

# Phylogenetic and Experimental Tests of Interactions among Mutualistic Plant Defense Traits in *Viburnum* (Adoxaceae)

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Submitted October 7, 2011; Accepted May 30, 2012; Electronically published August 22, 2012

Online enhancement: appendix. Dryad data: <http://dx.doi.org/10.5061/dryad.t4n0g>.

**ABSTRACT:** Plant traits that mediate mutualistic interactions are widespread, yet few studies have linked their macroevolutionary patterns with the ecological interactions they mediate. Here we merged phylogenetic and experimental approaches to investigate the evolution of two common mutualistic plant traits, extrafloral nectaries (EFNs) and leaf domatia. By using the flowering plant clade *Viburnum*, we tested whether macroevolutionary patterns support adaptive hypotheses and conducted field surveys and manipulative experiments to examine whether ecological interactions are concordant with evolutionary predictions. Phylogenetic reconstructions suggested that EFN-bearing species are monophyletic, whereas the evolution of domatia correlated with leaf production strategy (deciduous or evergreen) and climate. Domatia were also more common in the EFN clade, suggesting that the two traits may jointly mediate ecological interactions. This result was further investigated in a common-garden survey, where plants with domatia and EFNs on the leaf blade had more mutualistic mites than plants with other trait combinations, and in manipulative field experiments, where the traits additively increased mutualist abundance. Taken together, our results suggest that mutualistic traits in *Viburnum* are not ecologically independent, as they work in concert to attract and retain mutualists, and their long-term evolution may be influenced by complex interactions among multiple traits, mutualists, and geography.

**Keywords:** bed-and-breakfast hypothesis, mite, mutualism, indirect defense, *Viburnum*, extrafloral nectaries, leaf domatia.

## Introduction

Despite being widespread and ecologically important, plant traits that mediate mutualistic interactions are rarely studied in an integrative manner that spans phylogenetic and ecological scales (Bronstein et al. 2006). In plant-arthropod defense mutualisms, specialized plants traits provide food or shelter rewards to arthropods in return for protection against natural enemies. Two well-known examples of such traits are (1) extrafloral nectaries (EFNs),

which are plant organs that secrete sugary nectar and typically feed beneficial arthropods (Bentley 1977; Heil 2008); and (2) leaf domatia, which are small structures on the undersides of leaves that provide housing for predaceous or fungivorous mites (Lundström 1887; Romero and Benson 2005). Because traits such as EFNs and leaf domatia influence multiple trophic levels, their evolution can have strong impacts on community dynamics relative to other plant characters. Nonetheless, studies that directly link patterns in the evolution of these traits to their contemporary ecological function are rare (but see Karban et al. 1995; Heil et al. 2004).

Several hypotheses attempt to explain the evolution and phylogenetic distribution of mutualistic defense traits such as EFNs and leaf domatia. These hypotheses are primarily focused on two factors: (1) the adaptive value of mutualistic defense traits in certain biotic or abiotic environments (e.g., Bentley 1977; Schupp and Feener 1991; Heil and McKey 2003; Bronstein et al. 2006) and (2) evolutionary trade-offs or synergisms with other ecologically relevant plant traits (e.g., Heil 2004, 2008). Environmental hypotheses posit that large-scale geographic associations between mutualistic defense traits and environmental factors are adaptive. For example, the well-established pattern of increased EFN prevalence in the tropics is hypothesized to be driven by selective forces that are both biotic (e.g., increased abundance, species richness, and aggressiveness of mutualists) and abiotic (e.g., higher resource availability and longer growing seasons; Bentley 1977; Schupp and Feener 1991; Heil and McKey 2003; Bronstein et al. 2006). Not all defense mutualisms are more prevalent in the tropics, however: leaf tuft domatia are more frequently found in temperate plant species, a pattern hypothesized to be driven by increased risk of mite desiccation in temperate climates (Romero and Benson 2005).

A second set of hypotheses focuses on evolutionary trade-offs or synergisms and suggests that the origin, maintenance, and breakdown of indirect defensive traits are

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influenced by interactions with other plant traits rather than environmental factors alone. For example, indirect defenses are hypothesized to positively associate with leaf longevity (Bronstein et al. 2006) because plants with short-lived leaves are predicted to invest less in defenses than those with longer-lived leaves (Coley 1988). Another hypothesis suggests that multiple indirect defensive traits interact with one another in a way that influences their ecological functions and, ultimately, their evolution (Heil 2008). For example, if two defensive traits are consistently ecologically redundant and costly, they are predicted to be negatively correlated across a phylogeny (Agrawal et al. 2010). Alternatively, if traits exhibit additive or synergistic ecological effects that are sustained over time, they may show positive correlated evolution. Heil and McKey (2003) suggested that mutualistic interactions are stronger and more likely to be maintained over time when several traits are found together, because mutualists are provided with multiple rewards (e.g., food and housing). Similarly, Fiala and Maschwitz (1992) suggested that ant domatia, in addition to food-based rewards, are an important component in the evolution of obligate defense mutualisms in *Macaranga*. In another example using *Viburnum tinus*, EFNs (Walter and O'Dowd 1995) and leaf domatia (Grostal and O'Dowd 1994) were each separately shown to increase the abundance of mutualistic mites living on leaves. Indeed, many striking defense mutualisms occur on plants that reward their mutualists with several distinct beneficial traits (Heil 2008), and yet their joint evolution has been little studied. In particular, no study has examined the combined impacts of EFNs and leaf domatia on mutualists, although these structures are frequently found together and have been demonstrated separately to increase mutualist abundance, decrease herbivore and/or pathogen load, and subsequently, decrease the risk of plant damage (see reviews by Bentley 1977; Walter 1996; Heil and McKey 2003; Bronstein et al. 2006).

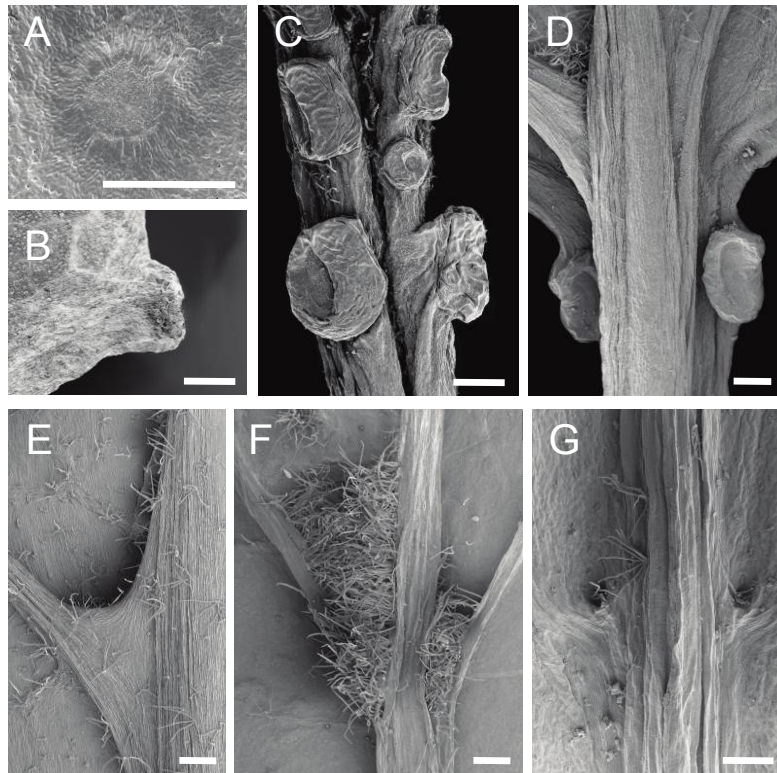
A comprehensive evaluation of these adaptation, trade-off, and synergism hypotheses requires an explicit evolutionary framework. Phylogenetic tests can determine whether defense-related traits have been conserved despite lineage shifts into new environments or state changes in other relevant plant traits. Rather than supporting adaptation to local conditions or evolutionary trade-offs and synergisms, these phylogenetic patterns may reflect genetic or developmental constraints or chance association. Alternatively, patterns in the geographic distribution of traits that are interpreted as adaptive may actually reflect differences in diversification rates between clades. Although such shifts in diversification may correlate with the particular defense traits under consideration here, they may also relate to other uninvestigated traits or to biogeographic movements (e.g., Moore and Donoghue 2007).

When historical patterns of macroevolution are concordant with contemporary ecological dynamics, hypotheses that link pattern with process are more strongly supported (Losos 1996, 2009; Weber and Agrawal 2012). Alternatively, the lack of concordance suggests that either conditions have changed substantially over time or hypotheses need to be further refined (Losos 1996, 2009). In this study, we integrate comparative phylogenetics with field surveys and manipulative experiments to examine the combined evolution of mutualistic defense traits in *Viburnum*, a widespread clade of shrubs and small trees. Specifically, we ask the following questions: (1) Are patterns of mutualistic trait evolution consistent with existing adaptive and trade-off or synergism hypotheses? In particular, do traits show correlated evolution with one another, with other relevant plant traits, or with environments hypothesized to influence their evolution? And (2) do field surveys and manipulative experiments of the two traits demonstrate ecological interactions that are consistent with our interpretations of trait evolution patterns?

## Methods

*Viburnum* (Adoxaceae, Dipsacales) is a clade of ~170 species of understory shrubs and small trees. *Viburnum* naturally occurs in temperate forests around the Northern Hemisphere, with extensions into the Southern Hemisphere in tropical forests of Southeast Asia and cloud forests of South America. Its two modern centers of species diversity are in Eastern Asia and Latin America (Losos 1996; Winkworth and Donoghue 2005; Moore and Donoghue 2007, 2009; Clement and Donoghue 2011). Many *Viburnum* species are popular ornamentals that are common in developed landscapes.

In order to determine the taxonomic distribution of EFNs and leaf domatia in *Viburnum*, we examined herbarium specimens in the Bailey Hortorium of Cornell University, the herbaria of the Yale Peabody Museum of Natural History, the Missouri Botanical Garden, the New York Botanical Garden, the Field Museum of Natural History, Harvard University, and Oregon State University, as well as the personal collections of M.J.D. The presence or absence of EFNs or domatia was determined visually, with the help of a dissecting microscope when necessary. Leaf domatia were classified into "tuft," "pit," and "cave" types (figs. 1E–1G, 2A, 2B) according to O'Dowd and Willson (1989), and EFNs were categorized according to their position on the leaf (figs. 1A–1D, 2C, 2D): (i) as glands (modified teeth) on the leaf margins, typically at the base of the leaf blade; (ii) embedded in the lower (abaxial) surface of the leaf blade, often near the base of the blade (hereafter referred as "laminar"); (iii) at the intersection of the leaf margin and petiole; and (iv) on the petiole.



**Figure 1:** Scanning electron micrographs of representative mutualistic leaf traits on dried *Viburnum* leaves: A, laminar extrafloral nectary (EFN) from *V. dilatatum*; B, marginal EFN from *V. dentatum*; C, petiole EFNs from *V. trilobum*; D, petiole-laminar-junction EFNs from *V. edule*; E, cave domatium from *V. sulcatum*; F, tuft domatia from *V. trilobum*; and G, pit domatia from *V. odoratissimum*. Scale bars indicate 500  $\mu\text{m}$ .

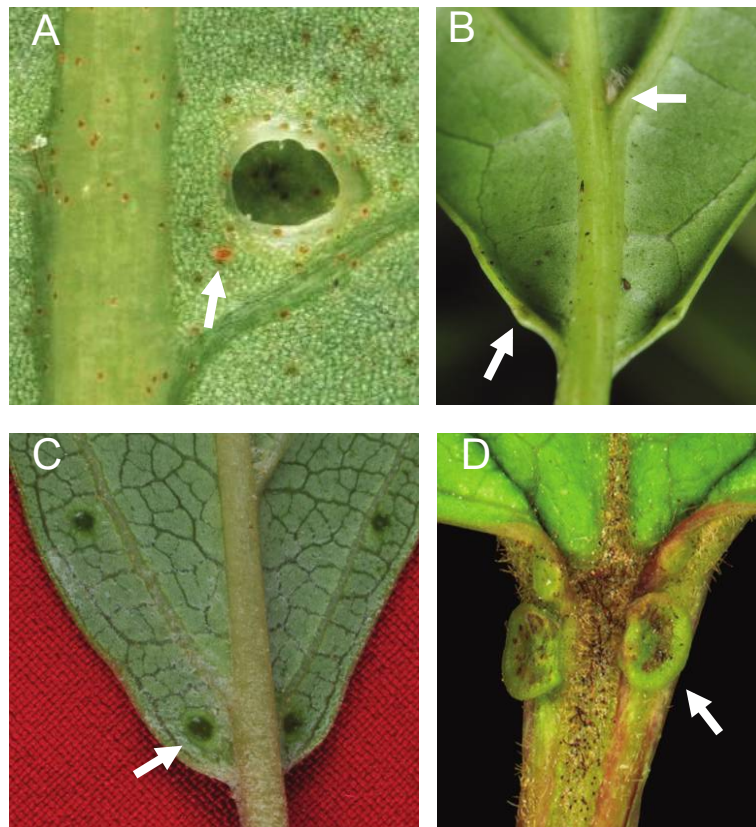
Sugar excretion was verified on live specimens of individuals from each of the four EFN forms, using glucose test strips (Clinistix, Miles Laboratories, Elkhart, IN), and on preserved specimens via the presence of sooty mold on or surrounding the glands (appendix, available online; Pemberton 1990, 1998).

Climate and leaf production strategy were assessed on the basis of specimen collection localities, field observations by M.J.D., and published species descriptions (Rehder 1908; Morton 1933; Kern 1951; Hsu 1975; Donoghue 1983; Hara 1983; Jones 1983; Yang 1994). To test for predicted associations between traits and latitude or habitat, species were assigned to one of three habitat categories: (1) “tropical,” including wet subtropical to tropical forests, in generally mountainous regions but at lower elevations (generally <1,700 m), and with limited temperature seasonality; (2) “cloud,” including montane cloud forests at southern latitudes, at generally higher elevations (mostly >2,000 m), and experiencing periodic colder temperatures but not prolonged temperature seasonality; and (3) “tem-

perate,” including temperate deciduous and boreal forests, at higher latitudes, with strong and prolonged seasonality. Additionally, in order to test the predicted correlation between longer leaf life spans and indirect defense traits, species were assigned to one of three leafing strategies: (1) “evergreen,” in which plants maintain their leaves year-round and individual leaves last for more than one season; (2) “leaf exchangers,” in which plants flush and lose their leaves asynchronously and may have short, sporadic periods of leaflessness (i.e., semideciduousness); and (3) “seasonally deciduous,” in which plants synchronously lose their leaves for a prolonged period each year.

#### *Phylogenetic Methods*

To evaluate patterns of EFN and domatia evolution, we utilized a recently published phylogenetic tree for 90 *Viburnum* species (Clement and Donoghue 2011). This tree was obtained from a Bayesian analysis of a combined molecular data set that included the chloroplast coding



**Figure 2:** Photographs of mutualistic traits from live specimens of several *Viburnum* species. A, Close-up of a pit domatium from *V. becarri*. Arrow points to a mite near the opening of the domatium. B, Basal, abaxial leaf surface of *V. cinnamomifolium*. Arrows point to a tuft domatium and a marginal extrafloral nectary (EFN). C, Basal, abaxial leaf surface of *V. coriaceum*. Arrow points to one of the lamina EFNs. D, Petiolar EFNs on *V. opulus*. Arrow points to one EFN. Photographs: A–C are by Patrick Sweeney, copyright 2011, Peabody Museum of Natural History; D is by Gary Fawless, Cofrin Center for Biodiversity.

regions *matK*, *ndhF*, and *rbcL* (4,104 bp); the chloroplast noncoding regions *petB–petD*, *rpl32–trnL<sup>(UAG)</sup>*, *trnH–psbA*, *trnC–ycf6*, *trnK*, and *trnS–trnG* (4,806 bp); and the nuclear ribosomal internal transcribed spacer region (642 bp). All of the DNA sequences used by Clement and Donoghue (2011) are available in Genbank (<http://www.ncbi.nlm.nih.gov/genbank>), and all of their data sets and trees can be downloaded from TreeBASE (<http://www.treebase.org>; S10714). All comparative analyses were conducted by using the majority consensus of the post-burn-in posterior distribution of Bayesian trees. For each analysis, the tree was pruned (retaining branch lengths) to include the species for which we had character information (appendix).

We inferred ancestral character states and estimated the number of evolutionary transitions in our traits using the maximum likelihood (ML) and maximum parsimony (MP) optimization criteria in the program Mesquite (Maddison and Maddison 2010). We evaluated the phylogenetic signal of the traits by using a Monte Carlo test

of trait conservatism (Webb et al. 2002; Crisp et al. 2009), comparing the MP number of transitions in our observed data with a distribution of MP character transitions from 10,000 random reshufflings of our tip states. If the observed MP number of state changes fell below the 95% confidence limit of the randomized distribution, then the observed data were significantly more conserved than would be expected by chance. This approach preserves the phylogenetic relationships of our taxa, as well as the number of species assigned to each character state, while varying the distribution of character states across the tree.

We tested for correlated evolution among plant traits with Pagel's test of discrete character correlation (Pagel 1994), using the Pagel94 module in Mesquite. This test asks whether the evolution of one binary character is dependent on the state of another binary character in a phylogeny. Specifically, the analysis compares the likelihoods of independent and dependent models of character evolution with Monte Carlo tests of simulated data. We ran

the simulation for 10,000 sets, with each simulation set having 50 optimizer iterations. For correlation analyses, climate and leafing strategy were grouped into binary categories. For leaf production analyses, seasonally deciduous species and leaf exchangers were together considered to be deciduous, in contrast to evergreen species. For climate analyses, temperate and cloud species were considered to experience significant cold periods, as opposed to the tropical species, which experienced no cold season.

#### *Common-Garden Survey*

In order to examine whether species with indirect defensive traits had more beneficial mites than species lacking those traits and which trait combinations foster the highest mite abundance, we surveyed mite populations on 16 species of *Viburnum* in an untreated common garden of mature shrubs in Ithaca, New York, that was established in 1999. Of the 16 species in the garden, seven lacked both leaf domatia and EFNs (*V. carlessii*, *V. cassinoides*, *V. lantana*, *V. lentago*, *V. macrocephalum*, *V. nudum*, *V. prunifolium*), two lacked EFNs but had tuft domatia (*V. plicatum*, *V. sieboldii*), two had laminar EFNs and tuft domatia (*V. dilatatum*, *V. setigerum*), two had marginal EFNs and tuft domatia (*V. dentatum*, *V. rafinesquianum*), and three had petiolar EFNs and tuft domatia (*V. opulus*, *V. sargentii*, *V. trilobum*). Thus, we compared mite abundance on species that lacked mutualistic traits with that on species that had only domatia or that had domatia paired with petiolar, marginal, or laminar EFNs.

We collected 10 leaves from each species (two to 10 mature shrubs sampled per species; mean  $n = 7$ ) in July of 2010. Leaves were placed in moist paper towels, transported to the laboratory on ice, and surveyed for mites under a dissecting scope. Mites found on leaves were counted, sorted into morphospecies, and stored in 75% ethyl alcohol. Representatives from each morphospecies were mounted in Hoyer's solution on microscope slides and later identified to taxonomic family (Krantz and Walter 2009). Because mites within families typically have a conserved diet range (i.e., mycophagous, predaceous, herbivorous; Krantz and Walter 2009), we used family as an indication of their potential interactions with the plant. We compared the abundance of mycophagous or predaceous mites on plants with different combinations of mutualistic traits using a nested ANOVA with species nested within mutualistic trait combination that was performed in R (R Development Core Team 2010).

#### *Field Manipulations*

We experimentally addressed the independent and joint impacts of leaf domatia and laminar versus marginal EFNs

on beneficial mite numbers, using natural populations of two species: *Viburnum acerifolium*, which has tuft domatia and a small, single pair of laminar EFNs on either side of the midrib, and *Viburnum dentatum*, which has tuft domatia and two to six small EFNs on the basal quarter of the leaf margin. On the basis of common-garden results and the physical locations of the nectaries, we predicted that the nectaries would more drastically increase mite abundance in *V. acerifolium* because of the close proximity of laminar nectaries to leaf domatia (~3 mm). Because natural populations of these two species are found in slightly different habitats, experiments using *V. dentatum* were conducted in an old-field community (42°30'1.44"N, 76°26'8.52"W), whereas experiments using *V. acerifolium* were performed in the Cornell University Polson Preserve, a deciduous forest understory (42°30'2.34"N, 76°26'8.49"W). For both species populations were large, with hundreds of plants in the surrounding areas, and so we selected relatively small plants for the experiment (10–30 cm in height and with two to 10 leaves) in order to easily manipulate all of the leaves on a plant.

We used a two-way factorial design to experimentally test whether the effects of EFNs and leaf domatia on mite population numbers are additive, redundant, or synergistic. We blocked mutualistic structures by filling EFNs and/or domatia with tree-pruning tar (Tanglefoot Asphalt Pruning Sealer, Contech Enterprises, Victoria, BC). Controls received the same amount of tar, which was applied as droplets ~0.8 cm to the side of each EFN or domatium. By using these methods, we created the following treatments: (1) both EFNs and leaf domatia blocked, (2) EFNs blocked and tar control placed near domatia, (3) domatia blocked and tar control placed near EFNs, and (4) tar control placed near EFNs and domatia. Visual inspections made under a dissecting microscope verified the effectiveness of these treatments in blocking nectar flow and domatium openings. All leaves on the plant were manipulated. We censused leaves 3 weeks after imposing the manipulation by removing one leaf per replicate plant, wrapping each leaf individually in a paper towel, and transporting wrapped leaves to the lab in plastic bags placed in a cooler with ice. Mites were counted under a dissecting microscope and preserved in 75% ethyl alcohol.

Because we observed ants visiting *V. dentatum* leaves, we conducted an additional experiment to determine whether the presence of EFNs increased ant abundance on *V. dentatum*, using different plants in the same population. We blocked EFNs on all of the leaves of small plants ( $n = 25$ ) by covering glands with tree-pruning tar, and we created an additional 25 control plants with tar placed near but not blocking the glands. Ant visitation was counted twice per day over the subsequent 3 days. For all experiments, mite and ant numbers were averaged for each

plant and treatments were compared in R, using a Poisson-distributed general linear ANOVA.

## Results

### *Phylogenetic Analyses*

We examined 400 herbarium specimens representing 90 of the ~170 species of *Viburnum* (one to nine specimens per species; mean  $n = 5$ ; appendix). We observed that EFNs were present in 57% of the species included in the phylogeny. When present, EFNs occurred in four distinct locations: on the leaf margin (25% of the 90 species in the phylogeny), on the abaxial leaf lamina (26%), on the petiole (4%), and at the intersection of the petiole and the leaf margin (2%). Leaf domatia were present in 65% of the species sampled. When present, domatia always occurred in the abaxial primary and secondary vein axils. Domatia occurred in three distinct forms: tuft domatia (58% of the 90 species in the phylogeny), which are dense concentrations of trichomes often associated with a depression of the leaf surface; cave domatia (4%), which consist of a flap of leaf tissue extending out over the vein axils, creating a wide opening; and pit domatia (3%), which consist of a cavity covered by a dome raised above the leaf lamina, with a small, pore-like opening. These major positions or types of traits were consistent across multiple collections of the same species, with the exception of *Viburnum odoratissimum*, in which the majority of leaf domatia were pits but where several individual domatia combined pit characteristics with a tuft- or cave-like appearance.

The phylogenetic distribution of the climate trait (fig. 3) is consistent with the interpretation suggested by Clement and Donoghue (2011) that *Viburnum* originally diversified in montane tropical forests in Southeast Asia and then made a number of subsequent movements into temperate forests. This is suggested by the early divergence of the tropical clades and their appearance in a number of cases as sister to temperate radiations (as opposed to their being nested well within temperate lineages). However, solely on the basis of this optimization, it is also possible that *Viburnum* originated in temperate forests and made several movements into tropical habitats. Although additional data are needed to choose between these alternative hypotheses, it is clear in either case that there have been multiple movements between these two habitats. In contrast, the shift into cloud forest habitats occurred within the *Oreinodototinus* clade (the clade that contains *V. dentatum*–*V. blandum* in fig. 3) as it moved south into the mountains of Latin America (Moore and Donoghue 2007, 2009; Clement and Donoghue 2011).

The phylogenetic distribution of the leaf production

character generally mirrors the climate character. Evergreen species are largely tropical, seasonally deciduous species are found mainly in temperate forests, and leaf exchangers (i.e., species that flush and lose their leaves asynchronously) are found in cloud forests. The prime exception is the *Tinus* clade (the clade that contains *V. atrocyaneum*–*V. tinus* in fig. 3): these plants are evergreen despite living mainly in temperate forests. Again, our reconstruction suggests multiple shifts among leaf production categories.

Both the parsimony method and the ML method reconstructed, with high confidence, a single origination of EFNs in *Viburnum* (fig. 4A). Furthermore, the presence of EFNs was significantly conserved despite evolutionary shifts in climate and leaf production strategy (Monte Carlo simulation,  $P < .001$ ). The reconstructions suggested that EFNs originated on the leaf margin along the branch subtending the large *Imbricotinus* clade of Winkworth and Donoghue (2005; Clement and Donoghue 2011; *V. acerifolium*–*V. tinus* in fig. 3). Reconstructions suggest that in the *Opulus* clade (*V. edule*–*V. trilobum* in fig. 3), EFNs migrated to the juncture of the petiole in the *V. edule*–*V. koreanum* clade and entirely onto the distal end of the petiole in the *V. opulus*–*V. sargentii*–*V. trilobum* clade. Separately, our reconstructions implied that EFNs migrated from the marginal position to the leaf lamina in the asyet-unnamed clade (*V. acerifolium*–*V. ternatum* in fig. 3) that includes the *Succodontotinus*, *Lobata*, *Sambucina*, and *Coriacea* clades (Clement and Donoghue 2011). Regarding the position of EFNs on the leaf surface, we found only a single instance of homoplasy: namely, the independent acquisition of petiolar nectaries in the circumboreal *Opulus* clade and in the South American species *V. toronis*.

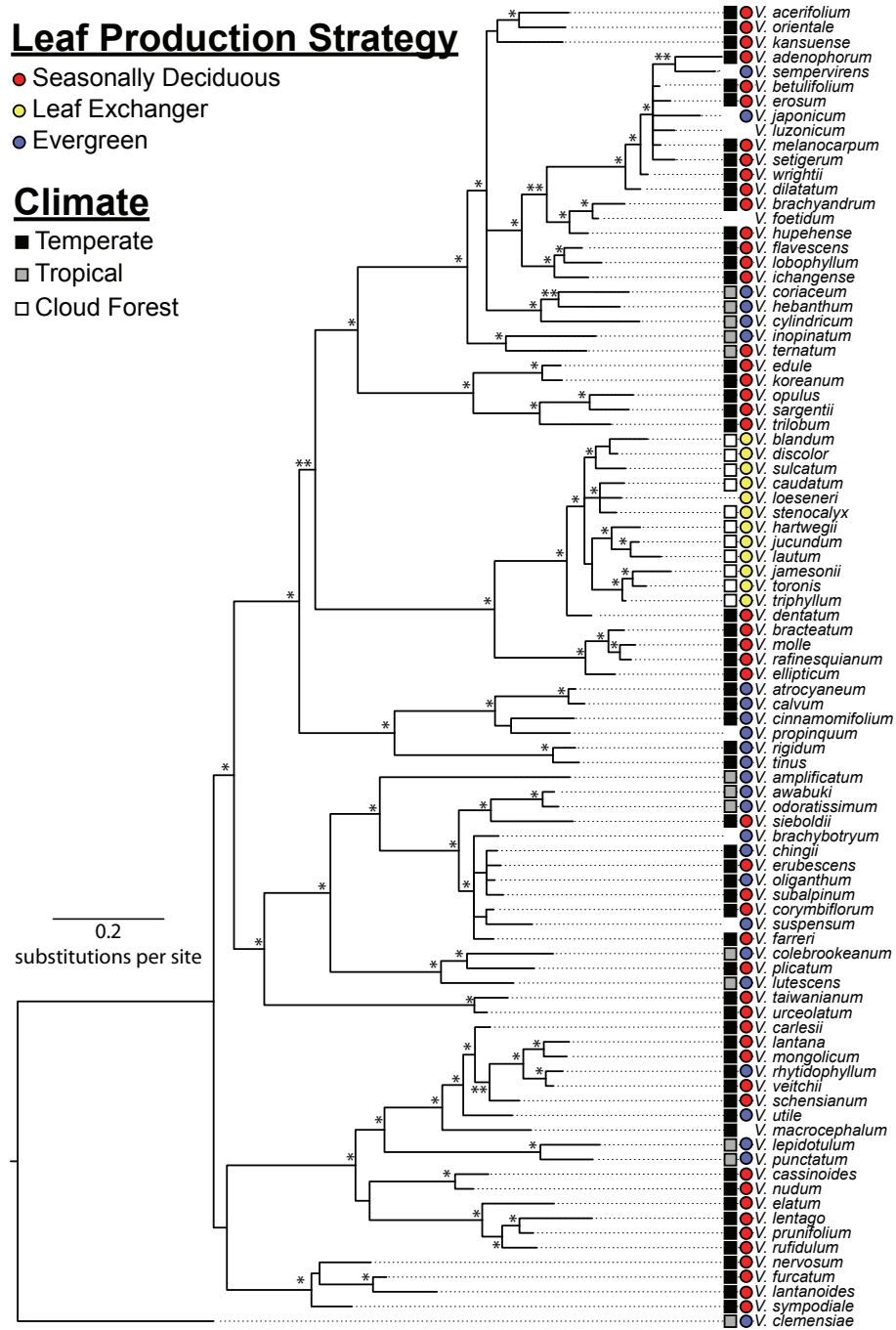
In contrast to EFNs, leaf domatia were more evolutionarily labile, showing considerable homoplasy despite exhibiting phylogenetic signal overall ( $P < .05$ ; fig. 4B). Domatia were evolutionarily correlated with both deciduousness (domatia were more likely to be gained in deciduous than evergreen clades; Pagel's test  $D = 6.07$ ,  $P = .014$ ) and temperate habitats (domatia were more likely to be gained in temperate rather than cloud forest or tropical clades;  $D = 5.93$ ,  $P = .013$ ). When we separated the domatia into the three morphological classes, tuft domatia exhibited these same patterns, being strongly evolutionarily correlated with deciduous ( $D = 10.148$ ,  $P < .001$ ) and temperate habitats ( $D = 6.083$ ,  $P = .018$ ). However, the other forms of domatia deviated from this pattern. Pit domatia, which are present in only three species included in our analyses (*V. clemensiae*, *V. odoratissimum*, *V. awabuki*), originated in two tropical evergreen lineages. Cave domatia, represented by four species in our analyses (*V. mongolicum*, *V. sulcatum*, *V. taiwanianum*, *V. urceolatum*), evolved twice in seasonally deciduous tem-

## Leaf Production Strategy

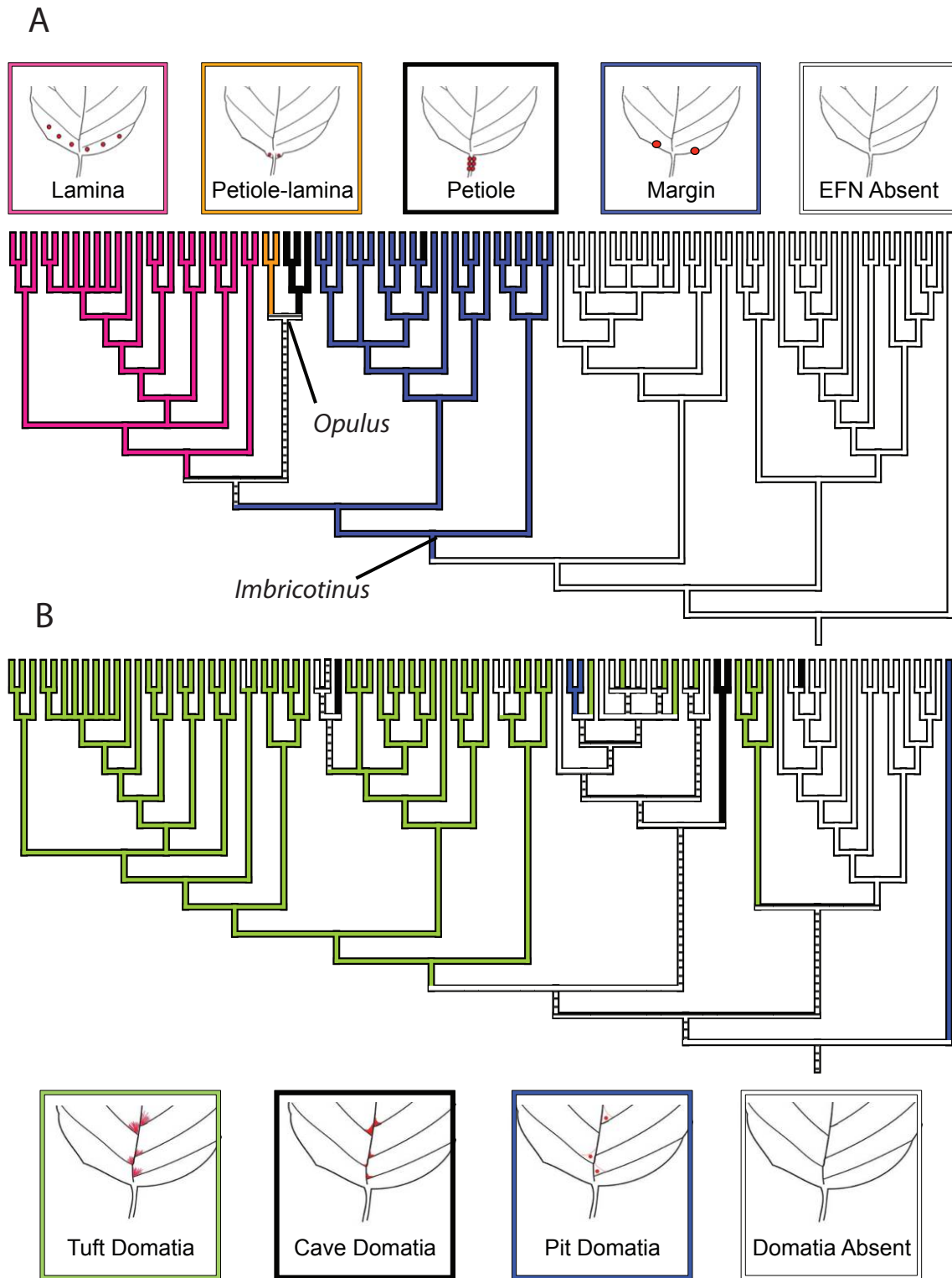
- Seasonally Deciduous
- Leaf Exchanger
- Evergreen

## Climate

- Temperate
- Tropical
- Cloud Forest



**Figure 3:** Bayesian majority rule consensus tree of *Viburnum* based on nine chloroplast genes and nuclear ribosomal internal transcribed spacer sequences, showing the distribution of climate (squares) and leaf production strategy (circles). Branch lengths are drawn proportional to genetic distance. Posterior probabilities greater than .95 or between .9 and .94 are indicated above the branches with an asterisk or two asterisks, respectively. The traits of some species were excluded because of uncertainty in scoring (appendix, available online).



**Figure 4:** Phylogenetic patterns in indirect defensive traits in 90 species of *Viburnum*. Shown are the most parsimonious ancestral character state reconstructions for extrafloral nectaries (EFNs; A) and leaf domatia (B). Red marks on the leaf line drawings indicate the general locations and shapes of the traits.



perate lineages and once in a leaf exchanger cloud forest lineage. Overall, cave domatia evolved independently of climate and leaf production traits (for climate:  $D = 1.93$ ,  $P = .23$ ; for deciduousness:  $D = 1.67$ ,  $P = .35$ ).

Finally, leaf domatia and EFNs were evolutionarily correlated across *Viburnum* ( $D = 2.89$ ,  $P = .032$ ). In particular, the EFN clade (fig. 4A) has significantly more domatia-bearing branches (fig. 4B) than do the several clades in which EFNs are lacking. This result suggests that EFNs and domatia are not ecologically redundant but may instead have an additive or synergistic ecological impact. In order to investigate this hypothesis, we performed common-garden surveys and experimental manipulations investigating the joint and independent impacts of EFNs and leaf domatia on mutualist populations.

#### *Common-Garden and Field Manipulation Results*

In our common-garden survey of *Viburnum* species, mite diversity was largely dominated by the mycophagous family Tydeidae (80.7%), but it also included predatory Phytoseiidae (16.7%), mycophagous and more rarely phytophagous Oribatidae (1.7%), and phytophagous Tetranychidae (0.87%). Multiple mite lineages were sometimes found within the same domatium, but more frequently mites from a single lineage occupied a given domatium. The abundance of mutualistic (mycophagous or predatory) mites on leaves depended on the types of mutualistic traits present (nested ANOVA,  $F_{4,11} = 48.598$ ,  $P < .0001$ ). Leaves that lacked both traits had ~93% fewer mites than leaves with tuft domatia and petiole EFNs, marginal EFNs, or no EFNs (post hoc Tukey HSD tests; fig. 5). However, species with tuft domatia and laminar EFNs had well over twice as many mites than plants of any of the other trait combinations (fig. 5).

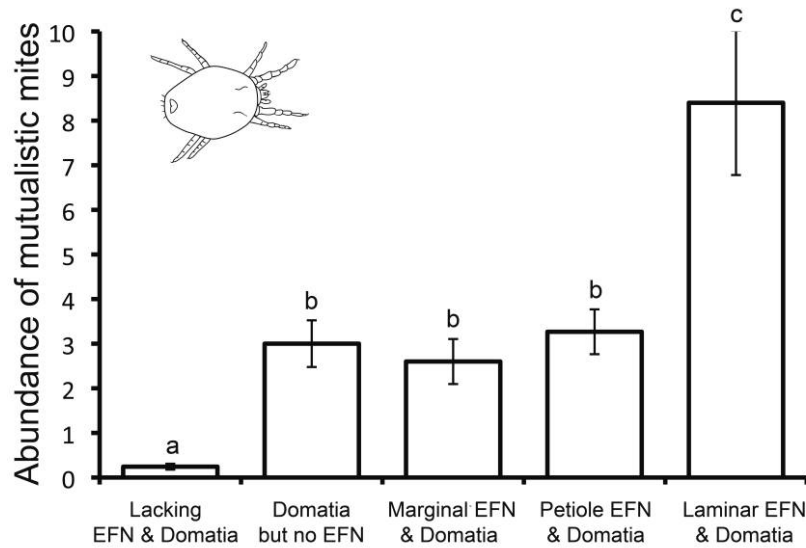
In manipulative experiments with natural populations, mites found on leaves were predominantly of the same mycophagous and predatory families reported above. Considerably more mites were found on the control leaves of *V. acerifolium* (mean, 10.66) than *V. dentatum* (mean, 3.48). For *V. acerifolium*, mite populations decreased additively in the absence of each indirect defensive trait (fig. 6A); blocking either EFNs or leaf domatia decreased mite populations by 47% and 62%, respectively, whereas blocking both traits reduced mite populations by 78%. Ants were not observed on *V. acerifolium*. For *V. dentatum*, mite populations also decreased (by 89%) in the absence of domatia (fig. 6B) but were not affected by the absence of marginal EFNs (fig. 6B). Visitation to *V. dentatum* leaves by ants (*Formica podzolica*) decreased by 57% when EFNs were blocked (inset in fig. 6B; generalized linear ANOVA,  $F_{1,46} = 3.74$ ,  $P = .052$ ).

#### **Discussion**

The study of historical evolutionary processes is inherently challenging, because past events cannot be directly observed or experimentally manipulated. Instead, it is necessary to assess whether multiple lines of evidence are consistent with hypotheses concerning the drivers of historical patterns (for a more detailed discussion of the implications and assumptions of this general approach, see Losos 2009). Here we integrate information on large-scale patterns of character evolution with experimental evidence of trait function to examine and generate hypotheses about the evolutionary ecology of two traits that commonly mediate mutualistic interactions between plants and arthropods (extrafloral nectaries and leaf domatia). We report phylogenetic patterns that are consistent with both the environmental adaptation and trait-interaction hypotheses for the evolutionary drivers of these mutualistic traits. In particular, phylogenetic trait correlations have led to the hypothesis that domatia and EFNs are not ecologically redundant but instead may interact to attract and retain higher abundances of arthropod mutualists to leaves. This hypothesis was further investigated in both a common-garden study and in manipulative field experiments with natural populations: plants with EFNs and leaf domatia attracted more mutualistic arthropods (mites and ants) than did plants without both traits. Together, our data reveal that mutualistic traits in *Viburnum* are not ecologically independent and are consistent with the hypothesis that their long-term evolution is influenced by complex interactions among multiple traits, mutualists, and geography.

#### *Geographic Hypotheses and Trait Conservatism*

Large-scale geographic patterns in trait distributions have led to the hypothesis that mutualistic plant traits are locally adapted to certain regions. From this comes the prediction that specific environmental variables should correlate, at least in part, with trait gains and losses over evolutionary time. Across 90 species of *Viburnum*, we found that leaf domatia were evolutionarily correlated with the climate and leaf production strategy of a species, in support of adaptive hypotheses on the basis of regional survey data (Willson 1991; Walter 1996; O'Dowd and Willson 1997; O'Dowd and Pemberton 1998). Tuft domatia were highly correlated with temperate deciduous clades, whereas pit domatia were associated with tropical evergreen lineages. Pit domatia appear to have originated within at least two widely separated tropical lineages. However, this number is likely an underestimation, because pit domatia are known in several species that have not yet been included in molecular phylogenetic analyses but that are likely to



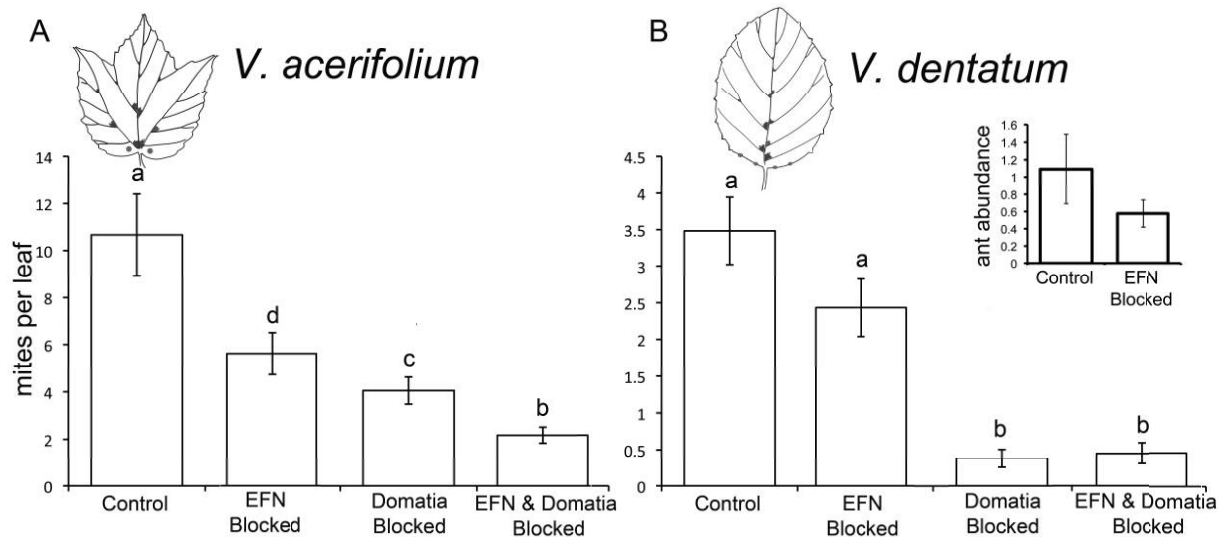
**Figure 5:** Abundance (mean  $\pm$  SE) of mites per leaf on 16 species of *Viburnum* with different combinations of mutualistic plant traits. Different letters represent significant differences ( $P < .05$ ) determined by post hoc Tukey HSD tests. Of the 16 species included, seven lacked both leaf domatia and extrafloral nectaries (EFNs), two lacked EFNs but had tuft domatia, two had laminar EFNs and tuft domatia, two had marginal EFNs and tuft domatia, and three had petiolar EFNs and tuft domatia (see text). The line drawing represents a mycophagous tydeid mite, which was the dominant group in our survey.

belong to additional clades. Specifically, pit domatia have been documented in the Bornean species *V. vernicosum*, which is a member of *Megalotinus* subsection *Sambucina*, and in *V. beccarii* from Peninsular Malaysia, which is thought to be related to members of *Megalotinus* subsection *Coriacea* (Kern 1951; Clement and Donoghue 2011; see fig. 3). Pit domatia have also been reported in *V. glaberrimum*, *V. cornutidens*, and *V. platyphyllum* (Kern 1951; Brouwer and Clifford 1990), which are additional members of subsection *Coriacea* from the Philippines. Together these species likely add at least two additional origins of pit domatia in tropical *Viburnum* clades.

Although regional surveys of woody plant communities have described associations of temperate climates with tuft domatia and tropical climates with pit domatia (Walter 1996), few studies have explicitly attempted to explain these geographic associations, perhaps because of the inconspicuous nature of these traits. However, the convergent patterns in our data support the idea that these traits are adaptive in particular environments. Studies that directly compare the ecology of different forms of domatia are rare, however, and as of yet have lent little insight into the potential selective forces driving this pattern. For example, tuft and pit domatia house similar abundances of mutualistic mites in the forests of Papua New Guinea, North Queensland, and Victoria (O'Dowd and Willson 1989), and tuft and pit domatia have separately been

shown to enhance populations of predatory arthropods (O'Dowd 1994; Agrawal and Karban 1997; O'Dowd and Pemberton 1998), reduce intraguild predation among predatory arthropods (Ferreira et al. 2008), and reduce disease incidence via the enhancement of mycophagous mites (Norton et al. 2000, 2001; Romero and Benson 2005). We expect that more detailed studies will reveal the distinct selective drivers of divergent forms of leaf domatia in different climates. In this context, we note the need to consider other leaf characteristics as potential correlates with domatia evolution. For example, because tuft domatia are constructed of trichomes, the evolution of trichomes elsewhere on leaf surfaces may be relevant.

Adaptive hypotheses concerning the geographic and phylogenetic distribution of EFNs are better developed. In particular, geographic surveys suggest that EFN-bearing species are more abundant at lower latitudes, leading to the hypothesis that EFNs are tropical adaptations whose evolution is driven by increases in ant diversity and herbivore pressure (Bentley 1977; Schupp and Feener 1991; Heil and McKey 2003; Bronstein et al. 2006). However, to our knowledge, no study has tested the relationship between EFNs and tropicality using an explicitly phylogenetic framework, thereby controlling for shared evolutionary history and determining whether latitudinal trends are caused by the high diversity of tropical lineages or repeated evolution. In *Viburnum*, we found that EFNs marked a



**Figure 6:** Abundance (mean  $\pm$  SE) of mites per leaf on *Viburnum acerifolium* (A) and *Viburnum dentatum* (B) in which mutualistic plant traits were factorially blocked. Shaded portions on leaf illustrations mark the positions of EFNs and leaf domatia for each species. Letters represent significant differences ( $P < .05$ ) according to post hoc Tukey HSD tests comparing means among treatments within each species (but not among *V. acerifolium* and *V. dentatum*). The inset in B shows abundance (mean  $\pm$  SE) of ants on *V. dentatum* for which EFNs were blocked and on tar controls (data from a separate experiment).

single large clade and have been retained in all members of this clade despite multiple shifts between different geographic regions and environments (fig. 4A). Furthermore, the largest EFNs in *Viburnum* (located on the petiole in members of the *Opulus* clade) evolved at high latitudes, and in boreal rather than tropical environments. EFNs are also highly conserved in other clades, including *Acacia* (Heil et al. 2004; Gomez-Acevedo et al. 2010), *Gossypium* (Rudgers et al. 2004), *Populus* (Keeler 2008), and *Senna* (Marazzi et al. 2006), yet no study has explicitly addressed whether the origin or persistence of EFNs in these other clades is correlated with tropicality. Indeed, our data suggest that EFN evolution is not necessarily tied to the increased ant diversity and herbivore pressure found in the tropics and that alternative hypotheses are required to explain their phylogenetic distribution.

#### Interactions among Mutualistic Plant Traits

Several hypotheses suggest that mutualistic traits may interact in ways that could potentially influence their evolution. For example, the presence of multiple traits that attract or retain mutualists may be redundant and costly (Oliver et al. 2008), resulting in a negative evolutionary correlation if it is consistent over time. Alternatively, multiple mutualistic traits might interact synergistically or additively (Heil 2008), which could result in positive evolutionary associations. The trade-off and synergism

hypotheses are not mutually exclusive of the geographic hypotheses discussed above, and they can be evaluated by integrating information about a trait's evolutionary history with the results of studies of the ecological effects of the two traits.

We found a positive evolutionary correlation between EFNs and leaf domatia in *Viburnum*, suggesting that these traits may have beneficial ecological effects when found together, rather than being ecologically redundant. The results of our common-garden study and manipulative experiments were consistent with this “bed-and-breakfast” hypothesis in that there was increased abundance of mutualists when both food and housing rewards were present. Specifically, our results revealed that laminar EFNs, which are positioned closer than marginal EFNs to leaf domatia, function with domatia to increase mite populations by offering both food (EFNs) and housing (leaf domatia) rewards to mutualists. However, different morphological positions of EFNs attract different types and abundances of arthropod mutualists, and neither marginal nor petiolar nectaries appear to influence mite abundance (and instead may attract ants).

Although our common-garden and experimental study data are consistent with this interpretation, it should be noted that we obtained these data from only a limited number of *Viburnum* species (common garden,  $n = 16$  species; experimental,  $n = 2$  species). Regardless, these results yield a testable hypothesis for our inferred evolu-

tionary “migration” of EFNs from the leaf margin onto the leaf lamina (hence, closer to the domatia situated in the major vein axils). Further investigation of this hypothesis would require a more direct test of the relationship between mutualist abundance and plant performance in this system, perhaps coupled with measures of natural selection. This is especially important given that the benefit a plant is expected to receive with increasing mutualist abundance is predicted to eventually taper off or decrease rather than necessarily continuously increase (Holland et al. 2002; Morris et al. 2010). Nonetheless, we note that quantitatively similar changes in the abundance of the dominant mite family found in our study (Tydeidae) have been demonstrated to negatively impact powdery mildew growth on the leaves of riverbank grape in domatia-blocking experiments in central New York. In these experiments, increased mite abundances of up to 25 mites per leaf translated into steep decreases in leaf mildew, and after this point there was little further benefit (Norton et al. 2000). Thus, it is reasonable to expect that the range of mite numbers found in our study (figs. 5, 6) fall well within the range of increasing benefits with increasing mutualist abundance.

We speculate that a positive coupling of housing and food (“bed-and-breakfast”) rewards may be more widespread than previously believed, perhaps especially in northern temperate ecosystems. Similar to leaf domatia, sugar-secreting laminar EFNs are common but remarkably inconspicuous, and they seem to have escaped the attention of many botanists. It is noteworthy, for example, that they have been largely overlooked in the taxonomic literature on *Viburnum*, despite their presence in 37 of the species examined here. Indeed, on the basis of a combination of the latest phylogenetic and taxonomic information, we estimate that some form of EFN is present in 107 (~63%) of the ~170 species of *Viburnum*. Additionally, mite visits to EFNs have been sporadically recorded in the literature for over a decade (Walter 1996; van Rijn and Tanigoshi 1999), and the presence of both EFNs and tuft domatia have been separately shown to increase mutualistic mite abundance on *Viburnum tinus* leaves (Grostal and O’Dowd 1994; Walter and O’Dowd 1995). A formal survey estimating the number of plant species bearing both EFNs and mite domatia has not been conducted, but several well-known and broadly dispersed genera include species with both traits, such as *Prunus* and *Populus* (Brouwer and Clifford 1990; Keeler 2008). However, a replicated study examining multiple lineages that have laminar EFNs and tuft domatia is ultimately needed to evaluate the generality of the bed-and-breakfast hypothesis and, more specifically, the influences of the relative positions and types of EFNs and domatia on the functioning of the entire system.

### Integrating Experimental and Phylogenetic Comparative Methods

Despite repeated calls for the integration of historical and experimental approaches (Brooks and McLennan 1991; Losos 1996; Weber and Agrawal 2012), phylogenetic comparative studies of macroevolutionary patterns and ecological studies that use extant taxa are still typically conducted in isolation. In part, this is due to the challenges associated with jointly interpreting historical and contemporary results given that biological systems change over time. However, integrating multiple lines of evidence ultimately leads to a reciprocally informative process through which hypotheses continue to be evaluated and refined with the addition of multiple types of information (Losos 1996). Strong support for a causal hypothesis is obtained when these disparate types of information are consistent. Nonetheless, a lack of consistency between historical and contemporary results is also revealing, because it suggests that the hypothesized process is not currently at play in the contemporary system being studied. In this way, conflicting results can clarify the original causal hypothesis by initiating investigations into how current and historical populations differ or by facilitating the formulation of alternative hypotheses.

By integrating phylogenetic and experimental methods, we were able to generate and test complex hypotheses about the forces influencing the long-term evolutionary dynamics of two ecologically important plant traits. We found evidence for bed-and-breakfast interactions over a backdrop of evolutionary conservatism in EFNs and strong associations between the presence of domatia and both climate and leaf production strategy. Ultimately, our study supports roles for trait conservatism, adaptation to environmental factors, and interactions among plant mutualistic characters as the drivers of the evolution of mutualistic traits in *Viburnum*. Our finding that EFNs and domatia are phylogenetically correlated is concordant with experimental data demonstrating that these traits are not ecologically redundant but instead work in concert to attract and retain arthropod mutualists. Importantly, our experiments reciprocally inform phylogenetic studies, suggesting a possible (and testable) causal explanation for the evolutionary shifts that we have documented in the position of EFNs.

### Acknowledgments

We thank S. Cook, E. Edwards, A. Erwin, M. Geber, B. Gould, H. Greene, G. Loeb, I. Lovette, and I. Singh for valuable discussion and comments on this manuscript; A. Stalter and P. Sweeney for help with accessing specimens;

G. Fewless and P. Sweeney for photographs; C. Bliss and G. Desurmont for help in the field; S. Colby, S. Hesler, G. Loeb, and K. Wentworth for help with mite identification; and the Cornell Plantations Natural Areas for access to the Polson Preserve. The common garden used in this study was originally established by Paul Weston. M.G.W. was funded by a Sigma Xi Grant in Aid of Research, the SSE Rosemary Grant Award, a Kieker Fellowship Research Grant, and a National Science Foundation (NSF) Graduate Research Fellowship. This work was also supported by NSF grants DEB-1118783 (to A.A.A.) and IOS-0842800 (to M.J.D.).

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Associate Editor: Elizabeth Elle  
Editor: Judith L. Bronstein



An ant (bodyguard) drinking from an extrafloral nectary of a *Viburnum*. Photograph by Gaylord Desurmont.