DNA PROFILING OF *Blumea balsamifera* (L.) DC. USING DIRECT AMPLIFICATION OF LENGTH POLYMORPHISMS (DALP) ANALYSIS

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JOSHUA CHIN

This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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University Malaysia Sarawak

ABSTRACT

Studying DNA polymorphism in medicinal plant such as *Blumea balsamifera* is valuable for genetic manipulation such as trait improvement. There is a need to establish a suitable genomic DNA extraction protocol, as the plant is high in phenolic compounds, which can inhibit PCR reaction. Thus, three extraction methods were carried out. Subsequently, the DNA extraction method using high PVP percentage (6 %) was determined to be the most effective, yielding intact DNA of high quality, with insignificant levels of contaminations. Using that method, DNA from four locations were extracted and then subjected to PCR amplification using DALP analysis to generate DNA profiles. The selective forward primer DALP 221, paired with DALPR reverse primer successfully generated multiple banding patterns. Ten different DNA bands were generated ranging from 150 bp to 1500 bp and the DALP profile was reproducible. Four constant bands were observed in all the locations. DNA samples from Asa Jaya and Bau have the same banding patterns. DNA samples from Samarahan and Kuching have different banding patterns compared to those from Asa Jaya and Bau. Three bands were absent in Samarahan’s DNA sample but present in the other locations. Instead, two different bands, not observed in other locations, were present in DNA sample from Kuching. DNA sample from Kuching has the same seven of the eight bands that were shared among DNA samples from Asa Jaya and Bau. However, there was an absence of a band observed in Kuching’s DNA sample. Thus, Samarahan and Kuching exhibit certain degree of DNA variations. However, further verification through statistical analysis is needed to confirm DNA polymorphism in future studies.

Keywords: Phenolic compounds, PVP, DALP.

ABSTRAK


Kata kunci: Sebatian fenolik, PVP, DALP.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgement</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Abstrak</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vi</td>
</tr>
</tbody>
</table>

## CHAPTER I  INTRODUCTION

1.0 Introduction

1.0.1 Characteristics and morphological properties of \( B. \text{balsamifera} \)

1.0.2 Geographical distributions of \( B. \text{balsamifera} \).

1.0.3 Medicinal properties and treatment uses of \( B. \text{balsamifera} \).

1.0.4 Genomic DNA extraction

1.0.5 DNA profiling

1.0.6 Direct Amplification of Length Polymorphism

1.1 Objectives

## CHAPTER II  LITERATURE REVIEWS

2.0 Phytochemical studies of \( B. \text{balsamifera} \) chemical constituents

2.1 DNA profiling using DALP

2.2 DNA extraction of plant tissues high in polyphenolics
CHAPTER III MATERIAL & METHODOLOGY

3.0 Sample collection 12
3.1 Genomic DNA extraction using PVP 12
3.2 DNA separation and visualization 13
3.3 DNA quantification 13
3.4 PCR amplification 14
3.5 PCR product separation 16

CHAPTER IV RESULTS

4.0 DNA quantification through spectrophotometer readings 17
4.1 Agarose gel electrophoresis for genomic DNA visualization 19
4.2 Results of PCR amplification using DALP 20

CHAPTER V DISCUSSIONS

5.0 Comparisons of the three genomic DNA extraction methods 22
5.1 PCR amplification optimisation 25
5.2 Banding patterns of amplified PCR products 26

CHAPTER VI CONCLUSION

6.0 Conclusion and recommendations for future studies 29

REFERENCES 30
APPENDIX I 35
APPENDIX II 36
APPENDIX III 37
LISTS OF TABLES

Table 1  
Primer sequence  
Table 2  
PCR reaction components  
Table 3  
Spectrophotometer readings of the extracted genomic DNA from the three extraction methods  
Table 4  
Calculated DNA concentration of DNA extracts  
Table 5  
Band scoring results

LISTS OF FIGURES

Figure 1  
Pictures of *B. balsamifera*  
Figure 2  
Agarose gel analysis (1% agarose) of extracted genomic DNA  
Figure 3  
PCR products amplified using the forward primer DALP 221 separated in a 1.5% agarose gel  
Figure 4  
Representative diagram of amplified PCR products using primer DALP221
**LISTS OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CIA</td>
<td>Chloroform: Isoamyl alcohol</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>M</td>
<td>molar or molarity</td>
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</tr>
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<td>MgCl₂</td>
<td>magnesium chloride</td>
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<td>microliter</td>
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<tr>
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<td>milimolar</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>pmol</td>
<td>picomolar</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td><em>Taq</em></td>
<td><em>Thermus aquaticus</em> DNA polymerase</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris-hydrochloric acid</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
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<td>V</td>
<td>volts</td>
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</table>
CHAPTER I
INTRODUCTION

1.0 INTRODUCTION

1.0.1 Characteristics and morphological properties of *Blumea balsamifera* (L.) DC.

*B. balsamifera* of the family *Compositae* is a medicinal plant species that thrives in open space of secondary forest and bamboo forest, while best grows in moist soil (Materia Medika Indonesia, 1979). Among the Malays in Malaysia, it is known as ‘Capa’, ‘Sembong’ or by its vernacular synonym ‘Telinga kerbau’ (Werner, 2002). It is also named as ‘Ngai camphor’ in English, ‘Chapa’ and ‘Sembung’ (Goh et al., 1995). It normally grows between one meter to two meter tall, but sometimes able to grow up measuring four meters tall (Zhari et al., 1999).

Its light green and elliptic-lanceolate leaves are thick, yet soft and very hairy (Zhari et al., 1999) with velvet-like texture. Its upper leaves are without petioles whereas the lower leaves have petioles (Materia Medika Indonesia, 1979). This plant has a large inflorescence with plenty flower heads while its reduced leaves run along the main axis of the stalk (Goh et al., 1995). The leaves have strong, distinct and stinging aromatic smell, especially after the leaf surface has been rubbed on.
Figure 1 (A to C): Pictures of *Blumea balsamifera*, known locally as ‘Sembong’ in Malay language.

Source A: Porsea Herbal Techno-Catalog (n.d.)

Source B: Forest Department Sarawak (n.d.).

Source C: Ecoport (2001)
1.0.2 Geographical distributions of *B. balsamifera*.

*B. balsamifera* can be found in India, Philippines, Indonesia, and Malaysia (Materia Medika Indonesia, 1979 and Zhari et al., 1999). In fact, it is most commonly found in Southeast Asia whereby the sunny tropical weather provides suitable growth conditions for the plant to thrive (Zhari et al., 1999).

1.0.3 Medicinal properties and treatment uses of *B. balsamifera*.

Leaves of *B. balsamifera* are rich in secondary metabolites that mainly are responsible in rendering the plant to possess medicinal values. Examples of the secondary metabolites are such as stearoptene, which is identical to camphor oil (Dey, 1984), monoterpenes, triterpenes, sesquiterpenes and flavones (classed under the group of heterocyclic compounds responsible for leaf colour pigments and possess antioxidant properties) (Fazilatun et al., 2004). The leaves also contain blumealactones, borneol, xanthoxylin (Zhari et al., 1999), flavonoids and alkaloids (Goh et al., 1995).

These chemical constituents are responsible for the plant’s medicinal properties such as diuretic, antioxidant, antitumor, antihistamine, capable of reducing gall bladder cholesterol and acting as bile acid/lecithin production stimulant (Pizzorno et al., 2002 and Goh et al., 1995). It has been used traditionally in folk medicine for treatments such as reducing heatiness, improving circulation and treating diarrhoea (Goh et al., 1995). The Malays have been traditionally using
the leaves of to treat inflammation of heart, headache, cuts, loss of appetite and fever (Werner, 2002).

1.0.4 Genomic DNA extraction

Extracting pure DNA from medicinal plants would pose potential difficulties. This is because medicinal plants are known for their high content of secondary metabolites such as terpenes, alkaloids and phenolics, which are largely accountable for the medicinal properties in the plants. These chemical constituents usually co-precipitate and interact irreversibly with nucleic acids, making extraction of pure genomic DNA difficult (Katterman and Shattuck, 1983; cited in Das et al., 2006). As noted earlier, B. balsamifera contain high amounts of phenolic compounds and other secondary metabolites that are powerful oxidizing agents and can affect the yield and purity of DNA extracted (Khanuja et al., 1999). Consequently, there is a need to establish a suitable DNA extraction methodology to overcome this problem.

Hence, in this study, three DNA extraction methodologies were carried out to seek the most effective extraction methodology. Firstly was the genomic DNA extraction using high percentage of polyvinylpyrrolidone PVP developed by Kim et al. (1997). Secondly, was the modified CTAB method by Doyle and Doyle (1990). The third methodology was the rapid DNA extraction methodology by Mukherjee (1999). Eventually, the methodology based on Kim et al. (1997), with some modifications, was determined to be the most suitable and subsequently used for the rest of this study conduct.
1.0.5 DNA profiling

After obtaining a good quality DNA, the DNA can be further manipulated using PCR to generate multi-banded DNA fingerprints, referred to as DNA profile, which is unique DNA profile of an individual organism. According to Brown (2001), DNA profiling is a technique that detects the variation/differences at multiple loci of a genome as a mean of DNA sequence-based identification. This display of sequence variation is called polymorphism. The polymorphism event can be due to single base mutation, insertion, or deletion (Aitken et. al., 1994) and can be detected by the presence or absence of a DNA fragment or by the lengths of the DNA fragments (Desmarais et. al., 1998).

No two individuals of the same species have the same exact DNA profile. Thus, the unique DNA profile generated can specifically characterise the DNA make-up of an organism, assess the polymorphism level among individuals and determine intra-species or inter-species variations. The characterization of an individual species through DNA profiling can then be the foundation of comprehending its biological information from the molecular and genetic perspective (Bruno, 1996), thus giving insights into a plant functional biology systems such the works of specific gene functions. Subsequently, this provides an avenue for plant breeding programme that can be used for generating plants with desired or beneficial traits (Bruno, 1996). Thus, by considering the potential benefit of B. balsamifera in the aspects commercial cultivation and its application in medicine and health, characterising its DNA is a stepping-stone for achieving these benefits.
1.0.6 Direct Amplification of Length Polymorphisms (DALP)

A suitable genetic marker system is needed in order to generate a DNA profile and to assess its genetic variation. Hence, in this study, the DALP analysis was used. It is a genetic marker system developed for generating new genetic markers of many species as well as assessing their genetic variation (Desmarais et. al., 1998). Moreover, DALP is a codominant marker system, which distinguishes recessive alleles from dominant alleles (Perrot-Minnot et. al., 2000). This technique employs the utilization of a set of selective forward primers harbouring a similar 5' core sequence (M13 Universal sequencing primer) with additional 3' bases, pairing with a common reverse primer. Moreover, by using the same set of forward primer and reverse primer, sequencing of the polymorphic loci can be performed directly.

1.1 OBJECTIVES

The objectives of this study are to:

i. isolate total genomic DNA of *B. balsamifera* and subsequently to determine the suitable extraction protocol.

ii. generate DNA banding pattern profile *B. balsamifera*

iii. determine the presence of variation among *B. balsamifera* individuals from different locations based on the DNA banding pattern generated.
CHAPTER II
LITERATURE REVIEWS

2.0 Phytochemical studies of *B. balsamifera* chemical constituents

As described earlier, *B. balsamifera* possesses many medicinal properties ranging from treating diarrhoea to treating kidney stones. This plant species has been used in the pharmaceutical and dietary supplement production. In the Philippines, its usage as health supplement has been approved by the Bureau of Food and Drugs and registered as drug for pharmaceutical commercialisation (Department of Science and Technology - Philippines Council for Health Research and Development, 2003). The health supplements are also generally prescribed by the doctors there for ailment treatments. With such medical importance, many phytochemical studies has been done to identify and test the chemical constituents of *B. balsamifera* that are responsible for its medicinal properties such as free radical scavenging activity and subsequently leading to the development of new drugs for various disease treatments (Fazilatun *et. al.*, 2004).

All studies to date on *B. balsamifera* mainly had been focusing on the chemical constituents’ identification, characterisation and analysis. Xu *et. al.* (1993) did a study on blumeatin; a secondary metabolite derived from the plant. They tested on the rat models and results showed blumeatin has the capability to prevent liver injury caused by the CC14 carcinogenic agent and thioacetamide, which is suspected to be another potential carcinogen.
They concluded their study stating that blumeatin can potentially provide defensive capability against liver injury.

Furthermore, a phytochemical study on flavonoids derived from *B. balsamifera* by Fazilatun *et. al.* (2004) reported that the flavonoids of this plant extract showed strong antioxidant properties. In another study done by Ali *et. al.* (2005), they had successfully isolated a new chemical constituent named 3-*O-7"*-biluteolin. The newly found chemical constituent is a new biflavonoid, a class of important antioxidants. It would be of great interest if genetic-based molecular biology studies on the chemical constituents could find ways in enhancing these chemical productions as well as coming up with high yielding plant cultivars. Thus, studying its DNA profile would pave way into this beneficial area.

2.1 DNA profiling using Direct Amplification of Length Polymorphisms (DALP)

DALP technique has been successfully applied in researches on different classes of organisms. Desmarais and his co-researches (1998) did a research on the detection of wide polymorphic loci to generate new DNA markers of different classes of organisms such as mice, tilapia fish, barb, birds, mussels, mite and also λ virus. Studies on plant using DALP also had been done like the development of cultivated sunflower (*H. annuus* L) genome (Langar *et. al.*, 2002). These show that DALP can be applied in genetic studies upon a wide range of different organisms.
As there is no nucleotide sequence information of this plant, it is important to use a marker system that is able to overlook this criterion. Since DALP is a simple and rapid DNA marker analysis, requiring no restriction digestion procedure and needs no prior sequence information of the loci or their nature (Desmarais et. al. 1998), it is suitable for obtaining the DNA profile of this plant. Navajas and Fenton (2000) mentioned that DALP enables the detection of more polymorphic loci, meaning that a large number of DNA markers can be potentially generated. In fact, DALP only isolates polymorphic loci (Navajas and Fenton, 2000), thus implying that DALP is specific in detecting polymorphic loci.

The study on new DNA marker development using DALP by Arnaud-Haond et. al. (2002) was successful in generating DNA markers of black-lipped oyster *Pinctada margaritifera* and other *Pinctada* species. The polymorphic loci can be sequenced directly in a single step as this technique employs the same set of forward selective primer and reverse primer in the sequencing step.

By studying its DNA profile, subsequently gene marker for this plant can be developed. Consequently, the information gained can be further be used to look at the gene control and expression levels of enzymes that involved in the metabolic processes of these medically important chemical productions. For example, the study of genetic diversity of the medicinal plant species *Podophyllum peltatum* L., known as North American mayapple, seek to assess its DNA profile and analyse its population genetic variation (Lata et. al., 2002 in Janick and Whipkey, 2002). With that, it enables the determination of mayapple cultivars with desirable genetic material that enhances the production of the medicinally important podophyllotoxin,
subsequently leading to its commercial propagation and cultivation (Lata et. al., 2002 in Janick and Whipkey, 2002). This shows the future prospect of developing better medicinally important plant cultivars such as *B. balsamifera* for medicinal applications as well as its commercial production.

2.2 DNA extraction of plant tissues high in phenolic compounds

Obtaining good quality genomic DNA from plants is much harder due to the presence of secondary metabolites, particularly polyphenolic compounds (Henry, 2001). As *B. balsamifera* is known to contain high amounts of phenolic compounds, three types of genomic DNA extraction methodologies were chosen to find out the best methodology that is able to yield significant amounts of DNA of good quality.

The first methodology is the method using a high PVP percentage based on the protocol by Kim et. al. (1997). It reportedly able to yield high quality intact DNA extracted from plants, especially those containing substantial amount of phenolic compounds, by using a PVP concentration of 6%, whereby PVP is noted for its ability to defend against contamination of phenolic compounds, found in high amounts in most plant tissues (Henry, 2001).

Secondly is the CTAB methodology based on the protocol by Doyle and Doyle (1990), which is a common methodology for plant DNA extraction protocol (Sambrook et. al., 1989). This CTAB methodology uses a higher concentration of sodium chloride (NaCl) at the concentration of 1.4 M (Appendix II) in order to remove polysaccharides. It is because NaCl
concentration higher than 0.5 M when combined with CTAB was reportedly able to remove polysaccharides effectively (Puchooa, 2004).

The third methodology is the rapid extraction method developed by Mukherjee (1999) that enables precipitation of DNA to be done in a single centrifugation step (Appendix III). The methodology reduces the many centrifugation steps and the alcohol extraction (Mukherjee, 1999).
CHAPTER III
MATERIALS AND METHODOLOGIES

3.0 Sample collection

Leave samples of *B. balsamifera* were collected from Kuching, Bau, Kota Samarahan and Asa Jaya. Young leaves were used in the DNA isolation procedures. The leaves were initially washed with distilled water and 70% ethanol. They were then stored at -80°C to preserve the plants' quality and freshness.

3.1 Genomic DNA extraction using PVP method (*Kim et. al. 1997*) (Refer to Appendix I for chemical details).

This extraction method, with slight modifications performed in this study, has been determined to be the most suitable DNA extraction method and subsequently used for extracting DNA from all the plant samples for PCR amplification. Firstly, leaf samples were grounded with liquid nitrogen. The resulting powders were then transferred into 1.5 ml centrifuge tubes containing 300 μl of extraction buffer, 5 μl of 1% (v/v) β-mercaptoethanol and fresh PVP (6% of final volume) each. The content mixture was mixed by flicking. Then, the mixture was incubated at 37°C for one hour. One-half volume of 7.5 M ammonium acetate was then added. Subsequently, the mixture was incubated for 30 minutes on ice. Equal volume of CIA (24:1), approximately 500 μl, was then added. Then, centrifugation was performed at 11200 rpm at 4°C for 10 minutes.
The resulting supernatant from each mixture was then transferred into new tubes. Next, one volume of isopropanol was included and left for DNA precipitation at -20°C for overnight. After incubation, the mixture was centrifuged at 11,200 rpm for 10 minutes. The resulting supernatant was discarded and the DNA pellet was washed with 80% ethanol. Following that, centrifugation was performed at 13,000 rpm for 2 minutes at 4°C. The washing and the subsequent centrifugation were then repeated. The pellet was air-dried and re-suspended with 30 μl of distilled water.

3.2 DNA separation and visualization

Extracted DNA samples were separated on a 1.0 % agarose gel (Agarose LE Analytical Grade, Promega). The gel was stained with ethidium bromide and the DNA was separated by electrophoresis at 110 V in 1X TAE buffer for approximately 2 hours. Subsequently, the stained gel was visualized by using UV-gel image acquisition camera (Gel Doc 1000; Bio-Rad Laboratories).

3.3 DNA quantification

Concentration and purity level of the extracted DNA samples were measured using a spectrophotometer (Ultrospec® 1100 pro.). 5 μl of sample DNA was diluted with 495 μl of distilled water in quartz cuvette tube. Absorbance values at the wavelengths of 260 nm (A₂₆₀) and 280 nm (A₂₈₀) were determined. The ratio of A₂₆₀/A₂₈₀ was measured for purity determination for each DNA sample. Ratio value within the range of 1.8 to 2.0 indicates high
purity of the DNA extracted, with 2.0 being the highest in purity, meaning the DNA is free from phenol or protein contaminants (Brown, 1990).

The extracted DNA concentrations were calculated using the formula shown below:

\[
\text{DNA concentration (µg/µl)} = A_{260} \times 50 \, \text{µg / ml x Dilution factor}
\]

3.4 PCR amplification

A forward selective primer (DALP 221) harbouring the core sequence of M13 universal sequencing primer paired with a M13 reverse primer designated DALPR were used (Refer to Table 1). PCR amplification were performed in final volumes of 25 µl containing 1X PCR buffer (Yeastem Biotech), 100 µM dNTP (Promega), 1.5 U Taq DNA polymerase (Yeastem Biotech) in 1.5 mM magnesium chloride, 20 pmol of each primers (1st Base) and 100 ng of genomic DNA. A total of 40 PCR amplification cycles were performed in a Biometra T-Gradient PCR machine. The cycling parameters were performed as follows: 94°C initial denaturation for 3 minutes; 94°C denaturation for 1 minute, 45°C annealing for 1.5 minutes, 72°C extension for 2 minutes; 72°C final extension for 7 minutes.
Table 1: Universal-sequencing primers (U.S.P.) The bold sequences indicate the -40 U.S.P core sequence.

<table>
<thead>
<tr>
<th>Reverse Primer</th>
<th>DALPR</th>
<th>TTTCAACACAGGAAACAGCTATGAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selective Forward Primer</td>
<td>DALP 221</td>
<td>GTTTCCAGTCACGACGC</td>
</tr>
</tbody>
</table>

Table 2: The PCR components for a 25 µl final PCR reaction volume.

<table>
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<tr>
<th>Reagents</th>
<th>Final concentration</th>
<th>Volume (µl)</th>
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<tr>
<td>Sterilised water</td>
<td>-</td>
<td>10.5</td>
</tr>
<tr>
<td>10X PCR buffer (with 1mM MgCl₂)</td>
<td>1X</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200 µM</td>
<td>5</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.5 mM</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>20 pmol</td>
<td>2</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>20 pmol</td>
<td>2</td>
</tr>
<tr>
<td><em>Tag</em> DNA polymerase</td>
<td>1.5 U</td>
<td>1.5</td>
</tr>
<tr>
<td>DNA template</td>
<td>100 ng</td>
<td>1</td>
</tr>
</tbody>
</table>
3.5 PCR product separation and visualisation

The PCR products were separated on a 1.5% agarose gel (Agarose LE Analytical Grade, Promega) at 90 V for approximately 3 hours. The gel was stained with ethidium bromide, destained using distilled water, and visualised using a UV transilluminator. The resulting gel image was captured using Polaroid 667 film.