THE EVOLUTION OF MUTUALISTIC DEFENSE TRAITS IN PLANTS

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Marjorie Gail Weber

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THE EVOLUTION OF MUTUALISTIC DEFENSE TRAITS IN PLANTS

Marjorie Gail Weber, Ph. D.

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Plant traits that mediate mutualistic interactions as a mode of defense are pervasive, have originated independently many times within angiosperms, and are highly variable across taxa. My dissertation research examines the evolutionary ecology of two common plant traits that mediate defense mutualisms in plants: extrafloral nectaries (EFNs), plant organs that secrete small volumes of nectar, thereby attracting predacious arthropods to leaves, and (2) leaf domatia, small structures on the undersides of leaves that provide housing for predacious or fungivorous mites. Because traits like EFNs and domatia influence multiple trophic levels, their evolution can have strong impacts on community dynamics relative to other plant characters. Nonetheless, studies that directly link the ecological effects of these traits with their evolutionary dynamics are rare.

BIOGRAPHICAL SKETCH

Marjorie Weber was born in Grosse Pointe, Michigan. She received a BA in Biology from Lewis and Clark College in 2007.

Dedicated to my family, friends, and to Gideon

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Abstract

Recent progress in the development of phylogenetic comparative methods and access to molecular phylogenies has made the joint use of comparative analyses and manipulative experiments more accessible and revealing than ever before. Here, we provide a roadmap for linking comparative phylogenetic patterns with ecological experiments to test hypotheses across ecological and evolutionary scales. As examples, we consider five cornerstones of ecological and evolutionary research: tests of adaptation, tradeoffs and synergisms among traits, coevolution due to species interactions, trait influences on lineage diversification, and community assembly and composition. Although several scenarios can result in a lack of concordance between historical patterns and contemporary experiments, we argue that coupling phylogenetic and experimental methods is an increasingly revealing approach to hypothesis testing in evolutionary ecology.

Key words: community ecology; comparative biology; coevolution and cospeciation; convergence; correlated evolution; key innovation; phylogenetic field experiment;

phylogenetic signal; species coexistence; adaptive radiation

Why integrate phylogenies and experiments?

The joint application of comparative phylogenetic and experimental methods has been advocated since at least the 1990's as a way to generate and evaluate causal hypotheses in evolutionary ecology (e.g., (Brooks and McLennan 1991; Losos 1996; McLennan 1991)). Merging of these two approaches can be particularly revealing due to the complimentary insights they provide: phylogenetic comparative methods allow for the identification of broad scale patterns across many taxa over long periods of time, while experimental manipulations allow for tests of mechanistic hypotheses implicated in driving those patterns. Furthermore, the joint use of phylogenetic and experimental methods can address common interpretational drawbacks of using one method alone. For example, when used in isolation, comparative phylogenetic studies stop short of rigorously evaluating the ecological mechanisms suggested by their results. Conversely, experimental results can be interpreted as general patterns without being placed in a broader evolutionary framework. We revisit the call for integration of comparative phylogenetics and experiments, and discuss the potential of this merger to facilitate novel links between historical patterns and ecological processes given the significant methodological leaps made over the last decade. Rather than broadly survey across all empirical and comparative approaches, we focus specifically on the integration of manipulative ecological experiments (used to investigate causal arguments) with recently developed phylogenetic comparative analyses (that allow for historical inference), as their joint use represents a growing frontier in evolutionary

ecology and has not been extensively dealt with in a previous review.

We begin with a general conceptual framework for integrating manipulative experiments with comparative phylogenetics. However, because the methods, benefits, and challenges associated with coupling these approaches change with the hypothesis being addressed, we consider five major areas in evolutionary ecology: tests of adaptation, trait tradeoffs and synergisms, coevolution and cospeciation, trait influences on lineage diversification, and ecological community structure.

A conceptual roadmap for integrative, reciprocal hypothesis testing

When used in isolation, phylogenetic and experimental approaches can each generate hypotheses that are then testable using the alternative approach (Figure 1) (e.g., (Brooks and McLennan 1991; Jackson et al. 2002; Losos 1996; Scheiner 2010)). For example, phylogenetic patterns can suggest the existence of a causal mechanism (i.e., selection) that can then be investigated using manipulative experiments on contemporary populations (Figure 1A). A phylogenetic framework can also help researchers design these experiments, allowing for powerful, evolutionarily replicated tests (Figure 1B, Box 1). Similarly, experimental results can generate evolutionary hypotheses that are testable using phylogenetic comparative methods (Figure 1C-D), such as when a process is hypothesized to result in a specific macroevolutionary pattern.

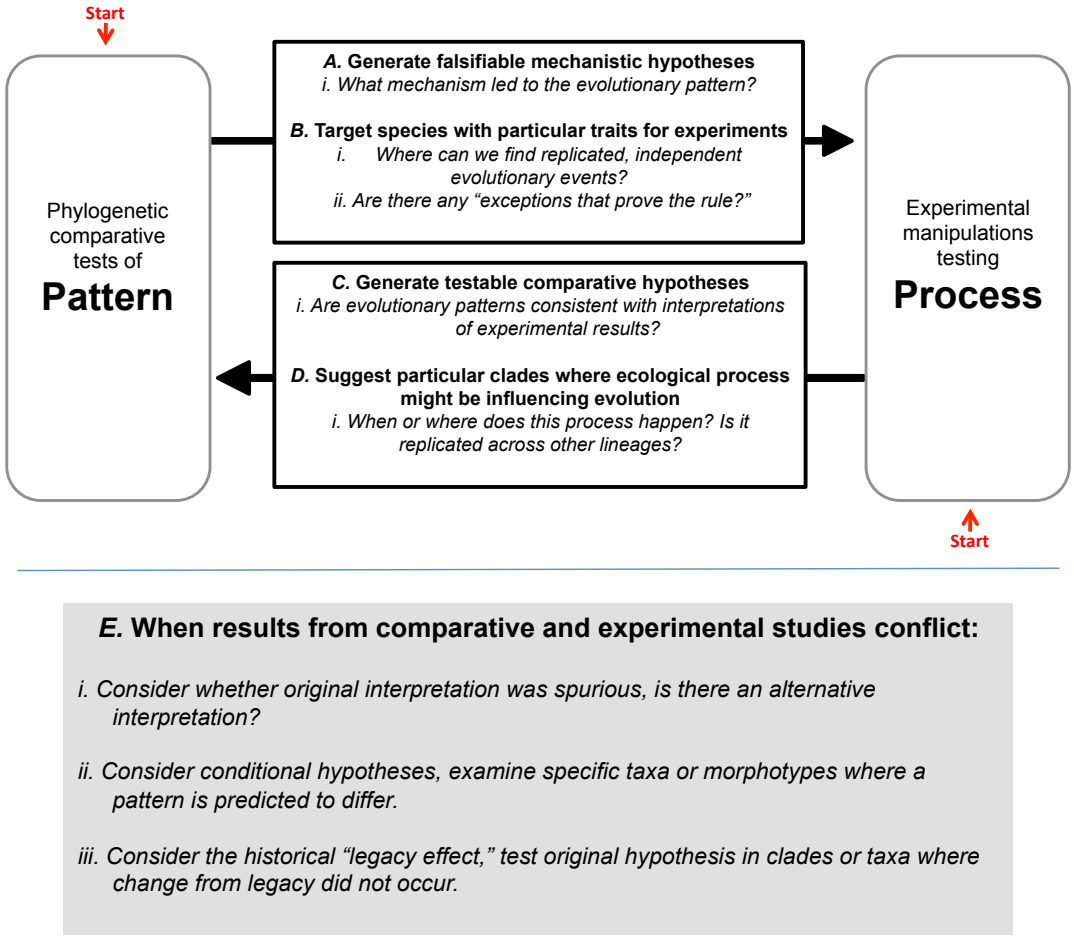

Figure 1: A schematic describing the iterative hypothesis testing using phylogenetic patterns and experiments in evolutionary ecology. Whether one begins with tests of pattern or process, results can lead to studies of the other type.

Despite the benefits of integrating approaches, reconciling results from contemporary and historical studies can present logistical and interpretational challenges. For example, comparing experimental results from multiple species within a clade often involves a common garden design, which can lead to variable results simply because species are removed from the ecological context in which they evolved. Furthermore, there can be interpretational issues when experimental and comparative results conflict (Figure 1E), as conflicting results can reflect a true

rejection of the original hypothesis, or can be the result of changes in the strength or direction of key forces over space or time (the "legacy effect", (Losos 1996)). Indeed, many factors that potentially influence population dynamics shift over time or space, such as species' ranges, habitat types, ecological interactions, and genotypic makeup. Regardless, the rejection of a historically derived hypothesis using current populations remains informative, as it suggests that the hypothesized process is not at play in the contemporary system. In this way, lack of concordance between historical and contemporary data can clarify a causal hypothesis, thereby allowing researchers to ask how current and historical populations differ or to formulate alternative hypotheses that better explain previous results (e.g., (Edwards and Smith 2010)).

Tests of adaptation through trait-environment associations

A great deal of research in evolutionary ecology is focused on identifying the adaptive value of traits under different conditions. Adaptive hypotheses are commonly investigated using a phylogenetic framework, whereby researchers ask whether traits and environments are evolutionarily correlated across a phylogeny. Indeed, comparative methods for identifying phylogenetic patterns consistent with adaptation have become increasingly rigorous and accessible over the last decade. Models of character evolution are becoming increasingly sophisticated, and are now easily implemented using open access statistical programs (e.g., (R Development Core Team 2012)). Bayesian and maximum likelihood methods are available to evaluate the fit of phylogenetic, character and habitat data to models of character evolution where, for example, traits and habitat are non-independent (Pagel 1994; Pagel and Meade 2006),

where traits are evolving according to different selective optima in different environments (Beaulieu et al. 2012; Butler and King 2004), or where rates of phenotypic evolution differ among clades in a phylogeny (e.g., (Revell et al. 2012; Slater et al. 2012)). These methods are being increasingly applied to a broad range of traits and taxonomic groups. Nonetheless, while phylogenetic patterns can be consistent with an adaptive signature, they do not adequately address causal hypotheses on their own, as they fail to evaluate the role of implicated selective agents. Pairing these studies with experiments that clarify the costs and benefits of traits in different environments can shed light on adaptive interpretations of phylogenetic patterns. Yet, if these cost-benefit experiments were presented in isolation, they could not be generalized or interpreted in a historical context (for examples of experiments that were intepreted differently using a historical framework, see (Autumn et al. 2002)).

Consider crypsis, for example, which is generally hypothesized to be an adaptation to avoid predation. In *Timema* walking sticks, a dorsal stripe is hypothesized to confer crypsis for insects on plants with needle-like leaves (Sandoval and Crespi 2008) (Figure 2A). Phylogenetic comparative analyses of trait evolution across *Timema* were consistent with this adaptive hypothesis, revealing that the origin of dorsal stripes was evolutionarily correlated with shifts onto plants with needle-like leaves (Figure 2C). However, experimental manipulations using extant walking sticks were ultimately needed to evaluate whether crypsis (the proposed mechanism) was present and likely driving the trait-environment association. Experimental comparisons of predation rates in closely related striped and non-striped *Timema* were

conducted, confirming that dorsal stripes confer crypsis and protection from predators. These studies support the interpretation of the phylogenetic pattern (Sandoval 1994) (Figure 2B), thus providing a clear example of how the integration of comparative and experimental approaches can reinforce an otherwise speculative adaptive hypothesis (for other examples, see (Castellanos et al. 2004; Losos et al. 2001; McPeek 1996; Wilson et al. 2004)).

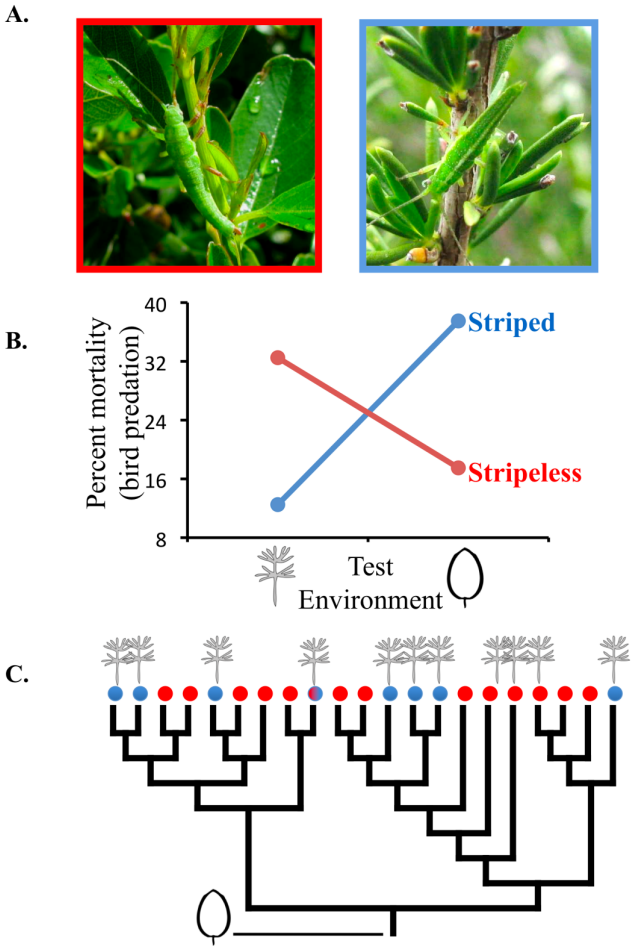

Figure 2. Dorsal stripe morphology as an adaptation to predation in *Timema* walking sticks. (A) Two morphs of *T. cristinae* on their respective host plants: striped (blue outline) and stripeless (red outline) (photos by Aaron Comeault). (B) A manipulative ecological experiment measuring selection on individuals with and without stripes in both habitats (Sandoval 1994). In the presence of bird predators, the striped morph has higher fitness on plants with needle-like leaves, whereas the stripeless morph has

higher fitness on broad leaves. Fitness of the morphs is equivalent on the two plant types in the absence of predators (Nosil 2007). (C) To test for the broad scale consequences of such divergent selection, a phylogenetic study of all 21 species of *Timema* was conducted. *Timema* walking sticks evolved a dorsal stripe 5 or 6 times independently, each time associated with a shift to needle-like leaves. Species with dorsal stripes are marked with a blue dot, while those without stripes are marked in red. Use of broad leaf host plants is ancestral, with repeated host-shifts to plants with needle-like leaves associated with the evolution of a dorsal stripe. Species with members that shifted to needle-like leaves are marked with grey plants above tips (bottom panel redrawn from (Sandoval and Crespi 2008)).

Experimental tests of adaptive hypotheses often take the form of reciprocal transplants or manipulations using closely related but divergent species pairs. For example, Fine *et al.* (2004; 2006) conducted reciprocal transplants using ten pairs of closely related Amazonian tree species that differed in their soil habitat (nutrient-poor sand versus nutrient-rich clay) to test the hypothesis that plant defensive strategy is adapted to resource availability. Plant species repeatedly differentiated in their chemistry over evolutionary time, and species from low nutrient soils had greater levels of defensive chemicals than species from high nutrient habitats (Fine et al. 2004; Fine et al. 2006). In this case, however, the two environments differed along both biotic (herbivore) and abiotic (resource) axes and neither the trait-environment correlation nor the transplant experiment was sufficient to determine the ecological basis of trait differentiation. Experimental manipulations were ultimately needed, and confirmed that plants performed equally well in both habitats in the absence of herbivores, implicating habitat specific herbivory, rather than resource availability *per se*, as the mechanism driving the relationship, a conclusion that was unreachable in the absence of integration.

Maladaptation and invasion success

With *a priori* information from both approaches, one can design studies that merge comparative approaches and manipulative experiments to test a targeted adaptive hypothesis. For example, *Viburnum* spp. shrubs in Europe evolved with a damaging beetle herbivore, while North American species have, until recently, lived beetle-free. Consistent with an adaptive hypothesis, a phylogenetic field experiment on sixteen species of *Viburnum* demonstrated that North American species have consistently (and convergently) lower defenses against beetles than their non-native congeners (Desurmont et al. 2011). Experiments further confirmed that lower plant defenses were critical for the success of the invasive insect pest, resulting in North American species being more susceptible than species from the insect's native range. Thus, the integration of historical patterns with ecological experiments revealed how a trait-environment mismatch can cause the proliferation of pests, potentially driving a species invasion.

Tradeoffs, synergisms and trait interactions over time

Many hypotheses in ecology and evolutionary biology address covariation among multiple traits. For example, life-history theory predicts that progeny size and number should tradeoff due to allocation of limiting resources (Messina and Fox 2001). Other traits are predicted to show negative correlations for adaptive reasons: when one trait is employed, the other is disfavored by natural selection (Agrawal et al. 2010). By contrast, when two traits function additively or synergistically, we expect natural selection to favor their correlated evolution. Evolutionary changes in one trait

are also sometimes predicted to be dependent on changes in another trait. For example, the evolution of gregariousness in caterpillars was hypothesized to originate after warning coloration, because gregariousness is only thought to be advantageous in visibly non-palatable animals (Sillen-Tullberg 1988). Such sequential events are also important when one trait is hypothesized to evolve by modification of another ecologically relevant trait (e.g., (Armbruster et al. 1997)).

Comparative studies of trait-trait interactions frequently employ phylogenetic methods to test whether traits are evolutionarily correlated. Indeed, new methods have recently been developed to test for phylogenetic patterns that are consistent with evolutionary non-independence of multiple traits (e.g., (Butler and King 2004; Hansen et al. 2008; Revell and Collar 2009)). In general, the same phylogenetic comparative methods used to evaluate adaptive signatures (discussed above) are used to identify patterns consistent with trait-trait correlations. Despite the increased sophistication of these methods, however, interpreting evolutionary correlations on their own is exceedingly difficult, as correlated evolution can be caused by several different processes, including selection for particular trait combinations as well as genetic or developmental constraints (Armbruster and Schwaegerle 1996; Harvey and Pagel 1991). Coupling phylogenetic comparative results with experiments testing whether various trait pairings differentially influence fitness can distinguish between these scenarios. For example, to address why pollination and defense traits frequently show correlated evolution in plants, Herrera et al. (2002) experimentally asked whether individuals possessing particular combinations of these traits had higher fitness than plants with other trait combinations. They found repeatable non-additive fitness effects

of the traits, consistent with the interpretation that selection is driving correlated evolution (for another example, see (Armbruster 2002)).

Many ecological studies experimentally demonstrate how multiple traits function together in particular populations, but fail to test whether these ecological interactions are persistent or powerful enough to influence long-term evolution. Phenotypic or genetic (e.g., gene silencing) manipulations of multiple traits using a full-factorial design are particularly powerful because the statistical interaction term (in analysis of variance) indicates whether traits have an additive or non-additive effect. When traits have their greatest ecological impact together, and if these interactions affect individual fitness, they can evolve in a positively correlated fashion, a hypothesis that is testable using the phylogenetic comparative methods cited above. For example, by merging experimental and phylogenetic tests, we found that plant traits providing food and housing rewards to arthropod bodyguards exhibit complementary ecological effects and were evolutionarily correlated, consistent with the hypothesis that the ecological benefit of having both types of traits drives their evolutionary overlap (Weber et al. *in revision*).

Using exceptions to prove the rule

A novel approach to studying ecological and evolutionary trait interactions is to generate hypotheses based on cases where the evolution of two traits which frequently evolve together has become decoupled (Figure 3). Lineages that deviate from correlated evolution can then be targeted in experiments that test hypotheses about the cause of the original correlation. Arnqvist and Rowe (2002) used this

approach in water striders to evaluate whether correlations in male and female secondary sexual morphology were driven by an evolutionary arms race between the sexes. They compared comparative phylogenetic results with outcomes of experimental matings, and found that species whose traits deviate from correlated evolution also had imbalanced behavioral interactions (with one sex having the behavioral upper hand), thereby supporting the evolutionary arms race hypothesis. Examples such as this, which creatively make use of exceptions to, or deviations from, evolutionary patterns to design rigorous experimental tests, are remarkably rare and yet hold tremendous potential for progress in evolutionary ecology. We predict this approach will prove particularly promising given recent increases in access to large online organismal trait databases (e.g., The Worldwide Leaf Economics Spectrum (Wright et al. 2004), TRY- A Global Database of Plant Traits (Kattge et al. 2011)).

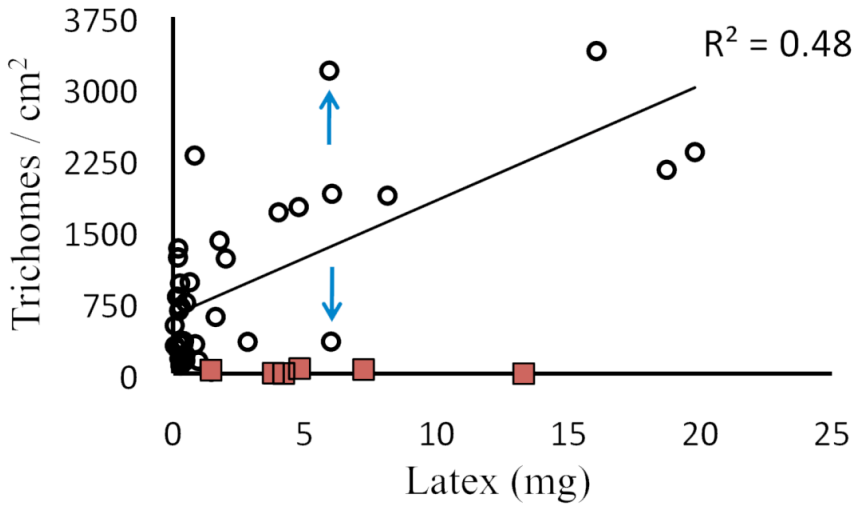

Figure 3. Deviations from correlated evolution can decouple traits that are hypothesized to intact ecologically (Arnqvist and Rowe 2002), suggesting species to target in future experimental studies. An example of correlated evolution between foliar trichome densities and latex production (mg of latex exuded when cut) across 44

species of milkweeds (*Asclepias* spp.) (data from (Agrawal et al. 2009)). If an ecological process is hypothesized to drive an evolutionary correlation between two traits, then species or lineages that deviate from that correlation are also expected to deviate ecologically. Note that six species (in red) are excluded from the regression; although these species have few trichomes, they are the only species with leaf surface wax crystals, leading to the hypothesis that wax crystals function ecophysiologically as trichomes (Agrawal et al. 2009), which was tested using experiments. In addition, blue arrows indicate potential target species for ecological experiments due to their deviation from the overall pattern.

Coevolution and cospeciation

Coevolution (i.e., reciprocal evolution between species leading to diversification) has long been suspected in systems where species interact with high specificity (Ehrlich and Raven 1964). Generally speaking, specialized species interactions are hypothesized to result in coevolution when they increase the ecological or geographic structure of populations, thereby promoting differentiation (Schluter 2000). For example, interactions between South Hills crossbills and Rocky Mountain lodgepole pine were hypothesized to result in specialized beak morphology and vocalization (Edelaar and Benkman 2006), which in turn promoted assortative mating and nearly complete reproductive isolation between bird populations (Smith and Benkman 2007). Coevolution is a provocative hypothesis, but it is exceedingly difficult to test whether specific interactions were important in the evolution of diversity. However, we gain confidence in coevolutionary claims when patterns are presented alongside experimental evidence of ecological factors implicated in driving coevolution, such as specialization and differentiation.

Traditionally, parallel phylogenies have been used to identify potential cases of coevolution. Parallel phylogenies represent highly specific associations between

clades of organisms, with early diverging species in one clade associating with basal species of the other clade, and progressively derived taxa similarly sharing an association (resulting in a pattern where, when held side by side, phylogenies appear as mirror images). Indeed, groups such as vertebrate hosts and their parasitic lice (Figure 4) (e.g., (Clayton et al. 2003)), plants and their pollinating seed parasites(e.g., (Smith et al. 2008)), and metabolically codependent symbionts (e.g., (Clark et al. 2000)) have parallel phylogenies. Nonetheless, this pattern, termed cospeciation, is also caused by joint vicariance of both groups, and ecological interactions between the species need not be invoked. In other words, species can have parallel phylogenies because they have similar biogeographical histories (due to habitat sharing), rather than because of specific coevolutionary interactions (e.g., yuccas and yucca moths (Althoff et al. 2012)).

Other aspects of phylogenetic congruence can also be useful in inferring whether ecological interactions drove cospeciation. First, time-calibrated phylogenies can be used to elucidate the temporal sequence of divergences, potentially ruling out coevolution. For example, in associations between leaf-cutter ants and lepiotaceous fungi, fungal lineages diverged well before ants, with ants acquiring fungi relatively recently (Mikheyev et al. 2010). Thus, ants might be evolutionarily tracking fungi or fungi might have subsequently spread through the ant lineages, but no reciprocal interaction was likely involved in cospeciation. This approach holds particular promise, as methods for calibrating phylogenies using fossil or geographic data have greatly improved in the last decade, and it is now possible to estimate calibrated phylogenies incorporating uncertainty in both node dating and tree topology using

Bayesian inference (Drummond and Rambaut 2007). Furthermore, patterns of congruence between phylogenies generally involve some element of incongruence, which itself can be informative. For example, partner switching could pinpoint instances where species deviated from an overall pattern of coevolution and inform further investigations into the drivers of host shifts. However, while multiple lines of phylogenetic evidence can be consistent with coevolutionary hypotheses, integration with ecological information is required for researchers to implicate ecological factors as drivers of coevolutonary patterns.

Experimental studies can be a powerful tool for testing coevolutionary interpretations of phylogenetic patterns (see examples in, (Thompson 2005)). For example, if coevolving lineages are hypothesized to cause phenotypic matching between interacting species (e.g., the correspondence in body size between birds and their louse parasites, (Clayton et al. 2003)) (Figure 4), then reciprocal transplant experiments can confirm that host switching is indeed limited by matched traits. For birds and lice, both body size and defensive preening behavior were shown to be important in maintaining specificity (Clayton et al. 2003) (Figure 4B and 4C). Additionally, if coevolution is occurring, tradeoffs in fitness are expected when specialized species interact with close relatives of their usual host. Experimental evidence of these tradeoffs (e.g., host use in parasites, reward collection in mutualists, or the ability to resist competitors) is also consistent with specialization contributing to divergence. Although experiments do not necessarily imply that the interactions contributed to divergence *per se*, they can rigorously evaluate whether other lines of evidence are consistent with the hypothesis that a pattern of cospeciation is directly

influenced by the ecological interaction and that phenotypes constrain the range of available partners (for other examples, see (Koskella and Lively 2007; Miller and Pitnick 2002; Toju and Sota 2006)).

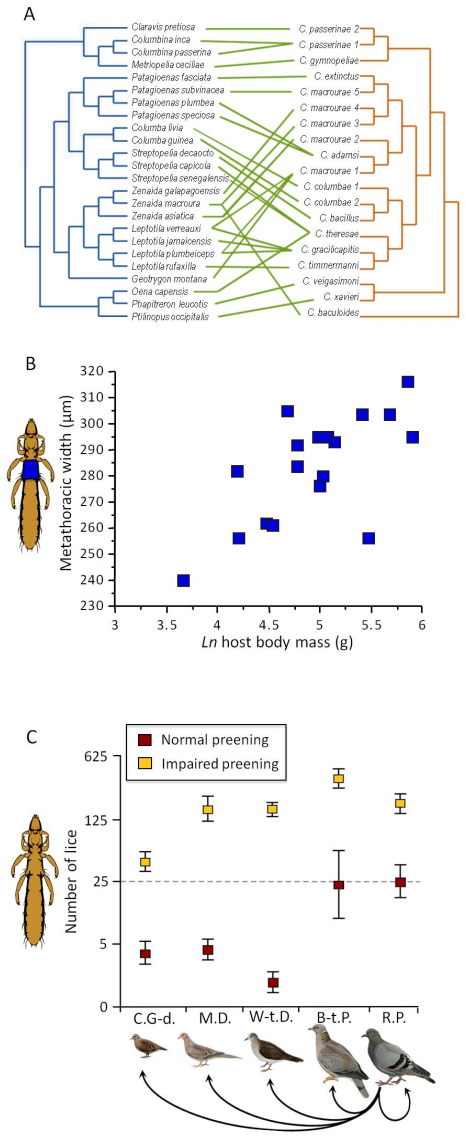

Figure 4. Merging of phylogenetic and experimental approaches in the study of host-parasite macroevolution. Despite being able to feed and proliferate on all birds, feather lice are preened from birds that are unmatched for size, suggesting that host defense reinforces cospeciation by preventing host switches. A) Phylogenies of Columbiform birds and their feather lice in the genus *Columbicola,* showing a pattern of

cospeciation. Green lines show host–parasite associations. B) Host and parasite body size show correlated evolution, suggesting that physical constraints might be a driver of cospeciation. C) Population sizes (mean ± SE) of lice (*C. columbae*) transferred to novel host species. Dotted line represents the native host (Rock Pigeon, R.P., *Columba livia*). Host abbreviations: C.G-d., Common Ground-dove (*Columbina passerina*), M.D., Mourning Dove (*Zenaida macroura*), W-t.D., White-tipped Dove (*Leptotila verreauxi*), B-t.P., Band-tailed Pigeon (*Patagioenas fasciata*). Modified from (Clayton et al. 2003) courtesy of the authors.

Trait influences on lineage diversification

Organismal traits are frequently implicated in influencing the species richness of particular clades, either positively (in the case of key innovations or adaptive radiations) or negatively (in the case of evolutionary dead-ends). Examples of these traits range from nectar spurs and self-fertilization in plants (Hodges 1997; Takebayashi and Morrell 2001) to incisor growth in rodents (Wilson 1951). Recently, a surge of powerful comparative phylogenetic methods have been developed to address whether a given trait is associated with changes in clade diversification rates, such as model fitting and comparison approaches that utilize maximum likelihood and Bayesian methods to evaluate if and where on a phylogeny diversification rates might have shifted (FitzJohn 2010; Moore and Donoghue 2009; Rabosky 2006). However, while these methods can test for evolutionary patterns consistent with hypotheses linking traits with diversification rate shifts, they do not evaluate causation or address hypothesized mechanisms (e.g., (Armbruster and Muchhala 2009)) (Box 1). Thus, experimental manipulations are ultimately needed to evaluate whether specific causal relationships are present (Donoghue 2005).

Traits are hypothesized to influence diversification rates via mechanisms such as changes in reproductive or ecological specialization, changes in population density,

and escape from competition via invasion into new adaptive zones (1995). Each of these mechanisms are testable using experiments under the right conditions; nonetheless, long generation times or slow rates of evolution can pose substantial logistical challenges to applying experimentation in this way (Barraclough et al. 1998). In some cases, empirical tests of mechanistic hypotheses linking traits to species diversity have been pursued, and several traits (such as nectar spurs (Fulton and Hodges 1999; Hodges and Arnold 1995), sexual dichromatism (Barraclough et al. 1995; Uy et al. 2009), and viviparity (Schrader and Travis 2008; Slowinski and Guyer 1993)) have been evaluated using a combination of both phylogenetic and experimental methods.

For traits not involved in mate choice and reproduction, the links between key innovations and mechanisms of population differentiation are less clear and rarely experimentally explored. For example, plant defense theory led Farrell *et al.* (Farrell et al. 1991) to hypothesize that defensive canals (carrying latex or resin) promoted increased speciation rates in plants. The hypothesized mechanism for this association was that latex decreased herbivore pressure, which in turn allowed for larger population sizes and lower risk of extinction. To evaluate this hypothesis, Farrell *et al.* tested for a macroevolutionary association between defensive canals and increased diversification rates using sister clade comparisons, and found that in thirteen out of sixteen plant lineages, clades with canals had more species than their sister clades without canals. However, a number of ecological and evolutionary processes could account for this pattern (Heard and Hauser 1995; McPeek 1996; Rabosky 2009), and thus targeted experiments are needed to test whether additional evidence supports the

proposed ecological mechanisms. Experimental manipulations could address whether the presence of latex does decrease herbivore pressure, and whether decreasing herbivore pressure alters population sizes or diversification, key steps that are necessary if Farrell *et al's* hypothesis is correct. These tests could include intraspecific selection experiments or experiments that follow population-level impacts of a transgenic trait manipulation. Although studies have linked the production of latex to reduced herbivory and plant fitness (Agrawal 2005), little work has focused on the putative link between latex and increased population density or rates of genetic differentiation.

Diversifying approaches to studying diversification

Indeed, many traits implicated in influencing clade diversification rates still remain to be investigated using both experimental and modern comparative methods. For example, altered beak morphology promotes assortative mating and reproductive isolation in some bird populations (Smith and Benkman 2007), and associative learning influences the genetic differentiation of apple maggot flies (Feder et al. 1994), both of which could increase diversification rates. Phylogenetic tests could be used to address whether these traits are associated with altered diversification rates. Alternatively, population variation can be used to elucidate whether a trait is associated with varied population structure, size, or geographic range (e.g., (Wagner and McCune 2009)). Coupling this approach with transplant experiments could demonstrate the importance of a trait in colonization, establishment, and success in novel environments, moving beyond the realm of correlates as evidence for

diversification hypotheses.

Phylogenetic structure in ecological communities

Understanding the assembly and subsequent structure of communities has been a central pursuit of ecology. Research traditions were established early in this field, including approaches that utilized experimental manipulations of species interactions (e.g., (Connell 1961)) and those that evaluated patterns generated by historical processes (e.g., (Diamond 1975)). Both approaches argued that the relatedness of the species that make up a community is non-random and cited species interactions (often competition) as a major driver of community structure.

The increased availability of molecular phylogenies has led to a resurgence of interest in the relationship between species relatedness and patterns of co-occurrence in communities (Cavender-Bares et al. 2009; Strauss et al. 2006). It is now possible to incorporate information on trait evolution and the relatedness of species in a community (e.g, (Ingram and Shurin 2009; Sargent and Ackerly 2008)) to generate hypotheses about the forces driving community assembly (Figure 5). For example, when ecologically relevant traits are phylogenetically conserved or show a strong phylogenetic signal (e.g., (Burns and Strauss 2011a)) and species in a community are "overdispersed" (the co-occurrence of more distantly related species than expected at random), negative ecological interactions among phenotypically similar relatives are frequently hypothesized to have driven community assembly (Figure 5) (Cavender-Bares et al. 2004). However, several different processes can result in the same trait evolution and phylogenetic community structure pattern (Vamosi et al. 2009), and

thus, experiments are ultimately needed to evaluate whether these hypothesized processes are consistent with the ecological dynamics currently operating in a community.

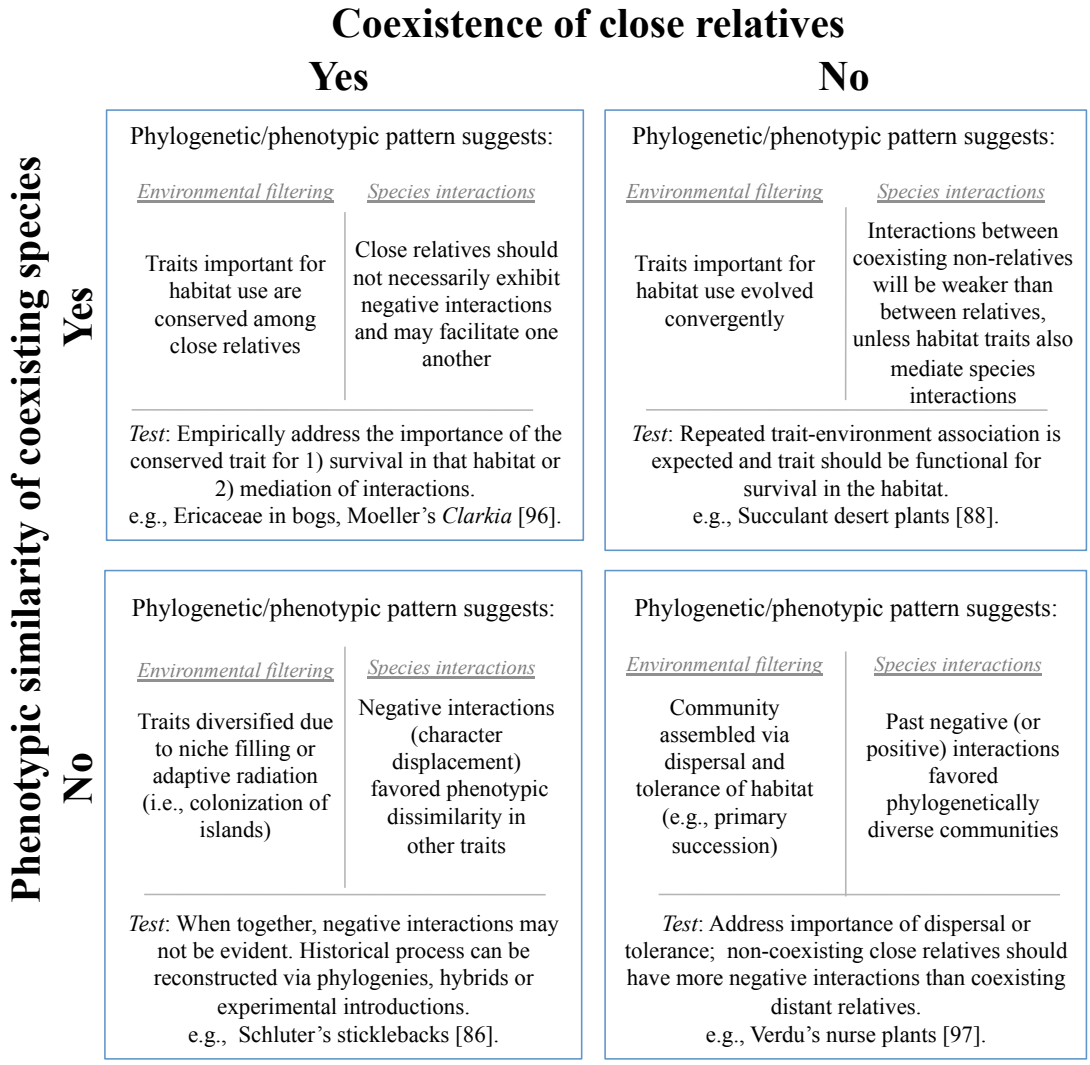

Figure 5. Information about the relatedness and phenotypic similarity of species in a community can provide insights into processes frequently hypothesized to drive community composition (see, (Cavender-Bares et al. 2004; Sargent and Ackerly 2008; Vamosi et al. 2009)). Under certain scenarios, traits that are relevant to environmental filtering (i.e., are necessary for species to live in particular abiotic environments) can show different patterns than those traits that are related to species interactions. Note that only the "no-yes" pairings involve trait evolution during diversification: in the bottom-left trait evolution is frequently hypothesized to be driven by species interactions and in the top-right by to adaptation to the environment. In both cases the

"no-yes" traits are predicted to show weaker phylogenetic signal than the like-pairings (yes-yes and no-no). The like-pairings focus on assembly of communities once the species have evolved, presumably in allopatry. Experimental tests on extant communities can clarify the mechanisms that generate community structure.

A phylogenetic signal for species interactions is suggestive, but not definitive evidence, that species interactions influenced community structure (Burns and Strauss 2011a). For example, in a meta-analysis of experiments, Cahill et al. (2008) reported that competition between eudicot plant species is more intense among close relatives as compared to distant relatives. In manipulative experiments, plant competition is reduced with increasing phylogenetic distance (i.e., more distantly related species are more productive when grown together) (Burns and Strauss 2011b; Cadotte et al. 2008). For herbivory and disease, it is similarly the case that closely related plants often share parasites, which could potentially lead to overdispersion (Futuyma and Agrawal 2009). However, positive species interactions can also have a phylogenetic signal [80], and negative interactions between close relatives should not be assumed *a priori*. Studies that match patterns of co-occurrence with experimental manipulations testing for increased negative interactions among related species can close the loop between pattern and process, demonstrating the importance of species interactions for generating community structure (Vamosi et al. 2009).

Rather than phylogenetic overdisperison, phylogenetic clustering (closely related species coexisting more often than expected) is often hypothesized to reflect habitat filtering or character displacement among coexisting close relatives, especially when paired with certain patterns of trait evolution (Figure 5). Unlike the general

prediction outlined above, here experiments using closely related species are expected to reveal relatively low levels of competition or survivorship in reciprocal transplant experiments. In order to demonstrate whether these processes lead to clustering, experimentally reconstructing the history of events can be a powerful approach. For example, Schluter (2003) experimentally demonstrated that closely related competitor species drove natural selection towards divergent ecologies in a target population of threespine sticklebacks. Similarly, experimental work has demonstrated that competition leads to diversifying selection in microbes (Meyer and Kassen 2007). Coupling these experiments with reciprocal transplants could identify the relative effects of different processes, such as environmental filtering, facilitation, and competition, in driving phylogentic clustering.

Convergent evolution and traits with low phylogenetic signal (e.g., (Arakaki et al. 2011)) can also generate non-intuitive links between species interactions and phylogenetic community structure (Figure 5) (Cavender-Bares et al. 2004). For example, in plant communities herbivore pressure is frequently hypothesized to drive phenotypic overdispersion (e.g., (Becerra 2007; Kursar et al. 2009)) because herbivores often host-shift onto chemically similar plant species. More generally, overdispersion of defense phenotypes suggests that apparent competition (negative interactions between species via shared enemies) was important in generating community structure. Although tests of this idea have not been conducted, the experiments should be straightforward. Among co-occurring species, those that are phenotypically similar are predicted to experience stronger enemy-mediated interactions than those that are phenotypically dissimilar, regardless of phylogenetic

relatedness (Becerra 1997; Becerra 2007; Kursar et al. 2009).

An expanding frontier in evolutionary ecology

Ultimately, deep historical events can never be directly observed or manipulated, and there are thus no definitive means by which we can directly test many evolutionary hypotheses. Instead, evolutionary ecologists must be willing to integrate multiple lines of evidence as they evaluate the plausibility of their causal hypotheses given all the information (see Losos' "evolutionary detective" (Losos 2009)). Here we have argued that integrating phylogenetic analyses and manipulative experiments is a revealing and rapidly growing approach to evaluating hypotheses that link evolutionary patterns (e.g., clade diversification rates, correlated character evolution, cospeciation, phylogenetic community structure) with mechanistic causes (e.g., population fragmentation, ecological tradeoffs, specialization, species interactions). Experimental approaches, such as reciprocal transplants or phenotypic manipulations, can elucidate ecological processes that are operating for a single (or small number of) species at a given place and time, but the generality and long-term evolutionary consequences of that ecological scenario remain unclear. In comparative phylogenetic analyses, a large-scale evolutionary pattern can suggest hypotheses about ecological process, but the analyses themselves do not address specific mechanisms, and experimental methods are ultimately required to evaluate causal scenarios and avoid evolutionary "storytelling". The combined application of phylogenetic and experimental methods can greatly enhance the process of testing and refining hypotheses, and such integration should increasingly be utilized in the pursuit of

strong inference in evolutionary ecology.

Box 1. Replication in causal hypothesis testing using phylogenies and experiments

Replication is critical for identifying causal relationships at all levels of biological organization. First, because organisms and the environments they live in are not static, but rather plastic, variable, and evolving, replication is essential if we are to capture accurate measures of biological phenomena. Second, replication allows for the use of statistical inference in biological research, both in manipulative experiments and correlational analyses. Although the use of replication is standard in traditional ecological studies, the importance of replication in phylogenetic hypothesis testing is debated, an incongruence between disciplines that is perhaps due to the fact that many important evolutionary events truly only occur once. However, single evolutionary events are not sufficient tests of causal hypotheses on their own, and more rigorous evaluation requires integration with other types of information, such as targeted experiments or the identification of evolutionary replication if it exists.

In research on key innovations, for example, studies continue to implicate one-time instances of trait evolution as causal agents in accelerated diversification rates (e.g., (Marazzi and Sanderson 2010)). There are severe limitations to this single-case approach. For instance, evolutionary transitions in a particular trait are almost always associated with other changes in additional traits or with transitions in the ecology of organisms (e.g., radiation into a novel habitat) and, without replication or experimentation, it is impossible to distinguish between these confounding effects (Barraclough et al. 1998). Although powerful statistical methods have recently been

developed to directly test if and where on a phylogeny shifts in diversification rates occurred (e.g., (Moore and Donoghue 2009)), and while these approaches can reject the underlying hypothesis, they only circumstantially address the causes of shifts in diversification rate and do not address the relative importance of multiple factors associated with these shifts. Indeed, hypothesis testing in biology typically focuses on rejecting the null hypothesis, which is difficult without experimentation and replication.

In cases of unique apomorphies, studies are restricted to one-time evolutionary events and thus necessarily lack replication. In these situations, rigorous models explicitly incorporating interactions between factors can provide potential for building strong evidence of evolutionary links in the absence of replication. Additionally, experimental and case-study based mechanistic evidence for associations between traits and diversification can shed light on these relationships.

To summarize, it is not possible to determine cause and effect in phylogenetic studies without experimentation and replication. Because phylogenetic analyses are focused on examining evolutionary patterns rather than process, if there are confounding factors, then cause (and the relationship itself) becomes questionable. However, experiments addressing causal hypotheses coupled with evolutionary independence gained through explicit statistical consideration of phylogeny can provide increased evidence for causal explanations and should thus generally be implemented whenever it is feasible to do so.

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CHAPTER 1

PHYLOGENETIC AND EXPERIMENTAL TESTS OF INTERACTIONS AMONG MUTUALISTIC PLANT DEFENSE TRAITS IN VIBURNUM (ADOXACEAE).[1,2]

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ABSTRACT

Plant traits that mediate mutualistic interactions are widespread, yet few studies link 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. 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 evolutionarily correlated with EFNs, 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 that their long-term evolution may be influenced by complex interactions among multiple traits, mutualists, and geography.

Introduction

Despite being wide-spread and ecologically important, plant traits that mediate mutualistic interactions are rarely studied in an integrative manner spanning 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 (hereafter EFNs), plant organs that secrete sugary nectar and typically feed beneficial arthropods (Bentley 1977; Heil 2008), and (2) leaf domatia, small structures on the undersides of leaves that provide housing for predacious or fungivorous mites (Lundström 1887; Romero and Benson 2005). Because traits like 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 contemporary ecological function are rare (but see Heil et al. 2004; Karban et al. 1995).

Several hypotheses attempt to explain the evolution and phylogenetic distribution of mutualistic defense traits such as EFNs and leaf domatia. These hypotheses have primarily focused on two factors: (1) their adaptive value in certain biotic or abiotic environments (e.g., Bentley 1977; Bronstein et al. 2006; Heil and McKey 2003; Schupp and Feener 1991), and (2) evolutionary trade-offs or synergisms

with other ecologically relevant plant traits (e.g., Heil 2004; Heil 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; Bronstein et al. 2006; Heil and McKey 2003; Schupp and Feener 1991). Not all defense mutualisms are more prevalent in the tropics, however: leaf 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 focus on evolutionary tradeoffs or synergisms, and suggest that the origin, maintenance and breakdown of indirect defensive traits is influenced by interactions with other plant traits rather than with 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 function 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. For example,

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, were 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 shown separately 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 they are frequently found together and have both been separately shown to increase mutualist abundance, which in turn can decrease herbivore and/or pathogen load and subsequently decrease the risk of plant damage (see reviews by Bentley, 1977; Walter, 1996; Bronstein *et al*, 2006; Heil & McKey, 2003).

A comprehensive evaluation of these adaption, tradeoff, and synergism hypotheses requires an evolutionary framework. Phylogenetic tests can determine whether defense-related traits have been conserved despite lineage shifts into new environments or despite state changes in other relevant plant traits. Rather than supporting adaptation to local conditions or evolutionary tradeoffs/synergisms, some phylogenetic patterns could reflect genetic or developmental constraints or chance association. Alternatively, patterns in the geographic distribution of traits 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, 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. Alternatively, the lack of concordance suggests that either conditions have changed substantially over time, or that hypotheses need to be further refined (Losos 1996; Losos 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 tradeoff/synergism hypotheses? Specifically, 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?

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 in cloud forests of South America. Its two modern centers of species diversity are in Eastern Asia and Latin America (Clement and

Donoghue 2011; Losos 1996; Moore and Donoghue 2007; Moore and Donoghue 2009; Winkworth and Donoghue 2005). Many *Viburnum* species are popular ornamentals and 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 (BH), the herbaria of the Yale Peabody Museum of Natural History (YU), the Missouri Botanical Garden (MO), the New York Botanical Garden (NY), the Field Museum of Natural History (F), Harvard University (A, GH), and Oregon State University (OSC), as well as the personal collections of MJD. 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 (figure 1 E-G, figure 2 A-B) according to O'Dowd and Willson (1989), and EFNs were categorized according to their position on the leaf (figure 1 A-D, figure 2 C-D): (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, we refer to these as 'laminar'); (iii) at the intersection of the leaf margin and petiole; and (iv) on the petiole. Sugar excretion was verified on live specimens of individuals from each of the four EFN forms using glucose test strips (Clinistix, Miles Lab., Elkhart, Indiana, USA), and on preserved specimens via the presence of sooty mold on or surrounding the glands (Appendix A, Pemberton 1990; Pemberton 1998).

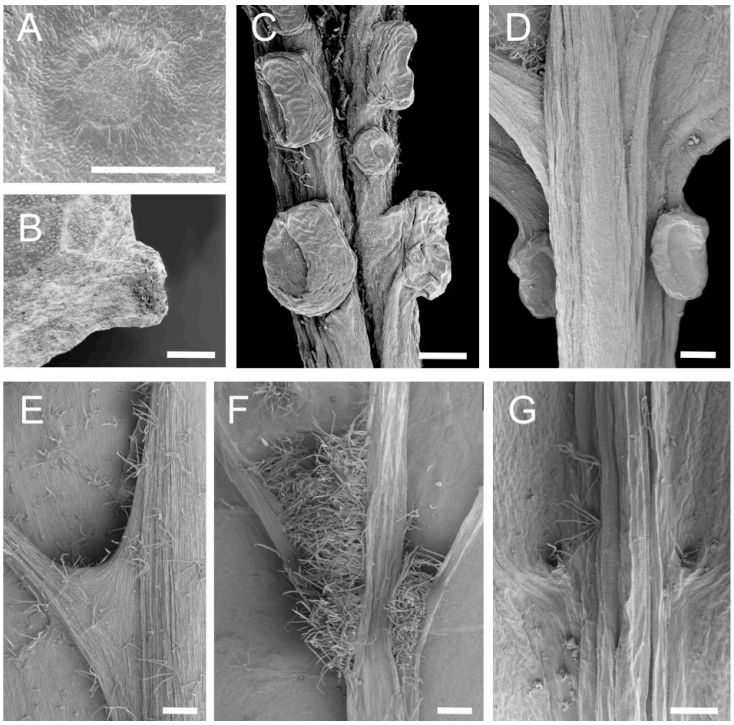

Figure 1: Scanning electron micrographs of representative mutualistic leaf traits on dried *Viburnum* leaves: (A) laminar EFN from *V. dilatatum*, (B) marginal EFN from *V. dentatum,* (C) petiole EFNs from *V. trilobum,* (D) petiole-laminar junction EFNs from *V. edule,* (E) cave domatium from *V. sulcatum,* (F) tuft domatia from *V. trilobum*, and (G) pit domatia from *V. odoratissimum.* Scale bars = 500μm.

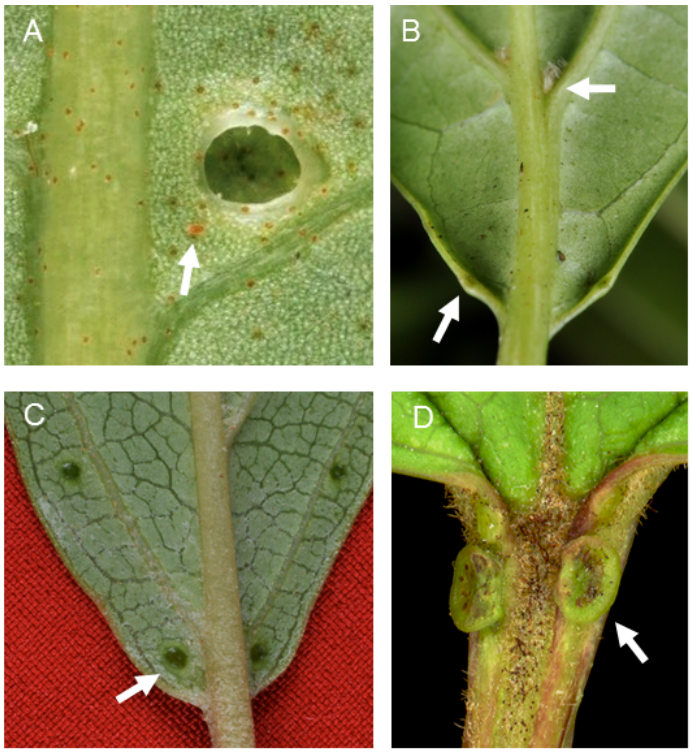

Figure 2: Photographs of mutualistic traits from live specimens of several *Viburnum* species. (A) close-up of a pit domatium from *V. becarri.* Arrow points to a mite near the opening of the domatium. (B) Basal, abaxial leaf surface of *V. cinnamomifolium.* Arrows point to a tuft domatium and a marginal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

Climate and leaf production strategy were assessed based on specimen collection localities, published species descriptions (Donoghue 1983; Hara 1983; Hsu 1975; Jones 1983; Kern 1951; Morton 1933; Rehder 1908; Yang 1994), and field observations by MJD. To test for predicted associations between traits and latitude/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 meters), and with limited temperature seasonality; (2) "cloud," including montane cloud forests at southern latitudes, at generally higher

elevations (mostly >2000 meters), and experiencing periodic colder temperatures but not prolonged temperature seasonality, and (3) "temperate," including deciduous temperate 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 a season; (2) "leaf exchangers," in which plants flush and lose their leaves asynchronously, and may have short, sporadic periods of leaflessness (i.e., semi-deciduousness); 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 in a Baysian analysis of a combined molecular dataset that included the chloroplast coding regions *mat*K, *ndh*F, and *rbc*L (4104 bp), the chloroplast non-coding regions *pet*B-*pet*D, *rpl*32-*trn*L$^{(UAG)}$, *trn*H-*psb*A, *trn*C-*ycf*6, *trn*K, *trn*S-*trn*G; (4806 bp), and the nuclear ribosomal Internal Transcribed Spacer region (ITS; 642 bp). All of the DNA sequences used by Clement and Donoghue (2011) are available in Genbank (www.ncbi.nlm.nih.gov/genbank), and all of their datasets and trees can be downloaded from TreeBASE (www.treebase.org; S10714). All comparative analyses were conducted using the majority consensus of the post-burnin 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 A).

We inferred ancestral character states and estimated the number of evolutionary transitions in our traits using maximum likelihood (ML) and parsimony (MP) optimization criterion in the program Mesquite (Maddison and Maddison 2010). We evaluated the phylogenetic signal of the traits using a Monte Carlo test of trait conservatism (Crisp et al. 2009; Webb et al. 2002), 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 one would expect 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 using 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 upon 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 the "evergreen" species. For climate analyses, "temperate"

and "cloud" species were considered to experience significant cold periods, as opposed to the "tropical" species without a cold season.

Common Garden Survey

In order to examine whether species with indirect defensive traits had more beneficial mites than species lacking the traits, and which trait combinations foster the highest mite abundance, we surveyed mite populations on 16 *Viburnum* species in an untreated common garden of mature *Viburnum* shrubs in Ithaca, NY, established in 1999. Of the 16 species in the garden, seven species 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 petioliar EFNs and tuft domatia (*V. opulus, V. sargentii, V. trilobum*). Thus, we compared mite abundance on species that lacked mutualistic traits with species that had only domatia, or had domatia paired with petoliar, marginal, or laminar EFNs.

We collected ten leaves from each species (2-10 mature shrubs sampled per species, mean n = 7) in July of 2010. Leaves were placed in moist paper towels, transported to the lab on ice, and surveyed for mites under a dissecting scope. Mites found on leaves were counted, sorted into morphospecies and stored in 75% EtOH. Representatives from each morphospecies were mounted in Hoyers solution on microscope slides and later identified to their 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, performed in R (R Development Core Team 2010).

Field Manipulations

We experimentally addressed the independent and joint impact of leaf domatia and laminar verses marginal EFN on beneficial mite numbers using natural populations of two species*: V. acerifolium*, which has tuft domatia and a small, single pair of laminar EFNs on either side of the midrib, and *V. dentatum*, which has tuft domatia and 2-6 small EFNs on the basal quarter of the leaf margin. Based on common garden results and the physical location of the nectaries, we predicted that the nectaries would more drastically increase mite abundance in *V. acerifolium* due to the close proximity (with ≈3mm) of laminar nectaries to leaf domatia. 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 1.44N, 76 26 8.52W), whereas experiments using *V. acerifolium* were performed in the Cornell University Polson Preserve, a deciduous forest understory (42 30 2.34N, 76 26 8.49W). For both species, populations were large, with hundreds of plants in the surrounding areas, and we selected relatively small plants for the experiment (10-30cm in height and with 2-10 leaves) in order to easily manipulate all 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 placed as droplets ≈0.8 cm to the side of each EFNs or domatium. Using these methods, we created the following treatments: (1) both EFNs and leaf domatia blocked, (2) EFNs blocked and tar control near domatia, (3) domatia blocked and tar control near EFNs, and (4) tar control near both EFNs and domatia. Visual inspections under a dissecting microscope verified the effectiveness of these treatments in blocking nectar flow and domatia 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 placed in a plastic bag, and transporting them to the lab in a cooler. Mites were counted under a dissecting microscope and preserved in 75% EtOH.

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 leaves of small plants (n=25) by covering glands with tree pruning tar, and created an additional 25 control plants with tar 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 using a Poisson distributed general linear ANOVA in R.

Results

Phylogenetic analyses

We examined 400 herbarium specimens representing 90 of the ~170 species

of *Viburnum* (1-9 specimens per species, mean n=5) (Appendix A). 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 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), dense concentrations of trichomes, often associated with a depression of the leaf surface; cave domatia (4%), a flap of leaf tissue extending out over the vein axils with a wide opening; and pit domatia (3%), a cavity covered by a dome raised above the leaf lamina, with a small pore-like opening. These major positions/types of traits were consistent across multiple collections of the same species, with the exception of *V. odortatissimum*, where the majority of leaf domatia were pits, but several individual domatia combined pit characteristics with a tuft- or cave-like appearance.

The phylogenetic distribution of the climate trait (figure 3) is consistent with the interpretation suggested by Clement and Donoghue (2011) that *Viburnum* diversified originally in montane tropical forests in southeast Asia, with 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, based solely on this optimization it is also possible that *Viburnum* originated in temperate forests followed by 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 figure 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 mainly in temperate forests, and leaf exchangers (i.e., species that flush and lose their leaves asynchronously) are in cloud forests. The prime exception is the *Tinus* clade (the clade that contains *V. atrocyaneum* to *V. tinus* in figure 3); these plants are evergreen despite mainly living in temperate forests. Again, our reconstruction suggests multiple shifts among leaf production categories.

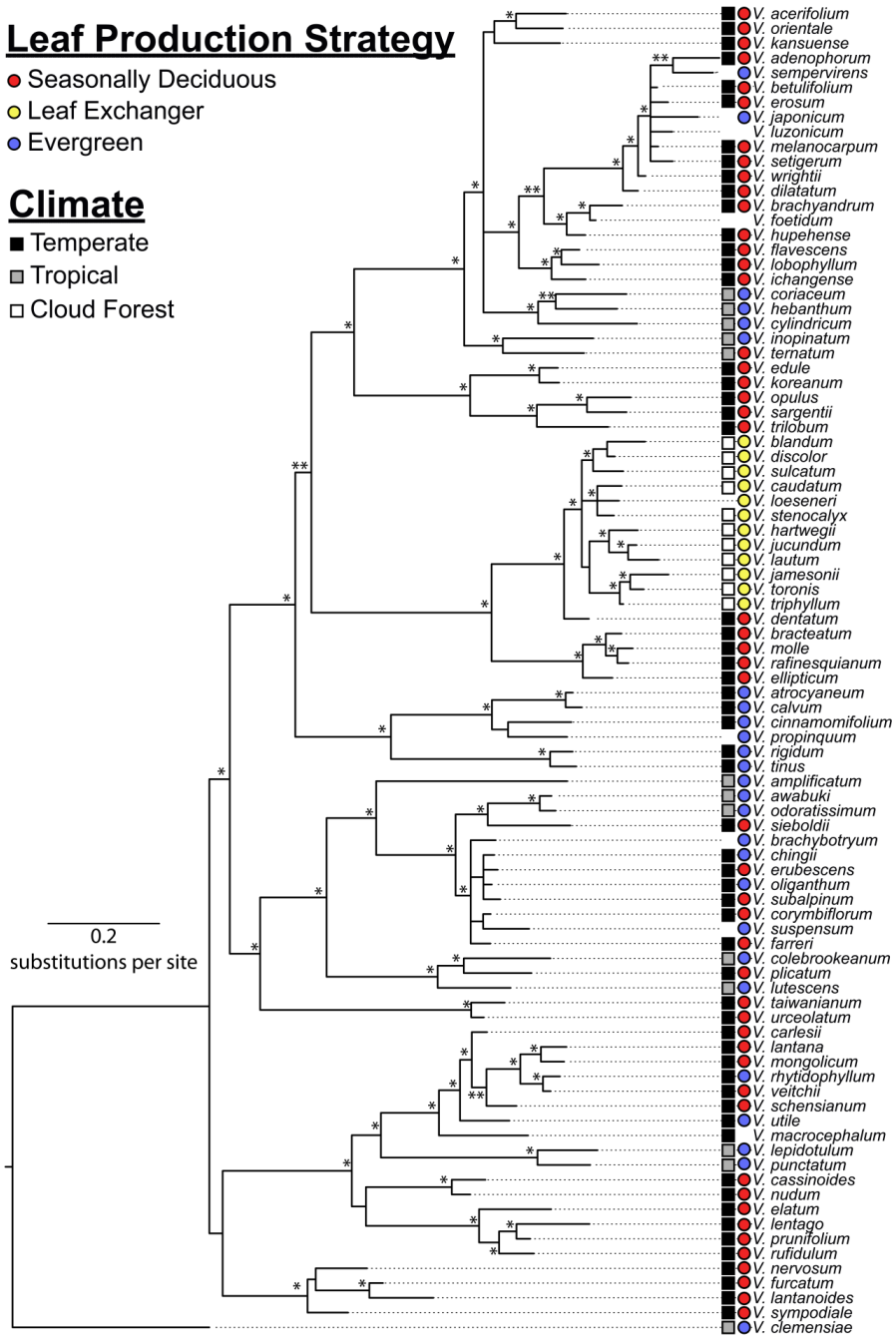

Figure 3: Bayesian majority rule consensus tree of *Viburnum* based on nine chloroplast genes and nuclear ribosomal ITS sequences showing the distribution of climate (squares) and leaf production strategy (circles). Branch lengths are drawn proportional to genetic distance. Posterior probabilities greater than 0.95 or between 0.9-0.94 are indicated above the branches with a "*" or "**", respectively. Some species have traits excluded due to uncertainty in scorings (Appendix A).

Both parsimony and ML methods reconstructed, with high confidence, a single origination of EFNs in *Viburnum* (figure 4 A). Furthermore, the presence of EFNs was significantly conserved despite evolutionary shifts in climate and leaf production strategy (monte carlo simulation, $p<0.001$). 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* to *V. tinus* in figure 3). In the *Opulus* clade (*V. edule* to *V. trilobum* in figure 3) reconstructions suggested that 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 into the leaf lamina in the as yet un-named clade (from *V. acerifolium* to *V. ternatum* in figure 3) that includes the *Succodontotinus*, *Lobata*, *Sambucina*, and *Coriacea* clades (Clement and Donoghue, 2011). Regarding the position of EFNs on the leaf surface, we find 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 ($p<0.05$) (figure 4 B). Domatia were evolutionarily correlated with both deciduousness (domatia were more likely to be gained in deciduous than in evergreen clades, Pagel's test D=6.07, p=0.014) and with temperate habitats (domatia were more likely to be gained in temperate rather than cloud forest or tropical clades, D=5.93, p=0.013). 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<0.001) and temperate habitats (D=6.083, p=0.018). 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 evergreen, tropical lineages. Cave domatia, represented by four species (*V. mongolicum, V. sulcatum, V. taiwanianum, V. urceolatum*), evolved twice in temperate, seasonally deciduous lineages, and once in a cloud forest, leaf exchanger lineage. Overall, cave domatia evolved independently of climate and leaf production traits (climate, D=1.93, p=0.23; deciduousness, D=1.67, p=0.35).

Finally, leaf domatia and EFNs were evolutionarily correlated across *Viburnum* (D=2.89, p=0.032). In particular, the EFN clade (figure 4 A) has significantly more domatia bearing branches (figure 4 B) 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.

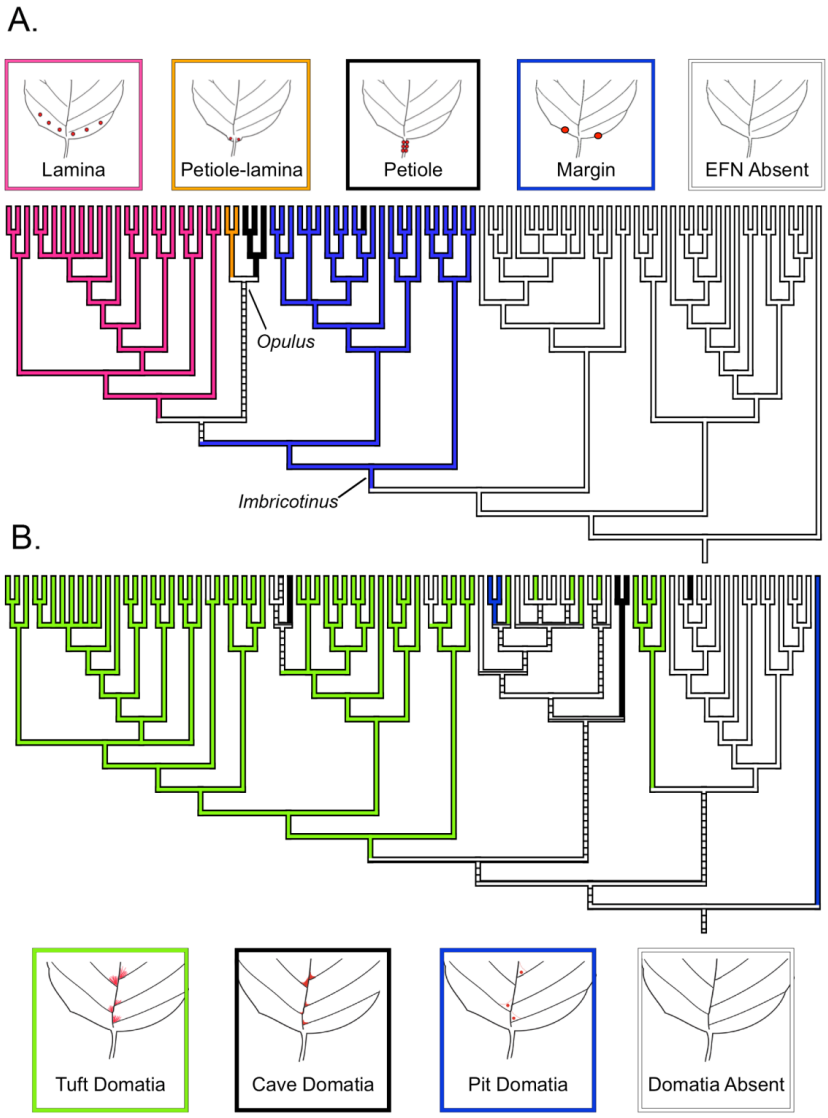

Figure 4: Phylogenetic patterns in indirect defensive traits in 90 species of *Viburnum*. Shown are the most parsimonious ancestral character state reconstructions for EFNs (above) and leaf domatia (below). Red marks on the leaf line drawings depict the general location and shape of the traits.

Common Garden & Field Manipulation Results

In our common garden survey of *Viburnum* species, mite diversity was largely dominated by the mycophagous family Tydeidae (80.7%), but 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 often 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, $p<0.0001$). Leaves that lacked both traits had ≈93% fewer mites than leaves with tuft domatia and no EFNs, petioler EFNs, or marginal EFNs (post-hoc Tukey HSD tests, figure 5). However, species with tuft domatia coupled with laminar EFNs had well over two-fold more mites than any of the other trait combinations (figure 5).

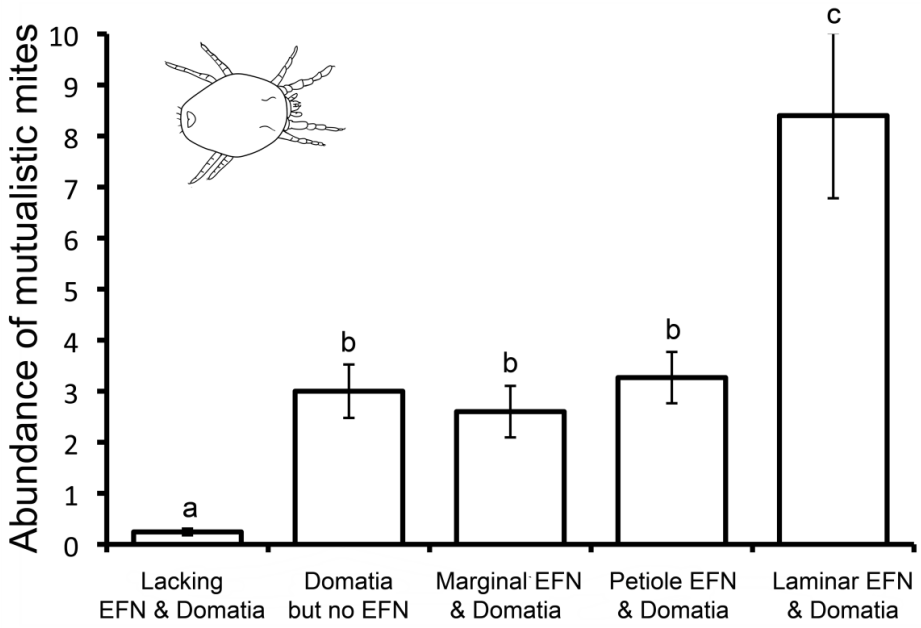

Figure 5: Abundance (mean ± se) of mites per leaf on16 species of *Viburnum* with different combinations of mutualistic plant traits. Different letters represent significant differences ($p<0.05$) based on post-hoc Tukey HSD tests. Of the 16 species included, seven species lacked both leaf domatia and 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 petioliar EFNs and tuft domatia (see text). The line drawing represents a mycophagous Tydeid mite, the dominant group in our survey.

In manipulative experiments on natural populations, mites found on leaves were dominated by the same mycophagous and predatory families as 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 (figure 6 A); 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 (89%) in the absence of domatia (figure 6 B), but mite populations were not affected

by the absence of marginal EFNs (figure 6 B). Visitation to *V. dentatum* leaves by ants (*Formica podzolica*) decreased by 57% when EFNs were blocked (figure 6 B inset, Generalized linear ANOVA, $F_{1,46}$=3.74, p=0.052).

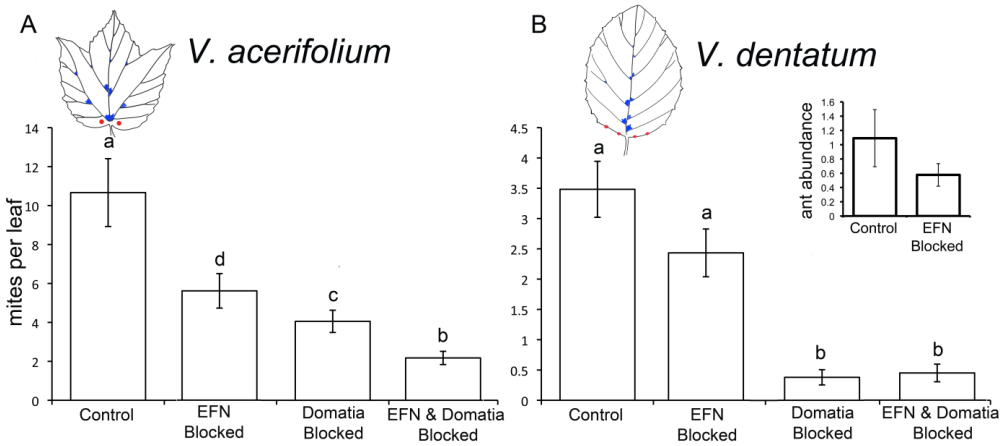

Figure 6: Abundance (mean ± se) of mites per leaf on (A) *V. acerifolum* and (B) *V. dentatum*, with mutualistic plant traits factorially blocked. Red coloration on leaf illustrations marks the position of EFNs for that species; blue coloration marks tuft domatia. Letters represent significant differences ($p<0.05$) based on post-hoc Tukey HSD tests comparing means among treatments within each species (but not among *V. acerifolium* and *V. dentatum* experiments). The inset in B shows abundance (mean ± se) of ants on *V. dentatum* with EFNs blocked and tar controls (data from a separate experiment).

Discussion

The study of historical evolutionary processes is inherently challenging, as 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 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 consistent with both environmental adaptation and trait-interaction hypotheses as evolutionary drivers of these mutualistic traits. In particular, phylogenetic trait correlations led to the hypothesis that domatia and EFNs are not ecologically redundant, but 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 using natural populations, where plants with EFNs and leaf domatia attracted more mutualistic arthropods (mites and ants) than 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 & trait conservatism

Large-scale geographic patterns in trait distributions have led to the hypothesis that mutualistic plant traits are locally adapted to certain regions, generating 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 species' climate and leaf production strategy, supporting adaptive hypotheses based in regional survey data (O'Dowd and Pemberton 1998; O'Dowd and Willson 1997; Walter 1996; Willson 1991). 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 underestimate, as pit domatia are known in several species that have not yet been included in molecular phylogenetic analyses but which are likely to belong to additional clades. Specifically, pit domatia have been documented in the Bornean species *V. vernicosum*, a member of *Megalotinus* subsection *Sambucina*, and in *V. beccarii* from Penninsular Malaysia, which is thought to be related to members of *Megalotinus* subsection *Coriacea* (Kern, 1951; Clement and Donoghue, 2011; see figure 3). Pit domatia have also been reported in *V. glaberrimum, V. cornutidens,* and *V. platyphyllum* (Brouwer and Clifford 1990; Kern 1951), 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 the association 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 traits' inconspicuous nature. However, the convergent patterns in our data support the idea that these traits are adaptive in particular environments. Studies directly comparing the ecology of different forms of domatia are rare, however, and as yet lend little insight into 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 both tuft and pit domatia have been separately shown to enhance populations of predatory arthropods (Agrawal and Karban 1997; O'Dowd 1994; 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. 2001; Norton et al. 2000; 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 increased ant diversity and herbivore pressure (Bentley 1977; Bronstein et al. 2006; Heil and McKey 2003; Schupp and Feener 1991). 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 single large clade, and have been retained in all members of this clade despite multiple shifts between different geographic regions and environments (figure 4 A). Furthermore, the largest EFNs in *Viburnum* (placed on the petiole in members of the *Opulus* clade) evolved at high latitudes, in boreal rather than tropical environments. EFNs are also highly conserved in other clades, including *Acacia* (Gomez-Acevedo et al. 2010; Heil et al. 2004), *Gossypium* (Rudgers et al. 2004), *Populus* (Keeler 2008), and *Senna* (Marazzi

et al. 2006), yet no other study to date explicitly addressed whether the origin or persistence of EFNs 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, having multiple traits that attract or retain mutualists may be redundant and costly (Oliver et al. 2008), resulting in a negative evolutionary correlation if consistent over time. Alternatively, multiple mutualistic traits might interact synergistically or additively (Heil 2008), which could result in positive evolutionary associations. These tradeoff and synergism hypotheses are not mutually exclusive of the geographic hypotheses discussed above, and can be evaluated by integrating information about trait evolutionary history with studies of the ecological effects of the two traits.

We found a positive evolutionary correlation between EFNs and leaf domatia in *Viburnum*, suggesting that the traits may have especially 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, and revealed that different morphological positions of EFNs attract different types and abundances of arthropod mutualists. Specifically, our results are consistent with the hypothesis that laminar EFNs, which are positioned closer to leaf domatia than marginal EFNs, function with domatia to increase mite

populations, and that neither marginal nor petiolar nectaries appear to influence mite abundance but may instead attract ants. It should be noted, however, that while our common garden and experimental data are consistent with this interpretation, we have obtained such data from a limited number *Viburnum* species (common garden n=16 species; experiment n=2 species). Regardless, these results yield a testable hypothesis for our inferred evolutionary "migration" of EFNs from the leaf margin into 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 predicted benefit a plant is expected to receive with increasing mutualist abundance has the potential to eventually taper off or decrease rather than necessarily continuously increase (Holland et al. 2002; Morris et al. 2010). Nonetheless, we note that quantatively similar changes in the abundance of the dominant mite family found in our study (Tydeidae) have been demonstrated to negatively impact powerdery mildew growth on leaves of Riverbank Grape in domatia blocking experiments in Central New York (Norton et al. 2000). In particular, increased mite abundances of up to 25 mites per leaf translated into a steep decrease in leaf mildew, after which 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 (Figures 5, 6) fall well within the range of increasing mutualist benefits.

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. Like leaf domatia, sugar-secreting laminar EFNs are common, but remarkably inconspicuous, and 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, based on a combination of the latest phylogenetic and taxonomic information, we estimate that EFNs are present 107 of the ~170 species of *Viburnum* (~63%). Mite visits to EFNs have, nonetheless, been sporadically recorded in the literature for over a decade (van Rijn and Tanigoshi 1999; Walter 1996), and the presence of both EFNs and tuft domatia were 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 have species with both traits, including *Prunus*, *Populus*, and *Quercus* (Brouwer and Clifford 1990; Keeler 2008). However, a replicated study examining multiple lineages with laminar EFNs and tuft domatia is ultimately needed to evaluate the generality of the bed and breakfast hypothesis, and, more specifically, the influence of the relative position 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 *in review*), phylogenetic comparative studies of macroevolutionary pattern and ecological studies using extant taxa are still typically conducted in isolation. This is due, in part, 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 are continuously evaluated and refined with the addition of multiple types of information (Weber and Agrawal *in review*). 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 informative, as it suggests that the hypothesized process is not at play in the contemporary system. In this way, conflicting results can lead to the clarification of the original causal hypothesis (by asking how current and historical populations differ) or 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 domatia and both climate and leaf production strategy. Ultimately, we found evidence for trait conservatism, adaptation to environmental factors, and interactions among plant mutualistic characters as 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 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.

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CHAPTER 2

THE PHYLOGENETIC DISTRIBUTION OF EXTRAFLORAL NECTARIES IN PLANTS[1]

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Abstract

• *Background and Aims:* Understanding the evolutionary patterns of ecologically relevant traits is a central goal in plant biology. However, for most important traits, we lack the comprehensive understanding of their taxonomic distribution needed to evaluate their evolutionary mode and tempo across the tree of life. Here we evaluate the broad phylogenetic patterns of a common plant-defence trait found across vascular plants: extrafloral nectaries (EFNs), plant glands that secrete nectar and are located outside the flower. EFNs typically defend plants indirectly by attracting invertebrate predators who reduce herbivory.

• *Methods:* We compiled records of EFNs published over the last 135 years. After accounting for changes in taxonomy, we used phylogenetic comparative methods to evaluate patterns of EFN evolution using a phylogeny of over 55,000 species of vascular plants. Using comparisons of parametric and non-parametric models, we estimated the true number of species with EFNs likely to exist beyond the current list.

• *Key Results:* To date, EFNs have been reported in 3,941 species representing 745 genera in 108 families, about 1-2% of vascular plant species and ~21% of families. They are found in 33 of 65 angiosperm orders. Foliar nectaries are known in four of 36

fern families. Extrafloral nectaries are unknown in basal angiosperms, magnoliids and gymnosperms. They occur throughout monocotyledons, yet most EFNs are found within eudicots, with the bulk of species with EFNs being rosids. Phylogenetic analyses strongly support the repeated gain and loss of EFNs across plant clades, especially in more derived dicot families, and suggest that EFNs are found in a minimum of 457 independent lineages. However, model selection methods estimate that the number of unreported cases of EFNs may be as high as the number of species already reported.

• *Conclusions:* Extrafloral nectaries are widespread and evolutionarily labile traits that have repeatedly evolved a remarkable number of times in vascular plants. Our current understanding of the phylogenetic patterns of EFNs makes them powerful candidates for future work exploring the drivers of their evolutionary origins, shifts, and losses.

Keywords: Extrafloral, extranuptial, foliar, nectary, extrafloral nectary, phylogeny, taxonomy, distribution, mutualism, angiosperms, rosids, asteriids

Introduction

Extrafloral nectaries (hereafter EFNs) are plant glands that secrete sugar, water and amino-acids (first called "extranuptual nectaries" Delpino 1886). Unlike floral nectaries, which function primarily in pollination, EFNs are commonly implicated in indirect plant defence, as they attract invertebrate predators whose presence and activity can reduce herbivory (Bentley 1977). A large body of research has focused on understanding the ecology and physiology of EFNs, and they are frequently featured in studies of facultative mutualisms and indirect plant defence (Bronstein et al. 2006; Heil and McKey 2003). However, our ability to formulate and evaluate hypotheses about the evolutionary origins and ecological drivers of EFNs is limited by the lack of detailed knowledge concerning their phylogenetic distribution across plant clades. Here, we synthesize reports of EFNs in the literature and analyse the phylogenetic patterns of EFNs across vascular plants using modern comparative methods. Our goal is to provide a comprehensive evaluation of how many plants have EFNs and how they are distributed and evolving across plants, to identify regions of the plant tree-of-life that are in particular need of finer-scale studies of EFNs, and to facilitate the formulation of general hypotheses about the drivers of EFN evolution.

Extrafloral nectaries are highly diverse morphologically and include glandular structures that differ considerably in their location, size, and form (Figure 1). They have been described on almost every aboveground plant part, including leaves, petioles, bud bracts, stipules, stems, cotyledons, fruits, and the outside of sepals (Elias 1983). They include structures that range from single-cell nectar secreting hairs,

"formless" glandular tissue, complex raised cups, and shallow bowl-like depressions, and they range from highly vascularized to completely lacking vascularization (Elias 1983). The high diversity of plant structures that fall under the name EFN is due in part to their definition, which is generally based on ecological function (nectaries not involved in pollination, Delpino 1886), rather than their location, structure or developmental origin *per se* (nectaries on plant parts not related to the flower Caspary 1848). The present, weakly-defined categories make the formulation of general hypotheses about the drivers of EFN evolution challenging, as different types of EFNs may not be homologous or may be influenced by different ecological and developmental factors. By incorporating a phylogenetic perspective in studies of EFN diversity, however, we can begin to disentangle the evolutionary history of origin, loss, and morphological transition in EFNs, leading to informed hypotheses about the homology of various forms of this functionally defined trait. Furthermore, establishing the phylogenetic distribution of a trait will facilitate future testing of specific hypotheses concerning the ecological drivers of EFN evolution. For example, are there evolutionary correlations between EFN types and ecological factors previously predicted to influence EFN evolution, such as resource availability (Heil and McKey 2003; McKey 1989; Schupp and Feener 1991), and mutualist abundance or aggressiveness (see Schupp and Feener 1991)? Or, does the origin or loss of EFN correlate with other plant traits predicted to influence their ecology, such as a vine-like growth habit (Koptur 1992), or the presence of additional mutualist rewards such as domatia (Weber et al. 2012)?

Figure 1: EFNs are diverse within and between taxa, and are found throughout vascular plants. From upper left: (1) ants feeding on the foliar nectaries of a fern frond, *Dyrnaria quercifolia*. (2-3) Monocots: sepal nectaries on *Dendrobium gatton* and an unidentified Orchid (4) Malvales: sepal nectaries on *Hibiscus* sp. (5-6) Solanales: ant feeding on the foliar/petiolar nectaries on *Ipomoea carnea*, sepal nectaries on *Ipomoea alba*. (7-9) Dipsacales: foliar nectaries on *Viburnum coriaceum*, marginal nectaries on *V. cinnamomifolium,* and petiolar nectaries on *V. opulus*. Photograph 1 by Obsidian Soul, 2 by Suzanne Koptur, 3-7 by K.H.K., 7-8 by Patrick Sweeney © 2011 Peabody Museum of Natural History, 9 by Gary Fawless, Cofrin Center for Biodiversity.

Reports of EFNs have been published since at least the 1870's (Belt 1874; Darwin 1877; Poulsen 1877), but potentially as early as 1762 (Hall cited in Bentley 1977). Many reports are from taxonomic species descriptions, where EFNs are frequently noted as characters relevant to identification (e.g., Fryxell 1978; Killip 1938). Extrafloral nectaries have also been the specific subject of numerous morphological and anatomical studies (e.g., Bonnier 1879; Lüttge 1971). Several studies have extensively documented EFN prevalence by surveying specific habitats and locations,

including Costa Rican forest and riparian habitats (Bentley 1976) and the cerrado of southeast Brazil (Machado et al. 2008; Oliveira and Leitao-Filho 1987), east Asia (Pemberton 1998), and various temperate habitats (Keeler 1980). In some cases, detailed surveys of EFN presence and morphology have been conducted within specific plant clades, for example *Macaranga* (Euphorbiaceae) (Fiala and Maschwitz 1991), *Viburnum* (Adoxaceae) (Weber et al. 2012), and *Senna* (Leguminosae, Cassiinae) (Marazzi et al. 2006; Marazzi and Sanderson 2010). Together, these sources contribute to the growing list of plant taxa known to have EFNs.

Over the past century, there have been several reviews of the taxonomic distribution of plants reported to have EFNs at the time of publication. Delpino (1886) calculated that 2,900 species had "extranuptial nectaries" and discussed their taxonomic and geographic distribution. Later, Zimmermann (1932) published an assessment of the distribution of plants with EFNs together with a categorization of nectary structure. Almost fifty years later, Bentley (1977) provided an abridged version of this report and included additional tropical families newly found to harbour EFNs. Elias (1983) added still more families to the growing list in his evaluation of terminology used to describe different morphological types of EFNs. In 1992, Koptur provided the most recent published synthesis of families and genera with EFNs (Koptur 1992). However, all authors of these past reviews stressed that the number of species reported to have EFNs was likely to grow as new taxa were examined, and that our understanding of how EFNs are distributed across plants families is likely to change as additional species are evaluated. Indeed, the continued accumulation of additional reports of EFNs published over the last two decades, as well as substantial

updates in plant systematics over the last century, merit a re-evaluation of how many plants have EFNs, and how they are dispersed across the plant phylogeny.

Previous reviews have noted that EFNs appear to be a phylogenetically widespread plant trait and hypothesized that they have been gained and lost many times independently across the plant tree of life (Bentley 1977; Bronstein et al. 2006). Such a pattern would suggest that EFNs exhibit high evolutionary convergence, consistent with their distribution being influenced by forces such as adaptation to ecological factors. Recent technological advances in building and analysing large phylogenies allow us to evaluate these hypotheses in a broad phylogenetic context. Thus, by consolidating published reports of EFNs over the last 135 years, we provide a summary of the taxonomic distribution of plants reported as having EFNs and assess their evolutionary patterns using widely sampled phylogenies of vascular plants. In particular, we analyse the taxonomic and phylogenetic patterns of plants reported to have EFNs, discuss what insights these patterns provide concerning the drivers of EFN evolution, and provide statistical estimates of how these patterns may change with the discovery of new EFN taxa in the future.

Material And Methods

To document the taxonomic distribution of plants described as having EFNs, K.H.K. began compiling published reports and personal observations of plants with EFNs in the late 1970s (Keeler 2008). This "World List of Plants with Extrafloral Nectaries" (hereafter, World List), includes information on the genus, species and family of all plants reported to have EFNs over the last 135 years from 1877 (Poulsen 1877) to January 2012. This list incorporates previously published lists of EFN-

bearing taxa (Elias 1983; Koptur 1992; Schnell et al. 1963; Zimmermann 1932). When available, the position of the EFN on the plant, the species' common name, and the plant's growth habit were also included. All information in the World List was deposited in an open-source, online database (Keeler 2008) that is publicly available as a resource for those studying EFNs.

Because the taxonomic nomenclature of plants in the World List has changed since many of the original reports were published, we updated all names of plants documented as having EFNs to their current Angiosperm Phylogeny Group (Angiosperm Phylogeny Group 2009) taxonomic classifications. To do this, we cross-referenced the World List with the International Plant Names Index (The International Plant Names Index 2008), the World Checklist of Selected Plant Families (WCSP 2012), and Tropicos (Tropicos.org 2012) using Plantminer (Gustavo et al. 2010). All synonyms were replaced with current accepted names for analyses. We further curated the list by omitting EFN reports in which we had low confidence. We omitted (1) families with only a single, unpublished report of EFNs: Aristolochiaceae and Clusiaceae; (2) families with a single, published report of EFNs that could not be confirmed: Alismataceae (Schnell et al. 1963), Ancistrocladaceae (Metcalfe 1951 in Koptur 1992), Annonaceae (Koptur 1992), Bruneliliaceae (Watson and Dallwitz 1992), Caryophyllaceae (Bentley 1977), Goodeniaceae (Bentley 1977), Hydrangeaceae (Zimmermann 1932), Icacinaceae (Koptur 1992), Musaceae (Koptur 1992), Olacaeae (Metcalf and Chalk 1971) and Stryracaceae (Vesque 1886); and (3) genera without any species identification, if they could not be confirmed by other means (26 genera). Additionally, Nepi *et al.* (2009) call the nectaries of *Gnetum*

cuspidaturm extrafloral, but because Kato *et al.,* (1995) demonstrated their function in pollinator reward, so we omit them here. While these omissions risk potentially discarding taxa with EFNs, they conservatively deal with potential false positive EFN reports.

Estimating the total number of species with EFNs

Because our understanding of the taxonomic and phylogenetic distribution of EFNs is likely to change as more species with EFNs are discovered, we used a model comparison framework to estimate the number of unreported cases of EFNs likely to exist beyond the current publication list. In particular, we utilised a general methodology designed for the estimation of total number of classes in a population from observed frequency count data (Bunge 2011). For each species in the World List (other than the omitted cases above), we obtained a "frequency count" based on the number of times EFNs have been reported for that species in the literature. We obtained the publication count for each species in the World List using the following search terms in Google Scholar: the genus name, the species name, and *either* (1) "extrafloral nectar*" (2) "extranuptial nectar*," (3) "foliar nectar*," or (4) "bract* nectar*." This count included any works in the Google Scholar database as of March 1st, 2012 that fit our search criteria, including academic books, journal, conference abstracts, dissertations, theses, and peer reviewed articles. Our search did not discriminate between multiple publications from the same or different authors (for example, two publications from the same lab were treated the same as two publications from two different labs). Using this publication "count data," we estimated the total number of species with EFNs, represented as the sum of the

number of reported and the estimated number of as-yet-unreported species with EFNs, using the CatchAll (Bunge 2011), a program for analysing frequency count data from incidence-based samples. CatchAll uses maximum likelihood estimates and a combined heuristic/statistical model-selection algorithm to compare diversity estimate models across multiple levels of outlier deletion. We compared five non-parametric models (Good-Turing, Chao1, ACE, ACE1, and Chao-Bunge gamma-Poisson), and five parametric models (Poisson, single exponential-mixed Poisson, and mixtures-of-2, 3 and 4-exponentials-mixed-Poisson) to find the best fitting estimate of total EFN richness (Bunge et al. 2012).

Phylogenetic methods

In order to visualize the large-scale phylogenetic distribution of plant families reported to contain at least one species with EFNs, we mapped the presence/absence of species with EFNs on the family level mega-tree from the Angiosperm Phylogeny Group (APGIII). The APGIII tree (Angiosperm Phylogeny Group 2009) is a compilation of previously published plant phylogenies, and is intended to give the current best estimate of relationships among all plant families. We incorporated branch lengths using the program Phylocom (BLADJ; Webb et al. 2008) according to age estimates from Wikström et al (2001) based on fossil records and non-parametric rate smoothing estimates. Patterns of EFN distribution across plant families were graphically displayed using iTOL (Letunic and Bork 2007).

While the APGIII tree allows for a broad view of EFN distribution across seed plants, a phylogeny with finer scale resolution is required to evaluate metrics such as phylogenetic signal. Thus, we calculated phylogenetic signal using a trimmed version

of a consensus tree of maximum likelihood phylogenies of 55,437 seed plant species constructed using the gene regions *atpB, matK, trnK, trnL, rbcl,* and *ITS* (Smith et al. 2009). Taxa were selected for inclusion in this phylogeny in an unrelated study (Smith et al. 2009) and thus should be neutral with respect to EFN presence (see Sage et al. 2011 for similar approach). For analyses, the 55,337 species tree was trimmed so that each genus was represented by only one tip (9,745 genera) using the drop.tip function in the R package APE (Paradis et al. 2004) which preserves topology and branch lengths. Using the trimmed phylogeny, we evaluated the phylogenetic signal of EFNs via the estimation of Fritz & Pervis' *D* for binary traits, which is a measure of sister-clade differences in a discrete character state for a given phylogeny (Fritz and Purvis 2010). An estimated *D* of 1 represents a distribution of binary traits that is random with respect to the phylogeny, where as a *D* of 0 represents a distribution expected under Brownian motion (Fritz and Purvis 2010). Similarly, a *D* of greater than 1 is more over-dispersed than expected at random, while a negative *D* is more phylogenetically clumped than expected under Brownian motion (Fritz and Purvis 2010). Using the R package caper (Orme et al. 2011; R Development Core Team 2012), we calculated *D* for the presence of EFNs and, in order to assess significance, compared our estimate with simulated distributions of *D* under (1) randomly reshuffled trait values across the tips of the tree, and (2) trait evolution under Brownian motion. Each simulation included 1,000 permutations. 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 inferred the number of gains and losses needed to explain the distribution of EFNs on the trimmed phylogeny from Smith et al (2009) using stochastic character mapping (Huelsenbeck et al. 2003) in SIMMAP (Bollback 2006) and maximum parsimony criteria in GLOOME (Cohen et al. 2010). Maximum parsimony calculates the fewest number of evolutionary gains and losses needed to explain the distribution of EFNs on the phylogeny, where the relative cost of gains and losses are equal. Stochastic character mapping infers the probability and expected number of gains and losses in EFNs based on the phylogeny and an underlying probabilistic model of character evolution. Unlike parsimony methods, stochastic character mapping can incorporate branch lengths information and allows for multiple state changes to occur on a single branch. We utilized a beta distribution (starting alpha =1, k=31) on the bias prior for the two-state frequencies prior, and a gamma distribution (starting alpha=1.25, beta=0.25, k=90) on the overall rate prior. The number of evolutionary events (gains 0->1, and losses 1->0) was estimated via the simulation of 1,000 stochastic mutational maps, with each map having 20 draws from the prior distribution. We estimated the rate of gain and loss of EFNs across the phylogeny using maximum likelihood optimization and compared one and two rate markov models (Pagel 1994) in the diversitree package in R (R Development Core Team 2012).

Results

We found reports of EFNs in 3,941 species of vascular plants representing 745 genera in 108 families (Table 1). Foliar nectaries have been reported in four fern families (39 species from 7 genera), but are not known from bryophytes,

gymnosperms, basal angiosperms and magnoliids. Extrafloral nectaries occur in a variety of monocotyledons (260 species from 82 genera representing 15 families), including some true grasses (22 species from 5 genera), various dioscorea (71 species), and many orchids (77 species in 45 genera). Extrafloral nectaries are most common in eudicots (3,642 species in 654 genera representing 89 families), with over half of all species with reported EFNs (2,342 species) belonging to the rosid I clade (Table 1). Extrafloral nectaries have not been reported in the Apiales. The families with the most EFNs are Fabaceae (1,069 out of ~19,500 species, in Fabales), Passifloraceae (438 out of ~935 species, in Malpighiales) and Malvaceae (301 out of ~4,225 species, in Malvales), while the genera with the most EFNs are *Passiflora* (322 species, Passifloraceae), *Inga* (294 species, Fabaceae), and *Acacia* (*sensu latu* 204 species, Fabaceae).

Table 1. Taxonomic distribution of EFN reports in major clades of vascular plants. Numbers of families, genera and species in each order are from (Stevens 2012) APGIII. If Stevens (2012) reports a range of counts for a clade, we used the highest number given. Where available, EFN location is provided, L=leaf, Pt=Petiole, Sp=stipules, Sm=stem, Pd= pedicels, peduncles or stems of inflorescence, Sl=sepals/calyx/perianth/tepals/floral bracts/cataphylls, Br=Leaf bracts/leaf buds, and F=fruit. Basal angiosperms include the Amborellales, Nymphaeales and Austrobaileales. The only family with EFNs that is not included here is Boranginaceae, an unplaced Asteroid family that includes two EFN species from the same genus (*Cordia dentata* and *C. spinescens*).

Major clade(s) / **Order**	**Families with EFN** / Total Families (%)	**Genera with EFN** / Total Genera (%)	**Species with EFN/** Total Species (%)	**EFN location**
Basal Tracheophytes	**0** (0)	**0** (0)	**0** (0)	-
Ferns				
Cyatheales	**1**/8 (12.5)	**2**/15 (13)	**2**/663 (0.3)	L

Polypodiales	**3**/15 (20)	**7**/252 (2)	**37**/6962 (0.1)	L
Gymnosperms	**0**/15 (0)	**0**/79 (0)	**0**/850 (0)	-
Basal Angiosperms	**0**/7 (0)	**0**/12 (0)	**0**/175 (0)	-
Magnoliids	**0**/5 (0)	**0**/154 (0)	**0**/2929 (0)	-
Monocotyledons				
Alismatales	**2**/14 (14)	**4**/166 (2.4)	**9**/4560 (0.20)	L Pt Sm Pd Br
Asparagales	**4**/14 (28)	**51**/1122 (4.5)	**106**/26070 (0.40)	L Se Pd F
Commelinales	**1**/5 (20)	**1**/68 (1.5)	**1**/812 (0.12)	Se
Dioscoreales	**1**/5 (20)	**2**/21 (9.5)	**71**/1037 (6.8)	L Pt
Liliales	**2**/11 (18)	**3**/67 (4.5)	**7**/1558 (0.45)	L Sm Se
Poales	**3**/17 (18)	**11**/997 (1.1)	**35**/18325 (0.22)	L Pt Se Pd Br
Zingiberales	**2**/8 (25)	**10**/92 (11)	**31**/2111 (1.5)	L Pt Pd Se Br
Basal Eudicots				
Proteales	**1**/4 (25)	**6**/85 (7.1)	**6**/1710 (0.41)	L
Ranunculales	**2**/7 (29)	**3**/199 (1.5)	**4**/4445 (0.09)	L
Rosid I: Fabidae				
Cucurbitales	**1**/7 (14)	**23**/129 (18)	**41**/2295 (1.8)	L Pt Sm Pd Se
Fabales	**3**/4 (75)	**113**/754 (15)	**1020**/20055 (5.2)	L Pt Sp Sm Pd Se
Fagales	**1**/8 (12.5)	**1**/33 (3.0)	**2**/1055 (0.19)	Br
Malpighiales	**13**/39 (33)	**136**/716 (19)	**1028**/15935 (6.5)	L Pt Sp Sm Pd Se
Oxalidales	**2**/7 (29)	**2**/60 (3.3)	**2**/1815 (0.17)	L
Rosales	**3**/9 (33)	**18**/261 (6.9)	**249**/7725 (3.2)	L Pt Sp Sm Se
Rosid II: Malvidae				
Brassicales	**2**/17 (12)	**3**/398 (0.75)	**6**/4765 (0.13)	L F
Crossosomatales	**1**/7 (14)	**1**/12 (8.3)	**1**/66 (1.5)	L
Malvales	**4**/10 (40)	**59**/338 (17)	**305**/6005 (5.3)	L Pt Sp Pd Se
Myrtales	**5**/9 (56)	**18**/380 (4.5)	**69**/11027 (0.63)	L Pt Sm Pd
Picramniales	**1**/1 (100)	**1**/2 (50)	**1**/46 (2.2)	L
Sapindales	**5**/9 (56)	**32**/471 (6.6)	**57**/6070 (0.92)	L Pt Sm Sp Se F
Other Core Eudicots				
Caryophyllales	**9**/34 (26)	**32**/811 (3.9)	**116**/11510 (0.95)	L Pt Sm Se Ae F
Ericales	**8**/25 (32)	**22**/346 (6.9)	**170**/11515 (1.5)	L Pt Se Pd
Santalales	**1**/13 (7.7)	**1**/151 (0.66)	**2**/1992 (0.10)	Pd

Saxifragales	**2**/15 (13)	**2**/112 (1.8)	**4**/2470 (0.16)	Se
Vitales	**1**/1 (100)	**3**/14 (21)	**8**/850 (0.94)	St Sm
Asterid I: Solanidae				
Gentianales	**3**/5 (60)	**37**/1118 (3.3)	**55**/16637 (0.34)	L Pt Sm Pd Se
Lamiales	**13**/23 (52)	**96**/1059 (9.6)	**292**/23810 (1.2)	L Pt Sm Pd Se F
Solanales	**2**/5 (40)	**19**/165 (12)	**107**/4080 (2.7)	L Pt Sm Pd Se
Asterid II: Asteridae				
Aquifoliales	**1**/5 (20)	**1**/21 (4.8)	**1**/536 (0.19)	L
Asterales	**3**/11 (27)	**22**/1743 (1.5)	**46**/26870 (0.19)	L Se
Dipsacales	**1**/7 (14)	**2**/45 (4.4)	**48**/1090 (4.4)	L Pt St

Estimating the total number of species with EFNs

Comparisons of frequency count estimation models based on searches of publication records suggest that the total number of plants with EFNs are best explained by a model with a finite mixture of three geometric distributions (Table 2). The goodness of fit statistics GOF5 (the corrected X^2 p-values) for the four best models were well over 0.01, indicating they displayed good fit to the data. The non-parametric and other parametric models (Poisson, inverse Gaussian, negative binomial, and log-normal mixed Poisson) provided inferior fits. The best fitting model, estimated the total number of species with EFNs to be 8,184 species (SE ± 392) species. The next three best fitting models provided slightly higher, but quantitatively similar, estimates of total number of species with EFNs (8,314 ± 460, 8,318 ± 492, and 8,482 ± 423, respectively).

Table 2. Predicted total number of species with EFNs from model estimates. Tau = the upper frequency cutoff, SE= the standard error of the estimate, lower and upper CB = the lower and upper 95% confidence bound, respectively. The model with the best overall fit is listed first (Best Model), followed by five alternative models. "Best, Model 2, Model 3" = top three selected parametric models; "Non-P 1-2" = top non-

parametric models, “1, 2, & 3 Mixed Exp. Poiss.” = Models with stochastic abundance distribution as a mixture of one, two or three exponentials and a two-geometrics mixed Poisson distribution; “SE” = standard error; “CB” = 95% confidence bound.

	Model	Tau	Estimated Total Sp	SE	Lower CB	Upper CB
Best Model	3 Mixed Exp. Poiss.	102	8184	392	7473.4	9013.9
Model 2	4 Mixed Exp. Poiss.	240	8318	492.8	7440.3	9379
Model 3	2 Mixed Exp. Poiss.	17	8483	423.4	7716.6	9380.6
Non-P 1	Chao1	2	5348	146	5081	5654.3
Non-P 2	ACE1	10	7336	281.4	6821.1	7926.1

Phylogenetic patterns

Plant species reported to possess EFNs are widely scattered across vascular plant lineages (Figure 2). The distribution of EFNs across vascular plants exhibited a moderate level of phylogenetic signal (D=0.56). Simulation tests indicated that, while the phylogenetic pattern differed significantly from the Brownian expectation (probability of estimating D under Brownian evolution < 0.001), it also differed significantly from 1 (probability of estimating D with random phylogenetic structure <0.001).

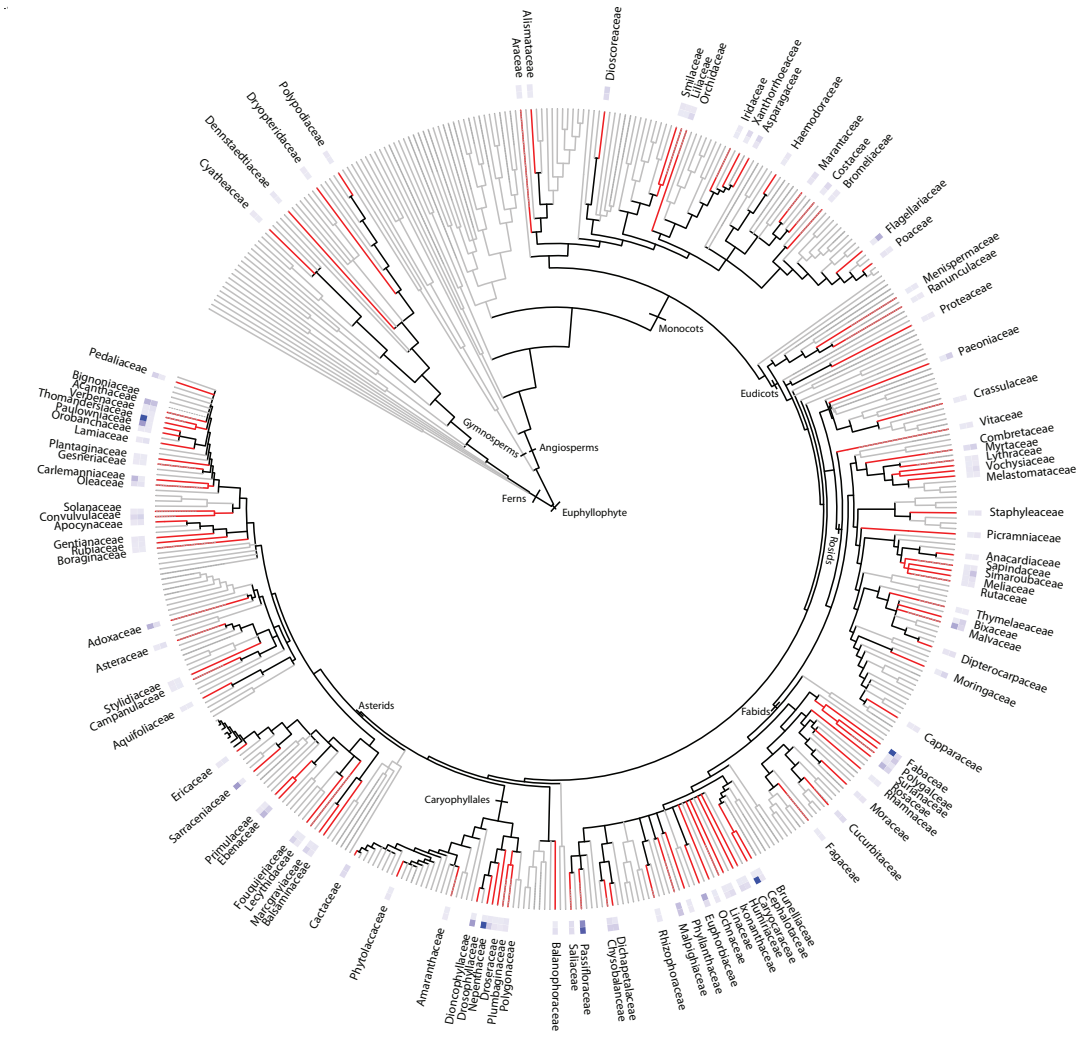

Figure 2: The phylogenetic distribution of plant families containing species reported to have EFNs (Angiosperm Phylogeny Working Group 2011). Branch lengths are according to fossil information from Wilkström *et al* (2001). Family names are only given for those families with EFNs. Ancestral branches leading only to families containing reports of EFNs are coloured in red. Branches whose daughters include EFNs and non-EFN families are indicated in black. Blue boxes surrounding the phylogeny are shaded according to the number of species with EFNs in that family (inner ring), and the percent of species with EFNs in that family (outer ring), with light shading representing low numbers and dark shading representing high numbers. The number of species with EFNs in a family (inner ring) range 1,069 species (Fabaceae) to 0 species. The percent of species with EFNs (outer ring) ranges from 100% (Cephalotaceae, Thomadersiaceae, Drosophyllaceae) to 0%.

Phylogenetic analyses strongly support a high number of repeated gain and loss of EFNs across plant clades. Parsimony methods revealed that a minimum of 457 independent gains and 41 losses are required to explain the distribution of EFNs across a broadly sample seed plant phylogeny. Using stochastic character mapping methods, however, the expected number of gains and losses was estimated to be much higher: 701 gains and 316 losses, respectively.

Discussion

The phylogenetic and taxonomic distribution of EFN reports in plants

To date, EFNs have been reported in ~1.0% to 1.8% of flowering plant species, depending on total plant species estimates (Govaerts 2001; Scotland and Wortley 2003). However, we estimate that the number of unreported cases of EFNs is as high as the number already reported, suggesting that in total, EFNs may be present in ~2.0% to 3.6% of flowering plants. Almost all the currently reported species with EFNs are angiosperms (99.7%, the remaining 0.3% being ferns) and 93% of those are eudicots. The majority of EFNs are found in two orders of rosids: the Malpighiales (26.0% of all EFN reports) and the Fabales (25.8% of all reports). Although EFNs are widespread, almost half of the angiosperm orders (33 out of 65) lack them, and EFNs appear to be entirely absent in gymnosperms, early angiosperms, and early ferns. Reports of species with EFNs represent at least 457 independent lineages that are widely distributed across vascular plants. Models of their evolution strongly support a high number of independent evolutionary gains and losses of EFNs across plant clades, especially in more derived eudicot families. Taken together, our study

confirms that EFNs are a relatively common and phylogenetically widespread plant trait. Their broad distribution, repeated evolution, and moderate phylogenetic signal across vascular plants are consistent with ecological research suggesting that selection and trait conservatism have a role in shaping their distribution.

Phylogenetic analyses using Smith's (2009) broadly-sampled vascular plant phylogeny revealed that the distribution of EFNs displays a moderate phylogenetic effect. We found that EFN evolution deviates significantly from the pattern expected under strict Brownian motion, a result that reflects the many single reports of EFN-bearing species in otherwise EFN-free genera, families or even orders. On the other hand, the distribution of EFNs across plants also deviates significantly from a pattern that is random in regards to phylogeny, and obvious instances of phylogenetic clustering do exist. Most notably, the majority of all species with EFNs (59%) are found in one major clade of rosids (Table 1). Extrafloral nectaries are also frequently phylogenetically clustered within smaller clades. For instance, they are present on all species of at least two small (non-monotypic) families (the Ebenaceae and the Thomandersiaceae), and in all, or virtually all, species of some genera (*Passiflora* Passifloraceae, *Inga* Fabaceae, *Populus* Salicaceae, *Gossypium* Malvaceae). In at least two genera with a mix of EFN and non-EFN species, clade-specific phylogenetic studies have demonstrated that the EFN bearing species form monophyletic groups (Senna Marazzi and Sanderson 2010; Viburnum Weber et al. 2012). Additionally, in some families and genera—for example Fabaceae, Polygonaceae, and *Senna*,—there is enough clustering of plants with EFNs to utilize the trait as an informative taxonomic character (Fryxell 1978; Marazzi et al. 2006; Sanchez et al. 2009).

Our phylogenetic assessment strongly agrees with previous work suggesting that EFNs are an evolutionarily "labile" trait that have evolved convergently many times in plants (Bentley 1977; Elias 1983; Koptur 1992). Furthermore, EFNs repeatedly arise on the same plant parts in distantly related clades, particularly the stipules, bracts, lower leaf surface, leaf margins, and leaf petioles (see Table 1). This pattern is striking, and future investigations into its causes (i.e., adaptation, constraint) are warranted. While the results described here offer a first estimation of the total number of evolutionary events needed to explain EFN distributions in plants, they should be considered as rough estimates of the actual number of EFN evolutionary gains and losses that have occurred during the evolutionary history of living plant lineages. Ideally, the accumulation of targeted studies conducted at narrower taxonomic scales, with finer phylogenetic resolution, will further refine our understanding of the precise number and type of evolutionary transitions that have occurred in EFN evolution across the vascular plant phylogeny.

Future directions in studying EFN evolution

The list of plant taxa with EFNs has been steadily growing since the first reports before 1900. Here, new published reports of EFN species along with changes in taxonomy bring the list up to 108 families of flowering plants containing 745 genera. The accumulation of this type of character and natural history information is crucial for synthetic investigations of their distribution and evolutionary history. As new species with EFNs continue to be discovered, our understanding of their broad distributional patterns will undoubtedly continue to shift. Indeed, model selection

methods estimate that that the number of unreported cases of EFN may be higher than the number of species already reported. Thus, we suggest that studies documenting EFN presence and absence in additional plant groups will be particularly valuable in the future of EFN evolutionary biology (Table 3). These studies will be particularly important in large families already rich in plants reported to have EFNs, such as the Bignoniaceae (140 out of 800 species reported with EFN), Euphorbiaceae (286 out of 5,735 species), and Malvaceae (293 out of 4425 species). We hypothesize these already EFN-rich clades will prove to contain the bulk of as yet unreported EFN taxa. Inconspicuous EFN morphologies are also likely underrepresented in the literature, and studies that systematically check for specific EFN structures or locations (such as formless or small laminar EFN, Figure 1) will likely be successful in uncovering additional taxa.

Table 3. Areas in need research in the study of EFN evolution and distribution in plants. Examples of specific hypotheses for each area are provided, along with a suggested approach and citations of example studies, where available.

General Area	Examples of hypotheses	Suggested Approach
Elucidating patterns of EFN evolution	(1) EFNs have a higher rate of evolution in certain clades (e.g, legumes, McKey 1989)	Evaluate EFNs presence or confirm absence on herbarium and live specimens, place distribution into an explicit phylogenetic context (e.g., Marazzi et al. 2006; Marazzi and Sanderson 2010), fit multi-rate and single-rate models of character evolution.
Ecological and environmental drivers of EFN evolution	(1) EFNs are tropical adaptations (Koptur 1992) (2) EFNs evolve in response to resource availability (Heil and McKey 2003; McKey 1989; Schupp and Feener 1991) (3) Mutualist abundance and/or aggression is a driver of EFN gain/loss (see Keeler 1985; Schupp and Feener 1991) (4) EFNs evolved as defences against ant-homoptera mutualisms (Becerra and Venable 1989)	Comparative phylogenetic studies that test for correlations between EFNs and putative adaptive factor (e.g., Weber et al. 2012). Are origins and losses in EFNs correlated with: Moves into and out of the tropics? Carbon-rich habitats with continuously flushing leaves? Habitats with high mutualist abundance/aggression? Susceptibility to ant-tended homoptera? A coupling of identifying phylogenetic patterns and experimental manipulations may be particularly useful in untangling ecological drivers of EFN evolution (Weber and Agrawal 2012).
Evolutionary interactions between EFNs and other plant	(1) EFNs are more likely to evolve in vines than other growth habits (Bentley 1977; Koptur 1992)	Comparative phylogenetic studies that test for correlations between EFNs and traits while accounting for shared history (e.g., Rudgers et al.

constraints)	Stewart and Keeler 1988) (3) Some EFNs, especially small laminar EFNs have evolved with mite domatia, providing *Bed and Breakfast* for predaceous and fungivorous mites (Weber et al. 2012).	while positive correlations suggest synergisms or adaptive trait pairings.
Assessing EFN origin and homology among nectary types	(1) EFNs within eudicots are homologous and share common genetic controls (Lee et al. 2005a) (2) EFNs and floral nectaries are homologous (3) Hydathodes are evolutionary precursors to EFNs (Elias and Gelband 1977)	Phylogenetically informed developmental genetics (e.g., Lee et al. 2005a)

Whereas the overall number of plants reported to have EFNs will undoubtedly increase with additional surveys, some reports of EFNs may eventually prove to be false positives. Not all extrafloral plant glands are nectaries (hydathodes and lacticifers, in particular, have been confused with EFNs) and tests for sugar secretion were not always performed before reporting a gland as an EFN (for an example of sugar testing, see Pemberton 1998). Thus, additional studies documenting sugar secretion will be necessary. Further, because the functions of the majority of EFNs have not been studied, most current discussions (including this one) can not distinguish between EFNs that function in plant defence and those that reward pollinators (e.g., in Euphorbia and Australian acacias, Knox et al. 1985) or attract prey (e.g., on carnivorous plants such as Droseraceae, Nepenthaceae, and Sarraceniaceae). Future analyses should consider how the phylogenetic distribution of EFNs relates to variation in their functions.

Studies that pair clade-specific surveys of EFN presence and absence with phylogenetic comparative analyses hold particular promise for revealing the drivers of EFN evolution (Table 3). For example, testing for phylogenetic correlations between EFNs and ecological factors hypothesized to influence EFN evolution (such as nutrient availability, ant abundance or aggressiveness; Bentley 1977; Heil and McKey 2003; Schupp and Feener 1991) can evaluate whether evolutionary patterns are constant with ecological adaptation hypotheses (e.g., Weber et al. 2012). Similarly, phylogenetic model testing can be used to ask whether EFNs are more likely to evolve in clades with certain plant traits than clades without those traits. This approach can be applied to the investigation of trade-off hypotheses (e.g. indirect defensive traits,

Rudgers et al. 2004), or tests of predispositions (e.g., EFNs evolve more frequently in vines than in other growth habits, Bentley 1977). Studies that test for these patterns across multiple independent clades will be particularly influential in revealing general drivers of EFN evolution. Indeed, many plant clades with species reported to have EFNs already have published phylogenies available that could be used in comparative analyses of this sort (Table 4).

Table 4: Examples of genera with EFNs and published phylogenies, good candidates to include in replicated phylogenetic comparative studies of EFN evolution.

Genus	**Family**	**Most recent published phylogeny**
Ruellia	Acanthaceae	(Tripp et al. 2008)
Viburnum	Adoxaceae	(Clement and Donoghue 2011)
Philodendron	*Araceae*	*(Gauthier et al. 2008)*
Impatiens	Balsaminaceae	(Janssens et al. 2006)
Catalpa	Bignoniaceae	(Li 2008)
Centaurea	Compositae	(Garcia-Jacas et al. 2001)
Helianthus	Compositae	(Timme et al. 2007)
Dioscorea	Dioscoreaceae	(Wilkin et al. 2005)
Shorea	Dipterocarpaceae	(Kamiya et al. 2005)
Croton	Euphorbiaceae	(Berry et al. 2005)
Mallotus	Euphorbiaceae	(Sierra et al. 2010)
Manihot	Euphorbiaceae	(Chacun et al. 2008)
Acacia	Fabaceae	(Miller and Bayer 2001)
Senna	Fabaceae	(Marazzi and Sanderson 2010)
Byttneria	Malvaceae	(Whitlock and Hale 2011)
Gossypium	Malvaceae	(Cronn et al. 2002)
Hibiscus	Malvaceae	(Pfeil et al. 2002)
Ficus	Moraceae	(Jousselin et al. 2003)
Adenia	Passifloraceae	(Hearn 2006)

Turnera	Passifloraceae	(Truyens et al. 2005)
Prunus	Rosaceae	(Shaw and Small 2004)
Populus	Salicaceae	(Cervera et al. 2005)

Finally, an understanding of the drivers of macroevolutionary patterns in EFNs will require assessments of trait homology across variable EFN forms. Most research to date on this topic has focused on orthologs of the *CRABS CLAW* gene, which is necessary for nectary development in *Arabidopsis* (Baum et al. 2001; Bowman and Smyth 1999; Lee et al. 2005b). Indeed, *CRABS CLAW* holds promise for unravelling the genetic control of EFN across the core eudicots. Lee *et al*. (2005a) found that *CRABS CLAW* is conserved across species of rosids and asterids, despite their having morphologically different nectary structures (floral and extrafloral). This pattern suggests that EFN in core eudicots may share a common ontology despite being highly modified over evolutionary time. However, there is no evidence of *CRABS CLAW* activity the EFNs of basal eudicots (Lee et al. 2005a), and we are unaware of studies that examine this relationship in ferns or monocots. Furthermore, while *CRABS CLAW* is necessary and can be sufficient for floral nectary formation in core eudicots, nectary formation on non-floral tissue requires the modification of several genes other than *CRABS CLAW* (Lee et al. 2005a). Thus, an understanding of the genetic drivers of the origin, loss, and modification of EFN across the plant-tree of life will require more detailed evolutionary developmental genetic studies that incorporate non-eudicot clades (Table 3).

Conclusions

Extrafloral nectaries are relatively common and broadly distributed plant traits that often function to mediate ecologically widespread mutualistic interactions. They have originated and been lost a great number of times across vascular plants. They have evolved in ferns, monocotyledons and many eudicots, and repeatedly make evolutionary shifts onto similar locations (e.g., leaves, stipules, petioles) in disparate plant clades. Because of their widespread phylogenetic distribution and their ability to mediate mutualistic interactions between plants and arthropods, EFNs are a powerful trait for inclusion in comparative studies linking phylogenetic patterns to ecological hypotheses.

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CHAPTER 3

DEFENSE MUTUALISMS ENHANCE PLANT DIVERSIFICATION[1]

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Abstract: The ability of plants to form mutualistic relationships with animal-defenders has long been suspected to influence their evolutionary success, both by decreasing extinction risk and by increasing opportunity for speciation through an expanded realized niche. However, the hypothesis that defense mutualism consistently enhance plant diversification across lineages has not been well-tested due to a lack of phenotypic and phylogenetic information. Using a global analysis, we show that among the >100 vascular plant families in which species have evolved plant organs that recruit arthropod mutualists, extrafloral nectaries, there are two-fold higher diversification rates than in families that lack species with extrafloral nectaries. Zooming in on six distantly related plant clades, trait-dependent diversification models confirmed the tendency for lineages with extrafloral nectaries to display increased rates of diversification. These results were consistent across methodological approaches. Inference using reversible-jump MCMC to model the placement and number of diversification rate shifts revealed that these high EFN rates were driven by an increased number of positive rate shifts following EFN evolution as compared to sister clades, suggesting that EFNs may be indirect facilitators of diversification. This replicated analysis indicates that defense mutualisms put lineages on a path towards increased diversification rates within and between clades, and is concordant with the

hypothesis that deep macroevolutionary patterns of plant diversity are impacted by mutualistic interactions with animals.

Keywords: Mutualism, extrafloral nectaries, key innovation, lineage diversification rates, plant defense, macroevolution, phylogenetics, plant-insect interactions.

Significance Statement: Plants that provide food and housing to animals in return for defense against enemies are classic examples of mutualistic partnerships in nature. Here we show that the evolution of such plant-animal mutualisms also leads to a trajectory of accelerated accumulation of plant species in the lineages that participate in these cooperative interactions. We find that the evolution of plant organs (extrafloral nectaries) that facilitate mutualisms with animal defenders were repeatedly followed by increased rates of diversification across distantly related plant lineages. These results suggest that, by enabling ecological interactions with animals, the convergent evolution of relatively simple glands changed the course of plant evolution towards greater protection from pests and accelerated rates of biodiversity generation.

Introduction

Ever since the key innovation hypothesis was first proposed in the 1940s (Miller 1949; Simpson 1944), the origination of novel traits has been a popular yet controversial explanation for the exceptional disparity in species richness observed across clades in the tree of life. Despite decades of research linking traits to

diversification, we have remarkably few examples of traits that have been convincingly demonstrated to repeatedly spur diversification across independent, distantly related groups. Notable exceptions include a number of ecologically important traits mediating interactions between plants and animals (Farrell et al. 1991; Hodges 1997; Lengyel et al. 2009; Sargent 2004), suggesting that these interactions may be particularly important drivers of macroevolutionary patterns. Here, we test the hypothesis that plant defense mutualisms — a widespread and classically studied ecological interaction whereby plants provide food rewards to arthropod bodyguards in return for protection against natural enemies (Janzen 1966) —increase the evolutionary diversification rate of the plant lineages that participate in them. The morphological traits that mediate defense mutualisms represent well-studied examples of characters hypothesized to expand a plant's niche via interactions with mutualists and influence species success in various environmental contexts (Boucher 1985). Although the costs and benefits of participating in a defense mutualism are well-known (Heil and McKey 2003), the hypothesis that the ecological impact of defense mutualism leaves a predictable macroevolutionary signature, increasing lineage diversification within and among clades of plants, has only been examined in a single genus (Marazzi and Sanderson 2010).

Defense mutualisms may impact plant speciation and extinction rates via several mechanisms. Unlike the evolution of traits related to reproduction, which more intuitively impact lineage diversification (e.g., Barraclough et al. 1995; Sargent 2004), the direct mechanisms by which defense mutualisms are hypothesized to influence diversification are less obvious. One direct mechanisms is a decreased incidence of

damage and disease due to an enhanced defensive repertoire, which may allow for increased population sizes and, in turn, lower extinction rates (Farrell et al. 1991). Additionally, by expanding the realized niche (Bruno et al. 2003), defense mutualisms may broaden the range of habitats a plant can occupy (Marazzi and Sanderson 2010), thereby increasing instances of allopatric speciation.

However, in addition to these direct mechanisms, the evolution of mutualistic traits may also facilitate diversification indirectly. First, if niche expansion results in the successful occupation of more environments, mutualistic traits may increase the probability a lineage will encounter conditions ripe with ecological opportunity (e.g., new adaptive zone), which in turn will drive increases in diversification. In other words, the evolution of a trait may *enable* subsequent diversification via increasing exposure to new environments, some of which will harbor external drivers of radiation, such as the uplift of a mountain range or unoccupied niches. Second, the evolution of defense mutualisms may free up resources for the plant and thereby facilitate the evolution of other innovative traits that subsequently enhance diversification. These indirect effects need not be contingent on the existence of the direct effects mentioned above, and represent a largely overlooked hypothesis concerning how traits can impact diversification (De Queiroz 2002; Donoghue 2005).

We suggest that indirect impacts of trait evolution on diversification should be reflected in a phylogenetic pattern where the origination of the trait is followed by an increased probability of subsequent, downstream rate shifts relative to clades that lack the trait (Figure S1). Because the indirect effect of the trait is contingent upon additional conditions (e.g., ecological opportunity, the evolution of another trait), there

may be a substantial lag between the origin of the trait and rate shifts. Alternatively, a direct effect of the trait on diversification rate is consistent with a pattern whereby a sustained rate shift occurs concomitantly with, or on the same branch as, the origin of the trait on the phylogeny (Figure S1). Direct and indirect patterns are not mutually exclusive, and both patterns may be detectable on a single phylogeny (Figure S1).

We focus on the macroevolutionary consequences of the repeated origination of extrafloral nectaries (EFNs) - nectar-secreting glands found on non-floral plant tissues that provide food for a wide array of beneficial bodyguard arthropods (Koptur 1992). EFNs are well-studied ecologically, with their only known function being defense against herbivores and microbial pathogens by attracting natural enemies (Bentley 1977). Such features have evolved hundreds of times and occur in about a quarter of all vascular plant families (Weber and Keeler 2013). Here, we first ask whether, across all vascular plants, families containing species with EFNs are associated with higher diversification rates than families without EFNs. We then focus in on the phylogenetic history and evolution of EFNs in six distantly related plant clades to evaluate whether EFNs are linked, directly or indirectly, to increased lineage diversification rates. As such, this study represents a replicated, multi-scale test of the macroevolutionary consequences of a convergently evolved and ecologically important mutualistic trait.

Results & Discussion:

In a global analysis of vascular plant families, we combined published records of EFN occurrence (Weber and Keeler 2013) with fossil calibrated mega-trees

(Angiosperm Phylogeny Group 2009; Wikström et al. 2001; Zanne et al. 2013) to compare net diversification rates across families with and without EFNs. Overall net diversification rates were >2-fold higher among the 108 families that contain instances of species with EFN species compared to the ~300 families without EFN species (Figure 1, Table S2). Because our current knowledge likely underestimates the number of families with EFNs by ~7% (Weber and Keeler 2013), we repeated this analysis with randomized inclusion of EFNs in otherwise non-EFN families, and found the initial result to be robust to missing information (Figure S2). Additionally, we found no evidence that EFN-bearing clades were older, suggesting they did not have more time to accumulate species than clades without EFNs (Figure S2). Finally, phylogenetic non-independence did not confound estimates of the relationship between EFNs and species richness, as the evolution of EFNs was significantly unstructured compared to the null expectation of Brownian motion evolution (APGIII: $D=0.74$, $p<0.001$; Zanne: $D=0.62$, $p=0.009$).

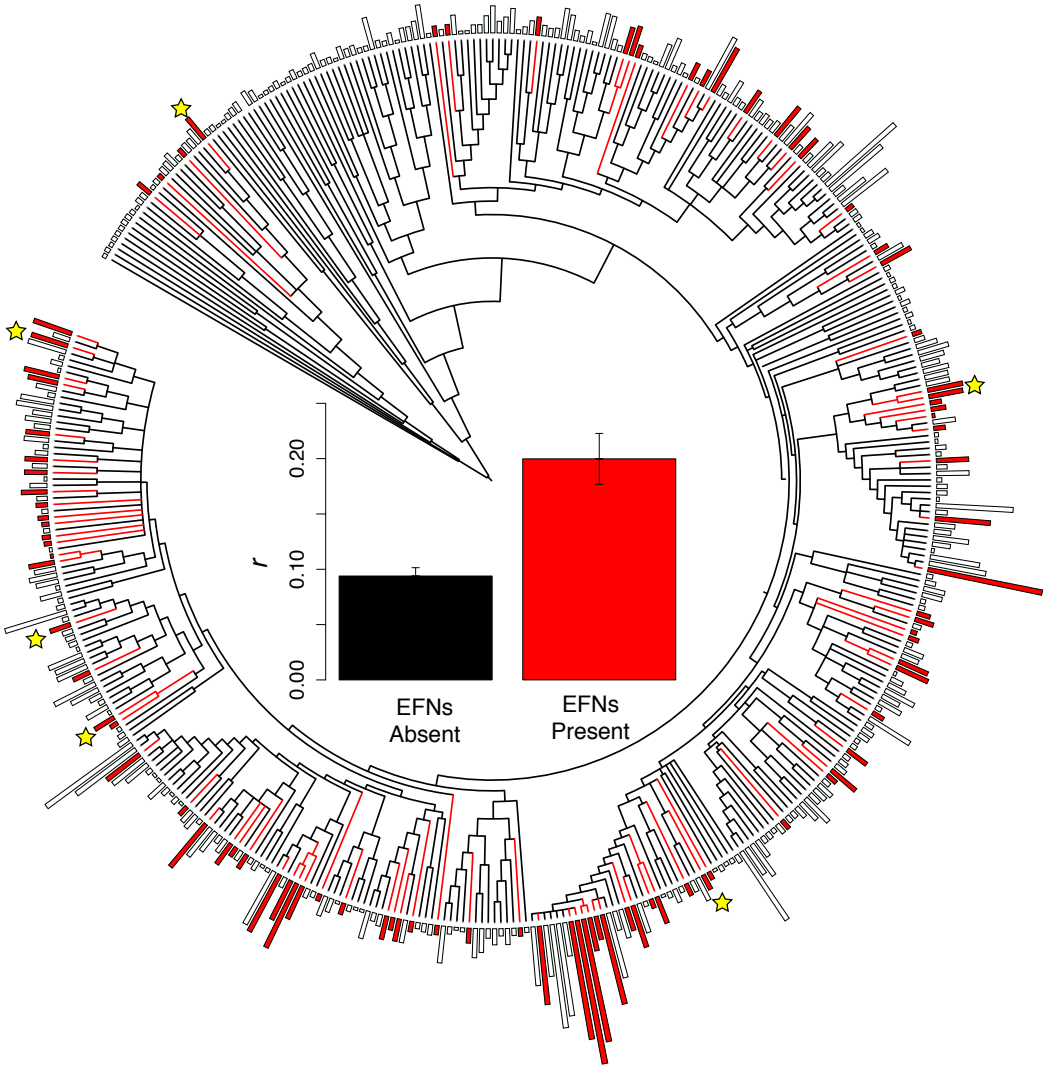

Figure 1: Phylogeny of vascular plant families (APGIII, Angiosperm Phylogeny Group 2009), with families containing species with EFNs colored red. Outer bars correspond to the age-standardized number of species (i.e., (number of species)/(age of plant family in millions of years). Yellow stars mark the six clades analyzed subsequently in this study. Inset: mean diversification rate (r) ± standard error of families with and without species with EFNs calculated according to the method of Magallon and Sanderson (2001) assuming no extinction. For F and P statistics and calculations with additional extinction fractions for both megatrees, see Table S2.

Results from our global analysis are consistent with a pattern where, across the hundreds of independent origins of EFNs, there is a net positive effect of this

mutualistic trait on rates of species diversification. Nonetheless, these results should be interpreted with caution due to the scale of this analysis. In particular, at this broad level it is not possible to directly link shifts in diversification with the origin and loss of EFNs. Additionally, cases of EFNs may be more likely to be reported in speciose families simply because of their relatively large size, creating a sampling effect.

To address the limitations of the global analysis, and to examine the direct versus indirect evolutionary consequence of EFN evolution, we pursued analyses at a finer taxonomic scale by reconstructing the evolution of EFNs in six distantly related plant clades (stars in Figure 1): *Byttneria* (order Malvales), *Senna* (Fabales)*, Turnera* (Malpighaiales), *Viburnum* (Dipsacales), *Polygoneae* (Caryophyllales) and *Pleopeltis* (Polypodiales). We selected these clades because they had recently published phylogenies, were known to contain species with and without EFNs based on descriptions in the literature (Marazzi et al. 2006; Marazzi and Sanderson 2010; Mercedes Arbo and Espert 2009; Otto et al. 2009; Schuster et al. 2011; Weber et al. 2012; Weber and Keeler 2013; Whitlock and Hale 2011), and were distantly related to one another. For *Senna*, which was previously investigated for a link between EFN and increased diversification rates (Marazzi and Sanderson 2010), we added recently published records on EFNs in an additional clade (Marazzi et al. 2013). Together these six plant groups encompass over 350 million years of evolution since diverging from a common ancestor, contain a wide variety of growth forms and life-history strategies, and occupy diverse habitats globally.

For each lineage, we first investigated whether a macroevolutionary model invoking state-dependent diversification rates (BiSSE; FitzJohn et al. 2009; Maddison

et al. 2007) explained the phylogenetic distribution of EFNs and diversification patterns. We found that EFNs were associated with higher mean net diversification rates (speciation rate – extinction rate) compared with lineages lacking EFNs in all six plant groups (Figure 2). We assessed statistical significance according to the percentile of an observed zero difference in state dependent net diversification rates according to the post-burnin MCMC interval. Differences in rates were below the 0.05 (one-tailed) percentile for *Pleopeltis* ($p < 0.001$), *Turnera* ($p = 0.049$), and *Viburnum* ($p = 0.008$), below 0.1 for *Senna* ($p = 0.06$), and non-significant for *Polygoneae* ($p=0.13$), and *Byttneria* ($p = 0.43$) (Figure 2 inset). Combining the probabilities from the individual clades indicated that there was a significant overall positive association between EFNs and diversification rate ($Z=4.087$; $P<0.001$). In simulations where EFNs evolved independent of rate shifts, we found type-one error rates ranging from 3-34% for individual topologies (Figure S3). However, down-weighting the observed p-values by the probability of seeing observed results in simulations still resulted in an overall combined probability of less than 0.001 ($Z=4.045$). We also confirmed the BiSSE patterns using an alternative methodological approach which paired marginal ancestral state reconstructions of EFNs (FitzJohn 2012) with a recently developed reversible jump Bayesian framework for modeling diversification rates (BAMM; Rabosky 2014). Consistent with results from BiSSE, we found that branches subtending nodes reconstructed with a high probability of EFNs had higher mean net diversification rate estimates than branches lacking EFNs in the same four out of six lineages examined (Table 1). Overall, the broad pattern across clades is consistent with hypothesis that EFNs play a role in increased plant diversification.

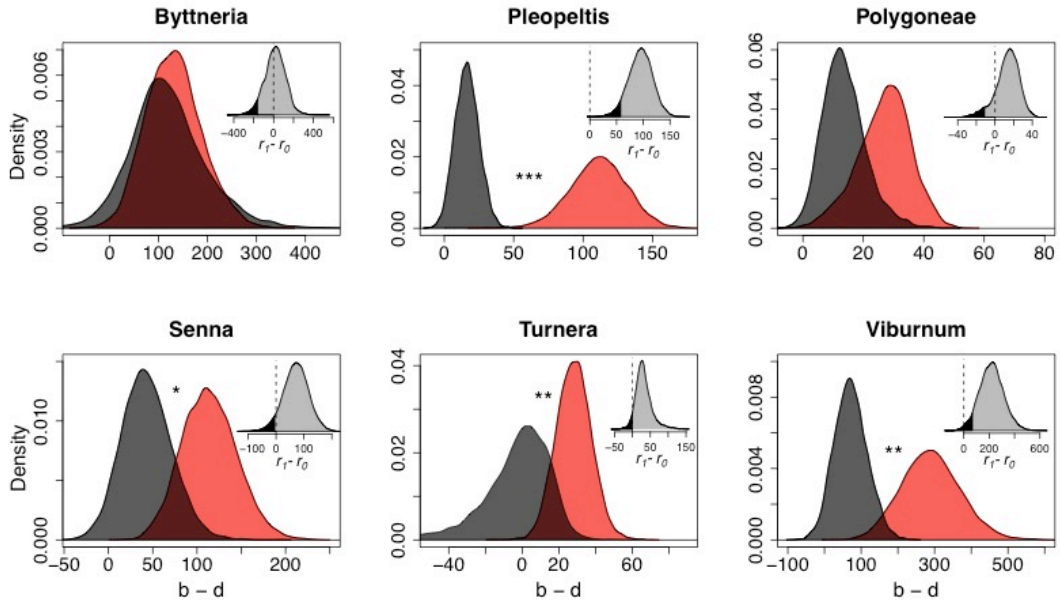

Figure 2: Marginal distribution of net-diversification rate (speciation – extinction) parameters in EFN present (red) and EFN-absent (black) clades from an analysis using the Bayesian implementation of BiSSE (FitzJohn et al. 2009; Maddison et al. 2007) on Maximum Clade Credibility trees with median node heights from BEAST analyses for each lineage. Significance symbols: *** >0.001, ** >0.5, * > 0.1. Inset histogram represents the joint marginal distribution of the difference between EFN and non-EFN diversification rates, with the >0.05% probability quantile shaded dark grey and a dotted line at zero.

To test whether the increased rates of diversification associated with EFNs showed patterns that supported direct or indirect effects on diversification, we utilized the BAMM framework (Rabosky 2014) to model the number and placement of rate shifts on each phylogeny with respect to the marginal probability of EFN presence or absence. We found that, across our six clades, rate shifts were rarely placed with high confidence on the same branch as EFN transition events. Instead, the shifts that were responsible for the increased net diversification rate in EFN clades commonly

occurred with some delay after the inferred origins of the trait (Figure 3). Additionally, consistent with the hypothesis that defense mutualisms are favored by natural selection and may not be easily lost, rates of EFN gain were estimated as higher than rates of EFN loss. In four of the six clades (*Viburnum, Senna, Pleopeltis, Polygoneae*), EFN loss was estimated as near zero, in one clade (*Byttneria*) the rate of gain was >3.5-fold higher than the rate of loss, and in one clade (*Turnera*) the rate of loss was estimated as higher than the rate of gain (Table S4, S5).

Table 1: Clade-specific mean (and standard deviation) net diversification rate, number of rate shifts/time, and shift density estimates obtained for EFN and non-EFN clades from BAMM. Sister clade comparisons were not possible for *Byttneria* and *Turnera* because of tree shape.

	Whole trees		Sister clades					
	r_1	r_0	Shifts /time$_{1sis}$	Shifts /time$_{0sis}$	r_{1sis}	r_{0sis}	Shift density$_{1sis}$	Shift density$_{0sis}$
Byttneria	172.25 (53.3)	**212.40 (105.24)**	-	-	-	-	-	-
Pleopeltis	**65.11 (29.56)**	31.5 (9.88)	**3.5 (2.45)**	3 (2.16)	**65.11 (21.18)**	46.19 (18.59)	1.8 (0.93)	**2.63 (1.36)**
Polygoneae	23.66 (7.26)	**33.41 (6.83)**	2.5 (1.87)	**5.5 (3.02)**	23.66 (7.26)	**39.38 (7.73)**	**2.08 (1.0)**	1.31 (0.41)
Senna	**113.54 (27.24)**	50.70 (28.15)	**4.6 (3.2)**	1 (1)	**113.54 (27.24)**	67.27 (39.34)	7.02 (2.93)	**30.78 (10.28)**
Turnera	**24.23 (6.42)**	18.22 (7.22)	-	-	-	-	-	-
Viburnum	**266.6 (67.92)**	114.4 (43.86)	**5 (3.32)**	2.5 (1.87)	**266.6 (67.92)**	184.42 (79.34)	20.02 (7.35)	**30.81 (12.79)**

In four groups, tree shape and the distribution of EFNs allowed for an additional comparison of the number of rate shifts that occurred in sister clades with and without EFNs. This allowed us to ask whether EFN clades contain more rate shifts than their non-EFN sister clades while controlling for clade age. Indeed, we found that in three of the four plant groups examined, rate shifts were estimated to have occurred

more frequently in EFN compared with non-EFN sister clades (Figure 3, Table 1). These same three groups displayed significant associations between EFNs and diversification rates in BiSSE analyses. The exception was *Polygoneae,* which was non-significant in the BiSSE analyses and displayed more rate shifts in the non-EFN lineage as compared to the EFN sister clade. Sister clade comparisons of net diversification rates in the four groups mirror these results and reveal the directionality of the shifts: in three groups (*Pleopeltis, Viburnum,* and *Senna*) sister clades with EFN had higher net diversification rates than sister clades without EFNs, while the opposite pattern was true for *Polygoneae* (Table 1). Because rate shifts are positive in these cases, each shift results in an increase in the total number of branches in EFN clades relative to non-EFN sister clades. Thus, while EFNs are associated with a higher total number of shifts, the density of rate shifts (number of shifts / total branch length) is not higher in these clades. Together, these phylogenetic patterns across plant groups are concordant with the hypothesis that EFNs are often indirect enablers of increased lineage diversification rates, as they are associated with higher diversification rates caused by increased instances of delayed positive rate shifts.

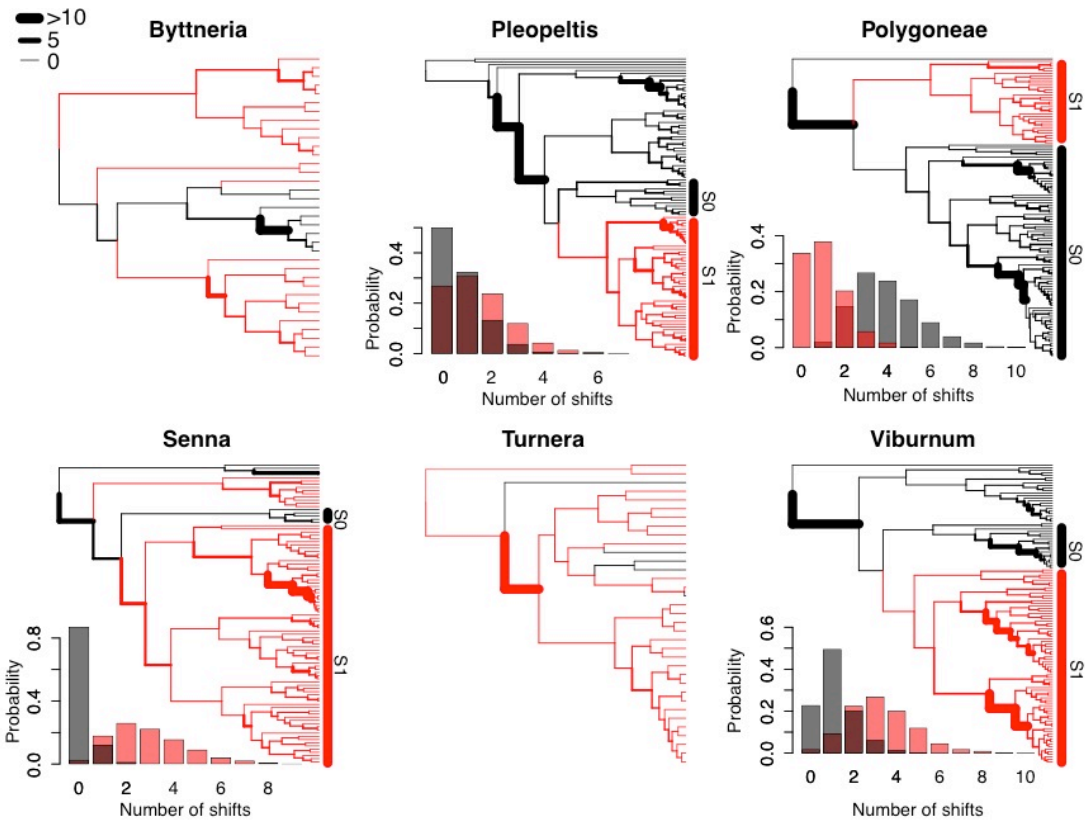

Figure 3: Diversification rate shifts in the EFN and non-EFN clades of six plant clades. For each group, the Maximum Clade Credibility trees are shown with branches subtending nodes with high marginal probability of EFNs reconstructed in red and branches subtending non-EFN nodes are shown in black. Branch widths are scaled to Bayes factors, representing confidence that a shift occurred on that branch. Bars to the left of phylogenies display the EFN (S1) and non-EFN (S0) sister clades used in sister-clade comparisons. Insert histograms display the posterior distribution of the number of shifts in the EFN clade (in red) and the sister non-EFN clade (in grey).

Our results within and among clades suggest that extrafloral nectaries, which are ecologically important, common, and functionally convergent traits across vascular plants, repeatedly set plant lineages on a path towards higher rates of lineage diversification. At fine phylogenetic scales, EFNs were generally associated with higher incidence of positive, but delayed, diversification rate shifts. This suggests that

EFN's facilitation of diversification may be contingent on other factors (Donoghue 2005), such as developmental differences in EFN types, the presence or absence of other morphological traits in the clades in which they are found, or the environmental conditions in which they occur. Thus, EFNs on their own may not be causally linked with immediate increased ecological opportunity**,** but rather may serve as indirect innovations, enabling the probability of subsequent shifts. Despite the fine-scale variation seen among the six clades studied here, we found a consistent pattern of EFNs evolving in families with higher diversification across all vascular plants. Thus, although the ecological impacts of EFNs are variable in space and time (Bronstein et al. 2006), macroevolutionary patterns of EFNs are consistent across phylogenetic scales.

Other traits hypothesized to increase diversification rates have also shown a delayed association with rate shifts (e.g., C4 photosynthesis (Spriggs et al. 2014), mammary glands (Bininda-Emonds et al. 2007), complete metamorphism (Nel et al. 2007)), suggesting that this pattern may be widespread. However, testing causal hypotheses that link a trait with a delayed set of rate shifts can be challenging due to the possibility of interceding traits and evolutionary transitions in the same area of the phylogeny. This is why evolutionary replication is key, as it increases our confidence that a particular trait may or may not be playing a role, as well as allows us to disentangle the complex ways in which traits interact with each other and the environment to impact diversification. For example, in the case of EFNs, clades such as *Polygoneae*, which displayed fewer shifts in EFN than non-EFN lineages, provide opportunities for testing hypotheses about the factors that explain differential effects

of EFNs on diversification rates (De Queiroz 2002; Weber and Agrawal 2012). Indeed, there are many reasons why the impact of a trait on diversification could be dampened or delayed based on ecological context. Nonetheless, for EFNs, our work within and among clades shows that over deep time, these important defensive traits may impact diversification and, ultimately, the diversity of plant species.

Methods:

Vascular plant family analysis

To test for a global association between defense mutualism and plant diversification, we compared net diversification rates of vascular plant families with and without species with EFNs. Families were scored as either containing or not containing accounts of at least one species with EFNs based on previous work (Weber and Keeler 2013). We utilized two published mega-tree phylogenies. The first was the APGIII mega-tree from the Angiosperm Phylogeny Group (Angiosperm Phylogeny Group 2009) with branch lengths adjusted according to fossil-based age estimates from Wikström *et al* (Wikström et al. 2001) using parametric rate-smoothing estimates with the program Phylocom (Webb et al. 2008) The APGIII tree is a compilation of previously published plant phylogenies and gives the most up-to-date estimate of relationships. We time-calibrated the APGIII phylogeny by adjusting branch lengths according to fossil-based age estimates from Wikström *et al* (Wikström et al. 2001) using parametric rate-smoothing estimates with the program Phylocom (Webb et al. 2008). The second was a rooted vascular plant mega-tree published by Zanne et al.

(Zanne et al. 2013), which was calibrated according to divergence time estimates from Soltis et al.'s (Soltis et al. 2011) broadly sampled molecular phylogeny and 39 fossil calibration points (Zanne et al. 2013). For analyses, this tree was trimmed so that each family was represented by only one tip using the drop.tip function in the R package APE (Paradis et al. 2004) which preserves topology and branch lengths. Genera were assigned to families according to the supplementary data in Zanne et al (Zanne et al. 2013).

We calculated net diversification rate using Magallon & Sanderson's (2001) method implemented in GEIGER (Harmon et al. 2008). We calculated rates based on species richness and median crown clade ages from both APG-III and Zanne trees. We repeated calculations for four values of *e*, the extinction rate expressed as a fraction of the speciation rate: 0, 0.1, 0.5 and 0.9. The number of species in each family was taken from Stevens (Stevens 2012), if a range of species counts for a family was reported, we used the highest number given. The difference in mean net diversification rate of families with and without EFNs was then analyzed by a two-way ANOVA. Because our current knowledge likely underestimates the number of families with EFNs by 4-9% (Weber and Keeler 2013), we repeated this analysis 10,000 times, each time converting 10 (an additional ~9% of current total) randomly selected EFN-absent families to EFN-present families in order to conservatively simulate the discovery of new families with EFN.

We tested for phylogenetic signal in the presence of species with EFNs in a family via the estimation of Fritz & Pervis' *D* for binary traits, which is a measure of sister-clade differences in a discrete character state for a given phylogeny (Fritz and

Purvis 2010). An estimated *D* of 1 implies a distribution that is random with respect to the phylogeny, where as a *D* of 0 implies a distribution expected under Brownian motion (Fritz and Purvis 2010). Using the R package caper (Orme et al. 2011), we calculated *D* for the presence of EFNs and, in order to assess significance, compared our estimate with simulated distributions of *D* under (1) randomly reshuffled trait values across the tips of the tree, and (2) trait evolution under Brownian motion. Each simulation included 10,000 permutations. This approach preserves the phylogenetic relationships of families, as well as the number of families assigned to each character state, while varying the distribution of character states across the tree.

Clade-level Analyses

We selected six vascular plant clades for phylogenetic comparative analyses that (1) had sequence representation in Genbank and (2) were known to contain species with and without EFNs based on descriptions in the literature: *Byttneria* (Malvaceae), *Pleopeltis* (Polypodiaceae), *Polygoneae* (Polygoneaceae), *Senna* (Fabaceae), *Turnera* (Passifloraceae) and *Viburnum* (Adoxaceae). Each of these clades represents an independent evolutionary origin of EFNs, and together they span a large portion of the angiosperm tree of life (Figure 1).

Phylogenetic inference

We reconstructed a distribution of time-calibrated phylogenies separately for each genus using Bayesian methods in order to include the highest possible number of species and because the most recently published phylogenies of our clades of interest were frequently not ultrametric. Sequence availability for each group was evaluated

and sequences were obtained from Genbank using PhyLoTA Browser (rel. 1.5) (Sanderson et al. 2008). Molecular markers were chosen for inclusion in phylogenetic analyses if they were sampled for over 30% of the species available in genbank (accession numbers deposited in Treebase #16059). Outgroup taxa were selected based on the most recent published phylogeny, or from the parent cluster in PhyLoTA based on overlapping sequence coverage with ingroup taxa. Nucleotide sequences were aligned using the L-INS-I strategy in MAFFT v.6 (Katoh and Toh 2008) with a gap opening penalty of 1.53 and a 0.0 offset value using the R (R Development Core Team 2012) package Phyloch (Heibl 2008). We trimmed aligned sequence ends to minimize missing data among taxa, and checked alignments by hand. We used jModeltest (Posada 2008) to determine appropriate substitution models for each partition based on Akaike's Information Criterion (AIC) and estimated starting parameters for Bayesian Inference implemented through the R package phanghorn (Schliep 2011).

For each of the six clades, we estimated the joint posterior distribution of topologies and relative node divergence times using three independent Bayesian MCMC searches in BEAST (Drummond et al. 2012). Each marker was partitioned with its own unlinked previously estimated substitution model. We utilized one uncorrelated exponential relaxed clock model to estimate node heights for all of the partitions. For each clade, three MCMC searches were run for 100,000,000 generations sampled every 10,000 generations using a random starting tree. Trees were rooted by constraining the in-group to be monophyletic. Convergence of each Bayesian run was assessed by plotting the log-likelihood of sampled trees and

parameters using Tracer v.1.5 (Rambaut and Drummond 2007). The first 25% of sampled trees were removed from each run as a burnin. A maximum clade credibility tree was identified from the combined output of the three MCMC runs using LogCombiner (Rambaut and Drummond 2012a) and TreeAnnotator (Rambaut and Drummond 2012b).

Character state assignment

Presence or absence of EFNs was coded as a discrete, binary character state. The distribution of EFNs within each clade was evaluated using previous publication records (Marazzi et al. 2006; Marazzi and Sanderson 2010; Mercedes Arbo and Espert 2009; Otto et al. 2009; Schuster et al. 2011; Weber et al. 2012; Weber and Keeler 2013; Whitlock and Hale 2011) and herbarium specimens from the Bailey Hortorium of Cornell University (BH), the herbaria of the Yale Peabody Museum of Natural History (YU), and digitized specimens in the JSTOR Global Plants database (JSTOR 2013). The world list of plants extrafloral nectary database can be accessed at www.extrafloralnectaries.org.

Lineage Diversification Analyses

We evaluated whether net diversification rates in each clade were dependent on EFN state using BiSSE (Maddison et al. 2007) and BAMM (Rabosky 2014). Outgroup taxa and multiple individuals per species were pruned from the trees for each analysis (maximum clade credibility trees and 1,000 trees randomly chosen from the posterior distribution of trees), so that resulting phylogenies contained only one sample per species.

We used the BiSSE (Maddison et al. 2007) state dependent speciation and extinction model to estimate net diversification (speciation - extinction) rates in lineages with and without extrafloral nectaries. We implemented the Bayesian BiSSE MCMC algorithm in the diversitree package in R (FitzJohn 2012). Because some species are missing from our phylogenies for each our six groups, we accounted for missing taxa in these analyses by including information on the proportions of taxa (included and missing) assigned to each character state (FitzJohn et al. 2009) using species descriptions and clade estimates from previous publications (Clement and Donoghue 2012; Marazzi and Sanderson 2010; Mercedes Arbo and Espert 2009; Otto et al. 2009; Schuster et al. 2011; Whitlock and Hale 2011). In cases where the character state of missing species was unknown, we assumed the proportions of character states in our known samples were representative (Table S3).

To test whether EFN and non-EFN lineages had different diversification rates, we utilized Bayesian MCMC BiSSE analyses on the maximum clade credibility tree for each clade using exponential priors for all parameters were estimated using the starting.point.bisse() function in diverstree with a mean of twice the state-independent net diversification rate. Initial models were fit using heuristic parameter starting points estimated from a constant-rate birth-death model. We assumed an MK model of evolution for the trait in all cases, despite the trait distribution. First, a primary MCMC was run for each clade for 1,000 generations with an arbitrary tuning parameter of 0.1. We used the posterior parameter distributions of these initial MCMC runs to estimate tuning parameters of the final MCMC analyses, which each ran for 10,000 generations. Significance was assessed according to the credible set of the differences

between state dependent net diversification rates. Finally, in order to gain insight into the probability of seeing our results if EFNs were evolving independent of rate shift location, we conducted ML-BiSSE analyses across the same topologies using simulated trait data. EFN tip states were simulated as discrete characters 100 times for each topology using an MK2 model according to parameter estimates fit using the observed trait data using the fitDiscrete function in the R package GEIGER (Harmon et al. 2008). Simulations with less than five species in each state were rejected in order to condition on having more than a small number of species in one state. Root state for simulations were determined using a random draw from a binomial distribution with a probability of successfully drawing a state proportional to the marginal of that state at the root in the ancestral reconstruction using the asr.bisse function in diversitree (FitzJohn 2012). We used a weighted Z-test (Whitlock 2005) to combine probabilities from the six BiSSE analyses, both with and without weights based on the 1/the probability of seeing our observed p-value in simulations (to account for type-1 error rate).

To examine (1) whether lineages with EFNs contained more rate shifts than lineages without EFNs, (2) whether EFN lineages had a higher density of rate shifts than non-EFN clades, and (3) where rate shifts occurred on phylogenies in relation to EFN origination or loss, we utilized BAMM (Bayesian Analysis of Macroevolutionary Mixtures) program (Rabosky 2014) and the package BAMMtools (Rabosky et al. 2014). For each clade, we performed three BAMM runs on the MCC phylogeny from the log-combined BEAST analyses in order to avoid getting stuck in local optima. Each BAMM was run for 100,000,000 MCMC generations, sampling parameters

every 50,000 generations. We accounted for incomplete sampling in each clade according to diversity estimates from publications (Clement and Donoghue 2012; Marazzi and Sanderson 2010; Mercedes Arbo and Espert 2009; Otto et al. 2009; Schuster et al. 2011; Whitlock and Hale 2011). We ran Bayesian MEDUSA-like models, where the rate of speciation and extinction were constant within shift regimes by setting the updateRateLabdaShift and lamdaShift0 parameters to 0. We computed tree appropriate rate priors using the setBAMMpriors function in BAMMtools, and utilized a flattened PoissonRatePrior of 0.1 and a minimum clade size for rate shifts (minCladeSizeforShift) of 2. We assessed convergence of the three BAMM runs for each clade by assuring the effective sample sizes of log-likelihoods, number of processes, and evolutionary rate parameters were greater than 500 using the CODA library (Plummer et al. 2006).

We assigned the presence or absence of EFNs to clades according to the probability of each state at internal nodes using the asr.marginal function in diversitree (FitzJohn 2012), which performs marginal reconstructions of ancestral states for each node. In order to account for potentially misleading effects of trait-associated diversification rates, we reconstructed ancestral states of EFNs under the BiSSE model, which was fit for each clade using the using the make.bisse and find.mle functions, with starting parameter guesses of the mean parameter estimates from mcmc BiSSE analysese. Using these marginal reconstructions, we asked whether branches subtending nodes with a high probability of being in the EFN state have a higher net diversification rate than branches in the non-EFN state. Diversification rates were calculated using the getcladerate() function in BAMMtools on BAMMobjects

pruned to include only EFN or only non-EFN taxa using the subtreeBAMM function.

We visualized the probability of rate shifts on branches of the tree by scaling the edge widths of each plotted phylogeny according to the Bayes factor associated with that branch using the bayesFactorBranches function. This method corrects for differences in branch length or biases introduced by the rate number prior distribution. We estimated the number of shifts in sister clades with and without EFNs for groups that had sister groups with and without EFNs that included at least 2 species each. We used the subtreeBAMM function to extract sister clades from the original BAMM objects. Shift density for each extracted sister subclade was calculated by dividing each sample from the subclade's posterior by the sum of branch lengths for that subclade.

Data availability

Phylogenies are deposited on TreeBase (#16059). Character states and genbank accession numbers are deposited in DataDryad. R scripts are available on request from the first author.

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APPENDIX

Chapter One Supplementary Materials:

Online Appendix A: Character and herbarium information for the 92 *Viburnum* species used in this study.

Specimens examined are from the personal collection of MJD (M) and the following herbaria: Cornell Baily Hortorium (BH), Yale Peabody Museum (YU), the Gray and Arnold Arboretum collections within the Harvard University Herbaria (HUH), Oregon State University (OSC), the New York Botanical Garden (NY), the Field Museum of Natural History (F), and the Missouri Botanical Garden (MO). Species are grouped into three habitat types, (1) "tropical," including wet subtropical to tropical forests, in generally mountainous regions but at lower elevations (generally <1,700 meters), and with limited temperature seasonality; (2) "cloud," including montane cloud forests at southern latitudes, at generally higher elevations (mostly >2000 meters), and experiencing periodic colder temperatures but not prolonged temperature seasonality, and (3) "temperate," including deciduous temperate and boreal forests with strong and prolonged seasonality. Species were also assigned to one of three leaf production categories: (1) "evergreen," in which plants maintain their leaves year-round and individual leaves last for more than a season; (2) "leaf exchangers," in which plants flush and lose their leaves asynchronously, and may have short, sporadic periods of leaflessness; and (3) "seasonally deciduous," in which plants synchronously lose their leaves for a prolonged period each year. * = gland checked for sugar on live specimen. ^{o} = species in common garden. ‡ = sooty mold present on preserved specimens. Domatia denoted as "Absent^{+}" represent species with densely tomentose abaxial leaf surfaces. "Pet-Mar" = petiole margin junction. A complete list of herbarium specimens sampled for this paper (n=405) is available from the authors.

Species	Herbaria (n)	EFNs	EFN Location	Leaf Domatia	Domatia Type	Habitat	Leaf Production Strategy
V. acerifolium L.*	BH (5)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. adenophorum Smith	HUH (1)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. amplificatum Kern	(Kern 1951)	Absent	Absent	Absent	Absent	Tropical	Evergreen
V. atrocyaneum Clarke	BH, HUH, MO (4)	Present	Margin	Absent	Absent	Temperate	Evergreen
V. awabuki Koch	BH, HUH, YU (4)	Absent	Absent	Present	Pit	Tropical	Evergreen
V. betulifolium Batal.	BH, HUH (5)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. blandum Morton	YU (1)	Present	Margin	Absent	Absent	Cloud	Leaf Exchangers
V. brachyandrum Nakai	HUH (1)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. brachybotryum Hemsl.	M (1)	Absent	Absent	Absent	Absent	?	Evergreen
V. bracteatum Rehd.	BH (3)	Present	Margin	Present	Tuft	Temperate	S. Deciduous
V. calvum Rehder	M, YU (2)	Present	Margin	Absent	Absent	Temperate	Evergreen
V. carlesii Hemsl.º	BH (5)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. cassinoides L.º	BH, M (8)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. caudatum Greenm.	M, YU (3)	Present	Margin	Present	Tuft	Cloud	Leaf Exchangers
V. chingii Hsu	M (1)	Absent	Absent	Absent	Absent	Temperate	Evergreen
V. cinnamomifolium Rehd.	BH, M (3)	Present	Margin	Present	Tuft	Temperate	Evergreen
V. clemensiae Kern	M (1)	Absent	Absent	Present	Pit	Tropical	Evergreen
V. colebrookeanum Wall.	BH, HUH, M (5)	Absent	Absent	Absent	Absent	Tropical	Evergreen
V. coriaceum Blume	BH, HUH, MO, YU (5)	Present	Lamina	Present	Tuft	Tropical	Evergreen
V. corymbiflorum Hsu	(Yang 1994)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. cylindricum Buch. Ham.	BH, M, YU (7)	Present	Lamina	Present	Tuft	Tropical	Evergreen
V. davidii Franchet*	BH (5)	Present	Margin	Present	Tuft	Temperate	Evergreen
V. dentatum L.º*	BH (6)	Present	Margin	Present	Tuft	Temperate	S. Deciduous
V. dilatatum Thurnb.º*	BH (6)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. discolor Benth.‡	M, MO, YU (9)	Present	Margin	Absent+	Absent+	Cloud	Leaf Exchangers
V. edule Michx. Raf.*	BH, M, OSC (9)	Present	Pet-Mar	Present	Tuft	Temperate	S. Deciduous
V. elatum Benth	BH, M (3)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. ellipticum Hook	BH, M (6)	Present	Margin	Present	Tuft	Temperate	S. Deciduous
V. erosum Thunb.	BH, M (7)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. erubescens Wall.	BH, M (6)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. farreri Stearn	BH (3)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous

V. flavescens Smith	HUH (2)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. foetidum Wall.	BH, NY (5)	Present	Lamina	Present	Tuft	?	?
V. furcatum Blume	BH (5)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. hartwegii Benth	BH, MO, F (6)	Present	Margin	Present	Tuft	Cloud	Leaf Exchangers
V. hebanthum W. & A.	HUH, NY (3)	Present	Lamina	Present	Tuft	Tropical	Evergreen
V. hupehense Rehder‡	BH (4)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. ichangense Rehder	BH, HUH, (5)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. inopinatum Craib.	HUH, MO, M (3)	Present	Lamina	Absent	Absent	Tropical	Evergreen
V. jamesonii Oerst.‡	HUH, MO (5)	Present	Margin	Absent^{+}	Absent^{+}	Cloud	Leaf Exchangers
V. japonicum Spreng.	BH, M (7)	Present	Lamina	Present	Tuft	?	Evergreen
V. jucundum Morton	HUH, MO, NY (6)	Present	Margin	Absent^{+}	Absent^{+}	Cloud	Leaf Exchangers
V. kansuense Batalin	HUH (2)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. koreanum Nakai	(Yang 1994)	Present	Pet-Mar	Present	Tuft	Temperate	S. Deciduous
V. lantana L.^{o}	BH (7)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. lantanoides Michx.	BH (5)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. lautum Morton	M (1)	Present	Margin	Present	Tuft	Cloud	Leaf Exchangers
V. lentago L.^{o}	BH (7)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. lepidotulum Merrill	MO (2)	Absent	Absent	Absent	Absent	Tropical	Evergreen
V. lobophyllum Wilson	BH, HUH (5)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. loeseneri Graebn.	NY (1)	Present	Margin	Absent^{+}	Absent^{+}	?	Leaf Exchangers
V. lutescens Bl.	BH, HUH (5)	Absent	Absent	Absent	Absent	Tropical	Evergreen
V. luzonicum Rolfe	HUH, MO (5)	Present	Lamina	Present	Tuft	?	?
V. macrocephalum Fort.^{o}	BH, HUH (5)	Absent	Absent	Absent	Absent	Temperate	?
V. melanocarpum Hsu	(Yang 1994)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. molle Michx.	BH (5)	Present	Margin	Present	Tuft	Temperate	S. Deciduous
V. mongolicum Rehder	BH, HUH (4)	Absent	Absent	Present	Cave	Temperate	S. Deciduous
V. nervosum D. Don	BH, M, MO (5)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. nudum L.^{o}	BH (7)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. odoratissimum Ker.Gawler	BH (6)	Absent	Absent	Present	Pit	Tropical	Evergreen
V. oliganthum Batal.	BH, HUH (3)	Absent	Absent	Absent	Absent	Temperate	Evergreen
V. opulus L.^{o}*	BH, YU (7)	Present	Petiole	Present	Tuft	Temperate	S. Deciduous
V. orientale Pall.	BH, YU (3)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. plicatum Thurnb.^{o}	BH, HUH, YU (5)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. propinquum Hemsl.	BH (5)	Present	Margin	Present	Tuft	?	Evergreen
V. prunifolium L.^{o}	BH (7)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. punctatum Buch. Ham	HUH (5)	Absent	Absent	Absent	Absent	Tropical	Evergreen
V. rafinesquianum Schult.^{o}	BH (7)	Present	Margin	Present	Tuft	Temperate	S. Deciduous
V. rhytidophyllum Hemsl.	BH, HUH (5)	Absent	Absent	Absent^{+}	Absent^{+}	Temperate	Evergreen
V. rigidum Vent.‡	BH, YU (5)	Present	Margin	Present	Tuft	Temperate	Evergreen

V. rufidulum Raf.	BH (5)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. sargentii Koehne[o]*	BH (8)	Present	Petiole	Present	Tuft	Temperate	S. Deciduous
V. schensianum Maxim.	BH, HUH (3)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. sempervirens Koch.	BH (5)	Present	Lamina	Present	Tuft	?	Evergreen
V. setigerum Hance[o]*	BH (7)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous
V. sieboldii Miq.[o]	BH (7)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. stenocalyx Hemsl.	BH, MO, NY, YU (7)	Present	Margin	Present	Tuft	Cloud	Leaf Exchangers
V. subalpinum Hand. Mazz.	HUH (1)	Absent	Absent	Absent	Absent	Temperate	Deciduous
V. sulcatum Hemsl.	M (2)	Present	Margin	Present	Cave	Cloud	Leaf Exchangers
V. suspensum Lindl.	BH (6)	Absent	Absent	Present	Tuft	?	Evergreen
V. sympodiale Graebn.	HUH (2)	Absent	Absent	Present	Tuft	Temperate	S. Deciduous
V. taiwanianum Hayata	BH, HUH (5)	Absent	Absent	Present	Cave	Temperate	S. Deciduous
V. ternatum Rehder	MO (2)	Present	Lamina	Present	Tuft	Tropical	S. Deciduous
V. tinus L.*	BH, YU (8)	Present	Margin	Present	Tuft	Temperate	Evergreen
V. toronis Killip & Smith‡	MO (3)	Present	Petiole	Present	Tuft	Cloud	Leaf Exchangers
V. trilobum Marshall[o]*	BH (7)	Present	Petiole	Present	Tuft	Temperate	S. Deciduous
V. triphyllum Benth.	HUH, MO (5)	Present	Margin	Present	Tuft	Cloud	Leaf Exchangers
V. urceolatum Hara	BH, HUH (6)	Absent	Absent	Present	Cave	Temperate	S. Deciduous
V. utile Hemsl.	BH, HUH (3)	Absent	Absent	Absent[+]	Absent[+]	Temperate	Evergreen
V. veitchii Hemsl.	BH (4)	Absent	Absent	Absent	Absent	Temperate	S. Deciduous
V. wrightii Miq.	BH, HUH (5)	Present	Lamina	Present	Tuft	Temperate	S. Deciduous

Chapter Three Supplementary Materials:

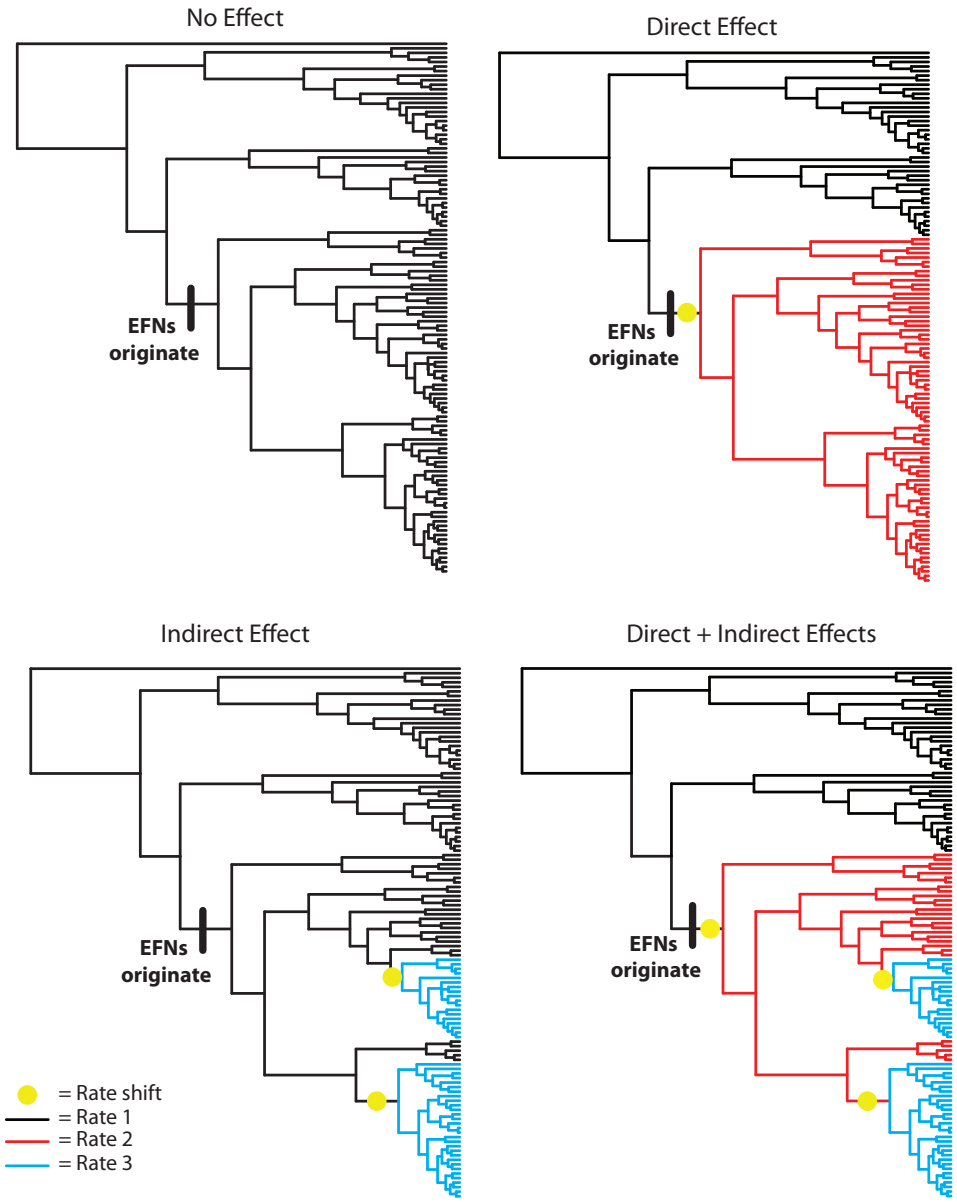

Figure S1. Phylogenetic patterns consistent with about direct and indirect effects of EFNs (or any trait) on lineage diversification. State dependent analyses such as BiSSE compare no effect versus net changes in diversification (top row); a net change in diversification may be due to direct or indirect mechanisms. Models estimating the number and placement of rate shifts on a topology, such as BAMM, place shifts without expectations from a priori hypotheses (lower row), thus having the ability to distinguish between direct and indirect hypotheses based on the timing and number of rate shifts.

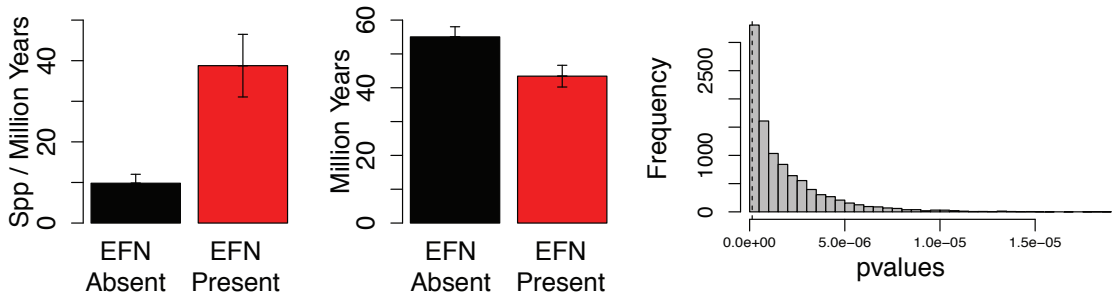

Fig. S2. Mean (± standard error) species per million years (A), and mean (± standard error) age (B), of families without EFNs (black) versus with EFNs (red). (C) Histogram of p-values from 10,000 tests that each randomly "discover" EFNs in ten non-EFN families. Dotted line marks the p-value from the non-simulated test.

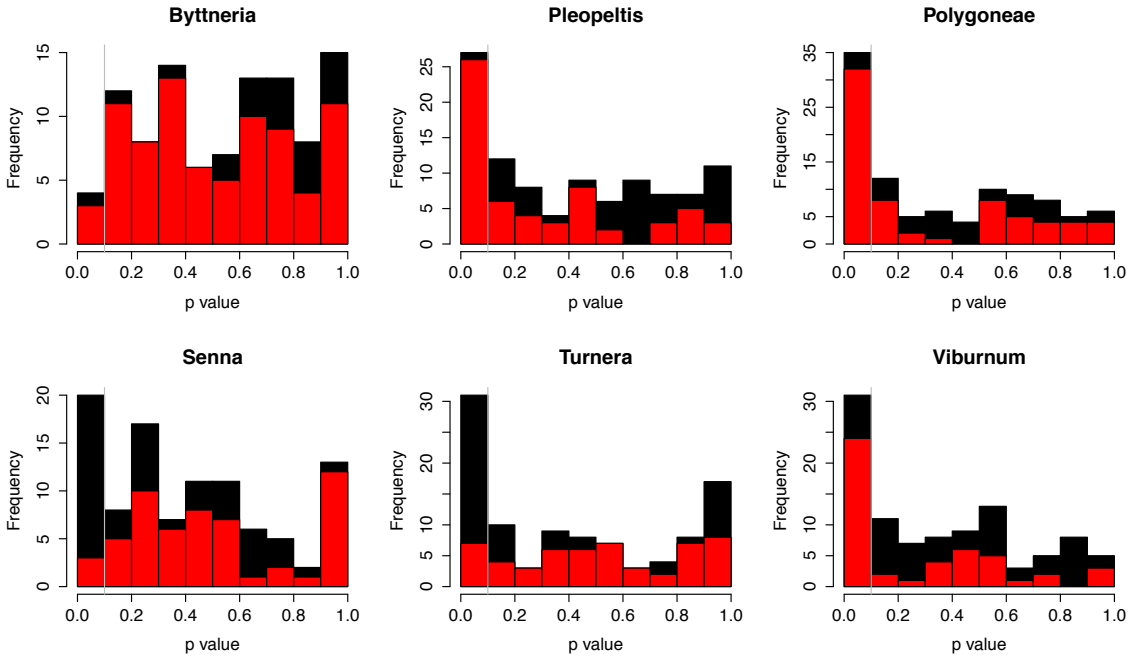

Fig. S2. Distribution of p-values from ML-BiSSE analyses on simulated data. Black bars represent the total frequency of p-values from the simulations (regardless of the directionality of the outcome). Red bars represent only those simulations where EFN rates were higher than non-EFN rates. Grey line marks the 0.1 significance level.

Table S2: Relationship between net diversification rates and the presence of species with EFNs in a family. Diversification rates were calculated following the method of Magallon and Sanderson (2001) assuming no extinction (e=0), and extinction fractions of 0.1,0.5, and 0.9. Ages were derived from the APGIII megatree (above) and the Zanne et al, 2014 megatree (below). In all analyses, diversification rates are higher in families that contain at least one species with EFNs. Mean diversification rates (standard errors) for families that contain instances of EFNs (+) and those that do no (-), F statistics, and P values are reported for each test.

APG-III

e	**+ EFN**	**- EFN**	**F** (1, 423)	**P**
0	0.2 (0.023)	0.094 (0.008)	32.06	2.76e-08***
0.1	0.199 (0.023)	0.094 (0.008)	32.09	2.73e-08***
0.5	0.194 (0.022)	0.1 (0.007)	26.05	5.24e-07***
0.9	0.143 (0.017)	0.066 (0.005)	29.49	9.98e-08***

Zanne et al. 2014 Megatree

e	**+ EFN**	**- EFN**	**F** (1,414)	**P**
0	0.084 (0.005)	0.039 (0.002)	80.62	*<2e-16***
0.1	0.083 (0.005)	0.039 (0.002)	80.67	*<2e-16***
0.5	0.082 (0.005)	0.04 (0.002)	71.42	6.2e-16***
0.9	0.061 (0.004)	0.027 (0.002)	82.11	*<2e-16***

Table S3: Number of species included in the phylogenetic analyses for each clade, along with estimates for total numbers of species. Numbers of species with and without EFNs EFNs are included parenthetically.

	Phylogeny *(EFN+/EFN-)*	**Total** *(EFN+/EFN-)*
Byttneria	**35** *(27/8)*	**204** *(141/63)*
Pleopeltis	**125** *(35/90)*	**173** *(75/98)*
Polygoneae	**104** *(49/55)*	**160** *(89/71)*
Senna	**94** *(85/9)*	**400** *(362/38)*
Turnera	**35** *(30/5)*	**137** *(129/8)*
Viburnum	**117** *(71/41)*	**165** *(103/62)*

Table S4: Summary of parameter estimates from post-burnin MCMC for BiSSE analyses. The mean (and standard deviation) are reported. EFN absent = 0, EFN present = 1.

	Byttneria	*Pleopeltis*	*Polygoneae*	*Senna*	*Turnera*	*Viburnum*
λ_0	232.9 (85.97)	128.56 (27.58)	77.44 (13.34)	144.51 (57.58)	15.4 (11.2)	456.75 (108.47)
λ_1	288.07 (78.79)	146.12 (23.59)	39.62 (8.42)	387.92 (74.28)	48.6 (11.2)	760.13 (138.1)
μ_0	115.6 (95.45)	112.19 (30.96)	63.77 (16.81)	101.14 (68.22)	18.66 (17.57)	385.24 (128.79)
μ_1	157.32 (103.45)	35.03 (28.67)	12.73 (11.19)	273.79 (92.55)	20.46 (14.91)	470.07 (184.43)
q_{01}	54.13 (42.69)	0.56 (0.48)	0.44 (0.36)	18.54 (13.51)	11.22 (12.51)	7.38 (6.5)
q_{10}	10.82 (11.28)	2.68 (2.64)	1.39 (1.47)	1.83 (1.85)	12.37 (8.9)	5 (5.16)
p_1	85.35 (2.08)	282.17 (2.1)	304.76 (2.22)	298.01 (2.03)	27.69 (2.27)	486.22 (1.94)
r_1-r_0	13.46 (103.92)	94.72 (22.15)	13.23 (12.55)	70.75 (44.77)	31.39 (23.3)	218.55 (93.48)

Table S5: Summary of results from from post-burnin MCMC for BAMM analyses. The mean (and standard deviation) are reported. EFN absent = 0, EFN present = 1.

	Byttneria	*Pleopeltis*	*Polygoneae*	*Senna*	*Turnera*	*Viburnum*
λ_0	644.56 (247.65)	*90.02 (21.67)*	64.32 (10.4)	233.51 (75.93)	41.30 (14.01)	418.37 (101.61)
λ_1	569.4 (163.55)	146.97 (29.56)	44.37 (10.39)	360.80 (78.53)	51.91 (12.22)	591.77 (97.47)
μ_0	432.15 (283.84)	58.52 (27.12)	30.9 (13.82)	182.8 (80.97)	23.08 (15.44)	304.17 (121.68)
μ_1	397.14 (187.07)	81.86 (39.55)	20.71 (12.77)	247.26 (93.18)	27.67 (14.4)	325.15 (129.5)
q_{01}	14.71	0.31	0.25	6.33	3.73	3.087
q_{10}	4.69	<0.001	0.001	<0.001	8.04	<0.001
r_1-r_0	-40.15 (107.74)	33.61 (20.02)	-9.76 (8.93)	62.83 (35.57)	6.01 (6.55)	152.43 (70.2)