

1 Running Head: Chemical diversity in forest trees

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3 Title: Comparative metabolomics of forest communities: Species differences in foliar chemistry
4 are greater in the tropics.

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Abstract

Interspecific variation in the secondary metabolites of plants constrains host specificity of insect herbivores and microbial pathogens. The intensity and specificity of these plant-pest interactions is widely believed to increase towards the Equator, leading to the prediction that secondary metabolites should differ more among co-occurring plant species in tropical communities than in temperate communities. 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 evaluated how pairwise metabolomic similarity varies phylogenetically. Leaf metabolomes exhibited clear phylogenetic signal for the temperate plot, with high similarity among congeneric species. In contrast, leaf metabolomes lacked phylogenetic signal for the tropical plot, with low similarity among congeners. Our results suggest that species differences in secondary chemistry comprise important axes of niche differentiation among tropical trees, especially within species-rich genera, and that the contribution of species differences in secondary chemistry to niche differences increases towards the equator in forest tree communities.

Keywords: chemical ecology, forest ecology, mass spectrometry, molecular network, anti-herbivore defense, species coexistence, Barro Colorado Island.

41 Recent innovations in metabolomics promise new insights into the causes of the well-known
42 latitudinal gradient in plant diversity. Wallace (1878) and Dobzhansky (1950) proposed that
43 biotic interactions comprise a stronger selective force than the physical environment in the
44 tropics, and herbivore and pathogen pressure is indeed greater in the tropics than at higher
45 latitudes (Coley and Barone 1996, Schemske et al. 2009, Lim *et al.* 2015). Ehrlich and Raven
46 (1964) hypothesized that coevolution between herbivores and pathogens and plant defenses
47 drives diversification of plants and their natural enemies. If latitudinal variation in selection
48 exerted by plant enemies contributes to greater tropical plant diversity, tropical plants should be
49 better defended and have more variable defenses than temperate plants. Several authors have
50 tested this prediction with respect to quantitative investment in chemical defenses, such as
51 tannins and phenolic compounds (e.g. Coley and Aide 1991), but a recent meta-analysis (Moles
52 *et al.* 2011a) and a multi-site empirical study (Moles *et al.* 2011b) found no support for the
53 prediction that tropical plants are better defended.

54 Qualitative differences in the small-molecule metabolite profiles, or metabolomes, of
55 plants may play an important role in generating and maintaining species diversity by
56 constraining the host ranges of plant enemies. Herbivore host ranges are narrower in the tropics
57 than at higher latitudes (Dyer *et al.* 2007), and focused studies of tropical tree genera have found
58 that congeneric species are often remarkably divergent in secondary chemistry (Becerra 1997,
59 Kursar *et al.* 2009, Fine *et al.* 2013, Richards *et al.* 2015, Salazar *et al.* 2016, Sedio *et al.* 2017).
60 Such differences in secondary metabolites may allow closely related species to carve out
61 “niches” defined by the insects and microbes they support, and those they avoid. If the
62 importance of biotic interactions in shaping plant communities varies over latitude, temperate
63 and tropical forests may differ in the extent to which co-occurring species differ with respect to

64 secondary metabolites. Few studies have considered interspecific metabolomic variation among
65 temperate forest plants (Agrawal *et al.* 2009, Mason *et al.* 2016), and none has compared such
66 variation in a temperate and a tropical forest at the community scale.

67 Many thousands of plant compounds influence their biotic interactions. The structures of
68 most plant metabolites remain unknown (Wang *et al.* 2016) and any given compound is likely to
69 be shared by few species in a community. This combination of vast chemical diversity, unknown
70 molecular structure, and rarity of secondary metabolites has precluded the pursuit of comparative
71 metabolomics at the large taxonomic scales necessary for the study of whole communities (Sedio
72 2017). However, recent innovations in mass spectrometry (MS) bioinformatics make it possible
73 to compare the structures of thousands of unknown metabolites from diverse chemical classes in
74 hundreds of plant species simultaneously. Here, we quantify the structural similarity of all
75 compounds, including the many unidentified compounds (Wang *et al.* 2016). We then quantify
76 chemical similarity for all pairwise combinations of species, incorporating shared compounds
77 and the structural similarity of compounds unique to one species in each pair (Sedio *et al.* 2017).

78 We assess chemical similarity among 138 tropical and 65 temperate plant species to
79 assess differences in chemical diversity and phylogenetic signal. We compare a tropical moist
80 forest in Panama (9° 9' N) and a temperate deciduous forest in Maryland (38° 53' N), USA. We
81 ask to what extent these forests differ with respect to interspecific metabolomic variation and
82 phylogenetic signal in interspecific metabolomic variation. If the role of secondary metabolites
83 in defining species niche differences increases toward the Equator, we expect interspecific
84 metabolomic variation to be greater at our tropical site than our temperate site. If selection for
85 chemical divergence increases toward the Equator, we expect chemical dissimilarity to

86 accumulate more rapidly and phylogenetic signal in metabolomic similarity to be weaker at our
87 tropical site than our temperate site.

88 **Materials and Methods**

89 *Study Sites and Species*

90 Barro Colorado Island (BCI), Panama (9^o 9' N, 79^o 51' W) supports tropical moist forest.
91 The 2010 census of a 50-ha forest dynamics plot (FDP) recorded 301 species with individuals \geq
92 1 cm in diameter at breast height (DBH) (Condit (1998)). We sampled 138 species, including the
93 48 most abundant species, and every species in seven of the eight most species-rich woody
94 genera (*Eugenia* (4 species), *Inga* (17), *Miconia* (12), *Ocotea* (9), *Piper* (11), *Protium* (5) and
95 *Psychotria* (21)). Several of these species-rich genera are paraphyletic but form monophyletic
96 clades when subsidiary genera are merged (Erickson *et al.* 2014). Hence, these figures include
97 *Clidemia* and *Leandra* among the *Miconia*, *Cinnamomum* and *Nectandra* among the *Ocotea*
98 (Erickson *et al.* 2014), *Tetragastris* among the *Protium* (Fine *et al.* 2014), and *Carapichea* and
99 *Palicourea* among the *Psychotria* (Nepokroeff *et al.* 1999). We refer to these monophyletic
100 clades by the most species-rich generic name on BCI. The 138 species represent 89% of the
101 stems \geq 1 cm DBH recorded in the 2010 census.

102 The Smithsonian Environmental Research Center (SERC) in Edgewater, MD (38^o 53' N,
103 76^o 33' W) supports temperate deciduous forest. The 2014 census of a 16-ha FDP recorded 69
104 species with individuals \geq 1 cm DBH. We sampled all 18 introduced species recorded in the FDP
105 and 47 native species, including all species in the three most species-rich genera [*Carya* (3
106 species), *Quercus* (8), and *Viburnum* (3)]. The 47 native species represent 99% of the native
107 stems \geq 1 cm DBH in the FDP.

108 *Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)*

109 We collected expanding, un lignified leaves from the shaded understory for 611 randomly
110 chosen individuals of the 203 focal species between April and August 2014. We stored samples
111 on ice immediately and at -80°C within five hours. Sedio *et al.* (2017, in press) describe
112 chemical extraction and analysis methods. Briefly, 100 mg of homogenized leaf tissue was
113 extracted twice with 700 μL 90:10 methanol:water at pH 5 for 10 min. This solvent extracts
114 small molecules of a wide range in polarity. Mild acidity aids the extraction of alkaloids. We
115 used ultra high-performance liquid chromatography, electrospray ionization and molecular
116 fragmentation, and tandem mass spectrometry (MS/MS) to analyze extracts (Sedio *et al.* in
117 press) and the Global Natural Products Social (GNPS) Molecular Networking software to cluster
118 the MS/MS spectra into consensus spectra that represent unique molecular structures (Wang et
119 al. 2016). We refer to consensus spectra as compounds throughout.

120 Molecular networks that capture the structural similarity of unknown compounds are
121 possible because molecules with similar structures fragment into many of the same sub-
122 structures. Thus, the similarity of mass to charge ratio (m/z) of the fragments of two molecules
123 reflects their structural similarity. We quantified structural similarity for every pair of
124 compounds as the cosine of the angle between vectors defined by the m/z values of their
125 constituent fragments (Wang *et al.* 2016). Cosine values < 0.6 are unlikely to reflect meaningful
126 levels of chemical structural similarity and were omitted from molecular networks (Watrous *et*
127 *al.* 2016). Our MS data and network can be found at

128 <http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=d1f7f083fa554f2c9608f238c1ccda0e>.

129 *Chemical Structural and Compositional Similarity (CSCS)*

130 Sedio *et al.* (2017) developed a metric that quantifies chemical structural-compositional
131 similarity (CSCS) over all compounds in two species. Conventional similarity indices such as

132 Bray-Curtis incorporate shared compounds, but ignore structural similarity of unshared
133 compounds. In contrast, CSCS incorporates the structural similarity of compounds that are
134 unique to each species. A simple example illustrates the implications. Compounds x and y are
135 structurally similar (cosine ≥ 0.6). Species A contains compound x but not y , and species B
136 contains y but not x . In this example, compounds x and y contribute zero to Bray-Curtis
137 similarity, but make a positive contribution to CSCS based on their structural similarity.

138 CSCS weights every pairwise combination of compounds in two species by the product
139 of their similarity (cosine score if ≥ 0.6 or 0 otherwise) and their proportional ion intensity in
140 each species (Sedio *et al.* 2017). To calculate proportional ion intensities, we calculated mean
141 ion intensities for every compound over all individuals and standardized by the summed means
142 for each species. We calculated CSCS for all 20,503 pairs of species. We also recorded the
143 chemical similarity between each species and its nearest neighbor in chemical space by selecting
144 the greatest CSCS value for each species. We refer to this metric as nearest-neighbor CSCS
145 (CSCS_{nn}).

146 *Statistical Analyses*

147 To generate phylogenies for each forest, we pruned the ForestGEO-CTFS mega-
148 phylogeny (Erickson *et al.* 2014) to the 126 and 34 species present in the mega-phylogeny and
149 our BCI and SERC data, respectively (Fig. S1). This excludes species introduced to SERC. We
150 performed phylogenetic ANOVA with the R package ‘geiger’ (Harmon *et al.* 2008) to determine
151 whether CSCS_{nn} differs between forests. To determine whether CSCS differs between forests, we
152 performed ANOVA on random draws of independent pairs of species. CSCS differed
153 significantly between forests if 95% of 10,000 ANOVAs were significant.

154 To evaluate the relationship between phylogeny and metabolomic similarity, we
155 calculated mean CSCS for all pairs of species descended from each node in the phylogeny. We
156 refer to this metric as $CSCS_{mrca}$, where MRCA refers to most recent common ancestor. Figure S2
157 illustrates this calculation. To evaluate phylogenetic signal, we regressed $CSCS_{mrca}$ against log-
158 transformed phylogenetic distance.

159 To test for differences in the chemical space occupied by two groups of species, we first
160 used non-metric multidimensional scaling (NMDS) to reduce the molecular network to two
161 dimensions (using the ‘MASS’ package in R, Venables and Ripley 2002). We then compared the
162 observed difference in area occupied by the two groups with the distribution of differences
163 generated by 10,000 randomizations. Randomizations reassigned species over columns of the
164 pairwise CSCS matrix. The chemical space occupied by two groups differed significantly if the
165 observed difference in area was greater than 95% of randomized differences.

166 All analyses excluded the 18 introduced species at SERC. Appendix S1 presents results
167 of analyses that include the introduced species.

168 **Results**

169 We detected 126,746 compounds, ranging from 107.06 to 2,174.66 Daltons (Da), in foliar
170 extracts of 185 native species from BCI and SERC. The GNPS database of natural products
171 (Wang et al. 2016) included 130 matches with these compounds. The matches include
172 flavonoids, piperazines, quinoline alkaloids, indole alkaloids, and terpenoids, classes of plant
173 secondary metabolites known to include anti-herbivore defenses (Fig. 1). Networks of
174 compounds linked by cosine scores ≥ 0.6 ranged in size from 2 to 23,029 compounds, and
175 95,407 compounds had cosine scores < 0.6 with every other compound (Fig. 1). In many
176 instances, compounds unique to one or a few species comprise subnetworks of structurally

177 similar compounds (Sedio et al. 2017). Such clusters of structurally similar compounds may
178 represent structural precursors or alternative products from shared metabolic pathways.

179 The tropical, BCI species exhibited lower chemical similarity ($p < 0.0001$; Fig. 2a) and
180 lower chemical similarity to their nearest neighbor in chemical space (PGLS ANOVA $F_{1,158} =$
181 45.78 , $p < 0.0001$; Fig. 2b) than the temperate, SERC species. The largest genera made an
182 important contribution to these site differences, with $CSCS$ and $CSCS_{nn}$ being much lower for
183 the seven most species-rich BCI genera than for the three species-rich SERC genera (Fig. 2c-d).

184 Among BCI species, $CSCS_{mrca}$ was unrelated to log-transformed phylogenetic distance of
185 most recent common ancestors ($t = -1.28$, $df = 123$, $p = 0.205$; Fig. 3a), indicating a strong
186 tendency for chemical divergence among closely related species in this tropical forest. Among
187 SERC species, $CSCS_{mrca}$ was strongly related to phylogeny ($t = -3.59$, $df = 31$, $p = 0.001$; Fig.
188 3b), indicating that closely related species have similar metabolomes.

189 The NMDS ordination illustrates the chemical space represented by 138 BCI species and
190 47 native SERC species (Fig. 4a). Species comprising the largest BCI genera occupy a greater
191 area in chemical space than the remaining BCI species ($p < 0.001$; Figs. 4b, 4c and 4e). In
192 contrast, species comprising the largest SERC genera do not comprise a greater chemical space
193 than the remaining SERC species ($p = 0.707$; Fig. 4d). Results were qualitatively similar for
194 analyses that included the 18 introduced SERC species (Appendix S1).

195 Discussion

196 There are fundamental chemical differences between trees from tropical, Panama and
197 temperate, Maryland. The tropical tree species are chemically more distinctive (or dissimilar)
198 when compared to most recent common ancestors (Figs. 3a,b), to the most chemically similar
199 species (Figs. 2b, 4a), and over all species (Figs. 2a, 4a). Chemical similarity was also

200 consistently lower among species-rich tropical genera than among species-rich temperate genera
201 (Figs. 2c, 2d, 4b, 4c and 4d). These results are consistent with the hypothesis that plant-enemy
202 interactions are more intense in the tropics, leading to rapid evolution of phytochemical diversity
203 in tropical versus temperate trees.

204 The contrasting relationships between chemical similarity and phylogenetic distance for
205 most recent common ancestors (Fig. 3) suggest contrasting selection regimes. In the BCI
206 community, chemical similarity and phylogenetic distance are decoupled (Fig. 3a). This suggests
207 chemical differences accrue rapidly at speciation events or with selection for divergence among
208 closely related species. In the SERC community, chemical similarity and log-transformed
209 phylogenetic distance are linearly related. This exponential decay of chemical similarity suggests
210 a constant rate of chemical divergence over time. This marked contrast in phylogenetic signal
211 suggests that selection for chemical divergence among close relatives is stronger in the tropical
212 community and weaker in the temperate community.

213 The absence of phylogenetic signal in foliar metabolomic similarity among BCI tree
214 species presents a stark contrast with leaf functional traits such as mass per area; tissue density;
215 lamina toughness; vein toughness; cellulose, lignin, nitrogen, phosphorus and potassium content;
216 and carbon-to-nitrogen ratio, all of which exhibit phylogenetic signal (Lebrija-Trejos *et al.*
217 2014). This contrast suggests that leaf chemical traits diverge more rapidly than leaf functional
218 traits during or shortly after speciation in tropical trees and is consistent with the hypothesis that
219 reciprocal coevolution between plants and their enemies promotes diversification, especially at
220 low latitudes (Ehrlich and Raven 1964, Schemske *et al.* 2009).

221 The hypothesis that biotic interactions are more intense in the tropics and contribute to
222 the global latitudinal diversity gradient has seen much recent controversy (Moles *et al.* 2011a,b).

223 A key prediction of this hypothesis is that plants should be better defended at lower latitudes
224 (Schemske et al. 2009). Recent evaluations of this prediction have focused on quantitative
225 investment in defense, with mixed results (Coley and Aide 1991, Moles et al. 2011a,b). In
226 contrast, our data suggest that qualitative chemical differences are greater among tropical species
227 than among temperate species. Qualitative differences in chemical defenses have the potential to
228 constrain the host ranges of herbivores and pathogens, enabling enemy-based niches, and may be
229 especially important among members of species-rich tree genera that otherwise share similar
230 niches (e.g. Kursar *et al.* 2009, Sedio *et al.* 2012). These qualitative differences evolved more
231 rapidly for a tropical community than a temperate community (Fig. 3). Thus, our results suggest
232 selection for divergence in secondary metabolites is greater in tropical than in temperate plants,
233 even if quantitative investment is not (e.g. Moles *et al.* 2011a,b).

234 The extension of our conclusions beyond one tropical and one temperate forest to
235 understand global ecological patterns will require comparative forest metabolomics of multiple
236 sites along broad latitudinal gradients using consistent methods. Ideally, these sites would
237 include several biogeographic regions. By enabling the study of hundreds of thousands of
238 metabolites in hundreds of plant species, the forest metabolomic approach presented here
239 promises to enable a more mechanistic understanding of the role that interspecific chemical
240 variation plays in niche partitioning among co-occurring species and in lineage diversification at
241 community, biogeographic, and macroevolutionary scales (Sedio 2017). Ultimately, integrating
242 forest metabolomics with plant-enemy associations, recruitment dynamics, and phylogeny over
243 geographically diverse sites will provide a critical test of the hypothesis that chemically mediated
244 biotic interactions are a primary contributor to global patterns of plant diversity.

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323

324 Fig. 1. Molecular network of 36,223 compounds in leaves of tree and shrub species from SERC,
325 Maryland and BCI, Panama. Nodes represent compounds. Links between nodes represent
326 structural similarity between compounds indicated by cosine similarity scores ≥ 0.6 . Colors
327 represent compounds found 79 species in seven large genera at BCI (light blue), another 59 BCI
328 species (dark blue), 14 species in three large genera at SERC (yellow) and another 33 SERC
329 species (orange). The 130 known compounds identified chemical classes (e.g. 'flavonoids'). We
330 severed links with cosine scores < 0.8 to break the largest network into smaller networks for
331 visualization. Three subnetworks are highlighted at right to illustrate compound matches to
332 GNPS libraries. Matched compounds are I) ReSpect:PS043007 Puerarin, II) ReSpect:PM007810
333 3'-O-Methyluteolin 6-C-glucoside, III) ReSpect:PS086308 Orientin, IV) GNPS:Vitexin,
334 ReSpect:PM007805 Isoorientin, VI) GNPS:Orientin, VII) GNPS:Hexanoside of (iso)orientin,
335 VIII) GNPS:Pentoside of (iso)vitexin, IX) Massbank:PB006223 Vitexin-2''-O-rhamnoside, X)
336 GNPS:Soyasaponin I, XI) GNPS:MLS000111555-01! Tetrahydroalstonine, XII)
337 GNPS:Yohimbine.
338

339 Fig. 2. Tree and shrub species are chemically more similar at SERC and less similar at BCI.
340 Chemical similarity for all pairwise combinations of species from BCI and from SERC (panel a)
341 and of congeners from ten large genera (b). Chemical similarity between nearest neighbors in
342 chemical space ($CSCS_{nn}$) for all species from BCI and from SERC (c) and for congeners from
343 ten large genera (d).

344

345 Fig. 3. Relationships between the mean chemical similarity of species descended from each node
346 (or most recent common ancestor, $CSCS_{mrca}$) and log-transformed phylogenetic distance for BCI
347 (panel a) and SERC (b). The dashed and solid red lines represent insignificant and significant
348 linear regressions, respectively. The calculation of $CSCS_{mrca}$ is illustrated in Fig. S2.

349

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

358

359 Fig. 1.

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362 Fig. 2.

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366 Fig. 3.

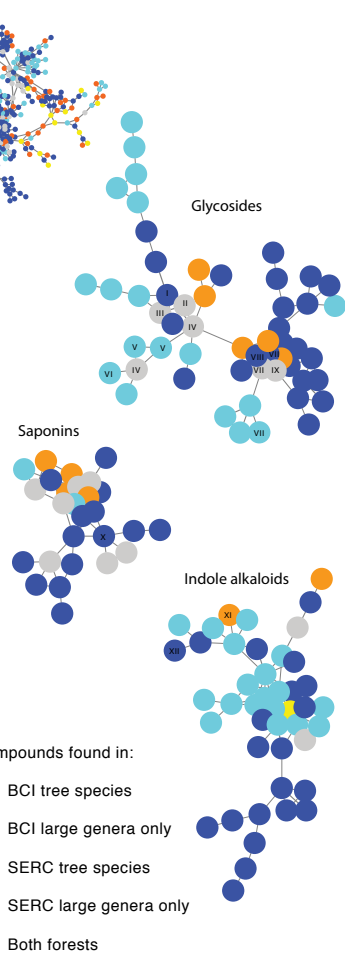
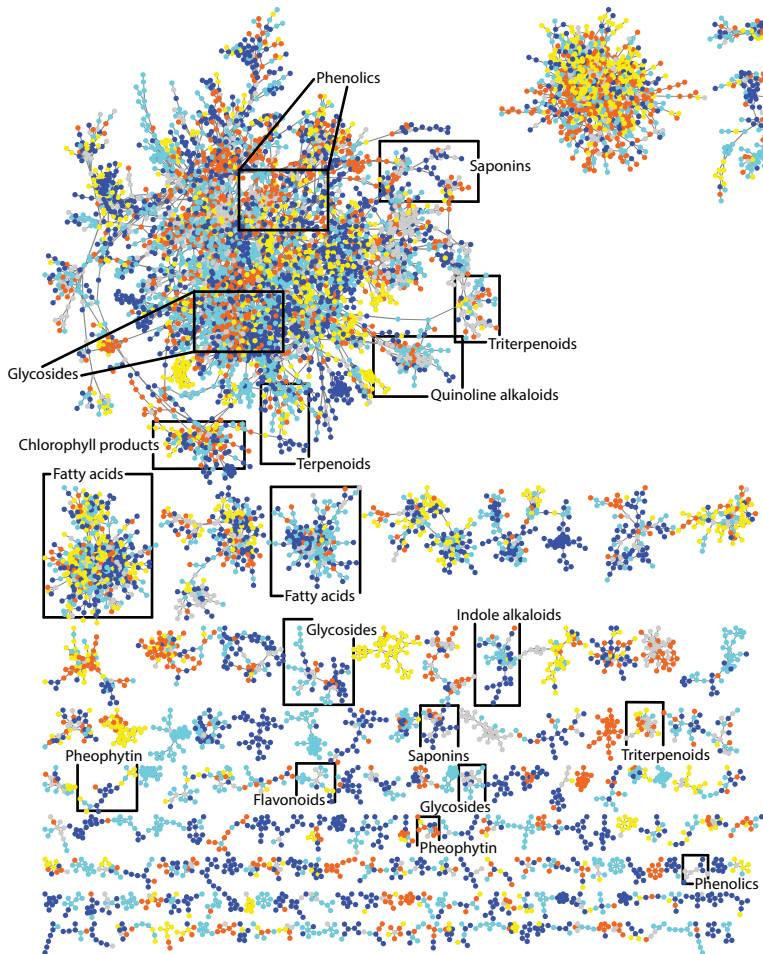
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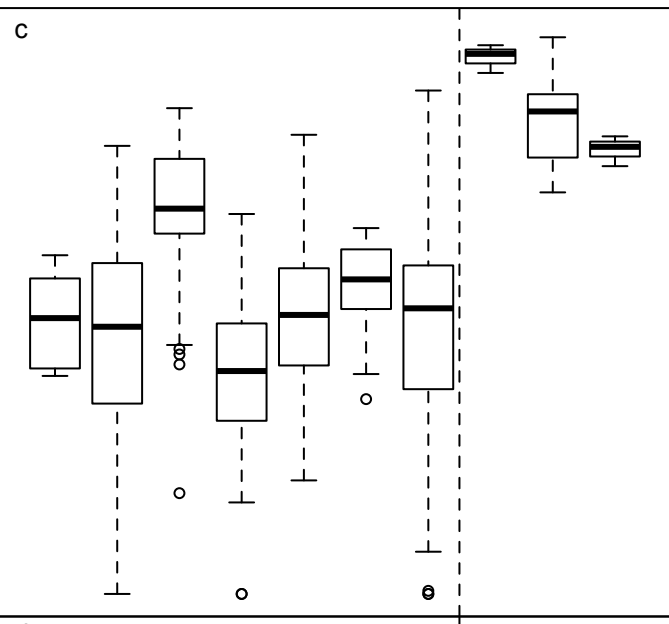
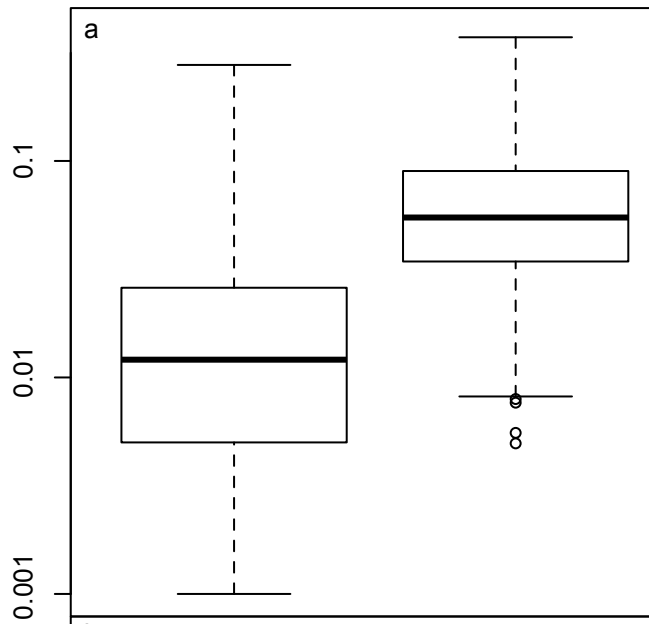
369

370

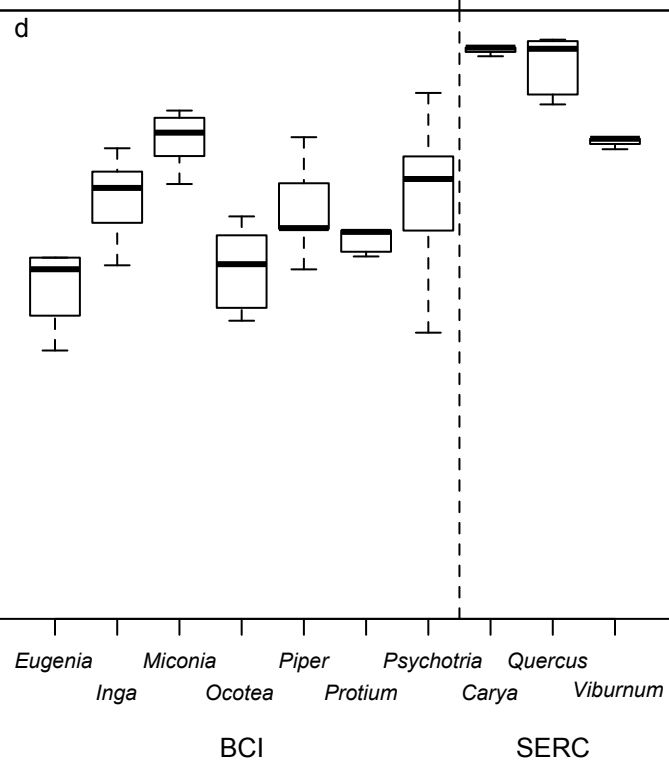
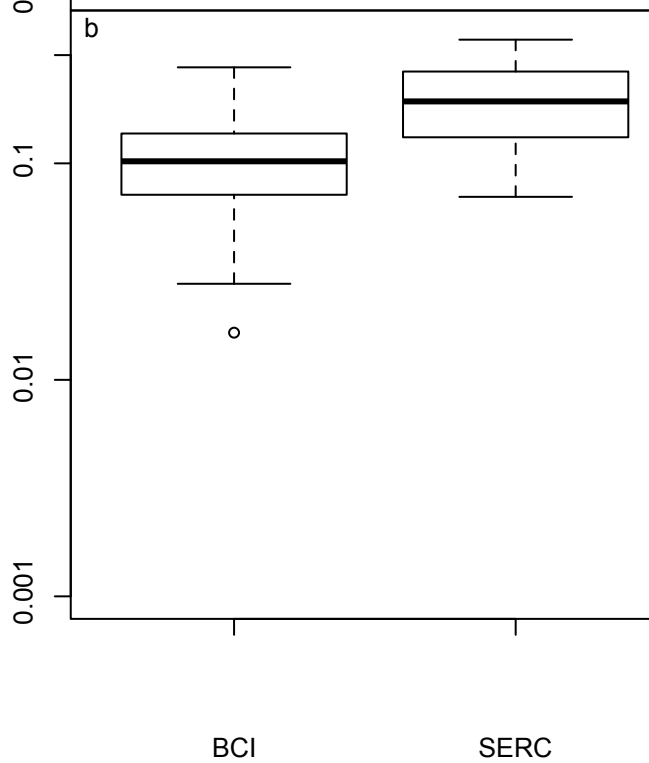
371 Fig. 4



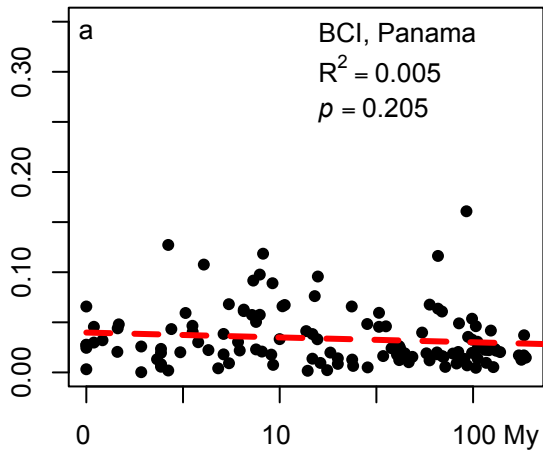
All pairwise CSCS



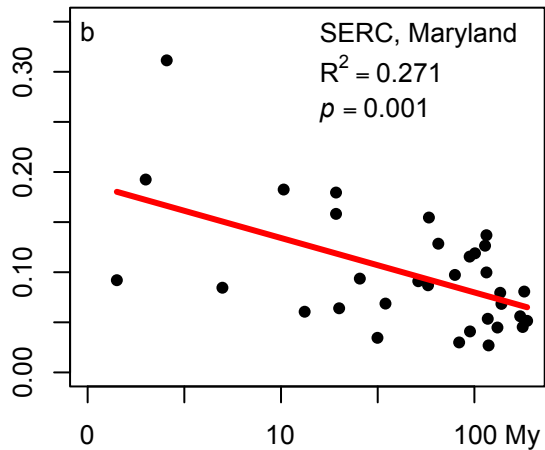
Nearest-neighbor CSCS



Chemical similarity (CSCSmrca)



Phylogenetic distance



Phylogenetic distance

