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Comparative genomics and transcriptomics to analyze fruiting body development in filamentous ascomycetes

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Data availability:

Raw sequence data generated in this study were submitted to the NCBI SRA (*A. nigricans* genome sequencing and transcriptome sequencing for annotation, accession numbers SRP082924 and SRP082925) and GEO databases (*A. nigricans* transcriptome data, accession number GSE92315). The *A. nigricans* whole genome shotgun project has been deposited at

DDBJ/EMBL/GenBank under the accession SSHT00000000. The version described in this manuscript is version SSHT01000000.

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ABSTRACT

Many filamentous ascomycetes develop three-dimensional fruiting bodies for production and dispersal of sexual spores. Fruiting bodies are among the most complex structures differentiated by ascomycetes; however, the molecular mechanisms underlying this process are insufficiently understood. Previous comparative transcriptomics analyses of fruiting body development in different ascomycetes suggested that there might be a core set of genes that are transcriptionally regulated in a similar manner across species. Conserved patterns of gene expression can be indicative of functional relevance, and therefore such a set of genes might constitute promising candidates for functional analyses. In this study, we have sequenced the genome of the Pezizomycete *Ascodesmis nigricans*, and performed comparative transcriptomics of developing fruiting bodies of this fungus, the Pezizomycete *Pyronema confluens*, and the Sordariomycete *Sordaria macospora*. With only 27 Mb, the *A. nigricans* genome is the smallest Pezizomycete genome sequenced to date. Comparative transcriptomics indicated that gene expression patterns in developing fruiting bodies of the three species are more similar to each other than to non-sexual hyphae of the same species. An analysis of 83 genes that are upregulated only during fruiting body development in all three species revealed ~~22~~ 23 genes encoding proteins with predicted roles in vesicle transport, the endomembrane system, or transport across membranes, and 13 genes encoding proteins with predicted roles in chromatin organization or the regulation of gene expression. Among four genes chosen for functional analysis by deletion in *S. macrospora*, three were shown to be involved in fruiting body formation, including two predicted chromatin modifier genes.

INTRODUCTION

The ability to develop complex multicellular structures evolved several times independently in eukaryotes (Knoll 2011; Niklas 2014). Within the fungi (Eumycota), complex multicellular structures evolved at least twice and possibly up to eleven times. Fungal multicellular structures are often

involved in sexual development, e.g. the fruiting bodies of basidiomycetes and filamentous ascomycetes, which most likely evolved independently (Knoll 2011; Nagy 2017; Nagy *et al.* 2018; Varga *et al.* 2019). Fruiting bodies function in the production and dispersal of sexual spores, and contain a number of cell types that are not found in vegetative mycelium (Bistis *et al.* 2003; Han 2009; Kües 2000; Lord and Read 2011; Pöggeler *et al.* 2018). The molecular mechanisms regulating fruiting body development in filamentous ascomycetes have been studied in recent decades mostly using model organisms from the *Sordariomycetes* or *Eurotiomycetes*, e.g. *Neurospora crassa*, *Sordaria macrospora*, *Fusarium graminearum (Gibberella zeae)*, *Trichoderma reesei*, and *Aspergillus nidulans*, which are able to produce fruiting bodies under laboratory conditions and are amenable to classical and molecular genetics (Pöggeler *et al.* 2018). With the advent of next generation sequencing techniques, sequencing of genomes and transcriptomes of non-model species became feasible, allowing comparative genomics and transcriptomics analyses of fruiting body development in different fungal groups (Nowrousian 2014; Nowrousian 2018). In a previous study, we sequenced the genome and several transcriptomes of different developmental stages from *Pyronema confluens*, which belongs to the early-diverging lineage of *Pezizomycetes* (Traeger *et al.* 2013). A comparative analysis of *P. confluens* transcriptome data with transcriptomes from different developmental stages of *S. macrospora* suggested that gene expression during sexual development might be conserved to some degree, and that similar tissues from different species might have more similar expression patterns than different tissues within a species (Teichert *et al.* 2012; Traeger *et al.* 2013). However, at the time of this analysis, fruiting body-specific transcriptomes were available for *S. macrospora*, while for *P. confluens*, only total sexual mycelia were analyzed, which contain fruiting bodies and the surrounding non-sexual hyphae. Recently, fruiting body-specific transcriptomes were generated for *P. confluens* (Murat *et al.* 2018), and in the present study, we sequenced the genome and several transcriptomes for the Pezizomycete *Ascodesmis nigricans*, including fruiting body transcriptomes

that were used for a comparative study with *S. macrospora* and *P. confluens*.

Like *P. confluens*, *A. nigricans* is a member of the *Pezizomycetes*, an early-diverging group of filamentous ascomycetes. The *Pezizomycetes* form fruiting bodies called apothecia, which are often disk-like in appearance with the spore-containing asci (meiosporangia) exposed on top of the fruiting body. However, several *Pezizomycetes* lineages harbor ectomycorrhizal truffle species that form subterranean fruiting bodies with a complex morphology (Hansen and Pfister 2006; Murat *et al.* 2018). Only few *Pezizomycetes* are able to produce fruiting bodies under laboratory conditions. This has hampered the genetic and molecular analysis of sexual development in this group. An exception is *P. confluens*, which is able to produce fruiting bodies in the laboratory within one week (Claussen 1912; Moore and Korf 1963; Traeger *et al.* 2013). *A. nigricans* also produces fruiting bodies ~~within a week~~ under laboratory conditions ~~(Figure 1)~~, and similar to *P. confluens*, this species is homothallic (self-fertile) and therefore does not need a mating partner for sexual development (Obrist 1961; Van Brummelen 1981). ~~However, while *P. confluens* needs light for fruiting body formation , *A. nigricans* can form fruiting bodies independent of light (Figure S1).~~ *A. nigricans* is a coprophilic fungus (Obrist 1961), and in this it is similar to the Sordariomycete *S. macrospora* (Kück *et al.* 2009), whereas *P. confluens* is a soil-living saprobe (Seaver 1909). Under laboratory conditions, the three species *A. nigricans*, *P. confluens*, and *S. macrospora* display very similar life cycles as they are all homothallic and able to form fruiting bodies within a week ~~(Figure S2)~~. Furthermore, none of the three species forms conidia (asexual spores); therefore, changes in gene expression patterns during sexual reproduction are not obscured by changes related to asexual sporulation. Thus, they are suitable model organisms for a comparative study of gene expression during fruiting body development in filamentous ascomycetes.

Another reason for sequencing the *A. nigricans* genome was the analysis of its genome size and repeat content. Previous studies of eight *Pezizomycetes* genomes showed that they are overall rather large for filamentous fungi, the smallest genomes being those of saprotrophic

species (48-60 Mb for *Morchella importuna*, *P. confluens*, and *Ascobolus immersus*), whereas five analyzed truffle species have genomes ranging from 63 to 192 Mb due to repeat expansion (Martin *et al.* 2010a; Murat *et al.* 2018; Traeger *et al.* 2013). However, so far the sequenced genomes cover mostly two of the three major phylogenetic lineages within the *Pezizomycetes*, with the third lineage represented only by the genome of *P. confluens* (Hansen and Pfister 2006; Murat *et al.* 2018). *A. nigricans* is also a member of this third lineage, even though it is only distantly related to *P. confluens* (Hansen and Pfister 2006). Therefore, analysis of the *A. nigricans* genome will improve the phylogenetic coverage for *Pezizomycetes* genomes, and also improve the coverage of *Pezizomycetes* with a non-mycorrhizal life style.

Another point of interest in the *A. nigricans* genome is the organization of the mating type (*MAT*) locus. *MAT* loci in filamentous ascomycetes contain various genes that are central regulators of sexual development. In heterothallic (self-sterile) ascomycetes, each strain possesses one of two non-allelic versions (idiomorphs) of a single *MAT* locus, named *MAT1-1* and *MAT1-2*. These loci usually contain (among others) the *MAT1-1-1* and *MAT1-2-1* genes, which encode transcription factors with a conserved alpha domain and high-mobility group (HMG) domain, respectively. In contrast, homothallic ascomycetes carry both *MAT* loci within a single genome. The two loci can be fused together, located within close proximity, or located on separate chromosomes (Bennett and Turgeon 2016; Billiard *et al.* 2011; Debuchy *et al.* 2010; Pöggeler *et al.* 2018). In *P. confluens*, homologs of the core *MAT* genes *MAT1-1-1* and *MAT1-2-1* were found, as expected for a homothallic ascomycete. However, other genes that are often part of the *MAT* loci in other ascomycetes were neither found near *MAT1-1-1* or *MAT1-2-1* in this species, nor in the *MAT* loci of the heterothallic Pezizomycete *Tuber melanosporum* (Rubini *et al.* 2011; Traeger *et al.* 2013). In addition, of the two genes *apn2* and *sla2* that often flank the *MAT* locus in more derived lineages of filamentous ascomycetes (Pöggeler *et al.* 2018), only *apn2* was identified in proximity to the *P. confluens MAT* locus, whereas none of these genes flanks the *MAT* loci of *T. melanosporum* (Rubini *et al.* 2011; Traeger *et al.* 2013). It is not clear if

the *MAT* loci of *T. melanosporum* and *P. confluens* represent basal or derived *MAT* configurations, therefore the analysis of additional *Pezizomycetes MAT* loci is of great interest for the analysis of the evolution of sexual development in fungi.

In this study, we sequenced the genome of *A. nigricans*, and generated transcriptomes for vegetative and sexual mycelia, as well as for developing fruiting bodies that were isolated from the surrounding mycelium by laser microdissection. The transcriptomics data were used for a comparative analysis with RNA-seq data from mycelia and developing fruiting bodies of *P. confluens* and *S. macrospora* to identify conserved core groups of genes that are differentially regulated during sexual development. Several differentially expressed genes were functionally characterized to address their roles during fruiting body morphogenesis by generating corresponding deletion mutants in *S. macrospora*.

Materials and Methods

Strains, culture conditions and genetic crosses

A. nigricans and *S. macrospora* strains used in this study are given in Table 1. *A. nigricans* was grown on cornmeal medium (BMM) (Esser 1982), RFA medium (rabbit food agar, 25 g of rabbit food pellets were boiled in 1 l A. dest., set to cool for 30 min, filtered through cotton, and autoclaved), or V8 medium (50 ml vegetable juice per liter, pH 5.2) at 25 °C. *S. macrospora* was grown on cornmeal medium (BMM, "Biomalz-Mais-Medium") or minimal medium (SWG, "Sordaria Westergaard's") at 25 °C as described (Esser 1982; Nowrousian *et al.* 2005). Both media support vigorous fruiting body formation. Transformation protocols and protocols for genetic crosses for *S. macrospora* were as described previously (Dirschnabel *et al.* 2014; Esser 1982; Nowrousian *et al.* 1999). To observe hyphal fusions, strains were grown on minimal medium (MM) with cellophane, which allows sparse hyphal growth for better visualization of individual hyphae (Rech *et al.* 2007). For microscopy, strains were inoculated for 2 to 10 d on glass slides with thin layer of BMM with 0.8 %

agar (Engh *et al.* 2007). Quantification of linear growth was performed on BMM or SWG using petri dishes with inoculation at the edge. The growth front was marked over 3 - 5 d every 24 h, experiments were performed as triplicate.

DNA preparation, sequencing, and assembly of the *A. nigricans* genome

Genomic DNA from *A. nigricans* strain CBS 389.68 was prepared for sequencing as described for *P. confluens* (Traeger *et al.* 2013). One 270 bp insert library (2 x 150 bp paired-end sequencing) and one 4 kb mate-pair library (2 x 100 bp paired-end sequencing) were sequenced on an Illumina HiSeq 2500. Illumina fastq files were filtered for artifacts/process contamination. Post-processed genomic reads were assembled with AllPathsLG v.R49403 (Gnerre *et al.* 2011).

Genome annotation and analysis of repeat content

RNA-Seq reads for annotation (for RNA preparation and sequencing, see below) were assembled into consensus sequences using Rnnotator v. 3.3.2 (Martin *et al.* 2010b). The assembled consensus RNA sequence data was mapped to genome assembly using alignments of 90% identity and 85% coverage or higher to assess genome completeness at 97.91%. The genome was annotated using the JGI Annotation pipeline and made available via JGI fungal genome portal MycoCosm (jgi.doe.gov/fungi) (Grigoriev *et al.* 2014).

Analysis of transposable elements and other repeats in the *A. nigricans* genome assembly was performed as described (Traeger *et al.* 2013) with RepeatMasker (A.F.A. Smit, R. Hubley, P. Green; www.repeatmasker.org) based on the RepbaseUpdate library (Jurka *et al.* 2005) and a library of *de novo*-identified *A. nigricans* repeat consensus sequences that was generated by RepeatModeler (A.F.A. Smit, R. Hubley; www.repeatmasker.org/RepeatModeler.html). An overview of assembly and annotation statistics is given in Table 2.

Laser microdissection, RNA preparation and RNA-seq

For RNA preparation, *A. nigricans* strain CBS 389.68 was grown in liquid RFA or V8 medium as surface cultures (in petri dishes without shaking) or from submerged cultures (in 100 ml flasks shaken at 130 rpm) at 25 °C. RNA preparation was performed as described (Nowrousian and Kück 2006). For annotation purposes, total RNA from mycelia grown for 3 d and 5 d as surface cultures in RFA and V8 was combined and sequenced on an Illumina HiSeq 2000 (2 x 150 bp paired-end sequencing). For quantification of gene expression, RNA was extracted from total vegetative and sexual mycelia as well as from young fruiting bodies isolated by laser microdissection. Total vegetative and sexual mycelia were obtained by growing *A. nigricans* as described above in submerged cultures and surface cultures, respectively, in 20 ml RFA medium for 4 d at 25 °C. For laser microdissection of young fruiting bodies, *A. nigricans* was grown on microdissection slides coated with 150-200 µl RFA (with 0.8 % agar) for 3 d at 25 °C. Fixation of slides, laser microdissection, RNA preparation and linear RNA amplification were as described (Teichert *et al.* 2012). Approximately 230 microdissected young fruiting bodies were combined for each RNA extraction. For each condition (vegetative mycelium, sexual mycelium, and young fruiting bodies), two independent biological replicates were performed. The corresponding RNAs were sequenced on an Illumina HiSeq 2500 (51 bp single-end sequencing) by GATC (Konstanz, Germany).

Synteny analysis

An orthology-based analysis of synteny was performed as described before (Traeger *et al.* 2013) by determining orthologs for all *A. nigricans* proteins in the predicted proteomes of *P. confluens* and *T. melanosporum* by reciprocal BLAST analysis (Altschul *et al.* 1997), and using custom-made Perl scripts based on BioPerl modules (Stajich *et al.* 2002) to determine the positions of corresponding orthologous genes on sequenced contigs.

Phylogenomics analysis

The predicted proteomes of *A. nigricans* and the following 19 other fungal species were used for the reconstruction of the phylome using the phylomeDB pipeline (Huerta-Cepas *et al.* 2011): *Agaricus bisporus* (Morin *et al.* 2012), *Arthrobotrys oligospora* (Yang *et al.* 2011), *Blumeria graminis* (Spanu *et al.* 2010), *Coccidioides immitis* (Sharpton *et al.* 2009), *Emericella nidulans* (Galagan *et al.* 2005), *Fusarium graminearum* ~~*Gibberella zeae*~~ (Cuomo *et al.* 2007), *Laccaria bicolor* (Martin *et al.* 2008), *Mycosphaerella graminicola* (Goodwin *et al.* 2011), *Neosartorya fischeri* (Fedorova *et al.* 2008), *Neurospora crassa* (Galagan *et al.* 2003), *Phaeosphaeria nodorum* (Hane *et al.* 2007), *Pyronema confluens* (Traeger *et al.* 2013), *Saccharomyces cerevisiae* (Goffeau *et al.* 1996), *Schizosaccharomyces pombe* (Wood *et al.* 2002), *Sclerotinia sclerotiorum* (Amselem *et al.* 2011), *Sordaria macrospora* (Nowrousian *et al.* 2010), *Taphrina deformans* (Cissé *et al.* 2013), *Tuber melanosporum* (Murat *et al.* 2018), *Yarrowia lipolytica* (Dujon *et al.* 2004). All alignments and trees are available in phylomeDB (www.phylomeDB.org) (Huerta-Cepas *et al.* 2014). For each gene encoded in *A. nigricans*, a Smith-Waterman search was performed against a proteome database containing the proteome information of the selected species. We used an e-value threshold <1e-05 and a continuous overlap of 50% over the query sequence for the detection of homologs. We limited the number of hits included in a tree to the closest 150 homologs per gene. We used three different aligners for the multiple sequence alignments of the homologous sequences (forward and reversed versions of the sequences): MUSCLE (Edgar 2004), MAFFT (Katoh *et al.* 2005) and KALIGN (Lassmann and Sonnhammer 2005). The final six alignments were combined using M-COFFEE (Wallace *et al.* 2006) and then trimAl to trim the alignment (consistency cut-off of 0.16667 and -gt >0.1) (Capella-Gutierrez *et al.* 2009). We used PhyML v3 for ML trees (Guindon *et al.* 2010). Branch support was analyzed using an aLTR (approximate likelihood ratio test) parametric test based on a chi-square distribution. We used a discrete gamma-distribution with three rates categories in all the cases (estimating the gamma parameter from the data). We scanned this phylome using a previously-described algorithm for duplication detection (Huerta-Cepas *et al.* 2010). Using FatiGO (Al-

Shahrour *et al.* 2007) we analyzed the gene enrichment of the genes duplicated at each branch of the species tree. To reconstruct the species tree, 143 genes that had one-to-one orthologs in each of the selected species were trimmed and then the alignments were concatenated. The final alignment had 108,319 nucleotide positions. To reconstruct the ML species tree for each alignment we used RaxML version 7.2.6, model Protgammalg and 100 bootstrap support (Stamatakis 2006). Finally, a consensus tree using Phylip and a super-tree using Duptree (Wehe *et al.* 2008) with a parsimony strategy from all single gene tree was created.

Quantitative analysis of gene expression in *A. nigricans* based on RNA-seq data, and comparative transcriptomics analysis of *A. nigricans*, *P. confluens*, and *S. macrospora*

Analysis of RNA-seq data from *A. nigricans* was done as described previously with minor modifications (Teichert *et al.* 2012; Traeger *et al.* 2013). Briefly, reads were trimmed with custom-made Perl programs to remove reads with nondetermined nucleotides, remove polyA or polyT stretches from end and start of reads, respectively, and trim reads from 3' and 5' ends until a base quality of ≥10 was reached. Trimmed reads of at least 40 bases were used for mapping to the *A. nigricans* genome using Tophat v2.0.11 (Trapnell *et al.* 2010). Reads mapping to annotated features were counted as described (Teichert *et al.* 2012), and quantitative analysis of gene expression was performed with DESeq2 (Love *et al.* 2014).

For comparative transcriptomics analyses of the three species *A. nigricans*, *P. confluens*, and *S. macrospora*, orthologs between *A. nigricans* and the other two species were determined by reciprocal BLAST analysis. ~~A total of 4791 genes were found with orthologs in all three species.~~ Read counts for each ortholog in the three species were obtained from RNA-seq data from this study as well as previous analyses of *P. confluens* (Murat *et al.* 2018; Traeger *et al.* 2013) and *S. macrospora* (Teichert *et al.* 2012). RNA-seq samples included in the analysis are given in Table 3. A combined analysis of read counts for all orthologs in all conditions was performed with DESeq2 (Love *et al.* 2014).

Analysis of the mating type region in several *A. nigricans* strains

DNA fragments from the mating type regions of the *A. nigricans* wild type strains given in Table 1 were amplified with primer combinations Anig_mat1/Anig_mat2, Anig_mat3/Anig_mat4, and Anig_mat5/Anig_mat6 (Table S1) and sequenced with Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The resulting overlapping fragments of 1 kb each cover the *MAT1-1-1* gene and flanking regions of 0.7 kb up- and 0.8 kb downstream.

Cloning procedures

Plasmids for generating gene deletion strains and complementation experiments in *S. macrospora* were cloned by homologous recombination in yeast as described (Colot *et al.* 2006). Oligonucleotides used for generating PCR products for cloning procedures are given in Table S1, plasmids are given in Table S2. Deletion cassettes for *SMAC_01829* (*spt3*), *SMAC_04946* (*scm1*), *SMAC_06113* (*aod5*), and *SMAC_06770* were generated by amplifying ~1 kb genomic regions upstream and downstream of the corresponding genes or including coding regions if the neighboring genes are closer than 1 kb (for *spt3* and *SMAC_06770*). PCR fragments were then cloned to flank the *hph* gene conferring hygromycin resistance (Nowrousian and Cebula 2005). Plasmid pN_1829.3-GFP contains the *spt3* and *egfp* open reading frames flanked by the *spt3* 5' untranslated regions (UTR) and 440 bp upstream of the 5' UTR, and the 3' UTR and 144 bp downstream of the 3' UTR in pRSnat, which confers nourseothricin resistance in *S. macrospora* (Klix *et al.* 2010). Plasmids pOE_1829.3-GFP and pSMAC_06113_EGFP carry the open reading frames of *spt3* and *aod5*, respectively, in fusion with a C-terminal *egfp* under control of the *Aspergillus nidulans gpd* promoter and *trpC* terminator.

Generation of gene deletion strains in *S. macrospora*

Deletion strains for *SMAC_01829* (*spt3*), *SMAC_04946* (*scm1*), *SMAC_06113* (*aod5*), and *SMAC_06770* were generated by transforming the deletion cassette (upstream and downstream regions flanking the *hph*

gene, obtained by restriction digest of the corresponding gene deletion plasmid and gel elution) into a Δku70 strain as described previously (Pöggeler and Kück 2006). Hygromycin resistant primary transformants were verified for insertion of the deletion cassette by PCR and Southern blot analysis, and knockout strains were crossed against the spore color mutant fus (Nowrousian *et al.* 2012) to obtain homokaryotic ascospore isolates carrying the deletion allele in a genetic background without the Δku70 allele.

Stereomicroscopy and microscopy

For top and side view of cultures, stereomicroscope Stemi 2000-C (Zeiss, Jena, Germany) was used. Images were captured with an AxioCam ERc5s (Zeiss, Jena, Germany) and Zen2Core (v2.5; Zeiss, Jena, Germany). Fluorescence and light microscopic investigations were carried out with an AxioImager microscope (Zeiss, Jena, Germany). Fluorescence was studied using Chroma (Bellows Falls, VT, USA) filter set 41017 (HQ470/40, HQ525/50, Q495lp) for detection of EGFP, and set 49008 (EG560/40x, ET630/75m, T585lp) for the detection of mRFP. Images were captured with a Photometrix Cool SnapHQ camera (Roper Scientific) and MetaMorph (Universal Imaging). Recorded images were edited with MetaMorph and Adobe Photoshop CS6. Light microscopy of ascus rossetts and ascospores were carried out with AxioPhot (Zeiss, Jena, Germany) and an AxioCam. ZEN (v2.3, blue edition; Zeiss, Jena, Germany) was used as software for taking images.

Data availability

Raw sequence data generated in this study were submitted to the NCBI SRA (*A. nigricans* genome sequencing and transcriptome sequencing for annotation, accession numbers SRP082924 and SRP082925) and GEO databases (*A. nigricans* transcriptome data, accession number GSE92315). The *A. nigricans* whole genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession SSHT00000000. The version described in this manuscript is version SSHT01000000. Supplemental

Figures S1-~~S10~~S11, and supplemental Tables S1-S6 were uploaded to figshare.

RESULTS

Sequencing and assembly of the *A. nigricans* genome

A. nigricans is a homothallic Pezizomycete that produces fruiting bodies within a week under laboratory conditions (Figure 1). However, while *P. confluens* needs light for fruiting body formation (Claussen 1912; Traeger *et al.* 2013), *A. nigricans* can form fruiting bodies independent of light (Figure S1), and under laboratory conditions has a life cycle that is very similar to those of *P. confluens* and *S. macrospora* (Figure S2), making it a suitable species to be included in comparative transcriptomics analyses of fruiting body formation.

The genome of *A. nigricans* strain CBS 389.68 was sequenced as part of the 1000 Fungal Genomes project (http://1000.fungalgenomes.org) (Grigoriev *et al.* 2011; Grigoriev *et al.* 2014). The assembly consists of 176 scaffolds with a total size of 27 Mb and 9,622 predicted protein-coding genes (Table 2). BLASTP searches with a eukaryotic core gene set were used to determine completeness of the gene space as described previously (Parra *et al.* 2009). All of the 248 single-copy core genes were present among the predicted *A. nigricans* genes, suggesting that the assembly covers the complete gene space. With 27 Mb, the *A. nigricans* genome is the smallest Pezizomycete genome sequenced to date. However, it contains about the same number of genes with a similar amount of coding sequence as the more than seven times larger genome of *Tuber magnatum*, the largest Pezizomycete genome currently known (Murat *et al.* 2018), as well as the genome of *P. confluens*, the closest sequenced relative of *A. nigricans* (Table 2). Part of the smaller genome size of *A. nigricans* can be attributed to much fewer repeat sequences compared to other *Pezizomycetes* (Table 2). Furthermore, intron sequences also cover less sequence space in the *A. nigricans* genome than in other *Pezizomycetes* (Table 2). Overall, the *A. nigricans* genome is more

compact with respect to non-coding features than other *Pezizomycetes* genomes but retains the same coding capacity.

To assess the evolution of *A. nigricans* genes and their homologs across 19 other sequenced fungi, we reconstructed their evolutionary histories using the phylomeDB pipeline (Huerta-Cepas *et al.* 2011). We reconstructed the evolutionary relationship of the selected species based on concatenating the alignments of 143 genes that were present in a single copy in all the species analyzed and building a super-tree combining all individual gene trees from the phylome (see Material and Methods). The resulting phylogeny confirms that *P. confluens* and *A. nigricans* are sister species within the *Pezizomycetes*, with the *Tuber* species, represented by *T. melanosporum*, on a separate branch within the *Pezizomycetes* lineage (Figure 2).

An analysis of synteny between the genomes of *A. nigricans* and other *Pezizomycetes* showed little conservation in gene order, both at the level of scaffolds as well as for small genomic regions of two or three genes (Figure S3). Interestingly, the number of syntenic gene pairs or triplets that *A. nigricans* shares with *P. confluens* is lower than the same numbers for *P. confluens* and *T. melanosporum*, even though *A. nigricans* and *P. confluens* are more closely related to each other than to *T. melanosporum* (Figure 2). One possible explanation might be that the reduction of genome size observed in *A. nigricans* was achieved through extensive genome restructuring involving multiple translocations.

Analysis of the mating type locus of *A. nigricans*

The genome of the homothallic Pezizomycete *P. confluens* contains the two *MAT* genes *MAT1-1-1* and *MAT1-2-1*, which is typical in homothallic ascomycetes (Traeger *et al.* 2013). In contrast, the *A. nigricans* genome contains only one *MAT* gene, namely *MAT1-1-1* (Figure 3). TBLASTN searches in the *A. nigricans* genome also failed to discover a *MAT1-2-1* homolog. Interestingly, the *A. nigricans MAT1-1-1* gene is located in the vicinity of two genes, *APN2* and locus tag *50832*, that are linked to *MAT1-2-1* in *P. confluens* (Figure 3). Furthermore, several repeat regions are flanking the *MAT* gene as well as *APN2* in *A. nigricans* (Figure 3). One

hypothesis to explain these findings might be that a common ancestor of *A. nigricans* and *P. confluens* carried a *MAT* locus with both *MAT1-1-1* and *MAT1-2-1*, and that a recombination/duplication event separated the *MAT* genes in *P. confluens*, whereas repeat-induced recombination led to the deletion of *MAT1-2-1* in *A. nigricans*.

To verify that the region occupied by *MAT1-1-1* in strain CBS 389.68 is the same in other *A. nigricans* strains, the region between the genes flanking *MAT1-1-1* was amplified by PCR from four *A. nigricans* wild type strains (including CBS389.68, Table 1, Figure 3) and sequenced by Sanger sequencing. All four strains carry the *MAT1-1-1* gene in this genomic location, therefore this *MAT* configuration is present in all analyzed *A. nigricans* strains so far.

Genes for secondary metabolism in *A. nigricans*

Most genomes of higher filamentous ascomycetes carry multiple genes for the biosynthesis of polyketides and non-ribosomal peptides, two major classes of secondary metabolites in fungi (Brakhage 2013; Bushley and Turgeon 2010; Keller 2019; Kroken *et al.* 2003; Teichert and Nowrousian 2011). However, previous analyses of the *P. confluens* genome revealed only seven non-ribosomal peptide synthase (NRPS) genes and one polyketide synthase (PKS) gene in this species, much fewer than in the genomes of higher filamentous ascomycetes (Traeger *et al.* 2013). An analysis of the predicted *A. nigricans* proteins revealed five putative NRPSs, but no PKS (Table S3). Thus, *A. nigricans* lacks even a homolog for the single type I PKS gene present in the *P. confluens* genome, and the single type III PKS gene present in the genomes of higher filamentous ascomycetes is ~~lacking~~ missing in both *P. confluens* and *A. nigricans*.

One of the five NRPS genes in the *A. nigricans* genome encodes a siderophore NRPS also found in other fungal genomes (Table S3). There are three putative alpha-aminoadipate reductase (AAR) NRPSs, which are typical fungal NRPSs involved in amino acid biosynthesis. Most fungi have only one AAR gene, an exception is *P. confluens* with five genes (Bushley and Turgeon 2010; Traeger *et al.* 2013). Thus, the three AAR gene homologs in *A. nigricans* suggest that this gene family expansion might be

present throughout the *P. confluens*/*A. nigricans* lineage of *Pezizomycetes*. The fifth NRPS gene in *A. nigricans*, *proteinId396591*, encodes a putative NRPS of unknown function (Table S3). There is one NRPS of unknown function encoded in *P. confluens*, too, but its domain architecture is different from *proteinId396591*, therefore these genes might not be orthologs.

Comparative transcriptomics of fruiting body development in *A. nigricans*, *P. confluens*, and *S. macrospora*

To analyze global changes in gene expression during sexual development in *A. nigricans*, we sequenced transcriptomes from three developmental stages by RNA-seq (Table 3, Table S4). To obtain total vegetative mycelia, *A. nigricans* was grown in submerged cultures, which prevents the formation of sexual structures. For total sexual mycelia, *A. nigricans* was grown as surface cultures, and the developing fruiting bodies as well as the surrounding non-sexual mycelium was harvested for RNA extraction. To obtain RNA solely from developing fruiting bodies, we used laser microdissection to isolate young fruiting bodies from the surrounding mycelium as described previously (Teichert *et al.* 2012). RNA-seq data from similar developmental stages are available for *P. confluens* and *S. macrospora* (Murat *et al.* 2018; Teichert *et al.* 2012; Traeger *et al.* 2013) (Table 3), and we used these for comparative transcriptomics analyses with *A. nigricans*.

To address the question if orthologous genes in the *A. nigricans* and *P. confluens* (*Pezizomycetes*) as well as in *S. macrospora* (*Sordariomycetes*) show similar expression patterns during fruiting body formation, we analyzed gene expression for the 4,791 genes for which putative orthologs were found in all three species (Table S5). In a previous study of *S. macrospora*, expression patterns in developing fruiting bodies differed much more from total vegetative and total sexual mycelia than the total mycelial samples differed from each other (Teichert *et al.* 2012). This trend is confirmed when analyzing data from orthologs in the three species (Figure 4). In all cases, the number of differentially expressed genes is much higher when comparing fruiting body samples versus

sexually competent mycelium than in a comparison of vegetative versus sexually competent mycelium. These data suggest that the expression patterns in sexual mycelia are dominated by the non-sexual hyphae that make up the bulk of the mycelium rather than by the developing fruiting bodies. The results also indicate that fruiting bodies of filamentous ascomycetes significantly restructure their transcriptome in the transition from vegetative hyphal growth to the development of fruiting bodies.

Clustering of correlation coefficients based on gene expression ratios for comparisons of fruiting bodies or vegetative mycelium versus sexual mycelium showed that comparisons involving fruiting bodies for all three species group together and are separated from the comparisons of vegetative versus sexual mycelia (Figure S4). This confirms the trend described above, namely that fruiting bodies have distinct transcriptomes compared to non-sexual hyphae (Figure 4). Furthermore, it suggests that there might be conserved gene expression patterns during sexual development in filamentous ascomycetes.

Next, we identified genes that are differentially regulated in developing fruiting bodies in all three species. There are 83 genes that are upregulated, and 114 genes that are downregulated in developing fruiting bodies of all three species, but not differentially regulated in other comparisons (Figure 5, Table S6). Among the downregulated genes are 16 genes with predicted functions in protein synthesis or turnover, and another 16 genes with predicted roles in protein phosphorylation/dephosphorylation or signal transduction (Table S6). It is possible that downregulation of such genes is an essential step during fruiting body formation, and in-depths analyses of these genes might be of interest for future studies. However, in this study ~~W~~we focused on the genes that are upregulated specifically in developing fruiting bodies in all three species, as these might have conserved roles in sexual development in filamentous ascomycetes. An analysis of putative functions based on conserved domains among the upregulated genes showed that there are ~~22~~ 23 genes encoding proteins with predicted roles in vesicle transport, the endomembrane system, or transport across membranes. This group of genes might be of interest for future functional analyses.

Among the upregulated genes during fruiting body formation in *A. nigricans*, *P. confluens*, and *S. macrospora* are also 13 genes encoding proteins with predicted roles in chromatin organization or the regulation of gene expression (Figure 5, Table S6). As the transition from vegetative growth to fruiting body development requires a drastic restructuring of the transcriptome, transcription factors and chromatin modifiers are expected to play pivotal roles in this transition. A number of specific transcription factors have already been shown to be involved in sexual development in filamentous ascomycetes, whereas the role of chromatin modifiers in this process is less well understood (Nowrousian 2018; Pöggeler *et al.* 2018). To learn more about the roles of genes with evolutionary conserved expression patterns, we chose four of these genes for functional analysis through gene deletion.

Functional analysis of genes with evolutionary conserved expression patterns during development

Functional analysis of four genes with conserved expression patterns was carried out in *S. macrospora*, because for this filamentous ascomycete molecular techniques like transformation and gene deletion systems are available (Engh *et al.* 2010; Teichert *et al.* 2014). The candidates were chosen from the genes with conserved upregulation during sexual development based on their predicted functions in other species or presence of conserved domains. Among the four genes that were chosen for deletion in *S. macrospora*, one (*SMAC_06770*) has a predicted function within the endomembrane system, whereas the other three (*SMAC_01829*, *SMAC_04946*, and *SMAC_06113*) are predicted to be involved in regulating transcription or chromatin organization.

SMAC_06770 encodes a homolog to the *S. cerevisiae ALG11* gene, which encodes glycolipid 2-alpha-mannosyltransferase, an enzyme involved in protein glycosylation in the endoplasmic reticulum (ER) through formation of glycosylation intermediates on the cytosolic side of the ER (Cipollo *et al.* 2001). Deletion of *ALG11* in *S. cerevisiae* leads to poor growth at 25 °C, and a temperature-sensitive lethality at 37 °C (Cipollo *et al.* 2001). Deletion of *SMAC_06770* in *S. macrospora* resulted in transformants that

grew very poorly and were unable to form fruiting bodies (Figure S5). Thus, the gene appears to be involved in basic cellular processes besides sexual development in *S. macrospora*, similar to *S. cerevisiae*.

SMAC_06113 is orthologous to the *N. crassa aod-5* gene, which regulates transcription of the gene encoding alternative oxidase (AOX) (Chae *et al.* 2007; Chae and Nargang 2009). AOD-5 consists of two domains, a GAL-4 like domain at the N-terminus and a central PAS domain that might be involved in protein-protein interactions. Deletion of *SMAC_06113* in *S. macrospora* did not result in any defects in sexual developmental under laboratory conditions. The strain was fully fertile, similar to the wild type (Figures S6 and S7). Because of the homology of *SMAC_06113* to *N. crassa aod-5*, we tested growth of the deletion strain and complemented transformants on antimycin A. This drug inhibits the electron transport through complex III in mitochondria, and consequently alternative oxidase expression is induced for respiration (Descheneau *et al.* 2005). Similar to *N. crassa* aod mutants, the *S. macrospora SMAC_06113* deletion strain was not able to grow in presents of antimycin A, in contrast to the wild type and a complemented strain (Figure S7). Therefore, *SMAC_06113* was named *aod5* (*alternative oxidase 5*). Fluorescence microscopy with strains expressing an *aod5-egfp* fusion showed that *aod5* localizes to nucleus as expected for a transcription factor (Figure S7).

Earlier studies with chromatin modifiers *asf1*, *cac2*, *crc1*, and *rtt106* revealed only *asf1* as essential for sexual reproduction in *S. macrospora*, whereas *cac2* and *rtt106* might have redundant function under nutrient deprivation (Gesing *et al.* 2012; Schumacher *et al.* 2018). Here we chose another putative chromatin modifier encoded by *SMAC_04946* for functional analysis. *SMAC_04946* encodes a protein with a conserved SAS4 domain. In *S. cerevisiae,* Sas4 is described as part of SAS complex (something about silencing) together with Sas2 and Sas5 (Sutton *et al.* 2003), and was found to interact with Asf1p (Osada *et al.* 2001). However, DELTA-BLASTp searches did not reveal clear homologs for Sas2 and Sas5 in *S. macrospora*, and the SAS4 domain is the only part of the SMAC_04946 protein that is conserved in *S. macrospora* compared to

yeast. Hence, we named the gene *scm1* (*sas4-domain chromatin modifier*) and analyzed if deletion of *scm1* results in any phenotype (Figure S8). Similar to the deletion of several other chromatin modifiers (*cac2*, *crc1* and *rtt106*) (Gesing *et al.* 2012; Schumacher *et al.* 2018), the Δscm1 mutant was fertile after 7 d on BMM and SWG (Figure 6). To address the question if there might be redundancy of SCM1 and other chromatin modifiers, we generated double deletion strains by genetic crossing (Figure S9). However, none of the –double mutants of *scm1* with *cac2*, *crc1*, or *rtt106* had a developmental phenotype (Figure 6). This is similar to double mutants involving *cac2*, *crc1*, or *rtt106*, which were generated previously, and all of which are fertile on BMM medium (Schumacher *et al.* 2018) (Figure S10). Therefore, we performed crosses to obtain triple and quadruple deletion strains (Figure S9). All possible triple mutant combinations of *scm1*, *cac2*, *crc1*, and *rtt106* showed at least reduced fertility up to sterility (Figure 6). While the Δcrc1/Δrtt106/Δscm1 mutant formed perithecia and even discharged some spores, all triple mutants with Δcac2 background are sterile. The triple mutants Δscm1/Δcac2/Δrtt106 and Δcac2/Δcrc1/Δrtt106 formed few immature fruiting bodies without a perithecial neck, sometimes with a few immature spores inside (Figure 6). However, the spores were not discharged even after 21 d on BMM. Δscm1Δcac2Δcrc1 formed only protoperithecia. The quadruple mutant showed a phenotype comparable to so-called pro- mutants (Teichert *et al.* 2014), forming only small protoperithecia (Figure 6).

The fourth gene we chose for further analysis was *SMAC_01829* encoding a homolog to the SPT3 subunit of the SAGA complex, a conserved eukaryotic transcriptional co-activator complex (Helmlinger and Tora 2017; Spedale *et al.* 2012). The SAGA complex is well characterized in yeast, and for filamentous fungi a deletion strain of *spt3* was analyzed in *Fusarium graminearum* (Gao *et al.* 2014; Timmers and Tora 2005). In *S. macrospora*, deletion of *spt3* results in a most conspicuous phenotype (Figure 7, Figure ~~S10~~S11). Δspt3 strains grow significantly slower than the wild type on both full medium (BMM) and minimal medium (SWG) (Figure 7B). The Δspt3 mutant is still able to undergo hyphal fusion (Figure 7C);

however, hyphal morphology is different from the wild type in older hyphae, with intrahyphal growth occurring in swollen hyphae (Figure 7C). Besides the vegetative phenotype, deletion of *spt3* leads to sterility with only few non-pigmented, often submerged protoperithecia (Figure 7A and 7D). Hyphae that make up the protoperithecia are less densely packed than in the wild type. The formation of fruiting bodies and ascospores was restored in complemented transformants on BMM with *spt3* under native and constitutive promoter within 10 days (Figure 7A and 7D). However, only complemented strains with *spt3* under a constitutive promotor were able to discharge spores. On SWG medium, complementation did not result in fertile strains even after 14 days, but in formation of more pigmented protoperithecia and few perithecia (Figure 7A). The growth rate was also only partially restored in complemented transformants (Figure 7C). The transformants carry ectopically integrated complementation plasmids, and it is possible that the native chromatin environment is required for a fully functional *spt3*.

Discussion

The *Ascodesmis nigricans* genome is small and gene-dense

Fruiting body morphogenesis in ascomycetes is a complex process that requires the concerted action of a large number of genes. Molecular studies with several model organisms have led to the identification of many such developmental genes, but the degree to which fruiting body development is conserved at the morphological and molecular level is not yet clear (Pöggeler *et al.* 2018). One way to address this question is by comparative transcriptomics to test if gene expression patterns are conserved across species. In this study, we sequenced the genome of the Pezizomycete *A. nigricans*, and generated several transcriptomes that were used in comparative transcriptomics analyses with two other ascomycetes. ~~*A. nigricans* was chosen for this study, because it is a member of a *Pezizomycetes* lineage with few sequenced genomes, and has a short, homothallic life cycle that can be completed under laboratory~~

conditions. In this respect, *A. nigricans* is similar to the other two species used for comparative transcriptomics, *P. confluens* and *S. macrospora*. Thus, we were able to compare transcriptome data from three species with very similar life cycles, but which are only distantly related.

Compared to previously sequenced Pezizomycete genomes, the *A. nigricans* genome is rather small. With 27 Mb, it is only about half the size of the *M. importuna* genome, the smallest of the previously sequenced Pezizomycete genomes (Murat *et al.* 2018). However, it has retained a coding capacity similar to other, much larger Pezizomycete genomes. The size differences are caused mainly by a higher amount of repeats in other *Pezizomycetes*, but non-coding regions like introns also make up a smaller part of the genome in *A. nigricans*. The differences in genome size could be explained by the expansion of repeats and non-coding regions including introns in the other *Pezizomycetes*, or by genome reduction processes specific to *A. nigricans*, or both. The finding that microsynteny is higher between *P. confluens* and *T. melanosporum* than between *P. confluens* and the more closely related *A. nigricans* might support a hypothesis of genome size reduction involving major restructuring in *A. nigricans*. In addition, the low amount of repeats in *A. nigricans* might indicate that *A. nigricans* has effective mechanisms to prevent repeat expansion. In fungi, several genome defense mechanisms are known. These include RNA interference, RIP (repeat-induced point mutations), MIP (methylation induced premeiotically), and MSUD (meiotic silencing by unpaired DNA) . The mechanistically related processes of RIP and MIP were discovered in the Sordariomycete *N. crassa* and the Pezizomycete *A. immersus*, respectively , and both species possess very low repeat contents in their genomes , similar to *A. nigricans*. Two homologous genes, *masc1* and *rid* involved in MIP and RIP in *A. immersus* and *N. crassa*, respectively, encode predicted cytosine methyltransferases . A *masc1/rid* homolog can also be found in *A. nigricans* (proteinId394667). However, it has been noted previously that *masc1/rid* homologs are widespread in filamentous ascomycetes and might play a role during sexual development, whereas active MIP or RIP silencing processes are not necessarily associated with their presence . Thus, whether MIP/RIP-like

~~processes or other genome defense mechanisms are active in *A. nigricans* remains to be elucidated.~~

One group of genes usually present in the genomes of filamentous ascomycetes, but absent in *A. nigricans*, are polyketide synthase (PKS) genes. This is unusual even for *Pezizomycetes*, which have fewer secondary metabolism genes than other Pezizomycotina, with *T. melanosporum* harboring two, and *P. confluens* containing only one PKS gene (Martin *et al.* 2010a; Teichert and Nowrousian 2011; Traeger *et al.* 2013). Given their phylogenetic relationships, the most parsimonious explanation would be the presence of (at least) one PKS gene in the common ancestor of Pezizomycetes, which was lost in the lineage leading to *A. nigricans*. The NRPS gene content of *A. nigricans* is more typical of filamentous ascomycetes, even though the number of NRPS genes is small, similar to other *Pezizomycetes*. ~~Of the five NRPS genes, one is predicted to be involved in siderophore biosynthesis, while three are putative alpha-aminoadipate reductases involved in amino acid biosynthesis. *P. confluens* has five AAR genes, and since most fungi harbor only one AAR gene, it is possible that this gene family expanded in the *Ascodesmis/Pyronema* lineage. The analysis of additional *Pezizomycetes* genomes is needed to find out if this gene family showed less expansion in *A. nigricans* or underwent expansion and subsequent reduction during a general genome size reduction in this species.~~

The mating type locus of the homothallic *A. nigricans* contains a single *MAT1-1-1* gene

Another unusual feature of the *A. nigricans* genome is its mating type region. *A. nigricans* is homothallic, and most homothallic filamentous ascomycetes harbor a *MAT1-1-1* gene and a *MAT1-2-1* gene in their genome. However, there is no indication of a *MAT1-2-1* gene in the *A. nigricans* genome, and the *MAT1-1-1* region is the same in three additional strains analyzed. Thus, *A. nigricans* apparently manages sexual reproduction with a single idiomorph carrying a single *MAT* gene. While unusual, there are other cases of such unisexual mating in filamentous ascomycetes, where all nuclei carry the same single *MAT* idiomorph

(Bennett and Turgeon 2016). One example is *Neurospora africana*, a homothallic species that carries a *MAT1-1* idiomorph, but no *MAT1-2*-related gene, and similar findings were made for several other homothallic *Neurospora* species, and possibly for homothallic species of the Dothideomycete genus *Stemphylium* (Gioti *et al.* 2012; Glass *et al.* 1990; Glass *et al.* 1988; Inderbitzin *et al.* 2005; Wik *et al.* 2008). In the homothallic Sordariomycete *Huntiella moniliformis*, unisexual reproduction takes place with just a *MAT1-2* idiomorph (Wilson *et al.* 2015). In the homothallic *S. macrospora*, *MAT1-1-1* is present, but dispensable for sexual development, whereas *MAT1-2-1* is required together with *MAT1-1-2* (Klix *et al.* 2010; Pöggeler *et al.* 2006b). Unisexual mating can also occur in heterothallic species, if one or both mating types are capable of sexual reproduction on their own. This was demonstrated, for example, for the *MAT* A mating type of the Sordariomycete *Sordaria brevicollis*, for *MAT* **a** cells of the ascomycete yeast *Candida albicans*, and for *MAT* α cells of the basidiomycete *Cryptococcus neoformans* (Alby *et al.* 2009; Lin *et al.* 2005; Robertson *et al.* 1998). Thus, it might formally be possible that *A. nigricans* is heterothallic with the ability of (at least) one mating type to undergo unisexual mating, because currently only four strains have been analyzed for their mating types, making it possible that additional mating types exist in the population. Another hypothesis to explain the single-gene mating type locus of *A. nigricans* might be that the species is indeed homothallic, and that the loss of the *MAT1-2-1* gene might be related to a reduction in morphological complexity of the fruiting body. It has been hypothesized previously that the morphologically simple fruiting bodies of the *Pyronema* and *Ascodesmis* lineages are reduced forms that evolved independently from more complex apothecia in other Pezizomycete lineages (Hansen and Pfister 2006). Since mating type genes can have functions other than the actual mating (Bennett and Turgeon 2016; Böhm *et al.* 2013), it is possible that a less complex fruiting body morphology can be sustained with a reduced complement of mating type genes.

Comparative transcriptomics of fruiting body development in three ascomycetes reveals conserved patterns of gene expression

~~With the genome sequence of *A. nigricans* available and having established laser microdissection of developing fruiting bodies for this species, we were able to analyze transcriptomes of different developmental stages, and perform for comparative analyses.~~ Comparative transcriptomics can be used to identify conserved patterns of gene expression in different species, or conversely to identify species-specific expression patterns that might help to explain, for example, morphological differences between species (Brawand *et al.* 2011; Romero *et al.* 2012; Stuart *et al.* 2003). In fungi, the latter approach was applied in comparative transcriptomics studies of Sordariomycete species from the *Fusarium* and *Neurospora* lineages. While expression patterns for many groups of genes or functional categories were similar, distinct differences in gene expression could be used to identify genes involved in species-specific morphological transitions (Lehr *et al.* 2014; Sikhakolli *et al.* 2012; Trail *et al.* 2017). In basidiomycete mushrooms, several comparative transcriptomics studies revealed a certain degree of conservation of gene expression during mushroom formation in several *Agaricomycetes*, including genes for cell wall remodeling, adhesion, signal transduction, transcription factors, and protein degradation *(Almási et al. 2019; Krizsán et al. 2019; Morin et al. 2012; Ohm et al. 2010; Plaza et al. 2014)*. To address the question if conserved patterns of gene expression can be found during fruiting body development in distantly related filamentous ascomycetes, we compared transcriptomes from mycelia and young fruiting bodies from *A. nigricans*, *P. confluens*, and *S. macrospora*. The three species represent different *Pezizomycotina* lineages, but have similar life styles in that they are homothallic and do not produce any asexual spores, facilitating sexual development-specific transcriptome analyses. Our results indicate that transcriptomes of developing fruiting bodies are distinct from mycelial samples in all three species, and furthermore are more similar between species than fruiting body transcriptomes are compared to mycelial samples from the same species. This confirms preliminary results based on comparisons of mycelia of *P. confluens* with fruiting bodies and mycelia of *S. macrospora* (Traeger *et al.* 2013). Similar tissue- or development-specific conserved expression

patterns of protein-coding genes have been noted previously in animals (Levin *et al.* 2016; Marlétaz *et al.* 2018; Necsulea and Kaessmann 2014).

To identify genes that might play a role in fruiting body development, we identified genes that were upregulated during fruiting body development in all three analyzed species, but which were not differentially regulated in other analyzed conditions. Among the 83 identified genes, ~~22~~ 23 encode proteins with predicted roles in vesicle transport, the endomembrane system, or transport across membranes. Genes that encode proteins involved in cellular transport were also enriched among genes that are expressed during fruiting body development of three *Neurospora* species (Lehr *et al.* 2014). Interestingly, a recent study on *Neolecta irregularis*, a member of the early-diverging ascomycete group of *Taphrinomycetes*, showed that genes involved in the functions of diverse endomembrane systems are conserved in *N. irregularis* and the *Pezizomycotina* (filamentous ascomycetes), all of which form fruiting bodies, but not in ascomycete yeasts that do not form fruiting bodies (Nguyen *et al.* 2017). The fruiting bodies of *Neolecta* and the *Pezizomycotina* most likely evolved independently, but based on a common set of genes in the last common ancestor of ascomycetes. It is possible that the evolution of complex multicellular structures with similar functions selected for similar cellular machineries (Nguyen *et al.* 2017). One reason might be that fruiting body formation requires a metabolically "competent" mycelium that transfers nutrients to the developing fruiting body (Pöggeler *et al.* 2006a; Wessels 1993). Such a transfer might need a specialized complement of genes managing the transport of large amounts of nutrients. Another, not mutually exclusive, explanation could be the requirement for building cells with specialized cell wall structures, e.g. asci, ascospores, or the non-sexual cells of the fruiting body. Again, specialized groups of genes involved in transport processes might be required for these purposes.

In addition to genes involved in transport processes, 13 genes with predicted roles in chromatin organization or the regulation of gene expression are among the genes upregulated during fruiting body development in the three species. The differentiation of fruiting bodies

entails a drastic restructuring of the transcriptome as evidenced by the greatly different transcriptome profiles of fruiting bodies and non-sexual mycelia. Thus, it is likely that the combined actions of chromatin modifiers and specific transcription factors prepare the cells for the transition to sexual development (Pöggeler *et al.* 2018). Enrichment of genes involved in transcription was also found among genes preferentially expressed during fruiting body morphogenesis of three *Neurospora* species, and in a comparative transcriptomics analysis of *S. macrospora* and *F. graminearum* (Gesing *et al.* 2012; Lehr *et al.* 2014). Genes involved in transcription might in turn regulate the expression of genes important for cell differentiation, e.g. genes for managing endomembrane systems as described above. In *S. macrospora*, the transcription factor gene *pro44* was found to be upregulated during fruiting body development (Teichert *et al.* 2012). Subsequent transcriptome analysis of a *pro44* deletion mutant showed that genes involved in cellular transport were downregulated in developing fruiting bodies of the mutant strain (Schumacher *et al.* 2018).

Functional characterization of genes with conserved expression patterns reveals roles in fruiting body development

Our functional characterization of four genes with evolutionary conserved transcriptional upregulation during fruiting body formation showed that three of them indeed play a role during sexual development. The exception is *aod5*, the homolog of *N. crassa aod-5*, which encodes a transcription factor involved in regulating the expression of the alternative oxidase gene *aod-1* (Chae *et al.* 2007; Chae and Nargang 2009). The corresponding *S. macrospora aod5* mutant shows a growth defect on antimycin A, similar to *N. crassa*, but no defects in sexual development. In *N. crassa*, AOD-5 interacts with another transcription factor, AOD-2, to activate *aod-1* transcription (Chae *et al.* 2007; Chae and Nargang 2009), and one might speculate that an *aod-2* homolog in *S. macrospora* might carry out some functions of *aod5* during development. However, the *aod-2* ortholog of *S. macrospora*, *SMAC_04081*, is not transcriptionally upregulated during development, in contrast to *aod5*.

For the other genes that were functionally characterized, involvement in fruiting body differentiation could be confirmed. However, for one of the genes, the putative glycolipid 2-alpha-mannosyltransferase *SMAC_06770*, sterility of the deletion mutant accompanies a severe growth defect. A growth phenotype was also reported for the corresponding *S. cerevisiae* mutant (Cipollo *et al.* 2001). The sterility of the *S. macrospora* deletion strains might therefore not be a specific effect related to development but caused by the overall growth defect.

Deletion of the putative chromatin modifier gene *scm1* did not result in a developmental phenotype in a single mutant or double mutants with chromatin modifier genes *cac2*, *crc1*, and *rtt106*. However, analysis of all possible triple mutants as well as the quadruple mutant revealed developmental defects ranging from impaired spore formation and discharge to complete lack of perithecia and spore production. While *cac2* and *rtt106* are homologs to histone H3/H4 chaperones of other eukaryotes, and *crc1* is predicted to encode a subunit of the chromatin remodeling complexes RSC or SWI/SNR (Avvakumov *et al.* 2011; Schumacher *et al.* 2018; Wilson *et al.* 2006), the molecular role of *scm1* is not yet clear. However, it is unlikely that the four chromatin modifiers act in the same protein complexes or regulatory pathways, therefore the lack of developmental phenotypes in the double mutants might indicate that there is a certain redundancy in the molecular mechanisms priming chromatin for its cellular functions. Another, not mutually exclusive, explanation might be that the chromatin structure needs to be drastically reshaped for successful fruiting body development, and that this restructuring needs most, but not all chromatin modifying activities to be available. Future experiments using techniques like Hi-C to analyze three-dimensional chromatin organization during development will help to address these hypotheses (Mota-Gómez and Lupiáñez 2019).

The deletion mutant of the gene for the predicted SAGA complex subunit SPT3 is sterile in addition to a mycelial growth defect, and these phenotypes are similar to the *spt3* mutant of *F. graminearum* (Gao *et al.* 2014). The SAGA complex is a multi-subunit transcriptional co-activator that performs multiple functions, e.g. histone modification and interaction

with transcriptional activators (Helmlinger and Tora 2017; Spedale *et al.* 2012). These activities are carried out by distinct modules within the complex, and SPT3 is part of the TBP (TATA-binding protein) binding module (Helmlinger and Tora 2017). The modularity of the complex allows sharing of the modules between SAGA and other complexes (Helmlinger and Tora 2017), and additional studies will be required to address the role of transcriptional co-activator complexes and other chromatin modifiers during sexual development in fungi.

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TABLES

Table 1. Fungal strains used in this study.

Strain	Relevant genotype and phenotype	Reference or source
Ascodesmis nigricans strains		
CBS 389.68	wild type	CBS-KNAW
CBS 704.96	wild type	CBS-KNAW
CBS 114.53	wild type	CBS-KNAW
CBS 163.74	wild type	CBS-KNAW
Sordaria macrospora strains		
wild type	wild type	AMB[a]
fus	spore color mutant	(Nowrousian *et al.* 2012)
S96888	Δku70	(Pöggeler and Kück 2006)
S110115	Δrtt106; fertile	(Gesing *et al.* 2012)
S110235	Δcac2; fertile	(Gesing *et al.* 2012)
S123704	Δcrc1; fertile	(Schumacher *et al.* 2018)
S111081	Δrtt106, Δcac2, fus; fertile	(Schumacher *et al.* 2018)
S111094	Δrtt106, Δcac2; fertile	(Schumacher *et al.* 2018)
S128347	Δcrc1, Δrtt106; fertile	(Schumacher *et al.* 2018)
S128175	Δcrc1, Δcac2, fus; fertile	(Schumacher *et al.* 2018)
S155732	Δscm1, fus; fertile	this study
S155906	Δscm1; fertile	this study
S156325	Δscm1, Δcac2; fertile	this study
S156391	Δscm1, Δcrc1, fus; fertile	this study
S156436	Δscm1, Δ rtt106; fertile	this study
RL1637	Δcac2, Δcrc1, Δrtt106; sterile	this study
RL1648	Δcrc1, Δrtt106, Δscm1; partially fertile	this study
RL1737	Δcac2, Δcrc1, Δscm1; sterile	this study
RL1738	Δcac2, Δcrc1, Δscm1; sterile	this study
RL1761	Δcac2, Δcrc1, Δscm1; sterile	this study
RL1987	Δcac2, Δrtt106, Δscm1; sterile	this study
RL1923	Δcac2, Δcrc1, Δrtt106, Δscm1; sterile	this study
RL1924	Δcac2, Δcrc1, Δrtt106, Δscm1; sterile	this study
RL1957	Δcac2, Δcrc1, Δrtt106, Δscm1; sterile	this study
S153858	Δspt3; sterile	this study
S155241	Δspt3; sterile	this study

	RL1164	Δspt3 + pOE_1829.3_GFP; fertile	this study
	RL1184	Δspt3 + pOE_1829.3_GFP; fertile	this study
	RL1493	Δspt3 + pN_1829.3_GFP; partially fertile	this study
	RL1509	Δspt3 + pN_1829.3_GFP; partially fertile	this study
	SJBK 1 AS8	Δaod-5; fertile	this study
	SJBK 19.2 AS9	Δaod-5 + pSMAC_06113_EGFP; fertile	this study

[a]AMB: Culture collection Allgemeine und Molekulare Botanik, Ruhr-Universität, Bochum, Germany

Table 2. Genome assembly statistics for three Pezizomycetes.

	A. nigricans	***P. confluens***[1]	***T. magnatum***[2]
assembly size (Mb)	27	50	192
no. of scaffolds	176	1,588	1,283
N50 (Mb)	0.49	0.14	1.81
repeats (Mb)	1	6	111
repeats (%)	4	12	58
predicted genes	9,622	13,369	9,433
coding regions (Mb)	12.1	14.6	11.5
coding regions (%)	44.3	29.2	6.0
introns (Mb)	1.5	2.5	2.4
introns (%)	5.6	5.1	1.2

[1]genome data from (Traeger *et al.* 2013), [2]genome data from (Murat *et al.* 2018)

Table 3. RNA-seq data analyzed in this study. For each condition, two independent biological replicates were analyzed, except for *P. confluens* protoapothecia, where three independent biological replicates were analyzed.

condition	GEO accession number	Reference
Ascodesmis nigricans		
vegetative mycelium	GSE92315	this study
sexual mycelium[1]	GSE92315	this study
protoapothecia[2]	GSE92315	this study
Pyronema confluens		
vegmix[3]	GSE41631	(Traeger *et al.* 2013)
DD[3]	GSE41631	(Traeger *et al.* 2013)
sexual mycelium[1]	GSE41631	(Traeger *et al.* 2013)
protoapothecia[2]	GSE61274	(Murat *et al.* 2018)
Sordaria macrospora		
vegetative mycelium	GSE33668	(Teichert *et al.* 2012)
sexual mycelium[1]	GSE33668	(Teichert *et al.* 2012)
wild type protoperithecia[2]	GSE33668	(Teichert *et al.* 2012)

[1]sexual mycelium represents mycelia including embedded developing fruiting bodies.

[2]protoapothecia and protoperithecia are young fruiting bodies isolated by laser microdissection.

[3]conditions vegmix (combined RNA from several growth conditions that allow only vegetative growth) and DD (growth in darkness) represent mycelia that cannot develop sexual structures.

FIGURE LEGENDS

Figure 1. Life cycle of *A. nigricans* under continuous illumination and laboratory conditions. Strain CBS 389.68 was grown on microscopic slides with RFA medium (with 0.8 % agar) for 1 to 6 d in constant light. After 1 d, a mycelium of septated hyphae is formed. After 2 d, apothecia initials can be observed that contain swollen young asci after 3 d (arrows). Immature, hyaline spores can be observed within asci after 4 d. Spores become pigmented during maturation after 5 d. Mature spores are released from eight-spored asci after 6 d. Development of mycelium and apothecia is the same in constant darkness (Figure S1). Scale bar represents 20 µm and is the same in all images.

Figure 2. Species tree of 20 fungal species based on phylome reconstruction. The species tree was built based on 143 single-copy, widespread genes (see Materials and Methods for details). All nodes are maximally supported by 100 % bootstrap. The scale bar gives substitutions per site.

Figure 3. Comparison of the mating type loci of *A. nigricans* and *P. confluens*. Orthologs of two genes that are linked to *MAT1-2-1* in *P. confluens* (*APN2*, shown in yellow, and *PCON_08388*, shown in green) are linked to *MAT1-1-1* in *A. nigricans*. No *MAT1-2-1* homolog was detected in *A. nigricans*. Genes shown in white do not have orthologs within the mating type regions. Repeat regions around the *A. nigricans MAT* locus are shown in red. The region around the *A. nigricans MAT1-1-1* amplified by PCR from several *A. nigricans* strains is indicated by a horizontal black bar. The predicted genes encoding helicase domain proteins adjacent to *MAT1-1-1* were manually annotated on scaffold 13 with the coordinates [join(17159..17356,17407..17700)] and [join(19850..21059,21107..21250,21298..22613)].

Figure 4. Comparative analysis of gene expression during development in *A. nigricans* (*A.n.*), *P. confluens* (*P.c.*), and *S. macrospora* (*S.m.*). The graphs show $\log_2$ fold change values versus mean expression for all genes with orthologs in all three species. In each graph, expression during fruiting body formation (protoapothecia or protoperithecia) or expression during vegetative growth (veg or vegmix) is compared to expression in total sexual mycelium from the respective species. The analysis was done with DESeq2, genes in red are genes that are differentially expressed with an adjusted p-value <0.1.

Figure 5. Expression ratios of orthologs that are up- or downregulated in young fruiting bodies of *A. nigricans* (*A.n.*), *P. confluens* (*P.c.*), and *S. macrospora* (*S.m.*), but not differentially regulated in other conditions. The heatmaps were generated based on hierarchical clustering of $\log_2$ fold changes. The heatmap on the left shows genes that are up- or downregulated in young fruiting bodies, the heatmap on the right shows only genes that are upregulated in young fruiting bodies. The corresponding *S. macrospora* locus tags for selected genes are indicated on the right. Locus tags shown in gray correspond to genes that are predicted to be involved in vesicle transport, the endomembrane system, or transport across membranes. Locus tags shown in black correspond to genes predicted to be involved in chromatin organization or regulation of gene expression.

Figure 6. Phenotypes of single, double, triple, and quadruple chromatin modifier mutants of *S. macrospora*. The strains were grown for 7 d on BMM. Gene deletion of *scm1* results in a fully fertile strain, which only sometimes forms perithecia lying on the side. Double deletion strains of *scm1* with *cac2*, *crc2*, or *rtt106* are also fully fertile after 7 d (the Δscm1/Δcrc1/fus mutant produces brown ascospores due to the presence of the spore color mutation fus). Triple and quadruple chromatin modifier deletion strains showed reduced fertility up to sterility. While Δscm1/Δcrc1/Δrtt106 was able to form perithecia and discharge spores, all three triple mutants containing Δcac2 were sterile. Although sometimes forming

immature fruiting bodies with few spores inside, Δscm1/Δcac2/Δrtt106 and Δcac2/Δcrc1/Δrtt106 never discharged spores (strains were observed for 21 d). Δscm1/Δcac2/Δcrc1 forms few enlarged protoperithecia, but no spores. The quadruple mutant showed a phenotype comparable to so-called pro mutants forming only protoperithecia, and therefore is sterile. Scale bars for top and side view 500 µm, for ascus rosettes and spores 100 µm.

Figure 7. Phenotypic characterization of *S. macrospora* Δspt3 and complemented strains. **A.** Overview of strains grown on BMM and SWG for 7 d and 14 d (details on the right for each strain). Δspt3 is sterile on both media and forms only few non-pigmented protoperithecia. Complemented strains under native promoter (Δspt3::na-*spt3-egfp*) and constitutive promoter (Δspt3::Pgpd-*spt3-egfp*) form perithecia on BMM, but need longer (10 d compared to 7 d in the wild type) to become fertile and discharcharge spores. On minimal medium (SWG), complemented strains did not form mature perithecia even after 14 d. **B.** The growth rate of Δspt3 is significantly reduced on BMM and SWG compared to the wild type. Complemented strains grow faster than the mutant strain, but not as fast as the wild type. **C.** Hyphal fusion and hyphal morphology of Δspt3. The mutant strain is able to form hyphal anastomoses (red arrowheads). In older mycelium, Δspt3 forms enlarged hyphae, which start to grow into dead hyphae (intrahyphal growth, yellow arrowheads). **D.** Detail of fruiting body development on BMM. Protoperithecia of Δspt3 are non-pigmented and less compact than wild type protoperithecia. Ascogonia were not found on the agar surface, where they are formed in the wild type, because protoperithecia in the mutant were mostly formed below the agar surface. Consequently, ascogonia are present within the agar, but difficult to detect there due to their small size and lack of pigmentation. The deletion strain never formed pigmented protoperithecia or perithecia. The complemented strains formed perithecia after 8-10 d. Only the complemented strain with *spt3* expressed from a constitutive promoter discharged spores after 10 d; however, both complemented strains formed spores within the perithecia. Scale bar for ascogonia and young

protoperithecia 20 µm. Scale bar for pigmented protoperithecia and perithecia 100 µm unless indicated otherwise. Scale bar for ascus rosettes 40 µm.