that makes the meaning of the word 'polymorphism' is many forms.

RFLP may caused by the alteration of the DNA structure or mutation. This mutation may arise in many ways that include insertion or deletion of nucleotides, inversion, translocation, transversions and transitions. The mutation will alter the relative position of the restriction endonuclease recognition sequences (Walker and Rapley, 1997). The concept of RFLP is based on the constructing and observing the restriction map that developed by monitoring the digestion of DNA segments by single or multiple restriction endonucleases. Any mutations that occur will lead to RFLP and it can be detected by examining the profile of the restriction fragments generated during the digestion.

One of the most widely use of RFLP is as the genetic marker for genetic analysis (Bains, 1993). According to Bhattramakki and Rafalski (2001), RFLP is one of the DNA molecular marker that chosen for comparative genomic studies. Besides that, RFLP can be used for tracing the transmission of gene or DNA segment through a family tree via the pattern of polymorphic

alleles linked to it (Summer and Phillips, 1991). RFLP also may be used for identifying, localising and tracking gene markers based on the concept how and where the polymorphisms occur within a genome. According to Walker and Rapley (1997), RFLP is the fundamental of the genetic fingerprinting process.

3.4.1 Restriction Enzyme Digestion

Restriction enzymes are the endonucleases that recognize short and specific DNA sequences and catalyse the cleavage of the double-stranded DNA (Roberts, 1992). Restriction enzyme is widely used especially in the recombinant DNA technology applications. The RFLP analysis is one of the applications that require the involvement of the restriction enzyme.

According to McClean (1998), plant DNA differs from the animal DNA. Studies have found that the 5-methyl cytosine (5-mC) component is occurring more frequently in plant comparing to animal. Plant has more than 25% of 5-mC compared to animal, which has about 2% to 7% 5-mC. 5-mC occurs at 70% to 80% of the 5'-CG-3' dinucleotides and percentage of occurrence in the plant is 3.4% compared to 0.5% to 1.0% in animal. Furthermore, 5-mC also occurs at the 5'-CXG-3' sequence in plant but does not happen in animal. The percentage of methylation of these sequences is about 80% for 5'-CAG-3'sequence and 50% for 5'-CCG-3' sequence.

This methyl group will protect the specific DNA site from the restriction enzyme cleavage. Therefore suitable restriction enzyme must be selected for digesting the plant DNA.

According to McClean (1998), some of the restriction enzymes that are suggested to use for sequence cutting in plant are the *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I (Table 1).

Table 1: Six-base Cutter Restriction Enzyme for Digestion Analysis and The Cutting Sequence

Restriction Enzyme	Cutting Sequence
*Eco*RI	G ↑ GATCC
*Eco*RV	GAT ↑ ATC
*Hin*dIII	A ↑ AGCTT
*Xba*I	T ↑ CTAGA

Complete digestion is crucial for interpreting the RFLP patterns. The restriction enzyme activity is affected by several factors that include the pH, concentration, types of ion in buffer, and the temperature of the reaction (Aquadro *et al.*, 1992). The expected restriction mixture is consists of the sterile water, buffer, the DNA and restriction enzyme. The mixture will later undergo incubation at the temperature suitable for the enzyme until it is inactivated either by heat treatment at 65 °C for about 10 minutes or by phenol extraction.

3.5 AGAROSE GEL ELECTROPHORESIS

Electrophoresis is a commonly used technique for separation of nucleic acids. The most commonly used medium for separation is either the agarose or polyacrylamide. Sample DNA isolated from different parts of plant that digested by specific restriction enzyme were separated using the agarose gel electrophoresis (AGE). Agarose gel is the ideal matrix for size fractioning of the resulting restriction enzyme digested DNA fragments by electrophoresis. Two buffers that commonly used for agarose gel electrophoresis include the TAE (Tris-Acetate EDTA) and TBE (Tris-Borate EDTA) buffer. The separated DNA samples will be stained using the ethidium bromide and visualized under UV light (Aquadro *et al.*, 1992).

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CHAPTER 4

MATERIALS AND METHODS

4.1 SAMPLE COLLECTION

The sampling areas of the *Blumea balsamifera* DC include the First Division and Eight Division of Sarawak. The First Division is Kuching, Bau and Serian meanwhile the Eight Division consists of Kota Samarahan, Serian and Asajaya. The sampling locations for this research are shown in Table 2. The sampling was carried out in places where the plant expected to grow such as by the roadsides, secondary bushes, small farms, plantation estates, and villages.

Table 2: Locations for *B. balsamifera* sampling

Location	Area
Kampung Meranek	Kota Samarahan
BDC Stakan Jaya	Kuching
Semengok	Kuching
Kampung Suba Buan	Bau
Pusat Latihan Staf UNIMAS, Suba Bau	Bau

The sampling was done by taking the branches with leaves and placed it in a plastic bag. Then, the collected samples were stored in the cold storage room in order to prevent degradation.

4.2 TOTAL GENOMIC DNA EXTRACTION

Four DNA extraction methods had been analysed in this study for selection of the best method for yielding high quality and quantity of DNA. The methods were Graham and Henry method (Henry, 2001), Thompson and Henry method (Henry, 2001), Aljanabi and Martinez method (Aljanabi and Martinez, 1997) and Doyle and Doyle method (Doyle, 1987). Doyle and Doyle method was selected and used for genomic DNA extraction from *Blumea balsamifera* DC.

4.2.1 DNA extraction based on modified Doyle and Doyle method (1987)

Solutions preparation:

200ml of Extraction buffer

The extraction buffer should consist of 100mM Tris-HCl, 20mM EDTA, 1.4M NaCl, 2% CTAB and 1% PVP (Polyvinylpyrrolidone). For the solution preparation, 20ml of 1M Tris-HCl, 8ml of 0.5M EDTA, 56ml of 5M NaCl, 4g of CTAB and 2g of PVP were required. Once the solution was mixed, it was autoclaved and stored at room temperature.

250ml of Chloroform: Isoamyl Alcohol (CIA) 24:1

240 ml of chloroform was mixed with 10 ml of isoamylalcohol in a 250 ml volumetric flask. The preparation was done under the fume hood. The solution was stored at room temperature.

250ml of TE (Tris-EDTA) Buffer

2.5 ml of 1 M Tris-HCl pH 8.0 and 0.5 ml of 0.5 M EDTA pH 8.0 was added to 247ml of distilled water. The solution was then autoclaved and stored at room temperature.