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Evolution of asexual and sexual reproduction in the aspergilli

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Abstract: *Aspergillus nidulans* has long-been used as a model organism to gain insights into the genetic basis of asexual and sexual developmental processes both in other members of the genus *Aspergillus*, and filamentous fungi in general. Paradigms have been established concerning the regulatory mechanisms of conidial development. However, recent studies have shown considerable genome divergence in the fungal kingdom, questioning the general applicability of findings from *Aspergillus*, and certain longstanding evolutionary theories have been questioned. The phylogenetic distribution of key regulatory elements of asexual reproduction in *A. nidulans* was investigated in a broad taxonomic range of fungi. This revealed that some proteins were well conserved in the *Pezizomycotina* (*e.g.* AbaA, FlbA, FluG, NsdD, MedA, and some velvet proteins), suggesting similar developmental roles. However, other elements (*e.g.* BrlA) had a more restricted distribution solely in the *Eurotiomycetes*, and it appears that the genetic control of sporulation seems to be more complex in the aspergilli than in some other taxonomic groups of the *Pezizomycotina*. The evolution of the velvet protein family is discussed based on the history of expansion and contraction events in the early divergent fungi. Heterologous expression of the *A. nidulans abaA* gene in *Monascus ruber* failed to induce development of complete conidiophores as seen in the aspergilli, but did result in increased conidial production. The absence of many components of the asexual developmental pathway from members of the *Saccharomycotina* supports the hypothesis that differences in the complexity of their spore formation is due in part to the increased diversity of the sporulation machinery evident in the *Pezizomycotina*. Investigations were also made into the evolution of sex and sexuality in the aspergilli. *MAT* loci were identified from the heterothallic *Aspergillus* (*Emericella*) *heterothallicus* and *Aspergillus* (*Neosartorya*) *fennelliae* and the homothallic *Aspergillus pseudoglaucus* (=*Eurotium repens*). A consistent architecture of the *MAT* locus was seen in these and other heterothallic aspergilli whereas much variation was seen in the arrangement of *MAT* loci in homothallic aspergilli. This suggested that it is most likely that the common ancestor of the aspergilli exhibited a heterothallic breeding system. Finally, the supposed prevalence of asexuality in the aspergilli was examined. Investigations were made using *A. clavatus* as a representative 'asexual' species. It was possible to induce a sexual cycle in *A. clavatus* given the correct *MAT1-1* and *MAT1-2* partners and environmental conditions, with recombination confirmed utilising molecular markers. This indicated that sexual reproduction might be possible in many supposedly asexual aspergilli and beyond, providing general insights into the nature of asexuality in fungi.

Key words: *abaA*, Asexuality, *Aspergillus nidulans*, *brlA*, Conidiation, Conidiophore, Development, Mating type, Sporulation, *velvet*.

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INTRODUCTION

Members of the genus *Aspergillus* have long-been used as model organisms to study developmental processes in filamentous fungi. This is due to their ease of cultivation and manipulation under laboratory conditions, the well-characterised morphology of asexual spore development, and the fact that they exhibit both homothallic (self-fertile) and heterothallic (obligate out-crossing) sexual breeding systems (Krijgsheld *et al.* 2013). The homothallic species *A. nidulans* in particular has been used extensively for investigations into the genetic basis of asexual and sexual sporulation, following its establishment as a model organism by Pontecorvo (1953). Studies have revealed a series of genetic pathways governing asexual and sexual reproduction, with ongoing research using a variety of –omic techniques to gain ever deeper insights into the precise molecular mechanisms of these pathways (Park & Yu 2012, Dyer & O'Gorman 2012).

Results from studies with *A. nidulans* have been considered to provide insights that are applicable to sporulation processes in the aspergilli as a whole, as well as being of relevance to the *Pezizomycotina* in general. However, it is possible that some aspects of the genetic regulation may be restricted to *A. nidulans* and its close relatives. Whole genome sequence has recently become available from both a wide taxonomic range of the aspergilli (de Vries *et al.* 2017) and the fungal kingdom in general, revealing considerable genome divergence within fungi. This now offers the opportunity to assess how widespread aspects of the regulatory pathways of reproduction in *A. nidulans* are in a broad biodiversity of fungi, as well as addressing certain specific questions concerning the control and evolution of asexual and sexual development in the aspergilli. These issues are investigated in detail in the present study under the common theme of reproduction, looking first at asexual and then later at sexual reproduction. Findings from both sets of analyses reveal how data obtained from *Aspergillus* species may, or may not, be of general relevance to understanding reproductive processes in other fungal taxa. The results presented follow on from initial work reported in the comparative genomics analysis of de Vries *et al.* (2017).

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Studies in Mycology

Asexual development in *Aspergillus*

Aspergillus species are well known for the prolific production of asexual spores termed conidia. These are produced from conidiophores with a characteristic aspergillum-like morphology consisting of a foot cell, stalk and vesicle bearing metulae and phialides with radiating conidia (Fig. 1A), although rare exceptions do exist within the aspergilli with different conidial head morphologies (Yu 2010, Samson *et al.* 2014). *A. nidulans* has been used as a model for the study of conidiation for many decades and consequently considerable knowledge has been accumulated about the regulatory pathways involved in this species (Adams *et al.* 1998, Etxebeste *et al.* 2010, Park & Yu 2012). The initiation of conidiation involves the regulation of thousands of genes in *A. nidulans* (Garzia *et al.* 2013, Cánovas *et al.* 2014), of which there are a series of upstream activators and negative repressors, central regulators, as well as light-responsive and velvet regulators [Fig. 1B; also see Fig. 2A of de Vries *et al.* (2017)].

The initiation of the conidial developmental pathway in *A. nidulans* is controlled by upstream developmental activators (UDAs), which consist of three genetic cascades containing the *flbA* (fluffy low BrlA expression), *flbB*/D/E and *flbC* genes. Upstream of the *flbB/D/E* and *flbC* modules lies *fluG*, which is an activator of the *flb* modules (Fig. 1B). FluG is responsible for the synthesis of an endogenous diffusible factor, with the meroterpenoid compound dehydroaustinol shown to induce conidiation in a Δ*fluG* mutant (Rodriguez-Urra *et al.* 2012). FluG is involved in the repression of the activity of SfgA (Seo *et al.* 2006). This step is crucial to initiate the conidiation machinery because SfgA itself is a repressor of the fluffy genes (Seo *et al.* 2006). Further repressors of conidiation active during vegetative growth are NsdD, VosA and two G-protein signalling pathways (Seo *et al.* 2006, Park & Yu 2012, Lee *et al.* 2014, 2016).

The expression of the various fluffy genes ultimately activates the central regulatory pathway (CRP) composed sequentially of *brlA*, *abaA* and *wetA* (Adams *et al.* 1998, Etxebeste *et al.* 2010, Park & Yu 2012) (Fig. 1B). Deletion of any of these genes leads to particular blocks in the proper development of conidiophores, resulting in abnormal morphologies termed *bristle, abacus* and *wet-white*, respectively (Yu 2010). The first genome sequencing projects of the aspergilli demonstrated that the CRP was also present in species such as *A. fumigatus* and *A. niger*, and it was suggested that the same pathway observed in *A. nidulans* was likely to control asexual sporulation in these species as well (Pel *et al.* 2007, Samson *et al.* 2009, Yu 2010). Most recently, de Vries *et al.* (2017) investigated the presence of CRP in a broader range of *Aspergillus* and *Pezizomycotina* species. BrlA seemed to be limited to the *Eurotiales*, suggesting a specific role for conidiation in this group. By contrast, WetA was widely distributed in the *Pezizomycotina*, suggesting a general function for the synthesis of spore cell wall layers. Meanwhile, AbaA was widespread in the *Ascomycota*, *Basidiomycota*, and *Mucoromycota*, suggesting other general functions in fungal development. However, intriguingly the *abaA* gene was missing from *Monascus ruber* (a close relative of the aspergilli) and was not uniformly present in the fungal kingdom (de Vries *et al.* 2017).

Other proteins also influence conidial formation in *A. nidulans*. These include the transcription factors StuA and MedA, both of which have been termed developmental modifiers because they are required for the development of proper conidiophore morphology (Busby *et al.* 1996, Wu & Miller 1997). A further major group is the velvet (Vel) proteins, which have been implicated in the regulation of developmental processes, and also secondary metabolism, and which are specific to fungi (Bayram & Braus 2012). The members of this family are characterized by the velvet domain comprising approximately 150 amino acids with a fold resembling the Rel homology domain (RHD) of the mammalian transcription factor NF-κB (Ahmed *et al.* 2013). The velvet proteins act downstream of the light receptor proteins LreA, LreB and FphA in *A. nidulans* to either promote or repress asexual or sexual reproduction, depending on the specific VeA, VelB or VelC protein (Bayram & Braus, 2012, Dyer & O'Gorman 2012). The velvet regulators can interact both with each other and also with non-velvet proteins to control development and conidiation (Bayram & Braus, 2012).

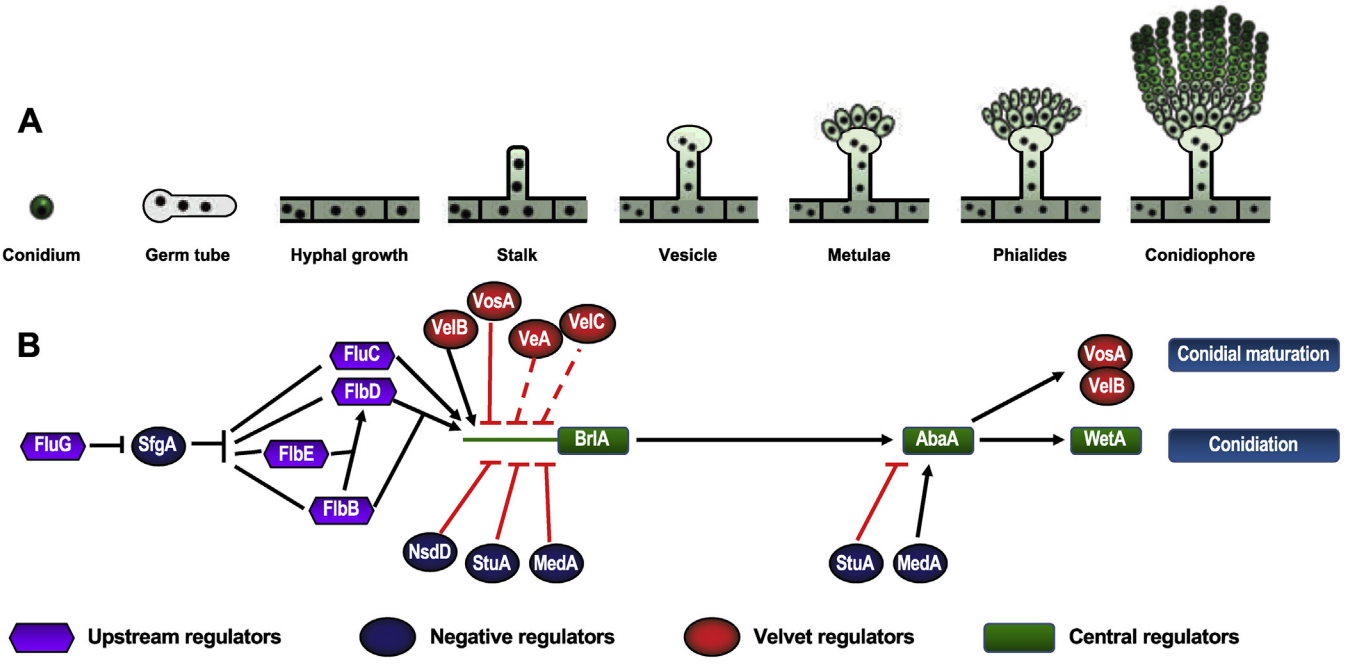


Fig. 1. (A) A schematic presentation of conidiophore development of in *A. nidulans*. (B) A genetic model of the regulation of conidiophore development.

Given this background, a main aim of the present study was to study the phylogenetic distribution of known regulators of conidiation in *A. nidulans* to determine how widespread the action of these proteins might be in the fungal kingdom. This included an analysis of the upstream regulators and repressors, the central regulatory pathway, and the possible expansion or contraction of the velvet family proteins. A second specific aim was to heterologously express *abaA* in *M. ruber*, to see to what extent this might impact on conidiophore morphology given the close taxonomic relatedness to the aspergilli and that *abaA* appears to be absent from the *M. ruber* genome whilst all other regulators are present (Vries *et al.* 2017).

Sexual development in *Aspergillus*

Whereas asexual reproduction is observed universally in the aspergilli (Raper & Fennell 1965, Samson *et al.* 2014), sexual reproduction has only been observed in approximately 36 % of species (Dyer & O'Gorman 2011). Where sex occurs, it leads to the formation of ascospores within enclosed cleistothecia, which break down at maturity to passively release the sexual spores. The *Aspergillus* species with described sexual cycles are overwhelmingly homothallic in nature, with a ratio of approximately 13:1 homothallic: heterothallic taxa (Dyer & O'Gorman 2012). Despite the supposed monophyly of *Aspergillus* there is nevertheless a surprising diversity in the morphology of sexual structures within the genus compared to the more limited variation seen in conidial development (Samson *et al.* 2014). Up to 12 different sexual genera have been phylogenetically associated with *Aspergillus* asexual forms, being distinguished by morphological aspects of the cleistothecia such as the wall (peridium) composition and colour, and whether cleistothecia develop within larger surrounding sclerotia (Dyer 2007, Peterson 2008, Samson & Varga 2010, Dyer & O'Gorman 2012).

Numerous studies have been undertaken with *A. nidulans* to determine the genetic basis of sexual development, with over 70 genes now identified as having roles in various stages of the sexual process. These have been divided into genes encoding proteins involved with perception of environmental signals, mating and signal transduction, transcription factors and other regulatory proteins, endogenous physiological processes, and ascospore production and maturation (Dyer & O'Gorman 2012). Of particular note was the discovery that the breeding system of particular species is governed by the presence of mating-type (*MAT*) genes (Dyer *et al.* 2016). The genome of the homothallic model species *A. nidulans* was found to contain both *MAT1* and *MAT2* mating-type genes encoding alpha-domain and high-mobility group (HMG) domain transcription factors, respectively (Galagan *et al.* 2005, Paoletti *et al.* 2007). A similar discovery was later made for the homothallic *Aspergillus (Neosartorya) fischeri* and *Aspergillus (Petromyces) alliaceus* (Rydholm *et al.* 2007, Ramirez-Prado *et al.* 2008). Deletion of either *MAT* gene led to loss of self-fertility in *A. nidulans*, although deletion mutants were still able to outcross in a heterothallic fashion (Paoletti *et al.* 2007). Related work led to the identification of complementary *MAT1-1* and *MAT1-2* isolates in species such as *A. fumigatus, A. parasiticus, A. flavus* and *A. lentulus* (Paoletti *et al.* 2005, O'Gorman *et al.* 2009, Horn *et al.* 2009a,b, Swilaiman *et al.* 2013). In these instances, isolates were found to have an idiomorphic *MAT* locus containing either a *MAT1-1-1* alpha domain or a *MAT1-2-1* HMG mating-type gene, respectively. Under suitable conditions a sexual cycle could be induced in all of these species, with successful crossing requiring isolates of compatible mating type to be present. This provided clear evidence of a heterothallic breeding system in all of these species, determined by the presence of either *MAT1-1-1* or *MAT1-2-1* genes in the genome of individual isolates. *MAT* genes were also shown to exhibit cross-species activity and influence gene expression in asexual species when heterologous genes were used in host *MAT* gene replacement experiments (Grobe & Krappmann 2008, Pyrzak *et al.* 2008, Wada *et al.* 2012).

The observation that homothallism predominates in the genus has been used as evidence to suggest that this breeding system was ancestral in the aspergilli, with subsequent conversion to heterothallism through loss of self-fertility in the relatively few known heterothallic species (Geiser *et al.* 1996, 1998, Varga *et al.* 2000). Furthermore, the fact that the majority of the aspergilli are only known to reproduce by asexual means has led to the theory that sexual reproduction (meiosis) has been lost multiple times in this group due to evolutionary selection for asexuality (Geiser *et al.* 1996). These hypotheses have been applied more generally to the evolution of sex and asexuality in fungi (Dyer & Kück 2017).

Given this background, an additional main aim of the present study was to determine whether homothallism is truly ancestral in the aspergilli, or whether the genus has heterothallic origins, building on recent findings by de Vries *et al.* (2017). To address this question, we examined how *MAT* locus architecture varied throughout the aspergilli, including the cloning of *MAT* loci from the first two ever identified heterothallic *Aspergillus* species, *Aspergillus (Emericella) heterothallicus* (Kwon & Raper 1967) and *Aspergillus (Neosartorya) fennelliae* (Kwon & Kim 1974), as well as the homothallic *Aspergillus pseudoglaucus* (=*Eurotium repens*) (Chen *et al.* 2017a,b). A further specific aim was to determine whether asexuality truly dominates in the aspergilli, or whether supposed 'asexual' species might retain the potential for sexual reproduction. To address this, an analysis of *MAT* gene presence and recent breakthroughs in inducing sexual reproduction was used to investigate whether sex might be possible in *A. clavatus*, which lacks a known sexual morph, as a representative of the asexual aspergilli. This species was chosen due to its medical importance both as an opportunistic pathogen and producer of antibiotics (Bergel *et al.* 1944, Suzuki *et al.* 1971, Varga *et al.* 2007), as well as its importance as a spoilage organism (Varga *et al.* 2003). Indeed, these studies overall are of possible biotechnological and fundamental significance given that the sexual cycle provides a valuable tool for strain improvement and genetic analysis (Ashton & Dyer 2016), so it would be of great benefit if sex could be induced in supposed 'asexual' aspergilli.

MATERIALS AND METHODS

Bioinformatic analyses

Supplementary Table 1 lists species employed in bioinformatic analyses in this study. Filtered gene model derived proteomes were downloaded from the Mycocosm site (https://genome.jgi.doe.gov/programs/fungi/index.jsf) (Grigoriev *et al.* 2014). OrthoFinder version 2.2.0 (Emms & Kelly 2015) with default options was used to assess orthology among the 54 fungal proteomes. The resulting species tree was modified with Archaeopteryx (Han

& Zmasek 2009) to fit the Mycocosm site evolutionary tree of the fungi, and a re-run of Orthofinder with the modified tree was used to estimate the reconciled trees for each orthogroup. Data from *Aspergillus nidulans* was used as a reference to find the orthogroups for the FluG, FlbA-E, SfgA, NsdD, MedA, StuA, VelA-C and VosA regulatory proteins of conidation. The reconciled trees were used to ascertain the orthology-paralogy relationships among the members of the same orthogroup. Protein domains were annotated employing the NCBI web Cd-search tool (Marchler-Bauer & Bryant 2004) against the CDD database. Domain architectures and trees were visualized with Domosaics (Moore *et al.* 2014).

To construct trees including basal fungi, proteins were first identified from the Mycocosm site. Multiple alignments were then made with Clustal Omega (Sievers *et al.* 2011), and maximum-likelihood trees were generated using the IQ-Tree server (Trifinopoulos *et al.* 2016) and drawn in iTOL (Letunic & Bork 2016). Branches were evaluated by 1 000 ultrafast bootstrap replicates and by the SH-aLRT test. Best model selection was carried out by the ModelFinder option included at the IQ-Tree server (Kalyaanamoorthy *et al.* 2017). Additional blastp and tblastn searches were conducted using the NCBI, JGI-Mycocosm (Grigoriev *et al.* 2014) and FungiDB (Stajich *et al.* 2012) databases where necessary.

For AbaA, BrlA and WetA, protein sequences from *A. nidulans* were used to query their homologues using the HMMER 3.1b2 package (http://www.hmmer.org/). The cut-off E values for homologues of AbaA, BrlA and WetA were set at e^{-5}, e^{-100} and e^{-5}, respectively. The homologues were aligned by MUSCLE (Edgar 2004) and then submitted to Weblogo (http://weblogo.threeplusone.com/create.cgi) to generate the conserved motifs.

Genetic manipulation of *abaA* in *Monascus ruber*

A heterologous gene expression approach was used to determine the effect of *abaA* expression in *Monascus ruber*. *A. nidulans* isolate FGSC A4 (Fungal Genetics Stock Center, USA) was used as the donor of the *abaA* gene and maintained on PDA slants at 30 °C. *Escherichia coli* DH 5α and *Agrobacterium tumefaciens* EHA105 were used for hosting plasmids and were cultivated in LB medium at 28 °C.

Briefly, the *abaA* gene (AN0422) was amplified from *A. nidulans* FGSC A4, while the *trpC* promoter and terminal fragments were cloned from the plasmid pSKH, and the selective marker gene *neoR* for neomycin resistance was cloned from the plasmid pKN1 (Li *et al.* 2010). These four DNA fragments were fused by double-joint PCR as illustrated in Supplementary Fig. 12 (Yu *et al.* 2004). Primers used in this study are listed in Supplementary Table 2. The fused fragment was then introduced into the expression vector pCAMBIA3300 via the vector pMD19-T. The recombinant vector carrying the *abaA* expression cassette was next introduced into the genome of *M. ruber* isolate M7 by *Agrobacterium tumefaciens*-mediated transformation according to Shao *et al.* (2009). Transformants were selected on potato dextrose agar (PDA) medium containing 15 mg/mL G418 (Sigma-Aldrich, Shanghai, China). Gene integration was confirmed by PCR, cDNA sequencing and Southern blotting. Southern blot assays were performed according to protocols for the DIG-High Prime DNA Labeling & Detection Starter kit I (Roche, Mannheim, Germany). To prepare probes, fragments from the open reading frame of *abaA* and the selective marker gene *neoR* were amplified with primer pairs abaA-F/abaA-R, and neo-F/neo-R (Supplementary Table 2), respectively, and then labelled with digoxin after purification with a DNA gel extraction kit (Sangon Biotech, China). In order to verify the relative expression level of *abaA* in the selected mutants, quantitative real-time RT-PCR was performed using β-actin as a reference gene (Liu *et al.* 2014). The wild-type and mutants were cultivated at 28 °C on potato dextrose agar (PDA) and an Olympus BH2 compound microscope with differential interference contrast optics was used to take photomicrographs of resulting phenotypes.

Identification and sequencing of the *MAT* locus of *Aspergillus* (*Emericella*) *heterothallicus*

Isolates 50-3 and 50-5 of *A. heterothallicus* (=*Emericella heterothallica*) were obtained from the BDUN culture collection at The University of Nottingham. These were derived from single sporing of the reference isolates WB4982 (*MAT-A*) and WB5086 (*MAT-a*), from Kwon & Raper (1967). Isolates were cultivated in malt extract liquid media (20 g malt extract powder, 1 g peptone per L distilled water) at 28 °C, and genomic DNA extracted using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions.

To characterise the *MAT* loci, a bridging strategy was used involving PCR with degenerate primers of the internal *MAT* genes as well as amplification of genes known to be conserved in the external flanking regions of the *MAT* locus. Initially, fragments of the *MAT1-1* and *MAT1-2* genes were amplified from *A. heterothallicus* isolates 50-5 and 50-3, respectively, utilising the degenerate primer sets MAT5-6 and MAT3-4, and MAT5-7 and MAT3-5 respectively [these primers designed for *Eurotiomycete* fungi; Houbraken & Dyer (2015)] using PCR conditions described by Eagle (2009). Resultant PCR products were gel extracted, ligated into plasmid pTOPO4, then cloned into *E. coli* prior to DNA sequencing. In parallel, fragments of the *SLA2* and *APN2* genes [known to flank either side of the *MAT* locus in many *Pezizomyoctina* species (Debuchy & Turgeon 2006, Dyer *et al.* 2016)] from isolates 50-5 and 50-3 were amplified and sequenced in a similar fashion using SLA2 and APN2 degenerate primer sets designed against conserved sequence found in the genomes of available *Aspergillus* species (primers used for SLA2 were aaSLA2: AYMGNGARATGGCNGAYYTNGARG and SLA2R: CRTANSDNGGNSWNGCRTTYTG; for APN2 primers used were aaAPN2: CARMGNAARGAYYTNMGNGAYGAYATG and APN2R: GGRTANCCNCCNAYYTGNYKNTC), using PCR conditions described by Eagle (2009). This allowed the subsequent design of species-specific non-degenerate primers for each of the resulting *MAT1-1, MAT1-2, SLA2* and *APN2* gene fragments. Primers were designed from the *SLA2* and *APN2* fragments to be orientated inwards towards the *MAT* locus, and pairs of outward primers were designed from the *MAT1-1* and *MAT1-2* fragments [see Eagle (2009) for specific details]. This allowed production of a SLA2 to *MAT* amplicon, and a separate *MAT* to *APN2* amplicon (amplifying outwards from either the *MAT1-1-1* or *MAT1-2-1* fragments). The resulting products were sequenced by chromosome walking (Eagle 2009). Resulting sequence was interrogated by PSORT II (http://psort.nibb.ac.jp/) and TFSITESCAN (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) programs for the presence of nuclear-targeting and promoter-region motifs.

Identification and sequencing of the *MAT* loci from *Aspergillus (Neosartorya) fennelliae* and *Aspergillus pseudoglaucus*

Isolates 54-1 (CBS 410.89, *MATA*) and 54-2 (CBS 411.89, *MATa*) of *A. (Neosartorya) fennelliae* were obtained from the BDUN culture collection at The University of Nottingham. Both were originally isolated from Marine Sludge in Japan, 1981 (Takada & Udagawa 1985). Similar procedures were used to identify the *MAT* locus as described above for *A. heterothallicus.* A bridging strategy was used based on initial degenerate PCR of fragments of the *MAT, SLA2* and *APN2* genes. This allowed the design of species-specific primers, which were used to amplify SLA2 to *MAT* and separate *MAT* to *APN2* amplicons, allowing chromosome walking of the entire *MAT* region. Full experimental details are provided in Eagle (2009).

For studies of *A. pseudoglaucus* (=*Eurotium repens*) two isolates were obtained from the BDUN culture collection (University of Nottingham), namely 51-1 (origin unknown) and 51-2 (CBS 529.65) originally isolated in 1965 from *Prunus domestica* in France (Peterson 2008). Attempts were made to identify *MAT* loci as described above for *A. heterothallicus* and *A. fennelliae* using the bridging strategy with degenerate PCR of fragments of the *MAT, SLA2* and *APN2* genes. However, it was also necessary to use thermal asymmetric interlaced (TAIL) PCR in combination with the use of the *MAT* degenerate primers to get sufficient *MAT* locus sequence (Arie *et al.* 1997). Successive rounds of TAIL-PCR were performed with degenerate PCR primers to extend the region around the *MAT* gene fragment (Liu & Whittier 1995). Sequence data was pooled from isolates 51-1 and 51-2 to ensure consistency. See Eagle (2009) for full experimental details.

Sexual biology of *Aspergillus clavatus*: mating-type diagnostic assay

Twenty isolates of *A. clavatus* from worldwide locations were obtained from the BDUN collection at the University of Nottingham (isolates 65-1 to 65-20; Supplementary Table 3). Isolates were maintained on *Aspergillus* complete agar or liquid media (ACM) (Paoletti *et al.* 2005) at 28 °C, and genomic DNA extracted using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions.

To study the potential for sexual reproduction in *A. clavatus* it was first necessary to elucidate the mating types of these isolates in order that directed crosses could be set up. Putative *MAT1-1-1* gene sequence data was obtained from The Broad Institute *A. clavatus* genome screening project (http://www.broad.mit.edu/tools/data/seq.html) (gene locus ID: ACLA_034110) and specific MAT1-1-1 primers were designed from within this sequence to detect the presence of isolates of the *MAT1-1* genotype (primers AclM1F: CAGTTGTTCGTAGCAGACGGGG, and AclM1R: CGGTGGAGTATGCTTTGGCGAGG). In parallel, attempts were made to amplify a fragment of the *MAT1-2-1* gene sequence using degenerate primers MAT5-7 and MAT3-5 (Houbraken & Dyer 2015) utilising PCR conditions described by Eagle (2009). The resulting putative *MAT1-2-1* amplicon from isolate 65-13 was cloned and sequenced. The same bridging strategy as used in *A. heterothallicus* (see above), involving chromosome walking in from the *SLA2* and *APN2* flanking genes, was then used to clone and sequence the entire *MAT1-2* region of *A. clavatus* from isolate 65-13 [see Eagle (2009) for specific details; GenBank accession MH401197]. This allowed the design of *MAT1-2-1* specific primers within the *MAT1-2* idiomorph to detect the presence of isolates of the *MAT1-2* genotype (primers AclM2F: ATCAAGGCTCTTCGTGTCATGC, and AclM2R: ATGCTTCTTCTTCATATCTTCTGCC).

The resulting *MAT* primer sets were used in PCR as diagnostic tools to determine mating type in a screen of genomic DNA from the remaining isolates of *A. clavatus*. Amplifications were performed using 25 µL reaction volumes containing 2.5 µL 10X PCR Buffer (containing 20 mM $MgCl_2$), 0.2 µL (25 mM each) dNTPs, 2.5 µL (10 µM) of the respective *MAT* forward and reverse primers, 0.2 µL FastStartTaq Polymerase (Roche, UK), ~50 ng genomic DNA, and ultrapure water to a final volume of 25 µL. PCR was performed on a Techne Genius thermal cycler, using an initial denaturation step of 94 °C for 5 min; 35 cycles consisting of 1 min at 94 °C, 1 min at 60 °C for *MAT1-1* and 55 °C for *MAT1-2*, and 1 min at 72 °C; followed by a final extension step at 72 °C for 5 min (all steps were performed at a ramp rate of 70 °C/min). Resultant PCR products were resolved on 1.5 % agarose gels and visualized by ethidium bromide staining. The hypothesis of a 1:1 ratio of mating types in the worldwide sample population and ascospore progeny was tested using χ^2 and contingency χ^2 tests (Fisher 1938).

Sexual biology of *Aspergillus clavatus*: crossing and progeny analysis

Crosses were then set up in 9 cm Petri dishes between six *MAT1-1* strains and three *MAT1-2* strains of *A. clavatus* which were inoculated in all possible pair wise combinations (n = 18), following the barrage crossing procedures of O'Gorman *et al.* (2009). All crosses were set up on oatmeal agar (Robert *et al.* 2007; pinhead, Odlums, Ireland) in triplicate, sealed with Nescofilm® and incubated at 25 °C, 28 °C or 30 °C in the dark. The crosses were examined periodically for the presence of cleistothecia for up to five months, using a Nikon-SMZ-2B dissection microscope.

Attempts were then made to isolate ascospore progeny from putative mature cleistothecia. The fruit bodies were cleaned by rolling on 4 % tap water agar to remove adhering conidia as described by Todd *et al.* (2007). Intact cleistothecia were then added to 500 µL of 0.05 % (v/v) Tween 80 and heat treated at 69 °C for 10 min to inactivate any adhering conidia, with the assumption that the peridium of the cleistothecia served as a barrier to protect the ascospores to some extent (higher temperatures and longer periods were found to kill the ascospores as well; data not shown). The cleistothecia were centrifuged, the supernatant discarded and then 50 µL of 0.05 % (v/v) Tween 80 was added and cleistothecia ruptured by squashing with a needle tip (Todd *et al.* 2007). The solution was then brought up to 500 µL by addition of 0.05 % Tween 80 (v/v) and vortex-mixed for 1 min to release the ascospores. One hundred µL of a 5×10^5 ascospore mL^{-1} suspension was then spread inoculated onto ACM plates (Paoletti *et al.* 2005), which were incubated at 37 °C for 14 h. Single spore cultures were established on ACM by transferring individual germinating ascospore with a LaRu lens cutter attached to a Nikon-OPTIPHOT microscope.

The segregation of five genetic markers was then examined in the ascospore offspring using RAPD-PCR fingerprinting as

previously described (Murtagh *et al.* 1999, O'Gorman *et al.* 2009, Swilaiman *et al.* 2013). An initial screen of sixteen RAPD primers revealed four (OPC20, 0PT18, UBC90, OPQ6; sequences available on request) that yielded polymorphisms suitable for genotyping. Finally, cleistothecia were examined by scanning electron microscopy as described by Swilaiman *et al.* (2013).

RESULTS AND DISCUSSION

Reproduction via the formation of spores is a property seen throughout the fungal kingdom, which presumably arose early on in the evolution of many different lineages. The ability to produce both a tremendous abundance of asexual and/or sexual spores, combined with the possibility of the long-distance dispersal of these propagules, helps account for the ecological success and widespread occurrence of members of the fungal kingdom (Golan & Pringle 2017). The formation of asexual and sexual spores is in a balance controlled by both environmental factors and intracellular signals (Adams *et al.* 1998, Rodríguez-Romero *et al.* 2010, Ruger-Herreros *et al.* 2011, Cánovas *et al.* 2016, Marcos *et al.* 2016). It is therefore of both academic and applied significance to understand the genetic controls of asexual and sexual development, with the prospect of exploiting such knowledge to control detrimental species whilst promoting growth of beneficial species.

In terms of filamentous fungi, *A. nidulans* and *Neurospora crassa* have been the most widely used models to study developmental processes up to this point. Research with *A. nidulans* in particular has established paradigms for the genetic regulation of asexual and sexual reproduction (Adams *et al.* 1998, Braus *et al.* 2002, Han & Han 2010, Etxebeste *et al.* 2010, Dyer & O'Gorman 2012, Park & Yu 2012), as well as the aspergilli in general being used to propose hypotheses concerning the evolution of asexuality and sexual breeding systems (Geiser *et al.* 1996, Geiser *et al.* 1998, Varga *et al.* 2000, Galagan *et al.* 2005, Dyer 2007, Dyer & O'Gorman 2012). However, it has become apparent that there can be significant divergence at the genome level even within a single fungal genus (Galagan *et al.* 2005). Therefore, the present study was undertaken to assess the phylogenetic distribution of the regulatory pathways of asexual reproduction in a broad taxonomic range of fungi, to gain some indication of their prevalence. In parallel some long-standing questions concerning the control and evolution of sexual development in the aspergilli were addressed. Overall it was found that some features seen in *A. nidulans* indeed appear to be of relevance to a wide biodiversity of fungi. However, some other features are much less conserved, even within the *Eurotiomycetes*, and some hypotheses about the origins of sex and asexuality in the aspergilli appear to be incorrect, as will now be described.

Bioinformatic analysis of asexual development in *Aspergillus*

An *A. nidulans*-centric approach was used to study the phylogenetic distribution and molecular features of known regulators of conidiation from this species. This involved screening for the presence of a series of upstream activators and repressors, central regulators, as well as velvet regulators (Fig. 1B) in 54 fungal species including 16 *Aspergillus* species, related *Ascomycota* and more distant *Basidiomycota* and *Mucoromycota* (Fig. 2). This analysis complements and builds on the findings presented by de Vries *et al.* (2017).

Upstream activators and repressors

With respect to conidiation upregulators, the *A. nidulans* FluG upstream activator protein was found to possess two characteristic domains, a GlnA domain (glutamine synthetase), and a metallo-dependent hydrolase domain, belonging to the amidohydrolase superfamily (Supplementary Fig. 1). Homologues of the *fluG* orthogroup were found in the majority of *Ascomycota*, possibly linked to a role in conidiation as seen in *A. nidulans*. More distant orthologues were also found in the *Basidiomycota*, although not in the *Mucoromycota* (Fig. 2A). Phylogenetic analysis further showed that this orthogroup can be divided into two large groups. One of these encompasses species with proteins that possess only the GlnA domain, with the other containing homologues that have both domains described above. The only exceptions were a subtree encompassing four basidiomycetes and the ascomycete *Penicilium chrysogenum*, in which proteins only contained the metallo-dependent hydrolase domain. These five species possess proteins that are likely to have lost the GlnA domain after the FluG orthologues became separated from the rest of the species in this orthogroup. In some species of the *Pezizomycotina fluG* has been lost, specifically in *Cladosporium, Botrytis, Trichoderma* and *Magnaporthe*. It was already noted by de Vries *et al.* (2017) that almost half of the *Aspergillus* species analysed possess two copies of the *fluG* gene, possibly suggesting more differentiated regulation of development in these species.

Regarding conidiation repressors, the *A. nidulans* SfgA repressor protein also has two specific domains: a Gal4-type $Zn(II)_2Cys_6$ type transcription factor, which consists of two helices organized around the $Zn(II)_2Cys_6$ motif, and a fungal transcription factor regulatory middle homology region, which is present in the large family of fungal zinc cluster transcription factors that contain an N-terminal GAL4-like DNA-binding domain (Supplementary Fig. 2). SfgA was found to be present exclusively in the *Eurotiomycetes*, being conserved in all *Aspergillus* species as well as being present in *Monascus, Histoplasma,* and *Penicillium*, although not in *Talaromyces* species (Fig. 2B). All the other *Pezizomycotina* lack homologues of *sfgA* (*Dothideomycetes, Sordariomycetes, Lecanoromycetes* etc.). By contrast, the NsdD repressor protein (a GATA-type zinc-finger transcription factor) whilst also being present in all the *Eurotiomycetes* had a broader distribution in many other *Ascomycota* (Fig. 2B). Interestingly *A. wentii* and *A. luchuensis* have two paralogs of *nsdD*, which likely appeared independently by gene duplication. Some other members of the *Pezizomycotina*, such as certain *Sordariomycetes and Leotiomycetes*, contain shorter copies of *nsdD*, which are likely homologues of *AnnsdD* as they cluster together (Supplementary Fig. 3). Indeed, deletion of *nsdD* orthologues in *Fusarium* (*csm-1*) and *Botrytis* (*ltf1*) increases conidiation (Schumacher *et al.* 2014, Niehaus *et al.* 2017) as has been reported for *A. nidulans* (Lee *et al.* 2016).

The absence of the *A. nidulans* SfgA and NsdD repressors of conidiation in some other *Ascomycota* indicates that in such groups the induction of conidiation employs a different mechanism than the derepression exerted by the FluG factor as seen in

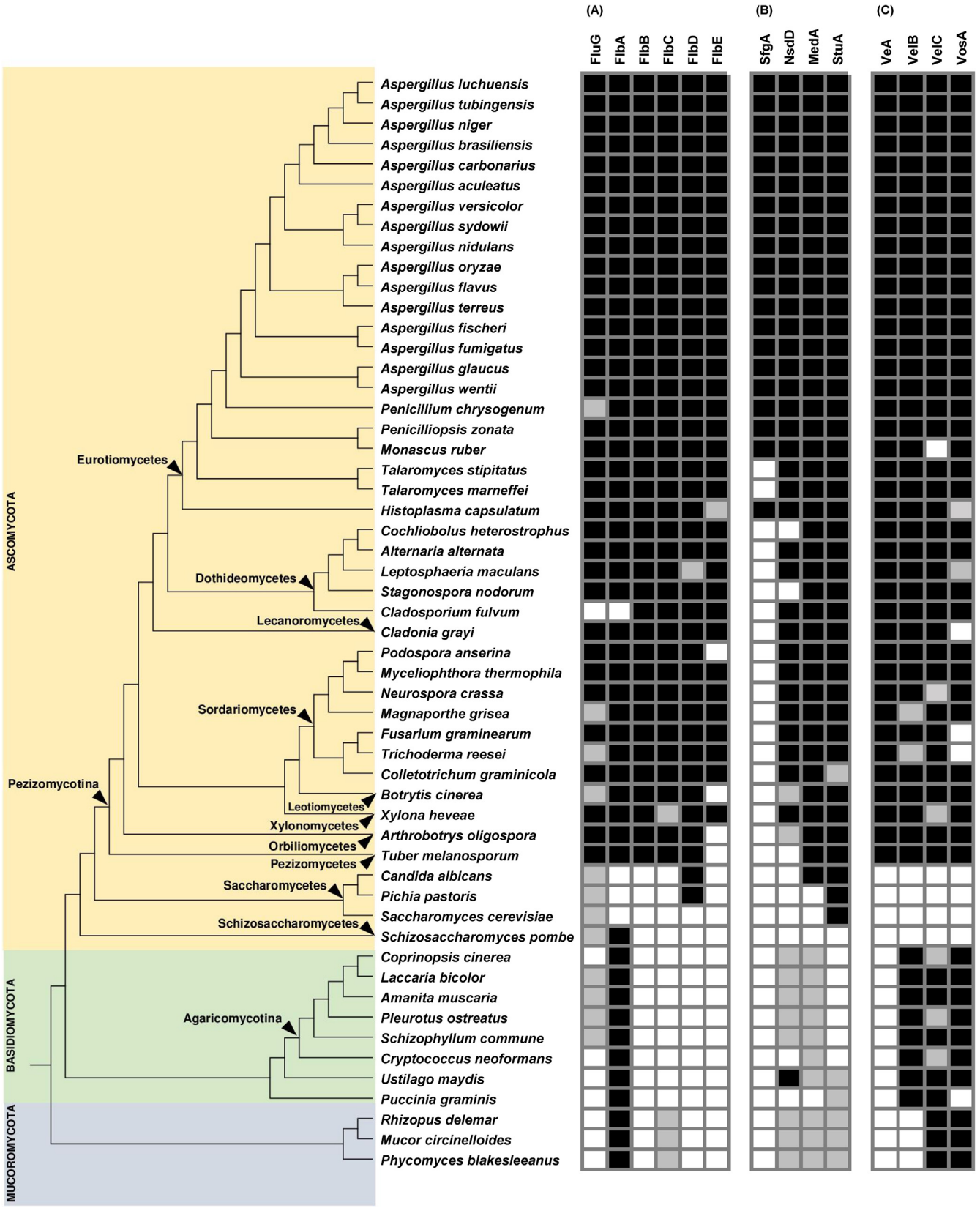


Fig. 2. Distribution of proteins involved in the regulation of conidiation using *A. nidulans* proteins as bait. (A) Fluffy genes involved in the activation of the central developmental pathway of conidiation. (B) Repressors of conidiation and developmental modifiers. (C) Velvet proteins. The phylogenetic tree of species was estimated using Orthofinder. Black squares denote presence; white squares denote absence; and grey squares denote that the presence of proteins that Orthofinder predicts to belong to the orthogroup but they are either truncated with respect to the *A. nidulans* respective homologue or do not fulfill the domain architecture requirements. Absence in the *Ascomycota* clade was confirmed by tblastn searches in the corresponding genomes using the corresponding *A. nidulans* homologue.

A. nidulans. Alternatively, given that in many of these cases a homologue of *fluG* is present in the genome, it is possible that FluG derepression occurs by some other mechanism, or even that FluG directly activates elements of a downstream conidiation pathway. Interestingly *Fusarium* can undergo microconidiation in liquid media under standard growth conditions (López-Berges *et al.* 2013) and contains a *fluG* homologue but not an *sfgA* homologue. Although it could be argued that the absence of *sfgA*

allows fungi to conidiate in liquid media, this is not the case for other *Sordariomycetes* also lacking *sfgA*, such as *N. crassa*, in which induction of conidation requires growth on a solid surface or particular starvation conditions (Berlin & Yanofsky 1985). In this group of organisms, repression of conidiation in *P. chrysogenum* poses an interesting case, as it lacks a complete homologue of *fluG*, but it has homologues of both *sfgA* and *nsdD* repressors. Analysis by tblastn against all the *Penicillium* taxon in NCBI revealed that some *Penicillium* species contain a complete *fluG* homologue, while some other species contain N-terminal or C-terminal truncated versions (data not shown).

The next set of results of the bioinformatic analyses concerned the remaining members of the *fluffy* group of genes, which promote asexual conidiation. FlbA is a regulator of the G-protein signalling (Yu *et al.* 1996). Accordingly, FlbA contains three different domains (Supplementary Fig. 4): two DEP domains that are responsible for mediating intracellular protein targeting and regulation of protein stability in the cell, and a RGS (Regulator of G-protein Signalling) domain that is an essential part of FlbA because it is involved in the cellular signalling events downstream of G-protein coupled receptors (GPCRs). The DEP domain is present in many signalling molecules, including RGS proteins. This pathway signals through a cAMP-PKA, which is broadly distributed in eukaryotes, and therefore it was expected that most fungal species would contain an *flbA* homologue. Indeed, FlbA was found to be highly conserved, appearing in all of the species included in this study with the exception of the *Saccharomycotina*, that have lost one of the domains during their evolution and, surprisingly, *Cladosporium* in which there are no homologues (Fig. 2A). By contrast, *N. crassa* and *F. graminearum* contain two copies of *flbA*. In the phylogenetic tree two subtrees were observed (Supplementary Fig. 4): the first one has species with just two of the three domains, and the second one has members where all three domains are conserved. We assume that this second subtree comprises species with proteins most orthologous to *A. nidulans* FlbA.

FlbC is a C_2H_2 zinc finger transcription factor involved in binding directly to the cis-regulatory element of *brlA* and inducing its expression (Kwon *et al.* 2010). FlbC is included in a very large orthogroup encompassing other C_2H_2 zinc finger transcription factors (*e.g.* BrlA). It was found to be well conserved throughout the *Pezizomycotina*, appearing in almost all the species studied (Fig. 2A). In this orthogroup, it was possible to further differentiate the orthologues of FlbC from paralogous proteins involved in other biological processes thanks to the domain architecture combined with the clustering pattern (Supplementary Fig. 5). According to this strategy, FlbC is present in all species of *Pezizomycotina* (except in *Xilomycetes*) and *Mucoromycota*, however was absent from the *Basidiomycota* and some *Ascomycota* such as the Taphrinomycotina and *Saccharomycotina*. Deletion of *flbC* in *Aspergillus* species and some Sordariomycetes is consistent with a broad role in fungal development. For example, deletion of *flbC* in *Fusarium* resulted in reduced conidiation, whilst in *N. crassa* and *M. oryzae flbC* mutants showed a reduction in aerial hyphae in addition to reduced conidiation levels (Son *et al*. 2014a,b, Malapi-Wight *et al*. 2014, Cao *et al*. 2016, Matheis *et al*. 2017, Boni *et al*. 2018). Overexpression of *flbC* in *A. nidulans* produced abnormal vesicles-like structures at the tips (Kwon *et al*. 2010), which suggests a possible role in the blastic development of the conidiophores.

In the other FluG-dependent pathway in *A. nidulans*, FlbE interacts with FlbB at the fungal tip in a process necessary to activate FlbB (Herrero-Garcia *et al.* 2015), and then FlbB induces FlbD (Fig. 1). FlbB and FlbD form a heterodimer that activates the expression of *brlA* in a cooperative way (Garzia *et al.* 2010). FlbB contains a basic leucine zipper (bZIP) domain of DNA binding (Etxebeste *et al.* 2008). The bZIP structural motif contains a basic region and a leucine zipper, composed of alpha helices with leucine residues 7 amino acids apart, which stabilize dimerization with a parallel leucine zipper domain. Analysis of the FlbB phylogenetic distribution revealed that it is found exclusively in the *Pezizomycotina* (Fig. 2A), and a duplication event is evident that divides the tree into two main subtrees (Supplementary Fig. 6). The upper one contains all the orthologues of *AnflbB*, whilst the lower one contains other bZIP proteins of the *Ascomycota*, which suggests the presence of paralogous proteins, which may have acquired new functions during evolution.

FlbD has been reported to possess a Myb-like DNA-binding domain (Wieser & Adams 1995). Myb DNA binding domains display extraordinary similarity to SANT domains, which are involved in histone tail binding and remodelling of nucleosomes (Boyer *et al.* 2004). Our search for domains using the Cd-search tool against the CDD database showed that FlbD has a SANT domain (Supplementary Fig. 7), which opens the possibility that the role of FlbD is to re-model the chromatin at the *brlA* promoter to allow its expression. The distribution of *flbD* perfectly matches the distribution of *flbB*, with the exception of *Leptosphaeria* (Fig. 2A), which contains two truncated versions clustering together in the *Dothideomycetes* cluster but with different domain architecture, which points to a different role. *Trichoderma* has a truncated version containing only the myb-like DNA binding domain and no additional sequence. Interestingly, despite the perfectly matching *flbB* and *flbD* distribution, deletion of *flbB* in *Fusarium* and *N. crassa* did not show any phenotype in conidiation (Son *et al*. 2014a,b, Carrillo *et al*. 2017). On the other hand, homologues of *flbD* are essential for the development of the conidiophores in *Fusarium* and *Magnaporthe* (Kim *et al.* 2014b, Son *et al*. 2014a, Dong *et al*. 2015, Matheis *et al.* 2017), and for filamentous growth in *Candida* (Homann *et al.* 2009), suggesting that FlbD can also operate without forming a heterodimer with FlbB. In *Aspergillus, flbD* can also function in a *flbB*-independent manner orchestrating the formation of the external tissue (peridium) of the fruiting body (cleistothecia) during sexual development (Arratia-Quijada *et al.* 2012). In addition to the activation of *brlA* expression, which is not present in *Sordariomycetes* (see below), it was reported that FlbB may also be a key factor in the transition from metulae to phialide in *A. nidulans* (Etxebeste *et al*. 2009). *Sordariomycetes* contain phialides but not metulae, which can explain the lack of phenotype of the *flbB* mutants. Although the asexual developmental structures of *N. crassa* are more simple than the *Aspergillus* ones, *N. crassa* displays a complex ontogeny with the formation of blasto-arthrospores during macroconidiation (Cole 1986). Microconidiation in *N. crassa* ressembles more the development of conidiophores in *Aspergillus* (Springer 1993). The *flbD* homologue in *N. crassa* poses another interesting case: it complements the orthlogous mutation of *A. nidulans*, but deletion in *N. crassa* does not show any phenotype (Shen *et al.* 1998) exposing again the differences in the ontogeny between these organisms.

FlbE has no known domains. In general, the distribution of *flbE* also matches the distribution of *flbB* and *flbD*, with the exception of *Podospora*. However, *flbE* seems to be absent from

the *Leotiomycetes, Orbiliomycetes and Pezizomycetes* examined (Fig. 2A). Further analysis showed that *Botrytis* has a homologue with low homology in the N-terminal part, and *Cladonia* has two putative copies (one of them shorter). The absence of *flbE* in some taxa suggest that in these cases, FlbB must be activated in a different way than in *A. nidulans*, whether this still happens at the tip or not remains unknown.

Regarding the developmental modifiers in *A. nidulans*, StuA contains a basic helix-loop-helix (bHLH)-like structure of the APSES domain (Dutton *et al.* 1997). Members of this family participate in developmental processes and cell cycle progression. A StuA homologue was present in all the *Pezizomycotina* and possible orthologues were detected in the *Mucoromycota*, which also showed an expansion of the number of copies. However, StuA was generally absent in the *Basidiomycota*, except for possible retention in *Ustilago* (Fig. 2B and Supplementary Fig. 8). The domain architecture is very diverse in this orthogroup. Some members have a Sec23_BS domain (sandwich domain) characteristic of SNARE proteins. Some others contain PAT1 domain (topoisomerase II-associated protein), required for accurate chromosomal transmission in yeast. In the *Saccharomycotina* two *stuA* homologues of the APSES family were found. In yeast these homologues (PHD1 and SOK2) are involved in pseudohyphal growth, a process with some similarities to the formation of sterigmata cells in the aspergilli, and in *Candida* EFG1 is involved in hyphal growth and the white-phase cell type (Stoldt *et al*. 1997). The fission yeast *Schizosaccharomyces pombe* has two APSES proteins involved in cell cycle (Zhu *et al*. 1997), which are non-ortholgous to the developmental APSES regulators of other fungi developing more complex structures. Although the deletion of *stuA* homologues results in a decreased conidiation in *Fusarium, Magnaporthe, Aspergillus, Talaromyces* and *Neurospora*, the morphological defects are different. In *Magnaporthe* and *Fusarium stuA* seems to be involved in the development of the conidiophore. Macroconidia are produced from intercalarly phialides, rather than from the conidiophore in *Fusarium*. No difference in morphology was observed between the mutant and the wild type in *Magnaporthe* (Ohara & Tsuge 2004, Nishimura *et al*. 2009). In *Aspergillus* and *Talaromyces stuA* mutants showed shorter stalks and absence of metulae and phialides. In this case, a few conidia arise directly from the stalk (Miller *et al*. 1992, Borneman *et al*. 2002). Borneman *et al*. (1992) suggested that StuA controls developmental processes requiring budding, which is in agreement with the non-filamentous phenotype of the deletion mutant of *Ustilago* (García-Pedrajas *et al*. 2010) and matches the blastic development of the conidiophores in these fungi. In the case of *N. crassa*, the deletion mutant was not characterized in depth with respect to conidiation. The mutant showed a stunted appearance, conidiating close to the agar surface (Aramayo *et al*. 1996). If StuA truly orchestrates blastic development, then residual conidiation of *N. crassa* could arise during the secondary arthric development of macroconidia. A deeper characterization of the mutant phenotype is required.

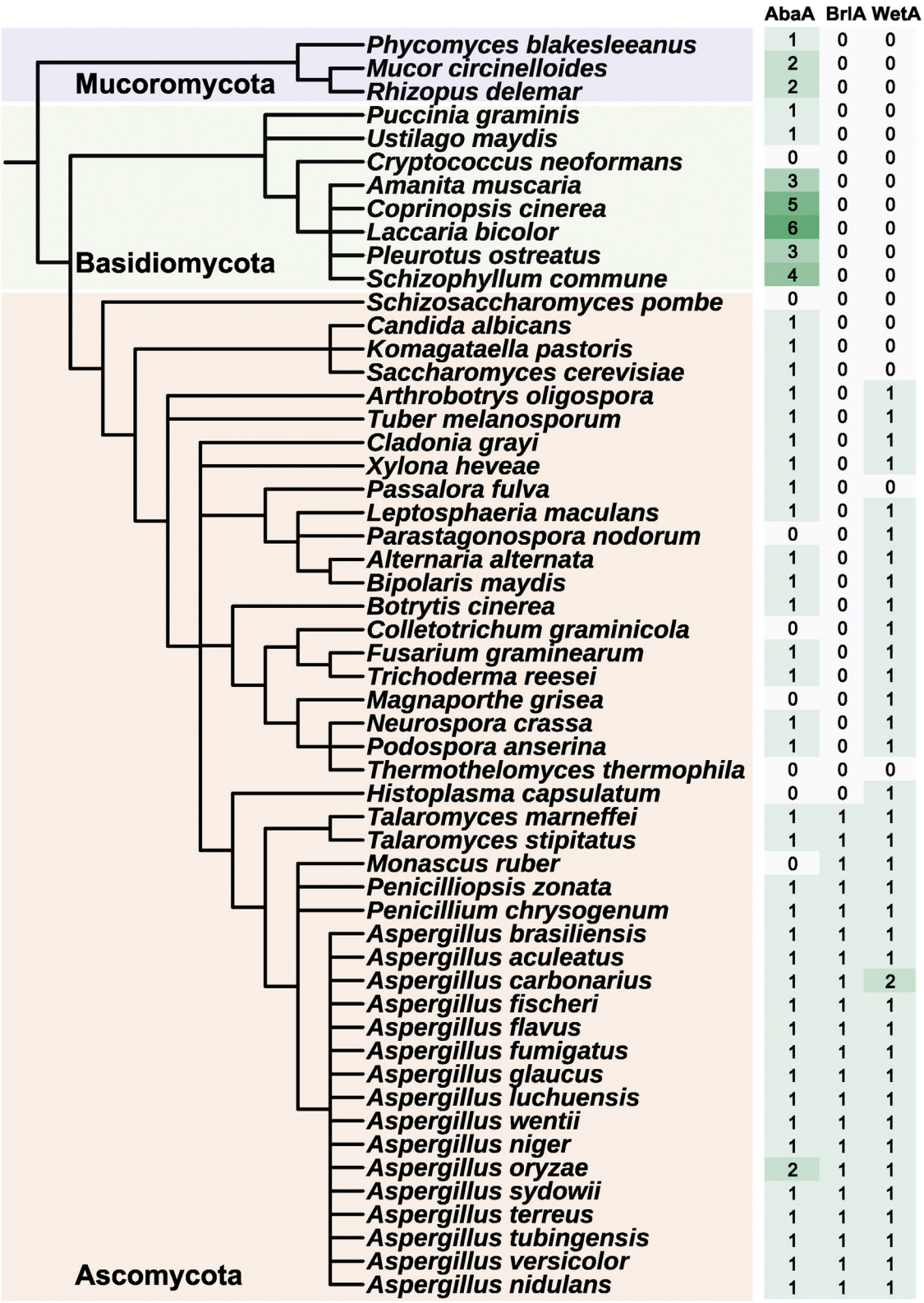
The final developmental modifier MedA, showed a taxonomic distribution similar to *stuA* (Fig. 2B). The *medA* homologues of the *Pezizomycotina* seemed to form a distinct cluster (Supplementary Fig. 9), which does not clarify whether homologues in the *Zygomycotina* and some *Basidiomycotina* have a similar role to that seen in *A. nidulans*. Furthermore, *medA* mutants show different phenotypes depending on the species. In all cases, the levels of conidiation are affected. Whereas in *A. nidulans*, deletion of *medA* produces conidiophores with multiple layers of sterigmata cells (metulae and phialides), in *A. fumigatus* the medusoid aspect was not observed, although mutants still produced a few conidia (Gems & Clutterbuck 1994, Gravelat et al. 2010). In *Fusarium* and *Magnaporthe*, the deletion of *medA* produced a switch to acropetal conidiation with aberrant conidiophores (Lau & Hamer 1998, Ohara *et al*. 2004). In *N. crassa* deletion of the *medA* homologue, *acon-3*, blocked the budding process resulting in major hyphal constrictions (Springer 1993). In contrast, in *U. maydis medA* mutants grew normally by budding but were incapable of forming conjugation tubes and filamentation (Chacko & Gold 2012). Taken together, it appears that *medA* homologues contribute to coordinate the switch between filamentous elongation and budding division, but have contrasting roles depending on the species. In agreement with this, monomorphic yeasts (such as *S. cerevisiae* and *S. pombe*) lack a *medA* homologue whereas fungi belonging to other taxonomic groups, that are capable of developing complex reproductive structures, contain an orthologue.

Central regulatory pathway

BrlA, AbaA and WetA have been identified as the central regulators for asexual development in *A. nidulans* (Adams *et al.* 1998, Park & Yu 2012). These transcription factors regulate mRNA expression of genes associated with initiation, elongation, and termination of conidiation (Park & Yu 2012). The bioinformatic analysis reported in de Vries *et al.* (2017) looked especially at the occurrence of the central regulatory pathway (*brlA* → *abaA* → *wetA*) in the *Eurotiomycete* genomes under investigation. It was found that whereas only one or two elements of the pathway were present in the *Ascomycota* in general, that all elements of the pathway were present as a central conserved feature in all *Aspergillus, Penicillium* and *Talaromyces* species examined. This bioinformatic analysis was extended in the present study to include further outgroup species and also an examination of motifs present in the central regulatory proteins.

It was again found that the central regulatory genes *brlA, abaA* and *wetA* are highly conserved in *Aspergillus* species, and also that conserved DNA binding motifs are present in these central regulatory proteins (Fig. 3A). For example, BrlA orthologues were found to be highly conserved in *Aspergillus* and *Penicillium* species and other *Eurotiomycetes*. However, BrlA was absent from all other *Ascomycota*, *Basidiomycota* and *Mucoromycota* (Fig. 3A). This suggests that BrlA plays a role in the initiation of conidiation specifically in Eurotiales fungi. BrlA orthologues contain a fungal specific C_2H_2 domain for DNA binding activity (Adams *et al.* 1988). By contrast, AbaA orthologues could be found in most fungi including both filamentous and yeast-like members of the *Ascomycota*, suggesting that AbaA is not only involved in conidiophore development but also might have other general functions for fungal morphogenesis. Interestingly, several *Basidiomycota* such as *Schizophyllum commune*, *Laccaria bicolor*, and *Coprinopsis cinerea* were found to contain more than one AbaA orthologue, whereas *A. oryzae* was the only member of the aspergilli to contain two AbaA homologues. It was also confirmed that *Monascus ruber*, a close relative of the aspergilli, lacks an AbaA homologue correlating with a change in conidiation morphology (Hawksworth & Pitt 1983, Wong & Chien 1986). AbaA orthologues were found to contain a TEA domain with a DNA binding motif (Burglin 1991, Andrianopoulos & Timberlake 1994). TEA domains contain three alpha-helices, two helices with possible DNA binding

A


AbaA BrlA WetA
Mucoromycota
Basidiomycota
Ascomycota
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Puccinia graminis 1 0 0
Ustilago maydis 1 0 0
Cryptococcus neoformans 0 0 0
Amanita muscaria 3 0 0
Coprinopsis cinerea 5 0 0
Laccaria bicolor 6 0 0
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Schizophyllum commune 4 0 0
Schizosaccharomyces pombe 0 0 0
Candida albicans 1 0 0
Komagataella pastoris 1 0 0
Saccharomyces cerevisiae 1 0 0
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Tuber melanosporum 1 0 1
Cladonia grayi 1 0 1
Xylona heveae 1 0 1
Passalora fulva 1 0 0
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Parastagonospora nodorum 0 0 1
Alternaria alternata 1 0 1
Bipolaris maydis 1 0 1
Botrytis cinerea 1 0 1
Colletotrichum graminicola 0 0 1
Fusarium graminearum 1 0 1
Trichoderma reesei 1 0 1
Magnaporthe grisea 0 0 1
Neurospora crassa 1 0 1
Podospora anserina 1 0 1
Thermothelomyces thermophila 0 0 0
Histoplasma capsulatum 0 0 1
Talaromyces marneffei 1 1 1
Talaromyces stipitatus 1 1 1
Monascus ruber 0 1 1
Penicilliopsis zonata 1 1 1
Penicillium chrysogenum 1 1 1
Aspergillus brasiliensis 1 1 1
Aspergillus aculeatus 1 1 1
Aspergillus carbonarius 1 1 2
Aspergillus fischeri 1 1 1
Aspergillus flavus 1 1 1
Aspergillus fumigatus 1 1 1
Aspergillus glaucus 1 1 1
Aspergillus luchuensis 1 1 1
Aspergillus wentii 1 1 1
Aspergillus niger 1 1 1
Aspergillus oryzae 2 1 1
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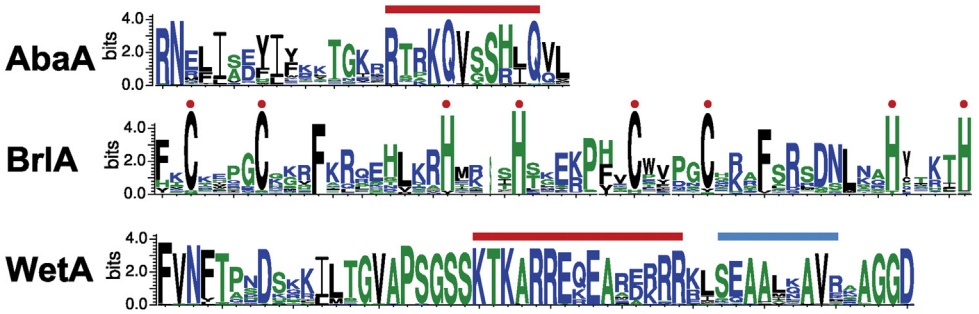
Aspergillus terreus 1 1 1
Aspergillus tubingensis 1 1 1
Aspergillus versicolor 1 1 1
Aspergillus nidulans 1 1 1

B


AbaA
BrlA
WetA
bits
4.0
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activity being in the N-terminus of the domain and whose sequences are quite diverse in fungi. The remaining helix is highly conserved in most fungi, and is thought to be a nuclear localisation signal (Fig. 3B, with the conserved RTRKQVSSHLQ sequence shown by a red bar). WetA orthologues were also present throughout the *Pezizomycotina*. However, *Saccharomycetes*, including *Candida albicans*, *Pichia pastoris*, and *Saccharomyces cerevisiae* did not contain WetA orthologues (Fig. 3A). WetA orthologues were found to contain a conserved ESC1/WetA-related domain containing a putative 16 amino acid nuclear localization signal and a 9 amino acid transcription activation domain (shown by red and blue bars, respectively, in Fig. 3B) (Marshall & Timberlake 1991, Son *et al*. 2014a,b, Wu *et al.* 2017).

Thus, the central regulatory pathway from *A. nidulans* appears to be a defining feature of the aspergilli as a whole and it appears likely that the pathway functions in a similar fashion throughout the genus. AbaA and WetA elements of the pathway are also more widely present in the fungal kingdom where it can be speculated that they are also involved in developmental processes such as sporulation, although this awaits experimental confirmation.

Velvet regulatory proteins: phylogenetic distribution and expansion/contraction of the velvet family

Although the velvet proteins have been mainly characterized in *Aspergillus*, they were found to be present across several different fungal taxa (Fig. 2C). In the aspergilli, all species included in this study contained one copy of *veA*, *velB*, *velC* and *vosA*, with the exception of *A. flavus and A. oryzae*, which contain a duplication of *vosA* (Supplementary Figs 10 & 11). It is rather interesting that *T. marneffei*, *T. stipitatus* and *P. zonata* also have duplications of *vosA*. There are two possible explanations: an early duplication of *vosA* in the *Eurotiomycetes* followed by lost of one of the paralogues in those species that only contain one copy. The second possibility is that independent duplications have led to the *vosA* paralogues found in these species. This second possibility appears to be more parsimonious due to the following observations. The duplication of *vosA* seen in *A. flavus* and *A. oryzae* is not present in the closely related species *A. terreus*, which suggests that the duplication occurred after the separation of the *A. terreus* and *A. flavus/A. oryzae* clades. The presence of two copies in *A. bombycis* and *A. nomius*, which form a monophyletic group together with *A. flavus* and *A. oryzae*, but not in *A. brevijanus* and *A. terreus* confirms this hypothesis. Indeed, the *velC* genes from *A. flavus* and *A. oryzae* appear to be separated from the rest of the aspergilli *velC* homologues (Supplementary Fig. 11). Both *T. marneffei* and *T. stipitatus* contain *vosA* paralogues that cluster according to the species, which points to a duplication event occurring independently after the separation of both species. This is further supported by observations of single *vosA* copies in the other three *Talaromyces* species available at the Mycocosm site. In particular *T. aculeatus* is in the same monophyletic group with *T. marneffei* but not with *T. stipitatus*, supporting this hypothesis. Taking all these observations above together, it suggests that for unknown reasons *vosA* has a higher tendency for gene expansions than the other velvet proteins in the *Eurotiomycetes*. Indeed, using the blastp search tool against the *Eurotiomycetes* database at the JGI website, we found two independent duplications of *veA* in *Penicillium*, no duplications of *velB*, five independent duplications of *velC* (two in *Aspergillus* and three in *Penicillium*) and 6-7 independent duplications of *vosA* (two in *Aspergillus*, one in *Penicilliopsis*, two in *Talaromyces*, and 1-2 in *Paecilomyces*).

Velvet proteins are specific to fungi (Bayram & Braus 2012) and seem to be widely distributed in this kingdom as they can be found in all the *Eurotiomycete* species included in this study (Fig. 2C). In order to study in further detail the distribution and evolution of the velvet proteins, we also included early divergent fungi in the analysis, using the velvet domain of AnVeA as a bait to search for homologues in Mycocosm (Grigoriev *et al.* 2014), and selected all the homologues found from two random species of each fungal phyla (except for the *Ascomycota*, where the model fungi *A. nidulans* and *N. crassa* were purposefully selected and those in which only one species is available in the Mycocosm database) (Fig. 4). Our initial searches and further interrogation using FungiDB (Stajich *et al.* 2012) could not identify velvet homologues in the 20 species belonging to six different genera of the oomycetes deposited in the databases. Similarly, no homologues could be found in the six species belonging to four different genera of the *Microsporidia*. However, two velvet proteins were found in the only species of *Cryptomycota* available on the JGI database, suggesting that either *Microsporidia* lost their velvet genes or the *Cryptomycota* have acquired these genes. The two homologues in *Cryptomycota* are short proteins of 239 and 247 amino acids displaying low similarity between each other (29 % identity and 46 % positives in 205 amino acids according to the blast search), and both contain the velvet domain encompassing most of the protein length. These two copies lie in separate clades in the tree and show a basal location in the branches in agreement with the presumed evolutionary history of the *Cryptomycota*. One of the clades contains the *Cryptomycota* velvet protein 1114 and the *veA* and *vosA* homologues of *A. nidulans* and *N. crassa* (Fig. 4). Homologues in the *vosA* clade appeared relatively early (in *Blastocladiomycota*) but seem to be absent in many basal phyla. The *vosA* and *veA* clades form a monophyletic group suggesting that *vosA* may have evolved from *veA*. Indeed the domain structure of VeA and VosA shares the N-terminal localization of the velvet domain, which is different to the domain organisation seen in VelB and VelC (Bayram & Braus, 2012). The clade containing the other *Cryptomycota* velvet protein (2092) is not well resolved in the tree and contain subtrees with non-characterized velvet homologues corresponding to basal fungi and basidiomycetes, and another subtree with the *velB* homologues of *A. nidulans* and *N. crassa*. Homologues in the *velB* clade appear later in Zoopagomycotina. The *velC*-like homologues encompass a non-monophyletic group of genes that are not well resolved in the tree. Supplementary Fig. 11 also shows a paraphyletic group of the *velC*-like homologues in the 54 fungal species under analysis, in which the homologues in the *Mucoromycota*, *Basidiomycota* and the rest of the *Pezizomycotina* that do not belong to the *Eurotiomycetes* form a separate group. *A. nidulans velC* is located in the base of the so-called unassigned velvets and the *velB* clade, which makes it difficult to draw conclusions. Available

Fig. 3. Orthogroup containing the BrlA, AbaA, and WetA proteins. (A) Distribution of the BrlA, AbaA, and WetA proteins for asexual sporulation across different fungal taxa. (B) Sequence logos of the DNA binding motifs of AbaA, BrlA, and WetA from the fungi examined. Red bars indicate nuclear localization signals (NLS) and the blue bar is the transcription activation domain. Amino acids indicated with red spots are associated with DNA binding activity.

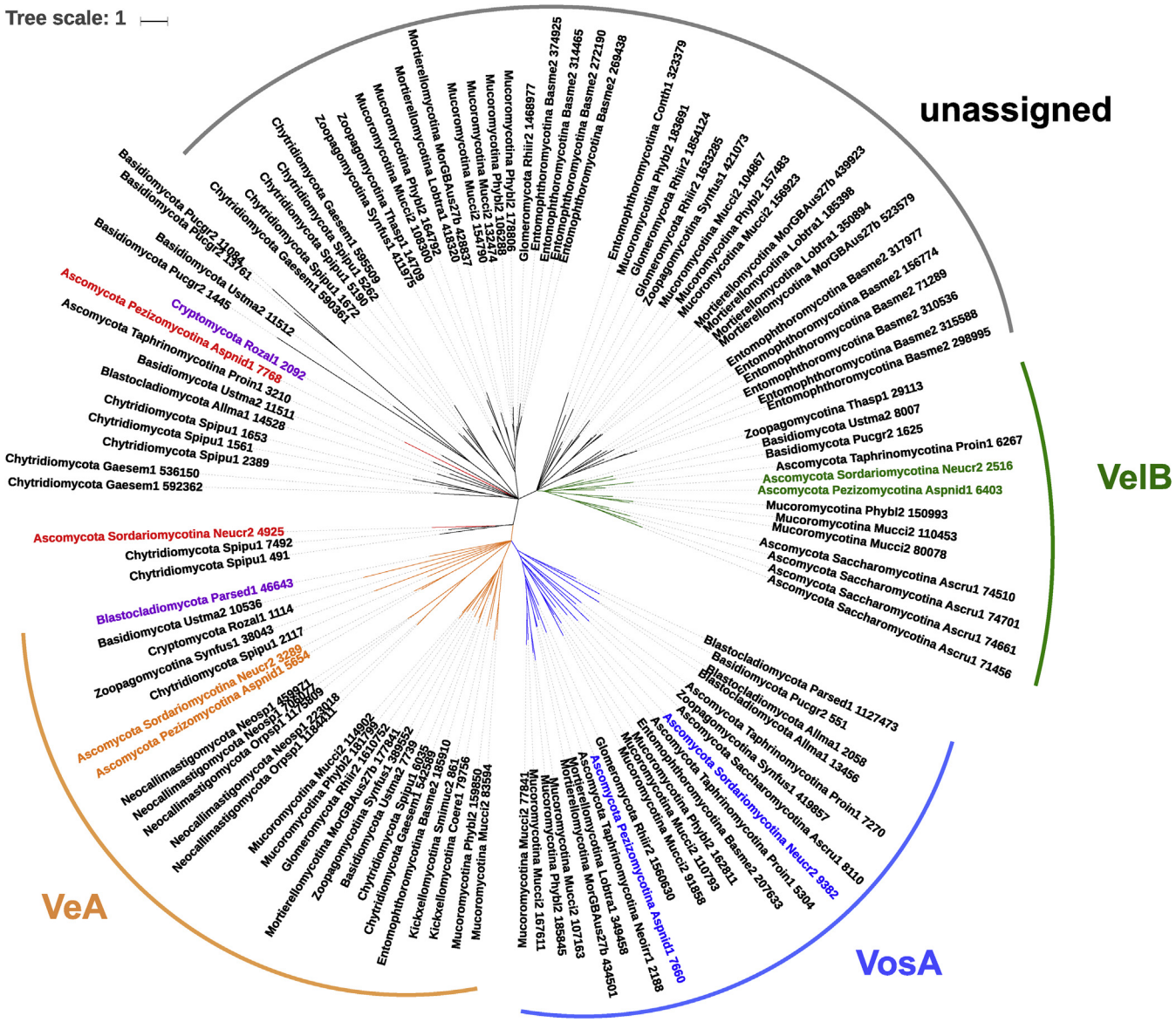

Fig. 4. Phylogenetic tree of the velvet proteins in fungi. The proteins of *A. nidulans* and *N. crassa* are coloured as follows: VeA, orange; VelB, green; VelC, red; VosA, blue. The branches of the clades containing those homologues are coloured accordingly. Branches and proteins from the basal fungus of the *Cryptomycota* phyla are coloured in purple. Black branches represent clades not assigned to any of the above homologues. Branches with bootstrap values lower than 0.8 have been collapsed and are not resolved in the tree.

data do not help either to predict a general function for them. For example, deletion of *velC* does not have any observable phenotype in *N. crassa* (manuscript in preparation), but it shows a decrease in conidiation, and affects appressoria and plant penetration in *M. oryzae* (Kim *et al.* 2014a). By contrast, deletion of *velC* in *A. nidulans* produced increased conidiation and reduced number of cleistothecia (Park *et al.* 2014). In the absence of more genomic sequences of the early divergent fungi of the *Cryptomycota* and *Blastocladiomycota* phyla, and homologues in the *Microsporidia*, it seems that the *Cryptomycota* homologue in this clade could be the closest form to the origin of the *velB/C* homologues. The tree shows an increasing expansion of the velvet family from *Cryptomycota* up to the *Mucoromycota* and then a contraction in the *Basidiomycota* and *Ascomycota*.

Overview of asexual development

Taking the results of the bioinformatic analyses above as a whole, a few key observations can be made. Firstly, the fact that many components of the asexual developmental pathway of *A. nidulans* are absent from members of the *Saccharomycotina* supports the hypothesis that the difference in their cellular complexity is due in part to the increased diversity in the sporulation machinery seen in the *Pezizomycotina* (Lengeler *et al.* 2000). Thus, many species in the *Saccharomycotina* are unicellular microorganisms, incapable of developing complex multicellular structures (such as conidiophores) or are only able to develop rudimentary ones (*e.g.* pseudohyphal growth of *S. cerevisiae*) (Gancedo 2001, Sudbery 2011). Upstream regulators of conidiation in the aspergilli are also missing from the *Basidiomycotina*, many of which undergo only sexual reproduction as part of their life cycle. Secondly, the model derived for asexual development in *A. nidulans* seems generally applicable to the aspergilli and most *Eurotiomycete* species, based at least on conservation of the regulatory proteins. Whereas in other members of the *Pezizomycotina*, homologues of the *Aspergillus* regulators of conidiation seem to be generally conserved, but perform somewhat different biological roles to accomplish the diverse ontogeny observed in this fungal group. Finally, based on

present knowledge it appears that the activation of sporulation seems to be more complex in the aspergilli than in some other taxonomic groupings of the *Pezizomycotina*, where one or more repressors of conidiation seem to be absent. Considering the evolution of asexual reproduction in the aspergilli, at least two possibilities seem possible. There might have been an acquisition of increased genetic complexity leading to the extant developmental program seen in the aspergilli, and/or the existence of convergent but different genetic strategies to control the onset of sporulation in other taxa. Linked to this, *Monascus* provides a particularly interesting example as it was found to contain all the upstream genetic regulatory machinery, including the conidiation suppressors and activators of *A. nidulans* (Fig. 2). It also has homologues for *brlA* and *wetA* of the central developmental pathway (Figs 1B & 3). However, critically it lacks the middle genetic element *abaA*, which is responsible for the differentiation of the phialides (Sewall *et al.* 1990). This led to the following section of experimental work.

Genetic manipulation of *abaA* in *Monascus ruber*

Members of the genus *Monascus* are used in the production of Asian foodstuffs and are phylogentically very closely related to the aspergilli (Chen *et al.* 2015, 2017a,b). However, *Monascus* has a distinct morphology regarding the development of asexual conidia. Asexual spores are produced either direct from hyphae or produced laterally on short pedicels either singly or in short chains (Hawksworth & Pitt 1983, Wong & Chien 1986). The bioinformatic analyses of de Vries *et al.* (2017) found that the genome of *M. ruber* contains all the standard genetic regulatory machinery for conidial production seen in the aspergilli. However, it lacks *abaA* from the central developmental pathway, which is responsible for the differentiation of the phialides in *A. nidulans* (Sewall *et al.* 1990). The conidiophore in *M. ruber* M7 can be likened to a single string of an abacus on the vesicle, whereas that in *A. nidulans* resembles several strings of an abacus emerging from a swollen vesicle and the phialides *i.e.* the conidiophore of *M. ruber* differs in that it lacks the production of a swollen vesicle and the metulae and phialides seen in *A. nidulans*. It was therefore tempting to speculate that the presence or absence of the *abaA* gene might be a significant contributory factor to the difference in conidiation form between *Monascus* and the aspergilli. We therefore examined whether heterologous expression of *A. nidulans abaA* in *M. ruber* might lead to a change in conidiation form, perhaps similar to that seen elsewhere in the *Eurotiales*.

A total of 21 *M. ruber* transformant strains were obtained in which expression of *A. nidulans abaA* was confirmed by PCR, cDNA sequencing and Southern blotting (Supplementary Figs 13 & 14). Nine were found to have one copy, ten possessed two copies, and two contained three copies of *abaA*. Among these, two strains (1 and 22) with one copy of *abaA*, two strains (3 and 8) with two copies, and two strains (11 and 14) with three gene copies were selected for further investigation. In order to verify the relative expression level of *abaA* in the selected mutants, quantitative real-time RT-PCR was performed. Results showed that the relative expression level was positively correlated with *abaA* gene copy number (Supplementary Fig. 15). The conidial morphology was then examined. This revealed that among the six *abaA* expression strains, most conidiophores were similar to the M7 parent, with no obvious change in micro-morphology or colony macro-morphology, although exceptionally a small number of conidiophores were observed in which one to three conidia were born in two-three-way branches at the top of vesicles (Fig. 5; Supplementary Fig. 16). By contrast, conidial counts showed that production of conidia was significantly increased in some mutants, which was positively correlated with *abaA* gene copy number, compared to the parental WT (Fig. 6). There was also evidence of earlier germination rates and increased resistance of spores to external stressors in the *abaA* expression strains, as well as changes in the proteome as a result of *abaA* expression (Supplementary Figs 17–19).

In conclusion, the heterologous expression of *abaA* in *M. ruber* had some effect on conidial formation, but it failed to lead to a branching conidiophore form as seen in *Aspergillus* or *Penicillium*. Given that AbaA is present in many other members of the *Pezizomycotina* (Fig. 3) it seems that most likely that gene loss has occurred in the ancestor of *Monascus* that diverged from an ancestor of the aspergilli, and that *Monascus* species have then adapted the regulation of the central pathway accordingly. Further evidence for this hypothesis is that the *Monascus* genome includes *brlA*, which otherwise only has a narrow distribution in the *Eurotiales*.

Evolution of sexual breeding systems in *Aspergillus*

There has been longstanding interest in the evolution and control of sexual reproduction in the fungal kingdom since the earliest reports of different sexes and self-fertility in fungi by Blakeslee in the early 1900s, who introduced the terms homothallism and heterothallism (Houbraken & Dyer 2015). Since then the study of fungal sex has been used to gain insights into the evolution of sex and transitions between self-fertility and cross-fertility that occur throughout the eukaryotic tree of life (Lee *et al.* 2010, Heitman *et al.* 2013, Heitman 2015).

Given that both homothallic and heterothallic breeding systems are widespread in the fungal kingdom, one particular question that has arisen and long-been debated in fungal biology is whether homothallism or heterothallism might be the ancestral sexual state (*e.g.* Whitehouse 1949, Metzenberg & Glass 1990). This is both of fundamental interest, but also has practical ramifications for the exploitation of fungal sex for breeding purposes (Ashton & Dyer 2016). It has been argued that given the long time scales and vast evolutionary distances separating extant species from common evolutionary ancestors, that at best any features of present day sex will be derived. Despite this, it is suggested that the original form of sexual reproduction may have been unisexual (unifactoral), with sexes superimposed as a later feature (Nieuwenhuis *et al.* 2013, Heitman 2015). In practice then, models for the evolution of sexual breeding system might be at best, and most reliably, applied with any certainty to related groups of extant taxa. It is also noteworthy that investigations into the evolution of sex in fungi have been greatly assisted over the past 20 years by the molecular identification of mating-type loci, which have been found to be responsible for transitions in modes of sexual reproduction (Heitman *et al.* 2013, Dyer *et al.* 2016).

In the case of the *Pezizomycotina*, different models for the evolution of sexual breeding systems were proposed in the 1990s. The fact that the vast majority of known *Aspergillus* sexual species are homothallic, combined with phylogenetic

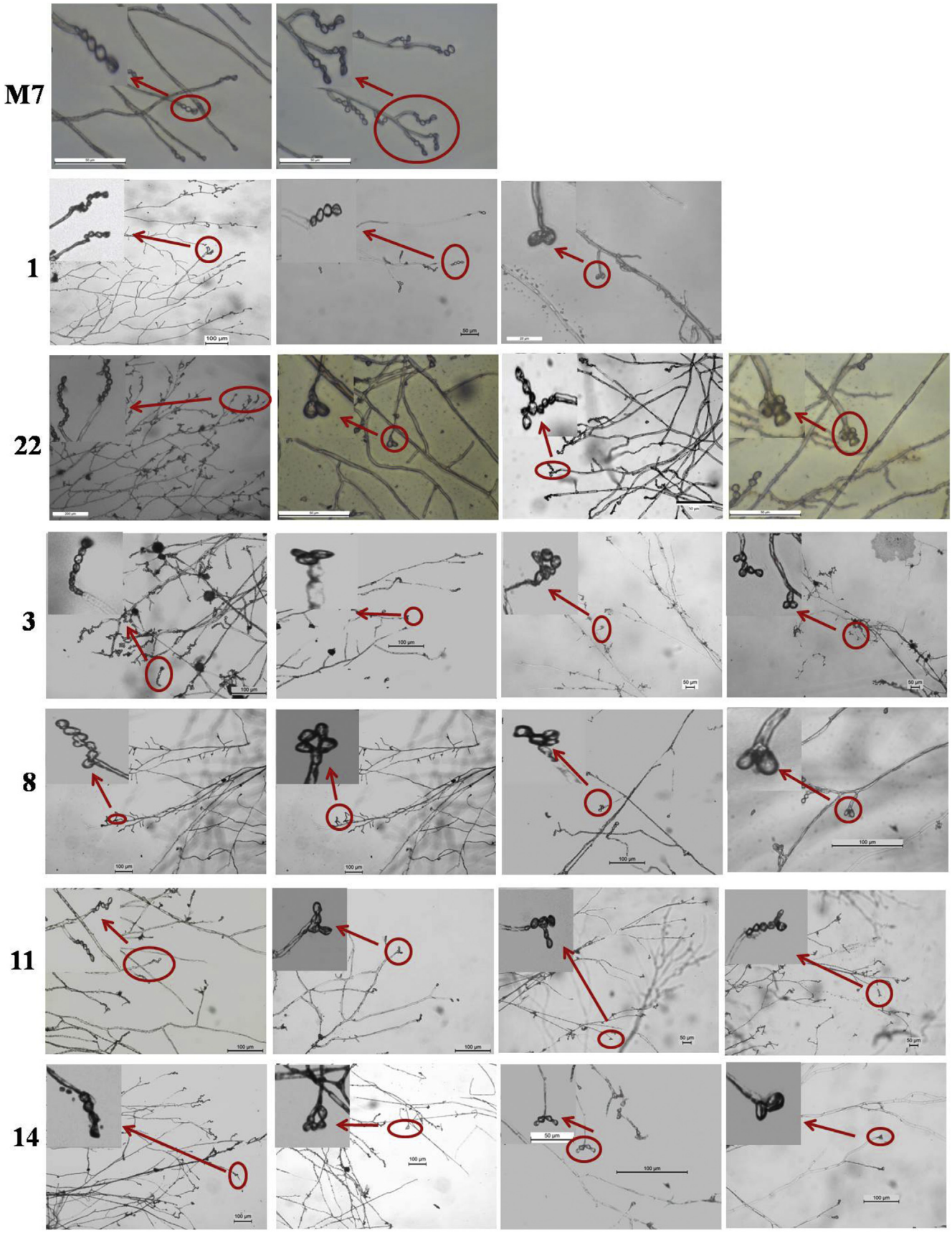

Fig. 5. Conidial morphology of wide-type *M. ruber* M7 (two images) and transformants (1, 22, 3, 8, 11, 14) (three images) expressing one to three copies of the *A. nidulans abaA* gene. Conidiophores are circled in red, with magnified images shown by the inset arrow. Strains were incubated on PDA (potato dextrose agar) medium at 28 °C, with pre-sterilized glass microscope oblique cutting in the medium. After 4 d, coverslips were taken to observe the conidia morphology under the microscope. Scale bars as indicated.

reconstruction analysis, led Geiser *et al.* (1996, 1998) to propose that this group was derived from a homothallic ancestor. This contrasted with evidence from *Cochliobolus* species that evolution of homothallism from a heterothallic ancestral strategy was more likely (Yun *et al.* 1999). This was based on the observation that whereas heterothallic species from the genus exhibited a consistent, conserved arrangement of mating-type genes at the *MAT* locus, that homothallic species instead had a variable

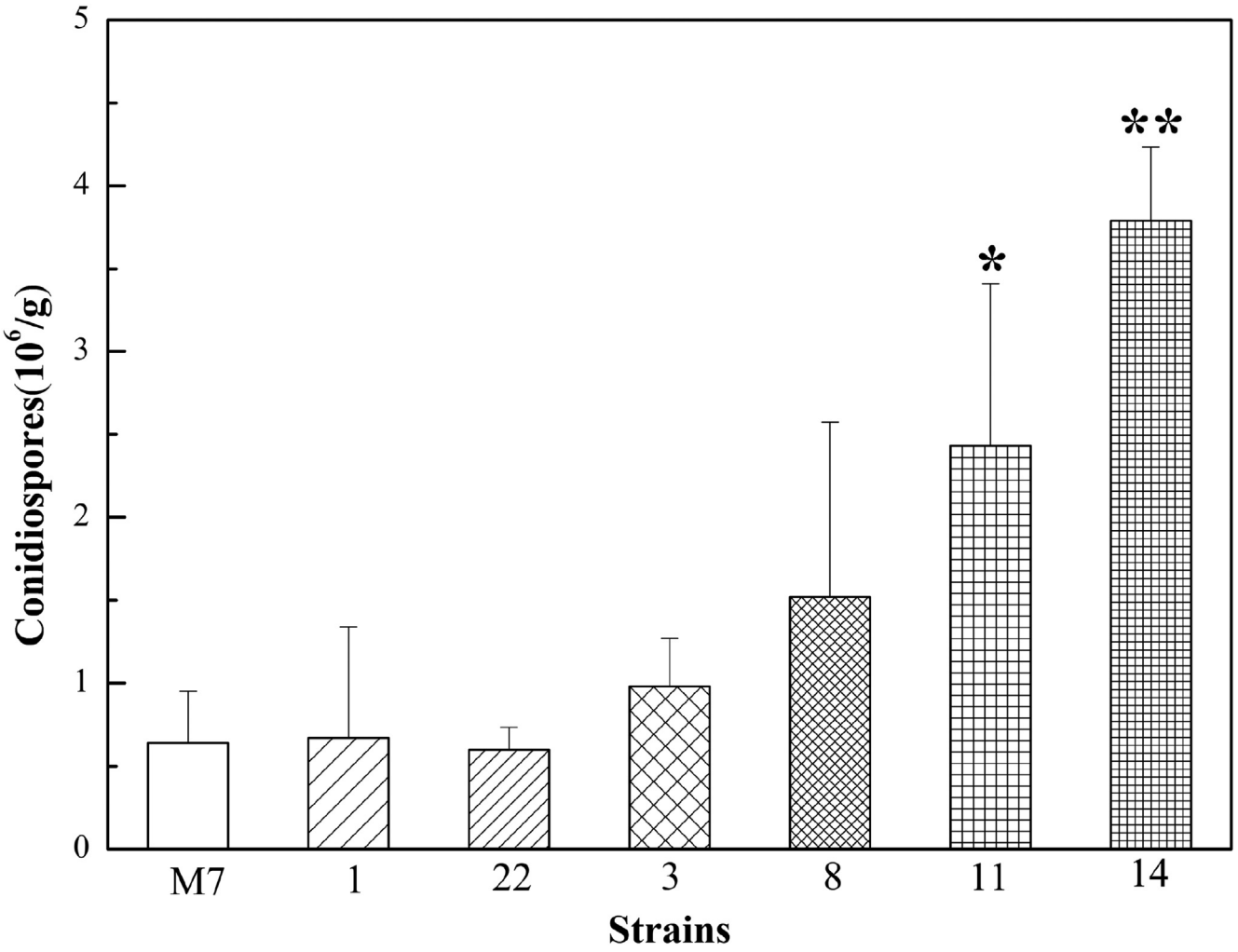

Fig. 6. Comparison of conidia production between *M. ruber* isolate M7 and transformant strains expressing the *A. nidulans abaA* gene. Strains were cultivated on PDA plates for 10 d before harvesting and counting of conidia. Error bars represent SD. ANOVA analysis of conidial counts was performed, with statistically significant differences between *M. ruber* isolate M7 and the transformant strains indicated: * represents $p < 0.05$ and ** represents $p < 0.001$.

arrangement of *MAT* genes both in terms of gene arrangement, order and orientation. It was therefore argued that the most parsimonious explanation was that homothallic species arose independently from heterothallic ancestors sharing a common *MAT* locus structure, accounting for the subsequent variation in homothallic *MAT* locus arrangement but consistent heterothallic *MAT* arrangement (Yun *et al.* 1999). There was also further evidence of sequential *MAT* gene insertions conferring homothallism in some species. It was later suggested that heterothallism is also the most likely ancestral mating state of members of the genus *Stemphylium*, which is closely related to *Cochliobolus* (Inderbitzin *et al.* 2005). It was hypothesized that homothallic members had arisen by an inversion and fusion event of an ancestral heterothallic *MAT* loci. In parallel it has been suggested that the ancestor of all extant ascomycete yeast species may have had a heterothallic mating strategy (Butler 2007).

Therefore the genus *Aspergillus* seemed to be the exception in having arisen from a homothallic ancestor. This apparent anomaly was investigated in the bioinformatic analysis of de Vries *et al.* (2017), where it was found that all of the presumed asexual species were found to contain either a *MAT1-1-1* or *MAT1-2-1* mating-type gene, consistent with the presence of either *MAT1-1* or *MAT1-2* idiomorphs. Adjacent gene synteny was also conserved across all species, again consistent with heterothallism (Dyer *et al.* 2016). This indicated that heterothallism might be widespread in the aspergilli, bringing into question the supposed homothallic origins of the genus. In the present study, *MAT* loci were therefore experimentally cloned from a further series of representative heterothallic and homothallic *Aspergillus* species, to determine whether observations of *MAT* locus structure could provide a more definitive insight following the approach of Yun *et al.* (1999).

Identification of MAT loci from A. heterothallicus, A. fennelliae and A. pseudoglaucus

In the case of the heterothallic *A. heterothallicus,* putative *MAT-1-1*, *MAT1-2-1, SLA2* and *APN2* gene fragments were successfully amplified using PCR with degenerate primers. Utilising the bridging strategy, it was then possible to amplify an entire *MAT1-1* idiomorph region from isolate 50-5 containing a putative *MAT1-1-1* gene, flanked by the *SLA2* and *APN2* genes (Fig. 7). Sequence analysis of the region revealed the presence of a 1 139 bp open reading frame (ORF), including one putative intron, which was predicted to encode a 362 amino acid MAT1-1-1 protein with a characteristic alpha-box domain (GenBank accession MH401192). Analysis of the putative MAT1-1 protein revealed no clear nuclear targeting signals. Similarly, the bridging strategy was also used to amplify an entire *MAT1-2* idiomorph region from isolate 50-3 containing a putative *MAT1-2* gene, again flanked by the *SLA2* and *APN2* genes (Fig. 7). Sequence analysis of the

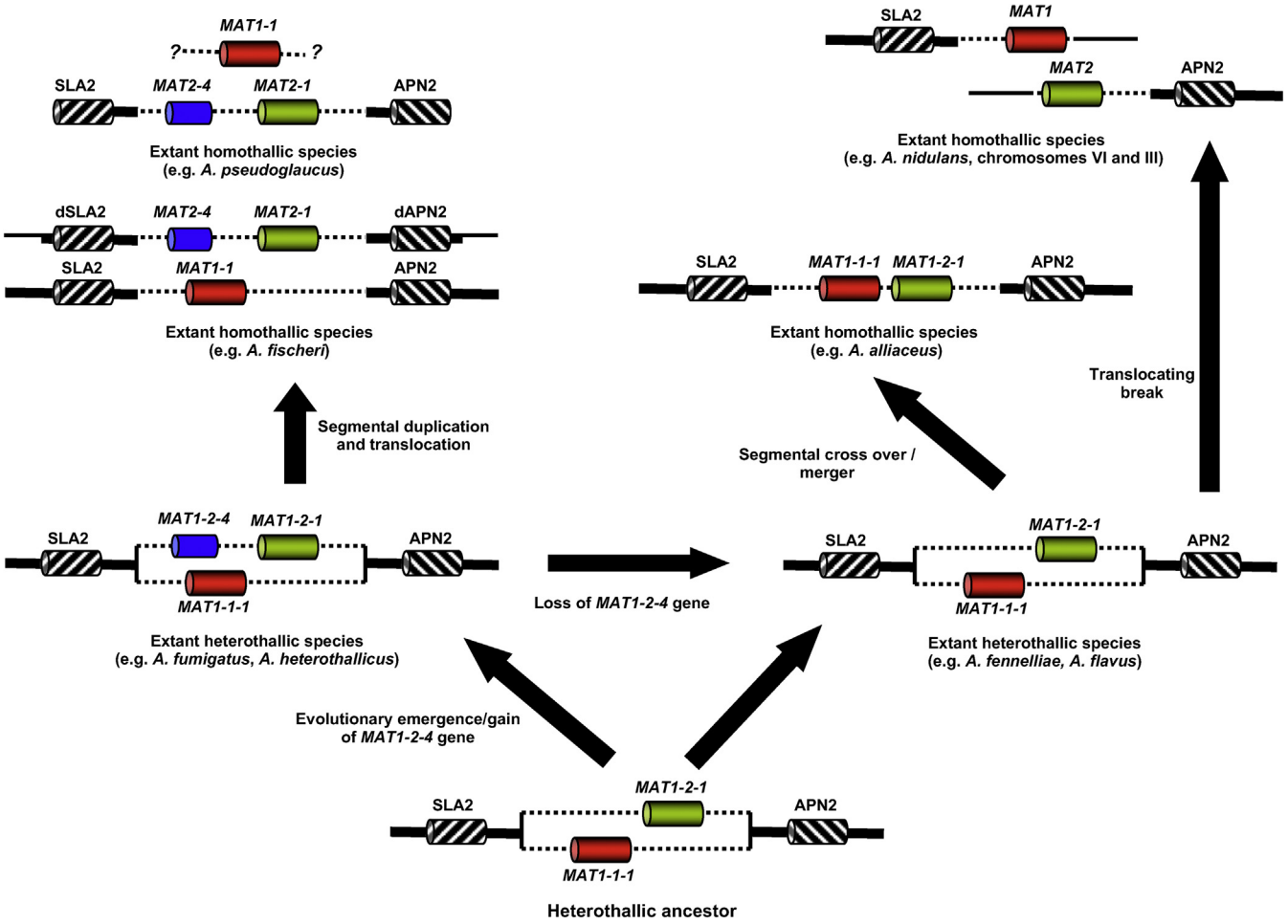

Fig. 7. Model proposed to explain the evolution of *MAT* loci and breeding systems in *Aspergillus* species from a heterothallic ancestor (not to scale). Adapted in part from Dyer (2007). Mating-type genes are shown in colour: *MAT1-1-1* family α-domain in red, *MAT1-2-1* HMG family in green, and *MAT1-2-4* family in blue. Note that gene nomenclature varies between heterothallic and homothallic species due to presence of idiomorphs only in the former species. Flanking genes (SLA2 and APN2) are shown by diagonal hatching. Dotted lines indicate idiomorph region; heavy bold lines indicate conserved sequence flanking the idiomorph region; suffix 'd' indicates disabled pseudogene (Rydholm *et al.* 2007). Note that the illustration does not show all genes present in the flanking regions (*e.g.* an APC gene is also present in some species, but syntenic order varies according to species). Furthermore, only limited sequence is available from the *A. pseudoglaucus MAT1-1* gene region, as indicated by question marks.

region revealed the presence of a 1 075 bp ORF including two introns, which was predicted to encode a 321 amino acid MAT1-2 protein (GenBank accession MH401191). Analysis of the putative MAT1-2 protein revealed three nuclear targeting signals (KKKH at position 182, RKRR at position 202 and PSERKRR at position 199) upstream of the start site. Possession of nuclear targeting sites is consistent with a role of the *MAT1-2-1* gene as a transcriptional activator (Dyer *et al.* 2016). Further analysis of the *MAT1-2* idiomorph region of isolate 50-3 also revealed, intriguingly, the presence of an additional putative ORF which shared high homology with the recently described *MAT1-2-4* gene identified from *A. fumigatus* (Yu *et al.* 2017). The *A. heterothallicus MAT1-2-4* gene was located between the *SLA2* and *MAT1-2-1* gene (Fig. 7) and comprised a 771 bp ORF (including one putative intron), which was predicted to encode a 242 amino acid MAT1-2-4 protein (GenBank accession MH401191). Analysis of the MAT1-2-4 protein revealed no clear targeting signals, only a transcriptional activator TATA box sequence 35 bp upstream of the start site.

The bridging strategy, using PCR with degenerate primers of *MAT-1-1*, *MAT1-2-1, SLA2* and *APN2* and consequent chromosome walking, was also used successfully to amplify entire *MAT1-1* and *MAT1-2* idiomorph regions from heterothallic *A. fennelliae* isolates 54-1 and 54-2, respectively. These *MAT* loci were again flanked by the *SLA2* and *APN2* genes (Fig. 7). Sequence analysis of the *MAT1-1* idiomorph revealed the presence of a 1 160 bp ORF containing one putative intron, which was predicted to encode a 369 amino acid MAT1-1-1 protein (GenBank accession MH401193). Analysis of the putative MAT1-1-1 protein revealed one nuclear localisation sequence (KKKP at position 82), consistent with a role for the *MAT1-1-1* gene as a transcriptional activator (Dyer *et al.* 2016). Analysis of the *MAT1-2* idiomorph revealed a 1 072 bp ORF, containing two putative introns, which was predicted to encode a 322 amino acid MAT1-2-1 protein (GenBank accession MH401194). Analysis of the putative MAT1-2-1 protein revealed three nuclear localisation signals (KKKH at position 183, RKRR at position 203 and PSERKRR at position 200). However, unlike *A. heterothallicus* no *MAT1-2-4* gene was found in the region adjoining the *MAT1-2-1* gene.

Finally, the bridging strategy was used successfully to identify a 9 437 bp SLA2 to APN2 region from isolates 51-1 and 51-2 of the homothallic *A. pseudoglaucus* (Fig. 7). This region was found to contain a 1 078 bp *MAT1-2-1* gene homologue, which contained two putative introns and was predicted to encode a 321 amino acid MAT1-2-1 protein (an alternative possible ATG start site was also detected 7 amino acids inwards of the proposed MAT1-2-1 start site) (GenBank accession MH401195). Analysis of the putative MAT1-2-1 protein revealed three nuclear targeting signals (KKKH at position 183, KKRR at position 203 and PYEKKRR at position 200 upstream of the start site). Further

analysis of the *MAT* region also revealed the presence of a putative *MAT1-2-4* gene homologue, containing one putative intron and which was predicted to encode a 242 amino acid MAT1-2-4 protein (Fig. 7), as reported by Yu *et al.* (2017). However, no evidence of a *MAT1-1-1* gene was found in the *SLA2* to *APN2* region. Despite this, a 151 bp fragment of a putative *MAT1-1-1* family gene was successfully amplified from *A. pseudoglaucus* by PCR with degenerate primers MAT5-6 and MAT3-4. TAIL-PCR was therefore used to chromosome walk upstream and downstream of this fragment. In total, 1 598 bp of sequence from the *MAT* region was obtained, which was found to include a 1 125 bp putative *MAT1-1-1* family gene, which contained one putative intron and was predicted to encode a 356 amino acid MAT1-1-1 family protein (GenBank accession MH401196). Analysis of the putative MAT1-1-1 family protein revealed three nuclear targeting signals (KKRR at position 83, KRRR at position 84 and RRRP at position 85) upstream of the putative start site. There was no obvious sequence homology to the previous SLA2-APN2 *A. pseudoglaucus MAT* gene region. Given the presence of two apparently independent *MAT* loci, these regions were therefore named *MAT1* (containing the alpha domain encoding gene) and *MAT2* (containing the HMG-domain encoding gene) to recognise their separate locations, consistent with the nomenclature of *A. nidulans* and *A.* (*Neosartorya*) *fischeri* as recommended by G. Turgeon (Fig. 7) (Turgeon & Yoder 2000, Paoletti *et al.* 2007, Rydholm *et al.* 2007). As a result the *MAT1-1-1* alpha domain family gene was named simply *MAT1-1* (as there were no alternative idiomorphs in this species), the *MAT1-2-1* HMG domain family gene was named *MAT2-1,* and the novel *MAT1-2-4* family gene was named *MAT2-4* (Fig. 7) for consistency with previous work (Turgeon & Yoder 2000, Paoletti *et al.* 2007, Rydholm *et al.* 2007, Yu *et al.* 2017). For further background see Wilken *et al.* (2017), who have recently proposed updated nomenclature for *MAT* genes.

RNA expression studies were also undertaken with all of the *MAT* genes identified from *A. heterothallicus, A. fennelliae* and *A. pseudoglaucus.* All of the genes were found to be expressed under the conditions assayed, except for the *MAT1-2-4* genes of *A. heterothallicus* and *A. pseudoglaucus* (Eagle 2009).

Implications of MAT *loci structure for evolution of sex in the aspergilli*

Results of the present study provided clear evidence that the heterothallic *A. heterothallicus* and *A. fennelliae* shared the same general genomic arrangement of *MAT* loci as seen previously in the heterothallic *A. fumigatus* and the asexual aspergilli studied by de Vries *et al.* (2017) (Fig. 7). The term 'proto-heterothallic' has been suggested to be used for such latter species where evidence of heterothallism is present, but a sexual cycle has yet to be demonstrated (Houbraken & Dyer 2015). By contrast, results from the homothallic *A. pseudoglaucus* added further evidence of a variety of *MAT* gene arrangements seen in homothallic *Aspergillus* species (Fig. 7). For example, there is evidence of a translocating break leading to the arrangement of *MAT* loci in *A. nidulans* (Galagan *et al.* 2005, Paoletti *et al.* 2007), both alpha- and HMG-domains at the same single *MAT* locus in *A.* (*Petromyces*) *alliaceus* (Ramirez-Prado *et al.* 2008), and a localised *MAT* region duplication and then translocation in *A. fischeri* (Rydholm *et al.* 2007). The precise situation in *A. pseudoglaucus* has yet to be determined as only limited sequence could be cloned adjacent to the *MAT1-1* gene, but there would appear to be two independent *MAT* loci present. Following the logic of Yun *et al.* (1999), it can therefore be strongly argued that it is most likely that the common ancestor of the aspergilli exhibited a heterothallic breeding species. This is on the basis of the general consistency of the heterothallic *MAT* locus arrangement in the aspergilli, but divergence in the homothallic *MAT* arrangement (Fig. 7). Thus, it can be envisaged that new *Aspergillus* species arose as sub groups, containing both *MAT1-1* and *MAT1-2* isolates, which gradually diverged from each other. Within such groups there might then be occasional evolutionary selection for homothallism (*e.g.* Murtagh *et al.* 2000) and the different forms of homothallic *MAT* loci would then arise as a result of spontaneous mutation, accounting for the inconsistency in their organisation.

One caveat to this conclusion is the recent discovery of the *MAT1-2-4* gene in the *MAT1-2* idiomorph of a diverse taxonomic range of 10 *Aspergillus* species including *A. fumigatus* and *A. pseudoglaucus* (Yu *et al.* 2017), and now *A. heterothallicus* as well (Fig. 7). The gene was shown to be functional in *A. fumigatus* where gene deletion led to an inability to mate (Yu *et al.* 2017). However, this gene has not so far been detected or described from most other aspergilli (including *A. nidulans*) and was absent from the *MAT1-2* idiomorphs of *A. fennelliae* and *A. clavatus* sequenced in the present study. Given the taxonomic divergence between *A. fumigatus, A. pseudoglaucus*, *A. heterothallicus* and other species where the gene has been detected [*e.g. A. versicolor* and *A. carbonarius*; Yu *et al.* (2017)] it might be expected that *MAT1-2-4* gene was a conserved ancestral feature of the *MAT* loci of the aspergilli. So, one possibility is that there have been multiple independent losses of this gene in the evolutionary history of the group (Fig. 7). A number of *MAT* genes specific to certain groups of the *Pezizomycotina* have now been described (Wilken *et al.* 2017). A further caveat is that a fragment of the *MAT1-2* gene was found bordering the *MAT1-1* idiomorph of *A. fumigatus* (Paoletti *et al.* 2005) and the related *A. lentulus* (Swilaiman *et al.* 2013), which could indicate evolution from a homothallic ancestor containing both *MAT1-1* and *MAT1-2* genes (Galagan *et al.* 2005). However, bioinformatic analysis of the *MAT* regions of many other aspergilli indicates this to be an unusual occurrence thereby not discrediting the hypothesis of a common heterothallic ancestor. One final consideration is that the apparent predominance of homothallism in the aspergilli is due to the considerable bias in the numbers of homothallic species with *Emericella* and *Eurotium* sexual states that have been described [see Table 1 of Dyer & O'Gorman (2012)]. If these are excluded and asexual species included, then there is a bias instead towards heterothallism.

Table 1. Number of cleistothecia produced by crosses of *A. clavatus* on oatmeal agar medium at 25 °C in the dark after 10 weeks.

Crosses		Number of cleistothecia		
		MAT1-2		
		65-16	65-19	65-20
MAT1-1	65-2	+	+	–
	65-7	–	+	–
	65-8	+	–	–
	56-10	+	–	–
	65-14	+++++	+	–
	65-18	++++	+	–

Ratings indicate the mean number of cleistothecia produced from three replicate crosses on Oatmeal agar in 9 cm diameter Petri dishes: -none; +,1–19; ++, 20–39; ++++, 60–79; +++++, 80–100.

Asexuality in the aspergilli and sexual reproduction in *A. clavatus*

Despite the many supposed benefits of sexual reproduction, approximately 20 % of all fungal species are only known to reproduce by asexual means (Hawksworth *et al.* 1995, Taylor *et al.* 1999, Dyer & Kück 2017). The genus *Aspergillus* is particularly well known for the predominance of asexual species. Based on the presence of meiotic and mitotic taxa in a series of different *Aspergillus* phylogenetic clades, Geiser *et al.* (1996) suggested that asexual fungi are recent derivatives from older meiotic lineages. However, although there might be short-term benefits there would also be long-term costs and Geiser *et al.* (1996) suggested that the asexual lineages would be more susceptible to extinction. Thus, the aspergilli have been seen to provide a model for the evolution of asexuality in fungi. However, there have been a number of breakthroughs over the past decade indicating that asexuality might not after all be dominant in the genus *Aspergillus.* Based on results of population genetic analyses, the presence of sex-related genes (as detected in genome sequencing projects), the presence of isolates of complementary mating type, and the induction of sexual reproduction in certain high-profile 'asexual' species, it has been argued that a 'sexual revolution' is occurring in the genus *Aspergillus* and the closely related genus *Penicillium* (Dyer & O'Gorman 2011). As a result, the prevalence of asexuality in the genus is being questioned.

To investigate whether asexual species might have a cryptic sexual cycle, de Vries *et al.* (2017) investigated whether 'sex-related' genes involved with mating processes were present and functionally expressed in supposed 'asexual' aspergilli. All of the presumed asexual species examined were found to contain either a *MAT1-1* or *MAT1-2* idiomorph as well as genes encoding putative pheromone receptors and a pheromone precursor. Furthermore, when the species were grown under conditions conducive to sexual reproduction in the aspergilli it was found that the mating-type, pheromone precursor and receptor genes were expressed in all of the asexual species in the same way as known sexual species. These results suggested the possibility of inducing the sexual cycle in species of applied importance.

This work was extended in the present study by assessing whether it was possible to induce sexual reproduction in the supposed asexual species *A. clavatus* (Varga *et al.* 2007). The MAT PCR diagnostic using primer pairs AclM1F with AclM1R, or AclM2F with AclM2R successfully amplified putative *MAT* gene fragments from all 20 worldwide isolates of *A. clavatus*. Amplicons of the predicted 244 bp size for *MAT1-1* genotypes and 388 bp for the *MAT1-2* genotypes were produced in different isolates (Supplementary Fig. 20) indicating a heterothallic breeding system. The overall mating-type distribution did not deviate significantly from a 1:1 ratio [(45 % *MAT1-1*) n = 9, 55 % *MAT1-2* n = 11; X^2 = 0.80; n = 20; P = 0.654, (p > 0.05)]. When isolates were grouped according to geographic origin there was also no significant difference in the *MAT* distribution. Based on the equal distribution of mating types, it can be assumed that these populations previously or currently are propagating sexually in their original habitats (Dyer & O'Gorman 2012).

Nine isolates of *A. clavatus* (six *MAT1-1* and three *MAT1-2*) from different geographic origins were then crossed in all possible pairings under a range of different temperatures on oat meal agar, which had previously been used to induce the sexual cycles of *A. fumigatus* and *A. lentulus* (O'Gorman *et al.* 2009, Swilaiman *et al.* 2013). Significantly, after four weeks of incubation cleistothecia were observed in three crosses at temperatures between at 25 °C to 30 °C; all contained asci and ascospores when crushed. The cleistothecia formed along the barrage zones between isolates of opposite mating types (Fig. 8). Cleistothecia were superficial, subglobose to ovoid (315−[513]−692 µm), hard, yellowish-brown saffron colour (fawn), uniloculate, nonostiolate covered by dense aerial hyphae, maturing gradually from the centre outward after 4 wk; covered by dense aerial hyphae stromatal peridium. Asci were 8-spored irregularly disposed globose to subglobose, and ascospores hyaline, lenticular (6.0−[6.5]−7.0 µm), variously sculptured, with two equatorial crests. The cleistothecia were similar to those seen in *Aspergillus acanthosporus* in which the species produce hard and sclerotioid, fawn nonostiolate, unilocular stromata that take 3–4 wk to mature and which at maturity contain hyaline ascospores that have two equatorial ridges (Udagawa & Uchiyama 2002).

Cleistothecia continued to develop in other crosses such that after 10 wk- of incubation cleistothecia were formed in 9 of the attempted 18 crosses (Table 1). Greatest fertility, in terms of number of cleistothecia, was observed in cultures incubated at 25 °C. This was slightly below the optimum temperatures previously described for *A. lentulus* and *A. fumigatus* of 28 °C and 30 °C, respectively (O'Gorman *et al.* 2009, Swilaiman *et al.* 2013). No additional cleistothecia were observed in any of the crosses at any of the three incubation temperatures when cultures were incubated for a further five months. A few features of the crossing data were noteworthy. Firstly, where cleistothecia formed there was a large variation in isolate fertility depending on the mating partners, with isolate 65-16 (*MAT1-2*) generally producing the highest number of cleistothecia whilst one isolate (from India) was sterile with all mating partners (Table 1). Secondly, there were relatively few cleistothecia produced overall, with 78 % of the nine fertile crosses producing less than 20 cleistothecia per 9 cm Petri dish, and no crosses producing more than 100 cleistothecia under the incubation conditions. Finally, some crosses were more flexible in their temperature requirement for crossing than others. For example, isolates 65-2, 65-8, 65-10, and 65-14 produced cleistothecia at both 25 and 28 °C, whereas isolates 65-7 and 65-18 were fertile only at 25 °C.

An analysis of recombination was then conducted to confirm that meiosis had taken place, using ascospore-derived progeny from the representative cross 65-14 × 65-16. There was clear evidence of recombination of genetic markers as a result of the sexual cycle. Analysis of the progeny using the PCR mating-type diagnostic revealed a near 1:1 segregation ratio of mating type [*MAT1-1:MAT1-2* = 6:6; χ^2 = 0.33; p = 0.563, (p > 0.05)]. Moreover, the RAPD analysis showed that the majority of these progeny had recombinant genotypes based on four RAPD-PCR markers. Combining the *MAT* and the RAPD data revealed that 83 % of the progeny had unique genotypes (Table 2). A representative gel of segregation patterns of the RAPD markers is shown in Supplementary Fig. 21. Note that loss of RAPD markers was observed in some offspring as described elsewhere (Dyer *et al.* 1996).

The discovery of sexual reproduction in *A. clavatus* is significant because it provides further evidence that sexual reproduction might be possible in supposedly 'asexual' aspergilli and beyond if partners of compatible mating type and the correct environmental conditions can be identified. Therefore, the original argument of the apparent evolution of asexuality in fungi based on the prevalence of asexuality in the aspergilli (Geiser *et al.* 1996) has been superseded given new evidence about the possibilities for sexual

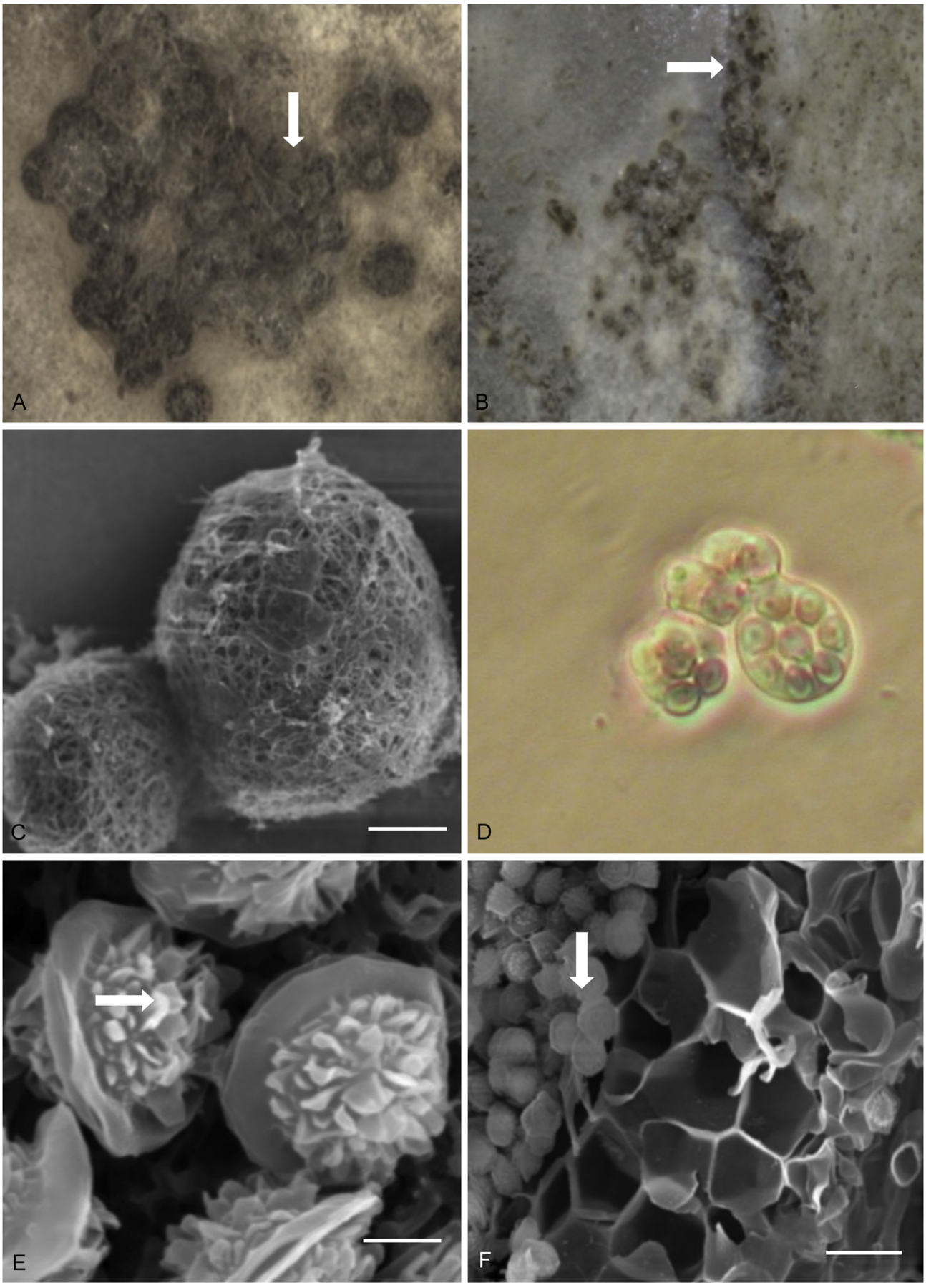

Fig 8. A, B. Sexual reproductive structures in *A. clavatus*. Paired cultures of isolates 65-14 × 65-16 on oatmeal agar showing formation of fawn to dark brown cleistothecia (arrowed) along the barrage zones following four weeks incubation at 25 °C. **C.** SEM micrograph of a cleistothecium showing the interwoven hyphae that form the peridial wall. Scale bar = 100 µm. **D.** A photomicrograph of 8-spored asci. **E.** SEM micrograph of lenticular ascospores (white arrow) Scale bar = 1 µm. **F.** Close-up of the peridium of interwoven hyphae with group of ascospores, (white arrow). Scale bar = 10 µm.

Table 2. Genotypes[1] in the parental isolates and 12 ascospore progeny of a cross between *A. clavatus* isolates 65-14 × 65-16.

Isolate	Mating type	RAPD band[2]				Genotype[3]
		OPC 20	OPT 18	UBC 90	OPQ6	
65-14	*MAT1-1*	−	−	+	+	P1
65-16	*MATl-2*	+	+	−	+	P2
14-16-2	*MAT1-2*	+	+	−	−	A
14-16-4	*MAT1-1*	+	−	+	+	B
14-16-5	*MAT1-1*	−	+	+	−	C
14-16-7	*MAT1-2*	+	+	−	−	D
14-16-8	*MAT1-2*	−	+	+	+	E
14-16-9	*MAT1-2*	−	−	+	+	F
14-16-10	*MAT1-1*	+	−	−	−	G
14-16-11	*MAT1-2*	+	+	−	−	A
14-16-13	*MAT1-1*	+	−	−	+	H
14-16-14	*MAT1-1*	−	−	+	+	P1
14-16-18	*MAT1-1*	−	−	−	+	J
14-16-19	*MAT1-1*	−	−	+	+	P1
P-value (2- tailed)[4]		1.00	0.072	1.00	0.56	
Contingency χ^2 [5,6]	0.771 (1)					

[1] Genotypic characterisation based on mating type and RAPD-PCR bands.
[2] RAPD-PCR bands amplified using Operon primers, OMT1 or R108 . '+' and '−' denotes presence or absence, respectively, of particular amplicons.
[3] The genotype of each progeny isolate, defined by unique combinations of mating-type and RAPD markers as distinct from the parental isolates (designated P1 and P2), is identified by a different letter of the alphabet.
[4] Fisher's exact test for deviation from the null hypothesis of independent assortment of mating-type and RAPD markers in the progeny (*i.e.* a 1:1:1:1 *MAT1-1*+:*MAT1-1*-:*MAT1-2*+:*MAT1-2*- ratio for each RAPD marker). Fisher's exact test was used instead of the χ^2 test because the expected frequencies were <5.
[5] To test for deviation from the null hypothesis of independent assortment of mating-type and RAPD markers in the progeny (*i.e.* an overall 1:1:1:1 *MAT1-1*+:*MAT1-1*-:*MAT1-2*+:*MAT1-2*-ratio for the sum of the RAPD markers).
[6] Number in parenthesis indicates the degree of freedom.

reproduction in these asexual aspergilli from recent (Dyer & O'Gorman 2011, 2012) and the present studies. Indeed, the discovery of sexual reproduction in *A. clavatus* is consistent with phylogenetic work demonstrating that the known sexual species *A. acanthosporus* clusters together with *A. clavatus*, which hinted at the possibility of sexual reproduction in the latter species (Peterson 2000). In addition, one species in the section Clavati, *Aspergillus ingratus*, has been reported to produce saffron-coloured sclerotia when incubated in the dark, representing a possible stage of sexual development (Yaguchi *et al.* 1993). It is noted under the 'one fungus, one name' convention (Hawksworth *et al.* 2011) that no new *Neocarpenteles* or other name is now presented for *A. clavatus,* instead the original *A. clavatus* epithet is applied to the holomorph.

CONCLUSIONS

Members of the genus *Aspergillus* will no doubt continue to be used as model fungi for a variety of reasons such as their ease of growth under laboratory conditions, the availability of classical genetic and molecular resources, and the economic and biotechnological importance of many of the species (Bennett 2010). Overall results of the present study indicate that results gained with *A. nidulans* can provide insights into asexual and sexual developmental processes certainly within the aspergilli, and also to the broader fungi kingdom to some extent. However, the growing appreciation of genome diversity in fungi indicates that caution must be exercised before making assumptions based simply on studies in *A. nidulans*. Also it cannot be assumed that just because homologues of genes are present in different taxa that they have the same functional/mechanistic action. This normally requires some experimental validation. There is also one final irony from the present study. Although some previous theories about the evolution of sexual reproduction in the aspergilli now appear to be incorrect and not applicable to fungi in general, new discoveries about the potential for sexual reproduction in asexual species in the aspergilli now provide a novel model for fungi in general. Therefore *Aspergillus* has arguably reclaimed its role in the vanguard of fungal biology in this instance.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.simyco.2018.10.002.

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