



SNP-based method for the genetic identification of ramin *Gonystylus* spp. timber and products: applied research meeting CITES enforcement needs

Rob Ogden^{1,5,*}, H. Noel McGough², Robyn S. Cowan², Lilian Chua³,
Madeleine Groves², Ross McEwing^{4,5}

¹Wildlife DNA Services, Tepnel, Appleton Parkway, Livingston EH54 7EZ, UK

²Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK

³Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia

⁴TRACE Wildlife Forensics Network, Science Campus, Deiniol Rd, Bangor LL57 2UW, UK

⁵School of Biological Sciences, Bangor University, Deiniol Road, Bangor LL57 2UW, UK

ABSTRACT: We describe the development of a genetic assay for the identification of the tropical hardwood ramin *Gonystylus* spp., a CITES-listed genus subject to illegal international trade. Samples representing different ramin species, confamilial genera and morphologically similar taxa were obtained. DNA extraction from leaf material and wood products was achieved using commercially available kits. Five chloroplast genes were examined for Single Nucleotide Polymorphism (SNP) loci capable of discriminating ramin. A locus within the *matK* gene was selected and a TaqMan[®] assay designed for sample genotyping. The assay was validated against different species to demonstrate its specificity and reproducibility. The final assay provides a robust, cost-effective, transferable method for identifying processed ramin. The research represents a feasibility study, addressing each of the steps required to develop a genetic identification assay for enforcement use; however, it should be noted that further work is required to produce a fully validated forensic identification tool.

KEY WORDS: Illegal trade · Logging · Forensics · Endangered species · Species identification

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INTRODUCTION

The illegal international trade in flora and fauna leads to loss of habitat and biodiversity on a global scale, as well as sustaining organised crime. Despite increasing efforts to implement trade regulations, fundamental issues such as the species identification of traded products continue to hamper law enforcement. In cases where external anatomical characteristics have been lost, forensic DNA techniques are now being used to identify items being smuggled or sold illegally. Genetic identification of species has been applied to a number of Convention on International

Trade in Endangered Species (CITES) enforcement issues, including illegal fishing (Magnussen et al. 2007), the ivory trade (Wasser et al. 2004) and trade in traditional Asian medicines (Linacre et al. 2003). Applications to timber identification have long been recognised but until recently such approaches have been restricted by technical limitations. Advances in DNA recovery (Deguilloux et al. 2002, Asif & Cannon 2005), coupled with the increase in availability of genomic sequence data through initiatives such as the Barcoding of Life (Chase et al. 2007, Hollingsworth 2008), have now paved the way for the development of forensic genetic timber-identification methods.

*Email: rogden@tepnelscientific.com

The current method for the identification of traded timber and processed wood products is anatomical analysis. Identification of single samples can be performed quickly and reliably by expert wood anatomists, and the technique is routinely employed at specialist research institutes in several countries, including the UK, the USA and Germany. The recent development of a computer-based anatomical key (Richter et al. 2008) has also improved the potential for initial identification of many traded timber species by enforcement officials. Despite these resources, identification of certain species remains problematic; with a growing need to routinely screen large numbers of samples, a complementary technique is required. The development of a DNA-based technique, employed as a standard laboratory tool, capable of high throughput analysis and of delivering categorical identification for forensic application would enhance the ability of both commercial and government authorities to regulate the trade. Where necessary, such techniques would also support the enforcement of national and international legislation, allowing exporting and importing countries to identify and prosecute illegal movements, greatly increasing our chances of ensuring a sustainable trade in timber.

Ramin *Gonystylus* spp. is a light tropical hardwood tree of swamp forests with a centre of diversity in the island of Borneo (Tawan 2004). The distribution includes Brunei Darussalam, Fiji, India (Nicobar Islands), Indonesia (Kalimantan and Sumatra), Malaysia (Peninsular Malaysia, Sabah, Sarawak), Papua New Guinea, the Philippines and the Solomon Islands (T. M. A. Utteridge pers. comm.). The *Gonystylus* genus includes about 30 species (Tawan 2004), although a full taxonomic review to confirm the exact status of described species is currently required (T. M. A. Utteridge pers. comm.). Of these species some 10 taxa are reported to have uses as timber or medicine (some taxa produce Agar) (CITES 2002). A number of species have been reported in international trade, with *G. bancanus* supplying, by far, the greater part of the market. Lim et al. (2004) estimated the annual trade in ramin to be worth

more than US \$100 million yr⁻¹. A proposal submitted by Indonesia to list the genus *Gonystylus* on Appendix 2 of CITES was adopted by consensus at the 13th meeting of the CITES Conference of the Parties in Bangkok, Thailand in October 2004 (Gray 2005). The generic listing was proposed to allow adequate identification using traditional wood anatomy techniques. All parts and derivatives of ramin wood were subject to CITES regulation to match the international market.

The European Union (EU) is a major importer of ramin, as is the USA. Within the EU, Italy imports the largest portion of ramin; other major importers include the UK, the Netherlands, Germany, Belgium, and Spain (Affre et al. 2004). Due to its high market value, ramin is subject to illegal trade into and within the EU, often in the form of finished products (Fig. 1) that are difficult or too numerous to identify anatomically. The EU also re-exports ramin products, such as dowels, picture frames and paint brushes. The trade is dominated and hence characterised by the movement of large numbers of finished products. It was the first trade of this type to be subject to CITES control (Affre et al. 2004).

We present here the research and development undertaken to produce a validated method for the forensic genetic identification of ramin *Gonystylus* spp. timber and wood products for use by enforcement agencies (customs/border inspection), and traders of timber and wood products. The project was designed as a pilot study to provide proof of concept for the development of an applied assay. It therefore had a limited scope, targeting a single genus, but including all stages in the production of an assay, from sample collection and DNA extraction through to marker selection, assay design and validation. Importantly, the research was driven by the needs of the end user, in this case, Her Majesty's (HM) Revenue and Customs (UK). This approach led to criteria governing the assay being specified from the outset, including considerations of assay transferability, per-sample cost, sample capacity, speed of assay and robustness of the technique to provide reliable evidence for legal prosecution.



Fig. 1. Examples of previously identified processed ramin timber products: (a) window blind toggle; (b) garden trowel handle; (c) panel of window blind

MATERIALS AND METHODS

Sample collection. Ramin: Authenticated samples were obtained for 7 species of ramin including all of those commonly traded. Leaf tissue was obtained from the Forest Research Institute of Malaysia under CITES licence. Wood samples were provided from the collection at Royal Botanical Gardens (RBG) Kew. In addition, a number of samples of processed wood products were donated by HM Revenue and Customs CITES team. These had been stored following seizure and had been identified anatomically by wood anatomists at RBG Kew as originating from ramin species.

Genetically similar taxa: The genus *Gonystylus*, belongs to the family Thymeleaceae, subfamily Gony-styloideae, which also includes the genera *Solmsia*, *Arnhemia* and *Lethedon* (Van der Bank et al. 2002). Representatives of each genus (*S. calophylla*, *A. cryptantha* and *L. cernua*) were obtained from the DNA bank at RBG Kew to compare with *Gonystylus* spp. at the same genetic marker.

Anatomically similar taxa: A total of 17 additional species were selected for validation of the ramin identification assay on the basis that their timber and or processed products may be confused with ramin during anatomical analysis. All samples were donated by the Economic Botany Collection at RBG Kew.

DNA extraction. DNA extraction was initially attempted on freshly dried leaf tissue, followed by timber samples and finally worked wood products in a dedicated (PCR-free) laboratory. Extraction was performed directly on leaf tissue. Wood material was prepared by initially sanding samples to remove the outer wood layer where chemical deposits and exogenous DNA contamination would likely be present. Sub-samples of wood were then taken using a hacksaw, sterilised between samples. Extraction controls were carried through to PCR amplification.

Two commercial kits designed for plant material were used, the Nucleon Phytopure[®] kit (Tepnel Life Sciences) based on a simple salt extraction method and the GMO BioKit[®] (Tepnel Biosystems) based on magnetic bead DNA capture. Manufacturer's protocols were followed throughout.

The quality of DNA recovered from both the leaf and processed timber was assessed through PCR amplification. The DNA recovered from processed wood was expected to be of inferior quality (reduced yield and fragmented) compared to fresh leaf material. The extent of the fragmentation was assessed using PCR amplification of 2 different sequence length products. The first fragment was an 800 bp fragment from the chloroplast *matK* gene amplified using the universal primers developed for this purpose; the second fragment was a 300 bp product (more than the product

length required for the final DNA test) from the same gene using specifically designed primers based on ramin DNA sequences. The primer sequences are as follows: Large Fragment Forward Primer (LFFP), CCTATCCATCTGGAAATCTTAG (*matk2.1*); LF reverse P, GTTCTAGCACAAGAAAGTCG (*matk5*); Small FFP (SFFP), CGTGTGGTCTCAATCAGGAA (*matk415f*); SFRP, CGATGCGTTACAAAATTCG (*matk637r*). PCR reaction conditions were: 20 µl reaction containing 0.25 units ABgene Thermo-Start DNA Polymerase, 1× Thermo-Start[®] reaction buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP, 20 pmol primer and 2.0 µl of template DNA. PCR was performed on a PTC-200 MJ Research thermocycler under the following parameters: 95°C for 15 min; 40 cycles of 94°C for 60 s, 48°C for 60 s, 72°C for 60 s; followed by 72°C for 5 min. Amplification products were visualised under UV light following electrophoresis on an ethidium bromide-stained 2% agarose gel.

Genetic marker selection. Five candidate partial gene regions present in plastid DNA were selected for investigation as potential discriminatory markers: *matK*, *rpoC1*, *rpoB*, *accD* and *ndhJ*. DNA sequences were initially produced for 2 *Gonystylus* species (*G. macrophyllus* and *G. borneensis*) and the 3 con-familial genera to identify inter and intra-genus Single Nucleotide Polymorphisms (SNPs). Following the selection of a single target gene region, the reference samples for the remaining 5 *Gonystylus* species were sequenced to confirm that SNP genotypes were conserved within the genus. The barcoding primer sequences and PCR reaction conditions used to amplify each region are published at www.kew.org/barcoding/protocols.html. PCR products were cleaned using QIAquick silica columns (Qiagen) and sequenced using Bigdye 3.1 terminator chemistry on an ABI 3730 following manufacturer's protocols (Applied Biosystems). The sequences were aligned to allow identification of SNPs that vary among species. The availability of diagnostic SNPs was then used to select the genetic marker to be targeted during assay development.

Assay design. The SNP genotyping method selected for the assay was a TaqMan[®] probe (Applied Biosystems). Three candidate SNPs were assessed for their suitability as TaqMan[®] target loci. Primers and probes were subsequently designed for a single SNP locus using the proprietary software, Primer Express (ABI). The total target sequence was only 95 bp long and consisted of the following primers and probes: primer 1, 5'-GGAAAATCTATTCTCGCTTCAAAGA-3'; primer 2, 5'-TTATGGCAATATCATTTTTACGTGTG-3' and probe, 5'-ACGCCTCTTCTrATG-3'. The assay was run under standard TaqMan[®] PCR conditions on an ABI 7500 machine. In addition to positive ramin and non-ramin

samples, a mixed sample was created from a 1:1 ratio of ramin and non-ramin DNA and run alongside the test samples and no-template controls (NTCs).

Validation. The resulting TaqMan[®] assay was investigated to examine its performance with different tissue types (leaf vs. heart wood), different species (17 lookalike species) and different DNA concentrations. Standardised conditions were employed to assess assay performance. In addition to validation of the assay itself, a positive control was developed to demonstrate the presence of DNA in samples that show no result with the TaqMan[®] probe, i.e. for species that are highly genetically distinct from ramin. The control consisted of a chloroplast microsatellite locus (CCMP2) approximately twice as long as the TaqMan[®] target region (~189 bp), amplified under published conditions (Weising & Gardener 1999), that amplifies in most angiosperms.

RESULTS

Sample collection. A full list of the samples obtained is shown in Table 1. These samples were sufficient to develop the assay within the remit of this pilot project. Examples of the processed timber products used are shown in Fig. 1.

DNA extraction. DNA was successfully recovered and PCR-amplified from both fresh plant material and 3 specimens of processed/worked ramin. In all cases, the short (300 bp) region could be amplified; however, the large (800 bp) fragment was only amplified in fresh leaf material, as expected. Both extraction kits enabled amplification of the target loci.

Genetic marker selection. Evaluation of the DNA sequences produced for each of the 5 candidate gene regions indicated that the marker *matK* contained the highest number of SNPs potentially capable of distinguishing ramin from non-ramin samples (Table 2). Of the 5 SNP positions identified, 3 were conserved across all 7 *Gonystylus* species; 1 of these was then selected for the design of a TaqMan[®] assay on the basis of probe stringency. The assay performed as predicted, correctly discriminating ramin from non-ramin samples. Sample identification was definitive in all cases, with distinct clusters formed for both SNP genotypes (Fig. 2). The mixed sample and no-template controls were positioned as expected. To process 24 samples takes approximately 2 h.

Validation. The assay worked with DNA recovered from all sample types tested, including the worked products. Results were obtained at DNA concentrations down to 0.01 ng μl^{-1} . No false positives were observed across the 20 non-ramin species tested (3 genetically similar and 17 anatomically similar). 'No

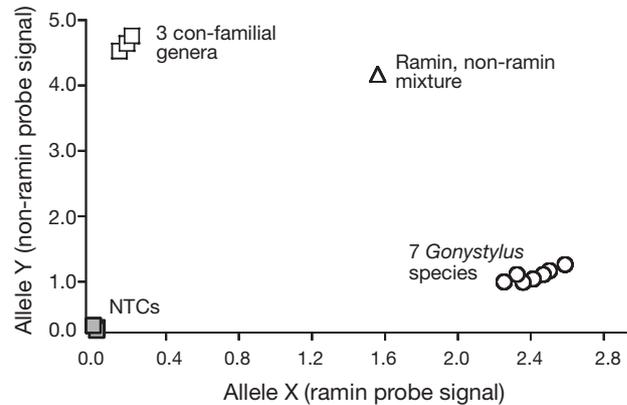


Fig. 2. Results of the TaqMan[®] assay showing differentiation of the 7 ramin *Gonystylus* spp. from the 3 genetically related species. The assay is based on allele-specific fluorescent probes that are incorporated during PCR according to the nucleotide base present at the Single Nucleotide Polymorphism (SNP) (1 colour for each allele). The genotype of each sample is then obtained by measuring the fluorescent intensity of the probes. In the ramin assay, the x-axis displays the signal from the ramin allele and the y-axis the non-ramin allele. Triangle represents a 1:1 DNA mixture control, shaded squares are no-template controls (NTCs)

result' was recorded for all 17 lookalike species; however, 15 of these amplified with the microsatellite control, demonstrating the presence of amplifiable wood DNA.

DISCUSSION

This study has demonstrated the potential to efficiently combine a series of modern genetic analysis techniques in order to produce a working assay suitable for direct application as an enforcement tool. The ability to genetically discriminate traded ramin from non-ramin wood provides a complementary technique to current anatomical methods. The use of standard commercially available extraction methods and the TaqMan[®] genotyping platform offers a high-throughput solution that can be applied worldwide with minimal training. It is estimated that 96 samples could be readily processed by 1 person in a single day, dramatically reducing time costs relative to current methods. This in turn will facilitate larger scale trade monitoring and survey effort, providing the tools required to adequately enforce the CITES listing and deter the illegal movement of ramin.

The scope of the work undertaken was limited to a proof of concept model, and further work is planned to increase the utility of the assay produced. Heavily processed wood products, such as heat-treated plywood, need to be tested to examine the performance of the

Table 1. All samples provided for the project, showing type, provider and geographic origin of samples. HMRC: Her Majesty's Revenue and Customs CITES Team; FRIM: Forest Research Institute of Malaysia; RBGK: Royal Botanic Gardens, Kew

Category Species	Sample type	Sample provider	Geographic provenance
Ramin — reference sample			
<i>Gonystylus maingayi</i>	Leaf	FRIM	Malaysia
<i>Gonystylus bancanus</i>	Leaf	FRIM	Malaysia
<i>Gonystylus affinis</i>	Leaf	FRIM	Malaysia
<i>Gonystylus confusus</i>	Leaf	FRIM	Malaysia
<i>Gonystylus brunnescens</i>	Leaf	FRIM	Malaysia
<i>Gonystylus macrophyllus</i>	DNA	RBGK	Indonesia
<i>Gonystylus borneensis</i>	DNA	RBGK	Brunei
Ramin — case sample			
<i>Gonystylus</i> spp.	Worked timber	HMRC	Unknown
<i>Gonystylus</i> spp.	Worked timber	HMRC	Unknown
<i>Gonystylus</i> spp.	Worked timber	HMRC	Unknown
Genetically similar			
<i>Solmsia calophylla</i>	DNA	RBGK	Australia
<i>Arnhemia cryptantha</i>	DNA	RBGK	Unknown
<i>Lethedon cernua</i>	DNA	RBGK	New Caledonia
Morphologically similar			
<i>Alstonia macrophylla</i>	Wood	RBGK	Malaysia
<i>Alstonia macrophylla</i>	Wood	RBGK	Sri Lanka
<i>Alstonia scholans</i>	Wood	RBGK	Sri Lanka
<i>Alstonia scholans</i>	Wood	RBGK	Malaysia
<i>Anthocephalus cadamba</i>	Wood	RBGK	Western New Guinea
<i>Anthocephalus cadamba</i>	Wood	RBGK	Malaysia
<i>Anthocephalus monandaefolius</i>	Wood	RBGK	India
<i>Brosimum alicastrum</i>	Wood	RBGK	Mexico
<i>Brosimum alicastrum</i>	Wood	RBGK	Trinidad
<i>Brosimum alicastrum</i>	Wood	RBGK	Jamaica
<i>Brosimum alicastrum</i>	Wood	RBGK	USA
<i>Brosimum paraense</i>	Wood	RBGK	USA
<i>Dyera costulata</i>	Wood	RBGK	Malaysia
<i>Dyera costulata</i>	Wood	RBGK	Malaysia
<i>Dyera costulata</i>	Wood	RBGK	North Borneo
<i>Intsia baken</i>	Wood	RBGK	Malaysia
<i>Intsia bijuga</i>	Wood	RBGK	North Borneo
<i>Intsia palembanica</i>	Wood	RBGK	Malaysia
<i>Jacaranda copaia</i>	Wood	RBGK	Panama
<i>Jacaranda copaia</i>	Wood	RBGK	Guyana
<i>Jacaranda copaia</i>	Wood	RBGK	Unknown
<i>Jacaranda copaia</i>	Wood	RBGK	Surinam
<i>Jacaranda copaia</i>	Wood	RBGK	Guyana
<i>Jacaranda copaia</i>	Wood	RBGK	Unknown
<i>Jacaranda copaia</i>	Wood	RBGK	Surinam
<i>Jacaranda copaia</i>	Wood	RBGK	Unknown
<i>Quassia amara</i>	DNA	RBGK	Unknown
<i>Shorea accuminata</i>	Wood	RBGK	Malaysia
<i>Shorea leprosula</i>	Wood	RBGK	Malaysia
<i>Shorea panvistipulata</i>	Wood	RBGK	Sarawak
<i>Shorea platyclados</i>	Wood	RBGK	Malaysia
<i>Shorea platyclados</i>	Wood	RBGK	North Borneo
<i>Simaruba amara</i>	Wood	RBGK	Guyana
<i>Simaruba amara</i>	Wood	RBGK	Trinidad and Tobago
<i>Simaruba amara</i>	Wood	RBGK	Brazil

assay on a wider range of case-type samples. Further, genetically related and anatomically similar taxa should also be tested, focussing on more species within the con-familial genera and additional *Gonystylus* species sampled from a broader geographic range. The use of a single SNP to identify ramin demonstrates the

strength of the technique, but the inclusion of further SNPs showing the same diagnostic pattern would increase confidence in the assay without impacting heavily on practical implementation.

The development of this assay builds on recent research seeking to apply molecular genetic tech-

Table 2. Detail of the variable nucleotide positions observed at each of the 5 genes for 5 reference samples. Numbers shown are base pairs from the start of the consensus gene sequence for all 5 species. Gene sequence lengths were 300 bp (*accD*), 737 bp (*matK*), 432 bp (*ndhJ*), 426 bp (*rpoB*) and 619 bp (*rpoC*). GenBank accession nos. are given in the same order as the genes in the previous columns. Potential ramin diagnostic Single Nucleotide Polymorphisms (SNPs) are identified by an asterisk

Species	Variable positions at each locus					GenBank Accession No.s
	<i>accD</i>	<i>matK</i>	<i>ndhJ</i>	<i>rpoB</i>	<i>rpoC1</i>	
	22	11112222334455556666667	1	1112223	11122233	
	44	401692237166602661344793	78	682562598	17906959	
	09	166881659940760128408711	66	695639271	11877256	
<i>Gonystylus macrophyllus</i>	TG	GCCACTCAAAAAGTGGGCGATTCC	GT	TCATGCCTC	TCGATACA	FJ379770, FJ379775, FJ379780 FJ379785, FJ379792
<i>Gonystylus borneensis</i>	TG	GCCACTCAAAAAGTGGGCAATTCC	GT	TCATGCCTC	TCGATCCA	FJ379774, FJ379779, FJ379784 FJ379789, FJ379794
<i>Solmsia calophylla</i>	TA	AACAATCGAAGATCGAGCAGTGCT	CC	CTATGCCTT	CYRAYMGA	FJ379771, FJ379776, FJ379781 FJ379786, FJ379790
<i>Arnhemia cryptantha</i>	TA	GATGCCTGCAGCTCTATGCGTGTC	GC	CTTCAATGT	CTGGCCGA	FJ379772, FJ379777, FJ379782 FJ379787, FJ379791
<i>Lethedon cernua</i>	GG	GACACTCAACGACCGAGCAGCGCC	GC	CTATGCCTC	CCAATCGC	FJ379773, FJ379778, FJ379783 FJ379788, FJ379793
Ramin discriminatory SNPs		* * * * *	*	**	* *	

niques to plant identification for trade control. Several publications have described the use of short fragment-length sequencing as an effective method for analysing degraded DNA (Deguilloux et al. 2004, Ziegenhagen et al. 2005, CITES 2006) and the use of real-time PCR has also been highlighted (Gugerli et al. 2005). The current study draws together many methodological advances within a forensic framework and suggests how the future application of genetic research to trade enforcement can be achieved.

In the broader enforcement context, the use of forensic genetic techniques for timber identification can be seen as complementary to existing anatomical identification techniques. With the development of new genetic assays, work should also be undertaken to develop an integrated approach to timber identification that can be implemented on a global scale, providing a suite of tools for enforcement officers.

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*Editorial responsibility: Brendan Godley,
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