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PHYLOGENETIC TRENDS IN PHENOLIC METABOLISM OF MILKWEEDS (*ASCLEPIAS*): EVIDENCE FOR ESCALATION

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Although plant-defense theory has long predicted patterns of chemical defense across taxa, we know remarkably little about the evolution of defense, especially in the context of directional phylogenetic trends. Here we contrast the production of phenolics and cardenolides in 35 species of milkweeds (*Asclepias* and *Gomphocarpus*). Maximum-likelihood analyses of character evolution revealed three major patterns. First, consistent with the defense-escalation hypothesis, the diversification of the milkweeds was associated with a trend for increasing phenolic production; this pattern was reversed (a declining evolutionary trend) for cardenolides, toxins sequestered by specialist herbivores. Second, phylogenetically independent correlations existed among phenolic classes across species. For example, coumaric acid derivatives showed negatively correlated evolution with caffeic acid derivatives, and this was likely driven by the fact that the former are used as precursors for the latter. In contrast, coumaric acid derivatives were positively correlated with flavonoids, consistent with competition for the precursor *p*-coumaric acid. Finally, of the phenolic classes, only flavonoids showed correlated evolution (positive) with cardenolides, consistent with a physiological and evolutionary link between the two via malonate. Thus, this study presents a rigorous test of the defense-escalation hypothesis and a novel phylogenetic approach to understanding the long-term persistence of physiological constraints on secondary metabolism.

KEY WORDS: **Apocynaceae, cardenolide, coevolution, evolutionary trend, flavonoid, plant-defense theory.**

Secondary plant metabolites are defined by their lack of a role in primary metabolism (resource acquisition and allocation) and are often implicated in defense against herbivores. For example, cardenolides are well-known toxins, primarily produced in plants from the Apocynaceae (Malcolm 1991). Phenolic compounds, produced widely in plants, have been implicated in many "secondary" ecological roles, including modulating interactions with insects and microbes, and screening ultraviolet light (Matsuki 1996; Cooper-Driver and Bhattacharya 1998; Taiz and Zeiger 2006). Despite their diversity of structure, broad taxonomic distribution, importance in ecology, and long history in phytochemical research, we know remarkably little about the origin of and macroevolutionary change in such secondary metabolites (Malcolm 1991; Aguilar-Ortigoza and Sosa 2004; Nyman and Julkunen-Tiitto 2005).

Comparative analyses in a phylogenetic context can yield novel insights into previously untested evolutionary hypotheses (Farrell et al. 1991; Agrawal 2007). For example, recent work

has suggested (1) evolutionary trends in the production of secondary metabolites (Becerra 1997; Wink 2003; Aguilar-Ortigoza and Sosa 2004; Agrawal 2007), (2) various levels of evolutionary integration within branches of biosynthetic pathways (Armbruster 2002; Rausher 2006), and (3) trade-offs or positive covariation between classes of secondary metabolites produced across species within a clade (Kursar and Coley 2003; Rudgers et al. 2004; Agrawal and Fishbein 2006; Fine et al. 2006; Agrawal 2007). In particular, examining evolutionary patterns of expression within and across secondary metabolite classes is a novel and complementary approach to studying the physiological mechanisms of metabolite production within a species.

Classic hypotheses predicted that secondary metabolite production would be associated with the degree of phylogenetic nesting, with an "escalation of defense" associated with increasingly derived taxa (Vermeij 1994). We define escalation as a directional trend for increased antiherbivore traits during the diversification of a plant lineage (Agrawal and Fishbein 2008). This prediction was made explicitly by Vermeij and is a special case of Ehrlich and Raven's "escape and radiate" hypothesis in which the evolution of novel traits that promote speciation is incremental and directional through the diversification process (Ehrlich and Raven 1964; Agrawal 2007). Ehrlich and Raven's hypothesis was formulated in the context of the evolution of novel traits, followed by adaptive radiation (i.e., an increase in the diversity of defensive traits during diversification). Alternatively, the escalation hypothesis outlined by Vermeij is focused more on the intensity or quantity of particular defenses exhibited through the diversification process. Of course, both processes may be important. Although incremental and directional trends are often invoked in other areas of evolutionary biology (Mooers et al. 1999; Pagel 1999, Alroy 2000; Adamowicz and Purvis 2006; Moen 2006; Jablonski 2008), such trends have not been rigorously tested in the evolution of plant defense (but see Agrawal & Fishbein 2008).

Typically most work on plant secondary metabolite biosynthetic integration (i.e., trade-offs and synergism in the flux through pathways) occurs on model species in which the flow of specific compounds can be followed, or competition for a particular enzymatic precursor can be identified (Keinanen et al. 1999; Kao et al. 2002; Laskar et al. 2006; Scalliet et al. 2006). This physiological approach has the advantage of being mechanistic and specific to the allocation patterns that occur within a species. However, an approach that considers patterns that exist across species addresses a different question about the long-term persistence and convergent evolution of particular compounds or the associations between branches of biosynthetic pathways (Liscombe et al. 2005; Pelser et al. 2005; Agrawal 2007). In other words, do physiological constraints engender evolutionary trade-offs? And, do associations between biosynthetic pathways persist over evolutionary time, or are they eroded by natural selection or nonadaptive processes rendering pathways independent?

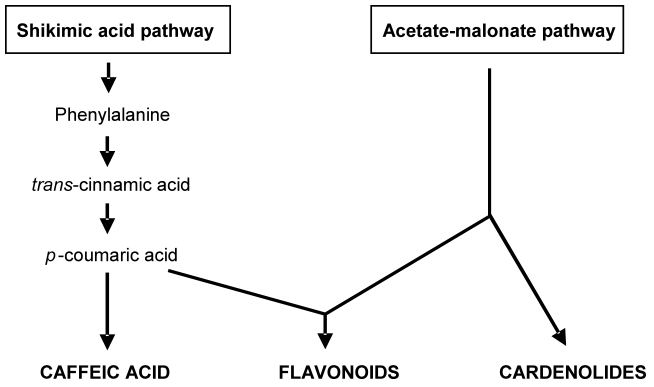

Figure 1. A simplified view of the biosynthesis of two classes of phenolic compounds and cardenolides via the shikimic acid pathway and the acetate-malonate pathway (based on Groeneveld 1999; Taiz and Zeiger 2006).

Figure 1 depicts a simplified view of phenolic metabolism in plants, highlighting the intersection of the shikimic acid and acetate-malonate pathways, the latter of which is responsible for cardenolide production. Given that caffeic acid derivatives and flavonoids are products of *p*-coumaric acid, it would be no surprise if the production of these two classes were negatively correlated within a species. Here the hypothesis is that production of one class depletes precursors necessary for the other, and this could be demonstrated with detailed physiological or enzymatic measurements. The finding of such a trade-off across related species, however, has an entirely different interpretation. If such a pattern persists over the history of a clade, the interpretation is either (1) a strong physiological constraint exists that is unbreakable even over speciational timescales, or (2) the relationship is adaptive and has been maintained by natural selection. A third scenario is that, although the trait values have dramatically changed in the lineage, there has not been selection on phenolic allocation per se, and the relationship is indirectly driven by pleiotropy; this seems improbable based on the likely costliness of the traits.

We addressed the evolution and escalation of secondary metabolism in the milkweeds (*Asclepias*) by growing 35 species of plants from a seed in a common environment and analyzing their contents for major phenolic classes (coumaric acid derivatives, caffeic acid derivatives, and flavonoids). In addition to total phenolics, we report on specific flavonoids (quercetin glycosides) that are known oviposition stimulants for the monarch butterfly (Haribal and Renwick 1998). We also assessed the leaf-cardenolide content in each species to identify escalation of these compounds, and potential correlations with flavonoids, which share a biosynthetic link via malonate (Groeneveld et al. 1990; Groeneveld 1999; Taiz and Zeiger 2006). We predicted varying degrees of evolutionary escalation of cardenolides and

phenolics because of their different modes of action. Cardenolides are typically considered "qualitative defenses" (i.e., toxic to generalist herbivores at low doses, although tolerated by most specialists; Feeny 1976); nonetheless, cardenolides, like other classic qualitative compounds, tend to provide resistance against specialists at higher doses (Zalucki et al. 2001; Agrawal and Kurashige 2003; Agrawal 2005). Phenolics, however, are typically (although not always) considered quantitative defenses (i.e., predicted to have dose-dependent effects on herbivores irrespective of the level of specialization; Feeny 1976). Thus, given that milkweeds have long been associated with a specialized fauna of herbivores that sequester cardenolides (Brower and Glazier 1975; Scudder et al. 1986; Farrell and Mitter 1998; Fordyce and Malcolm 2000; Mooney et al. 2008), we predicted weaker evidence for escalation of cardenolides than phenolics.

Materials and Methods

PLANT GROWTH

Asclepias in the narrow sense includes about 130 species in North America, including Mesoamerica and the Caribbean, and six additional species endemic to South America, excluding all species native to Africa (Woodson 1954; Bollwinkel 1969). Seeds of 35 species (33 North American *Asclepias* and two of African *Gomphocarpus*, selected from the sister group to *Asclepias*; Agrawal and Fishbein 2008; M. Fishbein, unpubl. data) were collected by the authors, colleagues, or from native plant nurseries. Seeds were synchronously germinated after stratifying moistened seeds at 4°C for two weeks. Seeds were planted in 500 mL square pots and grown in Metro-Mix soil (Scotts-Sierra Horticultural Products, Marysville, OH) in a controlled environment growth room with approximately 400-μmol/m^2/sec photosynthetic active light. Although replication varied slightly, we had an average of five plants per species; plants were totally randomized in the chamber. Plants were watered as needed and all of the above-ground biomass was harvested after 30 days of growth. Plant tissues were frozen, oven-dried at 50°C, and ground to a fine powder using a Retch mixer-mill (Retsch, Haan, Germany).

Although our common environment for all species maximizes control over plant age, history, and environmental variation, these milkweeds occupy a diversity of habitats in nature. In a separate study, we have been examining phenotypic plasticity of 12 milkweed species to various environmental factors. Indeed there is phenotypic plasticity in cardenolides and other defensive traits; however, this variation is overwhelmingly trumped by consistent differences between species (S. Cook et al., unpubl. data). Additionally, we compare our measures of phenolic variation among species with that from a previous study by another researcher and find consistency (see "Characterization of Phenolic Compounds").

EXTRACTION

Ground milkweed tissues (20-mg material per sample) were extracted three times (3 × 1 h) with 500-μl acetone/water (7/3, v/v) on a planary shaker. After extraction, acetone was evaporated from the combined extract in vacuo with an Eppendorf concentrator (Hamburg, Germany). The freeze-dried aqueous phase of the extract was dissolved in 1 mL water, the supernatant of the centrifuged (10 min at 2500 *g*) sample was filtered through a 0.45 μm PTFE filter and kept frozen at −20°C until analyzed with high-performance liquid chromatography with diode array detector (HPLC–DAD).

ANALYSIS OF PHENOLICS WITH HPLC–DAD

Analysis of milkweed leaf extracts was performed at 280 nm and 349 nm with Merck–Hitachi's LaChrom HPLC system (Merck–Hitachi, Tokyo, Japan). Column and chromatographic conditions were as described earlier (Salminen et al. 1999), except that 0.1 M H_3PO_4 was replaced with 0.05 M H_3PO_4. Phenolic compounds were quantified using coumaric acid, chlorogenic acid, and quercetin as external standards.

CHARACTERIZATION OF PHENOLIC COMPOUNDS

A large variety of individual phenolic compounds was found in the 35 species of milkweed; up to 76 HPLC peaks were found in individual samples. We thus classified the compounds into different phenolic subclasses on the basis of their UV spectra. No hydrolysable tannins were found in any of the milkweed samples. In contrast, we found that all the major milkweed phenolics could be classified into three subclasses: (1) coumaric acid derivatives, (2) caffeic acid derivatives, and (3) flavonoid glycosides. Because the number of individual compounds was numerous, for simplicity we present pooled values of phenolics within each subclass. In addition, we present data on the diversity of phenolics, which were calculated as the total number of distinct phenolic peaks. Because quercetin glycosides were a substantial fraction of milkweed-flavonoid glycosides (67%), and are known as important oviposition stimulants for Monarch butterflies (*Danaus plexippus*; Haribal and Renwick 1998), they were quantified as a separate subclass of flavonoid glycosides. Our quantification of quercetin glycosides mirrored that of Harribal and Renwick (1998); for the seven species studied in common, our resulting measures were correlated (Spearman's $\rho = 0.75$, $P = 0.05$).

CARDENOLIDE ANALYSIS

Powdered plant material from the same experiment were analyzed for their cardenolide content using a spectrophotometric assay described in Agrawal (2004b). Data are reported in digitoxin equivalents. We recently validated our spectrophotometric method against HPLC methods and found a concordance between the two ($n = 15$, $r = 0.82$, $P < 0.001$, S. Rasmann and A. Agrawal, unpubl. data)

LEAF-DRYING EXPERIMENT

Because in our main experiment we used oven-dried leaf material for phenolic assays, we conducted an additional experiment with four species (*A. angustifolia*, *A. curassavica*, *A. linaria*, and *G. physocarpus*) to address the potential impact of oven-drying leaves on phenolic yields. We collected leaves from five plants of each species (except *A. angustifolia* for which we had four plants) and divided the leaves of each plant into a group that was oven-dried as before and a group that was lyophilized. We then ground, extracted, and analyzed the phenolics as above. Our method of drying did not strongly alter the phenolic yields compared to lyophilization, and the four species showed consistent differences ($F_{3,30} = 15.158$, $P < 0.001$). Although there was an average of 12.5% reduction in total phenolics in our oven-drying treatment, this difference was not significant ($F_{1,30} = 1.512$, $P = 0.228$), and more importantly, there was no interaction between drying method and species $F_{3,30} = 1.500$, $P = 0.235$).

STATISTICAL ANALYSES

Differences in defense investment between species were analyzed with one-way analysis of variance (ANOVA) in JMP (ver. 6). Species means are presented in the Appendix. Pairwise associations between the trait levels measured in each species were performed with Pearson product–moment correlations. In one case, we employed stepwise procedures to delineate the influence of multiple predictor variables (i.e., the impact of coumaric and caffeic acid derivatives on flavonoids) using raw data; results were confirmed with phylogenetically independent contrasts (PICs). These correlation analyses did not distinguish the contributions of shared ancestry and independent adaptation and will henceforth be referred to as "raw" correlations. PIC analyses were conducted using a pruned phylogeny of *Asclepias* (Agrawal and Fishbein 2008; Fig. 2). Briefly, the phylogeny was estimated using Bayesian inference implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) from a dataset of 145 samples (including nearly all species of *Asclepias*), 20 representatives of the African sister group (including *Gomphocarpus*), and representatives of four outgroup genera (M. Fishbein, unpubl. data). Sequence data were obtained from three noncoding regions of the chloroplast genome (*rpl16* intron, *trnC–rpoB* intergenic spacer, and *trnS–trnG* spacer/*trnG* intron; Agrawal and Fishbein 2008). Branch lengths were estimated by maximum likelihood (ML), using PAUP* version 4.0b10 (Swofford 2002), on the 50% majority-rule consensus of trees sampled from the stationary distribution of a Markov Chain Monte Carlo exploration of tree space. Following optimization of branch lengths for the full dataset, branches were pruned to create a phylogram for the 35 taxa sampled for this study. For particular analyses in which phenotypic data were not obtained from all 35 species, trees were pruned further to include only those taxa for which phenotypic traits were measured.

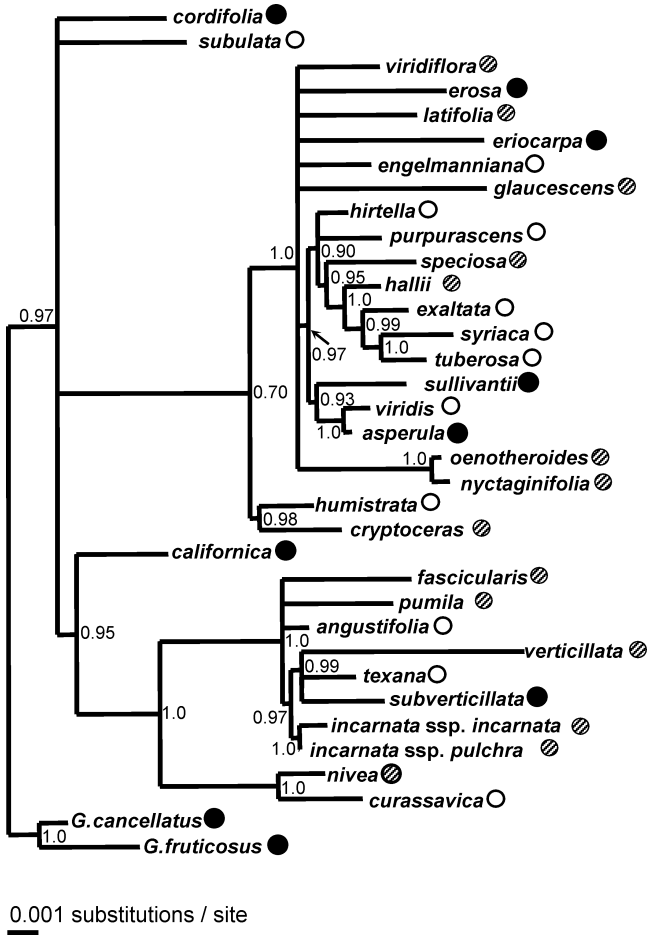

Figure 2. Phylogram of 35 species of *Asclepias* and *Gomphocarpus* examined in this study (Agrawal and Fishbein 2008). The topology was pruned from the 50% majority-rule consensus of trees sampled from the stationary distribution in a Bayesian analysis of 145 species; branch lengths were optimized by maximum likelihood. Numbers indicate Bayesian posterior probabilities. Shown at the tips are circles indicating levels of quercetin glycosides (oviposition stimulants for the monarch butterfly, percent dry tissue) in leaves; open circle, low (0.006–0.070), hatched circle, medium (0.080–0.140), and black circle, high (0.161–0.229).

To reduce potential bias due to shared evolutionary history in estimates of character correlation, we calculated phylogenetically independent correlations with phANOVA (ver. 1.1, available from www.herbivory.com, under "lab members, Lajeunesse") and the pruned phylogeny of *Asclepias*. phANOVA uses the generalized least squares (GLS) method for calculating independent contrasts (the correlation between PICs is a special case of this statistical framework; Rohlf 2006). The GLS method codes the phylogeny as a variance–covariance matrix (**V**) to account for the correlated relationships between species (e.g., all off diagonals of the matrix contain the shared root-to-tip molecular branch length distances between each species; Martins and Hansen 1997). phANOVA has the advantage over other GLS methods in that it can handle

unresolved nodes in the phylogeny. Because our best estimate of the phylogeny is not fully bifurcating (Fig. 2), the degrees of freedom for statistical tests involving PICs were conservatively adjusted down by the number of polytomies (following Purvis and Garland 1993).

We tested hypotheses of defense escalation in a ML framework using Pagel's Continuous implemented in BayesTraits (Pagel 1999; Freckleton et al. 2002). Using GLS, models of trait evolution differing in complexity (i.e., number of parameters) can be compared using a likelihood-ratio (LR) test in which LR = 2[log-likelihood of the better-fitting model − log-likelihood of the worse-fitting model]. Under the assumption of model equivalence, the LR statistic should be chi-square distributed with one degree of freedom (because only a single parameter is altered between the models compared). For example, the fit of a constant–variance random walk model (i.e., Brownian motion) can be compared to models in which directional trends are assessed by regressing the path length (from root to tip) against trait values. The benefit of this GLS framework is that the regression of path length against phylogeny is estimated while accounting for any phylogenetic nonindependence. Because our best estimate of the phylogeny had four polytomies, we conducted all analyses on 1000 randomly resolved phylogenies (branch length of resolved nodes set arbitrarily low to 0.00001) with 95% confidence intervals for all parameters estimated by the bias-corrected percentile method (Efron and Tibshirani 1993).

Prior to evaluating directional trends, we first estimated the extent of phylogenetic signal present in each trait (i.e., trait similarity due to shared ancestry) modeled by the parameter λ (Freckleton et al. 2002). This parameter is estimated using Model A (random walk only) in Continuous. A λ of 1 indicates trait covariance is consistent with a random walk model given the tree topology and branch lengths and 0 indicates no influence of shared ancestry on trait values (Freckleton et al. 2002). Unlike traditional independent contrast analyses, such as that employed above for PICs, here λ is used to scale the analyses for the level of phylogenetic signal.

Directional trends were assessed on trees with two measures of branch length: (1) a phylogram with ML branch lengths estimated from the DNA sequence data, corresponding to a gradual model of trait evolution and (2) a cladogram with all branches set to unity, corresponding to a speciational model of trait evolution (Moen 2006). In the latter case, branch length is equivalent to the number of intervening nodes between the root and a tip. Model A (from above) is compared to Model B in Continuous, in which a directional random walk is modeled by two parameters (λ that accounts for the influence of shared ancestry on the trait and β that estimates the slope of linear, directional change). The latter can be thought of as a phylogenetically independent regression of the trait of interest against total path length from the root to the tips. In other words, any "dominant direction of evolutionary change" is assessed (Pagel 1999). We report the estimated ancestral state (α) and the slope (β) of the regression of path length on each trait value.

Models A and B were compared on trees assuming both gradual evolution (phylogram) and speciational evolution (cladogram) and parameter estimates are reported for the best-fitting model in each case. The trees modeling gradual and speciational evolution differ in the assumed mode of character evolution. Gradual evolution is modeled as occurring in proportion to branch lengths estimated from the presumably neutral variation in noncoding cpDNA. Thus, there is an implicit assumption that rates of change in the defense traits of interest are in proportion to rates of change in the molecular sequence data. Alternatively, speciational evolution is modeled as occurring in proportion to the number of speciation events. Under this model, there is an implicit assumption that character change is concentrated at speciation events.

Results

Total phenolics as determined by HPLC were relatively low in concentration, but varied well over threefold among species (range: 0.25–0.86 percent dry tissue, $F_{34,141} = 10.041$, $P < 0.001$). Quercetin glycosides varied nearly 40-fold across species (range 0.006–0.23, $F_{34,141} = 10.082$, $P < 0.001$) and were very weakly correlated (negatively) with total phenolics (raw correlation, $r^2 = 0.113$, $F_{1,33} = 4.220$, $P = 0.048$). Across all taxa, total phenolics were composed of 54% ($\pm$4.2) caffeic acid derivatives, 10% ($\pm$1.7) coumaric acid derivatives, and 36% ($\pm$3.4) flavonoids (no hydrolysable tannins were found). Only caffeic acid derivatives were correlated (positively) with total phenolics (raw analysis on species means, $r^2 = 0.71$, $F_{1,33} = 80.787$, $P < 0.001$). Cardenolides varied over 14-fold across the milkweed species (range: 0.025–0.353 percent dry tissue, $r^2 = 0.281$, $F_{34,204} = 2.344$, $P < 0.001$). Cardenolides showed a level of phylogenetic conservatism consistent with the random walk model, whereas that for most phenolic classes was lower or not different from zero (i.e., no phylogenetic signal) (Table 1). In addition to those phenolic classes listed in Table 1, we found that the phylogenetic signal was moderate for caffeic acid derivatives ($\lambda = 0.80$, LR = 7.76, $P = 0.005$), coumaric acid derivatives ($\lambda = 0.82$, LR = 2.91, $P = 0.089$), and flavonoids ($\lambda = 0.52$, LR = 2.54, $P = 0.111$). The reported P-values are for tests against $\lambda = 0$; all estimated values of λ were significantly less than 1 at $P < 0.05$, data not shown). In all cases, the upper and lower 95% confidence intervals did not differ from the mean value at two decimal places, indicating little sensitivity to phylogenetic uncertainty around unresolved nodes.

Table 1. Maximum-likelihood estimates (MLEs) of phylogenetic conservatism (λ), ancestral state (α), and directional trends (β) in the evolution of cardenolides and phenolics. A λ value of 1 indicates phylogenetic conservatism consistent with a random walk on the tree topology and the significance is tested against the fit of a model with λ = 0. Directional trends are assessed separately for gradual and speciational models of evolution; in each case the directional model is tested against a random walk without a trend. λ, α, and β are reported for gradual models (which were better fitting than the speciational model in all cases). Significance of model parameters is evaluated by likelihood ratio (LR) tests. In most analyses, bootstrap replicates estimating uncertainty due to polytomies resulted in identical values to two decimal places; where this was not the case, upper and lower confidence intervals are provided for the LR and *P*-values.

	MLE	LR	*P*	Interpretation
Total cardenolides				
λ	0.88	16.94	**<0.001**	phylogenetic conservatism
Gradual trend		3.98	**0.046**	gradual trend
speciational trend		0.02	0.887	no directional trend
α	0.34			
β	−13.30			declining
Total phenolics				
λ	0.35	1.54	0.215	no phylogenetic conservatism
Gradual trend		7.02	**0.008**	gradual trend
speciational trend		5.82–14.39	**0.016 – <0.001**	speciational trend
α	0.33			
β	14.24			increasing
Phenolic diversity				
λ	0.45		0.083	little phylogenetic conservatism
gradual trend		5.52	**0.019**	gradual trend
speciational trend		3.24–8.05	**0.071–0.005**	equivocal speciational trend
α	46.15			
β	−257.18			declining
Quercetin glycosides				
λ	0.63	8.16	**0.004**	some phylogenetic conservatism
gradual trend		0.66	0.417	no directional trend
speciational trend		0.124–3.792	0.725–0.052	no directional trend
α	1.69			
β	n/a			

DEFENSE ESCALATION

A gradual model of evolution supported a strong, directional trend for total cardenolides and phenolics (Table 1, Fig. 3). The direction of these effects was opposing, however, with cardenolides decreasing as *Asclepias* diversified, whereas phenolics increased. Only in the former case was there evidence of phylogenetic conservatism (i.e., $\lambda > 0$), and the GLS analysis of trends accounted for this influence. Our best estimates for the ancestral states for phenolics and cardenolides were nearly equivalent on a percentage dry mass basis, as were the respective (opposing) slopes of the directional trends. Although both the raw and GLS slopes were significant, the GLS slopes were substantially steeper (Fig. 3, Table 1). The analyses revealed similar evolutionary trends for both the gradual and speciational models of character evolution, although the former were more often statistically significant (Table 1).

We conducted two additional tests of evolutionary trends in phenolic metabolism (Table 1). Quercetin glycosides showed moderate phylogenetic conservatism and no directional evolutionary trend (see Fig. 2). We found evidence for a decline in the number of phenolic compounds as *Asclepias* diversified (species had between 39 and 49 different phenolics), and this result was stronger in the gradual model (Table 1). Thus, although total phenolic metabolism escalates, this comes with a concomitant decline in the diversity of compounds produced.

In other analyses with these same species, we have shown that several traits unrelated to defense do not show patterns of directional evolutionary change (Agrawal and Fishbein 2008); this is critical, as it indicates that directional evolutionary patterns are not an artifact of the tree topology or other spurious factors that could generate apparent trends across all characters using our analytic methods.

CORRELATIONS BETWEEN DEFENSIVE CLASSES

Coumaric and caffeic acid derivatives showed negatively correlated evolution across species (phylogenetic independent contrast,

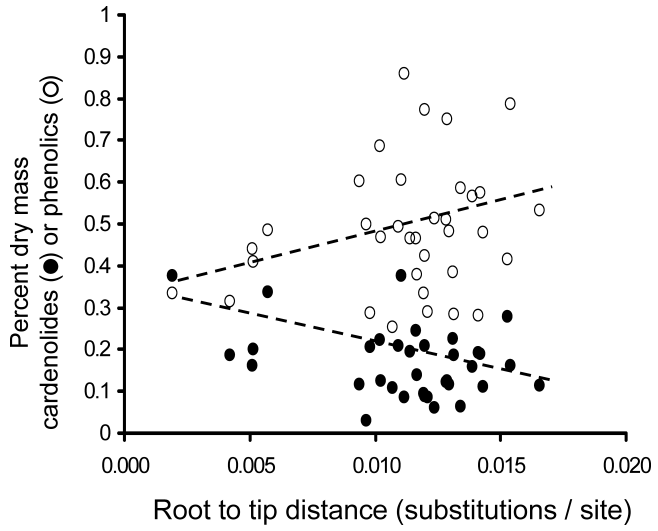

Figure 3. Relationships between the root to tip phylogenetic distance and percent dry mass of total cardenolides and total phenolics across 35 milkweed species. Shown are species means from approximately seven replicate individuals of each species grown from seed in a common environment. Slopes are indicated for phylogenetically controlled analyses (Table 1).

PIC: $r^2 = 0.165$, $F_{1,29} = 6.527$, $P = 0.016$). We next used a stepwise procedure (on raw data) to predict flavonoid levels based on coumaric and caffeic acid derivatives. This analysis revealed that only caffeic acid derivatives predicted flavonoids, and this association was negative (data not shown); a PIC confirmed negatively correlated evolution of the levels of these compounds ($r^2 = 0.238$, $F_{1,29} = 10.322$, $P = 0.003$). Indeed, flavonoids and caffeic acid derivatives share a common precursor (*p*-coumaric acid), suggesting competition for this intermediate. We tested for associations among the three phenolic classes and cardenolides using stepwise multiple regression, and found that only flavonoids and cardenolides were correlated (positively) (stepwise procedure on raw data not shown; confirmed with a pairwise PIC: $r^2 = 0.293$, $F_{1,29} = 13.693$, $P < 0.001$) (Fig. 4).

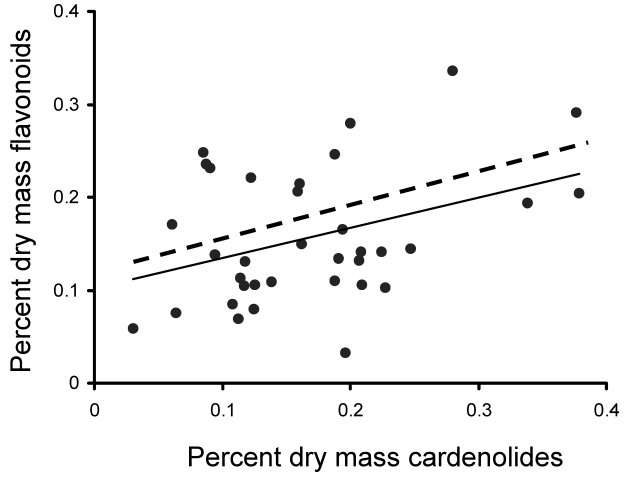

Figure 4. Correlation between percent dry mass cardenolides and flavonoids across 35 milkweed species. Shown are species means from approximately seven replicate individuals of each species grown from seed in a common environment. Shown are the raw (solid) and phylogenetically independent (dashed) slopes.

Discussion

Our results show three novel evolutionary patterns in the production of plant secondary metabolites. First, within the radiation of *Asclepias* we found a directional trend for increasing investment in phenolic metabolism, but the reverse for phenolic diversity and production of total cardenolides (i.e., evolutionary decline in investment). Second, phenolic subclasses showed correlated evolution consistent with trade-offs and synergism across milkweed species. Third, cardenolides and flavonoids, which are linked via the acetate-malonate pathway, showed positively correlated evolution. The fact that each of these relationships was upheld after accounting for the evolutionary history of *Asclepias* demonstrates that the associations have evolved repeatedly. Indeed, the persistence of such relationships is indicative that, rather than limiting evolution, strong constraints have resulted in a significant level of correlated evolution and adaptation. The importance of this evolutionary pattern is that the relationship between cardenolides and flavonoids has not been broken over the many speciation events that generated the 130 species of *Asclepias*. Costs associated with the production of such secondary metabolites minimize the possibility of passive indirect evolution of these traits (Coley et al. 1985; Strauss et al. 2002; Fine et al. 2006). Below we elaborate on the conceptual issues and context of each of these findings.

In Ehrlich and Raven's (1964) coevolutionary model, it was the evolution of novel-resistance mechanisms that fosters diversification (i.e., a key-innovation sensu Farrell et al. 1991; Hodges and Arnold 1995). A variation on this model, proposed by Vermeij (1994), predicted that the novel-resistance mechanisms may be incrementally increasing rather than new compounds per se (and directional) during the diversification of a clade. Accordingly, this suggests an escalation, either in terms of potency or diversity of secondary metabolites, in species that diverged relatively late in the radiation. Vermeij did not specifically suggest that escalation of traits contributes to diversification, but the inference of "escalation during diversification" versus "escalation causing diversification" is not distinguishable by the pattern elucidated by the methods we employed. In other words, the finding of a directional trend does not necessarily indicate that the trait itself promoted speciation. Although recently developed phylogenetic methods have been proposed to specifically test for a causal

link between continuous trait variation and diversification rates (Paradis 2005; Freckleton et al. 2008), these methods are computationally identical to Pagel's (1999) method for assessing directional trends using a speciational model (Table 1, R. P. Freckleton, pers. comm.).

Futuyma (1987) has argued that directional evolutionary trends may have little to do with adaptive traits causing diversification, but rather the reverse, that the process of speciation may be required to preserve directional change in adaptive trait values. Such directional change could otherwise be lost due to gene flow between populations or the relatively ephemeral nature (and perhaps small size) of local populations (Wright 1931). As discussed above, we do not yet have the methods to distinguish between the key-innovation hypothesis of adaptation, which facilitates speciation, and Futuyma's view in which speciation allows for the preservation of directionally changing adaptations. In general, only speciational models (not gradual models) will be consistent with an impact of traits on diversification. We note that only a gradual model was consistent with the progressive decline in cardenolides (Table 1). We interpret this result to indicate the speciation per se was not strongly caused by or accelerated the evolution of cardenolides. Phylogenetic trends in phenolic concentration and diversity were less clear, as both gradual and speciational models showed significant directional trends.

Several other issues pervade in the assessment and interpretation of directional phylogenetic trends. For example, the taxonomic scale at which we might expect to see a pattern of escalation is unclear. It may be that each innovation is followed by a burst of speciation, in which case a pattern of escalation may be difficult to detect in a species-level phylogeny. Alternatively, escalation may occur with each bifurcation in a phylogeny (Futuyma 1987). Additionally, innovations need not be incremental or directional; indeed, much of the literature on coevolution has focused on the evolution of novel traits (Berenbaum 1983; Armbruster 1997; Farrell and Mitter 1998). These empirical data are few and were not analyzed in a formal phylogenetic context, but they were in general agreement with the escalation hypothesis (Berenbaum 1983; Armbruster 1997; Farrell and Mitter 1998). More recent analyses with *Asclepias* show patterns of both escalation and decline of different plant-defense strategies (Agrawal and Fishbein 2008; Agrawal et al. 2008). Describing the evolutionary patterns of defense traits is ultimately an empirical question that will benefit from analyses of both the quantity and diversity of various defense strategies at multiple phylogenetic scales (Agrawal 2007).

In the current study, although the diversity of phenolics declined, total phenolic metabolism increased. The specific functional roles of each of the many phenolics are largely unknown. However, the progressive reduction in cardenolides could be a direct response to herbivory by specialized herbivores, most of which sequester cardenolides for their own fitness benefit (Brower and Glazier 1975; Scudder et al. 1986; Farrell and Mitter 1998; Fordyce and Malcolm 2000; Mooney et al. 2008). We are cautious in the adaptive interpretation of this phylogenetic decline because, even though milkweed specialist herbivores sequester cardenolides, there is still substantial evidence that they are negatively impacted by these same compounds (Zalucki et al. 2001; Agrawal 2004a, 2005).

EVOLUTIONARY INTEGRATION OF SECONDARY METABOLISM

Despite the fact that phenolics progressively increased and cardenolides progressively decreased during the evolutionary history of the milkweeds, these two classes of compounds did not show negatively correlated evolution overall ($P = 0.298$). This result indicates that the evolutionary patterns in these classes were not constrained by their biosynthetic integration. On the one hand, cardenolides showed a pattern of evolution strictly corresponding to a gradual model (Table 1), and this trend was linear (Fig. 3). The evolution of phenolics, on the other hand, was explained well by both gradual and speciational models. Thus, although cardenolides and phenolics clearly show opposing trends in their directional evolutionary change, each of these phytochemical groups has shown distinct patterns in trait evolution.

We found strong evidence for evolutionary integration among phenolic classes and across flavonoids and cardenolides. Where two classes of compounds share a common precursor, it is not uncommon to find a trade-off in expression within species (Gershenzon and Croteau 1992; Burbulis and Winkel-Shirley 1999; Keinanen et al. 1999; Nyman and Julkunen-Tiitto 2005). Conversely, changes in the level of a common precursor may cause similar effects in the expression of multiple products by simply changing the overall flux through the pathway, causing the levels of the products to be positively correlated (Martens and Mithofer 2005). Both of these findings have come primarily from within-species physiological studies. Across milkweed species, the same positive and negative evolutionary correlations are found at various branching points in the pathways. Indeed, caffeic acid derivatives and flavonoids share *p*-coumaric acid as a precursor, and there appears to be evolutionary competition for this precursor.

Although the biosynthetic production of cardenolides have not been widely studied, there is compelling evidence from work on *A. curassavica* (included in this study) that acetate and malonate are critical in cardenolide biosynthesis (Groeneveld et al. 1990; Groeneveld 1999). Indeed, the acetate-malonate pathway intersects with the shikimic acid pathway, providing the C6 aromatic ring for the production of flavonoids (Andersen et al. 2006). Our results suggest that positive flux through the acetate-malonate pathway results in greater levels of both flavonoids and cardenolides. This does not necessarily mean there is no physiological

competition for precursors, but rather that species are jointly evolving investment in both end products, possibly through investment in the precursor itself.

CONCLUSION

The phylogenetic revolution is allowing, for the first time, rigorous tests of classic plant-defense theory, and more fundamentally, discovery of morphological and physiolgoical trends in the evolution of life (Becerra 1997; Thaler and Karban 1997; Wink 2003; Heil et al. 2004; Agrawal and Fishbein 2008). Convergence nested within a background of phylogenetic conservatism appears to be the rule in most macroevolutionary studies of morphological and physiological traits. Within and across biosynthetic pathways, plant-defensive traits are evolving in a coordinated fashion. Correlations between traits persist, with some exceptions, and substantial deviation in a particular relationship suggests that it is not driven primarily by a physiological constraint. That there are evolutionary patterns in the expression of defense traits is perhaps the best evidence we have for phytochemicals as long-term evolutionary adaptations. We have identified both decline and escalation in the evolution of plant defenses; this finding necessitates a revaluation of plant-defense theories, potentially requiring a syndromes perspective, and one that involves consideration of multiple-selective agents on plants (Kursar and Coley 2003; Agrawal and Fishbein 2006; Agrawal et al. 2008).

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Appendix. **Raw data for the cardenolide and phenolic measures (percent dry mass) of the 35 species of milkweed. Reported are means from approximately seven replicate individuals from each species grown from a seed in a common environment and harvested at the same time.**

	Leaf cardenolides	Total phenolics	Coumaric acid derivatives	Caffeic acid derivatives	Flavonoids	Quercetin glycosides	Number of phenolic compounds
Asclepias angustifolia Schweigg.	0.030	0.501	0.013	0.430	0.059	0.009	26.0
A. asperula (Decne.) Woodson ssp. *asperula*	0.376	0.607	0.127	0.189	0.291	0.182	30.9
A. californica Greene ssp. *californica*	0.200	0.411	0.009	0.122	0.280	0.210	30.8
A. cordifolia (Benth.) Jeps.	0.161	0.441	0.068	0.159	0.215	0.198	32.2
A. cryptoceras S. Watson ssp. *cryptoceras*	0.108	0.254	0.022	0.147	0.085	0.080	31.7
A. curassavica L.	0.196	0.466	0.018	0.416	0.032	0.028	27.4
A. engelmanniana Woodson	0.139	0.381	0.032	0.240	0.109	0.062	27.0
A. eriocarpa Torr.	0.280	0.417	0.080	0.002	0.336	0.171	32.0
A. erosa Torr.	0.194	0.282	0.000	0.116	0.165	0.161	23.0
A. exaltata L.	0.125	0.751	0.014	0.657	0.079	0.006	23.8
A. fascicularis Decne.	0.118	0.484	0.014	0.339	0.131	0.124	18.8
A. glaucescens Kunth	0.162	0.788	0.000	0.638	0.149	0.110	29.6
A. hallii A. Gray	0.209	0.425	0.006	0.313	0.106	0.102	24.2
A. hirtella (Pennell) Woodson	0.208	0.494	0.054	0.299	0.142	0.019	27.3
A. humistrata Walter	0.206	0.288	0.044	0.112	0.132	0.069	29.7
A. incarnata L. spp. *incarnata*	0.126	0.470	0.012	0.352	0.106	0.088	30.8
A. tuberosa L.	0.064	0.586	0.035	0.475	0.076	0.069	27.6
A. *latifolia* (Torr.) Raf.	0.188	0.286	0.062	0.113	0.110	0.099	24.0
A. nivea L.	0.225	0.688	0.060	0.487	0.141	0.140	23.4
A. nyctaginifolia A. Gray	0.191	0.576	0.056	0.385	0.134	0.121	25.4
A. oenotheroides Schtldl. & Cham.	0.159	0.566	0.135	0.225	0.206	0.107	31.0
A. incarnata L. ssp. *pulchra* (Ehrh. ex Willd.) Woodson	0.117	0.603	0.026	0.474	0.104	0.103	30.8
A. pumila (A. Gray) Vail	0.061	0.514	0.032	0.312	0.171	0.127	33.9
A. purpurascens L.	0.090	0.774	0.090	0.453	0.232	0.060	27.4
A. speciosa Torr.	0.227	0.386	0.012	0.272	0.102	0.094	32.0
A. subulata Decne.	0.338	0.487	0.115	0.178	0.194	0.026	29.5
A. subverticillata (A. Gray) Vail	0.085	0.291	0.022	0.021	0.249	0.176	28.6
A. sullivantii Torr.	0.123	0.511	0.080	0.209	0.221	0.220	30.3
A. syriaca L.	0.113	0.482	0.039	0.373	0.069	0.065	26.7
A. texana A. Heller	0.088	0.860	0.021	0.604	0.235	0.010	22.8
A. verticillata L.	0.114	0.533	0.046	0.374	0.114	0.107	32.8
A. viridiflora Raf.	0.095	0.335	0.153	0.044	0.139	0.131	30.4
A. viridis Walter	0.247	0.466	0.116	0.205	0.145	0.012	24.0
Gomphocarpus cancellatus (Burm. f.)	0.378	0.336	0.000	0.132	0.204	0.198	25.6
G. fruticosus (L.) W.T. Aiton ssp. *fruticosus*	0.187	0.315	0.065	0.004	0.246	0.229	26.2