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Comparative foliar metabolomics of a tropical and a temperate forest community

Brian E. Sedio [1,2,4,5] John D. Parker,[3] Sean M. McMahon,[3] and S. Joseph Wright [1]

[1]*Smithsonian Tropical Research Institute, Apartado 0843–03092, Balboa, Ancón, Republic of Panama*
[2]*Center for Biodiversity and Drug Discovery, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología, Apartado 0843-01103, Ciudad del Saber, Ancón, Republic of Panama*
[3]*Smithsonian Environmental Research Center, 647 Contees Wharf Road, Edgewater, Mary Land 21037 USA*

Abstract. Plant enemies that attack chemically similar host species are thought to mediate competitive exclusion of chemically similar plants and select for chemical divergence among closely related species. This hypothesis predicts that plant defenses should diverge rapidly, minimizing phylogenetic signal. To evaluate this prediction, we quantified metabolomic similarity for 203 tree species that represent >89% of all individuals in large forest plots in Maryland and Panama. We constructed molecular networks based on mass spectrometry of all 203 species, quantified metabolomic similarity for all pairwise combinations of species, and used phylogenetically independent contrasts to evaluate how pairwise metabolomic similarity varies phylogenetically. Leaf metabolomes exhibited clear phylogenetic signal for the temperate plot, which is inconsistent with the prediction. In contrast, leaf metabolomes lacked phylogenetic signal for the tropical plot, with particularly low metabolomic similarity among congeners. In addition, community-wide variation in metabolomes was much greater for the tropical community, with single tropical genera supporting greater metabolomic variation than the entire temperate community. Our results are consistent with the hypothesis that stronger plant-enemy interactions lead to more rapid divergence and greater metabolomic variation in tropical than temperate plants. Additional community-level foliar metabolomes will be required from tropical and temperate forests to evaluate this hypothesis.

Key words: *anti-herbivore defense; Barro Colorado Island; chemical ecology; forest ecology; mass spectrometry; molecular network; species coexistence.*

Introduction

Foundational hypotheses in community ecology and evolution posit a central role for plant secondary metabolites in the maintenance and generation of species richness in plant communities. Qualitative differences in chemical defenses against herbivores and pathogens can distinguish plant species in the eyes of their natural enemies, allowing plant species to carve out "niches" defined by the insects and microbes they support and those they avoid. Gillett (1962) first proposed that the resulting reduction in shared enemies might reduce enemy-mediated negative interactions among heterospecific individuals, and hence facilitate coexistence. Ehrlich and Raven (1964) extended the concept to macroevolution, proposing that selection for novel defenses drives reciprocal coevolution, and ultimately speciation, in plants and their enemies.

If biotic interactions have played a strong role in shaping plant communities, co-occurring species should exhibit interspecific variation in secondary metabolites, even amongst close relatives. Focused studies of tropical tree genera have found that congeneric species are often remarkably divergent in secondary metabolites (Becerra 1997, Kursar et al. 2009, Fine et al. 2013, Richards et al. 2015, Salazar et al. 2016, Sedio et al. 2017). However, few studies have pursued untargeted metabolomics in a community or evolutionary context (Macel et al. 2014, Richards et al. 2015, Mason et al. 2016, Sedio et al. 2017) and none has examined interspecific metabolomic variation in a forest at the community scale.

Many thousands of compounds influence plant-animal interactions. Most plants produce complex mixtures of metabolically diverse secondary metabolites with multiple functions, and the structures and identities of most secondary metabolites remain unknown (Wang et al. 2016). This combination of vast diversity, unknown structure and function, and rarity of secondary metabolites has precluded the pursuit of comparative metabolomics at the large taxonomic scales necessary for the study of whole communities (Sedio 2017). However, recent innovations in mass spectrometry (MS) informatics make it possible to compare the structures of thousands of unknown metabolites from diverse chemical classes in hundreds of plant species simultaneously. Here, we quantify the structural similarity of all

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[4] Present address: Department of Integrative Biology, University of Texas at Austin, Austin, Texas 78712 USA.
[5] E-mail: SedioB@si.edu

compounds, including the many unidentified compounds, in methanol extracts of leaf tissue. We then quantify chemical similarity for all pairwise combinations of species, incorporating shared compounds and the structural similarity of compounds unique to one species in each pair (Sedio et al. 2017). We assess interspecific chemical similarity among 138 tropical and 65 temperate plant species from forests in Panama and Maryland, USA, respectively. Within each community, we ask to what extent foliar metabolomes differ among species, including congeneric species, and whether they exhibit phylogenetic signal. If biotic interactions have played a strong role in shaping the secondary metabolomes of these plant communities, co-occurring species should exhibit interspecific variation in secondary metabolites and phylogenetic signal should be largely absent.

Materials and Methods

Study sites and species

Barro Colorado Island (BCI), Panama (9°9′ N, 79°51′ W), supports tropical moist forest. The 2010 census of a 50-ha forest dynamics plot (FDP) recorded 301 species with individuals ≥1 cm in diameter at breast height (DBH; Condit 1998). We sampled 138 species, including the 48 most abundant species, and every species in seven of the eight most species-rich woody genera (*Eugenia* [4 species], *Inga* [17], *Miconia* [12], *Ocotea* [9], *Piper* [11], *Protium* [5] and *Psychotria* [21]). Several of these genera are paraphyletic but form monophyletic clades when subsidiary genera are merged (Erickson et al. 2014). Hence, these figures include *Clidemia* and *Leandra* among the *Miconia*, *Nectandra* among the *Ocotea* (Erickson et al. 2014), *Tetragastris* among the *Protium* (Daly and Fine in press), and *Carapichea* and *Palicourea* among the *Psychotria* (Nepokroeff et al. 1999). We refer to these clades by the most species-rich generic name on BCI. The 138 species represent 89% of the stems ≥1 cm DBH in the 2010 census.

The Smithsonian Environmental Research Center (SERC) in Edgewater, MD (38°53′ N, 76°33′ W), supports temperate deciduous forest. The 2014 census of a 16-ha FDP recorded 69 species with individuals ≥1 cm DBH. We sampled all 18 invasive species and 47 native species, including all species in the three most species-rich genera (*Carya* [3 species], *Quercus* [8], and *Viburnum* [3]). The 47 native species represent 99% of the native stems ≥1 cm DBH in the FDP.

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

We collected expanding, unlignified leaves from the shaded understory between April and August 2014 from 611 randomly chosen individuals of the 203 focal species, providing a mean of 3 individuals per species and a range of 1–10; 152 species were represented by ≥3 individuals. Sedio et al. (2017, 2018) describe chemical methods in detail. Briefly, we extracted 100 mg of homogenized leaf tissue twice with 700 μL 90:10 methanol: water at pH 5 for 10 min. This solvent extracts small molecules of a wide range in polarity. Mild acidity aids the extraction of alkaloids. We used ultra high-performance liquid chromatography, electrospray ionization and molecular fragmentation, and tandem mass spectrometry (MS/MS) to analyze extracts (Sedio et al. 2017, 2018) and the Global Natural Products Social (GNPS) Molecular Networking tool to cluster the MS/MS spectra into consensus spectra that represent unique structures (see Appendix S1: Table S1; Wang et al. 2016). We refer to consensus spectra as compounds throughout.

Molecules with similar structures fragment into many of the same sub-structures. Thus, the similarity of mass to charge ratio (m/z) of the fragments of two molecules reflects their structural similarity. We quantified structural similarity for every pair of compounds from all 203 species as the cosine of the angle between vectors defined by the m/z values of their constituent fragments (Wang et al. 2016). Cosine values <0.6 are unlikely to reflect meaningful levels of chemical structural similarity and were zeroed (Wang et al. 2016). Our MS data can be found at http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=d1f7f083fa554f2c9608f238c1ccda0e.

Our methods detect both primary metabolites involved in core metabolic pathways, which tend to be conserved across most plants, and secondary metabolites involved in defense. Secondary metabolites will dominate the combined metabolomes of 203 species due to their greater diversity and much greater interspecific variability (Salminen and Karonen 2011).

Chemical structural and compositional similarity (CSCS)

Sedio et al. (2017) developed a metric that quantifies chemical structural-compositional similarity (CSCS) over all compounds in two species. Conventional similarity indices such as Bray-Curtis incorporate shared compounds, but ignore structural similarity of unique compounds. In contrast, CSCS incorporates the structural similarity of compounds that are unique to each species. A simple example illustrates the implications. Compounds x and y are structurally similar (cosine ≥0.6). Species A contains compound x but not y, and species 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 based on their structural similarity.

Chemical structural-compositional similarity weights the similarity (cosine score if ≥0.6 or 0 otherwise) of every pairwise combination of compounds in two species by the product of their proportional ion intensities in each species (Sedio et al. 2017). Proportional ion intensities equal mean ion intensities over conspecific individuals for each compound standardized by the summed means for each species. We calculated CSCS for all 20,503 pairs of species. We also recorded the chemical similarity between each species and its nearest neighbor in chemical space by selecting the greatest CSCS value for each species. We refer to this metric as nearest-neighbor CSCS ($CSCS_{nn}$).

Statistical analyses

We used phylogenetically independent contrasts (PICs) to test for relationships between chemical similarity and phylogenetic distance. Each PIC equals the mean CSCS for all pairs of species descended from each node in the phylogeny. We refer to this metric as $CSCS_{mrca}$, where MRCA refers to

most recent common ancestor. This calculation is illustrated in Appendix S1: Fig. S1. To evaluate phylogenetic signal, we regressed $CSCS_{mrca}$ against log-transformed phylogenetic distance. To generate the phylogenies, we pruned the Forest-GEO-CTFS mega-phylogeny (Erickson et al. 2014) to the 126 and 34 species present in our BCI and SERC data, respectively (Appendix S1: Fig. S2).

To determine whether CSCS differed between forests, we performed a one-way ANOVA with forest (Maryland or Panama) as the effect. To overcome the statistical dependence of metrics calculated for pairwise combinations of species, we performed the ANOVA on 10,000 random draws of independent pairs of species. CSCS differed significantly between forests if 95% of the 10,000 ANOVAs were significant. To determine whether $CSCS_{nn}$ differed between forests, we performed phylogenetic ANOVA using 'geiger' (Harmon et al. 2008).

To test for differences in the chemical space occupied by two groups of species, we first used non-metric multidimensional scaling (NMDS) to reduce the molecular network to two dimensions (using the 'MASS' package in R; Venables and Ripley 2002). We then compared the observed difference in area occupied by the two groups with the distribution of differences generated by 10,000 randomizations. Randomizations reassigned species to groups. The chemical space occupied by two groups differed significantly if the observed difference in area was greater than 95% of randomized differences. All analyses excluded the 18 introduced species at SERC. Appendix S1 presents results of analyses that include the introduced species.

Results

We detected 126,746 compounds, ranging from 107.06 to 2,174.66 Daltons (Da), in foliar extracts of 185 native species from BCI and SERC. The GNPS database of natural products (Wang et al. 2016) included 130 matches with these compounds. Matches include flavonoids, piperazines, quinoline alkaloids, indole alkaloids, and terpenoids, classes of secondary metabolites known to include anti-herbivore defenses, as well as some primary metabolites, including chlorophyll and simple carbohydrates (Fig. 1). Networks of compounds linked by cosine scores ≥ 0.6 ranged in size from 2 to 23,029 compounds, and 95,407 compounds had cosine scores <0.6 with every other compound (Fig. 1). In many instances, compounds unique to one or a few species comprise subnetworks of structurally similar compounds (Sedio et al. 2017). Such clusters of structurally similar compounds might represent structural precursors or alternative products from shared metabolic pathways.

Phylogenetic signal differed between sites. For BCI, $CSCS_{mrca}$ was unrelated to log-transformed phylogenetic distance ($t = -1.28$, df = 123, $P = 0.205$; Fig. 2a), indicating a strong tendency for chemical divergence among closely related species. For SERC, $CSCS_{mrca}$ was strongly related to phylogeny ($t = -3.59$, df = 31, $P = 0.001$; Fig. 2b), indicating that closely related species have similar metabolomes. Chemical compositional similarity was phylogenetically conserved for SERC but not for BCI species.

The strong site difference in phylogenetic signal suggests closer examination of site differences in chemical similarity. Chemical similarity ($P < 0.0001$; Fig. 3a) and chemical similarity to the nearest neighbor in chemical space ($P < 0.0001$; Fig. 3b) were both lower for BCI species than for SERC species (Appendix S1: Tables S2, S3). The largest genera made an important contribution to these site differences, with CSCS and $CSCS_{nn}$ being much lower for the seven species-rich BCI genera than for the three species-rich SERC genera (Fig. 3c, d).

The NMDS ordination illustrates the chemical space represented by 138 BCI species and 47 native SERC species (Fig. 4a). Species comprising the largest BCI genera occupied a greater area in chemical space than the remaining BCI species ($P < 0.001$; Figs. 4b, c, e). In contrast, species comprising the largest SERC genera did not comprise a greater chemical space than the remaining SERC species ($P = 0.707$; Fig. 4d). Results were qualitatively similar for analyses that included the 18 introduced SERC species (Appendix S1).

Discussion

Plant enemies that attack chemically similar host species are thought to mediate competitive exclusion of chemically similar plant species (Gillett 1962, Sedio and Ostling 2013, Salazar et al. 2016) and select for chemical divergence among closely related plants (Becerra 1997, Kursar et al. 2009). This hypothesis predicts that plant defenses should diverge rapidly, minimizing phylogenetic signal. Leaf metabolomes lacked phylogenetic signal for the BCI community, which is consistent with the hypothesis. Selection for divergence among close relatives, competitive exclusion of chemically similar close relatives, or both might drive the absence of phylogenetic signal at BCI. Phenotypic plasticity is less likely to be important because metabolomic variation is much greater between than within species at BCI (Sedio et al. 2017). In contrast to BCI, leaf metabolomes exhibited clear phylogenetic signal for the temperate SERC community, which is inconsistent with the hypothesis that plant enemies exert strong selection for divergence among closely related plants. This suggests an interesting difference between tropical and temperate plant communities.

Dobzhansky (1950) proposed that biotic interactions comprise a stronger selective force than the physical environment in the tropics. Indeed, herbivore and pathogen pressure is greater, and host ranges narrower, in the tropics than at higher latitudes (Coley and Barone 1996, Dyer et al. 2007, Schemske et al. 2009, Lim et al. 2015). If natural enemies exert stronger selection for chemical defense and divergence in the tropics, then tropical plants should exhibit greater quantitative investment in chemical defenses and/or greater chemical dissimilarity than temperate plants (Coley and Aide 1991).

There are fundamental chemical differences between trees from tropical Panama and temperate Maryland. The tropical tree species were much less chemically similar when compared to closely related species (Fig. 2), to the most chemically similar species (Figs. 3b, 4a), and over all species (Figs. 3a, 4a). Chemical similarity was also consistently lower among species-rich tropical genera than among species-rich temperate genera (Figs. 3c, d, 4b–d). These results are consistent with the hypothesis that plant-enemy interactions are stronger in the tropics, leading to rapid evolution of phytochemical diversity in tropical trees.

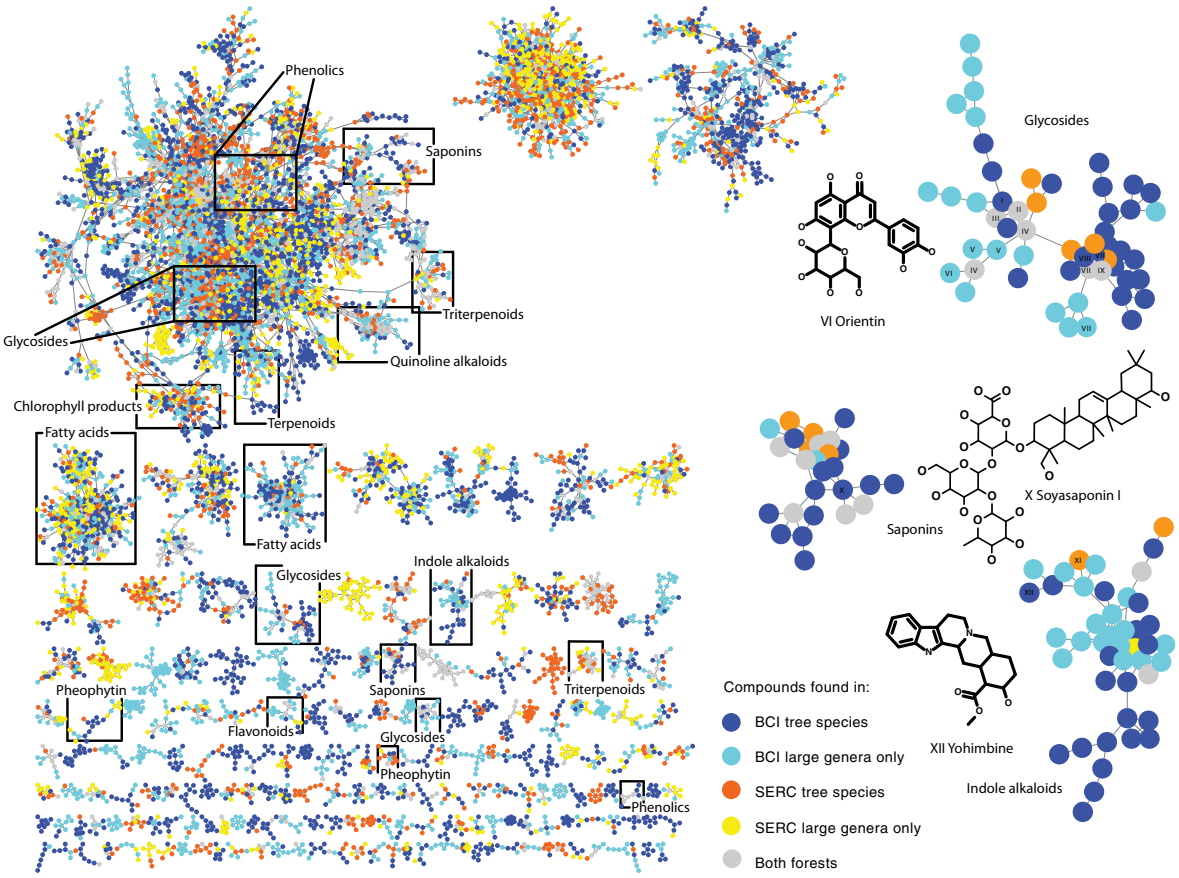

FIG. 1. Molecular network of 36,223 compounds in leaves of tree and shrub species from SERC, Maryland and BCI, Panama. Nodes represent compounds. Links between nodes represent structural similarity between compounds indicated by cosine similarity scores ≥0.6. Colors represent compounds found 79 species in seven large genera at BCI (light blue), another 59 BCI species (dark blue), 14 species in three large genera at SERC (yellow) and another 33 SERC species (orange). Matches to 130 known compounds identified chemical classes (e.g., 'flavonoids'). We severed links with cosine scores <0.8 to break the largest network into smaller networks for visualization. Three subnetworks are highlighted at right to illustrate compound matches to GNPS libraries. Matched compounds are I) ReSpect:PS043007 Puerarin, II) ReSpect:PM007810 3′-O-Methylluteolin 6-C-glucoside, III) ReSpect:PS086308 Orientin, IV) GNPS:Vitexin, ReSpect:PM007805 Isoorientin, VI) GNPS:Orientin, VII) GNPS:Hexanoside of (iso)orientin, VIII) GNPS:Pentoside of (iso)vitexin, IX) Massbank:PB006223 Vitexin-2″-O-rhamnoside, X) GNPS:Soyasaponin I, XI) GNPS:MLS000111555-01! Tetrahydroalstonine, XII) GNPS:Yohimbine.

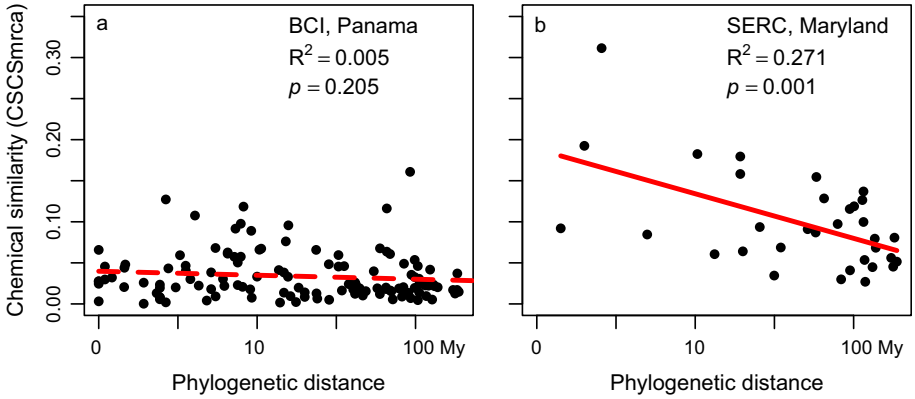

FIG. 2. Relationships between the mean chemical similarity of species descended from each node (or most recent common ancestor, $CSCS_{mrca}$) and log-transformed phylogenetic distance for BCI (panel a) and SERC (b). The dashed and solid red lines represent insignificant and significant linear regressions, respectively. The calculation of $CSCS_{mrca}$ is illustrated in Appendix S1: Fig. S1.

The contrasting relationships between chemical similarity and phylogenetic distance between sites (Fig. 2) suggest contrasting community assembly and selection regimes. Chemical similarity and phylogenetic distance were decoupled at BCI (Fig. 2a). This suggests chemical differences accrue rapidly at speciation events or with selection for divergence among

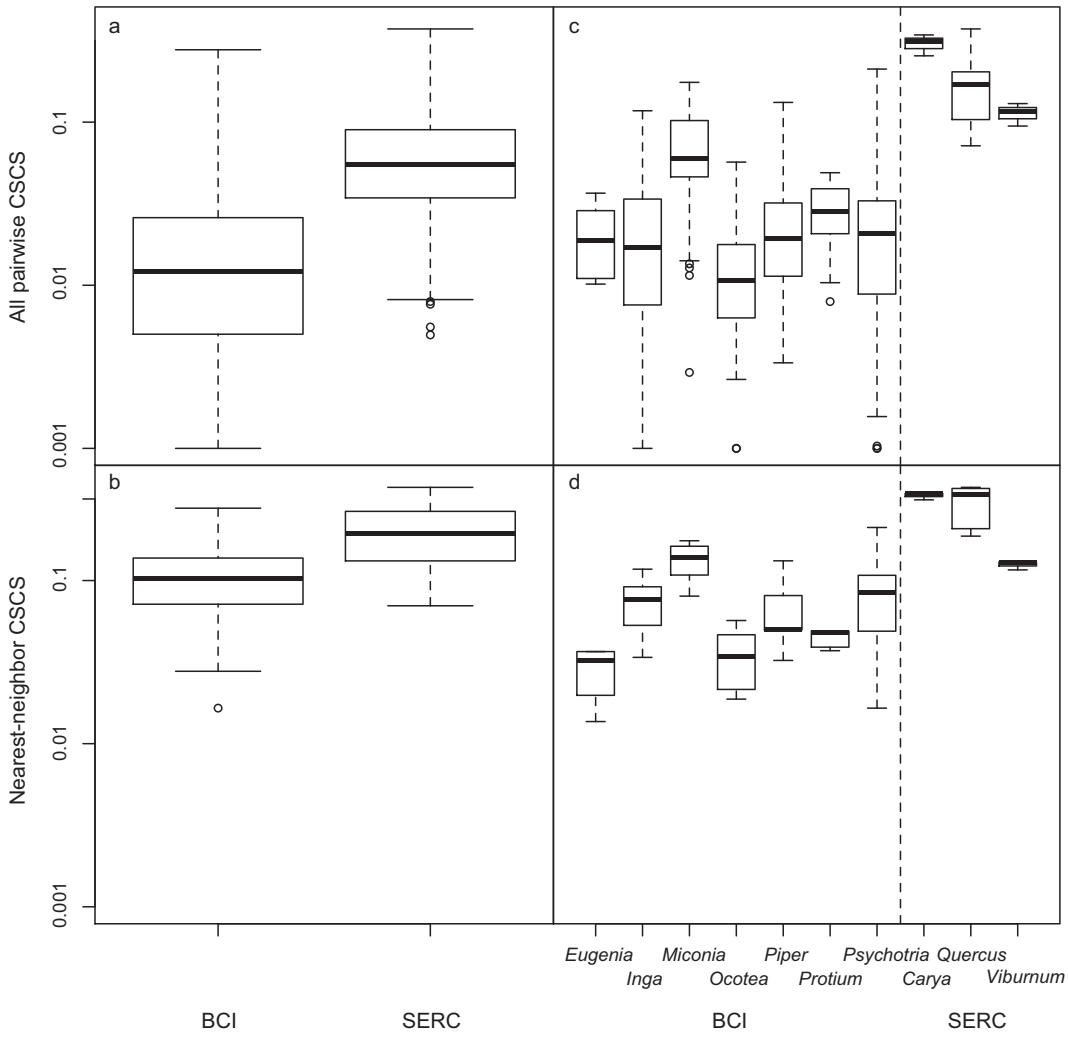

FIG. 3. Tree and shrub species are chemically more similar at SERC and less similar at BCI. Chemical similarity for all pairwise combinations of species from BCI and from SERC (panel a) and of congeners from ten large genera (b). Chemical similarity between nearest neighbors in chemical space ($CSCS_{nn}$) for all species from BCI and from SERC (c) and for congeners from ten large genera (d).

closely related species. In contrast, chemical similarity and log-transformed phylogenetic distance were linearly related at SERC. This exponential decay of chemical similarity suggests a constant net rate of chemical divergence over time (Ricklefs 2007). This marked contrast in phylogenetic signal suggests that selection for chemical divergence among close relatives or competitive exclusion by chemical similarity is stronger in the tropical than in the temperate community.

The absence of phylogenetic signal in foliar metabolomic similarity among BCI tree species presents a stark contrast with leaf functional traits such as mass per area; tissue density; lamina toughness; vein toughness; cellulose, lignin, nitrogen, phosphorus and potassium content; and carbon-to-nitrogen ratio, all of which exhibit phylogenetic signal (Lebrija-Trejos et al. 2014). This contrast suggests that leaf metabolomes diverge more rapidly than leaf functional traits during or shortly after speciation in tropical trees and is consistent with the hypothesis that traits with a greater capacity for reciprocal coevolution between plants and their enemies promote diversification, especially at low latitudes (Schemske et al. 2009).

The hypothesis that biotic interactions are more intense in the tropics and contribute to the global latitudinal diversity gradient has seen recent controversy (Moles et al. 2011*a*, *b*, Anstett et al. 2016). A key prediction of this hypothesis is that plants should invest more in defense or exhibit greater novelty in defense at lower latitudes (Schemske et al. 2009). Recent evaluations of this prediction have focused on quantitative investment in defense, with mixed results (Coley and Aide 1991, Moles et al. 2011*a*, *b*). In contrast, our data suggest that qualitative chemical differences are greater among tropical species than among temperate species. Qualitative differences in chemical defenses have the potential to constrain the host ranges of herbivores and pathogens, enabling enemy-based niches, and may be especially important among members of species-rich tree genera that otherwise share similar niches (Kursar et al. 2009, Sedio et al. 2012, Salazar et al. 2016). Our results suggest that these qualitative differences evolved more rapidly for a tropical than a temperate community (Fig. 2), and hence that selection for divergence in secondary metabolites is greater in tropical than in temperate plants, even if quantitative investment is not (Moles et al. 2011*a*, *b*).

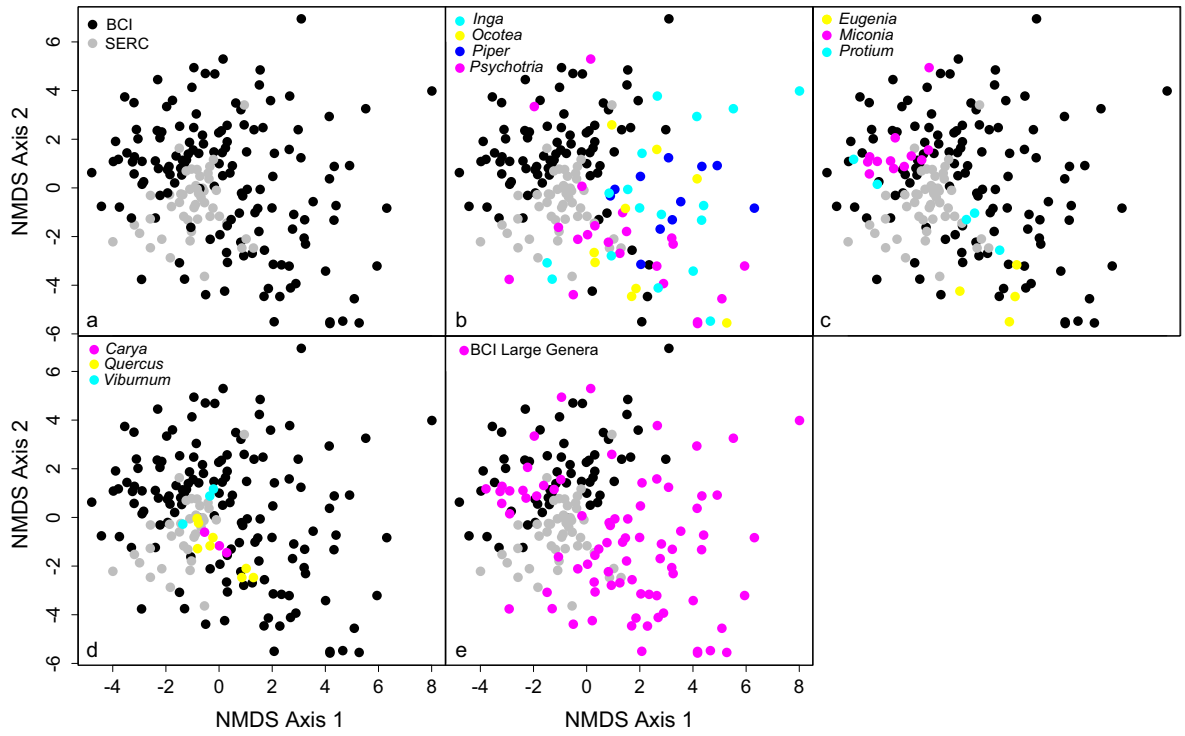

FIG. 4. Non-metric multidimensional scaling of pairwise CSCS chemical similarity for 185 tree and shrub species. Each point represents one species, and the distances between points reflect the pairwise CSCS similarity between all pairs of species, represented in two dimensions. The 185 species include 138 species from Barro Colorado Island, Panama (black points in all panels plus colored points in panels b, c and e) and 47 native species from the Smithsonian Environmental Research Center, Maryland (gray points in all panels plus colored points in d). Colors represent seven of the largest genera at BCI (b,c), the three largest genera at SERC (d), or all seven large BCI genera (e).

The extension of our conclusions beyond one tropical and one temperate forest to understand global ecological patterns will require comparative forest metabolomics of multiple sites along broad latitudinal gradients using consistent methods. Ideally, these sites would include several biogeographic regions. By permitting the study of hundreds of thousands of metabolites in hundreds of species, the metabolomics approach presented here enables a more mechanistic understanding of the role that chemical variation plays in niche partitioning and in lineage diversification at community, biogeographic, and macroevolutionary scales (Sedio 2017). Ultimately, collaborative efforts to integrate metabolomics with plant-enemy associations, plant performance relative to chemical similarity of neighbors, and phylogeny over geographically diverse sites will be necessary to test the hypothesis that chemically mediated biotic interactions are a primary contributor to global patterns of plant diversity.

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Supporting Information

Additional supporting information may be found in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ecy.2533/suppinfo

Data Availability

Data from this publication can be found on the University of California San Diego MassIVE Repository at: http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=d1f7f083fa554f2c9608f238c1ccda0e