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HOST MICROBE INTERACTIONS

Culture-Free Survey Reveals Diverse and Distinctive Fungal Communities Associated with Developing Figs (*Ficus* spp.) in Panama

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Abstract The ancient association of figs (*Ficus* spp.) and their pollinating wasps (fig wasps; Chalcidoidea, Hymenoptera) is one of the most interdependent plant–insect mutualisms known. In addition to pollinating wasps, a diverse community of organisms develops within the microcosm of the fig inflorescence and fruit. To better understand the multipartite context of the fig–fig wasp association, we used a culture-free approach to examine fungal communities associated with syconia of six species of *Ficus* and their pollinating wasps in lowland Panama. Diverse fungi were recovered from surface-sterilized flowers of all *Ficus* species, including gall- and seed flowers at four developmental stages. Fungal communities in syconia and on pollinating wasps were similar, dominated by diverse and previously unknown Saccharomycotina, and distinct from leaf- and stem endophyte communities in the same region. Before pollination, fungal communities were similar between gall- and seed flowers and among *Ficus* species. However, fungal communities differed significantly in flowers after pollination vs. before pollination, and between anciently diverged lineages of *Ficus* with active vs. passive pollination syndromes. Within groups of relatively closely related figs, there was little evidence for strict-sense host specificity between figs and particular fungal species. Instead, mixing of fungal communities among related figs, coupled with evidence for possible transfer by pollinating wasps, is consistent with recent suggestions of pollinator mixing within syconia. In turn, changes in fungal communities during fig development and ripening suggest an unexplored role of yeasts in the context of the fig–pollinator wasp mutualism.

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Introduction

Mutualisms are a feature of every ecosystem and increasingly are recognized as a driving force in the diversification of life on earth [14, 40]. Often characterized as bipartite exchanges of commodities such as nutrition, protection, or enhanced reproductive success [e.g., 11, 20, 41, 48, 66, 86], mutualisms exist within communities of species that can shape the currency or rate of exchange between partners [15, 62, 72]. Ecologists increasingly appreciate that mutualisms should be interpreted in a multipartite context [e.g., 1, 38, 88], which often reveals previously unexplored components of even the most classic two-partner associations [e.g., 22, 43, 63, 78].

Fig trees (*Ficus*, Moraceae) and their pollinating wasps (fig wasps; Chalcidoidea, Hymenoptera) share a coevolutionary history that spans up to 90 million years [56, 57, 71]. Their interactions represent some of the most interdependent plant–insect mutualisms known [49, 54]. With the exception

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of parthenocarpic figs used in agriculture, *Ficus* spp. depend solely on fig wasps to transfer their pollen from tree to tree, and the larvae of fig wasps can only develop within fig flowers.

When a female fig wasp enters a receptive fig (syconium), she encounters hundreds of flowers arranged in two layers. Flowers that receive pollen yield a fig seed, whereas those receiving an egg develop into a gall that provides nutrition to the wasp's offspring at the expense of that flower's seed production [41, 87]. Some species of wasps pollinate actively, storing pollen in specialized pockets and fertilizing flowers individually [50]. However, species in the basal lineages of fig-pollinating wasps do so passively, fertilizing inflorescences haphazardly as pollen brushes off the wasp's body [50]. Passive pollination is considered the ancestral condition in *Ficus*, with ca. 60 million years separating the New World actively- and passively pollinated clades [47, 50].

Regardless of pollination syndrome, female fig wasps (foundresses) consistently choose the inner ring of flowers (hereafter, gall flowers), rather than the flowers closer to the syconium wall (hereafter, seed flowers), for oviposition [80]. The reason for this preference is not known, but explanations such as limited ovipositor length and parasitoid avoidance have been refuted [see 13, 25, 30]. The observation that foundresses consistently oviposit in only ~50 % of available flowers despite having sufficient eggs to deposit in more, and thus die after realizing only a portion of their reproductive potential [29, see also 41], led West and Herre [88] to suggest that some flowers may be impervious to ovipositioning and/or gall development. The mechanism by which these "unbeatable seed flowers" [sensu 88] differ from gall flowers is not known, but preference against them is strong: with few exceptions, even non-pollinating wasps, which oviposit from outside the syconium and do not pollinate figs, preferentially use gall flowers even though the outer ring of seed flowers is more accessible [88]. Structural features of flowers such as ovary position or style length do not explain the selective avoidance of seed flowers by pollinators or parasitic wasps [13, 80], prompting us to explore alternative explanations.

In addition to pollinating wasps, a diverse community of organisms develops within the microcosm of the syconium (e.g., non-pollinating wasps, nematodes, and mites) [39, 84, 88]. Microbial communities associated with developing syconia are an especially unexplored aspect of the fig–wasp mutualism with potential implications for oviposition choice both at the level of *Ficus* species and tissue type (gall- vs. seed flowers). Female-pollinating wasps use volatile cues to identify receptive figs of the appropriate species and developmental stage for oviposition [35, 79, 85, 87]. Some plant-associated microbes influence oviposition behavior of insects by altering volatile signals [17, 46, 81], and the roles of microbes in gall formation, host plant selection by herbivores, and plant nutritional quality are well recognized [9, 32, 64, 67, 70]. Previous studies have detected yeasts in cultivated figs [e.g., 59, 60], and recent work suggests that these fungi influence volatile signatures of mature figs, with effects on frugivory by bats [73]. However, despite several surveys of fungal communities within leaves and rotting fruits of fig trees [24, 59, 60, 75, 83], fungal communities within developing syconia of non-domesticated *Ficus* spp. have not been studied previously.

We used a culture-free approach to examine the diversity and composition of fungal communities associated with fig flowers at four developmental stages. Sampling encompassed six species of *Ficus* and their pollinating wasps, including both actively and passively pollinated figs from a lowland, moist tropical forest in Panama. Here we examine fungal communities among *Ficus* species, gall- and seed flowers, and developmental stages of syconia to ask: (1) do fungal communities differ among *Ficus* spp., such that they may play a role in pollinator attraction to particular species of *Ficus*? (2) Do communities differ in gall- vs. seed flowers, such that they may influence oviposition by pollinators? (3) Do communities differ in syconia as a function of developmental stage, such that they may cue pollinators to indicate the conclusion of receptivity or frugivores to indicate ripeness?

Materials and Methods

In January–April 2010, developing figs from one mature individual of each of six species of *Ficus* were collected at Barro Colorado National Monument, Panama (BCNM; 9°9′ N, 79°51′ W; 25 m above sea level; for a full site description see [53]). Focal species represent both actively pollinated species (*Ficus costaricana*, *Ficus obtusifolia*, *Ficus popenoei*, and *Ficus triangle*; subgenus *Urostigma*, section *Americana*) pollinated by *Pegoscapus* spp., and passively pollinated species (*Ficus insipida*, *Ficus maxima*; subgenus *Pharmacosycea*, section *Pharmacosycea*) pollinated by *Tetrapus* spp. [12, 57]. All surveyed trees were located at the edge of Lake Gatún, where their readily accessible canopies overhang the water. Trees were separated by a mean of 2.6 km (±1.8 km) (Supplemental Fig. 1).

Intact, apparently healthy syconia were collected in four developmental stages. Receptive syconia (hereafter, receptive or pre-pollination) contained fully developed flowers but had not yet been entered by a pollinating wasp (typically a span of 24 to 72 h after gall- and seed flowers differentiate) [80]. Early post-pollination syconia (hereafter, early) were collected after a pollinating wasp had entered and oviposited but before larvae pupated (a span of 1 to 2 weeks) [80]. Late post-pollination

syconia (hereafter, late) were collected after galls and seeds within the fig had developed fully and wasps had pupated (a span of 2 to 4 weeks) [80]. Ripe fruits were collected after female wasps emerged from the fig but before a fruit dropped from the tree (a span of 1 to 4 days; EOM personal observation) [80]. Collections were staggered such that figs at different developmental stages were harvested at the same time to decouple date of collection from developmental stage.

Wasps were collected from late post-pollination figs at BCNM in June–August 2009 (wasps from *F. obtusifolia*, *F. maxima*, and *F. popenoei*; stored in sterile SDS buffer at −20 °C) and April 2005 (wasps from *F. insipida*, *F. costaricana*, and *F. triangle*; stored in 70 % ethanol at 4 °C). Wasps were collected from different individual trees than those sampled above but from the same area surrounding Lake Gatún (Machado, unpublished data).

DNA Extraction, PCR, and Sequencing

Figs were stored at 4 °C in sealed plastic bags and processed within 24 h after collection. Gall- and seed flowers from each syconium were separated with sterile microforceps under a dissecting microscope and stored separately in 70 % ethanol at −20 °C. Flowers were surface-sterilized by sequential immersion in 95 % ethanol (30 s), 10 % bleach (0.5 % NaOCl; 2 min), and 70 % ethanol (2 min) [7] followed by three rinses with sterile distilled water. This method removed exogenous DNA that might have contaminated samples in the lab from flower surfaces [29]. Wasps were not surface-sterilized so that fungi on wasps' cuticles could be evaluated [28].

Each sample of fig tissue, defined as a 0.2-ml tube containing gall- or seed flowers collected at the same time from one to three syconia from the same tree, was ground in liquid nitrogen prior to extraction of total genomic DNA using the Qiagen DNeasy Plant Mini Kit (Germantown, MD; manufacturer's protocol). Three wasps per species were pooled prior to DNA extraction with the Qiagen Puregene Core Kit A (Germantown, MD; manufacturer's protocol).

The largely fungal-specific primer ITS1F and nonselective primer LR3 (CTTGGTCAT TTAGAGGAAGTAA and GGTCCGTGTTTCAAGAC, respectively) [31, 82] were used to PCR-amplify the fungal nuclear ribosomal internal-transcribed spacers and 5.8S gene (ITS; ca. 600 bp) and an adjacent portion of the nuclear ribosomal large subunit (LSU; ca. 500 bp) as a single fragment. Each 25 μl reaction mixture contained 12.5 μl GoTaq® Green Master Mix (Madison, WI), 1 μl of each primer (5 μM), 2 μl of DNA template, and 8.5 μl of PCR-quality H_2O. Reactions were run on an Eppendorf Mastercycler ep gradient S thermocycler (Hamburg, Germany) with the following program: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 1 min; and 68 °C for 8 min. Ethidium bromide was used to visualize DNA bands on 1.2 % agarose gels. Positive controls containing verified fungal DNA, and negative controls containing sterile distilled water in place of DNA template, were run with every PCR. Any reaction set with a failure in either control was removed from the study.

Positive products were cloned using the Stratagene StrataClone PCR Cloning Kit (La Jolla, CA) using the manufacturer's protocol, followed by PCR with primers T3 and T7. Up to 15 positive clones per sample were chosen haphazardly for sequencing. PCR products were cleaned by adding 0.2 μl of NEB calf intestinal phosphatase and 0.2 μl of NEB exonuclease I to each sample, vortexing for 30 s, and incubating for 15 min at 37 °C followed by 15 min at 80 °C (J. Stavrinides, personal communication).

Products were sequenced bidirectionally at the UAGC sequencing facility at The University of Arizona on an Applied Biosystems 3730xl DNA Analyzer (Foster City, CA). Contigs were assembled and basecalls verified manually based on chromatograms in Sequencher v. 4.5 (Gene Codes, Ann Arbor, MI). No chimeric sequences were detected. Sequences have been deposited in GenBank under accession numbers JX174729-JX175042.

Ecological Analyses

Operational taxonomic units (OTU) were defined on the basis of 95 % sequence similarity over shared sequence lengths with a criterion of at least 40 % overlap using Sequencher 4.5 [6], which estimates OTU that are congruent with species-level clades of tropical plant-associated fungi [76]. To select representative clones for phylogenetic analyses, we chose one member of each group from figs or wasps as defined by 99 % sequence similarity (following [29]). This approach allows for minor sequencing errors while still capturing the genotypic diversity of the sample.

Species accumulation curves, bootstrap estimates, and diversity (measured as Fisher's α, which is robust to variation in sample size [27]) were inferred in EstimateS v. 8.2.0 (http://viceroy.eeb.uconn.edu/estimates). Similarity among partitions of the fungal community was assessed in PAST v. 2.06 [37] or EstimateS v. 8.2.0 using OTU (based on 95 % sequence similarity, as above) that were found more than once (i.e., non-singletons). Similarity values were calculated using Jaccard's index (JGR, based on presence/absence data) and the Morisita index (MGR, based on incidence). Indices were compared statistically using analysis of similarity (ANOSIM; [19]) with visualization by non-metric multidimensional scaling in PAST v. 2.06 [37] or Wilcoxon tests in JMP v. 8.0.1 (www.jmp.com).

Comparison with Non-Syconia Endophyte Communities

To assess the distinctiveness of syconia-associated fungi relative to fungi occurring in symbiosis with other aerial plant parts, one representative of each non-singleton OTU obtained from figs was compared against a database of 5,010 ITS-partial LSU sequences representing 581 OTU of leaf and stem endophytes from central Panama [3–5, 7, 8, 42, del Olmo, unpublished data; Arnold, unpublished data]. Sequence data represented isolates obtained in culture and sequences obtained by cloning, as mentioned above, from healthy tissues of 258 species representing 190 genera and 28 families of vascular plants (including *Ficus*) in sites throughout central Panama in the wet and dry seasons of 1999–2010. Of these, 4,061 sequences represented fungi that were isolated (3,671 isolates) or directly sequenced (390 clones) from diverse plants at BCNM. Data from figs and wasps were compared against the endophyte data in Sequencher as described above to determine groups with 95 and 100 % sequence similarity.

Phylogenetic Analyses

Fungal ITS-partial LSU sequences from figs and wasps were compared to the NCBI non-redundant database by the basic local alignment search tool (BLASTn) [2] to estimate taxonomic placement at the class level and above and to establish taxon sampling for phylogenetic analyses. The 5.8S and LSU portion of one representative sequence of each unique genotype obtained from each sample of fig flowers (defined by 99 % overall sequence similarity; N=81 sequences) was aligned using MAFFT v. 6 [51] with 37 reference sequences selected from the top BLASTn hits obtained from GenBank. The alignment was adjusted manually and ambiguously aligned regions were excluded in Mesquite v. 2.74 [58]. The alignment is accessioned at TreeBase under accession 12698.

Phylogenetic relationships were inferred using maximum likelihood (ML) in RAxML [74] and Bayesian MCMCMC in Mr. Bayes v. 3.1.2 (seven million generations, two chains, each initiated with random trees, and sampling every 1,000th tree) [45] using GTR+I+γ, determined to be the best-fitting model of evolution based on comparisons of the Akaike information criterion in ModelTest 3.7 [68]. Topological support was evaluated further by 1,000 ML bootstrap replicates. Output from MrBayes was filtered to remove the burn-in, defined as the sample of the posterior for which the standard deviation of the split frequencies was >0.01, and a majority rule consensus was constructed from 5.2 million trees in Mesquite.

Phylogenetic diversity of fungi was assessed with UniFrac [55] using the uncollapsed, most likely tree (Supplemental Fig. 2). The UniFrac metric determines the fraction of the total branch length that is unique or shared by two communities, with statistical support determined by 1,000 permutations [55]. UniFrac scores were assessed using both unweighted (based on presence/absence) and weighted (based on relative abundance) analyses.

To determine the placement of sequences obtained from wasps, 80 sequences were integrated into the alignment described above using MAFFT v. 6 [51]. Phylogenetic relationships were inferred using ML in RAxML [74] using GTR+I+γ, as mentioned above. Topological support was evaluated by 1,000 ML bootstrap replicates.

Results

Fungi were detected in every sample of syconia tissue from six species of *Ficus* in lowland Panama, and from all samples of wasps associated with these fig species (Table 1). A total of 234 ITS-partial LSU sequences representing 26 samples of fig flower tissue yielded 23 OTU (based on 95 % sequence similarity; Fisher's α=8.72; 30.4 % singletons) and 81 genotypes (based on 99 % sequence similarity). A total of 80 sequences from six samples of wasps yielded 9 OTU (Fisher's α=10.02; 5.0 % singletons) and 19 genotypes. Comparison of the bootstrap estimate of total species richness with the 95 % confidence interval around observed richness indicated that our sampling effort was statistically sufficient to capture the total estimated OTU richness for each fig species, both flower types, each developmental stage, and the wasps evaluated here (Fig. 1; Supplemental Figs. 3 and 4), providing a robust basis for community comparisons.

Community Structure Inferred from Fungal OTU

Relative to fungal communities found in living stems and leaves of vascular plants of the region, fungal communities in figs and pollinating fig wasps were highly distinct. No sequences of fungi found in syconia were 100 % identical to a previously recorded leaf- or stem endophyte. None of the Saccharomycotina OTU found in our surveys was detected previously as an endophyte using culture-based- or culture-free methods. Five of 27 OTU from syconia and wasps were 95 % similar to leaf- or stem endophytes (OTU 4, 6, 8, 10, and 11); all were clones with top BLAST matches for Pezizomycotina, which made up <10 % of sequences found in the present survey (see Fig. 2).

Fungal communities from figs strongly resembled those recovered from pollinating wasps both in terms of presence/absence and relative abundance of fungal OTU (JGR: p=0.9228; MGR: p=0.8875). Overall, the

Table 1 Fungi obtained via cloning from syconia at four developmental stages, representing six species of *Ficus* as well as their pollinating wasps in lowland Panama

	Sequences (samples)	Species represented	OTU (95 % CI)	Bootstrap estimate	Fisher's alpha (SD)
Ficus flower survey					
Fig species					
Passively pollinated					
F. insipida	63 (6)	–	11 (7.5–14.5)	12.2	3.86 (0.80)
F. maxima	19 (4)	–	6 (4.7–7.3)	6.6	3.02 (1.10)
Actively pollinated					
F. costaricana	20 (2)	–	4 (2.7–5.3)	4.5	1.50 (0.55)
F. obtusifolia	45 (5)	–	12 (9.5–14.5)	14.0	5.35 (1.27)
F. popenoei	24 (4)	–	5 (4.1–5.9)	5.6	1.92 (0.63)
F. triangle	63 (6)	–	10 (7.5–12.5)	10.9	3.35 (0.71)
Life stage					
Receptive	61 (8)	5	13 (10.4–15.6)	14.0	5.06 (1.04)
Early	74 (8)	5	14 (10.6–17.4)	15.8	5.11 (0.97)
Late	87 (8)	4	14 (9.6–18.4)	15.7	4.72 (0.85)
Ripe	12 (2)	2	1 (1.0–1.0)	1.0	–
Flower type					
Gall	127 (13)	6	22 (20.2–23.8)	23.8	7.68 (1.13)
Seed	107 (13)	6	21 (15.1–26.9)	24.3	7.81 (1.23)
Overall	234 (26)	6	29 (25.7–32.3)	32.8	8.72 (1.00)
Pollinating fig wasp survey					
Overall	80 (6)	6	8 (4.7–11.3)	9.3	2.22 (0.46)

Columns indicate the number of sequences obtained; the number of species represented in each pool (as relevant); fungal OTU richness; bootstrap estimate of fungal OTU richness; and diversity (Fisher's alpha) for each flower type, life stage, fig species, and survey

combined fig and wasp data sets included 27 OTU; of these, 12 were found in both fig- and fig–wasp surveys despite the different timing of sampling and the collection of material from different individual trees (Fig. 2; Supplemental Fig. 5).

Fungal community composition did not differ significantly among species of *Ficus* (range of JGR=0.1995–1.00, $p>0.05$ in all cases; MGR=0.1545–1.00; $p>0.05$ in all cases; data not shown). Fungal diversity, defined as Fisher's alpha, was similar among fig species overall ($F_{5,22}=0.67$, $p=0.6536$; Table 1). Fungal communities were especially similar among syconia of different species in the receptive phase (e.g., Fig. 3, Table 2) but differed markedly after pollination as described below.

Composition of fungal communities did not differ significantly between gall- and seed flowers overall (JGR=0.2685, $p=0.2671$; MGR=0.5571, $p=0.5605$; Supplemental Fig. 6), and diversity was similar between flowers of each type ($t_{22}=-0.33$, $p=0.7442$). However, even though fungal communities were especially similar between gall- and seed flowers in the receptive phase, they diverged markedly after pollination (Table 3).

Diversity of fungi was similar among receptive, early, and late stages of syconium development ($F_{2,22}=0.43$, $p=0.6536$; sequencing success from ripe figs was too limited to draw conclusions). However, communities of fungi differed as a function of the developmental stage of syconia in two ways. First, communities in receptive flowers differed significantly from those of pollinated flowers (Fig. 3; ripe figs excluded and early- and late pollinated flowers pooled for analysis because they were highly similar: JGR: $p=0.3512$; MGR: $p=0.3777$). Second, actively and passively pollinated fig species, which differ in their pollinating wasps, had highly similar communities prior to entrance by pollinators (JGR: $p=0.8550$; MGR: $p=0.5713$) but differed significantly after pollination (JGR: $p=0.0303$; MGR: $p=0.0185$; Fig. 3b).

Community Structure Inferred from Phylogenetic Analyses

Phylogenetic analyses of fungal communities from figs and fig wasps (Fig. 2; see also Supplementary Fig. 2) corroborate OTU analyses in six ways. First, taxonomic placement of syconia- and wasp-associated fungi reveals their distinctiveness relative to foliar- and stem endophyte communities in central Panama. The 27 OTU recovered here encompass at least five classes of fungi, including Basidiomycota (Microtyromycetes and Tremellomycetes, two OTU, and

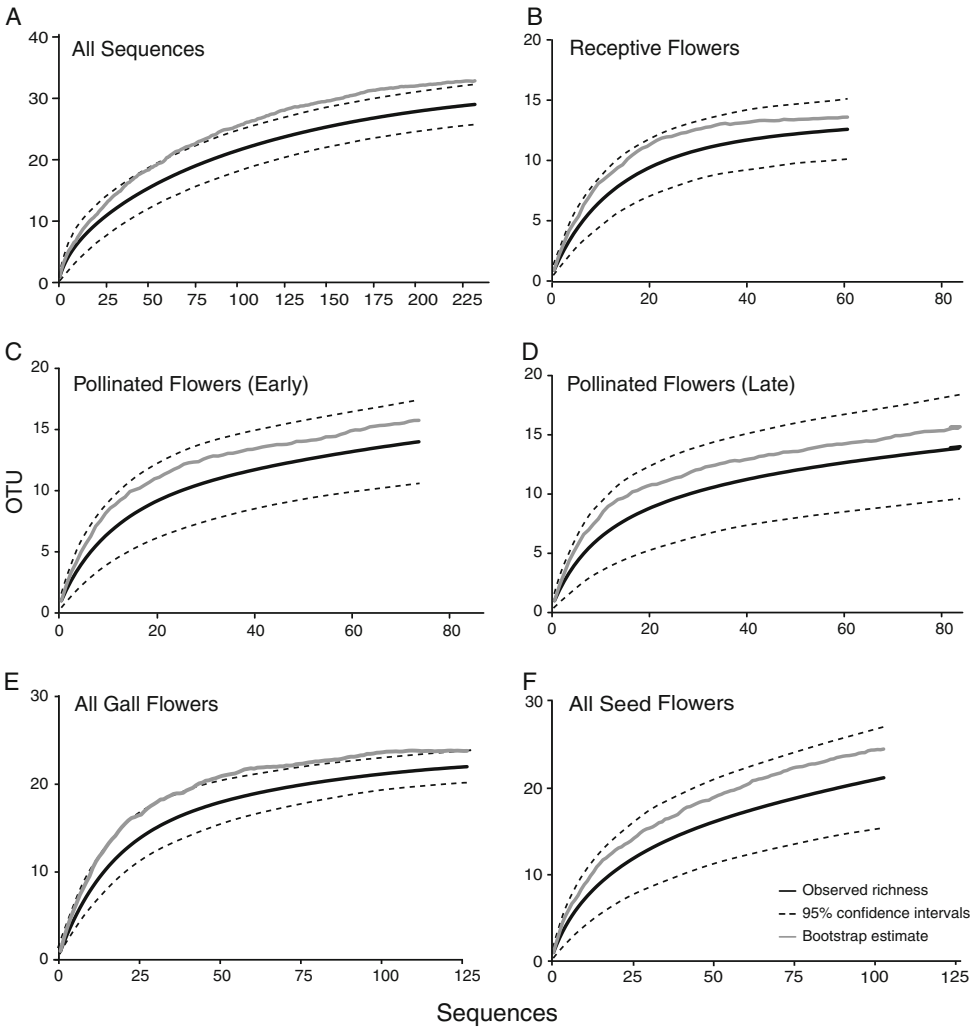

Figure 1 Accumulation of operational taxonomic units (OTU) of fungi from *Ficus* syconia, 95 % confidence intervals, and bootstrap estimates of richness based on ITS-LSU OTU (defined by 95 % sequence similarity) for **a** combined sequences from the entire survey (234 sequences); **b** receptive flowers; **c** early stage pollinated flowers; **d** late stage pollinated flowers; **e** gall flowers (from both receptive and pollinated syconia); and **f** seed flowers (from both receptive and pollinated syconia) of six species of *Ficus* in lowland Panama

seven sequences) and Ascomycota (primarily Saccharomycotina (Saccharomycetes), encompassing 15 OTU and 281 sequences overall and, to a lesser extent, Pezizomycotina, including Dothideomycetes (6 OTU, 20 sequences), Sordariomycetes (two OTU, three sequences), Eurotiomycetes (one OTU, one sequence), and one OTU of uncertain affinity (three OTU, two sequences)). In contrast, leaf- and stem endophyte communities are strongly dominated by Pezizomycotina in lowland Panamanian forests (>98 % of sequences in all surveys to date, with particular dominance by Sordariomycetes, followed by Dothideomycetes and Eurotiomycetes [3–8, 42, Arnold, unpublished data]). None of the common clades of yeasts recovered from syconia has been found in surveys of foliar endophytes in Panama.

Second, all non-singletons obtained from the pollinating wasp survey grouped with phylotypes of fungi known to date only from syconia (Fig. 2, Supplemental Fig. 5). The four singletons from wasps were placed with strong support within a distinctive lineage of syconia-associated yeasts (Fig. 2, Supplementary Fig. 5 clade containing OTU 19–23). This lineage encompasses the most common OTU in our surveys (172 sequences) and includes no sequence with >81 % affinity for any sequence data available through GenBank.

Third, syconia of all species of *Ficus* harbored phylogenetically similar fungal communities (Unifrac analysis of uncollapsed tree containing samples from receptive and pollinated figs: p=0.15–1.00 in presence/absence analyses; p=0.30–1.00 in weighted analyses; Supplementary Fig. 2). No strict-sense host specificity was observed at either the community level or within individual OTU. For example, the most common phylotypes (OTU 21 and 23, Saccharomycotina) occurred in every species, flower type, and developmental stage and also were found in samples from wasps (Fig. 2, Supplemental Fig. 2). Wasps were frequently found with fungi that were not recovered from their natal species of fig and instead were known from other fig species (Fig. 2).

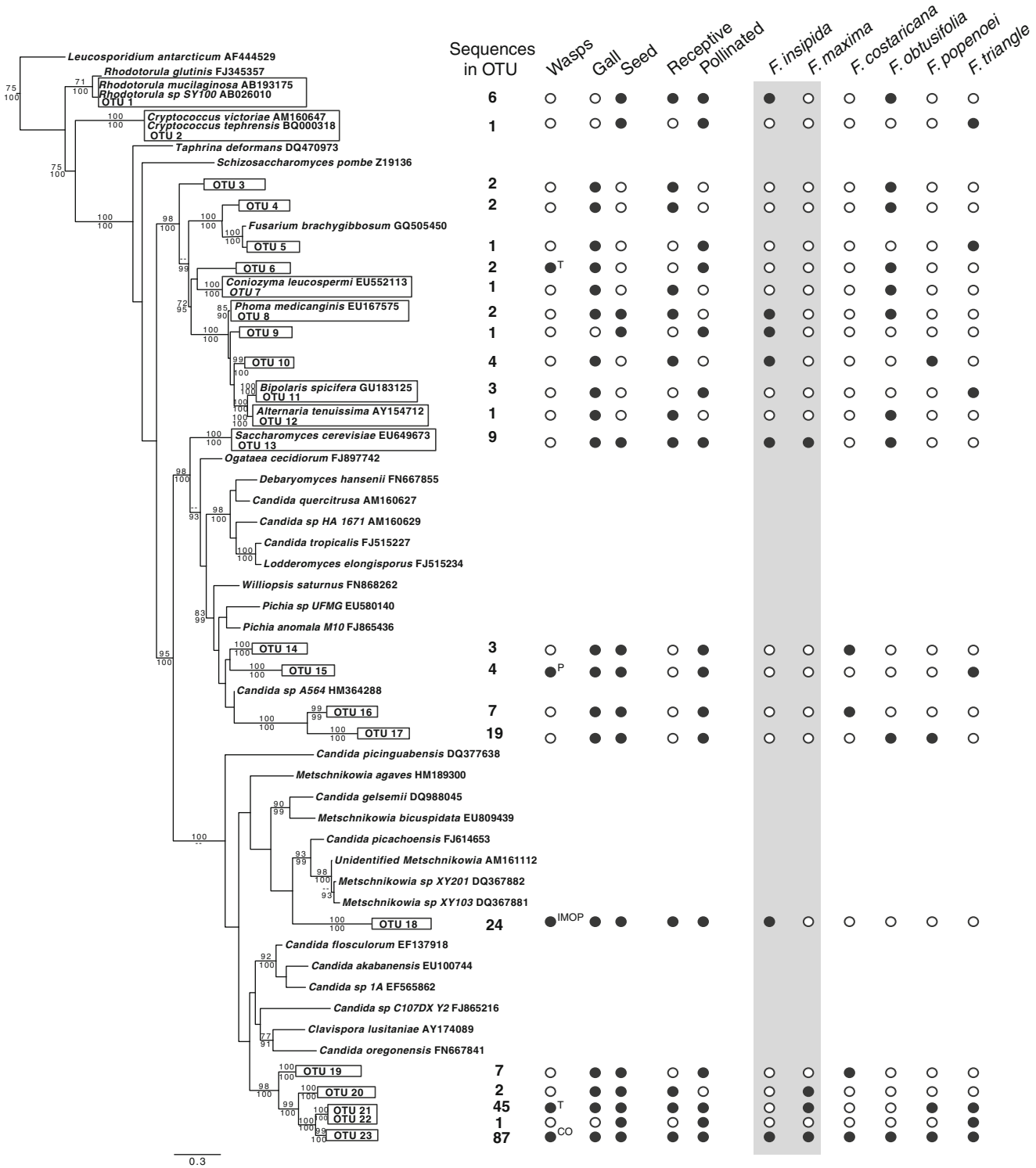

Figure 2 Most likely tree resulting from RAxML analyses of 5.8S and partial LSU sequence data, including results of maximum likelihood bootstrap (≥70 %; *above branches*) and Bayesian posterior probabilities (≥90 %, *below branches*), revealing the phylogenetic placement of fungal OTU recovered from figs in six species of *Ficus* in Panama. *Leucosporidium antarcticum* was included as an outgroup. Accession numbers are listed for all sequences obtained from GenBank. *Black boxes* indicate 95 % sequence similarity groups. *Columns* indicate the number of sequences in each OTU, followed by *darkened circles* that indicate presence of the OTU in the survey of pollinating wasps, each flower type, broad developmental stage, or fig species. *Superscripts in the wasp column* indicate from which *Ficus* species the pollinating wasps were collected: *C=F. costaricana*, *O=F. obtusifolia*, *P=F. popenoei*, *T=F. triangle*, *I=F. insipida*, and *M=F. maxima. Gray area* indicates passively pollinated fig species; all others are actively pollinated

Figure 3 Similarity of fungal communities between **a** early vs. late pollinated flowers, and receptive vs. pollinated flowers, and **b** actively vs. passively pollinated flowers before (receptive) and after (pollinated) visitation by wasps. *Asterisks* indicate significant differences in communities (alpha=0.05). Analyses are based on non-singleton OTU; significance was assessed by ANOSIM of Jaccard's index (based on presence/absence only) and the Morisita index (based on abundance)

Fourth, communities in gall- and seed flowers did not differ significantly in overall phylogenetic composition (Unifrac analysis of uncollapsed tree containing receptive and pollinated figs: p=0.61 in presence/absence analyses; p=0.99 in weighted analyses). Overall, only one non-singleton phylotype was found uniquely in seed flowers (OTU 1, Basidiomycota). All of the Saccharomycotina phylotypes found more than once were found in both seed and gall flowers (N=9; Fig. 2). Most of the non-singleton Pezizomycotina were found only in gall flowers, including OTU that were found both before and after pollination (Fig. 2). However, Pezizomycotina were relatively rare.

Fifth, receptive and pollinated flowers differed significantly in the phylogenetic structure of their fungal assemblages (Table 2). Across the entire dataset, 11 OTU were found only in pollinated flowers (Fig. 2). Seven of these OTU were recovered more than once (OTU 6 and 11, Dothideomycetes; OTU 14–17 and 19, Saccharomycotina). All members of the well-sampled clade containing OTU 14, 15, 16, and 17 (affinity for *Candida* sp. 564; Fig. 2) were found in syconia only after pollination (Fig. 2). This clade was recovered only from actively pollinated figs and from wasps associated with *F. popenoei*, an actively pollinated species.

Table 2 Fungal community structure in fig syconia differed as a function of pollination status and, after pollination, between actively vs. passively pollinated species. Bold indicates p≤0.05. Data are p values from weighted and unweighted analyses of phylogenetic structure in UniFrac, based on the tree shown in Supplemental Fig. 5

	Unweighted for abundance	Weighted for abundance
Pollinated vs. receptive flowers	**0.0100**	0.0880
Actively vs. passively pollinated flowers:		
Receptive flowers	0.5300	0.8030
Pollinated flowers	**0.0030**	**0.0260**

Sixth, pollinated flowers of actively vs. passively pollinated figs differed significantly in the phylogenetic structure of their fungal assemblages (Table 2). Although communities in figs of each type were not mutually exclusive, they differed in relative abundance of several OTU (Supplementary Fig. 2). The clade containing OTU 14–17 was found only in pollinated flowers of actively pollinated species, where it occurred in both gall- and seed flowers.

Discussion

We used a culture-free approach to characterize fungal communities associated with pollinating fig wasps and figs in six species of *Ficus* at four developmental stages in Panama, with special attention to evaluating community structure among fig species, between gall- and seed flowers, and as

Table 3 Fungal communities are more similar between gall and seed flowers in receptive figs than in figs after pollination. Data represent the mean and standard error for all pairwise comparisons among fig species at the receptive stage and after pollination (including early and late post-pollination stages). N indicating the number of comparisons from which means were computed. p values are based on (1) direct comparisons using nonparametric statistics (p_{all}) as shown below and (2) comparisons based on permutations to equalize sample sizes in which 16 similarity values were drawn at random from the pollinated fig data set, and means compared against the observed values from receptive figs. Bold font highlights significant values

	Receptive (N)	Pollinated (N)	p_{all}	p_{rand}
Jaccard	0.22±0.02 (16)	0.11±0.03 (70)	**0.0009**[a]	0.0604[b]
Morisita	0.60±0.09 (16)	0.19±0.04 (70)	**0.0001**[c]	**0.0001**[d]

[a] X^2 =11.1, df=1
[b] t=1.9, df=999
[c] X^2 =14.6, df=1
[d] t=5.1, df=999

a function of developmental stage. To our knowledge this is the first examination of fungal communities associated with non-agricultural figs within their natural range and the first in a tropical forest.

Our survey revealed that fungi were common and taxonomically diverse in apparently healthy syconia of six species of *Ficus* (Table 1). In contrast to Miller and Phaff's [59] conclusion that figs such as Calimyrna have essentially sterile internal tissues prior to visitation by pollinators, we found that fungi were present both before and after visits by pollinators. Many syconia-associated fungi were found in or on fig wasps (Fig. 2, Supplemental Fig. 2). These fungi never were observed in culture-based surveys of the same fig tissue (data not shown), which may suggest specialized nutrient requirements and/or growth conditions.

Fungi associated with syconia and their wasps were distinct at the community level relative to those recorded in foliage and stems of diverse vascular plants in lowland Panama, and in some cases, have been found to date only in association with figs. Fig- and wasp-associated fungal communities were especially rich in several clades of Saccharomycotina that are unique relative to sequenced strains available through GenBank (see clades containing OTU 14-23) and have not been recorded previously in aerial plant parts. The most common yeasts observed here were present in all focal species of *Ficus*, both flower types, and all pre-ripening developmental stages, but we observed marked differences at the community level as a function of receptive vs. pollinated status and, after pollination, as a function of pollination type (active vs. passive; Figs. 2 and 3).

Our OTU-level and phylogenetic analyses reveal that fungal communities did not differ significantly among *Ficus* species prior to pollination, suggesting that fungal communities likely do not play a decisive role in host–species identification or selection by foundresses (Fig. 3 and Table 2). This contrasts with the yeast/cactophilic *Drosophila* system [10], wherein volatiles from individual yeast species selectively attract particular species of flies. Fig wasps require volatile signals produced by the fig tree to identify their host species at the correct developmental stage [44, 85]. Similar communities of yeasts in different species of *Ficus*, which change markedly after pollination, may provide a non-species-specific indicator to fig wasps that syconia are receptive, perhaps via amplification of the fig's volatile repertoire [as in the case of nectar-inhabiting yeasts and some foliar endophytes; 23, 34, 46, 69]. The source of the pre-pollination fungal community is currently unknown. It is possible that fungi of the same genotype or OTU differ in functional traits or could be distinguished at loci that evolve more quickly than this portion of the ribosomal repeat [16, 65]. However, previous studies indicate that variation in the ITS region can distinguish yeast species [e.g., 18] and some have used only LSU data to conclude that the same yeast was present in different life stages of a focal insect [e.g., 33]. In future work, we will assess whether members of clades that appear to be closely related based on the analyses presented here differ functionally among fig species.

Similarly, our surveys revealed that gall- and seed flowers had similar communities before pollination, leading us to suggest that fungi likely are not the drivers of oviposition choice between these flower types (Table 3 and Supplemental Fig. 6). The occurrence of Pezizomycotina preferentially in gall flowers is intriguing, but in general these fungi were found at low abundances, such that it is difficult to distinguish rarity from apparent specificity. At present, we conclude that the presence, absence, and composition of fungal assemblages studied here do not appear to contribute to "unbeatable seeds" in figs [sensu 88].

By decoupling timing from developmental stage, our study reveals that fungal communities differ markedly among developmental stages of figs, with three results of note. First, communities differed significantly in receptive vs. pollinated figs (Fig. 3). These observations are consistent with a possible role of fungi in providing volatile cues that indicate a termination of receptivity for pollinators and an onset of ripeness for frugivorous seed dispersers.

Second, communities in gall- and seed flowers differed following pollination, consistent with the introduction of fungi by female wasps (Table 3). Fig-pollinating wasps live an average of 2 to 3 days after leaving the natal fig [26, 52], spending most of that time in the airstream above the forest canopy in search of compatible fig species in a receptive state. They do not ingest any plant material during this time [21]. Thus fungi recovered from pollinating wasps and pollinated fig flowers may be transmitted by fig wasps from their natal syconia. We found that wasps frequently carried fungi that had been found in the syconia of species that were not their natal species but were present in other *Ficus* surveyed. This could reflect undersampling of the fungal community in the syconia; however, our sampling reached statistical sufficiency for the figs evaluated here. Thus our observations are consistent with pollinator mixing among closely related figs that share the same pollination syndrome [57, 61] and suggest host–species generalism of many yeasts recovered here.

Third, fungal communities differ in pollinated flowers of passively and actively pollinated fig species, which have been separated by at least 60 million years in the New World clades (Fig 3) [56, 71]. This is consistent with introduction and maintenance of different fungal communities by wasps of each clade, which may frequently host-switch within the actively and passively pollinated clades but not between the two clades [57, 61, Machado, unpublished]. However, some fungi do occur in figs of both clades; mechanisms by which they

may occur in both, even when pollinators do not mix, remain to be resolved. Notably, some fungi found in receptive flowers were allied phylogenetically with the Basidiomycetous yeast *Rhodotorula mucilaginosa*, which was cultured from a cultivated fig in Japan (*Ficus carica* cv. Masui-Dofin and Horaishi) [36]. This suggests a geographically and taxonomically broad association with figs that merits further study.

Our study provides a first evaluation of the diversity and affiliations of microfungi in developing figs and their pollinating wasps. Although strong evidence was not obtained to indicate a fungal role in host identification or oviposition preference, the differences observed here for receptive and post-pollination figs, and figs with different pollination syndromes, set the stage for exploring their interactions with the iconic fig- and fig–wasp partnership. Increasingly the study of mutualisms has been expanded to include additional participating members, rather than the bipartite interactions of mutualistic partners alone [8, 22, 77]. The classic fig–fig wasp mutualism operates in the context of the microbial associations of each partner, which may play yet unexplored but important roles in this otherwise well-studied association.

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