Bacterial endosymbionts influence host sexuality and reveal reproductive genes of early divergent fungi.
Bacterial endosymbionts influence host sexuality and reveal reproductive genes of early divergent fungi

Many heritable mutualisms, in which beneficial symbionts are transmitted vertically between host generations, originate as antagonisms with parasite dispersal constrained by the host. Only after the parasite gains control over its transmission is the symbiosis expected to transition from antagonism to mutualism. Here, we explore this prediction in the mutualism between the fungus *Rhizopus microsporus* (*Rm*, Mucoromycotina) and a beta-proteobacterium *Burkholderia*, which controls host asexual reproduction. We show that reproductive addiction of *Rm* to endobacteria extends to mating, and is mediated by the symbiont gaining transcriptional control of the fungal *ras2* gene, which encodes a GTPase central to fungal reproductive development. We also discover candidate G-protein-coupled receptors for the perception of trisporic acids, mating pheromones unique to Mucoromycotina. Our results demonstrate that regulating host asexual proliferation and modifying its sexual reproduction are sufficient for the symbiont’s control of its own transmission, needed for antagonism-to-mutualism transition in heritable symbioses. These properties establish the *Rm-Burkholderia* symbiosis as a powerful system for identifying reproductive genes in Mucoromycotina.
Heritable mutualisms are a source of major evolutionary innovations\(^5\). However, their evolution remains elusive. Evolutionary theory suggests that many heritable mutualisms originate as antagonisms in which parasite dispersal is controlled by the host\(^6\). A transition to mutualism requires the parasite to dominate the coevolutionary race with the host by establishing control over its own transmission. However, few symbioses exist where this prediction can be explored. One such system is the mutualism between a soil fungus *Rhizopus microsporus* (Rm, Mucoromycotina) and a beta-proteobacterium *Burkholderia*, which controls asexual proliferation of its host\(^3\). Like many other Mucoromycotina, the Rm hosts of *Burkholderia* thrive as soil saprotrophs. They can cause food spoilage, infect plants\(^4\), and act as opportunistic pathogens of immune-compromised humans\(^8\). While the evolutionary history of the Rm-*Burkholderia* symbiosis is uncertain, present-day antagonistic interactions of *Burkholderia* endobacteria with nonhost Rm isolates naturally free of endobacteria\(^3\) suggest that it originated as an antagonism. In the Rm-*Burkholderia* mutualism, the partners can be separated, cultivated independently, and reassembled to form a functional symbiosis in which the endobacteria reside directly in the host cytoplasm\(^3\). *Burkholderia* cells are transmitted via sporangiospores, which are asexual propagules produced by the host\(^3\). Sporangiospores are generated continuously throughout colony growth in favorable environmental conditions, disperse asexually, and germinate rapidly.

In addition to asexual propagation, fungi, like most other eukaryotes, engage in sexual reproduction. In *Mucoromycotina*, sex involves the union of gametangia, leading to the formation of a zygospore\(^7\). In heterothallic species, such as *Rm*, two compatible strains, sex *plus* (sexP) and sex *minus* (sexM), are required for mating to be successful\(^7\). Partner recognition and progression of mating are mediated by trisporic acids and their precursors\(^8,\)\(^9\), which act as sex pheromones and are synthesized in a cooperative manner from intermediates provided by the complementary mating partner\(^10\). Due to their recalcitrance to genetic analysis and manipulation, *Mucoromycotina* are one of the least explored major lineages of fungi, with only few reproductive genes characterized functionally thus far\(^11\). To test the hypothesis that the *Burkholderia* endobacteria control sexual reproduction of the *Rm* host and identify the control mechanism, we mated fungi that harbored endosymbionts or were cured of them, followed by transcriptional profiling and phylogenomic analyses utilizing the wealth of information on sexual reproduction in *Dikarya*, a lineage uniting Ascomycota and Basidiomycota. We discovered that endobacteria modify sexual reproduction of *Rm*, and generated insights into the reproductive biology of *Mucoromycotina*.

### Results

#### Diversity and natural loss of endobacteria.

Previous observations indicated that only a single bacterial cell was present per sporangiospore in one of the *Rm* strains, CBS112285\(^2\). We found that bacterial loads in other strains differ, varying from on average three cells per sporangiospore in strain ATCC 52813 to four in ATCC 52814 (Supplementary Fig. 1). This observation suggests phenotypic diversity among host–symbiont pairings.

The role of endobacteria in asexual proliferation of *Rm* was evidenced by the loss of sporulation in mycelia treated with antibiotics that eradicate *Burkholderia*\(^3\). We observed that endobacteria and asexual reproduction can be lost spontaneously after as few as four generations of propagating the fungus via single spores, and after 12 generations of propagation via mycelial fragments (Supplementary Table 1). These patterns indicate that in nature, hosts can become naturally cleared of their endosymbionts, and the loss of sporulation can be attributed to endosymbiont absence.

#### Not all zygospores are populated by endobacteria.

All asexual sporangiospores formed by *Rm* strains hosting endobacteria appear to harbor the symbiont. To test whether the same is true for sexually produced zygospores, we mated *Rm* strains ATCC 52813 sexP and ATCC 52814 sexM, which both naturally contain

![Fig. 1](image-url) Impact of *Burkholderia* endobacteria on the reproductive biology of the *Rm* host. Successful mating between sex-compatible *Rm* B(+) strains: a ATCC 52813 and ATCC 52814, and b ATCC 52813 and ATCC 52811. c Complete loss of mating between B(−) isolates ATCC 52813 and ATCC 52814. d Restricted mating between B(−) isolates ATCC 52813 and ATCC 52811, with zygospores formed in the area indicated by an arrow. Accumulation of zygospores and β-carotene in the zone of interaction between B(+) mates: e ATCC 52813 and ATCC 52814, scale bar 100 μm, and f ATCC 52813 and ATCC 52811, scale bar 100 μm. g No sexual structures or β-carotene are apparent in the zone of interaction between B(−) mates ATCC 52813 and ATCC 52814 that show total loss of mating; scale bar 500 μm. h Rare zygospores produced during an interaction between B(−) mates ATCC 52813 and ATCC 52811 that resulted in restricted mating; scale bar 100 μm. i Effects of endobacteria and exogenous dibutyryl cAMP on the formation of zygospores during mating between B(+) ATCC 52813 and ATCC 52814 vs. mating between B(−) ATCC 52813 and ATCC 52814 showing limited zygospore formation. Increased concentrations of cAMP reduced the rate of zygospore formation in interactions between B(−) mates that were capable of restricted mating (Student post hoc test of the interaction between bacterial presence and cAMP level in two-way ANOVA, *P* = 0.02), whereas the decrease in zygospore formation in interactions between B(+) mates was not statistically significant. Ten mating interactions were examined per condition. B(+), endobacteria present; B(−), endobacteria absent; error bars represent s.e.m.
endobacteria in their mycelia. We then surveyed zygospores for bacterial presence by PCR targeting their 23S rRNA gene. *Burkholderia* was detected in 40% (±6 s.e.m.) of zygospores, suggesting that the rate of symbiont transmission through the sexual pathway is substantially lower than through the asexual pathway.

**Endobacteria modify fungal mating.** To test the hypothesis that *Burkholderia* endobacteria control sexual reproduction of *Rm*, we examined the interactions of wild-type B(+) strains that harbored endobacteria and B(−) isolates that were cured of endosymbionts: (1) ATCC 52813 sexP with ATCC 52814 sexM, (2) ATCC 52813 sexP with ATCC 52811 sexM, and (3) ATCC 62417 sexP with ATCC 52811 sexM. We found that in all pairs, bacteria impacted fungal ability to reproduce sexually. They either controlled it completely, with removal of endobacteria leading to total loss of mating, or incompletely, with removal of endobacteria leading to a reduced zygospore yield (Fig. 1). These two outcomes did not appear to be specific to the strains that were mated. Moreover, loss of mating was not a consequence of vigor reduction in cured isolates, as, with the exception of ATCC 62417, which grew poorly after the loss of endobacteria, the differences in the rate of mycelial expansion between the B(−) and B (+) mycelia were largely negligible (Supplementary Fig. 2).

**Attempts to restore fungal mating.** To examine whether the loss of host reproduction upon removal of endobacteria was reversible, we investigated the consequences of reintroducing endosymbionts into the cured host isolates that exhibited total loss of mating. Reintroduction of endobacteria restored both asexual and sexual reproduction regardless of whether bacteria were introduced into their original native hosts or nonnative hosts, with all pairwise combinations of source bacteria and target fungal strains across ATCC 52811, ATCC 52813, ATCC 52814, and ATCC 62417 yielding reproduction. However, asexual reproduction was restored immediately after reinfection, restoring the ability to mate required an additional step of exposing the reinfected isolates to extreme cold (−80 °C), which eliminated fungal hyphae while preserving sporangiospores. This observation suggested that there was a subtle mechanistic difference in how endosymbionts interact with asexual vs. sexual reproduction of the fungus. We also attempted to restore *Rm* sexual reproduction in the absence of endosymbionts by exposing compatible B(−) mates to extracts of mated mycelia and to environmental conditions known to affect reproduction in Mucoromycotina (Supplementary Table 2). None of these treatments restored mating or asexual propagation in cured fungi, suggesting that for reproduction, the host is addicted to bacterial factors.

**Gene networks responsible for mating in Mucoromycotina.** To investigate the genetic underpinnings of fungal reproductive responses to endobacteria, we conducted an RNA-seq experiment assessing global gene expression patterns under six different conditions: (1) *Rm* ATCC 52813 B(+) grown alone, (2) ATCC 52813 B(−) grown alone, (3) ATCC 52814 B(+) grown alone, (4)
candidate trisporic acid receptors. Present; B(+)-mates measured in three replicate cultures per time point. Error bars represent s.e.m. Rm (orange) and fi et al.6) to facilitate mapping of RNA-seq reads to each host.

Because the knowledge of molecular mechanisms underlying processes in these fungi.

ATCC 52814 B(−) grown alone, (5) both B(+) mates grown together, and (6) both B(−) mates grown together, with sexual reproduction completely absent. We sequenced the genomes of

We found that in Rm, 2124 genes were differentially expressed (DE) at a significant level during sexual reproduction relative to asexual growth and sporulation, with 1496 genes upregulated and 628 downregulated (false-discovery rate (FDR) corrected \( P \leq 0.05 \), identified using DESeq12; Supplementary Data 1, Supplementary Data 4, respectively). Candidate trisporic acid receptor.

Expression levels of the candidate tar genes in Rm. Three biological replicates were examined per condition. B(+)-endobacteria present; B(−)-endobacteria absent; error bars represent s.e.m. Changes of the candidate tar gene expression patterns during the progression of mating in Rm B(+) mates measured in three replicate cultures per time point. Error bars represent s.e.m.

ADHESION GPCR D1 AGRD1_HUMAN (SW:Q65N62)

G Protein coupled bile acid receptor 1 GPBAR_HUMAN (SW:Q14836)

Free fatty acid receptor 4 FFAR4_HUMAN (SW:Q65N62)

G Protein coupled bile acid receptor 1 GPBAR_HUMAN (SW:Q8TDU6)

Fig. 3 Candidate trisporic acid receptors are class C GPCRs. a) Maximum-likelihood phylogeny of seven-transmembrane domains from early-diverging fungi (orange) and five different classes of G-protein-coupled receptors (GPCRs): A (purple), B1 (light blue), B2 (dark blue), F (pink), as well as the fungal specific class D pheromone receptors (green). Fungal protein identifiers include species and strain designation, as well as GenBank accession number or JGI protein ID. Nonfungal proteins have GPCRdb74 identifiers and UniProtKB75 (SW) accession numbers. Support values are displayed above branches. The GPCR sequence alignment and the complete phylogeny include are supplemented in Supplementary Data 3 and Supplementary Data 4, respectively. TAR, candidate trisporic acid receptor. B(a) expression levels of the candidate tar genes in Rm. Three biological replicates were examined per condition. B(+)-endobacteria present; B(−)-endobacteria absent; error bars represent s.e.m. C) Changes of the candidate tar gene expression patterns during the progression of mating in Rm B(+) mates measured in three replicate cultures per time point. Error bars represent s.e.m.

We found that in Rm, 2124 genes were differentially expressed (DE) at a significant level during sexual reproduction relative to asexual growth and sporulation, with 1496 genes upregulated and 628 downregulated (false-discovery rate (FDR) corrected \( P \leq 0.05 \), identified using DESeq12; Supplementary Data 1, Supplementary Data 4, respectively). Ortholog clustering revealed that, while many of these genes were conserved across most fungi (57.95%), a substantial proportion were either restricted to Mucoromycotina (42.04%) or even unique to the Rm lineage (11.67%); Fig. 2, Supplementary
receptors (GPCRs) are involved in the perception of mating pheromones in other fungi\(^ {17} \) and animals\(^ {18} \), we explored \(Rm\) genes upregulated during sexual reproduction in search of GPCRs unique to the Mucoromyotina lineage. We found three such candidate trisporic acid receptor (\(\text{t}ar\)) genes, encoding class C seven-transmembrane domain GPCRs, that were present in the genomes of \(ATCC\ 52813\) and \(ATCC\ 52814\) (Fig. 3a). Through qRT-PCR, we confirmed that expression of these three candidate genes was also upregulated during mating interactions of a different pair of \(Rm\) mates, \(ATCC\ 52813\) and \(ATCC\ 52811\) (Fig. 3b). In addition, we established that the expression pattern of each gene changes during the progression of mating (Fig. 3c). Through phylogeny reconstructions, we determined that these \(G\)PCRs cluster with animal \(\gamma\)-aminobutyric acid (\(\text{GABA}\)) receptors, a group related to animal retinoic acid-inducible class \(C\) GPCRs (Fig. 3a).

**Effects of endobacteria on regulation of \(Rm\) mating.** Only 80 of the 2124 \(Rm\) sex-related transcripts were impacted by the symbiont presence in the hosts (Supplementary Fig. 3, Supplementary Data 1). One of the most striking observations was an \(-12\)-fold downregulation in the absence of endobacteria of an ortholog of \(\text{ras}\) 2, which encodes a small GTPase protein. Furthermore, \(\text{ras}\) 2 was downregulated in fungi cured of bacteria growing alone vegetatively and upregulated during active asexual proliferation. Despite the expansion of the \(\text{ras}\) gene family in Mucoromyotina
relative to Dikarya (Fig. 4a), only the expression of the Rm ras2-1 gene (protein ID 223475 and 28648 in Rm ATCC 52813 and ATCC 52814, respectively) was affected by endobacteria. Ras2 is conserved across all fungi except Ascomycota yeasts (Fig. 4a). In the basidiomycete Ustilago maydis, it controls the initiation of the mating pheromone MAPK cascade19. Ras2 is also involved in asexual reproduction, as shown in ascomycetes in which repression of ras2 has a negative impact on sporulation20, 21. To examine whether the role of ras2-1 extends to mating in other Mucoromycotina, we conducted qRT-PCR on vegetative mycelia vs. mating interactions involving ATCC 1216b sexP and CBS 277.49 sexM strains of Mucor circinelloides, and found that expression of ras2-1 was elevated during mating relative to vegetative growth (Fig. 4b). Finally, we examined expression levels of ras2-1 in mating interactions between Rm ATCC 52813 and ATCC 52811 focusing on patterns exhibited by cured mates that experience total loss of mating vs. restricted mating. Like in the RNA-seq experiment, the levels of ras2 expression were lower in interactions between B(−) mates compared to those between B (+) mates (Fig. 4c). However, they did not differ between interactions of B(−) mates exhibiting total loss of mating vs. restricted mating (Fig. 4c), a pattern consistent with incomplete control of mating by the endobacteria.

In addition to regulating the pheromone MAPK cascade, in U. maydis, Ras2 interacts with the cyclic adenosine monophosphate (cAMP) signaling pathway and controls morphogenesis19. cAMP is a secondary messenger that, in coordination with the pheromone MAPK cascade, affects sexual development in many fungi, albeit often with contrasting effects22. We explored the impact of exogenous cAMP on Rm mating by exposing B(+) and B(−) mates to 0 mM, 1 mM, and 2 mM dibutyryl cAMP. We found that increased concentrations of cAMP reduced the rate of zygospore formation in the interactions between B(−) mates that were capable of restricted mating (Student post hoc test of the interaction between bacterial presence and cAMP level in two-way ANOVA, P = 0.02), whereas the decrease in zygospore formation in interactions between B(+) mates was not statistically significant (Fig. 1i). These results suggest that elevated levels of cAMP interfered with mating in Rm, and the endosymbiont presence buffered the negative effects of high cAMP levels on sexual reproduction.

Discussion

We found that Rm is highly dependent for survival on the Burkholderia endobacteria. Although the fungus can grow vegetatively after endobacteria are lost naturally or eradicated with antibiotics23, it is unable to proliferate asexually and its ability to reproduce sexually is severely compromised. These patterns indicate that in the Rm-Burkholderia mutualism, the endosymbiont controls its own vertical transmission, which is a prerequisite for the antagonism-to-mutualism transition in heritable symbioses2. Remarkably, less than half of zygospores formed during mating interactions of wild hosts harbor endobacteria. However, as zygospore germination is extremely difficult to achieve under laboratory conditions24, it remains untested whether zygospore functionality is affected by endosymbiont absence. Zygospore germination occurs via a sporangium resembling the asexual sporangium. Therefore, it cannot be excluded that endobacteria-free zygospores may fail to germinate, further reinforcing endosymbiont dominance over host reproduction. Endosymbiont ability to control its own transmission is expected to facilitate reciprocal selection between the partners, leading to utilization of symbiont services by the host2. In the Rm-Burkholderia symbiosis, these services include endosymbiont-mediated synthesis of rhizoxin, a potent toxin that enables pathogenesis of plants by Rm25, 26.

In addition to facilitating the antagonism-to-mutualism transition, the role of endobacteria in regulating Rm asexual and sexual reproduction is consistent with the addiction model of mutualism evolution27, 28. According to this model, a host population that interacts with an antagonistic symbiont should develop mechanisms to compensate for its negative effects and become addicted to the symbiont’s continued presence2. In the case of the Rm-Burkholderia symbiosis, endobacteria have hijacked an indispensable component of the host’s developmental machinery by gaining control over expression of ras2-1, encoding a G-protein responsible for the reproductive development in Dikarya29, 30. The exact mechanism of bacterial control over the ras2-1 expression and the evolutionary trajectory that lead to it are unknown. However, as stimulation of ras signaling induces programmed cell death in other fungi31, 32, it is possible that in the ancestrally antagonistic relationship between Rm and Burkholderia33, establishing control over the ras2-1 expression by endosymbiosis was an important component of coevolution between partners, leading to adaptive changes in host regulation of ras2-1 signaling (Supplementary Fig. 4). Evolutionary theory predicts further that once a mutualism is established, the host is favored to control mixing of symbionts29. Such control is expected to reduce harmful competition among symbionts for the host resources. It remains to be investigated whether endobacteria are able to mix in the Rm-Burkholderia symbiosis.

We exploited the endosymbiont control over Rm reproduction to reconstruct the key reproductive pathways across the fungal kingdom, including Mucoromycota, Ascomycota, and Basidiomycota. Since many of these genes were experimentally studied in Dikarya, we were able to augment our findings with information on conservation of their sex-related function. Using this approach, we uncovered candidate genes that may be involved in perception of trisporic acid pheromones in Mucoromycotina. Unlike class D GPCRs responsible for pheromone sensing in Dikarya30, which are absent from Rm, these candidate receptors appear to represent class C GPCRs. They are encoded by genes that are conserved across all Mucoromycotina, upregulated during sexual reproduction in R. microsporus, absent from higher fungi, and closely related to retinoic acid-sensing GPCRs in animal systems. Similar to trisporic acid, retinoic acid is derived from β-carotene and is essential for the initiation of meiosis in animals31, 32. Further functional analyses are now required to test the hypothesis that these C GPCRs interact with and transduce trisporic acid pheromone signals.

Overall, our findings indicate that in the Rm-Burkholderia symbiosis, endobacteria control their vertical transmission by regulating asexual proliferation of the host and impacting its mating. Such control appears to be sufficient to have mediated the antagonism-to-mutualism transition in this heritable symbiosis. Symbiont presence correlated with expression levels of the host ras2-1 gene, a major regulator of both sexual and asexual reproduction in fungi, suggesting that endobacteria influence its activity. Finally, we took advantage of the symbiont impact on host mating to make inferences about reproductive genes in Mucoromycotina, a group of fungi recalcitrant to genetic analysis. In the process, we discovered candidate trisporic acid receptors, TARs, that may be responsible for perception of trisporic acid sex pheromones uniquely utilized by this group of fungi.

Methods

Symbiont strains, culture conditions, and loss of endobacteria. Strains ATCC 5281, ATCC 52813, ATCC 52814, and ATCC 62417 were cultivated on half (1/2) or full-strength potato dextrose agar (PDA) containing 2 g L−1 potato extract, 10 g L−1 dextrose, and 15 g L−1 agar. Plates were sealed with Parafilm M (Pechiney Plastic Products, Chicago, IL, USA) with mild compression, and incubated at 22 °C under constant light conditions. Conditions in the light provide sufficient illumination for mycelial growth, allowing the symbionts to colonize the PDA surface. For loss of endobacteria, mycelial plugs (1 cm diameter) from the edge of actively growing ATCC 52813 and ATCC 52814 colonies were transferred to half-strength PDA without endobacteria and incubated in the light for 8 weeks. Mycelial plugs from these agar plates were transferred to fresh half-strength PDA and incubated in the dark for 3 weeks to prevent light-mediated growth of endobacteria. Mycelial plugs from the endobacteria-free agar plates were then transferred to fresh half-strength PDA in the dark and incubated for 3 more weeks to confirm the absence of endobacteria.

Determination of endoglycosidase activity. Endoglycosidase activity was determined by using cell walls from mycelial plugs grown at 22 °C in the light on half-strength PDA containing 1 g L−1 dextrose and 2 g L−1 potato extract. Mycelial plugs were removed from the agar plates and placed into 25 mL Eppendorf tubes containing 1 mL of half-strength PDA without endobacteria. Mycelial plugs from the endobacteria-free agar plates were then transferred to fresh half-strength PDA in the dark and incubated for 3 more weeks to confirm the absence of endobacteria.
Cotton endobacteria with antibiotics as described in Partida-Martinez and Packaging Company, unless otherwise noted. In addition to curing fungi of NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-02052-8 ARTICLE Hertweck23, we experimented with the impact of their subculturing by individual of 16 negative controls. PCR was performed on the 1/20-diluted WGA products for multiple PCR reactions per zygospore. A volume of 1 ampli.

Visualization of endobacteria in DNA was then transferred onto a sterile 1.5% water agar and zygospores placed in 10% w/v chloramine T (Sigma) for 20 min to kill hyphae. The tuft was ATCC 52813 and ATCC 52814 were conducted on PDA (Sigma) as described bacterial Burlholderia. Fungal RNA was removed with the Human/ Mouse/Rat Ribo-Zero RNA Removal Kit (Epipemic), whereas bacterial RNA was treated with the Gram-negative bacteria Ribo-Zero RNA Removal Kit (Epipemic). After RNA removal, sequencing libraries were constructed using the TruSeq RNA Sample Preparation Kit (Illunina) and sequenced at the Cornell University Bio-technology Resource Center using the Illumina Hi-Seq 100 bp-paired end platform.

RNA-seq. Six different conditions were compared in Rm ATCC 52813 and ATCC 52814: B(+) and B(−) mates grown alone, as well as B(+) and B(−) partners grown together, respectively. We chose to analyze the interaction between B(−) mates that exhibited total loss of mating to maximize our chances of identifying genes impacted by bacteria during sexual reproduction. For each condition, fungal plugs were placed at the edge of the 1/2 PDA plate and harvested after six days, when opposite B(+) mates were undergoing sexual reproduction. Each condition had two biological replicates, each consisting of five culture plates, which were pooled prior to RNA extraction. Total RNA was extracted from a 2.5-cm-wide strip of mycelium from the middle of the plate where most mating occurred, using the Ambion ToTALLY Total RNA Isolation Kit (Life Technologies) to recover both bacterial and fungal transcrips. Fungal RNA was removed with the Human/ Mouse/Rat Ribo-Zero RNA Removal Kit (Epipemic), whereas bacterial RNA was treated with the Gram-negative bacteria Ribo-Zero RNA Removal Kit (Epipemic). After RNA removal, sequencing libraries were constructed using the TruSeq RNA Sample Preparation Kit (Illulina) and sequenced at the Cornell University Bio-technology Resource Center using the Illumina Hi-Seq 100 bp-paired end platform.

illuminas reads were quality controlled using BBduk (https://sourceforge.net/ projects/bbmap/), and then mapped to either the Rm ATCC 52813 or ATCC 52814 genomes (depending on the sample) using TopHat227. HTSeq was used to collect gene expression data per sample, followed by exploration of differential expression across several comparisons using DESeq2; (1) ATCC 52813 B(+) vs. ATCC 52813 B(−), (2) ATCC 52814 B(+) vs. ATCC 52814 B(−), (3) both B(+) mates grown together (active mating) vs. both B(−) mates grown together (no mating), and (4) both B(+) mates grown alone vs. both B(+) mates grown together (active mating). This approach enabled us to identify genes that were significantly DE in both mates due to (1) endobacteria presence and (2) sexual reproduction, as well as assess the overlap between them. For comparisons involving both mates, we mapped reads to a combined assembly of both genomes, and since these strains are so closely related, we allowed reads mapped twice to be retained for downstream Deseq analysis. Genes with an adjusted P < 0.05 were considered DE.

qRT-PCR. Matting interactions were set up as above between Rm ATCC 52813 sexP and ATCC 52811 sexM. Expression of ras2-1 was examined in (1) B(+) mating cultures, (2) cultures of B(−) mates exhibiting complete loss of mating, and (3) cultures of B(−) mates exhibiting restricted levels of mating. Expression of candidate tar1, tar2, and tar3 genes was measured in (B(+) and B(−) mates grown alone, as well as during mating interactions, with a particular focus on B(+) mating effect; or on mating cultures that reached three lines of contact. We mapped reads to a combined assembly of both genomes, and since these strains are so closely related, we allowed reads mapped twice to be retained for downstream Deseq analysis. Genes with an adjusted P < 0.05 were considered DE.

rDNA-internal transduction and cultivation of endobacteria. Young fungal mycelium (1–2 days old) containing endobacteria was finely chopped in 500-μL Luria-Bertani (LB) broth, and pressed gently to release cellular contents into the broth, followed by filtration through a 2-μm filter to remove fungal debris. Varying amounts of filtrate were added to LB plates containing 10 mL 5°C. glycerol and 100 mg mL−1 cycloheximide. Single colonies were isolated and grown at 30°C either on LB agar or in 5-mL LB broth incubated at 250 rpm. To reinfect fungi with endobacteria, a plug of agar was removed from 1/2 PDA using the upper end of a P-1000 pipette and replaced with a plug of LB. Bacterial inoculum was placed on the LB agar plug, and a plug containing caged fungus was either positioned directly on bacterial cells, or somewhere nearby on the plate.

Detection of endobacteria in Rm zygospores. Mating interactions between Rm ATCC 52813 and ATCC 52814 were conducted on PDA (Sigma) as described above, and zygospores were analyzed after 7 days of incubation in the dark at 30°C. A tuft of mycelium containing zygospores was removed from the mating zone and placed in 10% w/v chloramine T (Sigma) for 20 min to kill hyphae. The tuft was subsequently transferred to sterile water and shaken slowly for 5 min at room temperature, followed by two additional water washes lasting for 20 min. The mycelial tuft was then transferred onto a sterile 1.5% water agar and zygospores were collected with sterile forceps, taking care to remove all attached hyphae. A total of 80 zygospores from two separate mating interactions were transferred individually into 0.2-mL PCR tubes, crushed, and subjected to whole-genome amplification (WGA) with the Illustra GenomiPhi Kit v2 (GE) to generate template DNA libraries. The resulting WGA products per zygospore were pooled to a volume of 1 mL of 10 μL of PCR master mix. The qRT-PCR experiment was performed in triplicate. Probes and primers were Rm ras2-1 TaqMan® probe 5′-6 (5′-6) AGT CTT CCT GAC TAG TGT TAA A-3′, Rm ras2-1 reverse primer 5′-GCC GAA AAG CAT TTT TCA TGT A-3′, Rm ras2-1 forward primer 5′-CCT TCC GCG TGC TTC ATC TTA TGT AGA A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GCG TGC TTC ATC TTA TGT AGA A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′. Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 forward primer 5′-GCC GTC TGT ATC TTA TGT A-3′, Rm ras2-1 reverse primer 5′-GCC GTC TGT ATC TTA TGT A-3′.
reverse primer 5′-TGC TGA CTT CTC GAC GAA AT-3′. Rm ATCC 52813 gene encoding hypothetical protein 72589 served as an internal standard for ras2-1 and tar normalization, with TaqMan® probe 5′-[FAM]-AG TGG TGC TTT FTA ACA CGG [Tamra-Q3], forward primer 5′-AGG AAT TTA TCT GCA AAA ATA TGA A-3′, and reverse primer 5′-GAT CCC ACG CAG AGA AGC AT-3′. This gene was not affected by the presence/absence of endobacteria and displayed a high level of constitutive expression in the RNA-seq data. Mc actin gene (105861) was also used for normalization in Mc ras2-1 reactions with TaqMan® probe 5′-[FAM]-AG TGC AAC TGT TCT GTC ACTT [Tamra-Q3], forward primer 5′-GCA GGA ATC ACA AAA CGT ATG AAC-3′, and reverse primer 5′-GGT GTG TAT CGC CTG CAT TTC C-3′.

Identification of sex-relevant genes across fungi: To identify sex-related genes conserved across fungi, we queried the genomes of Saccharomyces cerevisiae YJ7895, Schizosaccharomyces pombe 927h4, Aspergillus nidulans FGSC A4, Coccidiobolus histolitica C4H, Neurospora crassa OR74A (N150), Ustilago maydis 521, Cryptococcus neoformans var. neoformans B-5001A, Batrachochytrium dendrobatidis JAM1 (genome-gji.doe.gov), Mucor circinelloides CBS 277.49, Phycomyces blakesleeanus NRRL15555, R. delemar 99-880-86, and Rm ATCC 52813 (genome.gij doe.gov) and ATCC 52814. We additionally included transcriptomic data from Rhizopus disappearrrris DAOM181602. OrthoMCL90 was conducted using default parameters (minimum E value cutoff of 1e-5, inflation 1.5) to cluster genes across all of these genomes and orthologous groups. From the clustering of S. cerevisiae, H. polymorpha, N. crassa, and U. maydis, we extracted a collection of genes with also validated in reproductive phenotypes as a tool for further characterization of the resulting clusters, and to assess whether these known genes were also altered in expression due to reproduction in Rm. For Ras phylogeny reconstruction, full-length amino acid sequences were aligned using MUSCLE12 under default parameters, and the phylogeny was constructed with MrBayes36 under the mixed amino acid substitution model with I rate variation run for 2 million generations. For GPCR phylogeny reconstruction, we extracted protein sequences for the seven-transmembrane domain, aligned them with MUSCLE, and reconstructed phylogeny using FastTree38 under the WAG substitution model39 with I rate variation.

Rm genome sequencing, assembly, and annotation. The Rm ATCC 52813 genome was sequenced using the Illumina platform. Two Illumina libraries were sequenced: (1) fragment library with 270-bp insert size in 2 x 150-bp reads, and (2) 5.2-kb-long mate pair in 2 x 100-bp reads. Each fastq file was QC filtered for artifact/process contamination and subsequently assembled with AllPath-LG R41043, resulting in 26-Mb assembly in 131 scaffolds and 773 contigs, with an average 143.6x read depth coverage. The assembled genome was annotated using the JGI annotation pipeline56, which combines several gene prediction and functional annotation methods, and integrates the annotated genome into JGI web-based resource for fungal comparative genomics, MycoCosm (http://genome.gi doe.gov/fungi)37.

Before gene prediction, assembly scaffolds were masked using RepeatMasker87, Repbase library38, and the most frequent (>150 times) repeats recognized by RepeatScout86. The following combination of gene predictors was run on the masked assembly: (1) ab initio, including Fgenes85 and GeneMark82, (2) homology-based, including Fgenes85 and GeneWise88 seeded by BLASTX alignments against the NCBI NR database, and (3) transcriptome based, including Fgenes and COMBEST86. In addition to protein-coding genes, tRNAs were predicted using tRNAscan-SE89. All predicted proteins were functionally annotated using SignalP90 for signal sequences, TMHMM91 for transmembrane domains, InterProScan92 for integrated collection of functional and structural protein domains, and protein alignments to NCBI NR, SwissProt93, EGG99 for metabolic pathways, and KOG90 for eukaryotic clusters of orthologs. InterPro93 and SwissProt92 hits were used to map Gene Ontology terms94. For each genomic locus, the best representative gene model was selected based on a combination of protein homology and transcriptome support, which resulted in the final set of 10,905 gene models reported in this study. Coverage of gene models by BLAT alignments of transcriptome assemblies to the genome assembly resulted in 85% of models being covered over at least 75% of their length, and 67% of models 100% covered. The complete set (100%) of eukaryotic core genes from the CEGMA dataset95 was found in Rm, indicating a reasonably complete genome assembly.

Data availability. The transcriptome data are available at the NCBI GEO database under the accession number GSE75644. The Rhizopus microsporus ATCC 52813 Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under accession JOSV00000000. All other relevant data are available in this article and its Supplementary Information files, or from the corresponding author upon request.
32. Koubova, J. et al. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc. Natl Acad. Sci. USA 103, 2474–2479 (2006).

33. Bianchi, N. V. Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus Gigaspora margarita through generation of vegetative spores. Appl. Environ. Microb. 70, 3600–3608 (2004).

34. Mond, S. J., Toomer, K. H., Morton, J. B., Lekberg, Y. & Pawlowska, T. E. Evolutionary stability in a 400-million-year-old heritable facultative mutualism. Proc. Natl Acad. Sci. USA 112, 2554–2567 (2015).

35. Bass, H. W., Marshall, W. F., Sedat, J. W., Agard, D. A. & Cande, W. Z. Telomeres cluster de novo before the initiation of synapsis: a three-dimensional spatial analysis of telomere positions before and during meiotic phase. J. Cell Biol. 137, 5–18 (1997).

36. Van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A. & Gianinazzi-Pearson, V. Characterization of root colonization profiles by a mycosymbiont community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7, 879–887 (1998).

37. Kim, D. et al. TopHat2: accurate alignment of transcripts in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36 (2013).

38. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169 (2015).

39. Wei, W. et al. Genome sequencing and comparative analysis of Cryptococcus neoformans. Genome Biol. 33, W116–W120 (2005).

40. UniProt Consortium. Activities at the Universal Protein Resource (UniProt). Nucleic Acids Res. 42, D191–D218 (2014).

41. Van Bel, P., Beutin, J. et al. Expansion of signal transduction pathways in fungi by insertions, deletions and gene fusions. PLoS Genet. 9, e1003233 (2013).

42. Galagan, J. E. et al. The genome sequence of the filamentous fungus Neurospora crassa. Nature 422, 859–868 (2003).

43. Kamper, J. et al. Insights from the genome of the biotrophic fungal plant pathogen Ustilago maydis. Nature 444, 97–101 (2006).

44. Stajich, J. E. et al. Insights into evolution of multicellular fungi from the assembled genomes of the mushroom Coprinopsis cinerea (Coprinus cinereus). Proc. Natl Acad. Sci. USA 107, 11889–11894 (2010).

45. Löffler, B. J. et al. The genome of the basidiomycetous yeast and human pathogen Cryptococcus neoformans. Science 307, 1321–1324 (2005).

46. Corrochano, L. M. et al. Expansion of signal transduction pathways in fungi by extensive genome duplication. Curr. Biol. 26, 1577–1584 (2016).

47. Ma, L. J. et al. Genomic analysis of the basal lineage fungus Rhizopus oryzae reveals a whole-genome duplication. PLoS Genet. 6, e1000549 (2009).

48. Tisserant, E. et al. The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. New Phytol. 193, 755–769 (2012).

49. Li, L., Stoeckert, C. J. & Roos, D. S. OrthoMCL: identification and evolutionary classification of ortholog families in large genomes. Genome Res. 18, 113 (2008).

50. Salamov, A. A. & Solovyev, V. V. Ab initio gene prediction of Drosophila melanogaster DNA. Genome Res. 10, S16–S22 (2000).

51. Ter-Hovhannisyan, V., Lomsadze, A., Chernoff, Y. O. & Borodovsky, M. Gene prediction in novel fungal genomes using an ab initio algorithm with unsupervised training. Genome Res. 18, 1979–1990 (2008).

52. Zhou, K. et al. Alternative splicing acting as a bridge in evolution. Stem Cell Investig. 2, 19 (2015).

53. Mondo, S. J., Toomer, K. H., Morton, J. B., Lekberg, Y. & Pawlowska, T. E. Evolutionary stability in a 400-million-year-old heritable facultative mutualism. Proc. Natl Acad. Sci. USA 112, 2554–2567 (2015).

54. Koubova, J. et al. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc. Natl Acad. Sci. USA 103, 2474–2479 (2006).

55. Gnerre, S. et al. High-quality draft assemblies of mammalian genomes from massively parallel sequence data. Proc. Natl Acad. Sci. USA 108, 1513–1518 (2011).

56. Salamov, A. A. & Solovyev, V. V. Ab initio gene prediction of Drosophila melanogaster DNA. Genome Res. 10, S16–S22 (2000).

57. Grigoriev, I. V., Martinez, D. A. & Salamov, A. A. Fungal genomic annotation. Appl. Mycol. Biotechnol. 6, 123–142 (2006).

58. Grigoriev, I. V. et al. MycoCosm portal: gearing up for 1000 fungal genomes. Nucleic Acids Res. 42, D499–D704 (2014).

59. Jurka, J. et al. Repbase Update, a database of eukaryotic repetitive elements. Cytogenet. Genome Res. 110, 462–467 (2005).

60. Price, A. L., Jones, N. C. & Pevzner, P. A. De novo identification of repeat families in large genomes. Bioinformatics 25, 142 (2006).