CHAPTER 5

DNA EXTRACTION FROM DRY WOOD OF *NEOBALANOCARPUS HEIMII* (DIPTEROCARPACEAE) FOR FORENSIC DNA PROFILING AND TIMBER TRACKING

5.1 INTRODUCTION

Neobalanocarpus heimii or locally known as chengal is endemic but widely distributed in Peninsular Malaysia. It is found in diverse localities, on low-lying flat land as well as on hills of up to 900 m (Symington, 1943). *Neobalanocarpus heimii* produces a naturally, highly durable wood and is among the strongest timbers in the world. It is used for heavy constructions, bridges, boats, buildings, and wherever strength is considered essential (Thomas, 1953). Under the IUCN Red List of Threaten Species, it was assigned under the vulnerable category due to a decline in the area of its distribution, the extent of occurrence and/or quality of habitat, and actual or potential levels of exploitation (Chua, 1998). Owing to the high demand for its valuable timber, *N. heimii* is subjected to illegal logging and this species might become endangered in the near future.

The use of inbuilt unique properties of DNA within a timber could serve as an important technical element in forensic forestry to support the determination of identity and provenance (Asia Forest Partnership, 2005). In combating illegal logging and monitoring forest certification, a chloroplast DNA (cpDNA) marker showing enough geographical structure could be used to differentiate the origin of one source of timber from another (Deguilloux *et al*., 2003; Tnah *et al*., 2009), while a highly polymorphic nuclear short tandem repeat (nSTR) marker could be used to generate DNA profiling databases for individual identification, in which an illegal log could be matched to its original stump (Tnah *et al*., 2010). Herein, the timber can be tracked ‘from logging to customer’. For the moment though, DNA profiling and population identification databases were established for *N. heimii* to be served as DNA authenticity tool for

forensic forestry in Peninsular Malaysia (Tnah *et al*., 2009, 2010). However, the fundamental challenge to use these DNA track-back systems relies on the possibility to extract DNA from dry wood.

Differential DNA access in various parts of wood tissues would determine the possibility to retrieve DNA from dry wood (Deguilloux *et al*., 2002). The cross-section of a tree generally comprises the inner bark, cambium, sapwood and heartwood tissues. The inner bark, also known as phloem is the living tissue which translocates food from the leaves to growing part of the tree. Cambium is composed mainly of living tissue, while sapwood is composed both of living and dead tissues (Forest Products Laboratory, 1999). In the heartwood, all cells are dead and empty, but certain short fragments are still attached on the cell wall (Cano, 1996). Empirical studies revealed that extraction from the cambium and sapwood tissues yielded a considerably amount of DNA for PCR amplification. However, only a small amount of DNA was retrievable via heartwood tissue though it is the most valuable part (Deguilloux *et al*., 2002; Rachmayanti *et al*., 2009).

Extraction of DNA from fresh tissues is routine in studies of tropical forest species, yet extraction from dry wood has not been fully explored. Only a few studies have demonstrated the potential for extracting DNA from wood, for instance, Robinia (De Filippis & Magel, 1998), oak (Dumolin-Lapègue *et al*., 1999; Deguilloux *et al*., 2002), *Gonystylus bancanus* (Asif & Cannon, 2005) and dipterocarps (Rachmayanti *et al*., 2006, 2009; Yoshida *et al*., 2007). Accumulated evidence shows that the extraction of DNA from dry wood will not be straightforward (Deguilloux *et al*., 2002; Asif & Cannon, 2005). In fact, extractions of high quality DNA and amplification from dry wood samples are hindered by several factors, including inappropriate preservation, exposure to natural degenerative process, microorganism decay and presence of PCR inhibitors; hence only small amount of low quality DNA is retrievable (Lee & Cooper, 1995; Cano, 1996; Deguilloux *et al*., 2002; Shepherd *et al*., 2002). In addition, in many cases of illegal logging or illegal timber trade, different forms of wood product are

seized. For instance, an intact form of log or raw wood is usually seized from a logging concession, while lumber that is supplied in the trading market is processed to the form of sawn or semi-finished wood product, e.g. plywood, veneer, hardboard, beam, etc. All these factors would seem to have a strong effect on the subsequent DNA availability.

In order to provide a general guideline for DNA authenticity testing established for *N. heimii*, the study was designed to evaluate the potential for extracting DNA from logs and stumps after felling, and the accessibility of the extracted DNA to nuclear and chloroplast genome. Specifically, the study aimed (i) to identify the best DNA extraction protocol from different parts of wood tissues (cambium, sapwood and heartwood), (ii) to determine the optimal preservation period of wood for DNA extraction, (iii) to assess the effect of amplicon size and genome's copy number on the PCR amplification success rate on DNA extracted from wood and (iv) to evaluate the feasibility to extract DNA from heat-treated lumber.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Two large individual trees (dbh > 30 cm) of *N. heimii* in the Forest Research Institute Malaysia (FRIM) were sacrificed to determine the best DNA extraction protocol from wood. After felling, the logs were placed under a shed while the stumps remained in the field. The DNAs were extracted from the cambium, sapwood and heartwood tissues of both the logs and stumps immediately after felling and after keeping for two, four and six weeks, and three, six, nine and 12 months. To evaluate the feasibility of extracting DNA from heat-treated wood, a small portion of the fresh log was cut into seven pieces of lumber and dried in an oven at 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C. As a positive control, DNA was also extracted from leaf samples immediately after felling to infer conformity of nuclear genotypes and chloroplast sequences.

5.2.2 DNA extraction protocol

Three DNA extraction protocols were tested in this study. These were the DNeasy Plant Mini Kit (Qiagen), modified CTAB protocol (Murray & Thompson, 1980) and modified CTAB with PTB (*N*-phenacylthiazolium bromide) protocol. The sapwood and heartwood tissues were drilled using sterile drill bit and wood shavings were filtered using sieve and only fine wood powders were collected for DNA extraction. Leaf and cambium tissues were homogenized into fine powder with liquid nitrogen using Miller IFM-150 homogenizer (Iwatani). To prevent contamination, the surface of leaf and wood tissues were cleaned with diluted bleach and all the DNA extractions were performed under sterile conditions in separate dedicated areas. In addition, blank extractions were performed simultaneously, starting out with an empty reaction tube containing just extraction buffer, and were treated exactly the same for the rest of the analysis.

The DNeasy Plant Mini Kit (Qiagen kit)

Approximately 100 mg of leaf or wood tissues (fine powder) were mixed with 400 µl of Buffer AP1 and 4 µl of RNase A. The mixture was vortexed and incubated for 10 min at 65 °C. The mixture was mixed three times by inverting the tube during incubation. A total of 130 µl of Buffer AP2 was added to each tube, mixed and incubated for 5 min on ice. The lysate was then pipetted into a QIAshredder Mini spin column in a 2 ml collection tube, and centrifuged for 2 min at 14,000 rpm. The flow-through fraction was transferred into a new tube without disturbing the pellet; 1.5 volumes of Buffer AP3/E was subsequently added and mixed by pipetting. Then, 650 µl of the mixture was transferred into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged for 1 min at 8,000 rpm. The flow-through was discarded and the step was repeated with the remaining sample. This was followed by adding 500 µl of Buffer AW and centrifuging for 1 min at 8,000 rpm. The flow-through was discarded. Another 500 µl of Buffer AW was added and centrifuged for 2 min at 14,000 rpm. The spin column was transferred to a new 1.5 ml microcentrifuge tube and 100 µl of Buffer AE was added for elution. The spin column was incubated for 5 min at room temperature and centrifuged

for 1 min at 8,000 rpm. Finally, this was followed by a repeat elution to give a total elution volume of 100 µl.

The modified CTAB protocol (Murray & Thompson, 1980)

Approximately 5 g of leaf or wood tissues (fine powder) was mixed with 20 ml of prewarmed (60 °C) CTAB (2%) extraction buffer (20 mM EDTA; 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 1% PVP-40; 1.5% β-mercapthethanol) in a 50 ml tube. The homogenate was incubated at 60 °C for 30 min. Then, an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed gently for about 15 min, followed by centrifugation at 3,000 rpm for 10 min. The extraction step was repeated with equal volume of chloroform-isoamyl alcohol (24:1). The supernatant was then transferred to a new 50 ml tube and the DNA was precipitated from the aqueous phase with 0.6 volume of cold isopropanol. The mixture was pelleted by centrifuging at 3,000 rpm for 10 min. Subsequently, the supernatant was removed and the DNA pellet was transferred into a 1.5 ml tube containing 1 ml of wash buffer (76% ethanol, 10 mM NH_4OAc); the tube was subsequently centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the DNA pellet was dried and redissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The extracted DNA was further purified using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH).

The modified CTAB with PTB protocol

Total DNA was extracted using the same procedure of the modified CTAB protocol (Murray & Thompson, 1980), except with the addition of 5.0 ml of 0.1 M PTB (Prime Organics) in the extraction buffer.

5.2.3 DNA quantification

Total DNA was quantified using NanoDrop 1000 Spectrophotometer (Thermo Scientific). Two microlitres of DNA samples was used to check the quantity and purity of DNA. DNA was further quantified using agarose gel electrophoresis. Ten microlitres

of DNA samples was electrophoresed on 0.85% agarose gel against MassRuler DNA ladder (Fermentas).

5.2.4 PCR amplification, genotyping and sequencing

A series of PCR primers specific to nuclear and chloroplast genomes was used to test the quality of all DNA samples extracted from leaf and wood tissues. PCR amplification and genotyping were performed for 12 nSTR loci (*Nhe004*, *Nhe005*, *Nhe011*, *Nhe015*, *Nhe018*, *Hbi161*, *Sle392*, *Sle605*, *Slu044a*, *Shc03*, *Shc04* and *Shc07*) which showed specific amplification, single-locus mode of inheritance, absence of mononucleotide repeat motifs and null alleles in *N. heimii* (Tnah *et al*., 2010). PCR amplifications were performed in 10 μL reaction mixture, consisting of approximately 10 ng of template DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP, and 0.5 U of *Taq* DNA polymerase (Promega). The reaction mixture was subjected to amplification using a GeneAmp PCR System 9700 (Applied Biosystems), for an initial denaturing step of 94 °C for 3 min, 40 cycles of 94 °C for 1 min, 45–50 °C annealing temperature for 30 s, and 72 °C for 30 s, followed by 72 °C for 7 min. The PCR products were electrophoresed along with GeneScan ROX 400 (Applied Biosystems) internal size standard and run on the ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele sizes were assigned against the internal size standard and individuals were genotyped using GENEMAPPER softwares version 4.0 (Applied Biosystems).

PCR amplifications and DNA sequencing were performed for four cpDNA regions which showed intraspecific variability in *N. heimii* (Tnah *et al*., 2009): *trn*L intron, *trn*G intron, *trn*K intron and *psb*K-*trn*S spacer. PCR amplifications were performed with 20 μL of PCR reaction mixture, consisting of approximately 10 ng of template DNA, 50 mM of KCl, 20 mM of Tris–HCl (pH 8.0), 1.5 mM of $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP, and 1 U of *Taq* DNA polymerase (Promega). All reaction mixtures were subjected to amplification using a GeneAmp PCR System 9700, for an initial denaturing step of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 50–55 °C

annealing temperature for 1 min, and 72 °C for 1 min. This was followed by further primer extension at 72 °C for 8 min. The PCR products were purified using the MinElute PCR Purification Kit (Qiagen) and sequenced in both directions using the BigDye Terminator Sequencing Kit (Applied Biosystems) based on the standard dideoxy-mediated chain termination method. The sequencing thermal profile was 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min on a GeneAmp PCR System 9700. Sequencing reactions were purified using ethanol precipitation and run on the ABI 3130xl Genetic Analyzer. Sequencing data were edited and assembled using CODONCODE ALIGNER version 2.0 (CodonCode Corporation).

5.3 RESULTS

5.3.1 DNA extraction protocols for cambium, sapwood and heartwood

The Qiagen kit, CTAB, and CTAB with PTB protocols yielded average concentrations of 721, 451 and 152 ng/µL of DNA from the cambium, sapwood and heartwood fresh tissues, respectively (Figure 5.1). Similarly, after 12 months of preservation, the extraction performed on the cambium tissue yielded higher amounts of DNA, whereas DNA retrieved from the sapwood and heartwood tissues could not be visualised from agarose gel (Figure 5.1). Overall, the efficacy of DNA extraction was higher for the cambium and sapwood than for heartwood tissues.

The results for PCR amplification tested on the DNA extracted using the Qiagen kit, CTAB, and CTAB with PTB protocols for logs are shown in Table 5.1. Out of a total 384 separate PCR amplification tests, the Qiagen kit yielded 76.3% of PCR amplification success rate, whilst the CTAB with PTB, and CTAB protocols yielded 72.9% and 49.7%, respectively. For stumps, the CTAB with PTB protocol yielded 88.8% of PCR amplification success rate, whilst the Qiagen kit and CTAB protocol yielded 78.1% and 69.3%, respectively (Table 5.2). In terms of tissue types, the Qiagen kit yielded higher PCR amplification rates from the cambium tissue, while the CTAB

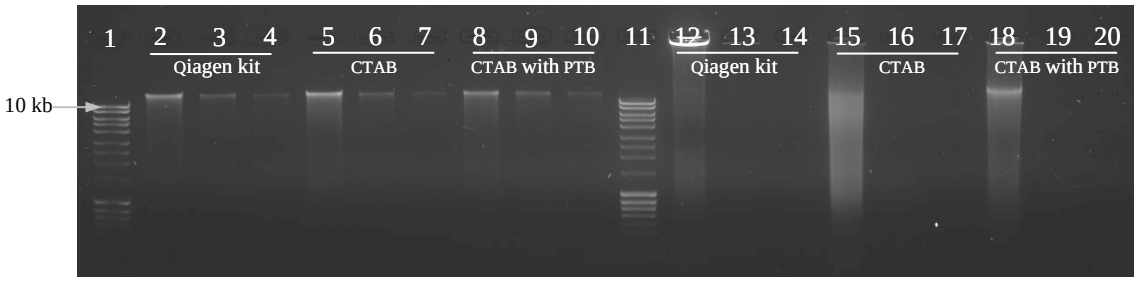


Figure 5.1: Agarose gel (0.85%) showing total DNA extracted from the cambium, sapwood and heartwood tissues using three extraction methods performed immediately after felling: the Qiagen kit (lane 2: cambium, lane 3: sapwood and lane 4: heartwood); the CTAB method (lane 5: cambium, lane 6: sapwood and lane 7: heartwood); the CTAB with PTB method (lane 8: cambium, lane 9: sapwood and lane 10: heartwood) and after 12 months preservation: the Qiagen kit (lane 12: cambium, lane 13: sapwood and lane 14: heartwood); the CTAB method (lane 15: cambium, lane 16: sapwood and lane 17: heartwood); the CTAB with PTB method (lane 18: cambium, lane 19: sapwood and lane 20: heartwood). Lane 1 and 11 denote for MassRuler DNA Ladder (Fermentas).

Table 5.1: Results of PCR amplification comparing three DNA extraction methods: the Qiagen kit, CTAB, and CTAB with PTB protocols for logs. Logs are labelled as 1–8. Number 1 corresponds to logs immediately felled, numbers 2–8 correspond to logs preserved for two, four, six weeks and three, six, nine and 12 months, respectively (numbers indicate amplification and dashes (-) indicate no amplification).

Primer	Qiagen			CTAB			CTAB with PTB		
	Cambium	Sapwood	Heartwood	Cambium	Sapwood	Heartwood	Cambium	Sapwood	Heartwood
Nhe004	1234567-	12345678	1234----	1234----	12345678	123-----	1234----	1234567-	123-----
Nhe005	1234567-	12345678	--------	123-----	12345678	12345678	123456--	1234567-	12345678
Nhe011	1234567-	12345678	--------	1234----	12345678	12345678	123456--	12345678	12345678
Nhe015	1234567-	12345678	12345678	1234----	12345678	12345678	123456--	12345678	12------
Nhe018	1234567-	12345678	--------	1234----	12345678	--------	123456--	12345678	--------
Hbi161	1234----	12345678	12345678	1234567-	12345678	1234----	1234----	12345678	12345678
Sle392	1234567-	12345678	--------	1-------	12345678	--------	1234----	12345678	123-----
Sle605	1234567-	12345678	12345678	1234----	12345678	--------	123456--	12345678	12345678
Slu044a	1234567-	12345678	--------	1234----	12345678	--------	1234----	12345678	--------
Shc03	1234567-	12345678	--------	1234----	12345678	--------	1234----	12345678	1-------
Shc04	1234567-	12345678	12345678	1-------	12345678	12345678	1234----	12345678	12345678
Shc07	1234567-	12345678	--------	1-------	12345678	12345678	1234----	12345678	12345678
*trn*L	1234567-	12345678	1234----	1234----	12345678	12345678	1234----	12345678	12345678
*trn*G	1234567-	1234----	1234----	1-------	1234----	1-------	1234----	1234----	1-------
*trn*K	1234567-	12345678	12345678	1-------	12345678	12345678	1-------	12345678	12345678
*psb*K-*trn*S	1234567-	12345678	12345678	1-------	12345678	12345678	1234----	12345678	12345678

Table 5.2: Results of PCR amplification comparing three DNA extraction methods: the Qiagen kit, CTAB and CTAB, with PTB protocols for stumps. Stumps are labelled as 1–8. Number 1 corresponds to stumps of trees immediately felled, numbers 2–8 correspond to stumps preserved for two, four, six weeks and three, six, nine and 12 months, respectively (numbers indicate amplification and dashes (-) indicate no amplification).

Primer	Qiagen			CTAB			CTAB with PTB		
	Cambium	Sapwood	Heartwood	Cambium	Sapwood	Heartwood	Cambium	Sapwood	Heartwood
Nhe004	12345678	12345---	1-------	1234----	12345678	1-------	123456--	12345678	12345---
Nhe005	12345678	12345678	--------	1234----	12345678	12345678	12345678	1234567-	12345678
Nhe011	12345678	12345---	--------	1234----	12345678	12345678	12345678	12345678	12345678
Nhe015	12345678	12345678	12345---	1234----	12345678	12345678	12345678	12345678	123456--
Nhe018	12345678	12345678	--------	1234----	123456--	--------	12345678	12345678	--------
Hbi161	12345678	12345678	123456--	12345678	12345678	1234----	12345678	12345678	12345---
Sle392	12345678	12345---	--------	1234----	123456--	--------	123456--	12345678	12345---
Sle605	12345678	12345678	12345678	1234----	1234567-	1234567	12345678	12345678	12345678
Slu044a	12345678	12345---	--------	1234----	123456--	--------	12345678	12345678	--------
Shc03	12345678	123456--	--------	1234----	12345678	--------	12345678	12345678	1-------
Shc04	12345678	12345678	12345678	1234----	123456--	12345678	12345678	12345678	12345678
Shc07	12345678	123456--	--------	1234----	123456--	1234----	12345678	12345678	12345678
*trn*L	12345678	12345678	12345---	1234----	12345678	12345678	12345678	12345678	12345678
*trn*G	12345678	12345678	12345---	1234----	12345678	12345678	123456--	12345678	12345678
*trn*K	12345678	12345678	12345678	1234----	12345678	12345678	123456--	12345678	12345678
*psb*K-*trn*S	12345678	12345678	12345678	1234----	12345678	12345678	12345678	12345678	12345678

with PTB protocol showed higher amplification rates in the sapwood and heartwood tissues (Figure 5.2).

5.3.2 Optimal preservation period of wood for DNA extraction

Optimal preservation periods of the wood tissues for logs and stumps were studied for 12 months after felling. For logs, results suggested that the DNA extracted from the cambium and sapwood tissues was only well preserved within six weeks after felling (Figure 5.3). In chloroplast genome, all cpDNA was 100% amplified from the cambium, sapwood and heartwood tissues within six weeks period. Similarly, in nuclear genome, all nSTR was only perfectly retrievable from the cambium and sapwood tissues within six weeks, while the heartwood yielded inconsistent genotyping results. By contrast, the DNA retrieved via stumps showed better preservation, in which the DNA extracted from the cambium tissue was well preserved within six months, whilst that from the sapwood tissue was well preserved within nine months after felling (Figure 5.4). In chloroplast genome, all cpDNA was 100% amplified from the cambium, sapwood and heartwood tissues within six months period. In nuclear genome, all nSTR was only perfectly retrievable from the cambium and sapwood tissues within six months, while the heartwood yielded inconsistent genotyping results. Overall, the PCR amplification success rates decreased rapidly six weeks after felling for DNA retrieved from logs, while it was six months after felling for DNA retrieved from stumps.

5.3.3 Effect of amplicon size and genome's copy number

The effects of amplicon size and genome's copy number on the PCR amplification success rate for logs and stumps are shown in Figure 5.5. In nSTR, smaller amplicon size (93–195 bp) yielded almost 100% amplification success rate, while larger amplicon size (281 bp) yielded 80% amplification success rate. Exception was observed in several amplicon sizes (133–161 bp), which yielded only 60–70% amplification success rate. On the other hand, the amplification success rate clearly increased as the copy number of the targeted genome increased. For instance,

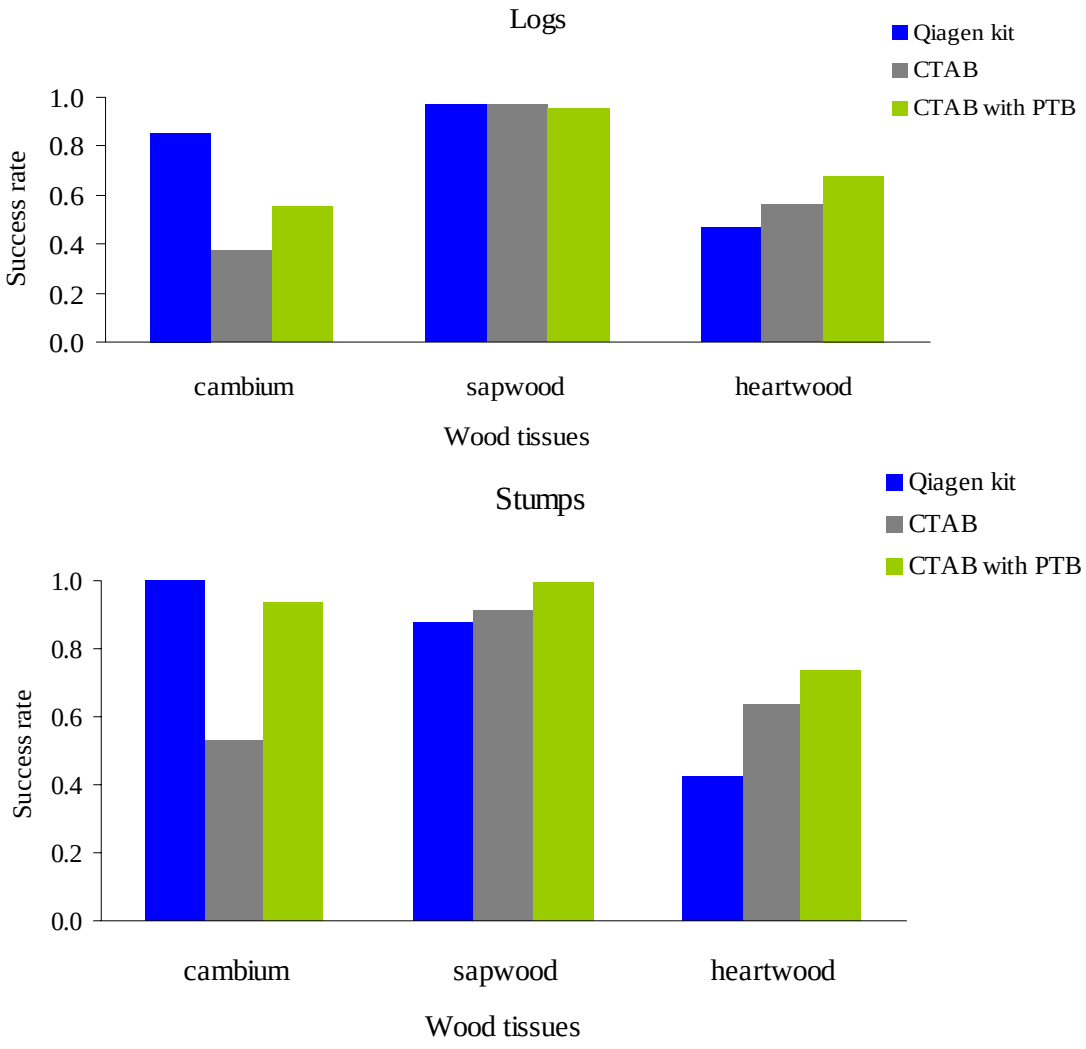


Figure 5.2: Three different DNA extraction methods were compared for logs and stumps using the cambium, sapwood and heartwood tissues.

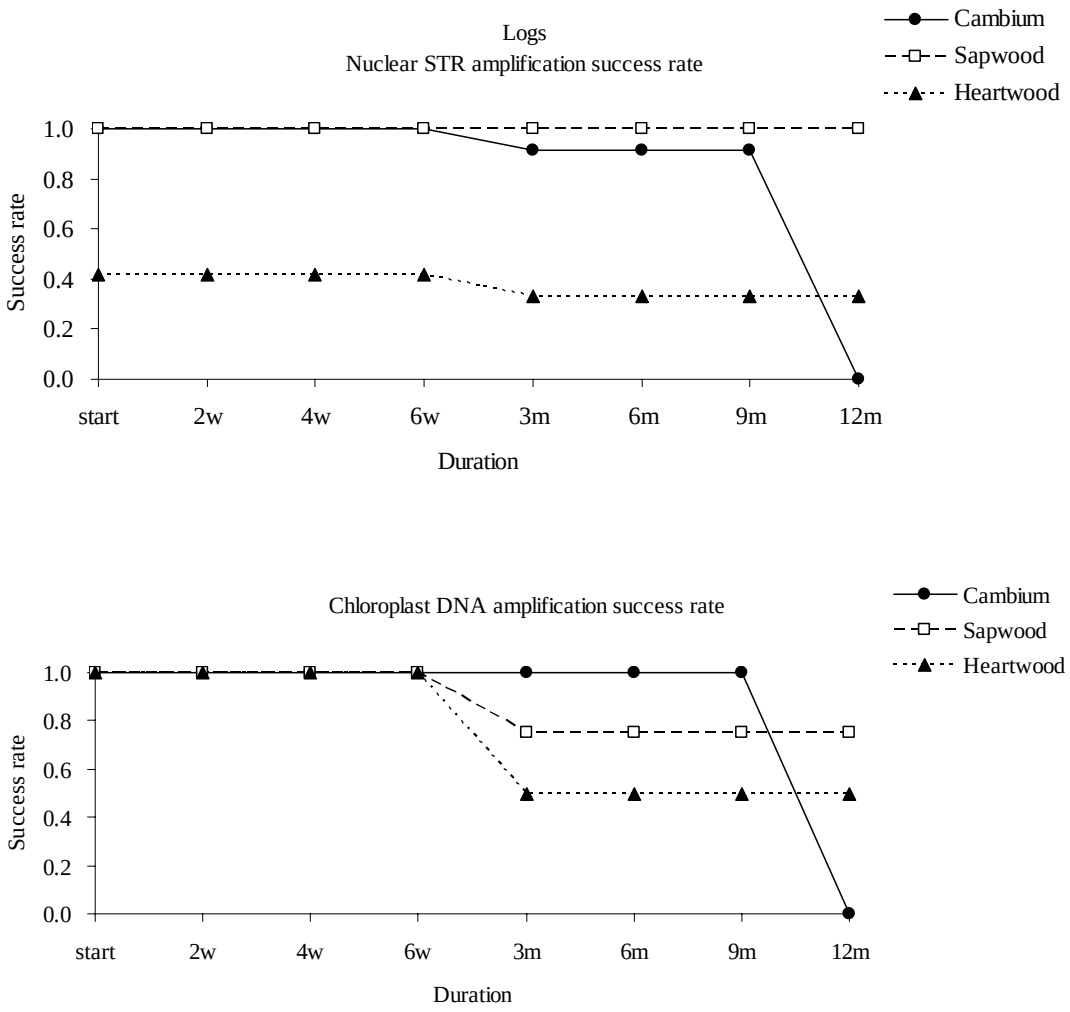


Figure 5.3: PCR amplification success rates of DNA extracted from the cambium, sapwood and heartwood tissues for logs using the Qiagen kit. The amplifications were performed for nuclear and chloroplast regions immediately after felling (start) and two (2w), four (4w), six weeks (6w) and three (3m), six (6m), nine (9m) and 12 months (12m) of preservation.

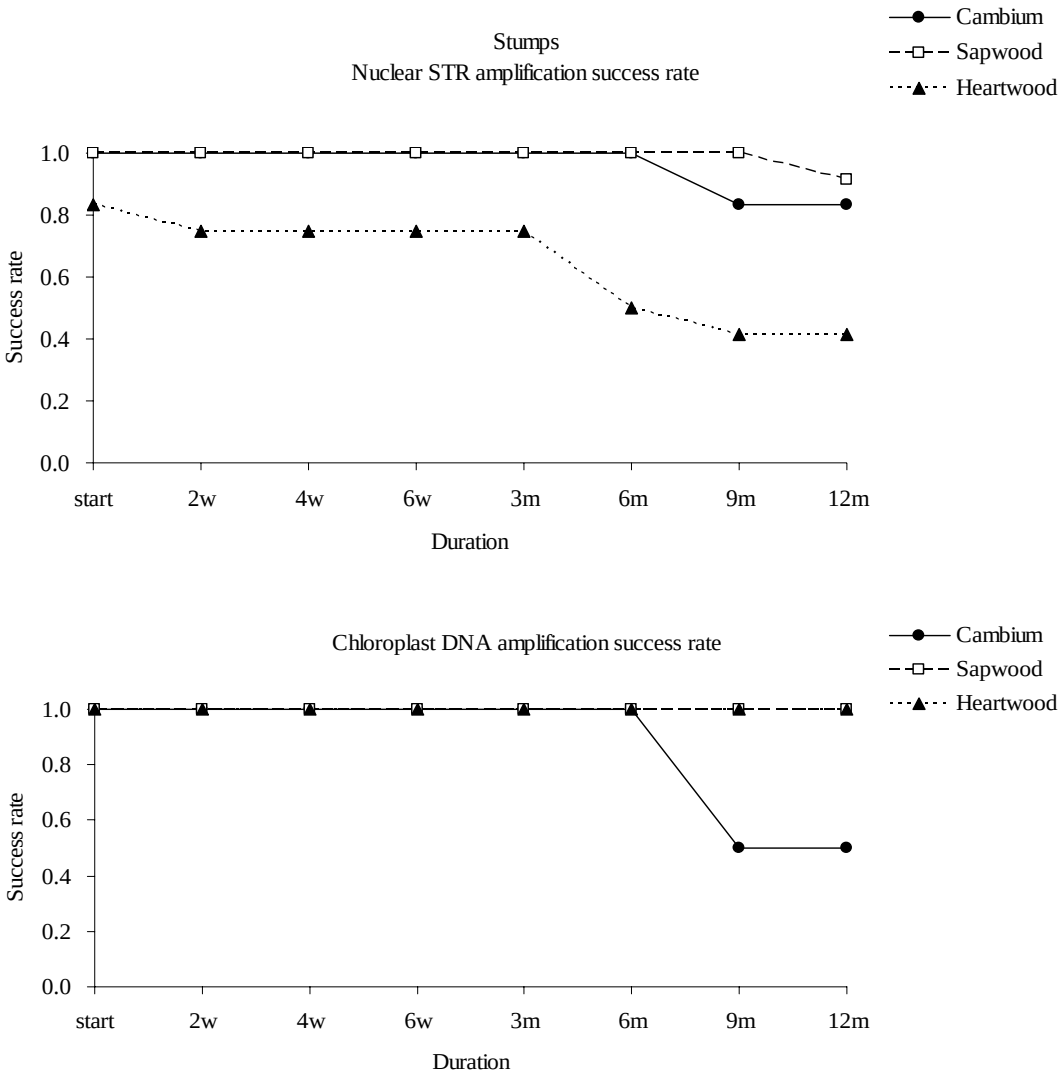


Figure 5.4: PCR amplification success rates of DNA extracted from the cambium, sapwood and heartwood tissues for stumps using the CTAB with PTB method. The amplifications were performed for nuclear and chloroplast regions immediately after felling (start) and two (2w), four (4w), six weeks (6w) and three (3m), six (6m), nine (9m) and 12 months (12m) of preservation.

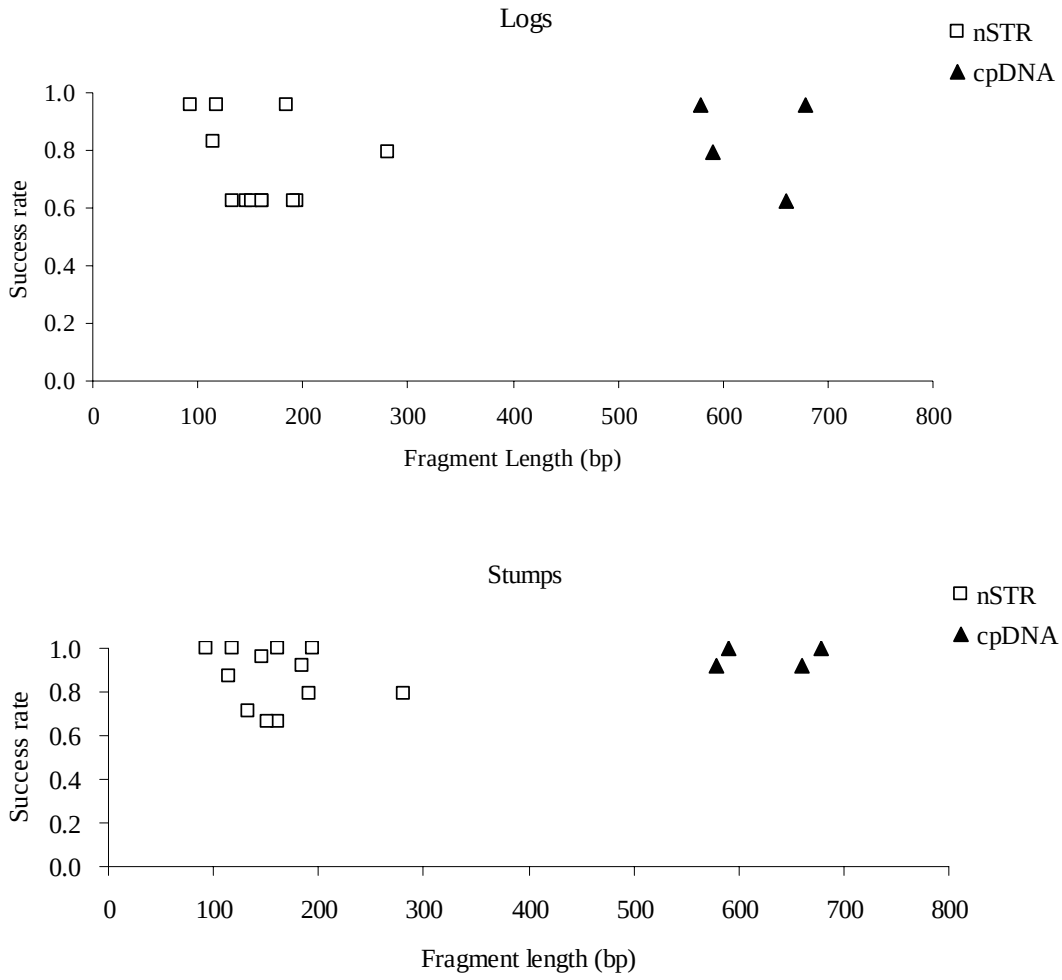


Figure 5.5: PCR amplification success rate related to amplified fragment length and genome's copy number for logs and stumps (open squares indicate nuclear STR and black triangles indicate chloroplast DNA).

chloroplast genome yielded 60–100% amplification success rate though larger amplicon sizes (579–679 bp) were used. Overall, it appears that the amplicon size might not account for the PCR amplification success rate and chloroplast genome yielded higher amplification success rate compared with nuclear genome.

5.3.4 Effect on heat-treated lumber

In heat-treated lumber, only a low quantity of DNA could be recovered (average concentration of 52.77 ng/µl) and it could not be visualized by ethidium bromide staining of agarose gel (data not shown). Yet, based on the CTAB with PTB protocol, the PCR amplifications showed that both the nuclear and chloroplast regions could be retrieved from lumber that was heat-treated at 40 °C to 100 °C (Table 5.3). Nevertheless, the phenomena of allelic dropout and inconsistency of genotyping were noted for some of the nSTR regions (Figure 5.6). For instance, allele dropout was observed in locus *Nhe011*, in which the genotype of control was heterozygote (179/197), while the genotype of heat-treated lumber was found to be in the form of homozygote (197/197). Inconsistency of genotyping was observed in loci *Hbi161*, *Sle392* and *Shc07*.

5.4 DISCUSSION

The present study showed that the efficacy of DNA extraction was higher for the cambium and sapwood than for the heartwood tissues. This might indicate that both the cambium and sapwood tissues can be a good source of DNA. Similarly, Deguilloux *et al*. (2002) and Rachmayanti *et al*. (2009) also reported that the PCR amplification success rates were higher with DNA extracted from the outer part of the logs, from cambium to sapwood. The differential of amplification success rates may be simply explained by the gradual transformation of wood cells during aging (Fengel, 1970). The cambium is composed mainly of living tissue, while the sapwood is composed both of living and dead tissues. In the heartwood, all cells are dead and empty. Although it is possible to recover DNA from the heartwood, the problems of

Table 5.3: PCR amplification tested on DNA extracted from lumber that had undergone drying process from 40 °C to 100 °C using the CTAB with PTB method. Symbol '+' indicates amplification.

Primer	Drying temperature (° C) of lumbers						
	40	50	60	70	80	90	100
Twelve nuclear STRs	+	+	+	+	+	+	+
*trn*L	+	+	+	+	+	+	+
*trn*G	+	+	+	+	+	+	+
*trn*K	+	+	+	+	+	+	+
*psb*K-*trn*S	+	+	+	+	+	+	+

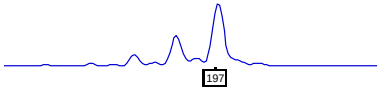
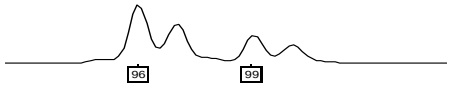
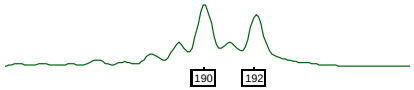
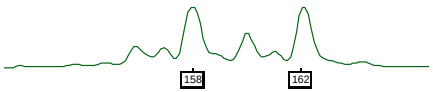
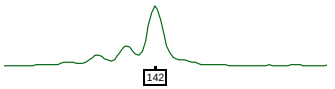
Locus	Genotypes of nuclear STR	
	Control	Heat-treated lumber
Nhe011	179/197 	197/197 
Hbi161	109/115 	96/99 
Sle392	190/192 	182/190 
Shc07	158/162 	142/142 

Figure 5.6: The problems of allelic dropout and inconsistency of genotyping were observed for DNA amplified from heat-treated lumber using nuclear STR. Genotypes of loci *Nhe011*, *Hbi161*, *Sle392* and *Shc07* for control and heat-treated lumber are shown.

amplification failure, allelic dropout and inconsistent genotyping might limit the applicability of heartwood tissues for authenticity testing using DNA.

The present study also showed that the Qiagen kit yielded higher PCR amplification success rates in the cambium tissue. The CTAB with PTB protocol showed higher amplification rates in the sapwood and heartwood tissues, while the CTAB protocol generally showed low PCR amplification success rates in all the tissue types. Numerous reports have demonstrated the effectiveness of the Qiagen kit to retrieve DNA from dry wood (Dumolin-Lapègue *et al*., 1999; Deguilloux *et al*., 2002; Rachmayanti *et al*., 2006, 2009; Yoshida *et al*., 2007), though Asif and Cannon (2005) have commented that the kit yielded low quality and quantity of DNA. For the CTAB with PTB protocol, addition of PTB in the CTAB protocol might improve the DNA yields by cleaving sugar-derived protein cross-links and help to release the entrapped DNA (Poinar *et al*., 1998; Asif & Cannon, 2005). The PTB has primarily been used to extract DNA from ancient samples, such as Neanderthal human remains (Krings *et al*., 1997) and ancient bone (Kelman & Kelman, 1999).

The presence of Maillard products in the DNA extracts or impurities such as terpenes, polyphenolics and polysaccharides might also affect DNA quality (Shepherd *et al*., 2002). Particularly, polyphenolic compounds such as stilbenoids are abundant in the Dipterocarpaceae (Tanaka *et al*., 2001; Ito *et al*., 2003), and are believed to be the main PCR inhibitory substances. To improvise the CTAB with PTB protocol, the inclusion of bovine serum albumin (BSA) in the extraction buffer or alcohol precipitation of DNA in the presence of high NaCl concentration might further enhance the efficiency of polysaccharide and polyphenolic removal (Crowley *et al*., 2003; Tibbits *et al*., 2006, Tsumura *et al*., 2010).

The period of preservation could have strong effect on the total DNA retrieved from dry wood, as the quality and quantity of DNA are likely to decrease throughout the year. Deguilloux *et al*. (2002) demonstrated the possibility to retrieve a relatively

long fragment of DNA (1,500 bp) from sapwood tissue even after storing for three years. However, in this study, the PCR amplification success rate rapidly decreased after six weeks of felling for logs and six months for stumps. The most likely explanation is that the stumps could be alive for several months and sometimes be able to regenerate into new trees due to the existing root structure. Hence, most of the wood tissues in the stumps are still well preserved though exposure of water, UV and microorganisms could probably degrade part of the DNA molecules (Murmanis *et al*., 1987; Cano, 1996). In contrast for logs, once a tree is cut, the quality and quantity of the DNA in the logs will quickly diminish and more likely be degraded into smaller fragments (Bär *et al*., 1988; Cano, 1996). The optimal period of preservation determined in this study could serve as a useful guide for DNA authentic testing on *N. heimii*. As a whole, for *N. heimii*, in order to safeguard the intactness of the DNA, it is recommended that DNA extraction be carried out within six weeks after felling for logs and six months after felling for stumps.

Previous studies reported that the increase of amplicon size would have adverse effect on the PCR amplification success rate (Pääbo, 1990; Deguilloux *et al*., 2003; Poinar *et al*., 2003; Rachmayanti *et al*., 2009). In the circumstance of highly degraded DNA, numerous studies have reported on the poor amplification for the larger amplicon size that ranged 300–500 bp (Wallin *et al*., 1998; Cotton *et al*., 2000; Krenke *et al*., 2002). However, in the present study, there was no obvious relationship among amplicon size and PCR amplification success rate. This might be due to the relatively short fragment length of nSTR region (93–281 bp) being used in this study. Alternatively, it could be also obscured by differential degradation mechanism of the DNA sequences in a particular genome.

The present study also showed that chloroplast genome yielded higher amplification success rate compared with nuclear genome. By referring to the PCR amplification from stumps, all cpDNA regions (approximately 600–700bp) were 90–100% retrievable, while nSTR region only yielded moderate amplification success rate,

although the fragment length of the 12 nSTR is relatively short (approximately 93–281bp). One possible explanation is that chloroplast genomes are present in multiple copies per cell, and greatly more abundant than single-copy nuclear genomes, which leads to higher success rate in amplification.

Normally in the sawmill, the logs are cut into pieces of lumber with various dimensions and subsequently dried at ordinary atmospheric temperatures (air drying), or in a kiln at controlled temperatures raised artificially above atmospheric temperature but not usually above 100 °C (Malaysia Timber Council, 2006). Drying lumber is one approach of adding value to sawn products from the primary wood processing industries (Haque, 2002), but in the context of DNA extraction, heating may lead to degradation of DNA or inhibition of PCR amplification. In this study, however, the PCR amplifications showed that both the nuclear and chloroplast regions could be retrieved from lumbers that were heat-treated at 40 °C to 100 °C, although some of the nSTR regions were embedded with severe allelic dropout problem and inconsistent genotype profile. Similarly, Rachmayanti *et al*. (2009) demonstrated the possibility to retrieve chloroplast region from processed dipterocarp wood (either in the form of sawn wood, glued wood or framed wood), though lower PCR success rate was observed. Also, Yoshida *et al*. (2007) showed the ability to recover DNA from heat-treated wood from 60 °C to 140 °C. This might indicate that although heating diminishes the presence of DNA in the wood tissues, a small amount of quality DNA can still be retrieved from heated wood, in particular from chloroplast genome. Thus, the feasibility to retrieve chloroplast region from heat-treated lumber signifies the potential for performing DNA testing for provenance traceability of processed wood.

In summary, there are several findings obtained from the study should be incorporated in a guideline for DNA authenticity testing established for *N. heimii*: (i) the efficacy of DNA extraction was higher for the cambium and sapwood than for the heartwood tissues; (ii) the wood DNA extracted from the CTAB with PTB protocol and Qiagen kit yielded higher PCR amplification rates; (iii) the DNA extraction should

be carried out within six weeks after felling for logs and six months after felling for stumps; (iv) there is no obvious relationship between amplicon size and PCR amplification success rate, and chloroplast genome yielded higher amplification success rate compared with nuclear genome; (v) only chloroplast regions could be perfectly retrieved from heat-treated lumber. In short, the guideline obtained from this study are ready to be used together with the population and individual identification databases developed by Tnah *et al*. (2009, 2010) for timber tracking and forensic DNA profiling of *N. heimii* in Peninsular Malaysia.