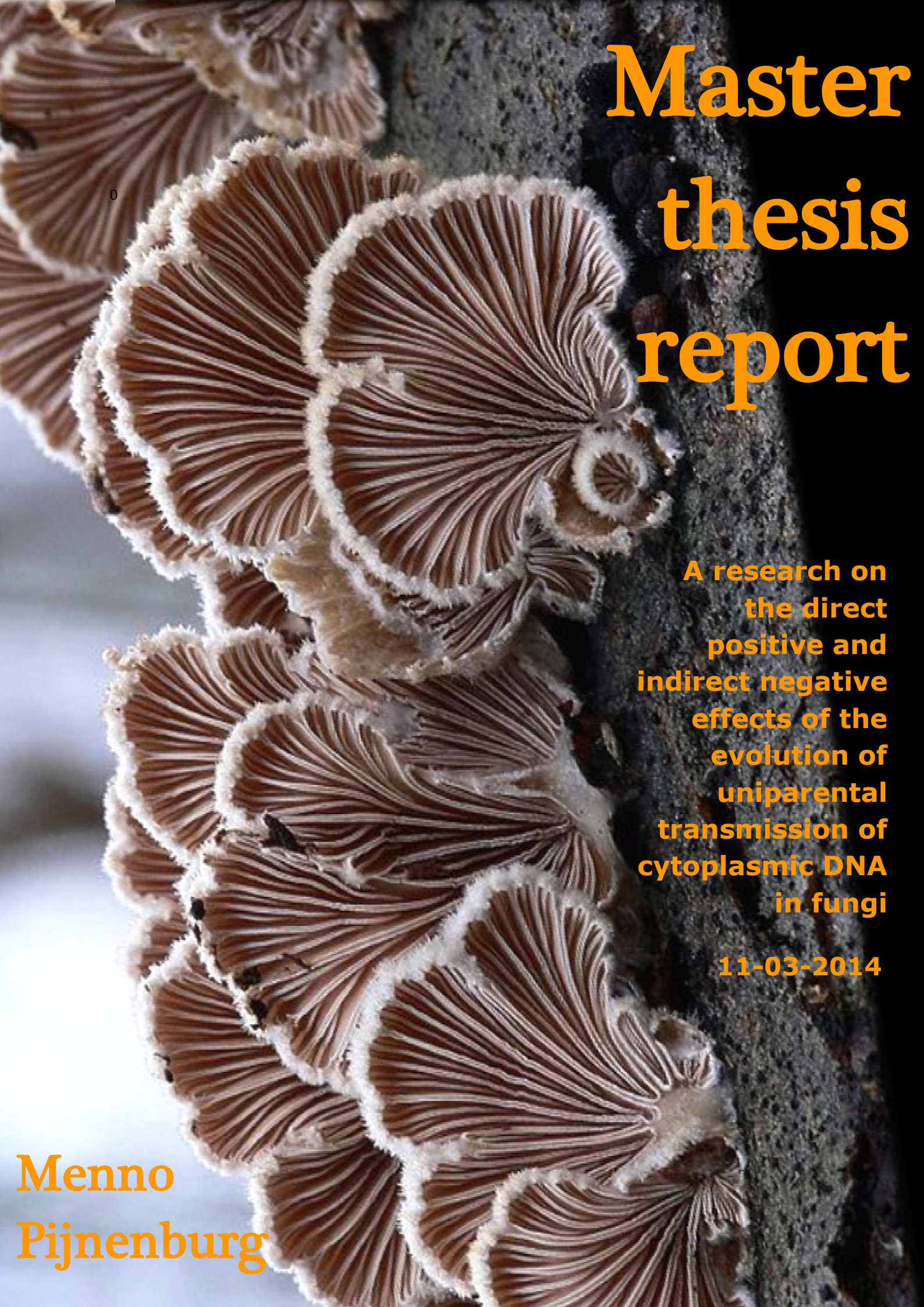

Master thesis report

A research on the direct positive and indirect negative effects of the evolution of uniparental transmission of cytoplasmic DNA in fungi

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Uniparental inheritance, direct benefits and indirect consequences

Researching the origin of uniparental inheritance

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Abstract

There is a discrepancy between the interests of nuclear and cytoplasmic genomes when it comes to sexual replication. Both are selected for maximal proliferation, but this is not attained in the same way. In plants, evidence has been reported that the mitochondria can evolve selfish mechanisms that improve their proliferation at the cost of overall fitness of the organism. We suppose these selfish mechanisms have evolved as a result of the uniparental transmission of cytoplasmic DNA. The evolution of uniparental inheritance is hard to explain, as most of the beneficial effects seem mostly relevant on a longer term. Long term beneficial effects are not very relevant for evolution. In this research we use the model system *Schizophyllum commune* to test whether cytoplasmic mixing leads to short term negative fitness effects for the organism. This research does not report evidence for direct negative effects of cytoplasmic mixing. However, we hypothesize that future experiments may well confirm that uniparental transmission has evolved to circumvent these supposed direct negative effects, which in turn may have led to the reported armed peace between the nuclear and cytoplasmic genomes. In fact, here we present data that confirms with a high statistical significance that nucleo-mitochondrial interactions have an effect on organismal fitness.

Introduction

The cell is a marvel of complexity and unity. The whole of it, be it an amoeba, part of an animal or part of a plant, seems to boil down to survival of the individual. This conclusion does not quite cut the truth though. Rather, the individual is designed to protect and propagate its blueprint, its unique DNA sequence. Everything works together so that the individual can transmit (part of) its DNA to a brand new being. By extending through generations, a DNA sequence can survive longer than the singular individual would last. However, an essential flaw is overlooked in the seemingly perfect design to continuously transmit DNA as effectively as possible through the ages. Every kind of eukaryotic cell, and thus every kind of individual contains multiple kinds of DNA, and that brings a problem. One set of DNA is located in the nucleus. Another is in the mitochondria. Additional ones can be those of the chloroplasts in plants, or present plasmids in bacteria. Excepting plasmids, which can also readily be transmitted horizontally to other individuals, all these genomes are propagated mainly to new generations. Basically, it seems that if the individual proliferates, every genome get its fair share of survival. The new cell needs nuclear DNA, mitochondria and, in plants, chloroplasts. Plasmids may also contain useful features, though they may not be strictly necessary for survival. So everything seems quite well organised and harmonious. But it is not.

Genomic conflicts

Different interests

Though the general problem we will assign here exists in both sexual and asexual species, we will only consider sexual proliferation, where DNA from two parents is combined and a new individual with traits from both parents is born. Though both parents' DNA is proliferated in this way, the cytoplasmic DNA (of the mitochondria, chloroplasts and plasmids) is usually only passed down through the mother. In other words, as they are only abated by a female progeny, a male individual is a dead end for these cytoplasmic genomes. In plants, gynodioecy, the existence of both hermaphrodite and female plants within one species, has been proven [2]. Gynodioecy is probably a consequence induced by the fact that males are dead ends for mitochondria. It has been described decades ago, though the cause long eluded science. The most recent theory is that this phenomenon is a result of interactions between cytoplasmic and nuclear DNA [3]. For the cytoplasmic genomes this induced male sterility is beneficial, as the plant has more energy to spend on its female proliferation. Of course, the change will be deleterious for the nuclear genome of the plant since, as this is transmitted equally via the male and female route and thus favours a fifty-fifty sex ratio. In the long run,

when the sex ratio becomes more and more female biased, the evolutionary bounty for a counter-mutation against male sterility increases. Population wide, a few plants acting out their male function may be sufficient to fertilize all female plants, but the selective advantage of being able to reproduce as a male becomes larger and larger with an increased female pool size. Selection for a nucleus with a mutation that counters the male sterility can be expected sooner or later. The initial mitochondrial mutation will be neutralized, and sexual balance can be restored. This situation is the basis of a continuous to and fro between cytoplasmic and nuclear DNA. Both are selected for maximal proliferation. It is this kind of conflict that stands at the basis of the projects that this thesis assesses. The cases are not restricted to the occurrence of male sterility, but it suits as a valid example.

Uniparental inheritance

We know that these intergenomic conflicts are a consequence of the different modes of inheritance between the nuclear and the mitochondrial DNA, but not all species have this uniparental inheritance of cytoplasmic DNA. In *Saccharomyces cerevisiae* for example, both parents contribute to the cytoplasmic DNA of the offspring after cellular fusion. There are many species in fact, in which biparental inheritance may occur, though it happens infrequently [4]. Even when biparental inheritance occurs, it is very common for any given cell to contain only one type of mitochondria. Remarkable, because individually both inherited types of mitochondria could probably persist in the cytoplasm. However, with some thought the necessity of only one cytoplasm persisting per cell becomes apparent.

Imagine a scenario where both mother and father would transmit their cytoplasms, which would both persist in the offspring. After a one generation, the offspring of two homoplasmic individuals would be heteroplasmic (their cytoplasm would not be homogenous). One generation later, each individual would transmit both cytoplasms to their offspring, simply because all cytoplasms would be present in all cells, including gametes, resulting in individuals with four types of cytoplasm per cell. This way, cytoplasmic types would accumulate in a cell through generations. The only logical answer to this scenario (still assuming all cytoplasms persist in the cell) would be selection of one of the present cytoplasms for reproduction, automatically resulting in intercellular competition for selection. So let us assume another situation, where this time both cytoplasms are inherited, but after a time only one cytoplasmic type remains in each cell. Which cytoplasm will persist can be decided in a number of ways. The most obvious one is a process of drift, where at random one type of mitochondria persists. Two others are either a rather passive form of competition, like a selective advantage for one of the mitochondria at mitosis, or a more active competitive.

Both options for the maintenance of biparental inheritance result in intercellular competition. One competition would resolve which cytoplasm is selected for transmission, the other competition would resolve which cytoplasm persists in the homoplasmic individual. Therefore, both these mechanisms would select partly for which cytoplasm is most competitive, not which results in highest fitness for the individual. Uniparental inheritance prevents the need for all these conflicts, so it makes sense that uniparental inheritance is selected for. However, the beneficial effects brought to this system by introducing uniparental inheritance are not direct. There is an advantage to not having cytoplasms accumulate, but it does not automatically increase fitness. Except if the heteroplasmic state is deleterious compared to a homoplasmic state.

In 2012, a research was conducted in which heteroplasmy was artificially induced in mouse offspring [5]. The heteroplasmic individuals showed significant metabolic and cognitive impairments, suggesting the induced heteroplasmy had direct negative effects. Similarly, in this research we will research direct negative effects of heteroplasmy, but in a more basic way. We induced the heteroplasmy in a much simpler organism, and monitored its fitness. Observed negative effects could help explain how uniparental inheritance evolved. The global two-part question we try to answer is: **Has uniparental transmission evolved by preventing direct negative effects of cytoplasmic mixing, and thereby given chance to indirect intergenomic conflicts to appear?**

The model organism

Schizophyllum commune (henceforth referred to as *S. commune*) was chosen to experiment in for this research. Chief among the reasons for choosing this organism is that it has actually been a model organism amongst basidiomycetes for a long time [6]. Accordingly, its genome has been sequenced [7], and general information about the organism is easy to obtain. Additionally, the organism is hermaphroditic, its cytoplasmic contents are easily manipulated and its rapid growth is favourable for testing. These aspects are particularly important for this specific research.

Species specifics

The fungus belongs to the group of homobasidiomycetes, distinct from heterobasidiomycetes by the fact that they have aseptate basidia. These fungi follow a rather distinct life-cycle in which they form spores that can develop into monokaryons if they land in a favourable environment. The fungus feeds on dead wood, so it is there the monokaryon forms and colonizes substrate by radial outgrowth of hyphae. It will continue to do so until it either runs out of substrate or comes in contact with a compatible spore, monokaryon or dikaryon, and fuses with it. The two individuals can either be compatible, or incompatible, as researched by J.R. Raper in 1958 [8]. This compatibility could be compared to mammal genders. A mating type (gender) cannot fertilize an individual of the same mating type. Compared to mating types however, mating types are much more numerous. As there are many mating types, there is a higher chance of a fertile union. After mating the whole is named a dikaryon or heterokaryon, a state in which each cell has two nuclei. This stage of development is unique to fungi; in plants and mammals the dikaryotic phase is basically skipped, as there is instantaneous fusion of nuclei leading to a diploid nucleus.

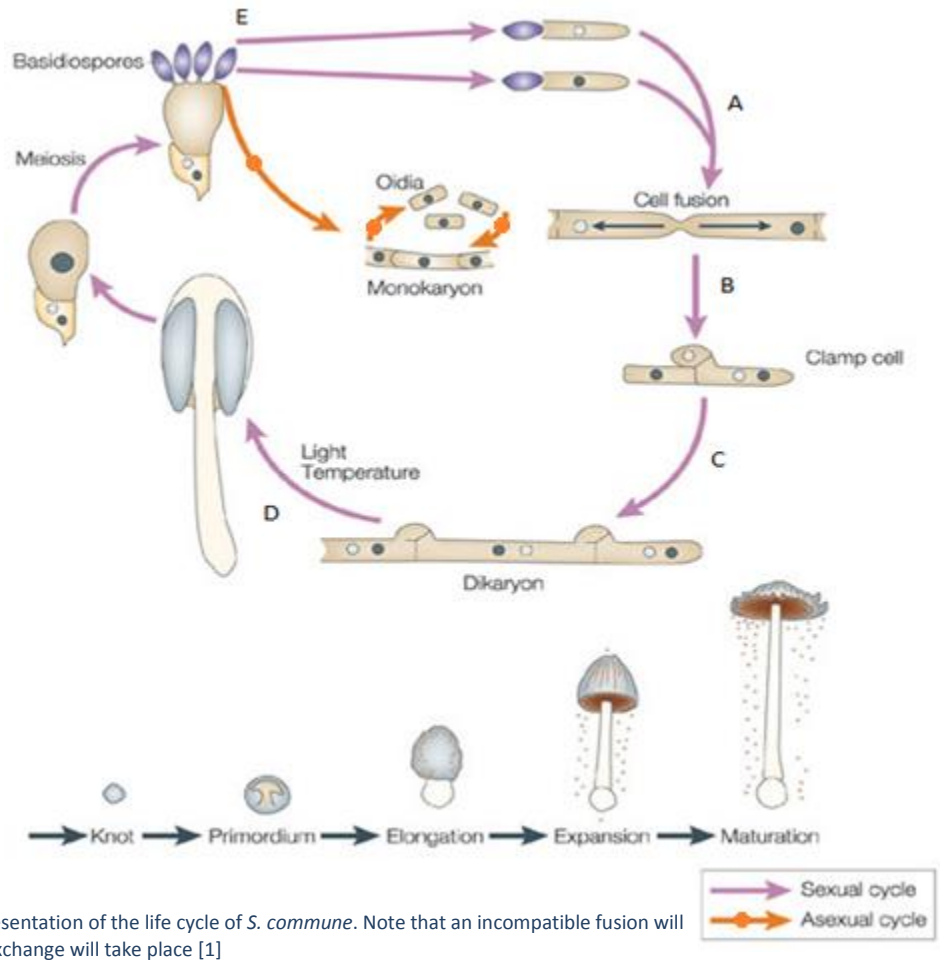

Figure 1. A schematic representation of the life cycle of *S. commune*. Note that an incompatible fusion will stop at step A. No nuclear exchange will take place [1]

Though fusion basically always happens when hyphae make contact, mating can only occur when two individuals of different mating type meet, as formerly mentioned. The fused cell contains two haploid nuclei, and is a collection of all material that was present in the former two cells, including the nuclear and mitochondrial genomes. If mating types are compatible, the nuclei can now divide and subsequently move to the next cell compartment. The invading nuclei will keep dividing and traveling through the mycelium until all cells within the mycelium have been fertilized (Fig 1, sign A). Nuclei travel through the mycelium at a rate of approximately two mm per hour [9] until every cell on the way has a copy of both nuclei [10]. Even a small monokaryon with only a few nuclei can fertilize the entirety of a large monokaryon. Therefore, the process of fertilization is probably performed by a small amount of nuclei which are constantly generated by division [11]. The cell walls supposedly allows only the passage of foreign nuclei, and only once [10]. For this research, it is important to note that only the paternal nucleus is transmitted in this way, and that the cytoplasmic DNA remains behind in the maternal monokaryon [12].

A fertilized mycelium can be visually recognized from an unfertilized monokaryon because cells of a dikaryotic mycelium show clamp connections. Clamp connections are regulated by the genes that also determine monokaryon compatibility. They are structures that enable the dikaryotic cell to constituently form new cells with the right number of copies of each nucleus [13]. We suppose this to be achieved by first growing the clamp, an extrusion of cell wall, somewhere around the middle of the cell. The cell then divides, isolating one nuclear copy is isolated, while the other three remain in the part with the preformed clamp. Next, the two unique nuclei pair, and the unpaired nucleus moves into the newly formed clamp (figure 1, sign B), while the other identical nucleus stays in the isolated cellular part. The clamp with the nucleus in it separates itself from the newly formed cell by forming a new part of cell wall, and subsequently fuses with the new cell (figure 1, sign C), releasing the second nucleus into the cytoplasm. This results in two cells, with identical genetic material. The clamp connection that facilitated the division remains visible.

In a dikaryon, the two nuclei run the homeostasis of the fungus, like one nucleus did before fertilization, or a fused nucleus does for mammal or plant cells. In the dikaryon, the two nuclei also regulate the frucification. When the new spores are created in the basidia (fig 1 sign D-E), there is recombination of genetic material. The nuclei fuse and form a diploid nucleus, which is copied once, and then divides. After the nuclei have divided, the new nuclei divide once more, resulting in four unique nuclei. The four nuclei derived from the combined nuclear material inhabit the spores that are released to find new substrate to colonize [6].

Genomic conflicts in S. commune

A few aspects of the lifecycle of *S. commune* make it an especially nice organism to perform this research in. First of all, several intergenomic conflicts can be observed in *S. commune*:

1. Internuclear interactions (Nuc-Nuc):

   The most obvious conflict in the fused or fertilized cell for these fungi is that between the two nuclei. If the nuclei are compatible, the two have to work together in order to fructify and thus procreate as efficiently as possible. In some areas however, competition will arise even here. An example is the process in which the dikaryon fertilizes an adjacent monokaryon. Here, only one nucleus can be donated, opening a window for competition.
2. Intermitochondrial interactions (Mit-Mit):

   When two cells from separate monokaryons fuse, the new cell has two distinct kinds of mitochondria. This so-called heteroplasmic state, however, seems to be rather unstable. The fused cells allow a unique chance to observe possible mitochondrial competition. By isolating the right cells and growing them we can experiment with the longevity of this heteroplasmic state, and how it is lost.

3. Nucleo-Mitochondrial interactions (Nuc-Mit):

   After fertilization, both the paternal and maternal nucleus are present in one cell, but the cell retains the maternal cytoplasm. After nuclear fusion and division, spores carrying parts of the genetic material develop into a new monokaryon which contain the old maternal cytoplasm with a new unique nucleus. As was mentioned before, nucleo-mitochondrial conflicts are expected to be present in every natural monokaryon, but most of these will probably be hidden by reciprocal countermutations. New combinations of cytoplasm with nuclei may allow these interactions to show.

A Nuc-Nuc interaction can be observed whenever two compatible monokaryons fuse and exchange nuclei. The nuclei can be in conflict for several reasons, most observable of which is which nucleus gets to execute the paternal role if the dikaryon should contact another monokaryon. The Mit-Mit and Mit-Nuc interactions are slightly harder to observe. Mitochondria do not usually come into contact with each other except in fused cells. The effects of mitochondria and nuclei separate from each other are hard to isolate as they are basically part of the same individual. After sporulation, freshly recombined nuclei may bring some of these interactions to the surface, but this is hardly reproducible. Instead, it is possible to swap the entire nucleus of two individuals into each other's cytoplasmic background.

Cytoplasmic swapping

The cytoplasm of a monokaryon can be transferred to another in a process called protoplasting. The cell wall of a dikaryon is enzymatically weakened such that the cytoplasm can leak out [1414]. The cytoplasm will still be contained by a membrane, so instead of just diffusing out, membrane-enveloped parts will bud off. In budding off, it is possible to isolate parts of the dikaryon. The yielded 'protoplasts' will all contain the cytoplasm of the dikaryon, but nuclear contents may vary. Four different kinds of protoplasts can be found and distinguished between in this fashion:

1. A protoplast without nucleus. Contains the maternal cytoplasm without any nuclei. This protoplast will not grow out radially after regeneration, since the nucleus is lacking.
2. A dikaryotic protoplast. Contains the maternal cytoplasm and both the maternal and paternal nuclei. This protoplast can be distinguished from other protoplasts because it will, after regeneration, show clamp connections in its mycelium.
3. A monokaryotic protoplast. Containing the maternal cytoplasm and the maternal nucleus. This protoplast can be distinguished from other protoplasts because it forms no clamp connections by itself, nor when it is crossed with a monokaryon containing the maternal nuclear genotype.
4. A different monokaryotic protoplast. Containing the maternal cytoplasm and the paternal nucleus. This protoplast can be distinguished from other protoplasts because it forms no clamp connections by itself, but it will when it is crossed with a monokaryon containing the maternal nuclear genotype.

In this way, from a basis of seven different monokaryons, forty-nine unique monokaryons can be created. That is each of the seven nuclei in each of the seven cytoplasmic backgrounds. This set of seven naturally occurring and forty-two artificially combined monokaryons grants an opportunity to better observe each of the three previously mentioned intergenomic interactions. We can easily observe hidden Nuc-Mit interactions, simply by allowing the monokaryons to grow or fertilize other monokaryons, noting any oddities. Nuc-Nuc interactions can be observed more cleanly then before, as there is no distortion in the measurements by the cytoplasmic background. Even Mit-Mit interactions can be analysed more cleanly. We can make mycelial crosses in which the two monokaryons have identical nuclei but distinct mitochondria. Now, we can observe the effects of heteroplasmy in fused cells while preventing the formation of a dikaryon.

It should be mentioned that only forty-seven of the forty-nine monokaryons were created. It turns out monokaryon M and F share one mating type. This was not noticed until testing already started. Therefore, no results are found for monokaryon M*f* and F*m* (notation here and henceforth in this article; if monokaryon is coded M*f*, then M = genotype nucleus, *f* = genotype cytoplasm). The monokaryons were created as a preliminary step for this research. Though we have the methods to create the last two monokaryons, we have not yet done so.

Materials and methods

The protocol and raw data for each experiment can be found in the supplements section.

Monokaryons genotypes

The monokaryons used in this research can be divided into two sets. Set 1 consists of a total of forty-seven unique monokaryons, derived from seven natural occurring samples, originating mostly from Europe. Set 2 consists of four natural variants of *S. commune* gathered over several continents. Set 2 was added later in this research to increase the genetic diversity in the available samples.

Set 1: code geographic origin.

A: Netherlands
B: Netherlands
C: Germany
E: Netherlands
F: Netherlands
G: Brazil
M: North Amerika (original sequenced lab-strain H4-8 (descendant from 4-40; Ohm et al 2010. Nature Biotechn; matA43 matB41; FGSC no. 9210))

Set 2: code geographic origin.

K: Cascade (Australia)
P: La Palma (Afrika)
Q: Tattone (Corsica)
V: Irkutsk

Monokaryon fitness test

The first experiment that was conducted was the monokaryon fitness test. This results of this test give information on Nuc-Mit interactions in the original and created monokaryons. The test was very straightforward, put mycelium on an SMM plate and monitor how fit each monokaryon is. The fitness measure chosen for this research was radial outgrowth. With increasing radial outgrowth of a monokaryon, the chance to encounter another monokaryon increases. Therefore, radial outgrowth should be a good measure for general fitness. We expect intergenomic conflicts to show in monokaryons where a nucleus is paired with mitochondria from a geographically far removed monokaryon. This is based on the assumption that these genotypes have long remained separate from each other. Therefore there is a larger chance that not all selfish mitochondrial mutations have been countered by the nuclear genome. This is expected to show as negative epistasis, resulting in a lower phenotype than would be expected by looking at performance of the individual genotypes. Based on the monokaryon fitness test results, we can select samples to be used in other experiments, such as a assay in which gynodioecy or mitochondrial competition may be observed. The hypothesis we want to test in this experiment is: **The cytoplasm evolves selfish mechanisms to promote its own proliferation. During co-evolution, nuclei that counter these mutations will be selected for.**

This experiment is backed up by a verification of cytoplasm. In the process of creating cytoplasmic swaps, the cytoplasmic type of all protoplasts is assumed to maternal type. The nuclear type of protoplasts is checked to make sure the right nucleus has been isolated, but the same is never done for the cytoplasm. Since no verification has ever been done during protoplasting, we cannot say with certainty that each cytoplasmic swap is the one we think it is.

In order to have a greater degree of certainty, all monokaryons were tested for their genotype by a KASPar PCR assay. The mitochondria of each monokaryon are identifiable by genetic code. A selection of SNP's designed to distinguish between all cytoplasmic genotypes was made by Bart Nieuwenhuis in 2013. Table 1 shows the codes which correlate to each cytoplasmic type.

Table 1. Cytoplasm identification set*

	474 (c/g)	6280 (c/t)	15534 (a/c)	30738 (t/c)	43198(t/g)	43418 (c/a)
a	C	T	C	C	C	A
b	C	C	C	C	C	A
c	G	C		C	T	
e	C	T	C	C	C	A
f	C	T	C	C	C	A
g	G	C	C	C	C	A
m	C	C	A	T	T	C

*Set of six SNP's designed to distinguish between as many of the available cytoplasms as possible. Column one correlates with the code of the monokaryon the cytoplasm originates from. Row one first shows the genetic location of the individual SNP's, and follows by listing the different nucleotides the KASPar PCR can recognize. The rest of the cells indicate the result of the KASPar assay with the specified SNP primer for that mitochondrial strain. No indicated result suggests the primer does not fit the DNA for that sample because of mutations either in the DNA the primer codes for, resulting in no amplification and therefore no signal, or because neither of the two recognizable nucleotides were detected, resulting in no signal.

Direct effect of cytoplasmic mixing – approach 1

This experiment focusses on the Mit-Mit interactions. As mentioned before, presence of multiple kinds of mitochondria within one cell may provoke some kind of interaction, or a form of competition. To test whether or not mitochondrial competition has a negative effect on monokaryon fitness, we utilize the fact that monokaryons with the same nucleus can fuse without fertilizing each other. In this way, the particular cells that fused will contain two types of mitochondria

For this assay we want to maximize cellular fusion. In order to do this, we fragment mycelia, and mix the fragmented bits in high concentration. Fragmenting is done with a rod mixer, shredding the mycelium into tiny bits. All bits can start growing again, increasing the amount of fusion that takes place, and therefore increasing the amount of heteroplasmic cells. After fragmentation, the rod mixer had to be cauterized to make sure the next mycelium would not be infected with the formerly fragmented. A small test to monitor the effect of cauterization was performed. In this test, the rod mixer was used to fragment some mycelia, and subsequently dipped into a flask with fresh liquid medium. The medium was incubated for a week, and monitored for mycelial growth. This test showed that only when the mixer was cauterized for four times in a row, no mycelial growth was observed. Three consecutive cauterizations caused mycelial growth in one out of three media. If the mixer was cauterized only once or twice, over half the media the mixer was dipped into became infected. Therefore, the process of fragmentation became a bit tedious.

Next, the fragmented mix is grown on a cellophane layer on solid medium. Cellophane will allow diffusion of nutrients towards the fungi, but will not allow the fungi to grow through the layer. After several days of growth, the cellophane layer with fungi on it is separated from the growth medium, dried and weighed. The increase in weight is a measure for the fitness, as it represents the growth.

If the average growth of the individual monokaryons exceeds the growth of the mixture, this suggests cytoplasmic mixing imposes a toll on the fitness of the individual. Part of the elegance of this experiment is that it can be repeated with up to seven mitochondrial types present. Unfortunately, this boils down to one hundred and three different combinations, all of which would have to be tested several times over, so a subset has been defined for the premises of this research. We have chosen to work with four different cytoplasms within one nuclear background, testing them in single culture (as a blanc), triple cytoplasmic mixture, and the combination of all four cytoplasms. We chose not to try the double cytoplasmic mixture as it would add many samples without giving much new information. If the approach works for double mixture, it should also work for triple, if not work better.

Two nuclear genotypes were chosen for the test, based on their performance in the monokaryon fitness test. Nuclear type A was chosen with cytoplasmic swaps A*b*, A*c*, A*f* and A*g* because these cytoplasms induced large fluctuations of fitness in this nuclear background. Nuclear type M on the other hand, was chosen with cytoplasmic swaps M*a*, M*c*, M*e* and M*g* because of how stable fitness remained with these different cytoplasms. These options should yield the most interesting results. The hypothesis we want to test in this experiment is: **Induced heteroplasmy yields direct deleterious effects for the fitness of an individual.**

Direct effect of cytoplasmic mixing – approach 2

A second approach to gather information on a possible fitness toll imposed upon an individual by occurrence of heteroplasmy. Test 1 for the direct effects of cytoplasmic mixing is rather a shot in the dark. We do not know if conducting the experiment in this fashion will yield any reproducible results. For this reason, we keep test 2 as a back-up. Test 2 has been used before and is very simple in its principle.

In this experiment, we bring two monokaryons on the same nutrient source, a few centimetres distanced from each other. Both monokaryons are allowed to grow radially outwards, until they meet, and the tips of the hyphae fuse. The goal of the experiment is to isolate three distinct cell-types. Two of these types are collected from anywhere in the individual monokaryons. The last type is isolated from the interaction zone, the thin line where hyphae have fused. The cells from the interaction zone must contain cytoplasm from both monokaryons, as the cells have fused in their entirety. If we isolate cells from this interaction zone and bring them onto a new SMM plate, we can suppose the heteroplasmy will remain stable. Speeds of radial growth can be compared between the cells isolated from the interaction zone, and those from the individual monokaryons.

Thanks to the set of cytoplasmic swaps we can test the effects of heteroplasmy very cleanly. If, for example, we fuse monokaryon A*a* with monokaryon A*b*, the cells in the interaction zone will be of type A*ab*. The cytoplasmic swaps allow us to induce fusion without the monokaryons fertilizing each other. Therefore, the observed effects may be assumed to be due to the composition of the cytoplasm. Like approach 1, the hypothesis we want to test here is also: **Induced heteroplasmy yields direct deleterious effects for the fitness of an individual.**

Dikaryon fructification test

The dikaryon fructification assay focusses on the differences in fructification between two halves of the same dikaryon. After fertilization every cell in the dikaryon has an identical collection of nuclear DNA, differences in fructification may occur in this assay on behalf of a few matters.

1. The monokaryon that grew before fertilization is the base level from which the dikaryon-half must fructify. As the monokaryons differ, this may induce differences in fructification. The maternal nucleus shapes the environment in which the paternal nucleus must execute its role. This may reduce the effect the invading nucleus can have on the fructification, leading to differences.

2. The dikaryon-halves in this experiment had different cytoplasms. One cytoplasm may have been more efficient than another, and therefore one half of the dikaryon may have fructified more easily than the other.
3. Half of the dikaryon (let's call it half A) may fructify relatively slowly because its partner-half (half B) is somehow actively suppressing half A's fructification.

If suppression as described in point 3 is a fact, it probably hails from the cytoplasm. There is no evident reason for the nuclear genome of half A to suppress the fructification of half B, since it would basically be slowing down its own procreation. The same is not true for the cytoplasm. If the cytoplasm of half A could, for example, induce leaching of nutrients from half B, this process would promote the spread of cytoplasm A and consequently slow the spread of cytoplasm B, considerably increasing fitness of cytoplasm A. Differences in fructification can therefore be observed between the halves of a dikaryon. In this experiment we have scored for absolute fructification, moment of fructification, absolute sporulation, and have tried to make a rough estimation of the intensity of sporulation by eyeballing the quantity of spores formed. In figure 2a we see the minimal fructification structure that must be observed in order to be scored as fructified. The hypothesis we want to test in this experiment is: **Fructification of both halves of a dikaryon does not always proceed in a parallel fashion, even though the nuclear DNA present is equal.**

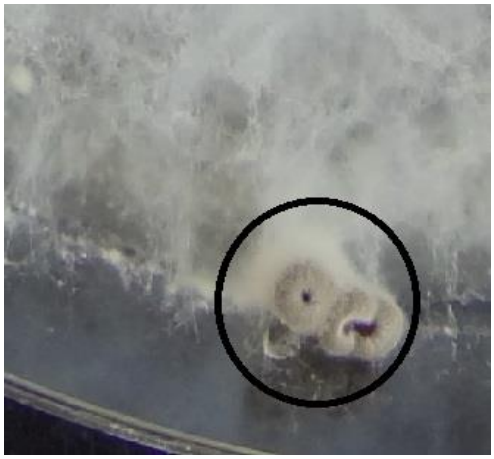

Figure 2a. minimal fructification structure.

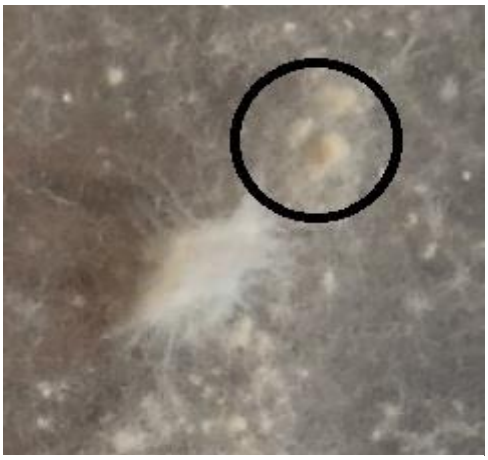

Figure 2b. Minimal fructification not observed.

In **figure 2a**, a fruiting body is shown that has opened up, and is therefore considered fructified. In **figure 2b**, a fruiting body is shown that has not been opened up, and is therefore scored negative. In **figure 2c**, a full-grown mushroom is shown. In **figure 2d**, a mycelium is shown where only the bottom half has fructified. These pictures were taken from the gynodioecy experiment. Large photographs can be found in the supplementary photographs.

Figure 2c. full-grown and sporulating mushroom.

Figure 2d. half fructified dikaryon.

A threshold had to be set for fructification to be scored as positive, as some mycelia seemed to be about to fructify for long periods without really doing so. These samples were scored negative until the observed fruiting bodies eventually opened up, as described. Figure 2b shows a structure that has not opened up, and is therefore not scored as fructified.

To increase the chance to find interesting differences in fructification pattern, four extra monokaryons were added to the collection. With increased genetic divergence, the chance to find differences in fructification-pattern between the halves of a dikaryon should increase. These monokaryons have been gathered from all over the world. For this experiment we have chosen monokaryons from every continent available to cross with each other.

Cytoplasm induced gynodioecy

This test was aimed to visualise hidden interactions between nuclear and mitochondrial DNA. In this test, we crossed compatible monokaryons and monitored all crosses to see if any couples only fructify in one half of the dikaryon. Each cross consisted of a cytoplasmic swap of monokaryon B, crossed with a monokaryon from another continent.

Normally hidden interaction could be expected to show because the nuclei in the monokaryons have not been able to co-evolve with the newly introduced mitochondria. Other interactions between the mitochondria and the nucleus that are usually hidden may also come to the surface. Results of this assay were scored in the form of absolute fructification. The hypothesis we want to test in this experiment is: **By crossing cytoplasmic swaps with other monokaryons, normally hidden intergenomic conflicts like gynodioecy can be consistently observed.**

Results

Monokaryon fitness test

Several parameters were monitored in the Monokaryon fitness test, all relevant of which can be found in Appendix A. The data has been condensed to only contain the measured radial outgrowth. This data was corrected by a zero measurement, radial outgrowth two days after inoculation. We chose to use data gathered two days after the zero-measurement even though data gathered on day three is available, as several mycelia were limited in their growth by the edge of the plate at that point, distorting the data. To highlight the relative fitness of each sample and the robustness of different nuclear types, a colour scale has been applied to the table.

Table 2. Radial outgrowth results monokaryon fitness assay*

	A	B	C	E	F	G	M
a	1.32 ± 0.11	~~1.21 ± 0.13~~	1.03 ± 0.23	0.89 ± 0.20	1.60 ± 0.04	1.54 ± 0.04	1.79 ± 0.14
~~***b***~~	~~0.72 ± 0.07~~	~~1.64 ± 0.20~~	1.23 ± 0.04	2.13 ± 0.10	1.48 ± 0.09	1.60 ± 0.05	1.69 ± 0.06
c	1.37 ± 0.05	1.40 ± 0.13	1.26 ± 0.02	1.20 ± 0.13	~~1.47 ± 0.05~~	1.62 ± 0.10	1.59 ± 0.05#
e	1.29 ± 0.13	1.93 ± 0.07	1.29 ± 0.04	1.68 ± 0.12	1.50 ± 0.14	1.46 ± 0.08	1.70 ± 0.23
f	2.13 ± 0.19	1.73 ± 0.06	~~1.29 ± 0.10~~	1.56 ± 0.15	1.52 ± 0.08	1.54 ± 0.05	
g	1.55 ± 0.12	1.66 ± 0.17	1.23 ± 0.03	1.67 ± 0.14	1.50 ± 0.02	1.66 ± 0.19	1.57 ± 0.07#
m	1.64 ± 0.02	1.33 ± 0.09	1.31 ± 0.09	1.64 ± 0.14		1.57 ± 0.00	1.94 ± 0.10

* Column one represents the cytoplasm each monokaryon contains, while row one shows the present type of nucleus. The values in this table indicate the maximal radial outgrowth of the measured sample in centimetres, three days after the zero measurement was taken. For easier interpretation, the table contains a colour scale, ranging from red to green with increased growth. Each value is the average of three replicates, the standard error of which is indicated. All values marked with a # were obtained from monokaryons that showed odd results in the KASPar PCR, but not sufficiently so to show they had the wrong cytoplasm. Struck-through values indicate samples tested, but whose cytoplasmic identity was proven wrong with the KASPar PCR's. A black cell means the monokaryon was not tested.

Data from Table 2 was derived from the tested monokaryons, but since the monokaryons were created we never verified the genotype of their cytoplasm. The complete set of results from the several KASPar PCRs that were run can be found in Appendix B. The first KASPar PCR was run with a minimal amount of SNP's sufficient to identify all cytoplasms. Unfortunately, according to the test not all samples contained the cytoplasm they were expected to contain. These samples were submitted to a second, more thorough test with six SNP's, to verify they were really not what they should be. Table 3 shows these samples and the results of both assays.

Table 3. results of the 2nd KASPar PCR*

Sample	Code LITERATURE	Code FOUND 1st run	Code FOUND 2nd run	Cytoplasm	Code stock FOUND
B***a***	CTCCCA	CC//C/	CCCCCA	*b*	?CC?C?
A***b***	CCCCCA	?T//C/	?TCCCA	*a*/*e*/*f*	CCCCCA
B***b***	CCCCCA	GC//C/	GCCCCA	*G*	CCCCCA
F***c***	GC?CT?	?T//C/	CTCCCA	*a*/*e*/*f*	CTCCCA
C***f***	CTCCCA	GC//?/	GCCCTA	*c*	GCCCTA
M***g***	GCCCCA	GC//(C/T)/	GCCC?A	*g*	GCCCCA
M***c***	GC?CT?	GC//C/	GC?CTA	*c*	GCCCTA

* Column 'Sample' shows the nuclear type is present, and what cytoplasm the monokaryon should contain. Column 'Code LITERATURE' shows the code that should be found in the KASPar PCR if the cytoplasm is correct. Columns 'Code FOUND 1st/2nd run' show the result of the KASPar PCR in both runs. Column 'Cytoplasm' shows what cytoplasm the KASPar assays show the monokaryon contains. Column 'Code stock FOUND' shows the result of a final KASP on an old stock. A '?' indicates the KASPar assay gave no clear result for the SNP. A '/' indicates the SNP was not tested for in that run. The symbols correspond with SNP's 474, 6280, 15534, 30738, 43198, 43418.

From table 3 we can deduce that samples B*a*, A*b*, B*b*, F*c* and C*f* have wrongfully assigned cytoplasms. No real wrong values were found for samples M*c* and M*g*, so these have not been marked as wrong in the end. The samples with incorrect cytoplasms have not been taken into account for the statistical analysis.

A statistical test was done in order to see whether or not the combination of nucleus and cytoplasm really mattered for the fitness. For the statistical analysis, the data gathered on day 3 (represented in Table 2) after the zero had been indicated was chosen. Results are shown in table 4.

Table 4. Statistal analysis of the monokaryon growth test

Tests of Between-Subjects Effects					
Dependent Variable:	MaxDay5				
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13,883[a]	41	.339	12.842	.000
Intercept	600.473	1	600.473	22773.259	.000
ContentNuc	4.098	6	.683	25.900	.000
ContentMit	2.435	6	.406	15.392	.000
ContentNuc * ContentMit	7.074	29	.244	9.251	.000
Error	2.215	84	.026		
Total	626.994	126			
Corrected Total	16.098	125			
a. R Squared = ,862 (Adjusted R Squared = ,795)					

Direct effect of cytoplasmic mixing – approach 1

Figure 3 shows the final results of the first approach to measure the effect of cytoplasmic mixing. The important data of this test have been condensed into one figure, showing the total dry weight of the combined cellophane and mycelium. In the test we scored several more parameters, the relevant of which can be found in Appendix C. Two nuclear types were submitted to a cytoplasmic mixing assay. Nuclear type A and nuclear type M, indicated as blue resp. red in figure 3. Of both nuclear types, four samples with different cytoplasms were chosen to be tested in this assay. Not all combinations of these four cytoplasms were tested. Instead of choosing every combination, we only report on the ones with a single, three and four combined cytoplasms. The single cultures have been tested with four replicates, the multiple cultures have been tested with five. Some of the cultures were contaminated and could therefore not be tested, so here and there a replicate is missing.

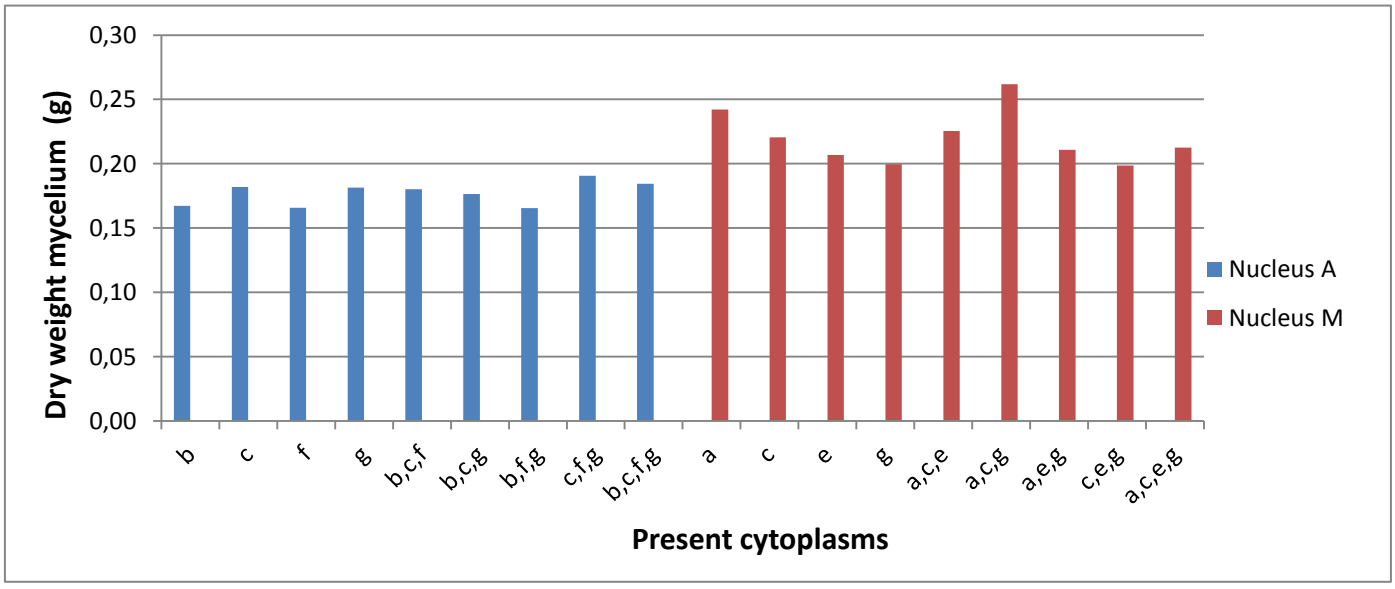

Figure 1. Weight of the mixed fragmented colonies after growth and subsequent drying.

Direct effect of cytoplasmic mixing – approach 2

Table 5 represents the radial outgrowth of all samples that showed growth in this test. Samples were taken from one Petri-dish on which monokaryon Ab was combined with monokaryon A*f*. Samples 'A*b*' and 'A*f*' were taken from their respective colonies, whereas samples 'Barrage' were taken from the interaction zone. Though twenty-two samples were taken, only twelve turned out to show growth. Because the nuclear genotypes were the same, these monokaryons did not fuse to form a dikaryon, so each mycelial sample grew out as a monokaryon, heteroplasmic or homoplasmic. Five days passed before the monokaryons grew large enough for a zero measurement. The measurements were done 2, 3 and 4 days after the zero was marked.

Table 5. Results of the second cytoplasmic mixing experiment*

Content	Measurement one	Measurement two	Measurement three
A*b*	0.86	1.18	1.48
A*b*	0.64	1.15	1.37
A*b*	0.5	0.69	0.98
A*f*	1.12	1.64	2.21
A*f*	1.08	1.65	2.31
A*f*	1.09	1.78	Limited by dish
A*f*	1.02	1.6	2.2
Barrage	1.4	2.33	Limited by dish
Barrage	0.62	0.95	1.52
Barrage	0.68	1.1	1.44
Barrage	0.83	1.37	1.62
Barrage	0.84	1.1	1.55

* Values indicate the radial cumulative outgrowth measured each day, from the indicated zero, in centimetres. Zero score was done on day five. The outgrowth data was collected with this zero as a basis, scored over the next three days.

For the statistical analysis, measurement 2 was chosen, as this represents the largest radial outgrowth where all samples could still properly be measured. The analysis, which does not confirm that the evidence for direct negative effects of heteroplasmy is statistically relevant (Significance = 0.138), is found directly below.

Table 6. Statistical analysis of data represented in table 5

Tests of Between-Subjects Effects					
Dependent Variable: Measurement2					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,750a	2	,375	2,488	**,138**
Intercept	20,817	1	20,817	138,161	,000
Content	,750	2	,375	2,488	**,138**
Error	1,356	9	,151		
Total	24,821	12			
Corrected Total	2,106	11			
a. R Squared = ,356 (Adjusted R Squared = ,213)					

Dikaryon fructification test

In this test, the samples were monitored strictly every day for twelve days, excepting day eight and nine. The data gathered through this experiment can be found in appendix E. The results show significant differences in moment of fructification, and observed pattern between the halves of many dikaryons. Some interesting observations are stated below and images are shown in figure 4.

- Halves showed differences in the formation of the mushrooms.
    - Phenotype of the mushrooms formed was always very similar.
    - Different localization of mushrooms (grown from the edge or from the mycelium
- Some halves continued growth after fertilization while the other did not.
- One half could not fructify at all, no minimal fructification structures observed.

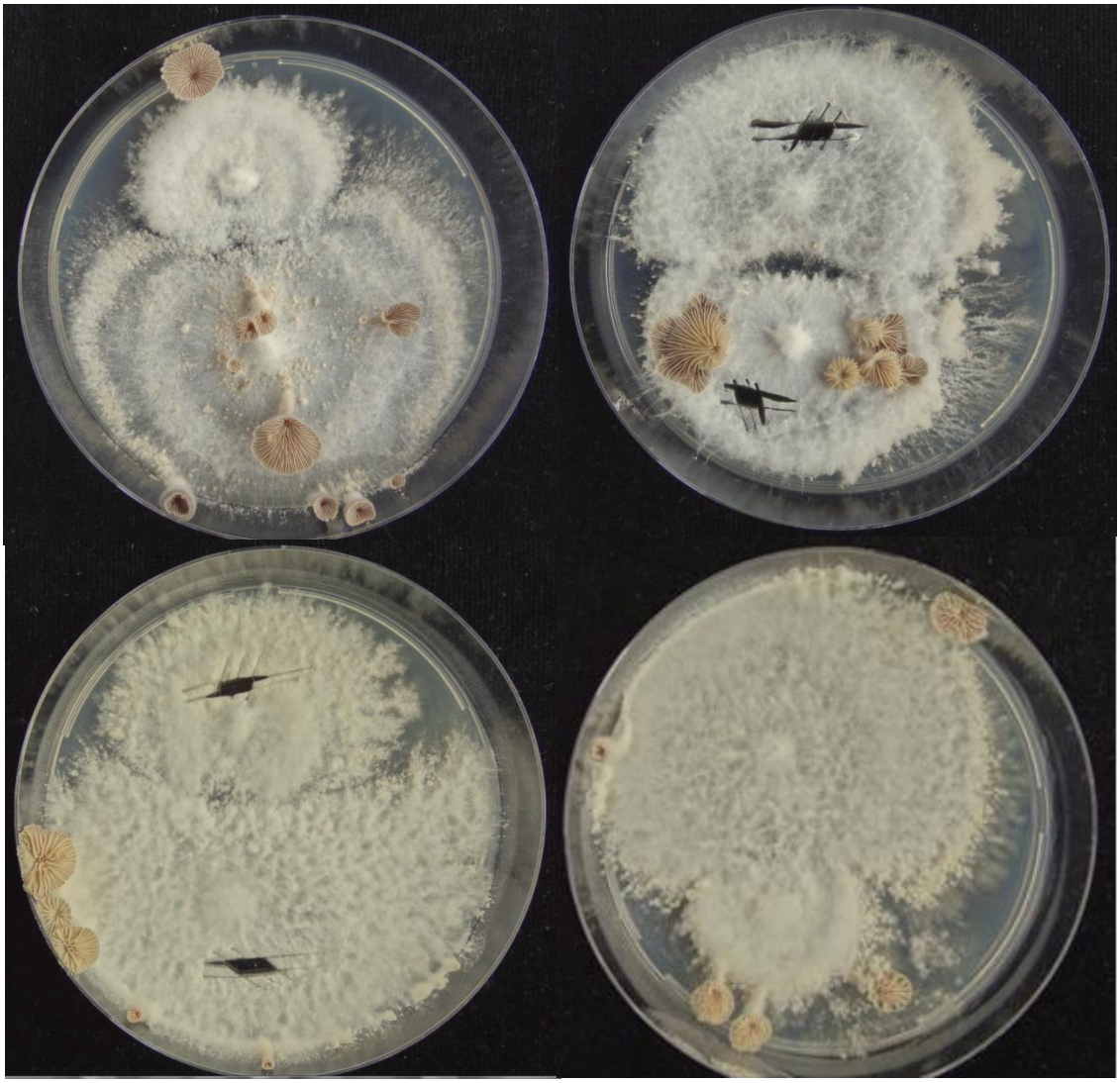

Figure 4. Compilation of the inequalities found amongst the halves of the formed dikaryons in this test. **Top left: Cross PQ1**, shows how the halves of a dikaryon can have mushrooms grow in completely different places. **Top right: cross BQ2**, shows how one half (the bottom half) of the mycelium may continue growing while the mycelium is exposed to light, while this not observed for the other half. **Bottom left: Cross PK2**, shows how one half of the dikaryon may not fructify while the other half does. **Bottom right: Cross BP3**, both sides have fructified, show similar mushroom localization, and both continue growing while exposed to light

Figure 5a shows a dikaryon, the mycelium of which originally grew from monokaryon P, and was crossed with monokaryon M*m*. Figure 5b shows the complete dikaryon. New plates were inoculated with small pieces of mycelium from both halves of the mycelium to see whether the differences in fructification pattern observed in figure 5a and 5b were stable. of mycelium from both halves of the dikaryon. Figure 5c and 5d show the fructification pattern of these new inoculations.

Figure 5a. Fructification pattern Part P dikaryon MP.

Figure 5c. Fructification pattern dikaryon MP, grown from mycelium of part M.

Figure 5a shows the fructification pattern of monokaryon P crossed with monokaryon Mm. The complete dikaryon can be seen in **figure 5b** (FRUCT cross MP3)**.** Some mycelium was cut from the dikaryon for a new inoculation. The fructification pattern that was observed from the mycelium that grew out of these new inoculations can be seen in **figure 5c** (taken from part P) and **figure 5d** (taken from part M). Large complete pictures can be found in appendix G.

Figure 5b. Clear difference between two halves of the same dikaryon.

Figure 5d. Fructification pattern dikaryon MP, grown from mycelium of part P.

On close examination, we can see that in figure 5b the top half of the dikaryon was showing signs of fructification. Figure 6a-d shows a similar example, but through all twelve days of monitoring, dikaryon crosses MQ showed not a single sign of fructification.

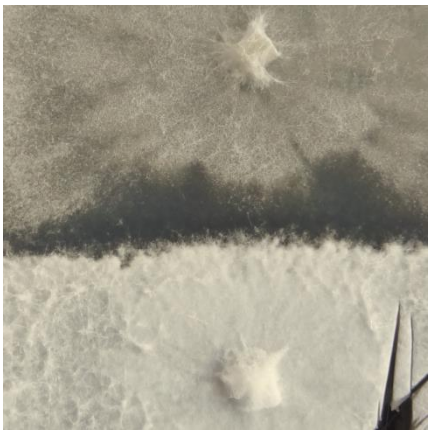

Figure 6a. No fructification observed in dikaryon MQ.

Figure 6c. Fructification pattern dikaryon MQ, grown from mycelium of part M.

Figure 6a shows the lack of fructification pattern in both halves of dikaryon MQ. The complete dikaryon can be seen in **figure 6b** (FRUCT cross MQ1). Some mycelium was cut from the dikaryon for a new inoculation. The fructification pattern that was observed from the mycelium that grew out of these new inoculations can be seen **in figure 6c** (taken from part Q) and **figure 6d** (taken from part M). Large complete pictures can be found in appendix G.

Figure 6b. Picture of complete mycelium MQ.

Figure 6c. Fructification pattern dikaryon MQ, grown from mycelium of part Q.

Suppressed fructification of one half of the mycelium (or the entire mycelium for cross MQ) was extremely reproducible through the assay. However, after the suppressed parts were inoculated on a new plate, all dikaryons showed formation of fruiting bodies, and sporulation.

Cytoplasm induced gynodioecy test

After almost three weeks of growth in the 27°C light growth chamber, final scoring for the cytoplasm induced gynodioecy was done. Scoring was done only for absolute fructification, as previously described, this means looking for the minimal fruiting body structure indicated in figure 2a. If fructification occurred, this is indicated with Yes, if it did not this is indicated with No, as shown in Table 7.

Table 7. Absolute fructification results for the cytoplasm induced gynodioecy experiment*

Cross	Fruct B part	Fruct other part
B*a* G 1	**No**	Yes
B*a* G 2	**No**	Yes
B*b* G 1	**No**	Yes
B*b* G 2	Yes	Yes
B*c* G 1	Yes	Yes
B*c* G 2	**No**	Yes
B*e* G 1	Yes	Yes
B*e* G 2	Yes	Yes
B*a* M 1	Yes	**No**
B*a* M 2	Yes	**No**
B*b* M 1	Yes	Yes
B*b* M 2	X	X
B*c* M 1	**No**	Yes
B*c* M 2	**No**	**No**
B*e* M 1	Yes	Yes
B*e* M 2	Yes	Yes
B*a* K 1	Yes	Yes
B*a* K 2	Yes	**No**
B*b* K 1	Yes	**No**
B*b* K 2	Yes	**No**
B*c* K 1	Yes	Yes
B*c* K 2	Yes	Yes
B*e* K 1	Yes	**No**
B*e* K 2	Yes	Yes

Cross	Fruct B part	Fruct other part
B*a* V 1	Yes	Yes
B*a* V 2	Yes	Yes
B*b* V 1	Yes	**No**
B*b* V 2	**No**	Yes
B*c* V 1	Yes	**No**
B*c* V 2	Yes	Yes
B*e* V 1	**No**	Yes
B*e* V 2	**No**	Yes
B*a* Q 1	**No**	Yes
B*a* Q 2	Yes	**No**
B*b* Q 1	**No**	Yes
B*b* Q 2	**No**	Yes
B*c* Q 1	**No**	Yes
B*c* Q 2	**No**	Yes
B*e* Q 1	Yes	Yes
B*e* Q 2	**No**	Yes
B*a* P 1	Yes	Yes
B*a* P2	Yes	Yes
B*b* P 1	Yes	Yes
B*b* P 2	Yes	Yes
B*c* P 1	Yes	**No**
B*c* P 2	**No**	Yes
B*e* P 1	Yes	Yes
B*e* P 2	**No**	Yes

* The genotypes crossed are shown in the column 'Cross'. The content in this column consist of a two-symbol code which stands for nucleus B with its specific cytoplasmic content, followed by a single symbol which represents the other monokaryon genotype of the cross. The 1 or 2 indicates the replicate, 1 or 2. The other columns list whether or not fructification was observed at all for each half of the dikaryon with yes or **no**. An X in a cell means the sample was not measured.

Plates B*a* V 1 and B*b* M 2 had infections early on. These infections were probably of *Aspergillus nidulans*, so they had to be removed quick, before sporulation could occur. Removal was done by cutting away the SMM with the infection, wide around the contaminated area. Unfortunately, plate B*b* M 1 had to be discarded later due to too heavy infection. Infection did not return on plate B*a* V 1. The results show very little consistency. Though many plates showed inhibition of growth on one side of the dikaryon, relatively few of these were seen on both replicates. Suppression of the cytoplasmic swap side was observed slightly more often than of the other side (17 to 11 times). This means that our hypothesis is definitely not confirmed here.

Discussion

Here, special mention will be made of everything out of the ordinary in the tests, and the test results will be interpreted.

Monokaryon fitness test

While monitoring the radial outgrowth, a difference in outgrowth appearance was noted. One of three types of outgrowth could be assigned to the plates. Type 1: a hair-like translucent phase. Type 2: white fuzzy mycelium with an additional phase appearing like an opaque disc. Type 3: white fuzzy mycelium followed by a phase appearing like an opaque disc, and on the very outer edge another translucent disc-like phase. Though we have not been able to distinguish between the occurrence of type 2 and type 3 outgrowth, over the course of the project we have started to associate type 1 outgrowth with a nutrient source that lacks thiamine. However, in the entire set, only four samples showed this type of mycelium, rendering the bad medium option unlikely. The used set of 200 Petri-dishes was poured from four bottles, each yielding approximately fifty plates, and used at random. It is possible however, as only 141 plates were used.

A statistical analysis of the results shows that both the cytoplasmic and the nuclear composition of the mycelium had a significant effect on the speed of radial outgrowth ($P > 0.001$). Moreover, the analysis shows that the interaction between cytoplasmic and the nuclear composition was also highly significant ($P < 0.001$). This suggests that the mitochondria and the nucleus can be adapted to each other to a certain degree. This may be just a matter of metabolic fine-tuning, or we could be measuring actual Nuc-Mit interactions. In this analysis, the data gathered from samples in which the cytoplasm later turned out to be different than expected was not considered.

The data in table 2 suggest some interesting notions. For instance, nuclei C, F and G seem to have a greater buffering capacity against the changes that mitochondria induce than others, meaning the change in cytoplasm influences the phenotype to a lesser degree here. This may mean these nuclei have a wide spectrum of cytoplasm-induced interactions countered. Also, though we assume the original nucleus in a monokaryon to be well adapted to its cytoplasm, some cytoplasmic swaps show significantly higher fitness than the original cytoplasm did in its nuclear backgrounds. This is observed in monokaryons A and E (it is also observed in monokaryon B, but the original B*b* was not included in this test, according to the KASP assay).

Another point is that even though monokaryons G and M have been collected from locations geographically far removed from genotypes A, B, C, E and F, their mitochondria seem to perform just fine in basically any nuclear background. Additionally, it is worth noticing that the fitness of monokaryon Mm far exceeds the cytoplasmic swaps into nuclear background M. This may suggest that the interactions cytoplasm m will have in a nuclear background are countered by the other nuclear genomes, whereas nucleus M does not counter a common interaction cytoplasms *a*, *b*, *c*, *e*, *f*, and *g* induce.

The DNA verification experiment was planned as a backing-up of the monokaryon fitness test, designed only to show all monokaryons carried the correct cytoplasm. It is to our good fortune that we did check this, as several monokaryons turned out to be miss-identified. For future research it might be beneficial to perform such a confirmation test beforehand instead of after, preventing an incomplete set as published here.

In the KASPar PCR, we could not tell cytoplasmic genotypes A, E and F apart with the SNP's used. This, and the very narrow distribution of the locations where the samples were collected, could have indicated these cytoplasms were more or less identical. The results of the monokaryon growth test argue against this assumption. One of the cytoplasms often causes drops or elevations of fitness where the others maintain an average phenotype. Cytoplasm *a* shows a large drop in phenotype in nuclear background F. For cytoplasm *e*, a peak in fitness is observed in nuclear background B. Cytoplasm *f* shows a great elevation in fitness in nuclear

background A. Now that we know this, it remains relevant to find out whether the cytoplasms assigned *a*, *e* or *f* by the KASP assay are really *a*, *e* or *f*.

Direct effect of cytoplasmic mixing – approach 1

The results of this approach to find direct effects of cytoplasmic mixing are relatively random. The values used in the graph are taken as an average of three, four or five replicates. The single cultures were tested with four replicates, the mixtures with five, though for some mixtures one replicate may have been lost due to contamination. The dry weight of each individual sample can be found in appendix III. In the data listed, severe fluctuations are observed. Most tested genotypes, even the single ones, seem somehow to have two levels of phenotype. One yields a high, and one yields a low dry weight. What caused this two-level system is yet unclear.

The assay was performed with a large number of replicates, so many replicates were chosen because the assay contains many contamination-sensitive steps. In the cauterization test performed, we assessed the number of times the rod mixer should be cauterized after having fragmented a mycelium, before it could be used again. Even if the mixer was cauterized thoroughly for three times, one in three flasks containing liquid medium it was dipped in showed mycelial growth after three days. Therefore, every time a flask would be opened to be handled there was a normal chance of infection, and an additional chance because the very act of progressing flask was a source of infection. Now, in a cytoplasmic mixing experiment it is not absolutely horrible if some little, mycelium from one genotype is put into another bottle. In fact, because of the cauterization done, the mycelium probably needs to regenerate somewhat, further minimalizing the effect. However, if the contaminating mycelium happened to be of another nuclear type than the mycelium in the bottle (which is possible, as two nuclear types were tested simultaneously), the monokaryon might become a dikaryon. A dikaryon grows faster. Perhaps this explains why many of the mixes showed two levels of phenotype. One could be a monokaryon, one a dikaryon. Unfortunately, the mixtures were not observed for formation of clamps, so we cannot report on this theory.

Altogether, approach 1 of the cytoplasmic mixing analysis took a lot of preparation, was a lot of work and gave rather random results. Additionally, another approach for the cytoplasmic mixing experiment is available. therefore, approach 1 was not further pursued.

Direct effect of cytoplasmic mixing – approach 2

This test shows that mycelial inoculation onto a new plate with a sterile toothpick can work. 12 of 22 plates showed growth, while inoculation was very tentatively done. Inoculating by toothpick has a great chance of giving the best results, as the very small surface of the pick increases the chance of picking up only the heteroplasmic cells. Unfortunately we probably picked up a few cells from mycelium A*f* though, when we were aiming for the interaction zone. This can be clearly seen from the first row with barrage content in table 5. The growth here far exceeds any of the other mycelia grown from the interaction zone and, oddly enough, has also grown much larger than the average sample picked up from monokaryon A*f*. But alas, there it is, and we must assume it comes from the interaction zone as it comes from this group. Statistical analysis of the data now shows that there is no significant effect on growth when cytoplasmic mixing occurs.

This weakness of the test may be partly overcome by isolating complete DNA from the grown mycelia and running a KASPar PCR on it. The KASP assay will give an insight in the present cytoplasm(s). In fact, even if the results of this test had been more favourable to our hypothesis, this verification should probably have been done. Unfortunately, at the time the DNA isolation and KASPar PCR were giving a lot of trouble, and therefore the extra effort of verifying these cytoplasms was not taken.

This insight, however, sets the stage for a problem. It is hard to, in a convenient manner, prove whether or not inoculation from the interaction zone has been done accurately. The homoplasmic part can be picked, grown,

isolated and identified easily, but what do we expect to find from the heteroplasmic part? If we were to pick up cells here and grow them, isolate their DNA and find heteroplasmy, this only tells us that both cytoplasms are present. What this does not tell us, is that no part of the isolated mycelium is homoplasmic, though this could be true if picking is done inaccurately. Our (unverified) hypothesis still states that homoplasmic mycelium grows more rapidly, so growth results of a combination of heteroplasmic and homoplasmic outgrowth would only give information on the aspects of the homoplasmic mycelium, as this would grow out fastest and be measured. If DNA is isolated and cytoplasms are identified from both the inner mycelium and the outer edge of the mycelium, heteroplasmy may be verified more accurately. However, even this way is not waterproof, as it is possible that previously heteroplasmic mycelium on the outer edge has lost its heteroplasmy. This situation may be indicated by sectored growth, where heteroplasmy is lost.

If heteroplasmy is lost, that is a valuable result in itself, but the observation in itself is hard to distinguish from inaccurate cell-picking. These two situations are hard to distinguish between, either inaccurate picking or loss of heteroplasmy can cause a pattern in which the middle of the mycelium is heteroplasmic and the outside is not. A way should be found to be utterly sure the cells that are plated out are heteroplasmic. Perhaps this calls for a combination of approach 1 and approach 2 mentioned in this research. In this combined approach, heteroplasmy should be ensured by fragmentation and mixing of mycelia, before the mycelium is brought onto a plate to grow out radially. The growth rate could then be measured relative to mycelium that has undergone the same treatment, but is not mixed and therefore homoplasmic.

The best result to hope for is a DNA identification from the edge of a mycelium to turn out heteroplasmic after radial outgrowth measurements are taken. If this result is found, there is no reason to doubt, or further verify the data.

Dikaryon fructification test

The fructification test gave some interesting results. Huge differences were found in the manner and moment of fructification between the halves of some dikaryons. Though the nuclear DNA is identical after fertilization, there are still differences within the dikaryon which may explain the discrepancy in fructification. These differences can be ascribed to three factors:

1. The cytoplasm present in both halves of the mycelium contain different mtDNA.
2. The mycelium which is to fructify is grown as a monokaryon, setting a base from which fructification must occur. One monokaryon may facilitate fructification more than another. This is a non-inheritable form of maternal imprinting.
3. The paternal nucleus comes into an environment created by the maternal nucleus. This means the effect the paternal nucleus can have on the fructification may be limited. This is a heritable form of maternal imprinting.

These inequalities may explain the observed differences in fructification that were observed in the test. Of course, the observed differences may be due to the factors in the mycelium (present cytoplasm, maternal nucleus and paternal nucleus), but the adjacent mycelium should not be overlooked. Some fructification patterns suggest a sort of active suppression by the physically adjacent mycelium. After the dikaryons grew, and many fructified, small parts of many of the (partly) unfructified dikaryons were put on a new plate to see if they would fructify there. The dikaryons would now form mycelium as a dikaryon (dikaryon-grown—dikaryons) as opposed to the way they grew in the test initially, where the mycelium was formed as monokaryon (monokaryon-grown-dikaryon). Most of the mycelium in the first test remained monokaryon-grown, as many mycelia halted radial outgrowth when they were put in the 27°C light stove. If no growth would be observed again, It would be arguable that the problem might be internal, and not induced by the adjacent mycelium.

After all mycelia were put on a new plate and had grown for a while, they were put in the 27°C light stove and were given another chance to fructify. The fructification patterns observed from these dikaryon-grown-dikaryons were similar over the two halves, as observed in figure 5c & 5d, and 6c & 6d. The halves of the dikaryon shown in figure 5 now show very similar patterns, though they looked and behaved completely different when they fructified as monokaryon-grown-dikaryons. The monokaryon-grown-dikaryon in figure 6 did not even show the slightest sign of fructification, but sporulated readily when grown isolated.

The results of figure 5 suggest that the way the mycelium forms (dikaryon or monokaryon-grown) is of great importance for the fructification. Figure 6 suggests that active suppression of the other half of the dikaryon may also be involved. In other crosses both monokaryon-grown-dikaryons that started out as monokaryon M or monokaryon Q fructified readily, but when they were paired with each other neither do so. When the mycelium is grown isolated however, fructification simply occurs. This may be due to the dikaryon previously being monokaryon-grown, but it is also probable that these two genotypes simply suppress each other's growth. As mentioned before, such an interaction would probably be favourable only to the cytoplasm, as neither nucleus gains fitness by suppressing the procreation of the other half of the dikaryon. Whether the cytoplasm causes this interaction or not, at the level of dikaryon-grown-dikaryons, no difference induced by the cytoplasm has been observed yet.

Cytoplasm induced gynodioecy test

If indeed the dikaryon fructification test shows us that the cytoplasm can suppress the fructification of one part of the mycelium, this should probably have been found in this test as well. As mentioned before several times, there is no apparent fitness advantage for the nucleus in suppressing the fructification of the other half of the dikaryon, and therefore we assume there should probably be selection for a nuclear type with a counter-mutation to such a mitochondrial function. The counter-mutation, if it exists, would only be found in nuclei that have co-evolved with a cytoplasm that exhibits such an effect, therefore, if the effect is not there, the counter will not be there. Because of this, the cytoplasmic swap is the best way to bring such a mitochondrial interaction to the surface, as the nucleus will now be pooled together with a cytoplasm it has probably not co-evolved with. The dikaryon fructification test further suggests that if the suppression really is a thing, it is dependent on both halves of the dikaryon, otherwise it would have been consistently found in pairings with the suppressing monokaryon, which was not the case anywhere. Considering the option that it is dependent on both halves of the dikaryon, it is important to try to get a great genetic variance in the test, so that the cytoplasmic swaps have the largest chance to have their effect.

The results, however, show little consistency in this test. If anything, the monokaryons-grown-dikaryons formed by the cytoplasmic swaps are suppressed much more often than those formed by the partner (17 dikaryon-halves of the cytoplasmic swaps were suppressed, while the partner was suppressed only 11 times). Monokaryon-grown-dikaryon swap B*a* did slightly more suppressing than the other swaps, while B*b* was slightly more often than the other swaps, but none of that was significant. One consistent and interesting observation is that monokaryon-grown-dikaryon genotype G was suppressed 0 times, in both fructification tests.

It is unfortunate that this test was already begun before the results of the KASP assay were interpreted. Had this been done earlier, the swaps of monokaryon B would not have been chosen. As it is now, we do not know the cytoplasm of 2 of the 4 monokaryons used. Then again, though this is not optimal for the analysis, it does not matter too much for the test. What is required here is cytoplasms with nuclei that have not co-evolved, which we still probably have.

Conclusion

So back to the hypothesis: **Uniparental transmission evolved by preventing direct negative effects of cytoplasmic mixing, and has thereby given chance to indirect intergenomic conflicts to appear.**

Well, unfortunately we cannot report significant evidence that mixing of cytoplasms is deleterious to monokaryon fitness. However, we have been able to verify that the genotype of the cytoplasm matters for the fitness of the monokaryon, and moreover, we have proof that interactions between the cytoplasm and the nucleus also have a significant effect on monokaryon fitness. It is possible that these interactions between the mitochondria and the nucleus have evolved in a genomic to- and fro between the cytoplasm and the nucleus, and are normally hidden by nuclear counter-mutations. This interplay between the present genomes within one cell may still have been selected for by the cytoplasm to better transmit itself, a discrepancy caused by uniparental transmission.

What's more, the dikaryon fitness test shows that there are very interesting interactions to be seen when monokaryons of large genetic divergence are crossed. These interactions may partly be caused by the cytoplasm of the tested monokaryons. Much information could be gained by for instance testing the cross that resulted in fructification for neither side of the dikaryon again with cytoplasmic swaps. This may answer the question whether the observed effects are caused by the cytoplasm, maternal imprinting or perhaps simply by circumstance. The cytoplasm induced gynodioecy test gave very random results. It will be interesting to see if consistency in fructification can be found in a larger crossing scheme including cytoplasmic swaps.

Future research

To further increase knowledge on genomic conflicts and the origin of uniparental inheritance we suggest the following steps to be taken.

- Verify whether all dikaryon-halves in the cytoplasm induced gynodioecy test will fructify if grown, and allowed a chance for fructification on a new plate in isolation.
- To make the cytoplasmic swaps for the new set of globally collected monokaryons, and test them in a growth analysis. Will the same effects be observed that were seen in this research?
- Can the cytoplasms of the monokaryons of set 2 be identified with the same set of SNP's used for identification of the cytoplasms of set 1?
- A follow-up study should be done for the test for direct effects of cytoplasmic mixing on monokaryon fitness. A way should be found not only to test for the effects of mixing, but also to verify the state of the cytoplasm of the resulting monokaryons. We suggest a very precise test with sterile toothpicks, in which cells are taken from the interaction zone of two monokaryons, and inoculated on a nutrient source with a layer of cellophane (with controls from both sides of course). Subsequently, mycelium must be isolated from the outer edge of all of these mycelia, and DNA should be islated and tested for cytoplasmic identity.
    - The consistency with which the KASPar PCR yields a heteroplasmic result, are a measure for how accurate the inoculation method is.
    - The consistency with which the KASPar PCR yields a homoplasmic result, is a measure for inoculation inaccuracy combined with a the chance that heteroplasmy might already have been lost again. These two options can be distinguished by also isolating and identifying mycelium from the inner area of the mycelium, where there is less chance that heteroplasmy was completely lost already.
- If the cytoplasmic mixing follow-up shows heteroplasmy to be stable, tests can be done for how many cytoplasms can be pooled together in one monokaryon, and if this gives more direct negative effects for the fitness of the monokaryon
- If the cytoplasmic mixing follow-up shows heteroplasmy to be instable, tests can be done for a hierarchy in cytoplasmic competition. Are some cytoplasms lost more easily than others? Are these cytoplasms correlated with a lower relative fitness in the monokaryon growth test?
- Perhaps approach 1 to test for the direct effect of cytoplasmic mixing was stopped too short. It could be run again on a small scale. If the two-level phenotype is observed again, the mycelia could be tested for clamp connections. Maybe the multiple mixtures simply grew larger because they had a larger chance to have become a dikaryon.
    - It must be said that even if the test will work, it is still a huge hassle to run, so maybe it's not worth it.
- Do dikaryons fructify differently under same-temperature circumstances when light exposure is varied? In the dikaryon fructification test, many monokaryon-grown-dikaryons simply stopped growing from the moment they entered the stove where they were exposed to light. Does a 12 hours light 12 hours dark cycle change this, resulting in radial outgrowth for the dikaryons?
    - Some of these dikaryons did not fructify from the monokaryon grown mycelium, but did fructify from the dikaryon grown mycelium. Perhaps the light dark cycle will allow some more dikaryons to fructify, because they can grow dikaryon mycelium.

An evolution experiment can also be done. This experiment would be aimed to learn what happens when the mtDNA and the nuclear DNA adapt to each other. When a cytoplasmic swap is made (A*a* and B*b* are combined into A*b* and B*a*), we often see a low phenotype (in radial outgrowth, the measure we take). This fitness-dip can be due to some uncompensated intergenomic conflicts between the cytoplasm and the nucleus, or simply due to fine-tuning still to occur.

The first step of the experiment would be to accurately measure fitness of all four monokaryons. For the evolution experiment, the focus should be on the swaps, but the original monokaryons should also be taken into account as blanks. During the experiment, selection should be done for the mycelium with the largest fitness. If fitness initially is growth, the section of the mycelium with the largest growth speed should always be inoculated through to the next plate. This should be done for all four monokaryons, so even A*a* and B*b* should be allowed to evolve, as controls. In the tables below, a scenario has been laid out. The table 'Controls' contains only the standard monokaryon and its evolved form, tested for fitness. Here, A*a* and A*a* evolved are two different samples, just to see if ste standard monokaryon also changed during the test. Fitness is standardized to A*a* here, with a fitness of 1. B*b* is supposed to be slightly less fit, but that's arbitrary.

Controls	**A*a***	**A*a* evolved**	**B*b***	**B*b* evolved**
Initial fitness	1	/	0.9	/
End fitness	1	1	0.9	0.9

The table 'Swaps' shows the expected phenotype of the cytoplasmic swaps, before and after evolution. Initially the phenotype is expected to be low, especially because the cytoplasmic swap chosen for this research should be one with a low phenotype. We are looking to see genetic improvement, so the lower it starts, the better. Later, other phenotypes can be tested for their evolution, too. It will be interesting to see how a high phenotype changes. After the experiment, the fitness level is supposed to have increased, though there is no certainty of this.

Swaps	**A*b***	**B*a***
Initial fitness	0.5	0.6
End fitness	0.7	0.8

Lastly, the table, the re-swaps, is the table that matters. The re-swaps are the cytoplasms swapped back to their original nucleus. No assumptions are made yet as to the results here, but different end results give different conclusions. If A*a*' has a lowered phenotype compared to A*a*, that means the mutations that increased the fitness of the swap were mostly fine-tuning. If the phenotype of A*a*' remains high, this suggests that perhaps some intergenomic conflicts that reduced the fitness of nucleus B in the swap have been countered by the nuclear genome, increasing the fitness of the swap. The same goes for B*b*'.

Re-swapped	**A*a*'**	**B*b*'**
End fitness	?	?

To see just how far fine-tuning can take the fitness up, the evolving cytoplasm could regularly be swapped back into a fresh, unadapted nuclear background. This is a tedious process however, since no dikaryon BB can be formed, the cytoplasm would have to be swapped into another nuclear background first, and then back into a fresh B type. It would also be impossible to discriminate between the two types of protoclones.

References

1. Casselton, L. and M. Zolan, *The art and design of genetic screens: filamentous fungi.* Nat Rev Genet, 2002. **3**(9): p. 683-97.
2. Lewis, D. and L.K. Crowe, *The Genetics and Evolution of Gynodioecy.* Evolution, 1956. **10**(2): p. 115-125.
3. Saumitoulaprade, P., J. Cuguen, and P. Vernet, *Cytoplasmic Male-Sterility in Plants - Molecular Evidence and the Nucleocytoplasmic Conflict.* Trends Ecol Evol, 1994. **9**(11): p. 431-435.
4. Hansen, A.K., et al., *Paternal, maternal, and biparental inheritance of the chloroplast genome in Passiflora (Passifloraceae): Implications for phylogenetic studies.* American Journal of Botany, 2007. **94**(1): p. 42-46.
5. Sharpley, M.S., et al., *Heteroplasmy of mouse mtDNA is genetically unstable and results in altered behavior and cognition.* Cell, 2012. **151**(2): p. 333-43.
6. Raper, J.R. and P.G. Miles, *The Genetics of Schizophyllum Commune.* Genetics, 1958. **43**(3): p. 530-46.
7. Ohm, R.A., et al., *Genome sequence of the model mushroom Schizophyllum commune.* Nature Biotechnology, 2010. **28**(9): p. 957-U10.
8. Raper, J.R., G.S. Krongelb, and M.G. Baxter, *The Number and Distribution of Incompatibility Factors in Schizophyllum.* American Naturalist, 1958. **92**(865): p. 221-232.
9. Niederpruem, D.J., *Direct Studies of Dikaryotization in Schizophyllum-Commune .1. Live Inter-Cellular Nuclear Migration Patterns.* Archives of Microbiology, 1980. **128**(2): p. 162-171.
10. Gladfelter, A. and J. Berman, *Dancing genomes: fungal nuclear positioning.* Nat Rev Microbiol, 2009. **7**(12): p. 875-86.
11. Adams, T.J.H., et al., *A Species-Specific Method of Analyzing Populations of Basidiospores.* Transactions of the British Mycological Society, 1984. **82**(Mar): p. 359-361.
12. Nieuwenhuis, B.P., A.J. Debets, and D.K. Aanen, *Sexual selection in mushroom-forming basidiomycetes.* Proc Biol Sci, 2011. **278**(1702): p. 152-7.
13. Raudaskoski, M. and E. Kothe, *Basidiomycete mating type genes and pheromone signaling.* Eukaryot Cell, 2010. **9**(6): p. 847-59.
14. de Vries, O.M. and J.G. Wessels, *Release of protoplasts from Schizophyllum commune by combined action of purified -1,3-glucanase and chitinase derived from Trichoderma viride.* J Gen Microbiol, 1973. **76**(2): p. 319-30.

Supplements

For each experiment the appendix shows the protocol followed by a table with all relevant found results, if not all values were displayed in the 'results' section of the report. The tables with the results are followed by a legend.

Appendix A - Monokaryon growth test

Protocol

All inoculations must be done near a Bunsen burner.

Step 1: Isolate all monokaryons to be tested. Grow the monokaryons in duplo on SMM at 27°C over two nights to amass sufficient material and be certain an infection won't mess up the timing. Growing the monokaryons in this way ensures they are all in the same phase of growth.

Step 2: Pour and label SMM plates in a random manner (random code since there is no need to know which colony is which). Check for infections the next day to prevent unnecessary trouble. Additionally, this way you can start working early on the next day. 180 plates will be poured for this particular assay.

Step 3: Place the colonies to be crossed on the SMM nutrient source. If a colony touches the SMM on more than one place, consider replacing the plate. Small blocks of mycelium will do. Each block of mycelium will come from any white fluffy part of the mycelium, excepting the spot where the plate was inoculated. If possible, land the mycelium-side on the SMM-plate, instead of the SMM-side on the SMM-plate. This way, the mycelium can start growing out radially instantly. Each monokaryon is tested in triplet.

Step 4: Grow the mycelia in a 27°C stove until every mycelium has clearly shown radial outgrowth. Outline the extremities of the mycelium with a coloured marker. This mark indicates zero growth. Place the mycelia back in the 27°C stove.

Scoring: Score growth two, four and five days after the zero mark has been indicated by ways of outlining the extremities of the mycelial outgrowth. Use a different colour for each successive day. Data is collected by measuring distance between the 'zero' outline and each other outline, in a straight line through the inoculation point.

Scoring will be done as follows: As this is a quantitative test, special care must be taken to treat all colonies the same while scoring. All boxes will be taken out of the 27°C stove at the same time. The boxes will all be opened for the full time they remain out of the stove, whether they are being scored at that moment or not. This way all plates are treated equally long, in a similar manner. Check the plates at the same time each day. After scoring, all boxes are closed and replaced in a random manner in the 27°C stove.

Materials used:

Machinery

- Stove
- Bunsen burner

Material per genotype to be tested

- 2 x 9cm Petri dish SMM

Additional material, per tested cross

- 3 x 9cm Petri dish SMM

Data

Table 8. Raw data monokaryon growth assay

Sample #	Content	Outgrowth Type	Conaminated	Wall Contained	Corr Max day 2	Corr Max day 3
535	Aa	3	Yes	No	1.43	2.06
593	Aa	/	No	No	1.36	1.99
642	Aa	/	No	No	1.16	1.87
Avg		**3**			**1.32**	**1.97**
St. Dev					**0.11**	**0.08**
St. Error					**0.07**	**0.05**
566	Ab	2	No	No	0.82	1.03
567	Ab	2	No	No	0.67	0.91
608	Ab	2	No	No	0.66	0.82
Avg		**2**			**0.72**	**0.92**
St. Dev					**0.07**	**0.09**
St. Error					**0.04**	**0.05**
506	Ac	3	No	No	1.40	1.95
552	Ac	3	No	No	1.30	1.87
592	Ac	/	No	No	1.41	2.05
Avg		**3**			**1.37**	**1.96**
St. Dev					**0.05**	**0.07**
St. Error					**0.03**	**0.04**
534	Ae	3	No	No	1.37	2.10
553	Ae	3	Yes	No	1.39	2.00
618	Ae	/	No	No	1.10	1.70
Avg		**3**			**1.29**	**1.93**
St. Dev					**0.13**	**0.17**
St. Error					**0.08**	**0.10**
523	Af	3	No	No	2.13	2.77
551	Af	/	No	Yes	2.36	3.11
626	Af	/	No	Yes	1.90	2.89
Avg		**Probably 3**			**2.13**	**2.92**
St. Dev					**0.19**	**0.14**
St. Error					**0.11**	**0.08**
516	Ag	3	No	No	1.72	2.37
582	Ag	/	No	No	1.48	2.25
607	Ag	/	No	No	1.45	2.28
Avg		**Probably 3**			**1.55**	**2.30**
St. Dev					**0.12**	**0.05**
St. Error					**0.07**	**0.03**
533	Am	1	No	No	1.62	2.22
581	Am	/	No	No	1.67	2.43
609	Am	/	No	No	1.63	2.19
Avg		**?**			**1.64**	**2.28**
St. Dev					**0.02**	**0.11**
St. Error					**0.01**	**0.06**

Sample #	Content	Outgrowth Type	Conaminated	Wall Contained	Corr Max day 2	Corr Max day 3
514	Ba	3	Yes	No	1.35	2.16
555	Ba	2	No	No	1.04	1.57
536	Ba	2	No	No	1.23	1.63
Avg		**Probably 2**			**1.21**	**1.79**
St. Dev					**0.13**	**0.27**
St. Error					**0.07**	**0.15**
522	Bb	3	No	No	1.89	2.61
584	Bb	3	No	No	1.62	2.41
625	Bb	/	No	No	1.40	2.42
Avg		**3**			**1.64**	**2.48**
St. Dev					**0.20**	**0.09**
St. Error					**0.12**	**0.05**
544	Bc	3	No	No	1.25	2.03
583	Bc	/	No	No	1.39	2.08
599	Bc	/	No	No	1.57	2.19
Avg		**Probably 3**			**1.40**	**2.10**
St. Dev					**0.13**	**0.07**
St. Error					**0.08**	**0.04**
554	Be	/	No	No	1.91	2.85
565	Be	3	No	No	2.03	2.67
591	Be	/	No	No	1.86	2.76
Avg		**Probably 3**			**1.93**	**2.76**
St. Dev					**0.07**	**0.07**
St. Error					**0.04**	**0.04**
515	Bf	3	No	No	1.78	2.54
556	Bf	/	Yes	No	1.76	2.67
638	Bf	/	No	No	1.65	2.35
Avg		**Probably 3**			**1.73**	**2.52**
St. Dev					**0.06**	**0.13**
St. Error					**0.03**	**0.08**
543	Bg	3	No	No	1.52	2.38
598	Bg	/	No	No	1.55	2.23
617	Bg	/	No	No	1.90	2.74
Avg		**Probably 3**			**1.66**	**2.45**
St. Dev					**0.17**	**0.21**
St. Error					**0.10**	**0.12**
564	Bm	3	No	No	1.20	1.93
576	Bm	3	No	No	1.39	2.23
590	Bm	/	Yes	No	1.39	2.24
Avg		**3**			**1.33**	**2.13**
St. Dev					**0.09**	**0.14**
St. Error					**0.05**	**0.08**

Sample #	Content	Outgrowth Type	Conaminated	Wall Contained	Corr Max day 2	Corr Max day 3
513	Ca	3	No	No	1.19	1.71
579	Ca	2	No	No	0.71	1.86
585	Ca	2	No	No	1.19	1.74
Avg		**Probably 2**			**1.03**	**1.77**
St. Dev					**0.23**	**0.06**
St. Error					**0.13**	**0.04**
524	Cb	3	No	No	1.27	1.92
559	Cb	2	No	No	1.23	1.60
600	Cb	2	No	No	1.18	1.99
Avg		**Probably 2**			**1.23**	**1.84**
St. Dev					**0.04**	**0.17**
St. Error					**0.02**	**0.10**
505	Cc	3	No	No	1.29	1.86
578	Cc	2	No	No	1.23	2.16
635	Cc	2	No	No	1.26	1.70
Avg		**Probably 2**			**1.26**	**1.91**
St. Dev					**0.02**	**0.19**
St. Error					**0.01**	**0.11**
586	Ce	2	No	No	1.32	1.74
627	Ce	2	No	No	1.23	1.82
629	Ce	2	Yes	No	1.32	1.91
Avg		**2**			**1.29**	**1.82**
St. Dev					**0.04**	**0.07**
St. Error					**0.02**	**0.04**
557	Cf	2	No	No	1.34	1.81
558	Cf	2	No	No	1.15	1.72
639	Cf	2	No	No	1.38	1.91
Avg		**2**			**1.29**	**1.81**
St. Dev					**0.10**	**0.08**
St. Error					**0.06**	**0.04**
614	Cg	2	Yes	No	1.23	1.71
631	Cg	2	No	No	1.19	1.84
537	Cg	2	No	No	1.26	1.76
Avg		**2**			**1.23**	**1.77**
St. Dev					**0.03**	**0.05**
St. Error					**0.02**	**0.03**
577	Cm	2	No	No	1.30	1.79
597	Cm	2	No	No	1.20	1.68
634	Cm	2	No	No	1.42	1.86
Avg		**2**			**1.31**	**1.78**
St. Dev					**0.09**	**0.07**
St. Error					**0.05**	**0.04**

Sample #	Content	Outgrowth Type	Conaminated	Wall Contained	Corr Max day 2	Corr Max day 3
502	Ea	1	No	No	1.15	1.49
568	Ea	1	No	No	0.84	0.99
571	Ea	1	No	No	0.67	1.01
Avg		**1**			**0.89**	**1.16**
St. Dev					**0.20**	**0.23**
St. Error					**0.11**	**0.13**
501	Eb	3	No	Yes	2.22	3.17
546	Eb	3	No	No	1.98	2.63
550	Eb	3	No	No	2.18	2.66
Avg		**3**			**2.13**	**2.82**
St. Dev					**0.10**	**0.25**
St. Error					**0.06**	**0.14**
517	Ec	3	No	No	1.38	2.50
603	Ec	2	No	No	1.10	1.60
610	Ec	2	No	No	1.11	1.63
Avg		**Probably 2**			**1.20**	**1.91**
St. Dev					**0.13**	**0.42**
St. Error					**0.07**	**0.24**
518	Ee	3	No	No	1.78	2.61
620	Ee	/	No	No	1.51	2.39
632	Ee	/	No	No	1.75	2.47
Avg		**Probably 3**			**1.68**	**2.49**
St. Dev					**0.12**	**0.09**
St. Error					**0.07**	**0.05**
530	Ef	3	No	No	1.65	2.56
569	Ef	3	No	No	1.69	2.38
570	Ef	3	No	No	1.35	2.58
Avg		**3**			**1.56**	**2.51**
St. Dev					**0.15**	**0.09**
St. Error					**0.09**	**0.05**
508	Eg	2	No	Yes	1.86	2.57
621	Eg	/	No	Yes	1.63	2.53
633	Eg	/	No	No	1.52	2.25
Avg		**?**			**1.67**	**2.45**
St. Dev					**0.14**	**0.14**
St. Error					**0.08**	**0.08**
549	Em	3	No	No	1.84	2.47
619	Em	/	No	No	1.59	2.33
641	Em	/	No	No	1.50	2.33
Avg		**Probably 3**			**1.64**	**2.38**
St. Dev					**0.14**	**0.07**
St. Error					**0.08**	**0.04**

Sample #	Content	Outgrowth Type	Conaminated	Wall Contained	Corr Max day 2	Corr Max day 3
528	Fa	2	No	No	1.64	2.18
575	Fa	2	No	No	1.54	2.15
630	Fa	/	No	No	1.63	2.25
Avg		**2**			**1.60**	**2.19**
St. Dev					**0.04**	**0.04**
St. Error					**0.03**	**0.02**
527	Fb	2	No	No	1.38	1.99
542	Fb	2	No	No	1.59	2.16
613	Fb	/	No	No	1.48	2.08
Avg		**2**			**1.48**	**2.08**
St. Dev					**0.09**	**0.07**
St. Error					**0.05**	**0.04**
602	Fc	/	No	No	1.40	2.05
589	Fc	/	No	No	1.53	2.06
574	Fc	2	No	No	1.47	2.00
Avg		**?**			**1.47**	**2.04**
St. Dev					**0.05**	**0.03**
St. Error					**0.03**	**0.02**
510	Fe	3	No	No	1.65	2.22
529	Fe	2	No	No	1.50	2.18
541	Fe	2	No	No	1.34	2.08
Avg		**Probably 2**			**1.50**	**2.16**
St. Dev					**0.13**	**0.06**
St. Error					**0.07**	**0.03**
563	Ff	3	No	No	1.63	2.21
637	Ff	/	No	No	1.48	2.11
640	Ff	/	No	No	1.46	2.12
Avg		**Probably 3**			**1.52**	**2.15**
St. Dev					**0.08**	**0.04**
St. Error					**0.04**	**0.03**
509	Fg	2	No	No	1.48	2.12
562	Fg	/	No	No	1.53	2.03
624	Fg	/	No	No	1.48	2.18
Avg		**?**			**1.50**	**2.11**
St. Dev					**0.02**	**0.06**
St. Error					**0.01**	**0.04**

Sample #	Content	Outgrowth Type	Contaminated	Wall Contained	Corr Max day 2	Corr Max day 3
526	Ga	3	No	No	1.48	2.13
503	Ga	3	No	No	1.56	2.18
587	Ga	/	No	No	1.57	2.24
Avg		**3**			**1.54**	**2.18**
St. Dev					**0.04**	**0.04**
St. Error					**0.02**	**0.03**
525	Gb	3	Yes	Yes	1.62	2.37
538	Gb	3	No	No	1.53	2.23
560	Gb	3	No	No	1.64	2.20
Avg		**3**			**1.60**	**2.27**
St. Dev					**0.05**	**0.07**
St. Error					**0.03**	**0.04**
588	Gc	/	No	No	1.50	2.17
601	Gc	/	No	No	1.75	2.26
615	Gc	/	No	No	1.62	2.11
Avg		**?**			**1.62**	**2.18**
St. Dev					**0.10**	**0.06**
St. Error					**0.06**	**0.04**
504	Ge	3	No	No	1.49	2.02
511	Ge	3	No	No	1.55	2.00
628	Ge	2	No	No	1.35	1.74
Avg		**Probably 3**			**1.46**	**1.92**
St. Dev					**0.08**	**0.13**
St. Error					**0.05**	**0.07**
539	Gf	3	No	No	1.50	2.25
580	Gf	/	No	No	1.51	2.27
595	Gf	/	No	No	1.61	2.10
Avg		**Probably 3**			**1.54**	**2.21**
St. Dev					**0.05**	**0.08**
St. Error					**0.03**	**0.04**
512	Gg	3	No	No	1.87	2.35
540	Gg	3	No	No	1.42	2.17
561	Gg	3	No	No	1.70	2.21
Avg		**3**			**1.66**	**2.24**
St. Dev					**0.19**	**0.08**
St. Error					**0.11**	**0.04**
596	Gm	/	No	No	1.57	2.25
616	Gm	/	No	No	1.58	2.13
636	Gm	/	No	No	1.57	2.35
Avg		**?**			**1.57**	**2.24**
St. Dev					**0.00**	**0.09**
St. Error					**0.00**	**0.05**

Sample #	Content	Outgrowth Type	Contaminated	Wall Contained	Corr Max day 2	Corr Max day 3
622	Ma	/	No	No	1.68	2.25
519	Ma	3	No	No	1.99	2.58
548	Ma	3	No	No	1.69	2.40
Avg		**3**			**1.79**	**2.41**
St. Dev					**0.14**	**0.13**
St. Error					**0.08**	**0.08**
507	Mb	3	No	No	1.75	2.64
531	Mb	3	No	No	1.60	2.29
594	Mb	/	No	No	1.71	2.33
Avg		**3**			**1.69**	**2.42**
St. Dev					**0.06**	**0.16**
St. Error					**0.04**	**0.09**
520	Mc	3	Yes	No	1.65	2.56
573	Mc	/	No	No	1.54	2.12
604	Mc	/	Yes	No	1.57	2.35
Avg		**probably 3**			**1.59**	**2.34**
St. Dev					**0.05**	**0.18**
St. Error					**0.03**	**0.10**
532	Me	3	No	No	1.60	2.24
547	Me	3	No	No	1.49	2.25
611	Me	/	No	No	2.02	2.44
Avg		**3**			**1.70**	**2.31**
St. Dev					**0.23**	**0.09**
St. Error					**0.13**	**0.05**
572	Mg	/	No	No	1.52	2.23
605	Mg	/	No	No	1.53	2.20
612	Mg	/	Yes	No	1.67	2.38
Avg		**?**			**1.57**	**2.27**
St. Dev					**0.07**	**0.08**
St. Error					**0.04**	**0.05**
521	Mm	3	No	Yes	2.03	2.85
606	Mm	/	No	No	1.99	2.96
623	Mm	/	No	No	1.80	2.79
Avg		**Probably 3**			**1.94**	**2.87**
St. Dev					**0.10**	**0.07**
St. Error					**0.06**	**0.04**

Table 8 (displayed above) shows all relevant data for monokaryon fitness test. Column one shows the random value of the rows displayed like random sample code, average, calculated standard deviation or calculated standard error. Column two shows monokaryon genotype. Column three shows type of outgrowth observed. Column four shows whether or not the mycelium was in contact with a contamination. Column five shows whether or not measuring the mycelial outgrowth was ever limited by the wall of the Petri-dish. Column six and seven show the radial outgrowth two and three days after the zero level was scored. Black cells contain no information.

Appendix B – Cytoplasm identification test

Protocol

KASPAR snp detection (see chapter 3 of thesis for primers used).

SNPs are identified using KASPar v4.0 SNP Genotyping Systems (KBioscience, Hoddesdon, England). This is a simple and very cheap method to test for snps. Only the primers have to be developed for the right location. The company used to have a program for that, but they figured out they can make more money with designing primers than just supplying consumables, so that program is not available anymore. I might still have a copy, and maybe some other people too (though with our upgrade to Windows7 the program stopped working, so an old computer is needed). The idea is that you do a PCR with a mix of two forward primers and a universal revers. The two fwd differ at the 5' and 3'side. At the 3' with one basepair (the SNP of interest) and at the 5' they have different tails. To the tails a dye can stick (a different flurophore for each tail) which results in a quantitative reading. By looking at the relative expression of the two different florophores can be deduced if the genotype is snp1, snp2 or heterozygous.

Primers used can be found on the next page.
Primers are very long, because the mtDNA has a very high AT content.
I had to change the standard protocol a bit, to make it work for mtDNA
DNA used needs to be clean. I used the Qiagen plant mini dnEasy kit for this.
Use 10 times diluted DNA
Per sample:

- 5ul DNA
- 5ul KASP 2X mix
- 0.08 ul Essay-mix
- 0.14 ul $MgCl_2$ 50mM as provided by KBiosciences to increase concentration to 2.2mM

Essay-mix is:

- 30 ul reverse common primer
- 46 ul ddH_2O

I used a CFX96 PCR machine from Bio-Rad Laboratories and the green 96well microtiter plates that come with it.
The standard KASPar thermocycling protocol was optimized for low GC content

- 94°C 15 min
- Cycle 9 times
  - 94°C for 20"
  - 65°C for 1' with decrement of 0.7°C per cycle
- Cycle 34 times
  - 94°C for 20"
  - 57°C for 1'
- 20°C for 30"
- Do measurements for VIC and FAM
- 50°C 1'

The 'allelic discrimination' tool of CFX Manager Software (V2.0, Bio-Rad Laboratories) can be used to determine the SNP allele for each strain.

Data

Table 9. 1st run KASPar PCR data and interpretation

Sample	747 (G/C)	6280 (T/C)	43198 (T/C)	Code	Cytoplasm
Aa	2	1	2	CTC	*a/e/f*
Ba	2	2	2	CCC	Test Again
Ca	2	1	2	CTC	*a/e/f*
Ea	0	1	2	XTC	*a/e/f*
Fa	2	1	2	CTC	*a/e/f*
Ga	2	1	2	CTC	*a/e/f*
Ma	2	1	2	CTC	*a/e/f*
Ab	0	2	2	XCC	Test Again
Bb	1	2	2	GCC	Test Again
Cb	2	2	2	CCC	*b*
Eb	2	2	2	CCC	*b*
Fb	2	2	2	CCC	*b*
Gb	2	2	2	CCC	*b*
Mb	2	2	2	CCC	*b*
Ac	1	2	1	GCT	*c*
Bc	1	2	1	GCT	*c*
Cc	1	2	1	GCT	*c*
Ec	1	2	1	GCT	*c*
Fc	0	1	2	XTC	Test Again
Gc	1	2	1	GCT	*c*
Mc	1	2	2	GCC	Test Again
Ae	2	1	2	CTC	*a/e/f*
Be	2	1	2	CTC	*a/e/f*
Ce	0	0	0	XXX	Test Again
Ee	2	1	2	CTC	*a/e/f*
Fe	2	1	2	CTC	*a/e/f*
Ge	2	1	2	CTC	*a/e/f*
Me	2	1	0	CTX	Test Again
Af	2	1	2	CTC	*a/e/f*
Bf	2	1	2	CTC	*a/e/f*
Cf	1	2	0	GCX	Test Again
Ef	2	1	2	CTC	*a/e/f*
Ff	2	1	2	CTC	*a/e/f*
Gf	2	1	2	CTC	*a/e/f*
Ag	1	2	2	GCC	*g*
Bg	1	2	2	GCC	*g*
Cg	1	2	2	GCC	*g*
Eg	1	2	2	GCC	*g*
Fg	1	2	2	GCC	*g*
Gg	1	2	1	GCT	*c*
Mg	1	2	DOUBLE	GC(C/T)	Test Again
Am	2	2	1	CCT	*m*
Bm	2	2	1	CCT	*m*

Cm	1	2	2	GCC	*g*
Em	2	2	1	CCT	*m*
Gm	2	2	0	CC(C/T)	Test Again
Mm	2	2	1	CCT	*m*
BB	BB	BB	BB	BBB	*BLANC*

Table 9 (displayed above) shows all relevant data for the second run of the 'KASPar PCR, cytoplasm genotyping test'. Column one shows the genotype of the monokaryon from which DNA was isolated. Column two, three and four show the result of the PCR, concluding either allele 1 or allele 2 is present in the DNA at the location of the SNP. If a 0 is indicated, no sufficiently high value was found to decide either way. If 1+2 is indicated, both alleles were indicated to be present. Column five shows column two, three and four collected into a code. Column six shows the code indicated in column five interpreted into a conclusion. The conclusion can either be a cytoplasmic genotype, or a decision to repeat the KASPar assay again. A red cell indicates a counter-intuitive value, whereas an orange value indicates a value that is only unexpected.

Table 10. 2nd run KASPar PCR data and interpretation

	747	6280	15534	30738	43198	43418	Code FOUND 2nd run	Code FOUND 1st run	Code LITERATURE	Cytoplasm
Ba	2	2	2	2	2	1	CCCCCA	CC//C/	CTCCCA	*B*
Ab	0	1	2	2	2	1	?TCCCA	XT//C/	CCCCCA	*a/e/f*
Bb	1	2	2	2	2	1	GCCCCA	GC//C/	CCCCCA	*g*
Fc	2	1	2	2	2	1	CTCCCA	XT//C/	GC?CT?	*a/e/f*
Mc	1	2	0	2	1	1	GC?CTA	GC//C/	GC?CT?	*c*
Me	2	1	2	2	2	1	CTCCCA	CT//X/	CTCCCA	*e*
Cf	1	2	2	2	1	1	GCCCTA	GC//X/	CTCCCA	*c*
Mg	1	2	2	2	1+2	1	GCCC?A	GC//(C/T)/	GCCCCA	*g*
Gm	2	2	1	1	1+2	2	CCAT?C	CC//(C/T)/	CCATTC	*m*
Ce	2	1	2	2	2	1	CTCCCA		CTCCCA	*e*

Table 10 (displayed above) shows all relevant data for the first run of the 'KASPar PCR, cytoplasm genotyping test'. Column one shows the genotype of the monokaryon from which DNA was isolated. Column two to seven show the result of the PCR, concluding either allele 1 or allele 2 is present in the DNA at the location of the SNP. If instead a 0 is indicated, no sufficiently high value was found to decide either way. If 1+2 is indicated, both alleles were indicated to be present. Column eight shows column two to seven collected into a code. Column nine shows the result of the first run. Column ten shows the code that should be found. Column eleven shows an interpretation of the two PCR runs, into the conclusion whether the sample contains the wrong cytoplasm or not. All samples with a cytoplasm that was incorrect was not taken into account for the statistical analysis.

Appendix C - Direct effects of cytoplasmic mixing – approach 1

Protocol

All inoculations must be done near a Bunsen burner.

Step 1: Pour 5cm Petri dish SMM plates, label with the colony which will be placed on it. two plates must be poured per colony to be tested so contamination would probably be no issue. Inoculate the plates with a small block of mycelium + agar from a recently grown or refrigerated colony, or a frozen stock. Grow the colonies at 27°C.

Step 2: Prepare cellophane layers for the plates to be tested by cutting disks with a diameter of about eight cm. Autoclave the cellophane disks in a water-filled glass Petri-dish wrapped with aluminium foil. As the placing of the cellophane layers is a pretty contamination-sensitive process, five disks of cellophane should be cut per combination to be tested.

Step 3: Pour the plates on which the cultures will be tested. These must be 9cm Petri dishes, poured with SMM. The plates should not be labelled yet. Five plates should be poured per combination to be tested. Put a layer of cellophane on each of the plates. Put the prepared plates in the 27°C stove overnight to check for contaminations. In addition, pour three extra 9cm SMM Petri dishes per genotype to be tested. These plates will be used to correct the results, by showing the original amount of colony forming units (CFU) for each suspension.

Step 4: The colonies should have grown sufficient material after two days. Cut the SMM + mycelium in half and put one half in a 500 ml sterile glass bottle with 100 ml liquid SMM medium. Use the rod mixer to fragment the mycelia into small parts in about twenty-five seconds. If the same rod mixer is to be utilized for a next colony, it is advised to dip the rod into 90% alcohol and cauterize it several times, before switching genotype. Put the suspension (in either the capped glass flask or a sterile Erlenmeyer) in a shake-stove overnight (27°C, 110 RPM) to allow the mycelia to regenerate and grow.

Step 4: Fragment the regenerated mycelia again for twenty seconds, then make a dilution series (10, 100, 1000) of the suspension. Put 50 µl of these suspensions on the SMM plates without cellophane (in duplo). Spread the suspension on the plate with 2 mm glass pearls. Once completed, place these plates in a 27°C stove overnight.

Step 5: Take a new Eppendorf and make the combinations by adding equal amounts of suspension to each Eppendorf. Here you can combine any number of monokaryons. Just make sure they all have the same nucleus, or the entire thing will become a dikaryon, significantly influencing the analysis. Vortex the combined suspension.

Step 6: Plate out the combinations by putting 50 µl of suspension on a randomly labelled SMM + cellophane plate, and spreading with 2 mm glass pearls. Make sure the random code + combination is written down somewhere. Place the plates in a 27°C stove to grow.

Step 7: Count the amount of CFU's per genotype two or three days after inoculation. This is done by taking a plate with not too many colonies to count from the dilution series. From this number we can calculate the original amount of CFU's per suspension.

Scoring: Three or four days after inoculation, peel the cellophane layers from the SMM plates with the combinations. Put the cellophane layer in an empty Petri dish labelled identically as its original plate. Store the dishes with the cellophane layers in a cooled environment in an unlidded box to dry the cellophane + mycelium. A few days later, weigh every individual cellophane layer. The weight is a measure for the amount of growth that has taken place. This may be influenced by cytoplasms mixing.

Materials used:

Machinery

- Rod mixer
- Shake-stove
- Vortex unit
- 4 decimal weighing unit

Material per genotype

- 500 ml sterile bottle
- 250 ml sterile Erlenmeyer flask
- 150 ml SMM liquid medium
- 2 x 5cm Petri dish SMM
- 6 x 9cm Petri dish SMM
- 3 x 1.5 ml Eppendorf tube

Additional material, per tested subset

- 5 x 9cm Petri dish SMM + cellophane
- Glass pearls 2 mm size (5-10 per Petri-dish)
- 1 x 1.5 ml Eppendorf tube

Data

Table 11. Raw data direct effect of cytoplasmic mixing – approach 1

Content*	Dry Weight Plate + mycelium	Weight empty Plate	Dry Weight mycelium	Average Dry Weight	ST. Dev Dry Weight
A*b*	13.8332	13.6918	0.1414	**0.1673**	**0.0285**
A*b*	13.9185	13.7271	0.1914		
A*b*	13.8084	13.6719	0.1365		
A*b*	13.9735	13.7738	0.1997		
A*c*	13.9427	13.7418	0.2009	**0.1818**	**0.0317**
A*c*	13.8938	13.6816	0.2122		
A*c*	13.8455	13.716	0.1295		
A*c*	13.8857	13.7011	0.1846		
A*f*	13.8871	13.7135	0.1736	**0.1657**	**0.0195**
A*f*	13.8384	13.6995	0.1389		
A*f*	13.8763	13.6916	0.1847		
A*g*	13.8623	13.7238	0.1385	**0.1814**	**0.032**
A*g*	13.9531	13.7377	0.2154		
A*g*	13.875	13.6847	0.1903		
A*bcf*	13.8807	13.6806	0.2001	**0.1802**	**0.0215**
A*bcf*	13.9112	13.7174	0.1938		
A*bcf*	13.8311	13.6919	0.1392		
A*bcf*	13.9188	13.7387	0.1801		
A*bcf*	13.8774	13.6897	0.1877		
A*bcg*	13.8863	13.6895	0.1968	**0.1765**	**0.0241**
A*bcg*	13.8769	13.6832	0.1937		
A*bcg*	13.8209	13.6869	0.134		
A*bcg*	13.8784	13.6858	0.1926		
A*bcg*	13.8438	13.6782	0.1656		
A*bfg*	13.8709	13.7298	0.1411	**0.1655**	**0.03**
A*bfg*	13.8903	13.7378	0.1525		
A*bfg*	13.8405	13.7038	0.1367		
A*bfg*	13.9358	13.7562	0.1796		
A*bfg*	13.9102	13.6927	0.2175		
A*cfg*	13.8813	13.7004	0.1809	**0.1906**	**0.0434**
A*cfg*	13.8705	13.7288	0.1417		
A*cfg*	13.9744	13.7026	0.2718		
A*cfg*	13.9228	13.7365	0.1863		
A*cfg*	13.8624	13.6899	0.1725		
A*bcfg*	13.9583	13.7636	0.1947	**0.1843**	**0.0106**
A*bcfg*	13.9065	13.7129	0.1936		
A*bcfg*	13.8818	13.7017	0.1801		
A*bcfg*	13.8736	13.7047	0.1689		

Content*	Dry Weight Plate + mycelium	Weight empty Plate	Dry Weight mycelium	Average Dry Weight	ST. Dev Dry Weight
M*a*	13.9754	13.7134	0.262	**0.242**	**0.0546**
M*a*	13.9735	13.6911	0.2824		
M*a*	14.0376	13.7622	0.2754		
M*a*	13.836	13.6877	0.1483		
M*c*	13.9983	13.7156	0.2827	**0.2205**	**0.0636**
M*c*	13.9606	13.7148	0.2458		
M*c*	13.89	13.7569	0.1331		
M*e*	13.9462	13.6924	0.2538	**0.2069**	**0.0577**
M*e*	13.9934	13.7192	0.2742		
M*e*	13.896	13.74	0.156		
M*e*	13.8461	13.7024	0.1437		
M*g*	13.9744	13.733	0.2414	**0.1996**	**0.0438**
M*g*	13.8598	13.7207	0.1391		
M*g*	13.9268	13.7086	0.2182		
M*ace*	14.0308	13.7514	0.2794	**0.2255**	**0.0679**
M*ace*	14.0038	13.7138	0.29		
M*ace*	13.9982	13.7255	0.2727		
M*ace*	13.8783	13.7339	0.1444		
M*ace*	13.8582	13.7174	0.1408		
M*acg*	14.1454	13.7068	0.4386	**0.2619**	**0.1076**
M*acg*	14.0315	13.7314	0.3001		
M*acg*	13.8519	13.7042	0.1477		
M*acg*	13.9991	13.7279	0.2712		
M*acg*	13.8684	13.7166	0.1518		
M*aeg*	13.9955	13.6862	0.3093	**0.2109**	**0.0602**
M*aeg*	13.8254	13.6765	0.1489		
M*aeg*	13.8796	13.6706	0.209		
M*aeg*	13.9572	13.7189	0.2383		
M*aeg*	13.8732	13.7242	0.149		
M*ceg*	13.9778	13.7053	0.2725	**0.1986**	**0.0614**
M*ceg*	13.8316	13.6829	0.1487		
M*ceg*	13.9262	13.6812	0.245		
M*ceg*	13.8611	13.7331	0.128		
M*aceg*	14.0122	13.7858	0.2264	**0.2125**	**0.0728**
M*aceg*	14.0261	13.7251	0.301		
M*aceg*	13.8645	13.7413	0.1232		
M*aceg*	13.8696	13.7357	0.1339		
M*aceg*	14.0195	13.7414	0.2781		

Table 11 (displayed above) shows all relevant data for test 'Direct effect of cytoplasmic mixing – approach 1'. Column one represents the combination of cytoplasmic swaps present in the mixture put on plate. The first symbol represents the nuclear type present in all monokaryons in the mix. The other symbols indicate the cytoplasms that have been pooled togethe. Column two shows the weight of the Petri-dish in which the drying step was done, with the dried cellophane and mycelium still in it. Column three shows the weight of the empty Petri-dish. Column four shows the weight of the cellophane + mycelium, calculated by subtracting the value of column three from the value of column two. Column five and six show the average dry weight and standard deviation of the scored value. All weighed values are shown in grams. Some samples were not taken into account because they were contaminated.

Appendix D - Direct effects of cytoplasmic mixing – approach 2

Protocol

All inoculations must be done near a Bunsen burner.

Step 1: Pour and label SMM plates in a cognitive manner (no random code since you have to know which colony is where). Mark the spot where each colony must land. Make sure the marks are all approximately three cm distanced from each other. This way each monokaryon has sufficient chance to settle before the whole becomes a dikaryon.

Step 2: Place on these plates the to-be-tested blocks of SMM + mycelium from recently grown or refrigerated cultures, or frozen stocks. For extra contrast it is best to use cytoplasmic swaps, monokaryons with the same nucleus but a different cytoplasm. Place the plates in a 27°C stove for two days.

Step 3: Pour and label SMM plates in a random manner (random code since there is no need to know which colony is which). Pour 11 x 9cm plates per tested combination (three plates for each individual colony, 5 for the interaction zone, as there is a good chance the inoculation fails).

Step 4: As soon as the colonies have reached each other, take samples from colony 1, colony 2, and the interaction zone. Sampling is done by dipping a sterile toothpick in the mycelium, then dipping it thoroughly in the middle of the fresh 9cm plate.

Sampling from the interaction zone must be done carefully. By working precisely the chance to only isolate fused cells increases. Sampling from the unfused mycelia can be done without much precision, and may even be done by cutting a small block of SMM + mycelium and transferring that.

Step 5: Grow the mycelia in a 27°C stove until every mycelium has clearly shown radial outgrowth. Outline the extremities of the mycelium with a coloured marker. This mark indicates zero growth. Place the mycelia back in the 27°C stove.

Scoring: Score growth two, four and five days after the zero mark has been indicated by ways of outlining the extremities of the mycelial outgrowth. We advise to use a different colour for each successive day. Data is collected by measuring distance between the 'zero' outline and each other outline, in a straight line through the inoculation point. If sector growth is observed, make a note of it. This may be an indication of loss of the heteroplasmic state.

Materials used:

Machinery

- Stove
- Bunsen burner

Material per tested cross

- 12 x 9cm Petri dish SMM
- 5 to 11 sterile toothpicks

Appendix E - Dikaryon fructification test

Protocol

Table 12. Crossing scheme for the dikaryon fitness test. In both column and row one the monokaryons from all over the world, which were crossed, are listed.

	B	G	M	P	Q	K
G	B G					
M	B M	G M				
P	B P	G P	M P			
Q	B Q	G Q	M Q	P Q		
K	B K	G K	M K	P K	Q K	
V	B V	G V	M V	P V	Q V	K V

All inoculations were done near a Bunsen burner.

Step 1: Isolate all monokaryons to be tested. Grow the monokaryons in duplo on SMM at 27°C over two nights to amass sufficient material and be certain an infection won't mess up the timing. Growing the monokaryons in this way ensures they are all in the same phase of growth.

Step 2: Pour and label SMM plates in a cognitive manner (no random code since you have to know which colony is where). Mark the spot where each colony must land. Make sure the marks are all approximately 2 cm distanced from each other. This way each monokaryon has sufficient chance to settle before the whole becomes a dikaryon. put them in 27°C overnight. Check for infections the next day to prevent unnecessary trouble. Additionally, this way you can start working early on the next day. One hundred SMM plates will be poured for this particular assay.

Step 3: Place the colonies to be crossed on the SMM nutrient source on the indicated place. If a colony touches the SMM on more than one place, consider replacing the plate. Small blocks of mycelium will do. Each block of mycelium will come from white fluffy part of the mycelium, excepting the spot where the plate was inoculated. If possible, land the mycelium-side on the SMM-plate, instead of the SMM-side on the SMM-plate. This way, the mycelium can start growing out radially instantly. Each cross is done in triplet.

Step 4: Grow the mycelia in a dark 27°C environment until every mycelium has clearly fertilized the other. Relocate the crosses to a 27°C lighted stove. Preferably, do not stack more than two plates on top of each other so that each plate receives sufficient light. Make sure all plates are in boxes. Do not put parafilm on the outside of the dish, or too much CO2 will accumulate.

Scoring: Mark the beginning of mushroom formation, the manner of mushroom formation (some to many and the beginning and quantity of spore production (day + many or few). This collective data forms the basis of how well the dikaryon fructifies. For this assay, we score mushrooms not as soon as a fruiting body is observed, but as soon as a fruiting body start to open up. If no fructification is observed, check for clamp connections with a microscope (400x).

Scoring will be done as follows: As this is a quantitative test, special care must be taken to treat all colonies the same while scoring. All boxes will be taken out of the 27°C lighted stove at the same time. The boxes will all be opened for the full time they remain out of the stove, whether they are being scored at that moment or not. Check the plates at the same time each day. After scoring, all boxes are closed and replaced in a random manner in the 27°C stove.

Materials used:

Machinery

- Stove
- Lighted stove (24 hours light cycle)
- Bunsen burner
- Microscope (400x magnification)

Material per genotype to be tested

- 2 x 9cm Petri dish SMM

Additional material, per tested cross

- 3 x 9cm Petri dish SMM

Data

Table 13. Relevant data dikaryon fructification test

Maternal Nucleus	Paternal Nucleus	Mushroom formation	Spore formation
B	G	Day 5	Day 6
B	G	Day 5	Day 6
B	G	Day 5	Day 6
B	M	Day 5	Day 5
B	M	Day 6	Day 6
B	M	Day 5	Day 6
B	P	Day 10	Day 10
B	P	Day 10	Day 10
B	P	Day 10	Day 10
B	Q	Never occurred	Never occurred
B	Q	Never occurred	Never occurred
B	Q	Never occurred	Never occurred
B	K	Day 6	Day 10
B	K	Day 6	Day 7
B	K	Day 6	Day 10
B	V	Never occurred	Never occurred
B	V	Day 10	Day 11
B	V	Never occurred	Never occurred
G	M	Day 3	Day 4
G	M	Day 3	Day 4
G	M	Day 4	Day 4
G	P	Day 7	Day 10
G	P	Day 7	Day 10
G	P	Day 7	Day 10
G	Q	Day 10	Day 10
G	Q	Day 7	Day 10
G	Q	Day 6	Day 7
G	K	Day 4	Day 6
G	K	Day 5	Day 6
G	K	Day 6	Day 6
G	V	Day 7	Day 10
G	V	Day 6	Day 7
G	V	Day 6	Day 10
G	B	Day 6	Day 10
G	B	Day 5	Day 6
G	B	Day 5	Day 6
K	V	Day 10	Day 10
K	V	Day 5	Day 6
K	V	Day 5	Day 7
K	B	Day 7	Never occurred

Maternal Nucleus	Paternal Nucleus	Mushroom formation	Spore formation
K	B	Day 6	Day 10
K	B	Day 6	Day 10
K	G	Day 6	Day 7
K	G	Day 6	Day 7
K	G	Day 6	Day 7
K	M	Day 5	Day 6
K	M	Day 5	Day 5
K	M	Day 6	Day 7
K	P	Day 10	Day 10
K	P	Day 10	Day 10
K	P	Day 6	Day 7
K	Q	Day 5	Day 6
K	Q	Day 4	Day 5
K	Q	Day 4	Day 7
M	P	Day 3	Day 6
M	P	Day 4	Day 6
M	P	Day 3	Never occurred
M	Q	Never occurred	Never occurred
M	Q	Never occurred	Never occurred
M	Q	Never occurred	Never occurred
M	K	Day 4	Day 5
M	K	Day 5	Day 5
M	K	Day 4	Day 5
M	V	Never occurred	Never occurred
M	V	Never occurred	Never occurred
M	V	Day 5	Day 6
M	B	Day 5	Day 6
M	B	Day 5	Day 6
M	B	Day 5	Day 6
M	G	Day 4	Day 4
M	G	Day 4	Day 5
M	G	Day 4	Day 6
P	Q	Day 10	Day 10
P	Q	Day 10	Day 10
P	Q	Never occurred	Never occurred
P	K	Never occurred	Never occurred
P	K	Never occurred	Never occurred
P	K	Day 5	Day 6
P	V	Day 7	Day 10
P	V	Day 10	Day 10
P	V	Day 7	Day 10
P	B	Day 10	Day 10
P	B	Day 10	Day 10
P	B	Day 7	Day 10
P	G	Never occurred	Never occurred

Maternal Nucleus	Paternal Nucleus	Mushroom formation	Spore formation
P	G	Day 10	Day 10
P	G	Never occurred	Never occurred
P	M	Day 4	Day 5
P	M	Day 4	Day 6
P	M	Day 4	Day 6
Q	K	Day 5	Day 6
Q	K	Day 4	Day 6
Q	K	Day 5	Day 6
Q	V	Day 10	Day 10
Q	V	Day 6	Day 10
Q	V	Day 4	Day 10
Q	B	Day 5	Day 6
Q	B	Day 5	Day 6
Q	B	Day 5	Day 5
Q	G	Day 6	Day 6
Q	G	Day 6	Day 7
Q	G	Day 6	Day 7
Q	M	Never occurred	Never occurred
Q	M	Never occurred	Never occurred
Q	M	Never occurred	Never occurred
Q	P	Day 6	Day 10
Q	P	Day 7	Day 10
Q	P	Day 6	Day 10
V	B	Day 10	Day 10
V	B	Day 10	Day 11
V	B	Day 10	Day 10
V	G	Day 5	Day 6
V	G	Day 6	Day 7
V	G	Day 10	Day 10
V	M	Day 4	Day 5
V	M	Day 3	Day 4
V	M	Day 4	Day 5
V	P	Day 7	Day 10
V	P	Day 6	Day 7
V	P	Day 6	Day 7
V	Q	Day 4	Day 7
V	Q	Day 7	Day 10
V	Q	Day 4	Day 10
V	K	Day 4	Day 5
V	K	Day 3	Day 5
V	K	Day 3	Day 5

Table 13 (displayed above) shows all relevant data for test 'Dikaryon fructification test'. Column one shows the maternal nuclear type of the observed dikaryon-half. Column two shows the paternal nuclear type with which the monokaryon of column one was crossed. Column three shows the first day the minimal fructification structure was observed. Column four shows the first day sporulation was observed.

Appendix F - Cytoplasm induced gynodioecy test

Protocol

Table 14. Monokaryon crossing scheme*

	G	M	K	V	Q	P
B*a*	B*a* G	B*a* M	B*a* K	B*a* V	B*a* Q	B*a* P
B*b*	B*b* G	B*b* M	B*b* K	B*b* V	B*b* Q	B*b* P
B*c*	B*c* G	B*c* M	B*c* K	B*c* V	B*c* Q	B*c* P
B*e*	B*e* G	B*e* M	B*e* K	B*e* V	B*e* Q	B*e* P

*Column one shows the different cytoplasmic swaps that will be crossed with monokaryons collected from all over the world, listed in row one. All other cells show the crosses to be made.

All inoculations were done near a Bunsen burner.

Step 1: Isolate all monokaryons to be tested. Grow the monokaryons in duplo on SMM at 27°C over two nights to amass sufficient material and be certain an infection won't mess up the timing. Growing the monokaryons in this way ensures they are all in the same phase of growth.

Step 2: Pour and label SMM plates in a cognitive manner (no random code since you have to know which colony is where). Mark the spot where each colony must land. Make sure the marks are all approximately two cm distanced from each other. This way each monokaryon has sufficient chance to settle before the whole becomes a dikaryon. put them in 27°C overnight. Check for infections the next day to prevent unnecessary trouble. Additionally, this way you can start working early on the next day. 60 plates will be poured for this particular assay.

Step 3: Place the colonies to be crossed on the SMM nutrient source on the indicated place. If a colony touches the SMM on more than one place, consider replacing the plate. Small blocks of mycelium will do. Each block of mycelium will come from any white fluffy part of the mycelium, excepting the spot where the plate was inoculated. If possible, land the mycelium-side on the SMM-plate, instead of the SMM-side on the SMM-plate. This way, the mycelium can start growing out radially instantly. Each cross is done in triplet.

Step 4: Grow the mycelia in a dark 27°C environment until every mycelium has clearly fertilized the other. Relocate the crosses to a 27°C lighted stove. Preferably, do not stack more than two plates on top of each other so that each plate receives sufficient light. Make sure all plates are in boxes. Do not put parafilm on the outside of the dish, or too much CO2 will accumulate. Check plates for clamps if fertilization is not obvious.

Scoring: Mark which part of the dikaryon fructifies. There is no need to monitor this day by day. Simply score one time after ten to twelve days. As this is a qualitative assay, there is no need for special treatment while scoring.

Materials used:

Machinery

- Stove
- Lighted stove (24 hours light cycle)
- Bunsen burner
- Microscope (400x magnification)

Material per genotype to be tested

- 2 x 9cm Petri dish SMM

Additional material, per tested cross

- 2 x 9cm Petri dish SMM

Appendix G - Supplementary photographs

Some photographs used in this report were either rather small or zoomed in. Here we display all those complete photographs.

Figure 2a complete. Original cross: Ba + Q

Figure 2b complete. Original picture: Be + Q

Figure 2c complete. Original cross Bb + Q replicate 1

Figure 2d complete. Original cross Bb + Q replicate 2

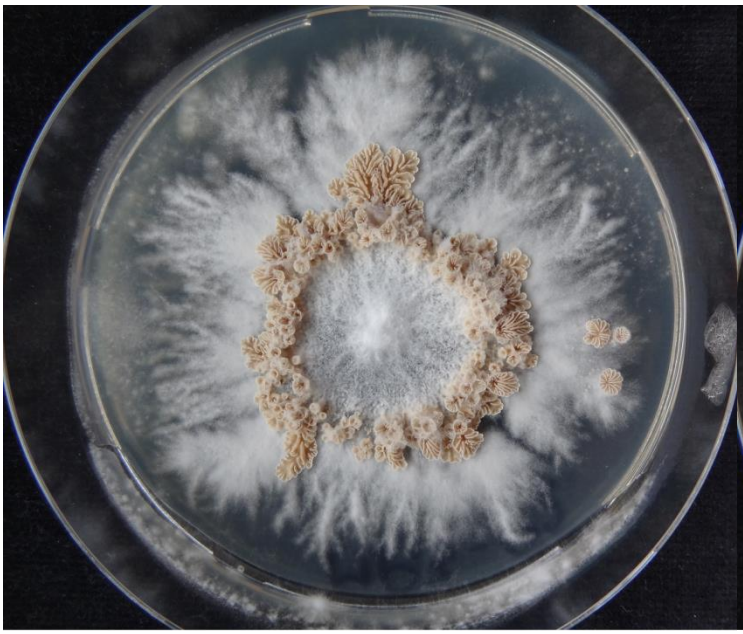

Figure 5c complete (Dikaryon MP, part M isolated and grown)

Figure 5d complete (Dikaryon MP, part P isolated and grown)

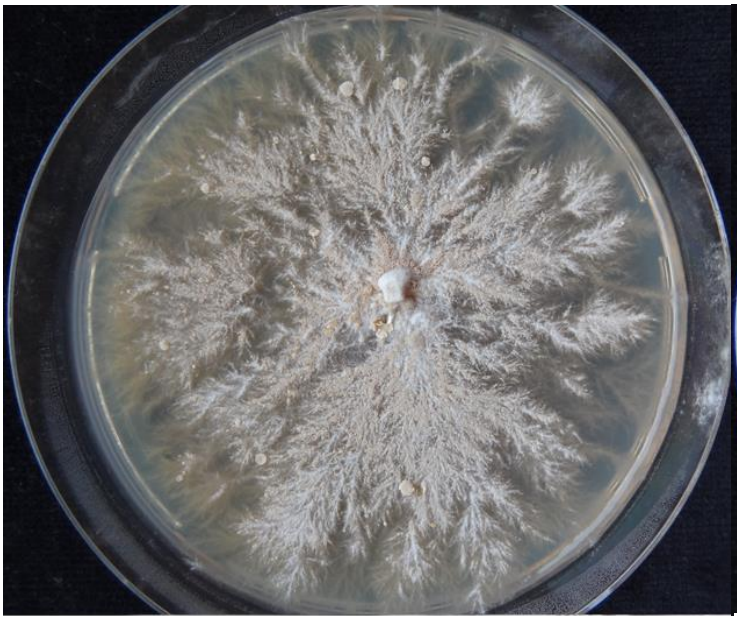

Figure 6c complete (Dikaryon MQ, part M isolated and grown)

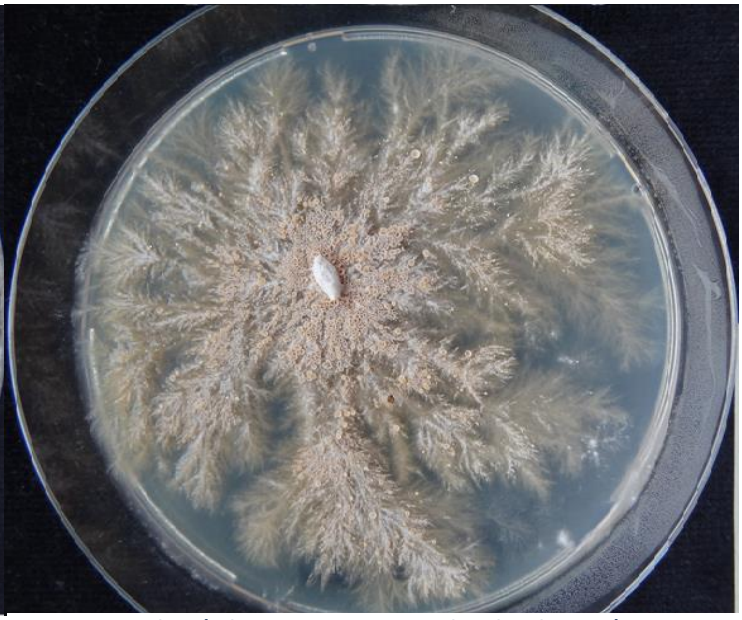

Figure 6c complete (Dikaryon MQ, part Q isolated and grown)

Appendix H - General protocols and used media

As described by Bart Nieuwenhuis.

Protoplast generation for isolation of protoclones

Based on protocol as described in (de Vries & Wessels 1972)

Materials

All per sample

50 plates (SMM + MgSO4 0.5M)
Liquid SMM in Erlemeyer
Blender Turrax with sterile mixer
500ml $MgSO_4$ 1M
500ml $MgSO_4$ 0.5M
100ml Sorbitol (1M)
MQ water
4 Büchner funnels
Glass funnel with sterile glass wool
100ml $MgSO_4$ 0.5M
penicillin/streptomycin (100mg/ml)

Procedure

Mycelium preparation

- Grow *S. commune* mycelium at 25°C in MM in 100ml Erlenmeyer on a shaker at 200 rpm.
- Macerate the mycelium the day before harvest for 15 seconds in a Waring blender.
- Grow the macerate at 25°C in 100 ml MM in 500ml Erlenmeyer on a shaker at 200 rpm for ~20h.
- When needed the culture can be diluted with MM after elimination of part of it and grown further.
- Harvest the mycelium by centrifugation of part of the culture in a sterile 50 ml plastic tube
- Wash once with 1M $MgSO_4$ and spin down again.
- Reduce the mycelial pellet to 5 ml.

Lysis

- Make a solution of 10 ml 1M $MgSO_4$ adjust to pH5.8 using 30 mM malate buffer and supplement with 20mg lyzing enzymes (Applied Plant Research, Wageningen UR, The Netherlands)
- Filter sterilize this solution with 0.45µm filter
- Resuspend the mycelium well in the lysing solution and incubated for 2,5 hours at 30°C (or at 24°C?)
- Add one volume sterile water and incubate the tube further for another hour.

Isolation

- Centrifuge the tube for 5 minutes at 360 g (1400 rpm in our swing out) and transfer the supernatant to a fresh tube.
- Discard left over small pieces of mycelium by sieving the suspension trough sterile glass wool.
- Add one volume sorbitol (1M) and mix well. Put the tube to rest for 15 minutes.
- Centrifuge the tube for 15 minutes at 2200 g (3500 rpm in our swing out).
- Discard the supernatant and carefully resuspend the protoplasts in the pellet into 25ml Mg SO_4 (0.5M).

Regeneration

- Count protoplasts with a counting chamber
- Centrifuge again and resuspend in 500µl $MgSO_4$ (0.5M)
- Make a 6 fold dilution series for 5 times:
  - 50µl in 250µl $MgSO_4$ + penicillin/streptomycin (1mg/ml)
  - Repeat this 5 times
- Plate 20 µl of each dilution on 0.5M $MgSO_4$ + M M plates
- Incubate the plates at 25°C overnight

DNA isolation

Qaigen Mini Plant kit

Use one colony of about three cm diameter grown on SMM with cellophane.
Utilize according to manufacturer's protocol.
Yields low amounts of DNA.

Changes or remarks for Qaigen protocol:

- Make sure the lysis buffer has not formed precipitate on the bottom of the flask, if it has, put it in the microwave for a few seconds and shake.
- Use bead-beater three times twenty seconds, freezing samples in liquid nitrogen in between.
- Add 4 µl of proteinase K solution to the lysis step for slightly better results.
- Put samples in heating block for thirty minutes during lysis-step. 65°C and 550 RPM.
- If samples seem very cohesive after a few minutes in the heating block, add one extra volume of lysic buffer, otherwise sample may be lost.
- Storing samples in -80 freezer for some months may improve results

Minimal medium: Schizophyllum Commune SMM

(Dons *et al.* 1979)

Dissolve in demi-water:

	Per *l*
Glucose	20 g
KH_2PO_4	0.46 g
K_2HPO_4. 3 H_2O	1.28 g
$MgSO_4$. 7 H_2O	0.5 g
Trace elements solution	1 ml
$FeCl_3$ solution (i.e. 5 mg)	1 ml (=5mg)
L-Asparagin	1.5 g
Agar	20 g (2%)

Add after sterilization:

Filter sterilized	1.2 ml
Thiamine 10mg/100ml	

SMM+$MgSO_4$

(Sonnenberg & Wessels 1987)
This medium is used for protoplast recovery.

Dissolve in demi-water:

	Per *l*
Glucose	20 g
KH_2PO_4	0.46 g
K_2HPO_4. 3 H_2O	1.28 g
$MgSO_4$. 7 H_2O	240 g
Trace elements solution	1 ml
$FeCl_3$ solution (i.e. 5 mg)	1 ml (=5mg)
L-Asparagin	1.5 g
Agar	20 g (2%)

Add after sterilization:

Filter sterilized	1.2 ml
Thiamine 10mg/100ml	

Pour at ~60°C. At lower temperatures the medium becomes turbid due to crystallisation of salt.
Do not autoclave twice, as the agar will not solidify again.
The medium can be melted again, but should be poured at ~65°C because the medium is even more likely to occur than with fresh medium.

Trace elements stock solution

(Dons *et al.* 1979) [i.e. Whitaker 1951]

Dissolve in demi-water

	Per *l*
HBO_3	60 mg
$(NH_4)_6Mo_7O_{24}$. 4 H_2O	40 mg
$CuSO_4$. 5 H_2O	0.2 g
$ZnSO_4$. 7 H_2O	2.0 g
$MnSO_4$. 4 H_2O	0.1 g
$CoCl_2$. 6 H_2O	0.4 g
$Ca(NO_3)_2$. 4 H_2O	1.2 g