Ambrosia beetle *Premnobius cavipennis* (Scolytinae: Ipini) carries highly divergent ascomycotan ambrosia fungus, *Afroraffaelea ambrosiae* gen. nov. sp. nov. (Ophiostomatales)

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Abstract

Ambrosia beetles and fungi represent an interesting and economically important symbiosis, but the vast majority of ambrosia fungi remain unexplored, hindering research, management of pathogens, and mitigation of invasive species. Beetles in the subtribe Premnobiini are one example of an entire beetle lineage whose fungal symbionts have never been studied. Here, we identify one dominant fungal symbiont of *Premnobius cavipennis* by using fungus culturing, community sequencing, microtome sectioning and micro-CT scanning of mycangia.

Phylogenetic analyses of combined 18S and 28S rDNA and β -tubulin sequences revealed a highly divergent fungal lineage within Ophiostomatales, *Afroraffaelea ambrosiae* gen. nov. et sp. nov. The newly described fungal lineage represents another origin of the symbiosis within the Kingdom Fungi, adding to our understanding of the geographic ancestry of ambrosia fungi.

Premnobius cavipennis possesses pharyngeal mycangia which appear restrictive in fungus selection. This ambrosia beetle-fungus association has remained stable even after invasions into non-native regions.

Key words: *Premnobius*; Ophiostomatales; pharyngeal mycangium; Illumina MiSeq, *Raffaelea*, fungus-farming, symbiosis

Introduction

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) are diverse, with over 3,200 species and at least 13 independently evolved origins (Farrell et al. 2001, Jordal and Cognato 2012, Kirkendall et al. 2015). Their fungal mutualists, which are cultivated for food inside trees, are poorly understood by comparison. At least seven independent ambrosial origins in fungi are known (Endoh et al. 2011, Kasson et al. 2013, Dreaden et al. 2014, Li et al. 2015, Mayers et al. 2015), but less than 1% of ambrosia beetle symbionts have been resolved using DNA typing. Most of the ambrosia fungi that are known have only been recorded from non-indigenous (exotic) pest beetle populations while the majority of native and economically insignificant beetles remain unstudied. The paucity of information on symbiont identity and ecology has been a hurdle for researchers trying to predict habits of invasive ambrosia communities (Carrillo et al. 2014), test coevolutionary theories (Mueller and Gerardo 2002), or generate insights for improving human agriculture (Mueller et al. 2005).

In the past century, international trade has brought more than 60 non-native scolytine species to the United States, with exotic wood-boring beetles being one of the most common and increasingly-intercepted insects at U.S. ports of travel (Haack et al. 2014, Smith and Cognato 2015). The arrival frequency of such imports, and the potential introduction of a virulent tree pathogen, make research on exotic ambrosia fungi urgent. In some cases, prior data on the identity and ecology of ambrosia fungi could prevent ecological and economic damage. Around 2002, the redbay ambrosia beetle *Xyleborus glabratus* was introduced to the U.S., and its pathogenic fungal symbiont *Raffaelea lauricola* began causing mass decline of redbay trees (*Persea borbonia*) and other lauraceous plants (Fraedrich et al. 2008). Because the fungus was

new to science and its interaction with host trees was unknown at the time of its discovery, no preventative action was triggered. In other cases, illumination of symbiont identity and ecological habits can inspire future research. The newly discovered basidiomycete *Flavodon ambrosius* transported by *Ambrosiodmus* and *Ambrosiophilus* ambrosia beetles allows the beetle to reach much larger colonies and persist in wood much longer compared to other ambrosia beetles carrying ascomycetes (Li et al. 2015, Kasson et al. 2016, Simmons et al. 2016b). Although this symbiosis does not warrant phytosanitary concern, it does have a significant potential for research. In all cases, symbiont identification is the first step to making predictions about the behavior, function and potential impact of the symbiosis.

The subtribe Premnobiini is one example of an entire ambrosia beetle clade whose fungal symbionts have never been identified. Aside from 26 beetle species descriptions and some notes on gallery construction and inbreeding, very little is known about Premnobiini beetles (Browne 1961, Wood 1982, Cognato 2013). The two constituent genera *Premnobius* and *Premnophilus* have evolved and diversified in Africa. The most well-known species, *Premnobius cavipennis*, is now globally distributed in tropical biomes (Wood 1982, Bright and Skidmore 1997). The African distribution of the majority of Premnobiini suggests that *P. cavipennis* attained its pantropical distribution by human assistance. Records indicate *P. cavipennis* has been present in the neotropics since at least 1950 (Browne 1961). There may have been multiple introductions since the morphologically uniform species has multiple distinct genotypes (Cognato 2013). It is also possible that *P. cavipennis* comprise several cryptic species as in the ambrosia beetle *Euwallacea fornicatus* (O'Donnell et al. 2015), and that a pantropical distribution was attained through ancient dispersals, similar to some *Xyleborus* ambrosia beetles (Gohli et al. 2016).

Sampling of *P. cavipennis* fungal symbionts could help uncover the beetle's biogeographic history, and help explain its success at colonizing several continents.

The phylogenetic placement of *Premnobius* within the tribe Ipini also makes it a useful system for examining the transition from phloem feeding to obligate symbiosis. Premnobiini ambrosia beetles are polyphagous in terms of host trees (Wood 1982), but may carry highly specific fungal symbionts like most other ambrosia beetles. In contrast, most species from the parental clade Ipini are bark beetles, highly specific to their host trees (Wood 1982), with little or no known relationship with fungi, and the fungi isolated from them are usually non-specific commensals (Yamaoka et al. 1997, Kopper et al. 2004). This indicates that Premnobiini are an independent origin of the ambrosia symbiosis within a bark beetle clade (Alamouti et al. 2007, Alamouti et al. 2009, Lieutier et al. 2016). A comparative analysis of Premnobiini and its sister taxa, and a contrasting analysis of similarly evolved ambrosia clades, could illuminate how these specific associations evolve. *Premnobius* is also a useful lineage to investigate symbiont maintenance because beetles in this genus possess a pharyngeal mycangium, which appears to be similar to the pre-oral mycangia found in the more distantly related Xyleborini ambrosia beetles (Shedl 1962, Francke-Grosmann 1967). Whether or not the two mycangial types are homologous is not known. If the two mycangia represent a case of morphological convergence, comparisons of their function and structure may provide insights into the mechanisms of control over symbiont communities.

In this study, we sought to identify the fungal symbionts within the mycangium of *P. cavipennis* using fungal culturing, community DNA metabarcoding, and histology. To strengthen evidence for the new association, we also updated the description of the pharyngeal mycangium

where the fungi are stored, which has only been documented by a single sketch from more than 50 years ago (Shedl 1962).

Materials and methods

Quantitative culturing of fungi from beetle mycangia

Seven adult female *P. cavipennis* specimens used for fungal isolation and culturing were collected from an ethanol-baited light trap at the Montgomery Botanical Center in Coral Gables, FL on August 12th, 2015. The specimens were stored alive at room temperature with water-moistened sterile paper towels for 2 d after collection.

Immediately preceding fungal isolation from mycangia, live beetles were washed by vortexing for 10 sec in a sterile solution of 1mL water and one drop of Tween 80. A second wash was performed using a solution of only sterile water. To compare fungal communities in different body parts, washed and dried beetles were held with forceps under a dissecting microscope while the head, thorax, and abdomen was separated using a sterile scalpel as previously described (Fraedrich et al. 2008, Kasson et al. 2013). The three respective body segments were each transferred into 2mL microcentrifuge tubes containing 1mL of PBS (phosphate buffer solution), and crushed using sterilized micropestels. Each tube containing a macerated body segment was then serially diluted and plated at concentrations of 1/10, 1/100, and 1/1000 on PDA (potato dextrose agar) media. Fungi were allowed to grow at 25°C for 5-7 d before sub-culturing. Representative subcultures of the dominant morphotype recovered from heads were cryopreserved on PDA slants submerged in 15% glycerol stored in the -80°C HULCR lab collection at the University of Florida (UF) and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extraction, amplification, library preparation, and sequencing

Four fungal isolates recovered from heads of Florida-collected *P. cavipennis* were included in DNA studies. Extraction of genomic DNA from fungal cultures was performed by scraping approximately 5-10 µg of mycelium from pure cultures and adding it to 20 µL extraction solution from the Extract-N-Amplify Plant PCR kit (Sigma-Aldrich). Samples were then incubated at 96°C for 15 min. Following incubation, 20 µL of 3% BSA (bovine serum albumin) solution was added, vortexed, and spun down. The upper 20 µL of supernatant was used as the PCR template.

PCR amplification for Sanger sequencing was performed on portions of the 18S small subunit and 28S large subunit ribosomal DNA (rDNA) loci using the primer pair NS1/NS4 (White et al. 1990) and LR0R/LR5 (Vilgalys and Hester 1990) and on a portion of the β -tubulin (β T) coding gene using Bt2a/Bt2b (O'Donnell and Cigelnik 1997). Final PCR volumes of 25 µL consisted of 1.25U *Taq* Polymerase (New England Biolabs), 200 µM each dNTP, 10 pmol of each primer, and 5% DMSO (V/V). Amplified products were cleaned using the ExoSAP-IT (Affymetrix Inc) kit according to manufacturer's instructions. Sequencing was performed on ABI PRISM 377XL or 3130XL by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (UF).

In addition to Sanger sequencing of live cultures, a complementary approach of community metabarcoding of preserved mycangia was utilized. For DNA extraction from mycangial communities, one cryopreserved *P. cavipennis* specimen collected during 2014 in Java, and two specimens collected during 2013 in Honduras, were used. The beetle heads were first aseptically removed and crushed using a micropestle. DNA from each macerated head was extracted using the UltraClean Microbial DNA (MoBio) kit following the manufacturer's

protocol. The MiSeq library was constructed via two PCR steps (O'Donnell et al 2016). The first-step 28S marker PCR used the template primers LR0R and JH-LSU-369rc (Li et al. 2015), and the following cycling conditions: 95°C-6 min, (94°C-30 s, 52°C-45 s, 72°C-1:30 min) 25x, 72°C-5 min. This PCR was repeated using 5 uL PCR amplicon in place of DNA template. A second step PCR using 1 µL of this library was used in a final PCR for eight cycles to anneal Illumina sequencing adapters following the Illumina metabarcoding protocol. DNA concentrations from each library were measured using PicoGreen fluorescent dye from the Quant-iT kit on a Realplex cycler (Eppendorf), and samples were made equimolar at a concentration of 23 ng/µL prior to pooling. Pooled libraries were cleaned using the AMPure XP kit. A 15% PhiX library was added. Paired-end fungal community sequencing was performed at 300 cycles/read in an Illumina Miseq at the ICBR) at UF. Metabarcoding sequence data was analyzed in QIIME 1.8.0 (Caporaso et al. 2010), and raw sequence data were deposited in NCBI Sequence Read Archive under accession number **SRP072278**.

Phylogenetic analyses

Sequences of 18S rDNA, 28S rDNA, and β T with introns 3/4/5 removed (Supplementary Table 1), were aligned in Geneious (Geneious version 9.1.5). The alignment was divided into five partitions for phylogenetic consideration: one partition for each rDNA locus and for each of the three codon positions in the protein encoding β T. The Akaike information criterion in jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) was used to select the nucleotide substitution model for each partition, and analyses continued with a concatenated data matrix. Maximum likelihood (ML) phylogenetic analyses were conducted in GARLI 2.01 (Zwickl 2006) with the recommended partition parameters to determine the best tree topology and bootstrap support values from 500 search replicates, which were summarized in SumTrees (Sukumaran & Holder

2010). Bayesian posterior probabilities (BPP) were estimated with the same partition parameters in an analysis conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), in which two runs of four chains each were executed simultaneously for 5,000,000 generations, with sampling every 500 generations. SumTrees was used to compute BPP from a summary of 7501 trees retained after a burn-in of the first 2500 trees collected. Bootstrap support was assessed in Garli on the University of Florida HiPerGator 2.0.

Mycangium presence and content

Adult female *P. cavipennis* specimens first underwent exploratory dissections to investigate the presence of a mycangium or fungal masses suggestive of propagule sequestration. Specimens were collected from ethanol baited traps in Honduras and stored in the Hulcr lab cryopreserved collection until their use. Only heads were examined based on a previous report of the cephalic mycangia (Shedl 1962), and based on a single, abundant morphotype recovered from all beetle heads used in fungal culturing. Fungal masses found inside the beetle's head were transferred to a microscope slide and observed under oil emersion in a compound microscope to document the presence and morphology of fungal cells.

Five additional female *P. cavipennis* specimens from the same collection were assessed for the presence of mycangia using a microtome. Heads were prepared as previously described by Li et al. (2015) and Kasson et al. (2016). Heads were orientated with the most anterior end facing downward to permit transverse sectioning and simultaneous bilateral visualization of the mycangia. 10 µm transverse sections were cut using a Microm HM 325 rotary microtome (Walldorf, Germany). Selected slides confirmed by immediate viewing were dried at 60°C for 24 h, double-stained with Harris-hematoxylin and eosin-phloxine by hand, and examined and photographed using a Nikon Eclipse E600 compound microscope (Nikon Instruments, Melville,

New York) equipped with a Nikon Digital Sight DS-Ri1 high-resolution microscope camera and Nikon NIS-Elements BR 3.2 imaging software.

For a non-destructive 3-D visualization of the mycangia, an adult female was scanned with a Phoenix v|tome|x M (GE's Measurement & Control business, Boston, USA) at the University of Florida's Nanoscale Research Facility. A 180kv x-ray tube with a diamond-tungsten target was used with the following settings: 50 kv, 200 mA, a two second detector time, averaging of 3 images per rotation and a resulting voxel resolution of 1.89 μ m. Raw x-ray data were processed using GE's proprietary datos|x software v 2.3 to produce a series of tomogram images. These Micro-CT image stacks were then viewed, sectioned, measured and analyzed using VG StudioMax 3.0 (Volume Graphics, Heidelberg, Germany). Final figures were prepared with Photoshop and Illustrator (CS5, Adobe).

Fungal morphology on agar medium

To determine optimal growth temperature, four subcultures of the dominant fungal morphotype of *P. cavipennis* were grown concurrently on PDA at 15, 20, 25, 30, and 35°C. In attempts to induce sporulation, additional culturing was performed at 25°C on various nutrient agars used in the cultivation of ascomycotan fungi including: malt extract agar (MEA), glucose yeast extract agar (GYEA), carnation leaf agar (CLA), *Ophiostoma* selective agar (OSA, Harrington 1981) with cycloheximide, Sabouraud dextrose agar (SDA), PDA with autoclaved scolytine beetle fragments, PDA with a moist twig fragments, PDA amended with sawdust, and near-ultraviolet light exposure. Three subcultures of isolate Hulcr 9747 were grown on these media for 7-14 d to induce sporulation. Mating tests were performed using four strains (six pairs) on 2% MEA.

Results

Culturing of fungi from beetle mycangia

All seven *P. cavipennis* macerated heads yielded a single fungal morphotype on PDA with an average 2,200 CFUs (colony forming units) with a range from 1,400-3,000 CFUs. The morphology of hyphae, conidiophores and conidia resembled Ophiostomatales but did not conform to any genus known to us. Colony macromorphology on PDA at 25°C was white, velvety, 60-70 mm after 10 d, turning brown to hazel after 10-14 d (Fig. 2). Optimal growth of pure cultures of the dominant morphotype on PDA occurred at 25°C. Despite significant efforts to induce sporulation, no reproductive structures were observed. No fungal growth was recorded at 15°C, 35°C, nor on media amended with cycloheximide.

Sequencing and phylogenetic analysis

Four isolates cultured from *P. cavipennis* in Florida and one dominant fungus amplified from *P. cavipennis* mycangia from Java yielded 28S sequences. The 28S sequences had very high G/C content and many repeating regions. All 28S sequences generated from each isolate were different, but >99% similar to each other. A GenBank BLAST query of 28S sequences from isolate Hulcr 9747 yielded a top match with *Raffaelea* sp. PL1001 (accession **KJ909293**) at 83% similarity and the closest match of the respective 18S sequence was *R. subfusca* C2335 (accession **KJ909306**) at 97% similarity. Phylogenetic analysis of the combined 18S, 28S, and β T sequences revealed the *P. cavipennis* mycosymbionts represent a monophyletic lineage within the Ophiostomatales (Fig. 1).

Fungal community amplicon analysis

The community metabarcoding approach yielded 1,583,464 total reads. After quality filtering, 527,370 reads remained and were included in analysis. When sequence similarity of 99% was set, 289 OTUs were produced. The most prevalent OTU comprised 45.5% of reads, and was identified as the same dominant *P. cavipennis* mycosymbiont morphotype recovered from culturing, based on phylogenetic analysis (Java MiSeq LSU, Fig 1). A representative sequence was deposited in GenBank (accession [SRP072278](#)). BLAST queries of the representative in GenBank resulted in two accessions with 91% similarity and 100% query coverage:

Ophiocordyceps gracilis (accession [HM119587](#)) and *Raffaelea arxii* (accession [EU984298](#)). The next dominant OTU comprised 15.2% of reads and was identified by a BLAST query as close to an uncultured fungal endophyte in the Pezizomycotina (accession [KP335416.1](#)). The next dominant OTU formed 3.2% of reads most similar to a *Phialemonium* sp. (accession [HM060271](#)); remaining OTUs comprised less than 3% read abundance and were composed of other fungi and yeasts.

Examination of mycangia

Paired fungal sacs were first observed during exploratory dissections within *P. cavipennis* heads. Each fungal mass was nestled within tissues just posterior to the emarginate notch of the beetle's eyes, adjacent to the pharynx. These paired pharyngeal mycangia were then confirmed in microtome cross-section images (Fig. 3) and micro-CT scans (Fig 4). Serial transverse sections from the heads of all five female *P. cavipennis* heads confirmed the presence of the paired pharyngeal mycangia.

Taxonomy

Afroraffaelea C Bateman, YT Huang, & DR Simmons, gen. nov.

Mycobank MB 816236

Aerial hyphae present, subhyaline, smooth, septate, greatly branched, often perpendicularly; thickened and melanized intercalarily; distal hyphae denticulate. Immersed hyphae subhyaline, smooth, constricted at septa, frequently branched, with tapering distal ends. Monillioid mycelia occurring in peptone-containing medium, fragmented unicellularly or bicellularly. Monophyletic lineage within the Ophiostomataceae.

Type species—

Afroraffaelea ambrosiae CC Bateman, YT Huang & DR Simmons

Etymology—

Prefix *Afro-* indicating its likely African origin, and “*raffaelea*” to recognize the ecological similarity to the closely related (though probably not monophyletic) ambrosia fungus genus *Raffaelea*.

Afroraffaelea ambrosiae C Bateman, YT Huang, & DR Simmons, sp. nov.

Fig. 3

Mycobank MB 816237

Colony margin effuse, white, velvet, 60-70 mm on PDA at 25°C after 10 d, turning brown to hazel after 10-14 d. Reverse firstly subhyaline to whitish, becoming brown to dark in old culture. Aerial hyphae abundant, subhyaline, smooth, septate, often branched perpendicularly; distal hyphae denticulate; thickened and intercalary melanized with age. Immersed hyphae subhyaline,

smooth, septate, somewhat constricted at septa, branched, distally tapering to the end. Monillioid mycelia occurring in peptone-containing medium, fragmented unicellularly or bicellularly. No sporulation or reproductive structures were observed.

Type—

USA, Florida, Miami-Dade County, Coral Gables (25°39'37.8"N 80°16'47.0"W): isolated from pharyngeal mycangia of *Premnobius cavipennis* collected at an ethanol-baited light trap, August 12th, 2015 (Holotype-BPI 910155-, Ex-type culture CBS 141678 = CMW 48331 = Hulcr 9747, additional cultures CBS 141679 = CMW 48332 = Hulcr 9759, CBS 141680 = CMW 48333 = Hulcr 9765, CBS 141681 = CMW 48334 = 9772). Genbank accessions: 18S rDNA, **KX620931**; 28S rDNA, **KX620930**; β T, **KX620929**;

Etymology—

Epithet *ambrosiae* to acknowledge species' engagement in a symbiosis with an ambrosia beetle.

Note—

A. ambrosiae is characterized by a distinctly fluffy colony, which is a character that has not been recorded for species of Ophiostomatales. No ascocarp was produced in our mating study. Attempts to stimulate the sporulation, e.g. MEA medium with sterilized wood chips, ground-up bark beetle, near-ultraviolet light treatment and other types of media failed to induce sporulation in our isolates. Notably, the monillioid hyphae occurring in peptone-containing media were also observed in microtome sections of beetle mycangia. The fragmentation of monillioid hyphae is a common convergent feature in ambrosia fungi, typically expressed during transport in the mycangium and possibly facilitating beetle feeding.

Discussion

A novel species of fungus described here, *Afroraffaelea ambrosiae*, is the dominant fungal symbiont of *Premnobius cavipennis*, based on culturing, community sequencing, and

histological methods. No other fungi were recovered in comparable abundance or prevalence by any of the methods used, indicating the relationship between beetle and fungus may be highly specific. This hypothesis is consistent with our confirmation of the fungus from both Floridian and Javan beetle populations. *Afroraffaelea* represents a divergent branch within the Ophiostomatales, though its placement within the family is robust and does not warrant the description at any higher taxonomic level at this time.

This is the first report describing the identity of fungi associated with Premnobiini beetles, and appears to represent another origin of the ambrosia symbiosis within Kingdom Fungi. As an independent origin, *Afroraffaelea* brings the total number of known fungal ambrosia lineages to at least eight. There is still a greater number of known origins among the beetles (thirteen confirmed, Farrell et al. 2001, Jordal and Cognato 2012, Kirkendall et al. 2015), but most beetle lineages have never had their fungal mutualists identified. As more fungal communities are investigated within the beetles, we speculate that the number of ambrosial origins within Fungi may actually exceed the number within Coleoptera. Some beetle clades are already known to associate with fungi from multiple ambrosial origins. One example is the Xyleborini, which contains beetles associated with disparate fungal mutualists in the genera *Flavodon*, *Raffaelea*, and *Fusarium*. Conversely, *Raffaelea* s.l. ambrosia fungi are widespread across several unrelated beetle tribes including Corthylini, Xyleborini, and Platypodinae beetles. However, it is likely that these *Raffaelea* s.l. symbionts will be placed into several new genera, further increasing the number of independent origins of fungal mutualists (Musvuugwa et al. 2015). Questions remain as to why some groups associate with a greater number of symbiont clades, and how the symbiosis is maintained across these diverse groups.

The biogeographic history of these organisms may show how the association is maintained or lost over time. Phylogenetic analyses indicate that *A. ambrosiae* may share a common ancestor with *Fragosphaeria* (Fig. 1), a genus found in dead and dying trees and loosely associated with galleries of Scolytine beetles (Shear 1923, Chesters 1935). However, low bootstrap support at the node uniting *Fragosphaeria* and *A. ambrosiae* indicates an uncertain position within the Ophiostomatales. Explorations of fungi associated with other Premnobiini beetles as well as isolates from *P. cavipennis* across its invaded range should help resolve the placement of *Afroraffaelea* within the order and may reduce the amount of divergence we observed from related taxa.

The substantial divergence between *Afroraffaelea* and other Ophiostomatales is probably a result of long separate evolution, not a methodological artifact. Premnobiini are thought to have evolved in Africa, possibly apart from other obligate Ophiostomatales-associated beetles (Wood 1982, Wood 2007, Bright and Skidmore 1997). Corthylini, the oldest ambrosia beetle clade which sometimes carry *Raffaelea* s.l. symbionts, originated in the Americas, while Xyleborini and Platypodinae associated with *Raffaelea* s.l. may have originated in Austral-Asia where they are most diverse (Jordal 2000, Cognato et al. 2013, Jordal 2015). Furthermore, our phylogeny yielded topologies highly similar to studies using a range of conserved (18S, De Beer et al. 2013) to divergent markers (β T, Dreaden et al. 2014, Ando et al. 2016).

The inability of *A. ambrosiae* to grow in the presence of cycloheximide further distinguishes it from *Raffaelea* and many other Ophiostomatales, which are known to tolerate the compound (Harrington 1981). We speculate that the strong divergence, coupled with the absence of this fungus from well-sampled beetles which carry promiscuous other Ophiostomatales

symbionts, indicates *Afroraffaelea* symbionts are highly coadapted to their *Premnobius* beetle partners, or *vice versa*.

The confirmation of the same fungus in *P. cavipennis* mycangia from several non-native regions is significant from the invasion biology perspective. Colonization of non-native regions and integration into non-native ambrosia communities do not seem to change the beetle-fungus association for symbioses that are already specific, such as *Premnobius-Afroraffaelea* (this paper) or *Xylosandrus-Ambrosiella* (Mayers et al., 2015). Horizontal transfer does occur in these groups, but at narrowly diffuse scales within genera, with no initiation of phylogenetically novel relationships. The opposite - mixing between beetle and fungal species in newly colonized regions - appears to be true for promiscuous symbioses, such as *Xyleborus-Raffaelea* (Carrillo et al., 2014, Simmons et al., 2016a) or *Euwallacea-Fusarium* (O'Donnell et al. 2015). Many of these clades have undergone horizontal transfers and symbiont switches with previously non-ambrosial beetles/fungi. The dynamic relationships that can be observed across the ambrosia symbiosis suggests that each clade of beetles or fungi has unique characters that serve to maintain the relationship. More rigorous sampling of underrepresented fungal communities would aid in designing experiments on the maintenance of these symbioses.

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Figures

Fig 1. Best ML tree from GARLI analysis of 18S, 28S rDNA, and β T (introns 3/4/5 removed) data matrix of Sordariomycetidae with Xylariales outgroup. Values at nodes represent ML bootstrap percentages $\geq 70\%$ from a summary of 500 replicates, and branches in bold represent BPP $\geq 85\%$.

Fig. 2. A-B. Colony on PDA at 8th day. C-D. Colony on PDA after 14 d. E-F. Aerial hyphae, showing intercalary melanization and denticulate distal hyphae. G-H. Immersed hyphae. I-J. Monilliod hyphae.

Fig 3. Transverse cross sections of adult female *Premnobius cavipennis* head showing the content of the paired mycangia. (A) Section showing the entire head with paired mycangia, each between the compound eye and the brain; (B) Compacted mycangial inoculum as an aggregation of monilliod hyphae; and (C) Loose monilliod hyphae from mycangial packet. Bars equal 25 μ m.
Key: CG, cerebral ganglia; E, compound eye; M, mycangia (paired).

Fig. 4 Micro-CT scan of adult female *Premnobius cavipennis* showing a three-dimensional mycangium structure which opens to the pharynx, shown from an (A) anterior aspect with scale and (B) lateral aspect.

Table S1. Species of *Sordariomycetidae* and *Xylariales* from De Beer *et al.* 2013 used for phylogenetic analyses, and information on new species in **bold**.

Figure 2

Figure 3

Figure 4