ISOLATION OF DNA FROM UNPROCESSED AND PROCESSED WOOD OF DIPTEROCARPACEAE

Dissertation

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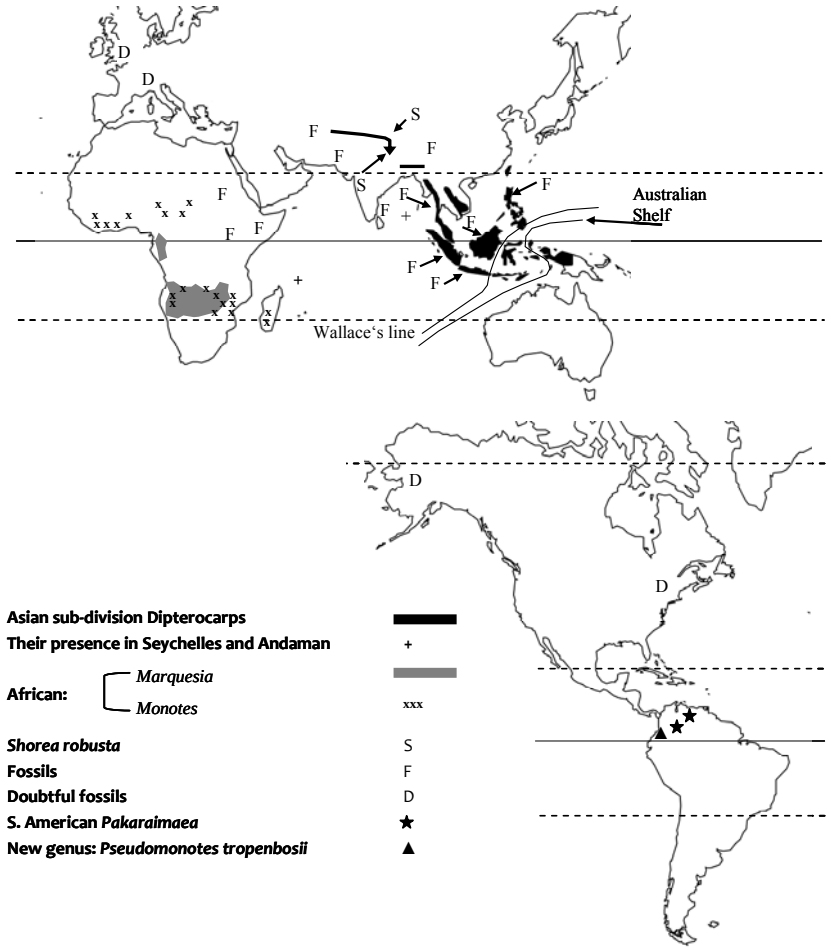
1. General Introduction

1.1. Family Dipterocarpaceae

Dipterocarpaceae ("dipterocarps") is a species-rich tree family dominating Southeast Asia's tropical forests. The distribution patterns of this family reflect routes of colonization and past climatic conditions. They are distributed over the tropical belt of three continents of South America (Guyana, Venezuela and a part of the Colombian Amazon), Africa (in the northern hemisphere, from Mali in the west to Sudan in the east; in the southern hemisphere, south of the Congolese rain forests; and at an insular part in Madagascar) and Asia (from the Seychelles, Sri Lanka and India northeastwards to southern China and the Batan Islands, and southeastwards to New Guinea and D'Entrecasteaux Island) (Fig. 1) (MAURY-LECHON and CURTET, 1998). This family consists of three subfamilies: 1) **Pakaraimoideae** (one genus, one species). Genus *Pakaraimaea* is confined to South America (Guyana and Venezuela); 2) **Monotoideae** (three genera, about 40 species). The genus *Marquesia* grows in Africa, *Monotes* are distributed across Africa and Madagascar, and *Pseudomonotes* is found in the Amazonian Colombia of South America; 3) **Dipterocarpoideae** (13 genera, about 470 species). Dipterocarpoideae can be classified into two groups: **Dipterocarpeae** group (8 genera: *Anisoptera, Cotylelobium, Dipterocarpus, Stemonoporus, Upuna, Vateria, Vateriopsis, Vatica*) and **Shoreae** group (5 genera: *Dryobalanops, Hopea, Neobalanocarpus, Parashorea, Shorea*). This subfamily is distributed in Asia and shows much higher species diversity compared to other subfamilies in Africa and South America. The centre of species diversity is reached in Borneo (approximately 267 species) followed by Peninsular Malaysia (approximately 155 species) (ASHTON, 1982; LONDOÑO *et al.*, 1995; MAURY-LECHON and CURTET, 1998).

In many Asian forests, which are regarded as a centre of global biodiversity, dipterocarps cover more than 50% of all trees including the majority of emergent trees of the canopy (FINKELDEY *et al.*, 2007). Dipterocarps predominate the international tropical timber market (trade names: meranti, balau for *Shorea* spp., Keruing for *Dipterocarpus* spp., kapur for *Dryobalanops*, etc.), and therefore play an important role in the economy of many of the Southeast Asian countries. They also constitute important timbers for domestic needs in the seasonal evergreen forests of Asia. Additionally, these forests are sources of a variety of minor products such as nuts, resin, dammar, camphor, tannin, etc., on which the rural people and many forest dwellers are directly dependent for their survival. However, in many regions dipterocarps are critically endangered due to forest destruction and non-sustainable forest management (APPANAH and TURNBULL, 1998; SHIVA and JANTAN, 1998).

Figure 1. Distribution of Dipterocarpaceae (MAURY-LECHON and CURTET, 1998).



1.2. Deforestation and illegal logging

Forest destruction and degradation remain major threats to global biodiversity and cause enormous environmental damage in particular in developing countries (FINKELDEY *et al.*, 2007). Forest losses are reported between 14 and 16 million hectares yearly. Most of them are in tropical regions, where the highest density of species is accommodated. Deforestation contributes to global warming, for 20 to 25 % of global CO_2 emissions. Several causes of deforestation are land use conversion (e.g. expansion of agriculture, livestock farming, bio-fuel and paper industry), settlements or infrastructure, exploitation of mineral resources and use of wood (both legal and illegal) (ZAHNEN, 2008; FAO, 2007). One third of global deforestation was caused by logging more than 50% of which was illegally done (BRACK, 2003). Global damages caused by illegal timber logging cost approximately 150 billion € per year as assessed by the Organization for Economic Co-operation and Development (OECD). According to this assessment approximately 50 % of timber exports from the Amazon Basin, Central Africa, South-East Asia and the Russian Federation originated from illegal logging (DEGEN and FLADUNG, 2008).

Many organizations such as donors, communities, governments and industry/wood product producers have initiated activities to combat illegal logging. Several associations of wood product manufacturers have released policy statements, in which member companies are committed to sourcing their wood or timber from legal and well-managed forests (AF&PA, 2002; CEPI, 2002; FPAC, 2002; ICFPA, 2002 and 2007; TTF, 2003). Governments have also taken similar initiatives which range from procurement policies to the signature of memoranda of understanding between countries to procurement policies of major do-it-yourself centers. An example for an initiative is the Action Plan for Forest Law Enforcement, Governance and Trade (FLEGT) adopted by the EU in 2003 to address the problem of illegal logging and related trade. The Action Plan blends measures in producer and consumer countries to facilitate trade of legal timber, and aims to eliminate illegal timber from trade within the EU (EUROPEAN UNION, 2002; SMITH, 2002; COMMISSION OF THE EUROPEAN COMMUNITIES, 2003). Another example is the policy of the Malaysian government to prohibit the import of logs from Indonesia, in order to assure purchasers that Malaysian wood products are taken from legal sources (MALAYSIAN TIMBER COUNCIL, 2002). Non-government organizations (NGOs) such as the Environmental Investigation Agency, Global Forest Watch, Greenpeace, TELAPAK etc. have played an important role as credible third-parties to ensure the correctness and effectiveness of the program taken by governments and industries. Due to their independency from government and industry and their credibility in the public, they are able to act as watchdogs working both in detecting illegal logging activities and in raising awareness of the issue (SMITH, 2002). In addition, the NGOs have proposed programs or methods to develop and implement sustainable forest management practices such as forest certification. A forest enterprise which has been certified as working in accordance to the principles of sustainability can be recognized by the logo of the certifying agency by end-consumers (FINKELDEY *et al.*, 2007). Different certification schemes have been proposed, but the most widely recognized schemes are the Program for the Endorsement of Forest Certification Schemes (PEFC; http://www.pefc.org) and, in particular in tropical forests, the Forestry Stewardship Council (FSC; http://www.fsc.org/fsc) (CAULEY *et al.*, 2001).

A core part of any illegal logging detection and monitoring program must be field investigations (SMITH, 2002). A crucial component of any successful and efficient certification scheme is the chain of custody, i.e. the path taken by raw materials of wood, processed materials and products from the forest to the consumer. Attempts to manipulate the system by including wood from non-sustainable forest management in "certified" products became increasingly lucrative with increased marketing potential and commercial success of products from certified forest enterprises (DYKSTRA *et al.*, 2003a and 2003b; FINKELDEY *et al.*, 2007). An example is

smuggling of illegal wood products from Indonesia to Malaysia (EIA and TELAPAK, 2004). An evidence of the origin of timber or even finished wood products from a legal logging activity, i.e. from one or several certified forestry enterprise(s) is necessary. Therefore, the development of tools to test the origin of wood particularly to check the plausibility of statements concerning the origin of wood from a certain forest enterprise is required. The observation of genetic traits offers great potential since genetic information at the DNA-level is a non-manipulable and hence a trustworthy trait to evaluate any biological material at various levels (FINKELDEY *et al.*, 2007).

1.3. Molecular genetic tools for the verification of wood origin

The use of molecular genetic tools for origin identification of ancient and modern humans is widely reported either at the individual level or at the population level. The potency of uniparentally inherited DNA markers, such as mitochondrial DNA (mtDNA) which is inherited only through the females of the previous generation (maternal lineage), was shown by Vernesi *et al.* (2001) in an investigation of the regional origin of the evangelist Luke (an early Christian leader). At the population level, the frequency of the 9-bp deletion in the cytochrome c oxydase subunit II (CoII/tRNA^{Lys}) of the mtDNA (together with other mitochondrial markers) was used to investigate the colonization of the Pacific islands and the American continent. While the 9-bp deletion is absent in many Caucasian and many African populations, a frequency of 10-60% exists in Southeast Asia, the Pacific islands and native North American populations. However, in South American native populations, frequencies of between 90-100% are found for the deletion. In ancient DNA analysis this may be used, for example, to identify mummies from uncertain geographical origin. Beside mtDNA markers, Y-chromosomal DNA, which is inherited through the paternal lineage, is also widely used as a valuable tool for human migration research, because of regional variation of these haplotype frequencies (HUMMEL, 2003).

Genetic methods can be also useful to infer species identity and are promising tools to control the geographic origin of logged timber. A spatial genetic structure for most species in natural forests could be observed, due to recolonisation after the last glacial periods and limited pollen and seed dispersal. Genetic inventories using extensive and systematic sampling over the whole species distribution area are the basis to identify the geographic region of timber origin. Hence, two basic conditions need to be met in order to apply molecular genetic methods to infer the origin of wood: First, protocols need to be developed to extract DNA in sufficient quantity and quality from unprocessed or processed wood. Second, diagnostic DNA markers need to be identified, i.e., genetic inventories with a high spatial resolution are needed (FINKELDEY *et al.*, 2007; DEGEN and FLADUNG, 2008).

1.3.1. Diagnostic DNA markers

Patterns of natural genetic differentiation among populations of forest trees have been shaped by the joint effects of evolutionary factors. Over time genetic structure at different spatial scales can result from limited pollen and seed dispersal within a population, limited gene flow between populations or the historic disruption of a once continuous range of a species by climatic or geographic changes. Each of these changes will produce genetic discontinuities or clines within a species that can be identified using a range of DNA-based markers. "Informative" DNA markers can be identified at various levels from large provenance regions to smaller subunits of populations for most species, if large scale inventories involving many samples and populations are undertaken. In many cases, only a combination of several informative markers will allow to come to a reliable conclusion with regard to the putative origin of tested wood. Maternally inherited markers (mtDNA or, in case of angiosperms, chloroplast DNA (cpDNA) markers) are particularly useful due to their frequently higher levels of population differentiation, because pollen movement is more widespread as compared to the movement of seeds in most species (PETIT *et al.*, 2003; FINKELDEY *et al.*, 2007; LOWE, 2008).

The spatial distribution of cpDNA haplotypes has been studied in much detail for European oaks, *Quercus* spp. A clear geographical pattern among naturally regenerated oak populations in Europa has been observed (PETIT *et al.*, 2002 and 2003). For European oaks of the section Lepidobalanus, in particular for *Quercus petraea* and *Q. robur*, the identification of the origin of wood by means of molecular genetic markers is well-advanced (DEGUILLOUX *et al.*, 2003). For tropical trees, several phylogeographic studies (genetic structuring due to historical gene flow and discontinuity processes) and broad scale population genetic differentiation have highlighted significant genetic structure across the native range of many trees (LOWE, 2008). A wide range of DNA marker systems was applied: SSRs (Simple Sequence Repeat) and RAPD (Random Amplified Polymorphic DNA) for *Swietenia macrophylla* (mahogany) in the geographical range of Central America and southern Brazil (NOVICK *et al.*, 2003; LEMES *et al.*, 2003; GILLIES *et al.*, 1999); cpDNA and AFLP (Amplified Fragment Length Polymorphism) for *Cedrela odorata* (Spanish cedar) in Central America (Costa Rica) (CAVERS *et al.*, 2003 and 2005); cpDNA and AFLPs for *Hagenia abyssinica* (African redwood / Rosewood) in Ethiopia (TAYE, 2008; TAYE *et al.*, 2009). In case of the tree family Dipterocarpaceae, a phylogeographic study (variation between geographic regions) was reported by Cao *et al.* (2006). The AFLP marker technique was applied to analyze the variation in seven *Shorea parvifolia* and *Shorea leprosula* populations from Borneo and from Sumatra. Large genetic variation was observed both within and among populations for both species (CAO *et al.*, 2006). AFLP markers showing strong differentiation among the islands of Sumatra and Borneo were successfully converted to simple SCAR (Sequence Characterized Amplified Region) markers (NURONIAH, 2009). This marker allows to unambiguously assign material of these common dipterocarps to one of the two main Indonesian islands (FINKELDEY *et al.*, 2007 and 2008). Beside phylogeographic, many phylogenetic studies (variation

between species) have been performed in dipterocarps using different nuclear and chloroplast DNA markers (DAYANANDAN *et al.*, 1999; MORTON *et al.*, 1999; GAMAGE *et al.*, 2003 and 2006; KAMIYA *et al.*, 1998; KAJITA *et al.*, 1998, LI *et al.*, 2004; YULITA *et al.*, 2005; INDRIOKO *et al.*, 2006). It was observed that the combination of different markers allows an unambiguous identification of the species. Since some dipterocarp species are present in a relatively restricted geographic range (i.e. endemic species), the identification of species becomes a prerequisite to test the correctness of declaration of wood from unknown origin. For example, *Upuna borneensis*, *Shorea fallax* and *Anisoptera reticulate,* the endemic species of northern Borneo, could be characterized by several diagnostic (species-specific) cpDNA markers (INDRIOKO *et al.*, 2006). In addition, species identification is an important requirement in particular for endangered taxa of the very diverse dipterocarp family (FINKELDEY *et al.*, 2007 and 2008).

1.3.2. Wood DNA extraction

A standard and uncomplicated DNA extraction methodology for plant leaf, bud, root and other "living" tissue has been widely used and is now enhanced by application of semi- or fully automatic equipment (e.g. QIAGEN extraction kits combined with robotic workstations supporting vacuum or centrifuge components). DNA extraction from freshly harvested wood incorporating cambium tissue has also been found to yield DNA of high quality comparable to that from leaf material (COLPAERT *et al.*, 2005). This is not the case for dried wood tissue, in which DNA extraction has been found to be more problematic (LOWE, 2008). Much work has been done on oak timber of different ages and preserved under different conditions (DEGUILLOUX *et al.*, 2002, 2003 and 2004). In special designed laboratories using contamination-exclusion techniques, DNA fragments of up to 500 bp from ancient sources of timber that was up to 3600 years old had been amplified (GUGERLI *et al.*, 2005).

Main obstacles for DNA extraction from wood and wood products as compared to other plant tissues are: (1) Physical, as mechanical treatments applied to disrupt a hard wood tissue cause overheating that may lead to irreversible DNA degradation (FINKELDEY *et al.*, 2007; RACHMAYANTI *et al.*, 2006). (2) Chemical, as numerous agents and wood compounds potentially inhibit DNA extraction or result in low-quality DNA not suitable for amplification by PCR (LEE and COOPER, 1995; RACHMAYANTI *et al.*, 2009). (3) Biological. Decomposition of wood by fungi and microorganisms due to long periods of inappropriate storage results in degradation of wood DNA and provides an alternative source of DNA from decaying organisms (FINKELDEY *et al.*, 2007; LINDHAL *et al.*, 1993). (4) Age, as degeneration of DNA will start after the death of a plant cell (the death of wood tissue), thus the size of DNA fragments which can be amplified is expected to continuously decrease (DEGUILLOUX *et al.*, 2002; RACHMAYANTI *et al.*, 2009).

1.4. Research objectives

Sustainable management of dipterocarp forests is feasible, if harvesting is carefully controlled and natural regeneration promoted (LAMPRECHT, 1986). 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 as well as their associated species. Studies to identify "informative" DNA markers for species identification and for geographical differentiation of dipterocarps have been started and will continuously proceed (FINKELDEY *et al.*, 2007 and 2008). Thus, the objective of this research is to establish DNA extraction methods for wood of Dipterocarpaceae. Wood probes of different species, age and type (unprocessed and processed) collected from different countries were included in this study for the optimization of DNA isolation methods.

1.5. Research methodology

In this research the following methodology was applied:

1. Test of several published DNA isolation methods and commercial kits for processed and unprocessed wood.
2. Analysis of extracted DNA to confirm isolation success.
3. Modification of methods to improve DNA isolation success.
4. Selection and verification of the best or the most efficient method.
5. Application of the selected method to isolate DNA from wood of different species, types and origins.
6. Quantitative and qualitative analysis of DNA extracted from different zones of dipterocarp wood as well as analysis of the PCR inhibitor content.

1.6. Summary of results

The optimized DNeasy Plant Mini Kit (Qiagen) method reported in Rachmayanti *et al.* (2006) is considered as the most efficient method and used to isolate DNA from dipterocarp woods of different species, type (unprocessed and processed), age and origin. It was used also for the study to analyze the quantity and quality of DNA extracted from different zones of dipterocarp woods and to analyze the PCR inhibitor content in the extracts. This method is considered simple, due to its size (small scale procedure) and its applicability to be carried out in a standard equipped genetic laboratory. Nevertheless it gives an adequate DNA quality & quantity for PCR amplification and other down stream molecular methodologies such as DNA genotyping, cloning and sequencing. The addition of polyvinylpyrrolidone (PVP) into the lysis buffer is an important

step in this method, since it could reduce PCR inhibition significantly (*see* Appendix 6 and RACHMAYANTI *et al.*, 2006). To verify the method, DNA from wood and leaf collected from the same tree were isolated and then analyzed by PCR amplification, microsatellite genotyping and sequencing. The result shows that DNA extracted from wood and leaf of the same tree are identical and Blast analysis at Genebank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that DNA sequences belong to expected regions (RACHMAYANTI *et al.*, 2006 and 2009).

The success of DNA isolation was confirmed by PCR amplification of DNA extracted from wood. The DNA isolation method is applicable for dipterocarp wood (332 samples) as well as for non-dipterocarp wood (74 samples). A study of chloroplast DNA markers using three primer pairs amplifying three fragments of different length (short fragment, *ccmp*2, approximately 150 bp; middle length fragment, *trn*L, approximately 600 bp; long fragment, *trn*LF, approximately 1,100 bp) showed that successful amplification was achieved in 369 out of 406 PCR reactions (90.9%) for the short fragment, in 319 out of 406 reactions (78.6%) for the middle length fragment and in 234 out of 406 reactions (57.6%) for the long fragment. Thus, the average success rate for the three fragments was 75.7% (*see* RACHMAYANTI *et al.*, 2009: Fig. 3). Analysis of DNA extracts from three different zones of wood, i.e. outer rings of sapwood (**a**), transition rings of sapwood and heartwood (**m**) and inner rings of heartwood (**i**), shows that : 1) genomic DNA in wood samples is generally degraded into small fragments. It is confirmed by the tendency observed in all DNA extracts of **a**, **m** and **i**, that the amplification success rate for the longer fragment (1.1 kb) is lower than for shorter fragments (0.6 kb and 0.15 kb); 2) DNA quantity is decreasing along the wood regions from outer sapwood to inner heartwood. This is indicated by the decreasing PCR success rate for all fragments (short, middle and long) along regions **a**, **m** and **i**; 3) DNA quality is lower along the wood regions from outer sapwood to inner heartwood, as DNA degradation level increases from outer sapwood to inner heartwood. This is concluded by comparing the decreasing level of PCR success rate between long, middle and short fragments along regions **a**, **m** and **i**. The decrease of PCR success rate for the long fragment along regions **a**, **m** and **i** is much higher than for middle-length and short fragments (*see* RACHMAYANTI *et al.*, 2009: Fig. 1); 4), the content of PCR inhibitory substances is decreasing from the outer sapwood to the inner heartwood (*see* RACHMAYANTI *et al.*, 2009: Fig. 2).

2. General Discussion

2.1. Wood samples and success rate of DNA amplification

Wood of different species, types (unprocessed and processed), regional origin and condition were analyzed in this study. A total of 406 wood samples consisting of 332 dipterocarp woods and 74 non-dipterocarp woods were analyzed (RACHMAYANTI *et al.*, 2009). PCR amplifications were done successfully (100% success rate for short, middle and long DNA fragments) for the following samples: all dipterocarp samples from Vietnam (n=40), all non-dipterocarp samples of *Tectona grandis* (n=12), *Prunus arborea* (n=1), *Strombosia ceylanica* (n=1), *Populus* spp. (n=25), *Taxus baccata* (n=2) and *Salix* spp. (n=1). Furthermore PCR inhibition was not found in undiluted DNA extracts for these samples (*see* Appendix 2 and RACHMAYANTI *et al.*, 2009: Fig. 5). In case of dipterocarp samples from Indonesia (n=38) and the Philippines (n=50) as well as for woods from Costa Rica (n=3) and *Pinus sylvestris* (n=12) PCR inhibition was found in DNA extracts using PCR inhibitory tests (see Appendix 5 and RACHMAYANTI *et al.*, 2009: Fig. 2). This inhibitory reaction could be eliminated by 10 or 20 times dilution of wood DNA extracts. In this case a successful amplification of the long fragment (1.1 kb of *trn*LF) could be achieved by a standard PCR (RACHMAYANTI *et al.*, 2006). For some other samples dilution until 40 times (1:40) or higher was needed to remove the PCR inhibition. However, a successful PCR amplification of the middle length and the long fragment (0.6 kb of *trn*L and 1.1 kb of *trn*LF)) was almost impossible. For dipterocarp samples from Thailand (n=53) and for non-dipterocarp samples of *Eusideroxylon zwageri* (n=2) and *Picea abies* (n=8) PCR success rate for the long fragment (*trn*LF) was lower than 100% (85%, 50% and 25% respectively; *see* Appendix 2 and RACHMAYANTI *et al.*, 2009: Fig. 5). In some samples a successful PCR amplification of the long fragment could not be achieved even though PCR inhibition was low or absent. Presumably the genomic DNA in these samples was already strongly degraded due to: 1) processing of wood samples (dried wood shavings in case of dipterocarp samples from Thailand); 2) long storage of wood samples (4 years in case of *Eusideroxylon zwageri*); 3) inappropriate wood storage conditions, i.e. wet or moist storage so that almost the whole wood was covered by wood decaying organisms like fungi (in case of *Picea abies*). Low amplification success rate was obtained in the samples of Meranti (*Shorea*; dipterocarp wood) and Abeche (*Triplochiton scleroxylon*) from wood enterprises (75 – 85% for the short fragment of *ccmp*2, 42 – 49% for the middle length fragment of *trn*L and about 15% for the long fragment of *trn*LF; *see* RACHMAYANTI *et al.*, 2009: Fig. 4 and Fig. 5), although the inhibition test revealed no PCR inhibitory substances in the DNA extracts. High degradation levels of the genomic wood DNA due to long storage including

shipment from producer to consumer countries and processing of wood such as gluing, pressurize and heating etc. are suspected to be the cause of these results. However, the use of short DNA fragments as molecular markers for the identification of processed wood seems to be feasible in most cases.

2.2. Test of DNA isolation methods and analysis of DNA extract

Five DNA isolation methods were examined for several wood samples: 1) Qiagen, DNeasy 96 Plant Kit; 2) Qiagen, DNeasy Plant Mini Kit; 3) Plant- Molzym Kit; 4) Qiagen, EZ1 DNA Tissue Kit with Biorobot EZ1 Workstation; 5) Qiagen, MagAttract 96 DNA Plant Core Kit, thereby using the 96-Well Magnet Type A (www.Qiagen.com) or using a magnetic particle concentrator from Dynal Biotech (www.dynalbiotech.com). Additional methods were applied to analyze DNA extracts or to proof the isolation success. Electrophoresis of total DNA on agarose gels gave non-interpretable results, since for most samples DNA could not be visualized using UV light. The following reasons are considered to be the causes: 1) the DNA yield is to low to be visualized by this technique; 2) Wood DNA is strongly degraded into small DNA fragments, thus on the gel only a slight smear throughout the lane is visible under UV light. Using spectrophotometry, DNA yield could be quantified if the extract is pure enough as indicated by the optical density (OD) ratios of λ260/280 and λ260/230 are 1.8-2.0 and 2.0, respectively (SAMBROOK *et al.*, 1989; Tataurov *et al.*, 2008). In this study, spectrophotometrical analysis applied to wood DNA extracts resulted in very low ratios of λ260/280 and λ260/230 in many cases (in 76% of the tested samples). This indicates high impurity of the DNA extracts due to other plant substances such as proteins, aromatic groups, phenols, carbohydrates and also probably due to inhibitory substances derived from decomposing plant material which are potentially found in dead wood tissues such as humic acid, tannins, etc. (HUMMEL, 2003; FINKELDEY *et al.*, 2007). Therefore, accurately measuring the DNA quantity by spectrophotometry is not possible for wood extracts. A better and reproducible technique to ensure DNA isolation success is by PCR amplification of extracted DNA using different primer pairs which amplify DNA fragments of different lengths followed by genotyping and/or sequencing (RACHMAYANTI *et al.*, 2006 and 2009).

2.3. Modification and evaluation of DNA isolation methods

Many modifications were tested for each method in order to improve DNA isolation success. The scope of modification includes: 1) the amount of wood (20, 50, 100 and 200 mg); 2) disruption techniques consisting of two steps. First, cutting by a scalpel or drilling by a bore machine in order to get small pieces of wood or shavings. Second, grinding of wood shavings by

a mortar or by mixer mills (Retsch) using different sizes of milling beads and jars (such as a Ø 3 mm of tungsten carbide bead or a Ø 5 mm stainless steel bead in a 2 ml Eppendorf tube and a Ø 12 mm agate ball in a 10 ml agate jar) and different milling frequencies and durations (such as 1 x 1.2 min at 20 Hz by Retsch type MM300 and 1 x 5 min at 75 frequency unit or 2 x 5 min at 70 frequency unit by Retsch type MM2); 3) lysis procedure including modification of the lysis buffer and incubation. Both of buffers provided by kits and other buffers such as 0.5 M EDTA pH 8.0 and cetyltrimethyl-ammoniumbromid (CTAB) were tested (TEL-ZUR *et al.*, 1999; KHANUJA *et al.*, 1999). In addition, mixtures of kit lysis buffers with polyvinylpyrrolidone (PVP, until 5% (w/v)) were tested (Appendix 6). Modifications of lysis incubation was done by prolonging the incubation time up to 2 nights and by shaking or vertical rotating of the tube containing the lysis mixture; 4) DNA purification by comparing the spin column procedure of the DNeasy plant mini kit (Qiagen) with the magnetic-based procedure of the MagAttract 96 plant core kit (Qiagen) and the Dynal Biotech kit (RACHMAYANTI *et al.*, 2006); 5) DNA elution was performed by separation of the second eluate from the first one.

A modified DNeasy Plant Mini Kit (Qiagen) protocol as reported in Rachmayanti *et al.* (2006) was considered as the best and most efficient method, because it is relative simple and cheap (a small scale procedure and applicable to standard equipments of genetic laboratory). Nevertheless it gives an adequate DNA quality & quantity for PCR amplification and other down stream molecular methodologies (DNA genotyping, cloning and sequencing). The addition of polyvinylpyrrolidone (PVP) into the lysis buffer is an important step in this method, since it could reduce PCR inhibitors significantly (RACHMAYANTI *et al.*, 2006). PVP can bind polyphenols and tannins, - chemical substances which inhibit Taq polymerase activity (HUMMEL, 2003) -, that may be found abundantly in dead wood tissue derived from the degradation of plant biomolecules such as lignin (FINKELDEY *et al.*, 2007). During preparation of wood samples by any mechanical treatment, the overheating of wood material should be avoided. Frequently incubating the samples on ice or freezing of the sample in liquid nitrogen is required. It is also important that the powder of milled wood is dissolved or mixed thoroughly with the lysis buffer for achieving effective lysis. This can be done by vortexing and then by shaking or vertically rotating the tube with the mixture inside during overnight lysis incubation (*see* RACHMAYANTI *et al.*, 2006). Powder of some wood samples however adsorbed the buffer solution with relatively high capacity (like a sponge), thus completely dissolving or mixing of wood powder with 500 µL of lysis buffer (*see* RACHMAYANTI *et al.*, 2006: DNA isolation protocol, lysis) was not possible. In this case higher volumes of lysis buffer (600, 800, 1000 or 1200 µL) were added to the powder until a good suspension was attained by shaking or rotating during overnight incubation. Since the incubation was carried out with a vertical tube rotation

and under a relatively high temperature, i.e., 65 ^{0}C, the tube should be closed very well in order to prevent leaking or spilling out of the lysis mixture and also to prevent the evaporation of the buffer solution. Sealing of the tube lid by parafilm was sometimes required (dependent on the tube quality) to close it safely. Grinding of wood samples using Retsch mixer mills employing a stainless steal bead (*see* RACHMAYANTI *et al.*, 2006: DNA isolation protocol, step 3 – 6) might have caused a crack in the lid of the tube (dependent on tube quality), thus the wood mills should be transferred to a new tube before lysis.

2.4. Verification of method and study of DNA extracts from different wood zones

The method was verified by comparing DNA isolated from wood and leaf collected from the same tree. Wood DNA was isolated following the modified method of DNeasy Plant Mini Kit (Qiagen) as described in RACHMAYANTI *et al.* (2006), while leaf DNA was isolated following the standard kit procedure. Nevertheless, the same procedure of PCR amplification, genotyping, cloning and sequencing was applied to both DNAs, isolated from wood and leaf. Wood as well as leave samples were collected from 48 dipterocarp trees and 6 chloroplast DNA regions were analyzed (*ccmp*1, *ccmp*2, *ccmp*3, *ccmp*6, *ccmp*10 and *trn*F). The results show that DNA extracted from wood and leaf from the same tree are identical and Blast analysis at Genebank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that DNA sequences belong to expected regions (*see* Appendix 3 and RACHMAYANTI *et al.*, 2006 and 2009).

A total of 18 wood samples from Indonesia with disk diameters between 19.0 until 32.0 cm were included in the study of DNA extracts from different wood zones: outer rings of sapwood (**a**), rings in the transition zone between sapwood and heartwood (**m**) and inner rings of heartwood (**i**). Eleven of them, - which showed no PCR inhibition when their undiluted extracts were tested,- were included in a comparative study of DNA quantity and quality of these three wood zones. The remaining samples were used for the analysis of PCR inhibitor content in different wood zones (*see* RACHMAYANTI *et al.*, 2009: Table 2.A and 2.B). This study was performed based on the PCR amplification technique using three primer pairs which amplified DNA of different lengths (*ccmp*2, c. 150 bp; *trn*L, c. 600 bp; *trn*LF, c. 1,100 bp). The results suggest that DNA quantity and quality is higher along the wood zones from inner heartwood to outer sapwood (lower DNA degradation level from inner heartwood to outer sapwood) and the content of PCR inhibitory substances is increasing from the inner heartwood to the outer sapwood (*see* Appendix 4 – 5 and RACHMAYANTI *et al.*, 2009: Fig. 1 and 2).

2.5. Authenticity of results

PCR is a sensitive technique which is capable of generating large amounts of amplified products from as little as single cells (HUMMEL, 2003), therefore contamination is a serious hazard in applying PCR especially for processed wood or wood products considered as a poor DNA source material. The occurrence of contaminating target sequences in an extract sample containing only small amounts of wood DNA may result in many PCR contamination problems such as unexpected amplicons (double bands) or irreproducibility of PCR. Therefore it is important to construct and to maintain sterile work conditions. In this study efforts have been made to combat contamination problems such as: i) preparation of wood samples, DNA isolation and PCR amplification were carried out in separated places or rooms; ii) use of different or separated micropipettes for DNA isolation and for PCR amplification; iii) sterilization of pipette tips, samples and PCR tubes under UV light (λ 320 nm, distance about 10 – 20 cm) for 20 minutes to remove any contaminating DNA (HUMMEL, 2003); iv), regular cleaning of the surface of laboratory benches (working place) and apparatus using reagents for the removal of DNA contaminations (DNA-Exitus Plus, AppliChem GmbH) as well as aquabidest and EtOH (70%); v) cleaning of mixer mill beads with liquid detergent (Alconox powder diluted in water with 6 times higher concentration as prescribe), then with aquabidest and EtOH (70%); vi) use of sterile gloves during working and a laboratory suit. To ensure that amplification was subjected only to wood DNA and not the contaminant, DNA validation was always performed using sets of control samples consisting of positive and negative controls. The positive control indicates that the reaction was set up properly and all parameters were suitably adjusted. In this study, good quality dipterocarp leaf DNA was used as positive control for PCR reactions. The negative controls have been used to observe systematic contamination. Two categories of negative controls were tested in this study: the extraction blanks which allowed all reagents used (during the entire DNA extraction phase until PCR) to be monitored and the no-template controls (sterile aqua bidest as PCR template) which confirmed only those reagents used in the amplification reaction mix.

3. Conclusions and Outlook

The modified method of Qiagen DNeasy Plant Mini Kit as described in RACHMAYANTI et al. (2006) is considered as the most efficient method which gives an adequate DNA quality & quantity for PCR amplification and is recommended to be firstly applied for any investigation involving wood DNA. In order to reduce PCR inhibitor substances, adding polyvinylpyrrolidone up to 2.6% (w/v) into the lysis buffer is recommended for a routine DNA isolation, since it has

been tested for 406 wood samples and gives a good results (high DNA amplification success rate as described in RACHMAYANTI et al., 2009). If required, inhibitor content could be further reduced by diluting DNA extract up to 10, 20 or 40 times to be used as PCR template. Amplification success of DNA isolated from wood depends on several factors: the length of the amplified target (the shorter the amplicon, the higher the success rate), wood species (strong PCR inhibitor substances in some species), wood zone (higher DNA quantity, lower DNA degradation, but higher PCR inhibitory content found in extracts from sapwood than from heartwood), wood storage conditions after felling until DNA extraction (wood decaying increases wood DNA degradation and provides microbial DNA contamination).

The EZ1 DNA Tissue Kit from Qiagen (see General Discussion, Chapter 2.2) is also considered as a good method for DNA isolation from wood. This method is simpler, since most steps could be done automatically employing a robotic workstation (Biorobot EZ1 Workstation). However, this procedure is relatively expensive.

Since DNA of wood is highly and randomly degraded, short DNA fragments are recommended to be employed in genetic investigation such as diagnostic PCR primers (SCARs [Sequence Characterized Amplified Region], RFLPs [Restriction Fragment Length Polymorphism], microsatellites) or sequencing in order to find diagnostic SNPs (Single Nucleotide Polymorphism). Therefore genetic investigation methods requiring high genomic DNA quality like RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) techniques (WEISING et al., 2005) are not appropriate.

In many cases wood decaying could not be prevented or recognized, therefore employing very specific PCR-primers is strongly recommended, i.e., primers which amplify only plant DNA and not DNA from decaying microorganisms. If available, leaf DNA from the same tree or the same species or family as the investigated wood should be used as positive control of PCR to confirm the PCR results.

Studies on common wood processing techniques in industries (plywood, window frames, wood chips or particle boards etc.) is required in order to analyze the effect of each wood treatment such as heating, gluing, pressing, impregnating etc. on the wood DNA degradation.

Based on the above conclusion the following research topics are proposed:

<u>1. Identification of Industrial process causing degradations of DNA content in wood.</u>

DNA content in the wood could be strongly degraded due to certain industrial process which in turn could significantly lower the success of wood origin investigation by means of molecular genetics. This study could be carried out by applying of the new proposed DNA Isolation method (RACHMAYANTI, et al., 2006) to a certain wood, which is included in the whole of process chain in the wood and timber industry. A small pieces of wood sample for DNA isolation should be taken at every industrial step starting from raw materials (saw log or veneer log), intermediate form for example after log storage (wet and/or dry), debarking and cutting (as sawmills, veneer, plywood mills, panel, etc.), as well as after F-joining (finger jointing), glue-lam (Glue laminated), moulding and engineered wood veneering until end products such as flooring, joinery, furniture, parts, etc. Through analysing and comparing DNA extracts from those process steps, the identification of any steps that effectively reduce or destruct DNA content in the wood could be done. This effort could be directed toward the improvement of the industrial processes and trading of woods.

2. Database development of the diagnostic marker for the identification of dipterocarp wood.

This database should provide alternative of DNA markers for specific identification from sub-family, genus until species of dipterocarpaceae as well as its geographical origin. The database could be developed by collecting known diagnostic DNA markers for dipterocarps (SCARs, RFLPs, microsatellites, SNPs, etc.) from eligible sources such as genebank, published papers or journals, etc. The database could be then extended by doing research to find any additional specific marker. After all, as an interface to the user, computer software can be developed that read the input (DNA marker), compare it to the database and finally provide the information regarding the identity of the wood.

4. Summary

Dipterocarpaceae ("dipterocarps") is a species-rich tree family distributed over the tropical belt of three continents of South America, Africa and Asia. This family consists of three subfamilies (Pakaraimoideae, Monotoideae and Dipterocarpoideae), 17 genera and more than 470 species. Dipterocarps predominate the international tropical timber market (trade names: meranti, balau for *Shorea* spp., keruing for *Dipterocarpus* spp., kapur for *Dryobalanops*, etc.), and therefore play an important role in the economy of many Southeast Asian countries. However, in many regions dipterocarps are critically endangered due to forest destruction and non-sustainable forest management.

Forest destruction and degradation (deforestation) remain major threats to the global biodiversity and cause enormous environmental damage in particular in tropical regions, where the highest density of species is accommodated. One third of global deforestation was caused by logging, in which more than 50% was illegally done (Brack, 2003). Many activities have been initiated by government or non-government organizations to fight illegal logging. Several associations of wood-product manufacturers have released policy statements, in which member companies are committed to sourcing their wood or timber from legal and well-managed forests. Non-government organizations (NGOs) have proposed programs or methods to develop and implement sustainable forest management practices such as forest certification. However, a core part of any illegal logging detection and monitoring program must be field investigations. Chain of custody, i.e. the path taken by raw materials of wood, processed materials and products from the forest to the consumer, is a crucial component in this investigation. An evidence of the origin of timber or even finished wood products from a legal logging activity, i.e. from one or several legal and well-managed forest(s) is necessary. Therefore, the development of tools to test the origin of wood particularly to check the plausibility of statements concerning the origin of wood from a certain forest enterprise is required.

Two basic conditions need to be met in order to apply molecular genetic methods: First, protocols need to be developed to extract DNA in sufficient quantity and quality from unprocessed or processed wood. Second, diagnostic DNA markers need to be identified, i.e., genetic inventories with a high spatial resolution. Studies to identify "informative" DNA markers for species identification and for geographical differentiation of dipterocarps have been started and will continuously proceed (FINKELDEY *et al.*, 2007 and 2008). Thus, the objective of this research is to establish DNA extraction methods for wood of Dipterocarpaceae. Wood probes of different species, age and type (unprocessed and processed) collected from different countries were included in this study for the optimization of DNA isolation methods.

This research was started by the test of several published DNA isolation methods and commercial kits for extraction of DNA from several processed and unprocessed woods. Wood extracts were then analyzed by PCR amplification to confirm the success of DNA isolation. Some methods, which showed successful wood-DNA extraction, were selected to be modified in order to improve the DNA isolation success, i.e. to get a better DNA quality and quantity. Finally, the best or the most efficient method was selected to be applied in the further study that is described as follows: Firstly, verification of the method by comparing the DNA extracted from wood and leaf collected from the same tree. PCR amplification, genotyping of microsatellite fragments and sequencing were carried out to analyze the extracted DNA. Secondly, investigation

of the applicability of the method to isolate DNA from wood of different species, types and origins. Thirdly, comparative study of the DNA quantity and quality in extracts of different zones of dipterocarp wood (outer rings of sapwood, rings in the transition zone between sapwood and heartwood and inner rings of heartwood) as well as study of the PCR inhibitor content.

The optimized DNeasy Plant Mini Kit (Qiagen) method reported in Rachmayanti *et al.* (2006) is considered as the most efficient method. This method is considered simple, due to its size (small scale procedure) and its applicability to be carried out in a standard equipped genetic laboratory. Nevertheless it gives an adequate DNA quality & quantity for PCR amplification and other down stream molecular methodologies such as DNA genotyping, cloning and sequencing. The addition of polyvinylpyrrolidone (PVP) into the lysis buffer is an important step in this method, since it could reduce PCR inhibition significantly. To verify the method, DNA from wood and leaf collected from the same tree were isolated and then analyzed by PCR amplification, microsatellite genotyping and sequencing. The result shows that DNA extracted from wood and leaf of the same tree are identical and Blast analysis at Genebank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that DNA sequences belong to expected regions.

Wood of different species, types (unprocessed and processed), regional origin and condition were analyzed in this study. A total of 406 wood samples consisting of 332 dipterocarp woods and 74 non-dipterocarp woods were analyzed. The success of DNA isolation was confirmed by PCR amplification of DNA extracted from wood. A study of chloroplast DNA markers using three primer pairs amplifying three fragments of different length (short fragment, *ccmp*2, approximately 150 bp; middle length fragment, *trn*L, approximately 600 bp; long fragment, *trn*LF, approximately 1,100 bp) showed that successful amplification was achieved in 369 out of 406 PCR reactions (90.9%) for the short fragment, in 319 out of 406 reactions (78.6%) for the middle length fragment and in 234 out of 406 reactions (57.6%) for the long fragment. Thus, the average success rate for the three fragments was 75.7%.

A total of 18 wood samples from Indonesia with disk diameters between 19.0 until 32.0 cm were included in the comparative study of DNA quantity and quality in extracts of different wood zones: outer rings of sapwood (**a**), rings in the transition zone between sapwood and heartwood (**m**) and inner rings of heartwood (**i**). This study was performed based on the PCR amplification technique using three primer pairs which amplified DNA of different lengths (*ccmp*2, 150 bp; *trn*L, 600 bp; *trn*LF, 1,100 bp). The results show that: 1) genomic DNA in wood samples is generally degraded into small fragments. It is confirmed by the tendency observed in all DNA extracts of **a**, **m** and **i**, that the amplification success rate for the longer fragment (1.1 kb) is lower

than for shorter fragments (0.6 kb and 0.15 kb); 2) DNA quantity is increasing along the wood regions from inner heartwood to outer sapwood; 3) DNA quality is higher along the wood regions from inner heartwood to outer sapwood, as DNA degradation level increases from outer sapwood to inner heartwood.; 4) the content of PCR inhibitory substances is increasing from the inner heartwood to the outer sapwood.

5. Zusammenfassung

Bei den Dipterocarpaceen handelt es sich um eine artenreiche Familie von Bäumen, die im tropischen Gürtel auf den Kontinenten Südamerika, Afrika und Asien beheimatet ist. Diese Familie besteht aus drei Unterfamilien (Pakaraimoideae, Monotoideae und Dipterocarpoideae), 17 Gattungen und über 470 Arten. Die Dipterocarpaceae werden häufig auf Tropenholzmärkten gehandelt und nehmen somit eine bedeutende Stellung in der Wirtschaft vieler südostasiatischer Staaten ein. In vielen Regionen sind die Dipterocarpaceen jedoch erheblich gefährdet aufgrund von Entwaldung und nicht nachhaltiger Bewirtschaftung.

Entwaldung stellt die hauptsächliche Bedrohung der globalen biologischen Vielfalt dar und verursacht erhebliche Umweltzerstörung insbesondere in den Tropenregionen, die die höchste Artendichte beherbergen. Ein Drittel der weltweiten Entwaldung ist durch Holzeinschlag bedingt, der zu über 50% illegal erfolgt (Brack, 2003). Gegen den illegalen Holzeinschlag wurden viele Maßnahmen von Regierungs- und Nichtregierungsorganisationen ergriffen. Einige Verbände der Holzindustrie haben Richtlinien erlassen, die ihre Mitglieder verpflichten, nur Holz aus legaler und nachhaltiger Wirtschaft zu beziehen. Nichtregierungsorganisationen (NGOs) haben Programme und Methoden vorgeschlagen, die der Entwicklung und Implementierung nachhaltiger Wirtschaftspraxis dienen, so die Waldzertifizierung. Kern jeglicher Programme zur Erkennung des illegalen Einschlags muß jedoch in einer Überwachung vor Ort bestehen. Eine lückenlose Überwachungskette ist notwendiger Bestandteil solcher Programme, so daß die Herkunft des Rohstoffes Holz aus nachhaltiger Holzwirtschaft über die Verarbeitungsschritte bis zum Endprodukt nachvollzogen werden kann. Hieraus ergibt sich die Notwendigkeit, Werkzeuge zu entwickeln, die die Bestimmung der Herkunft des Holzes ermöglichen und damit die Prüfung der Glaubwürdigkeit von Herkunftsbescheinigungen erlauben. Methoden der molekularen Genetik erweisen sich als nützlich, die Identität einer Art zu bestätigen und sind vielversprechende Werkzeuge der Bestimmung der geographischen Herkunft des geschlagenen Holzes.

Zur Anwendung des molekulargenetischen Ansatzes müssen zwei Bedingungen erfüllt sein: Zum Einen müssen Protokolle zur Isolierung von DNA in hinreichender Menge und Qualität aus rohem oder bearbeitetem Holz entwickelt werden. Zum Zweiten sind diagnostische DNA-Marker zu identifizieren, die eine hohe räumliche Auflösung aufweisen müssen. Arbeiten zur Identifizierung „informativer" DNA-Marker zur Identifizierung der Art und geographischer Differenzierung der Dipterocarpaceae wurden begonnen und laufend fortgeführt (Finkeldey *et al.*, 2007 und 2008). Das Ziel dieser Arbeit ist die Etablierung von Methoden der DNA Extraktion für das Holz der Dipterocarpaceae. Proben verschiedener Arten, Alter, Typ (roh oder bearbeitet) wurden in verschieden Ländern zur Optimierung der Methoden gesammelt.

Zum Beginn dieser Arbeit wurden mehrere veröffentlichte Methoden der DNA Isolierung sowie kommerzielle Extraktionskits an verschiedenen Proben von rohem und bearbeitetem Holz untersucht. Die Extrakte wurden mittels PCR analysiert, um den Erfolg der Extraktion nachzuweisen. Einige Methoden, die sich als erfolgreich erwiesen, wurden zwecks Optimierung ausgewählt, um bessere Ausbeute und Qualität der DNA zu erhalten. Schließlich wurde die beste oder effizienteste Methode zur Anwendung im weiteren Verlauf dieser Arbeit bestimmt: Zunächst erfolgte die Überprüfung der Methode durch Vergleich der aus Blättern und Holz des selben Baumes extrahierten DNA. PCR Amplifizierung, Genotypisierung der Mikrosatellitenfragmente und Sequenzierung dienten der Analyse der isolierten DNA. Weiterhin wurde die Anwendbarkeit der Methode zur Isolierung von DNA aus Holz unterschiedlicher Arten, Typen und Herkunft untersucht. Zuletzt wurden vergleichende Untersuchungen bezüglich der Menge und Qualität von DNA aus Extrakten aus unterschiedlichen Zonen des Holzes (Splintholz, Übergang zwischen Splint- und Kernholz, Kernholz) sowie Gehalt an PCR Inhibitoren angestellt.

Die optimierte DNeasy Plant mini Kit (Qiagen) Methode (Rachmayanti *et al.*, 2006) wird als die effizienteste Methode angesehen. Diese Methode überzeugt durch den kleinen Maßstab und die Anwendbarkeit in einem standardmäßig ausgestatten Genlabor. Nichtsdestotrotz erbringt sie eine hinreichende Menge und Qualität für PCR Amplifizierung und weitere nachfolgende Methoden wie DNA Genotypisierung, Klonierung und Sequenzierung. Die Zugabe von Polyvinylpyrrolidon (PVP) zum Lysepuffer ist wichtiger Bestandteil dieser Methode, da hierdurch die Hemmung der PCR signifikant vermindert werden kann. Zur Überprüfung der Methode wurde DNA von Holz und Blättern des selben Baumes isoliert und mittels PCR Amplifizierung, Genotypisierung der Mikrosatellitenfragmente sowie Sequenzierung untersucht. Das Ergebnis zeigt, daß die aus Blättern und Holz isolierte DNA identisch ist. Die Blast-Suche in der GenBank bestätigte die Zugehörigkeit der DNA-Sequenzen der erwarteten genomischen Regionen.

Holzproben unterschiedlicher Arten, Typen (roh und bearbeitet), geographischer Herkunft und Zustand wurden in dieser Arbeit untersucht. Insgesamt 406 Proben, bestehend aus 332 Proben von Dipterocarpaceae und 74 von anderen Holzarten wurden analysiert. Die Extrakte wurden mittels PCR analysiert um den Erfolg der Extraktion nachzuweisen. Eine Untersuchung mit Chloroplasten-DNA-Marker unter Verwendung dreier Primerpaare zur Amplifizierung dreier Fragmente unterschiedlicher Länge (kurzes Fragment, *ccmp*2, etwa 150 bp; mittellanges Fragment, *trn*L, etwa 600 bp; langes Fagment, *trn*LF, etwa 1,100 bp) zeigte erfolgreiche Amplifizierung in 369 von 406 PCR Experimenten (90,9%) beim kurzen Fragment, 319 von 406 PCR Experimenten (76,6%) beim mittellangen Fragment und 234 von 406 PCR Experimenten (57,6%) beim langen Fragment. Der durchschnittliche Erfolg für die drei Fragmente lag bei 75,7%.

Insgesamt 18 Holzproben aus Indonesien mit Querschnittsdurchmessern von 19,0 bis 32,0 cm wurden vergleichend untersucht hinsichlich der DNA Menge und Qualität in Extrakten der verschiedenen Zonen des Holzes: Splintholz (a), Übergangszone von Splint- zu Kernholz (m) und Kernholz (i). Die Untersuchung wurde unter Verwendung von PCR mit drei Primerpaaren zur Amplifizierung von DNA Abschnitten unterschiedlicher Längen durchgeführt *(ccmp*2, 150 bp; *trn*L, 600 bp; *trn*LF, 1,100 bp). Das Ergebnis zeigt, daß: 1) Genomische DNA in Holzproben im Allgemeinen in kürzere Fragmente zerfällt. Dies bestätigt sich durch die Beobachtung, daß bei allen Extrakten von a, m und i die Erfolgsrate der Amplifizierung des längeren Fragments niedriger liegt als bei den kürzeren und mittellangen Fragmenten. 2) Die DNA Menge im Verlauf vom Kernholz zum Splintholz zunimmt. 3) Die DNA Qualität vom Kernholz zum Splintholz zunimmt, da das Ausmaß des Zerfalls der DNA vom Splintholz zum Kernholz größer wird. 4) Der Gehalt an PCR Hemmstoffen vom Kernholz zum Splintholz zunimmt.

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Paper I

Extraction, Amplification and Characterization of Wood DNA from Dipterocarpaceae

Authors: Yanti Rachmayanti, Ludger Leinemann, Oliver Gailing and Reiner Finkeldey

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(Journal homepage: http://pubs.nrc-cnrc.gc.ca/ispmb/PR24-01.html).

Abstract. A successful DNA extraction from wood yielding appropriate DNA quality for PCR amplification allows molecular genetic investigations of wood tissue. Genotypes, the origin of sampled material, and species can be identified based on an investigation of wood if suitable information on genetic variation patterns within and among species is available. Potential applications are in forensics and in the control of the timber and wood trade. We extracted DNA from wood of Dipterocarpaceae, a family that dominates rainforests and comprises many important timber species in Southeast Asia. Several different DNA isolation techniques were compared and optimized for wood samples from natural populations and from wood processing enterprises. The quality of the DNA was tested by spectrophotometry, PCR amplification, and PCR inhibitor tests. An average DNA yield of 2.2 µg was obtained per 50-100 mg of dried wood sample. Chloroplast DNA (cpDNA) regions of different length were amenable to PCR amplification from the extracted DNA. Modification of DNA isolation techniques by the addition of polyvinylpyrrolidone (PVP) addition up to 3.1% into lysis buffer reduced PCR inhibition effectively. In order to evaluate the extraction method, we analyzed leaves and wood from the same tree by PCR amplification, genotyping and sequencing of chloroplast microsatellites.

Key words: chloroplast microsatellites, Dipterocarpaceae, DNA extraction, genotyping, PCR amplification, PCR inhibitor, PVP, sequencing, wood.

Abbreviations: *ccmp*, consensus chloroplast microsatellite primer; cpDNA, chloroplast DNA; cpSSR, chloroplast simple sequence repeat.

Paper II

DNA from processed and unprocessed wood: Factors influencing the isolation success

Authors: Yanti Rachmayanti, Ludger Leinemann, Oliver Gailing and Reiner Finkeldey

This paper was received at 27 September 2008, received in revised form 17 December 2008, accepted at 6 January 2009, published online at 05 February 2009 in "Forensic Science International: Genetics", volume 3, pages 185-192 (doi:10.1016/j.fsigen.2009.01.002).

Abstract. Molecular genetic markers have numerous potential applications in environmental forensics if DNA can be isolated from 'difficult' non-human biological material such as hairs, feathers, or wood. The identification of the origin of wood is particularly important in order to identify illegally harvested and traded timber and wood products. We describe success rates of DNA isolation from wood based on a simple, previously published extraction protocol. The protocol was used to isolate DNA from a total of 406 wood samples, mainly of the important tropical tree family Dipterocarpaceae. The reliability of the extraction method was confirmed by comparing fragment sizes and sequences after isolation of DNA from leaves and wood of the same trees. We observed the success of amplification of chloroplast DNA (cpDNA) fragments of different lengths by means of PCR, investigated key factors influencing PCR, and conducted inhibitor tests for a subset of the samples. The average rate of successful PCR amplification was 75.7%. Main factors influencing the success of PCR amplification were the size of the amplified fragment and the processing status of the wood. Short fragments and unprocessed wood resulted in higher success rates. The success rate was also dependent on the age (storage duration) of the wood probe and on the investigated species. Amplification success was higher if DNA was isolated from outer sapwood (without cambium) in comparison to DNA isolated from the transition zone between sapwood and heartwood and the inner heartwood. However, inhibitor tests also indicated more PCR inhibitory substances in the outer sapwood in comparison to transition wood and heartwood. The addition of polyvinylpyrolidone (PVP) to the lysis buffer proved to be highly efficient to improve the amplification success if inhibitory substances were present.

Keywords: timber, wood products, DNA isolation, identification of origin, Dipterocarpaceae, conservation of tropical forests.

Paper III

Molecular genetic tools for the identification of the origin of wood

Authors: Reiner Finkeldey, Yanti Rachmayanti and Oliver Gailing

This paper was published in the book "Wood Production, Wood Technology and Biotechnological Impacts", Ursula Kües (editor), Universitätsverlag Göttingen, pages 143-158, Göttingen 2007 (ISBN: 978-3-940344-11-3, homepage: http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf)

Introduction. Primary determinants of the value of wood are its dimension as well as its physical and chemical properties. However, additional features apart from those directly related to wood processing and conversion became increasingly important in the recent past. In particular, the management of forests during wood formation became an issue relevant for the trade of wood and wood products on worldwide markets (Bennett 2001). Wood in forests managed against the principles of sustainability can be harvested at lower costs for the forestry enterprise, but marketing of wood or wood products from non-sustainable forestry operations is becoming increasingly difficult. In Europe, the import and sale of tropical timber is greatly promoted by a proof of an origin from a company devoted to the principles of sustainability. The certification of wood, wood products, or businesses involved in forestry, wood harvest, and wood processing is of particular importance in this context.

Paper IV

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

Authors: Reiner Finkeldey, Yanti Rachmayanti, Hani Nuroniah, Nga Phi Nguyen, C. Cao and Oliver Gailing

This paper was published in Proceedings of the International Workshop "Fingerprinting Methods for the Identification of Timber Origins", Oct. 8-9 2007, Bonn, Germany, Bernd Degen (Ed.), Landbauforschung, vTI Agriculture & Forestry Research, p 20-27, Braunschweig 2008 (ISSN: 0376-0723, ISBN: 978-3-86576-046-3, http://www.wwf.de/fileadmin/fm-wwf/pdf_neu/Proceedings_Koenigswinter_BMELV_WWF.pdf)

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 elution 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.

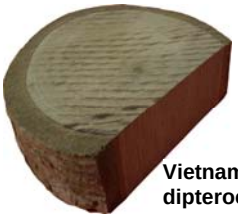
Keywords: timber origin, DNA extraction, DNA marker, genetic variation, tropical tree, Dipterocarpaceae

Appendix 1

Pictures of some wood samples

Wood collected from forest (unprocessed wood)

Indonesian dipterocarp



Vietnamese dipterocarp

Philippine dipterocarp

Thai dipterocarp

Borneo ironwood / *Eusideroxylon zwageri*

Scots pine / *Pinus sylvestris*

Poplar / Populus sp.

Indonesian teak / *Tectona grandis*

Thai teak

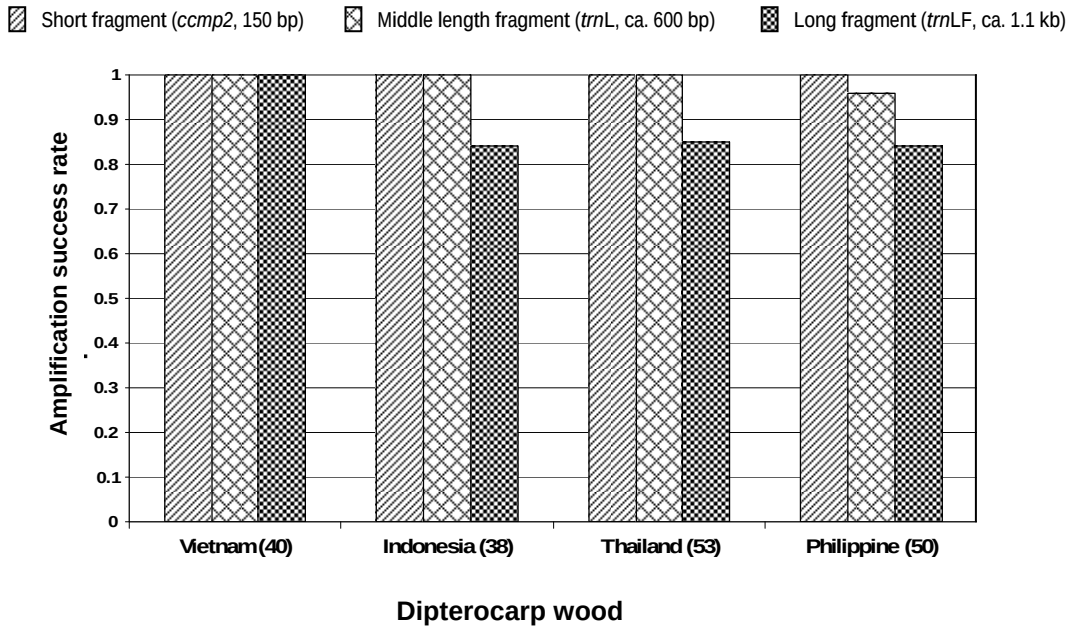
Wood collected from enterprises (processed wood)

Meranti

Obeche / *Triplochiton scleroxylon*

Appendix 2

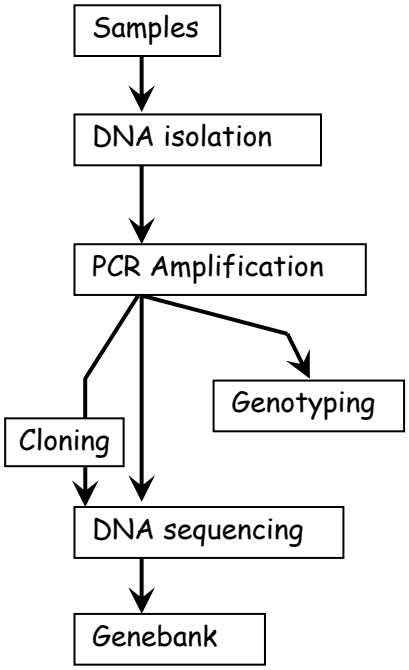
Diagram of successful PCR in unprocessed dipterocarps from different countries



Appendix 3

Verification of DNA isolation method

Verification schema



Wood & Leaf from the same tree (total = 43 samples).

Wood: modified method as described in Rachmayanti *et al*., 2006.
Leaf : DNeasy Plant Mini Kit (Qiagen).

PCR procedure described in Rachmayanti *et al*., 2006. Total of 6 cpDNA regions were amplified: 5 *ccmp* microsatellites (100 – 150 bp) and *trnF* (± 400 bp). PCR primers described in Rachmayanti *et al*., 2006 and 2009.

Genotyping procedure described in Rachmayanti *et al*., 2006. Five *ccmp* microsatellites were examined (*ccmp*1, *ccmp*2, *ccmp*3, *ccmp*6, *ccmp*10).

Sequencing procedure described in Rachmayanti *et al*., 2006. Sequencing initiated by cloning applied to three DNA fragments (*ccmp*2, *ccmp*6, *ccmp*10) and direct sequencing applied to *trnF*.

Verification results

Genotyping and sequencing results confirmed that DNA extracted from wood and leaf from the same tree are identical. *ccmp* microsatellite fragments amplified from wood DNA have the same length as that from its corresponding leaf. The *trn*F sequence obtained from wood and leaf from the same tree are identical with 100% homology. A Blast search against the EMBL data base shows that all sequences belong to the expected regions. Genotyping results from each 25 wood and leaf samples were described in Rachmayanti *et al*., 2006, Table 3. Other results such as genotyping of other 18 samples (woods and leaves each, Rachmayanti *et al*., 2009), sequencing of *ccmp*2, *ccmp*6 and *ccmp*10 fragments from the *Dipterocarpus kerrii* (Rachmayanti *et al*., 2006) and sequencing of *trn*F fragments from 5 samples of dipterocarps (Rachmayanti *et al*., 2009) are presented below.

Genotyping of chloroplast microsatellites of wood (W) and leaf (L) samples from the same tree of dipterocarps. Eighteen dipterocarp trees including 14 different species were tested. Nine haplotypes were identified

			Fragment length (bps)															
		L	*ccmp* 2				*ccmp* 3									*ccmp* 10		
Species	***N***	**W**	**148**	**150**	**151**	**152**	**100**	**101**	**102**	**106**	**111**	**112**	**114**	**123**	**136**	**94**	**98**	**101**	**Haplotype**
Dipterocarpus elongatus	1	L	•							•							•		**A**
		W	•							•							•		**A**
Shorea johorensis	2	L		•				•										•	**B**
		W		•				•										•	**B**
Shorea leprosula	2	L		•				•										•	**B**
		W		•				•										•	**B**
Shorea palembanica	2	L		•				•										•	**B**
		W		•				•										•	**B**
Shorea parvifolia	1	L		•			•											•	**C**
		W		•			•											•	**C**
Shorea ovalis	1	L		•			•											•	**C**
		W		•			•											•	**C**
Shorea fallax	1	L		•					•									•	**D**
		W		•					•									•	**D**
Shorea laevis	1	L		•							•							•	**E**
		W		•							•							•	**E**
Shorea patoensis	1	L		•								•						•	**F**
		W		•								•						•	**F**
Shorea multiflora	1	L		•								•						•	**F**
		W		•								•						•	**F**
Shorea sp	1	L		•								•						•	**F**
		W		•								•						•	**F**
Shorea virescens	2	L				•								•				•	**G**
		W				•								•				•	**G**
Shorea sp	1	L			•								•					•	**H**
		W			•								•					•	**H**
Vatica oblongifolia	1	L		•											•	•			**I**
		W		•											•	•			**I**

Alligment of *ccmp2, ccmp6* and *ccmp10* fragments sequenced from wood and leaf samples of *Dipterocarpus kerrii*. Fragment from wood and its corresponding leaf showed 100% sequence homology.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....|....|
                    5          15         25         35         45         55         65         75
ccmp2 (leaf)   GATCCCGGAC GTAATCCTGG ACGCGAAGAA TAAGAAAAAA AAAGGCTTCC TTGCTTTATT TTTATATTTT TATAAATAGG
ccmp2 (wood)   GATCCCGGAC GTAATCCTGG ACGCGAAGAA TAAGAAAAAA AAAGGCTTCC TTGCTTTATT TTTATATTTT TATAAATAGG

ccmp6 (leaf)   CGATGCATAT GTAGAAAGCC CTTTTTTCTA GTATTTACTA GTGTATTTAC TCTTTCTTTC CTTGTTTCTT TCTATAGTGG
ccmp6 (wood)   CGATGCATAT GTAGAAAGCC CTTTTTTCTA GTATTTACTA GTGTATTTAC TCTTTCTTTC CTTGTTTCTT TCTATAGTGG

ccmp10 (leaf)  TTCGTCGGCG TAGTAAATAG GATAGAAATT TTCTTTCTTC GTTTTTACAA TTTACAAAAG AAAAAGAGGA GTTAATTTGT
ccmp10 (wood)  TTCGTCGGCG TAGTAAATAG GATAGAAATT TTCTTTCTTC GTTTTTACAA TTTACAAAAG AAAAAGAGGA GTTAATTTGT


                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    85         95        105        115        125        135        145
ccmp2 (leaf)   ATTAGGATTT GATGTATTCT ACTGTCAAAA ACCAAAACGG AAAGAGAGGG ATTCGAACCC TCGGTACGAT
ccmp2 (wood)   ATTAGGATTT GATGTATTCT ACTGTCAAAA ACCAAAACGG AAAGAGAGGG ATTCGAACCC TCGGTACGAT

ccmp6 (leaf)   AGATAGTCGC ACGTAATG
ccmp6 (wood)   AGATAGTCGC ACGTAATG

ccmp10 (leaf)  GACACGTTCA CTAAAAAAAA A
ccmp10 (wood)  GACACGTTCA CTAAAAAAAA A
```

Alignment of *trn*F fragments sequenced from wood and leaf samples of 5 dipterocarp trees (5 different species). Sequences from wood and its corresponding leaf showed 100% homology. Polymorphic sites among species are highlighted. "-" indicates insertion/deletion, "N"=missing data

```
                                10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Shorea contorta                 TATTTCTTTCTTTTATCCTACCCTTTCCCTTGTTAAGAGGTCAAAATTTCTTATGTTCATCCTATTCTTTTAGATTTTATACATTTTACAAACGTATCCG
Shorea almon                    TATTTCTTTCTTTTATCCTACCCTTTTCCTTGTTAAGAGGTCAAAATTTCTTATGTTCATCCTATTCTTTTAGATTTTATACATTTTACAAACGTATCCG
Parashorea malaanoman           TATTTCTTTCTTTTATCCTACCCTTTCCCTTGGTAAGAGGTCAAAATTTCTTATGTTCATCCTATTCTTTTAGATTTTATACATTTTACAAACGTATCCG
Hopea plagata                   TATTTCTTTCTTTTATCCTACCCTTTCCCTTGTTAAGAGGTCAAAATTTCTTATGTTCATCCTATTCTTTTAGATTTTATCCATTTTACAAACGTATCCG
Dipterocarpus kerrii            NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATGTCAAAATTTCTTATGTTCATCCTATTCTTTTACATTTTATACATTTTACAAACGTATCCG

                               110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Shorea contorta                 AGCAAAAAATTGTCTCTCTCTTATCAGATATCATACACAAGTCTTGTGATATATATGATATACGTACAAATGAGCATCGGAATACATACCCCTTTGAATG
Shorea almon                    AGCAAAAAAATATCTCTCTCTTATTAGATATCATACACAAGTCTTGTGATATATATGAT----GTACAAATGAGCATCGGAATACATACCCCTTTGAATG
Parashorea malaanoman           AGCAAAAAATTTTCTCTCTCTTATCAGATATCATACACAAGTCTTGTGATATATATGATATACGTACAAATGGGCATCGGAATACATACCCCTTTGAATG
Hopea plagata                   AGCAAAAAATTTTCTCTCTCTT--------TCATACACAAGTCTTGTGATATATATGATATACGTACAAATGAGCATCGGAATACATACCCCTTTGAATG
Dipterocarpus kerrii            AGTAACAAAATTTCTCTCTTTTATCAGATATCATACGCAAGCCCTGTGATATATATGATATACGTACAAATGAGCATCGGAATACATACCCCTTTGAATG

                               210       220       230       240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Shorea contorta                 ATTCACAATCCATATCATTACTCATACTGAAACTGACAAAGTATTCTCTTTGAAGATCCAAGAAAATTCCCTGCGAGACTCTTCTTTTAATACTTTTTT-
Shorea almon                    ATTCACAATCCATATCATTACTCATACTGAAACTGACAAAGTATTCTCTTTGAAGATCCAAGAAAATTCCCTGCGAGACTCTTCTTTTAATACTTTTTTT
Parashorea malaanoman           ATTCACAATCCATATCATTACTCATACTGAAACTGACAAAGTATTCTCTTTGAAGATCCAAGAAAATTCCCTGCGAGACTCTTCTTTTAATACTTTTTT-
Hopea plagata                   ATTCACAATCCATATCATTACTCATACTGAAACTGACAAAGTCTTTTCTTTGAAGATCCAAGAAAATCCCCTGCGAGACTCTTCTTTTAATACTTTTTT-
Dipterocarpus kerrii            ATTCACAATCCATATCATTACTCATACTGAAACTGACAAAGTATTCTCTTTGAAGATCCAAGAAAATCCCCTACGAGACTCTTCTTTTAAGACTTTTTT-

                               310       320       330       340       350       360       370       380
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Shorea contorta                 GTCTCTTTTTTTTTTTAATTGACATAGACCCAATCCTCTAGTAAAATGAGGATGATGCATCGTTAATGGTCAGGATAGCTC
Shorea almon                    GTCTCTTTTTTTTTT-AATTGACATAGACCCAATCCTCTAGTAAAATGAGGATGATGCATCGTTAATGGTCAGGATAGCTC
Parashorea malaanoman           GTCTCTTTTTTTTTTTAATTGACATAGACCCAATCCTCTAATAAAATGAGGNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Hopea plagata                   GTCTCTTTTTTTTTTAATTGACATAGACCCAATCCTCTAGTAAAATGAGGATGATGCATCGT-AATGGTCAGGATAGCTC
Dipterocarpus kerrii            GTCTCTTTTTTTTTTAATTGACATAGACCAAATCCTCTAGTAAAATGAGGATGATNNNNNNNNNNNNNNNNNNNNNNNN
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Appendix 4

PCR amplification of DNA extracted from three different wood zones

PCR amplification procedure described in Rachmayanti *et al*., 2009 including its corresponding diagram (Fig.1).

No	Species name	Disk Diameter (cm)	Wood Zone	PCR result					
				1st Eluate of wood extract			2nd Eluate of wood extract		
				ccmp 2	*trn* L	*trn* LF	*ccmp* 2	*trn* L	*trn* LF
1	***Shorea palembanica* Miq.**	24.5	a	+	+	+	+	+	+
			m	+	+	+	+	+	+
			i	+	+	-	+	+	-
2	***Dipterocarpus elongatus* Korth.**	29.5	a	+	+	+	+	+	+
			m	+	+	+	+	+	+
			i	+	+	-	+	-	-
3	***Shorea* sp.**	22.5	a	+	+	+	+	+	+
			m	+	+	+	+	+	+
			i	+	+	-	-	-	-
4	***Vatica oblongifolia* Hook. F.**	25.5	a	+	+	+	+	+	+
			m	+	+	+	+	+	+
			i	+	+	+	+	+	+
5	***Shorea leprosula Miq.***	20	a	+	+	-	+	+	+
			m	+	-	-	-	-	-
			i	+	-	-	-	-	-
6	***Shorea johorensis* Foxw**	21.5	a	+	+	-	+	+	+
			m	+	-	-	-	-	-
			i	+	-	-	-	-	-
7	***Shorea virescens* Parijs**	22	a	+	+	-	+	+	+
			m	+	+	-	+	+	+
			i	+	-	-	-	-	-
8	***Shorea johorensis* Foxw**	22.5	a	+	+	-	+	+	+
			m	+	-	-	+	-	-
			i	+	-	-	-	-	-
9	**Shorea leprosula Miq.**	23	a	+	+	+	+	+	+
			m	-	-	-	-	-	-
			i	-	-	-	-	-	-
10	***Shorea palembanica* Miq.**	19	a	+	+	-	+	+	-
			m	+	+	+	+	+	-
			i	+	+	-	+	+	-
11	***Shorea virescens* Parijs**	24.5	a	+	+	+	+	+	+
			m	+	+	+	+	+	+
			i	+	-	-	+	-	-
	Total of successful PCR amplification :		a	11	11	6	11	11	10
			m	10	7	6	8	7	6
			i	10	5	1	5	3	1

a = outer rings of sapwood; m = rings transition of sapwood and heartwood; i = inner rings of heartwood

Appendix 5

PCR inhibition by DNA extracts from three different wood zones

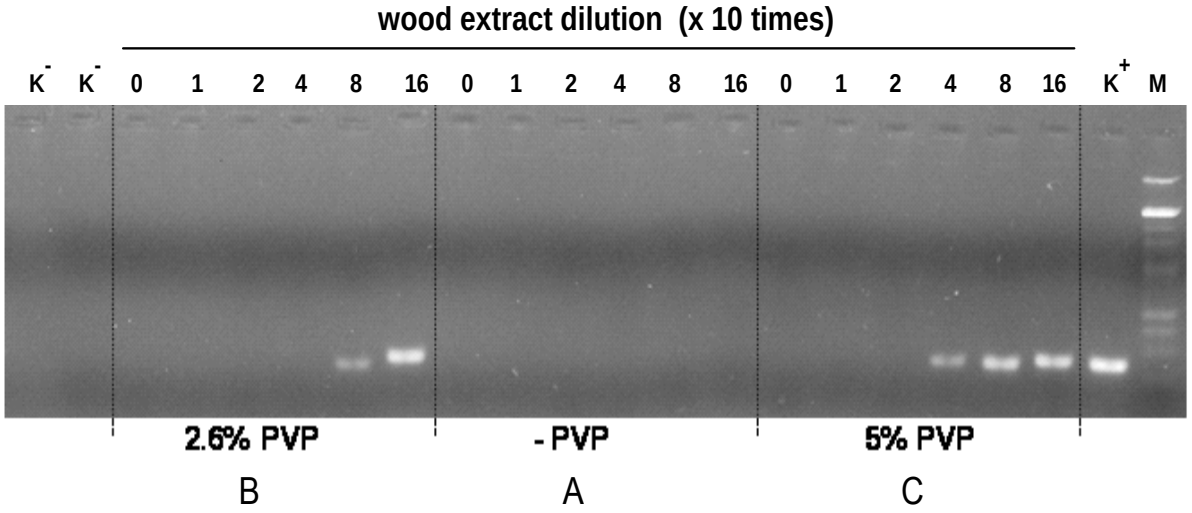
PCR amplification procedure described in Rachmayanti *et al*., 2009 including its corresponding diagram (Fig.2). Inhibition of PCR by Wood DNA extracts. + means unsuccessful amplification (approved PCR inhibition). - means successful amplification (no PCR inhibition)

No	Species name	Disk Diameter (cm)	Wood zone	Dilution of wood DNA extract						
				undiluted	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
1	*Shorea ovalis* (Korth.) Blume	32	a	+	+	+	-	-	-	-
			m	+	-	-	-	-	-	-
			i	+	-	-	-	-	-	-
2	*Shorea laevis* Ridl.	28.5	a	+	-	-	-	-	-	-
			m	+	-	-	-	-	-	-
			i	+	-	-	-	-	-	-
3	*Shorea patoensis Ashton*	30.5	a	+	+	-	-	-	-	-
			m	+	+	-	-	-	-	-
			i	+	+	-	-	-	-	-
4	*Shorea parvifolia* Dyer	24	a	+	-	-	-	-	-	-
			m	-	-	-	-	-	-	-
			i	-	-	-	-	-	-	-
5	*Shorea fallax* Meijer	22.5	a	+	-	-	-	-	-	-
			m	-	-	-	-	-	-	-
			i	-	-	-	-	-	-	-
6	*Shorea multiflora* (Burck) Sym.	22	a	+	+	+	+	+	+	-
			m	+	+	+	+	-	-	-
			i	+	+	-	-	-	-	-
7	*Shorea* sp.	24	a	+	+	+	+	-	-	-
			m	+	+	-	-	-	-	-
			i	+	-	-	-	-	-	-
Total of PCR inhibition :			a	7	4	3	2	1	1	0
			m	5	3	1	1	0	0	0
			i	5	2	0	0	0	0	0

a = outer rings sapwood; m = transition rings of sapwood and heartwood; i = inner rings of heartwood

Appendix 6

Analysis of PVP (polyvinylpyrrolidone) treatment on PCR inhibition



PCR inhibitory test:

Electrophoresis of PCR products on leaf DNA mixed by wood DNA (*Shorea* sp.) extracted with 3 different PVP treatments: without PVP (A), with 2.6% (w/v) PVP (B) and 5% (w/v) PVP (C). PCR Template were leaf DNA mixed with each 0, 10, 20, 40, 80 and 160 times diluted DNA extract from wood. PCR primer = *ccmp*2 (fragment length approximately 150 bp); K+ = PCR positive control (template = leaf DNA); M = DNA size standard; K- = PCR negative control (no template).

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]) were compared (see Rachmayanti *et al.*, 2006, step 8a of DNA isolation method). 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 figure above shows that DNA extraction without PVP treatment (0% PVP, A) left a high content of PCR inhibitory substances so that even 160 times diluted wood extract inhibited the PCR reaction. Figure B shows that after 80 times dilution of the extract with PVP addition up to 2.6% (w/v) the amplification of leaf DNA was successful. The addition of 5% (w/v) of PVP shows that a 40 times diluted extract had no inhibitory effect (Fig. C). This test shows that PVP addition to the lysis buffer can effectively reduce the PCR inhibition in the DNA extract.

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PUBLICATION

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