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RESEARCH PAPER

Direct analysis in real-time (DART) time-of-flight mass spectrometry (TOFMS) of wood reveals distinct chemical signatures of two species of *Afzelia*

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Abstract

• ***Key message*** **Distinct chemical fingerprints of the wood of *Afzelia pachyloba* and *A. bipindensis* demonstrated an effective method for identifying these two commercially important species. Direct analysis in real-time (DART) time-of-flight mass spectrometry (TOFMS) allowed high-throughput examination of chemotypes with vast potential in taxonomic, ecological, and forensic research of wood.**

• ***Context*** *Afzelia* is a genus of valuable tropical timber trees. Accurate identification of wood is required for the prevention of illicit timber trade as well as for certification purposes in the forest and wood products industry. For many years, particular interest has been focused on attempts to distinguish the wood of *A. bipindensis* Harms from *A. pachyloba* Harms due to substantial differences in the commercial values of these two species.

• ***Aims*** We investigated if wood chemical signatures and microscopy could identify the wood of *A. bipindensis* and *A. pachyloba*.

• ***Methods*** We used two approaches, namely metabolome profiling by direct analysis in real-time (DART) time-of-flight mass spectrometry (TOFMS) and wood microstructure by light microscopy and SEM. In all, we analyzed samples from 89 trees of *A. bipindensis*, and *A. pachyloba*.

• ***Results*** The two species could not be separated by the IAWA standard microscopic wood features. SEM analysis showed considerable variation in the morphology of vestured pits; however, this variation was not species-specific. In contrast, DART-TOFMS followed by unsupervised statistics (Discriminant Analysis of Principal Components) showed distinct metabolome signatures of the two species.

• ***Conclusion*** DART-TOFMS provides a rapid method for wood identification that can be easily applied to small heartwood samples. Time- and cost-effective classification of wood chemotypes by DART-TOFMS can have potential applications in various research questions in forestry, wood science, tree-ecophysiology, and forensics.

Keywords *Afzelia* · DART · TOFMS · Illegal logging · Vestured pits · Wood identification

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1 Introduction

1.1 Taxonomy and economic importance of *Afzelia*

Afzelia, an important genus of big trees in tropical forestry, is composed of twelve species in the large family of Fabaceae (subfamily Caesalpinioideae). Eight of the species are distributed in Africa (*Afzelia africana* Pers., *A. bella* Harms, *A. bipindensis* Harms, *A. bracteata* Benth., *A. pachyloba* Harms, *A. parviflora* (Vahl) Hepper, *A. peturei* De Wild., *A. quanzensis* Welw), and four in SE Asia (*A. javanica* (Miq.) J. Leonard, *A. martabanica* (Prain) J. Leonard, *A. rhomboidea* (Blanco) S. Vidal, *A. xylocarpa* (Kurz) Craib). This genus has wide geographic distribution in a broad range of tropical forest types and ecological conditions which have led to variations in morphological traits and within species genetic differentiation between populations (Pakkad et al. 2014; Jinga and Ashley 2018). The nomenclature of *Afzelia* species is intricate because a significant number of synonyms have historically been used as well as a homonym genus name "*Afzelia*" for some unrelated *Seymeria* species of Orobanchaceae (The Plant list). The *Afzelia* timber mostly originates from Africa with major exporters Cameroon, Côte d'Ivoire, and Ghana. The wood is heavy with a mean density of 0.8 g/cm^3 (SD = 0.06) (Gérard and Louppe 2012). Properties are similar to merbau, another timber of high demand from the closely related *Intsia* sp. from South-East Asia. The quality of *Afzelia* timber is comparable to highly prized wood species such as teak (*Tectona grandis* L.f.), African makore (*Tieghemella heckelii* (A. Chev.) Roberty), and douka (*T. africana* Pierre). The wood is valued for dimensional stability, low shrinkage rates during drying, and superior natural durability, as well as for having a high aesthetic value. It is suitable for a wide range of interior and exterior applications which include the most demanding and economically important applications such as doors, stairs, and frames, but it can also be used for shingles, boat building, mine props, railway sleepers, flooring, furniture, veneer, musical instruments, and sporting goods. The heartwood is highly resistant to fungal, termite, and borer attacks and moderately resistant to marine borers (Gérard and Louppe 2012). Special industrial applications have been reported, such as for chemical tanks or machine parts, because of the neutral pH of wood, low susceptibility to variations in humidity, and resistance to acids and bases. Similar to the related *Intsia* species, the xylem vessels may contain yellow crystalline deposits that affect the aesthetic look of products (Koch et al. 2006).

Except for timber, the species of *Afzelia* as legumes are valued in the agro-forestry for their nitrogen fixation ability which enriches the soil. However, the natural populations are threatened by habitat loss as well as overexploitation because harvesting has greatly exceeded the regeneration rate of these trees (Adomou et al. 2010). Therefore, the conservation status of the *Afzelia* species needs critical investigation in most countries. For example, while high genetic diversity of *A. xylocarpa* is still harbored in Thailand (Pakkad et al. 2014), low levels of genetic diversity are occurring for *A. xylocarpa* in Vietnam and *A. quanzensis* in Africa. This is likely due to isolated populations and declining numbers of mature individuals (Thanh et al. 2012; Jinga and Ashley 2018). At present, IUCN Red List of Threatened Species designates *A. africana*, *A. bipindensis*, *A. pachyloba*, and *A. rhomboidea* as "vulnerable" and A. *xylocarpa* as "endangered" (IUCN Red List of Threatened Species 2017). *A. quanzensis* is a protected species in South Africa and is also regionally listed by IUCN as vulnerable in Malawi (Golding 2002).

1.2 Interspecific diversity of *Afzelia* species and methods of wood identification

Variations in terms of habitat, ecology, growth rate, and population genetics of *Afzelia* species from different regions of Africa have been recorded, and the market value of *Afzelia* timber from different countries varies depending on origin and species (Gérard and Louppe 2012; Jinga and Ashley 2018). For instance, the price of *A. bipindensis* can be twice the price of *A. pachyloba*. However, the natural distribution of these two species overlaps, and identification based on geographic origin can be dubious. While identification of *Afzelia* wood has long been demanded by the international trade of timber, closely related species cannot be separated via comparative wood anatomical analysis. A popular conception postulates that the heartwood of *A. bipindensis* is not fluorescent under UV light while *A. pachyloba* exhibits yellow fluorescence. However, a variety of wood surface fluorescence patterns can be observed, and the scientific basis for using fluorescence for separation of these two species remains unverified. While producers, traders, consumers, and conservationists are aware of the variation in the value of different *Afzelia* wood, there is no established methodology for wood identification at the species level. Hence, the wood of all species of *Afzelia* is still typically mixed and traded under common names such as "doussie" or "pod mahogany" regardless of the botanical name, provenance, and variation in properties. The identification of *Afzelia* wood species is becoming increasingly important also for conservation purposes because of the vulnerable and endangered status of some of the species.

Novel methods for accurate wood identification have been intensively developed during the last two decades, such as DNA genotyping (Lemes et al. 2010; Tnah et al. 2010; Jolivet and Degen, 2012; Höltken et al. 2012, Degen et al. 2013; Hartvig 2015), machine vision using morphological features (Hermanson and Wiedenhoeft 2011; Rosa da Silva et al. 2017; Hermanson 2017; Ravindran et al. 2018; Kobayashi et al. 2019), near-infrared (NIR) spectroscopy (Pastore et al. 2011, Tsuchikawa et al. 2003; Braga et al. 2011; Bergo et al. 2016), and DART-TOFMS and chemometry (Espinoza et al. 2014, 2015; Deklerck et al. 2017, 2019, 2020; Paredes-Villanueva et al. 2018). These modern methods to date have only been evaluated and applied on a limited number of species that do not include *Afzelia* spp. DNA-Barcoding (plastid sequences) is a well-established method for biological species or geographic origin of timber identification (Deguilloux et al. 2003; Hollingsworth et al. 2011, Hartvig et al. 2015, Caron et al. 2019). Microsatellite markers were found to provide high resolution for species identification in forensic studies of timber (Nowakowska 2011, Tereba et al. 2017, Blanc-Jolivet et al. 2018). However, the xylem cells containing DNA constitute tiny proportions in most woody species, particularly in heartwood, the commercial part of the wood. Old or processed wood materials such as plywood may still contain short fragments of DNA that can be amplified by PCR (Tsumura et al. 2010). Nevertheless, extracting sufficient amount of DNA from wood products is currently challenging, time-consuming, and expensive. On the other hand, DART-TOFMS requires small (a few mm^3) slivers of heartwood specimens and gives an accurate profile of the metabolites of the sample (Espinoza et al. 2014, 2015). Moreover, the collection of DART-TOFMS data of wood is quick, cost effective, and relatively non-destructive. Once a statistically sufficient number of samples is analyzed, the resulting fingerprints show a common pattern that can be used to identify unknown samples (Musah et al. 2015). Metabolite profiles obtained by DART-TOFMS for wood identification are routinely used by the U.S. Fish and Wildlife Service to differentiate between CITES-listed and some look-alike timber species (Lancaster and Espinoza 2012; Espinoza et al. 2014, 2015; McClure et al. 2015, Evans et al. 2017).

The large Fabaceae family includes a great number of commercially and ecologically important species that remain difficult to identify using wood material (Höhn 1999; Normand and Paquis 1976; Wheeler 2011; Gérard et al. 2011; Gérard and Louppe 2012; Richter et al. 2014). Understanding the genetic and ecological variation of a wood species requires comprehensive research material. Scientific wood collections (xylaria) that store curated wood material can aid in wood identification and a wide range of research questions in botany, forestry, and wood science. Therefore, xylaria have been established with enormous efforts over long periods of time. The xylarium at the Royal Museum for Central Africa in Tervuren, Belgium (RMCA), contains about 80,000 specimens, many more than 100 years old. Commercial timber identification often does not require species-level resolution because typically a group of similar species represents a single commercial product (i.e., doussie, merbau). However, wood identification for nature conservation, or forensic purposes, requires accuracy at the species-level. Hence, it is essential to ensure that the xylarium specimens' biological species are properly identified. Vouchers that are linked to the acquired material are usually relied upon to provide assurance. However, vouchers may not be available for a significant number of wood specimens, particularly for the older ones. Nevertheless, unvouchered specimens often represent rare taxa and may have high value in terms of the wood block's size and preservation quality. Verification of such specimens by DNA analysis would be time-consuming and expensive. Our goal is to establish a cost-effective wood identification method to validate wood specimens of *Afzelia bipindensis* and *A. pachyloba*. We hypothesized that chemical fingerprinting by DART-TOFMS as well as microstructure of vestured pits can distinguish between these two commercially important species of *Afzelia*.

2 Material and methods

2.1 Plant material

Wood samples from two species of major economic importance, namely, *Afzelia bipindensis* and *A. pachyloba*, were provided by the Royal Museum for Central Africa in Tervuren, Belgium, and by the U.S. Fish and Wildlife Service in Ashland, Oregon. In total, heartwood samples from 50 trees of *A. bipindensis* and 39 trees of *A. pachyloba* were investigated for chemotypes (Table 1). For wood anatomical analysis, each of the two species was represented with five specimens as follows: *A. pachyloba* (Tw62, Tw3901, Tw52893, Tw52910, and Tw52911) and *A. bipindensis* (Tw27823, Tw45117, Tw52902, Tw52906, Tw52907). The species have been botanically identified at the time of collection. Herbarium vouchers are available for four specimens of *A. bipindensis*, and one of *A. pachyloba* (Table 1).

2.2 Anatomy methods

Sample blocks and permanent wood slides representing five individuals per species were used for comparative SEM and light microscopy of wood taxonomic features. The wood characteristics were determined according to the IAWA

Table 1 Description of the *Afzelia* wood specimens investigated for chemical profiles

Species	*Afzelia bipindensis*	*Afzelia pachyloba*
Origin of species	West and Central Africa: Nigeria, Uganda, DR Congo, Angola, Zambia	West and Central Africa
Conservation status by the IUCN Red List of Threatened Species as of July 2020	Vulnerable	Vulnerable
Xylarium acquisition Numbers Italics: vouchered specimens	($n=50$) *WD140197_Tw3956* *WD140200_Tw886* *WD140206_Tw3898* *WD140207_Tw2417* WD140283_TW54566 WD140286_TW54663 WD140305-2_TW54676 WD140306_Tw54672 WD140368_Tw65081 WD140379_Tw54201 WD140380_Tw54202 WD140383_Tw54205 WD140384_Tw54206 WD140385_Tw54207 WD140386_Tw54208 WD140387_Tw54209 WD140388_Tw54210 WD140389_Tw54211 WD140391_Tw54213 WD140392_Tw54214 WD140394_Tw54216 WD190014_Tw1567 WD190025_Tw53810 WD190026_Tw53809 WD190027_Tw53811 WD190028_Tw55825 WD190030_Tw54155 WD190034_Tw53563 WD190036_Tw54126 WD190046_Tw53566 WD190049_Tw53560 WD190050_Tw53579 WD190052_Tw53778 WD190058_Tw53767 WD190059_Tw54567 WD190063_Tw54669 WD190065_Tw53559 WD190072_Tw53751 WD190074_Tw54138 WD190075_Tw54670 WD190076_Tw54569 WD190077_Tw53766 WD190091_Tw53530 WD190137_Tw54145 WD190138_Tw54673 WD190139_Tw54665 WD190141_Tw54144 WD190143_Tw54671 WD190146_TW55119 WD190147_TW48453	($n=39$) WD140205_Tw11260; *WD140222_Tw62C;* WD140229-1_TW43998 WD140296_Tw54564; WD140299_Tw54233; WD140300-2_Tw54232; WD140302_Tw54235; WD140303_Tw54234; WD140304_Tw54754; WD140308_Tw54756; WD140398_Tw54221; WD140399_Tw54222; WD140401_Tw54225; WD140402_Tw54226_X; WD140403_Tw54227; WD140404_Tw54228; WD140405_Tw54229; WD140406_Tw54230; WD190010_Tw3901; WD190032_Tw53758; WD190033_Tw54153; WD190037_Tw54563; WD190042_Tw53777; WD190045_Tw54231; WD190051_Tw53764; WD190056_Tw53534; WD190061_Tw53568; WD190062_Tw53752; WD190064_Tw53707; WD190066_Tw50840; WD190067_Tw53753; WD190068_Tw53776; WD190069_Tw54164; WD190070_Tw53756; WD190071_Tw53699; WD190085_Tw53697; WD190089_Tw53564; WD190136_Tw54169; WD190150_Tw56985

list of wood microscopic taxonomic features (IAWA Committee 1989). Based on preliminary screening of the variability of wood features of *Afzelia* species, we focused our quantitative analysis on xylem vessel tangential diameters (lumens of cells) and on ray dimensions (in transverse and in tangential sections, respectively). Sample preparation for scanning electron microscopy (SEM) followed the procedures described by Kitin et al. (2009) and Dié et al (2012). In brief, small wood blocks were cut to 7 mm (longitudinal direction) × 2 mm (radial) × 5 mm (tangential) or 7 mm (longitudinal direction) × 5 mm (radial) × 2 mm (tangential) and rinsed in water. The radial and the tangential surfaces were cut with a razor blade. Next, the wood blocks were inserted in a 5% sodium hypochlorite solution for 3 min. Then, they were rinsed three times in water and dehydrated using an ethanol series of increasing concentrations (25, 50, 75, and 100%) for 30 min in each concentration. After three changes in 100% ethanol for 15–20 min each time, blocks were air dried. The samples were coated with gold in a sputter coater and observed with a scanning electron microscope (JSM-6610LV; JEOL, Tokyo, Japan) operated at an accelerating voltage of 10 kV.

2.3 Chemistry methods

A detailed description of the DART-TOFMS ionization and analysis mechanism has been provided by Harris et al. (2011) and Cody (2013). Discussions of the development of metabolite profiling for wood identification are available by Espinoza et al. (2014, 2015), Finch et al. (2017), and Deklerck et al. (2017, 2019). In this study, we used DART and statistical classification methods as previously described by Lancaster and Espinoza (2012), Espinoza et al. (2015), and McClure et al. (2015). A small sliver from the heartwood of each sample (Table 1) was held in a heated to 350 °C helium gas stream of a DART-SVP ion source (IonSense, Saugus, Massachusetts, USA) coupled to a JEOL AccuTOF 4G LC Plus mass spectrometer (JEOL USA, Peabody, Massachusetts, USA). Mass spectra of the emitted wood compounds were acquired in positive ion mode over the mass range of m/z 60 to 1000. Poly(ethylene glycol) 600 (Ultra Scientific, Kingstown, Rhode Island, USA) was used as a mass calibration standard after every fifth sample. The DART source parameters settings were the same as described by Espinoza et al. (2015) and Evans et al. (2017). TSSPro3 (Shrader Analytical Labs, Detroit, Michigan, USA) data processing software was used to export the mass-calibrated, centroided mass spectra for further analysis. The spectral data includes estimated mass-to-charge ratios (m/z) and relative molecule abundance (Kitin et al. 2020). Heat maps and statistical analysis of the datasets were conducted using the Mass Mountaineer version 2 software (RBC Software, Peabody, Massachusetts, USA, massmountaineer.com) using a tolerance of 5 mDa and 1% threshold. The classification algorithms of Mass Mountaineer were used to calculate the principal components of each dataset for classification by discriminant analysis of principal components (DAPC) using diagnostic ions. DAPC is a multivariate method that identifies and describes clusters of genetically related individuals (Jombart et al. 2010). It has gained popularity for exploring structures of datasets without a priori knowledge or assumptions of class memberships. By inspection of the heat maps, we manually selected ions that showed higher intensity in one of the species but lower in the other as described by Deklerck et al. (2017). Deklerck et al. (2017) established that minimum of 50 ions were needed for achieving the highest accuracy for wood identification of several species by different classification methods. To assess model accuracy, leave-one-out cross-validation (LOOCV) was employed. With this test, each sample is successively omitted from the entire training set (n) and placed as an unknown for comparison against the remaining training set ($n - 1$ samples). The LOOCV result represents an average of the n errors (validation analyses repeated n times) and provides an unbiased metric of how well the model performs in evaluating the distance from the cluster mean of each sample that is omitted. The LOOCV estimate is a constant for a given dataset, and the percentage of cases that are reliably classified reflects the degree to which the samples yield consistent information. For unknown specimens, the Mass Mountaineer software can assign a probability estimate to the classification of the spectrum.

3 Results

3.1 Physical and anatomical properties of *Afzelia* wood

The two species exhibit similar macrostructural and physical characteristics. The heartwood color varies from pale to dark red-brown or orange-brown, becoming darker in older samples; it is distinct from the pale yellow sapwood. The width of the sapwood can be up to 5 cm, and sapwood was often present in wood specimens, necessitating its removal prior to the chemical analysis. The wood is heavy with a specific gravity ranging from 0.7 to 0.9 and is characterized by straight to interlocked grain and medium to coarse texture.

The two species share similar microscopic features. **Cross sectional view**: wood diffuse-porous with growth ring boundaries distinct or absent; typical tangential vessel diameter ranges from 100 to 260 µm (*A. pachyloba*: mean = 180 µm, variance = 2057, n = 225; *A. bipindensis*: mean = 211 µm, variance 2828, n = 280); vessel frequency ≤ 5 per square millimeter; axial parenchyma vasicentric, aliform, lozenge aliform,

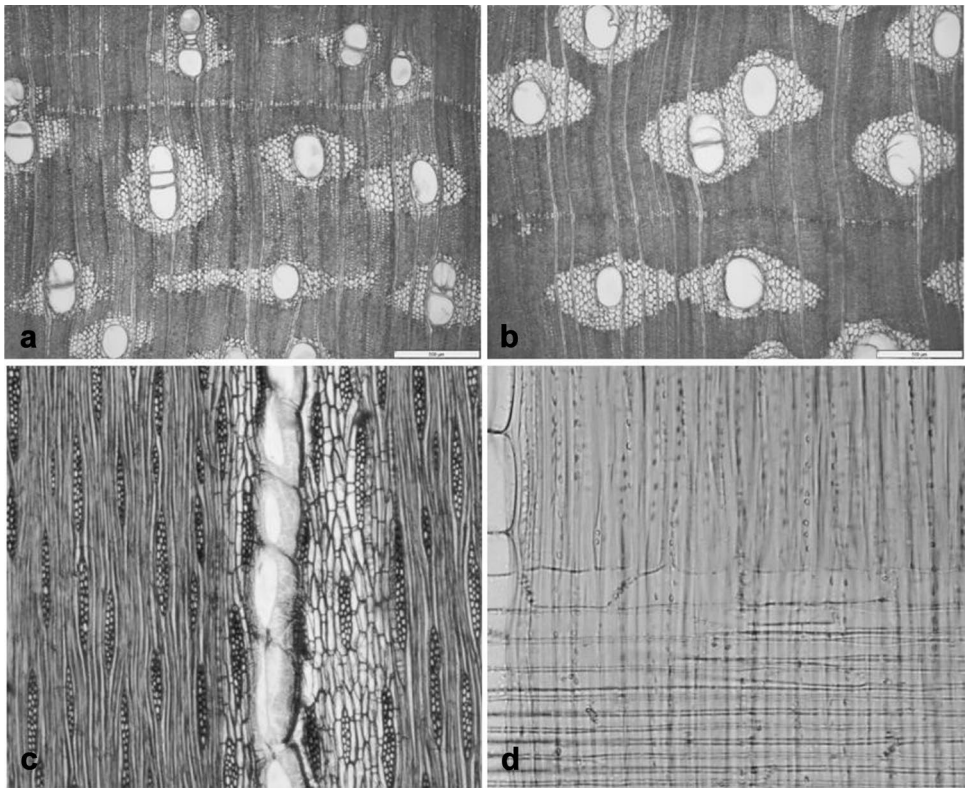


Fig. 1 Light microscopy of *Afzelia* wood. Transverse sections showing xylem vessels, axial parenchyma and rays in *A. pachyloba* (**a**) and *A. bipindensis* (**b**) Longitudinal-tangential section of *A. pachyloba* wood showing rays and axial parenchyma (**c**) Longitudinal-radial section of *A. bipindensis* showing ray cells and fibers with pitted fiber walls (**d**) Bars: 500 µm (**a**, **b**, **c**); 100 µm (**d**)

occasionally confluent or in marginal bands; and fibers thick-walled (Figs. 1a, b and 4). **Longitudinal-tangential view**: rays and axial parenchyma irregularly storied; ray frequency 4–12 per mm; axial parenchyma 2–4 cell per strand (Fig. 1c). The typical ray height ranges from 200 to 360 µm (*A. pachyloba*: mean = 238.7 µm, variance = 2730, $n = 292$; *A. bipindensis*: mean = 277.4 µm, variance 5893, $n = 477$). The typical ray width ranges from 1 to 3 cells (*A. pachyloba*: mean = 39.8 µm, variance 118,8, $n = 306$; *A. bipindensis*: mean = 38.5 µm, variance = 106.4, n = 423) (Figs. 2 and 4). Vessels with simple perforation plates; alternate intervessel pits, vestured (variable) and with pit membrane diameter (measured on dry wood) 4–7 µm (Figs. 2 and 4); vessel element length predominantly less than 350 µm; prismatic crystals present in chambered axial parenchyma cells. **Longitudinal-radial view:** fibers with simple to minutely bordered pits; ray cells procumbent either all of equal sizes or cell height in the marginal one or two rows variable and can be up to three times taller compared with cells in the middle of the ray (Fig. 1d); vessel-ray pits with distinct borders, sizes, and shapes similar to intervessel pits and vestures present. Characteristics of vestures, such as size, shape, and occurrence, were largely variable within a specimen, and even within single vessels (Fig. 3). The anatomical measurements of vessel tangential diameters, ray height, and

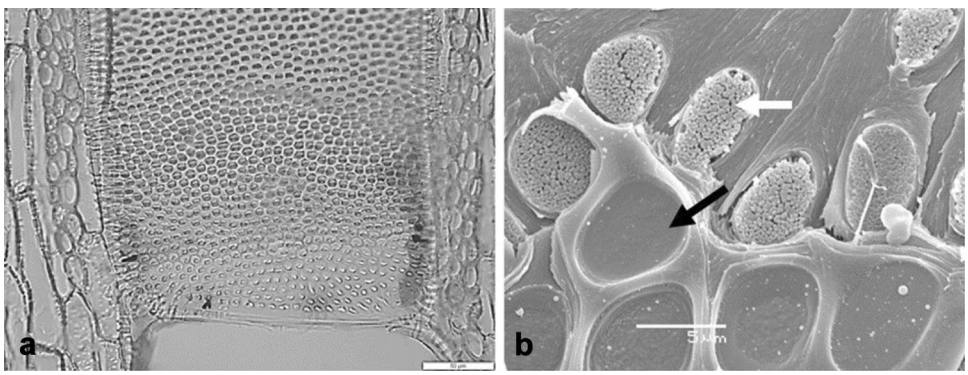


Fig. 2 Intervessel pits in tangential vessel walls of *Afzelia bipindensis*. Light microscopy showing alternate arrangement of minute pits (**a**) Scanning electron microscopy revealing vestures (white arrow) beneath pit membranes (black arrow) (**b**) Bars: 50 µm (**a**); 5 µm (**b**)

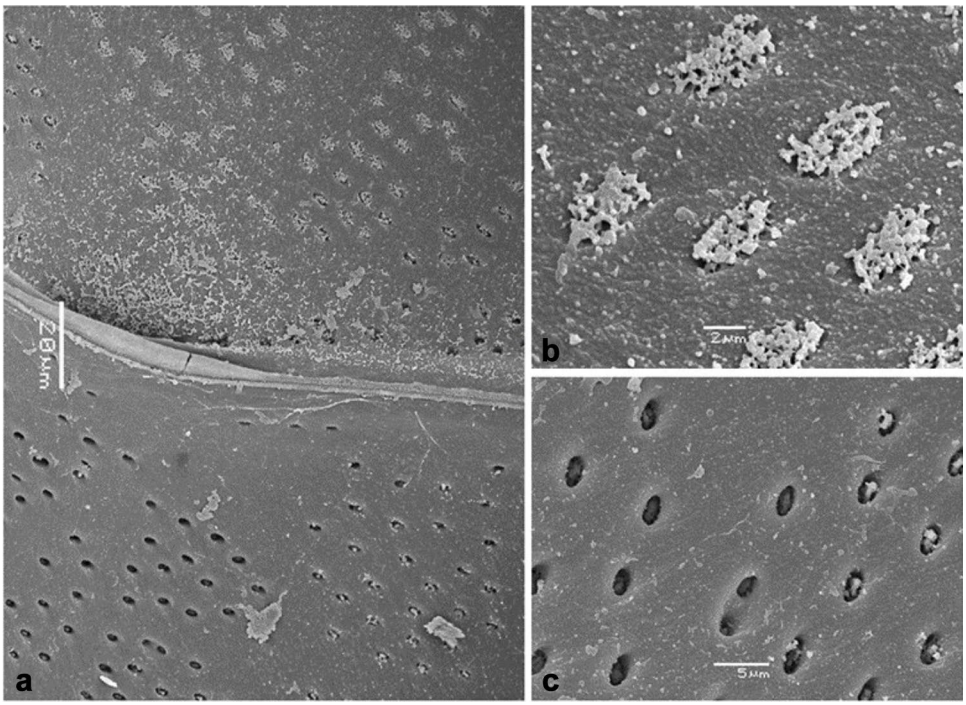


Fig. 3 Scanning electron microscopy showing variation in the structure of vessel-to-parenchyma pits in the inner side of vessel wall in *Afzelia bipindensis*. Adjacent areas of two cells (vessel elements) in the same vessel (**a**) The pits in the upper vessel element have abundant vestures (**a**, **b**) while the pits in the lower vessel element have few or no vestures (**a**, **c**) An enlarged view from the upper vessel element (**b**) An enlarged view from the lower vessel element (**c**) Bars: 20 µm (**a**); 2 µm (**b**); 5 µm (**c**)

ray width are shown in Fig. 4 and in Zenodo repository (Kitin et al. 2020).

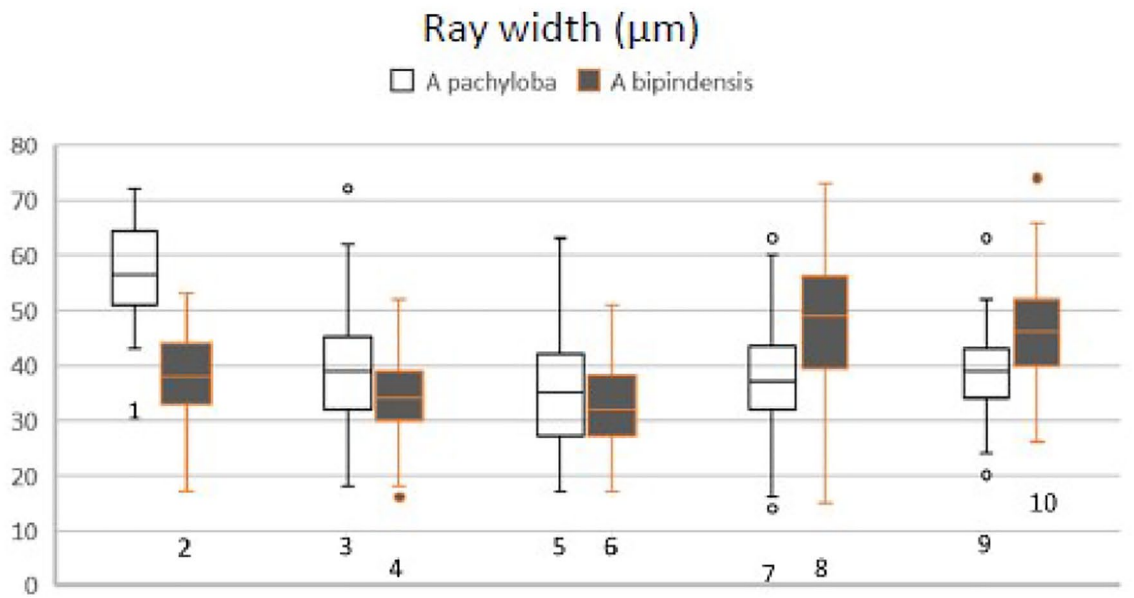
3.2 Chemistry

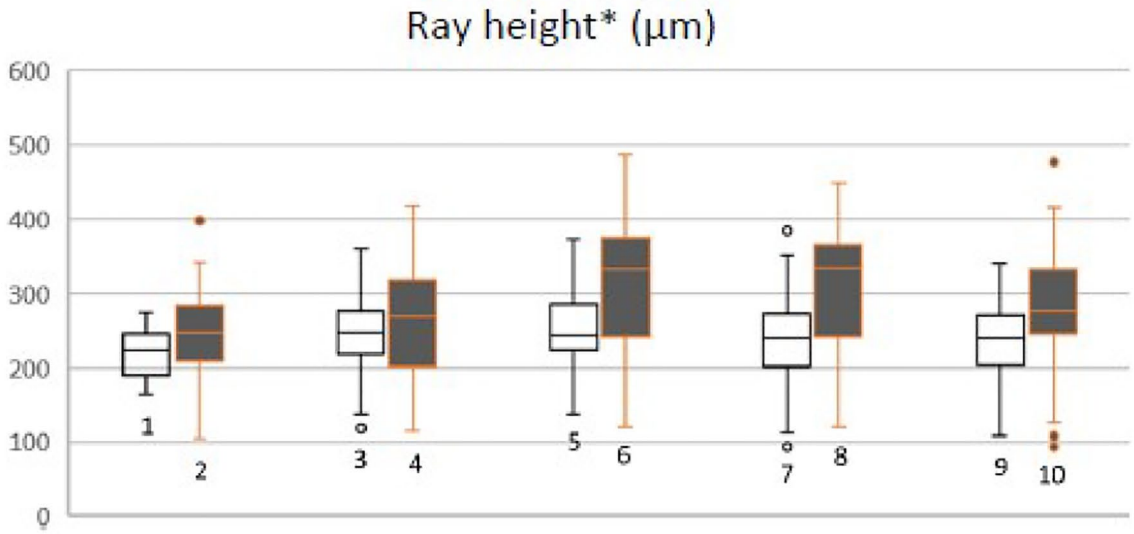
Tentatively identified chemical substances that are characteristic for both species are shown in Table 2. Examples of graphical spectra of molecular weights and relative abundance of the molecules in single specimens of *A. bipindensis* and *A. pachyloba* are shown in Fig. 5. The botanical identification of the two specimens represented in Fig. 5 is supported with vouchers. Although both species contain similar compounds, the relative abundance of each compound differs between the species. For example, 14, 15-dehydrocrepenynic acid is highly expressed in the *A. pachyloba* sample, while a compound with 309.2 m/z is more expressed in the *A. bipindensis* sample. Figure 6 is an overview of the entire experiment: a heat map graphical representation of the total number of specimens and mass spectra of individual samples. The intensity of the pixels in each line is a measure of the relative abundance of each molecule, i.e., the most intensely colored spots correspond to the highest bars (100%) in Fig. 5. The heat map demonstrates that the mass spectra profiles vary within species and between species. For instance, substances with the molecular mass in the region of 390–470 m/z were highly expressed in *A. pachyloba* but occurred in trace amounts in most of the *A. bipindensis* samples (Fig. 6). Heat maps can often visualize differences between species (Espinoza et al. 2015; Evans et al. 2017); however, in this case, molecules that are diagnostic for a species could not be easily perceived. By contrast, the DAPC statistical graph in Fig. 7 demonstrates that the mass spectra profiles of the two species were differentiated with 78% classification accuracy according to the LOOCV test.

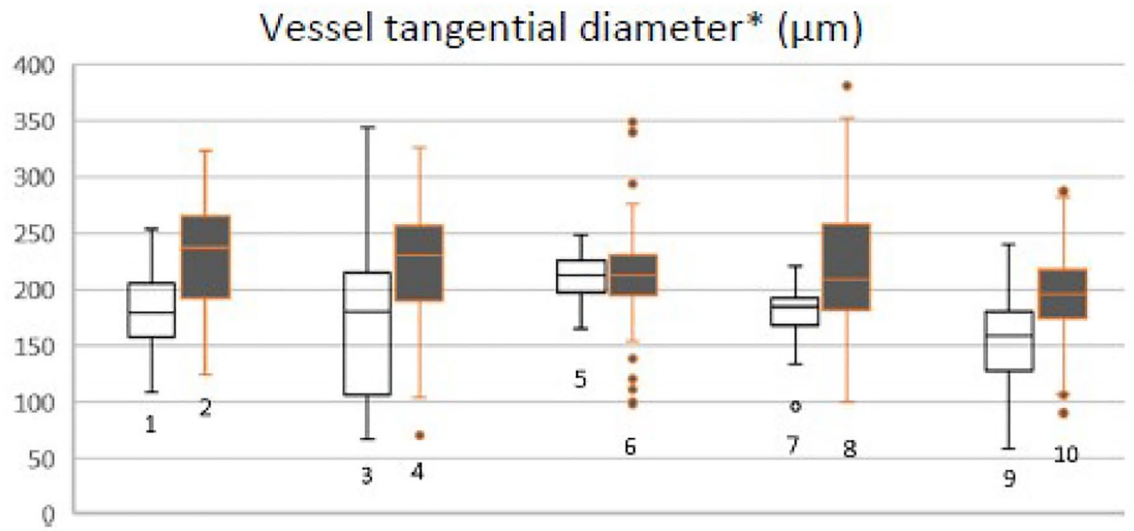
4 Discussion

4.1 Wood anatomy

Distinction to the species level by wood anatomical traits may be possible for some species as shown for other legumes (Gasson et al. 2010) or dipterocarps (Tsumura et al. 2010). Wood identification based on morphological features could be the only viable option when DNA or chemistry examination is not possible. However, achieving a species-level accuracy for wood identification has required high numbers of experimental trees, and in the case of dipterocarps, SEM investigation of the ultrastructure of xylem vessel walls (Tsumura et al. 2010). We focused on vestured pits because they are characteristic of many species of Fabaceae, and vestures are considered an essential taxonomic and eco-physiological wood character (Jansen et al. 2004). True vestures can potentially be confused with deposits of cell content such as remnants of cytoplasm, gums, or resins. Therefore, for revealing the true shape of vestures, we cleared the samples with alcohol and bleach according to the IAWA guidelines for observation of vestures (IAWA Committee, 1989).


Ray width (µm)
A pachyloba
A bipindensis
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10


Ray height* (µm)
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10


Vessel tangential diameter* (µm)
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10

◂**Fig. 4** Xylem vessel and ray dimensions of *Afzelia bipindensis* and *A. pachyloba*. The numbers indicate Tervuren xylarium specimens: Tw62 (1); Tw27823 (2); Tw52893 (3); Tw45117 (4); Tw52910 (5); Tw52902 (6); Tw52911 (7); Tw52906 (8); Tw3901 (9); Tw52907 (10). Ray width: *A. pachyloba*: mean = 39.8 µm, variance 118.8, $n = 306$; *A. bipindensis*: mean = 38.5, variance = 106.4, $n = 423$. Ray height: *A. pachyloba*: mean = 238.7 µm, variance = 2730, $n = 292$; *A. bipindensis*: mean = 277.4 µm, variance 5893, $n = 477$. Vessel tangential diameter: *A. pachyloba*: mean = 180 µm, variance = 2057, $n = 225$; *A. bipindensis*: mean = 211 µm, variance 2828, $n = 280$. The raw data is shown in Zenodo repository (Kitin et al 2020). Achieving a species-level identification accuracy is challenging because of the within-tree and between-tree variabilities of the quantitative characters (see Discussion)

Our preliminary observation by SEM of several *Afzelia* species indicated the occurrence of variation in the shape and size of vestures. Subsequent and more thorough investigation, however, failed to reveal consistent interspecific differences in the morphology of vestured pits. The abundance and morphology of vestures varied within species and even within individual vessels. Our description of taxonomic wood features was consistent with the features documented in the InsideWood website (InsideWood 2004-onwards; Wheeler 2011). The average vessel diameters and ray heights were slightly larger in the *A. bipindensis* samples that we studied (Fig. 4). Yet, we cannot confidently distinguish the two species by vessel diameter or ray height because of the substantial within-species variability and the relatively small number of trees studied. Previous studies have shown that the between-species variation of wood micro-features in *Afzelia*, such as vessel diameter, vessel frequency and arrangement, axial parenchyma pattern, frequency and arrangement of rays, ray height and width, intervessel pit size, and occurrence and shape of vestures, strongly overlaps

Table 2 Putatively identified characteristic molecules in the wood of both species, *A. bipindensis and A. pachyloba*, by comparing the mass-to-charge ratio of each molecule to a list of known molecules (Mass Mountaineer software)

Molecule name	Composition	Measured *m/z*
1-(2,5-dichlorophenyl)sulfonyl-4methylpiperazine	C11H14Cl2N2O2S	309.205
14, 15-Dehydrocrepenynic acid	C18H30O2	275.1978
Kaempferol	C15H10O6	287.0545

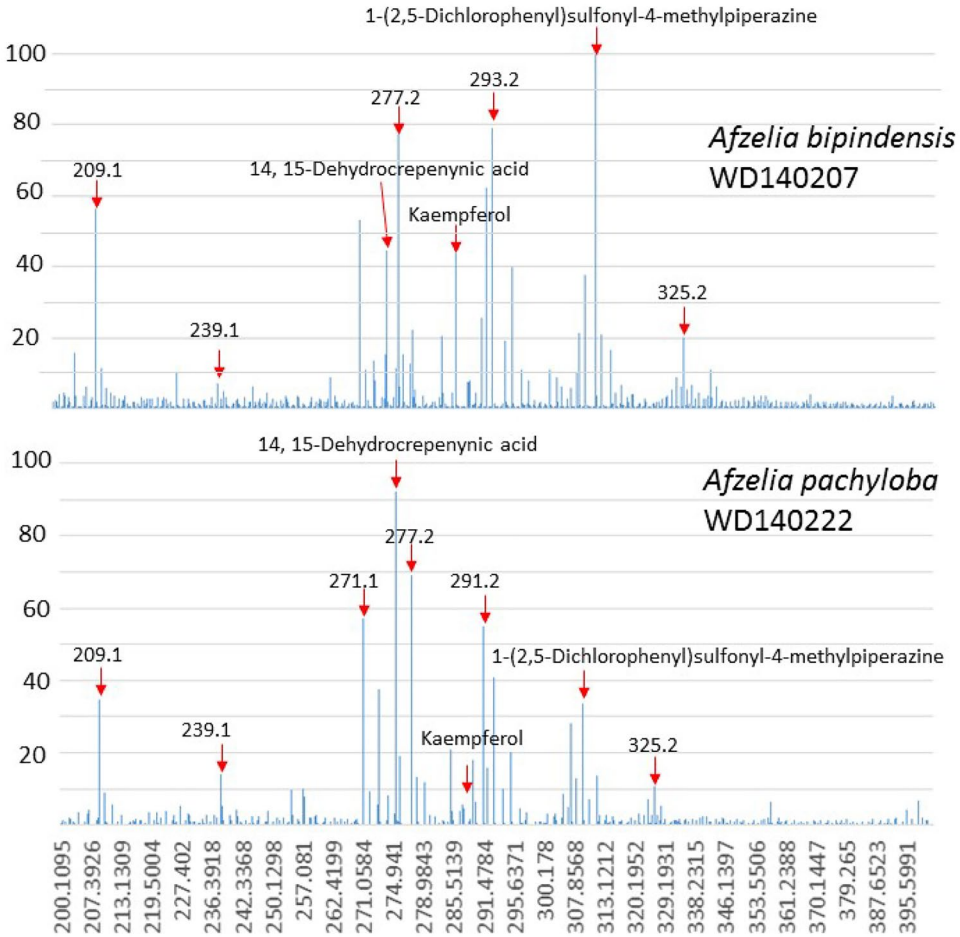


Fig. 5 DART-TOFMS spectra of two single specimens of *Afzelia bipindensis* (WD140207) and *A. pachyloba* (WD140222). The botanical identification of the two specimens is supported with vouchers. Each peak represents a different molecule, with its height normalized to that of the most abundant molecule. The horizontal axis shows the molecular mass of detected compounds, and the vertical axis shows the relative abundance of each molecule

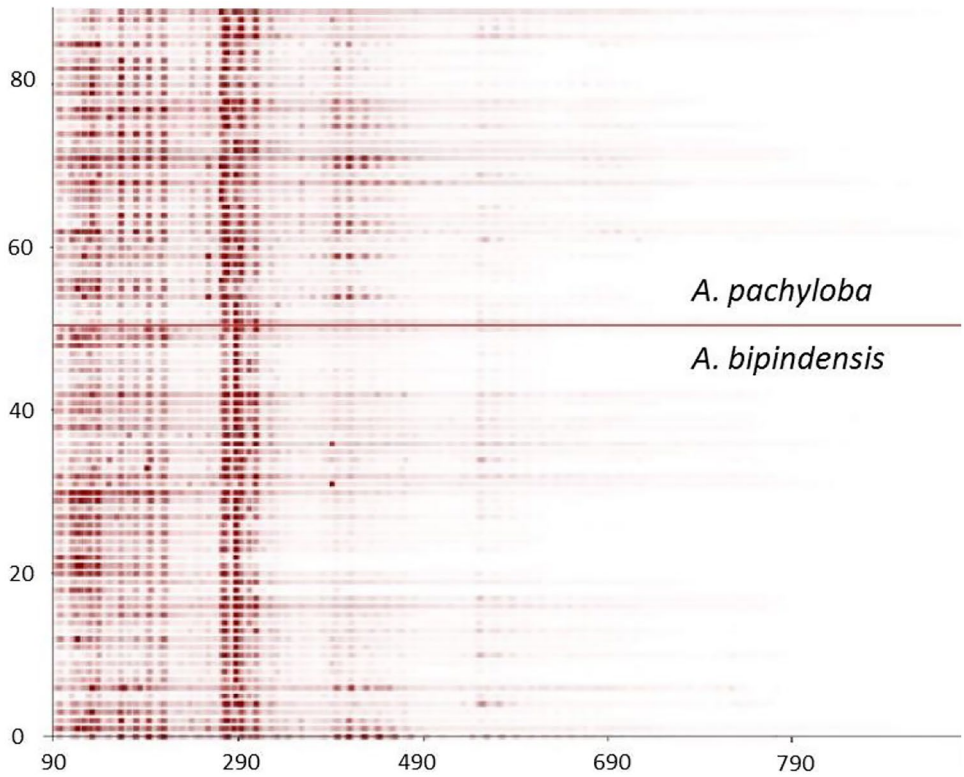


Fig. 6 Heat map of the samples from *Afzelia bipindensis* and *A. pachyloba*. The *x*-axis shows the molecular mass of detected chemical compounds and the *y*-axis shows the sequential number of each sample. The intensity of color in each individual spectrum (horizontal lines) indicates molecular abundance (higher abundance corresponds to darker color)

with the variation within species (Normand and Paquis 1976; Gérard and Louppe 2012; Wheeler 2011). For this reason, it has not been possible to identify *Afzelia* species using wood anatomical features. Microstructural traits, in particular porosity, vessel size, and cell wall pit size, are closely linked to hydraulic or biomechanical properties of wood (Baas and Miller 1985; Sperry et al. 2006; Lens et al. 2011; Christman et al. 2012; Beeckman 2016). Such traits typically vary within species depending on environmental conditions and genotype (Baas and Miller, 1985; Gasson et al. 2010; Gasson 2011, Tsumura et al. 2010). Even within individuals, microstructural traits related to xylem hydraulics may widely vary, reflecting environmental changes that can be seasonal or long-term shifts in the growth conditions of the tree. Therefore, anatomical investigation of the phenotypic variations of *Afzelia* wood might be promising in

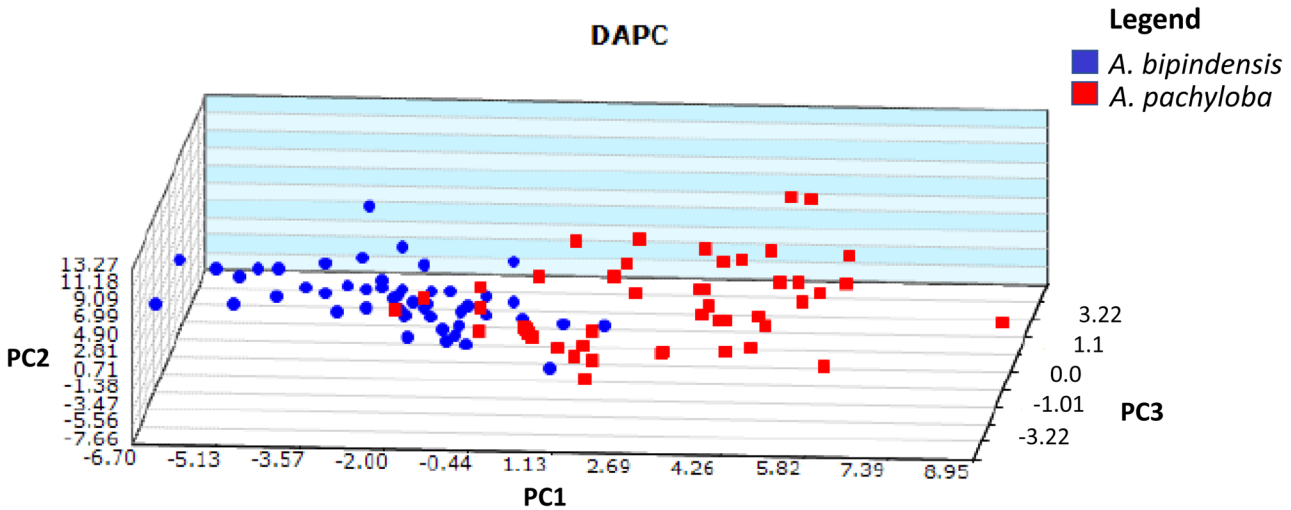


Fig. 7 Discriminant analysis of principal components using 53 ions of the chemotypes of *Afzelia bipindensis* and *A. pachyloba* (LOOCV = 78%)

ecological studies. On the other hand, microscopy analysis of wood for revealing species-specific taxonomic traits is challenging, particularly by SEM, as it may require extensive sampling of many trees as well as within single trees. We were unable to reveal at this time any species-specific taxonomic significance of the wood micro-features that we studied in these two species of *Afzelia*.

4.2 Chemical profiles

Wood of *Afzelia bipindensis* consists of about 46% cellulose, 27% hemicellulose, 21% lignin, 8% alkaloids (phenol, tannin, steroids, terpenoids, cyanogenic glycoside), 7% saponins, 4% crude proteins, 4% lipids, 2% flavonoids, pyroligneous acid, oxalate (Udeozo et al. 2018). As discussed elsewhere, the sapwood of *Afzelia* sp. is susceptible to insect and fungal attacks, while the heartwood is naturally durable due to the presence of protective compounds (Gérard and Louppe 2012; Udeozo et al. 2018). Logs may have crevices filled with a yellowish substance that is also accumulated in adjacent xylem vessels. The nature of the yellowish content in xylem vessels of *Afzelia* and the closely related *Intsia* has identified as kaempferol and other phenolic deposits (Koch et al. 2006). Alkaloids or flavonoids in wood, such as some of the raw compounds suggested by our study, may have important applications as medicinal drugs. Kaempferol, which has antimicrobial activities, was also one of the characteristic substances found in each of the two species in our study. 14, 15-Dehydrocrepenynic acid is another characteristic compound with potential applications as antimicrobial drug, and it can be also extracted from seed oil of *Afzelia quanzensis* (Gunstone et al. 1967). Dehydrocrepenynic acid is synthesized by plants and Basidiomycete fungi (Blacklock et al. 2010).

Although the two species contain similar chemical compounds, the relative abundance of chemicals and the chemical signatures by DART-TOFMS of heartwood were distinct in each of the two species. Our results confirm previous studies showing that DART-TOFMS can be used for wood identification of species from families with complex taxonomy such as Fabaceae and Meliaceae (Espinoza et al. 2014, 2015; McClure et al. 2015, Wiemann and Espinoza 2017, Deklerck et al. 2019). Previously, it was shown that the method is effective on both fresh and dry heartwood samples regardless of the sample position within a tree or the time of storage in xylarium (Finch et al. 2017; Deklerck et al. 2020). The accuracy of our classification model (78%) is satisfactory compared with the studies of other hardwood species. Deklerck et al. (2019) discussed possibilities for improving the species classification accuracy via optimization of pre-processing spectral parameters such as the mass tolerance for binning, the relative abundance cut-off thresholds, and the number of variables (ions). They presented an extensive analysis using supervised KDA statistics and the unsupervised random forest machine-learning algorithm on ten species of Meliaceae. By both methods, they achieved classification accuracies ranging from 78 to 82.2%. The classification accuracy, regardless of the statistical method, has a negative relationship with the variation of the chemical fingerprint within species. Within-species or between-species variation in wood chemistry can be influenced by both genetics and environmental change (Huber et al. 2005; Robinson et al. 2007; Finch et al. 2017). The heartwood metabolome profiles varied greatly in each of the two *Afzelia* species. Nevertheless, DARTTOFMS of 89 trees coupled with DAPC revealed markedly distinct heartwood chemotypes of *A. bipidensis* and *A. pachyloba*.

5 Conclusion

Neither of the two species could be separated using standard microscopic wood features by light microscopy or SEM. The anatomical analysis showed considerable variations in the morphology of vestured pits, vessel diameters, and ray height; however, this variation was not species-specific. In contrast, DART-TOFMS revealed distinct heartwood chemotypes of the two species of *Afzelia*. We conclude that DART-TOFMS provides a rapid method for wood identification that can be easily applied to small heartwood samples. Besides, DART-TOFMS can be useful for wood identification of species with a wide eco-geographical distribution that may have resulted in a heterogeneous wood structure. Future studies, to be abreast with environment protection laws, should aim at expanding the DART-TOFMS classification model by including reference samples from all *Afzelia* species and confusable look-alikes. Time- and cost-effective classification of wood chemotypes by DART-TOFMS can have potential applications in various research questions in forestry, wood science, tree-ecophysiology, and forensics. For example, wood metabolome information can be useful for evaluating the effect of environmental change on wood chemistry. Xylaria throughout the world can play a crucial role in tree species characterization and conservation by providing comprehensive and balanced reference data of wood types.

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HA, PMC. Resources: PK, EE, HB. Data curation: PK, EE, HB, PMC. Writing—original draft: PK, EE. Writing—review and editing: PK, EE, HB, HA, PMC. Visualization: PK, EE, PMC. Supervision: PK, EE, HB. Project administration: PK, EE, HB, HA. Funding acquisition: PK, EE, HB, HA.

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Data availability The datasets generated and/or analyzed during the current study are available in the Zenodo repository, http://doi.org/10.5281/zenodo.4293887

Compliance with ethical standards

Disclaimer The findings and conclusions in the article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors declare that they have not violated regulations of using the vulnerable species *Afzelia bipindensis* and *A. pachyloba* in the study.

The authors have obtained the approval of the U.S. Fish and Wildlife Service in the USA, the Forestry and Forest Products Research Institute in Japan, and the Royal Museum for Central Africa in Belgium for using wood samples of *Afzelia* in this study.

Consent for publication All the authors gave their informed consent to this publication and its content.

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