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Sources of variation in foliar secondary chemistry in a tropical forest tree community

Brian E. Sedio,[1,2,4] Juan C. Rojas Echeverri,[2] Cristopher A. Boya P.,[2,3] and S. Joseph Wright[1]

[1]*Smithsonian Tropical Research Institute, Apartado 0843–03092, Ancón, Panama*
[2]*Center for Biodiversity and Drug Discovery, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología, Apartado 0843-01103, Ciudad del Saber, Ancón, Panama*
[3]*Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur 522 510 India*

Abstract. Specialist herbivores and pathogens could induce negative conspecific density dependence among their hosts and thereby contribute to the diversity of plant communities. A small number of hyperdiverse genera comprise a large portion of tree diversity in tropical forests. These closely related congeners are likely to share natural enemies. Diverse defenses could still allow congeners to partition niche space defined by natural enemies, but interspecific differences in defenses would have to exceed intraspecific variation in defenses. We ask whether interspecific variation in secondary chemistry exceeds intraspecific variation for species from four hyperdiverse tropical tree genera. We used novel methods to quantify chemical structural similarity for all compounds present in methanol extracts of leaf tissue. We sought to maximize intraspecific variation by selecting conspecific leaves from different ontogenetic stages (expanding immature vs. fully hardened mature), different light environments (deep understory shade vs. large forest gaps), and different seasons (dry vs. wet). Chemical structural similarity differed with ontogeny, light environment, and season, but interspecific differences including those among congeneric species were much larger. Our results suggest that species differences in secondary chemistry are large relative to within-species variation, perhaps sufficiently large to permit niche segregation among congeneric tree species based on chemical defenses.

Key words: *anti-herbivore defense; chemical ecology;* Eugenia; Inga; *intraspecific variation; leaf ontogeny; mass spectrometry; molecular network;* Ocotea; Psychotria; *species coexistence.*

Introduction

Understanding how hundreds of ecologically and physiologically similar tree species coexist in tropical forests despite intense competition for light, water, and nutrients lies at the heart of efforts to understand what generates and maintains species diversity on the planet. Consensus is mounting that biotic interactions with specialist natural enemies comprise a key mechanism that limits the local recruitment of conspecific individuals, and thereby promotes diversity (Wright 2002, Leigh et al. 2004). Enemy–host interactions, particularly those of insect herbivores, are limited by the capacity for enemies to overcome plant defenses, including noxious secondary metabolites (Barrett and Heil 2012). Plant species with similar defenses are likely to share enemies and to experience competition mediated by those shared enemies (Sedio and Ostling 2013). Hence, species niche differences defined by secondary chemistry might contribute to the diversity of woody plant species occurring locally in tropical forests.

A small number of exceptionally species-rich genera comprise a substantial proportion of local plant diversity in many tropical forests (Gentry 1982). For example, the five most species-rich genera comprise nearly 25% of the woody plant species recorded on Barro Colorado Island (BCI), Panama (Foster and Hubbell 1990). As many as 22 species of *Pouteria* (Sapotaceae) can be found in a single hectare in Amazonian Ecuador, and the same number of *Inga* (Fabaceae) have been recorded in 0.16 ha (Valencia et al. 1994). Gentry (1982) referred to genera such as *Miconia* (Melastomataceae), *Piper* (Piperaceae), and *Psychotria* (Rubiaceae) as "species swarms" due to the high diversity and ecological similarity of local assemblages of congeneric species.

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[4]E-mail: sediob@si.edu

Locally diverse species swarms of congeneric woody plants pose a challenge to our emerging understanding of diversity maintenance in tropical forests because they are likely to share natural enemies (Novotny et al. 2002, Gilbert and Webb 2007) and therefore density-dependent recruitment limitation (Sedio and Ostling 2013). Case studies document secondary chemical differences among co-occurring species of *Bursera* and *Inga* (Becerra 2007, Kursar et al. 2009). Yet, it remains unclear whether co-occurring congeners exhibit broadly similar chemical profiles or exploit distinct defense-chemistry niches because intraspecific chemical variation has not been systematically explored and chemical overlap with other community members remains an unknown.

Assessing the secondary chemical niches of plant species is complicated by ecological variables that drive chemical differences within and among conspecific individuals. Leaf chemistry varies greatly over the ontogeny of a leaf, as young, expanding leaves rely on chemical defenses until they are mature enough for cell walls to harden and physical toughness to deter most herbivores (Coley and Barone 1996). Furthermore, many plants produce morphologically and physiologically distinct leaves in full sun vs. in deep shade, and these may differ in secondary chemistry (Valladares et al. 2000). Finally, most tropical forests experience rainfall seasonality, which subjects plants to drought stress during part of the year (Walsh 1996). Many insect herbivore life cycles are linked to high levels of leaf production early in the rainy season, providing a window to escape herbivores for plants able to produce leaves during the dry season (Aide 1992). Hence, both drought stress and reduced herbivore pressure may drive dry season changes in leaf defensive chemistry. If chemical defenses contribute to coexistence by distinguishing plant species in the eyes of their natural enemies, we expect chemical variation over developmental stages and environments to be small relative to interspecific variation.

The vast diversity of plant chemical defenses has precluded community-level studies of chemical ecology. We take advantage of novel methods to acquire and assemble mass spectra (MS) into molecular networks that quantify the chemical structural similarity of all compounds (Wang et al. 2016). The molecular networks quantify chemical similarities between samples even though few compounds are unambiguously identified, which is essential in chemically diverse and understudied tropical forests. We investigate the relative contributions of leaf ontogeny, light environment, and seasonality to variation in secondary chemistry for 11 focal species from the hyperdiverse tropical tree genera *Eugenia* (Myrtaceae), *Inga* (Fabaceae), *Ocotea* (Lauraceae), and *Psychotria* (Rubiaceae). We also evaluate the relative variation in secondary chemistry among conspecific individuals, congeneric species, and genera to assess the potential for chemical differences to facilitate ecological coexistence among 46 species from these four species swarms.

Materials and Methods

Study site, focal species, and genera

Barro Colorado Island (BCI), Panama (9°9′ N, 79°51′ W) supports tropical moist forest. The most abundant tree species, *Trichilia tuberculata*, comprises only 8% of canopy tree individuals. We sampled 46 species from four genus-level clades, including *Eugenia* (four species), *Inga* (14 species), *Ocotea* (including *Nectandra*; eight species) and *Psychotria* (including *Palicourea*; 20 species). The BCI community phylogeny suggests that *Ocotea* and *Psychotria* are paraphyletic, forming monophyletic clades only if subsidiary genera are merged (Kress et al. 2009). Hence, we include *Palicourea guianensis* among the *Psychotria*, and four species of *Nectandra* among the *Ocotea* and refer to these monophyletic clades simply as *Psychotria* and *Ocotea*, respectively. Together, these four genus-level clades comprise 11% of the 409 species of trees and shrubs recorded in the BCI forest (Kress et al. 2009.

Leaf collections

We collected 207 leaf samples from the 46 species mentioned above. To evaluate chemical variation with respect to leaf ontogeny, season, and light environment, we collected leaves from at least 17 individuals of one locally abundant species from each genus (*Eugenia oerstediana*, *Inga marginata*, *Ocotea whitei*, and *Psychotria acuminata*). To evaluate chemical variation among closely related species, we collected leaves from three additional species of the *Psychotria* clade (*Palicourea guianensis*, *Psychotria hoffmannseggiana*, and *P. horizontalis*) at two ontogenetic stages and two seasons and from two additional species of *Eugenia* (*E. galalonensis* and *E. nesiotica*) and two additional species of *Ocotea* (*O. cernua* and *O. oblonga*) at two ontogenetic stages. Appendix S1: Table S1 presents sample sizes for each species-variable combination.

We collected leaves between April and August 2014, placed them on ice immediately, and stored them at −80°C within three hours. Young leaves were still expanding and unlignified, whereas mature leaves had flushed and fully matured during the 2013 rainy season. Sun leaves were collected in full sun in forest treefall gaps, while shade leaves were collected from the deeply shaded understory. Dry season leaves were collected in April, near the end of the annual dry season, while wet season leaves were collected between June and August.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

We homogenized 100 mg of frozen leaf tissue in a ball mill (Qiagen TissueLyser, Hilden, Germany) and extracted the homogenate with 700 μL 90% methanol at pH 5 for 10 min. This solvent extracts small molecules of a wide range in polarity; mild acidity aids the extraction of alkaloids. The

solution was centrifuged, the supernatant was isolated, and the extraction was repeated on the remaining sample.

Samples were analyzed using ultra high-performance liquid chromatography (UHPLC), electrospray ionization and molecular fragmentation, and tandem mass spectrometry (MS/MS) of the molecular fragments, as described in Appendix S1. MS/MS spectra of fragmented molecules were clustered into consensus spectra that represent a single unique molecular structure. We refer to these consensus spectra as compounds. This process identified 3,662 compounds derived from the 207 leaf samples. Only 206 of the compounds matched a record in the Global Natural Products Social Molecular Networking database of natural products (Appendix S1: Table S2, Figs. S1–S7; database *available online*).[5] However, these library matches include flavonoids, quinolones, furochromenes, piperidines, indole alkaloids, and terpenoids: classes of plant secondary compounds known to include anti-herbivore defenses (Appendix S1: Table S2).

Molecular networks that capture the structural similarity of unknown compounds are possible because molecules with similar structures fragment into many of the same sub-structures. Thus, a comparison of the mass to charge ratio (m/z) of the fragments of two molecules reflects their structural similarity. Structural similarity was quantified for every pair of compounds as the cosine of the angle between vectors that comprise the m/z of their constituent fragments (Wang et al. 2016). Cosine ≥0.6 reflects meaningful similarity (Wang et al. 2016).

Chemical structural and compositional similarity (CSCS)

We used all pairwise combinations of compounds to calculate chemical structural and compositional similarity (CSCS) for each pairwise combination of the 207 samples. The chemical structural similarity (CSS) of all pairwise combinations of C compounds can be represented as a symmetrical C by C matrix

$$\mathbf{CSS} = \begin{bmatrix} \cos\Theta_{11} & \dots & \cos\Theta_{1C} \\ \dots & \dots & \dots \\ \cos\Theta_{C1} & \dots & \cos\Theta_{CC} \end{bmatrix} \quad (1)$$

where $\cos\Theta_{ab}$ is the structural similarity of compounds a and b (the cosine of the angle between the consensus tandem mass spectra of compounds a and b represented as vectors) and $0 \le \cos\Theta_{ab} \le 1$. Furthermore, $\cos\Theta_{aa} = 1$ for all compounds a, $\cos\Theta_{ab} = \cos\Theta_{ba}$ for all compounds $a \ne b$, and by convention, $\cos\Theta_{ab} = 0$ if $\cos\Theta_{ab} < 0.6$.

The chemical compositional similarity of all compounds accounts for variation in the ion intensity of compounds in samples A and B and is also a C by C matrix

$$\mathbf{AB}^{\mathrm{T}} = \begin{bmatrix} I_{1A} * I_{1B} & \dots & I_{1A} * I_{CB} \\ \dots & \dots & \dots \\ I_{CA} * I_{1B} & \dots & I_{CA} * I_{CB} \end{bmatrix} \quad (2)$$

where i_{cs} is the ion intensity of compound c in leaf sample s, $I_{cs} = i_{cs} / \sum_{c=1}^{C} i_{cs}$ is the ion intensity of compound c in sample s expressed as a proportion, and $\sum_{c=1}^{C} I_{cs} = 1$. $\mathbf{A} = \begin{bmatrix} I_{1A} \\ \vdots \\ I_{CA} \end{bmatrix}$ is a column vector of the proportional representation of all C compounds in leaf sample A, and $\mathbf{A}^{\mathrm{T}} = \begin{bmatrix} I_{1A} & \dots & I_{CA} \end{bmatrix}$ is the transpose of $\mathbf{A}$. Matrix entries are the products of the proportional representation of each compound in two leaf samples, A and B. Entries sum to one.

We combined these matrices to calculate the average structural similarity of all pairwise combinations of compounds weighted by their proportional representation in samples A and B. $\mathbf{CSS} \times \mathbf{AB}^{\mathrm{T}}$ is an entry-wise matrix product. It is a C by C matrix, whose entries are the products of the corresponding elements of $\mathbf{CSS}$ and $\mathbf{AB}^{\mathrm{T}}$ (for example, $\cos\Theta_{xy} \times I_{xA} \times I_{yB}$). The entries of $\mathbf{CSS} \times \mathbf{AB}^{\mathrm{T}}$ equal zero if $\cos\Theta_{xy} = 0$ or if compound x (y) is absent from sample A (B). Finally, the average chemical structural compositional similarity (CSCS) of all pairwise combinations of compounds weighted by their proportional representation in leaf samples A and B is the sum of the elements of $\mathrm{CSS} \times \mathrm{AB}^{\mathrm{T}}$ standardized by the maximum of ($\mathbf{CSS} \times \mathbf{AA}^{\mathrm{T}}$, $\mathbf{CSS} \times \mathbf{BB}^{\mathrm{T}}$).

We also calculated the Bray-Curtis similarity of compounds for each pair of samples using the R package vegan (Oksanen et al. 2009). Bray-Curtis similarity measures the compositional similarity of chemical compounds, weighted by their I_{CS} values while ignoring their structural relationships. In contrast, CSCS integrates I_{CS} values and structural similarity. Consider an example. Compounds x and y are structurally similar ($\cos\Theta_{xy} \ge 0.6$), sample A contains x but not y, and sample B contains y but not x. In this example, compounds x and y contribute zero to Bray-Curtis similarity but make a positive contribution to CSCS.

We calculated both the CSCS and Bray-Curtis similarity for every pair of samples. Given 207 samples, there are 21,321 sample pairs. Each sample is characterized by its species, leaf age, light environment, and season. We then calculated chemical similarities for pairs of samples that differed with respect to just one of these factors and measured the difference between within-factor (e.g., within leaf age) similarity and between-factor (e.g., between leaf ages) similarity. A permutation test is required to evaluate the significance of differences between within-factor and between-factor similarity because we consider all pairwise combinations of samples. We therefore randomized the assignment of factors (species, ontogenetic stage, light environment, and season) to samples and calculated distributions of all possible differences between within-factor and between-factor similarity. If the observed difference was >95% of the distribution of possible differences, the variable affected chemical similarity significantly. We examined the effect of each variable on all species considered together by combining P values using the weighted Z method (Whitlock 2005).

[5] gnps.ucsd.edu

Results

Networks of compounds linked by cosine scores ≥0.6 ranged in size from 2 to 1,685 compounds. The remaining 1,909 compounds had cosine scores <0.6 with every other compound found in the 207 leaf samples. For clarity of visualization only, we broke the largest network of 1,685 compounds into several smaller networks using Markov chain clustering, which identifies sub-clusters within a larger network based on connectivity. The R package MCL (Jäger 2015) with the inflation parameter equal to 1.5 identified 14 sub-clusters, which are included among the 89 clusters in Fig. 1.

Intraspecific chemical variation

Leaf chemical structural compositional similarity (CSCS) rarely differed significantly with leaf age, light environment or season. Immature and mature leaves differed significantly for zero of seven species (Table 1a). Leaves from large tree fall gaps and the deeply shaded understory differed significantly for two of seven species (Table 1b). And, wet- and dry-season leaves differed significantly for one of four species (Table 1c). Neither leaf age nor season had a significant effect when all species were considered together by combining *P* values with the weighted *Z* method (Table 1a, c). CSCS was greater within

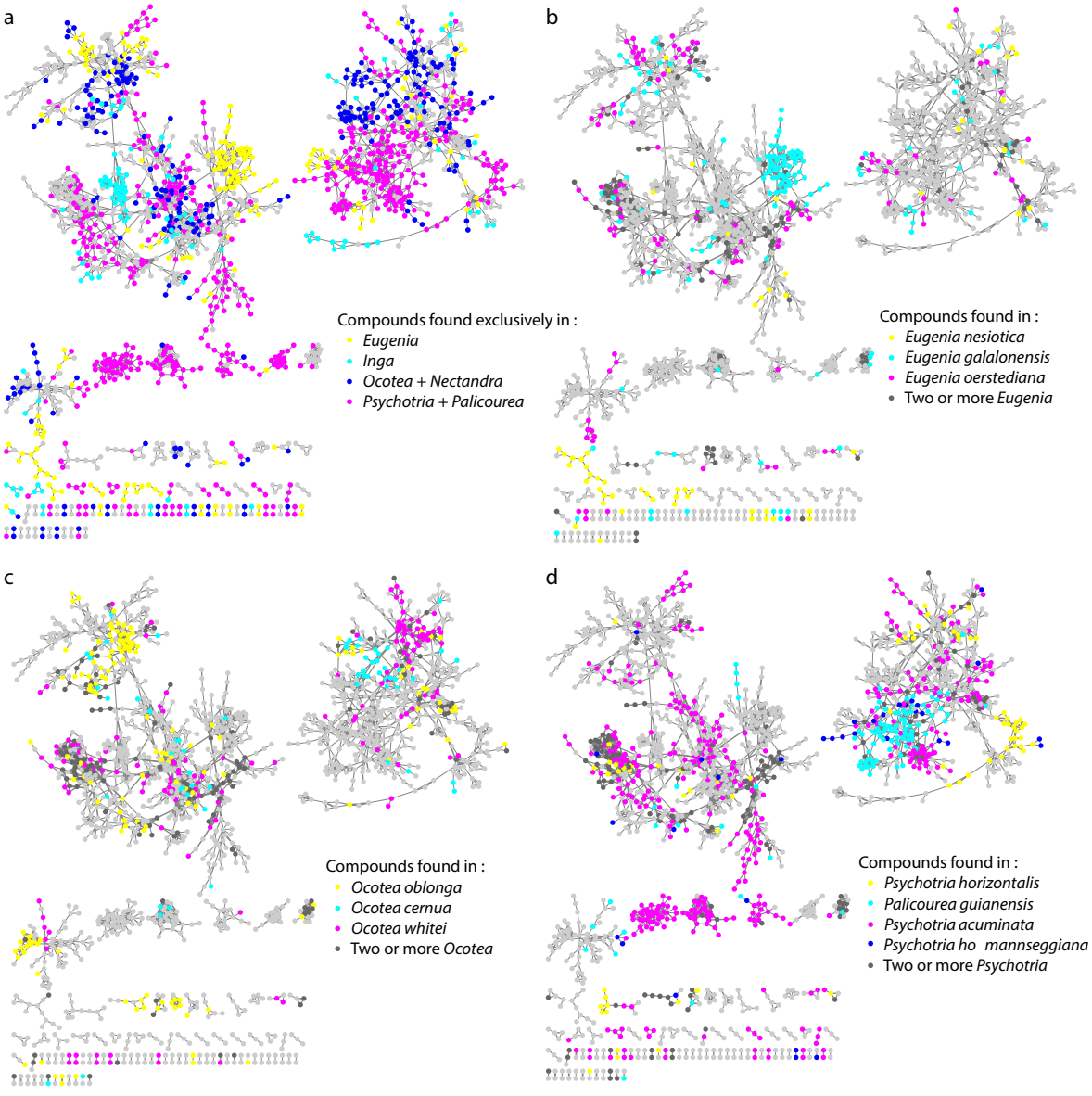

Fig. 1. Molecular network indicating the incidence of small molecules in congeneric species and in tree genera. Included are 1,824 compounds linked to at least one other compound by a cosine similarity score of ≥0.6. Nodes represent compounds (e.g., epicatechin); links between nodes indicate molecular structural similarity between compounds (e.g., epicatechin and epigallocatechin gallate).

TABLE 1. The effect of leaf age, light environment, and season on chemical structural compositional similarity (CSCS) and Bray-Curtis similarity (BC).

	CSCS				BC			
	Within	Between	Difference	*P*	Within	Between	Difference	*P*
a) Ontogeny								
E. oerstediana	0.23	0.18	0.05	0.149	0.12	0.10	0.02	0.227
I. marginata	0.34	0.30	0.04	0.102	0.15	0.13	0.02	0.124
O. whiteii	0.30	0.33	−0.04	0.874	0.14	0.16	−0.02	0.665
P. guianensis	0.41	0.38	0.03	0.168	**0.24**	**0.17**	**0.06**	**0.022**
P. acuminata	0.32	0.31	0.01	0.415	0.18	0.17	0.01	0.273
P. hoffmannseggiana	0.22	0.26	−0.03	0.807	0.13	0.13	0.01	0.305
P. horizontalis	0.29	0.31	−0.02	0.617	0.12	0.14	−0.01	0.590
Combined *P*†				0.329				**0.049**
b) Light environment								
E. oerstediana	0.23	0.25	−0.02	0.627	0.12	0.14	−0.02	0.812
I. marginata	0.34	0.30	0.05	0.148	**0.15**	**0.09**	**0.06**	**0.009**
O. whiteii	0.30	0.27	0.03	0.224	0.14	0.08	0.06	0.084
P. guianensis	**0.41**	**0.34**	**0.07**	**0.047**	**0.24**	**0.16**	**0.08**	**0.018**
P. acuminata	0.32	0.35	−0.03	0.736	0.18	0.18	0.00	0.481
P. hoffmannseggiana	0.22	0.19	0.03	0.172	0.13	0.12	0.02	0.141
P. horizontalis	**0.29**	**0.18**	**0.12**	**0.023**	**0.12**	**0.07**	**0.05**	**0.048**
Combined *P*†				**0.016**				**0.001**
c) Season								
E. oerstediana	0.31	0.29	0.02	0.311	0.17	0.15	0.02	0.229
I. marginata	0.43	0.45	−0.02	0.795	0.20	0.19	0.01	0.215
O. whiteii	0.30	0.25	0.05	0.211	0.15	0.12	0.03	0.238
P. acuminata	**0.33**	**0.23**	**0.11**	**0.044**	0.18	0.11	0.07	0.058
Combined *P*†				0.142				**0.028**

Notes: A permutation test was used to evaluate the null hypothesis that similarity was equal within vs. between categorical factors. Factors were immature vs. mature leaves for ontogeny, leaves growing in forest gaps vs. the shaded understory for light environment, and wet vs. dry for season. Genera are *Eugenia*, *Inga*, *Ocotea*, and *Psychotria*. Significant differences are indicated in bold.

†Combined *P* values indicate the significance of the effect of leaf age over all species using the weighted *Z* method.

than between light environments for five of seven species, and light environment had a significant effect when all species were considered together (Table 1b). CSCS values for conspecific leaves that differed with respect to age, light environment, or season were only marginally smaller than CSCS values for leaves that shared all of these factors (*cf*, first column vs. second and third columns in Fig. 2a and second through fourth columns in Appendix S1: Fig. S2a).

Leaf age, light environment, and season had stronger effects on Bray-Curtis chemical similarity. Leaf age was significant for one of seven species (Table 1a). Light environment was significant for three of seven species (Table 1b). And, season was marginally significant for one of four species (Table 1c). Within-factor Bray-Curtis chemical similarity was greater than between-factor similarity for 15 of 18 comparisons (Table 1). Leaf age, light environment, and season all had significant effects on Bray-Curtis similarity when all species were considered together by combining *P* values with the weighted *Z* method (Table 1). Although statistically significant, the quantitative difference in Bray-Curtis similarities was small for conspecific leaves that differed with respect to age, light environment, and season (*cf*, first column vs. second and third columns in Fig. 2b and second through fourth columns in Appendix S1: Fig. S2b).

Chemical variation among species and genera

Species differed greatly in compound richness and composition. The number of compounds detected ranged from 207 in *E. nesiotica* to 1,109 in *E. oerstediana* (Appendix S1: Table S3). More than 70% of the compounds detected in each genus was detected in a single congener for all four genera (Appendix S1: Table S4).

The molecular network revealed striking differences among genera and among congeneric species (Fig. 1). Congeneric species of *Eugenia*, *Ocotea*, and *Psychotria* were remarkably different chemically, whether measured in terms of CSCS (Table 2) or Bray-Curtis chemical similarity (Appendix S1: Table S5). Chemical differences were greater between congeneric species than between genera (Table 2), particularly with respect to CSCS. CSCS and Bray-Curtis similarity were much greater within than between species, even when comparing leaves that differed in leaf age, light environment, and season (pink vs. other colors in Fig. 2 and Appendix S1: Fig. S2).

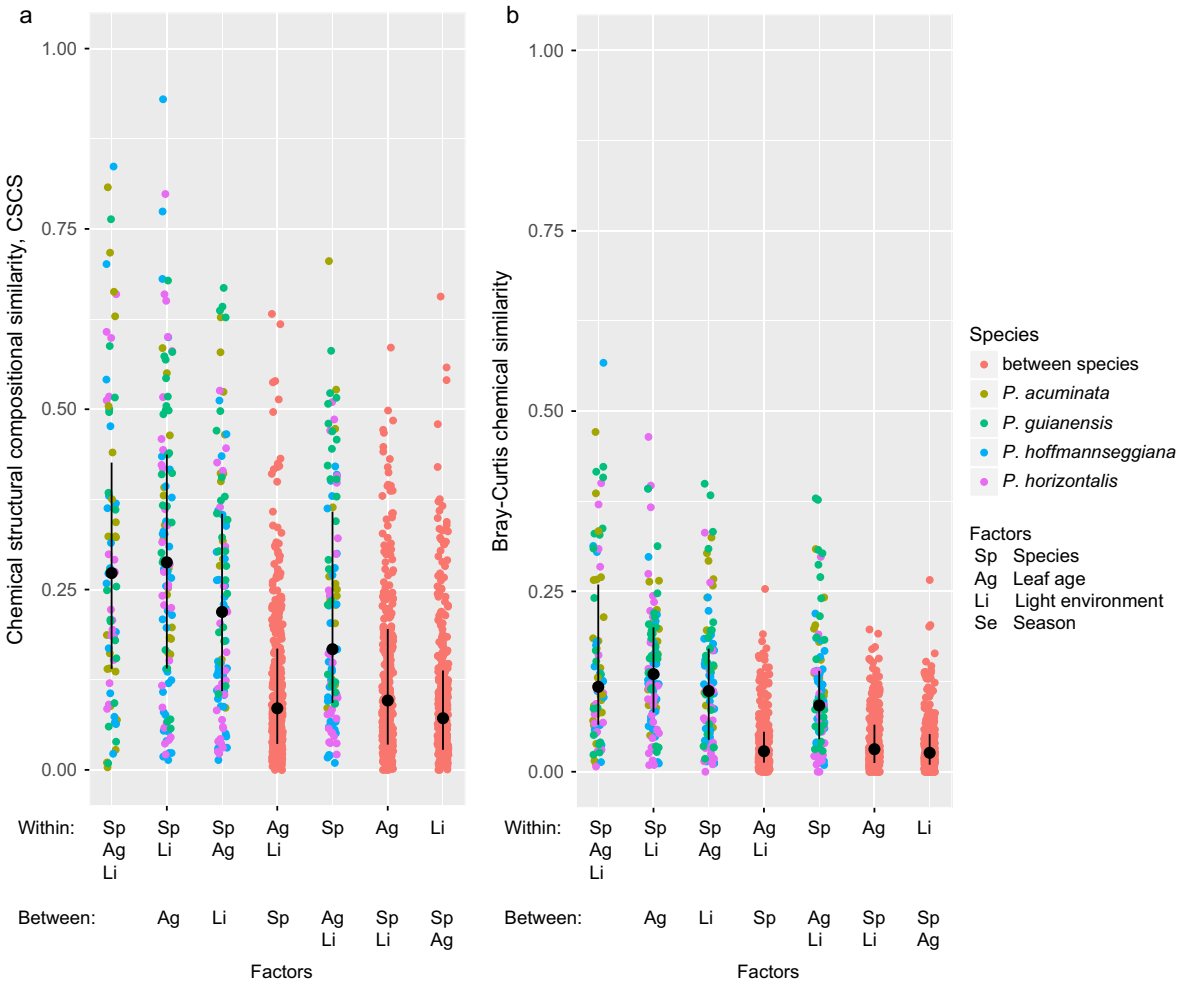

FIG. 2. Chemical similarity within and between leaf age, light environment, and species for four *Psychotria* species. Chemical similarity is represented by (a) chemical structural compositional similarity (CSCS) and (b) Bray-Curtis similarity. Each point represents the similarity between two leaf samples. Pairs of conspecific leaf samples are color coded by species. Pairs of heterospecific leaf samples are represented in pink. The horizontal axis label indicates whether leaf sample pairs plotted include pairs within or between the categorical variables leaf age, light environment, and species.

DISCUSSION

Intraspecific vs. interspecific chemical variation

Variation over ontogeny, light environment, and season does not obscure species differences in secondary chemistry (Figs. 1 and 2, Table 2). Fig. 1 clearly shows that congeneric species possess distinct compounds, and the compounds they possess tend to occur in distinct clusters of structurally related molecules in the molecular network. In Fig. 2 and Appendix S1: Fig. S2, the chemical similarity between pairs of conspecific individuals that differ in age, light environment, or season is marginally less than conspecific leaves sampled at the same age or environment. In contrast, the chemical similarity between heterospecific congeners is clearly much less than that between conspecific individuals, even for conspecifics sampled for different leaf ages or different environments (Table 2a, Fig. 2). Inducible chemical variation was not considered in this study, and may contribute to species differences independently of constitutive defenses (Haak et al. 2014).

Congeneric species of *Eugenia*, *Ocotea*, and *Psychotria* differ from one another to a greater extent than these genera differ from one another (Fig. 1, Table 2). This result occurs because, for a compound or cluster to be associated with variation among genera, it must be shared by species within a genus. Figs. 1 and 2a clearly indicate that congeneric species differ with respect to broad clusters of structurally related compounds and share few compounds.

Macroevolution of plant chemical defense

The focus of this analysis on variation within a small number of focal species does not lend itself to a

Table 2. Mean chemical structural compositional similarity (CSCS) within and between congeneric species and genera.

Genus	CSCS within	CSCS between	ΔCSCS	*P*
a) Inclusive				
Eugenia	0.20	0.09	0.11	<0.001
Ocotea	0.26	0.10	0.17	<0.001
Psychotria	0.27	0.12	0.15	<0.001
b) Young leaves				
Eugenia	0.25	0.07	0.18	<0.001
Ocotea	0.27	0.10	0.16	<0.001
Psychotria	0.25	0.11	0.14	<0.001
c) Across genera				
Genera	0.21	0.12	0.08	<0.001

Notes: Differences in mean chemical similarity between within-species and between-species sample pairs were all >99.9% of those calculated on permuted data sets. Part a includes variation over leaf age and light environment for three *Eugenia* species, three *Ocotea* species, and four *Psychotria* species. Part b includes variation over light environment within young leaves only for the same 10 species. Part c includes young-shade-wet season leaves for 4 *Eugenia*, 14 *Inga*, 8 *Ocotea*, and 20 *Psychotria* species.

phylogenetic analysis. Yet our results strongly suggest that even closely related species may differ substantially in their secondary chemistry. Divergence in chemistry among closely related species of *Eugenia*, *Ocotea*, and *Psychotria* would be consistent with earlier results for *Bursera*, *Inga*, and *Solanum* (Becerra 1997, Kursar et al. 2009, Haak et al. 2014), all of which exhibit an absence of phylogenetic signal in chemical defense within the genus. If phylogenetic diversification in hyperdiverse plant genera has been driven by selection for chemical divergence among closely related species (Ehrlich and Raven 1964), we would expect chemical traits to exhibit less phylogenetic signal within genera than do other functional traits that have not contributed as strongly to lineage diversification.

Finally, the dramatic differences in secondary chemistry we observed among congeneric species (Figs. 1 and 2) may have important implications for understanding natural enemy-mediated competition, and hence coexistence. Shared natural enemies are expected to mediate competitive exclusion among plant species (Sedio and Ostling 2013). The foliar metabolomes analyzed here likely contain compounds that function in defense as well as those that do not. Nevertheless, our results suggest that species differences in secondary chemistry are large relative to within-species variation, perhaps sufficiently large to permit niche segregation among congeneric tree species based on chemical defenses against insects and pathogens.

The vast majority of secondary metabolites in the present study, and in the BCI forest at large, remain unidentified and their functions unknown. Nevertheless, our results illustrate the capacity to compare metabolomes among understudied plant species made possible by mass spectrometry molecular networks. The integration of metabolomics and community ecology promises to greatly improve our understanding of chemical traits that may contribute to coexistence among members of "species swarms" of hyperdiverse tropical tree genera.

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