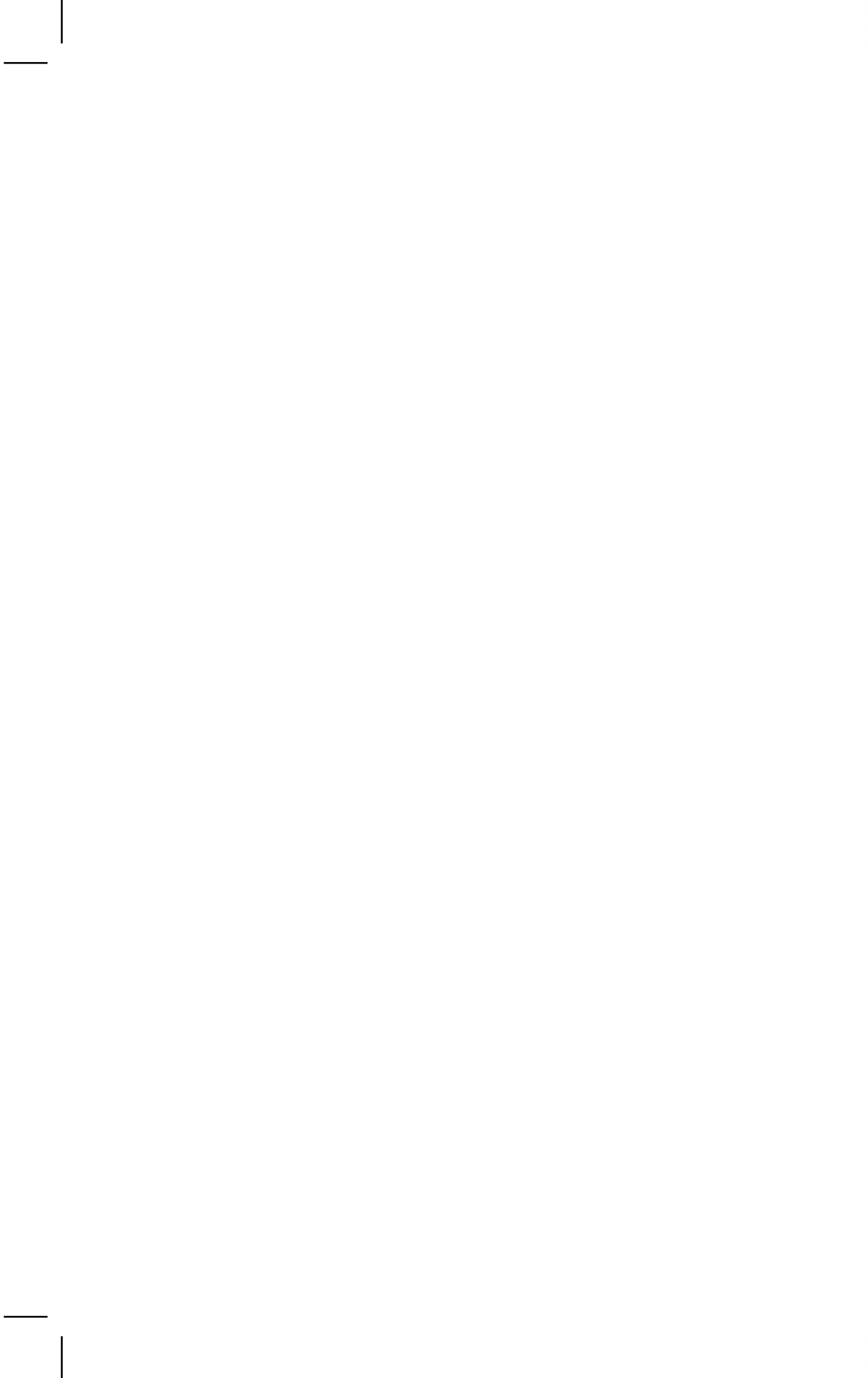


**STANDARD DNA EXTRACTION
PROTOCOL FOR
GONYSTYLUS SPECIES**

**BIBIAN DIWAY
LAI PEI SING
LUCY CHONG**





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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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Standard DNA extraction protocol for *Gonystylus* species/Bibian

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List of Abbreviations

DNA	Deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
PTB	N-phenacylthiazolium bromide
NaCl	Sodium chloride
PCR	Polymerase chain reaction
MgCl ₂	Magnesium chloride
dNTPs	Deoxynucleotides

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Summary

The DNA extraction from leaf and cambium of fresh wood samples is common especially for dipterocarp species. Often the extraction protocol used for timber trees is the CTAB extraction method which is normally modified to suit the species extracted. In this study we experimented with the modified CTAB extraction that could be applied not only for the DNA extraction from any *Gonystylus* species but also from any sample (leaf and wood) of *Gonystylus*.

The modification of extraction protocol focused on the elimination of polysaccharide contaminants from the DNA. Modification was done to obtain quality and suitable DNA for PCR and sequencing analysis for *Gonystylus* species. The DNA from leaf and wood samples of 21 *Gonystylus* species was extracted and purified with NaCl added in the CTAB buffer during the initial extraction and precipitation stage.

1.0 Introduction

The molecular DNA technology has been rapidly developing to verify species identification based on Genbank Blast search and to infer the origin of plant material. This has a good potential to enhance the regulation of timber trade especially for endangered and CITES-listed species, and their conservation. The fundamental challenge in this technology is to retrieve the DNA from the plant tissues. It is often very difficult and expensive particularly from wood samples.

Extracting DNA from the cambium of fresh wood samples is common and often successful for many species particularly for dipterocarp species. However, the extraction of DNA from other parts of the wood such as the heartwood and the sapwood is more complicated. It is possible to extract DNA from the sapwood as it contains both living and dead cells; however the DNA in general maybe highly degraded. Extracting DNA from the heartwood is hardest as the cells here are dead; it may be possible to do so with suitable protocols.

Deguilloux *et al.* (2002) successfully extracted and amplified DNA from all parts of the oak (*Quercus petraea*) logs including the heartwood suggesting that DNA from the dead heartwood could be amplified although with a lower success rate. A new extraction method for processed wood of *Gonystylus bancanus* was presented by Asif & Cannon (2005). The extraction was tested on fragments of furniture wood and samples of inner bark using cetyltrimethylammonium bromide (CTAB), DNAeasy Plant Mini Kit (QIAGEN) and N-phenacyl thiazolium bromide (PTB). However the

use of CTAB and the QIAGEN kit was not successful as the extracted DNA could not be amplified. Only extraction from processed wood and stem using PTB was successful. The extraction using CTAB or QIAGEN kit failed probably due to the presence of Millard products in the DNA extracts or impurities such as tannins, polyphenolics and polysaccharides (Shepherd *et al.* 2002). However by understanding the Millard products and other secondary compounds mechanism, the use of CTAB method with modification may be used to extract the DNA from wood. The extraction using PTB although successful takes longer time.

In this report, we only present the modified CTAB extraction protocol that was used successfully to extract DNA from leaf tissues and wood samples of *Gonystylus* species. Detailed experiments on the extraction of DNA from preserved wood samples stored under different preservation methods using this protocol are presented in another publication. The modified protocol was able to extract DNA from all *Gonystylus* species studied.

2.0 Materials and Methods

2.1 Plant samples

The DNA was extracted from leaf and wood samples collected from 13 populations in Sarawak: Kubah National Park, Gunung Gading National Park, Bako National Park, Lingga water catchment area, Upper Balui or Bakun Dam Area, Semenggoh Forest Reserve, Lambir Hills National Park, Lanjak Entimau Wildlife Sanctuary (LEWS), Bukit Mina Wildlife Corridor, Similajau National Park,

Mentawai in Gunung Mulu National Park, Biawak Village and Mukah Hill Forest Reserve (Figure 1). The leaf samples were preserved in silica gel and NaCl solution, while the wood samples from stem cuttings were stored for a duration of up to three months using the following methods:

1. Soaking in water
2. Air drying
3. Oven drying
4. Soaking in ethanol
5. Soaking in NaCl solution
6. Drying in silica gel



Figure 1: Ramin sampling locations in Sarawak. 1. Mentawai; 2. Lambir Hills National Park; 3. Similajau National Park; 4. Bukit Mina Wildlife Corridor; 5. Bakun Hydro-Electric dam ; 6. Mukah Hill; 7. Lanjak Entimau Wildlife Sanctuary; 8. Lingga water catchment area; 9. Bako National Park; 10. Semenggoh Forest Reserve; 11. Kubah National Park; 12. Biawak Village; 13. Gunung Gading National Park.

2.2 DNA extraction

The DNA was extracted from the samples using the QIAGEN plant kit and CTAB. The extraction using the QIAGEN kit produced low quality DNA. Thus only extraction using the CTAB method that produced higher quantity and quality of DNA is presented in this report.

Modified CTAB extraction protocol

1. A total of 5 ml CTAB buffer containing 100 mM Tris-HCl (pH8.0), 20 mM EDTA (pH8.0), 2.6 M NaCl, 3% CTAB, 1% PVP-40, 2% β -mercaptoethanol (added freshly) and 1 ml of 4 M NaCl (added freshly) was preheated in a 15 ml tube at 65°C for 30 minutes.
2. A total of 0.3 g of the tissue sample was ground in liquid nitrogen using mortar and pestle. The fine powder was then added to the tube which contained the preheated buffer and mixed well. The mixture was then incubated at 65°C for 30 minutes with occasional mixing.
3. After incubation, the mixture was centrifuged at 4,000 rpm for 10 minutes.
4. Then the mixture was extracted once using an equal amount of chloroform-isoamylalcohol (24:1). The two phases emulsified gently.
5. The two phases were separated by centrifuging the mixture at 4,000 rpm for 10 minutes.
6. The upper layer or the supernatant was then transferred to a new 15 ml tube.

7. A two-third amount of cold isopropanol and one-third amount of 4 M NaCl was added, mixed well and incubated overnight at -20°C.
8. The mixture was then centrifuged at 4,000 rpm for 15 minutes to pellet down the DNA.
9. Then the pellet was washed with 1 ml of 70 % ethanol and air-dried at room temperature.

2.3 DNA purification

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

2.4 PCR amplification and sequence analysis

A primer pair, trnF(F)-trnE(R) for chloroplast genome was used to test the DNA extracted from 21 *Gonystylus* species. The extracted DNA was amplified using Qiagen Type-it[®] Microsatellite PCR kit in the Palm Cycler (Corbett). The total PCR reaction volume of 20 µl contained 40 ng of genomic DNA, HotStarTaq[®] Plus DNA Polymerase, Type-it Microsatellite PCR buffer with 6 mM MgCl₂ and dNTPs. PCR reactions were carried using Gradient Palm Cycler[™] under the following conditions: 95°C for 5 minutes, followed by 28 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 90 seconds and extension at 72°C at 60 seconds, with final extension at 60°C for 30 minutes. DNA from three species (*G. bancanus*, *G. borneensis* and *G. brunnescens*) were selected for

sequencing. PCR fragments amplified using the DNA extracted from wood and leaves of these species were sent for sequencing. Sequences were resolved on a ABI 3130 xl Genetic Analyzer (Applied Biosystems, USA). The sequences were assembled and edited using Sequencher[®] version 5.0 sequence analysis software (Gene Codes Corporation, USA). Then, Pairwise alignment was done using Clustal W (www.genome.jp).

3.0 Results

3.1 DNA extraction and purification

The DNA extraction from leaves of several *Gonystylus* species yielded dirty yellow DNA with high viscosity (Figure 2). The viscosity showed that the leaves are rich in polysaccharides whereas the polyphenols oxidation and co-precipitation caused the browning of the DNA (Sablok *et al.*, 2009). In the agarose gel electrophoresis separation, the DNA samples in this condition caused smearing. This further suggests the presence of contaminants and degradation of the DNA (Sahu *et al.*, 2012). The presence of these contaminants would cause the failure in PCR amplification as they inhibit the action of enzymes, such as *Taq* polymerase (Moreira and Oliveira, 2010). Modification was done to obtain quality and suitable DNA for PCR and sequencing analysis for *Gonystylus* species.

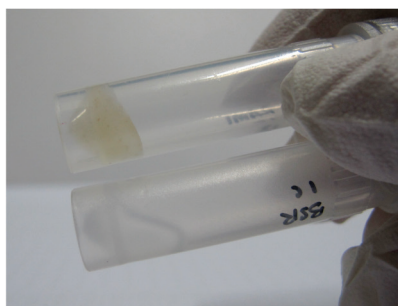


Figure 2: Dirty yellow DNA with high viscosity (upper tube) and clean DNA (bottom).

In order to overcome the problem of the presence of polysaccharides, 4 M NaCl was added in the CTAB buffer during the initial stage of extraction to remove the polysaccharides (Sahu *et al.*, 2012). Moreover, 4 M NaCl was also added during the precipitation of DNA as it facilitates the removal of polysaccharides by increasing

their solubility in isopropanol, and thus, only DNA will be precipitated (Sharma and Purohit, 2012). The viscosity of the DNA was greatly reduced; however, it was still present in few samples. The presence of viscosity in the DNA was finally reduced after purification. The DNA from leaf and wood samples for all 21 species was successfully extracted and purified using the same DNA extraction method in this study.

3.2 PCR amplification

As shown in Figure 3 and Figure 4, the amplification of DNA extracted from all 21 species of *Gonystylus* using the primer pair, trnF(F)-trnE(R) produced single and clear bands. The DNA extraction yielded PCR amplifiable DNA. Thus, the modified CTAB protocol was suitable for the extraction of DNA from wood and leaf samples for *Gonystylus* species.

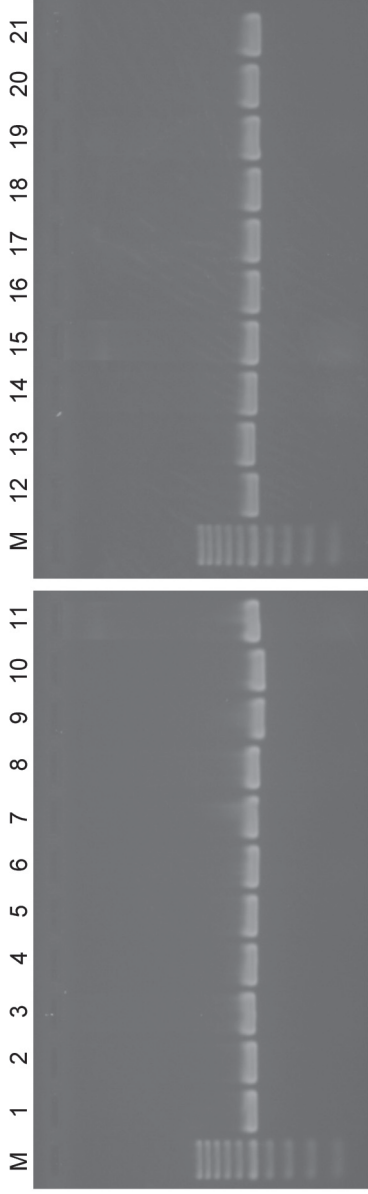


Figure 3: PCR amplification of DNA extracted from the leaf samples of 21 Ramin species. M: 100 bp DNA ladder; 1: *G. affinis* var *elegans*; 2: *G. affinis* ; 3: *G. areolatus*; 4: *G. augescens*; 5: *G. bancanus*; 6: *G. borneensis*; 7: *G. brunnescens*; 8: *G. calophylloides*; 9: *G. calophyllus*; 10: *G. consanguineus*; 11: *G. eximius*; 12: *G. forbesii*; 13: *G. keithii*; 14: *G. lucidulus*; 15: *G. maingayi*; 16: *G. micranthus*; 17: *G. othmanii*; 18: *G. spectabilis*; 19: *G. stenosepalus*; 20: *G. velutinus*; 21: *G. xylocarpus*.

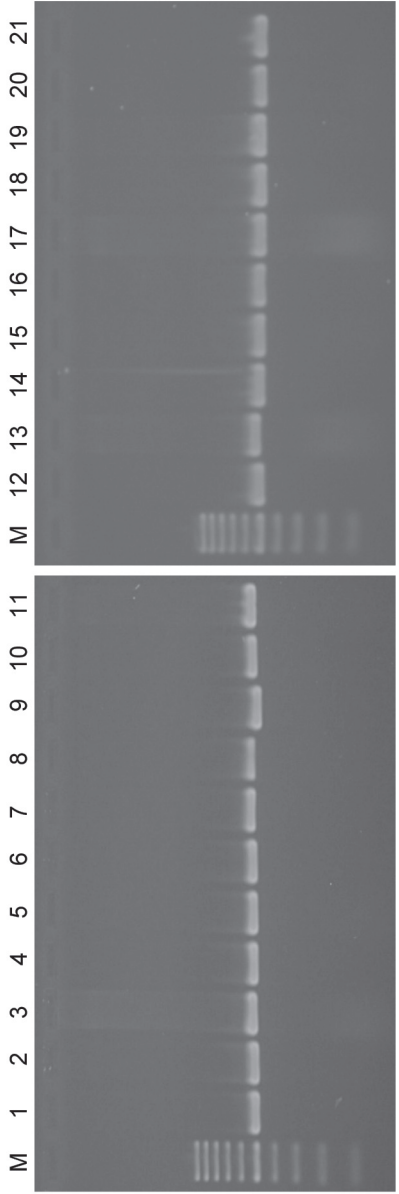


Figure 4: PCR amplification of DNA extracted from the wood samples of 21 Ramin species. M: 100 bp DNA ladder; 1: *G. affinis* var *elegans*; 2: *G. affinis*; 3: *G. areolatus*; 4: *G. augecens*; 5: *G. bancanus*; 6: *G. borneensis*; 7: *G. brunnescens*; 8: *G. calophylloides*; 9: *G. calophyllus*; 10: *G. consanguineus*; 11: *G. eximius*; 12: *G. forbesii*; 13: *G. keithii*; 14: *G. lucidulus*; 15: *G. maingayi*; 16: *G. micranthus*; 17: *G. othmanii*; 18: *G. spectabilis*; 19: *G. stenosepalus*; 20: *G. velutinus*; 21: *G. xylocarpus*.

3.3 Sequence analysis

PCR fragments of leaf and wood samples from the same species were sequenced and aligned (Figure 5 to Figure 7). The sequences obtained from the wood samples are all identical to the sequences obtained from the leaf samples. It is concluded that the DNA extracted from the wood samples is of good quality, similar to the DNA extracted from the leaves


```

G.borneensis_wood      TATCCCGACCATTACCAACACATCCTCATTTTAAATAGACGACTTGGGTCTATGTCAA
G.borneensis_leaf     TATCCCGACCATTACCAACACATCCTCATTTTAAATAGACGACTTGGGTCTATGTCAA
*****
G.borneensis_wood      TTAAAAATGAAAAAATCAAAAATAAAAATGAAAAAAGACGAAAAAAAAGTATTAAAAATAAAA
G.borneensis_leaf     TTAAAAATGAAAAAATCAAAAATAAAAATGAAAAAAGACGAAAAAAAAGTATTAAAAATAAAA
*****
G.borneensis_wood      GTAAAAAAAAGTAGTGAACCGGGAATTGATGGAATCTTCAAACCGGAAGACGTTGTAAAGTTT
G.borneensis_leaf     GTAAAAAAAAGTAGTGAACCGGGAATTGATGGAATCTTCAAACCGGAAGACGTTGTAAAGTTT
*****
G.borneensis_wood      CCGTCTGAGTAATGATATGGGATTTGAAATCAATTCAAAATGGGTATTCCTTGTCAAAGATGT
G.borneensis_leaf     CCGTCTGAGTAATGATATGGGATTTGAAATCAATTCAAAATGGGTATTCCTTGTCAAAGATGT
*****
G.borneensis_wood      CGATTTGTACGTATATTTGTGATAAGAGAAAAAATGATGCTCGGATACGCTTGTAAAAATG
G.borneensis_leaf     CGATTTGTACGTATATTTGTGATAAGAGAAAAAATGATGCTCGGATACGCTTGTAAAAATG
*****
G.borneensis_wood      TAAAAGAAATAGGATCAATGAGAAAACATAAGTAACTTTGAACCACCTAACAAAAAGGAGAGGG
G.borneensis_leaf     TAAAAGAAATAGGATCAATGAGAAAACATAAGTAACTTTGAACCACCTAACAAAAAGGAGAGGG
*****
G.borneensis_wood      TAGGATAAAAAAATAGGTACAAAATAAAAAAATAAGGTA
G.borneensis_leaf     TAGGATAAAAAAATAGGTACAAAATAAAAAAATAAGGTA
*****

```

Figure 5: Sequence alignment of PCR fragments of wood and leaf samples for *Gonystylus borneensis*.

G. brunnescens_wood	TATCCCGACCAITACCAACATCCTCCTCATTTTAAATAGACGACITGGGTCTATGTCAA
G. brunnescens_leaf	TATCCCGACCAITACCAACATCCTCCTCATTTTAAATAGACGACITGGGTCTATGTCAA

G. brunnescens_wood	TTAAAAATCAAAAAATCAAAATAAAATGAAAAAAGACGAAAAAAGTATTAAATATTTAAA
G. brunnescens_leaf	TTAAAAATCAAAAAATCAAAATAAAATGAAAAAAGACGAAAAAAGTATTAAATATTTAAA

G. brunnescens_wood	ATAAAAAGTCAAAAAAGTACTGAACGGGAATTGATGGAATCTTCAAACGGAACGACGTTGT
G. brunnescens_leaf	ATAAAAAGTCAAAAAAGTACTGAACGGGAATTGATGGAATCTTCAAACGGAACGACGTTGT

G. brunnescens_wood	AACTTCCGCTCGACTAATGATATGGAATTTGAAATCATTCAAATGGGTATTCCTTGGCTCAA
G. brunnescens_leaf	AACTTCCGCTCGACTAATGATATGGAATTTGAAATCATTCAAATGGGTATTCCTTGGCTCAA

G. brunnescens_wood	AGATGTCGATTTGTACGTATATTTGTGATAAGAGAAAAAATGATGCTCGGATACGCTTGT
G. brunnescens_leaf	AGATGTCGATTTGTACGTATATTTGTGATAAGAGAAAAAATGATGCTCGGATACGCTTGT

G. brunnescens_wood	AAAAATGTA AAAAGAAATAGGATGAAATGAGAAAGATAAGTACCTTTTGAACCACTAACAAAAAGG
G. brunnescens_leaf	AAAAATGTA AAAAGAAATAGGATGAAATGAGAAAGATAAGTACCTTTTGAACCACTAACAAAAAGG

G. brunnescens_wood	AGAGGGTAGGATAAAAAAATAGGTACAAAATAAAAAAATAAGGTA
G. brunnescens_leaf	AGAGGGTAGGATAAAAAAATAGGTACAAAATAAAAAAATAAGGTA

Figure 6: Sequence alignment of PCR fragments of wood and leaf samples from *Gonystylus brunnescens*.

G.bancanus_wood	TATCCCGACCATTACCACAACACATCCTCATTTTAAATAGACGACTTGGGTCTATGTCAA
G.bancanus_leaf	TATCCCGACCATTACCACAACACATCCTCATTTTAAATAGACGACTTGGGTCTATGTCAA

G.bancanus_wood	TTAAAAATCAAAAAATCAAAAATAAAAATCAAAAAAGACGAAAAAAAGTATTAAAAATAAAA
G.bancanus_leaf	TTAAAAATCAAAAAATCAAAAATAAAAATCAAAAAAGACGAAAAAAAGTATTAAAAATAAAA

G.bancanus_wood	GTAAAAAAAAGTAGTCAACCGGAATTGATGGAAATCTTCAAAAGCGAAGACGTTGTAAAGTIT
G.bancanus_leaf	GTAAAAAAAAGTAGTCAACCGGAATTGATGGAAATCTTCAAAAGCGAAGACGTTGTAAAGTIT

G.bancanus_wood	CCGTCTGACTAATGATATGGATTTGAATCATTCAAATGGGTATTCCTTGGCTCAAAGATGT
G.bancanus_leaf	CCGTCTGACTAATGATATGGATTTGAATCATTCAAATGGGTATTCCTTGGCTCAAAGATGT

G.bancanus_wood	CGATTTGTACGTATATTTGTGATAAGAGAAAAAATGATGCTCGGATACGCTTGTAAAAATG
G.bancanus_leaf	CGATTTGTACGTATATTTGTGATAAGAGAAAAAATGATGCTCGGATACGCTTGTAAAAATG

G.bancanus_wood	TAAAAAGATAGGATGAATGAGAAAAGATAAGTAACTTTGAACCACATAACAAAAGGAGAGGG
G.bancanus_leaf	TAAAAAGATAGGATGAATGAGAAAAGATAAGTAACTTTGAACCACATAACAAAAGGAGAGGG

G.bancanus_wood	TAGGATAAAAAAATAGGTAGAAAATAAAAAAATAAGTA
G.bancanus_leaf	TAGGATAAAAAAATAGGTAGAAAATAAAAAAATAAGTA

Figure 7: Sequence alignment of PCR fragments of wood and leaf samples from *Gonystylus bancanus*.

4.0 Conclusion

The potential of molecular DNA technology to identify species and geographical origin of species has given hope for the possibility to clarify the origin (illegal/legal) and species in timber trade particularly for CITES-listed timber species. The technology focuses on identifying DNA markers that are suitable for tracing timber. The early stage of the technology such as extracting DNA from plant tissues especially from wood samples is very challenging. High quality and quantity of DNA that is free from contaminants is required for the subsequent amplification and sequencing. Hence, suitable extraction protocol is crucial.

The modified CTAB protocol was used successfully to extract DNA from leaf and wood samples of all *Gonystylus* species under this project. The addition of 4 M NaCl in the CTAB buffer and during the isopropanol precipitation stage has greatly eliminated the presence of polysaccharides. The use of the modified CTAB protocol for *Gonystylus* species would enhance the analysis of assays for timber tracking in the future.

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