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Sexual and vegetative compatibility genes in the aspergilli

K. Pál[1, 2], A.D. van Diepeningen[1], J. Varga[2, 3], R.F. Hoekstra[1], P.S. Dyer[4] and A.J.M. Debets[1*]

[1]*Laboratory of Genetics, Plant Sciences, Wageningen University, Wageningen, The Netherlands;* [2]*University of Szeged, Faculty of Science and Informatics, Department of Microbiology, P.O. Box 533, Szeged, H-6701 Hungary;* [3]*CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands;* [4]*School of Biology, University of Nottingham, Nottingham NG7 2RD, U.K.*

**Correspondence*: Alfons J.M. Debets, fons.debets@wur.nl

Abstract: Gene flow within populations can occur by sexual and/or parasexual means. Analyses of experimental and *in silico* work are presented relevant to possible gene flow within the aspergilli. First, the discovery of mating-type (*MAT*) genes within certain species of *Aspergillus* is described. The implications for self-fertility, sexuality in supposedly asexual species and possible uses as phylogenetic markers are discussed. Second, the results of data mining for heterokaryon incompatibility (*het*) and programmed cell death (PCD) related genes in the genomes of two heterokaryon incompatible isolates of the asexual species *Aspergillus niger* are reported. *Het*-genes regulate the formation of anastomoses and heterokaryons, may protect resources and prevent the spread of infectious genetic elements. Depending on the *het* locus involved, hetero-allelism is not tolerated and fusion of genetically different individuals leads to growth inhibition or cell death. The high natural level of heterokaryon incompatibility in *A. niger* blocks parasexual analysis of the *het*-genes involved, but *in silico* experiments in the sequenced genomes allow us to identify putative *het*-genes. Homologous sequences to known *het*- and PCD-genes were compared between different sexual and asexual species including different *Aspergillus* species, *Sordariales* and the yeast *Saccharomyces cerevisiae*. Both *het*- and PCD-genes were well conserved in *A. niger*. However some point mutations and other small differences between the *het*-genes in the two *A. niger* isolates examined may hint to functions in heterokaryon incompatibility reactions.

Key words: apoptosis, ascomycete, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus niger*, heterokaryon incompatibility, *MAT*, mating type, *Neurospora crassa*, *Podospora anserina*, *Saccharomyces cerevisiae*, self/non-self recognition.

INTRODUCTION

Self and non-self recognition is a common requirement for all living organisms. Most taxa have developed specific systems to identify self and non-self. Fungi exhibit two types of compatibility systems based on self and non-self recognition, namely sexual compatibility and somatic or heterothallic (in)compatibility (Dyer *et al.* 1992, Leslie 1993). Such systems are of great significance as they govern the degree to which gene flow can occur between members of a species, with consequences for evolution and recognition of species (Taylor *et al.* 1999a). Maintaining gene flow within a species is advantageous as this may lead to increased genotypic variation allowing adaptation to changing environments (Milgroom 1996). However, unrestricted gene flow may lead to resource plundering by other genotypes (Debets & Griffiths 1998).

Many filamentous fungi are able to reproduce both sexually and asexually, depending on environmental conditions (Dyer & Paoletti 2005). In a nutritionally rich environment fungi generally produce mitotic spores (the anamorphic state), but when conditions become unfavourable for vegetative growth, they may initiate sexual reproduction (teleomorphic state). There are two main types of sexual breeding system evident in fungi: homothallism and heterothallism (Dyer *et al.* 1992). Homothallic strains are self-fertile, though may also be capable of outcrossing, whereas heterothallic strains are self-incompatible and require the presence of a compatible mating partner for the sexual cycle to occur. In addition there are numerous species which are apparently restricted to propagation by asexual means with no known sexual cycle (Taylor *et al.* 1999). Sexual compatibility in heterothallic fungi is governed by so called "mating-type" (*MAT*) genes, with two mating types MAT1-1 and MAT1-2 present in heterothallic filamentous ascomycetes (Turgeon & Yoder 2000). These differ according to DNA present at a single *MAT* locus, with highly divergent DNA sequence (termed an "idiomorph") present in isolates of opposite mating type. By convention MAT1-1 isolates contain an idiomorph including a *MAT* gene encoding a protein with an alpha-box domain. In contrast, MAT1-2 isolates contain an idiomorph including a *MAT* gene encoding a regulatory protein with a specific high mobility group (HMG) type DNA-binding domain (Turgeon & Yoder 2000). Mating-type genes regulate initial stages of the mating process such as pheromone signalling and plasmogamy leading to the production of ascogenous hyphae in ascomycetes, and may also have roles in later internuclear recognition (Lengeler *et al.* 2000, Coppin *et al*. 2005, Debuchy & Turgeon 2006). Intriguingly, *MAT* genes have also been shown to be required for sexual reproduction in homothallic ascomycetes (Paoletti *et al.* 2007). Mating-type genes also have other functions in some species, for example affecting vegetative incompatibility in *Neurospora crassa* (Glass *et al.* 1988).

The lack of a known sexual cycle does not mean automatically the lack of recombination. Indeed, there is evidence of genotypic diversity and gene flow in some supposedly asexual fungal species (Geiser *et al*. 1998, Paoletti *et al.* 2005). One possible explanation for such observations is that during the vegetative state of its life a fungus is able to undergo hyphal fusion, karyogamy and mitotic recombination, in the so-called "parasexual cycle" (Pontecorvo 1956). Finding an appropriate partner for mitotic recombination is a crucial aspect of the parasexual cycle, similarly to the sexual mating process. A complex set of heterokaryon-incompatibility genes and associated network of cellular machinery are responsible for the

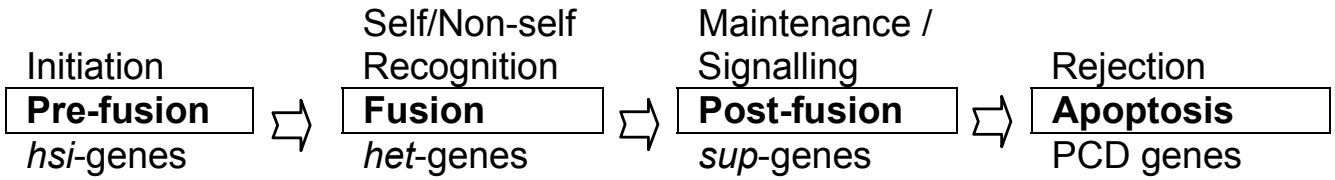

Fig. 1. Gene families involved in steps of vegetative incompatibility.

acceptance or rejection of partners in parasexuality (Glass *et al.* 2000, Saupe 2000).

There are four steps in this parasexual cycle, which can be distinguished by the gene sets governing the steps (Fig. 1) (Leslie and Zeller 1996). The initial step, named pre-fusion, is controlled by genes involved in pheromone production and receptors, and in heterokaryon self-incompatibility (*hsi* genes). The fusion step, in which the interhyphal anastomoses formed and results in a heteroplasmon, is governed by self/non-self recognition genes, like heterokaryon incompatibility (*het*) (Glass and Kuldau 1992) or vegetative incompatibility (*vic*) genes (Leslie 1993). Effects of *het*-genes can be influenced by modifier (*mod*) genes. After fusion the biochemical pathways leading to non-self recognition and cell death may be influenced by genes including suppressor (*sup*) genes, which can modify the signal. In the final step programmed cell death (PCD) genes initiate processes leading to apoptosis.

The precise function(s) of heterokaron incompatibility in fungi is not yet clear. There are three main theories to explain the existence of these vegetative self/non-self recognition systems in fungi. One theory, the so called allorecognition hypothesis, proposes that it is a *bona fide* reaction between genetically different individuals, which may limit the spread of harmful cytoplasmic or nuclear elements (Caten 1972) or prevent resource plundering (Debets and Griffiths 1998). In these examples the role of *het*-genes is to preserve genetic individuality. The second, alternative, theory suggests that *het*-genes simply arose accidentally during evolution. The existence of genes with dual function, like the *mat-a*/*mat-A* loci in *N. crassa* (Saupe 2000), supports the second theory. These genes sometimes behave as *het*-genes, whilst fulfilling other cellular functions at other times. Finally it has been suggested that vegetative incompatibility may promote the initiation of sexual reproduction in some species as a result of non-self recognition (Dyer *et al.* 1992).

There have been a limited number of studies attempting to test these hypotheses. For example, if *het*-genes are present to prevent formation of heterokaryons between genetically different individuals [i.e. to protect a local population from "invaders"] then an evolutionary trend towards generation of new alleles might be predicted, to avoid "pollution" of the isolates belonging to the same incompatibility groups (Saupe *et al.* 1994). However, population genetic modelling suggests that selection favouring more than a few alleles is expected to be extremely weak (Nauta and Hoekstra 1993). In contrast to positive selection in non-allelic systems, certain *het* loci are under maintaining/balancing selection. A well studied example is the *N. crassa het-c* with three known alleles. These alleles show trans-species polymorphism and balancing selection (Wu *et al.* 1998).

In this article, we describe experimental work assessing the occurrence of genes relating to mating and heterokaryon incompatibility processes in the aspergilli, and their possible roles in gene flow within species.

Occurrence of mating-type genes in the aspergilli

Although mating-type genes were first reported from filamentous fungi by Glass *et al.* in 1988, it has proved difficult to clone such genes by traditional molecular biology methods due to high sequence divergence between major ascomycete lineages. Indeed, the only regions conserved across divergent taxa are the alpha-domain in MAT1-1 family proteins, and the HMG-domain in MAT1-2 proteins, and even these show relatively poor sequence conservation [see examples in Debuchy & Turgeon (2006)]. This prevented initial attempts to identify *MAT* genes from *Aspergillus* species (e.g. Arie *et al.* 1997). However, two major developments in recent years have allowed mating-type genes to be identified from the aspergilli,. The first was the use of a degenerate-PCR approach, using primers designed to anneal to conserved sequence present at the HMG *MAT1-2* locus, to amplify *MAT1-2* sequence from species of *Aspergillus* (Singh *et al. 1999,* Paoletti *et al.* 2005, 2007). The second was the public release of genome sequence data from *certain species of Aspergillus*, meaning that whole genomes could be screened by bioinformatic approaches (e.g. BLAST searches) to determine the presence of *MAT* genes (Archer & Dyer 2004, Galagan *et al.* 2005, Pel *et al*. 2007). The use of these two techniques has lead to a series of major discoveries concerning sexuality and gene flow in *Aspergillus* species, with implications for species identity, as will be described.

Mating-type genes in sexual aspergilli

The first MAT gene to be reported from the aspergilli was an HMG-domain family gene from the homothallic sexual species *A. nidulans* (teleomorph *Emericella nidulans*), which was identified by a degenerate PCR approach (Dyer 2002; Paoletti *et al*. 2007). Subsequent BLAST searching of the *A. nidulans* genome revealed the presence of an alpha-domain family gene within the same genome (Dyer *et al.* 2003). The genes were found to be on different chromosomes and were named *MAT1* (alpha-domain on chromosome 6) and *MAT2* (HMG-domain on chromosome 3) to recognise the fact that they occupied different genetic loci (Turgeon & Yoder 2000, Paoletti *et al.* 2007). This compares to other homothallic ascomycetes in which alpha- and HMG-domain *MAT* genes, if both present, have most often been found linked at the same single *MAT* locus (DeBuchy & Turgeon 2006). It was suggested by Galagan *et al.* (2005) that the arrangement of *MAT* loci in *A. nidulans* may have arisen as a result of a chromosome break and translocation event(s) from an ancestral single *Aspergillus MAT* locus. The role of the *MAT* genes in *A. nidulans* was subsequently investigated by Paoletti *et al*. (2007) who used gene deletion/replacement, overexpression and RNA interference approaches to demonstrate that both *MAT1* and *MAT2* genes are required for completion of the sexual cycle in *A. nidulans*. Δ*MAT1* and Δ*MAT2* gene deletant mutants were unable to form ascospores,

although sterile cleistothecia were produced. This was a significant discovery, as it showed that *MAT* genes, normally associated with control of sexual compatibility in heterothallic species, are also required for sexual development in this model homothallic species. The only other *MAT* genes to be identified from a known sexual species of *Aspergillus* has been the identification of both alpha- and HMG-domain genes again together within the genome of the homothallic species *Neosartorya fischeri* (Rydholm *et al.* 2007). The genes were present at unlinked loci, and where therefore termed *MAT1* and *MAT2* respectively. However, the arrangement and synteny of *MAT* loci differed from that seen in *A. nidulans*, and it was suggested that homothallism in this species had arisen by a segmental chromosome duplication and translocation event (Rydholm *et al*. 2007). There have so far been no reports of *MAT* gene isolation from heterothallic *Aspergillus* species, reflecting the fact that the vast majority of sexual aspergilli are homothallic, with only four heterothallic species so far identified (Dyer 2007).

Mating-type genes in asexual aspergilli

Ironically, all the other *MAT* genes reported from the aspergilli have come from asexual species, which supposedly lack a sexual cycle. The detection of *MAT* genes in asexual species might appear surprising, but is thought to reflect the fact that asexual species have evolved from sexual ancestors by loss of sexuality (Geiser *et al.* 1996), therefore "sex-related" genes may be retained in the genome though prone to mutation and loss by genetic drift. Both genomic and experimental work was first used to identify apparently functional (i.e. lacking any frameshift or stop codon mutation) *MAT* genes from the opportunistic pathogen *A. fumigatus* (Pöggeler 2002, Varga 2003, Dyer and Paoletti 2005, Paoletti *et al.* 2005). The species was later shown to contain a complement of other genes required for sexual reproduction (Galagan *et al*. 2005; Nierman *et al*. 2005). Intriguingly a survey of 290 worldwide clinical and environmental isolates of *A. fumigatus*, using a newly-developed multiplex mating-type PCR diagnostic, revealed that all isolates contained either a *MAT1-1* alpha-domain gene or a *MAT1-2* HMG-domain gene and that *MAT1-1* and *MAT1-2* genotypes were present in a near 1:1 ratio (Paoletti *et al. 2005).* This resembled the pattern that might be expected to be seen in a heterothallic sexual species. Furthermore, Paoletti *et al*. (2005) showed that mating-type, pheromone-precursor and pheromone-receptor genes were expressed, again consistent with heterothallism. In parallel work using either degenerate PCR or genomic screening, MAT genes have also been identified from other "asexual" species including *A. oryzae* (Galagan *et* al. 2005), *A. niger* (Pel *et al.* 2007), *A. clavatus*, *A. sojae*, *A. flavus* and *A. parasiticus* (Dyer 2007). Significantly isolates of both *MAT1-1* and *MAT1-2* genotype have been detected in near equal number for many of these species, the only exception being from the *A. niger* “black aspergilli” group which shows a strong bias towards isolates containing an alpha-domain *MAT1-1* family gene (Dyer 2007; Varga, Kocsubé, Pál, Debets, Eyres, Baker, Samson & Dyer unpubl. data). *A. niger* was also shown to contain a complement of genes required for sexual reproduction, although possible mutation was evident in at least one gene (Pel *et al.* 2007).

Taken as a whole, these results are highly significant because they suggest that certain "asexual" aspergilli might retain a latent ability for sexual reproduction. Indeed, there is evidence from population genetic studies of *A. flavus* (Geiser *et al.* 1998) and *A. fumigatus* (Dyer & Paoletti 2005, Paoletti *et al.* 2005, Pringle *et al.* 2005) of high genetic diversity and genetic recombination within populations of these species. It is possible that this is a result of meiotic exchanges in the near past. However, there is also the tantalising possibility that these, and perhaps other aspergilli categorised as "asexual", might posses an extant cryptic sexual state which has yet to be identified. This perhaps might be a result of a slow decline in sexual fertility in the majority of isolates due to selection for asexuality, but the retention of fertile isolates as a sub-set of wild populations (Dyer & Paoletti 2005).

Mating-type genes and species identity

Because *MAT* genes evolve at a relatively fast rate it has been suggested that they might be particularly suited to phylogenetic analysis to resolve species identity and inter-species taxonomic relationships (Turgeon 1998). At present there is insufficient *MAT* sequence data available to allow meaningful phylogenies to be constructed in the aspergilli. A further obstacle is that it would be necessary to obtain homologous *MAT* sequence from all species under examination (e.g. *MAT1-2* gene sequence from all species) and for some species the necessary *MAT1-1* or *MAT1-2* data may be lacking as the majority of isolates might be of the opposite mating type. However, there remains the prospect that accumulating *MAT* data may provide a means to resolve closely related aspergilli taxa, complementing the use of other genes and markers presently used in phylogenetic studies.

Genetic control of heterokaryon incompatibility in ascomycete fungi

For filamentous fungi the establishment of hyphal anastomoses, both within and between individuals of the same species, is considered to be of high importance. A limitation to intermycelial fusions is heterokaryon incompatibility which is widespread among fungi and prevents the coexistence of genetically dissimilar nuclei within a common cytoplasm. The adaptive significance of heterokaryon incompatibility is unclear but it may serve to limit the spread of detrimental cytoplasmic or nuclear elements (Caten 1972) or prevent resource plundering (Debets & Griffiths 1998). A *het* locus can be any locus, at which heteroallelism is not tolerated in a heterokaryon (Saupe 2000). When heterokaryon-incompatible strains fuse, the resulting heterokaryotic hyphae are rapidly compartmentalised and destroyed (often with surrounding cells) or seriously inhibited in their growth, depending on the involved *het* locus. Heterokaryotic cells are often destroyed within 30 min after hyphal fusion. The process of destruction of hyphal compartments shows similarity at the microscopic level in different fungi, and some steps have common features even with multicellular metazoan programmed cell death (PCD) (Glass & Kaneko 2003).

Since heterokaryon incompatible strains can be sexually compatible, the number of *het*-genes that segregate from a cross can be deduced from the progeny. Genetic analysis of heterokaryon incompatibility was performed in a few sexual fungi. The number of identified *het*-genes varied with species. There are at least eight and maximum 18 *het* loci in *A. nidulans* (Anwar *et al.* 1993), six in *Cryphonectria parasitica* (Cortesi & Milgroom 1998), at least 11 in *N. crassa* (Glass *et al.* 2000) and nine in *P. anserina* (Saupe 2000). The majority of the incompatibility reactions is regulated by allelic systems, where two (e.g. *mat-a*/*mat-A* in *N. crassa*, Saupe 2000) or more alleles (e.g. *het-c* Groveland, Oakridge and Panama alleles in *N. crassa*, Sarkar *et al.* 2002) of the same locus interact. In other cases, two distinct loci trigger non-allelic incompatibility (e.g.

het-c/*het-e* in *P. anserina*, Saupe 2000). In non-allelic systems, incompatible alleles can be present in the same haploid nucleus in the progeny and thus vegetative incompatibily may occur also in homokaryotic cells, like in the *het-r*/*het-v* incompatibility in *P. anserina*. Such homokaryotic strains can be obtained for each non-allelic system, and are named self-incompatible (SI) strains (Bourges *et al.* 1998).

Identification of heterokaryon (in)compatibility related genes in the aspergilli

Unlike ascomycete species which have a sexual and usually also an asexual reproduction cycle, for many asexual aspergilli the only way to achieve (mitotic) recombination is via the parasexual cycle. There is evidence from studies of field isolates of species including *A. niger*, *A. terreus*, *A. versicolor*, *A. glaucus* and *E. nidulans* that heterokaryon incompatibility is widespread in the aspergilli (Croft & Jinks 1977). Mitotic recombination has been used for genetic analysis of mutants in an isogenic background of *A. niger* and the construction of a genetic map (Debets *et al.* 1993). But, natural isolates of black aspergilli are highly incompatible with each other, efficiently blocking virus transfer as well as the formation of heterokaryons (van Diepeningen *et al.* 1997). As a result, mitotic recombination between genetically dissimilar isolates is also blocked, so genetic analysis cannot reveal the genetic basis of heterokaryon incompatibility in *A. niger*.

In this study we present the results of data mining in the genomes of two heterokaryon incompatible isolates of the asexual *A. niger* species. We compared incompatibility and cell death related proteins of the two *A. niger* isolates with each other and similar proteins of related sexual (*A. nidulans*) and asexual aspergilli (*A. fumigatus*, *A. oryzae, A. terreus*), two members of the *Sordariales* (*P. anserina*, *N. crassa)* and the yeast *Saccharomyces cerevisiae*. Our analyses identified the major putative *het*-genes in the genome of *A. niger* and related aspergilli. These findings can be used for further functional analysis of candidate *het*-genes.

In silico comparison of yeasts and filamentous fungi

A list of known genes involved in either programmed cell death from *S. cerevisiae* or involved in heterokayon incompatibility and/or programmed cell death in *N. crassa* or *P. anserina* was constructed based on the literature (Table 1). Protein forms of genes were blasted against genomic databases: *A. fumigatus* preliminary sequence data was obtained from The Institute for Genomic Research website. The *A. nidulans*, *N. crassa* (release 7) and *A. terreus* sequence data were from the *Aspergillus nidulans*, *Neurospora crassa* and *Aspergillus terreus* Sequencing Projects, Broad Institute of MIT and Harvard. The *A. oryzae* sequences were available on the server of National Institute of Technology and Evaluation (NITE). The *P. anserina* genome was published by the Institut de Génétique et Microbiologie (Université de Paris-Sud XI / CNRS). *A. niger* ATCC1015 sequence data were produced by the US Department of Energy Joint Genome Institute and *A. niger* CBS513.88 sequence data by the DSM Research BV (Table 2).

Table 1. Genes used in this study involved in heterokaryon incompatibility in *N. crassa* and *P. anserina* and in Programmed Cell Death (PCD) in *S. cerevisiae*. All genes are given with their presumed function and references. ID numbers for *N. crassa* and *S. cerevisiae* proteins refer to the numbers given in their respective sequencing projects, the ID numbers for the *P. anserina* proteins were taken from GenBank. The table is an expanded v. of the table in Glass and Kaneko (2003).

Species	Class	Gene	Function	References
N. crassa				
	Heterokaryon incompatibility genes			
		het-6^{OR} (NCU03533.2)	allelic *het*-gene, TOL/HET-6/HET-E domain	Saupe 2000
		het-c (NCU3125.2)	allelic *het*-gene, signal peptide	Sarkar *et al.* 2002
				Saupe *et al.* 2000
		un-24 (NCU03539.2)	allelic *het*-gene, ribonucleotide reductase large subunit	Saupe 2000
				Smith *et al.* 2000
	Suppressor genes			
		tol (NCU04453.2)	TOL/HET-6/HET-E domain	Shiu & Glass 1999
		vib-1 (NCU03725.2)	regulation of conidiation and maybe of nrAPase	Xiang & Glass 2002
	Incompatibility related genes			
		ham-2 (NCU03727.2)	hyphal fusion, putative transmembrane protein	Xiang *et al.* 2002
		pin-c (NCU03494.2)	allelic gene with HET-domain, linked to *het-c*	Kaneko *et al*. 2006
		rnr-a (NCU07887.2)	suppresses *un-24* temperature sensitive mutation, ribonucleotide reductase small subunit	Kotierk & Smith 2001
P. anserina				
	Heterokaryon incompatibility genes			
		het-c2 (AAA20542)	nonallelic *het*-gene interacts with *het-d* and *het-e*, glycolipid transfer protein	Saupe *et al.* 1994
		het-d2y (AAL37301)	nonallelic *het*-gene against *het-c2*, GTP-binding, WD repeat, TOL/HET-6/HET-E domain	Espagne *et al.* 2002
		het-e4s (AAL37297)	nonallelic *het*-gene against *het-c2*, GTP-binding, WD repeat, TOL/HET-6/HET-E domain	Espagne *et al.* 2002

Table 1. (Continued).

Species	Class	Gene	Function	References
		het-S (AAB88771)	allelic *het*-gene, prion analog	Coustou *et al.* 1997
	Incompatibility related genes			
		idi-1 (AAC24119)	induced by *het-c/e* and *r/v* incompatibility, signal peptide	Dementhon *et al.* 2003
				Bourges *et al.* 1998
		idi-2 (AAC24120)	induced by *het-r/v* incompatibility, signal peptide	Bourges *et al.* 1998
		idi-3 (AAC24121)	induced by *het-c/e* and *r/v* incompatibility, signal peptide	Bourges *et al.* 1998
		idi-4 (*jlb-a*)	bZIP motif, putative trans-activation domain	Dementhon *et al.* 2004, 2005
		(AAT40415)		
		idi-6 (*pspA*)	induced by *het-c/e* and *r/v* incompatibility, subtilisin-like serine protease	Paoletti *et al.* 2001
		(AAC03564)		Reichard *et al.* 2000
		idi-7 (AAN41258)	ortholog of the *S. cerevisiae aut7p*	Pinan-Lucarre *et al.* 2003
	Modifier genes			
		mod-A (AAC25496)	modifier of *het-c*/*e*, *c*/*d* and *r*/*v* incompatibility, SH3-binding motif	Barreau *et al.* 1998
				Bourges *et al.* 1998
		mod-D (AAC24766)	modifier of *het-c*/*e* incompatibility, G protein α subunit	Loubradou *et al.* 1999
		mod-E (AAB97626)	modifier of *het-r*/*v* incompatibility, HSP90	Loubradou *et al.* 1997
S. cerevisiae				
	Programmed Cell Death genes			
		atp4 (YPL078C)	F_0F_1-ATPase	Matsuyama *et al.* 1998
		cdc48 (YDL126C)	cell division cycle, AAA ATPase, fusion of ER-derived vesicles	Madeo *et al.* 1997
		hel10 (YNL208W)	unknown	Ligr *et al.* 2001
		hel13 (YOR309C)	unknown	Ligr *et al.* 2001
		mca1/*yca1* (YOR197W)	metacaspase	Madeo *et al.* 2002
		nsr1 (YGR159C)	rRNA processing	Ligr *et al.* 2001
		ppa1 (YHR026W)	vacuolar H^+-ATPase	Ligr *et al.* 2001
		rsm23 (YGL129C)	mitochondrial small ribosomal unit	Madeo *et al.* 2002
		sar1 (YPL218W)	ER to Golgi transport	Ligr *et al.* 2001
		stm1 (YLR150W)	suppressor of *pop2* and *tom2*	Ligr *et al.* 2001
		tor1 (YJR066W)	regulation of cell death, phosphatidylinositol 3-kinase	Rohde *et al.* 2001
				Dementhon *et al.* 2003
				Fitzgibbon *et al.* 2005

Table 2. Genome databases and their websites used in this research.

Species	Strain	Website	References
A. fumigatus	Af293	http://tigrblast.tigr.org/er-blast/index.cgi?project=afu1	Nierman *et al.* 2005
A. nidulans	FGSC A4	http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Blast.html	Galagan *et al.* 2005
A. niger	CBS513.88	http://www.dsm.com/en_US/html/dfs/genomics_aniger.htm	Pel *et al.* 2007
A. niger	ATCC1015	http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Aspni1&advanced=1	DOE -JGI
A. oryzae	RIB40	http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao	Machida *et al.* 2005
A. terreus	NIH2624	http://www.broad.mit.edu/annotation/genome/aspergillus_terreus/Blast.html	Broad Institute of Harvard and MIT
N. crassa	OR74A	http://www.broad.mit.edu/annotation/genome/neurospora/Blast.html	Galagan *et al.* 2003
P. anserina	S	http://podospora.igmors.u-psud.fr/blast_ol.html	*P. anserina* genome project
S. cerevisiae	S288C	http://seq.yeastgenome.org/cgi-bin/nph-blast2sgd	*Saccharomyces* Genome Database

The *in silico* experiments performed with the different sequenced and available genomes show that the majority of the PCD genes from *S. cerevisiae* have homologs in the filamentous fungi, only *hel10* and *hel 13*, whose functions are unknown, are mostly missing (Table 3). In contrast the majority of vegetative incompatibility and cell death related genes from *N. crassa* and *P. anserina* can be found in the other filamentous fungi, but many of them, like *het-6*, *het-c*, *tol*, *vib-1*, *pin-c*, *het-c2*, *het-s*, *idi-1*, *idi-2*, *idi-3*, *idi-4* and *mod-A,* are missing in the baker"s yeast. Yeast and filamentous fungi diverged approximately 1.1 billion years ago (Cai *et al.* 2006) and yeast have a different, mainly unicellular, life style. Only the HET-D and HET-E proteins which have GTP binding capacity and the so-called WD (tryptophan-aspartate) repeats (Espagne *et al.* 2002) yielded many homologous sequences in *S. cerevisiae*. Of course these genes may have pleiotropic functions and may have a different function in *S. cerevisiae* than controlling anastomoses formation. The *idi-7* gene of *P. anserina* is classified as being an ortholog of the *S. cerevisiae aut7p* gene coding for a protein whose binding to the membrane represents an early step in vesicle formation (Lang *et al.* 1998). Not surprisingly this protein is quite well preserved between both filamentous fungi and yeast as are the genes coding for ribonucleotide reductases and serine proteases.

Some genes like the un-24 gene, known from *N. crassa* to be involved in heterokaryon incompatibility and coding for the ribonucleotide reductase large subunit, are well conserved in the filamentous fungi and have a highly similar homolog in *S. cerevisiae* (Supplementary Table 1). Whereas the putative transmembrane protein HAM-2 is very well conserved within the filamentous fungi, it differs considerably from the *S. cerevisiae* homolog.

These results show that most of the genes involved in programmed cell death are well conserved among both the filamentous fungi and the yeast *S. cerevisiae*, but that many genes involved in heterokaryon incompatibility are not.

In silico comparison of Sordariales and Eurotiales

N. crassa and *P. anserina* belong to the Sordariales, whereas the aspergilli belong to the Eurotiales. Comparing the incompatibility/apoptosis gene sets between these two groups of filamentous fungi (see Table 3 and Suppl. Table 1), the most remarkable difference is shown by *P. anserina*, bearing many more *idi* (induced during incompatibility) gene homologs than other fungi and missing the suppressor gene *stm1*. Among these fungi, *P. anserina* bears the most different gene set and the largest set of genes with *het*-domains: HET-6 has 35, HET-D 94, PIN-C 51 and TOL 48 homologs below the threshold of e^{-10}. Some of these homologous sequences overlap between the different genes due to their conserved *het*-domains. A possible explanation for this can be in its life cycle. *P. anserina* is a saprophytic fungus, which feeds on partially digested materials in the dung of herbivorous animals. As a coprophilic fungus it grows in synchrony and under rather high density with competitors for the same ephemeral and limited substrate. Therefore, the risk of exploitation or genetic infection by others may be relatively high in comparison to most other fungi. An efficient way to limit exchange of genetic materials is heterokaryon incompatibility, which process is governed by the so called *het*-genes. In *P. anserina* the majority of the incompatibility reactions is due to non-allelic interactions (exception is het *het-s*/*het-S* reaction). *Het-d* and *het-e* trigger incompatibility with the *het-c2*. Both *het-d* and *het-e* encode HET domain proteins, and due to the presence of this domain, these proteins seem to be responsible for nonself recognition in filamentous fungi, including *P. anserina* (Kaneko *et al.* 2006). Whether the huge number of HET domain protein homologs thus reflects the relative importance of fusion-rejection systems in the life history of *P. anserina* is unclear, though this has been suggested for coprophiles (Buss 1982). Of course, het-domain containing genes also may have other functions than just heterokaryon incompatibility reactions and there certainly seems to be a large family of het-domain genes.

Comparing the phylogenies of different proteins in the different species (see e.g. Fig. 2 with the HET-6 gene homologs from *A. niger* and *N. crassa*) one can see that the homologs of these genes show old polymorphisms. The sequences found in the *A. niger* strains are often very similar. The most similar homologs in *N. crassa* however, can be quite different from the *A. niger* sequences. Old duplications of the ancestor genes with possible new functions are also visible in the phylogenies.

Within the tested set of proteins, there are no exclusive proteins for members of the *Sordariales*. Only the MOD-A protein has but one hit in the aspergilli: the protein blast in *A. oryzae* resulted in one hit with only low similarity (e^{-11}) (Suppl. Table 1).

In silico comparison of the different aspergilli

S. cerevisiae PCD- and *N. crassa* and *P. anserina* HET-, modifier and suppressor protein sequences were used to search the genomes of *A. fumigatus* Af 293, *A. nidulans* FGSC A4, *A. oryzae* RIB40, and *A. terreus* NIH2624 for homologs (Table 2). Bi-directional best hit analyses were performed with as criterion for homologs an accepted E-value of $<e^{-10}$.

Comparing the different *Aspergillus* species for their putative heterokaryon incompatibility and cell death related proteins, there are no large differences between the species in the presence of certain proteins, in agreement with previous findings (Fedorova *et al.* 2005; Table 3). But, in the number of homologs of HET-6, TOL and TOL-related PIN-C proteins there is a surprisingly big difference between the asexual and (supposedly) sexual lines. Whereas there are at least 10 HET-6, 9 TOL and 11 PIN-C homologs in the asexual strains, in *A. nidulans* and *A. fumigatus* we found only 2 and 3 HET-6, 0 and 1 TOL, and 0 and 1 PIN-C homologs respectively (Suppl. Table 1). This finding could be explained by the capability of sex: *A. nidulans* is able to reproduce sexually, and there are indirect proofs for the presence of a sexual life cycle in *A. fumigatus* (Varga 2003, Paoletti *et al.* 2005). The other aspergilli (*A. niger*, *A. oryzae*, *A. terreus*) are known as asexual species. The question in this case is why do asexual species bear much more HET domain genes? As it was mentioned above these genes are the main components of non-allelic incompatibility, therefore there could be a disadvantage for fungi with a sexual cycle to have such genes.

Another reason could be the proposed function for heterokaryon incompatibility in limiting the spread of detrimental cytoplasmic or nuclear elements (Caten 1972). In *A. niger* and related black *Aspergillus* species dsRNA mycoviruses occur in approximately 10 % of the natural isolates. These mycoviruses are effectively transferred to all asexual spores (van Diepeningen *et al.* 2006). Tests showed that heterokaryon incompatibility indeed efficiently blocks the transfer of mycoviruses in these black aspergilli (van Diepeningen *et al.* 1997). In sexual *A. nidulans* no dsRNA viruses were found in nature and also here artificially introduced viruses efficiently find their way to the asexual spores. However, when sexual spores are produced the mycoviruses are excluded from the offspring (Coenen *et al.* 1997). Therefore *A. nidulans* has an extra option to get rid of parasitic elements through its sexual cycle and thus heterokaryon incompatibility may be less important between *A. nidulans* strains.

Table 3. HET and incompatibility related proteins in filamentous fungi and yeast. ID numbers for *N. crassa* and *S. cerevisiae* proteins refer to the numbers given in their respective sequencing projects, the ID numbers for the *P. anserina* proteins were taken from GenBank.

Species	Class	Protein	Species (Strains)								
			A. fumigatus (Af 293)	*A. nidulans* (FGSC A4)	*A. niger* (CBS513.88)	*A. niger* (ATCC*1015*)	*A. oryzae* (RIB40)	*A. terreus* (NIH 2624)	*N. crassa* (OR74A)	*P. anserina* (S)	*S. cerevisiae* (S288C)
N. crassa											
	Heterokaryon incompatibility genes										
		HET-6 (NCU03533.2)	●	●	●	●	●	●	●	●	
		HET-C (NCU03125.2)	●	●	●	●	●	●	●	●	
		UN-24 (NCU03539.2)	●	●	●	●	●	●	●	●	●
	Suppressor genes										
		TOL (NCU04453.2)	●		●	●	●	●	●	●	
		VIB-1 (NCU03725.2)	●	●	●	●	●	●	●	●	
	Incompatibility related genes										
		HAM-2 (NCU03727.2)	●	●	●	●	●	●	●	●	●
		PIN-C (NCU03494.2)	●		●	●	●	●	●	●	
		RNR-A (NCU07887.2)	●	●	●	●	●	●	●	●	●
P. anserina											
	Heterokaryon incompatibility genes										
		HET-C2 (AAA20542)	●	●	●	●	●	●	●	●	
		HET-D2Y (AAL37301)	●	●	●	●	●	●	●	●	●
		HET-E4S (AAL37297)	●	●	●	●	●	●	●	●	●
		HET-S (AAB88771)		●		●		●			
	Incompatibility related genes										
		IDI-1 (AAC24119)								●	
		IDI-2 (AAC24120)								●	
		IDI-3 (AAC24121)								●	
		IDI-4/ JLB-A (AAT40415)			●			●	●	●	
		IDI-6/ PSP (AAC03564)	●	●	●	●	●	●	●	●	●
		IDI-7 (AAN41258)	●	●	●	●	●	●	●	●	●
	Modifier genes										
		MOD-A (AAC25496)					●		●	●	
		MOD-D (AAC24766)	●	●	●	●	●	●	●	●	●
		MOD-E (AAB97626)	●	●	●	●	●	●	●	●	●
S.cerevisiae											
	Programmed Cell Death genes										
		ATP4 (YPL078C)	●	●	●	●	●	●	●	●	●
		CDC48 (YDL126C)	●	●	●	●	●	●	●	●	●
		HEL10 (YNL208W)	●		●						●
		HEL13 (YOR309C)			●						●
		MCA1/YCA1 (YOR197W)	●	●	●	●	●	●	●	●	●
		NSR1 (YGR159C)	●	●	●	●	●		●	●	●
		PPA1 (YHR026W)	●	●	●	●	●	●	●	●	●
		RSM23 (YGL129C)							●	●	●
		SAR1 (YPL218W)	●	●	●	●	●	●	●	●	●
		STM1 (YLR150W)	●	●	●	●	●	●	●		●
		TOR1 (YJR066W)	●	●	●	●	●	●	●	●	●

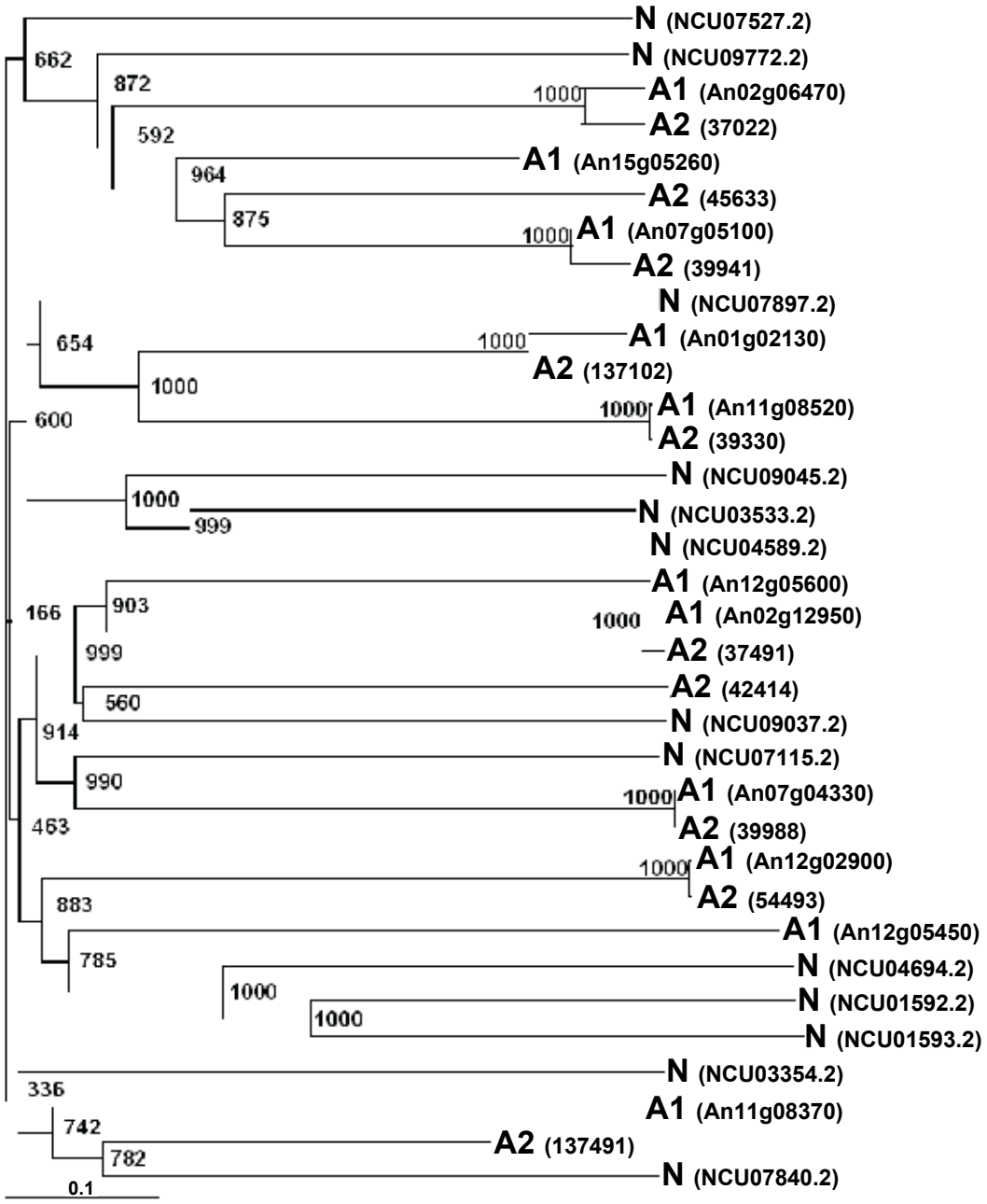

Fig. 2. Neighbour-joining tree of HET-6 proteins from *N. crassa* and two *A. nigers*. Strains on the tree: A1: *A. niger* CBS513.88; A2: *A. niger* ATCC1015; N: *N. crassa* OR74A. Accession numbers of the different homologs are given from the respective databases. Sequence alignment and bootstrapping was performed with ClustalX (Thompson *et al.* 1997). Trees were visualised by Treeview (Page, 1996).

In silico comparison of the two A. niger genomes

Using strains with different spore colors and different auxotrophic mutations or dominant resistances, one can test for the formation of heterokaryotic mycelium on media on which the single partners are unable to grow. Different mutant lines were isolated from DSM Research BV"s Strain CBS 513.88, DOE Joint Genome Institute"s culture collection strain ATCC1015 and our lab strain N400 (ATCC 9029; CBS 120.49), making it possible to test for heterokaryon incompatibility between these strains. Strains ATCC1015 and our laboratory strain N400 (ATCC 9029; CBS 120.49) proved heterokaryon compatible with one another. That they thus belong to the same vegetative compatibility group suggests that they share a common clonal ancestor. However, DSM Research BV"s Strain CBS 513.88 proved incompatible with the two other strains. Thus the two genomes sequenced by DSM and DOE Joint Genome Institute respectively are from heterokaryon incompatible strains. We searched the genomic databases of these two *A. niger* strains for incompatibility/apoptosis related genes (Table 2). For blastp searches we used the apoptosis-like PCD proteins of *S. cerevisiae* and the HET, modifier and suppressor protein sequences of *N. crassa* and *P. anserina* (Table 1). For validation of the identified *A. niger* sequences, a bi-directional best hit analysis was performed, using the polypeptide sequence of the identified *A. niger* ORFs as a query for a blastp search at the *N. crassa*, *P. anserina, S. cerevisiae* and GenBank database (http://ncbi.nih.gov/BLAST; Altschul *et al.* 1990). As criterion for homologs we used an accepted E-value of $<e^{-10}$.

Similarly to the other asexual aspergilli, we found a high number of HET domain proteins (PIN-C, HET-6, HET-D and TOL homologs). Nearly all *het*-genes were highly similar between both *A. niger* strains, differences were limited to a few substitutions but are potentially crucial for incompatibility reactions. However, the

Table 4. A comparison between the different heterokaryon incompatibility and programmed cell death related genes in the two sequenced *A. niger* genomes (CBS513.88 and ATCC1015). If two proteins differ in size, the longer one is the basis for counting percentage of identities, similarities and gaps. Gaps are counted only in the homologous region.

Function	Protein	Type and size of difference		Identities	Gaps
Heterokaryon incompatibility genes (*N. crassa* & *P. anserina*)					
	HET-6	only HET domain motifs are slightly conserved		–	–
	HET-C	indel: 622		791/793 (99 %)	1/793
		substitution: 196			(0 %)
	UN-24	no difference		(100 %)	(0 %)
	HET-C2	no difference		(100 %)	
	HET-D / HET-E	diverse proteins with WD40 repeats, but no remarkable similarity		–	
Suppressor genes (*N. crassa*)					
	TOL	very diverse proteins, with conserved HET domain motifs		–	–
	VIB-1	substitution: 195		585/586 (99 %)	(0 %)
Incompatibility related genes (*N. crassa* & *P. anserina*)					
	HAM-2	substitution: 756		1066/1067 (99 %)	(0 %)
	PIN-C	very diverse proteins, with conserved HET domain motifs		–	–
	RNR-A	no difference		(100 %)	(0 %)
	IDI-6 / PSP	1st pair: indel: 534		531/535 (99 %)	
	(2 alleles)	substitutions: 398, 525			
		2nd pair: substitutions: 398, 436–443, 450–459, 471–514		413/416 (99 %)	
	IDI-7	no difference		(100 %)	
Modifier genes (*P. anserina*)					
	MOD-D (2 alleles)	Members of the two allele pairs are 100 % identical, between the pairs there are some differences:			
		1–167 variable part, 1–60 and 168–360 more conserved region.			
	MOD-E	substitution: 244		672/702 (96 %)	(0 %)
Programmed Cell Death genes (*S. cerevisiae*)					
	ATP4	no difference		100 %	0 %
	CDC48	no difference		100 %	0 %
	HEL13	present only in CBS 513.88		–	–
	MCA1 / YCA1		1st pair	438/438 (100 %)	(0 %)
	(2 alleles)				
		2nd allele: indel region: 1–56, 75–104, 237, 443–447			
		subtitutions: 57–75, 105–106. 236, 440–442	2nd pair	341/441 (77 %)	1/333 (0 %)
	NSR1	diverse proteins, with short conserved motifs		–	–
	PPA1	no difference		100 %	0 %
	SAR1	no difference		100 %	0 %
	STM1	indel region: 8–13		297/303 (98 %)	6/303
					(1 %)
	TOR1	substitution: 16		2389/2390 (99 %)	(0 %)

two sequenced *A. niger* strains differ in their sets of heterokaryon incompatibility and apoptosis related genes. Although they largely possess the same gene set, strain CBS513.88 lacks a HET-S homologue and strain ATCC1015 lacks the IDI-4/JLB-A and HEL10 homologs (Table 3). In nearly all of their putative HET proteins the two *A. niger* isolates show little to no variation in sequence. The two strains are heterokaryon incompatible and the differences –sometimes only simple substitutions, sometimes small stretches of amino acids - in the known indel regions of some of the putative *het*-genes may explain the observed incompatibility reaction between the two strains (Table 4). For the *idi-6* (*psp*) genes two alleles are present, both very similar with very few substitutions. However, for the MCA1/YCA1 (a meta-caspase) one pair is completely identical, whereas the second pair shows more differences and is only 77 % identical.

The regions of these genes involved in self/non-self recognition may be under positive selection and single-amino-acid differences can be sufficient to trigger incompatibility (Saupe 2000). Thus, the

observed small differences may be an explanation for the observed heterokaryon incompatibility between the two *A. niger* strains. However, as both *P. anserina* and *N. crassa* seem to have selected different sets of heterokaryon incompatibility genes to block intermycelial transfer, aspergilli may use a completely different set of genes as well.

DISCUSSION

Little is known about the nature of gene flow in natural populations of aspergilli. However, there is clear evidence of recombination within populations (e.g. Geiser *et al.* 1994, Geiser *et al.* 1998, Paoletti *et al.* 2005). This may have arisen through sexual and/or parasexual means. The presence or absence of gene flow in populations has significant implications for speciation within the aspergilli, which may proceed at different rates depending on the presence of recombination or clonality (Taylor *et al.* 1999a).

In the particular case of the presumed asexual *A. niger* we used an *in silico* study to assess the genetic basis of heterokaryon incompatibility. Comparisons were made of genome sequences of two different *A. niger* strains that are heterokaryon incompatible, together with genome sequences of four closely related sexual and asexual species. We searched these databases with genes known to be involved in heterokaryon incompatibility or apoptosis in *P. anserina*, *N. crassa* and *S. cerevisiae*. Our aim was to find out whether the same genes may be involved in the incompatibility reactions between different *A. niger* isolates as the ones found to interact in *N. crassa* and *P. anserina*, fungi that have different sets of active *het*-genes. Few differences were found between the two sequenced *A. niger* genomes, but many of the known heterokaryon incompatibility genes were indeed present in the *A. niger* genomes. Some of the examined *het*-genes were even found to have many homologs.

Further practical research is needed to find a satisfying explanation for the high level of incompatibility in the natural populations of black aspergilli and to pinpoint functional *het*-genes in the species. As a result of our data mining, the sequences of the known incompatibility genes are available for functional analysis, to uncover the secrets of incompatibility between the natural isolates of black aspergilli.

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