Department of Genetics

Artificial Selection of sex chromosome

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Summary

In this project we looked at the effects of artificially assigning a sex chromosome on the emergence of sexually antagonistic genes, and studied at what rate deleterious mutations accumulate in the recombination block of this neo-sex chromosome. This was done using the pseudo-homothallic fungus *Neurospora tetrasperma*. This organism has two mating type chromosomes, mat-a and mat-A, each containing a large recombination block over the mating type genes. Normally the gene content is kept from becoming sexually antagonistic due to the fact that both mating type are used for all functions. In this project we forced a mating type to only be used for one specific function, in this case using ascospores as the sole fertilising agent. The opposite mating type would be static throughout the experiment, allowing the mating-type experiencing selection as the fertilising agent to adapt in absence of the normal sexual antagonistic arms race.

After allowing this evolutionary adaptation to continue for 16 generations homokaryons were isolated from the evolving lines and tested for growth speed, conidiospores production and ascospore in both male and female roles. During the homokaryon isolation a threefold increase in the number of homokaryons per isolate was observed compared to the ancestral lines. However the evolving mating type was only found at a frequency of 3% within the homokaryons. This 3% is a great deviation from the 50% presence that is expected, this expectation was based on the fact that for every mat-a homokaryotic spore there should also be a mat-A homokaryotic spore. This might be due to a germination issue which arose of the course of the evolution, as germination beyond initial budding is not required within the sexual cycle in the experiment, but is required to be picked up during the homokaryon isolation. Subsequent analysis of the homokaryons revealed that growth speed, asexual spore production and sexual spore production in the female role had remained at levels equal to that of the ancestral lines. Though sexual spore production using conidia as the fertilising agent resulted in increased ascospore production this cannot be attributed to adaptation of the evolved mating-type as it is found in all homokaryons.

On the whole we concluded that no significant decreases have occurred on any of the attributes that in this project were labelled "female" functions. As growth speed has been shown to still be at levels comparable with the ancestral lines. Asexual spore production of the evolved lines is at similar levels to the ancestral lines. Also the viability of the asexual spores as fertilising agents within the experiment appears to have increased significantly, rather than decreased as expected. In the female role sexual spore production is also still at comparable levels to the ancestral lines. In the project however two large gaps in knowledge still remain; what caused the large problems with the homokaryon isolation, and how has the male function of the ascospores developed over the course of the evolution experiment.

Acknowledgements

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Abbreviations

AMELX = Amelogenin gene located on the X chromosome
AMELY = Amelogenin gene located on the Y chromosome
DAZ = Deleted in azoospermia (gene family)
Igf = Insuline like growth factor
Mat = mating type
n = number of
PARs = Pseudo autosomal regions
SA = Sexual Antagonistic
SC medium = Synthetic crossing medium
SD = standard deviation
VMM = Vogel's minimal medium

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1. Introduction

This project seeks to provide further insight into sex chromosome evolution. It is therefore important to understand how sex chromosome evolution is currently understood. The introduction starts with an explanation as to why sex chromosome evolution occurs by explaining sexual antagonism through a series of examples. After this an in-depth explanation will be given about sex chromosome formation from the initial formation of the sex-determining gene up until shrinkage of the sex chromosome. Once the current state of sex chromosome evolution field has been discussed, the life cycle of the model organism, *Neurospora tetrasperma*, used in this project will be discussed and how it can be used to gain additional insight into sex chromosome evolution. Finally the goals and objectives of the project will be given.

1.1 Sexual Antagonism

When divergence between sexes occurs, new mutations may occur which are beneficial to one sex but harmful to the other sex. These new sexually antagonistic genes (SA genes) are hypothesized to play an important part in the evolution of sex chromosomes, sexual dimorphism and karyotypes. Sexual antagonism comes in two main branches: intra-locus and inter-locus competition.[1,2,3]

1.1.1 Intra-locus sexual antagonism

Intra-locus sexual antagonism occurs because of the different selective optima for traits and genes shared by both males and females on the autosomal part of the genome. This results in a "tug of war" between the two sexes which constrains the potential for adaptation in these shared genes, with both sexes trying to reach their respective optima. This causes sex-specific reduction in fitness. Though full resolution of intra-locus conflict is nearly impossible in the changing fitness landscape, several theories currently exist on the resolution of intra-locus sexual conflict.[4,5,6]

One theory on how this intra-locus competition can be resolved is by gene duplication events allowing each sex to diverge its own paralogs of the genes where these conflicts occur, leading to inter-locus sexual antagonism as the duplicates diverge. Interesting to note is that there are conflicting theories and evidence on how the duplicates accumulate on different chromosomes. Some state that female beneficial duplicates have a stronger tendency to accumulate on the X chromosome whereas male beneficial duplicates tend to accumulate on the autosomes. Because of problems accumulating on the Y sex chromosome due to the recombination block. Others find strong bias towards male beneficial duplicates accumulating on the male sex chromosome. Such as the male sperm producing traits occurring at high frequency only on the male sex chromosome. The resulting genes from these duplication and divergence

events, as well as other SA genes can be translocated to sex chromosome of the sex where they are most beneficial thereby further resolving any conflict by increasing or limiting, depending on the sex determination mechanism, the presence of the gene in the sex where it is beneficial whereas reducing it in the sex where it is not. Genes that are beneficial to male function would experience a great selective advantage if linked to the male sex chromosome due to its 100% presence in the male. If these genes were located on the autosome they would only be advantageous half the time and only one third of the time if they were X-linked. Whether this selective advantage makes fixation more likely remains to be modelled.[4,7]

An alternative way to resolve Intra-locus sexual conflict could be through DNA methylation and other epigenetic measures, thereby controlling potential expression of genes and allowing the organism to more closely reach the optimum. A known example of this are the insulin-like growth factors igf and igf2. These growth factors are silenced when inherited paternally. Within this system two forms of silencing occurs, one involves silencing several key igf promotors/enchancers through DNA-methylation. The second system utilizes Igf2r which is an antisense transcript to the maternal version. Thereby blocking expression of igf2. It is important to note that due to the nature of silencing this will only occur when the benefits to one sex outweigh the cost to the other.[4,8,9,10]

Another way to circumvent the intra-locus conflict is by allocating genders based on encountered conditions. In several species of lizards such as *Anolis sagrei* and *Uta stansburiana*, sex ratio is determined by the size of the male the female has mated with. The larger the male the more male offspring will be produced. This way the strongest genes will be transferred to the gender where they are the most liable to produce a positive outcome.[4]

The last way that will be covered in this introduction is resolution by alternative splicing. In this method the sex of the organism correlates to which splice sites will be used and subsequently determines final protein shape. Resulting in different proteins function based on the gender where it is present.[4]

1.1.2 Inter-locus sexual antagonism

As previously discussed inter-locus competition can be viewed as a solution to intra-locus competition, as well as a means to acquire further traits de novo which are sexually antagonistic. Inter-locus competition can be subdivided into three types: sex determining genes, directly harmful traits and indirectly harmful traits.[1,3,11]

The first and strongest sexually antagonistic trait would be a trait that would completely destroy the function of the other sex. This is precisely the case for a sex-determining gene. A sex-determining gene is, as the name implies, a gene or a set of genes which determine the sex of the organism.[1,11,12]

The second type are genes that confer a trait which is directly harmful to the other sex. One of the clearest examples of this is found in *Drosophila*. In this species the sperm of the male is highly toxic in order to kill the sperm of competing males. However it is also toxic to females, causing their death if exposed to a large quantity of sperm. A second example would be genes that boost male attractiveness to females, as these genes do not increase female reproductive success but do increase the chance of predation.[1,7,11,13]

A good example of both the third type, indirectly harmful traits, and how intra-locus competition can lead to inter-locus sexually antagonistic traits is teething in humans and other mammals. In mammal species where females are promiscuous, early teething would confer a fitness benefit to the mother. This would be attained by shortening the weaning duration and allowing the female to regain fertility. However the father would prefer later teething, as weaning promotes growth and the immune system increasing offspring survival and fitness, at the cost of monopolizing maternal resources. In some primates a strong correlation has been found between weaning age and the inter-birth interval of the mother. Furthermore in many primate species males tend to teeth later overall than females, though it should be noted that females develop faster than males on other fronts as well. During teething Amelogenin proteins form the scaffold for the accretion of tooth enamel. The genes encoding for Amelogenin have both and X (AMELX) and Y (AMELY) homolog. The Amelogenin genes serve several roles in regulating bone and cartilage growth depending on the splice products. The genes encoding for Amelogenin are predominantly expressed on the X chromosome in most mammals and only to a lesser extent on the Y chromosome. This expression on Y is puzzling as recombination between these homologs stopped 30-50 million years ago, plenty of time for the Y-gene to decay and lose all function. This would therefore indicate that some function still exist. Upon comparison of AMELX and AMELY it is revealed that AMELY misses a splice site, which causes disruption of one of the proteins required for the programmatic turnover in tooth eruption, causing AMELY to produce the sexually antagonistic phenotype of delayed teething.[7]

1.2 Sex chromosome formation

Sex chromosomes have arisen independently in multiple dioecious taxa from separate pairs of normal autosomes over the course of evolution. Because of this many structural and phylogenetic differences can be found between taxa such as mammals, insects, plants and birds. However many striking similarities can still be found. Chromosomes that are always in a hemizygous state are the Y chromosome in XY systems where male is hemizygous, and the W chromosome in ZW systems where the female is the hemizygous sex. In both systems the hemizygous sex chromosome has a strong tendency to be small, rich in repetitive sequence, and poor in genes. Their non-sex specific counterparts, the X and Z chromosomes, tend to show stronger similarities to autosomes in both size, form and genetic content. The consequence of this division in sex chromosomes is that the homozygous sex chromosome will occur at three times the frequency within the population given equal male female ratios. In many species dosage compensation has evolved in order to regulate gene expression between sexes on this autosome-like sex-chromosome. These strong parallel evolutions between sex chromosomes with different origins strongly suggest similar selective pressure being exerted by common factors which drive the evolution of sex chromosomes.[7,12,14,15]

Mammalian sex chromosomes are thought to have arisen from an ordinary pair of autosomes roughly 300 million years ago. Until then ambient temperature during embryonic development might have determined the sex of mammalian ancestors, as in many extant reptiles and other descendants of bony fish. This ceased to be useful for mammals and birds once homeotherms arose. This led to the emergence of two sex-determining genes in mammals from one progenitor gene: SOX3 and SRY. SRY gained and kept the male-determining function. The emergence of these two dominant and penetrant sex determining allele effectively rendered these two autosomes into sex chromosomes. An alternative theory proposes that the mammalian XY system is actually a ZW system that was overtaken via a Z autosome translocation in a ZZ male animal combined with the emergence of a dominant male sex determining gene.[7,14]

The emergence of one or multiple sex-determining genes has often been said to be the first step in sex-chromosome formation. Near this sex-determining region additional SA genes will then accumulate. The accumulation of SA genes near the sex-determining region of the chromosome brings the distinct advantage of increased presence in the sex where the SA gene is beneficial. If a gene is advantageous in the hemizygous sex and it translocates to the hemizygous sex chromosome it will then occur 100% of the time in the hemizygous sex. If a gene is beneficial to the homozygous sex and it translocates to the homozygous sex chromosome it will occur 66.6% of the time in the homozygous sex and 33.3% of the time in the hemizygous sex. This is a substantial increase from the normal 50% occurrence in the beneficial sex when present on an

autosome, and therefor allows for genes that are strongly sexually antagonistic to still be fixated in a population. A recent example of this is the DAZ gene. This multi-copy gene has recently crossed over from an autosome to the Y-chromosome. This gene has been shown to have a strong function in spermatogenesis, and appears to have been formed recently as it is not found in non-primates. Normal genes show little correlation between where they are on chromosomes and their function, and functionally related genes showing no correlation in occupying a particular chromosome or region. This however is different for the human Y chromosome which shows only a few distinct expression profiles to the extend where strong correlation is visible between in what tissue a gene is expressed and its function.[7,12,15]

1.2.1 Recombination Block formation

Once the sex determining genes and other SA genes have come to the same chromosome a large selective advantage can be gained by keeping these genes together. This is especially so for those genes that are directly harmful to the other sex when present. To this end a recombination block is formed. Though we will discuss the formation of the recombination block from the XY system, the same applies to ZW.

A recombination block will only be formed when there is more than one sexually antagonistic gene present on the neo-Y chromosome. The recombination block is primarily formed through inversions. In the human X and Y chromosome the non-recombining regions have expanded several times, each time converting a block of previously freely-recombining sequences into X- and Y- specific regions. The difference in gene order compared between the X and Y, with Y showing a much higher order of scrambling in the gene order, indicate that the recombination block was formed by serial, large scale inversions of much of the Y chromosome. This seems to have occurred four times in the evolution of the human Y chromosome. These inversions disrupted the alignment between the X and the Y, thereby disrupting recombination in that area.[7,15]

Translocations or centric fusions involving an autosome and a sex chromosome also play a role in the formation of the recombination block. These translocations or centric fusions can create linkage between SA genes on autosomes and the sex chromosomes, while at the same time creating sufficient sequence divergence to block recombination within the region. Translocations or centric fusions often occur at the onset of sex-chromosome formation and can start of a recombination block. A good example of this can be found in *Drosophila Miranda*. Here a Y-autosome fusion has occurred between the third chromosomes of its close relative *D. pseudoobscura.* The fusion causes the neo-Y chromosome to immediately stop recombining. A similar occurrence, but involving the X-chromosome has been found in *D. Americana* which is a close relative of *D. virilise*. Here an X-autosome fusion is either fixed or polymorphic in

some populations. In this last example the neo-Y chromosome is not physically attached to the Y, but co-segregates with the true Y chromosome from the X and the neo-X in males. The precise mechanics behind the centric fusion and translocations fall outside the scope of this project and will not be explained here.[7,15]

It is also conceivable that the recombination block can expand on its own, by virtue of recombination being rare at the very edge of the block. This results in sequence divergence over time, moving the boundary of the recombination block itself.[7,15]

As recombination is still required for cell division the non-recombining region is flanked by two pseudo autosomal regions (PARs), these serve as the recombining points during meiosis. These PARs therefore show a much larger rate of recombination then normal autosomal regions. The PAR's resemble autosomes in base composition, gene-density and diversity. The genes found in these PARs show little to no sexually antagonistic properties and most are exempted from X-chromosome inactivation as they are of equal dosage between the sex chromosomes.[7]

1.2.2 Consequences of the recombination Block

Genes in the non-recombining region have two distinct origins: evolution from the proto-Y genes, which were highly homologous to the proto-X chromosome, or recruitment from elsewhere in the genome. The fate of these genes falls in one of three categories: preservation in ancestral form, specialisation in male specific function or functional decay.

The first class of genes are those that are still homologous between the X and Y chromosomes. Genes may persist on both the X and Y chromosome non-recombining region if their function is crucial in both sexes such as for example housekeeping genes. In this case the genes continue to exist with little differentiation on both the X and Y chromosome. These homologous maintain an equivalent dosage in both sexes, should avoid X-inactivation and have equal function in both sexes. Experiments have observed protein to nucleotide divergence between such homologues to be significantly lower than in neighbouring loci. This is consistent with the idea of conserved functional similarities within these genes. [14, 15]

The second class are those genes with the recombination block that persist due to their direct advantage to the male sexual function and are very often sexually antagonistic, for example by playing a role in spermatogenesis or somatic masculinization. These genes differ significantly in function from their X homologues, which in all likelihood maintain their original function. Most class two genes have multiple copies along the Y chromosome. These multiple copies might be serving as a buffer against deleterious mutations. The long-repeat sequences found throughout hemizygous sex chromosomes might serve to induce further amplification, by mediating amplification

in repeat flanked genic regions. These genes often find their origin in a homologous pair where the X-homolog still has the original function and the Y-homolog has a new function often with a tissue specific expression.[14,15]

The third class of genes experience functional decay resulting from the increased genetic drift experienced by genes within the recombination block. In combination with accumulation of transposable elements and other deleterious mutations this often results in functional decay within the genes, as long as dosage compensation evolves this also happens to genes with critical functions. [14,15,16]

Many downsides, in addition to possible functional decay, are associated by having a recombination block. One of the main disadvantages is that new Y chromosomal alleles within the recombination block cannot move to other beneficial alleles that have occurred on the Y chromosome of another male. This limits the accumulation of new Y chromosome-advantageous alleles on the chromosome. The recombination block also shelters or enables the accumulation of non-essential or even parasitic retroviral and heterochromatic sequences. This results in the accumulation of transposable elements and other highly repetitive sequences, for which replication is unlikely to benefit the host. This and other forms of gene loss by genetic drift have been observed in most mammalian species as well as in some haploid systems where recombination block are present, such as *Chlamydomonas* and in some fungi. The combination of these factors, as a consequence of the recombination block, severely limits future evolution potential, and have been shown in *Drosophila* to threaten the long-term genetic integrity of a population and result in a slow shrinkage of sex chromosome. [7,14,15,16]

1.2.3 Sex chromosome shrinkage

The fact that the hemizygous sex chromosome degrades and can even be lost entirely is supported by several comparative studies in insects and vertebrates. In humans the difference between the X and Y chromosomes is about 105Mb. There is also a substantial difference in the number of genes, with the X having over a 1000 protein-coding genes and the Y only 45 unique proteins related to X, all others have deteriorated. It has been found in snakes that this degradation is an ongoing process. Within the snake families large differences can be found in the size of the W chromosome. In the *Python* genus the W is almost equal in size to the Z, whereas in *Viperidae* the W has differentiated from the Z to an extreme extent. This can also be found in some bird families. In mammals where the study is more in depth it can be seen on a gene level.[14]

Many models point to the recombination block as being the primary culprit for the shrinkage of the always hemizygous sex chromosomes. Genes within the recombination block accumulate null alleles because intact X homologues shelter/compensate for them, and without recombination to regenerate/purge them from the chromosomes

accumulation occurs. This becomes more profound if these null alleles are linked to beneficial alleles allowing the null mutations to hitchhike along with the beneficial mutation. Despite degeneration, some Y chromosomes like those found in the *Drosophilidae* have greatly expanded in size due to the translocations from autosomes.[7]

1.2.4 Dosage Compensation

One of the main consequences of the degeneration that occurs in hemizygous sex chromosomes is the up-regulation of these genes on the opposite chromosome. This results in a problem for the homozygous sex as an overdose of these transcripts would be produced. In order to prevent this, several dosage compensations systems have evolved. In mammals for example, one of the two X chromosomes is transcriptionally silenced in the early stages of embryonic development and remains that way for the rest of the organism's life. The molecular mechanisms behind the inactivation are complex and find their basis in epigenetic changes, involving the binding of untranslated RNA followed by the accumulation of histone modifications and DNA methylation. Comparison of the inactivation mechanisms found in humans with those found in distantly related marsupials has given insight in the evolutionary history of these systems. In placental mammals X chromosome inactivation is maternal, very stable yet slightly random. In marsupials however X chromosome inactivation is paternal, incomplete and tissue specific; also the molecular mechanisms underlying the silencing in marsupials is simpler, lacking DNA methylation, it however shares the histone modification mechanisms. A recent study showed that in humans many genes on the X-chromosome escape inactivation even in absence of the active Y partner. This leads to two interesting conclusions, the first conclusion is that the X-chromosome-inactivation system is continually expanding and is lagging behind on Y degradation. The second conclusion is that dosage compensation is not an extremely urgent requirement as females without the inactivation still maintain a fit phenotype.[14,15]

1.3 Model organism *Neurospora tetrasperma*

As can be seen in the previous section most of the knowledge regarding sex chromosome evolution stems from mammals and other higher organisms, rather than plants and fungi. However due to the shorter generation cycle of lower organisms, such as fungi, these provide an interesting model for evolutionary experiments.[17]

In fungi two main breeding systems exist, known as heterothallism and homothallism. A heterothallic species requires the interaction of two individuals of opposite mating type to complete the sexual cycle. Whereas a homothallic species is self-fertile and can complete the sexual cycle on its own. In this project we will use the model organism *Neurospora tetrasperma*. This is a pseudo-homothallic species, which means it has a two mating type system similar to heterothallic species, but has both mating-types within a single spore allowing it to be self-fertile. In order for these two mating types to reliably occur in a single spore a recombination block is required. The recombination block contains the mating type genes. This provides an interesting opportunity to study early sex-chromosome evolution, as SA genes could accumulate near the recombination block when sufficient sex-specific selective pressure is applied.[17,18,19,20,21,22]

The genus *Neurospora* is found in all moist tropical and subtropical areas in which it might be sought. It penetrates temperate climates in connection with human agricultural and commercial activities. In the past is has been a great contaminant in the bakery and sugar cane industry. *Neurospora* is conspicuous in nature as one of the first colonists in areas of burnt vegetation. This reflects the presence of ascospores, the products of the sexual cycle, which require heat activation prior to germination, as well as a well-developed capability of the organism to use cellulosic materials, particularly in moist hot habitats. The extension of the habitat of *Neurospora* to bakeries and areas where residue of sugar cane is collected is understandable on the same basis.[18]

As mentioned before the model organism *N. Tetrasperma* has a pseudo-homothallic lifecycle. Which can be subdivided in a sexual and an asexual cycle.

1.3.1 Asexual cycle

The lifecycle of *N. tetrasperma* can be seen in figure 1. The mycelium of the fungus is haploid. However the hyphae, the tubular filaments of the vegetative state, contain multiple nuclei which are not separated into different cells. This state is also known as coenocytic. The conidia are formed on aerial hyphae. Each macroconidium contains a multitude of haploid nuclei, with two nuclei being the most frequent. Upon coming into contact with suitable conditions the conidia will germinate by a germtube protruding from the conidia, forming the first hypha. This first hypha will continue to grow by tip extension and branching out forming a normal mycelium network. In order to strengthen the network cross walls are formed. Pores in the cross walls allow for the

exchange of cytoplasm, nuclei and organelles in young cells. This flow is restricted as the organism ages. When nutrients are exhausted aerial hyphae are formed, and by subsequent budding and segmentation yield macroconidia. The macroconidia are dispersed by wind, and on subsequent germination restart the cycle. In order to facilitate this wind dispersion the conidia are coated in a hydrophobic "rodlet" protein, which maintains the dryness of the spores allowing for easier dispersion. The macroconidia are also coated in an intense orange carotenoid pigment in order to further protect the conidia from light/UV-radiation.[18]

In addition to macroconidia, *N. tetrasperma* also produces microconidia. These microconidia are substantially smaller and uninucleate. Contrary to macroconidia these are extruded serially from small branched micro-conidiophores. The germination and subsequent growth occurs in the same fashion as with macroconidia, however the germination rate is substantially lower. The extend of this lower germination rate varies greatly between strains and the growth media used.[18]

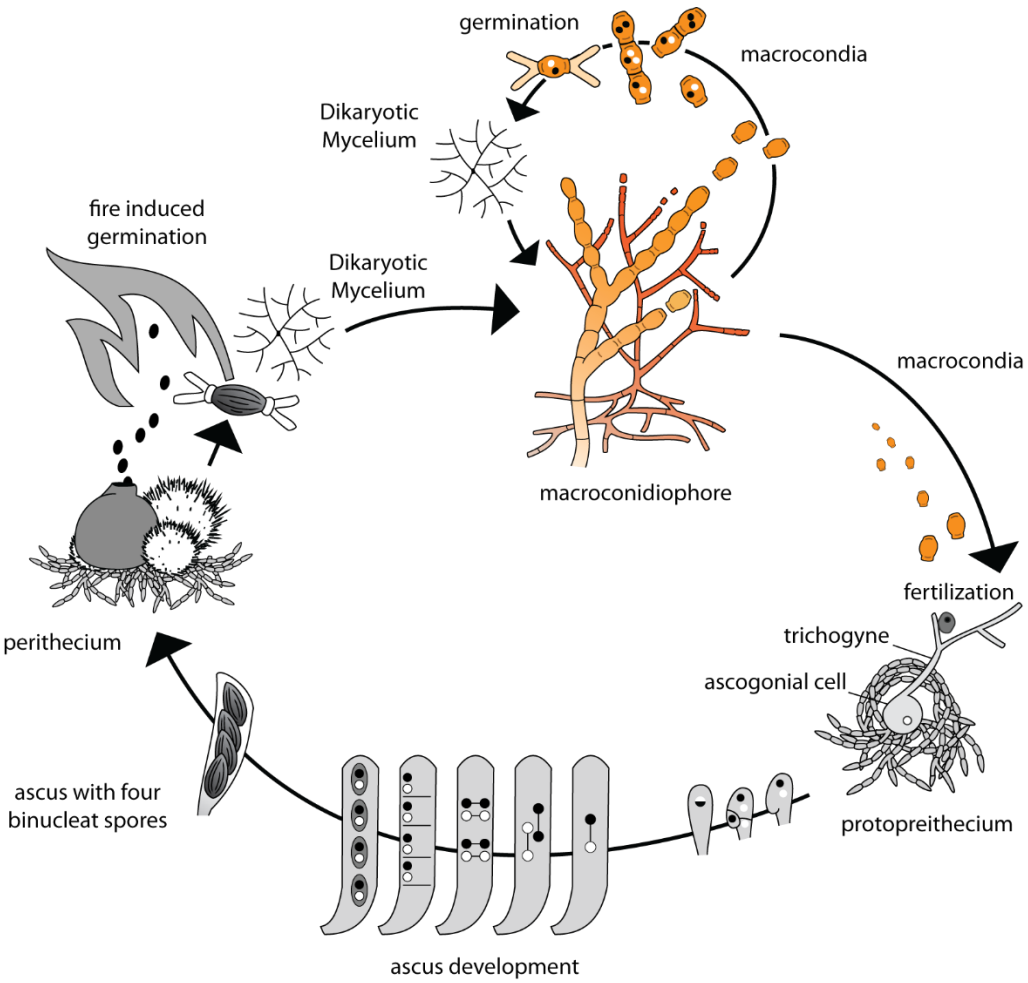


***Figure 1: Pseudo-homothallic lifecycle of* Neurospora tetrasperma.** *The cycle at the top is the asexual life cycle. Wherease the larger bottom cycle is the sexual cycle.*

1.3.2 Sexual cycle

The sexual cycle of all heterothallic and pseudo-homothallic *Neurospora* species require the presence of both mating type chromosomes. In the case of *N. tetrasperma* these are named mat-a and mat-A.[18,19,20]

The sexual cycle begins with the formation of a protoperithecium. Protoperithecia form in conditions of reduced nitrogen and carbon. Both of these, and especially nitrogen, are common limiting nutrients in nature. Protoperithecia begin to form as a small knot of hyphae around a few cells which will form the ascogonium. One of these cells functions as the female gamete. The hyphae around the proto-ascogonium form a protective barrier from which several trichogynes emerge. These find their origin in the female gamete and serve as contacts for the fertilizing agent. The trichogynes grow for a substantial distance and have the capability to branch out, looking for potential fertilizing agents. The fertilizing agent is typically a conidium which contains the opposite mating type. Hyphae and germinating ascospores can also serve as the fertilizing agent. Trichogynes respond to pheromones emitted by the fertilizing agent. Upon detection the trichogynes will grow towards the source until fusion occurs. Once fused plasmogamy occurs and a nucleus of the conidium or other fertilizing agent will travel along the trichogyne towards the ascogonial cell of the protoperithecium, where karyogamy and nuclear fusion subsequently occurs. This will trigger the formation of the asci within the protoperithecium. After meiosis and the mitotic division the ascus contains eight nuclei, four for each mating type. Each ascospores is cut around two nuclei, one of each mating type. This is accomplished by the recombination block around the matingtype genes and first meiotic division segregation (figure 1 and figure 2). During the second division segregation the spindles normally run parallel or overlap, and are at the right angle along the long axis of the ascus. One nucleus of each mating type is then enclosed by the ascospore wall resulting in ascospores which are heterokaryotic for mating type. Occasionally, three large ascospores are observed together with two smaller ascospores. In this event the smaller ascospores are homokaryotic. After the formation of the ascospores the asci further mature as the ascospores ripen.[16,18,19,22,23,24,25]

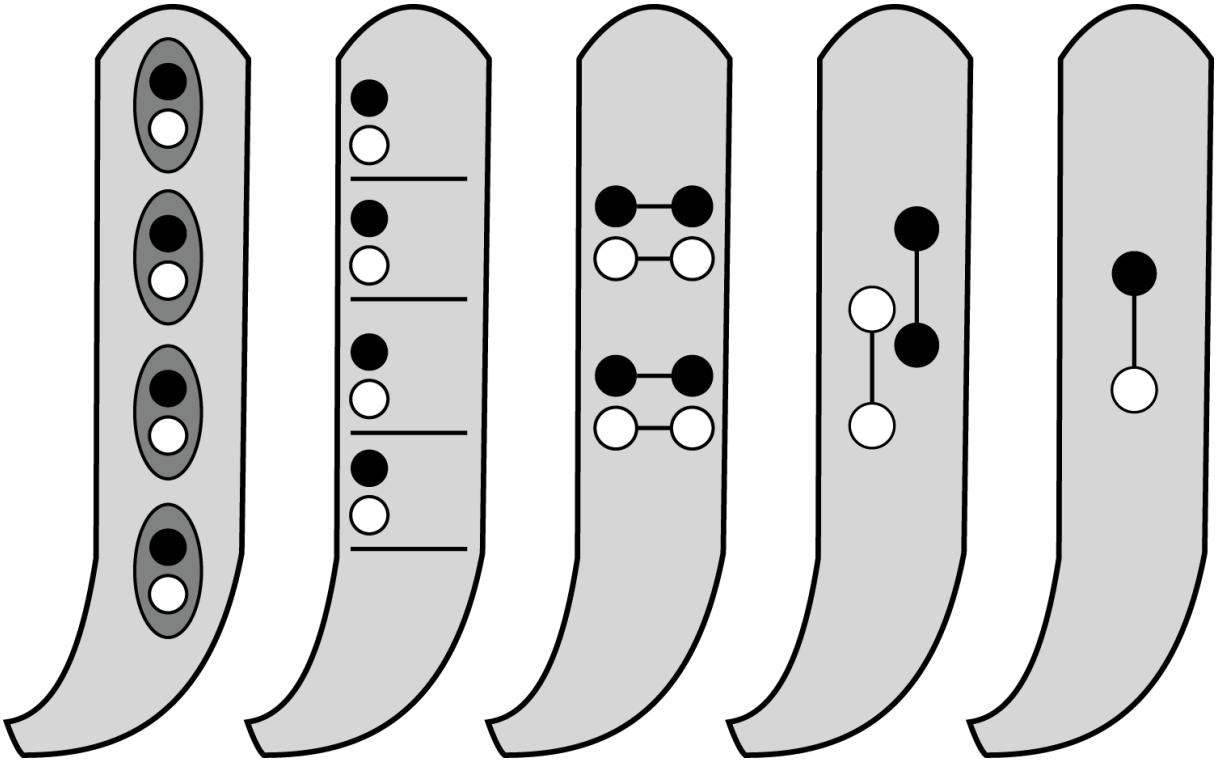


***Figure 2: Ascospore development in* Neurospora tetrasperma.** *The solid and open circles represent nuclei of mat-A and mat-a. which segregates at the first meiotic division and subsequently get packed in to 4 distinct ascospores.*[15]

In conjunction with the maturation of the asci, the perithecia itself enlarges and its walls become hard and melanized. At the top of the perithecium a beak like structure forms with a pore, also known as the ostiole. As this beak is formed it orients itself towards the light. The mature asci then insert their tip one at a time into the ostiole. Osmotic pressure within the ascus then causes the four ascospores to be launched from the perithecium adhering to the first thing they strike. This adhering is enabled by the gummy substance in which the ascospores were suspended while still in the ascus. The ascospores germinate upon a heat shock and grow in the same fashion as discussed earlier for conidia. Dormant ascospores can survive in the soil for extended periods of time even in absence of suitable nutrients. They can be activated later by fire or by chemicals released by fires such as furfural which seeps into the soil triggering germination.[18,19]

1.3.3 Genomic structure of mating type genes

In most *Neurospora* strains heterokaryons are blocked by heterokaryon incompatibility genes. In N. tetrasperma mating type heterokaryosis is a natural condition of the mycelium. This is permitted by an allele of a gene called "tol" in *N. tetrasperma* which, unlike for example, the *N. crassa* allele, overrides the effect of the mating-type genes in restricting heterokaryon formation. This, in combination with the 7Mbp recombination block on the mat chromosomes, allows for perfect segregation of both mat-types per ascospore. As the non-recombining region of the mat chromosome is limited it is still flanked with normal recombining regions. These regions can be easily equated to the pseudo autosomal regions found in fully fledged sex chromosomes. Also because the non-recombining region is relatively young (<6 MYA), no large scale degeneration has yet occurred, which is commonly observed in sex chromosomes of most other taxa. This degeneration is one of the main limiting factors in the study of sex chromosome evolution in those systems. These factors make the *N. tetrasperma* mating type chromosome's ideal for study into sex chromosomes, as they partake to recombination blocks.[18,22,26,27]

1.4 Objectives

The goal of this project is to test what effect artificially assigning a sex chromosome has on the emergence of sexually antagonistic genes, and at what rate deleterious mutations accumulate in this neo sex chromosome. This was done by forcing each of the mating-types to only be used for a single role. In this case the ascospores were used as the sole paternal fertilising agent. These ascospores were harvested, heatshocked and then immediately used to fertilise a static female line homozygous for the opposite mating type. This resulted in a lifecycle within the experiment that can be seen in figure 3. In this experimental cycle the static female line is supplied fresh in each cycle. It is then fertilised with a heatshocked ascospore from the previous cycle, which serves to fertilise the ascogonial cell. Because the ascospore is dikaryotic and the female is homokaryotic only the mating-type chromosome opposite to that of the female is utilised. Resulting in only one mating type chromosome being transmitted whereas the other is refreshed.

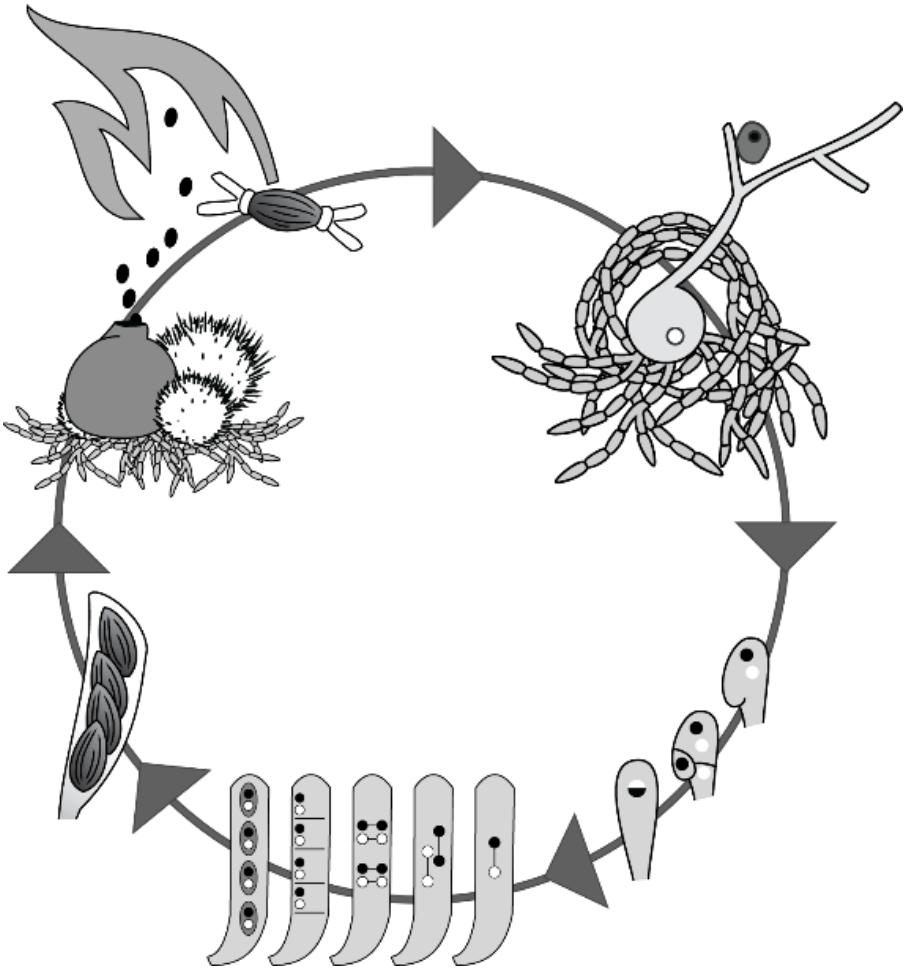


Figure 3. The lifecycle as experienced by the mating-type chromosome on which selection occurs. *In the experimental cycle the static female line is supplied fresh in each cycle. It is then fertilised with a heatshocked ascospore from the previous cycle, which serves to fertilise the ascogonial cell. Because the ascospore is dikaryotic and the female is homokaryotic only the mating-type chromosome opposite to that of the female is utilised. Resulting in only one mating type chromosome being transmitted whereas the other is refreshed.*

In this setup the same mating type chromosome is transferred each generation, whereas the other mating-type chromosome is refreshed every generation and the autosomes are refreshed every other generation on average. Because of this setup the mating type chromosome that is transferred is completely free to accumulate "male" beneficial "female" detrimental mutations. Male beneficial traits in this context would be traits that positively effect ascospores in their role as the sole fertilising agent. These new traits can in turn be detrimental for "female" functions as traits involved in these functions are not utilised over the course of the evolution experiment, allowing for highly sexually antagonistic male traits to develop. Female functions in this experiment are: germination rate, growth rate, asexual spore production and sexual spore production when the mating type is used in the maternal role.

Besides being free to develop detrimental traits for female functions, the large recombination block found on the mating type chromosome of *N. tetrasperma* will also allow for deleterious mutations to accumulate on any genes pertaining to female function within the recombination block. As no disadvantage will be experienced when these mutation accumulate we can expect this to further deteriorate female traits over the course of the evolution experiment.

As result of our experiments we therefore hypothesise an increase in ascospore production and in the competitive fitness of the rate at which ascospores fertilise the ascogonial cell compared to the ancestral lines. Whereas the removal of selective pressure on female traits will cause a reduction in germination rate, growth rate, asexual spore production and sexual spore production when the mating type is used in the maternal role. This will be the result of the creation of new male-beneficial female-detrimental traits, in combination with mutation accumulation, within the recombination block, in the genes which experience no selective pressure.

2. Material and Methods

2.1 Strains and media

In this project several strains of *Neurospora tetrasperma* were used, with different mating types and mutations (see Table 1). All strains were stored intermittently during the project at -20°C in small slanted tubes with Vogel's minimal medium (VMM), and stored shortly after the maturation of the conidia. A master stock was established on dry silica beads and stored at -80°C. The evolved lines were later stored at -80°C in 35% glycerol solution.

Table 1: *Strains used in this study*

	Mating type	**Genotype**	**Internal code**	**FGSC-number**	**Mutation**	**Papers**
Neurospora tetrasperma	**A**	**W11**	**NC-138**	**FGSC-9033**	**WT**	
Neurospora tetrasperma	**a**	**W12**	**NC-139**	**FGSC-9034**	**WT**	
Neurospora tetrasperma	**A**		**n/a**	**FGSC-1256**	**Albino (al-102)**	**Howe et al. 1966. Genetics 54:293-302**
Neurospora tetrasperma	**A**		**n/a**	**FGSC-7318**	**Peach (pe)**	
Neurospora tetrasperma	**a**		**n/a**	**FGSC-7319**	**Peach (pe)**	

All media used in this experiment can be found in Appendix I. All media used in the experiment are as dictated in Appendix I unless otherwise indicated. Such as the addition of sorbose or differing amounts of sucrose used.

2.2 Evolution experiment setup

The goal was to allow the evolving male line to develop male beneficial traits in absence of an evolutionary arms race. Therefore, static wild-type female lines were established for both mating types. These static lines were made by inoculating several small slanted tubes with VMM medium. The lines were then grown for four days at 24°C with a 12 hour day/night cycle. These lines were then stored at -20°C for later use.

The experiment had three male lines of each mating type evolving at each stage, six lines total. For the first generation three tubes of synthetic crossing medium (SC) were inoculated with conidia of each mating type. These served as the first females. The lines were then fertilised with conidia of the opposite mating-type. This was the only generation where conidia are used for the transfer of genetic material. Adjacent to this a new female line was inoculated on fresh SC medium. Both were then incubated at 24°C for seven days. The ascospores were then harvested with 3 mL saline solution, and vortexed to bring the spores in suspension. The resulting suspension was then transferred to a 2mL Eppendorf tube and judged for its ascospores content. If necessary the suspensions were concentrated. If concentrating the suspension was necessary this was done at 13000 rpm for 5 minutes, after which the saline supernatant was partially discarded. The ascospores suspension was then transferred to an Eppendorf tube and subjected to a heat shock of 60°C for 30 minutes. The slanted SC tube containing the new female was cleaned with a whet cotton swab. This was done to remove the conidia rich hyphen. The female was then fertilised with 500µL of the heatshocked ascospores suspension, and left to rest at an angle for a period no shorter than one hour. The fertilised females were then incubated for seven days at 24°C. A new female line was also inoculated from the same stocks. From this point on ascospores harvested at each generation served as the only genetic transfer agent. All conidia were destroyed during the aforementioned heat-shock administered to the ascospores suspension. This process was repeated over 16 generations.

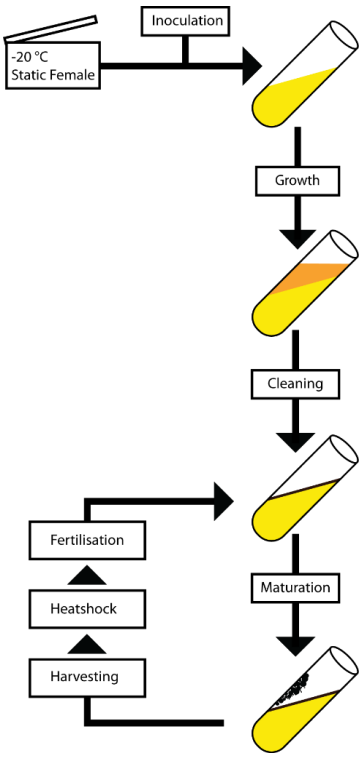


Figure 4: Evolution experimental setup

2.3 Biotin & Sugar utilisation experiment

As a reduction in ascospores production was observed, a test was developed to ascertain whether deterioration in the biotin stock was the cause of the observed reduction. This test consisted of increasing concentrations of biotin to compensate for any degradation that had occurred within the stock. For this a positive gradient of biotin concentrations were tested on standard SC medium. Ranging from the normal of 3µL of biotin solution (0.1mg/ml) and increasing at two fold increments to 10 fold the normal amount. When this experiment showed no changes it was repeated and redone with a fresh biotin stock.

As the biotin test showed no increase in ascospores production a second test was conducted with glucose and sucrose using a negative gradient. All other components in the SC medium were identical. The sugar concentration ranged from the normal of 1 mL of 2M sucrose, and decreased by gradient of: 0,75mL > 0,5mL > 0,25mL > 0,125mL 2M sucrose solution. A negative control of 2mL of 2M sucrose was also included.

When the negative control showed a positive increase in ascospores production the experiment was repeated with a positive gradient of: 1mL > 2mL > 3 mL > 5mL > 10mL of 2M sucrose. Results were compared to the outcome of the previous experiment and the 1mL baseline.

2.4 Homokaryon isolation

To get a clear picture on how the male function had developed, homokaryons were obtained. This was necessary to reduce the amount of interference and genetic compensation by the other mating type. Thus revealing any negative mutations that had occurred. To obtain homokaryons a diluted ascospores suspension, of 300 spores per 100 µL was plated on to 0.05% sorbose SC medium with 0.5% sucrose to keep the developing colonies as small as possible. The plates were incubated for a period of one to two days at 24°C. On each day colonies of an adequate size were isolated and transferred to a VMM slanted tube overlapping colonies were avoided. For each line no fewer than 60 colonies were picked. After four days of incubation the conidia produced were transferred to fresh VMM to test the karyotic state. This was done in duplicate. Incubation was done for fourteen days at 24 °C after which karyotic state was scored by the presence of ascospores. Mating-type of the homokaryons was determine by fertilising females of each mating type with conidia of the isolated homokaryons. These tubes were incubated for a period of fourteen days prior to scoring ascospores production. This same procedure was used to obtain an albino mat-A mutant. Only in the initial ascospores suspension ancestral mat-A was crossed with the albino mat-A mutant. A clear overview of the homokaryon isolation can be seen in figure 5.

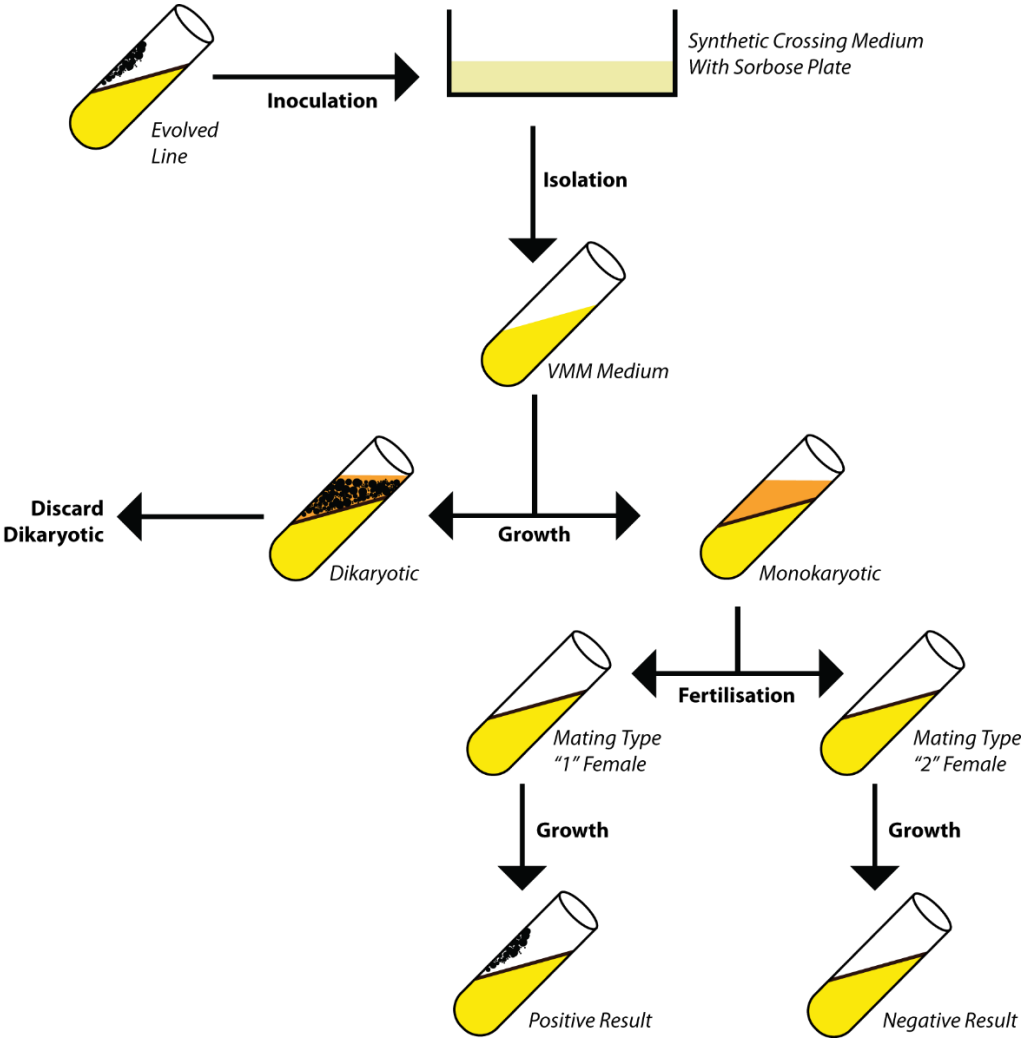


Figure 5: Homokaryon isolation setup

2.5 Growth rate

Growth rate was determined for all isolates using race tubes, pipet tubes filled horizontally with either of the two media to be tested. This was done by injecting 15mL of SC or VMM medium into the pipet. The pipet was then positioned horizontally for a period of 30 minutes until the medium had solidified. The experiment was performed in triplicate for all lines on both a low sucrose (0.5%) SC medium and normal sucrose (1.5%) VMM and SC medium. All tubes were inoculated with conidia from their associate line with a toothpick. A variable amount of spores was used as fluid could not be used for the inoculation. The tubes were then left to incubate in a dark incubator at 27°C for six days. Growth was marked on the tube at the end of each day, many line already reached the end of their race tube by day four, after which they were removed from the incubator to prevent cross contamination.

2.6 Spore production tests

In order to test changes in spore production, all lines were tested for both conidia and ascospore production. This was done on slanted tubes filled with VMM for conidia production, or SC medium for ascospores production. The tubes were inoculated with $1*10^6$ spores. This was done by making a conidia suspension and counting the number of spores in a counting chamber under a light microscope. The suspension was then adjusted to contain $1*10^6$ spores per 200µL. This was then used to inoculate the tubes, prior to each inoculation the suspension was vortexed to ensure even distribution of spores. These were then left to incubate for a period of four days at 24°C. The conidia suspension was then harvested and counted in a counting chamber under a light microscope. All lines were tested in triplicate. And all tests were counted in triplicate.

For the ascospores test a series of SC tubes were inoculated with $1*10^6$ spores of ancestral spores of both mating types to test male function. And a second series of tubes was inoculated with $1*10^6$ spores of each homokaryon isolated from the evolving lines to test for female function. After seven days of growth the tubes were then fertilised with 1 million conidia of the line to be tested for male function test. For the female function test the tubes were fertilised with $1*10^6$ spores of the ancestral of the opposite mating-type. After a further seven days of growth ascospores were harvested, diluted and counted on a water agar plate under a binocular. All tests were counted in triplicate.

3. Results

3.1 Effect of resources on the production of ascospores.

Over the course of the experiment it was noted that the production of ascospores was steadily declining. This started with a lower initial amount of ascospores already at generation one when compared to the previous trial run and further declined over the course of the experiment. At generation 10 the problem was solved.

A number of experiments were designed to bring back ascospore and conidia production to levels previously found during the trial run of the experiment. A list of potential causes was established based on previous experiments. Limitation of growth due to supply of biotin and supply of sugar were tested first. The initial list of potential causes stretched beyond the two variables tested within this project, but the problem was solved before these required testing. All results from these experiments relied on qualitative observations, no measurements were conducted.

3.1.1 Biotin

Previously found to be an issue in experiments on *Aspergillus*, the first component of the medium tested was biotin (Fons Debets, verbal consultation). Compound degradation of biotin had been found to have been the issue in those cases. Our first experiment was therefor to increase the biotin concentration to compensate for this degradation. After the fourteen day incubation period, all lines with increased biotin showed identical phenotype, ascospore production and conidia production to the control.

Due to the lack of any visible improvement between the strains a new batch of biotin was ordered from the supplier and the experiment repeated. This yielded the same results as with the previous batch of biotin. It was concluded that no biotin degradation had occurred.

3.1.2 Sugar concentration.

An experiment was set up to examine de influence of sugar concentration on the production of ascospores. The initial experiment consisted of reducing the sugar concentration. Previous experiments showed that a reduction in sugar concentration led to an increase in ascospores production. This trend continued to a concentration of 0.1% sucrose.

In this experiment, however, a decrease in ascospore production was observed with decreased sugar concentration, with minimal ascospores observed at the top of the tubes. This trend followed the reduction in sugar, with the lowest concentration in sucrose also having the lowest ascospore production. The negative control, which had double the sugar content from normal, showed an increase in ascospores production.

Further experiments with increasing sugar concentrations, resulted in ever increasing amounts of ascospores. This trend still held up at a 10-fold increase and is expected to continue beyond this point.

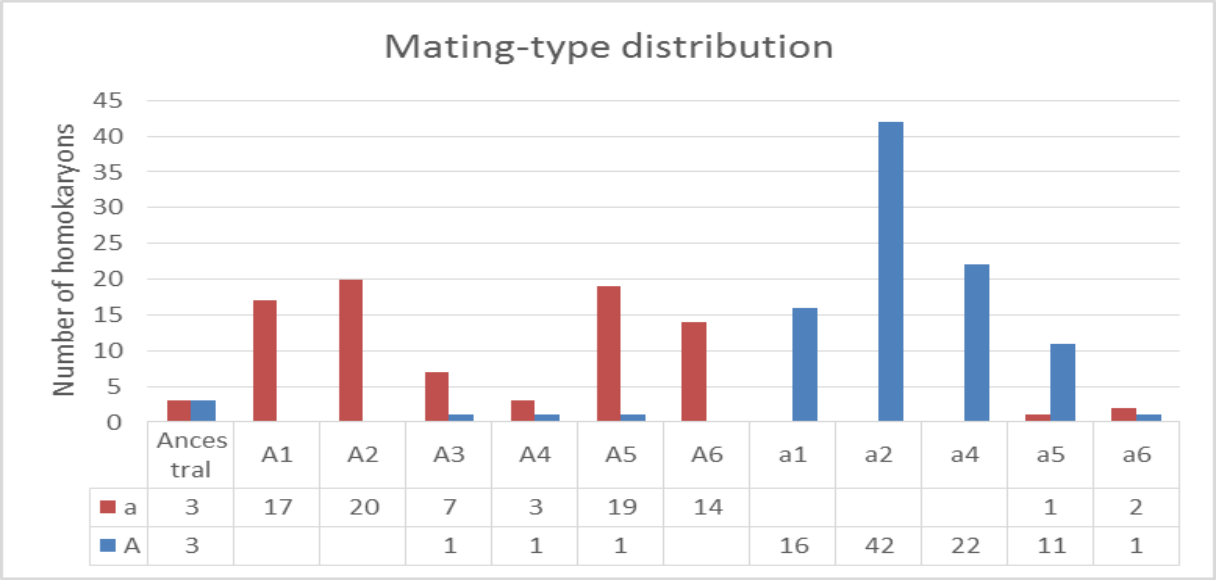
3.2 Homokaryon frequency in isolates.

A total of 2064 isolates were made. The total number of isolates and number of homokaryons obtained can be seen in Table 2.

Table 2: more homokaryons found per isolate in evolved lines then in ancestral lines. *Number of isolates compared to the number of homokaryons found in those isolates.*

	Number of isolates	Number of homokaryons	Percentage of homokaryons to isolates
Ancestral lines	192	6	3.1
Evolved lines	1872	178	9.5

The number of isolates required to obtain each homokaryon differed greatly between evolved and ancestral lines. The 192 isolates of the isolates of the ancestral cross 3.1% yielded homokaryons. Of the 1872 isolates of the evolved lines 9.5% yielded homokaryons. The number of homokaryons of each mating type for each line can be seen in Figure 6, the table directly underneath the graph provides numerical values for each point.



	Ancestral	A1	A2	A3	A4	A5	A6	a1	a2	a4	a5	a6
a	3	17	20	7	3	19	14				1	2
A	3			1	1	1		16	42	22	11	1

Figure 6: Half the evolving lines yielded appropriate mat-type progeny. *Number of isolates per evolving line. Mat-a is indicated in red and mat-A is indicated in blue. Actual numbers are listed in the table directly underneath the graph.*

In the homokaryons isolated from the ancestral line three were mat-A and three were mat-a. In the evolved lines half of the crosses yielded homokaryons of the mating type which had undergone selection. In the mat-A evolving lines A3, A4 and A5 yielded one mat-A homokaryon each. The other mat-A lines did not yield any homokaryons of the mat-A type. In the mat-a evolving lines a5 yielded one, and a6 two mat-a homokaryons. The other lines did not yield any homokaryons of the mat-a type. In the experimental setup homokaryons were isolated until a homokaryon of each mating type was obtained. If a homokaryon of each mating type from a line were obtained no more homokaryons would be isolated from that line. If a mat-type homokaryons was missing additional homokaryons were isolated of that line in the next round of the experiment. This resulted in large numbers of homokaryons being isolated of in those lines where a mating-type homokaryon was missing. Whereas those lines where both homokaryons were isolated within the first or second round have only minimal total number of isolated homokaryons.

Both mating types were found in equal frequencies among the ancestral homokaryons, following a 1:1 split. In the evolving lines which had undergone selection on the A mating type that mat-A was found in 3.61% of the homokaryons isolated. In the evolving lines which had undergone selection on mat-a, mat-a was found in 3.16% of the homokaryons. The number of homokaryons isolated and the number of homokaryons of the desired mating type can be seen in table 3.

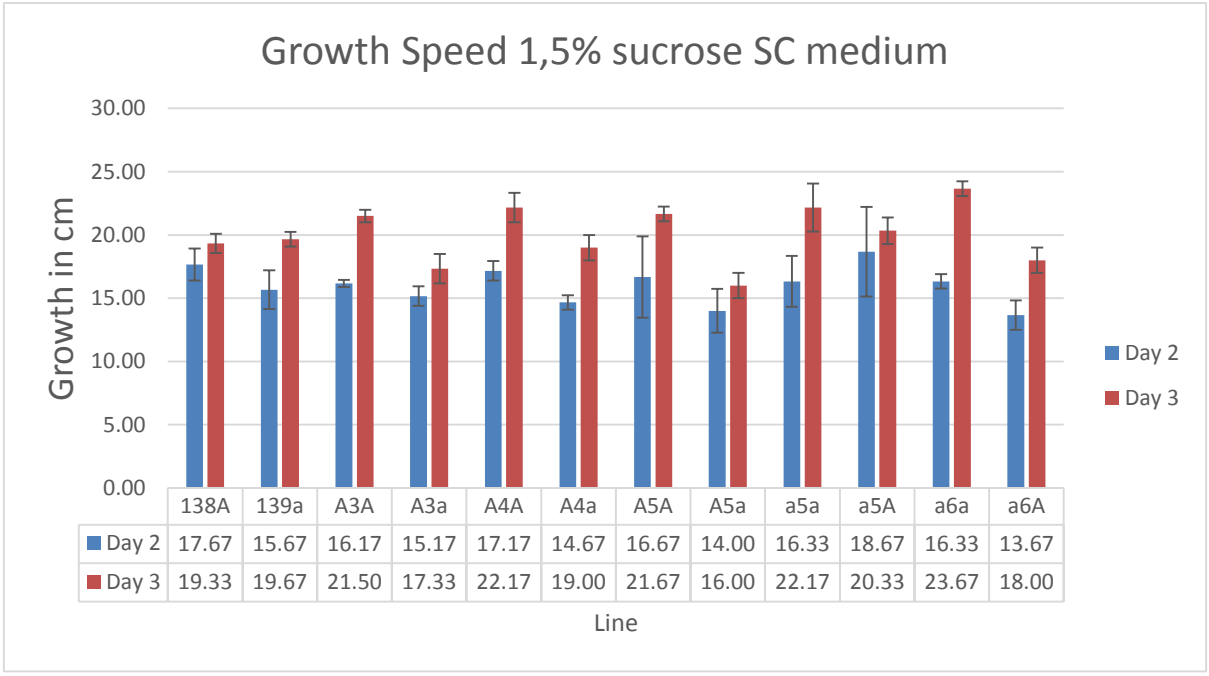
Table 3: Number of desired mating type homokaryons far lower than expected*. Number of isolated homokaryons compared the number of homokaryons of the desired mating type.*

	Number of homokaryons	Number of homokaryons Of desired mat-type	Percentage
Ancestral	6	3 of each	50
Mat-A Evolving lines	83	3	3.61
Mat-a Evolving lines	95	3	3.16

3.3 Growth rate

Growth rate was determined on synthetic crossing medium with either 1.5% sucrose or 0.5% sucrose and on Vogel's minimal medium with 1.5% sucrose. Day one has systematically been omitted from the result as growth rate data on the first day is unreliable due to germination. Focus is on day two and three as growth is at its optimum during this period. On day four the end of the race tube is reached cutting off further growth. In the case of the SC medium with 0.5% sucrose, day four could be included due to the lower growth rate on this medium.

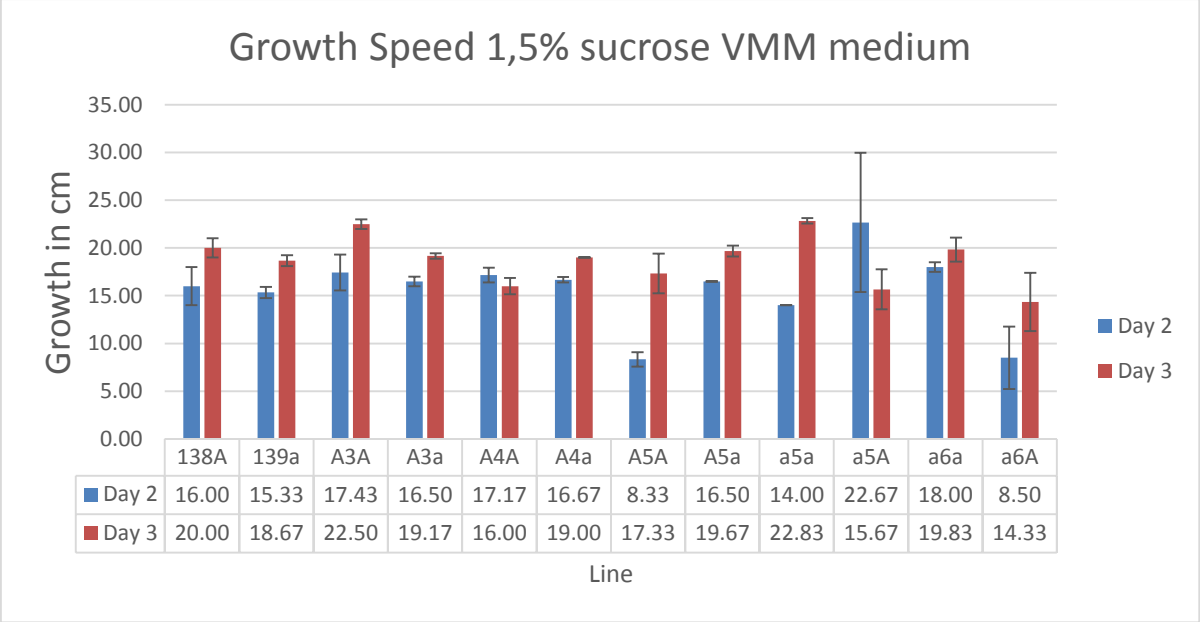
On SC medium with 1.5% sucrose, figure 7, the observed growth of the ancestral lines varied between 15.67 and 17.67 on day two, and between 19.33 and 19.67 on day three. No significant differences in growth speed were observed between the ancestral lines. In the evolving lines growth on day two varied between 13.67 and 18.67 cm, as can be seen in figure 7. Most of the evolved lines show a slight decrease in growth rate compared to the ancestral lines. This decrease is only significant in A3a and A4a. In the evolving lines growth on day three varied between 17.33 and 21.67 cm, as can be seen in figure 7. Seven of the evolving lines show a slight increase in growth rate on day three compared to the ancestral lines. However none of the differences is significant. In the evolving lines mat-A performance consistently better than mat-a in growth speed, when mat-A was selected for. In most lines this difference is significant and averages around a 2,5cm improvement over mat-a. Whereas mat-a performed slightly better in the cases where mat-a was selected for at an average of 2,2cm.



	138A	139a	A3A	A3a	A4A	A4a	A5A	A5a	a5a	a5A	a6a	a6A
Day 2	17.67	15.67	16.17	15.17	17.17	14.67	16.67	14.00	16.33	18.67	16.33	13.67
Day 3	19.33	19.67	21.50	17.33	22.17	19.00	21.67	16.00	22.17	20.33	23.67	18.00

Figure 7: Mat-A grows significantly faster than mat-a in all lines on SC medium with 1.5% sucrose*. Growth of the colony in cm on each day of all lines (day 2 in blue, day 3 in red). All bars are averages ± the mean SD.*

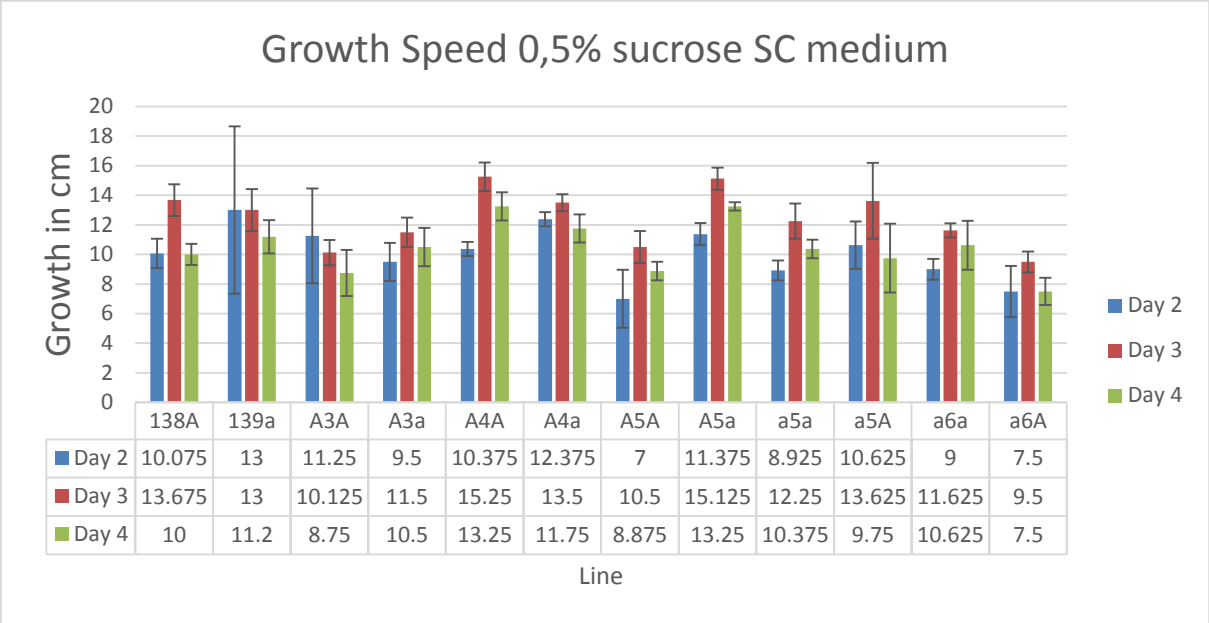
On VMM medium with 1.5% sucrose (figure 8) the observed growth of the ancestral lines varied between 16.67 and 15.33 on day two, and between 18.67 and 20.00 on day three. No significant difference in growth speed was observed between the ancestral lines. In the evolving lines growth on day two varied between 8.33 and 22.67 cm, as can be seen in figure 8. Most of the evolving lines show a slight decrease in growth rate compared to the ancestral lines. This decrease is only significant in A5A, a5a and a6a. In the evolved lines, growth on day three varied between 14.33 and 22.83 cm, as can be seen in figure 8. Two of the evolving lines (A3A and a5a) show a slight increase in growth rate on day three compared to the ancestral lines. Contrary to the 1.5% sucrose SC medium on VMM growth rate is higher in mat-a on average then in mat-A in almost all lines. In most lines this difference is significant and averages around a 3,5cm improvement over mat-A.



	138A	139a	A3A	A3a	A4A	A4a	A5A	A5a	a5a	a5A	a6a	a6A
Day 2	16.00	15.33	17.43	16.50	17.17	16.67	8.33	16.50	14.00	22.67	18.00	8.50
Day 3	20.00	18.67	22.50	19.17	16.00	19.00	17.33	19.67	22.83	15.67	19.83	14.33

Figure 8: Mat-a significantly higher growth rate on VMM then mat-A in the majority of the lines*. Growth of the colony in cm on each day of all lines (day 2 in blue, day 3 in red). All bars are averages ± the mean SD.*

On SC medium with low sucrose (0.5% sucrose) growth speed was greatly reduced when compared to both the 1.5% sucrose experiments. On day two the growth rate of all lines, both ancestral and evolved, was around 11cm per day. This increased to 13cm on day three. And decreased back down to 10cm on day four. No significant difference between the ancestral and the majority of the homokaryons was found, of both the evolved and non-evolved mating type (figure 9). Homokaryons a6A and A5A. Show a significantly reduced growth when compared to the ancestral lines growing around 7.5cm on days two and three and growing 10cm on day two.



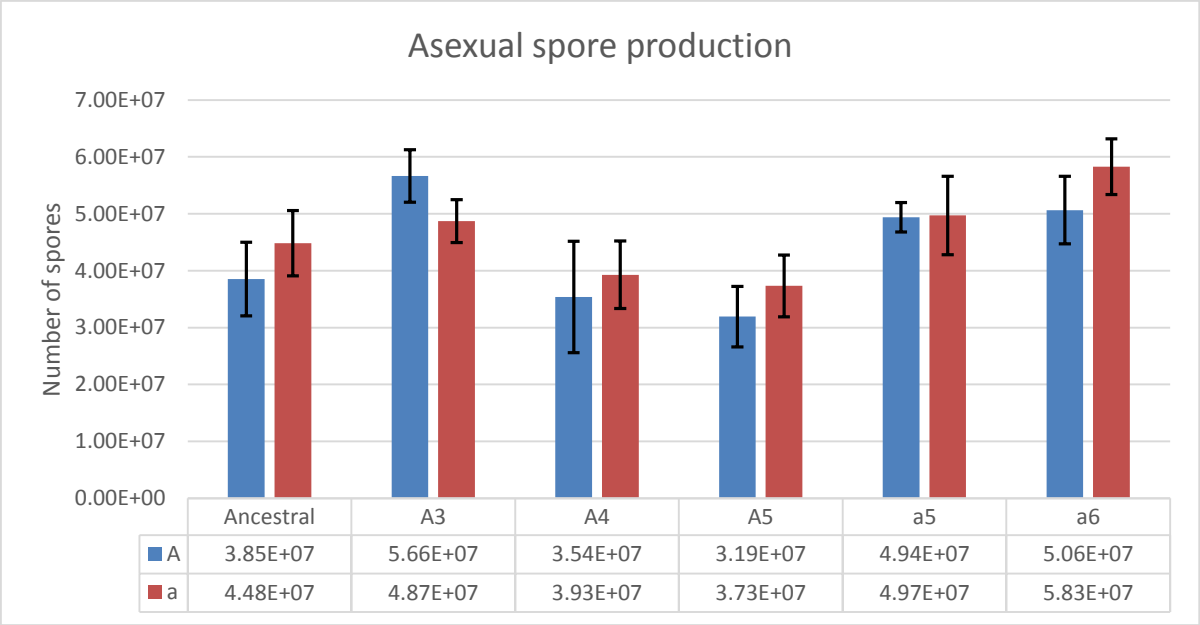
	138A	139a	A3A	A3a	A4A	A4a	A5A	A5a	a5a	a5A	a6a	a6A
Day 2	10.075	13	11.25	9.5	10.375	12.375	7	11.375	8.925	10.625	9	7.5
Day 3	13.675	13	10.125	11.5	15.25	13.5	10.5	15.125	12.25	13.625	11.625	9.5
Day 4	10	11.2	8.75	10.5	13.25	11.75	8.875	13.25	10.375	9.75	10.625	7.5

Figure 9: Mat-a growth speed slightly higher on average then mat-A. *Growth of the colony in cm on each day of all lines (day 2 in blue, day 3 in red & day 4 in green). All bars are averages ± the mean SD.*

The growth reduction observed in homokaryons a6A and A5A confirmed a finding when first isolating these homokaryons. It was noted, through qualitative observation, that a6A and A5A showed greatly reduced growth. Furthermore, it took significantly longer for these isolates to produce viable asexual conidia. Taking eleven days to reach a harvestable state rather than the normal seven days.

3.4 Spore production.

Spore production was determined in both the ancestral and all evolving lines. All tests were done in two biological replicates for evolving lines and three replicates for ancestral lines, all tests were done in triplicate. The tests revealed that the ancestral lines produced an average of $38.5*10^6$ conidia per small test tube in mat-A and $44.8*10^6$ in mat-a. Mat-a consistently produced slightly more spores on average than mat-A in evolving lines; A4 (mat-A $35.4*10^6$, mat-a $39.3*10^6$), A5 (mat-A $31.9*10^6$, mat-a $37.3*10^6$), a5 (mat-A $49.4*10^6$, mat-a $49.7*10^6$) and a6 (mat-A $50.6*10^6$, mat-a $58.3*10^6$). In the evolving lines A3 mat-A ($56.6*10^6$) produced on average slightly more spores then mat-a ($48.7*10^6$). Figure 10 shows the asexual spore production of both mating types. Significance of this data could not be established due to high standard deviations. There is a strong indication across the experiment that mat-a produces slightly more asexual spores then its mat-A counterpart.

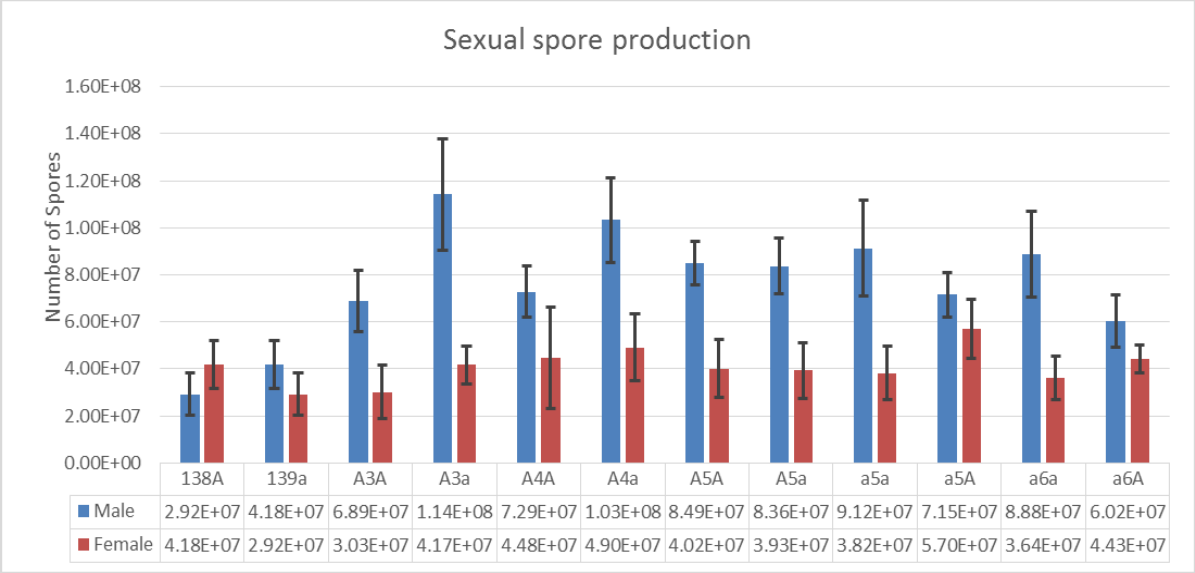


	Ancestral	A3	A4	A5	a5	a6
A	3.85E+07	5.66E+07	3.54E+07	3.19E+07	4.94E+07	5.06E+07
a	4.48E+07	4.87E+07	3.93E+07	3.73E+07	4.97E+07	5.83E+07

Figure 10: No significant changes in asexual spore production. *Number of spores produced by each line for each mating type. After four days of growth. Mat-A is depicted in blue and mat-a is indicated in red. Precise numbers can be seen in the table directly beneath the graphs. Bars are averages ± the mean SD.*

Sexual spore production can be subdivided into the way the evolved line was used. This subdivision consists of the male role in which conidia are used as the fertilising agent and used to fertilise the ancestral to produce the ascospores measured here. In the female role the homokaryons are used to grow the basis and are fertilised using conidia obtained from the ancestral line. In the male role the following quantities of ascospores were produced; ancestral (mat-A $29.2*10^6$, mat-a $41.8*10^6$), A3 (mat-A $68.9*10^6$, mat-a $114*10^6$), A4 (mat-A $72.9*10^6$, mat-a $103*10^6$), A5 (mat-A $84.9*10^6$, mat-a $83.6*10^6$), a5 (mat-A $71.5*10^6$, mat-a $91.2*10^6$) and a6 (mat-A $60.2*10^6$, mat-a $88.8*10^6$), see also Figure 11. A significant difference was observed in sexual spore production when the evolved lines were used in the male role compared to the ancestral lines. This increase was observed in both the mating-type that was selected for as well as the non-evolved mating-type. Results can be seen in figure 11. Furthermore in the male role mat-a consistently produced more spores than mat-A, This result, however, is not statistically significant.

In the female role the following quantities of ascospores were produced; ancestral (mat-A 41.8*10^6, mat-a 29.2*10^6), A3 (mat-A 30.3*10^6, mat-a 41.7*10^6), A4 (mat-A 44.8*10^6, mat-a 49.0*10^6), A5 (mat-A 40.2*10^6, mat-a 39.3*10^6), a5 (mat-A 57.0*10^6, mat-a 38.2*10^6) and a6 (mat-A 44.3*10^6, mat-a 36.4*10^6), see also Figure 11. In the female role no significant changes occurred upon comparison of the ancestor and the evolving lines.



	138A	139a	A3A	A3a	A4A	A4a	A5A	A5a	a5a	a5A	a6a	a6A
Male	2.92E+07	4.18E+07	6.89E+07	1.14E+08	7.29E+07	1.03E+08	8.49E+07	8.36E+07	9.12E+07	7.15E+07	8.88E+07	6.02E+07
Female	4.18E+07	2.92E+07	3.03E+07	4.17E+07	4.48E+07	4.90E+07	4.02E+07	3.93E+07	3.82E+07	5.70E+07	3.64E+07	4.43E+07

Figure 11: Significant increase in sexual spore production in all lines when in male role both evolved and non-evolved when compared to ancestral. No significant differences when in female role. *Number of spores produced by each line for each mating type. After 7 days of growth after fertilisation. When isolate is used in male role it is depicted in blue and female role is indicated in red. Precise numbers can be seen in the table directly beneath the graphs. Bars are averages ± the mean SD.*

4. Discussion, Conclusions and Recommendations

Increased sugar concentration leads to higher ascospores production.

Over the course of the generations, in the evolving lines, a greatly reduced spore production was observed. This lead to the hypothesis that biotin degradation had occurred in the biotin stock. An experiment was set up in which this hypothesis was tested. The biotin degradation experiment as described in 2.1 revealed that no degradation in the biotin stock had occurred. This hypothesis was confirmed as neither increasing the dosage nor replacing the stocks with fresh biotin had any effect on the spore production in the tested lines, as was described in 3.1.1.

Because the lack of spore production was not due to biotin degradation another hypothesis was formed that spore production would increase with a decrease in sucrose concentration. The subsequent experiments with sucrose as outlined in 2.1 revealed that a great reduction in ascospore production was observed below 1%. Whereas an increase in sucrose in the synthetic crossing medium increased the ascospores production. The increase in spore production was found to continue up to ten fold the original sugar content, equating to 5% sucrose. It was hypothesized that the increase will continue beyond this point, but this has not been tested. The result that increases in sugar content increases spore production contradicts earlier work shown by Davis. In this study it was found that a reduction in sugar concentration is beneficial to the sexual spore production. With the optimum lying at 0.5%. This advantage stems from an increased accessibility of the protoperithecia due to a decrease in aerial hyphen production, thereby allowing easier access of fertilising agents to the protoperithecium. In our experiment conidia and aerial hyphae were removed prior to fertilisation with conidia of the opposite mating type. This removed the advantage brought on by the reduced sugar content, and showed that higher sugar content does benefit spore production in a significant way, which is expected due to a higher abundance of resources in the sugar rich environment. Furthermore the increase in ascospore production was also observed in heterokaryons, the negative results of the homokaryon isolation experiments, and therefore is not limited to the homokaryons crosses. In the case of the heterokaryons the accessibility issue is also not present due to the aerial hyphen self-fertilising the protoperithecia.[18]

A 3% sucrose concentration was selected for subsequent growth as a compromise between cost and benefit, as higher concentration showed higher spore production but would result in higher cost for minimal benefit to the experiment. It is also important to note that because the experiment ran with low sucrose concentration for 10 generations some adaptation to the low sugar environment is to be expected. Though not necessarily harmful to the experimental setup, it is an additional selective pressure that can exert significant influence in the experiment. Genes involved in carbon metabolism located on

the mating-type chromosome could more likely adapt to the decrease in carbon availability than autosomal genes due to their high refresh rate experienced within the experiment.

Increase in homokaryon frequency in evolved lines

When starting the homokaryon isolation process it was hypothesized that homokaryons would occur at a rate of 20% of the asci which equals 5% of the ascospores, and that this would be equal between evolved and ancestral lines. This hypothesis was based on literature by Raju from 1992 which found this to be the average. What we found was that in the ancestral line homokaryotic ascospores were found in 3.1% of the cases. Which is slightly less than the 5% that was expected based on literature. The evolving lines produced homokaryotic progeny at a rate of 9.5%. Which is nearly double the amount expected. This higher number of homokaryotic ascospores is equal to the upper limits for ascospores at 10% found in work by Raju. Work by Corcoran et. al. in 2012 showed highly variable homokaryon production in conidia, ranging from 15.3% to 42.6%, within strains isolated at different locations. Though homokaryon production has been shown in multiple studies to be unstable between locations/strains, it is very interesting that in this study we found a threefold increase in homokaryon occurrence compared to the ancestral within the same strain, where it is expected to be stable. This could indicate that homokaryon production is a highly variable trait within the *Neurospora tetrasperma* species.[16,28]

Homokaryon isolation does not follow expected 1:1 segregation

Whereas normally heterokaryotic ascospores are formed by packaging two nuclei of opposite mating type, homokaryons are formed when the nuclei are packaged individually. Thus mat-a homokaryons and mat-A homokaryon are formed simultaneously in an ascus. This then leads to the expectation that a 1:1 ratio occurs of mating types in homokaryons. This is indeed found in the ancestral lines, however in the evolving lines a 30:1 ratio was found in favour of the non-evolved mating type. Research by Corcoran et. al, 2012, reported some variations in homokaryon frequency, on an individual basis ranging from the expected 1:1 to 3:1 with a few outliers at 17:1. On a location basis where the isolates were found the ration on average held at a 1:1.[28]

In this project however all evolved lines stem from a 1:1 ratio ancestors and result in a 30:1 ratio, biased towards the non-evolved mating type, at the end of the sixteen generations. The cause of this intriguing observation is expected to be in the germination rate of the ascospores. The ascospores are expected to still be formed and therefore an issue is possibly found in the germination rate. Upon initial morphological survey, in which perithecia were photo-activated to shoot ascospores on to a water agar

plate, no clear abnormalities were observed in ascospore shape. During this same experiment, which was to isolate the two smallest spores of five spored asci, it was noted that no clear grouping occurred and that spores were shot out in a diffuse pattern. Nor were any clear homokaryons found, based on size of the ascospores. During the follow up experiment utilising bulk isolates to acquire ascospores it was observed that germination occurred at varying delays, with some spores germinating at day one whereas other germinated at day three after the heatshock. In addition, great differences in colony size were observed, with some consisting of only one hyphen, whereas others formed full-fledged colonies several mm in diameter. The high variation in the way in which the spores germinate could indicate issues within this system. We therefore recommend that a germination test be performed, to determine whether the mating type skewness is indeed due to a decrease in germination of the ascospores. This could be done by crossing evolved lines with the opposite mating type ancestral, similar fashion done for the homokaryon isolation, and subsequently harvesting the ripe perithecia. The perithecia can then be opened up and the five spored asci acquired. The two smallest ascospores are homokaryons, one of each mating type, these homokaryons should then be isolated and plated out on SC medium, and there germination rate noted. The size difference has been shown to be a reliable method of determining karyosity by Raju 1992. The same experiment should be repeated then by crossing the known homokaryons with the ancestral and seeing if a higher germination rate is found in those ascospores for the isolated mating type.[16]

Another option would be to analyse the spores within the asci without actually plating them, in the event that a five spored asci had three normal size, one small and one extremely small ascospore. That would be a strong indication that the extremely small spore is in all likelihood malfunctioning and will not germinate. A few of the extremely small spores should still be plated to confirm they are indeed unable to germinate, with the small and normal size ascospores to serve as positive controls.[16]

Growthspeed of the homokaryons is equal to that of the ancestral lines

During the analysis of the isolated homokaryons we initially focussed on the female functions, such as growth, asexual spore production and sexual spore production in female role or utilising conidia in the male role. It was hypothesized that these functions would deteriorate over the course of the 16 generations of ascospore selection for only the male function. The growth speed was the first to be tested, as described in 2.5. Here no significant correlation was found between evolved mating types and changes in growth speed. As any significant changes when compared to the ancestral lines were mimicked in both mating types. Growth rate on day three was consistently higher than on day two across all experiments. Furthermore it was noted that on SC medium with

1.5% sucrose the faster mating type within the evolved lines was lightly correlated to the evolving mating type, which grew faster on average, however no significance could be established. On VMM medium mat-a grew at a slightly faster rate in all lines. On SC medium with 0.5% sucrose no significant differences were observed between mating types or lines. Though selection on low sugar had occurred for the first 10 generations this seems to have had no effect on growth rate. We hypothesize that this is due to the high refresh rate of the autosomes. Rendering any adaptations lost in a rather short timespan once selection shifted back to a higher sucrose environment from generations 11 to 16.

Asexual spore production is equal to that of the ancestors

The amount of asexual spores was hypothesized to deteriorate over the course of the evolution experiment. However no significant differences were found in the asexual spore (conidia) production. Though some minor differences can be seen in the averages, the large standard deviations found means no significance can be attributed to them. On the whole mat-a seems to produce 15-20% more spores than mat-A. This supports earlier data of Howe (1977). Increasing the number of biological duplicates in a repeat experiment would result in a larger data pool, thereby reducing the deviations found and giving a clearer indication as to significance of the found differences. An increase in the number of biological replications is therefore recommended for future experiments.

Increase found in male function of conidia

As the conidia are killed off by the heat shock treatment administered after harvesting the ascospores, it was hypothesized that the conidia would be exempt from any selective pressure. This could lead to accumulation of mutations in genes associated with conidia formation. Since conidia also function as spermatia we would therefor see a reduction in ascospore formation when conidia are used as a fertilising agent. However upon analysis of the isolated homokaryons significant increases in male function of the conidia was found, as can be seen in in section 3.4. This indicates the possibility conidia surviving the heat shock and experiencing selection as a fertilising agent. Previous research (Plesofsky, 1985) in *Neurospora crassa* showed that pre-exposure to temperatures around 45°C result in conidiospores being able to germinate at lethal temperatures of 50°C, though temperatures higher than this weren't tested, it does give rise to the possibility that conidia can survive heat shock treatment if exposed to either a pre-treatment or, in the event that heat shock equipment is faulty, a lower temperature is experienced. The conidiospores which go through this will experience severe selection on thermo tolerance as a result.[29,30,31]

Another important observation is that the increase in sexual spore production occurs in both the non-evolved mating type as well as the evolved mating type. This means that the beneficial mutation occurred on one of the autosomes. As the average increase in ascospore production is around two fold, this might be a strong enough advantage to lead to fixation within the populations even under the high autosomal refresh rates within the experiment.

In order to test these new hypotheses we recommend several tests to be conducted. First it should be tested whether the heat shock is indeed sufficient to prevent the conidia to be utilised as a fertilising agent. This would give insight in whether any selective pressure was exerted on conidia as a fertilising agent. This testing could be further expanded to see if any increases in thermo tolerance have emerged in the conidia of the evolved line. As thermo tolerance would be one of the main required traits with conidia functioning as a viable fertilising agent within the experiment. Secondly as the odds of beneficial mutations forming on autosomes in all five of the tested lines and fixating within all tested populations is relatively low. The experiment should be redone to make sure no errors were made. If the same outcome is then found it would mean that not only have conidia experienced a beneficial mutation which doubles sexual spore production but also that this mutation can be maintained within the autosome under extreme refresh pressure.

An experiment as to the performance of the male function of ascospores should also be performed as this was cut at the last moment due to time constrictions. This experiment was however crucial, as the ascospores experienced the main selective pressure over the course of the experiment. We would therefore expect the strongest increases in "male" function of the ascospores as a fertilising agents.

Sexual Spore production in female role equal to that of the ancestral lines

As the evolving mating type is never used as the female within the experiment all genes relating to protoperithecia development and genes related to ascogonial cell search mechanisms would no longer be under purifying selection, and thus susceptible to mutation accumulation. If there is a trade of female and male functions even antagonistic pleiotropic effects may be expected. However, no significant changes were observed between the female functions of evolving lines and the ancestral lines. Also between mating types the differences are minor, in a few cases mat-A lines yielded slightly better results in the female role, especially in the ancestral lines and a5 and a6.

Spore production favoured between mating types.

A mild association has been found between genotype and the quantity of spores produced. As can be observed in figure 10 and 11 it appears 139a favours conidia production and also its utilisation as a fertilising agent. Whereas 138A seems to favour ascospore production in the female role in the ancestral line and in three out of five evolved lines. This confirms results found by Down W H, et al. 1977, who found similar results. The significance of this could be improved by performing a large scale experiment utilising both ancestors, as the data in this project is only based on three biological replicates per line.

No significant decrease found in female function

On the whole we can conclude that no significant decreases have occurred on any of the attributes that we in this project have labelled "female" functions. As growth speed has been shown to still be at levels comparable with the ancestral lines. Asexual spore production of the evolved lines is at similar levels to the ancestral lines. Also the viability of the asexual spores as fertilising agents within the experiment appears to have increased significantly, rather than decreased as expected. In the female role sexual spore production is also still at comparable levels to the ancestral lines. In the project however two large gaps in knowledge still remain; what caused the large problems with the homokaryon isolation, and how has the male function of the ascospores developed over the course of the evolution experiment.

As noted in the section on homokaryon isolation a germination issue is suspected. This is a clear "female" attribute that might have been changed and would be a clear case of absence of selection at work. We therefore again strongly recommend its testing, as it would prove that absence of selection would result in deterioration of these attributes, as found in previous experiments by Howe and Rice.

In order to test ascospores in the male role an experiment identical to that used to test the conidia can be conducted, utilising ascospores rather than conidia as the fertilising agent. Due to the increased time required to acquire the ascospores this critical experiment was cut at the last moment. Here we strongly recommend doing ascospore experiments as many avenues for adaptation are open during evolution, relating to pheromones, morphological shape, ascospore production and competitive fitness. All these attributes could be tested as follow up experiments to this first analysis, and it is therefore a critical first step for further analysis.

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Appendix I: Media

SC+	100 mL	400mL
W & M	5 mL	20 mL
$MgSO_4$	1 mL	4 mL
NaCl	1 mL	4 mL
2M sucrose	750 µL	3 mL
$CaCl_2$ (0,13 g/mL)	100 µL	400 µL
Vogel's trace-elem	10 µL	40 µL
Biotine (0,1 mg/mL)	3 µL	12,5 µL
Add WA 2% till	100 mL	400 mL

VMM+	100 mL	400 mL
Vogel's Salt (50x)	2 mL	8 mL
2M glucose	5 mL	20 mL
Vogel's trace-elem	10 µL	40 µL
Biotine (0,1 mg/mL)	10 µL	40 µL
Add WA 2% till	100 mL	400 mL

Vogel's Salt (50x)	
H_2O	60mL initially
	adjusted to 90 mL at end
Na_3citrate.H_2O	12.5g
KH_2PO_4	25g
NH_4NO_3	10g
pH adjusted too 5,8	
Separate solutions	added after sterilisation
$CaCL_2.H_2O$	5g in 5 mL H2O
$MgSO_4.7H_2O$	10g in 5mL H2O

Vogel's trace-elem	
citric acid	5g
$ZnSO_4.7H_2O$	5g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1g
$CuSO_4.5H_2O$	0.25g
$MnSO_4.H_2O$	0.05g
H_3BO_3	0.05g
$NaMoO_4.2H_2O$	0.05g
H_2O	95 ml

W & M	
KNO_3	4g
K_2HPO_4	2.8g
KH_2PO_4	2g
H_2O	200mL

Other solutions	
$MgSO_4.7H_2O$	2g in 40mL H_2O
NaCl	2g in 40mL H_2O