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SHORT COMMUNICATION

Comparing the use of leaf and cambium tissue in a single genetic study of tropical trees

Sharon E. Zytynska · Michael F. Fay · Richard F. Preziosi

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Abstract Amplified fragment length polymorphisms (AFLPs) are a useful molecular tool for studying species with little available genetic information; however, since universal primers are used contaminant DNA from non-target organisms may also be amplified. Cambium tissue may contain fewer biotic contaminants or plant defense chemicals, than the more commonly used leaf tissue, and therefore be more suitable for use as a source of DNA when using universal primers. On the other hand, cambium tissue can be difficult to identify, yields low DNA and requires the bark of the tree to be damaged, thereby increasing the risk of introducing disease. We show that within two tropical tree species, there are few differences between AFLP profiles obtained from either cambium or leaf tissue from the same tree. We studied 50 *Brosimum alicastrum* individuals at 119 AFLP loci and 40 *Swietenia macrophylla* individuals at 112 AFLP loci. The matrix of Sørensen similarity indices between individual AFLP profiles for cambium samples was strongly correlated to the matrix for leaf samples in each species (Mantel test; *B. alicastrum* $r=0.815$, $P<0.001$; *S. macrophylla* $r=0.895$, $P<0.001$). The phylogenetic relationship between the trees studied did not differ dependent on tissue type used and therefore shows that both tissues can be used within a single study without introducing substantial error.

Keywords Tropical ecology · Ecological genetics · Molecular ecology

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S. E. Zytynska (✉) · R. F. Preziosi
Michael Smith Building, Faculty of Life Sciences,
University of Manchester,
Manchester M13 9PT, UK
e-mail: Sharon.zytynska@gmail.com

M. F. Fay
Jodrell Laboratory, Royal Botanic Gardens,
Kew TW9 3DS, UK

Present Address:
S. E. Zytynska
Department of Ecology and Ecosystem Management,
Technische Universität München,
Freising, Germany

Introduction

Genetic studies using amplified fragment length polymorphisms (AFLPs) use universal primers and thus require no previous investment in primer design and synthesis (Vos et al. 1995). The use of AFLPs is beneficial when little is known about a species; however, universal primers with broad applicability across numerous taxa can also result in the DNA of contaminants (for example endophytic fungi within leaf samples) being amplified alongside that of the target individual (Saar et al. 2001, but see Fay et al. 1999; Wilkinson et al. 2003). In 2005, Colpaert et al. described a method for extracting DNA from the cambium tissue of tropical trees and showed the DNA was suitable for use with AFLPs.

The cambial tissue of a tree is protected by the bark and therefore may be expected to contain fewer biotic contaminants (e.g. fungi) and defensive chemicals (e.g. phenols or tannins) compared to leaf material (Colpaert et al. 2005). There has been no direct study comparing leaf and cambium defensive chemicals. Current understanding suggests that leaf tissue contains more constitutive defensive chemicals that are always present, whereas cambium tissue

employs induced defensive chemicals against insect attack as it is protected by the constitutive physical defenses of the bark (Franceschi et al. 2005). Leaf tissue is currently the most commonly used source of DNA from plants due to the ease of extracting high yields of DNA using both traditional methods (e.g. CTAB; Doyle and Doyle 1987) and ready-made kits (e.g. DNeasy Plant Mini Kit from Qiagen). However, leaf material can often be inaccessible when studying tall trees, whereas cambium tissue is easily obtained at ground level making it a good candidate for use as a DNA source. Additionally, within a tropical ecosystem, diversity is high at all levels, and thus biotic contamination of leaf tissue may reduce the reliability of collected data, especially when using genetic markers such as AFLPs that utilize universal primers. Furthermore, compared to cambium tissue, leaves are expected to contain greater quantities of secondary metabolites, such as phenols, which can reduce the success of DNA extraction and downstream DNA restriction or amplification reactions (Khanuja et al. 1999). Cambium tissue could thus prove a better source of high quality DNA than leaf material; however, difficulties in identifying the cambium zone, thick bark and a porous and spongy cambium will reduce the sampling efficiency and the yield of DNA recovered (Colpaert et al. 2005). This leads to a necessary compromise between yield and quality, which must be made when considering the tissue to be used; leaf material yields more DNA, but cambium produces higher quality DNA. Cambium sampling also requires the bark to be damaged due to the invasive sampling method, and this could introduce disease to the tree (Pearce 1996). Certain methods of leaf collection may also damage the tree in such a way as to leave the tree vulnerable to disease; for example, shot-gun harvesting often breaks a branch and so may not be the preferred collection method for leaves. The method of rope climbing to collect leaf material could also potentially damage the bark at focal points if the tree is not rigged correctly, but the use of bark protectors can minimize this risk. Therefore, in many circumstances neither leaf nor cambium tissue is the ultimate preferred choice but if used together could advantageously increase samples sizes. It has also previously been suggested that AFLP profiles could differ between tissues within a single individual (Donini et al. 1997). However, we suggest that due to the nature of the undifferentiated cambium cells these concerns are unfounded. Indeed, a number of studies have successfully used multiple tissue types (Cloutier et al. 2007; Hanson et al. 2008; Duminil et al. 2006); however, no statistical analysis has been performed comparing the genetic relationship obtained among trees using the different tissues.

In this short communication, we explore whether the AFLP profiles obtained from different tissues within the same tree were more similar than samples from tissues among different trees, and we show that the genetic relationship among the trees is not dependant on tissue sample used. This shows that both leaf and cambium DNA can be used interchangeably in a single study, without introducing error from biotic contamination or genomic differences between tissues.

Materials and methods

Study area and tree species

All samples were collected at Las Cuevas Research Station within the Chiquibul National Park of Belize, Central America in 2007–2008. The tree species we investigated were the breadnut (*Brosimum alicastrum* Sw., Moraceae; $n=50$) and mahogany (*Swietenia macrophylla* King, Meliaceae; $n=40$). The study area was approximately 4 km^2 in broadleaf, deciduous forest.

Tissue collection and preservation

Leaf tissue was collected from each tree using rope-climbing techniques or a shotgun to shoot a branch. The surface of each leaf was wiped using 70% isopropanol to reduce the potential for contamination from surface biota. Each leaf was cut into thin strips and dried using grade12 silica gel (Sigma-Aldrich) in airtight plastic bags (Chase and Hills 1991). Self-indicating silica gel was used to show if the silica became saturated. Cambium tissue was collected from the trees using a leather punch (according to the methods by Colpaert et al. (2005)). To reduce the chance of disease introduction the plug of outer bark was replaced into the tree after cambium collection, and all equipment was cleaned using 70% isopropanol, before and after sampling. Cambial discs were dried using grade12 silica in O-ring tubes (Anachem Ltd.), with two crystals of self-indicating silica gel; the silica gel was replaced once, 1 day after the initial collection.

Genetic analysis

DNA was extracted using a modified 2× CTAB method (Doyle and Doyle 1987). We extracted DNA from 0.03 g of dried leaf and 0.06 g of dried cambium tissue (two of 9-mm×1-mm thickness discs), which were ground into fine powder before proceeding with the extraction. The extracted DNA was further cleaned using silica-based spin columns (Sigma-Aldrich) and finally eluted in 50 μl elution buffer. A NanoDrop® ND-1000 spectrophotometer was used to analyse the purity and quantity of DNA in the extractions. For the AFLPs, we used 500 ng of DNA, which was dried and resuspended in 5.5 μl of sterilized double-distilled (SDD) water.

*Mse*I and *Eco*RI restriction enzymes were used for the restriction-ligation stage: adaptor pairs were denatured at 95°C for 5 min and cooled for 10 min at room temperature. Then 1.1 μl of 10× T4 ligase buffer, 1.1 μl of 0.5 M NaCl, 0.55 μl of 1 mg/ml BSA, 0.02 μl of 50 U/μl *Mse*I, 0.2 μl of 25 U/μl *Eco*RI, 0.05 μl of 20 U/μl ligase, 1 μl of each of the denatured adaptor pairs and 0.48 μl of SDD water were added to each sample. The samples were incubated in a PCR machine at 37°C for 60 min, after which they were diluted 1:20 using 0.1 M TE buffer. The pre-selective amplification stage used the regular genome size pre-selective primer pairs from Applied Biosystems. For each sample, 7.5 μl of AFLP Core Mix (Applied Biosystems) and 0.5 μl of pre-selective primer pairs were added to 2 μl of diluted restriction ligation product. The pre-selective primer pairs were not denatured during this stage. The samples were amplified using a Gene Amp PCR System 9700 (heated at 94°C for 2 min, then for 20 cycles run at 94°C for 20 s, 56°C for 30 s and 72°C for 2 min, after which the samples were held at 60°C for 30 min before cooling to 4°C). Samples were then diluted 1:20 using 0.1 M TE buffer. The selective amplification primers were chosen (greatest number of bands) after a preliminary primer trial and consisted of three labeled primer combinations (one FAM™, one JOE™ and one NED™) per tree species. *B. alicastrum* primer combinations were ACA/CTG, ACG/CTC and AGC/CAT, and *S. macrophylla* primer combinations were ACT/CAG, AGG/CAT and AGC/CAC. The following were added: 1.5 μl of diluted pre-selective amplification product, 7.5 μl of AFLP Core Mix, 0.5 μl of the *Mse*I primer (Cxx) and 0.5 μl of the *Eco*RI primer (dye + Axx). The samples were amplified using a Gene Amp PCR System 9700 (heated at 94°C for 2 min, then for 10 cycles run at 94°C for 20 s, 66°C for 30 s and 72°C for 2 min, followed by 20 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min, after which the samples were held at 60°C for 30 min before cooling to 4°C). The samples were multiplexed and run on a Genetic Analyser ABI Prism 3100 (Applied Biosystems). For each well in the sequencing plate, 10 μl HiDi and 0.2 μl Genescan™ 500 Rox™ Size Standard were added. To this, undiluted selective amplification products were added (0.8 μl of the FAM™ or JOE™ primer combinations or 1.0 μl of the NED™ primer combination). The samples were denatured at 95°C for 5 min and cooled on ice for 10 min before being transferred to the genetic analyser.

Data analysis

The AFLP profiles were computationally analysed using Genotyper 2.0 (Applied Biosystems); peaks were automatically assigned but individually checked by eye to ensure correct assignment. Only bands between 50 and 500 bp were used, and those with unreliable assignment (for example low average peak height across all samples) were eliminated. A binary matrix was created for band presence (1) or absence (0) for each sample. In addition, a 5% error rate was used to eliminate spurious bands, where bands that occurred in less than 5% of the individuals were removed. Similarly, those bands present in at least 95% of the population were considered monomorphic. The results were analysed using CAP4 (*Community Analysis Package 4*, Version 4.1.3, Pisces Conservation Ltd., 2007); the similarity between each pair of samples was calculated using the Sørensen index:

$$S = \frac{2a}{(2a + b + c)} \quad (1)$$

where, a is the number of bands shared by each sample, b is the number of bands present in the first but not the second sample and c is the number of bands present in the second but not the first sample. This method computes the same 'genetic similarity' statistic as that by Nei and Li (1979). A mantel test (Legendre and Legendre 1998) was used to statistically analyse the association between the cambium and leaf samples of each individual (Sørensen similarity index values) using PASSaGE (Pattern Analysis, Spatial Statistics and Geographic Exegesis; Rosenberg and Anderson 2011). More specifically, we correlated the matrices of the pairwise Sørensen similarity values (between each pair of trees) for the cambium samples with the Sørensen similarity values for the leaf samples. Therefore, each cell in each matrix corresponded to the same pairwise comparison. The use of a Mantel test allows us to test the correlative relationship between two variables on pairwise data, as results from similarity indices. A highly significant correlation of +1 between the Sørensen similarity matrices of leaf and cambium would mean that, on average, there is a 1-unit increase in leaf values associated with a 1-unit increase in cambium values. Geographic distance was found not to influence the clustering relationship of the trees, and previous work by Zytynska et al. (2011) found that within the sampling area geographic distance was of limited influence.

Graphical representations of the data were made using boxplots in JMP® 8.0.2 to show the similarity values between leaf and cambium samples collected from the same individual (within); the cambium sample of a focal individual and the leaf samples of all other individuals (cambium to all); and finally, the leaf sample of a focal individual to the cambium samples of all other individuals (leaf to all). Clustering dendrograms (average linkage, '1-Sørensen') were constructed in CAP4 using 'hierarchical agglomerative cluster analysis' to determine if the tissue samples within a tree clustered more often than those between trees. The differences

in the number of AFLP band mismatches between the leaf and cambium samples were calculated for each individual. Tolerance levels for AFLP band mismatches for the cambium and leaf samples within an individual were calculated at 0% (no mismatches), 2.5%, 5% and 10%. These simply reveal the number of mismatches between these profiles and the error level at which they fall into. Grubb's outlier test (Grubb 1969) was used to detect any outliers within the number of mismatches (comparing leaf and cambium tissues) across the loci to show that no single locus was producing greater or fewer mismatches than another.

Results

A total of 50 *B. alicastrum* individuals were sampled, producing 119 loci (bands) from the AFLP analysis, of which 31 (26%) were monomorphic (i.e. every sample produced a band for this locus). Forty individuals were sampled from *S. macrophylla*, and these samples produced 112 loci from AFLP analysis with 42 (37.5%) monomorphic loci. From observation, the cambium of *B. alicastrum* was more distinct from the bark than for *S. macrophylla*, which also possessed a spongy cambium layer. In addition, the *B. alicastrum* leaves contain milk-like sap, whereas the *S. macrophylla* leaves did not. The DNA yield obtained from the samples differed between tissue type ($F_{1,\ 176}$= 77.9, P<0.001) but not tree species ($F_{1,\ 176}$=0.252, P=0.616) (Fig. 4 in ESM). Overall the leaf samples produced greater yield of DNA per gram of dried tissue. The DNA purity was consistent between leaf and cambium samples within tree species, indicating that there was little contamination among the samples (Table S1).

We found highly significant positive correlations between the Sørensen similarity values from the two different tissue samples (leaf and cambium) for both tree species, *B. alicastrum* (Mantel test: r=0.815, P<0.001) and *S. macrophylla* (Mantel test; r=0.895, P<0.001). The Sørensen similarity value for two AFLP profiles was greater for those within an individual compared to those between different individuals (Fig. 1). All of the leaf and cambium samples from the same individual clustered together for both *B. alicastrum* (Fig. 2) and *S. macrophylla* (Fig. 3). This shows they are more similar to each other than to a sample from another individual, and this means there is no change in the relationship between trees due to tissue type used.

Within *B. alicastrum* (n=50), there were five individuals for which the leaf and cambium AFLP profiles did not differ at all, and the greatest number of mismatching bands (i.e. where one sample shows presence of a band, or locus, and the other does not) was 15. Using band mismatch tolerance levels of 0%, 2.5%, 5% and 10%, we found that over half of the *B. alicastrum* individuals sampled (26/50)

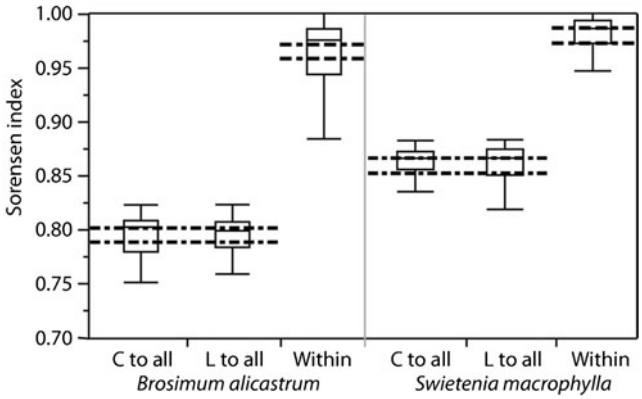

Fig. 1 The distribution of similarity values (Sørensen) between AFLP profiles of cambium and leaf tissue samples within the same tree (*within*) or different trees (cambium to all '*C to all*'; leaf to all '*L to all*'), on the two tree species studied. This shows that AFLP profiles are more similar from the different tissues within the same tree than among trees. Dotted lines represent 95% CI around the means for samples within, or from different, trees

were within the 2.5% tolerance level for band mismatches (Table 1). This means that 52% of the individuals produced leaf and cambium AFLP profiles with three or fewer band mismatches. Only two individuals were found to lie outside the 10% tolerance level, which corresponds to mismatches in more than 12 of the 119 scored loci in the AFLP analysis. The mismatched bands were randomly distributed across the loci with no locus producing a greater (or smaller) number of mismatched bands than any other (Grubb's outlier test, critical value Z=3.13, n=50, P>0.05).

Within *S. macrophylla* (n=40), there were seven individuals for which the leaf and cambium AFLP profiles did not differ at all, and the greatest number of mismatching bands between profiles within an individual was 11. The reproducibility of AFLP profiles from the cambium and leaf tissues of the same individual is high for this tree species, highlighted by 74% of individuals within the 2.5% tolerance level and 100% of individuals within the 10% tolerance level (Table 1). Again, the mismatched bands were distributed across the loci with no single locus producing a greater (or fewer) number of mismatched bands (Grubb's outlier test, critical value Z=3.05, n=41, P>0.05).

Discussion

This paper demonstrates that DNA samples extracted from the leaf and cambium tissue of the same tree produced, on average, more similar AFLP profiles to each other than to any other sample. The genetic relationships between the individuals within *B. alicastrum* and *S. macrophylla* were not altered when different tissue types were used in the analysis, confirmed by the clustering of leaf and cambium samples within each individual. Here we have considered

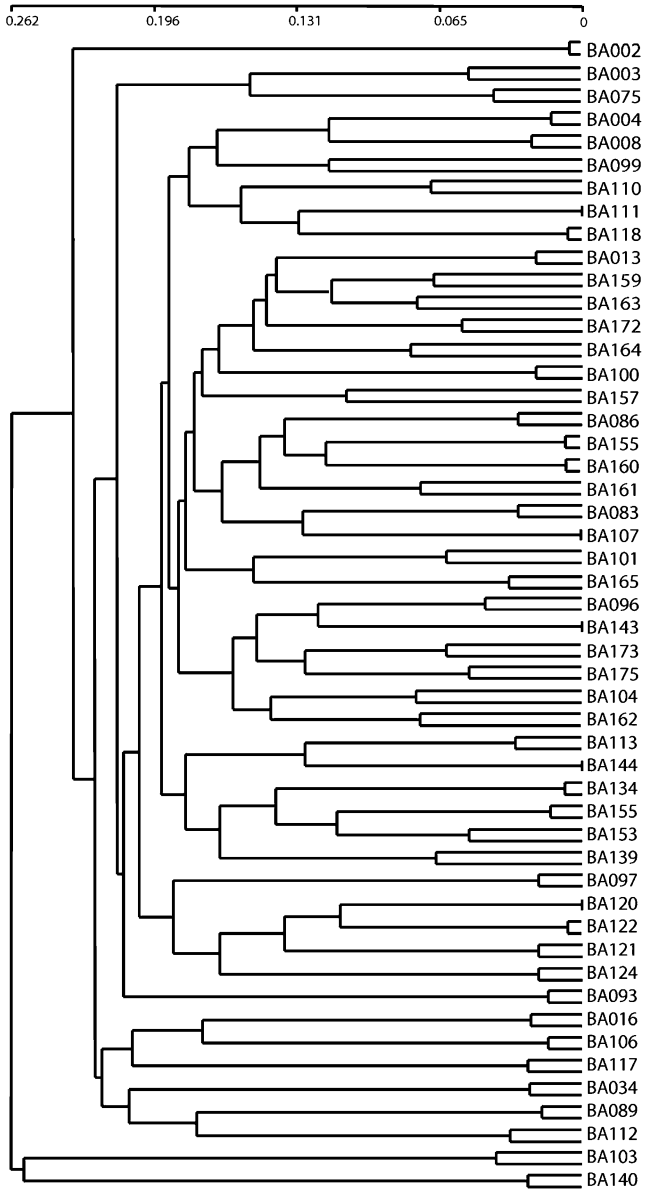

Fig. 2 Clustering relationship of leaf and cambium samples within 50 individual *Brosimum alicastrum* (*BA*) trees. BA *numbers* correspond to individual tree numbers, and the *x*-axis (*top*) shows the Sørensen index of dissimilarity (1-Sørensen)

the whole AFLP profile as a single trait, with each locus providing a small contribution to the resulting profile (as for similarity indices or genetic distances). By using analyses based on the whole profile, we begin to get closer to the quantitative genetic relationship between the trees (sensu Fisher 1918); however, a more refined estimate of the similarity between the trees could be uncovered through whole-genome sequencing. Each AFLP locus itself has a random component, but when statistically assessed together they are a stable indicator of the genetic relationship among trees. We did find mismatches of AFLP bands to occur between the leaf and cambium samples from the same tree. The error rates within this study are higher than have been previously shown to be achievable with AFLPs (Mueller and Wolfenbarger 1999), likely due to the few repeats made during the methods; however, we have shown that the phylogenetic relationship between the individuals is robust, and thus this increased error rate does not impede a study such as this. This suggests error during the methods or analysis and indicates that the use of AFLP analyses relying on band identity may not be as suitable as those using the whole profile as a single trait. Due to the complex nature of the AFLP method, small discrepancies can occur through experimental error during DNA extraction or amplification and create the differences observed (Jones et al. 1997; Blears et al. 1998). The use of an automated sequencer allows for weak bands to be detected; however, if the signal is particularly weak then the band will not be observed since it will fall below the baseline error rate. Methods that use band identity, such as for parentage analysis or clonal identification, will require a greater degree of accuracy in AFLP genotyping than is required when considering the profile as a whole. For such analyses, a greater number of primer combinations and repeated extractions/genotyping attempts will increase the accuracy. The comparison of AFLP profiles from multiple tissues provides a robust assessment of accuracy since the DNA is expected to be the same (assuming no genomic differences between tissues), but every stage from sample collection and preservation through to genotyping is distinct. While amplification of non-target DNA from leaf tissues in not always an issue (Wilkinson et al. 2003) the frequency is unknown and thus contamination is always a concern. If striking differences are found between samples, then further investigation can be done to determine if there is any DNA present from a non-target organism. This method could also be used as a primary identification tool if other methods such as sequencing are unavailable.

Our work shows that leaf and cambium tissue can be used interchangeably within a single genetic study when considering the AFLP profile as a single trait. Biotic contamination, from non-target organisms (Saar et al. 2001), or differences in the genome between tissues did not influence the genetic relationship between the trees studied. These results are widely applicable since the tree species studied reside in different tree families and show that, within a single study, the use of both cambium and leaf tissue will enable a greater sample number of trees to be studied. Climbing tall tropical trees to collect leaf material can be costly and incur risk but result in high quantities of extracted DNA, whereas the sampling of cambium material may be simple but introduce disease or produce low yields on DNA. The risk of disease introduction through cambium sampling can be reduced through careful collection methods; however, there is no study to date that quantitatively assesses the risk of disease

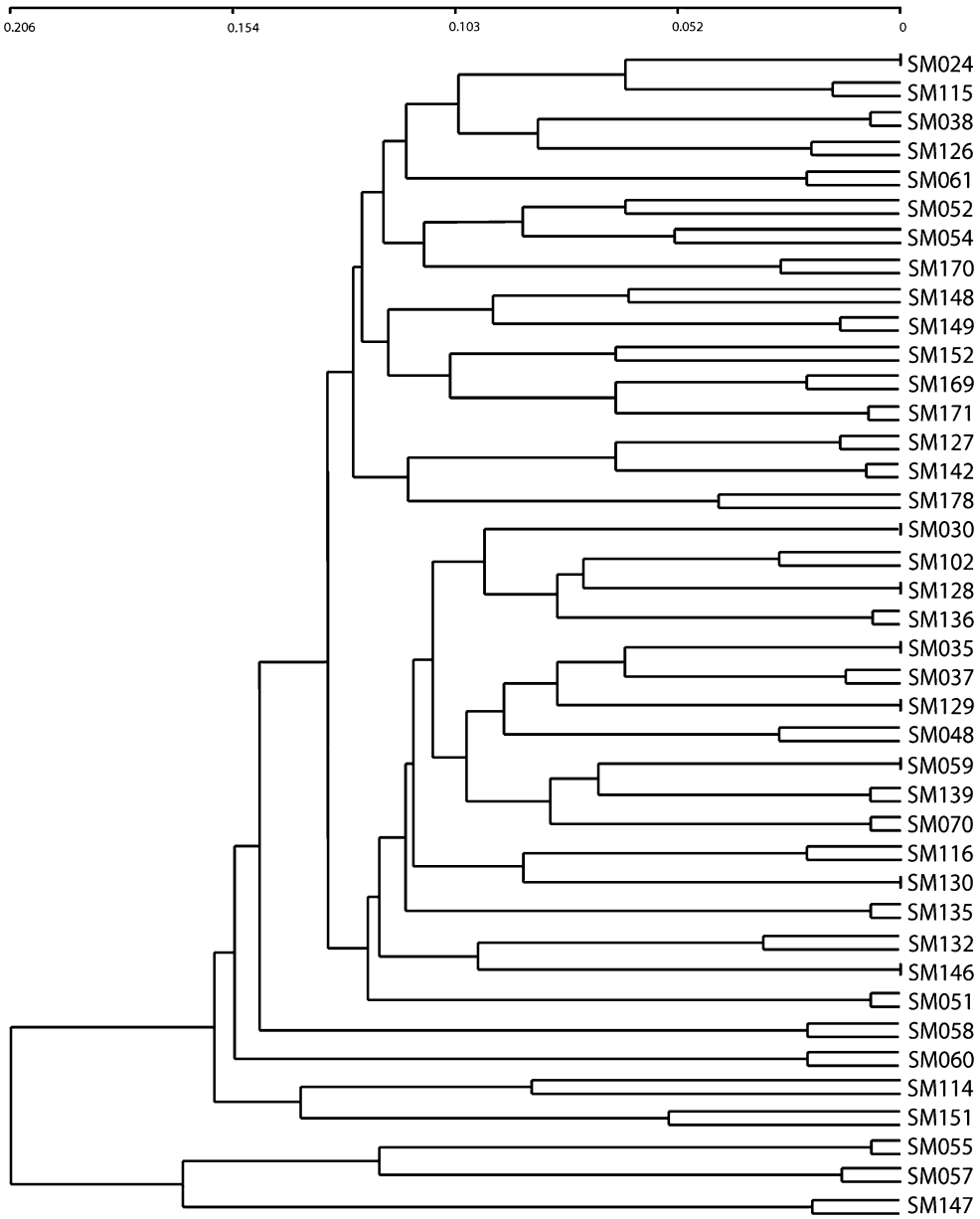

Fig. 3 Clustering relationship of leaf and cambium samples within 40 individual *Swietenia macrophylla* (*SM*) trees. SM *numbers* correspond to individual tree numbers, and the *x*-axis (*top*) shows the Sørensen index of dissimilarity (1-Sørensen)

introduction following cambium sampling in tropical forests, where optimal conditions for pathogenic growth can occur. The cambium samples in this study were collected in 2007–2008, and these trees have been returned

Table 1 Tolerance levels for AFLP band mismatches for cambium and leaf samples within an individual

Tolerance level (%)	*Brosimum alicastrum* (119 AFLP loci)		*Swietenia macrophylla* (112 AFLP loci)	
	No. mismatches	No. individuals (n=50)	No. mismatches	No. individuals (n=40)
0	0	5 (10%)	0	7 (18%)
2.5	0–3	26 (52%)	0–3	29 (73%)
5	0–6	33 (66%)	0–6	34 (85%)
10.0	0–12	48 (96%)	0–11	40 (100%)
> 10	> 12	2 (4%)	> 11	0 (0%)

Tolerance levels show the error rate of samples from within the same tree, such that at a 10% tolerance level one can expect up to 12 mismatches between AFLP profiles. A mismatch occurs when one sample has produced a band that the other sample has not

to in subsequent years (up to 2010) with no obvious incidence of disease (S. Zytynska pers. obs.).

We have shown that leaf tissue and cambium tissue can be used as a source of DNA for genetic analysis. Furthermore, we showed that a combination of these tissues could be used in a single genetic study of tree populations without compromising the accuracy of the phylogenetic relationships obtained among the trees studied. AFLPs are useful molecular tools and can be used in population and conservation genetics, systematics, biodiversity surveys and QTL mapping (Mueller and Wolfenbarger 1999). In addition, AFLPs require good quality DNA, and therefore the DNA recovered in this study would also be useful for whole-genome analyses such as restriction-site associated DNA (RAD; Baird et al. 2008).

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