DNA EXTRACTION FROM WOOD OF *GONYSTYLUS* SPECIES

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ITTO-CITES Activity: Use of DNA for Identification of *Gonystylus* species and Timber Geographical Origin in Sarawak

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List of Abbreviations

DNA	Deoxyribonucleic acid
NaCl	Sodium chloride
CTAB	Cetyltrimethylammonium bromide
PTB	N-phenacylthiazolium bromide
PCR	Polymerase chain reaction
$MgCl_2$	Magnesium chloride

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Summary

Ability to verify the origin of Ramin timber/timber product is very important in monitoring and control of the illegal activities related to timber trading. As plant population has generally strong genetic structure in space and unique geographic pattern of alleles, the use of molecular genetic tools to test timber origin is seen promising. Thus, it is very important that the DNA can be isolated from any part of Ramin trees to make the future use of DNA tracking system applicable for any product derived from Ramin timber.

Experiment to extract DNA from different sections of wood and preserved wood samples of Ramin was carried out using the modified CTAB extraction protocol. By using high salt (NaCl) precipitation, the polysaccharides contaminant that inhibited the Ramin DNA could be efficiently removed. The DNA extraction from preserved wood samples, inner bark and sapwood was successful even for samples stored up to three months.

1.0 Introduction

Gonystylus, one of the three genera under Thymelaeaceae is a tropical forest tree that is locally known as Melawis in Peninsular Malaysia, Gaharu Buaya and Medang Keladi in Borneo and Lanutan-Bagyo or Anauan in the Philippines (van Steenis, 1948). Commercially, the genus is well recognised and acceptable as Ramin. It is distributed over the South East Asia (in Malaysia, Indonesia, Brunei and the Philippines) Nicobar, Solomon and Fiji Island (Figure 1). The genus comprises 30 species with the highest diversity in Borneo, followed by Peninsular Malaysia and Indonesia. There are 27 species distributed in Sabah and Sarawak, mostly occurring in Sarawak (Tawan, 2004). However during this project only 21 species were identified from specimens collected from nine populations throughout Sarawak including the dominant species in trade, *Gonystylus bancanus* which is endemic to the peat swamp forest (PSF). The other species of *Gonystylus* occur in the lowland and hill mixed dipterocarp forest at altitude up to 700 m.a.s.l.

Ramin is a tropical light weight to moderately heavy hardwood timber. The timber is whitish in colour and in high demand for decorative uses, external building construction, planks cases, picture frames, billiard cues, blinds and baby cribs. However, the production and export volume of Ramin (*G. bancanus*) for Sarawak dropped drastically from 12, 1610 m^3 in 2006 to 3, 063.09 m^3 in 2008 (CITES, 2010). The reduction in export was due to the depletion of Ramin population, conversion of PSF to other land use and the enforcement of export quota.

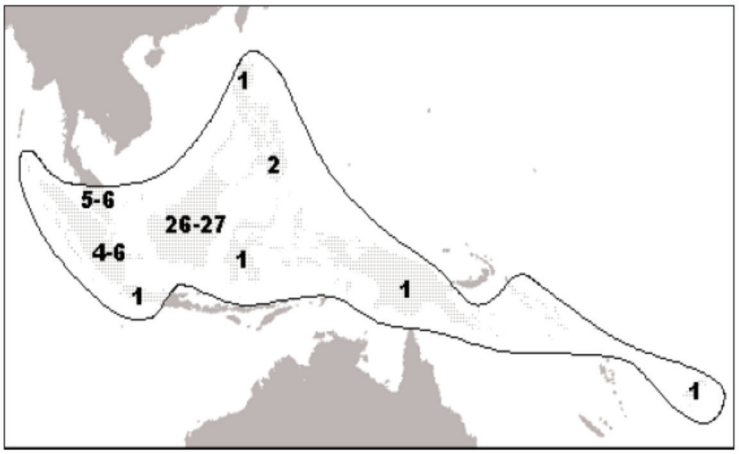

Figure 1: Distribution of the species of the genus *Gonystylus* – area bounded by the continuous line. Numerals indicate the number of species of *Gonystylus* known from the principal islands (*G. marcophyllus* is found throughout the range of the genus). (source: van Steenis, 1948)

Export destination		**Ramin product**	
Market	**Volume (M^3)**	**Product**	**Volume (M^3)**
China (including Hong Kong)	623	Sawn Timber	1,153
USA	454		
Japan	448	Dowel/mouldings	1,438
Others	1,066		
Total	2,591	Total	2,591

Source: CITES, 2010

Table 1: Export destination for Ramin products (January-November 2009) from Sarawak.

After the inclusion of Ramin (all parts and derivatives) in CITES Appendix II, control on the export of Ramin required verification by Customs on the CITES Export Permit. To enhance control and monitoring of the Ramin export, collaboration among various agencies involved like Forest Department, Royal Customs Department of Malaysia and Malaysia Maritime Enforcement Agency was established. To further strengthen the control measure on Ramin trade particularly involving the verification of species and legality of source, development of mechanism/technique to test and verify the species and origin of timber and/or wood products is required. Ability to verify the origin of Ramin timber/timber products would enable control of the illegal activities particularly those related to illegal Ramin from Indonesia laundered through Malaysia (EIA/Telapak, 2004 and EIA/Telapak, 2006).

Plant population generally has a strong genetic structure in space because its dispersal is usually limited. Thus, we often find a geographic pattern of alleles of plant populations, which could be unique locally. In other words, this geographic pattern of alleles can be used as the indicator of the plant's origin. Additionally, genetic traits at the DNA-level are non-manipulable and hence are highly reliable evidence to evaluate any biological material at various levels (Finkeldey *et al.* 2010). The use of molecular genetic tools to test timber origin is seen promising and trustworthy compared to other available identification tools as it has been used and widely reported in the origin identification of ancient and modern humans at the individual level or at the population level. In order to develop the identification system using the molecular DNA technique, the crucial

steps in the development of this system involve not only the identification of unique geographical pattern of alleles but also the isolation of DNA, in this case, from any part of the Ramin tree.

2.0 Research Objective

Isolation and optimisation of DNA from fresh and particularly processed wood is crucial. Extracting DNA from processed wood (dry wood) is harder than that from the fresh wood because the DNA of the dried wood is mostly degraded. Thus, we explored the most efficient method to extract DNA from wood stored under various conditions.

3.0 Materials and methods

3.1 Wood samples

Wood is divided into several parts: cambium, outer and inner sapwood, and outer and inner heartwood. The cambium comprises all living cells, the sapwood constitutes both living and dead cells whereas the cells in heartwood are all dead. Sapwood and heartwood are the most valuable part of the wood as most timber products consist of these parts; sometimes timber products are made only of heartwood.

We first examined if sample conditions such as drying methods and different parts of the stem, would influence the DNA extraction. Fresh wood (branches or twigs) samples were collected from Ramin

trees and were preserved using six preservation methods: under normal room temperature; oven-drying (~70°C) for 8 hours each day; soaking in water for 3 months; drying using silica gel; soaking in NaCl-CTAB and in 95% ethanol (Figure 2). The DNA was extracted weekly from these preserved samples for the first three weeks, thereafter monthly up to three months using the CTAB extraction protocol by Murray and Thompson (1980) with modification. Additional DNA extraction was carried out to test and compare the applicability of DNA extraction for different sections of the wood. For this purpose, only air-dried wood was used. The samples were preserved up to three months.

3.2 DNA purification

All DNA samples obtained were further purified with the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's protocol.

3.3 PCR amplification

The extracted DNA was applied in PCR amplification using Qiagen

at 60˚C for 30 minutes. The template was amplified with primer trnF (F): 5'-ATTTGAACTGGTGACACGAG-3' and trnE (R): 5'-GGTTCAAGTCCCTCTATCCC-3'. The expected fragment size is approximately 500 bp.

Figure 2: Preservation of ramin wood samples: 2a: air drying, 2b: Oven drying, 2c: Soaking in water, 2d: Soaking in 95% ethanol, 2e: Soaking in NaCl-CTAB and 2f: drying in silica gel.

4.0 Results and discussion

4.1 DNA extraction from preserved wood

The modified CTAB extraction method used for the DNA extraction from the leaves was also used for DNA extraction from the wood. Although we collected 21 *Gonystylus* species, only wood from *Gonystylus affinis* was tested. DNA of Ramin wood (twigs or branches) kept under various conditions were extracted (Figure 3) and amplified (Figure 4). By observing the agarose gel photos, DNA was successfully extracted from both inner bark and sapwood preserved in silica gel, NaCl-CTAB and air-drying for up to three months. However, the intensity of the DNA bands gradually decreased with time for wood preserved in 95% ethanol, oven-drying and water. This might be due to: over heating which resulted in irreversible degradation of DNA when the samples were oven-dried; and chemicals which inhibited DNA extraction or degradation of DNA after the death of the plant cell (Finkeldey *et al*., 2010; Rachmayanti *et al*., 2009).

Although DNA bands were not observed in the agarose gel for wood preserved in ethanol and oven dried after one month, DNA was detected using the spectrophotometer. This might indicate the presence of DNA in these samples although the concentration of DNA was low (Table 2a, b and c). For wood kept in silica gel and air-dried, the humidity and weight of the wood decreased with time and more DNA- containing cells were applied for DNA extraction even though samples with the same weight were used. Thus, the

concentrations of DNA extracted from wood preserved in silica gel and air-drying showed tremendous increase after one month.

The A260/280 ratio of 67% (24/36) of the samples was more than 2.0 and 19.4% (7/36) showed A260/230 ratio between 2.0 to 2.2. However, the quantity and quality of the DNA extracted from all preserved wood was adequate enough for PCR amplification as it showed positive amplification.

Previously, extraction of DNA from fresh and processed wood was done using CTAB which yielded low DNA and failed PCR amplification but was successful in N-phenacylthiazolium bromide (PTB) extraction (Asif and Cannon, 2005). He suggested that the failure in CTAB extraction could be due to the presence of impurities such as terpenes, polyphenolics and polysaccharides which were also mentioned by Shepherd *et al*, (2002). Ramin is known to have high polysaccharides content. The polysaccharides contaminant could be removed from plant DNA efficiently by using high salt (NaCl) precipitation (Fang *et al.*, 1992). In our experiment, the CTAB extraction solution was modified by increasing the concentration of NaCl to 4.0M. By applying the solution, all DNA extraction from the preserved wood samples was successful even for samples stored up to three months.

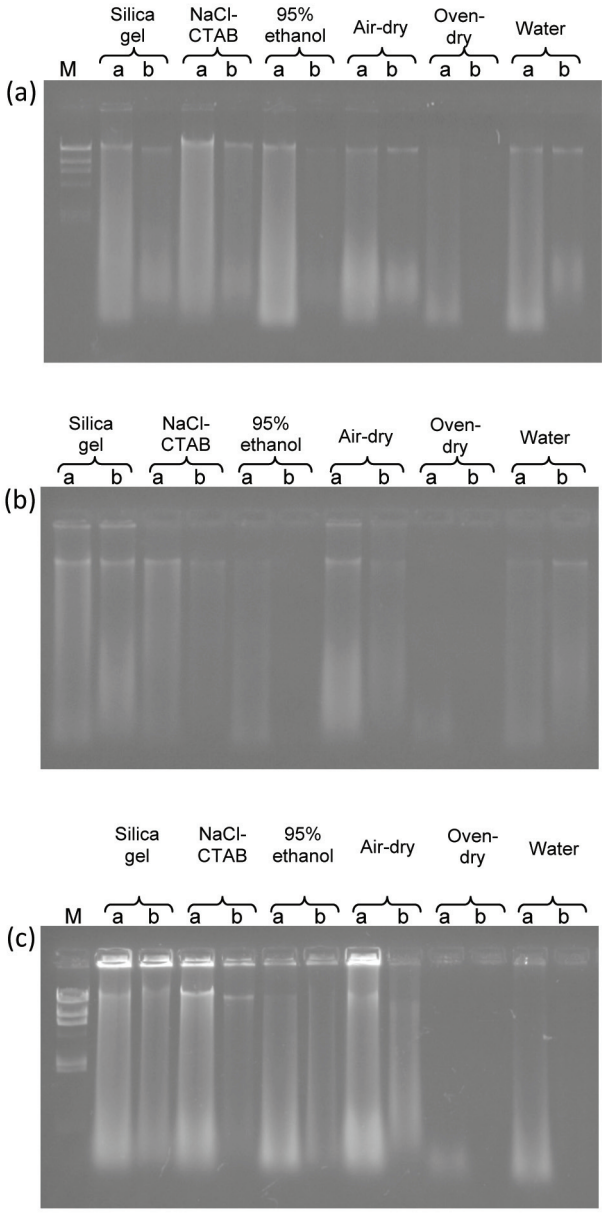

Figure 3: Agarose gel photo of DNA extracted from wood preserved under various methods after (a) 1 week, (b) 1 month and (c) 3 months. M: λ Hind III ladder; a: inner bark (cambium); b: sapwood.

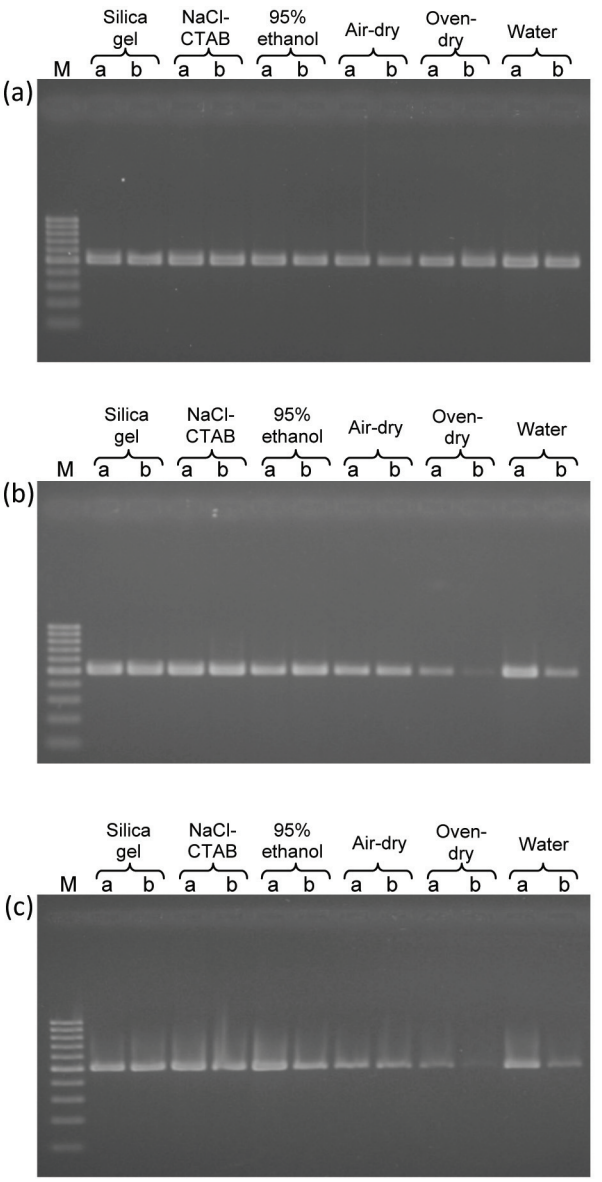

Figure 4: Agarose gel photo of PCR products from wood preserved under various methods after (a) 1 week, (b) 1 month and (c) 3 months. M: 100 bp ladder; a: inner bark (cambium); b: sapwood.

Table 2a: Spectrophotometer readings of DNA extracted from wood preserved in different methods for 1 week.

Sample	[DNA]	A260	A280	A260/A280	A260/A230
1 Week					
Silica gel - inner bark	71.50	1.43	0.71	2.01	1.82
- sapwood	47.8	0.96	0.51	1.89	1.41
NaCl-CTAB - inner bark	71.05	1.42	0.74	1.94	1.81
- sapwood	29.60	0.59	0.29	2.03	1.93
95% ethanol - inner bark	63.75	1.28	0.70	1.81	1.75
- sapwood	20.05	0.40	0.24	1.70	1.15
Air-dry - inner bark	61.25	1.23	0.62	1.98	1.81
- sapwood	44.65	0.89	0.47	1.90	1.48
Oven-dry - inner bark	17.90	0.36	0.22	1.65	0.92
- sapwood	12.10	0.24	0.19	1.30	0.67
Water - inner bark	36.70	0.73	0.42	1.76	1.48
- sapwood	27.80	0.56	0.26	2.15	1.99

Table 2b: Spectrophotometer readings of DNA extracted from wood preserved in different methods for 1 month.

Sample	[DNA]	A260	A280	A260/A280	A260/A230
1 month					
Silica gel - inner bark	177.50	3.55	1.78	1.99	2.19
- sapwood	233.45	4.67	2.23	2.10	2.33
NaCl-CTAB - inner bark	82.60	1.65	0.89	1.85	2.10
- sapwood	43.05	0.86	0.47	1.82	1.49
95% ethanol - inner bark	49.80	1.00	0.57	1.75	1.73
- sapwood	9.20	0.18	0.11	1.78	1.27
Air-dry - inner bark	252.50	5.05	2.42	2.08	2.20
- sapwood	145.80	2.92	1.42	2.06	2.09
Oven-dry - inner bark	22.55	0.45	0.26	1.75	1.32
- sapwood	13.70	0.27	0.18	1.53	0.73
Water - inner bark	53.85	1.08	0.58	1.85	1.88
- sapwood	128.80	2.58	1.25	2.07	2.28

Table 2c: Spectrophotometer readings of DNA extracted from wood preserved in different methods for 3 months.

Sample	[DNA]	A260	A280	A260/A280	A260/A230
3 months					
Silica gel - inner bark	225.20	4.50	2.21	2.04	2.13
- sapwood	138.95	2.78	1.34	2.07	2.07
NaCl-CTAB - inner bark	108.85	2.20	1.18	1.87	1.71
- sapwood	30.9	0.62	0.32	1.93	1.05
95% ethanol - inner bark	56.05	1.12	0.52	2.18	1.87
- sapwood	18.8	0.38	0.19	1.97	1.31
Air-dry - inner bark	268.1	5.36	2.58	2.08	2.05
- sapwood	84.00	1.68	0.85	1.98	1.72
Oven-dry - inner bark	34.15	0.68	0.43	1.59	0.59
- sapwood	13.60	0.27	0.19	1.47	0.62
Water - inner bark	36.40	0.73	0.41	1.76	1.17
- sapwood	11.35	0.23	0.15	1.47	0.49

4.2 Extraction from inner bark (cambium), sapwood and heartwood

Different parts of the wood were extracted using the modified CTAB method as PCR amplification using the extracted DNA was successful (Figure 5b). Similar with the findings by Asif and Cannon (2005), the DNA extracted could not be visualized by the ethidium bromide staining agarose gel (Figure 5a). This could be due to the low concentration of extracted DNA or that the DNA has degraded into small fragments (Rachmayanti *et al.*, 2009). However, the spectrophotometer detected the presence of DNA (Table 3) and the DNA extracted from all parts of the wood was sufficient for PCR amplification.

For pure DNA, the ratio of A260/280must be higher than 1.8 and the ratio of A260/230 should be between 2.0 to 2.2. Although the results obtained in the current experiment were not in this range except for A260/280 ratio for core heartwood, the extracted DNA is suitable for further analysis, such as PCR amplification, sequencing and DNA fingerprinting. The PCR products gradually decreased from the inner bark, sapwood to core heartwood as the intensity of PCR product bands gradually decreased. This is because higher DNA quantity and lower DNA degradation but higher PCR inhibitory content can be found from the inner bark and sapwood than from the heartwood (Rachmayanti *et al.*, 2009).

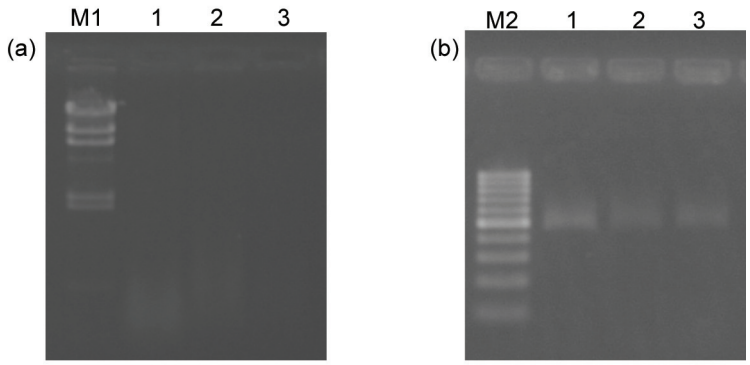

Figure 5: Gel photo of (a) DNA and (b) PCR product from different parts of the wood. M1: λ Hind III ladder; M2: 100 bp ladder; 1: inner bark (cambium); 2: sapwood; 3: core heartwood.

Sample	[DNA]	A260	A280	A260/A280	A260/A230
Inner bark (cambium)	197.50	3.95	0.75	5.30	0.28
Sapwood	23.40	0.47	0.22	2.16	0.42
Core heartwood	8.70	0.17	0.09	1.97	0.19

Table 3: Spectrophotometer readings of DNA extracted from different parts of the wood.

Additionally, the amount of DNA extracted is also dependent on the plant cells in each part of the wood. The cambium consists of living plant cells, whereas the sapwood consists of living and dead plant cells, and the heartwood consists of dead cells. The DNA extracted from all parts of the wood using the modified CTAB protocol could be amplified. This shows that the protocol has the potential for use in timber forensics.

5.0 Conclusion and recommendation

We examined the sample preservation methods using 95% ethanol, water, air-drying and oven-drying some of which represent the actual conditions of the logs after they have been felled: leaving under the sun at the log pond and transported using rivers, before processing in the mill. Transporting timber from the forest to the export point or importing countries will take time, sometimes years. The DNA would be mostly degraded but could still be extracted using appropriate protocols. One of the most crucial part for this study is to extract sufficient DNA for PCR amplification. Extracting DNA from fresh samples is routine but extracting DNA from preserved and processed wood is more complicated.

The present study using modified CTAB protocol succeeded in the extraction and amplification of DNA from the preserved wood stored up to three months. However, due to the project time frame, the protocol was only tested on wood preserved in the laboratory for up to three months. Longer period of wood preservation or using wood samples collected from the log ponds should be conducted to prove the effectiveness of the protocol developed.

In this study, we successfully developed the protocol to extract DNA from preserved wood of Ramin including from three important parts: cambium, sapwood and heartwood. Thus, the protocol can be used in the future for the testing of species identification and origin of Ramin timber.

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