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Mutualism with aggressive wood-degrading *Flavodon ambrosius* (Polyporales) facilitates niche expansion and communal social structure in *Ambrosiophilus* ambrosia beetles☆

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ABSTRACT

Most wood-boring insects compete with wood decaying basidiomycetes for woody biomass. One clade of ambrosia beetles gained access to rotten wood – an abundant resource unsuitable to most wood-boring insects – by evolving a farming-like mutualism with a white rot polypore. Here we show the mutualist of *Ambrosiodmus*/*Ambrosiophilus,* the polypore *Flavodon ambrosius,* is superior in lignocellulolytic capacity compared to Ascomycota ambrosia fungi and other white rot Basidiomycota. This mutualism facilitated the evolution of large, long-lived, communal colonies with overlapping generations and egg-laying by pre-dispersal progeny females. *F. ambrosius* resembles other white rot Polyporales in that it causes significant weight loss in wood decay assays and strong polyphenol oxidase reactions, indicative of lignin-modifying enzymes. The symbiosis is asymmetrical: there are many species of *Ambrosiodmus* and *Ambrosiophilus* but all use a single known species of *Flavodon*, which determines the ecological strategy of the entire insect clade.

1. Introduction

Wood is one of the most abundant natural organic compounds on Earth and a huge potential source of energy for heterotrophic organisms. However, very few animals have evolved the capacity to digest the lignocellulosic matrix, and thus lack access to a diverse number of polymeric carbohydrates. Many fungi do have the necessary enzymatic machinery, but lack the ability to access wood in living trees and often rely on wound sites to gain ingress into living trees (Pearce, 1996). Due to the complementary capabilities of animals and fungi, one of the most commonly evolved solutions for access to wood and utilization of plant substrates is mutualistic association (Mueller et al., 2005). In wood boring insects, association with fungal and bacterial mutualists is widespread. This includes associations with wood decay fungi, as with the fungus-growing termites *Macrotermes* and their mutualistic fungi, *Termitomyces* sp. (Hyodo et al., 2000). Although wood borers are capable of producing their own lignocellulolytic enzymes allowing for microbial-independent digestion of cellulose (Martin et al., 1991; Watanabe and Tokuda, 2010), association with fungi can provide superior or at least complementary catabolic capacity (Norris, 1980; Geib et al., 2008) and access to a larger volume of wood by taking advantage of fungal enzymes that selectively decompose the principal components of wood: cellulose, hemicellulose, and lignin. Access to the energy and nutrients sequestered in wood has, in part,

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facilitated the ecological and evolutionary success of wood borers, including adaptive species radiations and the development of an evolutionarily novel trait (i.e., mycangium). Access to recalcitrant compounds in wood has also led to the evolution of complex societies with hierarchical structure, and has helped establish wood borers as one of the foundational guilds and ecosystem engineers (Ellison et al., 2005) that exert strong positive effects on secondary colonizers (Calderón-Cortés et al., 2011). Insect-fungus symbioses specializing in using plant material, such as termites and leaf-cutter ants, support some of the largest colonies of animals on earth (Mueller and Gerardo, 2002).

One of the most well-known symbioses between wood boring insects and fungi are the fungus farming ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae). However, most ambrosia beetles are associated with fungi that are not capable of digesting lignocellulose and instead utilize less recalcitrant carbon compounds (Licht and Biedermann, 2012). These easily digestible carbon sources are typically available in a dead tree for only a short time after tree death. Consequently, the majority of ambrosia fungi and beetles are only able to colonize declining and freshly dead trees, and are not competitive in trees colonized by general wood-decay fungi. Furthermore, once a tree senesces and dies, the supplantation of mutualistic fungi by wood decaying basidiomycetes (Frankland, 1998) significantly limits the time the insects can remain in the wood. That in turn constrains the evolution of family and social dynamics among the insects: nearly all ambrosia beetle species are only able to develop a single generation on a given tree, and all new individuals must disperse. Very few ambrosia beetles display any more advanced levels of sociality (Kent and Simpson, 1992; Biedermann and Taborsky, 2011).

One exception among the subfamily Scolytinae is the recently discovered association between saproxylic *Ambrosiodmus* ambrosia beetles (Scolytinae: Xyleborini) and their mutualistic fungal symbiont *Flavodon ambrosius* (Basidiomycota: Polyporales) (Li et al., 2015; Simmons et al., 2016). This symbiosis between a wood borer and a wood-decay polypore led to several evolutionary and ecological advances: (1) *Ambrosiodmus* galleries and *Flavodon* persist and thrive even in wood so decayed that it is no longer usable by other ambrosia beetles and fungi; (2) a single species *F. ambrosius* appears to be conserved across different species of *Ambrosiodmus* on multiple continents; and (3) and the association has led to successful colonization of new regions by the symbiotic partners, as evidenced by the multiple Asian *Ambrosiodmus* now widespread across the U.S.A. (Atkinson, 2016). Field observations of the genus *Ambrosiophilus*, which is phylogenetically a sister genus to *Ambrosiodmus* (Hulcr and Cognato, 2009; Cognato et al., 2011), indicated that these beetles are widespread and may also carry a symbiotic fungus that is capable of decomposing lignocellulose. Similar to several non-native *Ambrosiodmus*, *Ambrosiophilus atratus* has spread rapidly throughout the Eastern U.S.A. since it was first detected in Maryland in 1983; it is now reportedly present in 29 states (Atkinson, 2016). In 2013, a second and previously undescribed *Ambrosiophilus* species, *Ambrosiophilus peregrinus,* a species presumed to be native to Asia, was reported from northern Georgia (Smith and Cognato, 2015). Similar to *Ambrosiodmus* (Faccoli et al., 2009; Li et al., 2015), the fungal mutualist of *Ambrosiophilus* appears to sustain large beetle colonies even in the presence of advanced decay and co-colonization by other wood decay fungi, long after tree death.

The novelty of *Ambrosiophilus* documented in this work is that the long-term availability of resources seems to have facilitated the largest colonies recorded from ambrosia beetles, overlapping generations, and a communal level of sociality (Kukuk and Schwarz, 1987), which is unique compared to behavior of all other known ambrosia beetles. Here we specifically address the identity of the *Ambrosiophilus* symbiont and its capacity to decompose lignocellulose compared to other ambrosial and non-ambrosial fungi. We also tested whether the *Ambrosiophilus* system satisfies the definition of a communal social system, by testing for the presence of overlap of generations, communal reproduction, and delayed dispersal by the new generation of females.

2. Methods

2.1. Sampling and isolation of fungi from host beetles and galleries

To isolate and identify the dominant fungal symbionts in *A. atratus*, individuals representing all life stages were carefully removed from an expansive multigenerational gallery found inside highly decayed wood of an *Ailanthus altissima* on the campus of West Virginia University in Morgantown, West Virginia, USA (39°38′ 01″N, 79° 57′ 02″W) (Fig. 1). A total of 67 mature adult females, 23 progeny females, 6 males, 15 larvae, 4 pupae, and 36 eggs were recovered from the active gallery. Many (possibly hundreds) additional individuals, representing all life stages were observed inside the wood but were not sampled. Sampling depth for the various life stages of *A. atratus* used in culture-dependent studies were as follows: four pupae, 15 larvae, three adult males, and 29 adult females. Sampling was carried out on WVU property and did not require permits.

Live extracted beetles and colonized gallery sections were transported to the laboratory in clean containers. Twenty nine females (a mix of parental and progeny females), three males, 15 larvae, and 4 pupae were surface-disinfected and used for serial dilution plating as previously described (Kasson et al., 2013). Adult males and females were separated into three body regions (head, pronotum, and abdomen) to resolve patterns of association between specific fungal morphotypes and body regions (Fraedrich et al., 2008; Kasson et al., 2013). The pupae and larvae were crushed whole. Colony forming units (CFUs) were tallied for each fungal morphotype and representative cultures were retained for long-term storage and DNA-based studies. Concurrent to serial dilution platings, six separate gallery scrapings of green cream–like fungal growth visible throughout the gallery system (Fig. 1A area of detail) were removed aseptically using a sterile teasing needle and added to 500 μl of sterile distilled water, vortexed, serially diluted, and plated on Glucose Yeast Extract Agar (GYE). Dominant morphotypes were quantified and purified by the methods described above. Following serial dilution plating, CFUs were tallied for each morphotype and representatives sub-cultured and retained for DNA studies. Additional subcultures were retained on Potato Dextrose Agar (PDA) slants for long-term storage in both the Kasson and Hulcr labs.

2.2. DNA extraction, amplification, and sequencing

Fungal DNA was extracted from dominant fungal morphotypes recovered from both beetles and the gallery substrate, as well as representative fungi used in wood decay assays (Table 1). Fungal isolates were transferred to Difco potato dextrose broth (PDB; BD and Co., Franklin Lakes, NJ, USA) and incubated for 7–10 d as previously described (Short et al., 2015). Following incubation, genomic DNA was extracted using a Wizard kit (Promega, Madison, WI, USA). DNA was suspended in 75 μl Tris-EDTA (TE) buffer preheated to 65 °C.

Sequences of the nuclear internal transcribed spacer region ITS1-5.8S-ITS2 (ITS) and nuclear 28S ribosomal DNA (rDNA) regions were amplified with the primer combinations ITS5/ITS4 (White et al., 1990) and LR0R/LR5 (Vilgalys and Hester, 1990) using Bio-Line PCR kits (Bioline USA Inc., Taunton, MA, USA) or ExTaq