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Interpretation and implementation of the Convention

Trade controls and marking

Timber identification

METABOLIC CHEMOTYPES OF CITES PROTECTED DALBERGIA TIMBERS FROM AFRICA, MADAGASCAR, AND ASIA

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Metabolic chemotypes of CITES protected *Dalbergia* timbers from Africa, Madagascar, and Asia

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RATIONALE: The genus *Dalbergia* includes approximately 250 species worldwide. Of these, 58 species are of economic importance and listed under CITES. Identification of illegal transnational timber trade is a challenge because logs or boards lack the typical descriptors used for species identification such as leaves and flowers; therefore, frequently the lowest taxonomic determination of these tree byproducts is genus. In this study, we explore the use of Direct Analysis in Real Time (DART) Time-Of-Flight Mass Spectrometry (TOFMS) in making species determinations of protected *Dalbergia* trees from Africa, Madagascar, and Asia.

METHODS: Metabolic profiles were collected using DART TOFMS from the heartwood of seven species and the sapwood of 17 species of *Dalbergia*. Also included in this study are 85 *Dalbergia* heartwood samples from Madagascar that were only identified to genus. In all, 21 species comprising 235 specimens were analyzed, the metabolic chemotypes were interpreted, and the spectra were analyzed using chemometric tools.

RESULTS: *Dalbergia cochinchinensis* and *Dalbergia* spp. from Madagascar (both CITES Appendix II) could be differentiated from each other and from the non-protected *Dalbergia latifolia* and *Dalbergia melanoxylon*.

CONCLUSIONS: DART TOFMS is a valuable high-throughput tool useful for making phytochemical classifications of *Dalbergia* spp. The data produced allows the protected Dalbergias from Madagascar to be distinguished and can differentiate closely related rosewood trees. Published in 2015. This article is a U.S. Government work and is in the public domain in the USA.

The genus *Dalbergia* (Leguminosae) contains approximately 250 tree species distributed worldwide around the tropics.[1,2] The wood of these taxa has a characteristic color and texture that makes it highly desirable, and they are referred to by the common name of rosewood. Historically, it has been used as a luxury wood to decorate European palaces and is still used to manufacture exclusive furniture and cabinetry. The increasing demand for rosewood is making illegal logging so lucrative that, despite the risks the loggers face, *Dalbergia* forests in Madagascar and Indochina are being decimated.[3] Between 2011 and 2013 over 90 illegal loggers were killed in Thailand by local authorities.[4] In September of 2014, 200 tons of *Dalbergia cochinchinensis* from Indochina was selling for $5.9 million US dollars.[5] The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)[6] lists 58 species of protected *Dalbergia*, of which 51 are endemic to Madagascar, one is from Indochina and the remaining six are from Central and South America.

The National Fish and Wildlife Forensics Laboratory of the U.S. Fish and Wildlife Service regularly receives requests to identify species of imported wood suspected of being in violation of CITES. Use of wood anatomical characters is the traditional means of identifying samples of timber. However, traditional methods do not work well for the separation of *Dalbergia* species because many of them have the same wood anatomy and the process of preparing thin sections for microscopic analysis is lengthy and difficult because of the hardness of the wood.[7] Wood anatomists are developing new ways to identify CITES listed timber species by using a 10× hand lens and online databases such as CITESwoodID[8] and InsideWood.[9] In cases where the evidence consists of shipping containers holding hundreds of logs or boards, the processing time of traditional wood anatomy identifications is an impediment for law enforcement prosecutions.

As early as 1963, Swain proposed that the chemical distribution of polyphenols extracted from heartwoods could be used to characterize family, genus or even species of plants.[10] This idea was extended by Hillis who reported the occurrence of polyphenols, lignans, stillbenoids, flavonoids and quinones, and other phytochemicals in sapwood and heartwood from many different trees.[11] However, it was not until the advent of modern mass spectrometers that this hypothesis could be tested in a practical manner. In 2013, Montero-Vargas *et al*. reported the classification of *Coffea* spp. trees by metabolic profiles collected on a direct-injection electrospray mass spectrometer and the data analysed by hierarchical cluster analysis.[12]

A new advance in wood metabolic profiling is the use of an open-air atmospheric ionization tool called Direct Analysis in Real Time (DART) Time-Of-Flight Mass Spectrometry (TOFMS). A detailed description of the scientific principles

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of DART TOFMS has been given by Cody *et al*.[13–15] DART TOFMS is particularly useful for analyzing solid materials; a thin wood specimen can be analyzed directly and the resulting spectrum shows all the phytochemicals extracted from the sample. DART TOFMS metabolic profiles, previously used to classify two species of American oak (*Quercus* spp.)[16] as well as four species of agarwood (*Aquilaria* spp.),[17,18] have been shown to be useful in determining species source of heartwood samples of *Dalbergias* from the Americas.[19,20]

Here we report on the application of DART TOFMS to *Dalbergia* timber identifications for species endemic to Africa, Madagascar, and Asia. Rosewoods (*Dalbergia* spp.) from Madagascar are in extraordinary demand.[21] A recent seizure in Singapore consisted of more than 29,000 rosewood logs (3000 tons of timber) suspected to have originated from Madagascar.[22]

Species determination of *Dalbergia* heartwood from Madagascar is hindered by the fact that there are no reference collections in the world that have all 51 Madagascar species. Additionally, in S.E. Asia, timber shipments are routinely mixed, so it is important for law enforcement to be able to distinguish illegal from legal timber and do so in a timely manner.

DART TOFMS metabolic chemotypes in conjunction with chemometric analysis can help the wood analyst to make unambiguous species determinations and can assist in mitigating the illegal timber trade of endangered trees.

EXPERIMENTAL

Specimens

Twenty-one species of *Dalbergia* from Africa, Madagascar and Asia were collected and included in this study are 85 *Dalbergia* heartwood samples from Madagascar that were only identified to genus. The sample size and scientific name with the appropriate authorities are listed in Table 1. The

Table 1. Reference samples and their source

Provenance	Species	CITES	Sapwood n	Heartwood n	Origin	Source
Africa	*Dalbergia melanoxylon* Guill. & Perr.	none		20	Tanzania	EIEH
Asia	*Dalbergia cochinchinensis* Pierre	App II		20	Laos	CW
				7	Thailand	EIEH
	Dalbergia latifolia Roxb.	none		20	India	
				20	Malaysia	EIEH
Madagascar	*Dalbergia baronii* Baker	App II		10		GW
	Dalbergia bathiei R.Vig.	App II	1		Fenoarivo Atsinanana	WSLI
	Dalbergia bracteolate Baker	App II	1		Morondava	WSLI
			1		Ambilobe	
	Dalbergia chapelieri Baill.	App II	1		Foulpointe	WSLI
			1		Morondava	
	Dalbergia greveana Baill.	App II	1		Ambilobe	WSLI
			1		Marovoay	
			1		Maevatanana	
	Dalbergia lemurica Bosser & R.Rabev.	App II	1		Morondava	WSLI
			1		Antsiranana	
	Dalbergia louvelii R.Vig.	App II	1		Mahabo	WSLI
	Dalbergia madagascariensis Vatke.	App II	1		Toamasina	WSLI
			1		Toamasina II	WSLI
			1		Antsiranana	WSLI
				20	Unknown	EIEH
	Dalbergia maritime R.Vig.	App II	1		Makirovana	WSLI
				3	unknown	GW
	Dalbergia mollis Bosser & R.Rabev.	App II	2		Marovoay	WSLI
	Dalbergia monticola Bosser & R.Rabev.	App II	1		Toamasina II	WSLI
	Dalbergia orientalis Bosser & R.Rabev.	App II	1		Toamasina	WSLI
			1		Sahamahitsy	
	Dalbergia pervillei Vatke.	App II	1		Ambilobe	WSLI
			1		Antsiranana	
	Dalbergia purpurascens Baill.	App II	1		Ambilobe	WSLI
			1		Antsiranana	
			1		Marovoay	
	Dalbergia suaresensis Baill.	App II	2		Antsiranana	WSLI
	Dalbergia trichocarpa Baker	App II	1		Marovoay	WSLI
	Dalbergia tricolor Drake	App II	1		Morondava	WSLI
	Dalbergia viguieri Bosser & R.Rabev.	App II	1		Toamasina	WSLI
	Dalbergia spp.	App II		85	unknown	OLE

Source code is described in the Experimental section.

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species were chosen because of their CITES protection or their commercial importance. In total, 235 specimens were obtained. The source of the specimens are: Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA (EIEH); Cook Woods, Klamath Falls, OR, USA (CW); Gilmer Wood Co., Portland, OR, USA (GW); Harisoa Ravaomanalina, ETH Zürich, Plant Ecological Genetics, Institute of Integrative Biology, Department of Environmental Sciences, Zurich, Switzerland (WSLI); National Fish and Wildlife Forensic Lab (OLE). The species sources of commercial wood samples were verified by keying out diagnostic macroscopic anatomical characters using a 10× hand lens and the databases found at CITESwoodID[8] for CITES listed species and InsideWood[9] for non-protected taxa.

Chemical methods

All of the samples were analyzed directly by DART-TOFMS by holding a wood sliver in the gas stream with no further sample preparation. Reference samples were run randomly, when received, since the collection of these rare specimens spanned over 5 years. A mass calibration standard of poly (ethylene glycol) 600 (Ultra, Kingstown, RI, USA) was run between every fifth sample. Mass spectra were acquired using a DART-SVP ion source (IonSense, Saugus, MA, USA) coupled to a JEOL AccuTOF time-of-flight mass spectrometer (JEOL USA, Peabody, MA, USA) in positive ion mode. The DART source parameters were: electrode 1 voltage, 150 V; electrode 2 voltage, 250 V; and gas heater temperature at 450 °C. The mass spectrometer settings included: ring lens voltage, 5 V; orifice 1 voltage, 20 V; orifice 2, 5 V; cone temperature, 120 °C; peaks voltage, 600 V; bias, 28 V; focus voltage, -120 V; reflectron voltage, 870 V; pusher voltage, 778 V; pulling voltage, -778 V; suppression voltage, 0.00 V; flight tube voltage, -7000 V; and detector voltage, 2300 V. Spectra were obtained over the mass range of m/z 60 to 1000 at 1 scan per second. The helium flow rate for the DART source was 2.0 mL s^{-1}. The resolving power of the mass spectrometer, as stated by the manufacturer, was 2.0 mDa.[17–19]

For accurate mass determinations, TSSPro3 (Shrader Analytical Labs, Detroit, MI, USA) data processing software was used relying on the centroided mass spectra for molecular formula determination. Heat-maps of the data spectra were created using the Mass Mountaineer software (RBC Software, Peabody, MA, USA). Kernel Discriminant Analysis (KDA) was performed using selected diagnostic ions at a tolerance of 5 mDa. KDA standard deviation was set at 150, and the KDA graph of the results was produced using K Means Clustering.

KDA is a supervised learning algorithm that relies on assigning each sample in a dataset to a class. The KDA algorithm then calculates the greatest separation between classes in a training set and maps these features into a non-linear higher-dimensional space. This allows points that cannot be linearly separated in a two-dimensional space to be separated.[23]

Model accuracy was assessed using ten specimens of *Dalbergia* from Madagascar that had not been used in the training and by leave-one-out cross-validation (LOOCV). LOOCV is an algorithmic process where each sample in the data set is successively removed from the training data and treated as an unknown. The software then assigns it to a class within the training dataset. An LOOCV of 100% indicates that every sample was correctly assigned.

RESULTS

Figure 1 shows a representative heartwood spectrum for each of the *Dalbergia* species tested and shows that they have different chemical profiles. Each peak in a spectrum corresponds to an ionized molecule in the wood sample. The exact molecular masses of the ions were compared against the KNApSAcK database[24] and a tentative assignment of the chemical metabolites could be inferred (data not shown).

Figure 2 is the heat-map of the mass spectral results of all the samples tested. The Y axis row corresponds to each sample, and the X axis is the molecular mass for the ions detected. The intensity of the color in a column is correlated to the quantity of the molecule in a spectrum; an intense color indicates a high concentration whereas a pale intensity indicates a lower concentration or ionization suppression effects. The heat-map demonstrates that the metabolic chemotypes of *D. melanoxylon*, *D. cochinchinensis*, and *D. latifolia* are consistent within species and are differentiable for the spectra of the other species tested.

The results of the specimens from Madagascar are more complex because of (1) small sample size for most of the species (n ≤3), (2) the presence of sapwood specimens, and (3) the large number (51) of CITES protected species from Madagascar. Figure 1(E) shows an example of a spectrum of *D. madagascariensis* and Fig. 1(D) is an example of a spectrum from a collection of 85 logs known to have Madagascar origin. This sample set is identified as "*Dalbergia* heartwood – Madagascar" in Fig. 2. It is significant to point out that the *D. madagascariensis* chemotype is different from the large sample set of logs, therefore implying that the species of these samples is absent in the reference database. For the three species for which we had significant sample size of heartwood (*D. madagascariensis*, *D. baronii*, *D. maritima*, and *Dalbergia* spp.) the heat-map shows a metabolic chemotype different than the sapwood samples.

Of the 18 sapwood samples obtained from Madagascar (See Table 1), 15 had fewer than three specimens. The sapwood samples are identified in the heat-map (Fig. 2) as "*Dalbergia* sapwood – Madagascar". It is readily apparent from the heat-map that the sapwood metabolic chemotypes are similar within the species tested, but also very distinct from the heartwood chemotypes because they lack the most prominent molecules detected in heartwood.

Chemometric analysis

The mass spectra of the African, Madagascar, and Asian samples were also analyzed by KDA; the results of the analysis are graphically shown in Fig. 3. Because of the number of species from Madagascar and the differing chemotypes between heartwood and sapwood, the samples from Madagascar were classified into two groups and labeled as "*D* spp. sapwood from Madagascar"and "*D* spp. heartwood from Madagascar". This supervised classification analysis demonstrates that the cluster of Madagascar specimens can be differentiated from the three other species. Clear separation was shown with 97.2% classification accuracy using LOOCV.

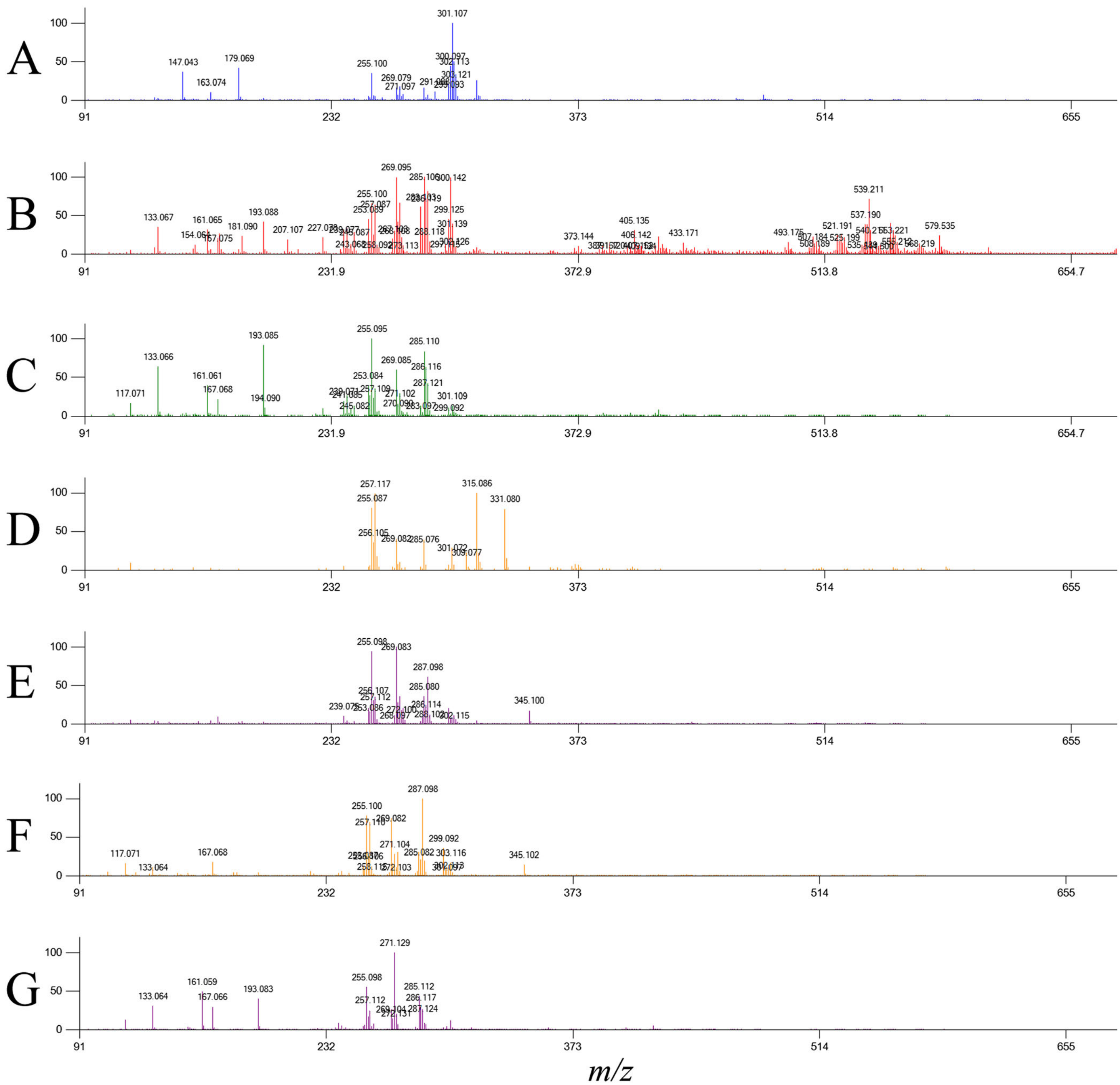

Figure 1. Representative spectra of *Dalbergia* heartwood: (A) *D. melanoxylon*, (B) *D. cochinchinensis*, (C) *D. latifolia*, (D) *Dalbergia* spp. from Madagascar, (E) *D. madagascariensis*, (F) *D. baronii*, and (G) *D. maritime*.

The KDA model also correctly identified the species source of ten heartwood specimens of *Dalbergia* spp. from Madagascar that had not been used in training the KDA model. Mass Mountaineer software assigned each of the unclassified spectra to the heartwood samples from Madagascar with probability estimates ranging from 76.6% to 99.99%. These samples are shown as the unclassified black dots in Fig. 3.

DISCUSSION

The evidence associated with the illegal trade of endangered trees is logs and timbers, and therefore species identification has to be determined from the heartwood alone. Under these circumstances, most wood anatomists are prepared to identify a wood sample to the genus level but not to species.[7]

The DART TOFMS metabolic chemotypes associated with *D. melanoxylon*, *D. cochinchinensis*, and *D. latifolia* specimens from Africa and Asia are unique, differentiable, and give robust information to make species determinations (Figs. 2 and 3).

From the analysis of the samples from Madagascar several inferences can be made: (1) the spectrum of the sapwood specimens is different from the heartwood (Fig. 2). This is not surprising since Hillis[11] reported that extractive concentrations in different tissues are unpredictable and not systematic or consistent across

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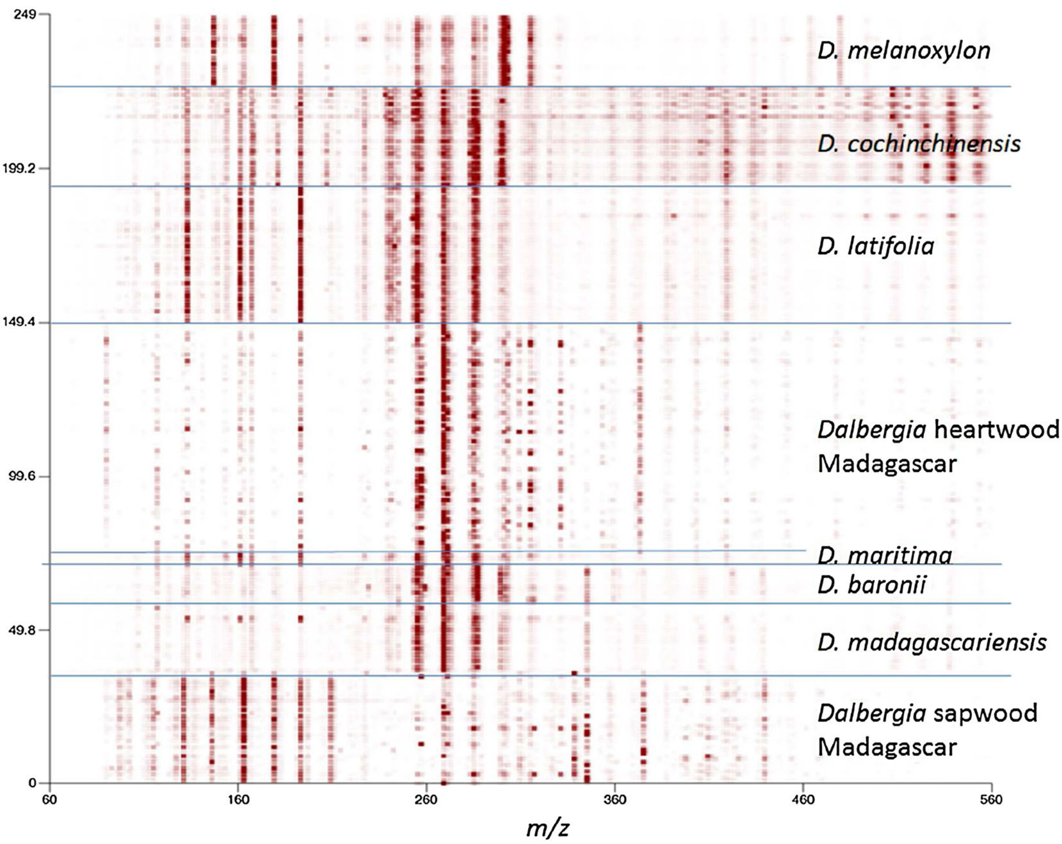

Figure 2. Heat-map of African and Asian *Dalbergia* species. The x axis is the molecular mass of compounds and the y axis is the sample number.

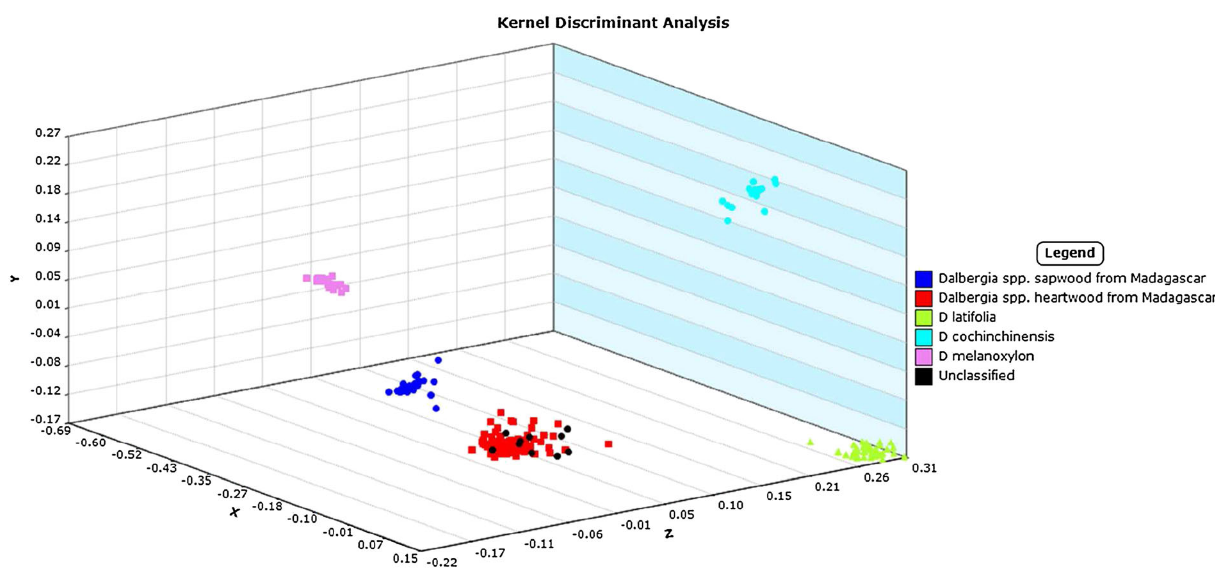

Figure 3. Kernel Discriminant Analysis of two African and two Asian *Dalbergia* species (LOOCV = 97.2%). Chemometric analysis was only conducted on taxa for which we had significant sample sizes.

species. However, in the case of illegal timber trade, this is not a problem because commercial wood is generally heartwood; (2) all the Madagascar sapwood samples (n = 37) have similar chemotypes regardless of species; (3) all the Madagascar heartwood samples (n = 122) have similar metabolic chemotypes regardless of species; (4) the chemotypes of the Madagascar heartwood specimens are distinct from *D. melanoxylon*, *D. cochinchinensis*, and *D. latifolia* (Fig. 2).

Anatomical descriptions for selected *Dalbergia* species have been documented and can be found in CITESwoodID[8] and InsideWood[25] but none of these databases has descriptions for all of the 51 Madagascar species. Given that all *Dalbergia* from Madagascar are protected, a classification based on metabolic profiles of phytochemicals using DART TOFMS and chemometric analysis can assist in determining the geographic provenance of Madagascar *Dalbergia* timber.

In summary, the heat-map graph and the KDA analysis demonstrate that DART TOFMS data is useful for inferring geographical provenance of *Dalbergia* spp. from Madagascar and in making species determinations in the case of *Dalbergia melanoxylon*, *Dalbergia cochinchinensis*, and *Dalbergia latifolia*. The analysis of wood by DART TOFMS for law enforcement purposes is a convenient tool to examine large numbers of specimens in a short period of time, and its use can supersede or augment traditional wood anatomical methods for making species identifications.

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