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Report

Shared Signatures of Parasitism and Phylogenomics Unite Cryptomycota and Microsporidia

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Summary

Fungi grow within their food, externally digesting it and absorbing nutrients across a semirigid chitinous cell wall. Members of the new phylum Cryptomycota were proposed to represent intermediate fungal forms, lacking a chitinous cell wall during feeding and known almost exclusively from ubiquitous environmental ribosomal RNA sequences that cluster at the base of the fungal tree [1, 2]. Here, we sequence the first Cryptomycotan genome (the water mold endoparasite *Rozella allomycis*) and unite the Cryptomycota with another group of endoparasites, the microsporidia, based on phylogenomics and shared genomic traits. We propose that Cryptomycota and microsporidia share a common endoparasitic ancestor, with the clade unified by a chitinous cell wall used to develop turgor pressure in the infection process [3, 4]. Shared genomic elements include a nucleotide transporter that is used by microsporidia for stealing energy in the form of ATP from their hosts [5]. *Rozella* harbors a mitochondrion that contains a very rapidly evolving genome and lacks complex I of the respiratory chain. These degenerate features are offset by the presence of nuclear genes for alternative respiratory pathways. The *Rozella* proteome has not undergone major contraction like microsporidia; instead, several classes have undergone expansion, such as host-effector, signal-transduction, and folding proteins.

Results and Discussion

The *Rozella* Genome Encodes Four Chitin Synthase Genes

Cryptomycota (also known as Rozellida) have recently been proposed to represent the earliest diverging branch of fungi whose diversity may be equal to that of the rest of the kingdom [1]. Some members of this phylum were hypothesized to lack a chitinous cell wall throughout their life cycle, and have thus been referred to as potential intermediate forms that diverged before the evolution of one of the defining features of the fungal kingdom, that of feeding across a chitinous cell wall. However, these claims are based primarily on microscopy and single-sequence analyses of environmental samples. By using a culture of the only described genus assigned to Cryptomycota, *Rozella*, we were able to perform in-depth investigations into the cell biology and genome content that were previously unfeasible.

Rozella allomycis can be cultivated as an obligate endoparasite of the water mold *Allomyces*. The parasite grows in the host as a naked, mitochondriate protoplast suspected of using phagocytosis to devour the cytoplasm of its host [6, 7]. For reproduction, it either stimulates the host to form a cell wall around developing zoosporangia containing motile zoospores or produces a thick-walled resting spore that stains positive for chitin or cellulose [8]. Using a combination of Illumina and Pacific Biosciences (PacBio) sequencing technologies, we assembled the genome of *Rozella* into 1,060 contigs totaling 11.86 Mbp. The genome is apparently diploid, with 3,972 high-quality heterozygous SNPs and 6,350 predicted genes. We identified four chitin synthase genes, all of which are in class IV or V/VII of division II [9]. Importantly, division II chitin synthases (Figure S1 and Table S1 available online) are known to be specific to fungi and microsporidia [10]. Among the division II chitin synthases of *Rozella*, one contains a myosin domain, a feature that could function in polarized growth during host invasion, similar to the development of the penetration tube in corn smut [11]. Previously, we showed that the inner wall of the resting spore was chitin positive using calcofluor white [8]; here, we show that the infective cyst form is also positive for N-acetyl-D-glucosamine (the primary sugar of chitin) and that the chitin stain is most intense at the penetration point (Figure 1).

Cryptomycota Are Related to Microsporidia

Cryptomycota may be related to two other groups, microsporidia and aphelids, both of which are endoparasites that also grow as naked protoplasts in their hosts [12, 13]. The phylogenetic placement of microsporidia has long been contentious and is confounded by an accelerated rate of evolution causing long branch attraction (LBA) [14], but a consensus has recently emerged that microsporidia are related to or derived from within basal fungi, with which they share the presence of a chitinous cell wall, but remarkably little else [15–17]. The only phylogenetic studies that have used protein-encoding gene sequences from both Cryptomycota and microsporidia have clustered these lineages together as the earliest branch of the fungal tree, but without robust statistical support [12, 13]. In the present study we generated a 200-gene concatenated supermatrix and estimated the phylogeny of 28 fungi and 11 outgroup species using maximum likelihood and Bayesian methods. These phylogenies strongly support *Rozella* + microsporidia as the earliest divergence within the fungi (Figure 2).

To test for LBA, we sequentially removed the most rapidly evolving sites [18]. After deleting an eighth, a quarter, and then half of the most quickly evolving sites, each tree and analysis method recovered strong support for *Rozella* + microsporidia, with bootstrap support increasing to 100% and Bayesian posterior probabilities remaining ≥ 0.99. We then tested whether alternative placements of the microsporidia could be statistically rejected using the approximately unbiased test. We were able to reject the placement of microsporidia at eight alternative locations ($p < 0.05$) (Figure 2); however, we were unable to statistically reject the possibility that

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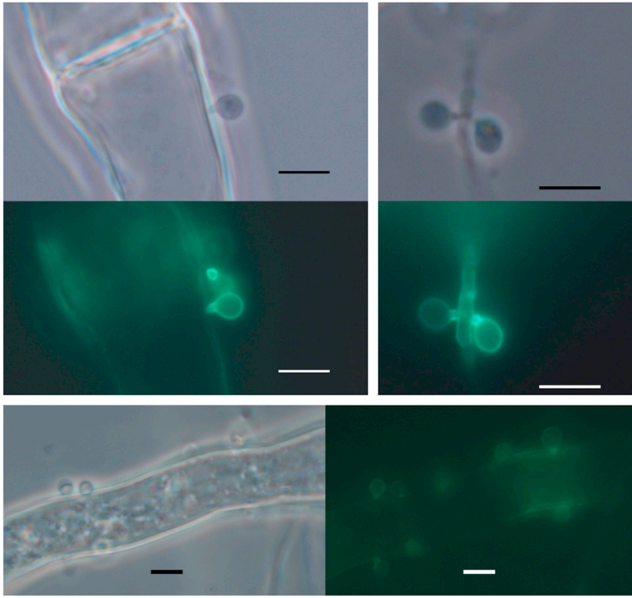

Figure 1. Rounded Cysts Stain with Wheat-Germ Agglutinin, Conjugated with Oregon Green

Shown are three slides in phase contrast and with fluorescence. Cysts were stained after 1 hr of infection of *Allomyces* with zoospores and show intense staining of the penetration peg. The scale bar represents 5 μm.

microsporidia branched just before Cryptomycota/*Rozella* diverged from the remaining fungi (p = 0.375). Using the approximately unbiased test, we also tested the support for the nine possible alternative placements of microsporidia under fast site removal. As sites were removed, the posterior probability for the alternative placement of microsporidia at the base of the fungi decreased, meaning that the alternative placement was statistically less likely (but not significantly worse) as fast sites were removed (Table S2).

Given the rapid evolution of microsporidian proteins, we searched for additional evidence of phylogenetic relatedness that does not suffer from LBA due to sequence evolution (i.e., genomic synapomorphies), and here we found that three genes previously thought to be exclusive to microsporidian genomes also have orthologs in *Rozella* (Figure S2). Interestingly, all of these were hypothesized to have originated from taxonomically diverse lineages by means of horizontal gene transfers to facilitate intracellular parasitism [19, 20]; they include the nucleotide phosphate transporters (NTTs; Pfam: PF03219), the nucleoside H^+ symporters (specific family of PF03825), and the chitinase class I genes (specific family of PF00182). The identification of these genes in *Rozella* represents an independent line of evidence for a close evolutionary link between Cryptomycota and microsporidia and indicates shared signatures of energy parasitism in the form of nucleotide and nucleoside transporters and genes for chitin degradation. Importantly, NTP transporters are known to be involved in the specific theft of ATP from the host in microsporidia and the intracellular parasitic prokaryotes (*Chlamydia*) from which the genes were originally transferred [5, 20].

The *Rozella* Mitochondrial Genome Shows Evidence of Degeneration

The capacity of microsporidia to steal ATP from their hosts using these bacterium-like NTTs has been linked with the degeneration of their mitochondrion. Specifically, this latter organelle can only be found as a vestigial, genome-less organelle called a mitosome [21]. By assembling the mitochondrial genome of *Rozella*, we found that its mitochondrial genome is degenerate; an intriguing finding that supports the idea that the capacity to import ATP results in drastic genome changes for the mitochondrion. Specifically, the mitochondrial genome of *Rozella* maps as a circular, 12-kbp-long, extremely AT-rich (86%) molecule that encodes a total of six known proteins, the small- and large-subunit ribosomal RNAs, and four transfer RNAs (tRNAs) (Figure 3). All genes are characterized by extreme sequence divergence and remarkably low GC content (e.g., *atp6* has only 9% GC). The mitochondrial genome also harbors a gene that requires one *trans*-splicing event, involving a group I intron located within the first subunit of cytochrome oxidase (Figure S3) at a position identical to that found in Placozoa and in more diverged fungal lineages (i.e., *Gigaspora* spp. [22]). The remaining protein genes encode components of the respiratory chain, except for complex I (i.e., these NADH dehydrogenase genes are absent from both the mitochondrial and nuclear genomes). The loss of complex I from both the mitochondrial and nuclear genomes is also

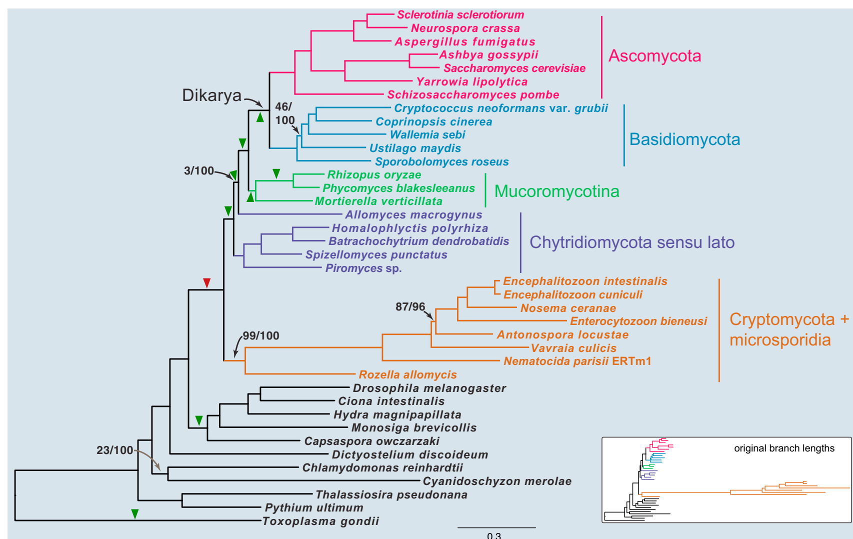

Figure 2. Phylogeny of Fungi Based on 200 Genes Shows *Rozella* and Microsporidia Form a Clade

All branches have 100% bootstrap and Bayesian support except the ones indicated (bootstrap/Bayes posterior probability). All microsporidian branches are reduced in length 4× for clarity. The inset shows the original tree with the unaltered branch lengths. Green triangles indicate an alternative placement of the microsporidia clade that is significantly worse (approximately unbiased test), and the red triangle shows a placement that is not significantly different.

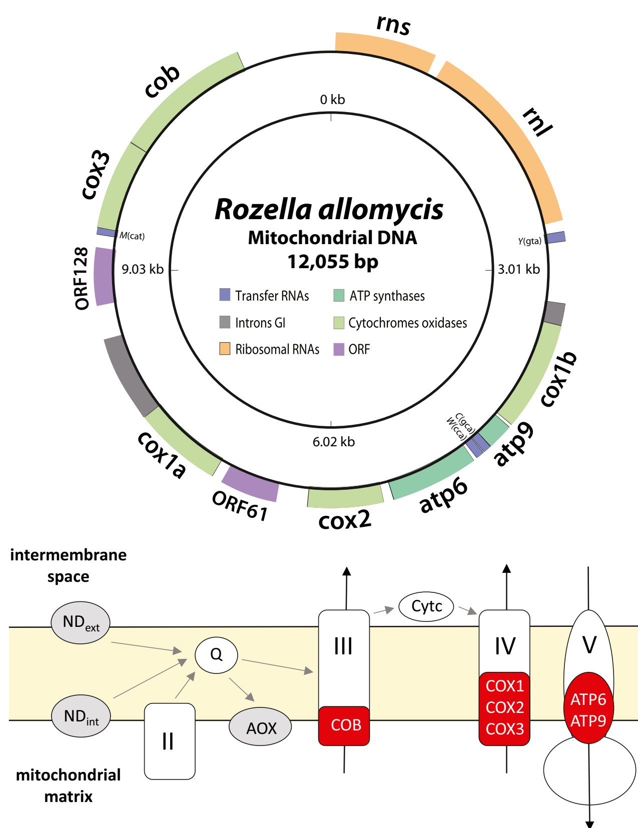

Figure 3. Reduced Mitochondrial Genome and Electron-Transport Chain in *Rozella*

Top: Genome map. Filled boxes represent genes, and group I introns are shown in gray. tRNA genes are shown according to their one-letter amino acid code, followed by their anticodon. Cox1 messenger RNA is generated by *trans*-splicing of Cox1a and Cox1b (Figure S3). All open reading frames (ORFs) longer than 60 codons are shown. Bottom: Schematic of potential respiratory chain pathways in *R. allomycis*. Subunits encoded in the mitochondrial genome are shown in red, and nucleus-encoded components are shown in either white, for complexes II–V of the oxidative phosphorylation pathway, or in gray, for the rotenone-insensitive (ND_{ext} and ND_{int}) and cyanide-insensitive (AOX) alternative pathways. The gray and black arrows represent the flow of electrons and protons, respectively. For complex I core components, only a 39 kDa subunit homolog was detected in the nuclear genome. Nuclear-encoded genes involved in the respiratory chain are listed in Table S4.

observed in apicomplexan mitochondrial genomes, such as *Plasmodium falciparum* [23], as well as in several Saccharomycetes, including the fungal species with the smallest mitochondrial genome, *Hanseniaspora uvarum* [24]. However, the loss of this complex may not dramatically impact the capacity of *Rozella* to produce ATP, given that this step of the respiratory chain can be bypassed through the involvement of several nuclear-encoded proteins in ways that are similar to those found in certain other eukaryotes. Specifically, *R. allomycis* encodes both external and internal NADH dehydrogenase genes and alternative oxidase (Table S3). These alternative means of regenerating NAD+ are unlinked to proton pumping in the mitochondrion, and thus generate less energy in the form of ATP. This may reflect a specialized lifestyle, as in apicomplexans for example, or alternatively, that the mitochondrion is deteriorating into a mitosome, as in microsporidial parasites [25, 26].

The *Rozella* Genome Lacks Many Genes for Primary Metabolism but Is Enriched for Signal-Transduction Genes

As expected for an obligate intracellular pathogen, the *Rozella* proteome is missing key components of primary metabolism. However, the *Rozella* genome encodes for the standard enzymes of the Krebs cycle and the proteins necessary for Fe-S cluster formation and heme biosynthesis, suggesting that the function of the mitochondrion is diverse and typical of other eukaryotes. Other key components of primary metabolism are more consistent with a lifestyle as an intracellular parasite, with core components of de novo nucleotide and oxidative phosphorylation missing (Figure 4). Overall, the portion of the proteome responsible for primary metabolism of *Rozella* is more similar to that of the apicomplexan parasites, *Plasmodium* and *Toxoplasma*, than that of microsporidia or other fungi. On the other hand, the amino acid metabolism of *R. allomycis* is more similar to that of Metazoa and Amoebozoa, perhaps suggestive of a phagotrophic mode of protein consumption and amino acid extraction.

Despite the large number of missing primary metabolism genes, the predicted *Rozella* proteome is larger than that of yeast (6,350 versus 5,770 proteins). This number of genes greatly exceeds that found in microsporidia, which typically ranges between 1,800 and 3,800 genes [28]. Using gene-ontology terms and Pfam domains of the 6,350 proteins, we sought to determine which biological categories are enriched in *Rozella*, and we found that proteins involved in protein-protein interactions (e.g., signal transduction, protein folding, kinases, and proteins with WD40 domains) are all enriched in percentage of the proteome (Figure S4; Table S4). We hypothesize that some of the protein-protein interaction domains are actually involved in the direct manipulation of host signaling or recycling of host proteins. In support of this argument, we identified 22 genes of the Crinkler family of effector proteins. Crinkler proteins are found in many symbiotic, microbial eukaryotes but are best known in oomycete plant pathogens as secreted proteins that translocate into the host cytoplasm or nucleus to induce plant cell death [29].

A Parasitic Root on the Fungal Tree of Life

Sequencing of the *Rozella* genome provides a glimpse into the early origin of fungi and their characteristics. Because Cryptomycota remained uncharacterized for so long, it may be speculated that they are largely unculturable fungi that parasitize hosts that are understudied or with little economic importance, such as diatoms or water molds [1, 8]. Given the recent data suggesting that Cryptomycota may be related to the algal parasites known as aphelids [13, 30], it is predicted that the earliest fungi lacked a cell wall in the trophic phase but retained the capacity for phagocytosis. Having a genome sequence of an aphelid would be very useful for ancestral-state reconstruction and gene-content analysis of the earliest fungal lineages.

Many Cryptomycota and aphelids appear to be aquatic and disperse by flagellated motile spores. A search for homologs of flagellum-associated proteins from the flagellated protozoan *Naegleria* genome [31] reveals a pattern of presence and absence of approximately 60 genes that can be found in the genomes of *Rozella* and zoosporic Chytridiomycota and Blastocladiomycota fungi, but not in Dikarya (Table S1). In nearly all cases wherein homologs of flagellar proteins were absent in Dikarya, they were similarly absent in the microsporidia, suggesting convergent losses of the flagellum. Although the primary radiation of terrestrial fungi is linked to what may

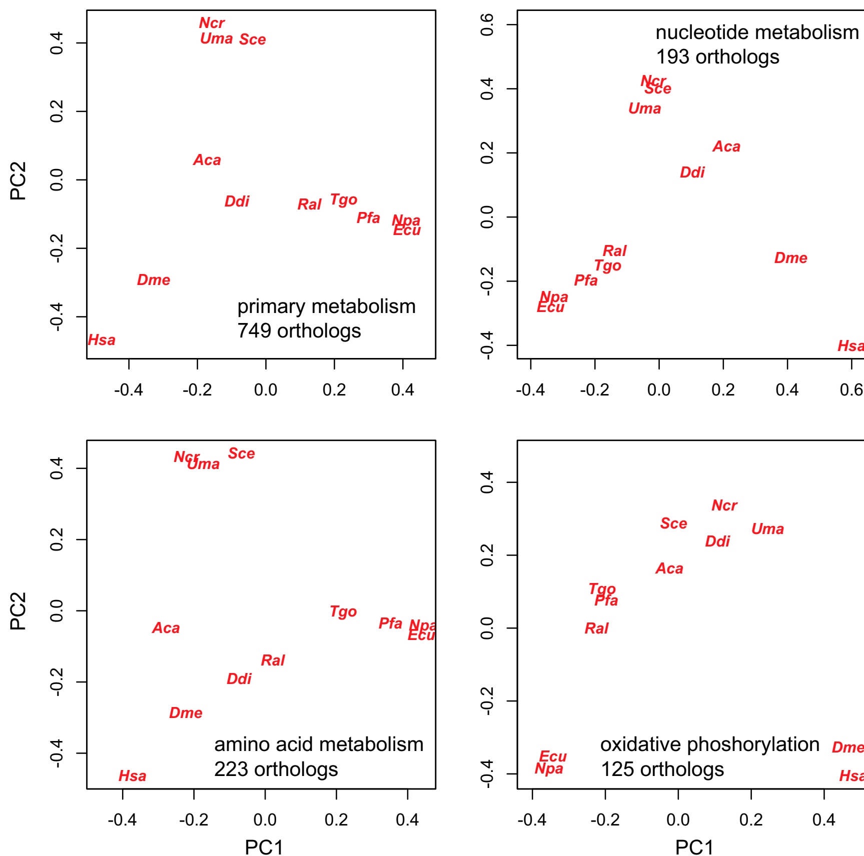

Figure 4. Principal-Component Analysis of the Presence and Absence of Primary Metabolism Genes in 12 Species

The 12 species analyzed were as follows: *Aca* (*Acanthamoeba castellanii*), *Ddi* (*Dictyostelium discoideum*), *Dme* (*Drosophila melanogaster*), *Ecu* (*Encephalitozoon cuniculi*), *Hsa* (*Homo sapiens*), *Ncr* (*Neurospora crassa*), *Npa* (*Nematocida parisii* ERTm1), *Pfa* (*Plasmodium falciparum*), *Ral* (*Rozella allomycis*), *Sce* (*Saccharomyces cerevisiae*), *Tgo* (*Toxoplasma gondii*), and *Uma* (*Ustilago maydis*). Orthologs and classification into functional groups were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG). We used pre-existing ortholog definitions for all species except *Aca*, *Npa*, and *Ral*. For those three species, we used the KEGG Automatic Annotation Server (KAAS) [27].

be a single loss of the flagellum [32, 33], it is clear from this study that multiple independent losses of the flagellum have occurred, with the one leading to the evolution of the microsporidian polar tube distinct from that associated with aerial dispersal in filamentous fungi.

In the majority of fungi, polarized apical cell growth involves using both turgor pressure and cytoskeletal forces to push on an elastic hyphal tip, revealing fungal protoplasm to be similar to an amoeba crawling within an extracellular chitinous tube [34]. In showing here that *Rozella* cysts actively produce a chitinous wall in the invasion process (Figure 1), we identify a characteristic that unites fungi with Cryptomycota, microsporidia, and possibly aphelids, namely, the use of a chitinous cell wall to generate turgor pressure during the polarized movement of the protoplasm. In *Rozella*, microsporidia, and aphelids, this involves the uptake of water into a cyst or spore, the formation of a posterior vacuole, and the forward injection of protoplasm into the host. The unification of the mysterious Cryptomycota with aphelids [13] and the well-known microsporidia, a group probably comprised of many more than the 1,300 described species [35], establishes a new, hyperdiverse clade of endoparasitic fungi near the root of the fungal tree that had already evolved a chitinous cell coat for both reproduction and invasion.

Experimental Procedures

Biological Material and Growth Conditions

R. allomycis isolate CSF55 was isolated from soil collected in a roadside drainage ditch in Hattiesburg, MI, USA. The soil was added to distilled water and baited with sterile hemp seeds. Coculturing of *R. allomycis* with its host *Allomyces* sp. was done using 1/8 strength Emerson's YpSs, with or without 2% agar. Zoospores of the parasite were harvested from the surface of Petri dishes by flooding with double-distilled H_2O.

DNA and RNA Sequencing

DNA was extracted from the zoospores using a standard protocol [36]. A genomic DNA library of fragments approximately 650 bp in size was prepared and run on 1/2 of a lane of an Illumina Genome Analyzer IIx at the University of Michigan DNA Sequencing Core, using paired-end reads of the fragments run to 159 bp cycles. A second genomic DNA library of fragment sizes of a mean length of 2 kbp was generated using a PacBio RS DNA Template Preparation Kit 1.0. The libraries were run in two SMRT cells of a PacBio RS analyzer at the University of Michigan DNA Sequencing Core.

Genome Assembly and Annotation

PacBio reads were postprocessed for correcting the high error rate of reads using the PacBioToCA module of the Celera Assembler v.7.0 [37] and assembled de novo into 4,866 contigs with an average size of 4 kb, with the largest contig being 81,317 bp. All PacBio reads and contigs were treated as long reads in the assembly process. De novo assembly was accomplished with velvet v.1.2.03 [38] using the short paired Illumina data and resolving repeats using the long PacBio reads.

The draft genome was assembled into 1,060 contigs of a total size of 11.86 Mbp. The largest contig was 719 kbp in size, the N50 was 58,027 bp, and the estimated fold coverage was 264X. The GC content was 34.5%, and the total number of bases in the assembly with Ns was 121,591. Genome annotation was accomplished using the MAKER pipeline 2.25 [39].

Phylogenetic Analysis

OrthoMCL was used to extract orthologous proteins from *Rozella* and 38 other species with an elevation value of 18. From the set of 35,947 orthologs, we discarded all orthologs that were not found in at least 36 of the 39 species, leaving 329. These orthologs were then filtered by removing those in which the multiple sequences were nonmonophyletic, leaving 200 orthologous proteins. The 200 proteins were concatenated into a supermatrix of 71,556 amino acids and analyzed using PhyloBayes 3.3 [40] with the CAT model for 500 hr using two chains and RAxML 7.0.4 [41] with the LG model.

Accession Numbers

Illumina and PacBio reads are available at the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under accession number SRA068184. The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under accession number ATJD00000000. The version described in this paper is version ATJD01000000.The mitochondrial genome of *R. allomycis* is deposited in GenBank under the accession number KC702881.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables, and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.057.

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