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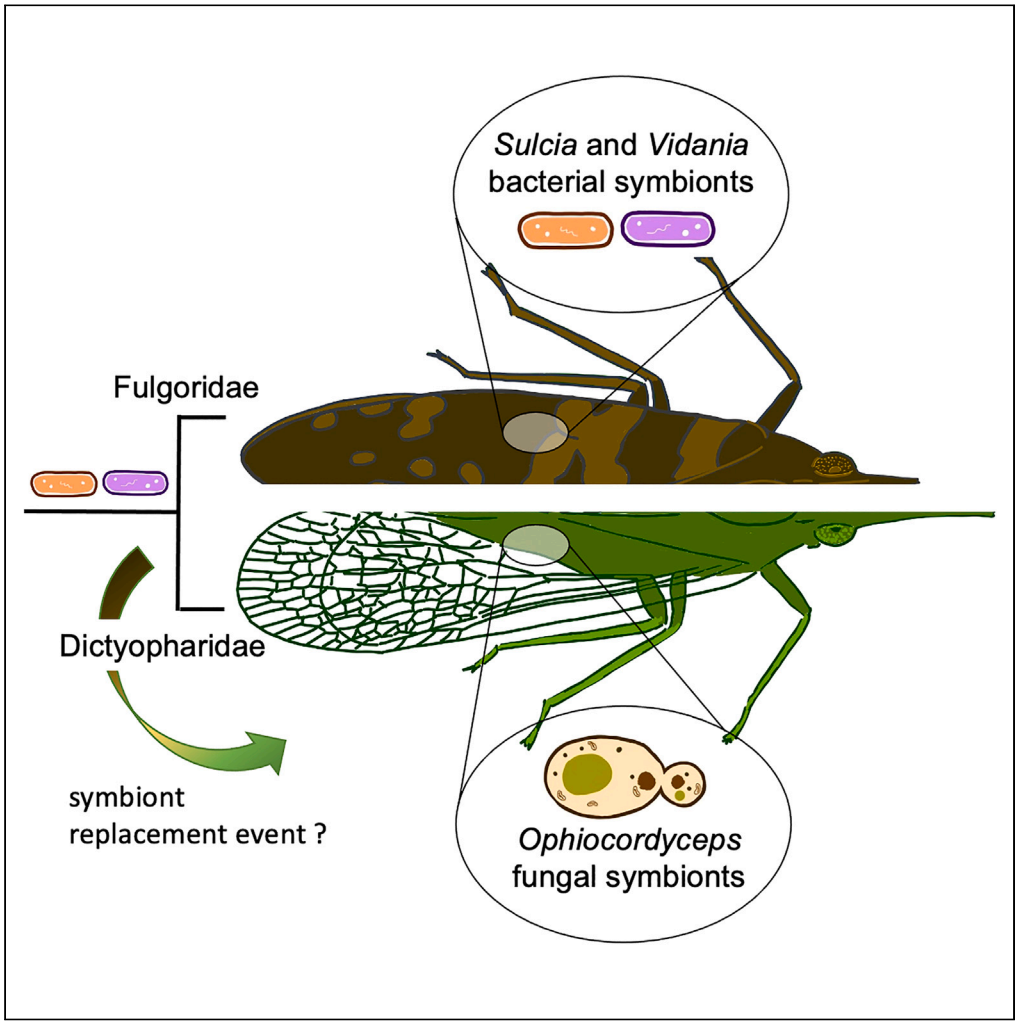

Ruby Siehl,
Katherine Vyhnal,
Shana K. Goffredi

sgoffredi@oxy.edu

Highlights

Five tropical insect families host yeast-like fungal symbionts instead of bacteria

The fungal endosymbionts are related to the lethal parasitic fungi *Ophiocordyceps*

The fungal cells reside intracellularly within the highly dynamic fat body

Eggs developing within female insects contain fungal cells, enabling coevolution

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Friendly fungi: Tropical insect families form partnerships with intracellular fungi related to pathogens

Ruby Siehl,[1] Katherine Vyhnal,[1] and Shana K. Goffredi[1,2,*]

SUMMARY

Sap-sucking insects fail to obtain vitamins, amino acids, and sterols from their plant diet. To compensate, obligate intracellular bacterial symbionts (usually *Sulcia* and *Vidania*) provide these missing nutrients. Notably, some planthoppers within the Fulgoromorpha (suborder Auchenorrhyncha) associate with intracellular fungi, which either accompany or replace the anciently associated bacterial partners. Planthopper-symbiont surveys, however, have only been conducted in limited temperate regions, thus necessitating examination of these relationships in the tropics, where insect and fungal diversity is high. Here, five tropical planthopper families host yeast-like endosymbionts related to the parasitic genus *Ophiocordyceps*. Fungal endosymbiont identity generally corresponded to host family, suggesting possible coevolution. Vertical transmission to offspring was supported by the occurrence of fungal cells in developing eggs. This serves as the most comprehensive tropical planthopper-symbiont survey to date, doubling the roster of known Fulgoromorpha species that host intracellular fungi and further elucidating the remarkable success of this diverse insect group.

INTRODUCTION

Members of the fungal genus *Ophiocordyceps* (Ascomycete; Hypocreales) are professional manipulators of insects. They consist of ~140 described species worldwide[1] and are known to infect at least nine insect orders, most commonly the Lepidoptera and Hemiptera, with great lethality.[2,3] This fungal genus rose to recent pop culture fame when depicted as the killer fungus in the critically acclaimed video game franchise and TV series *The Last of Us* (Sony Interactive Entertainment and HBO). Uncommonly, in at least three Hemipteran infraorders (Cicadomorpha [cicadas], Coccomorpha [scale insects], and Fulgoromorpha [planthoppers]), these fungi can exist internally without causing harm to the insect.[4–8] In these cases, the fungi are thought to provide nutritional benefits to the insect.[9–11] Little is known about how this lifestyle transition from pathogen to beneficial symbiont occurred, but it is clear that fungal pathogens represent a likely pool of new insect symbionts due to their ability to invade the host and avoid the immune system.[12,13]

Nearly all members of the Auchenorrhyncha (a suborder of the sap-sucking Hemiptera) are dietary specialists on plant sap and are known to have an especially high prevalence of internal symbionts in order to balance their carbohydrate-heavy, nutrient-deficient diet.[14] This role is normally filled by bacteria, mainly the genus *Sulcia* (phylum Bacteroidota), acquired ~300 MYA, along with a "secondary" betaproteobacteria (usually *Vidania, Zinderia,* and *Nasuia*) acquired more recently.[15–18] In some Auchenorrhyncha species, a fungal symbiont has completely replaced one or both bacterial symbionts.[17,19] Symbiont replacement can occur either because the ancient bacterial symbionts undergo massive gene loss that renders them ineffective or because the host shifts to different food sources that require a specific metabolic upgrade.[6,12,16,20] In cicadas and planthoppers, where the fungi have assumed the role of primary symbiont, they supplement the nutritional needs of the insect by providing vitamins, sterols, and amino acids, the latter via recycling of uric acid, a nitrogenous waste product of the insect.[9,10,21] Fungal cells primarily reside in the fat bodies, which appears optimal for the insect since fat bodies are involved in many metabolic functions, including protein production and storage of energy reserves in the form of lipids.[22]

When in the fat body tissue of an insect host, *Ophiocordyceps*, evolutionarily derived from highly lethal insect pathogens, appear to reproduce only via asexual budding.[5,23,24] This commitment to solely asexual reproduction presumably facilitates insect host survival, by limiting the most harmful, and often dramatic, fungal stromata growth. Because these symbiotic fungi retain a yeast-like budding morphology, they are often referred to as "yeast-like symbionts" (YLSs).[24–26] In the eggs of at least four temperate planthopper species, YLSs make their way into the oocytes, and undergo vertical transmission, much like the bacterial symbionts.[23,26–28]

Ophiocordyceps-allied fungal endosymbionts, acquired relatively recently by insects (~40–150 MYA), represent a fascinating twist in the dynamic nature of insect-microbe symbioses. However, nearly all publications to date involve temperate species. So far, 15 species of

[1]Occidental College, Los Angeles, CA, USA
[2]Lead contact
*Correspondence: sgoffredi@oxy.edu
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planthopper within the Fulgoromorpha, many from China, Australia, Japan, and Europe, have been shown to host internal fungal symbionts.[10,19,24,26,27,29] According to a highly comprehensive review,[30] only a single tropical planthopper species has been shown to possess YLSs. Matsuura et al.[6] and Brentassi and de la Fuente[30] suggest that future studies on insects from tropical and subtropical regions will likely uncover many more dynamic acquisitions and replacements of bacterial symbionts by fungi. The Fulgoromorpha is the ideal group with which to explore this theory, given past surveys of fungal presence in several of their temperate members, along with the high diversity of both planthoppers and *Ophiocordyceps* fungi in tropical rainforests.

This study sought to determine whether tropical planthoppers host fungal symbionts and whether they accompany or replace the more ancient bacterial symbionts. To that end, we examined 42 species from 13 insect families within the Auchenorrhyncha, collected from a lowland tropical rainforest in Costa Rica. Of these, 15 species within five closely related families within the infraorder Fulgoromorpha (Flatidae, Acanaloniidae, Issidae, Tropiduchidae, and Dictyopharidae) possessed fungal symbionts, nearly all replacing the more ancient *Sulcia* and *Vidania* symbionts observed in close relatives. These fungi were documented in the fat bodies of adults and nymphs, as well as eggs, in cases where these life stages were available. This survey of tropical insect symbioses provides new evidence for intimate fungal-insect associations and motivates the continued search for insect groups that have entered into partnerships with eukaryotic symbionts, especially those related to lethal pathogens.

RESULTS

Common bacterial symbionts detected in a range of planthopper families

Bacterial 16S rRNA gene barcoding performed on a total of 42 species within 13 families of the suborder Auchenorrhyncha revealed a common pattern in 17 planthopper species: the hosting of dual symbionts within the genus *Sulcia* and a second symbiont within the class Betaproteobacteria (ex. *Zinderia*, *Vidania*, and *Nasuia*; Figures 1 and S1). The insects that hosted both *Sulcia* and betaproteobacteria represented eight families, belonging to the planthopper infraorders Cicadomorpha (families Cercopidae, Cicadellidae, and Membracidae) and Fulgoromorpha (families Achilidae, Cixiidae, Fulgoridae, Nogodinidae, and Issidae). Three species of Derbidae lacked *Sulcia* and instead hosted only betaproteobacteria (*Zinderia* or *Vidania*), a pattern also observed by Michalik et al.[19] A single species of Cercopidae and two species of Cicadellidae hosted *Sulcia*, but no betaproteobacteria, and instead hosted bacteria from the genus *Morganella* (Gammaproteobacteria). Overall, 12 insect species were also associated with *Arsenophonus*, *Blochmannia*, *Sodalis*, or *Morganella*, all gammaproteobacteria known to be insect symbionts (Figure 1).[16,18,31] Additionally, 19 insect species were associated with *Wolbachia* or *Rickettsia*, common bacterial endoparasites of insects (Figure 1).[17,32–34] Finally, 15 species within four families of the Fulgoromorpha (Flatidae, Acanaloniidae, Tropiduchidae, and Dictyopharidae) lacked all known symbiotic bacteria (Figure 1).

Ophiocordyceps fungi detected in five planthopper families

Using diagnostic 18S rRNA gene surveys, the *Ophiocordyceps* (Ascomycota, Sordariomycetes, Hypocreales) was detected in all 15 Fulgoromorpha planthopper species that did not contain symbiotic bacteria, as well as two species of the Issidae, which also hosted *Sulcia* and *Vidania* (Figures 1 and S2). A phylogenetic analysis of the 18S rRNA gene revealed two separate clades of *Ophiocordyceps* within the tropical planthopper families that are closely related to one another (96.0%–96.8% similar) and to fungi that are pathogens of insects (Figure 2). Clade IA contains fungal symbionts of the tropical planthopper families Acanaloniidae, Flatidae, Issidae, and Tropiduchidae, along with the fungal symbiont of the temperate planthoppers *Metcalfa pruinosa* and *Acanalonia conica* (Figure 2).[19] Fungal symbionts within clade IA were highly similar to one another (97.6%–100% in 18S rRNA gene sequence) and formed a larger grouping with previously reported insect-associated fungi (denoted "IB"; Figure 2). The fungus was identical in two species of Flatidae in clade IA, collected in two consecutive years (2022 and 2023; Figure 2). Clade II contains the fungal symbionts of the tropical Dictyopharidae and their eggs (99.8% similarity), along with a fungal pathogen of insects (98% similarity).[36] The Fulgoromorpha fungal sequences were distinct from those recovered from two Cicadomorpha species (*Ledropsis* and *Tituria* sp.; ~95%–97% similarity).[4] Additionally, the fruiting body of a Lepidopteran-infecting fungal pathogen (Figure S3) was sequenced and found to be ~95%–96% similar to fungi in the tropical insect clades IA and II (Figure 2).

Ophiocordyceps is the sole fungus associated with Fulgoromorpha planthoppers

Other fungi, including *Hypomyces*, *Pichia*, *Candida*, and Saccharomycetales spp., have been occasionally detected in planthoppers.[37] Thus, in order to determine whether the *Ophiocordyceps* was the only fungus present in the planthoppers, we conducted additional amplicon sequencing on a subset of 10 fungi-positive species from all five insect families, determined via direct sequencing (described above), as well as four specimens from two families for which no fungi were detected (Figure S4). For seven fungi-positive species, *Ophiocordyceps* 18S rRNA genes represented ~82%–99% of the total recovered (1,400–31,000), whereas the rest were attributed to the insect host (Figure S4). In the Dictyopharidae species, however, fungal sequences comprised only 5%–12% of the recovered reads, with the remaining reads attributed to the insect host (Figure S4). Despite the lower ratio of fungus to insect sequences, the dictyopharid-associated fungi also belonged solely to the *Ophiocordyceps*, with all three species sharing the same *Ophiocordyceps* amplicon sequence variant (ASV). By contrast, each of the planthopper species within the tropical families Acanaloniidae, Flatidae, Issidae, and Tropiduchidae was associated with a unique *Ophiocordyceps* ASV (Figure S4). This result is in agreement with the phylogenetic analysis based on longer 18S rRNA sequences (Figure 2).

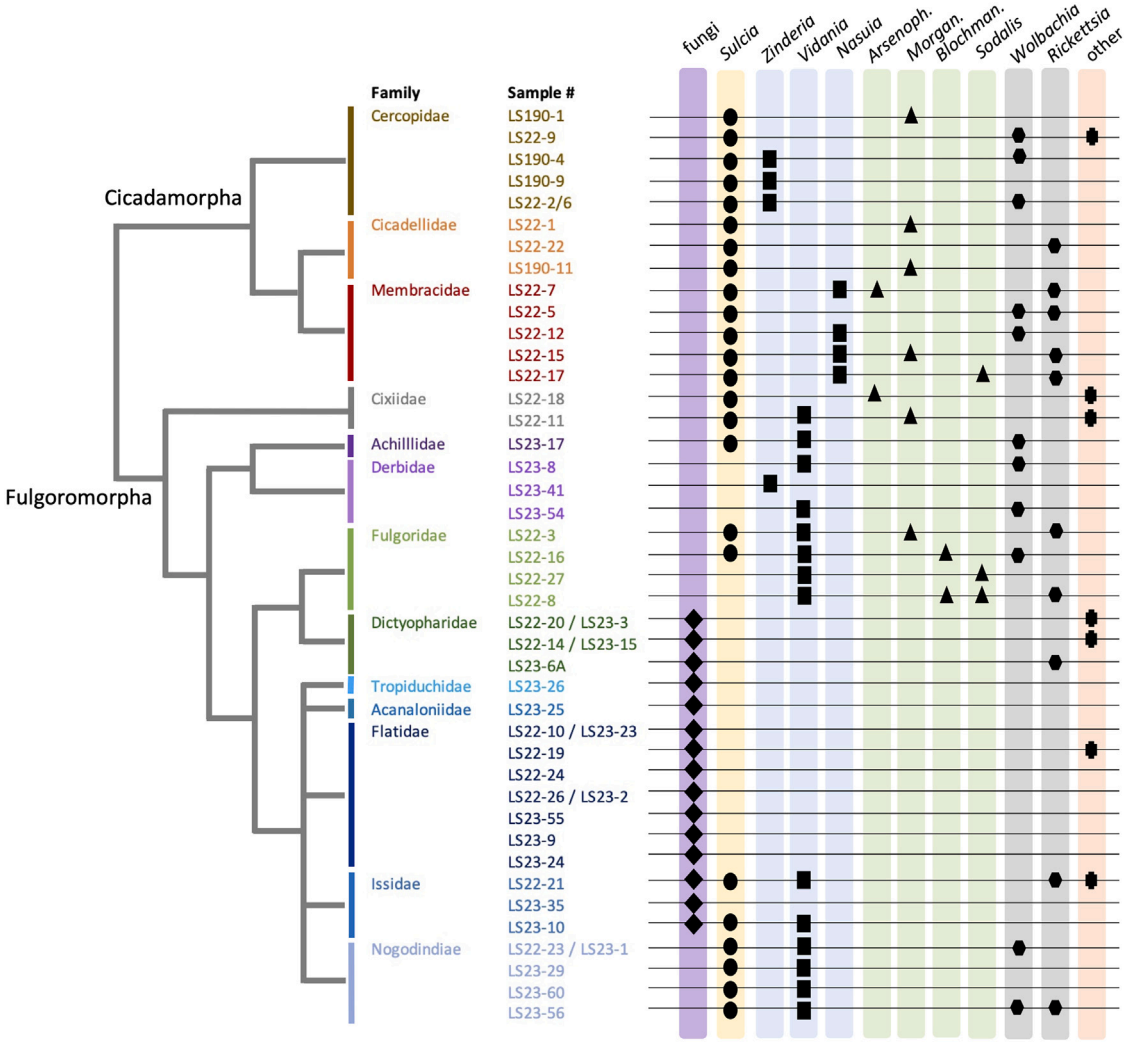

Figure 1. Symbiotic associations in 42 species (within 13 families) of the insect suborder Auchenorrhyncha

Grid symbols indicate the presence/absence of planthopper bacterial symbiont genera based on 16S rRNA gene barcoding. Betaproteobacteria genera are shaded in blue. Gammaproteobacteria genera are shaded in green. Parasitic genera are shaded in gray. Diamonds (highlighted in purple) show the detection of *Ophiocordyceps* fungal 18S rRNA genes, via specific diagnostic PCR primers, in 15 Fulgoromorpha planthopper species that did not contain symbiotic bacteria. The planthopper phylogeny, at left, is stylized and based on Bucher et al.[35] *Sulcia* was not recovered from two large fulgorid species, possibly a result of missing the symbiont-bearing tissue during dissection, prior to DNA extraction. "Other" includes *Herbaspirillum*, *Brachybacterium*, *Stenotrophomonas*, *and Actinobacteria*. *Arsenoph*, *Arsenophonus*; *Morgan*, *Morganella*; *Blochman*, *Blochmannia*.

Yeast-like symbionts are housed within planthopper fat body cells

Although molecular analysis allowed for the detection of fungal presence in different planthopper species, visualization of fungi within insect tissues confirmed their status as intracellular symbionts. Staining with lactophenol cotton blue served as the first visual confirmation of the presence of fungal symbionts in planthopper tissues (Figures S5A–S5C). Transmission electron microscopy revealed finer details of the fungal symbionts, including thick cell wall, conspicuous vacuoles, and nuclei (Figures S5D–S5F). Fluorescence *in situ* hybridization using an *Ophiocordyceps*-specific probe revealed fungal cells in the peripheral fat bodies of flatid and dictyopharid planthoppers (Figures 3, 4, and 5). Fungal cells present in both flatid adults and nymphs were oval shaped (~10 μm in length x 3–5 μm wide), densely populated within fat body cells, which are multinucleated due to fusion of neighboring fat body cells[38] (Figures 4 and S6). Many fungal cells appeared to be budding (Figures 4C and S5D), indicating reproduction in a yeast-like manner, as has been observed in other studies.[39,40] For this reason, from here forward we refer to the fungi as yeast-like symbionts (YLSs). Compared to the YLSs of the Flatidae, the Dictyopharidae YLSs were narrower (10 μm long x 2–3 μm wide), with slight differences in fluorescence (Figure 5C). In two dictyopharid adults, YLSs were not visually observed (even though fungi were detected via PCR amplification of the 18S rRNA gene). Many fewer YLSs were observed in the fat bodies of dictyopharids, compared to the flatid adults, which supports the lower ratio of fungus to insect host 18S rRNA gene, observed via barcoding, and described above (Figure S4).

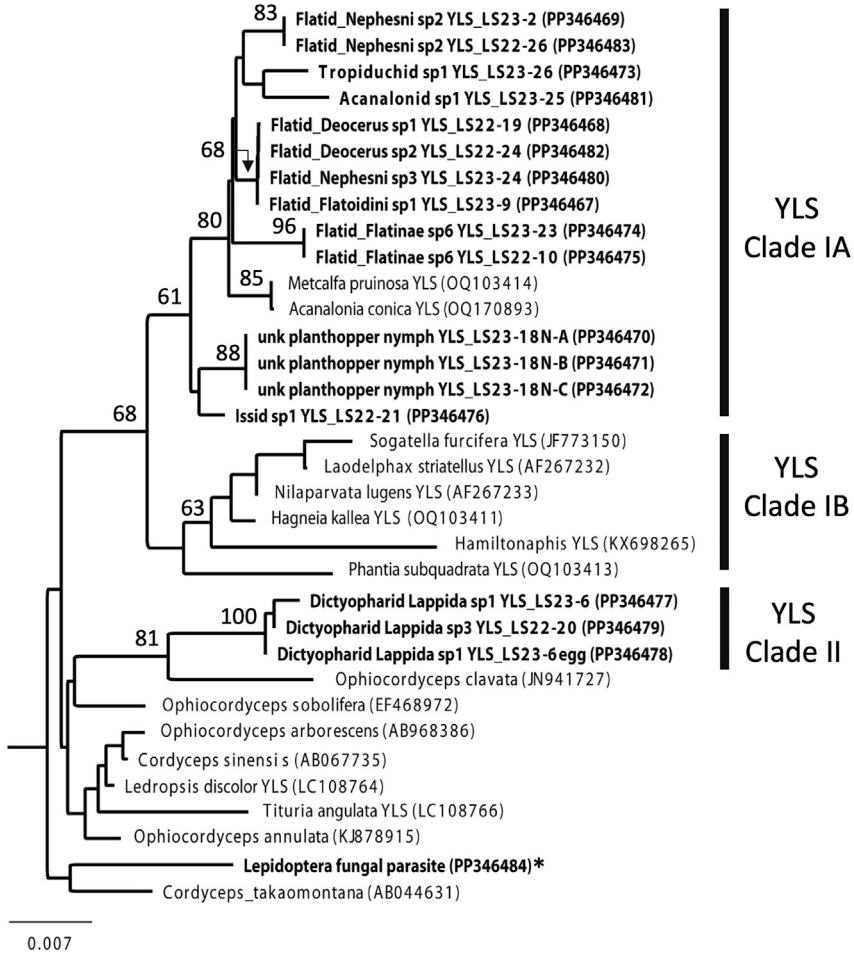

Figure 2. Phylogenetic relationships among the *Ophiocordyceps* fungal clades (Ascomycota; Sordariomycetes; Hypocreales) within tropical planthopper insects, based on the 18S rRNA gene

Taxa in bold denote those generated in this study. *Metarhizium anisopliae* was used as the outgroup (FJ609314; not shown). Numbers at nodes indicate bootstrap support (1,000 replicates, neighbor-joining, Tamura-Nei model), aligned using Geneious Prime 2021.2.2. Additional sequences from cultured representatives were obtained from GenBank, as were sequences from references Nishino et al.[4]; Suh et al.[24]; Michalik et al.[19] * = shown in Figure S3. YLS, yeast-like symbiont; unk, unknown. The scale bar represents percent divergence in the 18S rRNA gene.

For comparison, fluorescence *in situ* hybridization was also performed on the fruiting bodies of the Lepidopteran-infecting fungal pathogen sequenced in Figure 2. The visualization of these presumably sexual ascospore-forming structures (Figure S3) served as a contrast to the asexual, budding reproduction of the YLSs (Figures 4 and 5).

Yeast-like symbionts are vertically transmitted in dictyopharidae planthoppers

To determine possible maternal transmission of fungal cells, fluorescence *in situ* hybridization microscopy was performed on developing eggs removed from the abdominal cavity of female dictyopharids. Fungal cells (~20 per egg) were observed in a single layer along the egg surface, apparently enclosed in an outer membrane at the posterior pole of the egg, where the lateral oviduct connects[27] (Figures 5D–5G). YLSs associated with the developing eggs were slightly shorter, ranging in size from 3 to 8 μm in length x ~2 μm wide. The distinct phylogenetic placement of the Dictyopharidae YLS, separate from the other planthopper families, is consistent with principles of coevolution via vertical transmission in the oocytes.

DISCUSSION

Insect families within the suborder Auchenorrhyncha form complex symbiotic associations with numerous microorganisms, including both heritable bacteria and fungi. Hosting microorganisms internally allows these insects to exploit a nutrient-deficient diet of plant sap, which is high in carbohydrate content, but lacking in nitrogen.[41,42] In exchange for a stable, nutrient-rich niche, the symbionts are a source of endogenous amino acids and sterols in this extremely successful group of insects.[14,43] The unexplored regions of the tropics, with high levels of

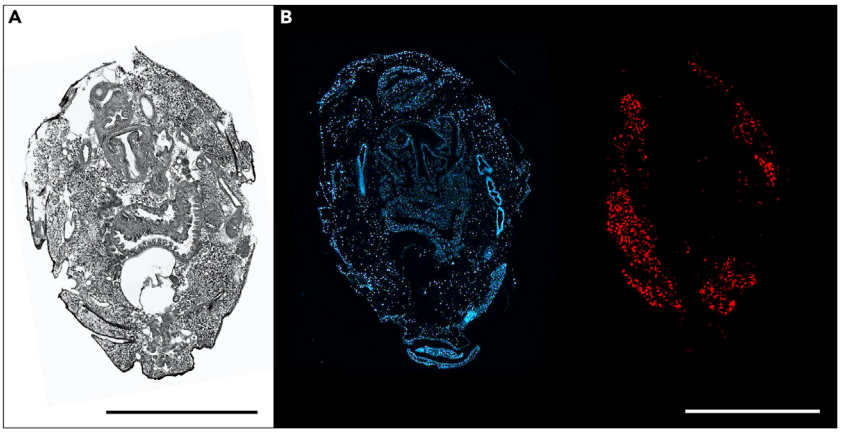

Figure 3. Visualization of internal fungal cells in the peripheral fat bodies of a flatid planthopper, *Flatinae* sp.

(A) Light microscopy of a Wright-stained transverse cross-section (5-μm thick) embedded in Steedman's resin.

(B) A corresponding 3-μm transverse cross-section stained with DAPI, revealing host cell nuclei (in blue, at left) and numerous fungal cells (in red, at right), hybridized with a fluorescence *in situ* hybridization probe designed to target the *Ophiocordyceps* (labeled with Cy5). The DAPI image was converted to cyan using the auto tone feature in Photoshop v21.1.0. Scale bars, 1mm.

insect diversity, are anticipated to host a great variety of insect-microbe associations.[6] As a group, the infraorder Fulgoromorpha is abundant in tropical rainforests, including Costa Rica, where this survey was conducted. Here, we surveyed 10 of 20 insect families within the Fulgoromorpha known to inhabit this region, and demonstrate that several of these tropical families associate exclusively with yeast-like fungi, related to filamentous pathogenic ascomycetes, housed inside of fat body cells. By documenting fungal symbionts in 15 new species of tropical insect, from five families, this survey doubles the number of Fulgoromorpha species and adds two new families (the Dictyopharidae and Tropiduchidae) to the roster of planthoppers known to associate with *Ophiocordyceps*, a genus that includes most insect entomopathogens, and usually infects with deadly consequences.

A 2023 analysis by Michalik et al.[19] demonstrated the presence of fungal symbionts in 11 temperate planthopper species but suggested that a more systematic sampling of families within the Auchenorrhyncha would allow a better understanding of the complicated evolutionary relationships between symbiont and insect hosts. Some of the seven Fulgoromorpha families are associated exclusively with *Ophiocordyceps* fungi, rather than bacteria. For example, all tropical species of Flatidae in our study, and two additional temperate species examined in previous studies,[19,26] hosted only fungi, which were identical in two insect species collected in multiple years. Conversely, some insect families show variability in fungal prevalence. For example, all tropical dictyopharid and tropiduchid species in the current study hosted fungal symbionts, whereas all temperate species hosted only bacterial symbionts.[19] Additionally, some tropical species within the Issidae hosted both fungi and bacteria simultaneously, which has also been documented for temperate issids,[19] cicadas[6] (Cicadomorpha), and scale insects[44] (Coccomorpha). This accompaniment of the bacteria (e.g., *Sulcia* and a betaproteobacteria) by the fungi in a single insect family suggests a possible transition from bacterial to fungal symbionts over time in the Auchenorrhyncha.

Among the Auchenorrhyncha surveyed in this study, evidence of coevolution between insect and fungal endosymbiont was observed on two different levels: between the Cicadomorpha and the Fulgoromorpha, and between families of the Fulgoromorpha. Within the tropical Fulgoromorpha, two fungal clades resulted from phylogenetic analysis of the 18S rRNA gene (and with distinct morphologies); one associated with the Dictyopharidae (Clade II in Figure 2), and the other associated with a group of four unresolved, but closely related, insect families that underwent a past rapid radiation event[35] (Clade IA in Figure 2). It follows that fungal symbionts would be similar in insects that diversified quickly and that an eventual pattern of family-level coevolution would emerge given enough time. We speculate that regional differences in host insect origin (e.g., temperate vs. tropical) might also be reflected in the fungal phylogeny, given that intermediate clade IB is composed of fungi from temperate insects in the families Delphacidae and Ricaniidae[19] (Figure 2). One exception to this idea, however, is the occurrence of temperate fungi, identical in two cosmopolitan insect host species, within the tropical clade IA. Additional surveys of Fulgoromorpha hosts and their *Ophiocordyceps* fungal symbionts would help determine whether this evolutionary differentiation between fungal symbionts relates to ecosystem characteristics or evolutionary history of the insect host.[30]

Fungal cells were distributed throughout peripheral fat bodies of both the Dictyopharidae and Flatidae planthoppers, a phenomenon observed in other planthopper studies.[19,27,29,39] This primary location appears significant, in that fat body tissue is involved in numerous important metabolic functions in insects.[22] Further, fat body reserves can sustain the insect during metamorphosis and egg laying and can, thus, change in volume depending on reproductive status. Relatedly, we noted that fat bodies containing fungi were much less abundant in the dictyopharid specimens, which were collected in June when individuals contained numerous mature eggs. In this case, perhaps the fat body reserves had been mobilized to the ovaries for egg development,[22] leaving behind a much more depleted tissue for the residence of

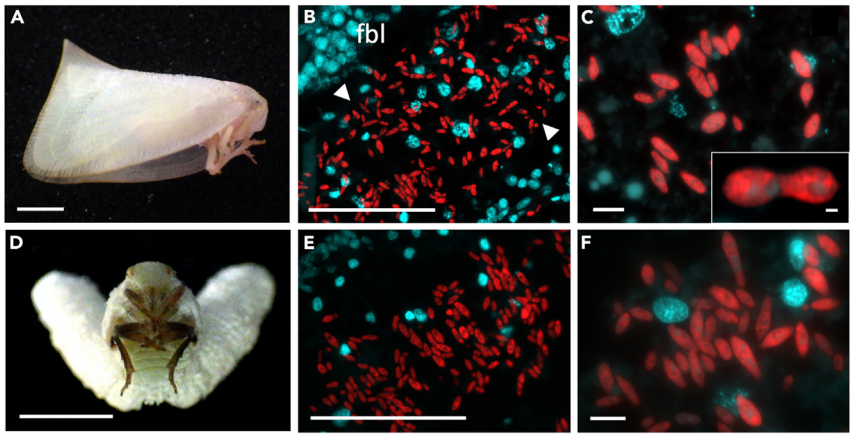

Figure 4. Visualization of internal fungal cells in Flatidae adult and nymph life stages

(A) Whole specimen of a flatid planthopper, *Flatinae* sp.

(B and C) Fluorescence *in situ* hybridization (FISH) of fungal cells within a multinucleated fat body cell, using an *Ophiocordyceps*-specific probe, shown in red (labeled with Cy5), counterstained with DAPI in cyan. Arrowheads in B delineate the outer membrane of the fat body cell. fbl, fat body lipids.

(C-inset) fungal cells budding.

(D) Whole specimen, flatid planthopper nymph, unknown genus, showing posterior exudate of white wax, common among planthopper nymphs.

(E and F) FISH imagery of fungal cells using the *Ophiocordyceps*-specific probe, shown in red, counterstained with DAPI in cyan. The DAPI channels were converted to cyan, for easier viewing, using the auto tone feature in Photoshop v21.1.0. Scale bars: (A), 5 mm. (B), 100 μm. (C), 10 μm (not including inset, 1 μm). (D), 5 mm. (E), 100 μm. (F), 10 μm.

fungi. This idea was further supported by the low percentage of fungal 18S rRNA sequences recovered from the three Dictyopharidae species, compared to the other fungi-hosting insect families, of which none contained developing eggs.

Yeast-like symbionts have been documented previously in the eggs of at least four planthopper species, suggesting that these fungi undergo vertical transmission, much like the bacterial symbionts.[23,26–28] We confirmed fungal cell presence in early life stages, including nymphs (for the Flatidae) and developing eggs (for the Dictyopharidae). Apparently, in temperate insects, fungi are released from fat body cells into the hemocoel via exocytosis, at which point they enter the developing eggs, ahead of yolk formation and egg shell deposition.[23,27] The nymphs then become infected as they hatch, with fungal cells already inhabiting the fat body tissue.[11,27] This vertical transmission via eggs likely contributes to the observed coevolutionary patterns, mentioned above, and implies that the fungi serve an essential role for their insect host.

Sexual reproduction of the fungi has, thus far, not been observed in any planthopper hosts.[26,40] Instead, the yeast-like symbionts propagate solely by budding, as was observed for the tropical planthoppers in this study. This modified reproductive strategy is supported by genomic analysis, where sexual-reproduction-related genes were not detected in temperate planthopper yeast-like symbionts, lost during evolution within the insect tissues.[21] Unlike other ascomycetes, lack of sexual reproduction by the insect-associated *Ophiocordyceps* likely allows for host insect survival, given that sexual reproduction and growth of hyphae would surely lead to host death. Fan et al.[21] suggest that this modified life history may be essential for the evolution of all eukaryotic symbionts more broadly.

In delphacid planthoppers, and cicadas, it has been demonstrated that the fungal symbionts support the insect host by providing sterols and amino acids, neither of which is available in the phloem diet.[6,9,10,41] Cholesterol is a precursor for the hormone ecdysone, which is essential for molting and metamorphosis in insects.[45] For the vast majority of insects within the Auchenorrhyncha (that have been reported), bacterial endosymbionts do not provide sterols. However, some planthoppers must apparently get these important cholesterol building blocks from their internal symbionts. Several early papers on two well-studied temperate delphacids provided evidence that the yeast-like fungal symbionts produce sterols, which the insect metabolizes into cholesterol.[9,46] By suppressing the fungal symbiont via heat treatment, they showed a reduction in total sterol content of the aposymbiotic insects, which would only survive and grow when supplied directly with dietary cholesterol or ecdysone. Many years later, by sequencing the genomes of both a planthopper and its fungal symbiont, Xue et al.[47] confirmed that the fungus possesses all of the genes necessary for sterol synthesis and that the partners complement each other in their metabolism of sterols.

Since plant sap is deficient in amino acids, planthoppers must also acquire these important nitrogen-containing compounds from their internal symbionts. Koyama et al.[48] demonstrated that withholding any of the 10 essential amino acids from the diet of *Nilaparvata lugens* nymphs failed to impact their development and growth, presumably because the internal fungi provide them instead. Like all insects, planthoppers synthesize uric acid as a nitrogenous waste product. Unlike other insects, however, uric acid is stored in their tissues, rather than excreted.[49] When rendered aposymbiotic via heat treatment, *N. lugens* experienced reduced uricase activity and a subsequent accumulation of uric acid, compared to symbiotic insects.[10] These studies suggest that uric acid is sequestered in fat body tissue, where it is metabolized by the uricase activity of the resident fungal symbionts, and eventually recycled into amino acids for the host. Xue et al.,[47] again, provided

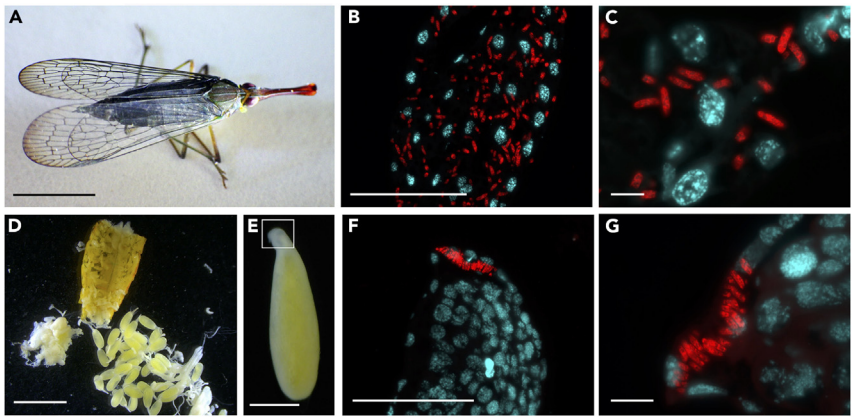

Figure 5. Visualization of internal fungal cells in Dictyopharidae adult and egg life stages

(A) Whole specimen of a dictyopharid planthopper, *Lappida* sp.

(B and C) Fluorescence *in situ* hybridization (FISH) of fungal cells within a multinucleated fat body cell, using an *Ophiocordyceps*-specific probe, shown in red (labeled with Cy5), counterstained with DAPI in cyan. Note slightly different morphology compared to the flatid fungi in Figure 4C.

(D) Ventral exoskeleton and viscera from a female dictyopharid abdomen, showing numerous mature eggs.

(E) Single egg. Box denotes the region in (F), at the posterior pole of the egg, near the lateral oviduct.

(F and G) FISH microscopy of fungal cells using the *Ophiocordyceps*-specific probe, shown in red, counterstained with DAPI in cyan. Fungal cells (~20 per egg) were aligned in a single layer along the egg surface, enclosed in a thin membrane. The DAPI channels were converted to cyan, for easier viewing, using the auto tone feature in Photoshop v21.1.0. Scale bars: (A), 10 mm. (B), 100 μm. (C), 10 μm. (D), 3 mm. (E), 500 μm. (F), 100 μm. (G), 10 μm.

genomic evidence for the presence of uricase genes in the fungal cells of *N. lugens*, as well as the metabolic capability of the fungus to synthesize all 10 essential amino acids missing from the insect genome. Recycling nitrogen in this fashion is likely beneficial for the survival of all sap-feeding insects, including the tropical planthoppers in this study.

Conclusion

Internal symbionts are critical to the enormous evolutionary success of insects, especially those utilizing imbalanced food sources, such as plant sap. The internal symbionts of the Auchenorrhyncha are typically bacteria (*Sulcia* and a secondary betaproteobacteria symbiont), which hold long-standing relationships with their host insects and provide nutritional support. However, in at least seven insect families within the infraorder Fulgoromorpha (two discovered in this study), fungal symbionts have replaced these bacterial symbionts and assumed the role of primary nutritional caregiver. The range of families examined in this study revealed the possible coevolution between insect hosts and their fungal symbionts, with clear differences between the infraorders Fulgoromorpha and Cicadomorpha and among families within the Fulgoromorpha. Some insect families hosting both bacteria and fungi appear to be in an intermediate stage in the evolution of this association. The current study effectively doubles the number of insect species known to host internal fungal symbionts (from 15to 30). These symbionts reside within fat body cells, an extremely dynamic tissue, and undergo vertical transmission from parent to offspring via the egg. Very few surveys have explored insect-fungal symbioses in tropical and subtropical regions. All of the newly documented species in the current study were collected from a tropical lowland rainforest in northern Costa Rica. Given the short time period of collection (~20 days in total) and the small geographic area of coverage of the study (<1600 ha), we presume that many more interesting examples of tropical insect-fungal relationships remain to be discovered. Thus, future studies should target an even wider range of families to better understand coevolution and symbiont replacement events in *Ophiocordyceps* symbioses, as well as the dynamics of eukaryote-eukaryote endosymbioses more broadly.

Limitations of the study

Metagenomic sequencing was not conducted. This approach could complement amplicon sequencing in the confirmation of absence of certain microbes in the insect tissues. Similarly, additionally microscopy could complement the amplicon data, but negative microscopy can be easily achieved in any tissue. Thus, amplicon sequencing of an entire specimen, including both fat bodies and bacteriomes, is expected to be more reliable to answer this question than microscopy or metagenomic sequencing. Fungal symbionts were localized to the fat bodies in all insects in this study; however, no microscopy was conducted on bacteriome tissue, largely due to lack of specimens. Thus, it is not known whether fungi also reside in bacteriome tissues broadly and for LS22-21, which harbors both bacterial and fungal symbionts, where *Sulcia* resides in this insect.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110674.

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AUTHOR CONTRIBUTIONS

R.S. and S.G. conceived of the study and designed experiments. R.S., S.G., and K.V. collected and dissected specimens and conducted sequencing work and analysis. R.S. and S.G. wrote the manuscript, and all authors made final comments on the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Panellus stipticus	Carolina Biological Supply	Cat# 156135
Sordaria fimicola	Carolina Biological Supply	Cat# 156290
Mahanarva costaricensis	This study	Cercopidae
Zulia vilior costaricensis	This study	Cercopidae
Prosapia simulans	This study	Cercopidae
Iphirhina limbata	This study	Cercopidae
Abana gigas	This study	Cicadellidae
Docalidia sp.	This study	Cicadellidae
Baleja rufofasciata	This study	Cicadellidae
Bolbonota sp.	This study	Membracidae
Poppea capricornis	This study	Membracidae
Darnis latior	This study	Membracidae
Adippe inaequalis	This study	Membracidae
Harmonides sp	This study	Membracidae
Oliarus sp.	This study	Cixiidae
Diareusa imitatrix	This study	Fulgoridae
Calyptoproctus sp.	This study	Fulgoridae
Sinuala sp.	This study	Fulgoridae
Phrictus quinqueparitus	This study	Fulgoridae
Omolicna sp.	This study	Derbidae
Derbe sp.	This study	Derbidae
Biolleyana costalis	This study	Nogodinidae
Biolleyana fenestra	This study	Nogodinidae
Biolleyana sp.	This study	Nogodinidae
Bladina sp.	This study	Nogodinidae
Thioniini sp.	This study	Issidae
Deocerus sp.	This study	Flatidae
Deocerus sp. 2	This study	Flatidae
Nephesini sp.	This study	Flatidae
Nephesini sp. 2	This study	Flatidae
Flatoidini sp.	This study	Flatidae
Flatinae sp.	This study	Flatidae
Flatini sp.	This study	Flatidae
Acanalonia sp.	This study	Acanaloniidae
Neurotmeta sp.	This study	Tropiduchidae
Lappida sp.	This study	Dictyopharidae
Lappida sp. 2	This study	Dictyopharidae
Hyalodictyon sp.	This study	Dictyopharidae
Deposited data		
BioProject	NCBI – GenBank	PRJNA1085985

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fungal sequence data	NCBI – GenBank	PP346467-PP346484
Chemicals, peptides, and recombinant proteins		
Polyethylene glycol distearate	Sigma	Cat# 305413
1-Hexadecanol	Sigma	Cat# 258741
Water, molecular grade	Sigma	Cat# W4502
Sodium Dodecyl Sulfate	Sigma	Cat# BP1311-200
cetyltrimethylammonium bromide	Sigma	Cat# H6269
polyvinylpyrrolidone	Sigma	Cat# PVP40
Ethylenediaminetetraacetic acid	Sigma	Cat# 03690
Chloroform:isoamyl alcohol	Sigma	Cat# C0549
Ethyl Alcohol	Pharmco	Cat# 111000200
Tris Hydrochloride	Fisher Scientific	Cat# BP1758
Sodium chloride	Fisher Scientific	Cat# S271
Phosphate buffered saline	Fisher Scientific	Cat# BP399-1
Formamide	Thermo Fisher Scientific	Cat# J67206
Glutaraldehyde	Ted Pella, Inc.	Cat# 18420
Paraformaldehyde	Ted Pella, Inc.	Cat# 15713
4′6′-diamidino- 2-phenylindole	Sigma	Cat# D8417
VectaShield Vibrance	Vector Laboratories, Inc.	Cat# H-1700
ThermoPolTaq Polymerase	New England Biolabs	Cat# M0267
Q5 Hot Start High-Fidelity Mix	New England Biolabs	Cat# M0494
lactophenol cotton blue	Carolina Biological Supply	Cat# 834445
Wright stain	Sigma	Cat# 45252
Critical commercial assays		
DNeasy Kit	Qiagen	Cat# 69504
Lysing Matrix E bead tubes	MP Biomedicals	Cat# 6914050
QuBit dsDNA BR kit	Invitrogen	Cat# Q32850
QuantIT PicoGreen dsDNA Assay	Thermo Fisher Scientific	Cat# P11496
MultiScreen Plate	Millipore	Cat# MSNU03010
Wizard SV Gel and PCR Clean-up System	Promega	Cat# A9281
Oligonucleotides		
515F; GTGYCAGCMGCCGCGGTAA	Caporaso et al.[50]	Bacterial 16S rRNA
806R; GGACTACHVGGGTWTCTAAT	Caporaso et al.[50]	Bacterial 16S rRNA
FR1; AICCATTCAATCGGTAIT	Vainio and Hantula[51]	Fungal 18S rRNA
FF390; CGATAACGAACGAGACCT	Vainio and Hantula[51]	Fungal 18S rRNA
Hyp760; CCTGCCTGGAGCACTCT	Matsuura et al.[6]	Fungal 18S rRNA
Y18F; CACAAGTTATCGTTTATTTGATAGCACCTTAC	Hemmati et al.[52]	Fungal 18S rRNA
Y18R; GGCTGCTGGCACCAGACTTGC	Hemmati et al.[52]	Fungal 18S rRNA
Nonsense: ACTCCTACGGGAGGCAGC	Wallner et al.[53]	Negative control – no target
Euk516; ACCAGACTTGCCCTCC	Amann et al.[54]	Eukaryote 18S rRNA

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
1269R: AAGAACGGCCATGCACCAC	López-García et al.[55]	Eukaryote 18S rRNA
Software		
R	R Core Team	www.r-project.org
Geneious Prime	N/A	www.geneious.com
CutAdapt v4.1	Martin[56]	cutadapt.readthedocs.io/en/v4.1/installation.html
FastQC v1.13	Babraham Bioinformatics group	www.bioinformatics.babraham.ac.uk/projects/fastqc
DADA2	Callahan et al.[57]	benjjneb.github.io/dada2
SILVA database v138.1	Quast et al.[58]	www.arb-silva.de/documentation/release-1381/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and commercial reagents should be directed to and will be fulfilled by the lead contact, Shana Goffredi (sgoffredi@oxy.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Sequencing data generated in this study have been deposited at the National Center for Biotechnology Information (NCBI) and are publicly available as of the date of publication under BioProject PRJNA1085985. Fungal 18S rRNA sequences generated in this study are available via GenBank accession numbers PP346467-PP346484.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Specimens were collected from La Selva Biological Research Station, Sarapiquí, Costa Rica (10°260 N, 83°590 W; Table S1; Figure S1) in May-June 2022 and 2023. A total of 42 specimens were collected, representing 13 families within the suborder Auchenorrhyncha (under permit number R-028-2022-OT- CONAGEBIO from the Costa Rican Ministerio De Ambiente Y Energía). Prior to dissection, all specimens were anesthetized for ~2-5 minutes at -20°C.[59] Samples for molecular work were dissected and preserved in 70% ethanol. Samples for microscopy were dissected in 1X phosphate buffered saline, preserved in 4% paraformaldehyde for 24-48 h, rinsed 3 times in phosphate buffered saline, and stored in 70% ethanol at -20°C.

METHOD DETAILS

DNA extraction

To account for difficulties in lysing fungal cell walls, four different DNA extraction methods that varied in bead type, buffers, and enzymes were tested on 50 mg of cultured fungi (*Panellus stipticus* and *Sordaria fimicola*, ordered from Carolina Biological Supply; Table S2). The protocol that consistently produced the highest DNA yields was the 2X cetyltrimethyl-ammonium bromide (CTAB) method, modified from.[60] Insect tissues were homogenized in Lysing Matrix E bead tubes (a mix of 0.1/1.4/4mm beads; MP Biomedicals, LLC, Irvine, CA), first dry for 5 minutes and then for 10 minutes with 1 ml of 2X CTAB buffer (2% CTAB, 1.4 M sodium chloride, 0.1 M Tris-HCl, 0.02 M EDTA, and 3% polyvinylpyrrolidone). After homogenization, bead tubes were centrifuged and the supernatant was incubated at 65°C for 1 hour. Following incubation, 500 µl of 24:1 chloroform:isoamyl alcohol mix was added, followed by centrifugation for 10 minutes at 13,000 g. The aqueous phase was recovered and mixed with 0.5 volumes 95% EtOH. DNA was then purified using the DNeasy Blood and Tissue Kit, following the manufacturer's instructions (Qiagen, Germantown MD). Total DNA was quantified via a Qubit 3.0 fluorometer using the dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Amplicon sequencing of both bacteria and fungi

For bacterial barcoding, the V4-V5 hypervariable region of the 16S rRNA gene was amplified using bacterial PCR primers (515F; 5′-GTGY CAGCMGCCGCGGTAA-3′ and 806R; 5′-GGACTACHVGGGTWTCTAAT-3′;[50]) with Illumina adapters on the 5′ end (San Diego, CA, United States). For fungal barcoding, a ~390-bp region of the 18S rRNA gene was amplified using fungal primers (FR1; 5′-AICCATTCAATCGGTAIT-3′ and FF390; 5′-CGATAACGAACGAGACCT-3′;[51,61]), with Illumina adapters on the 5′ end.

Each PCR product was secondarily barcoded with Illumina NexteraXT index v2 Primers that included unique 8-bp barcodes, with NEB Q5 Hot Start High-Fidelity Mix at an annealing temperature of 66°C for 11 cycles. Barcoded products were purified using Millipore-Sigma (St. Louis, MO, United States) MultiScreen Plate MSNU03010 with a vacuum manifold and quantified using the QuantIT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA) on a BioRad CFX96 Touch Real-Time PCR Detection System. Barcoded samples were combined in approximately equimolar amounts and purified again with Promega's Wizard SV Gel and PCR Clean-up System, and quantified again using the QuBit system. A single sample, usually 1500-3000 ng DNA total, was submitted to Laragen, Inc. (Culver City, CA, United States) for 2 × 250 bp paired-end analysis on the Illumina MiSeq platform with PhiX addition of 20%. The raw bacterial 16S rRNA barcode sequences and fungal 18S rRNA barcode sequences, along with metadata collected in this study, are available from the NCBI Small Read Archive (BioProject # PRJNA1085985).

Raw reads for both bacteria and fungi were processed according to.[62] Briefly, CutAdapt v4.1 was used to remove primer sequences, which allowed one error for every 10 bp in the primer sequence.[56] FastQC v1.13 was used to quality control the raw sequence data and identify trim cutoffs for both the forward and reverse reads, ahead of pairing. Raw sequences were then processed with DADA2 for initial quality trimming, error rate estimation, merging of read pairs, chimeric sequence removal, and community data matrix construction[57] and taxonomy was assigned to the processed amplicon sequence variants (ASVs at 100% identity) using the SILVA database v138.1[58] (Figure 1).

Direct sequencing of insect-associated fungi

A diagnostic screen for fungal presence was conducted on all 42 insect samples, using 18S rRNA primers specific for *Ophiocordyceps* symbionts in other insects (~300bp product using Y18F; 5′-CACAAGTTATCGTTTATTTGATAGCACCTTAC-3′ and Y18R; 5′-GGCTGCTGG-CAC CAGACTTGC-3′).[52] Thermal cycling conditions were as follows; 94°C: 5 min, followed by 30 cycles: 94°C, 64°C, and 72°C: 1 min each; and 5 min at 72°C. Note that most fungal primers also co-amplify host insect genes as well, and we found the Y18F/R pair to be the most reliable in terms of fungal amplification yields, out of 9 pairs attempted (Table S3). Extracts that showed a positive amplification product with the Y18F/R primer set, were then amplified with Y18F paired with the general eukaryote reverse primer 1269R (5′-AAGAACGGCCATGCACCAC-3′).[55] This amplification product was then sequenced directly using Sanger sequencing, via Laragen Inc., after which a phylogenetic analysis was conducted (Figure 2). 18S rRNA fungal sequences were submitted to GenBank under accession numbers PP346467-PP346484.

Microscopy

For microscopy, PFA-fixed samples were embedded in Steedman's wax (1 part cetyl alcohol: 9 parts polyethylene glycol (400) distearate, mixed at 60°C). An ethanol:wax gradient of 3:1, 2:1 and 1:1, and eventually 100% resin, was used to embed the samples (1h each treatment). Embedded samples were sectioned using a Leica RM2125 microtome and placed on Superfrost Plus slides. Sections were dewaxed in 100% ethanol rinses 3x.

A preliminary visualization of the fungal cells was performed on 5-μm sections with a fungi-specific lactophenol cotton blue stain. Following the protocol described by,[63] a drop of 70% EtOH and 1-2 drops of the stain were placed under a coverslip. Insect tissues were examined with transilluminated light using a Nikon E80i microscope with a Nikon DS-Qi1Mc high-sensitivity monochrome digital camera.

For fluorescence *in situ* hybridization (FISH) microscopy, we used a probe designed specifically to target the *Ophiocordyceps* (Hyp760; 5′-CCTGCCTGGAGCACTCT-3′),[6] labeled with the fluorochrome Cy5. Helper probes did not increase the fluorescent signal above that of the *Ophiocordyceps*-specific probe alone[8] (data not shown). A nonsense probe, also labeled with Cy5 (5′-ACTCCTACGGGAGGCAGC-3′),[53] was used as a negative control. FISH microscopy on a parasitized moth cadaver (Figure S3) was conducted with the general eukaryote probe Euk516 (3′-ACCAGACTTGCCCTCC-5′, labeled with Cy5).[54] All sections were incubated in hybridization buffer containing 50 nM probe at 46°C for 4–8 h, in a hybridization buffer (0.9 M NaCl, 0.02M Tris-HCl, 0.01% sodium dodecyl sulfate and 30% formamide) for 3 hours, followed by a 15 min wash at 48°C (0.1 M NaCl, 0.02 M Tris-HCl, 0.01% sodium dodecyl sulfate, 5 mM EDTA). Sections were counterstained with 4′6′-diamidino- 2-phenylindole (DAPI, 5 mg/ml) for 3 min, rinsed and mounted in VECTASHIELD® VibranceTM Antifade Mounting Medium (Vector Laboratories, Newark, CA). Sections were examined by epifluorescence microscopy using a Nikon E80i epifluorescence microscope with a Nikon DS-Qi1Mc high-sensitivity monochrome digital camera. As a reference, some 5-μm sections were also histologically examined via the Wright stain, using a 3-minute incubation, followed by thorough rinsing with distilled water.

Specimens for transmission electron microscopy were secondarily preserved in 3% glutaraldehyde buffered with 0.1 M phosphate and 0.3 M sucrose (pH 7.8). The fixed samples were dehydrated in a graded series of cold acetone, and embedded in epoxy resin (Oken Epok 812; Oken-shoji, Tokyo, Japan). Ultrathin sections (60-80 nm) were cut with a diamond knife, transferred to copper grids (50 mesh) that had been coated with Formvar membrane, stained with uranyl acetate and lead citrate, and observed with a Hitachi HT7700 TEM by Creative Bioarray (Shirley, NY).