Phylogenetic ecology of leaf surface traits in the milkweeds (Asclepias spp.): chemistry, ecophysiology, and insect behavior

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Summary

• The leaf surface is the contact point between plants and the environment and plays a crucial role in mediating biotic and abiotic interactions. Here, we took a phylogenetic approach to investigate the function, trade-offs, and evolution of leaf surface traits in the milkweeds (Asclepias).

• Across 47 species, we found trichome densities of up to 3000 trichomes cm\(^{-2}\) and epicuticular wax crystals (glaucousness) on 10 species. Glaucous species had a characteristic wax composition dominated by very-long-chain aldehydes.

• The ancestor of the milkweeds was probably a glaucous species, from which there have been several independent origins of glabrous and pubescent types. Trichomes and wax crystals showed negatively correlated evolution, with both surface types showing an affinity for arid habitats. Pubescent and glaucous milkweeds had a higher maximum photosynthetic rate and lower stomatal density than glabrous species. Pubescent and glaucous leaf surfaces impeded settling behavior of monarch caterpillars and aphids compared with glabrous species, although surface types did not show consistent differentiation in secondary chemistry.

• We hypothesize that pubescence and glaucousness have evolved as alternative mechanisms with similar functions. The glaucous type, however, appears to be ancestral, lost repeatedly, and never regained; we propose that trichomes are a more evolutionarily titratable strategy.

Introduction

The primary function of leaves is to turn light, water and carbon dioxide into chemical energy, and thus leaves are likely under strong selection to maximize physiological function in a plant’s habitat. However, given that plants are the direct or indirect source of energy for most animals and many microbes, the leaf surface should also be under strong selection to minimize attack by consumers. In this regard, two major leaf surface traits, trichomes (leaf hairs) and epicuticular wax crystals, may be key plant traits that have the potential to influence both photosynthesis and resistance to consumption. These traits have been intensively studied with respect to morphology, formation, chemical composition, and their functional role as adaptations to habitats and herbivores.

Trichomes have well-documented ecophysiological (e.g. Ehleringer & Clark, 1987; Sandquist & Ehleringer, 2003) and anti-herbivore (e.g. Coley, 1983; Mauricio & Rausher, 1997; Haddad & Hicks, 2000) functions. The presence of high-density, nonglandular trichomes has been correlated with plants growing in open, hot, and arid habitats, and in such situations, trichomes function to shade the leaf surface from ultraviolet (UV) light, reduce the heat load, and reduce absorbance of solar radiation (Ehleringer et al., 1976). In terms of resistance to herbivores, nonglandular trichomes may act as a physical barrier to movement and consumption for...
many invertebrate herbivores, although various insects have adaptations to overcome these effects (Malcolm, 1995; Fordyce & Agrawal, 2001; Agrawal, 2005).

Epicuticular waxes are also known to impart both ecophysiological (Schreiber & Riederer, 1996; Oliveira et al., 2003) and anti-herbivore (Eigenbrode & Espelie, 1995; Müller & Riederer, 2005) functions. Epicuticular wax crystals scatter light, giving the plant surface a glaucous (grayish) appearance, limiting penetration of UV light, as well as photosynthetically active radiation. This may benefit plants growing in open habitats where they receive high light loads (Schreiber & Riederer, 1996; Oliveira et al., 2003). In addition, wax crystals (and also some trichomes) increase water repellency at the leaf surface, which influences gas exchange and interactions with microorganisms (Müller & Riederer, 2005; Brewer & Nuñez, 2007). Although wax crystals themselves may promote the germination of some pathogenic fungi (Mendgen et al., 1996), glaucous leaves are less wettable and therefore more resistant to many microbes. Further, most invertebrates have difficulty walking on glaucous surfaces (Eigenbrode & Espelie, 1995; Eigenbrode et al., 1996; Riedel et al., 2007) and are often deterred by physical or chemical cues in epicuticular waxes (Müller & Riederer, 2005).

Although studies of natural selection on individual species can provide direct evidence for the present-day adaptive function of a particular leaf surface trait (e.g. Mauricio & Rausher, 1997; Sandquist & Ehleringer, 2003), there is also a rich tradition of using comparative approaches to infer longer term evolutionary dynamics and to draw general conclusions regarding the function and adaptive value of such traits (Coley, 1983; Ehleringer & Clark, 1987; Schreiber & Riederer, 1996; Markstäder et al., 2000; Oliveira et al., 2003). Historically, two previous limitations of the comparative approach have reduced its use and impact in studies of plant evolution and adaptation. First, phylogenetic information has frequently not been available or fully integrated into the study of plant adaptations. Such phylogenetic information allows us to recognize sets of species that have independently (or convergently) evolved particular traits. Much stronger evidence for an adaptive function can be extracted from the ecological comparison of these species than if convergent species were not compared (Armbruster, 1997; Wilson et al., 2006). Secondly, only rarely have transitions to multiple phenotypic states been studied simultaneously in the same lineage. For example, repeated transitions from glabrous (smooth) to glaucous or pubescent leaves in the same genus might reveal the ecological function of these divergent surface traits. Thus, multi-trait comparisons in the context of phylogenetic information allow for natural replication in assessing functional variation and its consequences at the evolutionary scale.

Milkweeds (Asclepias spp.), a diverse group of American perennial herbaceous plants, are an ideal clade for investigating leaf surface evolution and associated defensive and ecophysiological traits. Previously, we used micro- and macroevolutionary approaches to investigate the defensive ecology of this group (Agrawal, 2005, 2007; Agrawal & Fishbein, 2006; Agrawal et al., 2008). In addition to trichomes, two other traits have been strongly implicated in resistance to herbivores: cardenolides (toxic steroidal compounds that impact the function of Na+/K+-ATPases) and latex (a sticky, viscous substance that exudes upon tissue damage). We previously observed that the amount of defensive latex exuded and trichome density across 24 species of Asclepias showed correlated evolution (i.e. the positive correlation was significant after phylogenetic correction) (Agrawal & Fishbein, 2006). We specifically noted exceptions, however, of plants that were high in latex production but free of trichomes. Based on visual observations, we predicted that these species had a thick layer of epicuticular wax.

The central goal in this study was to employ phylogenetic information to study the evolutionary history and functional role of plant traits implicated in interactions with the biotic and abiotic environment. As such, we aimed to address long-standing adaptive hypotheses about convergence in leaf surface form and function (Givnish, 1987; Reich et al., 1999; Ackerly, 2004). We first attempted to categorize 47 Asclepias species as having glabrous, pubescent, or glaucous leaves. Trichome density is a quantitative trait that ranges from none (glabrous) to dense (pubescent), while glaucousness is a discrete trait – waxes are typically either inconspicuous or uniformly coat leaf surfaces with platelets imparting the appearance of a waxy bloom. After we characterized the surface structures using microscopy, we categorized species by their surface chemistry (all plants, glaucous or not, have surface waxes). Finally, we extended the analysis to potentially correlated variation in ecophysiological function (photosynthesis, water use efficiency, stomatal density, and carbon-to-nitrogen ratio), and defense investment (insect behavior, cardenolides, and phenolics). For the impact of leaf surface variation on insect behavior, we examined two common specialist herbivores of Asclepias spp., monarch butterflies (Danaus plexippus) and milkweed aphids (Aphis nerii). Thus, our goal was to take a multidisciplinary approach to understanding the ecology of species that have both diverged and converged in their habitat use, leaf surface traits, and biotic interactions (Chapin et al., 1993; Agrawal & Fishbein, 2006).

Materials and Methods

Natural history

The milkweeds (Asclepias spp.) are a monophyletic genus comprising c. 140 species in the Western Hemisphere; 130 species in North America and the Caribbean, and perhaps as many as 10 species in South America (Woodson, 1954; M. Fishbein et al., in press). Asclepias species have diversified into a variety of open habitats including wetlands, prairies, savannas, and deserts, although a few species grow in shady forest understories. Leaf shapes range from needle-like (e.g. Asclepias...
In this study, we focused on 47 milkweed species that live in open habitats and exhibit a range of leaf surface types (Supporting Information Table S1). For example, *Asclepias californica* (Greene), *Asclepias erosa* (Torr.), and *Asclepias eriocarpa* (Torr.) have dense trichomes and are typically associated with stony-soiled desert, or otherwise hot, arid environments in the western USA (Woodson, 1954). Another densely trichome-covered species, *Asclepias speciosa* (Torr.), occurs in arid environments, but is frequently found in disturbed sites and fields. Other species, including those that have glaucous leaves, inhabit dry soils, rocky slopes (*Asclepias glaucescens* (Kunth)), and sandy hills and ridges (*Asclepias humistrata* (Walter) and *Asclepias amplexicaulis* Sm.). Finally, many glabrous milkweeds are frequent in moist soils (*Asclepias angustifolia* (Schweigg.), *Asclepias incarnata* L. *ssp. incarnata* and *Asclepias perennis* (Walter)), and species that grow in moist forest understory (*A. angustifolia* and *Asclepias exaltata*).

**Plant growth**

Seeds were collected by the authors and colleagues, or purchased from native plant suppliers (see Acknowledgments), and synchronously germinated by moistening and stratifying at 4°C for at least 2 wk. Seedlings were planted in 500-ml plastic pots and grown in Metro Mix soil (Scotts-Sierra, Marysville, OH, USA). In different experiments, plants were grown from seed in a walk-in growth chamber, a greenhouse, or out of doors (see below). For any particular trait measure or bioassay, all plants were grown together as a cohort in a common environment, assigned to random locations, and assayed at a single time. Although replication varied slightly, for each assay we typically sampled five plants per species (see Table S1 for a complete list of the species used in each experiment).

**Leaf surface microscopy**

Trichome and wax crystal assessments were conducted on leaves from plants growing in a living collection maintained in the Cornell University glasshouses. Trichomes were counted from a leaf disc (28 mm²) under a dissecting microscope (summing the adaxial and abaxial sides) of one fully expanded leaf, each from at least three replicate individual plants of all species. Scanning electron microscopy was used to visualize epicuticular wax crystals. Leaf surface waxes come in many forms, but only crystals change the physical properties of the leaf. Briefly, a fresh, fully expanded leaf was collected from a single individual of each milkweed species. Pieces of leaves were mounted on aluminum holders, sputter-coated with gold-palladium and viewed at 200, 2000, and ×10 000 magnification (3 kV acceleration and typically a working distance of 15 mm). All species' leaves were examined on both the adaxial and abaxial sides.

**Cuticular waxes**

Because all plants (with or without epicuticular crystals) have surface waxes, we characterized the wax composition of a range of species that varied in trichome density and glaucosity. When plants had grown for 1 month and had over eight true leaves, a single young, but fully expanded leaf was harvested from each of three individuals of nine selected species (three from the extremes of glabrous, pubescent, and glaucous; Table S1, n = 27). Inasmuch as was possible, we chose species for these and subsequent analyses to maximize independent origins of the leaf surface traits (see Results). Cuticular wax was extracted from both sides of the leaf by dipping and rinsing the leaf in 100 ml of warm hexane (c. 50°C) for 30 s and repeating this process. The solutions were combined, n-tetracosane was added as an internal standard and the solvent was evaporated at room temperature. For analyses by gas chromatography (GC), the samples were resuspended in ~300 µl of CHCl₃, transferred to GC autosampler vials, dried under nitrogen, and derivatized with 10 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma) and 10 µl of pyridine (Fluka, Buchs, Switzerland) for 30 min at 70°C. Wax composition was analyzed using a capillary GC (5890N; Agilent; column 30 m HP-1, 0.32 mm i.d., dₕ = 0.1 µm, Santa Clara, CA, USA) with He carrier gas inlet pressure programmed for constant flow of 1.4 ml min⁻¹ with a mass spectrometric detector (5973N; Agilent). GC was carried out with temperature-programmed on-column injection and oven temperature set at 50°C for 2 min, raised by 40°C min⁻¹ to 200°C, held for 2 min at 200°C, raised by 3°C min⁻¹ to 320°C, and held for 30 min at 320°C. Individual wax components were identified by comparing their mass spectra with those of authentic standards and literature data. Quantitative analysis of wax mixtures was carried out using capillary GC with a flame ionization detector (FID) under the same conditions as above, but with CH₄ carrier gas inlet pressure regulated for constant flow of 2 ml min⁻¹. Leaves were digitally scanned for leaf area assessment, and wax coverage was calculated on a per area basis.

**Evolutionary history of leaf surface characteristics**

A phylogeny of *Asclepias* was generated from sequence data that were obtained from three noncoding regions of the chloroplast genome (*rpl16* intron, *trnC-rpoB* intergenic spacer, and *trnS-trnG* spacer/trnG intron; 4866 aligned sites) for 155 samples, including 120 of the c. 140 species of *Asclepias*, 20 representatives of the African sister group (a clade including 18 genera and 250 species), and representatives of four genera forming the outgroup (M. Fishbein et al., in press). The phylogeny was constructed by Bayesian inference implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), partitioning...
the data set by locus with the substitution model optimized independently for each partition using MrModeltest, version 2 (Nylander, 2004) and paup* 4b10 (Swofford, 2001). The GTR+I+F substitution model was selected for each partition and each model parameter was unlinked across partitions. Two independent runs of eight chains were conducted for $5 \times 10^6$ generations, with convergence on the stationary distribution evaluated by adequate swapping among chains, attainment of the standard deviation of split frequencies between runs < 0.015 and effective sample sizes of model parameters > 200 (using TRACER, version 1.4; Rambaut & Drummond, 2007), resulting in a burn-in of $1 \times 10^6$ generations. Trees were modified by pruning terminal branches leading to taxa for which phenotypic trait data were unavailable. The 47 species of Asclepias used in this study were selected primarily based on availability of seeds. However, this sample includes representatives of nine of the 13 major clades identified in analyses of the complete data set (M. Fishbein et al., in press), minimizing the potential for bias resulting from nonrandom sampling.

To estimate ancestral states, transition probabilities, and correlated evolution of leaf surface traits, we used maximum likelihood methods (Multistate and Continuous software) implemented in Pagel’s BayesTraits (Pagel, 1999a, 2007; Pagel & Meade, 2007). These methods use the generalized least squares (GLS) method, which codes the phylogeny as a variance-covariance matrix to account for the shared evolutionary history among species. Various models were compared using a likelihood ratio (LR) test, where $LR = 2(\log$-likelihood of the better fitting model – $\log$-likelihood of the worse fitting model). Here the LR statistic should be $\chi^2$ distributed with one degree of freedom (as long as a single parameter is altered between the two models) (Pagel & Meade, 2007). To account for uncertainty resulting from poorly supported nodes and point estimates of branch lengths, analyses were conducted on a set of 1000 phylogenies from the stationary distribution of samples generated in the Bayesian phylogenetic analysis (Fishbein et al., in press). To assess confidence in parameter estimates with respect to phylogenetic uncertainty, we calculated 95% credible sets by excluding the lowest and highest 2.5% of estimates across the 1000 tree samples from the stationary distribution (Huelsenbeck et al., 2000) and adopt the convention of not presenting these confidence values when they are very tight, that is, do not span > 5% of the mean value being calculated (and all values within the range have the same implication for statistical significance).

We first assessed the extent of phylogenetic signal for trichomes (i.e. trait similarity attributable to shared ancestry) using Pagel’s $\lambda$ (Freckleton et al., 2002). This parameter is estimated using Model A (random walk model only, as opposed to Model B, which incorporates a directional trend; see below). A $\lambda$ value of 1 corresponds to phylogenetic conservatism in which trait evolution is explained by the topology of the phylogeny and a random walk model; a $\lambda$ of 0 indicates no influence of shared ancestry on trait values (Freckleton et al., 2002). Probability values for the significance test of the maximum likelihood estimate of $\lambda$ against a value of zero are indicated with an asterisk (*) in the text indicates $P < 0.05$.

We further test for a directional trend in the expression of trichomes. Here, Model A is compared with Model B, a directional random walk, in which a linear, directional trend is modeled with an additional parameter, $\beta$ (Pagel, 1999, Agrawal & Fishbein, 2008). We also report the estimated ancestral state of the clade (6) for the best fitting model.

Because we found that leaf surfaces could be classified into one of three discrete categories (glabrous, pubescent, or glaucous), we used Pagel’s Multistate to estimate the ancestral state for leaf surface type and the transition probabilities among the three states. A model with estimated transition rates between leaf surface types (in each direction, for a total of six rates) was compared with a model where all rates were restricted to be equal. A difference of 2 log-likelihood units between the two models was used as a cut-off for significance (Pagel, 1999b).

We depict reconstructed ancestral states on the 50% majority rule consensus of trees sampled from the Bayesian stationary distribution using paup* 4b10 (Swofford, 2001) (Fig. 4). Reconstructions were conducted employing the 45 Asclepias species, leaving 43 possible ancestral nodes (plus the root); 31 nodes were resolved with > 50% posterior probability on the consensus tree. Most nodes on the consensus tree (23 of a total of 31) were present on all 1000 Bayesian trees sampled for ancestral state reconstructions. However, seven of the nodes were not present on 1–10% of the trees and one (the immediate ancestor of Asclepias cordifolia and A. linaria) was present in only 81% of the tree sample. Thus, reconstructions depict ancestral states reconstructed on the subset of the 1000 trees for which those nodes were present, in nearly all cases > 90% of the sampled trees. Two closely related species, Asclepias subulata and Asclepias albicans, were difficult to categorize because they both possess small, ephemeral leaves that are pubescent and lack epicuticular wax platelets; however, the stems of these species, which comprise the bulk of the plants’ photosynthetically active surface area, are covered with wax platelets and lack trichomes. We thus analyzed trait evolution under both character codings. Again, all analyses were conducted on 1000 phylogenies sampled from the stationary distribution of the Bayesian phylogenetic analysis.

ANOVA and phylogenetically independent analyses

Most analyses of differentiation among the leaf surface types were conducted as nested ANOVAs, with species nested within leaf surface type. To assess correlated evolution between other defense traits and the leaf surface types, we contrasted species means for defense traits against the surface category using Model A of the Continuous module of BayesTraits, which allows for one of the traits to be discrete (e.g. do glaucous species produce more latex than glabrous species?; Pagel, 1994, 2007). Here we estimated $\lambda$ simultaneously on
the two traits and used it as a scaling parameter in a "phylogenetically adjusted ANOVA" (PAA) using GLS (Pagel, 2007). The likelihood ratio test was again employed, comparing models with the correlation intact to one where the trait covariances were set to zero. In one analysis (to predict specific leaf area (SLA); see the next section 'Habitat shifts and evolution of leaf traits'), we used the Continuous Regression module to conduct a PAA with multiple predictor variables. Because of extreme power limitation (i.e. replication is the number of species in each category), such PAs were only conducted in analyses with substantial replication of species within each surface type. Thus, PAs were not conducted for hypothesis tests in which only representative samples from each surface category were studied (e.g. epicuticular wax chemistry).

Habitat shifts and evolution of leaf traits

To address correlated evolution between plant leaf surface traits and transitions to different habitat types, we categorized species by the aridity of the habitats where they are most commonly found. Although we did not have quantitative environmental data, we used our experience of observing natural populations to qualitatively classify the aridity of sites typically inhabited by each species. Our goal was to use SLA (surface area per unit mass) as a possible surrogate for the aridity of environments occupied by the species. Across large global data sets, species' SLA values are correlated with the aridity of the environment in which they grow (Givnish, 1987; Reich et al., 1999; Ackerly, 2004). In order to examine this association in Asclepias, we tested for differences in the SLA of a subset of species living in habitats that are extremely dry during the growing season (i.e. deserts, sand hills, rocky slopes in Mediterranean climates, plains grasslands, etc: A. albicans, A. californica, Asclepias cryptoceras, A. erosa, A. humistrata, Asclepias pumila, A. subulata, and Asclepias subverticillata), against those typically inhabiting extremely wet habitats (i.e. swamps, marshes, humid tropics, etc: Asclepias curassavica, A. incarnata ssp. incarnata, A. incarnata ssp. pulchra, Asclepias nivea, and A. perennis). We then asked if the SLAs of these species differed using a t-test and a phylogenetically controlled ANOVA using Pagel's Continuous. Given that we found that species living in dry habitats had substantially lower SLAs than species living in wet habitats (see Results), which is consistent with global data sets, we proceeded to utilize SLA as a surrogate for environmental aridity. Because leaf size has long been predicted as a trait that decreases with environmental aridity (Givnish, 1987; Ackerly, 2004), these data were additionally collected for all species and analyzed with trichomes to predict SLA.

Ecophysiology

The maximum photosynthetic rate ($A_{\text{max}}$) was measured on glasshouse-grown plants (usually three plants per species; Table S1) using a portable gas exchange system (Li-Cor 6400; Li-Cor, Inc., Lincoln, NE, USA) with CO$_2$, temperature, and light control. Cuvette conditions were held constant for all measurements (1200 µmol m$^{-2}$ s$^{-1}$ light, 25°C leaf temperature, and 375 ppm ambient CO$_2$ concentration). Stability of the measurement was defined as 2 min of constant photosynthetic rate, leaf conductance, and internal CO$_2$ concentration ($c_i$).

Stomatal density and aperture length were measured for 24 species by examining a single scanning electron micrograph per species. As such, we report the results of a one-way ANOVA for stomatal density (not a nested ANOVA), where the replicates for each surface type are a single measure of each of eight species.

Foliar carbon isotope ratio ($\delta^{13}C$, a measure of water use efficiency) and relative nitrogen amounts in leaves were estimated using isotope ratio mass spectrometry (IRMS). In two separate experiments, milkweed species were grown either in a growth chamber with ad libitum water (18 species in total, with at least five replicates of each), or out of doors in full sunlight (July) and under water stress conditions, with minimal water but no wilting (nine species, with six replicates of each).

These two conditions were employed to evaluate ecophysiology under stressed and unstressed conditions. All leaves were harvested, dried, and ground for isotopic measurement of $\delta^{13}C$ and total per cent nitrogen at the Cornell University Stable Isotope Laboratory. $\delta^{13}C$ provides an estimate of the ratio of leaf internal to external carbon dioxide concentrations, and thus integrates the average resistance to carbon gain and water loss over the lifetime of the leaf (Farquhar et al., 1989).

Insect behavior

To assess the walking and foraging behavior of newly arrived herbivores on milkweed plants, we assayed two specialists that have different feeding styles: monarch butterfly caterpillars (Danaus plexippus), which are leaf chewers, and milkweed aphids (Aphis nerii), which are phloem suckers. Both insects commonly encounter all of the milkweed species in the field. Five plants each of nine species (three species from each surface type; total $n = 45$) were grown from seed in a growth chamber and then assayed for herbivore behavioral response in a glasshouse on sunny days (Table S1). The milkweed species selected contained at least two independent origins of the glabrous and pubescent leaf surface types. Species and leaf surface types were tested in random order over a 5-d period.

For each assay we placed a single freshly hatched monarch larva or a wingless adult aphid near the apex of the plant. The times spent walking, sitting, and feeding were recorded for 30 min. For monarchs, the percentage of time spent walking was negatively correlated with the portion of time spent feeding ($n = 9$, $r = 0.66$, $P = 0.036$) and therefore only the former is presented. For aphids, the percentage of time spent walking positively correlated with the time spent settling ($n = 9$, $r = 0.92$, $P < 0.001$) and hence only the former is presented. To assess caterpillar behavior on nonhosts (i.e. non-milkweed plants), we placed a monarch larva or aphid on the species used in each experiment and measured the percentage of time spent settling and feeding.
extremely ‘resistant’ by comparison to the various *Asclepias* surface types), we examined monarch larvae as above on two replicate plants each of common evening primrose (*Oenothera biennis*), lima bean (*Phaseolus lunatus*), and a prostrate pigweed (*Portulaca* sp.). We used Pearson correlation tests to assess the impact of wax loads of individual milkweed species on insect behavior; additional analyses using phylogenetic GLS were conducted using Pagel’s Continuous.

**Latex**

We measured latex from 24 species by cutting the tip (0.5 cm) off the youngest fully expanded intact leaf and collecting the exuding latex onto a pre-weighed 1-cm disc of filter paper. Latex stopped flowing after ~10 s, all latex was absorbed on the filter paper, and this disc was placed in a pre-weighed microcentrifuge tube. Tubes were then weighed to estimate wet latex exudation per plant. More extensive chemical and evolutionary analyses of an expanded data set on latex are reported elsewhere (Agrawal *et al.*, 2008).

**Phenolics**

Data on total phenolics and specific flavonoids (quercetin glycosides) were assessed because these compounds act as oviposition stimulants for the monarch butterfly (Haribal & Renwick, 1998), and as sunscreens in a variety of other taxa (Hoque & Remus, 1999; Ryan *et al.*, 2001, 2002; Solovchenko & Merzlyak, 2003). Approximately five replicate plants from each of 24 species (total \(n = 117\)) were grown from seed in a growth chamber (Table S1). Here we focused on the association between leaf surface types and phenolics, while more details on the methods and results concerning the macroevolution of the phenolic metabolism of *Asclepias* species are reported in a separate paper (Agrawal *et al.*, 2009). Briefly, analysis of milkweed leaf extracts was performed at 280 and 349 nm with a 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 \(\mathrm{H}_3\mathrm{PO}_4\) was replaced with 0.05 M \(\mathrm{H}_3\mathrm{PO}_4\). Phenolic compounds were quantified using coumaric acid, chlorogenic acid and quercetin as standards.

**Cardenolides**

Cardenolides (cardiac glycosides) are bitter-tasting steroids that occur in most milkweed species in all tissues. These defensive secondary metabolites act by disrupting the sodium and potassium flux in cells, and have toxic effects on most animals (Malcolm, 1991). Powdered plant material from the same plants as for the phenolic assay (seven replicate plants from each of 24 species; total \(n = 170\)) were analyzed for their cardenolide content using a high-throughput spectrophotometric assay described in Agrawal (2004).

**Results**

**Leaf surface traits and classification of three surface types**

We investigated the upper and lower surfaces of the leaves of 47 milkweed species using scanning electron microscopy (SEM). Epicuticular wax crystals were found on 10 species on 10 species (Table S1). In all cases, similar upright platelet crystals formed dense networks on both the adaxial and abaxial sides. The epicuticular wax crystals gave the leaf surfaces of these species a glaucous appearance. All 47 species were also assessed for the density of trichomes on the adaxial and abaxial leaf surfaces using a dissecting microscope. Trichome densities ranged from 0 (*A. variegata*) to over 3000 trichomes \(\mathrm{cm}^{-2}\) (*A. erosa*). All trichomes were simple (rather than glandular or branched), but varied considerably in length, curvature and posture (appressed to erect; Figs 1, 2).

Based on the above findings, we hypothesized that species could be qualitatively categorized according to three leaf surface types: (1) glabrous, with < 300 trichomes \(\mathrm{cm}^{-2}\) and no wax crystals; (2) pubescent, with well above 300 trichomes \(\mathrm{cm}^{-2}\) and no wax crystals; and (3) glaucous, with the plant surface covered with a layer of epicuticular wax platelets but with few to no trichomes (Figs 1, 2). Although our range for ‘glaucous’ countinuitively includes densities up to 300 trichomes \(\mathrm{cm}^{-2}\), we employed this arbitrary cut-off for three reasons: (1) this allowed us to include the 10 least pubescent species (which were not glaucous) in this category; (2) most species in this group have trichomes concentrated primarily along the midrib, yielding an overall hairless appearance; and (3) the species in our sample that were categorized as ‘pubescent’ had three- to ninefold higher trichome densities than the average of those categorized as ‘glaucous’ (allowing a qualitative comparison along the gradient of pubescence). No species combined wax platelets with high trichome densities; thus *Asclepias* species exhibit only three of the four possible combinations of extreme wax platelet and trichome expression. We next statistically tested the discreteness of our proposed leaf surface categories. The glaucous milkweed species had the fewest trichomes (15 species tested; least squares (LS) means ± SE trichomes \(\mathrm{cm}^{-2}\): glabrous, 255 ± 109; pubescent, 2353 ± 112; glaucous, 27 ± 155; \(F_{1,48} = 116.254, P < 0.001\)), although we found no difference between glabrous and glaucous species (contrast: \(F_{1,48} = 1.449, P = 0.235\)). All pubescent species selected for subsequent experimental comparative analyses had > 1000 trichomes \(\mathrm{cm}^{-2}\).

**Cuticular wax composition**

Nine species were selected for wax analysis, three from each category defined above (glabrous, pubescent, and glaucous). Total wax loads ranged from 1.2 µg \(\mathrm{cm}^{-2}\) for glabrous *A. perennis* to 24.1 µg \(\mathrm{cm}^{-2}\) for pubescent *A. californica*. In
the analysis of wax loads, there was no effect of species nested within surface type, validating the surface type categorization. Note that high wax loads on pubescent species were not associated with platelets covering leaves (Fig. 1). Pubescent milkweed species had wax loads over five times higher than glabrous species (Table 1), but similar wax amounts to glaucous species (contrast $F_{1,18} = 0.192, P = 0.667$). Overall, differences in wax loads supported the classification of glabrous species as

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**Table 1** Analysis of variance for effects of leaf surface type and *Asclepias* species nested within surface type on leaf surface wax chemistry (total wax and per cent composition from each of seven classes of compounds)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Type</th>
<th>Glabrous</th>
<th>Pubescent</th>
<th>Glaucous</th>
<th>SE</th>
<th>Species (type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total wax</td>
<td>$F_{2,18}$</td>
<td>12.843***</td>
<td>3.3</td>
<td>17.1</td>
<td>18.4</td>
<td>2.3</td>
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<tr>
<td>Fatty aldehydes</td>
<td></td>
<td>245.128***</td>
<td>1.0</td>
<td>0</td>
<td>33.5</td>
<td>1.2</td>
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<tr>
<td>Alkanes$^\Phi$</td>
<td></td>
<td>16.297***</td>
<td>5.9</td>
<td>7.8</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Fatty alcohols</td>
<td></td>
<td>14.184***</td>
<td>32.9</td>
<td>13.4</td>
<td>21.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Alkyl esters$^\Phi$</td>
<td></td>
<td>81.082***</td>
<td>0</td>
<td>0</td>
<td>8.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Triterpenoid acids</td>
<td></td>
<td>237.167***</td>
<td>2.7</td>
<td>34.0</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>Triterpenoid alcohols</td>
<td></td>
<td>18.026***</td>
<td>18.5</td>
<td>10.0</td>
<td>2.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Triterpenoid acetates$^\Phi$</td>
<td></td>
<td>7.110**</td>
<td>0.3</td>
<td>4.4</td>
<td>0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Nine milkweed species, three in each type, were assayed. Least squares means and pooled standard error are presented for each leaf surface type. $^\Phi$, minor components, with $< 10\%$ of the composition in any species. ***, $P < 0.001$; **, $P < 0.01$. 

---

**Fig. 1** Scanning electron micrographs of leaf abaxial surfaces of three milkweed species: (a, b) ‘glabrous’ *Asclepias viridis*, (c, d) ‘pubescent’ *Asclepias eriocarpa*, and (e, f) ‘glaucous’ *Asclepias cryptoceras*. 

distinctly low in waxes, but did not provide a clear distinction between the other two leaf surface types. To further distinguish the leaf surface types, we next analyzed the specific chemical composition (quality) of waxes.

Overall, the leaf surface types differed in their wax composition (MANOVA on the four dominant classes: fatty aldehydes and alcohols, and triterpenoid alcohols and acids, surface type Wilks’ lambda \(= 0.002, F_{8,30} = 91.79, P < 0.001\); species(type), Wilks’ lambda \(= 0.001, F_{24,53} = 12.34, P < 0.001\)). Fatty aldehydes dominated the wax mixture of the three species characterized by epicuticular wax crystals (Table 1, Fig. 3). By contrast, aldehyde concentrations in leaf waxes of the other six species investigated were low or below the detection limit. The little wax produced by glabrous species was conspicuously dominated by alcohols (Table 1). Fatty alcohols were detected in the wax of all nine species, with relatively high concentrations for the six glabrous or glaucous species, as well as pubescent \(A.\) perennis. Various triterpenoid alcohols and acids were detected in the different wax mixtures, with relatively low concentrations in the three glaucous species and glabrous \(A.\) perennis; conspicuously high triterpenoid acid concentrations were found in the wax of pubescent \(A.\) californica and \(A.\) eriocarpa.

In addition, three minor compound classes further distinguished the wax mixtures from species of different surface types. For example, alkanes accounted for 6–8% of wax mixtures from glabrous and pubescent species (not different from each other; \(P = 0.151\)), but were essentially absent in glaucous species (Table 1). Conversely, alkyl esters, absent in waxes of pubescent and glabrous species, made up 8% of the mixtures from glaucous species. Finally, only pubescent species had detectable concentrations of triterpenoid acetates (4%).

Overall, the compound class composition of cuticular wax mixtures supported our species classification according to surface morphology. However, the species within surface types also had a significant effect on each of the constituent compound classes (Table 1, Fig. 3). In addition, the chain length distributions of aliphatic compounds within compound classes and the isomer distributions of triterpenoids were distinctive for all nine species (data not shown). Thus, beyond the main effect of surface types being significantly different from each other, there was substantial variation within surface types.

Fig. 2 Leaf abaxial surfaces of (a) three milkweed (\(Asclepias\)) species representing glabrous, pubescent, and glaucous species, respectively, and (b) leaf surface trichomes of three milkweed species; in addition to differences in density, note the different lengths and arrangements of trichomes (bars, 2 mm for b).

Fig. 3 Per cent composition of the four dominant chemical classes (mean ± SE) in the waxes of nine species of \(Asclepias\). Minor constituents are not shown here (see Table 1 and text).
Evolutionary history of leaf surface characteristics

Our survey of the evolutionary history of leaf traits includes approximately one-third of all *Asclepias* species (Fig. 4). Using the full data set on leaf trichomes (but not the outgroup species), we estimated the ancestral state for trichomes to be 922 trichomes cm$^{-2}$ (credible set 846–931 trichomes cm$^{-2}$). There was a significant level of phylogenetic signal for trichomes, consistent with the null model of Brownian motion evolution ($\lambda = 0.954^*$ ($^*$ indicates that $\lambda$ is significantly different from zero)). We found no evidence for a directional change in trichome density as *Asclepias* diversified ($\beta = 41916$, LR $= 0.203$, $P = 0.652$).

Leaf glaucousness and pubescence show an evolutionary trade-off ($\lambda = 1^*$, $r = -0.44$, LR for covariance estimated vs set to zero $= 9.60$, $P < 0.002$). Transitions toward increased pubescence were associated with the loss of glaucousness. A special case confirming this trade-off involves *A. subulata* and *A. albicans*, closely related species of the Sonoran Desert of the USA and Mexico, one of the most arid regions occupied by any milkweed species. These species have ephemeral, tiny, pubescent leaves (means of 662 and 3384 trichomes cm$^{-2}$, respectively), which lack epicuticular wax platelets; however, the stems of these species (which comprise the bulk of the plants’ photosynthetically active surface area) are covered with wax platelets, and lack trichomes.

Given this correlated evolution and the success of employing discrete designations to categorize the leaf surface types of the milkweeds, we proceeded to reconstruct ancestral states and estimate transition probabilities among the three leaf surface types. This analysis has the benefit of including glaucousness as a discrete character, while in the previous analyses glaucous and glabrous species were not distinguished – they both have very low trichome densities. Because it was unclear how to categorize *A. subulata* and *A. albicans*, we conducted alternative analyses with both species coded as either pubescent or glaucous.

Among the 1000 phylogeny samples, a glaucous ancestral state for *Asclepias* was almost universally favored by maximum likelihood (> 98% probability in 998 of the 1000 phylogenies), when *A. subulata* and *A. albicans* were coded as glaucous (Fig. 4). Analyses were essentially identical when both *A. albicans* and *A. subulata* were coded as pubescent. The only exception was that the ancestor of *Asclepias* and three additional nodes near the base declined from having nearly all phylogenies with > 98% probability of being glaucous to only 925 of the phylogenies having this probability; in the remaining 75 phylogenies these nodes had > 98% probability of being pubescent (see also Table 2). Thus, 7.5% of the sampled phylogenies imply a pubescent, rather than glaucous, ancestor for *Asclepias*, but only if the two desert shrubs with ephemeral leaves are coded as pubescent.

A model with unequal (maximum likelihood estimated) transition probabilities was a significantly better fit than a model with all six transition probabilities constrained to be equal (Table 2; difference in likelihood values for both codings of *A. albicans* and *A. subulata* $> 4.01$; a difference of 2 is the significance cut-off; Pagel, 1999b). As expected from the predominance of nonzero transition rates, independent origins were reconstructed for pubescent and glabrous types (Fig. 4). However, although glaucousness was lost several times, there is little evidence that it has ever been regained. The highest transition probabilities were from glaucous to pubescent and pubescent to glabrous (Table 2). However, for

### Table 2 Maximum likelihood estimates of transition rates between the leaf surface types during the evolutionary diversification of *Asclepias*

<table>
<thead>
<tr>
<th>Transition</th>
<th>Rate coefficient (1)</th>
<th>Rate coefficient (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pubescent → glaucous</td>
<td>0</td>
<td>8 (0–104)*1</td>
</tr>
<tr>
<td>Pubescent → glabrous</td>
<td>239 (233–252)</td>
<td>201 (172–213)</td>
</tr>
<tr>
<td>Glaucous → glabrous</td>
<td>72 (63–78)</td>
<td>72 (0–85)*2</td>
</tr>
<tr>
<td>Glaucous → pubescent</td>
<td>234 (226–246)</td>
<td>259 (0–291)*3</td>
</tr>
<tr>
<td>Glabrous → pubescent</td>
<td>190 (185–196)</td>
<td>175 (168–181)</td>
</tr>
<tr>
<td>Glabrous → glaucous</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Two rate coefficients estimated using the Multistate module of Pagel’sBayesTraits are presented: (1) *Asclepias subulata* and *Asclepias albicans* coded as glaucous, and (2) *A. subulata* and *A. albicans* coded as pubescent (see text for details). Means (95% credible sets) were estimated on 1000 phylogenies sampled from the stationary distribution of a Bayesian phylogenetic analysis (Huelsenbeck et al., 2000).

*Rate coefficients ranged widely for three transitions because of the alternate estimate of a pubescent ancestral state for *Asclepias* in 7.5% of the sampled phylogenies, resulting in broad credible sets; however, 92.5% of the 1000 phylogenies had values of 0 ($^*$), 67–87 ($^*$2), and 271–293 ($^*$3) for these rates.

---

Fig. 4 Fifty per cent majority rule consensus of phylogenies sampled from the stationary distribution of a Bayesian phylogenetic analysis of *Asclepias*, with branch lengths estimated by maximum likelihood. Probabilities of ancestral states of leaf surface type were estimated by maximum likelihood on 1000 phylogenies sampled from the stationary distribution and results are summarized on the consensus phylogeny as follows. If the likelihood of one ancestral state at a node exceeds 90% on all phylogenies (excluding those few for which the node was absent; see Materials and Methods), the descending branch is depicted as solid black (glabrous), dashed black (pubescent), or solid red (glaucous). If the likelihood of all three states at a node is < 90% on any of the phylogenies sampled, the descending branch is depicted as solid yellow, with a pie chart (black, glabrous; speckled, pubescent; red, glaucous) indicating the likelihood of each state averaged over the 1000 phylogenies, or subset for those nodes not present on all 1000 trees. Ancestral state reconstructions were conducted only for species of *Asclepias*; branches to related species in *Gomphocarpus*, for which both sampled species have leaves of the pubescent type, are depicted in solid blue. See text for further details.
Habitat shifts and evolution of leaf traits

SLA was nearly 70% higher in plant species from the wettest habitats compared with species from the driest habitats (n = 13 species listed in the Materials and Methods; mean ± SE: dry-habitat species, 0.204 ± 0.030 cm² mg⁻¹; wet-habitat species, 0.346 ± 0.038 cm² mg⁻¹; $F_{1,11} = 8.611$, $P = 0.014$). This result was somewhat biased by phylogenetic nonindependence, but remained marginally significant ($\lambda = 0.51^*$, $r = 0.49$, $LR = 3.64$, $P = 0.056$). We thus cautiously proceeded to use SLA as a proxy for the environmental aridity in the full data set (n = 43 species). We found a modest level of phylogenetic signal in SLA ($\lambda = 0.37^*$; this was significantly different from both 1 and 0). The ancestral state of SLA was estimated as 0.197, with no directional trend through the phylogeny ($\beta = 0.942$, $LR = 1.94$, $P = 0.163$). This inferred ancestral SLA value ranks 17th out of 43 extant species (from lowest to highest), a relatively low value indicative of a moderately dry ancestral habitat.

We next tested whether leaf surface traits and leaf size were predictive of our proxy for environmental aridity (SLA). In a multiple regression analysis (not corrected for phylogeny) with all species, 18% of the variance in SLA was explained by trichome density and leaf size (Table 3; $F_{2,40} = 4.406$, $P = 0.019$). The relationship between trichomes and leaf size with SLA remained significant in a phylogenetic GLS ANOVA (Table 3). Specifically, trichome density negatively predicted SLA (β = −0.00003, $P = 0.017$), while leaf size was positively associated with SLA ($B = 0.002$, $P = 0.054$) (leaf size and trichomes are not significantly correlated themselves). Finally, we examined a model where leaf glaucousness was included as a discrete trait, because glaucous leaf surfaces have few if any trichomes, but may be viewed as functionally similar to pubescent leaves in their ecological and ecophysiological effects. The results of this full model are substantially stronger (overall analysis, $R^2 = 0.26$; Fig. 5, Table 3), providing a significantly better fit of the data compared with the reduced model (general linear test; Netet et al., 1996; $F_{1,35} = 4.35$, $P = 0.044$); however, glaucousness was not significant in the phylogenetic GLS model (Table 3). Figure 5 shows the relationship between trichomes and leaf size with SLA, while highlighting that glaucous species do not follow the trend.

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**Table 3** Analysis of variance for the effects of trichome density, leaf size, and leaf glaucousness on specific leaf area (used as a proxy for environmental aridity of the plant species) for 43 milkweed (Asclepias) species

<table>
<thead>
<tr>
<th>Factor</th>
<th>ANOVA</th>
<th>Phylogenetic GLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>MS</td>
</tr>
<tr>
<td>Trichomes</td>
<td>1</td>
<td>0.056</td>
</tr>
<tr>
<td>Leaf size</td>
<td>1</td>
<td>0.023</td>
</tr>
<tr>
<td>Glaucousness</td>
<td>1</td>
<td>0.024</td>
</tr>
<tr>
<td>Error</td>
<td>39</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Phylogenetic generalized least squares (GLS) analyses were conducted using the Regression module of BayesTraits; λ was estimated at 0.29 for the full model (significantly different from both zero and 1) and LR is the likelihood ratio for the effect of each factor when removed from the full model (the credible set values from analyses on 1000 phylogenies sampled from the posterior distribution of a Bayesian phylogenetic analysis did not differ from the mean value at two decimal places).

Both codings, the magnitudes of the four nonzero transition rate coefficients were not statistically distinguishable; when they were constrained to be equal the likelihood of the model was very close to that of the unconstrained model (all differences < 0.5). Thus, only the zero probability transitions from glabrous or pubescent to glaucous were statistically different from the others. Except for the irreversible loss of glaucousness, the balanced transition probabilities suggest that directional trends in leaf surface evolution have not occurred.

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**Fig. 5** Relationship between (a) trichomes or (b) leaf size and residual specific leaf area (SLA). SLA is a strong predictor of habitat, with plants having a lower SLA typically coming from more arid habitats. Residual SLA indicates the residuals from the analyses to predict SLA using the other factor included in the model (i.e. (a) employs residuals from a model with leaf size predicting SLA). This data set excludes seven glaucous species (shown in gray circles, and not included in the regression line). Glaucousness evolutionarily trades off with pubescence and appears to be an alternative strategy to dense trichomes.
Ecophysiology

In the following analyses, because we assayed fewer species (with relatively extreme phenotypes), we return to analyzing the data with standard ANOVAs and no phylogenetic correction. Glabrous species had nearly half of the maximum photosynthetic rate ($A_{\text{max}}$) of either pubescent or glaucous species (Fig. 6; surface type: $F_{2,29} = 6.540, P = 0.005$; species within type: $F_{12,29} = 1.479, P = 0.189$). Note that the five species assayed in each leaf surface type did not vary significantly within the group. Although stomatal aperture length did not vary among surface types ($F_{2,21} = 0.916, P = 0.415$), stomatal density was twofold higher on glabrous species compared with pubescent and glaucous species (Fig. 6; $F_{12,21} = 6.598, P = 0.006$).

Foliar carbon isotope ratio and leaf nitrogen content were assessed in two experiments, one where plants were grown in a growth chamber and well watered, and a second where plants were grown in full sun, out of doors, with water stress. Foliar $\delta^{13}C$ is a measure of the long-term ratio of leaf internal to external CO$_2$ concentration and is influenced by both the resistance to CO$_2$ diffusion into the leaf and the rate of assimilation of CO$_2$ into cells from the cell walls within the leaf. More positive values of $\delta^{13}C$ correspond to greater resistance to diffusion (e.g. smaller stomatal aperture, lower stomatal density, increased boundary layer thickness as a result of larger leaves or dense trichomes, etc.) or a faster rate of carboxylation. Across all taxa, the foliar carbon isotope ratio was 20% lower when plants were grown in the growth chamber compared with out of doors (Fig. 6). However, despite significant differences between the experimental settings (Fig. 6; chamber experiment: surface type, $F_{2,29} = 17.257, P < 0.001$; species within type, $F_{12,29} = 6.038, P < 0.001$; full sun experiment: surface type, $F_{2,45} = 7.990, P = 0.001$; species within type, $F_{6,45} = 7.913, P < 0.001$), within each experiment foliar carbon isotope ratio only varied by 2–5% among the three leaf surface types. Indeed, variation among species within leaf surface types was greater than variation among types (a maximum of 15% among species; data not shown). Per cent nitrogen of leaf tissue was lowest for glabrous species in the growth chamber trial where plants were watered $ad$ $libitum$ ($F_{2,90} = 4.333, P = 0.016$; species within type, $F_{14,90} = 3.257, P < 0.001$). This pattern was not consistent, however, in the experiment where plants were water stressed and grown in full sun (Fig. 6; surface type, $F_{2,45} = 3.904, P = 0.027$; species within type, $F_{6,45} = 3.559, P = 0.006$). We note that our species pool was much smaller in the outdoor experiment (Table S1; 9 vs 18 species).

Insect behavior

First-instar monarch caterpillars spent considerably more time walking before settling to feed on pubescent and glaucous
species compared with glabrous species (Fig. 7; ls mean per cent time ± SE: glabrous, 19.410 ± 5.461; pubescent, 47.383 ± 5.262; glaucous, 29.467 ± 5.056; surface type, \( F_{2,33} = 7.05, P = 0.003 \); species nested within type, \( F_{6,33} = 3.99, P = 0.004 \)). Despite significant differences among the leaf surface types, considerable variation was observed among species within groups; of these, glabrous species were the most consistent (Fig. 7). In addition to the nine host Asclepias species, we also assayed monarch caterpillars on three nonhost species; caterpillars spent a greater percentage of their time walking on these nonhosts than on host species (59 ± 9%); compare with Fig. 7) and did not feed on any of the nonhosts. Walking speed was over 30% faster on glabrous species compared with the other leaf surface types (Fig. 7; ls mean cm min\(^{-1} \) ± SE: glabrous, 4.300 ± 0.269; pubescent, 3.175 ± 0.260; glaucous, 3.167 ± 0.260; surface type, \( F_{2,32} = 5.999, P = 0.006 \); species nested within type, \( F_{6,32} = 2.634, P = 0.034 \)).

We also used species means for wax loads and insect behavior to determine the impact of wax amounts irrespective of the surface type category. The relationship of wax load with the percentage of time spent walking was weak when phylogeny was not taken into account (\( n = 9, r = 0.638, P = 0.064 \)); however, the correlation using phylogenetic GLS was significant: \( \lambda = 1.024^*, r = 0.66, LR = 5.18, P = 0.023 \). Additionally, wax loads predicted monarch walking speed, with higher wax loads significantly slowing down monarchs (raw correlation, \( n = 9, r = −0.797, P = 0.010 \); correlation using phylogenetic GLS, \( \lambda = 0.997^*, r = −0.57, LR = 3.56, P = 0.059 \)).

*Apis nerii* spent >50% more time walking on pubescent and glaucous species compared with glabrous species, although the differences were not significant (Fig. 7; ls mean per cent time walking ± SE: glabrous, 19.279 ± 5.819; pubescent, 31.199 ± 5.819; glaucous, 37.819 ± 5.819; surface type, \( F_{2,30} = 2.49, P = 0.097 \); species nested within type, \( F_{6,30} = 0.815, P = 0.566 \)). Note that there was no significant effect of species nested within type, although, as in most of the analyses, glabrous and glaucous species were the least variable. When we explicitly examined species-level differences, we found that total wax load strongly positively correlated with the percentage of time aphids spent walking around before settling to feed (Fig. 8; raw correlation, \( n = 9, r = 0.877, P = 0.002 \); the correlation using phylogenetic GLS was the same: \( \lambda = 0.968^*, r = 0.91, LR = 16.28, P < 0.001 \). Although glabrous species had the lowest wax loads (Fig. 8), pubescent and glaucous species were intermixed, and wax load accurately predicted walking time.

**Correlated responses of leaf surface types and defense chemistry**

As predicted from previous results and hypotheses (Agrawal & Fishbein, 2006), glaucous and pubescent species had 14 times higher latex production, on average, than glabrous species (24 species tested; ls means ± SE (mg): glabrous, 0.403 ± 0.833; pubescent, 6.472 ± 0.779; glaucous, 4.668 ± 1.098; ANOVA on raw data \( F_{2,81} = 14.943, P < 0.001 \); pubescent and glaucous species, however, did not differ from each other (pairwise contrast on raw data: \( F_{2,81} = 1.708, P = 0.195 \)). Using phylogenetic GLS, glabrous species had less latex than glaucous species (\( \lambda = 1^*, LR = 11.70, P < 0.001 \)) or pubescent species (\( \lambda = 0.676^*, LR = 4.54, P = 0.033 \)), but glaucous and
pubescent species were not distinguishable ($\lambda = 0, \text{LR} = 1.46, P = 0.227$).

Differences in foliar phenolics among the leaf surface types were assessed for total phenolics and particular flavonoids (quercetin glycosides) determined by HPLC (Fig. 9). Overall, total phenolics were 25% higher in glabrous species compared with either pubescent or glaucous species, but there was substantial variation among species within each group (ls means $\pm$ SE mg g$^{-1}$ dry mass: glabrous, 5.386 $\pm$ 0.145; pubescent, 4.218 $\pm$ 0.146; glaucous, 4.563 $\pm$ 0.253; surface type, $F_{2,93} = 16.493, P < 0.001$; species nested within type, $F_{21,93} = 16.181, P < 0.001$). The least variable group was the pubescent species, with all but $A. purpurascens$ having low concentrations of phenolics (Fig. 9). This pattern was reversed for the quercetin glycosides, with over 50% higher concentrations in the pubescent and glaucous species compared with glabrous ones (ls means $\pm$ SE mg g$^{-1}$ SE: glabrous, 0.790 $\pm$ 0.076; pubescent, 1.210 $\pm$ 0.077; glaucous, 1.350 $\pm$ 0.133; surface type, $F_{2,93} = 10.639, P < 0.001$; species nested within type, $F_{21,93} = 7.959, P < 0.001$). However, again differences among leaf surface types are mitigated by a high level of variation among species within types (Fig. 9). Leaf cardenolides did not vary significantly among the leaf surface types (Fig. 9; ls means $\pm$ SE: glabrous, 1.436 $\pm$ 0.146; pubescent, 1.669 $\pm$ 0.151; glaucous, 1.489 $\pm$ 0.206; surface type, $F_{2,138} = 0.652, P = 0.523$; species nested within type, $F_{21,138} = 1.869, P = 0.018$).

**Discussion**

We performed phylogenetically informed comparative analyses of leaf surface structures of milkweeds, adopting a ‘syndromes’ approach to understanding both the direct consequences of leaf surface variation and correlated trait evolution (Chapin et al., 1993; Agrawal & Fishbein, 2006). By combining measures of functional traits with evaluation of interactions between plants and the biotic and abiotic environment, we present highly integrative research on leaf surface adaptations. Thus, our discussion of the results is framed as a series of adaptive hypotheses that seek to explain the patterns of divergence and convergence seen in leaf surface morphology across milkweed species.

Our results suggest that a major factor in the evolution of leaf surface traits in *Asclepias* has been an association with relatively arid environments; indeed, based on our phylogenetic reconstructions of SLA, the ancestral environment appears to have been a seasonally dry, warm climate that is characteristic of the environment of many extant species. The lineage probably invaded more moist habitats more than once (e.g. in the case of *A. exaltata*, and at least once in the case of several closely related species: *A. incarnata*, *A. curassavica* and *A. perennis*; see Fig. 4). We propose that aridity and high light have been the driving evolutionary forces underlying pubescent and wax-coated glaucous leaves, although the latter may have been the ancestral state and have been lost repeatedly in response to colonization of more mesic or shadier habitats. This interpretation is supported by the observation that species with dense trichomes or epicuticular wax platelets maintain the highest photosynthetic capacity in high-light environments, despite reflecting a considerable portion of photosynthetic radiation from their leaves and having the lowest stomatal density among the species investigated (Fig. 6). Also pointing to the importance of high-light environments to phenotypic evolution of *Asclepias* are high concentrations of quercetin glycosides that were detected in the leaves of pubescent and glaucous species (Fig. 9); these flavonoids function as sunscreens in many plants (Hoque & Remus, 1999; Ryan et al., 2001, 2002; Solovenko & Merzlyak, 2003). Interestingly, these same compounds also may make these species particularly attractive targets for oviposition by monarch butterflies, which are natural herbivores on all of the milkweeds and are attracted to quercetin glycosides (Haribal & Renwick, 1998). Given that our results indicate that trichomes and waxes decrease the speed and settling rate of monarchs and aphids, in addition to their effects on light reflectance, these surface features might also serve to discourage herbivores in the presence of otherwise highly attractive feeding opportunities (i.e. high quercetin glycoside signal).
Resistance to herbivory may take on increased importance in arid, low-nutrient environments, as the resources present in leaves may be more valuable and more costly to replace than in mesic or wetland habitats (Janzen, 1974; Fine et al., 2004). This increased value of leaves in dry environments is also underscored by the greater mass of tissue necessary to support a given unit of photosynthetic area in milkweed species adapted to dry environments (i.e. they have lower SLA). Thus, there is the potential for a selective environment in which multiple abiotic and biotic factors favor the evolution of similar leaf surface features (i.e. trichomes or wax to reflect high light and to discourage herbivory). Such concordant biotic and abiotic selection might partially explain the convergent evolution of certain leaf types and underlie the emergence of leaf type syndromes in *Asclepias*. We will develop these points in more detail in the discussion that follows.

**Composition and properties of *Asclepias* leaf surface waxes**

Several lines of evidence suggest distinct surface wax chemical compositions among our three groupings of glabrous, pubescent, and glaucous milkweed species. Both pubescent and glaucous species had six times higher wax coverage than glabrous species. However, the surface waxes of pubescent and glaucous species were distinct in that fatty aldehydes were only present in glaucous species (Fig. 3). Further, only pubescent taxa had detectable concentrations of triterpenoid acetates. In glabrous species, the limited amount of surface wax present was characterized by primary and tertiary alcohols. Despite this consistent differentiation among groups, for each of the constituent compound classes, the species term (nested within surface type) was significant, indicating substantial within-group variation (Table 1, Fig. 2). In particular, pubescent *A. speciosa* and glaucous *A. perennis* differed in their wax composition compared with the other species in their respective groupings.

It is generally accepted that single compounds or compound classes are responsible for the formation of epicuticular wax crystals (Baker, 1982). This was first inferred from comparative studies of diverse plant species, showing that the predominance of certain wax constituents was correlated with characteristic crystal shapes (Holloway et al., 1976). Conversely, crystals of various characteristic shapes could be generated in vitro by recrystallization of selected wax constituents (Jetter & Riederer, 1994, 1995). Thus, the current results on *Asclepias* wax mixtures indicate that fatty aldehydes are essential for the formation of wax crystals on the cuticle. This notion is supported by analogy with the well-studied trap surfaces of *Nepenthes* pitcher plants. The epicuticular wax crystals covering the slippery zone of the pitchers of these carnivorous plants are indistinguishable from the *Asclepias* crystals both in shape and in arrangement on the plant surface (Riedel et al., 2003, 2007). Furthermore, detailed chemical analyses of the *Nepenthes* crystals showed that they consist mainly of triacontanal, a C₃₀ wax aldehyde. We thus speculate that the slippery *Asclepias* surfaces are probably an example of chemical convergence of leaf surface properties for (at least some) similar proximate functions (i.e. slipperiness to arthropods) with different ultimate purposes (i.e. resource acquisition in *Nepenthes*, and resource defense in *Asclepias*).

Our extraction method yielded the complete cuticular waxes, comprising both intracuticular and epicuticular waxes, defined as two distinct layers deposited within or on top of the cutin polyester matrix, respectively (Jetter et al., 2000; Jeffree, 2006). The results can thus only be used to infer the qualitative make-up of the *Asclepias* wax crystals, but not their exact quantitative composition as the epicuticular and intracuticular layers may differ in composition. For example, it has been found that triterpenoid alcohols and acids tend to accumulate at high concentrations in the intracuticular wax, whereas the adjacent epicuticular wax contains lower percentages (or none) of these compounds (Jetter et al., 2000; Buschhaus et al., 2007).

We hypothesize that the triterpenoid compounds found in the total wax mixtures of glabrous and pubescent *Asclepias* species are also restricted to the intracuticular layer. This would mean that the epicuticular wax, that is, the outermost surface of the leaves of these species, had a composition dominated by fatty alcohols. Triterpenoids, known to affect insect behavior in a number of plant species (Gershenzon & Croteau, 1992), thus may not have directly contacted herbivores until feeding (unless in very small concentrations as a result of diffusion).

**Evolution of leaf surfaces**

The literature on chemotaxonomy has viewed leaf chemical traits as potential indicators of phylogenetic relationships. For example, there have been several attempts to use chemical profiles of cuticular waxes as taxonomic criteria to differentiate groups of species within plant families (Maffei, 1996) or genera (Maffei, 1996; Mimura et al., 1998; Medina et al., 2006). Flavonoids were also used to identify hybrids of *Asclepias* species in particular (Wyatt & Hunt, 1991). However, such chemical characteristics are unlikely to be neutral and, in fact, may often be adaptive, leading to the possibility of convergence (i.e. homoplasy). Several authors have therefore suggested that they are probably not useful as taxonomic characters (Wink, 2003; Pelser et al., 2005; Agrawal, 2007).

In the present study, instead of using chemical traits as data to infer phylogeny, we have used a phylogeny estimated from independent molecular sequence data to understand the evolution of leaf chemistry and its ecological consequences.

We employed maximum likelihood approaches to estimate ancestral states for continuous and discrete traits and to estimate evolutionary transition rates between leaf surface types. Because trichome density is a quantitative trait, with variation bridging our three leaf surface types, we initially assessed the evolution of trichomes independently. In this analysis, we
concluded that the ancestral milkweed was probably pubescent (i.e. ~1000 trichomes cm\(^{-2}\)) and we found no evidence for a consistent directional phenotypic trend as *Asclepias* diversified. However, the evolution of trichomes cannot be considered fully independent from that of other leaf surface characteristics, in part because trichomes and the wax platelets associated with glaucousness show strongly negatively correlated evolution. Despite this correlation, when glaucous species are excluded we still arrive at a similar estimation of ancestral trichome density (1623 trichomes cm\(^{-2}\)).

Additionally, we reconstructed the ancestral state for *Asclepias* and estimated transition probabilities among three discrete leaf surface states: glabrous, pubescent, and glaucous. Whether *A. albicans* and *A. subulata* were coded alternatively as glaucous (model 1, Table 1) or pubescent (model 2), we obtained similar results. The reconstructed ancestral state was decidedly glaucous under model 1, with several losses of glaucousness, which, once lost, was not regained. Under model 2 the ancestral state was also glaucous (100% probability in 92.5% of 1000 sampled phylogenies). A notable result emerging from both models was that the transitions from both glabrous and pubescent to glaucous had zero probabilities. By contrast, the largest transition probability was that from glaucous to pubescent surfaces (Table 2). Overall, while these patterns are suggestive of interesting evolutionary dynamics, we fully acknowledge that our confidence in the inferences drawn from these analyses is hampered in part by limited phylogenetic sampling (47 species, about one-third of the genus *Asclepias*; Fig. 4). Moreover, the present sampling is geographically and phylogenetically biased, with relatively few representatives of clades composed of species with mostly Mexican distributions. We have no reason to believe that the sampled species are unrepresentative of the genus as a whole, but future studies are necessary to validate this assumption. Nonetheless, the available data suggest that the ancestral state for milkweed leaf surface types, as well as SLA, was similar to that seen in extant species occupying arid environments, indicating that the genus may have originated in such environments (as opposed to mesic or wetland habitats where milkweeds also occur today).

Species’ values for SLA in global datasets are negatively correlated with the aridity of the environment in which plants grow (Givnish, 1987; Reich et al., 1999; Ackerly, 2004). Additionally, shaded plants within species often have leaves (via plasticity) that have fewer trichomes and higher SLA (Young & Smith, 1980; Morgan & Smith, 1981; McGuire & Agrawal, 2005). In other words, there is a phenotypic shift consistent with the notion that water stress is associated with reduced SLA values. Our data from *Asclepias* were consistent with this pattern, and we thus employed SLA as a surrogate for habitat aridity. We found a negative correlation between trichomes and SLA both here and in a previous field study (Agrawal & Fishbein, 2006). These results were robust in phylogenetically independent analyses, suggesting repeated and correlated evolution of pubescence and SLA in association with arid habitats (Table 3). This result for SLA is again consistent with aridity favoring small-leaved species (Givnish, 1987; Ackerly, 2004). Finally, the observation that glaucous species had dramatically lower SLA values than would be predicted based on their trichome densities suggests that glaucousness may aid in coping with environmental aridity; including glaucousness to predict SLA significantly improved the statistical model fit by 10%. We thus conclude that trichomes and glaucousness are alternative phenotypic strategies to achieve the same goals, including maintaining high rates of photosynthesis under potentially stressful high-light and high-temperature conditions, all the while helping to resist herbivores. Notably, the mean SLA of glaucous species, 0.197 (± 0.031) cm\(^2\) mg\(^{-1}\), is equivalent to the SLA of species with ~2000 trichomes cm\(^{-2}\).

**Leaf ecophysiology**

The leaf surface type is predicted to strongly influence the overall water relations and photosynthetic physiology of plants. Trichomes and surface wax crystals increase the depth of the boundary layer over a leaf surface. The depth of this still layer strongly influences the transport of matter and energy (water loss and carbon uptake by plants; Farquhar et al., 1982). For example, matter and energy are transported much faster under turbulent conditions than they are through the still air of a boundary layer where transport is solely accomplished via diffusion. Further, carbon dioxide and water vapor are transported by diffusion at different rates such that longer diffusional transport distances (i.e. a larger boundary layer) lead to more carbon transported per unit of water vapor lost. Consequently, while the overall magnitude of each flux decreases with increasing boundary layer, the efficiency increases. This dynamic, usually referred to as water use efficiency, is often maximized in plants associated with dry environments (Farquhar et al., 1989). However, a large boundary layer also decreases the transport of heat energy away from the leaf, leading to higher leaf temperatures. For this reason, many species associated with hot and arid environments have evolved small leaf sizes with smaller boundary layers. Overall it is apparent that plants must simultaneously manage both water loss and leaf temperature in arid environments, often evolving alternative leaf morphological characteristics (Givnish, 1987).

The comparative physiological measurements made in the present study show differences among species exhibiting the three leaf surface types, including lower maximum photosynthetic rate \(A_{\text{max}}\) and higher stomatal density in glabrous compared with glaucous or pubescent species (Fig. 6). We note that, although glaucousness is a discrete trait, trichomes are obviously continuous and highly variable in their densities; in our study, we primarily employed ‘pubescent’ species that were relatively extreme in having high trichome density (> 1000 trichomes cm\(^{-2}\)). Our results are consistent with across-
biome comparative measures which typically show higher $A_{\text{max}}$ in plant species from more arid regions (Reich et al., 1999). Similarly, other studies have reported trends toward greater stomatal densities found on plants in the wettest temperate habitats and decreased densities in species occupying xeric habitats (Brewer & Nuñez, 2007). Our data suggest that glabrous plants have the lowest resistance, at least in terms of the stomatal boundary, to $CO_2$ diffusion and yet the lowest maximum photosynthetic rate. In order to reconcile these two observations, there must be large differences in the carboxylation capacity of leaves. We predict that the rate of enzymatic fixation of carbon in the dark reactions has to be higher in glaucous and pubescent compared with glabrous species.

Data on foliar carbon isotope composition generally support the hypothesis that glaucous and pubescent species have higher carboxylation capacity than glabrous species (Fig. 7a). The foliar $\delta^{13}C$ composition is a reflection of the ratio of internal ($c_i$) to external ($c_a$) $CO_2$ concentration. The $c_i/c_a$ ratio is controlled by both the resistance to diffusion into the leaf and the rate of consumption of $CO_2$ within the leaf as a result of carboxylation. In our estimates of water use efficiency, we examined species likely to have very different resistances to $CO_2$ diffusion because of their leaf surface characteristics (i.e. all pubescent species had $>1000$ trichomes cm$^{-2}$). A reasonable a priori prediction would be that species with high diffusional resistances would exhibit more positive $\delta^{13}C$ values, which can be interpreted as higher water use efficiency. However, despite slight statistical variation among leaf surface types, the groups had remarkably similar $\delta^{13}C$ values to each other. This similarity was upheld under both well-watered and water-stressed conditions (Fig. 6). We hypothesize that the similarity of water use efficiency among leaf surface types is attributable to higher carboxylation capacities in glaucescent and pubescent species compared with those that are glabrous. Total leaf nitrogen content is often used as an estimate of total carboxylation capacity because, in most plants, the largest pool of leaf nitrogen is the enzyme Rubisco, the enzyme catalyzing the carboxylation of $CO_2$ (Lambers et al., 1998). In our study, leaf nitrogen content was higher in glaucous and pubescent compared with glabrous species only under well-watered conditions (Fig. 7b). The data may therefore suggest that the use of total leaf nitrogen as a proxy for carboxylation capacity is not appropriate for milkweeds. In particular, this disconnect may result from the presence of nitrogen-containing defensive compounds in the foliage of milkweeds that are not related to photosynthetic physiology. The appropriate measure would be the direct measurement of the enzyme Rubisco.

Müller & Riederer (2005) stated that ‘the most important feature of primary plant surfaces is, because of their hydrophobicity, the repellence of water, aqueous solutions, polar mucilages, and small organisms with polar coatings. ... due to cuticular wax crystals and sometimes also by papillose epidermal surfaces or trichomes.’ This is certainly supported for some groups, such as the Bromeliads, where trichomes and epicuticular wax powders serve important hydrophobic functions (Pierce et al., 2001). Hydrophobicity per se may contribute to gas exchange (i.e. boundary layer resistance) and interactions with pathogens, but is unlikely to affect interactions with herbivores or many of the other ecophysiological functions that are also strongly affected by leaf surface properties (Eigenbrode & Jetter, 2002). For example, it has been demonstrated that trichomes of Veratrum thapsus have multiple effects that go beyond hydrophobicity, including the reflection of UV light, which protect photosynthetic machinery, reduce water loss, and protect leaves from invertebrate herbivory (Woodman & Fernandes, 1991; Manetas, 2003).

Effects of leaf surface types on biotic interactions

In addition to their ecophysiological effects, trichomes and wax crystals have been intensively studied for their impacts on insect herbivores (Eigenbrode & Espelie, 1995; Hanley et al., 2007). Both types of surfaces strongly influence adult and larval behavior, disrupting attachment, and variously affecting feeding (Haddad & Hicks, 2000). For example, specialist beetles prefer to feed on alder (Alnus incana (L.)) leaves with naturally low densities of trichomes or leaves with trichomes that have been experimentally removed (Baur et al., 1991). In order to overcome the physical barrier posed by trichomes, many herbivores graze trichomes before feeding or cover them with a mat of silk to allow for freer movement and greater attachment (Rathcke & Poole, 1975; Fordyce & Agrawal, 2001; Medeiros & Moreira, 2005). First-instar monarch butterfly caterpillars must shave trichomes on pubescent species before consuming leaf tissue (observed in this study; Malcolm, 1995; Agrawal, 2005), and this probably plays a role in feeding site selection.

A distinct ecological function was first attributed to epicuticular wax crystals in studies of the slipperyzone of Nepenthes pitcher plants, where these waxes were hypothesized to play a pivotal role in the capture of insect prey (Knoll, 1914). This function was confirmed recently by detailed biomechanical studies, showing that microscopically rough, hydrophobic surfaces of diverse plant species indeed reduce the adhesion forces of insect feet and whole insects (Eigenbrode et al., 1999a; Federle et al., 2000). For example, epicuticular waxes have been well studied in Brassica crops, and evidence shows that wax blooms on glaucous surfaces reduce adult and larval feeding by some herbivores (Eigenbrode & Espelie, 1995; Eigenbrode et al., 2000). Nonetheless, lower net herbivory can often be achieved on ‘glossy’ Brassica lines, because predator attachment and movement are superior on these less slippery glabrous varieties (Eigenbrode et al., 1996, 1999b). Complementary work has revealed a similar set of interactions between herbivores and predators and pea (Pisum sativum) plants (White & Eigenbrode, 2000; Rutledge et al., 2003). In addition to their effects on the physical movements and consumption of plant tissue by herbivores, chemical compounds in leaf surface
waxes can also serve as both attractants and repellents (Eigenbrode & Espelie, 1995; Eigenbrode et al., 1999b). In our studies, monarch caterpillars were observed to fall off plants only three times, but in all cases this was on glaucous species. The slower speed of movement and reduced time to settling of milkweed herbivores we observed are consistent with an effect of glaucousness on insect attachment and movement. We therefore conclude that the effects of glaucous Asclepias species on insect behavior were attributable to surface slipperiness caused by wax crystals.

Both trichomes and wax crystals showed positive correlated evolution with latex exudation in the milkweeds. Latex is a potent defense that provides a physical barrier to consumption (i.e. chewing insects are faced with a mouthful, or more, of a viscous gooey substance), as well as delivery of several toxic substances (cardenolides, cysteine proteases, etc.) (Agrawal et al., 2008). Our finding of correlated evolution has two important implications. First, this result confirms our earlier speculation that plant defenses evolve as convergent suites of correlated traits, or syndromes (Agrawal & Fishbein, 2006). Second, along with other evidence presented in this paper, it appears that pubescence and glaucousness are functionally similar, with similar ecological interactions as well as correlated suites of trait evolution.

Concluding speculation

In Asclepias, the role of dense trichomes and wax platelets appears to be a complex interplay between biotic and abiotic factors. We have shown how milkweeds can be discretely categorized into leaf surface types, and that these groupings have distinct chemistries, and observed and predicted interactions with herbivorous insects. Nonetheless, the impact of the leaf surface types also extends to ecophysiological effects of gas exchange and, potentially, carboxylation capacity. In other words, the leaf surface type may correlate with other traits of the plant to form a strategy or syndrome that is associated with the particular environment in which species evolve. Ancestral milkweeds were probably glaucous and adapted to arid environments, although we cannot be sure as to the distinction between the benefits of glaucous leaves and those of densely trichome-covered leaves.

Why would species evolve from glaucous to pubescent forms (the most common transition), when by all accounts these species are similar in their ecologies? Our working hypothesis is that trichomes, unlike wax crystals, are remarkably variable in their densities on milkweeds, and this may allow greater lability in evolutionarily titrating a phenotype–environment match. Extant milkweeds have invaded wetlands, fields, and forest habitats. The loss of wax crystals occurred several times and transitions back to a glaucous state have not occurred (among sampled species). Trichome density thus probably evolved in response to particular biotic and abiotic environments, with the lowest densities found in mesic habitats. As Asclepias diversified and reinvaded extremely arid habitats, higher trichome densities evolved. Given the overall ‘phenotypic space’ available, there are a limited number of combinations of traits that seem to be viable in a given habitat; given the selection pressures of an environment, lineages are repeatedly pushed toward the same ‘solution’ or to alternative solutions that are apparently interchangeable.

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References

the genus *Clusia* from Panama. *Biochemical Systematics and Ecology* 34: 319–326.


**Nylander JAA. 2004.** MrModeltest2, version 2.3. Program distributed by the author. Uppsala, Sweden: Evolutionary Biology Centre, Uppsala University.


**Rambaut, A, Drummond, AJ. 2007.** *Tracer, version 1.4. Program distributed by the authors*. Uppsala, Sweden: Evolutionary Biology Centre, Uppsala University.


**Swofford DL. 2002.** PAUP*. Phylogenetic analysis using parsimony (*and other methods*), version 4.0b10, Sinauer, Sunderland, MA, USA.


**Woodson RE. 1954.** *The 47 species used for various measures in this study*. New Phytologist (2009) 183: 848–867