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Developing forensic tools for an African timber: Regional origin is revealed by genetic characteristics, but not by isotopic signature

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ABSTRACT

Combatting illegal timber trade requires forensic tools that independently verify claimed geographic origin of timber. Chemical and genetic wood characteristics are potentially suitable tools, but their performance at small spatial scales is unknown. Here we test whether stable isotopes and microsatellites can differentiate Tali timber (*Erythrophleum* spp.) at the level of forest concessions. We collected 394 wood samples from 134 individuals in five concessions in Cameroon and Congo Republic. The nearest neighbour concessions were 14 km apart and the furthest pair 836 km apart. We constructed genetic profiles using eight nuclear microsatellite markers and measured concentrations of $\delta^{18}O$, $\delta^{15}N$ and $\delta^{13}C$. We differentiated provenances using PCA (microsatellites), ANOVA and kernel discriminant analysis (isotopes). Next, we performed assignment tests using blind samples ($n = 12$, microsatellites) and leave one out cross validation (LOOCV, isotopes). Isotopic composition varied strongly within concessions and only $\delta^{13}C$ differed significantly between two concessions. As a result, LOOCV performed only marginally better than random. Genetic differentiation among provenances was also relatively low, but private alleles were commonly found. Bayesian clustering analysis correctly assigned 92% of the blind samples, including those of nearby concessions. Thus, Tali timber can be successfully assigned to the concession of origin using genetic markers, but not using isotopic composition. Isotopic differentiation may be possible at larger spatial scales or with stronger climatic or topographic variation. Our study shows that genetic analyses can differentiate the geographic origin of tropical timber at the scale of forest concessions, demonstrating their potential as forensic tools to enforce timber trade legislation.

1. Introduction

Despite numerous measures illegal logging is still a widespread phenomenon. It is estimated that 10–30% of the timber traded in the global market is illegally harvested (Nellemann, 2012). The problem of illegal timber trade is most predominant in the tropical forestry sector where an estimated 30–90% of the volume is illegally harvested (Hirschberger, 2008; Hoare, 2015). This illegal trade is detrimental to the economies of exporting countries, for example through tax evasion, and uncontrolled illegal logging poses a serious threat to rich and biodiverse tropical forests. To combat the illegal timber trade several legislative actions have been taken in key importing countries, including the EU timber regulation (EUTR) and amendment of the US Lacey Act (Lawson, 2015). In general these laws prohibit any illegally sourced timber from entering the domestic markets. Their implementation has resulted in an increasing demand for means to independently verify the legal status of timber, including forensic techniques.

So far the primary focus of forensic timber studies has been on the identification of timber species based on wood anatomical features (Gasson, 2011). Though useful in the struggle against illegal trade, wood anatomy alone is rarely sufficient when fraudulent claims of timber origin are suspected. Such fraudulent claims of origin, including forged forest management plans (INTERPOL, 2016), false export permits (Tacconi et al., 2016) and mixing of timber from different origins, are predominant ways of 'laundering' illegally sourced timber. False claims of geographic origin of tropical timber are likely the most important form of illegal trade in tropical timber. Therefore development

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of effective forensic techniques to verify claims of geographic origin are much needed (Dormontt et al., 2015). Such techniques would be particularly useful if their spatial resolution allows verifying timber sourced at the level of logging concessions, i.e., at the scale of 10–100 km (Jolivet and Degen, 2012).

Here we evaluate the potential suitability of two promising forensic techniques – stable isotopes and DNA microsatellites – to differentiate tropical timber sourced from different regions. Stable carbon, nitrogen and oxygen isotopes have been widely used for forensic tracing of various goods and products (Ehleringer et al., 2000; West et al., 2007), including trade in endangered species (Retief et al., 2014). Examples of isotope tracing from the forestry sector are scarce (but see: Horacek, 2012; Kagawa and Leavitt, 2010) and completely absent for the tropical forestry sector. The feasibility of DNA microsatellite tracing of tropical timber has been shown for a very limited number of tropical trees, e.g. *Swietenia* spp. (Mahogany) in South America (Degen et al., 2013), *Etandrophragma cylindricum* (Sapelli) in West Africa (Jolivet and Degen, 2012) and *Neobalanocarpus heimii* (Chengal) in Southeast Asia (Tnah et al., 2010). The study by Degen et al. (2013) focused on the assignment of country of origin, representing relatively large spatial scales, i.e. 100 s to 1000 s of kms. The study by Jolivet and Degen (2012) is the only known study at which origin was traced at the highly relevant concession level.

Forensic techniques to verify geographic origin likely have a minimal spatial scale of operation, because of limited spatial differentiation. For instance, genetic differentiation may be limited between nearby stands due to regular exchange of genetic material via seed or pollen (Bizoux et al., 2009). Likewise, low spatial variation in environmental conditions may cause limitations in spatial differentiation of isotopic composition (Wunder and Norris, 2008). Thus, an analysis of the spatial resolution at which the method can be applied is essential for any timber tracing method. Our study is the first to verify the potential of both genetic and isotopic timber tracing techniques to operate at the forest concession level.

We evaluate the potential of isotopic and genetic forensic approaches for one of the most traded African timbers, Tali, which is sourced from two species: *Erythrophleum suaveolens* and *E. ivorense*. Specifically, we addressed the following research questions: (1) Do wood isotopic and genetic signatures differ among provenances? (2) What is the spatial resolution at which these two methods can differentiate timber samples? (3) What is the success rate of assigning blind timber samples to their respective regions of origin? To this end we collected wood samples from four provenances in Cameroon and Congo (nearest neighbour concessions were 14 km apart and furthest pair were 836 km apart), measured genetic (microsatellites; (Duminil et al., 2011)) and isotopic characteristics ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$) of these samples and performed discriminant analyses to differentiate samples from different origins. We then used blind sample tests to evaluate the success of provenancing samples from unknown origin.

2. Material and methods

2.1. Study species

This study focuses on the timber sold under the trade name Tali, which is sourced from two botanically distinct species: *Erythrophleum suaveolens* (Guill. et Perr.) Brenan and *Erythrophleum ivorense* A.Chev. (for convenience we will from here on abbreviate the genus name when referring to the two species). These two closely related species are naturally occurring in West and Central Africa (Fig. 1). They are monoecious and likely insect-pollinated (Anaïs-Pasiphaé Gorel, 2015). *E. suaveolens* and *E. ivorense* diverged ~600 K years BP and within *E. suaveolens* two sub-populations can be distinguished that diverged 120 K and 60 K years BP (Duminil et al., 2015). While *E. ivorense* has a more coastal distribution and *E. suaveolens* a more inland distribution (see Fig. 1), they do occur in sympatry in a large part of their distribution (Duminil et al., 2010), including in the areas sampled in this study. Where the two species occur in sympatry, hybridization cannot be excluded (Duminil et al., 2010).

Tali wood is mostly shipped to Europe where it is applied in heavy construction works, such as sheet piling. In 2010, Cameroon exported an estimated 143,000 m^3 of Tali logs and sawnwood at an average price of 102 € per m^3 (ITTO, 2012). Tali is in the top-5 most valuable timbers for export in Cameroon. Neither of the two species yielding Tali has been classified as endangered, nor are they listed under CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora). Yet, the distribution region of these species in West and Central Africa is known for high incidence of illegal logging (Hirschberger, 2008; Hoare, 2015).

In two of our study sites (concessions 2 and 3) possibly only one of the species (*E. suaveolens*) occurs based on the distribution data (Fig. 1), while at another site (Concession 1) a mix of the two species may occur. Yet, the high degree of species mixing, the difficulty of separating these species (both in the field based on bark and leaf characteristics and in the lab based on wood anatomical characteristics) and the occurrence of identification errors in distribution data do not allow us to state with certainty which of our sampled trees belong to which species. Nevertheless, the fact that our samples are taken from two botanical species and that these species can possibly hybridize does not provide limitations to our study. We evaluate the potential for tracing of geographic origin of a tropical timber that is derived from multiple species (this is often the case in tropical timbers), and we do not conduct a population genetic study in a species conservation context. In addition, in terms of isotope signature, differences in isotope values between species may be smaller than those across sites (van der Sleen et al., 2015b). Clearly, the results of our study should not be used to derive conclusions about species-specific patterns.

2.2. Sample collection

We collected samples in five logging concessions (Fig. 1). In three concessions (1–3) we sampled more intensively and samples from these concessions were included in both the isotopic and genetic analyses. Samples from concessions 4 and 5 were included in the genetic analyses only. In July and August 2015, a total of 381 sapwood samples (triplicates) of 127 individual Tali trees were collected in Cameroon, in three concessions of Groupe Rougier Cameroon (concessions 1–3; Fig. 1; Table A.1). Fresh wood samples of three Tali trees (triplicates) were taken in the Danzer-Interholco IFO concession (concession 4) in Congo-Brazzaville in July 2016. All samples were collected following a standardized protocol, specifically designed to allow sample collection by non-scientific personnel. Sampling took place at multiple spatial scales (14–836 km; Table A.2) using a tiered approach. Samples were collected from stumps of large (> 60 cm diameter at breast height, dbh) trees, < 33 days after logging. Wood samples were solely collected from those trees identified as 'Tali' (so either *E suaveolens or E. ivorense*) by the botanist or prospector of the logging company. Per tree, three samples were taken at different locations along the circumference of the stem. These samples consisted of either ~1 cm^3 of fresh bark/sapwood samples stored in silica gel (concession 1–3) or ~10 cm^3 air-dried sap-/heartwood samples (concession 4). GPS-coordinates of the trees were taken and information, including dbh and estimated time since logging (days), were recorded on the provided field forms. Samples were sent to the Dendrolab at Wageningen University & Research, The Netherlands. Upon arrival of the samples superficial moulding was observed in ~50% of the samples stored in silica gel, any further decay of the wood was immediately stopped by adding extra silica gel to the bags. Finally, four single Tali heartwood samples previously collected in the northeast of Cameroon, were included (concession 5). These samples were collected in June 2011 in concession 11.001 of Transformation REEF Cameroon (TRC).

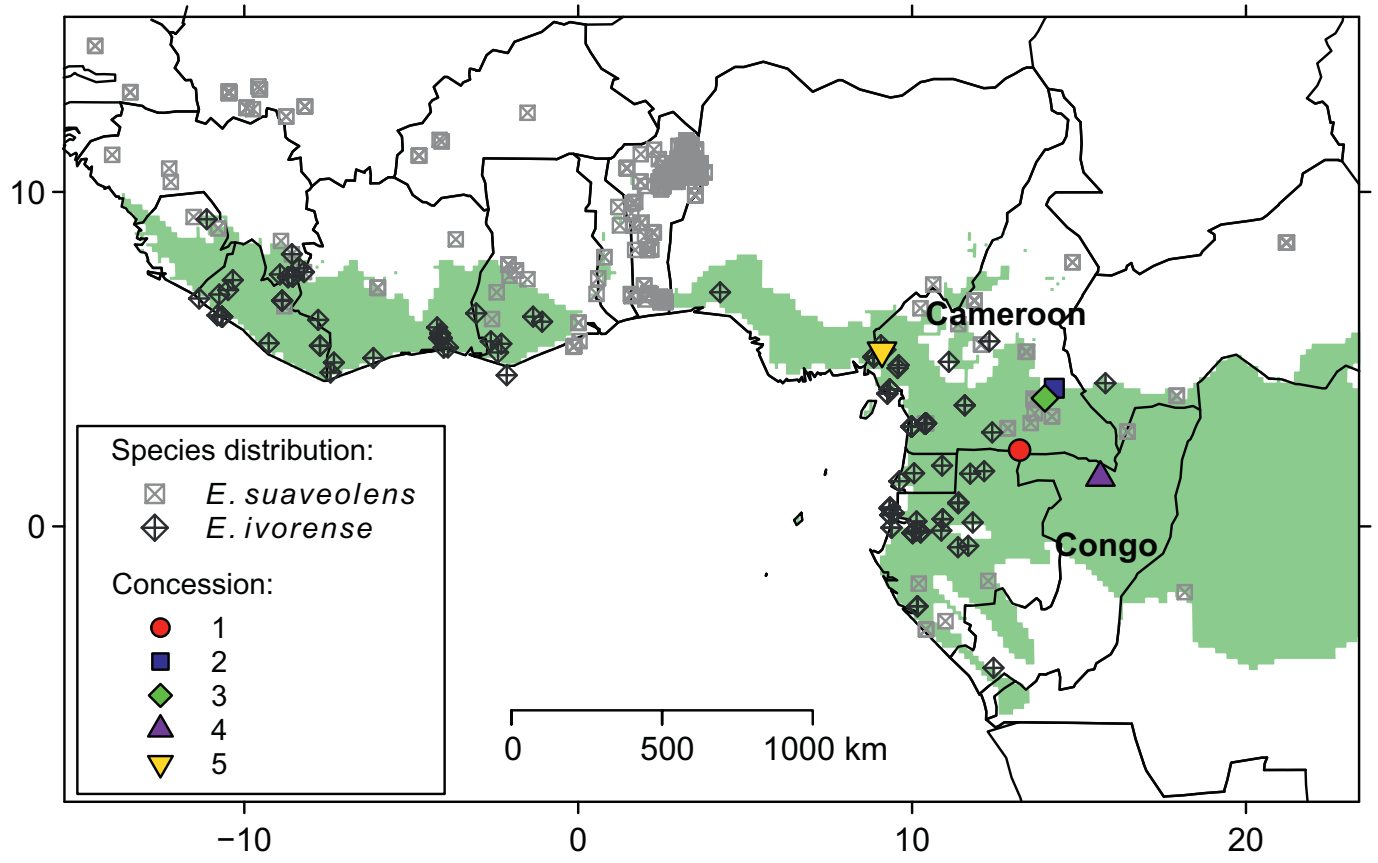

Fig. 1. Locations of the five forest concessions where samples were collected. Isotopic analyses were performed for concessions 1–3; genetic analyses for all concessions. Grey symbols represent documented occurrences of *Erythorphleum suaveolens* and *Erythrophleum ivorense* in Central and West Africa (Source distribution data: GBIF, 2016); green area is tropical forest cover (Source: Global Land Analysis and Discovery (GLAD), 2017).

2.3. Lab preparation – isotopes

The 127 samples collected in concessions 1–3 were included in the isotope analysis. Wood samples for $\delta^{13}C$ and $\delta^{18}O$ analysis were collected in the radial direction of the sample by taking ~25 mg of wood using a sharp knife. For $\delta^{15}N$ analysis approximately ~100 mg of wood was collected using a column drill with a 3 mm drill bit. The wood was pulverized with a mixer mill (Retsch MM301, Germany) using steel tubes and bullets, for 5 min at 25 rpm. We duplicated the analysis per tree to reduce the effect of outliers, assess measurement consistency among different samples of the same tree and minimize the chance of 'losing trees' in the analysis. Thus, we had 254 samples on which isotope analyses were conducted.

For $\delta^{13}C$ and $\delta^{18}O$ analysis cellulose extraction was performed following a modified protocol based on the Jayme-Wise method (Wieloch et al., 2011). Over a period of three days, the samples were first incubated for 4 h in a 5% NaOH solution to remove resins, fatty acids and tannins. Then the samples were incubated for a total of 34 h in a 7.5% $NaClO_2$ solution to remove lignin and dyes. Both extractions were run in a water bath at 60 °C. Crude cellulose samples were collected from the glass funnels using a pipette and mixed by pipetting and homogenized by vortexing the watery samples for five seconds. The samples were oven-dried for three days at 55 °C. Per sample ~0.3 mg of cellulose was subsequently weighed into each of 4 mm × 3.2 mm silver cups ($\delta^{18}O$) and ~1 mg of cellulose into 6 mm × 4 mm tin cups ($\delta^{13}C$).

For $\delta^{15}N$ analysis around 50 mg of fine wood powder was then collected in 2 ml Eppendorf safe lock tubes and soluble N compounds were extracted from the wood samples following (van der Sleen et al., 2015b). First we added 1 ml of a 1:1 toluene-ethanol mix for 4 h, followed by 1 ml of ethanol for 4 h. To rinse the samples 1 ml of demineralized water was added for 1 h. After adding the solution the samples were vortexed for 5 s and then place in an oven at 50 °C. Between each step the samples were centrifuged at 10000 rpm for 5 min and the supernatant decanted. The extraction was completed by oven-drying the samples for 18 h at 55 °C. Per sample ~10 mg of extracted wood powder was subsequently weighed into 6 mm × 4 mm tin cups.

The isotopic composition of the samples was determined using a mass spectrometer (Sercon Hydra 20-20) coupled to an elemental analyser (for ^{13}C and ^{15}N) and a high temperature furnace equipped with a glassy carbon reactor (for ^{18}O) at the Leicester Environmental Stable Isotope Laboratory. The carbon, nitrogen and oxygen stable isotope compositions were expressed in ‰ relative to V-PDB, the atmosphere and V-SMOW respectively.

2.4. Lab preparation – DNA

Wood shavings (100 mg) were collected from one sapwood sample per individual tree. To reduce the risk of contamination we first removed the outer layer of wood, second with clean material we drilled (slow speed to reduce heat) into the clean inner layer of the sample. Next the wood shaving were transported into XXTuff 2 ml screw cap tubes (Lab Services, USA), adding one 5 mm stainless steel bead (Qiagen, the Netherlands), then incubated on liquid nitrogen and ground to a fine powder at 25 Hz using a MixerMill MM300 (Retsch, Germany). DNA extraction was then performed on the wood powder following a protocol from Rachmayanti et al. (2006), but adding 2.6% polyvinylpyrrolidone (PVP) to the AP1 buffer of the DNeasy Plant Mini Kit (Qiagen, the Netherlands).

Genetic characterization was performed using a published set of nine nuclear microsatellite markers developed specifically to analyse

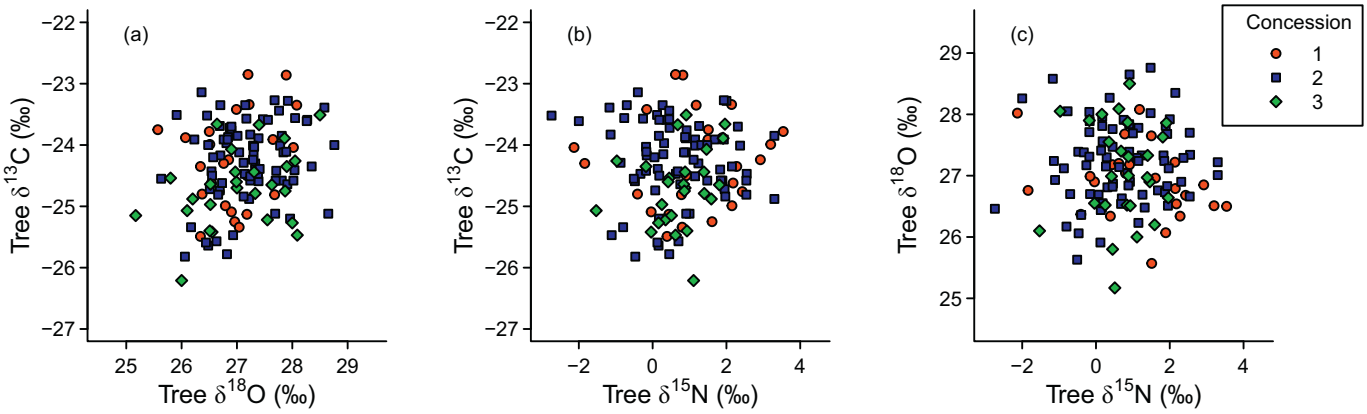

Fig. 2. Differentiation of concessions using isotopic data. Scatterplots contain mean values of isotopes per tree for $\delta^{18}O$ (‰) vs. V-SMOW (a,c), $\delta^{15}N$ (‰) vs. the atmosphere (bc) and $\delta^{13}C$ (‰) vs. V-PDB (a,b).

both *E. ivorense* and *E. suavolens* (Duminil et al., 2011). A full characterization including various checks for marker performance is available in Duminil et al. (2011). Based on a pilot study using tissue samples of varying quality (data not shown) we optimized the amplification protocol, resulting in minor changes of the amount of DNA template and primer concentrations. A full description of the final protocol is available as Appendix B. Allele detection was performed using an ABI 3730 capillary electrophoresis system (Thermo Fisher, USA). Allele scoring was performed using GeneMarker (SoftGenetics). Data quality was checked by testing for deviation from Hardy-Weinberg equilibrium (HWE) per provenance and linkage disequilibrium between pairs of markers, using Fstat v.2.9.3; (Goudet, 1995), resulting in the exclusion of data for one marker (Ery-14) that showed high amounts of missing values and strong deviations from HWE indicating the potential presence of null alleles. Duminil et al. (2011) also reported amplification problems for this marker, especially in *E. ivorense*. All analyses reported in this study were thus based on the remaining eight markers.

2.5. Data analysis – isotopes

All stable isotope data analyses were performed in R version 3.3.2 (R Core Team, 2016). The isotope data was first visually inspected for extreme outliers – none were observed. Next we calculated mean values per tree from the duplicates and scaled the isotope values so that they had a mean of 0 and a standard deviation of 1. We used Kernel Discriminate Analysis (KDA) to assess spatial clustering of the isotope data; package 'ks' (Duong, 2017). In this analysis scaled mean $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values per tree were used as test variables. Correct classification of each sample to its respective site (concessions 1–3) was evaluated using KDA. In a blind sample test using leave one out cross validation (LOOCV), per site *n*-1 was taken as a training dataset and the three remaining samples were assigned to one of the three sites. The success rate of assigning blind samples was tested in a 1000 bootstrap analysis. Because of the relatively small distance between concessions 2 and 3 (< 15 km) the KDA and LOOCV procedure was repeated by taking concessions 2 and 3 together.

2.6. Data analysis – DNA

The final dataset contained genetic profiles consisting of allele scores of individuals from concessions 1, 2, 3 and 4. Presence of private alleles (alleles occurring only in one provenance) and pairwise values of genetic differentiation among populations (Fst) were calculated using Fstat. To study differences in genetic composition among provenances in further detail, we performed PCA analysis based on binary presence-absence scores per individual per allele in PCOrd v.6.0 (McCune and Mefford, 1999). Finally, we performed a blind test to assess the success rate of DNA-based concession assignment using the reference dataset described above. For this purpose, 12 additional samples, originating from concessions included in the reference set as well as from a concession for which no reference data were available (concession 5; Fig. 1; Table A.1) were re-coded by the first author (MV) before handing them over to the laboratory staff (AdG and IL), so that the origin of these samples remained unknown throughout the laboratory and data analyses. The resulting genetic profiles were added to the reference dataset, and an assignment test was then performed for each of them using the Bayesian clustering software STRUCTURE (Pritchard et al., 2000). Concession origin of the reference samples was used as a prior to assist clustering. We used an admixture model with allele frequencies correlated among concessions. We ran three replicate model runs, each consisting of 1,000,000 MCMC iterations preceded by a burn-in period of 500,000 iterations. The assumed number of genetic populations K was fixed to 4. Assignment percentages per concession per individual were averaged over the three model runs. For each individual from the blind test set, the concession with the highest assignment percentage in the STRUCTURE output was accepted as the most likely origin, unless the individual was assigned to none of the reference concessions with a percentage above 33% (in which case the origin was reported as unknown). Additionally, we performed a manual assignment based on a strict exclusion criterion. That is, in case the allelic composition of a test sample showed one or more alleles that did not occur in our reference profiles for a particular concession, this concession was excluded as potential origin. In case one or more alleles were observed that did not occur at all in the reference dataset, sample origin was reported as most likely from a concession that was not present in the reference dataset (irrespective of the STRUCTURE-based assignment, which assumes the presence of reference data from all potential sources).

3. Results

3.1. Isotope analysis

For each of the 127 sample trees at least one wood stable isotope ratio was determined (249 $\delta^{18}O$, 254 $\delta^{15}N$ and 248 $\delta^{13}C$ values). Isotopic composition of Tali sapwood samples taken in concessions 1–3 were compared and differences tested. Overall the $\delta^{18}O$ values ranged from 25.2‰ to 28.8‰ (Fig. 2a). Mean tree $\delta^{15}N$ values ranged between −2.7‰ and 3.5‰ (Fig. 2b). Wood $\delta^{13}C$ values were ranging from −26.2‰ to −22.9‰ (Fig. 2c).

A MANOVA analysis revealed a significant difference of the combined isotopic composition among the three concessions ($F = 2.50$, $P = 0.02$). In spite of this difference, a large overlap of wood isotopic values is visible among the three concessions (Fig. 2).

When testing differences between concessions for isotopes

separately, we did not find evidence for significant differences in the $\delta^{18}O$ values of the wood among the three concessions (ANOVA $F = 1.67$, $P = 0.19$) and in the $\delta^{15}N$ signatures of the wood (ANOVA $F = 1.70$, $P = 0.19$). We did find, however, significant differences in wood $\delta^{13}C$ among the three concessions, (ANOVA $F = 4.37$, $P = 0.01$). Wood $\delta^{13}C$ was significantly lower in concession 3 compared to concession 2 (TukeyHSD $P = 0.01$). Comparison of wood $\delta^{13}C$ among concession 1 and 2 (TukeyHSD $P = 0.99$) and concession 1 and 3 (TukeyHSD $P = 0.07$) did not reveal any significant differences. Because wood $\delta^{13}C$ is known to be strongly associated with ontogenetic stage of the tree, we also tested for dbh differences among trees. We found no significant differences in dbh among the three concessions (ANOVA $F = 1.25$, $P = 0.29$).

We used KDA to produce a classification of each concession based on the isotopic composition of the timber. The KDA performed best in discerning the most heavily sampled concession (concession 2; $n = 76$). The classification success of samples from this concession was 99%. For concessions 1 and 3 the classifications were in 52% and 46% of the cases correct.

The blind sample test using LOOCV based on the original data correctly assigned on average 35% of the blind samples to their respective site (Table 1). This is only slightly higher than the 33% expected based on randomly assigning samples. Overall correct assignment of blind samples using LOOCV when taking the two neighbouring concessions, 2 and 3 (Fig. 1), together was similarly low (Table 1). In this case almost all of the blind samples (> 95%) were assigned to the combined concession 2/3.

3.2. DNA analysis

The final reference dataset contained microsatellite profiles originating from 115 trees (20 from concession 1, 68 from concession 2, 24 from concession 3 and 3 from concession 4). Profiles from ten individuals were deleted from the dataset because PCR amplification failed for more than one marker. The total number of alleles per marker observed in the reference dataset ranged from 4 (Ery-03) to 23 (Ery-07).

Pairwise genetic differentiation among concessions ranged from Fst = 0.026 to 0.032 between concessions 1, 2 and 3, but from Fst = 0.048 to 0.087 between these concessions and concession 4, suggesting relatively different allele frequencies in concession 4 but less so among the other provenances. Interestingly however, private alleles were commonly observed especially in these first three concessions. Based on eight markers, the total number of private alleles per concession was six in concession 1, 14 in in concession 2, three in concession 3 and none in concession 4. Typically, these private alleles were observed only in one or two individuals in the concession. As a result, in a PCA analysis based on allelic presence/absence, the first two axes explained only 14.5% of the variation and individuals from the same concession did not clearly cluster along these axes (Fig. 3). Yet, when using concession origin as a prior, a Bayesian clustering analysis (STRUCTURE) did assign all individuals in the reference set to the right concession (Fig. 4), likely based to a large extent on the presence of relatively large numbers of private alleles.

Table 1
Results of assignment tests based Kernel Discriminant Analysis (KDA) of the isotopic values. The Leave One Out Cross Valuation (LOOCV) was performed twice: for all three concessions and for a situation in which concessions 2 and 3 are combined because of their short distance to each other.

Concession	n	Three concessions		Concessions 2 and 3 merged	
		Prior probability	Correctly assigned	Prior probability	Correctly assigned
1	25	19.4%	12.6%	19.2%	7.7%
2	76	60.5%	87.9%	80.8%	95.8%
3	26	20.2%	4.5%		
Mean		33.3%	35.0%	50.0%	51.8%

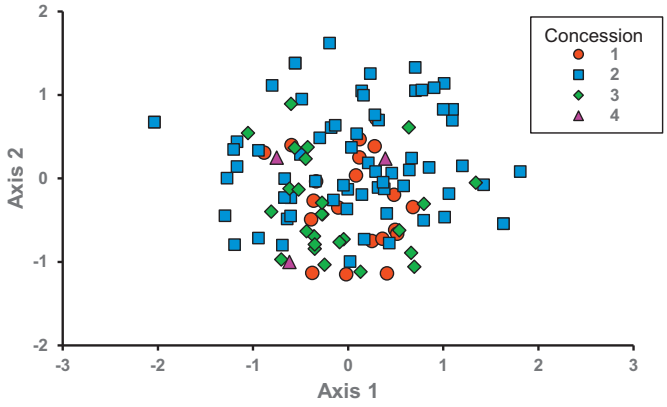

Fig. 3. Differentiation of concessions using genetic analyses. Results of Principle Component Analysis (PCA) analysis based on binary (presence/absence) allelic composition per tree. The first two axes together represent 14.5% of the total variation in the datasets.

In the blind sample test, PCR amplification failed for 2 of the 14 samples. These were both samples from trees outside the reference dataset. Out of the remaining 12 blind samples, 9 were correctly assigned to the reference concession from which they originated (Table 2). Two samples were correctly identified as originating from a provenance beyond the reference set, since multiple unknown alleles were observed (Table 2). The remaining sample was assigned with percentages between 21 and 26% to all four concessions in the STRUCTURE test, and its origin was therefore reported as unidentified. Thus, concession assignment was correct for 11 out of 12 samples (92%).

4. Discussion

Our findings show that microsatellite analyses allow correct assignment of timber samples to the concession of origin, even at short distances (< 20 km). In contrast, at the scales included in this study (14–216 km), the combined analysis of carbon, nitrogen and oxygen stable isotopes did not allow correct assignment.

4.1. Chemical timber tracing of Tali

This is the first study to assess the feasibility of using carbon, nitrogen and oxygen stable isotopes for provenancing tropical timber at a spatial scale of 10s to 100 s of kms. Our results indicate that spatial variation of wood isotopic profiles was insufficient to differentiate Tali provenances in south and south-eastern Cameroon. This result is probably caused by low spatial variation in environmental conditions that determine isotopic signatures of the wood. Our study was conducted in a continuous stretch of lowland tropical forest, with very limited topographic variation and a similar distances to the sea (400–500 km). A strong gradient in distance to the sea would have facilitated discrimination of wood samples based on $\delta^{18}O$ values (Aggarwal et al., 2010). Besides this we observed high variability in $\delta^{18}O$ values within trees and concessions, likely resulting from high year-to-year variation in $\delta^{18}O$ composition of rainwater (van der Sleen et al., 2015a). While we aimed to take wood bulk samples of multiple years to average over years, some of our samples were rather small. These samples consisted of wood formed in one or only a few years, increasing the among sample variation in $\delta^{18}O$ values. Unfortunately this effect could not be quantified because of poorly identifiable rings in Tali wood (Groenendijk et al., 2014).

The $\delta^{13}C$ composition of wood is strongly related to the ontogenetic stage of the tree and the local availability of light and water to the tree (van der Sleen et al., 2013). We tested for possible ontogenetic effects,

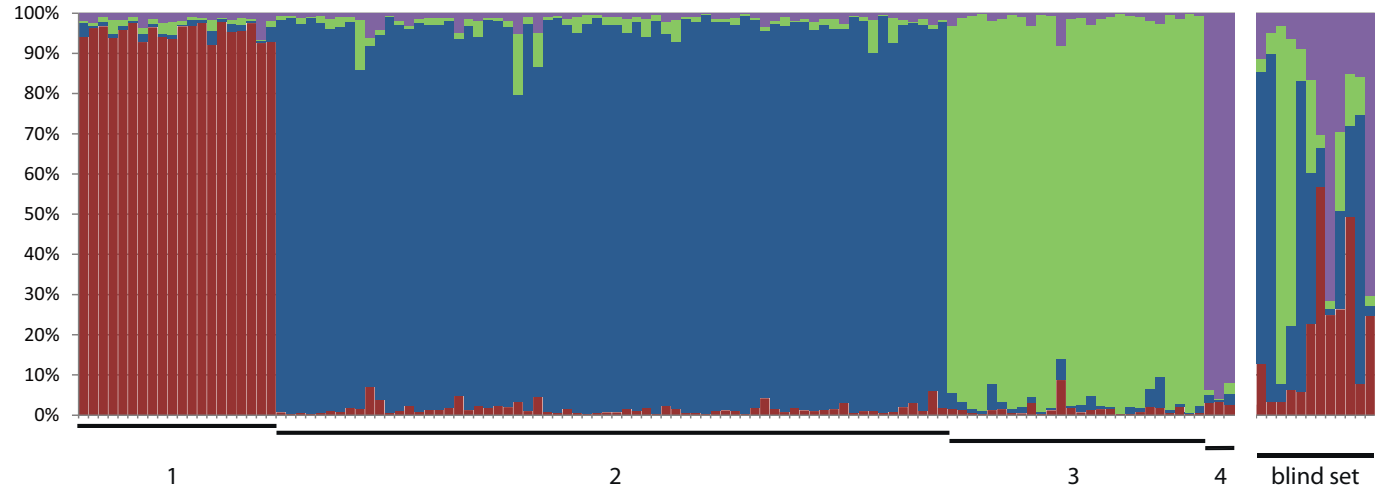

Fig. 4. Differentiation of concessions using genetic analyses and results of blind sample test. Concession assignment per individual based on Bayesian clustering of microsatellite data using STRUCTURE. Individuals are represented by vertical bars. Colours per individual represent percentage assignment to each of the four assumed genetic clusters (coding as in Fig. 1).

Table 2
Results of the blind sample test based on genetic analysis. Assigned concession origin was based on a combination of the presence of unknown alleles and an assignment test in STRUCTURE.

Sample	# Unknown alleles	STRUCTURE	Assigned origin	True origin	Correct?
TB002	0	2	2	2	Yes
TB003	0	2	2	2	Yes
TB004	0	3	3	3	Yes
TB006	0	3	3	3	Yes
TB007	0	2	2	2	Yes
TB008	0	2	2	2	Yes
TB009	0	1	1	1	Yes
TB010	3	4	Not in reference set	5	Yes
TB011	0	?	?	1	No
TB012	0	1	1	1	Yes
TB013	0	2	2	2	Yes
TB014	2	4	Not in reference set	5	Yes

i.e. the effect of dbh on $\delta^{13}C$ signature, to explain differences in wood $\delta^{13}C$ among concessions 2 and 3, but found no evidence for this. Differences in wood $\delta^{13}C$ between these sites could be related to variation in rainfall or soil water availability, but we have no information to assess this.

Plant tissue $\delta^{15}N$ concentration are mainly associated with atmospheric nitrogen deposition (van der Sleen et al., 2015b) and edaphic factors (Paolini et al., 2016). Atmospheric nitrogen deposition in the south of Cameroon is relatively low and the geographic variation is also low (Hietz et al., 2011; van der Sleen et al., 2015b). The area in which the three concessions are situated consists of a homogenous soil type of alluvial depositions with low nutrient availability. These factors likely explain the lack of clear clustering in $\delta^{15}N$ values among trees of different provenances.

In spite of the lack of clear regional clustering patterns in the isotope data presented here, we believe that stable isotopes are a promising technique for forensic timber tracing. For example variation in $\delta^{18}O$ of wood cellulose may be used to differentiate timber origin at larger spatial scales (> 1000 km). Discrimination of timber provenances may be facilitated if correlations are found between the $\delta^{18}O$ signature of wood and the $\delta^{18}O$ content of rainwater. Such relations provide potential for large-scale timber tracing as the amount of ^{18}O in rainwater consistently and predictably varies with distance to the sea, latitude and elevation (Bowen, 2010).

For fine-scale differentiation of timber, additional chemical techniques will be needed that more accurately reflect local soil chemistry and bedrock chemistry (Aggarwal et al., 2008). Potential candidates are wood strontium isotope (Sr^{87}/Sr^{86}) concentrations (English et al., 2001), rare earth elements (Joebstl et al., 2010) and trace elements (Ma et al., 2016). Proofs of principle to apply these last two techniques in timber provenancing do not exist in scientific literature yet.

4.2. Genetic timber tracing of Tali

A previous analysis of genetic structure within *E. suaveolens* and *E. ivorense* in Western Africa, based on the same marker set, showed limited spatial differentiation especially in the eastern part of the combined distribution areas of these species (Cameroon and Congo Brazzaville; Fst = 0.016) (Duminil et al., 2013). Our reference data from four concessions in this area support this result. In general the four concessions shared a strong overlap in dominant alleles, resulting in a limited spatial substructure, as illustrated by low explained variation and poor clustering in the PCA. We did, however, observe somewhat more differentiation than Duminil et al. (2013), at least for concession 4 (moderate pairwise Fst values up to 0.087). The Bayesian clustering algorithm successfully assigned almost all individuals from the blind test to the concession of origin. This assignment power likely depends on the presence of characteristic ('private') alleles, as one individual lacking such alleles was assigned with equal probability to all four reference concessions.

The fact that isolation by distance was only present in our dataset at the level of individuals within concessions, located only a few kilometres apart (results not shown), suggests that genetic exchange via pollen and seeds rarely exceeds this distance. The lack of long-distance dispersal at scales of tens to hundreds of kilometres (cf. Bizoux et al., 2009) may facilitate the ability to assign individuals to a specific concession in case reference data are available for all potential sources, as effectively shown in our study. It does hamper, however, the ability to use genetic distances to estimate the most likely region or origin in case reference data are only available for a few provenances. This means that for genetic tracing of Tali to be effective, a more exhaustive reference dataset must first be produced, also including samples from the eastern part of its distribution. At even larger spatial scales, Duminil et al. (2013) showed the existence of recognizable regional gene pools that are probably explained by climatic and ecological gradients (habitat diversification associated with Pleistocene refugia). This likely allows the assignment of unknown individuals to either the north-western or south-eastern part of the distribution range of *E. suaveolens*,

gene pools SW or SC respectively in Duminil et al. (2013), although this was never explicitly tested.

Degen et al. (2017) suggested that a nearest neighbour approach based on genetic distances may outperform a Bayesian clustering approach in assigning samples to the right region of origin. They also indicate, however, that his may only be true for datasets of species that show a relatively high spatial genetic differentiation (i.e. concessions or countries, of which the limits may not overlap with natural population boundaries). Our results indicate that for our study species, which showed limited spatial differentiation in any of the five provenances, the use of Bayesian clustering methods does allow correct assignment of the vast majority of samples.

4.3. Steps to develop forensic applications of genetic tracing for Tali

In all, microsatellites provide a valuable, fast and cost-effective tool for identifying geographic origin of timber samples, including tropical timbers (Degen et al., 2013; Jolivet and Degen, 2012; this study). Yet, the statistical power of inferences based on microsatellites remains relatively low due to the limited number of genetic markers, typically < 10 (Degen et al., 2013; Jolivet and Degen, 2012). The application of next-generation sequencing to identify large amounts of SNPs – variations in a single nucleotide that occur at a specific position in the genome – from the entire genome of tree species will likely allow a substantial increase in the statistical power of genetic tracing methods (Jardine et al., 2015). As such, it may yield increased potential for high resolution origin detection (i.e. to concession level).

We showed the potential to independently verify the geographic origin of Tali timber based on microsatellite analyses. Our blind test gives a 92% likelihood that Tali timber originates from the sampled concessions or not. This method can therefore be of direct use for timber trading companies to verify their Tali supply chain, and for European importers of Tali to use as an additional mitigating (i.e. risk-reducing) actions in the mandatory due diligence system of the EU timber regulation (EUTR).

In terms of forensic application, our study shows the *potential* of genetic tracing as a forensic tool for Tali. Several steps are needed to develop this genetic tracing method into a forensic tool that can be used in legal cases. First, although markers have been characterized (Duminil et al., 2011) and standard quality checks for population genetic datasets were performed to allow our current exploratory analyses, additional validation of marker performance will be required to fully comply with SWGDAM (Scientific Working Group on DNA Analysis Methods, 2016). This is particularly relevant since actual application will be based on heartwood rather sapwood samples, which typically contain DNA of much lower quality and quantity (Rachmayanti et al., 2009). A pilot study using an adapted extraction protocol has shown successful marker amplification (Appendix C), although multiple PCR replicates per sample will be required to avoid genotyping errors (multi-tube approach; Taberlet et al., 1996). Second, labs and protocols need to meet the rigorous criteria and international standards set in forensic science (e.g., those prescribed by SWGDAM), including accreditation of timber forensics labs, ring tests and a fully transparent chain of custody of timber samples used in the reference database. Third, the success rate of blind sample tests will need to be increased. This is possible by taking additional samples from these and additional sites, as this will improve the statistical power of statistical tests. In addition, it is recommended to increase the area covered by sampled concessions to cover the entire part of the distribution range of both *E. suaveolens* and *E. ivorense* and interpolate Tali genetic information. The resulting interpolated map will allow identifying the geographic origin of wood from any location, and thus is a strong improvement over the in-/exclusion results obtained in the tests performed so far.

5. Conclusions

Stable isotopes are probably not useful to discriminate the origin of tropical timber at the concession level. However, because of their low costs, relative simplicity in use and robustness for species differences, it is recommended to further assess the potential of chemical timber tracing for other species, regions and by using additional chemical elements. Microsatellite analysis provides a practical tool to discriminate timber at the concession level. This method is ready to be widely applied and address questions on the legal origin of timber. We believe that timber forensic are indispensable in the continued efforts to ascertain legal supply of tropical timber and the sustainable management of tropical forests.

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Appendix A

Table A.1
Description of the five concessions from which samples were obtained. For genetic analyses, we used samples from concessions 1–4 (triplicates) and blind samples from concession 5 (single samples). For isotopic analyses, we used samples from concessions 1–3.

Concession	Name	Logging company	Long (dec degr)	Lat (dec degr)	Elevation (m)	Year sample collection	*n* trees	Mean dbh ± sd (cm)	Dbh range (cm)
1	09003	Groupe Rougier	14.08	3.93	672	2015	25	90.5 ± 18.6	64.5–155.0
2	10054	Groupe Rougier	14.16	4.03	657	2015	76	87.7 ± 12.6	72.0–124.0
3	10056	Groupe Rougier	13.22	2.28	748	2015	26	84.5 ± 9.7	73.0–112.0
4	IFO2016	Danzer Interholco	15.64	1.46	450	2016	3	93.3 ± 7.6	85.0–100.0
5	11.001	TRC	9.10	5.25	180	2011	4	97.6 ± 8	59.4–123.0

Table A.2
Pairwise geographic distance (km) between all five concessions.

Concession	1	2	3	4
1	–			
2	216	–		
3	203	14	–	
4	284	322	317	–
5	562	579	573	836

Appendix B. Genotyping protocol

Genetic characterization was done using a set of nine nuclear microsatellite markers developed by Duminil et al. (2011). Amplification was performed in two multiplex reactions containing either five (TaliMP1) or four markers (TaliMP2). Primers were first mixed according to Table B.1. Both multiplex reactions were performed in volumes of 10 µl, using the Qiagen Multiplex Kit (reaction mixture as in Table B.2), and the same touchdown cycling protocol: 95 °C for 10 min, 10 cycles with 94 °C for 15 s, 180 s 59 °C minus 1 °C per cycle and 72 °C for 120 s, followed by 35 cycles with 94 °C for 15 s, 49 °C for 180 s and 72 °C for 120 s, followed by 60 °C for 30 min.

Table B.1
Concentrations of each primer in the primer mix.

Primer mix	Component	Concentration µM
TaliMP1	Ery-23_F_FAM	2,0
	Ery-23_R	2,0
	Ery-14_F_VIC	2,0
	Ery-14_R	2,0
	Ery-04_F_VIC	1,0
	Ery-04_R	1,0
	Ery-03_F_NED	3,0
	Ery-03_R	3,0
	Ery-18_F_PET	2,0
	Ery-18_R	2,0
TaliMP2	Ery-01_F_FAM	3,0
	Ery-01_R	3,0
	Ery-17_F_VIC	1,0
	Ery-17_R	1,0
	Ery-07_F_NED	1,5
	Ery-07_R	1,5
	Ery-06_F_PET	1,5
	Ery-06_R	1,5

Table B.2
PCR reaction mixture.

Component	Stock Conc.	Final Conc.	Volume 1 rxn (µl)
H2O	N/A	N/A	2,00
Qiagen Multiplex Master mix (EMM 2.0)	2×	1×	5,00
PrimerMix MP1 or MP2	10×	1×	1,00
DNA template	N/A	N/A	2,00
TOTAL			10,00

Appendix C. Protocol for DNA extraction from heartwood and genotyping results for heartwood

C.1. Description of extraction protocol

In August 2016, we extracted DNA from six fresh heartwood samples of Tali (three individuals with two replicates each) collected in July 2016 (see main text for collection details). We used an adapted version of the protocol as described in Rachmayanti et al. (2009), based on the DNeasy Plant Mini Kit (Qiagen), with the following additions and modifications:

C.1.1. Wood powdering

-Powdering of wood shavings (100 mg) was performed for 2 min at 25 Mz on a MixerMill MM300 (Retsch) apparatus. We used XXTuff 2 ml tubes (Lab Services, 330TX) to minimize damage to the tubes.

C.1.2. Lysis

- After adding the PVP (polyvinylpyrrolidone)/AP1 mixture and RNase, the tubes were incubated at 65 °C for 1 h using a ThermoMixer shaking at 1400 rpm.
- After 1 h, we added 80 µl of PTB (N-Phenacylthiazolium bromide; 0,1 M; supplier: Fluorochem), 40 µl of proteinase K (20 mg/ml; provided in DNeasy Plant Mini Kit) and 50 µl of DTT (Dithiothreitol; 1 M; supplier: VWR).
- This mixture was incubated overnight (16 h) at 56 °C using a ThermoMixer shaking at 1400 rpm.
- Temperature was then increased to 70 °C for a final incubation step of 15 min.
- 315 µl of buffer AP2 was then added before vortexing, incubation for 15 min at −20 °C and centrifuging for 5 min at 20,000*g*.

C.1.3. Purification

- Washing with AW2 in step 17 of the kit protocol was performed twice. If the membrane and/or eluate was significantly coloured, we washed an extra time with EtOH.

C.1.4. Elution

After transferring 50 µL of buffer AE to the spin column we incubated for 15 min before eluting a single time.

C.2. Potential for microsatellite genotyping

To test the potential to use the above derived DNA extracts from heartwood for microsatellite genotyping, we applied the PCR protocol for eight microsatellites described in the main text. As positive controls, we also genotyped two DNA extracts from sapwood of each of the same three individuals.

As shown in Table C.1, complete profiles could be obtained for all sapwood samples, and one heartwood sample of individuals S2 and S5. Near complete profiles were obtained for the second heartwood sample of these individuals. High amounts of missing values were obtained for heartwood samples of the third individual (S3). Sapwood samples of the same individual always showed identical profiles. Assuming that the sapwood profile is correct, results for the heartwood samples showed some cases of potential false alleles and/or allelic drop out (indicated in red in Table C.1). This is not uncommon in genotyping of samples with reduced DNA quantity, and can be solved by adopting a so-called multi-tube approach, in which a consensus profile is created from e.g. 3 to 6 replicated PCR reactions per sample (see. e.g. Taberlet et al., 1996).

Table C.1
Genotyping profile per marker (Ery_xx) per sample, and the percentage of markers for which alleles could be scored. Microsatellite genotyping is based on length variation of the amplified DNA fragments. The values presented below are fragment lengths, which represent allelic variants. Missing values (failed amplification) are indicated in blue. Potential genotyping errors are indicated in red.

Ind	Sample	Repl	Ery 23		Ery 04		Ery 03		Ery 18		Ery 01		Ery 17		Ery 07		Ery 06		% with result
S2	Sapwood	1	110	114	145	145	138	138	202	204	225	229	130	130	148	173	120	126	8 (100%)
		2	110	114	145	145	138	138	202	204	225	229	130	130	148	173	120	126	7 (88%)
	Heartwood	1	108	110	145	145	142	142	195	202	NA	NA	130	134	148	148	126	126	8 (100%)
		2	110	114	145	145	138	138	202	202	225	225	130	134	157	173	128	128	8 (100%)
S3	Sapwood	1	110	124	145	145	138	142	202	204	229	231	130	134	158	178	120	120	8 (100%)
		2	110	124	145	145	138	142	202	204	229	231	130	134	158	178	120	120	8 (100%)
	Heartwood	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	122	122	1 (12.5%)
		2	122	124	145	145	NA	NA	NA	NA	231	231	NA	NA	NA	NA	NA	NA	3 (37.5%)
S5	Sapwood	1	110	114	145	145	142	156	195	202	227	227	130	134	146	158	126	128	8 (100%)
		2	110	114	145	145	142	156	195	202	227	227	130	134	146	158	126	128	8 (100%)
	Heartwood	1	110	114	143	143	142	156	195	195	227	227	134	134	146	146	128	128	8 (100%)
		2	114	114	145	145	156	156	195	195	227	227	130	130	NA	NA	NA	NA	6 (75%)

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