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Authors: Anne D. van Diepeningen, Károly Pál, Theo A.J. van der Lee, Rolf F. Hoekstra, Alfons J.M. Debets

PII: S0953-7562(08)00239-6
DOI: 10.1016/j.mycres.2008.10.003
Reference: MYCRES 500

To appear in: *Mycological Research*

Received Date: 13 June 2008
Revised Date: 22 September 2008
Accepted Date: 13 October 2008

Please cite this article as: van Diepeningen AD , Pál K, van der Lee TAJ, Hoekstra RF, Debets AJM. The het-c heterokaryon incompatibility gene in Aspergillus niger, Mycological Research (2008), doi: 10.1016/j.mycres.2008.10.003

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<NOTE TO TYPESETTERS: Fig. 2A: double check final image **really** shows 500 bp marker as brighter, otherwise consider adding a simple arrow>

The *het-c* heterokaryon incompatibility gene in *Aspergillus niger*

Anne D. van Diepeningen[a,*†], Károly Pál[a,b]*, Theo A.J. van der Lee[c], Rolf F. Hoekstra[a], Alfons J.M. Debets[a]

[a]Laboratory of Genetics, Department of Plant Sciences, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, the Netherlands

[b] University of Szeged, Faculty of Science, Dept. of Microbiology, Szeged, H-6701 Hungary

[c] Plant Research International B.V., P.O. Box 16, 6700 AA Wageningen, the Netherlands

*Corresponding author. Tel.: +31 317 483142.

E-mail address: Anne.vanDiepeningen@wur.nl

†Both authors contributed equally to the paper.

Corresponding Editor: Paul Hooley

ABSTRACT

Heterokaryon incompatibility among *Aspergillus niger* strains is a widespread phenomenon that is observed as the inability to form stable heterokaryons. The genetic basis of heterokaryon incompatibility reactions is well established in some sexual filamentous fungi but largely unknown in presumed asexual species, such as *A. niger*. To test whether the genes that determine heterokaryon incompatibility in *Neurospora crassa,* such as *het-c*, *vib-1* and *pin-c,* have a similar function in *A. niger,* we performed a short *in silico* search for homologues of these genes in the *A. niger* and several related genomes. For *het-c*, *pin-c* and *vib-1* we did indeed identify putative orthologues. We then screened a genetically diverse worldwide collection of incompatible black aspergilli for polymorphisms in the *het-c*

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orthologue. No size variation was observed in the variable *het-c* indel region that determines the specificity in *N. crassa*. Sequence comparison showed only minor variation in the number of glutamine coding triplets. However, introduction of one of the three *N. crassa* alleles *(het-c2*) in *A. niger* by transformation resulted in an abortive phenotype, reminiscent of the heterokaryon incompatibility in *N. crassa*. We conclude that although the genes required are present and the *het-c* homologue could potentially function as a heterokaryon, incompatibility gene, *het-c,* has no direct function in heterokaryon incompatibility in *A. niger* because the necessary allelic variation is absent.

Keywords: vegetative incompatibility, *Neurospora crassa het-c*, *Aspergillus niger het-c*, protoplast transformation

INTRODUCTION

Filamentous fungi can form heterokaryons by anastomosis (fusion) of hyphal cells between genetically dissimilar mycelia. The phenomenon of heterokaryon (or vegetative-) incompatibility (HI) limits the formation of stable heterokaryotic cells between conspecifics and results in the death of the fused cells (Saupe 2000, Glass & Dementhon 2006). HI is considered to be a fungal self/non-self recognition system to counteract the negative consequences of hyphal fusion, such as the exchange of detrimental cytoplasmic elements (Caten 1972) or the risk of resource plundering (Debets & Griffiths, 1998). The genes that control HI are called *het* (heterokaryon incompatibility) (Glass & Kuldau 1992) or *vic* (for vegetative incompatibility) (Leslie 1993) genes. To assess HI in different filamentous fungi four methods have been commonly used: heterokaryon tests, partial diploid analysis, transformation, and confrontation (barrage) tests (for examples of each see Xiang & Glass 2004).

The best studied incompatibility gene is the *het-c* gene of *Neurospora crassa.* This locus is one of 11 identified *het* loci (Perkins 1988), and three different functional *het-c* alleles

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have been identified [*het-c1* (formerly known as *OR:* Oak Ridge), *het-c2* (*PA*: Panama), and *het-c3* (*GR*: Groveland)]. The *het-c* gene codes for a glycine-rich cell-wall protein (Saupe *et al.* 1996) and the specificity is dependent on an indel region encoding 34–48 amino acids (Mylyk 1976; Howlett *et al.* 1993; Saupe & Glass 1997). This *het-c* specificity region shows a high occurrence of non-synonymous substitutions (Wu *et al.*, 1998). *Het-c* interacts with closely linked *pin-c* ('partner for incompatibility' with *het-c*) in a non-allelic way (Kaneko *et al.* 2006). In addition, a suppressor gene that interacts with the *het-c* locus has been identified, the *vib-1* gene ('vegetative incompatibility blocked') (Xiang & Glass 2002).

In *N. crassa* the polymorphic *het-c* alleles appear in approximately equal amounts in the population. Several species and genera within the *Sordariaceae* share this *het-c* polymorphism. This, in combination with the high number of non-synonymous substitutions, is indicative of balancing selection on *het-c*, occurring already in an ancestral state (Wu *et al*., 1998). Homologues of *het-c, pin-c, and vib-1* have been identified in other filamentous fungi, e.g. in *Podospora anserina* where the *het-c* homologous gene is called *hch* (Saupe *et al.* 2000; Pal *et al*., 2007). In contrast to *N. crassa*, in *P. anserina* no polymorphism was detected and thus the *hch* gene may not function as a *het*-gene. However, heterologous expression of *N. crassa het-c* alleles in *P. anserina* lead to a growth defective phenotype reminiscent of that of the co-expression of incompatible *het-c* alleles in *N. crassa* (Saupe *et al.* 2000). Also in other fungi, such as *Aspergillus nidulans* (Davies *et al*. 2004) and *Fusarium proliferatum* and relatives (Kerényi *et al*., 2006), no *het-c* sequence variation was detected.

A. niger is a cosmopolitan mould with characteristic black conidiospores that is used for industrial purposes and occasionally acts as a pathogen. *A. niger* and other closely related black-spored aspergilli are asexual species with a functional parasexual cycle with a relatively high frequency of mitotic recombination that enables genetic analysis between isogenic and compatible isolates (Debets *et al*. 1993). Heterokaryon tests have shown a high incidence of HI among natural isolates (van Diepeningen *et al.* 1997). However, little is known about the genes involved in HI mechanisms in *A. niger*.

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In this study we performed some short *in silico* experiments with the genome sequences of two heterokaryon incompatible *A. niger* strains and seven other aspergilli, to discover whether *A. niger* and relatives also contain *het-c, vib-1,* and *pin-c* orthologues. Subsequently, we investigated whether 99 isolates of our black *Aspergillus* culture collection, belonging to the species *A. niger*, *A. tubingensis*, *A. carbonarius,* and *A. japonicus,* had a naturally occurring polymorphism at the *het-c* locus. Subsequently, we investigated the functionality of the *het-c* gene in the black aspergilli by introducing the three *N. crassa* alleles *het-c1, het-c2*, and *het-c3* in *A. niger.*

MATERIALS AND METHODS

Genome databases

Two *Aspergillus niger* genome databases were searched: the genome of culture collection strain CBS513.88 from DSM (Pel *et al*., 2007) and the genome of strain ATCC1015 from DOE Joint Genome Institute. These two strains are heterokaryon incompatible (Pal *et al*., 2007).

A. clavatus, A. flavus (Payne *et al*., 2006), *Neosartorya fischeri* (Fedorova *et al*., 2008), and *A. fumigatus* (Nierman *et al*., 2005) sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. *A. nidulans*, *Neurospora crassa* (Galagan *et al*, 2005), and *A. terreus* sequence data were from the *Aspergillus nidulans*, *Neurospora crassa* and *Aspergillus terreus* Sequencing Projects, respectively, of the Broad Institute of MIT and Harvard (http://www.broad.mit.edu). *A. oryzae* (Machida *et al*., 2005) sequences were available on the server of National Institute of Technology and Evaluation (NITE). A combined search in most of these *Aspergillus* genomes can now easily be done at the *Aspergillus* Comparative Database of the Broad Institute. The *Podospora anserina* genome was published by the Institut de Génétique et Microbiologie (Université de Paris-Sud XI / CNRS) (Espagne *et al*., 2008). *Saccharomyces cerevisiae* data were obtained from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

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Identification and comparison of het-c, vib-1, *and* pin-c *homologues*

The *Neurospora crassa het-c1*, *het-c2* and *het-c3* alleles (Mylyk 1976, Howlett *et al.* 1993, Saupe & Glass 1997), the *vib-1* (Xiang & Glass 2002), and *pin-c* genes (Kaneko *et al.* 2006) were used to identify homologue sequences in the aspergilli, *Podospora* and *Saccharomyces*, via blastp searches. For validation of the identified sequences a bi-directional best-hit analysis was performed with an accepted E-value of $<e^{-10}$ as the criterion for homologues.

Homologous polypeptides were aligned with ClustalX (Thompson *et al.* 1997) and graphics were enhanced with Boxshade 3.21 (written by Kay Hofmann and Michael D. Baron and available at http://www.ch.embnet.org/software/BOX_form.html). The degree of identity of proteins was described with blasting the proteins against each other by BLOSUM62 matrix (Tatusova & Madden 1999, at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi,).

Strains, characterization, and culture conditions

In total 98 wild-type, black *Aspergillus* strains and the laboratory strain N402 were tested for polymorphisms in the *het-c* gene. Strains were grown on complete medium (CM, according to Pontecorvo *et al.* 1953) supplemented with 1 mg l^{-1} $ZnSO_4$, $FeSO_4$, $MnCl_2$ and $CuSO_4$ at 30 °C. The wild-type strains were originally isolated exclusively on CM with 20 % tannic acid added, and represent the worldwide variation in black *Aspergillus* species, including isolates of *A. niger*, *A. tubingensis*, *A. japonicus,* and *A. carbonarius* and close relatives (van Diepeningen *et al.* 2004), and originated from all over the world (Table 1).

DNA extraction, PCR, and sequencing for het-c *polymorphism*

A 0.1 g mycelial mat, grown overnight on liquid CM, was frozen with liquid nitrogen in a 1.5 ml Eppendorf tube and disrupted with a bead beater. DNA was then isolated with phenol–chloroform extraction (van Diepeningen *et al.*, 2004).

Based on the *het-c* sequences of the DSM *Aspergillus niger* strain (CBS513.88), five sets of primers were developed with overlapping products that covered the complete gene: hetC-

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1for 5'-ATG GCT CCG AGA ATG AGT TT-3' and hetC-1rev 5'-TTT CTT GCT TCC ATG GGT ATA T-3' (expected product size 810 bp); hetC-2for 5'-TCT GCT TAC ATC AAG TAT AGC-3' and hetC-2rev 5'-GAT CGA AGT CCT GTA GAG C-3' (expected product size 673 bp); hetC-3for 5'- TCC GAA GCT CAG GAG TCT GCG-3' and hetC-3rev 5'-CAG CGC TGG GTG GTG GAA CA-3' (expected product size 657 bp); hetC-4for 5'-CGT TGC GCC TCG GGT TCT GTA-3' and hetC-4rev 5'-GCT TCT TCG GTC TCG CCT CTC G-3' (expected product size 480 bp), and finally, hetC-5for 5'- AAC AGC GCC TTC TCC AAT CT-3' and hetC-5rev 5'- TTC CCG CGT CCA TAA CTC G-3' (expected product size 407 bp).

PCR amplifications were performed in 25 µl volumes, with concentrations of the chemicals of the following: 0.35 units SuperTaq DNA Polymerase (HT Biotechnology, 5 units μl^{-1}), 200 µM dNTPs (Promega), 0.2 µM primers (MWG Biotech), 2.5 µl PCR buffer [10 mM Tris–HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % (w/v) Triton X-100, 0.01 % (w/v) gelatin]. The amplifications were done using a iCycler thermal cycler (Biorad), and an initial temperature of 95 °C for 1 min, 35 cycles at 95 °C for 1 min, 56–68 °C for 1 min, and 72 °C for 2 min were carried out with a final step at 72 °C for 5 min. Products were separated by 1 % TBE (tris–borate–EDTA) agarose electrophoresis. Sequencing reactions were performed on polymorphic PCR amplified *hetc*-4 fragments using the DYnamic ET Terminator Cycle Sequencing DETT reaction mix (Amersham Pharmacia Biotech) and analysed in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

Introducing Neurospora crassa het-c1*,* het-c2, *and* het-c3 *in Aspergillus niger*

pCB1004 plasmids containing the *Neurospora crassa het-c1, het-c2*, or *het-c3* alleles and the hygromycin-resistance marker *hygB,* were obtained from Sven Saupe's, Bordeaux (Saupe *et al.* 2000). Transformations were performed on the *Aspergillus niger* strain N814 (derived from N402; *cspA1*, *fwnA1*, *pyrA5* and *nicB5*), auxotrophic for uridine and sensitive to hygromycin. Protoplasts were co-transformed with two plasmids: pCB1004 plasmids contained one of the *N. crassa het-c* alleles and hygromycin resistance, and an autonomously replicating helper plasmid, pAB4-ARp1, which contained the *pyrG* gene for

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uridine auxotrophy, as described by Verdoes *et al.* (1994). Transformants were plated on selective media, amended with hygromycin (85 μg ml^{-1}). When upon introduction of a *het-c* allele no viable transformants were formed, HI was concluded to have occurred. In control transformations no pCB1004 plasmids were added.

Voucher material

The strains ATCC 1015 and CBS 513.88 of which the genomic sequences have been published, can be obtained from the ATCC and the Dutch CBS Fungal Biodiversity Centre in Utrecht, The Netherlands, respectively. All wild-type *Aspergillus niger* and related black Aspergillus strains used are part of the Wageningen Culture Collection of the Laboratory of Genetics, Wageningen University.

RESULTS

Identification and comparison of the het-c, pin-c and vib-1 *homologous sequences*

Blast analyses with the *Neurospora crassa* HET-C protein were done in nine genomes of different *Aspergillus* species, *N. crassa*, *Podospora anserina* and *Saccharomyces cerevisiae* (Table 2). In all the filamentous fungi one or two homologues of HET-C were found, but in *S. cerevisiae* none could be identified. The two sequenced *A. niger* genomes both contained only one homologue, the orthologue of the *het-c* gene, whereas the other species also contained a paralogous sequence. The paralogous sequence corresponded to the cytoplasmic TINC protein known from *A. nidulans* to interact with the 'never in mitosis A' NIMA, whereby together they have a putative role during nuclear membrane fusions (Davies *et al.*, 2004).

Comparison of the corresponding *het-c* genes showed that the *A. niger het-c* gene seems to contain four putative introns, whereas the *N. crassa het-c* contains only two introns, and the *P. anserina hch* gene and all other aspergilli contain three. The two HET-C proteins in the two heterokaryon-incompatible *A. niger* strains (Pal *et al.*, 2007), proved nearly

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identical: one substitution and one extra glutamine unit in a glutamine-rich region around position 620. The other aspergilli also contained a glutamine-rich repeat.

Comparison of the two *A. niger* HET-C alleles with the *N. crassa* HET-C1 and *P. anserina* HCH gene showed that conservation is strong at the N-terminal part of the proteins (first 530 amino acids of the *A. niger* proteins) and decreases toward the C-terminal end. The first 30 amino acids at the N-terminal part of the *A. niger* HET-C display similarity to the supposed signal peptide sequence found in the *N. crassa* HET-C (Fig 1A). However, the requirements to enter the secretory pathway are not completely fulfilled; the hydrophobic block is interrupted by glycine and the C-terminal end of the proposed signal sequence does not contain charged amino acids. Another similarity between the *A. niger* and *N. crassa* HET-C proteins is the presence of a heptad repeat structure (amino acids 440–472), with hydrophobic residues at positions **a** and **d** of the heptad (Fig 1B–C). This motif was found in protein domains with a coiled-coil structure, such as transcriptional regulators or myosin (Saupe *et al.* 1996).

There are two loci known to be involved in the *het*-c related incompatibility in *N. crassa*: *pin-c* and *vib-1*. In *N. crassa* the *pin-c* gene is linked to *het-c* and their proteins together interact with unlike *het-c*/*pin-c* alleles and trigger incompatibility. The *pin-c* gene is absent in *S. cerevisiae*, but various numbers of homologues can be found in the filamentous fungi (Table 2). The homology between the genes is mainly due to homology in the so-called HET domain of the *pin-c* gene. The two sequenced *A. niger* strains contained ten and 11 homologues, respectively, with E-values below the threshold of e^{-10}. However, no homologue linked with the *het-c* gene was found.

Screening with the *N. crassa* VIB-1 protein sequence (Kaneko *et al.* 2006) indicated that this protein is absent in *S. cerevisiae,* but one or two homologues are present in all the filamentous fungi analysed (Table 2). Both *A. niger* strains contained only the orthologue of the *N. crassa* VIB-1 protein.

Screening a population of wild-type black aspergilli for het-c *polymorphisms*

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Ninety-nine black *Aspergillus* isolates (Table 1) from our worldwide collection comprising different species and different geographic regions, including one lab strain, were tested for length polymorphism in the *het-c* gene as that was found for the three functional alleles in *Neurospora crassa*. Five primer pairs generating overlapping amplicons were developed that covered the complete *het-c* gene. We did not find size differences among the isolates in the indel region that determines specificity in *N. crassa*; however, in the region amplified with the *het-C4* primer pair, we found different sizes (480–550 bp) in the PCR products (Fig 2A). PCR products of this variably sized region were sequenced in nine randomly chosen strains, and the size polymorphism originates from a variable stretch of glutamine repeats (Fig 2B). The number of glutamine units did not show correlation with the species, e.g. *A. niger* and *A. tubingensis* and also the two sequenced *A. niger* strains vary in their number of glutamine units.

Testing the functionality of the Aspergillus niger het-c *gene*

To determine whether the *Aspergillus niger het-c* gene could function as a *het* gene and whether a HI reaction could be triggered by one of the known *het-c* alleles from *N. crassa*, we transformed protoplasts of *A. niger* with plasmids containing either of the three known alleles of *N. crassa het-c*. Our selection media contained hygromycin and no uridine, so only successfully co-transformed protoplasts could grow, which contained both the pCB1004 plasmid (with hygromycin resistance and one of the *N. crassa het-c* alleles) and the pAB4-ARp1 helper plasmid (with the wild type *pyrG* gene to compensate for uridine deficiency). Transformants containing a *het-c1* or *het-c3* all showed normal growth and spore production (Fig 3). However, transformants containing the *het-c2* allele showed severe growth inhibition, irregular growth, and a complete absence of conidiospores.

DISCUSSION

HI among *Aspergillus niger* strains is a widespread phenomenon that is observed as the inability to form stable heterokaryons. In several sexual filamentous fungi, such as

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Neurospora crassa and *Podospora anserina,* HI is a genetically determined process involving both allelic and non-allelic interactions. From sexual crosses between heterokaryon incompatible strains, the number of segregating HI genes (*het* loci) can be determined. For the (presumably) asexual *A. niger* the genetic basis of HI is unknown as genetic data from crosses cannot be obtained. In the asexual parasexual cycle, recombination can occur at a high frequency in *A. niger* (Debets *et al*. 1993), but this parasexual cycle is limited to compatible isolates, and therefore, can not be applied to genetic studies on incompatibility. The availability of two whole genome sequences of incompatible *A. niger* strains allows *in silico* screenings for polymorphisms in putative *het* genes, homologous to known *het* genes in sexual species (Pál *et al*., 2007).

In this study we analysed the genomes of two HI *A. niger* strains for the presence of polymorphisms in the sequence of the best studied *het* gene, the *het-c* gene of *N. crassa*, and the *pin-c* and *vib-1* genes known to interact with it. Concurring with Fedorova *et al.* (2005), we found little variation in these genes in the databases. *A. niger* proved to only contain the *het-c* orthologue and not the second close homologue found in all other aspergillus species. Furthermore, we screened for polymorphisms in the *het-c* gene among a representative sample of 99 wild-type *A. niger* and related black *Aspergillus* strains, but did not find differences in the regions that determine its specificity in *N. crassa.* However, through the introduction of the alleles from *N. crassa* by transformation, we demonstrated that the *het-c* gene could potentially be functional in HI reactions.

The availability of a genome sequence of two heterokaryon incompatible *A. niger* strains and other related sexual and asexual aspergilli makes it possible to screen for genes putatively involved in HI and programmed cell death (Pál *et al*., 2007). From various sexual filamentous fungi different sets of so-called *het* genes are known to be involved in this self/non-self recognition. The two *A. niger* strains have been screened for variation in a whole set of genes involved in HI, but the two were nearly always 99–100% identical in these known genes (Pál *et al*., 2007). So, either very small differences in known *het* genes from

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other species are responsible for HI in *A. niger*, or other undefined genes function as *het* genes.

In *N. crassa,* three different *het-c* alleles have been identified thus far, occurring in approximately equal ratios, in which the specificity is dependent on an indel encoding 30–48 amino acids (Mylyk 1976, Howlett *et al.* 1993, Saupe & Glass 1997, Wu *et al.*, 1998). Saupe *et al.* (2000) tested 11 natural *P. anserina* strains without finding evidence for polymorphism in the *het-c* homologue *hch*. We tested 99 black *Aspergillus* strains belonging to the species *A. carbonarius*, *A. japonicus*, *A. niger,* and *A. tubingensis*, that all proved to contain a *het-c* homologous gene, but we did not find any polymorphisms, let alone a balanced one, in the indel region that determines specificity in *N. crassa*. Overall, we found little variation located at the C-terminal end of the *het-c* gene. Sequencing of this variable region showed that this size difference is caused by different numbers of glutamine coding triplets in a glutamine-rich region, presumably without a HI function. Glutamine repeats with a variable number of glutamine residues might originate from DNA polymerase slippage (Zoghbi & Orr 2000). The function of the poly-glutamine repeat in the aspergilli is unknown.

That the *A. niger het-c* gene functions as the inducer of a HI reaction may be questionable, but as it is well-conserved it appears to be a functional gene and it may be a mediator of the HI reaction. The heterologous expression of the *N. crassa het-c2* in *A. niger* by transformation, but not of the other two *het-c* alleles showed a totally abortive phenotype, reminiscent of the incompatibility response suggesting possible involvement in HI reaction. However, there are another explanations for the cell-death phenomenon after introduction of the *N. crassa het-c2*allele; for example that it is toxic when expressed in *A. niger*. Our observation is similar to what has been found in *P. anserina* (Saupe *et al.* 2000). In *Fusarium proliferum* the *het-c* homologue, *hch,* shows a high level of homology to the *N. crassa het-c3* allele, but experiments changing this gene via a six amino acid insertion towards an *het-c1*-like allele and testing that for incompatibility reaction after transformation, did not give an incompatibility response (Kerényi *et al.*, 2006).

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A *het-c* homologous sequence was not only found in *A. niger,* but in all the aspergilli analysed. In *A. nidulans* the homologous protein of HET-C is called TINC, and is known to interact with the 'never in mitosis' NIMA protein thought to be involved in nuclear membrane fusion (Davies *et al*., 2004). NIMA is a G2-specific protein kinase. In *A. niger* TINC is missing, but an orthologue of the NIMA gene seems to be present. The high homology between the two paralogous proteins HET-C and TINC could suggest that in *A. niger* HET-C may be a NIMA interactor.

We also screened the genomic databases for different genes known to interact with the *het-c* gene in *N. crassa,* and we did find a highly similar orthologue gene for the *vib-1* (Xiang & Glass 2002) in the *A. niger* genome. For *pin-c* (Kaneko *et al.* 2006) we did find an orthologue with much lower homology, mainly in the conserved HET domain of the protein. In *N. crassa pin-c* and *het-c* are closely linked and essentially interact as non-allelic HI-genes, but the loci of *het-c* and the closest hit of the *pin-c* gene are located in different contigs in both *A. niger* genomes (e.g. contigs 6 and 11 in the *A. niger* ATCC1015 genome) at quite a distance. The incompatibility reactions observed after transformation of *A. niger* with the *het-c2* allele of *N. crassa*, show that *A. niger* can be induced to cell death. However, the absence of a closely linked *pin-C* orthologue might explain why the introduction of heterologous *het*-alleles only leads to mild incompatibility phenotypes (which might correspond to allelic *het-c/het-c* interactions).

We do not know anything about differences in expression of the *het-c* gene within or between species and whether that influences the strength of the HI reaction, but one can expect it may. Coenen *et al*. (1994) described the occurrence of so-called *partial-het* genes in *A. nidulans,* which segregated among progeny as single genes, but did not cause full apoptosis of resulting incompatible heterokaryons formed. This indicates that the type, and possibly the level, of expression of a gene may influence the resulting incompatibility reaction.

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Based on our findings it seems that *het-c* does not act as an allelic *het* gene in *A. niger*. Comparison of HET-C proteins from two different *A. niger* strains revealed only minor differences (two amino acids were different), and PCR analysis of 99 black *Aspergillus* strains taken from a global population only showed small variations in the number of glutamine residues. Yet, the introduction of the *het-c2* allele from *N. crassa* only yields abortive transformants, indicating that it may act as a HI mediator gene interacting with, as yet, unidentified polymorphic *het* genes. As different *het* genes from other species also did not seem to be involved in HI in *A. niger* (Pal *et al*., 2007), other, as yet, unknown genes must be engaged in triggering the HI reaction. Traditionally, HI has been considered a self/non-self recognition system to discriminate between fungal conspecific mycelia. The fact that fungi also encounter many other organisms has been largely overlooked as a driving force for the evolution of the HI recognition system. Interestingly, recently it was shown that fungal pathogens, such as *Pseudomonas syringae,* may use the fungal HI reactions caused by *het-c* and *pin-c* to induce cell death enabling the bacteria to feed on the fungal hyphae (Wichmann *et al*., 2008). Also in interactions with plants *het* genes may be relevant as, for instance, *Arabidopsis* has been shown to possess genes with high similarity to the known *het-c* gene in *P. anserina* (Brodersen *et al*., 2002). This insight could be of interest for various fungal species, from eurotiomycetes to sordariomycetes in which the homologue, such as *het-c,* is not involved in HI. Thus, although *A. niger* and relatives may perhaps not use the *het-c* homologue for intraspecies HI, it may play a role in inter-species interactions.

ACKNOWLEDGEMENTS

The authors thank Bertha Koopmanschap and Edu Holub for their invaluable help with the sequencing and protoplast fusions, respectively. We are grateful to Sven Saupe for providing us with the plasmids with the different *Neurospora crassa het-c* genes to transform *Aspergillus niger*. We thank DSM Research BV for the availability of the genome of the *A.*

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niger CBS513.88 strain and the DOE Joint Genome Institute for the genome of the *A. niger* ATCC1015 strain.

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Figure 1. Comparison of (A) signal peptide region and (B) coiled-coil heptade repeat structure between *Aspergillus niger* and *Neurospora crassa*. Legends for (A) and (B): grey box: hydrophobic residues (A, F, L, M, V, W); black box: negatively charged amino acid (D); bold: positively charged amino acids (H, K, R); italicized: polar, but uncharged residues (Q, S, T); underlined: identical amino acids in the same position. (C) is the helical wheel representation of the coiled-coil heptades.

Figure 2. Size polymorphisms in the *het-c* locus in black *Aspergillus* strains. (A) Photograph of a gel with different black *Aspergillus* strains showing polymorphisms in the *het-c* locus with primer pair hetC-4for and hetC-4rev. The expected product size is 480 bp, the isolated fragments ranged from *ca* 480 to 550 bp. N, *A. niger*; T, *A. tubingensis;* M, marker: 100bp ladder with extra bright band at 500 bp. (B) Protein sequences of the variable glutamine-rich region, probably caused by DNA polymerase slippage, in ten randomly sequenced strains.

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Figure 3. Photograph of Petri dishes each with 20 isolates of *A. niger*, transformed with either the *het-c1*, *het-c2,* or *het-c3* alleles of *Neurospora crassa*. The insets show single successfully co-transformed colonies. The *het-c1* and *het-c3* alleles yield viable conidiospores forming transformants among the non-transformed strains. The *het-c2* allele did not yield any vigorous transformants.

Table 1. The wild-type strains used in the experiments ordered as per black *Aspergillus* species

Species	Haplotype	Strains[a]			
Aspergillus carbonarius	C	Isr1.1.1			
A. japonicus	J	F2.1	G1.5	Ind1.3.2	M1.3
		F2.2	G2.1	Kam2.1	
		F4.1	G2.6	Kam7.4	
A. niger	1a	Bra2.4	F3.2	G5.7	**N402**
		F3.1	F3.3		
	1b	**Bra2.5**	G3.9	Ind1.8.10	Nep1.6
		Cam6.6	G4.2	Nep1.1	NZ2.1
		Eg1.13	G5.1	Nep1.2	NZ2.2
		Eg2.5	**Gu1.1**	Nep1.3	NZ3.3
		G1.1	**Ind1.6.17**	Nep1.4	NZ3.4
		G3.3	Ind1.8.1	Nep1.5	
	1c	**Bra2.2**	Can2.5	G2.9	**Ind1.7.3**
		Bra2.6	Can2.7	G3.6	NZ3.1
		Bra2.34	Eg1.1	Gu1.6	NZ3.5
		Can2.2	Eg1.6	Ind1.4.24	

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		Can2.4	F1.3	Ind1.5.3	
	n.d.	Ind3.1.10	Ind8.1.1		
A. tubingensis	2a	Bra2.23	Eg2.1	Isr4.1.1	Ned 1.1
		Can2.1	Eg2.4	M1.1	Ned13.1
		Can2.3	G2.12	M1.2	Ned14.2
		Can2.6	Ind1.8.6	M1.5	Ned15.3
		Can2.8	Isr1.1.2	M2.1	**Ned16.3**
		Can2.9	Isr2.1.2	M2.3	**Ned23.2**
		Eg1.8	Isr3.1.1	M2.5	NZ3.2
		Eg1.16			
	2b	Bra2.1	F1.1	G4.4	M2.2
		Bra2.3	F1.2	Ind1.1.1	M2.4
		Bra3.1			
	n.d.	Ind3.3.5			

The strains in which the *het-c4* region (Fig 1) was sequenced appear in bold.

[a] Origin of the strains: Bra, Brazil; Cam, Cameroon; Can, Canada; Eg, Egypt; F, France; G, Gabon, Gu, Equatorial Guinea; Ind, Indonesia; Isr, Israel; M, Maroc; N, Niger culture collection; Ned, Netherlands; Nep, Nepal; NZ, New Zealand.

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Table 2. Homologues of *Neurospora crassa* HET-C, PIN-C1, and VIB-1 proteins in different *Aspergillus* species, *N. crassa, Podospora anserina,* and *Saccharomyces cerevisiae*

		HET-C (EAA35748)[a]			PIN-C1 (EAA26535)			VIB-1 (EAA31128)		
Species	Strain	#	Identifier[b]	E-value	#	Identifier[b]	E-value	#	Identifier[b]	E-value
Aspergillus niger	CBS513.88	1	CAK42566	0.0	11	CAL00837	1e-46	1	CAK47109	1e-57
A. niger	ATCC1015	1	182955[c]	0.0	10	43178[c]	2e-38	1	193642[c]	1e-60
A. clavatus	NRRL 1	2	EAW14442/ EAW1086	0.0	2	EAW13637	7e-38	1	EAW15082	0.0
A. flavus	NRRL 3357	2	1866.m00219 / 2368.m00001[d]	0.0	16	1569.m00603[d]	5e-44	1	1739.m00454[d]	0.0
A. fumigatus	Af293	2	EAL93881 / EAL84637	0.0	1	EAL93522	1e-19	2	EAL85174	0.0
A. nidulans	FGSC A4	2	EAA64211/ EAA61900[f]	0.0	0	-		2	EAA64544	0.0
A. oryzae	RIB40	2	BAE60442/	0.0	12	BAE58855	1e-38	1	BAE65505	0.0

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			BAE64392							
A. terreus	NIH2624	2	EAU37424/ EAU38641	0.0	15	EAU31655	1e-38	1	EAU38663	0.0
Neurospora fischeri	NRRL 181	2	EAW19150/ EAW20992	0.0	0	-		1	EAW20054	0.0
N. crassa	OR74A	2	EAA35748/ EAA26644	0.0	35	EAA26535	0.0	2	EAA31128	1e-39
Podospora anserina	S	2	CAP71069/ CAP69442	0.0	51	CAP68343	7e-49	2	CAP73583	0.0
Saccharomyces cerevisiae	S288C	0	-	0.0	0	-		0	-	

For validation of the identified sequences a bi-directional best-hit analysis was performed with an accepted E-value of $<e^{-10}$ as the criterion for homologues. The number of homologous sequences are given per strain, the identifier of the best hit and it's E-value. For the HET-C protein identifiers of all homologous sequences are given.

[a]For the *het-c1*, *het-c2*, and *het-c3* alleles see AAB48349, AAF08294, and AAF08295 respectively.

[b]Identifiers are GenBank identifiers, unless stated otherwise.

[c]DOE Joint Genome Institute.

[d]Codes from www.aspergillusflavus.org

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A

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MAPRMSLGTHALLVLGLLLVLLPTQTWAFG    A. niger CBS513.88 HET-C
MTGLRIGWGSVLLVLALVLVVLPDKAAAFG    N. crassa HET-C^OR
1        10        20        30
```

B

```
VVRKLASIIEKIPGLEAIVEKITETLTVFVMSL    A. niger CBS513.88 HET-C
IVRSINNMIEKIPGLESLLEKISETLTAFILGL    N. crassa HET-C^OR
defgabcdefgabcdefgabcdefgabcdefga
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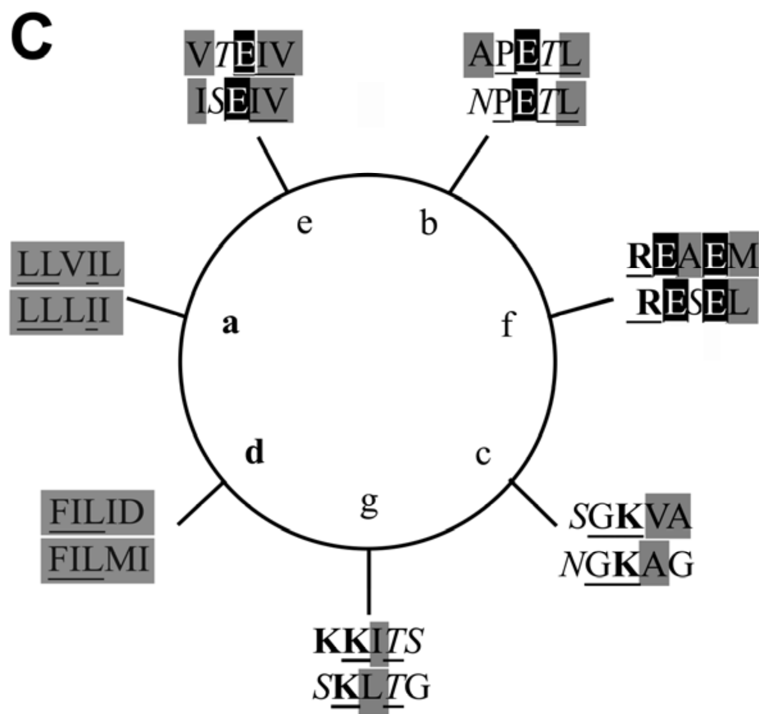

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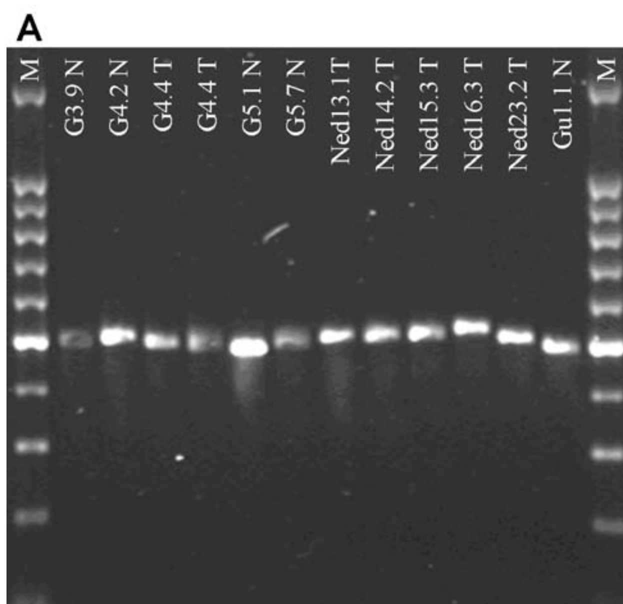

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B

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CBS513.88    1 FGAAGHSHGSQQQRPPQQQ----QQQQQQQQ---GSSPLPWDKLSSLPIP
Ned16.3      1 FGAAGHSHGSQQQRPPQQQ----QQQQQQQQQQQGSSPLPWDKLSSLPIP
Ind1.7.3     1 FGAAGHSHGSQQQRPPQQQ----QQQQQQ-----GSSPLPWDKLSSLPIP
N402         1 FGAAGHSHGSQQQRPPQQQ----QQQQQQQ----GSSPLPWDKLSSLPIP
Ned23.2      1 FGAAGHSHGSQQQRPPQQQ----QQQQQQQ----GSSPLPWDKLSSLPIP
Bra3.1       1 FGAAGHSHVSQQQRPPQQQ----QQQQ-------GSSPLPWDKLSSLPIP
Gu1.1        1 FSAAGHSHGSQQQRPPQQQ----QQQQQQ-----GSSPLPWDKLSSLPIP
Bra2.2       1 FSAAGHSHGSQQQRPPQQQRPPQQQQQQQ-----GSSPLPWDKLSSLPIP
Ind1.6.17    1 FSAAGHSHGSQQQRPPQQQ----QRSQQQ-----GSSPLPWDKLSSLPIP
Bra2.5       1 FSAAGHSHGSQQQRPPQQQ----Q----------GSSPLPWDKLSSLPIP
consensus    1 *.******.**********    *.....     ****************
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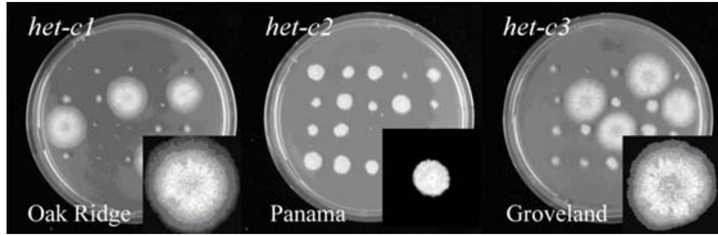

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