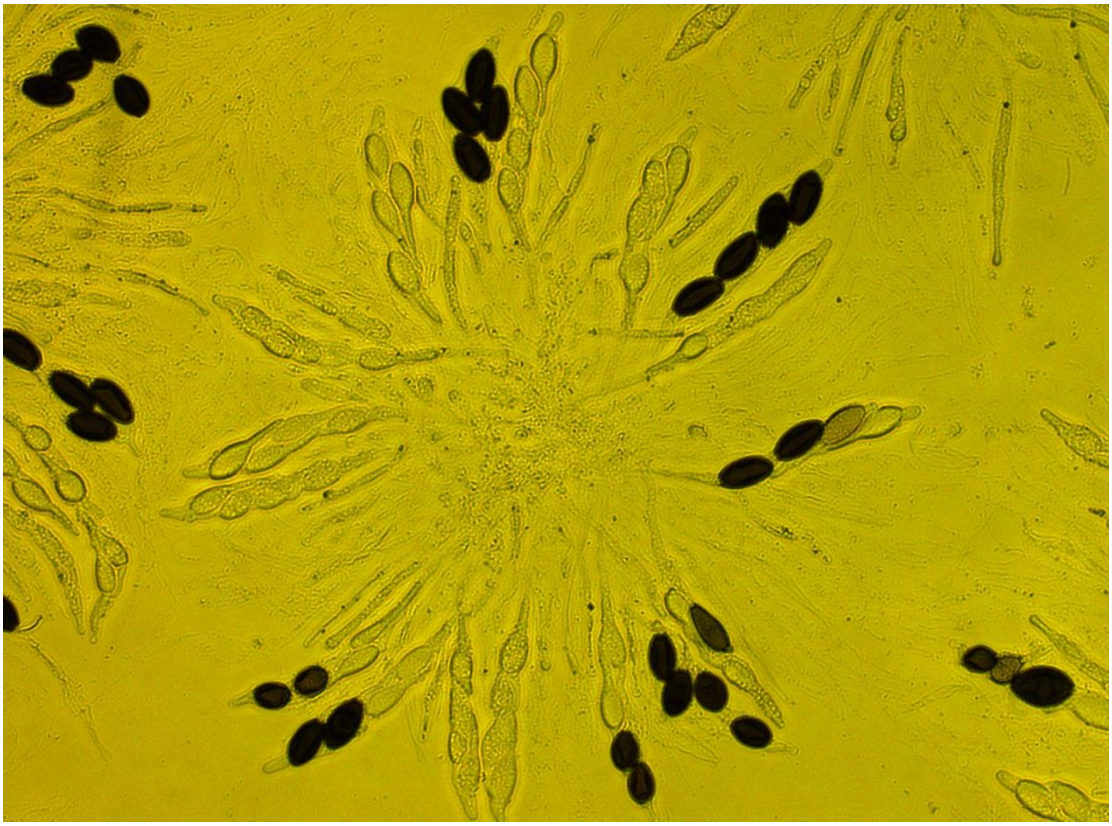
Interaction of Meiotic Drive Elements in *Podospora anserina*



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Abstract

Psks and [Het-s] are the two elements responsible for segregation distorter in *Podospora anserina*. When either of these elements is present in a cross to a sensitive strain, spore killing will occur where the surviving spores possess the killer element. The various Psk elements were previously classified based on their interaction. When strains with different Psk elements are crossed, either mutual resistance or dominant epistasis is observed, but the interaction of these Psk elements to [Het-s] has not been studied before. Here we study the interaction between [Het-s] and the other Psk elements. Strains were selected to represent each Psk-killer element of *het-S* genotype (wa 86 as Psk 1, wa 28 as Psk 2 , wa 47 as Psk 6), and crossed to [Het-s] strains Wa3 and pGPD het-s (a lab strain that overexpresses [Het-s]). Thus each strain will only have one killer element. Results showed that the interaction of the two killer elements are independent of one another when being confronted in an interaction. It was also deduced that pGPD het-s may be a dominant epistatic killer due to 83% formation of 2-spored asci when confronted with Psk 6. Interaction between Psk 6 and het-s, however gave a killing characteristic typical of Psk 6. No mutual interaction between the two killer elements were found.

Introduction

Meiotic drive, also known as segregation distorter or "preferential segregation", is the phenomenon that one member of a pair of alleles (or chromosomes) is transmitted to progeny in excess of the expected Mendelian fraction of 50%. Sandler and Novitski (1957) coined the term *meiotic drive*, but the first case of meiotic drive was observed by Gershensen (1928) in *Drosophila obscura* as a segregation distortion of the sexes. In his study, he found that some affected male *Drosophila sp.* could not bear males and in turn, produced up to 96% females. Later Rhoades (1942) observed 'preferential segregation' of one of the two chromosomes in corn that is heterozygous at chromosome 10.. In the cases mentioned above, clearly, these situations do not conform to the first law of Mendel which states that a gamete of a heterozygous individual will have an equal chance of being transmitted to the next generation. This is a very central principal that has governed our genetics understanding. However, when a population has one or several driving elements within the population, this causes a population to be off-balanced either in sex proportions or distribution of a gene(s). There are several meiotic drive phenomena observed in several populations of organisms namely, fruit fly, mice, mosquito among others. Little is known about the various mechanisms of meiotic drive. In fruit fly *Drosophila sp.* for example, a gamete carrying a segregation distorter gene will cause another gamete carrying a responder gene to be non-functional and therefore only gametes with a distorter gene will be transmitted to its progeny. In ascomycete fungi with ordered tetrads like *Podospora anserina* and *Neurospora spp* meiotic drive has been observed as spore killing in crosses between isolates from natural populations. However, its mechanism is likely different where it requires the allele carrying the killer element to also be resistant in order to prevent from killing its own self. Therefore, there are two elements required for resistance and ability to kill in order to survive and dominate the population (Hammond et al., 2012). It is noteworthy to state that the phenomenon of meiotic drive is not easily seen because more often than not it happens in an unobservable phenotype and it is often overlooked and it is often found coincidentally. The advantage of studying meiotic drive in spore-forming fungi such as *P.anserina* is that the phenotype can be observed directly in the spores and the genotype can be attributed to a single locus because of the haploid nature of the organism.

In the case of meiotic drive, one allele or in the case of *Podospora anserina,* one ascospore type is over-represented relative to the other. What is intriguing is that there are two very distinct and very different types of meiotic drive elements present in *P. anserina*. They are the [Het-s] prion which is also involved in heterokaryon incompatibility (a self/non-self-recognition system) and at least seven other *Podospora* spore-killers (*Psk*) of unknown function. There are quite a number of studies concerning the mode of action of each of them, however, so far the interaction between the two elements is not yet known. Both [Het-s] and *Psk* are capable of spore killing with [Het-s] through prion transmission (Debets et al., 2012) and *Psk* through a yet unknown mechanism. A toxin-antitoxin interaction has been suggested to explain the observation that from a *P.anserina* cross heterozygous for a Psk-element, only ascospores survive that carry at least one nucleus with the Psk-killer allele, whereas ascospores with only the Psk-sensitive alleles will abort (Van der Gaag, 2003). Interestingly, there are several *Psks* that have been classified; *Psk 1, Psk 2, Psk 3, Psk 4, Psk 5, Psk 6, and Psk 7.*

Spore killing by *het-s*

According to Debets et.al (2012), *het-s* allele that is capable of prion formation appear two times more frequent than *het-S* in a population of 112 individuals. With the *het-s* gene being the gene for vegetative incompatibility, no spore killing is observed when *P.anserina* is propagating in a vegetative manner or somatically with *het-S* strain. This is because the *het* genes including *het-s* need to be identical in all loci to be able to form heterokaryons . Hyphal fusion between [het-s] (i.e. a strain of *het-s* genotype with the prion protein) and *het-S* cultures (containing the antagonistic HET-S protein) result in barrage formation where one distinct line indicates dead cells from the fusion of the two strains. In sexual interaction between [het-s] and *het-S,* [Het-s] is able to kill ascospores of genotype *het-S*. Bernet (1965) observed that killing took place at a very restrictive temperature of 18°C when [Het-s] and *het-S* were crossed with [Het-s] acting as the maternal counterpart. In another study, Saupe (2000) found that there was 50% chance that the progeny are [Het-s*] (not having the prion) when the [Het-s] carrier is the father. In contrast, the proportion of progeny that are [Het-s*] are very low in a cross between a maternal *het-s* and paternal *het-S.* This shows that the prion was transmitted in the cytoplasm and since cytoplasm is

inherited maternally, there is a much greater chance of killing when [het-s] is carried maternally in a cross. Besides that, the low temperature factor is of course important in preventing the degradation of infectious protein such as prion and ensuring prion persistence in early asci (Dalstra et al., 2003). It is not however, the case in strains overexpressing *het*-s where regardless of temperature, *het*-s is able to penetrate completely where almost 96% of ascus showed killing. This, of course, does not happen naturally in the wild and the pGPD het-s strain is developed in the lab. Prion curing by *het-S* however had so far been hypothesized as one of the reasons *het-s* has not come to fixation in the natural population just yet.

Naturally, the *het-S* and *het-s* alleles act hand in hand for a balancing selection. Both alleles are actually 97% identical to each other according to Greenwald *et al.,* (2010). In his study he concluded that prion inhibition is not encoded by structure but rather in stability and in oligomerization properties. In an interaction between [Het-S] and [Het-s] when [Het-S] forms a prion seed or is incorporated into a [Het-s] fibril via its prion-forming domain (PFD), the B-structuring in this domain induces a change in its globular domain that produces an unstable molecule that is not able for fibril growth (Greenwald *et al*., 2010). Both alleles have its own PFD and an N-terminal globular domain, therefore, Het-S is able to protect itself from its own prion and also protect itself from the prion protein from Het-s (Greenwald *et al.,* 2010). This statement is supported by (Beisson-Schecroun 1962; Dalstra et al., 2003; Rizet, 1952) when they found that in vivo interactions between [Het-s] and [Het-S] can render [Het-s] free from its prion state. This can occur through a mechanism called prion curing which only happened in a sexual cross. In a vegetative interaction, [Het-S] can strongly inhibit mycelium propagation of [Het-s] (Beisson-Schecroun, 1962). From the statements above, we can conclude that [Het-S] can not only trigger cell death on itself in an interaction with [Het-s] but also prevent [Het-s] propagation in both sexual and vegetative interaction. This tells us that [Het-s] is not as infectious as we would think because it can only infect itself; [Het-s*] to [Het-s] and not [Het-S]. Therefore, it is noteworthy to know that there is a balancing interaction between the two alleles in the population with [Het-S] able to defend itself from [Het-s] and [Het-s] only able to infect its own kind and not converting [Het-S] to be one of them. However, is the defence stronger than the conversion? Is it [Het-s] or [Het-S] that mediates the killing? Test results performed by Balguerie et al. (2004) found that the globular domain of [Het-S] is essential for *het-s*-mediated Programmed Cell Death (PCD) of [Het-S]. Since the globular domain of [Het-S] and [Het-s] are specific for incompatibility, (Balguerie at al., 2004) deletion in this region of Het-S will automatically decrease the prion-inhibitory effect and incompatibility activity of Het-S. Thus, the Het-S globular domain is essential for PCD. The above information clearly illustrates that Het-S has two strategies to defend itself from het-s self-killing and prion inhibition but clearly, het-s does not have a defence mechanism against Het-S. It can be concluded that, het-s population will not be fixed due to the superiority of Het-S.

Spore killing by Psks

Another type of spore abortion in *P.anserina* is done by the different types of *Psk*-elements (*Psks*). These *Psk*s have a differential magnitude of killing depending on temperature and the type of *Psk* that they possessed. Therefore, with certain *Psk*s, full penetrance or complete killing will be observed while in some *Psk*s incomplete penetrance will be observed. An observation done by Van de Gaag et al. (2000) showed that *Psk* 1 has an ability to kill completely with full penetrance with the killing being at a very early stage of ascospore formation (Debets, personal communication) and *Psk* 2 in which killing is higher with increasing temperature. It is interesting to see how in different meiotic drive systems, the factors governing it is also different. As discussed in the previous paragraph, the *het-s/het-S* system requires low temperature to exhibit killing and on the other hand, at least some of the *Psk* systems require a higher temperature to function as a killer. It was really notable to see in *Psk 2* where a drop in temperature significantly decreased the percentage of two-spored asci. In the same findings of Van der Gaag (2000), among the spore killers *Psk* 1 and *Psk* 7 are the most superior with the ability to kill not only ascospore carrying the sensitive allele but also ascospore carrying other *Psk* allele types. Moreover, heterokaryotic ascospores that carry both killer and sensitive allele as a result of crossing over during meiosis I ended up surviving.

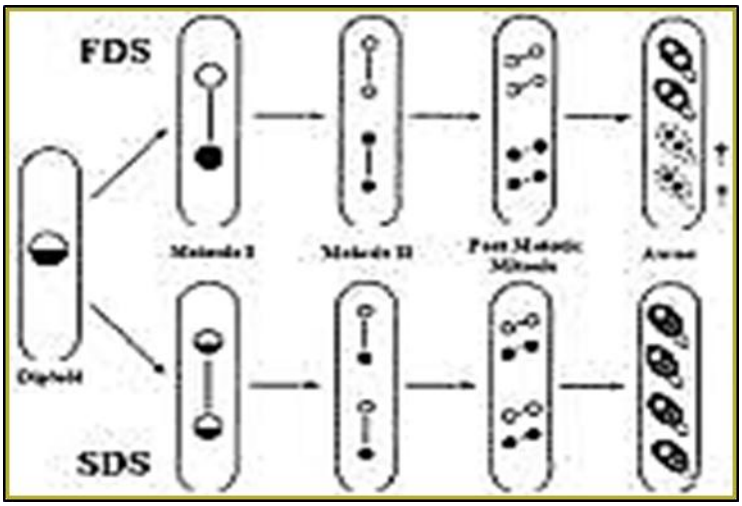

Figure 1: Indication of how First Division Segregation (FDS) and Second Division Segregation (SDS) affect the fate of ascospores with sensitive alleles. Ascospores are dikaryotic and are either homokaryotic or heterokaryotic for killer and sensitive alleles. Since only ascospores that carry a killer allele survive, sensitive alleles are rescued by a crossover since this results in SDS. The same situation also occurs with the *het-s* and *het-S* system where either a crossover between centromere and the *het-s/het-S* locus or prion curing of [het-s] *to* [het-s]* can result in normal four spore formation. Figure source: (Van der Gaag, 2000)

In a published book by Osiewacz (2002), he mentioned that this inactivating change is rather epigenetic than genetic. Since it has been shown that killing of ascospores is not the result of silencing, it could be true that no killing in heterokaryotic formation is the result of epigenetic by silencing.

Association of *Psks*, *het-S* and *het-s* alles in natural population of *P.anserina*

When two killers are present in one cross, we could not help but question if the two are linked. And, are they tightly linked or associated with one another? And if so, is the association between different spore killers the same in a natural population of *P.anserina,* 60% of Psk have an association with het-s. Among them, Psk 1 is largely associated to het-s with 90% of Psk 1 type-killers also having the other killer allele, het-s. Other Psks however, have a more balanced association with het-s. So far, only wa 86 is Psk 1 and het-S which suggests that recombination between them are very rare. This also indicates that Psk 1 and het-s might be tightly linked to each other. From the research carried out by Van der Gaag (2000), Psk 1 belongs to Linkage Group III. When crossed with a centromere linked marker of chromosome III, *Cs2, Psk 1* showed 0% of Non-Parental Ditypes (NPD) which means that there are no recombinants produced from the cross which indicates a very tight linkage. Therefore, since P*sk 1* is associated with *het-s,* the two have to be linked, in fact tightly linked to each other. And since for other Psks there is no linkage disequilibrium with het-s, it can be inferred that they are not so closely linked or *het-s* might even be in another linkage group. However, the latter hypothesis cannot be true due to the association established by *Psk 1* and *het-s.* This is supported by the findings of Van de Gaag (2000) that *Psk 1,Psk 2, Psk 5 and Psk 7* belonged to Linkage Group III. As of now, we can speculate that *Psk 1 and het-s* are localized close to the centromere and in close proximity to each other while *Psk 2,Psk 5* and *Psk 7* are localized a little further from *het-s.* Alternatively, the polymorphism is so ancient that over time the initial linkage disequilibrium has been lost.

With both systems of meiotic drive being clarified, it is the importance of this research to find out the interactions between the two elements when crossed together. This is interesting to see since both elements operate at a different level (optimum temperature *etc.*). Psks kill through toxin-antitoxin activity and *het-s*, on the other hand, kills via prion formation In this study we provided evidence that the two killer elements are independent of each other because they have different mode of killing. So far into this research, neither mutual resistance nor mutual sensitivity was found between the two killers. However, only one element will eventually wins or survives the interaction. In the interaction between het-s and Psk 1, 2 and 7, early killing of ascospores displays the actions of Psk-type killing. On the other

hand, when being confronted by pGPD het-s, the overexpressed version of het-s seemed to be taking the upper hand at killing the ascospores. The killing frequency increased by ten-fold when pGPD het-s is introduced within an interaction.

Materials and Methods

Growing strains and crossings:

Strains were selected from the -80 °C freezer. Strain obtained from mycelial growth is grown on $PASM_2$ while strains obtained from a spore suspension is grown on $PASM_2$ + acetate media. For the purpose of crossing, two monokaryotic (single mating type) strains are grown together in dung media following the confrontation method. Besides that, some crosses follow the spermatization method when either one of the strain is dikaryotic. However, it did not matter if both strains are monokaryotic to use spermatization procedure. This is done by pouring a suspension of microconidia (male) over a monokaryotic mycelium (female). This method usually follow suit when crosses combinations using the confrontation method did not yield any fruiting bodies.

Crosses are then put in the dark for 5-6 days at 27 °C until mycelium has covered the plate entirely before they are transferred into a lit chamber at 27 °C to start the formation of perithicia or fruiting bodies. Strains used are described in the table below:

Table 1: Strain name and genotype

Strain name	**Het-S/Het-s/killer type**
oct 1	Het-s background
wa 86	Psk 1/het-S
wa 28	Psk 2/het-S
wa 47	Psk 6/het-S
wa 3	het-s
wa 7	het-S
pGPD het-s	Overexpressed het-s

Dissection of spores

Under a dissecting microscope from Zeiss Stemi SV 6, Germany, perithicia were burst open to expose the rosette containing the spores. Spores were then transferred onto a glass slide that has been lubricated with glycerol. Excess glycerol was removed with a tissue paper after covering the slides with a glass cover. Edges were sealed with nail polish to preserve the slides longer.

Isolation and determination of single mating type

Double mating type spores were allowed to germinate. After perithicia are formed, the spores resembling and having a single mating type is grown on $PASM_2$ with acetate media for 2 days. Mating type is tested by growing it together with a strain of single mating type; positive and negative mating type. Only opposite mating types are able to germinate. Thus, mating type can be attributed to the spores in question where perithicia are formed.

Backcrosses

After a cross is established and perithicia are formed, two-spored asci that contain the surviving spores are collected and crossed back to both parents using spermatization procedure. If the spores survive the backcross then, the parent that it was crossed to was the one doing the killing.

Microscopy

Slides were prepared for imaging and counting purposes to quantify the killing activity for both crosses and backcrosses. Slides were analyzed using an imaging microscope by Zeiss AxioVision Imager A1. Images of the slides was captured using ZeissAxioCam IC c3 camera and visualized via AxioVision Rel 4.6 programme.

DNA Extraction

DNA was extracted from population wa 3, wa 7, wa 12, wa 86, wa 125 and wa 126 with the LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris-HCl, 0.5% SDS, pH 8) to give a cleaner PCR product. Mycelium was grown on PASM media covered with thin transparent cellophane for 2 to 3 days at 27°C. Mycelium was harvested while avoiding the heavily pigmented parts and stored in a 1.5 mL eppendorf. The harvested mycelium can be kept at -20°C or -80°C until further use. ±5 glass beads were added to the tubes containing the mycelium. Mycelium was frozen with liqiud Nitrogen and ground with a bead beater machine for 10 seconds. The freezing and grinding step was repeated for a second time. 1 mL of LETS buffer was added and mixed by vortexing. The aqueous phase of the mixture was centrifuged for 5 min at 13,000 rpm. 700 µL of the supernatant was transferred into a fresh 2 mL eppendorf tube where consequently 5 µL of Proteinase K was added and later incubated for 1 hour at 37°C. After that, 300 µL of phenol and 300 µL of SEVAG (chloroform:isoamyl = 24:1) were added to the same tube and the mixture was then centrifuged for 15 minutes at 13,000 rpm. 600 µL of the supernatant was transferred to a clean 1.5 mL eppendorf tube where 300 µL of SEVAG was added and then centrifuged for 15 minutes. 500 µL of the supernatant was transferred to another clean 1.5 mL eppendorf tube where 300 µL or 0.6 x volume of isopropanol was added to the mixture. At this point, the mixture can either be stored at -20°C for 15 minutes or -4°C overnight until the next steps. After that, the mixture was centrifuged for another 15 minutes at 13,000 rpm. Supernatant was discarded with a micropipette and 100 µL of 70% ice-cold ethanol was added to wash the pellet carefully (pellet must stay in place). Then, the mixture is centrifuged for 1 minute at 13,000 rpm. Supernatant was discarded and leftover liqiud is dried under a vacuum. Once dry, 50 µL of MiliQ water was added and stored at 4°C overnight to dissolve the DNA. The solution was made to be homogenous by mixing with a micropipette.

PCR

Polymerase Chain Reaction (PCR) was done on wa 12, wa 86, wa 125 and wa 125 populations to determine and to verify their het-S or her-s status with wa 3 (het-s) and wa 7 (het-S) as positive control. BioRad iCycler was used as a PCR machine with the following program:

Cycle 1 (1x) Step 1: 95°C for 4 mins

Cycle 2 (30x) Step 1: 94°C for 1 min

Step 2: 60°C for 1 min

Step 3: 72°C for 1:15 min

Cycle 3 (1x) Step 1: 72°C for 7 mins

Cycle 4 (1x) Step 1: 4°C for ∞

Primers used: Forward Primer: Het-s/S-1: 5'-TGACGGAGGAGCTCGGTTCG-3', Reverse Primer: Het-s/S/sx 2: 5'-CCTGGAAAGAAGCATGATGCCTTTC-3' Melting Temperature: 94°C, Annealing Temperature: 60°C.

Gel Electrophoresis

0.5 g of agarose gel was dissolved in 50 mL 1 x TAE buffer (1% agarose gel in TAE buffer). Agarose was dissolved by melting it in 1x TAE buffer in a microwave for 1 minute on full power. The melted mixture was cooled by running the Erlenmeyer flask under running tap water until the mixture is slightly warm to the touch. Then, 5 µL of Ethidium Bromide was added and the mixture is then poured into a 8 well tray that will accommodate 2 positive controls, 4 samples, 1 blank and 1 marker (λ. DNA digested with Bam H1, Eco R1 and Hind III). 2-5 µL of PCR product was applied to each corresponding well. Electrophoresis was run at 60 V for approximately 90 minutes or until the marker has moved ¾ of the way on the gel. Gel was analyzed with BioRad GelDoc™ XR+ using Image Lab Software as visualization tool.

Results

Crosses:

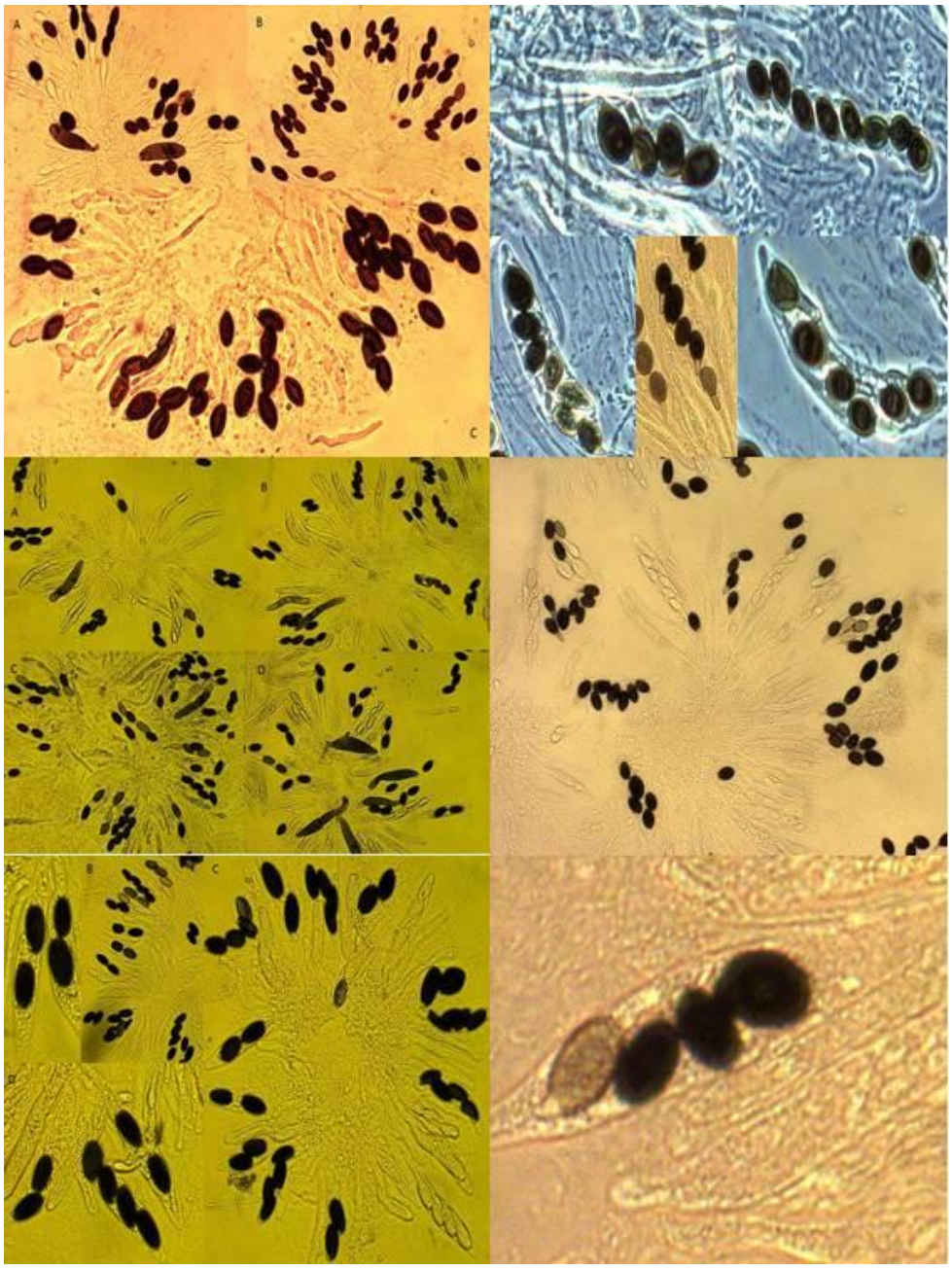

Figure 1: top-left; A cross involving wa 28 (Psk 2) and wa 7 (het-S), top right; Control crosses involving oct 1 (in het-s background) and pGPD het-s, middle left; A cross involving wa7 (het-S) and wa 86 (Psk 1) with wa 7 as a female, middle right; Cross with oct 1 (in het-s background) and wa 3 (het-s), bottom left; A cross involving wa 7 (het-S) and wa 28 (Psk 2) with wa 7 as the female and bottom right; Cross with oct 1 (in het-s background) and wa 47 (Psk 6)

Control crosses involving the oct 1 (in the het-s background) strain to pGPD het-s, wa 3 and wa 47 from top right to bottom right of Figure 1 respectively. Control crosses means that, it will reflect the behaviour of a killer element when it is crossed with another strain containing new killer element. The result of crosses with oct 1 (marker) strain and a killer gave penetrance or the ability of a killer element to give a high or low killing efficiency. For example, full penetrance killers will only display asci that only contains

up to 4 spores while incomplete penetrance killer will display a variant numbers of spores in a rosette from 4 to 8 spores in a rosette. A characteristic of the strain oct is that it is able to produce up to 8 spores in an ascus. Since it is double the amount of spores in asci the strain would normally produce, the space that it requires is quite limiting and it is evident to see especially in a cross between oct 1 and het-S, (Figure 1, middle right).

In a cross between oct 1 and pGPD het-s (top right, Figure 1) a variety of numbers of 4-8 spores are present in an ascus. This is why the cross showed a typical result of selfing where nearly all asci contain 8 spores. The effect of crossing a strain that overexpress het-s and a strain that does not, do not have a big impact on spore killing efficiency or giving a clue what the killing penetrance will be. It has been shown by Coustou, 1997 that crosses between het-s and pGPD het-s will only activate het-s0 (its non-prion form) to het-s (its active prion form). This also supports the statement made by Wickner *et al.* (2004) that prion protein are originated from a protein-encoding gene that have undergone changes such that it is able to alter the unaltered form of other proteins. In short, although no killing occurs, both strains are able to form prions and prion accumulation will double. Also, the cross between het-s and Psk 6 (middle right, Figure 1) showed a typical characteristics of selfing. Apart from that, in the cross between Psk 6 and oct-1 (bottom right, Figure 1), all asci showed evidence of killing. It was observed that only up to 4 spores were formed in the interaction. It can be inferred that Psk 6 may be a full penetrance killer. However, from previous study by Van de Gaag (2000), Psk 6 is also a less efficient killer because there were less 2 spored asci being formed compared to other Psk type of killers. This statement holds true for interaction between Psk 6 and het-s in Figure 2 (top left) and the killing also started early in the ascospore development. It seems as if Psk 6 becomes stronger when confronted with another type of antagonist.

In the left side of Figure 1 starting from top to bottom are crosses involving Psk 2 with het-S, Psk 1 with het-S and het-S with Psk 2 respectively. Crosses involving killers; Psk 1 and Psk 2 with non-killer; het-S could also serve as a control cross to show killing characteristics of the killers. In the cross between Psk 2 and het-S (Figure 1, top left), all asci showed a number of spores that are not more than 4. This showed that Psk 2 is full penetrance killer. It has been reported by Van de Gaag (2000) that Psk 2 type killing are influenced by temperature. The higher the temperature, the higher the killing frequency but change in temperature is only useful during perithicia formation to see a difference in killing percentage. The temperature that the cross is taking place is high enough to show a full penetrance killing characteristics. The same characteristic of killing was also observed when the maternal role is switched to het-S (Figure 1, bottom left). In the cross between Psk 1 and het-S (Figure 1, middle left) Psk 1 is also showing characteristics of a full penetrance killer. From the killing frequency which is 1.77 and 1.28 for Psk 1 and Psk 2 respectively, we can conclude that both are competent killers.

Table 2: Killing frequency in interactions between one and two killer elements

Crosses	2 spores	4 spores	Asci analyzed	Killing frequency
wa 28xwa 7	53	30	114	46.49
wa7xwa86	32	25	74	43.24
wa28xwa3	3	3	21	14.29
pGPD het-s x wa 47	34	2	41	82.93
wa3xwa47	42	33	144	29.17
wa7xwa47	150	170	539	27.83
wa47xwa3	28	17	70	40.00

Referring to Table 1, killing frequency within one killer interaction varied between 0.88-1.77 whereas, killing frequency within two killer interaction varies from 1.00-17.00. The killing frequency value is the ratio of 2-spored asci:4 spored asci. From the table, it was observed that the ratio is closer to 1 in one killer element interaction which might suggest a balancing selection by het-S. Introducing another killer element in an interaction will interfere with the balance.

From the table above, it could be deduced that that Psk2 and het-s may be mutually resistant to each other. They have a lower killing percentage when crossed with each other and higher killing percentage when crossed with sensitive strain, het-S. These may suggest that both alleles may be present in the ascus. According to Van der Gaag (2000) mutually resistant strains showed a much lower killing percentage when confronted with other killer strains but higher killing percentage when crossed with a sensitive cross. The same situation was observed in a cross between Psk 2 and het-s and in a cross between Psk 2 and het-S respectively.

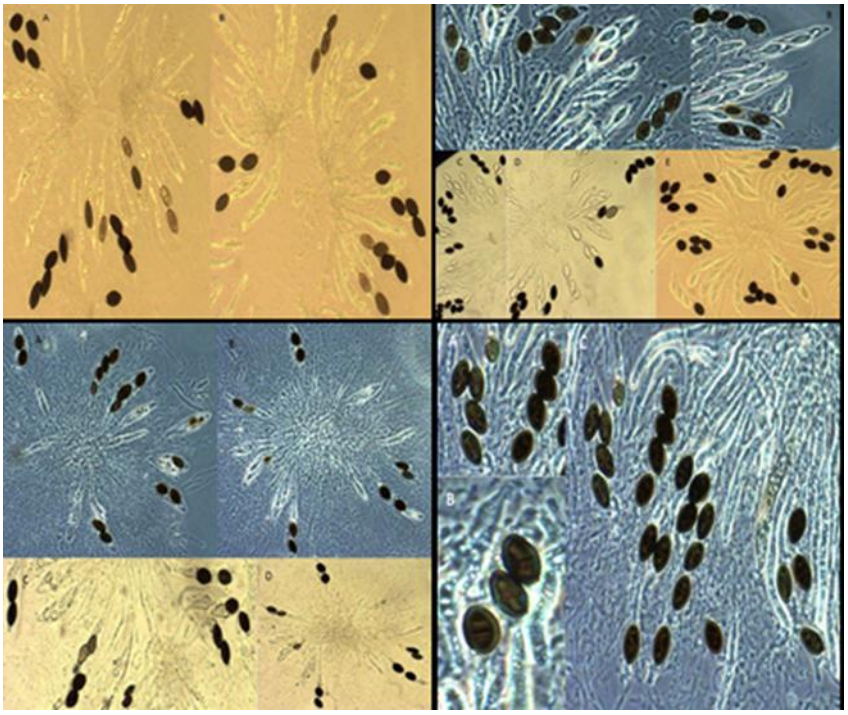

Figure 2: Top left: Cross involving wa 47 (Psk 6) and wa 3 (het-s) with Psk 6 acting as a female. Top right: A cross involving wa 47 (Psk 6) and wa 3 (het-s) with wa 3 as the female donor. Also, the frequency of two-spored asci to four-spored asci being quite low that might indicate a strain other than wa 3 did the killing, Bottom left: Cross involving wa 47 (Psk 6) and pGPD het-s with pGPD het-s as the female. Two killer elements in an interaction with very low frequency of 4-spores among all asci. Bottom right: Cross involving wa 28 (Psk 2) and wa 3 (het-s). Two killer elements in one interaction displaying not more than four ascospore in an ascus

In the top left and top right of Figure 2 are the reciprocal crosses between Psk 6 and het-s. The killing frequency, however, is in fact lower when maternal role is switched to het-s. As reported by Debets (2012), interaction between maternal het-s and paternal het-S will lead to an estimated value of 20% asci being aborted but that was in an interaction with its antagonist Het-S. The same principle might not hold true if het-s is confronted with a completely different set of antagonist; for example Psk 6. The results obtained have proven that indeed in an interaction with another killer, maternal het-s failed to dominate the confrontation. In a reciprocal crosses between Psk 6 and het-s, as low as 1.27 and 1.65 killing frequency value were obtained when het-s was the female donor and when Psk was the female donor respectively. This could only mean that Psk 6 managed to survive the interaction. Since there are so many factors involved in a cross, changing the temperature may give a slight change to the level of the killing frequency, even though het-s may give higher killing at lower temperature. However, it is doubtful if it will give a significant difference especially in an interaction with another killer element. This is because in previous literatures only killing frequency between het-s and het-S were evaluated for killing rate when temperatures were changed. Therefore, a defining comparison could not be made.

On the other hand, crosses involving Psk 6 and an overexpressing het-s strain (pGPD het-s) leads to a significant increase in killing frequency of 17 with 83% of two-spored asci being formed. A significant increase may be due to the result of pGPD het-s being a very strong killer and at the same time making it a possible dominant killer against Psks. However, there was not enough data to support this strong possibility but there was enough to prove that Psk 6 was not an epistatically dominant killer because other confrontational crosses involving Psk 6 did not give similar results as this cross did. Further confrontations involving pGPD het-s with other Psk type killers are required to support this hypothesis.

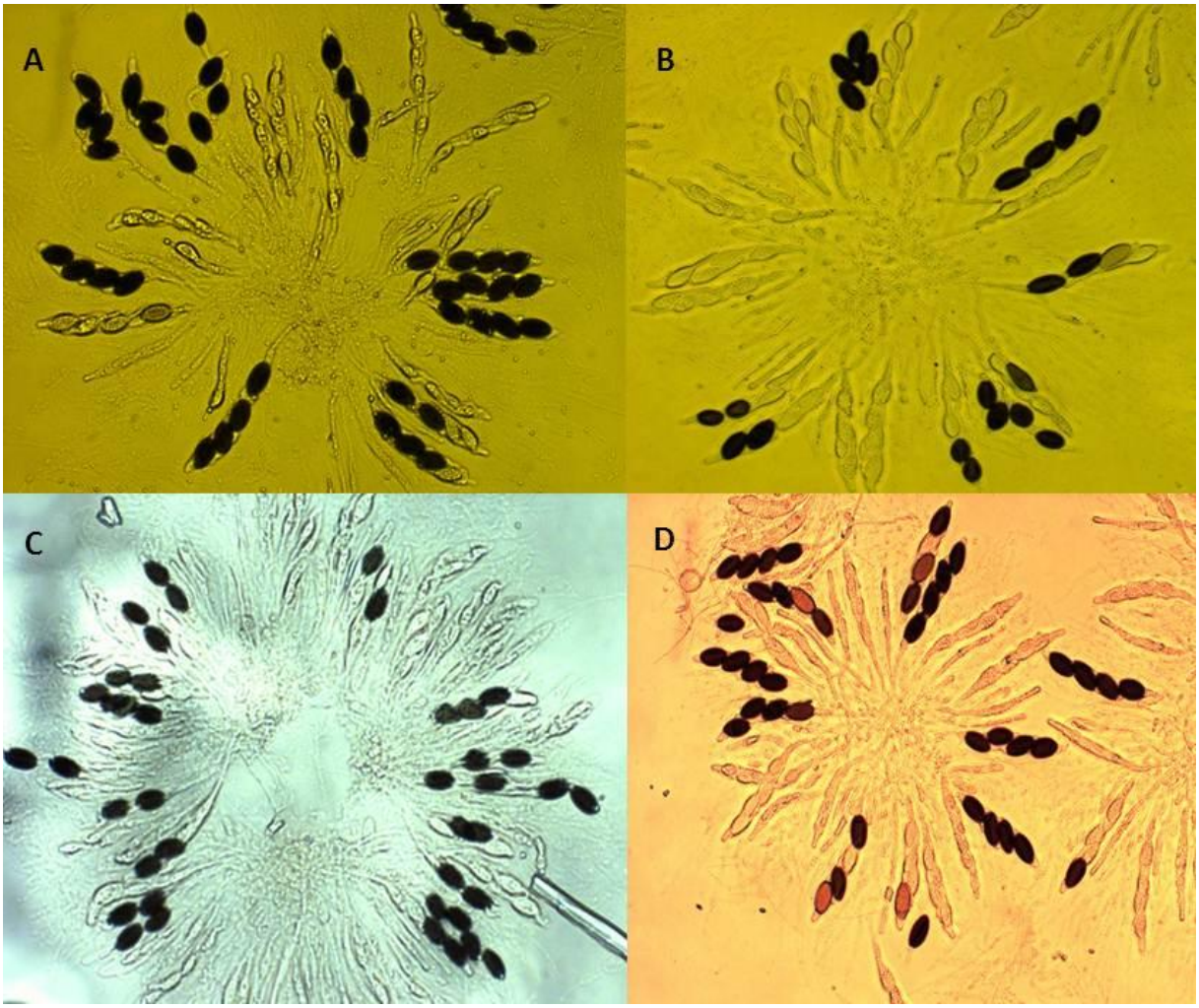

Figure 3: Backcrosses of progeny to its parent; A) asci produced from backcrosses of Psk 6 and pGPD het-s progeny to pGPD het-s, B) asci produced from backcrosses of Psk 6 and pGPD het-s progeny to Psk 6 , C) asci produced from backcross of Psk 6 and and het-S to het-S and D) asci produced from backcross of Psk 6 and het-s (Psk 6) progeny to het-s

Backcrossing is a classical technique in genetics. In a situation like meiotic drive, for example, backcrossing proved to be an effective way to determine the parent of which meiotic drive element is present. The idea behind it is by taking the surviving spores which can only have the allele from one of the parents who's doing the killing. By backcrossing the surviving spores to both parents, the resulting progeny will survive when it is crossed to the parent that share the same allele while in contrast, showed killing when the surviving spores did not share the same allele with another parent. That is normally the case in a normal interaction but sometimes the results may differ depending on the type of interaction the two parents may have. For instance, the progeny from mutually resistant parents may not show killing when they are backcrossed (Van de Gaag, 2000). In Figure 3, the backcrosses of progeny from Psk 6 and pGPD het-s to A) pGPD het-s and B) Psk 6 both showed evidence of killing which is unlikely. This may be due to crossing mistakes or the spores obtained for the backcrosses are heterokaryotic for

both alleles. This leads to an inconclusive statement to rule that indeed pGPD het-s is a dominant killer. In a backcross of 2-spored progeny of Psk 6 and het-S to het-S (Figure 3, C) there was also an evidence of killing. Unfortunately, reciprocal backcross to Psk 6 was not successful to rule out which parent that does the killing. It is likely that it was Psk 6 because het-S did not possess any killing characteristic. In a backcross involving 2-spored progeny from Psk 6 and het-s to het-s, there was also evidence of killing suggesting that Psk 6 was the culprit for that aborted spores. However, reciprocal backcross to Psk 6 was futile to prove that this is true.

Figure 4: Gel electrophoresis analysis for determining the status of het-s or het-S for 5 *Podospora anserina* strains from Wageningen. Lane 1: Ladder (Lambda DNA digested with Eco R1, Bam H1 and Hind III), Lane 2: het-s (positive control), Lane 3: het-S (positive control), Lane 4: wa 12, Lane 5: wa 86, Lane 6: wa 125, Lane 7: wa 126, Lane 8: Blank (negative control). Estimated size of het-s and het-S are 1800 bp and 2100 bp respectively.

In Figure 4, the het-s or het-S status was verified in each strain. There were some doubts about the status of het-S for wa 86 where its killer status is Psk 1. Almost 90% of Psk 1 are het-s and to find and later verify one population of Psk 1 (wa 86) as het-S showed that Psk 1 is highly associated with het-s. Besides that, for wa 12, wa 125 and wa 126, it was verified that they possess het-s, het-S and het-s respectively. The Psk status of wa 12, wa 125 and wa 126 is yet to be determined.

Discussions

We have some clues of the type of interaction involving Psk type killers and het-s type killers. From what we have derived from this study is the two elements are independent of one another. Psk type killing is hypothesized to have a toxin-antitoxin killing mechanism while het-s operated via prion synthesis as a killing mechanism (Debets, 2012). Clearly, the two elements operate at two different levels. Chances are, one or the other may dominate when put in a confrontation and it is highly unlikely that the two will

develop a symbiotic or mutually resistant interaction. As mentioned in a study done by Van de Gaag *et al.* (2000) there are three interactions; dominant epistasis, mutually resistant and mutually sensitive found when several Psk type killers were confronted by each other. For example Psk 1 and Psk 7 are epistatically dominant to Psk 2, 4 and 6 while still being mutually resistant of one another when confronted within an interaction. This unique characteristic of interaction among Psk type killers is what sparked the curiosity of finding out what type of interaction will be establishes when Psk type killers are confronted with het-s/S.

In some strains of *P. anserina*, fertility can be an issue to establish a good cross. It was found that in crosses involving Psk 2, spermatization seems to be the best method to accomplish it. As reported by Van de Gaag (2000), indeed in several strains of *P. anserina,* fertilizations involving wa 28 or Psk 2 seem to fail. Besides that, backcrossing of Psk 6 have been observed to be futile throughout this study and this could be due to fertility problems. Sometimes, crosses between two different strains may not yield desirable results, e.g; empty perithecia and empty ascus. Despite using both confrontation and spermatization method for crossing and backcrossing, there were little to no success accomplished. The same event had also been reported by Van de Gaag (2000) where backcrosses on Psk 4 and Psk 6 were less fertile. These findings were supported by Esser and Blaich (1994) sexual incompatibility are not only dependent upon vegetatitive incompatibility because there exist strains that are sexually compatible but vegetatively incompatible at the same time. In order to be able to mate, *Podospora anserina* needs to have compatible mating type, in other words, there have to be mat + and mat - strains to be able to cross sexually. However, there seem to be an unexplainable phenomena and factors that may influence compatibility between strains.

In another note, a high percentage of two-spored asci in some crosses suggests that the two killer elements are in close linkage with each other. They may also be clustered together like bacterial genes that share several common functions. As mentioned by Walton (2000), although genes for related functions are rarely found in close proximity in eukaryotes, gene clusters are regularly found in fungi, for instance, toxin-related genes and spore-specific genes. Therefore, it may not come as a surprise if het-s and some *Psk* related genes are in fact clustered together. From the results obtained thus far, it looks like the Psk-related genes and het-s genes are clustered together. Some confrontations between the elements gave a high percentage of two-spored asci from 50%-94%. The high number of two spored asci also reflects the high occurrence of First Division Segregation (FDS). First Division Segregation means no crossing over event will take place between the two killer allele. As a result, spores that will be formed will be homokaryotic for the killer allele. High frequency of FDS also means that recombination rate is low due to the close linkage of the killer alleles, thus, making the distribution skewed where more than 50% spores that survived will only have the killer genotype.

In relation to that is penetrance. Penetrance, in this context is the ability of one strain to completely dominate and change the outcome of an interaction or a fraction of a given genotype that indeed show the corresponding characteristic phenotype. In this study, complete penetrance status is given when the resulting asci that are formed from an interaction is only up to 4 spores. Variable number of spores formed from 4-8 will indicate incomplete penetrance. As opposed to FDS, Second Division Segregation resulted in incomplete penetrance. Non-parental Ditypes (NPD) that are formed as a result of recombination will give rise to rescue of the sensitive spores. In return, they will be able to survive the interaction. This unique interaction has been seen before with interaction of Psk strains to sensitive strains and het-s with het-S. It has also been reported by Van de Gaag (2000) one of the Psk type killers tested here which is Psk 6 has an incomplete penetrance. However, it looks like, Psk 6 has a complete penetrance when being confronted by het-s.

Crosses with oct 1 could show the killing characteristics and penetrance of a killer strain. Unfortunately, the oct 1 strain chosen was in the het-s background which is only useful to compare with other 2 killers interaction involving the same strain. For example, comparing oct 1 and Psk 6 with het-s and Psk 6. Ideally, both should give a comparable readings of killing percentage. However the readings were somewhat different at 0.33 and 1.27 respectively to warrant similarity in killing characteristic. However, variety in killing frequency of the same killer, Psk 6 may indicate dilution of the Psk 6 protein. As in the *het-s/het-S* model made by Bernet (1965). It was stated that in het-s/het-S system, prion proteins are diluted in asci that are produced later within the perithicium. As a result, the number of 4-spored asci and the killing percentage will vary among asci within the perithicia. The variability of killing behaviour in Psk 6 may be explained by dilution of Psk 6 killer molecules.

Last but not least is the association of Psk alleles and het-s/het-S alleles. From the past we have found approximately 90% of Psk 1 is greatly associated with het-s. Until today, the only strain of Psk 1 which is not het-s is from wa 86 population. Does this association of het-s to Psk 1 give an added fitness advantage to the population? Perhaps, after all, selfish genetic elements such as the two needs to be clustered together and transferred along the sexual cycle. Certainly, fitness and competition experiment are the best way to tell if indeed having two killer elements give an added advantage to a strain.

Conclusion

This research has certainly provided some clues for the interaction between meiotic drive elements in *Podospora anserina.* More studies have to be done to reveal the underlying mysteries surrounding this interaction. Modern methodology has to be used to its own advantages especially in determining the killer after an interaction is established. For example, primers for each Psk can be designed for Polymerase Chain Reaction (PCR) for ease of progeny genotyping in a backcross. The high incident of two-spored asci formed in a cross involving pGPD het-s may suggest that pGPD het-s is a strong killer and maybe an epistatically dominant one. On the other hand, Psk 6 is also a strong killer when confronted against het-s despite the reputation of being infertile in crosses.

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