Meiotic Regulators Ndt80 and Ime2 Have Different Roles in *Saccharomyces* and *Neurospora*

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ABSTRACT

Meiosis is a highly regulated process in eukaryotic species. The filamentous fungus *Neurospora crassa* has been shown to be missing homologs of a number of meiotic initiation genes conserved in *Saccharomyces cerevisiae*, but has three homologs of the well-characterized middle meiotic transcriptional regulator *NDT80*. In this study, we evaluated the role of all three *NDT80* homologs in the formation of female reproductive structures, sexual development, and meiosis. We found that none of the *NDT80* homologs were required for meiosis and that even the triple mutant was unaffected. However, strains containing mutations in NCU09915 (*fsd-1*) were defective in female sexual development and ascospore maturation. *vib-1* was a major regulator of protoperithecial development in *N. crassa*, and double mutants carrying deletions of both *vib-1* (NCU03725) and *fsd-1* exhibited a synergistic effect on the timing of female reproductive structure (protoperithecia) formation. We further evaluated the role of the *N. crassa* homolog of *IME2*, a kinase involved in initiation of meiosis in *S. cerevisiae*. Strains containing mutations in *ime-2* showed unregulated development of protoperithecia. Genetic analysis indicated that mutations in *vib-1* were epistatic to *ime-2*, suggesting that *IME-2* may negatively regulate *VIB-1* activity. Our data indicate that the *IME2/NDT80* pathway is not involved in meiosis in *N. crassa*, but rather regulates the formation of female reproductive structures.

MATING and meiosis in fungi entail a complex, highly regulated developmental process. Nutritional cues, as well as layers of transcriptional and post translational regulation accompany the initiation and completion of meiosis. Previous characterization of fungal meiosis focused primarily on the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, in which a cascade of transcriptional activity controls progression through early, middle, and late meiotic events (YAMAMOTO 1996; KASSIR *et al.* 2003). However, genome-sequencing data show that many filamentous fungi are missing key components of the meiotic regulatory machinery present in *S. cerevisiae* and *Schizosaccharomyces pombe* (BORKOVICH *et al.* 2004; GALAGAN *et al.* 2005; DONALDSON and SAVILLE 2008; BUTLER *et al.* 2009). For example, the *Neurospora crassa* genome contains homologs of *S. cerevisiae* meiotic regulators such as *IME2*, *RAD17*, and two of the *CLB* genes, yet homologs to *IME1*, *IME4*, *UME6*, and *SUM1* are absent (BORKOVICH *et al.* 2004). Further, even though homologs to meiotic genes are conserved, their

functions have diverged (XIANG and GLASS 2002; CHEN *et al.* 2004; KATZ *et al.* 2006; BAYRAM *et al.* 2009).

One such example is the *S. cerevisiae* meiotic transcription factor *NDT80*. Ndt80 belongs to the Ig-fold family of transcription factors (LAMOUREUX *et al.* 2002; MONTANO *et al.* 2002) and regulates the prophase-to-metaphase transition in *S. cerevisiae* (CHU and HERSKOWITZ 1998; HEPWORTH *et al.* 1998; TUNG *et al.* 2000; PAK and SEGALL 2002b). Ndt80 controls the expression of ~150–200 genes that regulate meiotic progression and spore formation (CHU and HERSKOWITZ 1998; CHU *et al.* 1998). *NDT80* expression is activated during mating by the transcriptional regulator Ime1, the protein kinase Ime2, and by Ndt80 itself (CHU and HERSKOWITZ 1998; SOPKO *et al.* 2002).

One of the three *NDT80* homologs (*vib-1*) in the filamentous ascomycete species *N. crassa* is required for heterokaryon incompatibility (HI). HI is a nonself recognition mechanism that is ubiquitous among filamentous ascomycete species (SAUPE 2000; DEMENTHON *et al.* 2006; AANEN *et al.* 2009). Hyphal fusion between strains that differ in allelic specificity at nonself recognition loci (called *het* loci) results in rapid compartmentalization and death of the fusion cell. Mutations in *vib-1* suppress cell death caused by allelic differences at a number of *het* loci (XIANG and GLASS 2002; XIANG and GLASS 2004; DEMENTHON *et al.* 2006). HI has been likened to a fungal immune system (PAOLETTI and SAUPE 2009) and has been shown to be important for

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.117184/DC1>.

Microarray data have been deposited at the Filamentous Fungal Gene Expression Database (experiment ID 49; <http://bioinfo.townsend.yale.edu/browse.jsp>).

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limiting the spread of mycoviruses and senescence plasmids throughout fungal populations (DEBETS *et al.* 1994; VAN DIEPENINGEN *et al.* 1997).

Two additional homologs of *NDT80*, NCU09915 and NCU04729, are present in the *N. crassa* genome (BORKOVICH *et al.* 2004). Here, we investigated whether NCU09915 and NCU04729 were involved in HI and whether any of the three *NDT80* homologs retained meiotic function in *N. crassa*. The *N. crassa* sexual cycle (Figure 1) differs quite significantly from that of *S. cerevisiae* (RAJU 1980, 2009). Although sexual cycle mutants have been molecularly characterized (POGgeler *et al.* 2006; BURNS *et al.* 2010; DEBUCHY *et al.* 2010) and homologs to known sexual development genes have been identified via a computational approach (BORKOVICH *et al.* 2004), a complete picture of the meiotic signaling cascade in *N. crassa* is still largely unknown. Our studies indicate that although the *NDT80* and *IME2* homologs have a role in female sexual development in *N. crassa*, strains containing mutations in these genes are unaffected in meiosis or meiotic progression.

MATERIALS AND METHODS

***N. crassa* strains and growth conditions:** Strains used in this study are listed in Table 1. Fungal Genetics Stock Center (FGSC) 11308, 11309, 12180, 12534, 17936, and 17937 were constructed by the Neurospora program project grant (COLOT *et al.* 2006) and obtained from the FGSC (McCLUSKEY 2003). Strains were grown on Vogel's minimal medium (VOGEL 1956) with supplements as required and were crossed on Westergaard's medium (WESTERGAARD and MITCHELL 1947). Heterokaryons were forced by plating strains with complementary auxotrophic markers on minimal media. Tubes used to measure aerial hyphae extension were prepared similar to COLOT *et al.* (2006). For quantification of protoperithecial formation, strains were grown on 2% water agar or Vogel's minimal media (VOGEL 1956) for 7–10 days on 35 × 10-mm plates, and 10 plates were inoculated per strain. Protoperithecia were counted using a stereomicroscope; numbers are an average of two to three experimental replicates (20–30 plates total). Statistical significance was calculated by performing unpaired *t*-tests using Microsoft Excel.

Phylogenetic analysis: Identification of homologs was done using BLAST (ALTSCHUL *et al.* 1990) searches of fungal genomes and for fungal genes annotated as having an Ndt80 DNA-binding domain (DBD). Sequences were aligned using ClustalW2 (THOMPSON *et al.* 2002) and viewed using BioEdit (HALL 1999). The DBD sequence was used to construct the phylogenetic tree as *NDT80* homologs are quite divergent outside the DBD. The best-fit nucleotide substitution model for the alignment was Hasegawa-Kishino-Yano with γ substitution rates estimated using FINDMODEL (TAO *et al.* 2005). Phylogenetic trees were created using MRBAYES 3.1 (RONQUIST and HUELSENBECK 2003) with four separate chains run for 500,000 generations. We used a burn-in value of 1000, and trees were sampled every 100 generations. Trees were viewed using Njplot (PERRIERE and GOUY 1996).

Fertilization assay: Fertilization assays were performed as previously described (BISTIS 1981; FLEISSNER *et al.* 2005). Either a Δ NCU09915 strain (FGSC 12534) or a wild-type strain (FGSC 2489) was used as the female and a histone H1-GFP-

FIGURE 1.—Sexual cycle of *N. crassa*. Protoperithecia form during nitrogen starvation and can be fertilized by conidia or hyphal fragments from the opposite mating type. Upon fertilization, parental nuclei remain apart and undergo karyogamy only after crozier structures have formed. Once karyogamy occurs and meiosis is complete, progeny undergo an additional mitosis, resulting in eight progeny (ascospores) in each ascus. Several hundred asci are present in each perithecium. Eventually, ascospores are shot from the perithecia and germinate, forming new, homokaryotic colonies. This figure was adapted and modified from FLEISSNER and GLASS (2006).

labeled strain (N2283) (FREITAG *et al.* 2004) was used as the male. Microconidial histone H1-GFP fluorescence was monitored approximately every 10–20 hr, and 90–100 trichogynes were examined for each strain. Percentage fusion was calculated as the number of successful trichogyne fusions at one time point compared to the total number of trichogynes examined.

Cell death assays: Cell death was assayed with the vital dye methylene blue. Forced heterokaryons were grown on minimal media for 2 days, transferred to minimal media overlaid with cellophane, and grown for an additional 2–3 days. Pieces of the cellophane with hyphae were cut out and stained with 0.003% methylene blue for 1–2 min. Heterokaryons were examined by light microscopy for cell death. Approximately 20 images were taken for each heterokaryon, and the number of cells stained with methylene blue was divided by the total number of cells. Numbers are an average of two replicates.

RNA extraction: RNA extractions were performed using the TRIzol method (Invitrogen). For extracting perithecial RNA, female strains were inoculated onto cellophane laid over Westergaard's plates. After fertilization and perithecial development, the entire sheet of cellophane with perithecia was removed and homogenized using a 6770 Freezer/Mill from SPEX CertiPrep Group. Homogenization cycling conditions used were three cycles of 1 min pre-cool, 1 min run time, and 1 min cool time with a speed of 15 cycles per second. TRIzol was added to the homogenized perithecial tissue and divided into 2-ml aliquots. Samples were further homogenized by adding 0.3 g of 0.5-mm silica beads and bead-beating on maximum speed for 1.5 min (Mini-BeadBeater-8, Biospec Products). Extracted RNA was treated with Ambion Turbo DNase for 30 min at 37° and cleaned using a Qiagen RNeasy kit. RNA sample quality was assessed via gel electrophoresis and a NanoDrop spectrophotometer (Thermo Scientific).

Quantitative reverse transcription PCR: Strains were inoculated onto slant tubes and grown at 25° for 1 week in constant light. Conidia were inoculated onto Westergaard's plates and grown at 25° in constant light for ~1 week (until protoperithecia were visible). Samples were collected before fertilization (time 0) and at 3, 5, 7, and 10 days later. RNA from two independent extractions for each treatment was pooled, and 75 ng of RNA was used as template in each quantitative reverse transcription PCR (qRT-PCR) reaction. qRT-PCR was carried out using either a Qiagen QuantiTect SYBR Green RT-

TABLE 1
Strains used in this study

Strain name	Genotype ^a	Origin or reference
FGSC 2489	<i>A</i>	FGSC
FGSC 6103	<i>his-3; A</i>	FGSC
FGSC 4564	<i>ad-3B cyl-1 a^{mt}</i>	FGSC
R15-7	<i>his-3; a</i>	DEMENTHON <i>et al.</i> (2006)
N2283	<i>his-3::Pccg-1-hH1-sgfp a</i>	FREITAG <i>et al.</i> (2004)
C9-2	<i>het-c2 pin-c2 thr-2 a</i>	SMITH <i>et al.</i> (2000)
C9-15	<i>het-c2 pin-c2 thr-2 A</i>	SMITH <i>et al.</i> (2000)
FGSC 11308	$\Delta vib-1 a$	FGSC
FGSC 11309	$\Delta vib-1 A$	FGSC
FGSC 12180	$\Delta NCU04729 a$	FGSC
FGSC 12534	$\Delta NCU09915 A$	FGSC
FGSC 17936	$\Delta ime-2 a$	FGSC
FGSC 17937	$\Delta ime-2 A$	FGSC
KD13-21	<i>his-3; $\Delta vib-1 A$</i>	DEMENTHON <i>et al.</i> (2006)
KD13-51	$\Delta vib-1; pan-2 A$	DEMENTHON <i>et al.</i> (2006)
KD13-33	$\Delta vib-1; pan-2 a$	DEMENTHON <i>et al.</i> (2006)
KD13-01	<i>het-c2 pin-c2 thr-2; $\Delta vib-1 a$</i>	DEMENTHON <i>et al.</i> (2006)
KD13-23	<i>his-3; het-c2 pin-c2; $\Delta vib-1; pan-2 A$</i>	DEMENTHON <i>et al.</i> (2006)
D49.5	$\Delta NCU09915; \Delta NCU04729 a$	FGSC 12180 $\times$ FGSC 12534
D9V.8 ^b	<i>his-3; $\Delta NCU09915; \Delta vib-1 A$</i>	KD13-23 $\times$ D49.5
D9V.12 ^b	$\Delta NCU09915; \Delta vib-1; pan-2 a$	KD13-23 $\times$ D49.5
D9V.18 ^b	$\Delta NCU09915; \Delta vib-1 a$	KD13-23 $\times$ D49.5
D49V.2 ^b	<i>his-3; $\Delta NCU09915; \Delta vib-1; \Delta NCU04729 A$</i>	KD13-23 $\times$ D49.5
D49V.17 ^b	$\Delta NCU09915; \Delta vib-1; \Delta NCU04729 a$	KD13-23 $\times$ D49.5
D9.4	<i>his-3; $\Delta NCU09915 a$</i>	FGSC 12534 $\times$ R15-7
D9.1	<i>het-c2 pin-c2 thr-2; $\Delta NCU09915 a$</i>	FGSC 12534 $\times$ C9-2
D4.3	<i>his-3; $\Delta NCU04729 A$</i>	FGSC 12180 $\times$ FGSC 6103
D4.9	<i>het-c2 pin-c2 thr-2; $\Delta NCU04729 A$</i>	FGSC 12180 $\times$ C9-15
D49.10	<i>his-3; $\Delta NCU09915; \Delta NCU04729 A$</i>	D49.5 $\times$ FGSC 6103
D49.01	<i>his-3; $\Delta NCU09915; \Delta NCU04729 a$</i>	D49.5 $\times$ FGSC 6103
D49.33	<i>thr-2; $\Delta NCU09915; \Delta NCU04729 A$</i>	D49.5 $\times$ C9-15
D49.28	<i>het-c2 pin-c2 thr-2; $\Delta NCU09915; \Delta NCU04729 a$</i>	D49.5 $\times$ C9-15
D9I.7	$\Delta ime-2; \Delta NCU09915 A$	FGSC 12534 $\times$ FGSC 17936
DVI.4	$\Delta ime-2; \Delta vib-1 a$	FGSC 11309 $\times$ FGSC 17936
D9VI.5	$\Delta ime-2; \Delta NCU09915; \Delta vib-1 A$	DVI.4 $\times$ FGSC 12534

FGSC, Fungal Genetics Stock Center.

^a Strains are of the *het-c1 pin-c1* genotype unless otherwise indicated.

^b *het-c pin-c* genotypes of these strains were not tested.

PCR kit (Figure 7) or an EXPRESS One-Step SYBR GreenER kit (Invitrogen) (supporting information, Figure S2) and an ABI 7300 real-time PCR system machine. Reactions were set up according to the manufacturer. Results were analyzed using ABI 7300 system software, version 1.3.0. For all experiments, qRT-PCR reactions were done in triplicate, actin was used as the endogenous control, and relative expression data were normalized to wild type.

Microarray hybridizations: Microarray construction and array hybridizations were performed according to TIAN *et al.* (2007). Equal amounts of DNase-treated RNA (between 10 and 20 μ g) were used for cDNA synthesis using the ChipShot Indirect cDNA Synthesis kit (Promega), performed according to the manufacturer. Hybridizations were performed using ProntoPlus kits (Promega) according to the manufacturer. Slides were scanned using an Axon GenePix 4000B scanner, and GenePix Pro 6 software (Molecular Devices) was used to analyze spot intensity in addition to manual edits. Data from two biological replicates were analyzed using BAGEL (Bayesian Analysis of Gene Expression Levels) (TOWNSEND and

HARTL 2002), and genes with significantly different expression levels >1.5 between wild-type and the homozygous deletion cross were analyzed. Expression patterns for 10 genes from the microarray results were confirmed using qRT-PCR from an independent experiment.

RESULTS

Multiple homologs of *NDT80* are present in ascomycete genomes: The *S. cerevisiae* genome contains a single copy of *NDT80*; however, many filamentous ascomycete genomes contain two to three *NDT80* homologs (Table S1). Phylogenetic analysis revealed that *NDT80* homologs fall into three well-supported groups within the Sordariomycetes (Figure 2), each containing a single *N. crassa* *NDT80* homolog [NCU03725 (*vib-1*) clade, NCU04729 clade, NCU09915 clade]. *S. cerevisiae* *NDT80*

FIGURE 2.—Bayesian consensus tree showing phylogenetic relationships of *NDT80* fungal homologs. A Bayesian tree for the Ndt80-binding domain in fungi was created using MRBAYES 3.1 (RONQUIST and HUELSENBECK 2003). Numbers above the nodes indicate posterior probabilities from the consensus tree, and the scale bar indicates the number of substitutions per nucleotide site. Gene locus name or accession number for each sequence is indicated in parentheses (see Table S1 for additional information on sequence sources).

forms an outgroup to other *NDT80* homologs, but groups more closely with the clade containing NCU09915. Of these homologs, only *vib-1* (NCU03725) and an *Aspergillus nidulans* homolog of *vib-1*, *xprG* (KATZ *et al.* 2006; ANID_01414; Figure 2), have been characterized. *vib-1* mutants suppress HI and display altered conidiation patterns, and, similar to the *xprG* mutant, *vib-1* mutants are deficient in protease secretion under nutrient-limited conditions (DEMENTHON *et al.* 2006).

Phenotypic analysis of *NDT80* homologs revealed that mutations in *vib-1* and NCU09915 affect protoperithelial development: First, we investigated whether strains containing deletion mutations in NCU09915 or NCU04729 (COLOT *et al.* 2006) showed similar vegetative phenotypes to that of $\Delta vib-1$ strains. *vib-1* loss-of-function mutants (FGSC 11309; see Table 1 for strain list) exhibit pinkish, deregulated conidiation with decreased aerial hyphae extension (XIANG and GLASS 2002; DEMENTHON *et al.* 2006). Deletion strains of NCU04729 (FGSC 12180), NCU09915 (FGSC 12534), or a double mutant ($\Delta NCU04729 \Delta NCU09915$; D49.5) showed no visible vegetative phenotype with respect to conidiation and aerial hyphae formation, and a triple-deletion strain ($\Delta NCU04729 \Delta NCU09915 \Delta vib-1$;

D49V.17) looked macroscopically identical to the *vib-1* mutant (Figure 3, A–L).

vib-1 mutations suppress programmed cell death due to HI (XIANG and GLASS 2002). *het* incompatible strains can be forced to grow as heterokaryons by using complementary auxotrophic markers. Incompatible heterokaryons are severely inhibited in growth rate, are aconidial, and have a high percentage of dead hyphal compartments. To assess whether mutations in NCU09915 and/or NCU04729 might affect/suppress HI, we constructed $\Delta NCU09915$ and $\Delta NCU04729$ deletion strains carrying complementary auxotrophic markers of either identical or incompatible *het-c pin-c* haplotype (Table 1). Wild-type incompatible heterokaryons had a death percentage of 30.3%, while heterokaryons of identical *het-c pin-c* specificity (compatible heterokaryons) showed 2% cell death across a colony (Figure 3, M and N). Incompatible heterokaryons carrying *vib-1* deletions were suppressed for HI (Figure 3P) and showed cell death rates of 1.7%. However, heterokaryons carrying homozygous deletions of NCU04729 or NCU09915 showed no effect on HI or cell death. The $\Delta NCU04729$ and $\Delta NCU09915$ incompatible heterokaryons exhibited a cell death rate of 29.5% and 26.7%, respectively. Incompatible and compatible $\Delta NCU04729 \Delta NCU09915$ double-mutant heterokaryons also looked identical to wild-type incompatible and compatible heterokaryons (Figure 3, Q and R) and had cell death rates of 34% and 5.9%, respectively.

Although we could not detect any phenotype of $\Delta NCU04729$ or $\Delta NCU09915$ deletion strains on minimal media, both $\Delta vib-1$ and $\Delta NCU09915$ strains showed a defect for formation of female reproductive structures (protoperithecia) when grown under nitrogen-poor conditions, which induces formation of these structures (WESTERGAARD and MITCHELL 1947; HIRSH 1954). Protoperithecia are spherical hyphal structures with specialized hyphae called trichogynes that are attracted to and fuse with a partner cell of the opposite mating type (Figure 1). The $\Delta vib-1$ mutant showed an ~80% reduction in the number of protoperithecia formed in a colony, while the $\Delta NCU09915$ mutant showed an ~60% reduction. A double mutant ($\Delta NCU09915 \Delta vib-1$; D9V.18) formed very few female reproductive structures (Figure 4A).

In a wild-type strain, protoperithecia generally form ~5–7 days post-inoculation on nitrogen-poor medium. The $\Delta vib-1$ and $\Delta NCU04729$ strains showed a similar timing for development of protoperithecia. Strains containing a deletion of NCU09915 were slightly delayed in the development of female reproductive structures (Figure 4B, $p < 0.001$), a phenotype that could be complemented in a heterokaryon with a strain (FGSC 4564) containing a wild-type copy of NCU09915. The $\Delta vib-1 \Delta NCU09915$ strain showed a significant delay in formation of the few protoperithecia observed in these strains (Figure 4B, $p < 0.01$); no additional

FIGURE 3.—Mutants in NCU09915 (*fsd-1*) and NCU04729 do not exhibit vegetative phenotypes, unlike *vib-1*. Δ *vib-1* (B and H; FGSC 11309), Δ NCU04729 (C and I; FGSC 12180), and Δ NCU09915 (D and J; FGSC 12534) strains were grown on minimal media and compared with wild type (A and G; FGSC 2489). Only Δ *vib-1* mutants display deregulated, pink conidiation. A Δ NCU04729 Δ NCU09915 mutant (E and K; D49.5) looks identical to wild type, and a triple mutant of all three paralogs (F and L; D49V.17) looks identical to the Δ *vib-1* mutant. Deletion strains of *vib-1* (H) or the triple mutant (L) had decreased aerial hyphae formation. Deletions in NCU09915 or NCU04729 do not suppress heterokaryon incompatibility. Wild-type compatible (M; FGSC 4564 +

FIGURE 4.—*vib-1* and NCU09915 (Δ *fsd-1*) mutants have defects in protoperithecial formation. (A) Mutant strains were evaluated for the numbers of protoperithecia formed. (B) Length of time required post-inoculation for protoperithecia formation. A Δ *fsd-1* Δ *vib-1* mutant (D9V.18) took almost twice as long as wild type or single mutants to initiate protoperithecia formation. The triple mutant (D49V.17) looked identical to strain D9V.18. This defect can be complemented in a heterokaryon with the helper strain FGSC 4564.

delay was observed in the triple mutant (Δ *vib-1* Δ NCU09915 Δ NCU04729). The delayed protoperithecial development in the double and triple mutants was also complemented in a heterokaryon with FGSC 4564.

NCU09915 deletion strains are female sterile: *NDT80* mutants in *S. cerevisiae* are blocked at the prophase-to-metaphase transition, arrest at pachytene, and do not sporulate (Xu *et al.* 1995). Thus, we investigated strains with mutations in *vib-1* or its paralogs for

FGSC6103) and incompatible (N; FGSC 4564 + C9-15) heterokaryons are shown for reference compared to Δ *vib-1* compatible (O; KD13-21 + KD13-51) and incompatible (P; KD13-33 + KD13-01) heterokaryons. Compatible (Q; D49.10 + D49.33) and incompatible (R; D49.01 + D49.28) heterokaryons of a Δ NCU04729 Δ NCU09915 double mutant look identical to wild-type compatible and incompatible heterokaryons.

FIGURE 5.—*fsd-1* is necessary for sexual development, but not for meiosis. Homozygous crosses were performed for wild type (A; FGSC 2489 × R15-7), $\Delta vib-1$ (B; FGSC 11309 × FGSC 11308), $\Delta NCU04729$ (C; FGSC 12180 × D4.3), and $\Delta fsd-1$ (D; FGSC 12534 × D9.4). Successful crossing is indicated by the formation of black perithecial structures, which are absent for the $\Delta fsd-1$ cross (D). This phenotype could be complemented in a heterokaryon with the helper strain FGSC 4564 [E; (FGSC 4564 + D9.4) × FGSC 12534]. $\Delta fsd-1 \Delta vib-1$ homozygous and $\Delta fsd-1 \Delta vib-1 \Delta NCU04729$ homozygous crosses exhibited an identical phenotype to $\Delta fsd-1$ homozygous crosses and could be complemented using FGSC 4564 [F; triple-deletion strain cross shown; (FGSC 4564 + D49V.2) × D49V.17]. Bar, 1 cm.

sexual cycle defects. Homozygous $\Delta vib-1$ and $\Delta NCU04729$ strains crossed successfully and looked identical to wild-type crosses (Figure 5, A–C). However, although the $\Delta NCU09915$ strain formed visibly normal-looking proto-perithecia, these did not develop into mature perithecia upon fertilization (Figure 5D). We therefore refer to $\Delta NCU09915$ as female sexual development-1 (*fsd-1*). These data suggested that perhaps *fsd-1* was an ortholog of *NDT80* and might regulate meiosis in *N. crassa*. However, the *fsd-1* homozygous deletion crosses arrested well before pachytene (RAJU 1980) and did not form croziers or any other distinguishable ascogenous tissues (data not shown). We therefore interrogated the $\Delta fsd-1$ mutant at

several steps during the sexual cycle to determine where the block in sexual development occurred.

To evaluate whether the $\Delta fsd-1$ mutant had normal mating behavior, we performed a fertilization assay (BISTIS 1981; FLEISSNER *et al.* 2005). The $\Delta fsd-1$ mutant successfully fused with and accepted nuclei from conidia of the opposite mating type in a manner indistinguishable from a wild-type cross (Figure S1). Thus, the $\Delta fsd-1$ mutant showed no defect in fertilization.

Next, we assessed whether the sexual development defect of the $\Delta fsd-1$ mutant could be complemented in a heterokaryon with a strain that can form female reproductive structures, but cannot participate in a cross.

FIGURE 6.—Homozygous $\Delta fsd-1$ deletion crosses exhibit a spore maturation defect. (A) Homozygous crosses of wild type (wt) (FGSC 2489 × R15-7), $\Delta vib-1$ (FGSC 11308 × FGSC 11309), $\Delta NCU04729$ (FGSC 12180 × D4.3), $\Delta fsd-1$ [(FGSC 4564 + D9.4) × FGSC 12534], $\Delta vib-1 \Delta fsd-1$ [(FGSC 4564 + D9V.12) × D9V.8], and $\Delta vib-1 \Delta fsd-1 \Delta NCU04729$ [(FGSC 4564 + D49V.2) × D49V.17] were assessed for the percentage of black *vs.* white spores. Any cross containing a deletion of $\Delta NCU09915$ exhibited a reduction to only 50% black ascospores. (B) Squashes of perithecia from 10 to 14 days post-fertilization are shown below the corresponding cross in A. (Bar, 65 μ m).

TABLE 2

Genes downregulated in an $\Delta fsd-1$ ($\Delta NCU09915$) homozygous cross compared to a wild-type cross

Gene ID	Annotation (Broad Institute)	FunCat (MIPS) ^b
NCU09559 ^a	Trehalose synthase (<i>cgg-9</i>)	Metabolism; energy; cell-type differentiation; cell rescue, defense, and virulence
NCU05858 ^a	Related to linoleate diol synthase	Metabolism
NCU06905 ^a	Probable tetrahydroxynaphthalene reductase	Metabolism
NCU09041 ^a	Probable L-xylulose reductase	Metabolism
NCU10997 ^a	con-8	Cell-type differentiation
NCU04334 ^a	Probable heat-shock protein 10	Protein fate; cell rescue, defense, and virulence
NCU04421 ^a	Annexin XIV ANX-14	Cellular transport; interaction with the environment
NCU04603 ^a	Related to rasp f7 allergen	Subcellular localization
NCU05651 ^a	Related to lectin	Subcellular localization; classification not yet clear-cut
NCU01108 ^a	Related to seed maturation protein pm25	Classification not yet clear-cut
NCU02329	Conserved hypothetical protein	Unclassified proteins
NCU05191	Conserved hypothetical protein	Unclassified proteins
NCU09196	Conserved hypothetical protein	Unclassified proteins
NCU00716	Putative protein	Unclassified proteins
NCU00878	Putative protein	Unclassified proteins
NCU02170	Putative protein	Unclassified proteins
NCU02944	Putative protein	Unclassified proteins
NCU03253	Hypothetical protein	Unclassified proteins
NCU05229	Hypothetical protein	Unclassified proteins
NCU05490	Hypothetical protein	Unclassified proteins
NCU06434	Putative protein	Unclassified proteins
NCU07345	Putative protein	Unclassified proteins
NCU07363	Putative protein	Unclassified proteins
NCU08095	Hypothetical protein	Unclassified proteins
NCU08282	Putative protein	Unclassified proteins
NCU08654	Hypothetical protein	Unclassified proteins
NCU09851	Hypothetical protein	Unclassified proteins
NCU09926	Hypothetical protein	Unclassified proteins

^a Indicates genes selected to be confirmed by qRT-PCR.^b Functional categories predicted from Munich Information Center for Protein Sequences (<http://www.helmholtz-muenchen.de/en/ibis>).

The FGSC 4564 “helper” strain has a mutation in its mating-type gene and is able to complement strains with defects in female reproductive structures/perithecial formation, but cannot complement mutations in genes required for ascogenous hyphae development or meiosis (PERKINS 1984; RAJU and LESLIE 1992). A heterokaryon between FGSC 4564 and $\Delta fsd-1$ crossed normally, indicating that mutations in $\Delta fsd-1$ specifically affect development of female reproductive structures post-fertilization (Figure 5E). An $\Delta fsd-1 \Delta vib-1 \Delta NCU04729$ strain was also female sterile (data not shown), a defect that was complemented in a heterokaryon with the helper strain (Figure 5F).

$\Delta fsd-1$ homozygous deletion crosses show a defect in spore maturation: Mutations in *NDT80* block progression through meiosis. Homozygous $\Delta fsd-1$ crosses (using the $\Delta fsd-1$ + FGSC 4564 as a female) underwent normal meiotic progression. Rosettes of asci in the $\Delta fsd-1$ homozygous crosses formed at a similar time to wild type and had an indistinguishable morphology (Figure 6B). However, the $\Delta fsd-1$ homozygous crosses exhibited a decreased number of mature ascospores (Figure 6A, $p < 0.05$) with ~50% of the ascospores

being white. White ascospores indicate either aneuploidy (PERKINS 1974) or defective spore maturation. To assess whether aneuploidy (and thus a meiotic defect) caused the increase in white ascospores in $\Delta fsd-1$ homozygous crosses, we used a similar method to Foss *et al.* (1993) that uses the albino mutation to visualize aneuploidy events. Albino-1 (*al-1*) mutants have white asexual spores (conidia). Homozygous $\Delta fsd-1$ crosses were performed where one parent carried the *al-1* mutation. If ascospore progeny have duplications of the *al-1* gene due to aneuploidy, these sexual progeny will break down into haploid albino (white) and wild-type (orange) colonies. However, all progeny from the homozygous $\Delta fsd-1$ crosses gave rise to either all white or all orange colonies. Thus, the increase in white ascospores observed in $\Delta fsd-1$ crosses is likely due to mis-regulation of spore maturation genes.

To test this hypothesis, we used whole-genome microarrays (KASUGA *et al.* 2005; TIAN *et al.* 2007) to assess transcriptional differences between a homozygous $\Delta fsd-1$ cross *vs.* a wild-type cross during spore maturation/melanization, which occurs ~7 days post-fertilization

FIGURE 7.—(A) The phenotype of an $\Delta ime-2$ strain (FGSC 17936) looks similar to wild-type (FGSC 2489) as shown in flasks, in perithecial squashes, and in perithecia formation. Bar, 65 μm . (B) qRT-PCR during a time course of sexual development (from 0 to 10 days post-fertilization) showing that *fsd-1* expression is unaffected in an $\Delta ime-2$ homozygous cross. Wild-type cross, FGSC 2489 $\times$ R15-7; $\Delta ime-2$ cross, FGSC 17936 $\times$ 17937; and $\Delta fsd-1$, (D9.4 + FGSC 4564) $\times$ FGSC 12534.

(RAJU 1980). RNA was harvested from 7-day-old perithecia to use for microarray analysis, and genes that showed >1.5 -fold increase in relative expression level in the wild-type cross as compared to the homozygous deletion cross are listed in Table 2 (microarray data available at the Filamentous Fungal Gene Expression Database (ZHANG and TOWNSEND 2010; experiment ID 49; <http://bioinfo.townsend.yale.edu/browse.jsp>). Of the 28 genes in Table 2, 10 were confirmed by Q-RT-PCR (Figure S2). Several of the genes exhibiting lower expression in the homozygous *fsd-1* deletion mutant cross have annotations that are consistent with a spore maturation phenotype. For example, NCU06905 is annotated as a tetrahydroxynaphthalene reductase, which may be involved in melanin biosynthesis (BELL and WHEELER 1986). Although there is no experimental evidence that *N. crassa* produces dihydroxynaphthalene melanins, it does contain homologs of the necessary enzymes for this pathway (LANGFELDER *et al.* 2003). NCU01108 is annotated as being similar to a plant “seed maturation protein pm25,” and a deletion strain shows decreased pigmentation in vegetative hyphae (Broad Institute, <http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html>). In addition, several genes previously shown to be involved in sexual development either in *N. crassa* or in other fungi exhibited decreased expression levels in $\Delta fsd-1$ crosses, including NCU05651 (NOWROUSIAN and CEBULA 2005), NCU05858 (GOODRICH-TANRIKULU *et al.* 1998), and NCU09559 (NI and YU 2007). The majority of the genes that were identified, however, are hypothetical.

In addition to genes involved in meiotic progression, Ndt80 also regulates genes involved in spore maturation (CHU and HERSKOWITZ 1998; CHU *et al.* 1998), suggesting that this feature may be conserved between Ndt80 and FSD-1. However, only 6 of the 28 genes in Table 2 had homologs in the *S. cerevisiae* genome (Saccharomyces Genome Database; <http://www.yeastgenome.org/>), and none were present in the NDT80 profiling data set (CHU *et al.* 1998; National Center for Biotechnology Information Gene Expression Omnibus #GDS104). These observations suggest that this pathway has diverged in yeast *vs.* filamentous fungi.

***fsd-1* is upregulated during meiosis, but this regulation is not dependent on *ime-2*:** In *S. cerevisiae*, transcription of *NDT80* is activated by *IME1* and, indirectly, by *IME2* (CHU and HERSKOWITZ 1998; HEPWORTH *et al.* 1998). Once expressed, Ndt80 activates its own expression through a positive feedback loop (CHU and HERSKOWITZ 1998). The *N. crassa* genome does not contain an *IME1* homolog, but does have one homolog of *IME2* (NCU01498) (BORKOVICH *et al.* 2004). *IME2* is a serine–threonine protein kinase that regulates the expression and activity of many early and late meiotic genes; *ime2* mutants do not sporulate (SMITH and MITCHELL 1989; HONIGBERG 2004). In contrast to *S. cerevisiae* *ime2* mutants, homozygous crosses between *N. crassa* strains containing a deletion in NCU01498 (*ime-2*) were fertile and exhibited no sexual cycle defects (Figure 7A). We evaluated whether *fsd-1* expression was regulated by *IME-2* in a wild-type cross *vs.* an $\Delta ime-2$ homozygous cross. Over a 10-day time course, the

FIGURE 8.—*ime-2* negatively regulates protoperithecial formation. The number of protoperithecia formed on water agar was evaluated for wild type (FGSC 2489) and for deletion strains ($\Delta ime-2$, FGSC 17937; $\Delta vib-1$, FGSC 11308; $\Delta fsd-1$, FGSC 12534; $\Delta ime-2 \Delta fsd-1$, D9I.7; $\Delta ime-2 \Delta vib-1$, DVI.4; and $\Delta ime-2 \Delta vib-1 \Delta fsd-1$, D9VI.5) and are shown on the left y-axis and by shaded bars. The number of protoperithecia was also evaluated for wild type and the *ime-2* deletion strain on minimal media. The number of protoperithecia formed for wild type was set to 100% and averaged 25/plate on water agar and 6/plate on minimal media. The timing of protoperithecia formation was also evaluated for these strains and is shown on the right y-axis and by solid lines.

developmental progression of both crosses was identical (data not shown). We observed an increase in expression level of *fsd-1* during sexual development (Figure 7B), consistent with its role in spore maturation. However, in an $\Delta ime-2$ homozygous deletion cross, the expression pattern of *fsd-1* was identical to that in a wild-type cross. In addition, the deletion of *fsd-1* had no effect on *ime-2* expression. Thus, in *N. crassa* there is no evidence that the homologs of *IME2* or *NDT80* regulate meiosis.

***ime-2* is a negative regulator of protoperithecial formation:** Although *ime-2* deletion strains were unaffected in sexual development, we observed that $\Delta ime-2$ strains produced significantly more protoperithecia than wild type (Figure 8), suggesting that *IME-2* negatively regulates protoperithecial development. In addition, $\Delta ime-2$ strains formed abundant protoperithecia on minimal media, conditions that repress protoperithecial development in wild type. These protoperithecia were fully functional, could be crossed, and formed ascospore progeny (data not shown). These data suggested that *IME-2* may regulate *VIB-1* or *FSD-1* because $\Delta vib-1$ and $\Delta fsd-1$ mutants showed a reduction in the number of protoperithecia (Figure 4A, Figure 8). Although the $\Delta fsd-1$ mutants showed a reduction in numbers of protoperithecia formed, the $\Delta ime-2 \Delta fsd-1$ mutant (D9I.7) looked identical to the $\Delta ime-2$ mutant itself (many protoperithecia). However, unlike the $\Delta ime-2 \Delta fsd-1$ mutants, the $\Delta ime-2 \Delta vib-1$ strain (DVI.4) exhibited $\Delta vib-1$ levels of protoperithecial formation (few perithecia). The triple-deletion strain ($\Delta ime-2 \Delta vib-1 \Delta fsd-1$; D9VI.5) also had a similar phenotype to the $\Delta vib-1$

FIGURE 9.—Model for genetic interaction of *vib-1*, *fsd-1*, and *ime-2*. See text for details.

mutant itself. These data indicate that mutations in *vib-1* are epistatic to *ime-2* and suggest that *IME-2* may regulate *VIB-1* activity. Mutations in *ime-2* alone did not affect the timing of protoperithecial development, nor did they affect the delayed protoperithecial phenotype of the $\Delta fsd-1 \Delta vib-1$ strain (compare $\Delta fsd-1 \Delta vib-1$ in Figure 4B to $\Delta ime-2 \Delta vib-1 \Delta fsd-1$ in Figure 8). Thus, an interplay among *FSD-1*, *VIB-1*, and *IME-2* acts as a major regulatory circuit for protoperithecial formation in *N. crassa* (Figure 9).

DISCUSSION

Meiosis and sporulation in fungi requires a sequential step of committed events that are highly regulated. In this study, we characterized the three *N. crassa* homologs of the *S. cerevisiae* meiotic regulator *NDT80*. *Ndt80* binds to the middle sporulation element (MSE) and activates expression of middle meiotic genes (CHU and HERSKOWITZ 1998; CHU *et al.* 1998); cells lacking *Ndt80* arrest at pachytene, prior to nuclear division in meiosis I (XU *et al.* 1995). From our data, it is clear that none of the *NDT80* homologs in *N. crassa* are required for meiosis, as even the triple mutant ($\Delta vib-1 \Delta fsd-1 \Delta NCU04729$) showed normal meiotic progression. Although the $\Delta fsd-1$ mutant failed to generate mature perithecia following fertilization, this defect was restored in a heterokaryon with a strain (FGSC 4564) that is able to complement female reproductive, but not meiotic, defects (PERKINS 1984). However, homozygous crosses between strains containing deletion mutations in *fsd-1* (NCU09915), the closest homolog to *NDT80* (Figure 2), showed a high percentage of white ascospores. These defects are apparently due to misregulation of genes associated with spore maturation, rather than a consequence of meiotic defects. Thus, *fsd-1* has both early and late roles in sexual development. *Ndt80* also regulates genes involved in sporulation/spore maturation in *S. cerevisiae* (CHU and HERSKOWITZ 1998; CHU *et al.* 1998). However, we did not detect an overlapping gene set involved in spore maturation between *S. cerevisiae* and *N. crassa*, suggesting that the target genes involved in these two processes may have substantially diverged. This hypothesis is supported by differences in sexual spore features in these two species.

Although not required for meiosis, we identified a role for *NDT80* homologs in the production of female reproductive structures in *N. crassa*, which are not formed in yeast species such as *S. cerevisiae*, *S. pombe* or *Candida albicans*. Mutations in both *vib-1* and *fsd-1* affected the timing and development of female reproductive structures prior to fertilization, while, for NCU04729, no phenotype or overlap in function with either *fsd-1* or *vib-1* was detected. *vib-1* and *fsd-1* have a synergistic interaction with respect to initiation of protoperithelial formation. The initiation of protoperithelial development in *N. crassa* is regulated by the availability of nitrogen (WESTERGAARD and MITCHELL 1947; HIRSH 1954). *vib-1* has previously been implicated in nutrient sensing and protease secretion in response to nitrogen starvation (DEMENTHON *et al.* 2006). We hypothesize that the nutrient sensing by VIB-1 and FSD-1 is important for the initiation of protoperithelial formation and that *fsd-1* can compensate for lack of *vib-1* function. Interestingly, *ndt80* Δ mutants that are blocked in pachytene can revert to mitotic growth if nutrients are replenished (XU *et al.* 1995). These data are consistent with the hypothesis that sensing nutritional signals may be a conserved feature of Ndt80 and its homologs (KATZ *et al.* 2006). In addition to the effects on the initiation of protoperithelial development, both *vib-1* and *fsd-1* mutants showed a significant decrease in numbers of protoperithecia produced per colony, particularly Δ *vib-1* mutants, suggesting that VIB-1 and FSD-1 are also involved in the development of female reproductive structures (Figure 9).

Surprisingly, a deletion strain of the protein kinase *ime-2*, a major positive regulator of meiosis in *S. cerevisiae*, was fertile. In *S. cerevisiae*, Ime2 activates *NDT80* expression by phosphorylation of the repressor Sum1, thereby removing Sum1 from *NDT80* promoters and allowing *NDT80* expression (PAK and SEGALL 2002a; AHMED *et al.* 2009). In *N. crassa*, Δ *ime-2* mutants were unaffected in expression of *fsd-1* during development of asci and ascospores. This data, in conjunction with the fact that *N. crassa* is missing homologs of several key, early meiotic initiation genes, indicate that the early steps of meiotic regulation in *N. crassa* have diverged significantly from that of *S. cerevisiae*. However, the *ime-2* deletion strain constitutively produced protoperithecia and produced several hundred-fold more protoperithecia per colony than a wild-type strain. These data indicate that, like *vib-1* and *fsd-1*, *ime-2* is involved in the regulation of female reproductive structure development and functions as a negative regulator. We hypothesize that the *ime-2* mutant fails to respond to the presence of nitrogen in the media and constitutively makes protoperithecia under conditions where nitrogen would normally suppress development of these structures. In *S. cerevisiae*, Ime2 positively regulates Ndt80 via phosphorylation (SOPKO *et al.* 2002). It is possible that regulatory interactions between IME-2 and the *NDT80* homologs,

VIB-1 and FSD-1, are conserved in *N. crassa*. This hypothesis is supported by our data showing that the phenotype of the Δ *ime-2* strain (constitutive production of protoperithecia) is suppressed in a Δ *vib-1* mutation, while the Δ *ime-2* Δ *fsd-1* mutant showed an identical phenotype to Δ *ime-2* mutants. Future experiments will test whether IME-2 directly regulates VIB-1 by phosphorylation (Figure 9).

The *NDT80* cascade regulating meiotic function in *S. cerevisiae* has also diverged in other fungi, even in closely related species such as *C. albicans*. In *C. albicans*, CaNdt80 controls the expression of *CDR1*, a drug efflux pump that contributes to resistance against certain antifungal compounds (CHEN *et al.* 2004), in addition to genes involved in filamentous growth (SELLAM *et al.* 2009, 2010). Like many other *NDT80* homologs, including those in *N. crassa*, the DNA-binding domain of CaNdt80 is similar to Ndt80, but the rest of the protein is divergent (WANG *et al.* 2006). In *A. nidulans*, the *NDT80* homolog *xprG* (ANID_01414; Figure 2) controls the production of proteases upon nutrient starvation (KATZ *et al.* 2006), similar to *vib-1* (DEMENTHON *et al.* 2006). *xprG* is regulated by two hexokinases; it is unknown whether these kinases have a regulatory role for other *NDT80* homologs (KATZ *et al.* 2000; BERNARDO *et al.* 2007). In *A. nidulans*, the *IME2* homolog, *imeB*, is involved in the sexual cycle by negatively regulating cleistothecial (fruiting sexual structure) production in response to light cues (BAYRAM *et al.* 2009). In addition, *imeB* is required for expression of the sterigmatocystin mycotoxin gene cluster. Many of these developmental programs in fungi respond to nutritional cues. These observations suggest that light/nutritional conditions may regulate *NDT80* homologs in diverse fungi, perhaps via *IME2* homologs, but that the output pathway can vary and can include developmental programs such as sexual development, conidiation, and filamentous growth, as well as protease secretion and mycotoxin production.

By comparative genomics, it is apparent that the machinery for regulatory aspects of meiosis is not conserved in fungi, including *N. crassa* (BORKOVICH *et al.* 2004), *Candida* spp. (TZUNG *et al.* 2001; BUTLER *et al.* 2009), *S. pombe* (MATA *et al.* 2002; MATA and BAHLER 2003), *Aspergillus* spp. (GALAGAN *et al.* 2005), and *Ustilago maydis* (DONALDSON and SAVILLE 2008). For example, genes encoding the major transcriptional regulator of meiosis in *S. cerevisiae*, Ime1, are absent in many fungal species. Ime1 integrates nutritional and cell cycle signals to initiate meiosis by regulating expression of early meiotic genes (HONIGBERG and PURNAPATRE 2003), including transcription of *IME2*. In addition, homologs of the Ndt80 regulator, Sum1, are absent in many fungal species; Sum1 binds to MSEs and represses expression of middle meiotic genes via competition with Ndt80 (PIERCE *et al.* 2003). The absence of these meiotic regulatory genes indicates that

different fungi may differentially regulate the sequential steps associated with sexual development and meiosis. Fungi have a diverse array of mechanisms for undergoing sexual development, and in *S. pombe*, it was found that species-specific genes were more likely to be upregulated during a complex developmental process such as mating and meiosis as compared to vegetative growth (MATA and BAHLER 2003). Variation in meiotic gene content among different fungi suggests that this process is highly specialized for each species (SHERWOOD and BENNETT 2009) and that, as species diverge and develop specific sexual structures and regulatory cues, their meiotic machinery becomes modified as well.

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GENETICS

Supporting Information

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**Interplay Between *NDT80* Homologs and the Protein Kinase IME-2
Regulates Sexual Development, but Not Meiosis,
in *Neurospora crassa***

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FIGURE S1.—Trichogyne assay for wild-type and $\Delta fcd-1$ crosses. Trichogyne assays over a time-course of 24-65 hours revealed that the $\Delta fcd-1$ deletion strain is not defective for trichogyne-microconidium fusion, as fusion levels for the mutant are not significantly different from wild-type throughout the time-course.

FIGURE S2.—Q-RT-PCR confirmation of microarray data. Ten genes were selected from the microarray dataset and confirmed independently via Q-RT-PCR; all ten of these genes were significantly up-regulated in a wild-type cross compared to a cross homozygous for an NCU09915 deletion, and the Q-RT-PCR data confirms this result. In addition, the Q-RT-PCR data confirms that although the female of the homozygous deletion cross was present as a heterokaryon with the helper strain, no NCU09915 (*fsd-1*) RNA is present in the perithecial tissue of this cross.

TABLE S1
List of fungal *NDT80* homologs

Species	Number of <i>NDT80</i> homologs	Gene ID	Source for sequences used in FIGURE 2
<i>Neurospora crassa</i>	3	NCU03725, NCU04729, NCU09915	Broad Institute ^a
<i>Podospora anserina</i>	3	Pa_2_9470, Pa_4_8710, Pa_1_22920	http://podospora.igmors.u-psud.fr/
<i>Chaetomium globosum</i>	2	CHGG_02523, CHGG_03914	Broad Institute ^a
<i>Magnaporthe grisea</i>	3	MGG_00729, MGG_00622, MGG_01475	Broad Institute ^a
<i>Fusarium graminearum</i>	3	FGSC_7157, FGSC_8397, FGSG_09709	
<i>Nectria haematococca</i>	3	NHAE_EEU44303, NHAE_EEU46886, NHAE_EEU40284	
<i>Botryotinia fuckeliana</i>	3	BC1G_00547, BC1G_00333, BC1G_11604	
<i>Aspergillus oryzae</i>	2	AO090103000018, AO090011000646	
<i>Aspergillus clavatus</i>	2	ACLA_057380, ACLA_068960	
<i>Neosartorya fischeri</i>	2	NFIA_096760, NFIA_085330	
<i>Aspergillus flavus</i>	1	AFLA_012100	
<i>Aspergillus fumigatus</i>	2	AFUA_8G04050, AFUA_1G00580	
<i>Aspergillus nidulans</i>	2	ANID_01414, ANID_6015	Broad Institute ^a
<i>Aspergillus niger</i>	3	An16g09130, An16g05190, An12g01840	Accession Numbers: XM_001398173, XM_001397790, XM_001395233
<i>Aspergillus terreus</i>	2	ATEG_00017, ATEG_04426	
<i>Penicillium chrysogenum</i>	3	Pchr 8303965, Pchr 8307369, Pchr 8315995	
<i>Talaromyces stipitatus</i>	4	TSTA 108960, TSTA 122060, TSTA 053280, TSTA 122410	
<i>Microsporum canis</i>	2	EEQ31782, EEQ31742	
<i>Coccidioides posadasii</i>	2	EER29657, EER25737	
<i>Unicinctocarpus reesii</i>	1	UREG 02151	
<i>Schlerotinium sclerotiorum</i>	3	SSIG_00692, SSIG_04059, SSIG_14485	
<i>Saccharomyces cerevisiae</i>	1	NDT80	SGD ^b
<i>Candida albicans</i>	3	CaNDT801, CaNDT802, CaO19.7521	
<i>Candida lusitanae</i>	3	CLUG 04000, CLUG 00404, CLUG 05634	
<i>Candida glabrata</i>	1	CAG62312.1	
<i>Ashbya gossypii</i>	1	AGR347W	
<i>Kluyveromyces lactis</i>	1	KLLA0F24420p	
<i>Yarrowia lipolytica</i>	2	YlipYALI0D24860p, YlipYALI0B14773p	

<i>Debaryomyces hansenii</i>	2	DEHA2E18304p, DEHA2A07282p
<i>Pichia guilliermondii</i>	3	PGUG 00339, PGUG 01255, PGUG 02096
<i>Pichia pastoris</i>	1	PAS 0362
<i>Schizosaccharomyces pombe</i>	0	-
<i>Coprinus cinereus</i>	0	-
<i>Phanerochaete chrysosporium</i>	0	-
<i>Ustilago maydis</i>	1	UM02775

^aBroad Institute Fungal Genome Initiative (<http://www.broadinstitute.org/science/projects/fungal-genome-initiative/fungal-genome-initiative>)

^bSaccharomyces Genome Database (<http://www.yeastgenome.org/>)