Patterns of Secondary Metabolite Allocation to Fruits and Seeds in *Piper reticulatum*

S. R. Whitehead · C. S. Jeffrey · M. D. Leonard · C. D. Dodson · L. A. Dyer · M. D. Bowers

Abstract Little is known about the evolution, diversity, and functional significance of secondary metabolites in reproductive plant parts, particularly fruits and seeds of plants in natural ecosystems. We compared the concentration and diversity of amides among six tissue types of *Piper reticulatum*: leaves, roots, flowers, unripe fruit pulp, ripe fruit pulp, and seeds. This represents the first detailed description of amides in *P. reticulatum*, and we identified 10 major and 3 minor compounds using GC/MS and NMR analysis. We also detected 30 additional unidentified minor amide components, many of which were restricted to one or a few plant parts. Seeds had the highest concentrations and the highest diversity of amides. Fruit pulp had intermediate concentrations and diversity that decreased with ripening. Leaves and roots had intermediate concentrations, but the lowest chemical diversity. In addition, to investigate the potential importance of amide concentration and diversity in plant defense, we measured leaf herbivory and seed damage in natural populations, and examined the relationships between amide occurrence and plant damage. We found no correlations between leaf damage and amide diversity or concentration, and no correlation between seed damage and amide concentration. The only relationship we detected was a negative correlation between seed damage and amide diversity. Together, our results provide evidence that there are strong selection pressures for fruit and seed defense independent of selection in vegetative tissues, and suggest a key role for chemical diversity in fruit-frugivore interactions.

Keywords Amides · Chemical diversity · Fruit secondary metabolites · Piperaceae · Seed dispersal · Toxic fruit

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Introduction

Plants employ a diverse arsenal of secondary metabolites as a defense against herbivores and pathogens. A long history of research has shown that these metabolites can increase plant fitness by reducing the preference and/or performance of a variety of antagonistic consumers (reviewed in Iason et al. 2012) or by influencing indirect interactions with natural enemies of antagonists (Price et al. 1980). However, the majority of research in this area has focused on secondary metabolites produced in leaves and their effects on leaf herbivores or pathogens, and less is known about the diversity and functional significance of secondary metabolites produced in other plant parts, such as flowers and fruits (Adler 2000; Cipollini and Levey 1997a; Tewksbury 2002). Because flowers and fruits often function primarily to attract mutualistic pollinators and seed dispersers, selective pressures in these tissues are likely to be qualitatively and quantitatively different from those in leaves, and can include conflicting

pressures from both mutualists and antagonists (Cazetta et al. 2008; Irwin et al. 2004; Kessler and Halitschke 2009; Tsahar et al. 2002). This study aimed to improve the basic understanding of the variation in diversity and abundance of secondary metabolites among different plant parts and its relationship to damage in natural populations. We focused in particular on fleshy fruit pulp and seeds, which have received relatively little attention in chemical ecology (Levey et al. 2007; Tewksbury 2002), but play an important role in plant fitness, and can contain a diversity of secondary metabolites (Cipollini and Levey 1997a; Herrera 1982; Whitehead and Bowers 2013a).

Although fruits, particularly agricultural species, often have been the subject of phytochemical investigations (e.g., Crozier et al. 2006; Moco et al. 2007; Suzuki and Waller 1985), it still is unclear how and why the abundances and diversity of secondary metabolites in wild fruits vary relative to other plant parts. There are a number of theoretical reasons to predict that plant allocation to the defense of fruits may be relatively high compared to leaves and other vegetative tissues (Cipollini et al. 2004; Levey et al. 2007). For example, optimal defense theory predicts that fruits should be among the most highly defended plant parts because: 1) fruits have a high fitness value due to their direct link to reproductive output, and 2) fruits may be at an increased risk of attack due to their high nutritional content (McCall and Fordyce 2010; McKey 1974; Rhoades and Cates 1976). In studies that have directly compared the concentrations of secondary metabolites in wild fruits and leaves, the allocation to fruits, and especially seeds, often has been shown to be relatively high compared to leaves (Alves et al. 2007; Brown et al. 2003; Johnson et al. 1985; Nelson et al. 1981; Whitehead and Bowers 2013b; Wink and Witte 1985; Zangerl and Rutledge 1996; but see Ehrlen and Eriksson 1993; Isman et al. 1977). In some cases, fruits also can contain a higher diversity of secondary metabolites than leaves (Kliebenstein et al. 2001; Whitehead and Bowers 2013b), which may increase the effectiveness of fruit defense if compounds interact synergistically or provide simultaneous defense against a diverse community of antagonists (Berenbaum 1985; Gershenzon et al. 2012). However, to better understand patterns of allocation to secondary metabolites in fruits, two factors must be considered: 1) differentiation between fleshy, animal-dispersed fruits and dry fruits that are dispersed abiotically; and 2) differentiation between seeds and the surrounding fruit tissue, which often have been combined for chemical analysis in previous studies.

Animal-mediated dispersal is a common feature of communities, with the percentage of animal-dispersed plants ranging up to 90 % in some tropical forests (Jordano 2000). Although the large majority of ecological studies that have provided quantitative comparisons of secondary metabolites in leaves and fruits have focused on abiotically-dispersed plants (Alves et al. 2007; Brown et al. 2003; Johnson et al. 1985; Nelson et al. 1981; Wink and Witte 1985; Zangerl and Rutledge 1996), potentially deterrent or toxic secondary metabolites also are found in fleshy, animal-dispersed fruits of numerous species (e.g., Cipollini et al. 2004; Izhaki 2002; Tewksbury et al. 2008; Whitehead and Bowers 2013b). One explanation for this apparent evolutionary paradox is that secondary metabolites in ripe, fleshy fruits are non-adaptive, and occur primarily as a pleiotropic consequence of strong selection for the defense of leaves, unripe fruits, or seeds (Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998; Whitehead and Poveda 2011). Alternatively, there are at least nine major hypotheses that propose adaptive pathways by which diverse mixes of ripe fruit secondary metabolites can evolve (Cipollini 2000; Cipollini and Levey 1997a; Rodríguez et al. 2013). Examples include selective pressures from mutualists, when secondary metabolites mediate dispersal effectiveness or function as association cues (Cipollini 2000; Rodríguez et al. 2013), or from antagonists, when secondary metabolites function to defend fruits against insect seed predators or fungal pathogens (Cipollini 2000). In particular, increasing evidence has shown that fruit secondary metabolites can play a key role in defense—in some cases leading to trade-offs in fruits between attraction of seed dispersers and defense against antagonists (Cazetta et al. 2008; Cipollini et al. 2004; Cipollini and Levey 1997a; Herrera 1982) and in other cases effectively defending fruits with minimal negative effects on mutualists (Cipollini and Levey 1997a; Struempf et al. 1999; Tewksbury and Nabhan 2001).

The second factor that must be considered to understand allocation of secondary metabolites to fleshy fruits is the potential difference in selective pressures between seeds and the surrounding pericarp and accessory tissues. However, in many phytochemical studies that examine secondary metabolites in fruits, entire fruits or infructescences are collected with no distinction between seeds and the surrounding tissues. In two cases where fruit pulp and seeds were compared, fruit pulp was found to have higher concentrations of secondary metabolites or higher toxicity to generalist fungi than seeds (Barnea et al. 1993; Beckman 2013). However, plant defense theory provides a number of reasons to predict that the opposite should be true. First, seeds provide a more direct link to reproductive output than any other tissue, and thus, a high relative allocation to the defense of seeds should maximize fitness benefits when defenses are costly to produce (McCall and Fordyce 2010; McKey 1974; Rhoades and Cates 1976). Second, seeds are at high risk of attack from both pre-dispersal and post-dispersal seed predators due to their concentrated supply of energy and nutrients, and often are exposed to predators for long periods of time prior to germination (Hulme 1998). Lastly, for fleshy fruits, there is a lower potential for ecological costs associated with the occurrence of deterrent secondary metabolites in seeds than in fruit pulp, because the seed itself is usually not a part of the reward for mutualist pollinators and seed dispersers (Eriksson and Ehrlen 1998).

To understand the adaptive significance of secondary metabolites that occur in fruits, and, more generally, the evolution of phytochemical diversity in a whole-plant context, studies are needed that provide detailed qualitative and quantitative comparisons of secondary metabolites in different plant parts, including vegetative tissues and reproductive tissues that experience differential selective pressures from mutualists and antagonists. Here, we compared the diversity and abundance of secondary metabolites in different plant parts of *Piper reticulatum* L. (Piperaceae), a large understory shrub that is common throughout the Neotropics and is dispersed primarily by frugivorous bats (Fleming 2004). Previous phytochemical research on *P. reticulatum* is limited, but a few compounds have been described from leaves, including the amides wisanidine and dihydrowisanidine, several sesquiterpenes, and two 5,6-dihydropyran-2-ones (Luz et al. 2003; Maxwell et al. 1998; Yamaguchi et al. 2011). We found no previous investigations of *P. reticulatum* fruits or other plant parts. Our analyses focused on amides, a diverse group of nitrogen (*N*)-containing metabolites that are the most abundant secondary metabolites in *P. reticulatum* leaves (Yamaguchi et al. 2011). Amides play a role in the defense of other *Piper* species against insect herbivores (Dyer et al. 2001, 2004a; Richards et al. 2010) and have a broad range of bioactivity in laboratory studies, including insecticidal, anti-fungal, and anti-bacterial effects (see e.g., Bernard et al. 1995; reviewed in do Nascimento et al. 2012). Thus, although amides also may have important non-defensive roles (e.g., effects on seed disperser foraging behavior or influences on seed development), their primary functions likely involve the defense of *P. reticulatum* against a variety of insect herbivores or pathogens that attack different plant parts. This study had two specific objectives: 1) To compare the diversity and abundances of amides in leaves, roots, flowers, unripe pulp, ripe pulp, and seeds of *P. reticulatum*; and 2) To examine the relationship between amide occurrence and patterns of leaf herbivory and seed damage in natural populations

Methods and Materials

Study System and Site All samples were collected at La Selva Biological Station, Heredia Province, Costa Rica. The site consists of premontane and tropical wet forest, as well as secondary forest and abandoned agricultural areas (McDade et al. 1994). La Selva is a center of diversity for the genus *Piper*, with 50+ species co-occurring (OTS 2012).

Piper reticulatum is a large rainforest understory shrub ranging from Honduras to Bolivia (Tropicos 2012), and is one of the most common *Piper* species in secondary forest and along trails at La Selva. Flowers are borne on distinct spike-shaped inflorescences that mature into infructescences

over a period of several months. Each infructescence contains ~100–300 individual fruits that ripen simultaneously, with the final ripening phase usually beginning mid-afternoon and lasting for several hours (SRW, personal observation). During the final ripening period, fruits soften and swell, but there is no color change from the pale green typical of unripe fruits. Individual trees can produce hundreds of infructescences that ripen sequentially, with anywhere from 1 to 20 infructescences maturing per day over the course of a fruiting peak that lasts for several weeks for an individual. Most ripe fruits are removed by bats on the first evening that they are ripe; those that are not removed are visibly beginning to rot on the following day, and they are usually not taken on the following evening (SRW, personal observation).

A small genus of fruit bats (*Carollia* spp., Phyllostomidae) is the primary disperser of *P. reticulatum* and most other *Piper* species in the Neotropics (Fleming 2004). Three *Carollia* species co-occur at La Selva and all feed on *P. reticulatum*: *C. perspicillata, C. sowelli,* and *C. castanea* (Fleming 1991; SRW personal observation). The *Carollia* bats provide the majority of seed dispersal services for *Piper,* and *Piper* fruits provide a year-round dietary staple for the bats (Fleming 2004). Olfactory cues appear to be the primary mechanism used by bats to locate fruits and distinguish between unripe and ripe fruits in close proximity (Laska 1990; Mikich et al. 2003; Thies et al. 1998). Entire infructescence spikes are removed in flight and carried to central feeding roosts, where the fruit is consumed from the central rachis of the spike (Thies et al. 1998; Fleming 2004).

Piper reticulatum is attacked by a variety of leaf herbivores at La Selva, including at least 24 species of lepidopteran larvae, the most abundant being *Quadrus cerialis* Stoll (Hesperiidae), a *Piper* specialist, and *Anacrusis nephrodes* Walsingham (Tortricidae), a generalist (Dyer and Gentry 2012). Fruits and seeds are attacked by hemipteran seed predators, especially *Sibaria englemani* Rolston (Pentatomidae), a *Piper* specialist (Greig 1993; SRW personal observation), as well as an abundant, but unidentified, dipteran larva that makes a leap from ripe fruits as they are removed from the plant and burrows into the soil to pupate (SRW personal observation). Microbial consumers also are likely important as fruit antagonists, as evidenced by the rapid decomposition of fruits once they reach final maturity (Thies and Kalko 2004; SRW personal observation).

Sample Collection We collected samples from 16 individuals of *Piper reticulatum* growing along trails and forest edges between July 10 and July 24, 2012. All trees were separated by a minimum of 25 m. From each individual, we collected leaves, roots, flowers (when available, for 12 of 16 individuals only), unripe fruits, and ripe fruits, always collecting all of the tissue types from a single tree at the same time. We collected

late in the afternoon and included only ripe fruits that had matured that day. Fruits soften and swell over a short period of time during ripening, and freshly ripened fruits are distinguishable from unripe fruits or day-old ripe fruits based on firmness. Samples were brought immediately back to the laboratory. From each individual, we took a subsample of each ripe infructescence (2–6 per plant) from which to sample the seeds by cutting small sections from the top, middle, and bottom of each spike. The seeds were gently washed in water to remove pulp and were stored separately from the remainder of the fruit prior to chemical analysis. The remainder of the ripe fruit was stored intact, and the seeds later were removed to obtain a pulp-only sample (see below). Samples of all plant parts were placed in paper packets and dried in silica gel. Dried samples were transported to the University of Colorado for analysis by gas chromatography combined with mass spectrometry (GC/MS) to determine amide diversity and concentration (see below). Leaves, roots, and cleaned seeds were ground to a fine powder with a coffee grinder and/or a sample mill (Tecator Cyclotec, FOSS North America, Inc). To obtain pulp-only samples from dried, intact ripe and unripe fruits, we ground the plant material through a fine mesh sieve to separate seeds from pulp. Seeds were removed from these samples and discarded, and the pulp was ground further to a fine powder with a coffee grinder.

Identification and Quantification of Amides by GC/MS To examine variation in amides among *P. reticulatum* individuals, we used a scaled-down version of extraction and quantification procedures as described in Dyer et al. (2004b). From each sample, ~100 mg (weighed to the nearest 0.1 mg) of dried plant material were extracted twice overnight in 7.5 ml ethanol, and the combined extracts were filtered to remove suspended material and evaporated to dryness under a stream of air. Samples then were re-suspended in 3 ml 3:1 water:ethanol, transferred to a separatory funnel, and partitioned three times against equal volumes of chloroform. The water partition was discarded, and the combined chloroform partitions (containing the amides) were evaporated to dryness. This extraction procedure resulted in an extract enriched in amides, although some other compounds were present as well. Samples were re-suspended in 1 ml dichloromethane, and piperine (obtained from Sigma-Aldrich Co.), an amide that does not occur in *P. reticulatum*, was added at a concentration of 0.75 mg/ml as an internal standard. Aliquots of 100 μl were then transferred to vials for analysis by GC/MS.

All samples were analyzed by using an HP Agilent 6890N GC coupled with an Agilent 5975C MS with an ion source of 70 eV at 230 °C. The instrument was equipped with a DB-5MS capillary column (30 m×0.25 mm i.d., 0.5 μm film thickness; Agilent Technologies, Santa Clara, CA, USA). Ultra-pure He was used as the carrier gas at a flow rate of

1.5 ml min^{-1}, a split flow ratio of 30:1, and a front inlet temperature of 280 °C. The following oven conditions were employed: initial temperature 50 °C, initial hold time 1 min; ramp 1: 15 °C min^{-1} to 275 °C, hold time 5 min; ramp 2: 20 °C min^{-1} to 320 °C, hold time 10 min. A blank sample (dichloromethane only) was run after every five samples to ensure there was no carryover between runs. Data were recorded and processed using MSD ChemStation software (version D.02.00.275). Quantities of individual compounds and of total amides were estimated based on peak areas in total ion current chromatograms and known concentrations of the internal standard, which showed a linear response across the range of concentrations present in our samples ($R^2 > 0.99$).

All compounds detected in the samples were first screened to identify potential amides based on assessment of spectra for characteristic fragmentation patterns of *N*-based compounds, in particular a molecular ion with an odd mass and ions corresponding to α-cleavage and McLafferty rearrangement (McLafferty and Turecek 1993). Typical fragmentation patterns were consistent with structural features common to amides that have been previously described from *Piper* species (do Nascimento et al. 2012). Tabulated mass spectral data and additional details on the tentative assignments of the minor amides **A**-**DD** are reported in Online Resource 1. For those compounds that we tentatively assigned as amides, we further compared our assessments with spectra of known amides in the Wiley-NIST 2005 library. Although most naturally-occurring amides are not in this database, we assessed compounds for similar structures using the automated substructure search feature, specifically searching for the presence of nitrogen (N), carbonyl (C = O), and amide (N-C = O) functional groups (Stein 1995). Furthermore, we checked that all putative amides had retention times between 12.5 min and 25 min, consistent with the range of retention times we have observed with known amides using identical instrument parameters. Although this rapid profiling approach cannot provide absolute certainty in compound identification, it is unlikely that the compounds we tentatively identified as amides belong to other compound classes for several reasons. First, our extraction and purification procedures would have removed many commonly occurring *N*-based plant compounds, such as most amino acids, based on polarity (Burroughs 1970). Second, most *N*-based compounds in plants, including primary metabolites (e.g., peptides, nucleic acids, and most amino acids) as well as other common classes of *N*-based secondary metabolites (e.g., non-protein amino acids, cyanogenic glycosides, glucosinolates, and most other classes of alkaloids), either cannot be analyzed using GC/MS or require derivatization prior to analysis due to their low volatility (Heaney et al. 1993; Hodisan et al. 1998; Seigler et al. 1993; Waterman et al. 1993). One other *N*-based defensive compound has been reported from *Piper reticulatum*, cyanobenzyl benzoate (Yamaguchi et al. 2011).

We did detect this compound in a number of leaf samples, but the retention time was not in the range of amides, and we did not include this compound in our statistical analyses of amide concentration and diversity. To ensure consistency in identification of unknown amides across multiple individuals and tissue types, once a compound was identified as an amide we added the mass spectrum to a user-created library of all compounds included in this study and identified repeated occurrence of the same compounds on the basis of matches with both library entries and retention times.

Structure Elucidation of Major Components Using ^{1}H-NMR Spectroscopy The structures of major amide components (defined as those compounds that contribute ≥2 % of the estimated total amides) and a few minor components were further confirmed using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) conducted at the University of Nevada. For ^{1}H-NMR analysis, composite samples were created using 5–6 individuals of *P. reticulatum* due to limited amounts of material in single samples. From each tissue type, 2 g of plant material were extracted twice with methanol (10 ml) with sonication (10 min, Branson 3510). The combined extracts were filtered and evaporated to dryness in a centrifugal evaporator (Savant SpeedVac SC210A, Thermo-Scientific) under reduced pressure. From the resulting residues, partial purification was performed for ripe fruit and leaf extracts by using medium pressure liquid chromatography (MPLC) with a pre-packed Biotage ZIP silica gel column [50 g (39×82 mm), 50 μm particle size] and eluting (10 ml/min) with an increasing gradient of ethyl acetate in hexanes. Like fractions (via TLC and UV 254 nm) were combined, evaporated under reduced pressure, and subjected to GC/MS and ^{1}H-NMR analysis. For NMR analysis, crude plant residues or isolated fractions were transferred to an NMR tube, and 1 ml of deuterated chloroform or d_4-methanol (Cambridge Isotope Laboratories) was added for ^{1}H-NMR analysis using a 400 MHz Varian instrument (Agilent Technologies). Isolated fractions were characterized by both ^{1}H-NMR and GC/MS analysis. Correlations between isolated compounds and the crude extracts for all plant parts were made through comparison of the GC/MS data from both samples. Additional details on our tentative assignments of the isolated amides (**1**–**13**) are reported in Online Resource 1.

Measurement of Leaf and Seed Damage in Natural Populations We measured standing levels of leaf herbivory and seed damage for each of the 16 sampled individuals of *P. reticulatum* at the time of collection for chemical analysis. To measure leaf herbivory, we took a sample of 10 fully expanded leaves from a variety of locations (different heights and exposures) on each individual plant, and we measured both the total leaf area and the area removed by using a leaf area meter (LI-COR LI-3100). The proportion of leaf area removed was averaged among leaves to estimate the herbivory level for each individual. To measure seed damage, we took subsamples of seeds from ripe fruits collected from each individual plant by cutting short (~2 cm) sections from various points along each infructescence. Where multiple ripe infructescences were collected from a single individual, subsamples from each infructescence were combined into one sample for each individual. The seeds were washed gently in water to remove pulp, and were sorted under a stereoscope into intact and damaged groups. Intact seeds were reddish-brown and rigid, whereas damaged seeds were visibly darker (appearing rotten) and soft (i.e., could be easily mashed with forceps). A minimum of 100 seeds were counted from each individual of *P. reticulatum* to estimate the proportion that were damaged vs. intact.

Statistical Analyses To examine intraplant variation in amides, we first compared the estimated total amide concentration and the chemical diversity (measured as the number of amides detected) among leaves, roots, flowers, unripe fruit pulp, ripe fruit pulp, and seeds. For analysis of total amide concentration, we used a linear mixed model with a normal distribution, with plant part specified as a fixed effect and plant identity as a random effect. The estimated amide concentration (% dry weight) was logit transformed prior to analysis to approximate a normal distribution (Warton and Hui 2011). For analysis of chemical diversity, we used a generalized linear mixed model with a Poisson distribution and the log-link function, again with plant part specified as a fixed effect and plant identity as a random effect (Bolker et al. 2009). For hypothesis testing, we compared these models to null models that included the random effect (plant identity) only using likelihood ratio tests (Bolker et al. 2009). When the model that included plant part provided a significantly better fit ($P<0.05$) to the data than the null model, we followed these analyses with a Tukey HSD *post-hoc* test to examine pairwise differences among plant parts. These analyses were conducted using the 'lme4' and 'multcomp' packages in the R Environment for Statistical Computing (Bates and Maechler 2010; Hothorn et al. 2011; R Development Core Team 2012).

To further examine overall similarities and differences among plant parts, we used multivariate non-metric multidimensional scaling analysis (NMDS), an ordination technique based on a dissimilarity matrix for all data that incorporates both the presence/absence and quantities of individual compounds (Minchin 1987). The ordination was based on the Bray-Curtis dissimilarity index and used 200 replicates with random starting coordinates. To determine the appropriate number of dimensions for the final solution, we generated a scree plot of the number of dimensions vs. the final stress, and found that further reductions in the final stress after two dimensions were small. This analysis was conducted using the 'vegan' package in R (Oksanen et al. 2010; R Development Core Team 2012).

To examine associations between amide concentration and patterns of leaf and seed damage in the field, we used non-parametric Kendall's rank correlations between leaf amide concentration or diversity and the proportion of leaf area removed, and between seed amide concentration or diversity and the proportion of damaged seeds. These analyses were conducted using the statistical software JMP v. 9.0.2 (2010).

Results

All parts of *P. reticulatum* contained amides, the most abundant of which was dihydrowisanidine (**1**), which occurred in all parts, although its relative abundance varied considerably (Table 1). This compound, nine additional major amides, and three minor amides were identified or tentatively identified using GC/MS and NMR data (Table 1, Fig. 1, Online Resource 1). The identified components together represented 92 % of the estimated total amides in leaves, 99 % in roots, 87 % in flowers, 90 % in unripe fruits, 86 % in ripe fruits, and 87 % in seeds (concentrations estimated as piperine [IS] equivalents; Table 1). In addition, we detected 30 additional minor compounds that were identified as amides based on their fragmentation patterns in mass spectra (McLafferty and Turecek 1993), but these compounds were not characterized. Many individual amides were unique to one or a few plant parts (Table 1). We also detected and identified one major component that occurred only in leaves and was not classified as an amide, cyanobenzyl benzoate (Yamaguchi et al. 2011).

The estimated total amide concentration (as a % dry weight) was significantly different among plant parts ($X^2 = 49.26$, $df = 5$, $P < 0.001$; Fig. 2a). Concentrations were highest in seeds, followed by flowers, unripe pulp, leaves, roots, and ripe pulp (Fig. 2a). Total amide diversity (i.e., the number of compounds detected) also was significantly different among plant parts ($X^2 = 269.83$, $df = 5$, $P < 0.001$; Fig. 2b). Chemical diversity was highest in seeds, followed by unripe pulp, flowers, ripe pulp, leaves, and roots (Fig. 2b). The NMDS analysis examining overall chemical similarity among plant parts revealed significant overlap in the chemical profiles of unripe pulp, ripe pulp, and seeds; however, leaves, flowers, and roots all formed distinct groups that were significantly different from other plant parts (2-D Final Stress=0.11, $R^2 = 0.43$, Fig. 3).

On average, we found that 4.6 % of leaf area was removed and 18.6 % of seeds were damaged (i.e., rotten). There were no significant correlations between the proportion of leaf area removed and either leaf amide concentration (Kendall's $\tau = -0.02$, $P = 0.93$) or diversity (Kendall's $\tau = 0.25$, $P = 0.20$). For seeds, we found no relationship between the proportion of damaged seeds and the seed amide concentration (Kendall's $\tau = -0.20$, $P = 0.30$), but we did find a marginally significant

negative correlation between the proportion of damaged seeds and seed amide diversity (Kendall's $\tau = -0.38$, $P = 0.055$; Fig. 4).

Discussion

The content and composition of secondary metabolites can be highly variable across different plant parts (McCall and Fordyce 2010), and it is clear that these compounds have a diversity of ecological functions that may mediate interactions with both mutualists and antagonists (Iason et al. 2012). This study provides a thorough examination of intraplant patterns of secondary metabolite allocation in a wild, fleshy-fruited species. We found that allocation to secondary metabolites in *P. reticulatum*, both in terms of total amide concentrations and chemical diversity, differed substantially among plant parts and was highest for seeds. In addition, we found that concentrations of amides in fruit pulp decreased with ripening, supporting the hypothesis that secondary metabolites retained in ripe fruit pulp can carry ecological costs. However, we found few correlations between amide concentration or diversity and leaf or seed damage in natural populations.

Our results showing strong differences in the composition of amides among plant parts (Table 1, Fig. 3) suggest that plants experience different selective pressures in different plant parts and are able to allocate secondary metabolites accordingly. In *P. reticulatum*, we found two major amides, *N*-isobutyleicosadienamide (**6**) and octadecadienoylpyrrolidine (**8**), and 27 minor amides that occurred in fruit pulp or seeds but never in leaves (Table 1). There also were two minor amides (Table 1), and one cyanogenic compound, cyanobenzyl benzoate, that occurred in leaves but never in fruits. These results do not support hypotheses that assume the occurrence of secondary metabolites in fruits or other reproductive tissues is primarily an ecological cost associated with the defense of other plant parts (Adler 2000; Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998). Although chemical trait evolution in different plant parts can be linked through shared genetics, hormonal regulation, biosynthetic pathways, and metabolism (Adler et al. 2006; Kessler and Halitschke 2009; Whitehead and Poveda 2011), our results show that a large percentage of compounds are tissue-specific, and, in particular, many compounds are unique to fruits or seeds. These patterns likely are the result of differential selective pressures across plant parts. For example, compounds that occur primarily in fruits, such as *N*-isobutyleicosadienamide (**6**), may play a key role in defending fruits against fungi associated with fruit rot, but have minimal benefits against leaf herbivores. Compounds that occur only in leaves, such as cyanobenzyl benzoate, may potentially have a defensive function against lepidopterans or other leaf

Table 1 Average estimated concentrations (% dry weight) of individual amides in different plant parts of *Piper reticulatum*

| Compound identity	% total[a]	RT[b]	Leaves	Roots	Flowers	Unripe	Ripe	Seeds
Major components:[c]								
Dihydrowisanidine (**1**)	30.905	17.00	0.078	0.700	0.072	0.419	0.277	0.694
Desmethoxydihydrowisanidine (**9**)	15.356	16.01	0.701	~	0.378	0.020	0.014	0.001
Methoxy dihydrotricholein (**2**)	8.411	19.74	0.003	0.006	0.030	0.170	0.090	0.311
Desmethoxywisanidine (**11**)	7.991	17.68	0.069	~	0.495	0.010	0.006	~
Methoxy tricholein B (**5**)	7.939	19.68	0.022	0.003	0.117	0.129	0.065	0.240
Octadecenoylpyrrolidine (**3**)	6.970	18.66	~	0.005	0.061	0.119	0.073	0.248
Methoxy tricholein A (**4**)	4.633	19.60	0.004	~	0.043	0.087	0.042	0.160
Iso-wisanidine (**10**)	2.942	19.94	0.009	0.015	0.019	0.045	0.012	0.113
Octadecadienoylpyrrolidine (**8**)	2.863	17.76	~	~	0.011	0.058	0.030	0.108
N-isobutyleicosadienamide (**6**)	2.781	19.47	~	~	0.004	0.056	0.029	0.113
Minor components:[d]								
A	1.044	16.78	0.017	~	0.045	0.001	0.006	0.005
N-isobutyleicosatrienamide (**7**)	0.880	19.37	~	~	0.006	0.014	0.008	0.036
B	0.815	21.97	~	~	~	~	~	0.059
C	0.811	21.09	~	~	~	0.010	0.006	0.043
D	0.658	16.88	0.005	~	0.001	0.008	0.009	0.025
E	0.653	21.96	~	~	~	0.025	0.014	0.009
F	0.648	22.19	~	~	~	0.009	0.001	0.037
G	0.526	18.49	~	~	0.006	0.006	0.004	0.022
H	0.451	27.08	~	~	0.013	0.007	0.001	0.012
I	0.432	18.37	~	~	~	0.006	0.003	0.022
J	0.353	21.66	~	~	~	0.012	0.014	~
K	0.341	21.61	~	~	~	~	~	0.025
L	0.328	15.75	0.024	~	~	~	~	~
M	0.301	16.68	0.002	~	0.020	~	~	~
N	0.171	24.38	~	~	~	0.005	~	0.008
O	0.110	20.62	~	~	~	~	~	0.008
Tetrahydrowisanidine (**12**)	0.099	15.83	~	~	~	0.002	0.002	0.003
Wisanidine (**13**)	0.079	17.99	~	~	0.006	~	~	~
P	0.079	18.23	~	0.003	~	~	0.003	~
Q	0.079	18.25	~	~	~	~	~	0.006
R	0.063	22.05	~	~	~	0.003	~	0.002
S	0.041	16.53	~	0.003	~	~	~	~
T	0.034	17.29	~	~	~	0.001	0.001	~
U	0.032	16.37	~	~	~	0.001	~	0.002
V	0.032	19.28	~	~	~	0.001	~	0.001
W	0.028	24.99	~	~	~	0.001	~	0.001
X	0.023	27.95	~	~	~	~	~	0.002
Y	0.021	13.03	0.002	~	~	~	~	~
Z	0.018	17.52	~	~	~	~	~	0.001
AA	0.015	15.72	~	~	~	~	~	0.001
BB	0.015	17.62	~	~	~	~	~	0.001
CC	0.014	14.69	~	~	~	~	~	~
DD	0.014	25.06	~	~	~	~	~	0.001
Average total concentration			0.936	0.735	1.326	1.223	0.709	2.318
Average amide diversity			4.19	1.50	7.71	10.75	8.07	13.20

[a] The estimated % of total amides contributed by each individual compound, averaged across plant parts

[b] *RT* retention time (min) in GC/MS analysis

[c] Major Components are defined as those compounds that contribute ≥2 % of the estimated total amides

[d] Minor Components are defined as those compounds that contribute <2 % of the estimated total amides

Fig. 1 Structures of amides in *Piper reticulatum*
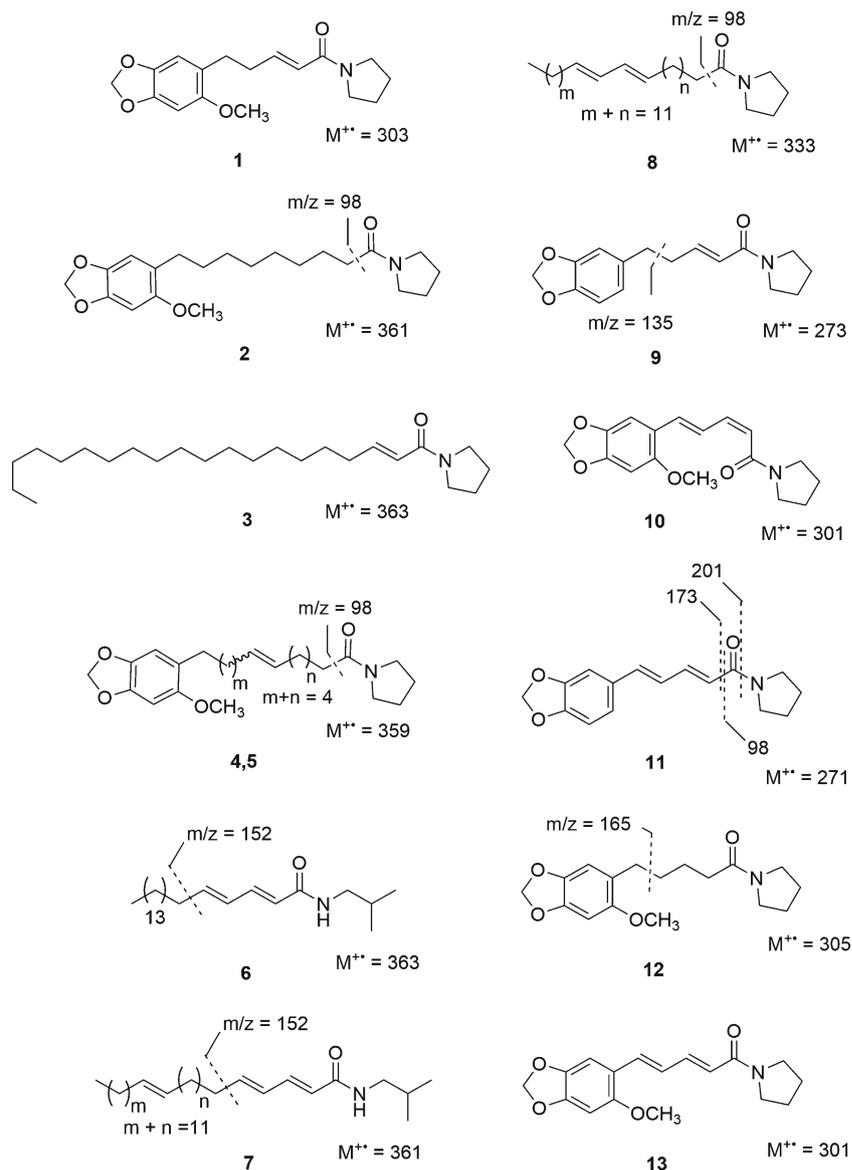
herbivores, but are excluded from fruits due to negative effects on seed-dispersing bats.

Although most studies that examine the role of secondary metabolites in ecological interactions focus on one or a few compounds, plants can produce hundreds of individual secondary metabolites and chemical diversity *per se* has been implicated as a force in determining the outcome of species interactions both on ecological and evolutionary timescales (Berenbaum and Zangerl 1996; Gershenzon et al. 2012). The chemical diversity of amides in *P. reticulatum* was higher for reproductive structures than for vegetative structures, with flowers, unripe pulp, ripe pulp, and seeds all containing higher numbers of detected compounds than leaves or roots (Fig. 2b). For roots in particular, chemical diversity was relatively low, with most samples dominated by a single compound, dihydrowisanidine (**1**) (Table 1). Because the production of

diverse mixtures of compounds can depend on increasingly complex metabolic pathways or additional enzymes (Gershenzon et al. 2012), our data suggest that the per gram dry weight investment in secondary metabolites by *P. reticulatum* is higher for reproductive structures than for vegetative structures, even if the concentrations are similar among tissue types. These results support the predictions of optimal defense theory, which suggests that allocation to the defense of reproductive tissues should be relatively high due to their high fitness value (McCall and Fordyce 2010; McKey 1974; Rhoades and Cates 1976). The chemical diversity in reproductive structures also may be in part explained by multiple selective pressures from antagonistic and mutualistic consumers in these tissue types. In particular, fruits often experience strong selective pressure from microbial consumers (Cazetta et al. 2008; Cipollini and Levey 1997b; Levey et al.
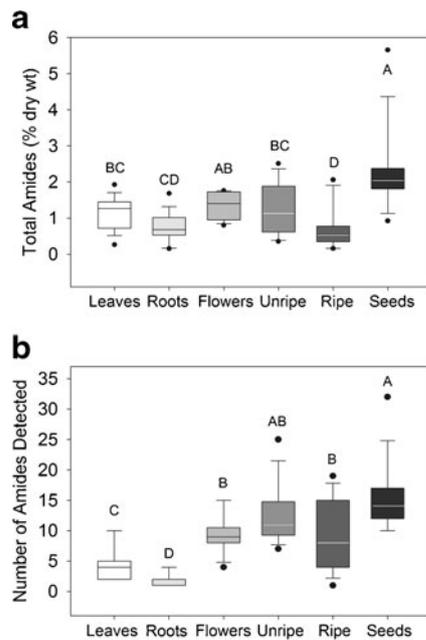
Fig. 2 Estimated total amide concentration (**a**) and diversity (**b**) in different plant parts of *Piper reticulatum*. *Box and whisker plots* show the median, 25th and 75th percentile, and range of total amide concentrations from $N=16$ plants. *Letters* represent significant differences from Tukey HSD *post-hoc* comparisons among plant parts
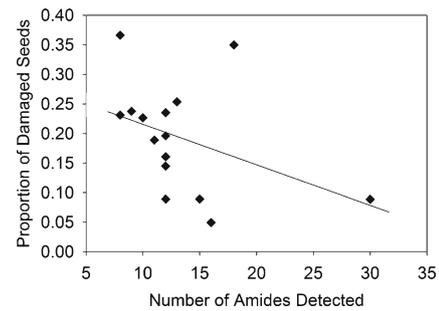

Fig. 4 Relationship between the amide diversity detected in *Piper reticulatum* seeds and the proportion of seeds that were damaged (Kendall's $\tau=-0.38$, $P=0.055$) for $N=16$ individuals

2007; Tewksbury et al. 2008), and one possibility is that the diverse community of fungi and bacteria involved in fruit and seed rot has played a role in the diversification of fruit secondary metabolites. Different chemical compounds may be bioactive against different consumers, or chemical diversity may increase the overall bioactivity of the mixture due to synergistic interactions among individual compounds (Dyer et al. 2003; Richards et al. 2010). Considering the number of individual amides that occur in reproductive structures, complex interactions among compounds seem inevitable and deserve further attention.

Fig. 3 Non-metric multidimensional scaling ordination plot of overall chemical similarity among plant parts (2-D Final Stress=0.11, $R^2=0.43$). *Ellipses* represent 95 % confidence intervals for group centroids (±1 SD)

Among plant parts, seeds had the highest detected chemical diversity and the highest concentration of amides (Fig. 2). Secondary metabolites play a role both in reducing seed predation risk (Tsahar et al. 2002; Whitehead and Bowers 2013a) and increasing seedling success (Ndakidemi and Dakora 2003). Average seed damage was relatively high (18.6 %), but variable, ranging from 5 % to 37 %, and our results showing a negative relationship between seed predation and chemical diversity (Fig. 4) suggest that plant investment in the defense of seeds may be a key factor mediating plant reproductive fitness in this system. However, it is also possible that seed secondary metabolites are selected for or maintained in part due to non-defensive roles, such as the regulation of germination or protection against oxidative stress (Baskin and Baskin 2004; Guo et al. 2003). Further research is necessary to answer the many questions related to the role of seed secondary metabolites in pre- and post-dispersal processes. In particular, we need information on how the chemical diversity of seeds may provide specific benefits either in interactions with the broad range of organisms that attack seeds (including vertebrates, invertebrates, and microbes) or in providing initial defense for plant offspring that arrive in different habitats with variable communities of antagonists and competitors.

In contrast to seeds, ripe fruit pulp had the lowest concentration of amides, almost two-fold lower than unripe fruit pulp and three-fold lower than seeds (Fig. 2a). This is the opposite of the trend reported by Barnea et al. (1993) that showed a higher concentration of metabolites in ripe fruit pulp than in seeds. One potential reason for the discrepancy is that the work by Barnea et al. (1993) was conducted with four bird-dispersed species, whereas our study was conducted with a bat-dispersed species. Birds have a high tolerance for many classes of secondary metabolites that can be deterrent or even highly toxic to mammals (e.g. Struempf et al. 1999; Tewksbury and Nabhan 2001). Our ongoing work seeks to quantify the effects of amides on bat foraging and feeding behavior, and preliminary results suggest that bats are deterred by a variety of amides (SRW, unpublished data). Pharmacological studies also have shown that amides can have diverse physiological effects on mammals, including effects on the digestive and

cardiovascular systems (Srinivasan 2007; de Araújo-Júnior et al. 2011). Thus, amides in ripe fruit pulp of *P. reticulatum* may represent significant costs in terms of reduced seed disperser preferences. Another possible explanation for the low amide concentration in ripe fruit pulp is that a re-allocation with ripening may reduce the overall physiological investment in defense, especially if compounds are shunted from fruit pulp to seeds during the ripening process. In addition, because ripe fruits of *P. reticulatum* generally are removed within hours of the final ripening phase (Thies and Kalko 2004; SRW personal observation), the risk of attack from antagonists is relatively low. The persistence time of fruits has been hypothesized to be a key factor in predicting interspecific variation in the occurrence patterns of fruit secondary metabolites, with more persistent fruits expected to exhibit higher levels of chemical defense (Cipollini and Levey 1997a). Overall, factors such as dispersal mode and persistence time may have resulted in selection for low levels of amides in the ripe fruit pulp of this species, but the patterns of allocation to ripe fruit pulp may differ substantially in different species.

In contrast to our expectations, we found few correlations between amide content and damage in natural populations. Because amides have a known role in leaf defense and are bioactive against a range of vertebrate, invertebrate, and microbial consumers (reviewed in Dyer et al. 2004b), it is likely that they also play a role in the chemical defense in this species. However, many factors may influence both the parasitism/herbivory rates and the production of secondary metabolites in natural populations, and thus, high variation in both variables likely makes the detection of relationships difficult. In addition, the detection of a relationship between leaf damage and amide content may have been limited by our measure of herbivory as leaf area removed, which restricts the detection of damage to chewing herbivores only, whereas our measure of seed damage was non-specific and may have been the result of damage from piercing/sucking insect seed predators as well as from pathogens. Although future research taking a more experimental approach or providing more detailed quantification of damage from different types of consumers could potentially reveal additional effects of amide identity and concentration on leaf or seed damage, the fact that the only relationship we detected was a negative correlation between chemical diversity and the proportion of damaged seeds suggests that, at least for seeds, chemical diversity may be a more important predictor of plant damage than concentration (Gershenzon et al. 2012).

The results of this study have added an interesting new element to our understanding of how plants allocate secondary metabolites to different plant parts, particularly fruits and seeds. We confirm that secondary metabolite concentrations and diversity can be higher in reproductive than in vegetative tissues, and that seeds in particular are highly defended. The phytochemical methods and results provided here should

allow the pursuit of additional questions related to the chemical ecology of interactions in *P. reticulatum*, which is an abundant and widespread Neotropical species. Future work should focus on the adaptive significance of secondary metabolite diversity, particularly in reproductive structures, and the role of seed chemical defense in pre- and post-dispersal processes that influence reproductive fitness and species distributions. Although decades of research have shown that plant chemistry plays a key role in plant-animal interactions, this field historically has been dominated by studies of leaf chemistry and leaf herbivores. Increased emphasis on how plant fitness is influenced by the chemistry of reproductive structures would have important implications for our understanding of both plant chemical trait evolution and the evolutionary ecology of mutualisms.

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