Phytochemistry 160 (2019) 61–70

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Volatile monoterpene 'fingerprints' of resinous *Protium* tree species in the Amazon rainforest

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ARTICLE INFO

Keywords:
Protium spp. (Burseraceae)
Tropical tree identification
Chemotaxonomy
Resins
Volatile organic compounds
Hyperdominant genus
Isoprenoids

ABSTRACT

Volatile terpenoid resins represent a diverse group of plant defense chemicals involved in defense against herbivory, abiotic stress, and communication. However, their composition in tropical forests remains poorly characterized. As a part of tree identification, the 'smell' of damaged trunks is widely used, but is highly subjective. Here, we analyzed trunk volatile monoterpene emissions from 15 species of the genus *Protium* in the central Amazon. By normalizing the abundances of 28 monoterpenes, 9 monoterpene 'fingerprint' patterns emerged, characterized by a distinct dominant monoterpene. While 4 of the 'fingerprint' patterns were composed of multiple species, 5 were composed of a single species. Moreover, among individuals of the same species, 6 species had a single 'fingerprint' pattern, while 9 species had two or more 'fingerprint' patterns among individuals. A comparison of 'fingerprints' between 2015 and 2017 from 15 individuals generally showed excellent agreement, demonstrating a strong dependence on species identity, but not time of collection. The results are consistent with a previous study that found multiple divergent copies of monoterpene synthase enzymes in *Protium*. We conclude that the monoterpene 'fingerprint' database has important implications for constraining *Protium* species identification and phylogenetic relationships and enhancing understanding of physiological and ecological functions of resins and their potential commercial applications.

1. Introduction

Tree resins consist of a mixture of defense chemicals, including terpenoids (Langenheim, 2003), that play major roles in tropical tree physiology, ecology and evolution (Zulak and Bohlmann, 2010), and are used in a variety of commercial products (Daly et al., 2010; Stacey et al., 2006). Terpenoid resins are synthesized within plastids of epithelial cells which surround resin ducts (Klock et al., 2005; Trapp and Croteau, 2001), and are highly abundant in vascular tissues (i.e. xylem and phloem) (Langenheim, 2003).

In the tropics it is estimated that about 10% of tree families synthesize resins (Langenheim, 1990). The Burseraceae family, widespread in tropical and subtropical regions, includes tree species characterized by high resin content in the trunks, and consists of approximately 750 species distributed in 19 genera (Correia, 1984; Daly et al., 2012). Resinous trees belonging to the Burseraceae family play an important role in the diversity and structure of tropical forests, representing 10–14% of species richness (Daly et al., 2012). Globally, the *Protium* genus is the most abundant of the Burseraceae family (Daly and Fine, 2011) and the second most hyperdominant genus in the Amazon (Ter Steege et al., 2013).

Terpenoid resins are dominated by volatile monoterpenes ($C_{10}H_{16}$) which have been shown to play important roles in ecological interactions within forested ecosystems including defense against natural enemies (Erbilgin et al., 2003). When submitted to mechanical damage by attacking herbivores and microbes, volatile monoterpenes in

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https://doi.org/10.1016/j.phytochem.2019.01.014
Received 3 July 2018; Received in revised form 18 January 2019; Accepted 24 January 2019
Available online 01 February 2019

exposed resin ducts are emitted to the atmosphere and play key roles in direct physical and chemical defenses (Phillips and Croteau, 1999). In addition, monoterpene resins are involved in indirect defenses through recruitment of herbivore predators and pheromone attraction/repelling of herbivores (Erbilgin and Raffa, 2001; Reddy and Guerrero, 2004).

By mediating plant-herbivore interactions, terpenoid resins are recognized as a critical factor in plant defense evolution (Langenheim, 2003; Phillips and Croteau, 1999). Thus, the development of a secretory canal system by plants is a result of the coevolution between plants and herbivores, and has been considered an evolutionary mechanism to spur plant diversification (Farrell et al., 1991). In addition, terpenoid resins represent an important economic component to society through its widespread use in medicine, cosmetics, biofuels, and solvents (Langenheim, 1990; Zulak and Bohlmann, 2010). For example, the usage of terpenoid resins extracted from frankincense and myrrh trees (both from the Burseraceae family) have been used in the treatment of several bacterial illnesses and diseases like cancer (Zhang et al., 2016).

The scent of tree resins is widely used to aid in the identification of tropical tree species (Ribeiro et al., 1999), where classic leaf taxonomic identification remains extremely challenging (Hopkins, 2007). This challenge primarily exists due to the high tree species diversity (e.g. 6727 in the Amazon (Cardoso et al., 2017)) and difficulties in canopy access for collecting plant vegetative and reproductive tissues (Hopkins, 2007). Although distinct scents from damaged trunks (e.g. cinnamon, sweet and spicy) have been empirically linked to specific species, this method remains highly subjective and with poor precision, because it depends on personal capacity to recognize and characterize each scent (Ribeiro et al., 1999). To address this problem, analytical laboratory techniques for identification of monoterpenes have been developed, including Gas Chromatography-Mass Spectrometry (GC-MS), and applied in temperate (Constable et al., 1999; Katoh and Croteau, 1998; Niogret et al., 2013) and tropical regions (Courtois et al., 2009, 2012). Current methods to evaluate monoterpene profiles are based on destructive bark sampling and require large air sample volumes (tens of liters) (Ortega and Helmig, 2008; Ortega et al., 2008). In addition, while high chemical diversity of VOC (Volatile Organic Compounds) emission from the bark of tropical species has been previously reported, there is little research on the volatile compounds responsible for the scent of tree resins from tropical species (Courtois et al., 2009, 2012). Courtois et al. (2009) showed that volatiles could be used to identify tropical tree species. Following up on this work, Courtois et al. (2012) showed that bark had a more distinctive and diverse blend of terpenes than did the leaves for most species. However, in the literature there are only a few publications on the characterization of *Protium* volatile profiles and, in most cases, previous studies were carried out on one or few species of this genus (Courtois et al., 2009, 2012). Therefore, a systematic study to characterize monoterpene bark composition in trunks across abundant and diverse tropical tree genera is lacking, in part by the lack of a simple and rapid in situ volatile collection method that avoids destructive sampling.

In this study, we aimed to develop and apply a new TD (Thermal Desorption) GC-MS (Gas Chromatography-Mass Spectrometry) methodology which allows for rapid in situ volatile collections of small volume (300 ml) headspace air samples from damaged tree trunks. Our primary objective was to provide the first systematic collection and analysis of volatile monoterpene compositions of tree trunk resins from 15 tree species among 77 individuals belonging to the genus *Protium* in an established forest transect in the central Amazon. We hypothesized that by normalizing the abundances of 28 monoterpenes in each individual, a unique *Protium* monoterpene 'fingerprint' pattern exists for each of the 15 species. We also hypothesized that relative to a strong dependence on species, a low dependence on time will be observed with *Protium* monoterpene 'fingerprints' from the same individual in 2015 similar to those in 2017. The results are discussed in terms of the implications for *Protium* tree species identification and physiological and ecological functions, evolutionary histories, and commercial applications of resins.

2. Results

2.1. Tree resin monoterpene emission composition

Across 15 species of *Protium*, 28 monoterpenes were identified in the 77 tree individuals analyzed (supporting information Table S1). They are: cyclofenchene, ξ-fenchene, β-thujene, tricyclene, α-pinene, α-fenchene, *d*-camphene, camphene, 5,5-dimethyl-1-propyl-1,3-cyclopentadiene, sabinene, β-myrcene, β-pinene, 2-carene, α-phellandrene, 3-carene, α-terpinene, trans-β-ocimene, *d*-limonene, β-phellandrene, cis-β-ocimene, eucalyptol, γ-terpinene, p-mentha-3,8-diene, α-terpinolene, isoterpinolene, 4-trans-6-cis-allocimene, terpenin-4-ol, and α-terpineol (Monoterpenes 1–28, respectively. Monoterpenes abbreviation: MTP).

2.2. Dominant monoterpene 'fingerprint' patterns

Following the analysis of resin monoterpene emissions collected from 77 individuals across 15 *Protium* species (see supporting information Table S2), monoterpene 'fingerprints' were generated by normalizing the relative abundances of 28 monoterpenes to the dominant monoterpene present. The results of this analysis revealed 9 monoterpene 'fingerprint' patterns, each one characterized by a distinct dominant monoterpene (Fig. 1), that included α-pinene, α-phellandrene, *d*-limonene, α-terpinolene, β-phellandrene, 3-carene, sabinene, camphene and β-myrcene.

In Figs. 2–4, representative monoterpene 'fingerprints' from *Protium* individuals for each of the 9 dominant 'fingerprint' patterns are shown, demonstrating the distinct monoterpene patterns encountered among species. Across the 15 species and 77 *Protium* individuals characterized for monoterpene 'fingerprints', α-pinene was the most common dominant monoterpene occurring in the majority of the individuals (44.2%), followed by α-phellandrene (23.4%), *d*-limonene (16.9%), and α-terpinolene (7.8%). In contrast, β-phellandrene, 3-carene, sabinene, camphene, and β-myrcene were much less common as dominant monoterpenes with an occurrence among individuals of 2.6%, 1.3%, 1.3%, 1.3%, and 1.3% respectively.

While 4 of the 'fingerprint' patterns were composed of multiple species, 5 were unique to single species, including *P. opacum* (β-phellandrene), *P. heptaphyllum* subsp. *ulei* (3-carene), *P. nitidifolium* (sabinene), *P. heptaphyllum* subsp. *ulei* (camphene) and *P. amazonicum* (β-myrcene). Moreover, among individuals of the same species, 6 species had a single 'fingerprint' pattern including *P. calendulinum, P. neglectum* and *P. opacum* subsp. *opacum* (α-pinene), *P. paniculatum* var. *paniculatum* and *P. paniculatum* var. *modestum* (α-phellandrene), and *P. paniculatum* var. *riedelianum* (α-terpinolene). In contrast, 9 species had two or more 'fingerprint' patterns among individuals including *P. amazonicum* (α-pinene, β-myrcene), *P. apiculatum* (α-pinene, *d*-limonene), *P. decandrum* (α-pinene, α-phellandrene), *P. hebetatum* var. 1 (α-pinene, α-phellandrene, *d*-limonene), *P. hebetatum* var. 2 [1] (α-pinene, α-phellandrene, *d*-limonene, α-terpinolene), *P. heptaphyllum* subsp. *ulei* (3-carene, camphene), *P. nitidifolium* (*d*-limonene, sabinene), *P. opacum* (α-pinene, β-phellandrene), and *P. strumosum* (α-pinene, α-terpinolene).

Therefore, although the same dominant monoterpene pattern could be observed among individuals in a single species (e.g. all 5 *P. calendulinum* individuals presented α-pinene as the dominant monoterpene pattern; all 7 *P. paniculatum* var. *modestum* individuals

[1] *Protium hebetatum* is known to represent two different morphotypes, which represent two species. While *hebetatum* var. 2 has shorter lateral petiolules, usually reddish in fresh leaves, usually pilose on rachis, lateral and terminal petiolules, *hebetatum* var. 1 has oblong leaflets, longer lateral petiolules, usually glabrous on rachis, lateral and terminal petiolules.

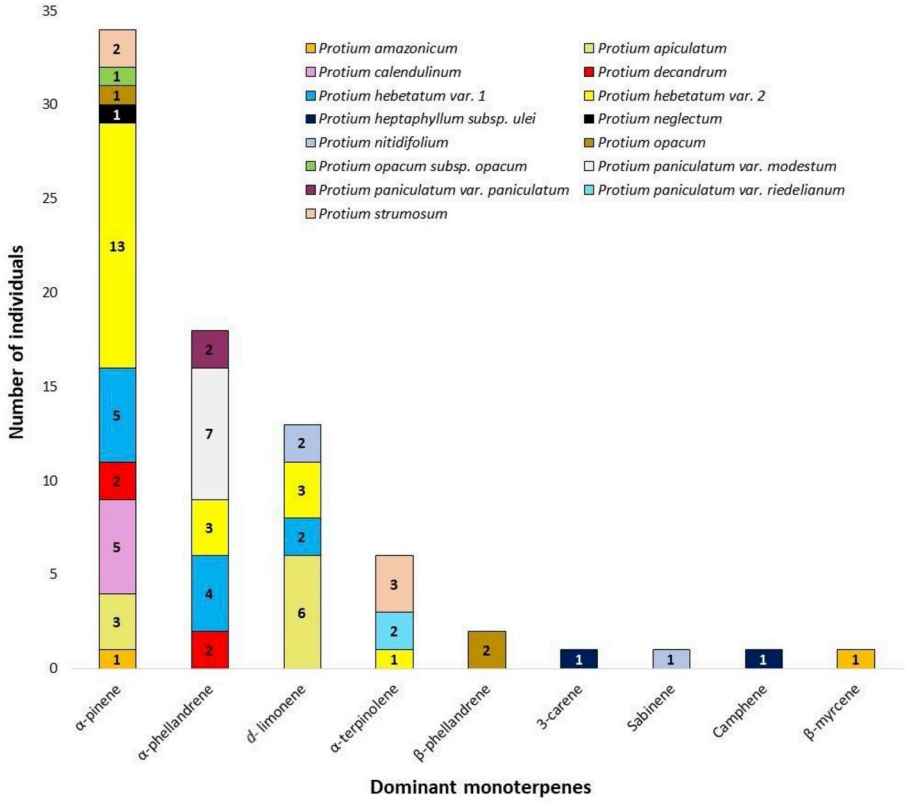

Fig. 1. Number of 'fingerprints' from each species of *Protium* studied organized according to the dominant monoterpene emitted from resins.

presented α-phellandrene as the dominant monoterpene pattern), two or more dominant monoterpene patterns were found in others (e.g. among 20 *P. hebetatum* var. 2 individuals, four different dominant monoterpene patterns were observed). Nonetheless, among species with multiple dominant monoterpene patterns among individuals, certain monoterpene patterns were more frequently observed. In *P. hebatatum* var. 2, for example, α-pinene was found to be the dominant monoterpene pattern in 13 out 20 individuals. However, for *P. hebetatum* var. 1, 5 out of 11 individuals had α-pinene as the dominant monoterpene pattern, 4 had α-phellandrene, and 2 had d-limonene. Out of 3 *P. nitidifolium* individuals, 2 presented *d*-limonene as dominant monoterpene pattern, and 1 presented sabinene.

2.3. Consistency of monoterpene 'fingerprints' collected in 2015 and 2017

Fifteen randomly chosen *Protium* individuals (tree ID 35, 39, 100, 277C, 306, 361, 385, 389, 391, 406, 435, 441, 492, 661, and 724) were used for repeat monoterpene 'fingerprint' analysis in 2017 of samples first collected in 2015. This permitted a test of the hypothesis that monoterpene 'fingerprints' are primarily determined by the genotype of the species, and do not vary substantially over time. In general, repeated monoterpene 'fingerprint' analysis of the same individual following the two-year period showed remarkable consistency (Fig. 5). As two examples, it is first noted that for tree 361 (*P. apiculatum*), α-pinene was the dominant monoterpene in 2015 as well as in 2017 (Fig. 5a). In addition, a similar relative abundance of other monoterpenes were observed in the repeated 'fingerprints' in 2015 and 2017, including β-thujene (10–40%), camphene (10–35%), sabinene (20–75%), α-terpinene (15–70%), *d*-limonene (30–65%), β-phellandrene (10–35%), γ-terpinene (18–58%), and α-terpinolene (10–100%). In a second example with tree 385 (*P. hebetatum* var. 2), *d*-limonene was observed as dominant monoterpene in both 2015 and 2017 (Fig. 5b), and similar relative abundance of other monoterpenes were observed in the repeated 'fingerprints' in 2015 and 2017 including α-pinene (15–45%) and α-phellandrene (5–80%). The tree 35 (*P. calendulinum*) showed α-pinene as the dominant monoterpene for collections in 2015 and 2017 and a very similar distribution of *d*-limonene (38–42%) and β-pinene (3–17%) (Fig. 5c).

In contrast, for tree 724 (*P. hebetatum* var. 2), the monoterpene 'fingerprints' between the years was less comparable (see supplementary File S3, α-pinene subfolder). In 2015, the dominant monoterpene was α-phellandrene, but in 2017, the dominant monoterpene was α-pinene. Nonetheless, the monoterpene 'fingerprints' between the both years presented similarities in the relative abundance of some monoterpenes including *d*-limonene (22–27%) and β-phellandrene (22–31%).

2.4. Comparison of Protium monoterpene 'fingerprints' between individuals

Of the 77 *Protium* individuals, we observed a consistency of *Protium* monoterpene 'fingerprints' that could be separated in two cases: a) similar monoterpene 'fingerprints' patterns between individuals of different species, b) similar monoterpene 'fingerprints' patterns between individuals of the same species. Graphical representations of example monoterpene 'fingerprints' showing consistency between individuals of different species (Figs. S2–S4) and the same species (Figs. S5–S6) is provided in the supporting information.

As an example of similar patterns between individuals from different species, the comparison of the monoterpene 'fingerprint' from *P. calendulinum* species (Tree 1408) and that of *P. hebetatum* var. 1 (Tree 277C) showed a strong consistency of monoterpene patterns (Figs. S2a and S2d). Both 'fingerprints' presented the same dominant monoterpene (α-pinene) and similar relative abundance for other monoterpenes, including camphene, β-pinene, and *d*-limonene (MTPs 8, 12 and 18, respectively).

In a second example, the monoterpene 'fingerprint' of a *P. paniculatum* var. *paniculatum* individual (Tree 1301) showed similarities with the 'fingerprint' of a second individual, from *P. paniculatum* var.

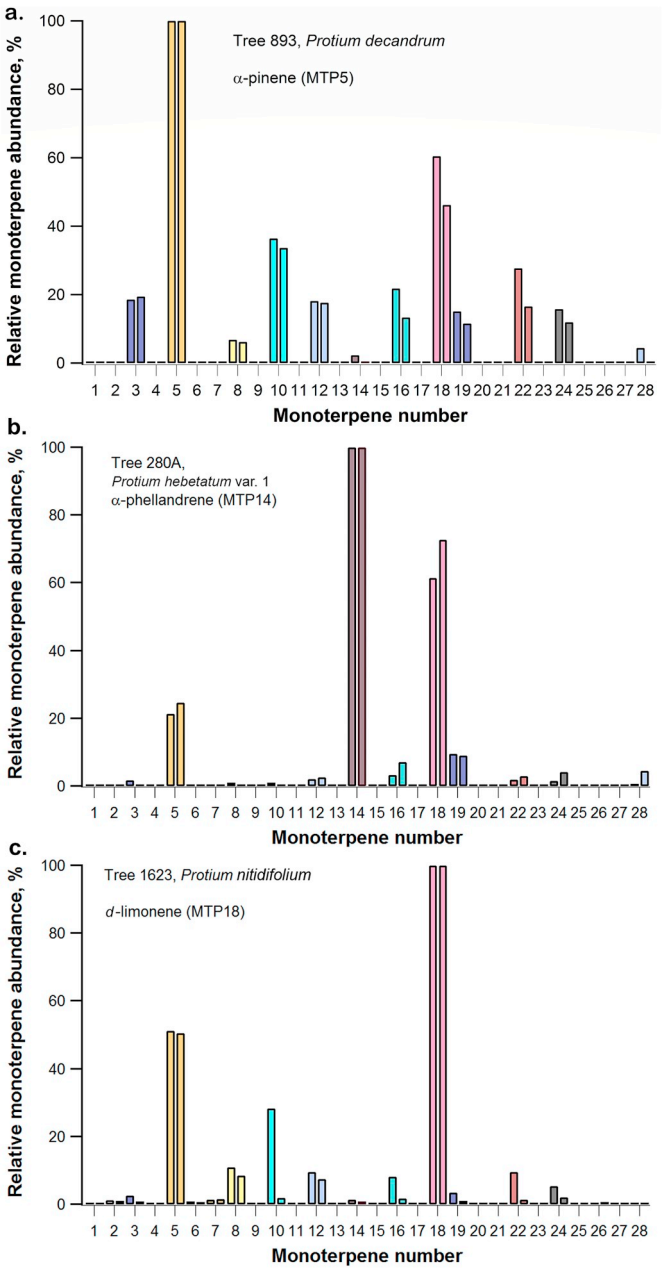

Fig. 2. Monoterpene 'fingerprints' from the trunks of three tree species in the central Amazon showing different dominant monoterpenes **a**) α-pinene, **b**) α-phellandrene, and **c)** *d*-limonene. Monoterpene number: 1. cyclofenchene, 2. ξ-fenchene, 3. β-thujene, 4. tricyclene, 5. α-pinene, 6. α-fenchene, 7. *d*-camphene, 8. camphene, 9. 5,5-dimethyl-1-propyl-1,3-cyclopentadiene, 10. sabinene, 11. β-myrcene, 12. β-pinene, 13. 2-carene, 14. α-phellandrene, 15. 3-carene, 16. α-terpinene, 17. trans-β-ocimene, 18. *d*-limonene, 19. β-phellandrene, 20. cis-β-ocimene, 21. eucalyptol, 22. γ-terpinene, 23. p-mentha-3,8-diene, 24. α-terpinolene, 25. isoterpinolene, 26. 4-trans-6-cis-allocimene, 27. terpenen-4-ol, 28. α-terpineol.

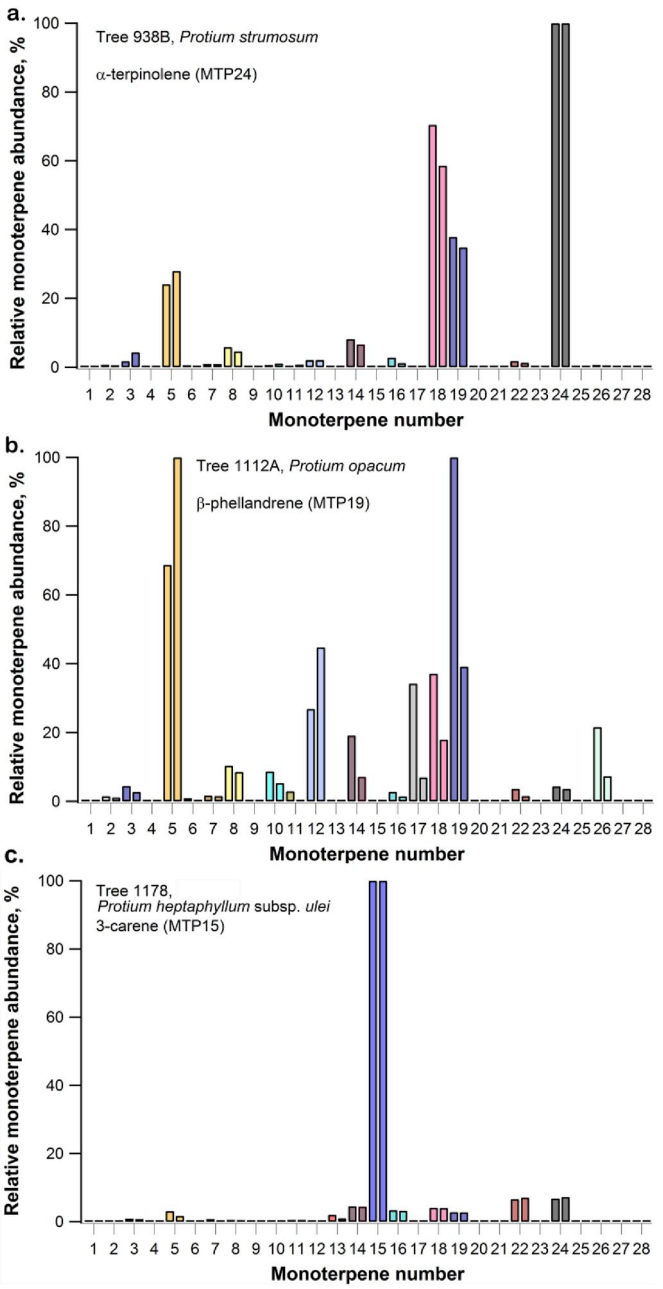

Fig. 3. Monoterpene 'fingerprints' from the trunks of three tree species in the central Amazon showing different dominant monoterpenes **a**) α-terpinolene, **b**) β-phellandrene, and **c**) 3-carene. Monoterpene number: 1. cyclofenchene, 2. ξ-fenchene, 3. β-thujene, 4. tricyclene, 5. α-pinene, 6. α-fenchene, 7. *d*-camphene, 8. camphene, 9. 5,5-dimethyl-1-propyl-1,3-cyclopentadiene, 10. sabinene, 11. β-myrcene, 12. β-pinene, 13. 2-carene, 14. α-phellandrene, 15. 3-carene, 16. α-terpinene, 17. trans-β-ocimene, 18. *d*-limonene, 19. β-phellandrene, 20. cis-β-ocimene, 21. eucalyptol, 22. γ-terpinene, 23. p-mentha-3,8-diene, 24. α-terpinolene, 25. isoterpinolene, 26. 4-trans-6-cis-allocimene, 27. terpenen-4-ol, 28. α-terpineol.

paniculatum species (Tree 157), with α-phellandrene as the dominant monoterpene in both cases (compare Fig. S2b with S2e). There was also a good similarity between the relative abundances of the monoterpenes α-terpinene, β-phellandrene, γ-terpinene, α-terpinolene and isoterpinolene (MTPs 16, 19, 22, 24 and 25, respectively). Another interesting example showed that the 'fingerprint' of a *P. apiculatum* (Tree 464) had a good similarity with the 'fingerprint' of a *P. nitidifolium* (Tree 334D) individual, with *d*-limonene as the dominant monoterpene (compare Fig. S2c with S2f). More examples of monoterpene 'fingerprints' similarities for individuals from different species can be observed in Figs. S3 and S4.

A comparison analysis of monoterpene 'fingerprints' with similar patterns between individuals from the same species was also conducted. Monoterpene 'fingerprints' from two individuals of *P. hebetatum* var. 2 (Trees 661 and 724) showed a consistency of monoterpene patterns (compare Fig. S5a with S5d), with α-pinene as the dominant monoterpene. Furthermore, for both 'fingerprints', the monoterpenes β-pinene, α-phellandrene and α-terpinene were detected. For another two

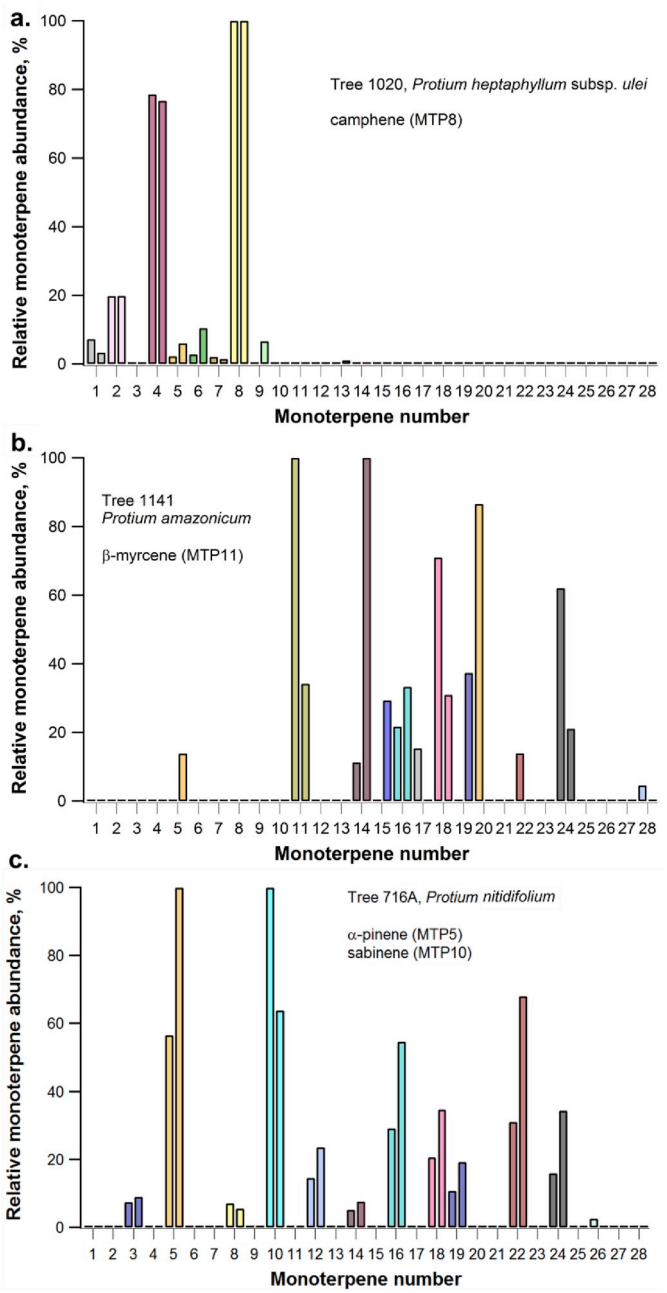

Fig. 4. Monoterpene 'fingerprints' from the trunks of three tree species in the central Amazon showing different dominant monoterpenes **a**) camphene, **b**) β-myrcene, and **c)** sabinene. Monoterpene number: 1. cyclofenchene, 2. ξ-fenchene, 3. β-thujene, 4. tricyclene, 5. α-pinene, 6. α-fenchene, 7. *d*-camphene, 8. camphene, 9. 5,5-dimethyl-1-propyl-1,3-cyclopentadiene, 10. sabinene, 11. β-myrcene, 12. β-pinene, 13. 2-carene, 14. α-phellandrene, 15. 3-carene, 16. α-terpinene, 17. trans-β-ocimene, 18. *d*-limonene, 19. β-phellandrene, 20. cis-β-ocimene, 21. eucalyptol, 22. γ-terpinene, 23. p-mentha-3,8-diene, 24. α-terpinolene, 25. isoterpinolene, 26. 4-trans-6-cis-allocimene, 27. terpenen-4-ol, 28. α-terpineol.

monoterpene 'fingerprints' (Trees 261 and 931B), also from *P. hebetatum* var. 2 species and with α-pinene as the dominant monoterpene, we can observe a consistency of monoterpenes occurrence and monoterpenes relative abundance (compare Fig. S5b with S5e). Other examples of similarities for 'fingerprints' from the same species are highlighted in Fig. S6 (a-f). In this figure, it is interesting to observe that four individuals from *P. paniculatum* var. *modestum* species presented strong similarities in their monoterpene 'fingerprints'. For example,

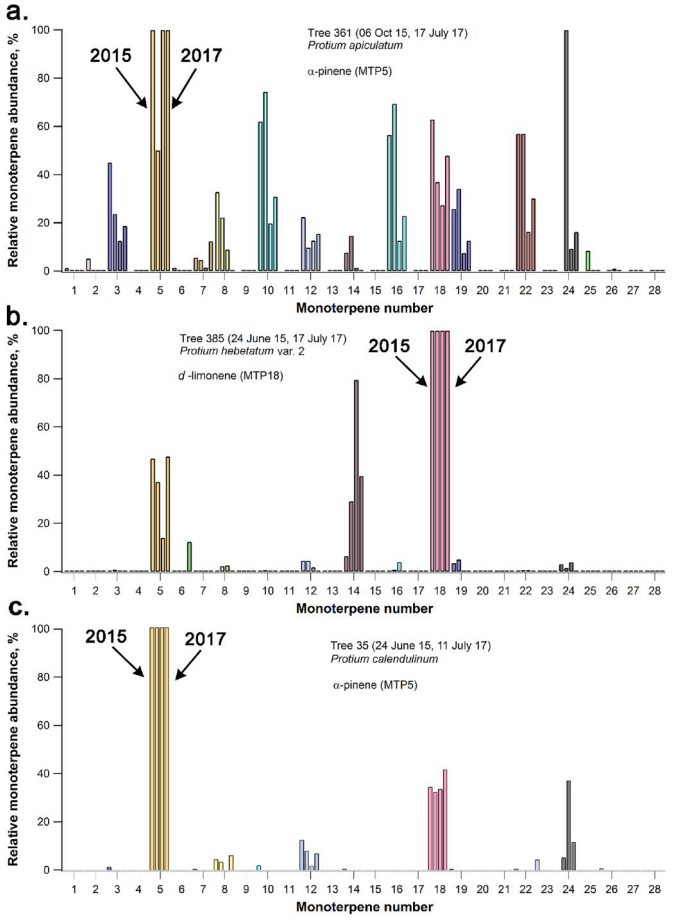

Fig. 5. Monoterpene 'fingerprints' from repeated collections in the years of 2015 and 2017, showing similar patterns with the same dominant monoterpenes observed **a**) α-pinene (*P. apiculatum*), **b**) *d*-limonene (*P. hebetatum* var. 2), and **c)** α-pinene (*P. calendulinum*). The first two vertical bars of each monoterpene represent the 2015 'fingerprint' and the 2 bars on the right represent the 2017 'fingerprint'. Monoterpene number: 1. cyclofenchene, 2. ξ-fenchene, 3. β-thujene, 4. tricyclene, 5. α-pinene, 6. α-fenchene, 7. *d*-camphene, 8. camphene, 9. 5,5-dimethyl-1-propyl-1,3-cyclopentadiene, 10. sabinene, 11. β-myrcene, 12. β-pinene, 13. 2-carene, 14. α-phellandrene, 15. 3-carene, 16. α-terpinene, 17. trans-β-ocimene, 18. *d*-limonene, 19. β-phellandrene, 20. cis-β-ocimene, 21. eucalyptol, 22. γ-terpinene, 23. p-mentha-3,8-diene, 24. α-terpinolene, 25. isoterpinolene, 26. 4-trans-6-cis-allocimene, 27. terpenen-4-ol, 28. α-terpineol.

both Trees 1289 and 1295 (Figs. S6a and S6b) presented the occurrence of monoterpenes 3-carene, α-terpinene, β-phellandrene, cis-β-ocimene and γ-terpinene, with strong relative abundance similarities of these monoterpenes. For these four 'fingerprints' from the same species, the same dominant monoterpene (α-phellandrene) was observed, as well.

3. Discussion

In this study, we present the first systematic study of monoterpene bark composition across an abundant genus (*Protium*) in a tropical ecosystem. Previous research on monoterpene bark composition was investigated in tree trunks and leaves of 195 individuals in French Guiana belonging to 55 species, including 4 *Protium* species (Courtois et al., 2009). α-pinene was the most common and present in 100% of species followed by *d*-limonene, 3-carene, β-myrcene, α-phellandrene, and camphene (present in 96%, 53%, 53%, 51%, and 47%, respectively of the species). The results are consistent with those in the present study on *Protium* terpenoid resins where we found α-pinene, *d*-limonene, and α-phellandrene as the most common monoterpenes to dominate stored

resin emissions in 77 species in the central Amazon, while 3-carene and β-myrcene were present as dominant monoterpenes in fewer species. As in the current study, Courtois et al. (2009) considered the important role of bark volatiles in characterizing tropical tree species, and found a high chemical diversity for species belonging to the Sapindales order, which includes the Burseraceae family, relative to other orders. Therefore, these results in French Guiana are consistent with our study in the Brazilian Amazon. A follow up study on monoterpenes provides evidence that bark of tropical species tend to have distinct and more diverse blend of volatile terpenes than leaves (Courtois et al., 2012). This more recent finding provides motivation for additional studies on the chemical composition of bark resins, and underlines the advantage of collecting samples from more accessible plant organs, as difficulties in canopy access represents an important constraint in the tropic (Hopkins, 2007).

In contrast to current methods to evaluate monoterpene profiles in tissue samples based on destructive bark sampling which require large air sample volumes (tens of liters) (Ortega and Helmig, 2008; Ortega et al., 2008), the sampling method developed and applied in this work was easily transferred into the field and proved to be simple, fast and convenient for the purpose of quantitative in-situ collection and characterization of the volatile monoterpene composition of trunk resins of different *Protium* species. This sampling method is based on a very short time of collection (timescale of minutes), and subsequent analysis by TD-GC-MS, followed by a simple normalization procedure to produce a library of monoterpene 'fingerprints' across 77 *Protium* individuals (supplementary File S1).

However, a number of limitations of our TD-GC-MS should be mentioned. First, a TD-GC-MS is an expensive and technical instrument, with TD sample analysis slow (one TD sample analyzed roughly every 45 min), and difficult to maintain in the tropics for long periods of time. However, the automated thermal desorption system employed allowed for all collected TD tube samples in the field (up to 100) to be analyzed sequentially without requiring user input. Near the end of the first year, a maintenance procedure of the MS required cutting a few cm off the end the column, and the column was replaced near the end of the second year. These procedures resulted in a slight shift (0.1–0.2 min) in the retention times of the monoterpenes, which required re-running analytical standards for compound identification verification. Regarding the calibration of the TD-GC-MS, most monoterpenes are unstable for long periods in gas cylinders, and so we instead applied an advanced method developed in our laboratory termed dynamic solution injection (DSI) using a custom monoterpene solution in methanol (Jardine et al., 2010). This calibration method is technical and few laboratories are set up to apply this method, which requires a nL-μL min^{-1} flow controlled liquid pump. Issues with identification of other monoterpenes not present in the standard stem from the fact that nearly all monoterpenes show a very similar mass spectra in conventional electron impact ion sources, making identification based solely on comparison with mass spectral libraries. Thus, the identification of monoterpenes not present in our liquid standard require future verification by the development of appropriate standards. Moreover, the GC-MS applied here is highly sensitive enabling on the one hand the analysis of very small air samples (300 mL). However, tree individuals with very high trunk resin content caused the molecular weight parent ion of monoterpenes (m/z 136) to saturate, requiring us to use the ion corresponding to the natural abundance of [$^{13}C_1$]monoterpenes (m/z 137) for analysis. Regarding sample collection onto TD tubes, the analysis of monoterpenes by TD-GC-MS was conducted within 1–2 days, but this not always possible in laboratories without a dedicated TD-GC-MS that may be utilized by multiple projects. Long storage time could potentially cause the monoterpenes to be lost and/or chemically transform. In addition, chemical transformations may have occurred during the heating steps associated with analysis by TD-GC-MS despite rigorously drying the samples under helium as a part of the automated analysis procedure. However, the use of inert sorbents in the TD tube likely minimized this possibility, but these potential transformations were not evaluated. In addition, atmospheric ozone could have been collected together with the monoterpenes and cause oxidation and loss during storage and analysis. However, ozone in the remote tropics is usually very low (< 40 ppb) and reaches a minimum near the ground (Jardine et al., 2011). Although low sample volumes (300 mL) collected near the ground likely minimized this potential artifact, this remains a limitation of the current methodology.

We present the concept of normalized monoterpene 'fingerprints', which we suggest can be used to help constrain the identity of unknown *Protium* species and therefore be used as a tool in *Protium* chemotaxonomy as a basis to address questions in ecology and evolution, physiology, and forest management (**Graphical Abstract**). It is important to note that the sampling strategy was not aimed at quantifying trunk monoterpene emission rates, and therefore require the use of a dynamic enclosure. Instead, a new rapid method is presented which normalizes the strong enhancement in monoterpene ambient concentrations in the headspace near a recently damaged trunk by the most abundant monoterpene to derive the 'monoterpene fingerprint'. Not only is this method simple and rapid, it is not dependent upon the absolute concentrations of the monoterpenes, which are rapidly diluted in the ambient air upon emissions which depends upon wind speeds and many other factors. In contrast, the relative abundance of each monoterpene in the ambient air in the trunk headspace reflects the composition of monoterpenes within the trunk resin. Repeated 'fingerprints' for the same individuals during 2015 and 2017 showed good agreement in most cases, suggesting that the monoterpene emission composition from the stored resins in *Protium* is highly sensitive to the individual and/or species studied and is less sensitive to annual variability.

We found that while 4 of the 'fingerprint' patterns were composed of multiple species, 5 were unique to single species. Thus, in some cases, individual *Protium* species were found to contain only a single monoterpene 'fingerprint' pattern that was only found in that species. However, the most common monterpene 'fingerprints' represented by α-pinene, α-phellandrene, *d*-limonene, and α-terpinolene, were present in 10, 5, 4, and 3 different species, respectively. When individuals of the same species with the same 'fingerprint' patterns were evaluated more closely, it was found that not only did they have the same dominant monoterpene, but also the relative abundance of the other monoterpenes present often compared well. This was also the case when individuals of different species with the same 'fingerprint' patterns were compared.

It should be noted that following the initial assignment of Protium species to individuals based on a previous forest transect survey, a re-analysis of the *Protium* taxonomy made by specialists on our team found that close to 50% of the species were incorrectly identified and several determined to be from other genera. Thus, uncertainty remains in the identifications from the remote Amazon field site, which was conducted using only images of scanned or photographed specimens rather than by specialists in the field.

Monoterpenes present in the resins are known to be produced by specific monoterpene synthase enzymes (Roach et al., 2014; Srividya et al., 2015). As 9 distinct monoterpene 'fingerprint' patterns emerged in our study, one possibility is that up to 9 distinct monoterpene synthase enzymes are present in the *Protium* genus, each producing a given blend of resin monoterpenes with a bias toward a particular monoterpene product. As 5 of the 'fingerprint' patterns were found to be unique to single species, one possibility is that each of these species contains a unique monoterpene synthase, not found in other *Protium* species. However, as the other 4 'fingerprint' patterns were found in three or more species, one possibility is that the same monoterpene synthase enzyme may be present in multiple species. Moreover, while 6 of the 15 *Protium* species had a single type of monoterpene 'fingerprint' pattern among individuals, 9 of the species had two or more monoterpene 'fingerprint' patterns occurring among individuals. This could be due to the potential that more than one monoterpene synthase

enzyme may be present among individuals of a single species. These possibilities are consistent with a previous study that reconstructed the phylogeny of the monoterpene synthase gene family (TPSb) for the *Protium* genus (Zapata and Fine, 2013). In this study, evidence was presented for one ancient and multiple more recent duplication events giving rise to roughly five copies of TPSb genes currently present in *Protium*. Therefore, the presence and expression of one or more TPSb genes may be responsible for determining the composition of stored monoterpenes present in trunk resins among *Protium* individuals. Future research should aim to make an explicit link between the *Protium* monoterpene genotypes, monoterpene synthase enzyme activity and production and blend of resin monoterpenes. At the leaf and ecosystem scales in the Amazon basin, large emission rates and elevated ambient concentrations of highly reactive trans-β-ocimene and cis-β-ocimene emissions were recently discovered including leaves of *Protium* species (Jardine et al., 2015, 2017). These highly reactive monoterpenes dominated by leaf and ecosystem monoterpene emissions during warm periods of the day and during the dry season were suggested to play important roles in the thermotolerance of photosynthesis by functioning as effective antioxidants within plants and as efficient atmospheric precursors of specialised organic aerosols (Jardine et al., 2015, 2017). As opposed to emissions from stored reservoirs (e.g. resins), $^{13}CO_2$ labeling demonstrated that tropical leaf monoterpene emissions derive from *de novo* biosynthesis linked with photosynthesis. In the present study on monoterpene composition of *Protium* trunk resins from 77 individuals, only small to negligible amounts of trans-β-ocimene and cis-β-ocimene were found in 4 and 3 individuals, respectively. Therefore, despite dominating leaf emissions from a number of species, these reactive monoterpenes were not one of the nine dominant monoterpenes observed as emissions from *Protium* trunk resins. This result may indicate that trans- and cis-β-ocimene are more characteristic of *de novo* biosynthesis in leaves rather than stored as resins in trunks, and/or that high temperatures regularly experienced in canopy leaves, but not trunks near the ground (Rey-Sánchez et al., 2016), are needed in order to form these highly reactive monoterpenes (Jardine et al., 2017).

Tropical trees allocate substantial resources to defense mechanisms to counter abiotic and biotic stresses (Courtois et al., 2009). While few studies have focused on tropical ecosystems, recent studies show that the blend of volatile monoterpenes produced represent a key physical and chemical strategy to counter natural enemies including herbivores and microbes (Courtois et al., 2009, 2012; Salazar et al., 2018). By mediating plant-herbivore and plant-microbe interactions, terpenoid resins play a major role in plant defense evolution (Langenheim, 2003; Phillips and Croteau, 1999) and diversification (Farrell et al., 1991). A recent study found strong evidence for the relationship between plant-herbivores interactions and the evolution of plant chemical diversity (defense chemicals) for Amazonian Protieae trees (Salazar et al., 2018). These authors found out the occurrence of 600 different defense chemicals (including terpenoids) in 31 species of *Protium* (in a recent study, Daly and Fine (2018) have transferred all species of the former genera *Crepidospermum* and *Tetragastris* to the genus *Protium*). Nonetheless, while the synthesis, storage, and emissions of these defense chemicals are known to vary with plant species and tissues (i.e. leaf versus stem) (Bracho-Nunez et al., 2013; Steeghs et al., 2004), the majority of research on tropical terpenoids have focused on leaf level emissions. Therefore, little knowledge exists on the diversity of monoterpenes in storage resins in the tropics and how resin monoterpene composition may vary across species within abundant tree genera like the *Protium* genus (Courtois et al., 2012). Therefore the characterization of 9 distinct monoterpene 'fingerprints', each represented by a specific dominant monoterpene, among *Protium* species in the central Amazon provides critical background information for understanding the chemical and physical landscape within trunk resins that may be encountered by herbivores that are prone to attack *Protium* tree trunks in the Neotropics. Each monoterpene has specific physical, chemical, and biological properties including volatility, reactivity (e.g. susceptibility to oxidation), and biological properties (i.e. antimicrobial and insecticide properties) which act to both flush and seal the injury while repelling/killing the invaders. For example, α-pinene, the most frequently encountered monoterpene in the *Protium* species studied, has been well characterized in its role in mediating the interactions between pine trees in North America and pine-feeding bark beetles (Pitman, 1971). However, α-pinene is known to both attract beetles in low atmospheric concentrations and be toxic to bark beetles in high concentrations (Seybold et al., 2006). In addition, bark beetle aggregation pheromones such as cis-verbenol can arise through oxidation of pine tree resin-derived α-pinene (and other monoterpenes) as well as de-novo beetle biosynthesis. These aggregation pheromones are used to signal a mass attack of the beetles on pines (Erbilgin et al., 2003), allowing a coordination of feeding and mating in time and space. α-phellendrene, the second most frequently encountered dominant monoterpene in the *Protium* species studied, is widely recognized as a potent biopesticide with strong insect toxicity and repellent activities (Evergetis et al., 2013), whereas *d*-limonene is reported to be an effective antifungal compound (Wilson et al., 1997). Additional research is needed in the study of which insect and microbial pathogens attack *Protium* species and how monoterpene resins mediate these multitrophic interactions through direct physical and chemical defenses, host selection and pheromone signaling (Trapp and Croteau, 2001). Nonetheless, *Protium* trees in the Amazon have been documented to suffer attacks by chrysomelid beetles and cicadellid hemipterans, and that these diverse interactions within the species may have helped spur species diversification and habitat-mediated speciation in these trees (Fine et al., 2013; Salazar et al., 2018). Furthermore, many *Protium* species are attacked by the larvae of two curculionid weevil genera, *Pappista* and *Piazurus*, which induce plant resin flow and the formation of characteristic resin lumps. Adult females lay eggs in the tree's bark, the larvae hatch and make a series of bore holes. Larvae feed in the phloem, cutting through resin canals in the inner bark releasing a flow of resin (Plowden et al., 2002). Successive layers of resin are exuded and harden forming a resin lump that can weigh 500 g. These lumps are collected by people and used for various purposes from many different Amazonian cultures (Plowden et al., 2002).

In Brazil, the exploitation and commercialization of non-timber products like resins has a large social and economic potential (Daly et al., 2010). However, despite numerous human uses of resins (Daly et al., 2012) including recent studies showing their application in the treatment of human diseases such as cancer (Zhang et al., 2016) there is still a vast potential to be explored in their commercial use for cosmetics, medicines, biofuels, flavoring agents and other renewable products. Knowledge of the monoterpene bark composition among the highly diverse and abundant *Protium* genus in the Neotropics will provide a basis for future commercial applications including those that attempt to genetically modify *Protium* species to maximize production of a particular monoterpene, blend of monoterpenes, or their chemical modified products (e.g. monoterpene alcohols used in perfumes). The commercialization of tree resins and extracts represents a large industry in Brazil, with emphasis on resins extracted from Pine trees as an important economic component in the southern regions of the country (Rodrigues et al., 2008). In the Amazon, the extraction of Copaíba (*Copaifera langsdorffii*) (Pieri et al., 2009) and Andiroba (*Carapa guianensis*) (Mendonça and Ferraz, 2007) essential oils are particularly active. Species from *Protium* genus also comprise an important and growing commercial source because of their pharmacological properties, especially in Amazon traditional medicine practices where resins are utilized as anti-inflammatory agents and in the treatment of rheumatism and lung and skin diseases (Correia, 1984; Machado et al., 2003). Furthermore, resins from *Protium* are used as wood adhesives (Vieira et al., 2014), and with potential use in other fields such as in the production of flavor and fragrances in the pharmaceutical and food industries. Therefore, the results of this study on the chemical composition of *Protium* tree resins will be valuable in efforts to explore

potential commercial applications by large established chemical companies, such as pharmaceuticals, and local populations of indigenous communities.

Finally, forest biodiversity has been shown to enhance ecosystem productivity, resilience to abiotic and biotic disturbances, and maximize resources available for human exploitation and economic development. Given the importance of *Protium* species in tropical forest biodiversity in the Neotropics, the results of our study may provide a useful tool for forest managers to help access *Protium* biodiversity and help evaluate management strategies aimed at maximizing diversity, carbon sequestration, and sustainable harvesting practices.

4. Conclusions

The Amazon forest, with vast biodiversity and territorial extension, cycles more carbon and water than any other terrestrial ecosystem on the planet. However, understanding the tree species and chemical composition of this rich biodiversity and how its products can sustainably benefit humans remains a major challenge. In this study, we present a new rapid field collection technique to characterize the composition of monoterpenes present in stem resins of 77 *Protium* individuals across 15 species in a primary rainforest ecosystem in the central Amazon rainforest. From the analysis of the database of monoterpene 'fingerprints' generated, 9 types of monoterpene 'fingerprint' patterns emerged, characterized by a distinct dominant monoterpene. A comparison of monoterpene 'fingerprints' between years from the same individuals showed excellent agreement, suggesting that the 'fingerprints' are highly sensitive to the individual/species, but show relatively low annual variability. We therefore suggest that the presented method can be used to help constrain the identity of unknown *Protium* species and therefore be used as a new tool in *Protium* chemotaxonomy. By characterizing the composition of monoterpene resins among *Protium* species in the central Amazon, the results will contribute to future *Protium* studies on plant-microbe and plant-insect interactions, phylogenetic relationships and evolutionary histories, atmospheric chemistry and land-surface climate interactions, and commercial uses of resins. Finally, knowledge of the distribution of specific monoterpene 'fingerprints' among *Protium* tree species will contribute to the conservation, management, and sustainable use of tropical ecosystems.

5. Experimental section

5.1. Site description

The study was conducted at the Tropical Silviculture Experimental Station (ZF-2); a 21,000 ha research reserve roughly 60 km NNW (North-Northwest) of Manaus, Brazil, managed by the National Institute for Amazon Research (INPA) (Fig. S1). The vegetation is classified as old-growth closed-canopy tropical evergreen forest, with a high tree species diversity and a dense understory (Chambers et al., 2001; Ribeiro et al., 1999), representative of mature forests in the Brazilian Amazon (Higuchi et al., 2004; Teixeira et al., 2007). The data was collected in a permanent plot known as North-South transect, covering an area of 20 m x 2500 m (5 ha) and located 33 km along the access dirt road. The North-South transect has diverse vegetation with 737 tree species belonging to 238 genera of 59 families, with the family Burseraceae represented by 27 species (surveyed in 2004) (Carneiro, 2004). According to the floristic data for this study area, there are 214 individual trees belonging to the *Protium* genus in 26 species, (Carneiro, 2004).

5.2. Volatile monoterpene sample collection

Trunk monoterpene resin composition analysis consisted of 18 expeditions to the field during 2015, 2016 and 2017 (the month/year and the season of collection for each *Protium* tree individual is listed in the File S1). Volatile monoterpene emissions were collected from trunks of 77 individuals from 15 *Protium* species, randomly chosen from the plateau, slope, and valley toposequence over the 5 ha of the North-South transect (Fig. S1). Monoterpene emissions from trunks were stimulated by removing a 1–2 cm^2 of the rhytidome (outer and inner bark) at a height of 1.2–1.8 m and immediately collected in the nearby headspace atmosphere on a commercial thermal desorption tube (TD packed with Tenax TA, Carbograph 1TD and Carboxen 1003, Markes International, UK) using a hand-held pump (Markes International, UK) placed 1–2 cm from the tree trunk. In order to verify that the observed monoterpenes derived from trunk emissions and not from the ambient air, one ambient air sample surrounding the trunk was collected, quantitatively trapped by passing 300 ml of air in 1.5 min through the thermal desorption tubes, prior to rhytidome removal roughly 1 m from the tree. The diameter at breast height (DBH) of *Protium* sampled trees was between 10.2 and 35.4 cm and all collections occurred between 8:00 and 15:00 local time. For each individual, a second TD tube sample was collected on the opposite side of the tree as a replicate. Although negligible peaks of several monoterpenes could be detected in ambient air samples (third TD tube sample) relative to the large peaks present in trunk air samples (first and second TD tube samples), monoterpenes were not detectable in TD tubes without sample collection (forth TD tube sample). Thus, the forth TD tube sample ruled out the potential for contamination of the TD tubes or analytical system.

5.3. Monoterpene analysis by TD-GC-MS

Following collection, the four tubes (first and second tube: trunk air samples; third tube: ambient air sample; forth tube: without sample collection) from each sample trees was returned to the analytical laboratory in Manaus, Brazil and analyzed for monoterpenes within two days using TD-GC-MS. Thermal desorption tubes were analyzed for monoterpenes using a thermal desorption system (TD-100, Markes International) interfaced with a gas chromatograph/electron impact mass spectrometer with a triple-axis detector (5975C series, Agilent Technologies, Santa Clara, CA, USA) at INPA, Manaus, Brazil, as previously described (Jardine et al., 2010). The GC-MS was calibrated to authentic monoterpene standards (99%, Sigma Aldrich, St. Louis, MO, USA) in methanol using the dynamic solution injection (DSI) technique (Jardine et al., 2010) by dynamic dilution with a hydrocarbon free air flow of 1.0 L min^{-1}. TD-GC-MS calibrations were conducted to establish retention times and identities of sample monoterpenes, with peak area responses demonstrated to be highly linear (Jardine et al., 2017). However, in this study, we did not calculate absolute monoterpene concentrations, but instead focused our analysis on normalized peak areas (see section d. Monoterpene 'fingerprints'). Out of the 28 monoterpenes identified from trunk resin samples, 11 of them were present in the methanol calibration solution. The other 17 monoterpenes were identified based only on mass spectral comparisons with the 2011 National Institute of Standards and Technology mass spectral database (see Table S1). Identification was based on the best match (> 90% confidence). During the three-year period of the experiment (2015–2017), maintenance on the GC-MS system due to cutting of the GC column once in 2015 and replacement of the GC column in 2016 caused a shift in the retention times. A list of the retention times used for each GC-MS sample file is given in File S1.

5.4. Monoterpene 'fingerprints'

The monoterpene 'fingerprints' for each trunk sample were determined by first calculating the peak area of the molecular weight ion (m/z 136) of each monoterpene present in the sample. For some species with very high resin content and high monoterpene emissions, m/z 137 corresponding to monoterpenes with a single ^{13}C-atom was used for the determination of peak areas for each monoterpene present in the sample. The reduced sensitivity of m/z 137 relative to 136 for

monoterpenes is due to an 11.1% natural abundance of monoterpenes with a single ^{13}C atom relative to monoterpenes with all ^{12}C atoms (Isotope distribution calculator, http://www.sisweb.com/mstools/isotope.htm). From the resulting peak areas, the monoterpene 'fingerprints' for each trunk sample was calculated by normalizing the peak areas of each monoterpene present with the most abundant monoterpene in the sample. Using this method, a detailed library of *Protium* monoterpenes emitted from the trunk resins was created for the 15 species, based on samples from 77 individuals, containing information on the individual tree species in the established forest transects in the central Amazon, raw data file name (GC-MS file), date of collection, type of sample (blank, ambient-air and trunk resin emissions), season (wet/dry), tree identification number, sample collection flow rate (ml min^{-1}), collection time (min), monoterpenes identified, absolute peak area and relative abundance (% area of dominant monoterpene) (File S1).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

We acknowledge the logistical and infrastructural support from the Central Office of the Large Scale Biosphere Atmosphere program (LBA) and from the Forest Management Laboratory (LMF) of the National Institute for Amazon Research (INPA), during field measurements, and the Brazilian National Council for Scientific and Technological Development (CNPq) for the financial support in students scholarships. This material is based upon work supported as part of the Next Generation Ecosystem Experiments-Tropics (NGEE-Tropics) funded by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research through contract No. DE-AC02-05CH11231 to LBNL, as part of DOE's Terrestrial Ecosystem Science Program. A special thanks to Izailene Saar in the Botany Department at INPA for drawing the graphical abstract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2019.01.014.

Formatting of funding sources

This material is based upon work supported as part of the Next Generation Ecosystem Experiments-Tropics (NGEE-Tropics) funded by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research through contract No. DE-AC02-05CH11231 to LBNL, as part of DOE's Terrestrial Ecosystem Science Program.

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