

# Phylogenetic escalation and decline of plant defense strategies

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As the basal resource in most food webs, plants have evolved myriad strategies to battle consumption by herbivores. Over the past 50 years, plant defense theories have been formulated to explain the remarkable variation in abundance, distribution, and diversity of secondary chemistry and other defensive traits. For example, classic theories of enemy-driven evolutionary dynamics have hypothesized that defensive traits escalate through the diversification process. Despite the fact that macroevolutionary patterns are an explicit part of defense theories, phylogenetic analyses have not been previously attempted to disentangle specific predictions concerning (i) investment in resistance traits, (ii) recovery after damage, and (iii) plant growth rate. We constructed a molecular phylogeny of 38 species of milkweed and tested four major predictions of defense theory using maximum-likelihood methods. We did not find support for the growth-rate hypothesis. Our key finding was a pattern of phyletic decline in the three most potent resistance traits (cardenolides, latex, and trichomes) and an escalation of regrowth ability. Our neontological approach complements more common paleontological approaches to discover directional trends in the evolution of life and points to the importance of natural enemies in the macroevolution of species. The finding of macroevolutionary escalating regrowth ability and declining resistance provides a window into the ongoing coevolutionary dynamics between plants and herbivores and suggests a revision of classic plant defense theory. Where plants are primarily consumed by specialist herbivores, regrowth (or tolerance) may be favored over resistance traits during the diversification process.

cardenolides | coevolution | macroevolutionary trends | milkweed *Asclepias* | plant defense theory

Milkweeds (*Asclepias* spp., Apocynaceae) are prime candidates for a clade-based approach to testing plant defense theories because of their well known defensive strategies and tremendous variation in expression of these traits (1–3). Three traits—cardenolides, latex, and trichomes—have been strongly implicated in providing milkweed resistance against herbivores. Each of these traits has been demonstrated to quantitatively affect the behavior (4, 5), performance (1, 6, 7), and abundance (8) of herbivores in the field. The resistance provided by these three traits occurs despite the fact that most milkweed herbivores are specialists and have adaptations to cope with each defense (4, 5, 9). Our central goal was thus to evaluate what factors predict variation in defense investment across the clade.

We tested four major predictions of classic plant defense theory using phylogenetically explicit analyses employing maximum-likelihood estimation of defense trait evolution (10, 11). First, do the individual resistance traits used by plants trade off due to redundancy, or do they repeatedly evolve together as a suite of covarying traits (12, 13)? Second, does plant growth rate covary with investment in resistance traits as predicted by resource availability theory (14, 15)? Third, do resistance and regrowth ability (a measure of tolerance) trade off as alternative defensive strategies to cope with herbivores (16)? And finally, as predicted by some (co)evolutionary theory, is there evidence for phenotypic escalation in defense trait expression as plant lin-

eages diversify (17–20)? This last prediction was made explicitly by Vermeij (19) and is a special case of Ehrlich and Raven's (17) "escape and radiate" hypothesis where the evolution of novel traits that promote speciation is incremental (and directional) through the diversification process.

To test our predictions, we grew replicate plants of each of 36 *Asclepias* species and two from the genus *Gomphocarpus* (representative of the sister clade to *Asclepias*) from seed in a common environment and measured cardenolides, latex, trichomes, growth rate, and regrowth ability after severe damage. Phylogenetically explicit analyses were conducted by using a molecular phylogeny of *Asclepias* estimated by using Bayesian inference (Fig. 1). The phylogeny was estimated from sequence data obtained from three noncoding regions of the chloroplast genome, and branch lengths were estimated by maximum likelihood.

## Results and Discussion

Contrary to prediction 1, we found no tradeoffs between the three most potent resistance traits (pairwise correlations: cardenolides–latex [raw correlation (rc):  $r = 0.279$ ,  $P = 0.094$ , phylogenetically corrected (pic):  $P = 0.317$ , cardenolides–trichomes (rc:  $r = 0.175$ ,  $P = 0.300$ , pic:  $P = 0.121$ ), latex–trichomes (rc:  $r = 0.647$ ,  $P < 0.001$ , pic:  $P = 0.001$ ]). Indeed, the strongly positive phylogenetically independent correlation between latex and trichomes is consistent with our previous field experiment on a subset of these species (1). Positive correlated evolution appears to be a general phenomenon in the evolution of plant defense against herbivory, because tradeoffs between resistance traits are relatively uncommon in inter- and intraspecific comparisons in other systems (1, 12, 21). Consequently, we sought to use a multivariate index of composite investment across the three traits. We used a recently proposed index, the sum of the Z scores for the three traits (22), which was also highly correlated with the factor scores of the first principal component estimated from the same data ( $r = 0.998$ ,  $P < 0.001$ ) [supporting information (SI) Table S1].

Resource-availability theory, which has been well supported in studies of tropical trees and some temperate herbaceous species, predicts that plant growth rate determines investment in resistance (14, 15, 23). However, despite >20-fold variation in plant growth rate, this did not explain investment in the composite resistance of milkweeds (Fig. 24). We do not interpret this result to mean that the production of resistance traits is cost-free, but rather that there is no evolutionary signature of intrinsic plant growth rate dictating investment in resistance traits across the clade.

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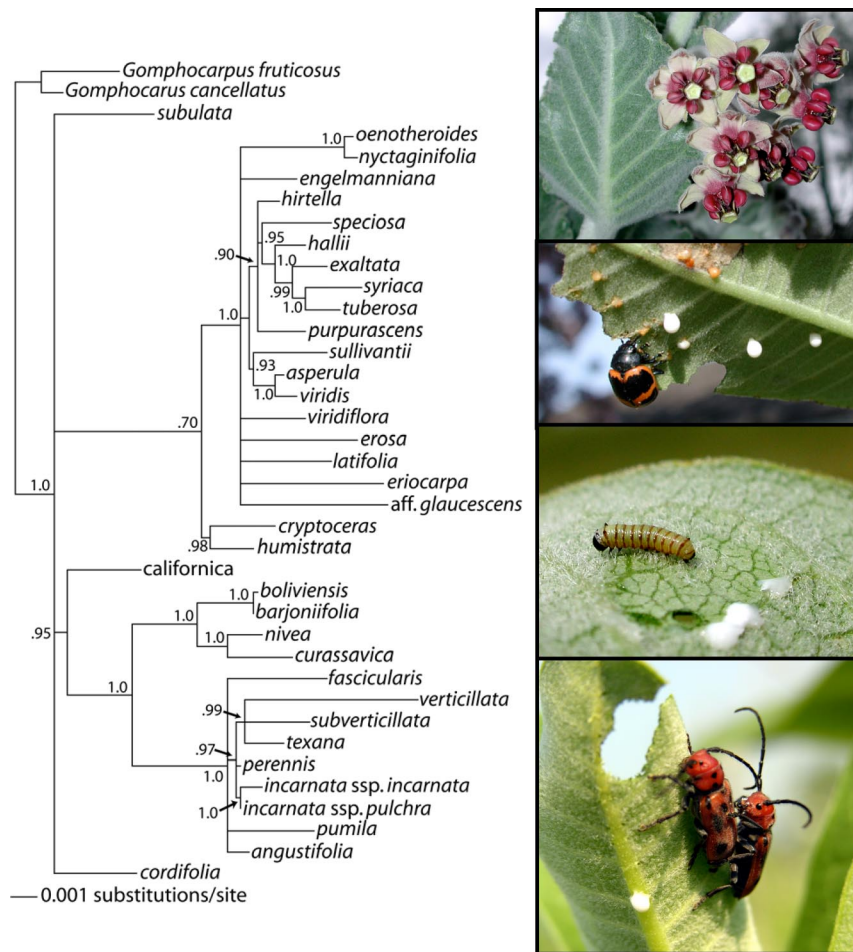
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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. are available in Table S4).

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**Fig. 1.** Phylogeny of 36 species of *Asclepias* and two of *Gomphocarpus*, with branch lengths estimated by maximum likelihood. DNA sequences from three, noncoding, plastid DNA regions (*rpl16* intron, *trnC-rpoB* spacer, *trnS-trnG* spacer/*trnG* intron) were analyzed. The phylogeny was inferred by Bayesian inference; the 50%-majority-rule consensus of trees sampled from the stationary distribution in an analysis of a broader sample of 145 species formed the starting tree from which the depicted tree was pruned (see *Materials and Methods*). Posterior probabilities ( $>0.5$ ) are indicated near nodes. Images on the right (top to bottom): *A. californica*, leaf beetle *Labidomera clivicollis* feeding on *A. syriaca* after deactivation of latex by clipping of the laticifers, Monarch butterfly caterpillar *Danaus plexippus* shaving trichomes of *A. syriaca*, and the longhorn beetle *Tetraopes tetraophthalmus* feeding after clipping the midrib of *A. syriaca*.

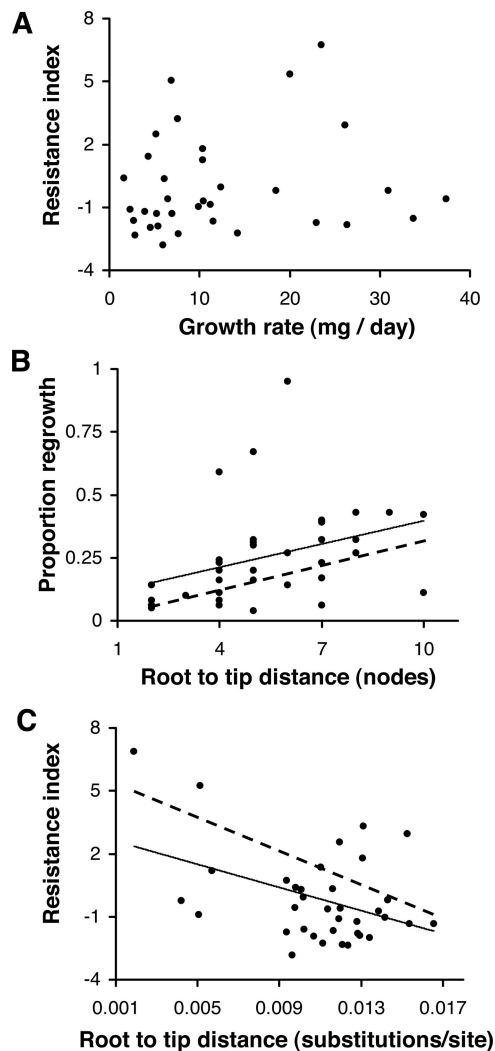
We tested two predictions about the ability of plants to regrow after damage. First, early models and intraspecific data from *A. syriaca* suggested that regrowth may be predicted by the root-to-shoot ratio, whereby those species that invest relatively more underground will be able to better recover from aboveground damage (3, 24). Indeed, we found that root-to-shoot ratio was a good predictor of regrowth, but that the association between high investment in roots and ability to regrow after damage was phylogenetically constrained (rc:  $r = 0.387$ ,  $P = 0.022$ , pic:  $P = 0.841$ ). Second, defense theories have suggested that investment in resistance traits and regrowth ability should trade off (14, 16). There is strong, but not universal, support for such tradeoffs within species; however, no previous test has taken a phylogenetic approach to test the comparative hypothesis. Although the sign of the relationship we observed was negative, we did not find a significant tradeoff between regrowth and composite resistance (rc:  $r = -0.243$ ,  $P = 0.165$ , pic:  $P = 0.371$ ).

Finally, we tested the prediction of macroevolutionary directional trends (19, 20) in defense investment as *Asclepias* diversified. Here, we used Pagel's method of assessing evolutionary trends for continuously varying traits by comparing a model of trait evolution that assumes a constant-variance random walk (i.e., Brownian motion) to one in which a directional trend is additionally assessed by regressing the path length (i.e., the

number of intervening nodes or molecular branch length from root to tip) against trait values. The benefit of this framework is that the regression of path length against the phenotype is estimated while correcting for phylogenetic nonindependence.

Our data were remarkably consistent with classic theory; however, the evolutionary escalation we report is in regrowth after damage but not in resistance traits (Fig. 2B). Indeed, the dominant evolutionary trend for our index of the three resistance traits was to strongly decline during the diversification of *Asclepias* (Fig. 2C). The directional trends for regrowth and resistance were not only divergent in their direction, but they also showed distinct patterns of trait evolution. The trend toward increased regrowth ability was best fit by a speciation model and was accompanied by extreme lability, with little apparent phylogenetic constraint. In contrast, the macroevolution of resistance traits, although it exhibited a significant reductive trend, was best fit by a gradual model and exhibited phylogenetic conservatism (Fig. 2B and C).

To assess whether these striking directional trends between plant defense phenotypes and phylogenetic path length are distinct from nondefense-related traits, we arbitrarily chose and examined macroevolutionary trends for three traits (from the same set of plants) unrelated to defense: leaf size, water use efficiency ( $\delta^{13}\text{C}$ , leaf carbon isotope ratio), and growth rate. None of these traits showed evidence for phyletic patterns (in either speciation or gradual models, the highest LR = 2.24,  $P = 0.134$ ; Table S2).



**Fig. 2.** Phylogenetic tests of plant defense theory. For illustrative purposes, raw data points (not corrected for phylogenetic non-independence) are shown for each milkweed species; significant slopes are shown for the raw data (solid lines) and phylogenetically independent correlations (dashed lines). (A) Plant growth rate (dry mass accumulation per day) does not predict investment in resistance (a multivariate summary of trait values for cardenolides, latex, and trichomes (raw correlation:  $r = 0.145$ ,  $P = 0.415$ , phylogenetically controlled:  $P = 0.708$ ). (B) Phenotypic escalation of regrowth ability, best fit is a speciation model with phylogenetic distance measured by number of intervening nodes,  $\lambda = 0.316$ , LR = 9.626 [ci 6.257–12.348],  $P = 0.002$ ,  $\alpha = 0.020$ ,  $\beta = 0.029$ . (C) Phenotypic decline of resistance; best fit is a gradual model with phylogenetic distance measured by expected substitutions per site,  $\lambda = 0.972$ , LR = 3.792 [ci 3.774–3.831],  $P = 0.05$ ,  $\alpha = 5.260$ ,  $\beta = -400.488$ . Speciation vs. gradual model fit was determined using the parameter  $\kappa$  (see *Materials and Methods*). Maximum-likelihood estimates of  $\kappa$  were not different from 0 for regrowth ability (estimated  $\kappa = 0.114$ , LR = 0.948,  $P = 0.330$ );  $\kappa$  was not different from 1 for the resistance index ( $\kappa = 0.673$ , LR = 1.645,  $P = 0.199$ ). In both cases, the preferred model was significantly better than the alternate.

Our results bear on the long-standing debates about the role of evolutionary history in the evolution of plant defense (17) and the predicted tradeoff between resistance and regrowth ability (14, 16). By integrating the two, we find that although an explicit relationship between resistance and regrowth ability is not evident in the extant species, a rigorous phylogenetic test of directional trends supports long-term escalation and decline in these divergent plant defense strategies. We propose that these countervailing evolutionary trends could be the result of the dominance of the milkweed herbivore fauna by specialist insects. Despite the function of

cardenolides, latex, and trichomes in reducing herbivory, each of the specialized herbivores employs several mechanisms to circumvent or attenuate the negative effects of these defensive traits (4, 5, 9). As a result, we find that herbivory on milkweeds is common, consistent across years, and often considerably intense.

Macroevolutionary relaxation of existing and poorly functioning resistance is thus predicted for plants with a herbivore fauna dominated by specialists. At least three nonexclusive alternatives could be favored in coevolution: (i) novel resistance traits with greater potency than those overcome by herbivores, (ii) escape in space or time, or (iii) escalation of regrowth to herbivores. Our data are consistent with milkweed herbivores favoring a shift in the defensive strategy away from resistance and toward increased regrowth ability via elevated root-to-shoot ratios. We speculate that early diverging species that retain lower regrowth ability may have either experienced lower levels of long-term herbivory by specialists or, more likely, have been constrained in their evolution and lagged in adaptation to the specialized herbivore fauna.

## Materials and Methods

**Plants and Traits.** Plants from the 38 species were grown from seed in a controlled environment growth room (400  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ , 12:12-h day/night cycle, 27°C/25°C), and traits were assessed by using standard protocols (five replicate plants per species). After 30 days of growth, all aboveground tissues directly above the cotyledons were harvested with scissors. Plant tissues were frozen, oven-dried at 50°C, weighed to assess growth rate, ground to a fine powder, and analyzed for cardenolides. Cardenolide concentration was assayed spectrophotometrically following the methods of Agrawal (7, 8). Although this method does not elucidate specific cardenolide peaks or their polarity, we have previously shown it to be a good predictor of resistance to Monarch larvae (8) and milkweed aphids (25).

Regrowth ability (an estimate of tolerance to herbivory) was measured as the percentage of aboveground biomass accumulated over a regrowth period (the 14 days subsequent to the initial harvest) relative to the initial growth. This simple standardized method of assessing plant recovery after severe defoliation was used because of the remarkably diverse growth forms and sizes produced by the 38 species. Root-to-shoot ratio was calculated by the dry mass of roots, washed free of soil, from the final harvest divided by the total aboveground biomass from the two harvests. Latex and trichomes were measured from a separate set of plants grown from seed and maintained in a living glasshouse collection at Cornell University. Again, a mean of five replicates was measured per species. Latex was measured by damaging one leaf tip per plant and collecting the exudate to determine its mass (8). Trichomes were counted from the same leaf position on a leaf disk (28  $\text{mm}^2$ ) under a dissecting microscope (summing the adaxial and abaxial sides).

**Phylogenetic Analysis.** We estimated the phylogeny of the 36 *Asclepias* and two *Gomphocarpus* species using DNA sequences obtained for a comprehensive phylogeny of the genus. The broader dataset contains 145 samples, including nearly all species of the wholly American *Asclepias* and 20 samples from various genera in the African sister group. The 36 species of *Asclepias* were selected primarily because of availability of seeds. However, this sample includes representatives of 9 of the 13 major clades identified in analyses of the complete dataset, minimizing the potential for bias due to nonrandom sampling. DNA sequences were obtained from three noncoding regions of the plastid genome: *rpl16* intron, *trnC-rpoB* intergenic spacer, and *trnS-trnG* spacer/*trnG* intron, by using standard procedures for DNA extraction, PCR amplification, and cycle-sequencing with dye terminators and automated fluorescent detection (26).

Reaction conditions and primer sequences are those used in the original studies employing these regions (27–30), except for the following modifications. Regardless of the thermal cycling conditions used in the original studies, PCR amplification of all three regions used the program developed specifically for the *rpl16* intron (30). *Asclepias*-specific primers were designed to optimally obtain complete sequences for both strands of the *rpl16* and *trnC-rpoB* regions (Table S3). Complete sequences were assembled and edited with Sequencher ver. 3.0 (Gene Codes) and the SeqMan II module of Lasergene ver. 6 (DNASTAR). Species sampled, voucher data, and GenBank accession numbers for each sequence are presented in Table S4.

Multiple sequence alignments for each region were obtained by eye with the aid of Se-Al version 2.0 (available from A. Rambaut, Se-Al: Sequence Alignment Editor at <http://evolve.zoo.ox.ac.uk/>) and MacClade version 4.08 (31). Many of the required gaps were easily interpreted as independent insertion/deletion (indel) events. However, each region contained at least one stretch of overlapping gaps

that could be aligned only with substantial ambiguity. For the analyses reported here, all gapped regions, whether the alignment was ambiguous or unambiguous, were omitted. There was no evidence of conflicting phylogenetic signal across the three regions (results not shown), so they were concatenated for simultaneous phylogenetic analysis.

The phylogeny of 38 species of *Asclepias* and *Gomphocarpus* was estimated by first analyzing the complete dataset of 145 concatenated sequences by using Bayesian inference, implemented by Metropolis-coupled Markov chain Monte Carlo simulation in MrBayes 3.1.2 (32). Each of the three plastid DNA regions was treated as an independently evolving partition in the Bayesian analysis. For each region, the best fitting evolutionary model was selected by using MrModeltest (33). For all three regions, this model was the GTR+I+ $\Gamma$  (34). Bayesian analysis with MrBayes used default formulations of uninformative priors on model parameters (e.g., Dirichlet distribution for relative substitution rates and base frequencies, bounded uniform distribution for proportion of invariable sites and gamma shape parameter for the distribution of among site rate variation, uniform distribution for topologies, and exponential distribution for branch lengths; see ref. 35), eight linked Markov chains per run with one cold and seven heated chains, and two simultaneous runs. Convergence and stationarity of the runs was assessed by checking for plateaus in the time series of parameter values and examining the standard deviation of split frequencies between the two runs. The simulations were carried out for  $2 \times 10^6$  generations of perturbing the model parameters (including the tree), which were logged every 100 generations. Based on the criteria stated above for convergence and stationarity, the first 300,000 generations (i.e., 3,000 trees) were discarded as the transient burn-in period.

The 50%-majority-rule consensus of trees sampled in the Bayesian phylogenetic analysis of 145 species was used to construct a phylogram, assuming the maximum likelihood estimates of model parameters, by using PAUP\* 4.0b10 (36). This phylogram was pruned to the appropriate taxon set (34–35 species) for subsequent analyses, preserving the branch lengths estimated with the full dataset.

**Macroevolutionary Trends Analyses.** We accounted for evolutionary history in our analyses with maximum likelihood (ML) methods using Pagel's Continuous implemented in BayesTraits (10) ([www.evolution.rdg.ac.uk](http://www.evolution.rdg.ac.uk)). Continuous was used both for phylogenetically independent correlations and to test the hypothesis of defense escalation. Continuous employs the generalized least-squares (GLS) method for calculating independent contrasts; here, we fixed all param-

eters so that the GLS framework produces the same result as traditional independent contrasts (10). 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 root-to-tip branch length distances shared between species). Because our best estimate of the phylogeny had four polytomies, we conducted all analyses of character evolution on 1,000 randomly resolved phylogenies and estimated 95% confidence intervals for parameters using the bias-corrected percentile method (37).

To assess directional trends, we first estimated the extent of phylogenetic conservatism (i.e., trait similarity due to shared ancestry) by using the parameter  $\lambda$  (10). A value of  $\lambda = 1$  indicates complete phylogenetic conservatism consistent with the tree topology and a random walk model of character evolution, whereas  $\lambda = 0$  indicates no influence of shared ancestry on trait values. Next, we evaluated whether a gradual or speciation model of evolution better fit the observed trait data. A parameter,  $\kappa$ , which differentially stretches or compresses individual branch lengths, is estimated by maximum likelihood (10). In Continuous, models are compared by using a likelihood ratio (LR) test (10). Using the LR test, we compared the estimated value of  $\kappa$  to 0 (corresponding to a speciation model, or cladogram with equal branch lengths) and 1 (corresponding to a gradual model, or phylogram, with branch lengths set to maximum-likelihood estimates from the DNA sequence data). We then used the best fitting model in analyses of evolutionary trends. We assessed trends by comparing a random walk model of character evolution on the best fitting gradual or speciation phylogenetic model to one with two additional parameters ( $\alpha$ , which estimates the ancestral state of the trait, and  $\beta$ , which estimates the rate of directional change). The latter model of character evolution can be thought of as a phylogenetically independent regression of the trait against total path length from the root of the tree to tips. A significantly better fit of the two-parameter model is consistent with a directional trend through the diversification of the lineage.

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