Evidence for both phylogenetic conservatism and lability in the evolution of secondary chemistry in a tropical angiosperm radiation

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Total word count: 6,461

Introduction word count: 1,344

Materials & Methods word count: 2,177

Results word count: 1,084

Discussion word count: 1,856

Number of figures: 4 (Figs. 1, 2, and 4 should be published in color)

Number of tables: 2

Supplementary material:

Table S1. Sampling information for all individuals.

Fig. S1. Results of the multivariate K test on 1000 permutations of all chemical regions.
Summary

Over evolutionary timescales, shifts in plant secondary chemistry may be associated with patterns of diversification in associated arthropods. Although foundational hypotheses of plant-insect codiversification and plant defense theory posit closely related plants should have similar chemical profiles, numerous studies have documented variation in the degree of phylogenetic signal, suggesting phytochemical evolution is more nuanced than initially assumed. We utilize proton nuclear magnetic resonance ($^1$H NMR) data, chemical classification, and genotyping-by-sequencing to resolve evolutionary relationships and characterize the evolution of secondary chemistry in the Neotropical plant clade Radula (Piper, Piperaceae). Sequencing data substantially improved phylogenetic resolution relative to past studies, and spectroscopic characterization revealed the presence of 35 metabolite classes. Broad metabolite classes displayed strong phylogenetic signal, whereas the crude $^1$H NMR spectra featured evolutionary lability in chemical resonances. Evolutionary correlations were detected in two pairs of compound classes (flavonoids with chalcones; $p$-alkenyl phenols with kavalactones), where the gain or loss of a class was dependent on the other’s state. Overall, the evolution of secondary chemistry in Radula is characterized by strong phylogenetic signal of broad compound classes and concomitant evolutionary lability of specialized chemical motifs, consistent with both classic evolutionary hypotheses and recent examinations of phytochemical evolution in young lineages.

Keywords: genotyping-by-sequencing, nuclear magnetic resonance ($^1$H NMR), phylogenetic comparative analyses, phylogenetic signal, phytochemistry, Piper, Radula
Introduction

Plant secondary chemistry affects plant-herbivore interactions at various stages throughout an insect’s lifespan: mixtures of compounds can shape adult oviposition preferences (Thompson & Pellmyr, 1991), specific chemical compounds can stimulate larval feeding (Bowers, 1983, 1984), plant chemistry can deter insect herbivores via toxicity or physiological disruptions (Malcolm, 1994; Zagrobelny et al., 2004), and sequestered metabolites can alter immune function against natural enemies (Smilanich et al., 2009; Richards et al., 2012). Plants capable of developing novel chemical defenses are hypothesized to accrue higher fitness in response to enemy release (e.g., Berenbaum, 1978), potentially resulting in the diversification of plant lineages with conserved chemical phenotypes (the escape and radiate hypothesis; Ehrlich & Raven, 1964; Thompson, 1989; reviewed by Janz, 2011). Coevolutionary hypotheses and plant defense theory (reviewed by Mithöfer & Boland, 2012) have yielded clear predictions that herbivory, additional trophic interactions, and resource availability shape the evolution of plant defenses, including secondary metabolites (Agrawal et al., 2009; Maron et al., 2019). However, an evolutionary response to these biotic and abiotic pressures could be complex and highly context-dependent.

Due in part to the enzymatic complexity of metabolic biosynthesis, phylogenetic conservatism is the null hypothesis for the evolution of plant secondary chemistry (Agrawal & Fishbein, 2006; Salazar et al., 2018). Indeed, expectations of phylogenetic conservatism appear to hold at deep evolutionary scales; for example, the family Solanaceae is characterized by the presence of tropane alkaloids (Griffin & Lin, 2000), though they are consistently present in only 3 of 19 tribes (Datureae, Hyoscyameae, Mandragoreae) and sporadically found elsewhere (Wink, 2003). Further, recent work suggests more classes of secondary metabolites are phylogenetically conserved in large seed plant clades (e.g., eudicots and superasterids) than at lower taxonomic scales (e.g., orders and families) (Zhang et al., 2020). However, at shallower scales, numerous studies provide evidence for evolutionary lability in chemical traits within genera (e.g., Becerra, 1997; Kursar et al., 2009; Agrawal et al., 2009; Rasmann & Agrawal, 2011; Salazar et al., 2016; Moreira et al., 2018; Allevato et al., 2019), suggesting that surveys of phytochemical variation within young plant lineages might yield variable perspectives on the evolution of secondary chemistry. Adding further complexity, many studies have found evidence for strong evolutionary associations among chemical classes (Kariñho-Betancourt et al., 2015; Boachon et al., 2018;
Allevato et al., 2019). For example, Johnson et al. (2014) found a strong positive correlation between flavonoids and phenolic diversity and a strong negative correlation between ellagitannins and flavonoids across a phylogeny of 26 evening primroses (Oenothera: Onagraceae). Such associations are relevant because they may reflect evolutionary constraints, and their causes may be varied. For example, positive associations may be associated with chemical defense syndromes (Agrawal & Fishbein, 2006; Agrawal, 2007) or synergistic effects of multiple classes on herbivore deterrence (Dyer et al., 2003; Richards et al., 2016). Alternatively, negative associations might be consistent with evolutionary tradeoffs or at least different optima in defense space (Agrawal, 2007; Johnson et al., 2014). By leveraging advances in organic chemistry and genomics, we stand to increase phylogenetic and metabolomic resolution to provide novel insight into the evolution of phytochemistry.

Recent advances in chemical ecology have improved perspectives on phytochemical diversity across a broad range of taxonomic groups and metabolite classes (Sedio, 2017; Dyer et al., 2018). High throughput processing of plant tissue, rapid advances in spectroscopy, and improved ordination and network analyses have enabled characterization of metabolomic variation across plant communities (Richards et al., 2016; Salazar et al., 2016, 2018; Dyer et al., 2018; Sedio et al., 2018; Ernst et al. 2019; Kang et al. 2019) and stand to enhance our understanding of phytochemical evolution across taxonomic scales (Sedio, 2017). Additionally, structural metabolomic approaches like $^1$H NMR can provide improved resolution of structural variation across a wide range of metabolite classes. Selection on the plant metabolome is inherently multivariate, arising from diverse herbivore communities and environmental conditions (Fine et al., 2006; Salazar et al., 2018), and even relatively small structural changes can impart disproportionate shifts in bioactivity. Thus, approaches that capture a larger proportion of the structural variation underlying phytochemical phenotypes could be well suited to addressing hypotheses concerning evolutionary patterns.

Next-generation sequencing data has reinvigorated phylogenetic analyses of traditionally challenging groups characterized by recent or rapid diversification (Wagner et al., 2013; Bagley et al., 2020; Léveillé-Bourret et al., 2020) as well as hybridization (Eaton & Ree, 2013; Carter et al., 2019; Hipp et al., 2020). Reduced representation DNA sequencing approaches [e.g., RADseq; genotyping-by-sequencing (GBS)] have been increasingly utilized in phylogenetic studies due to their ability to effectively sample large numbers of orthologous loci throughout the
genomes of non-model organisms without the need for prior genomic resources (Leaché & Oaks, 2017; Parchman et al., 2018). Nearly all such studies have reported increased topological accuracy and support compared with past phylogenetic inference based on smaller numbers of Sanger-sequenced loci (Herrera & Shank, 2016; Massatti et al., 2016; Du et al., 2020), especially when applied to diverse radiations (Wagner et al., 2013; Fernández-Mazuecos et al., 2017; Hamon et al., 2017; Paetzold et al., 2019). While reduced representation approaches have clear phylogenetic utility at relatively shallow time scales, they have also performed well for moderately deep divergence (Eaton et al., 2017; Du et al., 2020).

_Piper_ (Piperaceae) is a highly diverse, pantropical genus of nearly 2,600 accepted species (Callejas-Posada, 2020), with the highest diversity occurring in the Neotropics (Gentry, 1993; Martínez et al., 2015). Chemically, _Piper_ is impressively diverse (Parmar et al., 1997; Dyer & Palmer, 2004; Richards et al., 2015): chemical profiling in a modest number of taxa has yielded 667 different compounds from 11 distinct structural classes thus far (Parmar et al., 1997; Dyer et al., 2004; Kato & Furlan, 2007; Richards et al., 2018). This phytochemical diversity has likely contributed to the diversification of several herbivorous insect lineages that specialize on _Piper_, including most notably the geometrid moth genus _Eois_ (Strutzenberger et al., 2012; Wilson et al., 2012; Jahner et al., 2017). Furthermore, phytochemical variation in _Piper_ communities has been shown to shape tri-trophic interactions and the structure of tropical communities (Dyer et al., 2004; Glassmire et al., 2016; Richards et al., 2018). As a species-rich genus with abundant and ecologically consequential phytochemical variation, _Piper_ represents a valuable system for understanding how the history of diversification underlies the evolution of phytochemical variation.

_Piper_ is an old lineage (~72 Ma), yet most of its diversification occurred in the Neotropics during the last 30-40 My following Andean uplift and the emergence of Central America (Smith et al., 2008; Martínez et al., 2015). The largest clade of _Piper_, Radula, exemplifies this pattern, as much of its extant diversity (~450 species) arose relatively recently during the Miocene (Martínez et al., 2015). Such bouts of rapid and recent diversification have limited the efficacy of traditional Sanger sequencing methods to resolve the timing and tempo of diversification in _Piper_ (Jaramillo et al., 2008; Smith et al., 2008). Past phylogenetic analyses utilizing Sanger-sequenced nuclear and chloroplast regions have consistently inferred eleven major clades within _Piper_; however, phylogenetic resolution within these clades has been elusive.
(Jaramillo et al., 2008; Smith et al., 2008; Molina-Henao et al., 2016; Asmarayani, 2018).

Phylogenetic inference based on genome-wide data spanning a range of genealogical histories has recently improved phylogenetic resolution for diverse radiations (e.g., Wagner et al., 2013; Paetzold et al., 2019), and should facilitate an understanding of evolutionary patterns of phytochemical variation in *Piper* and their consequences for plant-insect codiversification.

We leveraged complementary phylogenomic, metabolite classification, and $^1$H NMR data sets to generate a *Piper* phylogeny and explore the evolution of secondary chemistry within the largest *Piper* clade (Radula). Specifically, our goals were to: 1) resolve the evolutionary relationships within the Radula clade of *Piper* included in this study; 2) characterize metabolomic variation across the genus and within Radula in particular; and 3) quantify the strength of phylogenetic signal and detect evolutionary associations in Radula secondary chemistry. Because secondary chemistry is an emergent composite phenotype of many traits that can evolve semi-independently, we expected to detect mixed strengths of phylogenetic signal and strong associations among a subset of traits over evolutionary time.

**Materials and Methods**

**Study system and sample collection**

For phylogenetic and chemical analyses, we collected leaf material from 71 individuals representing 65 Neotropical *Piper* species from the following clades: Churumayu ($N = 3$), Hemipodium ($N = 1$), Isophyllon ($N = 5$), Macrosthachys ($N = 4$), Peltobryon ($N = 2$), Pothomorphe ($N = 1$), Radula ($N = 44$), and Schilleria ($N = 5$). For chemical profiling and DNA sequencing, we collected the youngest, fully expanded leaves and dried them immediately with silica gel. Vouchers were pressed, dried, and deposited in one or more herbaria for future reference and species verification (Table S1). To investigate the evolution of phytochemistry at a relatively shallow evolutionary scale, we conducted the majority of our sampling within Radula (Martínez et al., 2015).

**Phylogenetic analyses**

Genome-wide polymorphism data was generated for 71 individuals for phylogenetic analyses. Either the same accession sampled for chemical analysis, or an individual from the same population as the one sampled, were sequenced with a genotyping-by-sequencing approach
(Parchman et al., 2012) that is analogous to ddRADseq (Peterson et al. 2012). Briefly, genomic DNA was digested with two restriction enzymes, *Eco*RI and *Mse*I. Sample-specific barcoded oligos containing Illumina adaptors were annealed to the *Eco*RI cut sites, and oligos containing the alternative Illumina adaptor were annealed to the *Mse*I cut sites. Fragments were PCR amplified and pooled for sequencing. The library was size-selected for fragments between 350 - 450 base pairs (bp) with the Pippin Prep System (Sage Sciences, Beverly, MA), and sequenced on two lanes of an Illumina HiSeq 2500 at the University of Texas Genome Sequencing and Analysis Facility (Austin, TX). Single-end, 100 bp, raw sequence data were filtered for contaminants (*E. coli*, *Phi*X, Illumina adaptors or primers) and low quality reads using bowtie2_db (Langmead & Salzberg, 2012) and a pipeline of bash and perl scripts (https://github.com/ncgr/tapioca). We used custom perl scripts to demultiplex our reads by individual and trim barcodes and restriction site-associated bases.

Assembly and initial filtering was conducted with *ipyRAD* v.0.7.30 (Eaton, 2014). *ipyRAD* was specifically designed to assemble RADseq data for phylogenetic applications, permits customization of clustering and filtering, and allows for indel variation among samples (Eaton, 2014). Because a suitable *Piper* genome was not available at the time of analysis, we generated a *de novo* consensus reference of sampled genomic regions with *ipyRAD*. Briefly, nucleotide sites with phred quality scores lower than 33 were treated as missing data. Sequences were clustered within individuals according to an 85% similarity threshold with *vsearch* (Rognes et al., 2016) and aligned with *muscle* (Edgar, 2004) to produce stacks of highly similar RADseq reads (hereafter, RADseq loci). The sequencing error rate and heterozygosity were jointly estimated for all RADseq loci with a depth >6, and these parameters informed statistical base calls according to a binomial model. Consensus sequences for each individual in the assembly were clustered once more, this time across individuals, and discarded if possessing >8 indels (max_Indels_locus), >50% heterozygous sites (max_shared_Hs_locus), or >20% variable sites (max_SNPs_locus). To reduce the amount of missing data in our alignment matrix, RADseq loci were retained if they were present in at least 50 of 71 samples. The nexus file of concatenated consensus sequences for each individual, including invariant sites, were used as input for the Bayesian phylogenetic methods described below. The nexus alignment as well as complete information on additional parameter settings for this analysis are archived at Dryad (https://doi.org/10.5061/dryad.j6q573nc7).

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To resolve patterns of diversification and to provide a foundation for investigating variation in the rates of phytochemical evolution, we estimated a rooted, calibrated tree according to a relaxed clock model in RevBayes v.1.0.12 (Höhna et al., 2016), which provides the ability to specify custom phylogenetic models for improved flexibility compared with other Bayesian approaches. The prior distribution on node ages was defined by a birth-death process in which the hyper priors on speciation and extinction rates were exponentially distributed with $\lambda = 10$. We relaxed the assumption of a global molecular clock by allowing each branch-rate variable to be drawn from a lognormal distribution. After comparing the relative fits of JC, HKY, GTR, and GTR+Gamma nucleotide substitution models with Bayes factors, we modeled DNA sequence evolution according to the best-fit HKY model. Eight independent MCMC chains were run for 100,000 generations with a burn-in of 1,000 generations and sampled every 10 generations. Chains were visually assessed for convergence with Tracer v.1.7.1 (Rambaut et al., 2018) and numerically assessed with effective sample sizes (ESS), the Gelman–Rubin convergence diagnostic (Gelman & Rubin, 1992), and by comparing the posterior probabilities of clades sampled between MCMC chains. The maximum clade credibility (MCC) tree provided the ultrametric fixed tree topology and relative node ages for phylogenetic comparative methods described below.

**Chemical profiling**

Crude proton nuclear magnetic resonance ($^1$H NMR) spectroscopy was chosen for chemotype mapping due to its ability to characterize subtle structural variation across a wide range of compound classes in a single, reproducible, non-destructive analysis (Richards et al., 2018). Briefly, after leaf samples were ground to fine powder, 2.00 g were transferred to a glass screw cap test tube with 10.0 ml of methanol, sonicated for 10 minutes, and filtered. This step was repeated and both filtrates were combined in a pre-weighed 20 ml scintillation vial. The solvent was removed in vacuo and dissolved in 0.6 ml methanol-$d_4$ for $^1$H NMR analysis. Extracts were analyzed on a Varian 400 MHz solution state NMR spectrometer with autosampler. Data were processed using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). Spectra from the crude extracts were aligned with the solvent peak (CD$_3$, $\delta = 3.31$ ppm), baseline corrected, phase corrected, and binned (0.04 ppm; 0.5 - 12 ppm). Solvent
and water peaks were removed and the binned spectra were normalized to a total area of 100.

This data set is referred to subsequently as “crude $^1$H NMR”.

In addition to crude $^1$H NMR spectral chemotyping, we further annotated and characterized samples based upon the presence or absence of compound classes and in some cases, specific compounds. To further gain structural resolution across the crude extracts that were sampled, aliquots of the $^1$H NMR extracts were diluted and subjected to GC-MS and LC-MS analysis. Crude extracts were classified using chemotaxonomic classifications outlined in Parmar’s comprehensive review of Piper phytochemistry (Parmar et al., 1997).

Presumptive compounds and compound classes were annotated based upon structural elucidation using $^1$H NMR, GC-MS fragmentation, and high-resolution LC-MS data. Comparison of the $^1$H NMR data to literature values of related compounds was used to increase confidence in these assignments. In some cases, crude 2D-NMR analysis was used to confirm structural classifications. Presence of a compound or compound class was determined based upon abundant and spectroscopically apparent evidence. This data set is referred to subsequently as “metabolite classes”.

Phylogenetic signal and evolution of metabolite classes

To assess whether metabolite classes were phylogenetically conserved across Radula, we quantified phylogenetic signal in these binary traits using the D statistic (Fritz & Purvis, 2010). The D statistic calculates the sum of sister-clade differences, $\Sigma d_{obs}$ (Felsenstein, 1985) for an observed tree and binary trait, and scales this value with the distributions of sums expected under two disparate evolutionary models, random and Brownian motion ($\Sigma d_{r}$ and $\Sigma d_{b}$, respectively), using the following equation:

$$D = \frac{[\Sigma d_{obs} - mean(\Sigma d_{b})]}{[mean(\Sigma d_{r}) - mean(\Sigma d_{b})]}$$

Thus, D is expected to equal 1 when the observed binary trait is distributed randomly, lacking phylogenetic signal, and is expected to equal 0 when it exhibits phylogenetic signal as expected under Brownian motion. Tests of phylogenetic signal with the D statistic are most accurate when the ratio of presences and absences is closer to 1:1 (Fritz and Purvis, 2010). We used the phylo.d function in the caper package (Orme et al., 2018) in R v.4.0.0 (R Core Team,
(2020) to calculate the observed D for a subset of binary traits that were sufficiently present across the phylogeny. This value was compared to a distribution of D values simulated under models of phylogenetic randomness (D = 1) and pure Brownian motion (D = 0) to determine whether the observed D differed from either zero or one.

To detect evolutionary associations among pairs of metabolite classes within Radula, we used Pagel’s (1994) method that models evolutionary changes in two binary traits, X and Y, as continuous-time Markov processes in which the probabilities of state transition at one trait may depend on the state at the other trait. Significant tests of correlated evolution were followed by tests of contingency, in which changes at X depend on the state of Y, or vice versa. Model fits, comparisons, and plots were performed with the `fitPagel` function in the `phytools` package (Revell, 2012) in R.

**Multivariate analyses of phylogenetic signal with crude ¹H NMR spectra**

While the analyses above based on broad classifications of structurally determined metabolites provide a coarse view of phytochemical evolution, these classifications are anchored to the foundations of plant secondary metabolite biosynthesis. Using ¹H NMR spectra as a raw chemotype should allow a more detailed multivariate perspective on phytochemical variation. Studies on other plant taxa have typically detected some signal and evolutionary correlations for broad classes of compounds but not necessarily for specific compounds or biologically active moieties, both of which can be inferred from ¹H NMR data. Multivariate approaches to phylogenetic comparative methods have provided insight into covarying suites of related traits, while simultaneously increasing the statistical power to detect phylogenetic signal (Zheng et al., 2009) and differences in trait means among taxa (Clavel et al., 2015). Indeed, these multivariate approaches might be particularly useful when exploring the evolution of complex phenotypes, like the plant metabolome, which exhibit trait covariances due to metabolomic or functional associations (Dyer et al., 2003; Richards et al., 2010; Fukushima et al., 2011). Here we utilize three multivariate methods to detect patterns of phylogenetic signal for 263 resonances found in the crude ¹H NMR data: 1) principal components analyses (PCA); 2) multiple regression on distance matrices (MRM); and 3) multivariate estimation of phylogenetic signal.

To visualize patterns of chemotypic variation across all sampled species from all clades, we first analyzed the ¹H NMR data with PCA using the `prcomp` function in R. If the major axes...
of metabolomic variation are phylogenetically conserved, the plotted species scores should be clustered by clade in a rotated principal component (PC) space. Alternatively, if metabolomic variation is randomly distributed across the phylogeny, there should be little to no clustering by clade (Klingenberg & Gidaszewski, 2010). The degree to which plant clade predicted chemical similarity was assessed using permutational multivariate analysis of variance (permanova; Anderson, 2001) in the vegan package (Oksanen et al., 2019) in R based on Euclidean distances of the first four PCs.

Mantel tests have been frequently used to assess the degree of phylogenetic signal in multivariate data (e.g., Cardini & Elton, 2008; Easson & Thacker, 2014; Salazar et al., 2018) by estimating the relationship between phylogenetic and phenotypic distances. Simulations under scenarios of measurement error have found instances where Mantel tests outperform traditional univariate methods in detecting phylogenetic signal, especially as the number of traits increases (Hardy & Pavoine, 2012). Because we were unable to account for measurement error in our study, we utilized MRM to examine the relationship between metabolomic and phylogenetic distance at two evolutionary scales (within Radula and across all clades). Euclidean distances were calculated with the crude $^1$H NMR spectra using the dist function in R, and two measures of phylogenetic distance were used as predictors: 1) Abouheif’s proximity (Abouheif, 1999; Pavoine et al., 2008) was calculated using the proxTips function in the adephylo package (Jombart et al., 2010) in R; and 2) the square root of patristic distance was calculated using the cophenet.phylo function in the ape package (Paradis et al., 2004) in R. MRM analyses were implemented using the MRM function with 1000 permutations in the ecodist package (Goslee & Urban, 2007) in R.

Since Blomberg et al.’s (2003) $K$ statistic exhibits higher statistical power to detect phylogenetic signal relative to Mantel tests (Harmon & Glor, 2010), we quantified phylogenetic signal of the crude $^1$H NMR at both evolutionary scales using a multivariate generalization of the $K$ statistic ($K_{\text{mult}}$; Adams, 2014) with the physignal function in the geomorph package (Adams et al., 2013) in R. The $K$ statistic provides a statistical estimate of phylogenetic signal relative to expectations under Brownian motion, where values of $K$ greater than 1 indicate phylogenetic signal greater than expected under Brownian motion, whereas values between 0 and 1 indicate less signal than expected under Brownian motion. Significance for the generalized $K$ statistic was assessed by permuting the $^1$H NMR peak data among the tips of the phylogeny for 999 iterations.
To determine whether the zero-inflated nature of the $^1$H NMR data influenced the detection of phylogenetic signal, we permuted our $^1$H NMR data set over 1000 iterations by randomly indexing our original $^1$H NMR data matrix. This permutation method preserves the original proportion of zeros in the matrix while obfuscating any observed phylogenetic signal. The generalized $K$ statistic test was calculated for each permutation, and our observed generalized $K$ statistic was compared to the null distribution of permuted values.

Results

Phylogenetic analyses

After contaminant filtering and demultiplexing, we retained ~313 million Illumina reads for phylogenetic analyses. Initial clustering, variant calling, and filtering clustered reads into 362,169 RADseq loci. There was a high proportion of missing data, presumably due to allelic dropout increasing with high levels of divergence among Piper clades. For Bayesian phylogenetic inference, we mitigated the influence of missing data by removing loci absent in >30% of samples. The final dataset for phylogenetic analyses consisted of 641 RADseq loci (~86 bp in length each) that housed 9,113 genetic variants (51% parsimony informative). Aligned loci were concatenated into a nexus alignment with missing data at 18.9% of sites.

Bayesian phylogenetic analysis of ddRADseq data resolved eight major Neotropical Piper clades with high posterior support (Fig. 1). While past phylogenetic studies supported the monophyly of seven of these eight clades (Macrostachys, Radula, Peltobryon, Pothomorphe, Hemipodion, Isophyllon, and Schilleria) (Jaramillo et al., 2008; Martínez et al., 2015), our analysis resolved an additional clade, Churumayu. Notably, Isophyllon and Churumayu were highly supported, monophyletic clades and not nested within Radula as was inferred in previous analyses (Jaramillo et al., 2008). Contrary to previous phylogenetic hypotheses of Piper (Jaramillo et al., 2008; Martínez et al., 2015), our analyses might suggest Churumayu is the most basal clade, but we caution that this node had very low posterior support (51%). Intragenic relationships below the clade level were highly resolved, with nearly all nodes exhibiting greater than 95% posterior support (Fig. 1), including within the diverse Radula clade (Fig. 1). Our phylogenetic hypothesis for Radula indicates three species ($P. hispidum$, $P. colonense$, $P. lucigaudens$) may be paraphyletic, reflecting past taxonomic uncertainty for these taxa.
Phytochemical variation in *Piper*

Nearly all common compound classes that have been previously reported in *Piper* were observed from our compound characterization analysis (Salehi *et al.*, 2019). This analysis revealed the presence of metabolite classes that are ubiquitous across plant families (lignans, flavonoids/chalcones, etc.) as well as classes that are specifically common in *Piper* (amides) (Fig. 2). Specific compound characterization revealed genus specific compounds and compound classes (piplartine, cenocladamide, crassinervic acid, kava lactones), as well as metabolites that are more rarely reported in plants (putrescine diamides, nerolidyl catechol, alkenyl phenols, anuramide peptides) (Fig. 2).

Metabolite phylogenetic signal and evolutionary associations

For all eight metabolite classes that were examined, estimates of D (Fritz & Purvis, 2010) were low and did not deviate from a null distribution generated under a scenario of Brownian motion (Table 1), consistent with phylogenetic signal. Two of the eight traits, phenolic glycosides and lignans, exhibited strong phylogenetic signal ($D < 0$), while the remaining six traits exhibited weak phylogenetic signal ($0 < D < 1$). Further, all metabolite classes had observed values of $D$ that differed from a null distribution generated under a phylogenetic randomness scenario (Table 1). The mean of the observed $D$ estimates for the metabolite classes was 0.04, with the largest $D$ statistic observed for the flavonoid class ($d_{obs} = 0.49$) and the smallest observed for the phenolic glycosides ($d_{obs} = -1.18$) (Table 1).

Evidence for correlated evolution was detected in two pairs of metabolite classes: 1) flavonoids and chalcones; and 2) $p$-alkenyl phenols and kavalactones/butenolides. For the first pair of traits, a model of contingency in which changes in chalcones depend on the state of flavonoids provided the best fit to the data (Table 2). In this model, when flavonoids are present, chalcone gains are almost two times more probable than chalcone losses; however, when flavonoids are absent, chalcone losses are much more probable than chalcone gains (Fig. 3). The alternative contingency model for this pair of traits (i.e., changes in flavonoids depend on the state of chalcone) was also a good fit to the data (Table 2). According to this model, when chalcones are present, flavonoid transitions are extremely probable, with flavonoid gains being approximately eight times more probable than flavonoid losses. Alternatively, when chalcones are absent, flavonoid losses are approximately five times more probable than flavonoid gains.
For the second pair of traits, \( p \)-alkenyl phenols and kavalactones/butenolides, the best fit model was one of interdependent correlated evolution in which changes in \( p \)-alkenyl phenol depend on the state of kavalactones/butenolides, and vice versa (Table 2). When kavalactones/butenolides are present, \( p \)-alkenyl phenol transitions are more probable than when they are absent, with the loss of \( p \)-alkenyl phenols being much more probable than the gain of \( p \)-alkenyl phenols under both scenarios. Alternatively, when \( p \)-alkenyl phenols are present, the loss of kavalactones/butenolides is extremely probable relative to the gain of kavalactones/butenolides, which is rarely observed. When \( p \)-alkenyl phenols are absent, kavalactones/butenolides are rarely gained or lost (Fig. 3).

**Phylogenetic signal in high-dimensional metabolomic data**

While broad metabolite classes uniformly exhibited at least moderate levels of phylogenetic signal, evidence for phylogenetic signal in multivariate analyses of the crude \(^1\)H NMR data was mixed. PCs 1 & 2 and 3 & 4 explained 47.89% and 17.16% of variance in the \(^1\)H NMR data, respectively, but showed little clustering by clade (Fig. 4a). Permutational multivariate analyses of variance were not significant for combinations of neither PC 1 & 2 (\( P = 0.635 \)) nor 3 & 4 (\( P = 0.445 \)), suggesting that different clades do not form distinct clusters in chemospace based on their \(^1\)H NMR spectra.

According to the MRM models, both patristic distance and Abouheif’s proximity significantly predict a small proportion of variation in chemical distance calculated among *Piper* samples from all clades (patristic: \( \beta = -6400.217, R^2 = 0.002, P = 0.005; \) Abouheif: \( \beta = 8.673, R^2 = 0.003, P = 0.001 \)) and among Radula samples only (patristic: \( \beta = -5480.108, R^2 = 0.004, P = 0.003; \) Abouheif: \( \beta = -6.456, R^2 = 0.002, P = 0.005 \)) (Fig. 4bc). Though explained variance is small, the slope coefficients for these significant relationships are negative, indicating that decreasing phylogenetic distance is associated with increasing chemical distance.

Analyses with the generalized \( K \) statistic (\( K_{\text{mult}} \); Adams, 2014) indicated lower levels of phylogenetic signal in the metabolomic data than expected under a Brownian motion model of evolution for *Piper* generally (\( K_{\text{mult}} = 0.1606, P = 0.001 \)) and for Radula specifically (\( K_{\text{mult}} = 0.1803, P = 0.001 \)). Still, the observed \( K_{\text{mult}} \) was higher than all \( K_{\text{mult}} \) values obtained with permutations of the \(^1\)H NMR dataset (Fig. S1). Additionally, few \( K_{\text{mult}} \) tests of the permuted data yielded significant \( P \)-values (4.4% of permutations), indicating that the estimate we observed,
though subtle and lower than Brownian motion expectations, was real and not a statistical artifact of zero-inflation in the data.

Discussion

Piper is a hyper-diverse lineage in which phytochemical variation has influenced evolutionary and ecological processes and shaped complex tropical communities (e.g., Salazar et al., 2016; Richards et al., 2018). However, there have been limitations in both the degree of phylogenetic resolution and the understanding of phytochemical variation in this group. Phylogenies inferred here with ddRADseq data substantially improved resolution and support compared to past studies of Piper, which were limited by interspecific variation in small numbers of Sanger-sequenced loci (Jaramillo et al., 2008; Smith et al., 2008; Martínez et al., 2015). Although the data set did not include members from all previously recognized groups, analyses resolved eight monophyletic Neotropical Piper clades, six of which have been inferred in previous analyses of the genus based on chloroplast psbJ-petA and ITS (Jaramillo et al., 2008; Martínez et al., 2015). Two of the eight clades, Churumayu and Isophyllon, had been previously nested within Radula (Jaramillo et al., 2008); however, our results suggest that they are independent monophyletic lineages (Fig. 1). Despite low support for several deep divergences, the phylogeny inferred here had strong resolution and support for recent relationships, including within Radula (Fig. 1), consistent with other recent reduced representation sequencing studies that have generated high quality phylogenies at shallow time scales (Massati et al., 2016; Eaton et al., 2017; Lecaudey et al., 2018; Paetzold et al., 2019). However, a potential limitation of such sequencing designs may include the recovery of fewer loci shared by more distantly related samples due to allelic dropout (Cariou et al. 2013; Cooke et al., 2016). It is possible that allelic dropout, potentially acerbated by strict filtering of missing data, led to weak support values for deep splits in the phylogeny, many of which occurred early in the history of the Neotropical Piper lineage (Martínez et al. 2015). Nonetheless, the resulting subset of data (641 loci; 9,113 SNPs) was sufficient for inferring a largely resolved phylogeny, highlighting the potential promise of reduced representation sequencing for resolving evolutionary histories even in groups spanning moderately deep divergence.

Comparative studies have taken diverse approaches to analyzing metabolomic data, each providing a unique perspective on the evolution of specialized metabolites (e.g., Salazar et al.,
2018; Sedio et al., 2018, 2019; Ernst et al. 2019; Kang et al. 2019). Here, we first characterized the presence/absence of 35 metabolite classes commonly used to categorize plant secondary compounds that are hierarchically nested into three levels of structural resolution. Specific categories at the lowest level of the hierarchy, representing specialized structural motifs or specific molecules, were rare across species and precluded tests of phylogenetic signal at our level of taxonomic sampling (Fig. 2). Despite not being able to test for phylogenetic signal, clustering is evident for more specific categories, such as crassinervic acid and prenylated flavonoids, which are only present in small subclades but include particularly effective defenses (Dyer & Palmer, 2004; Salehi et al., 2019). Alternatively, broader metabolite classes at intermediate and high positions in the hierarchy that are directly tied to fundamental secondary metabolite biosynthetic pathways were more abundant across species and exhibited moderate-high levels of phylogenetic signal across Radula (Table 1, Fig. 2). This pattern may be expected if initial biosynthetic steps are conserved over longer evolutionary scales, permitting the abundance of broad chemical classes, yet later stage modifications of these core structures are more evolutionarily labile, causing structural similarity to be low even among related species. Flavonoids are a good example of this pattern, with pathways that form the flavonoid scaffold being very conserved, as they are catalyzed by modified enzymes from ubiquitous metabolic pathways, but then subsequent biosynthetic steps (e.g., those catalyzed by p450 enzymes) modify these scaffolds (Yonekura-Sakakibara et al., 2019), yielding unique molecules towards the tips of evolutionary trees (Fig. 3E). For example, late-stage modification of common flavonoid scaffolds can result in the production of non-aromatic protoflavanoids. These compounds rarely occur across the plant kingdom and have only recently been found in one species of Piper (Freitas et al., 2014). Importantly, this subtle structural modification that leaves most of the flavonoid scaffold intact has been demonstrated to dramatically enhance the cytotoxic properties compared to that of the parent flavonoid (Hunyadi et al., 2014; Latif et al., 2020).

One key prediction from the escape and radiate hypothesis is that adaptive defensive traits should be phylogenetically conserved within the lineage they evolved, but this prediction has mostly been evaluated with broad classes of secondary metabolites at high taxonomic scales (e.g., Ehrlich & Raven, 1964; Moreira et al., 2018; Yonekura-Sakakibara et al., 2019; Zhang et al., 2020) rather than specific compounds in recent diversifications (e.g., Agrawal et al., 2009; Salazar et al., 2018; Allevato et al., 2019). A growing number of studies conducted at shallower
evolutionary scales suggest chemical traits may be evolutionarily labile and highlight the need for determining the level at which chemical defense is conserved, and which compound classes are more likely to exhibit phylogenetic signal and evolutionary correlations (Kursar et al., 2009; Sedio, 2013; Johnson et al., 2014; Salazar et al., 2016; Maldonado et al., 2017; Moreira et al., 2018). Further, an understanding of the phylogenetic scale of chemical trait conservation will enable insights into the drivers of herbivorous insect radiations, as the nature of codiversification in many of these lineages is likely structured by complex associations between geology, geography, chemical defense, and biotic interactions (Endara et al. 2017; Jahner et al. 2017). Our results are generally consistent with the predictions of signal (and conservatism) for broad classes of compounds, as well as the lack of signal for specific structures captured by $^1$H NMR data.

The $^1$H NMR data address a different set of hypotheses than data from categorization of individual molecules – peaks represent resonances associated with particular molecular structures rather than individual compounds, and the chemical shift (frequency), shape, and abundance of these resonances are extremely sensitive to subtle structural changes. $^1$H NMR spectroscopy easily detects a great range and subtle differences in compositional and structural complexity, including increasing size, asymmetry and oxidation states, that might be predicted to evolve in response to divergent selection across plant populations responding to different suites of enemies (Dyer et al., 2018). Low levels of phylogenetic signal in the $^1$H NMR data and evidence for phylogenetic overdispersion (Fig. 4) is also likely due to the fact that many molecular features of small defensive molecules have potentially evolved in a convergent manner across Piper, such as the kavalactones, $p$-alkenyl phenols, plaptartine, oxidized prenylated benzoic acids, chromanes, anuramide peptides, and phenethyl amides.

There are numerous limitations that could affect estimates of phylogenetic signal in comparative studies (reviewed by Kamilar & Cooper, 2013) that are relevant to the analyses presented here. First, incomplete taxon sampling and unresolved tree structure can substantially influence tests of phylogenetic signal and likely influenced our results to some degree. However, we made great effort to sample species from across the entire known phylogeny of Radula to reduce sampling bias, and more comprehensive genomic sampling produced enhanced phylogenetic resolution of the Radula clade, where we focused the majority of phylogenetic comparative methods. In addition, we were unable to quantify the measurement error associated
with the chemical traits within species (e.g., Johnson et al., 2014), which can decrease the
statistical power for detecting phylogenetic signal (Blomberg et al., 2003; Ives et al., 2007;
Hardy & Pavoine, 2012). It is also possible that environmental effects on our chemical traits
could bias estimates of phylogenetic signal and correlations (Ives et al., 2007).

The causes of correlated evolution, including linkage, epistasis, and selection, are
difficult to detect without careful approaches in quantitative genetics and population genomics.
Nevertheless, one advantage of examining the presence/absence of multiple classes of defensive
compounds in a phylogenetic context is that it is possible to test for expected patterns of
correlated evolution due to shared metabolic pathways (e.g., flavonoids and cardenolides;
Agrawal et al., 2009) or due to adaptive advantages of specific mixtures. Recent studies
detecting evolutionary associations among chemical traits (Johnson et al., 2014; Kariñho-Betancourt et al., 2015; Boachon et al., 2018) have posited that the branching structure of
metabolic pathways could potentially drive this pattern. If metabolite classes share a common
precursor, one might expect evolutionary tradeoffs and negative covariation. Alternatively, if
metabolite classes lie along the same metabolic pathway, an increase in one class may be
concomitant with increases in another (or vice versa) causing positive covariation among the
classes. There are also numerous empirical examples supporting the hypotheses that positive
correlations may be driven by functional redundancy (Jones & Firn, 1991; Romeo et al., 2013) or
selection for synergistic effects on herbivores (Dyer et al., 2003; Richards et al., 2010) rather
than the structural constraints of metabolism. Suites of covarying defensive traits, or defense
syndromes, have been detected in several plant genera (Becerra et al. 2001; Agrawal & Fishbein,
2006; Endara et al. 2017) and plant communities (Kursar & Coley, 2003), and have been
predominantly used to describe covariation among mechanical and chemical defenses. It is
interesting to note the correlated evolution of the flavones/chalcones and the $\mu$-alkenyl
phenols/kavalactones could be due to metabolic constraints, as well as possible adaptations via
synergistic (e.g., kavalactones in P. methysticum) or other mixture-associated defensive attributes
(reviewed in Dyer et al., 2018). Flavonoids and chalcones are directly linked biosynthetically,
such that the inherent reactivity of the chalcone moiety permits the enzymatic processes that
result in cyclization to the flavonoid scaffold (Fig. 3E). This strong biosynthetic tie predicts the
presence of one would depend on the other, and indeed our structural analysis found many cases
where both metabolite classes co-occurred in the same sample. Revealing the relationship
between the kavalactones and \( p \)-alkenyl phenols is more tenuous because both classes are less prevalent across our samples. Kavalactones and \( p \)-alkenyl phenols are dramatically different compounds that diverge at a much earlier branch point from a common cinnamic/coumaric acid precursor. Whereas one polyacetate chain extension pathway leads to the long-chain lipophilic substituent, characteristic of the \( p \)-alkenyl phenols, the other chain extension pathway conserves oxidation states through the chain extension process to produce the lactones (kavalactones or butenolides) through cyclization reactions (Fig. 3E). The overall outcome is different than the chalcone-flavonoid relationship; in this case, two dramatically different compounds are produced by divergence from a common early-stage biosynthetic precursor in contrast to the immediate biosynthetic precursor relationship between chalcones and flavonoids. Broader sampling across \( Piper \) and Radula will be necessary to confirm this unexpected relationship between kavalactones and \( p \)-alkenyl phenols.

### Conclusion

Here we sought to advance understanding of phylogenetic relationships within \( Piper \) while simultaneously investigating the mode and manner of phytochemical evolution in this group. In addition to generating a well-resolved phylogeny, our results support theoretical expectations that broad classes of compounds display higher degrees of phylogenetic conservatism than the more evolutionarily labile molecular features revealed by \( ^1H \) NMR data. In addition, trait associations observed in Radula can be used to pose functional hypotheses about genetic constraints or biases on phytochemical evolution and how these factors structure plant-animal interactions. Such investigations are one of the emerging frontiers in terrestrial ecology, and we hope that our study provides one example of how collaborative and multi-disciplinary research can progress in this area.

### Acknowledgements

This research was funded by the National Science Foundation (DEB-1145609 and DEB-1442103) to CJ, LAD, LAR, MLF, TLP, and AMS, by the National Science Foundation Graduate Research Award (Award No. 1650114) to KAU, and by FAPESP (Award No 2014/50316-7) to MJK. Fellowship support for KAU, KMO, and CSP and funding for chemical instrumentation and analysis was provided by the Hitchcock Center for Chemical Ecology at the
University of Nevada, Reno. We thank Jennifer L. McCracken for her assistance with the collection of GC-MS data for the categorical chemical characterization, and we thank Chris Feldman, Beth Leger, and Steve Vander Wall for their guidance during the earliest stages of this project.

Author contributions

MLF, LAD, AMS, CSJ, LAR, and TLP developed the original idea for the research and secured funding. EJT, MJK, and LFY collected specimens. EJT extracted DNA from plant specimens. KAU and TLP generated genotyping-by-sequencing libraries. KAU and JPJ analyzed the genetic data. KMO and LAR performed chemical extractions and analyses. CSJ, CSP, and CDD executed chemical annotation and structure determination. KAU and JPJ wrote the first draft of the manuscript, and all authors contributed to subsequent revisions.

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Table 1. Estimates of phylogenetic signal (D) (Purvis and Fritz, 2010) for a subset of metabolite classes (see Methods for explanation of subset). To ask whether traits evolved under scenarios of Brownian motion (D = 0) or phylogenetic randomness (D = 1), observed values of D were compared to null distributions of D modeled under each scenario.

<table>
<thead>
<tr>
<th>Metabolite class</th>
<th>Observed D</th>
<th>$\Sigma d_{\text{obs}}$</th>
<th>Randomness ($H_0$: $D=1$)</th>
<th>Brownian ($H_0$: $D=0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mean($\Sigma d_r$)</td>
<td>$P$</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>0.49</td>
<td>14.18</td>
<td>17.56</td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>Chalcones</td>
<td>0.39</td>
<td>9.77</td>
<td>12.18</td>
<td><strong>0.019</strong></td>
</tr>
<tr>
<td>Phenolic glycosides</td>
<td>-1.18</td>
<td>3.11</td>
<td>7.01</td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Lignans</td>
<td>-0.02</td>
<td>4.16</td>
<td>5.47</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td>PBA</td>
<td>0.22</td>
<td>12.40</td>
<td>17.51</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>$p$-alkenyl phenols</td>
<td>0.33</td>
<td>9.47</td>
<td>12.30</td>
<td><strong>0.010</strong></td>
</tr>
<tr>
<td>Kavalactones/butenolides</td>
<td>0.02</td>
<td>5.17</td>
<td>6.99</td>
<td><strong>0.027</strong></td>
</tr>
<tr>
<td>Piper amides</td>
<td>0.1</td>
<td>5.37</td>
<td>7.00</td>
<td><strong>0.033</strong></td>
</tr>
</tbody>
</table>
Table 2. Correlated evolution was detected in two pairs of metabolite classes with Pagel’s (1994) method: 1) chalcones and flavonoids; and 2) kavalactones/butenolides and \( p \)-alkenyl phenols. A model comparison framework was employed to evaluate four potential models of trait evolution using AIC: correlated evolution (transition rate in one trait depends on state at another, and vice versa); contingent change (transition rate in one trait depends on state at another, but not the converse); and independent evolution.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Model</th>
<th>AIC</th>
<th>Δ AIC</th>
<th>AIC weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalcones, flavonoids</td>
<td>Chalcones contingent on flavonoids</td>
<td>87.40</td>
<td>0</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Flavonoids contingent on chalcones</td>
<td>88.41</td>
<td>1.01</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Correlated evolution</td>
<td>90.54</td>
<td>3.14</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Independent evolution</td>
<td>95.32</td>
<td>7.92</td>
<td>0.01</td>
</tr>
<tr>
<td>Kavalactones/butenolides, ( p )-alkenyl phenols</td>
<td>Correlated evolution</td>
<td>62.35</td>
<td>0</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>( p )-alkenyl phenols contingent on kavalactones/butenolides</td>
<td>69.65</td>
<td>7.29</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Kavalactones/butenolides contingent on ( p )-alkenyl phenols</td>
<td>70.61</td>
<td>8.26</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Independent evolution</td>
<td>71.57</td>
<td>9.22</td>
<td>0.01</td>
</tr>
</tbody>
</table>
**Figure legends**

**Figure 1.** Maximum clade credibility tree of 48 species from the Radula clade of *Piper* and 23 outgroup species inferred with a Bayesian analysis of 641 concatenated RADseq loci (55,298 base pairs) comprising 9,113 genetic variants (of which 4,674 are parsimony informative). The outgroup taxa were sampled across multiple *Piper* clades: Isophyllon, Churumayu, Macrostromachys, Hemipodium, Peltobryon, Pothomorphe, and Schilleria. All nodes are supported by at least 95% posterior support except where noted with circles or labels. Blue circles indicate support values between 85-95%. Red circles indicate support values between 75-85%. Three nodes with less than 75% posterior support were given numerical support values. Blue bars at each node denote the 95% highest posterior density interval on node ages. Diversity of *Piper* with the clade they belong to in parentheses. Images of outgroups include A. *Piper hillianum* (Macrostromachys), B. *P. acutifolium* (Peltobryon), and C. *P. umbellatum* (Pothomorphe).

Examples of the Radula clade of *Piper* include D. *P. pseudofuligineum*, E. *P. concepcionis*, F. *P. disparipes*, G. *P. friedrichsthalii*, H. *P. dilatatum*, I. *P. bredemeyeri*, J. *P. immutatum*, K. *P. erubescentispicum*, and L. the widespread and often weedy *P. aduncum*.

**Figure 2.** Taxa comprise the columns of the matrix and are ordered according to their inferred phylogenetic relationships. Groups of columns are colored according to their designated *Piper* clade. Black circles within the phylogenetic tree designate nodes with posterior support values greater than 85%. Each row of the matrix represents a metabolite class which was detected from $^1$H NMR, GC-MS, and LC-MS data, with dark grey cells indicating the presence of that class in that taxa. Rows outlined in white indicate traits which were analyzed for phylogenetic signal in Radula. To the left of the matrix are representative compounds for a subset of metabolite classes which were detected in our samples.

**Figure 3.** Evolutionary associations were detected in two pairs of traits according to Pagel’s (1994) test of correlated evolution: 1) flavonoids and chalcones and 2) $p$-alkenyl phenols and kavalactones/butenolides. Filled shapes indicate presences and unfilled shapes indicate absences of flavonoids (circles), chalcones (squares), $p$-alkenyl phenols (diamonds), and
kavalactones/butenolides (triangles), respectively. The shapes used in the cophylogenetic plots (A and C) are repeated below (B and D) to depict four states comprising all combinations of presences and absences in the pair of traits. Arrows represent transition rates between states. **B.**

As both models of contingent change provided good fits to the flavonoid and chalcone data, both sets of transition rates are displayed, with the first set of values (bolded) corresponding to the best supported model (chalcone evolution contingent on flavonoid state) and the second set of values corresponding to the alternative contingency model (flavonoid evolution contingent on chalcone state). **D.** The best fit model to the p-alkenyl phenol and kavalactone/butenolide data was one of dependent evolution, where p-alkenyl phenol evolution is dependent on the state at the kavalactone/butanolide trait, and vice versa. Panel **E** illustrates the enzymatic processes and branch points along biosynthetic pathways that give rise to the four classes of metabolites.

Chalcones are immediate biosynthetic precursors of flavonoids, where the inherent reactivity of the chalcone moiety permits cyclization to the flavonoid scaffold. Subtle structural changes to the flavonoid scaffold caused by late-stage oxidation can produce protoflavonoids, a rare class of metabolite with potent cytotoxic activity. In contrast, the pathways of p-alkenyl phenols and kavalactones diverge much earlier and embark on distinct chain elongation pathways which lead to long-chain lipophilic substituent characteristic of the p-alkenyl phenols in one case, and lactones (kavalactones and butenolides) in the other case.

**Figure 4.** **A.** Chemospace of all 71 species constructed with the crude ¹H NMR data across 277 peaks. Point shapes and colors are formatted according to clade designation as portrayed in the phylogenetic tree in Figure 1. MRM analyses recovered significant negative relationships between phylogenetic and chemical distances calculated among samples from all clades (B), or from the Radula clade only (C); however, the proportion of variance explained was low for all tests.
Fig. 2
Fig. 3

A. Flavonoids
B. Chalcones

Absence
Presence

0/3.6

23.5/3.6

4.7/18.5

2.5/3.7

4.7/38190

2.5/301756

14.7/0.8

8.2/3.6

0/0.8


C. P-alkenyl/phenols
D. Kavalactones/Butenolides

Absence
Presence

0

5.4

0

179907

51.3

7.4

82991


E. Chemical structures and pathways:

- Flavonoids
- Chalcones
- P-alkenyl phenols
- Kavalactones/Butenolides

Chemical reactions:
- Chalcone synthesis
- Elongation of 3-methyl-CoA
- Elongation of 2-methyl-CoA
- Protoflavonoids (enhanced toxicity)
- Common biosynthetic precursor
- Lactonization

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Fig. 4

A

B

C