Horizontal Transfer of Genetic Elements in the Black *Aspergilli*

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Voor Abe en Steeph

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Introduction 'Horizontal Transfer of Genetic Elements in the black *Aspergilli*'.

In the next paragraphs different aspects of the 'horizontal transfer of genetic elements in the black *Aspergilli*' are introduced. First, horizontal transfer and its consequences for populations are discussed. Then the model species in these investigations, the black *Aspergilli* which form a complex of asexual filamentous fungi, are introduced. Heterokaryon incompatibility between strains forms a potential barrier to horizontal transfer. Next, different genetic elements are considered that may be transmitted in a horizontal way. Finally the outline of this thesis is described.

1.1 Population level consequences of horizontal transfer.

Two modes of transmission of genetic material can be distinguished: the vertical transmission of parent to (sexual and asexual) offspring and the horizontal (or lateral) transfer between two, not necessarily related, individuals. Genetic elements with exclusive vertical inheritance are not expected to become more frequent in a population unless they enhance the fitness (survival and/or reproducibility) of their host and are thus selected or show meiotic drive or biased segregation. Vertically transmitted elements which are neutral for their hosts will either get lost by genetic drift or get fixed, and those with deleterious effects would be selected against and almost always removed from the population.

Horizontal transfer of genetic information is of evolutionary importance since it leads to non-adaptive evolution; DNA or RNA sequences that have both vertical and horizontal transmission have the potential to increase in the population, even when they decrease the fitness of their host. Horizontal transfer may also lead to genetic recombination and this may be particularly important for organisms in which sexual recombination is absent or negligible. In bacteria, several mechanisms are known to facilitate horizontal transfer of genetic information: *Transduction* is bacteriophage mediated transfer, *Transformation*

involves the direct uptake of DNA-molecules and *Conjugation* involves the union of two bacterial cells.

The role of horizontal transfer in prokaryotes has now been well established, but the importance and extent of horizontal transmission in natural populations of filamentous fungi is not yet clear. Filamentous fungi also possess a potential mechanism for horizontal transfer: the ability to form anastomoses, making direct cytoplasmic contact between different hyphae. The formation via anastomoses of heteroplasmons (mixed cytoplasms), heterokaryons (mixed cytoplasms with different nuclei) and a (transient) diploid mycelium that after haploidisation results in haploids again is called the parasexual cycle. During the parasexual cycle mitotic recombination can lead to exchange of parts of chromosomes, and haploidisation of formed diploids can lead to reassortment of the chromosomes. Cytoplasmic elements may also recombine or be exchanged. Such a 'parasexual' recombination could be an important nuclear and cytoplasmic recombination mechanism for imperfect (asexual) fungi (Pontecorvo, 1956). It is, however, unknown to what extent the parasexual cycle that can be induced in the laboratory is a relevant event in nature. The formation of anastomoses depends on the heterokaryon compatibility between the mycelia involved (see §1.3). Incompatibility and the inability to produce viable heterokaryons protects the genetic integrity of the fungal individual and prevents the invasion of foreign genetic material.

This project aimed at elucidating rates of horizontal transfer in populations of the imperfect black *Aspergilli*. This complex of asexual fungi is introduced in the next paragraph. The heterokaryon (in)compatibility mechanisms that regulate the formation of anastomoses between mycelia, are introduced in paragraph 1.3. Paragraph 1.4 gives a list of possible genetic elements that may be transferred horizontally in a population. The concluding paragraph gives an outline of this thesis.

1.2 The black *Aspergilli*.

The name *Aspergillus* was introduced by Micheli in 1729 for moulds with a characteristic aspersory-(mop)-like organisation of the conidiophore with spores (c.f. Raper and Fennel, 1965). The first black-spored *Aspergillus*, '*Aspergillus niger*', was described by van Tieghem in 1867 as a fungus capable of using the plant polymer tannin as carbon source.

Over the years, several *Aspergilli* turned out to show the characteristic black pigmentation of conidial heads, see Figure 1.1. On the basis of morphological data three main groups can be distinguished; an *A. carbonarius* group, an *A. japonicus*/*A. aculeatus* group and a group centered around the most prevalent member, *A. niger* (the '*A. niger* aggregate') (Raper and Fennel, 1965). On the basis of molecular analyses using (ribosomal/mitochondrial) Restriction Fragment Length Polymorphisms (RFLPs), isozymes and Random Amplification of

	black *Aspergilli*				
morphology :	***A. carbonarius*** (Bain.)Thom	*A. niger* aggregate around *A. niger* Van Tieghem			***A. japonicus*** Saito / ***A. aculeatus*** Iizuka
sterigmata[a]:	biseriate	biseriate			single
conidia[a]:	large, multinucleate	relatively small			echinulate
RFLP:		***A. niger***	***A. tubingensis***	***A. brasiliensis***	
rRFLP:	C1-C2 [bc]	I-I' [b]	II-II' [b]		J [bc]
mtRFLP:	C1a-b &C2 [c]	1a-1e [d]	2a-2f [d]	3 [d]	J1-J7 [e]

Figure 1.1 Schematic view of recognisable types within the group of the black *Aspergilli* on basis of morphological and ribosomal and mitochondrial RFLP data. [a]) Raper and Fennel, 1965. [b]) Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993. [c]) Kevei *et al.*, 1996. [d]) Varga *et al.*, 1993; 1994a. [e]) Hamari *et al.*, 1997.

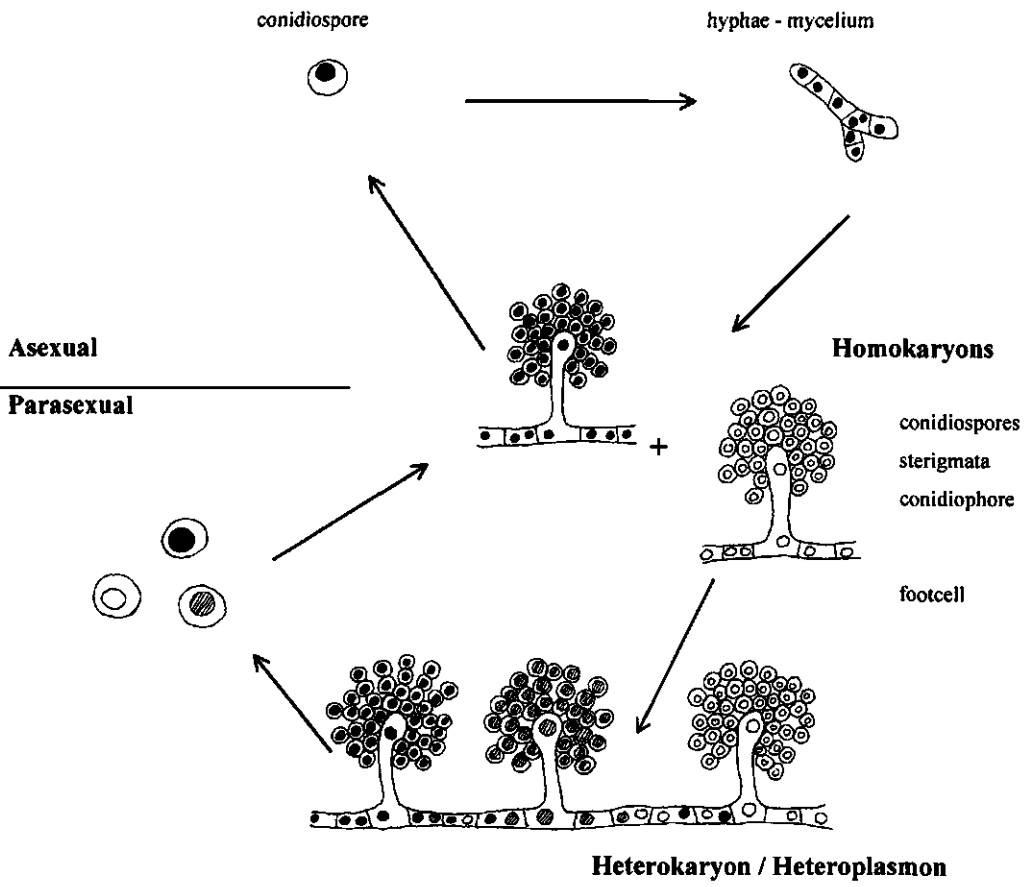


Figure 1.2 The asexual and parasexual life cycles of a black *Aspergillus*.

Polymorphic DNA (RAPDs) this *A. niger* aggregate can be further divided into two main groups *A. niger* and *A. tubingensis* and a smaller group *A. brasiliensis* (Kusters-van Someren *et al.* 1991, Mégnégneau *et al.*, 1993, Varga *et al.* 1993, 1994a).

The black *Aspergilli* are asexual, but under laboratory conditions vegetatively compatible strains can form heterozygous somatic diploids. Mitotic recombinants can be obtained via the so-called parasexual cycle (Pontecorvo *et al.*, 1953) (Figure 1.2). However, vegetative compatibility between natural isolates appears to be rare and it is unknown whether and to what extent parasexual recombination occurs in natural populations. For *A. niger* a mitotic map has been constructed by exploiting the parasexual cycle and an electrophoretic karyotype have been determined, recognising eight linkage groups (Debets *et al.*, 1990b; 1993; Verdoes *et al.*, 1994)

The black *Aspergilli* occur world-wide with a slight preference for tropical and subtropical areas (Rippel 1939, Raper & Fennel 1965, Domsch *et al.* 1980).The spores are distributed by air and the fungi - sometimes called soilborne - can be isolated from a large variety of substrates. The black *Aspergilli* are very versatile in their metabolism and are widely used in industry for the production of organic acids, enzymes and food fermentations (Lockwood, 1975; Underkofler, 1976; Wood, 1977). The widely used *A. niger* has a GRAS-status (Generally Recognised as Safe; US Food and Drug Administration), but occasionally plant and animal pathogenic strains are found. Some of the animal/human pathogenic *Aspergilli* are suggested to be infectiously transmitted (Polkey *et al.*, 1993).

1.3 Heterokaryon incompatibility, a potential barrier to horizontal transfer.

The first step in the parasexual cycle is the formation of a heteroplasmon-heterokaryon after anastomosis between hyphae of different strains. In many ascomycete fungi (see e.g. Glass and Kuldau, 1992; Leslie, 1993) this is controlled by heterokaryon (somatic or vegetative) incompatibility reactions. Prevention of the formation of s stable heteroplasmon may preclude horizontal transfer of genetic elements. Heterokaryon incompatibility in fungi can be studied in several ways (for examples see Fincham *et al.* 1979; Jennings and Rayner 1984; Perkins 1988; Glass and Kuldau 1992). The two most common phenotypes of heterokaryon incompatibility are the formation of a *barrage*, a zone of dying hyphae between mycelia (e.g. Rizet, 1952; Perkins, 1988) and the inability to form a prototrophic heterokaryon under forcing conditions. Often, complementation between different nitrate non-utilizing mutants is used to test for such prototrophic heterokaryon formation (Cove 1976; Correl *et al.* 1987; Joaquim and Rowe 1990; Brooker *et al.* 1991).

Leslie and Zeller (1996) have proposed a simple model distinguishing four different steps in a heterokaryon (in)compatibility reaction (see Figure 1.3). The initial pre-fusion step is under the control of genes like those involved in pheromone production and receptors and genes that can be involved in *h*eterokaryon *s*elf-*i*ncompatibility (*hsi*). The actual fusion step

is controlled by self/non-self recognition genes like some *het*erokaryon incompatibility genes (*het*). After fusion the cascade of reactions from non-self recognition to cell death may be influenced by several genes among which *sup*pressor-genes, modifying the signal. Finally apoptotic genes lead to cell death.

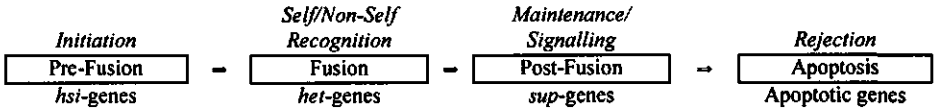


Figure 1.3 A simplified model identifying different steps in the heterokaryon incompatibility interaction process and genes that may be associated with these steps according to Leslie and Zeller (1996).

Absence of pre-fusion self-recognition or heterokaryon self-incompatibility has been found in several fungal species. In *Rhizoctonia solani*, non-self-anastomosing strains are a common phenomenon and are assumed to play a role in the decline of the disease caused by the fungus in monoculture (Hyakumachi & Ui, 1987). In several *Fusarium* species heterokaryon self-incompatible strains are regularly found and make up 1-2% of natural populations. These strains lack the ability to form heterokaryons with themselves and usually also with other strains (Correll *et al.*, 1989; Jacobson and Gordon, 1990; Campbell *et al.*, 1992; Hawthorne & Rees-George, 1996). In these strains hyphal branching *per se* does not appear to be affected but the number of hyphal fusions is drastically reduced.

Most genetic analysis has been done on the *het*-genes in several sexual ascomycetes. Here, both allelic and non-allelic *het*-gene systems have been found to be involved. In allelic systems an allelic difference between two strains at one *het*-locus is sufficient to cause an incompatibility reaction. In non-allelic interactions differences at two separate loci result in incompatibility (for a scheme see Bégueret *et al.*, 1994). The number of *het* genes in a population determines the number of vegetative compatibility groups (VCGs), within which heterokaryons can be readily formed. In a population with 10 bi-allelic *het* genes, in theory at least 2^{10} VCGs are possible. VCGs can serve as a natural means to subdivide populations of fungi that spend a large fraction of their life cycle reproducing asexually (Leslie and Klein, 1996). If selection acts to maintain a large number of VCGs within a population, either to reduce the spread of infectious elements (Caten, 1972; Hartl *et al.*, 1975; Nauta and Hoekstra, 1995) or due to values of individualism (Rayner, 1991), the frequency dependent selection may play an important role in maintaining many VCGs and heterozygous *het* loci in the population (Glass *et al.*, 1998; Wu *et al.*, 1998).

Some genes influencing the post fusion reactions have been identified. In *N. crassa* a number of 'post fusion gene' mutations can override heteroallelic incompatibility reactions at one or more *het* loci (Newmeyer, 1970; Arganoza *et al.*, 1994). In *P. anserina mod* genes were found that can inhibit allelic and/or non-allelic incompatibility genes (Boucherie and

Bernet, 1974; Durrens and Bernet, 1982; Bernet, 1992). Other mutations result in broader vegetative compatibility (*Podospora*: Bourges *et al.*, 1996; *Neurospora*: Jacobson *et al.*, 1995; *Fusarium*: Kuhn *et al.*, 1996; Zeller and Leslie, 1996). The final cell death is probably caused by proteases and cellulytic enzymes and their regulators in a characteristic manner that is conserved across plant and animal kingdoms (Wang *et al.*, 1996).

Many genes and processes are involved in the heterokaryon incompatibility reactions and also the strength of the reaction may vary (and thus also transfer possibilities could vary). Partial *het*-genes were identified in *A. nidulans* strains showing a weak heterokaryon by Coenen and co-workers (1994). Heterokaryons that result from protoplast fusions of otherwise vegetatively incompatible strains are often quite different from similar heterokaryons formed following hyphal anastomoses (Adams *et al.*, 1987; Stasz *et al.*, 1989; Molnár *et al.*, 1990). This indicates that some of these heterokaryon incompatibility reactions are based on cell wall or cell membrane components. Other reactions involve cytoplasmic components because in some instances mixing of cytoplasms leads to cell death (Wilson *et al.*, 1961; Williams and Wilson, 1966; Typas, 1983).

In *Aspergilli*, often no clear phenotypic heterokaryon incompatibility reactions can be observed, perhaps due to a low frequency of anastomoses. Nothing is known about the genetics of heterokaryon incompatibility in the black *Aspergilli*, because genetic analysis is difficult in this asexual species complex. The incompatibility is generally assumed to be similar to that in related sexual ascomycetes (mediated by *het*-genes etc.). In the *Aspergilli* complementation of different nitrate non-utilising mutants can be used as test for heterokaryon compatibility (Cove, 1976; Debets *et al.*, 1990). In species like *A. nidulans*, heterokaryon incompatibility can (partly) be overcome through the use of intraspecies protoplast fusion (Dales and Croft, 1977; Ferenczy *et al.*, 1977; Peberdy and Ferenczy, 1985). Coenen (1997) selected some *het*-gene suppressors in *A. nidulans*. Interspecies protoplast fusions between different *Aspergillus* species have also been successful in some cases (e.g. Bradshaw *et al.*, 1983; Kevei and Peberdy, 1984; Liang and Chen, 1987). Horn and Greene (1995) found heterokaryon self-incompatibility in two imperfect *Aspergillus* species: *Aspergillus flavus* and *Aspergillus parasiticus*.

1.4 Genetic elements that may be transmitted horizontally.

In this paragraph four different classes of genetic elements that may show horizontal transmission are discussed. The first class is the mitochondria. The second consists of the plasmids that in filamentous fungi are located in the mitochondria, although some nuclear and cytoplasmic plasmids have been found in yeasts. Some of the plasmids resemble mobile introns. The third type of elements discussed are nuclear transposable genetic elements.

Finally mycoviruses are discussed that are normally found in the cytoplasm, though some are associated with the mitochondria as well.

1.4.1 Mitochondria.

In eukaryotes the mitochondria are the site of ATP synthesis and the citric acid cycle. They contain circular double-stranded DNA genomes (mtDNA). The evolutionary origin of the mitochondrion is probably as an endosymbiontic prokaryote (Margulis, 1970; 1981; Gray *et al.*, 1984; Yang *et al.*, 1984; Cedergen *et al.*, 1988). In the course of evolution most of the genes of the mitochondrial genome have been transferred to the nucleus. Although the coding capacity of the mtDNA is rather conservative, remarkable size differences in mtDNA are observed in fungi ranging from a minimal size of about 17 kb in the yeast *Schizosaccharomyces pombe* (Zimmer *et al.*, 1984) to about 176 kb in *Agaricus bisporus* (Hintz *et al.*, 1985).

MtDNAs generally do not recombine because of their strictly uniparental inheritance (Birky 1978, 1983, 1994). In most sexual eukaryotes the mitochondria are inherited maternally, in asexuals transfer is clonal. However, low levels of paternal transmission (paternal leakage) have been described in *Armillaria* species (Smith *et al.*, 1990). Various mechanisms have been proposed to explain the predominantly uniparental transmission of mitochondria in sexual crosses: (1) an active digestion or methylation-restriction model, involving a post-fusion killing effect; (2) selective silencing of mitochondria of one of the parents; (3) a multicopy model with unequal numbers of mitochondria of the parents and (4) a special kind of anisogamy (Birky, 1994).

Recombination of mtDNA during the parasexual cycle was described for heterokaryon compatible *Aspergillus nidulans* strains (Rowlands and Turner, 1974, 1975). Also in other laboratory studies on fungi and in natural populations of *Armillaria gallica* recombination in mtDNA has been observed (Saville *et al.*, 1998). Mitochondrial transmission and recombination can also occur after protoplast fusion between heterokaryon incompatible *A. nidulans* strains and closely related species belonging to the section *Nidulantes* (Croft *et al.*, 1980; Earl *et al.*, 1981; Turner *et al.*, 1982; Croft and Dales, 1983, 1984; Gams *et al.*, 1985). The mixed mitochondrial population in heterokaryotic cells rapidly stabilised as homoplasmons (Croft *et al.*, 1980; Earl *et al.*, 1981). In (natural) isolates of *A. nidulans* no mtDNA (RFLP) polymorphisms were detected (Croft, 1987; Coenen *et al.*, 1996).

In the black *Aspergilli* the mtDNA restriction patterns show considerable variation. The mitochondrial genomes of both *A. carbonarius* and *A. japonicus* (~50 kb) are larger than those of the other black *Aspergilli* (~30-35 kb) (Varga *et al.*, 1994a). With mitochondrial RFLPs at least 3 distinct *A. carbonarius* types, 5 *A. niger* types, 6 *A. tubingensis* types, a *A.*

brasiliensis type and 7 types *A. japonicus/A. aculeatus* can be recognised (see Figure 1.1) (Varga *et al.*, 1993; 1994a; Kevei *et al.*, 1996; Hamari *et al.*, 1997).

To enable the study of mitochondrial recombination and transfer a mitochondrial oligomycin resistance has been selected in an *A. niger* culture collection strain N402 (mt-type 1a). Via protoplast fusions resistant mitochondrial recombinants and rare transfer of an unchanged parental mtRFLP profile from haplotype 1a to different black *Aspergillus* (1, 2 and 3) types could be selected (Kevei *et al.* 1997). Some of these strains could be used for anastomoses with nuclear isogenic oligomycin-sensitive strains. Spontaneous mitochondrial recombination was found in compatible combinations of strains. The mixed mitochondrial populations seemed to influence the compatibility reactions negatively: heterokaryon compatible strains with different mitochondria resulted less frequently in heterokaryons, which also showed poor growth in comparison to the control (Tóth *et al.* 1998).

1.4.2 Plasmids

Plasmids are (autonomously replicating) extrachromosomal DNA molecules. They replicate separately from the genome, but some can integrate covalently into the genome and replicate as part of genomic DNA. In 1967 the first plasmids were discovered in yeast; since the 80s they have been found in filamentous fungi as well. Since then plasmids are known to be relatively common in bacteria and fungi, whereas they occur rarely in plants and not at all in animals (Hardy, 1981; Esser *et al.*, 1986).

In fungi two types of plasmids are found: circular (covalently closed) and linear double-stranded DNA plasmids. Nearly all discovered plasmids, especially those in filamentous fungi, are located in the mitochondria (for reviews see Griffiths, 1995; Kempken, 1995b; Meinhardt *et al.*, 1990). So far the only natural nuclear plasmids found were the 2 μm plasmid of *Saccharomyces cerevisiae* and similar plasmids in related yeasts and the Ddp1 plasmid of the slime mould *Dictyostelium discoideum* (Esser *et al.*, 1986). The *killer*-plasmids in *Kluyveromyces lactis* are cytoplasmic.

The linear plasmids share several features: They code for both a DNA-polymerase and a RNA polymerase and contain terminal inverted repeats (TIRs), and are protected at their 5' ends by proteins. The DNA-polymerase is most similar to viral DNA polymerases. The RNA-polymerase in the mitochondrial plasmids resembles a bacteriophage RNA polymerase (possibly a remnant from the endosymbiotic origin of mitochondria). Also the TIRs are reminiscent of the genomes of some DNA viruses (Griffiths, 1995; Kempken, 1995b; Meinhardt *et al.*, 1990). The replication of the circular plasmids may involve a rolling circle mechanism, which would use a DNA polymerase. However, the circular Mauriceville plasmid in *Neurospora* was found to code for a reverse transcriptase (Michel and Lang,

1985) and could be a mobile intron, capable of insertion by reverse transcription, a property shared by retrotransposons (Lambowitz, 1989; Griffiths, 1995).

Little is known about the effects of plasmids on their host's phenotype. Exceptions are the linear and circular plasmids that by integrating into the mitochondrial genome cause senescence (or are associated with longevity) in *Neurospora* sp. and *Podospora anserina* and the *killer*-plasmids in *K. lactis*. However, for most plasmids no measurable effects have been observed yet, neither a negative effect caused by the genetic or metabolic burden placed on the host's mycelium, nor any selective advantage (Griffiths, 1995).

Transfer of the mitochondrial plasmids is generally together with the mitochondria and mitochondrial genome to the (sexual) offspring. Some paternal leakage of mitochondria and plasmids can occur and plasmids can also enter via the cytoplasmic 'back door' after anastomoses between mycelia (Van der Gaag *et al.*, 1998; Debets and Griffiths, 1998). In *Neurospora* plasmid-specific suppression mechanisms have been found in sexual crosses (Griffiths *et al.*, 1992). Between heterokaryon compatible *Neurospora* strains horizontal intermycelial transmission of plasmids occurs readily, but incompatibility slows this transfer (Griffiths *et al.*, 1990; Debets *et al.*, 1994). At asexual spore formation occasionally plasmids may fail to get included into a spore.

Some of the plasmids are widely distributed both within and between species. This could be due to vertical descent, but paternal transmission and horizontal transfer may also contribute. In some of the possible cases of horizontal transfer, the considerable amount of variation in related plasmids indicates that these transfers did not occur recently (Griffiths *et al.*, 1990; Collins and Saville, 1990; Kempken, 1995b). Most of the different plasmids were found to be distributed in patterns that were statistically independent, suggesting that the plasmids are freely mobile and can take up any association and coexist (Griffiths, 1995). An exception to the free distribution is the circular (satellite or defective) *Neurospora* plasmid VS that depends on the Varkud plasmid for its replication (Griffiths 1995). Griffiths and Yang (1995) have shown that circular and linear plasmids may recombine with one another.

In *Aspergillus* so far no natural plasmids have been detected, but 'artificial' plasmids have been obtained. Cloning vectors used to transform filamentous fungi are generally bacterial plasmids into which fungal genes have been inserted to act as selectable markers. Recently, a 6.1 inverted repeat sequence AMA1 (Autonomously Maintained in *Aspergillus*) has been isolated from *A. nidulans*. This AMA1 confers autonomous replication on plasmids that are normally strictly integrative (Gems *et al.*, 1991). Plasmids containing the AMA1 sequence increase the transformation frequency significantly in both *A. nidulans* (Gems *et al.*, 1991; Gems and Clutterbuck, 1993) and *A. niger* (Verdoes *et al.*, 1994). This laboratory-derived autonomously replicating (AR) plasmid (pAB4-ARp1) is confined to the nucleus

and appears to be mitotically highly unstable (Gems *et al.*, 1991; Verdoes *et al.*, 1994). In heterokaryons of *A. nidulans* and *A. niger* the AR plasmid can be transferred between nuclei (Aleksenko and Clutterbuck, 1995; Debets, 1998). Transfer could occur via the cytoplasm or perhaps more likely via transient contact between (dissimilar) nuclei (Debets, 1998). The described rate of AR plasmid transfer between nuclei indicates that when heterokaryons are formed exchanges between nuclei may occur very frequently.

1.4.3 Transposable Genetic Elements.

Transposable genetic elements are recently discovered mobile genetic units that can insert into a chromosome, exit and relocate. Transposable elements include insertion sequences, transposons, some phages, and controlling elements. They are ubiquitous in both prokaryotic and eukaryotic organisms and are a common cause of spontaneous genetic changes that can have wide ranging effects on the biology of the organisms (Döring and Starlinger, 1986; Green, 1988; Smith and Corces, 1991; McDonald 1993). Since the '90's functional transposable elements have been detected in fungi, especially in field isolates of phytopathogenic fungi characterised by a high level of genetic variability (Kistler and Miao, 1992; Dobinson and Hamer, 1993). The genetic variability observed in the asexual *Fusarium oxysporum* species has been postulated to be caused by the activity of transposable elements, of which many different types have been detected in the species (Daboussi *et al.*, 1992). Many authors suggest that the ubiquitous presence of transposable elements reflects a role in the speciation and adaptation of natural populations (review: McDonald, 1992).

The transposable elements are divided into two major classes based on their mode of propagation (Finnegan, 1989). The class I elements transpose by reverse transcription of an RNA intermediate. This class is subdivided into the retrotransposons, which have long terminal repeats (LTRs), and the LINE and SINE-like (Long and Short Interspersed Nuclear Elements) group of retroelements without LTRs. The second class of transposable elements transpose by a DNA-DNA mechanism and can be divided into elements with short inverted terminal repeats (ITRs) and elements with ITRs of variable length. Representatives of both classes of transposable elements are found in fungi. Some examples are listed in Table 1.1.

The two major strategies used to identify the transposable elements in fungi are (1) the characterisation of dispersed repetitive sequences and (2) the molecular analysis of spontaneous (instable) mutants. The former technique led to the identification of mainly retrotransposons and retroelements, whereas most of the class II DNA transposon of different types have been isolated after transposition in the nitrate reductase structural gene *niaD*.

Table 1.1 Classification of different transposable elements and some examples found in fungi.

Transposable elements		
Class I *reverse transcription via RNA*		**Class II** *transcription via DNA-DNA mechanism*
Retrotransposons (with Long Terminal Repeats)	**LINE** Long Interspersed Nuclear Elements	**elements with short ITRs** (Inverted Terminal Repeats)
Foret - F. oxysporum (Julien *et al.*, 1992) *grh - Magnaporthe grisea* (Dobinson *et al.*, 1993) *CfT-1 - Cladosporium fulvum* (McHale *et al.*, 1989, 1992) *Afut1 - Aspergillus fumigatus* (Neuvéglise *et al.*, 1996)	*Tad - Neurospora crassa* (Hamer *et al.*, 1989) *Palm - F. oxysporum* (Mouyna *et al.*, 1996)	*impala - F. oxysporum* (*Tc1/mariner* superfamily) (Langin *et al.*, 1994) *Ant1 - A. niger* (*Tc1/mariner* superfamily) (Glayzer *et al.*, 1995) *Fot1 - F. oxysporum* (*Fot1* family) (Daboussi *et al.*, 1992) *Pot1 - M. grisea* (*Fot1* family) (Kachroo *et al.*, 1994)
	SINE Short Interspersed Nuclear Elements	
	MGSR1 and *Mg-SINE - M. grisea* (Sone *et al.*, 1993, Kachroo *et al.*, 1995).	

Transposable elements have the ability to induce mutations due to their transposition. They can promote changes in gene expression, in gene sequence and probably in chromosome structure (Berg and Howe, 1989). In *N. crassa* insertion of the *Tad* element may create an unstable allele (Cambareri *et al.*, 1996). An alteration of transcription in the target gene was also demonstrated in *F. oxysporum* with the insertion of *Fot1* in the *niaD* gene (Daboussi and Langin, 1994). *Fot1* may also leave a footprint of two or three base pairs, leading to an often disfunctional protein due to frameshifts or an extra amino acid in the protein (Daboussi *et al.*, 1992). Karyotypes of fungi can be quite variable in several plant and human pathogens (Skinner *et al.*, 1991; Kistler and Miao, 1992). The translocations, deletions and duplications involved may be caused by inter- and intrachromosomal ectopic exchanges between transposable elements (Daboussi, 1996). So far in fungi only a small part of the existing transposable elements and the genome variation caused by them seem to have been detected. In *F. oxysporum* seven families have been identified, some of which seem concentrated in different genomic regions where they appear intermingled with and nested in other functional and degenerate transposable elements (Hua-Van *et al.*, 1998).

In some (sexual) species silencing processes may control the activity of transposable elements - of course sex and karyogamy may be the causes of infection. The Repeat-Induced Point mutation (RIP) process in *N. crassa* (Selker and Stevens, 1985; Selker *et al.*, 1993) and the Methylation Induced Premeiotically (MIP) process in *Ascobolus immersus* (Goyon and Faugeron, 1989; Rhounim *et al.*, 1992) deactivate linked and unlinked duplicated sequences. Species without sexual reproduction (and with strict vegetative incompatibility) may avoid transposable elements and may not need silencing processes.

Transfer of transposable elements may occur vertically as well as horizontally. The phylogenetic analysis of retrotransposons of the *gypsy* class shows that transmission of

transposable elements occurs vertically from parent to offspring, since a common ancestor of fungi obviously had retrotransposons (Daboussi, 1996). Some sporadic distributions of transposable elements in species or subgroups and similarities between elements in distant species indicate that horizontal transmission can occur as well (Kinsey, 1990a; Dobinson *et al.*, 1993; Capy *et al.*, 1994; Daboussi and Langin, 1994; Kempken *et al.*, 1998).

In *A. niger* three transposable elements have recently been isolated. The first, *A. niger* transposon-1 (*Ant1*) was isolated via transposon trapping within the coding region of the nitrate reductase gene (*niaD*) of *A. niger* strain N402 (Glayzer *et al.*, 1995). The element had inserted at a TA site and had duplicated the target site upon insertion. The element is 4798 bp long and contains 37 bp inverted, imperfect terminal repeats (ITRs). Sequence homology and structural features of the ORF1 open reading frame indicated that the element is related to the *Tc1/mariner* group of DNA transposons. Another sequence within the central region of the element showed similarity to the 3' coding and downstream untranslated region of the *amyA* gene of *A. niger*. *Ant1* was present as a single copy in the laboratory strain N402. Obviously this mobile transposon can change gene activities by insertion and by leaving (TA) footprints. It may also have the ability to transfer (parts of) nuclear genes.

The *Vader* transposable element was identified as well by screening unstable *niaD* mutants (Amutan *et al.*, 1996).The examined *A. niger* var. *awamori* strain used harboured approximately 15 copies of this element. *Vader* is 437 bp long and flanked by 44 bp inverted repeats (IR). Like *Ant1*, insertion of the *Vader* element causes a 2 bp (TA) duplication of the target sequence. The AT-rich *Vader* does not contain an open reading frame and hence it is deduced that the mobility of *Vader* is dependent upon a transposase activity present elsewhere in the genome.

The search for a transposase for the *Vader* element resulted in the discovery of a third transposable element: transposon *A. niger*-1 (*Tan1*) (Nyyssönen *et al.*, 1996). *Tan1* provides the transposase activity for the numerous mobile copies of *Vader* dispersed in the genome, but is only present in single copy in the genome. The *Tan1* element is 2.3 kb long and has a unique organisation: IR-ORF-IR-IR-*Vader*-IR with the same IR as detected around single *Vader* elements. The single open reading frame encodes an transposase homologous to that of members of the *Fot1* family, indicating that both *Tan1* and *Vader* are members of this family. The *Vader* element may act as an AT-rich terminator of transcription for the transposase gene. *Tan1* also duplicates TA at the target site.

All three detected transposable elements in *A. niger* duplicate the dinucleotide TA at the target site. *F. oxysporum Fot1* (Daboussi *et al.*, 1992), *M. grisea Pot2* (Kachroo *et al.*, 1994) and most of the members of the *Tc1* superfamily (Doak *et al.*, 1994) cause the same duplication. The open reading frames of transposases coded by these elements also share two

motifs: a so-called DE dipeptide and a D35E region. These common features of transposable elements in species belonging to unrelated taxa are an indication of a common mechanism of transposition (Doak *et al.*, 1994; Kachroo *et al.*, 1994). The common features may also indicate horizontal transfer of a progenitor transposon (Nyyssönen *et al.*, 1996).

1.4.4 Mycoviruses.

Viruses can be defined as infectious agents that are invisible with the light microscope, small enough to pass through a bacterial filter, lacking a metabolism of their own and depending on living host cells for their multiplication, but encoding some of the genes necessary for their own reproduction. Often the virus DNA or RNA is protected by a protein coat. Since the first discovery of a virus in a fungal species, it has become clear that mycoviruses and virus-like replicons occur commonly in fungi (Buck, 1986; 1998; Nuss and Koltin, 1990; Ghabrial, 1994; 1998).

The mycoviruses are exceptional in that they do not have an extracellular phase in their multiplication cycle and are transmitted only by intracellular routes. Most of the mycoviruses have double stranded RNA (dsRNA) genomes, but single stranded (ss) RNA and DNA genomes have also been described (Buck, 1986; 1998). Many fungal viruses are enclosed in protein capsids, but a significant number lack a protein coat. Some of the viruses without capsid are associated with lipid-rich cytoplasmic vesicles, with mitochondria, or are found as complexes with an RNA polymerase in the cytoplasm (for a list see Buck, 1998). A protein coat may be essential for viruses in general to survive outside the host cell. The mycoviruses do not need this function, and other essential functions for mycovirus capsid proteins have been described. Wickner (1996) describes a yeast virus capsid protein, that provides both protection in the form of a subcellular compartment for transcription and replication, and has a catalytic function in decapping host messenger RNA (mRNA) in favour of the viral mRNAs.

Table 1.2 Different types of fungal viruses and their characteristics.

dsRNA viruses		ssRNA viruses
Totiviridae	*Partitiviridae*	*Barniviridae*
isometric ø 30-40 nm 1 dsRNA segment ~ viral/protozoan *totiviridae* ~ selfreplicating mRNA	isometric ø 30-35 nm 2 dsRNA segments ~ plant *cryptoviruses*	bacilliform in cytoplasm/mitochondria ~ plant +strand RNA viruses
Reoviridae	*La France Virus*	*Leviviridae*-like
isometric ø 60 nm 11 dsRNA segments	isometric ø 34 nm 9 dsRNA segments	naked ~ RNA bacteriophages
Satellite and defective RNAs		

Different mycovirus types can be identified on the basis of presence and shape of the protein coat and of their genome organisation (see Table 1.2). The *Totiviridae* have isometric

particles 30-40 nm in diameter and contain a single species of dsRNA, coding for both the capsid protein and a RNA-dependent RNA polymerase. The *Partitiviridae* isometric particles are 30-35 nm and the two or three monocistronic segments of dsRNA are encapsidated separately. A possible *reovirus* was detected in *C. parasitica* (Enebak *et al.*, 1994). All its 11 dsRNA segments are present in approximately equimolar amounts and packed together in a 60 nm isometric particle. The nine dsRNA segments of the La France virus in *Agaricus bisporus* are also associated with an isometric particle (34 nm diameter) (Van der Lende *et al.*, 1994), but it is still unknown how these are organised.

A single-stranded RNA virus with a bacilliform capsid (*Barnaviridae*) has been found in *A. bisporus* (Revill *et al.*, 1994). The last group, another group of ss and dsRNA replicons coding only for a RNA-dependent RNA polymerase and without protein capsid, has been found in cytoplasm and/or mitochondria of fungi. These replicons seem to be related to positive-stranded RNA bacteriophages of the *Leviviridae* family (Buck, 1998).

Two types of extra RNA fragments, satellite and defective RNAs, can be detected in association with 'helper' viruses, on which they depend for their replication. Both RNAs can potentially interfere with the replication of their helper virus and are likely to be widespread in populations of dsRNA mycoviruses. They contribute to the complexity of dsRNA profiles from individual fungi (Buck, 1998). The satellite RNAs are comprised largely of sequences that are distinct from those of their helper virus (Mayo et al, 1995). The satellite RNAs may encode proteins or may be non-coding. Some protein toxins are encoded for by satellite RNAs (e.g. in *Saccharomyces cereviciae*, Wickner, 1996). The defective RNAs are derived from their helper viruses, generally by internal deletions (e.g. in *Cryphonectria parasitica* hypovirus, Tartaglia *et al.*, 1986; Shapira *et al.*, 1991).

The effects that dsRNA mycovirus infections have on their hosts vary and seem either caused by virally coded products or by disturbances of the cell metabolism. The killertoxins coded by some viruses or satellite RNAs, can have a selective advantage on infected organisms in crowded environments (e.g. Wickner, 1996). Some plant pathogenic fungi are known to become hypovirulent due to infections with mycoviruses, which may affect a large number of cell processes (e.g. Nuss, 1993;1996). Other viruses can cause serious crop losses as for instance in *A. bisporus* (La France disease)(Van der Lende *et al.*, 1996) or in *Pleurotus* spp. (Go *et al.*, 1992; Stobbs *et al.*, 1994). Such mycovirus infections can reduce their hosts' fitness. Because the effects of many mycoviruses are not yet known and/or less conspicuous, such infections are often considered 'cryptic'.

Mycoviruses may be transferred both vertically and horizontally. The vertical transmission of viruses into basidiospores and ascospores of yeasts generally occurs with high efficiency. The often restricted transmission into the ascospores of filamentous ascomycetes stands in sharp contrast (Buck, 1998). Mitochondrial dsRNAs inherit

maternally, though healthy dsRNA-free mitochondria may be transmitted preferentially (Polashok and Hillman, 1994; Rogers *et al.*, 1986a). The vertical transmission into asexual spores is generally efficient. Horizontal transmission by hyphal anastomosis is in general limited to individuals in the same or closely related species. In some fungi virus transmission is considered efficient between individuals in the same vegetative compatibility group, but restricted between individuals of different VC groups (Buck, 1998). Mixed infections with different viruses may be common (Buck, 1986). Generally, viruses are expected to be compatible in the same cell if they are sufficiently distinct so as not to compete for the same replication proteins (Buck, 1998).

Table 1.3 Mycovirus infected *Aspergillus* species and references.

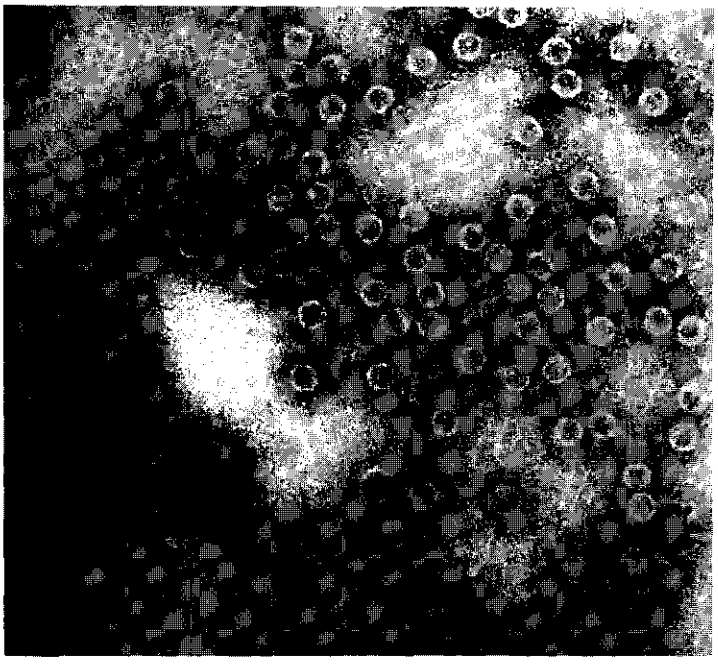
Aspergillus species		Author(s)
Asexual	*A. carbonarius*	Varga *et al.*, 1994b
	A. clavatus	Varga *et al.*, 1998
	A. flavus	Schmidt *et al.*, 1986; Elias and Cotty, 1996
	A. foetidus	Ratti and Buck, 1972; Buck and Ratti, 1975
	A. heteromorphus	Varga *et al.*, 1994b
	A. japonicus	*idem*
	A. niger	Buck *et al.*, 1973; Varga *et al.*, 1994b
	A. nomius	Elias and Cotty, 1996
	A. ochraceus	Kim and Bozarth, 1985; Varga *et al.*, 1998
	A. parasiticus	Elias and Cotty, 1996
	A. tamarii	*idem*
	A. tubingensis	Varga *et al.*, 1994b
Sexual	*Neosartorya hiratsukae*	Varga *et al.*, 1998
	Neosartorya quadricincta	*idem*
	Petromyces alliaceus	*idem*

The recognised groups of mycoviruses are probably of different evolutionary origins. Overall, the comparative sequence analysis strongly suggests that both RNA and DNA viruses have deep, archaic evolutionary roots both for genome structural organization and as regards certain genomic and protein domains (Holland and Domingo, 1998). In the *Totiviridae* comparisons of amino acid sequences of RNA-dependent RNA polymerases revealed relationships in viral and protozoal members of the *Totiviridae* (Bruenn, 1993). But also the theory of a cellular self-replicating mRNA as the origin of the monophyletic *Totiviruses* is attractive because of their apparent ancient origin, the close relationship among their RNA-dependent RNA polymerases, genome simplicity, and the ability to use host proteins efficiently (Holland and Domingo, 1998).The *Partitiviridae* of different fungi are distantly related to one another on comparison of amino acid sequences, but also show a relationship with plant cryptoviruses (Buck, 1998). On the other hand Holland and Domingo (1998) suggest that the *Partitiviruses* may originate from the *Totiviridae*. The ssRNA *Barnaviridae* appear to be distantly related to the plant positive-stranded RNA viruses of the genus *Luteovirus*, and the coatless ssRNA/dsRNA replicons resemble bacteriophage Qβ and

other *Leviviridae* (Buck, 1998). The modes of virus transmission suggest that mycoviruses could have been associated with a particular host species for long periods and that they co-evolved. But the observed relationships of viruses in different species or taxa also suggest the possibility of (rare) horizontal transmissions (Buck, 1998). The La France isometric virus appears to be of recent origin since it differs from its host in codon usage, but it may also have arisen from a *partitivirus* by acquiring additional genes (Holland and Domingo, 1998).

In the *Aspergilli* mycoviruses have mostly been found in species that are not known to reproduce sexually, but recently mycoviruses have also been detected in some sexual species (see Table 1.3). The viruses can be detected as dsRNA fragments after gel electrophoresis or as isometric particles with electron microscopy (see Figure 1.3). In most of these species the viruses are probably located in the cytoplasm. However, in *P. alliaceus* one viral fragment was located in the cytoplasm, another in the mitochondria and only the later transferred to the formed asci (Varga *et al.*, 1998). In general the viruses are readily transferred to the conidiospores, fairly commonly to ascospores formed by selfing but rarely to outcrossed ascospores (Coenen *et al.*, 1997; Varga *et al.*, 1998). In *A. nidulans* (*Emericella nidulans*) horizontal transfer can take place between vegetatively incompatible strains (Coenen *et al.*, 1997). In the black *Aspergilli* a large variety of dsRNA patterns was detected in infected strains (Varga *et al.*, 1994b).

Figure 1.3 Electron Microscopic view of isometric virus particles (ø 34-38 nm) in strain N076.



1.5 Outline of the Thesis.

Recently, different genetic elements were discovered in large numbers in fungi: mycoviruses, plasmids and transposable elements. Horizontal transfer between fungal strains carrying such possibly deleterious elements may have important consequences at the population level. It may lead to non-adaptive evolutionary processes, but also be responsible for genetic recombination in organisms lacking meiosis. Molecular approaches have become available for the detection of recombination in nuclei of species presumed to be asexual (e.g. Burt *et al.*, 1996) and between mitochondria, previously thought to be not recombining (e.g. Saville *et al.*, 1998).

This PhD project aimed at elucidating rates of horizontal transfer of genetic elements in natural populations of the black *Aspergilli*, a complex of imperfect species. In laboratory experiments isogenic black *Aspergilli* can be forced to recombine via the parasexual cycle, but little is known about recombination or genetic exchange in natural environments. To this end it is necessary to determine the genetic population structure, investigate what genetic elements are present in the population, how they affect individual fitness, and how and to what extent they can be transferred.

This first Chapter gives a general introduction to the asexual - possibly parasexual - black *Aspergilli*. It describes the mechanisms of heterokaryon incompatibility that seem to regulate the formation of anastomoses between hyphae of different mycelia and possibly control horizontal transfer rates, and gives an inventory of elements that may spread by horizontal transfer.

The second Chapter describes how natural populations of black *Aspergilli* were sampled world-wide and over several years, based on the special ability of black *Aspergilli* to degrade high concentrations of tannin. This resulted in a collection of over 600 isolates. The genetic structure of the populations was determined on the basis of mitochondrial RFLP patterns, and the occurrence of and variation in dsRNA mycoviruses was examined.

In Chapters 3, 4 and 5 the transfer of mycoviruses in the black *Aspergilli* and some other species is studied. Chapter 3 deals with heterokaryon incompatibility in a subset of the black *Aspergillus* isolates and its influence on virus transfer. 'Spontaneous' transfer of viruses is monitored between heterokaryon compatible and heterokaryon incompatible combinations of different black *Aspergillus* types in co-culture. In Chapter 4 the transfer within the black *Aspergilli* is compared with that in *Aspergillus nidulans* strains that were infected in the laboratory. Intra- and interspecies protoplast fusions between heterokaryon compatible and incompatible black *Aspergillus* strains and *A. nidulans* strains are described. Recombination of a mitochondrial oligomycin marker is used to ascertain cytoplasmic contact between the used black *Aspergilli*. The role of the heterokaryon incompatibility reactions in virus transfer is again examined and the possibility of virus resistance is tested.

The possibilities of interspecies virus transfer are further examined in Chapter 5, where protoplast fusions are described between naturally infected *Fusarium poae* strains and black *Aspergillus* strains.

Chapter 6 gives an inventory of the fitness effects of mycoviruses on different fitness-related traits like mycelial growth rate, spore production, and competitiveness. A model of the virus-infected black *Aspergillus* population is presented and the prerequisites for a stable virus infections in the population are discussed: deleterious infecting elements need horizontal transmission to be maintained in the population.

Recombination at the molecular level is tested in Chapter 7. A population of black *Aspergilli* is tested for the occurrence of (para)sexual recombination in nuclear genes. The phylogenies on the basis of sequences of several nuclear genes are also compared with sequence data of the *Ant1* transposon, mitochondrial and ribosomal RFLP data and mycovirus patterns, to test for molecular evidence of horizontal transfer of these elements. Chapter 8 summarises and discusses all results on horizontal transfer in natural populations of black *Aspergilli.*

2

Global epidemiology of black *Aspergilli* based on cytoplasmic elements.

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Abstract

A set of culture collection *Aspergillus* strains and black *Aspergilli* isolated on non-selective media were used to validate the use of media with 20% tannin for exclusive and complete selection of black *Aspergilli*. The 20% tannin medium proved useful for both quantitative and qualitative selection of all types of black *Aspergilli*. In this way 642 black *Aspergilli* from different populations were isolated from soil samples from different parts of the world, over a number of years. The density of black *Aspergilli* proved highest in tropical regions.

All isolates were classified according to their mitochondrial restriction fragment length polymorphism patterns, allowing recognition of different types of *A. carbonarius*, *A. japonicus*, and, within the *A. niger* aggregate, of different haplotypes of the two main groups *A. niger* and *A. tubingensis*. The most frequent *A. niger* and *A. tubingensis* haplotypes occur worldwide. Though *A. carbonarius* and *A. japonicus* were not found in all locations, they can occur in relatively high numbers.

Infections with dsRNA mycoviruses were found in approximately 10% of all strains, irrespective of sampling site, mitochondrial type, or year of sampling. This is one of the first studies on a global scale of the epidemiology of an asexual non-pathogenic fungal host and its mycovirus. The black *Aspergillus* population consists of many different clonal lineages with a highly efficient mode of dispersal, that obviously homogenizes the population world-wide and accounts for the high variety of strains per sampling site. The observed high density of black *Aspergilli* in connection with their unique ability to degrade high concentrations of tannin points to an important role in the nitrogen cycle in nature.

Introduction

The genetic population structure of a species provides valuable information on mating system, migration, and dispersion, and is indispensable for the control of pathogenic organisms. In general, random mating (sexual) populations are expected to show a higher degree of genotypic diversity than clonal asexual populations (Leung *et al.* 1993). The relative contributions of sexual and asexual reproduction will therefore influence the genetic structure of the population. The amount of gene flow will also contribute to the diversity.

The genetic epidemiology of fungi is relatively underdeveloped compared to plants and animals. The genetic population structure of a few, mainly plant pathogenic, fungal species has been determined with a variety of molecular techniques. For example, in *Ceratocystis fagacearum* low levels of variation were detected among isolates form a broad geographic area in the US. This was correlated with a recent introduction and founder effects (Kurdyla *et al.* 1995). The population of *Mycosphaerella graminicola* (*Septoria tritici*) proved to be more random-mating than clonal during the course of an epidemic and the variation in populations was similar around the world (McDonald *et al.* 1995 and Chen & McDonald 1996). In this paper we look at the populations of black *Aspergilli*.

The first '*Aspergillus niger*' was described by van Tieghem in 1867 as a fungus capable of using tannin as carbon source. Rippel reported in 1939 the exclusive selection of *Aspergillus niger* on high concentrations (20%) of tannin, but this characteristic seems to have been largely forgotten since. Nowadays *A. niger* is known to be part of a complex group of black imperfect filamentous fungi, many of which of industrial importance. The black *Aspergilli* show a wide range of variability in morphological and physiological characteristics, but share the characteristic of black conidiospores on aspersory-(mop)-like conidiophores (Raper & Fennel 1965, Al-Mussallam 1980). The spores of these mainly saprophytic but occasionally pathogenic fungi are distributed by air and the fungus can be isolated from a large variety of substrates. The black *Aspergilli* form a substantial part of the total *Aspergillus* and fungal populations (e.g. Manoharachary 1977, Rao & Venkateswarlu 1983, Ploetz *et al.* 1985, Misra & Jamil 1991). They occur world-wide with a preference for tropical and subtropical areas (Rippel 1939, Raper & Fennel 1965, Domsch *et al.* 1980).

Within the black *Aspergilli*, *Aspergillus carbonarius* and *Aspergillus japonicus* form two distinct types based on distinct morphological characters (Raper & Fennel 1965). The remaining strains or simply '*Aspergillus niger* aggregate', can be further divided into two main groups *Aspergillus niger* and *Aspergillus tubingensis* on the basis of molecular analyses like (ribosomal/mitochondrial) Restriction Fragment Length Polymorphisms (RFLPs), isozymes and Random Amplification of Polymorphic DNA (RAPDs) (Kusters-van Someren *et al.* 1991, Mégnégneau *et al.*, 1993, Varga *et al.* 1993, 1994a).

The black *Aspergilli* are asexual, but under laboratory conditions related, vegetative compatible strains can form heterozygous somatic diploids. Mitotic recombinants can be obtained via this so-called parasexual cycle (Pontecorvo *et al.*1953). However, vegetative compatibility between natural isolates is very rare (Van Diepeningen *et al.* 1997; Chapter 3) and it is unknown whether and to what extent parasexual recombination occurs in natural populations.

The aim of these experiments was to obtain a picture of the genetic variation in populations of the asexual black *Aspergilli* on a geographic scale. Several populations were sampled in different places worldwide by the selective isolation of all present black *Aspergilli*. The distribution of black *Aspergillus* types was based on mtRFLP type. The distribution of dsRNA viruses was also analysed and correlated with host mtDNA type.

As a first characterisation method for the populations we chose mitochondrial RFLPs. In general mtRFLPs show less variation than nuclear markers (Kurdyla et al 1995, McDonald et al 1995), but in the black *Aspergilli* the mitochondrial RFLP classification corresponds exactly to Mégnégneau's (*et al.* 1993) nuclear based classifications (personal data) and the technique is easy to perform on large numbers of isolates. Using mitochondrial RFLP data fourteen types can be detected within the two *niger*-aggregate groups (Varga *et al.* 1993, 1994a) and *A. carbonarius* and *A. japonicus* strains also have their characteristic mitochondrial RFLP's (Kevei *et al.* 1996, Hamari *et al.* 1997).

As second population characteristic dsRNA virus infections were used. Mycoviruses or fungal viruses are parasitic cytoplasmic elements that are frequently found in all classes of fungi, including *Aspergilli* (Buck 1986; 1998). Because dsRNA viruses are not viable outside the fungal mycelium, infection has to involve intermycelial cytoplasmic contact. The mycoviruses found in black *Aspergilli* consist of an isometric protein coat and variable dsRNA molecules (Varga *et al.* 1994b). No virus infected strains of sexual *Aspergillus nidulans* were found in nature (Coenen *et al.* 1997). However, in sexual and asexual *Aspergillus* isolates belonging to the sections *Fumigati* and *Circumdati* the frequency varied from 3.5 to 8.3 percent infected with mycoviruses (Varga *et al.* 1994b, 1998). In the *Aspergilli* of the section Flavi 10.9% was found to be infected (Elias & Cotty 1996); in contrast in the asexual *Fusarium poae* all tested isolates were infected (Fekete *et al.* 1995). The dsRNA mycoviruses in the black *Aspergilli* are stably maintained during subculturing without observed change in fragment patterns or loss of infection. The lateral transfer of viruses is blocked by the often found heterokaryon incompatibility between black *Aspergilli* (Van Diepeningen *et al.* 1997; Chapter 3). This incompatibility barrier seems much stronger than in other species and even between black *Aspergilli* and other species (Van Diepeningen *et al.* in press; Chapter 4).

The data on mtRFLPs and dsRNA mycovirus infections show a well-mixed global population with an infection frequency of approximately 10% in all black *Aspergillus* haplotypes. The absence of local differentiation and the amount of variation in populations can be an indication of the geneflow between populations.

Materials and Methods

Growth conditions and isolation of strains

All strains were incubated at 30°C and grown on either minimal medium (MM) or complete medium (CM) (Pontecorvo *et al.* 1953) with 1 $mg \cdot l^{-1}$ $ZnSO_4$, $FeSO_4$, $MnCl_2$ and $CuSO_4$ extra added. For the selective isolation of black *Aspergilli* 20% (W/V) tannin (Merck) was added to complete medium (CM + tan) (Rippel 1939). Samples of the undisturbed top-layer of soil and humus (5-50g) were collected worldwide between 1990 and 1995 and used as inoculum. Depending on the spore density in the samples, aliquots (0.01-1g) or dilutions were put on the selective CM + tan. The black *Aspergillus* colonies floating on this very acidous (pH≈2), liquid medium were further purified on solid MM. Each isolate was given a code indicating isolation site, year and number.

Strains

For the experiments three sets of black *Aspergilli* have been used. The first set contained culture collection strains obtained from the CBS (Baarn, the Netherlands). A list of these black *Aspergilli* and the colour mutants that we derived from these is given in Table 2.1. The second group consists of black *Aspergillus* strains isolated on non-selective media. This set contains 26 non-pathogenic strains isolated in and around hospitals in the Netherlands (Z 1.1- Z 2.25), 15 English strains (814-828) and 6 Indonesian strains (no numbers). The hospital strains are included in Table 2.2. The third and largest set of strains contains isolates selected on CM + tan from all over the world. An inventory of these strains is given in Table 2.2.

Nucleic acid (DNA and RNA) isolation

Total nucleic acids were isolated from fresh mycelial cultures (» 0.1 g wet weight), grown overnight from spores in liquid CM in test tubes. The mycelium was transferred to a 1.5 ml Eppendorf tube, frozen with liquid nitrogen and disrupted with a special pestle (size of a 0.5 ml Eppendorf tube, fitting exactly in the bigger vial). A phenol/chloroform extraction was performed after Maniatis *et al.* (1982). The nucleic acid isolations were used for determining virus content and characterisation of the mitochondrial haplotype.

Table 2.1 List of used culture collection strains with their CBS or ATCC number and name, available conidiospore colour mutants and the ability to grow on 20% tannin medium..

strain	code[a]	official name	colour mutants[b]	20% tannin
N050	CBS 111.26	*A. carbonarius*	*fwn, brn*	+
N051	CBS 112.80	*A. carbonarius*		+
N052	CBS 707.79	*A. ellipticus*		+
N053	CBS 677.79	*A. helicotrix*		+
N055	CBS 114.51	*A. japonicus*	*fwn, brn, whi, gry*	+
N056	CBS 621.78	*A. japonicus*		+
N057	CBS 172.66	*A. jap. aculeatus*	*fwn, brn, whi*	+
N058	CBS 115.80	*A. jap. aculeatus*		+
N059	CBS 554.65	*A. niger*	*fwn*	+
N061	CBS 134.48	*A. niger*		+
N062	CBS 557.65	*A. awamori*	*fwn, brn*	+
N063	CBS 563.65	*A. awamori*		+
N064	CBS 126.49	*A. phoenicis*	*fwn, brn*	+
N065	CBS 135.48	*A. phoenicis*		+
N066	CBS 136.52	*A. nanus*		+
N067	CBS 131.52	*A. nanus*	*fwn, brn, gry*	+
N068	CBS 139.52	*A. usami*	*fwn, brn*	+
N069	CBS 553.65	*A. usami*		+
N070	CBS 117.32	*A. intermedius*	*fwn, brn*	+
N071	CBS 118.35	*A. hennebergii*		+
N072	CBS 125.52	*A. hennebergii*		+
N073	CBS 558.65	*A. niger pulverulentus*		+
N074	CBS 425.65	*A. niger pulverulentus*		+
N075	CBS 121.28	*A. foetidus*		+
N076	CBS 681.78	*A. foetidus*	*fwn, brn*	+
N226	ATCC 1015	*A. niger*		+
N400	ATCC 9029	*A. niger*	*fwn, brn, gry, olv*	+
A001	-	*A. flavus*		-
A002	-	*A. ochraceus*		-
A003	-	*A. candidus*		-
A004	CBS 567.65	*A. candidus*		-
A005	CBS 225.80	*A. candidus*		-

[a] CBS = Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; ATCC = American Type Culture Collection, Rockville, MD USA. [b] conidiospore colours: *fwn* = fawn, *brn* = brown, *whi* = white, *gry* = grey and *olv* = olive green.

Determination of dsRNA virus content

To test the strains for virus infection, part of the total nucleic acid solution was run on a 0.8% agarose gel, stained with ethidiumbromide and examined by transillumination with UV. This technique clearly separates the DNA, dsRNA and ssRNA and the viral fragment patterns are easily distinguished without standard isolation of viruses by e.g. ultracentrifugation of mycelium. λHindIII/EcoRI or Boehringer DNA molecular weight marker X was used as molecular weight marker. The sizes of the dsRNA fragments were estimated correcting for the differences in mobility of DNA and dsRNA (Livshits *et al.* 1990). To confirm the dsRNA nature of the viruses, nucleic acid solutions were treated with DNase and RNase under low and high salt concentrations (Varga *et al.*, 1994b).

Table 2.2 List of the natural isolates; all strains were isolated on tannin except the strains isolated near Dutch hospitals. The sites, where soil/humus was collected, are listed from North to South. The number of black *Aspergillus* propagules per sample was also estimated on tannin medium.

Country (North-South)	sample year(s)	locations (total samples)		code & number of strains (total strains)		density (# sp/g.)
Brazil	1994	*Santarem, Campos de Jordão and Iguaçu*	(3)	B 2.1-4.2	(41)	50-75
Canada	1993-1994	*Vancouver and Gauley's Bay*	(3)	Can 1.1-3.1	(11)	0-2
The Netherlands	1991-1994	*Delft, Rotterdam, Wijlre, Nijmegen and Wageningen*	(27)	D 1.1-27.1	(58)	0-8
The Netherlands 2	1993-1994	*near Hospitals in Gouda & Rotterdam**	(8)	Z 1.1-2.25	(26)	-.-
France	1991	*Normandy*	(4)	F 1.1-4.1	(9)	0-2
Switzerland	1993	*Basel and Guarda*	(2)	CH 1.1-2.1	(4)	0-3
Egypt	1992	*Cairo*	(2)	Eg 1.1-2.5	(24)	8-10
Israël	1991	*Jerusalem and Haifa*	(4)	I 1.1-4.1	(6)	2-3
Morocco	1991	*Agadir*	(2)	M 1.1-2.5	(10)	25-85
Eq. Guinea	1994	*Cacoloondo river*	(1)	Gu 1.1-1.9	(9)	65
Gabon	1994	*Ngounié, Ogooué-Maritime and Moyen-Ogooué*	(5)	G 1.1-5.16	(85)	40-60
Kameroon	1994	*South-West and South provinces*	(6)	K 2.1-7.4	(42)	25-150
Indonesia	1990-1994	*Jakarta*	(12)	Ind 1.1.1-1.12.45	(320)	50-250
Malaysia	1995	*Penang*	(1)	Mal 1.1-1.6	(6)	-.-
Nepal	1993	*Annapûrna Massif*	(1)	Nep 1.1-1.6	(6)	4-6
Australia	1994	*Orpheus island*	(1)	Au 1.1-1.4	(4)	8-15
New Zealand	1994	*Cambridge*	(2)	NZ 1.1-2.5	(8)	6-12

* Non-pathogenic strains isolated in and around hospitals on non-selective media.

Mitochondrial characterisation

The mitochondrial haplotypes of strains were determined by restriction enzyme analysis with BglII and HindIII (Varga *et al.* 1993, 1994a). The digestion mixtures were examined on 0.8% agarose in the same way as the virus mixture. The isolation of mitochondria and subsequent restriction enzyme analysis of the mitochondrial DNA gave the same gel electrophoresis patterns as the direct digestion of total DNA extracts. Therefore, analysis was routinely done on total nucleic acid extract or, when virus patterns overlapped with the mitochondrial patterns, RNase treated total extract. BglII/HindIII patterns were compared with Varga's restriction patterns to determine *A. niger* (1) and *A. tubingensis* (2) types (for a survey of these patterns see Varga *et al.*, 1994a). Strains that didn't belong to either of these two classes, were further analysed with a single digestion of total DNA with EcoRI and compared with *A. carbonarius* (C) and *A. japonicus* (J) patterns (Kevei *et al.* 1996; Hamari *et al.*, 1997). All examined natural isolates fell in one of the four described main classes.

Results

Isolation on 20% Tannin

In 1939 Rippel described the selective isolation of *A. niger* strains on 20% tannin. To test whether this method allows reliable and exclusive isolation of black *Aspergilli* throughout the whole range of the *A. niger* species complex, we tested 79 black *Aspergilli* and some related *Aspergillus* species. The 32 culture collection strains used covered the whole *A. niger* complex,

Aspergillus species. The 32 culture collection strains used covered the whole *A. niger* complex, *A. carbonarius* and *A. japonicus* and related strains (Table 2.1). Twenty-six Dutch hospital strains, 6 Indonesian and 15 English strains, all of which were not isolated on tannin medium, made up the rest of the test panel. We found that all black *Aspergilli* as well as the colour mutants isolated from these, but none of the other *Aspergilli*, can utilise the tannin as sole carbon source in the 20% tannin medium. Some of the black *Aspergilli* can endure concentrations of 60-80 % tannin, whereas the related (non-black) *Aspergillus* species can grow on media with a maximum of 10% tannin. At lower concentrations of tannin, soil samples yield a whole range of yeasts and other fungi. Since the method allowed both quantitative (spore density) and qualitative sampling of all black *Aspergillus* propagules in a soil sample, we decided to examine the natural populations of black *Aspergilli* by using isolation on 20% tannin.

Interesting to note is the fact that most *Aspergillus* determination keys separate the efficient tannin-degrading black *Aspergilli* on their conidiospore colour from related strains: A colour mutant of a black *Aspergillus* would not be recognised as such but it still has the tannin degrading ability. We suggest the ability to degrade high tannin concentrations as a more reliable taxonomic character. Spontaneous colour mutants of the different black *Aspergillus* types are easy to obtain in the lab, yet none were found among the natural isolates. The resulting colour depends on both spore wall morphology and pigmentation. Fawn and brown mutants are easy to obtain in all black *Aspergillus* isolates. White mutants were only and easily isolated in *A. japonicus* types, despite extensive search for them in other types. Olive and grey were only occasionally obtained.

Quantitative Collection of Populations

Strains were isolated from undisturbed soil and humus samples collected between 1990 and 1995 from sites all over the world (Table 2.2). Isolations were done within a few days from arrival of the sample in the lab and samples were stored at 5°C to enable isolation of more strains. Media were inoculated with small quantities of the sample (0.01-1g) or with dilutions in saline (10-100x). After 5-7 days all colonies floating on the medium were collected. Although colour mutants can easily be selected in the lab, no natural colour mutants were selected. The isolates were coded with a letter code for the country, a number for the sampling site and a serial number for the colony in a sample. Four sites in Jakarta were sampled successively every two years. These sites were numbered consecutively (1990:1.1-1.4, 1992: 1.5-1.8 and 1994: 1.9-1.12). In total 642 black *Aspergillus* strains were isolated on 20% tannin from 80 sites in 16 different countries.

The variation in black *Aspergillus* spore density ranges from very few spores per gram substrate in Canada to sites with over 250 spores on average per gram in Indonesia (see Table

2.2). In general, the spore density is maximal around the equator and decreases with increasing latitude. This distribution is in accordance with the data of Rippel (1939), who found that *A. niger* spore densities in Europe - from Norway to Hungary - increase with temperature.

With a Coulter counter and channelyzer relative spore sizes were measured of all Indonesian strains isolated in 1992. The smallest spores after mtRFLP typing proved to belong to the *niger*-types (± 3.5µm), whereas relatively bigger spores could be either *niger* or *tubingensis*-types (± 5µm). No natural diploids were found among the strains, though one strain produced mainly binucleate spores (judged by DAPI staining).

Characterisation of strains and populations by mtRFLP

Spatial and temporal variation in and between (sub)populations was examined using mitochondrial Restriction Fragment Length Polymorphisms (mtRFLP's) and dsRNA virus infections (next paragraph). The mtRFLP technique is easy to perform and distinct types can be recognised (Varga *et al.* 1993, 1994a, Hamari *et al.* 1997, Kevei *et al.* 1996). The data agree with those of ribosomal RFLP's, isozyme analyses and nuclear RAPD's (as done by Kusters-van Someren *et al.* 1991, Mégnégneau *et al.* 1993) (unpublished data). Via protoplast fusions resistant mitochondrial recombinants from different black *Aspergillus* types can be selected (Kevei *et al.* 1997). However, spontaneous mitochondrial recombination was only found between isogenic - closely related - heterokaryon compatible strains (Tóth *et al.* 1998).

The data on the mtRFLP characterisation are given in Table 2.3. Per country and/or sampling site the number of isolates with certain mitochondrial types are given (*A. niger* types (1a-1d), *A. tubingensis* types (2a-2f), *A. japonicus* (J) and *A. carbonarius* (C)). No novel mitochondrial restriction patterns were found in the tested strains compared to those of Varga *et al.* (1993, 1994a), Hamari *et al.* (1997) and Kevei *et al.* (1996). The total number of isolates per sample, the world-wide totals and the virus infection incidence are also given in Table 2.3.

The results in Table 2.3 can be summarised as follows: The proportion of *A. niger* strains (the 1-types) and the proportion of *A. tubingensis* strains (the 2-types) in the populations are about the same, perhaps with a slight predominance for *A. tubingensis* types in more temperate zones. Together, *A. niger* and *A. tubingensis* form nearly 90% of the isolates. *A. japonicus* and *A. carbonarius* strains form the rest of the isolates, where *A. japonicus* reaches relatively high numbers only in Gabon and the Ind 1.4 sample.

Within *A. niger* and *A. tubingensis* the mitochondrial types 1b&c and 2a&b are the most common types world-wide and tend to represent the major part of each (sub)population. The other types may occur world-wide but form a smaller fraction of the populations.

Only two types with a limited distribution were found in this examination. Type 1d was discovered only once in Indonesia in 1992. The 1d mitochondrial type may have evolved recently out of 1c mitochondria since their RFLP patterns are quite similar. Mitochondrial type

Table 2.3 Population structure per site or country and year based on occurring mitochondrial RFLP types (distinguishing between types 1a-1d: *A. niger* types, types 2a-2f: *A. tubingensis* types, *A.jap: A. japonicus* and *A. car: A. carbonarius*). Virus infections are given in parenthesis.

		mitochondrial type *(virus infections)*												
place and year		*A. niger*				*A. tubingensis*						*A. jap*	*A.car*	total
		1a	1b	1c	1d	2a	2b	2c	2d	2e	2f			black *A*'s
Brazil	1994	3	8*(1)*	20*(1)*	-	5	5	-	-	-	-	-	-	41*(2)*
Canada	1993	-	-	4	-	6	-	-	-	-	-	-	-	10
Netherlands1	1991-4	1	6*(1)*	5*(2)*	-	40*(4)*	6	-	-	-	-	-	-	58*(7)*
Netherlands2	1993-4	5	4*(1)*	1	-	13*(1)*	1	1	1	-	-	-	-	26*(2)*
Netherlands total		6	10*(2)*	6*(2)*	-	53*(5)*	7	1	1	-	-	-	-	84*(9)*
France	1991	3	-	1	-	-	2	-	-	-	-	3	-	9
Zwitserland	1993	-	-	-	-	2	1	1	-	-	-	-	-	4
Egypt	1992	-	7	7	-	10	-	-	-	-	-	-	-	24
Israel	1991	-	1	-	-	4*(1)*	-	-	-	-	-	-	1	6*(1)*
Morocco	1991	-	-	-	-	6	2*(1)*	-	-	-	-	1	1	10*(1)*
Eq. Guinea	1994	-	6	3*(3)*	-	-	-	-	-	-	-	-	-	9*(3)*
Gabon	1994	3	24*(4)*	5*(1)*	-	4*(1)*	7	1	-	1*(1)*	-	40*(5)*	-	85*(12)*
Kameroon	1994	1	16*(1)*	6	-	1	2	6	-	-	-	9	1	42*(1)*
Ind 1.1	1990	-	1*(1)*	1	-	7	3	-	-	3	-	4	1	20*(1)*
Ind 1.2	1990	-	-	1	-	3	1	-	-	2	-	3	4	14
Ind 1.3	1990	-	1	3	-	-	-	-	-	-	-	2	-	6
Ind 1.4	1990	2	1	5*(1)*	-	4	7	-	-	1	-	10*(1)*	2*(1)*	32*(3)*
Indonesia 1990		2	3*(1)*	10*(1)*	-	14	11	-	-	6	-	19*(1)*	7*(1)*	72*(4)*
Ind 1.5	1992	1	6*(2)*	4	-	-	-	-	-	-	-	1	-	12*(2)*
Ind 1.6	1992	1	22*(1)*	2	-	4*(2)*	-	-	-	-	-	-	-	29*(3)*
Ind 1.7	1992	1	2	3*(1)*	-	-	-	1	1	-	-	2*(1)*	-	10*(2)*
Ind 1.8	1992	9*(1)*	22*(6)*	22*(2)*	1	12*(1)*	15	2	2*(1)*	-	-	-	-	85*(11)*
Indonesia 1992		12*(1)*	52*(9)*	31*(3)*	1	16*(3)*	15	3	3*(1)*	-	-	3*(1)*	-	136*(18)*
Ind 1.9	1994	3	4	7*(2)*	-	3*(1)*	2	-	3	4*(1)*	-	-	-	26*(4)*
Ind 1.10	1994	-	9	7	-	11*(3)*	2	-	-	-	1	-	-	30*(3)*
Ind 1.11	1994	4	1	5	-	-	-	1	-	-	-	-	-	11
Ind 1.12	1994	6	8*(2)*	14	-	6	3*(1)*	-	3*(1)*	4*(1)*	-	1	-	45*(5)*
Indonesia 1994		13	22*(2)*	33*(2)*	-	20*(4)*	7*(1)*	1	6*(1)*	8*(2)*	1	1	-	112*(12)*
Indonesia total		27*(1)*	77*(12)*	74*(6)*	1	50*(7)*	33*(1)*	4	9*(2)*	14*(2)*	1	23*(2)*	7*(1)*	320*(34)*
Malaysia	1995	-	5	-	-	1	-	-	-	-	-	-	-	6
Nepal	1993	-	6	-	-	-	-	-	-	-	-	-	-	6
Australia	1994	-	-	-	-	-	-	-	-	-	4	-	-	4
New Zealand	1994	-	7	-	-	1*(1)*	-	-	-	-	-	-	-	8*(1)*
World total		43*(1)*	167*(21)*	126*(13)*	1	143*(15)*	59*(2)*	13	10*(2)*	15*(2)*	5	76*(7)*	10*(1)*	668*(64)*

2f was so far only detected in Australia (this paper and Varga *et al.*, 1994a) and in 1994 also in Indonesia, suggesting a mitochondrial type of possibly Australian origin and with a recent expansion to Indonesia.

Four sites in Indonesia were sampled every two years; sample Ind1.8 was examined more extensively (85 strains); from the other sites roughly the same amount of soil was examined each year of sampling. Spore densities per sample stayed approximately the same. The first year relatively many *A. japonicus* and *A. carbonarius* strains were found. The distribution of the different haplotypes in the other two years was found to be more similar.

dsRNA Virus Infection

All isolates were tested for the presence of dsRNA viruses, which are easily and accurately detectable as bright and distinct bands with gel electrophoresis (see Figure 2.1). With electron microscopy virus particles could also be made visible in mycelium fractions of infected strains. In Table 2.3 the number of infected strains per sample and mitochondrial type is given. Viruses are present in nearly all black *Aspergillus* types found. Only the single 1d strain, the thirteen 2c and the five 2f-strains showed no virus infections.

The data on virus infection frequencies were tested with Chi^2-tests (Sokal & Rohlf 1995). Overall, dsRNA virus was present in 9.6% of the isolates in this assay. For the 95% confidence interval the lower and upper confidence limits can be set at 7.6% and 12% respectively. Only the small Equatorial Guinea sample deviates significantly from an overall infection frequency of approx. 10% infection. This average infection frequency of 10% seems to hold for all black *Aspergilli*, irrespective of their mitochondrial type, and the infections are evenly spread throughout the population worldwide.

There is considerable variation in the dsRNA patterns in the different strains (see Figure 2.1). In the 63 infected strains 50 different patterns were found. Sizes of the dsRNA fragments range from 0.5 to 4.4 kb, when calculated according to Livshits *et al.* 1990. In the examined infected black *Aspergilli* 1 to 8 different sized fragments were detected per strain. Some fragments are found as single infecting fragments in some strains (e.g. the fragment of approx. 4.4 kb in strains Z1.1, Ind 1.7.8 (Fig. 2.1: lanes 6&7) and M 2.2 or the fragment of approx. 3.2 kb in Ind 1.8.22 (lane 9)) and can be seen in other strains with extra bands (e.g. the 4.4 fragment in patterns in Fig. 2.1: lanes 2-5&10; the 3.2 kb fragment in the patterns in lanes 11&12). Sometimes more of the fragments similar in size to those that can occur on their own are found in the same strain (Strain N330 (lane10) contains both the 4.4 and the 3.2 kb fragment). These data indicate that multiple infection with different independent viruses and/or satellite and defective dsRNA fragments may occur in the black *Aspergilli*. If the similar sized fragments in the different strains are indeed related, it would mean that the different viruses (e.g the 2.2 kb virus) are well spread both throughout the world (Europe, Africa and Indonesia) and

throughout the different haplotypes (*niger*, *tubingensis* and *japonicus* types). If the similar sized fragments are not related, then the variety of dsRNAs is even more extensive.

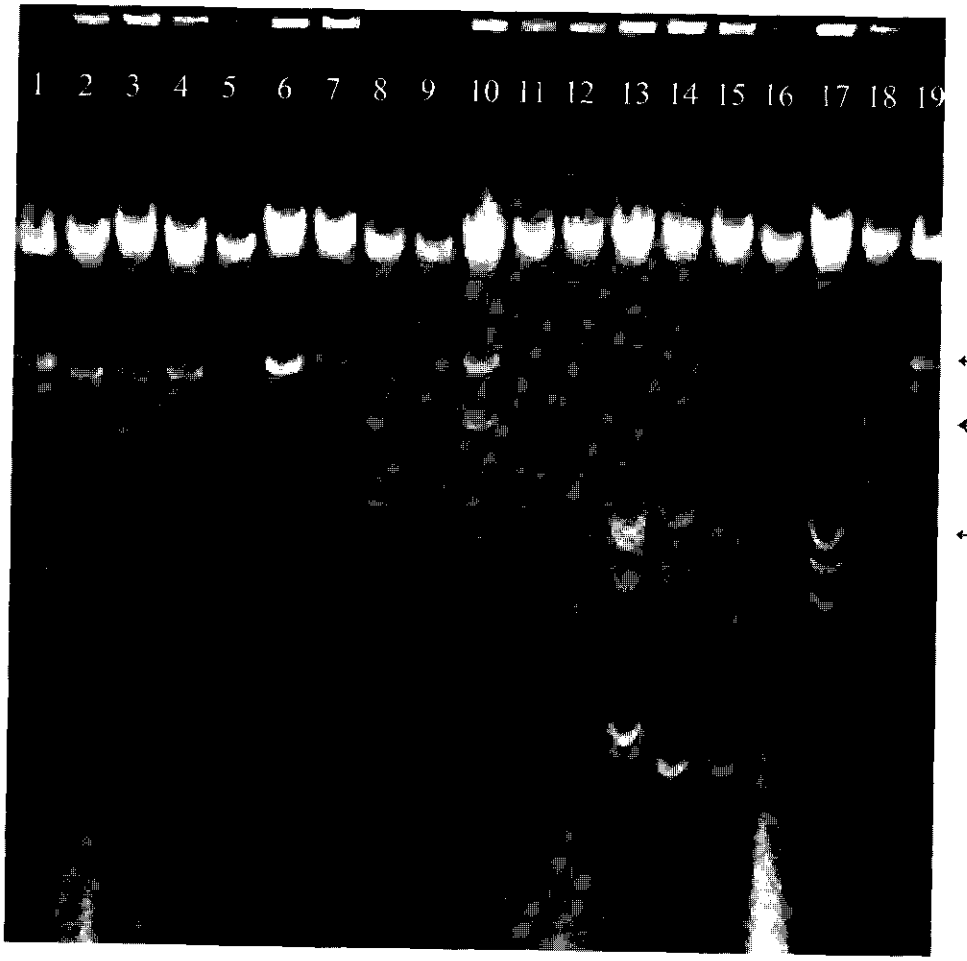

Figure 2.1. Gelelectrophoresis of total nucleic acid suspensions of 16 different virus infected strains. Lane 1,19: marker λHindIII/EcoRI, lane 2-18: strains (haplotype): Ind1.1.16(1b), Ind 1.8.7(1b), D14.1(2a), D14.4(2c), Z1.1(1b), Ind 1.7.8(1c)(= similar to pattern M2.2(2b)), Ind1.8.16(1b), Ind 1.8.22(1c), N330, Ind1.5.5(1b), Ind 1.8.16(1b), Ind 1.8.29(1a), Ind 1.4.32(J), NZ3.2(2a), Ind 1.10.15(2a), D 16.5(2a) and D19.1(2a).

Total variation

The variation in both mitochondrial types and virus infection in a sample can be large. Some of the sites with low density, like Equatorial Guinea and Nepal (sampled on a high altitude), show little variation in mitochondrial types in their population, and their isolates may be recent clones from a few parental strains. But, the soil sample Indonesia 1.8 for example contains 8 haplotypes and 11 infected strains with different virus patterns. In combination with

heterokaryon incompatibility data (Van Diepeningen *et al.* 1997; Chapter 3) one can assume that this soil sample consists mainly of different clonal lineages. Both high local variation and similarities worldwide are probably due to an efficient and wide-ranging aerial distribution of black *Aspergilli*.

Discussion

Isolation on 20% tannin

The first '*Aspergillus niger*' was described by van Tieghem (1867) as a fungus capable of using tannin as a carbon source. Tannins are acidous plant polymers that form strong complexes with proteins (*the tannin effect)* (e.g. Goldstein & Swain 1965). These complexes are difficult to mineralise and can control the availability of organic nitrogen in plant litter (Northup *et al.* 1995). Plants or plant parts can contain 5-20% tannin by weight, plants used for their tannin production even up to 40% (Clarke *et al.* 1949, Bollen & Lu 1969). The black *Aspergilli* and some related species are able to efficiently degrade the tannin by forming tannases. These extracellular enzymes have a combined esterase and depsidase activity (Haslam & Stangroom 1966). The special efficiency of tannin degrading (even in very high concentrations) suggests a unique ecological niche for the black *Aspergilli* in places where local tannin concentration reaches high values.

We tested 74 different strains encompassing the whole range of black *Aspergilli*, which were not selected on tannin. All black *Aspergilli* could grow on 20% tannin, but none of the related *Aspergilli* survived and utilised this concentration. It is an intriguing observation that all black *Aspergilli* share the resistance to tannin at concentrations of 20% and higher, whereas (closely) related *Aspergillus* species like *Aspergillus candidus* (Table 2.1) lack both the black spore colour and this efficient resistance, and can endure only up to 10% tannin maximum. All the colour mutants of black *Aspergilli* (olive, fawn, brown, grey or white coloured) that we isolated also show the feature of efficient tannin degrading, thus separating the black colour from the tannin degrading ability (Table 2.1). That no colour (or cell wall) mutants are found in nature, though they are easily selected in the laboratory, suggest a better survival and spread of black spores.The observation that some of *A. niger*-spores are relatively small may suggest that these could be more pathogenic in relation to e.g. aspergillosis.

Tannin selection enables qualitative as well as quantitative isolation of propagules, presumably conidiospores from different substrates. For our isolations we chose soil samples from sites all over the world as inoculum. The 74 samples yielded a total of 642 black *Aspergilli* on 20% tannin. The density of *A. niger* propagules varied from only a few per gram of wet soil in colder regions up to several hundreds in tropical regions. This number of propagules present in a quantity of soil gives an indication of the potential population density of

members of the black *Aspergillus* group. High densities may be a prerequisite for (somatic) genetic exchange.

Characterisation of strains and populations by mtRFLP

In *A. niger* mitochondrial RFLP's are an informative characteristic, yielding data in accordance with nuclear molecular data. In this examination the isolates could be characterised as belonging to twelve of these mtRFLP haplotypes. The populations sampled consisted mainly of strains from the *A. niger* aggregate; *A. niger*, *A. tubingensis*, and of smaller numbers of *A. carbonarius* and *A. japonicus*. *A. japonicus* was found in relatively high numbers in the 5 samples of Gabon and in Ind 1.4, suggesting locally favorable conditions. The virus infection patterns (2 patterns in five infected strains from different sites) in the Gabon population indicate that there is more than one clonal lines present, though each line may have been samped repeatedly.

Four mitochondrial types belonging to the *A. niger* aggregate (1b&c, 2a&b) are widespread and make up the majority of the examined local populations world-wide. The rare types 1d and 2f could be declining types or -more likely- recent characters: 1d seems to be a mutant from the 1c-mitochondria and 2f may be originally an Australian type, now also found in an Indonesian sample (1.10), indicating that the spores can be spread over large distances by air. Varga *et al.* (1994a) describe two more types that were not found in our experiments. New local lines may occur, but at the moment a few mitochondrial haplotypes are succesful worldwide.

The numbers of different clonal lines as displayed by haplotypes and virus infections indicate that some of the samples were relatively small. Local diversity in the populations is high, with little local specialisation. Globally, the population structure of the black *Aspergilli* shows relatively little temporal and spatial variation in mtRFLPs.

dsRNA Virus Infection

Mycoviral dsRNAs have to occur at a high enough frequency in the population and to be stable enough in clonal lineages to be useful genetic markers (McDonald 1997). No spontaneous loss was detected in clonal lineages (Van Diepeningen *et al.* 1997; Chapter 3) and selecting loss of viruses in young mycelium tips, racetubes or with cycloheximide treatment yielded only a partially cured strain in a line with exceptional phenotypic effect due to infection (Chapter 6). The infections therefore seem persistent enough in the black *Aspergilli*. The infection frequency in the population worldwide appears to be stable around ten percent.

When the infection data are combined with the data of mitochondrial classifications, viral infections appear not to be limited to certain types of black *Aspergilli*. The 19 representatives of three virus-free mitochondrial subclasses are most likely virus-free by chance; there is no

evidence for resistance to viruses in these classes. Using protoplast fusion experiments no evidence for resistance to viruses in black *Aspergillus* strains was found either (Van Diepeningen *et al.* 1998; Chapter 4).

Virus Variation

The variation in the detected viral dsRNA patterns is enormous in the black *Aspergilli.* Most mycoviruses are considered to be members of the toti- or partitiviridae. The essential information (for capsid protein and RNA dependent RNA polymerase) of toti-and partitiviruses lies on one or two dsRNA fragments respectively. These fragments can be accompanied by satellite and/or defective fragments, creating more variation (Buck 1998).

In the black *Aspergilli* several 'basic' fragments can be recognised (e.g. 4.4, 3.2 and 2.2 kb large) that can occur alone or in combination with other fragments. Some small differences in size of viral particles can also be detected with electron microscopy. This indicates that there are several different basic viruses present in the black *Aspergilli.* These types are spread throughout the populations and haplotypes independently. Protoplast fusion experiments show that coexistence of viruses in one mycelium can occur (van Diepeningen *et al.*, in press; Chapter 4) as is also suggested by these screening data. However, it remains to be proven that the similar sized fragments in the different strains are indeed related fragments and that other fragments may be degenerated or satellite fragments instead of basic viruses.

Several explanations can be put forward to explain the spread of infection throughout the black *Aspergillus* populations. Maybe the presence of mycovirus is not due to recent infection, but reflects an ancient association. The original parent of the black *Aspergilli* would then have been infected with several different viruses. It seems unlikely, however, that loss of (parts of the) infection and the accumulation of some defective/satellite fragments finally resulted in a population with such widespread similar infection patterns.

Another explanation for resemblances in infection patterns could be horizontal transfer of viruses between different black *Aspergillus* strains, implicating that there is some kind of contact between strains in nature.

Under laboratory conditions *A. niger* is capable of mitotic recombination in the so-called parasexual cycle, but this has never been observed in nature. The parasexual cycle consists of the following sequence of events: heterokaryon formation after hyphal fusion between genetically dissimilar colonies, formation of a heterozygous somatic diploid after fusion of unlike nuclei, and mitotic recombination by crossing over and/or nondisjunction. Heterokaryon formation is necessary for parasexual recombination, but heterokaryon incompatibility between strains is very common and seems to block the transfer of viruses in the black *Aspergilli* (Van Diepeningen *et al.* 1997; Chapter 3). The occurrence of similar infection patterns in distantly

related black *Aspergillus* types as seen in nature can not easily be explained by such horizontal transfer.

Yet another explanation for the variety in infections and the distribution within the range of black *Aspergilli* could be a repeated infection of black strains from other species. Hoffman *et al.* (1994) found interspecies transfer of a plasmid from a transgenic plant to an *A. niger* strain and Marienfeld et al (1997) reported the transfer of nucleic acid sequences of viral origin between fungi and plants. Similarities in dsRNA sizes and patterns in different species could be the result of (occasional) interspecies transfer.

A combination of these three ideas, coevolution, intra-, and interspecies transfer, could of course also be the cause of the spread of the mycoviral fragments through the population. The high densities of black *Aspergillus* and other species propagules in soil samples suggest that inter-and intraspecies encounters between strains may occur. A well exploited sample like Ind 1.8 shows a large variety in mitochondria as well as in virus infections, but it gives no evidence of any exchange.

Black Aspergillus *population structure*

This study is among the first to give a picture of the worldwide population structure of a fungus and of its dsRNA mycovirus infections. Little variation was detected in mitochondrial structure between the different populations. Occasionally, new local mitochondrial varieties occur. Four mitochondrial types now dominate the mondial population. Virus infections are widespread in *A. niger*, but the data give no definite answers about the origin(s) of infection or about the transfer of viruses within the population. The airborne distribution of the fungus may be the cause of the homogeneous distribution of all different types and viral infections. The effective dispersal and the enormous stock of black *Aspergillus* spores in the soil world-wide may also contribute to temporary stability of the population structure. The data suggest a well-mixed and relatively stable global population structure.

The combination of all these data on the isolated black *Aspergilli* yields a picture of a diverse eukaryote 'species' with a very wide distribution and an unique niche. The efficient ability to utilise tannin suggests a role in the nitrogen cycle. The asexual fungus can be found almost everywhere and in very high concentrations. But, in contrast to some pathogenic fungi, which show both sexual and asexual propagation, local black *Aspergillus* populations do not primarily consist of a limited number of successful clonal lineages. Instead there can be a large number of different clonal lines in a small sample, and potentially a great deal of competition between strains. This indicates that the sampled soil is not the substrate stimulating growth and spore production, but merely a stock of air dispersed spores waiting for better times to produce their own mycelium and large numbers of spores.

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3

Heterokaryon incompatibility blocks virus transfer among natural isolates of black *Aspergilli.*

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Abstract

Heterokaryon (also somatic or vegetative) incompatibility in black *Aspergillus* strains was examined using nitrate-nonutilising mutants selected on chlorate medium. Pairings of complementary mutants showed that somatic compatibility between different strains is exceptional in natural populations of the asexual black *Aspergilli.* Mycoviruses are present in a considerable fraction of the sampled natural population, but surprisingly, horizontal transfer of mycoviruses only occurs - at least under laboratory conditions - between the (very rare) compatible combinations of strains. Thus, unlike in other fungal species, somatic incompatibility in black *Aspergilli* efficiently blocks virus transfer. Viruses present in black *Aspergillus* isolates are highly efficiently transmitted to asexual progeny.

Introduction

In many ascomycete fungal species heterokaryon formation following anastomosis between hyphae of different strains is controlled by heterokaryon (also termed somatic or vegetative) incompatibility reactions. Genetic analysis of somatic incompatibility in several sexual ascomycete fungi has shown that it is generally caused by allelic differences between strains in one or more so-called *het*-genes (heterokaryon incompatibility genes). Somatic incompatibility in fungi can be studied in several ways (for examples see Fincham *et al.*, 1979; Jennings and Rayner, 1984; Perkins, 1988; Glass and Kuldau, 1992). Often complementation between different nitrate non-utilising mutants is used to test for

heterokaryon formation (Correl *et ,al.* 1987; Joaquim and Rowe, 1990; Brooker *et al.*, 1991). As in *Aspergillus nidulans*, as first demonstrated by Cove (1976) various complementing types of nitrate non-utilising mutations can be easily isolated in *A. niger* on the basis of resistance to chlorate (Debets *et al.*, 1990a). These mutants show leaky growth on NO_3^-, enabling formation and outgrowth of a vigorously growing heterokaryotic mycelium from two complementary compatible mutants inoculated in the same plate. Strains unable to form such a heterokaryon under these conditions are classified as heterokaryon incompatible.

In asexual fungi like members of the black *Aspergillus* aggregate the formation of a heterokaryon, followed by the formation of a diploid and subsequent haploidisation, is the only way to achieve (mitotic) recombination (parasexual cycle). Mitotic recombination has been used for genetic analysis of related mutants in an isogenic background of *A. niger* (Bos *et al.*, 1988; Debets *et al.*, 1990a). Since heterokaryon incompatibility in asexual fungi like the black *Aspergilli* prevents such genetic analysis, it remains unknown whether heterokaryon incompatibility is regulated by similar *het*-genes as in related sexual species.

The black *Aspergillus* aggregate consists of a complex of black *Aspergillus* species. On basis of morphological, RAPD, mitochondrial and ribosomal RFLP data the aggregate can be divided into several main groups - *Aspergillus carbonarius*, *Aspergillus japonicus*, *Aspergillus niger* and *Aspergillus tubingensis* (Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993; Varga *et al.*, 1993; 1994b). All black *Aspergillus* strains share the unique ability to grow on concentrations of 20% (w/v) tannin and on basis of this characteristic a wide range of black *Aspergilli* has been isolated from nature (Chapter 2). Most of the isolated black *Aspergillus* types occur world-wide and at some places in very high densities. About 10% of all these strains appeared to be infected with a variety of double stranded RNA mycoviruses (Varga *et al.*, 1993a; Chapter 2).

Horizontal transfer of cytoplasmic elements like viruses and mitochondrial plasmids is in many species limited, but not inhibited, by somatic incompatibility reactions (Caten, 1972, Anagnostakis and Day, 1979, Anagnostakis, 1983, Debets *et al.*, 1994). In *Endothia parasitica* the variation and stability of the dsRNA virus patterns is influenced by the vegetative incompatibility reactions between strains (Anagnostakis and Day, 1979). Virus transfer to fungal offspring seems mainly restricted to asexual progeny (Rawlinson *et al.*, 1973; Day *et al.*, 1977; Lecoq *et al.*, 1979; Rogers *et al.*, 1986b). Virus infection is only known to occur after hyphal anastomosis, not via infection by extracellular viruses. Virus transfer between black *Aspergillus* strains has only been detected after protoplast fusion between more distantly related strains (Lhoas, 1970; Varga *et al.*, 1994a).

In the present study we present the results of heterokaryon (in)compatibility experiments with nitrate non-utilising (*nia* and *cnx*) mutants of the whole range of black

Aspergillus field isolates. This provides an estimate of the rate of occurrence of heterokaryon formation under natural conditions. The transmission of viral dsRNA fragments to the conidiospores (vertical transfer) was also analysed. Furthermore we tested to what extent heterokaryon incompatibility is a barrier to the horizontal transfer of mycoviruses between different strains when grown in a mixed culture with or without selective pressure for heterokaryon formation. These experiments may help to understand the population dynamics of mycoviruses in black *Aspergilli*.

Materials and Methods

Strains.

A list of the used wild-type black *Aspergillus* strains is given in Table 3.1. The Indonesian (Ind) strains were isolated on selective medium with 20% (w/v) tannin from soil samples from 1990 and 1992 from Jakarta (Chapter 2). *A. niger* strains N400 (CBS 120-49 and ATCC 9029) and strain N062 (CBS 557.65) are culture collection strains. Strain Z 1.1 was obtained from a hospital in Gouda, the Netherlands. Strains are classified by their mitochondrial RFLP type (Varga *et al* 1993, 1994a; Chapter 2). Some of the strains (marked with *) are carrying dsRNA viruses (Varga *et al* .,1994b; Chapter 2).

Table 3.1. List of the wild-type black *Aspergillus* strains used. All Ind strains were isolated from a small yard in Jakarta, Indonesia. Strains N400 and N062 are culture-collection strains; strain Z 1.1 is a hospital isolate. Between brackets () the mitochondrial RFLP classification of the strains. * indicate dsRNA virus-infected strains, *½ is a partially cured strain (4 out of 6 bands lost).

Black *Aspergillus* strains employed							
Ind 1.2.15	(2b)	Ind 1.7.8	(1c)*	Ind 1.8.16+	(1b)*	Ind 1.8.34	(1a)
Ind 1.4.24	(1c)	Ind 1.7.9	(1a)	Ind 1.8.16-	(1b)*½	Ind 1.8.42	(2b)
Ind 1.4.29	(C)*	Ind 1.8.1	(1b)	Ind 1.8.19	(1b)*	Ind 1.8.47	(1a)
Ind 1.4.32	(J)*	Ind 1.8.2	(1a)	Ind 1.8.21	(1c)*	Ind 1.8.67	(1a)
Ind 1.5.5	(1b)*	Ind 1.8.3	(1b)	Ind 1.8.22	(1c)*	Ind 1.8.68	(1a)
Ind 1.5.7	(1a)	Ind 1.8.7	(1b)*	Ind 1.8.26	(2d)*	N400	(1a)
Ind 1.6.18	(1a)	Ind 1.8.10	(1b)*	Ind 1.8.29	(1a)*	N062	(1c)
Ind 1.6.19	(2a)*	Ind 1.8.11	(1b)*	Ind 1.8.30	(1a)	Z 1.1	(1c)*
Ind 1.6.23	(2a)*	Ind 1.8.13	(1c)	Ind 1.8.31	(1a)		

Culture conditions.

Complete medium (CM) was made as described by Pontecorvo *et al.* (1953) with 10 mM nitrate and/or 10 mM urea as nitrogen source and 1 mg/l $ZnSO_4$, $FeSO_4$, $MnCl_2$ and $CuSO_4$ extra added. Chlorate medium (CM+ClO_3) is CM + 200 mM $KClO_3$ and 10 mM urea. Minimal medium (MM) is an extra reduced form of Pontecorvo's minimal medium, with a composition as described by Coenen *et al.* (1994) without nitrogen source. As nitrogen sources for the (test)media 10 mM urea (U), 10 mM nitrate (N), 10 mM nitrite or 0.5 mM hypoxanthine were used. As supplements for *pro/arg*, *nic* and *pyr* strains final concentrations of 1 mM *arg*inine, *nic*otinamide and uridin (*pyr*imidine) or 2 mM *pro*line were used. *Pyr*

mutants were grown on CM with uridine and 0.8 mg/ml 5-Fluoro-orotic acid. All incubations were done at 30°C.

Isolation of mutants.

Fawn-coloured mutants (*fwn*) were selected on (supplemented) MM+N after irradiation of conidiospores with 120 J/m² Ultraviolet Light (UV). From strain N062 different shades of fawn and brown (*brn*) mutants were also isolated. Nitrate non-utilising chlorate resistant mutants were isolated on CM+ClO_3 and tested as described by Cove (1976) and Debets *et al.* (1990a) on MM with different nitrogen sources. *Cnx* (nitrate and hypoxanthine deficient) and *nia* (nitrate deficient) mutants of each strain were selected. To test the usefulness of the *cnx* and *nia* mutants heterokaryon self-compatibility tests were carried out: complementing mutants were successfully isolated in all natural isolates. The *pro/arg* (proline/arginine deficient) and *nic* (nicotinamide deficient) mutants of strain N062 were obtained after a dose of UV and screening on minimal and supplemented minimal media. The *pyr* mutants were selected on 5-Fluoro-orotic acid and tested on uridine deficiency.

Heterokaryon compatibility tests.

Pairs of strains distinguishable on colour and with complementing deficiency mutations were inoculated both individually and together on MM+N. The combination of no growth of individual strains and growth of a bi-coloured heterokaryon was declared compatible (Figure 3.1D). For most strains four possible combinations of mutants (strain1*nia* x strain2*fwn,cnx*; strain1*cnx* x strain2*fwn,nia*; strain1*fwn,nia* x strain2*cnx* and strain1*fwn,cnx* x strain2*nia*) were possible and at least two of these combinations were tested. The mutants used have been tested in self-compatibility reactions to exclude the possibility that heterokaryon incompatibility was due to marker effects.

Virus detection.

Total nucleic acids was isolated and tested for viral dsRNA fragment content with gel electrophoresis. For a scheme of virus infection patterns in black *Aspergillus* strains, as seen after gel electrophoresis see Varga *et al.* (1994a) or e.g. Chapter 2.

Virus stability.

Virus stability was tested in colonies from random single spores from infected strains by checking their mycovirus content (vertical transfer) and by continuous subcultering mycelia from infected strains and also testing those subcultures (sequential hyphal-tip isolation).

Virus infection (horizontal transfer) experiments.

Three sets of experiments were carried out. (1) Horizontal transfer in heterokaryon compatible combinations. Heterokaryons were constructed from heterokaryon compatible combinations of strains of which one or both contain virus. Subsequently homokaryons with parental phenotypes were re-isolated from these heterokaryons (conidiospores are uninucleate) and tested for their virus content. Heterokaryon incompatible combinations could not be tested by this method. (2) Horizontal transfer in combinations not selected for (transient) heterokaryon formation. Both heterokaryon compatible and incompatible combinations of strains could be used in this experiment. Pairs of strains distinguishable on basis of colour and chlorate resistance/nitrate deficiency were grown together for 6 weeks on minimal medium + urea (MM+U) in test tubes. A new layer of medium (1 ml) was added every week. Strains were separated and purified on the selective media CM+ClO_3 and MM+N. Both types of pure homokaryotic strains were checked for virus content afterwards. (3) Horizontal transfer not selected for (transient) heterokaryon formation and with mechanically disturbance. Experiments were carried out as in transfer experiment 2, but media and mycelia were disrupted with a scalpel on each of the three days before spores had been formed on fresh media. In this way we tested whether free cytoplasmic elements and wounds in mycelia would enhance the virus infection rate.

Results

Heterokaryon (in)compatibility

Heterokaryon (in)compatibility can be tested in black *Aspergillus* in several ways. Examples of these are shown in Figure 3.1. Figure 3.1A-C show heterokaryons growing from mixed mycelial mats; for Figure 3.1D strains were directly inoculated bothe separately and together on a Petri dish. In *A. niger* colour mutants are non-autonomous: colour mutations can complement in heterokaryons as shown in Figure 3.1A (see also Pontecorvo *et al.*, 1953; Lhoas, 1980) where fawn- and olive-coloured mutants complement to form black conidiospores. Figures 3.1B-D show compatible combinations of strains on selective media, where both strains are not able to grow separately but the combination of the two strains can. For these heterokaryons combinations between a black and fawn coloured strain were used for discrimination between the strains. In Figure 3.1B two auxotrophic strains are used (*lys*ine deficient and *trp* tryptophan deficient) on non-supplemented minimal medium. In Figure 3.1C a combination of an auxotrophic marker (*met*hionine) and a dominant nuclear oligomycine resistance marker (*oli*) in one of the strains is used to force heterokaryotic growth on MM with oligomycine. Two chlorate resistant stains with complementing nitrate deficiencies are used on minimal medium with nitrate as nitrogen source in Figure 3.1D. All strains in these examples are isogenic and originate from the N400 culture collection series.

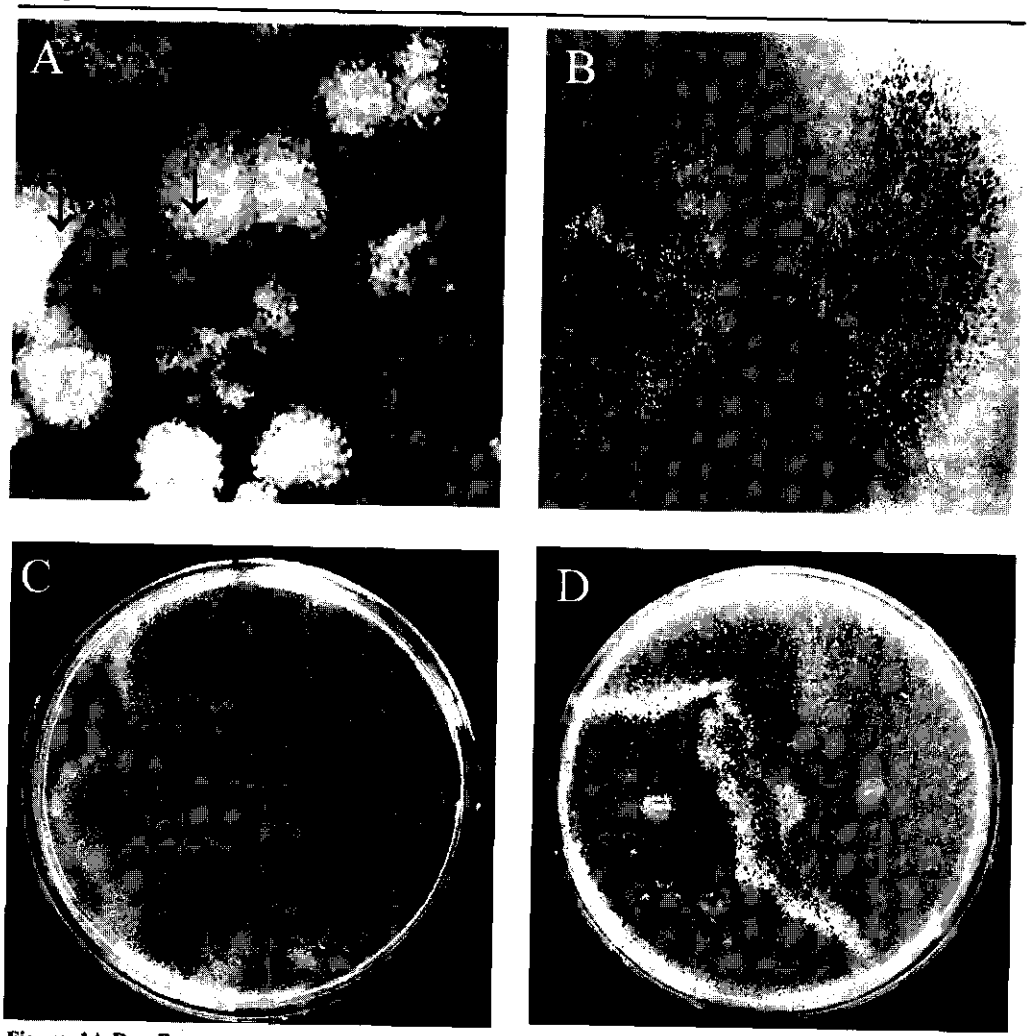

Figure 1A-D. Four ways to test for heterokaryon (in)compatibility; heterokaryons grown from mixed mycelium mats (**A-C**) or tested strains inoculated separately to the sites of the plate and together in the middle (**D**). In **B-D**, combinations of black- and fawn- coloured strains were used. **A** non-autonomous colour expression (←) between two different colour mutants (a fawn *fwn* and olive *olv* coloured strain). **B** combination of two auxotrophic strains (*met*hionine and *lys*ine) on non-supplemented MM. **C** combination of a strain with both an auxotrophic marker (*met*hionine) and a dominant oligomycine resistance (oli) with a wildtype strain on MM+oligomycine. **D** combination of two nitrate non-utilising strains (*cnx* and *nia*) on MM+N. All demonstrations with isogenic mutants of culture collection strain N400.

Of all isolated wild-type strains fawn (*fwn*) coloured mutants were isolated after a dose of ultra-violet light (UV). Of both black and fawn strains chlorate resistant/ nitrate deficient mutants were selected. Strains were tested on different nitrogen sources and both *nia* (nitrate deficient) and *cnx* (nitrate and hypoxanthine deficient) mutants were selected if possible for all strains (after Cove, 1976 and Debets *et al.*, 1990a). Combinations of complementing

mutant strains can show heterokaryotic growth on minimal medium with NO_3 as nitrogen source when the strains are compatible. All used natural isolates and strains N400 and Z1.1 were heterokaryon self-compatible (HSC).

Table 3.2 Mutants of self-incompatible strain N062 (CBS 557.65). *fwn* = fawn coloured spores, *brn* = brown coloured spores, *pro/arg* = proline or arginine-deficient, *nic* = nicotinamide-deficient, *nia* = chlorate-resistant/nitrate-deficient, *cnx* = chlorate-resistant/nitrate and hypoxanthine-deficient, *pyr* = 5- Fluoro-orotic acid-resistant/uridine deficient.

Strain	Mutations	Strain	Mutations
N062		N062-06	*lfwn*-1, *nia*-1
N062-01	*fwn*-1	N062-07	*dfwn*-1, *nia*-1
N062-02	*pro/arg*-1	N062-08	*lfwn*-1, *cnx*-1
N062-03	*nic*-1	N062-09	*dbrn*-2, *cnx*-1
N062-04	*nia*-1	N062-10	*fwn*-1, *nia*-1, *pyr*-1
N062-05	*cnx*-1	N062-11	*brn*-1, *cnx*-1, *pyr*-1

However, one tested culture collection strain, N062, did not show heterokaryotic growth of *nia* and *cnx* mutants. To avoid any special marker effects additional colour mutants (*d*ark and *l*ight shades of fawn and (dark)brown (*d*)*brn*) as well as extra deficiency mutants (*pro*line/*arg*inine, *nic*otinamide and *pyr* (uridine deficient) strains) of N062 were isolated. In Table 3.2 a list of mutant strains originating from N062 is given. None of the possible combinations of in principle complementary mutants of this strain showed heterokaryon growth. The colour mutants of strain N062 also lack the non-autonomous colour-expression evidence for heterokaryon compatibility when paired. We therfore conclude that this strain is heterokaryon self-incompatible (*hsi*).

The results of inter- and intrastrain pairings are listed in Table 3.3. In these experiments 26 strains spanning the whole range of black *Aspergilli* were tested for heterokaryon formation. Only very few combinations of strains showed compatible growth. Strain Indonesia 1.8.16 is represented twice in this table strain: once with virus infection, once in its cured form. Both forms are still compatible with one another. Strain Z1.1 was added in the heterokaryon compatibility tests because of its close resemblance to strain Ind 1.7.8 both in mitochondrial type as in virus infection (1 similar-sized band). Despite the resemblance the strains are heterokaryon incompatible.

A subset of the natural isolates consisting of strains with a similar mitochondrial type (1a) as the commonly used culture-collection strain N400 was also tested on heterokaryotic complementation. In this set (results shown in Table 3.4) only two strains both isolated from sample Indonesia 1.8 were heterokaryon compatible and probably isogenic.

Table 3.3 Heterokaryon (in)compatibility reactions between 26 strains covering the whole range of black *Aspergilli*. mt = mitochondrial type (Varga et al 1994b), + heterokaryon (self)compatible; - heterokaryon incompatible.

Strain	mt	Ind 1.8.2	Ind 1.8.29	Ind 1.8.1	Ind 1.8.3	Ind 1.5.5	Ind 1.8.7	Ind 1.8.10	Ind 1.8.11	Ind 1.8.19	Ind 1.8.16 +	Ind 1.8.16 -	Ind 1.8.13	Ind 1.4.24	Ind 1.8.21	Ind 1.8.22	Ind 1.7.8	Ind 1.8.9	Ind 1.6.19	Ind 1.6.23	Ind 1.8.42	Ind 1.2.15	Ind 1.8.26	Ind 1.4.32	Ind 1.4.29	N400	Z 1.1	N062
Ind 1.8.2	1a	+																										
Ind 1.8.29	1a	-	+																									
Ind 1.8.1	1b	-	-	+																								
Ind 1.8.3	1b	-	-	-	+																							
Ind 1.5.5	1b	-	-	+	-	+																						
Ind 1.8.7	1b	-	-	-	-	-	+																					
Ind 1.8.10	1b	-	-	-	-	-	-	+																				
Ind 1.8.11	1b	-	-	-	-	-	-	+	+																			
Ind 1.8.19	1b	-	-	-	-	-	-	-	-	+																		
Ind 1.8.16 +	1b	-	-	-	-	-	-	-	-	-	+																	
Ind 1.8.16 -	1b	-	-	-	-	-	-	-	-	-	+	+																
Ind 1.8.13	1c	-	-	-	-	-	-	-	-	-	-	-	+															
Ind 1.4.24	1c	-	-	-	-	-	-	-	-	-	-	-	-	+														
Ind 1.8.21	1c	-	-	-	-	-	-	-	-	-	-	-	-	-	+													
Ind 1.8.22	1c	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+												
Ind 1.7.8	1c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+											
Ind 1.8.9	1d	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+										
Ind 1.6.19	2a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+									
Ind 1.6.23	2a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+								
Ind 1.8.42	2b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+							
Ind 1.2.15	2b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+						
Ind 1.8.26	2d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+					
Ind 1.4.32	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+				
Ind 1.4.29	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+			
N400	1a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+		
Z 1.1	1b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
N062	1c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.4 Heterokaryon (in)compatibility reactions between 13 *A. niger* strains of mitochondrial type 1a. + Heterokaryon (self)compatible, - heterokaryon incompatible.

Strain	N 40 0	Ind 1.4 33	Ind 1.5 7	Ind 1.6 18	Ind 1.7 9	Ind 1.8 2	Ind 1.8 29	Ind 1.8 30	Ind 1.8 31	Ind 1.8 34	Ind 1.8 47	Ind 1.8 67	Ind 1.8 68
N400	+												
Ind 1.4.33	-	+											
Ind 1.5.7	-	-	+										
Ind 1.6.18	-	-	-	+									
Ind 1.7.9	-	-	-	-	+								
Ind 1.8.2	-	-	-	-	-	+							
Ind 1.8.29	-	-	-	-	-	-	+						
Ind 1.8.30	-	-	-	-	-	-	-	+					
Ind 1.8.31	-	-	-	-	-	-	-	-	+				
Ind 1.8.34	-	-	-	-	-	-	-	-	-	+			
Ind 1.8.47	-	-	-	-	-	-	-	-	-	-	+		
Ind 1.8.67	-	-	-	-	-	-	-	-	-	-	-	+	
Ind 1.8.68	-	-	-	-	-	-	-	-	-	-	-	+	+

Virus stability

From virus infected strains single-spore colonies were obtained from plated spore suspensions. Twenty-four to eighty colonies derived from strains Ind 1.5.5 (24), Ind 1.7.8 (24), Ind 1.8.7 (80), Ind1.8.16 (24) and Z 1.1 (24) were tested for their virus content. In all cases all progeny showed the same pattern of infection as the parental strain; no loss of any fragments nor addition of fragments was observed (data not shown). Thus vertical transfer is very efficient and clonally related isolates are expected to harbour identical viruses.

Other experiments tested the stability of viruses in mycelium during weeks of sub-culturing hyphal tips. Virus patterns were found to be stable in all strains during sub-cloning. Only the phenotypically exceptional strain Ind 1.8.16 could be cured of part of its virus this way: it lost four bands from the pattern of six bands and also its abnormal phenotype of non-sporulating sectors.

Virus transfer experiments

Transfer can be detected in those strain combinations where one of the strains is infected or where the two strains have different dsRNA patterns. Heterokaryon compatible combinations of strains showed transfer of the viruses (transfer experiment 1; Table 3.4). Also the re-infection of partially cured strain Ind 1.8.16 took place readily. Strains from heterokaryon incompatible combinations could not be recovered from these heterokaryon compatibility tests, because both mutants used in the incompatible mixture lack the ability to utilise the nitrate medium (MM+N) and fail to grow.

In preliminary transfer experiments between sets of strains without pressure for heterokaryotic growth, no transfer was detected after short periods of coculturing on different media. We were unsure if this was due to lack of transfer (possibilities), to resistance to

viruses or to non-detectable levels of dsRNA. Therefore we tested combinations after prolonged co-cultivation on minimal medium with urea (MM+U) to enhance transfer possibilities and to give the virus time to reach detectable levels throughout the mycelium after transfer (transfer experiments 2 and 3). After 6 weeks the strains were separated on selective media and subsequently tested for virus presence. In undisturbed combinations (transfer experiment 2) only virus transfer has been detected in the heterokaryon compatible combination (strains Ind 1.8.9 and Ind 1.8.22). The results of these experiments on horizontal transfer in combinations not selected for (transient) heterokaryon formation are listed in the left row (u) of each column in Table 3.5.

Table 3.5 Transfer experiments 2 and 3. Transfer between virus-free and virus-containing strains in the upper part of the table. In the lower part of the table combinations of two-virus-containing strains with different infection patterns. Left columns undisturbed (u), right columns mechanically disturbed (d) conditions (see Materials and Methods). * = virus infected strain; - = no transfer detected; p = partial transfer, not all dsRNA bands transferred; + = full transfer of virus pattern; n.d. = no acceptor selected; empty cell = transfer not detectable due to overlapping patterns. Alls strains were incompatible except for the combination Ind 1.8.9 and Ind 1.8.22.

Donor →		Ind		Ind		Ind		Ind		Ind		Ind	
Acceptor ↓		1.8.16*		1.8.7*		1.7.8*		1.8.22*		1.6.19*		.26*	
		(1b)		(1b)		(1c)		(1c)		(2a)		(2d)	
		(u)	(d)	(u)	(d)	(u)	(d)	(u)	(d)	(u)	(d)	(u)	(d)
Ind 1.8.2 *cnx*	(1a)	-	-	-	-	-	-	-	-	-	-	-	-
Ind 1.8.1 *cnx*	(1b)	-	-	-	-	-	-	-	-	-	-	-	-
Ind 1.8.13 *cnx*	(1c)	-	-	-	-	-	-	-	-	-	-	-	-
Ind 1.8.9 *cnx*	(1d)	-	p	-	-	-	-	+	+	-	-	-	-
Ind 1.8.39 *cnx*	(2a)	-	-	-	-	-	-	n.d.	-	-	-	-	-
Ind 1.8.42 *cnx*	(2b)	-	-	-	-	-	-	-	-	-	-	-	n.d.
Ind 1.7.6	(J)	n.d.	n.d.	-	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
Ind 1.5.5* *cnx*	(1b)			-	-	-	-			-	-	-	-
Ind 1.7.8* *cnx*	(1c)	-	-	-	-			-	-	-	-	-	-
Ind 1.8.22* *cnx*	(1c)			-	-	-	-			-	-		
Ind 1.6.19* *cnx*	(2a)			-	-	-	-	-	-				

The right row (d) of each column of Table 3.5 shows the results of similar experiments, but with mechanical disturbance of the mycelia during growth (transfer experiment 3). In these combinations free cytoplasm could be an extra source of infection in wounded mycelia. In one combination of incompatible strains partial infection occurred after this treatment. The largest fragment of the virus infecting strain Ind 1.8.16 (normally 6 bands) was detected in strain Ind 1.8.9, but caused no phenotypic effect. Test of transfer between two different virus containing strains yielded no multiply infected strains. Though in these cases the acceptor strains are clearly capable of harbouring viruses, no extra infection with different dsRNA fragments occurs. Not all combinations of virus infected strains could be tested due to overlapping virus patterns in some of them.

Discussion

The dsRNA viruses in the black *Aspergilli* do not show any phenotypic abnormalities, the only exception being the virus(es) in strain Ind 1.8.16 which produces non-sporulating sectors within the colony. Yet viruses may lower the fitness of their host even though they are seemingly neutral passengers. Without the possibility of horizontal virus transfer, even slight fitness effects would result in selection against virus-infected strains thus reducing the virus frequency in the fungal population. Yet, the global population of black *Aspergilli* shows an apparently stable infection frequency of approximately 10% (Chapter 2). Thus, one would expect that either the viruses have no negative effect on their host's fitness or that there is horizontal transfer. In this study we describe the stability of the viruses within their host and the limited possibilities for virus transfer in natural populations of black *Aspergilli.*

The dsRNA patterns of these mycoviruses vary between different host strains, but tests of virus stability both in mycelia and in the asexual progeny show that the virus patterns within a host line show no variation or loss of fragments. Only one infected strain, the only one in which the virus produced a phenotypic effect, lost part of its virus or one of its viruses after sequential sub-cultering of mycelia. All 24 randomly chosen conidiospores from this strain were found to be infected. In sexual species progeny from ascospores produced on infected mycelium can be free of virus, but for the infected asexual black *Aspergilli* there seems to be no escape.

The stability of mycoviruses outside their host appearss to be very low. Uptake experiments from purified virus particles in the media did not show any transfer via the medium (Van Diepeningen, unpublished data). Disruption of mycelia might occur regularly in nature, but partial transfer took place in only one strain in which the mycelia were repeatedly mechanically disrupted. A combination of wounded mycelium and a mixture of free cytoplasm around it may be the cause of this uptake of a single dsRNA fragment. The transferred fragment from donor strain Ind 1.8.16 forming a part of a more complex pattern can apparently cause a stable infection on its own in the acceptor strain Ind 1.8.9. The same fragment and three smaller bands are also the ones lost in the cured variant of Ind 1.8.16, while the two smallest fragments remain as a stable infection. This suggests that some dsRNA patterns may consist of more than one independent virus.

The variation in dsRNA patterns among infected strains and their stability within a host begs the question how such infection could become so widespread within the population. Information on transmission rates of viruses and on the fitness effects a virus has on its host, is clearly essential to answer this question.

To test for the horizontal spread of mycoviruses we first studied the occurrence of somatic (in)compatibility among natural black *Aspergillus* isolates, since in other species incompatibility can strongly reduce transfer of genetic elements by limiting the formation of

anastomoses and of transient heteroplasmons or heterokaryons. Chlorate resitance mutations are positive selectable and yield different deficiency mutations, making them very useful for compatibility tests. Isolationand characterisation of auxotrophic mutations is more laborious. Colour mutants (especially fawn) are easily detected by eye. A drawback of these muatations is the fact that colour complementation could be a consequence of cross-feeding possibly without heterokaryon formation (Lhoas, 1980) Dominant oligomycine resistance can only be used in combination with deficiencies or other dominant resistancies in the tester strain.

On our medium black *Aspergillus* strains produce on our medium no clear signs of anastomosis or antagonism when they meet; but the use of complementary mutants in vegetative compatibility tests works very well. All used strains were tested on their ability to form anastomoses by testing complementing mutants of each line. All strains were self-compatible except one culture collection strain, which appeared to be heterokaryon self-incompatible in all the combinations of mutants tested. Heterokaryon incompatibility was the rule between isolates though exceptionally strains were compatible, in which case also virus transfer occurred.

In separate experiments without heterokaryon forcing conditions, virus transfer between incompatible strains was not found even after prolonged cocultivation of strains on very minimal media. This suggests that in black *Aspergilli* heterokaryon incompatibility acts as a strict barrier for virus transfer under laboratory conditions, unlike other fungi like *Ceratocystus ulmi* and *Endothia parasitica* (Brasier 1984, Anagnostakis and Day 1979). Only when the mycelia are thoroughly disrupted was one of the virus fragments transferred in one experiment (from Ind 1.8.16 to Ind 1.8.9), so even when disruption occurs transfer takes place at a very low rate.

It is conceivable that infections of black *Aspergillus* populations originate from other fungi or other organisms. Hoffmann *et al.* (1994) did experiments growing transgenic plants together with a black *Aspergillus* strain under sterile conditions. Transfer of the resistance gene *hph* from four different plant species to an *A. niger* strain was reported. Kempken (1995a) found evidence for the transfer of a mitochondrial plasmid from *Ascobolus immersus* (a dicomycete) to *Podospora anserina* (a pyrenomycete), two fungi that share the same ecological niche. Perhaps the source of the wide-spread viruses in the black *Aspergilli* may have to be sought outside the species rather than through spread within the black *Aspergilli* and infections may occur rather frequent and in different backgrounds.

Another possibility would be that one or more viruses have co-evolved with their black *Aspergillus* hosts for a long time. Viruses may have lost their virulence and some hosts may have lost their virus and perhaps evolved resistance to viruses. However, virus transfer to the asexually produced offspring of black *Aspergillus* strains is very efficient: all 196 tested progeny contained all dsRNA fragments of the parental strain. Apparently virus loss through

conidiospores does not occur or else is very rare. In the few detected heterokaryon compatible combinations viruses were readily transferred and no virus resistance was observed. Strains with a virus infection did not receive any other bands showing that the heterokaryon incompatibility blocks the transfer to virus-competent strains.

For *Aspergillus nidulans* Butcher *et al.* (1972) postulated that isolates within a vegetative compatibility group (VCG) are more closely related than isolates belonging to different VCG's. But outcrossing may produce offspring belonging to different VCGs as a consequence of recombination between *het*-genes. In plant pathogens a close relationship between VCG and host pathogenicity or specificity is often found (Bosland & Williams 1987, Larkin *et al.* 1990, Ploetz & Correl 1988). In asexual non-recombining species all members of a VCG would be clonally related. In asexual *Aspergillus flavus* members of one VCG indeed share many characteristics regardless of geographic origin (Bayman & Cotty, 1993). In our study of 36 black *Aspergilli*, 33 originating from four sites in a single yard in Indonesia, strains often contained similar type mitochondria and sometimes similar mycovirus patterns, but were almost always heterokaryon incompatible. The occasional compatible combinations are closely related types on basis of their mitochondrial RFLPs (type 1d is supposedly a recent mutation of type 1c). Due to absence of recombination new VCG's are most likely the result of mutations.

Heterokaryon self-incompatibility could be a consequence of the generation of incompatibility by mutation, resulting in colonies where lack of anastomises limits the internal communication. Horn and Greene (1995) also found heterokaryon self-incompatibility in two other imperfect *Aspergillus* species: *Aspergillus flavus* and *Aspergillus parasiticus*. Decline in disease caused by *Rhizoctonia solani* in monoculture is a common phenomenon and is associated with th occurrence of non-self-anastomosing strains (Hyakumachi & Ui, 1987). The mechanism of anasomosis loss is unknown, but self-incompatible strains of *R. solani* are able to form anastomoses when a self-anastomosing strain is encountered. Some *Fusarium solani* (*Nectria haematococca*) heterokaryon self-incompatible (HSI) strains have been shown to form only a reduced number of hyphal fusions (Hawthorne & Rees-George, 1996). However, in all the tested combinations with *A. niger* strain N062 no compatibility reaction was detected and this was not due to any marker effects.

The extent of vegetative compatibility can be expressed by the ratio of the number of VCG's to the sample size (S/N). The two sets of compatibility tests yield an S/N of 22/26 (0.85) respectively 12/13 (0.92). The high diversity among strains from the same soil samplebreflects the effective aerial dispersion of the fungus. Horn and Greene (1995) also found higher numbers of VCG's per sample size for the air dispersed asexual *A. flavus* then for more restrictedly dispersing asexual *A. parasiticus*, which has a more-limited dispersal.

As a function of heterokaryon (in)compatibility both (inhibition of) nuclear recombination and (inhibition of) exchange of genetic material have been proposed (Glass and Kuldau, 1992). Our results indicate that in black *Aspergilli* somatic incompatibility completely blocks the transfer of (possibly deleterious) mycoviruses. Virus transfer is thus limited between somatically compatible combinations of identical, or closely related, black *Aspergillus* strains. Once infected there seems to be little escape. Black *Aspergilli* thus seem to have a more efficient barrier for the transfer of genetic elements than many (related) fungal species do.

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4

Intra- and interspecies virus transfer in *Aspergilli* via protoplast fusion.

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Abstract

Intra- and interspecies transfer of dsRNA viruses between black *Aspergilli* and *A. nidulans* strains has been investigated using protoplast fusion. We found interspecies transfer of virus in all combinations of black *Aspergillus* and *A. nidulans* strains and *vice versa.* Using the same conditions, intraspecies virus transfer among heterokaryon incompatible strains was also tested. Whereas such transfer was always found among *A. nidulans* strains, transfer among black *Aspergilli* was frequently unsuccessful. The lack of virus transfer between black *Aspergillus* isolates was further investigated by using a mitochondrial oligomycin resistance marker as a positive control for cytoplasmic exchange. These experiments showed independent transfer of the oligomycin resistance and dsRNA viruses during protoplast fusion of heterokaryon incompatible black *Aspergilli.* The inefficient transfer of dsRNA viruses between black *Aspergilli* is not caused by absolute resistance to viruses but may be related to heterokaryon incompatibility reactions that operate intraspecifically. Consequences for the dynamics of mycoviruses in populations of black *Aspergilli* are discussed.

Introduction

The black *Aspergilli* form a diverse group of asexual and black-spored *Aspergilli*, many of which are of industrial importance. The natural population structure of black *Aspergilli* seems very homogeneous worldwide when mitochondrial data are considered. In tropical regions these fungi can reach high densities (Chapter 2). Infections with various double-stranded (ds) RNA mycoviruses are common in black *Aspergilli* and nearly ten percent of

the population is infected (Chapter 2). Both *Aspergillus niger* and *Aspergillus tubingensis*, representing two closely related types of black *Aspergilli* (Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993; Varga *et al.*, 1993, 1994b), as well as less related black *Aspergillus* types, can harbour mycoviruses in nature (Varga *et al.*, 1994a, Van Diepeningen *et al.*, 1997; Chapter 3).

DsRNA viruses are commonly found in fungal species. Some viruses are connected with hypovirulence or killer-phenomena in their host, many are without known phenotypic effect (Buck, 1986). Only one virus in the black *Aspergilli* was found with a debilitating, phenotypic, effect (Chapter 6). Transfer of the mycoviruses between black *Aspergillus* strains has only been successful in heterokaryon compatible strains and via protoplast fusions (Lhoas, 1970, Liang *et al.*, 1983; Liang and Chen, 1987, Varga *et al.*, 1994a, Van Diepeningen *et al.*, 1997; Chapter 3). Direct transfer of these mycoviruses between mycelia is prevented by the heterokaryon incompatibility barrier, present between most strains (Van Diepeningen *et al.*, 1997; Chapter 3).

Nothing is known about the mechanism of heterokaryon incompatibility in black *Aspergilli.* It is generally assumed to be similar to that in related sexual ascomycetes, where the formation of heterokaryons and heteroplasmons following interstrain hyphal fusions (anastomoses) is regulated by heterokaryon incompatibility genes (*het*-genes). Both allelic and non-allelic *het*-gene systems have been found to be involved in heterokaryon formation. In allelic systems an allelic difference at one *het*-locus is sufficient to cause an incompatibility reaction. In non-allelic interactions differences at two separate loci result in incompatibility (for a scheme see Bégueret *et al.*, 1994). In *Neurospora crassa* and *Podospora anserina* several *het*-genes are identified to be responsible for cytoplasmic - postfusion- incompatibility reactions (Wilson *et al.*, 1961; Williams and Wilson, 1966; Begueret *et al.*, 1994). In many other species, it is unknown whether the self/nonself recognition is due to pre- and/or postfusion reactions.

In *Aspergilli*, often no clear phenotypic heterokaryon incompatibility reactions can be seen, perhaps due to a low frequency of anastomoses. Here, complementation of different nitrate non-utilising mutants can be used as test for heterokaryon compatibility (Cove, 1976; Debets *et al.*, 1990a). In species like *Aspergillus nidulans*, heterokaryon incompatibility can (partly) be overcome through the use of intraspecies protoplast fusion. Reassociation and recombination can be obtained via the transient heterokaryons and unstable diploids (Dales and Croft, 1977). Interspecies protoplast fusions between different *Aspergillus* species have also been successful in some cases (e.g. Bradshaw *et al.*, 1983; Kevei and Peberdy, 1984; Liang and Chen, 1987).

No viruses have been found in *Aspergillus nidulans* in nature. Previous experiments in our laboratory showed that a mycovirus from *A. niger* could be introduced into *A. nidulans*

by protoplast fusion relatively easily and that, in contrast to the experiments with black *Aspergilli*, spontaneous transfer between strains is not prevented by heterokaryon incompatibility (Coenen *et al.*, 1997). From this and other studies (Liang *et al.*, 1983, Liang and Chen, 1987, Varga *et al.*, 1994a), it was suggested that virus transfer after protoplast fusions occurs readily.

In this study, we investigate the transfer of mycoviruses following inter- and intraspecies protoplast fusion of different black *Aspergillus* (heterokaryon compatible and incompatible combinations) and *A. nidulans* strains (incompatible combinations). The protoplast fusion experiments described here show that the intra- and interspecies incompatibility barrier for viruses in natural isolates of black *Aspergilli* is stronger than the interspecies barrier between black *Aspergilli* and *Aspergillus nidulans*.

Materials and methods

Strains and mutations.

A list of the strains and mutations used for the virus transfer experiments is given in Table 4.1. The black *Aspergillus Ind* strains were isolated in 1992 from soil from one sampling site in Jakarta, Indonesia and characterised as *A. niger* (1-types) or *A. tubingensis* (2-type) on basis of mitochondrial RFLP (after Varga et al 1993, 1994b). Strains N400 (CBS

Table 4.1. Used black *Aspergilli* and *A. nidulans* strains. $^{+}$ = infected, the number indicates the virus dsRNA pattern; the numbering corresponds to the numbers in Fig 4.1A; mt*Oli* ^{r} = mitochondrial oligomycine resistance; *fwn* and *y* = fawn, yellow coloured spores respectively; *cnx* and *nia* = chlorate resistant/nitrate non-utilizing; *ade*$^{-}$, *arg*$^{-}$ and *pro*$^{-}$ = adenine, arginine and proline auxotroph; n.d. = not determined, SI = self-incompatible.

Donors	strain	mitotype		virus/mutations
black *Aspergilli*	N909	1a		--, *fwn, met*, mt*Oli* ^{r}
	Ind 1.5.5	1b		$^{+}$1, mt*Oli* ^{r}
	Ind 1.7.8	1c		$^{+}$2, mt*Oli* ^{r}
	Ind 1.8.3	1b		$^{+}$4, mt*Oli* ^{r}
	Ind 1.8.7	1b		$^{+}$
	Ind 1.8.16	1b		$^{+}$3, mt*Oli* ^{r}
	341 (CBS 223.43)	n.d.		$^{+}$4, *ade*$^{-}$, *arg*$^{-}$, *pro*$^{-}$
A. nidulans	701	nid.		$^{+}$4

acceptors	strain	mitotype		mutations
black *Aspergilli*	Ind 1.5.5	1b		$^{+}$1, *fwn, cnx*
	Ind 1.7.8	1c		$^{+}$2, *fwn, cnx*
	Ind 1.8.1	1b		*fwn, nia*
	Ind 1.8.3	1b		*fwn, nia*
	Ind 1.8.9	1d		*fwn, cnx*
	Ind 1.8.16	1b		$^{+}$3, *fwn, cnx*
	Ind 1.8.42	2a		*fwn, nia*
	N062(CBS 557.65)	1c	SI	*fwn, nia*
A. nidulans	701	nid.		*y, nia*
	702	nid.		*y, nia*
	703	nid.		*y, nia*
	704	nid.		*y, nia*

120.49 = ATCC 9029), 341 (CBS 223.43) and the heterokaryon self-incompatible strain N062 (CBS 557.65) are culture collection strains. Mutated strains N522 and N909 (see below) were derived from N400. The *A. nidulans* strains were isolated from soil samples from Birmingham, England in 1992 (Coenen *et al.* 1996).

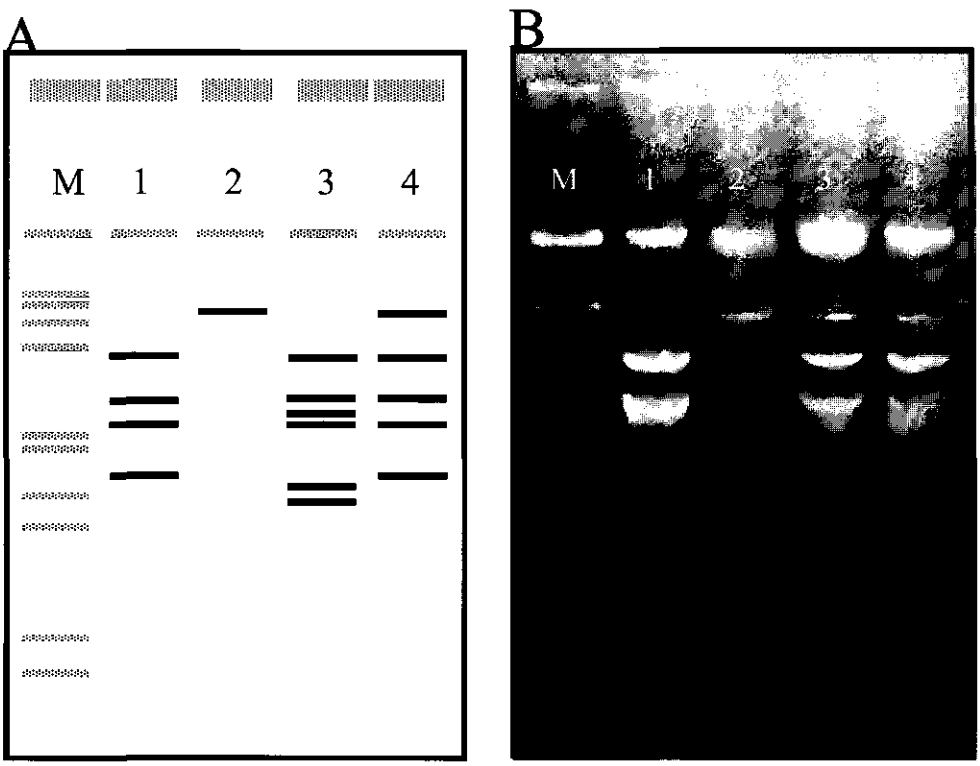

Figure 4.1.A Schematic picture of the infection patterns of different viruses: lane '1' Ind 1.5.5, lane '2' Ind 1.7.8, lane '3' Ind 1.8.16 and lane '4' strain 341. **B** Analysis of parental strains and fusion products: lane '1' strain Ind 1.8.16, lane '2' strain Ind 1.7.8, lanes '3'-'4' recombinant strains Ind 1.8.16 superinfected with the virus of strain Ind 1.7.8. M = marker λ*Eco*RI/*Hin*dIII.

The virus donor strains Ind 1.5.5, Ind 1.7.8, Ind 1.8.7, Ind 1.8.16 and 341 were selected on the basis of their varying infection patterns (Figure 4.1A). The varying infections consist of one to eight fragments, with some similar sized fragments in the different dsRNA patterns. Strain Ind 1.8.16 is known to have a deteriorating phenotypic effect, due to its virus infection. The other infected strains show no abnormal phenotype. The virus donor strain *A. nidulans* 701 was infected via protoplast fusion with *A. niger* strain 341. The *A. niger* strain Ind 1.8.3 was infected with the same virus via protoplast fusion with the infected *A. nidulans* strain 701. This way we constructed an *A. niger* donor strain infected with a virus that has gone through another host species: *A. nidulans*. The acceptor strains were either virus free or

contained viruses with dsRNA patterns dissimilar from that of the donor (differences detectable with gel electrophoresis).

From the strains colour, chlorate- and mitochondrial oligomycin resistant mutants were isolated to distinguish between them when used in the protoplast fusion experiments. The mutations used were: *fwn* = fawn-coloured conidiospores in the black *Aspergilli*, *y* = yellow-coloured conidiospores in *A. nidulans*, *nia* and *cnx* = chlorate resistant/nitrate (and hypoxanthine) non-utilizing and *ade*, *arg*, *met* and *pro* = adenine, argenine, methionine respectively proline auxotroph. Mt*Oli*^{r} is a mitochondrial oligomycin resistance. The mitochondrial oligomycin resistance in *A. niger* was originally isolated in strain N522 (*fwn*A1, *met*B11) from our collection derived from N400. This new strain was named N909 (*fwn*A1, *met*B11, mt*Oli*^{r}). For this two hundred oligomycin resistant colonies were screened in a heterokaryon test, only ten segregated independently from nuclear markers and were concluded to be mitochondrial mutants. Resistant mitochondria can be transferred between heterokaryon compatible strains.

The black *Aspergillus* donor strains containing mt*Oli*^{r} used in the experiments described in this paper, were constructed via protoplast fusion with collection strain N909 (*fwn*A1, *met*B11, mt*Oli*^{r}). The selection for oligomycin resistant wild-type strains and subsequent testing for virus content and nature (nuclear or mitochondrial) of the resistance marker in a heterokaryon test yielded suitable donor strains. Spontaneous oligomycin resistant colonies would be predominantly nuclear based resistances. We have observed nuclear oligomycin resistance in none of the tested colonies in our protoplast fusion experiments. Therefore, the mitochondrially based oligomycin resistant colonies are considered to be fusion products. Moreover in control experiments with oligomycin sensitive fusion partners we have never observed spontaneous resistance: four incompatible combinations of strains (non oligomycin resistant 'donor' strains Ind 1.7.8 and Ind 1.8.16 and acceptors Ind 1.8.1, *fwn*, *nia* and Ind 1.8.3, *fwn*, *nia*) were tested on the occurrence of spontaneous (mitochondrial) oligomycin resistance after protoplast fusion according to our protocol. No resistant colonies were found.

Heterokaryon (in)compatibility

All used *A. nidulans* and black *Aspergillus* strains, except strain N062, were heterokaryon self-compatible. Strain N062 lacks the ability to form anastomoses between its own hyphae. Between the *A. nidulans* strains there was no heterokaryon compatible combination of strains. Between the black *Aspergilli* only the combinations between the infected strain Ind 1.5.5 - virusfree Ind 1.8.1 and between infected Ind 1.8.3* and virusfree Ind 1.8.3 were heterokaryon compatible.

Culture conditions.

The complete medium (CM) used was made essentially as described by Pontecorvo *et al.* (1953) with 10 mM nitrate and/or 10 mM urea as nitrogen source and 1 mg/l $ZnSO_4$, $FeSO_4$, $MnCl_2$ and $CuSO_4$ added. The minimal medium (MM) was as described by Coenen *et al.* (1994) with nitrogen sources 10 mM urea (U) or 10 mM nitrate (N). Chlorate medium (+ClO_3) contained 200 mM $KClO_3$ and 10 mM urea. For protoplasts osmotically stabilised media with 1M sucrose were used, with a similar top layer with half the agar concentration. Mitochondrial oligomycin resistant mutants grow on 1 µg oligomycin/ml. For the recovery of oligomycin resistant protoplasts concentrations of 0.1 µg/ml in the top layer and of 1.0 µg/ml as concentration in bottom layer were used. For *ade*, *met*, *arg* and *pro* deficient strains final concentrations of 0.05 mM adenine, 0.05 mM methionine, 1 mM arginine or 2 mM proline respectively were used. All incubations were done at 30°C.

Protoplast fusion experiments.

Mycelium was harvested from an overnight culture in liquid MM + 0.1M glucose + 0.5% yeast extract + 0.2% casamino acids. Black *Aspergillus* strains were protoplasted with 1 mg/ml Novozym 234 in 0.7M NaCl, 0.2M $CaCl_2$ (2000 mOsm), *A. nidulans* with 2 mg/ml Novozym 234 in 0.8M NaCl, 0.075M $CaCl_2$ (1800 mOsm) for 1½-2 hours at 30°C. Suspensions were filtered over glasswool to remove mycelium and protoplasts were counted with a haemocytometer. Dilutions of black *Aspergilli* protoplasts were made in STC (1.4 M Sorbitol, 10 mM Tris, 50 mM $CaCl_2$; 2000 mOsm), for *A. nidulans* a similar buffer of 1800 mOsm was used. Protoplasts were pelleted by centrifugation during 5 minutes in a swing-out rotor at 1100 g.

Dilutions of protoplast suspensions of every strain were plated on normal and osmotically stabilized medium to test for protoplastation rates and protoplast survival. For each donor strain 24 colonies derived from single protoplasts were tested for presence of the virus.

Equal amounts of donor and acceptor protoplasts (10^6-10^7 each) were fused in a 30% w/v Polyethylene glycol 6000 (PEG6000), 50 mM $CaCl_2$ solution at 30°C for 30 minutes. Protoplasts were plated in a toplayer in osmotically stabilised media.

Virus transfer was tested in two ways: (I) analysis of the total protoplast fusion mixture (duplicated experiments) and (II) analysis of individual recombinant fusion products from the same fusion mixtures as in I (approximately 10 recombinants analysed).

(I) Total analysis. For testing qualitative virus transfer, the total mixture of donor and acceptor protoplasts was plated on osmotically stabilised medium with chlorate to select for chlorate resistant acceptor strains and against wild-type donor strains. After a week all conidia were harvested and transferred to fresh selective medium (+ ClO_3). This scheme was

repeated once. Cultures were then tested for contamination with donor (spore colour on supplemented medium/ growth on donor selective medium (MM + N)) and the purified acceptors (two per combination) were tested twice for virus content.

(II) Individual recombinant fusion product analysis. In intraspecific fusion experiments of the black *Aspergilli*, combinations of infected wild-type donor strains with mitochondrial oligomycin resistance and chlorate resistant/nitrate deficient acceptor strains were used. Recombinant products of single fusion events were selected by plating dilutions of the fusion mixture (I) on selective osmotically stabilised medium with oligomycin and chlorate. The mitochondrial nature of the oligomycin resistance was tested in heterokaryotic transfer to sensitive clones. Recombinant products were subcultured and tested for their virus content.

Virus detection.

Total nucleic acids were isolated via a phenol/chloroform extraction (Maniatis *et al.* 1982), extracts were tested for viral dsRNA fragment contents with gel electrophoresis (0.8% agarose), stained with Ethidium Bromide and visualised by UV transillumination. The infection patterns of the different viruses detected in this way are shown in Fig. 4.1.

Results

The protoplasts of the infected *Aspergillus* donor strains in the different fusion experiments (both black *Aspergilli* and *A. nidulans*) were tested for their virus content. All 24 colonies derived from single protoplasts from each donor contained their expected virus patterns. Thus it is assumed that in all interstrain protopast fusion experiments viruses are present in the initial fusion product.

Transfer of viruses by protoplast fusion was tested by analysis of the total protoplast fusion mixtures (see Materials and Methods). Per combination of strains two fusion mixtures were tested. The results of the various protoplast fusion experiments are given in Tables 4.2-5.

Virus Transfer from Black Aspergilli to A. nidulans

The protoplast fusions with black Aspergillus donors and *A. nidulans* acceptors are described in Table 4.2. Four different donor strains were used in combination with four different acceptor strains. One combination failed as only the donor strains could be recovered from the mixture and therefore transfer could not be tested. All other fifteen combinations showed virus transfer. In all cases, the complete virus pattern of the original donor was found in the acceptor (data not shown).

Table 4.2. Virus transfer via protoplast fusion from black *Aspergillus* donor strains to *A. nidulans* acceptor strains. + = succesfull transfer; n.d. = not determined, see text.

A. nidulans acceptors	black *Aspergillus* donors: 341	Ind 1.7.8	Ind 1.8.7	Ind 1.8.16
701, *nia*	+	+	+	+
702, *nia*	+	+	+	+
701, *nia*	+	+	n.d.	+
704, *nia*	+	+	+	+

Virus Transfer from A. nidulans To Black Aspergilli and A. nidulans

The results of the protoplast experiments with *A. nidulans* as donor are described in Tables 4.3 and 4.4 . Transfer between the heterokaryon incompatible *A. nidulans* strains was always successful (Table 4.3). Also the transfer from *A. nidulans* to *A. niger* and *A. tubingensis* strains was a complete success (Table 4.4). Even the heterokaryon self-incompatible strain N062-07 became infected. However, in contrast to the heterokaryon selfcompatible strains the mycelium of N062 was only partially infected. Some parts of the resulting culture appeared virus free through lack of intramycelial transfer. These transfer experiments with *A. nidulans* as donor yielded black *Aspergillus* strains that were infected via *A. nidulans*. The infected strain Ind 1.8.3* was subsequently used as donor in the transfers between black *Aspergillus* protoplasts. The results show that *A. nidulans* is an efficient donor to both other *A. nidulans* and black *Aspergillus* strains.

Table 4.3. Virus transfer via protoplast fusion between heterokaryon incompatible *A. nidulasn* strains. + = successful transfer.

A. nidulans acceptors	*A. nidulans* donor: 701
701, *nia*	+
702, *nia*	+
701, *nia*	+
704, *nia*	+

Table 4.4. Virus transfer via protoplast fusion between *A. nidulans* strain 701, infected with the virus from *A. niger* strain 341, and several black *Aspergillus* strains. + = successful transfer. +/- = partially infected: the infection is not spread throughout the whole mycelium.

black *Aspergillus* acceptors	*A. nidulans* donor: 701
Ind 1.8.1, *fwn, nia*	+
Ind 1.8.3, *fwn, nia*	+
Ind 1.8.9, *fwn, cnx*	+
Ind 1.8.42, *fwn, nia*	+
N062, *fwn, nia*	+/-

Limited Transfer Between Black Aspergilli

The data of transfer between the different black *Aspergilli* are shown in Table 4.5. The left columns (I) show the data of the total analysis experiments, the right columns (II) show

the data of combined transfer of virus and mitochondrial marker, which will be detailed in the next paragraph (the individual recombinant experiments). All strains belong to the *A. niger* type, except Ind 1.8.42 which is an *A. tubingensis* strain.

The results of the protoplast fusion experiments between black Aspergillus strains may be summarised as follows:

(1) Virus is transferred efficiently between protoplasts of heterokaryon compatible strains. Strain Ind 1.5.5 and Ind 1.8.1 are heterokaryon compatible and in protoplast fusion experiments virus transfers efficiently. Likewise, the isogenic infected and virus free strains Ind 1.8.3 are somatically compatible and virus is transferred in fusion experiments. This observation is consistent with our previous finding that virus is transferred between compatible isolates already during co-cultivation (Van Diepeningen *et al.*, 1997; Chapter 3).

(2) Virus transfer is inefficient in heterokaryon incompatible interactions. In the incompatible *niger-niger* and *niger-tubingensis* combinations (i.e. all combinations except Ind 1.8.1-Ind 1.5.5 and Ind 1.8.3-Ind 1.8.3) only about half of the transfer attempts were successful; in the combination Ind 1.8.1 - Ind 1.8.3 only one of the duplicates became infected, while the other remained virusfree. This inefficient transfer forms a contrast with the highly successful transfer of viruses in the *A. nidulans* experiments described above (Tables 4.2, 3 and 4).

Table 4.5. Virus transfer between different black *Aspergillus* strains. In the left columns (I) the results of the total analyses of the protoplast fusion mixtures, in the right columns (II) the analyses of individual recombinant fusion products; the number of virus containing colonies/total number of recombinant oligomycin resistant colonies tested. + = succesful (lumped) transfer; - = no transfer detected; +/- = one of the duplicate experiments succesful; X = not determined because the virus patterns are overlapping; v = only donor pattern present, acceptor pattern disappeared; * = virus originally from *A. niger* 341, transferred via *A. nidulans* 701.

back *Aspergillus* acceptors:	black *Aspergillus* donors: Ind 1.5.5		Ind 1.7.8		Ind 1.8.16		Ind 1.8.3*	
	(I)	(II)	(I)	(II)	(I)	(II)	(I)	(II)
Ind 1.8.1, *fwn, nia*	+	4/4	+	73/77	-	4/6	+/-	0/9
Ind 1.8.3, *fwn, nia*	-	0/10	-	0/10	+	4/10	+	10/10
Ind 1.8.9, *fwn, cnx*	-	10/10	+	4/10	+	0/10	-	12/12
Ind 1.8.42, *fwn, nia*	-	0/10	+	10/10	+	0/10	+	0/10
Ind 1.5.5, *fwn, cnx*	X	X	+	10/10	X	X	X	X
Ind 1.7.8, *fwn, cnx*	+	0/10	X	X	+	0/10	X	X
Ind 1.8.16, *fwn, cnx*	X	X	+	10/10v	X	X	X	X
N062, *fwn, nia*	-	0/10	-	0/6	-	0/2	-	0/8

(3) Different viruses can coexist in the same host. When the infected strain Ind 1.8.16 is fused with another infected strain, e.g. Ind 1.7.8, which has a different infection pattern, Ind 1.8.16 can be reisolated in which both viruses are present (see Figure 4.1B). Likewise, double infection of strain Ind 1.7.8 also occurs. Also, in the combinations of Ind 1.5.5 and Ind 1.7.8 such coinfections are obtained.

(4) Heterokaryon self-incompatibility and the resulting lack of intramycelial transport protects against virus infection from other black *Aspergillus* strains. The heterokaryon self-incompatible strain N062 was never infected when black *Aspergilli* are used as donor strains (Table 4.5). In the fusion experiments with *A. nidulans* as virus donor, infection did occur (Table 4.4).

(5) Viruses are not restricted to a specific host. Each of the viruses is capable of infecting other strains and even after cultivation in a new host (*A. nidulans*) the virus remains infective for black *Aspergilli* when returned to its original host. However, in our experiments not all strains became infected with each of the viruses. For instance the virus from strain Ind 1.8.16 infected strains Ind 1.8.3, Ind 1.8.9, Ind 1.8.42 and Ind 1.7.8, but it did not infect strains Ind 1.8.1 or N062. Strain Ind 1.8.1 on the other hand could be infected with each of the three other viruses used.

(6) Transfer of virus also depends on the donor host strain or donor-acceptor interaction. The virus from *A. niger* 341 can be transmitted via *A. nidulans* strain 701 to strain Ind 1.8.9. However, in a similar bulk mixing experiment with Ind 1.8.3* as donor the same virus is not transmitted to strain Ind 1.8.9.

Analysis of Individual Recombinant Black Aspergillus Fusion Products

Since the virus transfer was only successful in half of the total mixture experiments between black *Aspergilli*, we wanted to have a control for cytoplasmic contact during the black *Aspergilli* protoplast fusions. We decided to use the transfer of an mitochondrial oligomycin resistance as a selectable marker for cytoplasmic exchange between donor and acceptor strain. Absence of (detectable levels of) viruses in fusion products would then result from loss of the virus during outgrowth of the initial fusion product. Uninfected initial fusion products are unlikely because all tested donor protoplasts contained virus.

In our protoplast fusion experiments, all oligomycin resistances in the donor and acceptor strains were found to be mitochondrial, when checked in a heterokaryon test with isogenic strains with different markers. No spontaneous (mitochondrial) oligomycin resistances were found upon fusion of sensitive protoplasts or after PEG-treatment of acceptor protoplasts. This suggests that resistance was due to transfer of the mitochondrial marker and not to spontaneous novel resistance. In heterokaryon self-incompatible strains such tests are not possible: here mitochondrial rearrangements or mitochondrial replacements in combination with the oligomycine resistance could be used as indication for cytoplasmic contact (data not shown).

After the protoplast fusion, individual oligomycin resistant colonies were picked, checked for the mitochondrial nature of their resistance and tested for their virus content. Results are listed in the right columns of Table 4.5. The combination of strains Ind 1.7.8 and

Ind 1.8.1 was the first to be tested and many (77) colonies were checked. In later experiments approximately 10 colonies of each combination were tested.

Overall these more stringent selective protoplast fusions yielded the following results:
(1) The mitochondrial marker was transmitted in all combinations, suggesting that fusion took place. It also means that transfer of the mitochondrial marker and the necessary cytoplasmic contact is not *per se* lethal to the acceptor strains.
(2) In concordance with the previous experiments, the two heterokaryon compatible combinations (Ind 1.5.5 - In 1.8.1 and 1.8.3* - 1.8.3), both yielded 100% cotransfer of viruses with the oligomycin marker.
(3) Between heterokaryon incompatible strains there is independent transfer of the mitochondrial oligomycine resistance marker and dsRNA viruses during protoplast fusion. Cotransfer of viruses with the mitochondrial marker can take place in none, some or all of the tested oligomycin resistant recombinants. These transfer data can vary from the results in the bulk experiments.
(4) Coexistence of viruses was again observed in the combination of strains Ind 1.7.8 and Ind 1.5.5. In the fusion experiment of Ind 1.7.8 and Ind 1.8.16 the original (deleterious) virus of Ind 1.8.16 was replaced by the donor's virus.
(5) Oligomycin resistant colonies of the heterokaryon self-incompatible strain N062 could also be isolated, but could not be further tested in a heterokaryon test. In none of these colonies virus transfer was observed.

Discussion

We found about ten percent of the world-wide population of black *Aspergilli* to be infected with dsRNA mycoviruses (Chapter 2). The infection patterns, as detected in gel electrophoresis vary considerably in number and size of the dsRNAs, but appear stable in time. Understanding of this population structure requires information on virus transfer rate, virus stability and fitness consequences. This paper focuses on the virus transfer rate and virus stability in a new host. Previous studies in our laboratory have shown that horizontal transfer of these viruses is efficient between heterokaryon compatible strains but is effectively prevented by heterokaryon incompatibility (van Diepeningen *et al.* 1997; Chapter 3). By protoplast fusion it was possible to introduce mycovirus from a black *Aspergillus* isolate into *A. nidulans* in which no mycoviruses have been detected in nature (Coenen *et al.* 1997). In *A .nidulans*, heterokaryon incompatibility slowed down but did not prevent virus transfer, while viruses were transmitted via asexual conidiospores but not via sexual ascospores (Coenen *et al.* 1997). In this paper, we further analysed factors affecting the rate of virus transfer in black *Aspergilli* using protoplast fusions. We tested whether lack of

infectivity is due to the viruses themselves, due to specific resistance mechanisms in the host or due to the specific interaction between heterokaryon incompatible protoplasts.

Efficient transfer was obtained using protoplast fusion experiments from black *Aspergillus* strains to *A. nidulans* and *vice versa.* Between *A. nidulans* strains transfer was successful as well, but poor transfer took place between black *Aspergillus* isolates. In the bulk experiments all the protoplasts were lumped together and therefore these tests are not quantitative. All experiments were conducted in a similar way. Apparently under protoplast fusion conditions there is no absolute interspecies barrier between *A. nidulans* and the black *Aspergilli.* In *A. nidulans,* heterokaryon incompatibility forms a relative obstacle to virus transfer between strains (Coenen *et al.*, 1997) that can be totally overcome by protoplast fusion. However, in the various experiments with black *Aspergilli* only in approximately half of the cases transfer was demonstrated. The protoplast fusion experiments using total analysis of lumped protoplast cultures indicated that virus exchange between black *Aspergillus* and *A. nidulans* is more successful than between black *Aspergillus* isolates. But even when transfer of a mitochondrial oligomycin transfer involves cytoplasmic contact between black *Aspergilli,* only half of the virus transfers is successful.

Virus infectivity, multiple infection and absence of resistance

All the used viruses are capable of infecting both black *Aspergillus* and *A. nidulans* strains, so all are still functional viruses. Therefore absence of virus infectivity cannot explain failure of transfer. The variation and similarities in infection patterns in the black *Aspergilli* raises the question whether a pattern is caused by a single infection or composed of more then one virus and perhaps includes defective virus fragments. Via the protoplast fusions between infected strains with different gel electrophoresis patterns, composed patterns could be constructed, showing the possibility of multiple infection (as illustrated in Figure 4.1B).

Intrahost competition between viruses may cause the replacement of one virus by the other as seen in the oligomycin transfer experiments where the virus in acceptor Ind 1.8.16 was replaced by the virus from the donor Ind 1.7.8. The observed infection patterns in the natural isolates are all very stable when subcultured. The black *Aspergilli* are not resistant to viruses *per se*: in all isolates tested one virus or the other could be introduced via protoplast fusion, either from another black *Aspergillus* donor or from *A. nidulans*. These data suggest that absence of virus in the majority of natural isolates of black *Aspergilli* is not due to virus resistant genotypes, although variation between *Aspergillus* strains in resistance to specific dsRNAs cannot be excluded.

Independent transfer of cytoplasmic dsRNAs and mitochondrial oligomycin resistance

The question remains what causes the poor virus exchange between black *Aspergillus* strains. One can think of pre-fusion (I) and post-fusion and regeneration (II) events leading to non-infected mycelium.

(I) Pre-fusion exclusion of the virus. The total or partial absence of virus infection after protoplast fusion could formally have resulted from experimental artefacts. But, when donor protoplasts were tested for their virus content, all tested single (nucleate and viable) protoplast colonies were found to contain their virus(es). These controls demonstrate that virus must have been present in most if not all of initial fusion products. Furthermore, successful cytoplasmic contact between donor and acceptor protoplasts was ascertained by transfer of a mitochondrial oligomycin resistance. However, viruses were not detectable in a considerable fraction of the colonies derived from the fusion products. This suggests selective loss and elimination of the virus upon regeneration and outgrowth of the protoplasts. However, as stated above, there is no absolute general resistance in any of the black *Aspergillus* strains tested, and virus can be introduced very successfully in any black *Aspergillus* strain via protoplast fusion from *A. nidulans*.

(II) Post-fusion exclusion of virus. Alternatively, there may be elimination of dsRNA due to heterokaryon incompatibility reactions following cytoplasmic mixing in the fusion experiments. The severity of the incompatibility reaction can vary depending on type and number of *het*-genes involved. Evolutionary, the *het*-gene products of *A. nidulans* and black *Aspergilli* may be diverged, resulting in less specific recognition and few or no incompatibility reactions between the species after fusion.

The severity of the incompatibility reaction may also depend on the amount of cytoplasmic mixing. The tested viable donor protoplasts all contained virus, but the selected (oligomycin resistant) recombinants could be the results of fusions with small, possibly anucleate and virusfree protoplasts (combination of I and II). Transfer of large amounts of foreign cytoplasm may be lethal to the recipient. The selection for the mitochondrial marker, guarantees that there has been cytoplasmic contact, but not on what scale transfer has taken place.

Heterokaryon incompatibility

The role of heterokaryon incompatibility in nature is unknown. It has been suggested that heterokaryon compatibility may serve to enable recombination via the parasexual cycle and that heterokaryosis is a way for haploid fungi to enjoy the benefits of functional diploidy (Leslie, 1993). Prevention of heterokaryons may be beneficial in a natural population when incompatibility limits the transfer of possibly deleterious elements (e.g. Caten, 1972; Anagnostakis, 1983; Debets *et al.*, 1994). Alternatively, it may be essentially neutral

(Bégueret *et al.*, 1994). Van Diepeningen *et al.* (1997; Chapter 3) demonstrated that in cocultured black *Aspergilli* virus transfer is completely blocked by heterokaryon incompatibility, whereas between heterokaryon compatible strains viruses are readily transmitted. In *A. nidulans* heterokaryon incompatibility only slows down the rate of transfer (Coenen *et al.*, 1997). The experiments described in this paper suggest that heterokaryon incompatibility in black *Aspergilli* forms a more serious barrier to virus transfer that cannot always be overcome by protoplast fusion.

The heterokaryon self-incompatibility of strain N062 is a curiosity; all tested natural isolates of black Aspergilli were heterokaryon selfcompatible. Self-incompatible strains are not capable of fusion with isogenic and heterogenic hyphae and apparently this self-incompatibility causes the strain to be uninfectable via other black *Aspergillus* strains. Individual protoplasts can be infected via *A. nidulans*, but virus cannot spread between the isogenic self-incompatible colonies, which results in a patchy infection on the selection plates.

Virus dynamics

When the somatic incompatibility mechanisms differ, as might be between black *Aspergilli* and *A. nidulans*, a strong cytoplasmic incompatibility reaction may be absent. Transfer of cytoplasmic elements such as viruses may then occur after protoplast fusion. Whether spontaneous fusion of non-related mycelia occurs in nature remains unclear. Kempken (1995a) reported the transfer of a mitochondrial plasmid under semi-natural conditions from *Ascobolus immersus* to *Podospora anserina*, two fungi which inhabit the same ecological niche. Hoffmann *et al.* (1994) described the transfer of a resistance gene and other foreign sequences to *Aspergillus niger* during co-culture with transgenic plants. Interspecies transfer after contact seems therefore possible.

Mycoviruses in the black *Aspergilli* have a (small) deleterious effect on their host's fitness and are stable in the clonal lineages (Chapter 6). Intraspecies transfer is limited to the rare occurrence of heterokaryon compatibility among natural isolates (Van Diepeningen *et al.*, 1997; Chapter 3). Our results suggest that interspecies transfer could help explain the apparently stable virus infection throughout the black *Aspergillus* population. We therefore suggest that under natural conditions horizontal virus transfer should occur sufficiently frequent to offset the selective elimination of virus-infected strains. The unexpected observation that infection of black Aspergilli by *A. nidulans* is more successful than by other black *Aspergilli* may indicate that in nature the majority of these infections may come from other fungal species. Other authors also reported the ability of viruses to survive in new hosts or hinted at the relatedness of viruses in different host species (e.g. Lhoas, 1971; Kim and

Bozarth, 1985). A test of this hypothesis requires the quantitative estimation of the transfer rates and fitness effects of the dsRNA viruses involved, which will not be an easy task.

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5

Interspecies virus transfer via protoplast fusions between *Fusarium poae* and *Aspergillus niger* strains.

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Abstract

Protoplast fusion experiments were done between virus infected *Fusarium poae* strains and virusfree black *Aspergillus* strains. Partial and total transfer of *Fusarium* virus patterns occurred and the viruses survived in their new hosts. Protoplasting conditions can influence the transfer rate, but fusion can effectively bridge the differences between the two species. The mycoviruses are not restricted to their *Fusarium* host, but may have a broader host range. Also, similarities between the genome organisation of dsRNA mycoviruses and dsRNA patterns in different fungal species suggest a relatedness between these viruses, which could be the result of co-evolved infections or of interspecies transfer. Occasional interspecies transfer between species as suggested by these experiments could explain the scattered infection pattern seen throughout the population in the natural black *Aspergillus* population.

Introduction

Viruses are commonly found in animals, plants, bacteria and fungi. For instance, many plant viruses are found in connection with symptoms in their host. The majority of plant viruses have a single-stranded RNA genome, but some have double-stranded RNA or single

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or double stranded DNA genomes (Zaitlin and Hull, 1987). These plant viruses vary widely in their host ranges. The barley stripe mosaic virus (BSMV) is in nature limited to barley (Timian, 1974), whereas viruses like cucumber or tobacco mosaic virus (CMV and TMV), tomato spotted wild virus (TSWV) and tobacco ringspot virus (TRSV) have very wide host ranges (Matthews, 1991). For example, CMV can affect members of the *Cucurbitaceae*, *Solanaceae*, *Violaceae*, *Iridaceae* and others.

As more research on fungi is being conducted, an increasing number of 'myco'-viruses are discovered (Buck, 1986; 1998). Most of these mycoviruses have genomes of double-stranded (ds) RNA. Though most viruses in animals, plants and bacteria have an infectious extracellular phase in their multiplication cycle, mycoviruses are transmitted only via intracellular routes (Buck, 1998). Very little is known about the host ranges of these mycoviruses. Some mycoviruses from different hosts have similar sized particles and/or dsRNA fragments. Also (sequence) homology between viruses in distantly related fungal species has been found, suggesting relatedness of these viruses (Buck, 1998; Kim and Bozarth, 1985;Liang and Chen, 1990; Liang *et al.*, 1995). This could be due to long-lasting infections derived from a common ancestor, or due to more recent transfer events.

Fungal viruses vary in their effects: Killer viruses in yeast can be considered beneficial under high density conditions for their host (Dulfree and Bussey, 1979), whereas the mycoviruses in economically interesting species such as *Agaricus bisporus* (Hollings, 1962) and *Pleurotus* spp. (Go *et al.*, 1992; Stobbs *et al.*, 1994) are deleterious. Mycovirus in *Penicillium chrysogenum* can cause lysis of its host (Lemke *et al.*, 1973). Most of the mycoviruses, however, are cryptic in their effects on their host (Ghabrial, 1980). These mycoviruses spread mainly via asexual spores (Lecoq *et al.*, 1979) and often less efficiently via sexual spores (Rawlinson *et al.*, 1973; Day *et al.*, 1977; Roger *et al.*, 1986). Intraspecies transfer can be limited or blocked by the barrier formed by heterokaryon incompatibility reactions (Anagnostakis and Day, 1979; Brasier, 1984; Liu and Milgroom, 1996).

In the black *Aspergilli*, ten percent of the population world-wide is infected with a great variety of dsRNA mycoviruses, with similar infections in distantly related haplotypes (Varga *et al.*, 1994a; Chapter 2 & 7) The wide-spread heterokaryon incompatibility between different *Aspergillus niger* strains in nature blocks the intraspecies transfer of these viruses via direct hyphal contact in laboratory experiments (Van Diepeningen *et al.*, 1997; Chapter 3). Even in protoplast fusion experiments the black *Aspergillus* intraspecies transfer is limited (Van Diepeningen *et al.*, in press; Chapter 4). However, transmission via protoplast fusion from *A. niger* to *A. oryzae* and *A. ficuum* was mostly successful (Liang and Chen, 1987) and to naturally uninfected *A. nidulans* strains virus transfer was 100% successful (Van Diepeningen *et al.*, in press; Chapter 4). From a population dynamic point of view these viruses with (small) deleterious effects on fitness are expected to decline in the *A. niger*

population without (intraspecies) means of transfer, but the natural infection frequency is stable world-wide (Chapter 2).

Since extracellular mycoviruses are not infective, other routes of viral infection must be considered. When mycoviruses are transferred via transient interspecies hyphal contact or through (animal) vectors, such transfers could explain the observed extent of infection in a population with highly restricted intraspecies transfer. Mycoviruses could then have wider potential host ranges. In *Fusarium poae*, another ascomycete, all natural isolates were found to contain dsRNA viruses (Fekete *et al.*, 1995), some of which have similar sized fragments as *A. niger* viruses. In the experiments described in this paper we have tested the qualitative possibility of virus transfer and maintenance of several mycoviruses from *F. poae* in black *Aspergillus* strains. We have chosen infection via interspecies protoplast fusion as means of transfer.

Materials and Methods

Fungal isolates

The wild-type *F. poae* isolates A11, TAPO-18, TAPO-21 and TAPO-30, isolated from wheat kernels, were used as virus donors. The black *Aspergillus* strains used as acceptors were isolated from soil from Jakarta, Indonesia, on selective medium with 20% tannin. Ind 1.8.1, Ind 1.8.3 and Ind 1.8.9 were characterised as *A. niger* and Ind 1.8.42 as *A. tubingensis* on basis of their mitochondrial RFLP's (Varga *et al.*, 1993; 1994b). *Nia* chlorate resistant/nitrate deficient mutants of the black *Aspergilli* were used to enable direct selection of either donor or acceptor from the fusion mixtures (Van Diepeningen *et al.*, 1997; Chapter 3).

Culture Conditions

The *Fusarium* strains were grown on Czapek-Dox medium enriched with yeast extract, casamino acids and neopepton (3 g l^{-1} each). Liquid cultures were inoculated with 10^6 spores ml^{-1} and grown for 20 h at 25°C in a rotary shaker at 120 rev. min^{-1}. Mycelium was harvested by filtration and protoplasted with 2 mg ml^{-1} Novozym 234 or with Novozym in combination with Oerscovia both in 0.7M NaCl, 0.2M $CaCl_2$ (± 1800 mOsm) for 2-2½ h at 30°C. The '*Oerscovia*' lytic enzymes are excreted by *Oerscovia xanthineolytica* when grown on *A. nidulans* cell wall material. Portions of the enzyme preparations were freeze-dried and kept at -50°C (Bos and Slakhorst, 1981). Tested protoplasting mixtures with different concentrations of pure Novozym or Novozym in combination with either helicase, cytohelicase or cellulase yielded far fewer protoplasts than the combination of Novozym and Oerscovia.

The *Aspergilli* were grown on minimal medium (MM) with 0.1M glucose and 10mM urea and supplemented with 0.5 gl^{-1} yeast extract and 0.2 gl^{-1} casamino acids or in the case of strain Ind 1.8.3 on complete medium (CM) with 10mM urea (Pontecorvo *et al.*, 1953). Liquid cultures were inoculated with 10^6 spores ml^{-1} and grown for 20 h. at 30°C at 180 rev. min^{-1}. Harvested mycelium was protoplasted with 1 mg ml^{-1} Novozym 234 in 0.7M NaCl, 0.2M $CaCl_2$ (± 2000 mOsm) for 2-2½ h at 30°C.

Protoplast Fusion Experiments

Protoplast suspensions were filtered over glasswool to remove mycelium and protoplasts were counted with a haemocytometer. Dilutions of suspensions were made in STC (1.4M Sorbitol, 10mM Tris, 50 mM $CaCl_2$; 2000 mOsm). Equal amounts of donor and acceptor protoplasts (10^6-$0.5x10^7$) were fused in a 30% w/v PEG6000, 50 mM $CaCl_2$ solution for 45min at 30°C. The fusion mixture was gradually diluted to 1:1 with STC. These diluted fusion mixtures were directly plated in duplicate in a top-layer of osmotically stabilised medium containing 1M sucrose. Due to the strong aggregation and fusion of cells no distinct estimates of cell survival or number of cells per aggregate could be made. Adding 200mM $KClO_3$ and 10 mM urea enabled direct selection of the *Aspergillus* acceptors. After two selection rounds the fusion products were tested for contaminations with the donor on selective medium with NO_3^- as sole nitrogen source. The purified acceptors were tested for virus content. Both fusions and tests for virus content were done in duplicate.

dsRNA detection

Mycelium cultures were grown overnight and were powdered in liquid nitrogen. From these, total nucleic acids were isolated by a phenol/chloroform extraction (Maniatis *et al.*, 1982). Following agarose gel electrophoresis viral dsRNA fragments were stained with Ethidium Bromide and detected by UV transillumination (e.g. Figure 5.1). The dsRNA nature of the virus fragments was verified by treating the nucleic acid suspension with various nucleases (Fekete *et al.*, 1995).

Results

To test the donor *Fusarium* protoplasts for their virus content 24 single protoplast colonies of each donor were checked. All of these colonies contained the typical dsRNA banding pattern of the donor. In controls PEG-treatment of the black *A. niger* and *A. tubingensis* protoplasts never produced extra nucleic acid fragments. Occurrence of dsRNA viral fragments after fusion must therefore be the result of transfer. In total two sets of interspecies fusion experiments were done.

In the first set of experiments the *F. poae* strains were protoplasted with 2 μgml^{-1} Novozym 234 as protoplasting agent and the *A. niger* acceptor strains with 1 μgml^{-1} Novozym. The different *F. poae* strains yielded relatively few (max. $5*10^5$ protoplasts ml^{-1}), generally small, protoplasts. The *Aspergillus* strains yielded normal amounts of protoplasts ($1\text{-}5*10^7$ protoplasts ml^{-1}). The protoplasts of *Fusarium* strains A-11, TAPO-18 and TAPO-30 were collected and fused with protoplasts of *A. niger* strain Ind 1.8.9 and in case of *F. poae* strain TAPO-18 also with *A. niger* strain Ind 1.8.3. After two weeks of selective culturing the *A. niger* acceptor strains were tested for their virus content. Transfer of the complete virus pattern was detected in the combination of *F. poae* strain TAPO-18 and *A. niger* strain Ind 1.8.9 and transfer of only the largest virus fragment was detected in the combination of strains A-11 and the same acceptor strain (Figure 5.1). Only the third combination with acceptor Ind 1.8.9 showed no transfer at all.

Figure 5.1. Gel electrophoresis patterns of three sets of fusion products and parental strains. The first set shows parental *F. poae* strain TAPO-18 and two fusion products with *A.niger* Ind 1.8.9. Set two TAPO-30 and virus-free fusion products with Ind Ind 1.8.9. Set three: virus-free parental strain Ind 1.8.9, Donor strain A-11 and partially infected products.

Experiments were performed to increase the yield of protoplasts of the different *F. poae* strains. Mycelia of different age were protoplasted with different cell wall degrading enzymes or mixtures of these. Numbers of protoplasts were scored after various protoplasting periods. A mixture of Novozym and Oerscovia yielded the highest protoplast numbers ($1\text{-}5*10^7$ protoplasts ml^{-1}) under conditions as described in Material and Methods. Strain TAPO-18, successful in the first transfer experiments, performed poorly under the new conditions and was omitted from the second set of experiments.

The second set of fusion experiments was done under these new protoplasting conditions with Novozym and Oerscovia. Wild type *F. poae* donors were the strains A11, TAPO-21 and TAPO-30, and the two *A. niger* strains Ind 1.8.1 and Ind 1.8.9 and the *A. tubingensis* Ind 1.8.42 were used as acceptors. The results of these experiments are listed in Table 5.1 together with the results of the first experiment. These experiments with considerably more, differently obtained, protoplasts showed no detectable virus transfer, although the single donor protoplasts tested contained virus as normal.

Table 5.1. Virus transfer experiments via protoplast fusions between *F. poae* and *A. niger* strains. The experiments were done in duplicate; + = successful transfer, ± = partial transfer, – = no transfer, n.d.= not determined, [1] or [2] = first or second experiment.

A. niger acceptor strains	*F.poae* donor strains: A-11	TAPO-18	TAPO-21	TAPO-30
Ind 1.8.1	–[2]	n.d.	–[2]	–[2]
Ind 1.8.3	n.d.	–[1]	n.d.	n.d.
Ind 1.8.9	±[1]	+[1]	–[2]	–[1]
Ind 1.8.42	–[2]	n.d.	–[2]	–[2]

Discussion

In this study we have examined the possibility of virus transfer and maintenance following protoplast fusion between two distantly related fungal species. Successful transfer and stable maintenance would imply that mycoviruses do not have to be limited to one host species and may have a broader host range. In a previous study (Van Diepeningen *et al.*, in press; Chapter 4) interspecies virus transfer was more efficient from *A. niger* to *A. nidulans* and vice versa than intraspecies transfer from an *A. niger* isolate to other vegetatively incompatible *A. niger* isolates. However, although mycoviruses can be introduced and maintained in *A. nidulans*, natural isolates of *A. nidulans* are virus free (Coenen *et al.*, 1997). Transfer from *A. niger* to *A. oryzae* and *A. ficuum* was also shown to be possible (Liang and Chen, 1987). For species in which intraspecies virus transfer is blocked by intraspecies heterokaryon incompatibility barriers as in the black *Aspergilli* (Van Diepeningen *et al.*, 1997; Chapter 3), sources of infection could probably be found in other species.

The black *Aspergilli* and *F. poae* for example have some overlap in their ecological niches and consequently may co-occur. Infected *Fusarium poae* isolates could be a potential natural source of infection for *A. niger*. The black *Aspergilli* are mainly saprophytic, but

occasionally pathogenic and have a world-wide occurrence. Black *Aspergilli* are also frequently found on harvested products and their spores are distributed by air. *F. poae* is a secondary pathogen of small-grain cereals and both species occur soilborne.

Both *Aspergillus* and *Fusarium* species carry viruses in nature: Ten percent of the *A. niger* strains throughout the world contain dsRNA viruses, whereas most if not all *F. poae* strains are infected with dsRNA mycoviruses (Fekete *et al.*, 1995). Some of these viruses have similar sized dsRNA fragments as in *A. niger*, though their relationship is not yet confirmed on the basis of molecular data. In the *F. poae* population the vegetative incompatibility is less severe (Kerényi *et al.*, 1997) and it is not clear whether this vegetative incompatibility blocks transfer as in the black *Aspergilli.*

We took four naturally infected *F. poae* strains with different infection patterns as possible virus donors in protoplast fusion experiments and four virus-free members of the black *Aspergillus* aggregate as acceptors. These black *Aspergilli* could be classified as *A. niger* and *A. tubingensis* strains on the basis of molecular data. The *F. poae* strains were protoplasted with Novozym or a combination of Novozym and *Oerscovia.*

Virus transfer was observed in two combinations of strains. One was a complete transfer, the other a partial transfer of the respective dsRNA patterns of the donor strains. The *Oerscovia* enzyme seems to enhance the number and size of obtained protoplasts but also seem to affect the transfer negatively. Smaller protoplasts with less cytoplasm may result in fusion products with fewer incompatibility reactions and thus more surviving recombinants. This would be in accordance with the observations that in *A. niger-A. niger* fusions with strong intraspecies incompatibility barriers, transfer of an mitochondrial oligomycin resistance is not absolutely linked to virus transfer, but probably dependent on the size and thus survival of the fusion products. Due to clustering of protoplasts during PEG treatment no exact quantification of protoplast survival and transfer can be made. However, from a qualitative point of view, transfer is possible.

Compared to the transfer from *A. nidulans* to black *Aspergilli* and vice versa, the transfer from the *F. poae* strains to the black *Aspergilli* is less efficient. This could be explained by a stronger species barrier between *Fusarium* and *Aspergilli* or simply by differences in the effects of the protoplastation and fusion on the two species. Transformation of *Aspergillus* protoplasts with *Fusarium* viruses could be another way to test for the survival of viruses in a new host, but the technique excludes any influences of the direct fungal contact on transfer. Co-cultivation of the two species in Petri dishes on rich media resulted in rapid outgrowth and expulsion of one of the partners and no spontaneous transfer was detected. Poorer and more natural conditions may be necessary for these transfers. So although protoplast fusion is an unnatural way for virus transfer, these

experiments show the possibility of mycovirus survival in a new host after at least some transient cytoplasmic contact.

The transfer of only one dsRNA fragment of the pattern from *F. poae* strain A-11 to *A. niger* Ind 1.8.9 indicates that either this strain is infected by more than one virus or that the extra bands are not necessary. These extra fragments could be defective derivatives of the virus or satellite fragments (Buck, 1998). The native black *Aspergillus* viruses have a (small) deleterious effect on their host (Chapter 6). Whether the *Fusarium* viruses have such effects on *Fusarium* and/or *Aspergillus* hosts is unknown.

The experiments indicate that mycoviruses might indeed have broader host spectra than as yet assumed and that interspecies transfer in nature could be successful. Further molecular analyses is required to test whether this occurs/occurred in nature. The *Fusarium* and *Aspergillus* viruses consist of similar sized dsRNA fragments and the experiments described here showed that the *Fusarium* viruses can be maintained in new *Aspergillus* hosts. Perhaps such occasional interspecies transfer between species could result in a both geographically and genetically scattered infection pattern throughout the population as found in the black *Aspergillus* population. It could also explain the variation and spread of similar mycoviruses in the black *Aspergillus* species lacking intraspecies transfer. However, direct mycelial contact via anastomoses does not have to be the exclusive mode of dispersal for mycoviruses. Exchange of DNA from transgenic plants to *A. niger* has been demonstrated by Hoffmann *et al.* (1994). Marienfeld *et al.* (1997) reported to have evidence for the transfer of nucleic acid transfers between fungal viruses and plant mitochondria. Insect or nematode vectors could also be involved in mycovirus transfer as they are in the transfer of plant viruses. Once infected, an asexual fungal host obviously has no active mechanism against that virus infection.

Acknowledgements

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6

Fitness effects of mycoviruses in black *Aspergillus* strains.

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Abstract

Fitness effects of dsRNA mycoviruses on black *Aspergillus* strains were determined in isogenic infected and virus free strains that were constructed for this purpose. Comparison of the isogenic infected and virus-free strains showed small but detectable negative effects of the mycoviruses. One of the viruses causes a strong host phenotype with non-sporulating sectors. The fitness effects of the viruses were quantified on different media for mycelial growth rate (growth area)(~ 2%), spore production (~ 5%) and competition capacity of the host (~ 45%). A model is presented for the virus and host population interactions. This model predicts that for deleterious infections to cause a stable infection frequency, the horizontal transfer should equal the selective disadvantage and spontaneous loss. The consequences of the fitness effects on the population are discussed.

Introduction

Mycoviruses are widespread in fungi (Buck, 1986; 1998). They consist of an isometric or different formed protein coat and an often segmented double stranded RNA genome. Infection ratios vary per species. Mycoviruses have not been found in the sexual *Aspergillus nidulans* (0/112 isolates tested) (Coenen *et al.*, 1997). In sexual and asexual *Aspergillus* isolates belonging to the sections *Fumigati*, *Circumdati* and *Flavi* the infection frequency varies from 3.5 to 10.9% (Varga *et al.*, 1998; Elias and Cotty, 1996). In asexual *Fusarium poae* 100% of the population (55/55) is infected with mycoviruses displaying a great variety of dsRNA patterns (Fekete *et al.*, 1995). The vertical transfer of mycoviruses from parent to progeny is mainly through asexual spores (Lecoq *et al.*, 1979) and not or less efficiently via sexual spores (Rawlinson *et al.*, 1973; Day *et al.*, 1977; Rogers *et al.*, 1986b; Coenen *et al.*,

1997). Intraspecies transfer through direct mycelium contact can be limited or blocked by the barrier formed by heterokaryon incompatibility reactions (e.g. Anagnostakis and Day, 1979; Brasier, 1984; Liu and Milgroom, 1996). Little is known about interspecies transfer in nature and host ranges of the mycoviruses.

Most of the mycoviruses have no known effects on their host and live a 'cryptic' life (Ghabrial, 1980; 1996). Some can be considered beneficial to their host under certain conditions, like the killer viruses in yeasts and *Ustilago maydis* in high density populations. These killer viruses code for toxins killing uninfected strains, but protect infected strains, thus enhancing their and their host's relative proportion in the population (Koltin *et al.*, 1978; Wickner, 1991). Others, like the mycoviruses in economically important species like *Agaricus bisporus* (Hollings, 1962) and *Pleurotus* spp. (Go *et al.*, 1992; Stobbs *et al.*, 1994), are pathogens causing serious crop reductions.

Comparisons between infected and virusfree strains can be used to quantify the phenomena caused by the virus infections. Differences were observed between hypovirus infected and dsRNA-free strains of *Cryphonectria parasitica* (Elliston, 1985). Kazmierczak *et al.* (1996) attributed the cause of the reduced virulence, sporulation and pigmentation to the reduced accumulation of a small number of host mRNAs and proteins of especially four host genes under influence of the virus infection. In *Pleurotus florida* comparisons between different infected and virus free strains showed reduced growth and growth abnormalities, increased infections in culture beds and a reduction in fruitbody yield of circa 30% (Go *et al.*, 1992). Rinker *et al.* (1993) compared isogenic infected and virus free lines of *Pleurotus pulmonarius* and found reduced growth, no changes in carpophore morphology or colour, and a total reduction in yield of 50%.

The physiological effects of mycoviruses on their hosts are largely unknown. Perhaps the best characterised are the virus-produced killer toxins of *U. maydis*, which function by creating pores in the cell membrane and disrupting ion fluxes. Immunity to these toxins is conferred by the preprotoxins and their derivates or by nuclear resistance genes (Park *et al.*, 1994). In other fungi small viral effects may be caused by simple usage of metabolism and resources of their host. Ghabrial (1996) states that, as a rule, infections due to mycoviruses are both latent and persistent.

In the asexual black *Aspergilli* mycoviruses have a prevalence of 10% in natural populations and occur in many different haplotypes (Varga *et al.*, 1994b; Chapter 2). Vertical transmission of the variety of viral dsRNA fragments via conidiospores is highly efficient: all progeny contain the complete parental set of fragments and no spontaneous loss could be detected. In contrast, horizontal transfer is effectively blocked by the extensive heterokaryon incompatibility between strains (Van Diepeningen *et al.*, 1997; Chapter 3). Virusfree strains are not resistant to viruses since these can be introduced and maintained

successfully via intra- and interspecies protoplast fusion (Van Diepeningen *et al.*, 1998; Chapter 4). The (phenotypic) symptoms of virus infection are hidden in all but one of our infected (68) strains. In this paper we quantify the cost of such 'cryptic' virus infections in different black *Aspergillus* strains on three different fitness traits: mycelial growth rate ('vigour'), asexual spore production ('fecundity') and competition ability with respect to other strains ('competitiveness').

Estimates of the fitness effects and the rates of the different modes of transfer of a parasite allow model predictions of implications for a host population. Fine (Fine, 1975) specified that in an asexual host a parasite limited to vertical transfer cannot persist in a population if it lowers the fitness. Lipsitch *et al.* (1995) analysed a model for a parasite transmitting both vertically and horizontally. Their model predicts that if prevalence is high, most transmission will be vertical, but that horizontal transmission rates must be high to reach and maintain a stable equilibrium prevalence. For the black *Aspergilli* we present a simple population model and discuss the implications of the viral fitness effects and transmission limits for host and mycovirus.

Materials and Methods

Strains

The *Ind* strains used were isolated from soil samples from Indonesia on selective medium with 20% tannin (Van Diepeningen *et al.*, 1997; Chapter 3). Strain Z1.1 originated from a Dutch hospital. The strains were characterised based on their mitochondrial haplotypes according to Varga *et al.* (1993; 1994a). The strains were classified as *'Aspergillus niger'*-(1)-types, except strain Ind 1.8.42 which is a *'Aspergillus tubingensis'*-(2)-type. Both these types are members of the '*A. niger* aggregate' and only distinguishable with molecular markers.

Table 6.1. List of the strains used divided in originally virus free and infected strains. Strains characterised on mitochondrial haplotypes as *A. niger* (1-types) or *A. tubingensis* (2-type).

virus-free strains		infected strains		
strain	mt-type	strain	mt-type	virus-pattern
Ind 1.8.1 [a]	1b	Ind 1.4.24	1c	6
Ind 1.8.2	1a	Ind 1.5.5 [a]	1b	3
Ind 1.8.4	1c	Ind 1.7.8	1c	2
Ind 1.8.9	1d	Ind 1.8.7	1b	1
Ind 1.8.42	2b	Ind 1.8.16	1b	4 + 5[b]
		Z 1.1	1c	2

[a]) Strains Ind 1.8.1 and Ind 1.5.5 are heterokaryon compatible, all other combinations of strains are heterokaryon incompatible. [b]) Patterns of the infected and partially cured line respectively.

A list of the strains used with mitochondrial classification and virus content is given in Table 6.1. All pairwise combinations of these strains, except for the combination Ind 1.5.5 and Ind 1.8.1, are heterokaryon incompatible (Van Diepeningen *et al.*, 1997; Chapter 3).

Curing and Infecting

Isogenic lines with the same genetic wildtype background that only differ in their virus infection were constructed for the fitness experiments. From the naturally infected strains, strain Ind 1.8.16 could be cured from its virus by sequential hyphal tip isolation (two-daily subculturing of young hyphae over a period of three weeks). In the other strains this technique did not yield virus free lines. Addition of different amounts of the protein synthesis inhibitor cycloheximide hampered fungal growth, but did not cause loss of virus infections.

Infection of strains was obtained via spontaneous infection between the heterokaryon compatible strains (Van Diepeningen *et al.*, 1997; Chapter 3) or via protoplast fusion experiments between heterokaryon incompatible strains (Van Diepeningen *et al.*, 1998; Chapter 4). After infection, either spontaneous or via protoplast fusion, further transfer between isogenic (compatible) mutants can be obtained by co-culturing of these strains.

Media

Complete Medium (CM) and Minimal Medium (M) were made as described by Pontecorvo *et al.* (1953) with 10 mM nitrate as nitrogen source and 1 mg/l $ZnSO_4$, $FeSO_4$, $MnCl_2$ and $CuSO_4$ extra added. The more depleted Very Minimal Medium (MM) as described by Coenen *et al.* (1994), was also added with 10 mM nitrate. Water Agar (WA) consists of demiwater with 15 g/l agarose. In some linear growth tests 0.01 mM of the protein synthesis inhibitor cycloheximide (^{cyc}) was added to complete and minimal medium. All incubations took place at 30°C.

Fitness tests.

The strains used in the 3 different fitness tests were tested before and after the experiments on their virus content. The data of the fitness experiments were tested with a Student's t-test (Sokal and Rohlf, 1995).

1) Linear growth rate (vigour). Linear growth tests were done on (a selection of) all used media (CM, M, MM, WA, CM^{cyc} and M^{cyc}). Spores of a virusfree or infected strain were inoculated in the middle of a Petri dish or at the beginning of a race tube: a glass tube of 50 cm with raised ends, half filled over the whole length with medium. After a day the extent of the hyphal growth was marked and used as starting point. Linear growth was measured at fixed time intervals along two perpendicular axes (4 measurements) in the Petri

dishes or along the race tubes. Linear growth was converted to growth per day (linear growth rate). Virus stability was checked before and after growth. In some special race tubes with sampling holes mycelium could be sampled during growth. Isogenic virusfree and infected strains were compared to estimate the fitness effect of the virus infection.

2) Spore production (fecundity). Spore production was measured on complete and very minimal medium (CM and MM). The total spore production of strains was measured in Eppendorf tubes with 0.75 ml medium or in 0.5 cm^2 punches from full grown Petri dishes from growth rate experiments. Spores were inoculated with the tip of a needle. After 11-13 days of growth a spore suspension was made of the fully grown colonies in 0.5 ml Saline (0.8% NaCl) + 0.005% Tween vortexing 30 seconds. Further dilutions in Saline were made and spore numbers were counted in duplicate with a Coulter Counter (model ZF with channelyzer). The comparisons were made between isogenic virusfree and infected strains.

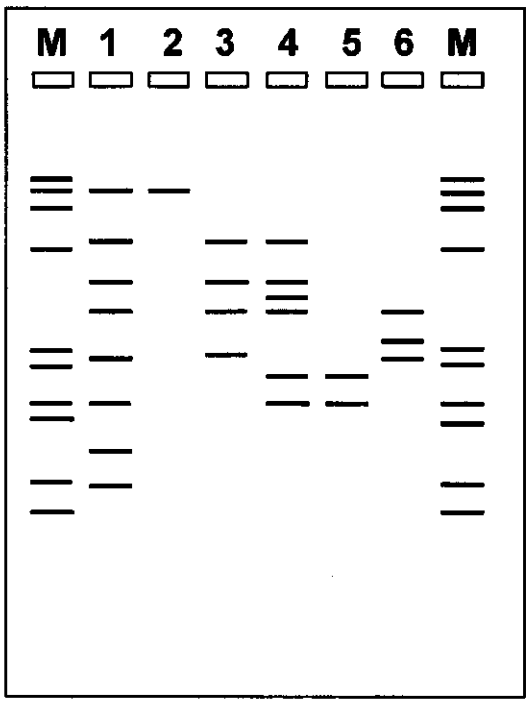

Figure 6.1. DsRNA mycovirus patterns of the infected strains. Lane 1 strain *Ind*1.8.7, lane 2 strain *Ind*1.7.8 (similar to infection in strain Z1.1), lane 3 strain *Ind*1.5.5, Lane 4 strain *Ind*1.8.16 (+), lane 5 strain *Ind*1.8.16(-) = partially cured, lane 6 strain *Ind*1.4.24. M's are molecular weight markers λ*Hin*dIII/*Eco*RI. Sizes of the dsRNA fragments range from 0.9 kb to 4.1kb (strain *Ind*1.8.7) (calculation of sizes on basis of DNA-marker sizes after Livshits et al. (1990)).

3) Competition experiments (competitiveness). The competition experiments were done on two media: complete and very minimal medium (CM and MM). Equal amounts of infected or virusfree wildtype strain and heterokaryon incompatible, fawn-coloured reference strain Ind 1.8.3 or Ind 1.8.9 (approx. 1000 viable spores each) were spread on medium in 9 cm ∅ Petri dishes. After five days all spores were harvested and dilutions in

Saline were plated on medium with 0.05% triton. The numbers of black and fawn colonies respectively were counted after 2-3 days. Ratios between reference strain and infected or virus-free strains were calculated and compared.

Virus detection

Total nucleic acid was isolated via a phenol/chloroform protocol and DNA, viral dsRNA and ssRNA were separated via gel electrophoresis (Maniatis *et al.*, 1982). The dsRNA nature of the viral fragments was confirmed by treating the nucleic acid suspensions with different nucleases under various salt concentrations (Fekete *et al.*, 1995; Varga *et al.*, 1993) Figure 6.1 shows the different dsRNA virus patterns of the used stains.

Results

To estimate the effects of a virus infection, isogenic lines which only differ in their infection need to be compared. Such strains were constructed in various ways. We first tried to cure strains of their viruses in order to produce such isogenic lines. A set of naturally infected strains was used for this. Spontaneous loss of virus in colonies grown from single spores could not be detected, nor did addition of the protein synthesis inhibitor cycloheximide yield cured lines, neither by the formation of cured spores nor by producing virus-free sectors in the colony. Because some infected strains do show a slightly ragged colony rim, we also tried sequential isolation of hyphal tips at the colony rim. This yielded one partly cured strain Ind 1.8.16, as shown in Figure 6.1, which gives an example of the virus infection patterns of the strains used in these experiments. The strong phenotypic effect due to the virus infection in strain Ind 1.8.16 is illustrated in Figure 6.2A. All other infected strains, which showed no apparent phenotypic effects, could not be cured of their virus by sequential hyphal tip isolation.

Infection of strains proved more useful than curing for obtaining isogenic virus-free and infected lines. In the case of heterokaryon compatible strains virus transfer took place spontaneously between co-cultured mycelia (e.g Ind 1.5.5 and Ind 1.8.1). The used strains were made to differ in a colour or resistance marker to discriminate between the partners. Between some heterokaryon incompatible combinations transfer could be obtained via protoplast fusion experiments (Van Diepeningen *et al.*, 1998; Chapter 4). Once established in a line, virus can be freely transmitted between the heterokaryon compatible mycelia of different isogenic (mutant) strains. In this way we obtained most isogenic wildtype strains with their virus infection as the only difference.

Fitness can be measured on different traits of the fungal colony. We checked the linear colony growth (1), the maximum spore production (2) and the success in competition (3).

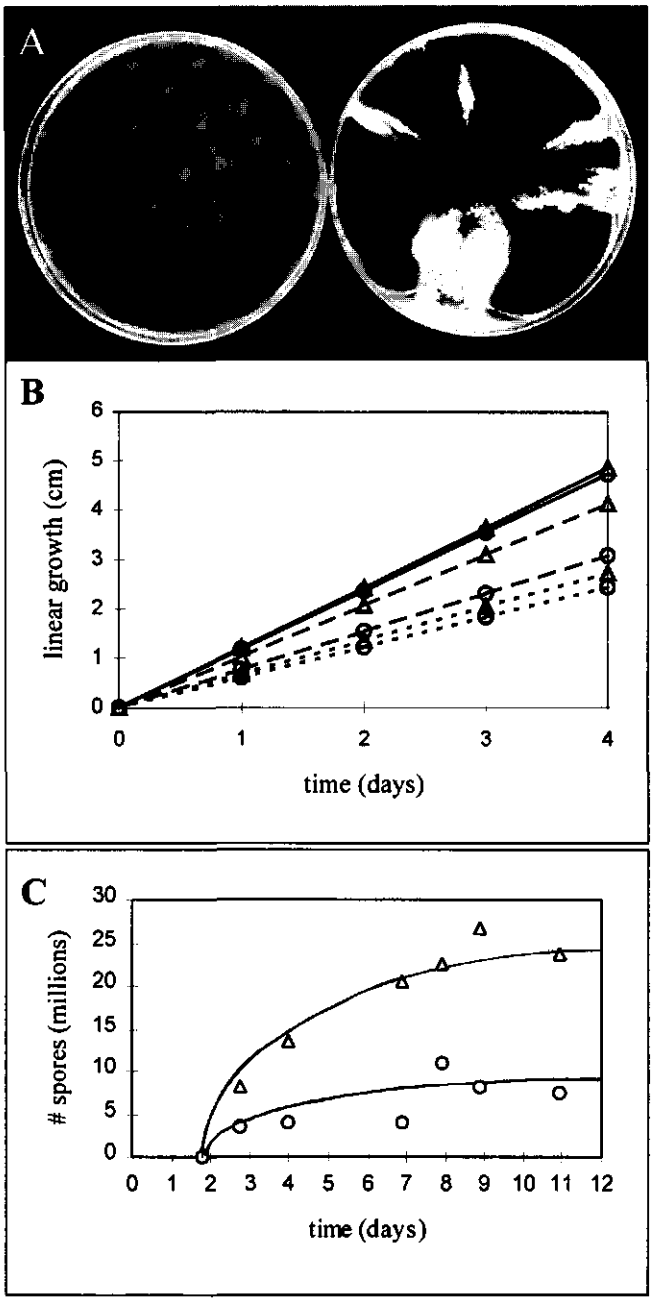

Figure 2A. Photograph of infected and virusfree strain Ind 1.8.16 grown on Petri dish. **B.** Linear growth of strain Ind 1.8.16 infected (O) and cured (Δ) on complete medium (CM; __), minimal medium (M; --) and very minimal medium (MM; ....). **C.** Spore production over time of strain Ind 1.8.16 on very minimal medium.

1). Vigour. Mature fungal colonies increase in radius at a linear rate or at least the hyphal extension is linear when measurements are made over intervals longer than several seconds (Trinci *et al.*, 1994). By measuring the growth of infected and virus-free lines over a time interval the viral effect on growth rate (vigour) can be determined on different media (e.g. the growth of Ind 1.8.16, infected and virusfree, in Figure 6.2B). Table 6.2 shows the radial growth rate measured in Petri dishes along four perpendicular axes. Longer periods of growth were measured in race tubes (Table 6.3). The rates of hyphal extension in both sets of experiments are comparable.

Table 6.2. Fitness effects of viruses on the daily linear growth rate [a] of their host in Petri dishes on different media. P-values calculated with Student's t-tests (* ≥ 90%, ** ≥ 95%, *** ≥ 99% reliability) .

Strain	Virus	medium	Growth rate virus free in cm day^{-1}	Growth rate infected in cm day^{-1}	Effect %	p-value (t-test)	
Ind 1.8.16	Ind 1.8.16	CM	1.21 ± 0.14 (50)	1.18 ± 0.12 (46)	- 2.6%	0.129	
		MM	1.03 ± 0.11 (48)	0.77 ± 0.10 (53)	-26 %	< 0.00	***
		M	0.68 ± 0.07 (119)	0.61 ± 0.11 (98)	-10 % (-5 -13%)[b]	< 0.00	***
		WA	0.65 ± 0.11 (75)	0.61 ± 0.05 (75)	- 6.6%	< 0.00	***
		CM^{cyc}	1.18 ± 0.08 (34)	1.15 ± 0.08 (36)	- 2.8%	0.047	**
		M^{cyc}	0.82 ± 0.07 (68)	0.80 ± 0.08 (102)	- 3.0%	0.025	**
Ind 1.8.16 *part. cured*	Ind 1.8.7	M	0.75 ± 0.06 (65)	0.73 ± 0.06 (42)	- 2.8%	0.043	**
Ind 1.8.1	Z 1.1	M	0.85 ± 0.04 (46)	0.84 ± 0.03 (43)	- 1.4%	0.070	*
Ind 1.8.2	Z 1.1	M	0.71 ± 0.04 (71)	0.70 ± 0.04 (60)	- 1.1%	0.129	
Ind 1.8.4	Ind 1.4.24	M	0.73 ± 0.06 (51)	0.72 ± 0.06 (70)	- 1.8%	0.089	*
	Ind 1.5.5	M	0.73 ± 0.06 (51)	0.74 ± 0.04 (47)	+ 0.8%	0.236	
	Ind 1.7.8	M	0.73 ± 0.06 (51)	0.71 ± 0.05 (59)	- 2.8%	0.020	**
Ind 1.8.9	Ind 1.5.5	M	0.77 ± 0.04 (80)	0.77 ± 0.06 (44)	+ 0.7%	0.295	
	Ind 1.8.7	M	0.77 ± 0.04 (80)	0.74 ± 0.05 (58)	- 3.8%	< 0.00	***
	Z 1.1	M	0.77 ± 0.04 (80)	0.77 ± 0.04 (58)	- 0.4%	0.306	
Ind 1.8.42	Ind 1.7.8	M	0.57 ± 0.03 (91)	0.55 ± 0.05 (95)	- 4.1%	< 0.00	***
	Z 1.1	M	0.57 ± 0.03 (91)	0.56 ± 0.06 (86)	- 2.2%	0.045	**

[a]) All growth rates are means ± standard deviation (number of measurements). [b]) Strain Ind 1.8.16 shows sectorial growth, effect differentiated between sporulating and non-sporulating sectors.

Table 6.3. Fitness effects of viruses on the daily linear growth rate [a] of their host in race tubes on different media. P-values calculated with Student's t-tests (* ≥ 90%, ** ≥ 95%, *** ≥ 99% reliability).

Strain	Virus	exp.	Growth rate virus free in cm day^{-1}	Growth rate infected in cm day^{-1}	Effect %	p-value	
Ind 1.8.16	Ind 1.8.16	CM-1	1.29 ± 0.13 (19)	1.23 ± 0.21 (12)	-5.4%	0.131	
		MM-1	--	0.84 ± 0.07 (17)	--	--	
Ind 1.8.1	Ind 1.5.5	M-1	0.97 ± 0.11 (58)	0.94 ± 0.10 (55)	-3.2%	0.062	*
		CM-1	1.38 ± 0.12 (60)	1.34 ± 0.13 (124)	-3.0%	0.013	**
	Ind 1.8.7	M-1	0.82 ± 0.06 (80)	0.80 ± 0.05 (66)	-1.8%	0.018	**
		M-2	0.87 ± 0.06 (31)	0.85 ± 0.14 (77)	-2.1%	0.048	**
Ind 1.8.9	Ind 1.5.5	M-1	0.75 ± 0.05 (84)	0.75 ± 0.07 (85)	-0.0%	0.428	
	Ind 1.8.7	M-1	0.75 ± 0.05 (84)	0.70 ± 0.07 (80)	-6.7%	< 0.00	***

[a]) All growth rates are means ± standard deviation (number of measurements).

Mycelial growth rate is determined by the concentration of nutrients in the medium and by genetic factors. Cured and infected lines of strain Ind 1.8.16 were tested on a selection of different media (Table 6.2). The largest differences in growth rate were observed on minimal and very minimal medium. On rich substrate like complete medium the effect of harbouring virus seems to be neutralized by the surplus of nutrients. On a very poor medium like water agar or media supplemented with cycloheximide the growth rates are so low that viral effects are harder to detect.

Most strains showed a small, though not always significant, reduction in growth rate of a few percent due to virus infection on complete or (very) minimal medium. Strain Ind 1.8.16 showed the highest growth reduction in non-sporulating sectors of the mycelium (Figure 6.2A) and a lower reduction in other parts of the mycelium (e.g. 13 vs. 5% reduction on MM). Analysis of a racetube of the infected Ind 1.8.16 strain showed that a temporary reduction in growth rate was correlated with an increase in the number of viruses in the mycelium (data not shown). At least in this strain the effects were correlated with the virus titer.

Table 6.4. Fitness effects of viruses on the spore production [a] of their host. Spore production measured in 1.5 ml Eppendorf tubes or in Petri dishes [b] on different media, counted with a Coulter counter. P-values calculated with Student's t-tests (* ≥ 90%, ** ≥ 95%, *** ≥ 99% reliability).

Strain	Virus	exp.	Spore production virus free per cm^{-2}	Spore production infected cm^{-2}	Effect %	p-value	
Ind 1.8.16	Ind 1.8.16	MM-1[b]	$2.92 \pm 1.12 \cdot 10^7$(5)	$0.75 \pm 0.29 \cdot 10^7$(5)	- 74%	0.002	***
		MM-2	$2.33 \pm 0.35 \cdot 10^7$(10)	$1.15 \pm 0.21 \cdot 10^7$(10)	- 51%	< 0.00	***
		MM-3	$2.44 \pm 0.55 \cdot 10^7$(15)	$0.88 \pm 0.36 \cdot 10^7$(15)	- 64%	0.005	***
		CM-1[b]	$2.32 \pm 0.88 \cdot 10^7$ (5)	$1.88 \pm 0.47 \cdot 10^7$ (5)	- 19%	0.175	
		CM-2	$2.66 \pm 0.28 \cdot 10^7$(10)	$2.10 \pm 0.19 \cdot 10^7$(10)	- 21%	0.001	***
Ind 1.8.1	Ind 1.5.5	MM-1	$1.41 \pm 0.15 \cdot 10^7$(12)	$1.27 \pm 0.22 \cdot 10^7$(12)	- 11%	0.006	***
		MM-2	$1.61 \pm 0.33 \cdot 10^7$(18)	$1.40 \pm 0.18 \cdot 10^7$(18)	- 15%	0.001	***
Ind 1.8.2	Ind 1.8.7	MM-1	$1.49 \pm 0.20 \cdot 10^7$ (8)	$1.63 \pm 0.19 \cdot 10^7$ (8)	+ 8%	0.023	**
		MM-2	$1.34 \pm 0.24 \cdot 10^7$(18)	$1.30 \pm 0.27 \cdot 10^7$(18)	- 3%	0.260	
Ind 1.8.4	Ind 1.5.5	MM-1	$1.37 \pm 0.19 \cdot 10^7$(12)	$1.28 \pm 0.20 \cdot 10^7$(12)	- 7%	0.132	
		MM-2	$1.40 \pm 0.15 \cdot 10^7$(16)	$1.35 \pm 0.22 \cdot 10^7$(16)	- 4%	0.154	
Ind 1.8.42	Ind 1.7.8	CM	$2.35 \pm 0.72 \cdot 10^7$ (4)	$1.02 \pm 0.51 \cdot 10^7$ (4)	- 57%	0.020	**
	Ind 1.8.16	CM	$2.35 \pm 0.72 \cdot 10^7$ (4)	$1.52 \pm 0.21 \cdot 10^7$ (4)	- 35%	0.052	*

[a]) All spore productions are means ± standard deviation (number of duplicate measurements). [b]) Spore production measured in Petri dishes.

2). Fecundity. The spore production (fecundity) of the different virus-free and infected strains were measured on two media: very minimal and complete medium. Mycelium of about one day old started producing conidiophores and subsequently conidiospores. The spore production followed a parabolic curve towards a maximum production depending on strain and nutrient availibility. Figure 6.2C shows the spore production of infected and virus-free strain Ind 1.8.16 over time. Maximum production in our experimental setting was

approached after 11-13 days. Table 6.4 lists the maximum numbers of spores for tested strains counted with a Coulter counter when the colonies were fully grown. The virus infections appeared to have a stronger impact on the spore production than on the growth rate.

3). Competitiveness. Competition experiments tested the ability of the strains to compete for the same resources. They tested for a combination of colony growth, spore production, resource use and other factors of importance under competition. A heterokaryon incompatible strain was used as reference strain to exclude heterokaryon formation and virus transfer. Very minimal and complete medium were again used as substrates. Plates were inoculated with equal amounts of viable spores of tester and reference strains. Table 5 lists the final ratios of the spores produced by the two strains after the competition. Again a strong negative effect on the host strain could be attributed to virus infections. No adverse effects on the competitor strain (as by killer-strains in yeast) were observed. The effects measured in these competition experiments were larger than those in the separate growth rate and spore production experiments.

Table 5. Competition experiments with infected and virusfree strains in comparison to a reference strain. The competition started with 1000 spores of each strain (1:1). The final ratios [a] of reference strain to (un)infected strain are given. P-values calculated with Student's t-tests (* ≥ 90%, ** ≥ 95%, *** ≥ 99% reliability).

Strain	Virus	Reference strain	exp.	ratio reference:uninfected	ratio reference:infected	Effect %	p-value	
Ind 1.8.16	Ind 1.8.16	Ind 1.8.3	MM	8.51 ± 4.42 : 1 (3)	> 105 : 1 (3)	- 91%	< 0.00	***
			CM	2.97 ± 1.22 : 1 (3)	3.10 ± 3.89 : 1 (3)	- 4%	0.471	
		Ind 1.8.9	MM	1.03 ± 0.62 : 1 (3)	9.59 ± 6.32 : 1 (3)	- 89%	0.004	***
			CM	2.43 ± 1.51 : 1 (3)	7.11 ± 3.48 : 1 (3)	- 83%	< 0.00	***
Ind 1.8.9	Ind 1.8.16	Ind 1.8.3	MM	4.67 ± 4.80 : 1 (3)	8.17 ± 4.65 : 1 (3)	- 43%	0.114	
			CM	1.43 ± 0.38 : 1 (3)	8.31 ± 5.16 : 1 (3)	- 83%	0.004	***
Ind 1.8.42	Ind 1.7.8	Ind 1.8.3	MM	9.24 ± 8.63 : 1 (2)	11.0 ± 4.95 : 1 (2)	- 16%	0.368	
			CM	7.13 ± 4.67 : 1 (2)	6.29 ± 3.37 : 1 (2)	+ 13 %	0.395	
		Ind 1.8.9	MM	8.48 ± 2.74 : 1 (2)	19.3 ± 8.24 : 1 (2)	- 55%	0.024	**
			CM	10.5 ± 3.19 : 1 (2)	23.8 ± 13.1 : 1 (2)	- 56%	0.048	**
	Ind 1.8.16	Ind 1.8.3	MM	9.24 ± 8.63 : 1 (2)	22.0 ± 10.6 : 1 (2)	- 58%	0.055	*
			CM	7.13 ± 4.67 : 1 (2)	8.99 ± 3.83 : 1 (2)	- 20%	0.293	
		Ind 1.8.9	MM	8.48 ± 2.74 : 1 (2)	19.7 ± 6.87 : 1 (2)	- 57%	0.012	**
			CM	10.5 ± 3.19 : 1 (2)	37.1 ± 8.52 : 1 (2)	- 72%	0.001	***

[a]) Final ratios of reference strain to (un)infected strain are means ± standard deviation (number of competition mixtures tested).

Model

The negative effects of ds RNA on fungal growth rate, spore production, and competitive ability in *A.niger* as found in our study suggest that novel infections should occur at a rate sufficient to counteract the expected decline of infected strains.

To obtain a better insight into the dynamics of ds RNA virus infection in *Aspergillus niger* populations, we analyzed a simple population model. In this model we can incorporate the fitness effects resulting from viral infection as estimated from our measurements, and study the effect of the rates of novel virus infection and of production of virus-free spores by infected strains.

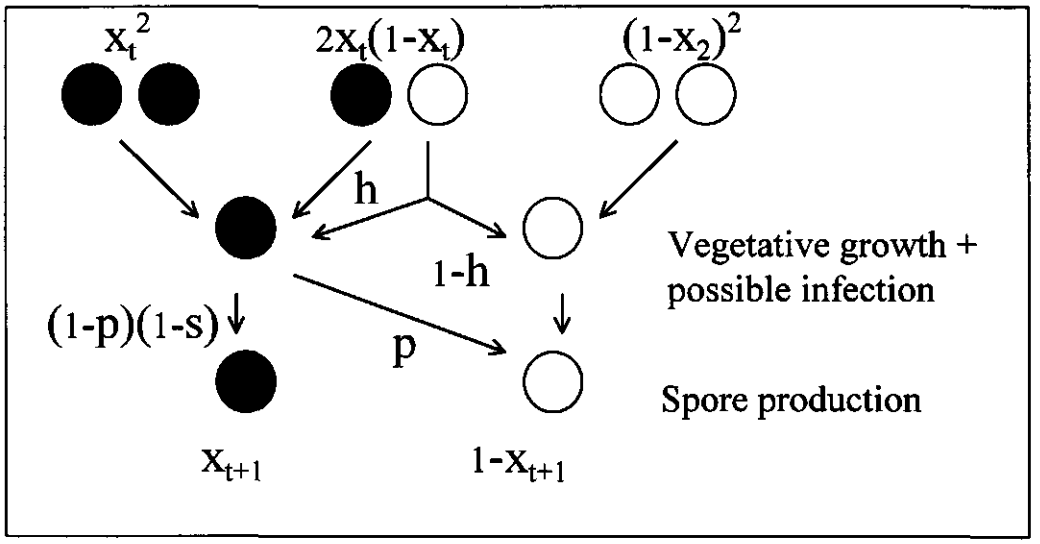

Figure 6.3. Schematic model of the black *Aspergillus* population dynamics. black = virus infection, x_t = fraction infected strains at generation t, h = rate of horizontal transmission to uninfected strains, s = selective disadvantage, p = spontaneous loss of virus.

We assume a population will consist of two types of colonies, either infected with ds RNA (relative frequency x) or uninfected (relative frequency $1-x$). Furthermore, we suppose that all fungal colonies are subject to the following life cycle (see Figure 6.3): upon germination they may encounter close contact with a conspecific during their vegetative growth; following this they sporulate, giving rise to the next generation. Pairwise contacts occur randomly, therefore two infected strains will meet with a probability proportional to x^2, two uninfected ones with probability proportional to $(1-x)^2$, and an infected strain will merge with an uninfected one with probability proportional to $2x(1-x)$. We assume that in the latter category close contact may result in infection of the virus-free colony with probability h, either as a consequence of anastomosis (in case of vegetative compatibility), or otherwise. Then all colonies sporulate; an infected colony produces $1-s$ times the number of spores from an uninfected colony. Finally we assume that a fraction p of the spores produced by an infected colony will carry no ds RNA (spontaneous 'curing').

From these assumptions we deduce the following equation for the change of the relative frequency of infected strains over one generation

$$x_{t+1} = \frac{(1-p)(1-s)x_t[1+h(1-x_t)]}{1-s(1+h)(1-p)\,x_t(1-x_t)}$$

Solving this equation analytically is possible but yields results that do not provide intuitive insight. Instead we provide the following summarizing conclusions based on linearization at sufficiently small values of x_t and standard stability analysis.

1. If $p = 0$ (there is no spontaneous curing and ds RNA is included in all spores produced by an infected colony), then a stable coexistence of infected and uninfected strains is not possible. Eventually there will only be uninfected strains (if $h < s[1-s]^{-1}$) or only infected strains (if $h > s[1-s]^{-1}$).

2. If $p > 0$ (there is at least some virus-free progeny from infected colonies), then two outcomes are possible:

(i) if $(1-p)(1-s)(1+h) > 1$ (if the rate of infection h is sufficiently high to compensate for the virus loss caused by spontaneous curing and by an impaired fitness of infected colonies), then a stable coexistence of infected and uninfected strains is possible;

(ii) if $(1-p)(1-s)(1+h) < 1$ (if the rate of infection h is too low), then the viruses are expected to disappear from the population.

Discussion

Isogenic infected and virusfree strains were constructed for the analyses of fitness consequences of mycoviruses. The different methods used to cure infected black *Aspergillus* strains yielded little results. Only the sequential isolation of hyphal tips resulted in a line that had lost part of the dsRNA infection pattern of the parental strain Ind 1.8.16. Together with the partial loss of the virus pattern this strain lost its abnormal phenotype (Fig 6.2A). However, all other isolations of young hyphal tips or single spores or treatment with cycloheximide yielded no cured lines. Infected lines could be created via virus transfer between some heterokaryon compatible strains or protoplast fusions between incompatible strains (Van Diepeningen *et al.*, 1998; Chapter 4). Once infected, these strains seem to have no active mechanisms to dispose of their virus(es). The omnipresent heterokaryon incompatibility between strains which strongly limits the virus transfer (Van Diepeningen *et al.*, 1997; Chapter 3) could very well be maintained to prevent such infections that are hard to get rid off.

Fitness comparisons between the infected and cured strain Ind 1.8.16 and other virus free and infected isogenic lines were done on different traits and media. The effects of the infection appear to depend on both nutrient availability of the medium and genetic background of fungal strain and virus infection. On rich media the viral effects can be masked by the abundance of available nutrients. On very poor medium the struggle for

growth reduces the measurable effect of infection. The effects caused by the viruses are easiest measured on media with intermediate nutrient availability.

The strongest effects in all comparisons were seen in the infected strain Ind 1.8.16, whose virus can reach extremely high titers in the mycelium. But, in strains without visible phenotypic effects a significant decrease in fitness could also be found. The effect of infection on linear growth rate in Ind 1.8.16 was -7.8% versus an average of -2.1% in all other infected strains when measured on the very minimal medium. In Ind 1.8.16 infection caused a reduction in maximum spore production of 63% on minimal medium and 20% on complete medium. In the other strains the reduction was on average 5.3% on MM and 46% on CM. In competition with a reference strain Ind 1.8.16 produced 90% fewer spores for the next generation on minimal medium due to its infection and 44% less on complete medium. For the other strains infection reduced the fitness in competition on average 43% and 44% on minimal and complete medium.

The relation between the different fitness traits seems to be as follows: spore production is a product of growth area (~ linear growth x linear growth x π) and the number of spores per colonised area. Therefore, the selective disadvantage on spore production (s_{sp}) appears to be proportional to the third power of the linear growth rate (s_{lg}). In competition with another uninfected strain deleterious effects of the virus on properties such as nutrient uptake, efficiency, and conversion further enlarge the total reduction in fitness.

Little is known about nutrient availability in the natural habitat of the black *Aspergilli*, but in all likelihood this availability is variable and discontinuous. Effects of viral infection may vary per location. In nature each strain will be surrounded by competitors: bacteria, fungi, nematodes etc. The success of a strain will depend on its colonizing abilities (e.g. growth rate), its competitive abilities (e.g. nutrient uptake) and its success in reproduction (e.g. formation of spores). Our data suggest that virus-infected strains will be less successful than uninfected strains in all these traits.

Viruses are widespread in fungi. In general they are supposed to have evolved in concert with their host and are usually associated with symptomless infections (Ghabrial, 1996). In these experiments, however, we have shown that there is a considerable cost to harbouring such presumed cryptic parasites. Most infected black *Aspergillus* strains show no immediate visible effect, but careful comparisons of infected and uninfected isogenic lines reveal the fitness costs of infection.

The black *Aspergillus* population seems to have a stable virus infection rate of 10% worldwide. These viruses can vary in number and size of their dsRNA fragments and can be accompanied by satellite and/or defective fragments (Buck, 1998). Infections with more than one virus are also possible. Similar fragments and patterns can be found in different mitochondrial haplotypes of strains indicating an ancient origin or horizontal transfer

(Chapters 2 & 7). In general, the mycoviruses are believed to be of ancient origin (Ghabrial, 1996).This begs the question how deleterious viruses can be maintained in fungal populations. Fine (Fine, 1975) already predicted the decline of a deleterious parasite with only vertical transfer.

Based on estimates of the selective disadvantage caused by virus infection one can use models to predict the population level consequences. If horizontal transfer of viruses occurs, our model predicts that horizontal transfer should be in balance with the selective disadvantage and spontaneous loss to achieve a stable infection frequency. In laboratory experiments no transfer of virus was found between heterokaryon incompatible strains (Van Diepeningen *et al.*, 1997; Chapter 3). Most pairs of strains isolated from nature are incompatible and no resistant isolates have been found. Based on the infection frequencies in different haplotypes of black *Aspergilli* and the frequencies of these types in a sampled population (Chapter 2), the chance for a strain to meet a compatible infected strain can be calculated to be less than 1 percent. Spontaneous loss of virus was not detected in any of the experiments, indicating that p will be very small. If the horizontal transfer rate h should equal the selective disadvantage s (ranging between -2% - 44% for the average strain), far more transfer should take place than was observed. Perhaps higher rates of intraspecies transfer occur in nature than under laboratory conditions, facilitated by certain conditions or because strains meet higher numbers of other black *Aspergillus* strains (which, however, would also intensify the competition).

Another, perhaps more likely, possibility would be that interspecies transfer is more frequent than sofar considered. Transfer via protoplast fusions between black *Aspergilli* and *A. nidulans* and vice versa is more frequent (~100% transfer) than transfer between black *Aspergillus* strains (~50% transfer) (Van Diepeningen *et al.*, 1998; Chapter 4). In addition, exchange of nucleic material from fungi to plants is reported to occur regularly. Hoffmann *et al.* (1994) showed that DNA from transgenic plants could be exchanged with *A. niger* and Marienfeld *et al.* (1997) reported to have evidence for the transfer of nucleic acid transfers between fungal viruses and plant mitochondria.

Regular intra- and/or interspecies transfer could explain the similarities in some infection patterns in different haplotypes of a species and even in members of different species. Transfer could involve transient cytoplasmic contact or perhaps another species acting as vector. Our results suggest that there is need for investigating possible interspecies transfer of viruses in fungi.

7

Recombination in phylogenies of nuclear genes and transposon in black *Aspergilli.*

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Abstract

Several methods can be used to detect recombination on the basis of molecular data; mosaic structures in the genome, the excess of homoplasies in the construction of a most parsimonious tree, and incongruencies between phylogenies based on different parts of the genome. The black *Aspergilli* are a complex of presumably asexual species, capable - at least in laboratories - of parasexual recombination. To discover whether recombination occurs in nature, parts of three genes coding for non-essential extracellular enzymes were sequenced in 21 black *Aspergillus* strains. Evidence was found for a very low level of nuclear recombination. Sequences of *Ant1* transposable element-homologues showed that this element has a spread within the population indicative of horizontal transmission independent of other nuclear genes.

Introduction

For many ascomycete fungi it is not known whether they have a sexual cycle since they do not show any direct evidence of sex (Anderson and Kohn, 1998). Next to sexual recombination, mitotic (parasexual) recombination is known to occur in fungi under laboratory conditions. The first step to parasexual recombination is the formation of a heteroplasmic and heterokaryotic mycelium after hyphal fusion of different genotypes. The ability of two mycelia to fuse is under the control of so-called heterokaryon (somatic/vegetative) incompatibility (*het*-) genes (Glass and Kuldau, 1992; Bégueret *et al.*, 1994). Populations often contain many different *het*-alleles that lead to different vegetative compatibility groups, between which parasexual recombination and transfer of cytoplasmic elements is limited (Caten, 1972, Anagnostakis and Day, 1979, Anagnostakis, 1983, Debets

et al., 1994; Van Diepeningen *et al.*, 1997). It is still unknown whether parasexual recombination occurs in nature.

The occurrence of (meiotic or mitotic) recombination has an important effect on the population structure (Anderson and Kohn, 1998). Several methods for detecting recombination are possible. (1) In bacteria one can look for a 'mosaic structure' in sequences, where recombination can lead to blocks of high similarity interspersed with blocks of high sequence divergence (Maynard Smith, 1992). (2) Another option, applicable both in prokaryotes and eukaryotes, is comparison of the number of homoplasies (similar changes at one site) in the most parsimonious tree with the number expected without recombination (homoplasy test) (Hudson and Kaplan, 1985; Maynard Smith and Smith, pers. comm.) (3) The third method compares phylogenies based on different sequences. The underlying theory states that in clonal lineages genotypic diversity is based purely on accumulation of mutations and that genealogies based on different parts of the non-recombining genome will be fully compatible. Recombination leads to shuffling of the mutations and thus to genealogies that are not consistent (Woese *et al.*, 1980; Dykhuizen and Green, 1991; Dykhuizen *et al.*, 1993; Hey and Kliman, 1994). The combination of (new) molecular techniques and phylogenetic analysis of detected polymorphisms has made it possible to detect recombination in presumed asexuals and, alternatively, to detect partitions in non-recombining (sub)populations in (para)sexual species (Burt *et al.*, 1996; Koufopanou *et al.*, 1997; Geiser *et al.*, 1998). The comparison of phylogenies of different host species and symbionts or parasites can in a similar manner be used to determine coevolution ('clonal') vs. horizontal transfer ('recombination').

The black *Aspergilli* form a complex of related asexual species, some of which of industrial and economicly importance. In nature both morphologically distinct types and types only distinguishable on molecular characters can be found conspecifically and in very high numbers (Chapter 2). Isogenic lines of *Aspergillus niger*, the most abundant member of the group, have been used in parasexual recombination studies which resulted in a linkage map (Debets *et al.*, 1990a; 1993). However, most of the natural isolates do not form heterokaryons with one another due to incompatibility reactions (Van Diepeningen et al., 1997), so it is questionable whether horizontal transfer of nuclear or cytoplasmic genetic material occurs at all. In black *Aspergilli* dsRNA mycoviruses have been found to affect approximately ten percent of the population (Varga *et al.*, 1994a; Van Diepeningen *et al.*, 1997, Chapter 3). Transfer of these viruses is limited to heterokaryon compatible strains and with insufficient intraspecies transfer, interspecies virus transfer may be necessary to explain the infection frequency (Chapter 3-6). In addition, three transposable elements have recently been characterised in different black *Aspergillus* species (Glayzer *et al.*, 1995; Amutan *et al.*, 1996; Nyyssönen *et al.*, 1996).

The experiments described here concentrate on two closely related black *Aspergilli*: *A. niger* and *A. tubingensis* and try to detect recombinational events. Both *Aspergillus* types are believed to be imperfect and in principle capable of parasexual recombination. The types can be distinguished on basis of ribosomal (Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993) and mitochondrial Restriction Fragment Length Polymorphisms (Varga *et al.*, 1993; 1994b). Many genes of the two have been cloned and sequenced and their sequences are available from public databases on the Internet. These genes can form the basis for comparisons of gene sequences and the construction of different gene genealogies. In our laboratory a large collection of natural black *Aspergilli* has been made by selecting for them from soil samples on high concentrations of tannin. Though experimental data and natural survey do not suggest any (para)sexual recombination or horizontal transfer, the molecular evidence for recombination and horizontal transfer may be found in these strains.

Materials and Methods

Strains.

For a list of the black *Aspergillus* strains used, see Table 7.1. N402 is a culture collection strain (derived from CBS 120-49 = ATCC 9029), Z 1.1 is a hospital isolate from Gouda, The Netherlands. The other strains are wildtype natural isolates selected on medium containing 20% (w/v) tannin. Strains Ind 1.1.16-1.8.42 were isolated from soil samples from Jakarta, Indonesia and G 1.3 is an isolate from Gabon (Van Diepeningen et al., 1997, Chapter 2 and 3).

Table 7.1. Strains used: N402 is a culture collection strain (derived from CBS 120-49 = ATCC 9029), Z 1.1 is a hospital isolate from Gouda, The Netherlands. Strains Ind 1.1.16-1.8.26 are wildtype isolates from Jakarta, Indonesia. G 1.3 is a wildtype isolate from Gabon. The wildtype isolates were isolated on selective medium with 20% tannin (Van Diepeningen et al., 1997).

Strain	Mt-type[a]	rRNA[b]	Virus	Strain	Mt-type	rRNA	Virus
N400	1a	I	-	Ind 1.8.3	1b	I'	-
G 1.3	2c	II	+	Ind 1.8.7	1b	I	+
Ind 1.1.16	1b	I	+	Ind 1.8.9	1d	I	-
Ind 1.2.15	2b	II	-	Ind 1.8.10	1b	I'	+
Ind 1.4.24	1c	I	+	Ind 1.8.11	1b	I	+
Ind 1.4.32	jap	jap	+	Ind 1.8.13	1c	I	-
Ind 1.5.5	1b	I	+	Ind 1.8.16	1b	I'	+
Ind 1.6.19	2a	II	+	Ind 1.8.22	1c	I	+
Ind 1.6.23	2a	II	+	Ind 1.8.26	2d	II	+
Ind 1.7.8	1c	I	+	Z 1.1	1b	I	+
Ind 1.8.2	1a	I'	-				

[a] mitochondrial Restriction Fragment Length Polymorphisms after Varga *et al.* (1993, 1994b), and [b] ribosomal classification after Kusters-van Someren *et al.* (1991) and Mégnégneau *et al.* (1993).

Total nucleic acids of these strains were isolated via a standard phenol/chloroform extraction (Maniatis *et al.* 1982; Chapter 2). The extracts of the strains were tested for viral

dsRNA fragment contents with gel electrophoresis (0.8% agarose), stained with Ethidium Bromide and visualised by UV transillumination.

The strains themselves were characterised as *A. niger* (1/I-types), *A. tubingensis* (2/II-types), *A. japonicus* (J) on the basis of *Bgl*II and *Hae*III digested mitochondrial Restriction Fragment Length Polymorphisms (RFLPs) (Varga *et al.*, 1993; 1994b; Hamari *et al.*, 1997) and on ribosomal RFLPs (Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993). The ribosomal typing was done on *Sma*I and on *EcoR*I digested DNA, after gel electrophoresis and southern blotting onto a Hybond-N^+ nucleic acid transfer filter (Amersham) (Mégnégneau *et al.*, 1993), the *Aspergillus nidulans* ribosomal repeat unit (pMN1) was used as probe with DIG-labelling and CSPD-detection (Boehringer Mannheim, user's manual). Mitochondrial haplotype, ribosomal type and virus infections are also listed in Table 7.1.

Sequencing and Analysis of Polymorphisms.

Primers were developed with the Generunner program, version 3.02 (Hastings software Inc., 1994) for three unlinked nuclear genes of black *Aspergilli* of which the nucleotide sequences are deposited in GenBank: *PgaII* coding for a polygalacturonase, *PepF* a peptidase and *PhyA* a phytase. The primers were designed for coding regions of the genes and when the gene was sequenced in more black *Aspergillus* strains, the primer regions were found to be identical in the different strains. For the transposable element *Ant1* two primer sets were made, resulting in the amplification of two partly overlapping sequences. The primers were made by Pharmacia and are listed in Table 7.2.

Table 7.2. Primers of the nuclear genes *PgaII*, *PepF* and *PhyA* and transposon *Ant1*, used in this study.

Gene	Accession numbers & Authors	Primer	Primer sequence 5' to 3'	Nucleotide site
pga II	X58894[a], X58893[a] and X 54146[b] (GenBank)	pga-1	CAGCGGAAAGAAGAAGCC	360-377
	[a]Bussink *et al.* (1991), [b]Ruttkowski *et al.* (1991)	pga-2	TGCTCACGGTGGAGTGTT	783-801
PepF	X79541[c] (GenBank)	pep-1	CTGGTTGTAGTTCCCTTG	1564-1582
	[c]van den Hombergh *et al.* (1994)	pep-2	GAGGGCATTGTTCTTCTG	2111-2128
PhyA	Z16414[d] and L02421[e] (GenBank)	phy-1	CTCATAGGCATCATGGGCGTCTC	12-34
	[d]van Hartingsveldt *et al.* (1993), [e]Piddington *et al.* (1993)	phy-2	CGTTCTGCTGGATCTCCTCAATG	396-418
Ant1	D486B15s[f] (EMBL)	ant1-1	GGCTGTAACCCAAGTGCTG	363-382
	[f]Glayzer *et al.* (1995)	ant1-2	GCCATCTTCCGCAGTGTTG	921-940
		ant2-1	GCCATATTTCTGTGGGAC	531-551
		ant2-2	ATCTTGACACCTGCTTGC	1010-1029

PCR amplifications were performed in 50 µl volume, with concentrations of the chemicals as recommended by the manufacturer and a final concentration of 2.5 µM $MgCl_2$ and 0.75 units Taqpolymerase per reaction (Promega). The amplifications were done in a

Techne Progene thermal cycler, cycling parameters set for 1 cycle 92/37/74° for 3/1/1 min., 40 cycles 92/37/74° for 1/1/3 min. and ending with 1 cycle 92/37/74° for 1/1/7 min.

PCR fragments were purified with the 'freeze-squeeze' method (Tautz *et al.*, 1983) (gene fragments) or PEG-cleaned (Rosenthal *et al.*, 1993) (transposon fragments). The fragments were then sequenced directly with one of the two primers with the Applied Biosystems Taq DyeDeoxy terminator cycle sequencing kit in a Perkin-Elmer thermal cycler and analyzed in an Applied Biosystems 373 DNA sequencer.

Sequences were aligned in Sequence Navigator (v. 1.0), a matrix with the polymorphisms was created and phylogenetic relationships using parsimony were inferred from these in PAUP 3.1.1 (Swofford, 1993) or PAUP 4.0b1 (test version, 1998). Alignment gaps were treated as data. All transformations were unordered and equally weighted.

Results

To address the question of recombination in the nuclei of the black *Aspergilli*, parts of three nuclear genes were sequenced in 21 strains, including an *A. japonicus* strain and sets of different *A. niger* (mtRFLP-types 1a-1d) and *A. tubingensis* (mtRFLP-types 2a, b, d & e) strains (see Table 7.1). The sequenced peptidase F gene (*PepF*) encodes a serine carboxypeptidase (van den Hombergh *et al.*, 1994). The phytase A gene (*PhyA*) catalyzes the hydrolysis of phytate to *myo*-inositol and inorganic phosphate (van Hartingsveldt *et al.*, 1993; Piddington *et al.*, 1993) and the polygalacturonase II gene (*PgalI*) is one of the genes in the complex degradation of pectin (Bussink *et al.*, 1991; Ruttkowski *et al.*, 1991). All three genes are non-essential extracellular degrading enzymes.

The polymorphic sites of the three genes in the 21 strains (for *PepF* 18 strains) are listed in Tables 7.3-5. Of the 983 nucleotide sites analysed, a high number, 172, were polymorphic (17.5%). Of these polymorphic sites 130 were parsimony informative and 36 had nonsynonymous substitutions (21 of which in parsimony informative sites). The sequenced introns contained a higher relative number of polymorphic sites than the exons. Of the different techniques to detect recombination the homoplasy test is less suitable to use for this data set because of the high number of polymorphic sites (Maynard Smith and Smith, pers. comm.).

Direct examination of the sequences of the three genes shows that three groups of strains can be recognised: two groups of *A. niger* (a 1a/1c/1d and a 1b-group on the basis of mitochondrial RFLP) and a group of *A. tubingensis* strains. In the first part of the *PgalI* gene a block of 20 polymorphic sites is found with 8 similar character states in five 1b-type strains and in the *A. tubingensis* group (Table 7.4; bold characters), whereas the remainder of the 1b-strain sequences resembles the other *A. niger* types. Thus, the sequenced *PgalI* gene shows a mosaic structure, indicative of an intragenic recombination event.

Table 7.3. Polymorphisms in the peptidaseF gene of 14 *A. niger* and 4 *A.tubingensis* strains and reference strain.

			peptidase F (*pepF*) - 367 nucleotide sites analysed
Nucleic acids			1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
Site[a]			6 6 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 8 8
			2 4 5 6 7 7 8 8 8 8 9 9 9 0 0 0 1 1 1 1 1 1 2 2 2 2 2 3 3 3 4 4 4 4 4 5 6 6 7 8 9 9 1 3
			2 0 2 4 0 3 8 8 8 9 4 6 9 1 2 6 2 4 6 7 8 9 2 3 4 6 7 9 0 7 8 0 2 4 7 7 7 3 9 8 7 6 9 4 5
			I[e] II[e] I[e] I[e]
Position[b]			3 3 3 3 3 3 * 3 3 3 3 3 3 3 3 3
Consensus			T T C T C T G T G T T T G T T G G T G T A T G C A T A C G C C A T T G G C T A C T C T G G
Strain	**mt**[c]	**r**[d]	
N400	1a	I	. . . . - - . . C . . C A . . A C . . . . G . . . . - . . . .
Ind1.8.2	1a	I'	. . . . - - . . C . . C A . . A C . . . . G . . . . - . . . .
Ind1.1.16	1b	I	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.5.5	1b	I	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.1	1b	I	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.3	1b	I'	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.7	1b	I	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.10	1b	I'	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.11	1b	I	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.16	1b	I'	. . . . - - C . . . C . . A C . . C G A - . . . . T . C . . T . G T A G . . .
Ind1.8.13	1c	I	. . . . - - . . C . . C A . . A C . . . . G . . . . - . . . .
Ind1.8.22	1c	I	. . . . - - . . C . . C A . . A C . . . . G . . . . - . . . .
Z1.1	1c	I	. . . . - - . . C . . C A . . A C . . . . G . . . . - . . . .
Ind1.8.9	1d	I	. . . . - - . . C . . C A . . A C . . . . G . . . . - . . . .
Ind1.6.23	2a	II	C A T C T C . T G . C . T . . . . G . C G A C - - T G . T A . C . . . . . C T T
Ind1.8.42	2b	II	C A T C T C T T G . C . T . . . . G . C G A . - - T G . T A . C . . . . . C T T
Ind1.8.26	2d	II	C A T C T C T T G . C . T . . . . G . C G A A - - T G . T A . C . . . . . C T T
G1.3	2e	II	C A . C T C T T C C C . T . . . . G . C G . A - - T G . T A . C . . . . . C . T
X79541			. . . . - - . - C . . C A . . A C . . . . G . . . . - . . . . . G . . .
Amino Acids			1 1 1 1 1 1 * 1 1 1 1 1 1 1 1 1
Site			4 4 5 5 5 5 6 6 6 7 7 7 7 8 8
			1 7 1 5 7 8 3 5 7 0 3 6 7 2 9
Consensus			P V Y N T N * D P G F G T A E K
Substitution			. . . . D N

[a] Site in the peptidase sequence according to Van den Hombergh et al. (1994), [b] Position in codon or intron (*), [c] mitochondrial RFLP type (Varga *et al.*, 1993; 1994b), [d] ribosomal RFLP type (Mégnégneau *et al.*, 1993),[e] deletion named after the previous nucleotide.

Table 7.3, continued. Polymorphisms in the peptidaseF gene.

		peptidase F (*pepF*), continued														
Nucleic Acids		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Site		8	8	8	8	8	8	9	9	9	9	9	9	9	9	9
		5	5	7	8	8	9	1	2	3	3	3	4	5	5	7
		9	8	1	0	3	2	3	8	0	4	7	3	2	6	3
Position*		3	3	3	3	3	3	3	3	2	3	3	3	3	1	3
Consensus		C	C	A	G	C	T	T	A	C	T	T	G	T	A	G
Strain																
N400	1a	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A
Ind1.8.2	1a	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A
Ind1.1.16	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
Ind1.5.5	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.1	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.3	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.7	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.10	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.11	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.16	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.4.24	1c	.	.	.	.	.	.	.								
Ind1.7.8	1c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.8.13	1c	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A
Ind1.8.22	1c	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A
Z1.1	1c	.	.	.	.	.	.	C	.	A	.	.	A	.	.	A
Ind1.8.9	1d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ind1.6.23	2a	A	.	.	A	A	.	C	C	.	G	C	.	C	.	.
Ind1.8.42	2b	A	.	G	A	A	.	C	C	.	G	C	.	C	.	.
Ind1.8.26	2d	A	.	G	A	A	.	C	C	.	G	C	.	C	.	.
G1.3	2e	.	T	.	A	T	C	C	C	.	G	C	.	C	.	.
X79541		.	.	.	.	.	.	.	.	.	.	.	A	.	.	A
Amino		1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Acids		9	0	0	0	0	0	1	2	2	2	2	2	2	3	3
Site		7	0	1	4	5	8	5	0	1	2	3	5	8	0	5
Consensus		I	I	K	K	I	T	R	I	S	A	A	L	N	T	L
Substitution		.	.	.	.	.	.	.	.	Y	.	.	.	.	P	.

Table 7.4. Polymorphisms in the polygalacturonase II gene of 16 *A. niger* and 5 *A.tubingensis* strains and reference strains.

Nucleic Acids		4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Site		0	0	1	1	2	2	3	3	5+5	6	6	6	7	7	7+8	8	8	8	9+9	9					
		2	7	2	5	4	7	3	9	3	4	0	1	7	5	8	9	1	4	7	8	5	6	9		
Position*		2	1	3	3	3	3	3	3	2+3	3	1	1	3	3	1+3	3	3	1	2+3	3					
Consensus		A	T	T	T	A	C	A	C	T	T	C	C	A	T	G	T	T	C	T	G	T	C	T		
Strain																										
Ind1.4.32	j	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
N400/*X58893*	1a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.8.2	1a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.1.16	1b	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		
Ind1.5.5	1b	.	.	C	C	G	.	G	.	.	.	G	G	.	.	C	.	T	.	A	.	.	.	.		
Ind1.8.3	1b	.	.	C	C	G	.	G	.	.	.	G	G	.	.	C	.	T	.	A	C	.	.	.		
Ind1.8.7	1b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.8.10	1b	.	.	C	C	G	.	G	.	.	.	G	G	.	.	C	.	T	.	A	.	.	.	.		
Ind1.8.11	1b	.	.	C	C	G	.	G	.	.	.	A	G	.	.	C	.	T	.	A	C	.	.	.		
Ind1.8.16	1b	.	.	C	C	G	.	G	.	.	.	G	G	.	.	C	.	T	.	A	C	.	.	.		
Ind1.4.24	1c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.7.8	1c	.	.	.	.	G	.	.	.	.	.	G	.	C	C	.	G	.	.	.	.	.	.	.		
Ind1.8.13	1c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.8.22	1c	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Z1.1	1c	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.8.9	1d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ind1.6.19	2a	.	.	C	.	G	T	.	.	C	.	.	G	G	C	C	C	G	T	.	A	C	.	C		
Ind1.6.23	2a	.	.	C	.	G	T	G	.	.	.	T	G	G	C	C	C	G	T	C	A	C	.	C		
Ind1.2.15	2b	.	.	C	.	G	T	G	.	.	.	T	G	G	C	C	C	G	T	C	A	C	.	C		
Ind1.8.26	2d	.	.	C	.	G	T	G	.	.	.	T	G	G	C	C	C	G	T	C	A	C	T	C		
G1.3	2e	.	.	C	.	G	T	G	T	C	C	G	.	G	C	T	C	G	T	C	A	C	G	C		
X54146/X58894		.	.	C	.	G	T	G	T	.	.	T	G	G	C	T	C	G	T	C	A	.	.	C		
Amino Acids		1	1	1	1	1	1	1	1	1		1	1	1	1	1	1		1	1	1	1		1		
Site		3	3	3	3	4	4	4	4	5		5	5	5	5	6	6		6	6	6	6		6		
		5	7	8	9	2	3	5	7	2		4	5	7	9	0	1		2	3	4	6		7		
Consensus		D	S	S	I	L	N	K	T	F		V	Q	N	I	T	L		T	D	I	I		N		
Substitution		V	A	.	.	.	.	.	.	S		.	E/KD	.	.	F/.	.		.	.	V	T		.		

Table 7.4. Continued. Polymorphisms in the polygalacturonase II gene.

		polygalacturonase II (*pgaII*) - 368 nucleotide sites analysed
Nucleic Acids		5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7
Site		0 0 1 1 1+1 1 2 4 4 4 5 5 6 7 8 8 1 1+1 3 3 3 3 4 4 4 4 4+5 5 5 5 6+6 6 6 8 9 2 6 3 4+4 6 6
		2 8 1 4 6 7 9 9 1 4 7 3 9 5 7 0 3 3 4 6 6 7 8 9 1 2 5 8 9 0 6 7 9 1 2 6 9 0 2 9 2 4 4 5 1 7
Position		3 3 3 3 2+3 2 3 3 3 3 3 3 3 3 3 3 3 1+3 * * * * * * * * * * * * * * * * * * 3 1 3 3 1+2 3 3
Consensus		T T C C C C A C G C T T G T T G T G G T G C T C G C T T G C T C G A T A G A C G T C G G C A
Strain		
Ind1.4.32	j	. . . . C . A . . . . G . . . .
N400/*X58893*	1a	. . . . C . G . . . . T . . . . G . . . . . . A . .
Ind1.8.2	1a	. . . . C . G . . . . T . . . . A A . .
Ind1.1.16	1b	. . . . C . A . . . .
Ind1.5.5	1b	. . . . T C . A . . . .
Ind1.8.3	1b	. . . . T T C . A . . . .
Ind1.8.7	1b	. . . . C . C . A . . . .
Ind1.8.10	1b	. . . . T C . A . . . .
Ind1.8.11	1b	. . . . T C . A . . . .
Ind1.8.16	1b	. . . . T C . A . . . .
Ind1.4.24	1c	. . . . A . . . .
Ind1.7.8	1c	. . . . G C A . . . . T . . . . T T G G . . . . A . .
Ind1.8.13	1c	. . . . C . G . . . . T . . . . A . .
Ind1.8.22	1c	. . . . C . G . . . . C . . . . T . . . . A . .
Z1.1	1c	. . . . C . G . . . . T . . . . A . .
Ind1.8.9	1d	. . . . C . G . C . . . . T . . . . A . .
Ind1.6.19	2a	. C T T G T . . A . C . C . C A G . A C A T C T . . C . A T . T C T G G . G T . C . A A . G
Ind1.6.23	2a	. C T T G T T . A T . . C . C A G . A C T T . T . . C . A T A T C T G G . G T . C . . A . G
Ind1.2.15	2b	. C T T G T T . A T . . C . C A G . A C . . . T . . C . A T . T C T G G . . T . C . . A . .
Ind1.8.26	2d	. C T T G T T . A T . . . . C A G . A C T T . T . . C . A T A T . T G G . G T . C . . A . G
G1.3	2e	C C T T . . T T . T . . C . C A G . . C . . . T . . C . A T . T T T G G A G . . . T . . T .
X54146/X58894		C C T T . . T . . T . . . . C A G . A C . . . T . A C G A T . T T T G G . G T A . T . . T G
Amino Acids		1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 * * * * * * * * * * * * * * * * * * 2 2 2 2 2 2 2
Site		6 7 7 7 7 7 7 8 8 8 8 8 9 9 9 0 0 1 1 2 4 3 3 3
		8 0 1 2 3 4 7 1 2 3 5 7 9 3 4 5 5 6 4 6 4 8 1 7 9
Consensus		N D G D T Q H G F D G S G I K P A V * * * * * * * * * * * * * * * * * * F S G G G V K
Substitution		. . . . S L . . . ./N. . I . . . . D/N . .

Table 7.5. Polymorphisms in the phytase A gene of 16 *A. niger* and 5 *A.tubingensis* strains and reference strains.

		phytase A (*phyA*) - 248 nucleotide sites analysed
Nucleic Acids		1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
Site		5 5 6 6 7 7 8 9 0 1 2 3 3 4+4 4 5 5+5 6 6 6 7 8 8 9 0 0 1+1+1 2 3 3 3+3 4 5 6 7 8+8
		0 5 3 4 2 7 5 1 9 6 8 1 5 2 3 9 5 7 8 2 7 8 5 0 7 4 0 3 6 6 7 8 2 0 3 5 6 5 4 9 5 0 1
Position*		3 2 1+2 1 3 2 2 2 3 3 3 1 2+3 3 3 2+3 1 3 1 2 1 2 3 3 3 3 1+2+3 1 3 3 2+3 3 3 3 3 2+3
Consensus		C C G C G G A C A T G G T T T C C C G T T T C A C C T T C C A A A C G T T C A C G A G
Strain		
Ind1.4.32	j	. . T C . . . . A C . . . G . . . . . . T . . . .
N400	1a	. . . . T . . T . . . .
Ind1.8.2	1a	. . . . T . . T . . . .
Ind1.1.16	1b	. . . . C . . . . T . . T . . . .
Ind1.5.5	1b	T T . . . . T . . . . G . . . .
Ind1.8.3	1b	T T . . . . T T . . . . G . . . .
Ind1.8.7	1b	. . . . . . G T . . . . . . G . A G T . C . T . T . . . . . . T . . A . . . G . . . .
Ind1.8.10	1b	T T . . . . T T . . . G . . . .
Ind1.8.11	1b	T T . . . . T . . . . G . . . .
Ind1.8.16	1b	T T . . . . T . . . . G . . . .
Ind1.4.24	1c	T T . . . . T . . . . G . . . .
Ind1.7.8	1c	. . . . A . . . . T . . . .
Ind1.8.13	1c	. . T C . . T . . . . T . . . .
Ind1.8.22	1c	. . . . G T . . . . G . . . . . . T . . . T C
Z1.1	1c	. . . . T . . . .
Ind1.8.9	1d	. . . . G T . . . . T . . . .
Ind1.6.19	2a	. . . . C . . . . T . . . . G . T . G . . G . . T . . C T . . A . .
Ind1.6.23	2a	. . . . C . . . . T . . . . G . T . G . . G . . T . . C T . . A . T
Ind1.2.15	2b	. T . . C A . . . . C . A C . G . . . A . . . . G . . G . . . . C . T . . A C .
Ind1.8.26	2d	. . . . T . . . . G . T . G . . G . . T . . C T . . A . .
G1.3	2e	. . . . C . . . . T . . A . C . . G T . . G . A G . . T . . C T G . . . .
L02421		. . . . C . . . . T . . . . C . . G . . . G . A G . . T . . C T G G . . .
Z16414		. . . . . . G . . . C . . . . T . . . . C . . G T . G G . A G . . T . . C T G . . . .
Amino Acids		1 1 2 2 2 2 3 3 3 4 4 4 4 4 5 5 5 5 5 5 6 6 6 6 6 6 7 7 7 7 7 8 8 8 9 9
Site		6 8 1 4 5 8 0 6 8 2 3 5 7 9 1 2 4 5 6 8 0 2 4 6 7 8 2 4 6 7 8 1 4 9 1 3
Consensus		V S A A S Q T Q Y S E S L G Y A F F S A K A S D V P Q T A Q V R A D K K
Substitution		. F S P .L/RS L . . . T ./P . st./= I . A = EG/V. E . . H/R S .=/H./A . . E . ./T

Examination of the *PhyA* data shows that in *A. niger* strain Ind 1.8.7 the mutation of a cytosine to adenine at site 255 resulted in a stopcodon at that location, leading to a non-functional 'pseudogene'. Without the selection for a functional gene the sequence obviously accumulated more mutations (reversions, unique mutations and mutations resulting in homoplasy). *A. tubingensis* strain Ind 1.2.15 also shows quite a number of mutations in *PhyA*, though there is no evidence in the sequenced part of the gene that this also resulted in a pseudogene. *PepF* shows most of its differences between the 3 groups and little within.

Recombination could be detected in gene genealogies. The genealogy of a locus is best described by the most parsimonious (MP) tree. Therefore, MP trees of the three separate genes and the combined data set were constructed by branch and bound searches in PAUP (Swofford, 1993; 1998). The mosaic *PgalI* gene was also divided into two subsets, *PgalI-1* containing the first 20 polymorphic sites, including the possibly recombined block, and *PgalI-2* with the remainder of the sequence. One MP tree for each data set is given in Figure 7.1.

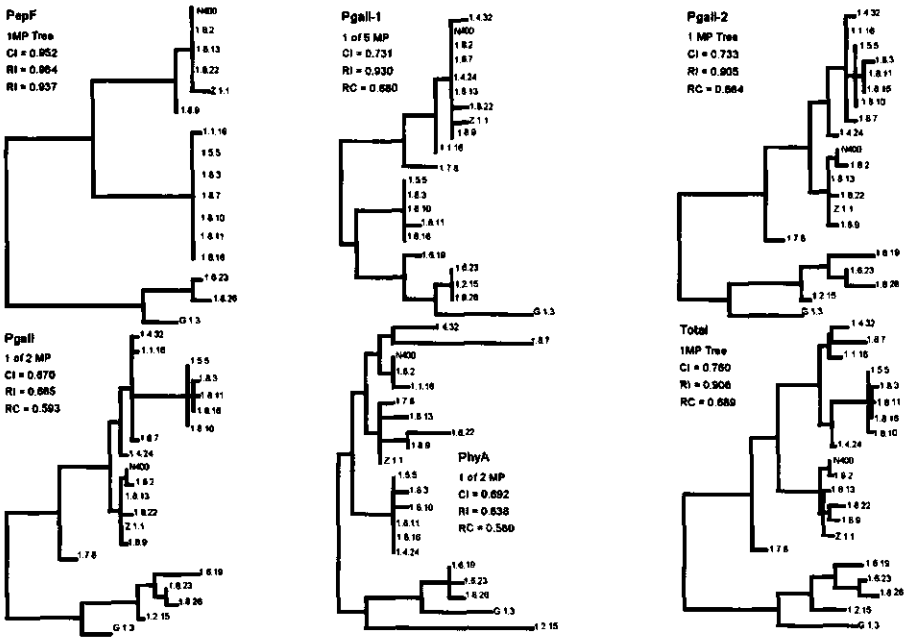

Figure 7.1. One MP tree from each of the three gene regions sequenced, from the two subsets of the *PgalI* gene (*PgalI*-1 and *PgalI*-2) and for the total data set. Trees are made on basis of all polymorphic characters and rooted with the *A. tubingensis* strains as outgroup. The CI= consistency index; RI= retention index and RC= rescaled consistency index are calculated on informative characters only.

All trees support the recognition of a separate group of *A. tubingensis* strains. The *A. niger* isolates fall into two groups: one containing the 1a, 1c and 1d mitochondrial haplotypes, the other the 1b haplotypes, where the *A. japonicus* strains is also most often

connected to. The clearest example of recombination is seen in the two subsets of the *PgaII* gene. A group of five 1b *A. niger* strains clusters in *PgaII-1* with the tubingensis strains, but in the *PgaII-2* tree this cluster is found further in the tree with other 1b *A. niger* isolates and the *A. japonicus* strain. In the combined *PgaII* tree these conflicting data result in a lowering of the consistency indices due to more apparent homoplasies.

Recombination leads to more apparent homoplasies in the genealogy. But, even when two sequences have diverged more and mutations have accumulated, more homoplasies are expected by chance (compare with the homoplasy test). Comparison of the actual number of steps of MP trees with the minimal tree length gives this number of apparent homoplasies. In Table 7.6 actual and minimum tree lengths are listed for the different genes and (sub)sets of strains. Obviously the black *Aspergilli* subgroups are not recombining freely, but very rarely some recombination may occur.

Table 7.6. Actual and minimum MP tree lengths - based on informative characters only - for the three genes, two subdivisions of *PgaII* and the *Ant1* transposon. (#) number of MP trees, '-' no informative characters.

	Total black *Aspergilli*			*A. niger*			*A. tubingensis*		
Locus	MP tree length (#)	Min. tree length	Excess steps	MP tree length (#)	Min. tree length	Excess steps	MP tree length (#)	Min. tree length	Excess steps
PepF	62 (1)	59	3	24 (1)	24	0	-	-	-
PgaII-1	*26 (5)*	*19*	*7*	*13 (5)*	*11*	*2*	*1 (1)*	*1*	*0*
PgaII-2	*59 (1)*	*44*	*15*	*14 (1)*	*12*	*2*	*4 (1)*	*4*	*0*
PgaII	93 (1)	63	30	29 (1)	23	6	6 (2)	5	1
PhyA	34 (5) +	25 +	9	13 (2) +	11 +	2	1 +	1 +	0
	189	147		66	58		7	6	
all three genes	199 -	189 =	10	72 -	66 =	6	7 -	7 =	0
		147 =	52		58 =	14		6 =	1

	A. niger 1a/1c/1d			*A. niger* 1b		
	MP tree length (#)	Min. tree length	Excess steps	MP tree length (#)	Min. tree length	Excess steps
PepF	-	-	-	-	-	-
PgaII-1	-	-	-	*10 (2)*	*10*	*0*
PgaII-2	*2 (1)*	*2*	*0*	*2 (1)*	*2*	*0*
PgaII	2 (1)	2	0	12 (1)	12	0
PhyA	3 (1) +	3 +	0	4 (1) +	4 +	0
	5	5		16	16	
all three genes	6 -	5 =	1	16 -	16 =	0
		5 =	1		16 =	0

The *Ant1* transposon was described as occurring in single copy in the genome of strain N402, a derivative of strain N400 (Glayzer *et al.*, 1995). Two sets of primers were developed for the sequence of the putative transposase. With the first primer set 8 strains were found to contain an *Ant1*-fragment, with the second set 9 of the 21 strains. Per strain the overlapping region of the two sequenced fragments matched exactly. The missing strain in the first

Table 7.7. Polymorphisms in the '*A. niger* transposon'-1 (*Ant1*).

A. niger transposon 1 - 521 nucleotide sites analysed

Nucleic Acids		primerset 1	both primersets
site		4 4 4 4 4 4 4 4 4 4 4 5 5 5	5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6
		3 3 3 3 4 4 4 6 6 7 7 0 0 2	4 5 5 5 6 6 6 7 7 7 8 8 1 1 1 1 1 2 3 3 3 3 4 5 5 5 5 6 6 6 6 7 7 8 8
		0 4 7 9 2 6 8 4 5 7 9 2 9 5	0 4 5 7 6 7 8 1 6 7 5 8 1 4 5 6 8 3 2 5 7 8 4 0 1 2 9 3 4 5 8 4 5 2 3
position		2 3 3 2 2 3 2 3 1 1 3 2 3 1	1 3 1 3 3 1 2 2 1 2 1 1 3 3 1 1 1 3 3 3 2 3 3 3 2 3 3 1 2 3 3 1 3 2 3
consensus		C C C A G G G A C A A A T G	T T C G A A C C A G C G T G A - A T G T T A T G A T C G A C T C T C A
strain			
Ind 1.4.32	J	T - T . A A A G T C G T C T	C C . C T T . T . . . . . A C - . C - G A T . A . C G A G . A . . T .
N400/*D486B15s*	1a	. . . G	. . . . . T . . . - . . . . . A C . . . . . G
Ind 1.8.2	1a	T - T . A A A G T C G T C T	C C . C T T . T . . . . . A C - . C - G A T . A . C G A G . A . . T .
Ind 1.8.3	1b	. . . . .	. . . . . - T . . . . . C . . . . .
Ind 1.8.11	1b	. . . . .	. . . . . - T . . . . . C . . . . .
Ind 1.4.24	1c	T - T . A A A G T C . T C G	. C . C T T . T . . . . . A . C - G A T . A . C G A G . A . . . .
Z 1.1	1c	? ? ? ? ? ? ? ? ? ? ? ? ? ?	. C T C T . A . C A T . C T . - . C - G A T . A C . G . G T . A C T .
Ind 1.6.23	2a	. . . . .	C . . . . . - T . . . . . C . . . . .
Ind 1.2.15	2b	. . . . .	. . . . . T . . . - . . . . . A C . . . . .

A. niger transposon 1

Nucleic Acids		both primersets	primerset 2
site		6 6 6 7 7 7 7 7 7 7 7 7	7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 9 9 9 9
		8 9 9 1 2 2 2 3 3 4 5 6	6 6 7 7 7 8 9 9 9 9 1 2 2 3 4 4 5 5 5 5 6 6 6 7 7 7 7 8 8 9 9 9 0 0 2 2
		6 5 7 6 1 2 5 3 7 9 1 5	6 9 2 3 9 5 1 4 6 7 5 4 6 0 4 9 2 4 5 7 0 3 9 1 5 8 9 1 9 0 7 9 1 2 2 3
position		3 3 2 3 2 3 3 2 3 3 2 1	2 2 2 3 3 3 3 3 2 3 3 3 2 3 2 1 1 3 1 3 3 3 3 2 3 3 1 3 2 3 1 3 2 3 2 3
consensus		T T G C C C A G A A G C	C T G C A A A C G G G C T G A A A T T T C C T A G T A A C T G G A T A A
strain			
Ind 1.4.32	J	G G A T . . C A . G A .	G . . A G G G T . A . T A . . C C A A G T . . G T A C T T . A A . C . .
N400/*D486B15s*	1a	. . . . .	. . . . . A A . . . . . T . . . . .
Ind 1.8.2	1a	G G A T . . C A . G A .	G . . A G G G T . A . T A . . C C A A G T . . G T A C T T . A A . C . .
Ind 1.8.3	1b	. . . . .	. . . . . C . . A . . . . .
Ind 1.8.11	1b	. . . . .	. . . . . C . . A . . . . .
Ind 1.4.24	1c	G G A T . . C A . G A .	G C . A G G G T . A . T A . . . T . A G T . . G T A . T T . A A C . . .
Z 1.1	1c	G C A T T A . A G G A T	. . A . G . G T . . . . . C . . G . . T T - G C A . T T G . . . . G C
Ind 1.6.23	2a	. . . . .	. . . . . C . . A . . . . .
Ind 1.2.15	2b	. . . . .	. . . . . A A . . . . . T . . . . .

dataset had mutations in the region of primer ant1-1. *Ant1* sequences are listed in Table 7.7. Of the 97 polymorphic sites, 72 were parsimony informative. The MP *Ant1* tree is shown in Figure 7.2; this tree yields only five apparent homoplasies and good consistency indices (CI = consistency index = 0.940, RI = retention index = 0.969 and RC = rescaled consistency index = 0.911). The topology of this *Ant1* MP tree is not concordant with the trees based on the nuclear genes (in Figure 7.2 without the pga1 data) and indicates horizontal transmission of the transposable element.

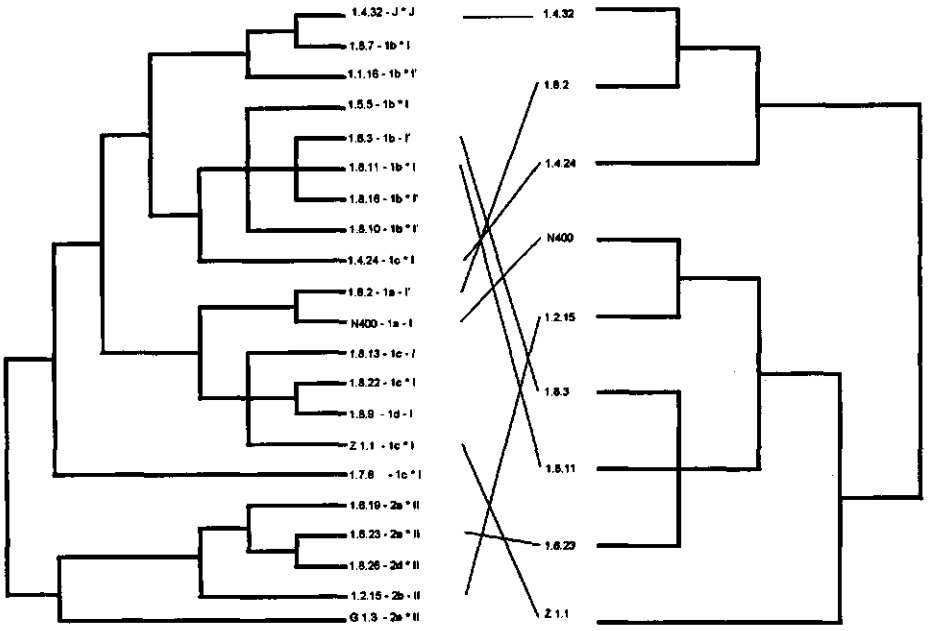

Figure 7.2. Combination of the MP phylogenetic tree of the three nuclear genes (without the *PgaII-1* data set) and the *Ant1* MP tree. Strains are listed with their mitochondrial haplotype, * = virus infection and ribosomal classification.

Figure 7.2 also includes data on mitochondrial and ribosomal RFLPs and virus infections. The set of strains in this experiments harboured many virus infected strains used in earlier experiments (Chapter 2-6) and propartionally more than the 10% infected found in nature. These previous experiments showed that the population structure in the black *Aspergilli* can only be explained by assuming (inter- and/or intraspecies) transmission of the mycoviruses. The mitochondrial RFLP data do not contradict the nuclear phylogenetic tree and both types 1a and 1d seem derived form haplotype 1c, which may in turn be derived from 1b or vice versa. The ribosomal classification of I and II types is in accordance with the split of *A. niger* and *A. tubingensis*, but the I and I' types occur in both subgroups of *A. niger*. This can be explained either by recombination or by the population having stayed polymorphic for this (~ multigenic) character for a long period of time.

Conclusions and discussion

The methods used for detecting recombination within the genome are based on the detection of data contradicting clonality. This can either be stretches of DNA showing a mosaic structure as found in bacteria, an unexpectedly large number of homoplasies necessary to produce a most parsimonious tree of a part of the genome, or incongruencies between phylogenies based on different parts of the genome. In this study three unlinked genes coding for extracellular degrading enzymes were partly sequenced in 21 different black *Aspergilli*. The genes showed a high level of divergence: 17.5% of the nucleotide sites were polymorphic, which may indicate a long history for the presumably asexual black *Aspergilli*.

Three main groups can be distinguished in the data: *A. niger* groups 1b and 1a/c/d and an *A. tubingensis* group. The morphological different *A. japonicus* seems most related to the 1b *A. niger* strains. Similarity within these groups is larger than that between them. Intragenic recombination in the *PgaII* is suggested by the mosaic structure of this gene. Homoplasies were detected both within MP trees of separate genes and in the MP tree of the total data set. The phylogenies of different (parts of) genes were not all congruent. Most data confirm asexuality in the black *Aspergilli* and the sequencing data largely support the trees based on mitochondrial and nuclear RFLPs. Some of the data are indicative of occasional (para)sexual recombination.

The most significant recombinational event in these data is found within the *PgaII* gene. The first part of this gene in five strains of the 1b-*A. niger* type seems to be derived from an *A. tubingensis* strain. From these data we can not conclude whether only a small fragment or the remainder of the chromosome was exchanged, or which mechanism has caused the recombination. Possible mechanisms could involve mitotic crossing over between chromosomes in a transient heterokaryotic diploid or transfer of genomic fragments mediated by, for instance, transposable elements. Also, dating the recombinational event is difficult. The variation between the five 1b-*A. niger* the *A. tubingensis* strains in the *PgaII-1* and *PgaII-2* subset of the sequence (assuming a similar molecular clock), suggests that this recombination event occurred a relatively long time ago (*PgaII-1* contains 75% percent of the variation found between the strains in *PgaII-2*). However the five *A. niger* strains vary little within this group and from the other 1b-types, which suggests a more recent introduction via an *A. tubingensis* strain that had diverged from the *A. tubingensis* strains in this study.

The *Ant1* transposon was discovered in strain N402 (derived from N400) through its active transposition in the *niaD* gene (Glayzer *et al.*, 1995). The primer sets were developed for overlapping stretches in the coding sequence of the putative transposase gene.

Comparison of the sequences of the different strains shows *Ant1*-homologues with both insertions and deletions, indicating that not all homologues have to be active transposons, or that the sequenced part is not that essential for functioning. Per strain the sequences obtained with either primerset in the overlapping parts exactly matched, suggesting that only one type of *Ant1* is present per strain, presumably in low-copy number.

Comparison of the phylogeny of the *Ant1* transposable element with those of the nuclear gene sequences, indicates that the element is most likely transferred (occasionally) between strains. Coevolution can not explain why very similar *Ant1*-types are found in distantly related strains (e.g. in *A. niger* types Ind 1.8.3 and Ind 1.8.11 and *A. tubingensis* Ind 1.6.23) or very dissimilar types in closely related strains (e.g. in N400 and Ind 1.8.2). *Ant1* is a transposon of the class that transposes by a DNA-DNA mechanism and contains short inverted terminal repeats (Glayzer *et al.*, 1995; Chapter 1). For *Tad*, a LINE-like element in *Neurospora* that transposes via reverse transcription via RNA (classI), transfer between nuclei has been found in heterokaryons. Because nuclei in heterokaryons of *Neurospora* do not normally fuse, *Tad* is supposed to have a cytoplasmic intermediate in its transposition (Kinsey, 1990b). Other studies indicate that horizontal transfer of transposable elements may occur (Kinsey, 1990a; Dobinson *et al.*, 1993; Capy *et al.*, 1994; Daboussi and Langin, 1994; Kempken *et al.*, 1998). Kempken and Kück (1998) recently found evidence for circular, possibly intermediate states of the class II transposon *Restless*. These elements were fused at the inverted repeat sites of the transposable elements and contained short insertions of up to 93 bp long. In our case, *Ant1* may be transferred between nuclei when they fuse, even without any further parasexual recombination, or may have a hypothetical and unknown cytoplasmic state that mediates transfer between nuclei.

Transposable elements have the ability to induce mutations because of their transposition and can promote changes in gene expression, in gene sequence, and probably in chromosome structure (Berg and Howe, 1989). They can also incorporate parts of their host's genome, as illustrated by the *Ant1* sequence that has strong similarities to the 3' coding and downstream untranslated region of the *amyA* gene of *A. niger* (Glayzer *et al.*, 1995). In transposable elements incorporated genomic parts may also recombine when transferred to another nucleus.

Earlier experiments showed that some inter- or intraspecies recombination should occur to explain the spread of viruses throughout the black *Aspergillus* population (Chapter 3-6). Tóth *et al.* (1998) showed that when different mitochondrial types are brought together recombination occurs readily. Mitochondria may fuse to recombine. In our data set we have no proof of such mitochondrial recombination. The presence of multiple copies of the mitochondrial genome per mitochondrion may perhaps obscure such recombination. The ribosomal classification of I and II types is in accordance with the split of *A. niger* and *A.*

tubingensis, but the I and I' designation must either have recombined or the population has remained polymorphic for this (~ multigenic) character for a longer period of time.

Most strains used in this study are wildtype isolates from the same site in Jakarta, Indonesia. The diversity in strains on this site for nuclear and cytoplasmic characters is enormous (Chapter 2 and this Chapter). However, strains N400, used for decennia as laboratory strain, and Z 1.1, a Dutch hospital isolate, fit in nicely with the Indonesian strains. This supports the idea that the black *Aspergilli* have an efficient aerial distribution and a rather homogeneous world-wide population (Chapter 2), and that due to a large 'spore bank' this population can be quite stable over time (Anderson and Kohn, 1998).

The black *Aspergilli* can be considered to be successful and diverse asexual organisms. We find evidence in the phylogenetic data described here for occasional parasexual recombination, perhaps through transient heterokaryon formation or transfer of chromosomal fragments. Nuclear and mitochondrial recombination via nuclear or mitochondrial fusions seem to occur at very low rates, but recombination via transposition of transposable elements may occur at a much higher frequency.

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8

General Discussion

8.1 Black *Aspergillus* population structure.

The imperfect filamentous black *Aspergilli* form a complex group of species. Some members such as *A. carbonarius* and *A. japonicus* can be recognized on morphological characteristics, other types such as *A. niger* and *A. tubingensis* can only be distinguished on the basis of different molecular markers (Raper and Fennel, 1965; Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993; Varga *et al.*, 1993; 1994a).

The black *Aspergilli* can be selectively isolated on media with 20% tannin from a large variety of substrates (Rippel, 1939). All black *Aspergillus* types share, next to their characteristic black conidiospore color, this ability to efficiently degrade high concentrations of the plant polymer tannin (Chapter 2). These tannins form complexes with proteins that are difficult to mineralize. Such complexes may control the availability of organic nitrogen in plant litter (Northup *et al.*, 1995). The black *Aspergilli* may thus, with their efficient tannin degrading system, occupy a special niche in the control of the natural nitrogen cycle.

For the experiments described in this thesis, populations of black *Aspergilli* were isolated from soil samples collected world-wide. The isolates from these populations were characterized with mitochondrial Restriction Fragment Length Polymorphisms (mtRFLPs) (Varga *et al.*, 1993; 1994a; Kevei *et al.*, 1996; Hamari *et al.*, 1997). Classifications based on MtRFLPs are mostly consistent with those based on other molecular techniques like ribosomal RFLPs, RAPDs and genealogies based on nuclear genes (Kusters-van Someren *et al.*, 1991; Mégnégneau *et al.*, 1993; Chapter 7).

In the sampled natural populations mixtures of *A. niger* types 1a-1d, *A. tubingensis* types 2a-2f and *A. carbonarius* and *A. japonicus* types were found. Especially the 1b & 1c *A. niger* and 2a & 2b *A. tubingensis* types occurred world-wide in high numbers. In general, several to many different haplotypes were present in local populations. Obviously, an efficient aerial distribution of the black conidiospores causes the wide distribution of haplotypes and the well-mixed population of spores in the soil (Chapter 2), leading to similar populations over large distances.

The black *Aspergilli* are considered to be soilborne fungi. Judging by the large numbers of different strains on a location, what we sampled was a sporebank full of conidiospores of different black *Aspergilli* rather than growing colonies. The presence of such a black *Aspergillus* sporebank (Anderson and Kohn, 1998) world-wide will also lead to a more stable population structure over time, depending on the survival of conidiospores.

Especially in warmer and more tropical regions high densities of black *Aspergilli* can be isolated per gram substrate (Chapter 2). Once conditions favor growth, large numbers of germinating spores present in such areas will have to compete for resources. In less dense (colder) areas the chances for different black *Aspergillus* strains to get in close contact are smaller. Estimates of the amount of black *Aspergillus* spores present world-wide can be made on the basis of the densities found: 10^{18}-10^{19} spores would probably be a conservative estimate (based on 1 spore per cm^3 in the top-layer world-wide).

8.2 Heterokaryon incompatibility.

When two strains grow close together, the possible fusion of their hyphae and survival of a formed heteroplasmon will depend on the (absence of) heterokaryon incompatibility reactions between them. For the exchange of nuclear and cytoplasmic genetic information which may result in infection of foreign genetic elements and parasexual recombination, (transient) contact between strains is necessary. Leslie and Zeller (1996) described different stadia in a heterokaryon incompatibility reaction: prefusion (under the control of *hsi*-genes), fusion (*het*-genes), postfusion (*sup*-genes) and finally apoptosis (apoptotic genes).

In the black *Aspergilli* nothing is known about the mechanism of incompatibility. Two strains in our collection show a heterokaryon self-incompatible phenotype and their heterokaryon formation (even between isogenic lines) is arrested in a prefusion stage. All other (50) examined strains are heterokaryon self-compatible, but unable to form heterokaryons with most other strains (Chapter 3). In these strains *het-* (heterokaryon incompatibility) genes and genes involved in later stages of the incompatibility reactions may be active (Chapters 3 and 4). In general the vehemence of an incompatibility reaction is assumed to depend on the number of genes involved. In the black *Aspergilli* many pre- and postfusion acting incompatibility genes and alleles could have evolved, which would explain the large number of vegetative compatibility groups and the often strong incompatibility reactions.

It is concievable that many extracellular, membrane, or intracellular protein that form complexes are involved in such incompatibility reactions. Some genes code for proteins that, when paired with a product of another allele or other gene (allelic and non-allelic incompatibility reactions), may either produce or lack a functional product, which leads to a cascade of reactions ending in apoptosis; these are strong *het*-genes. Other genes may not

lead to fatal reactions, but only influence the fitness of the formed heterokaryon as some 'partial' *het*-genes seem to do. Mutations in the systems for the recognition of other strains may result in pre-fusion incompatibility. In this way heterokaryon incompatibility could be a byproduct of mutations in functional genes and the existence of a large number of *'het'*-genes can be explained.

A positive (side-)effect of heterokaryon incompatibility for individual black *Aspergillus* strains could be protection from recombination or infection. The prevention of, or reduction in somatic transfer of, deleterious elements can maintain the *het*-genes in a population if these elements are more important than positive fitness effects from mitotic recombination via the parasexual cycle.

The chance of two random strains in a population being heterokaryon compatible is small (Chapter 2 and 3). Even in areas where large numbers of black *Aspergilli* are present the chance of successful heterokaryon formation is minimal. The probability of a genetic element transferring during a heterokaryon incompatibility reaction may depend on its physical size (larger elements may be more restricted) and its location in the cell (cytoplasm may be more accessible than nuclear or mitochondrial compartments). Our experiments on horizontal transfer show that some elements are not transferred, but that others seem to have spread throughout the population (see §8.3).

8.3 Recombination and horizontal transfer

8.3.1 Mitochondria

The mitochondria of the black *Aspergilli* can be classified by their restriction fragment length polymorphisms (mtRFLPs). The different species *A. carbonarius, A. japonicus, A. niger* and *A. tubingensis* can be distinguished and subgroups can be determined (Varga *et al.*, 1993; 1994a; Kevei *et al.*, 1996; Hamari *et al.*, 1997). The mtRFLP technique is easy to perform and large numbers of isolates could thus be characterised (Chapter 2).

Comparison of mtRFLP data with data of nuclear gene genealogies and ribosomal RFLPs (Chapter 7) confirms that the mitochondrial classifications are quite accurate. No horizontal transfer of mitochondria was detected in our set of natural isolates, though recombination between mitochondria and even exchange can be forced in protoplast fusions (Kevei *et al.*, 1997; Chapter 4). A mitochondrial based oligomycin resistance (difficult to obtain sponatneously) can be used as a transferable marker for cytoplamic contact.

Mutations (and of course recombination cannot be ruled out) may lead to new mitochondrial haplotypes. Our data suggest for instance that both types 1a and 1d are derived form the 1c-haplotype (Chapter 7). Tóth *et al.* (1998) showed that when different mitochondrial types are brought together recombination occurs readily. The presence of

multiple copies of the mitochondrial genome per mitochondrion and multiple mitochondria per fungal cell may limit detectable recombination in nature.

3.8.2 Transposable elements

So far three transposons have been described in black *Aspergillus* strains (Glayzer *et al.*, 1995; Amutan *et al.*, 1996; Nyyssönen *et al.*, 1996). The *Ant1* (*A. niger* transposon) transposon was discovered in strain N402 (derived from N400) through its active transposition in the *niaD* gene and is considered to be a single or low copy number class II (DNA-DNA) transposon (Glayzer *et al.*, 1995). Using two primer sets developed for the putative transposase gene of *Ant1*, *Ant1*-homologous sequences were found in nine out of 21 examined strains and nucleotide sequences were determined. Comparison of the sequences of the different strains shows *Ant1*-homologues with both insertions and deletions, indicating either that not all homologues have to be active transposons (perhaps silenced transposons or *pseudo*transposons?), or that the sequenced part is not essential for transposition.

Comparison of the phylogeny of the *Ant1* transposable element with those of nuclear gene sequences shows that *Ant1* is most likely (occasionally) transferred between strains, perhaps even between species. Co-evolution is not likely, since similar *Ant1* sequences are found in diverged black *Aspergilli* and, on the other hand, diverged sequences are found in closely related strains. A possible transmittable stage of a class II DNA-DNA transposon was recently described by Kempken and Kück (1998). They found ciruclar states of the transposon *Restless*, which were fused at the inverted repeat sites and contained short insertions of genomic DNA of up to 93 bp long. In our case, similar plasmid-like intermediate states of *Ant1* may either be transferred between nuclei when they fuse, even without any further parasexual recombination or may have a hypothetical cytoplasmic state that mediates transfer between nuclei. Transfer of an artificial and autonomously replicating plasmid in *A. niger* could occur via the cytoplasm or perhaps more likely via transient contact between (dissimilar) nuclei. The described rate of the AR plasmid transfer between nuclei indicates that when heterokaryons are formed exchanges between nuclei may occur very frequent (Debets, 1998).

Transposable elements have the ability to induce mutations because of their transposition and their footprints and can promote changes in gene expression, in gene sequence, and probably in chromosome structure (Berg and Howe, 1989). They can also incorporate parts of their host's genome, as illustrated by the *Restless* transposon (Kempken and Kück, 1998) and by the *Ant1* sequence that has strong similarities to the 3' coding and downstream untranslated region of the *amyA* gene of *A. niger* (Glayzer *et al.*, 1995). Transposition of class-II transposon within a genome is in general a rather rare incident, but

the genomic parts incorporated in transposable elements may be responsible for recombination within a nucleus and between different nuclei.

8.3.3 Black *Aspergillus* mycoviruses.

The major part of this thesis deals with mycoviruses in the black *Aspergilli* and their possibilities of horizontal transfer. These mycovirus infections, found world-wide in different black *Aspergillus* haplotypes in a frequency of approximately 10%, show a variety of dsRNA fragments and patterns in their infections (Chapter 2). Though no sequences of these viruses have been determined, their organization of dsRNA and particles suggest that they are related to the *Totiviridae*, but *Partitiviridae* may also be present. The 'basic' viruses containing one (or two) dsRNA fragments can be accompanied by both defective and satellite dsRNA fragments, which may account for the high variety and similarities in detected banding patterns. Multiple infections with mycoviruses can occur. Although the mycoviruses do have a protein coat they are not infectious outside the mycelium; perhaps the coat protects against degradation within the cytoplasm.

Infection with mycoviruses is not without fitness costs for black *Aspergillus* strains (Chapter 6): infected strains have a reduced growth rate, produce fewer spores and are less competitive with other strains when compared with isogenic virus free strains. One of the natural isolates (Ind 1.8.16) shows these effects in an extreme phenotype. Vertical transfer to the asexual spores is very efficient and no spontaneous loss was detected. Population genetic models predict that deleterious elements should disappear from a population, unless they have an extra route of transfer besides vertically transmission to the offspring.

Horizontal transfer may occur when two mycelia fuse. In many filamentous fungi the ability to form anastomoses between different strains is regulated by heterokaryon incompatibility or *het*-genes. Strains can be tested for heterokaryon compatibility in different ways. One of the most often used methods is the forcing of different chlorate resistant/nitrate non-utilizing strains to form a heterokaryon on medium containing nitrate as sole nitrogen source. Tests between black *Aspergillus* strains show that the majority of natural isolates are heterokaryon incompatible. This heterokaryon incompatibility may involve both pre- and postfusion reactions. Combinations of heterokaryon compatible and incompatible strains were tested for their ability to transfer mycoviruses between them. In compatible combinations mycoviruses were transmitted very easily, but in incompatible combinations no spontaneous transfer could be detected. When the mycelia were in close contact and regularly damaged transfer of a single dsRNA fragment was detected in one of the incompatible combinations (Chapter 3).

Some of the heterokaryon incompatibility reactions can be evaded by using protoplast fusions (Chapter 4). The cell wall and some membrane components of the incompatibility

reaction-network are thus bypassed. However, the intraspecies transfer of mycoviruses remains limited in the black *Aspergilli*, whereas interspecies transfer from black *Aspergilli* to *A. nidulans* and *vice versa* is successful. Obviously cytoplasmic components of the incompatibility reactions can also result in apoptotic reactions in the black *Aspergilli*. Exchange of a mitochondrial oligomycin resistance marker is not necessarily correlated with mycovirus transfer. This suggests that small amounts of cytoplasm can be survived in heterokaryon incompatibility reactions and that tranfer of the mitochondrial resistance does not imply that viruses are also transferred.

The divergence between the black *Aspergilli* and *A. nidulans* may also have resulted in diverged *het*-genes. While the species may perhaps very rarely form anastomoses in nature due to lack of recognition, postfusion reactions may have become less severe. This would explain why *A. niger* - *A. nidulans* interspecies protoplast fusions yield more virus transfer than intraspecies fusions between black *Aspergilli*.

Fusarium poae has a similar niche to the black *Aspergilli* and in natural isolates of this fungus mycoviruses were always (Fekete *et al.*, 1995). Similar sized dsRNA fragments occur in both species. After protoplast fusions between *F. poae* and black *Aspergillus* strains, black *Aspergillus* stains could be recovered which were infected with the *F. poae* viruses. This shows that at least the viruses are viable in their new host and thus that viable infections could occur in nature after interspecies contact.

A curious case is the heterokaryon self-incompatible strain N062. This strain does not form anastomoses with itself or any of the other tested strains. However, via protoplast fusion with *A. nidulans* viruses can be introduced, though these cannot spread between different colonies of strain N062. In effect this strain is immune to virus infections even from isogenic lines. Resistance to viruses was not observed in the other black *Aspergilli* used: all strains could be infected with virus, although the strength of the heterokaryotic incompatibility response influences the infection rate.

An alternative explanation for the wide-spreaded infection in the black *Aspergilli* could be an old infection that co-evolved with the species. This explanation is less likely because so many similar infection patterns would not be expected to occur in very diversified lines.

8.3.4 Nuclear recombination

In a heterokaryon, different nuclei can fuse to form a (transient) diploid. Upon haploidisation reassortment of chromosomes as well as mitotic crossing-overs may lead to recombination. This has been shown to occur between isogenic lines under selective laboratory conditions. In nature parasexual recombination may be limited by the almost omnipresent heterokaryon incompatibility between strains (Chapter 3). Recombination may

also be the result of exchanges with genetic elements of foreign origin such as transposable elements.

Remnants of recombination in nuclear genes may be detected by a mosaic structure, where blocks of high similarity are interspersed with blocks of high sequence divergence in the gene or genome (Maynard Smith, 1992). An unexpected large numbers of apparent homoplasies in a most parsimonious tree of a gene is also suggestive of recombination (Hudson and Kaplan, 1985; Maynard Smith and Smith, pers. comm.). A third method is based on inconsistencies in phylogenies of different parts of the genome (Woese *et al.*, 1980; Dykhuizen and Green, 1991; Dykhuizen *et al.*, 1993; Hey and Kliman, 1994). The last method revealed that (a considerable number of) recombination occurs in several fungal species previously presumed to be asexual (Burt *et al.*, 1996; Koufopanou *et al.*, 1997; Geiser *et al.*, 1998). The comparison of phylogenies of different host species and symbionts or parasites can in a similar manner be used to determine coevolution ('clonal') vs. horizontal transfer ('recombination').

Nuclear sequences of different extracellular non-essential genes were determined in a variety of black *Aspergillus* strains. Phylogenies based on these sequences have been compared. In the *pgaII* gene a presumably intragenic recombination event has lead to a mosaic structure, extra homoplasies and incongruencies between genealogies. Overall there seems to be some but very little recombination in the population. The black *Aspergilli* appear to be a largely clonal population with incidental recombination either through heterokaryon formation or through transfer of genetic elements with incorporated genomic parts (Chapter 7).

In one of the strains a non-functional pseudogene of phytaseA was sequenced, with a stopcodon in the sequenced part. Different phytase genes have been described in black *Aspergilli* and phytaseA is obviously not essential. However, without selection for a functional gene the defective *PhyA* locus accumulated much more mutations than sequenced parts in other strains with the same functional gene.

The ribosomal RFLP classification of I and II types is in accordance with the split of *A. niger* and *A. tubingensis*, but the I and I' types within *A. niger* must either have recombined or the population has remained polymorphic for this (~ multigenic) character over a longer period of time.

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Summary

This thesis deals with the horizontal transfer of genetic elements in the black *Aspergilli*. The black *Aspergilli* form a complex group of asexual species. All share a characteristic black conidiospore color and the ability to efficiently degrade tannin. Selective isolation of all different black *Aspergillus* types is possible on media with 20% tannin. Tannins can form complexes with proteins that are difficult to mineralize. Therefore, the strains may have a special niche in the control of the natural nitrogen cycle. Black *Aspergilli* occur worldwide and especially in warmer regions at high densities. The spores have an efficient aerial distribution, which produces a well-mixed sporebank in soil throughout the world.

Under laboratory conditions isogenic lines are capable, after hyphal fusions, to form a heteroplasmic heterokaryon and (transient) diploids. This so-called parasexual cycle can result in recombination via reassortment of chromosomes, mitotic crossing-over and/or exchange of cytoplasmic genetic elements. Most of the natural isolates are heterokaryon incompatible with one another and unable to form a stable heterokaryon. About the exact mechanism of the heterokaryon incompatibility reactions in the black *Aspergilli* little is known. Confrontations between heterokaryon self-incompatible strains suggest that prefusion genes are involved. The fact that protoplast fusions are partly able to overcome incompatibility reactions suggests that also fusion and postfusion genes are involved.

One of the cytoplasmic candidates for horizontal transfer is the mitochondrion. Different mitochondrial haplotypes can be distinguished, corresponding with different black *Aspergillus* types. No horizontal transfer or recombination of mitochondria was observed in our natural isolates, though in protoplast fusions mitochondria can recombine. In nature new mitochondrial types may result from mutations.

Most of the transfer experiments in this thesis were done with cytoplamsic dsRNA mycoviruses. In nature 10% of the population is infected with a variety of different dsRNA fragments of different viral origins. These mycoviruses can cause serious reductions in their host's fitness on traits as spore production and growth rate. Population genetic models predict that deleterious elements should disappear from a population, unless they have an extra way of transfer than just vertical transmission to offspring. Interspecies transfer of mycoviruses with species like *Fusarium poae* was in our experiments less difficult to achieve (and thus perhaps more likely in nature) than intraspecies transfer between different black *Aspergillus* types.

In a diploid both interchromosomal and intrachromosomal mitotic recombination could take place. However, molecular data suggests that there is little (para)sexual recombination in the black *Aspergillus* population, in contrast to other presumably asexual fungi tested so far. Recently transposable elements have been found in black *Aspergillus* strains. These do

seem to have transposed between different, quite unrelated strains. Circular intermediates of these transposable elements may also transfer little parts of genomic DNA, which may lead to some recombination. The size of the genetic elements may influence the chance on horizontal transfer during cell contact: no detectable transfer of mitochondria, very little of mycoviruses and some transfer of transposable elements.

Samenvatting

Dit proefschrift gaat over de horizontale overdracht van genetische informatie in de zwarte *Aspergilli.* Deze zwarte *Aspergilli* bestaan uit een complexe groep aseksuele soorten. Allemaal bezitten ze karakteristiek zwart gekleurde conidiosporen en de eigenschap om tannine efficiënt af te breken. Selectieve isolatie van alle verschillende typen zwarte *Aspergilli* is mogelijk met media die 20% tannine bevatten. Deze tanninen kunnen met eiwitten complexen vormen (die moeilijk af te breken zijn). Mogelijk hebben de zwarte *Aspergilli* daardoor een special niche in de stikstofcyclus in de natuur. De zwarte *Aspergilli* komen wereldwijd voor en kunnen vooral in warmere streken hoge dichtheden bereiken. De sporen hebben een efficiënte verspreiding via de lucht, wat zorgt voor een goed gemengde 'sporenbank' in de bodem over de hele wereld.

Onder laboratorium omstandigheden kunnen isogene stammen, na fusie van hyphe draden, een heteroplasmatisch heterokaryon en (tijdelijk) een diploïd vormen. Deze zogenaamde 'para'seksuele cyclus kan tot recombinatie leiden via onafhankelijke hergroepering van chromosomen, mitotische overkruising en/of uitwisseling van cytoplasmatisch genetische elementen. De meeste natuurlijke isolaten zijn echter niet in staat om met elkaar een stabiel heterokaryon te vormen en zijn dus heterokaryon incompatibel. Het precieze mechanisme waarlangs heterokaryon incompatibiliteitsreacties in de zwarte *Aspergilli* verlopen is niet bekend. Experimenten met heterokaryon zelf-incompatibele stammen geven een indicatie dat genen betrokken zijn bij de herkenning van andere stammen (prefusie). Dat protoplasten fusies een deel van de incompatibiliteitsreacties helpen omzeilen suggereert dat verder ook genen betrokken zijn bij de fusie zelf en de reacties daarna.

Het mitochondrium is een van de cytoplasmatische elementen die horizontaal zou kunnen overerven. Verschillende mitochondriale typen kunnen worden onderscheiden, die overeenkomen met de verschillende herkenbare zwarte *Aspergillus* typen. Horizontale overdracht of recombinatie van mitochondriën kon niet worden aangetoond in de bekeken isolaten, alhoewel dit wel kan gebeuren tijdens protoplasten fusies. Waarschijnlijk dat in de natuur nieuwe type mitochondriën ontstaan door mutaties.

De meeste experimenten beschreven in dit proefschrift zijn gedaan naar de overdracht van dsRNA schimmelvirussen. In de natuur is zo'n 10% van de populatie geïnfecteerd met een verscheidenheid aan dsRNA fragmenten van verschillende virale herkomst. Deze mycovirussen kunnen de 'fitness' van hun gastheren flink reduceren. Populatie genetische modellen voorspellen dat zulke fitnessverlagende elementen uit de populatie zullen verdwijnen tenzij ze niet alleen aan de nakomelingen worden overgedragen, maar ook horizontaal naar andere stammen worden overgedragen. Overdracht tussen zwarte

Aspergillus stammen en andere soorten als *Fusarium poae* bleek in onze experimenten gemakkelijker dan overdracht tussen verschillende zwarte *Aspergilli* onderling. Dit kan er op wijzen dat ook in de natuur overdracht tussen verschillende soorten gemakkelijker zou kunnen zijn.

In een diploïde kern kan mitotische recombinatie zowel binnen als tussen chromosomen plaatsvinden. Op grond van onze moleculaire data blijkt er echter weinig (para)seksuele recombinatie plaats te vinden in de populatie van verschillende zwarte *Aspergilli*. Dit is in tegenstelling tot andere geteste voorheen aseksueel geachte schimmels die wel bleken te recombineren. Onlangs zijn ook de eerste 'transposable elements' (nucleaire overdraagbare fragmenten) aangetoond in zwarte *Aspergillus* stammen. Deze overdraagbare elementen lijken wel te zijn uitgewisseld en zelfs tussen niet nauw verwante stammen. De circulaire tussenstadia van deze overdraagbare elementen kunnen stukjes genomisch DNA meenemen die elders zouden kunnen recombineren. De overdracht van genetische elementen tijdens celcontact zou bepaald kunnen worden door hun fysieke afmetingen: kern en mitochondrium wisselen niet waarneembaar uit, mycovirussen zelden en transposable elementen redelijk vaak.

Dankwoord

Inleiding

Bij de werkgroep microbiële/populatiegenetica van de vakgroep Erfelijkheidsleer wordt al jaren onderzoek gedaan aan verschillende filamenteuse schimmels. Rolf Hoekstra en Fons Debets boden mij binnen deze groep alle ruimte en steun om aan de genetica van de zwarte *Aspergilli* te werken en om in een fijne, vriendschappelijke omgeving een heleboel te leren. Behalve veel dank aan deze twee begeleiders, natuurlijk ook veel dank aan de 'vaste' werkgroepleden Klaas, Gerda, Marijke en Edu en andere mensen van de vakgroep Erfelijkheidsleer, die tesamen de omgeving voor dit promotieonderzoek vormden.

Materiaal en Methode

Het genetisch materiaal van de onderzoeker werd liefdevol samengebracht en grootgebracht door Abe en Steeph van Diepeningen-Nagelkerke, lieve ouders voor het leven. Ik ben heel blij dat pappa en mama altijd paraat staan voor me en dat er van het 'spreek-met-je-vader-en-het-komt-in-orde' nog altijd gebruik kan worden gemaakt.

Een groot deel van mijn erfelijke materiaal, en nog veel meer, wordt gedeeld met mijn kleine-grote zusje Jitske. Jip is altijd bereid om te helpen relativeren en zonodig met (gedeelde) chocolade de stemming te verbeteren. Zij merkte ook na een dagje meelopen op het lab fijntjes op dat wetenschappelijk onderzoek toch echt voor het grootste deel uit schoonmaken en opruimen bestaat.

De rest van het genetisch materiaal werd geleverd door verschillende zwarte *Aspergilli*, die te voorschijn zijn gekomen uit allerlei vieze zakjes en obscure potjes met grond, meegebracht of toegezonden vanuit allerlei mooie plekken ter wereld. Iedereen die een 'schimmeltje' heeft bijgedragen, bij deze bedankt.

Resultaten

Veel van de beschreven resultaten zouden er niet zijn geweest zonder de hulp van 'mijn' studenten en stagiaires. Heleen Broekhuis, Hanneke Jousma, Alex van Harn, Christina Rekers, Annemarie de Jong, Oscar van Marle, Marga Kluitenberg, Gaby Scholte en Robert Vellema hebben dan ook een 'significante' tot 'zeer significante' bijdrage geleverd aan het in dit proefschrift beschreven practische werk. Ik ben hen dan ook zeer dankbaar voor hun hulp, de gezellige tijd samen en hun vriendschap.

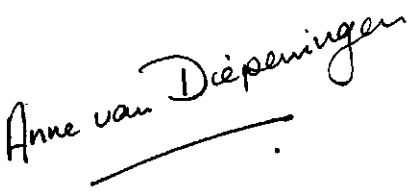
Also students (or some would say 'slaves') from abroad came to participate in my work and to join the joyfull laughter in the group. Roland Grey, David McLay en Jarkko Routtu thanks for what you did!

Bram van de Pas was misschien niet mijn student, maar ondertussen wel een goede vriend en van zijn resultaten heb ik goed gebruik kunnen maken. Olga en Grad hebben dit proefschrift op taalfouten nagelezen en er de komma's in geplaatst. En dan is er nog een hele rits 'oude' en 'nieuwe' collegas's; Maarten, Arjan, Alex, Marijn, Duur, Peter, Ronny, Judith, Henk en Menno die samen met tal van studenten de werkvloer opluisterden. Ook Eeke Anne en andere vrienden van buiten de vakgroep bedankt voor het luisteren en misschien kunnen we het nu weer eens over wat anders hebben dan over mijn proefschrift.

Discussie en Conclusies

Het fenotype wordt bepaald door de combinatie van genotype en milieu. Dit geldt ook voor het fenotype van een proefschrift. Dit proefschrift bevat net als de schimmel waarover het gaat acht 'linkage groups' en nog wat andere informatie. Op het verschijnen en de uiteindelijke vorm van dit proefschrift zijn veel mensen van invloed geweest, die ik hiervoor zeer erkentelijk ben. Allen daarvoor bedankt en in het bijzonder Marijn.

Weinig mensen zullen zo'n lief, gek en aardig vriendje hebben als ik. We leven (werken, wonen en knutselen) ondertussen al heel wat jaartjes samen en ik vrees dat ik niet meer zonder kan: Marijn, ik hou van je.



Curriculum Vitae

Op 20 april 1969 werd ik in Arnemuiden geboren. In dit Zeeuwse stadje bezocht ik de kleuterschool - waar ik een grote aversie kreeg tegen het gedwongen met de armen over elkaar zitten - en de lagere school, waar vrij lezen mijn favoriete 'vak' was. Aan de Stedelijke Scholengemeenschap te Middelburg behaalde ik in 1987 mijn gymnasium β diploma. In hetzelfde jaar begon ik met de studie biologie aan de Landbouwuniversiteit te Wageningen en sloot het eerste studiejaar af met de propedeuse (*cum laude*). In 1992 studeerde ik eveneens *cum laude* af met als afstudeervakken microbiologie en genetica en na een stage genetica aan het St. Patrick's College te Maynooth, Ierland.

Aansluitend begon ik als onderzoeker in opleiding in dienst van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO) met een promotieonderzoek bij het laboratorium voor Erfelijkheidsleer van de Landbouwuniversiteit. Ruim vierenhalf jaar onderzoek aan 'Horizontale overdracht van genetische informatie in de aseksuele schimmel *Aspergillus niger*' heeft geresulteerd in dit proefschrift.

Na mijn promotieonderzoek was ik een half jaar in dienst van het laboratorium voor Erfelijkheidsleer als toegevoegd docent en deed freelance wat 'computerwerk' en maakte examenvragen voor de module 'Evolutie' van de Open Universiteit. Per 1 januari 1999 treed ik in dienst als postdoc op een EG-gefinancieerd project aan oplosmiddelresistente stammen van de bacterie *Pseudomonas putida* bij het laboratorium voor Industriële Microbiologie van de Landbouwuniversiteit te Wageningen.

Het begon met een kleine cel, die splitste zich in twee
en even later zwom opeens de eerste vis in zee.
Toen hij pootjes kreeg, klom hij op het land.
En daarna ging hij ook nog vliegen.
Ja, Darwin had het goed gezien:
de bijbel bleek te liegen.

De eerste mens, een Sapiëns, werd later Neanderthaler.
Sindsdien is er niet veel gebeurd; hij werd alleen wat kaler.
Maar gisteren toen zag ik jou: een schoonheid, zo fantastisch!
Jij bent zo mooi, ik denk dat nou de evolutie af is.

Ja, Darwin kan tevreden zijn.
De evolutie is voltooid.
Zo machtig mooi was de mens nog nooit.
Jij hebt die taak volbracht
Miljoenen jaren lang gewacht,
maar nu jij er bent is het af!

Supporters, patsers, politici en wilde wegpiraten
Het is overal zo goed te zien: we stammen van de apen.
Het is dus maar goed dat jij er bent, als pleister op de wonde.
Of zou er toch een God bestaan,
die jou hier heeft gezonden?

Het begon met een kleine cel
en jij, jij bent het einde.

Pater Moeskroen, *Darwin kan tevreden zijn.*