On the evolution of allorecognition and somatic fusion in ascomycete filamentous fungi

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Chapter 1

General introduction

Eric Bastiaans

The evolution of cooperation

Cooperation can increase productivity or efficiency by exchange of goods and services with others. For example a house is built by a group of cooperating people. Each of these people is specialized and most productive in doing something different for example making a foundation, plumbing or roofing. By division of labour this house can be built much faster with less total work hours compared to when one person alone builds the same house. In nature, such division of labour can take extreme forms, such as in the social insects. Evolutionary theory predicts that individuals that can use available resources most efficiently for reproduction and survival in a certain environment will have the highest fitness, i.e. their genes will be transferred relatively more to the next generation. This will lead to selection of the best adjusted genotypes to that environment. By cooperating, individuals can potentially obtain a higher fitness than individuals that do not cooperate. Social insects like for example honeybees form colonies in which many sterile workers maintain the colony so that the queen of the colony can effectively reproduce. The workers themselves cannot reproduce which makes them true altruists that sacrifice their own reproduction for the benefit of the colony (Breed et al. 1982). Cooperation does not always involve division of labour as in the previous example, but cooperation can also be advantageous because a large group is simply more efficient to perform certain tasks than a single individual. For example, parasitic bacteria of the species *Pseudomonas aeruginosa* excrete siderophores, which are required for the uptake of iron (West and Buckling 2003). The excreted siderophores bind to insoluble iron after which the bacteria can take it up in their cells. Since the siderophores are excreted from the cell, they become a common good that all surrounding bacteria can use as well. If siderophores are produced by a large group this will be more efficient because relatively less siderophores will get lost by diffusion. Cooperation can be mutually beneficial, for example when there is a mechanism that enforces the receiver to cooperate as well. However, often cooperation is not mutually beneficial and gives a benefit for the receiver but is costly to the actor. This leads to a problem to explain the evolution of stable cooperation. Individuals that use the benefits provided by others, but do not contribute their fair share to cooperate, will have a selective advantage within a group of cooperating individuals. For example, in a group of siderophore producing bacteria it is advantageous not to invest costs in producing siderophores while using the siderophores produced by others. This way, every bacterium in the group has the same benefits from the common pool of siderophores but the non-producer has an advantage because it saves on the costs of producing siderophores. If this kind of selfish behaviour is a heritable mutation, it will be selected through natural selection, because of the relative advantage it provides to a carrier, until the social behaviour disappears completely. This theory, which predicts that cooperation is unstable because of its vulnerability to selfish or cheating individuals, is known as the tragedy of the commons (Hardin 1968) (figure 1-1, top panels).

Kin selection theory

The most widely accepted theory to explain how cooperation within a species can evolve stably is Hamilton's kin selection theory (Hamilton 1964a, b) also known as inclusive fitness theory. Essential in this theory is that cooperative behaviour or goods are directed with a higher probability to genetically related individuals, in such a way that the genes of the helper benefit more than the helping action costs. Hamilton's rule summarizes this in the simple formulized condition that an allele for cooperation can be selected if $rB > C$. Where r is the average relatedness of the actor to the individuals receiving the benefit, B is the fitness benefit received from cooperating and C is the fitness cost for the cooperating individual. In other words the Benefit of cooperation should always be higher than the costs, but the difference between cost and benefit can be smaller if help is directed towards more related individuals. This principle has been used regularly to try to explain cooperation as observed in nature for example in the social insects (Foster et al. 2006). Kin selection on itself is not always sufficient to explain stable evolution and may require additional mechanisms (Bourke 2011). For example several social insect species require active policing against cheaters to compromise for a reduced relatedness between members of the colony (Ratnieks et al. 2006).

The evolution of genetic kin recognition and Crozier's paradox

Kin selection requires a way to direct cooperation preferentially towards kin (genetically related individuals) rather than random individuals. One way is keeping kin together by population viscosity after propagation, as for example a bacterial colony on solid medium where bacteria stay next to each other after division. When individuals are more motile and are likely to meet non-kin, active kin discrimination is required for kin selection. Active kin discrimination can occur through the use of environmental or genetic cues (Grafen 1990). Whereas environmental cues, such as prior association or shared environment, are probably most important in higher organisms such as the social insects (Helanterä and Sundström 2007), kin discrimination in microorganisms usually involves genetic cues (West et al. 2007). Also in plants, some evidence for genetic kin recognition exists to regulate how roots of neighbouring plants interact, but little is known about possible mechanisms (Chen et al. 2012). Furthermore, kin recognition is also present in most multicellular organism to maintain relatedness among the cooperating cells within a multicellular individual (see next paragraph about multicellularity). Genetic kin recognition requires polymorphic recognition genes that have a higher probability to be shared between closely related individuals than between unrelated individuals. Crozier made a model based on colony forming marine invertebrates such as anemones, where neighbouring colonies can fuse if it they are clonally related, or actively compete by attacking each other if they are not closely related. This discrimination based on relatedness is genetically determined. Fusion between colonies is a cooperative process in which the individuals likely gain in fitness compared to actively competing with

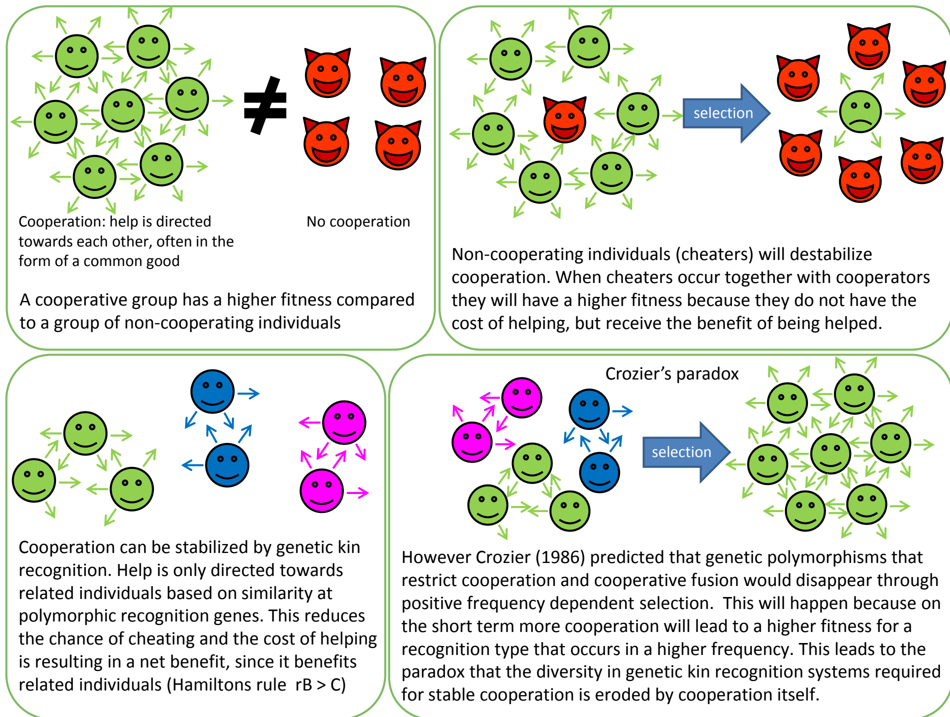

Figure 1 The evolution of cooperation and genetic kin-recognition

each other. Crozier's model predicted that the fitness advantage of cooperation by fusion favours common recognition alleles over rare alleles, since common alleles will receive more benefit from cooperation (Crozier 1986). This positive frequency dependent selection could eventually eliminate the recognition polymorphism in a population. This leads to a paradox: the advantage of cooperation erodes the recognition diversity on which that cooperation relies (see figure 1.1 for a schematic overview of cooperation, kin recognition and Crozier's paradox). This theoretical prediction is now sometimes referred to as "Crozier's paradox" or the "Crozier paradox" (Tsutsui et al. 2003; Aanen et al. 2008; Holman et al. 2013). In contrast to this theoretical prediction, recognition genes are often highly polymorphic. Crozier's model applies to all genetic recognition mechanisms that stabilize cooperative behaviours (Tsutsui et al. 2003; Aanen et al. 2008).

Multicellularity: cooperation between cells

Kin selection theory is also very important to explain the existence of multiple levels of selection in which a group of cooperating lower level entities forms a selectable higher level entity. During evolution several major transitions took place towards more complex life forms that evolve at a higher selection level than the elements they evolved from (Buss 1987; Maynard Smith and Szathmary 1995). For example, from simple cells complex cells evolved in which multiple mitochondria cooperate to increase the cell fitness and thus also the fitness of the group of mitochondria in that cell. From these complex cells the transition to multicellularity was next and took place multiple times (Grosberg and Strathmann 2007). Multicellularity essentially is an intensely cooperating group of cells that form an individual of a higher selection level. In order to maintain high fitness at the level of the multicellular individual it is very important that cooperation at the lower levels is stable. Cheating at the lower level, in the form of cells that increase their individual fitness at the cost of the fitness of the multicellular level, is a realistic threat as we see so often when cancer cells form life threatening tumours. Theory predicts that even more than other forms of cooperation, multicellularity is mainly stabilized by the high relatedness observed among cells of multicellular organisms (Buss 1987). This theory is supported by the fact that multicellular organisms have mechanisms to maintain high relatedness among the cells. Probably the most important mechanism that maintains high relatedness within multicellular organisms is the existence of regular single-celled bottlenecks in their lifecycle. The single cell divides via mitosis forming a clonally related group of cells that stay connected. So essentially a multicellular organism is an extremely viscous population of cells. Additionally, many organisms have a strict separation of the germline from the soma early in development. The somatic cells now are an evolutionarily dead end and can only contribute to their inclusive fitness by cooperating. As a consequence new mutations that arise in the soma cannot spread to the next generation. Another important property of most multicellular organisms that maintains the realised high relatedness is that they have genetic allorecognition mechanisms to regulate fusion, aggregation or grafting with conspecific cells or tissues (Buss 1982). Allorecognition restricts interactions to clonally related individuals and thus is an extreme form of kin-recognition. Allorecognition is most relevant for maintaining high relatedness in species for which aggregation or fusion is an important part of their life cycle, since this can lower the high relatedness obtained via a single-celled bottleneck. Aggregation of cells or fusion between groups of cells is a quick way to increase the size of a multicellular individual compared to mitotic division. Therefore aggregation or fusion can be beneficial to start a multicellular individual or to expand its size, as long as the risk of somatic cheating can be kept low. Thus, therefore it is expected that allorecognition is important in these species to maintain high relatedness and minimize the risk of cheating. Experimental evidence for the importance of high relatedness among the cells of multicellular organisms is scarce however.

Experimental models for the evolution of multicellularity and allorecognition

A popular model organism that is used for studying the evolution of multicellularity and the potential conflicts associated with it is *Dictyostelium discoideum*. This amoeba becomes multicellular via aggregation of single cells. During the main part of its lifecycle *Dictyostelium* lives as motile clonally dividing single cells. Under starvation conditions, however, the cells aggregate to form a multicellular fruiting body in which a part of the cells altruistically end up in a stalk structure to help the remaining cells disperse as spores. This division of labour is sensitive to cheating as a mutant that has a higher probability to become a spore will be selected. Therefore it is likely that high relatedness is important to maintain stable cooperation within the multicellular stage of *Dictystelium*. It has been shown that there is indeed a kind of kin discrimination in *D. discoideum* to increase relatedness within the aggregate (Ostrowski et al. 2008). However, the mechanism is not known yet and kin-discrimination is not very strong (Gilbert et al. 2012).
A popular model organism to study the evolution of allorecognition is *Botryllus schlosseri* and other marine colony-forming invertebrates. Although a lot is known about the molecular basis of allorecognition and the species are suitable for lab experiments, the model has not proved suitable to experimentally test and solve Crozier's paradox (Grosberg and Hart 2000; De Tomaso 2006; Rosengarten and Nicotra 2011).

Filamentous ascomycete fungi as a model for the evolution of multicellularity and allorecognition

In my thesis, I used filamentous ascomycete fungi as an experimental model to study the evolution of allorecognition and multicellularity because they combine interesting, easy to manipulate aspects of multicellularity and allorecognition. The already well-established fungal model organisms can give new insight and provide experimental support for existing theories because it has a well described very precise genetic allorecognition mechanism for different species, it can be easily handled and manipulated in laboratory experiments, relatedness within a multicellular individual can be easily manipulated during evolution experiments, important fitness components can easily be measured, relative fitness to a competitor can be determined and finally its lifecycle contains multicellular growth by mitotic expansion combined with optional fusion between individuals that is potentially both beneficial and costly. Especially the fact that fusion is optional makes ascomycete fungi an excellent model to study the effect of relatedness. In addition fungi are also of great economical and medical relevance, because of their wide usage in food industry, agriculture and chemical industry, and their negative role in human health and agricultural pests. For this reason, new knowledge about the evolution of fungi is likely to be also of use in the future for the application or control of fungi.
Although there is a substantial literature on the evolution of allorecognition and multicellularity, fungi are generally underrepresented in this literature. In a paper about somatic cheaters

in *Dictyostelium mucoroides*, Buss compiled a comprehensive list of phylogenetic groups that show evidence of a recognition mechanism to regulate somatic compatibility between individuals within a species, which included fungi, *Dictyostelium* species and marine colonial invertebrates (Buss 1982). In the same paper, he used fungi as an example for the costs and benefits of fusion. The problem to explain the diversity of the recognition or compatibility genes in fungi has been studied and discussed in the literature (Hartl et al. 1975; Nauta and Hoekstra 1994). However, more recent literature reviewing the evolution of allorecognition tends to neglect fungi as a potential model for the evolution of allorecognition (Tsutsui 2004; De Tomaso 2006). Aanen (Aanen et al. 2008) brought the fungi back in the spotlight as a model for the evolution of allorecognition. Although in some respects the growth of filamentous fungi differs from the growth of other multicellular organisms (see below), the evolutionary stability depends on cooperation between lower-level entities. Therefore, allorecognition is generally believed to have evolved as a protection against cheating nuclei, or as a side effect of selection for something else like pathogen recognition (see chapter 2). A third more fungal-specific hypothesis is that allorecognition is selected to prevent the spread of parasitic cytoplasmic elements such as deleterious mitochondrial plasmids and mycoviruses (van Diepeningen et al. 1997; Debets and Griffiths 1998; Brusini et al. 2011). This last hypothesis is not relevant to most other multicellular organisms because these groups have cell compartments, limiting the mobility of cytoplasmic elements, so that they can only spread together with their host cell, while fungi generally have less compartmentalisation (see next paragraph).

Fungal multinuclear lifecycle

In the life cycle of filamentous ascomycete fungi, individuals grow from sexually derived ascospores or asexual spores (see for example (Moore-Landecker 1996) for an overview of fungal biology). The primary mode of growth is via mitotic expansion. The spores contain a single or, more often, multiple haploid nuclei. After germination, these spores grow tube-like structures called hyphae that split up and fuse so that they radially grow into a network that forms the fungal colony called the mycelium. During growth nuclei divide mitotically within these hyphae. Although I consider the fungi to be multicellular, most ascomycete fungi do not have cells, as we see in other multicellular organisms. Fungal hyphae are compartmentalized by septa; however, the septa contain pores that allow cytoplasm and often nuclei to pass. This facilitates transport of molecules, organelles and nuclei to the place where they are required. So, instead of multicellular organisms, fungi could better be classified as multinuclear syncytia. This means that selection at the lower level does not take place at the level of cells, but rather at the level of nuclei (Roper et al. 2011). However, the nuclear genotype still determines the qualities of the local mycelium, so hyphal compartments with nuclei that improve the fitness qualities such as growth speed can be selected by outgrowing other hyphae. Many ascomycetes can form both sexual and asexual spores. The nuclei of

asexual spores or conidia are clonally derived from the nuclei in the mycelium and often grow soon after the mycelium is formed. Ascospores or sexual spores are a meiotic product, and usually require a compatible mate, but several ascomycete fungi are self-fertile. There is no separate germline for the formation of spores, which means that all nuclei in the mycelium can potentially end up in the spores.

Fungal colonies can fuse with neighbouring colonies

When two conspecific neighbouring colonies growing towards each other come in contact, their hyphae will fuse. The fungal allorecognition mechanism then determines whether the colonies are compatible and this fusion will be successful. In case of successful fusion, the two colonies will be connected and cytoplasm and nuclei can mix. In case of incompatibility, the fused hyphal compartments will be disconnected from their original mycelia by closure of their septa and enter a programmed cell death program (figure 1.2 and 2.1). The dead hyphal compartments now form a border between the incompatible colonies, which in some fungi is clearly visible under laboratory conditions as a so-called 'barrage'. The genetics of hyphal fusion has been well described in *Neurospora crassa* and many genes are involved that regulate homing of hyphae towards each other, and the initiation of fusion (Read et al. 2010). Mutants of these genes affect not only fusion of hyphae between two colonies but also fusion of hyphae within a colony. Therefore, these mutants cannot grow a network structure anymore, but rather form a treelike structure because hyphae only split up. As a result, these mutants often show aberrant growth and reduced spore production, probably because transport within the mycelium is less efficient. So fusion within a mycelium is an important component of fungal growth, while fusion between colonies is not required for a good functioning mycelium.

Allorecognition in fungi

The allorecognition mechanism has also been well described at the morphological and genetic level (Glass et al. 2000; Saupe 2000a; Aanen et al. 2010). Allorecognition in fungi has been variously described in the literature as vegetative incompatibility, somatic incompatibility and as heterokaryon incompatibility. The latter term refers to the fact that a heterokaryon, a mycelium with genetically different nuclei, with different allotypes, is not viable and will die, or segregate into different homokaryons. The recognition genes called *het*-genes or *vic*-genes are well described for the model species *Neurospora crassa* and *Podospora anserina,* and more recently for the phytopathogenic species *Cryphonectria parasitica* (Aanen et al. 2010; Choi et al. 2012). In general, each fungal species displays around ten unlinked *het*-loci, which in most cases have a limited number of alleles. If two individuals differ in at least one of these genes, they are of a different allotype resulting in somatic incompatibility. The high number of genes involved results in a high number of allotypes, so that in general two random natural isolates are incompatible, unless they are clonally related.

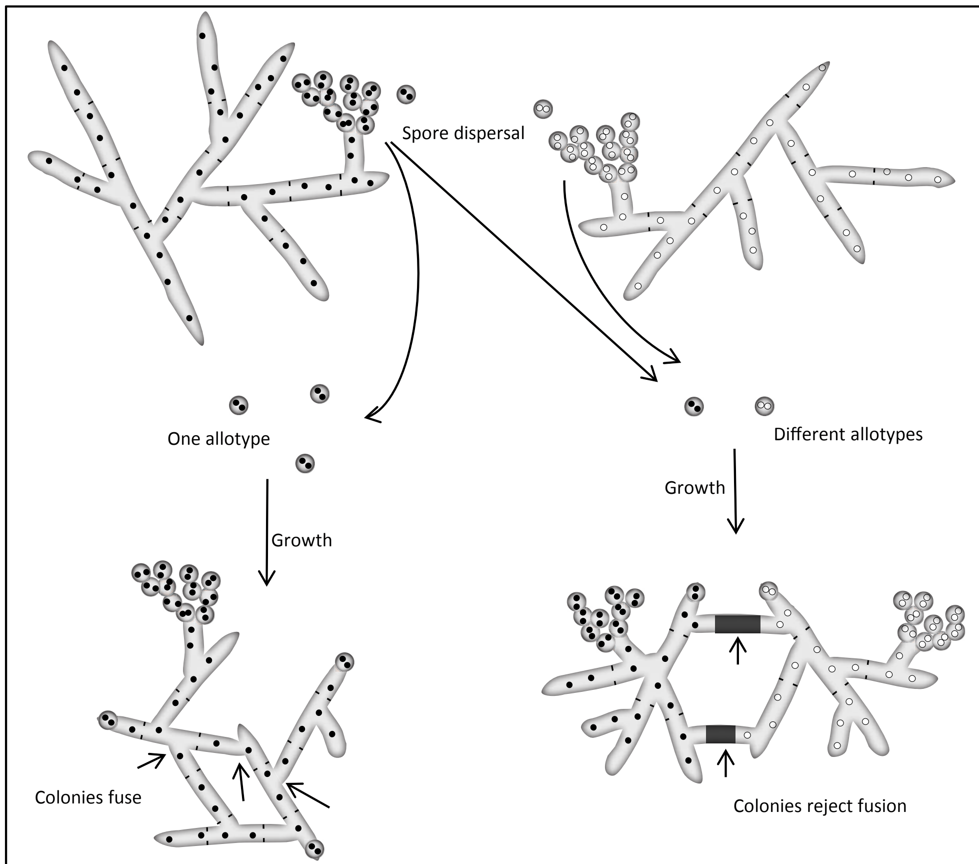

Figure 2 growth of the ascomycete colony with and without allorecognition
Sexual or asexual spores with one or more nuclei (black and white dots) develop on the mycelium and disperse. Spores can land somewhere alone (not shown), but they can also end up together with other conspecific spores. After the spores germinate the nuclei divide mitotically and the hyphae grow to form a network like colony. Hyphae from neighbouring colonies that come in contact always initiate fusion. If the colonies have the same allotype, fusion will be successful and a single coherent colony is formed in which nuclei and cytoplasm can move around freely (left side, arrows indicate fusion points). If the colonies have different allotypes the fused hyphal compartments are closed off from the rest of the colony and die, preventing mixing of cytoplasm and exchange of nuclei of the neighbouring colonies (right side, arrows indicate rejection of fusion).

The model species used in this thesis

Fungi have been studied extensively with a focus on their ecology, genetics and cell- and molecular biology. One reason to study fungi is that they are microbial eukaryotes that are very convenient to handle in the lab and generally have short lifecycles. Another important reason to study fungi is that they are of huge economical and medical importance. Because

of this several well-established fungal model organisms have been developed in the past century. However as a model to study various evolutionary theories, fungi were not used so often, but more recently fungi are frequently used in evolutionary research (e.g. Schoustra et al. 2006; Menkis et al. 2008; Nieuwenhuis and Aanen 2012).

In this thesis, I use the species *Neurospora crassa*, *Neurospora intermedia* and *Podospora anserina* because of their well-described allorecognition mechanism, and their establishment as a model for fusion and somatic incompatibility. Most of the research in this thesis involves experiments in which cultures that were grown under different conditions are compared. *Neurospora* is very suitable for lab experiments that can be kept simple and rapid by using asexual spores rather than sexual spores. For *Neurospora crassa* many strains are available with different allorecognition types. Also a fusion mutant is available which is used in this thesis to maintain high relatedness in evolution experiments and for testing the fitness effects of fusion. *Podospora anserina* does seem to have allorecognition genes that have evolutionary features different from other known allorecognition genes in fungi. I studied the diversity of one of these genes for this thesis.

Setup and aim of this thesis

This thesis is addressing the paradox posed by Crozier (Crozier 1986) that the allorecognition diversity required for stable cooperation is eroded by the selective pressure of cooperation itself. Importantly, Crozier's model did not take into account one key component of cooperation: the possibility of cheating, i.e. saving costs on contributing while still profiting (Grafen 1990). Using verbal arguments, Grafen demonstrated that the occurrence of cheating can maintain polymorphism at a kin recognition locus, because common alleles are hit harder when cheating arises. Another hypothesis from Crozier himself is that allorecognition diversity is maintained by selection for something else (Crozier 1986). He considered it most likely that the recognition loci had a role in immune defence, so they that were kept polymorphic because of host-parasite interactions.
We use fungi as a model to investigate how somatic fusion, stable multicellularity and allorecognition are linked. The main question addressed in this thesis is: What selective pressures drive the evolution of allorecognition in fungi? And closely related to that: Do allorecognition and the associated high-relatedness aid the evolution and maintenance of stable multicellularity in fungi?
For this, we first established that fusion indeed is beneficial and allorecognition thus costly, which causes selection pressure against allorecognition diversity. After this we present results from experiments that support several hypotheses that explain the evolution and maintenance of allorecognition diversity.

In **chapter 2** the problem of evolution of allorecognition is introduced: "The cost of allorecognition may reduce the genetic variation upon which allorecognition crucially relies, a prediction now known as Crozier's paradox". We introduce ascomycete fungi as a model and show that allorecognition in fungi can be costly compared to fusion. We then show experimental evidence that this cost will indeed cause positive frequency-dependent selection on allotypes, which leads to erosion of allotype diversity. We further show that there are two costs associated with allorecognition. First, the average size of the individual colonies in a culture decreases with allorecognition leading to a fitness reduction. Second, the fact that tissue is sacrificed in the antagonistic reaction to incompatible neighbour colonies imposes an extra cost. At the end of this chapter, we discuss the hypotheses that explain what maintains allorecognition diversity despite the costs associated with it.
In **chapter 3, 4, and 5** experiments are presented that support these hypotheses. In **chapter 3,** we address the hypothesis that by maintaining high relatedness among allorecognition provides protection against somatic cheaters that evolve from cooperative cells. We answer the question: How realistic is somatic cheating in the absence of allorecognition? We show in an evolution experiment with *Neurospora crassa* that cheating nuclei indeed evolve quickly

under low-relatedness conditions (unrestricted fusion, complete mixing and a high density), resulting in an increased competitive fitness, but a strong decrease in spore production of the culture. In **chapter 4** we address the hypothesis that allorecognition can restrict selection of parasitic cytoplasmic elements. For this, we use mitochondria that become defective under the influence of a mitochondrial plasmid, and then behave as selfish elements that cause senescence in *Neurospora*. These mitochondria represent cheating genotypes as well since they do not invest in respiratory function. We demonstrate how relatedness influences the selection of such cheating mitochondria, and finally show that allorecognition can prevent or delay senescence caused by these mitochondria. In **chapter 5** we address the hypothesis that allorecognition diversity can also be the result of selection for another function. We explore the diversity in a population of *Podospora* of an allorecognition gene that is much more polymorphic than other allorecognition genes in fungi. The data provide some support for a possible role of this gene in pathogen recognition. We discuss how this function can relate to its role in allorecognition.

This thesis ends with a general discussion in **chapter 6**. I summarize the conclusion on Croziers paradox applied to fungal ascomycete allorecognition, and reflect on future work that can be done to follow up the work in this thesis. In addition to this I reflect on the relevance of allorecognition in different phylogenetic groups is discussed. Finally I briefly discuss the relevance and potential applications for which this research may be relevant.

Chapter 2

Experimental demonstration of the benefits of somatic fusion and the consequences for allorecognition

Eric Bastiaans, Alfons J.M. Debets and Duur K. Aanen

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Abstract

Allorecognition, the ability to distinguish 'self' from 'non-self' based on allelic differences at allorecognition loci, is common in all domains of life. Allorecognition restricts the opportunities for social parasitism, and is therefore crucial for the evolution of cooperation. However, the maintenance of allorecognition diversity provides a paradox. If allorecognition is costly relative to cooperation, common alleles will be favoured. Thus, the cost of allorecognition may reduce the genetic variation upon which allorecognition crucially relies, a prediction now known as 'Crozier's paradox'. We establish the relative costs of allorecognition, and their consequences for the short-term evolution of recognition labels theoretically predicted by Crozier. We use fusion among colonies of the fungus *Neurospora crassa*, regulated by highly variable allorecognition genes, as an experimental model system. We demonstrate that fusion among colonies is mutually beneficial, relative to absence of fusion upon allorecognition. This benefit is due not only to absence of mutual antagonism, which occurs upon allorecognition, but also to an increase in colony size *per se*. We then experimentally demonstrate that the benefit of fusion selects against allorecognition diversity, as predicted by Crozier. We discuss what maintains allorecognition diversity.

Introduction

Allorecognition, the ability to distinguish 'self' from 'non-self', is common in all domains of life, ranging from bacteria to plants and animals (Tsutsui 2004; Chen et al. 2012; Pathak et al. 2013). It regulates the outcome of somatic fusion between different tissues, but also antagonism between individuals belonging to different colonies of social insects (Holman et al. 2013). Allorecognition is believed to be crucial for the evolution of cooperation, including cooperation among the cells of multicellular organisms, as it restricts the opportunities for social parasitism (Buss 1982; Buss 1987; Czaran et al. 2014).

The late Ross Crozier (Crozier 1986) identified a theoretical problem in explaining the evolution of allorecognition. Most allorecognition systems are based on highly diverse gene polymorphisms, often at multiple recognition loci (Laird et al. 2005; Aanen et al. 2010). Crozier argued that the relative costs of allorecognition will provide a selective force against the genetic diversity of cues required for allorecognition. If fusion provides a benefit, or if rejection is costly, the common allele will always be favoured, because it will fuse more often than rare alleles. Therefore, the relative costs of allorecognition 'eat up' the genetic variation upon which it crucially relies, a prediction now known as 'Crozier's paradox' (Crozier 1986; Rousset and Roze 2007; Aanen et al. 2008). However, the costs of allorecognition have rarely been experimentally established. In this article we established the relative costs of allorecognition compared to somatic fusion, and their consequences for the short-term evolution of recognition labels, as theoretically predicted by Crozier.

As a model system, we used the ascomycete fungus *Neurospora crassa*. Somatic growth of fungi differs from other multicellular organisms in several respects. First, fungal individuals consist of filaments (hyphae), which are branching and fusing regularly to form a dense, radially growing, network called the mycelium, or fungal colony (Moore-Landecker 1996). Furthermore, in contrast to most other multicellular organisms, cell compartmentalization is not very strong and in some fungi nuclei can freely move through parts of the colony (Moore-Landecker 1996). Therefore, the units among which cooperation can occur in the fungal colony are not the cells, but the haploid nuclei (Rayner 1991). Similar to almost all multicellular organisms, fungal colonies develop via mitotic divisions from a single-celled zygote, either a sexually or asexually produced spore, maximizing relatedness among nuclei (Maynard Smith and Szathmary 1995; Kondrashov 1997). However, fungal colonies can also increase in size through somatic fusion between germinating spores during colony establishment and between mature colonies (Roca et al. 2005b; Read et al. 2010). Somatic growth and fusion thus determine the size of the fungal colony.

The exact fitness consequences of fusion have not been studied in great detail yet, but fusion is likely to have a positive effect on fitness (Pringle et al. 2003; Richard et al. 2012; Simonin et al. 2012). Fusion in fungi is regulated by genetic allorecognition systems based on gene polymorphisms at several loci, restricting fusion almost exclusively to clonally related colonies (Glass et al. 2000; Saupe 2000a; Aanen et al. 2010). Successful fusion between colonies requires matching at all recognition loci, otherwise colonies are somatically incompatible and fusion will be interrupted by programmed cell death of the fused hyphal compartments at the border of two colonies (Fig. 1A).We hypothesized that the total fitness of successfully fusing colonies will be higher than that of incompatible colonies, due to two factors. First, allorecognition results in post-fusion cell death, presumably negatively affecting fitness. Second, there may also be a benefit of increased colony size *per se* increasing the benefit of successful fusion relative to allorecognition. To separate these two effects, we made use of a fusion mutant (Fleissner et al. 2005), in addition to using strains with different allotypes. Colonies of the fusion mutant lack the ability to fuse altogether and thus do not show antagonistic cell death. This allowed us to separate the effect on fitness of allorecognition and the effect on fitness of colony size *per se*.

The objectives of this study were i) to establish the costs of allorecognition relative to successful fusion; ii) to explain the relative costs of allorecognition by disentangling the benefit of fusion *per se* from the costs of antagonism following allorecognition, and iii) to experimentally demonstrate that the benefit of fusion causes positive frequency-dependent selection on the allorecognition types, causing erosion of allorecognition, as theoretically predicted by Crozier (Crozier 1986).

Material and methods

Strains and culturing conditions

The lifecycle of our model *Neurospora crassa* has a sexual and an asexual route (Davis 2000). The sexual cycle requires interaction between two strains from different mating type, and typically takes about two weeks under laboratory conditions. The asexual cycle, in contrast, does not require interaction between different mating types and requires only two days of growth after which abundant asexual spores are formed. All strains used were obtained from the Fungal Genetics Stock Center (FGSC) and strain numbers refer to the FGSC catalogue (McCluskey 2003). All experiments were performed at 25ºC with cycles of 12 hours light and 12 hours darkness, on Vogels Minimal Medium (VMM) with 2% sucrose as carbon source and solidified with 2% agar (Davis 2000). VMM contains all the necessary ingredients for optimal growth during the asexual lifecycle.

Testing the fitness of cultures with unrestricted fusion and cultures with restricted fusion due to allorecognition

To test whether there is a cost of allorecognition relative to successful fusion, we determined the effect of fusion on the yield of asexual spores, an important fitness parameter (Pringle and Taylor 2002). For this we compared the yield of cultures with varying degrees of fusion between colonies. We manipulated the degree of fusion in a culture, by varying the number of allotypes (isogenic except at some allorecognition genes). By mixing allotypes, fusion will be restricted by the allorecognition reaction resulting in a border of dead hyphae between colonies. In contrast, a culture grown from spores of a single allotype will form a single coherent colony, because all hyphae can successfully fuse (fig 1A). The assay was done with the following FGSC strains, all of a different allotype: 1423, 1424, 1427, 1428, 1430, 2657, 2660, and 2662. These strains are virtually isogenic through repeated backcrossing, except for selected differences at four allorecognition genes (Wilson and Garnjobst 1966). Mixed cultures were made with varying numbers of these strains in equal frequencies (eight, five, three, two and one; all in triplicate; Supplementary Table S1).

Experiments were done in glass tubes (150x17.5mm) with 7.5 ml of VMM medium solidified under an angle of approximately 60 degrees, resulting in a surface area of approximately 7.14 cm^2. Asexual spores were inoculated as 20µl suspension and spread out over the surface using a glass bead. The inoculation density used was $5*10^5$ asexual spores per culture ($\sim 7*10^4$ spores/cm^2). After seven days of growth, asexual spores were harvested by adding 5 ml of sterile water to the culture tube followed by thorough vortex mixing. This suspension was then used to estimate spore yields using a haemocytometer. A linear regression test with spore yield as dependent variable and fraction of successful fusion as independent variable was performed to test whether there is an effect of fusion on the production of asexual spores. The fraction of successful fusion for these cultures was calculated as 1 divided by the number of allotypes in the mixture.

Test to disentangle the effect of colony size per se and the allorecognition reaction on fitness

To investigate the effect of colony size on the cost of allorecognition we compared cultures with fusion restricted by allorecognition (eight allotypes) and cultures with unrestricted fusion (one allotype) at a range of inoculation densities. Increasing the inoculation density in a mix of allotypes will reduce both the average colony size and also increase the fraction of dying hyphae, but not in a culture with unrestricted fusion (fig2A).

With an additional experiment, we tested whether the effect of inoculation density on cultures with restricted fusion is caused by the restriction in size of the separate colonies only, or also by the increased proportion of dead hyphae. For this we used a fusion mutant, inoculated in the same range of inoculation densities as the mix of allotypes. The fusion mutant lacks

the ability to fuse, so that colony size is negatively correlated with density, but without the typical allorecognition reaction in the mixture of different allotypes (figure 2A). Density assays were done with the same strains as in the first experiment described here and with the fusion mutant *soft* (FGSC11293) (Fleissner et al. 2005).The fusion mutant is mostly isogenic to the other strains used here, except for a mutation in a gene necessary for hyphal fusion. The mix of eight strains, the monocultures from strain FGSC1427 and monocultures from the fusion mutant were grown at a range of inoculation densities from 4 to $4*10^7$ (4, 8, 20, 40, 80, 200, 400, $4*10^3$, $4*10^4$, $4*10^5$, $4*10^6$, $4*10^7$) asexual spores per culture (~0.56 to ~$5.6*10^6$ /cm^2). The other seven strains (FGSC 1423, 1424, 1428, 1430, 2657, 2660, and 2662) in monoculture were only grown at the two extreme densities of 4 and $4*10^7$, in order to test whether allorecognition is costly at these densities. All experiments were done using five replicates. Fitness was measured again as asexual spore yield similar to the first experiment described here.
For all three culture types (unrestricted fusion, allotype mix, and fusion mutant) a
linear regression with yield as dependent variable and ^{10}log inoculation density as independent variable was performed to test whether there is an effect of inoculation density on fitness. For densities 4 and $4*10^7$ a Tukey HSD test with spore yield as a variable was performed to see whether the eight strains grown in monoculture differed in spore yield from the allotype mix. After visual inspection of the graph of figure 2C showing the effect of density on the soft strain, we decided to test whether there is an effect of density on spore yield at the lower densities up to 400 spores per culture. We performed a similar linear regression on this subset of the data for the fusion mutant and the allotype mix cultures.

Testing for positive frequency dependent selection on allotypes

In order to test whether positive frequency dependent selection will reduce the diversity of allotypes, we tested how the relative fitness depends on allotype frequency. For this we determined the relative yield of a strain in two treatments: i) in competition with a single other allotype or ii) in competition with a group of four other allotypes.
We inoculated the tested strain in a 1:1 ratio with the competing strain or group of strains, and determined the yield of the tested strain, relative to the competing strain or group of strains. In the first treatment the tested strain was inoculated at the same frequency as the competing strain, so the allotype ratio is 1:1. In the second treatment, the competing group consisted of four allotypes so that the tested strain started with a four times higher frequency relative to the four allotypes in the competing group (with an allotype ratio of 4:1:1:1:1; Fig. 3A). By comparing the relative yield of the tested strain after competition in both treatments, we were able to test the prediction that positive frequency-dependent selection on the recognition types will erode allotype diversity. We used two colour markers, white (mutation) and orange (wildtype), to distinguish the two competing groups. We used four strains of each marker (FGSC numbers orange strains: 1422, 1436, 1437, 1455; FGSC numbers white strains: 1423,

1428, 1430 and 2656). These eight strains are all of different allotype based on differences at four allorecognition genes (Wilson and Garnjobst 1966). In treatment 1, each of these eight strains was grown at a 1:1 ratio, separately with all four strains with the other colour marker. In treatment 2, each of the eight strains was grown in a 4:1:1:1:1 ratio to all four strains from the other marker together. All competition experiments were replicated three times. To start competitions, asexual spores from the competing strains were spread on solid medium in glass tubes similar to the previous two experiments described here. The inoculation density was 10^6 asexual spores per culture (~$1.4*10^5/cm^2$). In order to determine the exact ratio of the competing groups, appropriate dilutions of the spore suspension used for inoculation were spread on counting plates and grown for about one week until colonies could be distinguished by their colour. Counting plates are Petri dishes with VMM adjusted by replacing sucrose with sorbose and adding 0.05% fructose and 0.05% glucose (sorbose-VMM) on which growth is restricted, so that single spore colonies remain small (Davis 2000).

Competition mixtures were grown for seven days under standard growth conditions. Afterwards, spores were harvested as described for the first experiment, after which appropriate dilutions were spread on counting plates to determine the ratio after competition. Relative yield (Ry) was calculated as the percentage of colonies from the tested strain (white or orange colonies) after competition corrected for deviations in the starting ratio from the intended 50 percent (Ry = (end percentage/start percentage)*50). The comparison between the two treatments was done in two ways. First all data were used to compare the two treatments in a Wilcoxon signed ranks test, with the null hypothesis being no difference between treatments. Second, for all eight strains tested, we compared treatment 1 and 2 in an independent samples Mann-Whitney U test with n=12 for treatment 1 and n=3 for treatment 2.

Results

Somatic fusion increases fitness.

We compared the spore yield of cultures with a single allotype, which have unrestricted fusion between colonies with the spore yield of cultures consisting of a mixture of allotypes, in which fusion is restricted. Unrestricted fusion between colonies will result in a single coherent network of hyphae. Adding different allotypes decreases the chance of successful fusion, resulting in restricted colony size and dying hyphae at the borders between incompatible colonies (Fig. 1A). We found a highly significant positive relationship between spore yield and the degree of fusion resulting from the degree of allotype diversity in a culture (Fig. 1B; linear regression; $F_{1,40}$=103.557, p<0.0005). These results imply that fusion provides a fitness advantage relative to failure to fuse, caused by allorecognition.

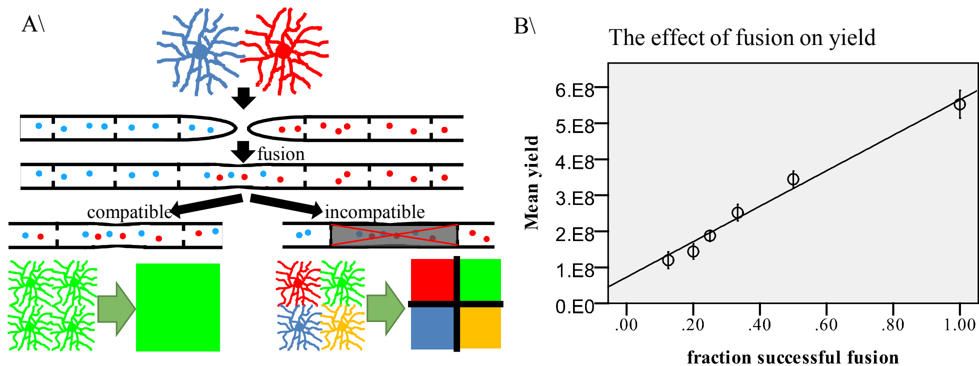

Figure 1 The consequences of fusion on fitness.
(A) Fungal fusion and allorecognition. The hyphae of filamentous ascomycete fungi not only fuse *within* a colony, but also between colonies of the same species. The outcome of such fusion is regulated by highly polymorphic recognition genes. If two fusing hyphae carry the same alleles at all of their recognition loci, fusion will be successful, and one coherent colony will be formed. However, if the two colonies differ at one or more of their recognition loci, the fused hyphal compartment will die, resulting in an interaction zone of dead cells and separate colonies. This allows us to experimentally manipulate the degree of successful fusion by inoculating mixtures of asexual spores with varying numbers of different allotypes. (B) Regression analysis showed a highly significant positive relationship between the fraction of successful fusion (x-axis) and average yield (number of asexual spores; y-axis).

The benefit of fusion is due to reduced cell death and increased colony size.

We hypothesized that the benefit of fusion depends on inoculation density, as the inoculation density determines the average colony size (of a single coherent mycelium) in a mixture of spores and the proportion of death hyphae as a result of the allorecognition reaction(Fig. 2A). Consistent with our hypothesis, we found a highly significant negative relationship between the inoculation density and yield for cultures consisting of eight allotypes to restrict fusion (linear regression; $F_{1,58}$=186,086, p<0.0005), but not for cultures with unrestricted fusion ($F_{1,58}$=0.11, p=0.918; Fig. 2B). The effect of allorecognition on fitness is not detected at very low densities. At the lowest density (~0.56 spores/cm^2) the culture with restricted fusion did not differ significantly from all eight allotypes grown in monoculture (unrestricted fusion) (Tukey HSD test; $p \geq 0.829$), while it differed significantly from all of the eight monocultures at the highest density (~5. 6*10^6 spores/cm^2) (Tukey HSD test; p<0.0005).

In principle, two effects may cause the fitness difference between fusion and restricted fusion in a culture grown at high densities. First, unrestricted fusion will lead to a single coherent colony, while restricted fusion will lead to smaller, isolated colonies (Fig. 2A). If the efficiency of reproduction or space and nutrient use depends on colony size, reproduction will be more efficient in large colonies than in small colonies, and thus be positively correlated with the degree of fusion. Especially for very small colonies it is easy to imagine that a colony can be too small to support specialized structures, like aerial hyphae, needed for spore

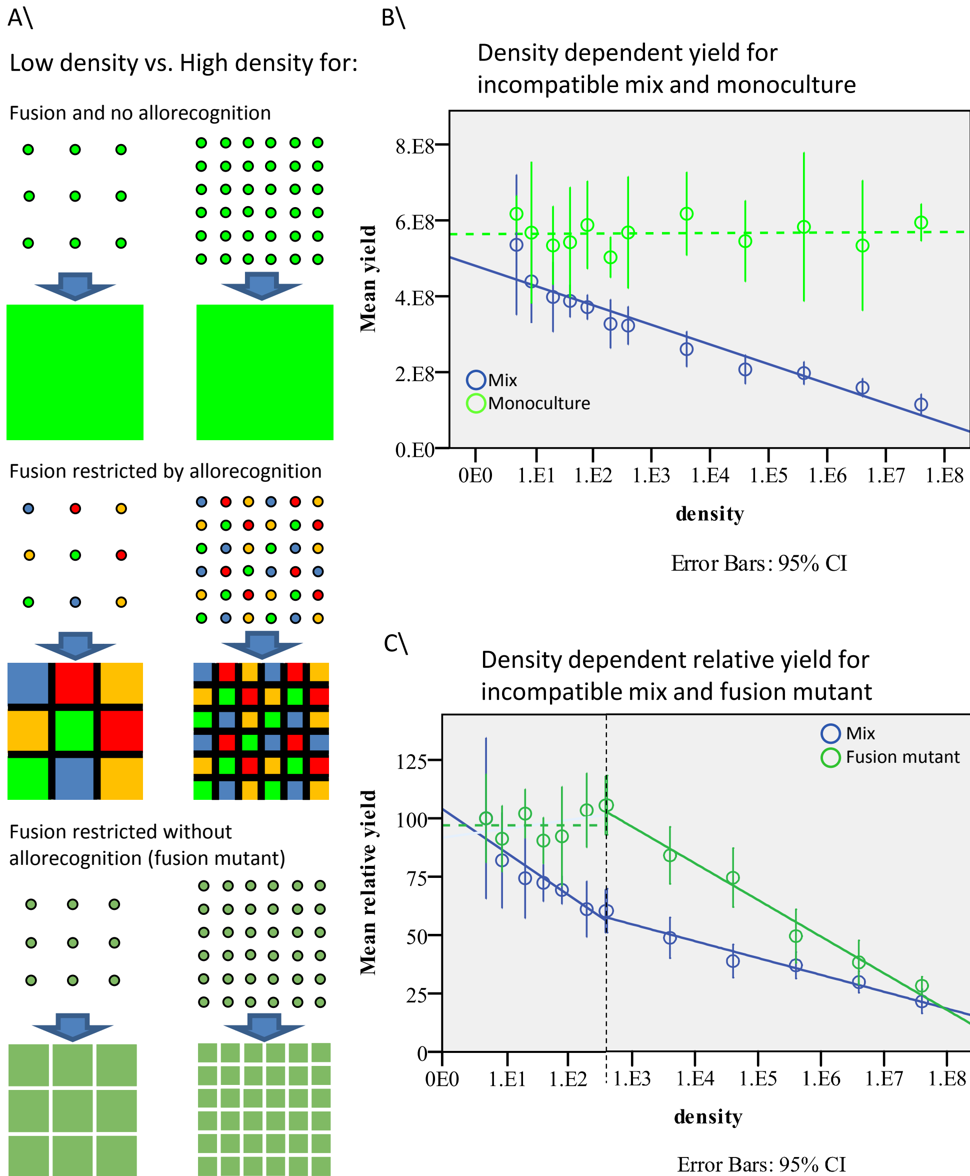

Figure 2 The effect on fitness of colony size and the allorecognition reaction under restricted and unrestricted fusion.
(A) Schematic representation of expected growth patterns in cultures with varying inoculation densities. When spores (circles) of one allotype are inoculated on a solid growth medium they will germinate and form colonies that will fuse into one coherent colony. With unrestricted fusion density will have no effect on the final colony size. If fusion is restricted in a mixture of different allotypes, colonies (shown as coloured squares) will be separated by zones of dead cell compartments (shown as black lines). A higher density will lead to smaller colonies and more dying material in the contact zones where hyphae fuse and die. The

hyphae of the fusion mutant we use cannot fuse. Since fusion is not initiated, there will not be a cell death reaction either. So spores will develop into separate colonies (shown as green squares) without zones of dead cells in between them. Therefore, for this mutant, a higher density will only lead to smaller colonies, but not to increased cell death. (B) Yield (number of asexual spores produced) as a function of inoculation density (total number of spores inoculated). Cultures grown from a mixture of allotypes (restricted fusion, blue circles) are compared to cultures grown from a single allotype (fusion, green circles). Regression line is shown as solid line when significant (linear regression $p<0.05$) or dotted line if no significant correlation was found. (C) Relative yield (asexual spore production standardized at 100 % at the lowest density) as a function of inoculation density (total spores inoculated). The relationship of cultures grown from a mix of allotypes (restricted fusion, blue circles) is compared to the relationship of cultures grown from the fusion mutant (restricted fusion without cell death, green circles). Separate regression lines are given for data points up to density 400 and for data points from density 400 and higher. The regression line is shown as solid line if significant (linear regression $p<0.05$) or dotted line if no significant relationship was found. Because the fusion mutant has a lower yield than wildtype strains, relative yield is used instead of yield to make comparison between the mixture and the fusion mutant easier.

formation. Second, under restricted fusion, there will be an increased frequency of cell death as a consequence of somatic incompatibility reactions (Fig. 2A). In contrast, successfully fusing colonies do not have this energy and resource loss. This antagonistic action may inflate the fitness effect of reduced colony size under allorecognition.

To disentangle the effect of these two factors, we compared the density-dependent fitness of mixed allotypes with the density-dependent fitness of a fusion-deficient mutant strain (Fleissner et al. 2005). Since fusion is not initiated at all in this mutant, there will not be a post-fusion allorecognition reaction with dying hyphae at the interaction zone between meeting colonies. However, increasing the inoculation density for such a strain will still result in smaller colonies (Fig. 2A). This fusion mutant thus allowed us to manipulate the average colony size without the associated disturbing effect of cell death. We found a highly significant linear relationship between yield and the ^{10}log of the inoculation density (linear regression; $F_{1,58} = 179.707$, $p<0.0005$) for the fusion mutant, similar to a mixture between incompatible allotypes . This supports the hypothesis that colony size is important for fungal fitness. However, in contrast to the allotype mixture, for the fusion mutant, the effect of density on yield is only observable from a minimum density of approximately 400 spores per tube. For the densities up to 400 we found no significant relationship between yield and inoculation density (linear regression; $F_{1,33} = 1.217$, $p=0.278$) for the fusion mutant (Fig. 2C). In contrast, in the experiments where fusion was restricted by allotype diversity, we did find a significant relationship between yield and inoculation density at these lower densities up to 400 (linear regression; $F_{1,33} = 25{,}197$, $p<0.0005$; Fig. 2C). Apparently, there is a colony size above which efficiency in terms of yield per surface area does not increase anymore because it is not restricted by colony size anymore. The fact that density changes up to 400 do have

an effect, if fusion is restricted by allotype diversity and not for the fusion mutant, shows that cell death plays an additional role for fitness. Cell death caused by the allorecognition reaction inflates the yield difference between unrestricted fusion and restricted fusion by allotype diversity. The antagonistic effect of allorecognition explains why already at low densities, yield starts to decline with increasing density in the mixture with incompatible allotypes.

Experimental demonstration of the erosion of allotype diversity resulting from fusion benefits.

Having shown that fusion between colonies is mutually beneficial and that there is a cost to allorecognition, we then used this model to test Crozier's predictions on the short-term evolution of genetic kin recognition. For this, we compared the relative fitness of a strain in two treatments: i) in competition with a single other allotype or ii) in competition with a group of four other allotypes (Fig. 3A).

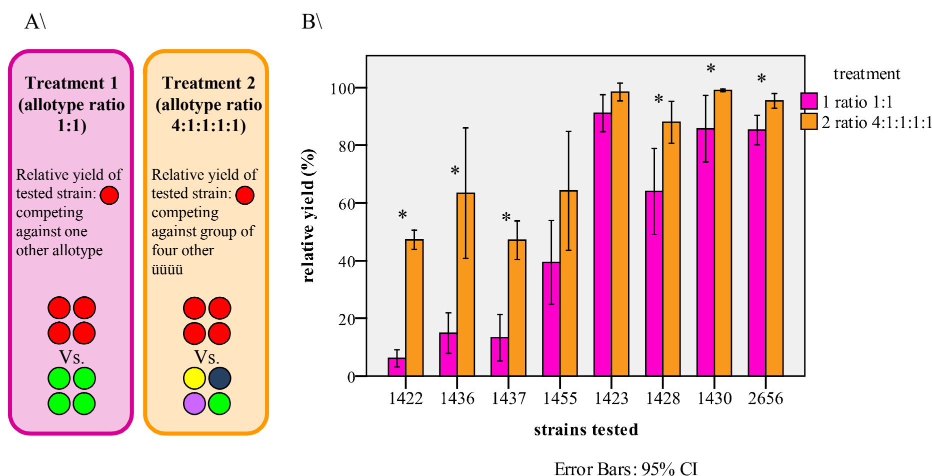

Figure 3 Does positive frequency dependent selection reduce allotype diversity in *N. crassa*?

(A) Experimental setup. A strain was mixed with a competitor in a tube with solid growth medium at a starting frequency of 50 percent in two treatments. In the first treatment the competitor consisted of a single strain with a different allotype, in the second treatment the competitor was a mixed group of four different allotypes. The fitness of the tested strain relative to its competitor was measured as the relative yield of the tested strain after one week of growth. (B) Column chart comparing the relative yield of all tested strains between the two treatments. A relative yield of 50% indicates unchanged frequency of the tested strain. Eight different strains were tested. Significant differences between treatment 1 and 2 (Mann-Whitney U test; $p<0.05$) are indicated with *.

For all eight strains used, we found that the yield of a strain relative to the competing group was higher when that strain's allotype had a relatively higher frequency than the allotypes of the competing group (treatment ii). Across all eight tested strains, this difference between treatments was highly significant (Wilcoxon signed ranks test, $p = 0.0039$) and for six of the eight strains tested individually the difference between treatments was significant as well (independent samples Mann-Whitney U test, $p < 0.05$ for all except for FGSC1455 p=0.18 and FGSC1423 p=0.070; Fig. 3B). This strongly supports the theoretical prediction that recognition types for fusion are under positive frequency-dependent selection and that the advantage of fusion can lead to erosion of allotype diversity. As can be seen in the results, the marker or associated genetic differences created a fitness difference between the groups when they competed at an equal frequency of allotypes (in treatment 1), resulting in deviations from the expected relative yield of 50%. However, since we focus here on the difference between the treatments and since we have used the strains with both markers both in the role of tested strain and in the role of competitor, we can rule out the possibility that a marker effect is responsible for these results.

Discussion

Using two approaches to manipulate the degree of fusion between fungal colonies, we have demonstrated experimentally that fusion among colonies is mutually beneficial. By comparing mixtures of incompatible strains, with mixtures of the fusion mutant under varying densities we could disentangle the causes of the relative benefit of fusion. First, using mixtures of incompatible strains, we demonstrated that the cell death reaction associated with allorecognition reduces yield. Second, using the fusion mutant in varying inoculation densities we could vary colony size in the absence of antagonistic cell death, and demonstrated that the benefit of fusion is also due to an increase in colony size *per se*. Finally, we experimentally showed that the benefits of fusion may generate positive frequency-dependent selection, reducing allorecognition diversity, as theoretically predicted by Crozier.

The benefit of fusion is density dependent.

Our experiments show that the relative advantage of fusion, and thus the strength of selection against allotype diversity, crucially depends on the inoculation density. In nature, the density with which fungal colonies are started can vary greatly. For *N. crassa*, we expect this to vary from single spore colonization, usually of a sexually produced spore, to high density inoculations with the massively produced asexual spores of identical allotype during local colonization after a fire (Perkins and Turner 1988). So we expect that fusion in *Neurospora* is indeed an important cooperative process that helps the fungus to colonize large areas quickly. As the results of this paper demonstrate, at the same time the benefit of fusion under these

circumstances will select against allotype diversity. It seems likely that in some other fungi with different ecologies and lifestyles, fusion between different colonies will not provide a strong benefit, since colonization of substrates often will occur at low densities. For these fungi, it has been proposed that the primary function of somatic fusion is fusion among the hyphae *within* the colony (Rayner et al. 1984; Rayner 1991; Aanen et al. 2008).

The relationship between size and fitness

To explain the benefit of increased colony size, we have to search for the benefits of multicellularity itself. These benefits are due to the benefit of cooperation among cells, giving a higher inclusive fitness to the group of cells, than the summed fitness of all cells individually (Buss 1987; Michod and Roze 2001; Grosberg and Strathmann 2007). One category of benefits of multicellularity is related to increases in size. For example, being bigger may provide protection against predation. Most importantly, multicellularity facilitates the evolution of altruistic strategies, where some cells reduce their chances to reproduce, by helping other, genetically related cells to reproduce. According to the size-complexity rule, bigger organisms generally have a greater division of labour than smaller ones, reflected in a higher number of cell types (Bonner 1993; Bonner 2003).

The positive correlation between yield and colony size that we found is consistent with the size-complexity rule, although it is unlikely that larger fungal colonies have a higher number of cell types and a higher degree of specialization. Rather, the benefit of increased colony size may be a consequence of a more fundamental relationship between size and efficiency. For example, there may be a fixed cost for the production of the supporting tissue of a fruiting structure. If a single colony has fewer resources than required for an optimal single fruiting structure, this implies that relatively more resources will be spent on supporting tissue, and fewer on spores. In support of this hypothesis, a positive correlation has been demonstrated between colony size and spore yield, but a negative correlation between colony size and the number of fruiting bodies in a different fungus (Aanen et al. 2009). Also in other fungi, it has been demonstrated that fusion can be mutually beneficial, as a positive correlation has been found between colony size and reproduction in lichens (Pringle et al. 2003). Furthermore, fusion between germinating spores of *N. crassa* has been found to increase the growth rate (Richard et al. 2012). In contrast to the latter study, which studied the consequences of fusion on radial growth rate comparing a wildtype strain and a fusion mutant, our study addressed the consequences of fusion on spore production mainly comparing cultures consisting of a single allotype with cultures consisting of different allotypes.

Mathematical models have shown that the formation of networks may provide a benefit by promoting the exchange of nutrients or information between different parts of the colony (Simonin et al. 2012). Especially in heterogeneous environments, this may be beneficial, as it allows the utilization by a single colony of a larger set of environmental conditions. Also under homogeneous conditions, cytoplasmic contact may be important, but it is unclear how this is related to the size of a colony.

Positive-frequency dependent selection erodes allorecognition diversity

We have demonstrated that the benefits of fusion select against allotype diversity. This selective process was predicted by Crozier (Crozier 1986). He specifically focused on somatic fusion between individuals of colony-forming marine invertebrate species, but his logic applies more generally to genetic recognition mechanisms that stabilize cooperative behaviours (Tsutsui et al. 2003; Aanen et al. 2008). Although short-term selection thus acts against allorecognition diversity, allorecognition is prevalent in nature, not only in fungi, but in a wide range of colony-forming organisms, such as clonal marine invertebrates like sponges and sea anemones. The widespread occurrence of allorecognition suggests that the disadvantages of fusion on average will be greater than the benefits (Grafen 1990; Nauta and Hoekstra 1994) or that something else is selecting for diversity on the allorecognition genes (Crozier 1986; Rousset and Roze 2007; Paoletti and Saupe 2009; Holman et al. 2013; Bastiaans et al. 2014b).

Importantly, our experiments did not consider any of the factors hypothesized to counteract the positive frequency-dependent selection effect of fusion benefits and thus maintaining allotype diversity. In fungi several of such mechanisms have been proposed. Most importantly, allorecognition may provide protection against somatic parasitism or cheating, i.e. genotypes (nuclei) that, analogously to cancer cells in metazoan bodies, exploit the soma (Nauta and Hoekstra 1994; Aanen et al. 2008; Czaran et al. 2014). Compared to most animals, fungi are expected to be more sensitive to somatic cheaters, as they do not have an early germline-soma differentiation so that mutant nuclei potentially can enter the spores and cheaters can infect other colonies via fusion. There is some experimental evidence for cheating (Ryan and Lederberg 1946; Davis 1960), although cheaters have not been found in natural isolates of fungi. A related hypothesis is that allorecognition in fungi has evolved as a protection against cytoplasmic parasitic elements, which can easily be transmitted via fusion (Debets et al. 1994). There is experimental evidence that restriction to fusion at least partially prevents transfer of cytoplasmic elements, such as plasmids, viruses and mitochondria (Debets et al. 1994; van Diepeningen et al. 1997; Debets and Griffiths 1998; Cortesi et al. 2001; Brusini et al. 2011; Bastiaans et al. 2014a). Recently, it has been proposed that allorecognition in fungi is a secondary consequence of selection for xenorecognition, i.e. recognition of parasites belonging to a different species, similar to allorecognition in animals (Paoletti and Saupe 2009). There is some evidence that this function may be important in *Podospora anserina* (Paoletti and Saupe 2009; Bastiaans et al. 2014b). It is likely that the risks of fusion proposed here are dependent on density as well, since lower densities will decrease the frequency of interaction between colonies. In general, lower densities will result in weaker selection pressure from both the risks and the advantages of fusion. Therefore, the evolution of allorecognition is likely to be density dependent.

Fungi have all the advantages of microorganisms for experimental studies of evolution and some of them have very well-characterized genetic recognition mechanisms. However, in contrast to single-celled organisms, their colonial organization and potential to fuse provide unique opportunities to study the evolution of one of the most extreme forms of cooperation, that is the sharing of a multicellular soma. We have shown here that increases in colony size in fungi can be beneficial and that this benefit provides a selective pressure against rare allotypes. The high diversity of allorecognition alleles observed in nature indicates that there is a delicate balance between these advantages of fusion between closely related colonies and the risks of infection with various kinds of somatic parasites by fusion with more distantly related colonies. It remains to be demonstrated which of these various proposed risk factors is most important to stabilize allorecognition diversity. Future work should therefore address the dynamics of allorecognition in multigenerational experiments of cultures with and without the various risk factors.

Acknowledgements

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Supplementary material

Supplementary Table S1

Strain combinations used in the mix and monocultures for determining the fitness of cultures with restricted and unrestricted fusion

culture	# of allotypes	FGSC strain numbers
mono1	1	1424
mono2	1	1427
mono3	1	2660
mix1	2	1423, 2657
mix2	2	1430, 2662
mix3	2	1428, 2662
mix4	3	1423, 1424, 2657
mix5	3	1427, 1430, 2662
mix6	3	1428, 2657, 2662
mix7	4	1423, 1424, 1428, 2657
mix8	4	1423, 1430, 2657, 2662
mix9	5	1423, 1424, 1430, 2657, 2662
mix10	5	1423, 1424, 1427, 1430, 2662
mix11	8	1423, 1424, 1427, 1428, 1430, 2657, 2660, 2662

Mono1 till mono3 are cultures with unrestricted fusion because only one allotype is present. Mix1 till mix11 are cultures with restricted fusion. All strains are of different allotype based on differences in 4 allotype genes, where two different strains differ at least for one of the allotype genes. Fusion becomes more restricted with more strains in the cultures.

Chapter 3

High relatedness protects multicellular growth against somatic cheaters

Eric Bastiaans, Alfons J. M. Debets, Duur K. Aanen

Abstract

In multicellular organisms, only a fraction of the cells reproduce, while the rest contributes to somatic functions only. This reproductive division of labour may favour cheating mutants, which contribute less to somatic functions but more to reproduction. High genetic relatedness has been proposed as the main factor stabilizing multicellular growth, but direct evidence for this hypothesis is absent. We experimentally evolved asexual populations of the fungus *Neurospora crassa* under conditions of low and high relatedness. While average spore production did not decrease in the high-relatedness lines, it significantly decreased in all low-relatedness replicate lines, on average three-fold. This yield reduction was caused by cheating mutants, with increased competitive fitness, but reduced somatic growth and spore production. The cheats owe their increased competitive fitness to somatic fusion with genotypes that invest more in somatic growth. These results experimentally demonstrate that high relatedness stabilizes multicellular cooperation.

Introduction

The evolution of multicellularity was one of the most significant major transitions in evolution (Buss 1987; Maynard Smith and Szathmary 1995; Queller 2000; Ispolatov et al. 2012). It allowed increases in size and complexity through division of labour between differentiated cellular lineages within an individual (Bonner 2003; Ispolatov et al. 2012). The evolutionarily most extreme division of labour was in reproduction. Only a fraction of the cells reproduce, whereas the majority supports the reproductive cells by contributing to somatic functions only. Reproductive division of labour leads to a potential conflict among the cells of a multicellular individual. A cheating variant with an increased probability to become a reproductive cell will be selected within the individual, even if this variant decreases individual fitness. The stability of multicellular growth thus depends on mechanisms to reduce the selective scope for cheats.

High genetic relatedness has been proposed as the fundamental factor contributing to the stability of multicellular growth (Maynard Smith and Szathmary 1995; Queller 2000; Fisher et al. 2013). In the vast majority of multicellular organisms, individuals develop from a single-celled zygote undergoing mitotic divisions. The resulting high genetic relatedness ($r = 1$, barring new mutations) implies that some of these cells can become sterile somatic tissues in the service of the other cells if this increases the total reproduction of the group of cells, i.e. if *benefits* > *costs* (Hamilton 1964a, b; Queller 2000). In further support of the importance of high relatedness, multicellular organisms have very precise allorecognition mechanisms, which restrict somatic fusion to closely related individuals, thus maintaining high relatedness among the cells of an individual (Grosberg and Strathmann 2007; Czaran et al. 2014).

Direct tests of the hypothesis that high relatedness stabilizes multicellular growth are rare. Organisms that arise via aggregation of solitary cells instead of clonal outgrowth from a single-celled zygote provide an opportunity to experimentally reduce among-cell relatedness. The best known example is *Dictyostelium*, which forms multicellular fruiting bodies via aggregation of single cells. It has been shown that cheaters can arise under low-relatedness conditions (Kuzdzal-Fick et al. 2011). However, direct comparisons between high and low-relatedness conditions under otherwise identical conditions have not been made, since relatedness cannot easily be controlled during an evolution experiment (Saxer et al. 2010).

In this article, we use the fungus *Neurospora crassa* as a model system to test the significance of genetic relatedness for the stability of multicellular growth. Fungi are intermediate between pure clonal development from a single-celled zygote and development via aggregation, because fungal individuals can increase in size by mitotic division, but also by fusion between the filaments of different individuals (Rayner 1991; Roca et al. 2005b; Read et al. 2010). This characteristic allowed us to vary relatedness. Filamentous fungi form filaments (hyphae) that are branching and fusing regularly to form a dense, radially growing network

called the mycelium. Each fragment can reproduce via the formation of sexual and asexual spores. In contrast to most other multicellular organisms, cell compartmentalization is not very strong and in some fungi nuclei can freely move through parts of the mycelium (Roper et al. 2011). Therefore, the cooperating units in the fungal colony primarily are the haploid nuclei (Rayner 1991).

To experimentally test the importance of high relatedness among nuclei for selection against cheating variants, we performed an evolution experiment, using two treatments, one maximizing and the other minimizing relatedness among the nuclei of individuals. In the maximal-relatedness treatment, we used a mutant that lacked the ability to fuse. In this treatment, each germinating spore forms a separate individual, which produces offspring. This maximizes the relatedness among the nuclei of a single individual, and minimizes the opportunities for cheating, as a variant selected within an individual will be tested for its effect on multicellular fitness in the next round of experimental evolution. The other treatment minimized relatedness by using a single allotype, a high inoculation density and complete mixing at each transfer. The use of a single allotype and a high inoculation density guaranteed fusion among all germinated spores, so that a single, coherent fungal colony was formed, essentially making the multicellular individual a common good. We predicted that these conditions favour cheating, as the link between investment in somatic 'helper' tissue and reproduction was minimized (figure 1).

Results

After 31 rounds of asexual reproduction, we determined the average spore yield of all cultures and compared it to the unevolved strain. In the high-relatedness treatment, spore production had not significantly changed (independent samples Mann-Whitney U test, p =0.599), but in the low-relatedness treatment spore production had significantly decreased, on average threefold (independent samples Mann-Whitney U test, p =0.23; figure 2A). To further investigate the changes in the low-relatedness evolution lines, we assessed variation among single-spore cultures of each line. All lines consisted of at least two, and sometimes more different phenotypes (Figure 2 B). One of the types of each line was similar to the original phenotype, producing abundant spores in monoculture. In contrast, the other types produced fewer spores in monoculture.

To characterize these different types in more detail, we isolated the different morphotypes for each line and tested them separately for two fitness components in monoculture, the production of asexual spores and the mycelial growth rate (Pringle and Taylor 2002). Spore production generally was lower for the deviating phenotypes, while it had remained unchanged for the types that resembled the ancestral phenotype (figure 2C). The mycelial growth rate showed a similar trend as spore yield for most types (supplemental figure) and

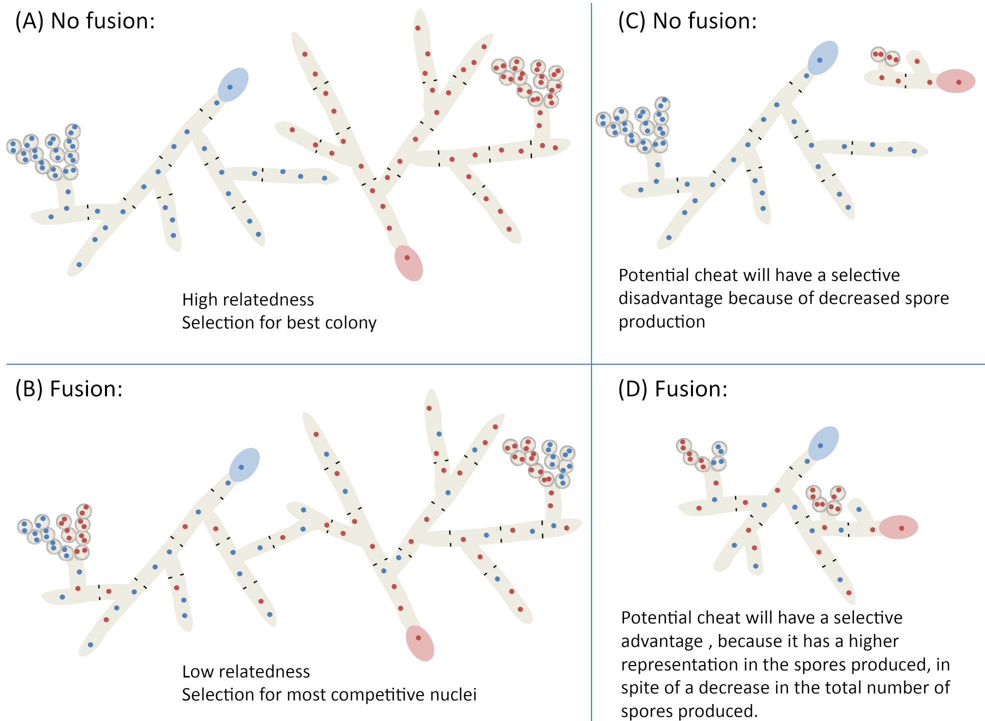


Figure 1 Model of the consequences of somatic fusion for relatedness and evolution of cheating.
After fungal spores (sexual or asexual) germinate they will form a colony by clonal division of their nuclei leading to high relatedness among the nuclei within a colony. (A) If fusion between colonies is not possible, nuclei in each colony are highly related. Nuclei between colonies are less related and therefore selection will take place between colonies, the colonies that grow the fastest and/or have the highest output of spores will be selected. (B) If colonies can fuse to neighbouring colonies, their nuclei will be mixed within the fused individual, lowering relatedness. Since all somatic resources are shared, selection will take place between the nuclei, which are in competition to use the somatic resources for their own reproduction. (C+D) If fusion is possible, a potential cheater can shift investment in somatic functions towards more reproduction, by cheating on somatic functions of neighbouring colonies thereby lowering the total yield of the fused colony. In contrast, if fusion is not possible, such a cheater would suffer a selective disadvantage because it cannot perform the somatic functions required to produce spores.

may thus explain the reduced spore production, at least partially.

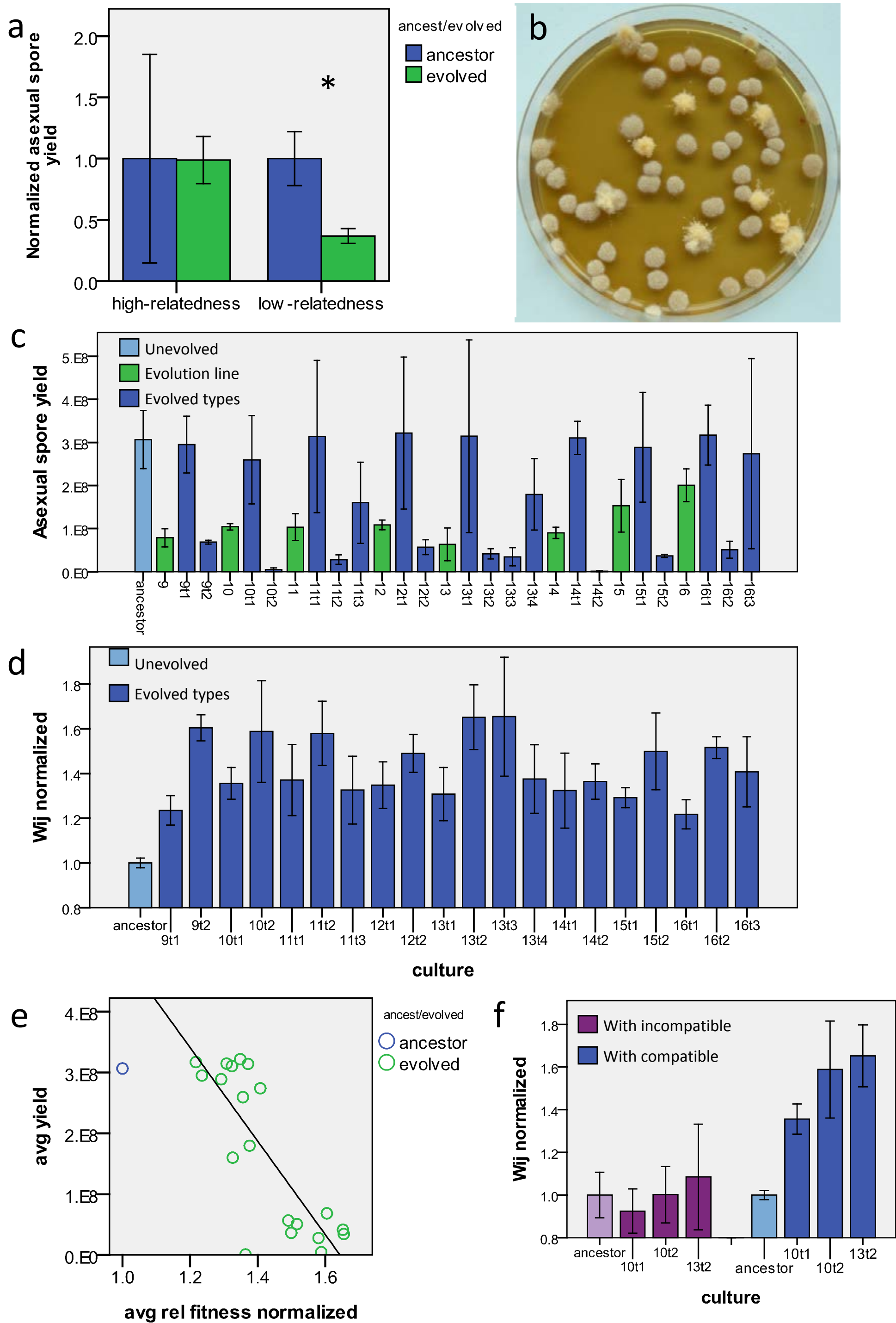
We then tested the morphotypes for their competitive fitness at a low starting frequency relative to the ancestor (1:9; figure 2D). All types had increased competitive fitness, and the types with reduced yield often had a higher competitive fitness than the types with unchanged yield. This suggests that the genotypes with a reduced spore yield are obligate cheaters: they invest less in somatic functions and thus are affected in their growth, but when they have access to the soma of an ancestral genotype, they can exploit this for their own benefit resulting in a

relatively higher fitness. Overall, we found a highly significant negative relationship between competitive fitness and yield in monoculture (linear regression; $F_{1,18} = 34.052$, $p<0.0005$; figure 2E). This trade-off is typical for obligate cheaters on a social trait, which cannot switch back to producing the common good, in this case the soma, in the absence of social types to cheat on (Ghoul et al. 2014). The negative effect on yield of the cheating mutation not only is observed when the cheater grows alone, but also in combination with social types, since the yield of evolved lines is intermediate between the yields in monoculture of the types they consist of (figure 2C). Within an evolution line, we call the morphotype that produces, when in monoculture, most spores 'social type', and the one or more types that in monoculture produce fewer spores 'cheater'.

We hypothesized that the evolution of cheating in this experiment depends on fusion between individuals. To test this hypothesis, we determined competitive fitness of three evolved types relative to a strain they could not fuse with. If increased competitive fitness depends on fusion, we predicted that competitive fitness would not be increased relative to this strain. Consistent with this hypothesis, we now did not find a significant difference in competitive fitness relative to the unevolved ancestor strain (figure 2F). This proves that increased competitive fitness in our evolution experiment depends on fusion with a social type.

To confirm that the cheating phenotype is caused by nuclear mutations, we back-crossed the typical cheat types to non-evolved types. In all crosses between evolved cheat and non-evolved phenotypes, progeny segregated 1:1 into the cheating phenotype and the non-evolved phenotype, as expected for single nuclear mutations. For two crosses (10.2 and 14.2) we found a third, difficult to distinguish type among the progeny, indicating that more than one mutation has occurred in these types. Additionally, these crosses revealed that the cheating types all have severely reduced female functioning, which is consistent with the hypothesis that these strains have reduced somatic investment required to support the growth of relatively costly female fruiting structures. The reciprocal cross, in which the cheater had the relatively cheap male role, did not show reduced fertility.

At the end of our evolution experiment, in all replicate lines a social type still coexisted with one or more cheating types. Since the cheater has a higher competitive fitness relative to the social types we hypothesized that the cheating genotype would increase in frequency during the evolution experiment until fixation. For three lines we analysed the frequency dynamics of the cheating type throughout the experiment (figure 3A). Based on the increasing trend we hypothesized that the cheating genotypes could go to fixation during subsequent evolution. We therefore continued the evolution experiment for these lines for another 10 transfers and measured the cheater frequency again to see if cheating genotypes would go to fixation (table 1). In one of the lines the cheater had gone to fixation indeed. However, in the two other lines both the cheating type and the social type were still present, although the frequencies fluctuated between generations, suggesting complex dynamics between cheat and social type. Stable coexistence of the different types could be explained if the interaction between types


a
Normalized asexual spore yield
2.0
1.5
1.0
0.5
0.0
*
high-relatedness
low -relatedness
ancest/ev olv ed
ancestor
evolved
b
c
Asexual spore yield
5.E8
4.E8
3.E8
2.E8
1.E8
0.E0
Unevolved
Evolution line
Evolved types
ancestor
9
9t1
9t2
10
10t1
10t2
11
11t1
11t2
11t3
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culture
e
avg yield
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3.E8
2.E8
1.E8
0.E0
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With incompatible
With compatible
ancestor
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ancestor
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13t2
culture

Figure 2 Yield and competitive fitness of the evolved lines and morphotypes found within these lines.
(A) Under high-relatedness conditions high spore production is maintained, while under low-relatedness conditions spore yield drops. Column chart comparing average yield of eight evolved lines per treatment before and after an estimated 205 asexual generations. The two treatments were selection with high relatedness among nuclei within a colony and selection with low relatedness among nuclei within a colony. Error bars depict 95% confidence intervals. Significant differences between yield before and after evolution (t-test $p<0.05$) are indicated with *. (B) Evolution under low-relatedness conditions results in polymorphism within lines. Photo of a plate with one week old colonies grown from asexual spores of one of the cultures evolved under low relatedness conditions. Bright orange colonies are sporulating, similar to the ancestral type, while the more pale colonies have reduced sporulation. (C) Yield of evolved lines and of the different morphotypes within these lines. Column chart comparing asexual spore yields of the low-relatedness lines and their morphotypes with the yield of the ancestral type. Error bars depict 95% confidence intervals. (D) Competitive fitness of evolved morphotypes has increased relative to the ancestor. Column chart comparing normalized competitive fitness of the morphotypes evolved under low relatedness relative to the ancestral strain. Error bars depict 95% confidence intervals. (E) There is a highly significant negative correlation between yield and competitive fitness. The graph shows average asexual spore yield plotted against competitive fitness relative to the ancestral strain. (F) Increased competitive fitness depends on somatic fusion. The column chart compares normalized competitive fitness of three selected morphotypes evolved under low-relatedness conditions relative to an ancestral strain with a different allotype with which fusion is not possible. For comparison, the data from competition of the same morphotypes with the ancestral strain of the same allotype is depicted in the right-hand graph. Error bars depict 95% confidence intervals.

were mutually beneficial. For example, in bacteria cross-feeding between different strains can lead to higher yield of mixed cultures than predicted by the average of the strains grown as monocultures (Pande et al. 2013). We can reject mutual benefits as the mechanism for stable coexistence, as the yield of all evolution lines was intermediate between the yields of the composite types in monoculture, and not higher, as predicted under the mutualism model. An alternative explanation for the stable coexistence of the different types is negative frequency-dependent selection. Negative frequency-dependent selection has been found in several studies on social cheating in microbes (e.g. (Velicer et al. 2000; Dugatkin et al. 2005)). Theory suggests that negative frequency-dependent selection is expected when there is population structuring and an association between the level of cooperation and population growth (Ross-Gillespie et al. 2007). Both conditions are fulfilled in our experiments. Clonal outgrowth of a spore will lead to local patches of clonally related nuclei, and cheating has a negative effect upon total spore yield. Especially under low inoculation density, there is more population structuring. Because of the emergence of cheaters in the course of the evolution experiment, the number of harvested spores, and consequently, the inoculation density, decreases, since we transfer a fixed fraction of the spores (figure 2A). A lower inoculation density leads to more clonal outgrowth and consequently a higher average relatedness among

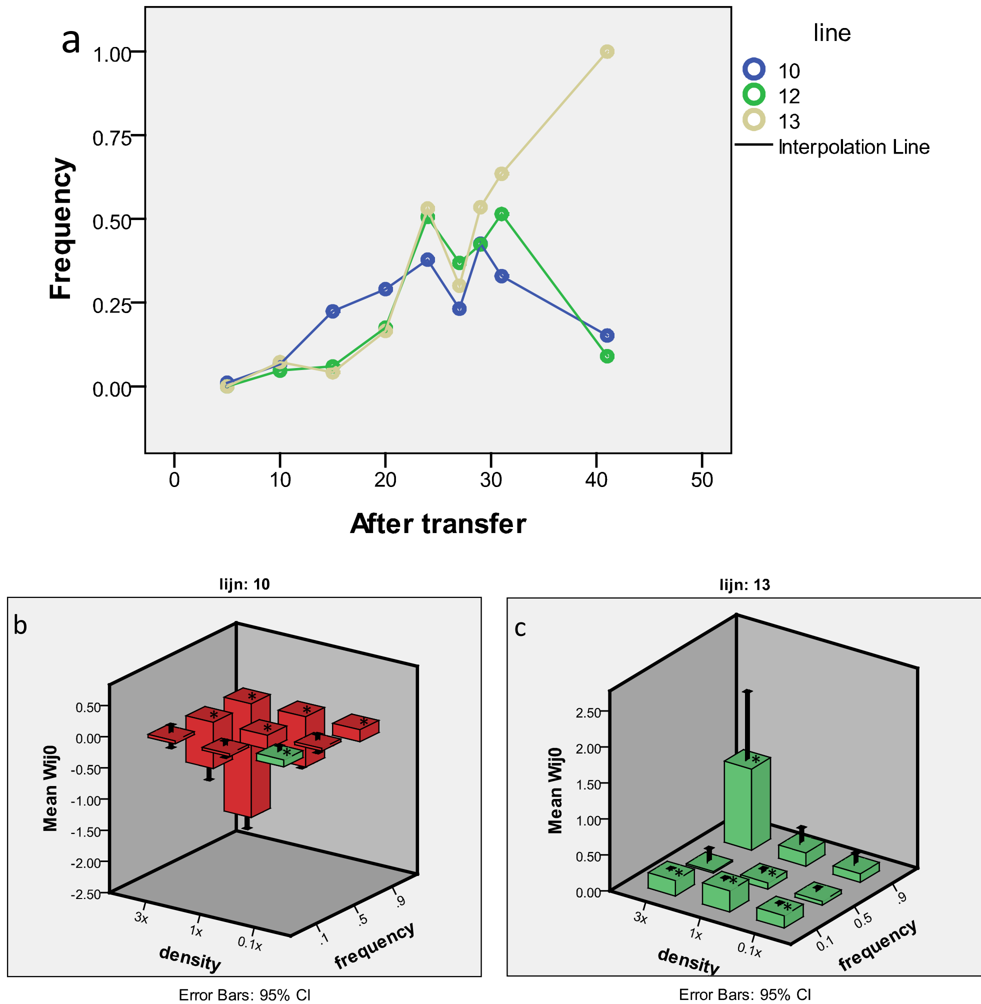

Figure 3 The effect of inoculation density and frequency on the relative fitness of cheaters.

(A) Graph showing the frequency changes of the cheat types during the evolution experiment in three lines. (B)+(C) Bar diagrams showing competitive fitness of the cheat types relative to the social types from line 10 and 13 as a function of inoculation density (x-axis) and inoculation frequency (z-axis)). On the y-axis relative fitness – 1 is depicted, so that positive and negative values represent a selective advantage or disadvantage to the competitor, respectively. Relative fitness is measured for different inoculation densities (x-axis) and different starting frequencies (z-axis). Starting density 1x is the density representative for the first few transfers of the evolution experiment ($4*10^7$ spores/ml). The other starting densities are 3 times concentrated (3x) and 10 times diluted (0.1x) compared to 1x. Significant differences from 0 (t-test $p<0.05$) are indicated with *.

Table 1 Frequencies of typical cheat types in each line at two time points

Line	After transfer 31	After transfer 41
9	0.89	0.35
10	0.29	0.15
11[a]	0.32	0.22
12	0.55	0.09
13[a]	0.62	1
14	0.2	0.09
15	0.15	0.04
16	0.15	0.39

[a] If a line contains more than 1 typical cheat type, their combined frequency is displayed because they are not distinguishable from each other on a counting plate.

neighbouring nuclei in the fungal individual, limiting the opportunities for cheaters. In other words, since cheating types derive their increased competitive fitness from somatic fusion with social types, their opportunities decrease with lower inoculation density.
To test for negative frequency-dependent selection and the effect of inoculation density, we measured the competitive fitness of two cheaters from two different lines in direct competition with the social type with which they were coexisting after transfer 31. Competitions were set up under three different frequencies of the cheater (0.1, 0.5 and 0.9) and at three different inoculation densities (initial density, 3X and 0.1X) in a full factorial design. These experiments showed a sharp contrast between line 10 and 13 (figure 3B and C), which explains the frequency changes of the cheat types in these lines. In line 10 we only measured a significant advantage for the cheating type for the combination low frequency and low density. This explains that the cheater remains at a low frequency. So for line 10, we confirm that negative frequency-dependent selection acts upon the cheater, although we do not precisely understand how. In line 13, on the other hand, the cheater always had an advantage relative to the more social type (significant in 5 of the 9 tested conditions) which fits the observation that this type went to fixation.

General discussion

Although fusion provides benefits relative to allorecognition due to increased individual size and the absence of costly antagonism (Bastiaans et al. 2015), our results demonstrate that high relatedness is crucial for the evolutionary stability of multicellular growth in fungi (Nauta and Hoekstra 1994; Czaran et al. 2014). Experimentally minimizing relatedness by free fusion, complete mixing of spores and a high inoculation density, resulted in selection of cheaters. These variants had shifted from the optimal division of labour for the highest

possible yield, to exploiting other individuals upon fusion. This resulted in a lower total yield of the composite individual, but a relatively higher representation in the produced spores relative to the cooperative genotype. In contrast, when high genetic relatedness was maintained through prevention of fusion and through recurrent single-celled bottlenecks, cheaters were not selected.

There are no known examples of cheating in *Neurospora* isolates from nature, suggesting that high genetic relatedness is maintained in nature, likely due to a variety of factors. First, single-celled bottlenecks are usually associated with dispersal, so that there is more opportunity for clonal outgrowth than in our experiments. Second, as in the vast majority of organisms that can fuse, highly diverse genetic allorecognition mechanisms exist in *Neurospora* (Glass et al. 2000; Saupe 2000a; Aanen et al. 2010), restricting somatic fusion to close relatives only, thus maintaining high relatedness among the nuclei of individuals.

The next step is to identify the mechanism of cheating of the mutants selected in our experiments. Earlier studies have demonstrated that loss-of-function mutants, for example for the uptake or production of an essential nutrient, can behave as cheaters in combination with wild-type genotypes. This shows that profiting from these functions provided by a partner individual can be capitalized for increased personal reproductive success (Ryan and Lederberg 1946; Davis 1960). Additionally, cheating may be based on faster replication of nuclei, leading to a higher probability to enter a spore, but reducing individual fitness, analogously to a cancerous mutant. Furthermore, screening of the high-relatedness control lines revealed that also under these conditions a few types with reduced sporulation had evolved (supplementary figure 2). Although we did not measure competitive fitness of these types, this result suggests that there are additional possibilities to cheat on neighbouring mycelia other than fusion, for example by profiting from extracellular enzymes for substrate degradation or nutrient uptake, produced and excreted by surrounding individuals (Allison 2005).

Control of conflicts is essential in every transition to a higher level of selection, not only the transition to multicellular growth, but also the transition from solitary individuals to highly integrated societies of social insects (Maynard Smith and Szathmary 1995). Our results experimentally demonstrate that high genetic relatedness is crucial for the formation of social groups. Additional mechanisms may contribute to the maintenance of social groups, once they are formed (Bourke 2011). For example, in higher organisms, such as mammals, policing mechanisms reduce the selective scope for cancerous mutations (Efeyan and Serrano 2007). Cell compartmentalization of organelles itself has been proposed as a mechanism to restrict the opportunities of cheating mutants (Buss 1987). Fungi are excellent model systems to experimentally study the importance of these various factors, as they vary in the extent to which there is cell compartmentalisation. Furthermore, the dual nature of multicellular development, both by clonal outgrowth and by fusion among individuals, facilitates precise manipulation of relatedness, as our experiments demonstrate.

Material and methods

Experimental evolution

To test whether the fungus *Neurospora crassa* is vulnerable to the evolution of somatic cheaters we performed an evolution experiment, in which a culture was transferred to fresh growth medium regularly to allow selection of new mutations.
To test whether relatedness among the nuclei of a single individual affects the evolution of cheating we compared selective conditions with high and low relatedness. Both selective conditions were similar in growth conditions such as medium, temperature and growth time. Importantly, at each transfer nuclei went through a single-celled bottleneck as asexual spores. These spores were inoculated evenly on solid medium at a high density (~$2.85*10^5$ per cm^2) with the result that during or soon after germination, germlings or young colonies were in contact with each other. The key difference between the two selective conditions was the ability of colonies to fuse with each other. Fusion counteracts increased relatedness within colonies after the single-celled bottleneck.
For the high-relatedness condition, we used a mutant strain, the hyphae of which lacked the ability to fuse. Each spore of this mutant formed a separate colony with clonally related nuclei, with its own reproductive output (figure 1A). Competition therefore mainly occurred between colonies, favouring genotypes with a high spore yield (figure 1A+C). This condition mimics a natural situation in which clonal outgrowth and limited fusion between colonies resulting from allorecognition, maintain high-relatedness among the nuclei of a single individual.
For the low relatedness condition, a regular strain was used, the hyphae of which are able to fuse. Since we used a single strain, all spores had the same allotype so that all germlings could fuse with surrounding germlings, resulting in a single coherent individual, shared by the nuclei of all germlings. Since there are no physical borders dividing different fungal individuals anymore, competition now primarily occurred at the level of nuclei, competing for the reproductive output of the shared individual, and less at the level of colonies (figure 1B). Initially, the nuclei were highly related (i.e. genetically similar), as they all descended from a single spore culture, but mutation will soon supply genetic diversity lowering relatedness among nuclei. New mutations could lead to a somatic cheater, defined as a variant that has a selective advantage within the mycelium by using somatic resources for its own replication while contributing less to somatic functions (figure 1C+D).

Strains were obtained from the Fungal Genetics Stock Center (FGSC) and strain numbers refer to the FGSC catalogue (McCluskey 2003). For the high-relatedness treatment the fusion mutant *soft* (FGSC11293) (Fleissner et al. 2005) was used. For the low-relatedness treatment a strain was selected from a cross between a standard lab strain (FGSC2489) and a strain

with multiple markers in the background of the standard lab strain (FGSC5130). The selected strain contained a mutation that induces inositol dependence for growth, functioning as a selective auxotrophic marker.

For both treatments, a culture that originated from a single spore was made to start the evolution lines, and asexual spores from these cultures were stored at -80°C in a glycerol/pepton solution (25% glycerol, and 7% Bacto™ neopepton (BD, Sparks, MD 21152 USA)). For both treatments eight parallel evolving cultures were started from this single spore culture. The growth medium used for experimental evolution was Vogels Minimal Medium (VMM) with 2% sucrose as carbon source and solidified with 2% agar (Davis 2000). VMM contains all the necessary ingredients for optimal growth during the asexual lifecycle.

Cultures were grown in glass tubes (150x17.5mm) with 7.5 ml of VMM medium solidified under an angle of ~60 degrees (slanted culture tubes), resulting in approximately 7 cm^2 of surface area. Evolution lines were maintained by serially transferring 1% of the produced asexual spores with growth phases in between transfers. Asexual spores were inoculated by transferring 50µl suspension with a micropipette and spread out this suspension over the surface with the same pipette tip. The first inoculation was done with a spore suspension made from a culture grown from asexual spores from the -80°C stock. To a 4 day old culture, 5 ml of sterile water was added and the tube was vortex mixed for ~15 seconds. The cultures were incubated for 3 or 4 days at 25ºC with cycles of 12 hours light and 12 hours darkness. After this, asexual spores were harvested from the cultures in 5 ml of sterile water by vortex mixing ~15 seconds. This yields roughly $4*10^7$ asexual spores per ml. From these spore suspensions 50 µl was then used to inoculate fresh culture tubes similar as the previous ones, resulting in a density of $~2.85*10^5$ asexual spores per cm^2. From the same suspensions 800ul was frozen at -80ºC in a glycerol/pepton solution. This serial transfer was then done twice per week alternating 3 and 4 days of growth in between transfers. Initially 31 transfers were done for all 16 evolution lines. If a simple binary fission model for division is assumed and 1% of the spores grow to 100% of the spores each transfer, then $\log_2 100 = 6.64$ generations or divisions occur between transfers. This equals 205 asexual generations after 31 transfers. This is a conservative estimate, since not all nuclei will end up in the spores and not all spores will go into suspension during spore harvesting and not all spores have equal fitness. Later the lines were continued with asexual spores from the lines that were frozen at -80°C after transfer 31, for ten more transfers up to a total of 41 transfers.

Testing spore yields

We determined the spore yield of all evolution lines in order to compare the effect on fitness between both treatments. The yield of asexual spores is an important fitness parameter for fungi (Pringle and Taylor 2002), which determines in our evolution lines how many spores are transferred to the next culture. To determine the yield we grew cultures in tubes under the same conditions as the evolution lines. For this, we spread spore suspension from the

samples frozen after transfer 31 on culture tubes. Spores from this culture were then used to make suspensions of $4*10^7$ asexual spores per ml of which 50 µl was spread on fresh slanted culture tubes. After four days of growth, asexual spores were harvested by adding 5 ml of sterile water to the culture tube followed by ~15 seconds of vortex mixing. This suspension was then used to estimate spore yields using a haemocytometer. Per line, the yield was determined for three replicate cultures.

Isolating morphotypes

We analysed colonies grown from single spores for morphological differences in order to find out whether the evolved cultures consist of more than one phenotype and isolate these different morphotypes. This was done by spreading diluted spore suspensions obtained from the evolved cultures after transfer 31 on counting plates. Counting plates are Petri dishes with VMM adjusted, by replacing sucrose with sorbose and adding 0.05% fructose and 0.05% glucose (sorbose-VMM) on which growth is restricted, so that single spore colonies remain small (Davis 2000). These plates were incubated for one week at 25°C with cycles of 12 hours light and 12 hours darkness to allow for full development of the phenotype. From each distinguishable morphotype (by eye or dissecting microscope) on these plates several colonies were picked up to grow in standard slanted culture tubes that were described before. These were grown for four days at 25°C with cycles of 12 hours light and 12 hours darkness after which cultures grown from similar morphotypes on plate were compared to make sure they were similar in morphology in the environment they were selected in. Diluted suspensions from these tube cultures were spread on counting plates again to make sure it was a pure culture and to confirm the phenotype was stable.

Testing linear growth rate and yield of the morphotypes

We tested the obtained morphotypes for yield of asexual spores in order to compare them to the evolved cultures they were part of. In addition we tested the linear growth rate to see if this trait is changed as well in the evolved types. Yield was determined in the same way as described above for the yield of the evolved cultures. Linear growth rate is defined as the distance a fungal culture can grow in one direction per time unit. To determine this we grew the morphotypes in disposable so-called race tubes that contain long stretches of agar. The race tube is made from a disposable 50 ml pipette in which 20 ml of VMM medium is solidified over the length of the pipette by laying the pipette horizontally on the bench during solidifying (White and Woodward 1995). The different morphotypes were inoculated in race tubes using an inoculum of $\sim 2*10^5$ asexual spores suspended in 20 µl of water. Growth in three replicate tubes was measured daily for three days.

Testing competitive fitness relative to ancestral strain

We tested the morphotypes from the evolved low-relatedness cultures for their fitness relative to an ancestral strain in direct competition. The ancestral competitor was selected from progeny from a cross between lab strain FGSC2489 and multiple marker strain FGSC5130. The strain is isogenic to the strain used in the evolution experiment except for a different selective marker, which is a pantothenic acid requirement. The competing strain is isogenic and thus of the same allotype, so that the evolved strain can fuse to its competitor similarly as during the evolution experiment. Competitive fitness was measured in a setting identical to the selective environment of the evolution experiment in a ratio of 1:9 (evolved morphotype:ancestral). This low frequency of the evolved morphotype was chosen to maximize potential benefits from exploiting the competitor. Competitions were setup between the evolved morphotypes and the competitor and, to correct for marker effects in the analysis, also between the ancestor of the evolution lines and the competitor with the other marker. The competition was setup in slanted culture tubes with VMM medium. Asexual spores from the morphotype and the ancestral strain were mixed in a ratio of 1:9 in a suspension of $4*10^7$ asexual spores per ml, of which 50 µl was used to spread on a slanted culture tube. The competition culture was then grown for four days at 25ºC with cycles of 12 hours light and 12 hours darkness. Asexual spores were suspended in 5 ml of water. To determine the numbers of each competitor before and after the competition, an appropriate dilution of this suspension was plated on five counting plates with sorbose VMM supplemented with pantothenic acid and five counting plates supplemented with inositol, allowing growth of only the ancestral competitor and only the evolved morphotype, respectively.

In addition, to test if fitness differences in competition with a strain depended on fusion with the tested strain, we randomly selected a few strains to compete with a strain they could not fuse with. Two typical cheat types morphotype 10.2 and 13.2 and one social morphotype 10.1 were chosen to test the competitive fitness in competition with a strain of a different allotype similarly as the first series of competitions. The competing strain is a strain with the same genetic background as the strain used in the evolution experiment except for differences at three allorecognition genes and a pantothenic acid requirement marker (FGSC1425, (Wilson and Garnjobst 1966)).

Competitive fitness was calculated as

$W_{ij} = Ln[N_i(t)/N_i(0)]/Ln[N_j(t)/N_j(0)]$ (Lenski et al. 1991).

Where W_{ij} is the fitness of strain i relative to strain j, N_i and N_j are the numbers of each competitor at the end and start of competition. N is determined based on the average amount of colonies counted on the counting plates before and after the competition corrected for the dilution and the volume plated. Competitive fitness was normalized to the fitness of the ancestral strain used for the experimental evolution experiment.

Determining frequency of cheater in evolution lines

We determined the frequency of the typical cheat type for all the lines evolved under low relatedness conditions (line 9 through 16) after transfer 31 and after transfer 41. For line 10, 12 and 13 we determined the cheater frequency at more time points in the evolution experiment to see how their frequency is changing in time. Frequency was determined by plating appropriate dilutions of spore suspensions obtained from the -80ºC samples made after each transfer on counting plates. These plates were incubated for one week at 25ºC with cycles of 12 hours light and 12 hours darkness to allow for full development of the phenotype. If more than one cheating morphotype existed in an evolved line, these were not easily distinguishable on the plate and thus were counted as one group.

Determining frequency and density dependence of competitive fitness

To test whether the competitive fitness of a cheater, relative to a social type is dependent on frequency and/or density we set up competitions directly between the cheat type and the social type originating from the same evolution line under various inoculation densities and various starting frequencies of the cheat type. This was done with the type 10.1 and 10.2 from line 10 and type 13.1 and 13.2 from line 13. The competitions were set up and analysed similar to the competitions with the ancestral strain described above, with the exception that the two competing strains now did not contain different markers. For this reason only one type of counting plate was used on which both types could be counted separately. The frequency and density were manipulated as follows. Enough spores were collected from 1 or multiple 7 day old cultures of the required types. For each type these were collected separately in suspension of $1.2*10^8$ spores/ml water. Much more than this was not possible because dense spore suspensions are difficult to handle and the cheat types produce very small numbers of spores. From these suspensions three mixtures of the two types were made with the cheating type at frequency 0.1, 0.5 and 0.9. These were then diluted three times to the $4*10^7$ spores/ml, which is similar to the concentration used in the previous competitions and represents the average starting density of cultures in the beginning of the evolution experiment. These diluted suspensions were then diluted ten times to $4*10^6$ spores/ml. This resulted in nine suspensions representing all possible combinations of three cheat frequencies and three starting densities.

Back crossings to ancestral type

All the typical cheat types were crossed with the standard lab strain in order to test whether the morphotype is caused by one or a few nuclear mutations. Crosses were set up on slanted tubes with synthetic crossing medium (Davis 2000) with 2% of sucrose as a carbon source. The lab strain we used necessarily was of the opposite mating type as this is required for a successful cross. As *N. crassa* is hermaphroditical, we performed both reciprocal crosses,

forcing the cheating type in the female role in one cross and in the male role in the other. For each cross the strain with the female role was inoculated by transferring some asexual spores to a fresh tube and then grown for about one week before it was fertilized with asexual spores of the strain in the male role. This fertilized culture was then grown for one to two weeks until mature fruiting bodies and sexual spores were visible.

Asexual spores were given the required heat shock at 60ºC for 30 minutes before an appropriate dilution was inoculated on two counting plates. These plates were incubated for one week at 25ºC with cycles of 12 hours light and 12 hours darkness to allow for full development of the phenotype.

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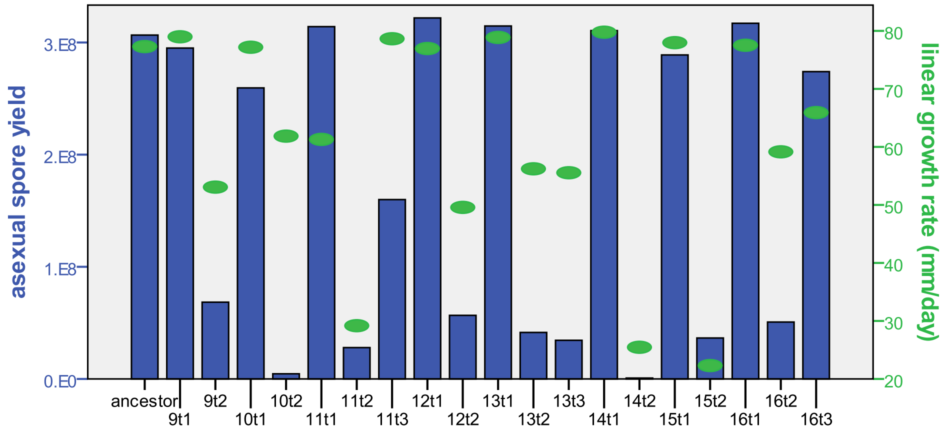
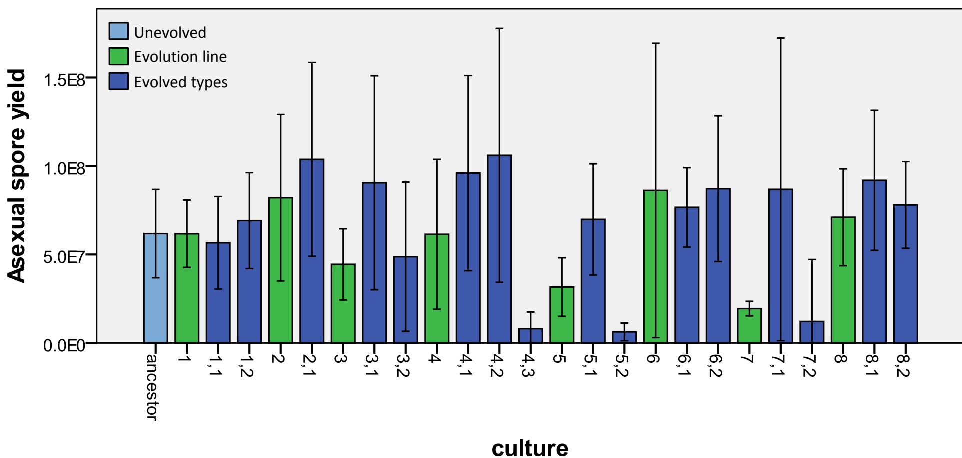
Supplementary material

Supplementary figure 1



Yield and linear growth rate are affected in a similar way for different morphotypes
Comparison between asexual spore yield and linear growth rate of the morphotypes isolated from the lines evolved under low-relatedness conditions. Blue bars indicate asexual spore yield. Green ellipsoids indicate linear growth rate in mm per day

Supplementary figure 2



Yield of lines evolved under high-relatedness conditions and of the different morphotypes within these lines.
Column chart comparing asexual spore yields of the high-relatedness lines and their morphotypes with the yield of the ancestral type. Error bars depict 95% confidence intervals.

Chapter 4

Regular bottlenecks and restrictions to somatic fusion prevent the accumulation of mitochondrial defects in *Neurospora*

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Abstract

The replication and segregation of the multi-copy mitochondrial DNA (mtDNA) are not under strict control of the nuclear DNA. Within-cell selection may thus favour variants with an intracellular selective advantage but a detrimental effect on cell fitness. High relatedness among the mtDNA variants of an individual is predicted to disfavour such deleterious selfish genetic elements, but experimental evidence for this hypothesis is scarce. We studied the effect of mtDNA relatedness on the opportunities for suppressive mtDNA variants in the fungus *Neurospora* carrying the mitochondrial mutator plasmid pKALILO (pKAL). During growth, this plasmid integrates into the mitochondrial genome, generating suppressive mtDNA variants. These mtDNA variants gradually replace the wild-type mtDNA, ultimately culminating in growth arrest and death. We show that regular sequestration of mtDNA variation is required for effective selection against suppressive mtDNA variants. First, bottlenecks in the number of mtDNA copies from which a *'Kalilo'* culture started significantly increased the maximum lifespan and variation in life span among cultures. Second, restrictions to somatic fusion among fungal individuals, either by using anastomosis-deficient mutants or by generating allotype diversity, prevented the accumulation of suppressive mtDNA variants. We discuss the implications of these results for the somatic accumulation of mitochondrial defects during ageing.

Introduction

Mitochondria are membrane-enclosed organelles with an ancient endosymbiotic origin, of an α-protobacterium in an archaebacterial host (Sagan 1967; Margulis 1993). Although most mitochondrial genes have been transferred to the nuclear genome, the mitochondria have retained a small genome separate from the nuclear DNA, the mtDNA. Cells typically contain many mitochondria, and each mitochondrion has multiple copies of mtDNA, resulting in hundreds of mtDNA copies per cell. As the replication of mtDNA and its segregation during mitotic and meiotic division are not under strict control of the nucleus, mtDNA variants with an intracellular selective advantage (e.g. by a replication benefit) can increase in frequency even if these decrease individual-level fitness (Hurst 1995). Such selfish mitochondrial mutants are well known from yeast (Taylor et al. 2002). With a frequency of about 1%, yeast cells mutate to form mini colonies containing far fewer cells than the wild type. The *petite* phenotype is caused by mtDNA mutations giving a replication benefit to the mutated mtDNA at the cost of reduced respiration. Therefore, mutated mtDNA molecules impose a cost to cell fitness and *petite* strains are selected against in competition with wild-type cells.

Selection within cells and among individuals (or cells) can thus act in opposite directions, creating a genetic conflict (Hurst et al. 1996; Taylor et al. 2002). Selection between individuals favours functional mtDNA genomes, while selection within cells may favour selfish, less functional, variants. Various mechanisms that maintain functional mtDNA by selecting against dysfunctional mtDNA variants have been identified. For example, uniparental transmission of mtDNA binds the fate of mtDNA with a single host lineage and restricts the opportunities for selfish mtDNA variants by preventing ‘infection’ of other lineages (Hurst and Hamilton 1992; Hoekstra 2000; Hadjivasiliou et al. 2013). Furthermore, a mtDNA bottleneck during egg cell formation has been shown in mice (Cao et al. 2007; Cree et al. 2008; Wolff et al. 2011), redistributing mtDNA variation from within cells to among cells, and facilitating selection among individuals (Bergstrom and Pritchard 1998), as well as purifying selection in the germline (Stewart et al. 2008). A unifying characteristic of these different mechanisms is that they increase the genetic relatedness among the mtDNAs within individuals. With relatedness among mtDNAs we refer to the genetic similarity of mtDNA copies relative to that expected from random combinations of mtDNA copies in the population where competitive interactions occur (Hamilton 1964a; Queller 1994). High within-individual relatedness exposes the effect of mtDNA mutations to selection at the level of the individual, facilitating efficient selection against selfish mtDNA variants (Cosmides and Tooby 1981; Taylor et al. 2001).

Although there is substantial circumstantial evidence for the significance of high relatedness among the mtDNA within an individual, direct evidence supporting this hypothesis is scarce (Taylor et al. 2002; Jasmin and Zeyl 2014). In this paper, we experimentally study

the significance of high relatedness among the mtDNAs within an individual for selection among individuals against dysfunctional selfish mtDNA variants. As a model system, we use mtDNA-related ageing in the fungal genus *Neurospora*. In these fungi, fitness can easily be measured (Pringle and Taylor 2002), and the syncytial nature of these fungi, the possibility of fusion between individuals and restrictions to such fusion by fusion-deficient mutants and allorecognition, allows us to manipulate the levels of selection acting on mtDNA.

Neurospora as a model system for studying levels of selection acting on mtDNA

Like many other modular organisms, the filamentous ascomycetes *N. crassa* and *N. intermedia* reproduce both sexually and asexually. Individuals normally develop from a single cell. In the sexual cycle they develop from a uninucleate ascospore and in the asexual cycle from a multinucleate macroconidium. New individuals may, however, also develop from multiple cells, for example from a number of macroconidia that fuse upon germination. Upon germination, spores form specialized hyphae called germ tubes, but also form anastomosis or fusion tubes (Roca et al. 2005a). The former ones differentiate into the vegetative hyphae of a fungal colony whereas the latter ones fuse with other tubes or hyphae (figure 1a) (Bistis et al. 2003). The hyphae of a growing colony constantly branch and fuse, ultimately generating an intricate network of hyphae called the mycelium, which makes up the fungal body. The mycelium is compartmentalized by septa, but cytoplasm and even nuclei can pass the septa through pores, so that the mycelium effectively is a syncytium. Hyphal fusion is especially important for communication and homeostasis within the mycelium (Glass et al. 2000). However, between genetically different individuals, fusion usually is heavily restricted. A single genetic difference at any of several recognition-loci results in compartmentalization and death of the fused hyphae. This form of allorecognition is generally known in fungi as somatic or heterokaryon incompatibility (Rayner et al. 1984; Glass and Kaneko 2003).
PKALILO (pKAL) is a linear mitochondrial plasmid found in the natural population of *Neurospora* species. It acts as an insertional mutagen and is associated with a senescence syndrome (Bertrand et al. 1985; Bertrand et al. 1986) Like almost all other fungi, *Neurospora* strains without the plasmid do not show senescence, meaning that their vegetative growth potentially is unlimited, but strains carrying the plasmid have a limited lifespan. In senescing cultures, the plasmid inserts into the mitochondrial genome, and it is often found at sites located within or near the mitochondrial rRNA loci. For yet unknown reasons, mtDNA molecules carrying an integrated copy of pKAL are ‘suppressive’, *i.e.* they accumulate during growth, gradually replacing the wild type molecules (Myers et al. 1989). Isolates carrying the mutator plasmid thus become deficient in functional mitochondrial ribosomes and ultimately die. Essentially, this means that the mutated mtDNA has become a selfish element that is selected, even though it comes at a cost for the multicellular mycelium at a higher level of selection.

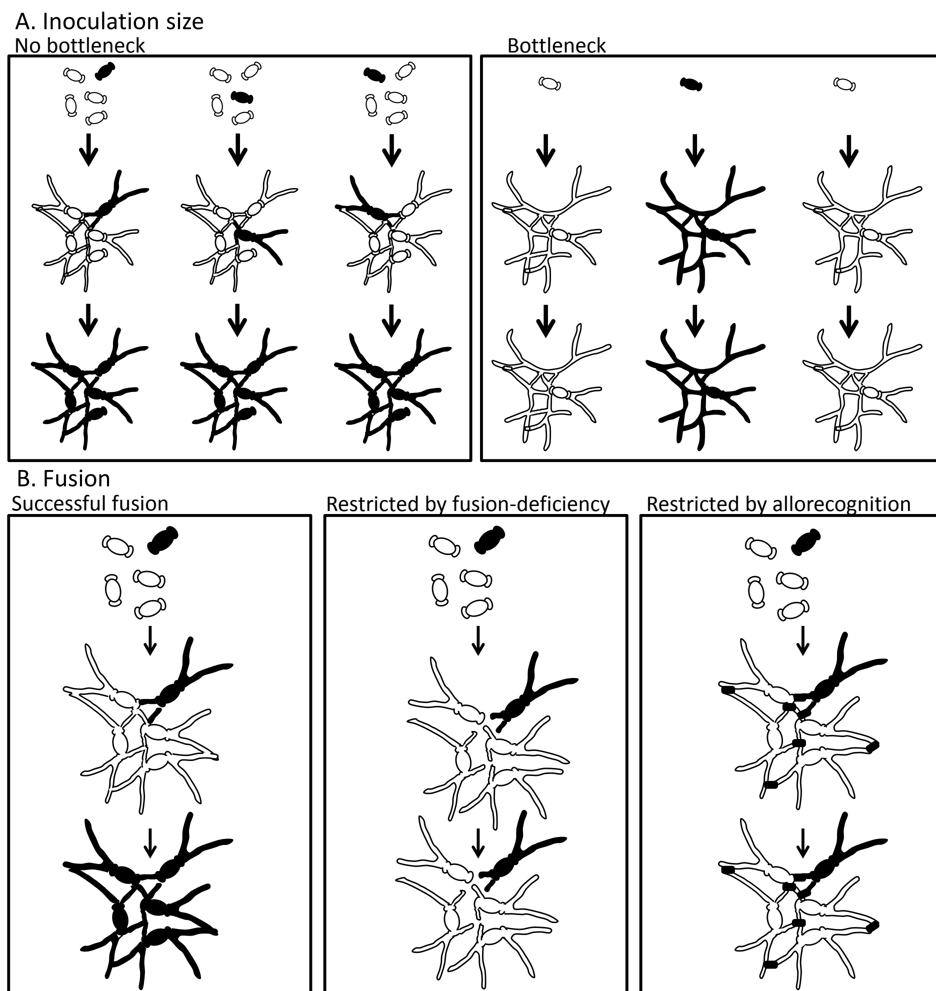

Figure 1 Conditions that influence the level at which natural selection on mitochondria acts and the consequences for the lifespan of cultures.
The figure schematically represents how conidia germinate and then fuse (or not) and form a mycelium. Black conidia and mycelia indicate a high frequency of suppressive mitochondria that will lead to senescence during somatic growth, while white conidia and mycelia indicate a low frequency or absence of suppressive mitochondria. (a) A large inoculation size leads to a relatively low relatedness among the mitochondria within a single mycelium, increasing the probability that suppressive mitochondria are present in the mycelium, which will cause senescence of the culture. In contrast, a smaller inoculation size creates a bottleneck and increases relatedness among the mtDNAs of a single mycelium, reducing the probability that suppressive mitochondria will be in the culture. (b) Successful fusion between conidia allows the exchange of mitochondria, and will transmit the suppressive mitochondria from any of the conidia with suppressive mitochondria to the entire mycelium. Restriction to fusion prevents exchange of mitochondria, creating an effective bottleneck of one conidium. Selection between mycelia growing from one spore will now disfavour mycelia grown from conidia with suppressive mitochondria. Fusion between mycelia and germinating spores can be restricted in two ways: using a fusion defective mutant or by allorecognition.

Senescence in *Neurospora* can be demonstrated using 'race-tubes' containing long slants of agar, where survival is measured in mm of growth or days of growth. Alternatively it can also be demonstrated by serially transferring large numbers of conidia. After a certain number of sub-cultures, usually within a matter of weeks, strains that carry the pKAL plasmid can no longer be propagated, in contrast to strains not carrying the pKAL plasmid, which do not show senescence at all, even after many sub-cultures. The pKAL plasmid can be seen as a truly parasitic element and presumably survives by virtue of its ability to spread horizontally. Though the system of vegetative incompatibility is effective most of the time, sometimes it does not completely prevent cytoplasmic exchange. Transmission of pKAL can thus sometimes be observed even between somatically incompatible isolates (Debets et al. 1994). Transmission of the senescent state has, however, never been observed between somatically incompatible isolates, while this easily occurs between compatible isolates. This shows that allorecognition effectively prevents the exchange of mtDNAs. Furthermore, during the formation of sexual spores, 'rejuvenation' occurs, meaning that suppressive mtDNAs are not transmitted to the sexual spores (Griffiths and Bertrand 1984; Myers et al. 1989).

This study

Here we systematically assess the significance of relatedness among mtDNA within individuals for the opportunities of dysfunctional selfish mtDNAs causing senescence. The mutagenic action of the linear plasmid pKALILO causes variation among mtDNAs resulting in lowered genetic similarity among the mtDNAs of a single mycelium. We manipulated mtDNA relatedness within mycelia by manipulating the bottleneck size of transferred conidia and the degree of fusion, using mixtures of incompatible strains, and fusion mutants. We show that high among-mtDNA relatedness within individuals facilitates selection among individuals against dysfunctional selfish mtDNAs.

Material and Methods

Strains and culturing conditions in test for the effect of somatic bottleneck size and defective fusion

For testing the effect of bottleneck size, a *Neurospora crassa* strain with standard Oak-Ridge genetic background was used, in which the prototypic pKAL plasmid was introduced via transient anastomosis and several rounds of introgression. To test the effect of defective fusion, *ham-2*^{RIP} cultures deficient in hyphal fusion (Xiang et al. 2002) were obtained by crossing the *ham-2*^{RIP} null allele (provided by C. Rasmussen) into a '*Kalilo'* background, using the pKAL-carrying partner as the female and the *ham-2*^{RIP} partner as a male. Crosses were done at 25°C, on Westergaard's medium (WM), according to the protocol given by Davis and de Serres (Davis et al. 1970). The progeny was screened for the presence of pKAL

using PCR as described further on.
Senescence was tested by serially sub-culturing the isolates in small tubes containing 1.3 ml of solid minimal medium (VMM), as previously described by Griffiths and Bertrand (Griffiths and Bertrand 1984), or by growing them in 50 cm long glass 'race-tubes' containing 18 ml of VMM, at 25 °C. To control for inoculum size, spores were harvested by adding water to the cultures and suspending the spores by vortex mixing. Then the spore densities of the obtained spore suspensions were estimated by counting, using a haemocytometer. With these estimates, the spore suspensions were diluted to obtain appropriate inoculum sizes (of 10^3-10^6 spores/10 µl). From these suspensions 10 ul was transferred to a tube with fresh solid medium.

Strains and culturing conditions in the test of the effects of allorecognition

To test the effects of allorecognition, wild *Neurospora intermedia* isolates collected from Hawaiian soil samples were used, described earlier by Maas et al. (Maas et al. 2005). Samples that contained pKALILO and had proven to senesce were selected. The conidia used were taken from serially transferred cultures in an advanced stage of ageing (two transfers before the cultures died). For this experiment the conidia were harvested in water. The suspensions were used to make the mixed cultures. Then suspensions from mixed strains and from single strains were inoculated by spreading the suspension evenly over the agar surface. All mixed and monocultures were grown as three replicate serial transferred cultures. Cultures were grown in 16x100 mm glass tubes containing 3.5ml of solid minimal medium (VMM) solidified under an angle of 60 degrees. Further serial transferring of these cultures was done in a similar way, since we hypothesised that spreading the conidia ensures that the fusion rates are lowered in the mixed cultures.

Detection of pKAL

For DNA extraction, cultures were grown in liquid VMM for ~24 hr at 25°. Mycelium was harvested, dried between filter paper, and ground using liquid nitrogen, followed by a standard phenol/chloroform-based DNA extraction (Sambrook et al. 1989). Prior to the addition of phenol and chloroform, samples were incubated for 1 hr at 37° with proteinase-K (100 µg/ml final concentration). Diagnostic PCRs were done using primers located within the open reading frame (ORF) of the plasmid: 5′-GGT GGA ATC TGT GAG CTA TA-3′ and 5′-TGC ATC TCC CTC TTC TTC AC-3' (Maas et al. 2005). Nested semi-random two-step (ST-)PCRs were done as described earlier (Maas et al. 2007).
Calculations of the Weibull parameter estimates
Weibull parameter estimates for k and λ were obtained using the Solver tool in Microsoft Excel which uses quasi-Newton methods to minimize the sum of squares but with derivatives replaced by finite differences.

Results

The survival rate of senescing 'Kalilo' cultures depends on vegetative 'bottleneck' size

Using the traditional way of demonstrating senescence in *Neurospora* (*i.e.* via the serial transfer of large numbers of macroconidia), pKAL carrying cultures generally survive for several weeks. The survival of these cultures fits well with a two-parameter Weibull function $S(x;\lambda,k)=\exp(-(x/\lambda)^k)$ (x is time to failure, $\lambda>0$ is the scale parameter and $k>0$ is the shape parameter) using an increasing failure rate (*i.e.* with a 'Weibull slope' or 'shape' parameter $k>1$). This essentially means that cultures rarely die early on in life and rarely survive much longer than average. The mortality rate sharply increases only after several weeks of sub-culturing (*i.e.* corresponding to an 'E-type' survival function that is typical for a genetically uniform population in a constant environment).

We manipulated the size of the vegetative bottleneck (*i.e.* inoculum size) in the serial sub-culturing tests, to see how this influences life span. We harvested and counted the number of spores, and then made transfers using appropriate dilutions to obtain inoculum sizes of 10^3,10^4 10^5 or 10^6 conidia. With 'bottleneck' width, median life span slightly increased; variance and maximum life span however strongly decreased (Table 1, figure 2). The maximum life span observed using 'narrow' somatic bottlenecks was almost three times that observed using 'wide' somatic bottlenecks (corresponding to 115 days or 49 subcultures versus 49 days or 21 subcultures, respectively).

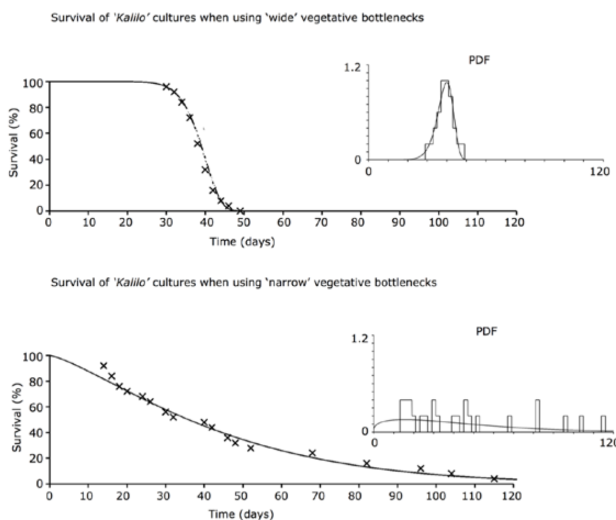

Figure 2 Survival of '*Kalilo*' cultures is strongly affected by vegetative bottleneck size. When sub-culturing is done using narrow vegetative bottlenecks ('wide' corresponds to 10^6; 'narrow' to 10^3 macroconidia/ inoculum), the probability density function (PDF, inset) effectively flattens out (with a shape parameter of k=1, see text), leading to a survivorship function that is typical of disease-related mortality. Descriptive statistics are given in Table 1.

Table 1. Effect of vegetative bottleneck size on the survival of '*Kalilo*' cultures in a 'serial sub-culturing test'.
Whereas median life span appears to increase with 'bottleneck' width, maximum life span and especially variance strongly decreases (see also Figure 2).

Bottleneck size	Life span							
	(# sub-cultures)				(# days)			
	Av. ± sd (n)	Median	Min.	Max.	Av. ± sd (n)	Median	Min.	Max.
10^3 conidia/inoc.	19 ± 16 (25)	9	6	49	45 ± 12 (25)	21	14	115
10^4 conidia/inoc.	8 ± 2 (25)	8	6	18	19 ± 5 (25)	18	14	42
10^5 conidia/inoc.	12 ± 1 (25)	12	9	15	27 ± 3 (25)	28	21	35
10^6 conidia/inoc.	17 ± 1 (25)	16	15	21	39 ± 3 (25)	38	35	49

Table 2. Estimates of the Weibull parameters of the survival curves of 'Kalilo' cultures for the tested bottleneck sizes in the 'serial sub-culturing test'

Bottleneck size	Weibull parameter estimates	
	'shape' parameter k (95% CI)	'scale' parameter λ (95% CI)
10^3 conidia/inoc.	1.3 (1.0-1.8)	49.7 (36.2-68.3)
10^4 conidia/inoc.	3.3 (2.6-4.2)	21.4 (18.9-24.4)
10^5 conidia/inoc.	8.5 (6.5-11.2)	28.9 (27.5-30.3)
10^6 conidia/inoc.	10.7 (8.4-13.8)	40.4 (38.8-42.0)

The width of the somatic bottlenecks strongly influenced the shape of the survival curve. With a decrease in the width of the somatic bottlenecks, estimates of the Weibull slope or 'shape' parameter k progressively decreased (Table 2). At the narrowest bottleneck size used, k no longer differed from one. This means that by narrowing the bottlenecks, the survival function was essentially reduced to an exponential one (*i.e.* with a hazard rate that is constant over time rather than increasing, see Figure 2). With these results we predicted that further reducing the bottleneck size and its frequency would reduce the chance of accumulating suppressive mtDNA variants even more, and thus increase the maximum lifespan. We tested this by reducing the frequency of somatic fusion.

The survival rate of senescing pKAL cultures depends on somatic fusion

Fusion between the conidia and mycelia in a starting culture brings the mitochondria of different individuals together in a fused individual and thus reduces the relatedness among mtDNA. We argued that, without the possibility to fuse, a culture consists of many separate mycelia each of which goes through a bottleneck of a single conidium during each transfer.

Such a small bottleneck maximizes the relatedness among the mtDNAs within individual mycelia, each transfer redistributing new mtDNA variation, arisen via mutation, across spores. This then enables the sequestering of suppressive mtDNA variants in the culture, and selection against them at the level of between-mycelia competition (figure 1b). We therefore tested whether a *ham2*^{RIP} strain, which is defective in fusion, would show suppression of pKAL-based senescence.

First, we tested this using 'race tubes' containing long slants of agar; in these cultures the single-cell bottleneck will only occur once when the culture is started. Life span of the *ham2*^{RIP} strain was increased about two-fold compared to the wildtype *ham-2* strain (Wald's Z=10.734, df=1, P=0.001; Table 3). We hypothesized that the effect of *ham-2* inactivation would be larger when using serial sub-culturing tests than when using 'race tubes': using 'race tubes', suppressive mtDNA variants would be able to invade the culture from behind the growth front through cytoplasmic contact, and due to the narrow growth front of a race tube, genetic drift and between-mycelium selection would quickly eliminate the variation between spores of the starting inoculum, even in the absence of fusion (figure 3). Using serial sub-culturing, this would not be the case: *Ham-2*^{RIP} strains would repeatedly go through an effective bottleneck of a single conidium. *Ham-2*^{RIP} spores that germinate do not fuse with any of their neighbours, potentially increasing the efficiency of sequestering suppressive mtDNA variants.

We therefore tested the effect of the *ham-2*^{RIP} null mutation also using 'serial sub-culturing'. The effect was much more pronounced than when using 'race tubes' (Table 3): None of the tested pKAL carrying *ham-2*^{RIP} cultures died within the time course of the experiment. Defective fusion in combination with serial transferring conidia thus effectively protected the mycelium against the deleterious effects of the plasmid. Using PCR we tested whether pKALILO was lost in the surviving *ham-2*^{RIP} cultures after 25 transfers. This was not the case.

Table 3. Effect of hyphal fusion on the survival of '*Kalilo*' cultures, using two different culturing methods.
On 'race tubes', fusion-defective *ham-2*^{RIP} strains live approximately two-fold longer than wild-type strains (Wald's Z=10.734, df=1, P=0.001). This difference is much more pronounced when using serial sub-culturing: Within the time frame of the experiment (approximately three months), we did not observe any stoppers among the serially sub-cultured *ham-2*^{RIP} lines.

Culturing method	Genotype	Life span							
		# subcultures				Days			
		Av ± sd (n)	Median	Min	Max	Av ± sd (n)	Median	Min	Max
Race tubes	**ham-2**	n/a	n/a	n/a	n/a	11.0 ± 2.8 (20)	11	8	15
	ham-2^{RIP}	n/a	n/a	n/a	n/a	18.0 ± 4.5 (20)	18	7	32
Serial sub-culturing	**ham-2**	9.9 ± 2.5 (20)	10	8	14	19.6 ± 4.9 (20)	20	16	28
	ham-2^{RIP}	>25 (20)	>25	>25	>25	>100 (20)	>100	>100	>100

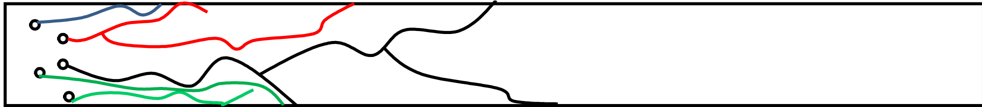

Figure 3
Schematic representation of mycelial growth of a fusion mutant in a racetube. A culture starts (on the left) with the inoculation of multiple conidia. These will form multiple mycelia competing at the growth front. Due to drift and selection for fast growth, the number of mycelia will decrease until only a single mycelium will be left at the growth front. At this point there will only be selection among the mitochondria within this mycelium.

Using semi-random two-step (ST-)PCR we tested whether the long-lived cultures contained inserts of the plasmid. This was not the case either. It thus appeared that pKAL was merely tolerated as an autonomously replicating plasmid and that suppressive mtDNA variants either did not arise, or, more probably, that they arose but were highly effectively selected against.

Allorecognition effectively protects against pKALILO driven senescence

Allorecognition potentially has a similar effect as reduced fusion rates. If conidia from cultures of different allotypes are mixed, they will have a reduced chance of successfully fusing to neighbouring conidia. Only if neighbouring conidia are from the same allotype successful fusion can occur (figure 1b). The more allotypes present in a culture, the lower the probability that neighbouring conidia are of the same allotype, so that the rate of fusion is inversely related to the number of allotypes present in a culture. With this in mind, we hypothesized that if multiple allotypes are present in a well-mixed culture, senescence could be postponed and even be prevented if sufficient allotypes were present, just as effective as in the *ham-2*^{RIP} cultures.
We found a clear effect of allotype diversity in a culture on senescence. In an experiment using cultures that were near the end of their lifespan, we found that, with one exception almost all monocultures aged after two or three more transfers. In sharp contrast, senescence was effectively delayed in cultures consisting of minimally three different allotypes (figure 4). Cultures consisting of a mix of three or more allotypes survived significantly longer than the monocultures of the strains used in these mixes (Mann-Whitney U test $p<0.05$). Four of nine of these mixed cultures still lived after 18 transfers when we stopped the experiment.

Discussion

The replication and segregation of mtDNA is not under strict control of the nuclear DNA. This provides opportunity to selfish mtDNA variants that bear a selective advantage within the cell but a deleterious effect on organismal fitness, to propagate, and leads to a conflict between two levels of selection. We experimentally addressed the importance of genetic relatedness

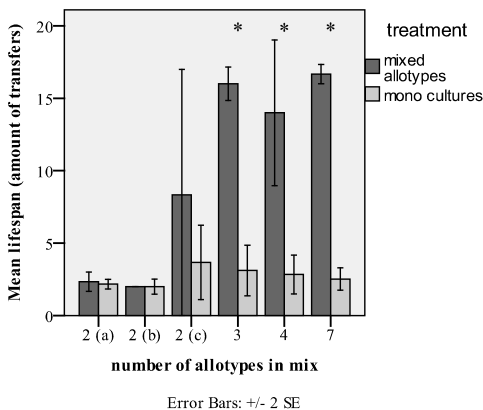

Figure 4
Bar diagram showing the effects on lifespan of allotype diversity in pKAL containing cultures that have been serially transferred. Cultures consisting of a mix of two, three, four or seven allotypes are compared to cultures that contain the same allotypes in monoculture. Survival is defined as number of transfers made from the start of the experiment until the culture died. Significant differences between mixed cultures and monocultures are indicated by * (Mann-Whitney U test $p<0.05$ $N=3$).

among the mitochondria of a single mycelium for selection against selfish mtDNAs using the ascomycete genus *Neurospora* as a model system.

High relatedness among mtDNA facilitates selection against selfish mtDNA

First, we varied relatedness among mitochondria by the number of conidia inoculated in serially transferred cultures. An mtDNA bottleneck resulted in more variation in lifespan and a longer maximum lifespan. We then effectively decreased the bottleneck to a single conidium by using a fusion defective mutant, so that conidia and mycelia cannot fuse. This prevented senescence in serially transferred cultures altogether. Relatedness among mitochondria within starting mycelia is maximized this way, and the suppressive mtDNA variants are sequestered and selected against due to their deleterious effect on mycelial fitness (figure 1). However, we showed that this fusion mutant *did* senesce in a race tube. In a race tube, there is only a single bottleneck during inoculation (figure 3). During subsequent growth, no further bottlenecks occur, and thus no further sequestration of different mtDNA variants is possible. Due to genetic drift and selection at the level of the mycelium, we expect a loss of individuals, so that ultimately within-mycelium selection in the initially most successful individual will dominate, which then will senesce (figure 3).

Finally we maximized relatedness among mtDNAs by allorecognition in a similar way as defective fusion does. In a situation where multiple allotypes occur together, neighbouring mycelia will often be of different allotype and thus not successfully fuse. We show that mixing at least three different allotypes in a serial culturing experiment effectively delays senescence. An interesting question for follow-up research is whether allotype diversity is maintained during the experiment, in which case the culture would be protected against senescence. Alternatively, due to drift and/or positive frequency dependent selection, allotypes are lost, in which case we will expect only a delay in senescence. We detected a large phenotypic

instability during the extended life of the mixed cultures. A culture sometimes seemed to be almost aged while after one or more transfers this culture appeared rejuvenated again. This suggests a delicate balance between the two levels of selection, viz. among mycelia and among mtDNA within mycelia.
A likely explanation for the difference in shape between the highest and lowest inoculum sizes is the potential number of suppressive mtDNAs. A large inoculum increases the probability that the inoculum contains a suppressive mtDNA variant. The frequency of this suppressive mtDNA will initially be small since it finds itself in a large inoculum but will gradually increase during the serial transfers until the culture becomes unviable. With a bottleneck genetic drift becomes more important. On the one hand, the probability that the inoculum contains a suppressive mtDNA variant is smaller. This explains why the maximum lifespan is much higher with a bottleneck. On the other hand, however, if the inoculum contains a suppressive mtDNA variant, it will have a higher starting frequency. As a consequence, senescence will then take place earlier in life. This explains why the minimum lifespan is reduced for cultures transferred with a small bottleneck.
These results provide experimental evidence for the importance of high relatedness among mtDNAs in an individual or cell to prevent the invasion of selfish mtDNA variants. In this system, a regular single-celled bottleneck is sufficient to protect individuals against the invasion by suppressive mtDNA that are constantly generated by the mutagenic action of the KALILO plasmid. If suppressive mtDNA is present in an individual it will increase in frequency during growth because of the selective advantage of this mtDNA within the individual. Many cells with multiple mtDNA copies per cell will be formed during the formation of single-celled dispersal units. The frequency of suppressive mtDNA will vary among these cells, resulting in high genetic similarity among the mtDNA copies within individuals originating from these spores relative to genetic similarity between these individuals. The resulting high within-mycelium relatedness among mtDNAs creates the opportunity for selection against suppressive mtDNAs at the level of the individual.

mtDNA evolution in fungi

The cells of the mycelium of *Neurospora* are incompletely closed and allow the passage of cytoplasm and even nuclei, which results in efficient exchange of mtDNA throughout an individual. This syncytial nature, also found in other filamentous fungi, increases the opportunities for suppressive mtDNA to spread through an individual. However, in nature *Neurospora* will go through a regular bottleneck of one cell or sometimes a small group of cells during both the vegetative and the sexual stage of its lifecycle. Furthermore, during sexual reproduction rejuvenation of the sexual spores occurs (Griffiths and Bertrand 1984; Myers et al. 1989). A recent study shows that also in yeast sexual reproduction reduces the frequency of respiratory deficient mtDNA variants (Jasmin and Zeyl 2014). A possible explanation is that meiosis requires respiration, providing a selective sieve against respiratory-deficient

mtDNA variants (Jasmin and Zeyl 2014).
MtDNA-induced senescence has been found in a few other fungi (Geydan et al. 2012). The evolution of senescence in fungi has been explained by their ecology: senescence usually occurs in species from ephemeral substrates, such as herbivore dung (Geydan et al. 2012). The ecology of *Neurospora* is consistent with this pattern, as strains with the KALILO plasmid have been isolated from sugar cane fields in Hawaii. On this island, fields are burnt yearly, extinguishing all mycelia and asexual spores, while triggering the germination of the sexual spores. External mortality causes thus limit the lifespan of the vegetative state in *Neurospora* in this habitat, reducing the power of natural selection with age, thus 'allowing' the evolution of senescence (Medawar 1952; Williams).

The benefits and risks of somatic fusion

Our results demonstrate that fusion between mycelia increases the possibilities for selfish mtDNA variants and thus poses a high risk to a mycelium. Fusion-deficient mutants are protected against selfish mtDNA variants and one could therefore ask why natural selection has not favoured such variants in nature. The reason is probably that fusion also provides benefits. For example, fusion of conidia increases the growth speed of a starting mycelium (Richard et al. 2012) and under conditions of high density, fusion results in a higher conidia yield (Bastiaans et al in preparation). Furthermore, the fusion mutant has a much lower spore production, indicating the importance of hyphal anastomosis *within* a fungal individual (Xiang et al. 2002).
However, somatic fusion carries risks because it provides opportunities to selfish genetic elements. Successful fusion in fungi is severely restricted by allorecognition. We show that allorecognition can prevent mitochondria-related ageing, suggesting that this may be an adaptation to prevent diseases caused by cytoplasmic selfish genetic elements (Brusini et al. 2011). *Vice versa*, cytoplasmic selfish genetic elements may contribute to the evolutionary stability of allorecognition (Nauta and Hoekstra 1994). However, the balance between selection favouring allorecognition and selection favouring fusion remains to be established (Crozier 1986; Aanen et al. 2008).

MtDNA evolution in other organisms

In contrast to fungi, in most animals, the opportunities for somatic fusion are much more restricted. Only in the sexual stage gametes fuse, and unrelated mtDNAs could be united in a single zygote. However, the vast majority of sexually reproducing organisms have uniparental transmission of mtDNA (Hurst and Hamilton 1992; Birky 1995), maintaining high relatedness. Furthermore, regular single-celled bottlenecks, usually in combination with an additional bottleneck for mtDNA (Cao et al. 2007; Cree et al. 2008), facilitate efficient selection against suppressive mtDNA.
Nevertheless, some aspects of human mitochondrial diseases are consistent with the existence

of conflicts between levels of selection (Taylor et al. 2002). Especially during somatic growth, mtDNA mutations may occur and accumulate within tissues and contribute to aging. Tissues may differ in the relative importance of within-cell selection among mtDNA variants versus among-cell selection. Long-lived cells that undergo few divisions and/or cells with high energetic demands that require large amounts of mitochondria are predicted to be most sensitive to accumulating deleterious mitochondrial mutations. Indeed, mtDNA abnormalities are most frequently found in tissues such as brain, heart and muscle (Chinnery et al. 2002; Krishnan et al. 2007). The model system explored in this study may be a good model for approaching mtDNA related diseases as an evolutionary problem of conflicts between the levels of selection.

Acknowledgements

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Chapter 5

Natural variation of heterokaryon incompatibility gene *het-c* in *Podospora anserina* reveals diversifying selection

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Abstract

In filamentous fungi, allorecognition takes the form of heterokayon incompatibility, a cell death reaction triggered when genetically distinct hyphae fuse. Heterokaryon incompatibility is controlled by specific loci termed *het*-loci. In this paper, we analysed the natural variation in one such fungal allorecognition determinant, the *het-c* heterokaryon incompatibility locus of the filamentous ascomycete *Podospora anserina.* The *het-c* locus determines an allogenic incompatibility reaction together with two unlinked loci termed *het-d* and *het-e.* Each *het-c* allele is incompatible with a specific subset of the *het-d* and *het-e* alleles. We analysed variability at the *het-c* locus in a population of 110 individuals, and in additional isolates from various localities. We identified a total of eleven *het-c* alleles which define seven distinct incompatibility specificity classes in combination with the known *het-d* and *het-e* alleles. We found that the *het-c* allorecognition gene of *P. anserina* is under diversifying selection. We find a highly unequal allele distribution of *het-c* in the population, which contrasts with the more balanced distribution of functional groups of *het-c* based on their allorecognition function. One explanation for the observed *het-c* diversity in the population is its function in allorecognition. However, alleles that are most efficient in allorecognition are rare. An alternative, and not exclusive explanation for the observed diversity is that *het-c* is involved in pathogen recognition. In *Arabidopsis thaliana*, a homolog of *het-c* is a pathogen effector target, supporting this hypothesis. We hypothesize that the *het-c* diversity in *P. anserina* results from both its functions in pathogen-defence, and allorecognition.

Introduction

Non-self recognition is essential to many aspects of inter-organismal interactions, from social behaviour to defence against pathogens (Boehm and Zufall 2006; Strassmann et al. 2011). Conspecific recognition occurs between individuals belonging to the same species while heterospecific recognition occurs between individuals from different species. Genes involved in non-self recognition often display specific evolutionary signatures such as balancing selection and fast evolution (Tiffin and Moeller 2006; Schierup and Vekemans 2008; Weedall and Conway 2010).

Filamentous fungi have the ability to undergo spontaneous somatic cell fusions. However, fusion between wild isolates usually results in a rejection response. This process is known as heterokaryon incompatibility (HI) or vegetative incompatibility. The fused hyphal compartments undergo cell death if the strains involved express incompatible alleles for one or more of the polymorphic heterokaryon incompatibility genes (*het*-genes); (for review see (Glass et al. 2000; Saupe 2000b; Aanen et al. 2010)).

Thus allorecognition in the form of heterokaryon incompatibility sets the boundaries for individuality, but it still is unclear why this individuality is important in the evolution of fungi (Aanen et al. 2008). One possible explanation is that allorecognition provides protection against parasitic nuclei (Hartl et al. 1975; Nauta and Hoekstra 1994; Debets and Griffiths 1998; Aanen et al. 2008; Brusini et al. 2011). Theoretical models have shown that such parasitism can select for allorecognition, as a protection against it (Nauta and Hoekstra 1996; Aanen et al. 2008; Aanen et al. 2010). The stable outcome is a high degree of allorecognition diversity in combination with very low levels of parasitism (Czaran et al. 2015). Another proposed function of allorecognition is protection against the spread of cytoplasmic replicons, such as deleterious mitochondrial plasmids and mycoviruses (Debets et al. 1994; van Diepeningen et al. 1997; Brusini et al. 2011). However, HI does not always provide a strong barrier against the spread of such elements (Debets et al. 1994; Cortesi et al. 2001).

In general, each fungal species displays around ten unlinked *het*-loci which in most cases have a limited number of alleles. This genetic constitution is sufficient to generate considerable numbers of vegetative compatibility groups (VCGs) (Wu et al. 1998; Powell et al. 2007). A VCG is defined as a group of strains which are all compatible with each other but incompatible with members of a different VCG. *Het*-genes have been characterized in the model ascomycetes *Neurospora crassa* and *Podospora anserina* (Aanen et al. 2010), and more recently in the phytopathogenic species *Cryphonectria parasitica,* causing chestnut blight (Choi et al. 2012). Many of the characterized *het*-genes encode proteins displaying a domain termed HET domain, which is involved in initiating cell death (Paoletti and Clave 2007). The level of polymorphism at *het*-loci is generally elevated (Turcq et al. 1991; Wu et al. 1998; Hall et al. 2010; Choi et al. 2012; Lafontaine and Smith 2012). In several cases,

it could be shown that these loci are under an evolutionary regimen that favours generation and maintenance of polymorphism. Notably, in *N. crassa het-C* and *het-6* show positive selection, trans-species polymorphism and balancing selection, in line with their proposed role in allorecognition (Wu et al. 1998; Micali and Smith 2006; Powell et al. 2007; Hall et al. 2010).

In *P. anserina*, the *het-c, het-d* and *het-e het*-loci define two incompatibility systems (*het-c/het-e* and *het-c/het-d*). Each locus is multiallelic and each *het-c* allele is incompatible with a subset of *het-d* and *het-e* alleles. *Het-d* and *het-e* encode STAND proteins (Leipe et al. 2004) that display a N-terminal HET cell-death effector domain, a central NACHT domain for fixation of NTPs and a C-terminal WD repeat domain (Saupe et al. 1995a; Espagne et al. 2002; Chevanne et al. 2009). The WD repeat domain is a ligand binding domain that defines specificity of interaction with the antagonistic HET-C partner (Espagne et al. 2002; Chevanne et al. 2009; Chevanne et al. 2010). *Het-d* and *het-e* are part of a large gene family termed *nwd*, comprising a total of ten members encoding proteins with a NACHT domain and the WD repeat domain. A subset of five of these genes encode proteins with an N-terminal HET domain (the *hnwd* genes). Positive selection was evidenced in the WD repeat region at the *het-d* and *het-e het*-loci (Paoletti et al. 2007). The evolution of the WD repeat encoding sequences is remarkable. The repeats of all members of the gene family undergo concerted evolution as repeat exchanges occur both within and between members of the gene family. Positive diversifying selection then operates on four specific codon positions of each 42 amino-acid long WD-repeat. These four codons correspond to amino acid positions that are predicted to be located at the interaction surface of the WD repeat β-propeller structure. This concerted evolution process allows for generation of new alleles at a high frequency (Chevanne et al. 2010). Because, the *hnwd*-genes are structurally homologous to STAND proteins acting as Pathogen Recognition Receptors (PRRs) in plants and animals, it was proposed that these genes could represent fungal PRRs (Paoletti and Saupe 2009). In that hypothesis, the observed diversity in these *het*-genes would be a consequence of diversifying selection for pathogens resistance, rather than of selection for conspecific allorecognition. The HI reaction induced by these *het*-genes would then be a by-product of their pathogen-driven diversification, and not a primary function.

Het-c, the interacting partner of the *het-e* and *het-d hnwd*-genes in HI encodes a glycolipid transfer protein (GLTP) (Saupe et al. 1995b). GLTPs are almost universally conserved in eukaryotes and bind and transfer various glycolipids between vesicles *in vitro*, but their biological function remains unclear (Mattjus 2009). Glycolipid binding and transfer activity of HET-C has been demonstrated *in vitro*, and the X-ray structure of the protein has been solved (Mattjus et al. 2003; Kenoth et al. 2010). In addition to its role in HI, HET-C has a function in the sexual cycle of *P. anserina* as its inactivation leads to defects in the formation of meiotic progeny (ascospores) (Saupe et al. 1994).

Originally, four *het-c* alleles (*het-c1* to *het-c4*) have been identified in a collection of 19 *P.*

anserina isolates, each one being characterized by its specific incompatibility pattern with the different *het-d* and *het-e* alleles (Bernet 1967; Saupe et al. 1995b). In this paper, we investigate the evolutionary features of this gene. We have screened a collection of *P. anserina* isolates of over 100 wild isolates collected in the area around Wageningen (the Netherlands), for polymorphisms and functional diversity at the *het-c* locus. Additionally, we screened six isolates originating from various places around the world. We show that *het-c* is highly polymorphic and describe eleven different *het-c* alleles corresponding to seven functional categories in HI. We identify several codons in *het-c* that are under positive diversifying selection. Hence, *het-c* like its partner genes *het-d* and *het-e* is under positive diversifying selection. Two alternate hypotheses to account for this positive selection in *het-c* (and *het-d / het-e*) are presented. As classically proposed, the allorecognition function of these genes might drive their rapid diversification. Alternatively, these genes could have a function in host-defence and pathogen-driven divergence might as a secondary consequence lead to HI.

Results

Screening for alleles

The *het-c* gene was PCR amplified and sequenced from isolates of the Wageningen *P. anserina* collection (van Diepeningen et al. 2008), along with six additional isolates from elsewhere. Among 110 isolates from the local population surrounding Wageningen we found eight different *het-c* alleles. Five of these alleles (*het-c5* to *het-c9*) were new and not previously described, whereas the other three were identical to the ones found previously in French isolates (Saupe et al. 1995b). Additionally six strains isolated from various places around the world yielded two additional *het-c* alleles (*het-c10* and *het-c11*). With the seven new alleles found in this study, the total number of known *het-c* alleles is now 11. A phylogenetic reconstruction of these sequences is presented in figure 1. Together, these alleles contain 50 polymorphic sites. Nine polymorphic sites are located in one of the two introns. Amongst the 41 remaining polymorphic sites, 6 polymorphisms are silent (see below and supplemental fig S1). The remaining differences lead to 26 polymorphic sites at the protein level (fig. 2A). We found no indel polymorphism in the *het-c* sequences.

Mapping of the polymorphic sites on the HET-C structure

Het-c encodes a glycolipid transfer protein and the structure of the HET-C protein has been solved by Kenoth et al. (Kenoth et al. 2010). HET-C displays a distinctive two-layer “sandwich-motif” dominated by α-helices. Alpha-helices α1, α2, α6 and α7 make up one layer while α3, α4, α5 and α8 make up the other layer. Amino acids forming the hydrophobic pocket accommodating the lipid or amino acids involved in binding the sugar moiety upon loading of the glycolipid have been identified (fig. 2). In addition, a computational approach

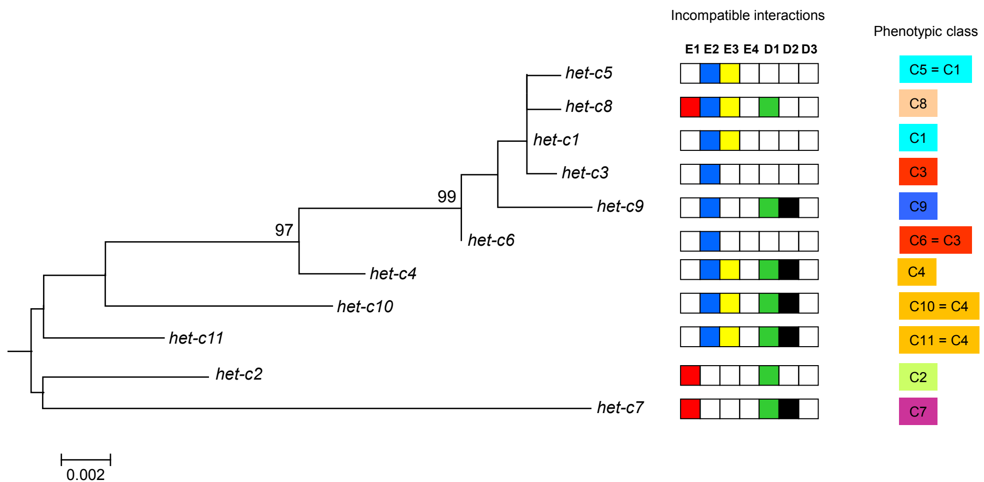

Figure 1
Phylogenetic relationship of the eleven *het-c* alleles identified in *P. anserina* in a Neighbour-Joining tree. Only bootstrap values above 95 are indicated. Branch lengths are drawn to scale according to evolutionary distances calculated as substitutions per site. In the middle panel the incompatible interactions of each *het-c* allele with the different *het-d* and *het-e* alleles are indicated by coloured boxes, white boxes indicating compatible reactions. The right panel indicates phenotypic classes as a combination of all incompatible reactions in reference to previously characterized *het-c1* to *het-c4* alleles.

was used to predict amino acids potentially involved in the binding of HET-C to membranes. An alignment of the 11 HET-C protein sequences presented in figure 2A reveals that the polymorphic positions are mainly clustered in two highly variable regions between positions 58 and 84 (six polymorphic sites) and positions 118 to 160 (twelve polymorphic sites) thereby confirming previous observations by Saupe *et al.* (Saupe et al. 1995b). Eighteen polymorphic positions are located in the α1, α2, α6, α7 layer of the protein, and 12 in the α3, α4, α5, α8 layer. Six of the remaining polymorphic sites are located in loop regions between α-helices. The polymorphic positions identified between the *het-c* alleles are distinct from the positions directly involved in lipid or sugar binding consistent with the necessity to maintain *het-c*-function in glycolipid transfer (fig. 2A). A number of polymorphic sites are immediately adjacent to residues involved in sugar binding. Polymorphic positions are located at the protein surface and importantly, they are found in the two distinct layers of the "sandwich-motif" and thus do not group as a single interaction surface (fig. 2B). The existence of these two distinct polymorphic interaction surfaces might be explained in a model postulating that the WD-repeat domain of HET-D and HET-E are organized as two distinct β-propellers forming a clamp-like structure around HET-C as occurs in the cytochrome *c*/APAF-1 interaction (Yu et al. 2005).

Specificity of the novel *het-c* alleles

To assess HI specificity of the newly identified *het-c* alleles, the alleles were PCR amplified along with promoter and terminator sequences, cloned and introduced by transformation in a Δhet-c *het-e4 het-d3* recipient strain. The *het-e4 het-d3* genotype was chosen for the recipient because these *het-e* and *het-d* alleles were found to be universally compatible with all previously identified *het-c* alleles. Each transformation resulted in a comparable number of transformants, suggesting compatibility between the transformed *het-c* alleles and genes expressed by the recipient strains, in particular the *nwd*-gene family members. Transformants were selected and presence of the transforming DNA was confirmed by PCR for at least two transformants for each *het-c* allele.

The seven new *het-c* alleles were tested for incompatibility with the seven known tester alleles for *het-d* and *het-e*. The results were compared with the patterns of the four original *het-c* alleles (fig. 1) (Bernet 1967; Saupe et al. 1994). Each of the 11 alleles is incompatible with at least one of the three *het-e* tester strains. As expected none of the alleles showed incompatibility to the *het-d3* or *het-e4*. The eleven *het-c* natural alleles can be grouped into seven different phenotypic classes based on their patterns of heterokaryon incompatibility, the new variant alleles defining three new phenotypic classes (fig. 1). The novel *het-c5* and *het-c6* alleles have the same interaction specificity as previously characterized for *het-c1* and *het-c3* respectively. Similarly, *het-c10* and *het-c11* have the same specificity as *het-c4*. This analysis thus identifies a total of seven functional categories in wild-type alleles. In a previous study, 10 artificial chimeric alleles yielding novel interaction patterns were generated by combining sequences from the four reference alleles (Saupe et al. 1995b), their interaction patterns are recalled in (supplemental fig. S2). The pattern of the novel *het-c7* allele was obtained for three of the artificial chimeric alleles. Two additional patterns are only seen in the chimeric artificial alleles.

Inspection of the compatibility of *het-c* alleles reveals existence of specific patterns in these interactions. First of note is the fact that all wild-type alleles produce at least one incompatibility reaction. In contrast to the *het-d* and *het*-e loci, no neutral alleles are found. All alleles are incompatible at least with either *het-e1* or *het-e2*. Incompatibilities with *het-e1* and *het-e2* are generally mutually exclusive. *Het-c8* represents an exception in that regard. Then, the incompatibility patterns reveal a hierarchy in the incompatibility reactions. For instance, wild-type alleles that are incompatible with *het-e1* are also incompatible with *het-d1* (only artificial *het-c2-2* is an exception). Similarly, alleles incompatible with *het-d2* are also incompatible with *het-d1* and alleles incompatible with *het-e3* are also incompatible with *het-e2*. In each case, the converse is not true*, i.e.* alleles incompatible with *het-e1* are not necessarily incompatible with *het-d1*.

The new alleles contribute to refinement of our understanding of the role of polymorphic

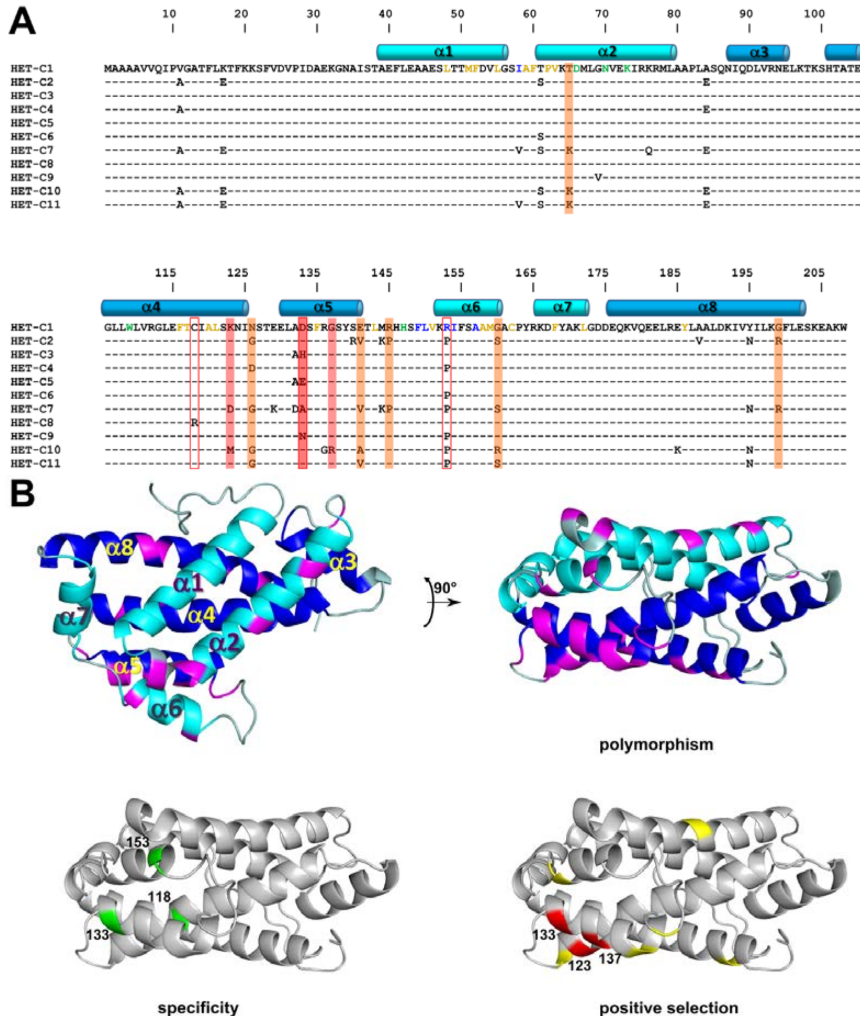

Figure 2

(A) Alignment of amino acid sequences encoded by the eleven known naturally occurring *het-c* alleles found in this study and previous studies. The amino acid sequence encoded by *het-c1* is shown completely, only sequence differences are given for the other *het-c* alleles. Position of the α-helices of HET-C is given in cyan and blue respectively for the helices forming the two layers of the sandwich motif. Residues coloured in yellow correspond to residues involved in lipid binding, in green to residues involved in sugar binding, in blue to residues proposed to interact with membranes. Residues boxed in red (118,133,153) are shown to be involved in allele specificity. Residues shadowed in red and orange correspond to residues under positive selection with a 99% and 95% confidence level respectively (B) Three-dimensional structure of HET-C protein (Kenoth et al. 2010). The α-helices that form the two layers of the sandwich motif (α1,2,6,7 and α3,4,5,8) are given in cyan and blue respectively. On the two top panels, polymorphic positions are given in magenta. The two lower panels identify the residues shown to be involved in allele specificity in green and the residues under positive selection in red for residues with a 99% confidence level and in yellow for residues with a 95% confidence level.

positions in allele specificity. By comparing the four initial alleles as well as chimeric alleles, Saupe and co-authors could identify that polymorphic position 133 and 153 are involved in allele specificity (Saupe et al. 1995b). This study now identified position 118 as also involved in the control of specificity. *het-c1* and *het-c8* have a distinct incompatibility pattern and differ by a single amino acid at position 118. Replacement of the cysteine at this position in *het-c1,* by a positively charged arginine in *het-c8,* leads to the acquisition of two additional HI reactions with *het-e1* and *het-d1*. The present data also confirm the role of the amino acid at position 133. Indeed alleles *het-c1*, *het-c3* and *het-c5* differ only by a single polymorphism at this position. The products of *het-c1* and *het-c5* differ by a conservative substitution (of aspartic to glutamic acid) at that position and present the same incompatibility specificity (incompatible with *het-e2* and *het-e3*). A histidine is found in the *het-c3* product and this allele product is incompatible only with *het-e2*. Thus, a histidine at amino acid position 133 in place of a negatively charged amino acid determines incompatibility with *het-e3*. This brings the number of positions identified as involved in defining incompatibility specificity to three (118, 133, 153). However, previous studies have shown that at least one polymorphic position in the N-terminal part of the protein between position 11 and 65 is also involved. The smallest combination of amino acid positions that could account for all differences in allele specificity is the seven amino acids at positions 17, 65, 84, 118, 126, 133, 153.
Of note is the fact that allele products with different protein sequences can display the same specificity. For instance, *het-c4* and *het-c10* display the same allelic specificity while differing by 10 amino acids. Similarly, *het-c7* and *het-c1-2* (a chimeric allele) are identical in specificity while differing also by 10 residues. This observation may indicate that some of the polymorphisms existing in *het-c* do not modify allele specificity or that these are specific to yet unknown alleles of *het-d* and *het-e*.

Escape from self-incompatibility caused by newly identified *het-c* alleles occurs by repeat loss in the antagonistic *hnwd*-gene

Self-incompatible strains caused by *hnwd*-genes escape HI most of the time through deletion of a variable number of WD repeats (Chevanne et al. 2010). A *het-d2* tester strain was crossed with a Δhet-c strain transformed with either *het-c9* or *het-c10*. Self-incompatible progeny were recovered and escape mutants were selected as previously described (Chevanne et al. 2010) and the length of the WD repeat domain of *het-d* analysed by PCR. As expected most of them displayed a WD repeat domain reduced in size compared to the starting allele (data not shown). Out of the 32 escaped mutants tested, 26 were reduced in size. Their estimated size varies between two and thirteen WD repeats, while the parental *het-d2* was estimated to have twelve WD repeats. Thus escape from the genetic conflict generated by introduction of a new *het-c* variant is resolved through the modification of the WD-repeat domain of the antagonistic *het-d* gene, providing further evidence that incompatibility indeed occurs between the variant *het-c* allele and the *hnwd* locus.

Distribution of *het-c* alleles in the population

Het-c2 is the most frequent allele representing 46% of the population (fig. 3A). Allele frequencies of *het-c1, -3* and *-5* are in the range of 15% followed by *het-c6* at a frequency of 5%. *het-c7, -8* and *-9* are rare alleles each found only once, while *het-c4*, *-10* and *-11* were absent from the Wageningen population sample. Alleles can be grouped by specificity-type into phenotypic classes. *Het-c5* belongs to the same phenotypic class as *het-c1* and likewise *het-c6* is phenotypically identical to *het-c3*. This classification into phenotypic classes reveals three abundant classes (C2, C1 and C3-type) representing respectively 46, 31 and 20% of the population (fig. 3B). We compared this phenotypic class distribution to the distribution found in a collection of 19 isolates collected in various locations in France in the 1940s (Supplemental fig. S3). In this collection also, the C2-type is the most abundant type followed by C1 and C3. The C4-type allele absent from the Wageningen population was found only twice in the French isolates.

As noted previously, the vast majority of the alleles are incompatible with either *het-e1* or *het-e2*. By grouping alleles by this criterion, it appears that the alleles from the Wageningen population form two equilibrated classes with 47% and 52% of the alleles incompatible with *het-e1* and *het-e2* respectively (the remaining 1% is incompatible with both *het-e1* and *het-e2*). Similarly, in the French collection of 19 strains, one finds 9 alleles incompatible with *het-e1* and 10 alleles incompatible with *het-e2*.

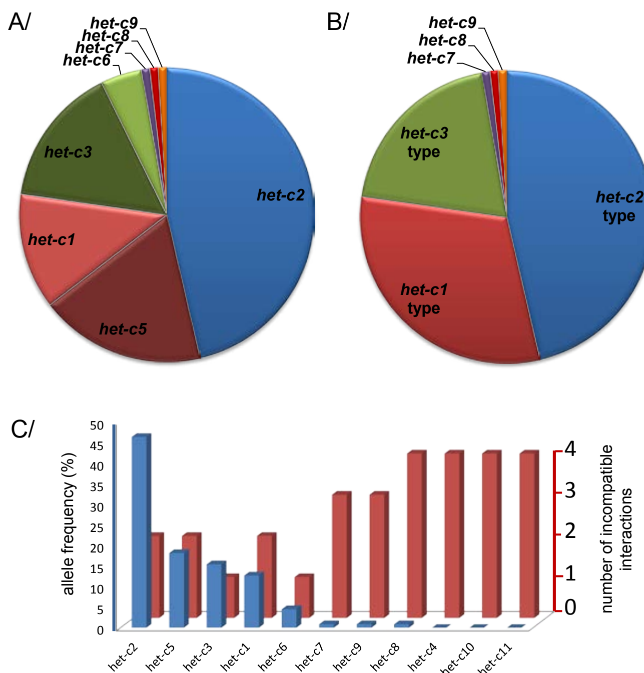

Figure 3
Allele frequency distribution for *het*-c in the Wageningen collection of *P.* anserina. (A) Pie chart showing frequency distribution of the eight different alleles found in the collection. (B) Pie chart grouping the different *het-c* alleles into functional groups based on their incompatibility pattern with known *het-d* and *het-e* alleles. (C) Column chart comparing for all known *het-c* alleles its allele frequencies in the Wageningen collection with the number of *het-d* and *het-e* alleles it shows an incompatible interaction with.

A phylogenetic reconstruction of the different *het-c* DNA sequences reveals two main clades (fig. 1). Although these clades are not well supported, it appears that alleles incompatible with *het-e2* comprise one clade, while two out of three alleles incompatible with *het-e1* comprise the other clade (fig. 1). Assuming that this phylogeny represents the historical relationship among these alleles, these data may suggest that these incompatibilities are ancestral and were maintained throughout evolution. Only *het-c8* seems to have gained incompatibility with *het-e2* secondarily. This situation seems to be different for incompatibility with *het-d1*, *het-d2* and *het-e3* that seem to have been gained or lost independently several times. However, the observed clustering of these different kinds of incompatibility interactions in the phylogeny may also be a direct consequence of sequence similarity among alleles with similar incompatibility interactions caused by positive selection for their function in these incompatibility interactions.
To find a possible cause for the unequal distribution of the *het-c* alleles and the absence of *het-c4 het-c10* and *het-c11*, we tried to find out if there is a relation with the number of incompatible reactions a *het-c* allele can cause. The *het-c* alleles differ by the number of incompatibility reactions with *het-d* and *het-e* alleles they produce. This number ranges from 1 (*e.g.* for *het-c3*) to 4 (*e.g.* for *het-c8*). There appears to be an inverse correlation between the number of incompatible interactions and allele frequency (fig. 3C). Interestingly, alleles that yield many incompatible reactions with the known *het-d* and *het-e* alleles are rare or absent from the population, suggesting the allorecognition function of *het-c* comes at a cost.
Natural populations of *P. anserina* are infected by a mitochondrial senescence plasmid pAL2-1 which affects life span in all tested conditions. Propagation of this deleterious plasmid was found to be limited by incompatibility based on a distinct allelic *het*-system (the *het-s/het-S* allele incompatibility system) (Debets et al. 2012). We have analysed the distribution of the senescence plasmid in relation with the *het-c* genotype (Supplemental fig S4). It appears that the *het-c2* class of isolates that occurs at the highest frequency has a higher frequency of pAL2-1 infections compared to the less frequent *het-c* classes. However, overall we found no significant correlation between the *het-c* genotype and the distribution of the pAL2-1 plasmid.

Het-c is subject to positive selection in *P. anserina*

Sequence analysis of the first four alleles unravelled unusual evolutionary features for *het-c*: more polymorphisms were identified within the open reading frame than in the introns or 5’ and 3’ non-coding sequences (Saupe et al. 1995b). In the present study we focused our analysis on the coding sequences. We found a total of 41 polymorphic sites between the *het-c* alleles in the ORF, only six of which correspond to synonymous substitutions. Four of these synonymous substitutions occur in *het-c7* (Supplemental data 1). These observations suggest that the *het-c* locus may be under positive diversifying selection. We have thus analysed *het-c* divergence using the HyPhy software package (Pond et al. 2005). The codon-

based substitution models developed by Nielsen and Yang (Nielsen and Yang 1998; Yang et al. 2000) were implemented within the HyPhy software package (Kosakovsky Pond and Muse 2005). These models, based on the ratios between non-synonymous and synonymous substitutions ω=dN/dS, allow for varying rates of substitutions between sites to account for varying selection forces. The analysis was conducted with the *het-c* allele data set. All models except Model 4 resulted in overall dN/dS ratio values between 1.12 and 1.48 (Table 2). Model 1 and 7 do not allow for $\omega > 1$. These results indicate that *het-c* evolves under positive selection in *P. anserina*. Comparing pairs of models (M1-M2) and (M7-M8) indicate that both models M2 and M8 identify codons under positive selection at the 99% confidence level. Posterior probabilities of model M2 reveal codons 123, 133 and 137 as positively selected at the 99% confidence level and codons 65, 126, 141, 145, 160 and 199 at the 95% confidence level. Model M8 identifies all of these codons as positively selected at the 99% confidence level, and additional codons 11, 17, 58, 69, 76, 84, 118, 129, 132, 140, 153, 185, 188, 195 as positively selected at the 95% confidence level. Other codons are under purifying selection. This indicates that the main force driving evolution of *het-c* alleles is positive diversifying selection acting on a limited number of codons. The positions under selection are indicated on the structure of the HET-C protein (fig. 2). Positively selected positions are located on the two opposite sides of the “sandwich-motif”. The three positions that show the strongest statistical support for positive selection (123, 133 and 137) group on the HET-C structure (fig. 2B). Among them, position 133 is proven experimentally to be involved in allele specificity.

We next asked whether positive selection is a general feature of GLTP encoding genes in fungi. Sequences from different fungal GLTPs were recovered after blastx searches of the fungal genome sequences in the NCBI Fungal Genome Central database using the *P. anserina het-c2* sequence as a query. The accession numbers for 37 sequences retrieved are listed in supplemental figure S5. The chosen sequences cover the subphylum of the Pezizomycotina. A protein guided alignment of the ORF sequences was generated with ClustalW, and a Neighbour-joining tree constructed using the MEGA4 package (Tamura et al. 2007; Supplemental fig. S5). All models found overall low dN/dS ratios of 0.15 to 0.34 (Table 1). We thus conclude from this analysis that GLTP encoding genes in filamentous fungi are evolving under purifying selection and that positive selection appears specific to *P. anserina*. This implies that in *P. anserina* the GLTP *het-c* has gained a function in non-self recognition in addition to its function as a GLTP.

It is relevant to note that Ellison and co-authors conducted an analysis of population genomics and local adaptation in *N. crassa*, a close relative to *P. anserina* (Ellison et al. 2011). This analysis examined Single Nucleotide Polymorphisms (SNPs) in the transcriptome of 48 wild isolates from the Caribbean basin, South America and Africa, constituting two recently

Table 1: Likelihood values and overall dN/dS ratio obtained for the Nielsen Yang models with the eleven *het-c* alleles from *P. anserina*.

Model	Log Likelihood	dN/dS
M0	-1183.50160	1.24
M1	-1179.03409	0.44
M2	-1168.45456	1.42
M3	-1168.43569	1.32
M4	-1170.53317	0.74
M5	-1169.07790	1.41
M6	-1169.06485	1.45
M7	-1179.73459	0.49
M8	-1168.64954	1.12
M9	-1169.06542	1.40
M10	-1169.06280	1.42
M11	-1169.07282	1.48
M12	-1168.47380	1.42
M13	-1169.06289	1.43

Table 2: Likelihood values and overall dN/dS ratio obtained for the Nielsen Yang models with the GLTP encoding genes from filamentous fungi.

Model	Log Likelihood	dN/dS
M0	-12323.53746	0.15
M1	-12153,94895	0.29
M2	-12153,74433	1.70
M3	-11960,78952	0.18
M4	-12193,55755	0.34
M5	-11961,46353	0.18
M6	-11961,46354	0.18
M7	-11959,66209	0.19
M8	-11953,08587	0.19
M9	-11959,35832	0.19
M10	-11959,66246	0.19
M11	-11960,89653	0.19
M12	-11958,65961	0.19
M13	-12006,13631	0.50

diverged populations and a number of outliers. They identified a list of 12 genes evolving under positive selection, which did not include NCU07947, the *N. crassa* orthologue of *het-c*. In the SNPs data, we identified 18 polymorphic positions in NCU07947, all corresponding to synonymous sites (Supplemental fig. S6). Thus, as for fungal GLTP encoding genes, it seems that at least in the populations analysed NCU07947 evolves under purifying selection. A similar genomic population analysis was conducted with two *Coccidioides* species (Ellison et al. 2011) and again we found that *Coccidioides het-c* orthologue appears to be evolving under purifying selection (Supplemental fig. S7). For a number of additional species *het-c* allele sequences are held in databases that again display essentially synonymous polymorphisms (supplemental fig. S8).

Overall it appears that fungal GLTPs encoding genes are under purifying selection while diversifying selection is operating specifically on *P. anserina het-c.*

Discussion

We show that *het-c*, involved in vegetative incompatibility with *het-d* and *het-e* and encoding a GLTP, contains several codons that are under positive diversifying selection leading to high levels of polymorphism. In addition we show that the fast evolving codons of *het-c* encode amino acids known not to be involved in glycolipid binding nor in forming the proposed membrane interface. Polymorphic residues are found in two regions lying at the surface on opposite sides of the protein structure. Positive selection appears to be specific to the *P. anserina het-c* gene as *het-c* orthologues in other fungal species evolve under purifying selection. We observed a high allelic diversity of *het-c* associated with an uneven allele distribution. However, the distribution of phenotypic classes, as defined by their incompatibility pattern with *het-d* and *het-e*, proves to be more balanced. The high level of diversity and polymorphism observed at the *het-c* locus was also observed at the antagonistic loci *het-d* and *het-e* (Paoletti et al. 2007; Chevanne et al. 2010) suggesting that *het-c, het-d* and *het-e* are under similar selective regimens.

Allorecognition function of *het-c* as a cause for diversifying selection

What is the cause of the diversifying selection acting on *het-c*? The simplest and most obvious hypothesis is that diversifying selection acting on *het-c* relates to the role of this gene in allorecognition. Classically it is proposed that allorecognition in fungi in the form of vegetative incompatibility may have evolved to provide individuals with a protection against nuclear parasites (Hartl et al. 1975; Nauta and Hoekstra 1994; Debets and Griffiths 1998; Aanen et al. 2008; Brusini et al. 2011) or deleterious cytoplasmic elements (Debets et al. 1994; van Diepeningen et al. 1997; Brusini et al. 2011). HI will limit cytoplasmic exchanges between individuals and thus limit propagation of deleterious cytoplasmic elements between

strains. The stable outcome of HI evolution is a high degree of allorecognition diversity in combination with low levels of parasitism (Brasier 1988; Muirhead et al. 2002; Czaran et al. 2014). In these systems diversity is maintained by balancing selection leading to an even distribution of incompatible alleles. A typical example of this situation is provided by the *het-c* and *het-6* incompatibility systems in the genus *Neurospora*, where highly polymorphic incompatible alleles are maintained in the population at these loci over long periods of time (Wu et al. 1998; Powell et al. 2007).

Natural populations of *P. anserina* are infected by a mitochondrial senescence plasmid pAL2-1 which affects life span in all tested conditions. Plasmid infection was found to be negatively correlated with allele frequency at the *het-s* locus meaning that strains bearing the less common *het-S* allele were less infected than those bearing the more frequent *het-s* allele (Debets et al. 2012). We found no clear evidence for biased distribution of pAL2-1 in strains bearing rare alleles of *het-c* yet. Since we are dealing here with a non-allelic system, efficiency of a *het-c* allele in preventing plasmid spreading in the population is not expected to be determined solely by its prevalence but also by the prevalence of the antagonistic *het-d* and *het-e* alleles (which is currently unknown for the Wageningen population).

Pathogen-driven divergence as a possible alternative cause for diversifying selection in *het-c*

An alternative hypothesis to explain *het-c* diversification stems from the studies on *ACD11*, the *het-c* homolog in *A. thaliana*. *ACD11* (accelerated cell death) was isolated as a gene whose inactivation initiates cell death in the form of a hypersensitive response (the pleiotropic inflammatory response associated with the immune response to viral or bacterial pathogens in higher plants). The *acd11* point mutant alleles affected in glycolipids binding suppress the *accelerated cell death* phenotype indicating that the cell death reaction cannot be directly attributed to a defect in glycolipid transfer activity (Petersen et al. 2008). Instead, this cell death reaction was equated to an autoimmune reaction and is currently interpreted in the frame of the "guard hypothesis". In this model, plant immune NB-LRR receptor proteins act as guards that survey a guardee, that is a host protein targeted by a pathogen effector molecule (Dangl and Jones 2001; Jones and Dangl 2006). The hypersensitive immune response is initiated when the receptor senses modification of the guardee by a pathogen effector molecule. It was found that the presence of the protein *NB-LRR* resistance gene *LAZ5* is required to initiate the *acd11* associated PCD reaction (Palma et al. 2010). This led to the suggestion that ACD11 might be a guardee targeted by a pathogen effector molecule. In support of this model is the fact that several proteins interacting with ACD11 were identified as direct targets of pathogen effectors (Dreze et al. 2011; Mukhtar et al. 2011). By analogy, *P. anserina,* HET-C might correspond to a guardee (pathogen effector target) under the surveillance of the *het-e* and *het-d* HNWD proteins which are structurally related to plant NB-LRR and animal NOD-like pathogen recognition receptors. Plant host targets

have been found to show diversifying selection consistent with the existence of a host-pathogen arms race (Kaschani et al. 2010). If *het-c* also represents a host target as proposed for ACD11, it may be speculated that diversifying selection in *het-c* is pathogen-driven. The fact that pathogen-driven divergence can lead to genetic conflicts is illustrated by the process of hybrid necrosis in plants. In *A. thaliana*, hybrid necrosis occurs in the progeny of crosses between incompatible isolates. This necrosis, caused by incompatible gene-to-gene interactions, involves a pathogen resistance gene of the NB-LRR type (Bomblies et al. 2007) and phenotypically mimics an inflammatory response. Hybrid necrosis is thus considered an auto-immune condition caused by divergence in genes with an immune function.

If *het-c* indeed is a pathogen-effector target, diversification of *het-c* might result from selection of variants insensitive to the pathogen effector. Hörger and co-authors (Hörger et al. 2012) have recently demonstrated that co-evolution between the guardee and its guard was the main force driving the evolution of the *RCR3* gene in the wild tomato species *Solanum peruvianum*. If HET-D and HET-E act as guards of HET-C, *het-c* could also diversify rapidly to match the fast evolution of these *hnwd* genes to maintain an optimum guard-guardee interaction.

P. anserina is likely to encounter many potential pathogens and parasites during its life. It grows on herbivore dung where many other organisms occur like bacteria, fungi, insects and nematodes. To grow and survive in such a hostile environment a good defence mechanism against all the potential enemies it encounters in the dung would seem beneficial. Although currently no pathogens of *P. anserina* are known, a number of fungal species have been described as interfering with *P. anserina*'s development (Silar 2005). Mycoviruses have long been described in the fungal kingdom (Dawe and Nuss 2012), and it is also not uncommon for bacteria to be pathogenic to fungi (Paoletti and Saupe 2009; Frey-Klett et al. 2011). Of note in this context, is the fact the transcriptional profile during the incompatibility response in Podospora is reminiscent of a defence response with induction of numerous toxins, secondary metabolism clusters and hydrolases (Bidard et al. 2013).

Het-c allele distribution and polymorphism

It is of note that all *het-c* identified in the present study are active in incompatibility although *het-c* alleles inactive in VI but retaining *het-c*-function in ascospore formation can be obtained artificially (Saupe et al. 1995b). If *het-c* HI is the non-adaptive accidental by-product of pathogen-driven divergence, one does not expect that variant alleles systematically trigger incompatibility. The fact that all *het-c* alleles show HI reactivity rather favours the notion that HI is indeed adaptive and that alleles are selected for that purpose. In addition, although *het-c* allele distribution is unbalanced, when considering *het-c* allele reactivity to *het-e1* and *het-e2*, functional incompatibility classes reach close to balanced distribution in populations and thus match expectations for an allorecognition system under balancing selection.

Whereas all identified *het-c* alleles display activity in incompatibility against at least one *het-d*

or *het-e* allele, the opposite is not true. Indeed, null *het-d* and *het-e* alleles for incompatibility exist (*het-e4* and *het-d3*). Bernet showed that these inactive alleles were widely represented in the 16 isolates from France (Bernet 1967): six isolates expressed the inactive *het-e4* allele, and 13 expressed the null *het-d3* allele (including five also expressing the *het-e4* null allele). An important consequence of the presence of null *het-d* and *het-e* alleles is that these incompatibility systems fail to split the population into so called vegetative compatibility groups (VCGs). Members of a VCG are compatible with each other and incompatible with members of other VCGs. In principle, individuals belonging to a given VCG will be protected from the infectious replicons present in isolates from different VCGs. Infection may spread even between incompatible strains when passing through an intermediate strain showing dual compatibility. The existence of such inactive alleles (at least in the Bernet collection) questions the notion that *het-c/het-d* and *het-c/het-e* incompatibility systems are adaptive. Yet, it might be that existence of such null alleles is an unavoidable consequence of the genetic instability of the WD-repeat regions of the *hnwd* genes (Chevanne et al. 2010).

Most isolates in the Wageningen population express the *het-c* alleles that are less efficient in allorecognition (*het-c1*, *het-c2*, *het-c3*, *het-c5*, and *het-c6*); these alleles only display either one or two incompatible interactions with the known *het-d* and *het-e* testers. In contrast, the most efficient *het-c* alleles in terms of allorecognition (*het-c4*, *het-c7*, *het-c8*, *het-c9*, *het-c10* and *het-c11*), incompatible with three or four tester strains, are either absent from the Wageningen population or found in only one isolate. Again, this is not expected if the allorecognition function is considered adaptive. The rare occurrence of *het-c* alleles leading to numerous incompatible interactions could be explained by the cost of the genetic conflicts such alleles trigger during the sexual cycle. Non-allelic systems in *P. anserina* also act as sexual incompatibility loci. Crosses between incompatible strains lead to partial or total sterility and the formation of self-incompatible lethal progeny (Bernet 1967). In an event of out-crossing, strains carrying *het-c* alleles leading to multiple incompatibilities (*i.e. het-c8*) might be disfavoured because they are unlikely to encounter a compatible sexual partner.

If polymorphism in *het-c* is solely driven by the allorecognition function, it might be expected that all variable positions impact allele specificity. Position 133 illustrates this principle. This position is the most variable in HET-C (with 5 distinct amino acids found in the different allele products) and it was indeed found to control allele specificity. Yet, part of the polymorphism in *het-c* is not accounted for in terms of functional variation in the incompatibility specificity. That is a number of polymorphisms do not lead to changes in the incompatibility pattern with the currently known *het-d* and *het-e* alleles. This is for instance illustrated by the fact that the *het-c4* and *het-c10* encoded proteins display the same allelic specificity while differing by 10 amino acids. Simply put, there is apparently more polymorphism in *het-c* than needed for the allorecognition function. Thus, positive selection detected on these codon positions may

not be caused by the HI function. One possibility is that these polymorphisms are the result of selection for pathogen defence. Another possibility is that the additional polymorphisms are compensatory mutations to maintain its GLTP function. Alternatively, we might have an incomplete picture of the incompatible interactions. Additional *het-d* and *het-e* alleles with different specificity types might exist in the Wageningen population. This last hypothesis appears quite likely considering that many more *het-c* alleles were identified in this study in addition to the original alleles identified in the Bernet collection that comprised only 16 strains. Finally, as mentioned before during outcrossing, self-incompatible progeny can arise. There will then be high selective pressure to resolve this conflict, so any mutations that can remove the self-incompatibility will be selected, and this could potentially lead to new polymorphisms. As we observed, resolution of such conflicts are more likely to generate polymorphisms in the WD repeat domains of *het-d* or *het-e* but will also on occasion produce mutations in *het-c*. This may also explain why we see some alleles at a very low frequency. They may have been selected upon an outcrossing event to escape self-incompatibility, while there is no subsequent selective benefit that would increase their frequency.

Clearly, the cause for rapid variation of *het-c* cannot at present be unambiguously defined. If selection for allorecognition function is apparently a satisfying proximal cause for this diversification, certain aspects in *het-c* allele distribution and polymorphism are not fully accounted for in this hypothesis. A more complete understanding of the evolution of *het-c* may have to await a full characterization of the allele constitution and distribution at the partner *het-d* and *het-e* alleles in the Wageningen population.

Incompatibility as an exaptation

The term exaptation was introduced to describe the re-use by natural selection of a structure or gene with a previously different purpose (Gould and Vrba 1982). Mating-type incompatibility in *N. crassa* may constitute an example of this form of exaptation: unlike in most Pezizomycetes, in *N. crassa* the mating-type locus has a secondary function as a heterokaryon incompatibility locus. The *het-s*-based incompatibility in *P. anserina* was also proposed to result from an exaptation event (Debets et al. 2012). Actually, considering that all HI systems characterized so far include at least a gene conserved across the fungal kingdom and not involved in HI in other species (Saupe et al. 2000; Kerényi et al. 2006; Micali and Smith 2006; van Diepeningen et al. 2009; Iotti et al. 2012), lineage specific exaptation of a conserved cellular function to ensure allorecognition function might be the rule rather than the exception. If members of the *het-c* and *het-d/e* are indeed involved in pathogen recognition in a guardee/guard relationship, it is possible that incompatibility between *het-e* (and *het-d*) and *het-c* variants has emerged as a by-product of this pathogen-driven diversification. As HI can be selectively advantageous in a number of conditions, from then on, selection might have acted on *het-c*/*het-e* to maintain and promote polymorphisms leading to incompatibility.

According to this last hypothesis, *het-c* would be under a dual selection regimen and diversification may represent the additive result of pathogen-driven divergence and selection associated with the allorecognition function.

Material and methods

Strains and culture conditions

P. anserina strains used for *het-c* screening are from the Wageningen collection of wild-type isolates and from six strains collected worldwide which were retrieved from the CBS, Utrecht, The Netherlands (www.cbs.knaw.nl). The strain numbers for the isolates retrieved from the CBS are: CBS124.78, CBS253.71, CBS333.63, CBS433.50, CBS455.64 and CBS102042. In total 110 isolates were used from the Wageningen collection of wild-type *P. anserina* strains, that were all collected at different locations near Wageningen between 1991 and 2001. Most strains have been isolated from rabbit, horse or sheep dung. Details on the sampling methods and locations of the Wageningen collection have been described earlier (van der Gaag et al. 1998; van Diepeningen et al. 2008).

DNA isolation, PCR, and sequencing

DNA was isolated from these strains by growing them in a Petri dish with *Podospora anserina* synthetic medium (PASM, described by Esser (Esser 1974)) covered with a layer of cellophane foil. After 2 days of growth at 27°C, mycelium was scraped off and put in a 1.5 ml Eppendorf tube, which was then frozen in liquid nitrogen immediately. Several glass beads (2-3 mm) were added after the material was frozen. The mycelium was ground two times ten seconds in a bead beater machine, in between the material was refrozen in liquid nitrogen and some new glass beads were added. The ground material was used in a standard phenol-chloroform extraction (Sambrook et al. 1989).
The *het-c* gene was amplified with standard PCR, using primers that bind roughly 500 bp before and after the transcribed sequence of *het-c*. Primers used were: 3' CGAAGGTGAAAACGAGACGA 5' and 3' ACCAAGGCTGGACCTGATA 5'. Based on the sequence of *het-c1* (GenBank: L36207) these primers are expected to give a product of 1792 basepairs. PCR was done with following cycle conditions: 5 min at 95°C followed by 35 cycles of 30s at 94°C, 30s at 50°C and 2 min at 72°C, followed by one cycle of 5 min at 72°C and a final hold step at 4°C. PCR products were cleaned using the Sigma GenElute PCR clean-Up Kit. Cleaned PCR products were sequenced using the value read service of MWG biotech. Sequencing was done in two directions using primers binding just outside the coding part of *het-c*. Primers used for sequencing were: 3' CAACCAACCTTCAACCAACC 5' and 3' CAGATGCGTATGCTTTTTGC 5'. These primers bind just outside the open reading frame of the *het-c* gene. Sequences were reliable up to 900 basepairs from the starting point.

Sequence data were handled with the DNAstar software package.
Sequences for *het-c5* to *het-c11* have been deposited in GenBank with accession numbers KF951052 until KF951058.

Sequence analyses

Alignments of the DNA and amino acid sequences were done with ClustalW (Larkin et al. 2007). Phylogenetic trees were constructed using the Neighbour-Joining method with the software package MEGA4 (Tamura et al. 2007). Bootstrap supports, expressed as a percentage, were calculated over 1000 replicates. Additionally the sequences were analysed for possible positive diversifying selection on *het-c* codons. To detect selection we used the methods developed by Yang et al. (Yang et al. 2000), which are implemented in the HyPhy software (Kosakovsky Pond and Muse 2005). Besides testing the *het-c* alleles, a set of 37 GLTP encoding genes from filamentous fungi found by a BLAST search of the Fungal Genome Database held at the NCBI, including one *P. anserina het-c* allele, was tested for positive diversifying selection. The sequence accession numbers are listed in Supplemental figure S8.

Transformation and testing functionality of new *het-c* alleles

Protoplasts were prepared and transformed as previously described (Bergès and Barreau 1989). The recipient Δ*het-c P. anserina* strain lacks the entire *het-c* ORF (Saupe et al. 1994). The PCR products made for sequencing of the new *het-c* variants were cloned in the pGEM-t easy vector (Promega). 7.5µg of the cloned *het-c* DNA was used to co-transform protoplasts of the Δ*het-c* strain with 1.5µg of the pPaBle plasmid conferring resistance to phleomycin (Coppin and Debuchy 2000). The transformed protoplasts were spread on synthetic medium with a high sucrose concentration and phleomycin for selection of transformants. After four to five days, transformants were transferred to a standard synthetic medium.
For each new *het-c* allele 18 transformants were tested for barrage formation with tester strains each expressing a different *het-e* or *het-d* allele. For each allele, two co-transformants have been checked for the presence of the introduced *het-c* allele by PCR using primers binding on the pGEM-t easy vector.

Analysis of WD-repeat number variation for escaped sectors of self-incompatible progeny

Crosses were set up between the tester strain for *het-d2* and two different transformants with the incompatible alleles *het-c9* and *10.* Self-incompatible progeny were selected and transferred to PASM with 6 g/l dihydrostreptomycin covered with a cellophane sheet, which allows growth of the self-incompatible colonies. The cellophane sheets were then transferred to PASM without dihydrostreptomycin after one day of growth. After several days escaping sectors were observed from these colonies. DNA was isolated from mycelium of these sectors

and PCR on the WD repeat region was performed as was described by Paoletti et al. (Paoletti et al. 2007).

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Supplementary material

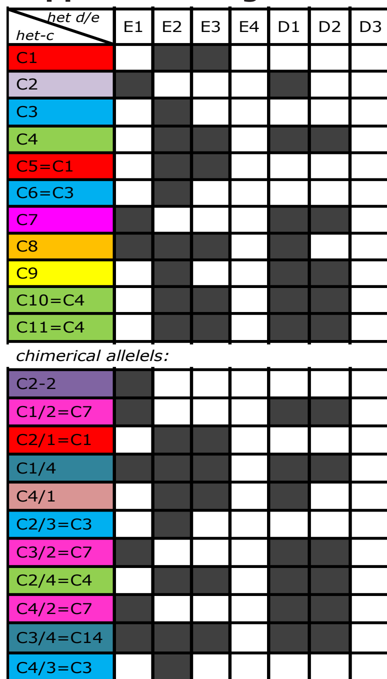
Supplemental figure 1

ClustalW alignment of the het-c alleles nucleic sequences. Sequences of the introns are indicated in italics. Only polymorphic sites are indicated compared to the het-c1 reference sequences for the variant alleles. Synonymous mutations are given in bold.

het-c1	1	ATGGCCGCCGCCGCCGTTGTCCAAAT**C**CCCGTCGGGGCTACCTTCCTTAAGACCTTCAAGAAGTCCTTCGTT	72
het-c2	1	-------------------------------C----------------G-----------------------	72
het-c3	1	--	72
het-c4	1	-------------------------------C--	72
het-c5	1	--	72
het-c6	1	--	72
het-c7	1	-------------------------------C----------------G-----------------------	72
het-c8	1	--	72
het-c9	1	--	72
het-c10	1	-------------------------------C----------------G-----------------------	72
het-c11	1	--------------------------**T**----C----------------G-----------------------	72
het-c1	73	GATGTCCCAATCGATGC**T**GAGAAGGGCAATGCCATCTCCACCGCCGAGTTCCTTGAGGCGGCCGAGTCCTTG	144
het-c2	73	--	144
het-c3	73	--	144
het-c4	73	-----------------**C**--	144
het-c5	73	--	144
het-c6	73	--	144
het-c7	73	-----------------**C**--	144
het-c8	73	--	144
het-c9	73	--	144
het-c10	73	-----------------**C**--	144
het-c11	73	-----------------**C**--	144
het-c1	145	ACCACCATGTTCGATGTGCTCGGCTCCATCGCCTTCACCCC**C**GTCAAGACGGATATGTTGGGCAACGTCGAG	216
het-c2	145	------------------------------------T-----------------------------------	216
het-c3	145	--	216
het-c4	145	------------------------------------T------------A----------------------	216
het-c5	145	--	216
het-c6	145	------------------------------------T-----------------------------------	216
het-c7	145	---------------------------G--------T------------A----------------------	216
het-c8	145	--	216
het-c9	145	---TT---------	216
het-c10	145	------------------------------------T----**T**-------A----------------------	216
het-c11	145	---------------------------G--------T------------A----------------------	216
het-c1	217	*GTGCGCTGCTATTATACCCGTTGGTTAGGACGTCAGCTAACCACCCTTTCCTGCTAG*AAAATTCGCAAGCGC	288
het-c2	217	------------------*T*---	288
het-c3	217	--	288
het-c4	217	--	288
het-c5	217	--	288
het-c6	217	--	288
het-c7	217	---------------------------*T*--*T*-----------------------------------C-----	288
het-c8	217	--	288
het-c9	217	--	288
het-c10	217	--	288
het-c11	217	--	288
het-c1	289	ATGCTTGCCGCCCCCCTCGCATCCCAGAACATCCAGGATCTTGTGAGGAACGAGCTCAAGACCAAAAGCCAT	360
het-c2	289	-------------------AG---	360
het-c3	289	--	360
het-c4	289	-------------------A--	360
het-c5	289	--	360
het-c6	289	--	360
het-c7	289	-------------------AG---	360
het-c8	289	--	360
het-c9	289	--	360
het-c10	289	-------------------A--	360
het-c11	289	-------------------AG---	360

```
het-c1    361 ACCGCGACGGAGGGGTTGCTGTGGCTGGTCAGGTGAGGGCGCCTTGATCTGTTCCATAACCAAGCCAAGTTA  432
het-c2    361 ----------------------------------------------------------G-----T-----C-  432
het-c3    361 ------------------------------------------------------------------------  432
het-c4    361 ---------------------------------------------------------------------CC-  432
het-c5    361 ------------------------------------------------------------------------  432
het-c6    361 ------------------------------------------------------------------------  432
het-c7    361 -----------------------------------------------------G----G-----T-------  432
het-c8    361 ------------------------------------------------------------------------  432
het-c9    361 ------------------------------------------------------------------------  432
het-c10   361 ----------------------------------------------------------G-----T-------  432
het-c11   361 ----------------------------------------------------------G-----T---A-C-  432

het-c1    433 CTGACAGTGGTCTGTGACAGGGGTCTCGAATTCACATGCATTGCTCTTAGCAAGAACATCAACTCAACAGAG  504
het-c2    433 ------------------------------------------------------------GG----------  504
het-c3    433 ------------------------------------------------------------------------  504
het-c4    433 ------------------------------------------------------------G-----------  504
het-c5    433 ------------------------------------------------------------------------  504
het-c6    433 ------------------------------------------------------------------------  504
het-c7    433 ---------------------------------------------------G-T------GG-------A--  504
het-c8    433 ------------------------------------C-----------------------------------  504
het-c9    433 ------------------------------------------------------------------------  504
het-c10   433 ----------------------------------------------------T-------GG----------  504
het-c11   433 ------------------------------------------------------------GG----------  504

het-c1    505 GAGCTCGCCGACTCCTTCCGCGGGTCTTACAGTGAGACTCTCATGCGACACCACAGCTTCCTGGTGAAGCGC  576
het-c2    505 --------------------------------G-T--------A--C-----------------------C-  576
het-c3    505 ---------C--------------------------------------------------------------  576
het-c4    505 ----------------------------------------------------------------------C-  576
het-c5    505 -----------G------------------------------------------------------------  576
het-c6    505 ----------------------------------------------------------------------C-  576
het-c7    505 -------A--C----------AA-----------T--------A--C-----------------------C-  576
het-c8    505 ------------------------------------------------------------------------  576
het-c9    505 ---------A------------------------------------------------------------C-  576
het-c10   505 ------------------G---------------C-----------------------------------C-  576
het-c11   505 ----------------------------------T-----------------------------------C-  576

het-c1    577 ATCTTCAGCGCCGCCATGGGCGCGTGCCCATACCGCAAGGACTTCTACGCCAAGCTTGGTGACGACGAGCAA  648
het-c2    577 ------------------A-----------------------------------------------------  648
het-c3    577 ------------------------------------------------------------------------  648
het-c4    577 ------------------------------------------------------------------------  648
het-c5    577 ------------------------------------------------------------------------  648
het-c6    577 ------------------------------------------------------------------------  648
het-c7    577 ------------------A----------------------------------------------T------  648
het-c8    577 ------------------------------------------------------------------------  648
het-c9    577 ------------------------------------------------------------------------  648
het-c10   577 ------------------A-A---------------------------------------------------  648
het-c11   577 ------------------A-----------------------------------------------------  648

het-c1    649 AAGGTTCAAGAGGAGCTTCGCGAATACCTTGCTGCTCTCGACAAGATCGTCTACATTCTCAAGGGATTCTTG  720
het-c2    649 -------------------------------T-------------------A-----------A--------  720
het-c3    649 ------------------------------------------------------------------------  720
het-c4    649 ------------------------------------------------------------------------  720
het-c5    649 ------------------------------------------------------------------------  720
het-c6    649 ------------------------------------------------------------------------  720
het-c7    649 --------------------------------------T--------T---A-----------C--------  720
het-c8    649 ------------------------------------------------------------------------  720
het-c9    649 ------------------------------------------------------------------------  720
het-c10   649 ---------------------A-----------------------------A--------------------  720
het-c11   649 ---------------------------------------------------A--------------------  720

het-c1    721 GAGAGCAAGGAGGCCAAGTGGTAA  744
het-c2    721 ------------------------  744
het-c3    721 ------------------------  744
het-c4    721 ------------------------  744
het-c5    721 ------------------------  744
het-c6    721 ------------------------  744
het-c7    721 ------------------------  744
het-c8    721 ------------------------  744
het-c9    721 ------------------------  744
het-c10   721 ------------------------  744
het-c11   721 ------------------------  744
```

Supplemental figure 2

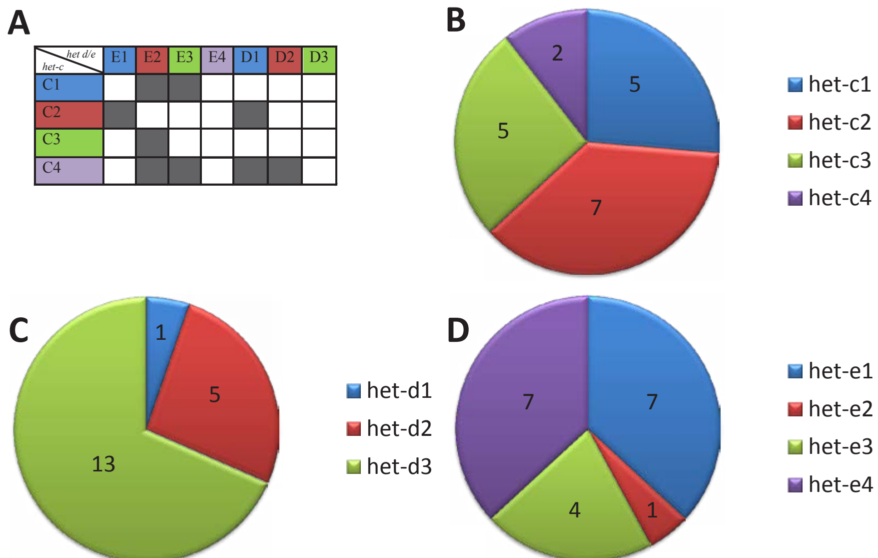


het-c \ *het d/e*	E1	E2	E3	E4	D1	D2	D3
C1		■	■				
C2	■				■		
C3		■					
C4		■	■		■	■	
C5=C1		■	■				
C6=C3		■					
C7	■				■	■	
C8	■	■	■		■		
C9		■			■	■	
C10=C4		■	■		■	■	
C11=C4		■	■		■	■	
chimerical allelels:							
C2-2	■						
C1/2=C7	■				■	■	
C2/1=C1		■	■				
C1/4	■	■	■		■	■	
C4/1		■	■		■		
C2/3=C3		■					
C3/2=C7	■				■	■	
C2/4=C4		■	■		■	■	
C4/2=C7	■				■	■	
C3/4=C14	■	■	■		■	■	
C4/3=C3		■					

Incompatibility patterns of the eleven known naturally occurring het-c alleles (data for het-c1 to het-c4 taken from (Bernet 1967) and of eleven chimeric alleles artificially constructed from het-c1 to het-c4 (taken from (Saupe, Turcq, Bégueret 1995)). Interactions were tested with the four available het-e and three het-d alleles. A dark grey square indicates an incompatible reaction was shown for this combination of alleles. If an allele has a similar incompatibility pattern as an allele earlier in the list, it is indicated by the "=" symbol followed by the allele number it shows similarity to. Alleles with the same incompatibility pattern are given the same background color.

Supplemental figure 3

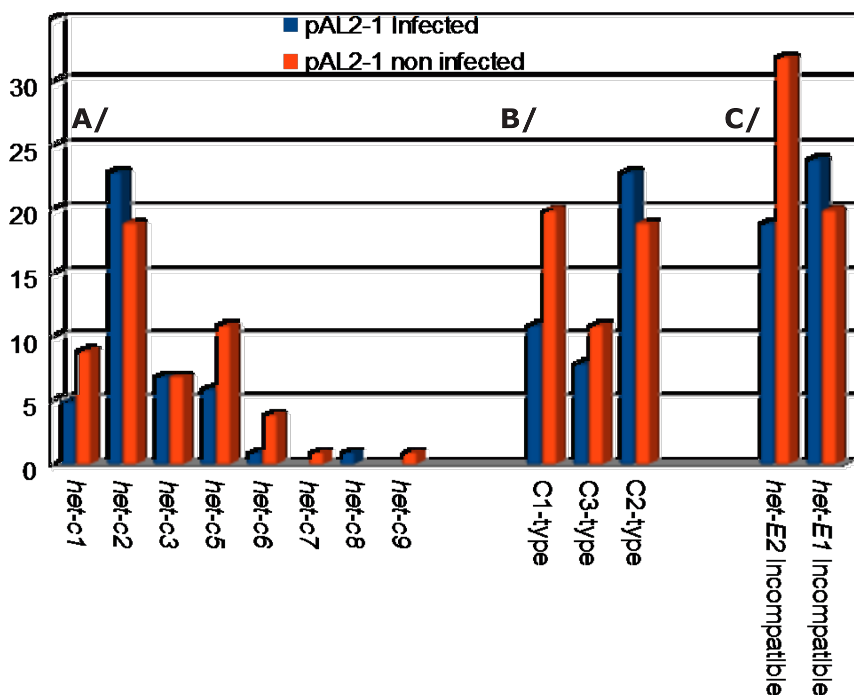
Distribution of het-c, -d and -e alleles in the P. anserina isolates collected in France in the 1940s. A\ incompatibility patterns of the four het-c alleles found in these isolates. B\ - D\ pie charts showing the distribution of alleles for respectively het-c, het-d and het-e. Numbers represent amount of isolates with this allele.



het-c \ *het d/e*	E1	E2	E3	E4	D1	D2	D3
C1		■	■				
C2	■				■		
C3		■					
C4		■	■		■	■	

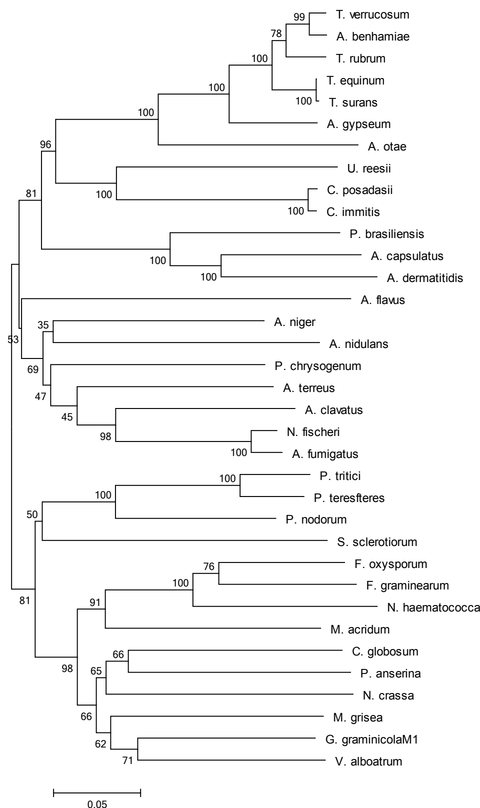
Supplemental figure 4:

Distribution of the pAL2-1 plasmid in relation with the het-c alleles, het-c phenotypic classes (C1, C2 or C3) or incompatibility with the het-e1 and het-e2 testers.
We combined our data on het-c allele distribution in the Wageningen P. anserina population with the distribution of the pAL2-1 senescence plasmid (van Diepeningen et al. 2008) resulting in a total of 95 isolates characterised for both sets of data. The proportion of infected and non-infected isolates is represented depending on A/ the het-c alleles expressed, B/ the C-type to which the het-c alleles belong, C/ the incompatibility with het-e1 or het-e2. In the latest panel the difference between infected and non-infected isolates is not significant (2 tail Fisher's exact test, p value=0.145).



Supplemental figure 5:

A/ Accession numbers for the GLTP encoding cDNAs from filamentous fungi used.
Podospora anserina het-c2 (U05236), *Chaetomium globosum* CBS148.51 (XM_001224167), *Glomerella graminicola* M1 (GG697350), *Verticillium albo_atrum* VaMs (XM_003001318), *Metarhizium acridum* CQMa102 (GL698531), *Neurospora crassa* OR74A (XM_956987), *Sclerotinia sclerotiorum* 1980 UF_70 (XM_001585194), *Nectria haematococca* mp VI77_13_4 (XM_003040028), *Phaeosphaeria nodorum* SN15 (XM_001796420), *Fusarium oxysporum* Fo5176 (AFQF01002826), *Arthroderma gypseum* CBS118893 (XM_003176546), *Trichophyton verrucosum* HKI0517 (XM_003022135), *Trichophyton rubrum* CBS118892 (XM_003238513), *Trichophyton equinum* CBS 127.97 (DS995722), *Pyrenophora tritici_ repentis* Pt_1C_BFP (XM_001935155), *Trichophyton tonsurans* CBS112818 (GG698505), *Pyrenophora teres f. teres* 0_1 (XM_003303860), *Arthroderma benhamiae* CBS112371 (XM_003016258), *Aspergillus terreus* NIH2624 (XM_001213225), *Penicillium chrysogenum* Wisconsin 54_1255 (XM_002559878), *Aspergillus niger* CBS51388 (XM_001400048), *Aspergillus clavatus* NRRL1 (XM_001271363), *Neosartorya fischeri* NRRL181 (XM_001263079), *Arthroderma otae* CBS113480 (XM_002849967), *Coccidioides posadasii* C735deltaSOWgp (XM_003066772), *Aspergillus fumigatus* Af293 (XM_749149), *Coccidioides immitis* RS (XM_001246321), *Paracoccidioides brasiliensis* Pb01 (XM_002791993), *Aspergillus nidulans* FGSCA4 (XM_655659), *Ajellomyces capsulatus* NAm1 (XM_001544035), *Uncinocarpus reesii* 1704 (XM_002540637), *Ajellomyces dermatitidis* SLH14081 (XM_002623113), *Aspergillus flavus* NRRL 3357 (AFL2G_03641), *Aspergillus oryzae* RIB40 (AO090012000776), *Fusarium graminearum* PH-1 (FG01974), *Magnaporthe grisea* (XM_364530).



B/ Protein guided Neighbor-joining phylogenetic tree constructed from the GLTPs encoding cDNAs.
Neighgour joining phylogenetic tree of the fungal GLTPs listed above. The sequences were aligned with ClustalW and the tree constructed with the package MEGA5.

Supplemental figure 6

Polymorphic sites identified in alleles of N. crassa NCU07947 orthologous to the P. anserina het-c gene. NCU07947 is located on the minus strand of supercontig 4 at coordinates 1260122-1260907. Polymorphisms are highlighted and represented on the reference sequence from N. crassa isolate OR74. Positions are relative to start of the ORF. Data are extracted from Ellison et al. (Ellison et al. 2011).

```
atgtctaccggccagatcccccccggaggtacctacctcaacaccctcaagcgttccttc
 M  S  T  G  Q  I  P  P  G  G  T  Y  L  N  T  L  K  R  S  F

                                   T96               t114
accgacgtccccgtccaggccgacaatgggaatgccattcccaccaccgagttcctcgag
 T  D  V  P  V  Q  A  D  N  G  N  A  I  P  T  T  E  F  L  E

                          T147                    t171
gctgccgagtcgctagtctccatcttcgatgtgctcggctctgccgccttctcccccgtc
 A  A  E  S  L  V  S  I  F  D  V  L  G  S  A  A  F  S  P  V

                                   T216     t225        t237
aagagcgacatgctcggcaatgtcgagaaaatccgccagcgtttccttgccgccccgacc
 K  S  D  M  L  G  N  V  E  K  I  R  Q  R  F  L  A  A  P  T

                                      G279  t285
gagtccgagactctccaggaccttgtgaacaacgagcaaaaggccaaggagaacaaggcc
 E  S  E  T  L  Q  D  L  V  N  N  E  Q  K  A  K  E  N  K  A

                                                  C351
ggccaggccctgctctggctcgtcaggggtctcgagttcacttgcaaaggtcttgccaac
 G  Q  A  L  L  W  L  V  R  G  L  E  F  T  C  K  G  L  A  N

                             c390  c396        t408
aacgtcgccgccgctgaccaggagctttctacctcgttccgcgctgcctacgatgtcacc
 N  V  A  A  A  D  Q  E  L  S  T  S  F  R  A  A  Y  D  V  T

        T429           t444  a450
ctcaagccccaccacagcttcctcatcaagcccatcttcagcgccgccatgagcgcttgc
 L  K  P  H  H  S  F  L  I  K  P  I  F  S  A  A  M  S  A  C

                    C501                                c537
ccttaccgcaaggacttctataccaagctcggtgacgaccaggacaaggtcaacgctcag
 P  Y  R  K  D  F  Y  T  K  L  G  D  D  Q  D  K  V  N  A  Q

ctcaaggagtacctcgcagctctcgagaactttgtcaacatcctcaaggccttcctcgac
 L  K  E  Y  L  A  A  L  E  N  F  V  N  I  L  K  A  F  L  D

agcaagggcatcaagaaatag
 S  K  G  I  K  K  -
```

Supplemental figure 7

Polymorphic sites identified in alleles of the locus Coccidioides species reported to the cDNA sequence of the locus CPSG_04807 from the C. posadasii RMSCC757/Silveira (access number EFW18121) orthologous to the P. anserina het-c gene. Synonymous polymorphisms are highlighted in pink and a single non synonymous polymorphism is highlighted in green and represented on the reference sequence from N. crassa isolate OR74. Positions are relative to the start of the gene. Two polymorphic sites not represented here are located in intronic sequences. Data are extracted from Ellison et al. (Ellison et al. 2011).

```
atggcttctgctgctgtgattcccgccgacggcacctggttcgacactatcaggaggtca
 M  A  S  A  A  V  I  P  A  D  G  T  W  F  D  T  I  R  R  S


ttcgccgatgtcccaatcaacgacaacggtatttccacaacggagttcctcgaggctgcc
 F  A  D  V  P  I  N  D  N  G  I  S  T  T  E  F  L  E  A  A

                 g138              g205
gaagctttggtgatgctatttgacctccttggctccgtcgcattcactccggtaaagaac
 E  A  L  V  M  L  F  D  L  L  G  S  V  A  F  T  P  V  K  N


gacttgctaggcaacatcaagaaaattcgtgaccgccagttggcagctccagcagaatcc
 D  L  L  G  N  I  K  K  I  R  D  R  Q  L  A  A  P  A  E  S

                             a376                       c403
gagactcttcagcaacttgttgtgaacgagctcaagactggaaagcacacggcgactgaa
 E  T  L  Q  Q  L  V  V  N  E  L  K  T  G  K  H  T  A  T  E


ggcctgctatggctggtccgtggtctcgacttcactgcccaagccctccgcctcaatctt
 G  L  L  W  L  V  R  G  L  D  F  T  A  Q  A  L  R  L  N  L

                    c553
tccgatcctgctgctgaacttttctacttctttccgtgctgcatacggcactaccttaaaa
 S  D  P  A  A  E  L  S  T  S  F  R  A  A  Y  G  T  T  L  K

           t604  g610
ccacatcacggcttgcttgtcaaaccgattttcagtgctgccatgtccgcaactccttat
 P  H  H  G  L  L  V  K  P  I  F  S  A  A  M  S  A  T  P  Y


agaaaggacttctatgccaagcttggtcaggacgctaccaaggtctcaactgcgatgaac
 R  K  D  F  Y  A  K  L  G  Q  D  A  T  K  V  S  T  A  M  N

g713          c727
atcgaaataactgctttggagaaagtagtaggaatcctccatgaattccttaaaagtcca
 I  E  I  T  A  L  E  K  V  V  G  I  L  H  E  F  L  K  S  P
 V

gcagccaaatggtga
 A  A  K  W
```

Supplemental figure 8

Different fungal species were checked for polymorphisms in their het-c homologues in different isolates. The alleles were identified by tblastn searches of the NCBI database using P. anserina HET-C2 as query. Positively selected sites predicted by model M2 are highlighted in red, additional sites predicted by model M8 are highlighted in grey.

A/ Paracoccidioides brasiliensis

P. brasiliensis PB01 (XP_002792039)
P. brasiliensis PB03 (EEH23613)

```
PB01              --MASTQVIPQGGTWFDTLNRGFYSVRIADD--NAISTTEFLEAAESLTT 46
PB03              --MASTQVIPQGGTWFDTLNRGFYNVRITDD--NAISTTEFLEAAESLTT 46
HET-C             MAAAAVVQIPAGATFLETFKKSFVDVPIDAEKGNAISTAEFLEAAESLTT 50
                     *:.  ** *.*:::*:::.* .* *  :  *****:***********

PB01              LFDLLGSVAFTPVKNDLLGNIKKIRDRQLAAPAESETLQALVLNELKAKK 96
PB03              LFDLLGSVAFTPVKNDLLGNIKKIRDRQLAAPAESETLQELVLNELKAKK 96
HET-C             MFDVLGSIAFSPVKTDMLGNVEKIRKRMLAAPLESQNIQDLVRNELKTKS 100
                  :**:***:**:***.*:***::***.* **** **:.:* ** ****:*.

PB01 PB01         NTATVGLLWLVRGLDFTAQALRHNISNPTDELSASFRVAYGKTLKPYHNF 146
PB03              NTATVGLLWLVRGLDFTAQALRHNISNPADELSASFRVAYGKTLKPYHNF 146
HET-C             HTATEGLLWLVRGLEFTCIALSKNIG-STEELADSFRGSYRVTLKPHHSF 149
                  :*** *********:**. ** :**. .::**: *** :*  ****:*.*

PB01              LIKPIFTAAMGATPYRKDFYANLGDDSVKSQAALELSTTSLEKIVSILKE 196
PB03              LIKPIFTAAMGATPYRKDFYAKLGDDSVKSQAALELSTTSLEKIVSILKE 196
HET-C             LVKPIFSAAMSACPYRKDFYAKLGDDEQKVQEELREYLVALDKIVNILKR 199
                  *:****:***.* ********:****. * *  *.   .:*:***.***.

PB01              FLETPEVKKAAS 208
PB03              FLETPEVKKAAS 208
HET-C             FLESKEAKW--- 208
                  ***: *.*
```

ClustalW alignment of HET-C2 protein and variant homologues from P. brasiliensis. Polymorphic sites between variants P. brasiliensis het-c homologues are indicated in yellow, while sites under diversifying selection are highlighted in grey. Accession numbers are indicated in brackets.

B/ Ajellomyces capsulatus

A. capsulatus Nam1 (XP_001544085)
A. capsulatus H88 (EGC47776)
A. capsulatus H143 (EER38624)

```
Nam1            --MANTQVIPEGGTWFDTLKRGFDAVPVADD--NAISTSEFLEAAESLTT 46
H88             --MANTQVIPEGGTWFDTLTRGFDAVPVADD--NAISTSEFLEAAESLTT 46
H143            --MANTQVIPEGGTWFDTLTRGFDAVPVADD--NAISTSEFLEAAESLTT 46
HET-C           MAAAAVVQIPAGATFLETFKKSFVDVPIDAEKGNAISTAEFLEAAESLTT 50
                * .  ** *.*:::*:.:.*  **:  :  *****:***********

Nam1            LF-----VAFTPVKNDLLGNIKKIRDRQLAAPAESETLQALVLNEIKAKK 91
H88             LFDLLGSVAFTPVKNDLLGNIKTACG-----TAESETLQALVLNEIKAKK 91
H143            LFDLLGSVAFTPVKNDLLGNIKKIRDRQLAAPAESETLQALVLNEIKAKK 96
HET-C           MFDVLGSIAFSPVKTDMLGNVEKIRKRMLAAPLESQNIQDLVRNELKTKS 100
                :*     :**:***.*:***::.        . **:.:* ** **:*:*.

Nam1            NTASVGFLWLVRGLDFTAKALRHNISFPNDELSTSFRAAYGDTLKPHHNF 141
H88             NTASVGFLWLVRGLDFTAKALRHNISFPNDELSTSFRAAYGDTLKPHHNF 141
H143            NTASVGFLWLVRGLDFTAKALRHNISFPNDELSTSFRSSLR--------- 137
HET-C           HTATEGLLWLVRGLEFTCIALSKNIG-STEELADSFRGSYRVTLKPHHSF 149
                :**: *:*******:**. ** :**. ..:**: ***.:

Nam1            LVKPIFVAAMGATPYRKDFYAKLGNDTAKCQAALELSTASLEKIVSILKE 191
H88             LVKPIFVAAMGATPYRKDFYAKLGNDPAKCQAALELSTASLEKIVSILKE 191
H143            --------------------------------------------------
HET-C           LVKPIFSAAMSACPYRKDFYAKLGDDEQKVQEELREYLVALDKIVNILKR 199

Nam1            FLETPEVKKACS 203
H88             FLETPEVKKACS 203
H143            ------------
HET-C           FLESKEAKW--- 208
```

ClustalW alignment of HET-C2 protein and variant homologues from A. capsulatus. Polymorphic sites between variants A. capsulatus het-c homologues are indicated in yellow, while sites under diversifying selection are highlighted in grey. Accession numbers are indicated in brackets.

C/ Ajellomyces dermatidis

ClustalW alignment of HET-C2 protein and variant homologues from A. dermatidis. Polymorphic sites between variants A. dermatidiis het-c homologues are indicated in yellow, while sites under diversifying selection are highlighted in grey. Accession numbers are indicated in brackets.

A. dermatidis 18188 (EGE78761)
A. dermatidis SLH14081 (XP_002623159)

```
18188           --MANTQVIPPGGTWFDTLQRGFDAVPVSDD--NAITTSEFLEAAEALTTLFDLLGSVAF 56
SLH14081        --MANTQVIPPGGTWFDTLQRGFDAVPVSDD--NAITTSEFLEAAEALTTLFAFTGANYT 56
HET-C           MAAAAVVQIPAGATFLETFKKSFVDVPIDAEKGNAISTAEFLEAAESLTTMFDVLGSIAF 60
                   * .  **.*.*:::*:::.*  **:. :  ***:*:*******:***:* . *:

18188           TPVKNDLLGNIKKIRDRQLAAPAESETLQALVLNELKAKKNTASGGLLWLLRGLDFTAKA 116
SLH14081        IPFQ--------KIRDRQLAAPAESETLQALVLNELKAKKNTASGGLLWLLRGLDFTAKA 108
HET-C           SPVKTDMLGNVEKIRKRMLAAPLESQNIQDLVRNELKTKSHTATEGLLWLVRGLEFTCIA 120
                 *.:        ***.* **** **:..:* ** ****:*.:**: *****:***:**. *

18188           LRHNISHPNEELSASFRAAYGDTLKPHHNFLVKPIFVAAMGATPYRKDFYRKLGDDPAKC 176
SLH14081        LRHNISHPNEELSASFRAAYGDTLKPHHNFLVKPIFVAAMGATPYRKDFYRKLGDDPAKC 168
HET-C           LSKNIGS-TEELADSFRGSYRVTLKPHHSFLVKPIFSAAMSACPYRKDFYAKLGDDEQKV 179
                * :**.  .***: ***.:*  ******.******* ***.* ******* *****  *

18188           QAALELSTASLEKIVSILKDFLETPEVKKAIS 208
SLH14081        QAALELSTASLEKIVSILKDFLETPEVKKAIS 200
HET-C           QEELREYLVALDKIVNILKRFLESKEAKW--- 208
                *  *.   .:*:***.*** ***: *.*
```

D/ Aspergillus fumigatus

ClustalW alignment of HET-C2 protein with homologues identified in A. fumigatus Af293 and Af1163 strains. No polymorphic sites were identified between these two allele expressed in the sequenced strains A1163 (AFUB_035370) and strain AF293 (Afu3g13820).

A. fumigatus A1163 (AFUB_035370)
A. fumigatus AF293 (Afu3g13820)

```
Af293           MAAA----IPPGGTWFDTLKRSFADVPIND---NGISTTEFLEAAESLVTLFDLLGSKCF 53
Af1163          MAAA----IPPGGTWFDTLKRSFADVPIND---NGISTTEFLEAAESLVTLFDLLGSKCF 53
het-c           MAAAAVVQIPAGATFLETFKKSFVDVPIDAEKGNAISTAEFLEAAESLTTMFDVLGSIAF 60
                ****    **.*.*:::*:*:**.****:     *.***:*********.*:**:*** .*

Af293           APVKNDLLGNIKKVRDRQLAAPAESETLQALVVNELKTGKHVATEGLLWLVRGLDFTVQA 113
Af1163          APVKNDLLGNIKKVRDRQLAAPAESETLQALVVNELKTGKHVATEGLLWLVRGLDFTVQA 113
het-c           SPVKTDMLGNVEKIRKRMLAAPLESQNIQDLVRNELKTKSHTATEGLLWLVRGLEFTCIA 120
                :***.*:***::*:*.* **** **:..:* ** ***** .*.***********:**  *

Af293           LRHNLDKETELSVSFREAYGNTLKPHHSFVVKPIFSAAMSATPYRKEFYEKLGSDSDKVN 173
Af1163          LRHNLDKETELSVSFREAYGNTLKPHHSFVVKPIFSAAMSATPYRKEFYEKLGSDSDKVN 173
het-c           LSKNIGSTEELADSFRGSYRVTLKPHHSFLVKPIFSAAMSACPYRKDFYAKLGDDEQKVQ 180
                * :*:..   **: *** :*  ********:*********** ****:** ***.*.:**:

Af293           VALKREVEALEKIVATLNAFMSSKEAKW 201
Af1163          VALKREVEALEKIVATLNAFMSSKEAKW 201
het-c           EELREYLVALDKIVNILKRFLESKEAKW 208
                  *:. : **:***  *: *:.******
```

Chapter 6

General discussion

Eric Bastiaans

Allorecognition can be explained by somatic cheating

The work described in this thesis demonstrates that Crozier's theoretical prediction about the evolution of genetic allorecognition applies to fungi: somatic fusion is beneficial and this benefit imposes selection against allotype diversity in *Neurospora crassa* (chapter 2) while stable cooperative growth relies on this allorecognition (chapter 3 and 4). We then experimentally demonstrate that somatic cheating evolves quickly in *N. crassa* in the absence of allorecognition (chapter 3). The cheaters that were selected at the level of nuclei had a devastating effect on the fitness of the level of the colony. This cost associated with cheating was proposed to invoke a selective pressure for the maintenance of allorecognition diversity that could counteract the benefits of fusion (Grafen 1990). The selection pressure of cheating was not anticipated by Crozier (Crozier 1986), and modelling a scenario that predicts the allotype diversity observed in nature has proven difficult for fungi (Nauta and Hoekstra 1994) and other organisms (Rousset and Roze 2007).

However, recently Czaran et al. (Czaran et al. 2014) presented a model that incorporated spatial structure, thereby creating a more realistic model. Their main finding was that with the right parameters the model predicts stable high allorecognition diversity, with no or almost no cheating genotypes present. This situation is comparable to the natural situation. In order to get this result from the model, cheating has to evolve 'easily' at a high frequency. This way, cheaters provide a selection pressure to maintain allorecognition polymorphism even though they are not detectable. In chapter 3, we have shown that this requirement is realistic, as we see that cheating evolves quickly in all parallel evolving cultures under low relatedness. This is an indication that there is a constant threat of cheating. We further demonstrated that even though these cheaters arise frequently by mutation, they cannot be selected if high relatedness is maintained by restricting fusion. One important parameter of the model was that the fitness loss of a cooperative genotype fused with cheaters should be dominant or co-dominant, i.e. the fitness (spore yield) of a heterokaryotic colony with cheat and cooperative nuclei should be lower than a colony with only cooperative nuclei. This condition was met by the different cheating genotypes we described in chapter 3. One outcome of our experimental evolution not similar to the model predictions was that cheaters in the model never coexisted with cooperative types for long times. We found that the competitive fitness of the cheat types is frequency dependent and in most cases the cheater does not go to fixation. In contrast, in the model when not sufficient allotype diversity emerged, cheaters always went to fixation. It would therefore be good if the model could be adjusted with parameters for the cheating type that are more in line with the cheaters that we find and see if the model still can predict high allorecognition diversity. I predict it will, since the cheaters already reduce colony fitness significantly at lower frequencies before they go to fixation.

Cheating mitochondria are comparable to cheating nuclei

In chapter 4, we showed that allorecognition can also prevent senescence in *Neurospora*, by maintaining a high relatedness among the mitochondria. The mutant cheating mitochondria that cause senescence are not fundamentally different from the cheating nuclei described in chapter 3. The lack of compartmentalization places the mitochondria at the same selection level as the nuclei as both kinds of organelle can move through the fungal colony (Roper et al. 2011). Therefore it is likely that the cost to the colony of cheating mitochondria poses a similar selection pressure to maintain allorecognition as cheating nuclear genotypes. In both cases the reproductive output of the colony is reduced.

Allorecognition diversity selected for another function?

In chapter 5 we found some support for Crozier's idea that allotype diversity can also be maintained by selection for a different function than allorecognition, i.e. as an immune system for pathogen recognition. Although it seems plausible that the *het-c, het-d* and *het-e* genes are involved in pathogen recognition, this remains to be proven and does not exclude that polymorphism in these genes is maintained both by selection provided by pathogens and allorecognition.

Relevance of allorecognition in multicellular organisms

We have shown that allorecognition in fungi is very important to maintain stable multicellular growth by preventing somatic cheaters. Allorecognition is important because it regulates fusion, a process that is common in fungi and also in colonial invertebrates, upon which Crozier based his model. Cheating has not been the focus of attention to explain allorecognition in fungi, probably because efficient allorecognition keeps cheating at such a low frequency that they have never been picked up from nature or recognized as such. Most other multicellular organisms have allorecognition as well. Just like in the fungi allorecognition has been difficult to explain because cheaters are not visible as a threat to stable multicellularity and also because fusion is not clearly visible the lifecycle.

Allorecognition in vertebrates prevents the transmission of cancers

The allorecognition mechanism of vertebrates is the major histocompatibility complex (MHC). The high diversity for MHC genes and its allorecognition function has been subject of discussion for a long time now (Medawar 1952, 1957; Buss and Green 1985; Hughes and Yeager 1998). An allorecognition mechanism does not seem relevant for organisms that do not have the opportunities to fuse in their lifecycle. For this reason, several theories were proposed to explain the existence of MHC polymorphism in vertebrates, which excluded selection for non-self recognition (Buss and Green 1985). It has been proposed that diversity was selected for something else such as its role in pathogen defence with allorecognition as

a by-product (Burnet 1970). It is now clear that formation of natural chimeras (individuals composed of different genotypes) is possible and does occur also in vertebrates, indicating that allorecognition can be functional in preventing such chimera (Mayr et al. 1979; Chen et al. 2013). In humans, many examples of chimera exist, always originated early in development during pregnancy from different zygotes, or from the zygote and the mother (Rinkevich 2001). These chimeras are likely occurring due to lowered allogenic reaction during pregnancy. This creates opportunities for somatic cheaters like cancers to spread as well. While cancers are often believed to be on an evolutionarily dead end as a result of the early germline soma separation, there are also several examples of transmissible cancers that can spread horizontally or vertically to conspecifics. An example in humans is a leukemic cancer that transmitted from mother to child. The cancer possessed mutations to evade immune and allorecognition response from the child (Isoda et al. 2009). Similarly, cancers have been transmitted during organ transplantations (Penn 1997), during which the allorecognition system is silenced artificially to prevent rejection of the new organ. Cancers can also spread in a later stage of life in a natural way. For example, the Tasmanian devil population is threatened with a cancer that spreads via their frequent biting behaviour (Pearse and Swift 2006), in dogs a sexually transmitted cancer is common around the world (Rebbeck et al. 2009) and in hamsters the transmission of leukemic cancer via mosquitos has been reported (Banfield et al. 1965). However, it must be emphasized that these three examples are rare exceptions. The occurrence of these transmissible cancers all coincide with lowered or assumed lowered allorecognition diversity due to inbreeding and population bottlenecks (Banfield et al. 1965; Siddle et al. 2007; Rebbeck et al. 2009). The previous examples show that allorecognition in vertebrates is very important to prevent the horizontal and vertical spread of cancers in vertebrates. The idea that cancers are at an evolutionarily dead end as a result from the separation of the germline from the soma is only true because of the additional protection from the MHC allorecognition.

Why is plant fusion not regulated by allorecognition?

So we know now that a single-celled bottleneck is not sufficient to maintain high relatedness within an individual in fungi and vertebrates, because fusion can decrease relatedness. Interestingly, plants do not distinguish self from non-self during artificial grafting (Biedrzycki and Bais 1981), while they do have a form of kin-recognition used to regulate root growth in order to cooperate or compete with neighbouring plants (Chen et al. 2012) and a highly polymorphic self/non-self recognition mechanism to prevent self-pollination (Takayama and Isogai 2005). The lack of incompatibility to form chimeras poses a theoretical problem since similar to other plant tissues plant tumours can also be grafted (Littau and Black 1952). However, although most described tumours spread over the plant, they only do so because they are induced by a virus or bacterial agent or a genetic disease (Matveeva et al. 2001). Spontaneous tumours in plants remain local, at the spot where they originated (Littau and

Black 1952) and are therefore usually not a major cost for plant fitness. This is a sharp contrast to vertebrate tumours, which quite commonly can disperse through the body. This difference can be explained by the structural differences between plants and animals (Buss 1987). The plant has cell walls which keep cells in place and the vascular system is designed to be free of particles. In contrast to that, vertebrate cells do not have rigid cell walls, making them more flexible and vertebrate blood vessels contain many cells that are important for the functioning of the organism.

Just as in fungi, in plants natural grafting or fusion can have advantages. For example, fusion of roots can make plants better rooted to the ground and expand their range for obtaining nutrients (Graham and Bormann 1966). More advantages of root fusion have been proposed, but risks of fusion are also acknowledged such as disease transmission and resource parasitism (Lev-Yadun 2011). Additionally, allorecognition for root-fusion may be less important for some plants with limited seed dispersal since they are likely to grow mostly next to related individuals (Lev-Yadun 2011). Perhaps in plants, in contrast to our findings for fungi, the selective advantages of fusion are greater than the disadvantages.

Tumours in fungi?

In plants and vertebrates, somatic cheaters often form tumours, i.e. local aberrant outgrowth from an individual. In fungi, typical tumour growth is not observed, as far as I know. This may have to do with the fact that the typical cheaters we expect can disperse within the mycelium. Because in plants and vertebrates tumour cells divide rapidly, they will mostly remain locally and thus form a tumour. However, in fungi rapidly dividing nuclei can spread in the mycelium thereby avoiding tumour formation and optimizing their opportunities by using resources from elsewhere in the colony.

What is the societal relevance of this research?

Fungi have wide economic and health importance (Moore-Landecker 1996). Industrial fermentation of fungi is used to produce useful substances. Many food products rely on fungi or contain fungi. Agriculture is often negatively affected by fungal pests (e.g. (Elad and Freeman 2002)), while mycorrhizal fungi are important for uptake of nutrients by crops and thus increase production (e.g. (Sawers et al. 2008)). Fungi are also of medical importance as fungal pathogens (e.g. (Bignell 2014)), but also the negative health effects when fungi occur indoors (e.g. (Dubey et al. 2011)). In the examples given above, frequently the fungi will grow in monoculture or with low genetic diversity. This is initially useful when we use fungi for our benefit and production increases because production increases in a monoculture (chapter 2), but the results described in this thesis demonstrate that these systems are vulnerable to invasion of cheating genotypes (chapter3+4). Harmful fungi such as agricultural pest and fungal disease also likely establish themselves as a monoculture as the substrates created by humans usually have low diversity. This helps the fungi to spread quickly, and the damage

will already be done before populations can be affected by cheating individuals. In theory, we could make use of a cheating genotype by letting it invade harmful fungal pests and infections thereby destroying cooperative growth of the pathogen and reducing its impact on a patient or crop. This has been suggested earlier for cheating bacteria, and mitochondria (Brown et al. 2009). Whether this last idea is realistic remains to be proven, and will require much more research. However, it is useful to understand what happens to fungi grown in monoculture, as this is often done in food production, industrial production systems using fungi in a fermenter, and in stock preservation. It is likely that fungal cultures used in these processes are affected by somatic cheating. The knowledge from this thesis is therefore useful in the search for the conditions to grow fungi for an optimal and sustainable production strategy.

Conclusion and further research

This thesis has provided experimental evidence for, on the one hand the benefits of fusion that select against allorecognition diversity and, on the other hand, for the possible costs of fusion that can maintain allorecognition diversity. The parameters of the model by Czaran et al. (2014) fit well with our results. However, it remains to be tested experimentally whether the cost of cheating is higher than the benefits of fusion shown in chapter 2. With the cheating genotypes available for *Neurospora* described in chapter 3, I have provided an excellent experimental system to test the effect of cheating on the evolution of allorecognition diversity. We do not know yet what mutations cause the cheating phenotype associated with slower growth and reduced number of asexual spores. Are they point mutations in genes associated with asexual spore mutation, nutrient uptake or metabolism and somehow give the nuclei a relative advantage to end up in the spores? Or are they large deletions which lead to faster mitotic division? Some laboratory mutants that behave as cheaters have been described that involve the uptake or production of molecules (Ryan and Lederberg 1946; Davis 1960). However, these are phenotypically different from the cheating phenotypes that we selected. Sequencing the genomes of the different evolved cheat types, will give insight in the mechanisms that make somatic cheating in ascomycete filamentous fungi possible. When the cheaters are well characterised, it will also be interesting to find out whether they really are rare in nature, or whether they are “invisible”, for example because they mostly are hidden in natural heterokaryons or because researchers never have recognized aberrant phenotypes as somatic cheaters.

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Summary

Cooperation -behaviour that benefits other individuals- can be beneficial at the level of the group. For example, a large group is better protected against predators than a small group, and a group of individuals dividing certain tasks may be more efficient than a group of individuals that perform all tasks separately. A cooperating group can thus reach a higher reproduction than a group of non-cooperating individuals. However, even though cooperation increases fitness at the level of the group, it is difficult to explain the evolution of cooperation: *within* a cooperative group, non-cooperative individuals, which still do profit from other, cooperating, individuals will have more resources to spend on reproduction, and thus are predicted to have a higher fitness. Natural selection will thus select these cheaters until there are no cooperating individuals left.

Hamilton's kin-selection theory predicts that stable cooperation can evolve when cooperation is directed with a higher probability towards genetically related individuals (kin) than towards unrelated individuals. In his formulized condition ($rB - C > 0$), cooperation is stable when the cost of helping (expressed as the number of offspring not produced because of the cooperative allele) is lower than the benefit (the number of additional offspring as a consequence of the cooperative allele), multiplied by the average relatedness of the individuals that receive the help. Kin-selection theory is not the only theory that can explain cooperation, but is generally accepted as an important factor in many forms of cooperation. One mechanism to direct help preferentially towards kin is population viscosity. Little dispersal results in progeny and ancestors staying close together in a group. If individuals are more motile, active kin discrimination becomes important. In order to direct cooperation preferentially towards kin, many organisms have developed specialized genetic kin recognition mechanisms, based on one or more polymorphic recognition loci. These organisms only cooperate with individuals that partially or fully match their own recognition genes. Crozier made a model based on marine invertebrates that form colonies. When colonies are close to each other they cooperatively fuse with neighbouring colonies when they are clonally related, or actively compete when they are less related. The decision to cooperate is based on genetic allorecognition. Crozier's model predicted that if fusion increases fitness, common alleles will be favoured since individuals with common recognition alleles will fuse more often. This will lead to selection of the most frequent recognition alleles until recognition polymorphism disappears completely. Thus, there is a cost of allorecognition that may reduce the genetic variation upon which allorecognition crucially relies, a prediction now known as 'Crozier's paradox'. An important hypothesis that can solve this paradox is to incorporate the effect of cheating. Cheating will lead to a cost to the group of cooperating individuals and therefore can impose selection pressure to maintain allorecognition. Another hypothesis is

that allorecognition diversity may be selected for another function.

Multicellularity is an extreme example of cooperation: the cells of an individual usually show division of labour, and altruism is strong because only a fraction of the cells reach the germline. A multicellular individual thus essentially is a cooperating group of cells, and evolution acts at different levels. The multicellular individual gets selected based on its fitness compared to the multicellular individuals it competes with, while at the same time the cells within the multicellular individual are under natural selection. This can lead to a potential conflict, where cheating cells evolve that have a higher fitness relative to other cells within the individual, but at the same time reduce the fitness of the multicellular individual. Theory predicts that a high relatedness among the cells of an individual reduces the opportunities for such cheating cells. Consistent with this hypothesis, there are some important mechanisms, which maintain high relatedness observed in multicellular organisms. One mechanism is regular single-celled bottlenecks in the lifecycle such as spores or seeds or zygotes from which multicellular individuals develop by mitotic division. Another important mechanism to maintain the high relatedness after the single-celled bottleneck is allorecognition to prevent fusion with non-self cells. Allorecognition is found in most multicellular organisms, but seems most relevant for organisms in which fusion between individuals or aggregation of cells is a notable part of their lifecycle.

In this thesis, I have used filamentous ascomycete fungi as a model for the evolution of stable multicellularity and allorecognition. The fungi have regular single-celled bottlenecks in the form of spore formation, from which they develop by clonal division of the nuclei to form a tubular network known as the fungal mycelium or colony. An interesting aspect of fungal growth is that the mycelium is not clearly divided into cell compartments, with the result that cytoplasm and nuclei can freely move through parts of the colony. This implies that organelles (nuclei, mitochondria), and not cells, are the main potential selective unit below the individual. Another important feature of fungi is that fungal colonies can fuse. Whether neighbouring colonies fuse or reject each other is determined by a highly polymorphic genetic allorecognition mechanism.

In this thesis, I have used these fungi to address the theoretical problem identified by Crozier, the evolutionary stability of genetic kin recognition. We first tested whether fusion between colonies indeed is beneficial compared to allorecognition, and whether this can lead to erosion of allorecognition diversity (chapter 2). We used the ascomycete fungus *Neurospora crassa*, a well-established model species for genetic research, of which numerous strains are available of different allotype. We found that cultures grown from a single allotype have a higher spore production than cultures grown from a mixture of different allotypes. This shows that fusion is beneficial relative to allorecognition. We determined the precise causes of this relative cost of allorecognition, by using a fusion mutant that partially mimics the effect of allorecognition. Colonies remain separated from each other, similar to colonies separated due to allorecognition. However, in contrast to confrontations between colonies

with a different allotype, in which part of the mycelium is sacrificed in a cell death reaction, in confrontations between different colonies of the fusion mutant, there is no active rejection. This comparison showed that the benefit of fusion is due not only to absence of mutual antagonism, which occurs upon allorecognition, but also to an increase in colony size per se. We then experimentally demonstrated that the benefit of fusion selects against allorecognition diversity, as predicted by Crozier. We show that there is a positive correlation between the frequency of an allotype and its competitive fitness, thus showing that positive frequency dependent selection acts on allotype diversity, thus leading to erosion of allotype diversity.
In the remaining part of the thesis, I have used different ascomycete fungus models to test various hypotheses to explain the evolutionary stability of allorecognition. One hypothesis considers allorecognition as a means to protect against cheating genotypes, genotypes that have a competitive advantage in combination with a wildtype genotype, but that reduce total reproductive output (chapter 3). According to recent theoretical models that simulate the evolution of allorecognition in combination with the possibility of somatic cheating, high allorecognition diversity can evolve in combination with low frequencies of cheating. The main condition is that cheating can evolve from cooperative genotypes. In order to test the hypothesis that cheating is a realistic threat to multicellular growth in fungi, we used an experimental evolution approach with *N. crassa* that maximised the potential for cheating genotypes by selecting under low relatedness, conditions: a high inoculation density, complete mixing at each transfer and in the absence of allorecognition. Within less than 300 generations, all eight replicate lines we evolved under these conditions significantly decreased their average asexual spore production. This yield reduction was caused by genotypes that matched the criteria for cheating: they had increased competitive fitness relative to a cooperative ancestral type, but spore production was significantly decreased when grown in mono culture or together with a cooperative type. A parallel control experiment, in which relatedness was kept high within the colony by using a fusion mutant, did not result in a reduction in asexual spore yield, showing that maintaining high relatedness provides efficient protection against cheating. From these results we can conclude that cheating can evolve quickly from cooperative genotypes, but that cheating only is selected when relatedness is low. This explains that cheating genotypes are generally not picked up from nature, since relatedness will usually be higher under natural conditions. First, the extremely high density used in our experiments is unlikely to occur in nature, so that there is more clonal outgrowth relative to fusion. Second, the high diversity of allorecognition alleles observed in nature will increase the average relatedness among the nuclei of a single individual. At the same time, the threat of cheating creates selection pressure to maintain allorecognition.
A different hypothesis, specific to fungi, is the possibility that allorecognition provides protection against cytoplasmic cheaters (chapter 4). Usually, mitochondria are restricted in their movement by cell compartments, so that there is selection at the level below that of the cells. In fungi, mitochondria can move through the mycelium similar to the nuclei. For

this reason, mitochondria can be selected within a fungal colony similar to the way nuclei can be selected within a fungal colony. We studied the evolutionary dynamics of mutant mitochondria that cause senescence in *Neurospora intermedia*, a species closely related to *N. crassa*. The mitochondria mutate under the influence of a natural occurring mitochondrial plasmid that acts as a mutagen. The mutated mitochondria have a selective advantage within the fungal colony, which allows them to increase in frequency at the cost of colony fitness. Once the mutated mitochondria reach a high frequency, the colony dies. Therefore, these mitochondrial mutants are typical cheaters, which increase their own relative fitness at the cost of the colony. We performed evolution experiments where we varied relatedness by varying fusion and bottleneck size. We show that reduction of the bottleneck size reduces the predictability of selection of mutant mitochondria. Then, we show that evolution with a fusion mutant effectively selects against mutant mitochondria and prevents senescence of the cultures. In a following experiment we then show that allorecognition can prevent or delay senescence in a similar way as what happens in cultures with a fusion mutant. These experiments confirmed that cheating mitochondrial genotypes provide a realistic threat to fungal multicellularity and that allorecognition can help keeping these mutants at a low frequency.

Although the selective pressure by cheating appears to be sufficient to maintain the allorecognition diversity observed in fungi, it does not exclude the hypothesis that allorecognition diversity can also be the result of selection for another function. In chapter 5, I describe the highly polymorphic *het-c* locus in *Podospora anserina*. The *het-c* locus determines allorecognition together with two unlinked loci termed *het-d* and *het-e*. Each *het-c* allele is incompatible with a specific subset of the *het-d* and *het-e* alleles. We found that the *het-c* allorecognition gene is under diversifying selection and more polymorphic than most other fungal allorecognition genes. Several aspects hint to a possible function in pathogen recognition for the *het-c*, *het-d* and *het-e* allorecognition system, such as its high variability and structural and sequence homologies to plant defence genes. Therefore, we argue that diversity in these genes may be selected for both maintaining allorecognition and pathogen recognition. The characteristics of these genes seem an exception and have not been found for other fungal allorecognition genes. The functioning of these genes in pathogen recognition and defence remains to be demonstrated. So although these results are interesting, cheating remains the most probable solution to explain the evolution of allorecognition diversity.

The results described in this thesis emphasize the influence of somatic cheating on the evolution of allorecognition in fungi. Fungi are economically and medically very important for society. Therefore, the results described in this thesis are very useful since they give new insight in how high relatedness can keep fungal growth stable if this is desired and how cheating might be useful to use against undesired fungal growth. Finally, I discuss that cheating is a risk in most multicellular organisms and that allorecognition is very important to prevent such cheating genotypes from spreading between individuals.

Nederlandse samenvatting

(Dutch summary)

De evolutietheorie leert ons dat de individuen die het beste zijn aangepast aan hun omgeving, de hoogste fitness hebben, dat wil zeggen dat zij de meeste gezonde nakomelingen hebben ten opzichte van anderen. Erfelijke eigenschappen zijn hierbij erg belangrijk, aangezien individuen met goede erfelijke eigenschappen deze door kunnen geven aan hun nakomelingen, die daar op hun beurt ook weer van kunnen profiteren in de vorm van een hogere fitness. Hierdoor nemen deze goede eigenschappen in frequentie toe, een proces bekend als natuurlijke selectie.
In de natuur kennen we veel voorbeelden van sociaal gedrag of samenwerking tussen soortgenoten, bijvoorbeeld leeuwen die samen op jacht gaan of een bijenkolonie waarin alle bijen samenwerken om zoveel mogelijk nakomelingen groot te brengen. Samenwerking kan ten koste gaan van de eigen fitness, maar is gunstig voor de fitness van anderen. Op het niveau van een groep kan samenwerking veel voordelen hebben, waardoor de gemiddelede fitness van alle individuen in de groep toeneemt. Een grote groep is bijvoorbeeld beter beschermd tegen roofdieren dan een klein groepje of één individu, en door taken te verdelen kan er specialisatie optreden, wat vaak een hogere efficiëntie heeft. Ondanks deze voordelen van samenwerking is het toch moeilijk om de evolutie van samenwerking te verklaren: Binnen een groep van samenwerkende individuen, hebben niet-samenwerkende individuen, die nog wel kunnen profiteren van het sociale gedrag van anderen, meer middelen over om te gebruiken voor hun eigen overleving en reproductie. Dit leidt tot de verwachting dat deze zogenaamde *valsspelers* of *sociale parasieten* dus een relatief hogere fitness zullen hebben dan de samenwerkende individuen en dat natuurlijke selectie de sociale parasieten zal selecteren totdat samenwerking helemaal verdwenen is.

Hamilton's kin-selectie theorie (kin = familie of verwanten) voorspelt dat stabiele samenwerking kan evolueren als samenwerking vooral gericht is op verwante individuen. In zijn model is samenwerking stabiel wanneer de kosten van meehelpen (gedefinieerd als de vermindering van het aantal nakomelingen) lager zijn dan de opbrengsten (= extra nakomelingen) vermenigvuldigd met de gemiddelde verwantschap met de individuen die profiteren van de hulp (bijvoorbeeld broers hebben een verwantschap van 0,5). Hoewel kin-selectie theorie niet de enige theorie om samenwerking te verklaren, is kin-selectie algemeen geaccepteerd als een belangrijke factor bij veel natuurlijke vormen van samenwerking. Een manier om te zorgen dat samenwerking gericht is op verwanten is door populatieviscositeit ('stroperigheid'). Bij hoge populatieviscositeit, dus als individuen zich weinig verplaatsen, zullen de nakomelingen van een ouder automatisch bij elkaar blijven in een groep,

waarbinnen samenwerking dus vooral tussen verwante individuen plaats zal vinden. Bij een lage populatieviscositeit, dus als individuen zich meer verplaatsen, is het belangrijk dat verwante individuen elkaar kunnen herkennen en hun samenwerkingsgedrag daarop aan kunnen passen. Veel soorten hebben daarom erfelijke herkenningsmechanismen gebaseerd op een of meer polymorfe genen (genen die verschillen vertonen tussen individuen van een populatie). Samenwerking vindt dan alleen plaats tussen individuen die (grotendeels) hetzelfde zijn voor alle herkenningsgenen. Dit leidt echter weer tot een nieuw dilemma: hoe kan herkenning stabiel evolueren?

Wijlen Ross Crozier maakte een eenvoudig model gebaseerd op zeeanemonen (zeediertjes die samenwerken door een kolonie te vormen). Wanneer de kolonies van deze anemonen dicht bij elkaar groeien, fuseren ze met elkaar wanneer de kolonies klonaal verwant zijn (genetisch identiek zoals tweelingen), maar vindt er een felle afweer plaats wanneer de kolonies genetisch verschillend zijn. De herkenning gebaseerd op klonale verwantschap is feitelijk onderscheid maken tussen soortgenoten die (genetisch) zelf en niet-zelf zijn, meestal allo-herkenning genoemd. Deze beslissing vindt plaats op basis van polymorfe herkennningsgenen. Het model van Crozier voorspelt dat wanneer fusie de fitness verhoogt, de meest voorkomende genvarianten voor herkenning een voordeel hebben omdat zij meer kans hebben om te kunnen fuseren. Hierdoor ontstaat er selectie voor de meest voorkomende genvariant die zal gaan toenemen in frequentie totdat er nog maar één variant over is en er dus geen onderscheid meer gemaakt kan worden. Kortom, hoewel herkenning belangrijk is voor stabiele samenwerking, zijn er ook kosten aan verbonden doordat herkenning de mogelijkheden tot samenwerking beperkt. De voorspelling dat de kosten van herkenning zorgen voor de verminderde genetische variatie in de herkenningsgenen waarvan herkenning afhankelijk is, is ook wel bekend als Croziers paradox.

In bovenstaand model is de mogelijkheid van sociale parasieten niet meegenomen. Een belangrijke hypothese om de paradox op te lossen is dat de kosten die parasieten zullen hebben op een groep wellicht een selectiedruk uitoefenen om variatie in herkenningsgenen te behouden. Een andere hypothese om Croziers paradox te verklaren, voorgesteld door Crozier zelf, is dat de diversiteit van herkenningsgenen wordt geselecteerd via natuurlijke selectie vanwege een andere functie en dat allo-herkenning een bijproduct is.

Een extreme vorm van samenwerking is meercelligheid: Cellen van een individu verdelen vaak de taken via specialisatie van cellen, en de meeste cellen offeren zich op, omdat slechts enkele gespecialiseerde geslachtscellen het nageslacht vormen. Een meercellig individu is dus eigenlijk een samenwerkende groep cellen, waarbij evolutie plaats kan vinden op verschillende niveaus. Een meercellig individu staat onder selectie op basis van zijn fitness ten opzichte van de andere meercellige individuen waar het mee in competitie is, maar

tegelijkertijd vindt er ook selectie plaats op het niveau van de cel binnen het meercellig individu. Dit kan tot een conflict leiden waarbij 'vals spelende' cellen evolueren die weliswaar een hogere fitness hebben binnen het organisme (bij voorbeeld meer celdelingen), maar op hetzelfde moment de fitness van het meercellige individu verlagen (bijvoorbeeld in het geval van kankercellen). Volgens de theorie zullen dit soort vals spelende cellen veel minder voorkomen wanneer de verwantschap tussen de cellen van een meercellig individu hoog is, omdat de valsspelers afhankelijk zijn van 'sociale cellen'. In lijn met deze hypothese zien we dan ook dat de overgrote meerderheid van meercellige organismen een of meer mechanismen hebben om de verwantschap tussen cellen hoog te houden. Het belangrijkste mechanisme is dat meercelligen regelmatig door een eencellig stadium gaan, van waaruit het meercellig individu groeit via klonale deling van die cel, waardoor alle cellen genetisch hetzelfde zijn, met uitzondering van nieuwe spontane mutaties. Daarnaast is het belangrijk dat fusie van cellen en weefsels tussen genetisch verschillende individuen voorkomen kan worden door genetische allo-herkenningsmechanismen. Allo-herkenning bestaat in veel meercelligen, maar lijkt toch het meest relevant voor organismen waar fusie tussen individuen een belangrijk onderdeel van de levenscyclus is.

Voor dit proefschrift heb ik filamenteuze schimmels als een model voor de evolutie van stabiele meercelligheid en de evolutie van allo-herkenning gebruikt. Deze schimmels gaan regelmatig door een eencellig stadium als sporen gevormd uit vegetatieve groei (klonale sporen) en uit paringen (seksuele sporen). Vanuit deze spore vormt de schimmel via klonale deling een netwerk van schimmeldraden bekend als de kolonie of het mycelium. De cellen in deze kolonie zijn niet geheel gesloten, waardoor de kernen en het cytoplasma zich vaak vrij door het mycelium kunnen bewegen. Technisch gezien zal selectie binnen de kolonie dus vooral plaatsvinden door competitie tussen kernen en cytoplasmatische elementen zoals mitochondriën en niet tussen hele cellen zoals bij andere meercelligen eerder het geval zal zijn. Een andere belangrijke eigenschap van schimmels is dat kolonies met elkaar kunnen fuseren. Een zeer divers genetisch herkenningsmechanisme reguleert dat fusie tussen klonaal verwante kolonies succesvol is en dat de fusie geblokkeerd wordt tussen niet klonaal verwante kolonies.

Voor dit proefschrift heb ik schimmels gebruikt om het theoretisch voorspelde dilemma betreffende de evolutie van genetische verwantschapsherkenning dat volgt uit Croziers model experimenteel te onderzoeken. Eerst heb ik onderzocht of fusie tussen kolonies inderdaad de fitness van een schimmelculture kan verhogen ten opzichte van geblokkeerde fusie door allo-herkenning en dus echt een uitbreiding van de meercellige samenwerking is, en of dit leidt tot de theoretisch voorspelde afbraak van de diversiteit in de herkenningsgenen benodigd voor de herkenning van niet-zelf (Hoofdstuk 2). Voor dit onderzoek heb ik de schimmel *Neuropora crassa* gebruikt, die veel voor onderzoek gebruikt wordt en waarvoor

veel verschillende isolaten beschikbaar zijn en waarvoor de methoden voor gebruik in laboratoriumexperimenten uitgebreid beschreven zijn. Een culture met één allotype (=herkenningstype) produceert veel meer sporen (meer nakomelingen dus hogere fitness) dan cultures waarin fusie tussen kolonies beperkt is doordat er verschillende herkenningstypen in gemixt zijn. Dit laat zien dat er inderdaad kosten verbonden zijn aan herkenning. Met extra experimenten heb ik vervolgens aangetoond dat deze kosten worden veroorzaakt door twee factoren: 1. beperking van fusie levert kleinere kolonies op (maar wel meer), die gezamenlijk minder sporen produceren dan minder grotere en 2. door het mechanisme dat de blokkade van fusie veroorzaakt, namelijk celsterfte op de grens tussen twee kolonies. Tenslotte heb ik in hoofdstuk 2 laten zien dat door de kosten van herkenning ten opzichte van fusie er inderdaad selectie is voor het meest voorkomende herkenningstype in een gemixte culture (omdat het meest voorkomende type meer kan fuseren) wat leidt tot het verdwijnen van de diversiteit in de herkenningsgenen zoals dat in het model van Crozier voorspeld wordt.

In de overige hoofdstukken beschrijf ik experimenten die verschillende hypothesen testen die kunnen verklaren waarom allo-herkenning toch stabiel is geëvolueerd, ondanks de korte-termijnkosten zoals theoretisch voorspeld door Crozier en experimenteel aangetoond in hoofdstuk 2. Hoofdstuk 3 behandelt de hypothese dat allo-herkenning bescherming kan bieden tegen selectie van sociale parasieten of valsspelers van de meercellige samenwerking. Dat zijn kernen die weliswaar op het niveau van competitie tussen de kernen binnen één kolonie een hogere fitness hebben, maar de fitness van de hele kolonie verlagen. Recente modellen die wel rekeningen houden met de mogelijkheid van sociale parasieten laten zien dat er wel een hoge diversiteit van allo-herkenningsgenen kan evolueren waarbij er een evenwicht ontstaat, waarbij sociale parasieten slechts in zeer lage frequentie voorkomen, maar daarmee voldoende selectiedruk veroorzaken voor het behoud van allo-herkenning. De belangrijkste voorwaarde voor dit model is dat deze sociale parasieten gemakkelijk kunnen evolueren via mutatie vanuit de sociale genetische typen. Deze voorwaarde hebben we getest door de *Neurospora* schimmel te laten evolueren in het laboratorium door het langdurig overzetten van cultures op vers groeimedium met periodes van groei tussendoor. Deze experimentele evolutie beslaat vele generaties die zich generatie op generatie beter kunnen aanpassen aan hun groei omstandigheden door natuurlijke selectie van de beste spontaan ontstane mutaties. In dit evolutie-experiment hebben we de verwantschap tussen kernen laag gemaakt door fusie tussen kolonies toe te laten door de afwezigheid van allo-herkenning. Hierdoor zullen nieuwe mutanten bij het overzetten van de culture als eencellige sporen snel weer kunnen fuseren met individuen die de mutatie niet hebben. Op deze manier heb ik in alle acht de parallel geëvolueerde cultures gevonden dat de sporeproductie (een belangrijke fitness maat voor de kolonie) gedaald was, gemiddeld met ongeveer 70%. Deze spectaculaire daling was veroorzaakt door genetische varianten die voldeden aan de beschrijving van een typische valsspeler: ze hadden een hogere fitness ten opzichte van het sociale voorouder-type maar

hun sporeproductie was significant gedaald wanneer ze alleen groeiden (doordat de kernen niet meer efficiënt samenwerken). Een controle evolutie-experiment waarbij fusie tussen kolonies niet mogelijk was, en dus de verwantschap tussen kernen van een kolonie hoog bleef, omdat deze steeds weer klonaal groeiden vanuit een enkele spore, liet geen daling zien van de sporeproductie. Dit toont aan dat hoge verwantschap tussen de kernen van schimmelkolonies dus erg belangrijk is om de invasie van valsspelers, die samenwerking in gevaar brengen, te voorkomen. We hebben hiermee laten zien dat sociale parasieten makkelijk kunnen evolueren in een samenwerkingsverband, maar alleen als de verwantschap niet beschermd is. Dit laatste verklaart dat we vals spelende types bij schimmels niet detecteren in de natuur, aangezien verwantschap binnen een kolonie hier hoog gehouden wordt. Maar het feit dat deze sociale parasieten wel erg makkelijk kunnen ontstaan zal wellicht voldoende selectiedruk kunnen genereren op het behoud van allo-herkenning diversiteit zoals waargenomen in de natuur.
Een andere hypothese die vooral opgaat voor schimmels, is dat allo-herkenning bescherming kan bieden tegen valsspelers op cytoplasmatisch niveau (hoofdstuk 4). Het cytoplasma bevat de mitochondriën die ook erfelijke eigenschappen hebben en dus kunnen evolueren. In de schimmel kunnen de mitochondriën zich net als de kernen vrij rond bewegen in de kolonie, waardoor ze theoretisch op de zelfde manier kunnen parasiteren als boven beschreven vals spelende kernen. Hoewel mitochondriën normaliter erg stabiel zijn, zijn er natuurlijke isolaten die een mutageen element bevatten dat er voor zorgt dat mitochondriën snel kunnen muteren tot sociale parasieten die in frequentie toenemen ten opzichte van het originele sociale type binnen een kolonie (dus een relatief hogere fitness hebben) terwijl de kolonie sterk in fitness achteruit gaat omdat de vals spelende mitochondriën niet meer hun functie in de energievoorziening van de kolonie vervullen. Dit fenomeen is beschreven als veroudering in schimmels, omdat de schimmels niet meer levensvatbaar zijn als na een langere periode van groei te weinig goede mitochondriën over zijn. De experimenten beschreven in hoofdstuk 4 laten zien dat ook hier verwantschap tussen de mitochondriën binnen de kolonies erg belangrijk is voor selectie tegen vals spelende mitochondriën. Eerst manipuleerden we het aantal sporen waarmee cultures overgezet worden (de flessenhals), waarbij een kleinere flessenhals een relatief hogere verwantschap binnen de kolonie veroorzaakt na fusie van de kolonies. Vervolgens manipuleerden we ook de fusie tussen kolonies in de cultures zoals in hoofdstuk 3, en laten daarmee zien dat allo-herkenning veroudering kan voorkomen of uitstellen bij *Neurospora*.

Hoewel het lijkt dat de selectieve druk door sociale parasieten zoals beschreven in hoofdstuk 3 en 4 voldoende is voor stabiele evolutie van allo-herkenning, sluit dit niet de hypothese uit dat allo-herkenningsdiversiteit is geëvolueerd onder selectiedruk voor een andere functie van deze genen, met allo-herkenning als bijeffect. In hoofdstuk 5 beschrijf ik de diversiteit van het gen "*het-c*". We vinden dat dit gen veel meer varianten heeft in natuurlijke populaties dan de meeste andere beschreven allo-herkenningsgenen. Dit is waarschijnlijk veel meer

diversiteit dan nodig is voor efficiënt onderscheid tussen zelf en niet-zelf. Dit en verschillende andere eigenschappen van dit gen en de genen waarmee het interacties heeft, duiden op een mogelijke functie van dit gen in pathogeen herkenning. Daarom beargumenteren we dat de diversiteit in dit gen primair geselecteerd is voor een functie in pathogeenherkenning en dat alloherkenning een bijeffect is. Echter, de eigenschappen van dit gen lijken een uitzondering te zijn ten opzichte van de meeste genen voor alloherkenning. Dus hoewel dit een erg interessante bevinding is, lijkt selectiedruk ten gevolge van verschillende typen sociale parasieten de meest waarschijnlijke verklaring voor de evolutie van allo-herkenning te zijn.

De resultaten uit dit proefschrift benadrukken de invloed van parasieten van de multicellulaire samenwerking op de evolutie van genetische allo-herkenning in schimmels. Schimmels zijn maatschappelijk gezien erg belangrijk in economisch en medisch opzicht, vanwege zowel de schade die ze toe kunnen brengen als de voordelen die ze op kunnen leveren, bij voorbeeld als producent van enzymen voor industriële toepassingen. Om die reden kunnen de resultaten uit dit proefschrift erg nuttig zijn, door bijvoorbeeld te onderzoeken hoe hoge verwantschap kan helpen de productie van schimmels te verhogen en te onderzoeken hoe we sociale parasieten kunnen gebruiken tegen schadelijke schimmels. Tot slot bediscussieer ik dat sociaal parasitisme een risico is in de meerderheid van de meercellige soorten en dat allo-herkenning erg belangrijk is om de verspreiding van sociale parasieten tegen te gaan zelfs als menging van cellen van verschillende individuen niet zo duidelijk aanwezig is in deze soorten als in schimmels.

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It seems most of the life during my PhD exists around the people from the Laboratory of

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Although this is perhaps kind of true, there were some people that probably did not directly contribute to this thesis but made life during PhD much more pleasant.

First I thank Hanna Johannesson for creating the opportunity to start a new project and a new job as a postdoc at Uppsala University. It can be somewhat stressful to have to finish the PhD thesis and think about the future at the same time, however discussion with you about future projects was inspiring.
I like to thank the “wageningse biologen vrienden”. Roland, Lucas, Tineke, Thomas, Diana, Marc, Arjan, Piet, Dieuwertje, Andre, Linnea, Annemarie, Shirley and Peiwei (waar is Joris eigenlijk?). I’m happy that we still see each other occasionally for example for drinking tea, beer, holiday, making walks, or at reunions.
Another activity I picked up during my PhD is gardening, before I was limited to growing tomatoes on my balcony, but thanks to a small piece of land in the garden group “de Ungel” I now extended the range of vegetables significantly. Thank you Ungel members for your creating a great gardening atmosphere to distract me from the PhD life.
Then there is the home life that was invaded by coworker, friend, and I guess part of the family by now Alex GrGr. Thank you Alex for being a great housemate. I also thank the many other house guests that made life much more interesting.
I’m sure I forgot to mention a lot of people here that are important to me, but
Then my real family papa, mama, Carla, Marcel, Lauke, Anneke, Lukas, Peter, Marissa, Kasper, and Karlijn thanks for being there and supporting me.
Finally thank you Jianhua for being in my life, you are my newest family, and I foresee lots of good times ahead of us.

Curriculum vitae

Eric Bastiaans was born on 24 July 1980 in Schiedam, the Netherlands. In 1998 he obtained his high school degree (VWO) from "Scholengemeenschap Aquamarijn", Vlaardingen. He started the study of Biology at Wageningen University in 1999. He followed the cell/ molecular specialisation. His first Master thesis project was performed at the Laboratory of Genetics of Wageningen University under supervision of Fons Debets and Marc Maas on the subject: Localization of linear plasmid pAL2-1 insertions in the mitochondrial genome of *Podospora anserina* by semi-random two step PCR, in association with ageing in this fungus. His second Master thesis project was also at the Laboratory of Genetics of Wageningen University on the subject of vegetative incompatibility of *Podospora anserina* under supervision of Fons Debets and Anne van Diepeningen. For this project one month was spent in the fungal genetics lab of the CNRS in Bordeaux, France to do part of the laboratory work where a collaboration was started with Mathieu Paoletti and Sven Saupe that continued during his PhD project. His master was finished with an internship project at the Fungal Genetics group at Uppsala University under supervision of Hanna Johannesson with the subject: Reproductive isolation in the fungus *Neurospora tetrasperma*, in association with the evolution of the mating type chromosome. After finishing his biology studies in 2008 he spent one month assisting in a research project of Hanna Johannesson (Uppsala University, Sweden), involving expression studies on different mating types in the fungus *Neurospora tetrasperma.* For this project the practical work was carried out in the Townsend Lab (Yale University, New Haven, CT, USA). In the same year he started the PhD project that has led to this thesis, under supervision of Duur Aanen and Fons Debets.

Publications

Bastiaans, E., D.K. Aanen, A.J.M. Debets, R.F. Hoekstra, B. Lestrade, and M.F.P.M. Maas. 2014a. Regular bottlenecks and restrictions to somatic fusion prevent the accumulation of mitochondrial defects in Neurospora. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**:20130448.

Bastiaans, E., A.J.M. Debets, and D. K. Aanen. 2015. Experimental demonstration of the benefits of somatic fusion and the consequences for allorecognition. *Evolution* **69**:1091-1099.

Bastiaans, E., A.J.M. Debets, and D. K. Aanen. in preparation. High relatedness stabilizes multicellular cooperation.

Bastiaans, E., A. J.M. Debets, D. K. Aanen, A. D. van Diepeningen, S. J. Saupe, and M. Paoletti. 2014b. Natural variation of heterokaryon incompatibility gene het-c in Podospora anserina reveals diversifying selection. *Molecular biology and evolution* **31**:962-974.

Chevanne, D., E. Bastiaans, A.J.M. Debets, S. Saupe, C. Clavé, and M. Paoletti. 2009. Identification of the *het-r* vegetative incompatibility gene of *Podospora anserina* as a member of the fast evolving *HNWD* gene family. *Current Genetics* **55**:93-102.

Menkis, A., E. Bastiaans, D. Jacobson, and H. Johannesson. 2009. Phylogenetic and biological species diversity within the Neurospora tetrasperma complex. *Journal of evolutionary biology* **22**:1923-1936.

Samils, N., A. Gioti, M. Karlsson, Y. Sun, T. Kasuga, E. Bastiaans, Z. Wang, N. Li, J. P. Townsend, and H. Johannesson. 2013. Sex-linked transcriptional divergence in the hermaphrodite fungus Neurospora tetrasperma. *Proceedings of the Royal Society B: Biological Sciences* **280**:20130862.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)
- Kin- and allorecognition in fungi and other organisms (2009)

Post-graduate courses (4.5 ECTS)
- Evolutionary workshop in the Swiss alps; University of Basel (ETH), Zürich (2012)
- Recent insights in mitochondrial evolution applied to health and aging; Lorentz Centre (2013)

Invited review of (unpublished) journal manuscript (2 ECTS)
- Bioassays: somatic incompatibility in fungi (2009)
- Project proposal (ANR, France): non-self recognition in fungi (2011)

Deficiency, refresh, brush-up courses (1.5 ECTS)
- Basic statistics (2011)
-

Competence strengthening / skills courses (1.5 ECTS)
- PhD Competence assessment; WGS (2009)
- Speed reading workshop; KLV (2012)
- Scientific publishing; WGS (2013)
- Voice matters; WGS (2013)
- Career assessment; WGS (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC PhD Weekend (2009)
- PE&RC Day: intelligent nature: on the origin of communication (2009)
- PE&RC Day: innovation for sustainability: what are the neighbours doing (2011)
- PE&RC Day: biomimicry – unlocking nature's secrets (2013)
- PE&RC Day: optimization of science: pressure and pleasure (2014)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)
- Experimental Evolution Discussion Group (EEDG) (2009-2014)
- Wageningen Evolution and Ecology Seminars (WEES) and masterclasses (2009-2014)
- Symposium: evolution in the laboratory; oral presentation (2013)
- Symposium: genetics of social life; oral presentation (2013)

International symposia, workshops and conferences (9 ECTS)
- 16th Annual European Meeting of PhD Students in Evolutionary Biology (EMPSEB); Wierzba, Poland (2010)
- 16th Congress of the International Union for the Study of Social Insects (IUSSI); Copenhagen, Denmark (2010)
- The annual meeting of the section Mycology of the Dutch Society for Microbiology (NVvM); Utrecht, the Netherlands (2010)
- 13th Congress of the European Society for Evolutionary Biology (ESEB); Tubingen, Germany (2011)
- 11th European Conference on Fungal Genetics (ECFG11); Marburg, Germany (2012)
- Neurospora satellite meeting to the 11th European Conference on Fungal genetics; Marburg, Germany (2012)
- The 26th Fungal genetics Conference at Asilomar (FGC), Asilomar conference grounds; Pacific Grove, CA, USA (2013)
- 14th Congress of the European Society for Evolutionary Biology (ESEB); Lisbon, Portugal (2013)
- Neurospora, Asilomar conference grounds; Pacific Grove, CA, USA (2014)

Lecturing / supervision of practical's / tutorials (3 ECTS)
- Genetic analysis trends and concepts (2013, 2014)

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