Wood identification by taxon specific primers

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Four different wood species (1, 2, 3, and 4) are provided to each Group (group1, group2, group3 and group4). The numbers 1, 2, 3, and 4 represent the following wood species: two African mahogany species, *Entandrophragma angolense* and *Khaya ivorensis* and two true mahogany species, *Swietenia macrophylla* and *Swietenia mahagoni*.
Following DNA extraction, PCR is performed using taxon-specific primers for the four wood species.

- *Entandrophragma angolense*: Entango for1 (forward primer) + Entango rev2 (reverse Primer)
- *Khaya ivorensis:* Khaya for1 (forward primer) + Khaya rev1 (reverse Primer)
- *Swietenia macrophylla*: Machum for1 (forward primer) + Machum rev1 (reverse Primer)
- *Swietenia mahagoni*: Mahogany for (forward primer) + Mahogany rev2 (reverse Primer)

A PCR amplification product is only formed when DNA from that specific wood species is used as a template.

The ITS-Region

Phylogenetic studies at the molecular level have shown that the rapidly evolving internal transcribed spacer (ITS) region of the ribosomal DNA is particularly well suited for the construction of relationships, especially at genus and species level. This region is therefore used to identify organisms. In contrast, highly conserved regions are useful for comparisons at the family level and beyond, such as 18S and 28S rDNA. The ITS region is located between these conserved 18S and 28S regions. It consists of the sub-regions ITS1 and ITS2, between which the conserved 5.8S rDNA is located. Sequences of the ITS region are very similar among the individuals of the same species and therefore easy to compare. Nevertheless, their variables are sufficient to distinguish between species. Since these are non-coding regions, they usually change much faster than coding regions during evolutionary processes. Thus, the ITS region is used as a barcode region in higher plants and fungi. The ITS region has a high replication number in the genome and is relatively small in size, 600 to 700 base pairs (bp). These two characteristics allow easy amplification of the region and the amplicon is valuable for phylogenetic studies. Due to the flanking conserved regions, it can be easily amplified using the universal primers for eukaryotes ITS1.1 and ITS4 (Fig. 1).
If the ITS sequence of a (wood) species is known, so-called taxon-specific primers (Tax for, Tax rev; Fig. 1) can be designed. With these primers, a PCR product is formed when DNA from this specific wood species is used as a template.

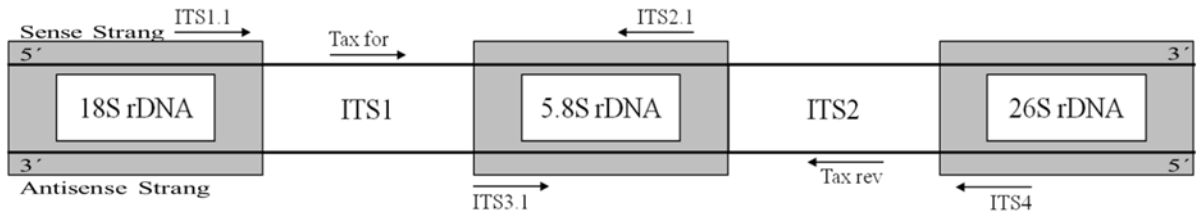


Fig. 1: Internal transcribed spacer region of ribosomal DNA with binding sites of the universal primers ITS1.1 (forward) and ITS4 (reverse), the wood-specific primers ITS 2.1 and ITS 3.1, as well as the taxon primers Tax for and Tax rev.

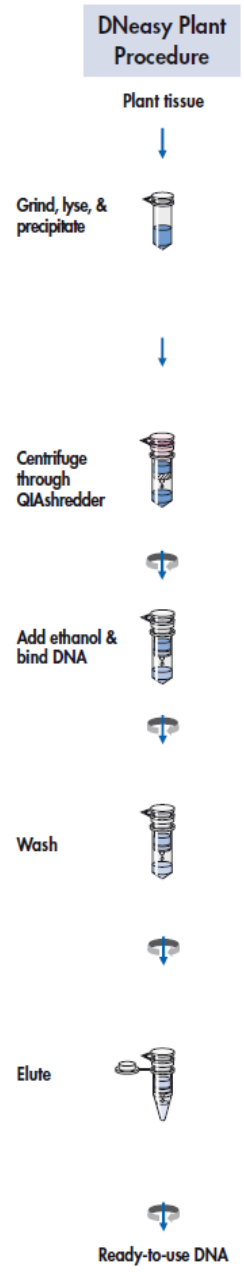
1) DNA Extraction

Approximately 50 mg of the collected and homogenized sample material is weighed into 2 ml of Eppicups for DNA extraction.

A detailed description of the extraction protocol (DNeasy® Plant Mini Kit, Qiagen) can be found in the corresponding manual.
https://www.qiagen.com/de/resources/resourcedetail?id=95dec8a9-ec37-4457-8884-5dedd8ba9448&lang=en)

Protocol:

1.) Weigh 50 mg of wood flour sample into a 2 ml tube.
2.) Add 600 µl Buffer **AP1**. Vortex and incubate for 10 min at 65°C (thermoblock). Vortex the tube 2–3 times during incubation.
→ *Cell-Lysis*
3.) Add 200 µl Buffer **P3**. Vortex and incubate for 5 min at -20°C.
→ *Precipitation of proteins and polysaccharides*
4.) Transfer the lysate into a "QIAshredder" spin column.
5.) Centrifuge for 2 min at 13000 rpm.
→ *Removal of cell debris, proteins, polysaccharides, etc.*
6.) Transfer the flow-through into a new tube without disturbing the pellet.
7.) Add 750 µl Buffer **AW1**, mix carefully by inverting the tube.
→ *"Precipitation" or reduction of the solubility of the DNA*
8.) Transfer 650 µl of the DNA solution to "DNeasy Mini-Spin" column
9.) Centrifuge for 1 min at 8000 rpm and discard the flow-through.
→ *DNA binds to the column*
10.) Transfer the remaining DNA solution to the column.
11.) Centrifuge for 1 min at 8000 rpm and discard the flow-through.
12.) Add 500 µl Buffer **AW2**, centrifuge for 30 sec at 8000 rpm and discard the flow-through.
→ *"Washing"*
13.) Repeat the last step.
→ *Second "washing"*
14.) Centrifuge for 1 min at 13000 rpm.
→ Drying the column
15.) Transfer the spin column to a new 1.5 ml tube.
16.) Add 30 µl Buffer **AE**. Incubate for 5 min at room temperature.
17.) Centrifuge for 1 min at 10000 rpm
→ *Eluting DNA from the column*
18.) Repeat the steps 16 and 17 with new 1.5 ml tubes.



Result:
Genomic DNA dissolved in 30 µl Buffer AE (2 tubes eluat 1 and eluat 2).
The DNA eluates are stored in the refrigerator at 4°C.

2) Concentration and quality of the DNA

The DNA concentration and quality is determined spectrophotometrically with the Nanodrop 2000 (Thermo Scientific). 1 µl of the extracted DNA is used to determine the absorption in the range of 230 – 350 nm. Nucleic acids absorb UV light at a wavelength of 260 nm, while proteins absorb at 280 nm, and phenolic components at 230 nm. Based on the absorption of the 260/280 and 260/230 ratios, conclusions can be drawn about the purity of the nucleic acid. Ratios in the range of 1.8 to 2.0 indicate a high DNA purity and thus quality.

3) Polymerase Chain Reaction (PCR) for amplification of the ITS-Region

PCR allows the amplification of minute amounts of a specific DNA-region. Although it was first discovered in 1983, today it is indispensable in the molecular biology.

3.1) The principle of PCR

PCR is dependent on heat-stable DNA polymerases. These enzymes are found in organisms that can survive in extreme conditions such as hot springs. The most commonly used enzyme is the polymerase from the bacterium *Thermophilus aquaticus*, abbreviated **"Taq"**. PCR allows a DNA fragment to be amplified that is located between two regions of known base sequences. At regions of known sequence, synthetic oligonucleotides (**primers**) anneal to the denatured DNA single strands. The primer sequences are designed to hybridize with the sense strand (forward primer) on the one side and the antisense strand (reverse primer) on the other side. At this point, the Taq polymerase starts synthesising the double-stranded DNA.
To separate the DNA double helix, the reaction mixture is briefly heated to 90°C (**denaturation/ melting**). Then follows the **annealing** step. At the primer specific annealing temperature between 40 and 65°C (X°C), the primers hybridize to their binding sites on the template. In the following step, the temperature is increased to 72°C (temperature optimum of Taq). This allows the fragments between the two primers to extend according to the template (**elongation**).

94°C – 4 min		
94°C – 30 sec	}	**35x**
X°C – 30 sec	}	
72°C – 30 sec	}	
72°C – 7 min		

This temperature profile is repeated cyclically. Since the elongation product of one cycle can serve as the starting material for the next, the DNA segment flanked by the primers is multiplied exponentially. With 35 cycles, one template can theoretically be amplified by 2^{35} times!

3.2) The discovery of PCR

The PCR was invented by Kary B. Mullis in 1983. It is reported that this ingenious inspiration came to him during a late-night drive on Route 101 in Northern California, when he was thinking about a completely different scientific problem. Since he was employed by Cetus at the time, he received $10,000 from this company for his lucrative idea (Cetus later sold the patent to Roche for $30 million). In 1993, Mullis was awarded the Nobel Prize in Chemistry.

3.3) Protocol for performing a PCR

Pipette the **x+1** fold volumes (**x** = number of PCR samples, this time **x=10**) of the components listed in Table 1 into a 1500 µl tube and mix gently (master mix). Then transfer 24 µl aliquots each into 200 µl tubes. After preheating the thermocycler, add the corresponding sample DNA (1.0 µl) to the tubes and start the PCR.

15.55 µl	distilled water
5.0 µl	Q-Solution
2.5 µl	10x-Buffer
0.5 µl	dNTP-Mix
0.15 µl	forward Primer
0.15 µl	reverse Primer
0.15 µl	Taq-Polymerase

Tab. 1: List of components and amount for each sample (24µl)

Primer combinations:
Group 1: Entango for1 (as forward primer) and Entango rev2 (as reverse Primer)
Group 2: Khaya for1 (as forward primer) and Khaya rev1 (as reverse Primer)
Group 3: Machum for1 (as forward primer) and Machum rev1 (as reverse Primer)
Group 4: Mahogany for (as forward primer) and Mahogany rev2 (as reverse Primer)

Annealing temperature for the specific primers used in the PCR and **length** of expected amplicons
55 °C for: Entango for1 (as forward primer) and Entango rev2 (as reverse Primer) **470 bp**
59 °C for: Khaya for1 (as forward primer) and Khaya rev2 (as reverse Primer) **230 bp**
60 °C for: Machum for1 (as forward primer) and Machum rev1 (as reverse Primer) **470 bp**
58 °C for: Mahogany for (as forward primer) and Mahogany rev2 (as reverse Primer) **300 bp**

4) Documentation of PCR-products

The documentation of the amplified PCR products is carried out by gel electrophoresis. The PCR product is mixed with a loading buffer and loaded onto a 2.0% agarose gel, followed by the application of an electric field to separate the PCR products. The amplicons are detected by staining with ethidium bromide (CAUTION! DANGEROUS TO HEALTH! HAZARD!) and detection under UV light.

1) Prepare a 2 % agarose gel with 0.5 % TAE buffer
2) Pipette 2.5 µl PCR product + approximately 1.0 µl loading buffer together
3) Pipette the mixture into the wells of the gel
4) Pipette 0.5 µl of Nippon Genetics 100bp Marker into the outer wells
5) Set gel electrophoresis at 135 V for 25 min.
6) Place the gel into ethidium bromide for 2 min (CAUTION! Etbr is TOXIC!!)
7) Rinse the gel in water bath for 20 minutes to destain the background

Agarose is a red seaweed polysaccharide that forms pores from 150 nm to 500 nm. This method is suitable for the separation of DNA fragments in the range of 0.5 to 15 kb. After applying an electric field, the DNA fragments (due to their negatively charged backbone) migrate through the pores to the positive pole. The migration rate is inversely proportional to the size of the fragment.
To prepare a 2.0% agarose gel, dissolve 2 g of agarose in 100 ml of TAE buffer (20 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0). The running buffer is as well a TAE buffer.

The visualization is performed under a UV light screen from Bio-RAD Laboratories GmbH, Munich.