

## REVIEW AND SYNTHESIS

# Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects

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### Abstract

Endosymbiosis is a pervasive, powerful force in arthropod evolution. In the recent literature, bacterial symbionts of insects have been shown to function as reproductive manipulators, nutritional mutualists and as defenders of their hosts. Fungi, like bacteria, are also frequently associated with insects. Initial estimates suggest that insect–fungal endosymbionts are hyperdiverse, yet there has been comparatively little research investigating the roles that fungi play in their insect hosts. In many systems in which the bacterial symbionts are well-characterized, the possible presence of fungi has been routinely ignored. Why has there been so little research on this important group of symbionts? Here, we explore the differences between fungal and bacterial endosymbiotic insect mutualists. We make predictions about why a bacterium or fungus might be found associated with an insect host given particular ecological, physiological, or evolutionary conditions. We also touch on the various hurdles for studying fungal vs. bacterial endosymbionts and potential future research directions.

### Keywords

Context-dependent associations, symbiont genomes, symbiosis, yeast-like symbionts.

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In this book we shall discuss only [animal hosts and plant guests] ... [this] includes the extraordinarily widespread and often fantastically complex bacterial and fungal symbiosis which has been set forth only within recent decades. p. 3.

P. Buchner 1965. Endosymbiosis of animals with plant microorganisms.

### INTRODUCTION

Microbial endosymbionts are widespread in insects, and bacterial symbionts are best understood. A recent literature analysis of *Wolbachia* infections suggested that 65% insect species are infected (Hilgenboecker *et al.* 2008). The best-characterized associations are those of bacteria that benefit their hosts nutritionally, and those that manipulate their hosts' reproduction. However, bacterial symbionts may play other roles in their hosts' biology, such as heat tolerance, or protection against parasitism and pathogens (reviewed in Moran *et al.* 2008).

Fungi are also frequently associated with insects. However, while there are some well-studied examples of insects associated with ectosymbiotic fungi, such as leafcutter ants

and termites and their respective basidiomycetous fungi, there has been comparatively little research investigating either the diversity or the roles of insect–fungal endosymbionts (See Box 1 for definitions and conventions used here).

Nearly 90 years ago, Paul Buchner, the father of symbiosis research, documented a remarkable array of both endosymbiotic fungal and bacterial associates of arthropods. In the English translation of this work, Buchner (1965) suggested that fungi, in addition to bacteria, could augment the nutrition of insects with deficient diets (e.g. blood, phloem and wood). This has been demonstrated in a few well-studied, obligate insect–fungal associations. The death-watch beetles, *Lasioderma serricorne* and *Stegobium paniceum*, harbour yeast-like symbionts (YLS) (Ascomycota: Pezizomycotina: *Symbiotaphrina* spp.) that provide sterols and also function in substrate detoxification (Shen & Dowd 1992; Noda & Kodama 1996). Clavicipitaceous YLS (Ascomycota: Pezizomycotina: Sordariomycetes: Hypocreales) are documented in three species of planthoppers (Noda & Koizumi 2003), as well as in some aphids (Fukatsu & Ishikawa 1996). In planthoppers, these fungal

**Box 1** Our use of terms and fungal definitions

We use the term 'symbiosis' in the broad sense, as the persistent living together of unlike organisms as de Bary (1879) defined it. Further, we use the term endosymbiont to mean an internal associate of the insect (the host) including the gut, and we qualify whether the symbiont is located between cells (inter-) or intracellularly within host cells. Given their habitat, it is not surprising that intercellular fungal symbionts are often compact unicellular yeasts or yeast-like forms (Vega & Dowd 2005). Ten orders within three fungal phyla (Basidiomycota, Ascomycota, Zygomycota) have converged on the yeast form. Researchers often refer to 'yeast-like fungi' or 'yeast-like symbionts' (YLS) for those fungal taxa that are not within the subphylum Saccharomycotina. For the purposes of this review, we follow this convention and provide more precise taxonomic information where known. Obligate associates are required by their hosts for survival and/or reproduction and facultative associates are non-essential.

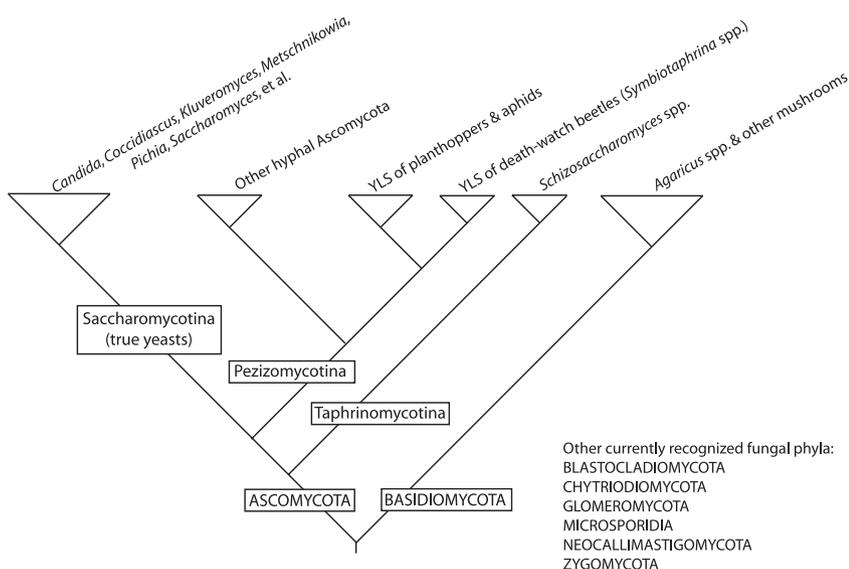
symbionts live within fat body cells and are involved in sterol biosynthesis and nitrogen-recycling (Sasaki *et al.* 1996; Noda & Koizumi 2003).

There is growing interest in understanding yeast diversity associated with insect hosts. In a literature survey, Vega & Dowd (2005) reported that 143 insect species, spanning eight orders, were infected with asymptomatic or beneficial yeasts. Suh *et al.* (2005) isolated 650 yeasts, including 200 undescribed taxa (representing a 20% increase in the number of described yeast species) from the guts of beetles in 27 different families. Most of the fungal taxa discovered were true yeasts (Ascomycota: Saccharomycotina); however, some were yeast-like Basidiomycota (see Fig. 1 for a simplified fungal phylogeny). In another study, Nguyen *et al.* (2007) isolated yeasts from the guts of lacewings, fishflies and craneflies, and found some apparent yeast-to-

insect species-specificity. This research suggests that diverse fungi have close associations with a broad range of insects.

More recent studies are finding context-dependent relationships of fungi with insects. For example, bark beetles' fungal partners may change in density or in the nature of their relationship with their host throughout the life cycle of the beetle (reviewed in Klepzig *et al.* 2009). As has been found in bacteria, relationships with fungi may also be ambiguous. For example, a heritable yeast in a parasitic wasp, long presumed to be a nutritional mutualist, was shown instead to incur a fitness cost for its host in individual laboratory fitness assays (Gibson & Hunter 2009b). While the fungus appeared to be parasitic in the laboratory, it could be beneficial in a context that has not yet been discovered. In general, beyond the documentation of insect-yeast associations, little is known about yeast transmission routes or the fitness consequences of these diverse fungal infections for their insect hosts.

Our goal in this review is to highlight how bacterial and fungal mutualists of insects differ, and to encourage further empirical work with fungal endosymbionts. First, we explore the evolutionary implications of insect-microbial symbiosis using the established theoretical framework of the dynamics of transmission. Next, we speculate about overarching differences in the nature of insect-fungal vs. bacterial endosymbiosis based on what we know about the physical and biochemical properties of each microbial group. For instance, are there particular conditions under which we expect to find a fungal rather than a bacterial partner? In the last section, we address why we think there has been a lag in the study of fungal associates of insects, in hopes of identifying the kinds of work that will light the path ahead. We also briefly touch on the current methods that are certain to transform this field in the near future.



**Figure 1** A simplified phylogeny of the Ascomycota and Basidiomycota highlighting the placement of the true yeasts and the majority of yeast-like symbionts known to be endosymbiotic mutualists of insects.

## Part I – trends in bacterial and fungal endosymbiosis

Currently, vertically transmitted, bacterial associates dominate the insect symbiosis literature (e.g. *Buchnera aphidicola*) (see Table 1 for definitions of transmission modes). The descriptions of insect–fungal symbioses, in contrast, show a pattern of associates that are primarily horizontally transmitted and facultative (e.g. undescribed *Candida* spp. associated with a range of insect hosts) (Suh *et al.* 2005). Here, we provide some examples that are consistent with these patterns for bacterial and fungal symbionts. We also document exceptions to these patterns in each microbial group.

Highly interdependent symbiont–host systems can result from selection for specialization combined with restricted gene flow and gene loss that render symbionts dependent on host metabolites (Moran *et al.* 2008). Paradigmatic examples have accumulated recently owing to the ability of molecular techniques to characterize fastidious microbes and to resolve historical relationships. For instance, a variety of obligate, transovarially transmitted symbionts have been shown to exhibit strict co-cladogenesis with a diverse array of invertebrate hosts (Moran *et al.* 2008). In these intracellular symbionts, there is evidence of degenerative genome evolution, with the loss of regulatory genes that free-living bacteria retain. This degeneration is due to genetic bottle-

necks and the restricted habitat inside host cells, which leaves few opportunities for the acquisition of new genes. Two extreme examples of this are the gammaproteobacterial endosymbionts that live within the betaproteobacterial symbionts (*Tremblaya princeps*) within bacteriocytes of their mealybug hosts (von Dohlen *et al.* 2001), and the bacterial symbionts found within the nuclei of planthopper host cells (Arneodo *et al.* 2008). Interestingly, in the few well-studied examples of intracellular, transovarially transmitted fungal symbionts (i.e. YLS of planthoppers, aphids and beetles) there is no evidence that they have co-diversified with their hosts nor lost genes (see Part III) in a manner similar to these examples of bacterial associates (Suh *et al.* 2001).

On the other side of the spectrum are horizontally transmitted bacteria that remain autonomous and cultivable in cell-free media. These symbionts may also contribute important benefits to their host, but have received less attention. For instance, in house crickets, bacteria (*Citrobacter*, *Klebsiella*, *Yersinia*, *Bacteroides* and *Fusobacterium*) break down polysaccharides in the host gut, and in silkworm larvae the bacterial associates (*Enterobacter* spp.) serve to resist colonization by pathogens (Dillon & Dillon 2004). In locusts, a bacterial symbiotic complement of *Pantoea agglomerans*, *Klebsiella* spp. and *Enterobacter* sp. is required for the production of social cohesion pheromones and

**Table 1** Transmission modes and general rules

	Vertical (VT)	Horizontal (HT)
Definition	Symbiont moves from mother to offspring	Symbionts move among hosts that may or may not be related
Effect	Symbiont must induce host to produce more infected daughters relative to the number of daughters produced by uninfected females (Bull 1983). Symbiont is therefore reliant on the success of the host which selects for decreased virulence and is associated with mutualism.	Symbiont is not reliant on the reproductive success of any individual host and instead can achieve greater fitness by infecting many unrelated individuals. This can select for virulence in the symbiont, and is often associated with pathogenesis.
Genetic exchange	Insect host can transmit clones, identical strains of the symbiont, or multiple symbiont strains, where recombination among symbiont types can occur. Although co-inherited symbionts could also compete for host resources (virulence), co-inheritance typically selects for co-operation among strains. This promotes host specialization and concordance between insect host and symbiont phylogenies.	Greater opportunities for genetic exchange with free-living microbes, as well as competition for host resources (virulence). This feature could also expand the ecological opportunities for the hosts of HT. This could limit host specialization and prevent strict concordance between insect host and symbiont phylogenies.
Exceptions	Many facultative, vertically transmitted symbionts do not show co-cladogenesis with their hosts. A low frequency of horizontal transmission can result in mis-matches between host and symbiont phylogenies. Although vertically transmitted symbionts may be mutualists, others may manipulate host reproduction in ways that increase their own transmission, e.g. <i>Wolbachia</i> (Hilgenboecker <i>et al.</i> 2008).	There are examples of mutualist microbes that are transmitted horizontally. For example, an alcid bug acquires a mutualist bacterial symbiont, <i>Burkholderia</i> , from the soil each generation. This bacterium causes an increase in the body weight and length in both males and females (Kikuchi <i>et al.</i> 2007).

phenolic compounds that inhibit fungal pathogens (Dillon & Dillon 2004). Finally, in antlions, *Enterobacter aerogenes* produces a salivary toxin used to quell these predators' prey (Yoshida *et al.* 2001). This bacterium is cultivable, and also an opportunistic pathogen of humans.

With respect to fungi, most new associations are presumed facultative, or are completely unknown (Vega & Dowd 2005). Given the large number of fungal endosymbionts being documented in insect guts (e.g. Suh *et al.* 2005) it is interesting that there appear to be some 'yeast generalists' that can form relationships with a number of diverse insect taxa. For instance, laboratory-reared adults of surface-sterilized parasitic wasps, *Anagyrus pseudococci* and *Leptomastix dactylopii* (both Hymenoptera: Encyrtidae) both yielded cultivable *Candida fermentati* (Ascomycota: Saccharomycotina) (100% sequence 28S rDNA sequence similarity, C. M. Gibson, unpublished data) as did mushroom-feeding beetles (Suh & Blackwell 2004) and scolytid coffee berry borer beetles (Vega *et al.* 2003). Similarly, *Candida (Picchia) guilliermondii* has been recovered from long-horned beetles (Nardon & Grenier 1989), scarab beetles (Vishniac & Johnson 1990), metallic wood-boring beetles (Phaff & Starmer 1987), fire ants (Ba *et al.* 1995), adrenid bees and a mushroom-feeding fly (Zacchi & Vaughn-Martini 2002). In long-horned beetles, *C. guilliermondii* exhibits tissue specificity; larval gut cells are continually reinfected and in pupal females, the yeast increases in density in the hindgut and then colonizes mycangia (external sacs) near the ovipositor (Nardon & Grenier 1989). Although the roles of *C. fermentati* and *C. guilliermondii* in their insect hosts are not yet understood, whether these yeasts are true generalists could be determined with reciprocal infection studies.

In contrast to the many observations of facultative insect–fungal associations that dominate the literature, there are intriguing cases of obligate fungal associations. For instance, there are YLS that occur in the hemocoel (extracellularly) of four aphid genera in the Ceratophidini (Ascomycota: Pezizomycotina: Sordariomycetes: Hypocreales). While aphids are thought to have co-diversified with the bacterial lineage *Buchnera* after an initial infection between 115 and 230 MYA, *Buchnera* is missing in these aphid lineages. It is presumed that the YLS have replaced *Buchnera* (Hongoh & Ishikawa 2000), and play a functionally equivalent role. However, the specific contributions and nutritional needs of these YLS are unknown. It would be interesting to determine how closely the YLS mimic *Buchnera* in these aphids, and how the hosts have adapted to a eukaryotic mutualist partner. The circumstances of this exchange may be difficult to reconstruct, but there is an example in another aphid in which it appears that a facultative bacterium has started to complement services provided by a degenerate *Buchnera* (Perez-Brocal *et al.* 2006). While, we would argue that bacteria and fungi generally

provide different services to their insect hosts, the former aphid example suggests these microbial partners may sometimes be interchangeable.

## Part II – contrasts between bacterial and fungal–insect associations

### *Why are there fewer intracellular fungal symbionts?*

Surprisingly, Buchner's (1965) curiosity about symbiont transmission to host cells did not extend to why an insect might host a fungal rather than a bacterial symbiont. He simply suggested that fungal symbionts were not as highly adapted, as they tended to be found free in the haemolymph or fat body rather than housed in particular host cells (p. 388). This is a key question, as intracellular life tends to favour intimacy – symbionts that live within cells are likely to be more specialized and may be transmitted to the next generation in the egg cytoplasm. Fungi are indeed often housed extracellularly, in co-opted organs, such as the diverticulum (a blind sac off of the gut) (e.g. of green lacewings), or external to the body, such as in external pockets known as mycangia (e.g. bark beetles or wood wasps). However, adaptation is apparent in most of these organs. For example, mycangia reflect dramatic changes to the external morphology of the insect cuticle, and may include internal glands that maintain axenic cultures of the fungal mutualists (Klepzig *et al.* 2009). Further, Buchner's presumption that bacterial associations were more ancient and therefore more complex than fungal associations, is not borne out in the current estimates of both types of associations. From molecular phylogenetic estimates, obligate bacterial associations date from 40 to 280 MYA (see Table S1 for known estimates). Fungi have also been associated with insects for many millions of years. There is fungal spore fossil evidence from arthropod (mites) and basal hexapods (collembola) faeces from the Silurian, 420 MYA (Alexopoulos *et al.* 1996; Labandeira 2007), and ancestral state reconstruction suggests that a fungal parasite of scale insects diversified 150 MYA (Ascomycota: Pezizomycotina: Sordariomycetes: Hypocreales) (Sung *et al.* 2008). Further, bark beetle fungal associations, *Ceratocystis* sp. and *Ophiostoma* sp., have been dated to 40–85 MY (Harrington 2005). Given the age of these associations, it seems likely that there has been sufficient time for the evolution of intracellular life in insect-inhabiting fungi, as there has been in bacteria.

An intuitive explanation for the relative dearth of intracellular, transovarially transmitted fungal associations relates to cell size. One might imagine that eukaryotic fungal symbiont cells are generally much larger than bacterial symbiont cells, and may not be easily packed into cells or eggs that may be, at the lower limit, 100 µm or less. However, the intuitive explanation is flawed; the

size ranges of bacterial and fungal symbionts overlap broadly. Buchner (1965) noted transovarially transmitted bacteria (his ‘x symbionts’) in fulgoroid planthoppers were up to 200 µm long (p. 692) and *Sulcia muelleri* (Buchner’s ‘a’ symbiont) can be up to 100 µm long (Moran *et al.* 2005). In contrast, the YLS of planthoppers range from 5 to 13 µm (Cheng & Hou 2001). Buchner (1965) also noted many other fungal symbionts that are sufficiently small to be intracellular and transovarially transmitted: for example, in the plant-feeding insects *Ledra aurita* (p. 351), a megamelinae (p. 392, Fig 215), the wax and tortoise scales (p. 232), *Kermes quercus* (p. 236), *Lecaniodiaspis africana* (p. 237), the mealybugs, *Rastrococcus* spp. and *Stictococcus* sp., (p. 239), and hormaphidine aphids (p. 328). Although the size is unknown, trichomycetes (Zygomycota) associated with black flies, are also transovarially transmitted (McCreadie *et al.* 2005). Given that cell sizes of symbiotic bacteria and fungi overlap and that they can both be intracellular and transovarially transmitted, we suggest that physiological size constraints are also not the major impediment for fungal intracellular life within insects.

The bulk of all fungal associates of insects currently being described are true yeasts in the Saccharomycotina (Suh *et al.* 2005) (see Fig. 1). As a general rule, these fungi are located among host cells (intercellularly). The known exceptions to this are *Candida* spp. (Ascomycota: Saccharomycotina) symbionts of long-horned beetles (Nardon & Grenier 1989) with no known function, and the yeast symbiont, *Coccidiascus legeri* (Ascomycota: Saccharomycotina), of *Drosophila*, that can speed development time and increase pupation success (Ebbert *et al.* 2003). In both instances, these true yeast symbionts are sequestered within host vacuoles (Lushbaugh *et al.* 1976; Nardon & Grenier 1989). This suggests a different relationship between host and Saccharomycotina symbiont relative to the intracellular Pezizomycotina or bacteria, neither of

which have not been found dwelling within host vacuoles. Contrary to Buchner’s (1965) supposition that fungi represent relatively recent or less well-adapted partners of their hosts, we suggest instead that the kinds of metabolic advantages that are typically conferred to insects by their fungal partners are better collected at arms length. This is in contrast to the relative ease with which obligate bacterial ‘inmates’ are domesticated. As we suggest in the following section, the extracellular digestive capability of many fungi may limit their integration into host cells. (See Table 2 for an overview of general trends of bacterial vs. fungal insect endosymbionts).

*Are there conditions under which fungi are more likely to be insect associates than bacteria?*

Insects have nutritional requirements much like those of vertebrates; they require an exogenous source of 10–14 amino acids, several B vitamins and specific fatty acids, as well as sterols (Vega & Dowd 2005). Bacteria and fungi appear to differ as ‘microbial brokers,’ both in terms of the dietary compounds that each can provide for their insect hosts as well as the byproducts and/or enzymes for the degradation of recalcitrant carbon sources such as cellulose or lignin.

Both fungal and bacterial symbionts of insects require external sources of nutrients. As a group, fungi in general secrete enzymes into their surroundings and absorb nutrients from them (Alexopoulos *et al.* 1996). Fungi can use a wide range of carbon sources such as methane (least complex) to lignin (most complex). Bacteria also exhibit diverse nutritional ecologies: some are autotrophs, deriving energy from light, while others can use inorganic compounds. The bacteria associated with insects, usually require organic materials from their hosts. In return, like fungi, bacteria have a wide range of potential metabolic offerings for their hosts.

**Table 2** Bacterial vs. fungal mutualist symbionts of insects: general trends

	Bacteria	Fungi
Cell size	0.3–200 µm	2–250 µm
Genome size	160–1600 kb	15 100–20 900 kb
Age of association	40–280 MYA	Fungal spores associated with arthropod faeces 420 MYA
Known nutritional roles	Vitamins, amino acid synthesis	N recycling, breakdown of xylose, toxins, sterol synthesis
Types of association	More often obligate	More often facultative
Study	Easier to name and sequence Antibiotics will generally ‘cure’ host of bacteria 16S rDNA PCR primers generally effective for amplification of bacteria within the insect	Naming requires Latin diagnosis of growth on a range of media No single antifungal agent likely to work against most symbionts rDNA primers may amplify insect as well as fungal DNA

Although Buchner (1965) thought that fungi were routinely leaking proteins (p. 802), they do in fact produce sugars and glycerol, to maintain a hypertonic state (the state of most microbes with rigid cell walls) (Heritage *et al.* 1996). Bacteria, in contrast, maintain hypertonicity by producing amino acids (Heritage *et al.* 1996). Given that these compounds are produced as byproducts by these microbial groups, one might expect that fungi could readily provide sugars and fats, and that bacteria could provide amino acids in cost-free mutualisms (Douglas 2008). Interestingly, these roles (fungi producing sugars and fats, and bacteria producing amino acids) seem to fit with the current picture of endosymbiotic microbial mutualisms in insects (see Table S1). In most instances of sap-feeding symbiosis, the obligate bacteria are providing amino acids that may have been produced initially for their own benefit. Further, there are several examples of insects associating with fungi for fats (see below).

Both bacteria and fungi provide nitrogen when their hosts digest them, but bacteria are nitrogen-rich relative to fungi. The peptidoglycan cell walls of bacteria, composed of amino acids and sugars, contribute to the relatively high (11.5–12.5%) percentage of nitrogen in dry weight of bacteria (Martin & Kukor 1984). This is in contrast to fungi, with cell walls of chitin, a polysaccharide. Yeasts are 7.5–8.5% nitrogen by dry weight and filamentous fungi are 2.0–8.0% (Martin & Kukor 1984). Bacteria can also serve their hosts by dissimilating uric acid for nitrogen uptake by the host, as *Blattabacterium* does for cockroaches (Wren & Cochran 1987) and *Blochmannia* does for ants (Degnan *et al.* 2005). Atmospheric nitrogen fixation however, appears restricted to protists and the bacterial and archaeal associates of termites and cockroaches (Nardi *et al.* 2002; Hogg 2005). Fungi have not been found to fix nitrogen, but they are able to aid in its uptake from the insect host's diet or directly provide amino acids for their hosts, as in the examples of the two species of death-watch beetle symbionts (Pant *et al.* 1960).

Bacteria and fungi are both able to make vitamins for their hosts. Although vitamins are typically considered the primary factors provided by symbionts to insect hosts feeding on blood diets, e.g. tsetse flies with bacteria (see Table S1), fungi could also produce vitamins for their hosts on non-blood diets. For instance, Gusteleva (1975) suggested that fungi can more readily synthesize B vitamins (e.g. B3 and B5) than bacteria associated with xylem-feeding insects. In experiments with the death-watch beetle (*L. serricornis*), Jurzitza (1969) showed that the YLS could provide vitamins, but required at least 9 of the 10 essential amino acids from their hosts. *Candida* species yeasts also provide vitamins for various long-horned beetles (Nardon & Grenier 1989), and this is also the likely role for some trichomycetes (Zygomycota) associated with black flies (McCreadie *et al.* 2005).

Fungi and other eukaryotes use endogenously produced sterols for membrane lipids, as hormone precursors and for regulating development genes (Douglas 2009). Bacteria differ in this regard, as only the mycoplasmas use sterols for cell membrane construction, and other bacteria use a functionally equivalent, but distinctly different class of compounds, hopanoids (Hogg 2005). Although *Wigglesworthia* of tsetse flies and *Blochmannia* of ants retain genes for fatty acid metabolism (Gil *et al.* 2003), to date, bacterial symbionts have not been found to contribute directly to sterol synthesis (Douglas 2009). The sterols produced by fungi, in contrast, are readily used by their insect associates, as there are a number of examples of this type of provisioning in the literature. Ba *et al.* (1995) found that yeasts synthesized sterols for fire ant larvae. Sterols are provided by YLS for death-watch beetles and planthoppers (Noda & Koizumi 2003), by *Botrytis cinerea*, the 'noble rot' fungus (Ascomycota: Pezizomycotina: Leotiomycetes) for grape berry moth larvae (Fermaud & Lemenn 1992) and by *Ophiostoma* sp. (Ascomycota: Pezizomycotina: Sordariomycetes: Ophiostomales) for spruce beetles (Klepzig *et al.* 2009). Insects that make the transition to direct consumption of plant tissues can use phytosterols to construct their own cholesterol. However, it is reasonable to assume that insects with limited access to dietary sterols (e.g. xylem feeders such as cicadas) might associate facultatively with fungi to fulfil any additional requirements.

Given their heterotrophic lifestyle and absorptive nutrition, it is not surprising that there are numerous records of fungi producing digestive enzymes to aid in insect host nutrition. We might expect this to be especially true of filamentous fungi or YLS (Pezizomycotina), as these fungi secrete digestive enzymes into their environment for the extracellular absorption of nutrients (Alexopoulos *et al.* 1996). These fungi then grow away from areas of digestion towards fresh nutrients. This is in contrast to the true yeast symbionts (Saccharomycotina). Fungi in this latter subphylum are typically sessile unicells that do not secrete enzymes for degradation lest they be trapped in their own 'erosion zones'. Brues & Glaser (1921) suggested that fungal associates in soft scale insects produce a proteolytic enzyme and a lipase, and Shen & Dowd (1992) showed that the YLS of death-watch beetles are able to produce a wide range of detoxification enzymes that degrade a variety of compounds such as plant allelochemicals, mycotoxins, insecticides and herbicides. Bacteria may also aid in the breakdown of host dietary compounds within the gut. Although the lower termites and the wood cockroach (*Cryptocercus* sp.) rely on protists for the digestion of cellulose, hindgut bacteria in higher termites, other cockroaches, beetles, crane flies and millipedes play this role (Douglas 2009). In fungi, cellulose breakdown usually occurs outside the host, as in the case of bark beetles with their fungal associates *Ceratocytis* spp. and

*Ophiostoma* spp. (both Ascomycota: Pezizomycotina: Sordariomycetes: Ophiostomales) (Harrington 2005), or *Sirex* woodwasps with their white rot fungus, *Amylostereum areolatum* (Basidiomycota: Agaricomycetes: Russulales: Stereaceae) (Slippers *et al.* 2003), or *Xiphydria* woodwasps and their *Daldinia decipiens* and *Entonaema cinnabarina* (both Ascomycota: Pezizomycotina) fungi (Srutka *et al.* 2007).

#### *The evolution of microbial symbiosis*

What can phylogenetic affinity tell us about the origins of symbiosis? As mentioned, most intimate intracellular, obligate fungal associates of insects are found in the Pezizomycotina, where other filamentous plant and insect pathogens are found (Suh *et al.* 2001). In contrast, the vast majority of what appear to be facultative associates are being recovered in the Saccharomycotina. Although the sample size is not yet large, it is at least possible that distinct origins of insect–fungal symbiosis could fall out along these subphyla of the Ascomycota, with mutualist symbionts arising from pathogenic ancestors in the Pezizomycotina and from commensal associations in the Saccharomycotina. The initiation of symbioses for both fungi and bacteria are unknown but may be informed by studies of invasion by pathogens and interactions with commensals of both microbial types.

Fungal pathogenicity and host invasion is dependent on a variety of mechanisms, such as host environment sensing, fungal cell wall surface features, biofilm formation, penetration by hyphae and the secretion of toxins or degradative enzymes. Yeasts can form biofilms either as unicells or as pseudohyphae and these biofilms exhibit increased resistance to antifungals (Kumamoto & Vences 2005). Fungal pathogen research is primarily focused on mammalian pathogens, however, and in insects, only hyphal penetration of the insect integument is well understood. Clearly, many gut fungi establish when ingested by the insect host, although it is not clear how specific fungal symbionts are maintained, while other fungi are digested and excreted. Arbuscular mycorrhizae secrete compounds that prepare the plant root for the symbiosis (Reiman 2008). It is not known whether insect associates have any similar mechanisms for negotiating the relationship. In bacteria, both mutualists and pathogens appear to invade hosts in a similar manner, with secretion systems for the delivery of proteins or toxins for invasion of host cells (Dale *et al.* 2002).

Clues to the origins of microbial endosymbiotic mutualists may be found in evolutionary reconstructions of their phylogenetic relationships. Five thousand species of fungi attack economically important plants (Willey *et al.* 2007); this is in stark contrast to the ~100 species of bacteria that are plant pathogens (Jackson 2009). Some of the fungi that attack plants are also beneficial associates of insects, such as the bark beetle associates, *Ceratocystis* spp. and *Ophiostoma*

spp. fungi (Harrington 2005), and the woodwasp fungi mentioned in the context of cellulose breakdown above (Slippers *et al.* 2003; Srutka *et al.* 2007). Similarly, Sung *et al.* (2008), using the phylogenetic method of ancestral state reconstruction, found that plant pathogenesis is the proposed ancestral state for a diverse array of fungi that attack insects in the Clavicipitaceae and Cordycepsaceae (both Ascomycota: Pezizomycotina: Sordariomycetes) (Sung *et al.* 2008). Some of the entomopathogenic fungi in the Clavicipitaceae gave rise to the YLS of planthoppers and aphids (Suh *et al.* 2001). Similarly, at least two bacteria mutualist symbionts (*Serratia insecticola* and *S. symbiotica*) are found within a genus of ubiquitous bacteria that also include species pathogenic to insects (*S. marcescens*).

Microbes that are in the same habitats with insects, such as those in plant tissue, could also be progenitor mutualists. Both bacteria and fungi can exist as non-pathogenic intercellular associates of plants, known as endophytes (Arnold & Lewis 2005), and sap fluxes house high microbial densities particularly of fungi, given that high sugar concentrations typically exclude bacteria (Deacon 2006). Buchner (1965) noted that the orthezid ensign scale insect, *Newsteadia floccosa*, made the transition to directly feeding on the endophytic fungi within its host plant (p. 798) and Arnold & Lewis (2005) point out that at least one insect pathogenic fungus, *Beauveria bassiana* (Ascomycota: Pezizomycotina: Clavicipitaceae), can be recovered as an endophyte from maize (see Vega 2008 for a recent review of other fungal entomopathogens that can live as endophytes). Interestingly, there is not yet any evidence of insect–fungal associates being recovered from soil, where many encounters with soil-dwelling saprotrophs must occur.

Symbionts may also be lineage-specific. Among fungal groups, insect associates appear concentrated in the Pezizomycotina and the Saccharomycotina of the Ascomycota (see Fig. 1), and have not yet been found in either of the two newest delineated fungal phyla, Glomeromycota and Neocallimastigomycota. The first group is arbuscular mycorrhizal associates of plant roots and the second group is anaerobic ruminant fungal associates. Similarly, most bacteria involved in heritable mutualist symbioses with insects are found in certain lineages of bacteria (alpha-, beta-, gamma-proteobacteria and Bacteroidetes; see Table S1). The features that restrict these ecological relationships and transitions among them may be revealed in the coming decade with the increasing use of molecular genetic techniques.

### Part III – methods for the study of microbial symbionts

Some of the earliest work on symbionts was conducted by researchers interested in insect development. For example, Blochmann, more than 120 years ago (1884), noted the

presence of bacteria in both *Camponotus* and *Formica* ants (Buchner 1965) (p. 23) but was hesitant to declare them bacteria. Leydig first described fungal symbionts of insects in 1854 in scale insects (Buchner 1965). Given that insect–fungal symbioses were being discovered contemporaneously with bacterial associates, why have the latter been so much more thoroughly studied? As we have addressed above, part of the answer may lie in the fact that many bacterial mutualists of insects are obligate; they are therefore present in every host examined, and they invite research into the mechanism of mutual dependence. Facultative associations may be maddeningly fickle, and if their phenotype in the host is context-dependent, they may easily evade functional classification. In this section, we consider two additional points: first, the methods for the manipulation and characterization of bacteria have been better resolved than those for fungi, due in large part to their greater evolutionary divergence from their insect hosts. Second, the insights that have amassed from genomic data are favouring bacteria, given that they represent much more tractable sequencing projects.

Morphological techniques alone have limited resolving power for distinguishing most microbes. Not only bacteria and fungi are usually morphologically homogenous, but also, distinctive morphologies may not necessarily be diagnostic; the same strain can exhibit different phenotypes under varying environmental conditions or during the development of sexual (fungi) or asexual spores (Alexopoulos *et al.* 1996). Recent advances in molecular techniques, therefore, have vastly enlarged our toolkit for the study of microorganisms. For amplifying bacterial partners of insects, 16S rDNA primers are widely used and there is seldom amplification of host DNA to confound sequencing results. Fungal amplification from insects, in contrast, can present great challenges. Insects and fungi share a more recent common ancestry, and consequently a higher degree of similarity in the 18S and 28S subunit rDNA genes that are the commonly used for fungal molecular phylogenetics. To circumvent this issue, most researchers use culture-dependent methods for their work on fungi within insects. Unfortunately, this approach excludes fastidious, intimate associates (Zhang *et al.* 2003). Further, although some investigators have had success with direct amplification of the standard rDNA genes from fungus-infected hosts (Zhang *et al.* 2003; Gibson & Hunter 2009a), these methods may not work for some host-associates due to low or variable densities of the fungal symbionts.

To conduct a general molecular methods-based survey for *in vivo* fungal symbionts, primers for an ergosterol synthesis gene could be developed. This sterol synthesis gene is unique to fungi and these methods have some precedent in the medical community (e.g. Bammert & Postel

2000). Unfortunately, these data would not be useful initially for taxonomic affinities, because this gene is not yet widely used. However, ergosterol primers could be used to denote the presence or absence of fungal associates. For insect samples that are positive for the presence of the ergosterol gene, further work could be done to dissect particular tissues thought to contain fungi, or more stringent PCR conditions for rDNA genes could be used.

Comparing aposymbiotic or uninfected hosts with symbiont-containing hosts remains the accepted standard for determining phenotypes in insect symbiosis research. The lack of effective and specific antifungal agents makes generating symbiont-free lines of fungus-infected insects more difficult than for bacteria. For bacteria, antibiotic treatment is relatively straightforward and a variety of methods are available that generally cause few side effects on the host. There are some similar general methods for treating fungi within insects and these include egg surface sterilization (for fungi inherited on the egg surface) (Jurzitza 1966), heat (Olsen & Hoy 2002), and antifungals (Gibson & Hunter 2005). Many antifungals are largely designed for human use and target fungal sterols or chitin. As chitin is the primary constituent of both fungal cell walls and insect cuticle, chitin-targeting compounds are likely to have detrimental consequences for the insect host, especially during development. Still others attack membrane components and, as such, are toxic for eukaryotes as a whole, e.g. amphotericin B and azoles. However there are some promising compounds from other *in vitro* trials and *in vivo* systems: *in vitro* trials with 2% sertaconazole nitrate (Ertaczo, OrthoNeutragen, Skillman, NJ 08558) (C. M. Gibson, unpublished data) and plant secondary compounds, such as tea tree oil (C. M. Gibson, unpublished data) have shown successful antifungal activity against the yeast symbiont isolated from *Comperia merceti* wasps. Further, *in vivo* work with 0.01% cycloheximide (Gibson & Hunter 2005), baldcypress extract (Jones 1981), and antifungals secreted by particular bacteria (Cardoza *et al.* 2006), have been successful in other systems.

Another major obstacle that has hindered insect–fungal symbiosis research is the current fungal nomenclatural system, especially with respect to unicellular or uncultivable fungal symbionts. Yeasts must be grown on a variety of traditional fermentation and assimilation media and the results described in a paragraph-long diagnosis in Latin before they can be formally named. This system prevents the naming of any uncultivable or fastidious fungi. In contrast, bacteriologists no longer require a specimen in the Bacteriology Culture Collection and have recently opted for the system of using ‘*Candidatus*’ for fastidious bacteria that have distinctive phenotypes (e.g. as insect symbionts) and for which we have only sequence data (Murray & Schleifer 1994). Given the diversity of yeasts and YLS being

recovered from insects (Suh *et al.* 2005), and the unlikely event that all of these organisms will be cultivable, a similar strategy for fungi would facilitate research into fungal symbionts.

Newcomers to fungal research should note that some methods commonly used for bacterial identification and cultivation may also suffice for at least some fungi. For instance, Gram-staining is a straightforward and effective means of determining whether particular cells are bacterial or fungal, as fungal cells stain tan rather than pink (Gram negative bacteria) or purple (Gram positive bacteria). Further, in the same way that bacterial isolates may be preserved in glycerol stocks, we have had similar success with this method to preserve cultivable fungal associates of insects (C. M. Gibson, unpublished data). As some fungal symbionts decline in growth and viability shortly after isolation from their hosts, any cultured samples should be made into longer term storage stocks as soon as possible. In the United States, samples may be archived with the National Centre For Agricultural Utilization Research, Peoria IL 61604 with additional pertinent data (e.g. GenBank accession numbers).

#### *Methods, theory, computational ability*

In the last two decades microbial ecology has been, and continues to be, transformed by molecular methods. There are now new ways of understanding microbes that allow for their characterization as distinct entities: high-throughput sequencing (e.g. pyrosequencing) is enabling relatively small teams of collaborators to sequence whole genomes, and contemporary phylogenetic methods now facilitate the identification of novel strains and species more readily (Nguyen *et al.* 2007). Annotation of these genomes can lead to a broader understanding of the interaction of the symbiont with its immediate host environment, as well as how the symbiotic host functions in nature. Genome analysis tells us what the symbiont produces that the host may use, as well as what the symbiont is lacking and must therefore be provided by the host. Symbiont toxin and detoxification genes may aid the symbiotic host in negotiating with its environment: detoxifying food, or responding to natural enemies with toxins. Further, genome analysis may be used for *in silico* (computer aided) design of culture media for bacteria previously considered fastidious (Lemos *et al.* 2003). The ability to culture a symbiont makes it much more tractable for study and transformation. We can also collect functional information about microbial dynamics both within their hosts and as whole community assemblies; for instance, microarray analyses allow for the comparison of expression profiles under various conditions (Mahadav *et al.* 2008).

Currently, at least 2 606 bacterial, 365 fungal (33% of them *Saccharomyces* species) and 93 arthropod (33% of them

*Drosophila* species) genome sequencing projects are completed or are in progress (accessed 2 December 2009, <http://www.genomesonline.org/>). Clearly bacteria dominate these numbers. Relative to bacteria, fungal genomes are less gene-dense, and both genes and genomes are much larger, due to repetitive DNA, and larger intergenic regions with regulatory elements (Keeling & Slamovits 2004). Also, in contrast to bacteria, lateral gene transfer appears less common in fungi. In the fully sequenced fungal genomes of *Yarrowia lipolytica*, *Kluyveromyces lactis*, and *Debaryomyces hansenii* (all Ascomycota: Saccharomycotina), lateral gene transfer appears to account for < 1% of total gene number (Dujon *et al.* 2004). Researchers instead cite gene duplication as playing a large role in the versatility of fungal genomes (Scannell *et al.* 2007), a factor which could promote larger genome sizes in fungi. The genomes of YLS of planthoppers were estimated at 17 000–20 000 kb (Noda & Kawahara 1995) and those of the death-watch beetles, *L. serricornis* and *Sitodrepa paniceum*, were estimated at 20 900 and 15 100 kb, respectively (Noda & Kawahara 1995). These obligate mutualist fungal genomes are therefore orders of magnitude larger than those of sequenced bacterial symbionts (e.g. *B. aphidicola* 640 kb, and see Table S1 for other examples). Interestingly, two obligate fungal associates show some similarities to obligate bacterial symbionts. However, these are the microsporidian, *Nosema locustae*, an intracellular parasite of insects with a genome size of 2 900 kb, and *Pneumocystis jirovecii* (= *carinii*) (Ascomycota: Taphrinomycotina), a pathogen of human lungs, with a 7 700 kb genome. In the first case, the microsporidian demonstrates gene densities that are comparable to those of obligate intracellular bacteria, (one gene/kb, as in *B. aphidicola*), in contrast to the free-living *Saccharomyces cerevisiae* or *Eremothecium gossypii*, which have one gene/2 kb) (Keeling & Slamovits 2004). Given that Microsporidia is a separate fungal phylum and Taphrinomycotina a separate subphylum relative to other fungal associates, it is unclear whether reductions in genomic architecture will hold for other symbiotic mutualists or pathogens in the Pezizomycotina or Saccharomycotina. It seems unlikely, however, as one might expect a correlation between genome size and cultivability, and fungal associates in these latter two groups typically maintain cultivability.

To date, the relative ease of manipulation and characterization has biased the majority of symbiosis research towards bacterial symbionts. This is clearly not a representative picture of insect–microbial symbiosis as a myriad of fungal examples are coming to light. The methods listed here are bound to expand and be extended beyond their current uses within the next decade. This will provide not only a more balanced view of both fungal and bacterial symbionts, but also allow the exploration of novel genes and gene products from a greater diversity of insect associates.

## CONCLUSIONS

Fungi are frequent and important associates of insects. In spite of being common, however, fungi are routinely ignored in investigations of insect symbioses – even as investigators of intracellular bacterial symbionts become more aware of the need to exclusively sample the bacterial symbiont community within a host before attributing a phenotype to any associate. It is a sobering fact that the last attempt to catalogue the complete symbiotic associates of plants and animals was made by Buchner and colleagues who were conducting their work early in the 20th century. Fungi will not be discovered with the same diagnostic tools (largely 16S rDNA surveys) that are now routine for bacterial symbionts, but fungal-specific genes, for example, ergosterol, may be promising candidates for development.

The diversity of fungal insect associates is daunting, as are the challenges of manipulating fungal infections. However, for some applications, fungal symbionts may be the very associations to focus on. For instance, fungi might represent superior opportunities for symbiotic control of pest insects (e.g. beetles, cockroaches, or ticks) because of their relative ease of cultivation. Cultivable microbes can be more readily transformed for constructing recombinant symbionts that result in changes in the host insect's phenotype (Douglas 2007) or for probiotic inoculation to exclude competitors (Miller 2008). In addition, some fungi appear to be relatively specific to particular insect species, and given that eukaryotes are less prone to lateral gene transfer, the risk of any transformed genes escaping into non-target organisms would also be lessened relative to bacterial symbionts.

More fundamentally, what insights could a better understanding of fungal symbionts provide? Although in some cases they are ecological equivalents, fungi often differ from bacteria in their relationship with their insect hosts. With some exceptions, current examples suggest that fungi are found in facultative relationships with their insect hosts more frequently than bacteria. We propose here that the extracellular digestion of fungi may make them more difficult to domesticate within host cells. This fact explains the difference between bacterial symbionts, which are more often intracellular, inherited, and/or obligate, and fungal symbionts, which are more often extracellular, horizontally transmitted, and facultative. As nutritional mutualists, fungi may be more likely to contribute sterols and sugars, relative to the amino acids commonly produced by bacterial associates. Both groups may produce vitamins; but currently fungi seem to produce a wider array of digestive and detoxification enzymes.

Finally, without more research, we cannot yet delimit the diversity of roles of fungal symbionts. We know that insects without their bacterial symbionts are likely to be fundamentally different than their symbiotic conspecifics, displaying reduced nutritional capacities, differing susceptibilities to

parasites or viruses, differing reproductive relationships, sex ratios, or behaviours (Moran *et al.* 2008). Yet for fungal symbionts, beyond a few elegant examples of intimate associations (e.g. YLS of planthoppers, aphids and beetles), and some surveys that hint at extraordinary diversity (Suh *et al.* 2005), we simply do not know what roles these widespread associates of insects may be playing.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1** Endosymbiotic microbial associates of insects with an emphasis on mutualists and organized by host nutritional ecology.

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