THE EVOLUTIONARY ECOLOGY OF AN INSECT-FUNGUS INTERACTION: *BOTRYOSPHAERIA DOTHIDEA*, SYMBIOTIC WITH THE GOLDENROD-GALLING MIDGE *ASTEROMYIA CARBONIFERA* (DIPTERA: CECIDOMYIIDAE)

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To my family, Michael, Carol, and Erin Janson. I will never be able to thank you enough.

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"We might not be the best people..." - Jack Donaghy
"But we're not the worst..." - Liz Lemon
"Graduate students are the worst." - Liz and Jack in unison
-30 Rock, National Broadcasting Company-

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CHAPTER I

INTRODUCTION

Charles Darwin, one of the forefathers of modern evolutionary theory, once wrote that "natural selection cannot possibly produce any modification in a species exclusively for the good of another species; although throughout nature one species incessantly takes advantage of, and profits by, the structures of others" (Darwin 1859). This statement was predicated on the assumption that the evolution of "selfless phenotypes" would entail such a steep fitness cost in a population where others were "selfish" that they could not possibly persist in evolutionary time. It was not until later that it was realized that mutual cooperation between two species could evolve under very specific conditions. Contrary to Darwin's initial thesis, selfless phenotypes can (and do) persist when selflessness *increases* the fitness of individuals relative to the rest of the population. Yet, ever the visionary, Darwin was simultaneously vindicated, as it was also made apparent that the evolution of selfless phenotypes invariably involves an exchange of crucial resources between two organisms, be it nutrients, protection, or increased opportunities for reproduction, and thus these phenotypes are never "exclusively for the good of another species" (Ollerton 2006). The notion of mutualism, or the interaction between two species which results in net benefit (fitness gain) for both, was born (Bronstein 1994). From mycorrhizal fungi and plants, to endocellular bacteria and insects, to clown fish and anenomes, the evolution of traits specifically to foster the selfless interaction between two species has evolved countless times and is now recognized as an essential component to life on earth.

Of course, Darwin is more famously remembered for his insights into the relationship between the process of natural selection and the creation of biological diversity, than for his musings on the improbability of biological altruism. Indeed, Darwin was one of the first to recognize the power of natural selection in not only creating ecological diversity, such as a group of island finches that feed on different seed resources, but species diversity itself. He was one of the first to ask, why was there not a single type of finch with a range of beak sizes for exploiting different resources? Why were there distinct finch "kinds" that appeared to have real breaks in gross morphology, food preferences, habitat preferences, and behavior? These questions would lead to the scientific quest for a deeper understanding of the process of speciation.

Before one can understand the processes that drive the creation of new species, it is essential to understand what a species is. Surprisingly, this is not the simplest of tasks, as even Darwin himself recognized that the idea of species was precariously close to being arbitrary, underscored by the literally dozens of species concepts that have been used in the literature (Coyne and Orr 2004). Today it is generally accepted that a species is a group of individuals that are sufficiently similar (phenotypically, behaviorally, ecologically) to be grouped as a single kind or variety, and whose similarity is maintained by reproductive barriers from other groups (Schluter 2009). What is most desirable about this definition is that it implicitly recognizes concepts now known to be important to the process of speciation, specifically natural selection and the evolution of reproductive isoaltion. Indeed, since the time of Darwin's central thesis, great strides have been made in understanding how natural selection creates diversity and two sweeping phenomena—believed to be largely responsible for most of the biological diversity on earth—have been described in detail: adaptive radiation and ecological speciation. In these two processes, the interaction between natural selection and speciation are most clearly defined, and

numerous studies have been carried out in order to better understand these evolutionary phenomena. Because they share basic features (the process of ecological speciation is inherent in adaptive radiation), here I will primarily focus on the process of adaptive radiation. Defined, adaptive radiation is the selection driven, rapid multiplication of an ancestral species/population into distinct ecological niches, which concomitantly creates species diversity (Schluter 2000; Gavrilets and Losos 2009). What makes adaptive radiations unique compared to other instances of lineage splitting is the ecological diversity created from a common ancestor, the speed at which the ecological diversity come to be, and the species diversity that frequently accompanies the ecological diversity.

There are at least five widely recognized, broadly overlapping models of adaptive radiation (Gavrilets and Losos 2009). The most familiar, and thus the model adopted here, is known as the "invasion of empty niches" model (Gavrilets and Losos 2009). In this particular model, there are a number of prerequisites in order for adaptive radiation to occur. The first of these is ecological opportunity, which is defined as available ecological niches not occupied by competing species and which is evolutionarily accessible by an ancestral species (Schluter 2000). Often, ecological opportunity opens when an ancestral species moves to a sparsely inhabited island (or other type of habitat, such as a novel host plant) or somehow obtains what is called a key innovation—a phenotypic novelty that allows for the exploitation of currently un- or underexploited ecological niches. Ecological opportunity is important in adaptive radiation because in order for selection to be important in evolutionary diversification, there must be something for an ancestral population to adapt to. In other words, ecological opportunity offers an end point towards which selection can pull populations. Given ecological opportunity, the second necessary scenario is divergent natural selection. Here, random mutation provides the raw

variation on which selections acts. Selection itself then pulls populations into open niches, which creates the ecological diversity characteristic of adaptive radiation and the ecological/phenotypic differences among species. It follows that for divergent selection to act, phenotypes key to exploiting novel niches must have sufficient genetic diversity and not be under any other significant genetic constraints. Over time, adaptation to novel ecological niches is linked to the evolution of reproductive isolation, be it premating, postmating, or both, and reinforcement may sometimes act to solidify reproductive barriers among adaptively divergent populations. Suffiecient amounts of reproductive isolation between or among diverging populations then works to maintain the ecological and species differences resulting from ecological opportunity and divergent natural selection.

The question now becomes how does adaptive radiation relate to mutualistic symbioses? In early formulations of adaptive radiation theory, species interactions, namely competitive interactions, were important because they acted in concert with divergent natural selection to drive nascent species into novel niches (Schluter 2000). Taking cues from island-based adaptive radiations, it was initially believed that ecology could only drive divergence and initial speciation in allopatry, and that new colonizers would be driven into novel niches in part because of resource overlap and character displacement (Lack 1947; Mayr 1963). However, in a recent review of the literature about the theory and reality of adaptive radiation, species interactions were not mentioned once in a list of the ten patterns of adaptive radiation (Gavrilets and Losos 2009). It appears that species interactions have fallen by the wayside, but perhaps that is because a potentially important set of species interactions have been, until recently, egregiously overlooked. As touched on above, ecological opportunity and phenotypic evolution are crucial to the adaptive diversification process, as phenotypes are the only thing that is “visible” to natural

selection and phenotypes are ultimately what results in the ecological diversity of adaptive radiations. Very recently, it has come to the attention of biologists that symbionts, especially mutualistic symbionts, can act as sources of phenotypic complexity/variation for their hosts (Moran 2007). In this way, mutualists could strongly affect the process of speciation (see Chapter II for a detailed treatment of the subject).

In this dissertation, I examine the symbiosis between the goldenrod-galling midge, *Asteromyia carbonifera* and its fungal associate, *Botryosphaeria dothidea* (see chapter IV for more details about the fungus). I will address specific aspects of the ecology and evolution of the interaction in an attempt to clarify how the relationship may have influenced the evolutionary diversification of one or both of the players. *A. carbonifera* is a member of the Cecidomyiidae, a monophyletic family of nematoceran flies (Diptera) that includes over 5000 species worldwide, making it one of the most biologically diverse families of flies on earth (Gangé 2004). "Primitive" gall midges are free-living fungus feeders, often consuming saprophytic fungi associated with detritus. However, most of gall midge diversity (and their common name) comes from their derived ecology of inducing galls on plants. Among the plant-galling cecidomyiids, species within several tribes, mostly prominently the Asphondylini, Lasiopterini, and Alycaulini, are also intimately associated with fungus (Bissett and Borkent 1988; Gagné 1989). The females of many species in these groups appear to actively collect and transport the asexual reproductive spores (conidia) of their fungal associates within specialized envaginations on their terminal abdominal segment (Borkent and Bisset 1985). During oviposition, females will deposit conidia at the oviposition site. The conidia germinate and fungal mycelia then proliferate internally throughout the gall structure, which most species appear to feed on in addition to plant tissue. Most of the species within these tribes, regardless of their fungal associations, form normal plant

galls that are constructed from insect-manipulated, hyperplasic and hypertrophic plant tissue (Gagné 1989). However, at least one species, *Asteromyia carbonifera* (Alycaulini), forms galls that are primary composed of fungus (Camp 1981). These fungal blister galls are found on the leaves of dozens of *Solidago* (goldenrod) species throughout much of North America (Gangé 1968; Stireman et al. 2008, 2010). As the gall matures, *A. carbonifera* larvae come to lay in small chambers entirely surrounded my fungal mycelium. Over time, a layer of the fungus tissue hardens slightly and forms a structure called the stroma, which may help protect the larvae from predators and parasitoids (Weis 1982a). Interestingly, *A. carbonifera* galls exist as at least four distinct "morphologies" (herein called morphs), which have been christened the crescent, cushion, flat, and irregular morphs (Gagné 1968; Crego et al. 1990; Stireman et al. 2008). These gall morphs not only differ in external shape and location on the leaf itself, but also in several other physical characteristics (Stireman et al. 2008). Two separate studies have indicated that individuals from particular gall morphs form partially to fully reproductively isolated populations, and, in the case of the crescent morph, separate species (Crego et al. 1990; Stireman et al. 2008). It appears that the phenotypic and evolutionary variation has been directed by the phenotypically diverse assemblage of parasitoids that attack *A. carbonifera* and other *Asteromyia* species (Weis 1982b; J.O. Stireman, pers. comm.), suggesting the main source of selection for this putative adaptive radiation. Moreover, in addition to gall morph associated speciation, there is additional evidence that *A. carbonifera* and other *Asteromyia* species are also diverging along host plant and other lines (e.g., Stireman et al. 2008, 2010), making this a true candidate for an adaptive radiation possibly influenced by a microbial symbiont.

First, in Chapter II, I review the literature and lay out verbal arguments as to how mutualists, especially microbial mutualists like bacteria and fungi, could influence the process of

adaptive radiation/ecological speciation. Historically, the study of ecological speciation/adaptive radiation, in which phenotypic and ecological diversity is created concurrently with evolutionary diversity (Schluter 2000), has given the most attention to ecologically significant phenotypes encoded in the genomes of the organisms undergoing diversification. However, recent studies have demonstrated that ecologically significant phenotypes can be encoded in the genomes of organisms that are intimately associated with another organism (e.g., their symbionts). In this way, symbionts can act as a source of phenotypic complexity/variation for their hosts (Moran 2007) and may affect the rate of diversification or final species abundance during adaptive radiations (either by preventing extinction or promoting lineage splitting). Direct evidence is scant, but some studies have demonstrated the power of microbial associates in acting as sources of exploitable phenotypic diversity (e.g., Hosokawa et al. 2007). Working under the framework that natural selection on ecologically important phenotypes is important in creating both ecological and evolutionary diversity (i.e., adaptive radiation), I examine the potential roles of microbes in the evolutionary diversification of their hosts.

Second, in Chapter III, I use sterols as biomarkers to examine the nutritional relationship between *B. dothidea* and *A. carbonifera* and the trophic position of *A. carbonifera*. In many microbial symbioses, the symbiont provisions essential, but difficult to acquire (based on host ecology), nutrients to its host. Past studies of insect-fungal symbioses have demonstrated that the fungal associate is often the primary food source, or an indispensible supplementary food source that provides essential nutrients not supplied by their primary food source. Often, that essential nutrient is sterols, as insects are unable to synthesize sterols *de novo* and must therefore obtain sterols exogenously. Moreover, plants, animals, and fungi use structurally disparate sterols as inserts in their cellular membranes and as precursors for steroid hormones (Behmer and Nes

2003). Finally, an insect's whole body sterols can be strongly influenced by the sterol composition of their food source. Therefore, sterols can be used as nutritional biomarkers in cases where an insect's food source is unknown, but could be fungus-based, plant-based, or animal-based. Past studies have also raised questions about the trophic level of *A. carbonifera*. One of the most prominent figures in cecidomyiid biology, Raymond Gagné has concluded through observational studies that *A. carbonifera* is an herbivore (Gagné 1968). Conversely, two insect-fungus interaction biologists later posited that *A. carbonifera* (in fact, all fungal symbiotic gall midges) are fungivores (Bissett and Borkent 1988). This presents the ideal opportunity to use sterols to determine *A. carbonifera*'s trophic position. Understanding the nutritional relationship between *A. carbonifera* and *B. dothidea* is important, because it relates directly to the verbal framework built in Chapter II. If *A. carbonifera* feeds on its fungal associate, then it can circumvent any detrimental nutritional or secondary chemical variation in potential host plants, therefore possibly facilitating host-plant shifts. If selection for host-plant specialization exists along other lines (e.g., cognitive constraints; Egan and Funk 2006), the fungal association may actually increase the rate or amount of diversification (see Chapter II). Moreover, I examine the sterol metabolic capacities of other common insect herbivores on *S. altissima* in order to clarify the role of constraint and convergence in sterol metabolism in insects. Since most insects use cholesterol as their primary sterol, and plants contain little to no cholesterol, they must metabolism phytosterols into cholesterol through enzymatic pathways. I hypothesize that natural selection should optimize these pathways in order to minimize energy usage and metabolize the optimal sterol for the insect.

Finally, in Chapter IV, I examine the evolutionary history and population genetics of the fungal sybmiont of *A. carbonifera*. In order for symbionts to be influential in adaptive

evolutionary diversification, they must act as a source of ecologically significant phenotypic variation. This often occurs when symbiont and host are involved in a heritable mutualism (i.e., vertically transmitted). The alignment of reproductive interests between associates is thought to facilitate the evolution of phenotypes that are beneficial for the host (Moran 2007), including phenotypes that could be important for adaptive radiation/ecological speciation. A more comprehensive understanding the evolutionary patterns of the fungal associate should simultaneously address two important questions about the interaction between *A. carbonifera* and *B. dothidea*: is the symbiont a source of phenotypic complexity/variation and is the association a heritable mutualism. These questions are important, because first, it is unclear which partner is primarily responsible for the observed variation in gall morphology. While it is evident that the phenotypes are mediated through the fungal associate, it is unknown if the midges are associated with genetically divergent fungal symbionts or if the midges themselves are somehow able to manipulate the fungus into specific gall conformations. If the fungus is directly responsible for gall morphology, this would represent one of the only known cases of an ectosymbiont acting as a source of phenotypic complexity/variation for their hosts. Second, I will address whether or not the fungus is involved in a heritable mutualism, which, while not necessary for stable mutualisms to exist, has by far been shown to be the rule rather than the exception, and may be important if a symbiont is to act as source of phenotypic complexity/variation (Moran 2007). Over the past 20 years, evidence has accumulated that microbes involved in heritable mutualisms show very distinct evolutionary patterns when compared to their free-living con- and heterospecifics, such as increased rates of molecular evolution, A+T biased genome, reciprocal codivserification with hosts, and reduced genome size (Wernegreen 2002). Given the intimacy of the interaction and the capacity for the fungus to

mediate gall morphology, I predict that the fungus associated with *A. carbonifera* should be a heritable mutualist and therefore should also exhibit evolutionary patterns that are characteristic of heritable microbial mutualisms. This pattern should hold even if the fungus is not directly responsible for the observed variation in gall morphology.

CHAPTER II

PHYTOPHAGOUS INSECT–MICROBE MUTUALISMS AND ADAPTIVE EVOLUTIONARY DIVERSIFICATION

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Abstract

Adaptive diversification is a process intrinsically tied to species interactions. Yet, the influence of most types of interspecific interactions on adaptive evolutionary diversification remains poorly understood. In particular, the role of mutualistic interactions in shaping adaptive radiations has been largely unexplored, despite the ubiquity of mutualisms and increasing evidence of their ecological and evolutionary importance. Our aim here is to encourage empirical inquiry into the relationship between mutualism and evolutionary diversification, using herbivorous insects and their microbial mutualists as exemplars. Phytophagous insects have long been used to test theories of evolutionary diversification; moreover, the diversification of a number of phytophagous insect lineages has been linked to mutualisms with microbes. In this perspective, we examine microbial mutualist mediation of ecological opportunity and ecologically based divergent natural selection for their insect hosts. We also explore the conditions and mechanisms by which microbial mutualists may either facilitate or impede adaptive evolutionary diversification. These include effects on the availability of novel host plants or adaptive zones, modifying host-associated fitness trade-offs during host shifts, creating or reducing enemy-free space, and, overall, shaping the evolution of ecological (host plant) specialization. Although the conceptual framework presented here is built on phytophagous insect–microbe mutualisms, many of the processes and predictions are broadly applicable to other mutualisms in which host ecology is altered by mutualistic interactions.

Introduction

Adaptive radiation is the rapid, selection-driven diversification of one ancestral lineage into many descendent lineages (Schluter 2000). This process may create much of life's ecological and phenotypic diversity; consequently, adaptive radiation has long provided the most comprehensive model for evolutionary diversification (Lack 1947; Simpson 1953; Schluter 1996b, 2000). Indeed, case studies of adaptive radiation provided the biological touchstones for the Modern Synthesis, and modern evolutionary theory that now links the Cambrian explosion to the radiation of Darwin's finches.

Interspecific interactions, mostly in the form of resource-based competition, are central to adaptive diversification, because they act in concert with environmental variation to promote or maintain species diversity (Schluter 2000; Rundle and Nosil 2005). Exemplary studies, however, have rarely considered the impact of interactions other than competition, particularly those involving multiple trophic levels (e.g., predator–prey interactions, symbiosis). Moreover, when noncompetitive species interactions have been explicitly considered, they are often restricted to specific contexts in which the ecological and evolutionary processes described do not necessarily extrapolate across taxa (e.g., adaptive radiation in plant–pollinator mutualisms in which floral traits subject to natural selection are directly involved in reproductive isolation; Johnson et al. 1998; Schluter 2000; Levin 2006 and references therein). Thus, there remains little understanding of the relationship between the process of adaptive evolutionary diversification and the diversity of other community interactions—an omission of sufficient magnitude to lead one biologist to describe this area as "one big, vacant adaptive zone in evolutionary ecological research" (Futuyma 2003).

Interspecific mutualisms, especially those involving prokaryotic and eukaryotic microbes, are ripe for exploration in this regard, because they are integral to the radiation of many of life's major lineages (Margulis 1981; Smith and Read 1997; Blackwell 2000; Moran 2002). All the more unusual then that studies of precisely how microbial mutualism promote innovation and spur diversification on smaller (ecological) time scales are practically nonexistent. Do microbial mutualisms facilitate the evolution of reproductive isolation and speciation through their influence on host ecology? Do microbial mutualisms shape the path of diversification after facilitating the invasion of novel ecological zones? If so, how? What are the ecological mechanisms underlying diversification for genomes bound by a history of coevolution? How can those mechanisms be discovered empirically?

Here we use the growing body of literature addressing the impact of microbial mutualism on host ecology to address several important implications for adaptive radiation, evolutionary diversification, and species interactions. By linking the growing body of research on the evolutionary ecology of mutualisms (Bronstein et al. 2006) to the evolutionary ecology of diversification (Schluter 2000; Funk et al. 2002; Rundle and Nosil 2005), we hope to provide a path toward a broader understanding of the process of adaptive diversification and its dependence on mutualistic interactions. To do so, we draw from two fields: evolutionary and ecological studies of plant-feeding insects, and the ecology of phytophagous insect–microbe mutualisms. We outline proposed mechanisms of adaptive evolutionary diversification for phytophagous insects, and investigate how explicit consideration of microbial mutualists may facilitate or inhibit these processes: for example, by promoting or inhibiting the colonization of novel host plants. In doing so, we also hope to foster the idea of adaptive diversification as a

process that may be frequently tied to the joint host plant exploitation phenotypes generated by insects and their microbial mutualists.

This perspective is divided into three main sections. First, we present an overview of microbial mutualisms in phytophagous insects, underscoring their prevalence and the diversity of benefits that such mutualists confer to their insect hosts. Next, we outline some of the most significant ways in which microbial mutualists can facilitate or impede host evolutionary diversification of their hosts by focusing on how the mutualists may affect two core aspects of adaptive evolutionary diversification: ecological opportunity and divergent selection. Finally, we outline key gaps in current knowledge that must be addressed to achieve more comprehensive understanding of the impact of mutualisms on adaptive evolutionary diversification.

Prevalence of phytophagous insect–microbe mutualisms and the benefits conferred by microbial mutualists to their insect hosts

Virtually every multicellular organism hosts beneficial microbes. For example, the digestive tracts of animals are rich with a diverse assemblage of bacterial species that may outnumber their own cells (Dillon and Dillon 2004). Plants host an equally impressive diversity of beneficial or potentially beneficial endophytic fungi (Marks and Clay 1990; Varma et al. 1999; Wagner and Lewis 2000; Redman et al. 2002; Arnold et al. 2003; Schardl et al. 2004; Arnold and Lutzoni 2007) and bacteria (Chanway 1998), in addition to their well-known mycorrhizal (Smith and Read 1997) and nitrogen-fixing bacterial (Gresshoff 1990) mutualists. Here, we focus on insects in part because many taxa have independently formed mutualistic associations with a variety of microbial taxa, ranging from bacteria to fungi to protozoans (Buchner 1965; Breznak 1982; Campbell 1990; Gullan and Cranston 1994; Moran 2002; Bourtzis and Miller 2003; Baumann 2005; Moran and Degnan 2006). Species in several of the

most diverse phytophagous insect families have been shown to maintain indigenous facultative or obligate mutualistic associations with microbes (Fig. 1). Some of the most prominent examples come from the plant-feeding Chrysomelidae (Coleoptera) (Peterson and Schalk 1994; Jolivet and Verma 2002), Curculionidae (Coleoptera) (Six 2003; Heddi and Nardon 2005), plant-galling Cecidomyiidae (Diptera) (Bissett and Borkent 1988; Gagn´e 1989), and virtually all plant-feeding hemipteran families (Baumann 2005; Moran et al. 2005c,d; Hosokawa et al. 2006; Takiya et al. 2006). The prevalence of microbial mutualism in certain phytophagous insect taxa, such as Lepidoptera, is more limited, but this perception may be due to the absence of evidence more than evidence of absence (Fermaud and Le Menn 1989, 1992; Roehrich and Boller 1991; McKillip et al. 1997; Mondy et al. 1998a,b; Mondy and Corio-Costet 2000; Broderick et al. 2004; Genta et al. 2006). For phytophagous insects, bacterial mutualisms predominate, but fungal mutualisms are also relatively common (Bisset and Borkent 1988; Fermaud and Le Menn 1989; Gagn´e 1989; Six 2003). To our knowledge, protistal mutualisms, although common in certain insect groups (e.g., xylophages; Yamin 1979; Breznak 1982), have not been described in insect species that feed on nonwoody plant tissue.

Microbes provide a vast array of services that mediate the interactions between phytophagous insects and their host plants and natural enemies. Indeed, the services rendered by microbial mutualists to their insect hosts can be remarkably varied (Smith and Douglas 1987; Saffo 1992; Moran and Telang 1998; Moran 2001; Bourtzis and Miller 2003; Dillon and Dillon 2004; Wernegreen 2005; Moran 2007). Many insect-microbial mutualisms involve nutritional provisioning by the microbe in return for the protected environment provided by the host insect's body (Ollerton 2006). Mutualistic bacteria synthesize limiting metabolites for growth and

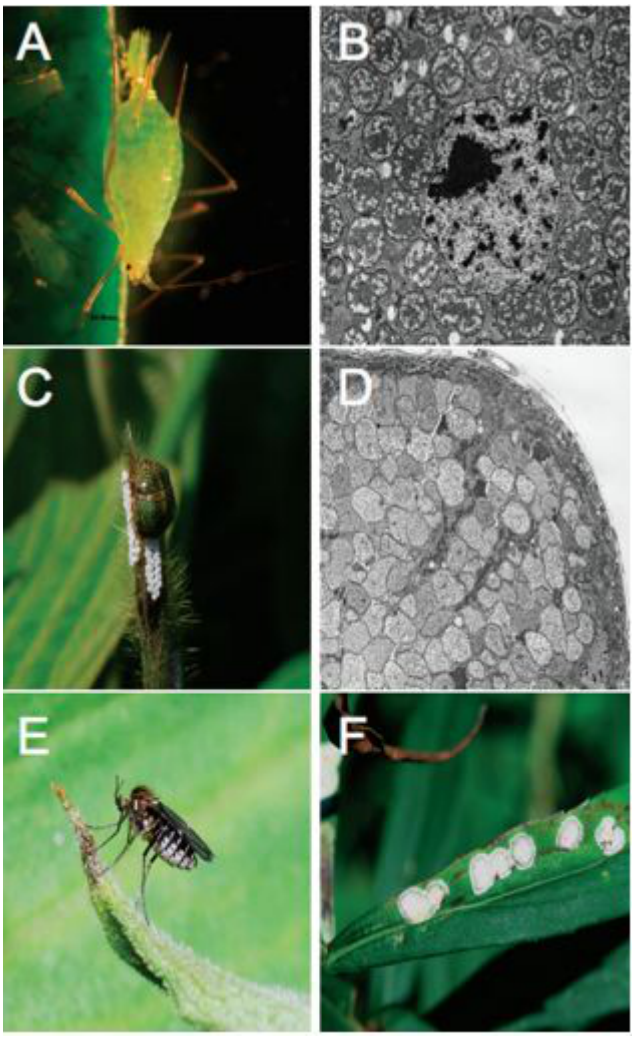

Figure 1. Examples of the diversity of insect–microbe mutualistic interactions. (A) A female *Acyrthosiphon pisum* (Hempitera: Aphididae) and her offspring. (N. Moran, University of Arizona) (B) Cells of *Buchnera aphidicola* from *A. pisum* localized in a specialized cell known as the mycetocyte. (N. Moran, University of Arizona) (C) A female *Megacopta punctatissima* (Hemiptera Plataspidae) depositing eggs and symbiont capsules in the field. (T. Hosokawa, National Insitute of Advanced Industrial Science and Technology, Japan (D) Candidtatus *Ishikawaella capsulata* cell in the midgut of a female *M. punctatissima*. (T. Hosokawa, National Insitute of Advanced Industrial Science and Technology, Japan) (E) *Asteromyia carbonifera* (Diptera: Ceciomyiidae) resting on a leaf of its host plant, *Solidago* sp. (J. Stireman, Wright State University) (F) Examples of *A. carbonifera galls*; gall structure is mediated by the fungal symbiont *Botryosphaeria* sp. (P. Abbot, Vanderbilt University).

nutrition (Douglas 1998; Moran et al. 2003; Dillon and Dillon 2004), assist in the breakdown or assimilation of recalcitrant plant compounds (Jones 1984; Genta et al. 2006), provision essential vitamins (Nakabachi and Ishikawa 1999), or recycle nitrogenous wastes (Whitehead et al. 1992; Gauderman et al. 2006). Fungal mutualists can provide a similar spectrum of services. For example, the yeast-like fungal endosymbionts of some hemipterans provide amino acids and other metabolic services, but can also synthesize sterols and enzymes for the degradation of plant material or allelochemical detoxification (Noda et al. 1979; Koyama 1985; Martin 1987; Dowd 1991; Shen and Dowd 1991; Sasaki et al. 1996; Mondy and Corio-Costet 2000; Wilkinson and Ishikawa 2001).Mutualistic microbes also provide nonnutritional services, such as preventing the colonization of pathogenic microbes by either mass action or active involvement in immune reactions (Dillon and Charnley 1988; Berg 1996; Six 2003; Ferrari et al. 2004; Loker et al. 2004; Scarborough et al. 2005); synthesizing various compounds and small molecules used by insects in social interactions (Dillon and Charnley 2002); increasing fitness in extreme abiotic environments (Chen et al. 2000; Montllor et al. 2002;Dunbar et al. 2007); overwhelming plant defenses (Paine et al. 1997); and providing protection from natural enemies (i.e., predators and parasitoids; Weis 1982b; Oliver et al. 2003, 2005, 2006).

Impact of microbial mutualists on adaptive evolutionary diversification in phytophagous insects

Most phytophagous insects tend to be host plant specialists, feeding and carrying out virtually all key life-history activities (e.g., mate-acquisition and reproduction) on one or a relatively small subset of closely related plant species (Futuyma 1991; Bernays and Chapman 1994; Thompson 1994; Novotny and Basset 2005). This host plant specialization is thought to stem from adaptation to variation among potential host plants in such factors as nutritional

quality, defensive chemistry, phenology, natural enemies, and competition, each of which can give rise to host plant associated fitness trade-offs that oppose the evolution of general diets (Bernays and Graham 1988; Futuyma and Moreno 1988; Jaenike 1990; Schluter 2000; Funk et al. 2002; Singer and Stireman 2005). Thus, adaptive diversification in phytophagous insects depends upon exploitation of ecological opportunity provided by novel host plants (i.e., plants that are generally free of enemies or competition, and which the insect has the genetic, morphological, and physiological capacity to exploit), allowing divergent natural selection between populations on alternative host plants to promote host plant (ecological) specialization and ultimately reproductive isolation (Schluter 2000; Funk et al. 2002; Kirkpatrick and Ravign´e 2002; Rundle and Nosil 2005). Finally, host plant use is often conservative, in that shifts to new hosts frequently reflect phylogenetic or chemical proximity to ancestral hosts (Ehrlich and Raven 1964; Futuyma et al. 1993, 1994, 1995; Becerra 1997; Janz and Nylin 1998; Winkler and Mitter 2007). By mediating the interactions between plants and insects, microbes can potentially influence both host plant-associated ecological opportunity and divergent natural selection, and thus the evolution of host plant specialization and reproductive isolation (Fig. 2).

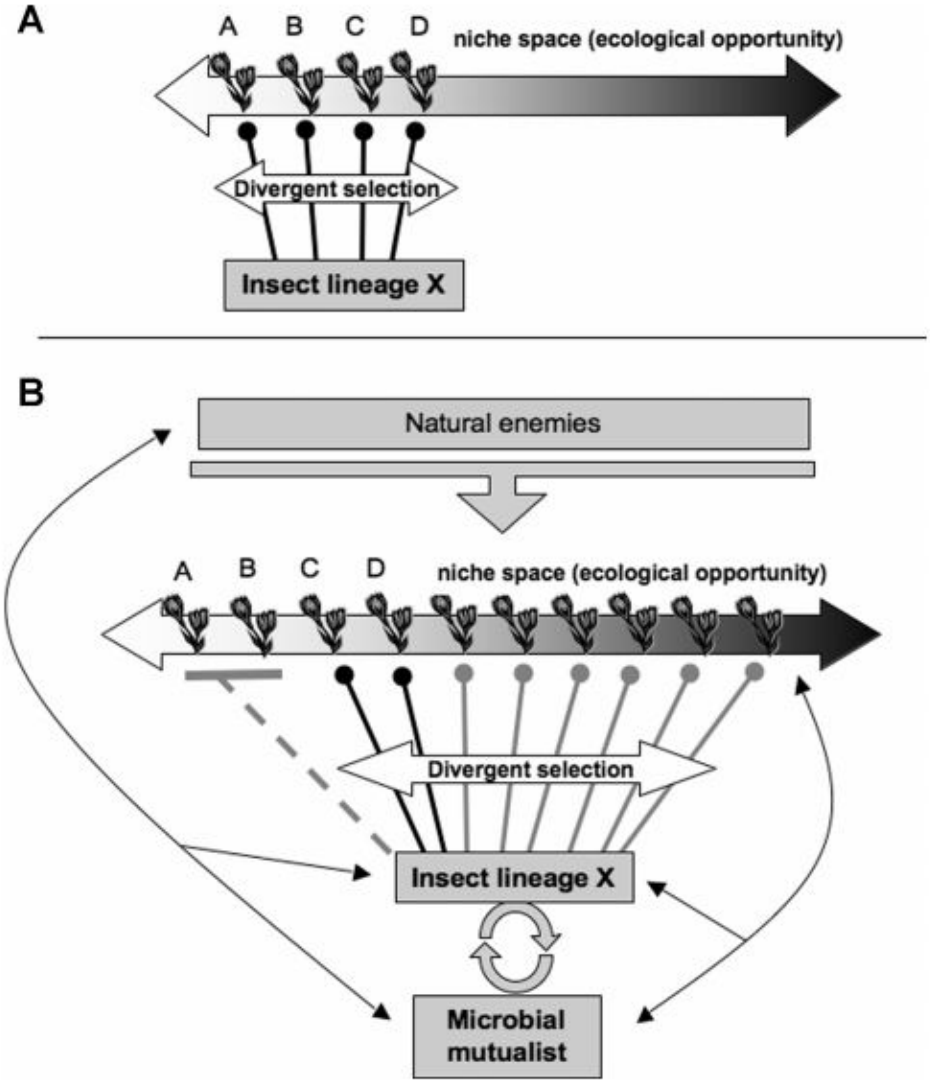

Figure 2. (A) The traditional model of an adaptive evolutionary radiation of an herbivore (lineage X) onto host plants or host plant resources. A fraction of the potential nice space is partitioned by groups (A-D) due to divergent natural selection arising out of the combination of ecological opportunity, environmental variation, competition, and fitness trade-offs on alternative resources/environments. (B) A multitrophic community perspective of an adaptive evolutionary raditation modulated by divergent natural selection among habitats/hosts and direct or indirect interactiosn with mutualists and natural enemies. Solid black arrows demonstrate that natural enemies and host plants interact with both insects and their microbial mutualsts, resulting in a "multigenomic" basis to host plant exploitations phenotypes. Mutualsts can either facilitate ecological opporunties (solida gray lines) or inhibit them (dashed line), depending on the nature of the interactions, the mode of microbial inheritance, and ultimately the process of host plant specialization (ecological specialization).

Modification of ecological opportunity by microbial mutualists: facilitative and inhibitory effects on diversification

Traditionally, ecological opportunity is loosely defined as evolutionary accessible resources little used by competing taxa (Schluter 2000). Because this definition is awkward to apply directly to the microbial partner in many insect–microbe mutualisms (e.g., bacterial endosymbioses), we will employ a modified definition. We define ecological opportunity as the capacity for an insect symbiotic with a microbial mutualist to establish and maintain a population on a novel host plant (niche). This definition makes the role of mutualistic interactions explicit by accounting for the fact that host plant exploitation phenotypes may require both the insect and its microbial mutualist(s). Thus, not only could enemies, competitors, and/or genetic constraints of either the insect or microbe restrict ecological opportunity, but the context dependency of microbe-insect interactions could also affect novel host plant invasions.

Facilitative effects: environmental buffering through microbial mutualists

Mutualistic symbioses may principally affect insect diversification by providing new ecological opportunity, via "environmental buffering." In essence, microbes can act as key innovations (Simpson 1953; Schulter 2000) for their insect hosts by opening ecological opportunity that would have been otherwise unexploitable (Fig. 3). For example, plants (and plant tissues) can vary widely in primary nutrient concentrations and compositions (Slansky and Rodriguez 1987; Simpson and Simpson 1990; cf. Berenbaum 1995; Behmer and Nes 2003; cf. Zangerl and Berenbaum 2004), impeding host plant shifts due to dietary inadequacies that may exist for maladapted herbivore genotypes (Simpson and Raubenheimer 1993; Bernays and Chapman 1994; Raubenheimer and Simpson 1997). However, for nutritional mutualisms, the dietary sufficiency of a novel host plant is determined by both insect and microbial partners.

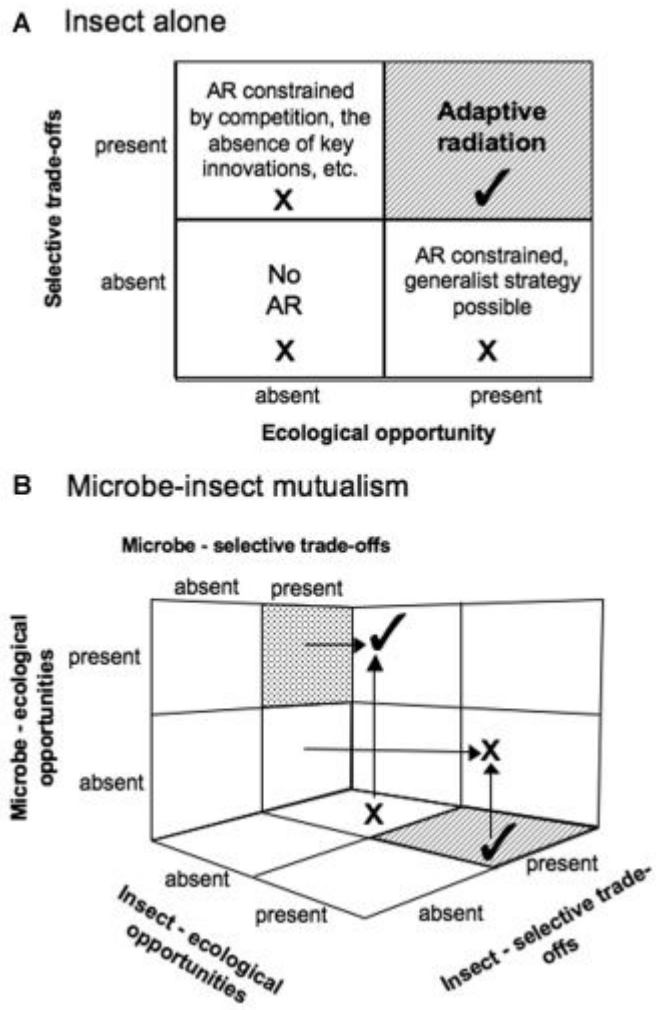

Figure 3. (A) Hatched box: radiations of phytophagous insects often occur via shifts onto novel host plant species on which they are competetiviely and evolutionarily viable (ecological opportunity is present) and between which divergent selection occurs (selective trade-offs are present). (B) The ecological opportunity and selective trade-offs experienced by microbial symbionts must be taken into account when considering the adaptive radiations if insect-microbe mutualisms. Stippled box: conditions favorable for the radiation of microbial partners can promte (check mark) or hinder (X) the radiation of insect hosts otherwise possessing or lacking the approparite ecological opportunities. Other configurations for the axes are easily imaginable (e.g., and axis for other trophic interactions, such as natural enemies, could be added).

Thus, microbes may furnish a dietary "buffer" that nurtures colonizing populations during the initial stages of niche or host plant shifts.

For example, plant-feeding insects vary widely in their ability to metabolize the numerous structural variants of phytosterols (Svoboda 1999; Behmer and Nes 2003), or may have dietary strategies that include feeding on plant tissue that lacks free sterols (Behmer and Nes 2003). Certain insect species appear to have overcome this difficulty by engaging in fungal mutualisms on their host plants, and metabolizing fungal sterols, rather than phytosterols. For example, the grape berry moth, *Lobesia botrana*, has a mutualistic association with *Botrytis cinerea*, a host plant generalist filamentous ascomycete fungus. *Lobesia botrana* larvae vector *B. cinerea* on their bodies (Fermaud and Le Menn 1992) and directly facilitate rapid infection and development on grape tissue through mechanical wounding of grape berries (Fermaud and Le Menn 1989; Mondy et al. 1998a,b). Fungal material is consumed in the process of grape consumption, and is a significant source of precursor sterols that the moth larvae use to synthesize hormones involved in growth and development (Mondy and Corio-Costet 2000). Thus, *Botrytis* could facilitate successful shifts by *Lobesia* onto novel plants or tissues by reducing at least one facet of the mismatch (nutritionally derived substrates for hormone metabolism) that occurs between maladapted insect genotypes and alternative host plants or tissues (Fogleman and Danielson 2001; Behmer and Nes 2003). Other potential examples of sterol-based, microbially mediated nutritional "buffering" come from the phloem-feeding bark beetles in the genus *Dendroctonus* (Bentz and Six 2006), and the fungus-associated gall midges in the genus *Asteromyia* (Janson et al. 2009).

However, insects need not consume their microbial mutualist to derive some nutritive benefit. For example, fungal infection can induce the accumulation of free amino acids,

nonstructural carbohydrates, and organic nitrogen-containing compounds inplant leaves (e.g., Holligan et al. 1973; Farrar and Lewis 1987; Potter 1987; Paul and Ayres 1988 Johnson et al. 2003), which may increase performance of folivorous herbivores by balancing carbohydrate–protein ratios (Bernays and Chapman 1994). Insect-vectored microbial mutualists that directly infect the plant immediately before or during insect attack have the potential to favorably modify plant nutritional quality (e.g., Ayres et al. 2000), which in turn can enable prolonged population persistence and opportunity for adaptive divergence on alternative host plants.

Similarly, microbes may buffer insects against novel plant secondary metabolites, or against increased concentrations of secondary metabolites during host plant shifts (Starmer and Aberdeen 1990; Douglas 1992; Fogleman and Danielson 2001). Despite the capacity for microbes to degrade organic molecules used by plants as defensive allelochemicals (e.g., Hemingway et al. 1977; Douglas 1994; Bhat et al. 1998; van der Vlugt-Bergmans and van der Werf 2001), concrete examples of this phenomenon in plant–herbivore–microbe interactions are rare. One potential example comes from the gypsy moth (*Lymantria dispar*), whose larvae are known to harbor at least 23 different phylotypes of midgut bacteria (Broderick et al. 2004). One phylotype (*Rhodococcus* sp.) is closely related to a species known to degrade monoterpenes (van der Vlugt-Bergmans and van der Werf 2001), a common plant allelochemical that is toxic to insect larvae and adults (Langheim1994), and may in part explain the relatively high tolerance of monoterpenes in gypsy moth larvae (Powell and Raffa 1999; Broderick et al. 2004; see also Genta et al. 2006). Moreover, recent studies have revealed that induced defenses used by plants to ward off different enemies (e.g., microorganisms versus insects) are subject to a significant degree of antagonistic “crosstalk” (Stout et al. 2006). Accordingly, a symbiotic microbe can induce plant resistance against other free-living microbes, but place susceptibility to it insect

host. Thus, instead of detoxifying secondary metabolites, specific microbial mutualists (e.g., fungal ectosymbionts like *Boytryosphaeria* mutualistic with *Asteromyia*) may directly interfere with the induction of insect herbivore-specific host plant defenses.

Microbial mutualists may be especially likely to provide such buffering when they are derived from phytopathogenic ancestors, and thus retain traits useful in host plant exploitation that can be co-opted by insects. This is the case for many fungal mutualists of plant-feeding insects (Bisset and Borkent 1988; Fermaud and Le Menn 1989; Krokene and Solheim 1998; Kluth et al. 2001, 2002; Six 2003; Vega and Dowd 2005; for bacterial examples see de Vries et al. 2001a,b, 2004; Kikuchi et al. 2007), which have wide host plant ranges and the capacity to colonize even distantly related plants relatively easily (Farr et al. 1989; Slippers et al. 2005; Gilbert and Webb 2007). For example, the gall midge *Asteromyia carbonifera* forms blister galls on over 65 species of *Solidago* in North America (Gagné 1968, 1989; T. Carr, unpubl. data). *Asteromyia carbonifera* galls are unusual in that they are composed primarily of fungal tissue and lack typical plant derived nutritive tissue (Bisset and Borkent 1988); in this case, a highly specific lineage of the filamentous ascomycete *Botryosphaeria dothidea*, which the midges vector in specialized pockets (mycangia) on their abdomens (Bissett and Borkent 1988). Most known noninsect associated *Botryosphaeria* species are plant pathogens/endophytes described from a broad array of higher plants, and found in nearly every region of the world (Smith 1934; Hepting 1971; Farr et al. 1989). Thus, like some intercellular and intracellular microbial mutualists (Dale et al. 2001), mutualisms such as those between gall midges and fungi may owe their evolutionary success to the microbial pathogenic trait precursors that facilitate host plant exploitation.

A general conceptual model of the buffering effects of microbial mutualists on herbivores can be described as follows. Host plant shifts and, ultimately, host plant specialization, require phenotypic trait values in herbivores that allow efficient exploitation of different plant resources (Schluter 2000), resulting in fitness landscapes with local optima that correspond to different trait combinations (Simpson 1944; Arnold et al. 2001). Thus, host plant shifts are akin to crossing valleys of character space that correspond to low population mean fitness. As with phenotypic plasticity and learning (Fear and Price 1998; Price et al. 2003; Paenke et al. 2007), mutualistic microbes may influence adaptive diversification by reshaping the fitness landscape around the peak occupied by an insect herbivore, determining in part the range of peaks in the local character space that are "within the realm of attraction" of the insect host (Price et al. 2003). Specifically, microbial mutualists may raise population mean fitness for a given range of variation in insect herbivorous traits, or move insect host trait values closer to the base of an unoccupied host plant determined adaptive peak, thus promoting peak shifts and population persistence on novel hosts. However, host shifts by herbivores may involve character evolution in the microbe as well, depending on aspects of the mutualism (e.g., the ability of the microbe to provision services across a wide range of plants). In essence, microbe-insect herbivore mutualisms may involve the coevolution of insect and microbe on two fitness surfaces. Ultimately, this process may promote diversification via host range expansion, especially if mutualisms facilitate more distant host shifts outside of conserved sets of hosts frequently observed among plant-feeding insects (e.g., Ehrlich and Raven 1964; Futuyma et al. 1993, 1994, 1995; Becerra 1997; Janz and Nylin 1998; Winkler and Mitter 2007).

Facilitative effects: reduction of insect genetic constraints by microbial mutualists

Microbial mutualists may provide ecological opportunity through alleviation of genetic constraints on host plant use. Theory suggests that lack of genetic variation for traits related to novel host plant exploitation limits adaptive evolution and host plant shifts in herbivorous insects (Lande 1979; Kirkpatrick and Lofsvold 1992; Futuyma et al. 1993, 1994, 1995; Janz et al. 2001; Blows and Hoffmann 2005). The limitations on an insect's genome will in part determine the adaptive response (both speed and phenotypic direction) to novel host plant-associated selection, and may often preclude the successful colonization of particular host plants or increase the likelihood of extinction upon shifts to novel hosts (Schluter 2000; Arnold et al. 2001). Given that phenotypes expressed by insects involved in mutualisms are the product of at least two genomes with some degree of historical independence, genetic constraints may be relaxed due to the existence of a potentially greater pool of ecologically significant genetic variation (Fig. 3). For example, a microbial population may exhibit substantial genetic variation for traits involved in plant allelochemical detoxification, although an insect population possesses little. By increasing the pool of independent genetic variation from which an insect population can draw, a microbial mutualist may facilitate colonization and population persistence on novel host plants. This is especially likely if host plant-associated adaptations are divided among insects and microbial mutualists along genetic lines of least resistance (i.e., greatest genetic variation; Schluter 1996a, 2000). Furthermore, because of the ability of asexual microbes to acquire genetic material through lateral gene transfer (in bacteria) or parasexual recombination (in fungi), insect–microbe mutualisms may express more genetic variation for certain host-use traits than insect herbivores without mutualists. Such recombination may be more important in microbial mutualists that are primarily facultative or horizontally transmitted (Dillon and Dillon 2004); evidence from bacterial symbionts suggests that obligate, vertically transmitted mutualists may frequently lose

such capabilities (e.g., *Buchnera*; Wernegreen and Moran 2001; Tamas et al. 2002; but see van Ham et al. 2000).

Inhibitory effects: microbial mutualist imposed limits on ecological opportunity

Although microbial mutualists may often possess the potential to open or expand ecological opportunity for their insect hosts, they may also limit ecological opportunity (Fig. 3). Microbes may render host plants more toxic, not less (Starmer and Aberdeen 1990), induce plant defenses synergistically, rather than antagonistically (Stout et al. 2006), or may be susceptible to species-specific plant allelochemicals (Jones 1981; Vega et al. 2003). Lack of genetic variation or adaptively plastic responses in microbial mutualists for traits coopted by insect hosts for host plant exploitation could retard insect adaptation to novel host plants, insect population growth, and, ultimately, population persistence.

A general theme in the modern study of mutualisms is that how these interactions function can depend upon the habitats or communities in which they occur (known as "conditionality" or "context dependency"; Thompson 1988; Bronstein 1994a,b; Chen et al. 2000; Agrawal 2001; Wilkinson et al. 2001; Montllor et al. 2002; Klepzig and Six 2004; Tsuchida et al. 2004; Thompson 2005; Bensadia et al. 2006). For example, in the western flower thrips, *Frankliniella occidentalis*, the gut microbe *Erwinia* sp. can be mutualistic or antagonistic, depending on which host plant the thrips is feeding (de Vries et al. 2004). In such cases, reduction in the level of mutualistic benefit across different host plants may preclude insects associated with mutualistic microbes from successfully exploiting specific host plants, despite the insect's capacity to recognize the plant species as a potential host. Context dependency may be especially important in mutualisms in which the microbe interfaces directly with the living

host plant (e.g., fungal ectosymbioses as seen in *Asteromyia* spp., *L. botrana*, *Dendroctonus* spp.), when the association is obligate and thus cannot be eliminated (e.g., some bacterial endosymbionts), or when novel microbial mutualist species or genotypes are difficult to acquire.

Modification of host plant-associated natural selection by microbial mutualists: facilitative and inhibitory effects on diversification

Fitness trade-offs among host plants form the basis for divergent selection and ultimately ecological specialization in phytophagous insects, from which reproductive isolation arises as a byproduct (Rice and Hostert 1993; Funk 1998; Feder and Filchak 1999; Schluter 2000; Nosil et al. 2002).Microbial mutualists that interact with the host plant or provide nutrients that are lacking in their insect host's diet may also exhibit host-associated fitness tradeoffs. In such cases, microbial mutualists may favor or hinder host plant-associated genetic differentiation in the insect, depending on whether fitness effects of a host plant on each mutualistic partner are positively or negatively correlated.

Facilitative effects: microbial mutualist promotion of host plant specialization

Vertical transmission coupled with the capacity for rapid adaptation of microbial mutualists may promote divergent selection on herbivores. In aphids, for example, host plants can possess marked variation in nutritional quality among and within species (e.g., Sandström and Pettersson 1994; Bernays and Klein 2002). Nutritional variation may be particularly important for phloem feeders like aphids that rely on a nutritionally imbalanced diet of marginal quality (Douglas 1998). Virtually all aphids depend on the bacterial endosymbiont *Buchnera aphidicola* to synthesize amino acids missing from their diet (Douglas and Prosser 1992; Douglas 1998). In several aphid lineages, genes involved in the rate-limiting step of tryptophan

and leucine production are located on exclusively vertically transmitted plasmids contained within their vertically transmitted primary endosymbionts (Lai et al. 1994; Bracho et al. 1995; Baumann et al. 1997; Rouhbakhsh et al. 1997; Silva et al. 1998; Wernegreen and Moran 2001). These plasmids show inter- and intraspecific variation in functional gene copy number and the number of amino acid biosynthesis pseudogenes (Baumann et al. 1995; Thao et al. 1998; Plague et al. 2003; Birkle et al. 2004; Moran and Degnan 2006). The observed variation in plasmid borne gene copy number among aphid lineages may be adaptive, resulting from selection favoring the functional inactivation of amino acid biosynthesis genes due to costs associated with overproduction of amino acids readily obtained from the diet (Atkinson 1977; Lai et al. 1996; Wernegreen and Moran 2000). Recent studies have also linked the inactivation of chromosomal endosymbiont amino acid biosynthesis genes in various aphid lineages to variation in the nutritional content of their host plant's phloem (Tamas et al. 2002; vanHamet al. 2003; Moran and Degnan 2006). Given the relatively rapid rate of molecular evolution in *Buchnera* nutrient provisioning genes (Moran et al. 1995; Moran 1996; Wernegreen et al. 2001), aphids may experience fitness trade-offs among host plants due to differential selection for reduction in functional amino acid synthesis genes. In this case, microbial adaptation to host plants with high levels of particular amino acids or their precursors could lower aphid fitness on host plants with lower levels of essential amino acids or their precursors (but see Birkle et al. 2002, 2004). This, in turn, could promote host plant specialization and adaptive diversification.

Direct empirical evidence for microbial mutualists contributing to insect host plant performance, and potentially host plant specialization, has been recently observed in the stinkbug species *Megacopta punctatissima* and *M. cribraria*. These species are closely related, exhibiting over 99% sequence identity in the mitochondrial 16S rRNA gene (Hosokawa et al. 2006). Yet,

they have distinct host plant preferences—*M. punctatissima* is a pest species that frequently feeds on leguminous crop plants in addition to wild legumes, whereas *M. cribraria* rarely feeds on domesticated plants. Hosokawa et al. (2007) discovered that this distinct host preference difference is reflected in host performance differences—field-collected *M. cribraria* performed poorly on soy (crop) plants relative to *M. punctatissima*. These two species are also known to harbor closely related, obligate, vertically transmitted proteobacterial gut bacteria in specialized midgut structures (Hosokawa et al. 2006). Hosokawa et al. (2007) discovered that when the mutualistic bacterial strain of *M. punctatissima* was transferred to *M. cribraria* individuals, their fitness on soy increased significantly, specifically due to increased egg hatch rate. Conversely, *M. punctatissima* individuals that received the mutualist strain of *M. cribraria* had significantly reduced egg hatch rates on soy. With this study, Hosokawa et al. (2007) demonstrated that host plant-associated performance in an insect can be directly tied to the genotype of its microbial mutualist. More such manipulative studies involving microbial mutualists (e.g., Koga et al. 2007) are crucial to understanding microbial contributions to insect host plant performance and ecological (host plant) specialization.

Microbial mutualists that cannot regulate their mutualistic services in the face of host plant-associated environmental variation may also generate long-term host plant-associated fitness trade-offs for their insect hosts. In such cases, successful exploitation of host plants by phytophagous insect hosts may come about only through genetically based adaptations in the microbes, and not through the ability of microbes to plastically regulate their mutualistic contributions to their insect host in the face of host plant-associated variation. Again, this is illustrated by the primary endosymbiont of aphids, which has generally lost the ability to fine-tune regulation of its amino acid biosynthetic genes because of the genetic decay associated with

the endosymbiotic lifestyle (Moran et al. 2003, 2005b; Moran and Degnan 2006). Thus, any adaptive response to variation in host plant nutritional quality is necessarily accomplished through either amplification or inactivation of nutrient provisioning genes, rather than through up- or down-regulation of those same genes. If such host plant-associated adaptation in the microbial mutualist results in fitness variation across host plants for the insect host, host plant specialization following host plant shifts may be favored in the insect host and host plant-specific adaptations may ensue.

Fitness trade-offs and the evolution of reproductive isolation may also arise when host plant-adapted phenotypes arise through genotype × genotype × environment interactions (Thompson 1987, 1988; Agrawal 2001; Wade 2007). Insect–microbe genotypic combinations will vary in their capacity to successfully exploit host plants, and, consequently, the pairing of a particular mutualist genotype with a particular insect genotype may result in high fitness on some host plants, but low fitness on others. Thus, if particular microbial mutualist genotypes are typically transmitted among genetically similar individuals (e.g., individuals occupying the same host plant), outcrossing between host plant-associated populations could result in the frequent generation of ecologically unfit insect microbe genotypic combinations, which in turn could lead to reinforcement of premating isolation. Insects involved in such mutualisms would also be subject to other causes of host plant-associated reproductive isolation, such as immigrant inviability (Nosil et al. 2005), further reducing gene flow among host plant associated populations.

The most informative studies on this topic have been performed on the pea aphid *Acyrthosiphon pisum*. *A. pisum* comprises a group of genetically and ecologically distinct host races that feed on leguminous hosts (e.g., Via 1999; Via et al. 2000). Surveys of natural *A. pisum*

populations have shown that certain host race populations harbor specific secondary bacterial species at unusually high frequencies (Tsuchida et al. 2002; Leonardo and Muiru 2003; Simon et al. 2003; Ferrari et al. 2004). Fitness variation across different host plants and environmental conditions is dependent on the presence or abundance of specific secondary symbionts (Chen et al. 2000; Koga et al. 2003; Leonardo and Muiru 2003; Tsuchida et al. 2004; Oliver et al. 2005; Russell and Moran 2006; but see Leonardo 2004). Here, the interaction between specific aphid and symbiont genotypes may have facilitated shifts onto novel host plants, allowing subsequent host plant-associated selection to promote host plant specialization and reproductive isolation. This hypothesis is supported by recent evidence that *Regiella insecticola* (pea aphid U-type symbiont [PAUS]) is causally involved in increasing host plant associated fitness in some pea aphid genotypes, but not others (Leonardo 2004; Tsuchida et al. 2004; Ferrari et al. 2007; see also Chen et al. 2000). Similarly, certain pea aphid × secondary symbiont genotype combinations have been shown to reduce the number of winged dispersal morphs and alter the timing of sexual offspring production (Leonardo and Mondor 2006). Thus, host-mutualist genotypic interactions can affect a variety of traits that may increase positive assortative mating, reduce gene flow among populations, and enhance selection for ecological (host plant) specialization.

Many phytophagous insects possess mutualistic associations with multiple microbial taxa (Six 2003; Broderick et al. 2004; Dillon and Dillon 2004; Moran et al. 2005a; Wu et al. 2006). Consequently, successful exploitation of host plants may be a function of the full combination of microbial and insect genotypes. Microbial mutualist communities that interact intimately within their insect host through the complex sharing of metabolic resources (e.g., Wu et al. 2006; McCutcheon and Moran 2007), or through complementary adaptations to host plants are particularly likely to facilitate host-associated reproductive isolation due to the challenge of

coordinating multiple genomes for optimal host plant performance and the many opportunities for intergenomic mismatching.

Inhibitory effects: reduction in host plant (ecological) specialization through microbial mutualists

Rather than fostering diversification, microbial mutualists also have the potential to inhibit divergent selection and subsequent diversification of herbivore populations. Mutualists may attenuate some selective pressures imposed on insects by host plants and preclude divergent adaptation to host plant-associated environmental variation.

For example, as described above, a microbial mutualist lineage may have a broader "host range" and express less phylogenetic conservatism in host plant "use" than its insect partner (e.g., it is able to perform mutualistic services, such as host plant allelochemical detoxification, across a relatively broad range of plants). The microbe may then act as an environmental buffer, with little influence on the variation in host-use patterns and diversification of phytophagous insects.Microbe-mediated buffering that inhibits evolutionary radiation is also likely when microbes that facilitate exploitation of particular host plants can be free-living or horizontally transmitted (e.g., gut microbiota; Dillon and Charnley 2002; Broderick et al. 2004; Kikuchi et al. 2007; secondary endosymbionts of aphids; Darby and Douglas 2003; Russell et al. 2003; Moran et al. 2005c; Russell and Moran 2005) and/or when the insect host is able to recognize many plants as suitable hosts. In this regard, microbial mutualists could provide a form of phenotypic plasticity for host use, favoring general diets over specialization, and hindering the process of host-associated diversification (Price et al. 2003; Moran 2007). Some generalist insect species may even take advantage of microbial amelioration of host-associated fitness trade-offs by managing the consortium of microbial mutualists best suited to each host plant (although we

know of no examples; Broderick et al. 2004; Dillon and Dillon 2004). Such management could be achieved through the induction of antibacterial genes that favor specific microbes when challenged with particular host plants or environmental conditions (e.g., Mittapalli et al. 2006).

Insect–microbe mutualisms in a tri-trophic context: facilitative and inhibitory effects on diversification

Pressure from natural enemies can facilitate or maintain host shifts in phytophagous insects, and thus can play an integral role in the evolution of host plant specialization and reproductive isolation (e.g., Brown et al. 1995; Murphy 2004; Nosil 2004; Singer and Stireman 2005; Nosil and Crespi 2006). In cases in which microbial mutualists mediate interactions between phytophagous insects and natural enemies, microbes may also play a significant role in adaptive diversification. For example, it was suggested by Weis (1982) that fungal mutualists of *Asteromyia* gall midges, which play a major role in gall formation, might also protect developing midge larvae from parasitoids by forming an impenetrable stroma (hyphal crust; see also Skuhravá and Skuhravy 1992). More recently, experimental evidence has mounted that the presence of specific secondary bacterial symbionts in pea aphids confers at least partial resistance to parasitoid attack (Oliver et al. 2003, 2005, 2006; Ferrari et al. 2004). However, there are also conditions under which a microbial mutualist may increase the vulnerability of its host to enemies, such as when a microbe produces apparent cues that can be used by enemies to locate host insects, or when microbial mutualists induce the release of natural enemy-attracting plant volatiles (Dillon and Dillon 2004; Sullivan and Berisford 2004).

If microbial mutualists frequently provide protection against generalist enemies, then the tri-trophic fitness advantages of host specialization in phytophagous insects (e.g., Bernays and Graham 1988; Bernays and Cornelius 1989; Dyer 1995, 1997) may be attenuated and broad host

ranges may be favored. Generalist host use patterns in ant-tended lycaenid butterflies that maintain obligate mutualisms with ants (Fiedler 1994) support this contention (Jaenike 1990). Conversely, microbial defenses may allow expansion into new niches formerly inaccessible to herbivores due to intense pressure from enemies, which could increase opportunities for divergent selection and ecological speciation (as argued for a marine isopod-bacterial association; Lindquist et al. 2005). In either case, explicit consideration of the role of mutualists in providing enemy-free space for herbivores (sensu Jeffries and Lawton 1984) will likely provide greater insight into the mechanisms by which herbivore diet breadth evolves and its consequences for adaptive diversification. However, it may be difficult to distinguish the effects of nutritional versus defensive benefits of microbial mutualists if in both cases one predicted outcome is broader host ranges and fewer opportunities for host plant related genetic differentiation.

Predictions concerning the effect of enemy–herbivore–mutualist interactions on diversification depend on the prey- or habitat-specificity of the natural enemies exerting selective pressure. Interactions between herbivore–microbe mutualisms and specialized enemies could serve to inhibit adaptive diversification, but under some circumstances, such enemy pressure may favor host plant shifts and host plant associated genetic differentiation. If an herbivore's dominant enemies are specialized to a particular herbivore–plant association (e.g., many parasitoids), shifts onto novel host plants may garner some degree of enemy-free space for herbivores (Lawton 1986; Gratton and Welter 1999; Singer and Stireman 2005). If microbial mutualists increase the frequency of viable host-shifts onto novel plant taxa by ameliorating nutritional or defensive barriers, and the shift is favored by enemy-free space, these two factors may act synergistically, thus ensuring that alleles associated with the novel host preference

increase in frequency. Under conditions in which the enemy or suite of enemies a herbivore faces is dependent upon host plant identity, selection by these enemies is likely to amplify preexisting trade-offs in host plant use and further encourage genetic differentiation of host plant associated populations.

Interactions between microbial mutualists and specialized enemies may also encourage diversification on a single host plant. For example, in the *Asteromyia–Botryosphaeria* gall midgefungal mutualism, at least four gall morphotypes can be found on a single *Solidago* species (*S. altissima*) (Gagné 1968; Crego et al. 1990). The gall morphs differ primarily in the thickness of the fungal hyphal mass and fungus-derived stroma, which in turn influences parasitism rates by wasps (Weis 1982b; Crego et al. 1990; T. Carr and J. O. Stireman, unpubl. data). Furthermore, allozyme (Crego et al. 1990), mtDNA (Stireman et al. 2008), and AFLP (Stireman et al. 2008) markers reveal that the midges exhibit non-trivial genetic differentiation with respect to gall morphotype. This coincidence of gall morph, parasitism frequency, and genetic differentiation suggests that the diversification of these midges has been driven by interactions between parasitoids and gall morphology mediated through the fungal mutualist.

Microbial mutualists may also provide protection from antagonistic or competitive microbes. For example, Scarborough et al. (2005) demonstrated that the presence of the secondary symbiont *R. insecticola* reduced post-attack sporulation of the aphid fungal entomopathogen *Pandora* (*Erynia*) *neoaphidis* (see also Ferrari et al. 2004). Similarly, specific fungal and bacterial associates of bark beetles provide protection against antagonistic fungi that frequently colonize their galleries (Six 2003; Cardoza et al. 2006). If the antimicrobial benefits conferred by a mutualist are independent of host plant genotype or species, microbial mutualists may facilitate host shifts or expansions by providing some degree of protection from antagonistic

microbes that may be associated with novel host plants. However, if the degree of microbial-mediated defense against antagonistic microbes is strongly associated with particular host plant species, host plant genotypes, or environmental conditions (e.g., Klepzig and Six 2004; Hofstetter et al. 2005), shifting hosts may reduce or eliminate the mutualist's defensive capabilities and render the insect host susceptible to antagonistic microbe attack. In the former case, the increased potential for shifting hosts may favor the long-term adaptive diversification of an insect population host by allowing colonization of novel host plants, but divergence may be discouraged by the ability to feed on many hosts. In the latter case, selection for greater specialization creates evolutionary trade-offs in host plant use, again potentially facilitating adaptive divergence of populations, but the reduced ability to explore novel host plants may inhibit diversification.

Conclusions and Future Directions

Many of the most prominent evolutionary radiations of organisms in the history of life appear to coincide with the origin of intimate mutualistic and antagonistic associations. Tightly coupled mutualistic associations have repeatedly resulted in key innovations allowing the invasion of novel adaptive zones (Margulis and Fester 1991; Maynard Smith and Szathm´ary 1995; Moran and Telang 1998). The present perspective was motivated by the disparity between the implications of this hypothesis for understanding organismal evolution, and the dearth of empirical investigation into the mechanistic and ecological basis for precisely how mutualisms might influence diversification. Modern evolutionary biology is poised to reveal these ecological mechanisms because the tools to characterize microbes in seemingly inaccessible communities are themselves becoming accessible (e.g., Venter et al. 2004). No longer must the ecologically

significant variation that microbes introduce in plant–insect interactions be averaged into the biotic backdrop framing herbivory. In addition, molecular and genomic surveys have given evolutionary biologists a widespread appreciation of the often surprising capacities of microbes for creative meddling in the evolution of eukaryotes (e.g.,Wolbachia; Charlat et al. 2003). These new insights into microbial symbiont biology suggest new mechanisms in the evolutionary ecology of insect-plant interactions (e.g., Broderick et al. 2003, 2006; Wade 2007).

A particularly productive area for evolutionary ecological studies would focus on the trade-offs between ecological opportunity and ecological specialization mediated by microbial mutualists. We have argued that horizontally transmitted/facultative mutualists may expand ecological opportunity for insect lineages due to their potential to move among insect genotypes and exchange genes with other microbes in host and nonhost microbial communities. However, horizontally transmitted/facultative mutualists might limit an insect host lineage's ability to specialize ecologically due to reduced heritability of these phenotypic benefits and the difficulty of building linkage disequilibrium between insect host genes and microbial mutualist genes involved in plant exploitation (Wade 2007). In this respect, the central tension regarding the role of microbes in evolutionary diversification seems analogous to the evolutionary consequences of learning and plasticity (Paenke et al. 2007). In both, the issue is the introduction of flexibility in the relation between phenotype and genotype. What we currently do not know empirically—in both cases as it turns out—is the effect of that flexibility on the response to selection under different ecological scenarios.

In contrast to horizontally transmitted and facultative mutualists, vertically transmitted, obligate mutualists may promote a rigid phenotype-to-genotype map and ultimately ecological specialization. The consequence is that the reduction in genetic exchange with other microbial

genotypes, coupled with coevolutionary adaptation and irreversible processes such as genome reduction, may ultimately limit the ecological opportunity they can provide for their hosts. It is uncertain how common such strict inheritance is for microbes at the plant–insect interface. Many insect lineages may engage in coevolutionary interactions with microbes that are neither strictly vertical nor horizontally transmitted, but rather exhibit characteristics of both depending on the spatial and temporal time scale (Thompson 2005; Mikheyev et al. 2006). There may be a tension between ecological specialization and opportunity even within organismal lineages coevolving with mutualist microbes, and the trade-off between the two might ultimately shape the patterns of adaptive radiation. Moreover, maintaining associations with both obligate/vertically transmitted and facultative/horizontally transmitted mutualists may strike a balance between these trade-offs, allowing for some degree of ecological specialization while maintaining the potential to facilitate exploration of the adaptive landscape.

All of this offers a great opportunity for evolutionary biologists to elucidate mechanisms that generate biodiversity. The genomic revolution has provided enormous new information about the astounding diversity of microbial species and their metabolic capacities, many of which are symbiotic with higher eukaryotes (Moran 2007). But evolutionary ecologists need not become microbiologists nor genomic biologists. Rather, the uniqueness of herbivorous insects—their sheer diversity, borne of their struggle to exploit plants and avoid enemies, and their consistent tendencies to recruit microbial partners-in-aid—means evolutionary ecologists are uniquely positioned to put the genomic revolution to work in pursuit of broad themes in ecological and evolutionary research. If one big, vacant adaptive zone in evolutionary ecological research is the role of diverse community interactions in the adaptive radiation of eukaryotes

(Futuyma 2003), then the study of herbivorous insects and their microbial mutualists is rich with unexploited opportunities.

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CHAPTER III

SAME HOST-PLANT, DIFFERENT STEROLS: VARIATION IN STEROL METABOLISM IN AN INSECT HERBIVORE COMMUNITY

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Running title: Sterol metabolic variation in an insect herbivore community

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Abstract

Insects lack the ability to synthesize sterols *de novo*, which are required as cell membrane inserts and as precursors for steroid hormones. Herbivorous insects typically utilize cholesterol as their primary sterol. However, plants rarely contain cholesterol and herbivorous insects must therefore produce cholesterol by metabolizing plant sterols. Previous studies have shown that insects generally display diversity in their ability to metabolize phytosterols into cholesterol. This, coupled with the structural variation in plant sterols, suggests that plant sterols could mediate numerous plant-insect interactions related to nutrition. Despite the biological importance of sterols, there has been no investigation of sterol metabolism in a naturally occurring herbivorous insect community. We therefore determined the neutral sterol profile of *Solidago altissima* L., six taxonomically and ecologically diverse herbivorous insect associates, and the fungal symbiont of one herbivore. Our results demonstrated that *S. altissima* contained Δ^7-sterols (spinasterol, 22-dihydrospinasterol, avenasterol, and 24-epifungisterol), and 85% of the sterol pool existed in a conjugated form. Despite feeding on a shared host plant, we observed significant variation among the herbivores in terms of their tissue sterol profiles, and significant variation in the cholesterol content of the various insects. Cholesterol was completely absent in two dipteran gall-formers, and at extremely low levels in a beetle. Cholesterol content was also highly variable three hemipteran phloem feeders, including substantial differences in two aphid species, despite being congeners. The fungal ectosymbiont of one dipteran gall former contained primarily ergosterol and an ergosterol precursor. The larvae and pupae of the symbiotic gall-former lacked phytosterols, phytosterol metabolites, or cholesterol, instead containing an ergosterol metabolite in addition to unmetabolized ergosterol and erogsterol precursors, demonstrating the crucial role that a fungal symbiont plays in their nutritional ecology. These

data are discussed in the context of sterol physiology and metabolism in insects, and the potential ecological and evolutionary implications.

Introduction

The study of the chemical ecology of plant-insect interactions has been dominated by investigations of plant secondary metabolites (synthesized chemical compounds that are not involved in basic metabolic functions) and inducible defenses (Howe and Jander 2008). More recently, greater scrutiny has been given to the role that plant primary compounds (nutrients and other molecules necessary to sustain growth and survival) play in plant-insect interactions (Berenbaum 1995; Zangerl and Berenbaum 2004). Plant primary nutrient composition has a genetic basis and can vary both spatially and temporally among and within species (cf. Zangerl and Berenbaum 2004), suggesting that the deterrent properties of primary chemistry could evolve in response to selection from insect herbivory (Berenbaum 1995). Even in the absence of a direct functional role in deterrence, the ecological implications of primary nutrient and chemistry variation are significant—numerous studies have demonstrated that primary nutrients can influence host plant selection, foraging, growth and performance, the composition of insect communities, and possibly the evolution of host plant specialization in herbivorous insects (reviewed in Berenbaum 1995).

One of the most significant primary nutrients for herbivorous insects is sterols. Sterols are triterpenoid steroid alcohols that are used by eukaryotic organisms to maintain cell membrane integrity, permeability, and fluidity, as precursors for steroid hormones, and for developmental gene regulation (Behmer and Nes 2003). For most insects studied to date, cholesterol (Fig. 4) is the primary sterol incorporated into cell membranes and a necessary precursor to steroid hormones (e.g., ecdysteroids in arthropods). However, unlike many other organisms, insects are unable to synthesize the steroid nucleus *de novo* and must obtain sterols (i.e., cholesterol) either directly or indirectly from their diets (Clayton 1964). For example, carnivorous insects are able

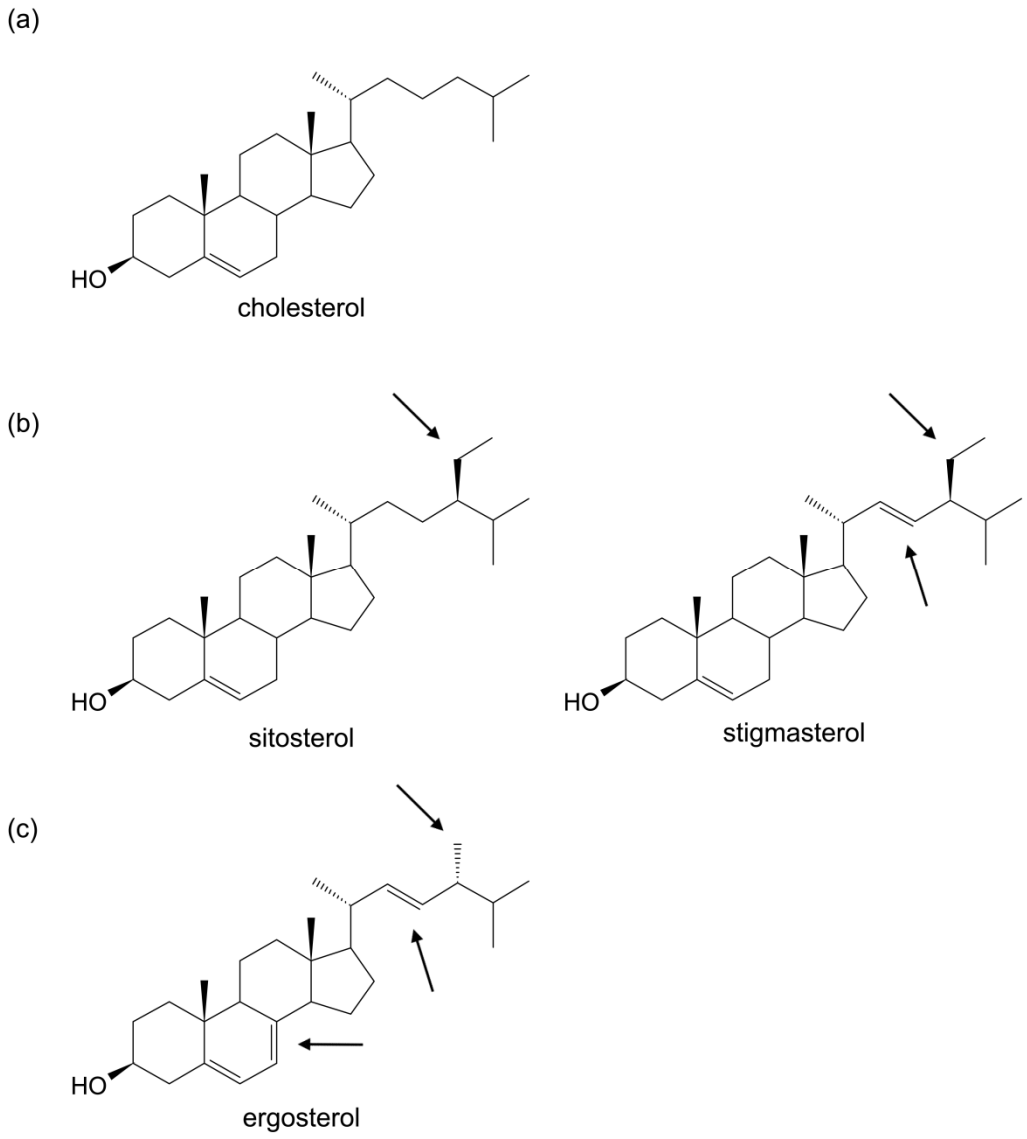

Figure 4. The chemical structures of the most common (a) animal, (b) plant, and (c) fungal sterols. The black arrows highlight structural differences of plant and fungal sterols relative to cholesterol.

to absorb and directly utilize the cholesterol contained in their prey's tissues. For herbivorous insects, however, obtaining cholesterol poses a challenge. Plants synthesize hundreds of different sterol molecules (collectively known as phytosterols), but often synthesize very little- to no cholesterol (Svoboda and Thompson 1985). Instead, the two most common phytosterols are sitosterol and stigmasterol (Fig. 4). Spinasterol is also prevalent, but much less common (Fig. 5). Phytosterols share gross structural similarities with cholesterol, but often have ethyl- or methyl-groups at carbon position 24 and/or double bonds at positions other than five in the tetracyclic nucleus (B-ring) (Figs. 4 and 5). Consequently, in order to obtain cholesterol from their diet, most herbivorous insects must metabolize ingested phytosterols into cholesterol or other cholesterol-like molecules.

Two key factors complicate cholesterol acquisition for herbivorous insects. First, some species are unable to convert certain structural classes of phytosterols into cholesterol. Grasshoppers (Orthoptera: Acrididae), for example, are unable convert sterols containing Δ^7 and/or Δ^{22} double bonds into cholesterol (see Figs. 4 and 5 for examples) (Behmer and Elias 1999a; Behmer et al. 1999b). Like grasshoppers, a number of insects are unable to dealkylate the hydrocarbon side chain, and/or remove double bonds on the side chain or within the tetracyclic nucleus (B-ring) of the sterol molecule (e.g., Svoboda et al. 1977; Kircher et al. 1984; Ritter 1984; Rees 1985; Svoboda et al. 1989; MacDonald et al. 1990). The inability of some insects to utilize and/or efficiently metabolize entire suites of phytosterols suggests that, in addition to their dietary necessity, the sterol physiology of plants could mediate a myriad of plant-insect ecological and community-level processes. The second is that, for reasons that are not entirely clear, plants frequently maintain a portion of their sterols in a conjugated form (Wojciechowski 1991; Moreau et al. 2002). Conjugated sterols are virtually identical to free sterols, except that

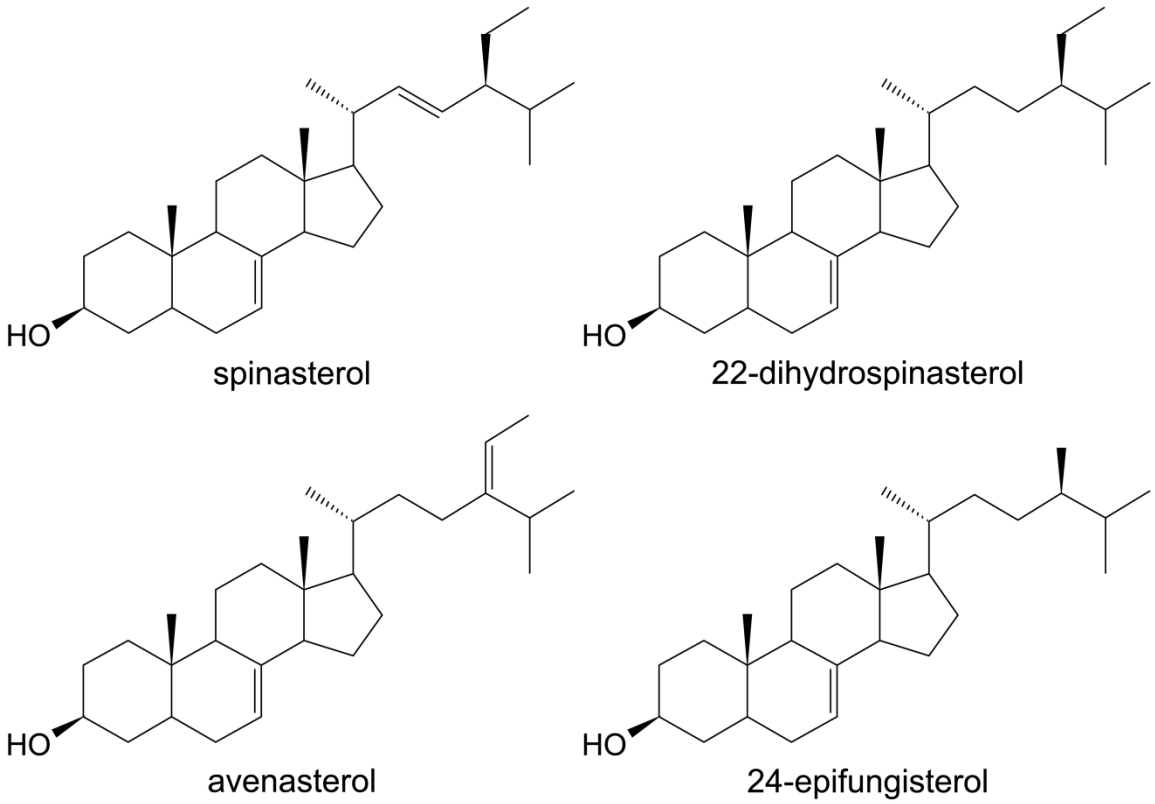

Figure 5. The chemical structures of the sterols detected in the vegetative tissues of *Solidago altissima*.

they are covalently bonded at the 3β-OH group to either fatty acids (steryl esters) or carbohydrates (steryl glycosides or acylated steryl glycosides; Moreau et al. 2002). The relative amount of conjugated sterols varies among plant species, and can even vary within species and within individuals under different environmental conditions, over time, or in different tissues (cf. Moreau et al. 2002). Conjugating phytosterols may also interfere with an insect's ability to absorb and/or metabolize those sterols, but relatively little is known about the ecological consequences of sterol conjugation.

Remarkably, despite decades of research on insect sterol metabolism, little is known about the ecology of plant-insect sterol physiology and metabolism. In particular, it is unknown how a community of herbivorous insects metabolizes the sterols found in their common host plant. Do communities of herbivores specializing on a host plant converge on a common sterol metabolic strategy? Or, does host plant variation along with variation in insect host breadth offer a variety of "sterol niches"? Are particular insect species constrained for their metabolism strategies and, if so, what patterns are evident? Here, we analyzed the sterol profile of the tall goldenrod, *Solidago altissima* L., and six of its common insect herbivores, including two gall-formers, and the fungal symbiont associated with one of the gall-formers. Specifically, we attempted to elucidate how insects from distinct taxonomic groups, with different phylogenetic histories, and different ways of obtaining nutrients (feeding guilds) metabolize the phytosterols in their shared diet. We ask how much these insects vary in the metabolic translation of host plant phytosterols, and what the observed variation reveals about the contribution of plant primary nutrients to plant-insect ecology. We predicted that insects that were phylogenetically related would exhibit similar metabolic capabilities, but that insects may diverge in sterol metabolic strategies as they diverge more generally in dietary strategies.

Our results show that: 1) Several insect species were unable to fully metabolize *S. altissima* sterols into cholesterol, despite many of them specializing or feeding regularly on *Solidago*. Thus, there appears to be significant constraint on the evolution of sterol metabolism in insects in that many insect species appeared to be unable to- or inefficient at converting phytosterols with Δ^7 double bonds to Δ^5 sterols. 2) Closely related species and those occupying the same feeding guild differed dramatically in sterol metabolism. 3) Fungal symbionts can provide a source of dietary sterols for their insect hosts. We discuss our results in the context of current knowledge of plant sterol physiology and insect sterol metabolism.

Methods and Materials

Collection sites

All collections were made in August-October of 2007/2008 at one of two sites. The first of these, Shelby Bottoms Greenway and Nature Park in Davidson County, Tennessee, USA (36.168°, -86.718°), is a protected 810 acre stretch of alluvial floodplain that runs parallel to the Cumberland River. This site is characterized by large stands of open prairie and wetlands surrounded by light forest. The plant community throughout the open prairies is dominated by various grasses and *S. altissima*, but also includes other asteraceous species, such as *Ambrosia*, *Bidens*, *Cirsium, Eupatorium*, and *Rudbeckia*. Some of the common non-asteraceous genera include *Asclepias*, *Daucus*, *Desmodium*, *Rubus*, *Solanum*, and *Vitis*.

The second site was Beaver Creek Wildlife Area North in Greene County, Ohio, USA (39.765°, -84.002°). Beaver Creek Wetland Wildlife Area North is a 380 acre portion of the protected Beaver Creek Wetland, specifically designated for hunting and fishing. This site

includes sections of open prairie, marshland, and lightly wooded areas. The open prairie is dominated by various grasses and *S. altissima*, but also includes numerous other herbaceous asteraceous genera, including *Ambrosia*, *Aster*, *Bidens*, *Conyza*, *Erigeron*, *Eupatorium*, *Euthamia*, *Rudbeckia*, and *Symphyotrichum*. Some of the common non-asteraceous genera include *Asclepias*, *Daucus*, *Desmodium*, *Rubus*, *Solanum*, and *Vitis*.

Study taxa

This study focused on the plant *S. altissima*, a subset of insects observed on it, and a fungus found associated with one of the insects. A brief description of each taxon follows.

The plant

Solidago altissima (Asteraceae) is an herbaceous, rhizomatous perennial that inhabits old fields and disturbed habitats throughout a large portion of North America. Its range extends from southwestern Canada, south through the eastern Rocky Mountains to southeastern Texas, and east to the Atlantic seaboard and coastal plains of the Gulf of Mexico. *S. altissima* ramets emerge from a mother rhizome in late spring, grow throughout the summer, and flower in late Aug.-early Oct. *S. altissima* prefers dryer, more circum-neutral soils, but is relatively tolerant of a wide variety of environmental conditions and is a dominant disturbed habitat species through a large portion of its range. *S. altissima* is attacked by over 100 species of herbivorous insects, including numerous non-specialists and a handful of *Solidago*/*Aster* specialists (Abrahamson and Weis 1997).

The insects

We focused on six locally abundant species that represented a range of feeding guilds, including a leaf-chewer, three phloem-feeders, and two gallers—one stem galler and one leaf galler. Our target insects were also taxonomically diverse, representing three orders and five families.

The leaf chewer we collected was *Trirhabda virgata* LeConte (Coleoptera: Chrysomelidae). This beetle specializes on plants in the genus *Solidago,* including the species *S. canadensis* L., *S. altissima, S. gigantea* Ait., *S. rugosa* Mill., *S. juncea* Ait., and *S. missouriensis* Nutt. (Swigoňová and Kjer 2004). It has also been recorded feeding on *Euthamia graminifolia* (L.) Nutt. and *Aster* spp., although these plants are unlikely to be consumed regularly (Messina and Root 1980). This beetle has a broad geographic range that extends from southern Canada through the United States east of the Great Plains.

We collected three phloem-feeders. The first was *Acutalis tartarea* Say (Hemiptera: Auchenorrhyncha: Membracidae). This treehopper specializes on species in the Asteraceae, including several *Solidago* species, *Ambrosia* spp. (putative preferred host), *Bidens bipinnata* L., and *Callistephus chinensis* (L.) Nees (Tsai and Kopp 1981). Records of it feeding on locust (*Robinia* spp.) also exist, but these records may be based on misidentifications (C. Dietrich, pers. comm.). It geographic range is broad, extending from southeastern Canada, throughout the eastern and southeastern US, and west to the Great Plains.

The other two phloem-feeders we collected are aphids in the genus *Uroleucon* (Hemiptera: Sternorrhyncha: Aphididae). The first of these, *U. luteolum* Willams, specializes on species in the Asteraceae, with *Solidago* spp. as its preferred hosts. It attacks several *Solidago* species and may attack species in the genera *Erigeron*, *Conyza*, and *Aster* (Blackman and Eastop

2006). Its range extends from southeastern Canada, throughout the eastern and southeastern USA, and west to Kansas and Nebraska.

The second aphid we collected was *U. nigrotuberculatum* Olive. It also appears to be an Asteraceae specialist, with *Solidago* spp. as its preferred hosts (Richards 1972). It may also occasionally attack *Zigadenus* spp. (Melanthiaceae) (Robinson 1985). Records from Japan, where it is invasive, indicates that it can feed on other members of the Asteraceae, including *Aster*, *Callistephus*, *Chrysanthemum*, and *Rudbeckia*, plus two other non-Asteraceae, *Zigadenus* spp. (Melanthiaceae) and *Oenothera erythrosepala* Borbás (Onagraceae) (Blackman and Eastop 2006). It is broadly distributed in North America, extending throughout much of southeastern Canada, the eastern USA into the midsouth, and as far west as Colorado.

Finally, two galling flies were collected. The first of these was *Eurosta solidaginis* subp. *solidaginis* (Diptera: Bracycera: Tephritidae), a stem-galling fly that specializes on only two *Solidago* species: *S. altissima* and *S. gigantea*. Rarely, *E. solidaginis* galls have also been recorded on *S. canadensis* and *S. rugosa* (Abrahamson and Weis 1997). *E. solidaginis*' range extends from southeastern Canada throughout most of the eastern USA, which includes New England west to North Dakota, south through northern Texas, and throughout the southeast to northern Florida.

The second fly was the leaf-galling midge *Asteromyia carbonifera* (Diptera: Nematocera: Cecidomyiidae). It too specializes on the genus *Solidago*, but has been collected on dozens of different goldenrod species (Gagné 1968; J.O. Stireman, T.G. Carr, pers. comm.). Like a number of other gall midges in the family Cecidomyiidae, *A. carbonifera* maintains a symbiotic association with a fungus (see below) (Bissett and Borkent 1988), which females actively transport to oviposition sites in specialized structure called the mycangium (Bissett and Borkent

1988). Unlike many other plant galling insects, the galls of *A. carbonifera* are not composed of plant tissue, but are rather made up almost entirely of fungal mycelium. Many of the biological details of the *Asteromyia*-fungus symbiosis are not well characterized, including any nutritional relationships between *A. carbonifera* and the fungus (Gagné 1968; Bissett and Borkent 1988). Its range extends throughout southern Canada, south through the United States into Mexico, and from coast to coast in the United States, but it is most common in northeastern North America.

The fungus

Botryosphaeria dothidea (Moug.) Ces. and De Not. (Ascomycota: Botryosphaeriales: Botryosphaeriaceae) has been identified as the fungal symbiont of *Asteromyia carbonifera* (Bissett and Borkent 1988; Janson et al. in press). It is a generalist, cosmopolitan plant pathogen and endophyte, that has been isolated from hundreds of plant genera in numerous families throughout the world (Farr et al. 1989; Smith et al. 1996). In addition to being associated with *A. carbonifera*, *Botryosphaeria* species/lineages appear to be associated with many cecidomyiids, and thus may represent a long evolutionary association with gall midges (Bissett and Borkent 1988).

Tissue collection for sterol analysis

Plant, insect and fungus were collected in the field and processed according to the following protocols.

S. altissima leaves were hand removed directly from ramets, placed in sealable plastic bags, and then transported in a chilled cooler. Leaves of all ages were picked, except those leaves

that had started to senesce. Leaves were immediately frozen whole at -80°C until analysis upon arrival to the laboratory.

The beetles, treehoppers, and aphids were collected directly from *S. altissima* plants in the field and killed by placing them into separate 50 ml plastic centrifuge tubes filled with 100% ethanol. The beetles and treehoppers were allowed to drop directly from the plants into the tubes, while aphids were removed using soft forceps. We collected insects without regard to their sex. For the beetles and treehoppers, only adults were available for collection. For aphids, we collected both nymphs and adults. The specimens were stored at 4°C in 100% ethanol until analysis.

We collected *E. solidaginis* galls by clipping them from *S. altissima* stems and transporting them in a chilled cooler. Galls were then dissected and larvae were killed and stored by placing them in a -80°C freezer until analysis. Entire leaves containing galls of *A. carbonifera* were collected directly from *Solidago* ramets and transported in a chilled cooler. Galls were dissected and larvae/pupae were killed and stored by placing them in a -80°C freezer until analysis. No adult flies were analyzed in this study.

In order to obtain enough tissue for analysis and to obtain uncontaminated sterol profiles for the fungal symbiont of *A. carbonifera*, sterol analysis was performed on mycelia from plated fungal cultures. *A. carbonifera* galls were excised from *S. altissima* leaves and surfaced sterilized with a sequential sterilization procedure (10 secs. in 95% ethanol, followed by 2 mins. in 10% bleach solution, and finally 2 mins. in 70% ethanol) and then placed on 2% malt extract agar until evidence of mycelial growth. Mycelia were then isolated using sterile technique and sub-cultured to obtain pure cultures. The identity of each fungal isolate analyzed was confirmed by phylogenetic reconstruction of the nrITS region (data not shown). Briefly, genomic DNA was

extracted using the method of Arnold and Lutzoni (2007). The nrITS region was then amplified in 20 μl PCR reactions (1x NEB standard PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM each primer, 1 M betaine, and 1 U NEB *Taq* DNA polymerase) using primers ITS4 and ITS5 (White et al. 1990) with a thermal cycler program that included 2 mins at 96°C, followed by 30 cycles of 30 secs at 94°C, 45 secs at 52°C, and 45 sec at 72°C, finishing with a 10 min extension at 72°C. Amplicons were treated with SAP/EXOI to remove residual dNTPs and primers, and then sequenced at the Vanderbilt University DNA Sequencing Core Facility (Nashville, TN, USA) or the University of Arizona Genetics Core (Tucson, AZ, USA). Phylogenetic reconstruction was performed in MEGA 4.02 (Tamura et al. 2007). Reconstruction was performed using maximum parsimony with the close-neighbor-interchange (CNI) algorithm where initial trees were obtained by random addition of sequences (10 replicates). Clade support was estimated by1000 bootstrap replications. The reconstruction included several *Botryosphaeria* spp. samples publically available on NCBI's GenBank, including sequences from the epitype specimen for *B. dothidea*. We employed a phylogenetic species concept and considered all isolates that belonged to the same monophyletic clade as the *B. dothidea* epitype sequence to be *B. dothidea* isolates. Only those isolates that met these criteria were used in sterol analysis.

Sterol extraction and analysis

Samples used for insect sterol extraction and analysis were comprised of hundreds to thousands of individual collected in the same geographic location at the same time of year (although in two separate calendar years). The qualitative sterol profile of each study species did not significantly vary across space or time, so the reported sterol profiles are an average

(numerous individuals, two geographic locations, different calendar years) sterol profile for the species in question. This approach was necessitated in part by the relatively small size of the study insects and the goal of the study, which was to characterize broad patterns of sterol metabolism in an insect herbivore community. The free-alcohol and conjugated sterol profiles for *S. altissima* were obtained from hundreds of leaves across numerous plants and the fungal sterol profile was obtained from 20 different fungal isolates.

Fresh frozen plant material, fresh fungal material, or ethanol preserved insect carcasses were extracted by obliteration using 8, #5 glass beads (Sigma, St. Louis, MO, USA) and a modified paint shaker. The obliteration was repeated 3 times for each sample in a 50 ml centrifuge tube, with constant shaking for 30 minutes in 30 ml of 95% ethanol. The ethanol fractions were removed, combined, evaporated to dryness, and the residue was resuspended in 70% methanol:water and the steroids were extracted from the 70% methanol:water phase with water equilibrated hexane. The steroid fraction was evaporated to dryness under nitrogen and subsequently resuspended in a minimal volume of hexane for subsequent concentration and cleaning by thin layer chromatography (TLC) on Silica G (Alltech, Nicholasville, KY, USA – 250 micron plate thickness) developed with toluene:ethyl acetate (9:1). The bands co-migrating with sterol standards were scraped from the TLC plate and extracted from the silica in anhydrous ethyl ether. The ether fraction was evaporated to dryness under nitrogen and the resulting film was resuspended in hexane. Sterols were concentrated by repeated injections and collection of sterol containing fractions from reverse-phase liquid chromatography (RP-HPLC) column. This was accomplished by eight, 50 μL injections per sample on an Apollo C18 high efficiency column (Alltech, Nicholasville, KY, USA) and eluted with acetonitrile:MeOH (9:1, 1 ml/min) at 38^{o}C.

Next, sterol fractions were collected, combined and evaporated to dryness under nitrogen and resuspended in 50 μL of hexane for subsequent injection on a gas chromatograph – mass spectrometer (GC-MS) for conclusive sterol identification. Steroids were identified and quantified by GC-MS using previously produced standard curves for all steroids. Authentic sterol standards were purchased commercially (Sigma, St. Louis, MO, USA) or in the case of the Δ^7 sterols, isolated and purified from spinach. Steroids were analyzed by GC-MS on a 6850 networked gas chromatograph (Technologies, Inc., Santa Clara, CA, USA) and a 5973 mass selective detector (Technologies, Inc., Santa Clara, CA, USA) using the following conditions: Inlet temp 260^{o}C, transfer line temp of 280^{o}C, and column oven temp programmed from 80 to 300^{o}C with the initial temp maintained for 1 min and the final temp for 20 min and a ramp rate of 30^{o}C/min. The column used was a glass capillary MS-5 column (30 m) (Restek, Bellefonte, PA, USA) with a film thickness of 0.25 mm. Helium at a flow rate of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temp of 250^{o}C and a quadrupole temp of 180^{o}C. All steroids were in agreement with authentic sterol standards at each of the separation, concentration and identification steps. We report (1) cholesterol, (2) spinasterol, (3) 22-dihydrospinasterol, (4) avenasterol, (5) ergosterol, (6) brassicasterol (ergosta-5,22-dien-3β-ol), (7) ergosta-7,22-dien-3β-ol, (8) 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol, (9) desmosterol, (10) lathosterol, (11) stigmasterol, (12) sitosterol and (13) 24-epifungisterol within insect, fungi, or higher plant tissues. The mass spectrum for each of the sterols was obtained and is consistent with previous studies. The prominent mass ions for all sterols are as follows: cholesterol m/z 386 [98%], 371 [50%], 368 [60%], 301 [56%], 275 [100%] and 255 [50%]; spinasterol maintains prominent mass ions of: m/z 412 [35%], 397 [25%], 369 [30%], 271 [95%] and 255 [100%]; 22-dihydrospinasterol produces the following

prominent mass ions: m/z 414 [99%], 399 [75%], 273 [75%] and 255 [100%]; avenasterol produces the following prominent mass ions: m/z 412 [25%], 397 [25%], 314 [90%], 271 [100%] and 255 [80%]; ergosterol produced the following prominent mass ions: m/z 396 [60%], 363 [100%], 271 [30%] and 253 [60%]; brassicasterol produced the following prominent mass ions: m/z 398 [100%], 383 [16%], 380 [20%], 271 [65%] and 255 [194%]; ergosta-7,22-dien-3-β-ol produced the following prominent mass ions: m/z 398 [25%], 383 [15%], 355 [10%], 271 [100%] and 255 [30%]; 7-dehydrodesmosterol produced the following prominent mass ions: m/z 382 [80%], 349 [100%], 323 [40%], 271 [10%] and 253 [30%]; lathosterol produced the following prominent mass ions: m/z 386 [100%], 371 [29%], 368 [3%], 273 [20%] and 255 [80%]; stigmasterol produced the following prominent mass ions: m/z 412 [60%], 397 [15%], 379 [10%], 271 [80%] and 255 [100%]; sitosterol produced the following prominent mass ions: m/z 414 [90%], 399 [60%], 396 [40%], 273 [60%] and 255 [100%]; 24-epifungisterol produced the following prominent mass ions: m/z 400 [50%], 385 [25%], 367 [10%], 273 [5%] and 255 [100%].

Throughout the paper, free steroids are those identifiable in the tissue without further processing following ethanol extraction, while steryl-ester pools were reported following a subsequent base or acid hydrolysis step. Base saponification includes the treatment of ethanol extracted sterols with a 5% ethanolic KOH solution at 70^{o}C for 12 hrs, while acid hydrolysis includes the treatment of ethanol extracted sterols with 0.5% H_2SO_4 in 95% ethanol at 50^{o}C for 12 hrs. Subsequently, the freed steroids were extracted with hexane, backwashed to neutrality and processed as described above for free sterols. Internal standard, consisting of 100 μg of cholestane, was added to select samples to determine extraction efficiency of the sterol extraction and concentration procedure. All data is represented as relative percentages of the total sterol

profile identified within the given organism because no organismal weights were recorded. Where there is no report of sterol conjugates, the limited starting mass of material hindered our ability to divide the sample for conjugate analysis.

Results

Solidago leaf sterol profile

In nature sterols can exist in either a free- or conjugated form, and in the majority of plants the dominant form is the free-form. In the leaves of *S. altissima*, however, approximately 85% of the total sterol profile was in the conjugated-form (Table 1), with the remaining 15% in the free-alcohol form. In terms of the composition of the free sterol pool, spinasterol (C-29 $\Delta^{7,22}$-sterol) was by far the dominant sterol (94%) (Table 1; Fig. 5). Three other free sterols were present, but at a low proportion of the total pool (Table 1; Fig. 5). In terms of the conjugated sterol pool, it consisted entirely of spinasterol. In this pool, approximately 63% of the spinasterol was conjugated to fatty acids/acetate, with the remaining fraction being conjugated to carbohydrates.

Externally feeding insects' sterol profiles

The sterol profiles of the four external feeding insects we examined showed tremendous variation in tissue sterol profile (Table 2; Figs. 4, 5, and 6) even though they were all collected directly from *S. altissima* plants.

The first insect listed in Table 2 is the chrysomelid beetle *Trirhabda virgata*. Almost 60% of its sterol profile consisted of unmetabolized *S. altissima* sterols, with spinasterol comprising

Table 1. Identification and relative percentages of sterols detected in the leaf tissue of *Solidago altissima*.

	sterol type (as relative % of specified sterol pool)			
sterol form	spinasterol	22-dihydrospinasterol	avenasterol	24-epifungisterol
free-alcohol (15*)	94	4	1	1
conjugated (85*)	100	-	-	-

*These numbers represent the relative percentages of the total sterol pool that were in the free-alcohol or conjugated form.

Table 2. Identification and relative percentages of sterols found in the tissues of four externally feeding insects collected on *Solidago altissima*.

	sterol type (as a % of the total sterol pool)							
sterol source	**(1)**	(2*)	(3*)	(4*)	(5)	(6)	(7)	(8)
Trirhabda virgata (Coleoptera: Chrysomelidae)	**1**	49	5	5	40	-	-	-
Acutalis tartarea (Hemiptera: Membracidae)	**40**	20	10	-	-	-	30	-
Uroleucon luteolum (Hemiptera: Aphidae)	**90**	-	-	-	3.3	3.3	3.3	-
Uroleucon nigrotuberculatum (Hemiptera: Aphidae)	**16.6**	45	-	-	15	6.6	6.6	10

(1) Cholesterol, (2) spinasterol, (3) 22-dihydrospinasterol, (4) avenasterol, (5) lathosterol, (6) sitosterol, (7) stigmasterol, and (8) 22-dihydrobrassicasterol. Sterols with asterisks are found in Solidago altissima (see Table 1). A dash denotes that the sterol was not detected in that insect. Values may not sum precisely to 100% because of rounding error.

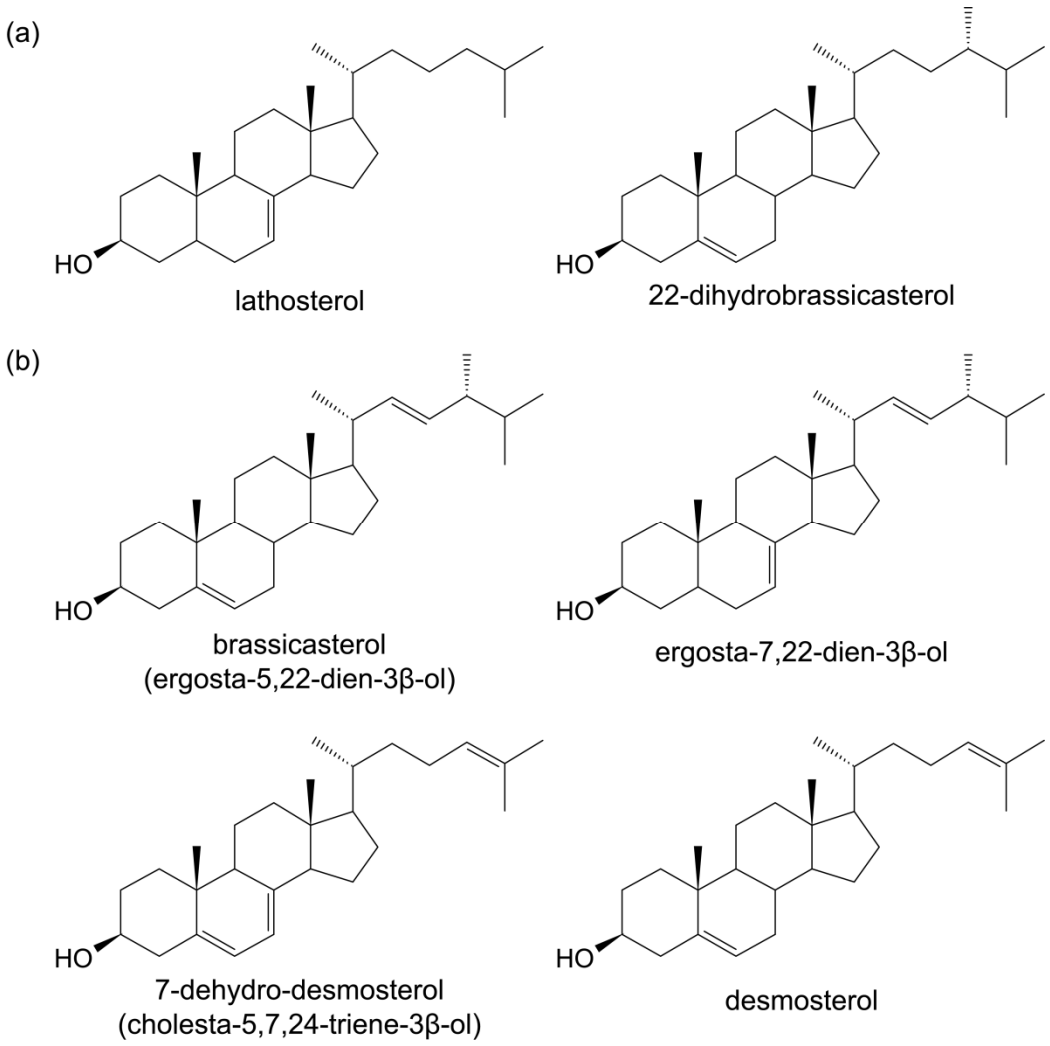

Figure 6. The chemical structures of the additional sterols detected in the tissues of the insects associated with *Solidago altissima*. The two sterols shown in panel (a) are sterol products derived from phytosterols, while those shown in panel (b) are sterol products derived from fungal sterols.

almost 50% of the total tissue sterol profile. Lathosterol (C-27 Δ^7-sterol), a potential metabolite of any *S. altissima* sterol following dealkylation, made up 40% of this beetle's tissue sterol profile. Only a very small amount of cholesterol (1%) was detected in this beetle.

We also observed multiple sterols in the tissues of the treehopper *A. tartarea*. Cholesterol (C-27 Δ^5-sterol) was the most abundant sterol (40%), but three unmetabolized plant sterols – stigmasterol (C-29 $\Delta^{5,22}$-sterol), spinasterol, and 22-dihydrospinasterol (C-29 Δ^7-sterol) – were also observed, at 30%, 20%, and 10%, respectively.

Interestingly the two aphids showed marked differences in sterol profile, even though they are congeners and were feeding on the same plant part. *U. luteolum* shows a very high cholesterol profile (90%), with the remaining 10% being divided equally among lathosterol, sitosterol (C-29 Δ^5-sterol), and stigmasterol. In contrast, *U. nigrotuberculatum* had much lower cholesterol content (just over 16%), and almost half of its tissue sterol profile was unmetabolized spinasterol. Four other sterols were observed in *U. nigrotuberculatum*. One of these was lathosterol (15%), which is likely generated by the aphid dealkylating spinasterol or other *S. altissima* sterols. The remaining three sterols were plant sterols (sitosterol, stigmasterol, and 22-dihydrobrassicasterol), but none were observed exceeding 10%.

Fungus sterol profile

The dominant sterol in *B. dothidea* was ergosterol at 95%. The other recorded sterol (ergosta-7,22-dien-3β-ol (5%)) may be a stable component of *B. dothidea*'s sterol biosynthesis pathway (Table 3; Figs. 4 and 6).

Table 3. Identification and relative percentages of sterols found in the tissues of two internally feeding insects and the fungal symbiont of *Asteroymia carbonifera* collected on *Solidago altissima*.

	sterol type (as a % of the total sterol pool)								
sterol source	**(1)**	(2*)	(3*)	(4*)	(5)	(6)	(7)	(8)	(9)
Botryosphaeria dothidea (Ascomycota: Botryosphaeriaceae)	-	-	-	-	95	-	5	-	-
Asteromyia carbonifera (Diptera: Nematocera: Cecidomyiidae)	-	-	-	-	21	6	34	36	3
Eurosta solidaginis (Diptera: Brachycera: Tephritidae)	-	50	25	20	-	-	-	-	5

(1) Cholesterol, (2) spinasterol, (3) 22-dihydrospinasterol, (4) avenasterol, (5) ergosterol, (6) brassicasterol (ergosta-5,22-dien-3β-ol), (7) ergosta-7,22-dien-3β-ol, and (8) 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol). Column nine represents a consolidation of other sterols found in smaller amounts (≤ 5%). Sterols with asterisks are found in Solidago altissima (see Table 1). A dash denotes that the sterol was not detected in that organism.

Internally feeding insects' sterol profiles

The sterol profile of the leaf-galling midge *A. carbonifera* was completely free of any of the phytosterols detected in *S. altissima*. Instead, the *A. carbonifera* profile contained ergosterol (C-28 $\Delta^{5,7,22}$) and ergosterol precursors/metabolites. This latter group of sterols included the C-27 ergosterol metabolite 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol), plus brassicasterol (ergosta-5,22-dien-3β-ol) and ergosta-7,22-dien-3β-ol (both are C-28 sterols). No detectable levels of cholesterol or 24-epifungisterol were found in *A. carbonifera*, although the common cholesterol precursor desmosterol (a C-27 $\Delta^{5,24}$-sterol) was found at very low levels (3%).

In complete contrast to the leaf-galling *A. carbonifera*, the sterol profile of the stem-galling fly *E. solidaginis* contained only phytosterols and phytosterol metabolites (Table 3; Figs. 5 and 6). The majority (95%) of its tissue sterol profile was unmetabolized plant sterols, including spinasterol, 22-dihydrospinasterol, and avenasterol. The remaining 5% was mostly an unidentified sterol, plus a small amount of lathosterol. No 24-epifungisterol or cholesterol was detected in *E. solidaginis* tissue.

Discussion

Because insects must acquire sterols exogenously, the acquisition and metabolism of sterols may be play important roles in insect ecology and community processes. Previous studies have demonstrated variation in sterol metabolism in insects, but have provided little ecological context. We characterized the sterol profiles of a natural insect herbivore community on the ecological model plant, the goldenrod *Solidago altissima*. Our data show that, despite sharing a host plant and a common sterol source, these insects exhibit marked variation in their sterol profiles, even between congeneric species. Part of this diversity is likely due to variation in host

breadth and feeding guild among study species. However, our data also highlight the likelihood of significant constraints on the evolution of sterol metabolism in insects: most species, even *Solidago* specialists, were very inefficient at- or unable to metabolize *S. altissima* sterols into cholesterol. Finally, our results also underscore the importance of fungi as sterol sources for plant-associated insects, as witnessed in a number of other species (Kok et al. 1970; Mondy and Corio-Costet 2000; Noda and Koizumi 2003).

Compared to most species in the Asteraceae, which typically produce Δ^5-sterols (Nes and McKean 1977), *S. altissima* appears somewhat unusual in producing Δ^7-sterols as its principal sterols. However, this result is consistent with results from a previous pollen study of *Solidago* spp. where the principal sterol detected was 22-dihydrospinasterol (as Δ^7-stigmasten-3β-ol) (Svoboda et al. 1983). Here, it appears that specific subclades within the Asteraceae may contain species that synthesize primarily Δ^7-sterols, while other clades contain species that synthesize both Δ^7- and Δ^5-sterols, or Δ^5-sterols exclusively. For example, *S. altissima* belongs to the subfamily Asteroideae (Panero and Funk 2008), and in the Asteroideae sterol profiles from at least one species from seven different tribes have been recorded. The principal sterols in four of these seven tribes (Anthemideae, Eupatorieae, Gnaphalieae, and Senecioneae) appear to be Δ^5-sterols. In contrast, two of the seven tribes (the Calenduleae and Heliantheae alliance) contain species that synthesize Δ^7-sterols (Nes and McKean 1977). Finally, *Aster scaber*, which like *Solidago* belongs to the tribe Astereae, appears to synthesize Δ^7-sterols (spinasterol) exclusively (Tada et al. 1974). Thus, in clades that are basal to Astereae (e.g., Calenduleae), species that synthesize Δ^5:Δ^7-sterol mixes or only Δ^5-sterols may be common, while within the more derived Astereae, Δ^7-sterols (e.g., spinasterol) may be more common.

The ecological and evolutionary significance of sterol profile convergence in plants is unclear, but there are tantalizing clues. Δ^7-sterols may confer important biochemical functions to the plant, and act in physiological adaptation to particular habitats (for example dry, saline environments; Salt et al. 1991; Behmer and Nes 2003). They may also act in defense against insects (Behmer and Nes, 2003). Many insects cannot efficiently metabolize/utilize Δ^7-sterols, (Clark and Bloch 1959; Ritter and Nes 1981; Ritter 1984; Behmer and Elias 2000), and some insect herbivores learn to avoid foods that contain large amounts of unsuitable sterols (Behmer and Elias 1999b; Behmer et al. 1999a). Convergence could therefore be a result of a history of intense selection from common insect herbivores that are unable to metabolize Δ^7-sterols (e.g., grasshoppers) and/or biochemical adaptation to novel habitats and/or niches.

A surprising finding was that *S. altissima* contained mostly conjugated sterols. Most plants conjugate free sterols, but only a relatively small percentage (Wojciechowski 1991; Moreau et al. 2002). The function of conjugated sterols in plants and how insects metabolize them remains an open question (Behmer and Nes 2003; Schaller 2003). However, one intriguing possibility is that conjugation could be a form of plant defense. Possibly, conjugation may prevent some insects from accessing needed sterols by keeping them into difficult-to-metabolize forms. Further work is necessary to determine if conjugated sterols are an effective insect defense and under what circumstances.

One of the most significant results is remarkable variation observed in cholesterol content between insect herbivores. The virtual lack of cholesterol in the beetle (*T. virgata*) is somewhat surprising, but this finding is consistent with work done on the alfalfa weevil, *Hypera postica* Gyllenahl, which also specializes on plants (alfalfa) that contain only Δ^7-sterols (MacDonald et al. 1990). That a relatively large amount of lathosterol was recovered from *T. virgata* tissue

demonstrates that side-chain dealkyalation likely occurs. Moreover, the fact that 99% of the tissue sterols recovered from *T. virgata* had a double bond at position seven (Δ^7) is consistent with the notion that many herbivorous insects lack the enzymes (specifically isomerases) necessary to completely convert Δ^7-sterols to Δ^5-sterols (Ritter1984; Rees 1985; MacDonald et al. 1990). The detection of low levels of cholesterol raises the possibility that *T. virgata* can convert a very small amount of Δ^7-sterol to cholesterol, or that an undetected Δ^5-sterol could have been dealkylated and converted to cholesterol. However, further work is necessary to differentiate metabolism from contamination (e.g., prior to sampling, *T. virgata* may have ingested some material (e.g. pollen) that contained a small amount of cholesterol).

The high percentage of Δ^7-sterols in *T. virgata* suggests that Δ^5-sterols (like cholesterol) are not necessarily the primary sterol required for growth and development, as seen in some grasshopper (Behmer and Elias 2000) and leaf-cutter ant species (Ritter et al. 1982). For insects that regularly encounter Δ^7-sterols, such as *S. altissima* specialists, it may be more metabolically efficient to forego metabolism to cholesterol and instead use dealkyalted Δ^7-sterols (e.g., lathosterol) and/or unmetabolized Δ^7-sterols for necessary biological functions. However, for some generalist insects, the inability to metabolize Δ^7-sterols from host-plants has an adverse effect on growth and development, likely due to difficulties associated with ecdysteroid biosynthesis (Ritter and Nes 1981; Behmer and Elias 1999a, 2000). A puzzling issue remains for specialist insects that feed on plants with Δ^7-sterols and cannot introduce Δ^5 double bonds, in that most insect ecdysteroid biosynthesis pathways appear to require $\Delta^{5,7}$ sterol intermediates (Rees 1985), which these species would be unable to synthesize.

Given the significant amounts of cholesterol and lathosterol recovered in our two aphid species, our results clearly indicate that aphids are capable of phytosterol dealkylation. However,

the relatively small amount of cholesterol and lathosterol, and high amounts of spinasterol and other phytosterols suggests that, in *U. nigrotuberculatum*, dealkylation capabilities may be relatively inefficient. Similar results were observed in another aphid species (*Schizaphis gramimum* Rondani) fed Δ^5-sterols (Campbell and Nes 1983). The distinct difference in sterol profiles observed in the two *Uroleucon* species is notable, but the precise reasons behind the difference cannot be determined by this study. *Uroleucon* is a large genus that includes species that vary widely in their host-plant specificity and life histories. *U. luteolum* may simply be more efficient than *U. nigrotuberculatum* at converting phytosterols to cholesterol. One previous study hinted that phytosterol metabolism can show minor variability within a species (Behmer and Grebenok 1998). Thus it is even more likely that substantial metabolic variation can exist between congeneric species.

Both *Uroleucon* species also contained detectable, albeit relatively low amounts of the Δ^5-sterols sitosterol and stigmasterol, neither of which was detected in *S. altissima* leaf tissue. Two possibilities may explain this result. First, recent evidence has shown that phloem phytosterol profiles do not always identically match that of the leaf tissue (S.T. Behmer, R.J. Grebenok, and A.E. Douglas, unpubl. data) and *Solidago* may synthesize some Δ^5-sterols that are more highly concentrated in the phloem (see also Svoboda et al. 1983). Sap-feeders like aphids may be particularly likely to sequester 'whole plant' nutrients, vitamins, and sterols not immediately present at the feeding site. The second explanation is that aphids collected for analysis may have recently been feeding on plants that contained Δ^5-sterols, stored those sterols in a conjugated (esterified) form for later metabolism, and passed a fraction of those sterols on to their offspring. Approximately 20% of the individuals in aphid samples were winged alates, which could have been migrants from other host plant species.

Interestingly, similar patterns were observed in the treehopper *Acutalis tartarea*. *A. tartarea* adults are pholem-feeders and highly mobile, so it is possible that the stigmasterol detected in their tissues was either transported by the *S. altissima* vascular tissues or obtained from a non-*S. altissima* plant species. A previous study of a planthopper (*Laodelphax striatellus* Fallen) suggests that species in the Auchenorrhyncha can dealkylate sterols (Noda and Koizumi 2003). It appears that *A. tartarea* may be inefficient at- or incapable of metabolizing sterols with Δ^7 and/or Δ^{22} double bonds, given the lack of lathosterol and detectable levels of stigmasterol, spinasterol, and 22-dihydrospinasterol detected in its tissues. Further work, including direct sterol analysis of phloem itself and manipulative field experiments on confined insects, will provide insights into the unusual sterol variability in the phloem-feeders.

The sterol profiles of the two dipteran gall-formers were dramatically different. *E. solidaginis* belongs to the family Tephrititdae, a large family of primarily herbivorous brachyceran flies. In addition to unmetabolized plant sterols, only trace levels of C-27 sterol were detected in larval tissues, suggesting that *E. solidaginis* is unable to dealkylate phytosterols. All brachyceran species studied to date are incapable of dealkylating C-28/C-29 sterols (Robbins 1963; Kircher et al. 1984; Svoboda et al. 1989). Lack of dealkylation is somewhat surprising, because species in herbivorous brachyceran families may frequently encounter low to non-existent levels of C-27 sterols in their plant-based diet. Moreover, fly larvae—especially the galling/leaf mining species common to the Brachycera—are relatively immobile, preventing them from incorporating behavioral mechanisms such as dietary mixing to cope with sterol inadequacies (e.g., Behmer and Elias 2000). Trace amounts of lathosterol in *E. solidaginis* may be transient components of *S. altissima*'s ecdysteroid biosynthesis pathway concentrated to detectable levels in *E. solidaginis* tissue. Other brachycerans (i.e., *Musca* and *Drosophila*) have

been shown to selectively absorb minute amounts of C-27 sterols from their diets for use in ecdysteroid production, as long as other sterols are present to act in structural roles (a phenomenon known as sterol sparing; Clark and Bloch 1959; Robbins 1963; Kircher et al. 1984; Feldhaufer et al. 1995). The capacity to successfully use C-28/29 phytosterols as membrane inserts may have been crucial for the shift some bracyceran species have made from basal feeding modes such as saprophagy/predation/parasitism/blood feeding (where C-27 sterols are abundant) to more derived herbivory (where C-27 sterols are relatively rare).

By contrast, *Asteromyia carbonifera*, a member of the midge family Cecidomyiidae, lacked plant-derived sterols altogether suggesting that it does not consume *S. altissima* tissue at all. Instead, it appears to acquire its sterols by consuming its fungal ectosymbiont. The presence of the C-27 ergosterol metabolite 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol) and the C-27 sterol desmosterol indicates that, like other nematocerans, *A. carbonifera* can dealkylate sterol side-chains (Svoboda et al. 1982). The dependency of *A. carbonifera* on its nutritional mutualist for dietary sterols adds to growing evidence that fungal mutualists can act as sources of dietary nutrients for plant-associated insects (Kok et al. 1970; Mondy and Corio-Costet 2000; Noda and Koizumi 2003). Indeed, many other cecidomyiids consume fungus as their principal food source (Bissett and Borkent 1988). Because *A. carbonifera* actively transports a single species of fungus for consumption (Bissett and Borkent 1988), this interaction demonstrates how fungal symbioses could potentially circumvent some level of "nutritional mismatching" between an insect and its host plant (Janson et al. 2008). Here, the phytosterol metabolic capacity of *A. carbonifera* is irrelevant, because it can simply metabolize the sterols provided by its symbiont on all plants that it is able to gall.

The physiology and biochemistry of plant and insect sterol synthesis and metabolism has been characterized in great detail over the past 50 years. What has been largely ignored is how plant sterol physiology and the limitations of insect sterol metabolism affect the ecology of plant-insect interactions. What are the proximate and ultimate causes for the marked variation we observe in phytosterol physiology and insect sterol metabolism? Are phytosterols important in structuring insect communities? Do sterols shape how insects compete on their host plants or act as chemical defenses, and, if so, how often and under what ecological and evolutionary circumstances? To address such questions, a deeper understanding of the functional significance of the variation in phytosterol profiles among many plant species is necessary (e.g., Omoloye and Vidal 2007). These questions might best be addressed by correlating environmental conditions with particular phytosterol profiles across numerous phylogenetically independent plant groups, and determining the sterol metabolic capabilities of insect herbivores that co-occur with these plants. Such an approach would provide clues as to how important phytosterols are as defense against herbivores. For example, trade-offs associated with sterol specialization may affect host plant specificity and the phylogenetic conservatism in host associations. Furthermore, it is an open question as to how frequently and at what rate sterol metabolic capabilities are gained or lost. Groups such as beetles, which have so far shown extreme variation in side-chain dealkylation, but consistency in B-ring metabolism (they are unable to introduce Δ^5 double bonds), may be especially amenable to such studies.

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CHAPTER IV

EVOLUTIONARY PATTERNS IN THE FUNGAL ECTOSYMBIONT OF THE GOLDENROD GALL MIDGE *ASTEROMYIA CARBONIFERA* (DIPTERA: CECIDOMYIIDAE) REVEAL SYMBIONT-MEDIATED PHENOTYPIC VARIATION WITHOUT COEVOLUTION

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Running title: Symbiont-mediated phenotypic diversity in a gall midge

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Abstract

Symbiosis with prokaryotic and eukaryotic microorganisms is a common feature of life for insects, and recent study has emphasized that microbial symbionts can be a source of adaptive phenotypic variation for their host. However, little is known about the circumstances in which coevolutionary interactions between host and symbiont contribute to phenotypic variation. Here, we investigated the phylogenetic, population genetic, and phenotypic patterns of the ectosymbiotic fungal associate of the evolutionary and ecologically radiating goldenrod galling midge, *Asteromyia carbonifera*. Phylogenetic reconstruction of ITS rRNA and partial EF1-α placed the fungal symbiont within the same clade as *Botryosphaeria dothidea*, a typically free-living, generalist plant pathogen and endophyte. Symbiont isolates from four divergent midge lineages and two geographic locations demonstrated a striking lack of parallel diversification with their host. Symbiotic *B. dothidea* also did not exhibit molecular evolutionary patterns common to microbial symbioses, such as substitution rate acceleration or A+T nucleotide bias. AFLP genotyping revealed within-lineage genetic diversity not observed in the nucleotide data, although this diversity was not clustered along host population or geographic lines. Three separate growth rate tests demonstrated that the symbiont-mediated phenotypic differences in gall structure observed in nature are not borne out in the absence of the plant and midge. This study demonstrates that phenotypic variation mediated by an ectosymbiotic association does not have to be the result of extensive coevolutionary interactions between a symbiont and its host. Moreover, this study shows that galling in insects is not relegated to manipulation of plant tissue, but also extends to manipulation of plant-infecting fungal tissue.

Introduction

The traditional view of adaptive evolution, championed starting as far back as the time of Darwin (1859), has held that phenotypic variation key to the adaptive process is encoded by the genetic material of the organism that is expressing the phenotype. More recently, it has come to light that a historically overlooked source of adaptive phenotypic variation for one organism may be the genes (and therefore the phenotypes) of a different organism (Oliver et al. 2005; Moran 2007; Janson et al. 2008). Specifically, organisms that maintain symbiotic associations may take advantage of the phenotypes expressed by their symbionts to exploit novel resources (Janson et al. 2008). Recent work has demonstrated that this scenario is common in the case of hereditary mutualisms, or associations in which a beneficial symbiotic associate is vertically transmitted from mother to offspring across host generations. In these cases, the long-standing association of specific host-symbiont lineages, and the alignment of reproductive interests, enhances the evolution of reciprocally beneficial adaptations. In turn, co-adaptation facilitates the use of a symbiont as a source of phenotypic variation for the host. This type of symbiosis has manifested in several ways, from simple single host-single symbiont associations to complex associations of several complementary associates residing in a single host, with the host taking advantage of different phenotypes offered by different symbionts (e.g., Baumann et al. 1995; Oliver et al. 2006; Wu et al. 2006; Sabree et al. 2009).

Some of the most common hereditary mutualisms in which the symbiont acts as a source for phenotypic diversity for the host exist between microbes and insects (Buchner 1965). Heritable mutualistic microbes provide crucial services to their hosts, such as nutritional compensation (Gündüz and Douglas 2009) and defense from natural enemies (Oliver et al. 2005). In addition to their phenotypic contributions, a common feature of heritable mutalisms is

the distinct genetic signature left in the genomes of the symbiont (e.g., Lutzoni and Pagel 1997; Wernegreen 2002; Hosokawa et al. 2006). For example, relative to non-symbiotic heterospecifics, hereditary microbial mutualists exhibit codiverisification with their host, accelerated rates of nucleotide substitution, genome-wide A+T nucleotide bias, reduced genome size, and increased fixation of slightly deleterious mutations (Wernegreen 2002). Indeed, some or all of these patterns have been observed in diverse microbial symbioses, including endocellular bacterial mutualisms (Moran et al. 1995; Moran 1996; Clark et al. 2000; Thao et al. 2000; Moran 2002), exocellular (e.g., gut) bacterial mutualisms (Hosokawa et al. 2006), and the fungal associate in lichens (Lutzoni and Pagel 1997). While these patterns do not characterize all stable microbial mutualisms (e.g., Wilkinson and Sherratt 2001; Kiers and van der Heijden 2006), their presence is a strong indicator that a microbial symbiont is a hereditary mutualist and a possible source of phenotypic variation for its host.

Despite rapidly increasing knowledge of the phenotypic benefits of microbial mutualisms, there is still relatively little known about purely ectosymbiotic microbial symbioses that are not involved in agricultural relationships with their hosts (see Mueller et al. 2005). Here, we define microbial ectosymbiotic mutualism as the intimate, mutually beneficial association between a microbe (bacteria, fungus, or protozoan) and a eukaryotic host, in which the microbial associate lives entirely outside of the host's body. Ectosymbiosis is relatively common in certain insect families/subfamilies, especially species that are intimately associated with fungi. Such groups include gall midges in the Cecidomyiinae (Bissett and Borkent 1988), ambrosia beetles in the Scolytinae and Platypodinae (Farrell et al. 2001; Six 2003), wood wasps in the Siciridae (Morgan 1968), fungus-farming termites in the Macrotermitinae, and fungus-farming ants in the Myrmicinae (Mueller et al. 2005). Ectosymbiotic mutualisms can differ from enodsymbiotic

mutualisms in their mode of transmission (opportunity for horizontal transmission and symbiont replacement), amount of exposure to "unprotected" (external) environments, increased opportunity for gene flow from other populations, and the overall intimacy of the interaction—all of which may in turn influence coevolutionary, evolutionary, and ecological processes of the symbiont, the evolution of co-adaptive complexes, and ultimately its capacity to act as a source of phenotypic variation.

Out of the above ectosymbioses, gall midges are unique. Gall midges have a long history of intimate association with fungi (Bissett and Borkent 1988; Roskam 2005). Ancestrally, gall midges are fungus feeding, consuming fungi found in association with detritus, with some species living in gall-like structures on fungus itself (Larew et al. 1987; Gagné 1989; Roskam 2005). The vast diversity of gall midges, however, received their common name for their phylogenetically derived lifestyle: inducing galls on living plant tissue. Many cecidomyiids induce typical plant galls and feed directly on hypertrophic/hyperplasic plant tissue (Gagné 1989; Harris et al. 2003). However, a number of plant-galling cecidomyiid species are symbiotic with fungi, most notably those species in the tribes Alycaulini, Asphondylini, and Lasiopterini (Gagné 1989, Rohfritsch 2008). These species directly introduce fungus, usually as reproductive spores, to galling sites (Borkent and Bissett 1985; Rohfritsch 2008). The fungus then proliferates inside of the gall structure and the developing midge larva feeds primarily or exclusively on fungus tissue (Bissett and Borket 1988; Rohfritsch 1997, 2008; Janson et al. 2009). There exists evidence of evolutionary transitions from strict fungus feeding (no plant galling), to plant-galling without a fungal symbiont and plant galling with a fungal symbiont, with additional evidence of independent gains and losses of fungal symbiosis in plant gallers (Roskam 2005; Stireman et al.

2010). Outside of strict fungus feeding being the ancestral character state, however, the precise order of these transitions is unclear.

Despite the unique nature of this symbiosis, little is known about the contribution of the fungus (or fungi) associated with any gall midge species to adaptive phenotypic variation exploited by the midge host. Moreover, little is known about how this mutualism behaves on an evolutionary timescale and what that reveals about the nature of the association. In this study, we address two main questions about the *A. carbonifera*-fungal ectosymbiosis in order to clarify the possible contribution of the fungal symbiont to the unusual phenotypic diversity observed in *A. carbonifera* (see below). First, we ask if the fungal symbiont exhibits evolutionary patterns that characterize most microbial symbioses. Here, we predict that, as seen in some other insect-fungus ectosymbioses, the fungus should show patterns common to microbial mutualisms, namely reciprocal co-diversification with its host, accelerated rates of molecular evolution, and A+T nucleotide bias. Second, we investigate whether the fungal symbiont is directly responsible for the observed phenotypic variation in gall structure seen in the gall midge species *Asteromyia carbonifera*. Here we predict that gall-morph specific groups of *A. carbonifera* will be associated with genetically divergent populations of fungus (from both free-living isolates and from each other) and that phenotypic differences observed in the field will manifest in culture. Answers to these questions will advance understanding of ectosymbioses, the contribution of symbionts to phenotypic variation, and provide answers to uninvestigated aspects of an unusual symbiosis between a gall-midge and a fungus.

Methods and Materials

Study organism

The goldenrod-galling midge *Asteromyia carbonifera* (Diptera: Cecidomyiidae) forms blister-like galls on the leaves of *Solidago* spp. throughout much of North America (Gagné 1968, 1989) (Fig. 7). *A. carbonifera* undergoes multiple generations each spring through the early fall, with the last generation overwintering in the gall as third-instar larvae (Gagné 1968; Weis 1982a). The following spring, the larvae pupate and eclose, reinitiating the life cycle. Adults are short lived, and are unlikely to survive for longer than 48 hours in nature (Weis et al. 1983; Harris et al. 2003; Yukawa and Rohrfritsch 2005). Shortly after emergence, females mate once and search for suitable oviposition sites (Gagné 1968, 1989).

A. carbonifera maintains an intimate association with a fungus (Borkent and Bissett 1985; Bissett and Borkent 1988; Gagné 1989). Borkent and Bissett (1985) provided evidence that females gather conidia and carry them in specialized invaginations (mycangium) in their terminal abdominal segment. Conidia are deposited along with eggs on *Solidago* leaves during oviposition. Conidia germinate soon after oviposition and fungal hyphae proliferate through the leaf tissue (Camp 1981; Borkent and Bissett 1985; Bissett and Borkent 1988). Unlike most insect-induced galls, *Asteromyia* galls do not exhibit any evidence of plant cell hyperplasy or hypertrophy (Camp 1981; Rohfritsch 2008). Instead, larvae come to lie in a central gall chamber, completely surrounded by mycelium with fungus tissue making up the majority of the gall structure (Camp 1981; Crego et al. 1990). During the course of gall maturation, a layer of mycelium differentiates into a relatively hard, carboniferous tissue (stroma), which appears to prevent successful attack from at least one species of hymenopteran parasitoid (Weis 1982b). *In vivo* sterol analysis (Janson et al. 2009) and *in vitro* culturing (Heath and Stireman, in press) indicate that *A. carbonifera* larvae feed exclusively on fungal tissue. Previously, the fungus was

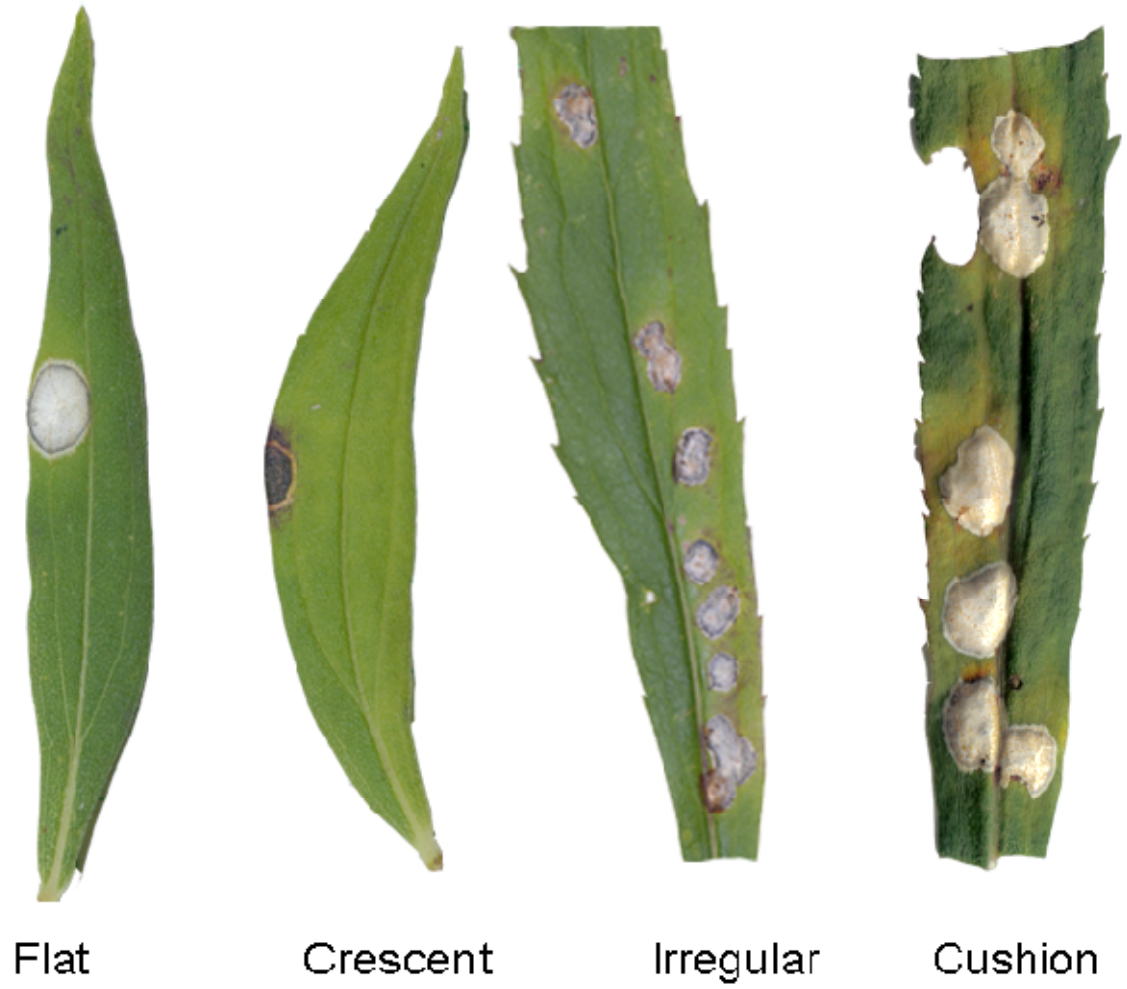

Figure 7. Examples of each of the four recognized gall morphs of *Asteromya carbonifera* on their most common hots plant, *Solidago altissima*. The color, shape, and placement of the galls in these examples are characterisitc of each gall morph.

identified as *Sclerotinium asteris* and later as *Macrophoma* sp. (Bissett and Borkent 1988; Roskam 2005)—a now defunct anamorphic (asexually reproducing) genus linked to the teleomorph (sexually reproducing) genus *Botryosphaeria* (Sutton 1980; Denman et al. 2000). These identifications were based on the morphology of conidia isolated from the mycangia of field collected females, and from the morphology of mycelia in culture and on the leaves of their host plants.

Crego et al. (1990) identified four morphologically distinct gall morphologies caused by *A. carbonifera* on the common old-field goldenrod *Solidago altissima* (as *S. canadensis*) in Illinois. They termed these morphologies "flat", "cushion", "crescent", and "irregular" (see also Gagné 1968; Stireman et al. 2008). In addition to differing in external shape, these galls consistently differ in the mean number of chambers per gall, the position of larvae within the gall, the position of the gall on the leaf, the thickness of the gall, and several other characteristics (Gagné 1968; Crego et al. 1990; Stireman et al. 2008). Each gall morph can be found sympatrically and syntopically, often co-occurring on the same ramet or occasionally the same leaf (Stireman et al. 2008). Two independent studies have demonstrated that *A. carbonifera* gall-morph-associated populations are partially to fully reproductively isolated (Crego et al. 1990; Stireman et al. 2008). Phenotypic diversity in *A. carbonifera* gall morphology is also correlated with variation in susceptibility to the various parasitoids that attack the galls (Weis 1982a; Stireman et al. 2008, unpubl. data), suggesting a possible adaptive basis to the phenotypic variation (Stone and Schönrogge 2003).

Fungus isolation

During the summers of 2005-2008, leaves containing *Asteromyia carbonifera* galls were collected from *Solidago* plants in several locations: local sites around Fredericton, New Brunswick, Canada; Dayton, Ohio, USA; Nebraska, USA; southern Illinois, USA, and southern and eastern Georgia, USA. At each location, we attempted to sample as many of the four gall morphs as possible and sample from the most dominant *Solidago* species (usually *Solidago altissima*). Briefly, leaves containing galls were placed into plastic bags, placed into a chilled cooler, and transported back to the lab where they were refrigerated until use. Photographs were taken of each gall prior to processing so that a record of the gall morph could be maintained. Leaves were rinsed briefly in running deionized water and then galls were excised from the leaf. Excised galls were surface sterilized by sequential immersion in 95% ethanol (10s), 10% Clorox solution (2 mins), and 70% ethanol (2 mins). Galls were then dried under sterile conditions and plated on 2% malt extract agar (MEA). Plates were sealed with parafilm, allowed to incubate at room temperature, and checked daily for any mycelial growth for up to 12 weeks. When mycelial growth was observed, a small agar plug was removed from the plate and transferred to a fresh 2% MEA plate. These subcultures were regularly checked for culture purity and subcultured when necessary. Because any fungi isolated from galls could be either the symbiont of *A. cabonifera* or incidental endophytes/pathogens, we also cultured field-collected *A. carbonifera* eggs, which frequently have conidia on their surface (Heath and Stireman, in press). Finally, we cloned ITS PCR amplicons from whole gall genomic DNA extractions (see below). We then matched the sequences of numerous egg-derived isolates and cloned direct-gall extractions to that of the gall-derived fungi to determine the focal species most likely to the symbiont of *A. carbonifera*. Pure cultures of gall-isolated fungi were allowed to grow at room temperature on 2% MEA for 4-6 weeks prior to genomic DNA extraction. To obtain tissue for

genomic DNA extraction for use in AFLP analysis, mycelium was scraped from MEA plates and placed into 14 mL tubes containing ~7 mL malt extract broth. Liquid cultures were allowed to grow for 4-7 days prior to genomic DNA extraction (see below).

Genomic DNA extraction and amplification for cloning and sequencing

Genomic DNA for use in sequencing and cloning was extracted using the method of Arnold and Lutzoni (2007). Two loci were targeted for sequencing: the internal transcribed spacer regions (including the full 5.8S subunit) of the nrDNA and partial elongation factor 1 alpha. These loci were chosen for their phylogenetic informativeness at potentially low levels of evolutionary divergence (White et al. 1990; Glass and Donaldson 1995) and their ability to be included in phylogenies with other publically available sequences. The ITS locus was amplified with the primers ITS4 and ITS5 (White et al. 1990). The primers EF1-728F and EF1-986R (Carbone and Kohn 1999) were used to amplify part of EF-1α. Genomic DNA was amplified in 15 or 20 μl reactions (10-20 ng genomic DNA, 1.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 μM forward and reverse primers). One molar betaine was added to the ITS PCR reactions because of the relatively high GC content of the ITS sequences. PCRs were performed with the following amplification program: 2 min at 96°C, 30 secs at 94°C, 45 secs at 52-54°C (depending on locus), 1 min at 72°C, followed by a 10 min final extension at 72°C, and an indefinite 4°C soak. All samples were checked for successful PCR amplification on a 1.5% agarose gel prior to sequencing.

PCR amplicons sequenced at the University of Arizona Genomic Analysis and Technology Core (GATC) or the Vanderbilt University DNA Sequencing Core. All PCR reactions were purified with an automated 96-well PCR purification system and then analyzed on

an ABI 3730xl DNA Analyzer at the GATC or treated with 1U each shrimp alkaline phosphotase/Exonuclease I if sequenced at Vanderbilt. PCR amplicons were sequenced in the 5' and 3' directions with the original amplification primers. ITS PCR amplicons from whole gall genomic extractions were cloned into chemically compotent *E. coli* cells using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Cloned amplicons were sequenced using M13 vector primer at the University of Arizona GATC.

Phylogenetic reconstruction and analysis of DNA sequence data

Contigs were assembled and edited from trace files in Sequencher 4.5 (Gene Codes Corp., Ann Arbor, MI). Each sequence was examined for miscalled bases and manually edited when necessary. The consensus sequences were then manually aligned in MacClade 4.08 (Maddison and Maddison 2005) and searched for variable sites across all isolates. No nucleotide substitutions were detected for all isolates at both loci (see results). Therefore, representative sequences for each locus from select *A. carbonifera* gall-morph isolates were chosen for use in phylogenetic reconstructions. Representative sequences for both loci were entered in BLASTn searches to approximate the genus-level identity of the symbiont. Congeneric and conspecific sequences were then obtained from GenBank and included in all phylogenetic reconstructions. All sequences were aligned using MUSCLE 3.7 (Edgar 2004) and manually adjusted in MacClade 4.08 when necessary.

Phylogenetic analysis of nucleotide sequence data was conducted by searching for trees using maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods. Prior to phylogenetic analysis, a partition homogeneity test (PHT) with 500 replicates was performed in PAUP* 4b10 (Swofford 2003) to determine if the two loci could be combined in a single

concatenated dataset. The PHT was non-significant (P = 0.848) and so all phylogenetic analyses were performed on the concatenated two-locus dataset.

The interspecific parsimony analysis was carried out in PAUP* 4b10. The parsimony analysis consisted of 100 replicate heuristic searches of trees generated by random stepwise addition using tree bisection reconnection (TBR) branch swapping. Alignment gaps were treated differently in two separate searches: the first as missing data, and the second as a 5th character state. All characters were equally weighted and unordered. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Because of the relatively large number of taxa and low level of sequence divergence in the intraspecific analysis, TNT 1.1 (Goloboff et al. 2008) was used to reconstruct the most parsimonious intraspecific tree. This parsimony analysis consisted of 100 replicate heurisitc searches generated by random stepwise addition using TBR branch swapping. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The MP search used four advanced MP tree searching algorithms (sectorial searching, parsimony ratchet, tree drifting, and tree fusing) with their default parameters. The robustness of the MP trees was evaluated with 1000 bootstrap replicates.

Prior to ML and Bayesian analysis, an appropriate model of nucleotide substitution was determined. Models of nucleotide substitution were selected according to the AIC using the program jModelTest 0.1.1 (Posada 2008). Maximum likelihood (ML) searches were conducted using PhyML 3.0 (Guindon and Gascuel 2003). Trees were obtained by 100 replicate heuristic ML searches of trees generated by creating an initial distance tree using the BIONJ algorithm. Branch swapping was performed using subtree pruning and regrafting (SPR). The interspecific data set employed a TPM1uf+G model of substitution (Kimura 1981) with a gamma shape parameter of 0.0960 (Ncat = 4). Equilibrium nucleotide frequencies were fixed at A=0.18982,

C=0.31143, G=0.24692, T=0.25183. Maximum likelihood was not performed on the intraspecific dataset. The robustness of the reconstructed tree was evaluated with 1000 bootstrap replicates using the aforementioned substitution model parameters.

Bayesian analysis was carried out in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2003). The interspecific dataset employed a HKY+G model of substitution with the transition/transversion ratio, equilibrium nucleotide frequencies, and gamma shape parameters optimized by MrBayes. The intraspecific dataset employed a HKY+I model of substitution with the transition/transversion ratio, equilibrium nucleotide frequencies, and proportion of invariant sites parameters optimized by MrBayes. Each analysis was run using four chains (one cold and three hot) for 2-4 million generations, until stationarity was reached. Trees were sampled every 1,000 generations and the first 25% of the sampled trees were discarded as burn-in. In all phylogenetic reconstructions, trees were either rooted with sequences from *Guignardia philoprina* (intrespecific tree) (Slippers et al. 2004) or *Botryosphaeria corticis* (intraspecific tree).

Tajima's relative rate test for three sequences (Tajima 1993) was performed in MEGA 4.02 using several different ingroup and outgroup taxa depending on the comparison being made. *P* values were standard Bonferroni corrected for multiple comparisons since the same symbiotic *B. dothidea* sequences were used in each comparison. Nucleotide composition was calculated in BioEdit 7.0.9.0 (Hall 1999) and statistically evaluated with a chi-square test in JMP 8.0.1 (SAS Institute, Cary, NC). Two additional loci were included in these tests that were not used for phylogenetic reconstruction due to their lack phylogenetic informativeness between free living and symbiotic fungus isolates (data not shown): partial 28S large ribosomal subunit (amplified

with primers LR0R and LR16; Moncalvo et al. 1993; Rehner and Samuels 1994) and partial beta-tubulin (amplified with the primers Bt2a and Bt2b; Glass and Donaldson 1995).

Genomic DNA extraction and amplification for AFLP analysis

Genomic DNA for use in AFLPs was extracted separately from the genomic DNA used for sequencing. A subset of isolates was chosen for DNA extraction for use in AFLP genotyping. Fungus tissue was removed from liquid culture (see above), dried under vacuum, flash-frozen in liquid nitrogen, crushed with a mortar and pestle, and then extracted with a CTAB extraction buffer containing beta-mercamptoethanol, followed by ethanol precipitation (Gibbons et al. in press). AFLP genotypes were generated using the methods of Vos et al. (1995), with some modifications. Briefly, approximately 150-300 ng of genomic DNA was digested with the restriction enzymes *Eco*R I and *Mse* I (NEB, Ipswitch, MA). AFLP adapters were ligated to the ends of genomic restriction fragments with T4 ligase (Roche, Palo Alto, CA). The samples were diluted five-fold and used as a template for pre-selective amplification. A pre-selective amplification was performed using two primers complementary to the AFLP adapters and the restriction site sequences (*Eco*+A primer and *Mse*+C primer) (Vos et al. 1995). Amplification conditions were: 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, for a total of 20 cycles, and then held at 10°C indefinitely. Samples were again diluted five-fold for selective amplification. One additional base was added to the primers for selective amplification and the forward primer was fluorescently labeled to visualize the DNA during migration through the gel. The following primer combinations were used in the selective amplification: 6FAM *Eco*R I+AA and *Mse*+CG, VIC *Eco*R I+AC and *Mse*+CT, PET *Eco*RI+AA and *Mse*+CC, and NED *Eco*RI+AT and *Mse*+CC. Selective amplification conditions were: 94°C for 2 min, 94°C for 30 s, 65°C for 30 s

(reduced by 1°C per cycle), 72°C for 1 min, repeat ten times excluding 94°C for 2 min; 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, repeat last cycle 30 times, and follow with 72°C for 30 min and holding at 10°C indefinitely. Selective AFLP reactions were poolplexed and run on an ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA) at the University of Arizona Genomic Analysis and Technology Core (Tucson, AZ).

Amplified fragment length polymorphism (AFLP) genotyping and analysis

Florescent AFLP fragment peaks were scored with Peak Scanner 1.0 (Applied Biosystems, Inc., Foster City, CA). Peak Scanner was set to filter out all peaks that were less than 100 bp, as fragments < 100 bp tend to exhibit significant size homoplasy (Vekemans et al. 2002). Fragment data obtained in Peak Scanner was standardized and filtered with the software package RawGeno 1.1-2 (Arrigo et al. 2008) implemented in the R 2.10.0 statistical software package (R Development Core Team, Vienna, Austria) to remove as much noise as possible from the AFLP dataset. Fragment bin size and peak filtering was performed with the following parameters: tolerance: 0.97, maximum bin width: 2 bp, minimum bin width: 0 bp, close bins: 0.05%, low intensity bins: 50 std RFU, low intensity peaks: 0.05%, low frequency bins: 4 Nblnds (~5% of the sample size). Phylogenetic analysis of the AFLP data set was conducted in PHYLIP 3.69 (Felsenstein 2005) by coding each fragment as a dichotomous character (absent: 0/present: 1) and searching for trees using maximum parsimony with the PARS module. Data was unweighted and the Wagner parsimony analysis consisted of 100 replicate random sequence additions and thorough searches for the best tree at each replicate. A maximum of 500000 trees were saved at each replicate. Robustness of reconstructed phylogenetic relationships was evaluated with 1000 bootstrap replicates.

To visualize any phylogenetic uncertainty and determine whether distance-based methods resulted in similar phylogenetic relationships, we created a splits diagram using SPLITSTREE 4.10 (Huson and Bryant 2006), which may provide a more appropriate representation of relationships at the intraspecific level. A splits diagram represents all inferred splits in a network diagram and is composed of parallel edges, rather than a pruned bifurcating tree representing only a consensus of the optimal tree or trees (Huson and Bryant 2006). We derived a Dice distance matrix from the binary AFLP data. A neighbor-joining (NJ) algorithm ('NeighborNet,' Bryant and Moulton 2004) was then used to construct an unrooted dendrogram, which is a visualization of the equal-angle split transformation we performed on the AFLP distance matrix.

F_{ST} was calculated for isolates over all gall morphotypes using AFLP-surv (Vekemans 2002). The significance of the F_{ST} values (i.e. significantly greater than zero) and 95% confidence intervals were calculated via permutation tests employing 1000 resamplings. The relative proportion of genetic variation explained by geography versus gall morph was assessed using analysis of molecular variance (AMOVA), as implemented in GenAlEx 6.2 (Peakall and Smouse 2006). The AMOVA was performed on a distance matrix calculated in GenAlEx 6.2 with the genetic distance formula for haploid binary data of Huff et al. (1993). Grouping levels were geographic location (OH vs. GA) and gall morph (crescent, cushion, flat, and irregular), and significance was established by 1000 permutations of the data. To determine if genetic recombination (sexual reproduction) occurs in the fungal symbiont of *A. carbonifera*, a statistical test on AFLP genotypes called the index of association (I_A; Maynard Smith et al. 1993; Taylor et al. 1999) was performed in LIAN 3.5 (Haubold and Hudson 2000). This method examines multilocus genotype data for a nonrandom statistical association among alleles at each locus (linkage disequilibrium). Linkage disequilibrium among large numbers of loci is suggestive of

low to non-existent levels of genetic recombination and thus asexual reproduction (Maynard Smith et al. 1993; Burt et al. 1996). Significance testing of the null hypothesis of linkage equilibrium was performed through a Monte Carlo simulation procedure that resampled the data without replacement 1,000 times (Haubold and Hudson 2000).

Growth rate assays

We also examined if the phenotypic differences observed in nature are an intrinsic property of the fungus or a product of the interaction with the midge/plant. In other words, we attempted to determine if there was a fungal genetic component to the phenotypic variation observed in the field. To do this, we subjected isolates from each of the four divergent midge lineages to three growth rate (culture) experiments. Prior to all growth rate tests, fungal isolates were allowed to acclimate to lab conditions by performing at least 12 weeks of continual subculturing. The first culture experiment involved growing isolates on unamended potato dextrose agar (PDA) in complete darkness. The second experiment involved growing isolates on PDA amended with varying amounts of KCl in order to assess growth under water stress (five treatments total; Kim et al. 2005). In the final experiment, isolates were grown on Czapek-Dox agar (CDA) with reduced concentrations (10% of standard recipe amount) of the nutrients phosphorous, carbon, and nitrogen, plus an unaltered control. The standard recipe for the CDA was as follows: 30 g/L sucrose, 3 g/L sodium nitrate, 0.5 g/L potassium chloride, 0.5 g/L magnesium sulfate heptahydrate, 0.01 g/L iron(II) sulfate heptahydrate, 1 g/L dipotassium hydrogen phosphate, 15 g/L agar. Three millimeter plugs of advancing mycelium were punched out of 2% MEA with a cork borer and transferred to a growth rate experimental plate (see below). Growth rates were then determined by taking two perpendicular measurements from the

edge of the plug to the edge of the advancing mycelium on days two, three, and four post-transfer using a digital caliper. The mean of these two measurements was then calculated and the daily growth rate (mm/day) was obtained by subtracting the mean growth of the previous day from the mean growth up to the measurement day. The two separate growth rate measurements (from day 2-3 and from day 3-4) were then averaged to get an overall mean growth rate for the replicate. The replicate growth rates were then averaged to get a mean growth rate for the isolate. Each isolate was replicated three times for each treatment. When necessary, as in the case of slowly growing isolates, measurements were taken from consecutive days beyond day four. Those isolates that grew too quickly were repeated so that two daily growth rates could be obtained and averaged. All tests were performed at 23°C ± 0.5°C and ambient humidity (~40%) in a temperature controlled environmental chamber. Growth rate data were natural log-transformed and analyzed with MANOVA in JMP 8.0.1. In the event of a significant MANOVA, univariate ANOVAs were performed.

Results

Fungal identity, phylogenetic reconstruction, and other evolutionary metrics

Based on BLASTn searches of ITS sequences, several fungal genera were isolated from plated *A. carbonifera* galls, including some common, cosmopolitan soil and plant-pathogenic fungi (Table 4). Most of these appeared to be co-occuring pathogens as some were isolated from ungalled *Solidago* leaves in addition to isolation from gall tissue (Table 4). Many of the non-*Botryosphaeria* species isolated in this study form leaf spots, which sometimes bare superficial

Table 4. Fungi isolated from whole *Asteromyia carbonifera* galls, *A. carbonifera* eggs, *Solidago* sp. leaves, and/or cloned from PCR amplifications of genomic DNA isolated from whole *A. carbonifera* galls. Numbers in parentheses indicate the number of confirmed (either by culture morphology or PCR amplification and sequencing of ITS) isolations of specific taxa out of the total number of confirmations in that category. If a particular category is absent from a fungal taxon, it indicates that taxon was not detected in that category.

Fungus taxon	Isolated from	Notes
Botryosphaeria dothidea	Plated *Asteromyia carbonifera* gall tissue (117/199) Plated *A. carbonifera* eggs (32/32) Cloned *A. carbonifera* whole gall genomic extractions (70/70)	Single phylogenetc lineage/sublineage in all cases (described in this paper)
Alternaria spp.	Plated *A. carbonifera* gall tissue (43/199) Plated *S. altissima* leaf tissue (12/25)	Multiple strains/species detected by PCR
Diaporthe sp.	Plated *A. carbonifera* gall tissue (29/199) Plated *S. altissima* leaf tissue (11/25)	Multiple strains/species detected by PCR
Collectotricum sp./*Glomerella* sp.	Plated *A. carbonifera* gall tissue (1/199)	Likely leaf spot misidentification
Nigrospora sp.	Plated *A. carbonifera* gall tissue (1/199)	
Phoma sp.	Plated *A. carbonifera* gall tissue (8/199) Plated *S. altissima* leaf tissue (2/25)	

resemblance to *A. carbonifera* galls, may have been misidentified as older *A. carbonifera* galls. Thus, many of the non-*Botryosphaeria* isolates may not have been associated with *A. carbonifera* galls. *Botryosphaeria* sp. was isolated from 31/31 plated *A. carbonifera* eggs and 70/70 cloned ITS PCR product that sequenced successfully (26 total failed sequencing reactions). Taken together, this indicated that the fungal associate of *A. carbonifera* belongs to the genus *Botryosphaeria*. Therefore, further phylogenetic analysis was performed using *Botryosphaeria* species obtained from GenBank.

The interspecific phylogenetic reconstruction (MP with gaps as 5th character states) included 39 isolates of 19 *Botryosphaeria* species. The aligned nucleotide dataset consisted of a total of 920 characters. Of those characters in the combined dataset, 386 were constant, 187 variable characters were parsimony uninformative, and 347 were parsimony informative. The MP search recovered six trees of length 1142. One of those MPTs is displayed in Fig. 8. Trees reconstructed with different methods were highly consistent, only differing in the placement of *B. tsugae* and *B. protearum*. The reconstructed trees were consistent with other phylogenetic trees reconstructed on the genus *Botryosphaeria*. All symbiotic isolates were placed within a clade that included the epitype specimen of *B. dothidea* (strain CMW 8000) with consistently high bootstrap and posterior probability support (Fig. 8). This clade included the species *B. populi*, which was previously described as a distinct species based on morphology (Phillips 2000), but was later deemed synonymous with *B. dothiea* (Phillips et al. 2005).

The intraspecific phylogenetic reconstruction (Fig. 9; MP tree with gaps treated as a 5th character state) on the concatenated ITS and Ef-1a datasets included 92 isolates of *B. dothidea*. The aligned dataset contained 764 characters. In total, 695 characters were constant, 40 variable sites were parsimony uninformative, and 25 variable sites were parsimony informative. The MP

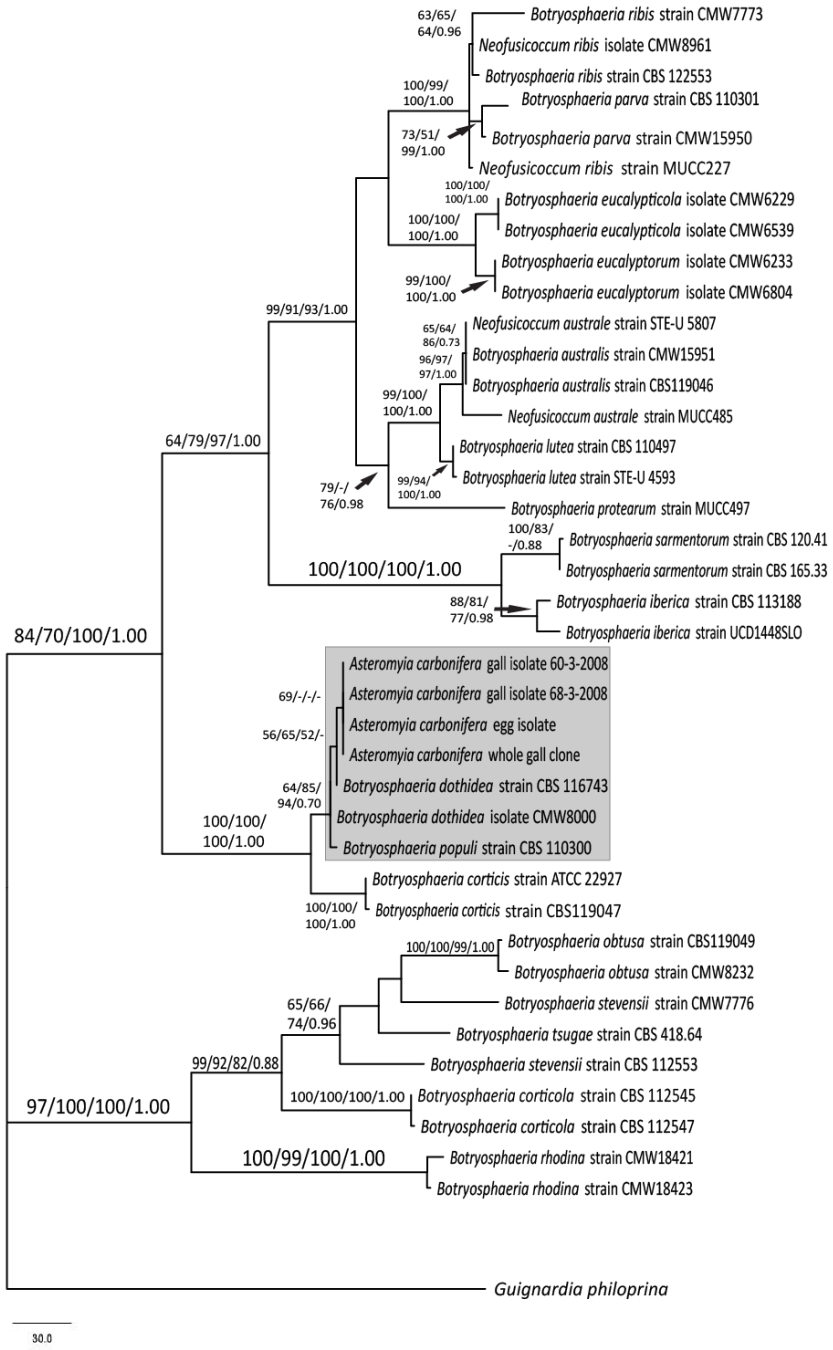

Figure 8. Phylogram of one of 18 most parsimonious interspecific trees reconstructed from the combined fungus ITS and EF-1α DNA sequences (gaps treated as a 5th character state). Numbers at nodes represent statistical support for each node. From left to right, the numbers are: bootstrap values, MP reconstruction with gaps as 5th character state; bootstrap values, MP reconstruction with gaps as missing data; bootstrap values, ML reconstruction; and posterior probabilities, Bayesian reconstruction. Nodes that have < 50% bootstrap support or < 0.50 posterior probability are shown with a dash or have no values at the node. The scale bar indicates the branch length that represents 30 nucleotide substitutions. Gray shading indicates isolates associated with *Asteromyia carbonifera*.

search recovered a total 76 trees of length 84. One of the most parsimonious trees is displayed in Fig. 9. The intraspecific tree contains two core monophyletic clades, one representing two isolates from South America and the other containing the bulk of the *B. dothidea* diversity. Within the main *B. dothidea* clades, there were several subclades, but few were supported highly. There was little evidence of host plant or geographic structure to the phylogeny, underscoring *B. dothidea*'s host plant generalist lifestyle and cosmopolitan distribution. All *A. carbonifera* symbiotic *B. dothidea* isolates belonged to a single clade, with the exception of three isolates from southeastern Canada, which formed a small subclade within the larger symbiotic clade. This subclade was reconstructed based on a two nucleotide indel that was not found in other isolates, which resulted in no statistical support for the clade when gaps were not treated as informative (Fig. 9). The *A. carbonifera* symbiont clade also included a few free-living isolates and the symbionts of several *Asphondylia* species native to South Africa and Australia.

Nucleotide composition varied among the four examined loci for both free living and symbiotic fungal isolates (Table 5). G+C content was higher than A+T content for all loci in both groups. Total A+T content was not elevated in *A. carbonifera*-associated isolates relative to free-living isolates (45.0% vs. 45.8%, respectively). Indeed, overall nucleotide composition did not significantly differ between free-living and symbiotic fungal isolates of *B. dothidea* ($\chi^2_3 = 0.31$; $P = 0.96$). Tajima's relative rate test (1993) revealed no evidence of nucleotide substitution rate differences for the symbiotic isolates compared to a free-living isolates (Table 6). There was, however, evidence of substitution rate increase in a fungus species that is involved in a lichen symbiosis (Table 6).

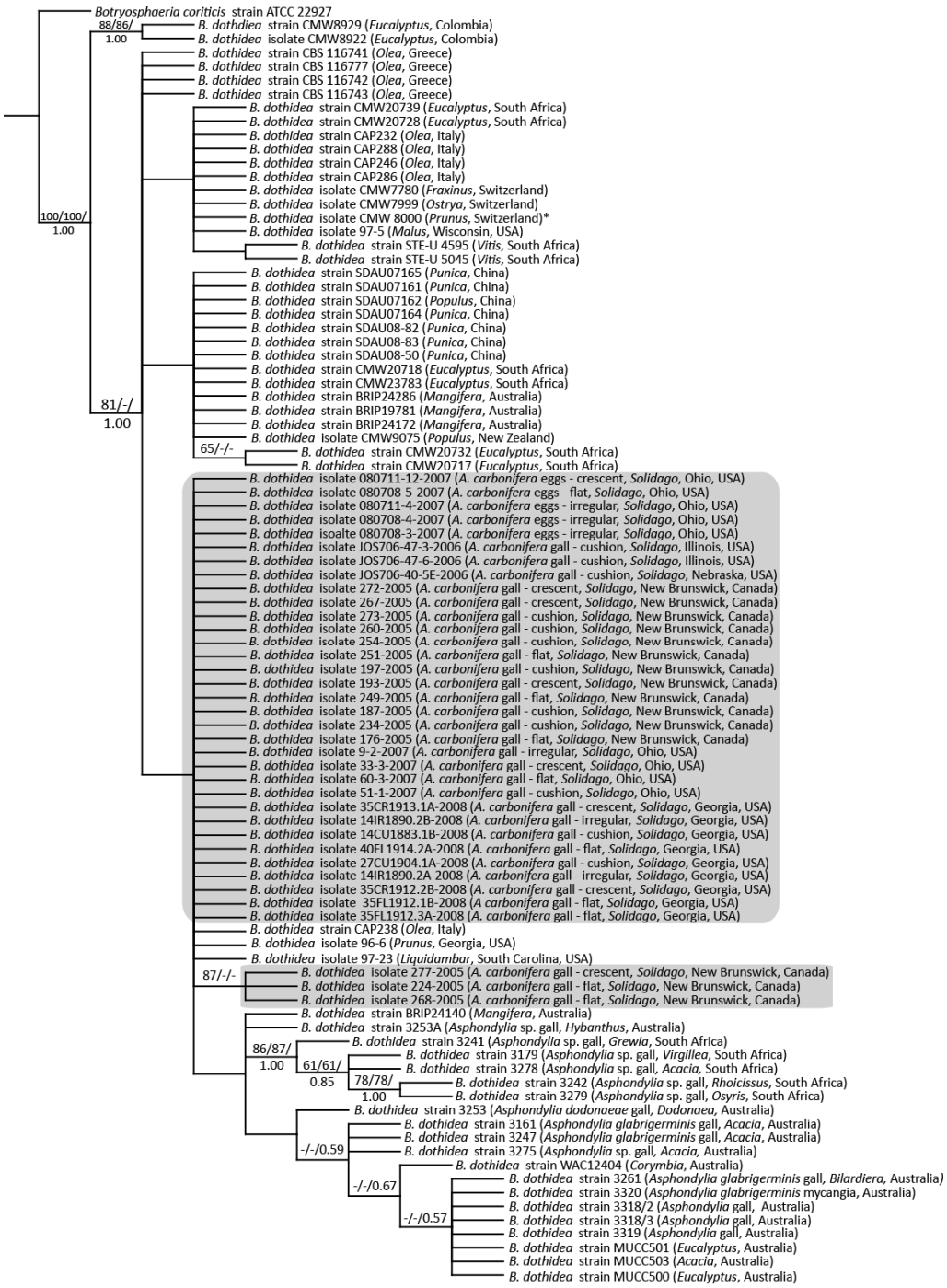

Figure 9. Cladogram of one of 76 most parsimonious intraspecific trees reconstructed from the combined fungus ITS and EF-1α DNA sequences (gaps treated as a 5th character state). The numbers at nodes represent statistical support for each node. From left to right, the numbers are: bootstrap values, MP reconstruction with gaps treated as a 5th character state; bootstrap values, MP reconstruction with gaps as missing data; and posterior probabilities, Bayesian reconstruction. Nodes that have < 50% bootstrap support or < 0.50 posterior probability are shown with a dash or have no values at the node. The asterisk denotes the epitype specimen for *Botryosphaeria dothidea* (isolate CMW8000). Gray shading indicates isolates associated with *Asteromyia carbonifera*.

Table 5. Mean percent nucleotide composition for four nuclear loci in free-living (collected from plant tissue and not in association with an insect) and *A. carbonifera* symbiotic isolates of *B. dothidea*. Sequences from all free-living isolates were obtained from GenBank and include isolates from several locations throughout the world found on numerous host plant genera. Sample sizes are as follows: free living isolates, 28S: N = 11; ITS: N = 242; beta-tubulin: N = 38; EF-1α: N = 79. Symbiotic *B. dothidea* isolates, 28S = 48; ITS: N = 150; beta-tubulin: N = 48; EF-1α: N = 96.

Locus	Nucleotide							
	%A		%C		%G		%T	
	Free-living	Symbiotic	Free-living	Symbiotic	Free-living	Symbiotic	Free-living	Symbiotic
Partial 28S LRSU	25.6	25.7	22.0	22.0	30.6	30.2	21.8	22.0
ITS nrDNA	21.7	23.4	29.2	27.5	25.8	25.9	23.3	23.3
Partial beta-tubulin	22.0	22.0	33.3	33.3	24.1	24.1	20.6	20.6
Partial EF-1α	19.2	19.9	29.8	29.6	26.6	25.6	24.5	24.9
Overall	22.7	23.3	27.9	27.4	27.1	26.8	22.3	22.5

Table 6. Results of Tajima's (1993) relative rate test for three sequences between various free-living and gall-midge symbiotic fungus isolates, and *Asteromyia carbonifera* symbiotic *Botryosphaeria dothidea*. Significance of the comparisons was determined by a Chi-square test with a standard Bonferroni correction. Sequences for all isolates not associated with *Asteromyia carbonifera* were retrieved from GenBank.

Lineage 1	Lineage 2	Outgroup	Gene	M_{iii}	M_{ijk}	m_{jii}	m_{iji}	m_{iij}	χ^2_1	P	Notes
Asphondylia symbiotic *Botryosphaeria dothidea* (Australia)	*Asteromyia carbonifera* symbiotic *Botryosphaeria dothidea* (North America)	*Guignardia philoprina*	ITS nrDNA	442	0	3	0	70	3.00	0.083	
			Partial translation elongation factor 1α	141	2	1	0	65	1.00	0.317	
Asphondylia symbiotic *B. dothidea* (South Africa)	*A. carbonifera* symbiotic *B. dothidea* (North America)	*G. philoprina*	ITS nrDNA	-	-	-	-	-	-	-	Test cannot be performed: no nucleotide variation
			Partial translation elongation factor 1α	138	4	4	2	61	0.67	0.414	
Lichenized (symbiotic) *Trypethelium* sp.	*A. carbonifera* symbiotic *B. dothidea* (North America)	*Aleuria aurantia*	Partial 28S large subunit nrDNA	419	24	69	34	38	11.89	<0.001*	Lichenized (symbiotic) *Trypethelium* sp. has the faster rate of evolution for both loci
			ITS nrDNA	255	19	76	40	36	11.17	<0.001*	
"Free-living" *Botryosphaeria corticis* (North America)†	*A. carbonifera* symbiotic *B. dothidea* (North America)	*G. philoprina*	Partial 28S large subunit nrDNA	567	0	2	1	44	0.33	0.564	
			ITS nrDNA	436	3	5	2	70	1.29	0.257	
			Partial beta-tubulin	322	2	3	0	96	3.00	0.083	
			Partial elongation factor 1α	172	1	1	1	54	0.00	1.000	
"Free-living"	*A. carbonifera* symbiotic	*Tuber*	ITS nrDNA	261	35	44	33	77	1.57	0.210	

Alternaria alternata†	*Botryosphaeria dothidea* (North America)	*indicum*							
			Partial beta-tubulin	107	10	33	22	30	2.20	0.138
			Partial translation elongation factor 1α	77	29	34	37	57	0.13	0.722

*: Significant comparison. †: "Free-living" indicates that these isolates were not found in association with an insect host, although they are plant pathogens/endophytes. M_{iii}: number of identical sites in all three lineages; M_{ijk}: number of unique sites in all three lineages; m_{jii}: number of sites unique to lineage one; m_{iji}: number of site unique to lineage two; m_{iij}: number of sites unique to lineage three (outgroup). All non-*Botryosphaeria dothidea* sequences were obtained from GenBank. Accession numbers for GenBank obtained sequences are as follows: *Asphondylia* symbiotic *B. dothidea* (Australia), ITS: EF614926; EF-1α: EF61494; *Guignardia philoprina*, 28S: DQ377878; ITS: FJ824768; beta-tubulin: FJ824779; EF-1α: FJ824773; *Asphondylia* symbiotic *B. dothidea* (South Africa), ITS: EF614918; EF-1α: EF614935; *Trypethelium* sp., 28S: AY584652; ITS: DQ782839; *Aleuria aurantia*, 28S: AY544654; ITS: DQ491495; *Botryosphaeria corticis*, 28S: EU673244; ITS: DQ299245; beta-tubulin: EU673107; EF-1α: EU017539; *Alternaria alternata*, ITS: AF347031; beta-tubulin: AY438647; EF-1α: *Tuber indicum*, ITS: DQ375511; beta-tubulin: DQ379288; EF-1α: DQ336315.

AFLP population genetics and phylogenetics

Quality filtering of our AFLP dataset removed an average of 19.6% of the initial fragments detected, leaving our final dataset consisting of 73 isolates and 1010 loci. The parsimony analysis reconstructed two most parsimonious trees (MPTs) of length 10461. These MPTs indicated that there were several distinct clusters of isolates, but these were not consistently associated with gall-morph or geographic location. Two free-living isolates were nested well within the symbiotic isolates. The bootstrap support for the AFLP parsimony tree was weak across most of the tree, except for some of the tips. The tips of the NeighborNet tree inferred using SPLITSTREE is highly consistent with the results from the parsimony analysis and provides a visual summary of the phylogenetic uncertainties (Fig. 10). Again, the pattern of genetic variation in the AFLP samples did not consistently correspond to geography or the morphology of the gall-morph of origin.

The overall F_{ST} among gall morph associated isolates was 0.0077, lower 95% CI: -0.0033, upper 95% CI: 0.0119 ($P = 0.11$), suggesting a lack of genetic structure among isolates from different *A. carbonifera* gall morphs. Pairwise F_{ST} among gall-morph associated isolates ranged from 0.0001-0.0150 (Table 7). Nei's genetic distance (Lynch and Milligan 1994) among gall-morph associated isolates ranged from 0.000-0.0051 (Table 7). In the AMOVA of AFLP data, the gall morph of origin was responsible for approximately 1% of the total genetic variation, whereas population (sampling site) was responsible for an additional 1% (Table 8). These variance components were not significantly greater than expected, as most of the genetic variation was found within gall morphs and within populations (98%).

The standardized index of association (I^S_A) for three separate geographic groupings of fungi is summarized in Table 9. This test indicated that the fungal symbiont of *A. carbonifera*

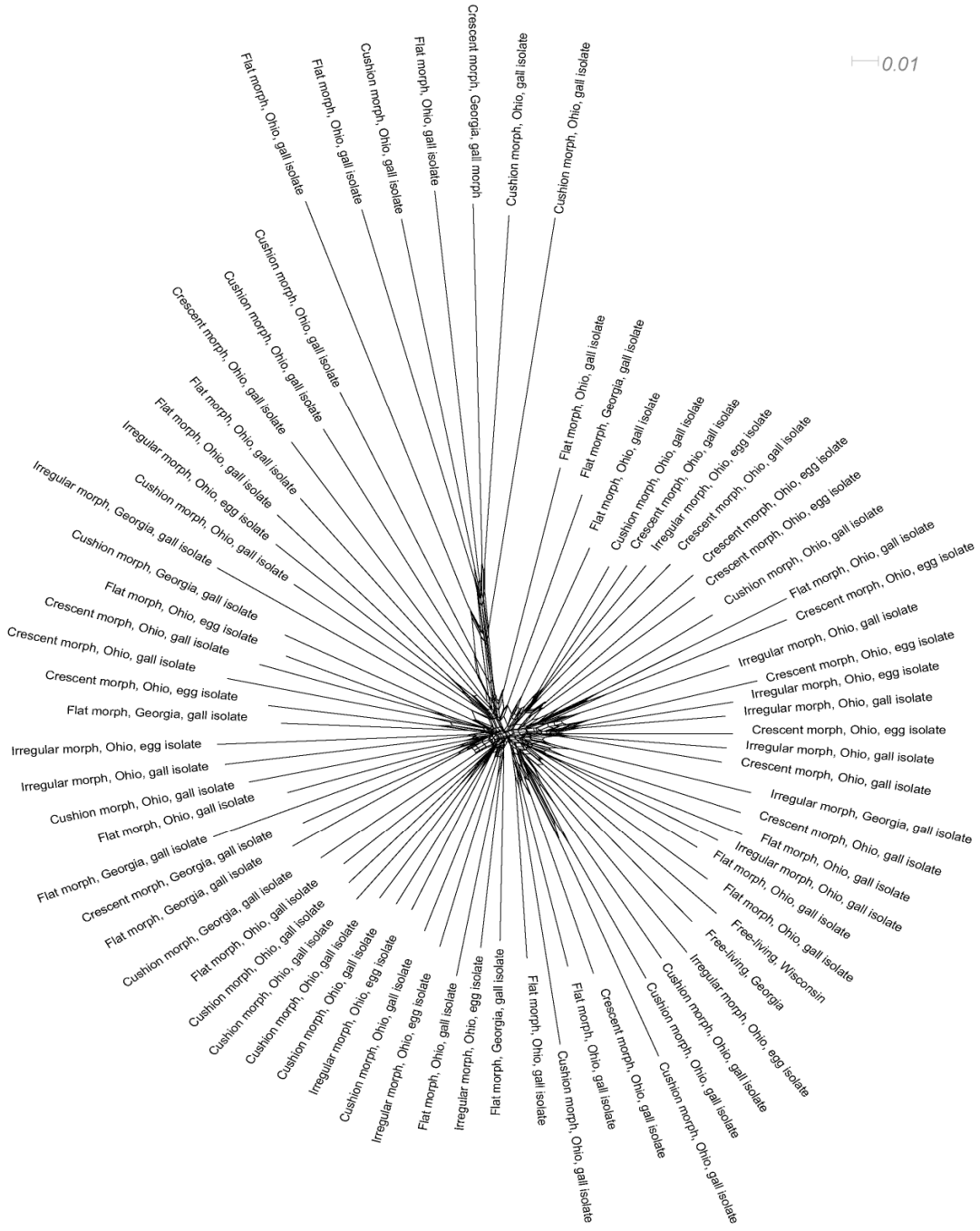

Figure 8. Intraspecific NeighborNet distance tree (based on Dice genetic distances) of AFLP markers showing phyletic relationships among fungal isolates from alternative gall morphs on *Solidago altissima*. Most isolates represented here were collected around south central OH and southeastern GA. Details on methods used to reconstruct this tree are detailed in the text of the Materials and Methods.

Table 7. Below the diagonal: Pairwise F_{ST} for fungal isolates associated with the four *Asteromyia carbonifera* gall morph populations. Above the diagonal: pairwise Nei's genetic distances for fungal isolates associated with the four *Asteromyia carbonifera* gall morph populations.

	Population			
	Crescent	Cushion	Flat	Irregular
Population				
Crescent		0.0022	0.0029	0.0018
Cushion	0.0062		0.0000	0.0044
Flat	0.0079	0.0001		0.0054
Irregular	0.0052	0.0123	0.0150	

Table 8. Summary of analysis of molecular variance (AMOVA) of amplified fragment length polymorphism (AFLP) data based on Euclidian genetic distances.

Source	d.f.	SS	MS	Estimated variance	%	Statistic	Value	P
Among geographic locales	1	165.696	165.696	1.234	1	Φ_{RT}	0.009	0.082
Among *A. carbonifera* gall morphs within geographic locales	6	883.696	147.283	1.240	1	Φ_{PR}	0.009	0.083
Within populations	65	8850.005	136.154	136.154	98	Φ_{PT}	0.018	0.033
Total	72	9899.397		138.627	100			

%: the percentage of variance explained by each sampling level. Significance of F statistics (molecular analogs of Fisher's F statistics) is based on 1000 permutations of samples.

Table 9. Summary of index of association (I^S_A) calculations and statistical testing.

Dataset	N	N loci	V_D	V_e	I^S_A	Mean genetic diversity	Monte Carlo simulations		
							Var(V_D)	L	P
Entire	73	1010	2933.35	177.30	0.0154	0.2723 ± 0.0047	211.95	203.28	<0.0001
OH only	62	1008	2788.68	176.43	0.0147	0.2723 ± 0.0048	231.20	203.26	<0.0001
GA only	11	789	4247.00	163.19	0.0318	0.3452 ± 0.0049	2036.50	252.63	<0.0001

N: sample size; N loci: number of AFLP loci included in the analysis; V_D: observed variance of the mismatch distribution; V_e: expected linkage equilibrium variance of the mismatch distribution; I^S_A: standardized index of association. See Haubold & Hudson (2000) for details.

shows linage disequilibrium that is greater than would be expected in a sexually reproducing population for two geographic divisions of the data and the entire combined dataset.

Phenotypic assays

The general appearance of each isolate in culture was homogenous across isolates from different gall morphologies and consistent with previously published descriptions of *Botryospaheria dothidea*. All cultures (on PDA in total darkness) were initially buff and then became olivaceous gray to black, with a sparse to moderately dense, appressed mycelieal mat and occasional aerial mycelium reaching the lid of the Petri dish. Outside of isolate-level variation in growth rate (see below), there were no obvious differences in the cultural appearance of the different gall-morph associated isolates.

The mean overall growth rate (on PDA in total darkness) for the fungal symbiont of *A. carbonifera* was 7.4 mm/day ± 0.2 (N = 93). The results of the growth rate tests for each gall morph associated set of isolates are detailed in Fig. 11a-c. There was no significant difference in growth rate among the isolates from each gall morph (MANOVA; $F_{3,48} = 0.5137$; $P = 0.6748$). There was evidence of non-identical growth rates among individual isolates, indicating that there is individual-level phenotypic variation for growth rate within the fungal symbiont of *A. carbonifera*. Growth rates for isolates were positively correlated within experiments (Table 10a, b), suggesting that a significant amount of the variation in growth rate is intrinsic to individual isolates, but not to groups of isolates from particular gall morphs.

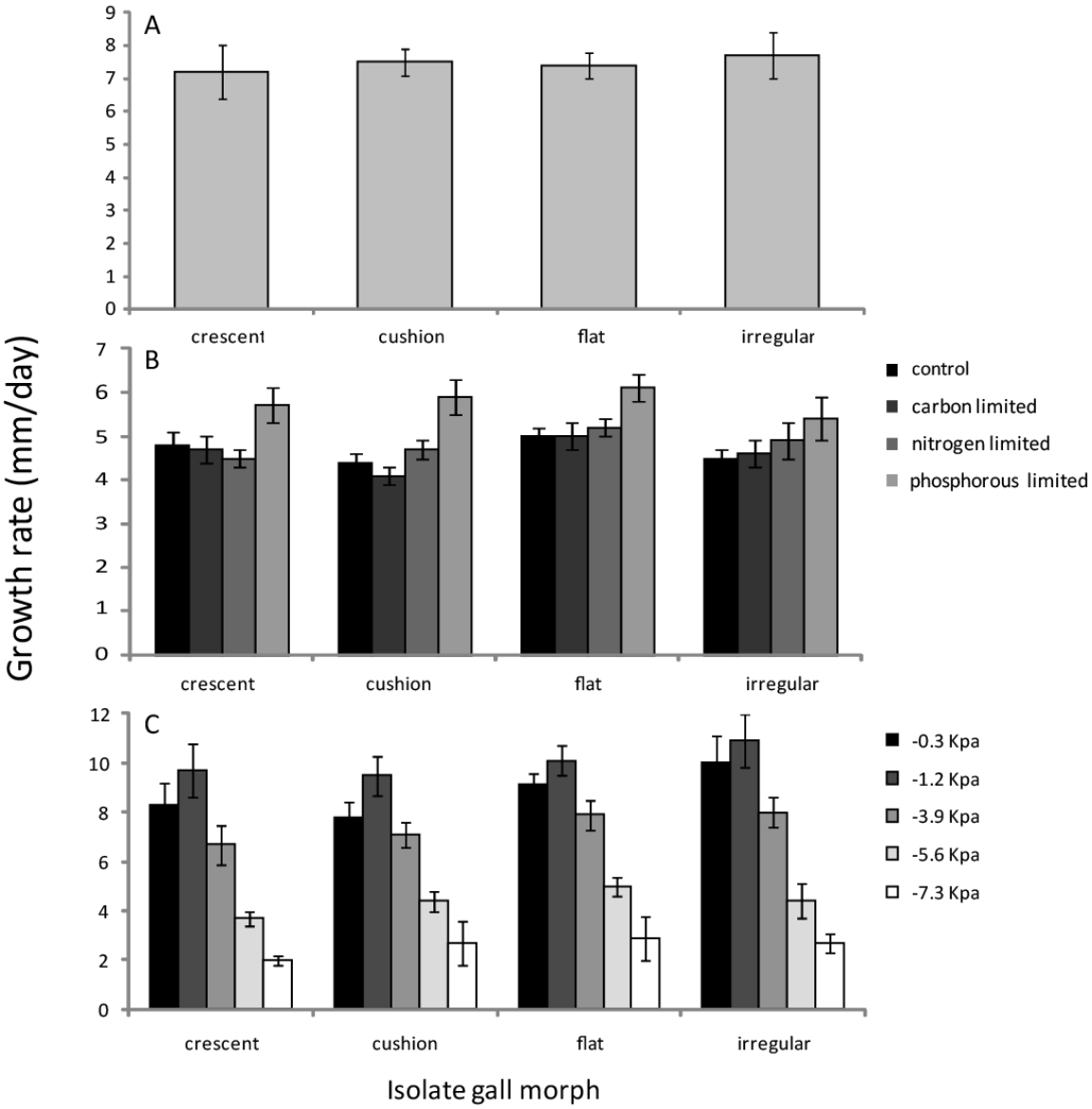

Figure 9. Growth rates of fungal isolates associated with different *Asteromyia carbonifera* gall morphs grown under three different culture conditions. Sample sizes are as follows: A.) standard growth rate; crescent, N = 14; cushion, N = 38; flat, N = 30, irregular, N = 11. B.) nutrient limitation test; crescent, N = 10; cushion, N = 20; flat, N = 20; irregular, N = 7; C.) water stress test; crescent, N = 9; cushion, N = 20; flat, N = 20; irregular, N = 7. See text for details.

Table 10. Pairwise correlations of growth rates for individual isolates across treatments for the water stress (top) and nutrient limitation (bottom) experiments. Below the diagonal are the parametric correlations on log transformed data, above the diagonal are the non-parametric correlations.

Treatment	-0.3	-1.2	-3.9	-5.6	-7.3
-0.3		0.6598***	0.6141***	0.2935	0.3398
-1.2	0.7713***		0.6076***	0.3008	0.1845
-3.9	0.7014***	0.7684***		0.6102***	0.5241***
-5.6	0.3854*	0.4902**	0.6939***		0.7885***
-7.3	0.4126*	0.3842*	0.5932***	0.8473***	

Treatment	Control	Carbon lim.	Nitrogen lim.	Phosphorus lim.
Control		0.4422**	0.3199	0.4676**
Carbon lim.	0.6003***		0.6143***	0.2890
Nitrogen lim.	0.4850**	0.7050***		0.4176*
Phosphorus lim.	0.5219***	0.3598*	0.4960***	

* = $P < 0.005$ for water stress data; $P < 0.0083$ for nutrient limitation (standard Bonferroni corrected *P*-values). ** = $P < 0.001$. *** = $P < 0.0001$.

Discussion

The methods used in the present study are similar to those used to investigate geneetic divergence the midge host, *Asteromyia carbonifera* (Crego et al. 1990; Stireman et al. 2008), in which it was shown that midges isolated from different gall morphologies represented at least genetically divergent populations or at most phylogenetically distinct lineages. Given the intimacy of the association, we predicted that the gall-phenotype and evolutionary diversity seen in *A. carbonifera* could result from coevolutionary hitchhiking of the host with divergent symbiont lineages, and that symbiotic *B. dothidea* would exhibit patterns that characterize microbial mutualism in which phenotypic variation is conferred from symbiont to host. However, our data did not support any of these expectations. These results underscore that, despite the abundance of insect-microbe ectosymbioses, relatively little is known about the evolutionary and population genetic patterns of the symbionts involved in ecotsymbioses, and the circumstances in which symbionts contribute to phenotypic diversity. The only other extensive studies on evolutionary patterns in ectosymbionts have been on the fungal associates of attine ants, but that particular association, in which the ants actively cultivate particular species of fungus in a way analogous to agriculture (Mueller et al. 2005), is not characteristic of all insect-microbe ectosymbioses. For gall midges, very little maintenance of the fungal associate is performed by the midge, and the microbial associate is not sequestered from the environment. We know of no other studies that have examined ectosymbionts as a source of phenotypic variation.

A long held tenet of symbiotic theory is that mutually beneficial associations cannot evolve or persist in the absence of strong, consistent coevolutionary interactions (Douglas 1997;

Palmer et al. 2000; West et al. 2002a,b). However, evidence is accumulating, including evidence from this study, that diffuse coevolution or uncoupled evolutionary histories may be common for symbioses, especially ectosymbioses (e.g., Kiers and van der Heijden 2006; Mikheyev et al. 2006, 2007). Even when the mutualism is not strictly hereditary (i.e., vertically transmitted), symbionts can be a source of phenotypic variation (e.g., Hosokawa et al. 2007).

Botryosphaeria dothidea *as a source of phenotypic variation and its influence on evolutionary divergence for* Asteromyia carbonifera

Results from this study, along with other lines of evidence, suggest that the observed variation in gall morphology is not the result of genetically-based phenotypic divergence in the fungal symbiont. These include: 1.) tightly coupled genetic and phenotypic (gall structure) divergence in *A. carbonifera* (Crego et al. 1990; Stireman et al. 2008), 2.) an association with a single lineage of *B. dothidea* and a lack of genetic and phenotypic divergence in the fungus along gall phenotype lines (this study), 3.) gall structures do not form in the absence of the midge or when the midge is removed (Heath and Stireman, in press), and 4.) normal development and maturation in the fungal symbiont is suppressed by the presence of the midge (Bissett and Borkent 1988; Adair et al. 2009). Thus, while the observed gall diversity within *A. carbonifera* is mediated by the symbiont, it is not the *source* of the diversity. Instead, *A. carbonifera* populations appear to be adapted to specifically manipulating fungal growth and developmental pathways, facilitating the observed phenotypic divergence in gall structure in association with a single species of fungus. This result is not surprising, given that in all gall forming insects, the gall structure is determined largely by insect genotype (Stone and Scönrogge 2003). Interestingly, it appears that *A. carbonifera* is the only species in the genus that is able to

manipulate *B. dothidea* to such a degree. Thus, symbiont mediated phenotypic variation in gall structure appears to be a recent evolutionary development.

Why this particular *A. carbonifera* galling novelty evolved remains an open question, specifically in light of the fact that insect symbiotic *B. dothidea* does not appear to be in a heritable mutualism. Yet, the results from this study shed some light on the observed phenotypic and evolutionary divergence in *A. carbonifera*. Recent evidence strongly suggests that the variation in gall structure is the result of diversifying selection from a phenotypically diverse assemblage of parasitoids (Weis 1982a; J.O. Stireman, unpubl. data). Therefore, phenotypic and evolutionary divergence in the midge may result from a combination of direct, top-down selection from parasitoids and extrinsic selection against interpopulation mating that causes the expression of maladapted gall phenotypes. The symbiotic association may also facilitate host-shifts, and therefore host-plant mediated evolutionary divergence (Stireman et al. 2010), because *A. carbonifera* would not have to contend directly with gall-induction resistance from its host plant. It has been posited that the fungal association may increase the host plant range of symbiotic gall midges compared to gall midge species that do not have fungal associations (Roskam 2005; see also Janson et al. 2008), although this has not been explicitly examined.

Implications for the evolution of gall midge-fungus associations

Phylogenetic analysis demonstrated that the symbiotic fungal associate of *A. carbonifera* is the filamentous ascomycete *Botryosphaeria dothidea* (Botryosphaeriales: Botryosphaeriaceae). *B. dothidea* is a cosmopolitan, opportunistic parasite (sometimes endophyte) of dozens of plant families throughout the world, including many economically important woody crop plants (Denman et al. 2000). It was posited by Bissett and Borkent (1988)

that all symbiotic gall midges are associated with an anamorphic (asexual) *Botryosphaeria* species and this study further supports that contention. *Botryosphaeria* species (mostly *dothidea*) have been found in association with a number of other gall midge species, including *Asphondylia* species in Australia, South Africa (Adair et al. 2009), and North America (J.B. Joy, pers. comm; I. Park, pers. comm.), other *Asteromyia* species in North America, and at least one *Lasioptera* species in Europe (Rohfritsch 1997).

In the context of the symbiosis, association with *B. dothidea* would be beneficial to gall midges due to *B. dothidea*'s relatively low host plant specificity, weak pathogenicity, and cosmopolitan distribution. In addition, it appears that *A. carbonifera* is mostly associated with a single lineage within *B. dothidea*, although a sublineage of symbiotic *B. dothidea* was found associated with a few individuals in southeastern Canada. The association between *A. carbonifera* and its symbiont exhibits a level of specificity that might not be expected in a fully ectosymbiotic association, especially since several nascent phylogenetic lineages comprise *B. dothidea* (e.g., this study, Adair et al. 2009). This is because non-endosymbiotic symbioses are believed to be especially susceptible to invasion and symbiont replacement (Buchner 1965). How *A. carbonifera* (or any gall midge) maintains this broad level of symbiotic specificity is unclear. As hypothesized for all gall midges, it appears that females actively collect conidia (asexual reproductive spores) from the environment sometime after eclosing as adults (Borkent and Bissett 1985; Bissett and Borkent 1988; Rohfritsch 2008). It also appears that they do not collect conidia from the fungus in their natal galls, since reproductive structures are rarely observed at the time of adult emergence (Bissett and Borkent 1988; Rohfritsch 2008; Adair et al. 2009). Therefore, females may collect conidia from fruiting fungus found on old galls in the leaf litter (Bissett and Borkent 1988) or from galls where resident midges/parasitoids have died

(Adair et al. 2009). In either case, broad specificity could be maintained if females only collect condia that are found in association with *Asteromyia* galls, but fine-scale symbiont transmission is, by definition, horizontal since females never collect conidia from their natal galls. Symbiont transmission that is broadly (but not strictly) vertical, in that an individual's offspring's symbionts are genetically highly similar to their own, has been deemed pseudo-vertical transmission (Wilkinson and Sherratt 2001). Pseudo-vertical transmission may be important in initiating and maintaining symbiotic integration in associations where strict vertical transmission is impossible or occurs inconsistently. The fact that this particular lineage of *B. dothidea* appears to exist outside of the association also suggests that females could collect conidia from *B. dothidea* that is not in association with *Asteromyia* galls, although how *A. carbonifera* would be able to distinguish the symbiotic lineage of *B. dothidea* from non-symbiotic lineages is unknown.

Both the intraspecific phylogenetic analysis and the AFLP data demonstrate that there is no consistent evolutionary divergence along gall-morph lines. This is somewhat surprising given the intimate nature of the interaction. Yet, the association of several insect lineages with a single, non-co-diversified (but not necessarily genetically homogenous) species of ectosymbiont does bear some resemblance to another well studied fungus ectosymbiosis: the attine ants (Silva-Pinhati et al. 2004; Mikheyev et al. 2006, 2007). Several ant species share non-co-diversified fungal associates, and the symbionts are shared among populations that are geographically isolated. In the attine ants, regular genetic recombination within the symbiotic associate and long distance dispersal appears to be important in maintaining symbiont homogeneity (Mikheyev et al. 2006). For *A. carbonifera*, our results indicate that the fungal associate experiences no detectable genetic recombination (it is likely strictly asexual in ecological time), and thus genetic "homogeneity" across divergent midge populations is likely maintained by regular horizontal

transmission and long distance dispersal of genotypes within an asexual symbiotic fungal lineage. The apparent lack of strict co-diversification among midge hosts (see Stireman et al. 2008) and fungal associates further supports the apparent lack of midge-host associated genetic structure, because strict vertical transmission coupled with asexuality should result in co-diversification of host and symbiont (Thao et al. 2000; Clark et al. 2005; Takiya et al. 2006). Occasional parasexual genetic recombination cannot be ruled out, however, as preliminary vegetative compatibility studies demonstrated that isolates from different gall morphs are compatible. The particular pattern of genetic diversity seen here may be caused by a single (or a small number of) mother genotype(s)/lineage(s) through which daughter genotype(s)/lineage(s) are continually produced and accumulate different neutral mutations in parallel, while the mother genotype(s)/lineage(s) continues to persist through time. Lack of strict vertical transmission and long distance dispersal of asexual genotypes then leads to the observed lack of structure and apparent lineage sharing compared to the significant structure observed in the host. The fungal symbionts of *Asphondylia* species from Australia and South Africa were similar to the *A. carbonifera* fungal associate in this regard, except that there was a well-supported phylogenetic split between the South African and Australian isolates (Adair et al. 2009). Thus, intracontinental long distance dispersal and horizontal transmission may be common for fungi associated with gall midges, while intercontinental dispersal may be less so. Local adaptation (to host plants, gall midge species, or environmental conditions) of particular *B. dothidea* lineages cannot be ruled out as a cause for the phylogenetic divergence observed in the Adair et al. (2009) study, although there was little evidence of such an occurrence in *Asteromyia*.

However, lack of symbiont diversification may have other explanations. For example, the loci used for phylogenetic reconstruction in the fungus may be evolving too slowly or there may

be isolated genomic islands of divergence that were not detected by the AFLPs. However, it is notable that the relative lack of phylogenetic structure extends to *B. dothidea* associated with conspecific gall midge populations, heterospecific gall midges species, and to free-living fungal conspecifics. Indeed, the relatively small amount of divergence within different biological divisions of *B. dothidea* is one of the most striking aspects of this system, especially when the symbiosis between gall midges and *B. dothidea* appears highly integrated. For example, mitochondrial sequence data (COI) show high mean sequence divergence between species of *Asphondylia* and *A. carbonifera*, averaging around 0.248 ± 0.033 substitutions per site (Jukes-Cantor corrected). Assuming a mitochondrial molecular clock of ~1.0×10^{-8} substitutions per site per year, or 0.010 substitutions per site per million years (Desalle et al. 1987; Brower 1994; Stireman et al. 2010), these two groups have been diverging for approximately 25 MY. However, the mean nrITS (ITS1 and ITS2) sequence divergence between *B. dothidea* associated with those two gall midge genera (Jukes-Cantor corrected) is only 0.002 ± 0.001 substitutions per site. If we assume that symbiotic lineages of *B. dothidea* have been associated with their host genera since their initial divergence, and have been vertically transmitted the length of the association, this result would suggest that *B. dothidea* has a substitution rate closer to 8×10^{-11} per site per year (or 0.00008 substitutions per site per MY), which is over two orders of magnitude slower than the estimated mean rate for ITS of ascomycetous fungi of $1.4 \pm 1.3 \times 10^{-9}$ substitutions per site per year (or 0.0014 substitutions per site per MY) (Kasuga et al. 2002). Conversely, if we assume that symbiotic *B. dothidea* has an ITS substitution rate closer to the estimated rate for fungi, then lineages of symbiotic *B. dothidea* have only been diverging for 0.143 MY—a significantly shorter amount of time than their host lineages. While low rates of molecular evolution in nuclear genes have been observed for some asexual eukaryotes (e.g., Schon et al. 1998),

microbial symbionts often have higher rates of substitution than their hosts (e.g., Moran et al. 1995).

Knowing this, what then could explain the disconcordance in evolutionary divergence between host and symbiont? Since asexual populations may eventually succumb to mutational meltdown (Lynch et al. 1993), it may be that gall midges regularly reacquire their symbiotic associates from at least occasionally recombining (perhaps free-living) stocks, or that particular symbiotic fungal lineages rise to fixation through selective sweeps after rare recombination events or through clonal interference after rare beneficial mutations. Thus, the current symbiotic *B. dothidea* lineage may simply have not been in association with *A. carbonifera* long enough to accumulate a large number of fixed nucleotide differences among host populations. The movement of the *Asteromyia* fungal symbiont between "free-living" and symbiotic states is further supported by the regular coexistence of free-living isolates and symbiotic isolates within monophyletic clades (Fig. 8). Evidence of symbiont reacquisition has been observed in the attine ants (Mueller et al. 1998; Schultz and Brady 2008), thus such a scenario would not be unprecedented. However, this does not adequately explain why there is very little genetic divergence between *B. dothidea* isolates that are symbiotic with deeply divergent and geographically isolated species of gall midges. In the case of recent symbiont reacquisition, numerous gall midge species would have to acquire the same lineage of *B. dothidea* at nearly the same time, which seems unlikely.

Comparisons to other microbial symbioses

A consistently striking result from other insect-microbial symbioses is that symbiotic microorganisms experience evolutionary forces that are distinct from their free-living hetero- and

conspecifics. For example, the heritable bacterial primary endosymbionts of aphids and the horizontally-transmitted bacterial gut symbionts of stinkbugs show evidence of accelerated rates of nucleotide substitution, co-diversification with hosts, and a genome-wide A+T nucleotide bias compared to their non-symbiotic and/or non-mutualistic relatives (Moran et al. 1995; Clark et al. 2000; Wernegreen 2002; Hosokawa et al. 2006; Kikuchi et al. 2009). The fungal associate in several lichen symbioses also exhibit accelerated substitution rates (Lutzoni and Pagel 1997). Although the causes of these patterns can vary from symbiosis to symbiosis, taken together, these patterns suggest some aspect of the demography, rates of mutation, or the stringency of selection are consistently different between free-living and mutualistic lineages. These patterns are not specific to strictly vertically-transmitted or purely endosymbiotic associations (Hosokawa et al. 2006), and so it is not unexpected that these patterns should appear in the case of *A. carbonifera* and *B. dothidea*. However, the results of this study suggest that the evolutionary forces experienced by free-living and symbiotic *B. dothidea* are not different. Why the fungi associated with *A. carbonifera* (and other gall-midge species) do not appear to experience unique demographic or inter-generation transmission processes remains an open question, but likely explanations can be put forth. For example, the stringency or kinds of selection external to the host-symbiotn interaction should not sigficantly differ between symbiotic and non-symbiotic *B. dothidea*, since the symbiosis does not appear to be protected or sequestered in any way. The lack of difference may be because of recent symbiont replacement from free-living stocks that have not had ample time to exhibit the above patterns. However, it is more like that the purely ectosymbiotic nature of the association, and the apparently homogenous biology of *B. dothidea* regardless of its symbiotic associations, is the key factor behind the similar evolutionary patterns. One final possibility is that *A. carbonifera* and *B. dothidea* are not involved in a mutualism, but

instead *A. carbonifera* (and other gall midge species) are parasitizing a normally free-living lineage of *B. dothidea*. The fungal symbiont serves as a source of nutrition (Janson et al. 2009) and defense from natural enemies (Weis 1982b; Stireman et al. 2008), and thus the benefits conferred to *A. carbonifera* from *B. dothidea* are clear. It has not been determined if *B. dothidea* receives similar benefits from the association. Such an explanation may underlie the apparent misalignment of reproductive interests between the two players and the diffuse coevolutionary histories.

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CHAPTER V

CONCLUSIONS

Microbial mutualism has become the focal point of intense empirical investigation over the past two decades, which has led to significant discoveries for the fields of ecology and evolutionary biology. Arguably, the most important outcome of these investigations is that it is now understood that microbes can be a repository of ecologically significant phenotypic variation for its host. While initially believed to be relegated to nutritional provisioning, it is now known that microbes can be the source of diverse phenotypes for their host, mediating both abiotic and biotic interactions (Moran 2007). Most importantly, hosts organisms are able to make use of genetic variation contained within their symbionts in ways that could drastically alter the strength and/or direction of selection, and, therefore, could influence the host's evolutionary trajectory and patterns of diversification (Janson et al. 2008). A second key outcome of empirical study is that it is now accepted that partially or fully ectosymbiotic associations often do not follow the traditional "rules" prescribed for the evolution and maintenance of stable symbioses, and yet are able to persist in evolutionary time (e.g., Kiers and van der Heijden 2006). Through these revelations, the gap in understanding between the observation of mutualistic contributions to phenotypic variation and the evolutionary consequences of microbial phenotypes is being closed.

The present body of work was set forth to elucidate the contribution of an ectosymbiotic microbial mutualism to the evolutionary diversification observed in its host and to better understand the general evolutionary ecology of ectosymbiosis. Foundational advancements

towards these goals were achieved and outlined in the results of this dissertation. Firstly, a theoretical framework was presented, which outlined how and when microbial mutualists could facilitate or impede adaptive evolutionary divergence (ecological speciation) within a host lineage, and the core hypothesis of that framework—that mutualists can act as a source of phenotypic variation that facilitates ecological shifts and, thus, lineage splitting—was examined. A key proposal of that study was that, in certain circumstances, microbes may act in a way that is either analogous to adaptive phenotypic plasticity, facilitating movement across adaptive landscapes (Agrawal 2001; Price et al. 2003), or changing the strength of selection as to render what would be ecological specialization into ecological generalism. This study was presented in a verbal framework, and a mathematical treatment of the subject would be beneficial in determining when the scenarios presented would facilitate or impede evolutionary diversification. Some questions that are especially pressing are: how important is the potential for symbiont replacement/loss (horizontal transmission) or extra-symbiotic genetic transfer in determining whether a symbiont acts as a facilitator or impediment to ecological and evolutionary diversification in hosts? With the evidence of genome erosion and the strength of drift, especially in the types of symbioses in which symbionts are most likely to act as sources of phenotypic variation (heritable mutualisms), over what length of evolutionary time can symbionts respond to selection both from their host and from the environment? And, how can selection promote host lineage splitting (evolution of reproductive isolation) when ecologically significant phenotypes are encoded within the symbiont genome? A testable hypothesis of this study is that symbiotic clades of gall midges should show distinctly different diversification patterns from their non-symbiotic relatives. Once determined if symbiotic lineages show higher or lower rates of diversification/extinction, specific hypotheses about why such patterns exist

could then be examined. A veritable mountain of work remains in order to determine if, how, and when mutualists have affected the evolution of their hosts, yet this is arguably the most tantalizing future direction of the present dissertation.

The most common relationship between insects and their microbial mutualists is that of nutrient provisioning from symbiont to host (Douglas 2009). We found that the *Asteromyia-Botryosphaeria* association is no exception (Janson et al. 2009). Indeed, in virtually all known insect-fungus associations, the fungus is the primary or exclusive food source for the insect. In addition, as seen in other insect-fungus associations, the fungus is the primary source of usable sterols for their insect hosts, which are unable to synthesize crucial sterols *de novo* (Behmer and Nes 2003; Janson et al. 2008, 2009). What is also evident from this study is that *A. carbonifera* appears to be an exclusive fungus feeder. This result would not be surprising, since primitive gall midges are strict fungivores, however, it is still unknown if the relationship between *A. carbonifera* and *B. dothidea* is obligate. It appears that *A. carbonifera* may be obligately dependent on *B. dothidea*. This question is important because there appears to be at least two independent losses of the fungal symbiont within the genus *Asteromyia*, including one clade that shares a recent common ancestor with *A. carbonifera* (Stireman et al. 2010). Thus, ancestral *Asteromyia* species either had the genetic capacity for relatively rapid shifts in nutritional physiology or they were able to feed on fungus and/or plants with similar efficacy, with some clades eventually shifting towards phytophagy over fungivory or vice versa. Further study could determine if *A. carbonifera* and other *Asteromyia* species have specific nutritional obligations and retrace the evolution of nutritional physiology in the genus. Answers to these questions would aid in understanding the consequences of rapid shifts in trophic position, as the ecology of *Asteromyia* species that have lost their fungal symbiont appears to be distinctly different from the

rest of the genus. In addition to the questions raised about *Asteromyia* and *Botryosphaeria*, this study raises general questions about sterol metabolism and physiological constraints in insects. The insect herbivore community on *S. altissima* showed marked variation in sterol metabolism, even demonstrating that some specialist insects are unable to metabolize their host plant sterols. This result highlights the importance of the somewhat overlooked primary nutrient dimension in plant-insect interactions. Given the structural diversity of phytosterols and the variation in insect sterol metabolism, sterols may, in some instances, be used as defensive compounds by plants. A greater focus of the evolution of sterol profiles and sterol metabolism capabilities in host plants and their herbivorous insects, respectively, would help clarify the possible role of sterols as a defensive chemical (Berenbaum 1995).

The final study of this dissertation examined several evolutionary aspects of the host-symbiont relationship. The results of this study were the most surprising and raise the most questions about the relationship between *A. carbonifera* and *B. dothidea*. Instead of observing evolutionary patterns that suggest an intimate mutualistic association, the results of this study suggest that the *B. dothidea-A. carbonifera* relationship may be relatively fluid, potentially involving symbiont loss, reacquisition, and regular horizontal transmission. Together, these results suggest that the *A. carbonifera-B. dothidea* relationship is perhaps more complicated than many other well studied animal-microbial symbioses. In order to further clarify the interaction, future studies should attempt to determine the kinds and magnitude (if any) of benefits that *A. carbonifera* confers to its fungal associate. Future studies should investigate whether the fungal symbiont of *A. carbonifera* exhibits the presently observed evolutionary patterns across all host plants and *Asteromyia* species. Some preliminary data suggests that other *Asteromyia* species are symbiotic with the same lineage of *B. dothidea* at *A. carbonifera*, but the population genetics of

the fungus associated with those *Asteromyia* species has not been investigated. Although, given the host-plant generalism of *B. dothidea*, it seems doubtful that the patterns would be significantly different from plant to plant or species to species. With the advent of the genomic revolution, there are now easily accessible tools that can be applied to the *Asteromyia-Botryosphaeria* relationship. For example, phenotypic variation in fungus-mediated gall morphology may not be the result of fixed genetic differences in the fungus, but rather by differences in gene expression as influenced by the midge. If expression variation in fungus associated with different midge populations could be consistently correlated with variation in gene expression in the midges themselves, this would be further convincing evidence that the midges are directly manipulating their symbionts. Direct manipulation of the midge and fungus has proven difficult (Heath and Stireman, in press), and this may be a more feasible approach. One exciting aspect this study is the striking similarities that the *A. carbonifera-B. dothidea* symbiosis appears to share with the attine ants and their fungal cultivars, despite of the obvious dissimilarities in ecology between the two groups. Any parallels between the two groups may signal that there are a distinct set of biological "rules" that govern fungal ectosymbioses. It would be especially interesting to see if the evolutionary patterns observed between free-living and symbiotic *B. dothidea* are similar to that between free-living and symbiotic fungi associated with attine ants (Vo et al. 2009). A comparative approach looking more closely at the free-living and symbiotic nascent clades of *B. dothidea* may answer some questions surrounding the evolution of fungal symbiosis in gall midges.

Finally, one small piece of data not included in the body of this dissertation is worth further investigation, as it may be important in understanding diversification within *A. carbonifera*. A non-trivial number of individuals (~20-40%, depending on the population) are

infected with *Spiroplasma* strain that belongs to a known male-killing clade (Hurst et al. 1999; Majerus et al. 1999). While the effects of male-killers on host diversification are not entirely clear, it is known the hybrid mating between infected and non-infected individuals can lead to male-killing, and, under some circumstances, could lead to the evolution of reproductive isolation between infected and uninfected populations (Jiggins et al. 2000). Clearly characterizing the phenotypic effects of the *Spiroplasma* symbiont and the population-level prevalence would be useful in determining the potential contribution of *Spiroplasma* to the evolutionary patterns of *A. carbonifera*. Moreover, understanding population and species level patterns of *Spiroplasma* infection may be important in understanding the evolution of an interesting genetic system seen in many cecidomyiids and their relatives. Gall midges have a genetic system called paternal genome elimination, which is the evolutionary equivalent to the haplodiploidy observed in many social hymenopterans (although the mechanism by which males fail to pass on their father's genomic contribution is vastly different) (Normark 2003). It has been posited that such genetic systems may arise in response to heritable male-killing microbial symbionts (Normark 2004; Ubeda and Normark 2006), and understanding the population and species-level patterns of infections and phenotypic effects of gall-midge associated *Spiroplasma* may provide insight into this hypothesis.

As with the many a nascent research program, this dissertation work raises more questions than it answers, but these studies do address some fundamental questions about the nature of a fascinating relationship between two vastly different, but intimately associated eukaryotes. What is certain is that the *Asteromyia-Botryosphaeria* association will provide an excellent system for studying ectosymbiotic insect-fungus interactions and evolutionary

diversification alone. It appears that it may also be the perfect system for investigating the relationship between of symbiont-mediate phenotypic diversity and evolutionary diversification.

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