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HOST MICROBE INTERACTIONS

Inherited Fungal and Bacterial Endosymbionts of a Parasitic Wasp and Its Cockroach Host

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Abstract Bacterial endosymbionts of insects are increasingly being recognized as common, diverse, and integral to the biology of their hosts. Inherited fungal symbionts have been largely overlooked, however, even though insect guts appear to be a key habitat for an incredible array of fungal diversity. Like bacteria, fungal symbionts also likely play important roles in the ecology and evolution of their insect associates. The objective of this study was to lay the foundations for understanding the roles of the vertically transmitted fungal and bacterial associates of both the brownbanded cockroach, *Supella longipalpa*, and its parasitic wasp, *Comperia merceti*. We used culture-dependent and culture-independent molecular methods and phylogenetic analyses in order to identify the symbionts. Two fungal associates of brownbanded cockroaches were found. To our knowledge, this is the first record of vertically transmitted fungal symbionts in the order Blattaria. The wasp was found to house a close relative of one of the cockroach fungi but no bacterial symbionts. Finally, the brownbanded cockroaches also harbored three lineages of bacterial symbionts: *Blattabacterium* and two lineages of *Wolbachia*, indicating the number of vertically transmitted symbionts in this insect may be as many as five.

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Introduction

Inherited microbial symbionts are very common in insects. The best-characterized associations are those of bacterial symbionts that benefit their hosts nutritionally (e.g., *Buchnera* [1], *Blochmannia* [2], and *Wigglesworthia* [3]) and those that manipulate their hosts' reproduction, including *Wolbachia* and *Cardinium* [4, 5]. However, symbionts may play other roles in their hosts' biology; for instance, some provide heat tolerance [6], while others protect against parasitism [7] or pathogens [8].

There are some well-studied examples of insects associated with fungi for nutritional benefits, such as leafcutter ants and their ectosymbiotic fungi and wood-boring beetles and their endosymbiotic fungi. However, given the frequency with which fungi are associated with insects [9], there has been comparatively little research investigating either the diversity or the roles that fungi play as insect endosymbionts. Given their habitat inside insect hosts, it is not surprising that these fungi are often compact unicellular yeasts or yeast-like forms [10].

In 1985, LeBeck [11] noted a vertically transmitted yeast-like symbiont in *Comperia merceti* (Hymenoptera: Encyrtidae), a parasitic wasp that specializes on brownbanded cockroaches, *Supella longipalpa* (Blattaria: Blattellidae). She noted that this fungus was passed from mother wasp to her offspring via the pedicel of eggs during oviposition into cockroach egg cases. She suggested that it was *Candida* sp. and hypothesized that it might be required to modify the host cockroach environment to aid the development of the young wasp larvae. The current research lays the groundwork for understanding the contributions of both the wasp and cockroach fungi in this system.

Methods

Insect Collection and Natural History

A culture of brownbanded cockroaches was started from feral individuals collected between 2003 and 2006 in the Marley building of the University of Arizona. The colony was maintained on autoclaved dog food (Science Diet) and sterile water. The *C. merceti* wasp culture was initiated from parasitized brownbanded cockroach egg cases sent from the University of Massachusetts (2003) and was maintained continuously on brownbanded cockroach egg cases, honey, and sterile water.

Brownbanded cockroaches are global pests, and their feces and cast exoskeletons are implicated in various respiratory problems (e.g., childhood asthma) [12]. Each brownbanded cockroach egg case, if unparasitized, can result in 18 nymphs. An adult female cockroach can lay an average of one egg case per week over the course of her 20-week life span [13]. Adult *C. merceti* wasps are used for biological control of these cockroaches and have locally extirpated populations [14]. Female wasps seek out cockroach host egg cases and lay from one to ten eggs (along with yeast cells on the egg pedicels) per host cockroach egg case ([15], C. Gibson, unpublished data). Wasp larvae hatch from their eggs within the cockroach egg case, feed on the fungal cells, and then move throughout the case consuming developing cockroach embryos. Wasps pupate and then chew a single exit hole out of the egg case. Wasps live an average of 11 days, with females laying approximately 21 offspring in an average of 2.4 egg cases (C. Gibson, unpublished data).

Fungal Isolation + DNA Extractions

Cockroach egg cases were rinsed in a 10% bleach (5.25% NaOCl), 70% ethanol, 20% sterile water solution (1 min) and then rinsed in sterile water (1 min) [16]. This sterile rinse water was plated as a control for verifying the sterility of external surfaces. Half of the insects dissected out of surface-sterilized egg cases (wasp larvae or unparasitized cockroach embryos) were plated on standard isolation media for isolating fungi and bacteria. These media were yeast-peptone-D-glucose agar (YPD; 0.5% hydrolyzed yeast, 1.0% peptone, 2% dextrose, 1.5% agar) and potato dextrose agar. Colonies were isolated after 1–2 weeks at 27°C, and successive isolations were performed at least once for purification. The other half of the insects dissected out of egg cases were used for DNA analysis. For DNA extraction, insect tissue (either wasp or cockroach) or a sample of a single colony isolate of cultivated fungus was placed in a 1.5 ml tube, lowered into liquid nitrogen, and then crushed with a sterile pestle. Subsequent steps followed the Qiagen DNEasy kit protocol for insect DNA extractions.

Polymerase Chain Reaction and Cloning

For screening bacterial symbionts, approximately 1,500 bp of 16S ribosomal DNA (rDNA) was amplified and sequenced using the universal primers 10f and 1507r [17]. For fungal screening, approximately 900 bp of 28S nuclear large subunit (LSU) rDNA was amplified and sequenced using primers LS1 and LR5 [18, 19]. These genes are widely used for surveys of fungi in general, as well as those associated with insects in particular [16, 20]. A 600-bp region in LSU rDNA is useful for determining yeast species [21]; taxa that are greater than 6 bp different in this region are considered unique [16]. Touchdown polymerase chain reaction (PCR) cycling conditions for amplifying fungal amplicons were as follows: 94°C for 2 min, 94°C for 1 min, 68°C for 1 min (−0.7°C per cycle down to 58°C) for 12 cycles and then 25 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 6 min. The reaction mix for each PCR run contained 16.95 μl sterile water, 3 μl 10× buffer (New England BioLabs), 2.4 μl 10 mM dNTPs, 1.5 μl 25 mM $MgCl_2$ (Eppendorf), 1.5 μl 5 pmol/μl of each of the forward and reverse primer, 3 μl of template DNA, and 0.15 μl Taq DNA Polymerase (New England BioLabs) for a total volume of 30 μl. PCR products were mixed with loading dye and SYBR green and were loaded onto a 1.5% agarose gel along with a 1 kb ladder (Invitrogen). Products were visualized under a UV transilluminator.

PCR products from in vivo extractions from both wasps and cockroaches were cloned using an Invitrogen pCR 2.1-TOPO cloning kit following the manufacturer's instructions. Sequencing was done with an Applied Biosystems 3730xl DNA Analyzer at the Genomic Analysis and Technology Core, University of Arizona. Successful sequences were returned for 19 cockroach fungal clones, 21 wasp fungal clones and 17 cockroach bacterial clones.

Phylogenetic Analysis

Sequences were aligned, and representative sequences were chosen for each symbiont type based on 100% sequence identity. These sequences were then used to find the top Basic Local Alignment Search Tool (BLASTn) matches from GenBank, based on the Expect score. Given that there are few Saccharomycotina LSU sequences past the D2 domain (~400 bp) in GenBank, the Saccharomycotina LSU read was truncated from 652 to 386 bp and the resulting top BLAST matches were used for the analysis. All sequences were aligned using the software MUSCLE with the default settings [22] and manually adjusted further with MacClade

v4.08 [23]. Regions with ambiguous alignment were excluded. Analyses were performed using MrBayes 3.1.2 [24] with two runs of the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method under the GTR+Γ+I model. This model was considered optimal by ModelTest [25] implemented in PAUP v. 4.0 [26]. In MrBayes, Four chains were run for more than 4,000,000 generations for each analysis with trees sampled every 1,000 generations. The generation after which chains had converged was found in Tracer v1.2 [27] (the point at which the average deviation of the split frequencies drops below 0.01), and trees previous to this were discarded as burn-in. A 50% majority-rule consensus tree with posterior probability support values and consensus branch lengths was computed for each analysis in MrBayes and viewed in Mesquite 1.12 (build i19) [28].

Results

We found both fungal and bacterial symbionts in brownbanded cockroaches via cloning from a sterile unparasitized cockroach egg case. The *Blattabacterium* recovered from brownbanded cockroaches (GenBank accession numbers: FJ152085–FJ152092) is identical to the *Blattabacterium* (EF423764, 750 bp) of a recent study [29] and appears to be fixed in the population (11 of 11 egg cases screened with diagnostic PCR, data not shown). The other bacteria amplified from the cockroaches in this study were *Wolbachia* (Proteobacteria) (GenBank accession numbers: FJ152093–FJ152101). From the phylogenetic analysis (Fig. 1), there are minimally two strains of *Wolbachia*. All 11 egg cases were infected with *Wolbachia* in a diagnostic screen with *wsp* primers, although it is unclear which strains were present in each egg case. None of these bacterial taxa was cultivable.

In addition to bacterial symbionts, we recovered two fungal symbionts via cloning from a sterile cockroach egg case. The first fungus, represented by clone e2 (Fig. 2), was uncultivable and was identified by molecular analysis alone based on the phylogenetic placement among its top BLAST hits. This fungus is in the Pezizomycotina, a subphylum of the Ascomycota (GenBank accession numbers: FJ152066–FJ152071, FJ152073–FJ152084). The Pezizomycotina phylogeny (Fig. 2) shows the cockroach fungal symbiont on a long, unsupported branch as a sister taxon to a group of nematode-trapping fungi. In an effort to discover whether this might be a fungal lineage associated with other cockroaches, specific primers were designed for the clade including this taxon and the nematode-trapping fungi. These primers were used to conduct PCR on nymphs or adults of three taxa which were maintained in the laboratory for more than 20 years, speckled cockroaches, *Nauphoeta cinerea* (Blaberidae), Madagascar hissing cockroaches, *Gromphadorhina portentosa* (Blaberidae), and American cockroaches, *Periplaneta americana* (Blattidae) and two taxa which were wild collected, German cockroaches, *Blattella germanica* (Blattellidae), and *Cryptocercus* sp. (Cryptocercidae). There were no close relatives of the clone e2 fungus found from any of these other cockroaches, although each of these other cockroaches, save the American cockroach, was associated with other, likely facultative gut-associated, Saccharomycotina fungi (data not shown). The specific primers were also used to amplify the Pezizomycota e2 sequence in 11 individual, sterilized brownbanded cockroach egg cases. We found that ten egg cases contained the e2 fungus which suggests that it is common but not fixed in brownbanded cockroaches.

The cockroaches housed an additional fungal symbiont (represented by clone d8; Genbank accession number: FJ152072) that, combined with the fungal associate of the wasps, forms the sister taxon to a poorly supported monophyletic group of *Candida* sp. and six species of *Metschnikowia*. These *Metschnikowia* spp. are also insect-associated yeasts in the Saccharomycotina (Fig. 3). This cockroach Saccharomycotina fungal symbiont was cultivable with some difficulty. Specific primers were designed for these fungi, and in a survey of individual, sterilized brownbanded cockroach egg cases, only one of 11 was infected.

With respect to the parasitic wasps, no bacteria were amplified. The only symbiont recovered with molecular or culture-based methods was a fungus in the Saccharomycotina (GenBank accession numbers: FJ152045–FJ152065). The wasp fungus, represented by clone c26, is 7 bp different in the ~650 bp sequence of LSU rDNA from the Saccharomycotina fungus recovered from the cockroaches and is therefore likely a novel species. The clone c26 fungus was readily cultivable on YPD medium, with colonies apparent after 1 week at 27°C. To further determine fungal identities, samples of both the cockroach and the wasp yeasts were sent to S.-O. Suh and M. Blackwell (Louisiana State University) for fermentation and assimilation tests. Neither of these isolates was able to be cultured on a wide enough range of fermentation and assimilation media to be described as new fungal species using traditional methods. A sample of the c26 yeast isolated from wasp larvae was accessioned with the National Center For Agricultural Utilization Research, Peoria IL 61604 (accession number: NRRL Y-48467).

Discussion

Our population of brownbanded cockroaches hosts at least three lineages of vertically transmitted bacterial symbionts and two fungal symbionts. The *Blattabacterium* (Bacteroidetes) symbiont has been found in fat body bacteriocytes

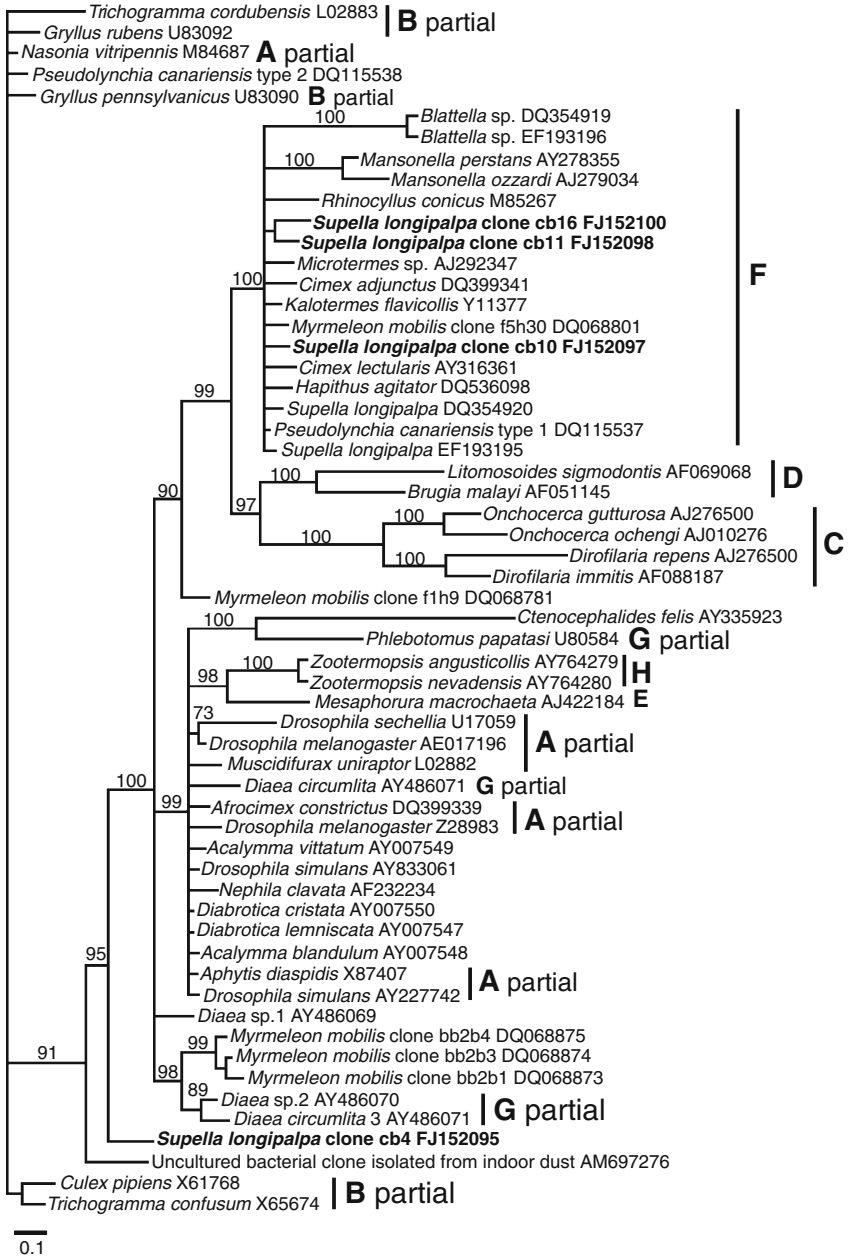

Figure 1 Bayesian 50% majority rule consensus tree of *Wolbachia* recovered from brownbanded cockroaches in this study (*names in bold*), their top BLAST hits from GenBank and additional taxa from the study of Vaishampayan et al. [29]. The tree is based on an alignment of 1,400 bp of 16S bacterial rDNA with a GTR+Γ+I model of evolution with two runs of the MCMCMC for 7,985,000 generations (4,791,000 generations were discarded as burn-in; the consensus tree was computed from 6,388 trees). Only those branches with posterior probabilities greater than 70 are labeled. Supergroup attributions follow those of Vaishampayan et al. [29] and are meant to highlight differences (taxa without supergroup attributions are GenBank accessions for the top 15 BLAST hits for *Wolbachia* recovered from this study). Names are those of host species followed by accession numbers. *Scale bar* represents substitutions per site

of nearly all cockroaches examined to date and plays critical roles in uric acid storage [30] and amino acid synthesis [31, 32]. Not surprisingly, the *Blattabacterium* from the Arizona (AZ) population of brownbanded cockroaches is identical to that found previously in India [29]. This symbiont has been shown to exhibit co-cladogenesis within cockroaches [33] and has been used as corroborating evidence that termites are likely social cockroaches [34].

The other bacterial symbionts found within brownbanded cockroaches are *Wolbachia* spp. *Wolbachia* can cause a range of reproductive manipulations in invertebrates, including parthenogenesis induction, cytoplasmic incompatibility, feminization, and male-killing (reviewed in [35]). The effect of *Wolbachia* in brownbanded cockroaches is unclear, and there is only one previous record of *Wolbachia* in any cockroach [29]. We have not observed the sex ratio bias that one would expect from male-killing or feminizing *Wolbachia* in our cockroach cultures. Cytoplasmic incompatibility is perhaps more likely, as it does not cause sex ratio distortion in diploid systems, and it is the dominant *Wolbachia* phenotype [36]. However in some cases, the phenotypic effects of *Wolbachia* have not been found [37], or *Wolbachia* persists as a mutualist [38].

It has recently been noted that the best method for understanding *Wolbachia* strains or supergroup attributions of *Wolbachia* is with the use of a Multi Locus Sequence Typing approach rather than 16S or *wsp* alone [39]. Given this, we have followed the supergroup attributions of the

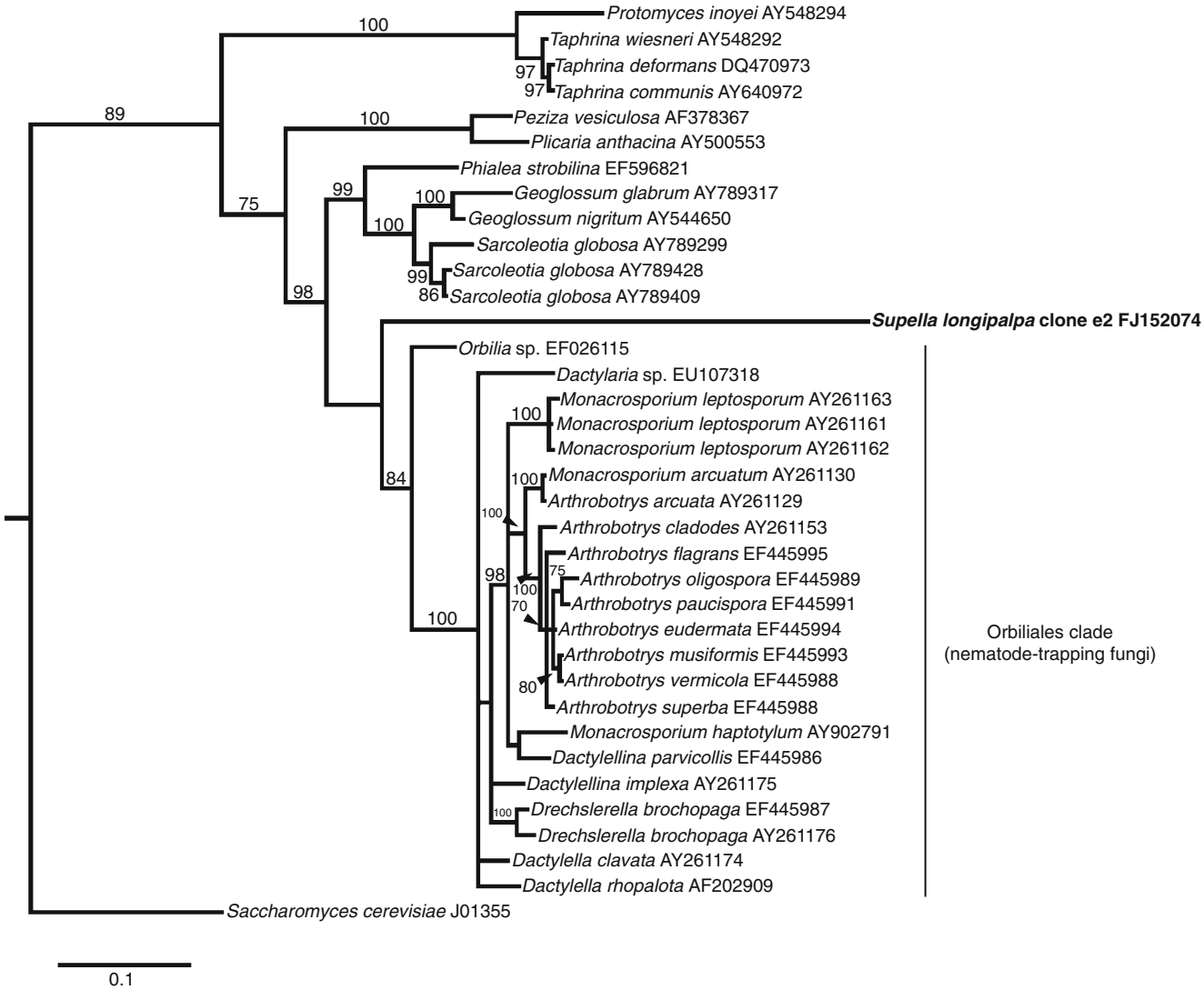

Figure 2 Bayesian 50% majority rule consensus tree of the fungus recovered from brownbanded cockroaches (*name in bold*) and its top BLAST hits from GenBank. The tree is based on an alignment of 900 bp of LSU fungal rDNA with a GTR+Γ+I model of evolution with two runs of the MCMCMC for 5,000,000 generations (1,565,000 generations were discarded as burn-in; the consensus tree was computed from 6,870 trees). Only those branches with posterior probabilities greater than 70 are labeled. This tree is constructed primarily with fungi from the subphylum Pezizomycotina (Ascomycota) and is rooted with *Saccharomyces cerevisiae* (Ascomycota: Saccharomycotina). Names are those of fungi followed by accession numbers. *Scale bar* represents substitutions per site

16S phylogenetic analysis (Fig. 1) of Vaishampayan et al. [29] in order to highlight supergroup affinities and variation rather than to attempt to assign strains to supergroups. One strain of *Wolbachia* from this study is found within a clade that forms the F supergroup (Fig. 1) and is 2 bp different (over the 764 bp sequence) from the Vaishampayan et al. [29] *Wolbachia* (DQ354920). A recent finding [40] suggested that strains in the F supergroup have high genetic diversity and no evidence of recombination and that it is therefore likely an older lineage than the A or B supergroups. In general, the F supergroup is the least common type of *Wolbachia* [40]. The other *Wolbachia* from brownbanded cockroaches is represented by clone cb4. This second strain of *Wolbachia* is 26 bp different from DQ354920 and is sister to a clade of a mixed set of supergroups.

It is not uncommon for insects to harbor multiple strains of *Wolbachia* [41–45]. Different *Wolbachia* strains may have different roles in their hosts [46]. It is unclear whether the population of brownbanded cockroaches studied by Vaishampayan et al. [29] was singly or multiply infected with *Wolbachia* strains; direct sequencing approaches are likely to underestimate the number of symbiont infections discovered. For the AZ population, further work is required to understand the phenotypes of the different *Wolbachia* strains, as well as their evolutionary history.

The two fungal symbionts of brownbanded cockroaches represent independent lineages. The one that appears most

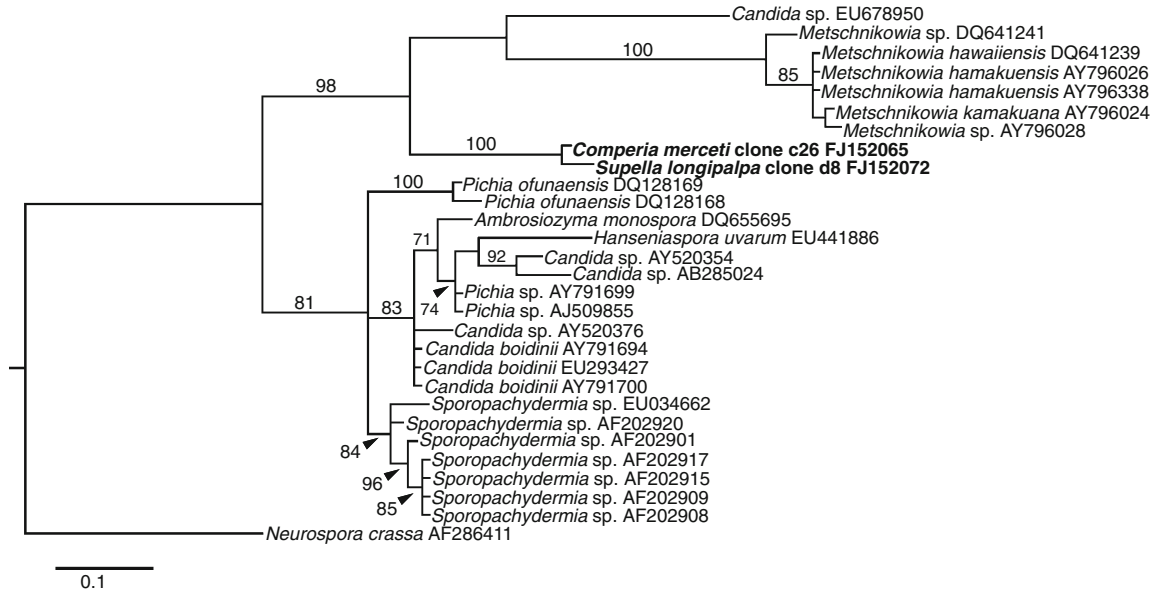

Figure 3 Bayesian 50% majority rule consensus tree of the fungi recovered from brownbanded cockroaches and the parasitic wasps that attack them (*names in bold*) and their top BLAST hits from GenBank. The tree is based on an alignment of 333 bp of LSU fungal rDNA with a GTR+Γ+I model of evolution with two runs of the MCMCMC for 9,034,000 generations (2,085,000 generations were discarded as burn-in; the consensus tree was computed from 13,898 trees). Only those branches with posterior probabilities greater than 70 are labeled. This tree is constructed primarily with fungi from the subphylum Saccharomycotina (Ascomycota) and is rooted with *Neurospora crassa* (Ascomycota: Pezizomycotina). Names are those of fungi followed by accession numbers. *Scale bar* represents substitutions per site

common in this population is also the most unusual. This fungus is in the Pezizomycotina where other filamentous ascomycotans are found. This is in contrast to the bulk of other known fungal associates of insects, which are in the Saccharomycotina [47]. The Pezizomycotina, or "higher" ascomycotans, can have complex morphologies and include the lichenized fungi and other well-known fungi such as morels, *Penicillium*, and *Aspergillus* [48].

The clone e2 fungus (Fig. 2) is on a long, unsupported branch as the sister taxon to a group of predaceous, nematode-trapping fungi. A long branch of this type is likely due to one of two causes. One possibility is that there has been rapid evolution in this lineage, relative to related taxa. This phenomenon is common in obligate insect associates [49]. Another possibility is that GenBank is simply not yet well populated with fungi related to the e2 fungus. It is clear that a vast number of fungi remain to be represented in GenBank: For instance, Porter et al. [48] recently found what appears to be a new subphylum of Ascomycota (a sister group to the monophyletic group of the Pezizomycotina and the Saccharomycotina) from a cloning survey of soil fungi. Although the nearest relatives of the cockroach e2 fungus appear to be nematode-trapping fungi, we have found no evidence of nematodes in our cockroach culture.

The other fungus from the cockroaches, represented by clone d8, is the sister taxon to the parasitic wasp fungus (clone c26, Fig. 3). Together, these two fungi form the sister taxon to a *Candida* sp. isolate and a group of *Metschnikowia* yeast isolates recovered from nitidulid beetles [50]. The roles of the closely related fungi in the cockroach and wasp hosts are unknown. The wasp and cockroach Saccharomycotina LSU sequences are only 7 bp different; however, after 21 generations of rearing yeast-free wasps on our brownbanded cockroaches, we have no evidence that wasps are ever infected with any of the bacterial or fungal symbionts found within their cockroach hosts (using diagnostic PCR, data not shown). Theory for strictly vertically transmitted symbionts requires their engagement in host fitness and evolution. Vertically transmitted symbionts must either contribute to the fitness of their hosts or manipulate host reproduction in order to invade and persist in host populations [51]. One question of interest is whether there is horizontal transmission among cockroaches, wasps, or between these insect species. The high frequency of fungal infection in the wasps suggests a more intimate association between wasp and fungus than the closely related fungal symbiont of the brownbanded cockroaches and its host.

The vertically transmitted fungal symbiont of *C. merceti* was originally noted by LeBeck [11]. Here we verified that the symbiotic complement of this wasp does indeed appear to be restricted to a single fungus, a yeast in the Saccharomycotina. LeBeck [15] proposed that the yeast was required to alter the host cockroach environment for the benefit of the developing wasp larvae. Our preliminary data do not support this hypothesis, as we have cultured

uninfected wasps for 2 years without any obvious fitness costs. Fitness experiments are underway to determine the consequences of yeast infection in these wasps.

While researchers increasingly recognize the abundance and importance of bacterial symbionts in insects, fungal symbionts have been largely unstudied. The discovery of two independent lineages of inherited fungi in an otherwise well-studied domestic insect pest suggests that other insect systems may similarly house intimate fungal associates. Domestic cockroach species can be important vectors for bacterial, fungal, and viral diseases [52]. Further, these cockroaches are major nuisance pests [53]. The identification of the full microbial community of these cockroaches is the first step towards understanding the role that symbionts play in their host's biology. Vertically transmitted symbionts, such as *Wolbachia*, may offer potential means of controlling pests, either by influencing life history directly [36] or by driving genes into these insects that lessen their pest status. Additionally, in non-urban settings, cockroaches perform important ecosystem services, such as cellulose degradation, and decomposition of other organic matter [54]. Symbionts may contribute to these processes, and understanding the mechanisms by which this occurs may allow us to manipulate their populations or to cultivate these mutualists (or their genes) for industrial processes, as is currently being researched for termites [55].

Here we have shown that laboratory-reared brown-banded cockroaches may host as many as five vertically transmitted lineages of symbionts from two domains of life. Outstanding questions include whether or not the fungal symbionts are specific symbionts of cockroaches or may be more generally associated with other insects, as has recently been shown for other fungal taxa associated with insects [47]. Also of interest is whether cockroaches in nature, unlike our well-fed laboratory-reared cockroaches, have more limited access to nutrients and therefore house an even greater variety of specific associates, as predicted by Thrall et al. [56].

A large body of work has established that diverse bacterial lineages are intimately associated with invertebrates and have profound impacts on the ecology and evolution of their hosts. Fungal partners of insects are also known to be diverse [9, 47], but their roles are little known. It is unlikely that these microbial consortia of insects are simply static menageries. Understanding the entire complement of microbes within insect hosts is the first step towards understanding the full range of potential interactions.

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