Correlation between anatomical/chemical wood properties and genetic markers as a means of wood certification

Dissertation

Submitted for the degree of Doctor of Philosophy (PhD) in the Faculty of Forest Sciences and Forest Ecology of Georg-August University of Goettingen, Germany

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http://resolver.sub.uni-goettingen.de/purl/?webdoc-1820

Date of examination: June 06, 2008

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes

Summary

Southeast Asian forests have high species diversity. Of the floral community, Dipterocarpaceae (dipterocarps) are the most well known. This tree family is extremely rich with more than 500 species in 17 genera and includes a considerably high fraction of the emergent and understory trees. This family is very important both from ecological and economic perspective.

Because of their economic significance the forests of the developing countries are being destroyed at an alarming rate. Illegal felling is one of the major causes. There is an increasing awareness of forest conservation issues. Therefore a forest certification scheme has been launched in the early 90's, which requires establishing a relationship between the end products that go to the consumers and the certified practices in the forest from where the products are originating from. Linking timber products to the place of origin is one of the main problems.

Besides, due to the high species diversity in the tropical forests, many species are quite difficult to distinguish and therefore they are assembled under single trade names. This is particularly true for dipterocarps. The most important timber species of this family are known under with only a few trade names.

This study was conducted to address the two problems by searching new methods for the detection of the origin of wood and species differentiation. In this study FTIR (Fourier Transform Infrared spectroscopy) was applied in combination with multivariate analyses and other methods to address these problems. For this purpose wood was irradiated with infrared and the chemical compounds present in the sample caused characteristic absorbance, known as the chemical fingerprint of that compound. By using the chemical fingerprint of wood with FTIR, attempts were made for the detection of the origin of wood. As a case study beech (*Fagus sylvatica*) wood collected from six different places in Germany and Luxemburg, was analysed since wood from different places of tropical countries was not available. FTIR in combination with PCA (Principal Component Analysis) or cluster analysis could clearly separate trees from five of the six sites. Only trees from two sites having similar edaphic and climatic condition were not separable. This shows that FTIR has the potential to differentiate same tree species originating from different growth areas.

To differentiate wood of five important timber species of dipterocarps, (*Dipterocarpus kerrii*, *Hopea plagata*, *Parashorea malaanoman*, *Shorea almon* and *Shorea contorta*) grown in a plantation, FTIR was used in combination with simple traits

like wood density and tree height for the separation of species. Multivariate methods applied to wood density and tree height could clearly separate 3 out of five species. One species of *S. contorta* was found to be mixed up with *P. malaanoman*. This result was confirmed with molecular marker, which also revealed same mixing of *P. malaanoman* and *S. contorta*. The chemical fingerprint of wood could separate species up to the genus level.

As durability of these five species was reported to differ, lignin was analysed as a trait associated with wood strength. The FTIR spectra of extracted lignin were measured. Differences in lignin content and composition were observed. Multivariate methods were applied to differentiate the five dipterocarp species by their FTIR spectra of extracted lignin. *H. plagata* and *D. kerrii* lignins differed from those of *P. malaanoman*, *S. almon* and *S. contorta*. This differentiation pattern reflected the differentiation pattern of wood. The lignin concentration was lowest in *H. plagata*. However, because of the high density of *H. plagata* wood, the lignin content per volume was highest in this species.

Some anatomical and biochemical traits were used for species separation. For this purpose traits like vessel frequency, average vessel diameter, vessel element length, predicted conductivity, fibre tracheid length and wall thickness, percentage of different cell elements, ratios of carbon/nitrogen and lignin/carbohydrate (1505/1738) were determined. Emphasis was given on vessel and fibre tracheid traits which are particularly important for water transport and support. Anatomical analysis revealed that in most cases, traits of *D. kerrii* and *H. plagata* were different from the other three species. *P. malaanoman* and the two *Shorea* species had similar traits in most instances. Using multivariate methods to differentiate the species by anatomical and biochemical traits gave the best results for *D. kerrii*, *H. plagata* and *S. contorta*. However *P. malaanoman* was found to be mixed up with *S. almon* only, underlining the close relationship among these three species.

It can be concluded that the chemical fingerprint of wood obtained by FTIR spectroscopy has the potential to be used for origin detection in different growth areas. However, further studies with species of tropical countries are needed. Besides, the use of simple traits proved to be useful for species separation and has got the potential to be used for taxonomic purposes. The close relationship between *P. malaanoman*, *S. almon* and *S. contorta* found by molecular analysis was also reflected when studying anatomical, and biochemical traits including lignin. Only a restricted number of species were included in this study due to the unavailability of timber species from the tropical countries. Further

studies on timber species from the tropical countries (same species from different provinces/country) would be interesting for future work.

Zusammenfassung

Die Wälder im Südosten Asiens zeichnen sich durch eine hohe Artenvielfalt aus. Die bekanntesten Baumarten aus Asien kommen aus der Familie der Dipterocarpaceae. Diese Familie ist mit mehr als 500 Arten in 17 Genera extrem artenreich. Dipterocarpaceae sind sowohl von großer ökologischen als auch ökonomischen Bedeutungen.

Aufgrund ihrer großen ökonomischen Bedeutung hat die Zerstörung der Wälder in den Entwicklungsländern beängstigend zugenommen. Die illegale Rodung der Wälder ist eine der Hauptursachen. Aber auch das Bewusstein für die Notwendigkeit von Wald Schutzprogrammen nimmt zu. In den frühen 90er Jahren des vorigen Jahrhunderts wurden Zertifizierungssysteme für Wälder eingeführt, welche es dem Konsumenten des Holzes ermöglichen, den Weg eines Produktes von der Fällung in einem zertifizierten Wald bis zum Endprodukt lückenlos zu verfolgen. Ein Hauptproblem ist allerdings die Herkunftsbestimmung von Holzprodukten.

Durch die große Artenvielfalt der tropischen Wälder ist es auch heute noch schwierig viele Arten genau zu bestimmen. Außerdem werden verschiedene Holzarten oft unter demselben Handelsnamen verkauft. Dies trifft insbesondere für die Dipterocarpaceae zu. Die wichtigsten Holzarten der Familie der Dipterocarpaceae sind nur unter wenigen Handelsnamen bekannt. Im Rahmen dieser Studie sollten mit Hilfe neuer Methoden, der FTIR (Fourier-Transform-Infrarpt-Spektroskopie) in Kombination mit Multivarianz- und anderen Analyseverfahren, sowohl die Baumarten als auch die Wuchsgebiete bestimmt werden.

Bei der FTIR-Spektroskopie werden Holzprobrn mit Infrarotlicht bestrahlt. Die verschiedenen chemischen Komponenten der Holzproben absorbieren Licht unterschiedlicher Wellenlänge, so dass ein charakteristisches Adsorptionsspektrum entsteht, der sogenannte chemische Fingerabdruck.

Als Fallbeispiel wurden Buchenproben (*Fagus sylvatica*) von 6 verschiedenen Standorten in Deutschland und Luxemburg mittels FTIR-Spektrokopie analysiert, da Holz von verschiedenen tropischen Standorten nicht verfügbar war. Mittels FTIR-Spektroskopie in Kombination mit PCA (Principal Component Analysis) oder Clusteranalyse konnten die Bäume von 5 der 6 Standorte deutlich unterschieden werden. Nur Bäume von 2 Standorten mit ähnlichen Boden- und klimatischen Bedingungen konnten nicht getrennt werden. Diese Ergebnisse zeigten, dass es möglich ist, mit FTIR-Spektroskopie Baumindividuen derselben Baumart von verschiedenen Standorten zu unterscheiden.

In einem weiteren Versuch wurden Holzproben der fünf ökonomisch wichtigsten Dipterocarpaceae-Arten (*Dipterocarpus kerrii*, *Hopea plagata*, *Parashorea malaanoman*, *Shorea almon* und *Shorea contorta*) von Plantagen mit Hilfe von FTIR analysiert sowie Holzdichte und Baumhöhe ermittelt. Mittels Multivarianzanalysen der Holzdichte und der Baumhöhe konnten deutlich 3 der 5 Baumarten voneinander unterschieden werden. *S. contorta* konnte nicht von *P. malaanoman* getrennt werden. Dies entsprach auch einer Untersuchung mit molekularen Markern. Auch mit dieser Methode konnten die beiden Arten nicht separiert werden. Die Methode des chemischen Fingerabdrucks konnte hingegen zur Artunterscheidung bei Holz bis zur Gattungsebene erfolgreich angewandt werden.

Die Beständigkeit der fünf tropischen Baumarten wird als sehr unterschiedlich beschrieben. Da Lignin die Dauerhaftigkeit von Holz beeinflusst, wurde es extrahiert und analysiert. FTIR Spektren des extrahierten Lignins wurden aufgenommen. Unterschiede im Ligningehalt und in der Ligninzusammensetzung wurden festgestellt. Multivarianzanalyse wurde durchgeführt, um die fünf Baumarten anhand ihrer FTIR Spektren von extrahiertem Lignin zu unterscheiden. Das Lignin von *H. plagata* und *D. kerrii* unterschied sich von dem von *P. malaanoman*, *S. almon* und *S. contorta*. *H. plagata* hatte die geringste Ligninkonzentration bezogen auf Gewichtseinheiten. Da dieses Holz aber die größte Dichte hat, war die Ligninkonzentration pro Volumeneinheit in dieser Baumart am höchsten.

Weiterhin wurden anatomische und biochemische Holzuntersuchungen zur Unterscheidung der Baumarten durchgeführt. Es wurde die Häufigkeit der Gefäße, der durchschnittliche Gefäßdurchmesser, die Gefäßelementlänge, die berechnete Leitfähigkeit, die Länge der Fasertracheiden, die Zellwanddicke, die Anteile der verschiedenen Zellelemente und das C/N- sowie das Lignin/Kohlenhydrat Verhältnis (1505/1738) bestimmt. Der Schwerpunkt lag auf den Gefäßen und den Fasertracheiden,

da sie besonders wichtig für den Wassertransport und die Holzfestigkeit sind. Die anatomischen Analysen ergaben, dass sich in den meisten Fällen *D. kerrii* und *H. plagata* von den anderen drei Arten unterschieden. *P. malaanoman* und die beiden Shoreaarten hatten meistens ähnliche Eigenschaften. Eine Multivarianzanalyse mit den anatomischen und biochemischen Eigenschaften der Baumarten ergab, dass sich *D. kerrii*, *H. plagata* und *S. contorta* unterschieden. *P. malaanoman* und *S. almon* konnten anhand dieser Analysen nicht unterschieden werden, was die enge Verwandtschaft dieser beiden Arten verdeutlicht.

Zusammenfassend lässt sich sagen, dass der chemische Fingerabdruck mittels FTIR-Spektroskopie eine Methode ist, um Holzproben aus verschiedenen Wuchsgebieten zu unterscheiden. Es sind aber noch weitere Untersuchungen mit unterschiedlichen tropischen Baumarten aus verschiedenen Gebieten notwendig. Die Anwendung einfacher Merkmale, wie z.B. Holzdichte und Baumhöhe, können verwendet werden, um tropischen Baumarten zu unterscheiden. Eine enge Verwandtschaft zwischen *P. malaanoman*, *S. almon* und *S. contorta* konnte sowohl mit molekularen Analysen als auch mit anatomischen und biochemischen Merkmalen nachgewiesen werden.

Nur eine begrenzte Anzahl an Baumarten wurden in dieser Studie analysiert, da nur wenige Proben tropischer Hölzer zur Verfügung standen. Für weiterführende Arbeiten wär die Analyse weiterer Holzproben der gleichen Baumarten aus anderen tropischen Ländern oder Provinzen interessant.

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1. Introduction

1.1. Biological and socio-economic background

1.1.1. Biodiversity of tree species in the tropics/ subtropics

Among the tropical rain forests in the world, the Indo-Malayan (Southeast Asia) rain forest is the second most extensive after the American rain forest centred on the Amazon basin (Whitemore 1984). The tropical rainforests in Southeast Asia are the most species rich in the world in terms of both plant and animal life. The extreme floristic richness is largely due to co-occurrence of a great number of species within the same community (Whitemore 1998).

Most tropical forests in Southeast Asia are dominated by a single species rich tree family, the dipterocarps (Dipterocarpaceae). The members of this family are exceedingly abundant in lowland forests of Southeast Asia, for example, in many areas, 80% of the emergent individuals and 40% of the under story trees are dipterocarps (Ashton 1982). They are one of the most important tree families both from an ecological and economic point of view (Lamprecht 1989). For example, the tropical forests of Borneo has been reported to contain 150-200 trees species (≥ cm dbh) per hectare, where dipterocarps are the dominant family representing 25% of the stems (120 stems ≥ dbh 10 cm ha^{-1}), with large size stems contributing 75-80% of the canopy and emergent trees (Appanah and Weinland 1993; Sist and Saridan 1999; Whitemore 1984). In the Philippines, the Dipterocarpaceae are widely distributed in forest areas of the country. In most cases they are found to be growing in groups with other species occurring in relatively dense stands (Lomibao 1973) and are generally medium to large in size (Ella et al. 1990). Fig. 1 shows two dipterocarp species growing with other species. Rojo (unpublished report cited by Lomibao 1973) reported the presence of 53 species of dipterocarps under 7 genera in the Philippines.

Fig. 1 Two dipterocarps species, *Dipterocarpus dyeri* on the left and *Shorea henryana* on the right (Source: Nga Nguyen Phi from the Institute of Forest Genetics and Forest Tree Breeding).

1.1.2. Commercial significance of Dipterocarpaceae

The OECD (2001) estimates that the global trade in timber worth more than US$150 billion per year with the EU being the world's largest importer. The biological and economic importance of Dipterocarpaceae lies in the extraordinary dominance of its members over vast areas in forests of Southeast Asia (Bawa 1998). The extracted volume of dipterocarps varies from 50-100 m^3 ha^{-1} (Sist et al. 2003). Currently the dipterocarps predominate the international tropical timber market and therefore play an important role in the economy of many of the Southeast Asian countries (Poore 1989; Soerianegara and Lemmens 1994; Whitmore 1984). This is especially true for the Philippines, which are famous for the dominance of this family in the lowland forests, where dipterocarps contribute 94% of the timber volume (Soerianegara and Lemmens 1994).

The Dipterocarpaceae are the backbone of Philippine forestry and contribute the most important timber producing family. The bulk of timber in commerce, both for domestic use and for export is supplied by this family (Ella et al. 1990; Lomibao 1973). In the present study five dipterocarps of the Philippines have been studied,

which are of high economic importance (Ashton 1982; Ella and Meniado 1992; Lomibao 1973; Newman et al. 1996). The timber of Dipterocarpaceae varies from soft and light to hard and heavy and they are widely used for construction purposes.

The Apitong group (*Dipterocarpus kerrii*) which comprises 9 species is the most common commercial structural timber of the Philippine. These species are practically found in all forest areas of the country with other dipterocarp species. A survey by Rojo et al. (1991) revealed that the Apitong group comprises the bulk of the volume of standing trees in the country's logged over forests. The wood is hard and is classified as general utility material and is used in places which require hard and heavy timbers.

The Yakal group (*Hopea plagata*) comprises 12 species of the genera Hopea and Shorea. Woods are used for high grade construction works and other installations requiring high strength and durability.

The Philippine mahogany group is derived from 7-9 traditional exportable species which include the genera Shorea, Parashorea and Pentacme. This name is given by the Philippine lumber and log producers. This group ranks first as source of log and lumber export comprising the bulk of lumber in the domestic market and veneer logs for the plywood mills. Furniture and cabinet makers value the beautiful colour and ribbon figure of these species. These species are used for cabinet work, ship planks for general construction purposes and the waste and residues are used for pulp and paper making.

The dipterocarps also constitute important timbers for the domestic needs in the seasonal evergreen forests of Asia. In addition, these forests are sources of a variety of minor products on which many forest dwellers are directly dependent for their survival (Panayotou and Ashton 1992).

Despite such eminence in the plant world, there has never been an attempt to assemble under one cover all the principal aspects of this exceptional family of trees. Much of the knowledge on the tree species within the Dipterocarpaceae exists in a disparate form even though research has been conducted on them for about a century. Apart from some classical work on their taxonomy, silviculture (Ashton 1982; Loñdono et al. 1995; Morton 1995; Troup 1921; Wyatt-Smith 1963), most other studies remain fragmented. A uniform comparative body of information on dipterocarps is still lacking (Appanah 1998). Since the presence of a large number of dipterocarp species makes them difficult to distinguish, many different species are

assembled under one name. The species rich genus *Shorea*, which contains more than 100 species, is differentiated into only a few trade names in many tropical countries like Indonesia (white meranti, yellow meranti, dark red and light red meranti) and the Philippines (red lulan-Philippine red mahogany and white lulan- Philippine light red mahogany) (Finkeldey et al. 2008; Newman et al. 1996).

1.1.3. The need of wood certification

The tropical countries are the major timber producing countries in the world. In many of these countries more than 50% of all logging is illegal, either completely or in many significant aspects (Contreras-Hermosilla 2002). Between 1980 and 1995 developing nations lost 200 million ha of forests (FAO 1999) and the area of 12.5 million ha of natural forest was lost world wide each year in between 1990-2000 (FAO 2001). Although accurate levels of economic losses are difficult to estimate, on a global level the World Bank (2002) estimates that the annual market value of losses resulting from illegal logging is at least US$10-15 billion. According to Forest Monitor (2001), approximately 50% of EU imports of timber and wood products may be illegally sourced. In Indonesia, a recent Greenpeace report (2003) puts the current level of illegal supplies to industry at 70-80% of the total wood supply. A Senate Committee in the Philippines estimated that the country lost as much as $1.8 billion per year from illegal logging during the 1980s (Callister 1992).

In many Southeast-Asian forests the dipterocarps are critically endangered due to illegal logging practice. Over the last 15 years, timber demand has been changing and harvesting is no longer confined to particular dipterocarp species (red merantis). Almost all dipterocarps are now considered commercial and this trend seems to be continuing. In addition, many forest products are considered quite valuable (Sheil et al. 2003).

The underlying causes of tropical deforestation and forest degradation are complex and vary from one country to another (IISD 1999). Illegal logging has mainly been reported in countries that have extensive areas of natural forests but now-a-days it has been observed in plantations in densely populated and well protected areas as well (Mir and Fraser 2003). Although the main impacts are observed in developing and emerging producer countries, illegal logging is sustained by actors in both industrialized and developing countries (Speechly 2003). A number of reasons

are responsible for illegal logging. Several authors have described the symbiotic relationship between illegal logging and corruption (Callister 1999; Contreras-Hermosilla 2001; Palmer 2001; Scotland et al. 2000). The drastic increase in the wood processing capacity due to the increased demand of people took place with little or no regard for the forest's resources' capacity to supply raw material in a sustainable manner (Mir and Fraser 2003). This is one of the major reasons of illegal logging. In addition poverty, unemployment, poor governance, poor law enforcement, lack of resources, alternative forms of land uses, mining, non sustainable forest management etc. contributed to the failure of policies and markets to direct resources in an equitable and efficient manner (Birikorang et al. 2001; Brack 2003; Mir and Fraser 2003; Speechly 2003).

The issues of illegal harvesting and trade of forest products have received substantial attention all over the world in recent years. Today forest certification plays an important role to develop and implement sustainable forest management practices in temperate, boreal and tropical forests. Among the several agencies, the Forestry Stewardship Council (FSC; http://www.fsc.org/en/) which is a voluntary certification scheme plays an important role in forest certification particularly in tropical forests (Cauley et al. 2001). The FSC is an international NGO comprised of environmental, social and industry interests. Since being founded in 1993, which was followed by other certification schemes, the FSC has developed a worldwide forest certification network (Takahshi et al. 2003).

Although the different certification schemes contain several important characteristics, the main objective of forest certification is to identify forestry enterprises working in accordance to commonly accepted principles of sustainable forest management. "Chain of Custody" (CoC) is an essential component in the certification scheme as it traces material from certified forests to the consumers in the market place. Its main objective is to ensure that only products from certified forests will be sold as certified products. In this way CoC plays an important role in maintaining customers confidence and in addition it also addresses the issues of illegal harvesting (Dykstra et al. 2003; Finkeldey et al. 2008; Wingate and McFarlane 2005).

1.1.4. Methods of wood certification

Currently several difficulties are faced by the EU enforcement authorities in controlling the entry of illegally-sourced timber and wood products into EU markets. The most important of them is the matter of identification: in most cases it is impossible to tell through simple inspection whether a particular shipment of timber or wood products has been produced illegally (Marijnissen 2003). In this context the development of suitable tools for the identification of the origin of wood has considerable scope for practical application within the context of forest certification. Timber source must be identified and the verification of the legal origin is also a must in wood certification processes. In the existing system of log tracking three different components are found. In the first component logs are identified by using various labelling technologies. The second component involves log segregation processes where logs from known sources are being separated from logs of unknown sources. In the third component documentation of the labelled logs are done (Dykstra et al. 2003). In recent years there have been some improvements in labelling technologies like Radio-Frequency Identification (RFID) transceivers, Microtaggant tracer paint, Chemical tracer paint, Eco-labelling etc. (Wingate and McFarlane 2005). Although several authors have mentioned the importance of chemical and genetic fingerprinting methods (Dykstra et al. 2003; Finkeldey et al. 2008), no known chemical or genetic fingerprinting technology has reached a stage of development that would insure the identification of the origin of wood in the wood certification process. Since the manipulation of genetic traits is impossible, genetic fingerprinting technology can play an important role in wood identification process (Finkeldey et al. 2008). However, genetic fingerprinting methods are usually very expensive and time consuming as they require extensive sample preparation in addition to laboratory facilities. Besides, difficulties of successful isolation and amplification of DNA from wood have been reported by several authors (Bär et al. 1988; Deguilloux et al. 2002; Lee and Cooper 1995; Lindhal 1993). Therefore, there is an urgent need for the development of cost-efficient and highly reliable new methods like studies on wood anatomy and chemistry as well as the use of spectroscopic techniques to infer origin of wood (Finkeldey et al. 2008).

1.2. Methods for wood analysis

1.2.1. Conventional methods

The conventional methods of wood analysis include traditional wet chemical analysis for quantitative analysis of wood properties such as gravimetry, titrimetry, staining etc. Although they are useful for obtaining information on wood, the possibilities of accurate molecular characterization of different samples are sometimes limited due to the structural complexity of certain molecules. For example, lignin is partially soluble in a wide range of solvents and due to covalent linking with polysaccharides it is quite difficult to extract lignin from wood materials with wet chemical analysis (Martínez et al. 1999). Besides, these methods are time-consuming, require larger amounts of samples for analyses and also result in a concomitant degradation of natural polymers during the use of dyes and labels for the detection of certain compounds (Naumann et al. 2008; Pandey 1999; Pandey and Pitman 2004; Salzer et al. 2000).

1.2.2. FTIR-ATR spectroscopy in wood analysis

FTIR is a widely used technique for wood analysis. Infrared light passed through the sample is characterized by various chemical linkages such as C-H, O-H, N-H, C=O, C-C etc. Infrared radiation results in bending, stretching and twisting of these linkages leading to characteristics reflectance pattern (Günzler and Gremlich 2002). Tentative assignment of these linkages to specific molecular bonds or functional groups is possible by this technique. In FTIR an absorption peak is defined as the highest absorption in a band. Shoulders are noticeable deviations in the spectral shape that do have a defined maximum (Shenk et al. 2001). FTIR techniques are quick, easy to perform, do not require extensive sample preparation; they are less expensive than many other methods and provide both qualitative and quantitative results on any kind of samples (Faix 1991; Moore and Owen 2001; Naumann et al. 2008; Schrader et al. 1998). The versatile uses of FTIR spectroscopy have been described in detail by Naumann et al. (2008). The finger print of a compound is a unique pattern indicating the presence of particular molecules. This characteristics pattern is used to identify chemicals and compounds. This is particularly true for

wood which is a complex polymer of several components therefore representing a finger print of major organic constituents (Rana et al. 2008). FTIR techniques have been reported to provide superior specificity because of their fingerprint capability. The functional groups in the fingerprint region (1800-600 cm^{-1}) contain a wealth of information which is unique for each molecule (Mansfield et al. 1999; Reeves et al. 2001; Willson 1999).

The invention of ATR (attenuated total reflection) in the mid 60's is another important step in the field of spectroscopic research. FTIR spectroscopy in combination with an attenuated total reflection (ATR) device is now being extensively used in many different fields of research. ATR is a reflection technique where the IR beam is directed through an internal reflection element. This internal reflection element is the ATR crystal which is usually made of elements with high refractive index like diamond/zinc selenide. After the sample has been pressed against the ATR crystal, IR light penetrates into the samples, gets reflected and the IR data from the sample can be obtained (Naumann et al. 2008). Fig. 1 shows the FTIR-ATR spectra of a hardwood (*Robinia pseudoacacia*) (A) and a softwood species (*Larix decidua*) (B) in the fingerprint region (1800-600 cm^{-1}). Distinction between hardwood and softwood is possible by spectral analysis of the characteristics peaks which have been marked by arrows.

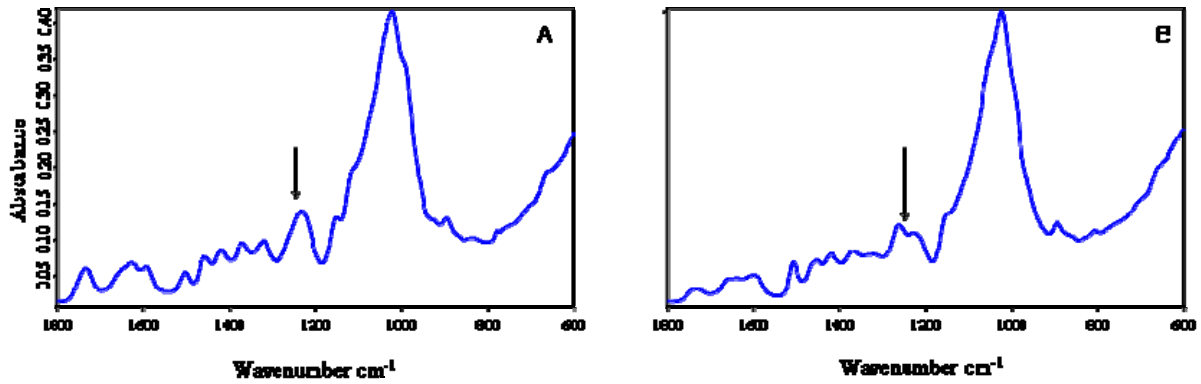

Fig. 2 FTIR-ATR spectra of one hardwood (A) (*Robinia pseudoacacia*) and one softwood (B) species (*Larix decidua*) in the finger print region collected from the Forest Botanical Garden. The arrows indicate typical peaks for hardwood and softwoods.

1.2.3. Multivariate methods in wood analysis

Wood is a complex polymer of cellulose, hemicellulose and lignin (Fengel and Wegener 2003). Absorption bands which are observed in wood during spectroscopic studies arise due to the combination of various vibrations (C-O, O-H, N-H). However signals from theses vibrations are quite similar and overlapping and in most cases bands cannot be directly assigned to a single component (Michell 1988; Owen and Thomas 1989; Shenk et al. 2001). Therefore, multivariate analyses are being used very often (Ferraz et al. 2000; Gierlinger et al. 2004). Multivariate methods provide ample opportunities for pre-treatment, evaluation and representation of huge and complex data structures. Univariate methods consider only a single property of a given object (e.g. a single intensity at a given wavenumber), multivariate methods can evaluate several properties of the objects at the same time (Naumann 2000). Among the most commonly used multivariate methods, principal component analysis (PCA) and hierarchical clustering have been reported in a number of spectroscopic studies (Dytham 2003; ; Gierlinger et al. 2004; Müller et al. 2006; Rana et al. 2008).

Principal component analysis (PCA) is a technique which compresses the main information in a set of variables into a lower number of variables in order to provide the maximum discrimination between individuals (Dytham 2003; Schimlek et al. 1996). In spectroscopic studies, one of the major difficulties is the handling of very large number of data points. For example in a mid infrared spectrum of wood measured at a resolution of 4 cm^{-1} from 4000-400 cm^{-1} 900 data points are available. However strong intercorrelation may exists between many of the data points and the relationship of interest may be hidden. PCA finds the most important variables in the set by studying the characteristics of the variation and accordingly it classifies the various groups of samples (Schimlek et al. 1996). In PCA there are three main components to the output: (1) the weightage applied to each of the variables to generate the principal axes, (2) the set of eigenvalues that shows how important the principal axes are (each axis will always explain less variation than the last) and (3) the position of the individuals on the axis. This information is used to generate the graphical display of the output (Dytham 2003).

By examining the factor loadings, the nature of variables compressed into the principal components can be discovered (Schimlek et al. 1996). The spectra of the identical or very similar products will be located closely to one another as a result of

their almost equal loadings, whereas spectra of the other substances or product grades are farther apart (Brunner et al. 1996). On of the simplest ways of using PCA is the score plot. The scores are the coordinates of the objects in a coordinate system defined by the direction of maximum variance, the principal components (Schimlek et al. 1996). In recent studies both two and three dimensional plots have been constructed for the differentiation of a number of species (Brunner et al. 1996; Naumann 2000; Schimlek et al. 1996).

The term cluster analysis is used for a huge range of techniques for the classification of individuals. It is being used very often in different fields like spectroscopy, taxonomy, community ecology, molecular and phylogenetic studies because of its potential to classify a huge range of samples (Dytham 2003). Cluster analysis procedures work in the following way: first, “n” objects are classified, second, closest lying objects are assembled in one cluster, third, measurement of the distance between the new cluster and all other objects are done, fourth, next nearest objects or clusters are searched. This process goes on until all the objects are assembled in one cluster. In this procedure a dendrogram is created that shows the merging process of classes/groups (Naumann 2000). Cluster analysis has been used to differentiate lignins and celluloses of different origin (Müller et al. 2006), to identify tree species growing on the same as well as on different sites (Gierlinger et al. 2004; Rana et al. 2008), and to identify fungi, bacteria and yeasts (Naumann 2000; Naumann et al. 2005). Fig. 3 shows a cluster of 4 hardwoods (*Robinia pseudoacacia*, *Fagus sylvatica*, *Fraxinus excelsior* and *Quercus robur*) and five softwoods (*Abies alba*, *Larix decidua*, *Picea abies*, *Pinus sylvestris* and *Pseudotsuga menziesii*) species. With cluster analysis it was possible to clearly separate the hardwood and the softwood species.

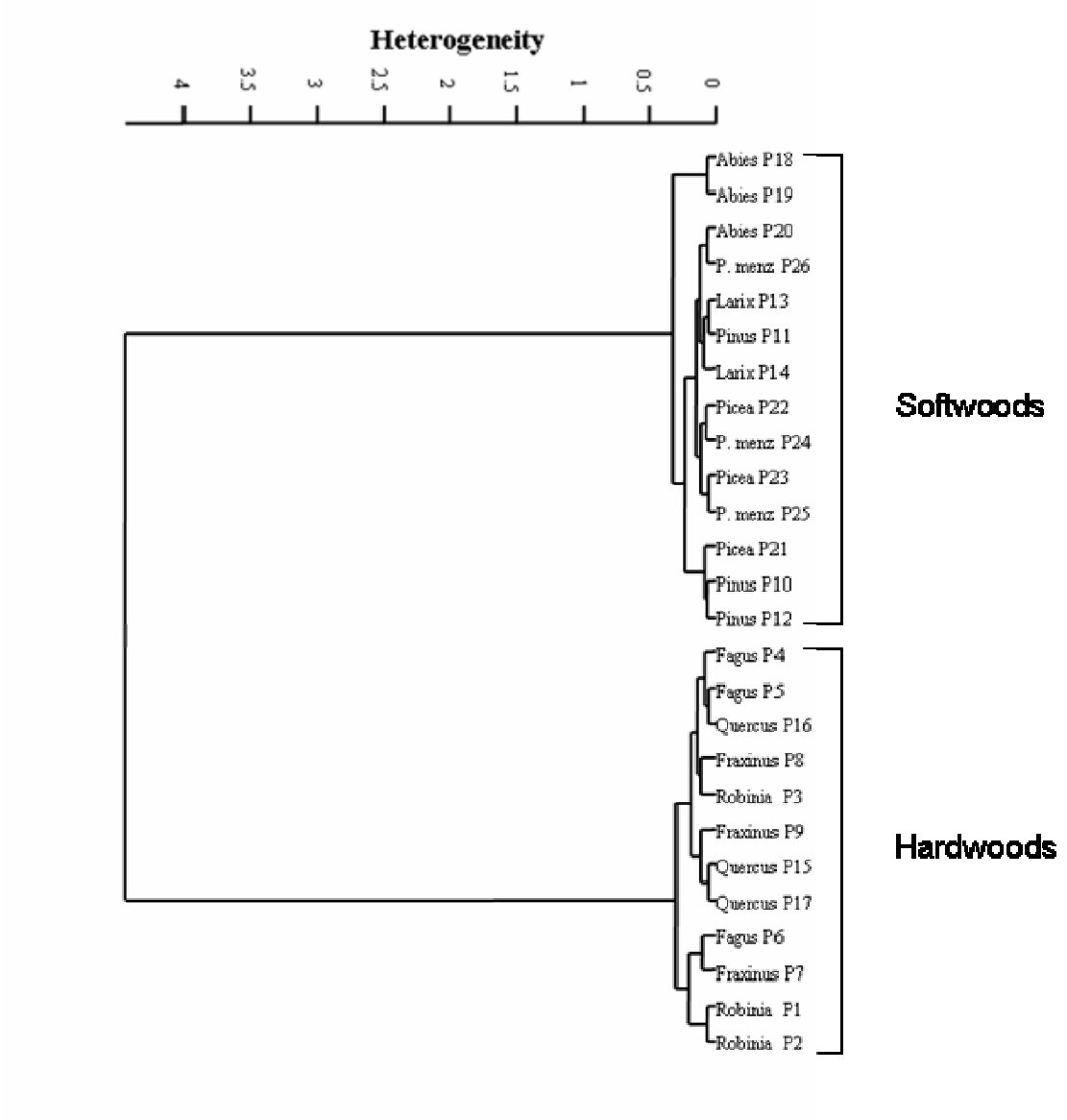

Fig. 3 Cluster analysis obtained by five softwood (*Abies alba*, *Larix decidua*, *Picea abies*, *Pinus sylvestris* and *Pseudotsuga menziesii*) and four hardwood species (*Robinia pseudoacacia*, *Fagus sylvatica*, *Fraxinus excelsior* and *Quercus robur*) collected from the Forest Botanical Garden. A clear separation of the hardwood and the softwood species were possible by clustering

1.3. Goal of the present study

Forest certification schemes are still in the developing stage. The goals of the present study were to investigate the potential of FTIR for the analysis of wood properties, wood certification and species differentiation.

In particular the following points were addressed:

(i) to test the potential of FTIR-ATR spectroscopy to distinguish the same species grown at different sites by using the "chemical fingerprint" of wood of these species;

(ii) to apply FTIR-ATR spectroscopy and simple traits to distinguish five different dipterocarp species grown at same site;

(iii) to compare chemical wood properties in five dipterocarp species;

(iv) to distinguish five dipterocarps on the basis of their anatomical and chemical wood properties.

To achieve these goals, the results of this work are projected in the following four independent chapters. In chapter 2, wood samples collected from six different sites in Germany and Luxembourg were analysed by FTIR-ATR spectroscopy to find out whether they are distinguishable. Beech trees (*Fagus sylvatica*) of different ages were used for this study since tropical species were not available. In chapter 3, five dipterocarp species (*Dipterocarpus kerrii*, *Hopea plagata*, *Parashorea malaanoman*, *Shorea almon* and *Shorea contorta*) grown at the same site in the Philippine were separated using height growth and wood density in combination with their chemical finger print and FTIR-ATR. The results were discussed in the context of phylogenetic data. In chapter 4, lignin was investigated histochemically and the lignin content was determined for the dipterocarps. A detailed comparison was made among the five species. In chapter 5, quantitative anatomical and biochemical traits were measured. Image analysis was used for anatomical quantification. It was tested whether the traits can be used to distinguish the different taxa.

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2. FTIR spectroscopy in combination with principal component analysis or cluster analysis as a tool to distinguish beech (*Fagus sylvatica* L.) trees grown at different sites

2.1. Abstract

FTIR spectroscopy was used to distinguish between beech (*Fagus sylvatica* L.) trees grown at six different sites; two in middle Germany close to Göttingen (Forest Botanical Garden and forest district Rheinhausen), three located in the south-west (two in Rhineland-Palatinate: forest districts Saarburg and Hochwald, and one in Luxembourg), and one in North-Rhine Westfalia. Detailed investigation of the spectra in the fingerprint region (1800-600 cm^{-1}) revealed 16 to 18 distinct peaks and shoulders, most of which were assignable to wavenumbers previously shown to represent wood compounds. Differences in peak heights and peak ratios indicated differences in wood composition of beech trees from different sites. To determine if the wood of individual trees could be distinguished, principal component analysis (PCA) and cluster analysis were performed using the first derivative of vector normalized or untreated spectra as input data. With both methods PCA and cluster analysis, trees from five of the six different sites were separated. It was not possible to distinguish between trees from Saarburg and Hochwald, where similar edaphic and climatic conditions existed, while spectra of wood from trees of all other areas clearly segregated. Wood collected at different positions in the stem (bottom, crown, centre and outer year rings) of trees grown at the same site was not distinguishable. Therefore, FTIR spectral analysis in combination with multivariate statistical methods can be used to distinguish wood of trees from different growth habitats. Extension of this method to other species may be of great interest for wood certification as it may be possible to distinguish wood, of a given species, originating from different regions.

Key words: certification, *Fagus sylvatica*, FTIR spectroscopy, multivariate statistical method, wood composition

2.2. Introduction

Infrared spectroscopy is among the most widely utilized techniques for determination of molecular structures and identification of compounds in biological samples (Ona et al. 1999). To investigate the chemical composition of a sample, it is irradiated with infrared. The absorbed energy results in stretch and deformation vibrations of specific molecular bonds, for example C-H, O-H, N-H, C=O, etc., which are characteristic for the chemical composition of the particular sample (Günzler and Gremlich 2002). The FTIR spectra of plant tissues, therefore, represent a finger-print of the major organic constituents such as carbohydrates, proteins, lipids, lignins and other aromatic or other abundant compounds.

Wood has been studied by FTIR for a long time (Kratzl and Tschamler 1952). Technological advance was achieved by the introduction of FTIR-attenuated total reflection (ATR) spectroscopy, which requires no further sample pre-treatment and, thus, is suitable as a high throughput method. The ATR crystal is pressed directly on wood or on milled wood powder and reflects the incoming radiation similar as a prism (Naumann et al. 2008). Fourier transformed infrared spectra have been used to characterise the chemistry of wood (Faix 1988; Faix 1991; Faix et al. 1991; Faix 1992; Fengel and Wegener 1989; Owen and Thomas 1989; Pandey 1999), the influence of fungi on wood and for the detection of fungi in wood (Naumann et al. 2005; Pandey and Pitman 2003; Pandey and Pitman 2004).

Besides the analysis of chemical wood composition, infrared spectroscopy was applied to distinguish tree species. For example, Wienhaus et al. (1988) were able to differentiate between softwoods and hardwoods because the chemical differences in lignin composition of conifers and angiosperms were easily detected by FTIR analyses. However, if differences in the chemical composition are small, the analysis of individual chemical components is insufficient to group a set of samples according to species. To be able to classify and compare spectra, multivariate statistical methods are necessary. For example, principal component analysis (PCA) has been used to distinguish very subtle spectral changes in cell walls (McCann et al. 1997).

Multivariate methods are now routinely used in combination with infrared spectroscopy. Using discriminant analysis, Chen et al. (1998) were able to identify small changes in cell wall properties. Wood degradation by wood rotting fungi was

monitored by FT near and middle infrared spectral changes; based on PCA analysis, regression models were developed to predict weight loss induced by fungal activity (Fackler et al. 2007). Using FT near infrared in combination with PCA, cluster analysis and soft independent modelling of class analogy, Gierlinger et al. (2004) were able to differentiate between European larch (*Larix decidua*), Japanese larch (*Larix kaempferi*) and hybrid larch (*Larix eurolepis*). Schimleck et al. (1996) discriminated between wood from different Eucalyptus species, different provenances and from the same Eucalyptus species grown on different sites by analysing NIR spectra by PCA. These examples underline that analysis of large spectral data sets by multivariate methods are powerful tools for various applications in wood analyses.

Currently, the demand on wood is increasing. It is often necessary to know from which growth area the wood is originating, e.g. to prevent trade with timber from illegal logging. Therefore, new methods for wood identification and certification are required. Simple methods that can distinguish wood from different growth areas would, therefore, be particularly useful for such applications. The goal of this study was to investigate the potential of FTIR spectroscopy in combination with different multivariate statistical methods to distinguish wood of the same species grown in different habitats. In the present case study, we used wood from beech (*Fagus sylvatica* L.) grown in six different areas in Germany as testing material. FTIR spectra of the wood samples were analysed by two unsupervised, exploratory classification methodologies, PCA and cluster analysis, respectively. To investigate the variability of the chemical finger-print within individual trees, wood samples obtained from the crown, the bottom of the trunk, and the inner and outer parts of stem disks were also analysed.

2.3. Materials and methods

2.3.1. Field sites and sampling

Samples were collected at six different sites, two in Rhineland-Palatinate, one in Luxembourg, one in North Rhine Westphalia, and two in Lower Saxony. Climatic and soil conditions have been summarized in Table 1. In Rhineland-Palatinate, samples were obtained from (a) forest office Saarburg (SAB) (formerly FA Saar-Hochwald) in the compartments 146 a l and 147 a, which are in a mountainous region

in a mixed stand of spruce (*Picea abies*, 15 % in clusters) and beech (*Fagus sylvatica*) and (b) forest office Hochwald (HOW) (formerly FA Hermeskeil) in compartment 126 a l in a mountainous region in a mixed stand of spruce (1% scattered) and beech. The beech trees originated from natural regeneration and showed intermediate growth (stem class III, 0). Beech trees of the same growth characteristics were sampled in the forest district Wiltz-Luxembourg (LUX), which belongs to the region of Clervaux and is close to the German city Trier in Rhineland-Palatinate. In Lower Saxony, samples were obtained from the forest office Reinhausen/Sattenhausen (REH), which belongs to the district "Göttinger Wald" from compartment 3153 b. The trees were located at a north slope in a mixed stand of beech and ash (*Fraxinus excelsior)*. They also belonged to the intermediate growth category (III). The Forest Botanical Garden (FBG) of the University of Göttingen belongs to the climatic area of Göttinger Wald and is located at a distance of 8.2 km from RHE. During installation of the FBG in the late 1960's, bedrock was covered with surface soil taken from the north of Göttingen (Anonymous 1971). In the city forest of Schmallenberg in North Rhine-Westphalia (SAL), the beech trees were grown in compartment 238 a. The forest stand, which was established by planting a mixture of *Fagus sylvatica* (40 %), *Abies grandis* (50 %) and *Pseudotsuga menziesii* (10 %), was located at west to northwest exposition at an altitude of 600 m on a steep slope.

In SAB, HOW, LUX, SAL and REH whole beech trees were cut down. In SAB, HOW and LUX wood samples were taken at the bottom of the stem with an increment borer (50 mm length, 7 mm width) from the inner part of tree trunk. In REH stem disks were cut at the bottom of the stem of which inner parts were used for analysis. In FBG branches, approximately 5-years-old, were used. In SAL the trees were felled and stem disks were cut at the bottom (0.3 m above ground) and in the crown (10 m above ground). The stem disks were transported to the laboratory, where inner parts (46- to 56-year-old wood) and outer parts (young wood 1- to 10-year-old) were cut out for further analysis. Only healthy trees with no apparent injury were used. At the sites SAB and HOW five individual trees and at all other sites 10 individual trees were used for sampling.

2.3.2. Sample preparation

Wood was debarked and dried at 30 to 35°C in a drying oven for two weeks until a constant weight was achieved. The samples were cut into small pieces with a cutter and powdered in a ball mill (MM2000, Retsch, Haan, Germany) for about 20 minutes to a fine powder.

2.3.3. FTIR-ATR measurements and multivariate data analyses

FTIR-ATR spectra of milled wood powder were recorded with the FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany) with a deuterium trigylcine sulfate detector and an attached ATR unit (DuraSamplIR, SensIR Europe, Warrington, England) at a resolution of 4 cm^{-1} in the range from 600 to 4000cm^{-1}. The powdered wood samples were pressed against the diamond crystal of the ATR device. A pressure applicator with a torque knob ensured that the pressure applied was the same for all measurements. For each sample 32 scans were conducted and averaged. Background scanning was done regularly after 15 to 20 min. For each sample five different sub samples were analysed and averaged to give a mean spectrum per individual tree.

The mean spectra of each individual tree were used for PCA and cluster analysis. Spectral data were evaluated using OPUS version 5.5 software (Bruker, Ettlingen, Germany). Cluster analysis and PCA were conducted by the software OPUS IDENT, version 5.5 (Bruker, Germany). Cluster analysis is an undirected, unbiased statistical method to analyse spectroscopic information. As unsupervised methodologies are model-free, unexpected as well as anticipated responses can be identified (Baumgartner et al. 1997; Salzer et al. 2000). Cluster analysis is used to identify regions of the sample that have a similar spectral response by clustering the spectra such that the differences in the intra-cluster spectral responses are minimized, while simultaneously maximizing the inter-cluster differences between spectral responses (Salzer et al. 2000). Satisfactory results on IR spectra of complex biological molecules have been obtained with Ward's algorithm, Euclidean distances or correlation coefficient calculation as a distance metrics (Helm et al. 1991a, b). Ward's method generates agglomerative clusters, i.e. groups of spectra according to similarity, using the increase in variance as a distance function. To cluster data according to homogeneous groups, only those two data sets are merged which show

the smallest growth in heterogeneity factor H, which can be calculated by different distance measures (e.g. correlation coefficients, Euclidean distances, Naumann, 2000). In our sample set, the first derivatives of the FTIR spectra in the region 1800-600 cm^{-1} were used for clustering. The best algorithms for cluster analysis and the optimal distance measure, indicated as heterogeneity in the figures, were found to be Ward's method and correlation coefficient as distance metric. This method was used to construct dendrogrammes.

PCA is a method for data reduction and gives information on the major components of the spectra that are dominant factors determining differences among samples. A typical spectrum contains several hundred data points (or variates); but these variates will normally be correlated with each other to some extent. PCA removes the redundancy of having many points varying in a correlated way by transforming the original data into a set of new, uncorrelated variates, termed the principal component (PC) scores (Lai et al. 1994; Briandet et al. 1996; Defernez et al. 1995; Kemsley et al. 1994). PCA identifies directions (principal components) along which the variance of the data is maximal. The effect of this process is to concentrate the sources of variability in the data into the first few PCs. Plots of the so called PC scores (projection on to PC axes) against one another can reveal clustering or structure in the data set (Limei et al. 1998; Cotrim et al. 1999) and may generate information about the major components responsible for variability in certain regions of the spectrum (Kawata et al. 1987; Mansfield et at. 1997). For PCA of our data set, the first derivative of vector normalized spectra in the region of 1800 to 600 cm^{-1} was used and the factor loadings were plotted.

2.4. Results

Beech wood showed 16 prominent peaks in the finger-print region of the wavenumbers from 1800 to 600 cm^{-1} (Fig. 1). According to published literature most of these peaks represent major cell wall components such as cellulose, hemicellulose, and lignin (Table 2). It is immediately apparent that the mean spectra from samples of the different sites displayed some pronounced differences; spectra for REH and FBG were strongly overlapping, also the spectra for SAB and HOW, whereas those of LUX and SAL were clearly separated from wood spectra of the other sites (Fig. 1). Closer inspection of the mean FBG spectrum revealed some distinct features in comparison

with the others such as small peaks (15 and 17) at wavenumbers 789 cm^{-1} and 871 cm^{-1}, respectively (Fig. 1). The region from 1660 to 1608 cm^{-1} also looked slightly different from that of the other spectra. These small differences may have been due to these trees being the youngest and that they represented branch wood. Despite many similarities across all spectra, analysis of peak ratios, which reflect relative shifts in chemical wood composition, revealed site-specific features. For example the ratios of peak 13-to-1, which represent roughly the ratio of aromatic compounds-to-xylans, changed from 8.5 (REH), 7.7 (LUX), 7.5 (FBG) to 6.7 (HOW and SAB) and 5.2 (SAL) suggesting that wood from SAL had a relatively higher content of xylans to lignin than those from the other sites. The only exceptions were SAB and HOW, which had the same peak ratios. These examples illustrate that the mean spectra contained specific information that might be useful to distinguish wood from most different sites.

To determine if the spectral differences were sufficient to permit grouping of individual trees according to their growth area, cluster analysis was conducted (Fig. 2). Two major clusters were observed, one formed by samples from SAL, the other by the other wood samples. This second subcluster was split into two 2^{nd} order subclusters separating wood from lower Saxony (REH and FBG) from that of the south-west geographic region encompassing SAB, HOW, and LUX. In the "Lower Saxony" branch of the cluster, REH and FBG formed two distinct 3^{rd} order subclusters (Fig. 2), although the geographic distance between the two sites was small. However, the sites REH and FBG differed strongly in soil conditions and, as outlined above, trees of different age were used (Table 1), which may have affected wood spectral properties. In the "south-west" branch of the cluster, samples from SAB and HOW were not discriminated. These two forest districts are very close to each other and have similar soil and climatic conditions (Table 1). Among ten trees from LUX, two were found to be mixed up in the subcluster formed by SAB and HOW, the remaining 8 samples formed a separate cluster (Fig. 2). Soil data were not available for LUX, but the climatic data indicate slightly drier and warmer conditions than in SAB and HOW (Table 1).

Since wood of different age was used, it was unclear whether age or other inherent factors might have contributed to the results shown in Fig. 2. To test for positional and age effects, wood disks from SAL trees were collected at the bottom (B) and in the crown (T) of felled stems and used to excise samples from the outer (O)

and inner year rings (I). FTIR spectra were analysed by cluster analysis as before but no separation according to height, wood age or tree number was achieved (Fig. 3). Furthermore the heterogeneity was about 15-times lower than that found for the comparison of wood from different growth areas (Fig. 2). This indicates that the major wood properties of trees grown at the same site were remarkable constant and not affected by age or position. Thus, the differences found between wood of trees from different sites must have been caused site-specific and not by tree-inherent factors. FTIR spectroscopy in combination with cluster analysis can be used to distinguish wood of the same species grown at different sites.

In addition, we employed PCA as another multivariate method to classify spectra. Figure 4 shows the PCA map constructed by three-dimensional projection of eigenvectors (factor spectra) 2, 3 and 4. Each point in the map represents a spectrum; all three factorial coordinates (factor loadings, see below) were used for data representation. By the trial and error, we found that the best model with three principal components was obtained when using the first derivative of vector normalized spectra in the fingerprint region (1800-600 cm^{-1} wavenumbers) as input data. Five different groups were distinguished representing six different sites of beech collection (Fig. 4). Only the spectra from SAB and HOW were mixed up, whereas all others were separated (Fig. 4). This result is similar to that obtained cluster analysis but leads to slightly improved separation of wood spectra from LUX and those of SAB/HOW.

Since the dendrogrammes or PCA maps (Fig. 2, 4) do not contain any information which chemical differences were responsible for the observed grouping, the factor loadings obtained by PCA were used to identify the most divergent wavenumbers (Fig. 5).The first factor loading (PC1) strongly resembled the first derivative of normalized mean spectra of beech wood (Fig. 5A) and thus, was not considered further. The second (PC2), third (PC3) and the fourth (PC4) factor loadings (Figs. 5B, C and D) revealed distinct differences to Fig. 5A . We assigned numbers and tentative chemical characteristics to the seven highest peaks for each of the three factors loadings (Fig. 5B, C, D, Table 3) similar as suggested by Tillmann (1996), who considered in his NIRS analysis the five highest peaks to represent the major differences. Most of the identified peaks in the factor loadings of our study corresponded to wavenumbers indicating ring vibrations of carbohydrates, but there were also some pointing to lignin (Table 3).

2.5. Discussion

A major finding of the present analysis was that FTIR-ATR spectroscopy in combination with PCA or with cluster analysis is suitable to discriminate between beech trees originating from different geographic growth regions. These regional differences were more important than e.g. wood age or wood position within the tree (Fig. 3). It is notable that wood from trees in the south-west geographic region (SAB+HOW and LUX) clustered or grouped more closely together in the cluster analysis and in PCA maps than that from other sites. The reason for the formation of these regional and local groups was probably related to a combination of climatic factors, soil properties and perhaps also differences in the genetic background of the trees. Environmental factors and inherited traits both influence wood properties and, thus, may have led to sufficient spectral deviations of wood from the different sites to enable separation. In our study we identified ring vibration of carbohydrates and also aromatic vibrations and C=O stretch as major factors determining differences in wood quality leading to the site-specific grouping in PCA.

The power of Fourier transform infrared spectroscopy in combination multivariate statistical methods has also shown for other applications, e.g. FTIR and PCA were used to discriminate wood species (Anttii 1999; Brunner et al. 1996; Gierlinger et al. 2004), soil organic matter and microbial communities from forest sites (Priha et al. 2001), to identify the origin of lignins (Cortrim et al. 1999), and to detect *Fusarium* fungi on maize (Kos et al. 2002). FTIR in combination with cluster analysis has been successfully applied to differentiate between Gram-positive and Gram-negative microorganisms (Salzer et al. 2000) and to differentiate between two different fungal species (Naumann et al. 2000). Interestingly, more complex spectral analyses were necessary to distinguish three species *(Larix decidua, L. kaempferi, L. eurolepis)* within one genus (Gierlinger et al. 2004) than to differentiate between growth areas of the same species as shown in this study. For 100% correct clustering of the larch species, Gierlinger et al. (2004) had to identify a narrow spectral region in which differences were most pronounced before subsequent cluster analysis. Since the larch trees were all from the same growth habitant, it is quite likely that environmental conditions have a more important influence on wood properties in phylogenetically closely related taxa than inherent species-related differences. This might render

discrimination of species from the same growth habitats more difficult than to distinguish between individuals from different environments.

The possibility to discriminate wood grown in different environments with a simple and rapid method is advantageous, if it is necessary to classify wood according to its provenience, for example for certification. However, we have also shown that some limitations exist since wood of trees from adjacent forest districts (SAB, HOW) was not discernable and that of trees from the same geographic region (LUX) showed some overlap with SAB/HOW wood in cluster analysis. In practical terms, this meant that 80% of the LUX trees were assigned correctly by cluster analysis, whereas PCA analysis enabled improved separation LUX and SAB/HOW, but not of SAB and HOW. If higher rates of accuracy were required, e.g. to identify trees from adjacent sites or the same growth area, this could be achieved by subjecting the remaining samples to genotyping by molecular analysis. Since molecular analyses are laborious and expensive, a pre-selection by FTIR-ATR in combination with PCA or cluster analysis would be useful to reduce sample numbers and, thus, costs. In conclusion, this study shows that FTIR-ATR spectroscopy together with PCA and cluster analyses have a potential for discrimination of wood from different growth habitats.

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Table 1: Description of the field sites. Climatic data represent a 4 year (2002-2005) mean.

Site (Abbreviation)	Geographic coordinates	Altitude (m asl)	Age of trees (years)	Precipitation (mm) annual sum	Precipitation (mm) growth * period	Air temperature (°C) annual mean	Air temperature (°C) Growth* period	Soil type
Saarburg (SAB)[1] Compartment 146 a l Compartment 147 a	49°31'N 06°31' E	590-675	108-128	>950	367	9.6	16.2	Soil derived from sandstone during the lower Devonian period, poor nutrient availability, high water availability
Hochwald (HOW)[1] Compartment 126 a l	49°31'N 06°31'E	660-700	143-174	>950	307	9.0	15.4	See SAB.
Wiltz-Luxembourg (LUX)[2]	49°46'N 07°03' E	>405	140	686	292	10.2	16.7	No data available
Reinhausen (REH)[3] Compartment 3153 b	51°26'N 10°01' E	301-350	147	728	313	9.4	16.6	Soil derived from weathered limestone loamy soil, high stone content, nutrient rich, mull type of humus
Forest Botanical Garden, University of Göttingen (FBG)[4]	51°33`N 9°57`E	219	27	728	313	9.4	16.6	Bedrock from the Triassic Garden, characterized by middle and lower limestone with soil layer of about 0.4m
Schmallenberg Compartment 238 a (SAL)	51°14'N 08°23 E	600	56	1200	>500	5.8	11.8	Brown soil with limited nutrient supply

*Growth period: May to September, the information for soil data was taken from 1) Bestandesblatt des Forsteinrichtungswerks des Landes Rheinland-Pfalz, 2003, 2) not available, climatic data were taken from Trier, which is the closed city to the forest site, 3) Otto (1991), 4) Roloff et al. 1993).

Table 2: Band assignments in the mid-infrared region of beech (*Fagus sylvatica*) wood powder.

Peak No.	Wave number (cm^{-1})	Band origin	Reference
1	1738	C=O stretch in unconjugated ketones, carbonyls and in ester groups (frequently of carbohydrate origin)	Faix (1991), Pandey and Pitman 2003)
2	1650	absorbed O-H and conjugated C-O	Pandey and Pitman (2003)
3	1596	aromatic skeletal vibration plus C=O stretch	Faix (1991), Faix et al. (1991)
4	1505	same as peak no. 3	Faix (1991)
5	1462	C-H deformation; asymmetric in $–CH_3$ and $–CH_2$-	Hergert (1971) Faix (1991)
6	1425	aromatic skeletal vibrations combined with C-H in plane deformation	Hergert (1971) Faix (1991),
7	1375	C-H deformation in cellulose and hemicellulose	Pandey and Pitman (2003)
8	1330	S ring plus G ring condensed	Faix (1991), Hergert (1971)
9	1268/1270	G ring plus C = O stretch	Faix (1991), Evans (1991)
10	1235	syringyl nuclei deformation combined with deformation of cellulose	Evans (1991)
11	1158	C-O-C vibration in cellulose and hemicellulose	Pandey and Pitman (2003)
12	1122	aromatic skeletal and C-O stretch	Pandey and Pitman (2003)
13	1030	aromatic C-H in plane deformation, guaiacyl type and C-O deformation, primary alcohol	Hergert (1971), Faix (1991)
14	898	C-H deformation in cellulose	Pandey and Pitman (2003)
15	871	no information	
16	840	aromatic C-H out-of-plane deformations of the 1,3,4,5-substituted rings associated with the syringyl nuclei	Evans (1991)
17	789	out of plane C-H deformation	Stuart (1997)
18	669	stretching vibration of C-S	Parker (1983)

Table 3: Band assignments of the second (PC2), third (PC3) and fourth (PC4) factor loadings obtained by Principal component analysis. The seven highest peaks are indicated for each factor loading. The numbers in parenthesis indicates the position according to height.

Wave number (cm^{-1})			Band origin	References
2^{nd} factor	3^{rd} factor	4^{th} factor		
1030 (1)			aromatic C-H in plane deformation, guaiacyl type and C-O deformation, primary alcohol	Hergert (1971), Faix (1991)
982 (2)			-HC = CH- out-of-plane deformation	Faix (1991)
1130 (3)			C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
1104 (4)			same as peak no. 3	Naumann et al. (1991), Faix et al. (1991)
1166 (5)			typical for HGS lignins; C=O in ester groups	Faix (1991)
1584 (6)			asymmetrical deformation of NH_3^+	Parker (1983)
1470 (7)			C-H deformations; asym. in $–CH^3$ and $–CH^2$-	Faix (1991)
	1086 (1)		C-O deformation in secondary alcohols and aliphatic ethers	Faix (1991)
	1044 (2)		C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
	1235 (3)		syringyl nuclei deformation combined with deformation of cellulose	Evans (1991)
	1166 (4)		typical for HGS lignins; C=O in ester groups	Faix (1991)
	989 (5)		-HC = CH- out-of-plane deformation	Faix (1991)
	948 (6)		C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
	1593 (7)		aromatic skeletal vibration plus C=O stretch	Faix (1991)
		785 (1)	no information available	
		1061 (2)	C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
		1097 (3)	C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
		879 (4)	no information available	
		906 (5)	C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
		939 (6)	C-O-C, C-O dominated by ring vibration of carbohydrates	Naumann et al. (1991), Faix et al. (1991)
		1615 (7)	asymmetrical stretching of NO_2	Parker (1983)

Figure Legends

Figure 1. Mean FTIR spectra of beech (*Fagus sylvatica* L.) wood from five different sites. Each spectrum is a mean of spectra from 10 individual trees sampled at Forest Botanical Garden (FBG, Göttingen), Rheinhausen (REH), Wiltz-Luxembourg (LUX), Schmallenberg (SAL), and from 5 individual trees sampled at Saarburg (SAB) and Hochwald (HOW), respectively. The different colours indicate different sampling sites. The different numbers refer to peaks in Table 2.

Figure 2. Cluster analysis of beech wood FTIR spectra from a total of 50 different trees grown at five different sites, FBG and REH close to Göttingen (Lower Saxony), SAB and HOW in Rhineland-Palatinate, LUX in Wiltz-Luxembourg and SAL in North Rhine Westfalia. First derivates of spectra were used for cluster analysis using Ward's algorithm and correlation coefficient distance.

Figure 3. Cluster analysis of beech wood FTIR spectra from samples collected at SAL. Wood was obtained from 10 trees in the crown (T) and the bottom of the stem (B) and from the inner (I) and the outer part of the stem wood. Data were analysed as described for fig. 2.

Figure 4. PCA map calculated from a data set of 50 IR spectra (first derivative of vector normalized data in the spectral range 1800-600 cm^{-1}) obtained from beech trees grown on 6 different sites. For projection of data, the factorial coordinates (factor loadings) PC 2, PC 3 and PC4 were used.

Figure 5. Factor loadings of the first derivative of normalized mean spectra of beech wood: (A) first, (B) second, (C) third and, (D) fourth factor loadings, respectively. The different numbers in B, C and D refer to peaks described in Table 3. The first seven highest peaks were assigned in B, C, D.

Fig. 1

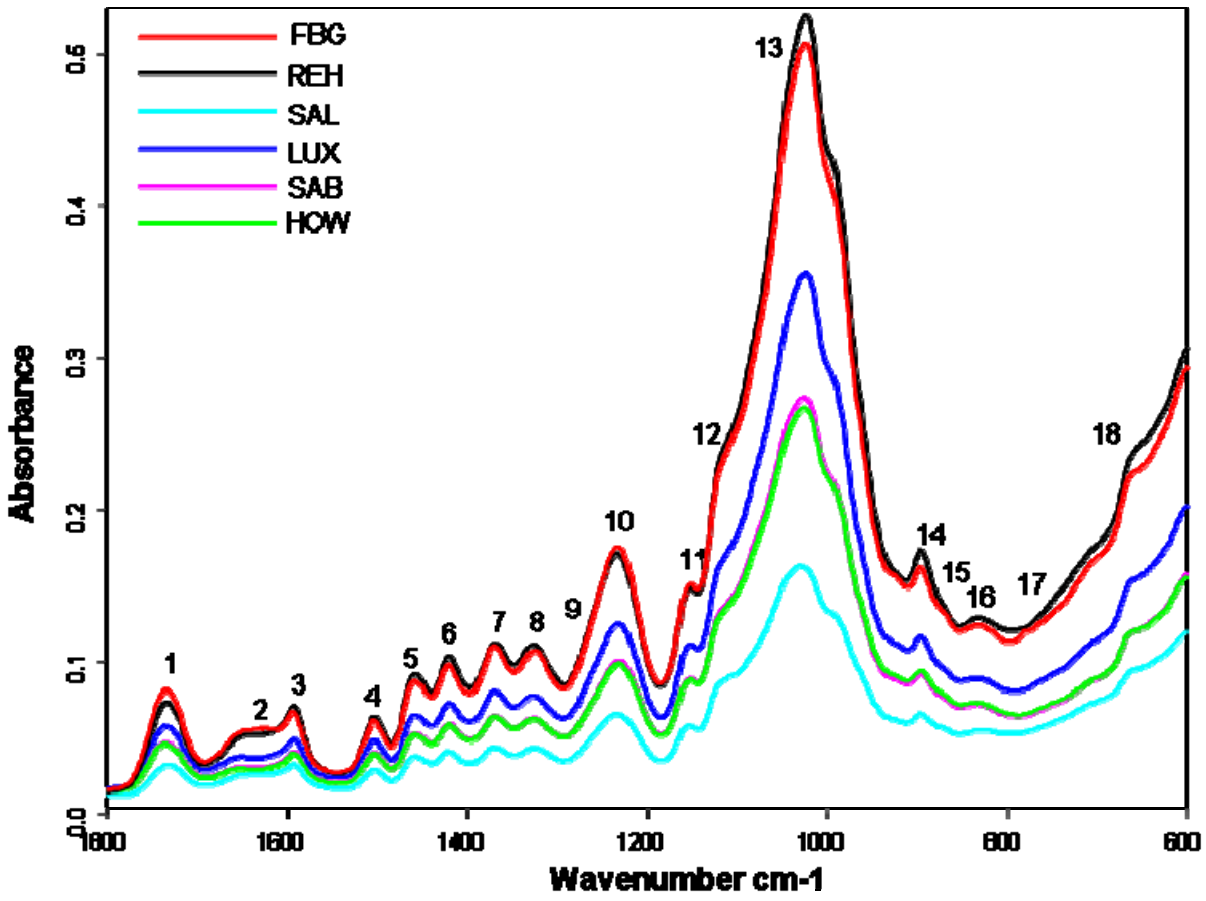

FBG
REH
SAL
LUX
SAB
HOW
Absorbance
0.0
0.1
0.2
0.3
0.4
0.6
1800
1600
1400
1200
1000
800
600
Wavenumber cm-1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Fig. 2

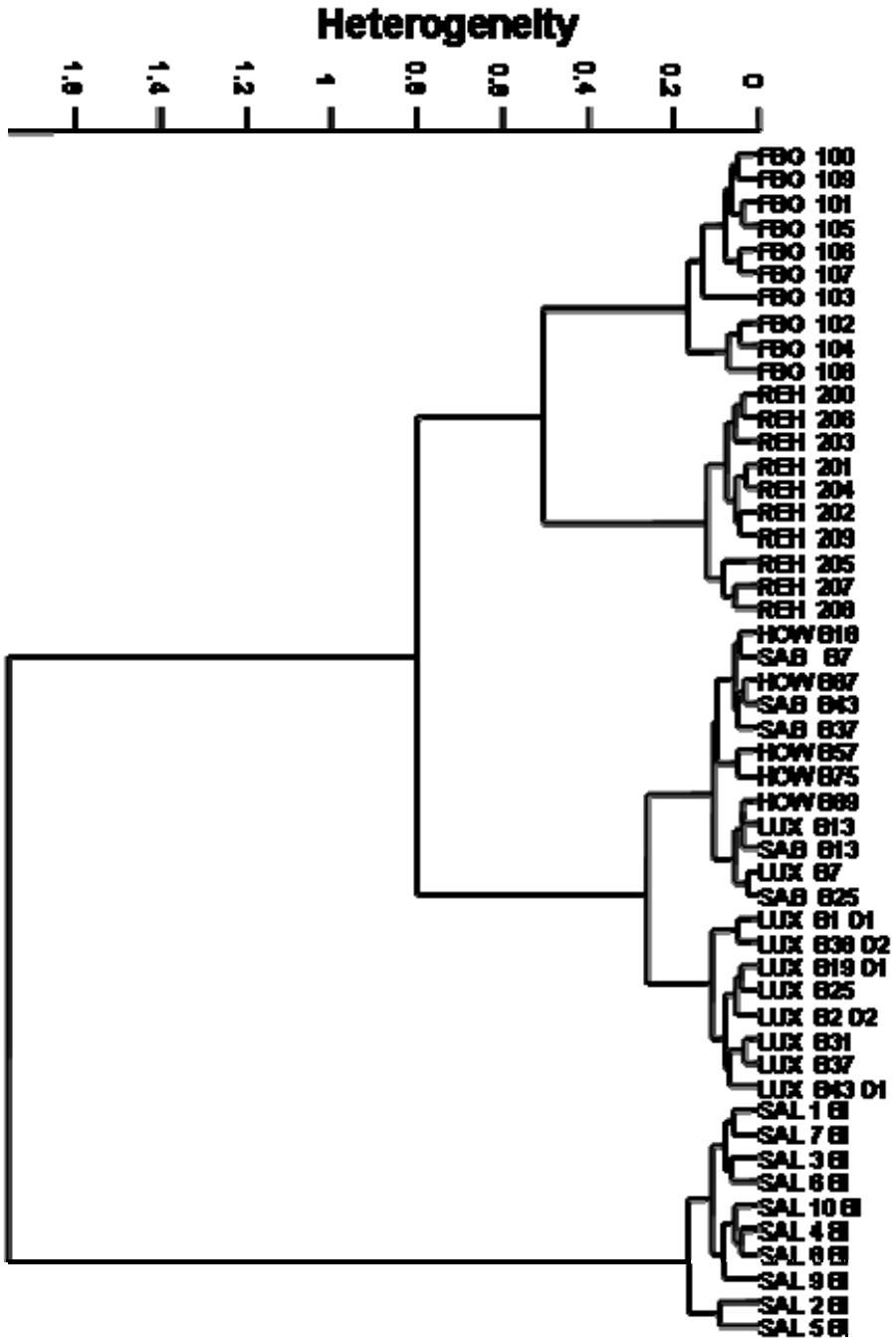
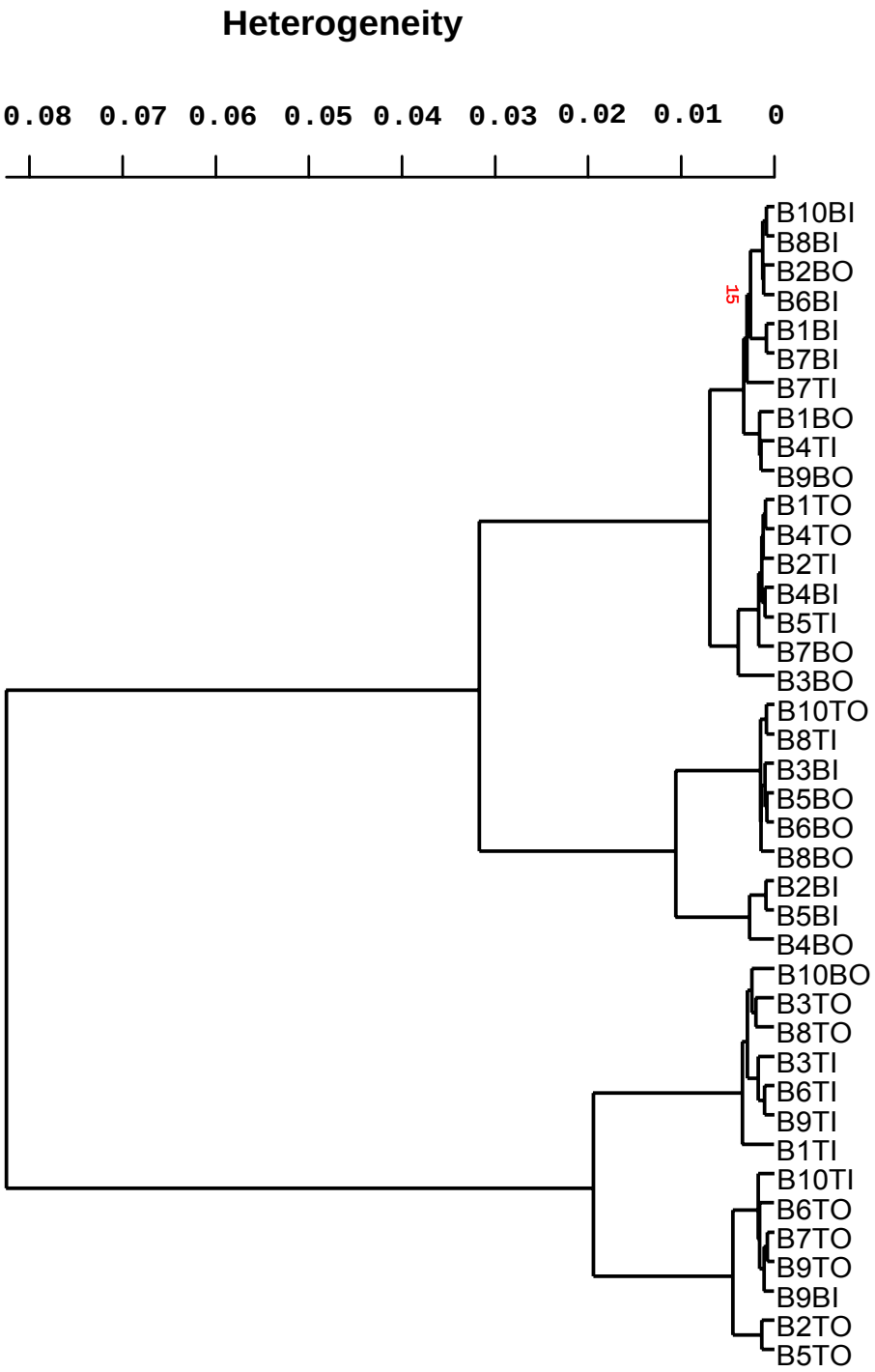

Heterogeneity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FBO 100
FBO 109
FBO 101
FBO 105
FBO 106
FBO 107
FBO 103
FBO 102
FBO 104
FBO 108
REH 200
REH 206
REH 203
REH 201
REH 204
REH 202
REH 209
REH 205
REH 207
REH 208
HOW B18
SAB B7
HOW B67
SAB B43
SAB B37
HOW B57
HOW B75
HOW B69
LUX B13
SAB B13
LUX B7
SAB B25
LUX B1 D1
LUX B36 D2
LUX B19 D1
LUX B25
LUX B2 D2
LUX B31
LUX B37
LUX B43 D1
SAL 1 B
SAL 7 B
SAL 3 B
SAL 6 B
SAL 10 B
SAL 4 B
SAL 8 B
SAL 9 B
SAL 2 B
SAL 5 B

Fig. 3


Heterogeneity
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15
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B8BI
B2BO
B6BI
B1BI
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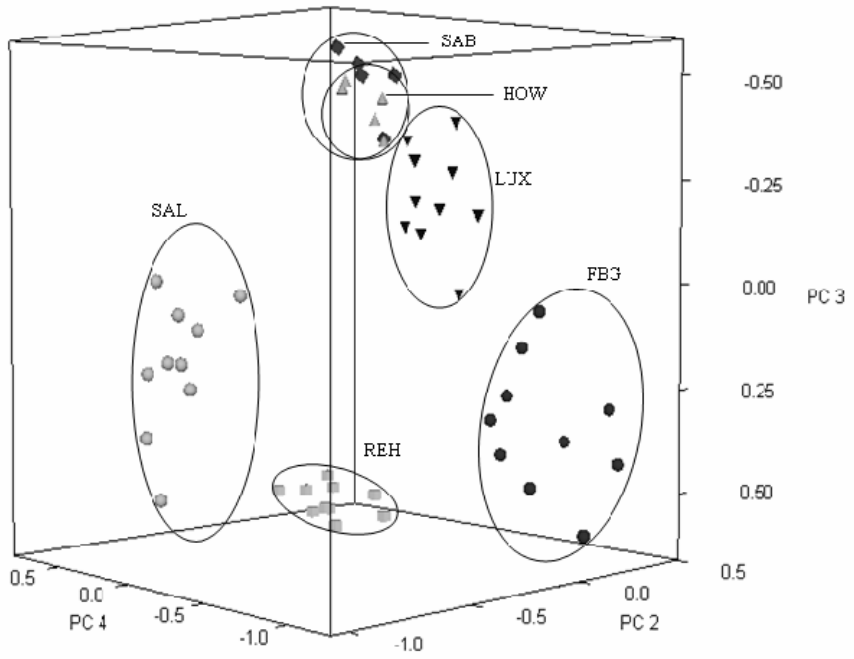
B7TI
B1BO
B4TI
B9BO
B1TO
B4TO
B2TI
B4BI
B5TI
B7BO
B3BO
B10TO
B8TI
B3BI
B5BO
B6BO
B8BO
B2BI
B5BI
B4BO
B10BO
B3TO
B8TO
B3TI
B6TI
B9TI
B1TI
B10TI
B6TO
B7TO
B9TO
B9BI
B2TO
B5TO

Fig. 4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HOW
LJX
SAL
FB3
REH
-0.50
-0.25
0.00
0.25
0.50
PC 3
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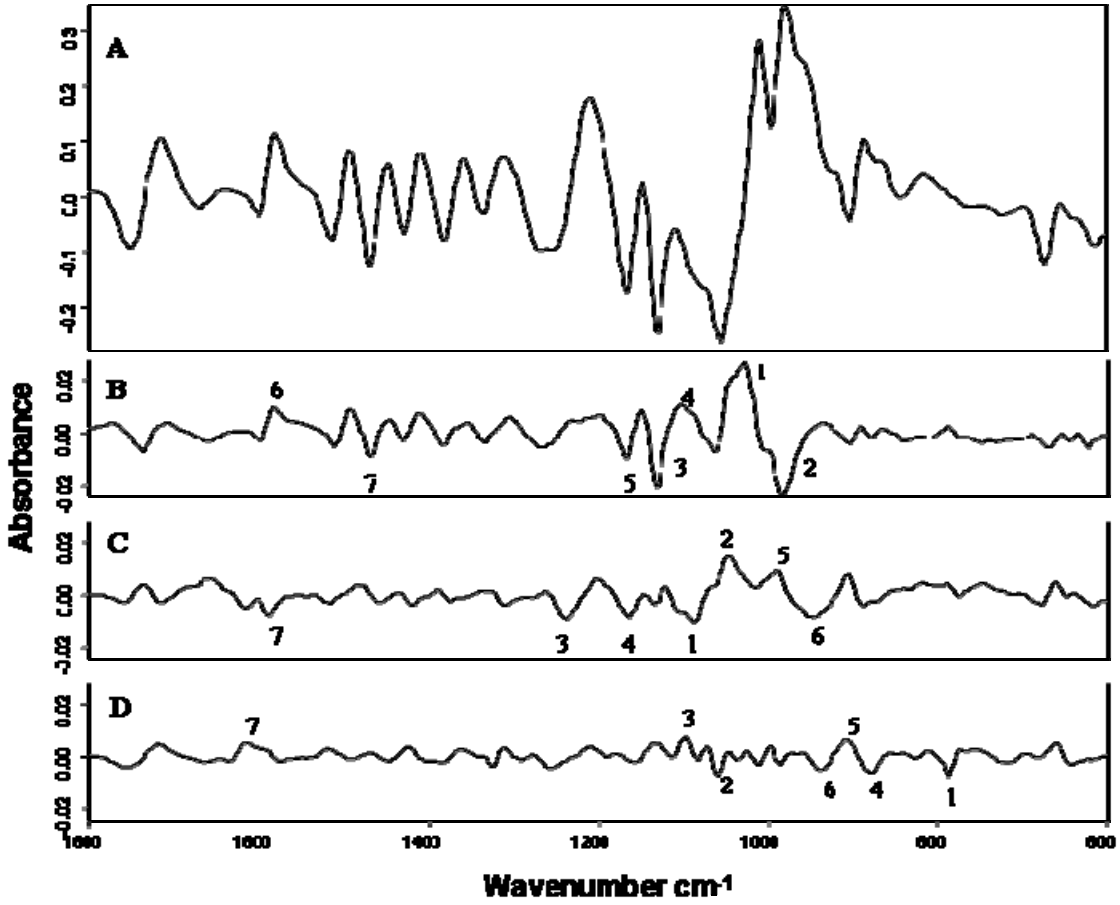
0.0
-0.5
-1.0
PC 4
-1.0
-0.5
0.0
0.5
PC 2

Fig. 5


A
B
C
D
Absorbance
Wavenumber cm-1
1800
1600
1400
1200
1000
800
600

3. Height growth, wood traits and molecular markers to distinguish five tree species of Dipterocarpaceae grown at same site

3.1. Abstract

The dipterocarps are of economic and ecological significance. To determine if simple traits can be used to distinguish wood of five representative species (*Dipterocarpus kerrii*, *Hopea plagata*, *Parashorea malaanoman*, *Shorea almon* and *Shorea contorta*), we determined tree height, wood density, Fourier transform infrared (FTIR) spectra of wood as a chemical fingerprint, and chloroplastic (cp) DNA restriction patterns as a molecular fingerprint. Wood was obtained from trees of the same age grown at the same plantation (Leyte, Philippines). Data were subjected to cluster analysis (tree height, wood density), principal component analysis (PCA) (FTIR spectra) and used for the construction of a phylogenetic tree (cpDNA-PCR). Comparison of the result of cluster analysis and PCA with the phylogeny of the taxa based on cpDNA variation showed similar differentiation patterns. *D. kerrii* and *H. plagata* were always clearly separated from the other three species. FITR in combination with PCA did not distinguish wood of the same genus (*Shorea* spp.), but resulted in mostly separated groups of *Parashorea* and *Shorea* with only little overlap, and clearly separated groups of *D. kerrii* and *H. plagata*. Cluster analyses on tree characters revealed a clear grouping of all samples according to species, except for *S. contorta*, which was partly mixed with the subcluster of *P. malaanoman*. Since the formation of clades obtained by cpDNA-PCR analysis of samples was similar to that found by cluster analysis, our data indicate that wood traits can be used for taxonomic purposes to distinguish distantly related tree species of the same family. Extension of this method may be of great interest for wood certification as it may be possible to distinguish wood of different species originating from same region.

Key words: Dipterocarpaceae, FTIR spectroscopy, cluster analysis, principal component analysis, certification, phylogeny, cpDNA.

3.2. Introduction

Dipterocarpaceae form an important pantropical tree family comprising three subfamilies according to recent classifications (Ashton 1982, Loñdono *et al*. 1995, Morton 1995) with 'Dipterocarpoideae' (13 genera, about 470 species) reaching from the Seychelles, SriLanka and Indian north-eastwards to southern China and the Batan inlands, and south-eastwards to New Guinea and D' Entrecasteaux Island, with 'Monotoideae' (3 genera, about 40 species) in Africa and South America, and the 'Pakaraimoideae' (1 genus, 1 species) confined to South America. Many tropical forests in Asia are dominated by dipterocarps (Dipterocarpoideae) and can be regarded as hot spots of global biological diversity (Cao *et al*. 2006). The subfamily Dipterocarpoideae is very species-rich and common in Asian evergreen and Monsoon forests whereas comparatively few species occur in the neotropics and in Africa. The Asian Dipterocarps have been classified into two tribes based on morphology, wood anatomy and cytology (Gottwald & Parameswaran 1966, Ashton 1982). The tribe Dipterocarpeae comprising the genera *Dipterocarpus*, *Anisoptera*, *Cotylelobium*, *Upuna*, and *Vatica*, is characterized by valvate fruit-sepals, scattered resin channels, and a basic chromosome number of 11. The tribe Shoreae includes the genera *Hopea*, *Parashorea*, and *Shorea*, which have imbricate fruit sepals, resin channels in tangential bands, and a common basic chromosome number of 7. The tribe Shoreae is richer in species than Dipterocarpeae due to a high number of taxa in the genera *Shorea* and *Hopea*. *Shorea* comprises more than 100 species in 11 sections and is more species rich than *Hopea* (Ashton 1982).

Dipterocarps are the most important commercial timber species in Southeast Asia. Indonesia supplied more than 70% of the world's demand for plywood, principally from dipterocarp species (Chong & Achmadi 1996). Consequently, the timber industries of many Southeast Asian countries critically depend on trees of the family of Dipterocarpaceae (Cao *et al*. 2006). Shorea is the largest and economically most important genus in this family (Seibert 1996). Mixed dipterocarp forests are the world's main source of hardwood timber (Whitemore 1984), especially since the end of the Second World War and therefore of considerable economic importance.

Several authors (Ashton 1982, Ella 1993, Lomibao 1973, Newman *et al*. 1996a, b, Newman *et al*. 1998) described growth and main features for the

Dipterocarps. The main features of the five important timber wood dipterocarps have been compiled in Table 1. They can mainly be distinguished by their bark colours and types, leaf shape and sometimes by fruits (*D. kerrii*). However, reliable species identification may require molecular genetic analysis. Molecular genetic markers can also be used to identify the origin of wood since the manipulation of genetic traits is impossible and since environmental conditions have no impact on variation patterns (Finkeldey *et al*. 2008). To date, the number of marker types and reference data are increasing (Finkeldey *et al*. 2007). Marker technologies based on DNA polymorphisms have been established in various disciplines such as phylogeny, taxonomy, ecology, genetics and breeding (Weising *et al*. 2005). DNA markers provide valuable tools for genetic analysis and breeding research in forest trees, and facilitate studies of genome organization, mating systems and genetic diversity (Arcade *et al*. 2000). The use of molecular markers in phylogenetic research has been adopted because of the simplicity in obtaining large amounts of data and the higher reliability as compared to morphological data for constructing phylogenetic trees (Chase *et al*. 1993). Phylogenetic analyses are helpful in understanding relationships among species or higher-level comparisons. They also provide many new insights into the origin and biogeography of different groups of plants (Strauss *et al*. 1992). The recent techniques developed for the evaluation and genetic manipulation of populations and individual trees include amplified fragment length polymorphism (AFLP, (Vos *et al*. 1995)), random amplified polymorphic DNA (RAPDs) (Williams *et al*. 1990), microsatellites, and restriction fragment length polymorphism (RFLPs) (Burley 2001). These methods have also been applied to clarify the phylogeny of Dipterocarpaceae, e.g. by using RFLP markers and nucleotide sequences of chloroplast cp(DNA) genes (Dayanandan *et al*. 1999, Indrioko *et al*. 2006, Kajita *et al*. 1998, Morton *et al*. 1999, Tsumura *et al*. 1996), RAPD markers (Rath *et al*. 1998) or partial sequences of nuclear genes (Kamiya *et al*. 2005).

Wood is the most versatile and widely used structural material to be used in both indoor and outdoor applications. Its ever increasing demand and the large gap between demand and supply is causing illegal felling and the destruction of forest resources in large parts in particular in developing countries of lower latitudes. While the forest area in industrialized nations increased during the last decade, considerable losses of forest cover in developing countries diminish the forested areas in all main regions of the tropics (FAO 2001). In order to limit illegal timber trading, the import

and sale of tropical timber is greatly promoted by a proof of an origin in industrialized countries. Wood identification plays an important role in this context. We have shown for the example of beech that wood of this species grown at different sites could be distinguished by Fourier transform infrared spectroscopy (FTIR) and multivariate statistical analysis (Rana *et al*. 2008, chapter 2). FTIR analysis reveals the chemical finger print of wood and has been used for many different applications in wood research, e.g. to distinguish different species (Brunner *et al*. 1996, Gierlinger *et al*. 2005, Schimleck *et al*. 1996). It is not known whether it is possible to distinguish wood of different dipterocarps by this method.

The main objective of this study was to find out whether simple characteristics like tree height, wood density and the chemical fingerprint of wood can be used as means to distinguish species and to clarify phylogenetic relationships among taxa. To investigate these questions, we determined tree height, wood density and the chemical fingerprint of wood by FTIR-ATR of five selected dipterocarps, *D. kerrii*, *H. plagata*, *P. malaanoman*, *S. almon* and *S. contorta*. These taxa were chosen because of their economic significance. The results of wood chemistry and other tree traits were compared with those obtained by molecular analysis of cpDNA restriction data for species identification.

3.3. Materials and methods

3.3.1. Field site and sampling

Samples were collected in a plantation on the island Leyte (Philippines) which is located between 9° 55′ N and 11°48′ N and 124° 17′ E to 125° 18′ E (Langenberger 2006). The local climate data from a weather station of the Philippines Atmospheric, Geophysical and Astronomical Service Administration (PAGASA, 7 m a. s. l.) on the campus of the Leyte State University show an average annual precipitation of 2586 mm (Langenberger 2003). The wettest months are November to January with a mean monthly precipitation of about 290 mm. The driest months are March to May with an average monthly precipitation of 95 to 133 mm. Annual as well as monthly variability of rainfall is high. Although the mean precipitation is within the range for evergreen rain forests as given by Walsh (1996), drought periods occur, especially during El

Niño Southern Oscillation events (Langenberger 2003). While seasonal rainfall patterns can be assumed to be similar for the lowlands and the mountain range, it is likely that the precipitation in the vicinity of Mt. Pangasugan's summit and its slopes, where the plantation of this study is located, is higher than the values measured at the PAGASA station due to orographic rains.

Stem samples of 5 Dipterocarpaceae species (five replicate trees of each) were collected from a plantation at the western foothills of Mt. Pangasugan, within the forest reserve of the Leyte State University. The area was hilly to some extent and samples were taken on the upper part of the slope. The trees were planted at the same time and had an age of 6 years at harvest. Only healthy trees with no apparent injury were used. Disks excised from each tree at breast height (1.3 m) were used for determination of wood density and FTIR-ATR analysis. Blocks were cut from the disks of cross sectional dimensions ranging from 7.3 cm^2 to 2.2 cm^2, because of the variation of the tree diameter. Blocks were about 3 cm in height. For each species five individual trees were used for sampling.

3.3.2. Wood density determination

Wood disks were used for density determination (excluding the bark). It was not possible to remove the pith because of the small disk size of *S. almon*. The volume of fresh wood samples was determined by Archimedes' principle (Hacke *et al*. 2000). The samples were immersed in a water-filled tray, which was placed on a balance. Displacement weight was converted to sample volume by the formula: displacement mass (g) / 0.998 (g cm^{-3}), where 0.998 g cm^{-3} is the density of water at 20°C. The dry mass of the wood samples was measured after drying for at least 7 days at 35°C. Wood density was calculated as the ratio of dry weight to volume of fresh wood.

3.3.3. Sample preparation, FTIR-ATR measurements and analysis

The same dried wood disks used for density determination were used for FTIR. The samples were hackled with a gripper to small pieces and powdered in a ball mill (Retsch, MM 2000 Hannover, Germany) for about 20 minutes at 60 u/min to a fine powder. The frequency was raised slowly over the next 5 min up to 90 u/min. FTIR-ATR spectra of milled wood powder were recorded with the FTIR spectrometer

Equinox 55 (Bruker Optics, Ettlingen, Germany) combined with an ATR unit (DuraSamplIR, SensIR Europe, Warrington, England) at a resolution of 4 cm^{-1} for 32 scans in the range from 600 to 4000 cm^{-1}. The powdered wood samples were pressed against the diamond crystal of the ATR device. A pressure applicator with a torque knob ensured that the pressure applied was the same for all measurements. A background spectrum of the clear window was recorded prior to acquisition of sample spectra. The spectrum of the background was subtracted from spectrum of the sample before conversion into absorbance units. For each sample five different sub-samples were analysed and averaged to give a mean spectrum per sample.

All mean spectra of the individual trees were used for principal component analysis (PCA). For PCA, the second derivatives of vector normalized spectra were used in two specific regions (1547-1481 and 1292-1182 cm^{-1}) of the spectra and the factor loading were calculated. Deviation, normalization and PCA (factor) analysis were performed using the software OPUS Ver. 6.5 (Bruker, Germany).

3.3.4. Chloroplast DNA analyses

The total DNA was extracted from about 1 cm^2 of silica gel dried leaf tissue using the DNeasy Plant Kit (Qiagen, GmbH, Hilden). DNA amount and quality was checked on a 0.8% agarose gel after staining with ethidium bromide (Sambrook *et al*. 1989). DNA was diluted 1:20 prior to PCR amplification.

Five cpDNA gene/interspecific region, *rbc*L, *pet*B, *psa*A (Tsumura *et al*. 1995) *and trn*LF (tRNA-Leu (UAA) - tRNA-Phe (GAA) (Taberlet *et al*. 1991) were amplified by PCR and digested with a total of seven restriction enzymes (*Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I, see (Indrioko *et al*. 2006). PCR conditions were chosen according to Tsumura *et al*. (1996) and Taberlet *et al*. (1991) with slight modifications. The restriction site data were transformed into a binary matrix (0/1). Length variants of restriction fragments were coded as multistate characters. A total of 117 informative characters were used as input for PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 for Macintosh (Swofford 1998).

The restriction site data were used to calculate a Neighbor joining tree (Saitou & Nei 1987) based on the mean number of pairwise character differences. Statistical support of the clades was tested by 1000 bootstrap replications.

3.3.5. Statistical analysis

Statistical analysis was performed with SAS (9.13 version, SAS Institute Inc. 2004, Cary, NC, USA) using analysis of variance (ANOVA), followed by Duncan's multiple range test. Data are indicated as means ± SD. Differences between parameter means were considered significant when the P – value of the ANOVA Duncan's multiple range test was less than 0.05. Different letters indicate significant difference. Cluster analysis was done by Ward's method using Euclidean square as distance metric with the programme Statgraphic Plus (Statistical Graphics Corporation, St. Louis, MO, USA). Tree height and wood density served as input data.

3.4. Results

3.4.1. Growth and wood characteristics

Significant differences in tree height and wood density were observed among the five dipterocarp tree species (Table 2). To determine if tree height and wood density were sufficient to permit grouping of these trees according to species, cluster analysis was conducted with these two parameters (Fig. 1). This analysis revealed two main subclusters, one for *H. plagata* and *D. kerrii* and the other for *P. malaanoman*, *S. almon* and *S. contorta*. *D. kerrii* and *H. plagata* were clearly separated in the second order sub-cluster. *S. almon* and *P. mlaanoman* were also found to form separate second order subclusters. Only one sample of *S. contorta* was mixed up with the *Parashorea* subcluster. These data show that the five even-aged species grown at the same site were distinguishable by simple tree characters such height and wood density. However, in practical terms, tree height will usually be strongly variable due to differences in age and growth environments and, thus, will only be applicable under certain, limited conditions.

3.4.2. The chemical fingerprint of wood

To investigate whether the organic wood composition contains spectral information to enable species separation, the chemical fingerprint of wood of the five dipterocarp species was analysed by FTIR spectroscopy (Fig. 2). The original spectra

were vector normalised and the second derivative was calculated (Fig. 2A). The resulting spectra showed several prominent peaks in the finger-print region of wavenumbers from 1800 to 600 cm^{-1} (Fig. 2A). Most of these peaks represent changes in absorbance caused by major cell wall components such as cellulose, hemicelluloses, and lignin. For example, the peak arising at 1626 cm^{-1} due to stretching vibration of C=O (Parker 1983). The peak at 1330(1320) cm^{-1} indicates syringyl ring breathing with CO stretching (Hergert 1971, Fengel & Wegener 2003) and the peaks between 1200 und 900 cm^{-1} are characteristic of the polysaccharide region (cellulose, hemicellulose), in which absorbance changes arise due to C-O-C and C-O ring vibrations of carbohydrates (Faix *et al*. 1991, Naumann *et al*. 1991). It is apparent that the mean spectra from samples of different species were overlapping in large regions.

Closer inspection of the mean *D. kerrii* and *H. plagata* spectra revealed some distinct features in comparison with the others. In the region around 1626 cm^{-1} *H. plagata* had a prominent peak and the spectrum of *D. kerrii* was different from those of the other species. The peak at 1330 cm^{-1} was displaced in *H. plagata* (1320 cm^{-1}). A small shoulder was observed in *H. plagata* at 1305 cm^{-1}. In the region between 1100-1020 cm^{-1}, 650-620 cm^{-1} and at 859 cm^{-1} both *D. kerrii* and *H. plagata* displayed different spectral patterns than the other species and at 797, 781 and 768 cm^{-1} distinct peaks were observed in *H. plagata* (Fig. 2A).

To investigate the spectra in-depths, PCA analysis was conducted. Figure 3 shows a PCA projection calculated from the spectral data sets of each individual tree. The plot has been constructed by three-dimensional projection of Eigenvectors (factor spectra) 1, 2 and 3 so that the intrinsic group or class structure of the whole data set can be inspected. Each point in the map represents a spectrum; all three factorial coordinates (factor loadings) were used for data representation (Fig. 3). The best model with three principal components was obtained when using second derivative, vector normalized spectra in the range of 1547-1481 cm^{-1} and 1292-1182 cm^{-1} as input data (Fig. 2 B, C). The PCs from *D. kerrii* and *H. plagata* formed two distinct groups which could be easily separated (Fig. 3). The PCs from *S. almon* and *S. contorta* did not form separate groups. The PCs from *P. malaanoman* partly overlapped with the two S*horea* species (Fig. 3).

To investigate the source of variation, factor loadings were analysed. The factor loading of the first factor had an Eigenvalue of 4.937, which explains 98.7%

(0.987) of the variation. The second and third factor explained 1.06% and 0.0016% of the variation, respectively. As figure 3 shows, all the samples were scored positive along the first factor loading (PC1) which had the highest percentage of variation. *H. plagata* samples were scoring positive, *D. kerrii* samples mostly negative and the remaining samples scored negative along the second factor loading (PC2) (Fig. 3). Along the third factor loading positive scoring was seen in *H. plagata*, partly positive and partly negative scoring was observed in *S. almon* and *S. contorta*. The samples of *P. malaanoman* and *D. kerrii* were completely in the negative range. We have assigned 7 peaks of the first and second factor loading, respectively (Fig. 2 B, C, Table 3). Tillmann (1996), who tested multivariate methods for FTIR analysis, used the five highest peaks to characterize major components contributing to sample variation. In our study the third factor loading was not considered because it accounted for a very low percentage of variation.

The band assignments of the factor loadings shown in Fig. 2 B and C indicated the existence of several important compounds (Table 3). Amide bands (peak 1 and 8) were found for both factors and lignin peaks at 1510 cm^{-1} for the second and at 1505 cm^{-1} for the first factor, respectively. The band at 1270 cm^{-1} which has been assigned as guaiacyl lignin (Fengel & Wegener 2003) was also present in both factors. Besides stretching vibrations of various compounds (mainly carbon in combination with other compounds) were found in both the factor loadings (Table 3). Although these data indicate that lignins, amides and some other compounds contributed to the variations between the species, it is also apparent that only three groups were separated by this analysis, *D. kerrii,* as representative of the tribe Dipterocarpeae, and *H. plagata,* a member of the Shoreae from the three other members of this tribe, namely *S. contorta, S. almon* and *P. malaanoman.*

3.4.3. Phylogenetic analysis with cpDNA restriction

To investigate the phylogenetic distance between the five species of this study, chloroplast DNA restriction analysis was performed (Fig. 4). The Neighbor Joining tree based on cpDNA restriction data (Fig. 4) showed a clear separation between *D. kerrii* (tribe Dipterocarpeae) and the tribe Shoreae. The Shoreae clade showed 100% bootstrap support and comprises, as expected, the species *H. plagata*, *S. contorta* , *S. almon* and *P. malaanoman*. While all samples of *Hopea plagata*, *Shorea almon*, and

Parashorea malaanoman formed each a distinct group with high bootstrap support, of the three samples analysed of *S. contorta* two formed a sister group to *Hopea plagata*, and one *S. contorta* sample was mixed in the *P. malaanoman* clade.

3.5. Discussion

Among the analysed tree species *H. plagata* belongs to heavy hardwood, *D. kerrii* to medium hardwood and the other three species belong to Philippine light red mahogany (Newman *et al*. 1996b). This classification was also evident from the measured wood densities (Table 2). According to published data *H. plagata* and *D. kerrii* reach tree heights less than those of *S. almon, S. contorta* and P. *malaanoman* (Table 1). In contrast to this, among the trees of this plantation, *S. almon* was the smallest (Table 2). In the wet tropical forests, the growth rate of sapling is associated with a number of factors like variation in leaf area and light level (Poorter 2001, Sterck *et al*. 1999,). Growth rate increases linearly with light interception, although there is substantial unexplained variation in growth (King *et al*. 2005). The dipterocarps vary greatly in growth rates and reach mature habit within 60 years under forest conditions; other tree species are usually shade tolerant and grow very slowly. The former have estimated life-spans of about 250, the later of probably more than 1000 years, judged on the basis of girth growth data (Ashton 1982). According to Whitemore (1984), *Dryobalanops aromatica*, a dipterocarp species can persist in deep shade for a prolonged period of time during seedling and sapling stage and responds quickly to increased light. Because of the high plasticity in growth response, tree height is strongly variable and can only be used as a tree character if growth rates are known or if trees of the same age are growing at the same sites as in the present study. Using wood density and tree height as two simple traits, we have been able to group the trees of this study according to species with the exception of *S. contorta*, which was partly mixed with the cluster of *P. malaanoman* (Fig. 1). A similar grouping was achieved using molecular techniques (Fig. 4).

The phylogenetic tree obtained by cpDNA restriction (Fig. 4) indicates clear separation between the two tribes, Dipterocarpeae and Shoreae as well at the level of the genus (Hopea vs. Shorea). A number of studies were performed to unravel the relationship between *Shorea* and *Parashorea* species. Molecular phylogenetic studies (e.g. Cao *et al*. 2006, Indrioko *et al*. 2006, Tsumura *et al*. 1996) and earlier

morphological examinations (Symington 1943) showed a close affinity between the genus *Parashorea* and the species-rich genus *Shorea*. Similar to our results on *S. contorta,* the diagnostic haplotypes of *Shorea fallax* fell into 5 different subclades throughout the clade of Red meranti in a phylogenetic tree derived from the nuclear gene *PgiC* suggesting interspecific hybridization or ancestral polymorphisms (Kamiya *et al*. 2005). These examples underline that in some cases it is difficult to distinguish unambiguously some tropical species, even with current molecular techniques.

To increase our knowledge on the traits of these major timber species, we analysed wood by FTIR spectroscopy, which yields a chemical finger-print. It was possible distinguish two tribes (Dipterocarpeae against Shoreae) and genera (*Hopea* vs *Shorea/Parashorea*). However it was not possible to distinguish species within a genus (two *Shorea* spp.) (Fig. 3). Our study of wood traits and growth parameters supports the close relationship between *Parashorea* and *Shorea* spp (Figs. 1, 3). The differentiation patterns observed by means of cluster analysis (Fig. 1) and PCA (Fig. 3) reflect phylogenetic relationships among dipterocarps as assessed by PCR-RFLPs of cpDNA (Fig. 4).

In previous studies, FTIR spectroscopy in combination with PCA has been successfully applied to discriminate some woody species (Brunner *et al*. 1996, Gierlinger *et al*. 2004). However, very complex spectral analyses were necessary to distinguish three species *(Larix decidua, L. kaempferi, L. eurolepis)* within one genus (Gierlinger *et al*. 2004) growing on the same area. For 100% correct clustering of the larch species, Gierlinger *et al*. (2004) identified a narrow spectral region in which the differences were most pronounced. Similarly, we had to focus on particular spectral regions (1547-1481 cm^{-1} and 1292-1182 cm^{-1}), where the differences were most pronounced (Fig. 2 B, C) to obtain reasonable species separation. Overall, it can be concluded that if differences are small and not well understood, a detailed spectroscopic analysis, followed by restriction to relevant wavenumbers leads to a classification by PCA with considerable success.

The possibility to discriminate wood grown in same environment with a simple and rapid method is advantageous, if it is necessary to classify wood according to its provenience, for example for certification. Although a higher accuracy can be achieved by subjecting samples to genotyping by molecular methods, which are more laborious and expensive, this study shows that wood traits also have a potential to be used for taxonomic purposes and to clarify phylogenetic relationship within tree

families.

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Table 1. Main features (including some physical characteristics and properties) for the identification of the main timber wood dipterocarp species.

Species	Tree height (m)	Diameter at breast height (cm)	Buttress	Outer bark	Inner bark	Bark thickness (mm)	Main diagnostic features
Dipterocarpus kerrii	30	130	if present, blunt	thin, grey, scaly	pinkish brown, brittle	no information available	rather small, shinning few-nerved, long-petioled leaves with few secondary nerves, the fallen stipules silky inside and the rather globose fruits
Hopea plagata	55	180	prominent	deep fissured bark	no information available	8	very hard and durable wood, leaves small, usually elliptical, thinly leathery , secondary nerves on lower leaf surface prominent but slender, upper leaf surface drying brown, mature fruit with two longer and upper shorter wings, number of stamens 32-38
Parashorea malaanoman	>60	~200	prominent	brown to almost black but greyish when exposed to sunlight	tan coloured with whitish vertical bands beneath the grooves	8	large size of trees, grey or almost black broadly fissured bark, large buttress, the medium-sized elliptic leaf with steeply ascending secondary nerves, matched-sized splinters burn charcoal to partial ash
Shorea almon	70	160	long and string	dark brown, dark brown , hard, ~0.6 mm thick, shallowly fissured	reddish yellow turning to reddish brown on exposure, fibrous, ~1.2 mm thick	11	the very dark or almost black bole, and the medium-sized, thin, slightly boat shaped leaves with a dense pale brown momentum beneath, match-sized splinters burn greyish or brown ash
Shorea contorta	50	180	present	brown to nearly black or grey when exposed to sunlight, V-fissured with vertical white strips of lenticels in the fissures	brown to slightly inkish, stringy	12	lower leaf surface smooth to touch; secondary nerves on ower leaf surface stoutly prominent, petals white, match-sized splinters burn white ash

Table 2. Plant material, height and wood density. Data indicate means (± SD, n= 5). Different letters in columns indicate significant differences at $P \leq 0.05$.

Timber name [a]	Species	Sample no.	Tree height (m)	Density (g cm^{-3})
Yakal	*Hopea plagata* (Blanco) S. Vidal	1-5	9.6 ± 1.14 A	0.97 ± 0.01 A
Minyak	*Dipterocarpus kerrii* King Damar	6-10	8.6 ± 0.89 A	0.70 ± 0.06 B
White Lulan	*Shorea contorta* Vidal	11-15	9.8 ± 1.30 A	0.40 ± 0.02 D
White Lulan bagtikan	*Parashorea malaanoman* (Blanco) Merr	16-20	7.2 ± 0.84 B	0.45 ± 0.03 C
Meranti	*Shorea almon* Foxw	21-25	4.1 ± 0.55 C	0.38 ± 0.03 D

[a]Kamiya *et al*. (2005) and Wood Density Database

Table 3: Band assignments of the first and second factor loadings obtained by principal component analysis. The numbers in parenthesis indicate their positions in Fig. 2 B, C respectively. Peak 1268 cm^{-1} is present in both factors (in first factor and second factor, 4 and 12 respectively). The first 7 peaks have been assigned in each factor loading.

Wave number (cm^{-1})	Band origin	Short comments	Reference
1524 (1)	N-H deformation	Secondary amide (upward direction)	Parker (1983)
1522 (8)	N-H deformation	Secondary amide (downward direction)	Parker (1983)
1510 (9)	aromatic skeletal vibration plus C=O stretch; S > G; G condensed > G etherified	Upward direction	Faix (1991), Fengel & Wegener (2003), Evans (1991)
1505 (2)	Same as peak 1510	Downward direction	Faix (1991), Fengel & Wegener (2003)
1496 (10)	C=S stretching	-N-C=S (downward direction)	Parker (1983)
1485 (11)	C=S stretching	-N-C=S (upward direction)	Parker (1983)
1286 (3)	Amide III	Protein (upward direction)	Naumann *et al*. (1991)
1270 (4/12)	guaiacyl ring breathing	Present in both factors. Downward and upward direction in first and second factor loading respectively	Fengel & Wegener (2003)
1251(5)	C-O stretching	CH_3COOR, acetic ester (upward direction)	Parker (1983)
1238 (13)	C-O stretching	Downward direction	Parker (1983)
1227 (6)	C-C plus C-O plus C=O stretch; G condensed > G etherified	Downward direction	Faix (1991)
1208(14)	C-N stretching vibration	Aliphatic amine (upwards direction)	Parker (1983)
1197 (7)	C-O-C, C-O dominated by ring vibration of carbohydrates		Naumann *et al*. (1991)

Figure legends

Figure 1. Cluster analysis of five species of dipterocarps from a total of 25 different trees. Hp (*H. plagata*), Dk (*D. kerrii*), Pm (*P. malaanoman*), Sc (*S. contorta*) and Sa (*S. almon*) were grown at the same site. The dendrogramme was constructed by Ward's method (Euclidian distance) using tree height and density (From Tab. 1).

Figure 2. Second derivative of vector normalized mean FTIR spectra of wood of five different species of dipterocarps from the same plantation (A). Each spectrum is a mean of spectra from 5 individual trees sampled from *D. kerrii*, *H. plagata*, *P. malaanoman*, *S. almon,* and *S. contorta*. The different colour indicates different tree species. (B) and (C) refer to first and second factor loadings, respectively, for PCA of five different dipterocarps. The arrows in B and C indicate the region (1480-1291 cm^{-1}) that has been omitted during principal component analysis. The different numbers in B and C refer to peaks described in Table 3. The first seven main peaks were assigned.

Figure 3. PCA map for five different dipterocarp tree species growing on the same site. For projection of data, the factorial coordinates (factor loadings) PC1, PC2 and PC3 are used, obtained from the second derivative of vector normalized data in two spectral ranges (1547-1481 and 1292-1182 cm^{-1}).

Figure 4. Phylogenetic analysis of five dipterocarp species. The neighbor joining tree constructed based on modified data of Villarin (2005). Bootstrap values in percent from 1000 replicates are indicated above the nodes. The tree is unrooted and branch lengths are shown.

Fig. 1

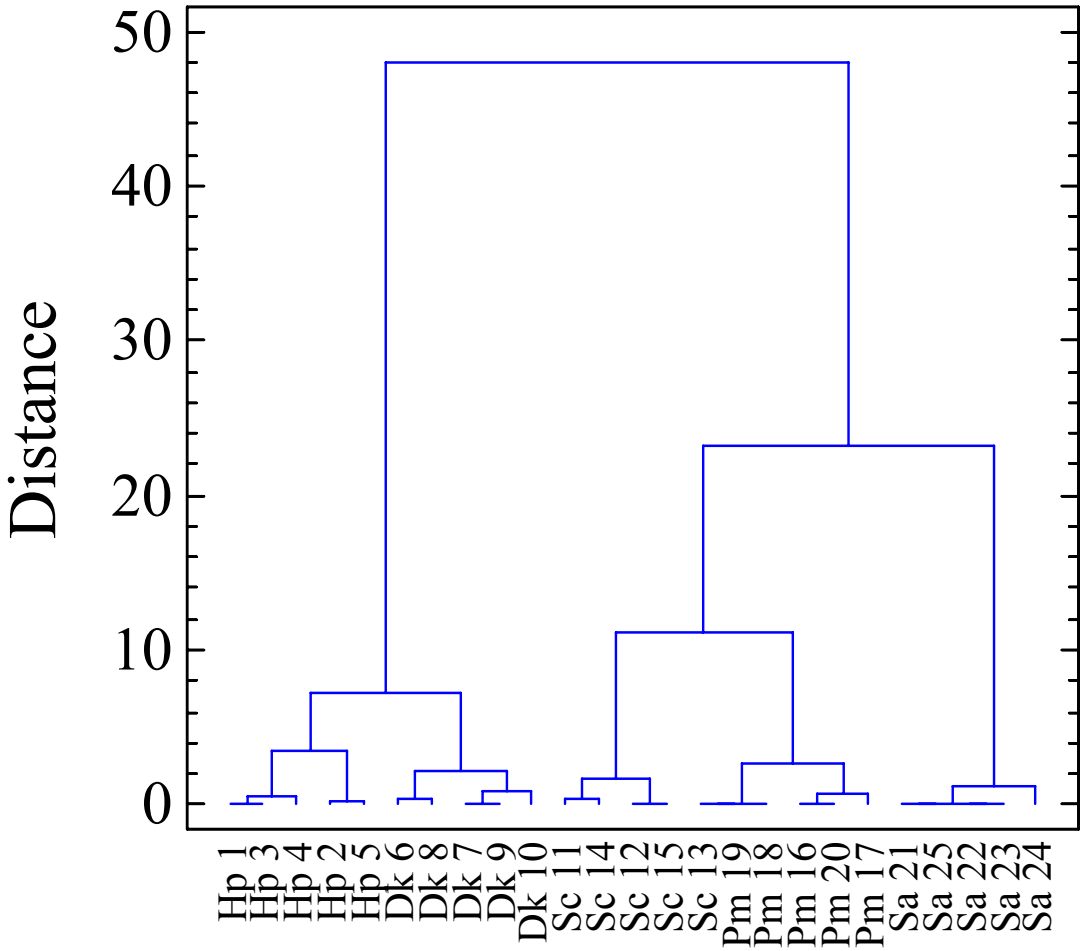

Distance
50
40
30
20
10
0
Hp 1
Hp 3
Hp 4
Hp 2
Hp 5
Dk 6
Dk 8
Dk 7
Dk 9
Dk 10
Sc 11
Sc 14
Sc 12
Sc 15
Sc 13
Pm 19
Pm 18
Pm 16
Pm 20
Pm 17
Sa 21
Sa 25
Sa 22
Sa 23
Sa 24

Fig. 2

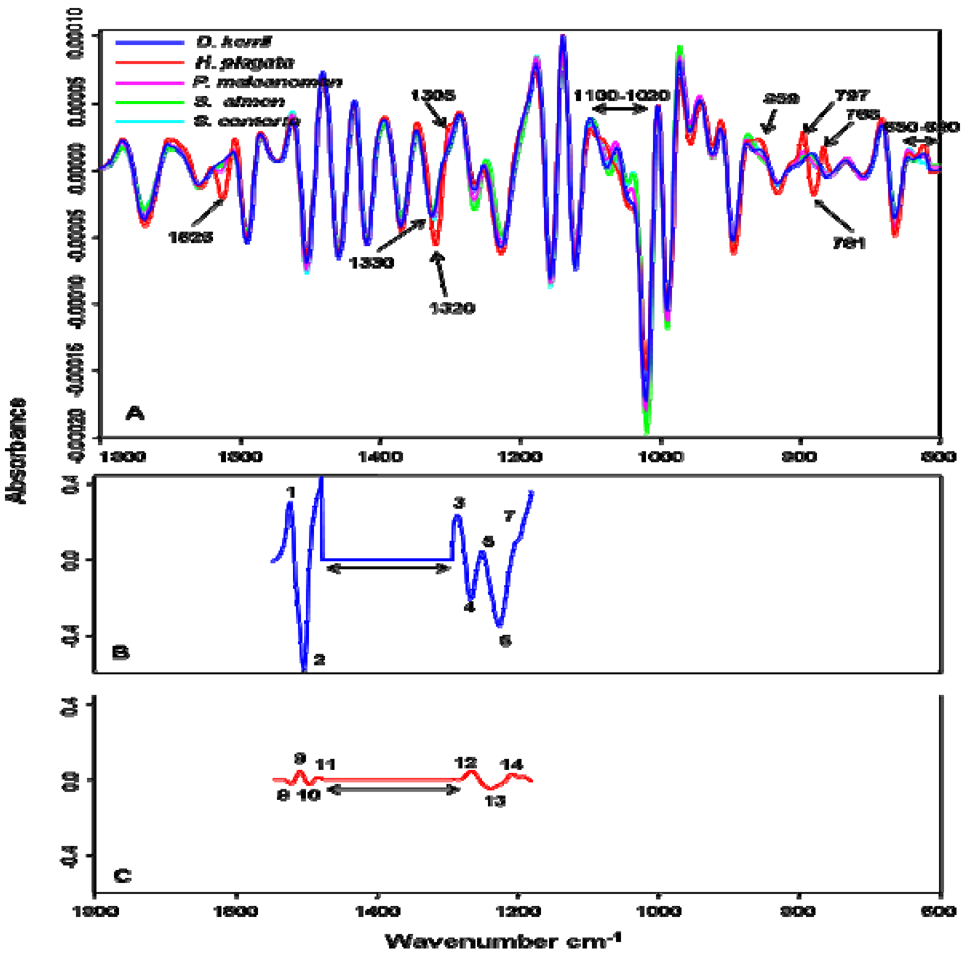

D. kerrii
H. plagata
P. melanomen
S. almon
S. comoris
1625
1330
1320
1305
1100-1020
859
797
788
761
650-620
A
B
C
Absorbance
Wavenumber cm-1

Fig. 3

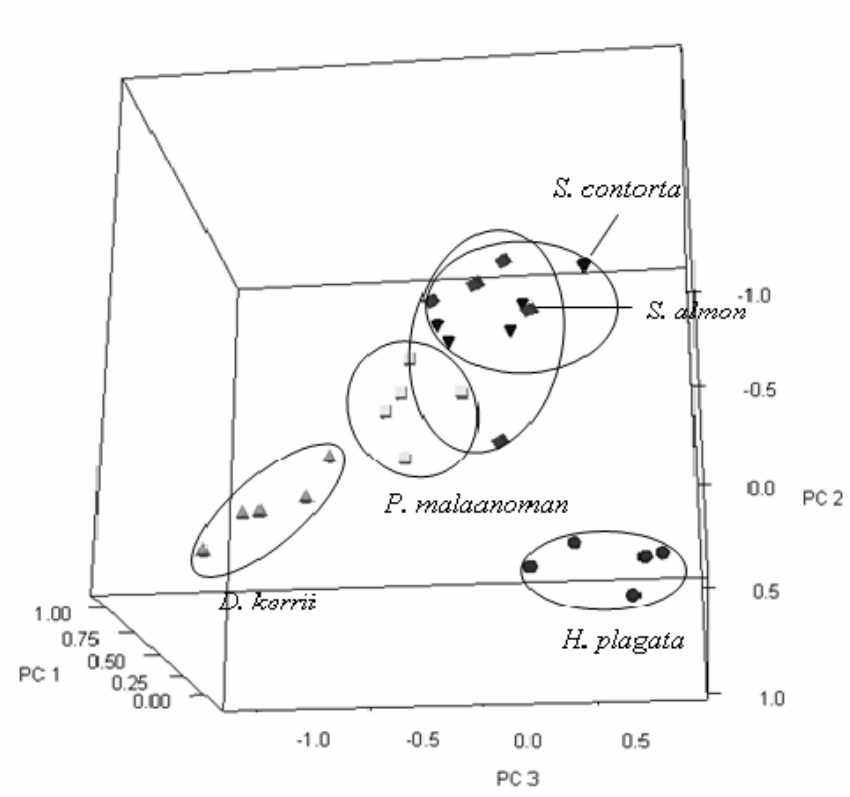
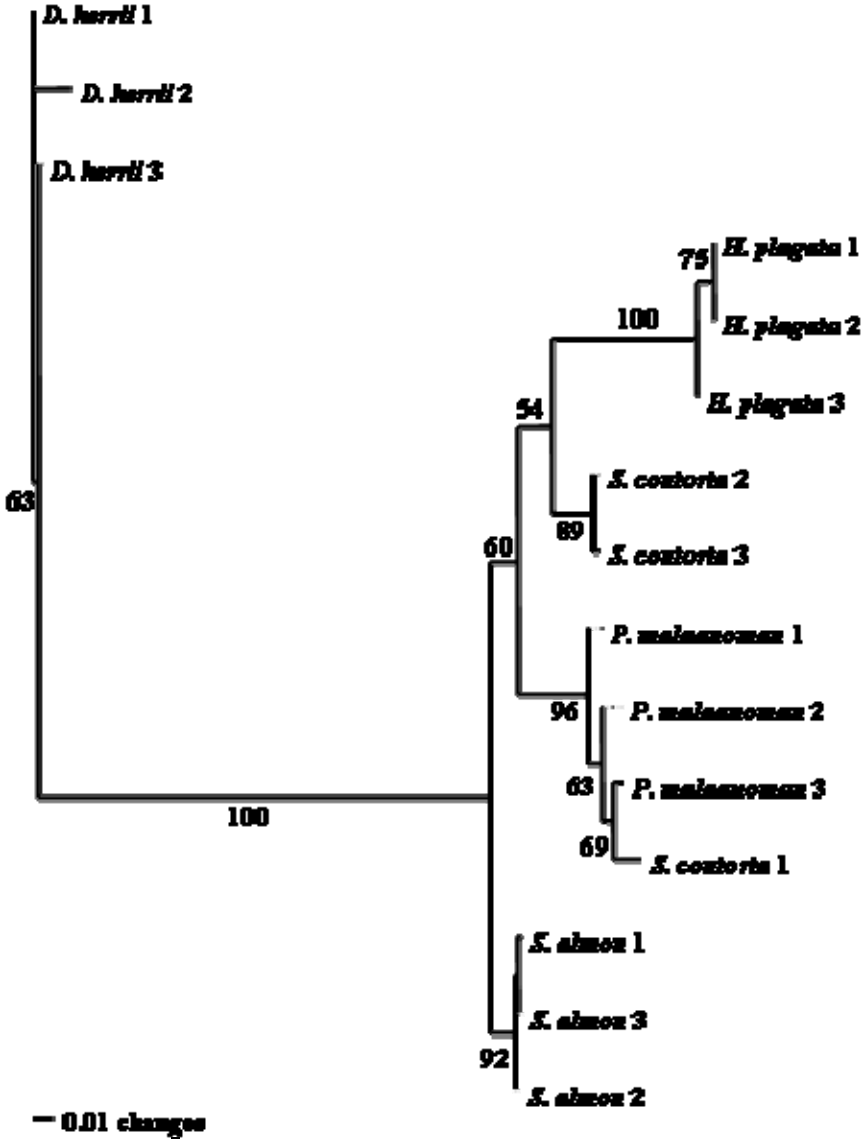

S. contorta
S. dimon
P. malaanoman
D. korrii
H. plagata
PC 1
1.00
0.75
0.50
0.25
0.00
PC 3
-1.0
-0.5
0.0
0.5
PC 2
-1.0
-0.5
0.0
0.5
1.0

Fig. 4


D. hurrii 1
D. hurrii 2
D. hurrii 3
H. pinguis 1
H. pinguis 2
H. pinguis 3
S. contorta 2
S. contorta 3
P. malanomum 1
P. malanomum 2
P. malanomum 3
S. contorta 1
S. almon 1
S. almon 3
S. almon 2
75
100
54
89
63
60
96
63
69
100
92
0.01 changes

4. Comparison of lignin in five tropical plantation species of the Dipterocarpaceae by histochemistry and Fourier Transform Infrared Spectroscopy

4.1. Abstract

The goal of this study was to characterize lignification of five dipterocarps (*D. kerrii*, *H. plagata*, *P. malaanoman*, *S. almon* and *S. contorta*), which are reported to be important timber species. Mäule and Wiesner staining were used for histochemical analysis and differences in G/S ratios in different species were compared to the reported durability of these species. *H. plagata* contained the lowest lignin concentration per dry mass but the highest per density. Detailed FTIR spectroscopic studies of wood and isolated lignin of *D. kerrii* and *H. plagata* revealed differences compared to the other species. The wood spectra of *P. malaanoman* and the *Shorea* species were similar; the lignin spectra of these species were also similar. The wood spectra and the Klason lignin spectra, both showed lower intensities of peaks of G-lignin in *D. kerrii* and *H. plagata* than those of the other three species leading to lower G/S ratio than in *P. malaanoman* and *Shorea* species. PCA of Klason lignin spectra separated lignins of *D. kerrii* and *H. plagata* from those of the other three species. This indicated the presence of different lignin constituents in *D. kerrii* and *H. plagata* compared with the later three species. The higher lignin densities in *D. kerrii* and *H. plagata* provided higher durability. Besides *H. plagata* lignin had spectral similarity with beech lignin from the temperate region.

Key words: Dipterocarp, Dipterocarpaceae, FTIR spectroscopy, Klason lignin, guaiacyl lignin, syringyl lignin.

4.2. Introduction

Forests cover one-third of the land surface of the earth and account for approximately 80% of the plant and approximately 40% of the soil carbon (Watson et al. 2000). In many Asian forests, which are regarded as a centre of global biodiversity, more than 50% of all trees, including the majority of emergent and trees of the canopy, are diptorocarps (Dipterocarpaceae) (Whitemore 1984). Dipterocarps are not only a keystone resource of wood in tropical Southeast Asia, but they are among the most important tropical timbers for trading (e.g., trade names: Meranti, Balau for *Shorea* spp., Keruing for *Dipterocarpus* spp., Kapur for *Dryobalanops*, etc. Whitmore 1984). This is especially true for the Philippines where dipterocarps contribute 94% of the timber volume (Soerianegara and Lemmens 1994).

Different species are being used for different purposes because of differences in their durability (Gierlinger et al. 2004 a, b). For example *H. plagata* has been reported to be very durable under weather-exposed conditions, including contact with the ground in wet tropics (Lomibao 1973; Newman et al. 1996). *D. kerrii* is an excellent construction timber but requires protective treatments if used outdoors in the tropics (Newman et al. 1996). The durability of *P. malaanoman*, *S. almon* and *S. contorta* is moderate. *P. malaanoman* and *S. almon* can attain a service life of 2-4 years in exposed conditions, whereas that of *S. contorta* is only 1-2 years (Newman et al. 1996). The reasons for these differences are unknown. In general, durability is provided by lignin and other extractives incorporated in the heartwood.

Lignins are the major polyphenolic compounds in wood, constituting about 20-30% of the dry mass. They are aromatic polymers of phenylpropanoid units entangled together with cellulose, hemicelluloses and pectin forming a three-dimensional network of high chemical stability (Sjöstörm and Alen 1998; Fengel and Wegener 2003). The incorporation of lignins renders plant cell walls mechanically rigid, water repellent and chemically resistant, thereby determining tissue quality. Lignins constitute a major defence against pathogens, insects, predators, UV-light and are important for wound healing (Polle et al. 1997; Whetten and Sederoff 1995; Whetten et al. 1998). Trees have an especial need for both structural support and water transport because of their high dimensions. This is especially true for tropical tree species, among which the dipterocarps belong to medium to large sized species (Ashton 1982; Newman et al. 1996).

Studies on lignin in tropical trees are very rare. A few studies have reported the lignin content to be in the range of 29.4-40.5% (W/W) (Fengel et al. 1983; Pastore et al. 2004; Saka 2001). In *Trema orientalis*, a fast growing hardwood species in tropical countries, wood contains lignin of about 45-50% (Jahan and Mun 2007). In another study on 36 Brazilian Amazon forest wood species, the range of acid insoluble and acid soluble lignin content was reported to be 26-37% and 0.7-1.7% respectively (Santana and Okino 2007). Only a few studies have reported on the lignin concentration of the seeds (11-44%) and leaf litter (35-42%) of some dipterocarp species (Kitayama et al. 2004; Nakagawa and Nakashizuka 2004). However, information on wood lignin of dipterocarps is lacking.

Currently, no unambiguous method for the quantification of lignin is available (Anterola and Lewis 2002; Fengel and Wegener 2003). Hardwood lignins are composed mainly of guaiacylpropane (G) units and syringylpropane (S) units, but may contain additionally other phenolic constituents at low concentrations (Sjöstörm 1993). The lignin content and G/S ratio of cell walls in hardwoods differ among cell-wall layers in individual cells (Fergus and Goring 1970a, 1970b; Fujii et al. 1987; Lewis and Yamamoto 1990; Mellerowicz et al. 2001; Musha and Goring 1975). The quantification of lignin is difficult not only because of its varying monomeric composition but also because lignins are covalently linked with cell wall carbohydrates, proteins, phenolics or other compounds. These compounds may interfere with determination of lignins leading to over-or underestimation (Monties 1989). One of the most frequently used methods for lignin determination is the Klason method (Schwanninger and Hinterstoisser 2002; Seca et al. 1998). This method is based on the removal of cellulose and hemicelluloses by acid extraction. The remaining lignin fraction is determined gravimetrically. Lignin content and composition are known to vary between the major groups of higher plants and between species (Freudenberg 1959, Sarkanen and Hergert 1971).

Histochemical staining indicates the presence of lignin and their localization within plant tissues (Lewis and Yamamoto 1990). By application of different staining reagents, like Mäule reagents or phloroglucinol/HCl, it is possible to distinguish between hardwood and softwood. The hardwoods react with a purple-red and the softwoods with a brownish colour to the Mäule stain (Takabe et al. 1992). This reaction allows visualization of the distribution of S-lignin in tissue without the destruction of cell walls (Fukazawa 1992). Meshitsuka and Nakano (1977, 1978,

1979) elucidated the mechanism of the Mäule colour reaction and concluded that a 3-methoxy ortho quinone structure generated from syringyl structures is responsible for the purple-red colour. Iiyama and Pant (1988) examined the mechanism of the Mäule colour reaction and suggested that the syringyl units reacted with free phenolic hydroxyl groups produced by the ß-ether cleavage during permanganate oxidation. The Mäule stain has been applied to investigate the distribution of syringyl lignins in annual rings (Takabe et al. 1992).

The phloroglucinol/HCl reagent (Wiesner stain) has been used to detect G-lignin in plant tissues. Although frequently cited as specific for coniferyl aldehyde (Sarkanen and Ludwig 1971), recently it has been stated that the reagent reacts with all three *p*-hydroxycinnamyl aldehydes (M. Jourdes et al. unpublished manuscript cited by Pomar et al. 2002). In contrast to the Wiesner reaction, the Mäule reagent can be used specifically to differentiate between S moieties (red) H and G moieties (brown) (Iiyama and Pant 1988; Nakano and Meshitsuka 1992; Patten et al. 2005).

FTIR spectroscopy is a further method to characterize wood, lignin and lignin derivatives. Absorption bands which arise in the lignin spectra can be assigned to different structural groups (Fengel and Wegener 2003). However, careful assignments of these bands are a prerequisite for useful results (Hergert 1971). In ligno-cellulose assessments of the lignin concentration as well as information on lignin molecular composition and inter-unit linkages are possible employing FTIR spectroscopy (Faix 1988; Martínez et al. 1999). FTIR spectroscopy has been used to identify lignins of different origin, to estimate the lignin/hemicellulose and guaiacyl/syringyl ratio, to study changes in lignin in wood during fungal attack, etc. (Cotrim et al. 1999; Faix 1991; Pandey and Pitman 2003; Sjöström 1993).

In the present study we investigated lignins of five important dipterocarp timber species, *D. kerrii*, *H. plagata*, *P. malaanoman*, *S. almon* and *S. contorta*. Wood was obtained from a plantation established for research (Leyte, Philippines). To characterise lignification, cross sections of the five species were stained with Wiesner and Mäule stains, respectively, to localize lignin in cell walls. The lignin content was quantified by the Klason method and the resulting fractions were characterised by FTIR spectroscopy and compared with their respective wood spectra. To find out whether the lignins differ in their composition, the FTIR spectra were subjected to principal component analysis and the major chemical constituents of the factor loadings contributing to spectral separation were identified.

4.3. Materials and methods

4.3.1. Field site and plant materials

Dipterocarp samples were collected in a plantation on the island Leyte (Philippines, 9° 55′ N and 11°48′ N and 124° 17′ E to 125° 18′ E, Langenberger, 2006). An average annual precipitation of 2586 mm has been reported (Local Climate Data, weather station of the Philippines Atmospheric, Geophysical and Astronomical Service Administration (PAGASA, 7 m a. s. l., Leyte State University, Langenberger, 2003). For detailed description of the field site, see chapter 3.

Stem samples of five dipterocarp species (*Dipterocarpus kerrii* King Damar, *Hopea plagata* (Blanco) S. Vidal, *Parashorea malaanoman* (Blanco) Merr, *Shorea almon* Foxw and *Shorea contorta* Vidal) were collected after felling of six-year-old trees grown in a plantation at the western foothills of Mt. Pangasugan, within the forest reserve of the Leyte State University. Only healthy trees with no apparent injury were used. Two stems disks were excised from each tree at 1.3 m above ground. One was used for FTIR-ATR analysis and Klason lignin determination and the other was used for histochemical analysis. For the latter purpose, disks were preserved in 70% ethanol in wide-mounted jars with lids. For further details, see chapter 3. For each species five individual trees were used for sampling.

Since the samples were very hard to section, especially those of *H. plagata*, small parts were taken from ethanol preserved disks and softened as described by (Wagenführ 1966). The disks were boiled for about 90 min in 30% glycerine. *Hopea plagata* wood was boiled for 150 min. After boiling the samples were kept in a glycerine/ethanol/water solution (30% ethanol, 30% glycerine, filled up to a final volume of 100 ml with distilled water) for more than 30 min before sectioning. For anatomical studies 30 µm thick wood cross sections of heartwood part were cut with a sledge microtome (Reichert-Jung, Heidelberg, Germany).

4.3.2. Lignin histochemistry

Sections were directly mounted in phloroglucinol/HCl solution (5.25g phloroglucinol (1, 3, 5-trihydroxybenzol) dissolved in 350 ml 95% ethanol and 175

ml concentrated HCl (25%) (adopted from Wiesner 1878 as modified by Eschrich 1976). Acidic phloroglucinol gives a red-pink product with cinnamyl aldehyde groups present in lignins (Vallet et al. 1996). Sections were treated with the Mäule reagent (Mäule 1901) (2% w/v potassium permanganate, 5% HCl and 1% NH_3) for the detection of the syringyl moieties (di-methoxylated residues) in lignin (Meshitsuka and Nakano, 1978, 1979). Stained sections were mounted in 60% glycerol for microscopy. Well stained sections and a micrometer scale were photographed under a light microscope (Axioplan, Zeiss, Oberkochen, Germany) with a digital camera (Nikon CoolPix 990, Nikon, Tokyo, Japan). Two replicates from each species were used for histochemical analysis. As the staining faded quickly, images were recorded within 20 minutes of reagent application. We used the term "fibre tracheid" instead of fibre according to the recommendation out-lined in Carlquist (1988).

4.3.3. Wood density determination

For wood density determination, see Chapter 3.

4.3.4. Sample preparation for FTIR spectroscopy and determination of Klason lignin

For The wood and lignin samples were milled to a fine powder in a ball mill as outlined in chapter 3.

4.3.5. Quantitative lignin determination by Klason method

The determination of acid-insoluble (Klason) lignin was adapted from Dence (1992). Dry ground plant powder material (400 mg) (W1) was suspended in 40 ml washing buffer (0.5M K_2PO_4/KH_2PO_4, pH 7.8, 0.5% Triton). The mixture was shaken slowly for 30 min at room temperature and centrifuged for 10 min (5000 g, 4 °C, Rotanta, 96R, Hettrich, Tittlingen). The precipitate was resuspended in 100 % methanol and the procedure was repeated three times (30 minutes for each step). The structural biomass was immersed by in 40 ml of 96% ethanol/cyclohexan (1/2, V/V) followed by incubation in a water bath for 6 h at 50 °C (Rettberg, Göttingen). During incubation, the samples were shaken every 30 min. The mixture was centrifuged (4500g, 10 min, 4 °C) and then washed again with 40 ml ethanol/ cyclohexan as

above. The pellet was washed with 20 ml 100% acetone and centrifuged. This washing step was repeated three times.

The precipitate was dried overnight under the fume hood, weighed, mixed with 8 ml 72% H_2SO_4 and incubated for 60 min at room temperature. The mixture was suspended in 200 ml distilled H_2O, autoclaved (1 h, 121 °C, 1 bar overpressure), cooled down to room temperature, filtered with a pre-weighed (W2) filter (Filter paper 1, Springfield Mill, Kent, England) and washed with distilled water. The filter containing the lignin was dried in an oven (24-48h, 70-80°C) and stored in a desiccator (60 min). Finally, the filter containing the lignin was weighted (W3) and Klason lignin was calculated as (W3-W2) x 100/W1.

4.3.6. FTIR-ATR spectroscopy of wood and Klason lignin and multivariate data analysis

FTIR-ATR spectra were recorded of milled wood powder and Klason lignin with the FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany) combined with an ATR unit (DuraSamplIR, SensIR Europe, Warrington, England) at a resolution of 4 cm^{-1} for 32 scans in the range from 600 to 4000 cm^{-1}. The samples were pressed against the diamond crystal of the ATR device. A pressure applicator with a torque knob ensured that the pressure applied was the same for all measurements. A background spectrum of the clear window was recorded prior to acquisition of sample spectra. The spectrum of the background was subtracted from the spectrum of the sample before conversion into absorbance units. For wood sample five different sub-samples and for each lignin sample three different sub-samples were analysed and averaged to give a mean spectrum per sample. For each species, wood and lignin samples from five individual trees were analysed.

Spectral data were evaluated using OPUS version 6.5 software (Bruker, Germany) in the fingerprint region of 1800-600 cm^{-1}. FTIR spectra of Klason lignin obtained for all tested trees were used for principal component analysis (PCA). The first derivation of vector normalized spectra was used in the range of 1800-1200 cm^{-1} for PCA analysis. The highest seven peaks in the first, second and the third factor loadings were assigned (Table 3). In fig. 2B, the baseline corrected Klason lignin spectra for each species (mean) were normalized by multiplying with corresponding mean lignin concentrations (Table 1). Peak heights were determined by using the peak

integration method (P). The baseline correction was done by the rubber-band method using 64 baseline points. All of theses functions were performed by using OPUS version 6.5 (Bruker Optics, Ettlingen, Germany).

4.3.7. Statistical analysis

Statistical tests were performed in SAS (9.13 version, SAS Institute Inc. 2004, Cary, NC, USA) using analysis of variance (ANOVA), followed by Duncan's multiple range test. Data were indicated as means ± SD. Differences between parameter means were considered significant when the P – value of the ANOVA Duncan's multiple range test was less than 0.05. Significant differences were marked by different letters.

4.4. Results

4.4.1. Lignin localization

In wood sections treated with Mäule reagent, cell walls containing only syringyl units or both guaiacyl and syringyl units turn predominantly reddish purple, whereas cell walls containing guaiacyl units remain yellowish or brownish in colour (Watanabe et al. 1997; Watanabe et al. 2004). In *D. kerrii* (Fig. 1A) and *H. plagata* (Fig. 1C), the Mäule reaction revealed dark reddish orange and vivid purplish red colours, respectively. *P. malaanoman* (Fig. 1E) and the two *Shorea* species, *S. almon* and *S. contorta* (Figs. 1G, 1I) expressed strongly red colour and the staining intensity in the latter three species was almost similar. Staining revealed the presence of more and less dense wood "zones" in all species except *D. kerrii* (Fig. 1). These zones were caused by thick and thin-walled fibre tracheids, which were less prominent in *H. plagata* (Fig. 1C) than the other three species (Figs. 1E, G, I).

Coniferyl alcohol is usually also an abundant compound in angiosperm wood. During lignification its precursor, coniferyl aldehyde, is incorporated into lignin in small amounts. Coniferyl aldehydes produce a strong red colouration with phloroglucinol/HCl (Dence 1992). Both *D. kerrii* (Fig. 1B) and *H. plagata* (Fig. 1D) expressed a moderate purplish - pink colour with phloroglucinol/HCl staining. The intensity of pink colour varied between bright and faded colours in the walls of fibre

tracheids of *H. plagata* (Fig. 1D). In *D. kerrii* sections were uniformly stained purplish with G-lignin (Fig. 1B). The most intense colouration with the Wiesner stain was found in *P. malaanoman* (Fig. 1F). Zonal variations in the staining intensities were observed in *P. malaanoman* (Fig. IF), *S. almon* (Fig. IH) and *S. contorta* (Fig. 1J) because of variations in thick-and thin-walled fibre tracheids. Among the five species the colour intensity of the Wiesner stain was the lowest in *S. contorta* (Fig. 1J). In *H. plagata* (Fig. 1D) and *P. malaanoman* (Fig. 1F) vessel walls stained much brighter than the surrounding area, probably indicating higher intensity of G-lignin in the vessel walls.

4.4.2. Lignin content

The differences in lignin staining may point to differences in lignin content. The Klason lignin concentration of *H. plagata* was significantly lower than that of the other four species (Table 1). No significant differences in lignin concentrations were found among *D. kerrii*, *P. malaanoman*, *S. almon* and *S. contorta* (Table 1).

These results were surprising at the first glance since the intense staining of the thick cell walls suggested the presence of higher lignin contents in *D. kerrii* and *H. plagata* than in the other three species. However, it must be considered that these five species differ in their wood densities, which is also evident from fig. 1. The cell walls in *D. kerrii* and *H. plagata* wood were very thick, almost without any lumen (Fig.1 A-D), whereas those of *P. malaanoman, S. almon* and *S. contorta* were much thinner (Fig. 1 E-J). This is supported by measured wood densities of these five species (*D. kerrii*: 700 to 910 kg m^{-3}, *H. plagata*: 800 to 1200 kg m^{-3}, *P. malaanoman:* 510 kg m^{-3} and *S. almon* and *S. contorta*: 450 to 460 kg m^{-3}, Newman et al. 1996). These data also correspond with the measured densities described in the previous chapter (Chapter 2, Table 2). Lignin content per volume was calculated for each of these species with the data from chapter 2, table 2. This analysis showed that lignin densities of *H. plagata* and *D. kerrii* were significantly higher than in the other three species (Table 1). Lignin densities of *P. malaanoman, S. almon* and *S. contorta* were about 2-times lower than that of *H. plagata* and did not show any significant difference among each other (Table 1).

4.4.3. The chemical fingerprint of wood and of lignin

Spectral analysis of dipterocarp wood showed some prominent peaks in the finger print regions of 1800-600 cm^{-1} (Fig. 2A). The peaks were numbered and assigned to chemical compounds, according to published literature (Table 2). Most of the observed peaks represent major cell wall components such as cellulose (1154, 898 cm^{-1}), hemicellulose (1738, 1022, 1057, 1090 cm^{-1}) and lignin (1596, 1505, 1270 cm^{-1}, Table 2). Although the wood spectra of the five different species were very similar, closer inspection revealed some differences in *D. kerrii* and in *H. plagata* (Fig. 2A) with the other three species. The spectra of *P. malaanoman*, *S. almon* and *S. contorta* appeared very similar (Fig. 2A). Most differences were observed in *H. plagata* compared to the other species. Peak 3 and peak 21 were present only in *H. plagata*. Guaiacyl peaks (peak 10) in *D. kerrii* and *H. plagata* were not as prominent as in the other three species. In *H. plagata* a shift of the syringyl peak (peak 9) was observed. The guaiacyl-to-syringyl (peak 10-to-peak 9) ratio was determined from the wood spectra (Table 1). The result corresponded to the lignin content determined by Klason method. In both cases *H. plagata* had the lowest value (Table 1).

To characterize the dipterocarp lignins further, FTIR spectra of Klason lignin were analysed (Fig. 2B). The lignin spectra of the five dipterocarps showed generally similar patterns. However, prominent differences in *H. plagata* and some minor differences in *D. kerrii* were observable (Fig 2B). The major difference observed in *H. plagata* was the height of peak 15 which was almost similar to peak 17. In *D. kerrii* and in *H. plagata* the intensity of peak 10 was lower than in the other three species (Fig. 2B). Attempts were made to determine the guaiacyl and syringyl ratio in the lignin spectra by considering the same peaks as used in the wood spectra. Due to the shift of the guaiacyl and the syringyl peaks in the lignin spectra, the G/S ratio was calculated by considering the peaks at 1265 and 1311 cm^{-1} respectively (Table 1). The correlation between these G/S ratios and those extracted from the wood spectra was $P = 0.029$. The G/S ratios corroborate the histochemical analysis by showing that *D. kerrii* and *H. plagata* lignins are composed of relatively less G- than S-moieties (Table 1).

4.4.4. Comparison between chemical fingerprint of wood and lignin spectra

Detailed comparisons between the wood spectra and the lignin spectra were made (Fig. 2 A, B). In the lignin spectra the absence of wood peaks 3 and 19, which arise due to C=O stretch and C-H deformation of cellulose respectively, is an indication of carbohydrate removal during lignin isolation (Fig. 2B, Table 2). The wood band 3, 21 characteristic of *H. plagata*, disappeared in the lignin spectrum of this species (Fig. 2B). The bands at positions 1596 and 1505 cm^{-1} in wood spectra (Fig. 2A) roughly corresponded to bands 1600 and 1496 cm^{-1} in the lignin spectra (Fig 2B). Peaks 6 and 7 of wood spectra were not displaced in the lignin spectra but peak 6 was the highest among peaks 4, 5, 6 and 7 (Table 2, Fig. 2). The 9^{th} peak at 1320-1330 cm^{-1} in wood spectra arising due to CO stretch and syringyl ring breathing (Table 2) was probably shifted in the lignin spectrum to position 1311 cm^{-1} at slightly lower wavenumbers (Fig. 2A, B). Similarly the G-lignin peaks (peak 10) of wood spectra (1270 cm^{-1}) were shifted to 1265 cm^{-1} in the lignin spectra. Both in wood (1270 cm^{-1}) and lignin (1265 cm^{-1}) spectra, the guaiacyl peaks at band 9, showed lower intensity in *D. kerrii* and *H. plagata* than the other three species (Fig. 2 A, B). The region from 1200-900 cm^{-1} has been considered as the polysaccharide region (Faix et al. 1991; Naumann et al. 1991). In this region 13, 14 and 17 peaks were present in both spectra, whereas 15, 16 and 18 peaks were visible only in the lignin spectra (Fig. 2 A, B).

Although the lignin spectra of the different species were generally similar, various differences were found, especially in the region between 900 and 1400cm^{-1} (Fig.2B). To find out if these differences were sufficient to distinguish the species by their lignin spectra, PCA was conducted. Figure 3 shows a PCA map calculated from a data set of 25 Klason lignin spectra collected from five different dipterocarps grown at the same site. The plot has been constructed by three-dimensional projection of Eigenvectors (factor spectra) 1, 2 and 3. Each point in the map represents a spectrum; all three factorial coordinates (factor loadings) were used for data representation (Fig. 3). The best model with three principal components was obtained when using the first derivation of vector normalized spectra in the range of 1800-1200 cm^{-1} as input data (Fig. 3). PC1, PC2 and PC3 were mainly responsible for separation of three distinct groups of data points: *D. kerrii* and *H. plagata* and the mixed group of *P.*

malaanoman, *S. almon* and *S. contorta*. This indicates that the chemical composition of the lignin fractions of the later three species did not differ.

To find out the source of variation, factor loadings of the PCA were analysed. The first, second and the third factor had Eigenvalues of 4.983, 0.013, and, 0.002 respectively and explaining 99.71%, 0.26% and 0.05% of the variances, respectively (Fig. 4). The highest seven peaks were assigned in these three factor loadings (Table 3). Band assignment of the factor loadings showed aromatic ring vibrations in all three factor loadings in addition to several stretching compounds (Table 3). Hydroxyl compounds were present in all three factor loadings. 1338 and 1351 cm^{-1} bands have been reported to disappear due to deuteration of guaiacyl compounds because of their hydroxyl character in the lignin model compounds (Hergert 1971).

4.5. Discussion

With FTIR much of the early works on lignin were concentrated on structure elucidation (Dence 1992; Hergert 1971; Fengel and Wegener 2003), but the recent works focus on the rapid quantitative analyses (Faix 1986; Schultz and Glasser 1986; Rodrigues et al. 1998). In the lignin spectra the height of peak 17 was always higher than that of peak 15 (Fig. 2B), except *H. plagata* where the reverse was true. Surprisingly, in the Klason lignin spectra of beech (*Fagus sylvatica*) samples (Müller G., personal communication, unpublished result), the similar characteristics peak height pattern that of *H. plagata* exists, and the G-lignin peaks were found to be very indistinct. In the lignin spectra of five dipterocarps, G-lignin peaks were less prominent in *D. kerrii* and *H. plagata* (Fig. 2B). The G/S ratios in lignin (Fig. 2B) of the above mentioned two species were significantly different from *Parashorea* and the two *Shorea* species whereas *H. plagata* had significantly different G/S ratio than others in wood (Fig. 2A), (Table 1). So it can be assumed that *H. plagata* and *D. kerrii* lignin are characterized by low G-lignin content which is probably similar to temperate region hardwood species lignin especially beech.

Shift of peaks 4, 5 and 20 were observed in the lignin spectra. In wood spectra their corresponding positions was 1596, 1505 and 834 cm^{-1} respectively whereas in lignin spectra they shifted to 1600, 1496 and 845 cm^{-1} respectively (Fig. 2A, B). Peak 4 and 5 arise due to aromatic vibration in lignins and peak 20 arises due to C-H out of

plane in G-units (Table 2). The same trend was observed by Müller et al. (2006) in the Klason lignin samples of grand fir (*Abies grandis*). This shift can be caused by inductive effects of the substituents (e. g. H_3CO) in the aromatic ring system of lignin (Pastuasiak 2003). The low and high absorbance of peak 4 and 12 respectively in lignin spectra is in accordance with Müller et al. (2006). The shift of syringyl peaks were observed in the lignin spectra at 1311 cm^{-1} (Fig. 2 A, B). In a study by Faix et al. (1994) the same trend was observed in *Tripochyton scleroxylon* lignin which is a diffuse porous tropical tree species. The shifting of peak 1 at a low absorbance in the lignin spectra is probably an indication that the dipterocarps contained low amount of phenolic hydroxyl groups (Faix 1987; Faix et al. 1992; Wegener and Strobel 1991) (Table 2, Fig. 2).

The lignin content of the dipterocarps is found to be within the range of tropical hardwood species (Nuopponen et al. 2006) (Table 1). The lignin concentration was lowest in *H. plagata* but the high density of *H. plagata* wood contributed to the highest lignin content per volume in this species (Table 1). Durability in tropical hardwoods is affected by the combination of density, content and composition of lignin and extractives (Nuopponen et al. 2006; Onuorah 2000). Among the five species the compressive strength of *H. plagata*, *P. malaanoman* and the two *Shorea* species are reported to be (at 12% moisture content) about 700 kg/cm^2, 470 kg/cm^2 and 415 kg/cm^2 respectively (Newman et al. 1996). The compressive strength of *D. kerrii* is unknown. It has been stated that better bioresistance is provided by higher amount of G-units (Nuopponen et al. 2006) although exceptions are also mentioned. This seems to be partially true for *S. contorta*, the most faded phloroglucinol/HCl staining was observed in this species (Fig. 1J), although in terms of lignin density, lignin content and G/S ratio it had no significant difference with *P. malaanoman* and *S. almon* (Table 1). However it does not seem to be true with the rest of the species. The very durable (*H. plagata*) and durable (*D. kerrii*) species had low intensity of G-lignin peaks in comparison with the other three species in both wood (Fig. 2A) and lignin (Fig. 2B) spectra and the staining intensity (phloroglucinol/HCl) was also found to be less prominent especially with *P. malaanoman* (Fig. 1B, D, F).

Nuopponen et al. (2006) concluded that the higher amount of G-lignin content is correlated with a higher amount of lignin content which is in accordance with the dipterocarp lignins (Table 1). The distinct Klason lignin spectral pattern of *H. plagata*

and *D. kerrii* is a clear indication of the presence of additional components (Fig 2A). In a number of studies multivariate analyses were used to separate lignin from different sources (Cotrim et al. 1999; Müller et al. 2006). PCA could clearly separate *D. kerrii* and *H. plagata* on the basis of their distinct components (Fig. 3). The remaining three species were mixed up revealing similar compounds in the lignin which was also expressed during the staining reactions (Figs. 1E-J, 3). PCA result of wood spectra (Chapter 2, Fig. 3) and Klason lignin spectra were similar indicating the wood and lignin compounds of *H. plagata* and *D. kerrii* were different than that of the remaining species. Cotrim et al. (1999) identified a specific region (1950-800 cm^{-1}) for getting the best result in PCA for the separation of lignin obtained from different treatments. Similarly we had to select a specific region of 1800-1200 cm^{-1} only to obtain the best result (Fig. 3).

Fig. 4 refers to the peaks which contributed to major variations among the species. The presence of aromatic skeletal vibration peaks in all of the factor loadings is an indication of lignin compounds and surprisingly they all contributed to very high intensity peaks indicating large variations. The peak around 1500 cm^{-1} has been assigned to lignin peak in a similar study by Nuopponen et al. (2006).

Higuchi (1990) has reported that the characteristics of the lignin macromolecule can prevent the hydrolysis of cellulose “in situ” by various organisms. However such characteristics of dipterocarps have not been reported so far. According to several authors (Zimmermann and Brown 1971; Zimmermann 1982), with increase in vessel size there is increased danger of pathogen attack. In our samples *H. plagata* had lowest average vessel diameter which was significantly different from the others (see chapter 5, Table 1).

It can be assumed that the highest compressive strength and extreme durability of *H. plagata* is due to their high lignin density and small vessel size which probably resists against degradation. *D. kerrii* is in a durability category having significantly lower lignin density than *H. plagata* but significantly higher lignin density than *P. malaanoman* and the two *Shorea* species (Table 1). This lignin density contributed *D. kerrii* to a very good structural timber. The utility of *D. kerrii* for various construction purposes have been reported in the literature (Newman et al. 1996; Lomibao 1973). It has been stated that the identification of minor differences by direct comparison of lignin spectra is quite difficult due to many overlapping bands (Cotrim et al. 1999;

Fengel and Wegener 2003). This is the first attempt to separate similar species depending on lignin compounds where no pre-treatment was involved.

4.6. References

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Table 1. Lignin concentrations, densities and G/S ratios in five tree species of Dipterocarpaceae. Lignin concentration was determined gravimetrically by Klason method. Density was obtained by multiplication with the corresponding wood densities. The G/S ratios are based on peak heights determined in FTIR spectre of wood and isolated Klason lignin, respectively. Data indicate means (± SD, n= 5). Different letters indicate significant differences at $P < 0.05$. The ratios are based on peak heights.

Species	Lignin (%)	Lignin densities (g m^{-3})	G/S in wood spectra 1270/(1320)1330	G/S in lignin spectra 1265/1311
Dipterocarpus kerrii	23.2 ± 1.49 b	163.72 ± 22.75 b	1.07 ± 0.02 b	0.97 ± 0.03 b
Hopea plagata	19.75 ± 1.00 a	192.06 ± 10.24 c	0.99 ± 0.08 a	0.93 ± 0.03 a
Parashorea malaanoman	23.27 ± 1.12 b	105.57 ± 10.44 a	1.11 ± 0.03 b	1.02 ± 0.02 c
Shorea almon	23.10 ± 1.08 b	88.41 ± 10.16 a	1.12 ± 0.03 b	1.06 ± 0.02 c
Shorea contorta	24.22 ± 0.94 b	97.39 ± 6.46 a	1.09 ± 0.03 b	1.04 ± 0.03 c

Number in parenthesis refers to peak position in *H. plagata*.

Table 2: Band assignments in the mid-infrared region of five dipterocarp species wood powder and Klason lignin. The values after slash indicate the shifted peak positions in lignin. Value in parenthesis indicates the peak position in *H. plagata* wood spectra.

Peak number		Wave number (cm^{-1})	Band origin	Short comments	Reference
Wood	Lignin				
1	1	1738/1713	C=O stretch in unconjugated ketones, carbonyls and in ester groups (frequently of carbohydrate origin)	conjugated aldehydes and carboxylic acids absorb around and below 1700 cm^{-1}	Faix (1991)
2	2	1663	C=O stretch in conjugated p-subst. aryl ketones		Faix (1991)
3	absent	1626	stretching vibration of C=O	nonconjugated C=C, appeared only in *H. plagata* in wood samples	Parker (1983)
4	4	1596/1600	aromatic skeletal vibration plus C=O stretch		Faix (1991), Faix et al. (1991)
5	5	1505/1496	aromatic skeletal vibrations; G>S		Faix (1991), Faix et al. (1991)
6	6	1462	C-H deformation; asymmetric in $–CH_3$ and $–CH_2$-		Hergert (1971), Faix (1991)
7	7	1421	in plane bending in C-H group	$C=CH_2$	Parker (1983)
8	8	1370	C-H deformations (symmetric)	in lignin spectrum it is present as a very weak shoulder	Hergert (1971), Fengel and Wegener (2003)
9	9	(1320)1330/1311	syringyl ring breathing with CO stretching	only in *H. plagata* the 1330 cm^{-1} peak was found to be shifted towards 1320 $cm^{-1.}$	Hergert (1971), Fengel and Wegener (2003)
10	10	1270/1265	guaiacyl ring breathing		Fengel and Wegener (2003)

11	11	1230	C-C plus C-O plus C=O stretch	in lignin spectrum it is present as a very weak shoulder	Faix (1991)
absent	12	1214	C-O in G ring		Müller et al. (2006)
13	13	1154	cellulose C-O-C		Collom and Carrilo (2005)
14	14	1107	C-O-C, C-O dominated by ring vibration of carbohydrates		Naumann et al. (1991), Faix et al. (1991)
absent	15	1090	Secondary hydroxyl groups		Hergert (1971)
absent	16	1057	primary hydroxyl groups		Hergert (1971)
17	17	1024	C-O-C, C-O dominated by ring vibration of carbohydrates		Naumann et al. (1991), Faix et al. (1991)
absent	18	912	same as peak no. 17		Naumann et al. (1991), Faix et al. (1991)
19	absent	898	C-H deformation in cellulose		Pandey and Pitman (2003)
20	20	834/845	C-H out-of-plane in position 2 and 6 of S and in all positions G units		Faix (1991)
21	absent	781	appeared only in *H. plagata* samples. No information available.		
22	22	665	no information available.		

Table 3: Band assignments of the second (PC1), third (PC2) and fourth (PC3) factor loadings obtained by principal component analysis. The seven highest peaks are indicated for each factor loading. The numbers in parenthesis indicates the position according to height.

Wave number (cm^{-1})			Band origin	References
1st factor	2nd factor	3rd factor		
1515(1)			Aromatic skeletal vibration	Hergert (1971), Fengel and Wegener (2003)
1444(2)			Stretching (sym) vibration of C-O, $-COO^-$, carboxylate	Parker (1983)
1470(3)			C-H deformations; asym. in $–CH^3$ and $–CH^2$-	Faix (1991), Fengel and Wegener (2003)
1580(4)			Interaction effects of C=N (plus C=C)	Parker (1983)
1411(5)			Stretching (sym) vibration of C-O, $-COO^-$, carboxylate	Parker (1983)
1488(6)			Stretching vibration of C=S	Parker (1983)
1338(7)			Hydroxyl compounds	Hergert (1971)
	1251(1)		C-O stretching (CH_3COOR, acetic ester (upward direction)	Parker (1983)
	1515(2)		Aromatic ring vibrations	Hergert (1971), Fengel and Wegener (2003)
	1282(3)		Stretching vibration of C-O	Parker (1983)
	1500(4)		Aromatic skeletal vibration	Faix (1991)
	1474(5)		Stretching vibration of C=S	Parker (1983)
	1450(6)		Stretching (sym) vibration of C-O, $-COO^-$, carboxylate	Parker (1983)
	1351(7)		Hydroxyl compounds	Hergert (1971)
		1500(1)	Aromatic skeletal vibration	Faix (1991)
		1474(2)	Stretching vibration of C=S	Parker (1983)
		1587(3)	Asymmetric deformation of NH_3^+	Parker (1983)
		1748(4)	Hydroxyl groups	Hergert (1971)
		1400(5)	C-O stretching (sym) of COO^-	Naumann et al. (1991)
		1732(6)	C=O stretch in unconjugated ketones, carbonyls and in ester groups (frequently of carbohydrate origin)	Faix (1991), Pandey and Pitman (2003)
		1244(7)	C=O and C-O vibrations of the acetyl groups in hardwood xylans	Harrington et al. (1964)

Figure Legends

Figure 1. Typical cross sections of the five dipterocarps, *D. kerrii* (A, B), *H. plagata* (C, D), *P. malaanoman* (E, F), *S. alomon* (G, H) and *S. contorta* (I, J) after Mäule (A, C, E, G, I) and phloroglucinol/HCl staining (B, D, F, H, J). The sections were 30 μm thick. The arrows in the figures indicate the zones where the fibre tracheids have very thick walls.

Figure 2. Mean FTIR spectra of wood (A) and lignin (B) of five different species of dipterocarps from the same site in the wavenumber range from 1800-600 cm^{-1}. Each spectrum is a mean of spectra from 5 individual trees sampled from *D. kerrii*, *H. plagata*, *P. malaanoman*, *S. almon*, *S. contorta*, respectively. The different numbers refer to peaks described in Table 2.

Figure 3. PCA map of Klason lignin from a data set of 25 IR spectra (first derivative of vector normalized data in the spectral range 1800-1200 cm^{-1}) obtained from five different dipterocarp species grown on the same site. For projection of data, the factorial coordinates (factor loadings) PC1, PC2 and PC3 were used.

Figure 4. Factor loadings of the first derivative of normalized Klason lignin spectra (1800-1200 cm^{-1}) of five different dipterocarp species: (A) first, (B) second and (C) third factor loadings respectively. The different numbers in A, B and C refer to peaks described in Table 3. The first seven highest peaks were assigned in A, B and C.

Fig. 1

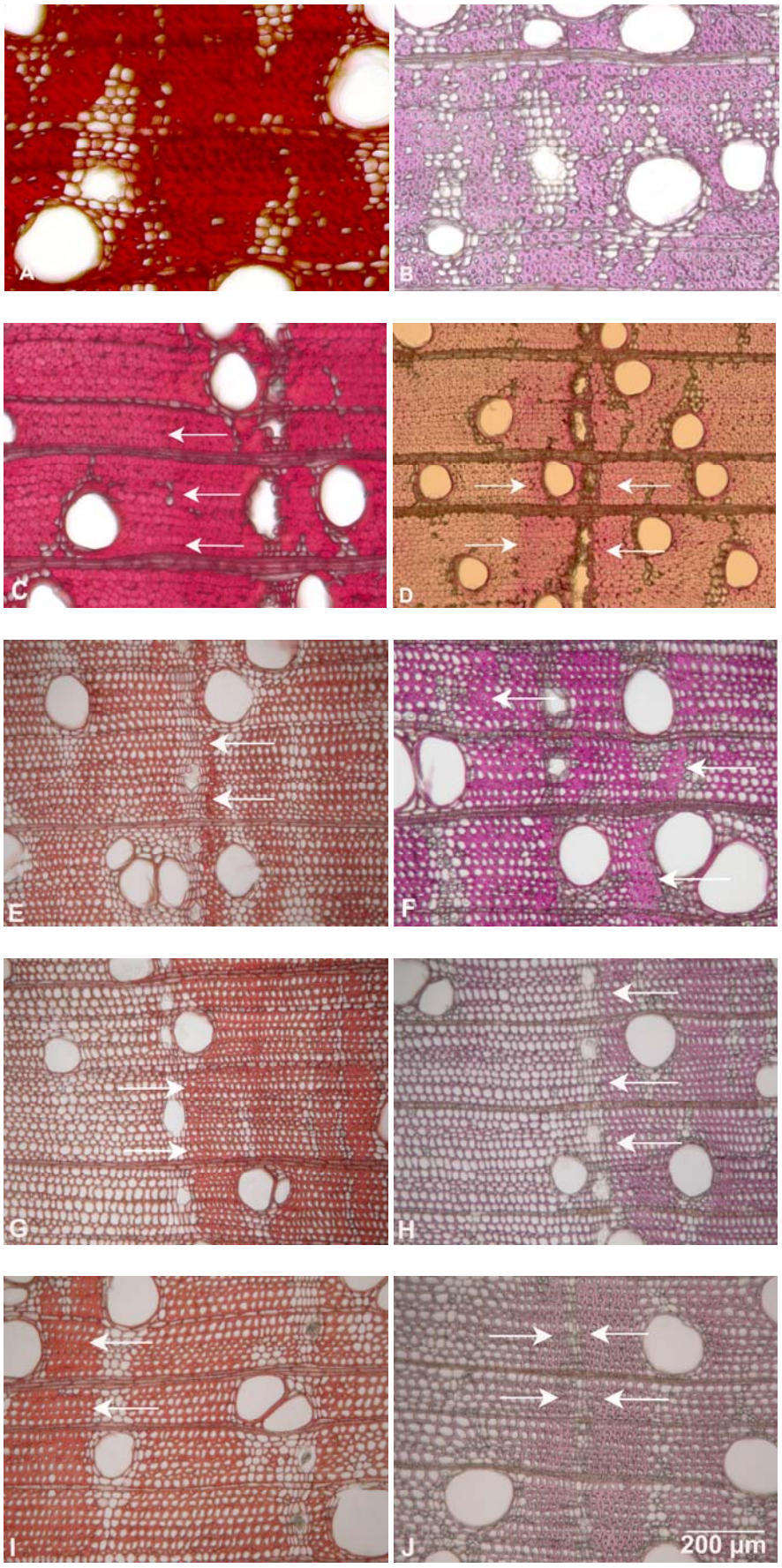


Fig. 2

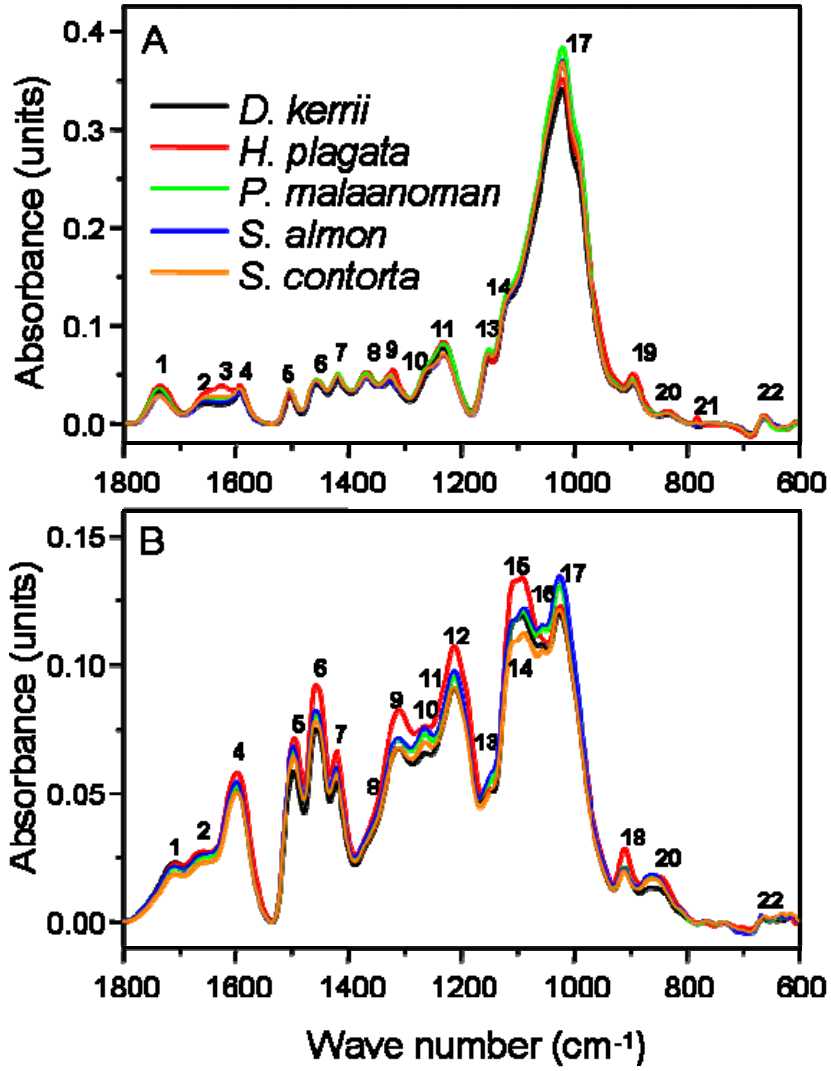

A
D. kerrii
H. plagata
P. malaanoman
S. almon
S. contorta
Absorbance (units)
0.4
0.3
0.2
0.1
0.0
1800
1600
1400
1200
1000
800
600
B
0.15
0.10
0.05
0.00
Absorbance (units)
Wave number (cm-1)

Fig. 3

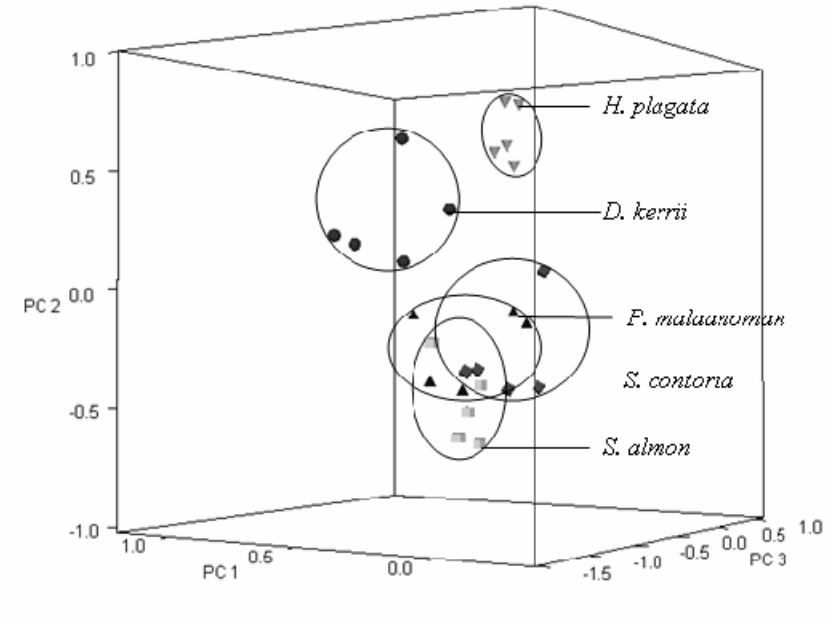
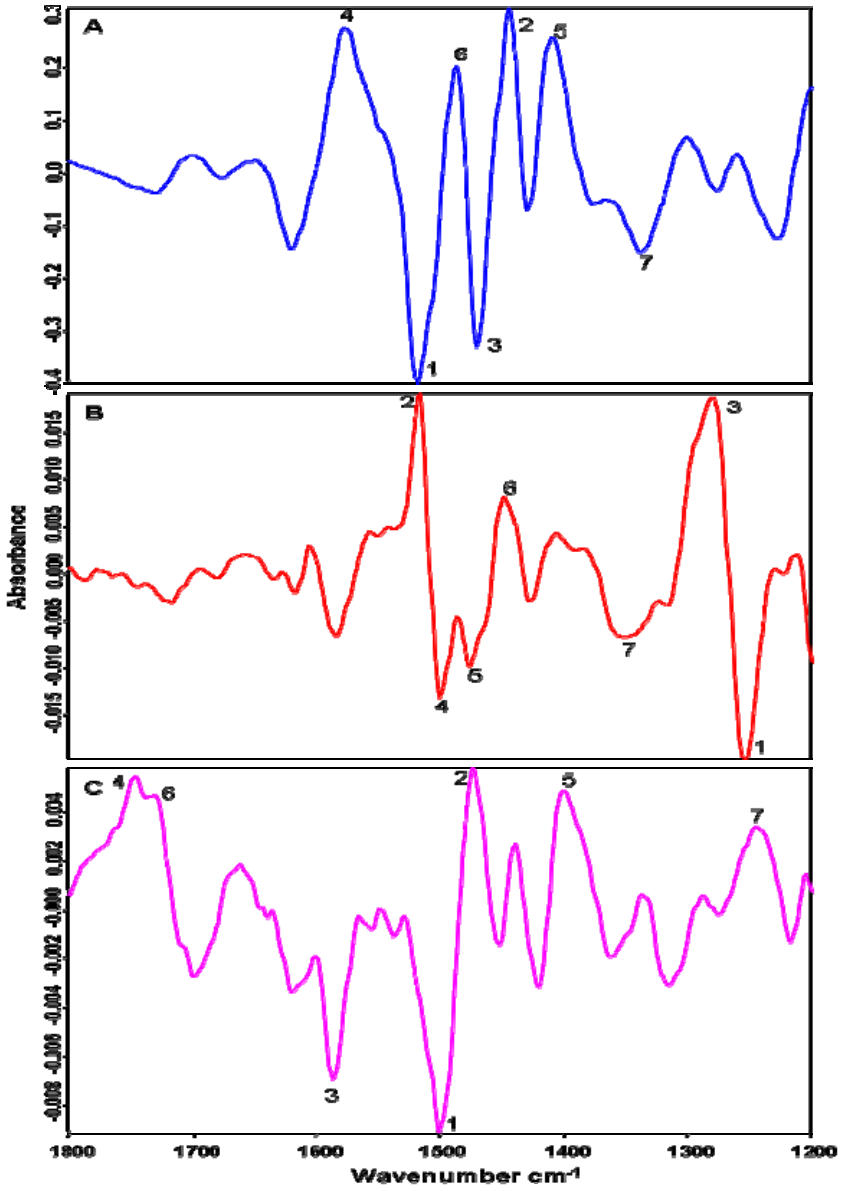

H. plagata
D. kerrii
P. malaanorum
S. contoria
S. almon
PC 1
PC 2
PC 3
1.0
0.5
0.0
-0.5
-1.0
-1.5

Fig. 4


A
B
C
Absorbance
Wavenumber cm-1
1800
1700
1600
1500
1400
1300
1200

5. Anatomical and biochemical traits to differentiate five dipterocarp tree species

5.1. Abstract

Species differentiation is a problem in the species-rich tropical forests. Many important commercial timber species are exported under common trade names. This is particularly true for dipterocarps which play an important role in the economy of many Southeast Asian countries. To address the issue of species differentiation some anatomical and biochemical traits were quantified. Five dipterocarp species (*Dipterocarpus kerrii*, *Hopea plagata*, *Parashorea malaanoman*, *Shorea almon* and *Shorea contorta*) grown on a plantation were used for this study. The anatomical and biochemical traits revealed close relationship among *P. malaanoman* and the two *Shorea* species. They had similar characteristics in many instances whereas *D. kerrii* and *H. plagata* differed in most of the instances. Two multivariate methods (cluster analysis and principal component analysis (PCA) were used for species separation based on anatomical and biochemical traits. Cluster analysis grouped all individuals correctly according to species, with one exception (one sample of *P. malaanoman* was found in the cluster of *S. almon*). PCA separated fewer distinct groups with *D. kerrii* and *H. plagata* only. The remaining three species were mixed up. The results suggest that the anatomical and biochemical traits have got the potential to be used in species differentiation purposes for distantly but not for closely related species.

Key words: Dipterocarps, anatomical traits, biochemical traits, multivariate analyses, cluster analysis, principal component analysis.

5.2. Introduction

The dominance of dipterocarp species in the Southeast Asian forests has been described in detail in the previous chapters. They are well known timber species for economic purposes. Sufficient knowledge of the anatomy of wood allows for better understanding of wood properties and solving specific problems related to wood identification or wood quality assessment (Ella and Meniado 1992). Several authors have described the anatomy of dipterocarps in detail (Ashton 1982; FAO 1985; Gottwald and Parameswaran 1966; Ella and Meniado 1992; Lomibao 1973; Newman et al. 1996). According to Gottwald and Parameswaran (1966) the great diversity of wood anatomy of the family Dipterocarpaceae provides valuable taxonomic characters at subfamilial, tribal, generic, intrageneric and sometimes at the species level. Quantitative anatomical analyses for species differentiation are still rare. Also other traits have only seldom been studied. For example, Nakagawa and Nakashizuka (2004) reported on some biochemical properties of seeds of some dipterocarp species: the nitrogen content ranged from 2.6-17.9% in these seeds. However, information on biochemical properties of wood of dipterocarp species is lacking.

Species differentiation is a major problem in the species rich tropical forest. A large number of commercially important dipterocarp species are recognized only under a few trade names though they possess different characteristics (see chapter 1). Attempts were made to distinguish five species of dipterocarps (*D. kerrii*, *H. plagata*, *P. malaanoman*, *S. almon* and *S. contorta*) by using anatomical traits like vessel frequency, average vessel diameter, vessel element length, predicted conductivity, fibre tracheid length and wall thickness, fraction of different cell elements along with some biochemical properties. The carbon and nitrogen content and lignin/carbohydrate ratio was determined for biochemical characterizations. The chemical fingerprint of wood determined by FTIR (for details see chapter 3) was used for the lignin carbohydrate ratio determination. The peaks at 1505 and at 1738 cm^{-1} represent lignin and carbohydrates respectively (Faix 1991; Fengel and Wegener 2003).

Vessel dimensions are among the most important wood traits. The combined effects of vessel lumen area/vessel diameter and vessel density affect the wood density in angiosperms (Preston et al. 2006). Different combinations of vessel diameter and vessel frequency have been proposed in determining wood density.

Wood with larger vessels is mechanically less stable than wood with smaller ones. Large vessels contribute to high hydraulic conductivity but at the same time they are more susceptible to embolism and perhaps less capable to recover from embolism (Bass 1986; Bass et al. 2004; Ewers 1985; Jacobson et al. 2005; Niklas 1994; Tyree et al. 1994; Zimmermann, 1983). In contrast, wood that is composed of small vessels spaced widely within a fibre matrix is very dense with low hydraulic conductivity (Preston et al. 2006).

Fifty percent of water conductivity can be contributed by relatively few large vessels, because of the 4^{th} – power of diameter effect as $L_p = r^4\pi / 8\eta$ (Zimmermann 1983). These larger vessels can occupy only a small share of the stem cross- section, with limited effect on wood density (Westoby and Wright 2004). Doubling the vessel frequency doubles the lumen area and merely doubles the hydraulic efficiency, whereas doubling the vessel diameter increases lumen area 4-fold and increases hydraulic efficiency by 16-fold (Bass et al. 2004).

In the lower latitude species, mechanical resistance as well as high conductivity can be achieved by the combination of bigger vessel diameters and thick walled fibres (Alves and Angyalossy- Alfonso 2002; Tyree et al. 1994). Therefore vessel evolution is likely to affect fibre evolution and vice versa (Bass et al. 2004). In angiosperms fewer vessels are required to achieve high conductive efficiency than in the tracheid bearing conifers. The space between the vessels is often occupied by supporting fibres (Hacke et al. 2001).

In emergent tropical trees, thin-walled fibres are more typical for rapidly growing early successional species and thick-walled fibres are more common in climax species (Swaine and Whitemore 1988). The fibres in the wood matrix appear to contribute directly to biomechanical strength, cavitation resistance and offer support against implosion (Bass et al. 2004; Berry and Roderick 2005; Jacobsen et al. 2005). The avoidance of implosion is thought to be the original function of thickened and lignified cell walls as they evolved in the first vascular plants (Raven 1987).

Vessel diameters in angiosperms range from 0.025-0.3 mm, most commonly more than 0.05 mm (Bass et al. 2004). On a global scale, vessel diameters tend to increase with decreasing latitude, that is, in warmer regions (Bass et al. 2004). Woodcock et al. (2000) found very large vessel diameters (>200 μm) and only a few narrow vessels in trees from the Amazon forests where temperatures do not fall below 0°C.

Taller trees are usually characterized by large vessels especially on moist soils (Preston et al. 2006). Vessel lengths vary from a few millimetres to several meters long (Zimmerman 1983). The vessels of diffuse-porous trees and shrubs are much narrower and are usually well under one meter in length (Zimmermann 1983; Ewers & Fisher 1989; Hacke and Sauter 1995; Kolb & Sperry 1999).

The abundance and distribution of parenchyma varies considerably within angiosperms. Some schemes suggesting functional significance in variation of parenchyma distribution and abundance have been devised (Braun 1970). According to Bass et al. (2004), the parenchyma cells which are present in most stems, do not have a direct role in transport but function in storage of photosynthates, water and minerals. According to Alves and Angyalossy-Alfonso (2002), in tropical forests where the forest species have high photosynthetic rates, abundant parenchyma is a characteristic of these tropical species. The combination of abundant parenchyma with septate fibre tracheids gives the huge potential for storage and metabolism.

The main goal of this study was to test whether it is possible to separate the five dipterocarp species on the basis of quantitative anatomical and biochemical traits which would be useful for species differentiation purposes.

5.3. Materials and methods

5.3.1. Field site and sampling

For detailed description of the field site and sampling see chapter 3. The same stem disks that were used for lignin histochemical analyses were used for anatomical analysis. Wood softening was carried out in the same manner as described in chapter 4.

5.3.2. Wood anatomy

Wood cross sections (30 µm) were made with a sledge microtome (Reichert-Jung, Heidelberg, Germany). Sections were stained for 10 min with toluidine blue (pH 7.0 w/v=0.05%) and then washed with Naphosphatebuffer (0.1M) (Robinson et al. 1987). They were then mounted in 60% glycerol for microscopy. Well stained sections and a micrometer scale were photographed under a light microscope

(Axioplan, Zeiss, Oberkochen, Germany) with a digital camera (Nikon CoolPix 990, Nikon, Tokyo, Japan). It was not possible to detect the parenchyma cells in the *Parashorea malaanoman* and the *Shorea* species with toluidine blue. We applied the IKI reaction (Johansen 1940; Eschrich 1976) for detection of starch. Consideration was also given on thin walled cells for identification of parenchyma cells. Zinc chloriodide (Jensen 1962) staining was also done which stains the cellulosic cells violet-blue and cutinised/suberinised cells are yellow.

5.3.3. Maceration

To determine the lengths of vessel elements and fibres tracheids the same stem segments used for cross sections were chosen. About 1 mm width of wood next to the cambium was discarded to avoid young xylem cells and the residual wood was cut longitudinally into pieces for chemical maceration in 30% H_2O_2 (Merck, Darmstadt, Germany) / Acetic acid (Merck, Darmstadt, Germany) 1: 1 after Franklin (1945) in Jansen et al. (1998). After 5 days of maceration at 60°C it was possible to separate the different elements. The segments were then preserved in 70% ethanol for further analysis. Parts from those segments were stained with toluidine blue (pH 7.0 w/v=0.05%) and mounted in 60% glycerol for microscopy. The short lengths of vessel elements (the short distance between the perforations) were measured after the definition of Chalk and Chattaway (1934).

5.3.4. Lignin/ carbohydrate peak ratio (1505/1738)

Wood powder was analysed by FTIR/ATR spectroscopy and the heights of peak at 1505 and at 1738 cm^{-1} were determined by using the peak integration method (P) OPUS, version 6.5 (Bruker Optics, Ettlingen, Germany). For details see chapter 4.

5.3.5. Carbon and nitrogen determination

For carbon and nitrogen determination, wood powder was used. Approximately 800 µg of wood powder (three replicates) were weighted to Zn capsules (5x9 mm), which were transferred to an Element analyzer CNS (vario EL, Elementer, Hanau, Germany).

5.3.6. Analysis of wood anatomy

Microphotographs of wood were analysed with the image processing program ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA; http//rsb.info.nih.gov/ij/) for the following parameters: diameter of vessel lumina (excluding the cell wall), area of ray parenchyma cells (including the cell wall as it was not possible to differentiate it from the cell lumina), vessel lumina, fibre tracheid lumina, thickness of double cell fibre tracheid wall (the wall between two adjacent fibre tracheid cells. Except *D. kerrii*, in the other four species tangential bands of axial canals were found and they contributed to a small proportion of the cell elements (Chapter 3, Fig. 1). Therefore representative parts (excluding axial canals) of cross sections were taken for *H. plagata*, *P. malaanoman*, *S. almon* and *S. contorta* for the ImageJ programme. This was unavoidable in *D. kerrii* which had scattered axial canals and they contributed to a small fragment of the total cell elements.

Ten measurements were taken for each tree and means were calculated. The number of vessels per unit area was determined. The unit area was set in ImageJ as 3.7431 mm^2. Fibre tracheid lumen area was determined in 0.0148 mm^2 as it was not possible to determine it in 3.7431 mm^2. Afterwards for calculating the fraction of fibre trachied lumen area the second formula was used.

The following equation was used to calculate the relative fibre tracheid lumen:
FL (%) = [Cell wall area **{**cell wall of (vessel +fibre tracheid + parenchyma)} + fibre tracheid lumen area] = [{total area – area of (vessel lumina + lumina of parenchyma cells + area of ray parenchyma cells)}*100]/ total area
% Fibre tracheid lumen area = {% of fibre lumen area on an area of 0.0148 mm^2 * % of (Cell wall area + fibre tracheid lumen area)}/100

The wood anatomical terms are used in accordance with the IAWA Committee (1989). For definition of fibre tracheids, axial canals we follow the IAWA Committee (1989), Carlquist (1988), Fahn (1990).

K_h is the total predicted hydraulic conductance calculated as the sum of the fourth power radii of all vessels in a section ($\sum r^4$). Predicted conductivity was determined as the sum of individual conductivities of vessels per area of xylem ($\sum r^4$ mm^{-2}) (Zimmermann 1983). Relative contribution (%) of vessel diameter classes to the predicted conductivity was calculated as {($\sum r^4$) (diameter class) x 100}/ $\sum r^4$ (all vessels).

5.3.7. Statistical analysis

Statistical analysis was performed with SAS (9.13 version, SAS Institute Inc. 2004, Cary, NC, USA) using analysis of variance (ANOVA), followed by Duncan's multiple range test. Data were indicated as means ± SD. Differences between means were considered significant when the P – value of the ANOVA Duncan's multiple range test was less than 0.05. Different letters indicate significant difference. Cluster analysis was done by Ward's method using Euclidean square as distance metric with the programme Statgraphic Plus (Statistical Graphics Corporation, St. Louis, MO, USA). Several wood traits were used for this purpose. PCA (principal component analysis) was performed by using the same software.

5.4. Results

5.4.1. Differences in cell elements among the five species

The cross sections of the five dipterocarps revealed some differences at the first glance (Fig. 1). For example the fibre tracheids of *D. kerrii* (Fig. 1A) and *H. plagata* (Fig. 1B) were very thick, almost without any lumen. The vessel diameters of *H. plagata* were smaller than those of the other species and the vessel frequency was high (Fig. 1B). Besides the axial parenchyma surrounding the vessels in *H. plagata* was also found to be less than those of the other species (Fig. 1B). Wood of *P. malaanoman, S. almon* and *S. contorta* were similar in their cross sections (Fig. 1C-E). To find out whether significant differences exist among cell elements of these five species quantitative anatomical analysis was carried out.

The five species showed significant structural differences in their normal wood composition (Table 1, Fig. 2). The secondary xylem of *D. kerrii* had the largest portion of vessel lumina, which was significantly different from that of the remaining four species (Fig. 2A). The highest vessel frequency in *H. plagata* did not accumulate to the largest portion of vessel lumina in *H. plagata*. This species did not show any significant difference compared with *P. malaanoman*, *S. almon* and *S. contorta* (Table 1, Fig. 2B).

An important feature affecting wood density is the thickness of the fibre wall. We measured the thickness of the walls between two adjacent fibre tracheid cells,

denominated as “double fibre tracheid wall thickness”. Significant differences were observed among the five species regarding this parameter. The higher double fibre wall thickness values of *D. kerrii* and *H. plagata* contributed to a higher fraction of cell wall per area than in those of the remaining three species (Table 1, Fig. 2). Although the fibre tracheid wall thickness values of *D. kerrii* and *H. plagata* did not differ significantly, the fraction of cell wall areas in these two species were significantly different from the remaining three species. (Table 1, Fig. 2). Similar trends are observed in *P. malaanoman* and *S. almon* (Table 1). The fibre wall thickness values did not vary significantly in *P. malaanoman* and the two *Shorea* species but the fraction of cell wall area varied significantly in *P. malaanoman* and *S. almon* (Fig. 1C-E). The fibre tracheid lumen fraction in *D. kerrii* and *H. plagata* was significantly different from the other three species (Fig. 2) due to their very thick fibre tracheid walls (Table 1, Figs. 1, 2). *P. malaanoman*, *S. almon* and *S. contorta* had significantly higher fractions of fibre tracheid lumen than *D. kerrii* and *H. plagata*. The decreased fraction of cell wall area was caused by increased fraction of lumen of fibre tracheids in *P. malaanoman* and the two *Shorea* species and the reverse was true for *D. kerrii* and *H. plagata* (Table 1, Fig. 2). The values of ray parenchyma fraction formed three groups in the five species (Fig. 2). Axial parenchyma fraction of *H. plagata* was significantly lower than in the other species. No significant difference was observed among the remaining four species (Fig. 2).

Similarly anatomical traits like average vessel diameter, vessel element length and fibre tracheid length also varied among the five species (Table 1). The vessel element length and fibre tracheid length of *D. kerrii* was significantly higher than in the others (Table 1). Fibre tracheid length of *H. plagata* and *S. contorta* was not significantly different, on the other hand no significant difference was observed between in *P. malaanoman*, and *S. almon* (Table 1).

5.4.2. Distribution of vessel length classes among the five species

Vessels of all five species were classified into 13 classes of 50 µm steps starting at vessel length of 100 µm. Only about 2.2% of the vessels of *D. kerrii* were > 700 µm (longest). 17.6% belonged to the class of 501-550 µm long vessels (Fig. 3a). *D. kerrii* did not contain vessels shorter than < 201 µm (Fig. 3a). In *H. plagata* vessels larger than > 650 µm were absent (Fig. 3b). 22.4% of the vessels belonged to the class

of 351-400 µm long vessels (Fig 3b). In *P. malaanoman* long vessels (> 700-601 µm) were also absent, whereas the 351-400 µm vessel length class contributed to 27.2% to all vessels (Fig. 3c). The vessel element length distribution of *S. almon* was irregular. 26.4% of all the vessel elements of *S. almon* belonged to the class of 351-400 µm long vessels (Fig. 3d). In *S. contorta* 10.19% of the vessels belonged to the class of 401-450 µm long vessels and the 101-150 µm length class was absent (Fig. 3e).

5.4.3. Vessel diameter and the distribution of vessel diameter classes among the five species

The vessel diameters of five dipterocarps were classified into 15 classes of 10 µm steps starting at 60 µm diameter. There was considerable variation in the distribution of vessel diameter classes among the five species (Fig. 4). *D. kerrii* had the largest range of vessel diameter values. No significant difference was observed in the vessel diameter range between *D. kerrii* and *S. contorta* (Table 1). The vessel diameter values of *P. malaanoman* and *S. almon* were significantly different from *D. kerrii* and *H. plagata* (Table 1). *P. malaanoman* and *S. contorta* did not have significant difference in vessel diameter values between them (Table 1). The 150-159 µm diameter class of *D. kerrii* was responsible for 21% of predicted conductivity (PC) (Fig. 4a). *H. plagata* had a very short range of vessel diameter class distribution, only seven diameter classes were observed (Fig. 4b). It had the lowest range of diameter values which was significantly different from the others (Table 1). The largest vessel diameter in this species was found to be within 120-129 µm diameter class (Fig. 4b). The highest PC (29%) was found to be represented by 100-109 µm diameter class. The PC of 90-99 µm diameter class and 110-119 µm diameter classes were almost the same (Fig. 4b). In *P. malaanoman* the 200-209 µm vessel diameter class was absent (Fig. 4c). The highest PC was observed in 130-139 µm diameter class. The PC of 140-149 µm and 160-169 µm diameter classes were almost similar (Fig. 4c). *S. almon* lacks the 160-169 µm to 180-189 µm diameter classes (Fig. 4d). The highest PC (27%) was observed in 120-129 µm diameter class (Fig. 4d). In *S. contorta* the 170-179 diameter class contributed to almost 23% of the predicted conductivity (Fig. 4e).

5.4.4. Biochemical analysis

For biochemical analysis carbon and nitrogen content were determined in the five species (Table 2). In addition the lignin to carbohydrate ratio was determined from FTIR spectra of the wood powder of five dipterocarps (Table 2). The carbon content of *H. plagata* was significantly different than that of the other four species. In term of nitrogen content no significant difference was observed among the five species. However when the nitrogen/carbon ratio was determined a significant difference was observed between *D. kerrii* and *H. plagata* (Table 2). The lignin/carbohydrate ratio revealed three different groups among the five species. In *H. plagata* the lignin/carbohydrate was significantly lower than that of the others.

5.4.5. Multivariate analyses

To find out whether the five dipterocarps could be separated on the basis of measured quantitative anatomical and biochemical traits two multivariate methods namely cluster analysis and principal component analysis (PCA) were performed. The cluster analysis obtained with five variables (average vessel diameter, lignin/carbohydrate ratio, predicted conductivity, fibre tracheid wall thickness and fraction of cell wall) could clearly separate *D. kerrii*, *H. plagata* and *S. contorta*. One sample of *P. malaanoman* was mixed up with *S. almon* (Fig. 5). With PCA clear separation of *D. kerrii* and *H. plagata* was possible (Fig. 6) by using eight variables (vessel frequency, portion of vessel lumina, fibre lumina, axial parenchyma cells, cell wall area, length of vessel element and fibre tracheid and carbon/nitrogen ratio). Eighty percent of the samples of *S. contorta* were assigned correctly. However *P. malaanoman* and *S. almon* were found to be mixed up (Fig. 6). The first and the second principal component were responsible for 49% and 24% of the variations, respectively.

5.5. Discussion

The five dipterocarp species showed significant structural differences in their cell components (Fig 2). The vessel element length distribution classes also varied among the five species (Fig. 3). The higher predicted conductivity of *D. kerrii* and *H. plagata*

is caused by higher vessel diameter classes whereas species with lower vessel diameter values showed higher predicted conductivity in the lower vessel diameter classes. *H. plagata*, which contained the significantly lowest value of vessel diameter showed the highest predicted conductivity in 100-109 μm diameter classes (Fig. 4). In a study by Willingen et al. (2000), four subtropical angiosperm tree species showed considerable variations in the distribution of vessel lengths and vessel diameter classes. A number of studies have been conducted on the xylem hydraulic characteristics of plants (Willingen et al. 2000; Hacke et al. 2000) of different habitats. It has been stated that smaller diameter vessels are more resistant to cavitation and resistant (thick walled) xylem is the characteristics of high wood density (Hacke et al. 2000). In a study in Bornean dipterocarp forests (Tyree et al. 1998), several dipterocarp species were studied for their vulnerability to drought-induced embolism. The dipterocarp species were found to be highly vulnerable to draught induced embolism. A weak but significant relationship was found between vessel diameter and vulnerability to embolism in this study. However studies on embolism on these particular five species have not been done so far. We assume the smallest diameter of *H. plagata* than the other species makes it more resistant to embolism than the remaining four species (Table 1).

The growth rate of the five dipterocarps has been reported in the literature (Ashton 1982; Newman 1996). The reported growth rate of *D. kerrii* and *H. plagata* is slower than *P. malaanoman* and the two S*horea* species. Among the five species *H. plagata* has the smallest growth rate, and because of the small size, the *Hopea* species never reach the forest canopy (Ashton 1982). According to the study of Swine and Whitemore (1988) slow growing species are characterized by thick walled fibres whereas the reverse is true for the fast growing species. The thickness of the fibre tracheids of the five dipterocarps is in accordance with the study (Table 1). Besides the anatomical properties the biochemical properties varied among the dipterocarps (Tables 1, 2) as well.

Overall it was observed that the traits of *D. kerrii* and *H. plagata* were different in many aspects and the remaining three species possessed similarity in most instances which was also reflected in the previous chapters (chapter 3, 4). With multivariate analysis, recently attempts were made to separate angiosperms of different habitats by using several important traits (Preston et al. 2006). However emphasis was given on wood density and plant height along with some other characteristics. We have already

shown the suitability of using wood density and height growth for species separation (see chapter 3). Here emphasis was given on characters that are particularly important for conduction and support, in addition with some biochemical traits which have not been reported in dipterocarp wood so far (Tables 1, 2). Multivariate methods could clearly separate 3 out of five species (Figs 5, 6) and the results are in accordance with the results of the previous chapters (chapter 3, 4) which indicate the suitability of these traits to be used for species differentiation purposes. The close relationship of *P. malaanoman* and the two *Shorea* species has been established once more in this work.

5.6. References

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Table 1. Anatomical characteristics of vessels and fibre tracheids in the secondary xylem of the five dipterocarps. Statistical results of the average diameter of vessel lumen (ADV), vessel element length (VEL), vessel frequency (VF), fibre tracheid length (FL) and thickness of the double fibre tracheid wall (TDFW). Data indicate means (± SD, n= 5 (VF), n=14 (ADV), n=25 (VEL, FL) and n=10 (TDFW). The values followed by different letters in the same column indicate significant differences at $P<0.05$.

Species	VF (number in mm^{-2})	ADV (µm)	VEL (µm)	FL (µm)	TDFW (µm)
D. kerrii	9.94 ± 2.49 A	157.91 ± 20.20 D	459.04 ± 29.59 D	778.04 ± 57.08 C	13.77± 2.89 B
H. plagata	19.13 ± 3.70 B	96.03± 8.73 A	378.12± 62.23 B (A)	692.53± 71.37 B	13.94± 1.28 B
P. malaanoman	8.18 ± 1.30 A	134.72± 17.65 C (B)	338.45± 33.48 A	527.30± 31.49 A	4.19± 0.51A
S. almon	8.92 ± 1.59 A	118.78± 6.44 C	369.45± 26.71B (A)	465.87± 44.17 A	2.93± 0.20 A
S. contorta	9.03 ± 1.58 A	151.36± 10.82 D (B)	402.50± 48.14 B	624.76± 51.63 B	4.03± 0.72 A

Table 2. Biochemical data of the five dipterocarps. Statistical results of carbon (C), nitrogen (N), C/N ratio and lignin/carbohydrate (1505/1738). Data indicate means (± SD, n= 5). Significant differences are indicated by different letters after the values at $P<0.05$.

Species	Carbon (%)	Nitrogen (%)	C/N	Lignin/ carbohydrate (1505/1738)
D. kerrii	47.62 ± 0.52 B	0.28 ± 0.06 A	172.88 ± 29.66 A	1.09 ± 0.03 B
H. plagata	46.53 ± 0.41 A	0.19 ± 0.09 A	271.62 ± 82.55 B	1.00 ± 0.04 A
P. malaanoman	48.04 ± 0.23 B	0.23 ± 0.09 A	228.09 ± 60.66 B(A)	1.16 ± 0.03 B
S. almon	47.94 ± 0.23 B	0.21 ± 0.06 A	239.42 ± 96.97 B(A)	1.26 ± 0.09 C
S. contorta	47.76 ± 0.25 B	0.23 ± 0.02 A	206.42 ± 23.57 B(A)	1.32 ± 0.02 C

Figure legends

Figure 1. Stem cross sections of five dipterocarp species. *D. kerrii* (A), *H. plagata* (B), *P. malaanoman* (C), *S. almon* (D) and *S. contorta* (E). As the detection of axial parenchyma cells were not possible in *P. malaanoman* and the *Shorea* species with toluidine blue, C-D were stained with IKI for starch detection in axial parenchyma during ImageJ analysis. *P. malaanoman* and the two *Shorea* species had cross sections with similar appearance, for this reason cell elements were indicated only in *P. malaanoman*. The different cell elements are shown by vessel element (VE), axial parenchyma (AP), ray parenchyma (RP), fibre tracheid (FT) and axial canal (AC) (only in *D. kerrii*).

Figure 2. Pie charts (A-E) showing the statistical results of the portions of cell wall area (PCWA), the portion of vessel lumen area (PVL), the portion of fibre tracheid lumen area (PFT), the portion of ray parenchyma area (PRP), the portion of axial parenchyma area (PAP) and the portion of axial canal (PAC) (only in *D. kerrii* in A) in five dipterocarp species.

Figure 3. Relative contribution (%) of vessel element length (VEL) classes in five dipterocarps. The smallest vessel element length class starts from 1 (100-150 μm). The highest length class is 13 (>700 μm). Data indicate means (± SD, n = 5).

Figure 4. Relative contribution (%) of vessel diameter classes (10μm) to predicted conductivity of the xylem in five dipterocarps. The lowest diameter class starts from 1 (60-69 μm). Data indicate means (± SD, n = 5).

Figure 5. Cluster analysis of five species of dipterocarps, Hp (*H. plagata*), Dk (*D. kerrii*), Pm (*P. malaanoman*), Sc (*S. contorta*) and Sa (*S. almon*) from a total of 25 different trees (five replicates of each). The dendrogramme was constructed by Ward's method (Euclidian distance) using values of average vessel diameter (μm), lignin/carbohydrate (1505/1738) ratio, predicted conductivity ($\sum r^4$), cell wall thickness (μm) and ray cell (%).

Figure 6. PCA map for five different dipterocarp tree species (five replicates of each).

The two dimensional plot was constructed by using eight variables, vessel frequency, vessel lumina (%), fibre tracheid lumina (%), axial parenchyma (%), cell wall area (%), vessel element length (μm), fibre length (μm) and C/N ratio.

Fig.1

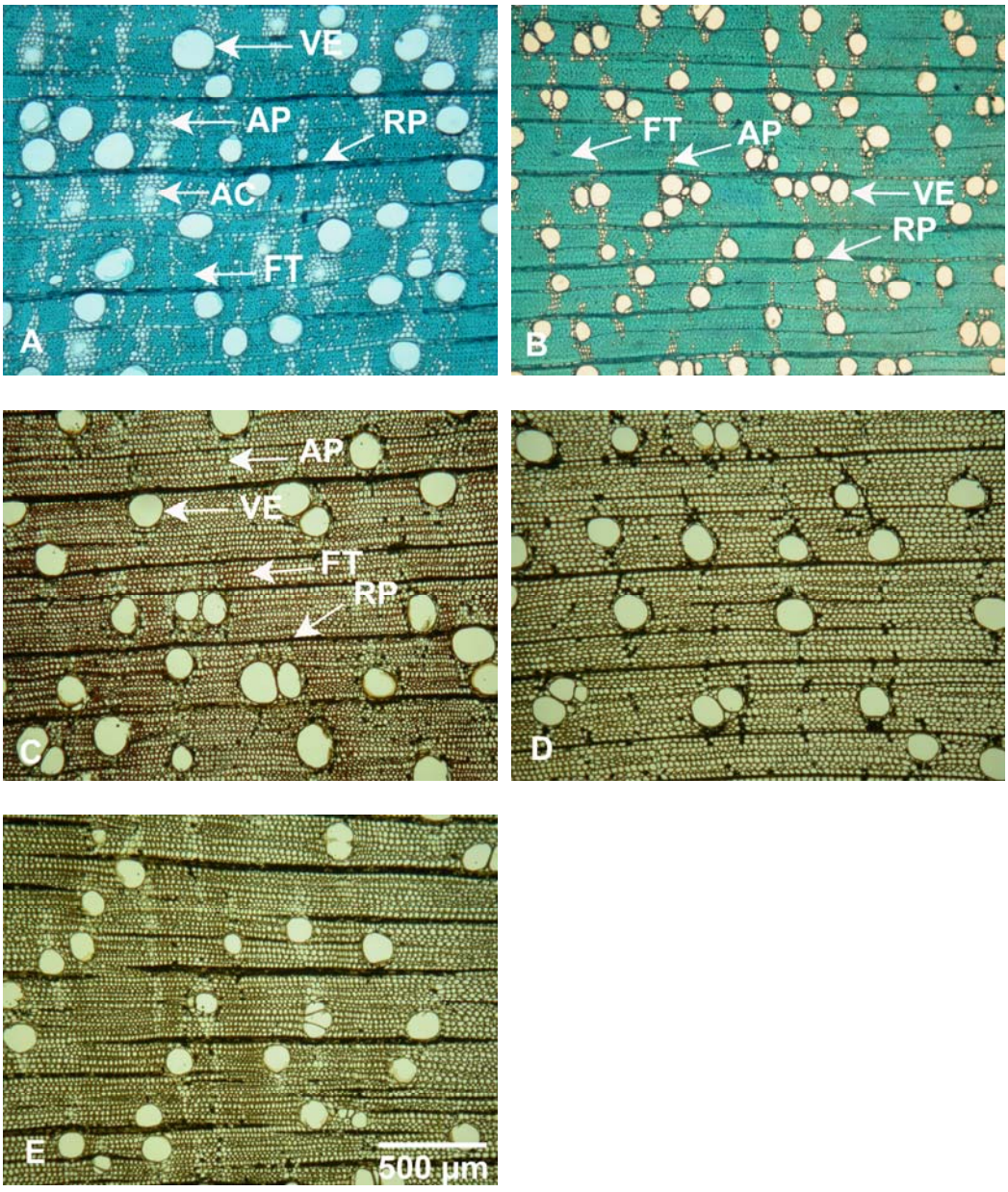


Fig. 2

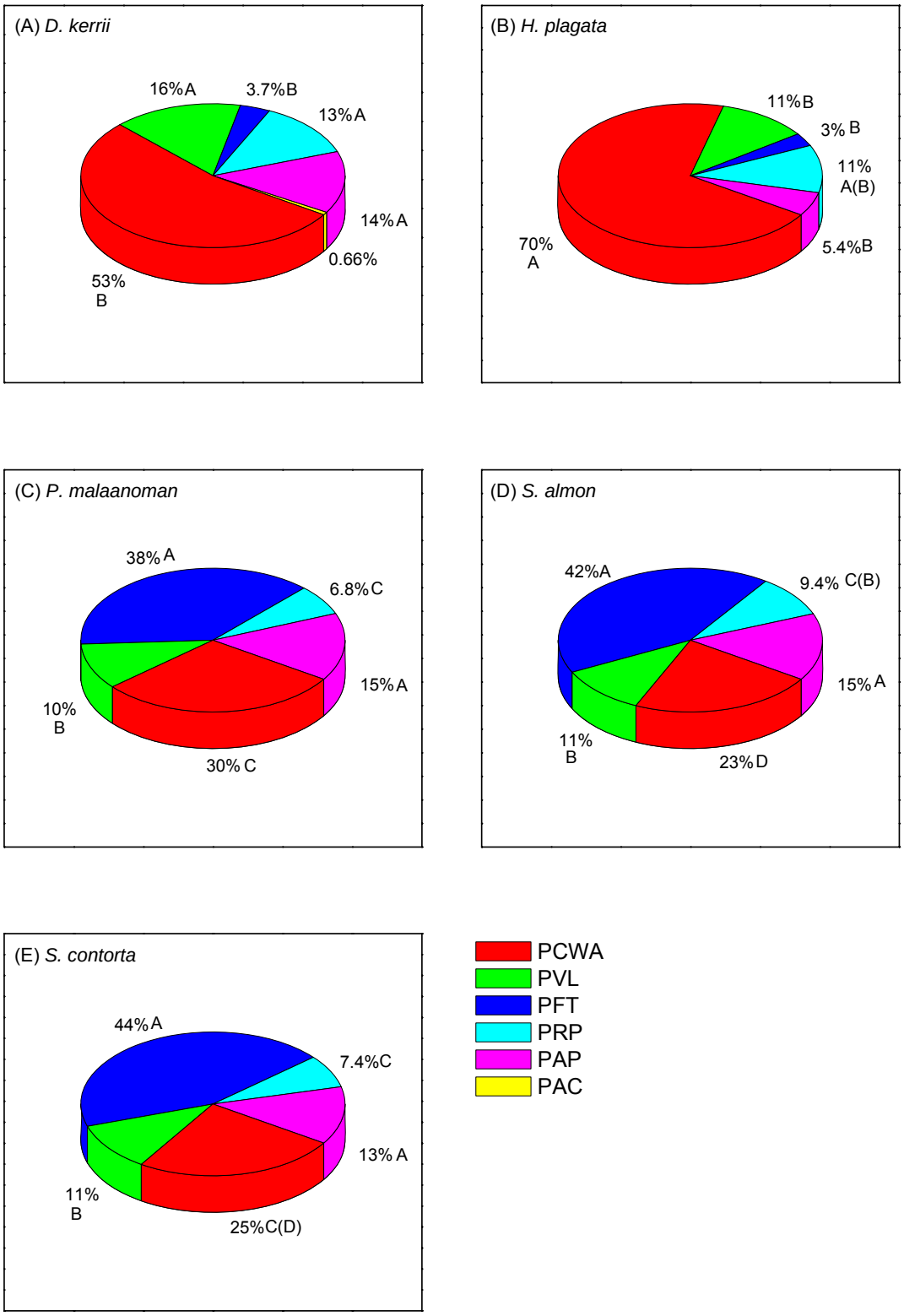

(A) D. kerrii
16%A
3.7%B
13%A
14%A
0.66%
53%
B
(B) H. plagata
11%B
3% B
11%
A(B)
5.4%B
70%
A
(C) P. malaanoman
38% A
6.8%C
15%A
10%
B
30% C
(D) S. almon
42%A
9.4% C(B)
15%A
11%
B
23%D
(E) S. contorta
44%A
7.4%C
13% A
11%
B
25%C(D)
PCWA
PVL
PFT
PRP
PAP
PAC

Fig. 3

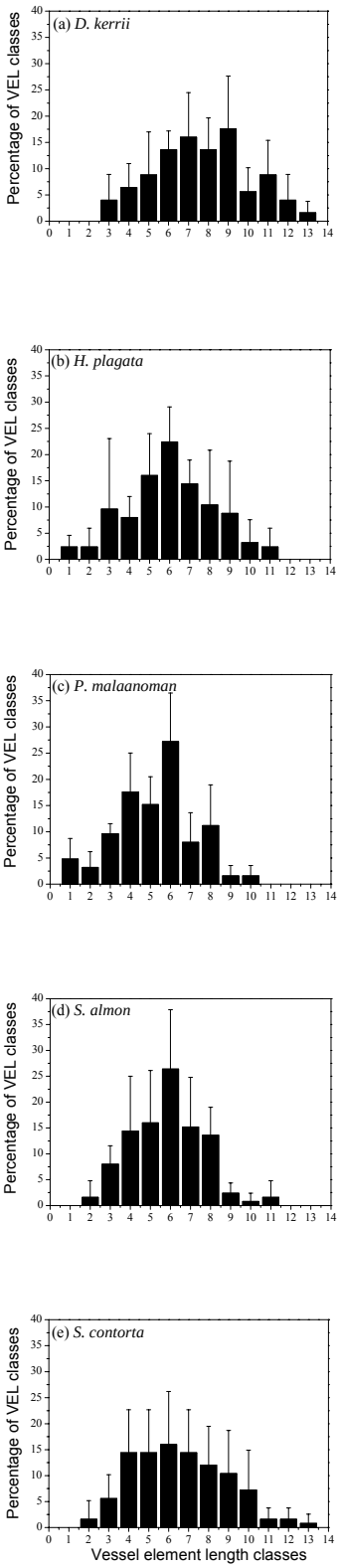

(a) D. kerrii
(b) H. plagata
(c) P. malaanoman
(d) S. almon
(e) S. contorta
Percentage of VEL classes
Vessel element length classes

Fig. 4

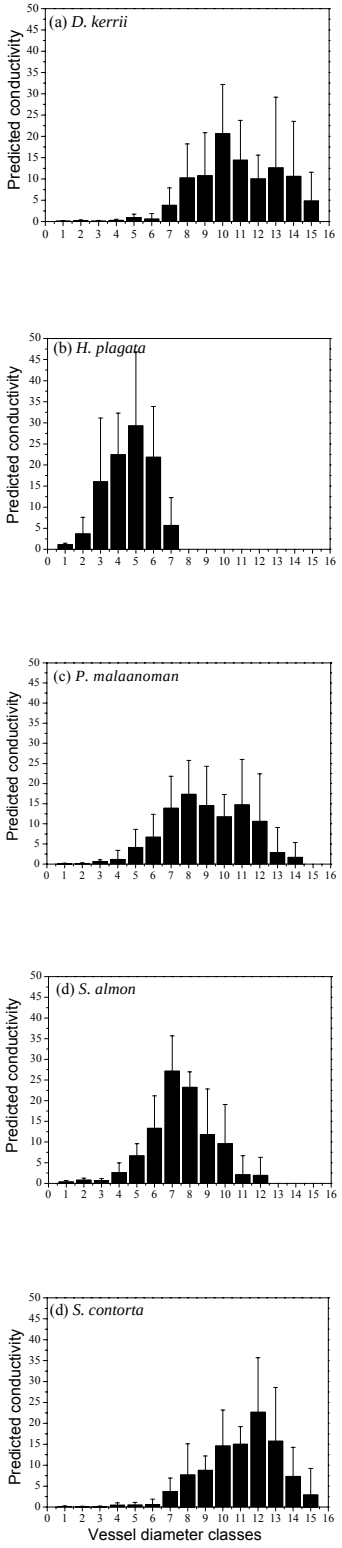

(a) D. kerrii
(b) H. plagata
(c) P. malaanoman
(d) S. almon
(d) S. contorta
Predicted conductivity
Vessel diameter classes

Fig. 5

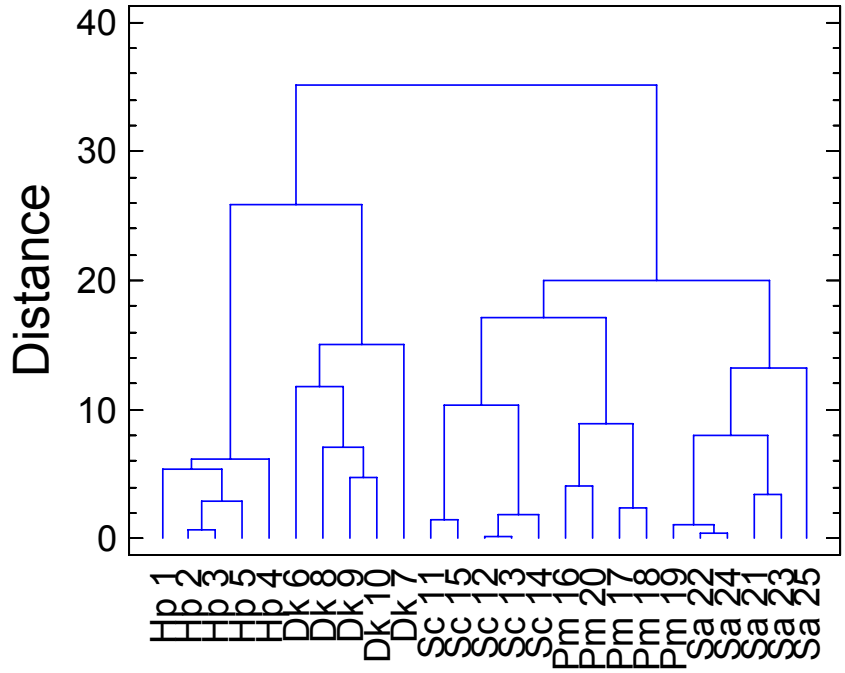
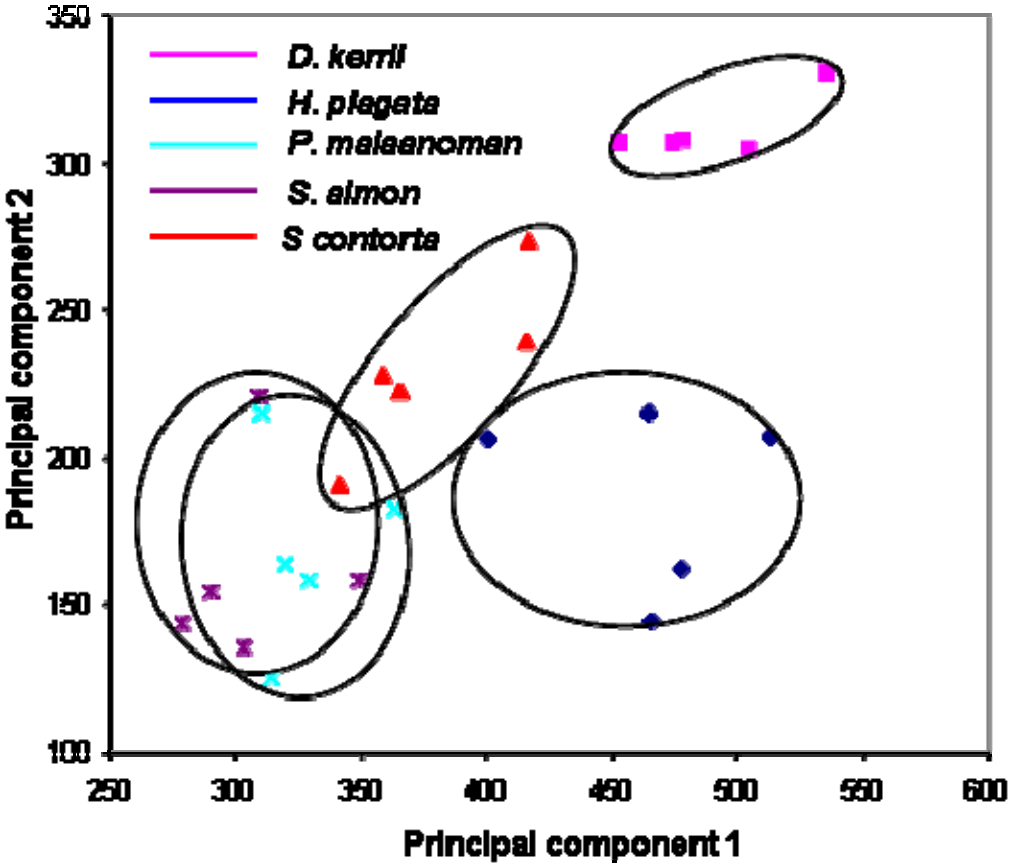

Distance
40
30
20
10
0
Hp1
Hp2
Hp3
Hp5
Hp4
Dk6
Dk8
Dk9
Dk10
Dk7
Dk11
Sc15
Sc12
Sc13
Sc14
Sc16
Pm20
Pm17
Pm18
Pm19
Pm22
Sa24
Sa21
Sa23
Sa25
Sa

Fig. 6


D. kerrii
H. plegata
P. malaanoman
S. almon
S contorta
Principal component 2
Principal component 1
350
300
250
200
150
100
250
300
350
400
450
500
550
600

Declaration

Some data shown in this thesis have been provided by colleagues:

Chapter 2: "FTIR spectroscopy in combination with principal component analysis or cluster analysis as a tool to distinguish beech (*Fagus sylvatica* L.) trees grown at different sites"

The beech sample data collected in the city forest of Schmallenberg in North Rhine-Westphalia (SAL) and cluster analysis (Fig. 3) was provided by Günter Müller under the guidance of Prof. Dr. Andrea Polle (Forest Botany and Tree Physiology, Büsgen-Institute, Georg-August-University Göttingen). This work is now in press "Holzforschung".

Chapter 3: "Height growth, wood traits and molecular markers to distinguish five tree species of Dipterocarpaceae grown at same site"

The data for Chloroplast DNA analysis and the analysis work was performed by Randy Alfabete Villarin and Dr. Oliver Gailing (Forest Genetics and Forest Tree breeding, Büsgen-Institute, Georg-August-University Göttingen) under the supervision of Prof. Dr. Reiner Finkeldey (Forest Genetics and Forest Tree breeding, Büsgen-Institute, Georg-August-University Göttingen).

Göttingen, 21.04.08

(Rumana Rana)

Acknowledgement

This thesis work was performed at the Institute of Forest Botany and Tree Physiology of the Georg-August University Göttingen in the period from 2004 to 2008 with the financial support from the DAAD (German Academic Exchange Service).

This work would not have been possible without the manifold support of various persons whom I would like to express gratefulness.

I am grateful to my supervisor Prof. Dr. Andrea Polle for accepting me as a PhD student. She offered me a work place in the laboratory which was a prerequisite for DAAD scholarship. Her continuous supervision and guidance in the lab work and in writing was essential to finish the PhD work. During the work the discussions between us were instructive and interesting and I was enlightened.

I am thankful to Prof. Dr. Reiner Finkeldey of the Institute of Forest Genetics and Forest Tree Breeding for being my second supervisor and for bringing my wood samples from the Phillipines.

I would like to thank Dr. Rosemarie Langenfeld-Heyser, who helped me a lot in the anatomical and histochemical studies throughout. Thanks to Dr. Annette Naumann for introducing me with FTIR-ATR spectroscopy and providing me technical support. I am grateful to Mr. V. Meng, Mr. E. Kreysern, Dr. R. Petercord and Prof. H. Spellmann for providing me beech wood samples from different sites. I am also grateful to Dr. Thomas Teichmann, Dr. Andrea Olbrich, Dr. Zhibin Luo and Günter Müller for their cooperation in discussing matters that was very helpful. My special thanks to Dr. Payam Fayyaz who helped me a lot with the ImageJ programme. I thank my colleagues-Gisbert Langer-Kettner, Gabriele Lehmann, Marainne Smiatacz, Michael Reichel, Bernd Kopka who have helped me a lot in various ways during my work.

I want to thank Christa Lang, Manika Mishra-Knyrim and Tanja Ducic for their wonderful gesture and social time besides the academic work which was very important for my stay.

I centainly have to thank my husband Md. Golam Rakkibu for his help and encouragement throughout the work. Especially during the final days of hard work, without his help things would have been very difficult.

My daughter's sacrifice and patience was an inspiration to my work. I am thankful to other family members especially to my parents for their effort in my education.

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