WWF

PROJECT REPORT

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Final project report

Fighting illegal logging through the introduction of a combination of the isotope method for identifying the origins of timber and DNA analysis for differentiation of tree species

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1 Reason for the project and the set goals

Forests stabilise the ecological balance of our planet. The global use and destruction of forests therefore creates a wide range of ecological problems. On the one hand, habitats are destroyed and the pace at which species become extinct has accelerated. On the other hand, clearing large forested areas has an additional and considerable impact on climate and material cycles. The UN Climate Council IPCC has calculated that 15 to 20% of the carbon dioxide around the world is caused by deforestation. Tropical countries, where the largest portion of forests is being lost, also have the greatest amount of biodiversity in their ecosystems, precisely in those locations where illegal logging is known to be destroying these habitats.

Many countries that export wood and wood products to Germany have a problem as direct or indirect transit countries with illegal timber from e.g. Southeast Asia, West and Central Africa or South America. Indonesia, for instance, is Germany's most important trading partner for wood and its products outside of the EU. The OECD (2007) assumes that around 73% of this timber originates from illegal logging. Indonesia is one of the biggest emitters of carbon dioxide in the world due to the loss of forest.

One of the European Union's goals is to stop the import of illegal timber. The FLEGT process (Forest Law Enforcement, Governance and Trade) which started in 2003 and in which Germany is an active participant, is intended to slow down the destruction of forest regions in the tropics and elsewhere. To this end, an action plan was created to combat illegal logging and trade with illegally logged timber. This plan includes, among other things, a provision of proving the legality of timber imported into the EU. Of particular significance here is the Timber Trade Law passed by the EU Council in October 2010 (Regulation (EU) 995/2010). This law stipulates, for example, that importers will have to identify the country of origin of the timber found in their products in the future.

Until now, however, the **declared origin** can only be verified, e.g. by authorities by means of shipping documents and not through an independent inspection process. The WWF considers this an important "loophole" because even though the law requires importers to know the origin of the timber, this requirement can only be seriously implemented and monitored if there are independent procedures for reliably verifying the declared wood origin. The isotope method described here can make an important international contribution to these efforts.

Illegal logging and trade of CITES-protected tropical timber species necessitates the development of unambiguous species identification methods applicable over the whole chain-of-custody. Due to the fact that many protected species can easily be mistaken for legally harvested tree species because of a very similar wood anatomical pattern and structure, DNA barcoding provides an interesting tool for species differentiation. As an integrated “barcode”, not susceptible to manipulation, DNA methods display a promising instrument to control the whole chain-of-custody.

2 Applicants and project partners

The application was submitted by:

Umweltstiftung WWF Germany

The WWF Germany is an independent, non-profit, non-affiliated foundation with legal capacity headquartered in Berlin. The address is:

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Project partners

1. TÜV Rheinland Agroisolab GmbH

Agroisolab GmbH is a spin-off of the Jülich GmbH research centre. It grew out of the "Stabile Isotope" research group under the direction of Dr. H. Förstel who has been working with the various ways of applying stabile isotopes for 25 years now.

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3 Short summary of the project

The goal of the project described here is to introduce the combination of the stabile isotope method for verifying the declared origin of timber and the DNA analysis for species identification using concrete examples. As the project initiator, the WWF aims to give all interested stakeholders (companies, authorities, NGOs, etc.) an easy, fast and relatively low-cost way to verify the declared origin and tree species.

Using the results of the previous project as its starting point, the project partner, TÜV-Rheinland Agroisolab, further developed and substantiated the **stabile isotope method** specifically for the tree species teak and mahogany. Evidence of the practical application of the method was thus also furnished for **verifying the declared origins of tropical wood**. The previous project funded by the DBU (Deutsche Bundesstiftung Umwelt - German Federal Foundation for the Environment) concentrated on Europe and north-western Russia.

The project consisted of five chronological steps:

- Development of plan for concession screening, i.e. details of sampling in at least four different regions of the hemisphere
- Further development of stabile isotope application using all stabile isotopes of the bioelements: D/H, $^{18}O/^{16}O$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ as well as the strontium isotopes $^{87}Sr/^{86}Sr$
- First test series to characterise selected cropland; second test series to assess the transferability of the results to other tree species
- Expansion of the application to display test data on Google Earth and incorporate genetics and stabile isotopes
- Check of suspicious samples / blind test

At the end of the project, German customs commissioned TÜV Rheinland Agroisolab GmbH to verify the declared origin of confiscated teak samples. The new possibilities developed in the project were able to be successfully tested in practice in this real-world example.

As part of this project, the University of Hamburg worked together with the Thünen Institute to provide a practical method for unambiguous **species differentiation** of the three mahogany tree species protected under CITES *Swietenia macrophylla, S. mahagoni* and *S. humilis* and of teak (*Tectona grandis*) using species-specific **DNA barcodes**. The screening and further specification for sampling was conducted in collaboration with the project partners.

In this report we will address the following topics:

(1) Special features of DNA extraction from wood and woody products

(2) Advantages of the chloroplast genome for the detection of informative DNA sequences for genetic barcoding

(3) The utilisation of the completely sequenced cpDNA genome of *Populus trichocarpa* (Black Cottonwood) as basis for the design of new PCR primers

(4) The development of barcoding sequences for an easy viable detection of the CITES protected tree species of the mahogany genus *Swietenia* (consisting of *S. macrophylla*, *S. mahagoni* and *S. humilis*) as well as *Tectona grandis* (teak)

In a first practical test of timber samples confiscated by German customs, we were able to successfully verify the reliability and efficiency of the developed DNA markers.

A **blind test** carried out in the final project phase by the WWF Germany to check the declared origin was very promising.

In a stakeholder process, the possibility of setting up a shared **database** was discussed. Test data from both methods and possibly also future data could be jointly managed and made available to interested groups in this database.

The **project results** were made available to other key stakeholders at an international fingerprinting conference in the last phase of the project and through other communication measures including a trade forum held at the CBD 2009 in Bonn.

4 Sampling

The project focussed on two tree species:

1. Teak
2. Mahogany (real mahogany, African mahogany)

4.1 Teak (*Tectona grandis*)

Genus: *Tectona*

Family: *Lamiaceae*

Geographic distribution according to Richter, RICHTER, H G and DALLWITZ, M J: India, Pakistan, Sri Lanka, Burma/Myanmar, Thailand, Laos, Vietnam, Cambodia and tropical Africa (plantation); teak is grown on plantations in several regions (Java, Africa, Central America)

Teak was chosen as a tree species because there has been an EU import ban since 2008 (EU) Regulation (194/2008) for wood and other products from Burma (Myanmar). Teak from Burma, in particular, plays a special role due to its particularly highly rated quality. Prospective customers in Europe are prepared to pay high prices for teak from this country (approx. USD10,000/m³). During the project, a case came to the attention of WWF Germany where a German company imported several m³ of Burma teak to Germany by air freight. This is an unusual means of transport for wood and can only be explained through the very high price.

The WWF presumes that high-value teak from Burma is imported into the EU and to Germany using false declarations of the country of origin. This presumption was confirmed, on the one hand, by a German timber company that confessed to the WWF in confidence that even after an EU import ban, trade in Burma teak would continue almost completely unaltered – but has since gone underground. On the other hand, the confiscation of teak by the customs office at the end of this project confirmed the suspicion expressed by the WWF.

4.2 Mahogany

1. *Swietenia macrophylla* (international trade names: big-leaf mahogany, Honduras mahogany, mahogany)
2. *Swietenia mahagoni* (international trade names: Cuban mahogany, mahogany, Spanish mahogany, West Indian mahogany)
3. *Swietenia humilis*

Geographic distribution according to RICHTER, H G and DALLWITZ, M J: Mexico and Central America and the tropics of South America (except for the Amazon).

Mahogany was chosen as another tree species because the WWF became aware that between January and August 2007 five times the amount of mahogany was exported from Germany than imported by analysing Eurostat figures. This was the case despite the fact that imports were consistently low in the previous years. These figures are not evidence of illegal imports but they are an indicator.

Protected status under CITES (Washington Convention on International Trade in Endangered Species of Wild Fauna and Flora): *S. humilis* and *S. mahagoni* in Annex II; *S. macrophylla* in Annex III (Costa Rica)

Mahogany is also an extremely expensive tree species. The WWF presumes that real mahogany is illegally imported to Germany e.g. through false declaration of the tree species. To do this, tree species that look the same and can be confused with one another (usually African mahogany) can be substituted for false declaration. Our suspicion that real mahogany is imported to Germany illegally was confirmed by an actual case at the end of this project (see chapter 7.3)

African tree species that can be confused with one another:

- *Khaya spp.* (international trade names: Khaya, African Mahogany)
- *Entandrophragma cylindricum* (international trade names: Sprague, Sapeli)
- *Entandrophragma utile* (international trade names: Dawe & Sprague, Sipo)

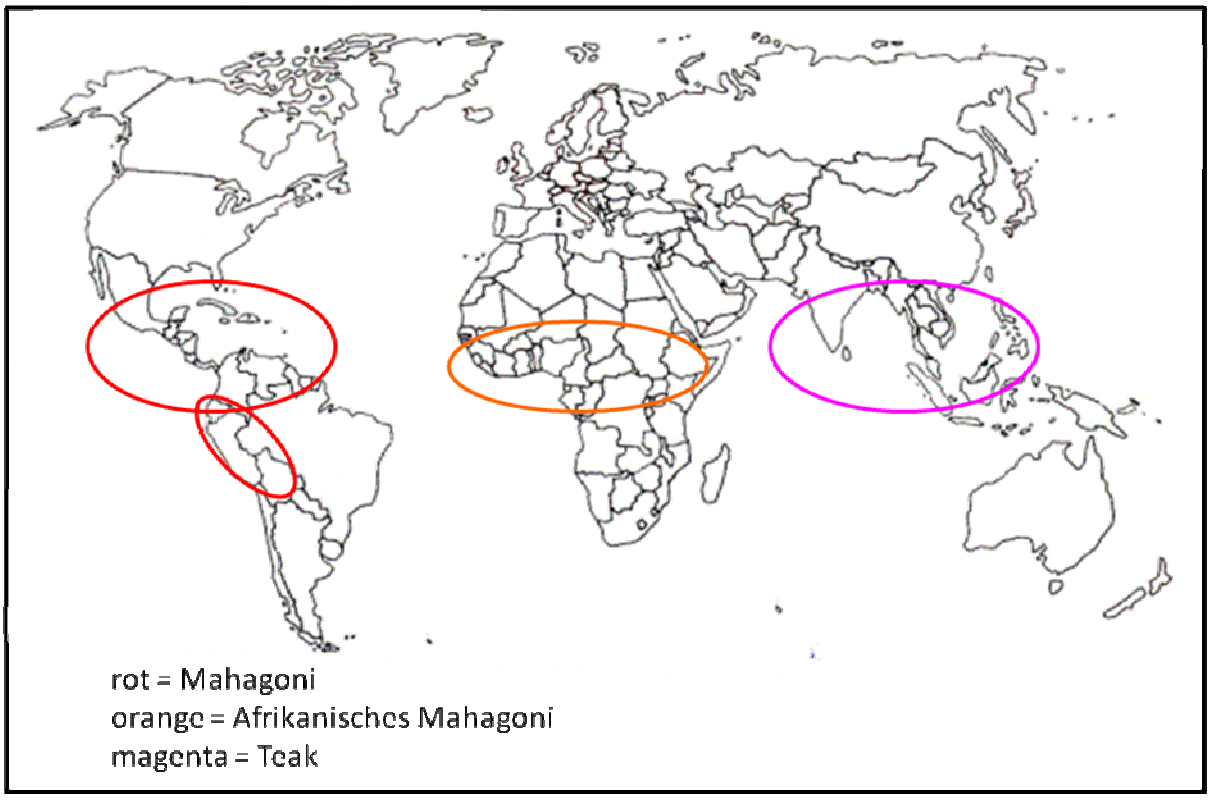


Figure 1: Natural distribution region for teak, mahogany, African mahogany

At the beginning of the project, there was a shared understanding that the wood samples should be collected in cooperation with companies or with the consent of the competent authorities and exported.

Region	Number of locations	Number of wood samples
Burma / Myanmar	15	24
Brazil	6	31
Costa Rica	5	25
Ecuador	21	21
Ghana		339
Guatemala	2	2
Honduras	35	74
India	7	34
Indonesia	2?	7
Java	27	124
Congo	26	37
Laos	7	35
Mexico	2	2
Panama	141	141
Papua New Guinea	4	18
Peru	70	70
Thailand	3	3
Vietnam	4?	18
Total:		1005

Figure 2: Wood samples collected during the project

Collecting the samples proved to be very difficult overall. In some cases, individual companies had to be persuaded over several months to participate in the project.

In other cases, it took a number of months to obtain the official permits and this process also required numerous forms to be filled out.

For these reasons, the total number of samples actually collected, which totalled 1,005, was considerably less than the 2000 samples planned.

During the last phase of the project, the opportunity arose to obtain wood samples from Ecuador and Colombia. The scientific advisory committee decided during its final meeting that the samples should be collected despite the late point in time even if it could no longer be guaranteed that the samples could analysed as part of the project. The wood samples will be available for subsequent analysis and, if necessary, for setting up an international database. All of the samples had not yet arrived by the end of the project (approx. 80 samples are still expected).

This means that more than 1,000 wood samples from 18 countries were available at the end of the project.

4.3 MTA (Material Transfer Agreement)

The "Material Transfer Agreement" (MTA) was developed to give both contract parties (the company that provides the samples on the one side and the project partners on the other) a secure foundation for collaboration. The MTA stipulated that the project partners could only use the wood samples provided for the purpose described in the project.

The following companies signed an MTA with the project:

1. Cooperacion para el Desarrollo Agraindustial (Panama)
2. Ecoforest S.A. (Panama)
3. Floresteca S.A. (Brazil)
4. Forest Finance Panama S.A. (Panama)
5. Reforestadora de la Cuenca Hidrografica del Rio Tapagrilla (Panama)
6. Geo Forestal S.A. (Panama)
7. Interholco AG (Congo; Switzerland)
8. Juan Pausa (Panama)
9. Norma Betzaida Solis Batista (Panama)
10. Perum Perhutani (Indonesia)
11. Precious Woods (Costa Rica; Switzerland)

5 Verifying the declared origin using the isotope method (Prof. Hilmar Förstel, Dr. Markus Boner)

5.1 Bases for the stable isotopes

5.1.1 Definition of stable isotopes

The elements in the periodic table are organised based on various types of atoms (nuclides) that, even though they have the same number of protons, possess different numbers of neutrons. This is reflected in the atomic weight. These types of nuclides are called isotopes (Greek *iso* = equal, *topos* = place). Chemically speaking, this fact has no further significance because ultimately only the protons and electrons of an element make up its chemical properties. However, a higher or a lower number of neutrons generally results in instable elements subject to radioactive decay. A very small number of these elements do not exhibit this atomic decay which is why they are called stable isotopes. Accordingly, radioactive elements belong to the unstable isotopes.

In nature, most chemical elements occur as isotope compounds. Only 21 elements are comprised solely of a single stable isotope. Generally, the elements with a higher atomic number have a higher number of isotopes. Elements with a lower atomic number, such as the "bioelements", have only a few stable isotopes.

The distribution and frequency of the stable isotopes, expressed as an atomic percentage, are not consistent particularly among the bioelements but are subject to slight fluctuations in nature that can be attributed to geochemical, geophysical, biochemical and biophysical fractionation processes. When the natural frequency of the stable isotopes is indicated, it is thus necessary to pay attention to the specified origin. The special characteristic of bioelements mentioned above (only two isotopes with a predominant isotope) makes it possible to indicate the natural variation of the isotope frequency in a key indicator that is explained in the following section and used in the further discussions.

5.1.2 Measurement notation

The isotope composition of the bioelements doesn't vary much which means that differences only occur starting with the second or third decimal place of the composition expressed as an atomic percentage.

Material	Relation in atomic-%		δ PDB [‰]
	^{12}C	^{13}C	
PDB	98,8887	1,1112	0
NBS-limestone Nr. 20	98,8899	1,1100	-1,1
Teplitz, limestone	98,8920	1,1079	-2,9
CO2 atmosphere	98,8972	1,1027	-7,7
C3 – sugar (beet)	98,9173	1,0826	-26,0

Figure 3: The relative frequency of ^{12}C and ^{13}C as an atomic percentage depending on the matrix using carbon as an example, slight deviations in the ^{13}C content are shown in various matrices. A deviation of an atomic percentage of +0.001 is equivalent to an enrichment of approximately 1‰.

For more than 60 years, the delta notation has been the standard measurement for this variation indicating the deviation from an international reference standard (for $^{13}C/^{12}C$: PDB). The isotope composition of the international reference standard serves as a reference point or zero value of the scale.

This is illustrated using oxygen as an example:

$$\delta^{18}O = \left(\frac{R_{pr} - R_{ref}}{R_{ref}}\right) * 1000$$

where $R_{pr} = [C^{16}O^{18}O] / [C^{16}O^{16}O]$ in the sample

and $R_{ref} = [C^{16}O^{18}O] / [C^{16}O^{16}O]$ in the international standard

In scientific literature, it has become standard to express this deviation in terms of per mil, or parts per thousand [‰] and is also applied accordingly to the other bioelements.

5.1.1 International reference standards

International reference materials that can be obtained from the IAEA (International Atomic Energy Agency, Vienna) are used as the reference standard for the delta notation. These primary standards [Gonfiantini 1978] define the delta scale as a reference point with their specific isotopic composition.

Name	Isotope	δ in [‰]	Remark
Primary standards			
PDB	^{13}C / ^{12}C	0	fossil squid (*Bellemnitella americana*) in South Carolina, USA, (not available any more)
SMOW	^{18}O / ^{16}O D / H	0 0	Standard Mean Ocean Water (not available any more)
CDT	^{34}S / ^{32}S	0	form Canyon diabolo meteorite
IAEA-N_2	$^{15}N/^{14}N$	0	Atmospheric nitrogen
Secondary standards			
IAEA-CH-7	^{13}C / ^{12}C	-31,8 v.s. PDB	polyethylene film (PEF)
IAEA-CH-6	^{13}C / ^{12}C	-10,4 v.s. PDB	Cane sugar
GISP	^{18}O / ^{16}O D / H	-24,8 v.s. VSMOW -189,5 v.s. VSMOW	Greenland Ice Sheet Precipitation
SLAP	^{18}O / ^{16}O D/H	-55,5 v.s. VSMOW -428,0 v.s. VSMOW	Standard Light Antarctic Precipitation
IAEA-S1	^{34}S / ^{32}S	-0,3 v.s. CDT	silver sulfide
IAEA-N-1	^{15}N / ^{14}N	+20,3 v.s. IAEA-N_2	ammonium sulphate
IAEA-N-2	^{15}N / ^{14}N	+4,7 v.s. IAEA-N_2	ammonium sulphate

Figure 4: Sample list of various isotope standards for the stable isotopes of the bioelements. The primary standards are only still available in residual stocks with the exception of the atmospheric nitrogen. The VSMOW (Vienna-Smow) is unique here. It is calibrated by the IAEA relative to the SMOW. The standards below were calibrated in international ring tests against these primary standards and can be acquired as alternatives from the IAEA.

Figure 4 shows that two "SMOW" standards are in use. The VSMOW is a calibrated, mixed standard based on the original SMOW. Herein lies one of the main problems of the delta notation. Enough of this "zero" standard should exist for it to be available over a long period of time. Unfortunately, various zero standards are no longer available today (PDB, SMOW) so that the following standards currently have to be used.

A "zero standard" is sufficient for determining the isotope ratios of carbon, nitrogen, oxygen and sulphur for the calibration of the analysis. The D/H isotope ratios, however, show enormous variation, the result being that extremely deviant samples are subject to considerable error in a one-point calibration. This problem is solved by establishing a VSMOW-SLAP scale [GONFIANTINI 1978]. The VSMOW continues to function as the "zero" standard here. The measurements of a heavily depleted standard (SLAP) and a "mean" standard (GISP), i.e. a standard that yields isotope ratios that are just about in the middle between SLAP and SMOW, were adjusted to it.

5.1.2 Measuring the stable isotopes

In addition to the NMR, mass spectrometry has proven particularly reliable as a method for measuring stable isotopes. The use of mass spectrometers is well-established and widespread in almost all fields of the natural sciences.

The application investigated here is the measurement of stable isotopes which is generally carried out using three types of mass spectrometers (TIMS, ICP-MS with multi-collector and IRMS) because they can detect the stable isotopes with the necessary accuracy and reproducibility. The first two can be used to determine the higher stable isotopes.

The stable isotopes of the strontium (^{87}Sr / ^{86}Sr) were determined accordingly using ICP-MS in an external project with the Jülich research centre.

The area of application of the IRMS systems (Isotope Ratio Mass Spectrometer) lies in determining the "light" elements, particularly the stable isotopes of the bioelements. For detection in the mass spectrometer, it is necessary to add the stable isotopes of the bioelements (N_2, CO_2, SO_2, H_2, CO) to the mass spectrometer in a gaseous state which is why sometimes the abbreviation GIRMS (gasIRMS) is used.

A detailed overview of the IRMS technology and its special features are available in different studies [POSSER 1993].

The following device configurations were used to measure the stable isotopes of the bioelements.

D/H and $^{18}O/^{16}O$ organic	High-temperature oven (Gero, 1550°C)	Isotope mass spectrometer (Isoprime)
^{13}C / ^{12}C	Element analyser NA 1500 (Carlo Erba)	Isotope mass spectrometer (Optima)
^{15}N / ^{14}N	Semi-element analyser Vario Isotope Cube (Elementar)	Isotope mass spectrometer (NU Instruments)
^{34}S / ^{32}S	Element analyser EA 3000 CN (Euro Vector)	Isotope mass spectrometer (Optima)
$^{87}Sr/^{86}Sr$	Microwave digestion	ICP mass spectrometer (Elan 6100)

Figure 5: Device combination to analyse the stable isotopes of the bioelements and the stable isotopes of strontium.

5.2 Application of the stable isotope method to verify geographic origin

The stable isotopes of the bioelements offer a wide range of possibilities for eliciting origin. The D/H and $^{18}O/^{16}O$ isotope ratios supply large-scale discriminant vectors. The $^{13}C/^{12}C$ and $^{34}S/^{32}S$ isotope ratios are diffuse in the information content and can be used for both local (small-scale) as well as regional differentiation. The $^{15}N/^{14}N$ and $^{34}S/^{32}S$ isotope ratios are to be regarded as geological parameters that generally describe local factors particularly for wood. Under the scope of this project, the goal was to incorporate all of the stable isotopes mentioned to make the best possible information content available for proof of origin.

The bases of these application options are described in brief in the following section.

5.2.1 Bases for the D/H and $^{18}O/^{16}O$ isotope ratios

Water is a basic element of life but only a small portion of the water volume, approx. 0.002%, can be used directly as surface freshwater. The largest portion by far can be found in the world's oceans with a volume of $1.37 * 10^9$ km^3. In terms of stable isotopes, the world's oceans are largely homogenous with respect to the isotopes $^{18}O/^{16}O$ and D/H. Consistent with this, the $^{18}O/^{16}O$ and D/H isotope ratios do not fluctuate much from zero [CRAIG 1965]. In contrast, freshwater shows considerable variation that span a range of +4 to –55‰ in the $^{18}O/^{16}O$ isotope value and a range of +40 to –500‰ in the D/H isotope value.

The $^{18}O/^{16}O$ isotope ratios are correlated here with the D/H isotope ratios. This correlation has established itself as the "meteoric water line" [CRAIG 1961] which is represented mathematically as:

$$\delta D = 8\delta^{18}O + 10$$

The physical basis of this variation is the Rayleigh fractionation that produces the isotope variation in the precipitation. In general, the heavy water falls as rain before the light water with a simultaneous depletion of heavy water from the cloud.

The mean isotope value in the water can be strictly correlated here with the mean annual temperature in a specific location.

Because there is a direct correlation of the $^{18}O/^{16}O$ and D/H isotope ratios of the cellulose to the water, the isotope ratios of the tree rings were also used for paleoclimatic studies [BURK 1981, YAPP 1982].

On the other hand, factors other than temperature affect the isotope ratios of the water. The continental effect, for example, is another important effect according to which the $^{18}O/^{16}O$ isotope ratios in water gradually decrease inland from the coast due to precipitation [CRAIG 1956]. Other factors influencing the distribution of isotopic compositions are found in the height above sea level (altitude effect), the geographic latitude (latitude effect) and the amount of rainfall (amount effect). This produces a varied picture for the $^{18}O/^{16}O$ and D/H isotope ratios in the global hydrological cycle.

Even though the $^{18}O/^{16}O$ and D/H isotope ratios of the cellulose reflect the water, they are still subject to various biochemical and distillative fractionations and influences. The $^{18}O/^{16}O$ isotope ratios of cellulose are generally enriched by +27 ‰ due to the immission of enriched oxygen from the atmospheric carbon dioxide. The D/H isotope ratios in the cellulose are affected even more. The signature for the water is generally retained but with different offsets that e.g. depend on the photosynthesis type (C3, C4 plants) [ZIEGLER 1976].

5.2.2 Bases for the $^{13}C/^{12}C$ isotope ratios

The carbon of the biomass is essentially a product of the photosynthesis of the plants and thus a result of the atmospheric carbon dioxide. Compared to the international reference standard (baseline), the carbon dioxide shows a depletion of –8‰ [KEELING 1995]. This value is not constant but depletes gradually due to the reduction in carbon dioxide produced by industrialisation over the last 200 years of –6.5‰ [LEUENBERGER 1992].

The isotope ratios of the atmospheric carbon dioxide are not found again in the plant. Instead, the biomass that forms shows considerable depletions in its $^{13}C/^{12}C$ isotope ratios. The type of photosynthesis system and the climate conditions essentially determine the degree of depletion variation.

The latter is the gas-water problem of the plants: If the stomata are wide open so that CO_2 absorption for photosynthesis is hindered as little as possible, the transpiration necessarily increases and thus the danger that the plant will dry out. If, however, the plant closes the

stomata to counteract this risk, CO_2 absorption is also blocked. The result is that the CO_2 in the space between cells is almost completely converted, the $^{13}C/^{12}C$ isotope ratios of the photosynthesis products are enriched.

Consequently, plants of one species have more enriched $^{13}C/^{12}C$ isotope ratios in their biomass in arid regions than in temperate regions [KÖRNER 1991, TREYDTE 2001].

5.2.3 Bases for the $^{15}N/^{14}N$ isotope ratios

The nitrogen pool in the plant biomass and thus the trees depends on the ratios of two nitrogen pools, that of the soil and that of the atmosphere. The nitrogen is generally absorbed by the plant as nitrate and, as a result of the nitrate reduction, can already be used by the glutamate synthesis cycle in the root or in the leaves.

The absorption of nitrate does not yield any isotope fractionations [YONEYAMA 1989], meaning that it can generally be assumed that the isotope ratios of the plant reflect the isotope ratios of the soil nitrate [YONEYAMA 1990; TURNER 1987].

Natural tree stocks frequently reflect depleted $^{15}N/^{14}N$ isotope ratios because the only nitrogen immission occurs through depleted nitrogen fallout.

Anthropogenic influences such as plant fertilisation [DOUGHTON 1991] are also expressed solely through this nitrogen fallout in the plant for natural tree stocks [SAVARD 2009].

5.2.4 Bases for the $^{34}S/^{32}S$ isotope ratios

Sulphur has stable isotopes in its natural form: ^{32}S (95%), ^{33}S (0.75%), ^{34}S (4.21%) and ^{36}S (0.002%). The only one that is currently significant for the analysis is the $^{34}S/^{32}S$ isotope ratio. Plants generally bind the primary quantity of sulphur in the amino acids cysteine and methionine. The available sulphate pool of the soil is used to accomplish this. Similar to nitrogen assimilation, no fractionation of the organically bound sulphur, or only a slight one (< 1 ‰), can be detected vis-à-vis the bioavailable sulphate [CHUKHROV 1980]. As a result, the isotope ratios of the sulphur in the wood largely mirror the geological conditions of a location. The isotope ratios of the sulphur in the soil can be heavily influenced by humans through deposits or leaching of sulphur compounds from the atmosphere. For instance, industrial combustion gases show enriched sulphur isotope ratios of up to +30‰. The "fallout" can be traced using the isotope change of the plant and the soil [WINNER 1978, KAWAMURA 2006].

5.2.5 Bases for the $^{87}Sr/^{86}Sr$ isotope ratios

The higher stable isotopes generally yield isotope patterns that can hardly be used to track origin. Due to the only slight differences in mass of the higher stable isotopes, no considerable kinetic isotope fractionations occur in nature, i.e. isotope patterns as a result of incomplete processes. A special case is the heavy isotope strontium: ^{87}Sr. It is formed in nature from the radioactive decay of the long-lived rubidium isotope ^{87}Rb. As a result, the $^{87}Sr/^{86}Sr$ isotope ratio reflects the age of the geology in a simplified form which in turn results in different regional isotope patterns of this strontium isotope in nature.

Accordingly, it can be used to determine the origin of agricultural products [SWOBODA 2007] as well as wood because this strontium is also absorbed by the plant as a trace element.

5.3 Sampling concept and sample preparation

5.3.1 Concept for taking wood samples

The identical sample protocol was used for both teak (*Tectona grandis*) and mahogany (*Meliaceae*). The general goal was to take drilled samples from both tree species at a height of 50 cm above the ground. The principle of examining at least 10 annual growth rings from the various reference samples was already applied in the previous project "Fichte" (Spruce) which concentrated on temperate and boreal regions. This takes into account that variations occur within the annual growth rings and these variations can be used as an index for the change in climate as is shown in the bases. However, there is a working hypothesis that the total of several annual growth rings produces a more or less robust mean value of the stable isotopes for a region.

Annual growth rings are not generally expected for tropical wood. As a result, a drilling depth or a length of the drill core of at least 10 cm was required.

Combined with the GPS coordinates, these prerequisites were the most important rules for taking samples. It was possible to follow these rules in most sample regions, particularly Java (see Figure 6). Another goal was to take more than one sample from defined sample sites. A complete overview of the defined sample sites is provided in Figure 7 to Figure 14. There are no GPS coordinates available from the countries of Vietnam, Indonesia, Peru, Costa Rica and Brazil.

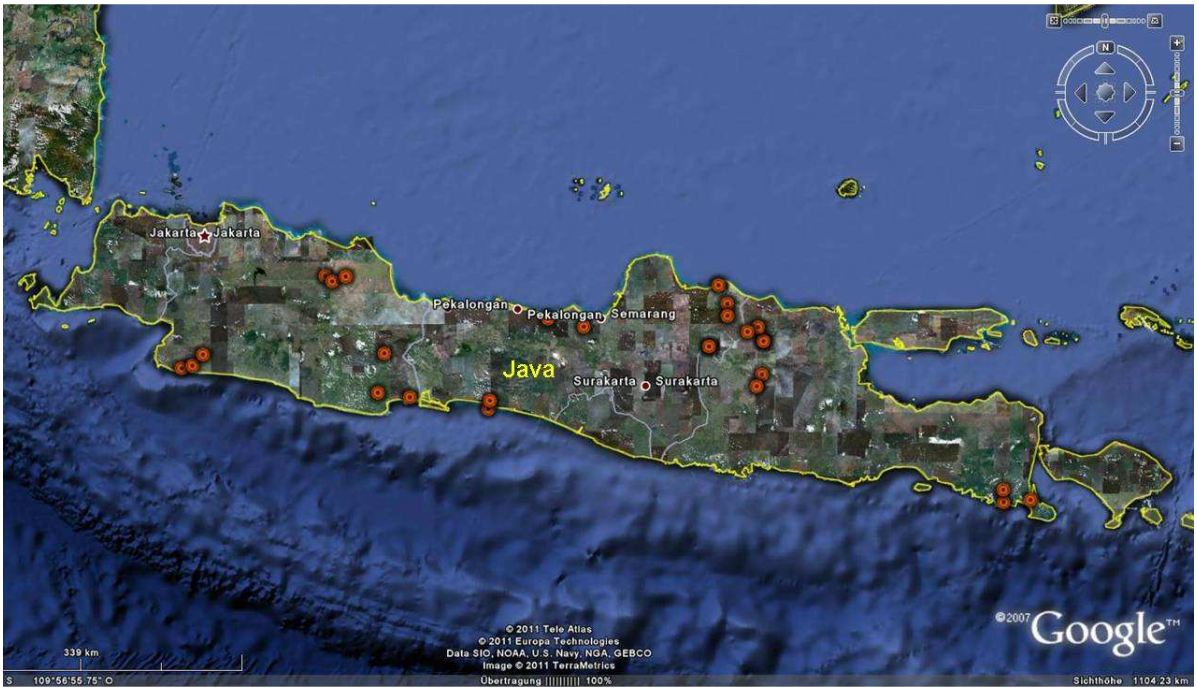


Figure 6: Sample sites for teak and mahogany in Java

Figure 7: Sample sites for teak in Papua New Guinea

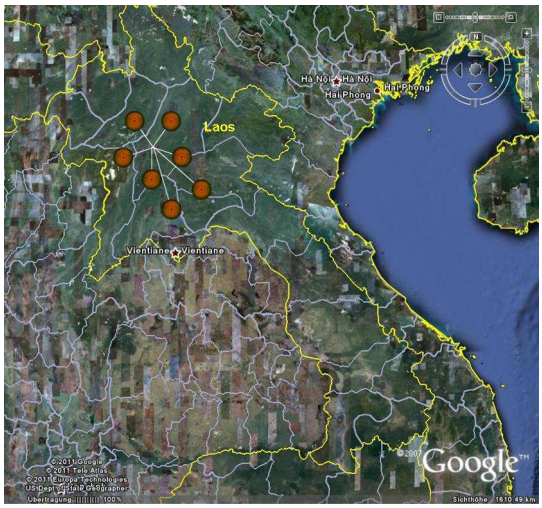


Figure8: Sample sites for teak in Laos

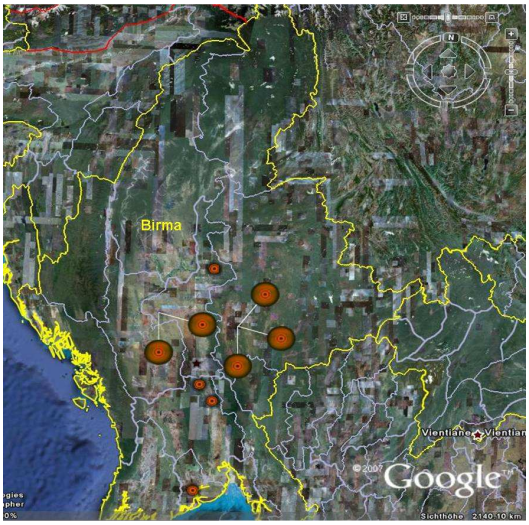


Figure9: Sample sites for teak in Burma

Figure10: Sample sites for teak in India

Figure11: Sample sites for mahogany in the Congo

Figure12: Sample sites for mahogany and teak in Ghana

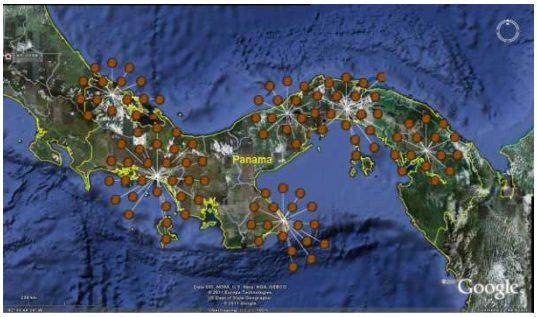


Figure 13: Sample sites for teak and mahogany in Panama

Figure14: Sample sites for teak in Panama

5.3.2 Preparation of the wood samples for the stable isotope analysis

The basic preparation process was applied from the first DBU project on this subject which focused on the tree species "Spruce". This process is described again below. However, it was necessary to optimise the pre-grinding because tropical wood, particularly teak and mahogany, are extremely hard compared to spruce. According to Brinell, teak has a hardness of 63 to 71 N/mm^2 compared to 12 N/mm^2 for spruce.

Consequently, wood preparation was enhanced by a Retsch SM100 centrifugal mill in the project. The wood samples are coarsely ground for preparation with the Retsch SM100 centrifugal mill after being dried at 103°C. Aliqouts weighing approx. 5g are extracted after grinding with apolar (dichlormethane) and polar (methanol) solvents in the Soxhlet for 5 hours. The purpose of this pre-cleaning is to completely condition the wood samples because e.g. teak can have different oil contents.

After a second drying phase at 103°C, the samples are finely ground in ball mills so that a homogenous amount of approx. 1 to 2 mg can be guaranteed. The D/H, $^{18}O/^{16}O$ and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios are directly determined from these prepared samples. Due to the extremely small quantity of nitrogen (<0.1%), measuring the $^{15}N/^{14}N$ isotope ratios represents a great obstacle to measuring the stable isotopes.

Based on the current level of knowledge, no stable isotope literature that focused on measuring wood samples could be referenced for this project. Tree analysis generally relies on nitrogen-rich parts of the tree such as leaves or needles.

As a result, the new generation of isotope mass spectrometers was used to measure the $^{15}N/^{14}N$ isotope ratios: NU-Horizon was used which guarantees a high level of linearity of isotope measurement even with the smallest quantities of nitrogen.

This isotope mass spectrometer was connected to a semi-element analyser which can analyse up to 100 mg of sample material. This is equivalent to approx. 10 times the quantity of the capacity of a "regular" element analyser. The aim was to transfer a measurable quantity of nitrogen to the isotope mass spectrometer using a larger quantity of sample material. It is, however, problematic that, for this large quantity of material, carbon dioxide can form as a product of incomplete combustion. A mass of 28 is also detected for this carbon monoxide, the same as for nitrogen, which can falsify the measurement. The carbon monoxide not only forms through combustion, it also forms directly through the breakdown of carbon dioxide in the mass spectrometer which is why all of the carbon dioxide must be removed prior to the measurement.

Figure 15: SM100 centrifugal mill for initial breakdown of wood samples

These requirements were satisfied by removing carbon dioxide with the patented trapping system made by the company Elementar and by using packed columns to separate carbon dioxide and nitrogen.

The measurement of the $^{34}S/^{32}S$ isotope ratio was combined with a concentration in which copper oxide and wood powder were carefully mixed in a 1:2 ratio to produce a sample amount weighing about 1 g which was then converted overnight in a muffle furnace at 350°C by adding doses of oxygen. On the one hand, the copper oxide supports the combustion as oxidans and, on the other, the sulphur dioxide that forms binds the sulphur as copper sulphate. The soluble copper sulphate can then be dissolved with water and measured right in the element analyser after drying.

The strontium isotopes were identified with concentrated nitric acid from the diluted eluent after a microwave digestion. A combustion temperature of 750°C was used for approx. 400 mg of wood sample for the microwave digestion.

A sub-aspect of the project was also the comparison of the hydrogen isotope ratios of all of the cellulose vis-à-vis to the nitrated cellulose of the wood samples. Various comparison samples were prepared for this sub-aspect in accordance with the specification from Brendel [BRENDEL, 2000].

5.4 Results of the isotope ratios of teak

Reference samples were supplied from 14 different countries (Figure 2) for the teak analysis. Overall, the reference samples were broken down into 7 countries in Asia, 5 countries in the Americas and 2 countries in Africa.

Sampling was ideal in Java (Figure 6). In addition to reference samples of 124 wood samples collected from almost the entire island, more in-depth information on the location, such as the distance to the ocean, was also provided.

This makes a more in-depth, detailed analysis of the region possible. Samples from many other regions could only be obtained in small areas of some countries. The resulting differentiations thus relate to these regional origins.

The discriminant analysis (DA) was used as a statistical analysis model to identify the possibility of differentiation. The goal of this discriminant analysis is to find a linear combination of variables or attributes to separate predefined, independent groups from one another. Separation takes place by identifying a specific number of measurable attributes of these objects and by establishing what is known as a discriminant function. Establishing or predicting the discriminant function is similar to predicting a regression function in regression analysis. The discriminant analysis then allocates a sample to one of several alternative groups based on its measurements.

An example of this is shown in the figure below:

von \ nach	Birma	Java	Laos	Gesamtwert	% korrekt
Birma	11	3	1	15	73,33%
Java	0	128	0	128	100,00%
Laos	0	0	30	30	100,00%
Gesamtwert	11	131	31	173	97,69%

Figure 16: Key statistical data for teak from Burma, Java and Laos; consistency of the samples (lines) with the references from the regions (columns)

A total of 15 samples are available from Burma, 128 from Java and 30 from Laos (total value). A discriminant function is calculated on the basis of these samples and it is tested using the following allocation (cluster analysis) of the individual samples to the three possible sample sets. This may give rise to misallocations, i.e. 3 samples from the Burma sample set can only be allocated to the sample set from Java because they are so similar. As a result, the probability of correct allocation is calculated on this basis.

5.4.1 Detailed analysis: Java

5.4.1.1 D/H and $^{18}O/^{16}O$ isotope ratios in Java

With an area of 127,000 km^2, the island of Java covers an area approx. 1/3 the size of Germany. The island generally extends between the 105° and the 115° eastern longitude. The extension between the southern latitude from 6° to 8° is small.

Because there is almost no change in latitude, the D/H and $^{18}O/^{16}O$ isotope ratios in the water are not expected to vary greatly as a result of the "latitude effect".

Direct water references from the Java region were unfortunately not available. For this reason, the model data in the OIPC database was used. This database is based on data and algorithms created by Bowen et al. [BOWEN 2002, 2003, 2005] that are suitable to interpolate the mean $^{18}O/^{16}O$ and D/H isotope ratios in the water for specific locations (Figure17).

Based on this data, the conclusion can be reached that the D/H and $^{18}O/^{16}O$ isotope ratios of the water only fluctuate in a narrow range from 1‰ in the $^{18}O/^{16}O$ and 9‰ in the D/H isotope ratios for the entire Java region.

Figure17: Calculated D/H and $^{18}O/^{16}O$ isotope ratios of groundwater in the Java region

The results of the D/H and $^{18}O/^{16}O$ isotope ratios of the samples in Java (Figure 18, Figure19) correspond to these figures. The mean values (n=5) for $^{18}O/^{16}O$ of the cellulose do not show any significant geographic trend toward enriched or depleted isotope ratios along the eastern longitude at the various locations with an R^2 of 0.23. In contrast, there is an identifiable trend for enriched D/H isotope ratios assumed along the eastern longitude for the D/H isotope ratios with an R^2 of 0.53 (Figure20).

There were no correlations along the southern latitude either for D/H or $^{18}O/^{16}O$. However, this could also be affected by the location of these reference samples because this eastern region is where many of the main sample sites are located.

The results have a special significance because the distance from the ocean was also supplied as additional information for the majority of samples.

To ensure that no single effect is given too much value, the samples were clustered based on distance and the mean value calculated for each group. This produced four clusters with different distances from the coast (REF _Ref297196022 \h).

These four individual mean values are normalised to the total mean value so that only the deviation is analysed. A divisor of 8 is also used for the D/H isotope ratios so that the $^{18}O/^{16}O$ and D/H isotope ratios can be directly compared to one another. The divisor 8 is derived from the "meteoric water line" (chapter 5.2.1) which describes the correlation of the D/H and $^{18}O/^{16}O$ isotope ratios in the groundwater in the precipitation and in the groundwater.

Taking into account the D/H and $^{18}O/^{16}O$ isotope ratios, a significant depletion can be observed compared to the regions farther from the coast. The $^{13}C/^{12}C$ isotope ratios, on the other hand, remain almost constant or show no tendency for the isotope ratios to enrich or deplete.

What is striking is the correlation $R^2 = 0.99$ of the $^{18}O/^{16}O$ isotope ratios to the D/H isotope ratios in the cellulose (REF _Ref297195560 \h). The correlation, however, no longer reflects the correlation of the "meteoric water line" but the slope factor was lowered to 6.5. This change in the correlation has already been proven in various other plants [DUNBAR 1983].

The $^{18}O/^{16}O$ isotope ratios are more enriched than the D/H isotope ratios through the effects of evaporation so that a slope of 2.5 was observed instead of 8 [EPSTEIN 1977]. There are different correlation lines for the plant between the "meteoric water line" and the evaporation line, here with the slope 6.5 (Figure22) due to the morphology, the physiology of the water balance and the climate conditions.

It can also be concluded here that even though there is a significant correlation of the $^{18}O/^{16}O$ and D/H isotope ratios (Figure22), the information content for the geographical statement of origin is different. This makes the correlation extremely useful because it also contains climate origin information independently of the isotopic composition of the initial water.

Figure 18. D/H isotope ratios of teak (cellulose) in Java

Figure19: $^{18}O/^{16}O$ isotope ratios of teak (cellulose) in Java

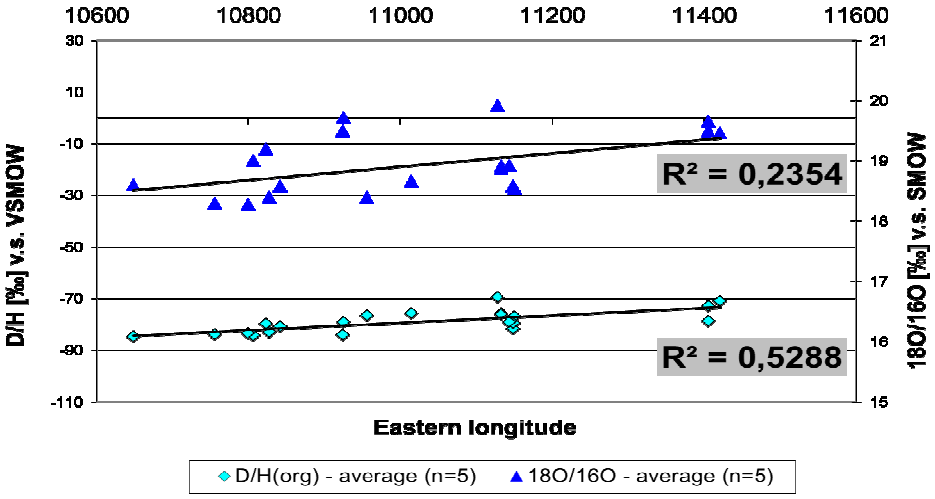


Figure20: Dependency of the D/H and $^{18}O/^{16}O$ isotope ratios of teak (cellulose) on longitude

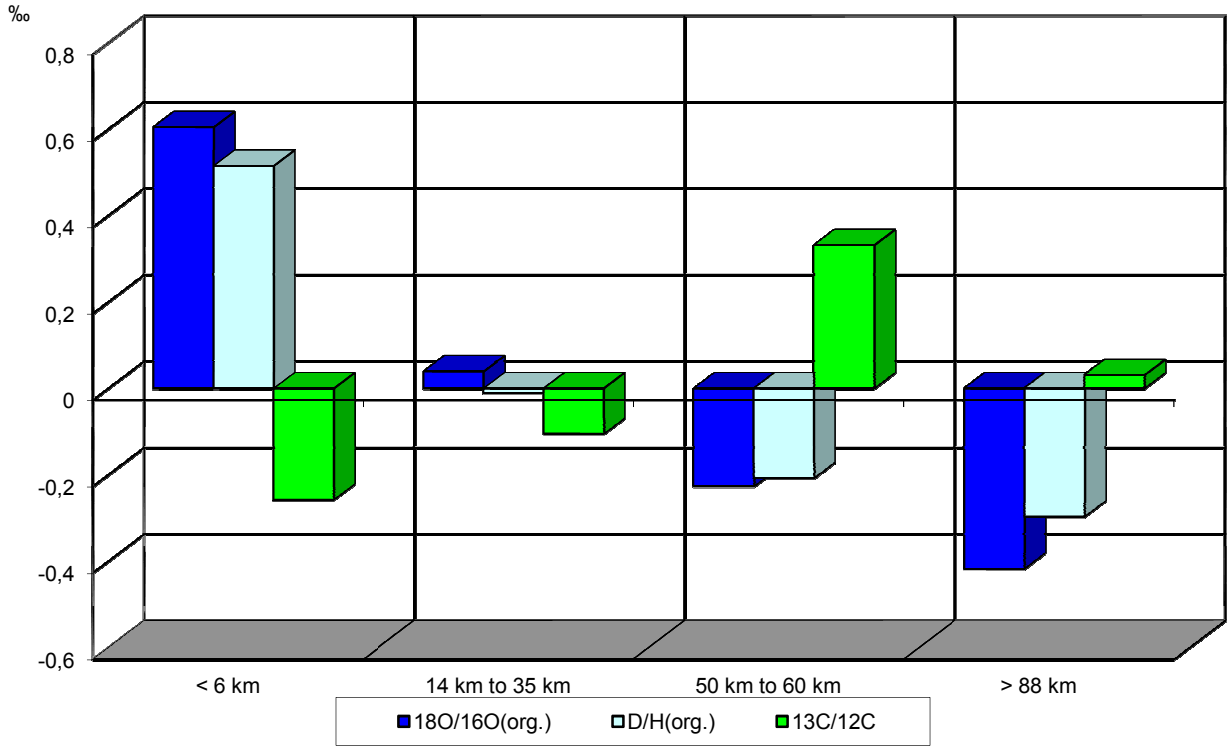


Figure21: Dependency of the $^{18}O/^{16}O$, D/H and $^{13}C/^{12}C$ isotope ratios in teak (cellulose) on the distance to the ocean in Java

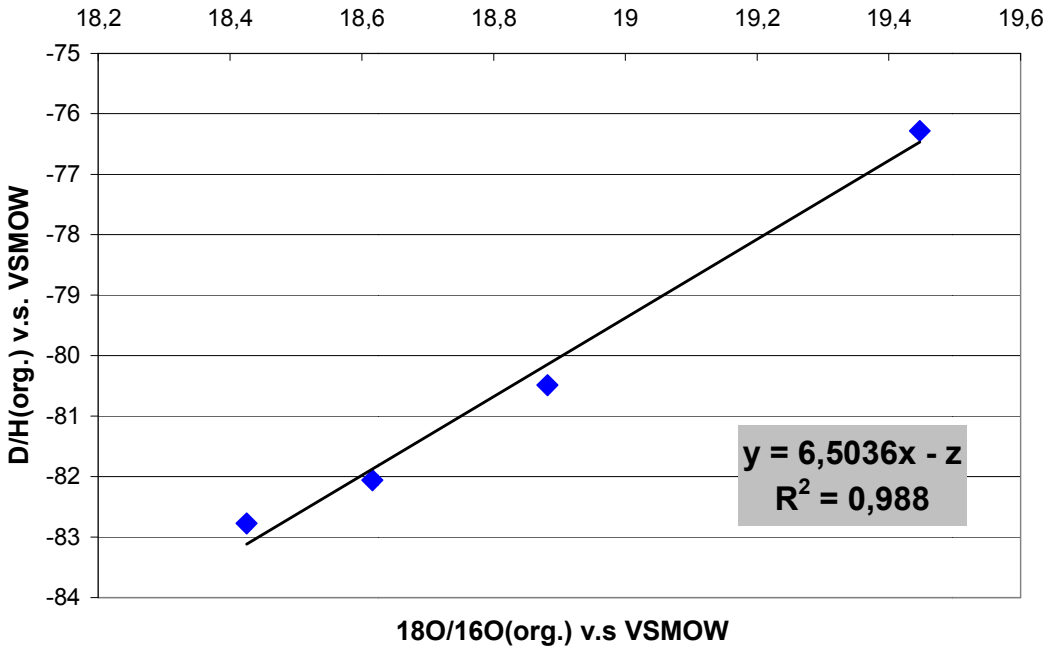


Figure22: Correlation of the D/H and $^{18}O/^{16}O$ isotope ratios of the four sample sets taking into consideration the distance to the ocean in Java

The higher enrichment of the $^{18}O/^{16}O$ isotope ratios compared to the D/H isotope ratios, particularly in the continental region of Java, could be a reason for the poor correlation of the $^{18}O/^{16}O$ isotope ratios along the eastern longitudes because the depletion that generally occurs (see D/H) is interfered with.

5.4.1.2 The $^{13}C/^{12}C$ and $^{87}Sr/^{86}Sr$ isotope ratios in Java

The $^{13}C/^{12}C$ isotope ratios in the reference samples yield a mean value of -26.3 +/- 0.6‰. There is no regionally specific trend (Figure23); this also applies to the continental effect mentioned above. Accordingly, Java cannot be further broken down using the $^{13}C/^{12}C$ isotope ratios. Instead the total mean value of -26.3 +/- 0.6‰ represents the basic signature for the entire island. A similar result was also obtained for strontium. There is again no regionally specific trend and the mean value for Java can currently be determined with 0.708 +/- 0.004 (Figure – the isotope ratios were factored with 1000 to provide a better representation).

Figure23: $^{13}C/^{12}C$ isotope ratios in teak (cellulose) in Java

Figure 24: Isotope ratios of strontium (87Sr/86Sr) in Java, factored with 1000

5.4.1.3 The $^{15}N/^{14}N$ und $^{34}S/^{32}S$ isotope ratios in Java

The nitrogen and sulphur isotope ratios generally reflect geological features of the soil.

Sulphur, in particular, shows considerable variation in Java, meaning that the $^{34}S/^{32}S$ isotope ratios span a large range. In line with this, there are both extremely depleted $^{34}S/^{32}S$ isotope ratios of -5‰ as well as significantly enriched $^{34}S/^{32}S$ isotope ratios of up to 13.8‰ (Figure 25). These types of enrichments are particularly typical for wood from South America.

On the one hand, this variation makes it difficult to determine origin because there is not a clear, unique pattern for Java. On the other hand, Java can be broken down more clearly into regions and allocation undertaken within Java on a small scale.

Small-scale allocation is also reflected in the respective measurements of the references from the individual regions (see Figure 25).

This enormous range of isotope ratios of more than 19‰ is not found again among the $^{15}N/^{14}N$ isotope ratios (Figure 26). On the contrary, there are significantly depleted or negative $^{15}N/^{14}N$ isotope rations across the entire region of Java. Without the exception of the coast reference at Semarang with a $^{15}N/^{14}N$ isotope ratio of +1.2‰, there is only a narrow range of significantly depleted $^{15}N/^{14}N$ isotope ratios between -2.9‰ and -0.1‰. There is not a regionally specific trend with the exception of the local characteristic already mentioned.

On average overall, depleted $^{15}N/^{14}N$ isotope ratios of -1.2 +/- 0.8‰ are to be assumed in Java.

The depleted $^{15}N/^{14}N$ isotope ratios thus result in a special signature for Java that is very useful in determining origin, see: Difference between Java and Burma.

Figure 25: Isotope ratios of sulphur (34S/32S) in Java

Figure26: Isotope ratios of nitrogen ($^{15}N/^{14}N$) in Java

5.4.1.4 Fluctuation range of the D/H and $^{18}O/^{16}O$ isotope ratios in the various sample sites

One of the sampling requirements was to take up to five samples from one location. This requirement was completely satisfied in Java in particular.

This requirement is useful for projecting the fluctuation range of the isotope ratios particularly for D/H and $^{18}O/^{16}O$.

The $^{15}N/^{14}N$ and $^{34}S/^{32}S$ isotope ratios, on the other hand, can yield large fluctuations locally. The isotope ratios are particularly interesting with respect to a large-scale discriminant parameter as was explained in the previous section.

Overall, there were narrow D/H and $^{18}O/^{16}O$ distributions for teak for the 26 sample sites (Figure 27, Figure 28).

Almost 50% of the samples yielded standard D/H deviations of ±3 to ±4‰ at the sample sites. A corresponding number have standard $^{18}O/^{16}O$ deviations between ±0.2 and ±0.4 ‰. If the error of reproducibility is taken into account, which is ±2 for D/H and +0.2 for $^{18}O/^{16}O$, it becomes evident that a majority of the samples lies within the measurement error and very constant isotope signatures are to be expected in the region.

The fluctuation range of the $^{18}O/^{16}O$ isotope ratios is slightly greater than that of the D/H isotope ratios here. The maximum standard deviation of the D/H isotope ratios of a sample site lies below +6‰; in contrast, the $^{18}O/^{16}O$ isotope ratios are below +1‰. If one assumes the usual correlation of the "meteoric water line", a maximum standard $^{18}O/^{16}O$ deviation of less than 0.8‰ can be expected in relation to the maximum D/H isotope ratios. In line with this, the $^{18}O/^{16}O$ isotope ratios are to be assessed as slightly inferior in their significance.

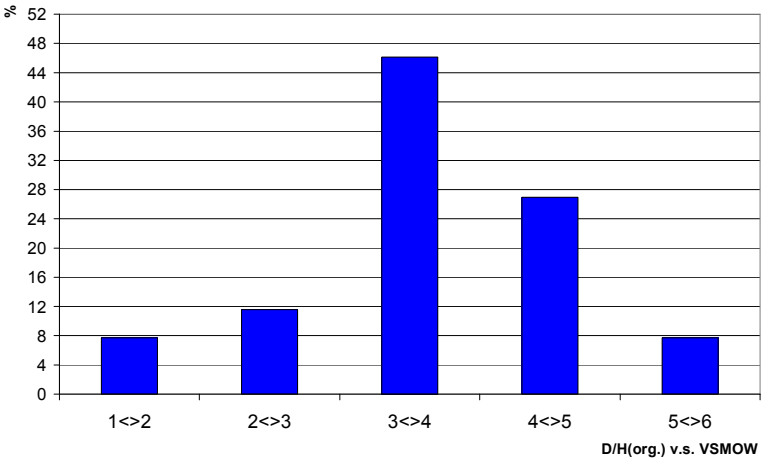


Figure 27: Standard deviation of the D/H isotope ratios of teak (cellulose) of the 26 sample sets

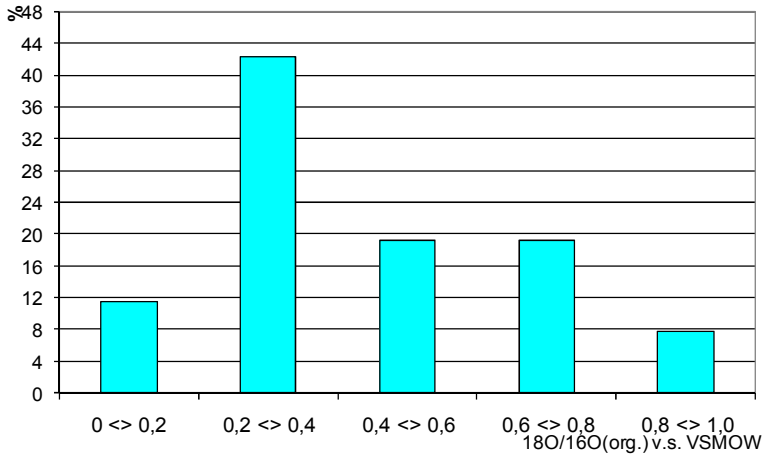


Figure 28: Standard deviation of the $^{18}O/^{16}O$ isotope ratios of teak (cellulose) of the 26 sample sets

5.4.2 Differentiation of teak from Asia particularly Java, Burma, Laos and India

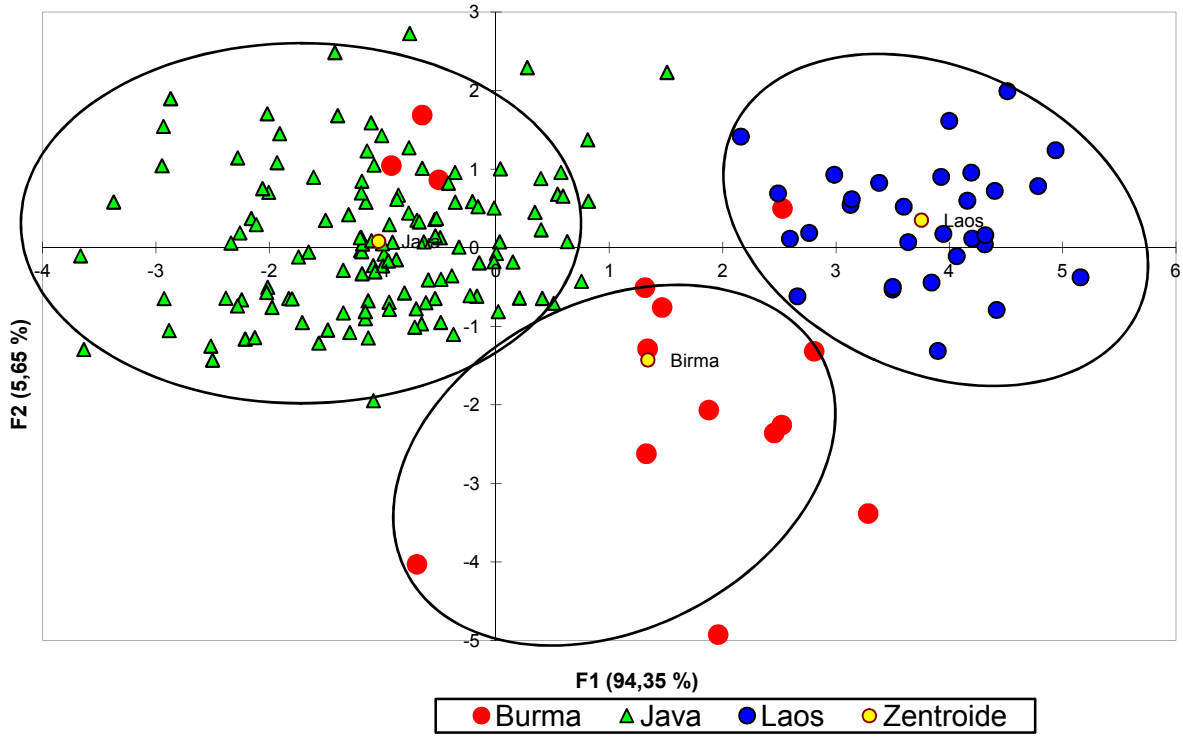
In addition to the references from Java, sampling for teak was expanded to other regions and other relevant countries where it is grown in Asia.

The differentiation and evidence of samples from Burma offer an important example of the possibilities of application.

According to Regulation (EC) 194/2008 of the Council from 25 February 2008, importing timber, particularly from Burma, to the European Union is no longer permitted. Old stocks in Germany can still, however, be sold. It stands to reason that three-way transport e.g. through Laos or Java, can continue to guarantee sales of Burma timber in the European Union.

There are currently 30 references available from Laos for differentiation. 11 samples were provided from Burma and the 128 teak samples from Java already mentioned above were also available.

Differentiation is largely possible using the stable isotopes of the five elements of hydrogen, nitrogen, carbon, oxygen and sulphur (Figure 28).



From \ to	Burma	Java	Laos	total	% correct
Birma	11	3	1	15	73,33%
Java	0	128	0	128	100,00%
Laos	0	0	30	30	100,00%
total	11	131	31	173	97,69%

Figure 29: Differentiation (DA) and key statistical data for teak from Burma, Java and Laos using stabile isotopes of the bioelements.

The table in Figure 28 shows that the samples from Java and Laos can always be significantly differentiated from one another. In contrast, three samples from Burma are misallocated to Java and one to Laos.

Currently, a discrimination rate of approx. 74% can be assumed for samples from Burma taking into account Laos and Java. This differentiation can be attributed, on the one hand, to the significantly enriched D/H isotope ratios of the samples from Burma compared to those from Laos. In Burma, there are on average D/H isotope ratios of -84 ± 5‰ compared to depleted D/H values in Laos of approx. -93 ± 5‰.

On the other hand, however, the D/H isotope ratios in Java and Burma are quite similar. Consequently, the differences in the $^{15}N/^{14}N$ isotope ratios must be indicated. In Java, as already shown above, depleted $^{15}N/^{14}N$ isotope ratios are to be expected. In contrast, the $^{15}N/^{14}N$ isotope ratios of the samples from Burma are significantly more positive or enriched (Figure 30). This can be used accordingly for further differentiation of the two countries.

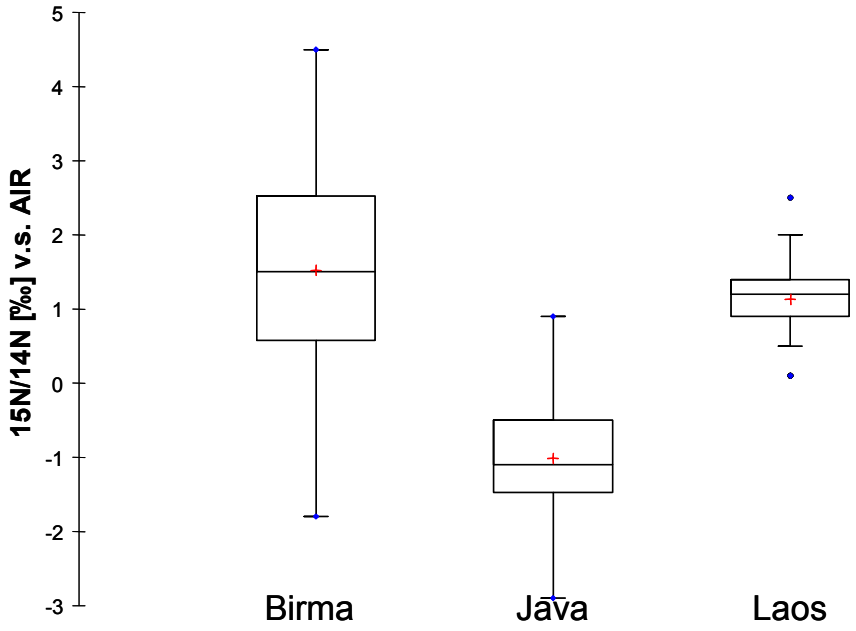
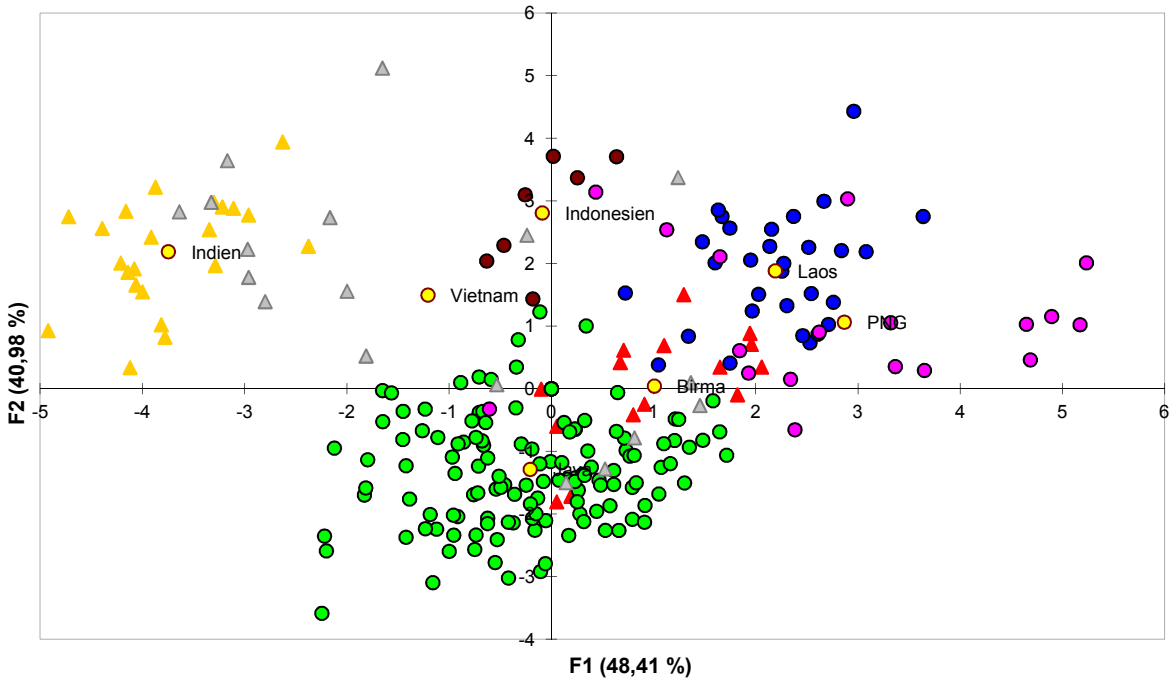


Figure 30: Box plots of the $^{15}N/^{14}N$ isotope ratios of teak in Burma, Java and Laos

5.4.3 *Further differentiation of teak from Asia taking into account India and Indonesia, Papua New Guinea and Vietnam*

If differentiation is expanded without restriction, the result is necessarily overlapping of the patterns (Figure 31). This can be explained by the fact that the isotope patterns reflect Gaussian distributions of the individual regions. The result is overlaps between the various regions for the individual isotopes. A key component of the isotope analysis and the evaluation is thus always the link to additional information or information about origin.

Aside from this, however, it must be emphasised that at least 5 of the 7 sampled countries have significant isotope patterns that guarantee good differentiation (see REF _Ref297196844 \h).



von \ nach	Burma	India	Indonesia	Java	Laos	PNG	Vietnam	total	% correct
Burma	6	0	0	6	1	2	0	15	40,00%
India	0	22	0	0	0	0	0	22	100,00%
Indonesia	0	0	6	0	1	0	0	7	85,71%
Java	0	0	0	127	1	0	0	127	99,25%
Laos	0	0	0	0	30	0	0	30	100,00%
PNG	1	0	1	1	1	14	0	18	77,78%
Vietnam	2	4	1	3	0	0	8	18	44,44%
total	9	26	8	142	34	16	8	238	89,50%

Figure 31: Differentiation (DA) and key statistical data for teak from 7 different Asian regions

In addition to Laos and Java, India in particular is to be referenced which has a discrimination rate of 100% compared to the other countries.

The $^{34}S/^{32}S$ isotope ratios of the sampled region in India are particularly relevant for this result. They yield a significant pattern with enriched $^{34}S/^{32}S$ values of +10.7 ± 0.7‰ on average. This makes it possible, for example, to significantly differentiate samples from India from samples from Vietnam (Figure 32). These enriched $^{34}S/^{32}S$ isotope ratios are also a unique feature compared to the other 5 Asian regions and are thus useful for differentiation.

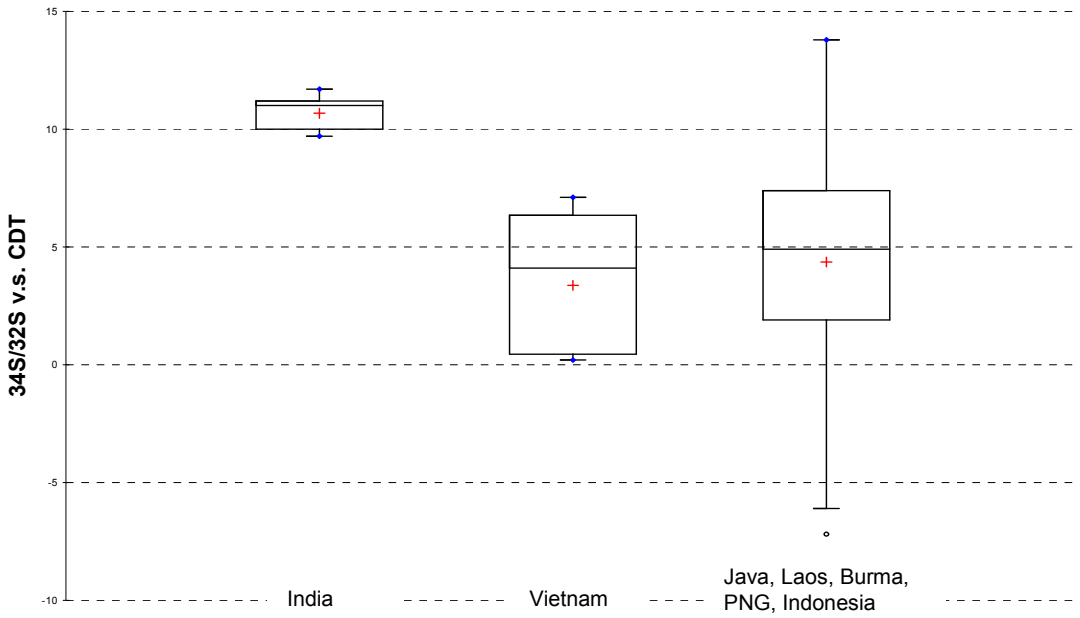
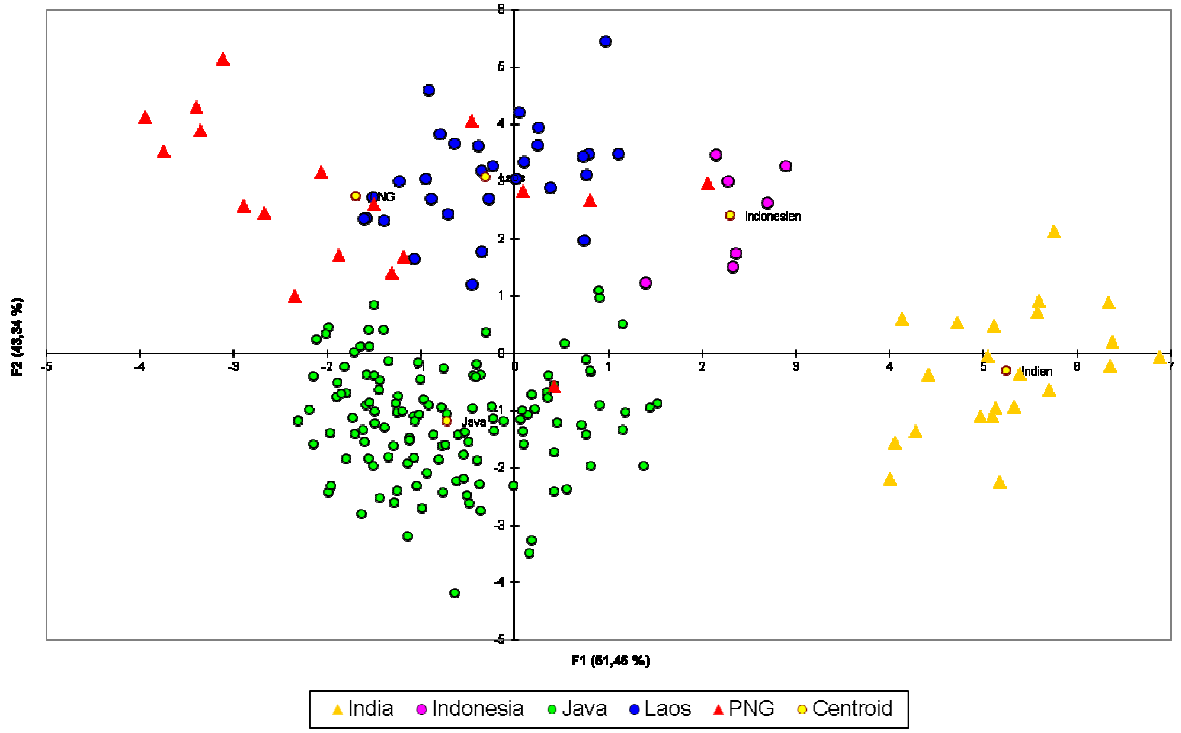


Figure 32: Box plots of the $^{34}S/^{32}S$ isotope ratios of India and Vietnam and other Asian countries

In addition to the three regions of Laos, Java and India, there are also good differentiation patterns in Indonesia and Papua New Guinea with probabilities of almost 78% (Papua New Guinea) and 86% (Indonesia).

If a possible evaluation is restricted solely to these five countries/regions, there is an extremely high discrimination rate of approx. 98% (Figure 33).



from \ to	India	Indonesia	Java	Laos	PNG	total	% correct
Indien	22	0	0	0	0	22	100,00%
Indonesien	0	7	0	0	0	7	100,00%
Java	0	0	127	1	0	128	99,22%
Laos	0	0	0	29	1	30	96,67%
PNG	0	1	1	1	15	18	83,33%
total	22	8	128	31	16	205	97,56%

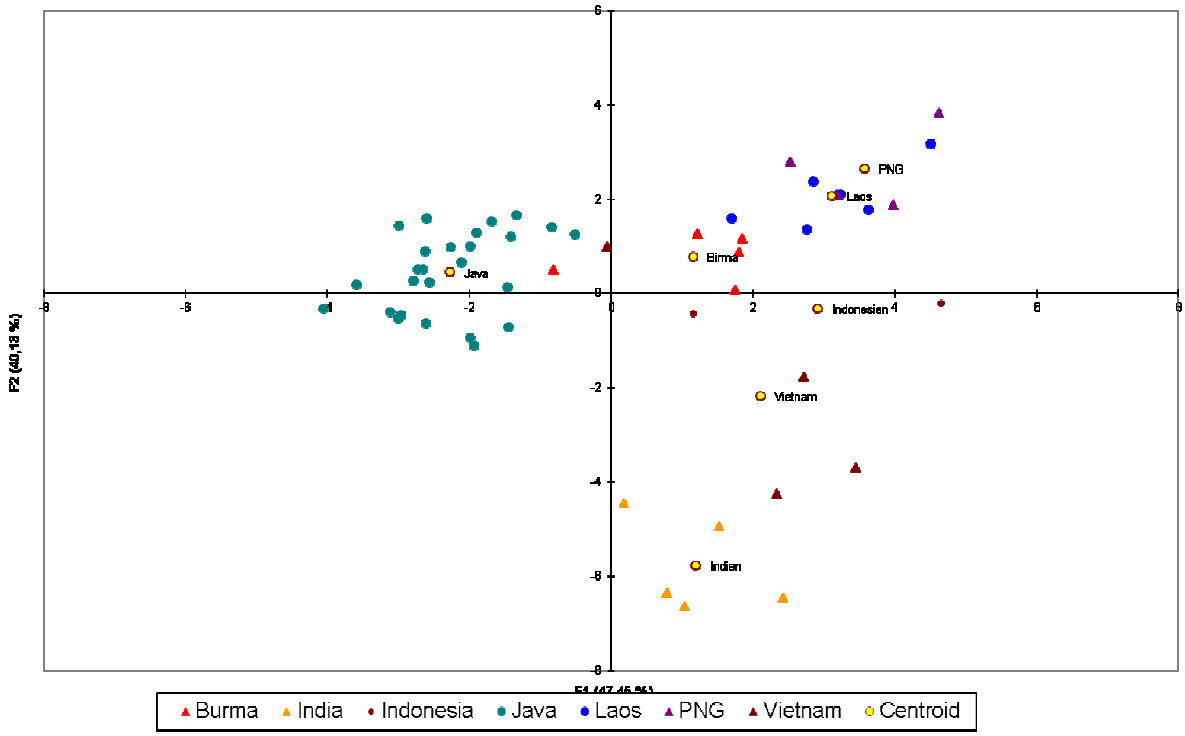
Figure 33: Differentiation (DA) and key statistical data for teak limited to 5 regions in Asia

The isotope patterns of Vietnam and Burma are still currently problematic and susceptible to error. There are overlaps and/or similarities in widely varying regions for both (Figure 33).

However, when the possible areas of origin are limited, differentiation can be considerably optimised. To improve differentiation, individual samples can be merged into mean values. For this reason, the goal was to obtain five references from each location in the various regions.

When up to five samples from a region are merged, differentiation is significantly better for the seven regions investigated in Asia (Figure 34).

The 52 different mean values yield an overall differentiation of more than 96%. Differentiation is now completely possible for the five regions already mentioned consisting of Laos, Java, India, Papua New Guinea and Indonesia. Only Burma and Vietnam still produce misallocations.



from \ to	Burma	India	Indonesia	Java	Laos	PNG	Vietnam	total	% correct
Burma	4	0	0	1	0	0	0	5	80,00%
India	0	5	0	0	0	0	0	5	100,00%
Indonesia	0	0	2	0	0	0	0	2	100,00%
Java	0	0	0	26	0	0	0	26	100,00%
Laos	0	0	0	0	6	0	0	6	100,00%
PNG	0	0	0	0	0	4	0	4	100,00%
Vietnam	1	0	0	0	0	0	3	4	75,00%
total	5	5	2	27	6	4	3	52	96,15%

Figure 34: Differentiation (DA) and key statistical data for teak limited to 5 regions in Asia

5.4.4 *Differentiation of teak from Latin America particularly Panama, Costa Rica, Honduras and Brazil*

Another focus of sampling for teak included regions in Latin America. Teak samples were provided particularly from the regions of Honduras, Costa Rica, Panama and Brazil

Taking into account all of the stable isotopes of the bioelements, 100% differentiation was only possible from the more than 112 references for Honduras at the beginning. This can be attributed to the isotope ratios of hydrogen (D/H) and oxygen ($^{18}O/^{16}O$) in the Honduras region (REF _Ref297197059 \h).

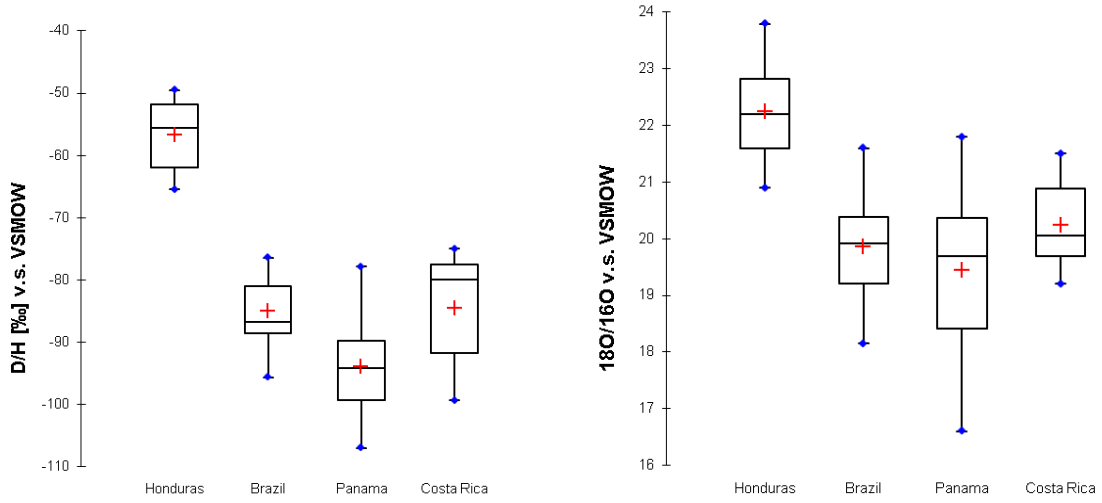
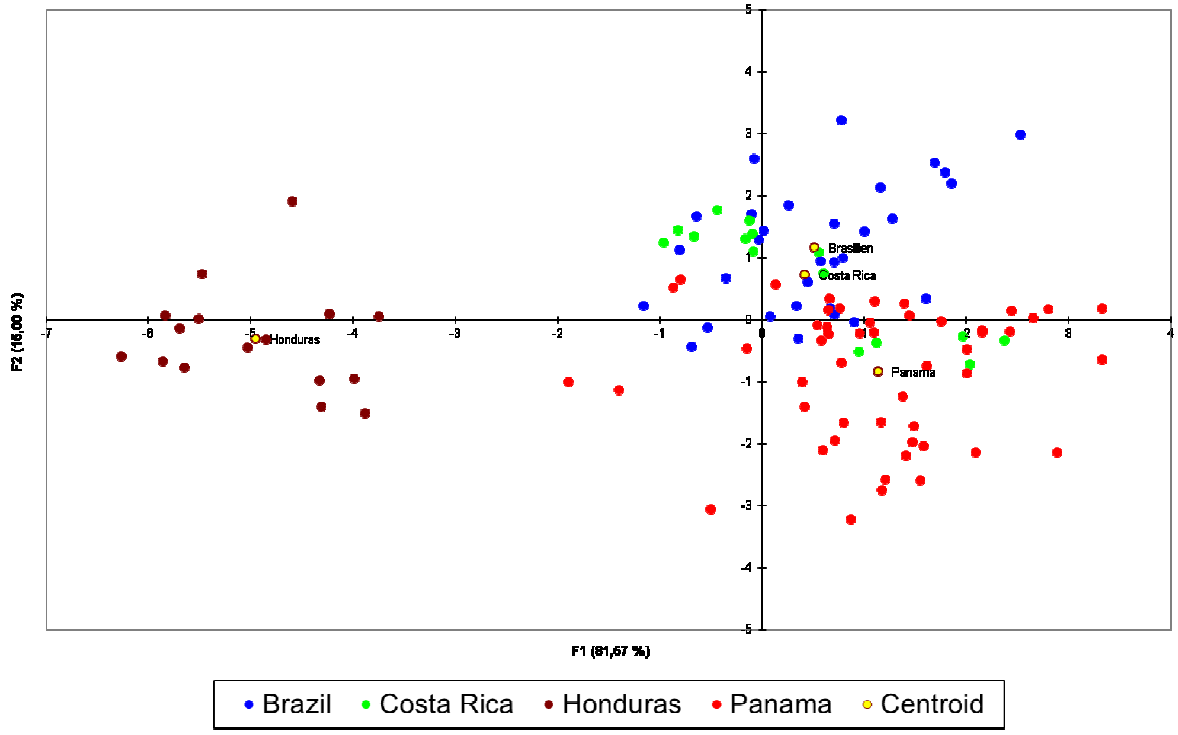


Figure 35: Box plots of the D/H and $^{18}O/^{16}O$ isotope ratios of the regions: Honduras, Brazil, Panama and Costa Rica in comparison

At a detail level, Honduras showed enriched values which vary significantly from the other regions particularly in the D/H isotope ratios and make complete differentiation in this region possible. A corresponding enrichment was also observed for the $^{18}O/^{16}O$ isotope ratios. However, the high significance of the D/H isotope ratios is lacking, meaning that the measurements overlap with those of the other three regions.

The three other regions are more difficult to differentiate or can only be differentiated incompletely using the stable isotopes of the bioelements (D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) (Figure 36). Only Panama has a clear isotope pattern with a probability of differentiation of more than 94% compared to the other regions.



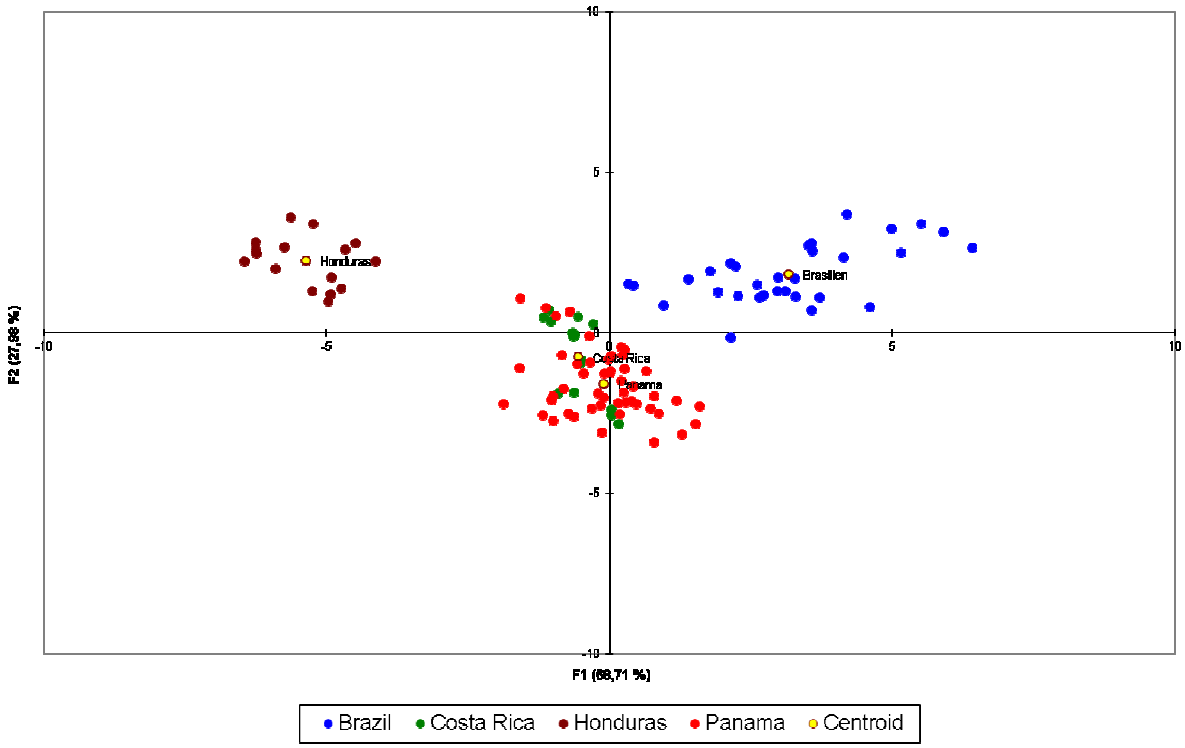
from \ to	Brazil	Costa Rica	Honduras	Panama	total	% correct
Brazil	22	1	0	8	31	70,97%
Costa Rica	2	8	0	5	15	53,33%
Honduras	0	0	16	0	16	100,00%
Panama	1	2	0	47	50	94,00%
total	25	11	16	60	112	83,04%

Figure 36: Differentiation (DA) and key statistical data for teak from 4 different regions in Latin America

Over the course of the project, it became evident that the isotope ratios of the strontium did not improve differentiation much so that they are often not shown.

There is an exception for differentiation of the four regions in Latin America. The strontium isotopes provide, particularly for the Brazilian samples, a significant improvement in differentiation.

This can be accounted for by the unusual enrichment in isotope ratios of the strontium of 0.726 ± 0.005 compared to 0.711 ± 0.006 for the other regions in Latin America. This improves the probability of differentiation from approximately 71% to 94% (REF _Ref297197089 \h).



from \ to	Brazil	Costa Rica	Honduras	Panama	total	% correct
Brazil	29	2	0	0	31	93,55%
Costa Rica	0	10	0	5	15	66,67%
Honduras	0	0	16	0	16	100,00%
Panama	0	3	0	47	50	94,00%
total	29	15	16	52	112	91,07%

Figure 37: Differentiation (DA) and key statistical data for teak from Latin America taking into account the ^{87}Sr / ^{86}Sr isotopes

This does not, however, apply for differentiation of samples from Panama and Costa Rica. Even taking into account the isotope ratios of the strontium, differentiation is still insufficient particularly for Costa Rica at 67%. A key problem in differentiation is the origin of the samples which is limited to a small area. If one decodes the various samples taken in Panama, it must be stressed that samples were taken only 50 km away from Costa Rica (Figure 38).

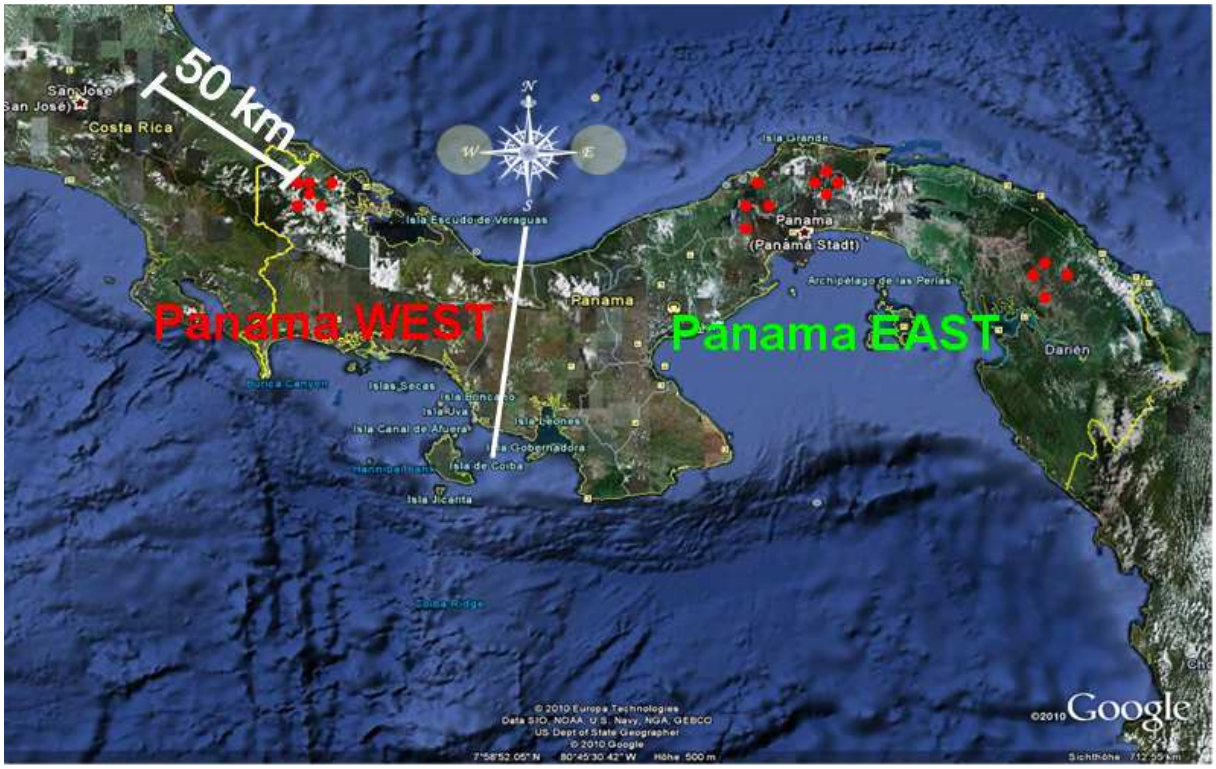
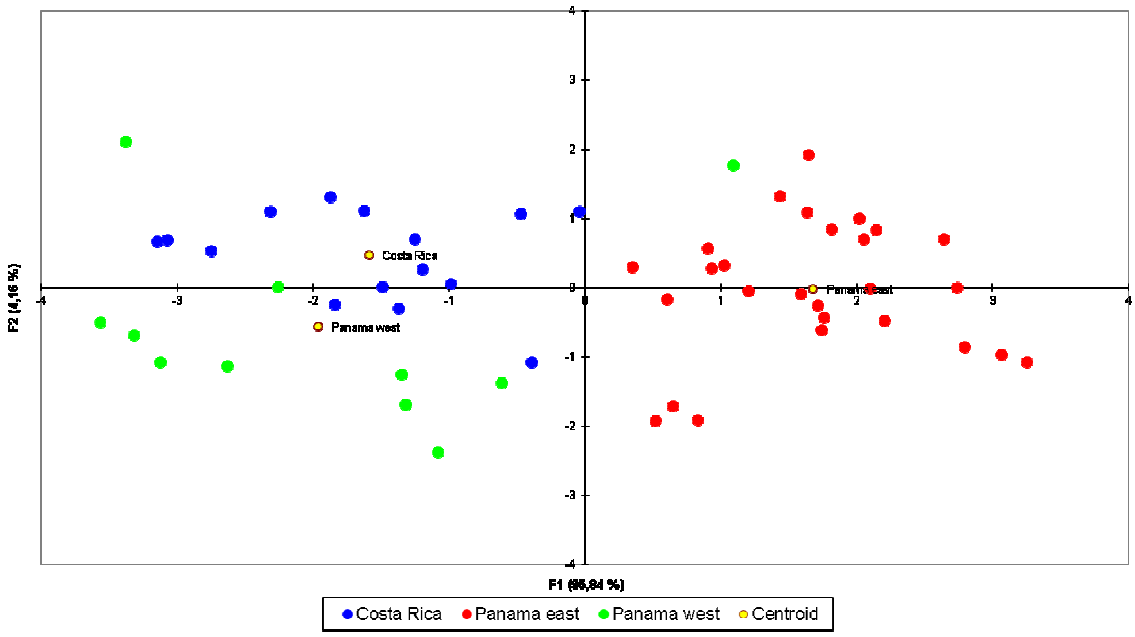


Figure 38: Sample sites in Panama and Costa Rica

If these small-area samples are taken into account and the references are divided into samples from Panama-West and Panama-East, the differentiation is significantly improved between these regions (Figure 39).

Dividing Panama into an eastern and western section takes into account the fact that the stable isotopes of oxygen and hydrogen only show significant changes across greater distances. The "geological" isotope such as nitrogen, sulphur and strontium can, however, but don't have to, guarantee small-scale differentiation. This is why it is often also useful to further sub-divide the isotope patterns of regions into geographic sub-groups.

Still, even with this breakdown into east and west groups, differentiation is at 92%, still insufficient to completely distinguish samples from the regions of Costa Rica and Panama.



from \ to	Costa Rica	Panama east	Panama west	total	% correct
Costa Rica	14	0	1	15	93,33%
Panama east	0	27	0	27	100,00%
Panama west	2	1	8	11	72,73%
total	16	28	9	53	92,45%

Figure 39: Differentiation (DA) and key statistical data of the regional breakdown of teak from Panama and Costa Rica.

To guarantee complete differentiation, linking to other chemical parameters or substances in the wood can be useful. Near-Infrared-Spectroscopy (NIR) offers a very interesting way to expand this analysis. This measurement is a quick and non-destructive method in which, for example, solids such as wood powder are placed in an infrared beam of near infrared and the reflection of the non-absorbed quantity is measured.

By using the energy-rich near infrared, molecule oscillations, particularly also of strong, chemical bonding, are stimulated such as covalent C-H carbon-hydrogen compounds. As a basic component to determine the chemical content, they characterise the organic-chemical and thus biochemical compounds and, even further, the agricultural product. They reproduce a chemical fingerprint that can be used for many purposes, in particular to determine content [NICOLAI 2007]. A number of basic and combination oscillations occur in this near-infrared range that result in distinct overlaps of the absorption bands. The spectrum can thus not be directly analysed. Instead the necessary information must be determined chemometrically using reference samples. Unlike the stable isotope method in which a physical fingerprint exists, in the NIR measurement, there is a chemical one. It is evident that this can also be a good way to help determine origin.

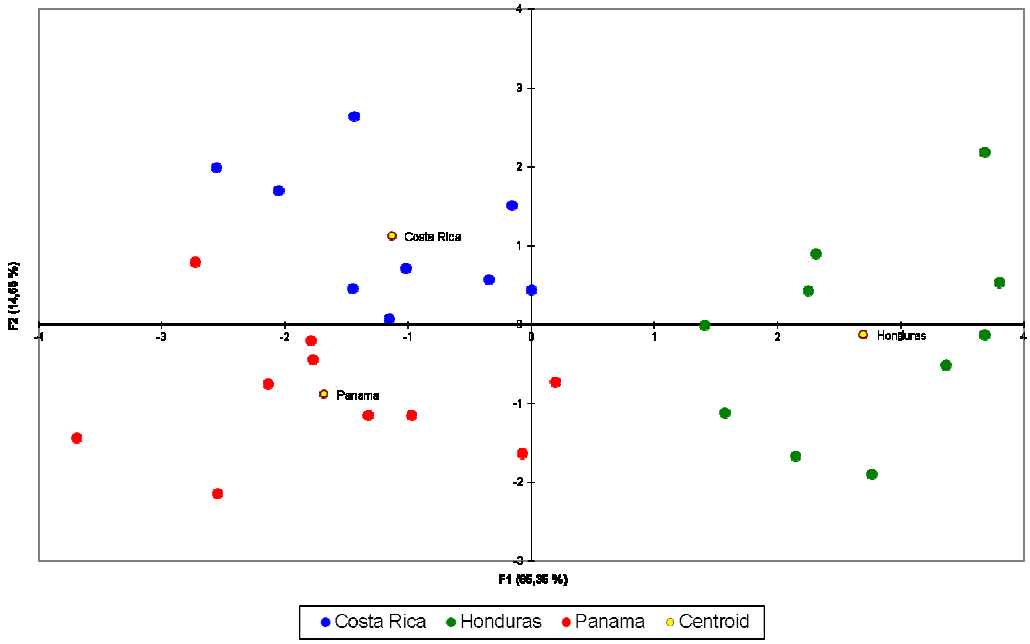
NIR measurement usually supplies a number of parameters across the spectrum. For instance, 2307 parameters were available from the frequency range of 3600 to 12500. Based on this, the frequency range of 3800 to 8000 was analysed and normalised with the slope variables.

The data quantity is reduced using a primary component analysis (PCA) so that in the end almost 90% of the original data information is available with 10 parameters from the primary component analysis.

It is necessary to concentrate the data quantity because otherwise the parameter diversity can easily lead to differentiation that is only the result of the high number of parameters and virtually every material can be distinguished from another.

29 samples from Costa Rica, Honduras and Panama were analysed in the NIR analysis.

These 29 samples could mostly be differentiated using the stable isotopes of the bioelements, in particular hydrogen, oxygen and carbon (Figure 40). Accordingly, the discrimination rate for the limited number of samples analysed is 93%.



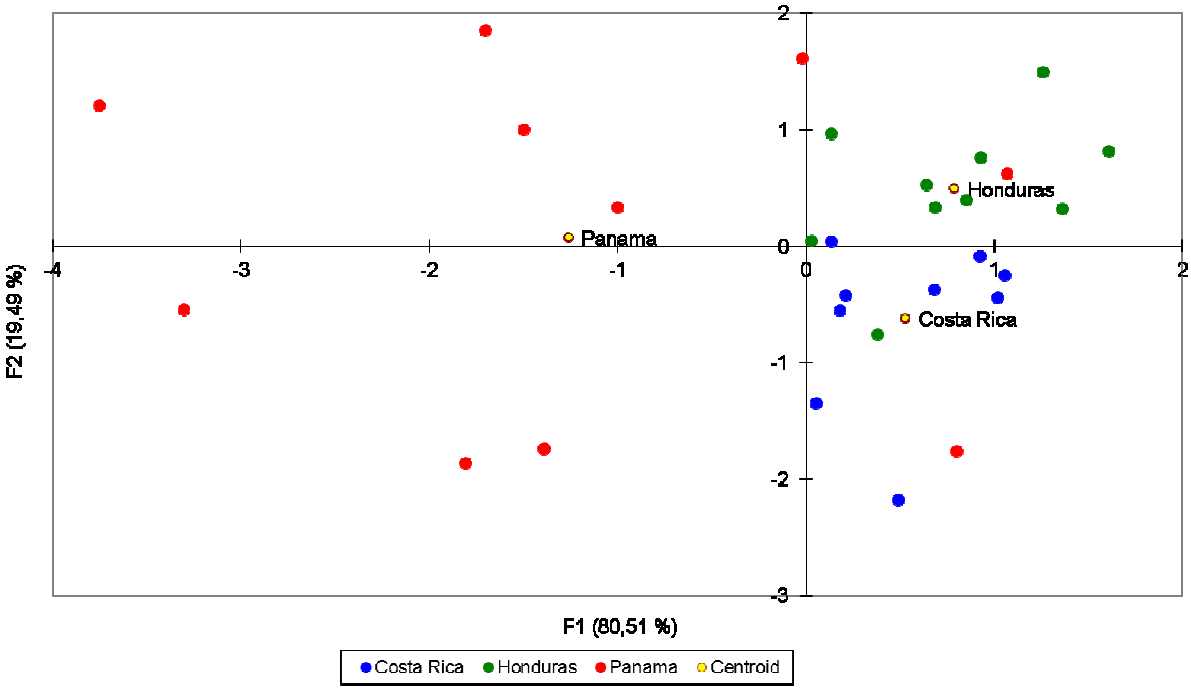
from \ to	Costa Rica	Honduras	Panama	total	% correct
Costa Rica	8	0	1	9	88,89%
Honduras	0	10	0	10	100,00%
Panama	1	0	9	10	90,00%
total	9	10	10	29	93,10%

Figure 40: Differentiation (DA) and key statistical data for teak from Costa Rica, Honduras and Panama selected for further differentiation with NIR

The samples were then also analysed with the 3 most important parameters from the PCA-NIR analysis. These 3 parameters contain a cumulative 62% of the original information from the NIR spectroscopy. The limitation to 3 parameters is primarily to allow for direct comparison of the significance of NIR and stable isotope data.

If only this NIR data is considered, there is no significant differentiation or differentiation quality is much worse (Figure 41). The limited data set only produces a discrimination rate of 75%. Differentiation is poor particularly for Panama and Costa Rica with a rate of between 66 and 70%.

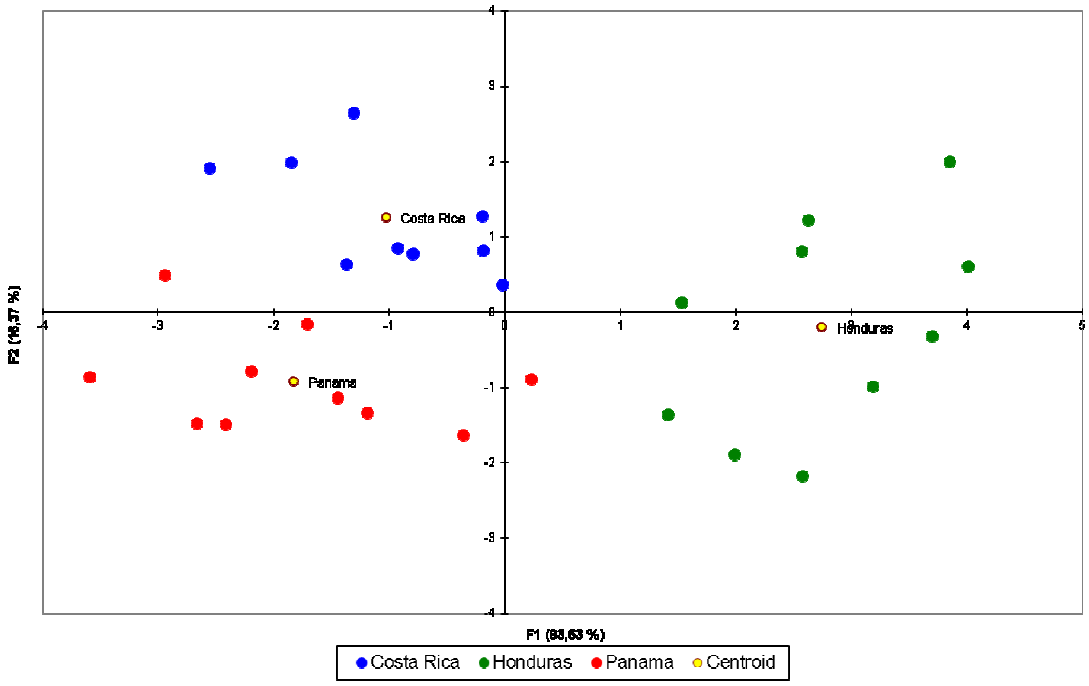
However, it should be emphasised that Honduras can be differentiated using the NIR data. In fact, the samples show a differentiation of 90%. From this, it can be concluded that the NIR analysis offers possibilities for improving differentiation in combination with the stable isotopes. However, this has to be further explored because the spectra themselves can only be evaluated through chemometric analysis.



from \ to	Costa Rica	Honduras	Panama	total	% correct
Costa Rica	6	3	0	9	66,67%
Honduras	1	9	0	10	90,00%
Panama	1	2	7	10	70,00%
total	8	14	7	29	75,86%

Figure 41: Differentiation (DA) and key statistical data of the NIR analysis from the selected 29 samples of teak from the regions Costa Rica, Honduras and Panama

Complete differentiation between Panama, Costa Rica and Honduras can be achieved by combining the three stable isotopes (D/H, $^{13}C/^{12}C$, $^{18}O/^{16}O$) with the most important parameters from the PCA-NIR analysis which contain 35% of the original information (Figure 42). Combining the two methods is currently still in the early stages and has to be tested as well as calibrated for other references.



from \ to	Costa Rica	Honduras	Panama	total	% correct
Costa Rica	9	0	0	9	100,00%
Honduras	0	10	0	10	100,00%
Panama	0	0	10	10	100,00%
total	9	10	10	29	100,00%

Figure 42: Differentiation (DA) and key statistical data from the NIR and the $^{13}C/^{12}C$, $^{18}O/^{16}O$ and D/H of the selected 29 samples of teak from the regions Costa Rica, Honduras and Panama

5.4.5 Combination of NIR and stable isotope method

Based on the results of the initial NIR analyses (see chapter 5.4.4), the NIR analysis was expanded to various other sampling regions for teak.

A total of 99 samples from 9 different countries in Asia and Central America were analysed.

These 99 samples can only be inadequately differentiated using the isotopes of the bioelements (D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$) in many cases. Accurate or good

allocations are only available for Laos (100%) and Honduras (100%) and, second to them, Brazil and Java (REF _Ref297197216 \h).

from \ to	Brazil	Costa Rica	Honduras	Indonesia	Java	Laos	PNG	Panama	Vietnam	total	% correct
Brazil	12	1	0	1	0	0	0	0	0	14	85,71%
Costa Rica	3	3	0	2	0	0	1	0	0	9	33,33%
Honduras	0	0	10	0	0	0	0	0	0	10	100,00%
Indonesia	1	0	0	6	1	0	1	0	0	9	66,67%
Java	2	0	0	0	12	0	0	0	0	14	85,71%
Laos	0	0	0	0	0	13	0	0	0	13	100,00%
PNG	0	0	0	2	0	0	6	2	0	10	60,00%
Panama	0	0	0	0	1	0	1	6	2	10	60,00%
Vietnam	0	1	1	1	0	0	0	3	4	10	40,00%
total	18	5	11	12	14	13	9	11	6	99	72,73%

Figure 43: Key statistical data of the discriminant analysis (DA) of the stable isotopes of the bioelements of teak from Latin America and Asia

Even when the 8 most important PCA-NIR parameters that contain more than 85% of the original information are used, better differentiation cannot be achieved using the NIR analysis (see Figure 44). On the contrary, the discrimination rate fell to 67%. The fact that no clear patterns occur makes this more difficult. 100% differentiation for at least Honduras and Java was always possible using the stable isotopes of the bioelements. For Brazil and Java, there were also good probabilities of differentiation above 85%. This type of significance of regional patterns does not occur when the NIR data used is analysed.

from \ to	Brazil	Costa Rica	Honduras	Indonesia	Java	Laos	PNG	Panama	Vietnam	total	% correct
Brazil	11	0	1	2	0	0	0	0	0	14	78,57%
Costa Rica	0	7	1	0	0	0	0	0	1	9	77,78%
Honduras	0	2	8	0	0	0	0	0	0	10	80,00%
Indonesia	0	0	1	6	0	0	2	0	0	9	66,67%
Java	0	3	0	0	10	1	0	0	0	14	71,43%
Laos	0	3	0	0	1	9	0	0	0	13	69,23%
PNG	2	0	2	0	0	1	3	2	0	10	30,00%
Panama	0	1	1	0	0	1	0	6	1	10	60,00%
Vietnam	0	0	2	1	0	0	0	0	7	10	70,00%
total	13	16	16	9	11	12	5	8	9	99	67,68%

Figure 44: Key statistical data of the discriminant analysis (DA) of the NIR analysis of teak from Latin America and Asia

Combining the stable isotopes of the bioelements with the 3 most important PCA-NIR parameters (contain 62% of the original information) produces, however, a different picture (Figure 42). The combination increases the discrimination rate considerably to almost 88% (Figure 45).

from \ to	Brazil	Costa Rica	Honduras	Indonesia	Java	Laos	PNG	Panama	Vietnam	total	% correct
Brazil	14	0	0	0	0	0	0	0	0	14	100,00%
Costa Rica	0	7	0	2	0	0	0	0	0	9	77,78%
Honduras	0	0	10	0	0	0	0	0	0	10	100,00%
Indonesia	0	0	0	7	1	0	1	0	0	9	77,78%
Java	1	0	0	0	13	0	0	0	0	14	92,86%
Laos	0	1	0	0	0	12	0	0	0	13	92,31%
PNG	0	0	0	0	0	0	10	0	0	10	100,00%
Panama	0	0	0	0	0	0	1	8	1	10	80,00%
Vietnam	0	1	1	0	0	0	0	2	6	10	60,00%
total	15	9	11	9	14	12	12	10	7	99	87,88%

Figure 45: Key statistical data of the discriminant analysis (DA) of the combination of the stable isotopes of the bioelements with the NIR analysis of teak from Latin America and Asia

Using this combination of 8 parameters, Brazil can now also be completely differentiated. Notwithstanding, however, it should be mentioned that this complete differentiation can also be achieved with the isotopes of the strontium because Brazil has the enriched isotope ratios of the strontium already listed.

What is remarkable is the significant improvement in the differentiation of Papua New Guinea that was increased from 30% to 100%.

Of course the number of parameters can be dramatically increased through the NIR spectroscopy at any time but there is a risk that the result is ever more detailed differentiation due to the increasing number of parameters. In further analyses, tests were conducted to determine which benefits the NIR analysis could have for improved differentiation. On the basis of these initial results, it can be assumed, however, that this NIR analysis is the ideal complement to the stable isotope data.

5.4.6 Differentiation of teak from Latin America under special consideration of teak from Ghana (Africa)

In addition to samples from Asia and Central America, teak samples were also supplied from Africa, Ghana in particular, as part of the project.

Extensive samples were taken in Ghana. There are currently 337 different wood samples from Ghana available. Along with 70 teak samples, the majority is made up of 207 mahogany samples (including African mahogany; Sapeli, Sipo, and Kaya). The other samples are wood samples from the *Malvaceae, Moraceae and Sapotaceae* families.

The 70 teak samples from Ghana have very enriched $^{18}O/^{16}O$ isotope ratios compared to the samples from Central America. This made it possible to largely differentiate the samples from Ghana from the samples from Latin America. There were only overlaps in the $^{18}O/^{16}O$ isotope patterns of the samples from Honduras and 100% differentiation with Honduras is thus not possible.

It is remarkable, however, that this significance of differentiation is not reflected in the D/H isotope ratios (Figure 46).

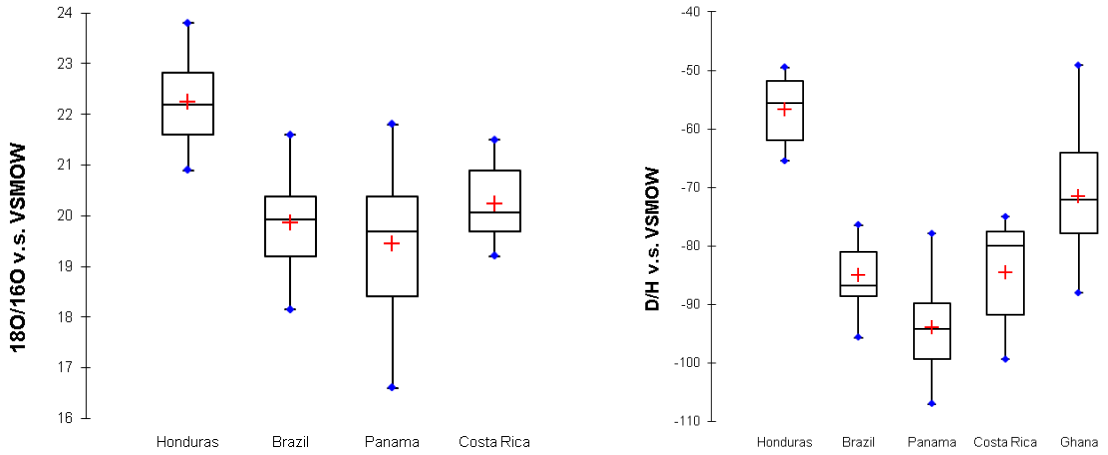


Figure 46: Box plots of the $^{18}O/^{16}O$ and D/H isotope ratios of the regions: Honduras, Brazil, Panama, Costa Rica and Ghana in comparison

Solely taking the D/H isotope ratios into consideration, it is only possible to ensure insufficient differentiation among the four regions in Latin America.

These various possibilities of differentiation and significance of the stable isotopes of the hydrogen (D/H) and oxygen ($^{18}O/^{16}O$) are completely the opposite when directly compared with the isotope patterns from Honduras. As already shown, it is not the $^{18}O/^{16}O$ but only the D/H isotope ratios of the samples from Honduras that have the necessary significance to guarantee 100% differentiation from the other Latin American samples.

This special characteristic is a solution for achieving almost 100% differentiation between Honduras and Ghana.

As already shown in the bases for the D/H and $^{18}O/^{16}O$ isotope ratios, there is a direct correlation (meteoric water line) of the stable isotopes in the water. This correlation is ultimately influenced or fractionated directly through the water balance and the biochemistry of the plant. If one considers that both countries have real teak (*Tectonia grandis*) and thus the biochemical prerequisites are identical, one reason for these differences can lie in the water balance of the trees influenced by the conditions in the respective region.

One possible indication of these types of differences is the changes in precipitation quantity. Examples are shown for the locations of Tegucigalpa (Honduras) and Accra (Ghana) (Figure 47).

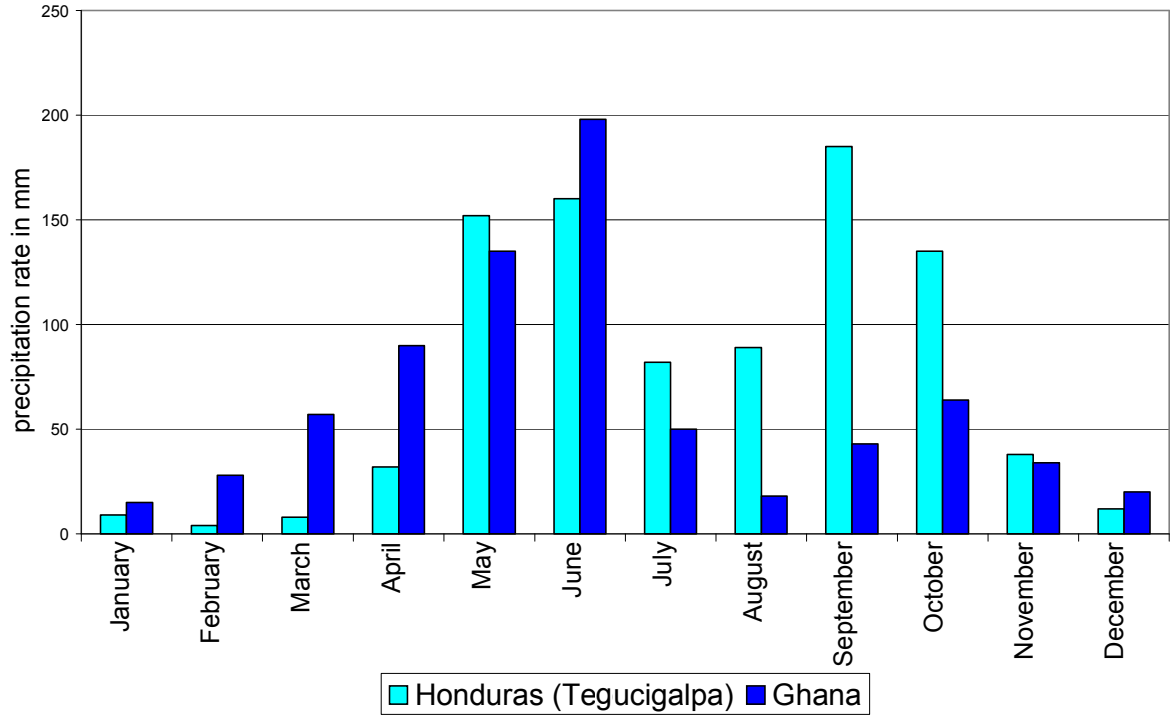


Figure 47: Sample annual rates of precipitation in Honduras and Ghana

The annual total precipitation rate is not very significant in this context. In this example, for instance, Honduras, with 906 mm, has a much higher rate of precipitation than Ghana with 752 mm.

A more critical factor is, however, that the teak tree, like all plants, needs a continuous supply of water. Because water is continuously lost through transpiration, the tissue water is enriched with the heavy isotopes of water and oxygen through an effect that corresponds to distillation.

Only in the three months of the year from April to June is the precipitation rate in Ghana comparable to the precipitation rate in Honduras. In the period from May to October, there is more water available over a longer time period in Honduras.

This ultimately affects transpiration and, accordingly, particularly the enrichment of the $^{18}O/^{16}O$ isotope ratios in the cellulose of the teak tree. It must be assumed that this is intensified by the regional temperature which is 26°C on average in Ghana and 22°C in Honduras.

This working hypothesis would certainly still need to be examined.

One result of the generally higher enrichment of the $^{18}O/^{16}O$ isotope ratios in the tissue water and then later also in the cellulose is necessarily a greater deviation of the correlation of the D/H and $^{18}O/^{16}O$ isotope ratios from the meteoric water line.

If the meteoric water line is now used as the water baseline in the regions, which is confirmed by experimental experience, it is possible to describe the deviation from this meteoric water line as the correlation number by reorganising the formula (in chapter 5.2.1).

This correlation number (d-excess) is expressed as: $\text{d-excess} = ((\delta D - 10)/8) - \delta^{18}O$

By determining the correlation number, Ghana can almost be differentiated 100% from Honduras and is thus a further improvement in differentiation over the previously described $^{18}O/^{16}O$ isotope ratios. (Figure 48).

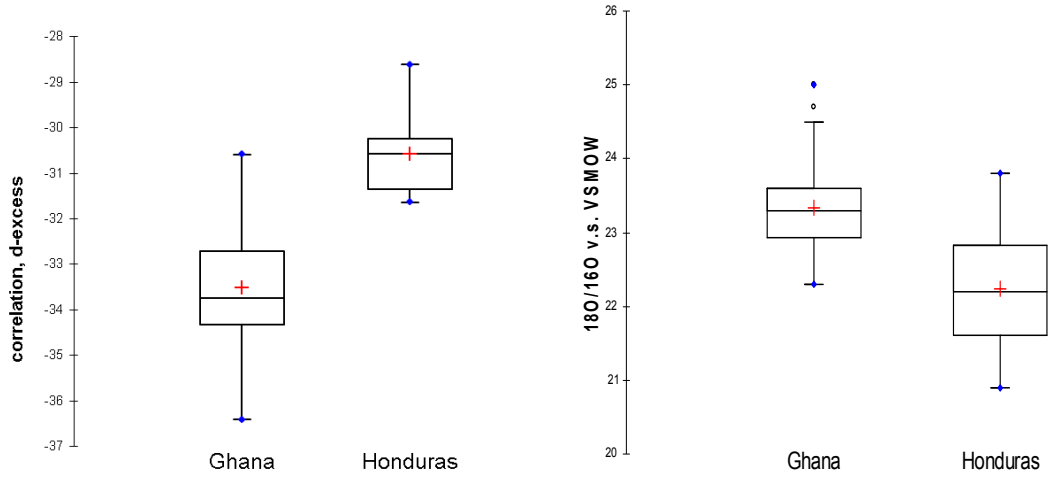
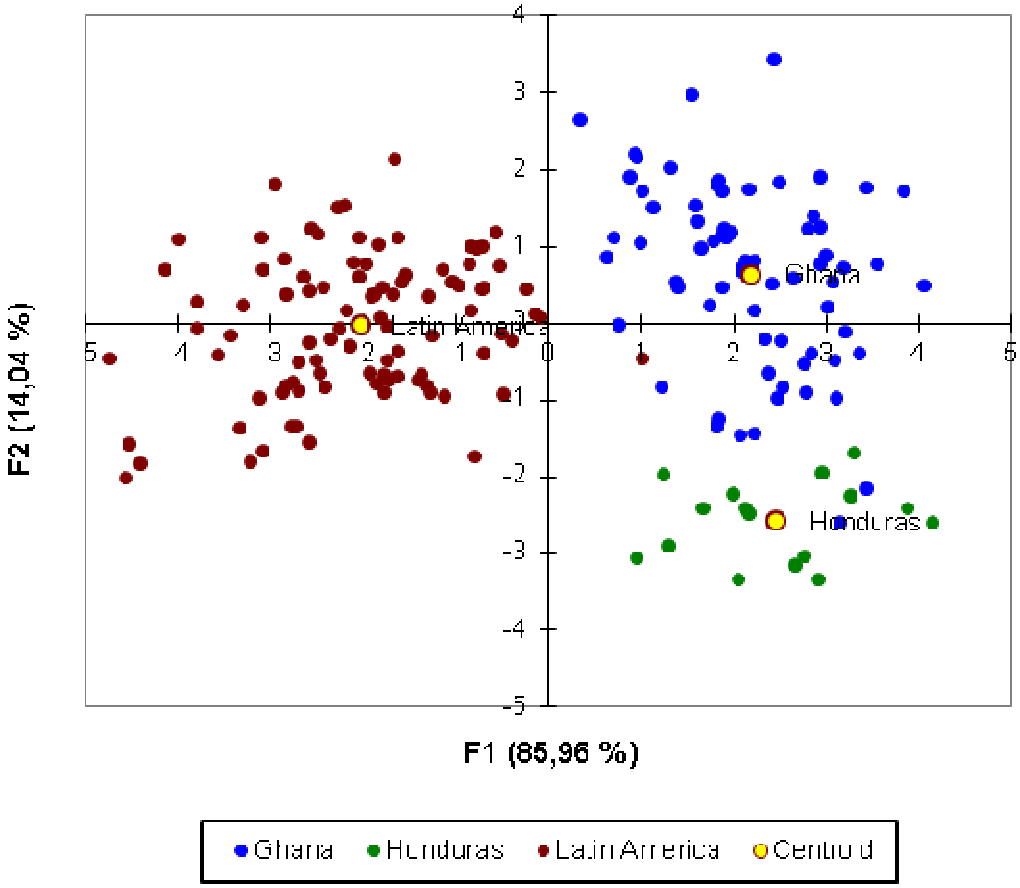


Figure 48: Box plot of the $^{18}O/^{16}O$ isotope ratios of teak (cellulose) of the regions: Ghana and Honduras compared to the box plot of the correlation figure of the $^{18}O/^{16}O$ and D/H isotope ratios (d-excess)

Taking into account all of the stable isotopes of the bioelements, a significant probability of differentiation of 95% results for the samples from Ghana (Figure 49) on the basis of this unique characteristic.

Within the sample sets from Ghana, there are only still 3 misallocations to Honduras from a total of 70 samples. One sample from Latin American is misallocated to the cluster from Honduras. Overall, a discrimination rate of almost 98% can be assumed from a significant possibility of differentiation for the samples from Ghana to Latin America.



from \ to	Ghana	Honduras	Latin America	total	% correct
Ghana	67	3	0	70	95,71%
Honduras	0	16	0	16	100,00%
Latin America	1	0	95	96	98,96%
total	68	19	95	182	97,80%

Figure 49: Differentiation (DA) and key statistical data for samples from Latin America particularly Honduras compared to samples from Ghana.

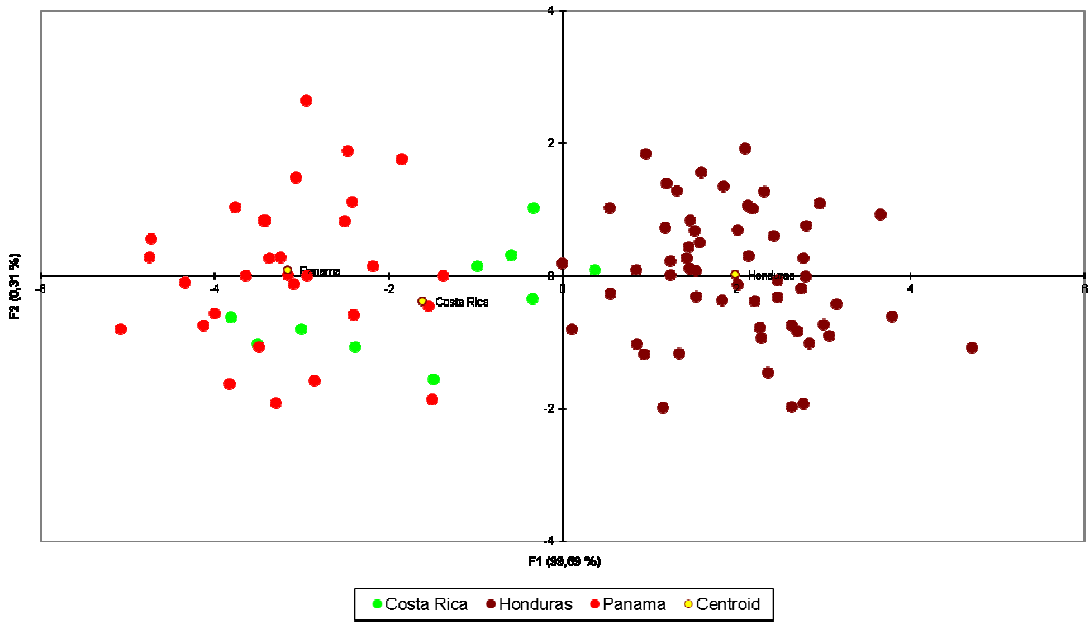
5.5 Differentiation of mahogany species from Latin America

In addition to teak, mahogany species were also included as a second variant in the samples. The origins of the samples generally concentrated on Central America. Almost 100 samples from Central America, in particular from Costa Rica, Panama and Honduras, were included in the analysis of the stable isotopes.

For the stable isotopes of the bioelements, options for differentiation similar to those described for teak exist.

Honduras can be 100% differentiated at any time among the three countries. There are significant overlaps between Panama and Costa Rica placing differentiation between these two regions only at 65% (Figure 50).

The NIR analysis may also be useful for resolving these overlaps but this could no longer be tested under the scope of the project.



from / to	Costa Rica	Honduras	Panama	total	% correct
Costa Rica	4	2	4	10	40,00%
Honduras	0	56	0	56	100,00%
Panama	3	0	27	30	90,00%
total	7	58	31	96	90,63%

Figure 50: Differentiation (DA) and key statistical data for the stable isotopes of the bioelements of mahogany samples from Costa Rica, Honduras and Panama in comparison

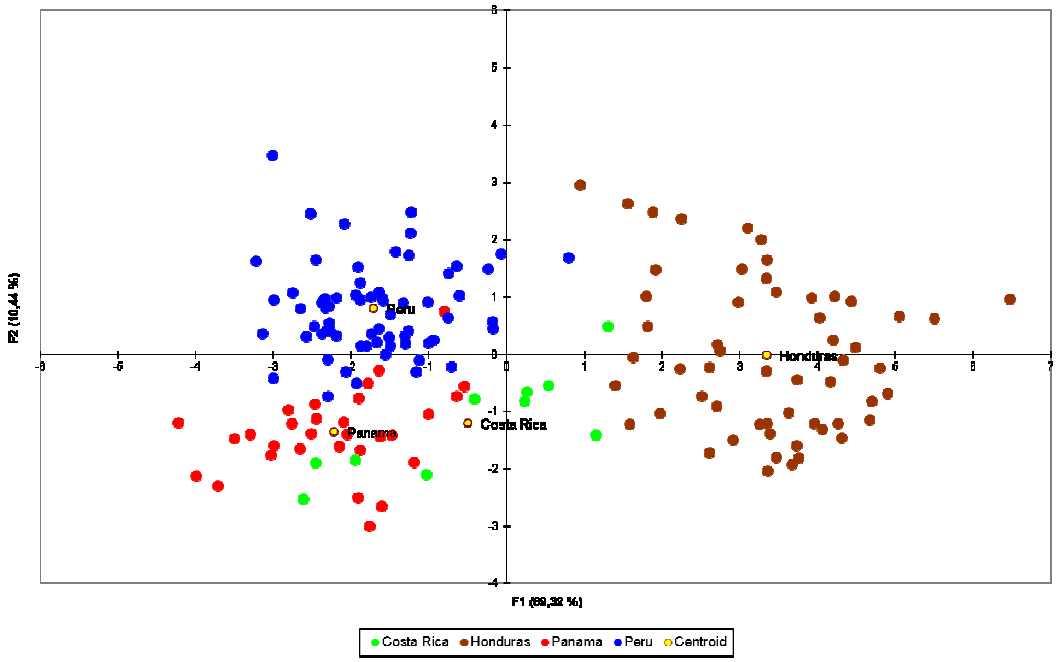
5.5.1 Differentiation of mahogany species from Central America particularly from Peru

Unlike the teak samples, the fourth region in Latin America was not Brazil but Peru. 70 mahogany samples (*Swietenia macrophylla*) were taken from this region.

In the D/H and $^{18}O/^{16}O$ isotope ratios, these samples with an average of -83.4‰ (D/H) and 19.6‰ ($^{18}O/^{16}O$) yielded values similar to Panama with -79.3‰ and 20.4‰. In general, the sample sets from Peru, however, showed depleted isotope ratios and thus varied significantly from the other three regions in Central America (see also the corresponding general depletion in the direction of Panama, Figure 51).

A key difference between these two sample sets can be found, however, in the $^{15}N/^{14}N$ isotope ratios. These vary significantly in Panama with isotope ration of on average 0.2‰ from the enriched $^{15}N/^{14}N$ isotope ratios in Peru that average +3.3 ‰.

Accordingly, a significant differentiation of 97% can also be achieved for the region of Peru (Figure 51).



from \ to	Costa Rica	Honduras	Panama	Peru	total	% correct
Costa Rica	4	2	4	0	10	40,00%
Honduras	0	55	0	1	56	98,21%
Panama	1	0	25	4	30	83,33%
Peru	0	0	2	66	68	97,06%
total	5	57	31	71	164	91,46%

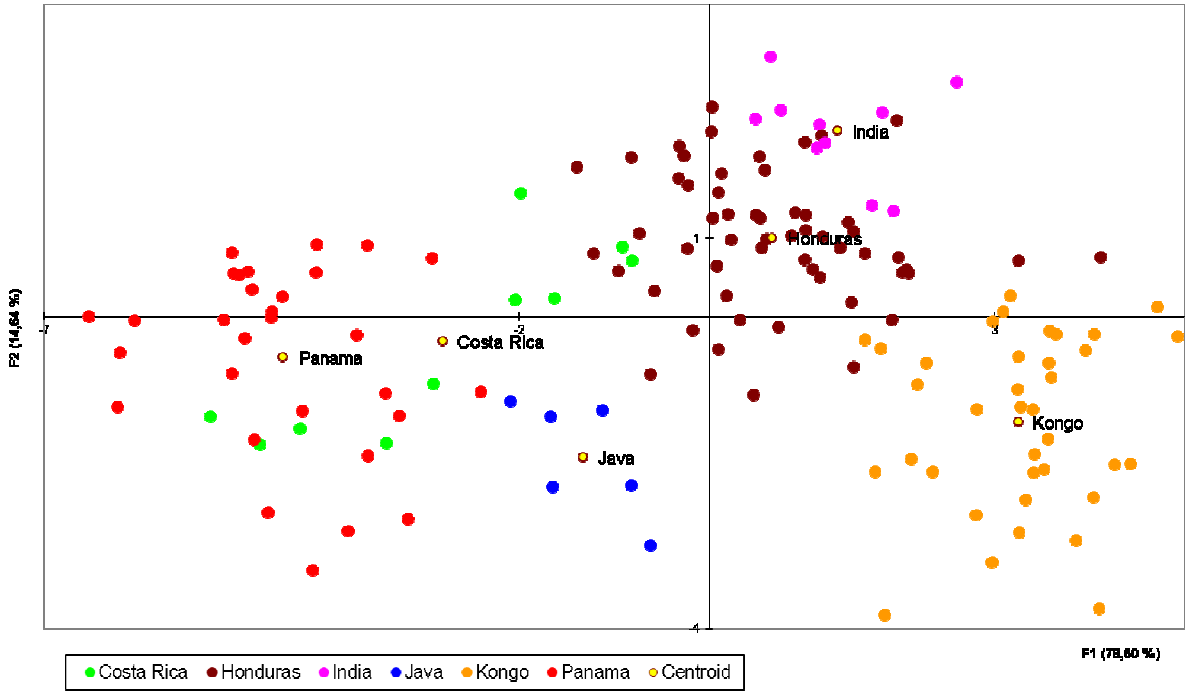
Figure 51: Differentiation (DA) and key statistical data for the stable isotopes of the bioelements of mahogany samples from Costa Rica, Honduras, Panama and Peru in comparison

5.5.2 Further differentiation of mahogany species from Central America taking into account the Congo, India and Ghana

In addition to the 166 mahogany samples from Central America, 37 mahogany samples from Congo (Africa), 10 from India and 6 from Java (Asia) were included in the analysis of the stable isotopes of the bioelements. There are hence 149 samples available from these 6 regions.

With the exception of Costa Rica already mentioned, there is a significant probability of differentiation for the six other regions (Figure52). Java and India yield isotope patterns that ensure complete differentiation. However, it must be pointed out that only a small number of samples are available from these two regions so that this high level of significance can only be seen as an initial base of knowledge.

The samples from the Congo also show significant differentiation of almost 95%. This corresponds to the knowledge level about the teak samples from Ghana that were also significantly different from the samples from Latin America.



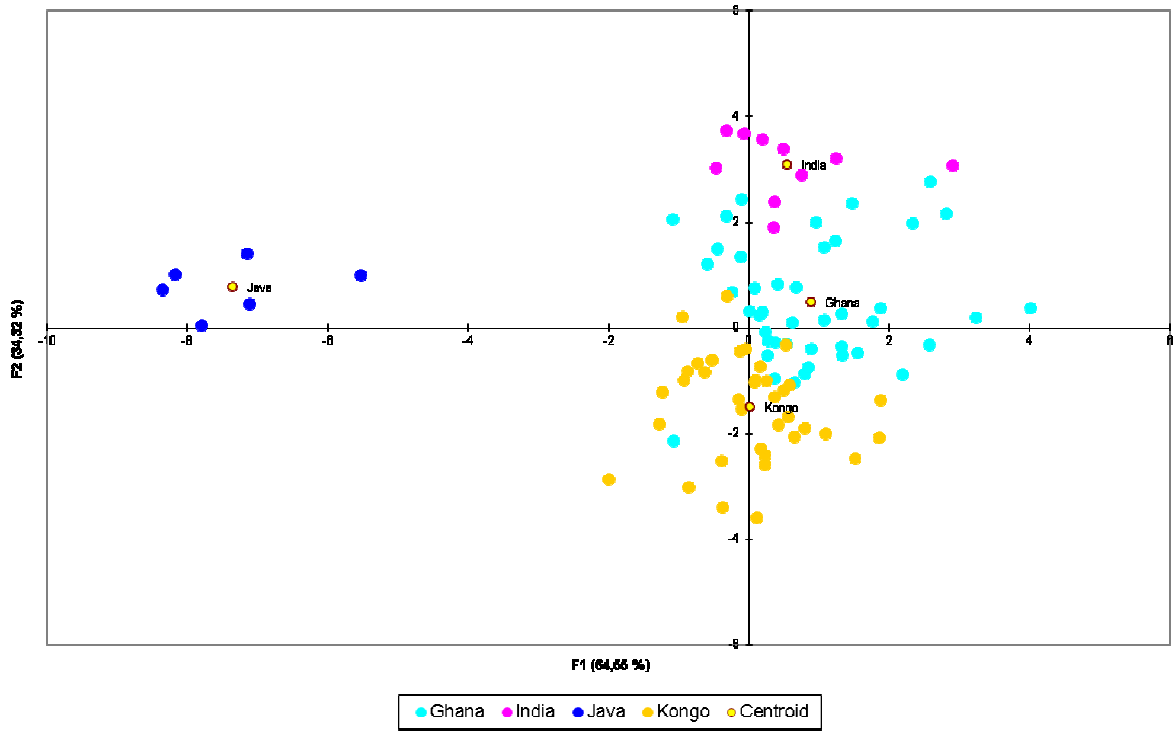
von \ nach	Costa Rica	Ghana	Honduras	Indien	Java	Kongo	Panama	Gesamtwert	% korrekt
Costa Rica	3	0	3	0	0	0	4	10	30,00%
Ghana	0	30	8	0	0	5	0	43	69,77%
Honduras	0	6	48	1	1	0	0	56	85,71%
Indien	0	0	0	10	0	0	0	10	100,00%
Java	0	0	0	0	6	0	0	6	100,00%
Kongo	0	5	1	0	0	31	0	37	83,78%
Panama	1	0	0	0	2	0	27	30	90,00%
Gesamtwert	4	41	60	11	9	36	31	192	80,73%

Figure52: Differentiation (DA) and key statistical data of the stable isotopes of the bioelements of mahogany samples from Latin America (Costa Rica, Panama, Honduras) as well as mahogany from Asia (India, Java) and Africa (Congo).

It was already mentioned in this context that extensive samples of mahogany species were provided particularly from Ghana.

Based on the enormous number of samples, it was possible to merge the samples into sample sets consisting of five samples taken from a sample site. This makes it possible to obtain a more robust sample set.

Certainly it is interesting in the first step to determine whether this sample set varies significantly from another African region: Congo. The probability here was determined to be approx. 90% (Figure 53). There are only 6 misallocations in the assignment of the samples/ sample sets from the Congo and Ghana. In addition, the regions of Java and India can be well-differentiated.



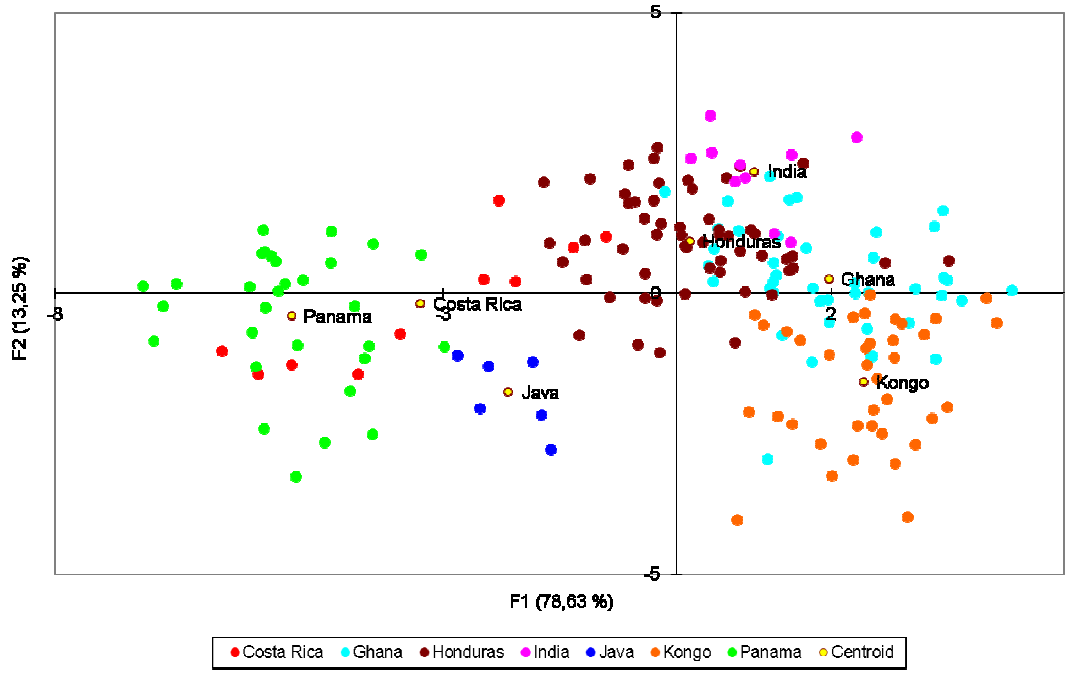
from \ to	Ghana	India	Java	Kongo	total	% correct
Ghana	38	2	0	3	43	88,37%
Indien	1	9	0	0	10	90,00%
Java	0	0	6	0	6	100,00%
Kongo	3	0	0	34	37	91,89%
total	42	11	6	37	96	90,63%

Figure 53: Differentiation (DA) and key statistical data for the stable isotopes of the bioelements of mahogany samples from Asia (India, Java) and Africa (Ghana, Congo)

Taking into account the samples from Central America, differentiation similar to teak emerges. Costa Rica and Panama can always be differentiated 100% from Ghana (Figure 54). However, the overlap of samples from Honduras and samples from Ghana is now significant. 8 samples/sample sets from Ghana have been misallocated to Honduras.

Taking into account the simultaneous overlap with the mahogany samples from the Congo, the discrimination rate is lower.

This is certainly somewhat contrary to the possibilities of differentiating teak from Ghana which could be differentiated from Honduras almost 100%. In summary, the possibility of differentiation for other tree species should always be verified particularly for regions where there tend to be overlaps.



from / to	Costa Rica	Ghana	Honduras	India	Java	Kongo	Panama	total	% correct
Costa Rica	3	0	3	0	0	0	4	10	30,00%
Ghana	0	30	8	0	0	5	0	43	69,77%
Honduras	0	6	48	1	1	0	0	56	85,71%
India	0	0	0	10	0	0	0	10	100,00%
Java	0	0	0	0	6	0	0	6	100,00%
Kongo	0	5	1	0	0	31	0	37	83,78%
Panama	1	0	0	0	2	0	27	30	90,00%
total	4	41	60	11	9	36	31	192	80,73%

Figure 54: Differentiation (DA) and key statistical data of the stable isotopes of the bioelements of mahogany samples from Latin America (Costa Rica, Panama, Honduras), Asia (India, Java), Africa (Congo) with special consideration for Ghana

5.5.3 *Comparison of the D/H and $^{18}O/^{16}O$ isotope ratios of teak and mahogany in a small region of Ghana*

As a result of the extensive samples from Ghana, it is also possible to directly compare the $^{18}O/^{16}O$ and D/H isotope ratios for teak and mahogany.

This comparison is useful because, even though a correlation to the water has to exist for both, this can have different characteristics or be subject to different fractionations depending on the water balance of the tree.

Of course, a direct comparison is only possible if both sample sets of teak and mahogany originate from a small region so that it can be assumed that the background situation is identical when considering the water.

In Ghana, two sample sites of teak and mahogany which are less than 10 km away from one another can be used (Figure 55).

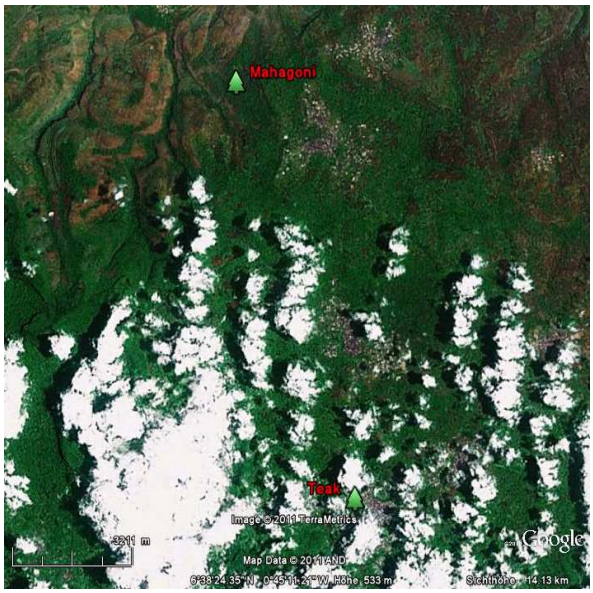


Figure 55: Sample sites (< 10 km) of teak and mahogany in small region of Ghana

There are 20 samples of teak and 10 samples of mahogany available for comparison from this small region. In the direct comparison of these two sample sets of teak and mahogany, it can be concluded that the sample set of mahogany yields enriched $^{18}O/^{16}O$ and D/H isotope ratios compared to the teak sample set. The median of the D/H isotope values of mahogany yields a value enriched by 5.6‰. The $^{18}O/^{16}O$ isotope value is 1.4‰ higher than the mean of teak (Figure 56).

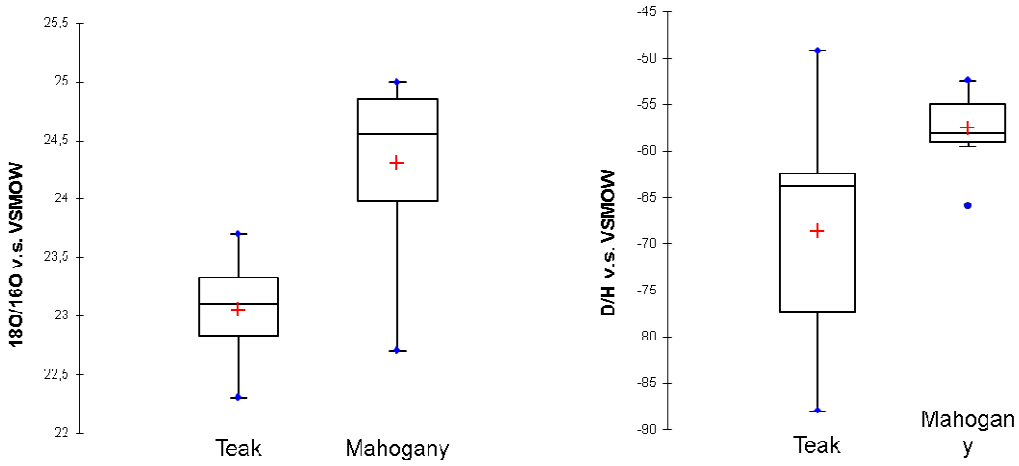


Figure 56: Box plot of the $^{18}O/^{16}O$ and D/H isotope ratios of teak and mahogany from Ghana in comparison (small-scale sampling)

This difference in the form of enrichment in the $^{18}O/^{16}O$ and D/H isotope ratios in mahogany samples is also seen when comparing teak and mahogany from other regional origins. Mahogany is also always more enriched on average than teak in Latin America, particularly in Panama, Costa Rica and Honduras as well as in Java (Figure 57, Figure 58). The enrichment in the $^{18}O/^{16}O$ and D/H isotope ratios lies in the range of the values that were also measured in Ghana. On average, there is always an enrichment for mahogany of 4 to 8‰ in the D/H and 0.8 to 1.6‰ in the $^{18}O/^{16}O$ isotope ratios.

Based on these differences, it is not possible to apply teak databases directly to new tree species. Because, however, a general relationship exists as is evident from the teak/mahogany example, a parameter can be determined by identifying comparison values of two tree species in the region that can enhance the existing data records.

A representative comparison always appears necessary, however, for this transfer parameter.

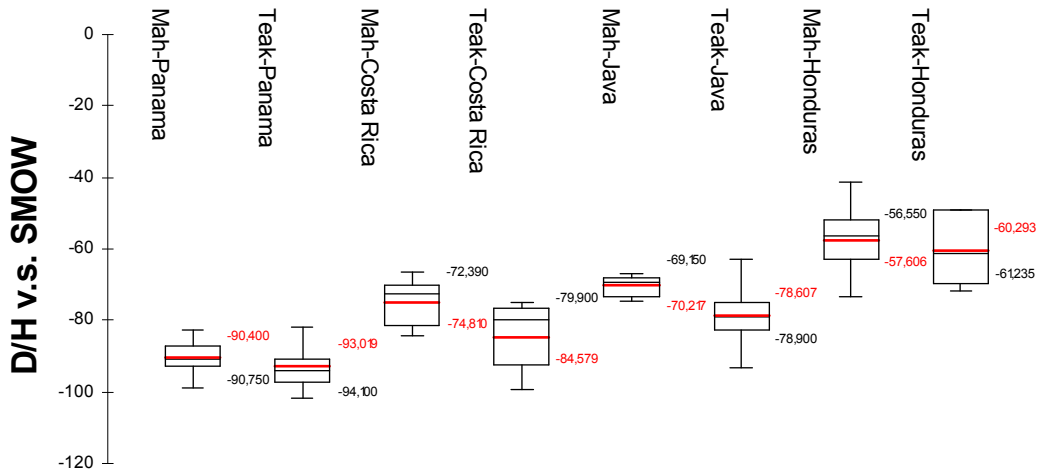


Figure 57: Box plot of the D/H isotope ratios of teak and mahogany from various origins

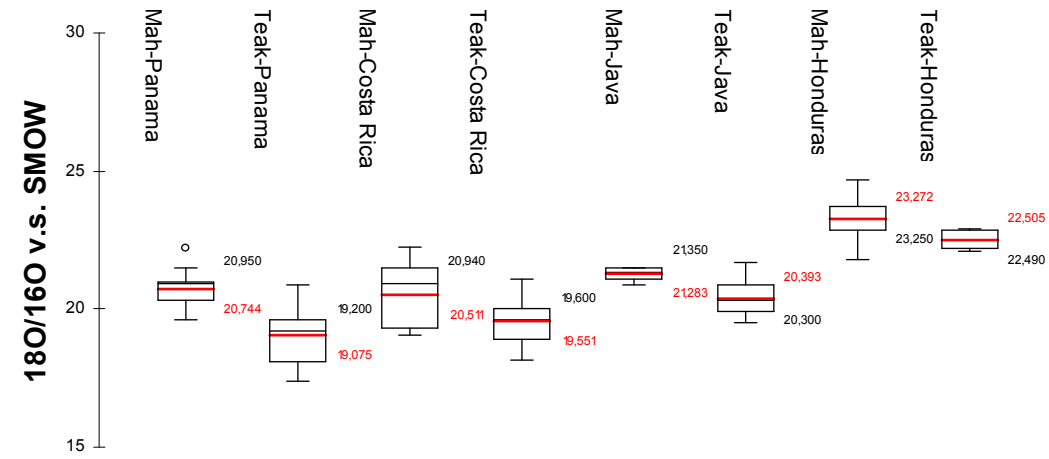


Figure 58: Box plot of the $^{18}O/^{16}O$ isotope ratios of teak and mahogany from various origins

6 Species identification using genetic barcoding methods (Dr. Aki M. Höltken)

6.1 Introduction

As a consequence of new legal regulations, substantial socio-economic & ecological damage and increasing public interest, information about the identity of forest products becomes more and more important. DNA-based information systems are a potentially revolutionary instrument to meet the continuously expanding demands of forest stakeholders for data and information on forestry resources. DNA profiles, comparable with a non-manipulable "integrated barcode", display a promising tool to control the whole chain-of-custody of timber and timber products.

One important field of application is the enforcement of the *Convention on International Trade in Endangered Species* (CITES). To ensure that international trade in timber species does not threaten their survival, more than 40 timber species have been listed in the current CITES regulations. Most of the identification procedures are based on visual descriptions of wood morphological characteristics. Although computer aided tools of macro- and microscopical wood features have been developed at the vTI (Johann Heinrich von Thünen-Institute, see KOCH et al 2008), we need further unambiguous species identification systems applicable over the whole chain-of-custody because many protected timbers can easily be mistaken for legally harvested tree species due to very similar wood anatomical structures. This is particularly the case for species of one genera (e.g. species within the genus *Swietenia*), but the differentiation of timbers of lower taxonomic relationship may also pose a challenge (KOCH et al. 2005, SCHMITZ-KRETSCHMER et al. 2006). To this regard, the establishment of DNA barcoding techniques will provide an interesting tool for species differentiation and, further, contribute to world wide initiated barcoding projects, e.g. "Barcode of Life" etc. (HÖLTKEN et al. submitted).

The aim of this project is the development of genetic tools for the differentiation of the timber species of the true mahogany genus (*Swietenia macrophylla* King, *S. mahagoni* (L.) Jacq. and *S. humilis* Zucc.) as well as teak (*Tectona grandis* L.f.). A further ultimate ambition was the design of unambiguous species determination methods that could be applied and run with low-cost equipment without the need for sequencing or capillary electrophoresis techniques

(HÖLTKEN et al., submitted). The presented results should provide a basis to develop further genetic barcoding sequences for the permanently increasing amount of tropical tree species on the international market.

6.2 Conceptual background

6.2.1 Technical challenges in timber DNA analysis

In the last years various protocols have been developed and published to extract DNA from wood, from recently logged (almost fresh) timber up to processed wood and woody products from different steps in the chain-of-custody. Most of the approaches are aligned to mitigate the effects of contamination of the samples with external DNA and to minimise further demolition of already degraded DNA sequences (DE FILIPPIS & MAGEL 1998, DEGUILLOUX et al. 2002, RACHMAYANTI et al. 2006, ASIF & CANNON 2007). Thus, DNA markers with the following characteristics have to be used:

(a) Low intraspecific but sufficient interspecific variability (species specific markers)

(b) Because of moulding conditions in tropical forests and potential fungal attacks in fresh plant and wood material, external (e.g. fungal) DNA should not disturb the genetic analyses.

(c) Because of the high degradation of the DNA, the fragments should be short in length (max. 400 bp.).

(d) The low yield of DNA following extraction requires a high-copy number of the target DNA fragments

These characteristics apply particularly to the DNA of chloroplasts (cpDNA), available in woody tissues in multiple copies per cell. Further, the ring structure of the cp-genome gives higher stability to the DNA molecule. The structure of the cpDNA of plants is thus comparable with the mitochondrial DNA (mtDNA), which can be analysed in old human or animal bones of several thousand years of age (KRAUSE et al. 2006, POINAR et al. 2006, ROGAEV et al. 2006). Preliminary tests using SSRs (microsatellites) resulted in higher amplification success for markers for the chloroplast than for the nuclear genome.

Further, because the cpDNA genome is maternally inherited (particularly in angiosperms, and thus in most of the tropical tree species), there is no recombination of the cp genome (in contrast to nuclear DNA during sexual reproduction). Therefore, the cpDNA is expected to show more variation between than within species and thus should be more suitable for species differentiation. Another important feature of cpDNA is that fungi do not possess this kind of DNA, so that moulding conditions cannot interfere genetic analyses.

6.2.2 The completely sequenced cpDNA genome of Populus trichocarpa *Torr. & Gray as a basis for the development of standard barcoding sequences*

In recent years, quite a few standard sequences taken from the chloroplast genome have been developed and discussed to barcode a vast amount of plant species (TABERLET et al. 2007; KRESS et al. 2005, KRESS & ERICKSON 2007, HEBERT et al 2003, HEBERT & GREGORY 2005). First studies were already made in the early 90s using introns of the *trn*L-*trn*F intergenic regions of the cpDNA to identify different plant species (TABERLET et al. 1991).

Nevertheless, the exclusive application of already available standard barcoding sequences turned out to be not sufficient to develop DNA based species differentiation systems, particularly in the case of wood material:

(a) We have to deal with timber samples differing in age as well as in the degree of advances in processing. Thus, we have to face problems such as low DNA quantity and quality. However, most of the standardised barcoding sequences are too long for amplification from woody material (800 up to more than 1000 bp.). Quite a few attempts on timber samples resulted in total failure of the PCR technique or an amplification of unspecific fragments not suitable for further analyses.

(b) The resolution between closely related species such as *Swietenia* sp. is too low, particularly if barcoding sequences of the coding regions are used (e.g. *rbc*L or *mat*K); see also comments of TABERLET et al. 2007 and MUELLNER et al. (2011).

These circumstances provoked us to develop a new set of barcoding sequences for species differentiation. The fact, that the chloroplast genome of black cottonwood *Populus*

trichocarpa Torr. & Gray has been totally sequenced (TUSKAN et al. 2006), a large amount of information, transferable on many other tree species, has become available.

Comparable to the nuclear genome, the chloroplast DNA is composed of coding regions (exons coding for proteins of the primary and secondary metabolism, protein biosynthesis etc.) as well as noncoding, intergenic spacer regions with no or unknown function (introns). Because genic sequences are relatively conserved across seed plants (and especially within angiosperms), all the PCR primers (=detection sites) for the amplification of non-coding regions were designed within these regions. All the primers used here were developed only for this study, although a few of the regions have been tried elsewhere.

Within these noncoding spacer regions we can expect a much higher degree of interspecific variation that might be useful to also differentiate closely related tree species. Altogether, we designed 22 primer pairs within the chloroplast genome to detect differences between the closely related *Swietenia* species (Figure 1).

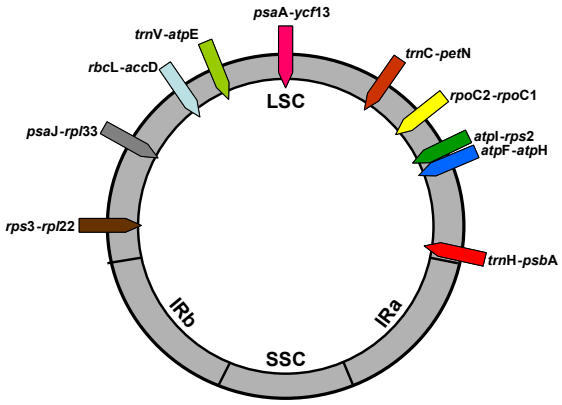


Figure 59: Intergenic loci of the Large-Single-Copy (LSC) region of the chloroplast genome used to differentiate the different *Swietenia* species (mahogany) as well as *Tectona grandis* (teak)

6.2.3 Technology transfer: Adaptation of the methods for a broader range of users

To make DNA based timber identification systems accessible to a broad range of users, cost effective technologies should be used applicable after short training courses for the staff. The vast amount of genetic information has to be reduced on only a few or even single diagnostic fragments that can be analysed with low-cost equipment in a short period of time. Differences between species should be made visible after a few technical steps in the lab. Only in the case of reasonable ground for suspecting illegal logging or illegal trade with CITES-protected species, further analyses have to be initiated for validation of the result. The process of species distinction with ready-for-use methods is explained in the box.

DNA-extraction: At the beginning of the genetic species identification process, nucleic acids have to be isolated from the woody samples. Commercial kits, with certain modifications to adapt the methods on wood material (see chapter 3.2, DNA isolation from wood samples) may help to simplify lab processes.

→ time for extraction: at least 4 to 6 hours

→ lab equipment: centrifuge

PCR: In this step the target DNA sequence with the decisive single nucleotide or fragment length difference is amplified. Besides specific PCR primers we need a PCR thermocycler.

→ time for PCR: about 2 to 4 hours

→ lab equipment: PCR-thermocycler

Detection of "Single Nucleotide Polymorphisms" using restriction enzymes: Restriction enzymes are one of the basic tools developed by molecular biologists several decades ago. These enzymes cut DNA fragments at very specific sites (here: species specific differences). For running this step, the PCR thermocycler can be used to ensure the optimal operation temperature of the restriction enzyme (37 to 60°C). Nowadays, fast-digest enzymes allow for the restriction of DNA fragments in less than 15 minutes.

→ time for restriction: 0.5 hours

→ lab equipment: heat block or PCR-thermocycler (see PCR)

Visualisation: Fragment length differences can be visualised on an agarose or a polyacrylamide gel.

→ time for electrophoresis: 1.5 to 3 hours

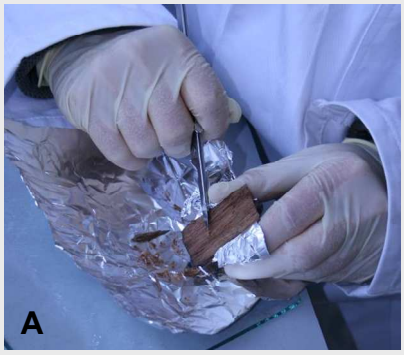
→ lab equipment: electrophoresis chamber, power supply, ultraviolet light, camera

Box: Ready-for-use DNA methods to identify CITES-protected timber species

1. Extraction of DNA isolates

A. Physical destruction of woody tissues
- Cutting thin slices from or wood shavings from timber samples
- Pulverization of wood fibres into a fine powder after freezing in liquid nitrogen

B. Chemical digestion
- Lysis of the cell walls and cell membranes
- DNA-purification: Removal of disturbing ingredients (PCR-inhibitors such as phenolic compounds (e.g. lignins), carbohydrates etc.)



2. PCR = polymerase chain reaction

Amplification of DNA target sequences: Artificial DNA sequences (so called primers, here in green) have been developed to identify DNA target regions with species specific differences (here in red: SNPs = single nucleotide polymorphisms).

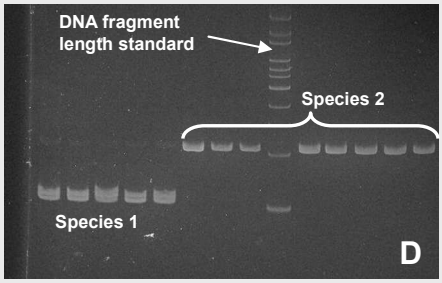
Species 1:
…**GTTATGCCGTAAAT**GTTGCTAGTTAAGTTATGCCGTAAATGTTAAGTTAT
GCCGTAAATG**A**ATTCAGTTAAGCCGTATGTTGCTAGT**TAAGTTATGCTTA**…

Species 2:
…**GTTATGCCGTAAAT**GTTGCTAGTTAAGTTATGCCGTAAATGTTAAGTTAT
GCCGTAAATG**C**ATTCAGTTAAGCCGTATGTTGCTAGT**TAAGTTATGCTTA**…

In only 15 to 50 µl reaction volume the target DNA fragments are amplified resulting in up to 2^{36} = 68 billion copies. The capacity of a PCR-machine (C): 96 or 384 Samples.

3. Restriction of DNA fragments and electrophoretic separation

Restriction endonucleases are enzymes that cut double- or single-stranded DNA at specific recognition nucleotide sequences known as restriction sites. A vast amount of different restriction enzymes is available with different restriction sites
In our example the restriction enzyme *Eco*RI cuts DNA at sites with the sequence GAATTC. The amplified fragment of species 1 will be cut and splitted in two shorter DNA sequences, the fragment of species 2 remains unchanged.
Different fragment lengths after restriction can be made visible on an agarose gel serving in the following as a genetic barcoding marker (D).



6.3 Material and Methods

6.3.1 Plant Material

Fresh or herbarium dried leaf material was used for method establishment (DNA of high quality and quantity). Analysed samples of the three *Swietenia* species, covering a large area of their natural distribution, are listed in table 1. Teak samples (*Tectona grandis*) originated from the greenhouse of the Johann Heinrich von Thünen Institute (vTI) in Hamburg-Lohbrügge and from plantations located on Java (Indonesia).

Timber samples for checking the applicability of the established methods (blind test) were obtained from a timber trading company in Hamburg (Theodor Nagel). Further, a first practical test was carried out on wood samples of the *Meliaceae* family, confiscated by German customs due to strong suspicion of illegal trade with CITES-protected *Swietenia* timber.

Nr.	Botanischer Name	Ort der Probenentnahme
5	*Swietenia mahagoni*	Gewächshaus vTI Lohbrügge
6	*Swietenia macrophylla*	Gewächshaus vTI Lohbrügge
13	*Swietenia mahagoni*	Botanischer Garten Universität Hamburg
14	*Swietenia macrophylla*	Botanischer Garten Universität Hamburg
15	*Swietenia macrophylla*	Botanischer Garten London
16	*Swietenia macrophylla*	Botanischer Garten Berlin
17	*Swietenia macrophylla*	Wilhelmina Zool.-Bot.Garten Stuttgart
18	*Swietenia mahagoni*	Wilhelmina Zool.-Bot.Garten Stuttgart
19	*Swietenia mahagoni*	Palmengarten Frankfurt am Main
20	*Swietenia macrophylla*	Palmengarten Frankfurt am Main
21	*Swietenia mahagoni*	Friedrich-Schiller-Universität Jena
22	*Swietenia macrophylla*	Friedrich-Schiller-Universität Jena
25	*Swietenia macrophylla*	Botanischer Garten Universität Tübingen
26	*Swietenia macrophylla*	Botanischer Garten Universität Tübingen
27	*Swietenia macrophylla*	Südamerika
28	*Swietenia macrophylla*	Südamerika
29	*Swietenia macrophylla*	Südamerika
30	*Swietenia macrophylla*	Südamerika
31	*Swietenia macrophylla*	Südamerika
32	*Swietenia macrophylla*	Südamerika
33	*Swietenia macrophylla*	Südamerika
34	*Swietenia macrophylla*	Südamerika
35	*Swietenia macrophylla*	Südamerika
36	*Swietenia macrophylla*	Südamerika
37	*Swietenia mahagoni*	Botanischer Garten München
38	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
39	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
40	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
41	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
42	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
43	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
44	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
45	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
46	*Swietenia macrophylla*	Herkunftsversuchsflächen, Mittelamerika
47	*Swietenia mahagoni*	Herkunftsversuchsflächen, Mittelamerika
48	*Swietenia mahagoni*	Herkunftsversuchsflächen, Mittelamerika
49	*Swietenia humilis*	Herkunftsversuchsflächen, Mittelamerika
50	*Swietenia humilis*	Herkunftsversuchsflächen, Mittelamerika
51	*Swietenia humilis*	Herkunftsversuchsflächen, Mittelamerika
52	*Swietenia macrophylla*	Herbarium, Botanischer Garten New York
53	*Swietenia macrophylla*	Herbarium, Botanischer Garten New York
54	*Swietenia macrophylla*	Herbarium, Botanischer Garten New York
55	*Swietenia macrophylla*	Herbarium, Botanischer Garten New York
56	*Swietenia mahagoni*	Herbarium, Botanischer Garten New York
57	*Swietenia mahagoni*	Herbarium, Botanischer Garten New York
58	*Swietenia humilis*	Herbarium, Botanischer Garten New York
59	*Swietenia humilis*	Herbarium, Botanischer Garten New York

Figure 60: Samples of the analysed three *Swietenia* species and their origin

6.3.2 DNA-isolation

Fresh leaf material: About 25-50 mg of a single leaf were ground to powder in a 2 mL microcentrifuge tube (Eppendorf) using a Mixer-mill apparatus Type MM 300 (Retsch). Into tubes with herbarium dried material only two stainless steel beeds were added, fresh leaf material was quick-frozen in liquid nitrogen before starting the powdering process. Total DNA was extracted, following a modified CTAB protocol by DOYLE & DOYLE (1987).

Wood samples: The treatment of woody tissues was carried out according to the recommendations of RACHMAYANTI et al. (2006). About 50 to 100 mg wood shavings were ground to a fine powder using the above mentioned Mixer-mill apparatus after incubation of the samples in liquid nitrogen for several minutes. The DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilgen, Germany) with some modifications. We added PVP to the AP1 buffer and incubated the mixture over night at 65°C instead of 1 h according to the manufacturers' recommendations. The DNA isolate was further purified using the *OneStep*™ PCR Inhibitor Removal Kit (Zymo Research, USA).

6.3.3 PCR

The PCR was carried out according to standard protocols. The reaction volume was 25 µl and contained 20 to 50 ng template, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.8 mM $MgCl_2$, 200 µM dNTPs, 1 unit Taq polymerase, 0.4 µM of the respective primer and, in some cases, BSA (bovine serum albumin). The PCR was carried out in thermocyclers made by the company Biometra (Göttingen) with an initial denaturation step at 94°C for 4 min, followed by 30 cycles with 94°C (1 min) of the respective annealing temperature (45 s up to 1 min, 55 to 65°C depending on the primer combination) and 72°C (1 to 2 min) and finally an elongation of 10 min also at 72°C.

6.3.4 Sequencing and sequence analysis

For sequencing, 25 µl of the PCR product was purified using the "High Pure PCR Product Purification" kit from Roche (Mannheim, Germany). For sequencing the service provided by StarSeq (Mainz, Germany) was used. The amount of DNA used for sequencing depended on the length of the fragments: for a fragment up to 200 bp 50 ng DNA was needed, for 500 bp 100 ng and for up to 1000 bp 200 ng DNA was used.

Obtained sequences were screened for single nucleotide polymorphisms (SNPs) and indels by using the software SeqMan 7.1.0 from DNAStar (Lasergene, GATC Biotech, Konstanz, Germany) and Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, USA). The fragment sites containing SNPs were checked for species specific restriction sites using the free software NEB cutter 2.0 from New England BioLabs Inc (Ipswich, USA).

6.4 Results DNA-based species identification

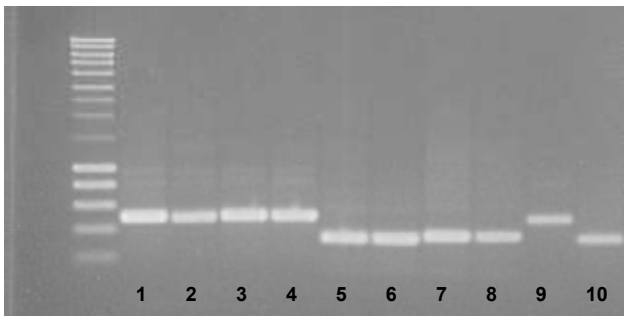
6.4.1 Differentiation Swietenia *sp.* –Tectona grandis

Between species with low taxonomic relationship, e.g. species belonging to different families, lower efforts and expenses are to be expected. A screening of different intergenic spacer regions of the chloroplast DNA and the comparison of the fragment lengths should be sufficient to develop a genetic barcoding method without the need for establishing a PCR-RFLP technique (see box). In Table 2 the best amplifying fragments are listed for both the three *Swietenia* species (*S. macrophylla*, *S. mahagoni* and *S. humilis*) belonging to the taxonomic order of *Sapinales* (family *Meliaceae*) and *Tectona grandis* belonging to the order *Lamiales* (family *Verbanaceae*, in former times *Lamiaceae*). Four amplification products showed the same fragment lengths; in three cases we found differences of 100 bp (*trn*C-*pet*N, *psa*A-*ycf*13) or 200 bp (*trn*H-*psb*A), respectively.

cpDNA-Locus	DNA-Fragmentlängen in Basenpaaren *Swietenia* sp.	*Tectona grandis*
*trn*H-*psb*A	350	500
*trn*G-*psb*K	550	550
*psb*K-*psb*I	600	600
*rps*2-*rpoC*2	700	700
*trn*C-*pet*N	800	900
*psa*A-*ycf*13	800	900
*psb*J-*psb*F	400	400

Figure 61: Fragment lengths of the seven best amplifying cpDNA loci for both, the three *Swietenia* species *S. macrophylla, S. mahagoni* and *S. humilis* (mahogany) as well in *Tectona grandis* (teak)

A rapid test using the PCR technique in combination with testing the fragment length differences on an agarose gel is demonstrated in Figure 2. *Tectona grandis* and *Swietenia* sp. using both, leaf and wood material, can clearly be distinguished on a 1.5% Agarose gel, running time about 1.5 hours.



lines 1-4: *Tectona grandis*, leaf material from different locations on Java

lines 5-8: *Swietenia macrophylla* (5, 6*), S. mahagoni* (7) and *S. humilis* (9), leaf material

line 9: *T. grandis* (wood sample)

line 10: *S. macrophylla* (wood sample)

Figure 62: Identification of fragment length differences between *Swietenia* sp. versus *Tectona grandis* using agarose gel electrophoresis of the *trn*H-*psb*A locus (chloroplast DNA)

This approach, using cpDNA markers, should be applicable also on other CITES-protected tree species and their exchange timbers with low taxonomic relationship, e.g. the differentiation of the two guaiak timber species *Guaiacum* sp. (CITES II) and *Tabebuia heptaphylla* with very similar wood anatomical structures. But also for conifers with a long evolutionary history, fragment length differences should be sufficient to develop genetic barcoding techniques (e.g. *Fitzroya cupressoides* (CITES I), *Thuja plicata*, *Sequoja sempervirens*).

6.4.2 ***Differentiation of the closely related tree species*** **Swietenia macrophylla, S. mahagoni *and* S. humilis**

6.4.2.1 Sequence differences

After amplifying the chosen noncoding cpDNA regions we sequenced the fragments in order to detect differences (SNPs or indels). Besides several SNPs and fragment length differences observed at various analysed cpDNA fragments, we found that *S. mahagoni* can easily be differentiated from the other two species *S. macrophylla* and *S. humilis*. The intergenic spacer region *trn*H-*psb*A was found to be the most suitable barcoding marker for species determination. This region is also emphasised as an official barcoding sequence by the Plant Working Group of the `Consortium of the Barcode of Life´ (CBOL).

Figure 3 shows a fraction of the sequence of the intergenic spacer *trn*H-*psb*A for the species *Swietenia macrophylla*, *S. humilis* and *S. mahagoni*. The substantial difference (here SNP = single nucleotide polymorphism) is visualised in figure 3. At position 94 of the amplified fragment, the nucleotide A (= adenine) in the species *S. mahagoni* is exchanged by C (= cytosine) in *S. macrophylla* and *S. humilis*.

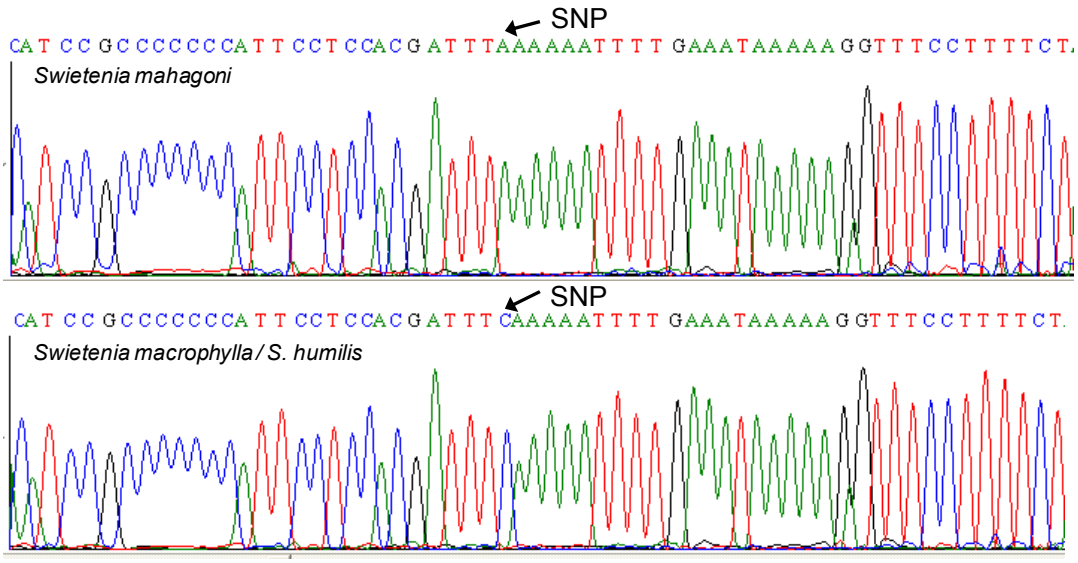


Figure63: Single nucleotide polymorphism (SNP) in the intergenic spacer region *trn*H-*psb*A of the cpDNA (arrows) between the mahogany species *Swietenia macrophylla* / *S. humilis* and *S. mahagoni*.

6.4.2.2 Adapted ready-for-use method

The outcome of this project is a single fragment within the intergenic spacer *trn*H-*psb*A, 246 bp long, for the differentiation of *Swietenia mahagoni* from *S. macrophylla* and *S. humilis*. because it displays the recognition site for the restriction enzyme *Dra*I in *Swietenia mahagoni* (TTTAAA) but not in *S. macrophylla* and *S. humilis* (TTTCAA). The result is visualised in figure 4. After digesting the amplicon with the mentioned restriction enzyme, *S. mahagoni* is cut into two fragments (94 and 152 bp long) whereas the amplicon of *S. macrophylla* and *S. humilis* remain unaffected. This procedure has been tested on all *Swietenia* samples listed in table 1.

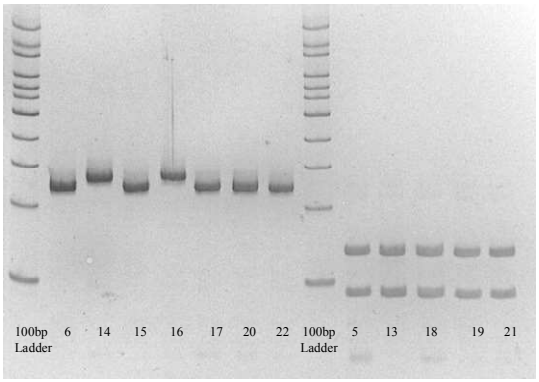


Figure 64: Ready-for-use PCR-RFLP method to genetically distinguish between *Swietenia mahagoni* and *S. macrophylla* / *S. humilis*; outcome of the electrophoresis of digested *trn*H-*psb*A fragments on a polyacrylamide gel

7 Blind test / practical tests

7.1 Blind test to verify the declared origin using the isotope method

The project included a blind test organised by the WWF to verify the functionality of the methods.

The particular challenge of the blind test was to structure the test to reflect practical application as closely as possible. For example, only one piece of wood from the respective location was provided to the laboratory for every test.

Overall, 13 of the 15 samples were correctly allocated.

One incorrect result was resolved directly after the test using near infrared (NIR) (blind test sample no. 3). The particular difficulty for blind test sample no. 3 was that the sample site was right next to the border of Costa Rica. A reference sample site was situated only 50 km away from the blind test sample site. The second incorrect evaluation was related to the fact that there were not yet any analysis results for the reference samples from the country in question (Ghana) (blind test sample no. 9) at the time of the analysis.

	Specified declaration/construed practical situation	Actual origin	Conclusion: Declared origin is…	Result report TÜV-Rheinland Agroisolab	Evaluation
1	Teak from Java	Panama	False	"False"	
2	Mahogany from Honduras	Panama	False	"False"	
3	Teak from Panama	Panama	True	"False"	
4	Teak from Panama	Laos	False	"False"	
5	Teak from Java	Laos	False	"False"	
6	Teak from Burma	Laos	False	"False"	
7	Teak from Laos	Laos	True	"True"	
8	Teak from Costa Rica	Laos	False	"False"	
9	Mahogany from Honduras	Ghana	False	"True"	
10	Teak from Java	Indonesia	True	"True"	
11	Teak from Laos	Indonesia	False	"False"	
12	Teak from Costa Rica	Indonesia	False	"False"	
13	Teak from Java	Burma	False	"False"	
14	Dealer says: Teak from Java; authorities presume Burma (question to laboratory: "How probable is Burma")	Burma (according to information from dealer)	False	"False"	*
15	Spruce from Germany / Bavaria	Russia (according to dealer information)	False	"False"	**

Figure 65: Overview of blind test

* This sample was not collected within the project but originates from a teak garden chair that was

purchased; dealer claims the wood is from Burma.

** This case does not concern tropical wood. This example was part of the blind test because it was shown as an example of an existing German building supply store at the international fingerprinting conference in Eschborn.

The positive result of the blind test underscores the potential of the stable isotope method in verifying the declared origin.

7.2 Practical test of isotope method: Verification of the declared origin of confiscated teak

The stabile isotope method was used to show further indications of the possible origin of teak confiscated by customs. In this case, imported teak wood is suspected of originating from Burma which is currently subject to a trade embargo.

A number of suspicious samples of various sample sets/batches was provided for the analysis. This ensures a robust mean value for evaluating the origin.

All stable isotopes of the bioelements were used in the test. Based on present knowledge, the result was that these suspicious samples were extremely similar to the reference samples from Burma (Figure 66).

Only one sample set comprised of only a single suspicious sample could not be directly allocated to Burma but shows isotope ratios with similarities to Java. Because this only involved a single sample, the possibility of an unclear result due to an extreme value cannot be ruled out.

A possible alternative origin was identified as part of the ongoing customs procedures. The region was not part of the current database. However, the stable isotope method was used to disprove this origin as a possibility using a direct reference sample from this region.

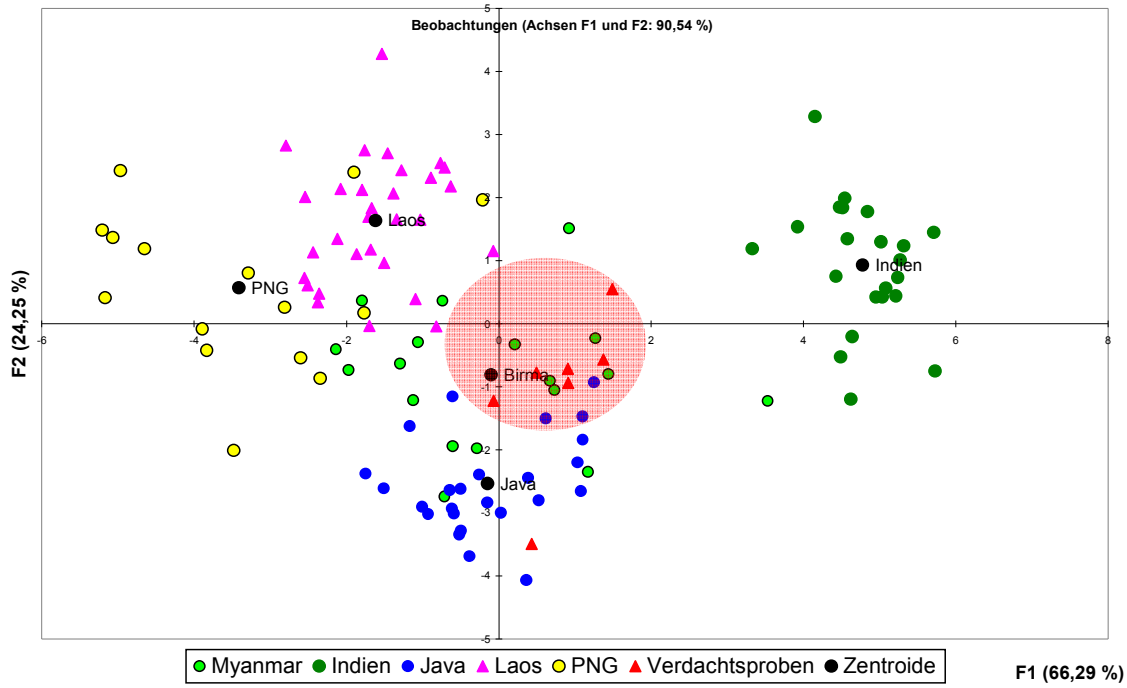
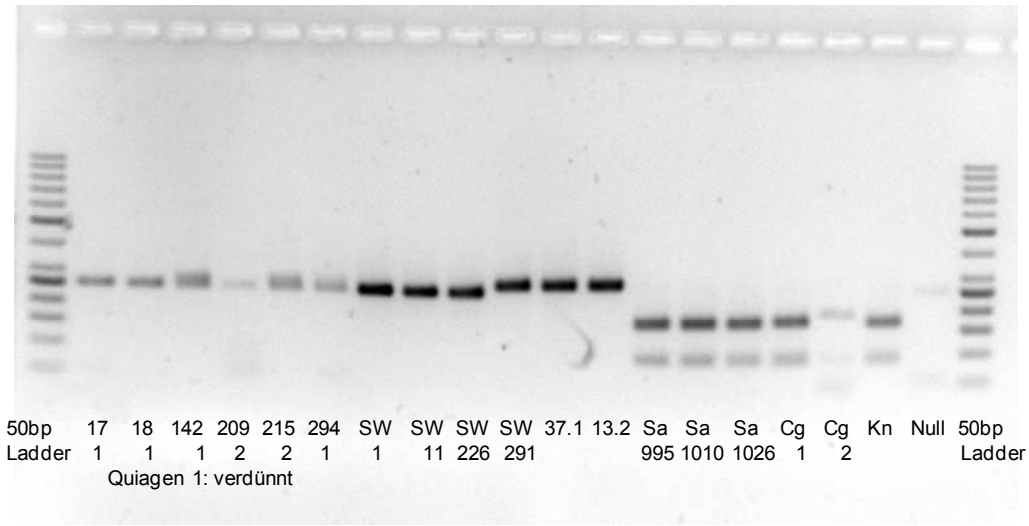


Figure 66: Practical test of confiscated teak samples suspected of originating from Burma/Myanmar (inside the red circle)

7.3 *First practical experience: A request from German customs*

A first successful practical test was conducted on timber samples confiscated by the German customs and previously determined as a member of the *Meliaceae* family (with strong suspicion on the CITES-protected *Swietenia*). The exact determination of the timber species was carried out in two steps:

(1) In the first instance we determined the correct genus of the samples to ensure that the confiscated timber belongs to the CITES-protected genus *Swietenia* and not to the morphologically very similar exchange timbers *Khaya*, *Entandrophragma* or *Carapa* (*Meliaceae* = mahogany family). This test was performed using a new PCR-RFLP method which will be available soon in a publication by Höltken et al. (2011, submitted), see figure 5.



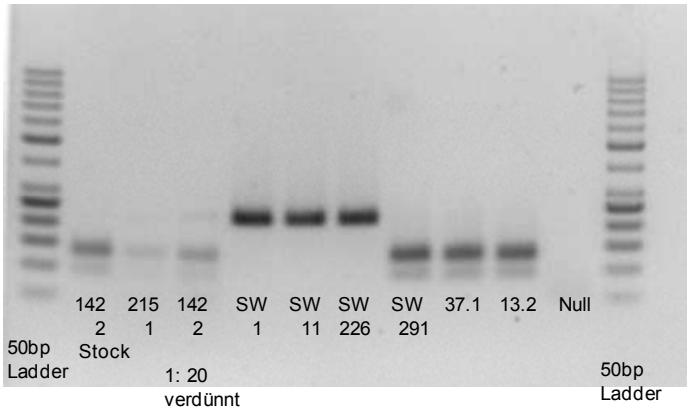
Proben 17, 18, 142, 209, 215 und 294 beschlagnahmte Holzproben

Referenzproben:

SW 1	*Swietenia macrophylla*
SW 11	*Swietenia macrophylla*
SW 226	*Swietenia macrophylla*
SW 291	*Swietenia mahagoni*
37.1	Swietenia mahagoni
13.2	Swietenia mahagoni
Sa 995	*Entandrophragma* sp.
Sa 1010	*Entandrophragma* sp.
Sa1026	*Entandrophragma* sp.
Cg 1	*Carapa guianensis*
Cg2	*Carapa guianensis*
Kn	*Khaya* sp.

Figure 67: Genus identification in the *Meliaceae* timber family; distinction between the CITES-protected genus *Swietenia* and the exchange timber genera *Entandrophragma* sp., *Khaya* sp. and *Carapa* sp. using PCR-RFLP technique and agarose gel electrophoresis (digestion of a *mat*K-*trn*K cpDNA fragment with the restriction enzyme *Msp*I, according to Höltken et al., submitted)

(2) After we could prove the genus of the timber samples (genus *Swietenia*), we determined the exact species name in a second step using the procedure described in chapter 4.2.2. In the particular case the correct species name of the timber was *Swietenia mahagoni* (Figure 6).



Proben 142 und 215 beschlagnahmte Holzproben

Referenzproben:

SW 1	Swietenia macrophylla
SW 11	Swietenia macrophylla
SW 226	Swietenia macrophylla
SW 291	Swietenia mahagoni
37.1	Swietenia mahagoni
13.2	Swietenia mahagoni

Figure 68: Species identification within the genus *Swietenia* using PCR-RFLP and agarose gel electrophoresis (digestion of a *trn*H-*psb*A cpDNA fragment with the restriction endonuclease *Dra*I (see chapter 4.2.2))

8 International reference database

One goal of the project was to determine the possibility of a joint database through a stakeholder process so that the project test data from both methods and possibly also future data could be jointly managed and made available to interested groups.

During the project, the German Federal Ministry for Consumer Protection, Food and Agriculture (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz - BMELV) expressed its support for setting up an international database.

Consequently, the WWF discussed, particularly with the Ministry, the general conditions for an international reference database and made recommendations based primarily on its experiences with the two fingerprinting projects financed by the DBU:

Information that should be stored with every data record

The information stored for the reference samples will subsequently serve as the basis for both fingerprinting methods. It is very important that this data is correct because otherwise there will be a systematic error causing all subsequent measurements or analyses to be incorrect. In light of this, WWF feels that it is not only important to store the core information such as GPS data and analysis results:

- Sample ID number (until now the IDs were specified without the system of the sample-takers)
- Country / region / location
- GPS data
- Information about company / organisation that provided the samples
- Name of the sample-taker
- Organisation for which the sample-taker works
- Organisation that commissioned the sample-taking
- Date sample taken
- Tree species (information from sample-taker / sample supplier)
- Tree species (unambiguously identified through XXX; e.g. DNA analysis vTI; microscopic identification vTI)
- Unusual features of the sample
- Comments from the sample-taker about the sample site
- DNA analysis results
- Isotope analysis result
- Analysis result of other methods

Information about the sample-taker

In striving to create the international database, it will be essential that wood samples are provided from an increasing number of sources. Private-sector companies can play an important role here. But how can it be ensured that false samples or incorrect GPS data are not stored in the database (this would be a "systematic error" that could result in a lot of confusion)? Let's assume that a company that is pursuing dishonest goals and has a concession next to a protected area submits samples from the protected area and claims that the samples came from the concession. The company could then process illegal timber from the protected area and falsely "document" that the timber originated from the concession by means of fingerprinting.

For this reason, the WWF feels that it is very important to also save the name of the sample-taker and the name of the organisation responsible for every wood sample in the data record. If it is discovered later on that there is something wrong with the samples, all of the references samples originating from this person or organisation could be deleted from the database and the organisation barred from providing future samples or from accessing data. This would be a sanctioning measure that would act as a deterrent.

The more the database grows, the higher the probability that false samples could be subsequently identified or would be noticed as a result of other reference samples from other people in the same region.

Another possibility for making the database reliable are random reference samples. An independent person could be commissioned by the institutions responsible for the database to take random samples in areas where wood samples with GPS data already exists. The result of the analysis of the random sample could then be compared with the reference data already stored in the database. If the analysis results are identical or at least very similar, this is evidence of the accuracy of the sample/data originally provided.

Information on tree species

It is extremely difficult to determine the tree species on-site. Identification is particularly difficult for tropical tree species. In some cases, there is a larger group of tree species behind the commercial name. The possibility can thus not be ruled out that a sample-taker provides information on the tree species that is intentionally or unintentionally incorrect or inaccurate. It thus appears to make sense to identify the tree species afterwards e.g. using genetic markers.

Ring tests

Laboratories that create analyses worldwide on the basis of data records from the database should also participate regularly in the ring tests so that a high level is ensured in verifying and tracking the declared timber origin.

Motivation for companies or countries to participate in setting up an international database

Several factors contribute to increasing the level of trust and acceptance in an openly accessible international database.

- Administration of the database by an international organisation accepted by all interest groups
- Headquartering the organisation in the southern hemisphere would be beneficial
- Security against hackers must be guaranteed
- Regulated access rights (users have to register)
- (Long-term) contracts keep the database from being commercialised (fees for data records cover the costs of maintaining the database)
- Access is open for all interest groups (authorities, companies, NGOs, etc.)

- Contractual certainty that no patents of the genetic material, etc. can arise from the sample material made available that could be later used at the expense of the country that provided the samples.

Other aspects that could further boost acceptance particularly among producer countries (and thus the willingness to support setup of a database):

- Opportunities for knowledge transfer (assistance setting up genetics and isotope laboratories in the producer countries, scientific conferences and exchange of information, etc.)

- Increased acceptance and interest among the producer countries if the EU uses these methods to inspect at borders in connection with the EU legislation.

- Incentives linked to Voluntary Partnership Agreements (VPA): Integration wood fingerprinting methods into the national system for controlling flows of wood reinforces this by offering additional inspection options. From an EU-PM dated July 2010: "Head of EU Delegation commends progress on development of innovative Wood Tracking System…. The WTS is being developed in context of the EU-Ghana Voluntary Partnership Agreement (VPA)….".
- If the EU countries themselves use fingerprinting methods to inspect imported timber, the interest of producer countries in having these inspection options and their own laboratories would likely grow.

The recommendations made by the WWF were discussed positively by the BMELV. There is generally consensus about the points listed for an international database. The BMELV pledged to finance an office which will help lay the foundations for an open international reference database for three years starting in 2011. The current plan is for the database to be under the auspices of Biodiversity International in Singapore.

The interest groups also discussed the database during the international conference in Eschborn. The following recommendations were made:

- Reliability of the sample material

- Merging of the statistics of both methods

- Merging of the reference data of both methods in a database

9 Conferences

9.1 GIZ/WWF - Wood fingerprinting conference; Eschborn (11/2010)

An international conference on wood fingerprinting was held in Eschborn (Germany) at the beginning of November 2010. The conference was organised by the GIZ (formerly the GTZ) and WWF Germany. The conference was funded by the German Environment Foundation (Deutsche Bundesstiftung Umwelt - DBU) and the European Union.

The conference entitled "Genetic and Isotope Fingerprinting Methods – Practical tools to verify the declared origin of wood" presented results from two projects that were near to completion at the time the conference was held.

1. The DBU financed project described here with the title "Fingerprinting of wood to fight illegal logging by introducing a combination of the isotope method for our origin identification of wood and the DNA analysis for species differentiation of wood".

2. A project carried out by the GIZ and funded by the EU. The project demonstrated the applicability of the methods at concession level for two tree species in Cameroon (FLEGT-Voluntary Partnership Agreement (VPA))

The goal of the conference was to present the experiences and results of the two projects and discuss them together in a forum. A further aim was to discuss the potential and future prerequisites for practical applicability. And, the technical requirements for setting up an international database were also to be discussed.

69 participants from 19 countries took part in the conference.

The project results from both projects were positively received by the participants. The presentations and discussions provided highly interesting new information on the status of fingerprinting methods and their applicability for all interest groups and representatives from producer countries, buyer countries, ministries, scientists and non-governmental organisations (NGOs). The discussion about the range of application, overlap and expansion to the new legal regulations of the EU to eliminate illegal timber but also discussions on the development of the methods were lively and productive. Numerous recommendations for practical use and further scientific work were compiled. The atmosphere at the end of the conference was extremely optimistic that these fingerprinting methods could and would play a role in the control of timber flows and in eliminating illegal timber.

A hardcopy summary was created in English and French. The text was also translated into Spanish.

The summary of the conference can be downloaded from http://www.wwf.de/themen/waelder/illegaler-holzeinschlag/.

The most important recommendations arising from the discussions at the conference:

- PR work to incorporate interest groups
- Support for national verification mechanisms in producer countries in conjunction with CITES and FLEGT. Here, priorities should be defined in the selection of the tree species
- Integration and national tracking and monitoring systems
- Capacity building in producer countries
- Coordination through regional organisations such as COMIFAC (Central African Forests Commission; commission des forets d'afrique centrale)
- Regular international conferences
- Further exchange of information on the setup and structure of the international database
- The considerable benefits of the methods for implementation of the CITES provisions should be made accessible to the CITES Plants Committee
- Cooperation with international organisations such as CITES, ITTO or FAO

In addition, there is a series of scientific recommendations to effectively structure further development of the methods.

9.2 World Bank - Potomac Forum and WRI/eia – Legal Forest Alliance Meeting (5/2011)

The Potomac Forum of the World Bank (WB) was held on illegal logging and trade in Washington DC on 4 May 2011. The Potomac Forum was founded in 1982 as a non-profit educational forum. The conferences and trainings cover a broad spectrum of educational services for US government and industry. Along with representatives of the World Bank, event participants included representatives of the US Forest Service, Society of American Foresters, USAID; US Department of Justice, US Department of State, International Wood Products Association and others. (http://www.potomacforum.org/)

The Forest Legality Alliance Meeting (FLA) took place in Washington DC on 5 May 2011. FLA is a joint initiative of the World Resources Institute (WRI) and the Environmental Investigation Agency (EIA) to reduce illegal logging. (http://www.forestlegality.org/)

The issue of wood fingerprinting was incorporated at both events. Information on progress in the status of development of wood fingerprinting through genetic markers and isotope methods was presented in various presentations, lectures and discussions.

In addition, there were also meetings and discussions both before and after the event with US government agency representatives on the issue of wood fingerprinting.

10 Publications and PR work

10.1 Press

- 2008: "Deutsche Welle" radio
- 2008: "Holzkurier" magazine
- 2008: "Holz-Revue" magazine
- 2008: "Stihl" magazine
- 2009: Trade forum at the CBD in Bonn 2009
- 2010: Informational flyer about the project described here
- 2011: DBU promotional film
- 2011: "Deutschlandradio-Kultur" radio

10.2 Scientific publications

- BONER M., HOFEM S., FÖRSTEL H.: Charakterisierung der geografischen Herkunft von Teak (Tectona grandis) mit Hilfe der naturlichen Variation der stabilen Isotope; (submitted)
- BONER M., HOFEM S., FÖRSTEL H.: Charakterisierung der geografischen Herkunft von echtem (Swietenia macrophylla) und afrikanischem (Khaya spec.) Mahagoni sowie anderen afrikanischen Holzarten mit Hilfe der naturlichen Variation der stabilen Isotope; (submitted)
- HÖLTKEN A.M., SCHRÖDER H., WISCHNEWSKI N., DEGEN B., MAGEL E. & FLADUNG M. (in press): "Development of DNA-based methods to identify CITES-protected tree species: A case study in the Meliaceae family." Holzforschung, International Journal of the Biology, Chemistry, Physics and Technology of Wood, deGruyter.
- University of Hamburg
  FELLINGHAUER D., HÖLTKEN A.M., DEGEN B., JOLIVET C. & FLADUNG M. (2010) DNA-Marker: Dem illegalen Holzhandel auf der Spur. (DNA Markers: On the Trail of Illegal Logging) Trade journal *Faszination Holz*, 1/10, 30-34.

DEGEN B. & HÖLTKEN A.M. (2011) DNA-Methoden zur Kontrolle von Holzart und Holzherkunft. (DNA Methods for Verifying Wood Species and Wood Origin). *Holz-Zentralblatt publication* 19, p. 416.

11 Summary and Outlook

11.1 Isotopes

In the project carried out, basic requirements for the development and establishment of effective databases to verify the origin of teak and mahogany species were defined. There are now a number of references from more than 14 different regions in Asia, Africa and Latin America available. Using these regions, it can be shown that the stable isotopes of the bioelements and strontium make it possible to significantly differentiate or even completely differentiate in many regions. Worthy of mention here are the regions of Asia including Laos, Java and India or Latin America with Honduras and Brazil. Also regions like Papua New Guinea, Peru or Congo/Ghana have unique stable isotope patterns that are adequately suitable for guaranteeing an origin analysis.

One special advantage of the stable isotope is the possibility of supplying origin statements simply using individual samples. This was demonstrated using the blind test. Of course, the error rate of allocation increases with a single sample. As with every analytical method, these "ambiguities" cannot be eliminated for the stable isotopes either. It is, however, possible on the one hand to minimise these "ambiguities" by creating a robust mean value of regions for suspicious samples (example: Asian regions). On the other hand, the discrimination rate of regions can be considerably improved with a small-scale clustering of data records (see the examples of Panama, Costa Rica).

These links with geographic information represent essential components for an effective origin database on the basis of stable isotopes because it is precisely this information that uncovers causalities in the stable isotope patterns (see dependency of the D/H isotope ratios on the distance to the coast, Java) and can thus be used for a detailed analysis.

In the future, the chemical fingerprint (NIR analysis) in particular can be a meaningful complement to the stable isotope analysis.

The quality of this first origin database can certainly be enhanced by the good blind test. Based on the two misallocations of the blind test, it can be concluded that outliers can exist that do not correspond to a declared sample set or can result from a misallocation due to lacking reference data records from unknown regions.

Both problems can certainly be remedied by continuously expanding the data records.

Using the two tree species of teak and mahogany, it can be demonstrated that the stable isotopes basically reflect the given conditions of the region particularly in the D/H and $^{18}O/^{16}O$ isotope ratios. The characterisation, however, depends on the tree species meaning

that even though it is possible to transfer reference data to other tree species, it must be, however, adjusted by a constant.

11.2 Species identification using genetic markers

Figure lists a series of CITES-protected tree species and the timber types that they can be easily confused with for which DNA-based techniques of species identification are urgently needed.

CITES-geschützte Holzarten			**Austausch-Holzarten**	
Wissenschaftlicher Name	**Handelsname**	**CITES-Status**	**Wissenschaftlicher Name**	**Handelsname**
Dalbergia retusa	Cocobolo	in prep.	*Dalbergia maritima*	Madagaskar Rosenholz
Dalbergia stevensonii			*Dalbergia spruceana*	Amazonas Rosenholz
			Dalbergia latifolia	Indisches Rosenholz
Dalbergia nigra	Brasilianisches Rosenholz	I	*Dalbergia spruceana*	Amazonas Rosenholz
			Dalbergia latifolia	Indisches Rosenholz
			Machaericum scleroxylon	Santos Rosenholz
Fitzroya cupressoides	Patagonische Zypresse	I	*Sequoja sempervirens*	Küstenmammutbaum
			Thuja plicata	Riesen-Lebensbaum
Pterocarpus santalinus	Padouk	II	*Pterocarpus indicus*	Amboina
			Pterocarpus soyauxii	Padouk

Figure 69: A selection of other protected tree species and the tree species that they can easily be confused with for which a genetic species identification method appears useful.

This work, however, brought to light another scientific aspect that is currently being investigated in more depth. The focus here is the taxonomic status of the three *Swietenia* species dealt with here. The fact that *S. macrophylla* cannot be distinguished from *S. humilis* using chloroplast DNA could be an initial indication they are not two reproductively isolated species but are only ecotypes of the same species. Investigations of ITS (Internal Transcribed Spacer) sequences (core DNA) are currently being carried out to confirm this hypothesis.

11.3 Outlook

The evaluation of the blind test and the practical test for confiscated teak to **verify the declared origin** demonstrates the excellent potential of the **stable isotope method**. Compared to the first project funded by the DBU that concentrated on Europe and north-west Russia, the challenge in verifying the declared origin by means of the isotope method was much more ambitious for this project. The reason is that the global water cycle begins at the equator and moves to the poles. Close to the equator, there are some regions with no clear primary wind directions meaning that the distribution of the natural isotopes is not uniform and thus the application of the isotope method is more difficult than, for example, in Germany. Even though this theoretic foundation was confirmed in the project, it was still possible to achieve good results in the tropics. The isotope method can thus be considered both for temperate, boreal and tropical regions as a method for verifying the declared origin.

The described approach to the development of low-budget methods to **identify tree species on the basis of chloroplast DNA** has produced promising results for the enforcement of international biodiversity regulations (CITES). The first practical experiences were also gathered from the wood confiscated by customs. An analysis conducted with the techniques described here was able to definitively prove a violation of CITES laws and made it possible to precisely determine the tree species. It is thus highly recommended to expand this method to other tree species.

Of course the techniques described here also represent an interesting and groundbreaking possibility for official monitoring for all other plant (and animal) species.

12 List of figures

13 Bibliography

ASIF M J & CANNON C H (2007): DNA extraction from processed wood: a case study for the identification of an endangered timber species (*Gonystylus bancanus*). *Plant Mol Biol Report* 23: 185-192.

BOWEN G J & REVENAUGH J (2003) Interpolating the isotopic composition of modern meteoric precipitation. *Water Resources Res* 39: 1299.

BOWEN G J & WASSENAAR L & HOBSON K A (2005): Global application of stable hydrogen and oxygen isotopes to wildlife forensics. *Oecologia* 143: 337-348.

BOWEN G J & WILKINSON B (2002): Spatial distribution of δ18O in meteoric precipitation. *Geology* 30: 315-318.

BURK R L & STUIVER M (1981): Oxygen isotope ratios in trees reflect mean annual temperature and humidity. *Science* 211: 1417-1419.

CHUKHROV et al. (1980): The isotopic composition of plant sulphur. *Org Geochem* 2: 69-75.

CRAIG H & GORDON L I (1965): Deuterium and oxygen 18 variations in the ocean and the marine atmosphere. *University of Rhode Island Publication*. Vol. 3: 277-374.

CRAIG H (1961): Isotopic variations in meteoric waters. *Science* 133: 1702-1703.

DE FILIPPIS L & MAGEL E (1998): Differences in genomic DNA extracted from bark and from wood of different zones in *Robinia* trees using RAPD-PCR. *Trees* 12: 377-384

DEGUILLOUX M F, PEMONGE M H & Petit R J (2002): Novel perspectives in wood certification and forensics: dry wood as a source of DNA. *Proc Roy Soc Lond Ser B-Biol Sci* 269: 1039-1046.

DEGUILLOUX MF, PEMONGE MH & PETIT RJ (2002): Novel perspectives in wood certification and forensics: dry wood as a source of DNA. *Proc Roy Soc Lond Ser B-Biol Sci* 269: 1039-1046.

DOUGHTON J.A. & SAFFIGNA P.G. & VALLIS I. (1991): Natural abundance of ^{15}N in barley as influenced by prior cropping or fallow, nitrogen fertilizer and tillage. *Aust J Agric Res* 42: 723-733.

DOYLE J J & DOYLE L J (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19: 11-15.

DUNBAR J. & WILSON A.T. (1983) Oxygen and hydrogen isotopes in fruit and vegetables juices. *Plant Physiol* 72: 725-727.

EPSTEIN S P THOMPSON P & YAPP C J (1977) Oxygen and hydrogen isotopic ratios in plant cellulose. *Science 198:* 1209-1215.

FRANCIS J K (1991): *Swietenia mahagoni Jacq.* West Indies mahogany. SO-ITF-46. Institute of Tropical Forestry. USDA-FS, Río Piedras, Puerto Rico. 7p.

HEBERT P D N & GREGORY T R (2005): The promise of DNA barcoding for taxonomy. *Syst Biol* 54: 852-859.

HEBERT P D N, CYWINSKA A, BALL S L & DE WAARD J R (2003): Biological identifications through DNA barcodes. *Proc Roy Soc Lond* 270:313-321.

HÖLTKEN A M, SCHRÖDER H, WISCHNEWSKI N, DEGEN B, MAGEL E. & FLADUNG M (submitted): Development of DNA based methods to identify CITES-protected timber species: A case study in the *Meliaceae* family.

KAWAMURA H et al. (2006): Isotopic evidence in tree rings for historical changes in atmospheric sulphur sources. *Environ Sci Technol* 40: 5750-5754.

KEELING C D et al. (1995): Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980. *Nature* 375: 666-670.

KOCH G, RICHTER H G & GEMBRUCH K (2005): CITESwoodID: Descriptions, illustrations, identification and information retrieval: CD in English and German. Version November 2005, Federal Institute for Agriculture and Forestry.

KÖRNER C et al. (1991): Carbon isotope discrimination by plants follows latitudinal and altitudinal trends. *Oecologia* 88: 30-40.

KRAUSE J, DEAR P H, POLLACK J L, SLATKIN M, SPRIGGS H, BARNES I, LISTER AM, EBERSBERGER I, PÄÄBO S & HOFREITER M (2006): Multiplex amplification of the mammoth mitochondrial genome and the evolution of *Elephantidae*. *Nature* 439:724–727.

KRESS W J & ERICKSON D L (2007): A two-locus global DNA barcode for land plants: The coding *rbc*L gene complements the non-coding *trn*H-*psb*A spacer region. *PLOS one* 6: e508.

KRESS W J, WURDACK K J, ZIMMER E A, WEIGT L A & JANZEN D H (2005): Use of barcodes to identify flowering plants. *Proc Natl Acad Sci USA* 102: 8369-8374.

LEHMANN S (2007): Schutz der Wälder -Nationale Verantwortung tragen und global handeln (Protecting the Forests - National responsibility and Global Action). BfN scripts 209 (Federal Agency for Nature Conservation - Bundesamt für Naturschutz).

LEUENBERGER M et al. (1992): Carbon isotope composition of atmospheric CO2 during the last ice age from an Antarctic ice core. *Nature* 357: 488-490.

MUELLNER A N, SCHAEFER H & LAHAYE, R (2011): Evaluation of candidate DNA barcoding loci for economically important timber species of the mahogany family (*Meliaceae*). *Mol Ecol Res*: doi:10.1111/j.1755-0998.2011.02984.x.

NICOLAI B.M. et al. (2007): Nondestructive measurement of fruits and vegetable quality by means of NIR spectroscopy: A review. *Postharvest Biol Techn* 46: 99-118.

POINAR H N, SCHWARZ C, Q I J, SHAPIRO B, MACPHEE R D E, BUIGUES B, TIKHONOV A, HUSON DH,TOMSHO L P, AUCH A, RAMPP M, MILLER W & SCHUSTER S C (2006): Metagenomics to paleogenomics: Large-scale sequencing of mammoth DNA. *Science* 311:392–394.

RACHMAYANTI Y, LEINEMANN L, GAILING O & FINKELDEY R (2006): Extraction, amplification and characterization of wood DNA from *Dipterocarpaceae*. *Plant Mol Biol Report* 24: 45-55.

RICHTER H G and. DALLWITZ M J (2000 onwards). 'Commercial timbers: descriptions, illustrations, identification, and information retrieval. In English, French, German & Spanish.' Version: 4 May 2000.

ROGAJEV E I, MOLIAKA Y K, MALYARCHUK B A, KONDRASHOV F A, DERENKO M V, CHUMAKOV I & GRIGORENKO A P (2006): Complete mitochondrial genome and phylogeny of Pleistocene mammoth *Mammuthus primigenius*. *PLoS Biol* 4:e73.

SAVARD M M, BEGIN C & SMIRNOFF A (2009): Isotopes reflect anthropogenic NOx emissions and climatic effets *Environ Sci Technol.* 43: 604-609.

SCHMITZ-KRETSCHMER H J, KOCH G & KRÄMER G (2006): Lupe, Notebook and the software CITES*wood*ID (Magnifying Glass, Notebook and the CITES woodID Software). *Holz-Zentralblatt publication* 132 : 484-485.

STYLES B T (1981): *Swietenioideae*. In: *Meliaceae*. Flora neotropica monograph. Pennington, STYLES T D & TAYLOR D A H (eds.). New York Botanical Garden. New York. USA. 28 : 359–418.

SWOBODA S. (2007): Identification of Marchfeld asparagus using Sr isotope ratio measurements by MC-ICP-MS. *Anal Bioanal Chem* 2: 487-494.

TABERLET P, COISSAC E, POMPANON F, GIELLY L, MIQUEL C, VALENTINI A, VERMUT T, CORTHIER G, BROCHMAN C & WILLERSLEV E (2007): Power and limitaions of the chloroplast *trn*L (UAA) intron for plant barcoding. *Nucleic Acids Res*: e14.

TABERLET P, GIELY L, PAUTOU G & BOUVET J (1991): Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17: 1105-1109.

TREYDTE K et al. (2001): The climatic significance of δ13C in subalpine spruces (Lötschental, Swiss Alps). *Tellus* 53B, 593-661.

TURNER G L et al. (1987) Differences in the natural abundance of ^{15}N in the extractable mineral nitrogen of cropped and fallowed surface soils. *Aust J Agric Res* 38: 15-25.

TUSKAN et al. (2006): The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313, 1596-1604.

PROSSER S J (1993): A novel magnetic sector mass spectrometer for isotope ratio determination of light gases. *Int J Mass Spectrom Ion Processes* 125: 241-266.

WINNER W E et al. (1978): Stable sulphur isotope analysis of SO_2 pollution impact on vegetation. *Oecologia*. 36: 351-361.

YAPP C & EPSTEIN S (1982): Climatic significance of the hydrogen isotope ratios in tree cellulose. *Nature* 297: 636–639.

YONEYAMA T et al. (1989): Fractionation of nitrogen isotopes during the uptake and assimilation of ammonia by plants. *Plant Cell Physiol* 21: 1211-1220.

YONEYAMA T et al. (1990): Variation of natural ^{15}N abundance of crops and soils in Japan with special reference to the effect of soil conditions and fertilizer application. *Soil Sci Plant Nutr* 36: 667-675.

ZIEGLER H et al. (1976): Hydrogen isotope discrimination in higher plants: correlation with photosynthetic pathway and environment. *Planta* 128: 85-92.