A New Genetic Linkage Map of the Zygomycete Fungus Phycomyces blakesleeanus

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Abstract

Phycomyces blakesleeanus is a member of the subphylum Mucoromycotina. A genetic map was constructed from 121 progeny of a cross between two wild type isolates of P. blakesleeanus with 134 markers. The markers were mostly PCR-RFLPs. Markers were located on 46 scaffolds of the genome sequence, covering more than 97% of the genome. Analysis of the alleles in the progeny revealed nine or 12 linkage groups, depending on the log of the odds (LOD) score, across 1583.4 cM at LOD 5. The linkage groups were overlaid on previous mapping data from crosses between mutants, aided by new identification of the mutations in primary metabolism mutant strains. The molecular marker map, the phenotype map and the genome sequence are overall congruent, with some exceptions. The new genetic map provides a genome-wide estimate for recombination, with the average of 33.2 kb per cM. This frequency is one piece of evidence for meiosis during zygospore development in Mucoromycotina species. At the same time as meiosis, transmission of non-recombinant chromosomes is also evident in the mating process in Phycomyces. The new map provides scaffold ordering for the genome sequence and a platform upon which to identify the genes in mutants that are affected in traits of interest, such as carotene biosynthesis, phototropism or gravitropism, using positional cloning.

Introduction

The fungus Phycomyces blakesleeanus is a member of the order Mucorales in the subphylum Mucoromycotina. By current estimates the basal lineages in the fungal kingdom, like the Mucoromycotina, represent less than 5% of all fungal species and yet hold the majority of the evolutionary history in this group of eukaryotes [1,2,3]. Estimating overall diversity at the DNA level or dating divergence is a challenge [4], although each lineage diverged prior to the split between the Ascomycota and Basidiomycota. Nevertheless, the early branches in the fungal kingdom receive relatively little research attention, despite our potential ability to understand the evolution of the fungi through their study.

This paucity in research effort is surprising in the context of the long research history associated with some Mucoromycotina species, especially on the mating properties of fungi. The first report of sex in fungi comes from the Mucorales [5,6,7]. The two major mating strategies in fungi, i.e. homothallism or heterothallicism, were defined in the Mucorales [8]. Diffusible pheromones in fungi were first identified from Mucor nuda [9]. However, the genes required for mating in the Mucorales are poorly understood, and completing the sexual cycle under laboratory conditions is often a challenge. In addition to these obstacles, many species are difficult to manipulate via the introduction of foreign DNA. For example, the current absence of a stable transformation system in Phyomycetes prevents the use of techniques such as cloning by complementation or gene function testing via disruption or RNAi-based silencing [10].

Phycomyces reproduces as a mycelium and forms spores either asexually or sexually. The asexual cycle includes the production of spores borne on sporangiophores that may rise 10–15 cm from the surface of the fungal colony. These long filaments are sensitive to many environmental conditions, including light that is a signal to trigger a phototropic response [11,12]. The sexual cycle leads to the production of zygospores and then progeny, a cycle defined by the 1920s as follows [13]. Two hyphae from each mating type encounter each other, the tips of which undergo a septation event to produce the equivalent of gametes. The two cells fuse to form the immature zygospore. The developing zygospore initially incorporates thousands of nuclei from the two original parents. During the course of zygospore maturation and dormancy, of two months to more than a year, most nuclei degrade. It is hypothesized that two nuclei, one from each parent, survive, fuse and undergo meiosis. Mitotic divisions then amplify the four meiotic products that form in a sporangium structure that develops out from the zygospore. However, analysis of genetic markers in progeny from crosses shows that the situation outlined above does not occur for all zygospores [14,15,16,17]. Furthermore, sex heterokaryons also arise, which are easily detected due to the production of the curled pseudophore structures that resemble gametangia [18]. Indeed, heterokaryon formation is used as a tool since they can be applied in complementation tests involving both nutritional and phototropic markers [19]. Because of the non-
Mendelian ratios of genetic markers in progeny from individual zygospores, the presence of meiosis in Phycomyces has been challenged, most recently in reference [17].

Strains of Phycomyces with mutant phenotypes were isolated by chemical mutagenesis. Whatever the mechanism(s) of recombination in the zygospore, individual genes could then be defined into complementation groups and their relative placement on chromosomes established through genetic linkage analysis. Over a two decade period this process led to the measurement of linkage distances based on segregation between these markers [20,21,22,23,24,25,26], including centromeres that were mapped with the equivalent of tetrad analysis [27,28]. The first genetic map was reported in 1987 [29], and the updated map was published in 1996 [29]. The construction of this map placed Phycomyces as the model for analysis of genetic segregation in the Mucoromycotina. For comparison, the next two best-studied species are Rhizopus stolonifer and Mucor hiemalis [30,31]. In these species, only two and six segregating markers have been examined. In M. hiemalis most products from the zygospore are a single genotype. The limited information from these two species illustrates how little is known about genetics in fungi, other than for the Dikarya which is the monophyletic lineage that encompasses the Ascomycota and Basidiomycota.

A major research focus on Phycomyces is its light-sensing abilities. The photoreceptive response of the sporangiophores is one of the best-studied traits in Phycomyces [11,32]. Analysis of this property included the generation of phototropism mutants via chemical mutagenesis, starting in the late 1960s [33,34]. Two of these genes, madA and madB, have been identified by predicting that they would be homologs of the photoreceptor complex genes wc-1 and wc-2 defined originally from Nematoda caressa [35,36]. An additional eight genes (madl-mad8) remain to be identified. Other mutants affect unidentified genes that control tropisms to gravity and wind, primary metabolism, the biosynthesis of β-carotene, spore germination, mating, and colony and sporangiophore morphology [37]. The primary goal of this project was to develop a genetic map covering the genome that could be used for map-based cloning to identify these unknown genes. At the same time, this map would serve additional purposes: to estimate the number of chromosomes in this species, provide a higher order genome scaffold assembly, and examine the genetic reduction process that occurs within the zygospore.

Table 1. Strains used in this study. NG: isolated after mutagenesis with N-methyl-N’-nitro-N-nitosoguanidine.

| Name         | Genotype | Parent and origin | Reference |
|--------------|----------|-------------------|-----------|
| NRRL1555     | Wild type (–) |                  |           |
| UBC21        | Wild type (+) |                  |           |
| A2           | furB (–) | NRRL1555, spontaneous mutant [27] | |
| A34          | furA madD (+) | B71 × C149 [28] | |
| A38          | carA furB (–) | A2 × C169 [28] | |
| A849         | pyrF (–) | NRRL1555+ NG [26] | |
| A851         | pyrF (–) | NRRL1555+ NG [26] | |
| A859         | pyrG (+) | A852 × UBC21 [26] | |
| A914         | lysA madl (–) | A637 × A845 [25] | |

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Table 2. Progeny generated for genetic analysis. The parent named “progeny 82A” is derived from the cross between UBC21 × NRRL1555.

| Parents                | Number of progeny | Reference |
|------------------------|-------------------|-----------|
| UBC21 (+) × NRRL1555 (–) | 121 (121 zygospores) | This study and [39] |
| UBC21 (+) × NRRL1555 (–) | 176 (from 8 zygospores) | This study |
| A34 (–) × NRRL1555 (–)  | 40 (40 zygospores) | This study |
| UBC21 (+) × A2 (–)      | 10 (10 zygospores) | This study |
| A34 (–) × progeny 82A (–) | 15 (15 zygospores) | This study |
| UBC21 (+) × A914 (–)    | 45 (45 zygospores) | [49] |

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Materials and Methods

Strains

Wild type strains were NRRL1555 (mating/sex type –) and UBC21 (+). The mutant strains are listed in table 1, and details about the progeny from crosses provided in table 2. Strains were cultured on potato dextrose agar (Difco; Franklin Lakes, NJ, USA). Crosses were established on V8 medium (5% V8 juice, 4% agar, pH 6) (Fig. 1A). Zygospores were transferred to damp filter paper (2 mm thickness) in deep Petri dishes (2 cm total height) that were sealed with parafilm. Zygospores germinated 2–4 months later. For most crosses, the germospores from a single zygospore were resuspended in 10 μl H₂O and heat shocked for 15 min at 45–46°C. The spore suspension was placed as a drop on a PDA plate, and the spores streaked to isolate single colonies. This was determined using a microscope prior to excision with a scalpel of the agar surrounding a single germinated cell. For a subset of zygospores, the spore suspension was plated as ten-fold serial dilutions on acidified minimal medium, that reduces colonial expansion [38]. Isolated colonies were subcultured onto PDA medium. 104 progeny from a cross between UBC21 and NRRL1555 had been generated previously [39], and 17 additional progeny were isolated to increase the population size. For scoring mating type, strains were crossed to NRRL1555 (–) or the congeneric strain A36 (+) that had been generated by backcrossing the (+) allele into the NRRL1555 strain background [40]. For the analysis of mutant auxotrophic phenotypes, these were scored by the presence or absence of growth on yeast nitrogen base (YNB) medium. Resistance to 5-fluorouracil was assessed on medium supplemented with 0.25 mg/ml of this chemical (Acros Organics; NJ, USA).

Design of Molecular Markers

The development of PCR-based restriction fragment length polymorphism markers (PCR-RFLPs) underwent refinement during the course of the project. Genome sequencing information has been publicly available for NRRL1555 since 2006. The initial markers were designed based on the comparison of DNA sequences of a small genomic library constructed from strain UBC21 with the sequence of strain NRRL1555 [39]. In several cases these markers required restriction enzymes with low efficiency in reactions that included PCR components. In other cases, the markers amplified an additional product. Both scenarios complicated interpretation of the alleles. Subsequently, strain UBC21 was sequenced with Illumina technology by the US Department of Energy Joint Genome Institute. Polymorphic single nucleotides (SNPs) were incorporated into the genome sequencing...
The majority of the molecular markers used amplification by PCR, and the alleles were defined by restriction digests of the PCR products. Prior to selection, 550–650 bp on either side of a polymorphic site were examined for additional restriction enzyme sites that could confound scoring of alleles after restriction digestion, and for copy number via BLASTn of the *Phycomyces* genome. 18–20 oligonucleotide primers were designed to amplify suitable regions. The sequences of the primers and their positions on the scaffolds of the *Phycomyces* genome release versions 1.1 and 2.0 are provided in table S1.

**Amplification of Molecular Markers**

Polymorphic regions were amplified by PCR using rTaq and ExTaq DNA polymerase (Takara, Otsu, Shiga, Japan). The conditions were 2 min 94°C, then 32 cycles of 20 s 94°C, 20 s 52–55°C, 1 min 72°C, and a final extension of 5 min at 72°C. PCR products were digested using the appropriate restriction enzyme (New England BioLabs, Ipswich, MA, USA) and fragments resolved on 0.8–1.2% agarose gels prepared in Tris-acetate-EDTA (TAE) buffer. Optimized conditions used 15 µl PCR volumes and the digestion of the PCR after amplification, directly in 25 µl reaction volumes. Exceptions to PCR-RFLPs are as follows. Primers for amplification of *sexM* and *sexP* produce a product specific for each sex type. Primers ai814-ai861, ai1070-ai1071 and ai904-ai905 generate amplicons of differing size that...
were resolved on 1.4–2.0% agarose gels. Primer set ai990–ai998 produces amplicons of different size that were resolved on 20% polyacrylamide Tris-borate-EDTA gels.

Identification of Genes

The genes \textit{furA}, \textit{lysA}, \textit{nicA}, \textit{pyrF} and \textit{pyrG} were predicted based on previous biochemical or phenotypic evidence, and on equivalent functions in other fungi. Homologs were identified in the genome sequence database, the genes amplified from mutant strains and sequenced (primer details are in table S2). Primers for \textit{lysA} were ALID0194–ALID202. Primers for \textit{furA} were ai857–ai858. Primers for \textit{pyrF} were ALID0449–450. Primers for \textit{pyrG} were ALID0035–ALID0036. Sequences were compared to the wild type sequence from the NRRL1555 genome by BLASTn.

Construction of Linkage Groups and Recombination Frequency Measurements

Allele information was recorded in Microsoft Excel (dataset S1) and exported to JoinMap 4.0 software [41]. Analyses utilized the multiple parameters available in the software. Based on the linkage groups and the maps obtained when compared to recombination frequencies between markers calculated manually, maximum likelihood methods were applied. Linkage between markers was first assessed by independence log of the odds (LOD). Recombination frequencies were calculated between markers in each linkage group assigned LOD 3 or higher. Linkage data were redrawn to scale in Canvas software for comparison to the physical distances between markers.

Results

A New Genetic Map of \textit{Phycomyces} Resolves Nine to Twelve Linkage Groups

A set of PCR-RFLP markers was used to develop a genetic map from a cross between two wild type strains of \textit{Phycomyces blakesleeanus}. 121 progeny were isolated, one from each of 121 zygospores of a cross between strains UBC21 (+) and NRRL1555 (−). Each progeny represents one of the outcomes of a fusion between the parents and the genetic reduction event from one zygospore (Fig. 1A). Markers were designed to the ends of each of the larger scaffolds in genome release version 1.1, then to the 50 largest scaffolds in version 2.0. Fig. 1B illustrates an example for the design of two primers, ALID1007–ALID1008, to amplify a region containing a HindIII site present in strain NRRL1555 and absent in strain UBC21. Five scaffolds (in version 2.0) did not have suitable locations for the design of markers, so are represented as the orphans (O) in figure 2.

The markers were amplified by PCR from the 121 progeny, digested with restriction enzyme, and alleles scored by resolving DNA on agarose gels (Fig. 1C). Four markers used size digested with restriction enzyme, and alleles scored by resolving the orphans (O) in figure 2.

The remaining 116 progeny were analyzed for independence log of odds (with maximum likelihood) to test for potential linkage between the markers. The analysis revealed nine linkage groups with support of LOD 3. These scores compare the probability of linkage between markers to obtaining the data by chance, expressed as logarithm base 10. LOD 3 means 1/1000 that the results obtained occur by chance. At LOD scores of 4 or 5, three groups supported at LOD 3 were split, giving 12 linkage groups in total. The recombination frequencies between the markers on each of the 12 linkage groups were calculated, generating a map summing 1503.4 cM. The smallest linkage group was four markers over 20.2 cM and the largest linkage group 20 markers over 308.6 cM. The linkage groups are currently numbered I – IX based on the LOD 3 values. For the three linkage groups that would be split at LOD 5, these are further distinguished as Ia and Ib, Va and Vb, and IXa and IXb.

The Phycomyces Genetic Map Correlates with the Physical Map

The genetic map and physical distances between the markers were compared (Fig. 2). The comparison reveals a range of recombination frequencies across the genome. Both maps are largely overlapping. Discrepancies between genetic and physical maps are located on scaffolds 1, 15, 19 and 20, where markers are found on more than one linkage group (LG). In addition, regions in which the linkage data suggest a possible inversion in the genome sequence assembly are found on LG I/scaffold 3 and LG IV/scaffold 5. These differences likely reflect the challenges of genome sequence assembly caused by repetitive elements in the genome, in that several of these breaks not present in the version 1.1 genome assembly.

Identification of Genes to Anchor the New Molecular Marker Map

Eight \textit{Phycomyces} genes have been mapped, which enables the incorporation of the previous genetics data with the new RFLP molecular marker map. In order to further strengthen these ties, additional genes with segregation information were sought. Predictions were made for \textit{furA}, \textit{furC}, \textit{nicA}, \textit{pyrF} and \textit{pyrG} based on the phenotypes of these mutant strains. Candidate genes were amplified from the equivalent mutants and sequenced. The nicotinic acid mutants of \textit{Phycomyces} are under separate investigation (Alcalde, Idrisur and Cerda-Olmedo, unpublished data), so are not discussed further here. Point mutations were found in all five genes, each causing a predicted amino acid substitution in a residue highly conserved in fungal homologs, and consistent with
the phenotypes of the strains (Fig. 3A, B). To strengthen the evidence of the mutations causing the phenotypes for the two lesser characterized genes, genetic linkage was established for the point mutations in furA and furB in crosses to wild type strains of the opposite mating type (Fig. 3C, 3D).

For furA, 45 progeny from a cross between strains UBC21 (+) and A914 (furA madD –) were inoculated onto yeast nitrogen base medium without amino acid supplements to score for the lysine auxotrophy. The furA gene was amplified and sequenced from 45 of these progeny. 25 progeny were wild type and all had the wild type furA sequence. 20 progeny were lysine auxotrophs and had the C-T mutation in the furA gene. That is, the furA candidate gene co-segregates with the mutant phenotype. Other markers, like a mutation affecting phototropism, segregated independently (Fig. 3C). The furA gene and mating type were previously reported to be linked [28]. The sex alleles of the progeny were determined by PCR amplification. In this cross no evidence of linkage was observed between furA and sex (22/45 or 48.9% recombinants).

For furB, 40 progeny were generated from a cross between strains A34 (furA madD +) and NRRL1555 (–), and the furA gene sequence and phenotype scored on media containing 5-fluorouracil (5-FU). The furA mutation confers resistance to this chemical. 19 progeny were able to grow on 5-FU, and all 19 contained the mutation in furA. 21 progeny were unable to grow on 5-FU, and all 21 contained the wild type furA allele. A34 also carries a mutation in the madD gene required for phototropic response. The phototropism mutation and resistance to 5-FU were tightly linked, consistent with the previous segregation data for these two genes [27,28]. The mating type was determined both by phenotype and by PCR amplification of the sex alleles, showing independent assortment of the sex locus with the furA and madD genes.

The furB gene was also sought. Like furA, mutation of furB confers resistance to 5-FU and furB is linked to madD [28]. Initially using genome version 1.1, scaffold 11 where furA is located was examined, but no obvious candidates were present. In the mapping data from the UBC21 × NRRL1555 cross, scaffold 45 is linked to scaffold 11: both scaffolds are part of scaffold 3 in genome release version 2.0. This region contains a gene (JGI ID 163361) encoding a protein similar to xanthine-guanine phosphoribosyltransferases Hpt1 and Xpt1 of S. cerevisiae, making it a candidate for furB. However, amplification and sequencing of the gene from strain A2 (furB), provided from two sources, did not reveal any mutations. Finally, sequencing the furB gene from the furB strain A2 and a derived strain A38 revealed that the identical mutation in the gene was present as seen in the furA mutant strain A34, suggesting that furA and furB are identical.

With the identification of the genes involved in primary metabolism, their positions in new map were compared to the previous mapping data (Fig. 4). Further, SNPs were identified between the NRRL1555 and UBC21 alleles of furA and pyrG. Markers were designed to amplify these regions, and the alleles scored in the 121 progeny of the cross between NRRL1555 and UBC21, enabling exact correlation between molecular and phenotypic markers. This comparison shows consistency with the maps made from crosses between mutant strains compared to this crossing system between two wild type strains. One exception is the linkage group previously defined for pyrG, furA, madD, furB and pyrF, which are split in the UBC21 × NRRL1555 data into three separate linkage groups, and the furA, pyrF and pyrG genes are also located on three different scaffolds in the genome sequence.

The lack of genetic linkage between furA-madD with the two pyr genes in the UBC21 × NRRL1555 cross was further explored in three additional crosses (Table 2). The furA and pyrG genes were amplified directly, and the two markers that flank pyrF were amplified. No linkage was observed for these markers for the progeny of UBC21 × A914, A2 × UBC21, or A34 × progeny 82A derived from the cross between UBC21 × NRRL1555 (Fig. 3C and dataset S2). Summing all progeny, the percentage of recombinants between furA and pyrF is 46%–47%; furA and pyrG is 49%, and pyrF and pyrG is 48–50%.

Irregular Gene Reduction Events Occur in Individual Zygospores

Previous analyses using phenotypic markers suggested that the reduction process in zygospores cannot be perfect meiosis. To examine this subject further, 176 progeny were isolated from eight single zygospores. Markers on each of the nine linkage groups (LOD score 3) were amplified and alleles assigned to the progeny (dataset S3). The expectation from meiosis is that each marker should be represented by each allele at equal frequency, and the markers on separate chromosomes segregate independently. Further, four genotypes should be observed per zygospore. Of the progeny from the eight zygospores examined only one produced four different genotypes. The progeny from the other zygospores provided evidence for amplification of one or two genotypes, while zygospore 1 yield progeny with markers all from strain UBC21. Furthermore, all eight zygospores produced progeny in which at least one allele only was present, rather than the expected 50:50 ratio. This result is consistent with previous observations on skews from normal proportions for crosses between UBC21 and NRRL1555 [42].

Based on the information of analysis from the eight zygospores, the data set of 121 progeny was re-examined for similar evidence of non-Mendelian reduction events. To be stringent, the data analyzed were the nine linkage groups supported at LOD 3 with the exception of the two smallest groups of five markers each (LG VII and LG VIII). There are 94 strains in which one or more linkage groups had markers from only one parent (Fig. 5, dataset S1). On the other hand, there is no progeny in which each linkage group showed segregation as a unit from each parent, although three of the progeny had most alleles derived from one parent.

Discussion

Mendelian genetic analysis is a powerful experimental tool available for research on eukaryotic organisms, and includes unique capabilities to address specific aspects about the behavior of chromosomes. Map-based cloning has led to the identification of genes to understand their functions in numerous eukaryotes. The ability to perform crosses is particularly important in organisms that have not been successfully transformed. The
Figure 3. Mutations identified in strains of Phycomyces. A. Phenotypes on potato dextrose agar (PDA), PDA +250 mg/L 5-fluorouracil, YNB minimal medium, and YNB supplemented with uracil (20 mg/L) or lysine (30 mg/L) after three days growth at room temperature. The PDA was supplemented with uracil to support growth of the *pyrF* and *pyrG* mutants. B. The positions (red lines and strain names) and nature (codons and amino acid residue substitutions) of the mutations in the corresponding genes in these strains. C. Segregation data for *lysA*. Parents were A914 (A) and for UBC21 (U). Alleles are: for auxotrophy are either A for *lysA* or WT for wild type, the C or T nucleotide in the *lysA* gene, for PCR-RFLPs A for *NRRL1555* and B UBC21, the *sex* gene is either *sexM* (M) or *sexP* (P), and the phototropism phenotype I for *madI* or WT for wild type. Progeny with both alleles are designated U and shaded blue and nt indicates a genotypic (rather than phenotypic) marker. D. Segregation data for *furA*. Parents were A34 (A) and NRRL1555 (N). Markers are: resistant (R) or sensitive (S) to 5-fluorouracil, the A or G nucleotide in the *furA* gene, *madD* (D) or wild type (WT) for phototropism, the sex phenotype is either (+) or (–) and equivalent PCR analysis for the *sexM* (M) or *sexP* (P) gene.
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primary aim of this study was to generate a genetic map that could be used as the framework for positional cloning of genes in the fungus *Phycomyces blakesleeanus*. Hundreds of mutants of this fungus have been isolated [37], yet due largely to the inability to transform DNA into this species only eight of those mutated genes have been identified. Secondary to this aim was to revisit the questions surrounding evidence for a sexual cycle in this fungus and, by extension, the Mucoromycotina.

We performed more than 16,000 PCRs to assign RFLP markers to 121 progeny from the cross between UBC21 (+) and NRRL1555 (–). The results show that recombination and crossing over occurs across the genome (Fig. 2; Fig. 5; dataset S1). This is an important observation for two reasons. First, crossover frequencies are one indicator of meiotic vs. mitotic recombination, discussed in more detail below. Second, recombination occurs across all linkage groups/chromosomes. Strain UBC21 has been recommended for genetic analysis since the 1970s. It has a short zygospore germination time (two months) when crossed with the laboratory standard strain NRRL1555, and is highly polymorphic with respect to NRRL1555 in which most mutants have been isolated. However, this divergence and the segregation ratios in progeny from crosses using this strain [40] were a concern in implementing it for map-based cloning. These segregation data suggest that this strain is suitable for map-based identification of genes, and may combine meiotic reduction with a proportion of non-recombinational transmission of the chromosomes, that together that would aid mapping.

Recombination rates within chromosomes of fungi vary between species. The *Phycomyces* estimate at 33.2 kb per cM is comparable to other sexual species. For example, this rate is the same as the mushroom-forming species *Coprinus cinereus* which is also 33 kb/cM [43] or plant pathogen *Gibberella zeae* which is 32 kb/cM [44]. *C. cinereus* is a model for meiosis in the basidiomycetes. *G. zeae* is a homothallic ascomycete whose ascospores are one form of inoculum during the disease cycle. Thus, the recombination frequency in the Mucoromycotina species *Phycomyces* is at a level observed in other fungi with well-established meiotic cycles.

The segregation data acquired here differ from the two previous approaches used for isolation of progeny of *Phycomyces*. One approach takes individual zygospores and scores all or many progeny derived from them. This has the power to map additional features of the *Phycomyces* genome, such as centromeres [14]. The second approach has been to pool the products from multiple zygospores, and treat the mix as in a random spore analysis. Our mapping approach used a single progeny taken from a single zygospore. This offers advantages by reducing physical manipulations or the analysis of large numbers of progeny.

Genes that have been sequenced and also mapped based on mutant phenotypes in *Phycomyces* are: *carRA* and *carB* that control biosynthesis of β-carotene [45,46]; the sex mating type locus [39]; *madA* and *madB* that encode a blue light photoreceptor complex [35,36]; *furA* and *furB* that are linked auxotrophic markers [47]; *pyrG* [26,48]; and recently *carS* for cleavage of β-carotene [49,50]. Knowledge of other genes would help link the two maps more thoroughly. We identified base pair substitution mutations in strains carrying *pyrF* and *pyrG* mutations (Fig. 3). We also identified mutations within *lysA* and *furA*. *lysA* bears a mutation within a gene

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**Figure 4. Comparison between genetic maps of *Phycomyces*.** The previous mapping data is dark blue, redrawn from reference [29]. The positions of the genes in the new RFLP map (pale blue) are based on the locations of the closest markers to the gene. Five additional linkage groups were established in previous studies and remain to be anchored to the RFLP-based map. doi:10.1371/journal.pone.0058931.g004
encoding γ-amino adipate reductase, the fifth step in the biosynthesis of lysine. furA bears a mutation within a gene encoding uracil phosphoribosyltransferase, required for salvage of pyrimidines. nicA was predicted to encode quinolinate phosphoribosyltransferase, the final step in the de novo biosynthesis of nicotinic acid from tryptophan [15]. A point mutation was identified in this homolog in a nicA mutant (Alcalde, Idnurm and Cerda- Olmedo, unpublished). Genetic segregation analysis of progeny from crosses between the lysA and furA mutants with a wild type parent show perfect co-segregation of the mutations and the corresponding phenotypes (Fig. 3C, D).

The new genetic map was aligned with the genetic map generated previously (Fig. 4). Overall, there was strong congruence between previous and current segregation data. This includes linkage between markers near purC, leuA and madA; lysA and sex; furA and madD. One exception was the pyrF and pyrG genes that were previously mapped to the same linkage group as furA, furB and madD [26]. In our analysis, both the pyrF and pyrG genes are unlinked to these genes. The reason behind this discrepancy is unknown for pyrG; the difference could be explained by a chromosomal rearrangement in the pyrG mutant strain used to establish the linkage. In the previous crosses pyrF was distant from the furA, madD and furB cluster at 39, 42.5 and 47 cM away, depending on the marker and progeny characterization used. These distances are not far from that expected for unlinked markers. Direct comparisons between the two mapping approaches should be used with caution since different methods were used to generate the progeny and calculate recombination distances. Figure 4 also shows mapped genes defined by mutant phenotypes that are yet to be identified: comparison of the maps could prioritize the targets for these unknown genes.

Recombination and meiosis in Phycomyces have been topics of investigation since Burgeff’s studies in the 1910s, and aided by phenotypic markers since the 1970s. Despite being the most amenable species for genetics in the Mucoromycotina, the events during a cross remain unclear, even with independent Mendelian analyses. For instance, Mehta and Cerda-Olmedo point out that their data and other segregation ratios are inconsistent with the model of one diploid nucleus undergoing a meiotic reduction [17], and that the evidence for meiosis in Phycomyces is scant. This is a valid point, although it is worth stressing that by similar criteria the evidence for meiosis is absent for most eukaryotes species, including many fungi.

To address the question of meiosis in Phycomyces further, we examined the evidence of crossing over on the establishing linkage groups. At least one cross-over event per chromosome is considered to stabilize chromosomes during meiosis [51]. Thus, the absence of recombination over a linkage group in one of the progeny may reflect nondisjunction of those chromosomes. We examined the UBC21 x NRRL1555 progeny set to look for examples of chromosomes with no recombination between the two parents (excluding the two smallest, VII and VIII), with the three progeny that inherited alleles primarily from UBC21 marked with *.

Figure 5. Graphical representation of allele segregation and recombination in 121 progeny. Each allele is colored green if from NRRL1555 (N), yellow if from UBC21 (U), blue if both, and grey if missing or ambiguous. The markers are divided into each linkage group (LOD score 3). The red boxes indicate progeny with one or more linkage groups with no recombination between the two parents (excluding the two smallest, VII and VIII), with the three progeny that inherited alleles primarily from UBC21 marked with *. doi:10.1371/journal.pone.0058931.g005
NRRL1555. Together with the two diploid or heterokaryotic strains, 79% have this pattern of segregation (Fig. 5). However, with the three exceptions in which the progeny were primarily the UBC21 genotype, there were no examples in which every linkage group had been contributed into the progeny without recombination.

Our data cannot fully resolve the nature of the genetic reduction event(s) within the Phycomyces zygospore. These cells do demonstrate recombination, but also suggest the process is not “perfect” meiosis, as has been noted previously. The germinated zygospores compatible with one meiosis in a previous four-factor cross was about 78% [14]. We favor a hypothesis that the process relies on meiosis, and that it is influenced by poor or mistimed aligned of chromosomes during meiosis I. This is based on the relatively high rates of recombination (i.e. 33.2 kb per cM) observed in this cross, which is considered poor for marker segregation compared to crosses between strains with a higher proportion of shared genetic material [40]. Either Phycomyces undergoes meiosis or has a mitotic recombination frequency orders of magnitude greater than other eukaryotes. The second piece of evidence for meiosis is the Phycomyces sex locus, that resembles sex-determining regions in both fungi and animals. The sexM and sexP genes are within idiomorphic regions of DNA surrounded by conserved DNA sequence [52]. Third, genes encoding proteins with dedicated roles in meiosis [53], such as homologs of Dmc1, Spo11, Hop2, Mxi1, Msh4/Msh5, are found in the Phycomyces genome. Thus, the recombination frequency we observe, at the same level seen in other fungi, provides an experimental piece of evidence for meiosis in Phycomyces, and by extension the potential for meiosis broadly in the order Mucorales.

A caveat in interpreting the segregation data to assess the presence of meiosis in Phycomyces is that crosses between UBC21 and NRRL1555 may not be representative of a normal interaction between wild strains of Phycomyces. The geographical origin and year of isolation of both strains is unknown beyond culture collection acquisition. Improvement in “tetrad” frequencies increases with introgression of the genetic background [40]. For other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis could reflect fitness defects for the other crosses, the observed genetic ratios that are skewed from those expected after meiosis...
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