ANNEX 9

Reporting of the control of chain of custody for two species of Khaya

CHAIN OF CUSTODY OF TIMBER RESOURCE IN SAMARTEX IN THE CONTEXT OF TRACKING SYSTEMS FOR LEGAL TIMBER IN GHANA

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Preamble

In recent years, there has been rapid development of international initiatives to deny market access to illegal timber. Governments in producer countries are being encouraged to establish reliable verification and monitoring systems in order to ensure that their timber exports have been legally sourced and produced.

The total estimated land area of Ghana is 23,854,000 ha (FRA 2010) out of which 7,448,000 ha (32.3%) of the vegetation falls within the High Forest Zone (HFZ) while the remaining 16,406,000 ha is savannah woodland. Timber production, which is about 5% of national GDP, is mainly from the HFZ of which about 1,630,000 ha is designated as forest reserves.

Over the last two decades, Ghana has developed and implemented comprehensive forest management systems including monitoring mechanisms such as harvesting, transporting, processing and exporting of timber and timber products as well as procedures for post auditing with the view of reducing leakages along the chain.

This paper highlights the main tracking process as well as challenges including efforts being made by the Forestry Commission (FC) of Ghana to improve and implement a more reliable monitoring and verification system for timber and timber products. This applies to Yoyo forest reserve a concession managed by Sarmartex Plywood and timber company ltd selected for a pilot study under the ITTO project PD 620/11 Rev.1 (M).

Timber production in Ghana may come either from Government designated forest reserves like Yoyo forest reserve where our pilot study was undertaken or from off-reserve areas. Each of these two sources of timber has different management and monitoring mechanism.

Timber Production from Yoyo Forest Reserve

Yoyo Forest reserve is managed for environmental protection or timber production and or both. Yoyo forest reserve covers an area of 231.31km^2 within the wet /moist Evergreen zone of the High Forest Zone in Ghana. It is one of the designated natural forest timber production reserve. Yoyo production Forest Reserve is sub divided into compartments (unit of management) with an average size of about 128 ha. There is a time line (Harvesting Schedule) for logging individual compartments. Logging history and static inventory of the production area is used as a guide in preparation of a Harvesting Schedule (HS) and every HS is prepared using a forty-year (40-yr) felling cycle.

Concessions (group of compartments) or Timber Utilization Contract (TUC) areas in the yoyo forest reserve where the pilot study was undertaken have been allocated to Sarmartex Timber and Plywood Company Ltd through competitive bidding process but regulation of timber cuts within a particular TUC area is done by the Forestry Commission.

Pre Logging Process in Yoyo Forest Reserve

1. **Stock Survey**: This activity is only initiated when a compartment is due for logging within the Yoyo reserve. Two processes are involved: A demarcation of the compartment due for logging, followed by enumeration of all timber species ≥50 cm dbh. Key activities under the enumeration process are the identification of the timber species, diameter measurement of the tree, the coordinates of the tree and assignment of a stock number for the tree (usually written below the point of felling with a scriber). The stock survey is done by the District Stock Survey Team of the Forest Services Division (FSD) of the FC.
2. **Check Survey**: This activity is a quality control measure by the Regional Team of the FSD. It involves a 5% check of the stock survey conducted by the Stock Survey Team.
3. **Stock Map Preparation**: A graphical representation of all the trees captured during stock survey. It shows the position and distribution of the trees within the compartment. This is done manually by the cartography unit, District FSD of Forestry commission.
4. **Yield Calculation**: It involves an application of a conservative formula to determine the number of stems of individual timber species that should be earmarked for felling within the compartment. The formulae are:

   $Z = 0.5Y + 0.2X$ normal formula

   $Z = 0.25Y + 0.2X$ reduced formula for species that have been overexploited in the past

   Where Z = the yield in number of stems, Y = the number of trees above felling limit, X = the number of trees in the 20 cm size class below the felling limit

5. **Yield Map Production**: This map shows only the spatial distribution of all trees in the proposed yield. It also shows the major skidding trails and log dumps for the TUC Holder (Sarmartex) to use during logging in the compartment. This is usually documented manually by the cartography unit, District FSD of Forestry Commission.
6. **Yield Approval**: The stock map, the proposed yield map and yield table are dispatched to the Regional FSD Office for scrutiny and approval.
7. **Yield Endorsement**: Here the Director of Resource Management Support Centre (RMSC) of the FC will vet the approved yield including environmental adherence and make changes if the need arise before a final consent is given for the TUC Holder

(Sarmartex) to start logging operation within the compartment. It is during this phase that softcopies of the final yield tables together with the yield map are released to both the Regional and District FSD offices for monitoring. A copy is also given to the TUC Holder (Sarmartex).

Logging and Transporting of Timber at Yoyo Forest Reserve

Tracking of timber begins from here. Sarmartex conducts his own felling and carting operations using the yield table and the yield map of the Yoyo Forest reserve as a guide. The following verification and monitoring procedures are conducted before the logs are conveyed to the mill:

- All trees felled are crosschecked by FSD Range Supervisor to make sure that the felled tree is part of the yield. Stock number, species name as well as the coordinates of the tree are the main decisive factors
- Tree bole volume parameters (diameter and length) are taken by the Range Supervisor before the felled tree is sectioned into logs by the logging team. These parameters are entered on a Tree Information Form (TIF) where individual trees volume are calculated to fulfill two key objectives:
    - To determine the stumpage value of the felled tree for the TUC Holder (Sarmartex) to pay
    - For tracking the logs that will be obtained from the felled tree to the mill
- Log Measurement and Conveyance Certificates (LMCCs): The District FSD office issues the LMCC to the Sarmartex before the logs could be transported to any destination within the country for milling. This conveyance certificates has other details such as the Reserve and compartment that the logs are coming from, stock numbers of the trees which were felled, the logs obtained from individual trees, their volumes etc
- Authentication of the LMCCs: this activity is done by a representative of the Timber Industry Development Division (TIDD) of the FC at the various road checkpoints and at the mills. Other checks at the mills that are closely monitored by both TIDD and RMSC are the Sawmill Entry Records and Recovery Records

Post Exploitation Checks in Yoyo Forest Reserve

This activity is usually conducted at Yoyo Forest Reserve at the end of a logging operation in a particular compartment by personnel from RMSC. Key activities here are:

1. Conformity to logging standards including compliance to environmental quality
2. All trees harvested are within the approved yield

Issuance of Compartment Closure Certificate in Yoyo Forest Reserve

This activity is initiated after post exploitation checkers are satisfied with the logging operation within the compartments of Yoyo Forest Reserve.

Penalties

When it is detected that some trees have been extracted outside the approved yield at Yoyo Forest Reserve, ten times the value of the tree is surcharged the TUC Holder (Sarmartex). When the offence is serious, the property mark of the Holder is suspended or revoked.

Mill Site Inspection at Yoyo Forest Reserve

Pre Mill: At the Mill a staff of TIDD will conduct log yard inspection at harvesting compartment to mop up leakages and to ensure that all logs are covered by LMCCs.

Contracts are then approved to ensure value for money and for the avoidance of discrepancies in production

Post Mill: Post milling inspection is conducted as a quality control measure at Yoyo, and to ensure that parcel information conforms to contract requirements.

Input-output analysis of logs and processed wood: This activity is done to ensure that logs for processing are from approved sources. It also monitors efficiency and recovery rates at the industry level.

Harbor Inspection: This is where final checks are conducted before the timber products are exported out of Ghana's shores

Reconciliation of harvesting data: The Resource management support centre (RMSC) of the FC will reconcile all harvesting and milling data by collating all trees harvested and milled at Yoyo Forest Reserve within the year as captured by various FC institutions (FSD, TIDD, and RMSC) to determine infractions. The infraction report is then sent to the Chief Executive of FC.

Challenges with the Existing Mode of Timber Tracking

1. Most of the tracking activities are done manually with a lot of paper work causing delays in delivery
2. Duplication of activities and documentation along the chain of custody among various organizations within FC making reconciliation cumbersome and difficult
3. The entire process is subject to human discretion

4. Errors and abuses are detected late along the chain making corrections and punishment ineffective.
5. Misrepresentation of species always difficult to be detected for processed wood. Thus, endangered species could be exploited and mixed up with species that could be traded.

The Way Forward To Enhance Forest Resource Monitoring in Ghana

The Wood Tracking System (WTS) under the Voluntary Partnership Agreement (VPA) aims at ensuring Forest Law Enforcement Governance and Trade (FLEGT). **Helveta**, a consulting agency in collaboration with FC have piloted a system that aimed at transforming the existing manual chain of custody into an electronic one that would improve efficiency and eliminates leakages and problems associated with the existing methods.

The electronic tracking process involves the use of Hand Held Computers to capture stock survey data, and transfer the data into a central station where yield determination, stock mapping and yield mapping would be automated. A new way of monitoring timber harvesting and transporting, processing and exporting were also piloted by HELVETA. However, most of the processes of the HELVETA system are under human discretion which cannot eliminate Errors and abuses associated with the manual tracking.

Currently, Ghana is a signatory to the VPA and is looking for internationally accepted technologies that could support the vigorous manual chain of custody to eliminate errors and abuses associated with the system. The DNA fingerprints technology for identifying species and source of timber when becomes operational could be used along the line of manual chain of custody to eliminate errors and abuses to ensure sustainable forest management and conservation of biodiversity of the forest resource estate in Ghana.

Project Report

M. PAULINI & A. M. HÖLTKEN

DNA-BASED SPECIES IDENTIFICATION AND TIMBER TRACKING OF *KHAYA IVORENSIS AND K. ANTHOTHECA* IN GHANA

in Frame of the ITTO project

"Development and implementation of a species and timber tracking system in Africa with DNA finderprints and stable isotopes (PD620/11 M (Rev. 1))"

Table of contents

1. Background

The timber company SAMARTEX and the Forestry Research Institute of Ghana (FORIG) in collaboration with the Resource Management Support Center of the Forestry Commission (FC) were seeking support from the company `Plant Genetic Diagnostics´ GmbH and the Thünen-Institute of Forest Genetics to implement a DNA-based wood tracking verification system. This should improve the overall effectiveness of the existing Wood Tracking System (WTS) set up in accordance with the Voluntary Partnership Agreement (VPA) and the due diligence obligations of SAMARTEX in frame of the EU timber regulation.

Illegal logging and trade in illegal timber continues to be a major threat to sustainable forest management in West Africa. The issue has attracted much attention from NGOs, policy makers and the media. Although legal instruments have been established to control logging and trade in illegal timber at both national and regional levels, these instruments are still lacking, providing ample opportunity for fraud and the continuation of illegal activities.

Over the last two decades, Ghana has developed and implemented comprehensive forest management systems, including monitoring mechanisms for harvesting, transportation, processing and exporting of timber and timber products. Many challenges to the effective execution of these systems remain including:

- A reliance on manual tracking activities generating a lot of paper work that causes delays in delivery,
- duplication of activities and documentation along the Chain-of-Custody amongst various organizations within the Forestry Commission making reconciliation cumbersome and difficult.
- The entire process is subject to human discretion and fraud is suspected to be commonplace.
- Errors and abuses are detected late along the chain making corrections and punishment ineffective.

DNA-based methods are now available that resolve these problems and provide timber companies and timber traders a high level of security. DNA provides a scientific, truly independent and infallible platform to validate Chain-of-Custody documentation and eliminate fraud. By comparing the individual genetic profile of a wood sample taken from a tree during the forest inventory with the genetic profile of a wood sample taken after harvesting at another point in the supply chain (e.g. log yard or mill), it is possible to independently validate the paper-based or electronic tracking system ensuring that correct trees have been harvested and processed.

In addition to the proof of correct and legal origin of the timber, the company SAMARTEX has much interest to know the exact botanical species of *Khaya* due to very similar and sometimes overlapping morphological traits making species identification by tree spotting in the field quite difficult (see table 1). This is an economically important information because there are clear differences in wood quality between *K. ivorensis* and *K. anthotheca* and thus needs to be known before felling the wrong trees causing further ecological and economical damage. Moreover, the EU-timber regulations ask for precise declarations of the botanical species.

Table 1: Most important morphological traits of *Khaya anthotheca* and *K. ivorensis*

Trait	*Khaya anthotheca*	*Khaya ivorensis*
Leaflets	2-4 pairs, 8-15 X 4-8 cm	4-7 pairs, 5-14 X 2-6 cm
Laterals	5-9 pairs	5-9 pairs
Ecology	Dry to moist semi-deciduous	Evergreen to moist semi-deciduous
Fruits	6-10 cm diameter, 4-5 valved, <3 (5) mm thick	4-7 cm diameter, 5 valved, <3 (5) mm thick
Bark	Smooth, often pale, with scattered scales	Rough, scaly (smooth when younger

Figure 1: Very similar morphology of *Khaya anthotheca* and *K. ivorensis*

In frame of the ITTO project "Development and implementation of a species identification and timber tracking system in Africa with DNA fingerprints and stable isotopes" we conducted a pilot study with the following three objectives:

1. Development of DNA-fingerprinting methods that can be used to identify the exact *Khaya* species before felling
2. Test of reliability of the DNA-based tree tracking system approach to control a chain-of-custody of *Khaya* timber

2. Material and Methods

2.1 Sampling of plant material

Cambium samples from 400 trees (*Khaya ivorensis* and *K. anthotheca*) were collected in the actual logging zone of the SAMARTEX concession (Fig. 2 and 3). The samples were stored in plastic bags or tubes with silica gel for quick dehydration and DNA conservation. Later, after the felling, wood and veneer samples were colleted at the saw mill and the veneer production facility. Most timber samples include only trees that were part of the before samples individuals in the logging area. After a mission in Ghana, all these timber samples were personally transported to Germany and passed over to PLANT GENETIC DIAGNOSTICS (PGD) Ltd.

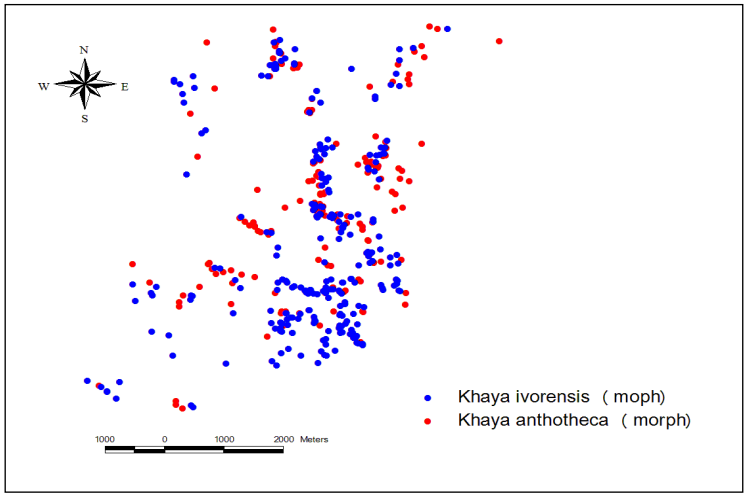

Figure 2: Distribution and morphological species identification of the sampled *Khaya* trees in the concession

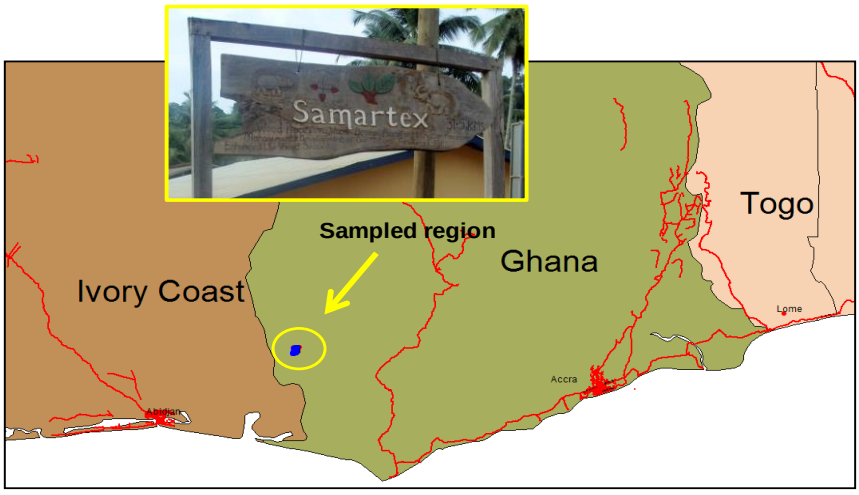

Figure 3: Location of the Samartex logging concession in Ghana

2.2 Lab work

2.2.1 DNA isolation

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 (DE FILIPPIS & MAGEL 1998, DEGUILLOUX et al. 2002, RACHMAYANTI et al. 2006, ASIF & CANNON 2007). In this study we used a new protocol which is applied for patent with partners from Germany and Australia and that was already used for up to 1000 years old oak timber samples. This approach is aligned to mitigate the effects of contamination of the samples with external DNA, to minimize further demolition of already degraded DNA sequences and to purify timber DNA from the majority of PCR inhibitors such as cellulose and hemicellulose, lignin (almost phenolic groups), waxes and different pigments and colours.

2.2.2 The selection of DNA markers

In many studies nuclear microsatellites, also known as simple sequence repeats (nSSRs), revealed to be the optimal choice of marker. Due to high variability and codominance this kind of marker is suitable for a combination of both, forensic identification purposes (e.g. individual fingerprints for the development of tracking systems to control chain-of-custody, LOWE et al. 2010) and species identification (HÖLTKEN et al. 2012, 2014). Furthermore, SSRs have been used to study spatial genetic structures and to develop geo-genetic reference maps. The latter method allows an independent determination of the geographic origin of timber without reference sampling during the timber chain-of-custody (see JOLIVET & DEGEN 2012).

Several sets of microsatellites have already been developed for different genera of the Meliaceae family such as *Khaya*, *Swietenia* and *Entandrophragma* (see KARAN et al. 2012, LEMES et al. 2002, LEMES et al. 2003, LI et al. 2010, SEXTON et al. 2010, WHITE & POWELL 1997 etc.). After testing a lot of primer pairs, six loci were assessed to be very suitable to deal with the above mentioned objectives.

Table 2: Primers before and after optimization (sequences, fragment length in base pairs, GC/AT-relation)

	primer information from literature			primer information after optimization		
SSR-Locus	primer-sequences	fragment length	GC/AT	primer-sequences	fragment length	GC/AT
Ks063	F: CAATATAAGGGACAATACTCTCA	206-268 bp	8 / 15	F: CAATATAAGGGACAATACTCTCA	209-228	8 / 15
	R: CAACATAGATCCATCGTGAGT		9 / 12	R: TATACAAACATAGATCCATCGT		8 / 14
Kse2_11	F: TTTCTACCAGTGCGGTCCCT	278-294 bp	11 / 9	F: TTTCTACCAGTGCGGTCCCT	252-270	11 / 9
	R: GTGCAAATTGTGCTGGGGTG		11 / 9	R: GTGCAAATTGTGCTGGGGTG		11 / 9
Kse1_14	F: TGTTCTCCCAGTTATGGCAGTG	159-193 bp	11 / 11	F: GTTCTCCCAGTTATGGCAGTG	134-166	11 / 10
	R: CCGGTGGAAGTGATTTGACCTT		11 / 11	R: CCGGTGGAAGTGATTTGACCT		11 / 10
Ks079	F: TTTCAACTCTTCAATCTTCATCT	87-115 bp	7 / 16	F: CAACTCTTCAATCTTCATCTGG	93-130	9 / 13
	R: GGCACTACCAATATTTTGTTTT		7 / 15	R: GAGACGGGGCACTACCAATA		11 / 9
Kse1_23	F: AATGAGTAATGACAAGAAAGTA	344-394 bp	6 / 16	F: GCTAATGAGTAATGACAAGAAAG	343-383	8 / 15
	R: AATTGGCGGATAGTTGATGT		8 / 12	R: GGCGGATAGTTGATGTATGC		10 / 10
Kse3_29	F: TTAGGCATAACCGAGGAAAC	193-229 bp	9 / 11	F: TAGGCATAACCGAGGAAACAG	168-195	10 / 11
	R: AAGGCTGTCATTGAAGATAGGAG		10 / 13	R: GCCTGTCATTGAAGATAGGAG		10 / 11

2.2.3 Optimisation of the chosen SSR-Markers for the analysis of timber samples

We designed new primers considering high degradation and low quality of DNA in timber. As shown in table 2, we reduced the sizes of several microsatellites fragments, we chose the primer nucleotide sequences in order to achieve high conformity of the annealing temperatures of forward and reverse primers (50-60°C), consisting of the nucleotides Guanin or Cytosin at their 3´- and 5´- ends allowing an optimized annealing of the primers to the single-stranded DNA template. Further, four of the used six microsatellites consist of repeating sequences of three base pairs of DNA providing a much more accurate amplification with lower numbers of "stutter" bands (ssrKS16, Ks063, Kse3-29, Ks079).

In the following we specified the information about the used SSR-fragments. The optimized primer pairs are visualized in grey and the SSR-units (sequence repeats) underlined:

Ks063; GenBank HQ200185.1

TACATATTGTTCTAAAAACAATATAAGGGACAATACTCTCATTATCTATTGATGGTACCTTTAACAATAATATGA
AATTTAAAAAATAATATTGCTATGTTAAAAATAATAAAGCAAATATTCAAAAGAATAACAATATATAGAACAAAA
TAATAATAATAATAATAATAATAATAATAATAAAGGACCTACTTATAATAAAATAAATAAATAAAAACTCAC
GATGGATCTATGTTGGTATATTTAGTTAGTTACAGTGCTACTTGTATTTTTT

Kse1-14; GenBank GU903058.1

TCATGTTCTCATGTTCTCCCAGTTATGGCAGTGGGGTAACCCTTAGGTGCATCATTGAATAACCAGCTTCATGCA
ACTCAGTATTTTTAGGCATAGGCATCCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATACCTCAAACA
AGGTCAAATCACTTCCACCGGTAATGTTCCACACAATTCCAGTAGCCCTCTCTATACCAAT

Kse1-23; GenBank GU903059.1

CAAAAGCCTGGAAGTTAATGCAAAAGGTTGAACTTAATTTGACGAAGCTAATGAGTAATGACAAGAAAGTATAAT
TCTATTTTCCTTGAATATGAATCAAAGAGATGGTTAATTTGTCCTATAGTTAGAAGGCAAGTGGCCAGGCTAGCA
AGGCTCAACAATAAATTTATATCGCACAAGCGCCATAACTCCACCATGAGTGAGAAGAAGAAGAAGAAGAAGAAG
AAGAAGAAGCAGAGTAAATTTATTGGTGAGGTTGGTGGAAGCCGCATCTACGTAATCTAGAAAGGCACCTTCCTC
TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGCATTTGCCTAGAATAATTA
GATTCCTCATTTAATGCATGGAGAAAAACCATTAAACGCATACATCAACTATCCGCCAATTTTGGTAAGTGCATT

Kse2-11; GenBank: GU903061.1

TAGAGTTCAAAAGGCCATGCACACAAGAAACAACACATACGAGTGCATAAGCCTATTTCTACCAGTGCGGTCCCT
CCTAAAATCATAACAAAAGATTGTGGATAATTGGCCTATAAAATTAACTGCAATTCATGAAGGGTTCAACAAACA
AGTCAAAGAAATTTAGTAAAGATTGGCAAATATTTTCAGACAATAAAGGGTGAGAGCCTGAAAACATTGCAGACA
AATCCATCCTTGTTAACCAATGTCAAAAATGTTACTAGCATGTGTGTGTGTGTGTGTGTGTGTCTCCCCGTAT
CACTCACACCCCAGCACAATTTGCACCTAACACAGCCTGATATATC

Kse3-29; GenBank GU903064.1

GCACTGGATTTAAGAGGCACAAAGACTTATTTAGGCATAACCGAGGAAACAGCCCACAAGCTTTACCTGATAAAT
TGCCAGAATCATCACACTCATCAACTTCCTCTTCACT*TTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTC*TATAT
CCTCCCCATAATGAGGTATGACAATAGACTTGTTAACAAGAGGCCTCCTATCTTCAATGACAGCCTTCATAACTT
CTCTTAGTTGCTCAATTGC

Ks079; GenBank: HQ200189.1

TTTTCAACTCTTCAATCTTCATCTGGACACTCTTCTACTTCTTCTTCTTCTTCTTCTTCTTCGCTCAGTTTCTGC
TTTCCAAAACAAAATATTGGTAGTGCCCCGTCTCGTATTTTGCCCCGCAAAAAGCCGACAGATTTTTTGATATGA

2.2.4 Testing amplification success of the chosen microsatellites

Amplification success of the chosen primer pairs was tested using three cambium samples from each of the two *Khaya* species (PCR-conditions in table 3A-D). As expected, the microsatellites with repeats of three base pairs yielded in more accurate amplification results than fragments consisting of two base pair repeats because of a lower amount of slippage events during polymerase activity. Two examples are given in Figure 4 (SSR-Loci Ks079, fig. 5a and b; Kse1-14, fig. 5c and d), showing the results of the fragment length detection on an ABI 3730 genetic analyzer. Altogether, the optimization of the primer pairs resulted in excellent amplification success.

Tables 3A – 3D: The PCR conditions are shown in Tables 2A to 2D.

SSR-Loci: Ks063, Kse2-11, Kse1-14, Kse3-29, Ks079

A

PCR-component	C stock	C final	V_R (µL)
PCR-buffer	10 X	1 X	1,50
$MgCl_2$	25 mM	1,75 mM	1,05
dNTPs	10 mM	0,2 mM	0,30
primer for	10 mM	0,2 mM	0,15
primer rev	10 mM	0,2 mM	0,15
polymerase	5 U/µL	0,4 U/µL	0,12
DNA	10 ng/µL	2 ng/µL	3,00
H_2O			8,73
	total reaction volume		15,00

B **PCR-programme**

First denaturation	94°C	3 min	
Denaturation	94°C	30 sec	
Annealing	57-59°C	45 sec	30 X
Elongation	72°C	1 min	
Final elongation	72°C	10 min	
Conservation	8°C	∞	

SSR-Locus: Kse1-23

C

PCR-component	C stock	C final	V_R (µL)
PCR-buffer	10 X	1 X	1,50
$MgCl_2$	25 mM	2,00 mM	1,20
dNTPs	10 mM	0,2 mM	0,30
primer for	10 mM	0,2 mM	0,15
primer rev	10 mM	0,2 mM	0,15
polymerase	5 U/µL	0,4 U/µL	0,12
DNA	10 ng/µL	2 ng/µL	3,00
H_2O			8,58
	total reaction volume		15,00

D **PCR-programme**

First denaturation	94°C	3 min	
Denaturation	94°C	30 sec	
Annealing	50°C	45 sec	30 X
Elongation	72°C	1 min	
Final elongation	72°C	10 min	
Conservation	8°C	∞	

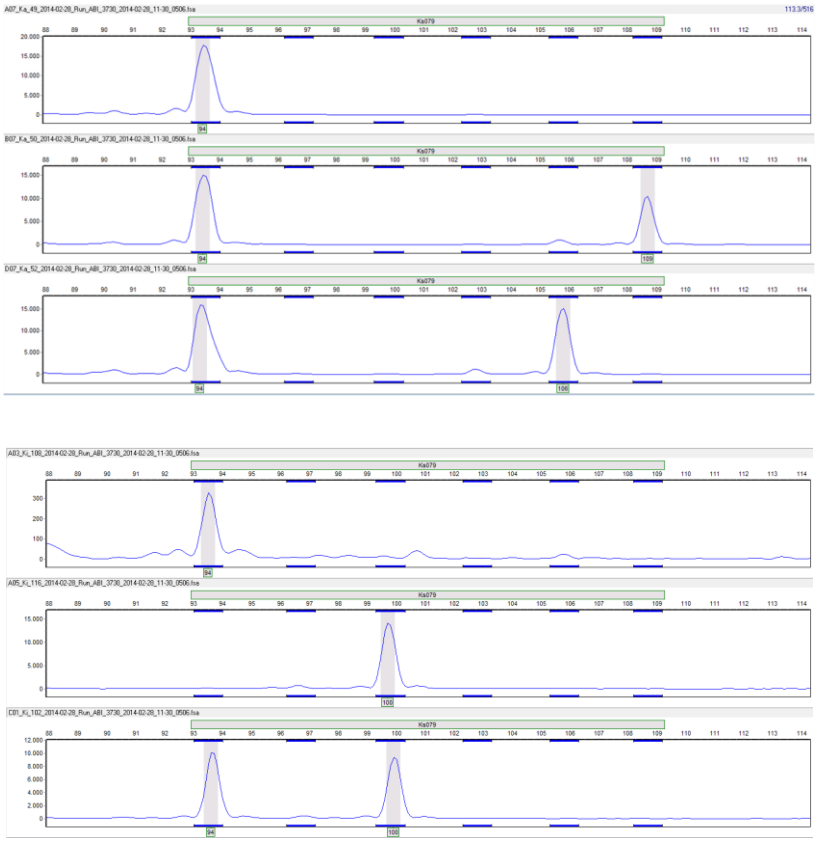

Figure 4a: Fragment lengths of three *Khaya anthotheca* (above) and three *K.ivorensis* (below) samples at the SSR locus Ks079 (3bp-repeats)

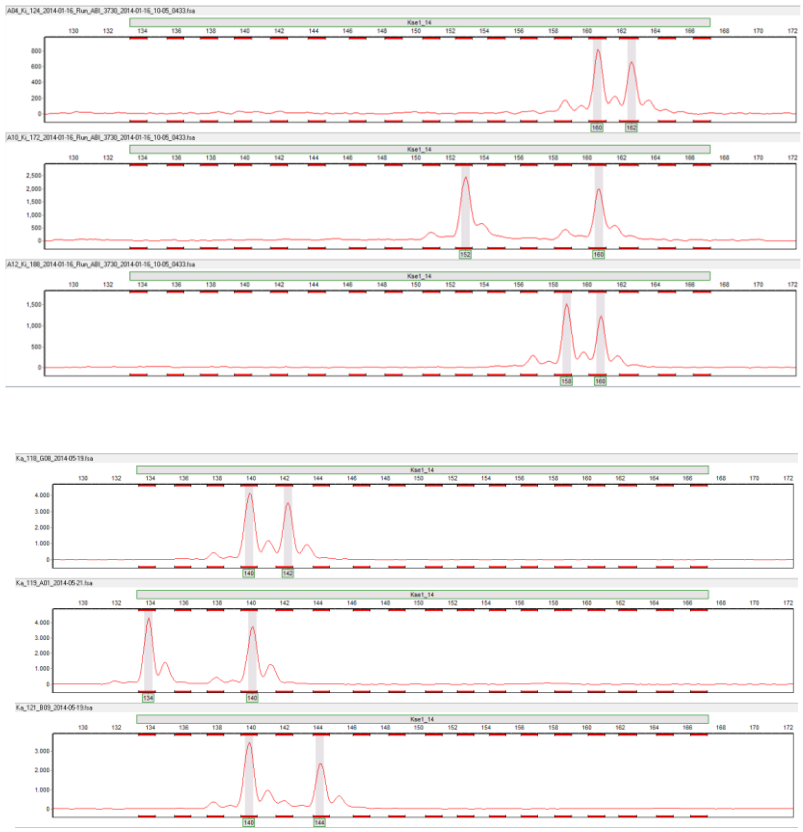

Figure 4b: Fragment lengths of three *Khaya anthotheca* (above) and three *K.ivorensis* (below) samples at the SSR locus Kse1-14 (2bp-repeats)

2.2.5 Population genetic calculations

2.2.5.1 Species identification and hybrid quantification

The assignment of individuals to reproductive groups was carried out using the software STRUCTURE 2.3.2 (PRITCHARD et al. 2009, FALUSH et al. 2003, 2007). This method is suitable to assort individuals to populations with restricted gene flow or to different taxonomic units (species or subspecies), but it also enables to detect hybrid swarms and to quantify the proportion of hybrids. The programme operates on a model-based Bayesian clustering method, which allows conclusions on population structures, reproductive conditions and mating systems, independent of predetermined clustering structures. In the process, the proportions of genetic admixture of each individual are quantified leading to an individual assignment to reproductive units or clusters.

2.2.5.2 Genetic profiles, diversity and differentiation

On the basis of the clustering results, allelic frequencies of the six analysed microsatellite loci were calculated for each of the reproductive group. Differences between clusters are highlighted by assembling the frequency information into allelic profiles (histograms).

Further, the genetic distances of the reproductive groups or clusters were characterized using the genetic differentiation parameter according to GREGORIUS (1974). This parameter measures the proportion of genetic types not shared by both of the populations. It equals half of the sum of the absolute differences of the frequencies of genetic types in populations X and Y. This distance parameter reaches its maximum value 1, if the two populations have no genetic types in common and its minimum value 0 if the two populations have identical genetic structures:

$$d_0 = 0{,}5 \sum_{i=1}^{n_k} |x_i - y_i|$$

x_i and y_i denotes the frequency of the i-th genetic type in population X and Y at the k-th locus

2.2.6 *Timber tracking*

The DNA-isolates of the wood samples, many of them part of the sampled trees in the logging area, were analysed using the six optimized SSR-markers for tracing back the timber chain-of-custody. Microsatellites are commonly accepted, particularly in forensic studies, due to their high variability and high exclusion probability.

3. Results and Discussion

Based on multilocus-SSR data (see appendix 1) we obtained information about the clustering of the genetically studied individuals into reproductive groups incl. hybridization as well as population genetic parameters of the clusters.

3.1 Reproductive groups

3.1.1 Reproductive groups based on genetic data

Compared to other closely related species, we found an exceptionally high genetic differentiation between two groups of individuals originating from a single area within the complex concession structure of the company SAMARTEX (figure 5). For example, in the two oak species *Quercus petraea* and *Q. pubescens*, native to Central and Southern Europe, for which species integrity is also presserved to a high extent, an estimation of species identity based on genetic markers has been shown to be more accurate on the population than on the individual level (HÖLTKEN et al. 2012). In the case of Khaya we are able to precicely differentiate single individuals into reproductively isolated and taxonomic groups. The proportion of potential hybrids is very low (<7%). This low value may also be interpreted as a result of sharing rare alleles by some individuals or as PCR-artefacts by polymerase slippage.

In conclusion, the genetic method developed in this study offers a reliable approach for a clear distinction of taxonomic groups on the individual level in a species mixture of *Khaya ivorensis* and *K. anthotheca*.

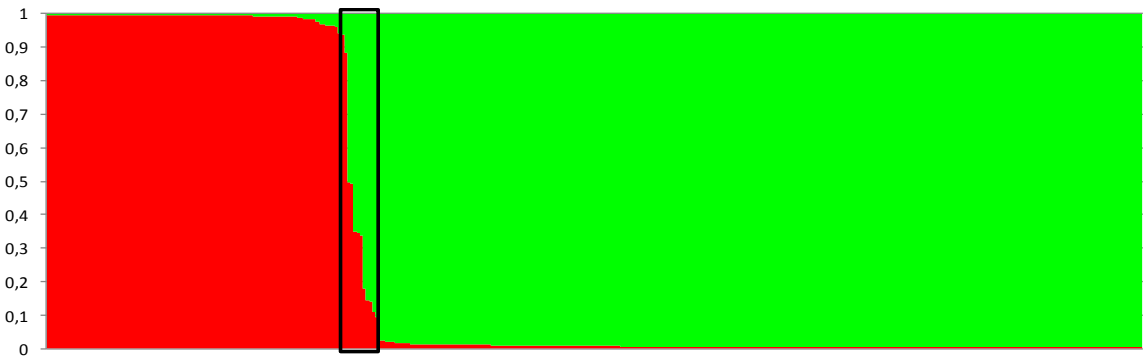

Figure 5: Genetic clustering of the analysed *Khaya* individuals based on STRUCTURE results of six SSR-loci; individuals ordered by genetic admixture

3.1.2 Differences of genetic and morphological characterization

In figure 6 the individuals are ordered by morphological species identification after the analysis of their genetic admixture. Individuals on the left side of the figure were phenologically characterized as *K. anthotheca*, on the right side as *K. ivorensis* (see arrows). The results clearly indicate that the conditions in the field are difficult for species identification by tree spotting, which may be a larger problems in tropical than in deciduous forests (tall trees, worse light conditions, dense shrub layer etc.). High error rates were detected for *K. anthotheca* (XXX%), a more accurate morphological species identifiation could be recorded for *K. ivorensis* (error rate XX%) with the more valuable timber.

In conclusion, this new and ready-to-use genetic approach offers a highly accurate „prescreening“ procedure before harvesting activities in order to evaluate the dominating *Khaya* species within concessions, reducing economical and ecological damage.

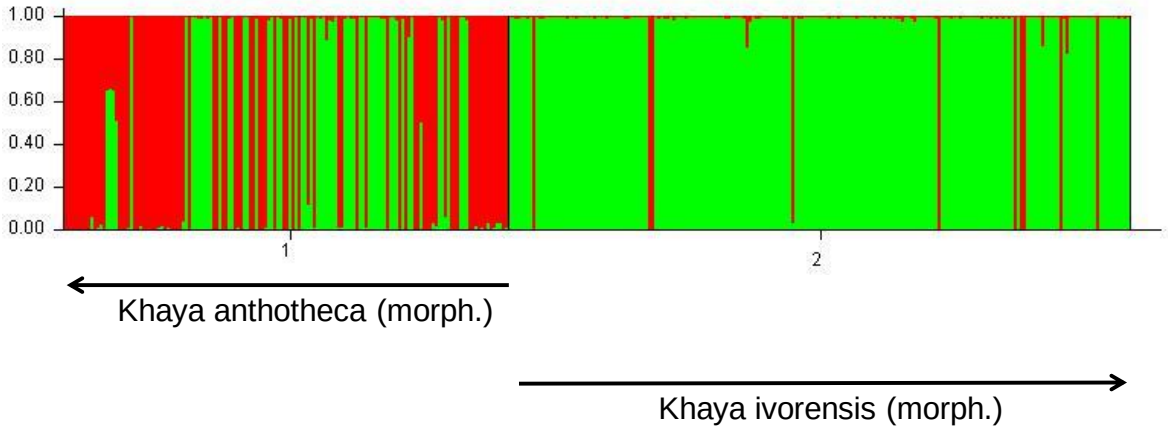

Figure 6: Genetic clustering of the analysed *Khaya* individuals based on STRUCTURE results of six SSR-loci; individuals ordered by morphological species identification; ■=*K. anthoteca* (genetic identification), ■=*K. ivorensis* (genetic identification)

3.2 Genetic structure of the reproductive units

Differences in allelic frequency profiles and a clear genetic differentiation underline the reliability of the developed genetic tool for differentiating the two species *Khaya ivorensis* and *K. anthotheca*.

In figure 7 the allele frequencies of the two reproductive units are shown for each of the six used microsatellite markers. For most of the loci we found two or more dominating alleles, which were found to be more or less fixed to one of the reproductive units (*K. ivorensis* or *K. anthotheca*). For example SSR-locus Ks063: *K. ivorensis* is almost fixed for the alleles 219 and 222, whereas alleles 212 and 215 dominate in *K. anthotheca*. The similar situation holds for the loci Kse2-11, Kse1-14 and Kse3-29. SSR-locus Kse1-23 shows a much higher variability but also low overlapping allelic frequencies. For Kse-079 we detected one dominating allele for both species (allele with 94 basepairs), but differentiating for the alleles 97 to 130.

Altogether, the genetic differentiation d_0 of the gene pool was 0.849. That means, 84.9% of the allelic variants have to be exchanged between the two reproductive groups to obtain identical population genetic structures. Single locus values for this parameters varied between 0.647 and 0.944. These are exceptionally high values for closely related species indicating a very restricted gene flow by hybridization effects (allele swamping).

This selection of microsatellite loci allows a reliable species identification using different statistical approaches. Here we used STRUCRURE 2.3.2 as basic methodology which clusters individuals into groups showing optimals Hardy-Weinberg conditions , but further procedures such as Geneclass based on gene frequencies should also offer very precise results using the developed genetic background data of this study.

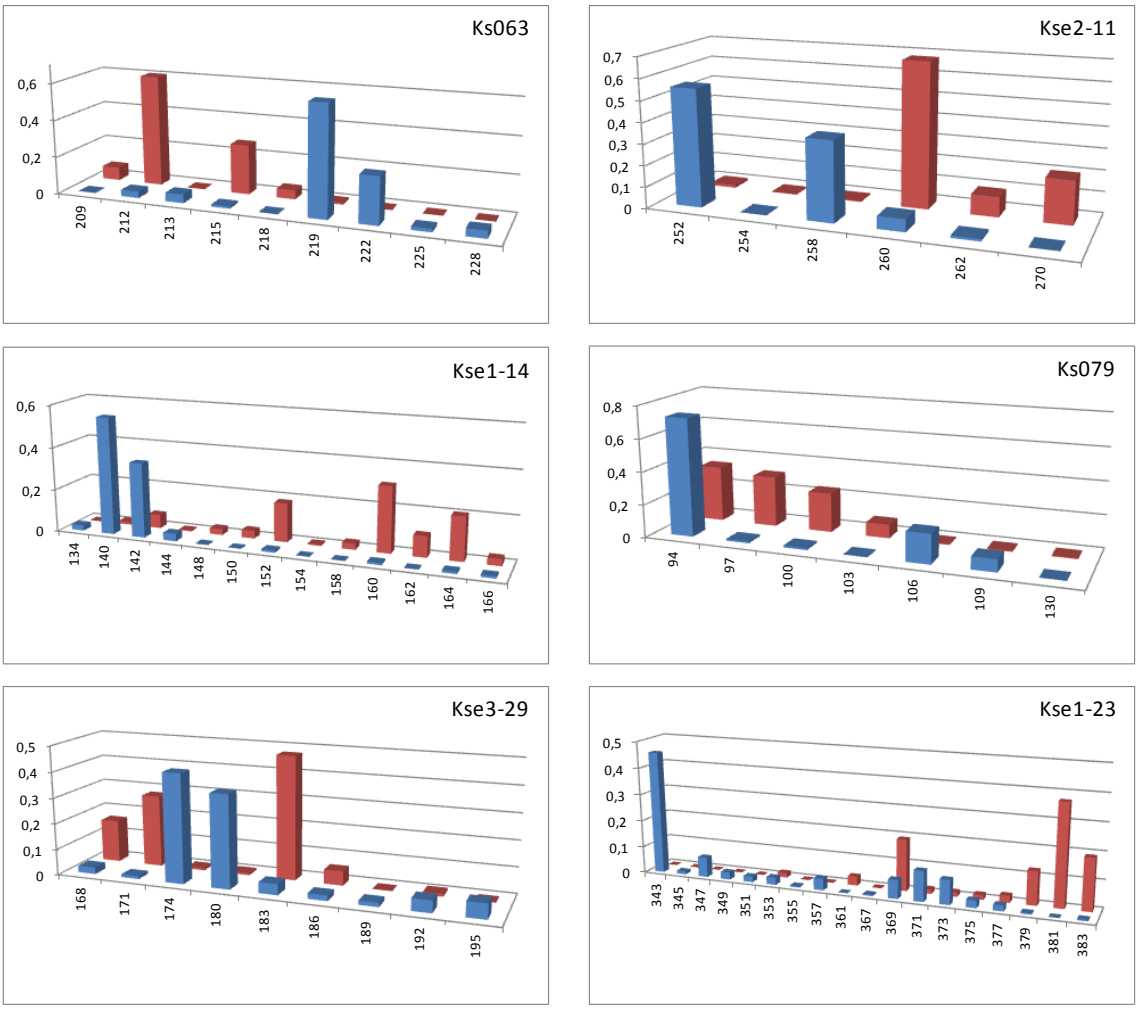

Figure 7: Allelic frequency profiles of the genetically identified reproductive groups in Khaya; ■= K. ivorensis, ■= K. anthotheca

3.3 Timber DNA analysis

More than 200 cambium and timber samples have been analyzed using the above optimized microsatellite primers. Table 4 represents a selection of the results of the timber tracking test. Cambium and corresponding timber samples (from the same putative tree) have been collected in order to evaluate the effectiveness of the chosen DNA marker system to work on woody tissues with low DNA quality and quantity.

Over all, about 50% of the timber samples could be genotyped at least at three of the six microsatellite loci. Due to the high variability of this marker system, this outcome should be enough to detect fraud in the documentation of the chain-of-custody of this high value timber (see serial no. 384, 420 in table 4). Nevertheless, the operators of this new timber tracking system should act with caution, because not all microsatellite fragments turned out to be stable. This is the case for SSR-locus Kse3-29. In several cases some alleles are not amplified at all (e.g. serial no. 213, 357, 421, 426) or the PCR generated shifts according to the length of the repeat motiv (3 bp).

To affirm the obtained results, two repetitions of the timber DNA analyses are recommended. Two further repetitions should be carried in the suspicious cases.

Table 4: A selection of the results concerning the *Khaya* timber tracking test; ■ PCR-misamplification due to low DNA quality; ■ clear wrong declaration of timber

Material	Serial No.	Kse063	Kse063	Kse2_11	Kse2_11	Kse1_14	Kse1_14	Ks079	Ks079	Kse3_29	Kse3_29	Kse1_23	Kse1_23
Kambium	213	215	215	260	270	164	166	100	100	171	183	-1	-1
Timber_1	213a_2	-1	-1	260	270	164	166	100	100	171	171	-1	-1
Timber_2	213b _1	215	215	260	270	164	166	-1	-1	183	183	-1	-1
Kambium	354	219	219	252	258	140	140	94	94	174	180	343	369
Timber_1	354a	219	219	252	258	140	140	-1	-1	-1		-1	-1
Timber_2	354b	219	219	-1	-1	140	140	-1	-1	-1	-1	-1	-1
Kambium	357	218	218	260	270	160	162	100	100	168	183	-1	-1
Timber_1	357a_1	218	218	260	270	160	162	-1	-1	183	183	-1	-1
Timber_2	357a_2	218	218	-1	-1	160	162	100	100	168	183	-1	-1
Timber_3	357b_1	218	218	260	270	160	162	-1	-1	183	183	-1	-1
Timber_4	357b_2	218	218	260	270	160	162	100	100	168	183	-1	-1
Kambium	361	212	212	260	270	152	160	94	94	183	183	383	383
Timber_1	361b_1	-1	-1	260	270	152	160	-1	-1	183	183	-1	-1
Timber_2	361b_2	-1	-1	260	270	152	160	-1	-1	183	183	-1	-1
Kambium	384	212	215	252	258	142	160	-1	-1	174	183	-1	-1
Timber_1	384a	-1	-1	270	270	152	166	-1	-1	183	183	-1	-1
Timber_2	384b	-1	-1	270	270	-1	-1	-1	-1	183	183	-1	-1
Kambium	406	212	218	260	270	150	164	97	97	168	171	-1	-1
Timber_1	406a_1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Timber_2	406a_2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Kambium	408	219	222	252	258	140	142	106	106	174	174	343	373
Timber_1	408a	-1	-1	252	258	140	142	-1	-1	174	174	343	373
Timber_2	408b	-1	-1	252	258	140	142	106	106	174	174	343	373
Kambium	409	212	215	260	260	150	164	100	103	168	171	-1	-1
Timber_1	409a	212	215	260	260	150	164	100	103	168	171	-1	-1
Timber_2	409b	212	215	260	260	150	164	100	103	168	171	-1	-1
Kambium	410	219	219	252	252	142	144	106	106	180	192	343	343
Timber_1	410a	219	219	252	252	142	144	106	106	180	192	343	343
Timber_2	410b	219	219	252	252	142	144	106	106	180	192	343	343
Kambium	411	212	215	252	258	158	162	-1	-1	183	186	-1	-1
Timber_1	411a	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Timber_2	411b	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Kambium	415	219	222	252	258	140	142	94	94	174	192	349	373
Timber_1	415a	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Timber_2	415b	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Kambium	416	212	212	260	270	152	164	-1	-1	171	183	-1	-1
Timber_1	416a	212	212	260	270	152	164	-1	-1	171	183	-1	-1
Kambium	419	212	212	260	260	148	164	-1	-1	171	183	-1	-1
Timber_1	419a	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Timber_2	419a	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Kambium	420	219	219	252	258	140	140	94	94	174	180	343	375
Timber_1	420a_1	212	212	-1	-1	152	164	-1	-1	-1	-1	-1	-1
Timber_2	420a_2	212	215	260	262	152	164	94	94	171	183	-1	-1
Kambium	421	222	222	252	258	140	142	94	94	168	195	343	369
Timber_1	421a_1	222	222	252	258	140	142	-1	-1	168	168	-1	-1
Timber_2	421b_2	222	222	252	258	-1	-1	94	94	168	195	343	369
Kambium	423	222	222	258	258	140	144	94	94	174	192	343	357
Timber_1	423a	222	222	258	258	140	144	94	94	174	192	343	357
Timber_2	423b	222	222	258	258	140	144	94	94	174	192	343	357
Kambium	426	212	212	260	270	162	164	-1	-1	171	171	-1	-1
Timber_1	426a_1	212	212	260	270	164	164	94	94	171	183	-1	-1
Timber_2	426a_2	212	212	260	270	164	164	-1	-1	171	171	-1	-1
Kambium	429	219	219	252	258	140	142	94	94	174	180	343	343
Timber_1	429a	-1	-1	252	258	140	142	94	94	174	180	343	343
Timber_2	429b	219	219	252	258	140	142	94	94	174	180	343	343
Kambium	430	219	219	258	258	140	142	106	106	174	186	343	373
Timber_1	430a	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Timber_2	430b	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Kambium	432	219	219	258	260	140	140	94	94	180	180	345	357
Timber_1	432a	219	219	-1	-1	140	140	-1	-1	-1	-1	-1	-1
Timber_2	432b	219	219	258	260	140	140	-1	-1	-1	-1	-1	-1
Kambium	435	-1	-1	252	258	140	142	106	106	168	180	343	371
Timber_1	435a_2	-1	-1	252	258	140	142	106	106	168	180	343	371
Timber_2	435b_1	219	219	252	258	140	142	-1	-1	168	183	-1	-1
Kambium	444	212	215	260	260	152	164	-1	-1	-1	-1	-1	-1
Timber_1	444a	212	215	260	260	152	164	-1	-1	168	168	-1	-1
Timber_2	444b	212	215	260	260	152	164	-1	-1	168	168	-1	-1

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