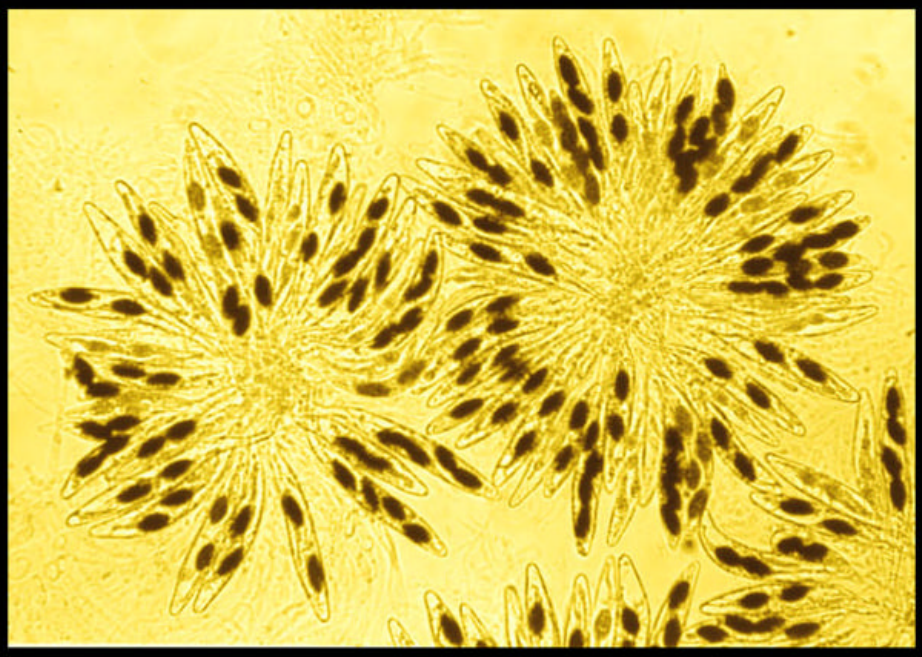
Genomic Conflicts in Podospora anserina



Marijn van der Gaag

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MARIJN VAN DER GAAG

Promotor: Prof. Dr. Rolf F. Hoekstra
Hoogleraar in de Genetica
Wageningen Universiteit

Copromotor: Dr. Ir. Alfons J. M. Debets
Universitair Hoofddocent
Laboratorium voor Erfelijkheidsleer
Wageningen Universiteit

Promotiecommissie:

Dr. Sven J. Saupe, Laboratoire Génétique Moleculaire des Champignons, Bordeaux, France
Prof. Dr. (Kuke) R. Bijlsma, Rijksuniversiteit Groningen
Prof. Dr. Piet Stam, Wageningen Universiteit
Prof. Dr. Pedro W.Crous, Centraal Bureau voor Schimmelcultures, Utrecht en Wageningen Universiteit
Dr. Ir. A. J. Termorshuizen, Wageningen Universiteit

Genomic conflicts in *Podospora anserina*

Genomische conflicten in *Podospora anserina*

Marijn van der Gaag

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Wageningen Universiteit,
Prof. Dr. M. J. Kropff
in het openbaar te verdedigen
op woensdag 19 oktober 2005
des namiddags te vier uur in de Aula.

van der Gaag, Marijn

Genomic conflics in *Podospora anserina* / Marijn van der Gaag
Thesis Wageningen University, with references – and summary in Dutch.

ISBN 90-8504-255-0

Subject headings: fungal genetics / linear plasmid / meiotic drive / *Podospora anserina* / spore killing.

Voor Alarik en Anne

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Chapter 1

General Introduction

"Mendelism is a magnificent invention for fairly testing genes in many combinations, like an elegant factorial experimental design. Yet it is vulnerable at many points and is in constant danger of subversion by cheaters that seem particularly adept at finding such points."

- J. F. Crow (1988)

In the next paragraphs the definition of genomic conflicts is introduced, followed by two examples of such conflicts that (also) exist in the ascomycete fungus *Podospora anserina*. These are genomic conflicts based on transfer of (parasitic) genetic elements and genomic conflicts based on deviations from Mendelian inheritance, also known as meiotic drive. Then characteristics of the fungus itself followed by several aspects of *Podospora* that influence these genomic conflicts are discussed. Finally, the outline of this thesis is described.

GENOMIC CONFLICTS …

The general view of the genome of an organism is an integrated and cooperative network of genes that is both viable and reproductive. Mutations in the genome that decrease the fitness of the organism are selected out, whereas mutations with beneficial effects on the fitness of the organism and its genome become established. However, a large proportion of the genome of eukaryotes is composed of selfish genetic elements (Hurst and Werren 2001). Selfish or 'parasitic' elements promote their own transmission relative to the other elements of the genome, but have neutral or even detrimental effects on the fitness of the organism. Hurst and Werren (2001) divide these common but diverse elements into four categories, based upon the mechanism by which they spread. These are:

- Autonomously replicating (mobile) elements, such as transposons and many fungal mitochondrial plasmids (Griffiths 1995).
- (Post) segregation distorters like meiotic drive elements, B chromosomes and killer plasmids.
- Sex-ratio distorters like cytoplasmic microorganisms that feminize hosts or kill males.
- Gene converting elements, such as homing endonucleases.

A genetic conflict, where the spread of one gene creates the context for the spread of another gene having the opposite effect and expressed in the same individual may result in a

genomic conflict. A genomic conflict arises when the effects of these selfish elements are opposite to the interests of the other parts of the genome. Basically, genetic conflicts are intra-individual, but become genomic when more (closely linked) genes become involved (Hurst *et al.* 1996).

Genomic conflicts are evolutionary interesting, because they can give insight in why or how certain genetic features have arisen. For instance meiotic drive elements can offer perspectives why 'fair' Mendelian inheritance is stable and to observe the evolution of mechanisms that counteract drive (Lyttle 1991, 1993). Recombination (random assortment of chromosomes and crossing over) might have its origin or is maintained to act against distorter genes by minimizing the size of linkage groups and hence to prevent the formation of potential drive allegiances by leaving the majority of genes freely recombining (Eshel 1985, Haig and Grafen 1991). Selfish elements are speculated to have influence on speciation by horizontal gene transfer (Rosewich and Kistler 2000) or by contributing to reproductive isolation, e.g. due to chromosomal rearrangements caused by segregation distorters or transposons or cytoplasmic incompatibility systems (Hurst and Werren 2001, Hurst *et al.* 1996). Furthermore, the genetic conflict between Mendelian nuclear genes and non-Mendelian sex-ratio distorters might have a role in the great variety of sex determination systems in plants and animals.

According to Hurst and Werren (2001) selfish genetic elements obey two common rules. The first rule is that the diversity of selfish genetic elements in a species is correlated with the rate of outcrossing. Inbreeding decreases the heterozygosity and lowers the opportunity for transmission between genomes. This also affects the possibilities for effective selfish behavior and accordingly the spread of the selfish element.

The second rule is that their phenotype is often shown in hybrids and between-population crosses, but that they are seldom observed in within-population crosses. The outcome of a genomic conflict within a population would either lead to removal or fixation of the selfish element, or even complete annihilation of the population (Hurst *et al.* 1996). A between-population cross can restore the genetic diversity for the element in question, resulting in renewed expression. To observe a genomic conflict within a population would mean that the conflict is not yet resolved or a condition of stalemate, as within *SD* of *Drosophila*, is reached (Hurst *et al.* 1996).

Fast termination of a conflict rarely produces any long-lasting effects, whereas extension of the conflict would provide more opportunities for modifiers, as well as secondary effects to appear. Suppressors are generally not cost-free and may not be restricted to counteracting the selfish element. A modifying allele can also be a neutral party and therefore not increase in frequency. Reappearance of conflicts can lead to a decrease in vulnerability to the action of selfish elements due to suppressors and modifiers of previous conflicts. For instance modifiers that increase recombination rate, would hamper spread of future drive elements (Hurst *et al.* 1996). Two examples of selfish elements used in this study, meiotic drive elements and (fungal) plasmids are discussed in more detail below.

Meiotic Drive (selfish chromosomal elements)

Meiotic drive is the mechanism by which one of the members of a pair of heterozygous alleles or chromosomes during meiotic divisions is transmitted into the progeny in excess of the expected 'fair' Mendelian proportion of 50%. Segregation distorters, the elements that perform meiotic drive are found in a wide range of organisms and usually involve a small number of interacting loci. Drive elements are coined 'ultraselfish' DNA, because they increase their frequency by actively promoting the destruction of the alternative allele (Crow 1988, Lyttle 1991). Segregation advantage arises at the population level, not at the individual level, where competition occurs among the gametes of single individuals (as in fungal drive systems, where the gametes are the offspring), and gamete loss results in less than proportional loss of individual fecundity. Logically, segregation distorter systems are more likely to be found in males of monogamous species, as the impact of gamete wastage is smallest (Lyttle 1991). Segregation distorters can be divided into two groups, autosomal or sex-linked. Well-studied examples of sex-ratio distortion are X chromosome drive (SR) in *Drosophila* and male drive (MD) in mosquitoes. Examples of autosomal drive are the *t*-haplotypes in mice, segregation distorter (SD) in *Drosophila* and Spore killer (*Sk, Psk*) in fungi (Lyttle 1991, Turner and Perkins 1991, Nauta *et al.* 1993).

Meiotic drive is initially limited in impact to population dynamics of the drive locus and those loci in close linkage. Alleles at these latter loci may exhibit indirect drive through genetic hitchhiking, and increase in frequency even though they might have negative effects on the individual fitness. Meiotic drive can also lead to loss of fecundity through gamete loss, as is the case with fungal drive systems (Nauta and Hoekstra 1993), or through reduced production of grandchildren in sex chromosome drive systems (Lyttle 1991). Both will force selection (and fixation) of unlinked suppressors of drive. The impact of sex-ratio distortion at the population level is stronger than autosomal drive and can lead to extinction, as one of the sexes is eliminated. In theory, autosomal distorters can become fixed without any detrimental effect on the population.

Theoretical studies on the population genetics of meiotic drive have made predictions on the evolution of the drive system, including target loci, drive modifiers and linkage arrangements (Hurst *et al.* 1996). A two-locus system might be expected, where the distorter factor produces a 'poison' and a second factor the antidote to this poison. For a drive system to be established there has to be tight linkage between the distorter and the target locus. Insensitive target alleles should be positioned in *cis* and the sensitive alleles in *trans* to the distorter allele. Tight linkage, either by close linkage to centromere or an inversion, will prevent the production of suicide chromosomes (Lyttle 1991). Elements consisting of one locus seem difficult to invoke, as the drive systems analyzed so far consist of gene complexes (Wu and Hammer 1990). In sex-ratio distorters tight linkage is already established by the sex chromosomes. Modifiers of distorters should also be in linkage disequilibrium; enhancer loci should be coupled and suppressors repulsed with the distorter allele. The occurrence of drive modifiers is dependent on the effect the distorter allele has on fitness of the gametes.

Suppressors, inhibiting meiotic drive elements occur where fertility is reduced or homozygous carriers are reduced viable/fertile (Hurst *et al.* 1996).

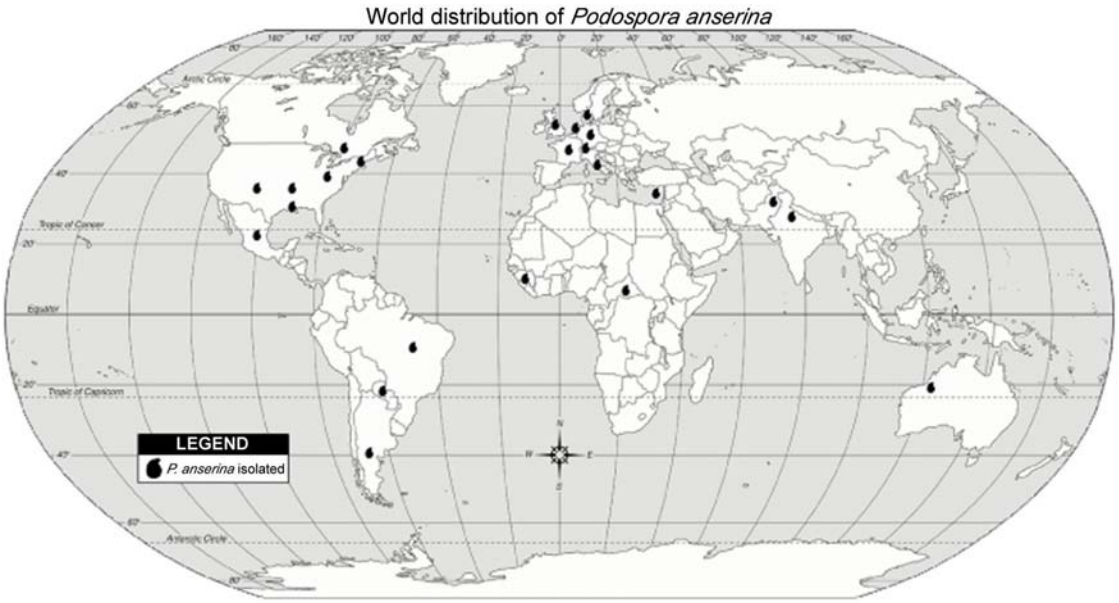
Different segregation distorters do not necessarily act via the same mechanism, as is shown by the autosomal drive systems in mice and *Drosophila* (Lyttle 1991, 1993). *t*-Haplotype wild-type allele bearing sperm of mice undergo a premature acrosome reaction which prevents them from binding to the egg. The differences found in drive sensitivity could be attributed to protein variants (multiple elements) that are associated with the *t*-haplotypes. The distorter locus *Tcd* acts premeiotically or is diffusible across cytoplasmic bridges, whereas the target *Tcr* acts postmeiotically. The *Drosophila* distorter *Sd* interferes with proper chromatin condensation during spermatogenesis and acts during meiosis I. Sensitivity increases with the repeat copy number of the target *Rsp.* The pairing of chromosomes has not been found necessary for distortion. The linked enhancer (*E(SD)*) and modifier (*M(SD)*) loci have impact on the distortion percentage (Lyttle 1993). The frequency of both meiotic drive systems in nature is 1-3% for SD (Lyttle 1991) and 6-20% for *t*-haplotype (Ardlie *et al.* 1998, Caroll *et al.* 2004).

Autosomal drive in fungi behaves slightly different, as the gametes are the progeny themselves. Here, the number of progeny bearing the distorter gene remains constant and drive is solely dependent on the loss of wild-type spores (Lyttle 1991). Due to their action these meiotic drive elements have been coined Spore killers (*Sk* in *Neurospora* and *Psk* in *Podospora*), as half of the spores within an ascus are aborted (Turner and Perkins 1991, Nauta *et al.* 1993, Raju 1994, 1996). Similar Spore killers have been found in *Cochliobolus heterostrophus* and *Giberella fujikuroi* (Raju 1996). Another difference is that fungi lack chromosomal sex determination and are hermaphroditic, and drive can be expressed in the female structures of both (sensitive and distorter) parents. Nauta and Hoekstra (1993) and Lyttle (1991) have shown that when the fungal drive element is rare, the impact on spore production is negligible in large populations, irrespective of the strength of the drive mechanism. However in small populations the distorter can reach fixation. This could explain why in *Neurospora Sk* alleles are rare or have reached fixation on island populations (Turner 2001). Although Spore killers show high percentage distortion, they show weak effective drive and suppression is expected to occur rapidly by the selection of insensitive target alleles (Lyttle 1991). Indeed insensitive targets are at the moment only found in fungi. Resistant strains to *Sk2* and *Sk3* have even been found in *Neurospora* species where no spore killer allele has been detected (Turner 2001). It has also been suggested that the transition of eight to four ascospores in *N. tetrasperma* is an adaptation to escape hybrid sterility resulting from meiotic drive (Raju and Perkins 1991).

Fungal plasmids and plasmid-like elements (autonomously replicating selfish elements)

Plasmids are small DNA molecules in cells of organisms. Normally they can replicate independently from the genome, but some are able to integrate within the genome and replicate as part of the genome. Plasmids are ubiquitous in bacteria and fungi, although some have been found in plants (Griffiths 1995, Meinhardt *et al.* 1997, Kempken *et al.* 1992).

FIGURE 1.1. --World distribution of *Podospora anserina.*
Distribution of *P. anserina* isolated from various herbivore dung sources world wide (CBS Stock List 2004, Moreau 1953, Mirza and Cain 1969 and Cannon *et al.* 1985).



Plasmids of yeasts reside mainly in the cytoplasm or the nuclear genome, whereas filamentous fungal plasmids are found in the mitochondria. Fungal plasmids can be divided into several categories: linear and circular, of which the first are the most common.

Linear plasmids range in size between 7 and 9 kb. They have an invertron structure with a terminal inverted repeat whose size is characteristic for each plasmid species. To each 5' end of the plasmid a protein is bound and they usually contain two large open reading frames that flank an intergenic region. Comparison of amino-acid sequences in the open reading frames suggest that they are of viral (adenovirus) or bacteriophage origin (Griffiths 1995, Rosewich and Kistler 2000). Circular plasmids are smaller and range from of 880 bp to 5 kb. They contain a DNA polymerase, a reverse transcriptase or they depend upon the polymerase activity of other plasmids to replicate. Also circular plasmids are suspected to be of viral origin (Griffiths 1995, Rosewich and Kistler 2000). Plasmid-like elements have much in common with circular plasmids. However they are part of the mitochondrial genome and are excised from it (Griffiths 1995). The senescence plasmids (senDNA-α, β and γ or pl-DNA) of *Podospora anserina* fall into this category.

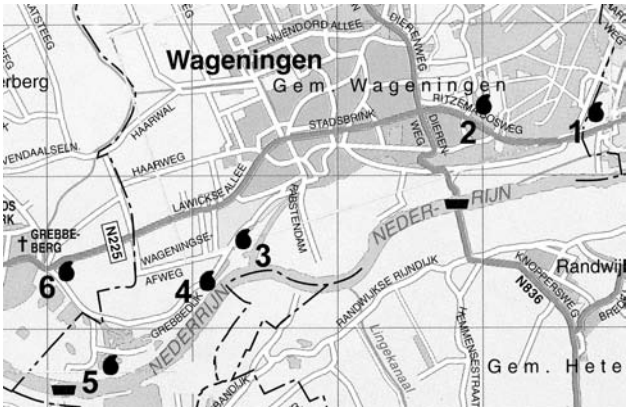
About half of the *Neurospora* species worldwide contain circular plasmids, which are able to cross species barriers (Natvig *et al.*1984). Also Yang and Griffiths (1993) and Arganoza *et al.* (1994) surveyed *Neurospora* strains for circular and linear plasmids and found them to be uncorrelated to their origin, giving the suggestion that they associate independently and are freely mobile. The relative ease of plasmid transfer between different isolates or even between species support this (Collins and Saville 1990). Linear (*zhisi*) and circular (*Harbin*) plasmids were even found to recombine with each other in *N. intermedia* giving rise to new plasmid species (Griffiths and Yang 1995). However comparison of

phylogenetic trees of plasmid sequence with evolutionary trees of the species in which they reside show that generally the pattern is in agreement with each other, supporting the idea that plasmids have evolved separately in diverging lineages and are of ancient origin (Rohe *et al.* 1992, Kempken *et al.* 1992). Rosewich and Kistler (2000) even suggest that eukaryotes that do not harbor any plasmids have eliminated them from their populations over evolutionary time.

All fungal plasmid and plasmid-like elements are transmitted through their mitochondria, usually via the maternal structures. There is virtually no paternal inheritance and cases of paternal leakage may be ascribed to transmission via heterokaryosis (Debets *et al.* 1994). Sometimes plasmids fail to be transmitted to the fungal progeny as found for the linear plasmid pAL2 in *Podospora* (Hermanns et al, 1994). The presence of plasmids and plasmid-like elements in high copy number in the mitochondria impose a genetic load. Despite this, most plasmids seem to have neutral or cryptic phenotypes. Only a few plasmids impose visible negative effects on their host. For instance, the linear plasmids *pKALILO* and *pMARANHAR* cause a senescent phenotype in *Neurospora* by inserting and recombining into the mitochondrial genome (Griffiths 1995). All wild-type *Podospora anserina* strains die through a well-studied process involving the excision and amplification of one or several mitochondrial regions resembling a plasmid. (Osiewacz 2002, Griffiths 1992). However, also some beneficial effects of plasmids have been discovered. The *KALILO* plasmid has been suggested to give some growth advantage under certain conditions (Bok and Griffiths 2000), whereas the linear plasmid pAL2-1 was found to be involved in the increase of lifespan Hermanns *et al*. 1994).

How these (potentially) harmful fungal selfish elements maintain themselves within a species is still an enigma. The average proportion of plasmids is generally low (around 10%) and plasmid loss is observed regularly (Rosewich and Kistler 2000). However, one could question whether the relative ease of horizontal transfer is sufficient for plasmid maintenance. Yeast plasmids have evolved mechanisms to regulate their copy number in the cell to counter the load they impose (Velmurugan *et al.* 2003), or they maintain themselves though a toxin-antitoxin addiction system (Cooper and Heineman 2000; Engelberg and Glaser 1999, Hayes 2003, Gerdes 2000).

FIGURE 1.2. -- Isolation spots of *P. anserina* around Wageningen, The Netherlands during 1991-1997.



Places of isolation are depicted by black perithecia:
(1) Oranje Nassau Oord
(2) Cow meadow (Zoomweg)
(3) Wageningen harbour
(4) Grebbedijk
(5) Blauwe Kamer nature reserve
(6) Grebbeberg.

… IN *PODOSPORA ANSERINA*

Podospora anserina (CES.) NIESSL, the fungus used in our studies, is a coprophilous ascomycete fungus, which is commonly found on dung of herbivores worldwide (Figure 1.1, CBS Stock List 2004, Moreau 1953, Mirza and Cain, 1969, Cannon *et al.* 1985). The genus name is derived from the characteristic ascospores, whereas the species name probably relates to the isolation from dung of geese. *P. anserina* was first described by Niesl in 1883 but is (in the older literature) also known under various other genus names like *Pleurage, Sordaria*, *Sphearia*, *Malinvernia* and *Bombaria* (Mirza and Cain 1969). The strains used in the studies described in this thesis are isolated from horse, sheep and rabbit dung around Wageningen, The Netherlands (Figure 1.2). Attempted isolations form other locations by us were unsuccessful (Table 1.1).

The life history of *P. anserina* is presented in Figure 1.3. Briefly, ascospores attached to vegetation are eaten by herbivores, pass through the intestines and are deposited on dung, their natural growth habitat. Within the dung, the ascospores germinate and form a mycelial network. On the mycelium phialides can be seen containing small single nucleus

TABLE 1.1

Sampling areas for *P. anserina* isolation used in this study.

Sampling area	Dung source	Year
Wageningen, The Netherlands [a]	sheep, rabbit, horse	1991-1997
Haringvreter, The Netherlands	rabbit	1993
Neeltje Jans, The Netherlands	rabbit	1993
Fort Rammekens, The Netherlands	rabbit	1993
Camarque, France	horse, rabbit	1993
St. Nicolaasga, The Netherlands	rabbit	1994
Groningen, The Netherlands	horse, rabbit	1994
Holland Park, London, UK	rabbit	1994
Arthurs seat, Edinburgh, UK	rabbit	1995
Dunnideer castle, Inch, UK	rabbit	1995
Stone circle, Daviot, UK	rabbit	1995
Mangoan, Leith, UK	sheep, rabbit	1995
Ascot, UK	horse	1996
Pembroke Coastal Park, UK	rabbit	1996
Roscommon castle, Ireland	rabbit	1996
Castle Baldwin, Carrowkeel, Ireland	rabbit	1996
Queen Meave's tomb, Ireland	rabbit	1996
Oss, The Netherlands	rabbit	1996
Oosterbeek, The Netherlands	rabbit, horse	1997
Sneekermeer, The Netherlands	rabbit	1997
Lauwersoog, The Netherlands	rabbit	1997
Krammersluis, The Netherlands	rabbit	1997
Hédevie, Bretagne, France	sheep	1997

[a] Only samples collected from Wageningen resulted in successful *P. anserina* isolations.

microconidia or spermatia, which act as male gametes in the sexual cycle (Figure 1.4a, Esser and Kuenen, 1967). These microconidia are not able to germinate and start a new colony. Upon nutrient exhaustion (Bernet 1991) or reaching the senescent state the fungus engages into the sexual cycle. The spermatia attract the trichogyne (female receptor) and fertilize the ascogenous mother cell within the protoperithecium. According to Bernet (1992), microconidia are not wind dispersed and make the sexual cycle a local affair. However, splash dispersal by raindrops seems a likely alternative. From the single ascogenous mother cell all asci are formed, in which nuclear fusion occurs followed by meiosis and the subsequent production of four dikaryotic ascospores per ascus (Coppin *et al.* 1997; Raju and Perkins 1994). The perithecium enlarges as up to 200 asci are formed (Zickler *et al.* 1995, Figure 1.4b). Ascospores are discharged per ascus from the perithecium towards the light and cling onto the grass with the sticky secondary appendages (Figure 1.4d), waiting to be harvested by herbivores. Discharged ascospores can reach a distance of 30 cm in wind-still glass tubes (van der Gaag, unpublished data). Further distances are probably reached when wind aided dispersal occurs as with other 'ground sporulating' ascomycetes (Ingold 1971, Bistis 1998). Perithecia start to appear after two weeks and will release the ascospores up to three weeks (Ingold 1971, Dix and Webster 1995, Wicklow 1981). A complete lifecycle will take approximately 1 week in the lab on copromes at the optimal growth temperature of 27° C (Esser 1974). A closely related species, *P. comata*, exists which has somewhat smaller perithecia and ascospores, and has a distinct mitochondrial type, but is interfertile with *P. anserina* (Padieu and Bernet 1967, Belcour *et al.* 1997).

FIGURE 1.3. -- Life history of *Podospora anserina.*

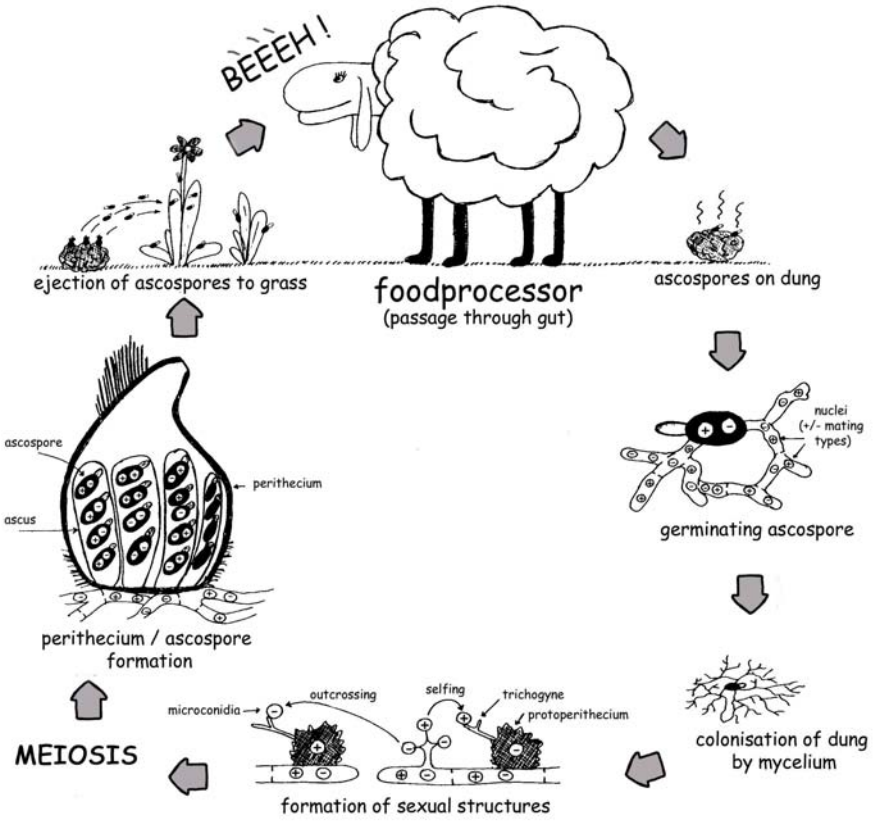


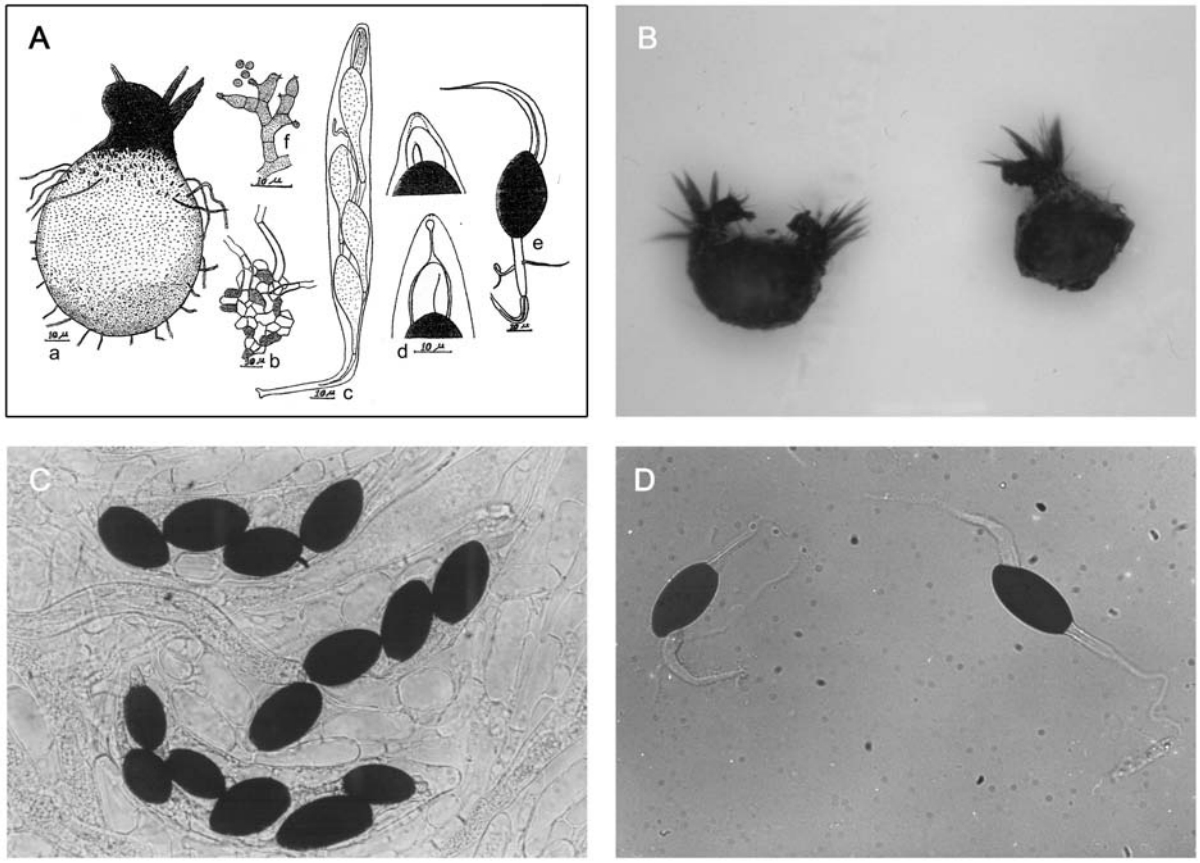
FIGURE 1.4. -- ***Podospora anserina***

(A) *P. anserina* characteristics as pictured by Mirza and Cain (1969). (a) perithecium (b) protoperithecium (c) ascus with young ascospores (d) apices of asci (e) ripe ascospore with podos and secondary appendages (f) phialides with spermatia.

(B) Two perithecia of *P. anserina*

(C) Asci of *P. anserina.* Two asci contain four binucleate ascospores and one ascus contains four uninucleate spores and two binucleate ascospores.

(D) Two ascospores with secondary appendages.



Podospora anserina is one of the few ascomycete fungi that are pseudo- or secondary homothallic. Secondary homothallic species are capable of completing their life cycle from a single sexual self-fertile ascospore, as with true homothallism. However, ascospores and mycelium are heterokaryotic and contain nuclei of two different mating types (mat+ and mat-). Both mating type nuclei are needed to initiate sexual reproduction. The formation of self-fertile ascospores in *P. anserina* is accomplished by strict regulation of second division segregation of the mating type (located opposite the centromere), coupled with non-overlapping second division spindles (Figure 1.5, Coppin *et al.* 1997, Raju and Perkins 1994). *P. anserina* regulates its second division frequency to 98% for mating type by positive chiasma interference (Esser and Kuenen 1967, Esser 1974). Though most ascospores are self-fertile in *Podospora*, two types of self-sterile ascospores occur. A small percentage (2%) of ascospores are self-sterile due to first division segregation of mating type. Another 1-2% of the asci contain some small uni-nucleate spores (Figure 1.4c). Usually two uni-nucleate

spores are produced instead of one bi-nucleate ascospore due to mispackage of the nuclei (Rizet and Engelmann 1949, Esser 1974). Both types of self-sterile ascospores rely on outcrossing to complete their lifecycle.

As mentioned in the previous paragraphs, several examples of genomic conflicts are present in *Podospora anserina*. However, some characteristics of *P. anserina* do influence the study of selfish elements in this species and they are briefly introduced hereafter.

….. ARE DEPENDENT UPON OTHER TRAITS.

Vegetative Incompatibility

Filamentous fungi are capable of undergoing hyphal fusion (anastomosis) between individuals to form vegetative heterokaryons that contain genetically different nuclei. However, if individuals differ in allelic specificity at vegetative (heterokaryon, somatic, or heterogenic) loci, the hyphal fusion cell is quickly compartimentalized and lysis is induced. The features of cell death are similar to those observed in programmed cell death (PCD) in multicellular eukaryotes (Glass *et al.* 2000, Bernet 1992). Vegetative incompatibility can to some extent restrict intracytoplasmic spread of harmful infectious particles as well as exploitation by aggressive genotypes (Glass *et al.* 2000, Saupe 2000). On the other hand, vegetative compatibility also offers potential benefits such as functional diploidy (for *P. anserina* this is the case for colonies arising from monokaryotic ascospores), mitotic recombination, improved communication or increased biomass for better resource exploitation or reproduction (Glass *et al.* 2000). Vegetative (in)compatibility seems to act like a non-self recognition system in filamentous fungi, which is expressed during vegetative growth, but arrested at the sexual stage (Glass and Kuldau 1992).

Hyphal anastomoses can be divided into three phases: pre-contact, post-contact and post-fusion. Pre-contact phase includes avoidance and attraction by chemotropy to respectively accumulating metabolites and changes in oxygen levels. The post-contact phase is defined by growth arrest, the breakdown of the hyphal cell wall, plasma membrane fusion and cytoplasmic mixing. The post-fusion phase encompasses the recognition and the breakdown machinery if self-recognition fails (Glass *et al.* 2000). Some phases of this recognition system are also required for cell fusion during mating. Several het-loci of *P. anserina* are involved in starvation and induction of female reproductive structures, as in yeast (Bernet 1991, Saupe 2000).

Vegetative incompatibility phenotypes are generally defined by the alleles at multiple *het (heterokaryon incompatibility)* -loci. The fungus *P. anserina* harbors two types of vegetative compatibility systems: an allelic type, in which five loci are involved, and three systems of an non-allelic type with five loci involved [a]. In an allelic interaction vegetative incompatibility is mediated by the alternative alleles of a single *het*-locus; a non-allelic incompatibility reaction is triggered by the interaction between specific alleles at two different loci (Bernet 1965, 1967, Saupe 2000). Molecularly, the interaction between alternative *het*-alleles is not well understood, as the *het* loci encode for very different gene products.

Heterocomplex formation between alternative HET polypeptides may act as a recognition complex and the formation of this complex could trigger the mediation of the biochemical and morphological aspects of vegetative incompatibility. Alternatively, the HET heterocomplex could act as a poison to the cell and thus actively mediate growth inhibition and death (Bégueret *et al.* 1994, Glass *et al.* 2000).

Polymorphisms for vegetative incompatibility are probably maintained by balancing selection, as evidence has been found in *Neurospora* (Wu *et al.* 1998), and is suspected in *Podospora* (Glass *et al.* 2000, Saupe *et al.* 1995). However Bégueret *et al.* (1994) suggested that het-genes were an evolutionary accident. In fungi that are self-fertile, such as the pseudo-homothalllic *P.anserina*, vegetative incompatibility may also provide an effective barrier to outbreeding by inviable combinations of *het*-genes and even favor speciation (Bernet 1965, Saupe 2000). Most isolated cultures of *P. anserina* are vegetative incompatible with each other (Esser and Blaich 1994, Esser 1971, Hamann and Osiewacz 2004)

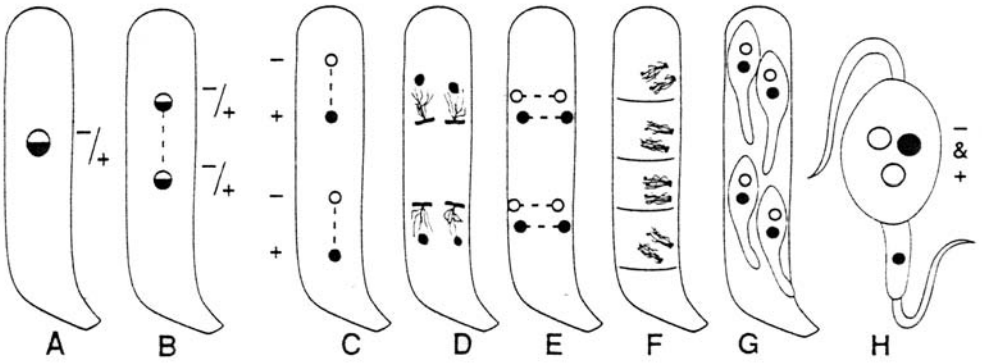
Sexual compatibility and Outcrossing rate.

Mating type can be viewed as a genetically determined sexual compatibility phenotype. Mating type is defined in terms of heterothallism (self-sterilty) in which gamete nuclei must come from parents of a different mating type. In homothallic fungi each strain is self-fertile, so sexual reproduction can involve genetically identical nuclei and mating type cannot be defined. In contrast, pseudo-homothallism refers to a system in which single strains are self-fertile, but in which the actual nuclei undergoing fusion are not identical (Kronstad and Staben, 1997). Sex in *P.anserina* is regulated by the ideomorphic mating type genes mat+ and mat-. Mating type genes have a role in mate recognition, fructification and in other heterothallic fungi also function as vegetative incompatibility loci (Arnaise *et al.* 1993). Over-expression of these genes leads to aberrant meiosis and abortion of ascospores (Zickler *et al.* 1995, Coppin and Debuchy 2000). Mutations in the mating type genes can lead to self-fertile strains (Arnaise *et al.* 2001).

Sexual reproduction in heterothallic fungi starts when the pheromone receptor (female organ: trichogyne, protoperithecium) senses the pheromone emitted by the fertilizing agent (male organ: spermatium). This would lead to a problematic situation in pseudo-homothallic and homothallic ascomycetes, where concomitant expression of receptor and pheromone genes in the same cell would lead to the two pheromone-receptor interactions, and improper inactivation of the pheromone response pathway (Bistis 1996, 1998). On the other hand this may also cause the sexual incompatible and semi-incompatible situations found in Podospora, when receptor and pheromone of outcrossing strains do not fit both ways or only one way (Coppin *et al.* 1997).

[a] Nomenclature of *het*-loci in *Podospora anserina* is confusing as they were named differently by research groups. According to Bernet (1965, 1967) the five allelic *het*-loci are *het-b, het-q, het-s, het-v* and *het-z,* and the three non-allelic combinations *het-c/het-d, het-c/het-e* and *het-r/het-v*. Esser named the allelic loci respectively *het-t, het-u, het-s, het-v* and *het-k*. The non-allelic combinations were named respectively *het-b/het-d, het-b/het-a* and *het-c/het-v* by Esser (Esser and Blaich 1994, Marcou *et al.* 1990). The Bernet annotation is used most in recent literature.

FIGURE 1.5. -- Meiosis and ascospore development in *Podospora anserina*.
(A) Nuclear fusion (B) First meiotic division (C) Telophase II; spindles are aligned in tandem (D) Interphase II; nuclei are aligned as pairs (E) Telophase III; third division spindles are paired and oriented across the ascus (F) Interphase III; the four pairs of nuclei are realigned regularly to one side of the ascus (G) Asci with four bi-nucleate young ascospores (H) One (of the four) ascospore after one further mitosis; one nucleus migrates into the 'podos'; additional mitosis result in a highly multinuclear spore. Mating types are represented by + and -. Figure taken from Raju and Perkins 1994.



The pseudo-homothallic nature of *P. anserina* provides the fungus with two options for sexual reproduction: selfing and outbreeding. Which type(s) play(s) a significant role in nature is currently unknown, but some prerequisites are needed for outcrossing. For instance, for outcrossing to occur other genotypes have to be present within a dung population (or in the vicinity). Furthermore, if alternative strains are present, they have to be sexually compatible. Sexual compatibility has been linked to non-allelic vegetative compatibility in *P. anserina* (Esser and Blaich 1994), but also both mating type and vegetative compatibility seem to use the same pathways (Kronstad and Staben 1997, Saupe 2000). Studies where different isolates were tested against each other for both sexual and vegetative compatibility showed that only a small fraction were sexually compatible with each other (Esser 1971, Bernet 1965, Hamann and Osiewacz 2004). Strains of *Neurospora tetrasperma* originating from different geographical regions even displayed severe forms of sexual dysfunction ranging from aborted spores, ascospore inviability and complete infertility. Factors underlying sexual dysfunction could be a too divergent genetic background, as well as differences in heterokaryon incompatibility genes (Jacobson 1995, Saenz *et al.* 2001)

Fungal senescence and longevity.

A fungal organism can roughly be viewed as a clone of mitotically derived cells that are capable of unlimited growth and propagation. Asexual spores can spread over vast geographical areas and a fungal colony can grow extensively. Generally, organismal death does not seem to be part of most fungal life cycles; however *Podospora anserina* is one of the exceptions (Osiewacz 2002, Griffiths 1992). The senescent phenotype of *P.anserina* is characterized by an age-related reduction of mycelial growth rate, a decrease in aerial hyphae

and an increase in the pigmentation of the distal mycelium (Rizet 1953). Each strain isolated from nature shows a unique limited lifespan, measured by the maximum growth length of the mycelium, which ranges from a few centimeters to several meters (Marcou 1961). Maternal inheritance and the ability to transfer the senescent state upon cytoplasmic contact pointed to the involvement of mitochondria in senescence (Marcou 1961). It was found that the senescent state correlated with the excision of circular plasmid-like elements from the mitochondrial genome. These *sen*DNA's originate from several regions in the mitochondrial genome and are called respectively *α, β, γ*, etc. or *pl*DNA. *α*-senDNA is most commonly excised and is identical in sequence to the first intron of cytochrome c oxidase (COX1) (Cummings *et al.* 1985). Each *sen*DNA is able to replicate autonomously, however these elements are nowadays seen as a product and not the cause of fungal senescence (Silliker and Cummmings 1990). Rather the molecular processes of respiration, which generates ROS radicals, destabilize the mitochondrial genome and give rise to the senescent state (Dufour *et al.* 2000).

Longevity mutants in *Podospora* have either nuclear genes inhibiting mitochondrial functioning, mitochondrial deletions of regions where *sen*DNAs are excised (and thus force the fungus to use another oxidative pathway, AOX), or possess an inserted linear plasmid pAL2 (Hermanns *et al.* 1994). Most other fungi that do not senesce may use the other pathway or are better able to regulate their respiratory pathways (Dufour *et al*. 2000). Longevity in *P. anserina* also correlates with calorie restriction, as with female sterility (Maas *et al.* 2004, Griffiths 1992). Differences in lifespan, aside from female sterility, may also have a significant effect on the reproduction. For instance, the shorter the lifespan of a fungal individual, the shorter the period of mycelial growth, which reduces the area inhabited by the fungal organism and resources available for sexual reproduction.

THE OUTLINE OF THIS THESIS.

Theories on the evolution of genetic conflicts need experimental and population genetic data to be validated. Models are necessarily oversimplifications of what is happening in nature. Processes in nature are very complex and under influence of many variable conditions. Therefore it is important to provide experimental and population data to test the relevance ot the models. Experimental data can also provide material for new theories on the evolution of the selfish elements. This thesis aims to provide population genetic data on genomic conflicts and to create a better understanding of the processes underlying the conflicts. The ascomycete fungus *Podospora anserina* is a suitable species to perform population studies on two types of selfish elements, segregation distorters and (linear) plasmids, as both are common in this species. In *P. anserina* meiotic drive elements were recently found in a population along sensitive strains, as well as some decades ago (Nauta *et al.* 1993, Padieu and Bernet 1967). Apparently drive has not reached fixation, or is somehow stable in this fungus. Also linear plasmids with presumably positive effects were found in the same population (Hermanns *et al.* 1994). This provides excellent opportunity to study the population dynamics of these

selfish elements in the field, also because no markers are needed, as drive can be measured directly on the progeny and linear plasmids are easily detected molecularly. Aside from the availability of selfish elements, the fungus has a short reproduction time, which enables us to follow it through many generations over the years. Furthermore, mycelium and spores can be stored in the freezer to be used later to compare different generations with another.

The second Chapter focuses on the population dynamics of linear plasmids in *Podospora anserina*. It describes the isolation of strains of *Podospora* in Wageningen over several years and the occurrence of pAL2-1 plasmid homologues in the population. Horizontal and vertical (sexual) plasmid transmission rates were determined in both vegetatively compatible and incompatible combinations. The life-span of all isolates was determined and was found to be generally uncorrelated with the presence of the plasmid.

Chapters 3, 4 and 5 study the meiotic drive factors found in *Podospora.* Chapter 3 deals with the determination of at least seven different types of segregation distorters found in the Wageningen population and some French isolates dating from 1937. Six meiotic drive elements were mapped to linkage group three of *Podospora.* Furthermore, the interaction of the Spore killers with each other was studied. Chapter 4 explores the possible relation between Spore killing and vegetative incompatibility. All spore-killer isolates remained vegetatively incompatible after repeated backcrossing to the same standard laboratory strain, suggesting a common mechanism. Chapter 5 further examines the possible mechanisms of segregation distortion in *Podospora anserina.* The role of methylation in Spore killing is tested, as well as temperature dependence of the distorter elements and the variable penetrance of sensitive spores to the Spore killer. All information of drive elements in *Podospora* is combined to discuss and propose a model, based on the toxin –antitoxin model of bacterial plasmids to explain meiotic drive in this fungus.

Next, Chapter 6 addresses several factors concerning the spread of selfish elements. Meiotic drive for instance is dependent upon outcrossing. The extent of outcrossing is estimated using the vegetative compatibility (VCG) groups and sexual compatibility reactions in the Wageningen population. The proportion of monokaryotic ascospores (which need outcrossing to produce offspring) for sensitive and spore killer strains is measured and the ability of outcrossing of dikaryotic ascospores (which are capable of selfing) and mycelium is examined. The results strongly suggest that outcrossing occurs regularly in *Podospora,* and is not limited to single mating type strains. Finally, Chapter 7 summarizes all the results on two genomic conflicts in *Podospora anserina* and discusses them in relation to recent findings.

Chapter 2

The dynamics of pAL2-1 homologous linear plasmids in *Podospora anserina.*

Marijn van der Gaag, Alfons J.M. Debets, Heinz D. Osiewacz and Rolf F. Hoekstra

Published in Molecular and General Genetics (1998:) 258:521-529.

ABSTRACT

A natural population of recently isolated *Podospora anserina* strains was screened for homologues of the linear longevity-inducing plasmid pAL2-1. Of the 78 wild-type isolates 14 hybridised with a pAL2-1 specific probe, half of which contained a single plasmid and the other half multiple plasmid copies (plasmid family). All strains did senesce normally, except one plasmid-containing strain. However, no inserted plasmid sequences were detected in the mitochondrial DNA, as was the case for the longevity inducing pAL2-1 plasmid.

Occasional loss of plasmids and of repeated plasmid sequences occurred during sexual transfer. Plasmid transmission was equally efficient for mono- and dikaryotic spores and independent of the genetic background of the strains. Furthermore, horizontal transfer experiments showed that the linear plasmid could easily infect plasmid-free strains. Horizontal transfer was even observed between strains showing a clear vegetative incompatibility response (barrage). The linear plasmids are inherited maternally, however paternal transmission was observed in crosses between confronted vegetative incompatible strains. Paternal transmission of the plasmid was never observed when using isolated spermatia for fertilisation, showing that mitochondrial plasmids can only get access to maternal sexual reproductive structures following horizontal transfer. These findings have implications for both the function of vegetative incompatibility in fungi and the maintenance of linear plasmids.

INTRODUCTION

Linear plasmids are genetic elements found in the cytoplasm and mitochondria of many lower and higher eukaryotes (for review see Griffiths 1995, Meinhardt *et al.* 1990). Although they have been studied extensively, little is known about their function. Most plasmids seem to be neutral passengers in their host, however in a few systems a specific role has been attributed to these elements. For instance, in yeasts linear cytoplasmic DNA elements have

been found which encode for a killer toxin and resistance against it (Stark and Boyd 1996, Worsham and Bolen 1990). In *Neurospora* two linear mitochondrial plasmids, *kalilo* and *maranhar*, are the causative agents of senescence. Here senescence was demonstrated to result from the integration of the plasmid into the mtDNA and the accumulation of defective mtDNA molecules (Bertrand *et al.* 1986, Court *et al.* 1991). In contrast, in a long-lived mutant of another ascomycete fungus, *Podospora anserina,* the presence of a linear plasmid is correlated with an increased lifespan (Hermanns and Osiewacz 1996, Osiewacz *et al.* 1989). Senescence in this fungus is related with (mt)DNA rearrangements and amplification of certain regions (senDNA α, β, γ or *pl*DNA) as tandemly repeated circular molecules (for review see Griffiths 1992).

Three major differences were found between this longevity mutant AL2 and the wild type strain A, from which it originated: First a delay in amplification of *α-sen*DNA was observed. Furthermore, a deletion of about 3.6 kb was detected, and finally a linear mitochondrial plasmid was found and characterised (Hermanns *et al.* 1994, Hermanns and Osiewacz 1994, Osiewacz *et al.* 1989). This 8.3 kb linear plasmid, named pAL2-1, contains long terminal inverted repeats (TIRs) of 975 bp and has two non-overlapping open reading frames of 3.6 and 2.8 kb located on opposite strands encoding respectively a DNA- and RNA-polymerase (Hermanns and Osiewacz 1992, Osiewacz *et al.* 1989). Also two giant linear plasmid-like DNAs of 50 and 70 kb were detected in strain AL2, composed of mtDNA and pAL2-1 specific sequences, which may act as intermediates in integration events of the linear plasmid pAL2-1 (Hermanns *et al.* 1995a). Transfer experiments of these plasmids to the wild-type A strains indicated that a specific insertion of the linear plasmid into the wild-type mitochondrial genome is responsible for the longevity phenotype (Hermanns and Osiewacz 1996).

Although much of the structure of pAL2-1 is known at the moment, the question of the origin of the plasmid remains unclear. Strain A of which mutant strain AL2 was derived, does not have linear plasmids, nor do the extensively screened isolates of an old collection of wild-type strains (Hermanns and Osiewacz 1994). However, one recently isolated wild-type *P.anserina* strain Wa6, did contain a family of plasmids with homology to the linear longevity plasmid pAL2-1 (Hermanns *et al.* 1995b).

Mitochondrial plasmids in fungi are generally stably transmitted, both mitotically and meiotically. However in *Neurospora intermedia,* Debets *et al.* (1995) found that the proportion of strains bearing plasmids was reduced due to the sexual cycle. Also in another ascomycete fungus, *Epichloë typhina*, incidental loss of mitochondrial plasmids to the ascospore progeny was observed (Chung *et al.* 1996). In crosses, plasmids are predominantly maternally transmitted, like other cytoplasmic elements. Again exceptions have been reported in fungi like male transmission and plasmid suppression (Chung *et al.* 1996, Griffiths *et al.* 1992, May and Taylor 1989, Yang and Griffiths 1993a, 1993b).

Reduction of the proportion of strains carrying linear plasmids may eventually lead to extinction of a plasmid species, unless the plasmid is able to (re)infect plasmid free strains. Horizontal transfer of cytoplasmic elements has been studied in several fungal species, often in combination with vegetative or heterokaryon incompatibility (Anagnostakis 1983, Caten

1972, Debets *et al.* 1994). The wide occurrence of vegetative (or heterokaryon) incompatibility genes in nature may represent an adaptive strategy, and one of its functions may be to protect fungal individuals against infection of parasitic cytoplasmic elements (Caten 1972, Esser and Blaich 1994, Glass and Kuldau 1992). In *P. anserina* up to 17 different allelic and non-allelic *het*-loci have been discovered (Bégueret *et al.* 1994, Esser and Blaich 1994, Glass and Kuldau 1992). Although vegetative incompatibility does not fully prevent infection of linear plasmids in the fungus *Neurospora*, it significantly slows down the spread of these elements (Debets *et al.* 1994).

In this study we present data of the screening for pAL2-1 plasmid homologues within recently isolated wild-type *P. anserina* strains. We also present the results of horizontal and vertical transfer experiments of the linear plasmid homologues. Furthermore, we correlate the presence of these plasmids with their population dynamics.

MATERIALS AND METHODS

Strains and culturing methods:

Wild-type *P. anserina* strains were isolated from fresh herbivore dung near Wageningen, the Netherlands, during 1991-1995 using sterilised horse dung as a semi-selective medium. Standard growth conditions and characteristics of strains s, A and AL2 were described previously (Esser 1974, Osiewacz *et al.* 1989). The wild-type strains Wa2, Wa3 and Wa6 have been studied previously by Hermanns *et al.* (1995b). Cultures for isolation of the DNA were grown for 3-5 days at 27°C in liquid complete medium (Esser 1974). All ascospores from selfings of the original isolated wild-type strains were collected as ascospore stock and stored at -50°C (Begel and Belcour 1991).

Characterisation of *P. anserina* strains:

New strains were tested for vegetative incompatibility reaction against laboratory strains and each other by mycelial confrontation on a culture dish (Esser and Blaich 1994). Lifespans (expressed as maximal growth length in cm) were determined in 50 cm long race tubes (Koll *et al.* 1984). Mycelium from dikaryotic spores from stock was used to check for presence of pAL2-1 linear plasmid homologues.

Genetic techniques:

Transmission of plasmids through ascospores was determined by Southern-blot analysis. For this purpose mono- and dikaryotic ascospores from selfed plasmid bearing strains, as well as dikaryotic spores from outcrosses with plasmid free strains were used. Crosses were made by confronting mycelia of opposite mating type on a culture dish, and/or by pouring a suspension of microconidia of one mating type over a proto-perithecial culture of the opposite mating type (Esser 1974). Strain Wa6 was used as plasmid donor, strain s and Wa2 as sexually compatible and Wa3 and Wa14 as sexually unilateral incompatible plasmid free strains.

Horizontal plasmid transfer experiments were performed by growing monokaryotic mycelia of plasmid free (acceptor) and plasmid bearing (donor) strains together on a culture dish with solid corn meal medium at 27°C (Esser 1974). After 3 weeks (to allow completion of the lifecycle) mycelial samples were taken from the acceptor strain at 0.5 and 2.5 cm distance from the mycelial contact zone and analysed by Southern-blotting. Strain Wa6 was used as donor strain, strain Wa2 as vegetative compatible acceptor and s, Wa3 and Wa14 as vegetative incompatible strains in reciprocal combinations of mating types.

Preparation and analysis of total DNA:

Small mycelial cultures were ground in liquid nitrogen and suspended in 1 ml DNA-extraction buffer (Yang and Griffiths 1993a). Cellular debris was separated by centrifugation for 30 seconds. Supernatant was treated with proteïnase K (0.5 mg/ml) for 1-2 h, followed by one phenol:chloroform:isoamylalcohol (25:24:1) extraction. The DNA was precipitated with 2-propanol, vacuum dried and redissolved in 100 µl MilliQ H_2O overnight at 4°C.

Standard methods were used for electrophoresis, Southern-blotting, in vitro labelling of nucleic acids and hybridisations (Sambrook *et al.* 1989). Digoxigenin labelling and hybridisation procedures were used according to Boehringer Mannheim. The *Xba*I X-3 fragment of pAL2-1 was used as a specific probe to detect homologous linear plasmids (Hermanns and Osiewacz 1994).

RESULTS

Characterisation of wild-type strains:

Wild-type strains Wa1 to Wa83 were isolated from rabbit, horse and sheep dung during 1991--1995 near Wageningen, the Netherlands. They were classified as *Podospora anserina* species by morphological criteria, described by Mirza and Cain (1969). This classification was verified genetically by crosses of each isolate to laboratory and wild-type strains of *P. anserina*. At the same time, vegetative compatibility of the isolates was tested by 'barrage' formation. A 'barrage' consists of a macroscopical clear zone resulting from lysis of fused hyphae (Esser and Blaich 1994). Each isolate was assigned a different strain number when it originated from a separate dung source, location, year, or when it was genetically different in vegetative compatibility.

Single mating type strains and ascospore stock were obtained by selfing of the original isolate. Ascospore stock remained frozen at -50°C, so only one sexual cycle had passed between the originally isolated wild-type strain and the strains tested for presence of linear plasmid, circumventing chances of plasmid loss during prolonged cultivation. To further reduce the possibility of plasmid loss, mycelium from three to five ascospores was pooled for DNA extraction. The pAL2-1 *Xba*I digest X-3 was used as a probe because it lacked the ORF-2 coding a RNA-polymerase, which was previously shown to hybridise to the high molecular mtDNA of other wild-type strains (Hermanns and Osiewacz 1994). Of the 78 wild-type strains 14 isolates hybridised with this fragment (Table 2.1, Figure 2.1), and 7 of them showed a similar plasmid family structure as found in the cultures from the monokaryotic

TABLE 2.1.
***Podospora anserina* strains used in this study.**

Plasmid containing strains				Plasmid-free strains					
Isolate[a]	Year[b]	Growth (cm)[c]	Plasmid	Isolate	Year	Growth (cm)	Isolate	Year	Growth (cm)
Wa1	1991	24.9	Family	Wa3	1991	22.4	Wa46	1993	37.8
Wa2		14.6	Family	Wa7	1993	25.5	Wa47		32.2
Wa4		26.7	Family	Wa8		20.2	Wa48		35.8
Wa5		25.6	Family	Wa9		19.3	Wa49		32.6
Wa6	1992	22.9	Family	Wa11		19.1	Wa50		28.2
Wa10	1993	17.7	Family	Wa12		23.7	Wa51		36.6
Wa18		20.1	Single	Wa13		26.6	Wa52		33.9
Wa30		10.4	Single	Wa14		20.2	Wa54		21.1
Wa32		94.2	Single	Wa15		19.0	Wa55		23.0
Wa34		15.8	Single	Wa16		21.8	Wa56		26.0
Wa35		47.2	Single	Wa17		19.3	Wa57		19.8
Wa45	1994	39.0	Single	Wa19		19.9	Wa58	1994	31.1
Wa53		35.3	Single	Wa20		10.3	Wa59		20.9
Wa70	1995	17.4	Family	Wa21		18.7	Wa60		25.0
AL2	1989	>227.2	Single	Wa22		28.8	Wa61		24.4
				Wa23		24.0	Wa62		21.2
				Wa24		26.7	Wa63		21.1
				Wa25		21.4	Wa64		23.8
				Wa26		20.2	Wa65		27.5
				Wa27		23.4	Wa66		26.4
				Wa28		13.0	Wa67		16.5
				Wa29		20.5	Wa68		21.9
				Wa31		19.6	Wa69		15.9
				Wa33		16.4	Wa71	1995	18.8
				Wa36		17.1	Wa72		22.1
				Wa37		12.4	Wa76		10.7
				Wa38		13.5	Wa77		38.3
				Wa39	1994	41.4	Wa78		14.4
				Wa40		38.4	Wa80		15.1
				Wa41		39.5	Wa81		16.7
				Wa42		34.9	Wa83		16.7
				Wa43		26.4	s	1937	27.3
				Wa44		38.7	A	1937	24.1

[a] Data concerning strains AL2, A and s were taken from Hermanns *et al.* (1995b). The type of pAL2-1 plasmid homologues is given for each strain if present. All Wa strains were isolated in Wageningen, the Netherlands, laboratory strains A and s were isolated in Normandy, France (Belcour *et al.* 1997, Deleu *et al.* 1993). Long-lived strain AL2 was derived from laboratory strain A (Osiewacz *et al.* 1989).

[b] The year of isolation is given for the first strain of that year in each column.

[c] Maximum growth length of strains is the mean value of two race tubes and given in cm.

spores of strain Wa6. Remarkably, plasmids were found in the dikaryotic strain Wa2 (Figure 2.1), where previously no plasmid was detected in the cultures derived from the monokaryotic

spores (Hermanns *et al.* 1995b). No plasmid signal was detected at the position of the high molecular weight mitochondrial genome, indicating that the linear plasmid was present as a free plasmid and not inserted into the mitochondrial genome (also see Figure 2.1). Also Hermanns *et al.* (1995b) reported no inserted plasmid sequences in the genome of wild-type strain Wa6.

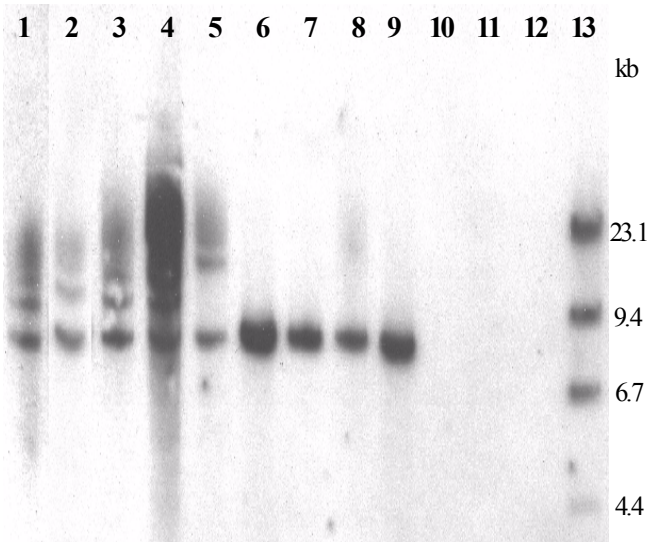
Of all wild-type strains, the lifespan was measured in race tubes to determine possible effects of the presence of pAL2-1 homologue plasmids on longevity (Table 2.1). All plasmid free strains showed the senescent phenotype, as did the pAL2-1 homologue plasmid containing strains. The only exception is strain Wa32, which did grow significantly longer than all the other wild-type isolates, but less if compared with the AL2 longevity mutant strain. Lifespan of the senescent strains is comparable with other lifespan measurements of wild-type strains, although minor differences occur due to environmental conditions (Hermanns *et al.* 1995b, Marcou 1961).

Efficiency of plasmid transfer:

The finding of a pAL2-1 homologue in the dikaryotic strain Wa2, while it was absent in the monokaryotic strains (Hermanns *et al.* 1995b), suggests that plasmids may fail to become included into the relatively small monokaryotic spores. Apart from cytoplasmic exclusion, reduction of the plasmid transfer efficiency could also have a genetic basis. To investigate genetic and cytoplasmic influences on the transfer efficiency of pAL2-1 plasmid homologues, mono- and dikaryotic offspring of selfed plasmid strains were tested.

Plasmid family transfer was examined in 31 monokaryotic spores of Wa6 and 14 monokaryotic spores of strain Wa2, as well as in 33 dikaryotic spores of Wa6 and 36 dikaryotic spores of Wa2. Table 2.2 shows that plasmid free progeny were found in both monokaryotic (average 24%) and dikaryotic ascospores (average 28%), indicating that plasmid transfer from parent to offspring is incomplete. Interestingly, not only loss of plasmid was found, also loss of sequences of the plasmid family could be observed. In cultures from

FIGURE 2.1. -- Example of linear plasmid detection in 12 isolates of *Podospora anserina* on a digoxigenin-labelled Southern blot.



The first five lanes show strains with a linear plasmid family, isolates in lanes 6-9 contain only a single plasmid, while strains in lane 10 - 12 are plasmid free. No plasmid signal is found at the size of the mitochondrial genome (80-102 kb). Strains used were Wa1 (lane 1), Wa2 (lane 2), Wa4 (lane 3), Wa6 (lane 4), Wa10 (lane 5), Wa18 (lane 6), Wa34 (lane 7), Wa45 (lane 8), Wa53 (lane 9), Wa3 (lane 10), Wa14 (lane 11), and s (lane 12). Lane 13 contains a lamda *Hin*dIII size marker.

TABLE 2.2.
Plasmid transfer of pAL2-1 homologues through selfings of seven *P. anserina* isolates.

Strain [a]		Percentage of plasmid containing spores (total spores)	
		Single plasmid	Plasmid family
Wa2	Monokaryotic	78.6 (14)	7.1 (14)
	Dikaryotic	47.2 (36)	36.1 (36)
Wa6 [b]	Monokaryotic	58.1 (31)	12.9 (31)
	Dikaryotic	60.6(33)	0 (33)
Wa18	Dikaryotic	100 (20)	-
Wa30	Dikaryotic	90 (20)	-
Wa32	Dikaryotic	71.4 (35)	-
Wa35	Dikaryotic	89.5 (19)	-
Wa53	Dikaryotic	80 (20)	-

[a] Strains Wa2 and Wa6 (plasmid family) were used to test transfer to mono- and dikaryotic ascospores. Strains Wa18, Wa30, Wa32, Wa35 and Wa53 (single plasmid) were used to test transfer to dikaryotic ascospores.
[b] Parental strain Wa6 only showed a single plasmid band, during testing of dikaryotic spores.

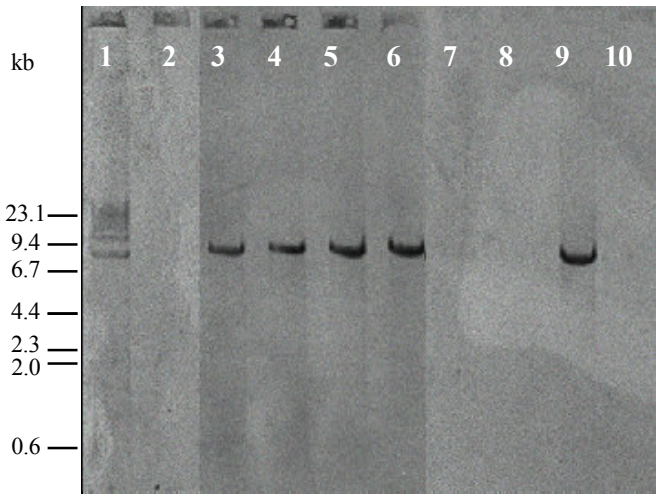
these spores, only the 'basic' plasmid pWa6-1 was detected.

Transfer efficiency of the single plasmid homologues to dikaryotic spores was tested in the same manner with 20 or more spores from five different isolates. Here we observed an average of 16% plasmid free offspring (Table 2.2).

Inheritance of pAL2-1 plasmid homologues:

Incomplete plasmid transfer to the progeny can be compensated by biparental inheritance, which allows transfer through both ascogonium (maternal) and microconidia (paternal). To examine this possibility, we investigated the inheritance of the mitochondrial plasmid family pWa6 in crosses with plasmid free strains. These crosses were also used to investigate the possible existence of plasmid suppressors (Griffiths *et al.* 1992, Yang and

FIGURE 2.2. -- Horizontal transfer between vegetative incompatible strains Wa6$^-$ and Wa3$^+$ as detected on a digoxigenin-labelled Southern blot.



The first lane contains the Wa6$^-$ donor strain showing the plasmid family, lane 2 the Wa3$^+$ acceptor strain before transfer. Lanes 3-6 are mycelial samples taken at 2.5 cm distance from the mycelial contact zone, lanes 7-10 contain samples taken at 0.5 cm distance. After transfer only the basic linear plasmid is found in the acceptor strain. Lambda DNA cut with *Hin*dIII was used as size marker.

TABLE 2.3.
Inheritance of the pAL2-1 homologue plasmid family (pWa6) in outcrosses.

Spores from cross [a]	Percentage of plasmid-containing spores (total number of spores)	
	Microconidial suspension	Mycelial confrontation
Sexual and vegetative compatible strains		
Wa2 [free] x Wa6	0 (97)	nd
Wa6 x Wa2 [free]	39.2 (90)	nd
Sexual and vegetative incompatible strains		
s x Wa6	0 (38)	0 (80)
Wa6 x s	40.9 (93)	27.5 (40)
Sexual unilaterally incompatible and vegetative. incompatible strains		
Wa3 x Wa6	0 (78)	38.2 (76)
Wa14 x Wa6	0 (38)	21.1 (38)

[a] Crosses were made with a microconidial suspension or by mycelial confrontation (in each cross, the maternal strain is listed first). Combinations of strains are vegetative compatible or incompatible, and sexual compatible or unilateral incompatible. Strain Wa2 [free] is a plasmid-free derivative of strain Wa2. Strain Wa6 contains a linear plasmid family. No parental determination of offspring (*nd*) could be made by mycelial confrontation in crosses between Wa6 and Wa2 [free] due to vegetative compatibility.

Griffiths 1993b). Crosses were performed in two ways: 1. by pouring microconidia onto single mating type cultures, and 2. by confronting mycelia of opposite mating type and taking ascospores from perithecia from both sides of the 'barrage'. In total, 38 up to 93 ascospores were tested for each cross (Table 2.3).

Fertilisation with microconidia produced only maternally inherited plasmid offspring. However, the ascospores produced by mycelial confrontation had also paternally inherited plasmids in crosses with strains Wa3 and Wa14. The difference in both results can be explained by the mechanism of the crosses. Mycelial confrontation opens up the possibility of horizontal plasmid transfer to the maternal mycelium before ascospore formation. Microconidia are only capable of fertilising the proto-perithecia, and no infection by mycelial contact could occur. Indeed, further horizontal transfer experiments described below confirmed our hypothesis.

Horizontal transfer of pWa6 plasmid family:

Besides biparental inheritance, (re)infection of strains by horizontal transfer is another possibility to compensate for plasmid loss. To investigate this, horizontal transfer experiments were performed using the pWa6 plasmid family in vegetatively compatible and allelic and non-allelic incompatible combinations of strains. Sexual compatibility of the Wa6 x s combination is unaffected, therefore vegetative incompatibility of these strains must result from an allelic mechanism. However, Wa6 in combination with Wa3 and Wa14 results in

TABLE 2.4.
Horizontal transfer of the pWa6 plasmid family to plasmid-free strains.

Donor and acceptor Strains [a]	Number of tissue samples at 0.5 cm			Number of tissue samples at 2.5 cm		
	Plasmid free	Single plasmid	Plasmid family	Plasmid free	Single plasmid	Plasmid family
Vegetative compatible pairings						
Wa6 x Wa2 [free]	1	6	1	2	6	0
Vegetative incompatible pairings (allelic)						
Wa6 x s	5	3	0	3	3	2
Vegetative incompatible pairings (non-allelic)						
Wa6 x Wa3	6	2	0	1	7	0
Wa6 x Wa14	3	2	3	3	3	2

[1] Eight acceptor tissue samples from two plates were taken at 0.5 and 2.5 cm distance from the mycelial contact zone between the donor and acceptor strain. Strain Wa2 [free] is a plasmid free derivative of strain Wa2. Strain Wa6 is donor and contains the linear plasmid family.

sexual unilateral incompatibility, pointing to the involvement of an non-allelic vegetative incompatibilty mechanism (Esser and Blaich 1994). Donor strains were tested after the horizontal transfer experiment, to exclude apparent transfer failure due to plasmid loss. The pWa6 plasmid family was found present in donor strains of all used donor-acceptor combinations. Table 2.4 shows that the linear plasmid was detected in almost all mycelial samples of the vegetative compatible acceptors. More surprising, the linear plasmid was also found in abundance in all three vegetative incompatible acceptors (Figure 2.2). Since donor and acceptor strains were of opposing mating type, and no fruiting bodies were found within the acceptor strain (except as normal next to the 'barrage'), it is very unlikely that hyphae of the donor strain have passed the vegetative incompatibility zone. The linear plasmid could not only be found close to the contact zone, but also further away, indicating an active transport of the plasmid through the mycelium of the acceptor strain.

Furthermore, loss of plasmid family members occurred during horizontal transfer of the plasmid. In most cases, only the smallest member of the family was transferred (Table 2.4, Figure 2.2). Thus, barrage formation does not restrict horizontal transfer of the linear plasmid pWa6, although transfer is not complete.

DISCUSSION

Linear plasmid presence in a natural population of *Podospora anserina:*

In this paper we have analysed the occurrence and transmission of homologues of the longevity inducing pAL2-1 plasmid in a recently isolated natural population of the ascomycete fungus *Podospora anserina.* We have sampled the *P. anserina* population of

Wageningen, the Netherlands, over 5 years, of which 3 years more extensively, resulting in a total of 78 new wild-type isolates. In each sampling year isolates with plasmids have been found. Overall 18% (14) of the strains contains a pAL2-1 homologue plasmid, half of which has a plasmid family, the other half containing only a single plasmid band. If one takes a closer look at the three more extensively sampled years of isolation, the proportion of plasmid containing strains varies approximately between 7% and 19%. The relatively high percentage of pAL2-1 homologues that have been found in a natural population is in contrast to the extensively subcultured laboratory strains of *P. anserina*, which failed to show any autonomous linear plasmids (Hermanns and Osiewacz 1994).

The presence of linear plasmids related to the longevity plasmid pAL2-1 did not have consequences for the lifespan of the wild-type strains and they do not cause conspicuous phenotypic effects. Only one of the 14 strains containing pAL2-1 homologues did have a significantly longer lifespan of approximately three times that of the other strains. However, Hermanns and Osiewacz (1996) found that only when pAL2-1 was inserted at a certain position into the mitochondrial genome, it converted the strain to its long-lived phenotype. The plasmids found in the population reported here were not detected in the mitochondrial genome and exist only in the free-living form. Also for strain Wa6 it has been proven that the linear plasmid family only was present in the free-living form (Hermanns *et al.* 1995b). The longevity of strain Wa32 must probably be attributed to other causes like mitochondrial rearrangements or nuclear mutations.

Only few other studies consider the actual proportion of linear plasmids within a natural population. Studies of linear plasmids in Hawaiian and Louisiana populations of *Neurospora* species show that frequencies of Kalilo plasmids between populations are quite different (Debets *et al.* 1995, Marcinko-Kuehn *et al.* 1994). World-wide comparison of linear plasmid frequencies in *Neurospora* (Arganoza *et al.* 1994), *Fusarium solani* f. sp. *cucurbitae* (Samac and Leong 1988) and *Epichloë typhina* (Mogen *et al.* 1991) show that the incidence of the pAL2-1 homologues is approximately the same of that of other linear plasmids in sexual ascomycete fungi, around 8-16%. The asexual fungus *Fusarium oxysporum* f. sp. *conglutinans*, a species related to ascomycetes, shows the much higher linear plasmid frequency of 100% (Kistler and Leong 1986). Interestingly, considerable plasmid loss is observed in the sexual cycle of *Neurospora*, but transmission to the asexual spores is found to be very efficient (Debets *et al.* 1995, Griffiths and Bertrand 1984, May and Taylor 1989, Yang and Griffiths 1993b).

Plasmid transfer through selfing:

The vast majority of *P. anserina* ascospores are dikaryotic, however the rare and smaller monokaryotic spores are used for genetic analysis. Our finding of plasmids in the dikaryotic spores of strain Wa2, while they were absent from the strains derived from monokaryotic spores could reflect a lower transmission efficiency to monokaryotic than to dikaryotic spores. Our analysis (R x C test of independence (Sokal and Rohlf 1995), $p = 0.58$) of vertical plasmid transfer efficiencies to mono- and dikaryotic spores did not confirm this idea. Transmission of plasmids to monokaryotic spores (76%) was as efficient as to dikaryotic

spores (72%). Also no significant difference in plasmid exclusion among the seven tested strains could be detected (R x C test of independence, p = 0.88), rejecting the possibility of a genetic influence. Therefore, loss of plasmids in selfed strains of *Podospora* is probably accidental.

Another remarkable result was the reduction of plasmid families to their smallest unit during sexual transmission. Each family member is in principle capable of generating the whole family (Hermanns *et al.* 1995b), and transfer of only one unit should lead to the reconstruction of the complete plasmid ladder. The loss of all repeated plasmid sequences must therefore be attributed to an unknown factor.

Loss of plasmids or plasmid sequences is regularly found in the sexual cycle and rarely during asexual propagation (Chung *et al.* 1996, Debets *et al.* 1995, Griffiths and Bertrand 1984, May and Taylor 1989, Yang and Griffiths 1993b). *P. anserina* is a secondary homothallic fungus and reproduces only sexually. Several mechanisms could be responsible for plasmid loss in the sexually produced ascospores. LA-kalDNA in *N. tetrasperma* is transmitted to 100% of the sexual offspring in one strain whereas 18% of the offspring of another strain remained plasmid free (Marcinko-Kuehn *et al.* 1994), suggesting a genetic influence on cytoplasmic transfer mechanisms, or plasmid proliferation characteristics leading to low copy numbers. Indeed, May and Taylor (1989) found that low copy number plasmids occasionally were lost. So far, only occasional loss of complete plasmids has so far only been observed in strains containing several plasmids (Chung *et al.* 1996).

Plasmid transfer through outcrossing:

We have tested the sexual transmission of the natural homologue (pWa6 plasmid family) of the longevity plasmid pAL2-1 using two crossing techniques. When we used microconidia for our crosses we found that the pWa6 plasmid family inherited strictly maternally, like the majority of fungal plasmids and cytoplasmic elements, but at a rather low rate of approximately 40%. In experiments that used mycelial confrontation to produce crosses, the result was strikingly different: A relatively high percentage of paternal transmission was found next to maternal inheritance. Hermanns *et al.* (1994) found only maternally inherited pAL2-1 plasmids, but he had used microconidia to establish crosses. The high percentage of paternal leakage could originate from horizontal transfer of the linear plasmid to the ‘plasmid free’ maternal mycelium before ascospore formation. Indeed, further experiments as discussed below showed that horizontal transfer of the linear plasmid had taken place in those cultures. These results imply that plasmids can gain direct access to progeny by invasion of the maternal tissue. Also Debets and Griffiths (1998) found evidence for such a transmission mode in *N. crassa*, where plasmid containing conidia fused with the maternal mycelium. Possibly also previous reports of paternal leakage in *E. typhina* (Chung *et al.* 1996), *N. crassa* (May and Taylor 1989) and in *N. intermedia* (Yang and Griffiths 1993a) could be explained this way.

Our finding of plasmid transfer efficiencies around 40% in outcrossings is rather low compared to the average 90% found in selfings. The reduction in transfer efficiency of nearly 50 % can be due to presence of suppressors, which activity only can be observed in crosses

between plasmid containing and plasmid free strains. Though the ascospores for these tests were sampled at random, they can reflect a 2:2 segregation of a centromere-linked autonomous suppressor in an ascus. Autonomous suppressors are active after the delineation of ascospores, which results in the segregation of the suppressor in the ascus (Griffiths *et al.* 1992, Yang and Griffiths 1993b). Analysis of suppressors in *P.anserina* is complicated by the heterokaryotic nature of the fungus. Not only linkage of the autonomous suppressor to the centromere is of importance here, but also if the suppressors are dominant or recessive. A non-autonomous plasmid suppressor, which acts only in a specific combination of two genotypes and on the cytoplasm at large, would have excluded the linear plasmid and not lowered the transfer efficiency (Yang and Griffiths 1993b). This type of suppression would show no segregation in an ascus and is apparently not present in the performed crosses in *Podospora*.

Horizontal transfer:

Our data of horizontal transfer of linear plasmids are surprising considering the fact that despite 'barrage' formation linear plasmids are transmitted rather effectively. The efficient horizontal transfer mechanism may (partly) make up the loss due to the observed inefficient vertical transfer, especially in outcrossing. Vegetative or heterokaryon incompatibility, due to allelic differences or non-allelic combinations in one or more *het*-genes, is associated with the formation of a 'barrage' in *Podospora* (Bégueret *et al.* 1994, Esser and Blaich 1994). Horizontal transfer of linear plasmids occurred readily in both allelic and non-allelic combinations. Vegetative incompatibility is often believed to function as a barrier against transfer of selfish genetic elements (Caten 1972). However, transfer of selfish elements, though limited by vegetative incompatibility, was also shown by various authors (Anagnostakis 1983, Debets *et al.* 1994).

Horizontal transfer has been proposed as a mechanism for spread of cytoplasmic elements between strains or even between different species. Non-random geographical distribution of plasmid homologues between fungal species is used as a strong argument for this theory (Arganoza *et al.* 1994), as well as molecular phylogenetic evidence (Kempken *et al.* 1992, Taylor *et al.* 1985). More recently, Kempken (1995) detected horizontal transfer of a linear plasmid experimentally between the related fungal species *Ascobolus immersus* (a discomycete) and *P.anserina* (a pyrenomycete). An effective horizontal spread, such as we have found in *P.anserina*, could also contribute to the spread and evolution of linear plasmids in fungal species.

ACKNOWLEDGEMENTS

This study was supported by the Life Science Foundation (SLW), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Chapter 3

Spore killing: Meiotic drive factors in a natural population of the fungus *Podospora anserina*

Marijn van der Gaag, Alfons J. M. Debets, Jessica Oosterhof, Marijke Slakhorst, Jessica A. G. M. Thijssen, and Rolf F. Hoekstra

Published in Genetics (2000): 156:593-605

ABSTRACT

In fungi, meiotic drive is observed as spore killing. In the secondarily homothallic ascomycete *Podospora anserina* it is characterized by the abortion of two of the four spores in the ascus. We have identified seven different types of meiotic drive elements (Spore killers). Among 99 isolates from nature, six of these meiotic drive elements occurred in a local population. Spore killers comprise 23% of the natural population of *P. anserina* in Wageningen, The Netherlands, sampled from 1991 to 1997. One Spore-killer type was also found in a French strain dating from 1937. All other isolates found so far are sensitive to spore killing. All seven Spore-killer types differ in the percentage of asci that show killing and in their mutual interactions. Interactions among Spore-killer types showed either mutual resistance or dominant epistasis. Most killer elements could be assigned to linkage group III but are not tightly linked to the centromere.

INTRODUCTION

Segregation distorters are genetic elements that show meiotic drive, a phenomenon in which one member of a pair of heterozygous alleles is transmitted in excess of the expected Mendelian ratio of 50% (Sandler and Novitski 1957, Lyttle 1991). Well-known examples of segregation distorters are the sex-ratio chromosomes (SR) in *Drosophila*, a male sex chromosomal drive system, and the *t*-haplotype in mice and *segregation distorter* (*SD*) in *Drosophila*, both male autosomal drive systems (Lyttle 1991). In *Drosophila* and mouse, the meiotic drive systems minimally involve two closely linked loci, a distorter and its *cis*-acting target. All distorters are associated with polymorphic chromosomal structures, such as inversions. Their ratio of distortion in these examples can exceed 90% and they are closely linked to the centromere. It is not known for most drive systems whether they involve two closely linked loci. Likewise, distortion ratios for *Drosophila* and mice in nature may vary

greatly. Meiotic drive systems in these organisms showing <90% distortion are harder to detect. Furthermore, classes of insensitive target or suppressor alleles have accumulated to counter these selfish elements (Lyttle 1991).

Meiotic drive allows deleterious alleles to spread through populations if the frequency gain from their segregation advantage more than compensates the frequency loss due to elimination by natural selection. Thus it threatens adaptive evolution and it is therefore of great interest to obtain information on the extent of meiotic drive in natural populations. This is not easy to study because in animals and plants a driving genetic element requires a specific phenotype to be observable. For this reason it is understandable that an appreciable number of known cases of meiotic drive involve genes affecting the sex ratio. However, fungi in which the haploid nuclei resulting from meiosis are linearly arranged within an ascus provide unique opportunities to analyze abnormal segregation, for precisely the same reason that they have played such a big role in the classical experiments by Lindegren and others on fundamental aspects of linkage, meiotic recombination, and gene conversion (see Whitehouse 1973, Perkins 1992). Any meiotic drive system in such fungi -- provided the elimination of the nuclei containing the nondriving allele occurs in an early stage after the completion of meiosis, as it does in all known meiotic drive systems -- will be observed in a cross between a driving and a sensitive strain as spore *killing*: the degeneration and early abortion of half the ascospores in a certain proportion of the asci. This is not the only distinguishing feature of drive systems in fungi. The ascospores are the products of meiosis as well as the progeny. Thus distortion in fungi also affects the number of offspring produced and reduces the fecundity, which has important consequences for the population genetics of meiotic drive in fungi (Nauta and Hoekstra 1993).

The earliest analysis of two segregation distorters in fungi, then called ascospore abortion factors, is by Padieu and Bernet (1967) in the ascomycete *Podospora*. Turner and Perkins (1979, 1991) identified such abortion factors in *Neurospora* as Spore killers. Other fungi in which distorters have been found are *Gibberella fujikuroi* (= *Fusarium moniliforme*) and *Cochliobolus heterostrophus* (see Raju 1994, 1996 for a review). However, the best-studied example of meiotic drive in ascomycetes is Spore killer (*Sk*) in *Neurospora*. Haploid Spore-killer strains of *Neurospora* were originally identified because asci always contained four viable black and four small inviable unpigmented spores in crosses with standard wild-type strains. All the viable spores carry the Sk^K allele. In crosses homozygous for a killer allele (Sk^K x Sk^K) each ascus contains eight viable black ascospores, as in normal sensitive crosses (Sk^S x Sk^S), indicating that killing occurs only in crosses heterozygous for the killing factor (Turner and Perkins 1979, 1991).

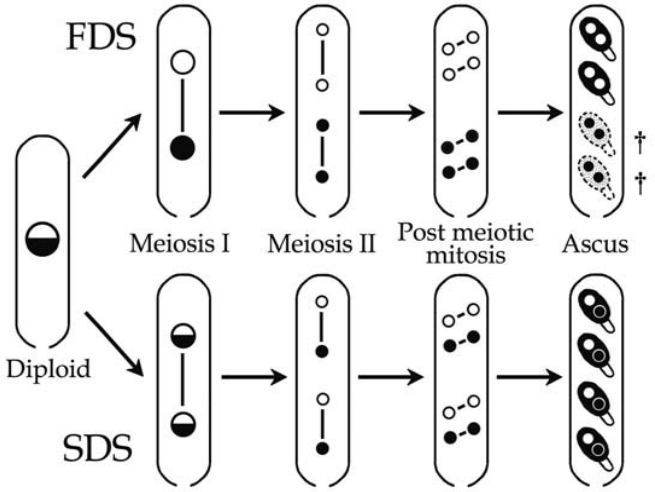
Several Spore-killer types have been characterized in *Neurospora*: $Sk\text{-}1^K$ from *N. sitophila* and $Sk\text{-}2^K$ and $Sk\text{-}3^K$ from *N. intermedia*. Only $Sk\text{-}1^K$ occurs widespread in nature (Turner and Perkins 1979). Both $Sk\text{-}2^K$ and $Sk\text{-}3^K$ were introgressed into the genetically better-characterized *N. crassa* and both mapped to a region of 30 map units across the centromere of linkage group III. This region, ~3% of the total genomic map, was found to contain a recombination block (Campbell and Turner 1987). No evidence was found for large inversions or chromosome rearrangements, though small inversions might exist between

markers (Bojko 1988, Turner and Perkins 1991). The killer complex must therefore be considered as a haplotype. Whether *Sk-1*^{K} is associated with a recombination block is unknown (Turner and Perkins 1979, 1991).

Meiosis is normal in crosses between Spore killers and sensitives. Both nuclear types coexist within the same ascus cytoplasm and ascus development is typical until after postmeiotic mitosis when the nuclei are enclosed by ascospore walls. Both nuclear types can coexist as well in vegetative heterokaryons, as is apparent from rare occasions when they are included together in the same ascospore (Raju 1979, Raju and Perkins 1991, Turner and Perkins 1991).

Sk-2 and *Sk-3* have been introgressed into the secondarily homothallic *N. tetrasperma*, which normally makes asci with four large spores that are heterokaryotic for mating type and any other centromere-linked markers that are heterozygous in the cross. Crosses of *N. tetrasperma* heterozygous for the centromere-linked killers *Sk-2* and *Sk-3* all produced four-spored asci as predicted from the behavior of these killers in the eight-spored species. The sensitive nuclei were protected in heterokaryotic *Sk*^{K} + *Sk*^{S} ascospores, but killing occurred in this species when exceptional small homokaryotic ascospores were formed (Raju and Perkins 1991).

FIGURE 3.1. -- Model to explain spore killing in *P. anserina* as the segregation of a meiotic drive element.



The figure shows a cross between two strains carrying a Spore killer (○) and a sensitive (●) allele. Two nonsister nuclei descending from a half tetrad are enclosed in each ascospore. FDS for the Spore-killer element results in an ascus with two surviving spores, each homokaryotic for the killer element, and two aborted spores, each homokaryotic for the sensitive alleles. SDS results in a four-spored ascus, in which each ascospore survives because it carries both a nucleus with the killer allele and one with the sensitive allele.

Podospora anserina grows on dung of herbivores and is also a secondarily homothallic ascomycete. It also produces four binucleate spores per ascus (Figure 3.1.). For the behavior of Spore killers in *P. anserina* the following aspects of ascospore formation are relevant.

(1) Programming of nuclear positioning in the *Podospora* ascus is such that following meiosis and postmeiotic mitosis, the two ascospores in one-half of the ascus each receive two nonsister nuclei descending from the two meiotic products from the same half tetrad. The other two ascospores each contain two nonsister nuclei from the other half tetrad. As a result, ascospores are homokaryotic for all markers showing first division segregation (FDS) and heterokaryotic for those markers that show second division segregation (SDS; see Esser 1974,

Raju and Perkins 1994). Due to an obligate single crossover between the centromere and the mating-type locus, (nearly) all spores are heterokaryotic for mating type (*mat*+ and *mat*-).

(2) In crosses heterozygous for a Spore-killer element, ascospores that receive only sensitive nuclei abort, whereas ascospores that are homo- or heterokaryotic for a killer nucleus survive. Therefore, the frequency of asci showing two viable and two inviable ascospores (two-spored asci) reflects the frequency of FDS for the Spore-killer element (Figure 3.1.). This fraction of two-spored asci is hereafter referred to as the spore-killing percentage. This study describes the results of our search for meiotic drive elements in a natural *P. anserina* population. We have identified and characterized seven different types of Spore killers, indicated by the abortion of half of the ascus progeny, among 99 recently isolated Dutch strains of *P. anserina* and 3 older isolates originating from France. Six of these Spore-killer types can be attributed with certainty to meiotic drive elements. We have also analyzed the interactions between the different killer types. Finally, mapping data of the Spore-killer elements are presented.

MATERIALS AND METHODS

Strains and culture methods:

P. anserina strain S, isolated in Normandy, France, in 1937 was used as a standard tester strain. Spore-killer strains Y and Z originated from Picardy, France, in 1937 (Belcour *et al.* 1997). *P. comata* Spore-killer strain T (Picardy, France, 1937) was previously described as *P. anserina* but was renamed later on the basis of morphology and mitochondrial type. It is interfertile with *P. anserina* (Padieu and Bernet 1967, Belcour *et al.* 1997). All other *P. anserina* Spore-killer strains (Wa numbers) were collected in Wageningen, The Netherlands, during 1991-1997 from dung (Chapter 2, Van der Gaag *et al.* 1998). Recombinants of the Spore-killer strains (XS numbers) with the genetic background of sensitive strain S were obtained by five recurrent backcrosses.

Marker strains used in this study were *Cs3* (16% SDS, LG I, cold sensitive), *136* (0.5% SDS, LG II, green spores), *Cs2* (3% SDS, LG III, cold sensitive), *187* (76% SDS, LG III, green spores), *rd1* (84% SDS, LG III, round spores), *Lys2* (0% SDS, LG IV, lysine requiring), *As7* (0% SDS, LG V, paromomycin resistant), *Cs18* (0-7% SDS, LG VI, cold sensitive), *Cs12* (0-3% SDS, LG VII, cold sensitive, paromomycin hypersensitive; Picard 1971, Marcou *et al.* 1990). All marker strains were derived from wild-type strain S.

Culture conditions and media have been described by Esser (1974). Cornmeal agar was used as a standard growth medium with 100 mg/liter lysine added for the *Lys2* marker. Tests for the *Lys2* marker were performed on minimal medium 2 (MM2) without lysine. Paromomycin hypersensitivity and resistance were tested on MM2 supplemented with 270 μg/ml paromomycin (Sigma, St. Louis). All cultures were grown at 27°. Cold sensitivity was tested at 11° (Picard-Bennoun and Le Coze 1980).

Crosses were performed on moistened copromes (horse dung tablets that were sterilized by γ-irradiation; Wood and Cooke 1984) on a sterile filter paper on top of the agar in a plate to improve crossing ability. Crossing occurred either by spermatization of monokaryotic

strains with microconidia or by confrontation of mycelia of opposing mating type. Backcrosses of progeny to the parental strains were performed using the spermatization technique.

TABLE 3.1.
Spore killer types found in isolates of *Podospora*

Spore-killer type	*P. anserina* strain	Year of isolation	% 2-spored asci (first cross)	% 2-spored asci (fifth backcross)
Psk-1	Wa1	1991	95.7 % (1480 asci)	90.9 % (956 asci)
	Wa2	1991	94.6 % (1460 asci)	ND [a]
	Wa6	1992	96.3 % (1112 asci)	93.6 % (109 asci)
	Wa12	1993	94.3 % (457 asci)	ND
	Wa52	1994	91.3 % (1412 asci)	89.0 % (845 asci)
	Wa53	1994	92.5 % (681 asci)	91.6 % (490 asci)
	Wa86	1997		
	Wa87	1997		
	Wa98	1997		
Psk-2	Wa28	1993	78.5 % (960 asci)	73.0 % (333 asci)
	Wa38	1993	77.2 % (228 asci)	77.7 % (323 asci)
	Wa49	1994	72.4 % (1204 asci)	82.5 % (388 asci)
	Wa85	1997		
	Wa97	1997		
Psk-3	Wa20	1993	Variable	ND
	Wa21	1993	Variable	ND
	Wa25	1993	Variable	ND
	Wa27	1993	Variable	ND
Psk-4	Wa46	1994	45.1 % (2671 asci)	ND
Psk-5	Y	1937	94.4 % (1239 asci)	96.0 % (250 asci)
Psk-6	Wa47	1994	45.7 % (1835 asci)	ND
	Wa89	1997		
	Wa90	1997		
Psk-7	Wa58	1994	53.6 % (2007 asci)	53.8 % (143 asci)
	Z	1937	48.9 % (6340 asci)	51.3 % (903 asci)
a_2 or (Sk-1)	T [b]	1937	70.8 % (861 asci)	ND

Isolates are classified in different killer types based on spore killing frequency (FDS) and killing interaction among Spore-killer isolates (Table 3.3.). Percentage of spore killing (FDS) is based on crosses to sensitive strain S (number of asci shown in parentheses). Backcrossed strains were obtained through five recurrent backcrosses with sensitive strain S. All Wa-strains were isolated around Wageningen, the Netherlands during 1991-1997. Strains T, Y and Z were isolated in Picardy, France during 1937. The 1997 Wa-strains were classified by interaction with other Spore killers.

[a] No backcrosses were made for these strains nor further backcrosses could be obtained due to infertility.

[b] Strain T was previously identified as *P. anserina* by Padieu and Bernet (1967), but reclassified *P. comata* based on morphological and molecular data (Belcour *et al*. 1997). Killer type classification of strain T was done by Turner and Perkins (1991).

Genetic mapping:

Methods of genetic analysis have been described by Esser and Kuenen 1967. In short, Spore-killer strains were crossed with centromere-linked marker strains to identify the linkage group. Two-spored asci resulting from FDS for the Spore-killer element (see Figure 3.1.) were tested for the occurrence of the marker. Such markers show FDS, and thus the two surviving ascospores will be homokaryotic either for the marker or the wild-type allele. Nonparental ditypes (NPD, *i.e.,* the two surviving spores show the marker) and parental ditypes (PD, *i.e.,* the two surviving spores are wild type) will be equally frequent in the progeny when killer and marker are unlinked. When linked, NPDs appear very rarely, because they require a four-chromatid double crossover between the killer locus and the marker. When using a centromere-linked tester strain, SDS for the marker is rare and can be neglected.

Markers more distal from the centromere were used for the establishment of linkage on chromosomal arms. Four-spored asci, resulting from SDS for the Spore-killer element, were tested for the absence or occurrence of the marker. A low percentage of SDS for only the Spore-killer element compared to SDS for both Spore killer and marker indicates linkage to that chromosomal arm (depending on the distance of the markers).

RESULTS

Spore killing in Podospora reflects meiotic drive:

To assess the extent of meiotic drive in a natural population we sampled the local *P. anserina* population of Wageningen, The Netherlands. During the period 1991-1997 we obtained a total of 99 new isolates of *P. anserina*. Species determination was based on morphological criteria, as well as on fertility with the standard strain S, or with wild-type strains sexually compatible with S (Chapter 2, Van der Gaag *et al.* 1998, Mirza and Cain 1969). We observed that 23 isolates produced up to 95% two-spored asci in these crosses, instead of the expected four-spored asci (Table 3.1, Figure 3.2). In addition to the new wild-type strains, we analyzed three French *Podospora* strains, T, Y, and Z, isolated in 1937 and showing similar spore abortion in specific crosses (Belcour *et al.* 1997). Strain T was previously characterized as a Spore-killer strain (Padieu and Bernet 1967, Turner and Perkins 1991) and was recently renamed *P. comata* on the basis of morphological and molecular data (Belcour *et al.* 1997). Progeny grown from two-spored and four-spored asci were backcrossed to both mating types of the parental strains to confirm that the observed spore killing is caused by meiotic drive. The results from such an analysis of strain Wa58 (*Psk-7*) are shown in Table 3.2 and may be summarized as follows:

1. Selfing of the progeny from two-spored ascus progeny always yielded normal four-spored asci (no abortion).
2. Backcrossing of the progeny of the two-spored asci to the S+ and S- strains showed spore killing, whereas backcrossing to the Wa58+ and Wa58- strains gave normal four-spored asci.
3. Selfing of the four-spored progeny showed ascospore abortion.

4. Backcrosses of the four-spored progeny produced spore killing in the backcross to S- and Wa58+ or to S+ and Wa58-, but not to both.
5. In addition to these observations, it must be added that there is no effect of the mating type or of the sexual role (maternal or paternal) of the strains involved.

These data support the meiotic drive model of spore killing as presented for *Neurospora* (Turner and Perkins 1979) and applied to the genetic system of *P. anserina* (Figure 3.1). Spores from a cross between a strain carrying a Spore-killer element and a sensitive strain would be either homokaryotic (*i.e.,* show FDS for the Spore-killer element, reflecting no crossover in the centromere proximal region) or heterokaryotic for the Spore-killer element (SDS, reflecting a crossover). Only spores carrying a Spore-killer nucleus survive; thus FDS for the Spore killer results in two-spored asci, the aborted spores carrying only sensitive alleles. SDS would result in four-spored asci. The sensitive nuclei in the heterokaryotic four-spored asci are viable as can be seen in the selfings and backcrosses. Results similar to those described in Table 3.2 were found for all other Spore-killer isolates that were tested. In all cases, standard strain S behaved as the sensitive isolate.

There are at least six different Spore-killer types in the Wageningen population of *P. anserina*:

The Spore-killer strains were initially classified on the basis of (1) spore killing frequency in a cross to a standard sensitive strain (FDS percentage) and (2) the interaction between the Spore killers (Table 3.1 and Table 3.3). In this way at least six types of Spore killers could be identified among the 99 natural isolates. An additional seventh type was discovered in the French *P. anserina* strain Y. All Spore-killer strains of the same type showed a constant and repeatable spore- killing frequency when crossed to strain S and

TABLE 3.2.
Progeny tests of cultures from two and four-spored asci of the cross Wa58 (*Psk-7*^{K}) x S (*Psk-7*^{S})

Ascospore no.	Occurrence of two-spored asci [a] in crosses x				Selfing	Inferred genotype of ascospores
	Wa58^{+} [b]	Wa58^{-}	S^{+}	S^{-}		
	2-spored asci (6 complete ascus progeny tested)					
1	No	No	Yes	Yes	No	*Psk-7*$^{K+}$ / *Psk-7*$^{K-}$
2	No	No	Yes	Yes	No	*Psk-7*$^{K+}$ / *Psk-7*$^{K-}$
	4-spored asci (5 complete ascus progeny tested)					
1	No	Yes	Yes	No	Yes	*Psk-7*$^{S+}$ / *Psk-7*$^{K-}$
2	No	Yes	Yes	No	Yes	*Psk-7*$^{S+}$ / *Psk-7*$^{K-}$
3	Yes	No	No	Yes	Yes	*Psk-7*$^{K+}$ / *Psk-7*$^{S-}$
4	Yes	No	No	Yes	Yes	*Psk-7*$^{K+}$ / *Psk-7*$^{S-}$

[a] With respect to the killing percentage of the killer strain, if killing occurs approximately half of the asci show two viable and two aborted ascospores, and the other asci have four viable ascospores. The percentage of two-spored asci is like that found in the parental cross.

[b] + en - refer to mating type of the nuclei in the ascospores or parental strains.

FIGURE 3.2. -- Rosettes of asci from crosses between *P. anserina* strains.
Asci with darker ascospores are more mature than those with lighter spores.

(A) A rosette from a normal cross showing only four-spored asci.
(B) A cross of Wa6 *(Psk-1)* x S showing a high percentage of two-spored asci.
(C) A cross of Wa28 *(Psk-2)* x S showing ~70% two-spored asci. An ascus containing two dikaryotic and two small monokaryotic killer spores can be seen (black arrow). Both monokaryotic sensitive spores have been aborted.
(D) A cross of Wa58 *(Psk-7)* x S showing 50% two-spored asci.
(E) A cross of Wa20 *(Psk-3)* x Wa16 showing rosettes with different killing percentages. Aborted spores are also visible within the asci (black arrows).
(F) A cross between Wa52 *(Psk-1)* x Wa58 *(Psk-7)* showing ~30% two-spored asci. The five-spored ascus (black arrow) indicates that both smaller mononucleate spores contain a killer locus (as expected for a parental ditype). A four-spored ascus (white arrow) containing a mononucleate spore indicates SDS for one of the killer loci, resulting in the segregation of an (aborted) sensitive nucleus.

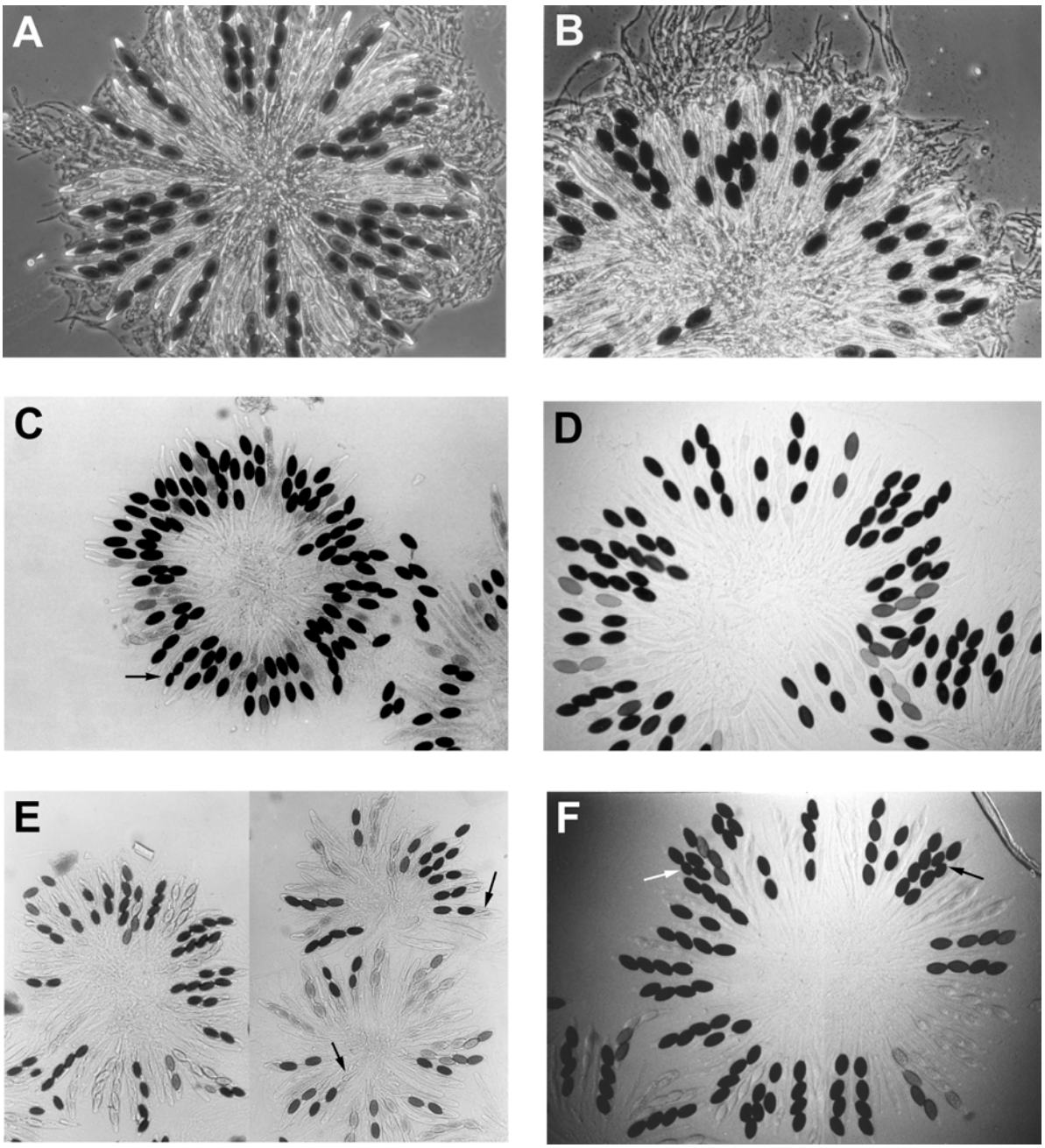


TABLE 3.3.
Interactions between different Spore-killer types.

Strains	*Psk-1* (Wa6)	*Psk-2* (Wa28)	*Psk-4* (Wa46)	*Psk-5* (Y)	*Psk-6* (Wa47)	*Psk-7* (Z)
Psk-1 (Wa6)	0 %					
Psk-2 (Wa28)	94.3 % (1594 asci)	0 %				
Psk-4 (Wa46)	90.1 % (243 asci)	62.5 % (557 asci)	0 %			
Psk-5 (Y)	25.5 % (825 asci)	92.4 % (607 asci)	89.9 % (715 asci)	0 %		
Psk-6 (Wa 47)	92.2 % (141 asci)	72.0 % (1009 asci)	26.5 % (895 asci)	93.8 % (727 asci)	0 %	
Psk-7 (Z)	23.2 % (992 asci)	53.4 % (1301 asci)	61.8 % (845 asci)	44.1 % (860 asci)	67.9 % (505 asci)	0%
Sensitive (S)	96.3 (1112 asci)	78.5 % (960 asci)	45.1 % (2671 asci)	94.4 % (1239 asci)	45.7% (1835 asci)	48.9% (6340 asci)

The percentage of two-spored asci is shown for crosses between one member of each Spore-killer type. Spore-killer strains of any one type have similar spore-killing percentages when crossed to a standard sensitive strain and do not show killing when intercrossed (Table 3.1). For comparison the percentage killing in a cross with strain S is also given. The number of asci analysed is shown between brackets.

absence of spore killing when intercrossed. When intercrossed (Table 3.3), however, Spore-killer strains of different types show killing, similar to the behavior of $Sk\text{-}2^K$ x $Sk\text{-}3^K$ in *Neurospora*.

Figure 3.2 shows some rosettes of a normal cross and different spore-killing reactions. The killer types *Psk-1* and *Psk-5* show the highest frequency of two-spored asci, >90% (Figure 3.2B). *Psk-4*, *Psk-6*, and *Psk-7* produce the lowest killing percentage; only half of the asci contain two spores; the remaining asci carry four spores as is the normal condition (Figure 3.2D). The *Psk-2* strain and *P. comata* strain T show intermediate levels of spore killing; ~75% of two-spored asci are found (Figure 3.2C). Spores homokaryotic for the sensitive allele can be observed only for a short time in these killer crosses. They completely degrade at the start of spore wall formation.

The group of *Psk-3* strains is different from the others in that the frequency of spore killing is highly variable among perithecia of the same cross (Figure 3.2E). Fruiting bodies with any combination of two- and four-spored asci can be observed. Furthermore, ascospore abortion is found only in crosses between specific strains and even between some *Psk-3* strains (Table 3.4). Another distinguishing feature of the *Psk-3* group is that the aborted spores do not disintegrate as in the other killer types, but remain in the asci as tiny, shriveled spores. Because of the erratic expression of spore abortion, further genetic analysis of these *Psk-3* isolates has not been performed. It is therefore not certain that this group contains true meiotic drive elements.

TABLE 3.4

Spore killing found in crosses with *Psk-3* isolates

Psk-3 Strains	Spore killing with sensitive strains	Spore killing with *Psk-3* members
Wa20	Wa14, Wa15, Wa16, Wa33	Wa25
Wa21	Wa15, Wa17, Wa18, Wa23, Wa26, Wa30, Wa41, Wa51, Wa57, Wa63	Wa25, Wa27
Wa25	Wa14, Wa17, Wa21, Wa26	Wa20, Wa27
Wa27	S, Wa3, Wa4, Wa14, Wa15, Wa16, Wa26, Wa50, Wa57, Wa63	Wa21, Wa25

All *Psk-3* strains show variable killing percentages within a cross.

Spore killers are stable upon recurrent backcrossing:

Several strains belonging to different Spore-killer types were backcrossed five times with the sensitive strain S to assess the stability of the Spore killers and, at the same time, to obtain a more identical genetic background and to increase fertility for further analysis. The fraction of two-spored asci of the fifth recurrent backcross did not differ from the percentage found in the first cross for *Psk-1*, *Psk-2*, *Psk-5*, and *Psk-7* (Table 3.1). These Spore-killer types all show a stable percentage of two-spored asci. We were not able to proceed in backcrossing *Psk-4* and *Psk-6* killers, owing to fertility problems.

High frequency and diversity of Spore killers in a natural population of *Podospora*:

The incidence of Spore-killer strains in the *P. anserina* population of Wageningen appears remarkably high. Of the 99 Wa strains isolated between 1991 and 1997, 23 contain a driving element. Spore killers were found during all years of isolation, except for 1995. In 1996 no strains were isolated. *Psk-1*, *Psk-2*, and *Psk-6* strains were found over several years in the population; *Psk-4* and *Psk-7* were isolated only in 1994 (Table 3.5).

Among six strains isolated in 1937 in Picardy, France, two contained a meiotic drive element. Also the *P. comata* Spore-killer strain T was isolated on that occasion (Belcour *et al.*

TABLE 3.5.

The occurrence of Spore-killer strains among natural isolates from the *P. anserina* population of Wageningen, The Netherlands

			Spore-killer type					
Year	No. of isolates	No. of Spore killers	*Psk-1*	*Psk-2*	*Psk-3*	*Psk-4*	*Psk-6*	*Psk-7*
1991	5	2	2	-	-	-	-	-
1992	1	1	1	-	-	-	-	-
1993	32	7	1	2	4	-	-	-
1994	31	6	2	1	-	1	1	1
1995	14	0	-	-	-	-	-	-
1997	16	7	3	2	-	-	2	-
Total	99	23	9	5	4	1	3	1

1997). No Spore-killer strains were reported among isolates from other French regions (L. Belcour, personal communication). Both the Dutch Spore-killer strain Wa58, isolated in 1994, and the French strain Z, originating in 1937, belong to the same killer type *Psk-7* (Table 3.1).

In contrast to *Neurospora*, no neutral strains, i.e., strains that are not killed but do not themselves kill, were found. However, only a selection of the Wageningen isolates before 1994 was tested against every new Spore-killer isolate. Exceptions are the strains from the *Psk-3* group that show killing behavior only in specific crosses (Table 3.4). Other nonkiller strains are sexually incompatible, or produce four-spored asci in crosses with *Psk-3* members, and seem to act like neutral strains. Among the different Spore-killer types some killer types are resistant to killing by other killer types. This is discussed below in more detail.

Spore-killer types show dominant epistatic or mutual resistant interactions:

We have crossed the Spore-killer strains to each other and measured the fraction of spore killing. The results of the initial crosses are shown in Table 3.3 for one representative of each killer type. Results for other strains were similar. No empty perithecia or completely aborted progeny were found in any of these crosses. Two types of interaction between killer elements were observed. (1) Most commonly, the fraction of two-spored asci was similar to that found for one of the parents when crossed to a sensitive tester strain. We refer to this type of interaction as dominant epistasis. For example, the cross between *Psk-1* and *Psk-2* shows the killing percentage characteristic of *Psk-1* (Table 3.3). Backcrosses of two-spored progeny

TABLE 3.6.

Progeny tests of cultures from two-spored asci of crosses between *Psk-1* and *Psk-7*

Ascospore No.	Occurrence of two-spored asci in backcrosses x							Inferred genotype of ascospores
	Psk-1^{+a}	*Psk-1*$^{-}$	*Psk-7*$^{+}$	*Psk-7*$^{-}$	S^{+}	S^{-}	Selfing	
Asci from Wa6 *(Psk-1)* x Wa58 *(Psk-7)* (progeny from two complete asci tested)								
1	No	No	ND	No	ND	ND	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
2	No	No	ND	No	ND	ND	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
Asci from Wa1 *(Psk-1) x Z (Psk-7)* (progeny from six complete asci tested)								
1	ND	No	No	No	Yes	Yes	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
2	ND	No	No	No	Yes	ND	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
Asci from Wa6 *(Psk-1)* x Z *(Psk-7)* (progeny from three complete asci tested)								
1	ND	No	ND	No	ND	Yes	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
2	ND	No	ND	No	ND	Yes	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
Asci from Wa52 *(Psk-1)* x Z *(Psk-7)* (progeny from five complete asci tested)								
1	No	No	No	ND	Yes	Yes	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$
2	No	No	No	ND	Yes	Yes	No	*Psk-1*^{K}/*Psk-7*$^{K-}$ *Psk-1*^{K}/*Psk-7*$^{K+}$

Crosses were performed and analysed as in Table 3.2. Initial killing percentages were 30.5% (Wa6 x Wa58; 525 asci), 15.5 % (Wa1 x Z; 774 asci), 23.2 % (Wa6 x Z; 992 asci) and 24.3 % (Wa52 x Z; 1671 asci). Crosses to sensitive strains produced the original percentage of asci that show killing. Not every test produced perithecia in all backcrosses with the parents (ND, no data due to infertility of the cross), but usually enough information could be extracted from the other test crosses. Results of crosses between other killer types are summarised in the text.

[a] + and - refer to mating type of the nuclei in the ascospores and strains used.

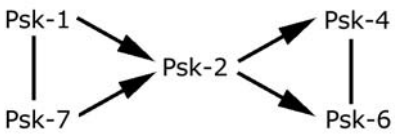
from these interactions produced results similar to those for backcrosses of two-spored progeny from *Psk-1* and a sensitive tester. (2) In some interactions between killers, a much lower percentage of two-spored asci was observed than was found for any of the parents when crossed to the standard testers. This type of interaction can be described as mutual resistance of the unlinked killer elements (for explanation see below). For example, in the cross *Psk-1* x *Psk-7* a relatively low percentage of two-spored asci was observed (Figure 3.2F and Table 3.3). We have analyzed the two-spored progeny by backcrosses to the parental Spore-killer strains, by selfing, and by crosses with a sensitive strain. The results of the analysis of the *Psk-1* x *Psk-7* progeny are shown in Table 3.6 as an example of mutually resistant Spore killers. These results can be summarized as follows:

1. Backcrosses with both parental strains did not show any killing. Every nucleus in the two-spored progeny therefore contains both killers.
2. Crosses with the sensitive strain produced a similar killing percentage as the original cross between *Psk-1* and *Psk-7* did. This verifies that the two surviving ascospores contain both killers.
3. Selfing of the progeny from two-spored asci yielded only four-spored asci. This is also consistent with the surviving ascospores being homokaryotic for both killer elements.

Spore killing between mutually resistant Spore killers is reminiscent of the interaction of unlinked duplicate genes. Ascospores survive when at least one killer element is present, and spore killing is limited to the NPD class of asci with FDS for both killer elements. In these asci, the surviving two spores carry both *Psk-1* and *Psk-7*. The two aborted spores carry neither. The low percentage (23%) of two-spored asci is consistent with the two killer loci being unlinked. Tightly linked Spore killers would have given a high percentage of two-spored asci. *Psk-4* and *Psk-6* were also found to be mutually resistant.

Not every backcross with each wild-type strain was fertile, but usually enough information could be extracted when backcrosses of other strains of the same killer type were taken into consideration. This was, however, not the case with analysis of the four-spored asci, although the incomplete results obtained in these backcrosses did not contradict the analysis of the two-spored asci.

The relation of the Spore-killer types found in Wageningen to each other is summarized in the scheme below:



The straight line represents a mutually resistant interaction, whereas the arrow indicates a dominant epistatic interaction. All Spore-killer types to the right of the arrowhead are sensitive to killing by the killer types on the left. Thus *Psk-7* and *Psk-1* are the most effective killer types in the Wageningen population. They are mutually resistant but kill *Psk-2, Psk-4*, and *Psk-6*. *Psk-4* and *Psk-6* are killed by all the other killer types but are mutually resistant.

TABLE 3.7.
Linkage group analysis of *P. anserina* Spore-killers types in crosses with centromere linked marker strains

Linkage group	Centromere marker	*Psk-1* (Wa6)	*Psk-2* (Wa38)	*Psk-4* (Wa46)	*Psk-5* (Y)	*Psk-6* (Wa47)	*Psk-7* (Z)
I	*Cs3*	19 / 56	24 / 45	13 / 61	50 / 113	31 / 140	89 / 218
II	*136*	500 / 892 [a]	424 / 719 [a]	116 / 224	87 / 172	255 / 418	172 / 341
III	*Cs2*	0 / 97 [b]	5 / 74 [b]	15 / 79	0 / 228 [b]	11 / 101 [b]	12 / 122 [b]
IV	*Lys2*	13 / 97	31 / 72	1 / 74 [b]	101 / 305	9 / 50	39 / 113
V	*As7*	11 / 57	18 / 40	ND [c]	11 / 30	9 / 12	28 / 141
VI	*Cs18*	23 / 57	30 / 69	25 / 81	19 / 45	35 / 94	43 / 193
VII	*Cs12*	16 / 53	44 / 74	12 / 25	21 / 48	14 / 40	100 / 232

Only data of one strain per Spore-killer type are shown. Other strains of the same Spore-killer type show identical results. Spore killers were crossed to marker strains carrying a centromere-linked marker. The fraction of NPD as the number of two-spored asci showing the centromere marker / total number of two-spored asci analysed is shown.

[a] Random spores are analysed.

[b] Linkage is indicated by a low fraction of nonparental ditype asci.

[c] ND, no data available due to infertility of the cross.

The French *Psk-5* Spore-killer type has a more complex interaction to the other killer types. *Psk-5* is sensitive to *Psk-7*, mutually resistant to *Psk-1*, and kills all other killer types.

Most Spore killers are assigned to LG III, but not tightly linked to the centromere:

All Spore-killer strains were crossed to centromere-linked marker strains to identify the linkage group of the killer element. The linkage analysis for one representative per Spore-killer type is presented in Table 3.7. Other strains of the same killer type showed similar results. All *Psk-1*, *Psk-2*, *Psk-5*, and *Psk-7* killer strains showed linkage to the linkage group III centromere marker *Cs2*, as indicated by the low percentage of NPD asci. Linkage could not be established with certainty for *Psk-6*, but LG III (10.8% NPD) seems more likely than LG IV (18% NPD). Spore-killer type *Psk-4* seems to be located on linkage group IV, although a possible location on LG V cannot be excluded. Not all unlinked markers, however, show an equal 50% segregation pattern (Table 3.7). This can be due to negative fitness aspects, which lead to an underrepresentation of the marker involved. Furthermore, interference as often observed in *P. anserina* may hamper linkage group assignment for certain genes (M. Picard, personal communication).

Psk-1 and *Psk-5* were crossed to a strain with two other markers on both sides of the LG III centromere to determine their location on the chromosome arm. A green spore marker, *187*, is located on the left arm of the chromosome, whereas *rd1*, a round spore marker, is situated on the right arm. Results of these crosses indicated a strong interference (Table 3.8). The SDS percentage for *rd1* was reduced from >80% to ~25% when a crossover for *Psk* occurred. This reduction was found in all combinations of killer and markers (data not shown). A control cross of *rd1* with *187* did not show this interference. Whether the

TABLE 3.8.
Linkage group analysis of *P. anserina* Spore-killer types in crosses with noncentromere-linked marker strains

		SDS gene 1		FDS gene 1					
		SDS gene 2 [a]	FDS gene 2	SDS gene 2	FDS gene 2		SDS %		
Parent 1	Parent 2	PD/NPD/T	T	T	PD	NPD	Gene 1	Gene 2	Total no. asci
Psk-1 crosses									
XS-Wa6-*rd1*	*187*	156	65	1537 [b]		391	10.3	70.6	2139
XS-Wa6-*rd1*	*187*	52	169	1741	126	71	10.3	83.8	2139
XS-Wa6-*rd1*	*187*	40	20	116	42	3	27.1	70.6	221 [c]
XS-Wa6-*187*	*rd1*	224	90	2258	615	-- [b]	9.9	71.3	3187
XS-Wa6-*187*	*rd1*	96	218	2509	218	116	9.9	81.7	3187
XS-Wa6-*187*	*rd1*	61	163	35	48	8	71.3	30.6	314 [c]
Psk-5 crosses									
XS-Y-*rd1*	*187*	37	14	552 [b]		121	7.1	72.5	723
XS-Y-*rd1*	*187*	7	44	605	62	6	7.1	84.6	723
XS-Y-*rd1*	*187*	5	2	32	10	2	13.7	72.5	51 [c]
Psk-7 crosses									
Wa58	*rd1-187*	55	17	97 [b]		15	39.1	76.4	184
Wa58	*rd1-187*	56	16	90	10	12	39.1	79.3	184
Wa58	*rd1-187*	44	12	11	4	1	77.8	76.4	72 [c]

The *Psk-1*, *Psk-5* and *Psk-7* Spore-killer strains were used in combinations with LG-III markers *187* (76 % SDS) and *rd1* (84 % SDS) to determine the location on the chromosome arm. Only data of one cross reciprocal for the markers are shown for *Psk-1*. Results were similar in all crosses with other possible combinations of used markers. Control crosses of markers with sensitive strains all showed normal segregation patterns. The ascus-type distribution is given for the underlined markers of each cross.

[a] The PD, NPD, and T classes can not be distinguished from each other in dikaryotic spores.

[b] No distinction can be made in two-spored asci for spores homokaryotic and heterokaryotic for the wild type, and the tetratype T is combined with either PD or NPD. The SDS percentage of the marker is therefore based on the four-spored asci.

[c] Percentages based on the four-spored asci (SDS for *Psk*).

interference is due to, or merely detected by, the presence of the Spore killer cannot be concluded from these data. These results can be explained by strong chromosomal interference across the centromere or by positive chromatid interference, if a crossover for *rd1* is followed by a specific second crossover involving the same chromatids. In the first case, the killer must be present on the left arm, whereas in the second case the killer is present on the right arm of the linkage group. Alternatively, centromere misdivision or occurrence of spindle overlap at the second division could simulate double crossovers across the centromere with positive interference (D. D. Perkins, personal communication).

Also, attempts were made to further localize *Psk-7* in crosses with LG III markers *rd1* and *187* (Table 3.8). Chromosomal arm linkage would be most strongly indicated by a low

tetratype (T) fraction of two-spored asci (FDS for *Psk-7*) for that marker. The T class for *187* is combined with the PD class, since neither can be distinguished phenotypically. If the PDs comprise a considerable part of the combined classes, linkage to the left arm is possible. The large T class for the *rd1* marker also indicates linkage to the left chromosomal arm.

DISCUSSION

Spore-killer types in Podospora:

We have found segregation distorters showing meiotic drive in natural isolates of the secondarily homothallic ascomycete *P. anserina*. These meiotic drive elements cause the abortion of two of the four spores in the ascus. The two surviving ascospores contain the distorter, whereas the aborted spores contain alleles sensitive to it. Other causes of ascospore abortion, such as translocations (Perkins 1974, Perkins and Barry 1977, Bronson 1988), lethal mutations (Delange 1981), or spore color mutants (Marrcou *et al.* 1990, Raju 1994), can be excluded. Reciprocal translocations are not expected to produce two-spored asci in *Podospora*, and nonreciprocal translocations could produce at most 50% of such asci. Lethal genotypes and spore color mutants can be excluded because both killer and sensitive strains behave normally during selfing and in homokaryotic condition. Our data on backcrosses, killing percentages, and localization of the killer complex demonstrate that the observed spore killing in *Podospora* is caused by meiotic drive elements.

Rescue of sensitive alleles was first shown in crosses of *Neurospora Sk-2* and *Sk-3* with the mutant *Banana*, which is sensitive to both killers. These crosses produce giant ascospores containing four killer and four sensitive nuclei of both types (Raju 1979). Also, when *Sk-2* and *Sk-3* were introgressed into pseudohomothallic *N. tetrasperma*, no killing was found in heterokaryotic ascospores. This fungus produces four binucleate spores, but unlike *P. anserina*, the spores are heterokaryotic for centromere-linked markers. The sensitive nuclei are saved here because tight linkage of the killer genes to the centromere ensures that every ascospore receives a killer nucleus (Raju and Perkins 1991). As in *Neurospora*, sensitive alleles can be rescued in *Podospora* by inclusion in an ascospore with a nucleus containing the killer allele. This is the case in four-spored asci. The sensitive nucleus proved fully functional in backcrosses of the four-spored progeny with the sensitive and killer parents and shows that no irreversible damage occurs to the nucleus during or after meiosis.

Segregation distortion in *Podospora* differs in an important common aspect from other meiotic drive systems found in nature (Lyttle 1991). Absence of tight linkage to the centromere was observed, which is reflected in the lower fraction of asci in which ascospores are killed in four of the seven Spore-killer types. Also the failure to detect resistant or suppressor alleles differs from the other drive systems. The *Podospora* ascospore abortion factors resemble in their behavior the Spore-killer complexes found in the sibling species *P. comata* (Padieu and Bernet 1967) and the heterothallic ascomycetes *C. heterostrophus* (Bronson *et al.* 1990), *G. fujikuroi* (= *F. moniliforme*; Kathariou and Spieth 1982), *N. intermedia*, and *N. sitophila* (Turner and Perkins 1979). However, a significant difference in the reproductive system exists between the secondarily homothallic *Podospora* and the other

ascomycetes, which are heterothallic. This sexual strategy has consequences for the presence of Spore killers. As in *N. tetrasperma*, each ascospore of *Podospora* contains two nuclei and is heterokaryotic for mating type. SDS for the Spore-killer locus results in shielding of the sensitive nucleus within the ascospores and the formation of a normal four-spored ascus whose spores are heterokaryotic for the Spore killer. Killing frequencies in this secondarily homothallic fungus can range from 0% (complete SDS) to 100% (complete FDS), as exemplified by this study. Thus, the percentage of SDS for the Spore-killer locus influences the percentage of two-spored asci found. In contrast, the ascospores of heterothallic ascomycetes are homokaryotic and a crossover only leads to a shift in the linear order of the nuclei in the ascus. All four sensitive nuclei are still killed, and the four spores containing the killer nuclei remain. Thus the percentage of SDS does not have any influence on the killing percentage in heterothallic fungi.

An SDS percentage of nearly 100% in *Podospora*, as found for the mating-type locus, would automatically lead to a nonkilling phenotype. This led Perkins and co-workers to propose that secondary homothallism (in *N. tetrasperma*) evolved as a mechanism to escape Spore-killer elements in the heterothallic precursor species (Turner and Perkins 1991, Raju and Perkins 1994). However, this proposition only holds for driving elements in *N. tetrasperma* that are closely linked to the centromere. A Spore killer located more distantly from the centromere would still give a spore-killing phenotype in *N. tetrasperma*, though such high frequencies as found in *P. anserina* would not be expected. No Spore-killer elements have been identified in *N. tetrasperma*. However, ascospore abortion is high in outcrosses between wild-collected *N. tetrasperma* strains (Jacobson 1995), and the basis of the ascospore death remains undetermined. Drive elements are not excluded.

We have identified seven different Spore-killer types, six of which occurred in a sample of 99 wild-collected strains from Wageningen, The Netherlands. *Psk-2* shows a percentage of killing comparable to the percentage found in *P. comata Sk-1* (or *-a2*; Padieu and Bernet 1967, Turner and Perkins 1991). It is probable that Spore-killer complexes found in *P. anserina* and *P. comata* are related, since the two species are relatively interfertile.

One set of Spore killers, the *Psk-3* group, possesses some unique properties different from other killer types. First, the two aborted spores remain visible within the ascus as small unpigmented spores together with the two normal-sized black ascospores. Second, the percentage of killing varies between fruiting bodies within the same cross. Last, *Psk-3* killers show the spore-killing phenotype only in crosses with specific strains. Most other strains are apparently resistant to *Psk-3* killing. This variable killing percentage superficially resembles the ascospore abortion found in *Podospora* by Bernet (1965) in crosses between strains S and s. Ascospore abortion occurred in the s perithecia at the restrictive temperature of 18° C. The amount of killing found varied over time; perithecia that were initiated later had a decreased amount of two-spored asci. No killing was found in the S perithecia, nor at normal growth temperatures. All the surviving spores in the two-spored asci belonged to the s genotype. The maternal effect is associated with the s prion, which is also involved in the heterokaryon incompatibility reaction in this fungus (Coustou *et al.* 1997). However, the *Psk-3* killing occurs at normal growth temperatures (27° C) and does not have a maternal effect.

In the heterothallic *G. fujikuroi*, a mixed Spore-killer type, Sk^{Mx}, was also found. This killer type causes the abortion of half of the spores in 23-70% of the asci. The remaining asci are normally eight-spored (Kathariou and Spieth 1982, Sidhu 1984). Crosses between Sk^{Mx} strains result in a variety of asci containing two, four, six, or eight viable spores (Sidhu 1984, 1988). Sk^{Mx} strains are also partially resistant to normal *Sk* strains. A variable killing percentage occurs also in *N. intermedia* with certain partially sensitive or resistant strains. Strains are called resistant in *Neurospora* when at least 25% of the asci contain eight spores, but the partially resistant strains found in nature produced at least 50% eight-spored asci (Turner 1977 and personal communication). It is possible that the killing reaction of the *Podospora Psk-3* group is caused by a few remaining partially resistant strains, whereas the other strains are fully resistant. The killing of the *Psk-3* group with other specific strains, but not the variability of killing, can also be explained by synthetic lethals (Thompson 1986), *i.e.,* epistatic genes that affect viability only in specific combinations.

Interaction between Spore-killer types:

The Spore-killer types found in *P. anserina* show either dominant epistasis or mutual resistance. In a dominant epistatic interaction one killer strain behaves like a killer and the other like a normal sensitive strain. In the Wageningen population, *Psk-1* and *Psk-7* show dominant epistasis to all other killer types, whereas *Psk-4* and *Psk-6* are sensitive to killing by all the other Spore killers. The dominant epistatic interaction resembles the interaction between Sk^{Mx} and *Sk* in *G. fujikuroi*, where Sk^{Mx} appears to be dominant epistatic to *Sk*, even though Sk^{Mx} kills less efficiently than *Sk* (Kathariou and Spieth 1982).

Interactions between mutually resistant Spore killers exhibit a much lower killing percentage than that observed in either parent when crossed to a normal sensitive. Ascospores from two-spored asci from these crosses are recombinant (NPD) types that now possess both killer elements. These recombinant double killers are less efficient distorters, since sensitive alleles can be rescued by each single killer type. We did not find double killer strains in our sample, but the strain studied by Padieu and Bernet (1967) contained two unlinked killer elements. We have localized six of the Spore-killer types by crossing killer strains with sensitive centromere-linked marker strains. Remarkably, almost all Spore-killer types are found in linkage group III. Also the *het-s* locus involved in the above-mentioned spore killing between strains s and S is located in linkage group III. The only exception, *Psk-4*, is probably in LG IV.

Recombination can easily occur between unlinked or distally linked killer types, as found for the interactions *Psk-4* x *Psk-6* and *Psk-1* x *Psk-7*. However, the observed recombination percentage of some interactions, *e.g.*, *Psk-1* x *Psk-5*, is hard to understand. Both killer types show a high percentage of FDS and are possibly located on the same arm of LG III. Normal recombination cannot produce such high recombination values for tightly linked markers. A very specific interference type has to be assumed, or perhaps some other factor interferes with the spore-killing pattern. In *C. heterostrophus*, the analysis of a Spore killer was complicated by the presence of a translocation (Taga *et al.* 1984, Bronson 1988, Bronson *et al.* 1990).

No mutually sensitive killer strains were found, in contrast to *N. intermedia*, in which Spore killer *Sk-2* and *Sk-3* kill each other when crossed (Turner and Perkins 1979, 1991). However, Turner and Perkins (1991) in their analysis of the data from Padieu and Bernet (1967) with the *P. comata* killer strains show that the results are consistent with mutual killing of the *a* and *b* genotypes. Apparently a mutually sensitive reaction may exist in *Podospora*, although such Spore-killer types have not been encountered in our *P. anserina* sample.

Natural populations:

Of the 99 newly isolated *P. anserina* strains from Wageningen, 23% contain a meiotic drive element. As argued in the Introduction, fungi with ordered tetrads linearly arranged in asci provide a unique possibility to observe the genome-wide extent of meiotic drive because any meiotic drive element present in a cross heterozygous for the driving allele will cause spore killing. Viewed in this way, meiotic drive can be concluded to be common in this population. On the other hand, assuming that the number of coding genes per genome is in the order of 10^4, the probability per locus of a segregation-distorting allele is in the order of 10^{-5}, implying that non-Mendelian segregation at nuclear loci is rare indeed. Data on spore killing in other fungal populations show roughly a similar picture. In *N. sitophila*, the overall incidence of *Sk-1* is 19%, but geographic regions exist where sensitive or killer strains were not obtained. The frequencies of *Sk-2* and *Sk-3* in *N. intermedia* in nature are extremely low and killer strains are restricted to the South-East Asian archipelago. The highest number of Spore killers was found in worldwide isolates of *G. fujikuroi* var. *moniliforme*. Here, a total frequency of 88% *Sk* and Sk^{Mx} killers was observed (Kathariou and Spieth 1982). However, a later study by Sidhu (1988) of midwestern United States isolates showed a reduced frequency of <50% *Sk* and Sk^{Mx}. For *G. fujikuroi* var. *subglutinans* Sidhu (1984) found results comparable to those of Kathariou and Spieth (1982). The worldwide incidence of Spore killers in *C. heterostrophus* is ~50% in Race O field isolates, but no killers have been found in the Race T isolates. Spore killers in Race O were restricted to the United States mainland and could be subdivided into regions that were polymorphic or consisted only of killer isolates (Bronson *et al.* 1990).

Most of the Spore-killer types originating from Wageningen could be recovered over several years, indicating a relative stability of the killer genes within the natural population. The finding of the *Psk-7* killer type in the recently isolated Dutch population and the French strains isolated almost 60 years earlier also supports the idea of a stable population of killers and sensitives. A prerequisite for the maintenance of a stable polymorphism of driving and sensitive alleles at a distorter locus in fungi is the existence of neutral or resistant strains as predicted by the model of Nauta and Hoekstra (1993). No such neutral strains have been found for *P. anserina* yet, though several killer types are resistant to other killer types. The same situation exists in *G. fujikuroi* where Sk^{mx} is partially resistant against *Sk* (Kathariou and Spieth 1982). No resistant types were observed in *C. heterostrophus* (Bronson *et al.* 1990) and strains resistant for $Sk\text{-}1^K$ are rare in *N. sitophila* (Turner and Perkins 1991, B. C. Turner, personal communication). In *N. intermedia*, fully and partially resistant strains to $Sk\text{-}2^K$ and $Sk\text{-}3^K$ or both were found in nature. Genes conferring resistance to killing have been mapped

closely linked to the killer complex. *r(Sk-2)-1* is at the left end of the recombination block, while two interacting resistance genes were mapped at loci flanking the right end of the recombination block. Widespread resistance for $Sk\text{-}2^K$ has been found in *N. crassa* and in *N. intermedia* even though the *Sk-2* killer haplotype has not been found in *N. crassa* (B. C. Turner, personal communication).

P. anserina and *N. tetrasperma* are able to reproduce by selfing and do not depend on outcrossing. Selfing protects the offspring from being harmed by Spore killers both because it avoids the introduction of killer elements from other strains and because the program of ascus development results in ascospores that are heterokaryotic for genes far from the centromere (*P. anserina*) or near the centromere (*N. tetrasperma*). Chances for meiotic drive by spore killing to occur depend on the occasional outcross of a sensitive strain with a Spore killer. This aspect of the reproductive system will affect the population genetics of meiotic drive and has not been taken into account in the model of spore killing analyzed by Nauta and Hoekstra (1993). Also the chromosomal location of the distorter locus relative to the centromere was not considered in this population genetics model. One would expect that any linked suppressor of recombination between a distorter and the centromere would be selected, since centromere-linked distorters are maximally effective. Remarkably, we have found some distorters with appreciable amounts of SDS. It is of interest to study further the population genetics of meiotic drive in *P. anserina*, not only taking the reproductive system and the location into account, but also the implications of interactions between abundant Spore-killer types for retention of sensitive alleles. Segregation distorters, once established in a population, may probably linger on for a longer time than in heterothallic species.

To understand the evolutionary consequences of spore killing, it is important to know more about the ecology of spore killing. In this study we detected Spore killers in roughly one-quarter of the natural isolates. However, all crosses were done under standardized laboratory conditions at a constant temperature of 27° C, while, *e.g.*, the *het-s* locus of *P. anserina* only shows meiotic drive when strain s is used as maternal parent in a cross to S at low temperature (18° C). It is important to analyze the effect of fluctuations in environmental conditions such as temperature on spore killing. Also fitness consequences of Spore killers should be studied; *e.g.*, are there differences in size or number of spores from perithecia of crosses homozygous or heterozygous for Spore killers as compared to sensitive strains?

Finally, a mechanistic understanding of spore killing awaits molecular characterization of the genes and gene products involved. At the same time such a molecular approach may yield insight into the evolutionary origins of meiotic drive in fungi.

ACKNOWLEDGEMENTS

We thank A. A. Haspels for assistance with the analysis of the Spore-killer strains; L. Belcour for providing us with the French Spore-killer strains T, Y, and Z; and M. Picard for the marker strains. Furthermore, many thanks to D. D. Perkins, N. B. Raju, and B. C. Turner for numerous helpful suggestions to improve the manuscript. This work was supported by a grant from the Dutch organization for scientific research (NWO-ALW).

Chapter 4

Spore killing in the fungus *Podospora anserina*: a connection between meiotic drive and vegetative incompatibility ?

Marijn van der Gaag, Alfons J. M. Debets and Rolf F. Hoekstra

Published in Genetica (2003): 117:59-65

ABSTRACT

Fungi in which the haploid nuclei resulting from meiosis are linearly arranged in asci provide unique opportunities to analyse abnormal segregation. Any meiotic drive system in such fungi will be observed in a cross between a driving and a sensitive strain as *spore killing*: the degeneration of half the ascospores in a certain proportion of the asci. In a sample of some 100 strains isolated from a single natural population we have discovered at least six different meiotic drive elements (van der Gaag *et al.* 2000, Chapter 3). Here we report results of research that was aimed at elucidating a possible correlation between meiotic drive and vegetative incompatibility in eight different Spore-killer strains from this population. We show that there is a strong correlation between these two phenotypes, although the precise genetic nature of the correlation is not yet clear. We discuss the implications of our results for the understanding of the population genetics of meiotic drive in *Podospora*.

INTRODUCTION

The standard mendelian segregation mechanism enables the fair transmission of alleles: both alleles at a locus are represented equally among the gametes formed by a heterozygote. However, cases of distortion of mendelian segregation by meiotic drive are known where particular alleles, chromosomal regions or chromosomes are over-represented in the gametes. This over-representation will cause such elements to spread in the population unless other selective effects working in the opposite direction form sufficient counterforce. Well-investigated cases of meiotic drive are *Segregation Distorter* in *Drosophila melanogaster* (Lyttle 1993) and the t-complex in mice (Silver 1993, Ardlie 1998). Perhaps not surprisingly, in both these systems there is indeed sufficient counterselection in the diploid phase against the driving elements, thus ensuring a stable maintenance of the driving element and its alleles.

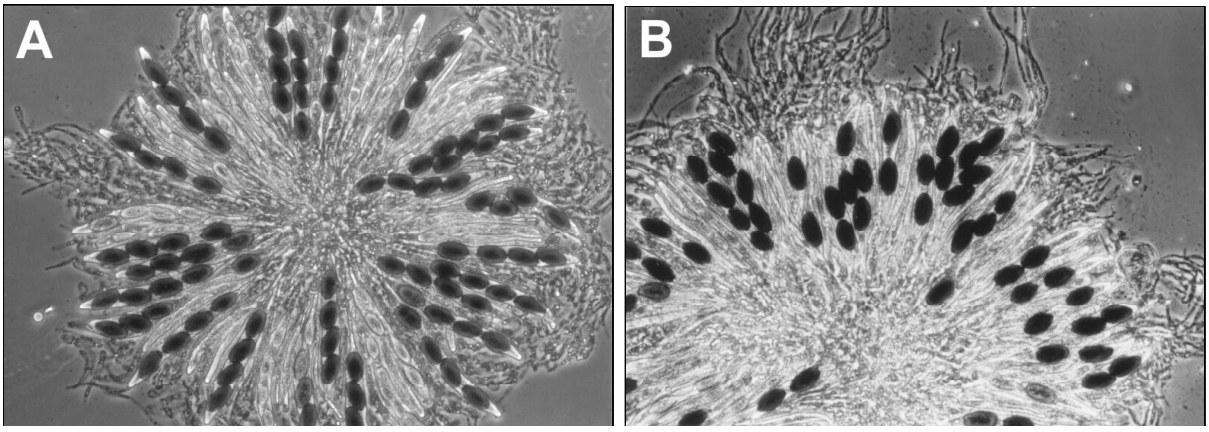
The stable polymorphism of course greatly extends the time period in which the effects associated with the meiotic drive can be observed. In the absence of sufficient counterselection the driving element is expected to sweep through the population relatively quickly and its effects are observable only in a short time interval. Meiotic drive can be viewed as an example of intra-genomic conflict. The driving gene or chromosome is selected for at the gamete level due to its segregation advantage but often selected against because of negative effects on individual fitness in the diploid state. Therefore, the inherent tendency to increase in frequency caused by the meiotic drive is in conflict with the interest of the rest of the genome.

In organisms that spend their vegetative life as haploids, such as most ascomycetous fungi, the population genetics of meiotic drive is rather different from the cases in *Drosphila* and mouse referred to above. In the first place, fitness effects of the driving gene in the diploid state are not likely because this phase is very short: the diploid zygote is not metabolically active and quickly undergoes meiosis to form four (or eight) so-called ascospores. Upon germination ascospores develop into a mycelium, the vegetative structure of the fungus, which is basically a network of hyphae containing haploid nuclei. An important

FIGURE 4.1. -- Rosettes of asci from crosses between *P.anserina* strains.

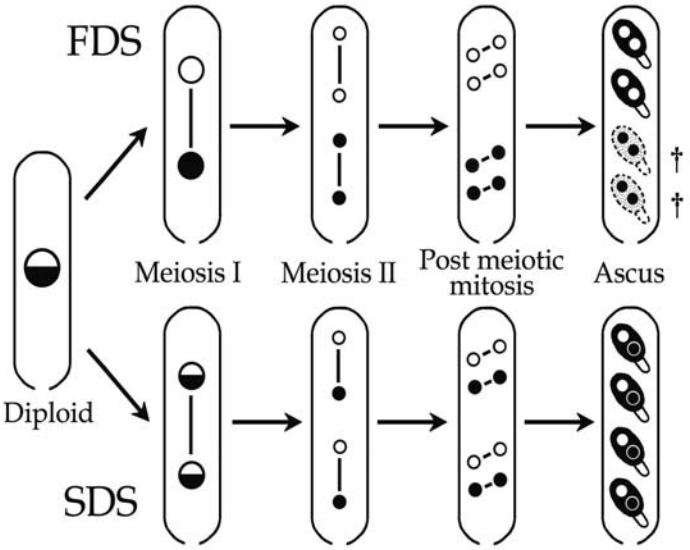
(A) Normal crosses of the secondarily homothallic ascomycete *P.anserina* show asci with four black oval spores. The two spores in one-half of the ascus each contain two nuclei representing the same half tetrad. The other two spores contain the nonsister nuclei from the other half tetrad. As a consequence, such spores will be heterokaryotic for markers that show second division segregation. First division segregation (FDS) of a marker gives rise to homokaryotic spores (Raju and Perkins 1994).

(B) Spore abortion of two of the four spores in an ascus is a consequence of FDS of the Spore-killer locus in crosses heterozygous for a Spore-killer element. The surviving spores are homokaryotic for the Spore-killer element as can be concluded from backcrosses to both parental strains: no spore killing observed in backcrosses to the Spore-killer strain, but similar frequency of two-spored asci in backcrosses to the sensitive parent. The spores receiving only the sensitive alleles are aborted. As can be seen, occasionally four-spored asci are formed. Genetic analysis of these spores showed that they are heterokaryotic for the Spore killer and that the sensitive nuclei are rescued in such spores. Thus, the fraction two-spored asci reflects the FDS frequency (see Figure 4.2 for a model).



consequence of the haploid lifestyle is that counterselection in the mycelial state against a driving gene would not produce a stable polymorphism, but an unstable one: either the driving gene or its allele increases to fixation. However, if next to a driving and a sensitive allele also a 'resistant' allele would occur in the population (insensitive to the driving allele but not driving itself), a stable polymorphism of three alleles (driving, resistant and sensitive) is theoretically possible (Nauta and Hoekstra 1993).

FIGURE 4.2. -- Model to explain spore killing in *P. anserina* as the segregation of a meiotic drive element.



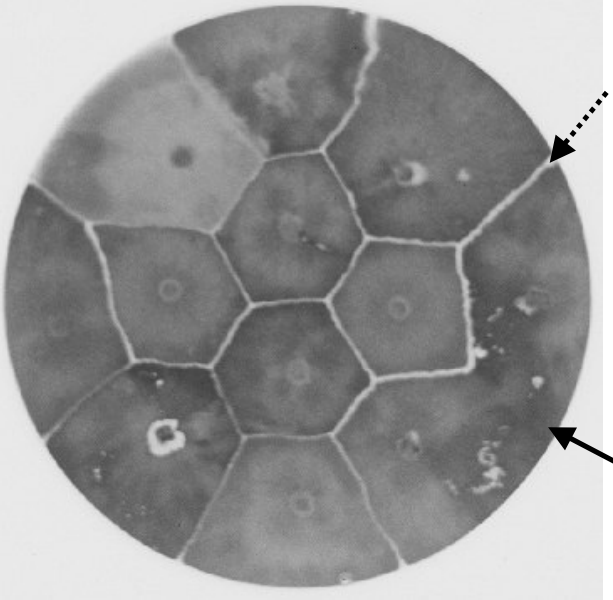
The figure shows a cross between two strains carrying a Spore killer (o) and a sensitive (•) allele. Nuclear migration and spore formation in *P.anserina* are such that two nonsister nuclei descending from a half tetrad are enclosed in each ascospore. First Division Segregation (FDS) for the Spore-killer element results in an ascus with two surviving spores, each homokaryotic for the killer element, and two aborted spores, each homokaryotic for the sensitive alleles. Second Division Segregation (SDS) results in a four-spored ascus, in which each ascospore survives because it carries both a nucleus with the killer allele and one with the sensitive allele. SDS results from a crossover between the centromere and the marker; when there is no crossover the marker shows FDS.

Another important and unique feature of meiotic drive in fungi (at least in those species which have asci in which the haploid meiotic products are arranged linearly) is its clear and easily observable phenotype, namely *spore killing*: the degeneration and early abortion of half the ascospores in a certain proportion of the asci in a fruiting body (Figures 4.1 and 4.2).

The appearance of meiotic drive as spore killing immediately shows another important difference with meiotic drive in diploids: here its effect is to reduce the number of ascospores (that is the number of offspring produced!), while in a diploid species the reduction of meiotic products (that is the number of gametes!) has little or no effect on the number of offspring. The reason is that genic meiotic drive mostly occurs only in males and males with half the normal number of sperm are still fertile. Despite the different population genetic consequences of meiotic drive in diploids and haploids, also in the latter case it does have the potential to generate intragenomic conflict: the driving gene is expected to lower fitness by reducing the number of offspring and perhaps also by other effects in the vegetative state, while it may nevertheless increase in frequency by distorting meiotic segregation. The two mentioned contrasts between meiotic drive in haploids and diploids decrease the scope for the establishment of driving genes in haploids. It is therefore quite remarkable that we have found at least six genetically different spore-killing elements (*i.e.* involving different loci) in a sample of about a hundred isolates from a single population of *Podospora anserina* (Chapter

3, van der Gaag *et al.* 2000). Though all *Podospora* strains are either sensitive to spore killing or contain a killer element (Chapter 3, van der Gaag *et al.* 2000), some strains resistant to spore killing are found in *Neurospora* (Turner 2001).

FIGURE 4.3. -- Barrage test of *P.anserina*.



Twelve strains are grown on Biomalt MM. The dashed arrow points to a clear zone between the colonies (*barrage*) indicating vegetative incompatibility, whereas the solid arrow shows a compatible interaction. As a consequence of vegetative incompatibility heterokaryosis is prevented, but the incompatibility response does not always completely prevent heteroplasmy. Mitochondrial plasmids and other parasitic cytoplasmic elements have been shown to be able to cross the incompatibility barrier, although with varying probability (Chapter 2, van der Gaag *et al.* 1998, Debets *et al.* 1994).

Vegetative incompatibility in fungi:

When different conspecific mycelia are growing close to each other, their hyphae may come into physical contact. Sometimes intermycelial anastomosis follows and if this occurs at a sufficiently large scale along their common border and the established connections are stably maintained, a genetically chimaeric mycelium results containing a mixture of the parental nuclei (hence the name heterokaryon) and cytoplasms (heteroplasmon). However, whether or not a confrontation between two conspecific individuals results in a heterokaryon depends on their genotype at a number of so-called het-loci. Only allelic identity at all het-loci allows heterokaryon formation. Strains obeying this rule are said to be vegetative compatible. Since the number of segregating het-loci (or vic loci, for vegetative incompatibility) in fungal populations often appears to be in the order of 10 with generally two alleles per locus (Saupe 2000), this criterion for heterokaryon formation will in practice restrict heterokaryosis mainly to clonally related individuals or to close kin. Thus vegetative incompatibility may in a loose sense be termed a self-nonself recognition system. In a confrontation between vegetative incompatible strains the hyphal cells in the contact zone are destroyed, creating a visible phenotype of the incompatibility reaction called ‘barrage’ (Figure 4.3).

A possible connection between spore killing and vegetative incompatibility?

In Podospora anserina one particular het-gene called het-s seems to be also involved in spore killing: at low temperature (18° C) spore killing results when strain s is used as maternal

parent in a cross to S (Bernet 1965). This apparent connection between vegetative incompatibility and spore killing has prompted us to hypothesize that spore killing might be the expression of the antagonistic vegetative incompatibility reaction at a stage in the life cycle when it is normally suppressed, namely during the sexual cycle.

In this article we report and discuss observations and experiments that we have carried out in order to further explore and understand the association between the phenomena of spore killing and vegetative incompatibility, and its potential contribution to the maintenance of intragenomic conflict as expressed by spore killing.

MATERIALS AND METHODS

Strains of *P.anserina*, culture conditions and genetic techniques were as described elsewhere (Chapter 2, van der Gaag *et al.* 1998, Chapter 3, van der Gaag *et al.* 2000). Strain S is of genotype *het-S* and is sensitive to all known Spore-killer elements.

RESULTS

The various Spore-killer strains of *P.anserina* have been classified in Spore-killer types (*Psk*) based on the spore-killing frequency when crossed to a standard sensitive strain (S) and the interaction to other Spore killers (Chapter 3, van der Gaag *et al.* 2000). Strains of the same killer type show no spore abortion when crossed and have a similar spore-killing frequency to strain S. Table 4.1 shows the characteristic spore-killing frequency of the strains used in the present investigation.

As mentioned in the introduction, ascospore abortion has also been reported as a pleiotropic effect of the vegetative (or heterokaryon) incompatibility locus *het-s/het-S* in heterozygous crosses under special conditions. We therefore decided to study the correlation between spore killing and vegetative incompatibility in our strains. Vegetative incompatibility can be tested by confronting colonies on special media. Vegetative incompatible interactions result in a clear zone between the colonies called *barrage*, whereas a compatible interaction shows typical anastomoses between the cultures and the absence of a *barrage* (Figure 4.3). All Spore-killer strains were initially (*i.e.* after their isolation from nature and prior to the backcrossing procedure described below) vegetative incompatible to strain S as well as to each other (data not shown). We performed seven backcrosses of eight Spore-killer strains to sensitive strain S as follows. Ascospores from two-spored asci were isolated after each cross and again crossed to strain S. The Spore-killer cultures obtained after seven successive backcrosses were tested for vegetative (in)compatibility, both among themselves and with strain S. The results of these incompatibility tests are listed in Table 4.1. As can be seen from this table all Spore-killer progeny (7XS) were still vegetative incompatible to strain S. On the other hand, strain derivatives belonging to the same Spore-killer group were compatible among themselves.

TABLE 4.1.
Vegetative compatibility interactions[a] between Spore killer and sensitive strains.

Spore-killer type		*Psk1*			*Psk2*		*Psk5*	*Psk7*		*Sensitive*	
	P.anserina strain [b]	7XS-Wa1	7XS-Wa6	7XS-Wa52	7XS-Wa28	7XS-Wa38	7XS-Y	7XS-Z	7XS-Wa58	S	Percentage two-spored asci [c]
Psk1	7XS-Wa1	+	+	+	-	-	+	+	+	-	96 %
	7XS-Wa6	+	+	+	-	-	+	+	+	-	96 %
	7XS-Wa52	+	+	+	-	-	+	+	+	-	91 %
Psk2	7XS-Wa28	-	-	-	+	+	-	-	-	-	79 %
	7XS-Wa38	-	-	-	+	+	-	-	-	-	77 %
Psk5	7XS-Y	+	+	+	-	-	+	+	+	-	94 %
Psk7	7XS-Z	+	+	+	-	-	+	+	+	-	49 %
	7XS-Wa58	+	+	+	-	-	+	+	+	-	54 %

[a] The vegetative compatible interactions are marked with a (+) sign and the vegetative incompatible interactions with a (-) sign.
[b] The Spore-killer strains (7XS) are the result of seven backcrosses to S.
[c] The spore-killing frequency of each Spore-killer strain when crossed to S is shown as the fraction two-spored asci.

DISCUSSION

In this Chapter we report results of experiments that were aimed to elucidate a possible connection between the phenomena of spore killing and vegetative incompatibility in the fungus *Podospora anserina*. The study has been motivated by the observation that the vegetative incompatibility locus *het*-s also produces spore killing at low temperature in certain crosses. Could perhaps the early destruction and abortion of half the meiotic products as occurs in spore killing be caused by an incompatibility response 'at the wrong moment' (namely during the sexual cycle when it normally is suppressed)?

Eight genetically different spore-killing strains belonging to four different Spore killer types (*Psk1, Psk2, Psk5* and *Psk7*) were subjected to seven consecutive backcrosses to a standard sensitive labstrain after which their mutual vegetative (in)compatibility relations were established. The backcrossing procedure involved the selection of offspring surviving a spore-killing event, which guaranteed that the sporekiller genotype was maintained throughout these seven generations. The results were unequivocal and striking: without exception all seven times backcrossed strains were still vegetative incompatible with strain S, despite a theoretical genetic identity of 99%. Since incompatibility points to at least one allelic difference at a *het*-locus between the strains involved, these findings imply that in all

strains the Spore killer element either functions itself as a *het*-gene, or is closely linked to a *het*-gene carrying an allele which differs from its homologue in the S-strain. Since the procedure of backcrossing forced the retention of offspring in which no recombination occurred between the Spore killer locus and the centromere (Figure 4.2), linkage to a *het*-allele will have been maintained in any case if this *het*-gene is located between the Spore killer locus and the centromere. All Spore killers involved as well as *het*-s are located on linkage group III (Chapter 3, van der Gaag *et al.* 2000), so genetic linkage is not excluded. The association between spore killing and vegetative incompatibility is further confirmed in the compatibility tests that we carried out between all pairwise combinations of the eight strains used. Without exception the seven times backcrossed strains belonging to the same Spore killer type were compatible. Strains of different Spore killer type were incompatible, except in the mutual combinations between *Psk1, Psk 5* and *Psk7*. This is in line with the fact that these three Spore killers, although genetically dissimilar, show mutual resistance with respect to spore killing. (Chapter 3, van der Gaag *et al.* 2000).

At present it is not yet possible to discriminate between two possible explanations of the strict association between vegetative incompatibility and spore killing. Either both phenomena are pleiotropic effects of the same locus, or the Spore killer genes are genetically closely linked to a *het*-gene. It is known that at least three *het*-genes are located on the same linkage group III as these four Spore killers (Marcou *et al.* 1990). In *Neurospora*, recombination is blocked in a large region (of 30 map units) that spans the centromere and contains the Spore-killer element *Sk-2* (Campbell and Turner 1987). In *Podospora*, there is strong interference of recombination in several regions (Marcou *et al.* 1990), but whether Spore killers in *Podospora* are associated with a recombination block is unknown. But either way, our results point to interesting implications for our understanding of the frequent occurrence of spore killing. There is evidence (Wu *et al.* 1998) that polymorphisms at a *het*-locus in *Neurospora* are ancient and probably maintained by some form of balancing selection. If this would apply more generally to other *het*-genes in fungi, closely linked Spore killer genes (which experience their drive in heterozygous condition), might strongly profit from the balancing selection maintaining the *het*-allele polymorphism at the neighbouring locus, provided the Spore killer locus and the vegetative incompatibility locus are in strong linkage disequilibrium. If the incompatibility and Spore killer phenotypes are expressions of the same genes, this explanation would even have more force: balancing natural selection on the incompatibility phenotype would automatically maintain the spore killing, or at least greatly extend the time period during which Spore killer loci remain polymorphic. If true, the intragenomic conflict resulting from the surprisingly high frequency of meiotic drive in the fungal population that we studied, would be a consequence of balancing selection at a closely linked (or the same) locus.

Chapter 5

Possible mechanisms of spore killing in *Podospora anserina*

Marijn van der Gaag, Wouter R. van der Meer, Marijke Slakhorst, Alfons J. M. Debets and Rolf F. Hoekstra.

ABSTRACT

We have investigated several possible models to explain the spore-killing mechanism in the ascomycete fungus *Podospora anserina*. *Podospora* Spore killers (*Psk*) are meiotic drive elements that cause the abortion of two of the four dikaryotic ascospores not containing the killer element. At present seven different *Psk* types are known in this fungus. The *Podospora* genome was screened for presence of fungal silencing genes that could play a role in spore killing. Homologues of all known genes involved in the RIP, MSUD and Quelling processes of *Neurospora* were found. The possible role of methylation during the killing process was excluded by experiments using the drug 5-azacytidine, which removes methylation from the genome and prevents *de-novo* methylation. No effect of the 5-azacytidine treatments was found on spore-killing frequency for all Spore-killer types.

Furthermore we analysed crosses of *Psk* with the *oct1* marker, which increases the amount of monokaryotic spores produced, to test the consequences of formation of dikaryotic ascospores on spore-killing expression. Spore-killer types *Psk-2*, *Psk-3* (Wa27) and *Psk-4*, but not *Psk-1, Psk-5, Psk-6* and *Psk-7*, produced asci containing more than four spores. In such asci sensitive nuclei were able to survive and resist the meiotic drive system, indicating incomplete penetrance of the spore-killing elements. Also the effect of low temperatures (22 ºC) on spore killing was tested. *Psk-2* dramatically decreased the percentage of killing at this temperature to almost zero. This has severe implications on the population genetics of meiotic drive in this fungus. Based on these and previous observations (Chapter 3, Chapter 4, van der Gaag *et al.* 2000, 2003) we propose that spore killing in *Podospora* may be an example of post-segregational killing due to Toxin-Antitoxin mechanisms.

INTRODUCTION

Meiotic drive in ascomycete fungi is observed as spore killing, the early abortion and degeneration of half the spores in a certain proportion of the asci. The remaining spores within the ascus all contain the distorter element. The ascospores are the products of meiosis

as well as the progeny. Therefore distortion in fungi not only affects the number of gametes produced, but also reduces the fecundity (Nauta and Hoekstra 1993). This contrasts fungal meiotic drive with drive systems found in other organisms such as Drosophila and mouse (Lyttle 1991) and makes fungi very suitable organisms to study the consequences and mechanisms of distortion.

In the secondary homothallic fungus *Podospora anserina* drive is observed by the abortion of two of the four spores in the ascus. Recently, seven different segregation distorters or Spore-killer elements (*Psk*) were discovered in *Podospora* (Chapter 3, van der Gaag *et al.* 2000). Though several more ascomycete fungi were reported to contain such elements, relatively little is known about the mechanism behind the abortion of spores (Raju 1994, 1996). Raju (1979) has studied the timing of spore abortion in *Neurospora* cytologically. He observed that the second meiotic division did not influence spore killing in *Neurospora intermedia* and that killing must be initiated after this division. The visible timing seems similar for *Cochliobolus heterostrophus*, but occurs at an earlier stage in *Gibberella fujikuroi* (Raju 1994), suggesting different mechanisms underlie spore killing. Another hint at the possible mechanisms of spore killing is the rescue of sensitive nuclei in *Podospora* and *Neurospora* when they are included into an ascospore with a nucleus containing the killer element (Chapter 3, van der Gaag *et al.* 2000, Turner and Perkins 1991). The following characteristics and evidence of *Podospora anserina* further define any model explaining the mechanism of spore killing in this fungus:

- *Podospora* normally produces four-spored asci. Each dikaryotic ascospore contains one nucleus carrying the *mat*+ and the other nucleus the *mat*– mating type. Sometimes two smaller uninucleate single mating type spores are produced instead of one dikaryotic spore (Raju and Perkins 1994, Zickler *et al.* 1995).
- All nuclei of one meiosis share the same cytoplasm from the start of meiosis until ascospore wall formation (Raju and Perkins 1994, Zickler *et al.* 1995).
- Mating is cytoplasmic anisogamous. Spermatia (male) are very small microconidia that carry little cytoplasmic content. Mitochondria are usually maternally inherited.
- First division segregation for the drive element results in spore killing, the abortion of the progeny containing only the sensitive allele. Second division segregation for the spore-killer locus results in four-spored asci, with each ascospore containing a killer and a sensitive nucleus. The sensitive nuclei are saved in these spores and remain fully functional in backcrosses. Sensitive nuclei can co-exist with killer nuclei in the vegetative state (Chapter 3, van der Gaag *et al.* 2000).
- Spore-killing percentages are stable for each Spore-killer type and vary between 50 and 95% two-spored asci. Only the *Psk-3* group shows an unstable percentage spore killing, varying with each perithecium in a cross (Chapter 3, van der Gaag *et al.* 2000).
- The Spore-killer locus is resistant against it's own killing action. Interaction between different killer types shows mutual resistance (i.e. spores containing either Spore killer survive; spores containing only sensitive alleles survive) or dominant epistatis (i.e. one of the killer types behaves like a sensitive)(Chapter 3, van der Gaag *et al.* 2000, Figure 3).

- Aborted spores are degraded rapidly in all Spore-killer types except in the *Psk-3* group (and also in *het-s* killing) where aborted spores remain present in the ascus (Chapter 3, van der Gaag *et al.* 2000, Bernet 1965).
- Vegetative incompatibility prevents fusion of mycelia displaying unequal *het*-alleles in somatic tissue. *Het*-genes are not expressed during the sexual cycle. All our spore-killer types are vegetative incompatible to each other and strain S. Only Psk-1, Psk-5 and Psk-7, which are mutually resistant, are in the same vegetative compatibility group (Chapter 3, Chapter 4, van der Gaag *et al.* 2000, 2003).

Several mechanisms have been suggested for the spore killing observed in sexual crosses between a Spore killer and a sensitive strain. They can be divided into two groups:

1. **Spore abortion due to abnormal regulation of gene expression in the sensitive nuclei in the sexual cycle.** For example the sensitive allele of an essential gene required for spore maturation, is not switched on, leading to a deficiency so that only cells carrying the killer allele mature.
2. **Spore abortion due to antagonistic interaction between the gene products of the Spore-killer and the sensitive allele**.

The **first** assumes that the Spore-killer element interferes with the gene-regulation of the sensitive nuclei after meiosis during spore formation, such that cells carrying only sensitive nuclei will not develop normally. Resistance may be the result of exclusive *trans*-activity of the closely linked killer element or there may be a variant resistant allele of the maturation gene. Turner and Perkins (1979) and Raju (1979) suggested for *Neurospora*, that alleles involved in spore killing were either not turned on or (not) shut down during meiosis. Such a misregulation could be mediated by imprinting of genes or by silencing of unpaired DNA. These silencing mechanisms act shortly after karyogamy when genes are paired (Figure 5.1A and B), but the effects would only become evident when the regulated genes are required and fail to function. Epigenetic silencing of genes is commonly found in fungi under processes like RIP (Repeat Induced Point mutation) in *Neurospora*, MIP (Methylation Induced Pre-meiotically) in *Ascobolus* and Quelling in *Neurospora* (see Faugeron 2000, Galagan and Selker 2004, Selker 2002, Pickford *et al.* 2002 for a review). Recently a RIP-like process has also been discovered in *Podospora anserina* (Graïa *et al.* 2001). Most of these processes involve the methylation of duplicated gene sequences and are thought to play a role in the protection of the genome against transposon activities, or in the regulation of transcription. Meiotic silencing by unpaired DNA (MSUD), also called transvection or *trans*-sensing effects, which occurs in *Neurospora* directly before meiosis, requires the pairing and proximity of the affected genes. A failure of pairing, for instance due to an inversion, results in the inactivation of both alleles and can lead to ascospore abortion. Silencing by MSUD (as well as Quelling) is mediated by dsRNAs and siRNAs and is related to RNAi in animals and PTGS in plants (Aramayo and Metzenberg 1996, Shiu *et al.* 2001, Lee *et al.* 2004).

The **second** group of mechanisms proposes that ascospore abortion is caused by interactions of gene products encoded by the Spore-killer and the sensitive allele. This requires that resistance, a modified target, must be coupled to the killer allele. An example of such a drive mechanism is *SD* of *Drosophila* and its interaction with the *Rsp* gene (Kusano *et al*. 2002). Another example of such a mechanism could be the Toxin-Antitoxin system (abbreviated as TA and also known as Post-Segregational Killing (PSK) system), which widely occurs in bacterial plasmids (Engelberg-Kulka and Glaser 1999, Cooper and Heineman 2000, Hayes 2003). If a plasmid encoding a TA system fails to be incorporated into the two daughter cells during cell division (vertical reproduction), the fate of the siblings is sealed. Bacterial cells not inheriting the plasmid are unable to produce the antitoxin against the more persistent toxin and die. In contrast, plasmid-containing daughter cells remain viable, through the continued expression of the antitoxin gene. The plasmid thereby ensures that the majority of cells in a population remain plasmid containing.

This TA model applied to *Podospora*, with a gene coding for a stable toxin together with a closely linked gene coding for a less stable antitoxin is shown in Figure 5.1C. A prerequisite for this TA model is the availability of separate daughter cells. This occurs only during the sexual stage of Podospora at ascospore delimitation. Before spore wall formation all nuclei share the same ascus environment, including toxins and anti-toxins.

An analogous example of a TA-model is found in *P. anserina* for the *het-s/S* vegetative incompatibility system (Figure 5.1D). This prion moderated incompatibility system was found responsible for a special type of meiotic drive (Bernet 1965, Dalstra *et al.* 2003). Bernet observed a variable percentage of ascospore abortion at the restrictive temperature of 18 ºC, between crosses of [Het s] and *het-S* strains, when the [Het-s] strain acted as the mother. Early formed asci showed spore killing, whereas later produced asci did not. Recently, Dalstra *et al.* (2003) reanalyzed these crosses and showed that the HET-s prion acts as the Spore killer to cells producing the non-prion HET-S product. Low temperatures ensured prion persistence in the early asci. Due to the inactivation of the *het-s* gene before the meiotic process no new pre-prion [Het-s*] products are formed which can be converted to the [Het-s] prion form. Therefore later produced asci are prion free and do not show spore killing. The use of a strain overproducing the prion element results in full spore killing, regardless of temperature or ascus stage. The prion acts in this example as the persistent toxin, whereas the presence of HET-S prevents the conversion of [Het-s*] to the prion form (in absence of the prion). This means *het-S* has a dual function as both target and antidote (Dalstra *et al.* 2003).

The involvement of *het*-genes in spore killing was proposed by van der Gaag (Chapter 4, 2003), based on the vegetative compatibility within each Spore-killer type and the vegetative incompatibility between *Psk*-types sharing the same genetic background, and was also suggested by Turner and Perkins (1991). Vegetative incompatibility or *het*-genes are usually inactive during the sexual stage (Bégueret *et al.*, 1994). At least nine *het*-genes with multiple alleles exist in *P. anserina* and are of allelic or (unlinked) non-allelic type. Non-allelic vegetative incompatibility genes can produce lethal ascospores containing two incompatible alleles by recombination (e.g. *het-e1* and *het-c1*, Figure 5.1E). Ascospores look normal; however shortly after germination the mycelium is destroyed by a lytic reaction

(Saupe 2000). However, the non-allelic type of ascospore abortion is an example of a synthetic lethal and does not resemble the genetics of spore killing found in *Podospora* (Chapter 3, van der Gaag *et al.* 2000).

Here we report on experiments to elucidate the mechanisms for spore killing in *Podospora anserina.* 1. We analysed the effect of the demethylating drug 5-azacitidine on spore-killing frequency for most *Psk* types. 2. We studied the consequence of the formation of dikaryotic spores for the expression of spore killing in seven Spore-killer types. 3. We analysed the expression of spore killing at lower temperature and 4. We looked for homologous silencing genes in the *Podospora* genome. Finally, models for the various Spore-killer elements are discussed based upon these and previous findings.

MATERIALS AND METHODS

Strains and culture methods:

Podospora anserina is a secondary homothallic ascomycete, which grows on dung of herbivores. Its lifecycle and culture conditions have been described by Esser (1974). *P. anserina.* Spore-killer strains were characterized previously (Chapter 3, van der Gaag *et al.* 2000). Other strains used in this study are standard wild-type strain *S* and *oct1* (68% SDS, LG-IV, up to eight spores). Both strains share the same genetic background and are sensitive to spore killing (Marcou *et al.* 1990, Chapter 3, van der Gaag *et al.* 2000).

Cornmeal agar was used as a standard growth medium (Esser 1974). Standard crosses were performed on moistened γ-sterilized copromes (horse dung tablets, Wood and Cooke 1984) on a sterile filter paper on top of an agar plate to improve crossing ability. Crossings occurred either by spermatisation of monokaryotic strains with microconidia or by confrontation of mycelia of opposing mating type. Culture growth was performed at 27 °C, or during testing of temperature dependence at 22 °C and 25 °C.

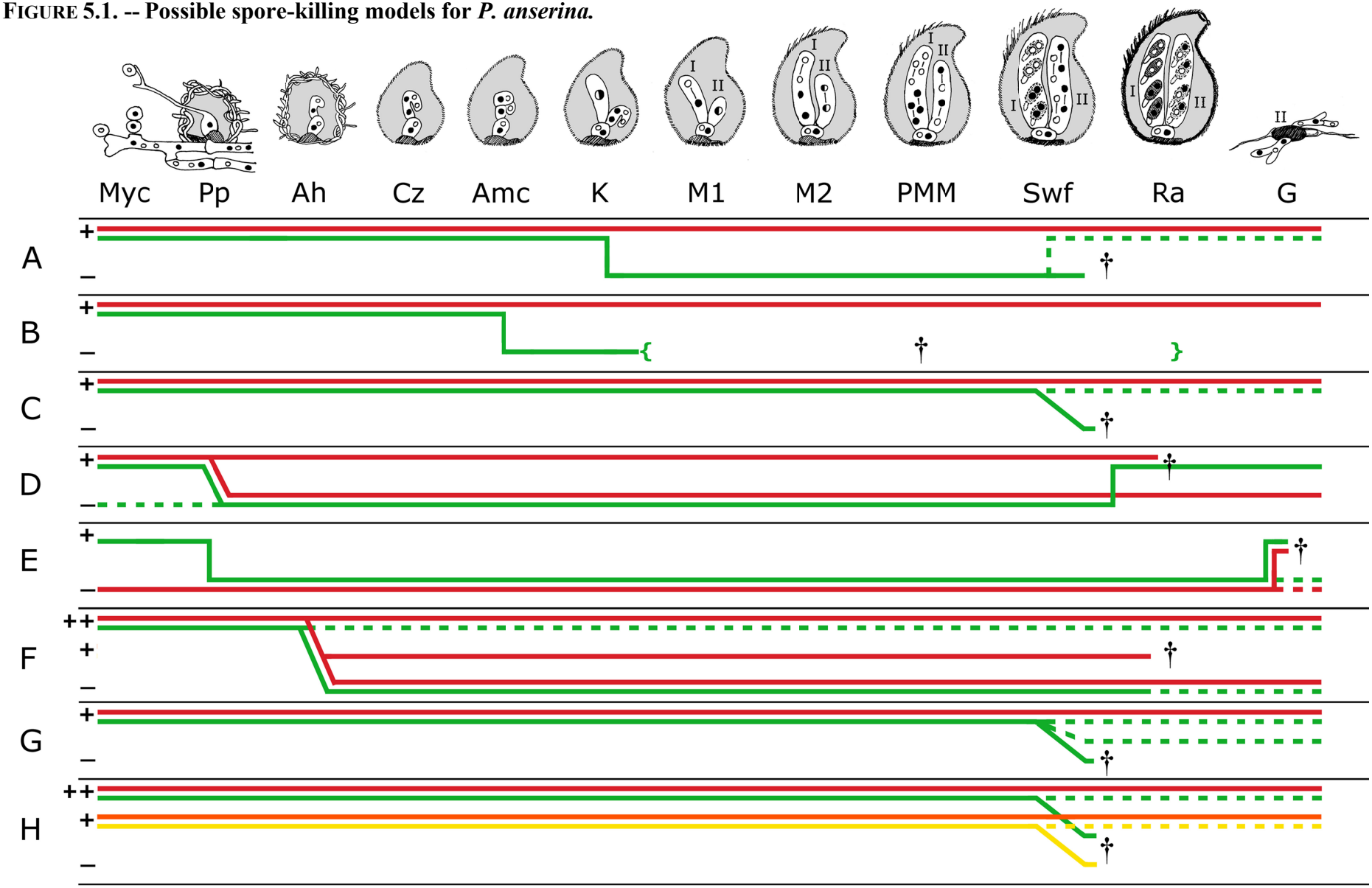
5-azacytidine tests:

Mycelium of Spore-killer and sensitive strains were grown on cornmeal agar plates (without copromes) supplemented with 30μM of 5-azacytidine. Crosses were performed by spermatisation. Fruiting bodies were scored on percentage spore killing (FDS) and compared with control crosses without 5-azacytidine.

Bioinformatics:

The protein sequences from *Neurospora crassa* and *Ascobolus immersus* involved in MSUD, Quelling, MIP and RIP (Faugeron 2000, Pickford *et al.* 2002, Tamaru and Selker 2001, 2003, Lee *et al.* 2003, Catalanotto *et al.* 2004, Freitag *et al.* 2004) were obtained from NCBI and searched with tBlastn against the *Podospora anserina* genome (release 1, http://podospora.igmors.u-psud.fr).

FIGURE 5.1. -- Possible spore-killing models for *P. anserina.*



Several spore-killing models are depicted against the stages of sexual development of *Podospora anserina*. Depicted developmental stages (after Raju and Perkins 1994, Zickler *et al.* 1995) are: Myc (mycelium), Pp (proto perithecium), Ah (ascogenous hyphae, first binucleate heterokaryotic cell), Cz (crozier formation), Amc (ascogenous mother cell (upper cell of crozier), K (karyogamy), M1 (meiosis 1), M2 (meiosis 2), PMM (post meiotic mitosis), Swf (ascospore delimitation/spore wall formation), Ra (Ripe ascospore) and G (ascospore germination).The ● and ○ represent nuclei containing different alleles for a (Spore-killer) gene, whereas the numbers I and II depict respectively a First Division Segregation ascus and a Second Division Segregation ascus for a (killer) gene.

Each model shows the activity (+ or -) of a (killer) gene, or presence (++, +) and absence (-) of gene product during the developmental stages when FDS takes place. The [†] signals the developmental stage where (spore-) killing occurs. The Spore-killer gene product is shown as a red line, green lines are complementary gene products. A dotted line shows the gene activity or product presence of the surviving spores, the strait line of the same color that of aborted spores.

(A) Meiotic silencing model. Meiotic silencing can occur by processes like MSUD (PTGS, RNAi) in *Neurospora.* Silencing of genes is mediated by diffusible small interfering RNA particles (siRNA) maintained by dsRNAs produced by the Spore killer (red line). MSUD needs chromosomal pairing and operates between karyogamy and the first meiotic division. Gene products necessary for ascospore development are degraded (green line). These genes can be involved anywhere from the stages of meiosis 2 (after the separation of killer and sensitive allele) to ascospore germination. The Spore killer carries an insensitive variant of this gene (dotted line). Similar silencing could also be achieved by persistent dsRNAs of a silencing mechanism of the vegetative stage, such as Quelling in *Neurospora*, during meiosis persist as long as the gene necessary for ascospore development is active.

(B) Methylation model. RIP and MIP are premeiotic gene silencing mechanisms and act between fertilization and karyogamy. DNA pairing structure is needed as substrate to confer gene silencing (by methylation). These silenced but necessary genes (green line) can be involved anywhere from the stages of Meiosis 2 (after the separation of killer and sensitive allele) to ascospore germination. The Spore killer retains a variant of the gene unaffected by silencing (red line).

(C) The Toxin-AntiToxin model as applied to *Podospora*. Toxin and AntiToxin genes are coupled. The Toxin (red line) is more persistent than the AntiToxin (green lines) and spores without the TA genes abort by AntiToxin depletion. TA can only act after ascospore delimitation, as the spore wall prevents rescue by the anti-toxin still produced by the other two spores (dotted line).

(D) The prion-gene model. The allelic *het-s/het-S* model where spore killing is caused by [Het-s] prion action. The prion transmission through meiosis is more efficient at 18º C. Activation of *het-S* (green line) in presence of [Het-s] molecules (red line) starts the lethal reaction leading to ascospore degradation. [Het-s] molecules can quickly switch the pre-prion [het-s*] into the prion form. This transition is prevented by *het-S* when prions are unavailable. Delution of prions below threshold level (dotted line) lead to normal spore formation in the later produced asci.

(E) Synthethic lethals model. Non-allelic vegetative incompatibility in *Podospora* (e.g. the incompatible combination of *het-e1*(red line) and *het-c1* (green line)). Vegetative incompatibility genes are not expressed during sexual stage. These genes are re-activated during spore germination and cause after a few hours the lysis of the mycelium carrying the antagonistic alleles. Alternative *het*-alleles do not cause incompatibility (dotted lines).

(F) *Psk-3* (Wa27) spore-killing model. The more persistent Toxin affects a process during ascospore formation. Both Toxin (red) and Antitoxin (green) are shut down between ascogenous hyphal state and ascogenous mother cell, and will be activated after spore wall formation. Dilution of Toxin and Antitoxin by the formation of new ascogenouus mother cells, below threshold levels will create the observed variability in ascospore abortion. Persistence of antitoxin above threshold level (dotted green line) saves spores from abortion, whereas diluted Toxin below threshold level fails to abort spores (dotted red line). Only spores with sufficient Toxin and insufficient Antitoxin are killed (strait lines).

(G) Spore-killing model for *Psk-2*, *Psk-4 and Psk-6*. Resembles the TA model applied for *Podospora*. After spore wall formation no new Antitoxin becomes available and present Antitoxin is degraded below threshold level leading to spore killing (green line). Ascospores are safed from abortion when the Antitoxin levels remain above threshold (low dotted line). As Toxins and Antitoxins are equally distributed within the ascus this results in a stable (non) killing percentage. The *Psk-2* Antitoxin level is stabilized at lower temperatures, leading to complete absence of spore killing (high dotted line).

(H) Spore-killing model for *Psk-1*, *Psk-5* and *Psk-7*. Same TA-model as in G. Toxin and Antitoxin of these different *Psk's* must be able to interact with each other (identical or identical group). Different sensitivity or amounts of Toxin (red and orange) and Antitoxin (green and yellow) explain the interactions between the *Psk*'s. The Spore-killer types are represented by the following colors: *Psk-7* (red and green lines), *Psk-1* (orange and green lines) and *Psk-5* (orange and yellow lines). Antitoxin represented by green produces enough Antitoxin for the Toxin levels of both red and orange, whereas the yellow Antitoxin levels only are sufficient for the orange Toxin level.

RESULTS

Methylation experiment:

We investigated the possibility that methylation of the sensitive allele during meiosis gives rise to spore killing. Methylated sequences are demethylated and *de novo* methylation is inhibited by the use of the drug 5-azacytidine in the growth media (Jones 1984). We have tested the dose of the drug at concentrations ranging from 5 to 300 μM and found that a concentration of 30 μM of 5-azacytidine provided a slight reduction in growth and perithecia formation. This concentration lies well within the range used in experiments with *Neurospora* (Irelan and Selker 1997, Selker and Stevens 1985) and *Ascobolus* (Rhounim *et al.* 1992).

Table 5.1 shows the percentages of two-spored asci observed with and without 5-azacytidine. We found no significant alteration of the percentage of spore killing within crosses between killers and sensitive strains, regardless of the killer type and killer strain used. Also no difference in killing percentage for the use of either strain neither as maternal parent, nor with mating type was found (data not shown). These results indicate that methylation is not involved in the killing process of the tested killer types.

Individual nuclei, Crosses with *oct1*:

The *oct1* marker causes an increase in monokaryotic spore production (Marcou *et al*. 1990). Crosses of a normal wild-type strain with the *oct1* marker strain mispackages the eight nuclei after the post-meiotic division and produces a range of asci containing four dikaryotic spores up to asci with eight monokaryotic spores (Figure 5.2A). Crosses of Spore-killer strains with the *oct1* marker strain allow us to follow the fate of individual nuclei, since most nuclei are packed into monokaryotic spores. The *oct1* marker also allows us to make a clearer comparison between heterothallic (e.g. *Neurospora crassa* and *N.intermedia*) and secondary homothallic (e.g. *P.anserina*) spore-killer species.

We observed two types of action (Table 5.2, Figure 5.2). Spore-killer types *Psk-1*, *Psk-5*, *Psk-6* and *Psk-7* aborted as expected all spores containing only sensitive nuclei. The

TABLE 5.1.
Killing behavior of Spore-killer types in crosses with 5-azacytidine.

Spore-killer type	Strain	Percentage two-spored asci	
		Without 5-azacytidine	5-azacytidine added [a]
Psk-1	Wa53	96 % (1112 asci)	94 % (319 asci)
Psk-2	Wa49	72 % (1204 asci)	80 % (267 asci)
Psk-4	Wa46	45 % (2671 asci)	44 % (109 asci)
Psk-5	Y	94 % (1239 asci)	94 % (379 asci)
Psk-6	Wa47	46 % (1835 asci)	37 % (303 asci)
Psk-7	Z	49 % (6340 asci)	51 % (284 asci)

Spore-killer types were crossed with sensitive strain S. The total number of asci analyzed is given between parentheses.

[a] use of 5-azacytidine reduced the growth rate and the amount of perithecia produced.

number of spores within an ascus never rose above four. First division segregation (FDS) for the Spore killer is observed by asci with two dikaryotic spores or one dikaryotic spore with two monokaryotic spores. Second division segregation (SDS) for the spore-killer locus is seen as a four-spored ascus in any configuration of dikaryotic and monokaryotic ascospores (Figure 5.2B). These asci show full penetrance of the Spore-killer action.

For Spore-killer types *Psk-2*, *Psk-3* and *Psk-4* another type of ascus could be observed as well, where the number of spores reached a higher number than four (Table 5.2, Figure 5.2C). In these asci no killing occurs and the number of nuclei in the ascus is always eight. This indicates that the Spore killer mode of action does not always reach high enough expression levels for the Spore-killer allele to fully penetrate the sensitive monokaryotic spores. Both full and incomplete penetrance could be observed within the same perithecium.

The percentage of killing was altered slightly for all *Psk* strains with the use of the *oct1* marker. However, this alteration can probably be attributed to the class of asci containing four monokaryotic or dikaryotic spores, which were omitted from the count. Asci containing four monokaryotic spores however could have originated either by FDS or SDS. Asci containing four dikaryotic spores can also originate from FDS or SDS, but only if incomplete penetrance occurs.

Table 5.2.
Killing behavior of Spore-killer types in crosses with *oct1*.

Spore-killer type	Strain	Cross with *S*	Cross with *oct1*	
		Percentage two-spored asci	Penetrance [a]	Percentage two-spored asci [b]
Psk-1	Wa1	96 % (1480 asci)	Full	88 % (239 asci)
	Wa6	96 % (1112 asci)	Full	87 % (629 asci)
	Wa52	91 % (1412 asci)	Full	87 % (943 asci)
Psk-2	Wa28	79 % (960 asci)	Incomplete	65 % (886 asci)
	Wa38	77 % (228 asci)	Incomplete	62 % (1220 asci)
	Wa49	72 % (1204 asci)	Incomplete	63 % (958 asci)
Psk-3	Wa27	Variable	Incomplete	Variable (low %)
Psk-4	Wa46	45 % (2671 asci)	Incomplete	42 % (99 asci)
Psk-5	Y	94 % (1239 asci)	Full	91 % (222 asci)
Psk-6	Wa47	46 % (1835 asci)	Full	59 % (204 asci)
Psk-7	Z	49 % (1835 asci)	Full	58 % (204 asci)
	Wa48	54 % (2007 asci)	Full	58 % (604 asci)

The total number of asci analyzed is given between parentheses.

[a] Asci with two, three or four ascospores show full penetrance, asci with more than four ascospores incomplete penetrance. A single perithecium can show both full and incompletely penetrance.

[b] Asci with 1 dikaryotic spore and two monokaryotic spores are counted as two-spored asci. Asci with four monokaryotic or four bikaryotic spores are omitted, since no distinction between FDS and SDS can be made. The occurrence of these four-spored asci is less than 10% and can explain the observed differences in killing percentage of the Spore killers to *S* and *oct1*.

Temperature experiment:

Segregation distortion by spore killing for the *het-s/S* system in *Podospora anserina* depends on low temperatures (Bernet 1965, Dalstra *et al*. 2003). We investigated whether a temperature-based effect also could be observed in our Spore-killer types. Crosses between killer and sensitive strains were continuously grown at 22, 25 and 27 degrees Celsius. No difference between killing action between 25° C and 27° C was found (data not shown).

However, contrary to the *het-s/S* system where killing increases at low temperatures, a spectacular decrease in killing occurs at temperatures of 22° C for *Psk-2* strains (Table 5.3, Figure 5.2F). To check the timeframe of the impact of low temperatures on spore killing, a temperature change from 22° C to 27° C and vice versa was applied during spermatisation. Table 5.3 clearly shows that low temperatures before fructification have no effect on the percentage two-spored asci. Striking changes in killing percentages were observed when the temperature changed from 27° C to 22° C during perithecium formation (data not shown). Perithecia contain normal percentages of two spored asci before the temperature change, but directly after the change the percentage of two spored asci decreases dramatically, especially in newly formed perithecia. Thus, the reduction in spore killing can be fully contributed to the lower temperature during perithecium development.

A cross of *Psk-2* with *oct1* at 22º C (Figure 5.2D) produced only asci containing four or more spores, showing that the reduction in spore killing cannot be attributed to a change in recombination frequency, and must be due to a change in the killing reaction itself.

***P. anserina* gene silencing homologues:**

Several genes involved in the silencing mechanisms of *Neurospora* and *Ascobolus immersus* have been cloned and sequenced. We have used the protein sequences to search for

Table 5.3.
Killing behavior of Spore-killer types at temperatures of 22° C and 27° C.

Spore killer-type	Strain	Percentage two-spored asci			
		27 ° C	22 ° C	22→27 ° C [a]	27→22 ° C [a]
Psk-1	Wa6	96 % (1112 asci)	92 % (1190 asci)	ND	ND
	Wa52	91 % (1412 asci)	98 % (1026 asci)	ND	ND
	Wa53	96 % (1112 asci)	93 % (1184 asci)	91 % (281 asci)	ND
Psk-2	Wa28	79 % (960 asci)	7 % (1736 asci)	65 % (418 asci)	ND
	Wa38	77 % (228 asci)	2 % (1914 asci)	52 % (258 asci)	8 % (171 asci)
	Wa49	72 % (1204 asci)	1 % (619 asci)	ND	ND
Psk-3	Wa27	Variable	5-33 % (324 asci)	61 % (262 asci)	ND
Psk-5	Y	94 % (1239 asci)	93 % (1383 asci)	ND	ND
Psk-7	Z	49 % (6340 asci)	48 % (1017 asci)	51 % (87 asci)	ND
	Wa58	54 % (2007 asci)	53 % (977 asci)	ND	ND

Spore-killer types were crossed with sensitive strain S. The total number of asci analyzed is given between parentheses.

[a] Crosses marked with ND are not performed. The temperature shift of 22 ºC to 27 ºC and vice versa took place when plates were crossed with spermatia of the opposite mating type.

FIGURE 5.2. -- Rosettes of asci from crosses between *P. anserina* strains.

Asci with darker ascospores are more mature than those with lighter spores.

(A) A rosette from a cross between a sensitive strain and the *oct1* marker at 27 ºC showing the asci containing up to eight ascospores. The number of nuclei within an ascus is always eight.

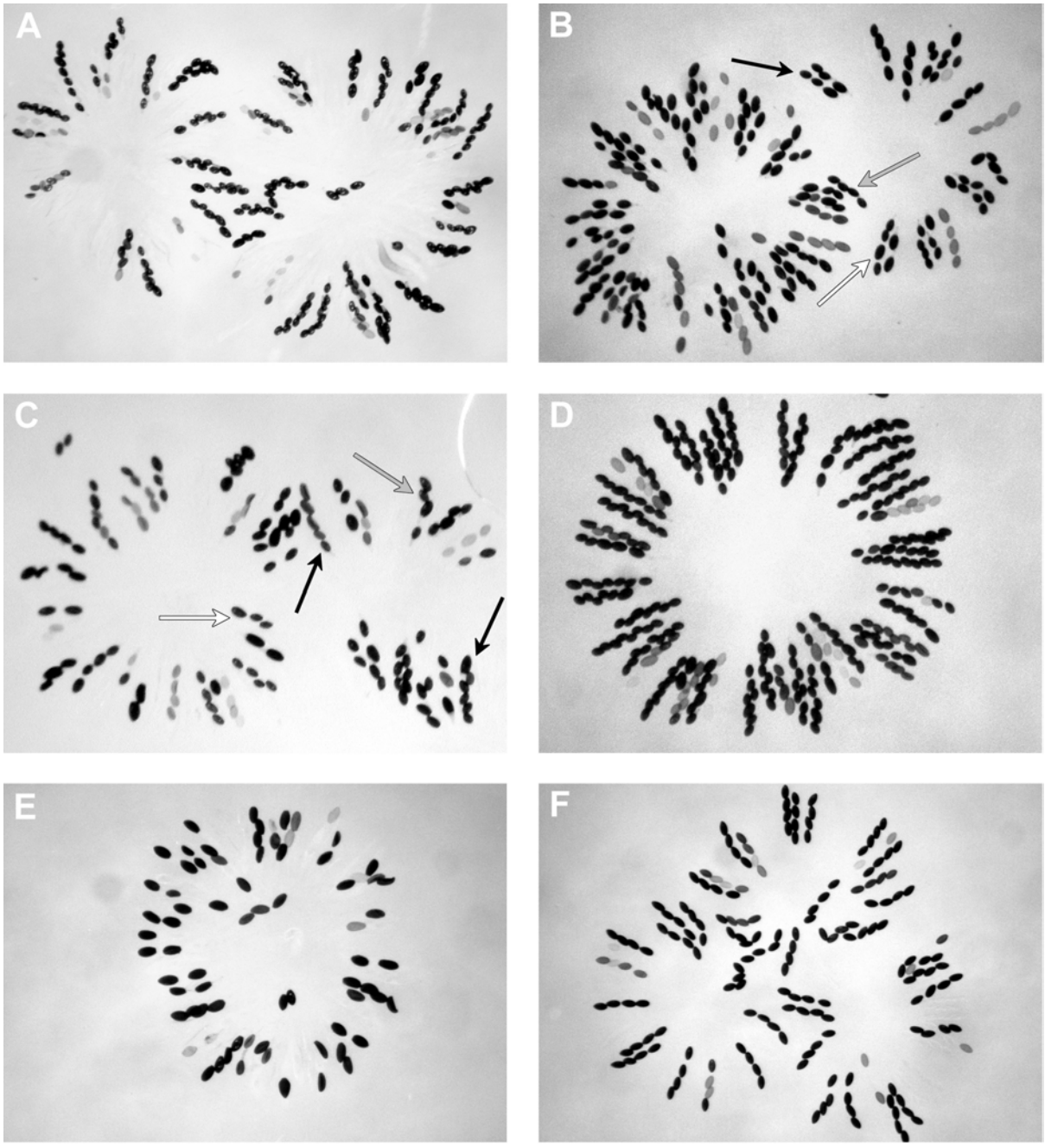
(B) A cross of *oct1* x XS-Z (*Psk-7*) at 27 ºC. Spore killing in these asci is fully penetrant, all sensitive ascospores are killed. Asci showing first division division segration for *Psk-7* contain two or three spores (black arrow). Second division segegrating asci always contain four ascospores (white arrow). Asci with four monokaryotic spores can originate from either FDS or SDS (grey arrow).

(C) Contents of one perithecium from a cross between *oct1* and XS-Wa28 (*Psk-2*) at 27 ºC. Some asci show incomplete penetrance (black arrow) and contain more than four spores and eight nuclei. Other asci behave normal and show the killing reaction (SDS: grey arrow; FDS: white arrow).

(D) A rosette from a cross between *oct1* and XS-Wa28 (*Psk-2*) at 22 ºC. Absence of spore killing. Asci all contain four to eight ascospores and eight nuclei.

(E) A rosette from a cross between sensitive strain S and XS-Wa28 (*Psk2*) at 27 ºC. ~70% two-spored asci can be seen.

(F) The same cross as (E) but at 22 ºC. All asci are four-spored, no spore killing occurs.



homologues in *P. anserina*, which need to be present if such mechanisms cause spore killing. We discovered genes with high similarity to the three known *Neurospora* genes in MSUD (sad-*1, sms-2* and *sms-3/dcl-1*; Lee *et al.* 2003), those involved in Quelling (*qde-1, qde-2, qde-3, dcl-1* and *dcl-2*; Pickford *et al.* 2002, Catalanotto *et al.* 2004), as well as to *dim-2, dim-5, hpo* and *rid* of *N. crassa* that are thought to be involved in RIP (Table 5.4; Kouzminova and Selker 2001, Freitag *et al.* 2002, 2004, Tamaru and Selker 2001). Lower or no sequence similarity was found for the *Ascobolus* methylation genes involved in MIP (Table 5.4; Faugeron 2000). Also a homologue for the RNA dependent RNA polymerase *rrp-3* was found, though this gene has not been connected to any silencing mechanism yet (Galagan *et al.* 2003).

DISCUSSION

Methylation does not play a role in spore killing.

Gene silencing in fungi is commonly associated with methylation. Four transgene-induced gene silencing mechanisms have been discovered in fungi, of which three involve methylation. Two of them, MIP and RIP, are mainly active during the sexual stage. RIP (repeat-induced point mutation) is found in *Neurospora* and acts premeiotically in the stage

Table 5.4.
***P. anserina* sequence homologues of known silencing genes in *Neurospora* and *Ascobolus*.**

Silencing gene	Putative function	Homologue in *P. anserina* contig	tblastn e-value
N. crassa			
sad-1	RdRP (MSUD)	2181/2080/1778	0.0
sms-2	Argonaute-like	2246	-170
dcl-1/sms-3	Dicer-like	1114	0.0
qde-1	RdRP (Quelling)	865	0.0
qde-2	Argonaute-like	469	0.0
qde-3	DNA-helicase	2168	0.0
dcl-2	Dicer-like	995	0.0
rrp-3	RdRP (unknown mechanism)	1206	0.0
req-2	DNA-helicase	2168	0.0
dim-2	DNA methyltransferase (RIP)	839	0.0
dim-5	Histone H3 lys9 methyltransferase	96	-131
hpo	HP-1 like heterochromatin protein	1296	-58
rid	Cytosine C5-DNA methyltransferase	190	-129
N. tetrasperma			
sad-1	RdRP (MSUD)	1778	-24
rid	Cytosine C5-DNA methyltransferase (RIP)	190	-53
A. immersus			
masc1	Cytosine C5-DNA methyltransferase (MIP)	190	-61
masc2	Cytosine C5-DNA-methyltransferase	No homologue	--

between fertilization and karyogamy and silences duplicated genes. The silencing action is a combination of C-G to T-A transitions and most often methylation (Galagan and Selker 2004, Selker 2002). Mutations of (previous) transitions lead to methylation, even if the sequence is not duplicated at the time of methylation (Selker 2002, Irelan and Selker 1997). If RIP is accompanied by methylation, the methylation extends far beyond the borders of sequence homology and possibly leads to silencing of nearby single copy genes (Irelan and Selker 1997). A closely related system to RIP exists in *Ascobolus immersus* and *Coprinus cinereus* and is called MIP (methylation induced premeiotically). MIP silences only by methylation and does not exhibit the C-T transitions. Furthermore methylation by MIP is reversible, because of the lack of mutations (Selker 2002). MIP can cause transfer of methylation among alleles (Colot *et al.* 1996).

As a possible spore-killing mechanism (see Figure 5.1B), MIP is more likely than RIP, because it is a reversible system. Sensitive nuclei from heterokaryotic spores are completely fertile (they produce four-spored asci) when crossed with sensitive strains (Chapter 3, van der Gaag 2000). This situation cannot occur when irreversible transitions have taken place. If killing occurs by a RIP mechanism most probably nearby genes (needed for ascospore production) are accidentally methylated due to a nearby RIP event (Irelan and Selker 1997). Recently, a mild RIP system (which was only detected at the molecular level and causes no phenotypic changes) has been found in *P. anserina*. Here, CT-mutations occur only at a level of 1% and after several rounds of sexual reproduction, whereas mutation in *N. crassa* appears at a much higher level (Graïa *et al*. 2001, Singer *et al*. 1995), although RIP-efficiency in *Podospora* can be increased (Bouhouche *et al*. 2004). Furthermore, RIP in *Podospora* lacks C-methylation and does not show a complete methylation of the *zeta-eta* region that is known to methylate rapidly in *Ascobolus* and *Neurospora* (Graïa *et al*. 2001).

Our experiment was aimed to test if absence of silencing by methylation would prevent spore killing to occur. The drug 5-azacytidine removes methylation from nucleotides and prevents *de novo* methylation. If methylation would be involved, then the asci of crosses between killer and sensitive strains should contain four normal ascospores when 5-azacytidine is used. Apart from side effects, possibly originating from activation of other previously methylated genes in the *Podospora* genome, no perithecia containing only four-spored asci were found. This indicates that methylation, either by MIP or RIP is not directly involved in spore killing. The finding of a mild RIP-like system in *Podospora* that acts only after repeated sexual crosses and without full methylation also suggest that methylation does not play a major role during spore killing in this fungus (Graïa *et al*. 2001). Not surprisingly, the homologous methylation genes we found in *P. anserina* were more related to the *Neurospora* genes involved in RIP than the *Ascobolus* methylation genes.

Genetic analysis of Spore killers using *oct-1* reveals incomplete penetrance for some Spore killers.

We have found no effect of the *oct1* marker on the percentage of two-spored asci for all Spore-killer types. The observed fractions of two-spored asci fall within the normal range for crosses between killer and sensitive strains. However, the asci ascribed to SDS for the Spore-

killer locus fall into two categories. This provides valuable insight into the possible mechanism and allows us to propose a model for spore killing for some Spore-killer types. Spore-killer types *Psk-2*, *Psk-3* (Wa27) and *Psk-4* show two types of asci for SDS. The first type of ascus contains ascospores representing eight nuclei regardless of the amount of spores (between 4 to 8 ascospores). All eight nuclei result from post-meiotic mitosis (Raju and Perkins 1994). The second type of ascus contains four ascospores, independent of the amount of nuclei (between 4 to 8 nuclei) in the ascus. In the first type sensitive nuclei within the (monokaryotic and dikaryotic) ascospores are unaffected by the killer nucleus, since ascospores containing only sensitive nuclei occur within the ascus. In the second type the sensitive nuclei are always killed when included in monokaryotic ascospores. This is similar to the situation ascribed to first division segregation where ascospores containing sensitive alleles are aborted. How can both types of sensitivities within a cross and even within a perithecium be explained, since conditions are identical? A pre-meiotic silencing mechanism such as MIP or RIP, implicates that no survival of ascospores containing only sensitive nuclei is possible, as the sensitive target genes are methylated. Since ascospores without killer-alleles were found in crosses with *oct1,* this rules out the involvement of an efficient RIP or MIP as a killing mechanism for *Psk-2*, *Psk-3* (Wa27) and *Psk-4.*

A possible explanation for this phenomenon could involve a threshold level of killer molecules (*e.g.* siRNA or Toxin molecules) that interact in the abortion of the spores. Our laboratory conditions could balance on this threshold level for molecules produced by Spore killers *Psk-2*, *Psk-3* (Wa27) and *Psk-4*. Then some asci will contain enough killer molecules available before ascospore delimitation resulting in spore killing and four-spored asci at SDS, and others not. Asci without sufficient killer molecules will produce four or more ascospores and eight nuclei. This also implicates that with first division segregation for the Spore killer and inadequate levels of killer molecules available, no spore abortion occurs. FDS for the Spore killer would then be masked as SDS. The killer locus of *Psk-2* and *Psk-4* should therefore be positioned closer to the centromere than previously estimated. Since no FDS for the spore-killing locus within previously examined four-spored asci was found (Chapter 3, van der Gaag *et al*. 2000), this shift towards the centromere must be marginal.

Since percentages of two-spored asci are evenly distributed among perithecia of different age in *Psk-2* and *Psk-4*, there can be no dilution of a protein or molecules, like in *het-s/S* spore killing (Bernet 1965). There, the prion protein responsible for spore killing is present in lower numbers in asci that are produced at a later stage within the perithecium. This results in more four-spored asci and lower percentages of killing in older perithecia, and a variable killing percentage among perithecia. From it can be inferred that the killer molecules of Spore-killer types *Psk-2* and *Psk-4* must be produced until after the formation of the ascus mothercell, the phase where the cell is separated from the rest of the fertilized mycelium. The *Psk-3* type of Spore killers is grouped on basis of the variable killing percentage between perithecia of the same cross (Chapter 3, van der Gaag *et al.* 2000). Variability in percentage of two-spored asci is also present in crosses of *Psk-3* with *oct1*. It is possible that *Psk-3* killer molecules are likewise diluted as in the *s/S* spore killing, explaining the variability of killing behavior.

A further distinction between Spore-killer types can be made based on the temperature. The *het-S* spore killing occurs only at low temperatures (18° C), due to the stabilizing effect of temperature on the transmission of the prion through the sexual cross (Dalstra *et al.* 2003). We found an opposite temperature effect for *Psk-2*: Almost absence of killing occurs at 22° C in contrast to normal killing percentages at higher temperatures. This sudden decrease in killing cannot be attributed to an increase of SDS frequency, as a cross with *oct1* at 22º C shows no aborted (monokaryotic) ascospores. Killing failure suggests a complete unavailability of killer molecules attributed to shutdown of production or maintenance, binding failure of the molecule to the target, or the presence of more (stable) antitoxin (if the mechanism is a TA-complex). The latter two seem more likely in terms of temperature effects on proteins.

The effect of low temperatures further reduces the effect of meiotic drive for *Psk-2*. Temperatures of 22° C and lower are common in the Netherlands, where most of our *Podospora* (Spore-killer) strains were isolated. All our other Spore-killer strains are not affected by temperature change. Also in *Neurospora* changes in temperature did not have an effect on spore-killing percentages (Raju 1979).

Silencing is probably unrelated to spore killing:

The two other silencing mechanisms found in fungi, quelling and MSUD, are reversible and do not need or use methylation to silence genes (Pickford *et al*. 2002, Shiu and Metzenberg 2002). Quelling has been found in *Neurospora* in the vegetative phase of the lifecycle and causes silencing of hyperhaploid genes. Methylation is found here, but not needed for quelling. Quelling is dominant in heterokaryotic strains (Pickford *et al*. 2002). Also MSUD (meiotic silencing by unpaired DNA, previously known as meiotic transvection) was discovered in *Neurospora.* It operates just after karyogamy and causes the silencing of all copies of any unpaired gene with its homolog, including homologous pairs that are themselves paired. MSUD silencing is restricted to the ascus, indicating that diffusion of transacting particles is limited. It can phenotypically be observed as ascospore abortion (Aramayo and Metzenberg 1996). Silencing by MSUD ends not later than ascospore germination, as the silenced genes are re-expressed during the vegetative stage (Shiu *et al*. 2001). Silencing is probably triggered by failure of meiotic recombination and transcription of the unpaired region. Methylated sequences therefore are unaffected by MSUD (Lee *et al.* 2004, Pratt *et al.* 2004). Both silencing mechanisms are related to PTGS in plants and RNAi in animals. Each one uses a different RNA-directed RNA polymerase (RdRP) that converts mRNA to an intermediate form of dsRNA. These dsRNAs are in turn cleaved into small interfering RNA (siRNA) molecules, which act as guides to the destruction of any mRNA of the original gene and prime the synthesis of more dsRNAs (Lee *et al.* 2004).

We found homologous genes for both quelling and MSUD in *P. anserina*, indicating that the silencing machinery is present in this fungus. Could these silencing mechanisms be responsible for spore killing? Several observations argue against MSUD as the mechanism of spore killing in *P. anserina*. Spore killing in *Podospora* aborts the progeny not containing the

killer locus, but those containing it are saved. As MSUD is ascus dominant, any dsRNA and siRNAs produced by a killer must not be able to affect the mRNA of the target locus of the Spore killer, or it would abort it's own spores. To prevent degradation, Spore-killer mRNA must either be unrelated to target sensitive mRNA, or unable to process dsRNAs and siRNAs. Failure of the Spore killer to maintain levels of dsRNA and siRNA could be caused by a (closely linked) defective *sms-2* or *sms-3/dcl-1* gene at ascospore formation. However, asci heterokaryotic for the Spore-killer locus would then be aborted as they contain the functional sensitive locus, meaning no four-spored asci in a cross between a Spore killer and a sensitive strain. Furthermore, the absence of killing at 22º C we found for *Psk-2* cannot be explained by small RNA-mediated silencing. Incomplete penetrance however could be explained by insufficient amounts of small RNAs available for complete silencing the sensitive target gene.

Finally, a cross between two different Spore killer types would have resulted in the abortion of no spores, if the target gene of both Spore killers is identical, or to all spores if the target gene is different, as killer function and resistance to it are tightly linked. So far, we found only unilateral or mutual resistant interactions between our Spore-killer strains and no complete abortion. For instance, Spore-killer types *Psk-1* and *Psk-7*, which were proven to be resistant to each other by genetic analysis of their progeny, are able to recombine and create sensitive spores (Chapter 3, van der Gaag *et al*. 2000). This necessitates the existence of (a) sensitive target(s), which in turn would be affected by the dsRNA resulting in complete abortion of ascospores originating by first division segregation (FDS) of both Spore killers. As we found these double FDS ascospores in our genetic analysis of Spore-killer interactions, spore killing by MSUD is probably not the case. As both MSUD and Quelling act through the same RNA intermediates, spore killing by prolonged or early quelling is likewise improbable. It should be noted that spore killing in *Neurospora* by *Sk* was found to be unaffected by MSUD, when tested as the possible killing mechanism (Shiu *et al*. 2001).

Models of spore killing:

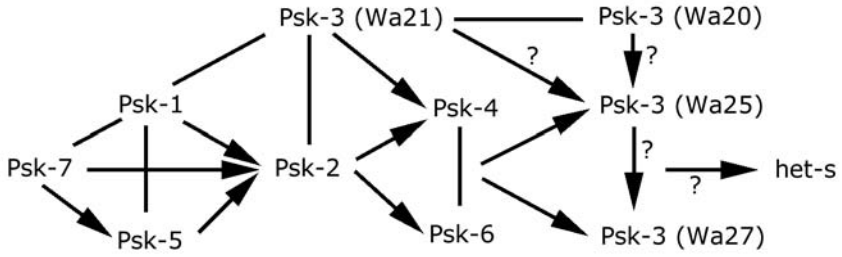
Based on the observed percentages of killing, interaction among Spore killers and the discussion above (which rejects the silencing models), we propose the following Toxin-Antitoxin models for the spore-killer types (see also Figure 5.1). Generally, toxin and antitoxin form closely linked cassettes, where the antitoxin is arranged first, regulated by a common mechanism (Hayes 2003). In the TA-models the toxin is more stable than the antagonist.

Spore killing for *Psk-3* (Wa27) resembles the prion type of spore-killing action (Figure 5.1F, 5.1D): Variability of spore-killing percentages, spore killing acts some time after ascospore delimitation (as shown by the presence of degenerated spores) and spore killing occurs only with specific wild-type strains (Chapter 3, van der Gaag *et al*. 2000). Here we have shown that (a specific) *Psk-3* is not temperature sensitive, and that not all first division segregation asci are affected by the killing. However, the *het-s* prion mechanism itself is not responsible for P*sk-3* (Wa27) spore killing. Strain Wa27 carries the *het-S* allele and not the *het-s* (prion) form (H. J. P. Dalstra, personal communication), and sensitive strains used in our

crosses display the *het-S* allele. This *Psk-3* type of action can be explained as a toxin-antitoxin situation where the toxin (and/or antitoxin) is stable enough (no temperature effects) to last through the sexual phase, but production is shut down during the sexual phase before separate cells are formed. Thus, a dilution effect of the toxin (and/or antitoxin) would occur, just like the prion in the *het-s* spore killing model (Dalstra *et al*. 2003), resulting in a variable killing percentage. In case of antitoxin dilution, the first asci within a perithecium are expected to show no spore killing, due to sufficient amounts antitoxin available (when the toxin target is also present), whereas later produced asci are prone to killing by antitoxin depletion. With toxin depletion the earlier asci will contain sufficient amounts of toxin to allow spore killing, whereas ascospores in subsequentially produced asci are saved by lack of sufficient toxin. Both (toxin and antitoxin depletions) situations could occur simultaneously depending on the stability of the toxin and antitoxin molecules. Fresh toxin-antitoxin production should start shortly after ascospore delimitation, to ensure survival of the ascospores carrying the TA cassette. The toxin probably targets a process occurring during ripening of the ascospores. Spore killing for the other *Psk-3* types can be based different, as they are also capable of killing among each other (Figure 5.3, Chapter 3, van der Gaag *et al*. 2000).

FIGURE 5.3. -- Relation of Spore-killer elements in *P. anserina.*

A straight line represents a mutual resistant interaction, whereas the arrow indicates a dominant epistatic interaction Chapter 3, van der Gaag *et al*. 2000). The relation towards *Psk-3* and *het-s* is unknown, as is the direction of dominant epistasis between *Psk-3* killers (shown by the question mark). *Psk-1*, *Psk-2*, *Psk-5* and *Psk-7* do not show a vegetative compatible reaction with *het-S*, suggesting they contain the *het-s* allele and cannot show *het-s* spore killing (Chapter 4, van der Gaag *et al*. 2003).



Spore-killing action in Spore-killer types *Psk-2* is temperature sensitive, no killing occurring at lower temperatures. Some first division segregating asci do not show spore killing at normal temperatures. Previously it was shown (Chapter 3, van der Gaag *et al*. 2000) that *Psk-2* killing acts shortly after ascospore delimitation, as sensitive ascospores can only be detected very shortly and degrade completely afterwards, and that killing percentage is stable at normal temperatures. The evidence suggests a TA-model for *Psk-2* where antitoxin still is produced during the sexual stage, at least until after the ascus mothercell is formed (Figure 5.1G). This ensures that toxin and antitoxin are present in every ascus mothercell and no dilution as in the prion / *Psk-3* model occurs. Toxin is more slowly degraded during meiosis and balances at the threshold level at ascospore delimitation, leading to a stable percentage of sensitive escapes (and consequently a stable killing percentage). A lower temperature

stabilizes the more stable antitoxin level sufficiently above the threshold level, and thus inhibits spore killing.

We have found the following evidence for *Psk-4*: It has a stable killing percentage of 50% and killing acts just after ascospore delimitation. Some *Psk-4* FDS asci show incomplete penetrance. This mode of action can be explained by the same model as *Psk-2* (Figure 5.1F), although temperature sensitivity of spore killing has not been measured for *Psk-4* (and *Psk-6*) and could be absent for these killer types. Spore-killer type *Psk-4* and *Psk-6* show a mutual resistant interaction (Chapter 3, van der Gaag *et al*. 2000). If *Psk-4* follows the TA-model, *Psk-6* probably does as well and would belong to the same toxin-antitoxin group. Antitoxin produced by *Psk-4* would thus prevent killing by *Psk-6* and vice versa. However antitoxin produced by *Psk-6* would be less stable than *Psk-4* antitoxin, or *Psk-6* toxin is more stable, as *Psk-6* does not show incomplete penetrance.

No additional information was found for Spore-killer types *Psk-1*, *Psk-5*and *Psk-7,* except that they are vegetatively compatible to each other (Chapter 4, van der Gaag *et al*. 2003). They have a stable killing percentage and show spore killing in all asci. Spore killing acts just after ascospore delimitation and no temperature effects are found. Furthermore complex interaction types between Spore-killer types *Psk-1*, *Psk-5* and *Psk-7* exist (Figure 5.3). *Psk-1* shows mutual resistance to *Psk-5* and *Psk-7*, where *Psk-7* is dominant epistatic to *Psk-5* (Chapter 3, van der Gaag *et al*. 2000). A model for these remaining killer types must also be able to explain the interactions. This kind of interaction can be explained by a TA-model (Figure 5.1H), where each *Psk* belongs to the same toxin-antitoxin group, but production levels or sensitivities of toxin and antitoxin differ. Here, *Psk-7* should produce both high amounts of toxin and antitoxin, *Psk-1* high amounts of antitoxin and low amounts of toxin, and *Psk-5* both low amounts of toxin and antitoxin. If the amount of toxin is higher than the antitoxin, killing occurs, whereas resistance to killing is found with equal or higher amounts of antitoxin. Antitoxin produced by *Psk-1*, *Psk-5* and *Psk-7* would be less stable, such that no antitoxin persists until the ascospore delimitation phase.

We could explain the behavior of our *Psk*-types as a post-segregational killing mechanism, with several variations on the Toxin-Antitoxin model. The presence of resistance genes closely linked to the Spore killer in *Neurospora* and the finding of resistant strains (producing only the antitoxin component), also support a TA model (Turner 2001, 2003). However the exact mode of action for each Spore-killer type still remains to be elucidated.

Chapter 6

Sexual Compatibility and Outcrossing in *Podospora anserina.*

Marijn van der Gaag, Anne D. van Diepeningen, Alfons J. M. Debets and Rolf F. Hoekstra

ABSTRACT

To assess the likelihood of outcrossing in the secondary homothallic ascomycete *Podospora anserina* we examined the extent of vegetative and sexual compatibility between wild-type strains. The number of vegetative compatibility groups (VCG's) in the population was estimated based on the incompatibility reactions between isolates in our survey. The estimated number of 107 VCG's exceeding the maximum number of 64 VCG's based on currently known *het*-loci, as well as the difference in sexual compatibility reactions of mating types of the same isolate suggest regular outcrossing in this fungus. We experimentally verified the options for outcrossing in *P. anserina.* Both monokaryotic and dikaryotic mycelial cultures proved capable of outcrossing, indicating the absence of a nonresponse by trichogynes. Outcrossing percentages between 1-5 percent were found in unmanipulated natural situations of ascospores on dung. The amount of monokaryotic ascospores found in Spore-killer (*Psk*) strains was significantly higher than in other isolates, which enhances outcrossing in these strains. Our findings contribute to the understanding of the population dynamics of the meiotic drive systems in *Podospora,* which cause the abortion of two of the four dikaryotic ascospores not containing the killer element, and are dependent on outcrossing for their action.

Introduction

Podospora anserina is one of the few ascomycete fungi that are pseudohomothallic or secondary homothallic. These species are capable of completing their life cycle from a single sexual self-fertile ascospore, as with true homothallism. However, ascospores and mycelium are heterokaryotic and contain nuclei of two different mating types (*mat*+ and *mat*-). Both mating-type nuclei are needed to initiate sexual reproduction. The formation of self-fertile ascospores in *P. anserina* (and also of *Podospora tetraspora* and *Gelasinospora tetrasperma*) is accomplished by strict regulation of second division segregation of the mating type (located distant from the centromere), coupled with non-overlapping second division spindles (Raju and Perkins 1994). The needed high second division frequency of 98% for mating type is

regulated by positive chiasma interference in *P. anserina* (Esser 1974). In contrast, the pseudohomothallic *Neurospora tetrasperma* achieves self-fertility by first division segregation of the mating type coupled with second division spindle overlap. Consequently the mating type is genetically linked to the centromere by suppressed recombination. (Raju 1992, Raju and Perkins 1994, Gallegos *et al.* 2000).

Though most ascospores are self-fertile in *Podospora*, two types of self-sterile ascospores occur. A small percentage (2%) of ascospores are self-sterile due to first division segregation of mating type. Here each ascospore contains two nuclei of the same mating type. Another 1-2% of the asci contain some small uni-nucleate spores. Usually two uni-nucleate spores are produced instead of one bi-nucleate ascospore due to mispackage of the nuclei (Rizet and Engelmann 1949, Esser 1974). Both types of self-sterile ascospores rely on outcrossing to complete their lifecycle. Self-sterile ascospores are more common in *N. tetrasperma* where as many as 10% of the ascospores are self-sterile and 20% of the (asexual) macroconidia receive only one mating type (Raju 1992).

However, outcrossing may not be restricted to the self-sterile mycelium. Theoretically, the dikaryotic mycelium could outcross as well, as a trichogyne (female receptor) from a dikaryotic mycelium might be able to accept a foreign spermatium. Experiments by Bistis (1996) in *N. tetrasperma* showed that trichogynes did not fuse to macroconidia in dikaryotic mycelium. However, the inability to outcross had probably more to do with the inhibitory effect of developing perithecia on the trichogyne (Bistis 1996), so this possibility cannot be rejected. Thus, pseudohomothallic species carry adequate capabilities for facultative outcrossing. As Raju (1992) remarked, pseudohomothallic species have "the best of both worlds", combining the potential for hybrid vigor and quick completion of the life cycle with the advantages of outbreeding.

Despite the advantages of the ability for both selfing and outbreeding, a study concerning outcrossing behaviour among *N. tetrasperma* strains showed that outcrossing resulted in varying degrees of sexual dysfunction, especially among geographically isolated regions (Jacobson 1995). Sexual dysfunction in this species ranged from complete infertility to diverse levels of ascospore abortion within perithecia. Some form of sexual dysfunction was found connected with *het-c*, a vegetative incompatibility gene (Saenz *et al.* 2001). However, molecular studies on *het-c* also revealed that *N. tetrasperma* sporadically outcrosses (Powell *et al.* 2001). Also in *Podospora anserina* specific combinations of non-allelic vegetative incompatibility alleles lead to absence of perithecia (Esser and Blaich 1994, Saupe *et al.* 1994, Loubradou *et al.* 1997).

Vegetative incompatibility is a self/nonself-recognition system in fungi, which is expressed during vegetative growth, but arrested at the sexual stage (Glass and Kuldau 1992). Vegetative incompatibility phenotypes are generally defined by the alleles at multiple *het*-loci. These *het*-loci are ubiquitous in ascomycete fungi, between 6 to 11 genes are commonly found per species (Saupe 2000). In most cases vegetative incompatibility appears to be controlled by allelic interactions in which two individuals are compatible only if they share the same alleles at the *het*-loci. As mentioned above, *P. anserina* also possesses a non-allelic vegetative incompatibility system: Five loci (*het-b, het-q, het-s, het-v* and *het-z*)[a] are involved

in the allelic system, and five loci in three non-allelic systems (*het-c/het-d, het-c/het-e* and *het-r/het-v*) [a] of which one locus also is involved in the allelic system (Bernet 1965, 1967, Saupe 2000, Marcou *et al.* 1990). A common hypothesis is that vegetative incompatibility polymorphisms are maintained by balancing selection (Wu *et al.* 1998) to restrict transmission of parasitic cytoplasmic elements (Caten 1972, Debets *et al.* 1994, van Diepeningen *et al.* 1997, Chapter 2, van der Gaag *et al.* 1998) or resource plundering (Debets and Griffiths 1998). Interestingly, a theoretical model by Nauta (1994) explains the existence of this type of combined vegetative and sexual incompatibility as an anti-meiotic drive system as it restricts outcrossing. *P. anserina* as well as several *Neurospora* species are known to contain multiple meiotic drive elements (Chapter 3, van der Gaag *et al.* 2000, Raju 1996). Studies on vegetative incompatibility groups (VCG's) have often been used to describe phenotypic diversity and structure in fungal populations. Inferences can be made from VCG-type survey data about recombination (outcrossing) or clonality (selfing) in natural populations (Milgroom and Cortesi 1999).

We have investigated vegetative and sexual compatibility interactions among our collection of wild-type *Podospora anserina* strains in order to assess the likelihood of outcrossing and the occurrence of sexual dysfunction in a natural population. Based on our compatibility survey we found evidence that *P. anserina* regularly outcrosses in nature. In vitro outcrossing experiments confirmed that double mating-type strains of *P.anserina* are able to outcross, indicating a high potential for recombination and meiotic drive.

[a] Nomenclature of *het*-loci according to Bernet (1965, 1967). Esser and Blaich (1994, Marcou *et al.* 1990) named the allelic loci respectively *het-t, het-u, het-s, het-v* and *het-k*. The non-allelic combinations were named respectively *het-b/het-d, het-b/het-a* and *het-c/het-v*.

MATERIALS AND METHODS

Testing sexual and vegetative compatibility:

Wild-type isolates of *P. anserina* were characterized previously (Chapter 3, van der Gaag *et al.* 2000). Standard laboratory strains S and s were included in the test for reference. Testing of isolates was performed on 20 cm Petri dishes with Biomalz-agar at 27 ºC (Esser 1974). Three single mating-type isolates were tested simultaneously against six other isolates (Figure 6.1). Standard, isolates were tested twice for each combination of strain and mating type.

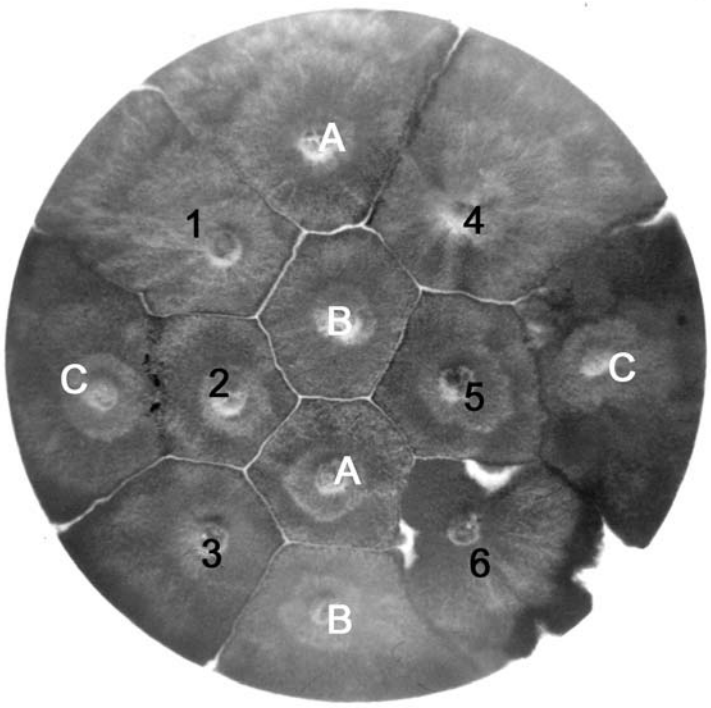
Vegetative incompatibility was scored when a barrage was visible, a clear zone of lysed mycelium between two isolates. Sexual compatibility was scored when (proto-)perithecia were present. Perithecia were opened and the contents were examined under a binocular. Four different types of sexual interaction were recognized:

1. **Full sexual compatibility** when perithecia contained only four ripe black ascospores per ascus.
2. **Ascospore abortion** when perithecia contained asci with less than four (ripe) ascospores. This includes abortion caused by Spore-killer strains.

3. **Perithecial dysfunction** when perithecia were barren or did not develop further than the proto-perithecial stage.

Strain combinations that did not show any perithecial development were scored as **fully sexually incompatible**.

FIGURE 6.1. -- Experimental setup of vegetative and sexual incompatibility tests of wild-type *P. anserina* isolates.



Monokaryotic strains 1-6 are tested against isolates A-C of the opposite mating type. A clear zone (e.g. strain 2 vs. strain A) designates a vegetative incompatible interaction ('barrage'); absence of such a zone indicates a vegetative compatible situation (strain 2 and 5 vs. strain C). Perithecial development can be seen on both sides of the barrage (strain 4 vs. strain A), on one side of the barrage (e.g. side strain 5 vs. strain A) or without the barrage (strain 2 vs. strain C). Noteworthy is the sexual incompatibility in combination with vegetative compatibility for strain 5 vs. strain C.

Outcrossing with monokaryotic cultures:

Monokaryotic cultures (both mating types) of Wa54, *136* (green spores, vegetative compatible with Wa54 (Marcou *et al.* 1990)) and Wa52 (*Psk-1* (Chapter 3, van der Gaag *et al.* 2000)) were grown on cornmeal agar at 27 °C (Esser 1974). Ten Petri dishes with single mating-type strains were used as maternal strains for each crossing pair and mating type. Microconidia were harvested with saline from dikaryotic strains and filtered on glasswool. Microconidia suspensions were counted by haemocytometer and equally mixed for the pairwise combinations used in the crossing. After two days exposure to light, monokaryotic cultures with protoperithecia were flooded with 0.5 ml saline containing approximately 2.5 * 10^6 of the microconidia mix. After a few days perithecia were dissected and examined by microscope for outcrossing. Outcrossing can be seen as either asci with spore killing or asci containing two green and two black ascospores. As a control to the crossing capability of the microconidia, single strain microconidia were applied to monokaryotic cultures to observe the formation of perithecia.

Outcrossing on dikaryotic cultures:

Dikaryotic mycelium of Spore-killer (Wa52, Wa6) and sensitive (Wa54, Wa3) strains was grown on cornmeal agar plates at 27 ºC and flooded with 0.5 ml saline containing approximately 2.5 * 10^6 microconidia after 2 days of exposure to light. Perithecia developed in flooded areas were dissected and examined by microscope for selfing (only four-spored asci) and outcrossing (predominantly two-spored asci).

Outcrossing between dikaryotic cultures:

Mixtures containing equal amounts of ascospores from strains *136* (green spores, S genetic background) with Wa6 (*Psk-1*), XS-Wa6 (*Psk-1,* S genetic background (Chapter 3, van der Gaag *et al.* 2000)) or Wa28 (*Psk-2*) were applied to cornmeal agar and grown at 27 ºC up to development of perithecia. The mixture contained approximately 1000 ascospores. An identical setup was used for ascospore combinations of Spore-killer (Wa6 and Wa28) and sensitive (S, Wa15, Wa30) strains on sterilized horse dung plates grown at 27 ºC.

A similar experiment used mycelium fragment mixtures of strains *136* (green spores) and XS-Wa6 (*Psk-1*). Mycelium fragments were harvested from dikaryotic mycelium with saline using a glass spatula. Saline suspensions were mixed, applied to cornmeal agar plates and grown at 27 ºC.

Perithecia from all experiments were dissected and examined by microscope for selfing and outcrossing. Outcrossing is scored when asci containing two ascospores (spore killing) are observed. As a control the germination and fructification of ascospores from single strains was tested on cornmeal agar plates.

Monokaryotic ascospore count:

Dikaryotic isolates of strains *136* (green spores), Wa5, Wa6 (*Psk-1*), Wa15, Wa28 (*Psk-2*), Wa30, Wa38 (*Psk-2*) and Wa52 (*Psk-1*) were grown in 5 cm petri dishes containing 18 ml Cornmeal agar (Esser 1974). Selfed perithecia were opened and mono and dikaryotic ascospores counted. Monokaryotic ascospore counts were pooled by Spore-killer type and compared with χ^2-tests.

RESULTS

Vegetative and Sexual Compatibility survey:

We have made a survey among 55 wild-type isolates from Wageningen, The Netherlands and two from Normandy, France in order to estimate the extent of vegetative and sexual compatibility among wild-type populations of *P. anserina*. The results of vegetative and sexual compatibility of each tested combination of monokaryotic strains is shown in Figure 6.2a-d. The Wageningen population of *P. anserina* is subdivided among sampling areas in this figure, which are (linearly) separated from each other by several kilometers. The results of our survey are used to assess the likelihood of outcrossing for this secondary homothallic ascomycete. We have tested all isolates among each other, except strains isolated in 1994. These were only tested against a subset of the strains isolated during 1991-1993, in

order to limit the amount of combinations to test that year. Some strain combinations did not yield any result (see Figure 6.2, open dots). This originated from either unavailability of the monokaryotic strains or the stagnation of growth during testing, for example due to the incoloris mutation (Bernet 1991).

In our survey we found an abundance of vegetative incompatibility reactions. Only 3.2 percent of all combinations between single mating-type strains proved vegetatively compatible (Figure 6.2, Tables 6.1 and 6.2). This excludes the combinations between reciprocal mating types of the same isolate, which all were uniformly vegetatively compatible. The percentage of vegetatively compatible reactions is comparable with those found by Esser (Esser 1971, Esser and Blaich 1994), also shown in Table 6.1.

Some cases of vegetative compatibility are remarkable, for instance strains Wa16, Wa36, Wa42 and Wa55. Vegetative compatibility tests show that they are likely in a single compatibility group, however strains Wa16 and Wa36, as well as Wa36 and Wa42 are vegetatively incompatible, while the other reactions are vegetatively compatible. These cases can be explained by different combinations of *het-c*, *het-d* and *het-e* alleles (Saupe *et al.* 1994).

Table 6.1
Percentage of vegetative and sexual compatibility between single mating-type (monokaryotic) *P. anserina* strains.

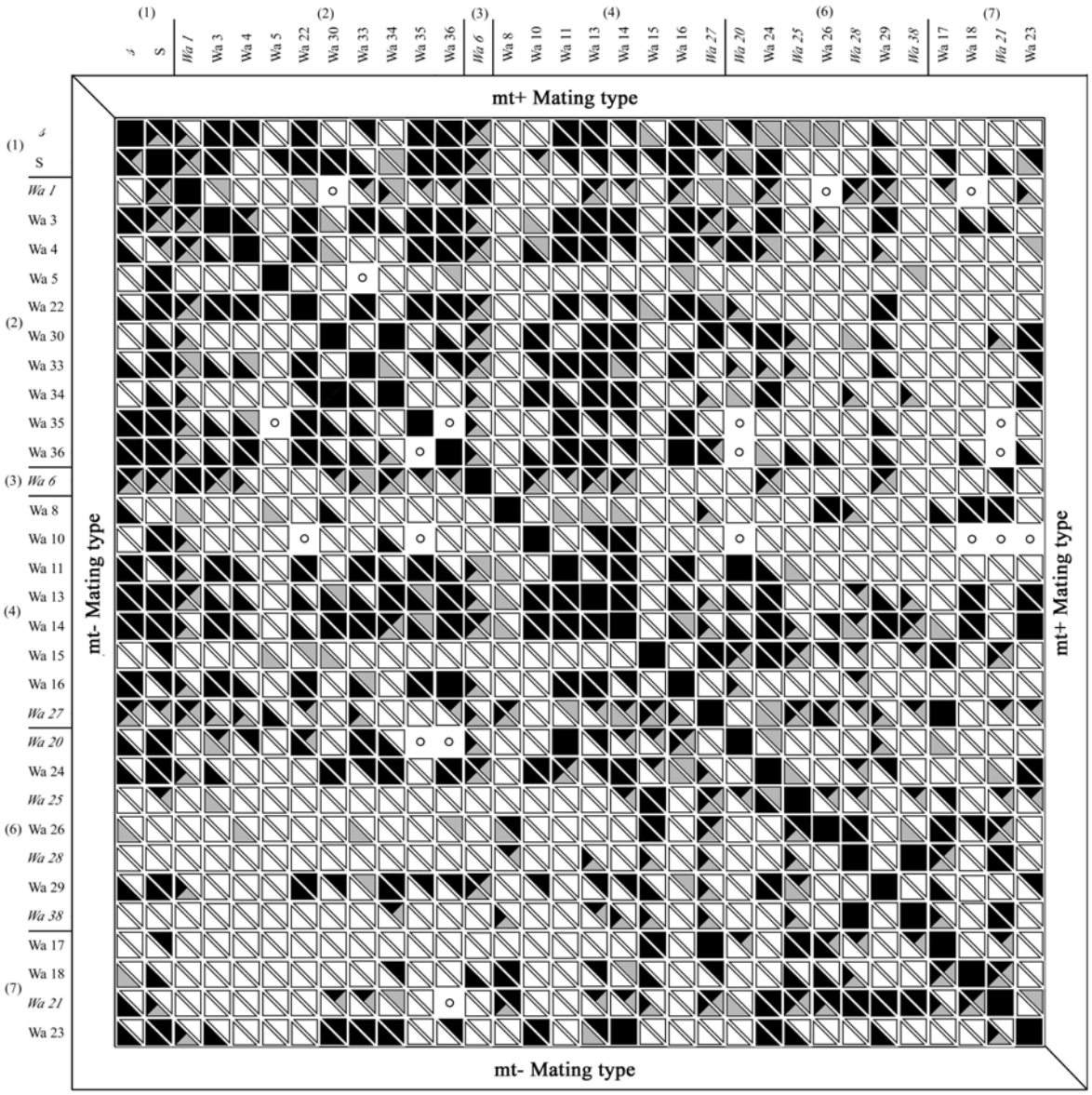
Vegetative compatibility [a]	Sexual compatibility [b, d]		Asci [c, d]		Percentage of strain combinations [f]		
					Wageningen		Esser [e]
+	-		-		0.7 % (18)	0.7 % (18)	0 % (0)
+	D		-		0 % (0)		
+	+		+		2.1 % (54)	2.5 % (63)	7.6 % (26)
+	+		A		0.4 % (9)		
-	-	-	-	-	43.7 % (1076)	52.1 % (1282)	43.9 % (150)
-	-	D	-	-	6.4 % (157)		
-	D	D	-	-	2.0 % (49)		
-	-	+	-	+	12.3 % (302)	28.2 % (692)	33.9 % (116)
-	-	+	-	A	12.7 % (312)		
-	D	+	-	+	1.7 % (42)		
-	D	+	-	A	1.5 % (36)		
-	+	+	+	+	10.2 % (251)	16.5 % (407)	14.6 % (50)
-	+	+	+	A	0.8 % (20)		
-	+	+	A	A	5.5 % (136)		

[a] Vegetatively compatible (+) or vegetatively incompatible (-) interactions.
[b] Fully sexually incompatible (-), perithecial dysfunction (D) or sexual compatible (+) interactions.
[c] Asci with ascospores abortion (A) or fully sexually compatible with black four-spored asci (+).
[d] A double row shows the results for both sides of the barrage (vegetative incompatibility reaction).
[e] The 19 wild-type strain combinations as examined by Esser (1971).
[f] Number of strain combinations shown in brackets, excluding the 57 (Wageningen) and 19 (Esser) selfing strain combinations.

FIGURE 6.2A. -- Vegetative incompatibility and mating reactions between wild-type monokaryons of *P. anserina* : *Interactions between strains isolated in 1991-1993.*

Strains are grouped by origin of isolation, and places of isolation geographically most close are placed next to each other. Isolation spots used are (1) Laboratory strains (Normandy, France - strains s and S), (2) Oranje Nassau Oord, (3) Cow meadow (4) Wageningen harbour, (5) Grebbedijk, (6) Blauwe Kamer nature reserve and (7) Grebbeberg. All places of isolation (2-7) are located around Wageningen, The Netherlands (see Figure 1.2 of Chapter 1). Spore-killer strains are represented by *italic* names.

Each square represents one cross between two isolates, the reciprocal counterpart can be found on the other side of the diagonal axis. Crosses represented by open dots are unresolved interactions (°). In case of a **vegetatively incompatible** situation, the square is divided into two separate triangles representing the two isolates (◪). A **vegetatively compatible** reaction has an undivided square (□). Sexual compatibility reactions are shown by different colors in the squares or triangles: A **fully sexually incompatible** reaction is shown as a white color (□), **perithecial dysfunction** is represented as a gray shade (▒), **ascospore abortion** is shown in bicolored gray and black (◪), and a **fully sexually compatible** interaction in black (■).



We scored the sexual compatibility reactions as four types, to roughly be able to compare them with the sexual dysfunction categories as described by Jacobson (1995). Two types, full sexual incompatibility and perithecial dysfunction, are sexual incompatibility types producing no offspring. The other two types, ascospore abortion and full sexual compatibility, describe levels of sexual compatibility. **Full sexual incompatibility** is defined as the visual absence of any perithecial development. **Perithecial dysfunction** is defined as the arrest of perithecial development; only protoperithecia or empty perithecia are observed. **Ascospore abortion** is defined as the abortion of one or more ascospores in asci of several perithecia. This includes ascospore abortion caused by meiotic drive elements (Spore killers). **Full sexual compatibility** is defined as the production of normal four-spored asci within the perithecia. Two different reactions can occur on both sides of a vegetative incompatibility or 'barrage' reaction (Figure 6.2), a reaction is called sexually semi-incompatible when only ascospores are produced on one side of the 'barrage'. A complete testing on sexual dysfunction as Jacobson (1995) did for *Neurospora tetrasperma*, including viability of ascospores and selfing of offspring was beyond the scope of our experiment. Almost half of all isolate combinations resulted in the production of mature black ascospores (Figure 6.2, Table 6.2). However, more than half of these interactions produce offspring only on side of the 'barrage' suggesting semi-incompatibility. The genetic basis of this has not been further

FIGURE 6.2B. -- Vegetative incompatibility and mating reactions between wild-type monokaryons of *P. anserina* : *Interactions between strains isolated in 1994.*

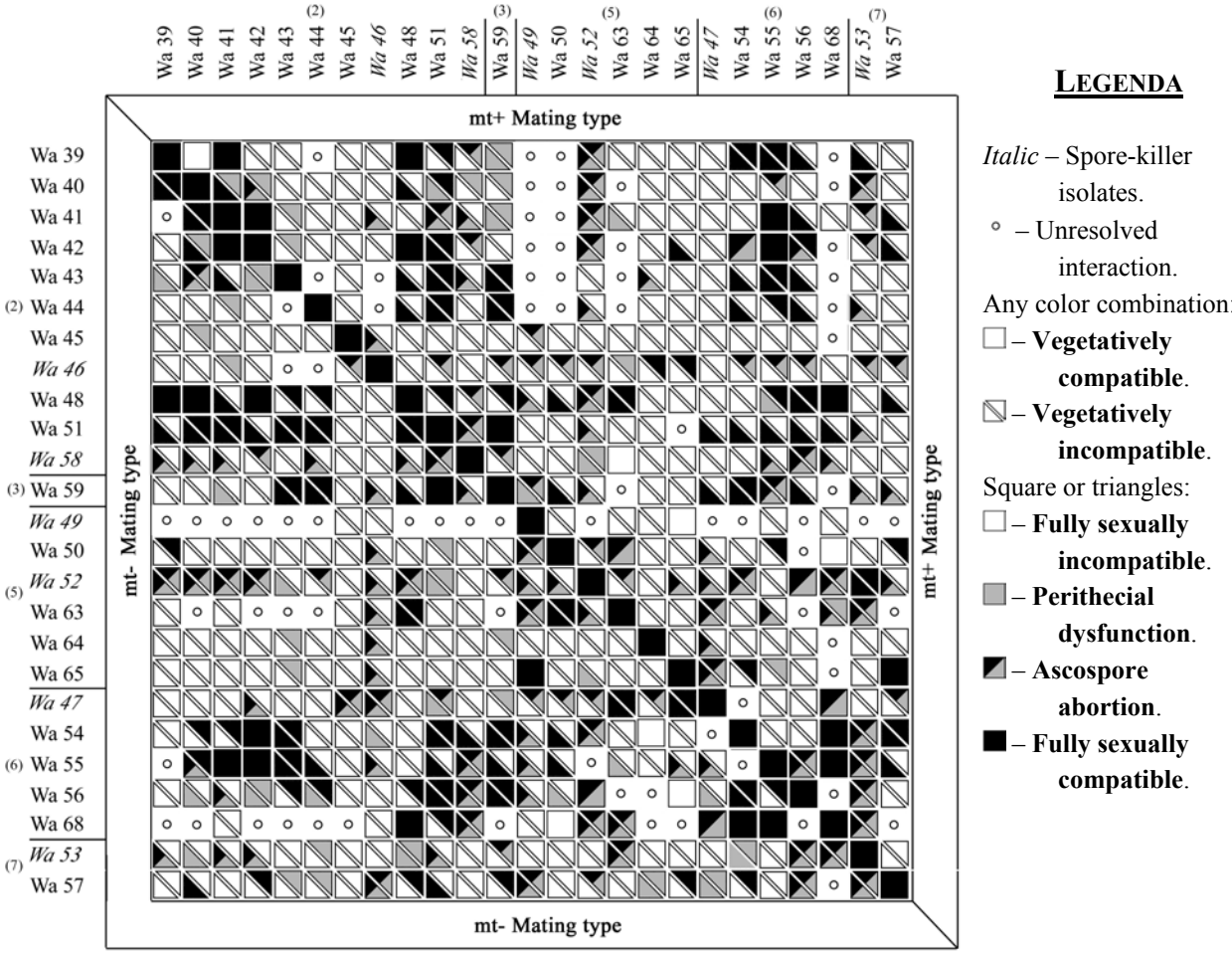
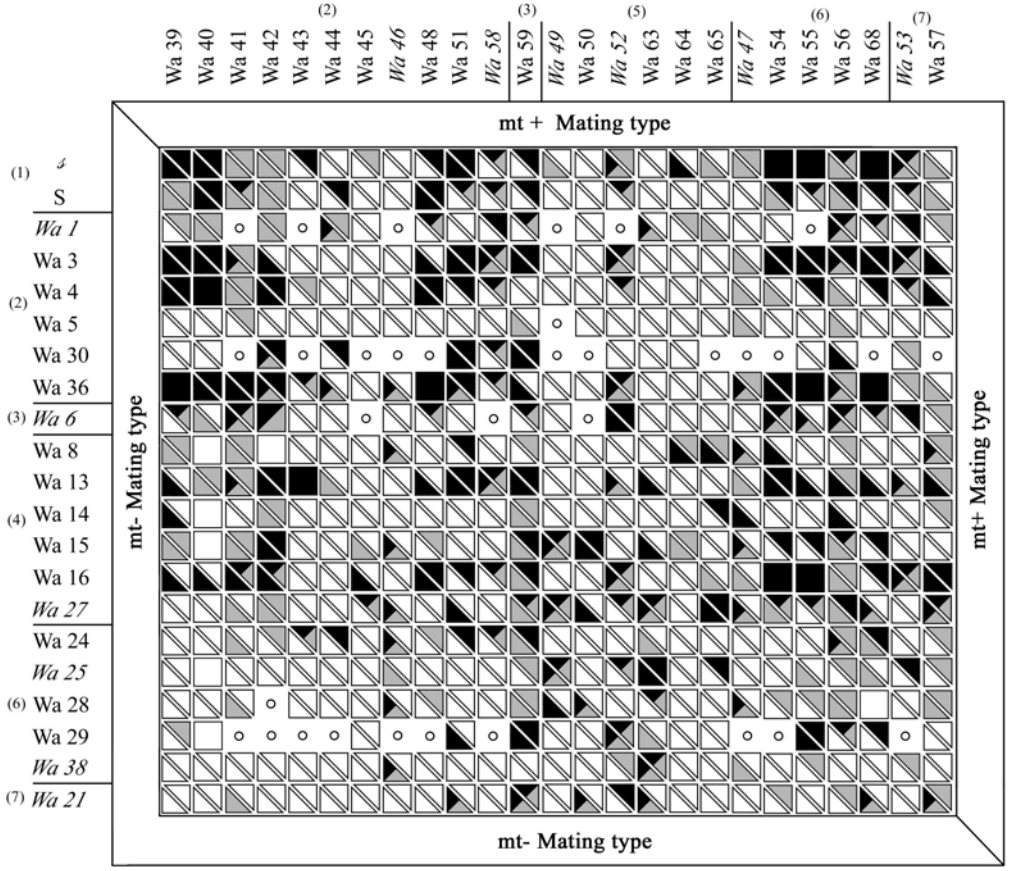


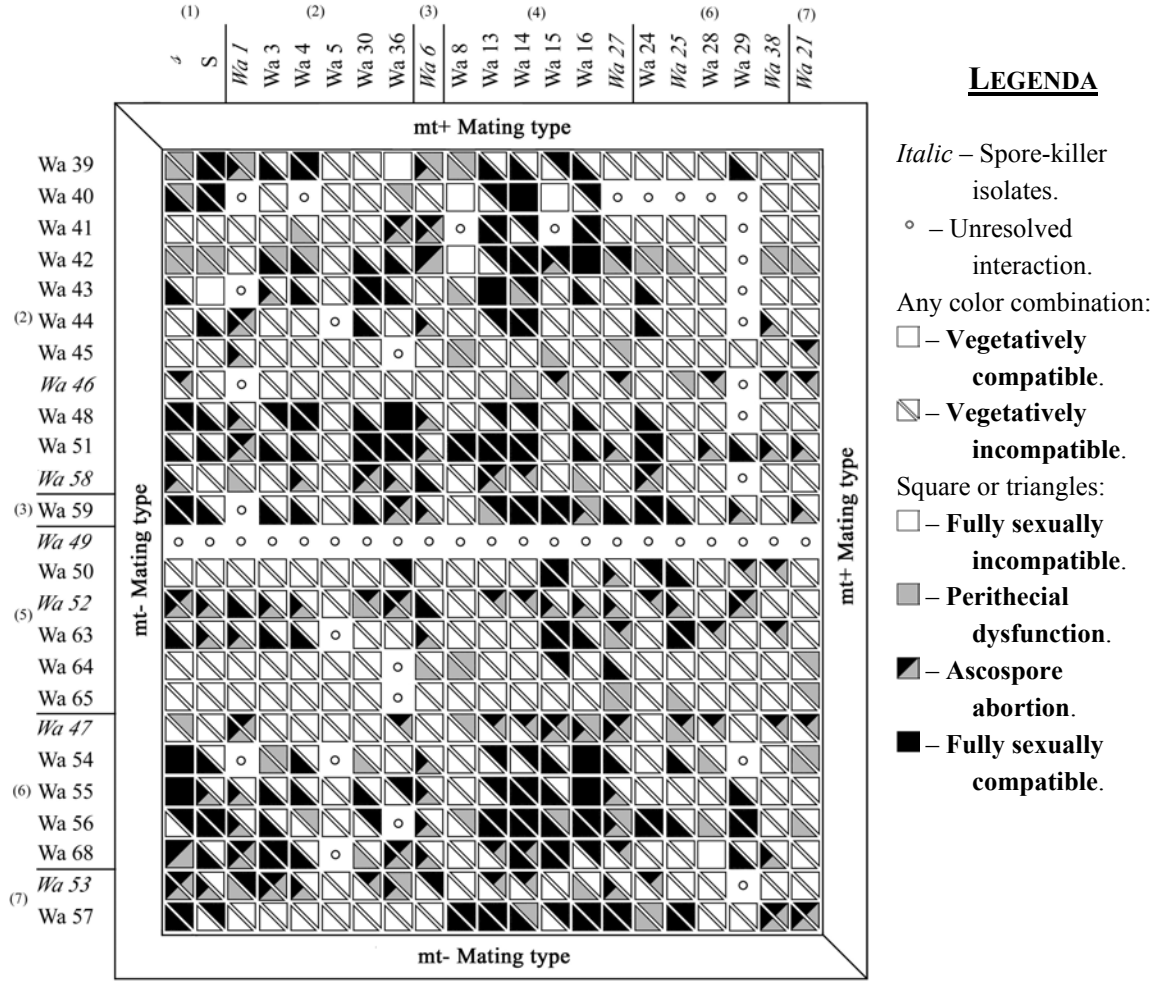
FIGURE 6.2C. -- Vegetative incompatibility and mating reactions between wild-type monokaryons of *P. anserina* : *Interactions between strains isolated in 1991-1993 and strains isolated in 1994.*



tested. A large proportion of strain combinations also suffer from ascospore abortion, though most can be attributed to Spore killers (*italic* strain names in Figure 6.2). The total percentage of sexual compatibility (including ascospore abortion) is slightly higher in the 19 wild-type strains of Esser (Table 6.1). However, our findings are probably underestimations as *Podospora anserina* strains cross better on dung, their natural substrate (Esser 1974, Chapter 3, van der Gaag 2000).

Some interesting observations could be made: The cross Wa68 x Wa50 is vegetatively compatible and sexually incompatible (Figures 6.1 and 6.2). This result cannot be explained by the sexual compatibility model proposed by Esser and Blaich (1994). Also, a sexual (semi-) compatibility result as shown, for example, by the three strain combinations of Wa1, Wa23 and compatibility result as shown, for example, by the three strain combinations of Wa1, Wa23 and Wa36 or Wa13, Wa27 and Wa38 (Figure 6.2) cannot be explained by combinations of the non-allelic incompatibility genes. According to the current non-allelic model (Bernet 1967, Esser 1971, Esser and Blaich 1994), sexual semi-incompatible combinations of strain X with Y and X with Z, where perithecia are formed on respectively side Y and X of the 'barrage', results in a sexually incompatible reaction between Y with Z.

FIGURE 6.2D. -- Vegetative incompatibility and mating reactions between wild-type monokaryons of *P. anserina* : *The reciprocal interactions of Figure 6.2C.*



However, we observed that all combinations resulted in the formation of perithecia on at least one side of the 'barrage'.

In order to establish whether our wild-type collection consisted of several subpopulations we compared the percentages of vegetative and sexual compatibility within and between sampling areas. No significant difference (χ^2 –test, $p>0.1$) could be found between the (grouped) sexual compatibility reactions within and between subpopulations of the Wageningen population. Only for vegetative compatibility a small difference in percentages ($p<0.001$) could be found (Figure 6.2, Table 6.2). The total view suggests that the Wageningen *P. anserina* population can be seen as a single population.

Evidence for outcrossing in nature:

Our data on sexual and vegetative compatibility can be used to obtain insight into the occurrence of selfing and outcrossing in *P. anserina* in two ways. First, by estimating the number of VCG's and comparing them with the maximum diversity of vegetative combination types under the current model of nine vegetative incompatibility loci (Saupe 2000). The diversity of VCG's in a population is a function of allelic diversity and

recombination among *het*-loci. Because ascomycetes are haploid, 2^k multilocus genotypes are possible, given two alleles at each of k polymorphic unlinked *het*-loci. Therefore potential genotypic diversity increases as k and allelic diversity increases (Milgroom and Cortesi 1999). Although nine *het*-loci are involved in vegetative incompatibility in *P. anserina* and three of the non-allelic *het*-loci contain multiple alleles (four for *het-c* and *het-e,* three for *het-d*), the maximum number of VCG's is far lower than expected on the basis of this number of loci and alleles. *Het-c/het-d* and *het-c/het-e* are self-lethal in half their allelic combinations and only the non-lethal combinations can exist within an isolate (Saupe *et al.* 1994). The remaining 23 non-lethal *het-c/het-d/het-e* strains give in more than half of their combined monokaryotic allelic combinations a vegetatively compatible reaction. However, each *het-c/het-d/het-e* strain can be compatibly linked to any other combination with help of another *het-c/het-d/het-e* strain. Therefore all will group to the same VCG. The other six *het*-loci account for 2^6 genotypes, resulting in a maximum of 64 possible VCG's.

Within our sample of 57 strains we found 33 VCG's. Isolates were assigned to a VCG if they were compatible with at least one other strain of this VCG group. To estimate the total

Table 6.2
Percentage of compatibility interactions within and between subpopulations of *P. anserina* strains.

Interaction type	Within Wageningen sampling areas	Between Wageningen sampling areas	Between Wageningen and Normandy [a]	Total of all areas [b]
Vegetative compatible	5.6 % (29) [g]	2.6 % (45) [g]	3.2 % (7)	3.2 % (81)
Full sexual compatible [c]	27.2 % (141)	23.5 % (404)	47.7 % (104)	26.4 % (1162)
Ascospore abortion [d]	20.4 % (106)	21.6 % (371)	15.6 % (34)	20.9 % (513)
Perithecial dysfunction [e]	12.9 % (67)	10.9 % (187)	13.8 % (30)	11.6 % (284)
Full sexual incommpatible	39.5 % (205)	44.0 % (757)	22.9 % (50)	41.2 % (1012)
Sexual semi-incompatible [f]	26.3 % (137)	27.8 % (479)	34.9 % (76)	28.2 % (692)

[a] Crosses between the Wageningen isolates (1991-1994) and strain S and s from Normandy (1937).

[b] The total of the complete population, excluding the 57 selfing combinations. The number of strain combinations is shown in brackets.

[c] A full sexual compatible reaction is counted on both sides of a 'barrage' in a vegetatively incompatible reaction. Also included is the portion that shows full sexual compatibility on one side and ascospore abortion (total 20 combinations), perithecial dysfunction (total 42 combinations) or full sexual incompatibility (total 302 combinations) on the other side of the 'barrage'.

[d] An ascospore abortion reaction is counted on both sides of a 'barrage' in a vegetatively incompatible reaction. Also included is the portion that shows ascospore abortion on one side and perithecial dysfunction (total 36 combinations) or full sexual incompatibility (total 312 combinations) on the other side of the 'barrage'.

[e] A perithecial dysfunction reaction is counted on both sides of a 'barrage' in a vegetatively incompatible reaction. Also included is the portion that shows perithecial dysfunction on one side and full sexual incompatibility (total 157 combinations) on the other side of the 'barrage'.

[f] Only the semi-incompatible interactions that produce ascospores are counted, including those showing ascospore abortion.

[g] χ^2-tests were performed between and within Wageningen subpopulations. Only significant different ($p<0.001$) combinations are noted.

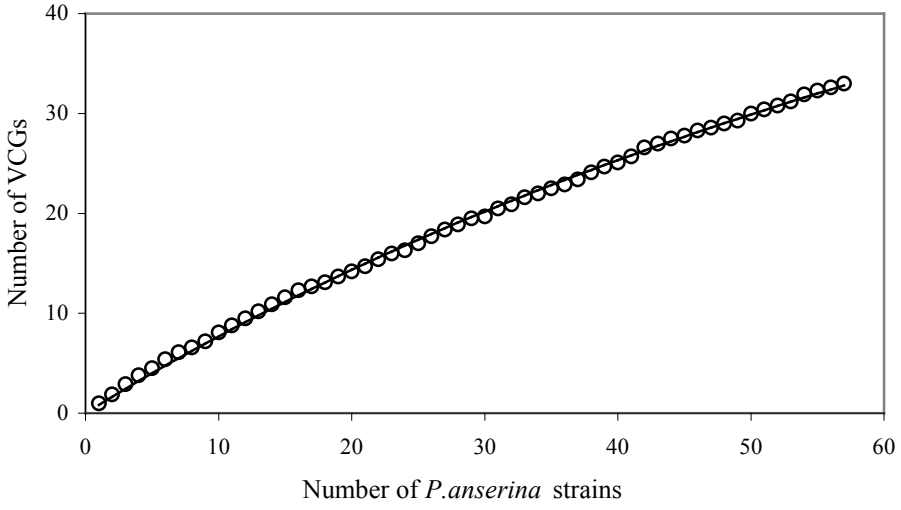
number of vegetative compatibility groups (VCG's) in our population, we used an accumulation curve where the cumulative number of VCG's were plotted against the (mean of our ten times) randomized number of sampled strains (Figure 6.3, Hughes *et al.* 2001). Using the parabolic shape of the accumulation curve we can determine the total amount of VCG's and see as well how our sample represents the total population. Curve extrapolation using the Michealis-Menten equation (Soberon and Llorente 1993) estimates an asymptote of 107 VCG's ($R^2 > 0.999$), which is more than the complete diversity of vegetative incompatibility combinations. The number of VCG's can be estimated as well by the non-parametric Chao1 formula (Hughes *et al.* 2001), resulting in 75 VCG's. However, Chao1 is known to underestimate in small samples, such as ours. The small size of our sample is also illustrated by the number of VCG's found, which is half of all possible VCG's.

Secondly, by using evidence of intrastrain variability (Merino *et al.* 1996). We measured intrastrain variability by comparing the strain interactions of the reciprocal matings. The occurrence of an outcrossing event could result in heterokaryotic offspring for the vegetative and sexual incompatibility alleles. Thus, monokaryotic ascospores of the same isolate can differ from each other for these loci in case of a recent outcrossing history. On the other hand recurrent selfing would lead to isogenic (monokaryotic) ascospores. If both monokaryotic mating types of a strain are identical for vegetative and sexual incompatibility loci, they must mirror their results along the mating type diagonal in Figure 6.2. However, we have found that more than 44 percent of the 1186 reciprocal combinations show a different reaction for sexual or vegetative compatibility. For example the combinations $Wa30^+$ x $Wa36^-$ and $Wa30^-$ x $Wa36^+$, or strains $Wa30^+$ x $Wa35^-$ and $Wa30^-$ x $Wa35^+$ do not show the same interaction. Differences in ascospore abortion were not incorporated into this percentage.

Thus, our findings strongly suggest that outcrossing occurs in a natural population of *P. anserina*. During our sampling from nature we once encountered a perithecium containing

FIGURE 6.3. -- Accumulation curve and fitting of estimation model of Vegetative Compatibility Groups (VCGs) in 57 strains of *P. anserina*.

The accumulation curve (open dots) is averaged among 10 random samplings of strains. The Michealis-Menten estimation model is shown as a closed line.



asci with aborted ascospores, suggesting that outcrossing had occured. Since the str ain derived from a two-spored ascus of this perithecium (Wa35) contains the *het-s* allele, but no *Psk* Spore-killer alleles, ascospore abortion can likely be attributed to the prion type of meiotic drive (Dalstra *et al.* 2003) during outcrossing.

Experimental outcrossing in *Podospora*:

Podospora produces single mating-type ascospores in relatively low frequencies, either as monokaryotic spores or by first division segregation of mating type. We have once sampled such a self-sterile isolate directly from nature (Wa12*, mat*+, *Psk-1*), which also happened to be a Spore killer. These single mating-type spores produce cultures that depend upon outcrossing to complete their sexual cycle and produce offspring. We performed experiments to examine the possibilities and circumstances for outcrossing in *Podospora.*

Rizet and Engelmann (1949) noted that strains differed in percentages of (single mating-type) monokaryotic spores produced. Therefore we first checked the frequencies of monokaryotic spores of several strains. A larger proportion of monokaryotic spores would mean a greater dependence upon other strains. This is especially interesting for Spore-killer isolates, since meiotic drive systems rely on outcrossing. We found varying percentages of monokaryotic spores among our strains, ranging from almost zero to 5.8% in strain Wa38 (Table 6.3). Based upon these strain observations, Spore-killer strains tend to make a significantly ($p < 0.001$) higher percentage of monokaryotic spores.

Next, we looked whether isogenic spermatia have an advantage to cross with monokaryotic cultures or not. We tested isogenic and non-isogenic vegetative compatible and incompatible combinations. The idea is that in isogenic crosses no conflict will arise from genetic heterozygosity. Furthermore, vegetatively compatible spermatia could be able to fuse with the mycelium as found in *Neurospora* (Debets and Griffiths 1998), whereas vegetative incompatible spermatia are not. Then, (isogenic) compatible spermatia would be able to

Table 6.3
Percentage of monokaryotic ascospores of *P. anserina* strains.

P.anserina strain	Monokaryotic ascospores	Total number of ascospores	χ^2-group[a]
136	2.6 %	538	a
Wa5	3.1 %	1580	
Wa15	0.2 %	3867	
Wa30	1.9 %	1394	
Wa6 (*Psk-1*)	1.6 %	2249	b
Wa52 (*Psk-1*)	4.8 %	951	
Wa38 (*Psk-2*)	5.8 %	1354	c
Wa28 (*Psk-2*)	5.0 %	1547	

[a] χ^2 –test performed between *non-Psk*, *Psk-1* and *Psk-2* groups. Results for each group were pooled. Noted groups are significantly different at $p<0.001$.

produce perithecia themselves (Debets and Griffiths 1998) or block fertilization by trichogynes with a nonresponse as found for double mating type mycelium in *N. tetrasperma* (Bistis 1996). Table 6.4 shows that the total percentages outcrossing found are close to 50%, indicating nopreference for outcrossing or selfing with isogenic and non-isogenic vegetatively compatible and incompatible microconidia. This can indicate that a nonresponse for (newly formed) double mating-type strains, as assumed in *N. tetrasperma*, does not exist in *Podospora*. Intriguing however, are the single mating-type differences within the strain combinations of Wa52/Wa54. Here the combination of $Wa52^{-}$ x $Wa54^{+}$ is exceptionally more successful, while the other mating types clearly prefer selfing (Table 6.4). This cannot be attributed to an uneven distribution of mating types in the microconidia mix, since mixtures were derived from a dikaryotic strain containing both mating types. Furthermore, the same spermatia solutions of each strain were used to make the different combinations, and the same difference between mating types cannot be seen in the other combinations. It is possible that both these strains are heterozygous (see also Figure 6.2) and therefore specific combinations of strains and mating-types are more successful.

While the relatively sparse single mating-type cultures need fertilization by other strains, the majority of *Podospora* cultures in nature are double mating type. They do not need outcrossing to produce their progeny, and as suggested by experiments in *Neurospora tetrasperma* (Bistis 1996), may not even be capable of fertilization by others. We performed experiments to find out whether dikaryotic strains of *Podospora* are able to perform outcrossing, and applied non-isogenic microconidia onto double mating-type mycelium. Our results (Table 6.5, microconidia data) clearly show that outcrossing is possible, even with vegetatively incompatible strain combinations. Only a low percentage of outcrossing is found, which can be due to a higher proportion of microconidia of the selfing strain. It is not possible to measure the amount of microconidia on the selfing strain, and accordingly adjust the outcrossing microconidia to equal amounts.

Table 6.4

Percentage of outcrossed perithecia on monokaryotic *P. anserina* cultures between vegetative compatible and incompatible strain combinations.

Maternal	Percentage outcrossing with microconidia mix			
P. anserina strain	Wa52/Wa54 [a,c]	Wa52/136 [a,c]	Wa54/136 [b,c]	Total [c]
Wa52+ *(Psk1)*	32.1 % (524)	45.6 % (579)		39.2 % (1103)
Wa52- *(Psk1)*	78.4 % (533)	42.7 % (575)		59.9 % (1108)
Total	55.4 % (1057)	44.2 % (1154)		49.6 % (2211)
136+		55.3 % (526)	63.1 % (528)	59.2 % (1054)
136-		42.1 % (733)	62.3 % (529)	50.8 % (1262)
Total		47.8 % (1259)	62.7 % (1057)	54.6 % (2316)
Wa54+	74.1 % (502)		40.5 % (237)	63.3 % (739)
Wa54-	35.3 % (433)		71.2 % (146)	44.4 % (579)
Total	56.1 % (935)		52.2 % (383)	55.0 % (1318)

[a] Vegetative incompatible combination of *P. anserina* strains.

[b] Vegetative compatible combination of *P. anserina* strains.

[c] The total number of perithecia examined per combination of strains is shown in brackets.

Table 6.5
Percentage of selfed and outcrossed perithecia on mixtures of dikaryotic *P. anserina* strains.

P. anserina strain A	*P. anserina* strain B	Percentage of perithecia			Total number of perithecia
		Selfing strain A	Selfing strain B	Outcrossing	
Microconidia B on strain A					
Wa52	Wa54	97.2 %	-	2.8 %	1558
Wa54	Wa52	94.9 %	-	6.1 %	1739
Wa3	Wa6	99 %	-	1 %	100
Ascospore mixture on cornmeal					
136 (green spores)	Wa6 (*Psk-1*)	40.7 %	57.6 %	1.7 %	578
	XS-Wa6 (*Psk-1*)	84.5 %	14.9 %	0.6 %	679
	Wa28 (*Psk-2*)	49.4 %	50.1 %	0.5 %	441
Ascospore mixture on dung					
Wa6 (*Psk-1*)	S	95.8 % [a]		4.2 %	72
	Wa15	100 %		0 %	33
	Wa28 (*Psk-2*)	98 %		2 %	51
	Wa30	100 %		0 %	43
Wa28 (*Psk-2*)	S	100 %		0 %	42
	Wa15	98 %		2 %	50
	Wa30	100 %		0 %	10
Mycelium mixture on cornmeal					
136	XS-Wa6	11.4 %	43.7 %	44.9 %	167

[a] No distinction can be made between selfings of strain A and strain B.

Having asserted that it is possible for both single and double mating-type strain to perform outcrossing we tried experimentally to assess the occurrence of outcrossing in an unforced situation of two strains. This was done using ascospore mixtures (containing the natural amount of single mating-type strains) and a mycelium mixture. Both experiments with ascospore mixtures (Table 6.5, ascospores on cornmeal and on dung) displayed outcrossing percentages ranging from zero and 4.2 percent. Three of the cases without outcrossing (Wa6/Wa15, Wa28/S and Wa28/Wa30) can probably be attributed to sexual incompatibility (see Figure 6.2; although the combination of Wa28 and Wa6 does show outcrossing, despite sexual incompatibility in the initial survey). Otherwise the small sampling rate on dung could have caused missing any outcrossed perithecia. Nevertheless, the ascospore experiments prove clearly that outcrossing can occur in an unmanipulated natural (dung) habitat. The mycelium experiment shows the remarkable high rate of outcrossing (45 %). The Petri dishes did show a close patchwork of green (strain *136*) and grey (strain XS-Wa6) cultures, enabling much contact between trychogynes and spermatia of both cultures. This experiment shows that extensive contact between and mixing of mycelial colonies increases the amount of outcrossing.

DISCUSSION

Can the estimated number of VCG's can be explained by the currently known *het*-genes?

Vegetative compatibility systems have been used to investigate genetic variation and population dynamics in fungi, both genetically and molecularly (Milgroom 1996, Milgroom and Cortesi 1999, Powell *et al.* 2001). We used our vegetative and sexual compatibility survey to examine genetic variation and population dynamics in *Podospora anserina*. Genetic diversity can be estimated from samples taken from nature and several methods are available for microbial organisms (Hughes *et al.* 2001). We have used two different methods to estimate the total of VCG's in our population. The first method is based upon the extrapolation of an accumulation curve and estimates 107 VCG's. Several equations can produce the same accumulation curve, but we found that the Michealis-Menten equation statistically best fitted our accumulation curve. This equation corresponds to the situation in *P. anserina* with a defined number of VCG's, describing a situation where the probability of adding a new VCG eventually vanishes, but increases with more sampling (Soberon and Llorente 1993). The Chao1 method, a non-parametric estimator, calculates 75 VCG's. Non-parametric estimators consider the proportion of VCG's that have been recaptured to those that have been observed only once. The consequence of this strategy is that in a small sample, such as our *Podospora* population, the number of VCG's will be underestimated (Hughes *et al.* 2001).

Both VCG estimates are higher than the maximum number of 64 VCG's, provided by the currently known alleles of the nine vegetative incompatibility loci. However it is probable that the estimated number of VCG's is an overrepresentation. Small samples will miss certain *het-c/het-d/het-e* combinations that would link several VCG groups together. Therefore small samples might show more VCG's than actually exist. Also the absence of some alleles of the non-allelic *het-c*, *het-d* or *het-e* loci in the population likewise results in more VCG's. If this is so the currently known *het*-genes can still account for the number of VCG's. On the other hand, drift as described in the sexual model of Nauta and Hoekstra (1996) may cause the number of VCG's in a population to be lower than the maximum number. Nauta and Hoekstra (1996) found that the number of VCG's in a population largely depends on population size, mutation rate (*i.e.* the appearance of new vegetative compatibility alleles) and frequency of sexual reproduction. Low amounts of sex (10-20%) compared to no sex sharply increase the number of VCG's for a given population size and mutation rate. If *P. anserina* is a strictly selfing fungus, it will need a considerable larger population size to reach the same number of VCG's. Population sizes are, contrary to VCG's, very difficult to measure. Figure 6.2 shows that no isolate behaves identical in both vegetative and sexual compatibility, determining them all to be different individuals and thus making the population size 'unestimatable'. Moreover the currently known *het*-loci would probably not be sufficient to produce our estimated number of VCG's, as only specific *het*-c, *het-d* and *het-e* alleles are needed in combinations with the other *het*-loci. There are however indications that more *het*-loci are present in *P. anserina,* as a *Neurospora het-c* homolog has been identified recently (Saupe

2000). It is unlikely that convergent evolution would have taken place for all *het*-alleles. The finding of identical *het*-alleles in diverging lines is usually seen as proof of outcrossing (Powell *et al.* 2001, Saupe 2000).

Sexual incompatibility in *P. anserina* not exclusively determined by non-allelic vegetative incompatibility:

We have found several results in our survey that cannot be explained by the current sexual compatibility dogma as posed by Esser and Blaich (1994). They state that "sexual incompatibility in interracial crosses is always linked to (non-allelic) vegetative incompatibility". Our results show several strain combinations that are both vegetatively compatible and sexually incompatible. Also some of the combinations of three strains do not fit in the current model of non-allelic *het*-genes (Bernet 1967, Esser 1971, Esser and Blaich 1994). Our results imply that other, non-*het*-genes are involved in sexual compatibility. For instance, sex pheromones that regulate mate recognition in ascomycetes may be involved (Debuchy 1999, Casselton 2002). A change in receptor or pheromone could lead to non-recognition and subsequently failure to produce ascospores. Gaseous pheromones produced by perithecia have been implicated to suppress perithecium development in several *Neurospora* species (Metzenberg 1993, Peleg *et al.* 1996). The setup of our survey was such that several strains were tested together for sexual compatibility. If an inhibitory effect of gas also exists in our survey, it would mean that the percentage of sexual compatibility is probably larger than we have measured, making a successful outcrossing even more likely. The perithecial dysfunction category in our survey is probably the most likely category to be affected by gas inhibition.

Sexual compatibility in *P. anserina*:

We have seen that *P. anserina* is capable of producing sexual offspring between isolates of different origin (our work, Bernet 1965, Esser and Blaich 1994). In fact, every strain can eventually cross with the other, making use of other strains as in between crosses. We did not find any differences in the four types of sexual compatibiltity interactions (Table 6.2) between sampling areas and must regard our strains as belonging to one population. Although our total sampling area is limited to a few square kilometers, results from more geographical distant isolates (originating from places across France and Germany) are similar (Table 6.1, Bernet 1965, Esser and Blaich 1994), suggesting one large geographical population. This contrasts to *Neurospora tetrasperma* where subpopulations can be ascribed to their geographic origin, although not enough to assign them to intersterility groups (Jacobson 1995).

We divided the results of our sexual compatibility survey into four categories to be able to compare them to the *N. tetrasperma* categories of reproductive success (Jacobson 1995). Although both species have pseudohomothallic lifecycle, their sexual compatibility results are highly dissimilar. Our **full sexual compatibility** group corresponds to his category 1-4, as they all produce black ascospores. Around 44% of *N. tetrasperma* combinations are sexually compatible, compared to 26% in *Podospora* (both full and semi-compatible, Table 6.2). Remarkably, the percentage of sexual compatibility is slightly higher (48%) between the

Wageningen strains and strain S/s from Normandy than in *N. tetrasperma* (Table 6.2). The **ascospore abortion** group (21%, Table 6.2) corresponds to category 5-7 and consists of 43% of the *Neurospora* strain combinations. The proportion of these *Neurospora* categories is much larger than our *Podospora* group. An important difference is that the largesse of abortion in *P. anserina* is due to Spore killers (Figure 6.2, italic isolates), whereas abortion in *N. tetrasperma* is not connected to meiotic drive elements (D. J. Jacobson, personal communication), which thus far have not been found in this fungus (Raju 1996, Turner 2001). Therefore most of the *Podospora* abortion category should be seen as sexual compatible. Group 7 (21 %) is the only category where *N. tetrasperma* is involved in sexual semi-incompatibility as well as ascospore abortion. Sexual semi-incompatibility is a common phenomenon in *Podospora* ascribed to non-allelic *het*-genes (28%, Table 6.2) and unrelated to ascospore abortion. Our **perithecial dysfunction** category does not have a separate class in *N. tetrasperma* (Jacobson 1995) and should be combined with the incompatible category (total 53%) and compared to the **full sexual incompatible** group (14%), which is notably lower. The differences in sexual compatibility of both ascomycetes will have an influence to their outcrossing potential.

We do not know the extent of inviability of the produced offspring of our crosses, nor of their capability to reproduce, as these traits were not tested. If an ascospore contains nuclei with conflicting *het*-alleles, it will eventually abort. Some combinations of *het*-genes are known to cause lysis a few hours after ascospore germination (Saupe 2000). Also incompatible combinations are found to split into monokaryotic entities, which rely on outcrossing to reproduce. We have estimated a considerable amount of VCG's in our population. This can have implications on the viability of spores, as it is likely that parents in an outcrossing situation differ from each other in several *het*-genes. However, *het*-genes do not have to segregate in such a way as to produce inviable combinations. If both nuclei of the ascospore remain homokaryotic for their *het*-genes, viable ascospores are produced. In other words if *het*-genes are located close to the centromere, outcrossing will be less problematic. In fact most of the *het*-genes have SDS percentages ranging from 4 to 18 % (Marcou *et al.* 1990, Esser 1974), which makes percentage of inviable ascospores due to vegetative incompatibility conflicts in a cross relatively low. This is illustrated by the fact that all our crosses involving the analysis of Spore-killer strains produced viable reproducing ascospores (Chapter 3, van der Gaag *et al.* 2000).

Meiotic drive elements increase outcrossing necessity:

The ability to produce sexual offspring is a prerequisite for outcrossing to occur, whereas outcrossing is a prerequisite for the occurrence of meiotic drive. Monokaryotic ascospore percentages are known to vary, but average around 1-2% (Rizet and Engelmann 1949). We observed that the percentage of monokaryotic ascospores is significantly higher in *Psk-1* and even more in *Psk-2* spore-killer strains than in sensitive strains. As monokaryotic strains are dependent upon other strains to produce offspring, this increases the potential for meiotic drive. Our result suggests that some traits involved in ascospore delimitation are linked to the Spore-killer elements.

Heterokaryotic strains of *P. anserina* are capable of outcrossing in nature:

We have demonstrated that self-fertile dikaryotic strains, containing both mating types, can act as a maternal parent to other isolates (Table 6.5). Not only did we find outcrossing experimentally, but also in an unmanipulated situation where an ascospores mixture was applied on dung. This contrasts with previous findings in *Podospora* and observations on *N. tetrasperma.* Rizet and Engelmann (1949) observed crosses of heterokaryotic versus monokaryotic strains in *P. anserina* and could only find outcrossed perithecia on the monokaryotic tissue. However, their technique of mycelial confrontation greatly reduces chances of finding outcrossed perithecia within a wealth of selfed fruiting bodies. Bistis (1996) could not detect fusion of trichogyne from dikaryotic *N. tetrasperma* with spermatia of other strains. He concluded that the fertilizing path of trichogyne and spermatia was blocked in heterokaryotic strains and only monokaryotic strains were able to use this path. However, he remarked that already (selfed) perithecia were developing on the strains he used for testing. Developing perithecia of *Neurospora* were found to excrete a gas that inhibits the development of new perithecia (Metzenberg 1993, Peleg *et al.* 1996). Therefore the nonresponse can likely be attributed to this emitted gas, and failure of fertilization may just have been a matter of ill timing.

The absence of a nonresponse for trichogynes in *Podospora* is also illustrated by our experiment of adding compatible and incompatible spermatia mixtures to a monokarytic parent (Table 6.4). No perithecial gas emission could intervene, as the maternal parent is single mating type. If the trichogyne were blocked in dikaryotic strains, then the incompatible mixture would be at a disadvantage. Fusion of the monokaryotic strain with an isogenic strain of the opposite mating type will produce a dikaryotic strain, revoking the trichogyne route to the incompatible strain. We did not find a conclusive preference for either strain in the mixtures, indicating the trichogyne route is still open and the nonresponse does not exist *Podospora* or blocking is minimal because of limited nuclear migration. Dodge (1936) observed outcrossing based nuclear migration in *P. anserina*, where monokaryotic cultures were invaded by non-spermatia forming cultures. Outcrossing following nuclear migration has also been found in *N. tetrasperma* and other ascomycetes (Dowding and Bakerspiel 1954, Lee and Taylor 1993). Nuclear migration is resticted to vegetative compatible combinations, as nuclear mixing is followed by lysis in incompatible combinations. Meiotic drive elements found by us remained incompatible to sensitive strains even after extensive backcrosses with these sensitive strains (Chapter 4, van der Gaag *et al.* 2003).

Outcrossing regularly occurs in natural populations of *Podospora anserina:*

We demonstrated that *P. anserina* strains could mate with other strains, regardless if they are mono- or heterokaryotic. Also we showed that outcrossing occurs in the natural habitat of *Podospora*, ascospores on dung, at relatively low frequencies. Further evidence for regular outcrossing is found in our survey on vegetative and sexual compatibility. Our estimated number of Vegetative Compatibility Groups exceeds the maximum number of VCG's, which implies that all combinations of the vegetative incompatibility loci and alleles

are needed to produce the estimated number of VCG's. All possible combinations of *het*-alleles are not expected to occur in a strictly selfing population, therefore outcrossing must take place. The estimated number of VCG's can only be explained in a selfing situation if more *het*-genes or alleles are present in *Podospora* than currently known. The theoretical model of vegetative incompatibility in ascomycetes of Nauta and Hoekstra (1996) shows as well that in a selfing population only a fraction of all VCG's is found. The number of VCG's present in a population only increases for very large population sizes. The population size of *Podospora* is hard to measure and fluctuates throughout the year due to environmental conditions. Another hint of outcrossing is the large percentage of intrastrain variability in the different sexual compatibility reactions between the mating types of the same isolate. Such a difference could point to heterozygosity of sexual compatibility related genes in the (dikaryotic) isolate, originating from a previous outcrossing event. Merino *et al.* (1996) attempted a RFLP-based approach to answer outcrossing rate in *N. tetrasperma,* but did not find much intrastrain variability for non-mating-type linkage groups. They concluded that *N. tetrasperma* is mainly selfing or crosses with close relatives. The large percentage of different compatibility reactions we found in *Podospora* points to the opposite; a regular occurrence of outcrossing. Differences in outcrossing behavior between the two species may be related to the ecological niche which they occupy. *Podospora* is a coprophilous fungus, of which the ascospores are sampled over a large area by passing herbivores and deposited in their dung. Chances of encountering a different individual within the substrate are therefore likely to occur. Adapting a strategy that also incorporates a successful outcrossing event, might help an individual to reproduce under limited resources for selfing. *Neurospora tetrasperma* on the other hand is a soil fungus, dependent on burned plant material as substrate (Davis 2000), and most likely encounters only nearby and (near-) identical individuals. Ascospores produced by (outcrossing) single mating type colonies of *N. tetrasperma* will then resemble selfed offspring. Incorporating regular outcrossing is not a necessity for this fungus. Further proof of outcrossing in *Podospora* could be obtained on VCG frequencies, which require larger population samples (Milgroom 1993, Milgroom and Cortesi 1999) or by using comparative molecular phylogenic studies on, for instance, *het*-gene sequences (Burt *et al.* 1996, Geiser *et al.* 1998).

Since every wild-type isolate proved viable in a selfing reaction (in order to create the single mating-type strains), sexual dysfunction does not seem to affect strains severely in the aftermath of an outcrossing event. Combined with the enormous potential for outcrossing in dikaryotic strains, an increased percentage of monokaryotic strains and small chances of interfering *het*-genes in ascospore viability, meiotic drive elements in *Podospora* have ample opportunity to execute their segregation distortion mechanism.

ACKNOWLEDGEMENTS

We would like to thank Marijke Slakhorst, Marcel Velner and Jarco Smeenk for their practical assistance, and Arjan de Visser for assistance with the extrapolation models.

Chapter 7

General Discussion

The Wageningen population of *P. anserina*.

Natural populations provide useful information for the study of the dynamics and evolution of selfish elements. The previous Chapters have described experiments on a natural population of *Podospora anserina*. We have sampled this ascomycete fungus around Wageningen, The Netherlands for six years (1991-1997), of which four years extensively. This resulted in a sample of more than 100 strains (Chapter 2, Chapter 3, Chapter 6). Isolation from dung samples, however, proved very laborious and our intended goal to follow the spread of selfish elements during a year could not be reached. Nevertheless, our data give a good view of the occurrence through several years, and make comparisons with other geographical areas possible. Considering the distribution among the sampling areas of the several traits studied in this thesis (sexual and vegetative compatibility in Chapter 6, segregation distorters in Chapter3 and linear plasmids in Chapter 2) the Wageningen isolates can be considered as belonging to one large population. Also the life-history of *Podospora anserina* supports the idea that the Wageningen population does not consist of several micropopulations. The dispersal mechanism and subsequent uptake of ascospores by foraging herbivores ensures mixing of different fungal individuals and placing them in a new geographically separated dung environment. Spread by flying herbivores, such as geese can distribute fungal ascospores over a very large area, which also increases possibilities for spread of (efficient) selfish elements. *Podospora anserina* isolates used in other population studies consist of few strains isolated from different regions, including some of unknown origin (Belcour *et al.* 1990, see Table 7.1 for an overview).

What fraction our population represents of the complete natural population of Wageningen is hard to measure. No difficulty is raised with multiple sampling of the same individual (clone), as *Podospora* produces only sexual ascospore offspring, and its lifecycle is restricted to the dung dropping it grows on (Rayner 1991). However continuous selfing would in principal lead to a clonal-like population, consisting of genetically identical fungal individuals (genets). On the other hand, selfing of a heterokaryotic (outcrossed) individual would keep on producing heterokaryotic offspring for several generations. In general, two sorts of fungal individuals make up the *Podospora* population.

Table 7.1
Other population studies in *Podospora anserina*

Studied trait	Strains [a]	Polymorphic	Reference
Spore killing, Sexual compatibility, Vegetative compatibility	D,E,F,H,M,O,s,S,W,*T*, He,Us1-8,Wü, Wa2,Wa3,Wa28,Wa58	Yes	Hamann and Osiewacz 2004
Mitochondria	A,B,C,D,E,F,H,M,S,U,V,W,X,Y,Z, *T.*	10 +*1* groups	Belcour *et al.* 1997
GT-Minisatellites	A,D,E,F,H,M,N,O,s,S,U,W,*T* He,Wü, Wa2,Wa3,Wa6	5 + *1* groups	Hamann *et al.* 1998
GT-Microsatellites	D,E,O,s, Wa2,Wa3,Wa6	No	Osiewacz *et al.* 1996
pat-Transposon	A,D,E,F,H,M,N,O,s,S,U,*T* He,Wü, Wa3,Wa6	No	Hamann *et al.* 2000a
yeti-Transposon	A,D,E,H,M,O,s,U,*T* He, Wa2	Yes	Hamann *et al.* 2000b
repa Element	A,B,D,E,H,N,R,s,S,U	Yes	Deleu *et al.* 1990
het-s	A,C,D,H,s,S,T,U,V,X	3 + *1* groups	Deleu *et al.* 1993
Sexual compatibility, Vegetative compatibility	A,B,C,D,E,F,G,H,K,L,M,N,s,S	Yes	Bernet 1965
Sexual compatibility, Vegetative compatibility	A,B,C,D,E,F,H,M,N,O,R,s,S,*T*,U,W He,Mü,Wü	Yes	Esser and Blaich 1994
Lifespan	A,B,C,D,E,F,G,H,M,N,S	Yes	Marcou 1961
Lifespan	A,E,N,s,S	Yes	Smith and Rubenstein 1973

[a] Strains are respectively from France (1937, single letter), Germany (double letters) and Netherlands (Wa-numbers). *P. comata* strains are given in italics.

1. The active organisms. For the coprophilous fungus *Podospora anserina* an active individual is most likely a colony (on dung) grown from a single ascospore. Dung is a discrete source of limited nutrients, limited size and isolated from other sources.
2. The passive organisms. All ascospores that are ejected on the field can be considered as the (gene) pool of potential individuals from which herbivores sample the next *Podospora* generation. This means that some ascospores could lie dormant for several years before being able to germinate.

We can only sample (the active organisms) from dung, as dung can sample from the passive organisms through herbivore consumption. Our isolates were considered ‘different’

individuals based on genetic (vegetative compatibility) and non-genetic criteria (year and place of isolation, or dung source (either not the same dropping or dung originating from another herbivore species)). More than one genetically different isolate has been sampled from the same dung source. VCG analysis (Chapter 6) shows that the maximum predicted number of groups (107 or 75) has not been reached by our sampling (33 groups). Our population represents only a third of the vegetative compatibility groups. Considering other genetic traits as well, such as lifespan (Chapter 2), sexual compatibility (Chapter 6) and presence of selfish elements (Chapter 2 and 3) each isolate indeed behaves as a genetically different individual. Therefore, no estimations can be made about the population size of *Podospora anserina* in Wageningen in terms of genetically different individuals (Hughes *et al.* 2001). It is however most probable that the Wageningen population is much larger than the theoretical population size used by Nauta and Hoekstra (1996) in their (sexual) vegetative compatibility model. Three outcrossed perithecia can in principle produce a thousand genetically different individuals, as approximately 200 asci are found per fruiting body and each ascus contains two different duplicate individuals (Zickler *et al.* 1995). Other, molecular methods have been used to distinguish *Podospora anserina* wild-type strains or to search for alternative alleles of *het*-genes (Table 7.1, Deleu *et al.* 1997). Belcour *et al.* (1997) could distinguish 10 polymorphic groups out of 15 strains based on mitochondrial restriction sites. Also repetitive elements such as minisatelites (Hamann *et al.* 1998) and the transposon derived *repa* element (Deleu *et al.* 1990, Hamann *et al.* 2000b) have been used to find polymorphisms between isolates. The closely related species *P. comata* (strain T) can be clearly distinguished by all these methods, and although relatively fertile with *P. anserina* indeed seems to be a different species. Some molecular methods, such as polymorphisms for GT-microsatellites (Osiewacz *et al.* 1996), the *Pat*-transposon (Hamann *et al.* 2000a), RAPD markers and several isozymes (unpublished data) proved not successful in distinguishing wild-type strains. Also AFLP mapping (unpublished data) hardly yielded any polymorphic markers from a cross between two divergent strains (*136* (Marcou *et al.* 1990) and Wa6 (Chapter 3)). These findings contrasts starkly with the variation found for instance for *het*-alleles. However, this variety might be selected for (Wu *et al.* 1998) giving a false impression of high genetic variation. Although a continuous history of distorter elements reaching fixation could have erased the genetic variation in large parts of the genome, the cause of the lack of variation is at the moment unresolved.

Distribution of selfish elements within populations of *P. anserina*.

The Wageningen population was screened for two types of selfish elements: Linear plasmids bearing sequence similarity to the pAL2-1 plasmid of *Podospora anserina* found by Hermanns *et al.* (1994), and meiotic drive elements, also known as Spore killers. Fourteen of 78 wild-type isolates hybridised with a pAL2-1 specific probe, half of which contained a single plasmid and the other half a family of multiple plasmid copies (Chapter 2). Recently, Maas *et al.* (2004) reanalysed some strains by PCR and found that two additional strains contained this plasmid, raising the total to 21 percent of the population (Table 7.2). Similar

plasmid screening of six French strains (Table 7.2) yielded no plasmid presence. The mutant strain AL2, from which the 'longevity' plasmid originated, was derived from strain A, and therefore that strain must also have contained the element at the time of mutagenesis. A similar reasoning can be made for strain s. Hermanns and Osiewacz (1994) performed a similarity search with the pAL2-1 sequence against the mitochondrial genome of strain s (Cummings *et al.* 1990) and found three inserted sequences bearing similarity to pAL2. Thus also an ancestor of strain s should once have contained this plasmid, though the timeframe of disappearance is unknown.

Plasmid-containing and plasmid-free strains were found along each other in sampling sites and through all sampled years, and form a stable polymorphism (Chapter 2). A longevity effect, as associated with the plasmid (Hermanns *et al.* 1994) could only be found for one plasmid-containing strain Wa32; all other plasmid-containing and plasmid-free strains did not show conspicuous phenotypic effects. However, no inserted plasmid sequences were detected in the mitochondrial DNA, as was the case in the longlived strain AL2-1, nor was the longevity effect as extreme as in strain AL2 (Chapter 2, Hermanns *et al.* 1994). Maas *et al.* (2004) found an opposite effect for pAL2-1 homologues under calorie restriction, where presence of this plasmid significantly decreased the lifespan. Here, these linear plasmids impose a negative effect on (growth) fitness when they are not integrated into a specific position in the mitochondrial genome.

Meiotic drive in ascomycete fungi is observed as spore killing, the abortion of half the progeny in a certain proportion of the asci. In *Podospora anserina* seven groups of meiotic drive (*Psk*) elements were discovered showing up to 95 percent of two-spored asci in the Wageningen population and some French strains (Chapter 3). An eighth type of meiotic drive, based on the *het-s* prion has been examined by Dalstra *et al.* (2003) and is only briefly discussed here. The Spore-killer strains were initially classified on the basis of (1) spore-killing frequency, the percentage asci showing meiotic drive, in a cross to a standard sensitive strain (*i.e*. the FDS percentage for the distorter element) and (2) the interaction between Spore killers. In this way at least six types of Spore killers could be identified among 99 isolates from Wageningen.

The aggregate group of *Psk-3* strains is different from the others in that the frequency of spore killing is highly variable among perithecia of the same cross and abortion is only found in crosses between specific strains. It is possible that this group actually consists of more meiotic drive elements (see Chapter 5, Figure 5.3), or some isolates do not represent true Spore killers. An additional Spore-killer type was discovered in the French *P. anserina* strain Y. All Spore-killer strains of the same type (except *Psk-3* and *het-s* Spore killers) show a repeatable spore-killing frequency when crossed to a standard sensitive strain and absence of spore killing when intercrossed. Spore-killer strains of different types show killing when intercrossed, with equal or lower killing percentage based on mutual resistance or dominant epistatic interactions (Chapter 3, Table 3.3).

Recently, Hamann and Osiewacz (2004) tested another set of *P. anserina* strains from nature and found an additional set of three Spore killers (Table 7.2) and initial attempts were

made to classify these Spore-killer isolates. Strain He displays the same killing percentage as found by us for *Psk-1* and *Psk-5,* and could be an identical type. It has not been determined if He shows killing with both types when intercrossed, as the only cross with *Psk-1* (strain Wa2) proved infertile and they did not try others or the *Psk-5* strain (Hamann and Osiewacz 2004). Strain Us3 shows on average a much lower killing percentage than Wa28 (*Psk-2*), but the intercross does not show killing, suggesting identical Spore-killer types. Spore killing by *Psk-2* is drastically influenced by temperature and shows incomplete penetrance (Chapter 5). This could in principle explain the difference in killing percentage between both strains and the variable killing percentage among crosses with different sensitive strains, as well as the behavior of the selfed offspring. On the other hand, if both killers are different types but found on the same linkage group and are mutual resistant to each other, killing could go undetected because of the sheltering effect in dikaryotic ascospores. A cross between both killers using the *oct1* marker could solve this question.

The last new meiotic drive element discovered by Hamann and Osiewacz (2004) is in the French strain O. It also shows a very variable killing percentage among the different sensitive strains, but does not show killing when intercrossed to *P. comata* strain T. The same reasoning as with Us3 and Wa28 can be used here, and other crosses must elucidate the relation of these Spore killers. Interestingly, strain O is molecularly distinct from *P. comata* strain T and groups with *P. anserina* (see Table 7.1 for references). We did not test by intercrossing whether our Spore-killer types were identical to the *Sk1*(T) element (Chapter 3). If both are the same killer type, then the same element is present in multiple species. This is relatively comparable to the situation in *Neurospora* where resistance against *Sk-2* and *Sk-3* is found in the (slightly) interfertile species *N. crassa* and *N. intermedia* (Turner 2001), and the fact that transfer is possible of the *Sk-2* and *Sk-3* distorters by introgression to *N. crassa* and *N. tetrasperma* (Raju and Perkins 1991, Campbell and Turner 1987).

Spore killers (excluding *het-s* drive) comprise 23% of the natural population of *P. anserina* in Wageningen sampled from 1991 to 1997 (Chapter 3, Table 7.2). Considering the arbitrary geographical distinction by countries, also in Germany and France meiotic drive is commonly found in *Podospora* (Table 7.2). This is however the overall picture and the percentages for each Spore-killer type are much lower. Identical drive elements could be found over several years and spread over a wide geographical area, for instance *Psk-7* was isolated in Wageningen, The Netherlands in 1994 and in Picardy, France in 1937 (Chapter 3). The percentage of Spore killers contrasts greatly with the occurence of *het-s* drive elements in nature (Bernet 1965, Dalstra *et al.* 2003). Approximately sixty percent of our population contains the *het-s* (Killer) allele. Curiously most plasmids containing isolates carry the *het-s* allele allele (Debets and Dalstra, unpublished data). A French population of nine strains consisted for 44% of *het-s* and 44% of *het-S* alleles (Deleu *et al.* 1993).

All non Spore-killer isolates from Wageningen are sensitive to spore killing, though slight variations in killing percentage are found in crosses with different sensitive wild-type isolates. Also full resistance was absent in French and German isolates (Chapter 3, Hamann and Osiewacz 2004). Hamann and Osiewacz did however notice significant variation in spore-killing percentage for *Psk-7* (The variation in killing percentage in presumed *Psk-2* and

Sk-1 strains (Hamann and Osiewacz 2004) can be due to reasons discussed above). It is quite possible that modifiers or suppressors for spore killing are present in some strains, reducing the frequency of ascospore abortion. Modifiers are expected to occur when selfish elements have a negative effect on fitness of the gametes (Hurst *et al.* 1996). Because Spore-killer elements show weak effective drive due to their effect on fecundity, suppression is even expected to occur rapidly by the selection of insensitive target alleles (Lyttle 1991) or by increasing the recombination rate (Hurst *et al.* 1996). Nauta and Hoekstra (1993) modeled fungal meiotic drive and found that a stable polymorphism of sensitives and Spore killers could only exist in the presence of resistant strains. Whether presence of modifiers would be enough to lead to a stable polymorphism has not been modeled. From our population data it must be concluded that meiotic drive elements in *Podospora* are ubiquitous and show a great variety. Although no strains resistant to Spore-killer types were found, they nevertheless seem to form a stable polymorphism in the population.

As both pAL2-like linear plasmids and Spore-killer elements are in a seemingly stable polymorphism in a natural population, the genomic conflict is still ongoing or is at stalemate (Hurst *et al.* 1996). In contrast, transposable elements found in *P. anserina* were found inactivated by RIP and genomic conflict here has ended in extinction of the selfish element. Degenerate and inactive traces of a DNA-transposon *Pat* was found present in multiple copies in all 16 isolates studied, originating from France, Germany and the Netherlands and including the *P. comata* strain T (Hamann *et al.* 2000a). Another degenerate LTR

Table 7.2
Geographic distribution of selfish elements in *Podospora.*

Origin of isolates	Isolates tested	Strains containing selfish elements	Percentage
Linear plasmids [c]			
Netherlands	Wa1-Wa83 (78 strains)	Table 2.1 (Chapter 2), Wa11, Wa26	21
France	A,D,E,F,S,s	AL2, s [b]	33
Spore killers [d]			
Netherlands	Wa1-Wa99	Table 3.1 (Chapter 3)	23
France	A,B,C,D,E,F,G,H,M,N,O,R,S,s ,*T,T'*,U,V,W,X,Y,Z [a,e]	Y,Z,O,*T,T'*	23
Germany	He,Mü,Us1-Us8,Wü	Us3, He	18
Het-s prion drive [f]			
Netherlands	Wa1-Wa99	55 Strains	56
France	A,C,D,H,s,S,*T*,U,V,X [a]	D,s,U,X	44

[a] *Podospora comata* strains are shown in *italics*, all other strains are *Podospora anserina.*
[b] Longlived strain AL2 is derived from strain A, therefore strain A should have contained a linear plasmid (Hermanns *et al.* 1994). Inserted plasmid sequences were detected in the mitochondrion of strain s, thus strain s should once (although not recently) have contained linear plasmid sequences (Hermanns and Osiewacz. 1994, Cummings *et al.* 1990).
[c] Linear plasmid data from Hermanns *et al.* (1995), Chapter 2 and Maas *et al.* (2004).
[d] Spore-killer data from Padieu and Bernet (1967), Chapter 3, Dalstra *et al.* (2003) and Hamann *et al.* (2004).
[e] Available French *Podospora* strains from Bernet (1965), Esser and Blaich (1994) and Belcour *et al.* (1997).
[f] *Het-s* data from Deleu *et al.* (1993) and Debets and Dalstra (unpublished data).

retrotransposon named *Yeti* as well as repeated sequence stretches (*repa*) derived from this transposable element were present in variable copy numbers in the same strains (Deleu *et al.* 1990, Hamann *et al.* 2000b).

Distribution of selfish elements in other species.

The linear plasmid pAL2-1 of *P. anserina*, formerly associated with longevity, has now also been shown to be associated with a senescence phenotype like the one caused by the pKALILO plasmid in *Neurospora intermedia* (Maas *et al.* 2004). PKALILO shows similar transfer characteristics as pAL2-1 in *Podospora* (Chapter 2): sometimes paternal transmission is found (Yang and Griffiths 1993a), loss through storage or subcultering occurs (Arganoza *et al.* 1994, He *et al.* 2000) and horizontal transfer among isolates is possible with remarkable ease in vegetative incompatible situations (Debets *et al.* 1994). Even inter-species transfer via introgression has been observed (Bok *et al.* 1999). Recently, Maas *et al.* (2005) found identical pKALILO plasmids in *N. intermedia* and *N. tetrasperma* from Hawaii. Only for pLA-KALILO identical plasmids were found before in different *Neurospora* species (He *et al.*2000). Although identical plasmids were found, plasmid characteristics seem host-specific. The senescence phenotype accumulated slower in *N. tetrasperma* than in *N. intermedia,* whereas the incidence of pKALILO is also lower in *N. tetrasperma* (22% vs. 47%). Unfortunately no information is available about the plasmid titre in these species during growth. A similar picture is observed for the presence of the circular pHANALEI plasmid (3% in *N. tetrasperma* vs 30% in *N. intermedia*). The occurrence of both plasmids was analysed previously by Debets *et al.* (1995). Comparison of both studies show that the occurrence of pKALILO remained the same, whereas the presence of pHANALEI dropped dramatically from around 74% in 1972 and 1976 to 30 percent in 1998 (Debets *et al.* 1995, Maas *et al.* 2005). We found fluctuations of plasmid frequencies as well in *P. anserina*, but this might be attributed to our smaller sample sizes for some years (Chapter 2).

Plasmid frequencies of pHANALEI and pKALILO are high for *N. intermedia* in Hawaii. A recent study of the circular pMADDUR plasmid showed also a high incidence of forty percent (149 isolates) in the Indian population of *Neurospora intermedia* (D'Souza *et al.* 2005). On average the incidence in other fungi is around 8-16%, incidence in *P. anserina* is only slightly higher (see Chapter2 for references).

Meiotic drive in fungi is most extensively studied in *Neurospora* spp. Turner (2001) described the worldwide occurrence of Spore killers, and resistance to them. Recently, new *Neurospora* spp. were collected from Southern Europe by D. J. Jacobson (personal communication). Among the *N. sitophila* strains a high incidence of 50% *Sk-1* Spore killers was found, many alongside sensitive isolates. This makes *Sk-1* among the most common segregation distorters in fungi (*Giberella Sk* is presumed to be in 80% of the isolates (Kathariou and Spieth 1982)), as well as the most widely distributed. More extensive sampling would probably reveal the occurrence of Spore killers in other regions. *Sk-1* can be seen as a successful drive element, conquering the world and probably on its way to fixation,

as only one resistant strain has been isolated (Turner 2001). Considering the mobility of fungal spores of *Neurospora* over large geographical areas, *Sk-1* has likely been around for many generations.

The opposite situation is found for *Sk-2* and *Sk-3,* which are rare among *N. intermedia* isolates and were only found a few times after extensive search in the Indonesian Archipel. Most likely *Sk-2* and *Sk-3* are on their way to extinction (Turner 2001), as resistance against both Spore killers commonly is found in all South-East Asia, Australia and Oceania. Resistance, as well as partial resistance to *Sk-2* is also found in the slightly interfertile species *N. crassa* (Turner 2001, 2003).

Compared to *Podospora*, in *Neurospora* spp. less meiotic drive elements are detected, while *Neurospora* Spore killers are the stronger distorters. In both species the fecundity is reduced, but sensitive nuclei in *Neurospora* (*i.e.* in the species where drive elements were detected) cannot be sheltered by second division segregation of the drive element. All asci in *Neurospora* show drive, as all ascospores are haploid, whereas in some *Psk* types only half of the asci show ascospore abortion due to the dikaryotic nature of the ascospores (Chapter 3, Raju 1994). Also *Podospora* may reproduce by selfing, instead of the obligate outcrossing of *Neurospora* (which will show drive when mating to a sensitive isolate). It is therefore possible that drive elements in *Neurospora* move faster towards fixation and go undetected.

The less studied fungal drive systems, *Sk* in *Cochliobolus heterostrophus* and *Sk* in *Gibberella fujikuroi* are both heterothallic like *Neurospora* (Raju 1994). Likewise, they cannot shelter sensitive nuclei and must outcross to produce ascospores. In *Gibberella* two Spore-killer types, Sk^{K} and Sk^{Mx}, are found in high frequency. The last type is an aggregate group like our *Psk-3* and can show different percentages four- and eight-spored asci in crosses with killers and sensitives, but also with other Sk^{Mx} strains (Kathariou and Spieth 1982, Sidhu 1984). Unfortunately, no complete genetic tests with crosses to both killer and sensitive, as well as the different Sk^{Mx} strains have been made. It is likely that Sk^{Mx} consists of different Spore killers, or suppressors of meiotic drive. Also the strains classified as Sk^{K} could in principle consist of both full resistants and Spore killers (Kathariou and Spieth 1984).

Stronger segregation distorters are more likely to quickly evolve suppressors with less deleterious effects than the distorter locus (Lyttle 1991, Hurst *et al.* 1996). It is possible that Sk^{Mx} represent such a class of (unlinked) suppressors, reducing the percentage of aborted ascospores, but still aborting sensitives. One could speculate if the presence of the large variety of Spore killers in *Podospora* with a hierarchical killing order (Chapter 3, Chapter 5) reflects the fact that they are suppressors of the other, with the negative side effect of being a segregation distorter (with spore killing ability) as well. This could very well be an example of an extended conflict or 'arms race' between Spore-killer types as described by Hurst *et al.* (1996).

Segregation distortion in *Podospora* differs in several important aspects from other autosomal meiotic drive systems found in nature (Lyttle 1991). Absence of tight linkage to the centromere is observed, which is reflected in the lower fraction of asci in which ascospores are killed in four of the Spore-killer types. Another aspect is the failure to detect full

resistance or suppressor alleles (see also discussion above). Drive in fungi is generally found in higher frequencies in natural populations than meiotic drive in animal systems, though the percentage of distorter genes involved is more or less the same, ranging between 1 in 10^3 and 10^4 genes (Table 7.3). This is a large percentage of the transcribed genome (and larger than assumed in Chapter 3), considering the following two things. (1) Not every gene is capable of showing drive, because their function or gene product cannot lead to ascospore abortion or they are unable to be expressed during meiosis. Furthermore, drive elements consist of linked two-gene pairs, which reduce the number of potential distorter loci (Wu and Hammer 1991). (2) Not all possible segregation distorters in a species are present within the same evolutionary time period. In the period of existence of the *Podospora* or *Neurospora* millions of years have past, enough to evolve many segregation distorters (Berbee and Taylor 1993, Davis 2000, Dettman et all. 2003a, 2003b). Some have become fixed within the population, others gone extinct, and some still await a mutation to appear in the population. The percentages of segregation distorters might even be higher as we simply could have overlooked some meiotic drive elements, like the recently uncovered *Om* locus in mice (Wu *et al.* 2005).

The population frequency of segregation distorters in animals is found to be stable but low. A systematic sampling of mice from nature and testing them with DNA markers for the frequency of *t*-haplotype, led to the remarkable finding of an average frequency of six percent *+/t* heterozygotes (Carroll *et al.* 2004). This is much lower than previously reported percentages of 15-25 percent (Ardlie *et al.* 1998, Lyttle 1991). The *t*-haplotype was present in 46% of the sampled populations and findings within a single population varied with respect to population size and stability. More variable and higher frequencies could be found at smaller populations (Ardlie *et al.* 1998). Negative female choice (*i.e.* a behavioral negative fitness effect) for *t*-bearing males contributed to the low frequency (Carroll *et al.* 2004). The other well-characterized autosomal distorter *SD* is found in multiple *Drosophila* species around the world. *SD* occurs as a stable polymorphism in nature with frequency of only 1-3% (Lyttle 1991).

Why does (autosomal) meiotic drive in animals exist in lower frequencies than in fungi? This seems a paradox, as there is stronger selection against meiotic drive in fungal haploid systems (Lyttle 1991). The main reason is probably the difficulty to detect autosomal segregation distorters in animals because they lack a recognizable phenotype. Other reasons could include behavioral aspects, like female choice and competition between partners (Carroll *et al.* 2004). In fungi mating is restricted to the (random) male gamete landing on the ascogonium. Waiting for another (better suited, non-disorter) gamete could result in loss of opportunity to reproduce sexually, due to aging or depletion of resources.

Hurst and Werren (2001, Chapter 1) stated that selfish genetic elements have two common rules. One rule states that the selfish phenotype is often shown in hybrids and between-population crosses, but that they are seldom observed in within-population crosses. For instance, spore killing as found in interstrain crosses of the fission yeast *S. pombe* (Kondrateva and Naumov 2001). However in this reported case, asci all contained four spores, with variable viability, and this presumed spore killing can probably be attributed to

Table 7.3
Percentage of known meiotic drive loci in predicted genes from genome sequencing.

Species	No. of predicted genes	No. of known meiotic drive elements	Percentage of genes
Drosophila spp.	13,676 (Misra *et al.* 2002)	4 *SR* loci, *SD* (Lyttle 1991)	0.04 %
Mus spp.	22,011 (Mouse Genome Sequencing Consortium, 2002)	3-6 *t*-loci (Lyttle 1991), *Om* (Wu *et al.* 2005), *Dr* (Agulnik *et al.* 1993),	0.04 %
Neurospora spp.	10,620 (Galagan *et al.* 2003)	3 *Sk* loci (Turner 2001)	0.03 %
Fusarium spp.	11,640 (*Fusarium graminearum* Sequencing Project. Broad Institute of MIT and Harvard (http://www.broad.mit.edu))	Sk^{K}, 4 Sk^{Mx} (Kathariou and Spieth 1982, Sidhu 1984) [a]	0.04 %
Podospora spp.	11,000 (Silar *et al.* 2003)	7 *Psk* loci, 2 *Sk*, *het-s* (Chapter 3, Padieu and Bernet 1967, Dalstra *et al.* 2003)	0.09 %

[a] The Sk^{Mx} are designated different loci based on killing percentage (~25, 50 and 70 % killing, Kathariou and Spieth (1982)) and Spore killer interaction (Sidhu 1984).

other forms of sexual dysfunction. For meiotic drive elements in *Podospora* (Chapter 3), and perhaps segregation distorters in general (Ardlie *et al.* 1998, Carroll *et al.* 2004, Lyttle 1991) this does not seem to be an absolute rule, as many drive elements are either stable or appear frequently within a population.

Models of spore killing in *P. anserina*.

Combining all characteristics of the Spore-killer types, we have investigated several possible models to explain meiotic drive in *Podospora anserina.* We could not explain spore killing as caused by any silencing mechanism, although homologous genes for RIP, MSUD and quelling were found in the genome (Chapter 5). The involvement of methylation during the killing process was experimentally ruled out as well (Chapter 5). In *Neurospora* both MSUD (Shiu *et al.* 2001) and methylation (N.B. Raju, personal communication) have been tested as a possible spore-killing mechanism, but found not to be involved in spore killing. On the contrary, Raju and coworkers (Raju *et al.* 2005) showed that Spore killers acted as suppressors of MSUD-silencing and resemble *Sad-1* in it's mode of action. Also the presence of MSUD homologous genes does not necessarily mean that they are expressed, as they were found inactive in *N. tetrasperma* (Jacobson and Raju 2005). Spore killing in *Podospora* can best be explained as a post-segregational killing (PSK) system due to Toxin-Antitoxin mechanisms (Chapter 5). These Toxin-Antitoxin mechanisms might very well originate from vegetative incompatibility genes. Among our Spore-killer strains is a strong correlation between these two phenotypes as Spore-killer types remain vegetative incompatible between each other after recurrent backcrosses, and are compatible within each resistant type (Chapter 4). Not all Spore killers in *Podospora* are correlated to vegetative incompatibility. Hamann and Osiewacz (2004) showed that the combination of Spore-killer strain O (presumably

identical to *P. comata* killer *Sk)* with sensitive strain D was vegetatively compatible, as observed by the absence of a barrage and a single line of perithecia. Also our Spore-killer types *Psk-4* and *Psk-6* could be vegetatively compatible, as we were unable to proceed backcrosses with strain S (Chapter 3).

The suggestion by Hamann and Osiewacz (2004) of spore killing resulting from or causing gene conversion should be taken with caution. The authors were unaware of temperature effects and penetrance of some Spore-killer types (Chapter 5), which can lead to analyses of FDS sensitive and killer ascospores, while assuming SDS for the Spore-killer element (discussed above). Also genetic analysis by dropping (single mating type) parental microconidia onto double mating type mycelium, could lead to analysis of selfed offspring (Chapter 6). Using microconidia from the dikaryotic offspring to backcross to single mating type parents would have circumvented this problem (Chapter 3, Chapter 6).

Most killer elements in *P. anserina* can be assigned to linkage group III but they are not tightly linked to the centromere (Chapter 3). Several vegetative compatibility genes are known on this linkage group (Marcou *et al.* 1990), which is one of the middle-sized chromosomes (4.9 Mb) (Javerzat *et al.* 1993). Spore-killer type *Psk-4* is likely located on linkage group IV, the smallest chromosome (3.8 Mb) (Chapter 3, Javerzat *et al.* 1993). Other fungal drive elements have also been mapped to linkage groups. In *Neurospora* both Spore killers *Sk-2* and *Sk-3* were assigned to linkage group III. Spore-killer Sk^K of *Gibberella fujikuroi* has also been located (LG V) and maps close to an RFLP marker (Xu *et al.* 1996). Combined with the genome sequencing of the related *Fusarium graminearum* (http://www.broad.mit.edu) this would make excellent opportunity for molecular analysis of this Spore-killer system. Also the distorter elements of *Podospora* and *Neurospora* await molecular characterization with the finished genome sequencing of *P. anserina* and *N. crassa* (Silar *et al.* 2003, Galagan *et al.* 2003), although the first still awaits a more thorough assembly and annotation. Data of linkage together with probable function in either vegetative compatibility, apoptosis or toxin-antitoxin mechanisms could point to candidate Spore-killer genes.

Theoretical studies show that a two-locus system is expected for a segregation distorter (Hurst *et al.* 1996, Wu and Hammer 1991). The Toxin-Antitoxin mechanism is based on a gene couple of killer and (in)sensitive target that need to be strongly linked. Strong linkage in *Neurospora* is archieved by a large recombination block across the centromere spanning 3% of the genetic map (Campbell and Turner 1987). *Gibberella* and *Podospora* do not show a (large) recombination block for Spore killers as *Neurospora*, but *Cochliobolus heterostrophus* does (Bronson *et al.* 1990). However both *Gibberella* and *Podospora* are known to show strong recombination interference, which could provide the same result (Xu *et al.* 1996, Kuenen 1962, Simonet 1973, Marcou 1979, M. Picard personal communication).

Sex and vegetative compatibility in *Podospora*.

The secondary homothallic ascomycete *Podospora anserina* is capable of both selfing and outcrossing (Chapter1, Chapter 6). Not only single mating type colonies, originating from

uninucleate ascospores or spores with FDS for mating type, proved capable of outcrossing, but also colonies rising from dikaryotic ascospores. This indicates that sexual reproduction uses the trichogyne irrespective of selfing or outcrossing and single or double mating-type cultures. This is in contrast to the nonresponse of trichogynes assumed in heterokaryotic cultures of the secondary homothallic *Neurospora tetrasperma* (Chapter 6, Bistis 1996). No preference for either selfing or outcrossing is found in *Podospora* (Chapter 6).

To assess the extent of outcrossing in nature we tested vegetative and sexual compatibility between wild-type strains. Two anomalies were observed during this survey: First, we found that sexual incompatibility not only depended on (non-allelic) vegetative incompatibility, as stated by Esser and Blaich (1994) since sexually incompatible but vegetatively compatible strain combinations were found. Secondly, sexual semi-incompatibility results of some strain combinations cannot be explained by the current (non-allelic) vegetative compatibility model. Therefore we must assume the existence of specific genes involved in sexual compatibility with no effect on vegetative compatibility (Chapter 6). The recent finding of pheromone precursors in *P. anserina* could be examples of such genes (Coppin *et al.* 2005).

The extent of vegetative compatibility is comparable what has been found in other wild-type strains of *Podospora* (see Table 7.1 for the isolates used), isolated in different regions of France and Germany by Bernet (1965) and Hamann and Osiewacz (2004), although Esser and Blaich (1994, Chapter 6) found higher percentages. Strains belonging to the same Vegetative Incompatibility Group (VCG) have been found across Europe (France and Germany (Würtenberg)). Sexual compatibility types are comparable to those found by Esser and Blaich (1994) and Hamann and Osiewacz (2004), but Bernet (1965) found a much higher percentage of sexual compatibility, increasing outcrossing possibilities.

The isolates used in our survey were sorted into 33 VCG's based on the incompatibility reactions. The number of existing VCG's in the population was then estimated using accumulation curve extrapolation (107 VCG's) and the (under-estimating) non-parametric Chao1 formula (75 VCG's) (Chapter 6). Under the current model of nine vegetative incompatibility loci in *Podospora* a maximum diversity of 117 vegetative combination types is possible (Saupe 2000). Both estimated numbers of VCG's need all available loci (and most alleles) to be recombining. Therefore convergent evolution of *het*-alleles is unlikely and outcrossing must occur. Outcrossing is also suggested by the difference in sexual compatibility reactions of mating types of the same isolate (Chapter 6). The other common rule of selfish elements (Hurst and Werren 2001) states that the diversity of selfish elements is correlated with the rate of outcrossing. The presence of many distorter elements in *Podospora* therefore implies regular occurring outcrossing. Furthermore, outcrossing in an unmanipulated environment has been confirmed by mixing two different strains on dung. Up to four percent of outcrossed perithecia could be found in these experiments. Extensive mycelial mixing of dikaryotic strains can further increase outcrossing percentages (Chapter 6).

Outcrossing needs two genetically different strains. The ecology of *P. anserina* assures that dung is supplied with a mixture of genetically different individuals (see discussion above). Such a genetic mixture improves chances of outcrossing to occur in nature.

The dynamics of selfish elements in *P. anserina*.

The dynamics of the two selfish elements studied in this thesis are very different from each other. This is mainly based on the fact that the linear plasmid is located in the highly mobile mitochondrion, whereas the meiotic drive elements are located in the nuclear genome. However, for both selfish elements it is necessary to have access to the sexual spores of *Podospora*, as they are the only gateway to their next generation.

Debets et al. (1995) gave an overview of factors that influence the dynamics of plasmids in fungi. Most of these also apply for the pAL2-1 plasmid homologues and are discussed below.

(1) Transmission to asexual spores does not apply to *P. anserina* as no asexual spores are produced and spread by conidia is irrelevant. The microconidia of *Podospora anserina* are not capable of germination and only function as male gametes. Plasmid transmission is usually 100 percent to the clonal spores of fungi that produce conidia (Debets *et al.* 1995, Giese *et al.* 1990, Baidyaroy *et al.* 2000).

(2) Mitochondrial inheritance in ascomycete fungi is mainly maternal (Coenen *et al.* 1996, Lee and Taylor 1993). Strict maternal plasmid inheritance has been found for the pAL2-1 (Chapter 2). Strict maternal inheritance implies that plasmids become lost in some outcrossing situations. No perithecia (and spores) are produced when the plasmid host contains a wrong set of non-allelic vegetative incompatibility genes, resulting in female sterility for the host (Chapter 6).

(3) Complete plasmids as well as plasmid family members are frequently lost in transmission to the sexual ascospores (Chapter 2). Plasmid transmission was equally efficient for mono- and dikaryotic spores and independent of the genetic background of the strains (Chapter 2). Loss of plasmids during the sexual cycle seems a common feature, as it is also found in other fungi and related to the related to the aggressiveness of the plasmid (Giese *et al.*2003, Debets *et al.* (1995). There is probably a bottleneck that excludes dysfunctional mitochondria (*e.g*. inserted plasmids) or mitochondria with a heavy plasmid load (*e.g.* plasmid families, senescence phenotype) resulting in spores without the selfish elements.

(4) Plasmid suppression could be active in *Podospora* as plasmid transfer was severely reduced to 40% in an outcrossing situation (Chapter 2). Due to the heterokaryotic nature of the offspring, suppressor action is dependent upon segregation and whether the suppressor is dominant or recessive. Furthermore is autosomal suppression dependent upon the outcrossing frequency with a parental suppressor strain (Yang and Griffiths 1993b).

(5) Horizontal transfer experiments showed that pAL2-1 easily could infect plasmid-free strains. Horizontal transfer was even observed between vegetative incompatible strains (Chapter 2). This observation is regularly found in nature for plasmids (Debets *et al.* 1994,

Baidyaroy *et al.* 2000, Giese *et al.* 2003), but also for viruses (Liu and Milgroom 1996) and even chromosomes (He *et al.* 1998). However, recent studies have shown that the strength of the vegetative incompatibility locus, rather than the number of loci involved, is the important factor restraining transmission of selfish elements. A weak *het*-locus with a delayed PCD (programmed cell death) response allows more time for the selfish element to pass to the new host (Baidyaroy *et al.* 2000, Cortesi *et al.* 2001, Biella *et al.* 2002). We do not know which *het*-alleles, aside from *het-s*/*het-S*, are present in our strains. It would be interesting to know to what extent each *het*-gene of *Podospora* contributes to the cytoplasmic barrier, and can prevent the seemingly efficient spread of pAL2-1.

The mode of horizontal transfer is another question. Mitochondria containing the plasmid could replace plasmid-free mitochondria (Lee and Taylor 1993), but also mitochondrial fusion can facilitate transfer as it occurs regularly and is essential in ascospore formation (Giese *et al.* 2003, Westermann and Prokish 2002).

(6) Somatic survival or host fitness indirectly affects the presence of the plasmid. A host that grows longer, occupies a larger area resulting in a greater number of perithecia and ascospores. The presence of pAL2-1 has been correlated with longevity for only two strains. Strain AL2 where the plasmid is integrated into a specific position in the mitochondrial genome (Hermanns *et al.* 1994) and strain Wa32 where no integration into the mitochondrion was detected (Chapter 2). Maas *et al.* (2004) did find a senescent phenotype correlated with pAL2-1 presence when culturing under calorie restriction.

One can argue if lifespan (measured by maximum growth (Chapter 2) or days (Maas *et al.* 2004)) is a relevant fitness measure in *Podospora*. Most dung droppings never reach sizes of 25 cm (the average maximum growth, Chapter 2) and the lifecycle on dung is completed within 2 weeks (Wicklow 1981, Dix and Webster 1995) including the ascopore germination step (which is not included using race tubes). Pringle and Taylor (2002) state that one fitness measure is usually appropriate when it is understood how different fitness factors are related to the ecology of a species. More relevant fitness markers for *P. anserina* could be growth speed during the initial growth phase or ascospore germination rate and speed (as measures for the ability to colonize the new dung), quickness of sexual production or ascospore production (the only offspring) and ascospore spread (as the ability to be included in the next generation).

The fluctuations in presence and high mobility of plasmids show that the population dynamics of fungal plasmids undergoes rapid change and changes through the years (Chapter 2, Maas *et al.* 2005). Although the pAL2-1 plasmid seems to compensate loss through horizontal transfer, most of the *Podospora* wild-type strains remain plasmid-free, even if they occur next to each other in a sample. Such coexistence has also been noted for a linear plasmid strain in *Erisyphe graminis* that was cultured together with a plasmid-free strain for 1.5 years without any horizontal transfer (Giese *et al.* 2003).

The dynamics of meiotic drive elements in *Podospora anserina*, is dependent upon several factors described below. Some are directly related to the heterokaryotic and secondary homothallic nature of the fungus.

(1) Fitness gain by abortion of ascospores not containing the segregation distorter. The gain is dependent upon the amount of FDS for the drive element. Lower percentages of FDS lead to more sheltering of sensitive nuclei and less effective drive. *E.g. Psk-4*, *Psk-6* and *Psk-7* are less efficient Spore killers than *Psk-1* and *Psk-5* (Chapter 3).

(2) Ecological factors like temperature have effects on the spore-killing efficiency, as found for *het-s* and *Psk-2* (Chapter 3, Bernet 1965).

(3) Outcrossing rate is the most important factor for segregation distortion in *Podospora*. Only outcrossing (initially) provides heterokaryosity for the Spore-killer element that leads to meiotic drive. If *Podospora* was restricted to selfing, the multitude of drive elements could only have been evolutionary accidents. We found evidence that *P. anserina* regularly outcrosses, but with relatively low percentages (Chapter 6).

(4) Spore-killer isolates probably enhance outcrossing rate as the percentage of monokaryotic ascospores in these strains is higher (Chapter 6). Another enhancing effect might be the close connection of Spore killers to *het*-genes which allows the distorter to profit from or to be maintained by the balancing selection of the vegetative incompatibility genes (Chapter 4, Wu *et al.* 1998).

(5) Genetic factors like suppressors or modifiers and resistance to meiotic drive elements, also lower the efficiency or counter drive. As suppressors do not have to be linked to the sensitive allele, nor are restricted to certain gene sets, they have more opportunities to evolve than distorters (Wu and Hammer 1991). Though no single resistance to spore killing was found, Spore-killer types were found to be dominant epistatic or mutually resistant against each other (Chapter 3). Findings by Hamann and Osiewacz (2004) suggest the presence of suppressors to spore killing. Infertility to Spore-killer strains acting as a barrier to drive, such as found in all strains of *Neurospora intermedia* from New Zealand and Mexico (Turner 2001) has not been detected in *Podospora*.

(6) Most meiotic drive models assume a negative fitness effect for the fungus associated with the distorter to balance the fitness gain by killing non-distorter offspring (Nauta and Hoekstra 1993). Prolonged polymorphism for distorter and sensitives increases the change of hitchhiking alleles with negative fitness effects (Hurst *et al.* 1996, Lyttle 1991). Such negative fitness effects were found for *t*-haplotypes in natural populations of mice (Caroll *et al.* 2004). No difference in variability of lifespan is found in *P. anserina* for Spore killers and sensitives (Chapter 2 for lifespan data, Chapter 3 for Spore-killer strains, excluding the *het-s* drive element). But again, it can be argued whether lifespan is a good fitness measure. A possible disadvantage for Spore killers is the fact that only two spores are left within the ascus. Ascospores tend to stick to each other, *i.e.* siblings stay close together in the release stage. Ballistic experiments with fungal spores show that larger projectiles reach further and are able to spread over a larger area (Ingold 1971). A preliminary experiment with *P. anserina* showed that two-spored bundles do not reach as far as four-spored projectiles.

What would be the time-scale for a segregation distorter to reach extinction, fixation or stability in a population? The fungal meiotic drive model used by Nauta and Hoekstra (1993) suggest that a conflict with only killers and sensitives is resolved in 30 generations. Assuming a life-cycle of two weeks for *P. anserina* would mean a maximum of 26 generations for this fungus per year. The actual number of generations is probably much lower as the optimum growth temperature of 27 °C is not reached throughout the year in the Netherlands. Notwithstanding seven years of sampling (a maximum of 182 generations) has not resolved the genomic conflict caused by Spore killers in *P. anserina.* Neither fixation nor extinction of the distorter element, but a seemingly stable polymorphism was detected as *Psk-7* has been present in Europe for 60 years (Chapter 3). Most likely, all mentioned factors as outcrossing rate, generation time, ecological effects and interaction between different Spore-killer types slow down or stabilize the outcome of the genomic conflict. Further prolongation of the conflict is probably caused by the passive pool of ascospores, which provide only a subset of the available individuals for the next generation (Chapter 1). A model taking these life-history traits into account should give better insight into the dynamics and probable outcome of this genomic conflict.

Evolutionary significance of selfish elements.

Evidence for horizontal transmission of plasmids among fungal species was found by Taylor *et al.* (1985) and Kempken *et al.* (1992) using evolutionary phylogenies. Transfer of plasmids and subsequent insertion into the mitochondrial genome also leads to the reorganization and evolution of mitochondrial genes. Rosewich and Kistler (2000) and He *et al.* (2000) pointed out that some cases of horizontal transfer among species also could be explained by introgression. However, the widespread occurrence of plasmids in fungi, while most other eukaryotes are devoid of them may be a sign that horizontal gene transfer in fungi is a more important evolutionary force than in other eukaryotes (Rosewich and Kistler 2000).

Horizontal transfer has been proposed as a mechanism to maintain gene clusters among bacterial taxa (Lawrence and Roth 1996). Although genes for related functions are rarely found in close proximity in eukaryotes, gene clusters are regularly found in fungi, *e.g.* genes related to toxin and antibiotics production, melanin biosynthesis or spore-specific genes (Keller and Hohn 1997, Orr and Timberlake1982, Kubo *et al.* 1996, Ahn and Walton 1996, Walton 2000). The definition of a gene cluster, ‘a close linkage of two or more genes that participate in a common metabolic or developmental pathway’ (Keller and Hohn 1997), closely resembles the prerequisites for a meiotic drive element; two (or more) strongly linked genes (*i.e.* the killer, the target and any enhancer loci) in a common developmental (*e.g.* ascospore formation) pathway. Horizontal transfer cannot explain fungal gene clusters and the force that maintains fungal clusters is unclear. Many fungi lack certain clusters while others contain functional unrelated genes (Keller and Hohn 1997, Walton 2000). A selfish meiotic drive element, however must maintain tight linkage between killing and resistance genes, and unrelated genes might hike along. Gene clusters involved in toxin production also confer resistance against the toxin, reminiscent of a TA-system like spore killing in *Podospora*

(Chapter 5, Walton 2000). The Spore-killer cluster is transferred as a unit through the sexual cycle and fixation of the distorter automatically leads to fixation of the cluster. Furthermore, a fungal species lacking a certain cluster might just not have evolved a segregation distorter for that specific pathway. It is possible that gene clusters are either ideal targets for newly occurring meiotic drive elements, or are relics from previous distorters who were successful upon reaching fixation.

Hitchhiking of genetic elements that confer a negative effect on the fitness of an individual has consequences on the fitness of the complete population when the Spore-killer cluster reaches fixation. In theory, repeated fixation of genetic elements with negative fitness effects could lead to extinction of the species (in competition with species occupying the same ecological niche). However, drive elements with strong negative fitness effects cannot invade a population (Nauta and Hoekstra 1993), thus many fixed distorters with small negative fitness effects are probably necessary to cause extinction. *Podospora* is a mainly selfing fungus (Chapter 6) and negative fitness effects will therefore impose a stronger load, as no fitness gain is obtained by the drive element in a selfing situation. Despite the abundant availability of meiotic drive elements in *Podospora anserina* (Chapter 3, Hamann and Osiewacz 2004), the fungus has so far managed to compensate any negative fitness effects, or the imposed negative fitness effects are negligible or non-existent. Fitness and competition experiments in *Podospora* could test the evolutionary importance of hitchhiking negative fitness elements.

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Summary

This thesis deals with genomic conflicts raised by selfish elements in the ascomycete fungus *Podospora anserina*. Genomic conflicts arise when the effects of the selfish elements are opposite to the interests of the other parts of the genome. Two types of selfish elements are studied as well as certain characteristics of *Podospora* involved in the population dynamics of these elements, such as vegetative and sexual incompatibility, senescence and outcrossing. The natural habitat of *Podospora anserina* is dung of herbivores where it has an optimum growth temperature of 27 °C. The fungus can only reproduce sexually and the ascospores are the products of meiosis as well as the next generation of the fungus. Perithecia or fruiting bodies contain asci with four linearly arranged ascospores, which provide unique opportunities to analyse abnormal segregation and makes this fungus one of the genetic model organisms. Most ascospores are capable of completing the lifecycle of the fungus, as they contain two nuclei, each with one of the two mating types. This fungal trait is called pseudo or secondairy homothallism, and it allows sexual offspring to be produced by either selfing or outcrossing. Sometimes smaller single mating type ascospores are formed containing one nucleus and less cytoplasmic content and mitochondria. Colonies from these spores must outcross with another isolate to produce offspring. The fungal isolates used in this thesis were sampled from dung around Wageningen, the Netherlands during 1990-1997, but also some older French isolates dating from 1937 were used.

The first selfish elements studied in Chapter 2 of this thesis are linear plasmids with homology to pAL2-1, a 8.3 kb plasmid previously found in this fungus. Linear plasmids are parasitic autonomous replicating genetic elements. In filamentous fungi they reside in the mitochondria. Most plasmids are cryptic or have a negative fitness effect on the host. However the pAL2 plasmid found in *P. anserina* has been associated with a longevity phenotype, though recently also negative effects were found. Homologous linear plasmids and plasmid families were present in 21 percent of the population and detected over several years. Half of the plasmid containing isolates possessed a single plasmid and the other half a plasmid family consisting of multiple plasmid copies. The lifespan of the isolates was generally uncorrelated with the presence of the plasmid. One isolate showed an increased lifespan, but no inserted plasmid sequences were detected in the mitochondrial DNA, as was the case for the longevity inducing pAL2-1 plasmid.

We have looked at the dynamics of plasmid transmission for these plasmids. Vertical transfer of the plasmids to the ascospores occurs only via the maternal line. This transfer is inefficient as up to 40% lose all plasmids in an outcrossing situation and up to 20% retain only the basic plasmid family member in presence of a plasmid family. No difference in

efficiency of plasmid transfer to the dikaryotic and the smaller monokaryotic ascospores was detected. Plasmid transmission was also found independent of the genetic background of the strains. Loss of plasmids via the sexual route is compensated by horizontal transmission of the plasmid through hyphal contact between different isolates.

Horizontal transfer occurs efficiently in both vegetative compatible and incompatible situations. Vegetative incompatibility can be macroscopically observed as a 'barrage', a contact zone of lysed hyphal cells. Vegetative incompatibility is thought to have evolved as a mechanism to hamper spread of mobile selfish elements and parasites. Our experiments show that vegetative incompatibility is not a perfect barrier against this type of selfish elements.

The other selfish elements studied in this thesis are meiotic drive factors or segregation distorters. Segregation distorters are transmitted into the progeny in excess of the fair Mendelian proportion of 50 %, by actively destructing the alternative allele. Genomic conflicts arise by hitchhiking of genes with deleterious fitness effects. Meiotic drive in *Podospora* is characterized by the abortion of two of the four spores in the ascus. Chapter 3 characterizes seven different groups of meiotic drive elements or Spore killer types that were identified. Among 99 isolates from nature, six of these meiotic drive elements occurred in our local population. All drive elements comprise 23% of the natural population of *P. anserina* in Wageningen, The Netherlands and most elements can be retrieved over the years. Spore-killer type *Psk-7* was also present in a French strain dating from 1937 and exists for more than 60 years. No resistance to meiotic drive was observed and all other isolates found so far are sensitive to spore killing. Each type of Spore killer differs in the percentage of asci that show killing, ranging from 50 to 95% two-spored asci, and in their mutual interactions. The aborted ascospores quickly degrade after spore wall formation, except for the *Psk-3* group where they remain visible as tiny shriveled ascospores, indicating different abortion factors or timing for killing. The *Psk-3* group also shows a variable percentage of two-spored asci within each perithecium. Spore-killer interactions show either mutual resistance (*i.e.* no abortion is found if an allele of either Spore killer is present) or dominant epistasis (*i.e.* one killer acts as a sensitive type). Genetic mapping could assign most Spore-killer types to linkage group III where they are not tightly linked to the centromere.

Several possible models that explain the spore killing mechanism in *Podospora anserina* were examined. Repeated backcrossing of Spore killers to the same sensitive isolate produces strains with the same genetic background. Chapter 4 shows that Spore killers that belong to the same killer type or show mutual resistance become vegetative compatible to each other during backcrosses. On the other hand, Spore killers that show dominant epistasis, as well as the sensitive strain remained vegetative incompatible. This suggests a common mechanism for spore killing, possibly related to vegetative incompatibility, although the precise genetic nature of the correlation is not yet clear. The *Podospora* genome was screened in Chapter 5 for homologues of genes known to be involved in silencing in fungi. Genes were found for all silencing mechanisms (RIP, MSUD, Quelling) known in the related fungus *Neurospora*. However, the possible role of silencing by methylation of genes during the killing process was excluded by experiments using the drug 5-azacytidine, which both

removes methylation and prevents *de-novo* methylation. No effect of the 5-azacytidine treatments was found on spore-killing frequency for all Spore-killer types. Chapter 5 also studied the consequences of formation of dikaryotic ascospores for expression of spore killing. Crosses were used of Spore killers with a marker that increases the number of monokaryotic spores to up to eight. Spore-killer types *Psk-2*, *Psk-3* (Wa27) and *Psk-4* produced some asci containing more than four spores in these crosses. In such asci sensitive nuclei were able to survive and resist the meiotic drive system, indicating incomplete penetrance of the spore-killing mechanism. Spore killing in the other killer types (*Psk-1, Psk-5, Psk-6* and *Psk-7*) were full penetrant; only asci with four or less spores could be detected. Here the killing mechanism works similar on all asci. Furthermore the effect of low temperatures (22 ºC) on spore killing was tested in this Chapter. *Psk-2* dramatically decreased the percentage of killing at this temperature to almost zero. Other Spore-killer types were not affected by temperature. Based on all characteristics and interactions of the Spore killers we propose that spore killing in *Podospora* may be an example of post-segregational killing due to Toxin-Antitoxin mechanisms. A Spore Killer produces both a persisting stable toxin and a less stable antitoxin. In ascospores where the Spore Killer is absent the antitoxin disappears more quickly than the toxin, leading to abortion of the spores. Variable killing percentages can be explained by the strict balance between toxin and antitoxin and the timing of shutdown of the genes involved.

Meiotic drive is only possible in an outcrossing situation. *Podospora* is in principle capable of both outcrossing and selfing. However to what extent the fungus outcrosses in nature is unknown. Chapter 6 assesses the likelihood of outcrossing in the secondary homothallic ascomycete *Podospora anserina.* We examined the extent of vegetative en sexual compatibility between wild type strains. The number of vegetative compatibility groups (VCG's) in the population was estimated based on the incompatibility reactions between isolates in our survey using accumulation curve extrapolation and the non-parametric Chao1 formula. The estimated number of VCG's compared to the maximum number of VCG's based on the currently known vegetative incompatibility genes suggest regular outcrossing in this fungus. Also the difference in sexual compatibility reactions of mating types of the same isolate assumes outcrossing takes place. Options for outcrossing in *P. anserina* were experimentally verified. Both single mating type monokaryotic and dikaryotic double mating type mycelial cultures proved capable of outcrossing, showing no preference for either genotype. This indicates that fertilization by selfing and outcrossing uses the same pathway. Outcrossing percentages between 1-5 percent were found in unmanipulated natural situations of ascospores on dung. The number of monokaryotic ascospores found in Spore-killer (*Psk*) strains was significantly higher than in other isolates, showing an enhancement to outcrossing in these strains.

The findings in this thesis contribute to the understanding of the population dynamics and evolution of two types of selfish elements, linear plasmids and segregation distorters, in the ascomycete fungus *Podospora anserina.* Furthermore it increases the knowledge on genomic conflicts caused by these types of selfish elements in general.

Samenvatting

Dit proefschrift handelt over genomische conflicten die veroorzaakt worden door zelfzuchtige elementen in de ascomycete schimmel *Podospora anserina*. Genomische conflicten ontstaan wanneer de effecten van de zelfzuchtige elementen tegengesteld zijn aan de belangen van de rest van het genoom. Twee soorten zelfzuchtige elementen zijn onderzocht in dit proefschrift, alsmede eigenschappen van *Podospora* die van belang zijn voor de populatie dynamiek van deze elementen, zoals vegetatieve en seksuele incompatibiliteit, veroudering en kruising. De natuurlijke leefomgeving van *Podospora* is de mest van herbivoren. De schimmel groeit hier optimaal bij een temperatuur van 27 °C. De schimmel kan zich alleen seksueel voortplanten en de ascosporen zijn zowel de producten van de meiose als de volgende generatie. De vruchtlichamen, ook wel perithecia genoemd, bevatten asci met vier lineair geordende ascosporen. Dit biedt unieke mogelijkheden tot genetische analyse van abnormale segregatiepatronen en hierdoor is de schimmel één van de genetische modelorganismen. Bijna iedere ascospore is in staat om de levenscyclus van de schimmel te voltooien omdat ze twee kernen, met elk één van de benodigde paringstypen bevatten. Deze eigenschap van de schimmel wordt pseudo- of secundaire homothallie genoemd, en het stelt de schimmel in staat seksuele nakomelingen te maken, met of zonder hulp van een andere schimmelpartner. Soms worden er kleinere éénkernige ascosporen geproduceerd met slechts één paringstype die ook minder cytoplasma en mitochondriën bevatten. Kolonies afkomstig van deze sporen moeten met een andere schimmel kruisen om nakomelingen te produceren. De in dit proefschrift gebruikte schimmelstammen zijn uit mest geïsoleerd die gedurende 1991-1997 is verzameld rond Wageningen. Daarnaast zijn enkele Franse standaard isolaten gebruikt die uit 1937 stammen.

De eerste soort zelfzuchtige elementen die bestudeerd zijn in Hoofdstuk 2, zijn lineaire plasmiden die homologie vertonen met het pAL2-1 plasmide. Dit plasmide is 8.3 kb groot en enige jaren eerder in deze schimmel gevonden. Lineaire plasmiden zijn parasitaire genetische elementen die zich zelfstandig kunnen vermenigvuldigen. In hyphe-vormende schimmels bevinden deze plasmiden zich in de mitochondriën. De meeste plasmiden hebben een onbekend of een negatief effect op de gezondheid van hun gastheer. Het pAL2-1 plasmide dat in *P. anserina* gevonden is wordt echter geassocieerd met langlevendheid, hoewel recent ook negatieve gezondheidsaspecten gevonden zijn. We hebben homologe lineaire plasmiden en plasmide families gevonden in 21 procent van de populatie, alsmede over meerdere jaren. De helft van de plasmiden bevattende isolaten bezat één enkele plasmide, de andere helft een uit meerdere kopieën bestaande plasmide familie. De levensduur van de schimmel-isolaten was niet gecorreleerd met de aanwezigheid van het plasmide. Slechts één isolaat bezat een

verlengde levensduur, maar hier was het plasmide niet geïnserteerd in het mitochondriële genoom zoals bij het langlevende plasmide pAL2-1.

De dynamiek van de overdracht van deze plasmiden hebben we nader onderzocht. Verticale overdracht naar de ascosporen vindt alleen plaats via de moederlijke lijn. Deze overdracht is echter inefficiënt; tot wel 40 procent van de ascosporen in een kruising verliest zijn plasmiden en tot 20 procent behoudt alleen de basis plasmide wanneer een plasmide familie aanwezig is. Er is geen verschil ontdekt in overdracht naar de tweekernige en de kleinere éénkernige sporen. Verder is de overdracht van plasmiden onafhankelijk bevonden van de genetische achtergrond van de isolaten. Verlies van plasmiden via de seksuele route wordt gecompenseerd door horizontale overdracht via hyphen contact tussen verschillende isolaten.

Horizontale overdracht is erg efficiënt in zowel vegetatief compatibele als incompatibele situaties. Vegetatieve incompatibiliteit is macroscopisch zichtbaar als een 'barrage' een contactzone van afgestorven hyphen. Het ontstaan van vegetatieve incompatibiliteit kan mogelijk verklaard worden als een mechanisme dat de verspreiding van mobiele zelfzuchtige elementen en parasieten tegenwerkt. Onze experimenten tonen echter aan dat vegetatieve incompatibiliteit geen perfecte barrière tegen dit soort zelfzuchtige elementen vormt.

Het andere type zelfzuchtige element dat in dit proefschrift is bestudeerd zijn segregatie verstoorders of meiotische 'drive' elementen. Segregatie verstoorders komen in meer dan de eerlijke 50 procent van genetische wetten Mendel in het nageslacht terecht, doordat ze gedurende de meiose actief degenen die de elementen niet dragen vernietigen. Genomische conflicten ontstaan hier doordat genen met negatieve gezondheidseffecten met de segregatie verstoorders mee kunnen liften. Meiotische 'drive' in *Podospora* kenmerkt zich door de abortie van twee van de vier sporen in de ascus. Hoofdstuk 3 karakteriseert de zeven verschillende groepen van segregatie verstoorders of 'Spore-Killer' typen die zijn ontdekt. Zes van deze Spore-killer groepen zijn aangetroffen in 99 isolaten uit Wageningen. In totaal bestaat 23 procent van de Wageningse *Podospora* populatie uit deze Spore killers en de meeste elementen kunnen over meerdere jaren worden teruggevonden. Spore-killer type *Psk-7* is ook in een Franse stam daterend uit 1937 gevonden en bestaat al ruim 60 jaar in deze schimmel. Er zijn geen resistente isolaten gevonden; alle overige isolaten zijn gevoelig voor ascospore abortie. Ieder Spore-killer type verschilt van elkaar in het percentage asci dat geaborteerde sporen laat zien, variërend van 50 tot 95 procent tweesporige asci, alsmede hun onderlinge interacties. De geaborteerde ascosporen vergaan vlak na de vorming van de spore wand, behalve voor *Psk-3* waar ze als kleine verschrompelde sporen zichtbaar blijven. Dit is een aanwijzing dat de betrokken factoren of de timing van abortie bij de Spore-killer typen onderling verschillen. De *Psk-3* groep laat tevens een variabel percentage tweesporige asci per vruchtlichaam zien. Interacties tussen de Spore-killer groepen laten ofwel onderlinge resistentie (dwz. er is geen abortie wanneer één van beide Spore killers aanwezig is in een spore) ofwel dominante epistasie (dwz. één Spore killer gedraagt zich als een sensitief in de

interactie). Genetische koppelings analyse plaatst de genen van de meeste Spore-killer typen op koppelingsgroep III van *Podospora*, maar niet gekoppeld aan het centromeer.

Verschillende mogelijke modellen die het mechanisme van ascospore abortie in *Podospora anserina* kunnen verklaren zijn onderzocht. Herhaaldelijk terugkruisen van Spore killers met hetzelfde sensitieve isolaat produceert stammen die een zelfde genetische achtergrond bezitten. Hoofdstuk 4 laat zien dat Spore killers die tot dezelfde groep of die onderling resistent zijn vegetatief compatibel worden gedurende de terugkruisingen. Dit in tegenstelling tot het sensitieve isolaat en de Spore killers die dominante epistasie vertonen, welke vegetatief incompatibel blijven. Dit suggereert dat ascospore abortie een gemeenschappelijk mechanisme bezit dat gerelateerd is aan vegetatieve incompatibiliteit. De precieze genetische rol van deze correlatie is echter nog niet duidelijk. Het genoom van *Podospora* is in Hoofdstuk 5 doorgelicht op homologe genen waarvan bekend is dat ze betrokken zijn bij verhinderen van de expressie (‘silencing’) van genen in schimmels. Er zijn genen gevonden voor alle bekende ‘silencing’ mechanismen (RIP, MSUD en Quelling) van de verwante schimmel *Neurospora*. De rol van ‘silencing’ door methylatie van genen als oorzaak van de abortie van ascosporen werd uitgesloten via experimenten met de stof 5-azacytidine. Deze stof verwijdert bestaande methylering en verhindert het optreden van nieuwe methylering van het genoom. Er is geen effect van 5-azacytidine op het percentage ascospore abortie bij alle Spore-killer typen gevonden. Hoofdstuk 5 onderzoekt ook de consequenties van de vorming van tweekernige sporen op de expressie van de ascospore abortie factoren. Hiervoor zijn kruisingen gebruikt met een marker die het aantal éénkernige sporen verhoogt tot een maximum van acht per ascus. Spore-killer typen *Psk-2*, *Psk-3* (Wa27) en *Psk-4* lieten in deze kruisingen ook enige asci zien waarin zich meer dan vier sporen bevonden. In dergelijke asci zijn de sensitieve kernen in staat het abortie proces te overleven en het meiotische ‘drive’ mechanisme te weerstaan. Dit wijst op een incomplete penetratie van de abortie factoren. Ascospore abortie in de andere Spore-killer groepen (*Psk-1*, *Psk-5*, *Psk-6* en *Psk-7*) kenmerkt zich als volledig penetrant; alleen asci met vier of minder sporen worden aangetroffen. Hier werken de abortie factoren gelijkmatig op alle asci. Verder is het effect van lage temperatuur (22 °C) op ascospore abortie in dit hoofdstuk getest. *Psk-2* liet een spectaculaire afname van het percentage asci met abortie tot vrijwel nul zien. De overige Spore-killer groepen hebben geen last van dit soort temperatuurseffecten. Alle karakteristieken en gegevens van de verschillende Spore-killer groepen samen genomen stellen we dat ascospore abortie in *Podospora* een voorbeeld is van post-segregationele killing, veroorzaakt door een Gif-Antigif systeem. De Spore killer produceert gelijktijdig zowel een persistent stabiel gif als een minder stabiel antigif. In ascosporen waar de Spore killer afwezig is verdwijnt het antigif sneller dan het gif, wat leidt tot abortie van de sporen. De variabele abortie percentages kunnen verklaard worden door een strikte balans tussen de hoeveelheden giffen en antigiffen, alsmede het tijdstip waarop de betrokken genen worden afgesloten.

Segregatie verstoring is alleen mogelijk in situatie waarbij kruising optreedt. *Podospora* is in principe in staat om zowel nakomelingen door zelfbevruchting als door kruising te verkrijgen, maar in welke mate de schimmel met een andere partner in de natuur kruist is

onbekend. Hoofdstuk 6 bestudeert de mate van kruising in de secundair homothallische schimmel *Podospora anserina*. We hebben een overzicht gemaakt van het voorkomen van vegetatieve en sexuele compatibiliteit in onze wild-type isolaten. Hieruit is het aantal vegetatieve compatibilieits groepen (VCG's) in de populatie geschat gebruikmakend van extrapolatie van de VCG accumulatie curve en de niet-parametrische Chao1 formule. Het geschatte aantal VCG's is vergeleken met het maximaal mogelijke aantal VCG's, gebaseerd op het momenteel bekende aantal vegetatieve incompatibiliteits genen, en suggereert dat kruising met andere partners in deze schimmel regelmatig voorkomt. Ook de gevonden verschillen in seksuele compatibiliteitsreacties tussen beide paringstypen van hetzelfde isolaat veronderstelt dat uitkruising plaats vindt. De verschillende mogelijkheden om te kruisen voor *P.anserina* zijn eveneens experimenteel geverifieerd in dit hoofdstuk. Zowel isolaten met één enkel paringstype als met een dubbel paringstype isolaten zijn in staat te kruisen en tonen geen voorkeur voor het eigen of het andere genotype. Dit is een indicatie dat bevruchting zowel bij kruising als zelfbevruchting gebruikt maakt van hetzelfde systeem. Het percentage kruising in een ongemanipuleerde situatie van ascospore mengsels op mest varieert tussen 1-5 procent. Het aantal éénkernige ascosporen dat wordt gevonden in Spore-killer isolaten ligt significant hoger dan in de sensitieve isolaten, hetgeen een versterking van het voorkomen van kruising in deze stammen betekent.

De bevindingen van dit proefschrift dragen bij tot het begrip van de populatie dynamiek en de evolutie van deze twee soorten zelfzuchtige elementen, de lineaire plasmiden en de segregatie verstoorders, in de ascomycete schimmel *Podospora anserina*. Bovendien vergroot het de kennis van genomische conflicten die door dit soort zelfzuchtige genetische elementen worden veroorzaakt.

Nawoord

Het voltooien van dit proefschrift is een kleine tienjarige evolutie vol met mutatie, selectie en selfish elements. Vele menselijke genomen hebben bijgedragen aan de totstandkoming van dit wetenschappelijke werk over het genoom van *Podospora*, zowel met praktische als mentale steun. Ik dank een ieder die aan dit proefschrift heeft bijgedragen. Een aantal van deze personen wil ik met name noemen.

Inge Haspels dank ik voor de isolatie van de eerste Wageningse *Podospora anserina* stam uit een konijnenkeutel, die tegelijkertijd de eerste Spore killer bleek te zijn. Dit leidde vervolgens tot het onderzoeksvoorstel waaraan ik zolang heb mogen werken.

Fons Debets dank ik bijzonder voor de continue stimulans en stroom ideeën die je aan het onderzoek hebt gegeven. Bovenal bleef je volhouden dat het de moeite waard was het proefschrift na al die jaren af te maken. Het afgelopen paar jaar heb ik dan ook op jouw kamer parasiterend mijn proefschrift in alle 'rust' kunnen voltooien. Rolf Hoekstra is een van de prettigste promotoren die een OIO zich kan wensen. Niet alleen voor de theoretische aspecten van de evolutie, maar zeker ook zijn 'unselfish' gastvrijheid moet genoemd worden.

Alle studenten en stagaires die in meer en mindere mate bijgedragen hebben aan het *Podospora* onderzoek wil ik bedanken voor hun inzet en, niet minder belangrijk, hun gezelligheid op het lab: Robin Hartman, Wouter van der Meer (voor alle serieuze en minder serieuze 'raket'proeven), Jessica Thijssen en Jessica Oosterhof, Marcel Velner, Jaap de Roode, Marlinde Hoek en Jarco Smeenk. Ook studenten van het vak evolutionaire ecologie hebben voor mij waardevol onderzoek geleverd, waar in dit proefschrift stukken van terug te vinden zijn. Mariska te Beest, Jorit Dekker, Peter Dijkstra, André van Esburg, Wilbert Flier, Marc Maas, Martijn Timmermans en Maurice van Veen dank ik voor hun *Podospora* resultaten in hun 'Annals of the Royal Talisker Society', 'Journal of Natural Born Killers' en 'Journal of Irreproduceble Results',

Zonder de gulle gaven van Ralf van Dijk zouden de Wageningse *Podospora*'s honger hebben moeten lijden. Hartelijk dank voor alle paardenmest die ik mocht halen van jou weide.

Natuurlijk mogen de mede-AIO en OIO's en overige medewerkers van de vakgroep Erfelijkheidheidsleer niet vergeten worden. Door hen werd het werken bijzonder plezierig gemaakt en heb ik me dan ook volkomen thuis gevoeld. Duur Aanen, Peter van Baarlen, Stefan Bosmans, Judith Bruggeman, Alex Coenen, Tamaz Czaran, Henk Dalstra, Michelle Habets, Edu Holub, Hans de Jong, Gerda Kobus, Aafke van der Kooi, Marc Maas, Anita Mulder, Maarten Nauta, Karoly Pal, Merijn Salverda, Ronny Schoonenberg, Siemen Schoustra, Marijke Slakhorst, Klaas Swart, Arjan de Visser, Bert Wennekes en alle anderen hiervoor bedankt.

Zonder hulp van de volgende vrienden zou het schrijven van het proefschrift nog veeeel langer hebben geduurd. Ze hebben belangeloos tijd vrij gemaakt om met Alarik te spelen en

op te passen. Caroline en Veronique Wilmer, Leendert en Lenie van der Gaag, Steeph en Jitske van Diepeningen, Bram, Katinka, Iris en Ariane van der Pas en Jannie, Bas, Zina en Sander Suverkropp hiervoor bijzonder bedankt.

Als allerbelangrijkste wil ik Anne en Alarik bedanken. Niet alleen voor hun bijdragen tot de totstandkoming van dit proefschrift, maar 'gewoon' door er altijd bij en voor mij te zijn. Zij beiden zijn het belangrijkste in mijn leven.

Marijn.

Curriculum vitae

Marijn van der Gaag is geboren op 23 november 1965 te Wageningen. Na het behalen van het VWO diploma aan het Streeklyceum te Ede begon hij in 1984 met de studie Plantenziektenkunde aan de Landbouwhogeschool te Wageningen. Gedurende 1988 zat hij in het bestuur van de studentenvereniging SSR-W. In 1990 studeerde hij af aan de Landbouw Universiteit Wageningen met afstudeervakken in de fytopathologie en plantenveredeling en een stage bij het Scottish Crop Research Institute te Invergowrie. Daarna was hij werkzaam als onderzoeker en microbiologisch analist op de vakgroep Erfelijkheidsleer van de Wageningen universiteit. In 1993 trad hij in dienst als OIO aan de leerstoelgroep Populatie en Schimmelgenetica van de Wageningen Universiteit, waar hij aan het onderwerp van dit proefschrift heeft gewerkt. In trad hij in dienst van Aqeuro Chain als database en applicatie programmeur voor het internationale soft en hardware bedrijf Unisys NV, waarvoor diverse detacherings opdrachten zijn uitgevoerd. Van 2002 tot 2004 werkte hij als bioinformaticus bij het cluster Applied Bioinformatics van Plant Research International PRI en de leerstoelgroep Genome Informatics van de Wageningen Universiteit. Daarnaast houdt Marijn zich ook bezig met meubelmaken, de Aziatische kookkunst, het ontwerpen van bordspelen en het rondlopen als ‘14e-eeuwse ridder’ in harnas.

Het onderzoek beschreven in dit proefschrift is uitgevoerd met een subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO-ALW).