Identification of the timber origin of tropical species by molecular genetic markers – the case of dipterocarps

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Abstract

Illegal logging continues to be a main cause for the destruction of tropical forest ecosystems. The development of non-manipulable tools to control the origin of timber and timber products from tropical tree species will greatly contribute to distinguish legally from illegally harvested wood. This will promote the marketing of tropical timber from sustainable managed forests and will eventually support the ban of illegally harvested material.

We tested the application of molecular genetic markers to identify the origin of tropical Dipterocarpaceae. Dipterocarps are a very species-rich family dominating tropical forests in South- and Southeast-Asia. They are the main source of tropical timber (trade name, for example, meranti) from this region. Since most species have a restricted distribution, species identification is an important and in many cases sufficient indication of the origin of timber.

In total, more than 3000 dipterocarps representing over 110 different species have been sampled. Sampling has been most intensive on the Indonesian islands of Borneo and Sumatra. Locations from Vietnam, Thailand, and the Philippines are represented as well. We developed a simple and reliable method to extract DNA from dipterocarp wood based on a frequently used extraction kit. The success and efficiency of the method to extract DNA of good quality for PCR amplification from freshly cut timber and processed wood products was tested. The success rate for amplification was influenced by the age of wood, the degree of processing, and inhibitory substances. It was possible to increase the success rate in many cases to 100% of all investigated samples by a careful selection of the amplified DNA fragment (fragment length; genomic origin, repeat number), appropriate dilution of template DNA, repeated eluation of DNA, and choice of the most suitable position for investigation (inner or outer wood). The method proved to be applicable for the majority of investigated dipterocarp wood samples and for most other investigated material as well.

In a parallel attempt, we developed markers to distinguish between closely related species from the same timber group and between geographic regions from widely distributed, common species. Species distinction is often possible by the investigation of cpDNA fragments of different length. The identification of the region of origin is hampered by a moderate degree of genetic differentiation for the two common dipterocarps Shorea leprosula and S. parvifolia. However, we observed strong geographic differentiation at several AFLP markers, which were converted to SCAR (Sequence Characterized Amplified Region) markers.

In summary, dipterocarps are suggested as a suitable group of species to implement a system for the identification of the origin of tropical timber.

Keywords: timber origin, DNA extraction, DNA marker, genetic variation, tropical tree, Dipterocarpaceae

1 Introduction

Forest destruction and degradation continue to be main threats to global biodiversity and cause severe environmental damage. Considerable losses of forest cover diminish the forested area in all main regions of the tropics (FAO 2001). Alarming rates of deforestation are reported for several tropical countries in Southeast-Asia. Several attempts to promote forest conservation and to combat deforestation aim at promoting the trade, marketing and use of timber and wood.
products from sustainably managed forests and to exclude illegally harvested timber from natural to apply tools allowing to proof the origin of wood and wood products or to reliably test the declaration of the origin of timber. Customs offices in producer and consumer countries, forest certification schemes as the Programme for the endorsement of Forest Certification Schemes, (PEFC, www.pefc.org/internet/html/index.htm) and the Forestry Stewardship Council (FSC, http://www.fsc.org/en/), forest enterprises producing timber according to the principles of sustainable forest management, as well as the timber industry and end consumers potentially benefit from improved methods to infer the origin of wood which might be potentially illegally harvested. Currently available methods only rarely allow to prove false declarations of the origin of wood in court. Thus, the development of innovative methods to test the proclaimed origin of timber based on non-manipulable characters is important in this context.

DNA is a stable and highly variable molecule. Hence, variation of DNA is potentially useful to conclude on the origin of any biological material. Two basic requirements need to be fulfilled in order to use DNA variation for the identification of the origin of wood: (i) it is possible to isolate DNA from wood of different age and processing status, and (ii) markers need to be developed which are informative with regard to the identification of the origin of wood.

We developed molecular tools to infer the origin of dipterocarps (Dipterocarpaceae). The family is pantropical with comparatively few species occurring in the neotropics and in Africa. The subfamily Dipterocarpoideae is very species-rich and common in Asian evergreen and Monsoon forests. The centre of species diversity is reached in Borneo (Kalimantan) with more than 260 described species; few species are native to forests east of the Wallace-Line.

More than 50% of all trees including the majority of emergents and trees of the canopy are dipterocarps in many natural forests in tropical Asia, which are regarded as a centre of global biodiversity. Dipterocarps are not only a key-stone resource in particular in tropical Southeast-Asia (Whitmore 1975), but they are also among the most important tropical timbers (trade names: meranti, balau for Shorea spp., kerau for Dipterocarpus spp., kapur for Dryobalanops, etc.). In many regions dipterocarps are critically endangered due to forest destruction and non-sustainable forest management leaving only secondary national and international markets. In this context, it is of prime importance to develop and forests of little commercial value after logging. Dipterocarps are rarely grown in plantations although attempts are made to establish man-made dipterocarp forests.

Sustainable management of dipterocarp forests is feasible, if harvesting is carefully controlled and natural regeneration promoted (Lamprecht 1986). Thus, the development of tools to identify dipterocarp wood from sustainably managed forests will contribute to the application of sustainable management practices and the conservation of dipterocarps and their associated species. The large number of commercial species needs to be taken into consideration for the development of tools for wood identification. For example, the wood from more than 100 species belonging to the species-rich genus Shorea is differentiated into only a few trade names (white meranti, yellow meranti, dark red and light red meranti).

We developed a simple protocol for the extraction of DNA from the wood of dipterocarps (Rachmayanti et al. 2006), which was tested for a large number of wood samples. We also investigated DNA variation among and within dipterocarp species in order to develop informative markers with regard to the origin of material.

## 2 Materials and methods

### 2.1 Material

Out of the 332 wood samples belonging to the family Dipterocarpaceae, 181 were collected from natural forests or plantations in South-East Asia, and 151 were from wood enterprises or wood processing facilities in Germany. At least 40 samples were taken in each of the following four countries: Thailand, Vietnam, Philippines, Indonesia. In each country, samples were collected at least at four different sites. The following taxa were sampled: Anisoptera, Dipterocarpus, Hopea, Parashorea, Shorea, and Vatica. Leaves collected from 25 dipterocarps trees and corresponding wood samples from Indonesia and the Philippines were used for the verification of the DNA extraction method. Wood from 74 tree species other than dipterocarps was included for comparisons.

Leaf material from over 3,000 dipterocarp trees was sampled throughout the Asian range of the family and is available for investigation. Most material is from natural forests in Indonesia; the
species-rich genus *Shorea* is particularly well-represented in these samples. A total of 116 different species were sampled in natural forests, botanical gardens, and plantations and arboreta.

3 DNA Isolation

DNA from wood was extracted with the DNeasy Plant Mini Kit (Qiagen) applying the same modifications and optimizations as reported in Rachmayanti et al. (2006). Prior to extraction the surface tissues (including cambium and bark) of wood samples were removed using a sawing machine to avoid contamination with other DNA. 50 – 100 mg of shavings produced by drilling of the clean inner part of wood samples were used for DNA extraction. Polyvinylpyrrolidone was added into the lysis buffer (step 8.a, Rachmayanti et al. 2006) up to 2.6% (w/v). The effect of PVP addition on PCR inhibition was tested for different concentrations. The last step of the extraction protocol (elution) was performed twice; the second eluate was collected separately from the first. Genomic DNA of leaf samples was extracted with the DNeasy Plant Mini Kit according to the manufacturer’s instructions (Qiagen).

DNA isolated from leaf and wood from the same tree were analyzed by PCR amplification, genotyping and sequencing. Chloroplast microsatellite primers (Weising and Gardner, 1999) were applied for genotyping. The intergenic spacer between *trnL* and *trnF* was amplified and sequenced as well. PCR amplification, genotyping and sequencing was carried out as reported by Rachmayanti et al. (2006) except that direct sequencing of purified PCR products (without cloning) was performed.

PCR amplification was performed to examine the success of DNA extraction from wood. Three primer pairs were chosen to amplify three fragments of different length (short fragment of about 100-200 bp, middle length fragment of about 500-600 bp and long fragment of about 1100bp). PCR–reaction mixtures were prepared and amplification protocols were used as reported previously (Rachmayanti et al. 2006).

In order to study the effectiveness of PVP addition on the reduction of PCR inhibitory substances, three DNA isolation methods differing in the concentration of PVP in the lysis buffer (without, with 2.6% and with 5.0 %[w/v]) PVP were compared (step 8a of DNA isolation method, Rachmayanti et al. 2006). PCR inhibitory tests were performed using the same parameters as in normal PCR except that mixed DNA (DNA extract from wood plus another known high-quality DNA) was applied as PCR template. A total of 2.5 μl volume of PCR template (2 μl of wood-DNA extract + 0.5 μl of high quality leaf DNA) was applied for each PCR reaction. In this PCR inhibitory test, a series of 2.5 μl volume of PCR templates was prepared. Each template contained 0.5 μl of leaf DNA mixed with 2 μl of undiluted, 10 times, 20 times, 40 times, 80 times and 160 times diluted wood DNA extract, respectively. This test was performed for the wood DNA extract from each isolation method.

The quantity and quality of the DNA extract from three different zones of wood were analyzed, i.e. zone a: outer sapwood (without cambium or bark), m: transition zone and zone i: inner heartwood. PCR amplification was performed on the first and the second eluate of each zone. Three markers with different fragment lengths (*ccmp2*, 0.15 kb; *trnL*, 0.6 kb; *tmLF*, 1.1 kb) were used for PCR. The PCR reaction mixture and the amplification protocol were carried out according to Rachmayanti et al. (2006). 2 μL of undiluted eluate was used as template in each PCR.

4 Genetic variation and marker development

Variation among species was assessed by means of PCR-RFLPs, cpSSRs, AFLPs, and sequencing. Methods are described in detail by Indrioko et al. (2006) for PCR-RFLPs and cpSSRs, and Cao et al. (2006a) for AFLPs. DNA sequence variation of selected cpDNA fragments and the ITS region is under investigation by Nuroniah et al. (2008; in prep.) and Nguyen et al. (2008; in prep.)

Intraspecific variation was assessed in detail for two common *Shorea* species in Indonesia: *S. leprosula* and *S. parvifolia* by means of AFLPs (Cao et al. 2006b). The conversion of AFLP markers to SCAR markers is described by Nuroniah et al. (2008; in prep.).

5 Results and discussion

5.1. DNA Isolation

In order to test the DNA isolation method, wood and leaf DNA extracts from the same tree were amplified with three chloroplast microsatellite primers and genotyped. The result shows that the microsatellite fragments of wood and leaf from the same tree have the same length. The chloroplast region *trnF* (approximately 400 bps)
was sequenced for five trees sampled in the Philippines in order to verify the method. The results show that the \textit{trnF} sequences obtained from wood and leaf from the same tree were identical (100\% homolog), but that the sequences differ among species. A Blast search against the EMBL database confirmed that the sequences belong to the expected genomic regions.

A concentration of 2.6\% (w/v) of PVP was routinely added to the lysis buffer in most extractions reported here. Results after the addition of 2.6\% PVP were generally satisfactory. However, the extraction of high quality DNA from a few wood samples was difficult due to strong PCR inhibition. These samples were used to evaluate the effect of different PVP additions to the lysis buffer on amplification success.

Three DNA isolation methods differentiated by the different PVP quantity added into the lysis buffer (0\%, 2.6\% and 5.0\% [w/v]) were compared. The effect of different PVP quantity on PCR inhibition was tested. DNA extraction without PVP treatment left a high content of PCR inhibitory substances so that even 160 times diluted wood extract inhibited the PCR reaction. The addition of 5\% (w/v) of PVP shows that a 40 times diluted extract had no inhibitory effect showing that PVP addition to the lysis buffer (step 8.a, Rachmayanti 2006) can effectively reduce PCR inhibition in the DNA extract.

Eleven wood samples from Indonesia were used for this analysis of DNA quantity and quality in different parts of the wood. Undiluted DNA extracts (both of 1st and 2nd eluate) from the three different zones of wood (outer sapwood (a), transition zone between sapwood and heartwood (m) and inner heartwood (i)) were used as template. PCR success rate was tested with the three chloroplast markers. In each PCR test a PCR positive control of dipterocarp leaf DNA and PCR negative controls of water were included (Fig. 1). An analysis of PCR success rates of the second eluate suggests that DNA quantity is decreasing along the wood regions from outer sapwood to inner heartwood. This is illustrated by the decreasing PCR success rate for short (0.15 kb), middle (0.6 kb) and long fragments (1.1 kb) along regions a (1.0; 1.0 and 0.91 for short, middle and long fragment, respectively), m (0.73; 0.64 and 0.55) and i. (0.45; 0.27 and 0.10). These results suggested that DNA quantity in the outer ring of sapwood (a) is higher than that in the middle rings (m), and lowest in the inner ring of heartwood (i).

Generally, the amplification success rate for the longer fragment (1.1 kb) is lower than for shorter fragments (0.6 kb and 0.15 kb). This tendency is observed on all DNA extracts of a, m and i suggesting that genomic DNA in wood samples is generally degraded into small fragments.

Further analysis of PCR inhibition on extracts from different parts of wood was performed on seven Indonesian wood samples which showed PCR inhibition after a first test. PCR inhibition was tested on the DNA extracts from three different parts (a, m and i) of each wood sample. PCR inhibition rate of concentrated DNA extracts from outer sapwood (a) is 100\%. However, in the case of undiluted DNA extracts from the transition zone (m) and from the inner ring of heartwood (i) the inhibitory rate drops to about 70\% (5 out of 7 samples). PCR inhibition in wood DNA extract was removed in zone i after 20 times dilution. In zone m after 80 times, and in zone a after 320 times dilution. These results suggest that the content of PCR inhibitory substances is decreasing from the outer sapwood to the inner heartwood.

The standard extraction protocol as described in Rachmayanti et al. (2006) with the addition of 2.6\% (w/v) PVP was used to extract DNA from a total of 332 dipterocarp wood samples. Shavings from outer sapwood were used for most unprocessed samples.

The success of DNA isolation was evaluated by applying PCR amplification to the DNA extracts instead of measuring DNA concentration spectrophotometrically since in many cases the ratio of A260/280 and A260/230 were very low indicating high impurity of the DNA extracts due to proteins, aromatic groups, phenols, carbohydrates or other substances.

Three chloroplast DNA markers which amplify DNA fragments of different length (0.15 kb for \textit{ccmp2}, 0.6 kb for \textit{trnL}, 1.1 kb for \textit{trnLF}) were applied for PCR amplification in order to evaluate DNA quality (degradation level of wood DNA).

Successful amplification was achieved in 369 out of 408 PCR reactions (90.9 \%) for the fragment \textit{ccmp2} (approximately 150 bps), in 319 out of 408 reactions (70.8 \%) for the \textit{trnL} fragment (approximately 600 bps), and in 234 out of 408 reactions (57.6 \%) for the \textit{trnLF} fragment (approximately 1,100 bps). Thus, the average success rate for the three fragments was 75.7 \%. All samples which were directly collected from natu
ral forests or plantations (unprocessed wood and storage duration until DNA extraction from 1 to 4 years) have a good result after PCR amplification, i.e. the PCR success rate is 100% for ccmp2 (short amplicon) and trnL (middle length amplicon) and 50% – 100% for trnLF depending on the geographic region or species. PCR amplification was highly successful (success rate 100% for short, middle and long amplicons) in all dipterocarps samples from Vietnam.

In case of dipterocarp samples from Indonesia and the Philippines PCR inhibition was found in DNA extracts of some samples in the PCR inhibitory test. The inhibitory effect could be reduced significantly by 10 or 20 times dilution of wood DNA extracts before performing PCR amplification. After dilution up to 1:20 a successful amplification of the long fragment (trnLF, 1.1 kb) could still be achieved in all samples. However, in some samples dilution until 80, 160 or even 320 times was needed to remove the PCR inhibition.

Low amplification success rate was obtained in the samples of Meranti (Shorea spp.) from wood enterprises (75 – 85% for short fragment of ccmp2, 42 – 49% for middle length fragment of trnL and about 15% for long fragment of trnLF), although the inhibition test revealed no PCR inhibitory substance in the DNA extract. This result might be caused by the high degradation level of the genomic DNA due to very long storage duration and processing of wood after shipment from producer to consumer countries (gluing, pressure, oven heating, etc.). However, the use of short DNA fragments as molecular markers for the identification of processed wood seems to be feasible in most cases even for processed wood.

5.2. Genetic variation and marker development

A phylogenetic tree based on the variation of cpDNA using PCR-RFLPs and cpSSRs (Indrioko et al. 2006) is consistent with the conventional taxonomy of the subfamily Dipterocarpoideae as described, for example, by Ashton (1982). Most species can be unambiguously recognized based on the observed cpDNA variation. Numerous diagnostic characters allow the assignment of samples to a particular taxon (species, genus, or tribe). The observation of diagnostic characters is particularly noteworthy for endemic species since their observation in a particular sample allow not only to identify the species, but gives at the same time information on the possible origin of the material, which is for obvious reasons restricted to the distribution area of the endemic species.

Tribe Dipterocarpaceae was further investigated by sequencing of the trnL intron and the trnL-trnF intergenic spacer region (Nguyen et al. 2008; Nguyen et al. in prep.). An unambiguous assignment of material up to the species level proved to be feasible based on the investigated sequences in almost all cases (Fig. 2).
Figure 2: One of the most parsimonous trees of tribe Dipterocarpaceae based on sequence variation of the cpDNA regions trnL and trnLF. Branch length above, bootstrap value below branches (Nguyen et al. 2008).
Intraspecific variation was assessed for selected taxa of the genus *Shorea* (Cao et al. submitted). All species revealed considerable levels of genetic diversity within populations (Table 1). The common Indonesian species *S. leprosula* and *S. parvifolia* were studied in particular detail. Preliminary results suggest low variation levels of cpDNA within species, and the absence of strong phylogeographic signals at cpDNA markers (Indrioko 2005). This observation severely impedes the identification of the origin of wood by this type of marker. However, ample genetic variation was observed both within and among populations at AFLPs for both species (Cao et al. 2006b).

<table>
<thead>
<tr>
<th>Pop. ID</th>
<th>Location</th>
<th>N</th>
<th>PPL</th>
<th>ne</th>
<th>He</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. parvifolia</em></td>
<td>NM</td>
<td>26</td>
<td>44.71%</td>
<td>1.447</td>
<td>1.176</td>
<td>0.110</td>
</tr>
<tr>
<td><em>S. acuminata</em></td>
<td>NM</td>
<td>32</td>
<td>49.41%</td>
<td>1.494</td>
<td>1.159</td>
<td>0.100</td>
</tr>
<tr>
<td><em>S. dasypylia</em></td>
<td>NM</td>
<td>20</td>
<td>55.29%</td>
<td>1.553</td>
<td>1.273</td>
<td>0.164</td>
</tr>
<tr>
<td><em>S. blumutensis</em></td>
<td>NM</td>
<td>21</td>
<td>62.35%</td>
<td>1.624</td>
<td>1.266</td>
<td>0.165</td>
</tr>
<tr>
<td><em>S. leprosula</em></td>
<td>NM</td>
<td>16</td>
<td>42.35%</td>
<td>1.424</td>
<td>1.224</td>
<td>0.134</td>
</tr>
<tr>
<td><em>S. macroptera</em></td>
<td>NM</td>
<td>26</td>
<td>52.94%</td>
<td>1.529</td>
<td>1.259</td>
<td>0.155</td>
</tr>
<tr>
<td>Mean</td>
<td>NM</td>
<td>24</td>
<td>51.18%</td>
<td>1.512</td>
<td>1.226</td>
<td>0.138</td>
</tr>
<tr>
<td><em>S. parvifolia</em></td>
<td>S</td>
<td>31</td>
<td>51.76%</td>
<td>1.518</td>
<td>1.199</td>
<td>0.122</td>
</tr>
<tr>
<td><em>S. leprosula</em></td>
<td>S</td>
<td>26</td>
<td>45.88%</td>
<td>1.459</td>
<td>1.192</td>
<td>0.115</td>
</tr>
<tr>
<td><em>S. palebanica</em></td>
<td>S</td>
<td>25</td>
<td>61.18%</td>
<td>1.612</td>
<td>1.245</td>
<td>0.149</td>
</tr>
<tr>
<td><em>S. platyclados</em></td>
<td>S</td>
<td>27</td>
<td>65.88%</td>
<td>1.659</td>
<td>1.235</td>
<td>0.144</td>
</tr>
<tr>
<td><em>S. johorensis</em></td>
<td>S</td>
<td>24</td>
<td>55.29%</td>
<td>1.553</td>
<td>1.183</td>
<td>0.115</td>
</tr>
<tr>
<td>Mean</td>
<td>S</td>
<td>27</td>
<td>56.00%</td>
<td>1.560</td>
<td>1.211</td>
<td>0.129</td>
</tr>
</tbody>
</table>

PPL, percentage of phenotypically polymorphic loci; ne, observed number of alleles per locus; ne', effective number of alleles per locus; He, Nei’s gene diversity; I, Shannon’s information index.

AFLP loci showing strong differentiation among the islands of Sumatra and Borneo were successfully converted to simple SCAR markers (Nuroniah et al. 2008; Nuroniah et al. in prep.). This marker allows to unambiguously assign material of this common dipterocarp to one of the two main Indonesian islands.

5.3. Conclusions

Dipterocarps are the most important group of timber trees from tropical Asia. The family Dipterocarpaceae contains both important common timber species and endangered taxa. Thus, the development of tools to identify the origin of dipterocarp wood deserves high priority within the context of both the international timber trade and biodiversity conservation.

We sampled material from more than 3000 dipterocarp trees belonging to 116 species with the main objective to develop tools for the identification of the origin of tropical timber. This material is a suitable basis for the development of reference data banks containing relevant genetic data on species and regions.

Simple and reliable methods to isolate DNA from wood of dipterocarps were developed which proved to be efficient for the isolation of DNA from wood of other tropical and temperate tree species as well. Untreated wood is a suitable source for DNA extraction even several months to years after felling. Success rates from treated wood are lower, but it is often possible to isolate short fragments of a few hundred bps even from processed wood. More systematic research is needed to investigate the effect of wood treatment on DNA isolation success.

Species identification is an important requirement in particular for endangered taxa of the very diverse dipterocarp family, and allows inferences on the possible origin of material due to the large number of endemic species. In most cases, variation of chloroplast DNA allows a reliable identification up to the species level.

Intraspecific variation is abundant, but mainly within populations. However, it is demanding, but possible to develop markers showing strong phylogeographic variation patterns within species. For example, a SCAR marker developed...
from an AFLP locus allows to distinguish *S. leprosula* trees from Sumatra island from trees of the same species growing on Borneo.

In summary, currently available tools allow to test the declaration of the origin of dipterocarp timber by means of molecular markers. The available techniques and the current knowledge will allow to identify false declarations in many, but not all cases. More research is required to develop informative markers showing strong intraspecific differentiation at the regional scale. The dipterocarps are a suitable group to prove the usefulness of molecular genetic markers to trace the origin of tropical timber.

6 References


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