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**Wood decay fungus *Flavodon ambrosius* (Basidiomycota: Polyporales) is widely farmed by two genera of ambrosia beetles**

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## Abstract

The ambrosia fungus *Flavodon ambrosius* is the primary nutritional mutualist of ambrosia beetles *Ambrosiodmus* and *Ambrosiophilus* in North America. *F. ambrosius* is the only known ambrosial basidiomycete, unique in its efficient lignocellulose degradation. *F. ambrosius* is associated with both native American beetle species and species introduced from Asia. It remains unknown whether *F. ambrosius* is strictly a North American fungus, or whether it is also associated with these ambrosia beetle genera on other continents. We isolated fungi from the mycangia and galleries of ambrosia beetles *Ambrosiodmus rubricollis*, *Ambrosiodmus minor*, *Ambrosiophilus atratus*, and *Ambrosiophilus subnepotulus* in China, South Korea, and Vietnam. Phylogenetic analyses suggest that all Asian and North American isolates represent a single haplotype. These results confirm *Flavodon ambrosius* as the exclusive mutualistic fungus of multiple *Ambrosiodmus* and *Ambrosiophilus* beetle species around the world, making it the most widespread known ambrosia fungus species, both geographically and in terms of the number of beetle species. The *Flavodon*-beetle symbiosis appears to employ an unusually strict mechanism for maintaining fidelity, compared to the symbioses of the related Xyleborini beetles, which mostly vector more dynamic fungal communities.

**Keywords:** ambrosia beetle; *Ambrosiodmus*; *Ambrosiophilus*; nuclear ITS rDNA; nuclear 28S rDNA; white rot

## 1 Introduction

Ambrosia fungi are obligate nutritional mutualists of wood-boring ambrosia beetles [Coleoptera: Curculionidae: Scolytinae and Platypodinae (Batra 1963, Hulcr and Stelinski 2017)]. The fungi are carried by the beetles into new tree hosts, where the beetles inoculate the newly-excavated tunnel systems with these fungi (Batra 1963, 1966; Six 2003). Of at least ten independent origins of the symbiosis within Fungi, all known ambrosia fungi lie within lineages of the phylum Ascomycota (Alamouti et al. 2009, Kolařík and Kirkendall 2010, Kasson et al. 2013, Dreaden et al. 2014, Mayers et al. 2015, Bateman et al. unpublished data). The only known exception is the *Flavodon ambrosius* D.R. Simmons, You Li, C.C. Bateman & Hulcr (Basidiomycota: Polyporales), which is not only the sole known basidiomycetous ambrosia fungus, but is also unique among other ambrosia fungi in its capacity to efficiently utilize cellulose and lignin (Kasson et al. 2016).

*F. ambrosius* is the primary mutualistic fungus of beetles in the genera *Ambrosiodmus* and *Ambrosiophilus* (Scolytinae: Xyleborini) (Li et al. 2015; Kasson et al. 2016; Simmons et al. 2016). These two beetle genera form a monophyletic clade (Storer et al., 2014), suggesting a single origin of *Flavodon* farming. Four beetle species have been recorded in association with *F. ambrosius*: *Ambrosiodmus lecontei*, *Ambrosiodmus minor*, *Ambrosiodmus rubricollis* (Li et al. 2015), and *Ambrosiophilus atratus* (Kasson et al. 2016). All of these species, except *Ambrosiodmus lecontei*, were recently introduced to North America from Asia (Bright 1968; Atkinson et al. 1990; Wood and Bright 1992) and are now established and common.

Many more species of *Ambrosiodmus* and *Ambrosiophilus* occur in Asia, Africa and South America, but their fungal symbionts have either not been studied (Wood 1982; Beaver and Liu 2010; Knížek 2011) or have been poorly identified (Yamashita 1966; Takagi 1967). Aside from *F. ambrosius*, the only described species of *Flavodon* are the saprophytic and free-living polypore species *F. flavus* (Klotzsch) Ryvarden (Ryvarden 1973), found in southern Asia, tropical Africa to South Africa, Australia, and Jamaica (Maas 1967; Ryvarden and Johansen 1980; Corner 1987; Miettinen et al. 2012), and “*F. cervinogilvus*” (Corner 1987), an invalid species first recorded from the eastern coast of the Island of Hawaii (Simmons et al. 2016). It is therefore unclear whether Asian

*Ambrosiodmus* and *Ambrosiophilus* 1) also farm *F. ambrosius*, 2) farm a different symbiotic species of *Flavodon*, or 3) farm a non-wood-decaying Ascomycete fungus species, as would be far more typical among ambrosia beetles.

The goal of this work was to test whether Asian *Ambrosiophilus* and *Ambrosiodmus* ambrosia beetle are associated with *F. ambrosius* in their native regions. The possibility of *F. ambrosius* playing the same symbiotic role around the world may indicate a scenario of unusual dominance of a single ambrosia fungus over many beetle species separated by continental-scale barriers and about 20 Ma (Jordal and Cognato 2012). Such strict associations between the *Ambrosiodmus/Ambrosiophilus* clade and their fungi would be in contrast to the related lineages of ambrosia beetles, namely *Xyleborus* and *Euwallacea*, most of which carry diverse and often loose symbiotic fungi in both native and non-native regions (Harrington et al. 2010, Hulcr and Cognato 2010; Kostovcik et al. 2015; O'Donnell et al. 2015; Ploetz et al. 2017). The results expand our understanding of the symbiotic relationship between basidiomycetous wood-decaying ambrosia fungi and their beetle vectors.

## 2 Materials and methods

Two *Ambrosiodmus* and two *Ambrosiophilus* species from Asia were sampled in four locations throughout China, South Korea, and Vietnam (October 2015 through May 2016; Table 1). All beetles were excised directly from active galleries in wood. In addition to gallery excision, *Ambrosiophilus atratus* was also caught in ethanol-baited traps in South Korea. To ensure finding the actual primary symbiont of each beetle species, we focused on fungi isolated strictly from the oral mycangia (excised by cutting off the frontal part of the head) and from active galleries of *Ambrosiodmus* and *Ambrosiophilus* beetles. Cultures obtained from beetle hosts were isolated by dilution-plating of mycangial contents and gallery wood chips on PDA medium, as described by Li et al. (2015). Colony forming units (CFU) of fungal isolation were recorded. Pure isolates were imported into, and studied in, a quarantine facility in Gainesville, FL, USA, under the USDA/APHIS permit No. P526P-16-02872. Beetle and fungus voucher specimens are preserved and stored at the Forest Entomology Laboratory, School of Forest Resources and Conservation, University of Florida, Gainesville, FL, USA.

DNA was extracted from pure subcultures. Sequences of the nuclear internal transcribed spacers ITS1-5.8S-ITS2 (ITS) and nuclear 28S ribosomal DNA (rDNA) regions were amplified. The primer pairs for PCR amplification were ITS1/ITS4 (White et al. 1990) and LROR/LR5 (Vilgalys and Hester 1990). PCR amplification of ITS and 28S followed protocols of Li et al. (2015). Sequencing of the PCR products in forward and reverse directions, and editing and assembling of the nucleotide sequences were accomplished following Simmons et al. (2016).

To place Asian *Flavodon* isolates within the known phylogenetic context, we selected 28S rDNA sequences from Simmons et al. (2016) and ITS rDNA sequences from Li et al. (2015), Kasson et al. (2016) and Miettinen et al. (2012), which included *F. ambrosius* and related taxa. We aligned these sequences with those from our cultured *Ambrosiodmus* and *Ambrosiophilus*-associated fungi using default settings in ClustalX 2.0 ([www.clustal.org](http://www.clustal.org); Hall 2013). The alignments are deposited in TreeBASE (Reviewer access URL: <http://purl.org/phylo/treebase/phyloids/study/TB2:S20605?x-access-code=a5b8cb957aff2c80c1985d844175f6ee&format=html>). Sequence evolution model for the Maximum likelihood (ML) analyses was selected using Mega 7 (Hall 2013) setting the initial tree for ML calculations to NJ/BioNJ. Tamura 3+G was selected for the ITS dataset and Kimura 2+G model was selected for the 28S dataset. ML analyses were also conducted with Mega 7, setting the number of fast bootstrap (BS) replicates to 1000.

### 3 Results and discussion

The unique ambrosia fungus *Flavodon ambrosius* was so far studied exclusively in the U.S. (Li et al. 2015; Kasson et al. 2016) but the majority of its vectors in the beetle genera *Ambrosiodmus* and *Ambrosiophilus* are not native in North America, and their greatest diversity occurs in Asia (Wood 1982; Knížek 2011). Our goal was to study whether these beetles also employ *F. ambrosius* as their primary symbiotic fungus in Asia where they are indigenous.

### 3.1 Identification and phylogenetic analyses of symbiotic fungus *Flavodon ambrosius*

The ML phylogenetic analyses of both the ITS and 28S markers recovered nearly identical topology for the *Flavodon* clade. The topology corroborated the placement of *F. ambrosius* in the genus *Flavodon* together with *F. flavus* in the phlebioid clade in the Polyporales (Li et al., 2015). All Asian *Flavodon* sequences were placed within the homogeneous *Flavodon ambrosius* clade, which consisted of sequences from the *F. ambrosius* holotype and other isolates from Florida and West Virginia, from both native and non-native North American beetle species. The *F. ambrosius* sequences did not show any divergence or lineages specific to different beetle vectors or locations.

The monophyly of all *F. ambrosius* isolates studied here strongly supports the hypothesis that beetles in the *Ambrosiodmus/Ambrosiophilus* clade share the same symbiotic fungus in Asia and North America. In total, five ambrosia beetle species in *Ambrosiophilus* and *Ambrosiodmus* are now confirmed to employ *F. ambrosius* as their primary symbiotic fungus (Li et al. 2015; Kasson et al. 2016; this study). The symbiosis between *F. ambrosius* and all its vectors has been maintained in both deep evolutionary time during the natural spread of ancestral *Ambrosiodmus* around the world, as well as during recent human-assisted spread and introductions.

The monophyly of *F. ambrosius* in two markers commonly used in fungal systematics indicates that all the isolates are members of the same phylogenetic species. The ITS region is typically variable enough to detect sub-species divergence in fungi of Basidiomycota (Lindner and Banik 2011; Schoch et al. 2012), but it did not recover any divergence within *F. ambrosius*. If multiple lineages exist within *F. ambrosius*, they are either unrecoverable using the two markers selected here, or additional *Flavodon* diversity occurs in geographic regions not sampled for this work, such as in South America.

All *Flavodon* strains isolated in this work were morphologically uniform. *F. ambrosius* isolated from *Ambrosiophilus atratus* in West Virginia displayed two types of colony morphology, but both were genetically identical (Kasson et al. 2016). An ambrosia fungus that was likely *Flavodon* was previously recorded from *Ambrosiodmus*

*rubricollis* from Japan (Yamashita 1966; Tagaki 1967) and described as “white wooly mycelia” without further identification. Isolates of this fungus were not available to us, but the brief description fits the morphology of *F. ambrosius*.

Most ambrosia beetles maintain their symbiont associations through time and through introductions into non-native regions. Mayers et al. (2015) suggests that in some groups, each beetle species is associated with a unique fungus species, and each fungus is typically only found associated with one beetle species. In other beetle clades, co-phylogenetic analysis has suggested that symbiotic partners may be frequently swapped over evolutionary time (O’Donnell et al. 2015). The association between *Ambrosiodmus/Ambrosiophilus* and *F. ambrosius* appears to represent an entirely different scenario. Our results suggest an association of a single fungus and many beetle species, a dominance of a single fungus that has been maintained throughout evolutionary history as the beetles have radiated into many species and spread around the world. Why has *F. ambrosius* not also radiated into diverse lineages of fungal symbionts that are each specialized for its own vector beetle, as has been observed in other closely related ambrosia beetle clades? Could the unique ecological traits of this sole known white-rotting basidiomycete ambrosia fungus explain these unique relationships? These questions offer avenues for future inquiry and remain at the forefront of symbiosis and co-evolutionary research.

### 3.2 Biology of fungus *Flavodon ambrosius*

The incidence and habits of *F. ambrosius* in Asia were consistent with those observed in North America. All branches containing *F. ambrosius* and their beetle vectors were notably decayed (Fig. 2A). In many cases, both *Ambrosiodmus* and *Ambrosiophilus* tunnel entrances were closely clustered, rather than being randomly distributed on the tree. For example, the *F. ambrosius* samples from China were collected from multiple galleries of *Ambrosiodmus rubricollis* and *Ambrosiophilus subnepotulus* found immediately adjacent to one another on the same branch. Galleries of both beetle species were covered by white filamentous fungal growth (Fig. 2D). These observations support the hypothesis that some *Ambrosiophilus* species are mycocleptic, colonizing the vicinity

of galleries established by other ambrosia beetles carrying the same fungus (Fig. 2B, C; Hulcr and Cognato 2010). The one species of *Ambrosiophilus* that has been well-studied (*A. atratus*) has oral mycangia with the capacity to transmit and preserve fungal inoculum, and frequently creates its own galleries (Kasson et al. 2016). However, the *Ambrosiophilus* species which are more often reported as mycocleptic have not been studied for their mycangia, and are typically found associated with ambrosia beetles from the genus *Beaverium* (Hulcr and Cognato 2010). This is the first report of multiple mycocleptic association between *Ambrosiophilus* and *Ambrosiodmus*.

The evolutionary transition of *Flavodon* from a free-living species to the ambrosial phenotype seen in *F. ambrosius* deserves future study. As in the USA, the Asian *F. ambrosius* is also known only from ambrosia beetles, and no fruiting body has been reported (Li et al. 2015; Kasson et al. 2016; Simmons et al. 2016). The closely related free-living species *F. flavus* is common in tropical regions of Asia, and frequently produces sexual fruiting bodies (Dai 2012). Our data do not allow to ascertain whether *F. ambrosius* participates in sexual reproduction, or whether the fungus is horizontally acquired by the beetles from free-living populations, but neither has been reported.

#### 4 Conclusions

This study suggests that a single species of the white-rot basidiomycete ambrosia fungus *F. ambrosius* is the primary associate of all *Ambrosiodmus* and *Ambrosiophilus* ambrosia beetles in the northern hemisphere. Many unexplored species of *Ambrosiodmus* occur throughout Africa and South America, but they were not available during this project. In addition, the genus *Beaverium*, widespread in Asia and Oceania, is also likely associated with *Flavodon*, as suggested by the frequent co-colonization by *Ambrosiophilus*. If all these beetles indeed carry *F. ambrosius* as their symbiont, it would make this fungus one of the most widespread fungal symbionts of animals.

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## Table and Figure legends

Table 1: The primary symbiont isolated from Asian Ambrosiodmus and Ambrosiophilus beetles and the NCBI/GenBank accession numbers of their ITS and 28S rDNA sequences. CFU: the average count of fungus cells in the mycangium calculated as the average count of colony-forming units on culture plates multiplied by the dilution factor.

Fig. 1: The best Maximum likelihood tree of *Flavodon* inferred from ITS and 28S rDNA datasets with source beetle genera and GenBank accession numbers. Values above nodes represent ML bootstrap percentages >50 % from a summary of 1000 replicates. The beetle vector genera are indicated by squares and circles.

Fig. 2: *Flavodon ambrosius* and ambrosia beetles collected from China. A: White-rot wood with galleries of ambrosia beetles. B: Gallery of *Ambrosiophilus subnepotulus* with *F. ambrosius*. C: Boring holes of *Ambrosiophilus* sp. (middle) and *Ambrosiodmus rubricollis* (right and left) on the bark. D: Galleries of *Ambrosiophilus subnepotulus* (right) and *Ambrosiodmus rubricollis* (left) on the same branch; Red arrows: *F. ambrosius*; Bars: 1 mm.

Table 1: The primary symbiont isolated from Asian *Ambrosiodmus* and *Ambrosiophilus* beetles and the NCBI/GenBank accession numbers of their ITS and 28S rDNA sequences. CFU: the average count of fungus cells in the mycangium calculated as the average count of colony-forming units on culture plates multiplied by the dilution factor.

| Taxon                     | Voucher no. | Locality                | Beetle vector                      | CFU   | Isolation body part | GenBank accession no. |          |
|---------------------------|-------------|-------------------------|------------------------------------|-------|---------------------|-----------------------|----------|
|                           |             |                         |                                    |       |                     | ITS                   | 28S      |
| <i>Flavodon ambrosius</i> | LL51        | Huaxi, Guizhou, China   | <i>Ambrosiodmus rubricollis</i>    | 16000 | oral mycangia       | LC216225              | LC215903 |
| <i>F. ambrosius</i>       | LL53        | Huaxi, Guizhou, China   | <i>Ambrosiodmus rubricollis</i>    | N/A   | gallery             | LC216226              | LC215904 |
| <i>F. ambrosius</i>       | LL70        | Nanming, Guizhou, China | <i>Ambrosiodmus rubricollis</i>    | 500   | oral mycangia       | LC216227              | LC215905 |
| <i>F. ambrosius</i>       | LL71        | Nanming, Guizhou, China | <i>Ambrosiophilus subnepotulus</i> | 1500  | oral mycangia       | LC216228              | LC215906 |
| <i>F. ambrosius</i>       | V12236      | Tam Đảo, Vietnam        | <i>Ambrosiodmus minor</i>          | 1500  | oral mycangia       | LC216229              | LC215907 |
| <i>F. ambrosius</i>       | V12544      | Gwangyang, South Korea  | <i>Ambrosiophilus atratus</i>      | 1100  | oral mycangia       | LC216230              | LC215908 |
| <i>F. ambrosius</i>       | V12546      | Gwangyang, South Korea  | <i>Ambrosiophilus atratus</i>      | 1000  | oral mycangia       | LC216231              | LC215909 |



