GTTN STANDARDS AND GUIDELINES

Version 1.0

Identification of timber species and geographic origin

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GLOBAL TIMBER TRACKING NETWORK

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Disclaimer

The following standards and guidelines have been prepared by Bioversity International, reviewed by and adopted by the Steering Committee members of the Project "Identification of Timber Species and Geographic Origin".

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BACKGROUND AND SCOPE

Unsustainable and illegal logging are driving deforestation and forest degradation worldwide. Estimates suggest that over 50% of wood exported from the Amazon, Central Africa, South East Asia and Russia is illegally harvested [1].

Accurate **timber species identification** and the tracking of their **geographic origins** along the chain-of-custody are fundamental and necessary to reduce illegal logging and associated trade.

New technologies combining DNA markers (useful also for species identification) and stable isotopes allow timber to be tracked using the intrinsic properties of the wood. They can complement existing tools effectively to detect and to remove illegal wood and wood products from the market; and be used as forensic proof to convict anyone involved in illegal logging activities.

The Global Timber Tracking Network (GTTN) led by Bioversity International is working with scientists and other stakeholders to elaborate a reference database of DNA and stable isotope fingerprints for priority timber species that will be used to identify species and track the origin of wood and wood products along the supply chain.

This document provides standards and guidelines for **timber tracking forensic analysis** of wood and wood products using DNA and stable isotopes. It includes sampling designs, samples handling and storage, laboratory practices, guidelines to conduct ring and blind tests. The Barcode of Life standards and guidelines are valid for timber species identification and are therefore not presented here.

SAMPLING STANDARDS AND GUIDELINES

Sampling design to build a reference database

Sampling should always be done across the whole distribution range of each species, if possible. Sampling size can vary depending on the density of the tree species, the methods and the marker types used, etc.

The minimum sample size recommended to elaborate a reference database is summarized in table 1. However, there is no upper limit to the number of samples and it is always better to take more samples whenever possible.

Table 1: Minimum sample size required to establish a reference database

Reference database based on		**Verification of origin at the level of**		
		Country and/or Region	**Concession (50x50 km²)**	
		Per population	**Per target concession**	**From neighboring concessions**
DNA	Chloroplast markers (microsatellites, gene sequences)	5	5	5
	Single locus nuclear DNA markers (Microsatellites, SNPs)	20	300	100
Stable isotopes		5 - 10	50	25

Verification of the country/region of origin

- If sampling size is low (i.e. tree density) when working with single locus DNA markers, the precision improves with the number of gene markers used. Investments should be made to develop more highly variable gene markers;
- Within a country, populations should be separated by at least 100 km and two trees should be separated by 100 m to minimise sampling of closely related trees, and to capture as much variation as possible;
- When working near borders of countries, samples should also be collected in neighbouring countries.

Verification of the concession of origin

- Samples should be collected in a grid pattern if tree density is uniform (at least 10 locations) within the target concession
- Additional samples should always be collected from neighbouring forest concessions or in the vicinity.

Sampling design for chain-of-custody certification

For each target species:

- Collect at least 500 reference samples at log storage areas;
- Collect an additional 250 samples distributed at two or more steps along the chain-of-custody (primary sawmills, primary sawmills, distributor, importer).

Samples collected at log storage areas, and along the chain-of-custody (primary sawmills, primary sawmills, distributor, importer) are genotyped and compared as described by Lowe et al. (2010) [2].

Samples, samples handling and storage

Leaf samples

- Collect two leaf samples (5cm² each; one sample for backup) and place them in separate ziplock bags with colour indicator silica gel (5 mL), replace the silica gel after few hours or once it has taken up the moisture from the leaf (change of color),
- Ensure that all ziplock bags are adequately labelled with GPS location, tree number, date and name of collector,
- Store all ziplock bags in a dry place (e.g. hermetic plastic container or in a room with air conditioning).

Cambium samples

- Punch a hole in the trunk of the tree,
- Remove at least 0.5 cm² of cambium (thin and humid layer between the bark and the wood) with a knife,
- Place the cambium in a 2 mL labelled tube containing 1.5 mL silica gel,
- Take a back up sample as explained above,
- Within 24 hours, transfer the drying cambium into a new tube containing 1.5 mL of fresh silica gel. Replace the silica gel if necessary until the cambium dries out completely.

Wood cores

- Trees must be drilled between 50 and 100 cm above ground,
- Drill (with increment corer or cordless screwdriver) between 10 and 20 cm depth of wood core perpendicularly towards the centre of the trunk to cut through as many rings as possible,
- When the bark is tick, it is recommended to remove it (e.g. with machete) before the drilling

- Take at least two cores from each tree for back up samples,
- Wrap cores in paper and put into a ziplock bag with silica gel.

Necessary data elements

- Clear and unique identification number for each sample
- Species name
- Population name
- GPS coordinates
- Date of collection
- Name of the collector
- Any relevant sampling site information.

LABORATORY STANDARDS

Protocols for DNA isolation, quantification and visualization from dry wood

Different protocols used to successfully extract DNA from wood tissues (cambium, sapwood, heartwood) and heat-treated wood are presented in Table 1 and detailed below

Table 1: DNA extraction protocols for dry wood

N°	Protocols	Species and references
1	DNeasy Plant Mini Kit (Qiagen)	*Neobalanocarpus heimii* [3], *Dipterocarps [4, 5], Entandrophragma cylindricum* [6], *Criptomeria japonica* [7], *Gonystylus bancanus* [8]; *Cunninghamia lanceolata [9]*
2	MagAttract 96 DNA Plant Core Kit (Qiagen)	
2	Modified CTAB	
4	CTAB with PTB protocol	

DNeasy Plant Mini Kit and MagAttract 96 DNA Plant Core Kit (Qiagen)

The protocol for DNA extraction from wood with the DNeasy Plant Mini Kit (Qiagen) or the MagAttract 96 DNA Plant Core Kit (Qiagen) applying modifications and optimizations as reported by [4] is presented below:

Reagents and chemicals

RNase A 100 mg/mL (Qiagen); polyvinylpyrrolidone (PVP40000, Roth); ethanol 100%; liquid nitrogen; DNeasy Plant Mini Kit (Qiagen); MagAttract 96 DNA Plant Core Kit (Qiagen).

Sample preparation

1. Remove surface tissues of wood sample under sterile conditions to avoid contamination with other plant DNA
2. Drill the clean inner part of wood or cut it using a sterile scalpel to produce small wood shavings. To prevent the sample from overheating during drilling, the drill bit should be frequently incubated on ice after each drilling, and washed with ethanol (70%)

Wood powdering

Grind the wood shavings into a fine powder using a Mixer-mill apparatus Type MM2 (Retsch) using the following steps:

3. Add 50-100 mg of wood shavings and one stainless steel bead (5 mm, Qiagen) to a 2 mL microcentrifuge tube. Place the microcentrifuge tube into an adapter rack for 5 reaction vials (Retsch).
4. Incubate the racks in liquid nitrogen for 5 min to freeze the wood samples.
5. Transfer the racks to the Mixer-mill apparatus and grind for 5 min at 75 units shaking-speed.
6. Remove adapter racks and re-incubate in liquid nitrogen for 5 min. Then repeat step 5. Shaking speed and time can be varied to produce a fine wood powder. Repeated incubation in liquid nitrogen is needed to keep the samples frozen during grinding.
7. Keep the samples frozen in liquid nitrogen or at –20°C until cell lysis.

Lysis

Apply the modifications described below to lysis:

8.a. Addition of PVP into AP1 lysis buffer of DNeasy Plant Mini Kit: Add PVP to the AP1 lysis buffer up to 3.1% (w/v); add 500-800 µL of the mixed buffer and 5-8 µL of RNase A to each tube containing a ground wood sample; vortex vigorously and incubate the mixture overnight at 65°C with shaking or vertical rotation; after incubation, add 162.5-260 µL of buffer AP2 to the lysate, mix by vortexing, then incubate for 15 min at –20°C; centrifuge for 5 min at 20,000 *g*.

b. Addition of PVP into RLT lysis buffer of MagAttract 96 DNA Plant Core Kit: Add PVP to the RLT lysis buffer up to 3.1% (w/v); add 500-800 µL of the mixed buffer to each ground wood sample; vortex vigorously until there are no remaining tissue clumps; centrifuge for 5 min at 20,000*g*.

DNA purification

Purify DNA in the supernatant using DNeasy Plant Mini Kit (for step 8a) or using MagAttract 96 DNA Plant Core Kit (for step 8b), using the magnetic particle concentrator Dynal MPC®-E-1 (DYNAL®) for 1.5 mL microtubes from Dynal Biotech.

9. Transfer the supernatant from step 8 to:

a. (Following step 8a.) the QIAshredder Mini Spin Column placed in a 2 mL collection tube and centrifuge for 2 min at 20,000*g*. Subsequent steps are continued following the kit protocol.

b. (Following step 8b) a 1.5 µL microcentrifuge tube containing a mix of buffer RB and MagAttract Suspension A (65 µL of buffer RB and 20 µL of MagAttract Suspension A for 200 µL of supernatant of step 8b); mix by vortexing; place the tube on the Dynal

MPC®-E-1 magnetic particles concentrator and remove the supernatant after magnetic separation. Subsequent steps (washing of DNA on magnetic particles) are continued following the kit protocol.

Elution

10. Transfer 50 µL of buffer AE to the spin column (from step 9a) or to the MagAttract particles (from step 9b) for the DNA elution. The elution is performed twice; the second eluate is collected separately from the first.

Modified cetyltrimethylammonium bromide (CTAB) protocol

Solutions

CTAB extraction buffer (100 mM Tris-HC1 (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 mM NaC1, 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. Grind 0.5 g of wood tissue in liquid nitrogen with Millser IFM 66D (Iwatani). Transfer the powder immediately into preheated (65°C) extraction buffer and mix thoroughly. Incubate the mixture for 2 h with occasional mixing.
2. Extract the mixture twice with equal volumes of chloroformisoamylalcohol (24:1), and emulsify 2 phases gently
3. Separate two phases by centrifuging at 5000 rpm (2300g) for 10 min at 4°C
4. Transfer the supernatant to a new 50-mL tube
5. Add an equal volume of cold isopropanol, mixed well, and incubate overnight at -40°C
6. Pellet down DNA by centrifuging at 5000 rpm (2300g) for 20 min at 4°C.
7. Wash the pellet by adding 1 mL of washing buffer, and repeat the process twice. Dry the pellet at room temperature and transfer it to a 1.5-mL Eppendorf tube
8. Wash the pellet twice with 70% ethanol and dry it at room temperature.

Purification of genomic DNA

Purify DNA samples extracted with the High Pure PCR Template Preparation Kit (Roche).

N-phenacylthiazolium bromide (PTB) protocol

A PTB-based DNA extraction protocol to extract DNA from plant tissues following the manufacturer's instructions (Prime Organics) with modifications is described below.

Protocol

1. Grind into powder 1 g of tissue of processed wood and 0.5 g of samples with a Millser IFM 66D (Iwatani) and liquid nitrogen,
2. Transfer the fine powder to 5 mL of 0.5 M EDTA and soak it for 48 h at room temperature to demineralize the wood tissues,
3. After demineralization, add 500 µtL (3 mg/mL) of proteinase K (FisherBiotech) and 1.0 mL of 0.1 M PTB (Prime Organics),
4. Mix thoroughly to homogenize the components. Incubate samples at 65°C for 12 h in a water bath. Do the extraction with equal volumes of phenol and chloroform, and follow by 2 subsequent extractions with equal volumes of chloroform and isoamylalcohol (24:1),
5. Precipitate the DNA by adding 2 vol of cold absolute ethanol and 500 µL of 7.5 M ammonium acetate. Store this solution at -40°C for 12 h and centrifuge it at 5000 rpm (2300g) for 20 rain at 4°C,
6. Wash the pellet twice with 80% ethanol and leave overnight in 80% ethanol,
7. Recover the pellet by centrifuging at 5000 rpm (2300g) and drying at room temperature.

Purification of genomic DNA

Extracted DNA samples are further purified with the High Pure PCR Template Preparation Kit (Roche).

Protocols for DNA fingerprinting

- Gene markers accepted for the identification of timber origin are single locus nuclear DNA markers (Microsatellites, SNPs, SCARs) and chloroplast markers (microsatellites, gene sequences)
- No dominant marker (AFLP, RAPD) data will be used in the GTTN database.
- **PCR amplification, genotyping and sequencing** should be performed with the kits available and used routinely in each participating lab.
- Lab equipment, thermal cyclers, capillary sequencers etc. shall be the same used by the other genetic labs conducting analyses.

Protocols for stable isotope fingerprinting

Stable isotopes commonly recommended for checking the origin are the light bio-elements: hydrogen (H), oxygen (O), carbon (C), nitrogen (N), sulphur (S) and the heavy stable isotope of strontium (Sr).

Reagents and chemicals

- Nonpolar solvent: methylene chloride (alternative: ether); polar solvent: methanol; copper oxide powder, distilled water.
- All chemicals should have a standard quality for analysis.
- Expansion for strontium: nitric acid (69 %, suprapur: Sr < 0,5ppb), hydrogen peroxide suprapur (30 %).

Equipment

Soxhlet apparatus, ball mill, combustion furnace e.g. (sulphur), microwave heater (strontium), laboratory-type drying cabinet.

Sample preparation

1. Grind the wood chips into a fine powder using a ball-mill apparatus.
2. Extract the powder in a soxhlet apparatus over 6 hours with nonpolar and polar solvent.
3. Dry the powder in a laboratory-type drying cabinet for at least 1 hour.
4. The samples should be stored in air-tight sample vials.
5. Expansion for strontium: burning of 2 to 4g of wood powder in a combustion furnace at a temperature of 750°C. The ash is transferred in a microwave heater with about 10ml nitric acid and 2ml hydrogen peroxide. Digestion temperature: >180°C for

>15min. Depending on the samples, a purification may be necessary to avoid isobaric influence. Regular purification with Sr resin (Sr-C20-A, Eichrom) is performed.

6. For samples with very low sulphur content, a concentration step is necessary. Therefore the pulverised timber samples will be mixed with copper oxide (mixing ratio: 1g sample + 0,5g copper oxide [purified powder!]) and ashed for 12 hours at 350°C. Afterwards the sample is mixed with 4 mL distilled water and heated in a locked vial for 1 hour at 100°C. The suspension is filtered and 30mg V_2O_5 (free of S) are added to the filtrate. Then the filtrate is dried in a laboratory-type drying cabinet at a temperature of 120°C.

Purification of copper oxide: It is essential to control the quality of copper oxide. The copper oxide has to be completely free of sulphur. Therefore copper oxide powder should be washed and filtrated several times with distilled water (e.g. 5 times).

Equipment and analysis of samples

Precision balance: resolution 0,001 to 0,01 mg, tin and silver capsules; exsiccator; light gaseous isotopic ratio mass spectrometers – IRMS (e.g. Thermo Scientific, Elementar, NU-Instruments) in combination with element analyser (EA) suitable for isotopic ratio analysis (e.g. Thermo Scientific, Elementar, Eurovector) or high temperature furnace in pyrolsis mode - HT-PyrOH (e.g. Hekatech, Elementar, Blisotec).Expansion Strontium: ICP-MS (e.g. Thermo Scientific)

1. The weight of the samples for analysis depends on the installed IRMS system. Normally the weight of samples is between 0.5 to 2mg. For HT-PyrOH silver capsules are recommended to avoid contamination of a oxidation. The samples in silver capsules should be stored in an exsiccator with silica gel.
2. D/H, $^{18}O/^{16}O$ measurement: HT-PyrOH with silicium carbide tube (Hekatech) or equivalent (carbon tube) filled with glassy carbon chips and coal powder. Working temperature for pyrolysis of >1450°C. Recommended to ensure an optimal pyrolysis: 1550°C. The autosampler should have a zero-blank quality (e.g. Blisotec zero blank autosampler).
3. $^{13}C/^{12}C$: EA with IRMS.
4. $^{15}N/^{14}N$: EA with IRMS. Because of low quantities of nitrogen an optimised oxidation is recommended. An addition of V2O5 (1:1) is helpful to ensure the optimal combustion. The carbon dioxide should be trapped with carbosorb or equivalent. A further addition of a packed column for CO separation is helpful if the optimal combustion could not be ensured.
5. $^{34}S/^{32}S$: EA with IRMS. It is recommended to measure the sulphur dioxide in one tube packing (oxidation and reduction in one tube) to avoid problems with SO3 and combustion water.
6. $^{87}Sr/^{86}SR$: ICP-MS (multi collector is recommended).
7. The system and working standards should be calibrated against at least two international working standards from IAEA or equivalent.

Barcoding protocols

The Barcode of life (http://www.barcodeoflife.org/) protocols for DNA barcode analysis are also recommended for timber species identification.

However, the practical application in timber tracking requires an adaptation of the methods to work for samples (wood and wood products) with degraded and low quantity of DNA: Additional DNA barcode loci (other than matK or rbcL) with shorter fragment length and more discriminatory power must be developed for many tree species.

A simplification of the laboratory procedures for use in small labs, without the need for sequencing or capillary electrophoresis techniques is recommended especially in timber producing countries where the risk of illegal logging is high, but where law enforcement agencies and national labs are poorly equipped. The simplification can be done with the use of PCR-RFLPs after detection of SNPs as described for few CITES-protected species [10].

GUIDELINES TO CONDUCT RING AND BLIND TESTS

Data for each timber species entered into the GTTN database must be validated before practical use; and participating labs must prove their ability to produce consistent and reliable results. These validation procedures are known as ring (to test uniformity of results among labs) and blind (to test the validity of data) tests.

Organization and overseeing storage and distribution of standard materials

- Sampling should be organised by the GTTN coordination without the involvement of participating labs
- Samples (leaf, cambium or wood core) should be collected, handled and stored according to the guidelines described above

The sampling design recommended for the blind tests is as follows:

- **For species identification,** all participating labs providing services for a particular species should receive samples collected from the same trees, and analyse them with the same DNA barcodes
- ***For the verification of geographic origin,*** all participating labs providing services for a particular species should receive samples collected from the same trees, and analyse them with the same gene markers or stable isotopes.

Validation of the results

Results of the tests should be collected by the GTTN coordination for analysis and comparison.

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