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Protocols

DNA Extraction From Processed Wood: A Case Study for the Identification of an Endangered Timber Species (*Gonystylus bancanus*)

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Abstract. We applied human forensic techniques to the extraction of whole genomic DNA from processed wood samples to explore the possibility of identifying an endangered tropical timber species by using DNA sequencing technology. High-yield and high-quality DNA samples were obtained from 2 commercial wood and 3 herbarium samples. Large PCR fragments ranging from 500-800 bp were successfully amplified from 2 chloroplast and 1 mitochondrial regions in all 5 samples, indicating limited degradation of the cytoplasmic genomes. DNA extraction from stem wood taken from herbarium specimens appeared superior to that from stem wood with bark intact or from leaf samples. DNA sequences from the *trn* regions allowed for easy identification of the focal species based on GenBank Blast search. Little sequence variation was observed in the 3 regions, with the mitochondrial *cox3* region completely conserved. Extraction of high-quality and large intact DNA fragments makes dry wood materials amenable to various DNA marker–based applications, including fingerprinting and historical approaches. By sampling stemwood, the wealth of historical information housed in international herbaria can be explored with minimal damage to taxonomically important features.

Key words: chloroplast, *Lithocarpus*, mitochondria, *N*-phenacylthiazolium bromide, Ramin, *Shorea*

Abbreviations: cpDNA, chloroplast DNA; CTAB, cetyl trimethyl ammonium bromide; mtDNA, mitochondrial DNA; PTB, *N*-phenacylthiazolium bromide.

Introduction

At a recent meeting of the International Tropical Timber Organization on phased approaches to certification, the need for external "third-party" verification of species identity and legal harvest was emphasized by trade officials, forest managers, and conservation groups (Simula, 2005). The use of genetic markers in this process has generally been viewed as being too difficult or expensive (Dykstra et al., 2002). However, molecular DNA technology has been rapidly developing, and many techniques that seemed unfeasible before are now routinely used. In anticipation of a new emergent technology, we have been exploring basic approaches

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for extracting whole genomic DNA from dry and processed wood, as part of a case study on an endangered species of tropical timber (*Gonystylus bancanus*). The ability to track timber resources from forest to marketplace is critically important for successful management and proper regulation of the timber trade, and the ability to reliably extract cooperative DNA from wood samples is a fundamental step in the application of genetic techniques to the timber trade (Deguilloux et al., 2003; Deguilloux et al., 2002; Gugerli et al., 2005).

Extraction of whole genomic DNA from fresh tissues is routine in studies of tropical forest species (Cannon and Manos, 2003; Kajita et al., 1998). DNA extracted from oak wood samples, by use of commercially available kits, was highly degraded with low yield (Dumolin-Lapegue et al., 1999). The use of these kits has not been fully demonstrated on the wide range of valuable tropical timber species. Previous work in our laboratory on a selection of plant groups did not yield high-quality DNA. Here we describe an effective extraction protocol that yields large amounts of high-quality and relatively intact whole genomic DNA. We demonstrate that such DNA can be obtained from dry processed wood samples purchased as an end-consumer in the United States and from heat-dried herbarium samples from the same selection of plant groups. We also clearly determine the taxonomic identity of ramin (*Gonystylus bancanus*) and stone oak (*Lithocarpus* sp.) wood samples from a partial chloroplast DNA sequence, verified against the public access GenBank.

Materials and Methods

Plant material

Whole genomic DNA was extracted from the following samples: dry processed dowel of *Gonystylus* purchased from a local craft retailer; a fragment of furniture wood unknown by species or origin; 2 herbarium samples of *Shorea* and *Lithocarpus* (portions of stems were cut off the herbarium sheet and bark was removed before grinding); 4 samples of inner bark from *Gonystylus* collected from Pahang, Malaysia (G2, G26), Sumatra, Indonesia (LOC3,5), and Borneo (Lg2).

DNA extraction

All samples and equipment were thoroughly cleaned with 70% ethanol before processing to reduce the risk of contamination. Genomic DNA from inner bark samples was extracted by a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Remaining samples were extracted by using CTAB, DNAeasy Mini Plant Kit (QIAGEN), and *N*-phenacylthiazolium bromide (PTB; Prime Organics).

CTAB extraction

Solutions

- CTAB extraction buffer: 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 mM NaCl, 3% CTAB, 1% polyvinylpyrrolidone, 0.2% mercaptoethanol

- Washing buffer: 76% ethanol, 10 mM ammonium acetate
- Chloroform-isoamylalcohol (24:1)
- Isopropanol
- 76% ethanol

Equipment
- Centrifuge
- Water bath
- 50-mL Fisherbrand tube
- 1.5-mL microcentrifuge Eppendorf tube

Protocol
1. 0.5 g of wood tissue was ground in liquid nitrogen with Millser IFM 66D (Iwatani). The powder was immediately transferred into preheated (65°C) extraction buffer and mixed thoroughly. The mixture was incubated for 2 h with occasional mixing.
2. The mixture was extracted twice with equal volumes of chloroform-isoamylalcohol (24:1), and 2 phases were emulsified gently.
3. Two phases were separated by centrifuging at 5000 rpm (2300*g*) for 10 min at 4°C.
4. The supernatant was transferred to a new 50-mL tube.
5. An equal volume of cold isopropanol was added, mixed well, and incubated overnight at –40°C.
6. DNA was pelleted down by centrifuging at 5000 rpm (2300*g*) for 20 min at 4°C.
7. The pellet was washed by adding 1 mL of washing buffer, and the process was repeated twice. The pellet was dried at room temperature and transferred to a 1.5-mL Eppendorf tube.
8. The pellet was twice washed with 70% ethanol and dried at room temperature.

Extraction with DNAeasy Mini Plant Kit

Genomic DNA was extracted by use of a DNAeasy Mini Plant Kit (QIAGEN) by strictly following the manufacturer's instructions.

PTB extraction

PTB has primarily been used for DNA extraction from ancient bone in paleontologic studies (Gugerli et al., 2005; Kelman and Kelman, 1999), from Neanderthal human remains (Krings et al., 1997), and from dung of the extinct ground sloth *Nothrotheriops shastensis* (Poinar et al., 1998). Here we describe a PTB-based DNA extraction protocol to extract DNA from plant tissues following the manufacturer's instructions (Prime Organics) with modifications.

Protocol

One gram of tissue of processed wood and 0.5 g of herbarium samples was ground into a powder with a Millser IFM 66D (Iwatani) and liquid nitrogen. The

Table 1. Chloroplast and mitochondrial primer pairs, sequences, amplification conditions, and product size amplified in *Gonystylus bancanus*. Primers *atpbE*, *rbcL*, *trnF*, *trnE* were used for chloroplast DNA and *cox3* for mitochondrial DNA amplification.

Primer Pair	Sequence (5′→3′)	Fragment Length, bp	Annealing Temperature, °C
atpbE (F)	GTGGAAACCCCGGGACGAA	800	55
rbcL (R)	TCTGTTTGTGGTGACATAAGTC		
trnF (F)	ATTTGAACTGGTGACACGAG	500	55
trnE (R)	GGTTCAAGTCCCTCTATCCC		
cox3 (F)	CCGTAGGAGGTGTGATGT	700	58
cox3 (R)	CTCCCCACCAATAGATAGAG		

fine powder was transferred to 5 mL of 0.5 M EDTA and soaked for 48 h at room temperature to demineralize the wood tissues. After demineralization, 500 µL (3 mg/mL) of proteinase K (FisherBiotech) and 1.0 mL of 0.1 M PTB (Prime Organics) were added. The mixture was mixed thoroughly to homogenize the components. Samples were incubated at 65°C for 12 h in a water bath. Extraction was done with equal volumes of phenol and chloroform, followed by 2 subsequent extractions with equal volumes of chloroform and isoamylalcohol (24:1). DNA was precipitated by adding 2 vol of cold absolute ethanol and 500 µL of 7.5 M ammonium acetate. This solution was stored at –40°C for 12 h and centrifuged at 5000 rpm (2300*g*) for 20 min at 4°C. The pellet was washed twice with 80% ethanol and left overnight in 80% ethanol. The pellet was recovered by centrifuging at 5000 rpm (2300*g*) and drying at room temperature.

Purification of genomic DNA

All DNA samples extracted by the 3 extraction protocols were further purified with the High Pure PCR Template Preparation Kit (Roche).

DNA quantification

Genomic DNA was quantified by measurement of sample absorbance at 260 nm with a NanoDrop Spectrophotometer. This instrument is highly sensitive and can measure low amounts of sample DNA (2 ng/µL). One microliter of DNA sample was submitted to check the quality and quantity of DNA. DNA was also quantified by agarose gel electrophoresis. Ten microliters of PTB-extracted DNA (200-500 ng) was electrophoresed on 1.2% agarose gels, stained with ethidium bromide, and visualized by UV fluorescence. The quantity of DNA was measured by comparing band intensity with that of standard amounts of DNA (Hi-Lo DNA marker; Minnesota Molecular).

PCR amplification and sequencing

We chose to amplify 1 mitochondrial DNA (mtDNA) and 2 chloroplast DNA (cpDNA)–noncoding regions (primer sequences in Table 1). PCR reactions were carried out on a thermocycler (Eppendorf) under the following conditions: 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at

55°C for 1 min for *atpbE*, *rbcL*, *trnE*, and *trnF*, and at 58°C for *cox3*, and extension at 72°C for 90 s, with a final extension at 72°C for 5 min. The total PCR reaction volume of 50 μL contained 50 ng of genomic DNA, 10 pmol of both primers, 1× PCR reaction buffer, 1.5 mM $MgCl_2$, and 0.5 U of Taq polymerase (Eppendorf). PCR products were visualized on 1.2% agarose gels. Each of the primer pairs amplified a product of the expected size. The PCR products were sequenced from both ends using their respective primers. These amplicons were purified with a QIAGEN MinElute PCR purification kit and were sequenced directly by using a CEQ dye terminator cycle sequencing kit (www.beckmancoulter.com) on a Beckman CEQ 2000. Sequence files were edited with the software Lasergene (DNA*DNASTAR) and aligned with CLUSTAL W software, version 1.6.

Results and Discussion

CTAB and the QIAGEN kit yielded low-quality and low quantities of DNA. These samples could not be amplified even though a detectable (<15 ng/μL) amount of DNA was present. DNA extracted from samples 1 and 2 with PTB could be visualized by ethidium bromide staining of agarose gels (20 ng/μL), whereas dry wood samples yielded high amounts of DNA (50 ng/μL). DNA appeared degraded in all samples, and fragment sizes ranged from 50 bp to 10.0 kbp. This difference between samples could be primarily due to the small quantity of sample used for extraction. Soaking wood tissues in EDTA leached out the water-soluble wood components and mineral moieties, as was evident from the change in buffer color. Earlier attempts to extract DNA from the herbarium leaf samples (by using CTAB and the QIAGEN kit; data not shown) yielded highly degraded and oxidized DNA that failed in subsequent PCR amplifications. The DNA extractions from stem wood, after the bark was removed, yielded samples that were successfully amplified in all 3 regions. The DNA in the stem wood may be better preserved in comparison to leaf tissues because the leaves go through a more dramatic and rapid change during the drying process than does stem wood.

Low DNA yield and failed PCR amplifications by use of CTAB or the QIAGEN kit could be due to the presence of Maillard products in the DNA extracts or impurities such as terpenes, polyphenolics, and polysaccharides (Shepherd et al., 2002). However, DNA oxidation is the significant byproduct of the Maillard reaction of protein and carbohydrates (Evershed et al., 1997). Maillard products are simply the condensation of reducing sugars with primary amines (proteins), which could significantly reduce the quality and yield of extracted DNA (Poinar et al., 1998). DNA extraction with PTB cleaves glucose-derived protein cross-links and helps to release the DNA that might have been trapped within sugar-derived condensation products.

PCR amplifications were successful in PTB-extracted samples (Figure 1). Both cpDNA and mtDNA regions were successfully amplified. One intergenic spacer region on the chloroplast genome (*atpbE* and *rbcL*) produced 800-bp-long amplicons, revealing that long DNA strands were still intact. Previous reports of DNA extraction from dry wood yielded only 300-bp amplification fragments

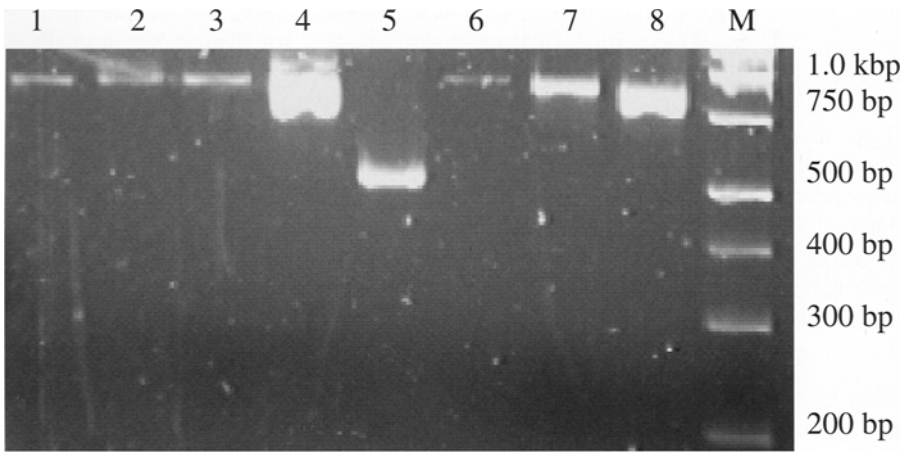

Figure 1. PCR amplification of the chloroplast DNA from processed wood and herbarium samples by using *atpbE* and *rbcL, trnF* and *trnE* primers. 1, 3: unknown processed wood sample amplified with *atpbE* and *rbcL* (800 bp); 2, 8: *Gonystylus*; 4, 5: *Lithocarpus* herbarium samples, amplified with *atpbE* and *rbcL* (800 bp) and *trnE* and *trnF* (500 bp) regions, respectively. 6, *Shorea*; 7, *Gonystylus* processed wood sample amplified with *atpbE* and *rbcL* (800 bp). Strong amplifications were observed both in fresh inner bark DNA sample and in processed wood.

(Dumolin-Lapegue et al., 1999). Our extractions from a wooden desk older than 50 years did not yield useful DNA, which may indicate that this discrepancy was due to the age of the wood used.

Potential problems with ancient or relatively damaged DNA are the amplification of sequences that include artifacts of DNA repair or polymerase errors during amplification (Dumolin-Lapegue et al., 1999). Comparison of samples on the basis of *trnE* and *trnF* cpDNA sequence regions revealed that DNA sequences from all ramin samples were identical to one another and closed matched those deposited in GenBank (Table 2), and the DNA sequence obtained from stem wood of the *Lithocarpus* sample was identical to that already sequenced in a larger phylogeographic study (Cannon and Manos, 2003). Furthermore, PCR controls and sequencing of 2 different reactions from the same dry wood sample yielded the same sequences, thus excluding the possibility of the results being artifacts of the PCR amplification process.

The successful extraction of useful DNA from various wood samples from a range of plant taxa for PCR amplification and DNA sequencing is a necessary first step in developing DNA fingerprinting techniques for the monitoring and management of the tropical timber trade. The ability to amplify both long and short regions of DNA will allow the inclusion of standard DNA sequencing loci, as well as traditional microsatellite loci, in the genetic database. The construction of an adequate database of DNA fingerprints for the large number of economically valuable timber species on the market in Southeast Asia (Danimihardja and Gandawidjaja, 1998; Soerianegara and Lemmens, 1994) will be greatly facilitated if DNA obtained from large international herbarium collections could be directly

Table 2. Chloroplast DNA sequence variation in *Gonystylus bancanus*: comparison of substitutions and insertions/deletions observed in the chloroplast DNA *trnE* and *trnF* regions.

	Sample				
Nucleotide Position	Unknown	Malaysia (G2)	Malaysia (G26)	Borneo (Lg2)	Indonesia (Loc3,5)
28	—	A	A	A	A
36	T	A	A	—	—
37	A	C	—	—	C
47	A	T	T	T	T
99	—	A	A	A	A
155	—	C	—	—	—
213	—	—	—	T	—
226	—	—	T	T	—
246	—	—	—	T	—
250	—	—	—	T	—
333	—	—	—	—	A
383	A	—	A	—	—
451	—	—	—	A	—
452	—	A	A	A	A
453	A	A	A	A	—
475	—	—	—	A	—

Mutations were observed at both inter- and intrapopulation levels. Bornean sample showed several insertions "T" clearly differentiated from Indonesian and Malaysian haplotypes.

incorporated, thus providing objective knowledge of prior conditions before timber harvest. Using a small piece of stem wood causes little to no damage to taxonomically informative characteristics of the herbarium sheet; therefore, our technique should be more acceptable to curators and collection managers. Our application of a forensic DNA extraction protocol, typically used for human remains, to wood samples indicates that high-quality and large quantities of whole genomic DNA can be obtained from wood samples and herbarium samples from a range of species and supplies a possible shortcut in the creation of DNA fingerprint databases as a verification tool in the timber certification process.

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