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Selectie arena in *Aspergillus nidulans*

vroege selectie van nakomelingen in een filamenteuse schimmel

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 26 november 2003
des namiddags te half twee in de Aula

Bruggeman, Judith

Selection arena in *Aspergillus nidulans* -early progeny choice in a filamentous fungus- / Judith Bruggeman
Thesis Wageningen University. –With references- With summary in Dutch

ISBN 90-5808-926-6.

aan mijn ouders

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Introduction

Prologue

A navel orange tree creates on average 200,000 buds. Only half the number of these buds proceed to the flower stage. Subsequently, one third of the flowers are purged, while two thirds of the flowers go on and invest in fruit formation. Then, nearly 99.7% of these fruit initials are aborted when they are still very tiny. Eventually, typical 400 fruits mature per tree (Kozlowski, 1973; p. 15).

In humans, 7 million oocytes have developed in week 20 of pregnancy. From that moment on, oocyte atresia starts and reduces the number of oocytes to about 1-2 million at birth and further reduces it to only 300,000 by puberty. So, by puberty over 95% of a female's potential germ-cell stock has been lost. Furthermore, only 400 of these will be released for potential fertilization (Baker, 1963; Morita and Tilly, 1999).

Some mammals have polyovulation. The plains viscacha (a rodent species from South America) sheds 50-845 ova per cycle. Although probably many are never fertilised, up to seven embryos are implanted. From these, generally two are born, while the others are resorbed. Reproduction in four species of elephant shrews also comprises polyovulation from 8-44 ova, while the common litter size in these species is two. These and more examples are reviewed in Birney and Baird (1985).

Quality control…

The above-mentioned examples suggest that reproduction involves a wasting of resources and energy. Already Darwin (1876) wondered why plants often produced many more flowers than they eventually developed into ripe fruits. Such wasting seems to be in conflict with natural selection which promotes survival and reproduction of organisms. However, overproduction of flowers, fruit initials and oocytes for future progeny provides an opportunity for early selection, for example based on the genetic quality. Such selection may reveal unfavourable genotypes before these enter the next generation and consequently prevent investment in these poor quality genotypes. In other words, energy for reproduction could chiefly be invested in progeny of high quality. Thus, provided that control of offspring quality is possible at the initial stages of reproduction, natural

selection could favour such a mechanism only if the benefits of high-quality progeny outweigh the cost of overproduction.

In sexually reproducing organisms, genetic information is transmitted with maximal uncertainty (the Mendelian Lottery) and therefore a 'low quality' allele (*e.g.*, a mutation) in a heterozygote has equal chance of being transmitted to offspring as its 'high quality' counterpart. At various stages of sexual reproduction (*i.e.*, at the germ-line stage, at the germ-cell stage, and at the zygote stage) quality control and selection can diminish the transmission of low quality alleles, thereby capitalising on the assumed benefits of sex (Otto and Lenormand, 2002). Non-Mendelian segregation of traits is an expected result of quality control (Korbecka *et al.*, 2002). Of course, offspring quality is also promoted through sexual selection - both at the level of mate choice and at the gamete level - but this lies outside the scope of this thesis. Quality control on reproduction may also operate in asexual organisms. However, potential benefit of such control is much smaller because the genetic variance among asexual offspring is affected only by mutation, while variance among sexual offspring is affected by both mutation and recombination.

In the following sections, I will present an overview of studies that provide strong indications for the existence of quality control in reproduction by means of overproduction and selection. Not surprisingly, all examples are from sexual reproduction routes.

… in germ-line cells

Typically, only a fraction of the progeny of initially established germ-line precursor cells contributes to the gametic population. Therefore, pre-gametic selection in the germ line forms the earliest opportunity for selection on quality of future progeny. Cells in the germ line undergo a process of proliferation and differentiation through mitotic divisions. Mutation, mitotic crossing-over and mitotic gene conversion can create genotypic diversity among these cells. Theoretical models have shown that cell-lineage selection in the germ line can influence the rate and fixation probabilities of new mutations; more specifically, such selection can boost the spread of beneficial mutations, hinder the spread of deleterious mutations, and reduce the genetic load imposed on the population

(Hastings, 1991; Otto and Hastings, 1998). In *Drosophila melanogaster*, selection seems to occur in mosaic germ-line populations (Extavour and Garcia-Bellido, 2001). In mice, a quality control mechanism appears to be operating during the premeiotic stages of spermatogenesis, such that only cells with low mutation numbers are allowed to proceed into meiosis (Walter *et al.*, 1998).

… in germ cells

The next stage of selection may operate at the level of the germ cells. Oocyte atresia in birds and mammals (described in the prologue for human females) may function as a 'genetic bottleneck' for mitochondrial inheritance to counteract the accumulation of mutations in mitochondria (Bergstrom and Pritchard, 1998). Oocyte atresia could delete oocytes with inferior or mutant mitochondrial genomes, thereby ensuring that only 'superior' mitochondria are passed from mother to offspring. The efficacy of potential selection is enlarged by the fact that human primordial germ cells contain only 1-30 mitochondria per cell, while a ripe oocyte has 100,000 mitochondria. Support for selection during atresia has come from a few studies. First, a comparative study among various animal species shows that a more severe mitochondrial bottleneck (measured as the minimum number of mitochondria in the primordial germ cells) correlates with a lower offspring number, and *vice versa*. Moreover, species with a more severe bottleneck experience higher percentages of atretic germ cells (Krakauer and Mira, 1999). Second, in murine oocytes with an inherently high apoptosis rate in vitro, this rate can be inhibited by injection of healthy mitochondria (Perez *et al.*, 2000). Finally, the decline in oocyte quality with age in humans has been related to mitochondrial dysfunction. Interestingly, microinjection of cytoplasm from a donor oocyte into an oocyte of a woman who suffers from compromised fertility 'rejuvenates' the recipient's egg and gives it a jump-start (Tilly, 2001). These three studies suggest a selection mechanism for mitochondrial quality during oocyte atresia. Yet, none of these studies actually demonstrate that atresia indeed selects against mitochondria with low genetic quality.

… in zygotes

The final stage of selection may operate shortly after fertilization. Which zygotes will get full support from the parent to develop into offspring? Several studies discuss the occurrence of spontaneous abortion in relation to this level of selection. In humans a highly significant degree of MHC (major histocompatibility complex) compatibility can be found in couples with a history of repetitive spontaneous abortions (Bolis *et al.*, 1985). Also in mice, conceptuses incompatible with their own MHC antigens seem to be preferred (Wedekind, 1994). Relaxed screening of embryos could explain the rising incidence of chromosomal abnormalities in live births, the falling incidence of normal embryos in spontaneous abortions, and the increased incidence of spontaneous abortions with maternal age in humans (Forbes, 1997). Some allele combinations are not viable and are selected against at the zygote level. For example, a specific heterozygous combination of alleles involved in neural tube development leads to neural tube defects. Many of the embryos carrying this combination are selectively aborted in humans (Joosten *et al.*, 2001). In coypu there seems to be selective abortion of entire litters with respect to quality and sex (Gosling, 1986). The botanical literature includes many examples where plant species produce far more ovules than they ever develop into seed (Stephenson, 1981). Some studies relate these observations to a quality control mechanism by trying to demonstrate that aborted ovules are potentially viable but would produce less vigorous progeny than non-aborted ovules would produce (Casper, 1988; Melser and Klinkhamer, 2001; Rocha and Stephenson, 1991; Stephenson and Winsor, 1986).

Selection arena

The present thesis focuses on offspring quality control at the zygote stage or soon thereafter. Selection at this stage is the most reliable of the levels discussed above because the zygote forms the real start of the progeny and contains the nuclear and mitochondrial genotypes and cytoplasm for the first time in combination. Besides, these genotypes are transcribed in the course of zygote development. Especially in sexually outcrossing organisms, offspring fitness depends on newly formed gene combinations. Interestingly in this regard is that the oocytes of most vertebrate and invertebrate species are in some stage

of meiotic arrest at the time of fertilisation. For example, human oocytes are arrested at meiosis I and complete it during ovulation (Buehr, 1997). Only upon fertilisation, meiosis II is completed and one haplotype will fuse with the fertilising sperm to form the zygote. Possibly, this mechanism ensures fusion of the best complementing haplotypes of egg and sperm.

Stearns (1987) formulated the selection arena hypothesis to study whether overproduction of zygotes could be explained as part of a quality control mechanism. This hypothesis states that overproduction of zygotes could be explained as part of a quality control mechanism which operates in the following manner: An enlarged array of zygotes is created of which only a genetically superior subset will fully develop; zygotes with a low future fitness fail, while zygotes with a high future fitness thrive. In this way, parental energy for reproduction is invested in the most promising zygotes. Essential in the hypothesis is that the potential members of the next generation are tested at a crucial moment, namely just before substantial parental investments start. This hypothesis further assumes that 1) zygotes are cheap to produce, 2) parental time, energy and/or risk are invested in the zygotes, 3) offspring vary in fitness, and 4) this fitness difference can be identified. Natural selection will favour such a selection arena only if the benefits of high-quality progeny outweigh the cost of overproduction. Especially if the initial cost of a zygote is low relative to the cost of an independent offspring, zygote overproduction will skyrocket (Kozlowski and Stearns, 1989). Selection pressure for overproduction of zygotes is less affected by the amount of genetic variation among the initial zygotes (Kozlowski and Stearns, 1989). In addition, Forbes and Mock (1998) modelled progeny choice to account for the evolution of offspring overproduction especially under different conditions of sibling rivalry. Their findings suggest similar conditions as those that Stearns gives: inexpensive forms of sibling rivalry, large cohorts of evenly matched offspring, and exaggerated variation in offspring genetic quality. How this quality should be evaluated was explored long ago in the botanical literature (Buchholz, 1922). Buchholz viewed ‘developmental selection’ through competition among progeny as a powerful mechanism for screening the best progeny. To be effective, such mechanism had to meet four requirements: competition must commence simultaneously, take place under uniform

conditions, measure comparable merit, and rigidly eliminate the great majority of individuals that fall below the standard. More recently, Møller (1997) discussed a similar idea of developmental selection.

In summary, several authors considered the overproduction of zygotes to be a mechanism for progeny choice. Furthermore, several examples have been described in the previous section which strongly suggest the operation of selection arenas in nature. Yet, although the idea of the selection arena is appealing and may be plausible, it has been difficult to actually demonstrate a mechanism of selection against low quality zygotes in favour of the production of higher quality ones. This is partly due to the complexity of the studied organisms and their life history traits.

Testing the selection arena hypothesis in the fungus *Aspergillus nidulans*

I propose that the fungus *Aspergillus nidulans* is highly suited to study the selection arena hypothesis: First, it fulfils the first three of the four conditions of the selection arena hypothesis. Second, I believe that the predicted result of the arena can indeed be tested in this fungus, namely that zygotes with a low future fitness fail, while zygotes with a high future fitness thrive. To start, I will present basic information on the biology of *A. nidulans* and explain its popularity as a model organism in genetical research (see also Fig. 1.1 and references in Adams *et al.*, 1998; Champe and Simon, 1992; Elliott, 1960; Pontecorvo, 1953). It is a simple eukaryotic organism consisting of thread-like filaments called hyphae that form a mycelial network. The fungus lives in soil in large parts of the world (Domsch *et al.*, 1993). It releases extracellular enzymes and feeds by absorption. We can easily culture the fungus in the laboratory on artificial medium in Petri dishes. Fungi are immobile, modular organisms with indeterminate growth in time and space by repeated iteration and branching of their hyphae. Therefore, in the lab, a new colony can be established just by transferring some mycelium to a new dish. *A. nidulans* produces enormous numbers of spores (more than 10^8 on one Petri dish). Most of these spores are asexual conidiospores produced via mitotic division in long chains on conidiophores. A smaller fraction of these spores are sexual ascospores produced via meiosis in spherical fruiting bodies, called cleistothecia.

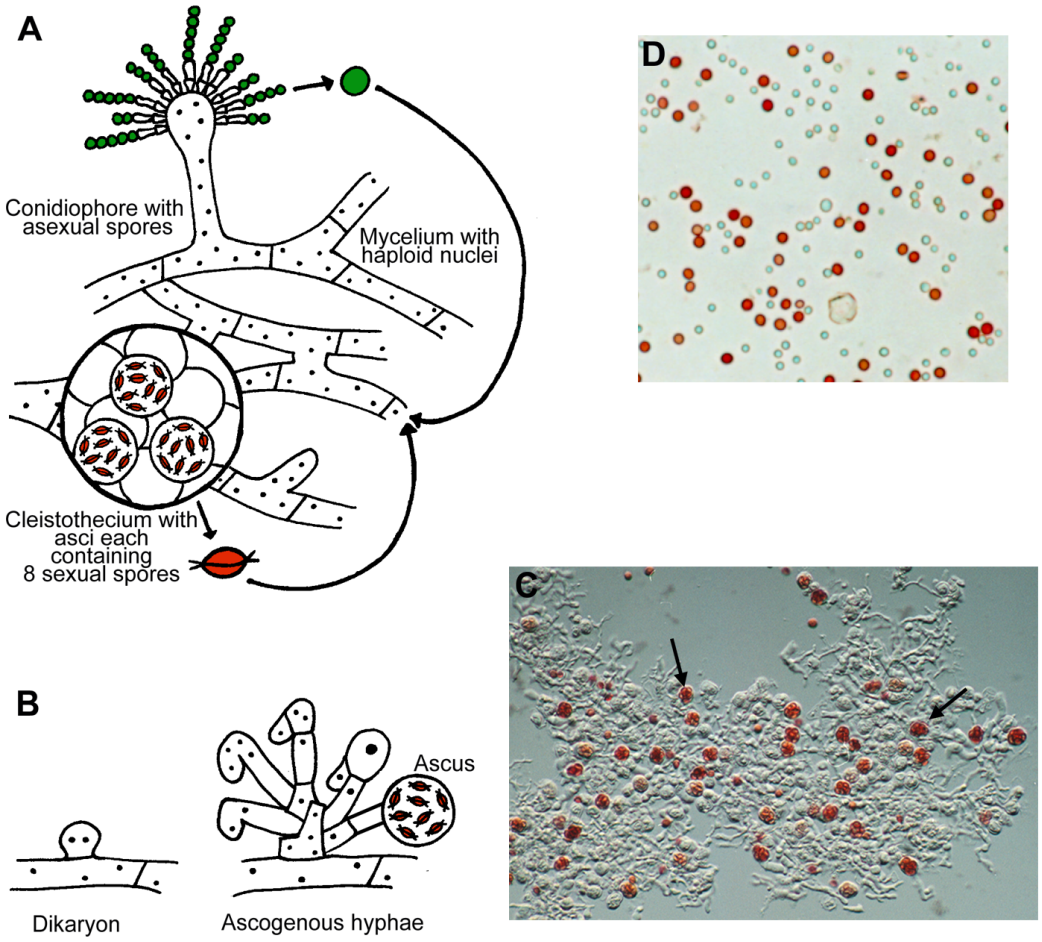


Figure 1.1 **The fungus *Aspergillus nidulans*.** (A) Lifecycle showing the asexual and sexual reproduction route. Asexual spore (conidiospore) production involves the development of a multinucleate footcell into a conidiophore which produces up to 10,000 asexual spores by mitotic divisions. The result of sexual reproduction is a spherical fruiting bodies, called cleistothecium, filled with up to 100,000 sexual spores, packed in 8-spored asci. At 37°C, asexual spore formation takes approximately 25 hours, while sexual spore formation takes 100-150 hours. In principle, each nucleus and cytoplasm from the mycelium can give rise to asexual and sexual spores. Both an asexual and a sexual spore can develop into a new mycelial network. (B) Sexual spore (ascospore) formation starts with fertilisation (selfing or outcrossing), dikaryon formation and proliferation of the dikaryotic tissue. This dikaryotic tissue differentiates into ascogenous hyphae, in which each of the 8-spored asci results from diploid formation of the same two nuclei. These diploid cells, the zygotes, directly undergo meiosis, followed by post-meiotic divisions. (C) Ascogenous hyphae with ripe asci (arrow). (D) The red spores are the sexual spores, the colourless spores are the asexual spores. Although the asexual spores contain a green pigment, this is not visible under the microscope.

One such fruiting body contains enough progeny for a complete genetic analysis. Furthermore, as this is a homothallic fungus, it can reproduce sexually by means of self-fertilisation, but outcrossing is also possible. Both the asexual and sexual generation times are short, respectively 25 and 100-150 hrs at 37°C. Spores can be preserved for a very long time on silicagel, or in glycerol/pepton at -80°C. The genotype is always expressed in the phenotype because the vegetative mycelium normally contains haploid nuclei. Both nuclear and mitochondrial mutations are easily isolated.

We will now explain in what way *A. nidulans* fulfils the first three of the four conditions of the selection arena hypothesis. A full-grown colony of *A. nidulans* carries many fruiting bodies. These fruiting bodies all start as a single dikaryotic cell in which two haploid nuclei are brought together upon fertilisation. Hence, the formation of such dikaryotic fruit initials, hereafter referred to as dikaryons, seems rather cheap in this fungus (condition 1). The pair of nuclei in the initial dikaryon proliferates into an extensive dikaryotic tissue by repeated synchronous mitotic divisions. Eventually, karyogamy in these dikaryotic cells gives rise to the thousands of identical actual zygotes. Each of these diploid zygotes subsequently goes through meiosis and a post-meiotic mitosis to form eight sexual spores that together fill up a fruiting body. This enormous proliferation is fuelled by stored glucans in the mycelium which have been produced early in development from easily accessible C-sources (Zonneveld, 1972). So, parental energy is invested in the proliferating dikaryons (condition 2). The dikaryons formed on an *A. nidulans* colony are often the result of selfing as this is a homothallic fungus, but outcrossing is also possible and does occur in natural populations (Geiser *et al.*, 1994). Even among the self-fertilized dikaryons variation may be present for the following reason: In principle all nuclei in a mycelium can end up in spores, as no special germ line exists. These nuclei can have differential fitness expectations as some nuclei carry novel mutations that have arisen spontaneously; other nuclei may be acquired from anastomosed mycelia. The cytoplasm can be heteroplasmic and can carry viruses, plasmids and abnormal mitochondria. Hence, potential offspring vary in fitness and it may therefore be adaptive to select which nuclei and cytoplasm from this coenocytic mycelium will start a new generation (condition 3). To conclude, a selection arena mechanism in *A. nidulans*

might consist of *overproduction* of dikaryotic fruit initials and *selection* on which of these will thrive to produce the thousands of zygotes and ultimately the sexual spores.

A selection arena may operate also in the asexual route of *A. nidulans*, but we expect it to be more stringent in the sexual route. First, selection arenas pay off more in a costly route: sexual spores are more expensive than asexual spores, as they are bigger and require a much longer developmental time (Champe and Simon, 1992). Second, the asexual route starts with a multinucleate footcell. Starting with a multinucleate footcell makes selection less effective than starting with a dikaryotic cell, by which the sexual route starts. In addition, the proliferation per initial in the sexual route is much more elaborate than the proliferation per initial in the asexual route. Finally, potential benefits of such offspring quality control are much larger in the sexual cycle where variation can be generated by recombination.

Outline of this thesis

In this thesis I will provide evidence for the operation of a selection arena in *A. nidulans* by showing that the predicted result of the selection arena hypothesis is fulfilled, namely that dikaryons with a low future fitness fail in proliferating into a ripe fruiting body, while dikaryons with a high future fitness succeed in it. We also speculate on how the fitness among dikaryons can be identified in this arena (condition 4 of the selection arena hypothesis).

Although the result of sexual reproduction in *A. nidulans* is a fruiting body filled with ascospores, the initiation of this fruiting body and especially the role of the nuclei and mitochondria from the two parental strains in its formation have remained unclear. Therefore, in **chapter 2** we report on experiments revealing these roles. We analyse the genetic constituents of cleistothecia from crosses between vegetatively compatible and incompatible parents and use genetic markers that enable us to determine the nuclear genotype of the cleistothecial wall, and the nuclear and mitochondrial genotype of the ascospores.

In the **chapters 3 and 4** we test the selection arena hypothesis in *A. nidulans* with emphasis on the predicted outcome, namely that dikaryons with a low future fitness fail in

proliferating into a ripe fruiting body, while dikaryons with a high future fitness succeed in it. We reformulate this into a testable prediction for this mycelial fungus: Nuclei with deleterious mutations will not be transmitted via the sexual route, or at least less easily than via the asexual route. A few auxotrophic mutations have been known to confer sexual self-sterility as pleiotropic effect under conditions of normal asexual sporulation. This self-sterility manifests itself in very tiny, empty cleistothecia. Such observation confirms our prediction. Therefore, in **chapter 3** we analyse 2 mitochondrial and 15 auxotrophic mutations for consequences on sexual and asexual reproduction. Each strain carrying an auxotrophic mutation is grown on a range of increasing supplementation for the required nutrient. These supplementations range from levels at which only mycelial growth is present to levels at which asexual and possibly sexual sporulation is abundant. We then examine the developmental state of the cleistothecia and count the number of asexual and sexual spores produced. If sexual self-sterility is the result of the selection arena, we expect this pleiotropic effect to be common for many mutations.

In **chapter 4** we take a different approach to test if nuclei with deleterious mutations will be transmitted less easily via the sexual route than via the asexual route. We now exploit the intriguing stable coexistence of asexual and sexual reproduction in *A. nidulans*. In the case of sexual self-fertilisation, offspring from the sexual and asexual pathway have the same genotype: in the sexual route, two identical haploid nuclei fuse to a diploid meiocyte only to produce the meiotic products that are genetically identical to the parental nuclei and to the asexually produced spores. We start a mutation accumulation experiment in 40 asexual and 40 sexual selfing lines. All lines will be derived from a single ancestral strain of *A. nidulans*. In these lines mutations are allowed to accumulate during 40 generations by single spore transfer. We thus create lines with each having a history of 40 successive bottlenecks of a single conidiospore or ascospore respectively. If nuclei with deleterious mutations are less easily transmitted via the sexual route than via the asexual route, we expect mutations to accumulate slower in the sexual route than in the asexual route. This will result in a higher fitness of the sexual mutation accumulation lines than of the asexual mutation accumulation lines. We use mycelial growth rate as fitness estimate in this study.

In **chapter 5** we compare mycelial growth rate with the alternative fitness estimate of spore production under competitive conditions. We encounter and discuss difficulties of fitness estimation in *A. nidulans* and in modular organisms in general, especially when they produce progeny (spores) via both a sexual and an asexual pathway.

In **chapter 6** we summarize the findings from this thesis and set out our view of how fitness differences among dikaryons can be identified in *A. nidulans*, which is the fourth condition of the selection arena hypothesis.

2

Male and female roles in crosses of *Aspergillus nidulans* as revealed by vegetatively incompatible parents

J. Bruggeman, A. J. M. Debets, K. Swart and R. F. Hoekstra, 2003.
Fungal Genetics and Biology 39, 136-141.

Abstract

To resolve the role of male and female nuclei and mitochondria in cleistothecium formation in the model organism *Aspergillus nidulans,* we analysed the genetic constituents of cleistothecia from crosses between vegetatively compatible and incompatible parents. We used markers that enabled us to determine the nuclear genotype of the cleistothecial wall and the nuclear and mitochondrial genotype of the ascospores. In compatible parents, nuclear genomes and cytoplasm usually mix in the vegetative hyphae prior to the formation of the sexual stage after which any cleistothecial composition is possible. In incompatible parents, the maternal strain contributes the nuclei for the cleistothecial wall and one nucleus as well as mitochondria for the ascospore origin. The paternal strain donates one nucleus for the ascospore origin. Only in crosses between vegetatively incompatible partners, it is possible to assign a female and male role to the parental strains. Our results confirm that the vegetative heterokaryotic stage is not a prerequisite for cleistothecium formation. Using this tool, we analysed sexual sporulation mutants for male or female sterility.

Introduction

It is of fundamental importance to know the female and male roles in sexual reproduction, as prerequisite to study evolutionary consequences such as genetic conflicts between the sexes, sexually antagonistic coevolution, genomic imprinting and timing of initial expression of female and male derived genes (Chapman and Partridge, 1996; Reik and Walter, 2001; Rice, 1996; Vielle-Calzada *et al.*, 2000).

In the homothallic filamentous ascomycete *Aspergillus nidulans* sexual reproduction results in the formation of fruiting bodies, or cleistothecia, each filled with thousands of 8-spored asci. The initiation of a fruiting body and especially - in the case of outcrossing - the role of the nuclei and mitochondria from the two parental strains in its formation remain unclear. Hence, in this genetically thoroughly explored model organism a tool to recognise the male and female strain in a cross is still lacking. Without such a tool, the study of the mentioned evolutionary consequences of male and female roles is severely hampered. Furthermore, without such a tool, mutations that confer self-sterility, *i.e.*, meiotic mutants, cannot be investigated for their effects on male or female fertility. Analysis of male and female roles is also relevant for investigating hybrid dysgenesis of transposons in *A. nidulans* (Li Destri Nicosia *et al.*, 2001), a phenomenon in which transposition after fertilisation depends on whether the male or female carries the transposable element. Knowing the exact contribution of the nuclei and mitochondria from the two parental strains in fruitbody formation is also important for unravelling the possible function of vegetative incompatibility in sexual crosses in this fungus.

The initial step in sexual reproduction in ascomycetes is the formation of a dikaryon: a cell in which two nuclei are brought together and share a common cytoplasm (Alexopoulos, 1962; Burnett, 1976). This process has never been observed in *A. nidulans* as no differentiated female and male (or recipient and donor) structures have been found. The dikaryon develops into ascogenous hyphae and these develop into asci, in which finally meiosis and an additional mitosis yield eight bi-nucleate haploid sexual spores (Elliott, 1960). All ascospores in a cleistothecium are either of crossed or of selfed origin (Pontecorvo, 1953), but rare exceptions exist (Hoffmann *et al.*, 2001). In addition, all the ascospores carry the same mitochondrial type and this is true in crosses between

vegetatively compatible and incompatible strains (Coenen *et al.*, 1996; Rowlands and Turner, 1976). The parent contributing the mitochondria is assigned the female role in analogy to the inheritance in other organisms. Most likely, two nuclei initiate the wall of the cleistothecium and these are not necessarily clonally related to those forming the ascogenous hyphae (Zonneveld, 1988). This was revealed using ascospore colour mutants. Earlier, these colour mutants were isolated and characterised by Apirion (1963). All the ascospores in a cleistothecium, regardless of their genotype, as well as the cleistothecial wall have the same colour, and the pair of nuclei of the cleistothecial wall determines this colour. Maternal inheritance has been suggested for the nuclei in the wall, but this was not firmly proven (Apirion, 1963; Rowlands and Turner, 1976).

Although a fragmentary image of the contribution of the nuclei and mitochondria from the two parental strains in cleistothecia formation arises from these studies, a complete picture is still lacking. To definitely reveal the role of male and female nuclei and mitochondria in cleistothecium formation, we analysed crosses between vegetatively compatible and incompatible strains, using markers that enabled us to determine in each cross the nuclear genotype of the cleistothecial wall and the nuclear and mitochondrial genotype of the ascospores. We show that in fruitbody formation one strain provides a nucleus with mitochondria (and thus cytoplasm) for the ascospore origin as well as the nuclei for the wall origin. This strain is assigned the maternal role. The other strain only donates one nucleus for the ascospore origin and thus fulfils the paternal role. This can only be investigated in vegetatively incompatible combinations. We used this information to investigate sexual sporulation mutants, which have recently been obtained and analysed (Swart *et al.*, 2001), for male or female sterility. Finally, we discuss our findings in relation to vegetative incompatibility, nuclear parasitism, and the usefulness of the mitochondrion as marker for the female parent in the fertilisation event.

Table 2.1

Strains of *A. nidulans* used in this study

Strain	Genotype
WG400	*clB6, anA1, adG14, pabaA1, yA2*
WG565	*wA2, blA1, adD3*
WG566	*pabaA1; wA2, blA1*
WG567	*yA2; clA4, methG1*
WG568	*yA2, biA1; clA4*
WG573	*wA2, blA1, adD3; mt camA112*
WG574	*pabaA1; wA2, blA1; mt camA112*
WG577	*yA2; clA4, methG1; mt camA112*
WG580	*yA2, biA1; clA4; mt camA112*
JC705/06	*pabaA1; wA2, blA1; nia; mt camA112*
JC705/07	*pabaA1; wA2, blA1; nia*
JC705/08	*wA2, blA1, adD3; cnx*
WG591	*fwA1; meiG12*
WG597	*fwA1; meiL18*
WG598	*fwA1; meiM19*
WG601	*fwA1; meiR23*

Materials and methods

Strains

The *A. nidulans* strains used in this study are shown in Table 2.1. The WG strains are Glasgow stock strains and belong to the heterokaryon compatibility group Glasgow (h-cGl). The JC strains belong to h-cB and are constructed for this study from crosses between the wild-type strain JC705 that belongs to h-cB (from the Birmingham collection of Dr. Jim Croft) and Glasgow strains. All strains carry appropriate markers to analyse the genetic origin of cleistothecia: fawn (*fwA1*), white (*wA3*) and yellow (*yA2*) are conidial colour markers, *wA3* and *yA2* can complement into the wild-type green colour to assess the genetic origin of the ascospores; blue (*blA1*) and colourless (*clA4, clB6*) are cleistothecial wall and non-autonomous ascospore colour markers, whereby blue and colourless complement each other resulting in wild-type red colour (Apirion, 1963); chloramphenicol resistance (*camA112*) is a mitochondrial marker. *Mei* are sexual sporu-

lation mutants. Other markers are also present, but dispensable for cleistothecium analysis (nomenclature can be found at http://www.fgsc.net/ of the Fungal Genetics Stock Centre).

Media and growth conditions

Complete Medium (CM) was essentially as described by Pontecorvo (1953) with per litre: 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 1 mg of each: $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$, 2 g neopepton, 1 g Casamino acids & yeast extract, 0.3 g ribonucleotides and 2 ml vitamin solution (0.1% riboflavin and nicotinic acid, 0.01% of each: thiamine, p-aminobenzoic acid and pantothenate, 0.05% pyridoxine, 0.0002% biotin), 15 g agar and 1% glucose, pH 5.8. Very Minimal Medium (VMM) contains per litre 0.5 g $NaNO_3$, 1 g KH_2PO_4/ K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.1 g KCl, 1 mg of each: $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$, 15 g agar and 1% glucose, pH 5.8. VMM was supplemented with the requirements to enable growth of auxotrophic strains: 0.1 mM adenine for the *ad* marker, 10 µM p-aminobenzoic acid for *paba*, 0.1 µM thiamine for *an*, 1mM methionine for *meth*, 0.015 µM biotin for *bi*, 10 mM urea for the nia and cnx marker. Chloramphenicol (CAM) medium contained 4g/L chloramphenicol. All cultures were grown at 37°C. Crosses were performed on supplemented VMM on which cleistothecium formation is fast (within seven days) and abundant.

Crosses

Conidiospores of parental strains were streaked crosswise on plates. Heterokaryon formation was not forced on these plates. To reveal the role of male and female nuclei and mitochondria in cleistothecium formation, we made incompatible and compatible crosses between strains of several genotypes to avoid genotype specific outcomes (Table 2.2).

To investigate sexual sporulation mutants for male or female sterility, we crossed four meiotic mutant strains to strain JC705/06. These combinations are all vegetatively incompatible.

Scoring of cleistothecia

From the crosses in Table 2.2, ripe cleistothecia were collected from well mixed regions on the plates and cleaned from adhering conidiospores and Hülle cells by rolling them over a 3% water agar plate after which they were crushed with a needle in 300 μL water in an Eppendorf tube. The cleistothecial wall and ascospore colour was then scored using a (dissecting) microscope. An aliquot of the ascospore suspension was spread and grown on a CM plate to assess the genetic origin of the ascospores based on the expressed conidiospore colour. A second aliquot was spread on CM + CAM to test for the presence or absence of CAM resistant mitochondria in the ascospores.

We analysed the crosses in which the meiotic mutants were involved as follows. Instead of investigating the cleistothecia individually - which is rather time consuming - we scraped cleistothecia from well mixed parental regions and put them in 10 ml 0.8% NaCl + 0.005% Tween. These harvested cleistothecia were vigorously shaken together with 1 millimetre ∅ glass beads on a GRIFFIN flask shaker for 15 minutes to break up the cleistothecia. Dilutions of these suspensions were plated and grown on MM. Subsequently, recombinant ascospores, recognised from their green colony colour, were investigated for carrying wild-type or CAM mitochondria. In this way, we established the maternal and paternal parent of this recombinant.

Results and discussion

In order to establish the maternal and paternal role in cleistothecium formation in *A. nidulans*, we made crosses between vegetatively compatible and incompatible strains and analysed the cleistothecia. We first determined the colour of each cleistothecium or of its ascospores as this revealed the nuclear origin of the wall. Colourless cleistothecia were easily recognised (Fig. 2.1A). Wild-type red and mutant-blue coloured cleistothecia could only be distinguished using a (dissecting) microscope by examining the colours of the ascospores (Fig. 2.1B and 2.1C). Next, by growing them and checking the expressed conidiospore colour we assessed the genetic origin of the ascospores (Fig 2.1D).

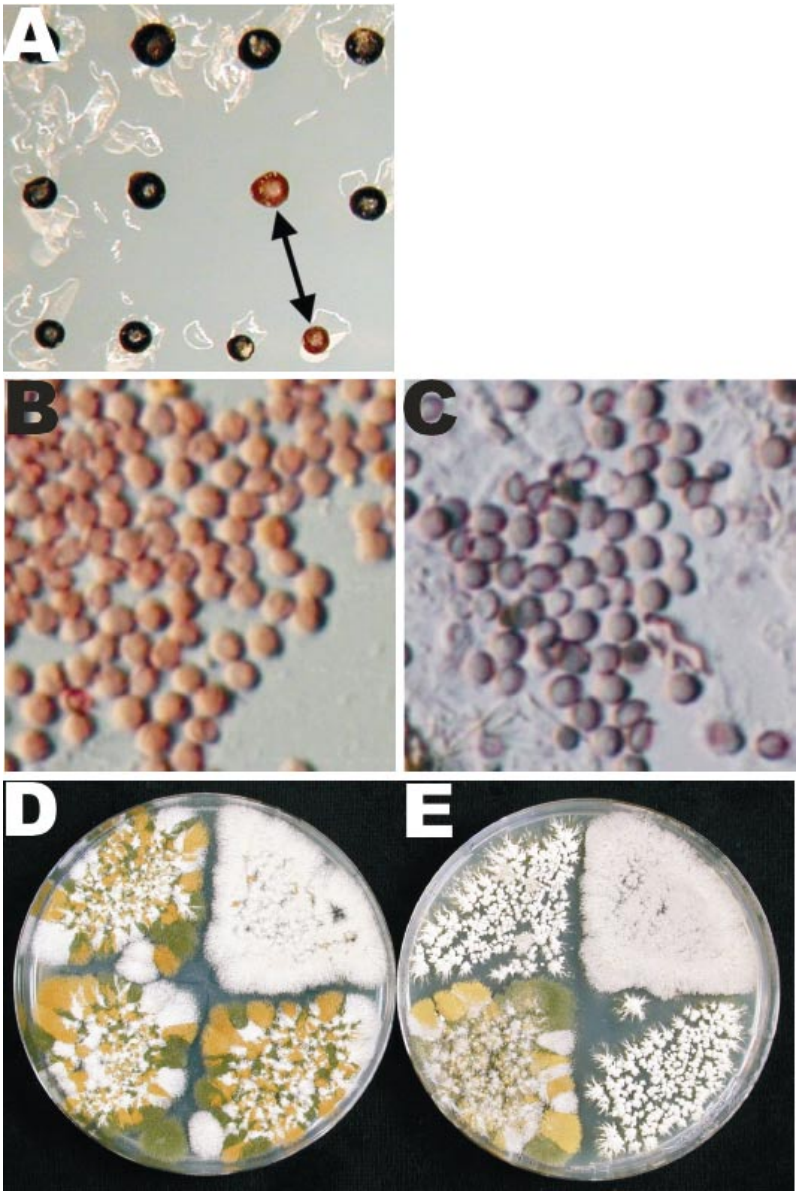


Figure 2.1 | **Cleistothecia from cross WG574 and WG400 are analysed for their genetic constituent.** (A) Colourless cleistothecia (arrow) are easily recognised and indicate that the nuclear genotype of the cleistothecial wall is of homokaryotic origin of strain WG400. Wild-type red ascospores (B) indicate heterokaryotic origin. Blue ascospores (C) indicate homokaryotic wall origin of strain WG574. (D) Ascospore suspensions were spread and grown on a CM plate to assess their genetic origin based on the expressed conidiospore colour. Three cleistothecia are of crossed origin; one cleistothecium is of parental type WG574 (upper right quadrant). (E) Aliquots are spread on medium with CAM to reveal the mitochondria transmitted in the cleistothecium. CAM resistant ascospores, which derived their mitochondria from WG574, grow and sporulate normally on these plates (upper right and lower left quadrants), while CAM sensitive ascospores, which derived their mitochondria from WG400, grow and sporulate poorly (upper left and lower right quadrants).

A second aliquot was spread on medium with chloramphenicol to test for the presence or absence of CAM resistant mitochondria in the ascospores (Fig. 2.1E).

After having determined these characters, we assigned each cleistothecium to a class based on its genetic constituents: The genetic origin of the wall nuclei can be homokaryotic from either parent (colourless or blue ascospores) or heterokaryotic (red ascospores); the genetic origin of the ascospore nuclei can be selfed from either parent (giving progeny with yellow or white conidiospore colour) or crossed (giving yellow, white and green progeny); and finally the genetic origin of the ascospore mitochondria can be from either parent (CAM sensitive or resistant ascospores). Therefore, 18 different types are theoretically possible. Each type has a mirror type based on the actual contribution of the two parental strains and when we combine those pairs nine classes of cleistothecia result (Table 2.2).

All nine classes were found in the crosses between compatible parents, whereas only two classes were found in the crosses between incompatible parents (Table 2.2). The seven classes (II and IV-IX) found only in the compatible combinations resulted from rearrangements of nuclei and mitochondria at the vegetative level. Following anastomosis, no association remains between parental nuclei and mitochondria and any cleistothecial composition is possible. Compelling evidence for this could be found in the following classes: In the classes V-VII the cleistothecial wall was heterokaryotic; in the classes II, IV and VIII the parent forming the cleistothecial wall did not donate the mitochondria; and in class VIII the female tissue of the cleistothecial wall was nursing ascospores of nuclear and mitochondrial genotype from the other parental strain.

In the incompatible crosses only two classes were found: class I and III. Class I comprised cleistothecia formed after sexual selfing of either parent. Class III comprised cleistothecia formed after sexual outcrossing between the two parental strains. Important to notice is that in this class the cleistothecial wall could be of either parent and thus was always homokaryotic. Furthermore, the parent forming this structure also always donated the mitochondria and one of the nuclei for the ascospore origin. This identifies the maternal contribution. The other strain only donates a nucleus for fertilisation and hence contributes to the origin of the ascospores, thereby fulfilling the paternal role. We firmly

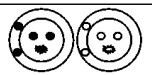
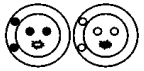
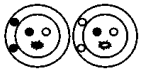
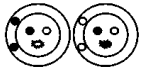
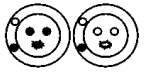
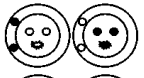
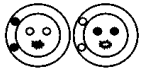
proved the homokaryotic origin of the cleistothecial wall due to our use of the colourless and blue cleistothecial wall markers. We never found a red cleistothecium among the 382 cleistothecia analysed, which would have indicated heterokaryotic origin of the cleistothecial wall. Earlier, homokaryotic origin of cleistothecial wall was assumed by Rowlands and Turner (1976), but not fully proven. They used the dominant wild-type red marker in combination with the mutant-blue marker, resulting in either red or blue cleistothecia and ascospores. The red cleistothecia and ascospores could have been the result of heterokaryotic mycelium that originated from the combination of a red and a blue marker (Apirion, 1963).

From our results, we learn that the male and female role in cleistothecium formation can only be revealed in crosses between vegetatively incompatible strains and that in these cases mitochondrial inheritance indicates the maternal parent. This information provides us with a tool to specify the maternal and paternal strain in a cross. We applied this tool to investigate several sexual sporulation mutants which have been recently obtained and analysed (Swart *et al.*, 2001). These mutants produce cleistothecia without ripe ascospores inside, as spore production is disturbed due to mutations in meiotic genes. All these mutations are recessive and therefore outcrossing does result in ripe ascospores. Earlier, in the fungus *Neurospora crassa* recessive mutants defective in the production of viable ascospores were isolated and some of these were female sterile (DeLange and Griffiths, 1980). In this study we tested some of the sexual sporulation mutants in *A. nidulans* for female or male sterility, by crossing them to a vegetatively incompatible partner carrying CAM resistant mitochondria. We found that the meiotic mutants WG591, WG597, WG598 and WG601 all could serve both as female and male, indicating no specific female or male sterility for these mutations.

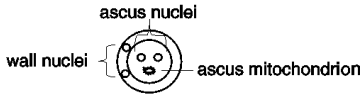
In combinations of compatible strains, mitochondrial inheritance is not any longer diagnostic of the maternal parent: nuclear genomes and cytoplasm often will mix in the vegetative hyphae prior to the formation of the sexual stage. Li Destri Nicosia *et al.* (2001) investigated hybrid dysgenesis in *A. nidulans*, a phenomenon in which transposition after fertilisation depends on whether the male or female carries the transposable element. Based on mitochondrial inheritance in compatible crosses, the

Table 2.2

Number of cleistothecia in the incompatible and compatible crosses and their assignment to the various classes

Class	Cleistothecia[a]	Incompatible crosses					Compatible crosses						Class explanation
		JC705/07 X WG580	JC705/06 X WG568	JC706/06 X WG400	JC705/08 X WG577	Sum (%)	WG565 X WG577	WG573 X WG567	WG566 X WG580	WG574 X WG400	WG573 X WG400	Sum (%)	
I		46 4[b]	70 58	82 6	64 30	360 (94%)	2 3	19 0	8 12	56 0	13 1	114 (37%)	selfed cleistothecia
II		0 0	0 0	0 0	0 0	-	0 0	11 0	0 0	14 0	0 0	25 (8%)	selfed cleistothecia; mitochondria of opposite partner
III		1 0	15 0	1 0	3 2	22 (6%)	28 0	9 0	0 0	10 0	42 0	89 (29%)	crossed cleistothecia
IV		0 0	0 0	0 0	0 0	-	8 0	3 0	3 0	11 0	20 0	45 (15%)	crossed cleistothecia; mitochondria not corresponding to nuclei in wall
V		0 0	0 0	0 0	0 0	-	0 0	0 0	0 0	2 1	0 1	4 (1%)	heterokaryotic wall; selfed ascospore origin
VI		0 0	0 0	0 0	0 0	-	0 0	0 0	0 0	3 0	0 0	3 (1%)	heterokaryotic wall; selfed ascospore origin; mitochondria of opposite partner
VII		0 0	0 0	0 0	0 0	-	0 0	0 0	0 0	7 8	1 0	16 (5%)	heterokaryotic wall; crossed ascospore origin
VIII		0 0	0 0	0 0	0 0	-	1 0	2 0	7 0	0 0	0 0	10 (3%)	selfed cleistothecia; wall nuclei of opposite partner
IX		0 0	0 0	0 0	0 0	-	3 0	0 0	1 0	0 0	0 0	4 (1%)	selfed cleistothecia; wall nuclei of opposite partner; mitochondria corresponding to nuclei in wall
						382 (100%)						310 (100%)	

[a] Each class contains two kinds of cleistothecia, referring to the actual contributions of the two parents.
Black filled symbols refer to a contribution from the first parent named in the cross and the unfilled symbols refer tot the second parent.



[b] The first number refers to the left cleistothecium in the drawing, the second number to the right one in the drawing.

authors concluded that hybrid dysgenesis was absent. However, we assume that their result exactly mirrors the mixing of mitochondria and nuclei in the mycelium of vegetatively compatible parents before sexual fertilisation. After mixing, no association remains between transposon carrying nuclei and their mitochondria, which makes it impossible to analyse hybrid dysgenesis in such a system.

Any cleistothecial composition is possible between vegetatively compatible partners, whereas only two classes of cleistothecia are possible between incompatible partners. This finding sheds interesting light on the function of vegetative incompatibility in regulating sexual reproduction in this fungus. Vegetative incompatibility in fungi is widespread and several biological functions have been proposed, mainly concerning prevention of horizontal transfer of cytoplasmic elements (Hoekstra, 2001). We observed that vegetative incompatibility prevented nuclear parasitism, as was proposed earlier for the fungus *Neurospora crassa* (Debets and Griffiths, 1998). Incompatibility ensures that the female tissue - *i.e.* the cleistothecial wall - and hence the female resources are used for a maternal nucleus of the same genotype and for a paternal nucleus. In vegetatively compatible partners, there is ample opportunity for nuclear parasitism. This is most compelling in class VIII in which the female tissue of the cleistothecial wall is nursing ascospores of exclusively paternal nuclear and mitochondrial genotype. That vegetative incompatibility is indeed functional in regulating sexual crosses is very clear in a heterothallic fungus like *N. crassa,* where the mating type locus also acts as a vegetative incompatibility locus (Newmeyer *et al.*, 1973).

Our data indicate that outcrossing between incompatible strains is a rather rare event: only 6% (22/382) of the cleistothecia are crossed. This is in agreement with an earlier study. Butcher (1968) made crosses between 17 incompatible wild-type strains. Hybrid cleistothecia were present in several strain combinations, but totally absent in other combinations. The mean crossing potential for these strains was 4%. Contrary to these findings, a study by Hoffmann *et al.* (2001) reports a cross between two incompatible strains that resulted only in outcrossed cleistothecia. However, these cleistothecia developed on forced heterokaryons, and one of the parents carried the genetic markers *pyrG89* and *pyroA*4 conferring it sexually self-sterile (own observations). In our

compatible crosses, 52% (160/310) of the cleistothecia are crossed. This is close to the expected percentage if nuclear recruitment for ascospore origin is a random process, which apparently is true in compatible strain combinations. However, when this percentage is calculated for individual crosses, it varies from 10% up to 80%. This variation is likely due to the markers used in the various crosses affecting the sexual cycle. For example, many crosses in our experiment lack various classes of cleistothecia (Table 2.2). In addition, strains carrying the *cl* allele (always the second strain named in each cross) seldom acted as female. This was the reason for us to use several strain combinations in both the incompatible and compatible crosses and pool the results to avoid strain specific outcomes. We propose that the phenomenon of 'relative heterothallism', first observed by Pontecorvo *et al.* (1953), is mainly due to the auxotrophic markers used in the crosses.

Our results confirm that the vegetative heterokaryotic stage is not a prerequisite for sexual crosses (Jinks *et al.*, 1966), and that fusion leading to sexual outcrossing - *i.e.* fertilisation - and anastomosis leading to vegetative hyphal fusion must be of two distinct types. This indicates the involvement of specialised structures in the fertilisation process in *A. nidulans*. For other *Aspergillus* species reproductive structures have been described (Benjamin, 1955), for example a coiled ascogonium and an antheridium in the *Aspergillus glaucus* group. In general, the fertilisation in this type of ascomycete involves female ascogonia and male antheridia: a single nucleus being introduced into the ascogonium from the antheridium (Alexopoulos, 1962). Although the morphology of the fertilisation process remains unknown, our results suggest a process similar to that in *Neurospora crassa* (Raju, 1980). We unravelled the role of male and female nuclei and mitochondria in cleistothecium formation, and our data indicate that vegetative incompatibility in *A. nidulans* is functional in preventing nuclear parasitism.

Acknowledgments

We thank Marijke Slakhorst for technical assistance. This work was supported by a grant from the Netherlands Organization for Scientific Research (NWO) to J.B., 805-36-355.

3

Selection arena in *Aspergillus nidulans*

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Accepted for publication in Fungal Genetics and Biology.

Abstract

The selection arena hypothesis states that overproduction of zygotes -a widespread phenomenon in animals and plants- can be explained as a mechanism of progeny choice. As a similar mechanism, the ascomycetous fungus *Aspergillus nidulans* may overproduce dikaryotic fruit initials, hereafter called dikaryons. Then, progeny choice might involve selection on which of these dikaryons will thrive to produce thousands of zygotes. These zygotes each produce eight sexual spores which together fill up one fruiting body. In this study, we test the selection arena hypothesis in this homothallic fungus that produces both sexual and asexual spores. We analysed 2 mitochondrial and 15 auxotrophic mutations for consequences on sexual and asexual reproduction. We found that many of these mutations confer sexual self-sterility as pleiotropic effect under conditions of normal asexual spore production. This confirms an important prediction of the selection arena, namely that dikaryons carrying a (slightly) deleterious mutation are not able to proliferate and produce sexual spores. The selection arena ensures that reproductive energy is invested mainly in dikaryons and thus sexual spores of good genetic quality.

Introduction

Many organisms produce more fertilised zygotes than they ever support in growth. As a consequence, only a subset of these zygotes will develop into new offspring. This seems to be in contradiction with the expected strong natural selection for juvenile survival. Nevertheless, the selection arena hypothesis – also called progeny choice hypothesis - can explain these observations (Stearns, 1987). It states that overproduction of zygotes could be explained as part of a quality control mechanism which operates in the following manner: An enlarged array of zygotes is created of which only a genetically superior subset will fully develop; zygotes with a low future fitness fail, while zygotes with a high future fitness thrive. In this way, parental energy for reproduction is invested in the most promising zygotes. Essential in the hypothesis is that the potential members of the next generation are tested at a crucial moment, namely just before substantial parental investments start. This hypothesis further assumes that 1) zygotes are cheap to produce, 2) parental time, energy and/or risk are invested in the zygotes, 3) offspring vary in fitness, and 4) this fitness difference can be identified. Natural selection will favour such a selection arena only if the benefits of high-quality progeny outweigh the cost of overproduction. Especially if the initial cost of a zygote is low relative to the cost of an independent offspring, zygote overproduction will skyrocket (Kozlowski and Stearns, 1989). Selection pressure for overproduction of zygotes is less affected by the amount of genetic variation among the initial zygotes (Kozlowski and Stearns, 1989).

Several studies discuss the occurrence of spontaneous abortion in relation to the selection arena theory. For example, selective embryo abortion is suspected in mammals. Mouse and man appear to prefer conceptuses incompatible with their own MHC (major histocompatibility complex) antigens (Bolis *et al.*, 1985; Wedekind, 1994). This has been extensively investigated in Hutterite woman, who experience increased fetal losses when married to men with similar HLA (human leucocyte antigens) alleles (Ober *et al.*, 1992; Ober, 1999). In coypu there seems to be selective abortion of entire litters with respect to quality and sex (Gosling, 1986). The botanical literature includes many examples where plant species produce far more ovules than they ever develop into seed (Stephenson, 1981). Some studies relate these observations to the selection arena theory by trying to

demonstrate that aborted ovules are potentially viable but would produce less vigorous progeny than non-aborted ovules would produce (Casper, 1988; Melser and Klinkhamer, 2001; Rocha and Stephenson, 1991; Stephenson and Winsor, 1986). However, plausible and appealing as the idea of the selection arena may be, it has been difficult to actually demonstrate a mechanism of selection against low quality zygotes in favour of the production of higher quality ones. This is partly due to the complexity of the studied organisms and their life history traits.

We propose that fungi are highly suited to study the selection arena hypothesis. The haploid filamentous ascomycete *Aspergillus nidulans* -a well-known genetic model organism- fulfils the first three of the four abovementioned conditions of the selection arena hypothesis (see also the lifecycle of this fungus in Fig. 1.1, and biology of this fungus in Adams *et al.*, 1998; Bruggeman *et al.*, 2003a; Champe and Simon, 1992; Elliott, 1960; Pontecorvo, 1953; Sohn and Yoon, 2002). A full-grown colony of *A. nidulans* carries many fruiting bodies. These fruiting bodies all start as a single dikaryotic cell in which two haploid nuclei are brought together upon fertilisation. Hence, the formation of such dikaryotic fruit initials, hereafter referred to as dikaryons, seems rather cheap in this fungus (condition 1). The pair of nuclei in the initial dikaryon proliferates into an extensive dikaryotic tissue by repeated synchronous mitotic divisions. Eventually, karyogamy in these dikaryotic cells gives rise to the thousands of identical actual zygotes. Each of these diploid zygotes subsequently goes through meiosis and a post-meiotic mitosis to form eight sexual spores that together fill up a fruiting body. This enormous proliferation is fuelled by stored glucans in the mycelium which have been produced early in development from easily accessible C-sources (Zonneveld, 1972). So, parental energy is invested in the proliferating dikaryons (condition 2). The dikaryons formed on an *A. nidulans* colony are often the result of selfing as this is a homothallic fungus, but outcrossing is also possible and does occur in natural populations (Geiser *et al.*, 1994). Even among the self-fertilized dikaryons variation may be present for the following reason: In principle all nuclei in a mycelium can end up in spores, as no special germ line exists. These nuclei can have differential fitness expectations as some nuclei carry novel mutations that have arisen spontaneously; other nuclei may be acquired from anastomosed

mycelia. The cytoplasm can be heteroplasmic and can carry viruses, plasmids and abnormal mitochondria. In conclusion, potential offspring vary in fitness and it may therefore be adaptive to select which nuclei and cytoplasm from this coenocytic mycelium will start a new generation (condition 3).

Recently, we have shown that slightly deleterious mutations accumulate at a lower rate in the sexual pathway than in the asexual pathway of *A. nidulans*, and argued that recombination is unlikely to have caused this advantage (Bruggeman *et al.*, 2003b). Probably, this resulted from a selection arena mechanism in the sexual route. In this study we will further examine the selection arena hypothesis in *A. nidulans,* with emphasis on the prediction of this theory, namely that zygotes with a low future fitness will fail, while zygotes with a high future fitness will thrive. We will test directly the important prediction of a selection arena in the sexual route that nuclei with deleterious mutations will not be transmitted via the sexual route, or at least less easily than via the asexual route. This direct testing makes *A. nidulans* a highly suitable organism to study the selection arena. We analyse numerous well-characterised mutations of *A. nidulans*, of which most are auxotrophic mutations, for consequences on sexual (by selfing or outcrossing) and asexual reproduction under various growth conditions. We also speculate on how the fitness differences among zygotes can be identified in this fungus. This is the fourth condition of the selection arena hypothesis. In addition, we discuss observations in other fungi that may be explained by the selection arena hypothesis.

Materials and methods

Strains

The mutations that were tested for consequences on sexual and asexual reproduction in *A. nidulans* are listed in Table 3.1, together with the strains that carry these markers. The WG and AN strains are Glasgow stock strains and belong to the heterokaryon compatibility group Glasgow (h-cGl). All these strains contain the *veA1* mutation. The JC strain belongs to h-cB and is $veA1^{+}$. mt means mitochondrial mutation.

Media and growth conditions

Minimal Medium (MM) was essentially as described by Pontecorvo (1953) with per litre: 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 1 mg of each: $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$, 15 g agar and 1% glucose, pH 5.8. All incubations were at 37°C in the dark. For each auxotrophic mutation that we investigated for consequences on sexual and asexual reproduction we prepared MM with a range of increasing supplementation for the required nutrient as shown in Table 3.1.

Analysis of mutations for consequences on sexual and asexual reproduction under various growth conditions

We tested 15 auxotrophic mutations and two mitochondrial mutations for consequences on sexual and asexual reproduction. Each strain carrying an auxotrophic mutation was grown on a range of increasing supplementation for the required nutrient in MM, as depicted in Table 3.1. These supplementations ranged from levels at which only mycelial growth was present to levels at which asexual and possibly sexual sporulation was abundant. We prepared for each treatment three replicate Petri dishes (90 mm diameter) and applied $1x10^4$ spores per Petri dish by spreading. The plates were then incubated for 12 days under dark conditions at 37°C. After incubation, we visually inspected the plates and in particular the developmental state of the cleistothecia. From each plate, we harvested the asexual spores and the cleistothecia in 10 ml 0.8% NaCl + 0.005% Tween using a spreader. These harvested spore suspensions were vigorously shaken together with 1 mm diameter glass beads on a GRIFFIN flask shaker for 15 minutes to break up the conidial chains and the cleistothecia. Subsequently, we counted the number of asexual and sexual spores in these samples using the Coulter counter. This particle counter distinguishes between the asexual and sexual spores, as these differ in size. Appropriate dilutions for the Coulter counter were made in ISOTON II, an azide-free balanced electrolyte solution. Data were analysed with CoulterAccucomp.

Table 3.1

***Aspergillus nidulans* mutations investigated in this study**

	Supplementation level of nutrient for auxotrophic mutation			
Mutation	Range used in this study[a]	FGSC[b]	Strain	Genotype
adD3	0, 0.1, 1, 2, 10 mM adenine	0.5 mM	WG27 JC705/08	*pabaA1, yA2; adD3* *wA2, blA1, adD3; cnx*
argA1	0, 1, 10, 30 mM arginine	4 mM	WG61	*biA1; argA1*
biA1	0, $1x10^{-5}$, $1x10^{-4}$, $2.5x10^{-4}$, $5x10^{-4}$ μM biotin	$8x10^{-2}$ μM	WG3	*biA1; acrA1*
hisB1	0, 0.01, 0.1, 1, 10, 30 mM histidine	4 mM	AN197	*hisB1, pabaA2, yA1*
luA1	0, 0.001, 0.01 mM leucine	4 mM	WG221	*luA1, yA2*
lysB5	0, 0.1, 1, 5, 10 mM lysine	4 mM	WG408	*dcl-1; pabaA1, yA2; lysB5; riboB2*
methG1	0, 0.1, 1, 5, 10 mM methionine	1 mM	WG337	*biA1; methG1*
ornA4	0, 0.001, 0.01, 0.1, 1 mM ornithine	4 mM	WG45	*biA1; ornA4*
pabaA1	0, 0.001, 0.01, 0.1, 1 μM p-aminobenzoic acid	5 μM	WG96	*yA2, pabaA1*
phenA2	0, 0.001, 0.01, 0.1, 1 mM phenylalanine	4 mM	WG30	*phenA2*
proA1	0, 0.01, 0.1, 1, 5 mM proline	4 mM	AN238	*proA1, biA1*
pyrD23	0, 0.01, 0.1, 1, 5, 10 mM uridine	0.2 mM	WG57	*pyrD23*
pyroA4	0, 0.01, 0.1, 1, 10, 100 μM pyridoxine	0.24 μM	WG94	*pabaA1; pyroA4*
riboB2	0, $1x10^{-4}$, 0.001, 0.01, 0.1, 1 mM riboflavin	$2x10^{-4}$ mM	WG553	*pabaA1, biA1; riboB2, chA1*
trpC801	0, 1, 5, 50 mM tryptophan	4 mM	WG292	*yA2, pabaA1; trpC801*
mt *camA112*	NA[c]	NA[c]	WG275	mt *camA112; wA3; pyroA4*
mt *oliA6*	NA[c]	NA[c]	WG273	mt *oliA6; pabaA1, yA2*

[a] These supplementations ranged from levels at which only mycelial growth was present to levels at which asexual and possibly sexual sporulation was abundant.

[b] Recommended supplementation level by the Fungal Genetics Stock Center (http://www.fgsc.net/).

[c] Not applicable.

To show that reproductive energy is directed to the highest quality dikaryons, we made a cross between two heterokaryon incompatible parents carrying respectively the self-sterility marker *adD3* and *riboB2* under low supplementation levels (0.1mM adenine and 0.01mM riboflavin). The use of heterokaryon incompatible strains prevents the formation of heterokaryotic maternal tissue and thus complementation of the deficiencies in that tissue. Complementation is therefore only possible in the dikaryotic tissue (Bruggeman *et al.*, 2003a). As a control, we also grew these strains on individual plates. From the plates with the individual strains as well as from the mixed plates, we picked randomly 13 cleistothecia and counted the numbers of spores inside, using a haemocytometer. In addition, an aliquot of these ascospores was grown to assess their genetic origin based on the expressed conidiospore colour: white or chartreuse conidiospores indicate a self-fertilised fruiting body from either parent; intermingled white and chartreuse conidiospores indicate a crossed fruiting body.

Results and discussion

If an effective selection arena operates during the sexual reproduction route of *A. nidulans*, then nuclei carrying deleterious mutations will not be transmitted via sexual spores, or at least less easily than via asexual spores. To investigate this, we analysed 2 mitochondrial and 15 auxotrophic mutations for consequences on sexual and asexual reproduction under various growth conditions. We found that many of these mutations confer sexual self-sterility as a pleiotropic effect, under conditions when mycelial growth and asexual sporulation are like the wild type (Table 3.2): cleistothecia were tiny and carried almost no ascospores inside. For the auxotrophic mutations, supply of the required nutrients in excess to what is required for normal asexual reproduction often could restore sexual self-fertility. So, sexual self-sterility is not absolute. In general, for completion of each successive step in the developmental sequence: germination → mycelial growth → asexual spore formation → sexual spore formation, the mutant strains required an increasing level of supplementation. This is illustrated with the example of the *riboB2* mutation (Fig. 3.1). Strain WG553 carries this mutation and was grown under increasing supplementation of the required nutrient: 0.001, 0.01, 0.1, and 1.0 mM riboflavin.

Table 3.2

Many mutations confer sexual self-sterility under conditions of normal asexual sporulation

Mutation	Mutation class	Sporulation		Reference
		Asexual	Sexual	
adD3	nucleotide auxotrophy	+	-	this study
argA1	amino acid auxotrophy	+	-	this study
argB12	amino acid auxotrophy	+	-	Serlupi Crescenzi *et al.*, 1983
biA1	vitamin auxotrophy	+	-	this study
hisB1	amino acid auxotrophy	+	-	this study; Busch *et al.*, 2001 Millington Ward *et al.*, 1984;
luA1	amino acid auxotrophy	+	+	this study
lysB5	amino acid auxotrophy	+	-	this study
methG1	amino acid auxotrophy	+	-	this study
ornA4	amino acid auxotrophy	+	+	this study
pabaA1	vitamin auxotrophy	+	-	this study
phenA2	amino acid auxotrophy	+	-	this study
proA1	amino acid auxotrophy	+	+	this study
pyrD23	nucleotide auxotrophy	+	-	this study
pyroA4	vitamin auxotrophy	+	-	this study
riboB2	vitamin auxotrophy	+	-	this study
trpC801	amino acid auxotrophy	+	-	this study; Eckert *et al.*, 1999; Käfer, 1977
yB1	yellow conidiospores	+	-	Kurtz and Champe, 1981
mt *camA112*	resistance	+	+	this study
mt *oliA6*	resistance	+	-	this study; Coenen *et al.*, 1996; Mason and Turner, 1975

Fig. 3.1A and B depict the intact and crushed cleistothecia, respectively. Note the tiny, almost empty cleistothecia under low supplementation and the big, full cleistothecia under high supplementation. In Fig. 3.1C, spore suspensions are depicted as seen under the microscope after we harvested all spores from a full-grown plate. The frequency distributions of spore diameters from these produced spores are depicted in Fig. 3.1D. The left top represents the asexual spore distribution and the right top represents the sexual spore distribution. Fig. 3.1C and D demonstrate that first asexual sporulation is restored, and only later sexual spore formation is restored.

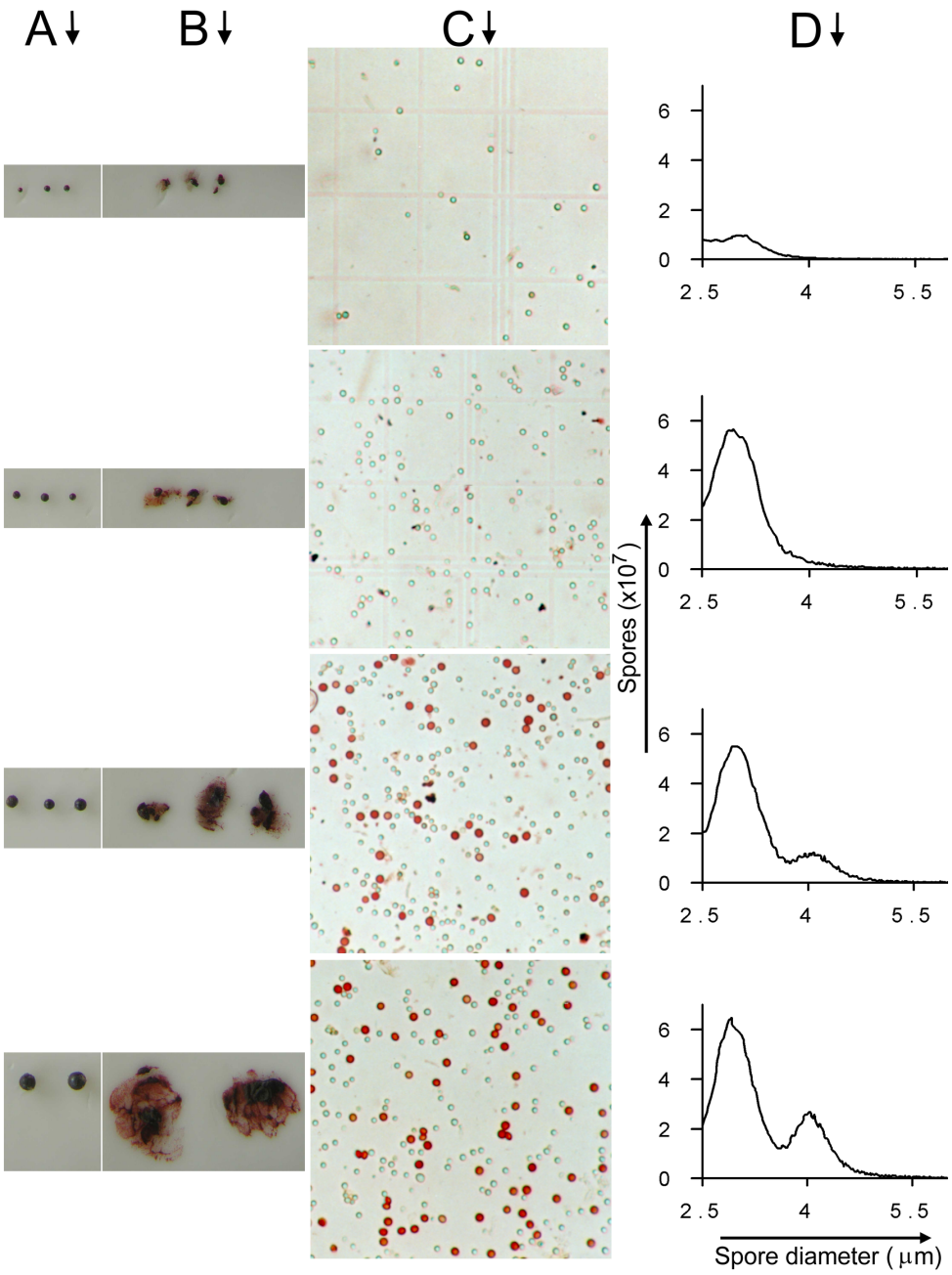

Figure 3.1 | **For completion of the successive steps in development of asexual and sexual sporulation, strains with auxotrophic mutations require increasing amounts of supplementation.** This is illustrated with figures from strain WG553 carrying the self-sterility mutation *riboB2* supplemented with increasing amounts of riboflavin: 0.001, 0.01, 0.1 and 1 mM. (A) Intact and (B) crushed cleistothecia. (C) Spore suspensions in haemocytometer, showing the light-gray asexual spores and the dark-grey sexual spores. (D) The frequency distribution of spore diameter from spores produced on one Petri dish. The left top identifies the distribution of the asexual spores and the right top identifies the distribution of the sexual spores.

Sexual spore production could not be restored in strains with the arginine, histidine, lysine and tryptophan deficiencies, although for *hisB1* and *trpC801*, restoration of spore formation has been reported earlier (Busch *et al.*, 2001; Eckert *et al.*, 1999).

For several mutations in *A. nidulans,* observations on sexual self-sterility have been made earlier in the literature. However, these individual observations were never interpreted as manifestation of a selection arena. In Table 3.2, our data are complemented with these data. The table shows that sexual self-sterility seems a general pleiotropic effect of biochemical mutations: 13 out of the 16 auxotrophic mutations showed differential effect of supplementation on asexual and sexual sporulation.

We suggest the mechanism of the selection arena to be as follows. An *A. nidulans* colony produces numerous initial dikaryons. Each of these dikaryons has the potential to proliferate into an extensive dikaryotic tissue, which eventually gives rise to the zygotes that produce the sexual spores. The selection arena involves selection on which of these dikaryons actually will go through this proliferation: We hypothesize that this proliferation is an autonomous process depending on the quality of the dikaryon. Only if the quality of the dikaryon enables itself sufficient metabolic vigour, the proliferation will be successful. One or more nuclear or cytoplasmic mutations in a dikaryon will negatively affect this quality and hence the proliferation. In this way, the selection arena selects against low quality dikaryons. Such developmental selection in organisms has also been hypothesised by Møller (1997).

Our data support the view that initial dikaryons have to develop highly autonomously in the selection arena and that dikaryons containing a pair of nuclei with uncomplemented metabolic deficiencies do not pass this arena and fail. One could object, however, that sexual self-sterility of auxotrophic markers is a direct consequence of the expression of these genes during or after meiosis. This appears unlikely for the following reasons. Self-sterility associated with auxotrophy gives rise to tiny fruiting bodies caused by the early (pre-meiotic) disruption of their development. In contrast, the fruiting bodies of meiotic mutants of *A. nidulans* have a normal size, as is expected from their late effect in the process of sexual spore formation (Swart *et al.*, 2001): after the huge proliferation of the dikaryotic tissue, meiosis is the second last division before the actual formation of the

spores. In the unicellular alga *Chlamydomonas reinhardtii* no parental investment in zygotes takes place and therefore a selection arena is not expected. In accordance with this, most auxotrophic markers have no discernible phenotypic effect among zygotes in this alga (Orr, 1991).

The pleiotropic effect of sexual self-sterility for many mutations in *A. nidulans* occurs under conditions when asexual sporulation is normal. This shows that the mycelium supports more easily asexual spore formation, and thus mutations are passed on via this route more easily. This finding was not unexpected for several reasons. First, selection arenas pay off more in a costly route. Sexual spores are more expensive than asexual spores, as they are bigger and require a much longer developmental time (Champe and Simon, 1992). In addition, the proliferation per initial in the sexual route is much more elaborate than per initial in the asexual route. Second, the sexual route starts with a dikaryotic cell; starting with a dikaryotic cell makes selection more effective than starting with a multinucleate footcell, by which the asexual route starts. Finally, potential benefits of such control are much larger in the sexual cycle where variation can be generated by recombination.

In the following paragraphs we show that energy for reproduction is preferentially invested in dikaryons with relative high fitness expectations. Although *A. nidulans* is a homothallic fungus and thus produces sexual spores mainly by selfing, outcrossing is possible though rare (Butcher, 1968). We performed a cross between two heterokaryon incompatible strains carrying the self-sterility mutations *adD3* and *riboB2* respectively. Heterokaryon incompatible strains were used to ensure that complementation is not possible at the somatic level but only possible at the (sexual) dikaryon level. The wall of a cleistothecium consists of vegetative tissue derived from the maternal strain, and only the dikaryotic cells inside the cleistothecium reflect the genotype of the progeny (Bruggeman *et al.*, 2003a). This explains that tiny abandoned cleistothecia seem normal at the outside, as this wall tissue is not subjected to the selection arena, but only the dikaryotic tissue inside. Parental-type cleistothecia in the analysed cross were small and carried only a few up till 100 ascospores under low supplementation levels. On the crossing plates strikingly big cleistothecia appeared between the small sterile selfed cleistothecia (Fig. 3.2A and B).

These big cleistothecia carried a mean (±SE) of 28,000 (± 6,000) with a maximum of 72,000 ascospores. All these big cleistothecia appeared to be crossed and since the two mutations *adD3* and *riboB2* are unlinked, 25 % of these spores would be of wild-type genotype. Such observation of preferential outcrossing of self-sterile strains in this characteristically selfing fungus was used by Bainbridge (1974) to easily obtain hybrid fruiting bodies. Also the long-standing observation of the phenomenon of relative heterothallism (Baracho and Azevedo, 1977) can be interpreted in the light of the selection arena. Relative heterothallism denotes that some strain combinations result in more than 50% crossed cleistothecia, higher than expected if mating is random (first observed by Pontecorvo (1953)).

The following provides another example of preferential investment in dikaryons with high-expected fitness: In *A. nidulans,* nuclear autonomous replicating plasmids have been constructed that have been used for high frequency transformation. This has been studied in a system with an *argB* or *trpC* auxotrophic strain carrying a plasmid with an intact *argB* or *trpC* gene. These plasmids seldom recombine with the *Aspergillus* genome during vegetative growth to give stable transformants (Gems *et al.*, 1991).

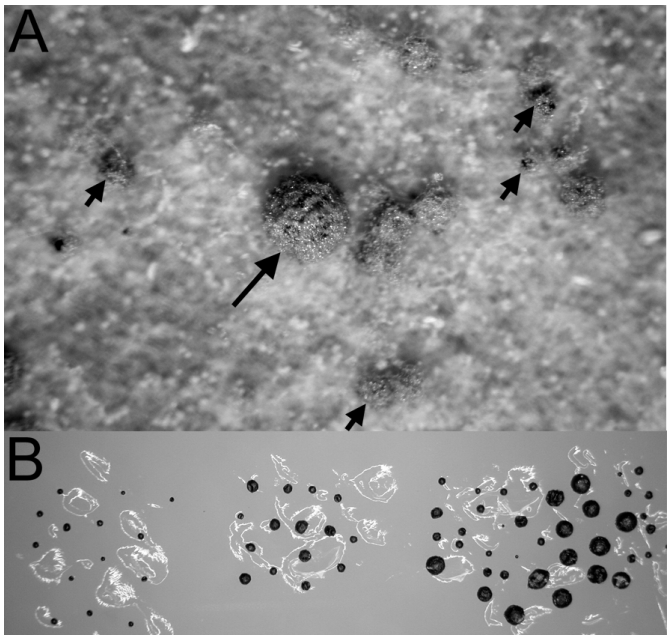

Figure 3.2 | **Mixing two strains on a Petri dish with the *adD3* marker and the *riboB2* marker yields small selfed and big crossed cleistothecia.** (A) A big crossed cleistothecia (long arrow) lies in between small selfed abandoned ones (short arrows). (B) Cleistothecia collected from the strains with the *adD3* and *riboB2* respectively (left and middle group) and cleistothecia collected from the crossing plates, showing the big crossed cleistothecia among the small self-fertilised ones (right group).

However, during sexual reproduction they are nearly always transmitted and even exhibit increased recombination (Aleksenko and Clutterbuck, 1995). We explain these observations with the selection arena hypothesis: dikaryons in which the *argB* or *trpC* mutation causing self-sterility is complemented by a plasmid carrying the intact gene are able to develop and will get full support from the mycelium. Those dikaryons lacking the plasmid with the wild-type allele will not be able to further develop.

We believe that the selection arena hypothesis may clarify many unexplained observations in mycelial fungi, indicating that this mechanism might be common in fungal biology: All natural isolates of the fungus *Podospora anserina* show senescence (Rizet, 1953; van der Gaag *et al.*, 1998) which is highly correlated with instability of the mitochondrial genome and the accumulation of circular mitochondrial DNA fragments during vegetative growth (Griffiths, 1992). Sexual progeny derived from such senescing cultures are again juvenile with normal mitochondria, a phenomenon termed *rejuvenation*. We hypothesize that only dikaryons that started with relatively healthy mitochondria are able to complete fruiting body development; this effectively selects against deleterious mitochondria. A similar operation of the selection arena is demonstrated by the fact that matings between mitochondrial mutant strains of *P. anserina* yield progeny with only wild-type mitochondria (Silliker *et al.*, 1996). Plausibly, transmission of the mutant mitochondria to ascospores is prevented such that only dikaryons containing wild-type recombinant mitochondrial DNA molecules could pass the selection arena.

It seems a general phenomenon in ascomycetous fungi that viruses are hardly or not transmitted into ascospores (Rogers *et al.*, 1988): for example in *A. nidulans* (Coenen *et al.*, 1997), in *Cryphonectria parasitica* (Anagnostakis, 1988), and in *Ophiostoma ulmi* (Rogers *et al.*, 1986). The selection arena hypothesis might account for this phenomenon. We speculate that virus-infected dikaryons display lower developmental vigour than virus-free dikaryons, and will not be sustained by the fungal mycelium. Finally, we believe that the selection arena hypothesis can clarify the observation that many mutations in *Neurospora crassa* cause female sterility or lead to reduced fertility (DeLange and Griffiths, 1980; Perkins *et al.*, 1982).

We conclude that a sexual selection arena operates in *A. nidulans* and that this may be a general phenomenon in fungal biology. The arena ensures that only the fittest nuclei and cytoplasm from the coenocytic mycelium end up in the sexual spores. Sexual spores allow the survival of environmental stresses, often accompanied by severe reduction in numbers and a subsequent building up of the population, while asexual spores enable fast occupation and utilization of locally available substrates. Moreover, the arena capitalises on the assumed benefits of recombination in the sexual pathway (Otto and Lenormand, 2002). Clearly, if a population periodically has to go through a bottleneck of relatively few surviving spores, genetic quality control should be highest among those spores: *i.e.* in the sexual cycle.

Acknowledgments

We thank Marijke Slakhorst for technical assistance. This work was supported by a grant from the Netherlands Organization for Scientific Research (NWO) to J.B., 805-36-355.

4

Sex slows down the accumulation of deleterious mutations in the homothallic fungus *Aspergillus nidulans*

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J. A. G. M. de Visser and R. F. Hoekstra, 2003.
Genetics 164, 479-485.

Abstract

Coexistence of sexual and asexual reproduction within the same individual is an intriguing problem, especially when it concerns homothallic haplonts, like the fungus *Aspergillus nidulans*. In this fungus asexual and sexual offspring have largely identical genotypes. This genetic model organism is an ideal tool to measure possible fitness effects of sex (compared to asex) resulting from causes other than recombination. In this article we show that in the sexual pathway slightly deleterious mutations accumulate at a lower rate than in the asexual pathway. This secondary sex advantage may contribute to the persistence of sexual spores in this fungus. We propose that this advantage results from intra-organismal selection of the fittest gametes or zygotes, which is more stringent in the costly sexual pathway.

Introduction

Many organisms are able to produce offspring both sexually and asexually (Bell, 1982). The apparent stability of this dual reproductive system raises questions about its functional significance. The coexistence of a sexual and an asexual reproductive pathway within the same individual is especially intriguing in homothallic organisms with predominantly haploid life cycles like many algae and fungi, because their sexual and asexual offspring have identical genotypes. A good example is the fungus *Aspergillus nidulans* (Fig. 1.1 on page 8). In its vegetative state, it consists of a mycelial colony that typically originates from a single haploid spore. All nuclei in this colony are therefore genetically identical, except for novel mutations that have arisen spontaneously during the growth of the mycelium. A mature colony produces offspring in the form of spores using both an asexual and a sexual pathway. The conidiospores are produced by mitotic division in 3 days after germination, while the ascospores are produced by zygote formation and subsequent meiosis after at least 7 days (Pontecorvo, 1953). The sexual ascospores are produced in fruiting bodies, or cleistothecia, each of which is the result of a single fertilization event and may contain up to 100,000 spores. Because *A. nidulans* is homothallic, a single colony can produce ascospores by self-fertilization. This implies that two identical haploid nuclei fuse to a diploid meiocyte only to produce meiotic products (the haploid ascospores) that are genetically identical to the parental nuclei and to the asexually produced conidiospores. Whatever fitness difference, if any, exists between sexually and asexually produced offspring in such a situation, it cannot be a consequence of genetic recombination, which is thought to be a key factor in the evolutionary success of sex (Otto and Lenormand, 2002). Thus, in *A. nidulans*, recombination in the sexual cycle has no genetic consequences in the case of selfing. Therefore, this genetic model organism is an ideal tool to measure possible fitness effects of sex (compared to asex) resulting from causes other than recombination. One such cause may be the early intra-organismal selection of potential offspring.

The existence of intra-organismal prezygotic (Extavour and Garcia-Bellido, 2001; Otto and Hastings, 1998; Walter *et al.*, 1998) and postzygotic mechanisms (Forbes and Mock, 1998; Gosling, 1986; Stearns, 1987; Stephenson and Winsor, 1986) to increase

offspring fitness has been suggested for several organisms with obligate sexuality. The key idea is that parents create an enlarged array of gametes and/or zygotes from which they choose a genetically superior subset. In this way, energy for reproduction is invested in the most promising zygotes. In principle, intra-organismal selection for offspring quality may occur in the sexual and the asexual cycle of *A. nidulans*.

Table 4.1

Differential restoration of asexual and sexual spore production in auxotrophic mutations

Mutation	Nutrient required	Supplementation for asexual spore formation (mM)	Supplementation for sexual spore formation (mM)	Reference
pyrD23	uridine	1	5	personal observation
adD3	adenine	0.1	2	personal observation
hisB	histidine	0.3	30	Busch *et al.* (2001)
trypC	tryptophan	4	20/30	Eckert *et al.* (1999)
pyroA4	pyridoxine	0.001	0.01	personal observation
riboB2	riboflavin	0.01	0.1	personal observation
yB	copper	0.0016	0.005	Kurtz and Champe (1981)

Interestingly, some auxotrophic mutations of *A. nidulans* confer sexual self-sterility as pleiotropic effect while asexual sporulation is normal. This self-sterility manifests itself in very tiny empty cleistothecia and has been described for the *hisB* locus (Millington Ward *et al.*, 1984; Busch *et al.*, 2001); the *argB* locus (Serlupi Crescenzi *et al.*, 1983); the *yB* locus (Kurtz and Champe, 1981), and the *trpC* locus (Yelton *et al.*, 1983; Eckert *et al.*, 1999). Restoration of sexual self-fertility needs higher supplementation of the deficient nutrient than restoration of asexual spore production needs. We tested other loci for this differential restoration of asexual and sexual fertility. Literature references and our own data point to the possibility of a more stringent selection in the sexual cycle than in the asexual cycle (Table 4.1).

This hypothesis predicts that in *A. nidulans* sexual spores produced by selfing will have a higher average fitness than that of the asexual spores with the same genotype. To test our hypothesis, we started a mutation accumulation experiment with 40 asexual and 40 sexual selfing lines, all derived from a common ancestral strain of *A. nidulans*. In these lines, mutations were allowed to accumulate during 40 generations by single-spore transfer. One generation refers to a full cycle of spore germination, mycelial colony growth, and (sexual or asexual) spore development. If intra-organismal selection operates more effectively in the sexual reproduction route, then the sexual lines are expected to carry a smaller load of deleterious mutations than the asexual lines carry. After 40 generations of mutation accumulation, the fitness of each line was estimated relative to that of the founder. Fitness was estimated by measuring the colony diameter grown in a fixed time period. For these measurements, we used stored conidiospores from the founder, the asexual, and the sexual lines respectively. We observed that mutations had accumulated in both groups, but with a significantly lower 'fitness impact' in the sexual lines. We discuss this finding and argue that genetic recombination can be excluded as an explanation for our result. Instead, we propose that this advantage of sex results from intra-organismal selection of the fittest gametes or zygotes, which is more stringent in the costly sexual pathway.

Materials and methods

Strain, media and culture conditions

The wild-type *Aspergillus nidulans* strain JC256 *veA*$^{+}$ was used in this study. This strain was isolated from cereal field soil in Hungary in 1981 (Birmingham Collection of Dr. J.H. Croft). Minimal medium (MM) and complete medium (CM) were essentially as described by Pontecorvo (1953). All incubations were done at 37°C. Asexual spore suspensions were prepared in saline (0.8% NaCl) + 0.005% Tween, and sexual spore suspensions were prepared in saline.

Mutation accumulation protocol

The mutation accumulation procedure is depicted in Fig. 4.1. The asexual and sexual mutation accumulation (MA) lines all started from one single founder colony of strain

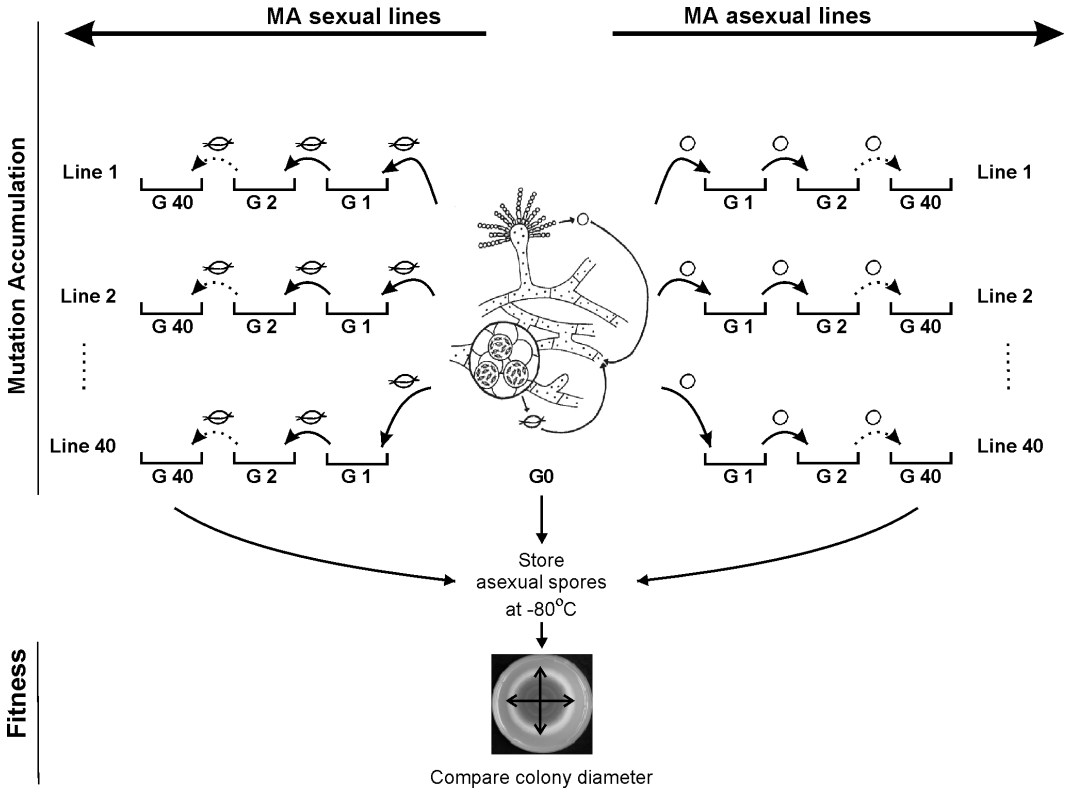

Figure 4.1 | **Representation of the mutation accumulation protocol.** Forty sexual and asexual lines were established from a single founder colony of *A. nidulans* and maintained during 40 generations. The successive generations were established by the inoculation of one single sexual or asexual spore from the preceding generation. As a fitness assay we measured the relative colony diameter. G, generation.

JC256. An *A. nidulans* colony develops asexual spores in 3 days after germination and sexual spores after at least 7 days. Therefore, in the asexual lines a new generation was started every 3 days while in the sexual lines a new generation was started every 12 days. For asexual MA, 40 CM plates were inoculated with an asexual spore suspension from the founder colony, so that individual colonies could originate from single spores. The colonies grown on these plates represented the first generation of the asexual MA lines. We refer to a generation as a full cycle of spore germination, mycelial colony growth, and spore development. After 3 days, from each of the 40 plates, a single colony was randomly chosen by taking the most central colony on the plate. Asexual spore suspensions were made from each of these 40 colonies, after which they were spread for

the second MA generation. This procedure was repeated every 3 days, until generation 40. This procedure creates for each line a history of 40 successive bottlenecks of a single conidiospore.

We started the sexual MA lines with 40 randomly chosen cleistothecia from the founder colony. The 40 cleistothecia were crushed in 1ml saline each. We inoculated 40 plates with these suspensions so that individual colonies could originate from single sexual spores. The colonies grown on these plates represented the first generation of the sexual MA lines. After 12 days, a sexually sporulating colony was randomly chosen on each of the 40 plates by taking the most central colony. From each of these 40 colonies, a cleistothecium was randomly chosen to start the next round of propagation. This procedure was repeated every 12 days until generation 40. This procedure creates for each line a history of 40 successive bottlenecks of a single ascospore. At regular intervals, asexual spore suspensions were stored at –80°C in glycerol/peptone (29%/0.67%) for both the sexual and the asexual lines.

Colony diameter measurements

After 40 generations of MA we measured, after 88 hr, colony diameter in millimeters of all MA lines relative to that of the founder (resulting in a relative colony diameter, RCD). For all lines (sexual as well as asexual, including the ancestor) we used stored asexual spores at -80°C for this fitness measurement. Measurements were performed on five replicate MM plates with point inoculation in the center of a plate using 10 µl asexual spore suspension obtained from 3-day-old cultures grown on CM. After 88 hr, we transferred all plates to 4°C, where, after 1hr rest, we measured diameters of each colony in two perpendicular directions. Mean RCD was calculated for each plate and used as the basic replicate fitness estimate in the analysis.

Data analysis

Differences between founder and mean sexual or asexual MA lines were tested one-tailed using a one-sample *t*-test. Among-group differences were analyzed using General Linear Model (GLM) in which the effect of groups (asexual or sexual) was treated as fixed and the effect of lines (nested within groups) as random effect. We used a Dunnett's test to compare all individual-line means with the ancestral value. Mutational parameters

were estimated with the classical Bateman-Mukai method, where U_{min} and s_{max} are derived from the decrease of average fitness and the increase of the genetic variance in fitness under the assumption of equal mutational effects (Mukai *et al.*, 1972). Bootstrap 95% confidence intervals for U_{min} en s_{max} were obtained from 1000 bootstrapped pseudovalues. Mutational parameters were also estimated using the maximum likelihood method (Keightley, 1994), which produces estimates of U and s themselves (instead of their limits) by assuming s to vary according to a gamma distribution. Confidence intervals of 95% for individual parameters were obtained by a drop of 2 log likelihood from the maximum. The kurtosis of this distribution is described by γ_2; with $\gamma_2 \rightarrow$infinity the distribution becomes increasingly leptokurtic. Differences between two parameter estimates are significant when their point estimates lie outside each other's 95% confidence interval

Results

After 40 generations of mutation accumulation, we found that the fitness of both the asexual lines and the sexual lines was significantly lower than that of the founder (asex: mean (SE) = 0.961 (0.0018); t_{38} = -12.78; $P < 0.001$; sex: mean (SE) = 0.973 (0.0020); t_{37} = -6.67; $P < 0.001$, Fig. 4.2). The sexual lines had a significantly higher fitness than the asexual lines ($F_{1,75}$ = 5.60; P = 0.021). Thus, mutations had accumulated in both groups, but with a significantly lower 'fitness impact' in the sexual lines. Moreover, 11 of the 38 sexual lines (29%) had a colony diameter significantly smaller than that of the founder, while this was true for 21 of the 39 asexual lines (54%). This difference between sexual and asexual lines was significant (χ^2_1 = 4.913; P = 0.027). These findings are consistent with our prediction that more effective intra-organismal selection in the sexual lines results in slowing down the accumulation of mutations. In addition, we found that the error variance of sexual lines is (slightly) lower than that of asexual lines ($F_{149,\ 147}$ = 1.3603, P = 0.031).

The higher fitness of sexual relative to asexual lines can be the result of a lower number of mutations that accumulated or a lower fitness effect of the mutations, or both. However, without *a priori* knowledge of the mechanism responsible for intra-organismal selection, we expect, first of all, a lower number of mutations to have accumulated. This is

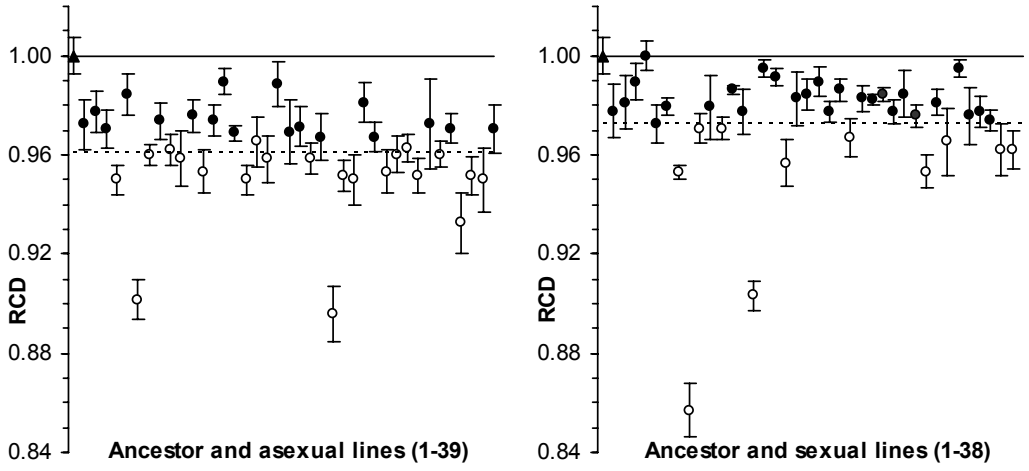

Figure 4.2 | **Colony diameter of the mutation accumulation lines relative to the ancestor (RCD).** Triangle represents the ancestor. Circles represent the MA lines, filled circles are lines not significantly different from ancestor; open circles are lines significantly different from ancestor. Error bars show standard errors. The horizontal solid line indicates the mean of the ancestor and the horizontal dashed line the mean of the asexual and sexual lines, respectively. (A) Asexual lines. (B) Sexual lines.

because *any* mechanism would reduce the number of mutations, while only a subset of mechanisms (namely those with selection bias toward mutations with large effect) would also reduce the average fitness effect. As a first approach to estimating the parameters underlying the mutation accumulation process, we used the classical Bateman-Mukai method (Mukai *et al.*, 1972). This method estimates the lower limit of the mutation rate ($U_{\min}$, per generation per whole genome) and the upper limit of the mean fitness effect of a mutation ($s_{\max}$). $U_{\min}$ and $s_{\max}$ are derived from the decrease of average fitness and the increase of the genetic variance in fitness under the assumption of equal mutational effects. This method, however, did not reveal significant differences between the asexual and sexual lines, as the point estimates did not lie outside each other's 95% confidence interval (Table 4.2A).

As a second approach, we used maximum-likelihood estimation, which estimates U and s, rather than U_{min} and s_{max}. This procedure assumes that mutational effects follow a gamma distribution, thus avoiding the unrealistic assumption of equal mutational effects.

Table 4.2

Parameters underlying the mutation accumulation process in the sexual and asexual lines

	A[a]		B[b]				C[c]			
Line	U_{min}	s_{max}	U	s	γ_2	LL	U	s	γ_2	LL
Sex	0.036 (0.0080, 0.19)	0.019 (0.0034, 0.049)	0.014 (0.0053, 0.029)	0.019[d]	11 (3.1, 59)	484.9	0.041 (0.016, 0.081)	0.0069[d]	33 (10, 159)	485.4
Asex	0.14 (0.042, 0.35)	0.0069 (0.0028, 0.017)	0.10 (0.057, 0.16)	0.0069[d]	2.8 (1, 10)	466.0	0.018 (0.0090, 0.037)	0.019[d]	1.1 (1, 28)	463.6

[a] U_{min} and s_{max} according to the Bateman-Mukai method. Bootstrap 95% confidence intervals for U_{min} and s_{max} were obtained from 1000 bootstrapped pseudovalues and are shown in parentheses.

[b] Maximum-likelihood estimation of U and γ_2, with s fixed at the Bateman-Mukai estimates for the corresponding lines. The value of the maximum log likelihood (LL) is indicated. Confidence intervals of 95% were obtained by a drop of 2 log likelihood from the maximum and are shown in parentheses.

[c] Maximum-likelihood estimation with s fixed at the Bateman-Mukai estimates from the other lines.

[d] s fixed.

In maximum-likelihood estimation, log likelihood is maximized for three parameters: U, s and γ_2, *i.e.*, the kurtosis of the gamma distribution. Maximization of log likelihood for all three parameters simultaneously resulted in an infinitely high U, an infinitely small s an an infinitely high γ_2 for both sexuals and asexuals. Fixing one of the parameters resulted in biologically more realistic parameter estimates. As we expected in any case effect on U, we fixed s by filling in the Bateman-Mukai estimates of s for sexuals and asexuals. In this way, we obtained maximum-likelihood estimates of U that are significantly lower for sexuals than for asexuals, as their point estimates lie outside each other's 95% confidence interval (Table 4.2B). However, since U and s are confounded in the estimation procedure, the lower U for sexuals could depend on the (nonsignificantly) higher estimate of s for this group. To exclude this possibility, we then fixed the same Bateman-Mukai estimate of s for both sexuals and asexuals. We used the low s estimated for asexuals for this test, since this value resulted in a higher likelihood than did the higher value estimated for the sexuals (see asexual lines in Table 4.2B, and sexual lines in Table 4.2C). In doing so, the estimated U was still significantly lower for sexuals. Hence, the mechanism causing the smaller load of deleterious mutations in the sexual lines reduces the number of mutations. Whether it also reduces the average fitness effect remains unclear.

The values we obtained for the mutation rate in *A. nidulans* agree with estimates in other organisms (Drake *et al.*, 1998). Based on a genome size of 3.10×10^7 bp, the Bateman-Mukai estimates of U_{min}, and 20 nuclear divisions between generations, we estimated the number of mutations per bp per nuclear division to be 2.26×10^{-10} for the asexual and 5.81×10^{-11} for the sexual lines.

Discussion

The stable coexistence of asexual and sexual reproduction in the homothallic fungus *A. nidulans* is an intriguing problem, since offspring from both pathways have the same genotype. We hypothesize that more stringent intra-organismal selection against deleterious mutations in the sexual than in the asexual reproduction route of *A. nidulans* contributes to the stability of the dual reproductive system. This selection would immediately give a higher fitness to the sexually produced spores than to the asexually

produced spores. We tested this hypothesis with a mutation accumulation experiment in 40 asexual and 40 sexual lines during 40 generations. As a fitness estimate we measured colony diameter relative to the ancestor. In the sexual lines, mutations accumulated with a significantly lower 'fitness impact' than those in the asexual lines, supporting our hypothesis of more efficient intra-organismal pre- and/or postzygotic selection in the sexual reproduction route. As a surprising result the error variance of sexual lines is (slightly) lower than that of asexual lines ($F_{149,\ 147} = 1.3603$, $P = 0.031$). This observation is consistent with the accumulation of more deleterious mutations in the asexuals if some mutations would cause these genotypes to be less robust against environmental variation (which is largely reflected by the error variance of the fitness assay). However, we do not have any direct evidence for this explanation.

Our experiment aimed to reveal a possible fitness difference between asexual lines and sexual lines, but not to reveal the actual mechanistic cause. However, from the fitness data we could estimate parameter values underlying the mutation accumulation process in the asexual and sexual lines. We found that the mechanism causing the smaller load of deleterious mutations in the sexual lines at least reduced the *number* of mutations. This is in agreement with the fact that all possible mechanisms should cause a lower U, while only a subset of mechanisms (*i.e.*, those with a bias against large mutations) would also cause a lower s.

Although we could not discriminate between intra-organismal prezygotic and postzygotic mechanisms, some observations favor postzygotic selection. Development of a dikaryon consists of a huge proliferation resulting in 100,000 or more ascospores. If this development is highly autonomous - for example resources from the parental mycelium are not readily available - efficient selection is possible since only mutation-free fruiting bodies will be able to complete development. As mentioned in the Introduction, the phenotypic effect of auxotrophic mutations is very suggestive in this respect. A low level of supplementation restores asexual spore formation, but does not allow the formation of cleistothecia: externally normal cleistothecia are formed, but these are tiny and do not contain ripe ascospores. Only higher supplementation allows full sexual development (Table 4.1).

The meiotic process of recombination itself is very unlikely to have caused our results. First, this process may be mutagenic itself (Russell and Russell, 1996; Watters and

Stadler, 1995). Second, in *A. nidulans*, self-fertilization is a local process and thus the two nuclei that form the basis of a sexual fruiting body must have a very recent common ancestor nucleus because they are located in the mycelium in each other's close vicinity. Recombination can only have genetic consequences in the highly unlikely event that these two nuclei carry at least two different mutations. And even then, all recombinant products will remain present in the tetrads formed since selection is absent at that stage. Consequently, in our protocol all recombinants had an equal chance to be picked for the next generation, so that the net genetic effect of recombination is zero. Last, recombinational repair of double-stranded DNA damage during the process of meiosis could not have caused our results because hyphal expansion can efficiently eliminate damaged nuclei.

Our result potentially could be explained by a lower number of nuclear divisions between generations in the sexual lines than in the asexual lines. However, the opposite is almost certainly the case: the longer sexual reproduction cycle - 12 days *vs.* 3 days in the asexual cycle - is very likely to reflect a higher number of divisions in this route.

Ascospores of *A. nidulans* contain two nuclei, resulting from a final mitosis in the ascospores, whereas asexual spores contain only one nucleus. Could this have any relevance for our findings? The two nuclei in an ascospore derive from a very recent mitotic division within the spore and are therefore identical with very high probability. Nevertheless, the binucleate condition may make a difference, for example, when the nuclei carry a mutation encoding reduced enzymatic function such that sufficient catalytic activity is provided by two copies of the mutant allele but not by a single copy. Thus some mutations may be transmitted via sexual spores but not via asexual spores. Therefore, the presence of two nuclei in the ascospores as opposed to a single nucleus in the conidiospores cannot contribute to an explanation of our results. Moreover, we have performed our fitness measurements exclusively on colonies that developed from asexual spores in both in the asexual and the sexual MA lines.

An important question concerns the generality of our results. Is *A. nidulans* idiosyncratic in this respect, or is the association of more stringent intra-organismal selection with the sexual cycle a feature of many organisms in which asexual and sexual reproduction coexist? It is difficult to answer this question due to the lack of suitable data. However, many mycelial fungi have basically the same reproductive biology as *A.*

nidulans, which is characterized by a quick production of very large numbers of conidiospores, followed by smaller numbers of ascospores. Ascospores are bigger than conidiospores, they require a longer development time, and under most conditions asexual sporulation is much more abundant than sexual sporulation (Adams *et al.*, 1998). Moreover, due to their protective spore wall, the sexual spores are more resistant than the asexual spores to adverse environmental conditions. The two types of spores seem to differ in their ecological role: conidiospores enable fast occupation and utilization of locally available substrate, while ascospores allow survival of environmental stresses, often accompanied by severe reduction in numbers and a subsequent building up of the population. It is therefore not unlikely that in many other mycelial fungi intra-organismal selection on offspring quality also will be stricter in the sexual cycle. Clearly, if a population periodically has to go through a bottleneck of relatively few surviving spores, genetic quality control should be highest among those spores, *i.e.*, in the sexual cycle.

We believe our study has revealed a novel functional aspect of sex in an organism with both sexual and asexual reproduction, namely more stringent screening for deleterious mutations in potential offspring in the sexual route than in the asexual route. This secundary sex advantage may form part of the explanation of the persistence of sexual spores in *A. nidulans*. Our findings imply that sexually produced offspring have a higher average fitness than that of asexually produced offspring. This difference may contribute to the somewhat puzzling stable coexistence of both reproductive modes in this homothallic haploid organism, in addition to the above-mentioned ecological specialisation of both types of spores. Although we speculate that the more autonomous (that is, less supported by the maternal mycelial resources) production of sexual spores compared with asexual spores is a possible explanation of our findings, the exact mechanism remains to be elucidated.

Acknowledgement

We thank P.D. Keightley for supplying his maximum-likelihood program and for advice. This work was supported by a grant (805-36-355) from the Netherlands Organization for Scientific Research (NWO) to J.B.

5

Fitness in a modular organism: the fungus *Aspergillus nidulans*

Abstract

Lifetime reproductive success is an unsuitable fitness estimate for the mycelial fungus *Aspergillus nidulans* for two major reasons. First, it concerns a modular organism in which growth is in principle indeterminate. Second, it produces both asexual and sexual spores. Therefore, offspring number cannot be derived in a straightforward way from spore production, as the two spore types have to be weighted differently in their contribution to fitness under various circumstances. Moreover, we show that this fungus flexibly adjusts the ratio of sexual and asexual spores. Instead, the rate of mycelial growth seems a good fitness estimate for this modular organism. The problems on fitness estimation discussed in this article apply not only to many fungi, but also to other modular organisms, such as plants with both asexual and sexual reproduction routes.

Introduction

Fitness is a central concept in the study of natural selection, population genetics and evolution. Yet, there exists no generally applicable definition of fitness or tool to quantify it (Murray, 1990; Stearns, 1992). Stearns (1992) adopts the following definition of fitness: the expected contribution of an allele, genotype or phenotype to future generations. Usually, individual fitness serves as starting point for quantifying fitness and is defined as the lifetime reproductive success, which is the total number of offspring produced by an individual. However, in many organisms, measuring total offspring number is not feasible or very laborious. Often, only indicators of individual fitness are measured such as body size, lifespan, *etc*.

Another complication of fitness measurement is encountered in modular organisms in which growth is indeterminate and characterized by repeated iteration of modules. The best-known modular organisms are plants and mycelial fungi, but also other taxa like corals, ascidians and bryozoans are modular. Often the modular growth form is combined with the option of clonal reproduction. In such organisms lifetime reproductive success is not a very meaningful concept especially as the term individual needs to be refined. The terms 'ramet' and 'genet' (Kays and Harper, 1974) are more useful than the term individual and have become accepted usage for the plant world; a genet is the genetic individual as produced via a zygote; clones or (physiologically independent) modules that form part of this genet are called ramets.

In mycelial fungi, the hypha is the basic module and can iterate itself indefinitely as long as conditions are favorable. Besides, many fungi propagate clonally by asexual spores or by mycelial fragmentation, but can also reproduce sexually. A number of studies have attempted to measure fungal fitness. In part, this concerned pathogenic fungi and questions related with prevention and treatment of the diseases caused by them. In these studies, often fitness parameters were measured that could explain various aspects of the biology of the pathogenic fungus. For example measuring the latent period, competition strength with other fungi, virulence, fungicide resistance, pathogenicity, *etc*. For an overview of such fitness estimates for the plant pathogenic fungi *Puccinia graminis* and *Botrytis cinerea* see Pringle and Taylor (2002). Only a few studies have estimated fitness in fungi in order to answer fundamental questions related to evolution and population genetics. For example, in one study that investigated interactions among mutations in

Aspergillus niger, fitness was estimated as mycelial growth rate (de Visser *et al.*, 1997). Mycelial growth rate and asexual spore production were highly correlated in this asexual fungus. Another study examined inbreeding depression in *Agaricus bisporus* (Xu, 1995). For this sexually reproducing fungus eight fitness estimates were assessed, all connected to the reproductive cycle: positive mycelial interactions, successful mating, heterokaryon growth rate, primordium formation, fertile fruiting body formation, time to first break, and number and weight of fruiting bodies. No more than three of these estimates (positive mycelial interactions, heterokaryon growth rate, and primordium formation) were higher in the outcrossed population. In addition, only two pairs of traits were significantly correlated: successful mating with mycelial interactions, and time to first break with number of fruiting bodies. Thus, different fitness estimates do not have to be correlated, but understanding the absence or presence of correlations also helps to understand the biology of the fungus.

In the present study we explore and discuss two different fitness estimates for the mycelial fungus *Aspergillus nidulans*, namely mycelial growth and spore production. This fungus is used as a genetic model organism and possesses an asexual and sexual spore production route (see Fig. 1.1). A colony starts with the germination of a spore. This subsequently develops into a radially symmetric mycelial network which expands indefinitely. After 25 hours, the first asexual spores are produced at the rim of the colony. When this burst of asexual spore formation ceases, sexual development commences with the formation of cleistothecia. In these fruiting bodies, sexual spore formation begins at 100-150 hours after the start of the new colony (Champe *et al.*, 1987). In a former study, we measured fitness in *A. nidulans* of asexual and sexual mutation accumulation (MA) lines relative to their common ancestor (Bruggeman *et al.*, 2003b). As fitness estimate for this modular organism we then used colony diameter after a fixed time period relative to that of the ancestor (RCD = relative colony diameter). However, as compared to RCD, spore production would be very well suited to measure under a competition regime. This is a harsh condition and potential differences in fitness between mutation accumulation lines and the ancestor are likely to be enlarged under harsh conditions (Kondrashov and Houle, 1994). Hence, in this study, we assessed asexual spore production of these same MA lines under a competition regime and compared this to the previously obtained fitness estimates based on RCD. Moreover, a fitness estimate including spore production seems

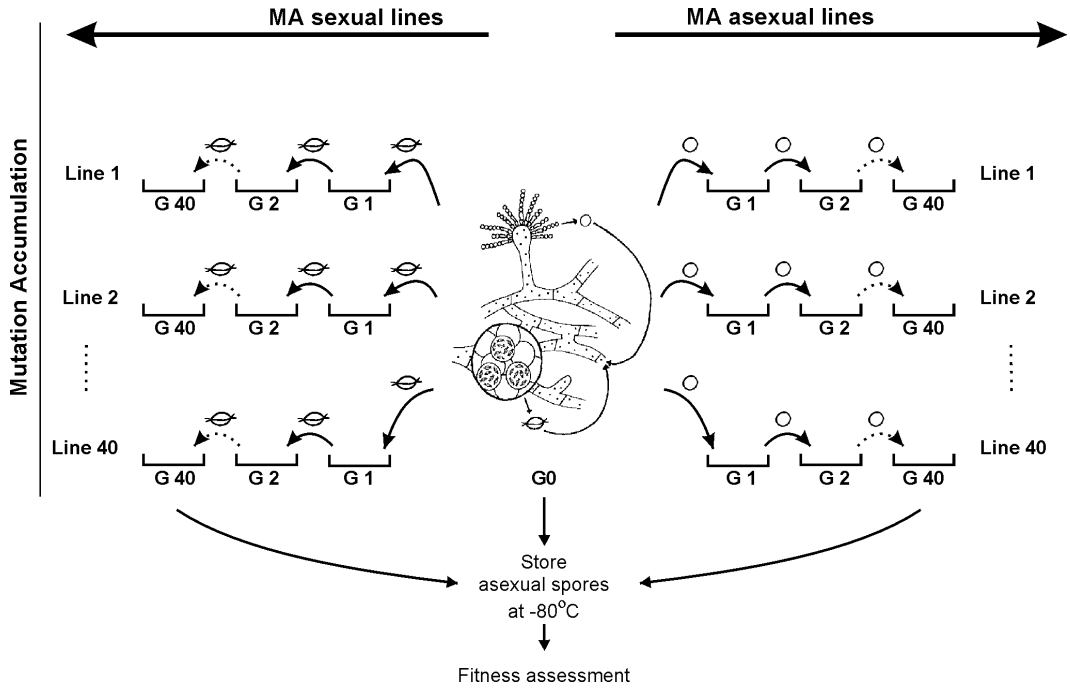

Figure 5.1 | **Representation of the mutation accumulation protocol.** Forty sexual and asexual lines were established from a single founder colony of *A. nidulans* and maintained during 40 generations. The successive generations were established by the inoculation of one single sexual or asexual spore from the preceding generation. G, generation.

to be closer related to the lifetime reproductive success preferentially used in unitary organisms.

Measuring fitness differences by means of competition is routinely employed in the bacterium *E. coli* (Lenski *et al.*, 1991) and is also employed in budding yeast (Zeyl and Graham, 1997) and viruses (Chao, 1990). Competition assays evaluate various components of fitness simultaneously. The competition assay as used in this study includes the following components: germination speed, colonization ability, mycelium growth rate, and spore production. The RCD used in our former study is only indicative of mycelium growth rate.

Since asexual spore production and mycelial growth rate are highly correlated in the closely related fungus *Aspergillus niger* (de Visser *et al.*, 1997), we hypothesize that both sexual and asexual spore production in *A. nidulans* are positively correlated with mycelial growth. Therefore, we expected differences between the mutation accumulation lines and

the ancestor similar to those found in our previous study based on RCD (Bruggeman *et al.*, 2003b): namely, a reduced competition strength for asexual spore production relative to the ancestor for both sexual and asexual lines, while the sexuals would still be fitter competitors than the asexuals would be. However, we did not find this result. Additional investigations revealed that the mutation accumulation protocol must have influenced the ratio of asexual to sexual spore production in the MA lines. We discuss the non-genetic nature of this phenomenon and review other factors affecting this ratio. Finally, we discuss our findings in the light of fitness measurement in this mycelial fungus and the implication for fitness assessment in any modular organism with both asexual and sexual offspring.

Materials and methods

Strains

The wild-type *A. nidulans* strain JC256 *veA*$^{+}$ with green conidiospores was used in this study. This strain was isolated from cereal field soil in Hungary in 1981 (Birmingham Collection of Dr. J.H. Croft). From this strain we constructed 40 asexual mutation accumulation lines and 40 sexual mutation accumulation lines, in which each line had a history of 40 successive bottlenecks of a single conidiospore or ascospore respectively (see Fig. 5.1). The procedure for these constructions have been previously described (Bruggeman *et al.*, 2003b). A spontaneous white mutant derived from JC256 was used in the competition experiments as the reference strain. This color marker did not affect fitness in competition with the green wild type (tested in pilot experiment, data not shown).

Media and culture conditions

Minimal medium (MM) was essentially as described by Pontecorvo (1953) with per liter: 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g of each $MgSO_4.7H_2O$ and KCl, 1 mg $FeSO_4$, $ZnSO_4$, $MnCl_2$ & $CuSO_4$, 15 g agar and 1% glucose. For complete medium (CM) the following extra ingredients were added: 2 g neopepton, 1 g Casamino acids, 1 g yeast extract , 0.3 g ribonucleotides and 2 ml vitamin solution (0.1% riboflavin & nicotinic acid, 0.01% thiamine, p-aminobenzoic acid & pantothenate, 0.05% pyridoxin, 0.0002% biotin),

pH 5.8. We used for all experiments stored asexual spores from the mutation accumulation lines at –80°C. Fitness was estimated on MM (to create a deprived environment).

At the start of the fitness assays, we made asexual spore suspensions that were vigorously shaken together with 1 millimeter dimater glass beads on a Griffin flask shaker for 15 minutes to break up the conidial chains. At the end of the fitness assays, we harvested all spores with a spreader from the plates in saline (0.8% NaCL) + Tween 0.005% suspensions. These were also shaken to break-up the conidial chains and, if present, to break-up the cleistothecia to release the sexual spores. For the competition experiments, we made viable counts of such suspensions on CM containing 0.05% Triton X-100. To count spores in the Coulter counter, we made appropriate dilutions of such suspensions in Isoton II (this is an azide free balanced electrolyte solution). Strains were incubated at 37°C in the dark.

Asexual spore production of the MA Lines under competition

In the competition experiments we compared the fitness of the 40 sexual and 40 asexual MA lines at generation 40 with the fitness of the founder strain JC257 by measuring the relative numbers of asexual spores produced when grown together with the reference strain during 94 hours. The competition experiment was performed in three blocks. Each block contained one replica of each line and six replicas of the founder. From all MA lines, the founder strain and the white-spored reference strain, we made asexual spore suspensions with a final concentration of $\pm 10^7$ spores/ml as measured with the Coulter counter. One ml from each MA line and the founder was mixed with 1 ml of the reference strain thus obtaining 81 competition mixtures. For each competition, 0.1 ml of the mixture was plated on a MM plate, resulting in $\pm 10^6$ spores on each competition plate. All plates were incubated at 37°C for 94 hours, after which the spores of the new generation were harvested.

We counted the initial (before competition) and the final (after competition) number of spores of the two competitors by making viable counts of appropriate dilutions. The two competitors were distinguished by their colony color (green or white). In total, we counted 154,942 colonies on 2,580 counting plates.

As a measure of fitness we used the relative fitness W_{ij} (Lenski *et al.*, 1991):

$$W_{ij} = \frac{\ln[N_i(1)/N_i(0)]}{\ln[N_j(1)/N_j(0)]}$$

where N_i are the number of spores of the MA lines or founder and N_j are the number of spores of the white-spored reference line; (0) and (1) indicates before and after competition. An ANOVA was performed on the three means of the founder blocks and this indicated that the second block had to be corrected for block effects. Consequently, a correction factor of 0.08685 was subtracted from all measurements in block two. This correction factor was calculated as the mean of the first and third founder block mean. Eventually, in the three blocks, W_{ij}'s of all lines were calculated relative to the founder mean in that block. Differences between W_{ij} of the founder mean and the sexual or asexual group mean were tested one-tailed using a one-sample *t*-test. Among-group differences were analyzed using General Linear Model (GLM) in which the effect of groups (asexual or sexual) was treated as fixed and the effect of lines (nested within groups) as random effect.

Analysis of investment in sexual spores of the MA lines

In the competition experiment described above, we measured competition strength after a short time span (94 hours), when only asexual spores had been produced. By assessing competition strength over a longer time span (310 hours), sexual spore production is included in the measurement as cleistothecia had appeared at that time. We calculated the relative fitness change between the first time point and the second time point, as this information is indicative for sexual spore production. This experiment was performed with one replica per line and six replicas for the founder. For the two treatments, relative fitness (W_{ij}) was calculated relative to the founder mean in that treatment, after which we calculated the relative fitness increase of each line ΔW_{ij}.

$$\Delta W_{ij} = \frac{W_{ij}(t=2) - W_{ij}(t=1)}{W_{ij}(t=1)}$$

ΔW_{ij} among-group differences were analyzed using GLM in which the effect of groups was treated as fixed effect. We used Tukey's method to compare all possible pairs of

group means. In our method to assess the relative fitness W_{ij}, only colony forming units are counted without recognizing their true sexual or asexual origin. Nevertheless, the Coulter counter allows distinguishing asexual and sexual spores in suspension as they differ in size. Therefore additionally, we raised a few of the asexual and sexual MA lines under non-competitive conditions and harvested all spores produced by these colonies at the two points in time. We counted the spores with one replica per line. Data were analyzed with Coulter Accucomp software.

Results

Asexual spore production under competition

We measured asexual spore production under competition with a reference strain of 40 asexual and 40 sexual mutation accumulation lines. As can be seen in Fig. 5.2 on page 66 the distribution of this fitness estimate was bimodal for the sexual lines, implying that there were two classes in the sexual lines. One class had a very low fitness and formed the left part of the bimodal W_{ij} distribution. The other class had a higher fitness and formed the right part of the bimodal W_{ij} distribution. This part coincided with the unimodal distribution of the asexual lines. Remarkably, we could phenotypically distinguish the two sexual classes on the basis of the presence or absence of a reddish-brown pigment in the medium of the competition plates (see Fig. 5.3). In each of the three replicates for this experiment, twenty-two sexual lines produced a reddish-brown secretion in the media and these lines were called sexual 'red' lines. The other eighteen had no such secretion and

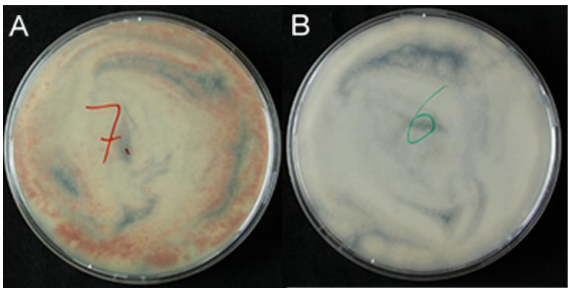

Figure 5.3 | **Red secretion in the medium.** (A) Example of the reddish-brown secretion in the medium of a competition plate of a sexual line. (B) In the competition plate of an asexual line the reddish-brown secretion is absent.

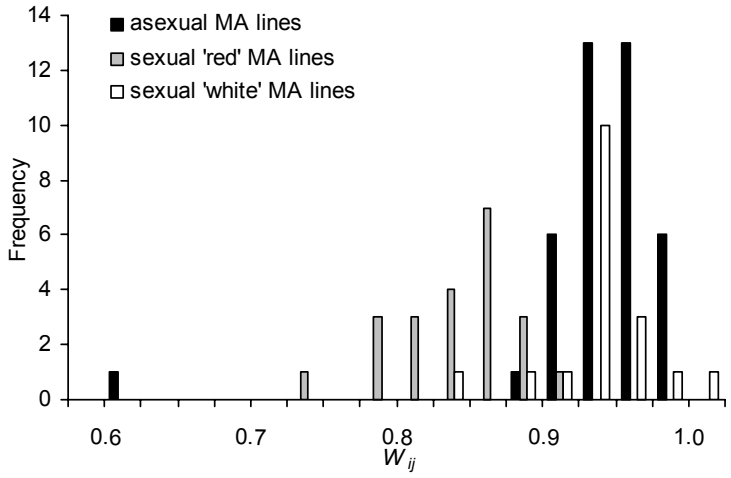

Figure 5.2 | **The frequency distribution of W_{ij}, showing the unimodal distribution in the asexual MA lines and the bimodal distribution in the sexual MA lines.** The frequency distribution of the sexual 'white' MA lines coincides with that of the asexual MA lines, whereas the distribution of the sexual 'red' MA lines is shifted to the left.

were called sexual 'white' lines. Neither the founder strain JC257, nor the asexual lines produced this pigment in the competition plates.

In Fig. 5.4 the mean W_{ij}'s of the founder, the sexual 'red' lines, the sexual 'white' lines and the asexual lines are plotted against the earlier obtained relative colony diameters (RCD) (Bruggeman *et al.*, 2003b). As consistent with the relative colony diameter measurements, the asexual lines, the sexual 'white' lines and the sexual 'red' lines had a lower W_{ij} than the founder: Asex: mean (SE) = 0.94256 (0.00896); (t_{39} = -6.41; $P < 0.001$). Sexual 'red' lines: mean (SE) = 0.84343 (0.00828); t_{21}= -18.90; $P < 0.001$. Sexual 'white' lines: mean (SE)= 0.93938 (0.00864); t_{17} = -7.01; $P < 0.001$. However, while the sexuals had a larger RCD than the asexuals, now the sexuals were the weaker competitors (GLM not shown). We could exclusively assign this weak competitive ability of the sexual lines to the 'red' lines among them; comparing the means of the asexual, sexual 'white' and sexual 'red' MA lines reveals a significant difference between them ($F_{2,39}$ = 25.60; $P < 0.001$). According to Tukey's test the sexual 'red' MA lines differ from the asexual and sexual 'white' MA lines, while the asexual and sexual 'white' MA lines do not differ from each other.

Analysis of investment of the MA lines in sexual spores

The reddish-brown secretion in the twenty-two sexual MA lines exactly coincided with very weak competition for asexual spore production. This suggested that these sexual lines would invest more in sexual spore production at the cost of asexual spore production. Therefore, we measured W_{ij} of the MA lines after a short time span - only competition for asexual spore production - and after a longer time span - competition for asexual and sexual spore production - to calculate the fitness change ΔW_{ij} (see Fig. 5.5). Significant differences occur among the mean ΔW_{ij} of the asexual, sexual 'red' and sexual 'white' MA lines ($F_{2,76} = 5.12$; $P = 0.008$). According to Tukey's test the sexual 'red' MA lines differ from the asexual and sexual 'white' MA lines, while the asexual and sexual 'white' MA lines do not differ from each other. We conclude that the sexual 'red' MA lines showed the highest ΔW_{ij}, probably due to higher sexual spore production.

For a decisive answer to this assumption we grew 10 asexual, 10 sexual 'red' and 10 sexual 'white' lines separately and counted in these lines the asexual and sexual spore production at the two time points, as was possible in the Coulter counter.

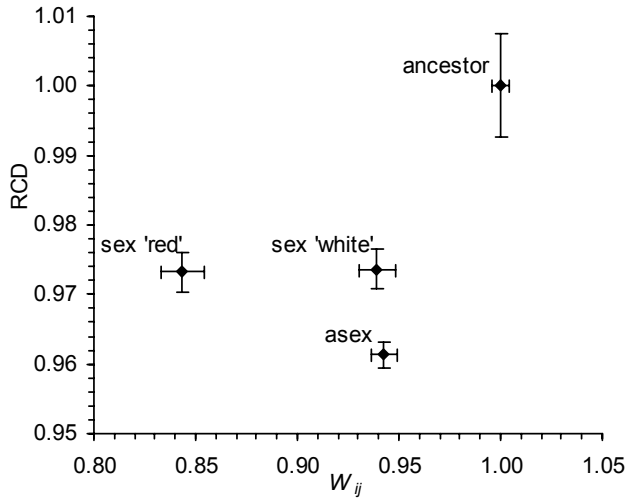

Figure 5.4 | ***W*$_{ij}$*'s* of sexual 'red' lines, sexual 'white' lines, asexual lines and ancestor are plotted against the previously obtained RCD's.** Mean ± SE values are shown.

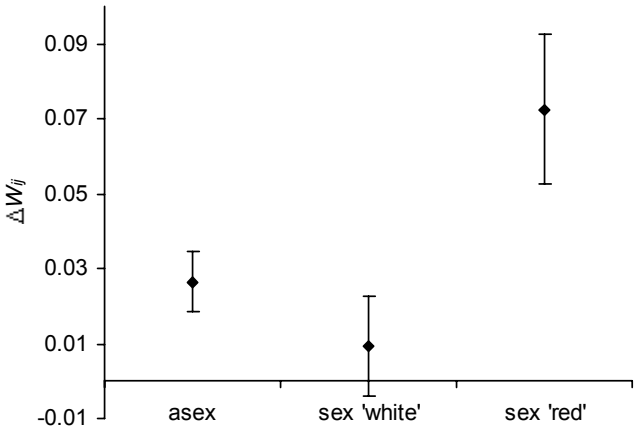

Figure 5.5 | **ΔW_{ij} of the asexual, sexual 'white' and sexual 'red' MA lines.** Mean ± SE values are shown.

Mean frequency distributions of spore diameter (the two tops in this distribution represent asexual and sexual spore distributions respectively) at two time points per group are depicted in Fig. 5.6. At the first time point, all three groups only invested in asexual spore production. Next, in the monitored time interval, the asexual and sexual 'white' lines invested in both asexual and sexual spore production. However, the 'red' sexual lines did not invest in asexual spore production in the monitored time interval. Instead, these lines invested only and more than the other lines in sexual spore production.

Discussion

In a former study, we compared mutation accumulation in asexual reproduction with that in sexual reproduction in this homothallic model fungus (Bruggeman *et al.*, 2003b). In that experiment, mutations were allowed to accumulate during 40 generations in 40 asexual reproducing lines and 40 sexual-selfing reproducing lines, all derived from a single founder colony. This procedure created for each line a history of 40 successive bottlenecks of a single asexual or sexual spore. Then, as a fitness estimate we measured colony diameter of all the mutation accumulation lines in a fixed time period relative to

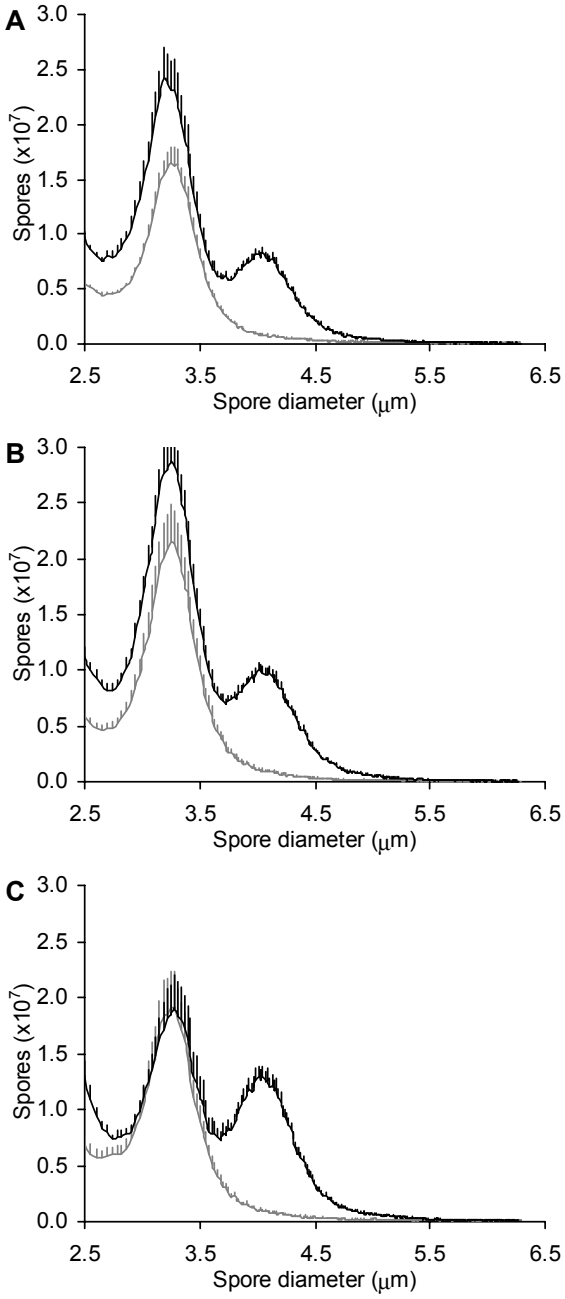

Figure 5.6 | **Frequency distribution of spore diameter from spores produced on a Petri dish as counted in the Coulter counter.** Left top represents the asexual spore distribution and the right top represents the sexual spore distribution. Gray line: time point 1; black line: time point 2. (A) Asexual lines. (B) Sexual 'white' lines. (C) Sexual 'red' lines. Mean + SE values are shown.

that of the ancestor (RCD). This trait was never selected in the mutation accumulation protocol. We found that mutations accumulated at a lower rate in the sexual lines than in the asexual lines, and we hypothesized that this was caused by more stringent intra-organismal selection in the sexual reproduction route.

In the present study, we used these MA lines to assess an additional fitness estimate and to determine the relationship between this fitness estimate and the previously employed relative colony diameter (RCD) estimate. As main additional fitness estimate for the mutation accumulation lines, we measured asexual spore production under a competition regime (W_{ij}) for reasons discussed in the introduction. The major result of this competition experiment was the bimodal fitness distribution of the sexual MA lines. Twenty-two of these 40 lines had a very low fitness and formed one part of the distribution, while 18 lines had a higher fitness and formed the other part of the distribution. This latter part coincided with the fitness distribution of the asexual lines. We found no correlation between the fitness estimate W_{ij} and the formerly obtained estimate based on RCD.

A reddish-brown pigment was present in the medium of the competition plates of the 22 sexual lines with very low W_{ij}. In *A. nidulans,* reddish-brown secretion produced by the hyphal cells is characteristic of sexually reproducing colonies (Mahony and Wilkie, 1958; Mahony and Wilkie, 1962) (see also Fig. 5.7). This reddish-brown secretion in a fraction of the sexual lines and its connection to very low competition strength for asexual spore

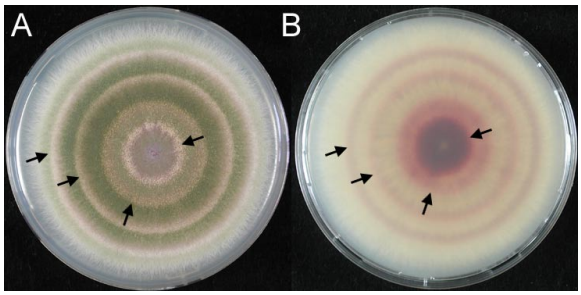

Figure 5.7 | **An *A. nidulans* colony alternates asexual and sexual spore production, which results in a circular pattern.** (A) The circular pattern is displayed at the front of the plate. (B) The circular pattern is also displayed at the back of the plate in the growth medium: each circle of reddish-brown secretion in the medium corresponds with a circle of sexual spore formation (indicated with arrows).

production, suggested that these lines had invested in sexual spore formation at the expense of asexual spore formation. This would imply a trade-off between sexual and asexual spore formation, which is predicted by life-history theory. A trade-off between mating efficiency and vegetative growth rate has been reported for the alga *Clamydomonas* (da Silva and Bell, 1992). Also, the plant *Allium vineale* allocates its reproductive energy to different reproductive modes according to trade-off principles (Ronsheim and Bever, 2000). For our study, two additional experiments confirmed such a trade-off hypothesis. First, during the time interval in which sexual spores are produced, the sexual 'red' lines had a significantly higher relative fitness increase than the asexual or sexual 'white' lines had (Fig. 5.5). Second, the Coulter counter revealed that sexual 'red' lines indeed produced fewer asexual spores and more sexual spores than the asexual or sexual 'white' lines produced (Fig. 5.6).

Thus, the experimental history of the mutation accumulation lines caused an unexpected result in 22 of the sexual lines. These lines became 'ultra-sexual' at the expense of asexual spore production, causing the chosen fitness estimate W_{ij} based on asexual spore production to become problematic. As a consequence of the difference in the ratio of asexual to sexual spore production between the 'red' and 'white' sexual lines, the two fitness measures RCD (relative colony diameter) and W_{ij} (asexual spore production under competitive conditions) were uncorrelated.

The nature and control of variation in the sexual phenotype in *A. nidulans* have been related to cytoplasmic factors (Croft, 1966; Mahony and Wilkie, 1962). Later, a sexual sporulation hormone -Psi factor- has been identified which mediates precocious sexual spore formation and inhibits asexual sporulation (Champe and El-Zayat, 1989; Champe *et al.*, 1987). Psi factor is an endogenous compound produced by *A. nidulans* which consists of three hydroxylated linoleic molecules, PsiA, PsiB and PsiC. The proportion of these three compounds controls the ratio of asexual to sexual spore development. Also plant seeds, when colonized by fungi, produce hydroperoxylinoleic acids. These factors resemble Psi factor and can also influence asexual and sexual sporulation in *A. nidulans* (Calvo *et al.*, 2001; Calvo *et al.*, 1999). Besides this Psi factor, also light influences the ratio of sexual to asexual spore production (Mooney *et al.*, 1990; Yager *et al.*, 1998). In wild-type strains, red light delays sexual development and induces conidium formation. Light, Psi factor, and seed resembling Psi factor, can only mediate their effect via an

unmutated *veA* gene. Recently, this gene has been identified and sequenced; over-expression of the gene caused the formation of increased numbers of sexual structures (Kim *et al.*, 2002).

We propose the following explanation for the 'ultra-sexual' phenotype in 22 of the sexual lines. The likelihood of acquiring a mutation resulting in this phenotype in each of these lines must be extremely small in an experiment in which each line has a history of 40 successive bottlenecks of a single ascospore. Therefore, this property in 22 of the sexual lines is most likely not genetic. Moreover, the ultra-sexual phenotype disappeared after consecutive reproduction via the asexual route and after sexual outcrossing (results not shown). We propose that our results can be explained by an epigenetic inheritance system in which the Psi hormone or a similar hormone is involved. The simplest epigenetic system is the steady-state inheritance system (Jablonca and Lamb, 1995, pp. 81-86). It is based on auto-regulation in which the gene product - in our case the Psi hormone or a similar hormone - acts as positive regulator of its own gene expression. The transmission of the functional state depends on the presence of a sufficient quantity of regulatory gene products -the hormone- in the spores. This system also explains the chance element involved: 22 of the sexual lines expressed the property. We want to emphasize that the 22 sexual 'red' lines were, as all the other lines, stored as asexual spore suspensions at –80°C and that the fitness measurements were always performed with fresh asexual spores grown from these stocks.

The active regulation of investment in asexual and sexual spore production under influences of diverse factors confirms the supposed different ecological and evolutionary roles for these two spore types. Ascospores are bigger than conidiospores, they require a longer developmental time, and under most conditions asexual spore production is much more abundant than sexual spore production (Adams *et al.*, 1998). Moreover, sexual spores in general are more resistant to adverse environmental conditions than asexual spores, due to their protective spore wall (Grishkan *et al.*, 2002). Last, asexual reproduction produces only clones, while sexual reproduction allows the production of recombinants in case of outcrossing. Clearly, under different conditions the two types of spores have to be weighted differently in their contribution to fitness: under some conditions mainly the asexual spores will give rise to offspring, while under other conditions most offspring will derive from sexual spores. Thus, although in principle

progeny number is the appropriate fitness measure, in a fungus with a flexible ratio between sexual and asexual spore production, offspring number cannot be derived in a straightforward way from spore production. Similar problems are to be expected in other modular organisms, and are indeed encountered in clonal plants (Pan and Price, 2002). Pan and Price (2002) plead for an integrated measure of clonal growth and seed production as fitness estimate in clonal plants, however they recognize the lack of integration so far. Our study shows that spore production as a measure of fitness is problematic and be better avoided. Instead, the rate of modular (mycelial) growth which we used previously (Bruggeman *et al.*, 2003b) should be preferred, and seems a good fitness estimate for a modular organism.

6

Summary and discussion

Selection arena

The selection arena hypothesis (Stearns, 1987) states that overproduction of zygotes –a widespread phenomenon in animals and plants- can be explained as a mechanism of quality control: An enlarged array of zygotes is created of which only a genetically superior subset will fully develop; zygotes with a low future fitness fail, while zygotes with a high future fitness thrive. In this way, parental energy for reproduction is invested in the most promising zygotes. This hypothesis further assumes that 1) zygotes are cheap to produce, 2) parental time, energy and/or risk are invested in the zygotes, 3) offspring vary in fitness, and 4) this fitness difference can be identified. In principle, quality control on future progeny is possible at earlier stages, for example at the germ-cell stage. However, selection at the zygote stage yields very reliable results because it contains the nuclear and mitochondrial genotype of the future progeny for the first time in combination. Yet, selection at this stage is in time before the parent starts major investments into the progeny.

In particular the botanical literature provides support for the selection arena hypothesis by trying to demonstrate that aborted ovules are potentially viable but would produce less vigorous progeny than non-aborted ovules would produce (Casper, 1988; Melser and Klinkhamer, 2001; Rocha and Stephenson, 1991; Stephenson and Winsor, 1986). Many of these studies employed experimental manipulations that can be questioned for their effect on zygote quality. Several authors speculated that spontaneous abortions in mammals signify the selection arena theory (Bolis *et al.*, 1985; Forbes, 1997; Gosling, 1986). However, it has been difficult to proof in mammals that these abortions are functional in selecting against low-quality zygotes in favour of high-quality ones.

Therefore, the present thesis set out to examine the selection arena hypothesis in the fungus *Aspergillus nidulans*. Related topics such as the male and female role in spore formation, and fitness estimation in this fungus were also investigated. We especially tested the predicted result of the arena in this fungus, namely that zygotes with a low future fitness would fail, while zygotes with a high future fitness would thrive. In **chapter 1** we put forward that this fungus fulfils the first three of the four conditions of the selection arena hypothesis. At the end of this chapter we set out of how fitness differences among future offspring can be identified, which is the fourth condition of the selection arena hypothesis.

A. nidulans has a typical fungal life cycle different from the life cycles of most plants and animals. It overproduces *dikaryotic fruit initials*, called dikaryons, just like animals and plants overproduce zygotes. Then, quality control might involve selection on which of these dikaryons will thrive to produce thousands of zygotes. These zygotes each produce eight sexual spores which together fill up one fruiting body.

We hypothesized to find a selection arena in this mycelial fungus because the selection arena can be a powerful tool to ensure offspring quality in such a modular organism. A mycelial network grows indefinitely and therefore the nuclei and mitochondria in its hyphae accumulate mutations. Deleterious nuclei and mitochondria may also be acquired from anastomosed mycelia. Moreover, the mycelium can be infected with deleterious viruses and plasmids (van Diepeningen, 1999; Meinhardt *et al.*, 1991). Any nucleus and cytoplasm from this mycelial network can end up in the spores since fungi have no germ line. The benefits of quality control seem obvious in such a system.

A. nidulans produces two spore types on its mycelial network, the asexual conidiospores and the sexual ascospores. Although the selection arena theory is usually mentioned in relation to sexual reproduction and zygotes, a similar mechanism of quality control may operate in the asexual route. However, in the sexual route of this fungus we expected *a priori* a more stringent selection for two reasons: investment in sexual spores is greater than that in asexual spores as they are bigger and require longer development time, and sexual spores are the survivors of environmental stresses that are used to re-establish a population (ecological role). Quality control should be the highest among those spores. Moreover, quality control on future progeny in the sexual cycle exploits the assumed benefits of recombination (Otto and Lenormand, 2002). Quality control is less pressing for asexual spores as these serve fast occupation and dispersal.

Chapter 2: The male and female role in ascospore formation

Only if the dikaryon passes the selection arena, it will eventually develop into a ripe fruiting body. *A. nidulans* is a thoroughly explored model organism in the fields of genetics, morphology and metabolism. However, the initiation of a fruiting body and especially - in the case of outcrossing - the roles of the nuclei and mitochondria from the two parental strains in its formation have remained unclear. In **chapter 2** we resolved

these roles by analysing the genetic constituents of these fruiting bodies (called cleistothecia) from crosses between vegetatively compatible and incompatible parents. We used markers that enabled us to determine the nuclear genotype of the cleistothecial wall and the nuclear and mitochondrial genotype of the ascospores. The result shows that in incompatible parents, the maternal strain contributes the nuclei for the cleistothecial wall and one nucleus as well as mitochondria for the ascospore origin. The paternal strain donates one nucleus for the ascospore origin. In compatible parents, nuclear genomes and cytoplasm usually mix in the vegetative hyphae prior to the formation of the sexual stage after which any cleistothecial composition is possible. Thus, the wall of the cleistothecium consists of vegetative tissue derived from the maternal strain; only the dikaryotic tissue inside the fruiting body is zygote tissue. Our findings are in accordance with a recent histological study on cleistothecium development, which reports that the ascogenous hyphae consist of a different tissue type than the surrounding network of sterile hyphae consist of (Sohn and Yoon, 2002). Until recently, fertilisation and the very early stages of the cleistothecium have not been observed. We detected a structure in *A. nidulans* with great similarity to the fertilisation structure described for the closely related *Aspergillus ruber* (Fig. 6.1). Also Sohn and Yoon (2002) depict the very early stages of cleistothecium formation in *A. nidulans*.

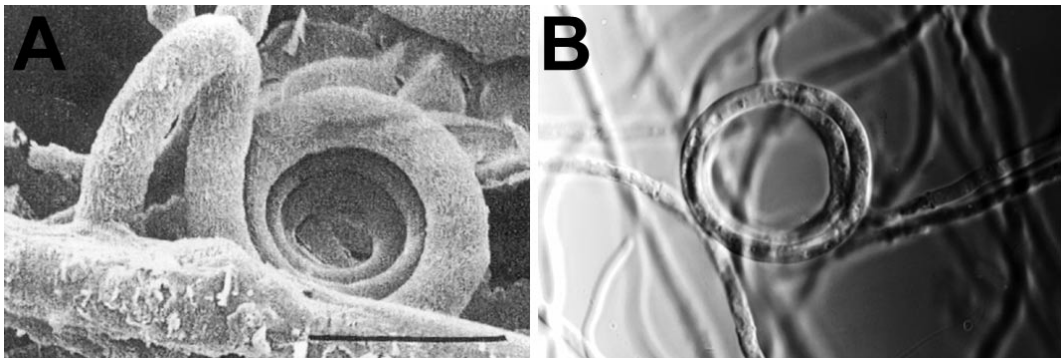

Figure 6.1 | **Ascogonial coils.** An ascogonial coil -the fertilisation structure- of *Aspergillus ruber (*from Champe and Simon (1992)). (B) We observed a similar structure in *A. nidulans*.

Chapter 3-5: Testing the selection arena in *Aspergillus nidulans*

In these chapters we tested the selection arena hypothesis in *A. nidulans* with emphasis on the predicted outcome, namely that zygotes with a low future fitness would fail, while zygotes with a high future fitness would thrive. We reformulated this into a testable prediction for this mycelial fungus: Nuclei with deleterious mutations will not be transmitted via the sexual route, or at least less easily than via the asexual route.

In **chapter 3,** we analysed 2 mitochondrial and 15 auxotrophic mutations for consequences on sexual and asexual reproduction. We found that many of these mutations confer sexual self-sterility as pleiotropic effect under conditions of normal asexual spore production: fruiting bodies were very tiny and contained hardly any ascospores. This confirms an important prediction of the selection arena, namely that dikaryons carrying a (slightly) deleterious mutation are not able to proliferate and produce sexual spores. So, no reproductive energy is invested in sexual spores carrying deleterious mutations, while asexual spore production is unaffected. Note that asexual and sexual spores would have carried the same genotype. Although self-sterility has been previously reported for a few mutations, these results have never been discussed as a manifestation of a selection arena (Busch *et al.*, 2001; Eckert *et al.*, 1999; Kurtz and Champe, 1981).

In **chapter 4** we tested the selection arena theory using a different approach. We exploited the coexistence of asexual and sexual reproduction in *A. nidulans*, especially in cases of sexual self-fertilisation, where offspring from the sexual and asexual pathway have the same genotype. Wild-type isolates preferentially self-fertilise (Butcher, 1968), but outcrossing is possible and does occur in nature (Geiser *et al.*, 1994). The selection arena hypothesis predicts that nuclei with deleterious mutations will not be transmitted via the sexual route, or at least less easily than via the asexual route. This implies then that the sexual spores - produced by selfing - will have a higher average fitness than the asexual spores with the same genotype. To investigate this, we started a mutation accumulation experiment with 40 asexual and 40 sexual selfing lines, all derived from one single ancestral strain of *A. nidulans*. In these lines, mutations were allowed to accumulate during 40 generations by single-spore transfer of respectively asexual and sexual spores. One generation refers to a full cycle of spore germination, mycelial colony growth, and (sexual or asexual) spore development. After 40 generations of mutation accumulation, the fitness of each line was estimated relative to that of the founder. Fitness was estimated

by measuring the colony diameter grown in a fixed time period (RCD = relative colony diameter). The results show that mutations accumulate in both groups, but, in accordance with the selection arena hypothesis, with a significantly lower 'fitness impact' in the sexual lines than in the asexual lines. We argue that genetic recombination can be excluded as an explanation for this result.

In **chapter 5** we estimated the fitness of the mutation accumulation lines using a different measure, namely asexual spore production under competitive conditions (W_{ij}). Such competition is harsh and provides a condition that likely amplifies potential differences in fitness between mutation accumulation lines and the ancestor. However, we did not find such a result. Instead, we found that the two fitness measures RCD (relative colony diameter) and W_{ij} (asexual spore production under competitive conditions) were uncorrelated. This appeared to be due to an 'ultra-sexual' phenotype in more than half of the sexual lines: these lines produced many sexual spores at the expense of asexual spores. The 'ultra-sexual' phenotype must have been caused by the experimental history in the mutation accumulation procedure and made the fitness estimate W_{ij} based on asexual spore production problematic. We discuss difficulties in fitness estimation in modular organism in general, especially when they produce progeny (spores) via both a sexual and an asexual pathway.

Mechanism of the selection arena

We confirmed the hypothesis to find a selection arena in the sexual cycle of the mycelial fungus *A. nidulans*. Although we did not aim to reveal the actual mechanism of this arena, in this section we will discuss its possible operation.

We envisage the selection arena as a genetic sieve that only allows passage of nuclei and cytoplasm with the highest fitness expectations. This sieve consists of two parts: bottlenecking and proliferation. The bottleneck phase consists of the creation of numerous initial dikaryons by a mycelial colony. The more dikaryons a colony creates, the more of its nuclei and cytoplasm will be exposed to selection. Usually, dikaryons start by recruitment of nuclei and cytoplasm from one mycelium (preferential selfing), but occasionally a male nucleus may be derived from another colony (rare outcrossing) (see also **chapter 2**). The proliferation phase consists of outgrowth of each of these fruiting

initials into a mature fruiting body. This outgrowth is fuelled by stored glucans in the mycelium which have been produced early in development from easily accessible carbon sources (Zonneveld, 1972). This proliferation seems to be a self-regulating mechanism controlled by two factors. The selection arena involves selection of which dikaryons are allowed to proliferate into mature fruiting bodies.

Off course, the most important factor for successful proliferation is the quality of the dikaryon itself. Dikaryons containing deleterious mutations or cytoplasm and thus being of low quality will not be able to complete development. In **chapter 3** we show that many biochemical mutations of *A. nidulans* confer sexual self-sterility as pleiotropic effect. In accordance with this, we show in **chapter 4** that deleterious mutations accumulate with a lower rate in the sexual cycle than in the asexual cycle. The dikaryons on self-sterile strains can develop into ripe fruiting bodies, provided that their quality is good, as we show that crossed dikaryons from two self-sterile strains do develop into ripe fruiting bodies (**chapter 3**).

The second factor is the condition of the maternal mycelium. For the biochemical mutations investigated in **chapter 3**, excess supply of the required nutrients could often restore development of the fruiting body. The importance of the maternal condition suggests that also bet hedging may play a role in sexual reproduction in *A. nidulans*. Bet hedging is a strategy adopted by organisms reproducing in unpredictable environments: under favourable conditions a maximum number of offspring is produced, but this number can easily be reduced under less favourable conditions without much energy expenditure. Although bet hedging may play a role in sexual reproduction, *A. nidulans* produces two spore types; offspring number is safeguarded by the quick production of large amounts of asexual spores. Another argument against bet-hedging being an important strategy in the sexual reproduction route of *A. nidulans*, is that progeny number is not only determined by the number of dikaryons a colony creates, but also largely by the extent of proliferation per dikaryon.

One could object, however, that sexual self-sterility of auxotrophic markers is a direct consequence of the expression of these genes during or after meiosis. This appears unlikely for the following reasons. Self-sterility associated with auxotrophy gives rise to tiny fruiting bodies caused by the early (pre-meiotic) disruption of their development. In contrast, the fruiting bodies of meiotic mutants of *A. nidulans* have a normal size, as is

expected from their late effect in the process of sexual spore formation (Swart *et al.*, 2001): after the huge proliferation of the dikaryotic tissue, meiosis is the second last division before the actual formation of the spores. In the unicellular alga *Chlamydomonas reinhardtii* no parental investment in zygotes takes place and therefore a selection arena is not expected. In accordance with this, most auxotrophic markers have no discernible phenotypic effect among zygotes in this alga (Orr, 1991).

In **chapter 4** we estimated three parameters underlying the mutation accumulation processes in the asexual and sexual lines: the mutation rate *(U*), the mean effect of a mutation *(s*), and the kurtosis of the gamma distribution of mutation effects (γ_2). We show that the selection arena reduces the *number* of transmitted mutations in the sexual spores compared with the number of transmitted mutations in the asexual spores. It remains unsolved whether the selection arena also reduced the *average fitness effect* of transmitted mutations in the sexual spores. However, we consider that mainly genes essential throughout the life cycle of the fungus will be tested for their quality during the proliferation of the dikaryon, especially the housekeeping genes. The biochemical mutations tested in **chapter 3** belong to this group of genes. Alleles that are lethal at particular stages of the life cycle - for example specific meiotic or mitotic mutations - are efficiently removed at those stages since *A. nidulans* is a haploid organism.

A final aspect of the mechanism of the selection arena that we would like to discuss concerns a trade-off between asexual and sexual spore production. In **chapter 5** we found evidence for a possible trade-off between asexual and sexual spore formation in *A. nidulans* and we reviewed the factors that are known to control the ratio of asexual to sexual spores. However, such trade-off was not found in the strains carrying mutations causing sexual self-sterility as pleiotropic effect (**chapter 3**): under self-sterile conditions asexual spore production was not higher than that under sexual self-fertile conditions. We believe that the ratio of asexual to sexual spores is determined early in the developmental program of *A. nidulans*, whereas the selection arena mechanism operates at a later stage. This is exemplified by the following: Under a 12 hr light/12 hr dark regime, a wild-type strain expands its colony while alternating asexual with sexual sectors. So, within these 12 hr, the ratio of asexual to sexual spores is decided upon. However, only after 40 hr of spore germination, the earliest stages of fruiting bodies are formed (Sohn and Yoon, 2002). At that stage, and shortly thereafter, the selection arena operates.

Generality of the selection arena and future research

The present thesis finds support for the operation of a selection arena in *A. nidulans*. This fungus, like other modular organisms, has no germ line in which the cells for the next generation are protected and safeguarded. Consequently, the selection arena could play an important role in quality control on future progeny. Accordingly, literature on selection arena mechanisms mostly concerns plants which are also modular organisms.

We believe that the selection arena hypothesis may clarify many unexplained observations in mycelial fungi. Some of these observations that require more detailed investigation are discussed below. All natural isolates of the fungus *Podospora anserina* show senescence (Rizet, 1953; van der Gaag *et al.*, 1998) which is highly correlated with instability of the mitochondrial genome and the accumulation of circular mitochondrial DNA fragments during vegetative growth (Griffiths, 1992). Sexual progeny derived from such senescing cultures are again juvenile with normal mitochondria, a phenomenon termed *rejuvenation*. We hypothesize that only dikaryons that started with relatively healthy mitochondria are able to complete fruiting body development; this effectively selects against deleterious mitochondria. A similar operation of the selection arena is demonstrated by the fact that matings between mitochondrial mutant strains of *P. anserina* yield progeny with only wild-type mitochondria (Silliker *et al.*, 1996). Plausibly, transmission of the mutant mitochondria to ascospores is prevented such that only dikaryons containing wild-type recombinant mitochondrial DNA molecules could pass the selection arena.

It seems a general phenomenon in ascomycetous fungi that viruses are hardly or not transmitted into ascospores (Rogers *et al.*, 1988): for example in *A. nidulans* (Coenen *et al.*, 1997), *Cryphonectria parasitica* (Anagnostakis, 1988), and *Ophiostoma ulmi* (Rogers *et al.*, 1986). The selection arena hypothesis might account for this phenomenon. We speculate that virus-infected dikaryons display lower developmental vigour than virus-free dikaryons, and will not be sustained by the fungal mycelium. Finally, we believe that the selection arena hypothesis can clarify the observation that many mutations in *Neurospora crassa* cause female sterility or lead to reduced fertility (DeLange and Griffiths, 1980; Perkins *et al.*, 1982).

Further research on the selection arena hypothesis can provide insight into the following questions:

- Which genes are expressed during the proliferation of the dikaryon and where do these gene expressions differ from those expressed during the asexual and vegetative phases?
- Does the selection arena prevent the transmission of any mutation or does it preferentially prevent the transmission of mutations with a large effect?
- Do different natural isolates of *A. nidulans* display genetic variation in selection arena stringency?

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Samenvatting

Selectie arena

Een zygote is de cel die wordt gevormd door het versmelten van twee geslachtscellen. Veel planten en dieren produceren meer zygoten dan zij tot volwaardige nakomelingen ontwikkelen. De selectie arena hypothese stelt dat deze overproductie van zygoten verklaard kan worden als een mechanisme van kwaliteitscontrole op de nakomelingen. Er worden met ópzet meer zygoten aangemaakt: alleen de genetisch superieure zygoten zullen ontwikkelen tot volwaardige nakomelingen, terwijl zygoten met een lage toekomstige fitness afvallen. Dit mechanisme zorgt ervoor dat energie voor reproductie zo optimaal mogelijk wordt aangewend en niet wordt verspild aan zygoten met slechte toekomstverwachtingen. De selectie arena hypothese veronderstelt de volgende randvoorwaarden: 1) de vorming van zygoten kost weinig energie, 2) de ouder investeert tijd, energie en/of risico in de zygoten, 3) er is variatie in fitness tussen de nakomelingen en 4) deze fitnessverschillen kunnen vroegtijdig worden opgespoord.

Kwaliteitscontrole op toekomstige nakomelingen is bijvoorbeeld ook mogelijk door selectie op geslachtscellen, want niet alle geslachtscellen worden ook daadwerkelijk gebruikt voor de vorming van zygoten. Echter, kwaliteitscontrole van zygoten is betrouwbaarder, omdat daarin voor het eerst de nucleaire en mitochondriële genotypen van de toekomstige nakomelingen samengebracht zijn. Bovendien is het nog op tijd voordat de ouder energie gaat investeren in de zygoten.

De selectie arena hypothese wordt vaak aangehaald in de plantenliteratuur. Planten en bomen produceren vaak veel meer vruchtbeginsels dan zij uiteindelijk tot rijpe vruchten

ontwikkelen. In deze literatuur wordt dan geprobeerd aan te tonen dat de geaborteerde vruchtbeginsels potentieel wel levensvatbaar zijn, maar genetisch minder goede nakomelingen zullen produceren dan de niet geaborteerde vruchtbeginsels. Spontane abortie bij zoogdieren, waaronder ook de mens, wordt soms in verband gebracht met de selectie arena hypothese. Echter, het blijft in alle gevallen zeer moeilijk om te bewijzen dat abortie gericht is tegen zygoten met een lage fitness ten gunste van zygoten met een hoge fitness.

In dit proefschrift heb ik de selectie arena hypothese in de schimmel *Aspergillus nidulans* onderzocht. De schimmel *A. nidulans* is geschikt om de hypothese van de selectie arena te onderzoeken, omdat deze schimmel zich goed leent voor experimentele manipulatie. Verder voldoet deze schimmel aan de eerste drie randvoorwaarden van de selectie arena hypothese. Dit leg ik uit in **hoofdstuk 1**. *A. nidulans* heeft een typische schimmellevenscyclus, die anders is dan die van de meeste planten en dieren. Zoals planten en dieren zygoten vormen, zo vormt deze schimmel dikaryotische vruchtbeginsels, die dikaryons worden genoemd. (Dit zijn cellen waarin twee kernen worden samengebracht. Dit kunnen twee identieke kernen zijn in het geval van zelfbevruchting of twee verschillende kernen in het geval van uitkruising.) Vervolgens wordt er geselecteerd welke van deze dikaryons zich zullen ontwikkelen tot duizenden zygoten. Deze zygoten produceren ieder acht seksuele sporen en bij elkaar vullen zij dan één vruchtlichaam, het cleistothecium geheten. Deze ontwikkeling is schematisch weergegeven in figuur 1.1B op bladzijde 8.

Een schimmel is een organisme dat bestaat uit draden, genaamd hyphen, die tezamen een myceliumnetwerk vormen. Deze hyphen kunnen oneindig doorgroeien, zowel in tijd als in ruimte. Onder andere hierdoor behoren schimmels tot de modulaire organismen (in plaats van tot de unitairen). De kernen en mitochondria in de hyphen accumuleren genetische mutaties tijdens deze groei. Ook kunnen de hyphen geïnfecteerd raken met virussen en plasmiden, of kunnen ze versmelten met hyphen van andere myceliumnetwerken. Omdat schimmels geen aparte kiembaan bezitten, kan elke kern en cytoplasma uit zo'n mycelium terechtkomen in de sporen. Juist in zo'n organisme kan het selectie arena mechanisme een belangrijk instrument zijn om de kwaliteit van de nakomelingen te bewaken en om energie voor reproductie zo efficiënt mogelijk aan te wenden.

A. nidulans produceert twee soorten sporen op zijn myceliumnetwerk, namelijk aseksuele conidiosporen en seksuele ascosporen. De selectie arena hypothese gaat meestal over seksuele reproductie, hoewel deze theorie net zo goed op aseksuele reproductie betrekking kan hebben. Echter, ik verwachtte *a-priori* een strengere selectie in de seksuele reproductie dan in de aseksuele reproductie: 1) seksuele sporen zijn duurder om te maken dan aseksuele sporen omdat ze groter zijn en meer ontwikkelingstijd vergen. 2) seksuele sporen overleven beter in stressvolle omstandigheden, waardoor zij de start vormen van een nieuw op te bouwen populatie. Juist voor zulke sporen verwacht je de strengste kwaliteitscontrole. Daarbij komt nog dat kwaliteitscontrole in de seksuele reproductie de veronderstelde voordelen van seksuele recombinatie extra uitbuit.

Hoofdstuk 2: De mannelijk en vrouwelijke rol in de vorming van vruchtlichamen

Slechts indien een dikaryon door de selectie arena komt, zal het zich ontwikkelen tot een volgroeid vruchtlichaam met sporen. *A. nidulans* dient als modeldier voor veel biologische gebieden zoals genetica, morfologie en metabolisme. Daarom is er al veel van zijn biologie opgehelderd. Echter, het eerste beginsel van een vruchtlichaam en de mannelijke en vrouwelijke rol van de twee ouderstammen in de vorming van zo'n vruchtlichaam, zijn onduidelijk gebleven. In **hoofdstuk 2** hebben we deze bijdragen opgehelderd middels het analyseren van de genetische samenstelling van vruchtlichamen van kruisingen tussen vegetatief compatible én van kruisingen tussen vegetatief incompatibele ouderstammen. Hiervoor gebruikten we markers die het nucleaire genotype van de wand van het vruchtlichaam konden identificeren en gebruikten we markers die het nucleaire en mitochondriële genotype van de ascosporen binnenin het vruchtlichaam konden identificeren.

De genetische samenstelling van vruchtlichamen van kruisingen tussen vegetatief incompatibele ouderstammen toonde het volgende aan: De ene ouderstam verschaft de kernen voor de start van de cleistotheciumwand en één kern en mitochondria voor de start van de ascosporen. Deze stam vervult de vrouwelijke rol. De andere stam doneert één kern voor de start van de ascosporen. Dit is de mannelijke stam. Wij ontdekten dat elke genetische samenstelling van vruchtlichamen mogelijk was in kruisingen tussen twee vegetatief compatibele ouderstammen. Dat komt omdat de kernen en het cytoplasma van

twee compatibele ouderstammen met elkaar mixen in de vegetatieve hyphen, nog voordat er een vruchtlichaam wordt gemaakt. Verder concluderen wij dat de wand van een cleistothecium bestaat uit vegetatief weefsel afkomstig van de moeder. Slechts het dikaryotische weefsel binnenin een vruchtlichaam is zygoten weefsel. Onze resultaten laten ook zien dat vegetatieve incompatibiliteit tussen twee stammen geen beletsel is voor seksuele interacties.

Hoofdstuk 3-5: Het testen van de selectie arena in *Aspergillus nidulans*

In deze hoofdstukken hebben we de selectie arena hypothese getest in *A. nidulans*. Hierbij hebben wij de nadruk gelegd op de voorspelling van deze hypothese, die luidt dat alleen de genetisch superieure dikaryons zullen ontwikkelen tot volwaardige nakomelingen, terwijl zygoten met een lage toekomstige fitness afvallen.

In **hoofdstuk 3** hebben we 2 mitochondriële en 15 auxotrophe mutaties onderzocht naar hun effect op seksuele en aseksuele sporulatie. We hebben ontdekt dat velen van deze mutaties leidden tot seksuele zelfsteriliteit terwijl aseksuele sporulatie normaal was: vruchtlichamen waren erg klein en bevatten nauwelijks seksuele sporen. Dit bevestigt de selectie arena hypothese, namelijk dat dikaryons met een nadelige mutatie niet in staat zijn zich te ontwikkelen en sporen te vormen. Er wordt dus geen energie geïnvesteerd in seksuele sporen met een mutatie, terwijl wel in aseksuele sporulatie wordt geïnvesteerd. Merk op dat de seksuele en de aseksuele sporen hetzelfde genotype hadden.

In **hoofdstuk 4** hebben we gebruik gemaakt van het opmerkelijke feit dat de zelfbevruchte seksuele sporen van een *A. nidulans* kolonie een zelfde genotype hebben als dat van de aseksuele sporen. Seksuele zelfbevruchting is een intrigerend fenomeen omdat het voordeel van seksuele voortplanting voornamelijk wordt gezocht in recombinatie. De selectie arena theorie voorspelt dat kernen met een nadelige mutatie niet zullen worden doorgegeven via de seksuele route, althans moeilijker dan via de aseksuele route. Dit impliceert dat de seksuele sporen van een kolonie een hogere fitness zullen hebben dan de aseksuele sporen met hetzelfde genotype. Om dit te onderzoeken hebben wij een mutatie accumulatie experiment uitgevoerd in 40 seksuele lijnen en 40 aseksuele lijnen, die allen afkomstig waren van één gemeenschappelijke voorouder. Deze lijnen werden gedurende 40 generaties voortgezet door overenten van slechts één enkele seksuele of aseksuele

spore. Op deze manier wordt selectie vermeden en zullen kleine nadelige mutaties ophopen in de lijnen. Na 40 generaties van mutatieophoping hebben we de fitness van elke lijn bepaald ten opzichte van de fitness van de voorouder. Als fitnessmaat bepaalden wij de koloniediameter gegroeid in een vast tijdsinterval (RCD = relatieve koloniediameter). Wij ontdekten dat zowel in de aseksuele als in de seksuele lijnen mutaties ophoopten. Dit gebeurde echter, overeenkomstig de selectie arena hypothese, met een significant lagere 'fitness impact' in de seksuele lijnen. We zetten uiteen dat recombinatie in de seksuele lijnen uitgesloten kan worden als oorzaak van dit resultaat.

In **hoofdstuk 5** hebben we de fitness van de mutatie accumulatie lijnen op een andere manier bepaald, namelijk via aseksuele sporenproductie onder competitieve omstandigheden (W_{ij}). Het is namelijk bekend dat competitieve omstandigheden de fitnessverschillen tussen mutatie accumulatie lijnen en voorouder kunnen vergroten. Wij vonden echter helemaal niet dit te verwachten resultaat. Integendeel, de twee fitnessmaten RCD en W_{ij} waren niet aan elkaar gecorreleerd. Dit werd veroorzaakt door een 'ultraseksueel' fenotype in meer dan de helft van de seksuele lijnen. Deze lijnen produceerden veel seksuele sporen ten koste van aseksuele sporen. Dit ultraseksuele fenotype is waarschijnlijk epi-genetisch en is veroorzaakt door de experimentele geschiedenis in de mutatie accumulatie ophoping. Deze bevindingen maken het gebruik van de fitnessmaat W_{ij} (gebaseerd op aseksuele sporenproductie) problematisch. We bediscussieerden de problemen van fitnessbepalingen in modulaire organismen in het algemeen, vooral wanneer zij zowel seksuele als aseksuele nakomelingen vormen.

Tot slot, dit proefschrift toont aan dat een selectie arena opereert in *A. nidulans*. Hoewel wij niet hebben onderzocht wat het eigenlijke mechanisme van de arena is, schetsen wij de volgende mogelijkheid: Een *A. nidulans* kolonie legt veel dikaryotische vruchtbeginsels aan. De selectie arena selecteert welke van deze dikaryons daadwerkelijk zullen ontwikkelen tot rijpe vruchtlichamen met sporen. Wij veronderstellen dat de ontwikkeling tot een rijp vruchtlichaam een autonoom proces is, afhankelijk van de eigen kwaliteit van het dikaryon. Slechts wanneer dikaryons genoeg eigen metabole potentie hebben, zullen zij zich succesvol kunnen ontwikkelen. Nucleaire of cytoplasmatische mutaties en virussen en plasmiden in het aangelegde dikaryon, kunnen de kwaliteit en dus de ontwikkeling van het dikaryon nadelig beïnvloeden. Op deze manier selecteert de

selectie arena tegen dit soort lage kwaliteit dikaryons, en voorkomt het dat energie voor reproductie aan hen wordt verspild.

Nawoord

Ik weet nog goed dat ik de advertentie voor mijn OIO-plaats bij de vakgroep Erfelijkheidsleer in de Volkskrant had gelezen en dacht; dat lijkt mij nog eens een leuk en interessant onderzoek. Toen wist ik hoegenaamd niets van schimmels en hun genetica. Nu heb ik dan na bijna 6 jaar werk dit proefschrift geschreven. Een proefschrift schrijven doe je niet alleen. Veel mensen van de vakgroep Erfelijkheidsleer hebben hun steentje bijgedragen. Fons Debets, als geestelijk vader van de selectie arena in *Aspergillus nidulans* heb jij mij geholpen in alle facetten van het onderzoek. Hartelijk dank voor je hulp bij het opzetten van experimenten, het werken in het lab en het structureren van de artikelen. Ook wil ik Rolf Hoekstra bedanken, mijn promotor. Onder jouw leiding is ons lab een prettige en ontspannen plek om onderzoek te doen. Ook was je altijd beschikbaar om het werk te bespreken en heb je de puntjes op de i gezet bij het schrijven van de artikelen. Marijke Slakhorst wil ik bedanken voor de praktische hulp bij bijna al mijn experimenten. Arjan de Visser bedank ik voor zijn bijdrage aan het mutatie accumulatie experiment. Met de hulp van Pieter Wijngaarden en 'zijn' computerprogramma zijn alle data netjes verwerkt en ben ik heel wat wijzer geworden over statistiek. Klaas Swart, bedankt voor het lezen en de verbeteringen van het Zonneveld manuscript. Drie MLO studenten hebben meegewerkt aan mijn project tijdens hun stage bij ons laboratorium, namelijk Simon Koopmans, Erwin Vergunst en Siti van Houten. Ook jullie bedankt! Aafke van der Kooi en Corrie Eekelder houden het secretariaat van Erfelijkheidsleer draaiende en Anita zorgt altijd maar weer voor schoon en gesteriliseerd glaswerk. Ook dank voor jullie ondersteuning.

Bedankt voor de goede tijd en vriendschap, ook alle andere mensen op het schimmellab: Siemen Schoustra, Peter van Baarlen, Henk dalstra, Karoly Pal, Marc Maas, Ronnie Schoonenberg, Duur Aanen, Anne van Diepeningen, Marijn van der Gaag, Edu Holub, Elaine Aparecida de Souza, Bertha Koopmanschap. Ik ben mijn promotieonderzoek gestart met de cursus Microbiële Genetica samen met Trudy van den Bosch. Leuk dat je mij bij wilt staan met de finish van dit onderzoek!

Als laatste gaat mijn dank uit naar Dennis. Jij moedigde mij altijd aan om door te zetten en was altijd belangstellend. Samen hebben we op een warme zondag in de koude kamer de koloniediameters van de mutatieaccumulatie lijnen gemeten en heb jij ook meegeholpen met het tellen van de 154.942 kolonies. Wat ik nu verder ga doen in het leven weet ik nog niet, maar ik doe het in ieder geval samen met jou!

Curriculum vitae

Judith Bruggeman

Op 6 januari 1973 ben ik geboren in Schiedam. Ik groeide op in het nabij gelegen dorp Maasland. Van 1985 tot 1991 fietste ik door weer en wind 12 km naar het Sint Stanislas College te Delft, waar ik het gymnasium-β diploma heb behaald. In 1991 ben ik begonnen aan de studie biologie aan de Vrije Universiteit (VU) Amsterdam, met als specialisaties Neurobiologie en Beleid, Bestuur & Natuurwetenschappen. De specialisatie Neurobiologie heb ik als volgt ingevuld: een histologisch onderzoek naar de lokalisatie van neuropeptiden in het centraal zenuwstelsel van de poelslak (VU); een scriptie over de rol van neuropeptiden in het zich ontwikkelende zenuwstelsel (VU), een scriptie over de invloed van het sympathische zenuwstelsel op het immuun systeem (TNO Preventie en Gezondheid); modelleren van de electrische activiteit van zenuwcellen op de computer (VU). De specialisatie Beleid, Bestuur & Natuurwetenschappen heb ik als volgt ingevuld: beleidsstage bij TNO Preventie en Gezondheid te Leiden, waar ik heb meegewerkt aan een project over Assistive Technology, vrij vertaald ‘technologie voor zelfstandig leven van ouderen, gehandicapten en chronisch zieken’. In 1996 haalde ik het doctoraal diploma. Vervolgens heb ik een half jaar gewerkt in het klinisch geneesmiddelen onderzoek als trial administrator bij IMRO te Berghem. In december 1997 ben ik begonnen als onderzoeker in opleiding bij de vakgroep Erfelijkheidsleer van Wageningen Universiteit. De resultaten van bijna 6 jaar werk staan in dit proefschrift beschreven.

The work presented in this thesis was carried out at Wageningen University, Laboratory of Genetics, the Netherlands, with financial support of the Netherlands Organization for Scientific Research (NWO), grant number 805-36-355.

Coverdesign by DINGO / Peter Oosterhout, Diemen

October 2003

Stellingen

1. De seksuele sporen van *Aspergillus nidulans* zijn van betere genetische kwaliteit dan de aseksuele sporen.
(Dit proefschrift)

2. Een kennelijk gebrek aan inzicht in het feit dat vegetatieve compatibiliteit tussen stammen invloed heeft op de genetische samenstelling van vruchtlichamen (Zonneveld. 1988. Trans. Brit. Mycol. Soc. 90, 369-373; Rowlands & Turner. 1976. Genet. Res. Camb. 28, 281-290; Dit proefschrift) leidt tot onjuiste of onvolledige conclusies in de publicaties van Hoffman *et al.* (2001. Genetics 157, 141-147) en Li Destri Nicosia *et al.* (2001. Mol. Microbiol. 39, 1330-1344).

3. De kans op een kind met een chromosomale afwijking neemt toe met de leeftijd van de moeder. De suggestie van Forbes (1997. Trends Ecol. Evol. 12, 446-450) dat dit verschijnsel veroorzaakt wordt door een selectie arena die met het ouder worden van de moeder minder streng wordt, is niet waarschijnlijk.

4. In een kruising tussen twee bijna isogene *Aspergillus nidulans* stammen vonden Hoffman *et al.* (2001. Genetics 157, 141-147) vruchtlichamen die niet alleen sporen afkomstig van uitkruising, maar ook sporen afkomstig van zelfbevruchting bevatten. Als dit waar is, moet de genetische kaart van *Aspergillus nidulans* worden herzien.

5. Tekstboeken over 'life history theory' richten zich voornamelijk op unitaire organismen en laten modulaire organismen onbehandeld. Daardoor is de algemene geldigheid van die theorie zeer twijfelachtig.

6. 'Onder alle wetenschappen zijn die het populairst, die het meest beïnvloed worden door de waan van de dag, de dwaze mode.' (Erasmus, Lof der zotheid) In het licht van deze 500 jaar oude observatie, is de oproep 'kies techniek' tot mislukken gedoemd.

7. De moderne hoogwaardige communicatiemiddelen garanderen geenszins hoogwaardige communicatie.

8. De overgewichtepidemie kan voor een deel opgelost worden door het in ere herstellen van de huishoudschool.

Stellingen behorende bij het proefschrift 'Selection arena in *Aspergillus nidulans*' door Judith Bruggeman, te verdedigen op 26 november 2003 te Wageningen.