CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES
OF WILD FAUNA AND FLORA

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Identification matters

THE DEVELOPMENT OF GENETIC TECHNIQUES FOR THE FORENSIC IDENTIFICATION OF *GONYSTYLUS* (RAMIN) TIMBER AND WOOD PRODUCTS

1. This document has been submitted by the Scientific Authority of the United Kingdom of Great Britain and Northern Ireland.

2. Introduction

   a) The trade in *Gonystylus* spp. is characterized by the movement of a wide range of parts and products across international borders. Identification of these parts and products has proved to be a challenge for CITES enforcement agencies in importing and exporting countries.

   b) The generic listing of *Gonystylus* spp. has facilitated the identification of traded material using traditional wood anatomy techniques, with individual species not being reliably distinguished. Anatomical identification of single samples can be performed quickly and reliably by expert wood anatomists. The recent development of the computer-based morphological key *CITESwoodID* (Richter, Gembruch and Koch, 2005), when combined with a two-day training course, has greatly improved the potential for risk analysis and initial identification of many traded timber species by enforcement officials. To complement these resources, there is a growing need for a rapid, high throughput, categorical identification method for ramin that can be employed as a standard laboratory tool, easily transferable among countries and capable of providing forensic evidence to court. To explore such options, the CITES Management Authority of the United Kingdom, the Department for Environment, Food and Rural Affairs (Defra) funded a pilot project to explore the use of genetic techniques in the identification of CITES listed timbers. The project was carried out by Wildlife DNA Services and the Royal Botanic Gardens, Kew, United Kingdom (United Kingdom CITES Scientific Authority for plants).

3. Goals

   a) The aim of this project was 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 commercial traders. 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 taxonomic scope, targeting a single genus, but included all stages in the production of a 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 Revenue and Customs (United Kingdom). This approach meant that criteria governing the practical application of the assay were specified from the outset, including considerations of

assay transferability, cost per sample, sample capacity, speed of assay and robustness of the technique to provide reliable evidence for legal prosecution.

b) The methods and results are presented in the Annex to this document in English and Spanish only.

4. Conclusions

The research demonstrated that samples of timber and wood products can be quickly and cost-effectively identified using widely available modern genetic analysis techniques. The development of such techniques is relatively straightforward and with the increasing availability of genetic data for many tree species, it should be possible to replicate this type of test for a large proportion of traded timbers. The analytical methods are easily transferable between laboratories and produce results that are sufficiently robust to be used as legal evidence.

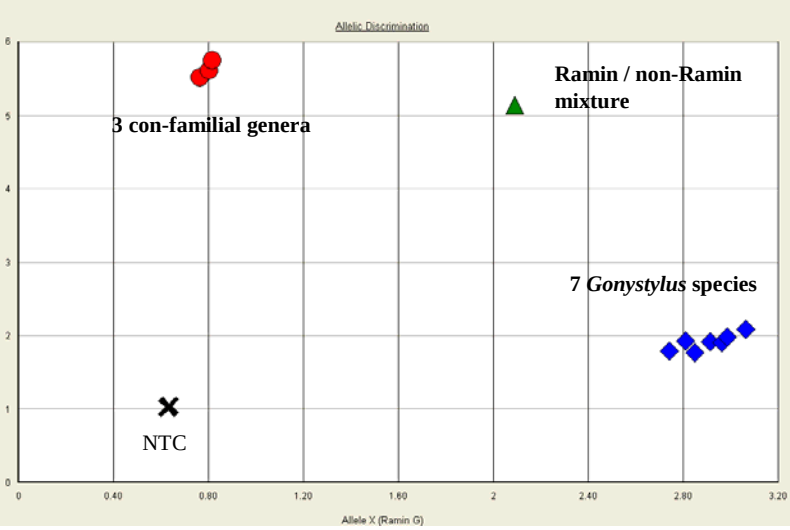

Figure 1: Results of the TaqMan assay showing differentiation of the seven ramin (*Gonystylus*) species from the three most closely related species. The triangle represents a 1:1 DNA mixture control, the crosses (bottom left) are negative controls.

5. Practical applications

In practice, there are three stages involved in the testing processes: sample collection, DNA extraction and genetic analysis. Sample collection is performed at the point of inspection and can be carried out by the investigating officer with minimal training and equipment. Samples are then transferred to a laboratory. DNA is extracted from the sample using a method that has been optimized during the project for several types of wood product. The subsequent genetic analysis stage is based on an industry standard technique and provides rapid unequivocal identification of ramin DNA. The total laboratory stage takes approximately six hours from start to finish with the capacity to process 96 (or on some instruments 384) samples simultaneously.

6. Resource requirements

Sample collection:	Basic equipment to remove ~1cm^3 of material, e.g. knife or saw. Enforcement officer training (1-2 hours)
Laboratory analysis:	Commercially available DNA extraction kits and reagents Real-time PCR genetic analysis system Freely available protocol (SOP) for performing the analysis

Facilities: For survey work, the test can be performed at any laboratory with the necessary equipment (>50 in the United Kingdom). For forensic investigation, the available facilities are limited to quality assured testing laboratories (>5 in the United Kingdom). Similar facilities are available worldwide.

Estimated Costs: *N.B. costs are subject to strong economies of scale*

Set-up costs for a lab to buy assay (1500 tests)	GBP 500.00
Per sample reagent costs (ex. staff time/overheads)	GBP 3.00
Realistic survey price for monitoring agency	GBP 20.00
Realistic forensic analysis price for enforcement	GBP 50.00

7. References

Richter H.G., Gembruch K, & Koch G. (2005). Software program: *CITESwoodID* version 1.0, *BFH German Federal Agency for Nature Conservation.* Available on CD-ROM.

Weising, K. & Gardener R.C. (1999). A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genome of dicotyledonous angiosperms. *Genome* 42, 9-19.

8. Acknowledgements

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9. Contact for further information

For further information on application of the test contact:
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10. Full details of the development and performance of the test are contained in an Annex to this paper, and are additionally to be published in a forthcoming edition of the journal, Endangered Species Research.

METHODS AND RESULTS OF THE PROJECT

Methods

Sample collection

1. Authenticated reference samples were obtained from material kindly provided by the Forest Research Institute of Malaysia (FRIM) and from the collection held at the Royal Botanic Gardens, Kew. The reference samples were divided into three categories: *Gonystylus* (seven target species including those commonly traded); genetically-related species (representatives of all three con-familial genera to *Gonystylus*); and anatomically similar species (examples of 17 species that may confound ramin identification). In addition, a range of sample types was obtained including dried leaf material, unprocessed timber and a variety of worked products such as dowels, window blinds and tool handles.

DNA-extraction

2. Two commercial DNA-extraction kits designed for plant material were tested: the Tepnel Nucleon Phytopure kit based on a simple salt extraction method, and the Tepnel GMO BioKit based on magnetic bead DNA capture. Both kits were employed following manufacturers' protocols. The quality of DNA recovered from both the leaf and processed timber was assessed through PCR amplification. The DNA 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 two different sequence length products (200 and 800 bp) from the plastid *matK* gene.

Genetic marker selection

3. Five candidate gene regions found in plastid DNA had been pre-selected for investigation as potential discriminatory markers for use as a universal plant DNA barcode: *matK, rpoC1, rpoB, accD and YCF5*. DNA sequences were produced for each region for the *Gonystylus* species samples and the three genetically-related species.

4. Sequences were aligned to allow identification of individual nucleotide positions that vary among species. These positions within the sequences, known as Single Nucleotide Polymorphisms (SNPs), were then evaluated to select SNPs that show the same DNA type across all ramin species, but different DNA types in non-ramin species. From the resulting diagnostic SNPs a single genetic marker was chosen for use in the assay design phase.

Assay development

5. The basis for the assay is a DNA identification method known as TaqMan, which allows the genetic variation at the target SNP to be detected using different coloured probes. The probes are then read by a machine that allows the test sample to be identified as originating from a ramin or non-ramin species.

Validation

6. The final TaqMan assay was investigated to examine its performance with different sample types, different species and different DNA concentrations. In addition to validation of the assay itself, an internal control was developed alongside the probe to demonstrate the presence of DNA in samples that show no result with the TaqMan probe. This may occur with samples that are very genetically distinct from ramin. The internal control consists of a second genetic marker, a plastid microsatellite locus (CCPR2) (Weising, & Gardener, 1999), that will give a standard result for almost all tree species to show that the test is working correctly.

Assay results

7. DNA was successfully recovered and PCR-amplified from both fresh plant material and specimens of worked ramin. In all cases the short DNA fragment was amplified; the large fragment was only amplified in fresh leaf material, as expected. The best performing DNA extraction technique was the Tepnel Phytopure kit based on DNA recovery and cost.

8. Evaluation of the DNA sequences produced for each of the five candidate gene regions indicated that the marker *matK* was most suitable for use in distinguishing ramin from non-ramin samples. The *matK* region contained three separate SNP positions that could discriminate the two groups.

9. The SNP assay performed as predicted, correctly discriminating ramin from non-ramin samples. Sample identification was definitive in all cases, with distinct clusters formed for both sequence types (Figure 1).

10. Validation tests showed that the assay worked with DNA recovered from all sample types tested, including the worked products. The assay displayed no false positive results across the 20 non-ramin species tested (three genetically similar and 17 morphologically similar). The TaqMan assay returns consistent results down to a level of 0.01ng/µl of template DNA.