

# 1 Combinations of *Spok* genes create 2 multiple meiotic drivers in *Podospora*

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9 **Abstract** Meiotic drive is the preferential transmission of a particular allele at a given locus  
10 during sexual reproduction. The phenomenon is observed as spore killing in a variety of fungal  
11 lineages, including *Podospora*. In natural populations of *Podospora anserina*, seven spore killers  
12 (*Psk*s) have been identified through classical genetic analyses. Here we show that the *Spok* gene  
13 family underlie the *Psk* spore killers. The combination of the various *Spok* genes at different  
14 chromosomal locations defines the spore killers and creates a killing hierarchy within the same  
15 population. We identify two novel *Spok* homologs that are located within a complex region (the  
16 *Spok* block) that reside in different chromosomal locations in given natural strains. We confirm that  
17 the individual SPOK proteins perform both the killing and resistance functions and show that these  
18 activities are dependent on distinct domains, a nuclease and a kinase domain respectively.  
19 Genomic data and phylogenetic analysis across ascomycetes suggest that the *Spok* genes disperse  
20 via cross-species transfer, and evolve by duplication and diversification within several lineages.

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## 22 Introduction

23 The genomes of all Eukaryotes harbour selfish genetic elements that employ a variety of mech-  
24 anisms to undermine the canonical modes of DNA replication and meiosis to bias their own  
25 transmission (*Werren et al., 1988; Burt and Trivers, 2009*). As the proliferation of these elements  
26 is independent from the regulated reproduction of the host organism, they can create conflict  
27 within the genome (*Rice and Holland, 1997*). Such intragenomic conflict is predicted by theory to  
28 spur an arms race between the genome and the elements, and consequently act as a major driver  
29 of evolutionary change (*Werren, 2011*). To understand the extent to which intragenomic conflict  
30 has shaped the evolution of genomes and populations it is crucial to identify the selfish genetic  
31 elements which are able to impact the dynamics of natural populations.

32 One important class of selfish genetic elements are known as meiotic drivers. These use  
33 a variety of mechanisms to hijack meiosis in order to bias their transmission to the gametes in  
34 proportions greater than 50% (*Sandler and Novitski, 1957*). This segregation distortion of alleles can  
35 be difficult to observe unless it is linked to an obvious phenotype such as sex (*Sandler and Novitski,*  
36 *1957; Helleu et al., 2014*), thus the prevalence of meiotic drive in nature is likely underestimated.  
37 Nevertheless, meiotic drive has been observed in many model systems, including *Drosophila*, *Mus*,

38 *Neurospora*, and *Zea mays*, suggesting that it is likely widespread across all major Eukaryotic groups  
39 (*Lindholm et al., 2016; Bravo Núñez et al., 2018*). Meiotic drive can be classified into three broad  
40 categories: female meiotic drive, sperm killing, and spore killing (*Lindholm et al., 2016*). Spore  
41 killing is found in ascomycete fungi and represents the most direct way to observe the presence  
42 of meiotic drive (*Turner and Perkins, 1991*). When a strain possessing a driving allele mates with  
43 a compatible strain that does not carry the allele (i.e., a sensitive strain), the meiotic products  
44 (ascospores) that carry the driving allele will induce the abortion of their sibling spores which do not  
45 have the allele. Spore killing is apparent in the sexual structures (asci) of the fungi as it results in half  
46 of the normal number of viable spores. Due to the haplontic life cycle of most fungi, spore killing  
47 is unusual among meiotic drivers as it is the only system where the offspring of an organism are  
48 killed by the drive (*Lyttle, 1991*). Additionally, with few exceptions (*Hammond et al., 2012; Svedberg*  
49 *et al., 2018*), spore killer elements appear to be governed by single loci that confer both killing and  
50 resistance (*Grognon et al., 2014; Nuckolls et al., 2017; Hu et al., 2017*), which is in contrast to the  
51 other well-studied drive systems that comprise genomic regions as large as entire chromosomes  
52 (*Larracuente and Presgraves, 2012; Hammer et al., 1989*).

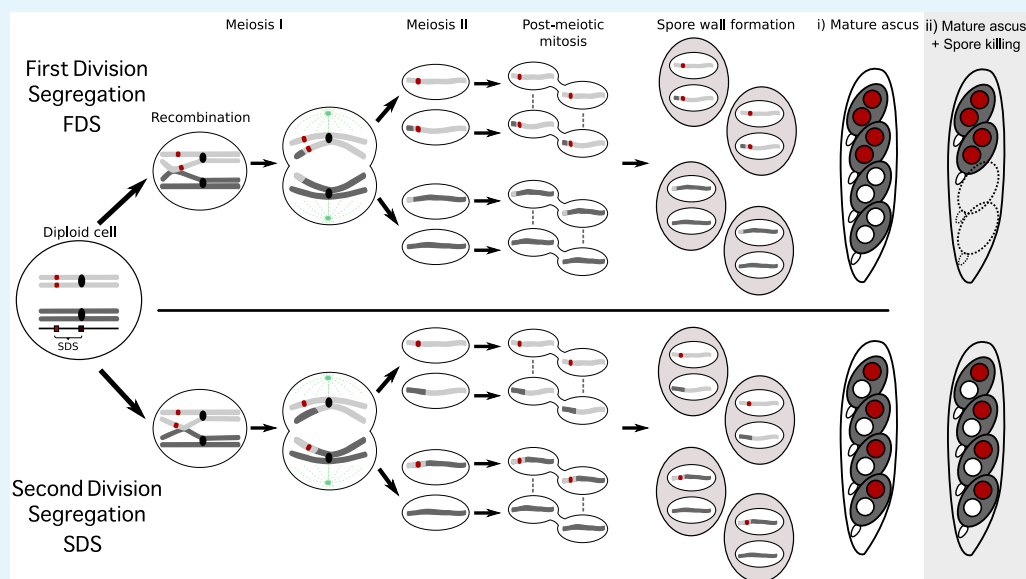
53 Meiotic drivers are often expected to reach fixation or extinction in populations relatively rapidly  
54 (*Crow, 1991*), at which point the effects of the drivers will no longer be observable. In agreement  
55 with this expectation, most drivers which have been described exhibit large shifts in frequencies in  
56 both time and space (*Lindholm et al., 2016; Carvalho and Vaz, 1999*). In the case of spore killers,  
57 multiple drivers have been found to coexist within a given species. The evolutionary dynamics of  
58 multiple drivers within species has not been thoroughly explored, but two contrasting examples  
59 are known. In genomes of *Schizosaccharomyces pombe*, numerous copies of both functional and  
60 pseudogenized versions of the *wtf* driver genes are found, suggesting that they duplicate readily,  
61 drive to high frequency in populations, and then lose their ability to kill (*Nuckolls et al., 2017; Hu*  
62 *et al., 2017*). In contrast, the two spore killers *Sk-2* and *Sk-3* of *Neurospora intermedia* have only been  
63 described in wild strains four times and once respectively, whereas resistance to spore killing is  
64 widespread (*Turner, 2001*). In neither of these cases, have the impact of multiple drivers coexisting  
65 in a single population been characterized.

66 Natural populations of the filamentous fungus *Podospora anserina* are known to host multiple  
67 spore killers (*Grognon et al., 2014; van der Gaag et al., 2000; Hamann and Osiewacz, 2004*) and  
68 hence, provide an ideal system for the investigation of interactions among drivers at the population  
69 level. The first spore killer gene described in *P. anserina* was *het-s*, a gene that is also involved in  
70 allorecognition (*Dalstra et al., 2003*). Another class of spore killer genes in *Podospora* are known as  
71 *Spok* genes. *Spok1* is only known from a single representative of the closely related species *P. comata*,  
72 while *Spok2* has been shown to exist in high frequency among strains of a French population of  
73 *P. anserina* (*Grognon et al., 2014*). *Spok1* is capable of killing in the presence of *Spok2*, but not vice  
74 versa, indicating a dominant epistatic relationship between the two genes. Using visual observation  
75 of spore killing in crosses among French and Dutch *P. anserina* strains, seven separate spore killers  
76 have been identified (*van der Gaag et al., 2000*). These are referred to as *Psk-1* through *Psk-7* and  
77 can be distinguished through classical genetic analysis, by observing the presence, absence and  
78 frequency of killed spores when the different spore killers are crossed to each other (**Box 1**). At  
79 the onset of this study, it was not known whether the *Psks* represent independent meiotic drive  
80 genes, or if they may be related to the *Spoks* and/or allorecognition loci. The *het-s* gene itself is not  
81 associated with the *Psks*, but allorecognition is correlated with *Psk* spore killing (*van der Gaag et al.,*  
82 **2003**). On the other hand, the relationship between the *Spoks* is reminiscent of the hierarchy of  
83 killing among the *Psks*, suggesting a possible connection between the activity of *Spok* genes and  
84 *Psks*.

115 The primary goal of this study was to determine the identity of the genes that are responsible

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## Box 1. Meiosis and spore killing in *Podospora*



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### Box 1 Figure 1. Meiosis in *Podospora*

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The seven separate *Psks* are defined by their spore killing percentage and mutual interactions. To understand how the spore killing percentages relate to the genotypes of the strains, it is necessary to first appreciate some of the fundamental aspects of *Podospora* biology. Within the fruiting body (perithecium), dikaryotic cells undergo karyogamy to produce a diploid nucleus and immediately enter meiosis. After meiosis, one round of post-meiotic mitosis occurs, resulting in eight daughter nuclei. The nuclei are packaged together with their non-sister nuclei from mitosis (dashed line) to generate dikaryotic, self-compatible spores. In a cross where the parental strains harbour two alternative alleles for a given gene of interest (one of which is indicated by the red mark on the chromosome), spores can be produced which are either homoallelic or heteroallelic for the gene, depending on the type of segregation. Specifically, if there is no recombination event between the gene and the centromere, the gene undergoes first division segregation (FDS) and the parental alleles co-segregate during meiosis I, generating homoallelic spores (i). FDS of a spore killing gene will thus result in a 2-spored ascus (ii). If there is a recombination event between the gene of interest and the centromere, second division segregation (SDS) occurs. In this case heteroallelic spores will be formed (i). For spore killing, a 4-spored ascus will still be produced as only one copy of the spore killer is required to provide resistance (ii). As SDS is reliant on recombination, the frequency of SDS relates to the relative distance from the centromere and can be used for linkage mapping. When there is spore killing, the percent of 2-spored asci is the frequency of FDS, and is referred to as "spore killing percentage". The *Psks* were described by crossing different strains and evaluating what their spore killing percentage is in each cross. The seven unique *Psks* were shown to interact in a complex hierarchy, showing either a dominance interaction, or mutual killing. Notably, crosses of strains carrying mutually resistant spore killers can still produce 2-spore asci if the killer loci are in different chromosomal locations (See **Appendix 1** for more details and **Figure 4–Figure Supplement 1** for a reproduction of the hierarchy presented in **van der Gaag et al. (2000)**).

116 for the *Psk* spore killers found in *P. anserina*, and whether they relate to known meiotic drive genes.  
117 We identified two novel *Spok* homologs (*Spok3* and *Spok4*) and showed that these two, together  
118 with the previously described *Spok2*, represent the genetic basis of the *Psk* spore killers. The novel  
119 *Spoks* occur in large complex regions that can be found in different genomic locations in different  
120 strains. Our results illuminate the underlying genetics of a polymorphic meiotic drive system and  
121 expand our knowledge regarding their mechanism of action.

## 122 Results

### 123 Genome assemblies

124 To investigate the genetic basis of spore killing in *P. anserina*, we generated high quality whole  
125 genome assemblies using a combination of long read (PacBio and MinION Oxford Nanopore) and  
126 short read (Illumina HiSeq) technologies. **Table 1** lists strains used for investigation. First, we  
127 selected strains from a natural population in Wageningen (Wa), the Netherlands, representing six  
128 of the previously described *Psk* spore killers (*van der Gaag et al., 2000*) along with a strain of a  
129 novel killing type (Wa100) that we referred to as *Psk-8*, and strain Wa63. Wa63 is of the same *Psk*  
130 type as the reference strain S, which we refer to herein as *Psk-S*. Additionally, we acquired and  
131 sequenced strains from the closely related *Podospora* species, *P. comata* (strain T) and *P. pauciseta*  
132 (CBS237.71). A strain labelled T (hereafter referred as T<sub>G</sub>) was kindly provided by Andrea Hamann  
133 and Heinz Osiewacz from the Goethe University Frankfurt and originates from the laboratory of  
134 Denise Marcou. However, as the genome sequence of T<sub>G</sub> did not match that reported by *Silar et al.*  
135 (*2018*), but instead is a strain of *P. anserina*, we included in our dataset another strain labelled T  
136 from the Wageningen Collection that was originally provided by the laboratory of Léon Belcour. We  
137 refer to this strain as T<sub>D</sub>, and sequenced it using only Illumina HiSeq. The genome of T<sub>D</sub> matches  
138 *Silar et al. (2018)* as the epitype of *P. comata* (See **Appendix 2** for further discussion).

139 The final assemblies (long-read technologies polished with Illumina HiSeq data) consist of 18  
140 to 53 scaffolds, from which the majority were either mitochondrial or rDNA in origin. Amongst  
141 the remaining scaffolds, the expected seven chromosomes were recovered in their entirety for  
142 almost all strains with PacBio data, and in up to 15 scaffolds with those sequenced using MinION  
143 (**Figure 1-source data 1**). Since the assemblies of each strain were produced from one haploid  
144 (monokaryotic) isolate, we will refer to specific genome assemblies with their strain name followed  
145 by their corresponding mating type, e.g. Wa63+.

### 146 Identification of novel *Spok* genes

147 By searching our assemblies for the *Spok2* sequence (presented by *Grognnet et al. (2014)*) using  
148 BLAST, we could confirm the presence of this *Spok* gene in the majority of strains, in agreement  
149 with *Grognnet et al. (2014)*. Furthermore, based on sequence similarity with *Spok2*, we identified two  
150 novel homologs that we refer to as *Spok3* and *Spok4*. Additionally, the BLAST searches recovered a  
151 pseudogenized *Spok* gene (*SpokΨ1*). The *Spok* gene content of the strains investigated in this study  
152 is reported in **Table 1**.

153 A schematic representation of the *Spok* homologs is shown in **Figure 1A**. We considered the  
154 *Spok2* sequence of S+, and the *Spok3* and *Spok4* sequences of Wa87+ as reference alleles for each  
155 homolog. Overall they show a high degree of conservation, including the 3' and 5' UTRs. A nucleotide  
156 alignment of the *Spok* genes' CDS revealed 130/2334 variable sites among the homologs (**Figure**  
157 **1-Figure Supplement 1** and **Figure 1-source data 1**). A relatively large proportion (67%, 87/130) of  
158 those result in amino acid changes and 74% are unique to one of the *Spok* homologs. There are  
159 also six indels among all the *Spok* genes including one at the 5' end of the ORF, which represents a

**Table 1.** List of all strains used in this study.

| Sample                        | Spore killer <sup>a</sup>     | Sequenced | Technology           | Mycelium         | <i>Spok</i> genes                                          | <i>Spok</i> block location |
|-------------------------------|-------------------------------|-----------|----------------------|------------------|------------------------------------------------------------|----------------------------|
| Natural Isolates              |                               |           |                      |                  |                                                            |                            |
| Wa21-                         | <i>Psk-2</i> ( <i>Psk-3</i> ) | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i> , <i>Spok3</i>                                | Pa_5_7950 – Pa_5_7960      |
| Wa28-                         | <i>Psk-2</i>                  | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i> , <i>Spok3</i>                                | Pa_5_7950 – Pa_5_7960      |
| Wa46+                         | naïve ( <i>Psk-4</i> )        | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>SpokΨ1</i>                                              | -                          |
| Wa53-                         | <i>Psk-1</i>                  | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Pa_3_945 – Pa_3_950        |
| Wa58-                         | <i>Psk-7</i>                  | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Pa_5_490 – Pa_5_470        |
| Wa63+                         | <i>Psk-5</i>                  | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i>                                               | -                          |
| Wa63-                         | <i>Psk-5</i>                  | RNA       | HiSeq 2500           | monokaryon       | <i>Spok2</i>                                               | -                          |
| Wa87+                         | <i>Psk-1</i>                  | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i> , <i>SpokΨ1</i> | Pa_3_945 – Pa_3_950        |
| Y+                            | <i>Psk-5</i>                  | DNA       | MinION<br>HiSeq 2500 | monokaryon       | <i>Spok3</i> , <i>Spok4</i>                                | Pa_3_945 – Pa_3_950        |
| Wa100+                        | <i>Psk-8</i>                  | DNA       | PacBio<br>HiSeq 2500 | monokaryon       | <i>Spok2</i> , <i>Spok4</i> , <i>SpokΨ1</i>                | Pa_5_490 – Pa_5_470        |
| T <sub>G</sub> +              | <i>Psk-5</i> ( <i>sk-1</i> )  | DNA       | MinION<br>HiSeq X    | monokaryon       | <i>Spok3</i> , <i>Spok3</i> , <i>Spok4</i>                 | Pa_3_945 – Pa_3_950        |
| CBS237.71-                    | <i>Psk-P1</i>                 | DNA       | MinION<br>HiSeq X    | monokaryon       | <i>Spok2</i> , <i>Spok3</i>                                | Pa_4_3420 – Pa_4_3410      |
| T <sub>D</sub> +              | <i>Psk-C1</i> ( <i>sk-1</i> ) | DNA       | HiSeq X              | monokaryon       | <i>Spok1</i>                                               | -                          |
| S+                            | <i>Psk-5</i>                  | DNA       | HiSeq X              | monokaryon       | <i>Spok2</i>                                               | -                          |
| S-                            | <i>Psk-5</i>                  | DNA       | HiSeq X              | monokaryon       | <i>Spok2</i>                                               | -                          |
| Wa47                          | naïve ( <i>Psk-6</i> )        | -         | -                    | -                | not sequenced                                              | -                          |
| Z                             | <i>Psk-7</i>                  | -         | -                    | -                | not sequenced                                              | -                          |
| s                             | <i>Psk-5</i>                  | -         | -                    | -                | not sequenced                                              | -                          |
| Us5                           | <i>Psk-5</i>                  | -         | -                    | -                | not sequenced                                              | -                          |
| Backcrosses to S <sup>c</sup> |                               |           |                      |                  |                                                            |                            |
| Psk1xS <sub>5</sub> - (Wa53)  | <i>Psk-1</i>                  | DNA       | HiSeq 2500           | monokaryon       | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Pa_3_945 – Pa_3_950        |
| Psk2xS <sub>5</sub> + (Wa28)  | <i>Psk-2</i>                  | DNA       | HiSeq 2500           | monokaryon       | <i>Spok2</i> , <i>Spok3</i>                                | Pa_5_7950 – Pa_5_7960      |
| Psk5xS <sub>5</sub> + (Y)     | <i>Psk-5</i> ( <i>Psk-1</i> ) | DNA       | HiSeq 2500           | monokaryon       | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Pa_3_945 – Pa_3_950        |
| Psk7xS <sub>5</sub> + (Wa58)  | <i>Psk-7</i>                  | DNA       | HiSeq 2500           | monokaryon       | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Pa_5_490 – Pa_5_470        |
| Psk1xS <sub>14</sub> - vs S   | <i>Psk-1</i>                  | RNA       | HiSeq 2500           | Selfing dikaryon | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Like parental              |
| Psk2xS <sub>14</sub> - vs S   | <i>Psk-2</i>                  | RNA       | HiSeq 2500           | Selfing dikaryon | <i>Spok2</i> , <i>Spok3</i>                                | Like parental              |
| Psk5xS <sub>14</sub> - vs S   | <i>Psk-1</i>                  | RNA       | HiSeq 2500           | Selfing dikaryon | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Like parental              |
| Psk7xS <sub>14</sub> - vs S   | <i>Psk-7</i>                  | RNA       | HiSeq 2500           | Selfing dikaryon | <i>Spok2</i> , <i>Spok3</i> , <i>Spok4</i>                 | Like parental              |

a Parentheses denote classification according to *van der Gaag et al. (2000)* when not in agreement with our phenotyping

b The *Spok* homologs present per strain were inferred with BLAST searches into genome assemblies or by inspecting RNAseq mapping. The *Spok* block is always located in an intergenic region, the flanking genes are given. The location of the *Spok* block in the S<sub>14</sub> backcrosses was not inferred from sequencing data.

c Parentheses denote parental spore killer strains

- Not applicable.



160 variable length repeat region, and one at the 3' end of the ORF shared by *Spok3* and *Spok4*. The 3'  
161 end indel induces a frameshift and changes the position of the stop codon (**Figure 1A**). *SpokΨ1* has a  
162 missing 5' end, multiple stop codons, and a discoglosse (Tc1/*mariner*-like) DNA transposon (**Espagne**  
163 **et al., 2008**) inserted in the coding region. Of particular interest, *SpokΨ1* has no deletions relative to  
164 the other *Spok* homologs, suggesting the indels in the functional *Spok* homologs represent derived  
165 deletions.

166 To aid in the identification of the meiotic drive genes, we gathered Illumina HiSeq data from  
167 the reference strain S together with four strains resulting from backcrossing of *Psk-1*, *Psk-2*, *Psk-5*,  
168 and *Psk-7* into S (**Table 1**). These and all other genomes sequenced with short-read data were  
169 assembled de novo using SPAdes. The resulting assemblies consisted of between 222 and 418  
170 scaffolds larger than 500bp, with a mean N50 of 227 kbp (Supplementary file 2). The coverage of  
171 the publicly available reference genome of the strain S+ (**Espagne et al., 2008**), hereafter referred to  
172 as Podan2, was above 98% for all of the SPAdes assemblies of *P. anserina*. When the filtered Illumina  
173 reads were mapped to Podan2, all samples had a depth of coverage above 75x (Supplementary file  
174 2). Taken together, our genome assemblies, resulting from both long and short-read data, are very  
175 comprehensive.

176 There is little allelic variation within the *Spok* homologs in the Wageningen population and the  
177 variants of the four homologs cluster phylogenetically (**Figure 1B and C**). The *Spok2* gene in the  
178 Wageningen strains are identical to the two alleles described in **Grogniet et al. (2014)**, with the  
179 exception of *Spok2* from Wa58- which has a single SNP that results in a D358N substitution. The  
180 *Spok2* allele of the French strain A, which shows resistance without killing (as reported by **Grogniet**  
181 **et al. (2014)**), was not found in any of our genomes. *Spok3* has five allelic variants, and the allelic  
182 variation of *Spok4* is reminiscent of *Spok2* with only Wa100+ and Wa58- having a single synonymous  
183 SNP (**Figure 1C**). Lastly, the three copies of *SpokΨ1* are all unique (**Figure 2-source data 2**).

184 Notably, a number of the variants of *Spok3* show signatures of gene conversion events (**Lazzaro**  
185 **and Clark, 2001**). Specifically, strain Y+ has three SNPs near the start of the gene that result in  
186 amino acid changes and match exactly those in *Spok2* (**Figure 1-Figure Supplement 1**). The Wa53+  
187 allele of *Spok3* has a series of SNPs (a track of 205 bp) that are identical to *Spok4*, but different from  
188 all other *Spok3* sequences, and three additional SNPs near the 5' end that also match *Spok4* (**Figure**  
189 **1-Figure Supplement 1**). The T<sub>G</sub>+ strain possesses two identical copies of *Spok3* (see **Methods**)  
190 that share the aforementioned tract with Wa53+, but which extends for an additional 217 bp  
191 (**Figure 1-Figure Supplement 1**). These chimeric *Spoks* are recovered from the final assemblies (pre-  
192 and post-Pilon polishing) with high long-read coverage (>30x), suggesting that our finding is not a  
193 bioinformatic artifact. The gene conversion events between *Spok* homologs are supported by the  
194 reticulation shown in a NeighborNet split network (**Figure 1B**) and by a significant recombination Phi  
195 test (199 informative sites,  $p = 1.528e-12$ ). A Maximum Likelihood phylogenetic analysis of the UTR  
196 sequences (defined by conservation across homologs) suggests that *Spok3* and *Spok4* are closely  
197 related (**Figure 1C**), which is at odds with the high structural similarity of the CDS of *Spok1* and *Spok4*  
198 (**Figure 1A**). Therefore, we cannot make any strong inference about the relationships between the  
199 *Spok* homologs from the sequence data.

200 The *Spok1* gene was previously identified from T<sub>D</sub> (**Grogniet et al., 2014**). No other strains  
201 investigated in this study were found to possess *Spok1*, indicating that it is likely not present in *P.*  
202 *anserina*. Remarkably, BLAST searches of the *Spok2* with the UTR sequences revealed the presence  
203 of a small piece (~156 bp long) of a presumably degraded *Spok* gene in the T<sub>D</sub> de novo assembly and  
204 on the chromosome 4 of the reference *P. comata* genome released by **Silar et al. (2018)**. This piece  
205 overlaps with the last amino acids of the CDS 3' end and it is flanked by an arthroleptis (solo LTR)  
206 retrotransposon on one side and by unknown sequence on the other. Due to the small size, it is  
207 unclear if this piece belongs to a novel *Spok* gene, but the location (between genes PODCO\_401390



208 and PODCO\_401400) does not align with any other known homolog. Strain CBS237.71 was formerly  
209 identified as *P. comata* and was reported to possess a *Spok* gene (*Grognet et al., 2014*). It has now  
210 been assigned to its own species, *P. pauciseta* (*Boucher et al., 2017*) and the sequencing reveals that  
211 the genome of this strain contains both *Spok3* and *Spok4* (*Figure 1B*).

## 212 **Backcrossing confirms the association of the *Spok* genes with the *Psks***

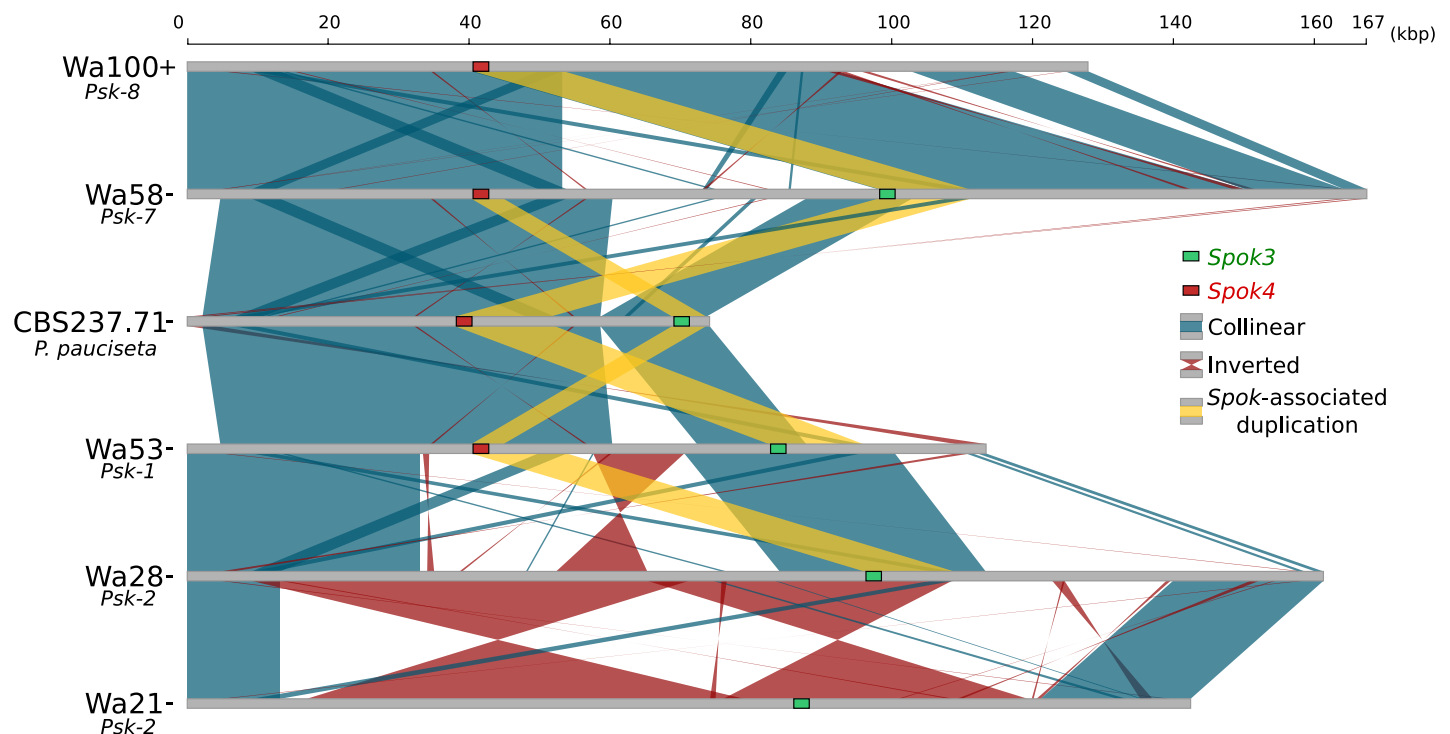
213 Four of the *Psk* spore killers were previously introgressed into the reference strain S through five  
214 successive backcrosses (*van der Gaag et al., 2000*) and are referred to here as Psk1xS<sub>5</sub>, Psk2xS<sub>5</sub>,  
215 Psk5xS<sub>5</sub>, and Psk7xS<sub>5</sub> (*Table 1*). Our Illumina data recovered in total 41482 filtered biallelic SNPs  
216 from the four S<sub>5</sub> backcrosses and the parental strains. All backcrossed strains show a few continuous  
217 tracts of SNPs from the killer parent (*Figure 2–Figure Supplement 3*). For example, Psk1xS<sub>5</sub>- has  
218 a long tract in chromosome 1 that represents the mat- mating type, which is expected since the  
219 published reference of S (Podan2), for which the SNPs are called, is of the opposite mating type  
220 (mat+). Importantly, the location of the *Spok* genes of each parental strain has a corresponding  
221 introgressed SNP tract in its S<sub>5</sub> backcross, while all backcrossed strains possess the *Spok2* gene  
222 from strain S (*Figure 2–Figure Supplement 3*). Notably, crossing results reveal that Psk5xS<sub>5</sub> has a  
223 *Psk-1* killing phenotype whereas all other S<sub>5</sub> backcrossed strains maintained the parental phenotype  
224 (*Figure 4–source data 1*). However as strain Y does not possess *Spok2*, the overall *Spok* content of Y  
225 is not the same as Psk5xS<sub>5</sub> (*Table 1*). These data suggest that the *Spok* content is responsible for  
226 the killer phenotype of the *Psks*.

227 As the various *Psk* types reflect specific *Spok* gene content, we can estimate the frequency of each  
228 *Spok* gene in the Wageningen population from *van der Gaag et al. (2000)*. We have determined  
229 the *Spok* gene composition for *Psk-1*, *Psk-2*, *Psk-4*, *Psk-5*, and *Psk-7*, as well as those previously  
230 considered as “sensitive”, now *Psk-5*. These account for 92/99 strains collected from Wageningen.  
231 The seven remaining strains were identified as either *Psk-3* or *Psk-6*. Our representative strain of  
232 *Psk-3* (Wa21) was shown to be *Psk-2*, and we are unable to comment on *Psk-6* as our representative  
233 strain (Wa47) behaves as *Psk-4* in test crosses (*Table 1*). Therefore we assume strains annotated as  
234 *Psk-4* possess no functional *Spok* genes (hereafter referred to as naïve) and omit all the *Psk-3* strains  
235 (except Wa21) and the *Psk-6* strains (except Wa47) from the analysis. Hence, *Spok2* is estimated to  
236 be in 98% of strains, *Spok3* in 17%, and *Spok4* in 11% of Dutch strains.

## 237 ***Spok* genes are found in complex regions associated with killer phenotypes**

238 While the *Spok* genes are often assembled into small fragmented contigs when obtained by using  
239 Illumina data alone, in the PacBio and MinION assemblies *Spok3* and *Spok4* are fully recovered  
240 within an inserted block of novel sequence (74–167 kbp depending on the strain), hereafter referred  
241 to as the *Spok* block. When present, the *Spok* block was never found more than once per genome  
242 and always contains at least one *Spok* gene. Whole genome alignments revealed that the *Spok*  
243 block has clear boundaries, and is localized at different chromosomal positions on chromosome 3  
244 or in either arm of chromosome 5 in different strains of *P. anserina* (*Table 1*). Importantly, these  
245 positions correspond with a single SNP tract from the S<sub>5</sub> backcrosses. In *P. pauciseta* (CBS237.71)  
246 the *Spok* block is found in chromosome 4. The *Spok* block of the different strains shares segments  
247 and overall structure (*Figure 2* and *Figure 2–Figure Supplement 1*), which suggests that they have a  
248 shared ancestry. However, complex rearrangements are found when aligning the block between  
249 the genomes. Within the *Spok* block, a given strain can harbour either or both of *Spok3* and *Spok4*  
250 and the regions containing the *Spok* genes appear to represent a duplication event (*Figure 2*). Strain  
251 T<sub>G</sub><sup>+</sup> shows an additional duplication which has resulted in a second copy of *Spok3* (*Figure 2–Figure*  
252 *Supplement 1*). While *Spok3* and *Spok4* are always found within the block, *Spok2* is never associated





**Figure 2.** Alignment of the *Spok* blocks from different strains. Grey bars represent the block sequences, blue vertical lines connect collinear regions between blocks, while red lines indicate inverted regions. The yellow lines show the region that is duplicated within the block surrounding *Spok3* (green) and *Spok4* (red).

**Figure 2-Figure supplement 1.** Alignment of *Psk-1/5 Spok* blocks.

**Figure 2-Figure supplement 2.** Dot plot showing synteny between the *SpokΨ1* region and the *Spok* block.

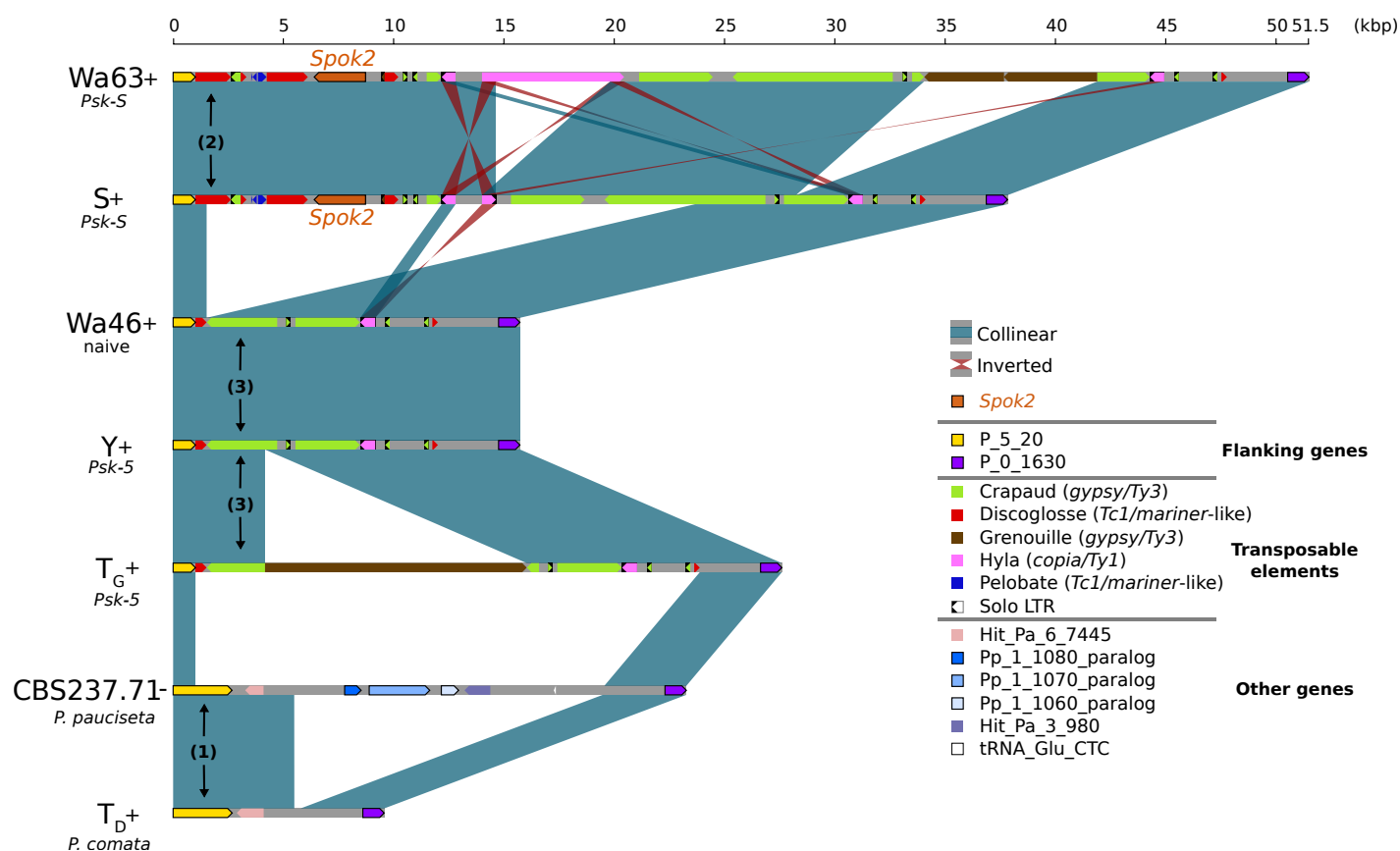
**Figure 2-Figure supplement 3.** Introgressed regions of the  $S_5$  backcrossed strains.

**Figure 2-source data 1.** Fasta file of the *Spok* block from all strains.

**Figure 2-source data 2.** Fasta file of the *SpokΨ1* region from all strains.

253 with a *Spok* block, but is found at the same location on chromosome 5 as previously described for  
 254 the reference strain S (*Grognat et al., 2014*). When present, *SpokΨ1* was found at a single position  
 255 in the right arm of chromosome 5. It is surrounded by numerous transposable elements (TEs), and  
 256 the region does not appear to be homologous to the *Spok* block (*Figure 2-Figure Supplement 2*).

257 In the few strains with no copy of *Spok2*, analysis of the region suggests that this is a result of  
 258 a one-time deletion (*Figure 3*). The annotation in the original reference genomes of  $T_D$  and S is  
 259 erroneous due to misassemblies and/or incomplete exon prediction, which were both corrected  
 260 using our own Illumina data, annotation pipeline, and validated with RNAseq expression data of  
 261  $T_D$ . First, the flanking gene P\_5\_20 (marked as (1) in *Figure 3*) in *P. pauciseta* (CBS237.71) and *P.*  
 262 *comata* ( $T_D$ ) is considerably longer than the *P. anserina* ortholog, which is truncated by a discoglosse  
 263 (Tc1/*mariner*-like) DNA transposon (2). In the strains without *Spok2* (Wa46, Y, and  $T_G$ ), this discoglosse  
 264 itself is interrupted and the sequence continues on the 3' end of a fragmented crapaud (*gypsy*/Ty3)  
 265 LTR element, which can be found in full length downstream of *Spok2* in the other strains. This  
 266 configuration implies that the absence of *Spok2* constitutes a deletion (3), rather than the ancestral  
 267 state within *P. anserina*. An alternative scenario would require multiple additional insertions and  
 268 deletions of TEs and *Spok2*.



**Figure 3.** Alignment of the *Spok2* locus in selected strains. The haplotypes are defined by the flanking genes P\_5\_20 and P\_0\_1630 located in chromosome 5 of the three sampled species. Every strain has a haplotype of different size, mainly due to differences in transposable element (TE) content. Within *P. anserina*, the TE variation across all sequenced strains occurs downstream of *Spok2*, as exemplified by strains Wa63 and S. The strains Wa46, Y and T<sub>G</sub> all lack *Spok2* and share break points. Notice that P\_5\_20 stands for the Pa\_5\_20 and PODCO\_500020 in the reference annotation of *P. anserina* and *P. comata*, respectively, while P\_0\_1630 stands for Pa\_0\_1630 and PODCO\_001630. As a note, *P. paucisetata* has a duplication of three genes in tandem from chromosome one (Pa\_1\_1080-60) between the flanking genes. Hit\_Pa\_X\_XXX genes stand for significant BLAST hits to genes of Podan2. TE nomenclature follows *Espagne et al. (2008)*.

**Figure 3-source data 1.** Fasta file of the *Spok2* region from all strains.

**Figure 3-source data 2.** Annotation file for TEs surrounding *Spok2*.

## 269 *Spok3* and *Spok4* function as meiotic drive genes

270 We constructed knock-in and knock-out strains to confirm that the newly discovered *Spok* homologs  
 271 *Spok3* and *Spok4* can induce spore killing on their own (**Table 2**), as previously shown for *Spok2*  
 272 by *Grognet et al. (2014)*. First, the *Spok2* gene was deleted from the strain s to create a  $\Delta Spok2$   
 273 strain for use with the knock-ins. A cross between s and the  $\Delta Spok2$  strain resulted in about ~40%  
 274 of 2-spored asci as previously reported by *Grognet et al. (2014)*, (80/197, 40.6%) (**Figure 4-Figure**  
 275 **Supplement 2B**). The *Spok3* and *Spok4* genes were inserted separately at the centromere-linked  
 276 *PaPKS1* locus (a gene controlling pigmentation of spores (*Coppin and Silar, 2007*)). A *Spok3::PaPKS1*  
 277  $\Delta Spok2$  x  $\Delta Spok2$  cross yielded almost 100% 2-spored asci with two white (unpigmented) spores  
 278 (118/119, 99.1%) (**Figure 4-Figure Supplement 2C**). Similarly, a *Spok4::PaPKS1*  $\Delta Spok2$  x  $\Delta Spok2$  cross  
 279 yielded almost 100% 2-spored asci with two white (unpigmented) spores (343/346, 99.1%) (**Figure**  
 280 **4-Figure Supplement 2D**), indicating that *Spok3* and *Spok4* function as spore killers when introduced  
 281 in a single copy at the *PaPKS1* locus.

**Table 2.** *Spok* gene content of genetically modified strains.

| Strains for genetic manipulations          | <i>Spok</i> genes   |
|--------------------------------------------|---------------------|
| $\Delta$ Ku70                              | <i>Spok2</i>        |
| s                                          | <i>Spok2</i>        |
| $\Delta$ <i>Spok2</i>                      | None                |
| <i>Spok3::PaPKS1</i> $\Delta$ <i>Spok2</i> | <i>Spok3</i>        |
| <i>Spok4::PaPKS1</i> $\Delta$ <i>Spok2</i> | <i>Spok4</i>        |
| <i>Spok3::PaPKS1d</i>                      | <i>Spok2, Spok3</i> |
| <i>Spok4::PaPKS1</i>                       | <i>Spok2, Spok4</i> |
| <i>Spok3::PaPKS1</i>                       | <i>Spok2, Spok3</i> |
| <i>Spok3</i> $\Delta$ i                    | <i>Spok3</i>        |
| <i>Spok3</i> D667A                         | <i>Spok3</i>        |
| C493A                                      | <i>Spok3</i>        |
| C497A                                      | <i>Spok3</i>        |
| C511A and C511S                            | <i>Spok3</i>        |
| K240A                                      | <i>Spok3</i>        |
| <i>Spok3</i> (1-490)                       | <i>Spok3</i>        |

$\Delta$ Ku70 and s were used exclusively for molecular work

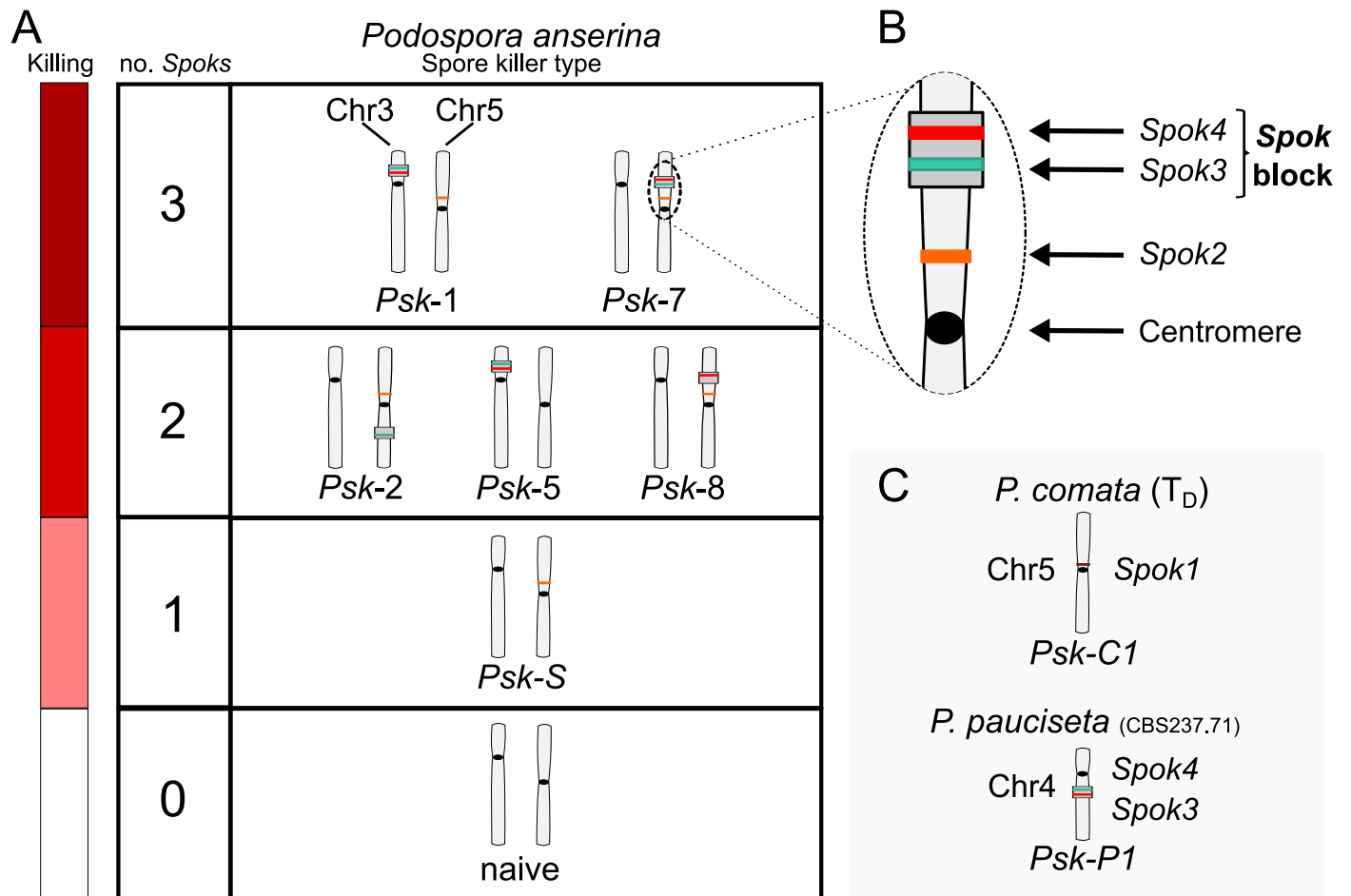
## 282 **The *P. anserina* *Spok* homologs are functionally independent**

283 To determine whether there are epistatic interactions among the *Spok* genes of *P. anserina*, pairwise  
284 crosses between the strains were conducted to determine which matings resulted in spore killing  
285 (**Figure 4–source data 1**). To assess any epistatic interaction between different killer types, dikaryotic  
286 F1 progeny that are homoallelic for the killing locus (**Box 1**) were selected, backcrossed to both  
287 parental strains, and were also allowed to self. Killing interactions were classified into one of the  
288 following categories.

- 289 1. Dominance interaction - Spore killing is observed when backcrossed to only one of the parental  
290 strains, and no spore killing is observed upon selfing.
- 291 2. Mutual resistance - No spore killing is observed when backcrossed to either parent nor when  
292 selfed.
- 293 3. Mutual killing - Spore killing is observed when backcrossed to either parent and/or when F1  
294 progeny are selfed.

295 As an example, in a cross between *Psk-1* and *Psk-7* there is spore killing. However the F1 progeny  
296 from this cross show no killing to either parent, satisfying condition 2. Thus they are mutually  
297 resistant, which is consistent with the fact that they carry the same three *Spok* genes. The reason  
298 spore killing is observed in the original cross is because the *Spok* block is located at different  
299 genomic positions. As a result, the *Spok* block can co-segregate during meiosis, leaving two spores  
300 without any *Spoks* and making them vulnerable to killing (see **Appendix 1** for a detailed explanation).

301 The results from these crosses are reported in **Figure 4–source data 1**. Note that we found  
302 several of the *Psk* designations of the strains to differ from those reported previously, and these  
303 discrepancies are shown in **Table 1**. From the epistatic interactions and killing percentages of the  
304 crosses, we construct a killing hierarchy (**Figure 4**) that also differs from that reported in **van der**  
305 **Gaag et al. (2000)** (**Figure 4–Figure Supplement 1**). In summary, our results show that *Spok2*, *Spok3*,  
306 and *Spok4* all act as spore killers and have no epistatic interactions with each other. The killing  
307 hierarchy observed in the Wageningen population of *P. anserina* is an emergent property of the  
308 presence and absence of the various *Spok* homologs in the different genomes. The rationale behind  
309 these conclusions is explained in detail below.



**Figure 4.** Interactions among the various *Psk* types and the occurrence of *Spok* genes. **A** The boxes represent hierarchical levels that increase in killing dominance from bottom to top, which correlates with the number of *Spok* genes that a strain possesses. Strains with three *Spok* genes induce spore killing of strains with only two *Spok* genes and show mutual resistance to each other. Strains with two *Spok* genes show mutual killing among themselves due to the different *Spok* genes and kill strains with only *Spok2*. Strains with one *Spok* kill strains with no *Spok* genes (naive strains). The chromosome diagrams depict the presence of the *Spok* genes and their location in the genome for the sequenced strains. **B** A zoomed in look at Chromosome 5 of a *Psk-7* strain demonstrating that *Spok3* and *Spok4* are present in the *Spok* block and *Spok2* is present at the standard location. **C** The closely related species *P. comata* and *P. pauciseta* also possess *Spok* genes, but at different locations. The *Spok* genes in *P. pauciseta* are present in a smaller *Spok* block, while *Spok1* is found on its own and exclusively in *P. comata*.

**Figure 4-Figure supplement 1.** Depiction of *Psk* killing hierarchy from van der Gaag et al. (2000).

**Figure 4-Figure supplement 2.** Images of spore killing between genetically modified strains.

**Figure 4-Figure supplement 3.** Results from pooled sequencing experiment of a cross between *Psk-1* and *Psk-5*.

**Figure 4-source data 1.** Table with killing percentages for all crosses tested between strains.

**Figure 4-source data 2.** Table with killing percentages for test crosses to determine epistatic interactions.

310 Spore killer types *Psk-1* and *Psk-7* reside at the top of the hierarchy, possess a *Spok* block with  
311 both *Spok3* and *Spok4*, and have *Spok2* (**Figure 4**). *Psk-2* and *Psk-8* are both dominant over *Psk-5*,  
312 which only has *Spok2*. *Psk-2* has a *Spok* block with just *Spok3* on the right arm of chromosome 5 and  
313 *Psk-8* has a *Spok* block with just *Spok4* at the same position as *Psk-7* on the left arm of chromosome  
314 5, indicating that *Spok2* does not provide resistance to either *Spok3* or *Spok4*. *Psk-1* and *Psk-7* are  
315 both dominant over *Psk-2* and *Psk-8*, indicating that *Spok3* does not provide resistance to *Spok4* and  
316 vice versa. The fact that *Psk-5* is capable of killing strains with no *Spok* genes (i.e. naïve) confirms  
317 previous results that *Spok2* alone is able to induce spore killing (**Figure 4**; and see **Grogniet et al.**  
318 **(2014)**).

319 *Psk-5* is a slightly more complicated case. It displays mutual killing with *Psk-1* and kills naïve  
320 strains, but *Psk-1* is dominant over *Psk-5*. *Psk-1* and *Psk-5* possess the same *Spok* block at the same  
321 location in Chromosome 3 (**Figure 4** and **Figure 2-Figure Supplement 1**), but *Psk-5* does not possess  
322 *Spok2*, suggesting that *Spok2* is responsible for killing in these crosses. If *Spok2* is responsible for  
323 killing when *Psk-1* is crossed with *Psk-5*, we expect the killing percentage to be the same as with  
324 crosses between *Psk-5* and naïve strains (~40%). However, these crosses consistently show only  
325 ~25% killing. To confirm that *Spok2* is responsible for killing in crosses between *Psk-1* and *Psk-5*, a  
326 pooled sequencing approach was employed. A cross was conducted between Wa87 (*Psk-1*) and Y  
327 (*Psk-5*), and spores from 2-spored (spore killing) and 4-spored asci (heteroallelic for killers) were  
328 collected and sequenced in separate pools. The 2-spored pool only contains SNPs from Wa87  
329 for a large portion of Chromosome 5, which includes the *Spok2* gene, whereas the 4-spored pool  
330 contains SNPs from both parents at this genomic location (**Figure 4-Figure Supplement 3**). As the  
331 2-spored asci are the result of FDS of the killing locus (**Box 1**), this result strongly suggests that *Spok2*  
332 is responsible for spore killing when *Psk-1* is crossed to *Psk-5* and thus that neither *Spok3* nor *Spok4*  
333 provides resistance against *Spok2*.

334 Of note, crosses between *Psk-1* and *Psk-5* often produce 3-spored asci and occasionally show  
335 erratic killing, which may contribute to the lower killing percentages. This phenomenon is also  
336 observed in crosses between *Psk-5* and naïve strains. We have been able to isolate a spore from a  
337 3-spored ascus in a cross between *Psk-5* and a naïve strain that has no copy of *Spok2* (**Appendix 2**).  
338 Therefore, the 3-spored asci are likely due to incomplete penetrance of the killing factor and  
339 supports the conclusion that the spore killing observed in these crosses is caused by the same gene,  
340 *Spok2*. This result is consistent with findings presented in the study by **van der Gaag (2005)** that  
341 provided independent evidence for incomplete penetrance of spore killing between S and Wa46  
342 (*Psk-5* and naïve).

343 The spore killing interactions of *Spok3* and *Spok4* cannot be dissociated from the *Spok* block  
344 with the use of wild or introgressed strains, so we made use of the aforementioned knock-in  
345 strains to confirm the independence of the *Spok* gene interactions from the *Spok* block. First,  
346 to confirm the killing interaction between *Spok3* and *Spok4*, we crossed a strain bearing *Spok4*  
347 at *PaPKS1* with a strain bearing *Spok3*. Because crosses homozygous for the *PaPKS1* deletion  
348 have poor fertility, we constructed a strain in which *Spok3* is inserted as a single copy at the  
349 *PaPKS1* locus but just downstream of the coding region (*Spok3::PaPKS1d*) in order to yield strains  
350 with normal pigmentation and normal fertility in crosses to *PaPKS1* deletion strains. In control  
351 crosses, the *Spok3::PaPKS1d* strain showed killing when crossed with a strain lacking *Spok3* but no  
352 killing when crossed with *Spok3::PaPKS1* (**Figure 4-Figure Supplement 2E and F**). The cross between  
353 *Spok3::PaPKS1d* and *Spok4::PaPKS1* yields asci with 4 aborted spores indicating mutual killing of  
354 *Spok3* and *Spok4* (**Figure 4-Figure Supplement 2G**). To determine the killing relation between *Spok2*  
355 and *Spok3*, a cross was conducted between *Spok3::PaPKS1* and s. This cross yielded mostly 2-spored  
356 asci with two unpigmented spores (163/165, 98.8%) (**Figure 4-Figure Supplement 2H**) indicating  
357 that *Spok3* kills in the presence of *Spok2*. Similarly, to determine the killing relation between *Spok2*



358 and *Spok4*, a cross was conducted between *Spok4::PaPKS1* and *s* (216/217, 99.5%) (**Figure 4–Figure**  
359 **Supplement 2I**). While these crosses indicate that *Spok2* does not confer resistance to *Spok3* and  
360 *Spok4* (*Spok3* and *Spok4* both kill *Spok2*), they do not allow us to determine as such whether *Spok3*  
361 or *Spok4* confer resistance to *Spok2*. To address this point, *Spok2* killing was analyzed in a cross  
362 homozygous for *Spok3* (*Spok3::PaPKS1* × *Spok3::PaPKS1d ΔSpok2*), which yielded 46% two-spored asci  
363 (143/310) confirming that *Spok2* killing occurs in the presence of *Spok3* (**Figure 4–Figure Supplement**  
364 **2J**). To determine if *Spok4* is resistant to *Spok2*, we made a *Spok4::PaPKS1* × *Spok4::PaPKS1 ΔSpok2*  
365 cross (11/24 two-spored asci) (**Figure 4–Figure Supplement 2K**). Although this genetic background is  
366 ill suited for determining killing frequency (because of the aforementioned effect of the homozygous  
367 *PaPKS1* deletion on fertility), presence of 2-spore asci suggests that *Spok4* does not confer resistance  
368 to *Spok2* killing. Overall, these results confirm the findings with the wild strains that *Spok2*, *Spok3*,  
369 and *Spok4* have no epistatic interactions, and imply that the *Spok* block does not augment the  
370 function of the *Spok* genes.

371 In contrast to the absence of epistatic interactions among *Spok* genes of *P. anserina*, *Spok1* of  
372 *P. comata* and *Spok2* do interact epistatically (**Grognet et al., 2014**). To determine if *Spok1* is also  
373 dominant to *Spok3* and *Spok4*, crosses were conducted between strain  $T_D$  and strains of *P. anserina*.  
374 Although  $T_D$  shows low fertility with *P. anserina* (**Boucher et al., 2017**), we were successful in mating  
375  $T_D$  to a number of the *P. anserina* strains of the different *Psk* spore killer types (**Figure 4–source**  
376 **data 1 and 2**). Often only few perithecia were produced with limited numbers of asci available to  
377 count, but despite this obstacle, the crosses clearly demonstrate that  $T_D$  is dominant to *Psk-5* and  
378 *Psk-2*, and is mutually resistant to *Psk-5*. This result implies that *Spok1* provides resistance to all  
379 of the *Spok* homologs in *P. anserina* and is capable of killing in the presence of *Spok2* and *Spok3*,  
380 but not *Spok4*. The mutual resistance with *Psk-5* also demonstrates that *Spok4* provides resistance  
381 against *Spok1*. Additional crosses were also conducted with the *P. pauciseta* strain CBS237.71, which  
382 confirms no epistatic interactions between *Spok3* and *Spok4* in this strain (**Figure 4–source data 1**  
383 **and 2**). As both  $T_D$  and CBS237.71 have unique spore killing phenotypes, we assign them the labels  
384 *Psk-C1* and *Psk-P1*, respectively.

### 385 **An intron in the 5' UTR is not required for spore killing**

386 To investigate if the *Spok* genes are expressed during spore killing, we conducted an additional nine  
387 backcrosses of the  $S_5$  strains to *S*, in order to generate  $S_{14}$  backcrossed strains (see **methods**). We  
388 produced RNAseq data of self-killing  $S_{14}$  cultures and mapped the reads to the final assemblies  
389 of the parental strains. The expression of the *Spok* genes is evident in this data and supports the  
390 presence of an intron in the 5' UTR of the *Spok* homologs (**Figure 1** and **Figure 1–Figure Supplement**  
391 **2**). Given its conservation across the *Spok* homologs and since the *wtf* spore killer system in *S. pombe*  
392 was described to involve two alternate transcripts of the same gene (**Hu et al., 2017; Nuckolls et al.,**  
393 **2017**), the role of this intron in the *Spok3* spore killing activity was investigated. The intron was  
394 deleted in the plasmid bearing the *Spok3::PaPKS1* deletion cassette by site directed mutagenesis  
395 and the modified plasmid was used to transform the  $\Delta Ku70 \Delta Spok2$  strain. Three transformants  
396 bearing the *Spok3* lacking the intron sequence (*Spok3 Δi*) were crossed to a  $\Delta Spok2$  strain. As in  
397 the control cross with wild type (*wt*) *Spok3*, in which close to 100% killing was found, we observed  
398 that 109/109 of the asci contained two unpigmented spores (**Figure 4–Figure Supplement 2L**). Thus,  
399 *Spok3 Δi* displays *wt* killing activity. We conclude from this experiment that the unspliced form of  
400 *Spok3* is not required for normal killing activity, nor does the killing and resistance function via an  
401 alternatively spliced form of this intron.

**Table 3.** Pairwise statistics between SPOK homologs. The  $d_N/d_S$  ratios, averaged across the coding region are shown below the diagonal, pairwise amino acid changes are shown above.

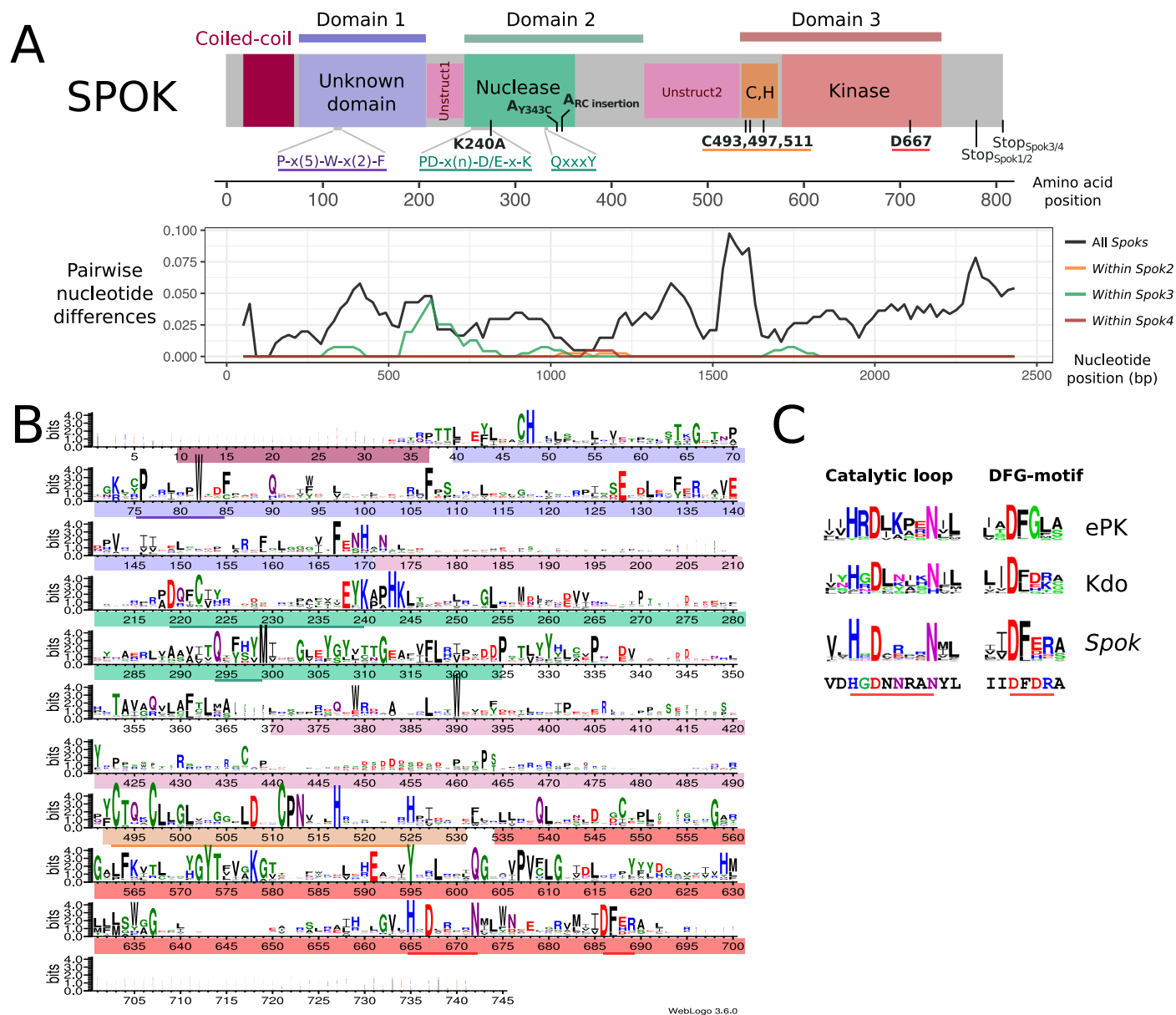
|       | SPOK4     | SPOK3     | SPOK2     | SPOK1 |
|-------|-----------|-----------|-----------|-------|
| SPOK4 | x         | 41        | 53        | 19    |
| SPOK3 | 0.8404081 | x         | 54        | 51    |
| SPOK2 | 0.9731409 | 0.9771488 | x         | 40    |
| SPOK1 | 0.6593501 | 0.7833958 | 0.7851462 | x     |

### 402 Functional annotation of SPOK3 predicts three ordered domains

403 In order to gain insights on the molecular function of the SPOK proteins, domain identification was  
404 performed with HHPred and a HMM profile based on an alignment of 282 *Spok3* homologs from  
405 various Ascomycota species. The SPOK3 protein was predicted to be composed of three folded  
406 domains (located at positions ~40 – 170, 210 – 400 and 490 – 700 in the protein) separated by two  
407 unstructured domains (~170 – 210 and 400 – 490) as shown in **Figure 5**. No functional identification  
408 was recovered for domain 1, however a coiled-coil motif was found in the N-terminal 40 amino  
409 acids and predicted to form a parallel dimer, which corresponds to the variable length repeat of the  
410 nucleotide sequences (**Figure 1A**). Domain 2 showed homology to a class of phosphodiesterase of  
411 the PD-(D/E)XK superfamily (~214 – 325) with the catalytic residues forming the PD-(D/E)XK motif  
412 spanning positions 219 to 240 in the SPOK3 sequence (**Steczkiwicz et al., 2012**). The best hit in  
413 HHPred was to the HsdR subunit of a type-I restriction enzyme from *Vibrio vulnificus* (**Uyen et al.,**  
414 **2009**). The sequences align in the catalytic core region in the PD-(D/E)XK motif and also around  
415 a QxxxY motif (294 – 298 in SPOK3) that was found to be important for nucleic acid binding and  
416 nuclease activity (**Sisáková et al., 2008**) (**Figure 5-Figure Supplement 2**).

417 Domain 3 was identified as a kinase domain (~539 – 700) as predicted previously by **Grognet**  
418 **et al. (2014)**. Additionally, a motif with a cluster of three highly conserved cysteine residues and  
419 histidine (C-x3-C-x13-C-x5-H-x7-H) reminiscent of zinc finger motifs was identified upstream of the  
420 kinase motif (**Figure 5**). As previously reported for *Spok2*, D667 was identified as the catalytic base  
421 residue in the catalytic loop (subdomain VIb) of the kinase domain. While kinases often use other  
422 proteins as substrates, they may also target small molecules (**Smith and King, 1995**). Inspection  
423 of the VIb and VII functional regions, which are informative regarding kinase substrate specificity,  
424 suggests that the *Spok*-kinase domain might be more closely related to eukaryotic-like kinases  
425 (ELKs) than to eukaryotic protein kinases (ePKs) raising the possibility that this kinase domain is not  
426 necessarily a protein kinase domain but could phosphorylate other substrates (**Steczkiwicz et al.,**  
427 **2012; Kannan et al., 2007**).

428 The SPOK proteins show a large degree of conservation among them and analyses of molecular  
429 evolution suggest that different domains of the protein evolve under different constraints. **Table 3**  
430 displays pairwise comparisons of the SPOK proteins. We tested whether any sites were evolving  
431 under positive selection using PAML 4.8 (**Yang, 2007**). The model of positive selection (M2) did not  
432 fit our data significantly better than its nested neutral model (M1). Furthermore, a likelihood test of  
433 model M3 (heterogenous site model) against the null model M0 (Homogeneous site model) showed  
434 no significant difference, which is likely due to the small number of sequences used in the analysis.  
435 In lieu of the site specific model, we calculated  $d_N/d_S$  ratios for the three predicted domains. The  
436 average  $d_N/d_S$  ratios of *Spok2*, *Spok3*, and *Spok4* are 2.70, 0.36, and 0.86 for domain 1, domain 2 and  
437 domain 3, respectively. This result suggests that domain 1 evolves under positive selection, domain  
438 2 under purifying selection, and domain 3 under neutral or weakly purifying selection in *P. anserina*.



### 439 **The killing and resistance functions can be attributed to separate domains**

440 The ability of the *Spoks* to perform both killer and resistance function with a single protein is  
441 unique among meiotic drive systems (*Bravo Núñez et al., 2018*). To investigate the role that the  
442 aforementioned domains may play in these two functions, we constructed a number of point  
443 mutations and truncation variants of *Spok3* and assayed their ability to kill or provide resistance  
444 in vegetative cells. We are able to determine that domain 2 is important for killing activity while  
445 domain 3 is important for resistance activity.

446 It was shown previously that the kinase domain of SPOK2 (*Figure 5*) is involved in the resistance  
447 function (*Grognet et al., 2014*). We generated a point mutant affected for the predicted catalytic as-  
448 partic acid residue of *Spok3* (D667A). The mutant allele was first used in transformation of a  $\Delta$ *Spok2*  
449 recipient strain. This *Spok3* D667A mutant allele leads to a drastic reduction in transformation  
450 efficiency (*Figure 4-source data 2*) while the *Spok3 wt* allele only moderately affects the number  
451 of transformants. Since this first approach results in random integration and potential multicopy  
452 insertion, we also attempted to introduce the mutant *Spok3* D667A allele as a single copy at the  
453 *PaPKS1* locus as described above for *wt Spok3*. The initial transformants were heterokaryotic and  
454 displayed sectors of abnormal growth that corresponded to unpigmented mycelium presumably  
455 containing nuclei with *Spok3* D667A that inserted at *PaPKS1*. Monokaryotic transformants could  
456 be recovered and were tested in killing activity in a cross to a  $\Delta$ *Spok2*. Four-spored asci with two  
457 white and two black spores were observed, suggesting that the D667A mutation abolishes spore  
458 killing. However, when the integrated *Spok3* allele was amplified by PCR and sequenced, it appeared  
459 that the allele presents a GAG to TAG mutation leading to a premature stop codon in position 282  
460 (E282stop). This result is consistent with the observation that *Spok3* D667A affects transformation  
461 efficiency and is toxic. Moreover, we detected expression of *Spok2* and *Spok1* in monokaryotic  
462 cultures (strains Wa63- and T<sub>D</sub>), suggesting that *Spok* activity is not restricted to the sexual cycle  
463 (*Figure 1-Figure Supplement 2*). No further attempts to insert the mutant allele at *PaPKS1* were  
464 made.

465 If toxicity of the *Spok3* D667A allele in vegetative cells is mechanistically related to spore  
466 killing, it is expected that this toxicity should be suppressed by *wt Spok3*. Therefore, we assessed  
467 whether *Spok3* D667A toxicity in vegetative cells is suppressed by co-expression with *wt Spok3*.  
468 Co-transformation experiments were set up with *Spok3* D667A used as the transformation vector in  
469 the presence or absence of *wt Spok3*. As in the previous experiment, *Spok3* D667A alone was found  
470 to affect transformation efficiency, but this effect was suppressed in co-transformations with *Spok3*  
471 (*Figure 4-source data 2*). This experiment confirms that *Spok3* D667A is only toxic in the absence of  
472 *Spok3*. Therefore, the *Spok*-related killing and resistance activities can be recapitulated in vegetative  
473 cells.

474 We also analyzed the role of the conserved cysteine cluster just upstream of the kinase domain.  
475 Three strains with point mutations in that region were constructed (a C493A C497A double mutant  
476 and C511A and C511S point mutants) and the mutant alleles were used in transformation assays  
477 as previously described for *Spok3* D667A. All three mutants reduced transformation efficiencies  
478 as compared to the controls and this effect was suppressed in co-transformations with *wt Spok3*  
479 (*Figure 4-source data 2*). These results suggest that the kinase domain and the cysteine-cluster  
480 region are both required for *Spok*-related resistance function but not for the killing activity. To  
481 test this, we constructed a truncated allele of *Spok3* which lacks these two regions: *Spok3*(1-490)  
482 (see *Figure 5-Figure Supplement 1*). The *Spok3*(1-490) allele drastically reduced transformation  
483 efficiencies and this effect was suppressed in co-transformations with *wt Spok3* (*Figure 4-source*  
484 *data 2*). If, as proposed here, the toxicity and suppression activities assayed in vegetative cells are  
485 mechanistically related to spore killing, then domain 3 appears to be required for the resistance  
486 function but dispensable for the killing activity which can be carried out by the N-terminal region of

487 the SPOK3 protein (domains 1 and 2).

488 Next we analyzed the role of the predicted nuclease domain (domain 2) in spore killing activ-  
489 ity. We generated a point mutant affected for the predicted catalytic core lysine residue (K240A).  
490 Introduction of this point mutation in the *Spok3*(1–490) allele abolished its killing activity in trans-  
491 formation assays (**Figure 4–source data 2**) suggesting that the nuclease domain is required for  
492 killing activity. The *Spok3* K240A mutant was then inserted at the *PaPKS1* locus and the resulting  
493 knock-in strain was crossed with a  $\Delta Spok2$  strain (to assay killing) and to a *Spok3::PaPKS1d* strain  
494 (to assay resistance) (**Figure 4–Figure Supplement 2M and N**). In the cross to  $\Delta Spok2$ , no killing  
495 was observed: the majority of the asci were four-spored with two white and two black spores  
496 (308/379, 81.2%) indicating that the K240A mutation abolishes spore-killing activity of *Spok3*. In  
497 the *Spok3* K240D::*PaPKS1* x *Spok3::PaPKS1d* cross, no killing was observed: the majority of the asci  
498 were four-spored with two white and two black spores (268/308, 87%). These crosses indicate that  
499 the *Spok3* K240A allele has lost killing ability but it has retained resistance. **Grognet et al. (2014)**  
500 reported that strain A bears a mutant allele of *Spok2* affected for killing but retaining resistance.  
501 The mutations in that allele fall in a conserved region of the nuclease domain (**Figure 5**) and map  
502 on predicted structural models in close vicinity of the catalytic lysine residue (K240 in SPOK3) and  
503 the other catalytic residues (**Figure 5–Figure Supplement 2**). Properties of the *Spok2* allele of strain  
504 A provide independent evidence that the nuclease domain of SPOK proteins is involved in killing  
505 activity but dispensable for resistance.

#### 506 **Phylogenetic distribution of *Spok* genes**

507 A search for closely related homologs of the *Spoks* across fungi reveals no closely related proteins  
508 among other members of the Sordariales. However, numerous species in the Hypocreales possess  
509 homologs, many of which have more than one putative copy per genome (**Figure 6**). Proteins  
510 with high similarity can also be found across other orders of the Sordariomycetes, namely the  
511 Xylariales and Glomerellales, as well as in one species of the Eurotiomycetes, *Polytolypa hystricis*  
512 (Onygenales). A maximum likelihood analysis of these sequences produced a phylogeny that  
513 can be robustly divided into two clades, one of which contains the NECHA\_82228 sequence from  
514 *Nectria haematococca* (Clade I), and the other which contains the *Podospora Spok* homologs (Clade  
515 II) (**Figure 6**). NECHA\_82228 was previously introduced into *P. anserina*, and the genetically modified  
516 strain produced empty asci when mated to a naïve strain, suggesting that it has a killing action  
517 (**Grognet et al., 2014**). Note that the sequences in Clade I are present in single copies per strain,  
518 except for *Fusarium oxysporum* f. sp. *pisi*, suggesting that they are all orthologs and hence, that  
519 the rate of gene duplications are low in this group. In contrast, many of the sequences in Clade  
520 II are present in multiple copies per strain. It is particularly notable how many *Spok* homologs  
521 are present in *F. oxysporum* and the number of copies that are found in each genome. Several of  
522 the duplicate *Spok* homologs are present on the lineage specific chromosomes of *Fusarium* that  
523 are often associated with pathogenicity (**Armitage et al., 2018**). The insect pathogens *Metarhizium*  
524 *rileyi* and *Cordyceps fumosorea* exhibit a number of divergent copies of *Spok* homologs with three  
525 and five copies respectively. This is in stark contrast to *Pseudomassariella vexata* and *Hirsutella*  
526 *minnesotensis* that have multiple, though nearly identical copies. The Clade II *Spok* homologs appear  
527 to diversify within each strain/species in much the same way as the *Spok* genes do in *Podospora*,  
528 with variable lengths of the coil-coil repeat region and frameshift mutations that relocate the stop  
529 codon. A few of the sequences may also represent pseudogenes as evident by premature stop  
530 codons and/or frameshifts, although this might also be the result of unidentified introns (**Figure 6**  
531 and **Figure 6–source data 1**).





## 532 Discussion

533 The identification of *Spok3* and *Spok4* has allowed us to explain the genomic basis for five of the  
534 seven *Psk* spore killer types found in natural populations of *P. anserina*. By our integrative approach  
535 of genomics, molecular biology and phenotyping, we have been able to demonstrate that the  
536 multiple drive elements genetically identified in *P. anserina* are not based on different underlying  
537 molecular mechanisms and/or specific gene interactions, but rather involve combinations of closely  
538 related driver genes belonging to the same *Spok* gene family. The *Spok* genes thus appear to be  
539 responsible for all identified drive elements in *Podospora*, with the exception of the *het-s* spore  
540 killing system.

### 541 The *Spok* Block

542 The presence of the complex *Spok* block presents a unique feature among the known meiotic  
543 drive systems. Often, meiotic drive elements occupy regions of suppressed recombination that  
544 span large tracts of chromosomes (Turner and Perkins, 1979; Hammer et al., 1989; Sandler et al.,  
545 1959) and co-occur with complex rearrangements (Harvey et al., 2014; Silver, 1993; Dyer et al.,  
546 2007; Svedberg et al., 2018). In these well-studied cases the elements of the drive mechanisms  
547 are encoded by separate genes within the region, and the rearrangements and suppression of  
548 recombination is expected to have evolved to ensure that the drive machinery (eg. the toxin and  
549 antitoxin genes) is inherited as one unit (Lyttle, 1991; Bravo Núñez et al., 2018). In *Podospora*, a  
550 single *Spok* gene is fully capable of driving, thus no region of suppressed recombination is required.  
551 Nevertheless, *Spok3* and *Spok4* are found in a large region that is not syntenic with the null allele.  
552 Hence, had the *Spok* genes not been previously identified from more placid genomic regions, the  
553 entire *Spok* block may have been misidentified as a driving haplotype with multiple interacting  
554 components. Considering that single-gene meiotic drivers might be more common than anticipated,  
555 it becomes necessary to question whether other drive systems located within complex regions and  
556 for which the genetics are not well known may also represent single gene drivers.

557 The relationship among the *Spoks* can provide insight as to the evolutionary history of the *Spok*  
558 block. The observation that *Spok3* and *Spok4* are both present in the *Spok* block in a duplicate  
559 region suggest that these represent homologs that formed via duplication. However, this scenario is  
560 contradicted by the finding that *Spok4* shares many features with *Spok1* of *P. comata*, yet not *Spok3*.  
561 It is possible that past hybridization between *P. anserina* and *P. comata* resulted in a transfer of *Spok4*  
562 to *P. comata* and that this gene has since diverged to become *Spok1*. In such a case, subsequent  
563 gene conversion between *Spok3* and *Spok4* would need to be invoked to explain certain features  
564 like the shared frameshift variant at the end of the CDS. If instead one assumes that the invasion of  
565 *Spok4* into *P. comata* (or of *Spok1* from *P. comata* to *P. anserina*) occurred prior to the duplication  
566 event that produced *Spok3* and *Spok4*, *Spok3* would have to mutate at a much higher rate than *Spok4*  
567 to explain the current pattern of divergence. Alternatively to duplication, *Spok3* and *Spok4* could be  
568 the result of divergence between different populations and ended up in their current distribution  
569 due to the fusion of two independent *Spok* blocks. Yet, another possible origin of *Spok3* and/or  
570 *Spok4* may be from another close relative, *P. pauciseta*, a scenario supported by our finding that the  
571 *P. pauciseta* strain CBS237.71 possess a *Spok* block with copies of both *Spok3* and *Spok4* that are  
572 nearly identical to the *P. anserina* alleles. Noteworthy, all possible scenarios outlined above invoke  
573 the introgression of *Spok* genes between species, most likely via hybridizations. Such interspecies  
574 interactions mediating the introgression of meiotic drive genes between species would not be a  
575 unique phenomenon to *Spok* genes of *Podospora*, as meiotic drive genes in *Drosophila* have been  
576 observed to cross species boundaries and erode barriers of reproduction (Meiklejohn et al., 2018).  
577 Further analyses of the genomes of populations of multiple *Podospora* species is needed in order  
578 to resolve the history of the *Spok* genes and the block.

579 At this stage, our data strongly suggest that the *Spok* block is moving in the genomes as a unit,  
580 but nevertheless, the mechanism of movement remains unknown. It may be hypothesized that  
581 movement of the block is achieved via an interaction with TEs at different genomic locations and  
582 non-allelic homologous recombination. This hypothesis is supported by the observation that the  
583 *Spok* genes outside of the *Spok* block, including *SpokΨ1*, are not located at the same position in the  
584 different species, and that they are often surrounded by similar TEs. Such movement may be under  
585 selection as matings between strains that have the same *Spok* genes but in different locations will  
586 result in spore killing. Furthermore, due to the idiosyncrasies of meiosis in *Podospora*, the position  
587 of the block may be under selection as the killing frequency is dependent on the frequency of  
588 crossing over with the centromere. Alternatively, the TEs may simply accumulate around the *Spok*  
589 genes because of a reduced efficacy of purifying selection at regions linked to the driver genes and  
590 that their presence *per se* increases the chance of rearrangements. As such, the role that TEs play in  
591 generating complex regions associated with meiotic drive should be investigated further in order to  
592 determine their importance to the evolution of drive.

### 593 **Molecular function of the *Spoks***

594 Spore killing systems display analogies to toxin-antitoxin (TA) systems in bacteria and it is interesting  
595 to note that many toxin families rely on nuclease activity (*Harms et al., 2018*). The contrast between  
596 our system and TA systems, however, resides in the fact that *Spok* toxin and antitoxin activities  
597 appear to be supported by the same protein molecule. While it is premature to propose a model  
598 for the molecular basis of *Spok*-gene drive, it can be stated that the kinase activity is able to  
599 counter the toxic activity of the nuclease domain of the same protein. One may hypothesize  
600 that autophosphorylation of the SPOK proteins relieves toxicity by inhibiting the nuclease activity.  
601 Alternatively, it could be that it is the phosphorylation of a distinct macromolecule or metabolite  
602 that nullifies toxicity. This last hypothesis is supported by the fact that the kinase domain of SPOK  
603 proteins resemble small molecule kinases more than protein kinases. In a simple model, the  
604 same molecule could be the target of both the kinase and nuclease activity. One can for instance  
605 imagine that the phosphorylation of the target would make it recalcitrant to the toxic action of  
606 the nuclease domain. All killing models have to explain why the proposed inhibitory activity of the  
607 kinase domain occurs only in spores bearing the *Spok* gene, yet suicidal point mutations can be  
608 rescued in trans (*Grognet et al., 2014*). The kinase and nuclease activity of the SPOK proteins might  
609 be differentially concentration-dependent, with the kinase activity favored at high SPOK-protein  
610 concentrations presumably occurring only in spores expressing the *Spok* gene. Alternatively, the  
611 possibility for kinase activity to protect against toxic activity of the nuclease domain might be  
612 temporally constrained during ascospore maturation so that spores exposed to SPOK proteins  
613 later in development (those not bearing *Spok* genes) might not benefit from the protective action.  
614 In addition to the yet unresolved mechanistic basis of killing and resistance, the characterization  
615 of the *Spok* gene function described here poses another puzzle. Since all SPOK products have an  
616 active kinase, it is not yet known what changes in sequence confer the hierarchical interactions  
617 among some *Spok* genes or why not all SPOKs are able to provide resistance to one another. One  
618 possibility is that the cellular targets for the nuclease and kinase activity differ for the different  
619 SPOK proteins.

620 The coil-coiled domain is likely involved in protein-protein interactions, based on studies of  
621 similar protein domains (*van Maldegem et al., 2015*). The fact that *Spok1* and *Spok4* have the  
622 same length repeat in this domain could imply that protein-protein interactions of this domain  
623 are important for resistance, as *Spok1* and *Spok4* are mutually resistant. This model would agree  
624 somewhat with the results of reporter constructs from *Grognet et al. (2014)* that showed an N-  
625 terminal mCherry tag on *Spok2* produced empty asci. As the adjacent unknown domain has  
626 signatures of positive selection, it is possible that the functional divergence observed between the

627 Spok proteins is due to mutations in this portion of the protein. In this model, domain 1 might  
628 be responsible for target specificity of the nuclease (and kinase) activity. The killing action itself is  
629 expected to be universal among the *Spoks* and is supported by the fact that this entire domain of  
630 *Spok3* from  $T_G$  is identical to *Spok4*, yet appears to retain *Spok3* functionality. The identification of  
631 the role of the nuclease domain in killing and of the kinase domain in resistance provides a first  
632 mechanistic insight into the dual role of *Spoks*. However, further dissection of the molecular action  
633 of these proteins is required to fully understand the molecular basis of *Spok* drive.

#### 634 **Absence of resistance**

635 One of the main factors that stands out in the *Podospora* system as compared to the other well  
636 studied spore killers is the lack of resistant strains. Only one strain of *P. anserina* (strain A) has  
637 ever been described as resistant (*Grognet et al., 2014*). The point mutations of *Spok3* induced  
638 in the laboratory imply that it is trivial to create a resistant strain, since only a single nucleotide  
639 change was required. Likewise, the resistant strain A *Spok2* is different from the reference allele  
640 only by two novel insertions. As such, the lack of resistance does not appear to be the result of a  
641 mechanistic constraint. Potentially, the current *Spok* gene distribution could be a relatively young  
642 phenomenon and resistance could evolve over time. Another possibility is that resistance itself is  
643 somehow costly to the organism and selected against. Additionally, it is puzzling that none of the  
644 *Spoks* in *P. anserina* show cross resistance. Intuitively, it would seem advantageous for novel *Spok*  
645 homologs to evolve new killing functions while maintaining resistance to the other *Spok* homologs.  
646 Again, the lack of cross-resistance does not solely appear to be the result of functional constraints,  
647 as *Spok1*, which is highly similar to *Spok4*, is resistant to all other *Spok* homologs. It is possible  
648 that it is more advantageous to combine multiple independent spore killers than to have a single  
649 broadly resistant gene. This option is supported by two observations presented in this study: the  
650 occurrence of the killing hierarchy and the association of *Spok3* and *Spok4*. The fact that *Spok3* and  
651 *Spok4* are present in the *Spok* block means that they are in tight linkage with each other. It may  
652 be the case that the linkage was selected for because it provided strains with the ability to drive  
653 against strains with just *Spok3* or just *Spok4*. However, this association could also be simply the  
654 result of a duplication without invoking selection. Whether the killing hierarchy we observe in *P.*  
655 *anserina* is due to a complex battle among the *Spok* homologs or a result of the existence of the  
656 *Spok* block will require further experimentation and mathematical modeling to resolve.

#### 657 **Evolutionary dynamics of the Spoks**

658 Some interesting aspects of meiotic drive in *Podospora* identified herein bears numerous parallel  
659 features to the *wtf* genes that are responsible for drive in *S. pombe*. There is no sequence similarity  
660 or conserved domains between the *Spok* and *wtf* genes, and *Podospora* and *Schizosaccharomyces*  
661 are only distantly related (~500 million years diverged) (*Wang et al., 2009; Prieto and Wedin, 2013*).  
662 Yet these systems display similar evolutionary dynamics within their respective species. Both of  
663 these systems are built of multiple members of gene families, that appear to duplicate, rapidly  
664 diverge to the point where they no longer show cross reactions (potentially with the aid of gene  
665 conversion), and then pseudogenize and become nonfunctional (*Bravo Núñez et al., 2018; Hu*  
666 *et al., 2017; Nuckolls et al., 2017*). Both systems also have close associations with TEs (*Bowen*  
667 *et al., 2003*). *Hu et al. (2017)* invoke LTR-mediated non-allelic homologous recombination as a  
668 possible mechanism for *wtf* gene deletion in a lab strain of *S. pombe*. While we provide evidence for  
669 the deletion of *Spok2*, it does not fit with expectation for being LTR-mediated, but as TEs are still  
670 accumulating in the region, other TE related processes may have been involved in the deletion.

671 The factors determining the abundance and diversity of multigene family meiotic drivers in a

672 species are the rates of gene duplication and loss, and time since origin. In the case of the *Spok*  
673 genes, we expect a low rate of deletion as they approach fixation, due to the dikaryotic nature of  
674 *Podospora*. Specifically, when first appearing, a deletion is only expected to be present in one of  
675 the two separate nuclear genomes maintained within a dikaryon. Any selfing event should erase  
676 (i.e. drive against) the deletion, meaning that in order to become homoallelic for a deletion, the  
677 strain would have to outcross with another individual with no *Spoks* or different *Spoks* from itself.  
678 Such outcrossing could allow deletions of *Spok3* and *Spok4*, but as *Spok2* is nearly fixed in the  
679 population, any outcrosses event should also lead to the deletion being eliminated by the driving  
680 action of *Spok2*. A possible solution to the paradoxical finding that *Spok2* appears to have been  
681 lost occasionally is that the incomplete penetrance of *Spok2* may have allowed spores that were  
682 homoallelic for the deletion to survive and persist. In this sense, *Spok2* fits the *wtf* model of driver  
683 turn over well, wherein it is beginning to lose killing function after becoming fixed in the population.  
684 *SpokΨ1* is missing the portion of the gene responsible for killing and the small *Spok* fragment of *P.*  
685 *comata* also corresponds to the resistance part of the gene. Both these observations suggest the  
686 killing domain may have been lost prior to these genes becoming fully pseudogenized and hints  
687 that they may have functioned as resistance genes.

688 It has been pointed out that spore killing may be a weak form of meiotic drive, since the  
689 transmission advantage is relative to the number of spores produced in a given cross, but there  
690 is no absolute increase at the population level (*Lyttle, 1991*). Hence, a spore killer requires an  
691 additional fitness advantage to reach fixation in a population (*Nauta and Hoekstra, 1993*). It is thus  
692 striking that *Spok2* is close to fixation in at least the French and Dutch populations, bringing into  
693 question the direct fitness effects of the *Spok* genes. On the other hand, the *Spok* block (and hence  
694 *Spok3* and *Spok4*) seems to be in relatively low frequency. It is possible that the rate at which the  
695 *Spok* block switches position is higher than the rate at which the *Spoks* can sweep to fixation. As  
696 such, the dynamics of *Spok* genes within the *Spok* block might differ from the *Spok2/wtf* life-cycle  
697 and may explain why spore killing is observed to be polymorphic in *P. anserina*. Additionally, *P.*  
698 *anserina* is capable of selfing, which may slow down the rate of fixation of the genes. Moreover, the  
699 vegetative and/or sexual expression of *Spok* genes might be deleterious in itself, and hence natural  
700 selection might be increasing or maintaining the frequency of strains without all *Spok* homologs.  
701 Overall, this complex system requires population genetic modelling to resolve the factors affecting  
702 the frequency of the *Spok* genes in populations of this fungus.

### 703 **Evolutionary history of the *Spok* gene family**

704 Looking more broadly at *Spok* genes across fungi for which genome sequences exist, it is rather  
705 interesting that *Spok* homologs are found in closely related orders, but not in other species of  
706 the Sordariales. This finding suggest that the *Spok* genes are transferred horizontally among  
707 evolutionarily disparate groups. This hypothesis is supported by the fact that the eurotiomycete  
708 *Polytolypa hystricis* possesses a closely related homolog to the *Podospora Spoks*. However, the  
709 phylogeny presented here shows that the homologs that group with the *Podospora Spoks* do  
710 generally agree with the known relationships among Sordariomycetes (*Maharachchikumbura et al.,*  
711 **2015**), suggesting that the *Spok* genes could be ancestral to the Sordariomycetes, but lost in most  
712 groups. Such a scenario would imply that there are long term consequences of possessing spore  
713 killer genes, even if they are fixed in the population.

714 Previously, proteins from *Nectria haematococca* and *Fusarium verticillioides* were identified as  
715 close homologs of the SPOK proteins, and it was demonstrated that the Necha\_82228 protein  
716 induces spore abortion in synthetic knock-ins of *P. anserina* (*Grognet et al., 2014*). Based on  
717 diversification patterns, the phylogeny presented here suggests that the *N. haematococca* and  
718 *F. verticillioides* sequences may represent orthologs that are conserved among the Hypocreales,



719 but do not represent meiotic drive genes since only one presumably orthologous copy is typically  
720 found. In contrast, the numerous closely related *Spok* homologs in *F. oxysporum* suggest that these  
721 genes could potentially be driving in this species. However, no sexual cycle has been observed  
722 in *F. oxysporum*. Given that we demonstrate vegetative killing with *Spok3*, it is possible that the  
723 *Fusarium Spoks* operate in vegetative tissue to ensure the maintenance of the pathogenic associated  
724 chromosomes. Alternatively, as *F. oxysporum* strains have been found with both mating type alleles  
725 (*O'Donnell et al., 2004*), there may be a cryptic sexual cycle in which the *Spok* homologs are active.

## 726 Conclusions

727 With this study, we have provided a robust connection between phenotype and genotype of spore  
728 killing in *P. anserina*. We showed that meiotic drive in *Podospora spp.* is governed by genes of the  
729 *Spok* family, a single locus drive system that confers both killing and resistance within a single  
730 protein, which synergize to create hierarchical dynamics by the combination of homologs at differ-  
731 ent genomic locations. The *Spok* genes are prone to duplication, diversification and movement in  
732 the genome. Furthermore, our results indicate that they likely evolved via cross-species transfer,  
733 highlighting potential risks with the release of synthetic gene drivers for biological control invading  
734 non-target species. Moreover, we present evidence that homologs of the *Spok* genes might have  
735 similar dynamics across other groups of fungi, including pathogenic strains of *Fusarium*. Taken to-  
736 gether, the *Spok* system provides insight into how the genome can harbour numerous independent  
737 elements enacting their own agendas and affecting the evolution of multiple taxa.

## 738 Methods

### 739 Fungal material

740 The fungal strains used in this study are listed in **Table 1** and were obtained from the collection  
741 maintained at the Laboratory of Genetics at Wageningen University (*van der Gaag et al., 2000*)  
742 and the University of Bordeaux. Strains with the “Wa” identifier were collected from the area  
743 around Wageningen between 1991 and 2000 (*Hermanns et al., 1995; van der Gaag et al., 1998,*  
744 **2000**). Strains S, Y, and Z were collected in France in 1937 (*Rizet, 1952; Belcour et al., 1997*). Strain  
745 S is commonly used as a wild type reference, and an annotated genome (*Espagne et al., 2008*) is  
746 publicly available at the Joint Genome Institute MycoCosm website ([https://genome.jgi.doe.gov/](https://genome.jgi.doe.gov/programs/fungi/index.jsf)  
747 [programs/fungi/index.jsf](https://genome.jgi.doe.gov/programs/fungi/index.jsf)) as “Podan2”. It remains unclear where exactly T<sub>D</sub> and T<sub>G</sub> were collected,  
748 given the labelling confusion.

749 Representative strains for the *Psk* spore killer types from the Wageningen collection were  
750 phenotyped to confirm the interactions described by *van der Gaag et al. (2000)*. Strains Wa87 and  
751 Wa53 were selected as representative of the *Psk-1* type, Wa28 for *Psk-2*, Wa21 for *Psk-3*, Wa46 for  
752 *Psk-4*, Y for *Psk-5*, Wa47 for *Psk-6*, and Wa58 for *Psk-7*. Strains S and Wa63 were used as reference  
753 strains and are annotated as *Psk-S*. Strain Wa58 mated poorly in general, so strain Z was used as  
754 a mating tester for the *Psk-7* spore killer type as well. For all crossing experiments and genome  
755 sequencing, we isolated self-sterile monokaryons (i.e., haploid strains containing only one nuclear  
756 type) from spontaneously produced 5-spored asci (*Rizet and Engelmann, 1949*), identified their  
757 mating type (mat<sup>+</sup> or mat<sup>-</sup>) by crossing them to tester strains, and annotated them with +/- signs  
758 accordingly.

## 759 **Culture and crossing conditions**

760 All crosses were performed on Petri-dishes with Henks Perfect barrage medium (HPM). This media  
761 is a modified recipe of PASM2 agar (van Diepeningen et al. 2008), where  $5 \text{ g L}^{-1}$  of dried horse dung  
762 are added prior to autoclaving. Strains were first grown on solid minimal medium, PASM0.2. For  
763 each cross, a small area of mycelia of each of two monokaryons was excised from the plates and  
764 transferred to HPM. Perithecia (fruiting bodies) form at the interface between sexually compatible  
765 mat+ and mat- monokaryons. Mature perithecia with fully developed ascospores were harvested  
766 after 8 – 11 days from which the percentage of 2-spored asci were evaluated to determine the killing  
767 percentage (**Box 1**). All cultures were incubated at 27 °C under 70% humidity for a 12:12 light/dark  
768 cycle. Barrage formation was also evaluated on HPM, whereby confrontations between mycelia of  
769 two different strains will produce a visible line of dead cells if they are vegetatively incompatible, for  
770 details see (van der Gaag, Debets, and Hoekstra 2003).

## 771 **DNA and RNA extraction and sequencing**

772 Culturing, extracting and sequencing genomic DNA using Illumina HiSeq

773 Monokaryotic strains of *P. anserina* were grown on plates of PASM0.2 covered with cellophane. The  
774 fungal material was harvested by scraping mycelium from the surface of the cellophane and placing  
775 80 mg to 100 mg of mycelium in 1.5 ml Eppendorf tubes, which were then stored at  $-20^\circ\text{C}$ . Whole  
776 genome DNA was extracted using the Fungal/Bacterial Microprep kit (Zymo, [www.zymo.com](http://www.zymo.com)) and  
777 sequenced at the SNP&SEQ Technology platform (SciLifeLab, Uppsala, Sweden), where paired-end  
778 libraries were prepared and sequenced with the Illumina HiSeq 2500 platform (125bp-long reads)  
779 or HiSeq X (150bp-long reads) (**Table 1**).

780 Culturing, extracting and sequencing genomic DNA using PacBio RSII

781 In order to generate high molecular weight DNA suitable for sequencing using PacBio, eight strains  
782 were grown on PASM0.2 for 5 – 7 days (**Table 1**). The agar with mycelium was cut into small  
783 pieces and used as inoculum for flasks containing 200 mL 3% malt extract solution, which were  
784 then incubated on a shaker for 10 – 14 days at 27 °C. The mycelia was filtered from the flasks, cut  
785 into small pieces and ~1 g was allotted into 2 ml tubes with screw-on caps, after which the tubes  
786 were stored at  $-20^\circ\text{C}$ . High molecular weight DNA was then extracted following the procedure  
787 described in **Sun et al. (2017)**. In brief, the mycelium was freeze-dried and then macerated, and  
788 DNA was extracted using Genomic Tip G-500 columns (Qiagen) and cleaned using the PowerClean  
789 DNA Clean-Up kit (MoBio Labs). The cleaned DNA was sequenced at the Uppsala Genome Center  
790 (SciLifeLab, Uppsala, Sweden) using the PacBio RSII platform (Pacific Biosciences). For each sample,  
791 10 kb libraries were prepared and sequenced using four SMRT cells and the C4 chemistry with P6  
792 polymerase.

793 MinION Oxford Nanopore sequencing

794 DNA extraction was performed as for the PacBio sequencing, except that the mycelia was dissected  
795 to remove the original agar inocula and the DNA was purified using magnetic beads (SpeedBeads,  
796 GE) then sequenced without further size-selection. Monokaryotic samples  $T_G+$  and CBS237.71- were  
797 sequenced first in a barcoded run on a R9.5.1 flowcell using the Oxford Nanopore Technologies  
798 (ONT) rapid barcoding kit (1.5  $\mu\text{l}$  RBK004 enzyme to 8.5  $\mu\text{l}$  DNA per reaction). Due to low tagmentation  
799 efficiency, we did additional sequencing for  $T_G+$  using the ligation sequencing kit (LSK108, R9.4.1  
800 flowcell). 500 ng DNA (25  $\mu\text{l}$ ) were mixed with 1.5  $\mu\text{l}$  NEB Ultra-II EP enzyme and 3.5  $\mu\text{l}$  NEB Ultra-II EP

801 buffer and incubated for 10 minutes at 20 °C and 10 minutes at 65 °C before addition of 20 µl AMX  
802 adaptor, 1 µl ligation enhancer, and 40 µl NEB Ultra-II ligase. After ligation the standard ONT washing  
803 and library loading protocol was followed and the sample was sequenced on a R9.4.1 flowcell. After  
804 sufficient sequencing depth had been achieved for sample T<sub>G</sub>, the flowcell was washed and the  
805 remaining barcoded samples were loaded to improve coverage also for sample CBS237.71. The  
806 sample Y+ yield less DNA (150 ng in 15 µl) and hence half the normal volume of adaptor was used  
807 (10 µl) and ligated using 20 µl Blunt/TA ligase for 15 minutes. Otherwise the standard protocol was  
808 followed, with sequencing done in a R9.4.1 flowcell. Basecalling and barcode split was done using  
809 Guppy 1.6 and Porechop (ONT) for all samples.

## 810 RNA sequencing

811 We generated transcriptomic data from dikaryotic strains that undergo spore killing during selfing.  
812 The S<sub>14</sub> backcrosses (see below) were mated to the strain S in order to obtain killer heteroallelic  
813 spores (from 4-spore asci) that were dissected from ripe fruiting bodies (see **Figure 1–Figure**  
814 **Supplement 2**). The spores were germinated in plates of PASM2 with 5 g L<sup>-1</sup> ammonium acetate  
815 added. Two days after germination, the culture was stored in PASM0.2 media at 4 °C to arrest  
816 growth. From that stock, we inoculated HPM plates with either a polycarbonate Track Etched 76 mm  
817 0.1 µm membrane disk (Poretics, GVS Life Sciences, USA)(Psk1xS<sub>5</sub> and Psk7xS<sub>5</sub>) or a cellophane  
818 layer (Psk2x<sub>5</sub> and Psk5x<sub>5</sub>) on top. The mycelium was grown for ~11 days and harvested for RNA  
819 extraction when the first spores were shot into the plate lid, ensuring several stages of fruiting  
820 body development. Note that *P. anserina* starts to degrade cellophane after ~6 days, and therefore  
821 the polycarbonate membrane allows for longer growing periods. Spore killing was independently  
822 confirmed on HPM plates inoculated without a membrane. Additionally, in order to improve gene  
823 annotation, we grew the strains Wa63- and T<sub>D</sub>+ on a cellophane layer on HPM for 11 and 7 days,  
824 respectively, to capture transcripts occurring during the monokaryotic phase.

825 The harvested mycelium was immediately frozen in liquid nitrogen and stored at –80 °C until  
826 RNA extraction. Next, 150 mg of frozen tissue were ground under liquid nitrogen and total RNA was  
827 extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quality of RNA was checked on  
828 the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). All RNA samples were treated with DNaseI  
829 (Thermo Scientific). Sequencing libraries were prepared using NEBNext Ultra Directional RNA Library  
830 Prep Kit for Illumina (New England Biolabs). The mRNA was selected by purifying polyA+ transcripts  
831 (NEBNext Poly(A) mRNA Magnetic Isolation Module, New England Biolabs). Finally, paired-end  
832 libraries were sequenced with Illumina HiSeq 2500 at the SNP&SEQ Technology platform.

## 833 Reads processing and genome assembly

834 For both DNA and RNA Illumina HiSeq reads, adapters were identified with cutadapt v. 1.13  
835 (**Martin, 2011**) and then trimmed using Trimmomatic 0.36 (**Bolger et al., 2014**) with the options  
836 ILLUMINACLIP:adapters.fasta:1:30:9 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:30.  
837 Only filtered reads with both forward and reverse were kept for downstream analyses. For short-  
838 read mapping, we used BWA v. 0.7.17 (**Li and Durbin, 2010**) with PCR duplicate marking of Picard v.  
839 2.18.11 (<http://broadinstitute.github.io/picard/>), followed by local indel re-aligning implemented in  
840 the Genome Analysis Toolkit (GATK) v. 3.7 (**Van der Auwera et al., 2013**). Mean depth of coverage  
841 was calculated with QualiMap v.2.2 (**Okonechnikov et al., 2016**).

842 The raw PacBio reads were filtered and assembled with the SMRT Analysis package and the  
843 HGAP 3.0 assembler (**Chin et al., 2013**). The resulting assembly was error-corrected (polished) with  
844 Pilon v. 1.17 (**Walker et al., 2014**) using the mapped filtered Illumina reads of the same strain. The  
845 samples sequenced with MinION were assembled using Minimap2 v. 2.11 (**Li, 2018**) and Miniasm

846 v. 0.2 (Li, 2016), polished twice with Racon v. 1.3.1 (Vaser et al., 2017) using the MinION reads, and  
847 further polished for five consecutive rounds of Pilon v. 1.22 using the Illumina reads as above.  
848 Additionally, DNA Illumina reads were assembled de novo for each sample using SPAdes v. 3.12.0  
849 (Bankevich et al., 2012; Antipov et al., 2015) using the k-mers 21,33,55,77 and the `-careful` option.  
850 Assemblies were evaluated using QUAST v. 4.6.3 (Mikheenko et al., 2016). Scaffolds were assigned  
851 chromosome numbers based on homology with Podan2. BLAST searches of the scaffolds in the  
852 final assembly of the strain CBS237.71 revealed contamination by a *Methylobacterium sp.* in the  
853 MinION data (but not in the Illumina data set). The scaffolds matching the bacterium were removed  
854 from the analysis.

855 The assembly of the *Spok* block was visually inspected by mapping the long reads (using Min-  
856 imap2) and the short reads (BWA) as above into the long-read polished assemblies. Since the  
857 MinION assemblies maintain some degree of sequencing error at repetitive regions that cannot be  
858 confidentially polished, we also assembled both types of reads into a hybrid assembly using SPAdes  
859 (same options as above) and, whenever different on short indels or SNPs but fully assembled, the  
860 sequence of the *Spok* genes was taken from the (low-error) hybrid assembly. The assembly of the  
861 *Spok* block of the T<sub>C</sub>+ strain was particularly challenging since the recovered MinION reads were  
862 relatively short. However, a few (<10) reads were long enough to cover the tandem duplication that  
863 contains *Spok3* (albeit with high nucleotide error rate in the assembly). The hybrid SPAdes assembly  
864 collapsed the duplication into a single copy. We therefore mapped the short reads into the hybrid  
865 assembly, confirming that the *Spok3* gene had doubled coverage and no SNPs, as expected from a  
866 perfect duplication.

867 Alignments of the assembled genomes were performed with the NUCmer script of the MUMmer  
868 package v. 4.0.0beta2 (Kurtz et al., 2004) using options `-b 200 -c 2000 -p -maxmatch`. The figures  
869 showing alignments of the *Spok* block and the *Spok2* region (Figure 2, Figure 3, and Figure 2-Figure  
870 Supplement 1) were generated by extracting the regions from each de novo assembly and aligning  
871 them in a pairwise fashion using MUMmer as described above. The MUMmer output were then  
872 visualized using a custom Python script.

## 873 Genome annotation

874 For annotation, we opted for gene prediction trained specifically on *P. anserina* genome features.  
875 We used the ab initio gene prediction programs GeneMark-ES v. 4.32 (Lomsadze et al., 2005;  
876 Ter-Hovhannisyan et al., 2008) and SNAP release 2013-06-16 (Korf, 2004). All the training process  
877 was done on the sample Wa28-, for which all chromosomes were assembled (see Results). The  
878 program GeneMark-ES was self-trained with the script `gmes_petap.pl` and the options `-funga1`  
879 `-max_intron 3000 -min_gene_prediction 120`. SNAP was trained as instructed in the tutorial of  
880 the MAKER pipeline v. 2.31.8 (Holt and Yandell, 2011), in (Campbell et al., 2014), and in the SNAP  
881 README file. First, we use the Podan2 transcripts and protein models as sole evidence to infer  
882 genes with MAKER (option `est2genome=1`) and then we had a first round of SNAP training. The  
883 resulting HMM file was used to re-run MAKER (`est2genome=0`) and to re-train SNAP, obtaining the  
884 final HMM training files.

885 A library of repetitive elements was constructed by collecting the reference *P. anserina* transpos-  
886 able elements described in Espagne et al. (2008) available in Genbank, and combining them with  
887 the fungal portion of Replibase version 20170127 (Bao et al., 2015), as well as the *Neurospora* library  
888 of Gioti et al. (2013). In order to produce transcript models we used STAR v. 2.6.1b (Dobin et al.,  
889 2013) with maximum intron length of 1000 to map the RNAseq reads of all samples, followed by  
890 processing with Cufflinks v. 2.2.1 (Trapnell et al., 2010). For the final genome annotation, we used  
891 MAKER v. 3.01.02 along with GeneMark-ES v. 4.33, SNAP release 2013-11-29, RepeatMasker v. 4.0.7

892 (<http://www.repeatmasker.org/>), BLAST suit 2.6.0+ (Camacho et al., 2009), Exonerate v. 2.2.0 (Slater  
893 and Birney, 2005), and tRNAscan-SE v. 1.3.1 (Lowe and Eddy, 1997). After preliminary testing, we  
894 chose the transcripts of Psk7xS<sub>14</sub> (mapped to the PacBio assembly of Wa58-) and Wa63- (PacBio  
895 assembly of the same strain) as EST evidence, and the Podan2 and T<sub>D</sub> (Silar et al., 2018) models as  
896 protein evidence. The MAKER models of relevant regions were manually curated by comparing with  
897 RNAseq mapping and coding sequences (CDS) produced with TransDecoder v. 5.5.0 (Haas et al.,  
898 2013) on the Cufflinks models.

899 We used blastn to localize possible copies of *Spok* genes in all genome assemblies. The *Spok2*  
900 (Pa\_5\_10) gene from Grognet et al. (2014) was selected as query. We named the new *Spok* genes  
901 (*Spok3* and *Spok4*) arbitrarily based on sequence similarity, as reflected in the Phylogenetic analyses  
902 (see below). Note that the existence of *Spok3* had previously been hypothesised by Grognet et al.  
903 (2014), however no DNA sequence was provided. Moreover, the strain Y, in which they identified it,  
904 contains both *Spok3* and *Spok4*.

### 905 Introgressions of the Spore-killing loci

906 Backcrossed strains of the various spore killer phenotypes were generated through five recurrent  
907 backcrosses to the reference strain S (S<sub>5</sub>) by van der Gaag et al. (2000). In the original study, the  
908 strains selected as spore killer parents were Wa53+ for *Psk-1*, Wa28- for *Psk-2*, Y+ for *Psk-5*, and  
909 Wa58- for *Psk-7*. The S<sub>5</sub> strains are annotated as Wa170 (*Psk-1*), Wa130 (*Psk-2*), Wa200 (*Psk-5*), and  
910 Wa180 (*Psk-7*) in the Wageningen Collection, however for the sake of clarity we refer to them as  
911 Psk1xS<sub>5</sub>, Psk2xS<sub>5</sub>, Psk5xS<sub>5</sub>, and Psk7xS<sub>5</sub>.

912 We sequenced the S<sub>5</sub> strains along with the reported parental strains using Illumina HiSeq  
913 2500. We mapped the reads to Podan2 as described above, followed by SNP calling using the  
914 HaplotypeCaller pipeline of GATK (options: -ploidy 1 -newQual -stand\_call\_conf 20.0). We re-  
915 moved sites that had missing data, that overlapped with repeated elements as defined by Re-  
916 peatMasker, or where all samples were different from the reference genome, using VCFtools v.  
917 0.1.16 (Danecek et al., 2011), BEDtools v. 2.27.1 (Quinlan and Hall, 2010), and BCFtools v. 1.9  
918 (Danecek and McCarthy, 2017), respectively. We plotted the density of filtered SNPs across the  
919 genome with the R packages vcfR (Knaus and Grünwald, 2016) and poppr (Knaus and Grünwald,  
920 2016; Kamvar et al., 2015). A full Snakemake (Köster and Rahmann, 2018) pipeline can be found at  
921 <https://github.com/johannessonlab/SpokPaper>. Notice that we sequenced both monokaryons of  
922 our strain S to account for the mutations that might have had occurred since the separation of the  
923 reference S strain in the laboratory of Espagne et al. (2008) and our S strain from the Wageningen  
924 Collection. These mutations should be present in the backcrosses, but they are independent from  
925 the spore killer elements.

926 Inspection of the introgressed tracks revealed that the variants of the backcross Psk1xS<sub>5</sub> do not  
927 match perfectly Wa53+ (the reported parent). Given that the *Spok* content is the same as Wa53+,  
928 the introgressed track co-occurs with the expected position of the *Spok* block in chromosome 3, and  
929 the fact that the phenotype of this backcross matches a *Psk-1* spore-killer type, we concluded that  
930 Wa170 (Psk1xS<sub>5</sub>) in the collection actually belongs to another of the *Psk-1* backcrosses described in  
931 the doctoral thesis of van der Gaag (2005), likely backcrossed from Wa52. Puzzling, an introgressed  
932 track in the chromosome 3 of the Psk2xS<sub>5</sub> strain does not match the expected parent (Wa28)  
933 either, both in SNPs and *het* genes alleles (Figure 2–Figure Supplement 3). However, other tracks in  
934 different chromosomes, including that of chromosome 5 where the *Psk-2 Spok* block can be found,  
935 do match Wa28. Likewise, Psk2xS<sub>5</sub> only has *Spok2* and *Spok3* copies like Wa28. Hence, we concluded  
936 that our results are not affected by these inconsistencies.



937 As reported by *van der Gaag et al. (2000)*, the  $S_5$  strains were generated by selecting ascospores  
938 from 2-spored asci of crosses between  $S$  and the spore killer parent. This procedure ensures that  
939 the offspring will be homozygous for alleles of the spore-killer parent from the spore killing locus  
940 to the centromere (**Box 1** and **Figure 2–Figure Supplement 3**). To eliminate as much background  
941 as possible from the spore killer parents in the backcrossed strains, nine additional backcrosses  
942 were conducted where ascospores were selected from 4-spored and 2-spored asci in alternating  
943 generations. Ascospores from the final generation were selected from 2-spored asci to ensure  
944 the strains would be homozygous at the spore-killing locus. These strains are the result of 14  
945 backcrosses to  $S$  ( $S_{14}$ ) and are annotated as Psk1x $S_{14}$ , Psk2x $S_{14}$ , Psk5x $S_{14}$ , and Psk7x $S_{14}$ . The  $S_5$  and  
946  $S_{14}$  strains were phenotyped by crossing the strains to their parents as well as other reference spore  
947 killer strains to confirm that the killing phenotypes remained unchanged after the backcrosses.

## 948 **Knock-out of *Spok2***

949 To knock-out *Spok2*, a 459 bp and a 495 bp fragment flanking the *Spok2* ORF downstream and  
950 upstream were obtained by PCR and cloned flanking the *hph* gene in the SKhph plasmid as blunt  
951 end fragments in a *EcoRV* site and a *SmaI* site. The deletion cassette was then amplified by PCR  
952 and used to transform a  $\Delta$ Ku70 strain (*El-Khoury et al., 2008*). Five transformants were screened  
953 for integration of the *hph* marker at *Spok2* by PCR and crossed to  $s$ . To purify the  $\Delta$ *Spok2* nuclei,  
954 a heterokaryotic binucleated  $\Delta$ *Spok2/Spok2* spore was recovered in a 2-spored ascus and used to  
955 fertilize the initial  $\Delta$ *Spok2* transformant (which may or may not be heterokaryotic). Uninucleated  
956 *hygR* resistant spores were then recovered from this cross.

## 957 **Construction of a disruption cassette to insert *Spok3* or *Spok4* in the *PaPKS1* locus**

958 To replace the ORF of the centromere-linked Pa\_2\_510 (*PaPKS1*) gene by one of the *Spok3* or  
959 *Spok4* genes (see **Results**), a disruption cassette was constructed as follows. A DNA fragment  
960 corresponding to the 700 bp upstream region of the *PaPKS1* ORF was amplified with oligonu-  
961 cleotides 5' tcgccgcgGCTAGGGGGTACTGATGGG 3' and 5' cacgcgccgcCTTGAAGCCTGTTGACGG  
962 3' (capital letters correspond to *P. anserina* genomic DNA sequences) and cloned in SKpBluescript  
963 vector (Stratagene) containing the nourseothricin-resistance gene *Nat* in the *EcoRV* site (vector  
964 named P1) using the *SacII/NotI* restriction enzymes (upstream from the *Nat* gene) to produce  
965 the P1UpstreamPKS1 vector. Then the 770 bp downstream of the *PaPKS1* ORF was amplified  
966 with oligonucleotides 5' tcgaagcttACAACAGTCATCGAACATG 3' and 5' gcggtcgacGGTACAATACGCC-  
967 CTAGTG 3' and cloned in the P1UpstreamPKS1 vector using the *HindIII/Sall* restriction enzymes  
968 (downstream from the *Nat* gene) to produce the P1UpstreamDownstreamPKS1 vector. Finally the  
969 *Spok3* and *Spok4* genes were amplified respectively from the Wa28 strain with oligonucleotides  
970 5' tcggcgccgcCACAGGAGCAGAGCTACGAC 3' and 5' gcgtctagaATATTTGGTACTTGGCGGC 3' and  
971 from the Wa87 strain with oligonucleotides 5' tcggcgccgcCACAGGAGCAGAGCTACGAC 3' and 5'  
972 gcgtctagaCAAGGTGCCGTGGAGTAAG 3' and cloned in the P1UpstreamDownstreamPKS1 vector  
973 using the *NotI/XbaI* restriction enzymes (between *PaPKS1* upstream region and *Nat* gene) to produce  
974 the P1UpstreamDownstreamPKS1\_*Spok3* or the P1UpstreamDownstreamPKS1\_*Spok4* vector so  
975 that the *Spok3/Spok4* and *Nat* genes are flanked by the upstream and downstream regions of  
976 *PaPKS1* ORF to allow *PaPKS1* ORF replacement by homologous recombination. The *Spok3* and  
977 *Spok4* amplified *Spok* genes contain the ORFs flanked with 983 bp upstream the start codon and  
978 460 bp downstream the stop codon for *Spok3*, and 984 bp upstream the start codon and 393  
979 bp downstream the stop codon for *Spok4*, allowing expression of *Spok* genes using their native  
980 promoter and terminator regions. The disruption cassette were then amplified from the final  
981 vectors using the most distal oligonucleotides 5' tcgccgcgGCTAGGGGGTACTGATGGG 3' and 5'  
982 gcggtcgacGGTACAATACGCCCTCAGTG 3' and named PKS1::*Spok3*\_nat-1 and PKS1::*Spok4*\_nat-1.

983 *P. anserina*  $\Delta Spok2$  ( $\Delta Pa\_5\_10$ ) strain was obtained after disruption of the gene Pa\_5\_10 and  
984 replacement of its ORF with the hygromycin-resistance gene *hph* in a  $\Delta Ku70$  strain. This strain was  
985 used as recipient strain for the disruption cassettes. We used 5  $\mu$ l of the cassettes for transfection  
986 and Nourseothricin resistant transformants were selected. As expected, most of the transformants  
987 were unpigmented and corresponded to insertion of *Spok3* or *Spok4* by replacement of *PaPKS1*.  
988 Gene replacement was verified by PCR.

## 989 Protein annotation methods

990 Prediction of unstructured regions was performed in SPOK3 with PrDOS with a 2% false positive  
991 setting (Ishida and Kinoshita, 2007). Coiled-coil prediction was performed with LOGICOIL (Vin-  
992 cent et al., 2012), CCHMM\_PROF (Bartoli et al., 2009) and Multicoil2 (Wolf et al., 1997). Domain  
993 prediction was performed using Gremlin (Balakrishnan et al., 2011) and RaptorX contact predict  
994 (Ma et al., 2015). Conserved residues were identified using Weblogo 3 (Crooks et al., 2004) with a  
995 Gremlin generated alignment as input. Domain identification was done with HHPred (Zimmermann  
996 et al., 2018).

997 In order to compare the diversity at the nucleotide level with the protein models, we calculated  
998 the average pairwise nucleotide differences (Nei and Li, 1979) for each bi-allelic site (correcting by  
999 the number of sites ( $n/(n - 1)$ ) while ignoring sites with gaps) on a *Spok* alignment (see below), using  
1000 overlapping windows of 100 bp and steps of 20 bp. This was performed on a selected representative  
1001 of each *Spok* homolog (*Spok2* of S, *Spok3* and *Spok4* of Wa87, and *Spok1* from T<sub>D</sub>), or for all the  
1002 alleles of each *Spok* within the *P. anserina* strains.

1003 Values of  $d_N/d_S$  were calculated using the seqinr package in R (Charif and Lobry, 2007). Align-  
1004 ments were manually trimmed to calculate separate values for each domain. Tests of sequence  
1005 evolution were conducted with paml 4.8 (Yang, 2007) using a star phylogeny of the *Spok* sequences.

## 1006 Phylogenetic analyses

1007 The final gene models of all the *Spok* genes in *Podospora spp.* were aligned along with the sequences  
1008 of *Spok2* and *Spok1* from Grognet et al. (2014) using MAFFT online version 7 (Kato et al., 2017)  
1009 with default settings (only one copy of *Spok3* from T<sub>G</sub> was used). The resulting alignment was  
1010 manually corrected taking into account the reading frame of the protein. Since the UTRs seem to be  
1011 conserved between paralogs, 654 (5' end) and 250 (3' end) bps of the flanking regions with respect to  
1012 *Spok2* were also included in the alignment. An unrooted split network was constructed in SplitsTree4  
1013 v. 4.14.16, build 26 Sep 2017 (Huson and Bryant, 2006) with a NeighborNet (Bryant and Moulton,  
1014 2002) distance transformation (uncorrected distances), and an EqualAngle splits transformation.  
1015 SplitsTree4 was used likewise to perform a Phi test for recombination (Bruen et al., 2006) using a  
1016 windows size of 100 and  $k = 6$ . Additionally, we used the BlackBox of RAXML-NG v. 0.6.0 (Kozlov  
1017 et al., 2018) to infer Maximum Likelihood phylogenetic trees of the nucleotide alignment of the 5'  
1018 UTR, the coding sequence (CDS), and the 3' UTR of the *Spok* homologs. We ran RAXML-NG with 10  
1019 parsimony and 10 random starting trees, a GTR + GAMMA (4 categories) substitution model, and  
1020 100 bootstrap pseudo-replicates for each analysis.

1021 In order to create a phylogeny of proteins closely related to the *Spok* genes in *Podospora* (and  
1022 hence likely to be meiotic drivers), the protein sequence of *Spok1* was used as a query against the  
1023 NCBI genome database. We collated all hits with e-values lower than Necha2\_82228, which has  
1024 been shown to have some spore killing functionality in *P. anserina* previously (Grognet et al., 2014),  
1025 with hit coverage greater than 75%, and no missing data (Ns) in the sequence. The sequences were  
1026 aligned using the codon-aware program MACSE v. 2.03 (Ranwez et al., 2018), with the representative

1027 *Podospora Spoks* set as “reliable” sequences (`-seq`), and the rest as “non reliable” (`-seq_1r`). Many  
1028 of the original gene models predict introns in the sequences, however no divergent regions were  
1029 apparent in the alignment and, even if present, MACSE tends to introduce compensatory frame  
1030 shifts. As such the entire gene alignment was used for the analysis. The resulting nucleotide  
1031 alignment was corrected manually, translated into amino acids, and trimmed with TrimAl v. 1.4.1  
1032 (*Capella-Gutiérrez et al., 2009*) using the `-gappyout` function. A Maximum likelihood tree was then  
1033 produced using IQ-TREE v. 1.6.8 (*Kalyaanamoorthy et al., 2017; Nguyen et al., 2015*) with extended  
1034 model selection (`-m MFP`) and 1000 standard bootstrap pseudo-replicates. The protein sequence  
1035 UV8b\_5543 of *Ustilaginoidea virens* was selected as outgroup based on a BioNJ tree made with  
1036 SeaView v. 4.5.4 (*Gouy et al., 2010*) of the Gremlin alignment described above.

### 1037 Pool-sequencing of *Psk-1* vs *Psk-5* progeny

1038 In order to confirm that *Spok2* is responsible of the killing between *Psk-5* and *Psk-1*, we conducted a  
1039 cross between the strains Wa87 and Y. When perithecia started shooting spores, we replaced the  
1040 lid of the cross plate with a water-agar plate upside-down, and let it sit for around an hour. Since  
1041 *P. anserina* spores from a single ascus are typically landing together, it is possible to distinguish  
1042 spores that came from an ascus with no killing (groups of four spores) from those that had killing  
1043 (groups of two). To improve germination rates, we scooped spore groups of the same ascus type  
1044 and deposited them together in a single plate of germination medium. After colonies became  
1045 visible, they were transferred into a PASM2 plate with a cellophane layer where they grew until DNA  
1046 extraction, followed by pool-sequencing with Illumina HiSeq X. In total 21 2-spore groups, and 63  
1047 4-spore groups were recovered.

1048 The resulting short reads were quality controlled and mapped to Podan2 as above. We used  
1049 GATK to call variants from the parental strains (treated as haploid) and the two pool-sequencing  
1050 databases (as diploids). We then extracted SNPs, removed sites with missing data, and attempted  
1051 to quantify the coverage frequency of the parental genotypes for each variant. The expectation  
1052 was that spore-killing (2-spore asci) would result in a long track of homozygosity (only one parental  
1053 genotype) around *Spok2*, as compared to the fully heterozygous 4-spore asci. A full Snakemake  
1054 pipeline is available at <https://github.com/johannessonlab/SpokPaper>.

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## 1101 Competing interests

1102 We declare no competing interests.

## 1103 Data availability

1104 The full CDS sequence and UTRs of *Spok3*, *Spok4*, and *SpokΨ1* (strain Wa87+) were deposited in  
1105 NCBI GenBank under the accession numbers MK521588, MK521589, and MK521590, respectively.  
1106 Raw sequencing reads were deposited on the NCBI SRA archive under the BioProject PRJNAXXXXXX.

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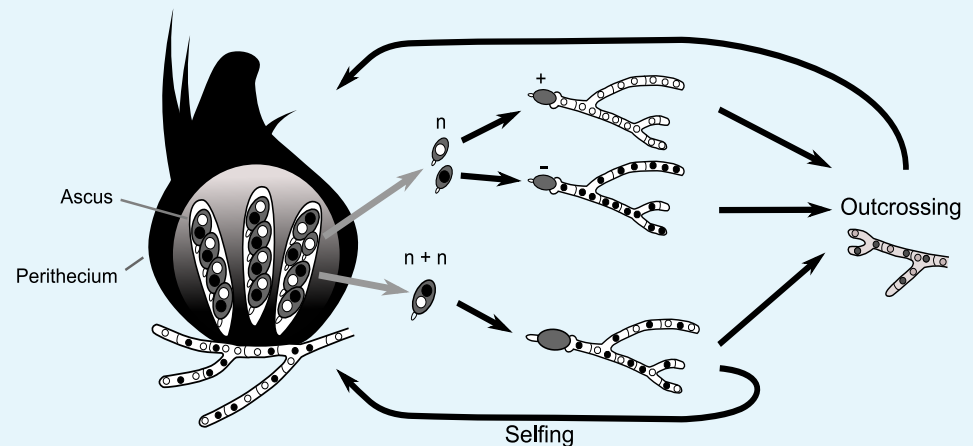
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1348 **Appendix 1**

1349 **The biology of *Podospora***

1350 The life cycle of *P. anserina* is an important factor to consider when discussing the meiotic  
1351 drive of the *Spok* genes. Although it has haploid nuclei, *P. anserina* maintains a dikaryotic  
1352 ( $n+n$ ) state throughout its entire lifecycle. Haploid nuclei of different mating-type are shown  
1353 as white and black circles within fungal cells. The fruiting body (perithecium) is generated  
1354 from dikaryotic ( $n+n$ ) mycelia, usually from a single individual strain. Within the perithecium,  
1355 the sexual cycle is completed to produce four dikaryotic ascospores per ascus. Occasionally,  
1356 atypical spore formation may occur and result in the production of five spores in an ascus, of  
1357 which two are small and monokaryotic ( $n$ ). These are self-sterile and need to outcross either  
1358 with a monokaryotic individual of the opposite mating type or with a dikaryotic individual to  
1359 complete the life cycle. Note that outcrossing may occur via mating between either siblings  
1360 or unrelated individuals of the opposite mating type. The monokaryotic spores are useful  
1361 for generating self-sterile (haploid) cultures for the purposes of sequencing and laboratory  
1362 mating. This intricate lifecycle is maintained by a strict meiotic process.



1363 **Appendix 1 Figure 1.** Simplified life cycle of *P. anserina*.  
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1366 **Two-locus spore killing interaction**

1368 The interaction between *Psk-1* and *Psk-7* provides a good example of how the meiotic drive  
1369 dynamics of *P. anserina* result in killing even though both *Psk-1* and *Psk-7* possess the same  
1370 *Spok* homologs. The three *Spok* homologs (*Spok2*, *Spok3*, and *Spok4*) are all present in  
1371 both *Psk-1* and *Psk-7*. The observed mutual resistance is thus due to the fact that the *Spok*  
1372 block (with *Spok3* and *Spok4*) is located on different chromosomes. Because chromosomes  
1373 segregate independently at meiosis the expected killing percentage can be calculated as:

$$0.5 * fk1 * fk2 = fsk \quad (1)$$

1375 where 0.5 is due to independent assortment of chromosomes,  $fk1$  is the killing percentage  
1376 of strain 1,  $fk2$  is the killing percentage of strain 2, and  $fsk$  is the spore killing frequency  
1377 observed between the two strains. For *Psk-1* crossed to *Psk-7* this equals 0.27. This agrees  
1378 well with the observed killing percentage of 23 – 27% (**Figure 4–Figure Supplement 1**).  
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## 1380 Appendix 2

### 1381 History of *Spore* killer research in *Podospora*

1382 Throughout the history of *spore* killing research in *Podospora*, a number of observations  
1383 have been made along with corresponding hypotheses. The discovery of *Spok3* and *Spok4*  
1384 provides us with the opportunity to reinterpret these data in light of the results presented  
1385 herein. Here we will address data from four important works: ((*Padieu and Bernet, 1967*;  
1386 *van der Gaag et al., 2000, 2003*; *Hamann and Osiewacz, 2004*).

### 1387 Inconsistencies among the *Psk* designations

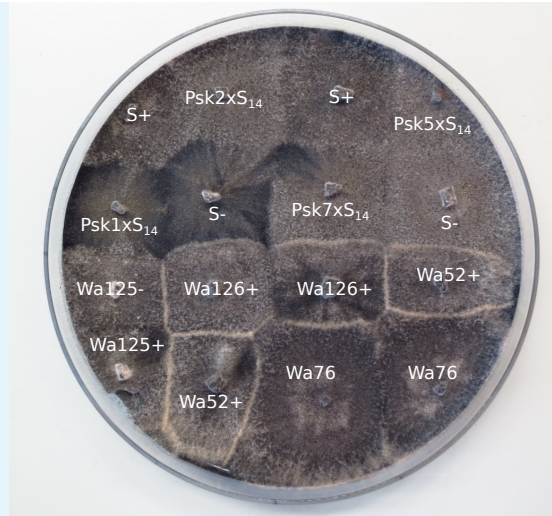
1388 Our phenotyping is in accordance with the results of (*van der Gaag et al., 2000*) for strains  
1389 Wa28, Wa53, Wa58, Wa63, Wa87, S, and Z, while contradictions were observed for Wa21,  
1390 Wa46, Wa47, and Y. Strain Wa21 was previously categorized as *Psk-3* which is typified by  
1391 inconsistent spore killing with *Psk-5* strains. Here we observed stable percentages and thus  
1392 consider Wa21 to be representative of *Psk-2*. The role of *Psk-3* as a spore killer has been in  
1393 doubt since its description (*van der Gaag et al., 2000*). This is in part due to the fact that  
1394 ascospores are not fully aborted as for the other spore killer types. Instead small transparent  
1395 ascospores can still be observed within the ascus. Here we were unable to find support for  
1396 this spore killer type and it has no clear correlation between its phenotype and any *Spok*  
1397 genes. We therefore find it likely that the effect is due to other incompatibility factors rather  
1398 than meiotic drive.

1399 We did not observe any spore killing in crosses between Wa46 (*Psk-4*) and Wa47 (*Psk-6*)  
1400 as reported in van der Gaag 2000. Two other strains had been annotated as *Psk-6*, Wa89  
1401 and Wa90, but no other strains were recorded as *Psk-4*. Unfortunately we were not able  
1402 to phenotype these strains and so we are unable to evaluate *Psk-6* further in this study.  
1403 In addition, results from crosses of *Psk-4* with a *Psk-5* strain (Wa63) reveals that there is a  
1404 dominance interaction between them with *Psk-5* killing *Psk-4*, which is the opposite of what  
1405 was proposed in *van der Gaag et al. (2000)*, i.e. that *Psk-5* kills *Psk-4*. Potentially, the original  
1406 interpretation was hindered by poor mating of the *Psk-4* strain with tester *Psk-5* strains.  
1407 Previously, strain Y was reported to have mutual resistance with *Psk-1*, be susceptible to  
1408 *Psk-7*, and dominant over all other types. Here we report that Y is susceptible to *Psk-1* and  
1409 *Psk-7*, and has mutual killing with all other types, except for crosses with naïve strains where  
1410 it is dominant.

### 1411 Allorecognition (*het*) genes and spore killing

1412 As the *het-s* gene is capable of causing both vegetative incompatibility and spore killing, it  
1413 was hypothesized that the *Psk* loci may be as well. The S<sub>5</sub> strains all demonstrate barrage  
1414 formation (symptomatic of vegetative incompatibility) with strain S (*van der Gaag et al.,*  
1415 *2003*). However when additional backcrosses were performed to generate S<sub>14</sub> strains, no  
1416 barrages were observed (*Figure 1*). This indicates that the spore killing types do not directly  
1417 affect vegetative incompatibility or vice versa, but may be linked to loci which do. Note that  
1418 the S<sub>5</sub> strains contain multiple genomic regions that are not isogenic with S, some of which  
1419 contain known allorecognition genes (*Figure 2-Figure Supplement 3*).





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**Appendix 2 Figure 1.** Barrage tests of the  $S_{14}$  strains. Strains Wa126, Wa76, Wa52, and Wa125 are wild isolates of *P. anserina* in the Wageningen collection. The thick white lines of mycelia demonstrate a barrage, which is indicative of heterokaryotic incompatibility in fungi. No barrages are seen among the  $S_{14}$  strains.

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### Incomplete penetrance of *Spok2*

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To investigate the nature of the 3-spored asci, tetrad dissections were conducted with asci from crosses between the *Psk-S* strains Wa63 and Us5, and the naïve strain Wa46. If the 3-spored asci were the result of a 4-spored ascus in which one of the spores aborted, all three spores should be heteroallelic for *Spok2*. If the 3-spored asci are the result of incomplete penetrance of the killing factor, two spores should be homoallelic for *Spok2* while the other spore should have no copy of *Spok2*. Unfortunately, spores from the crosses had very low germination rates (1/15 for Wa63 x Wa46 and 1/12 for Us5 x Wa46) as compared to other crosses (generally close to 100% germination). The progeny from the successfully germinated spores were backcrossed to the parental strains and also allowed to self to infer their *Spok2* genotype. Crosses with the Wa63/Wa46 progeny revealed it to be homoallelic for *Spok2*, and crosses with the Us5/Wa46 progeny revealed it to have no copy of *Spok2*. Both of these observations are consistent with the hypothesis for incomplete penetrance of *Spok2*.

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### Strain T and the original reports of spore killing in *Podospora*

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The strain T has featured prominently in a number of important publications on spore killing in *Podospora*. It was one of the two strains investigated in the original description of spore killing by **Padieu and Bernet (1967)** (translated and reinterpreted by **Turner and Perkins (1991)**), it was the strain in which *Spok1* was described **Grognet et al. (2014)**, and it was part of an investigation of spore killing in German strains of *Podospora* (**Hamann and Osiewacz, 2004**). Our results clearly demonstrate that two strains labeled as T ( $T_G$  and  $T_D$  herein) are not only different strains, but are different species. The description of spore killing in **Padieu and Bernet (1967)** matches our observations of crosses between  $T_G$  and the *Psk-S* strain Wa63, including incomplete penetrance as implied by the presence of 3-spored asci. Thus, we believe  $T_G$  to be representative of the original T strain. In light of this, we reinterpret the results of both **Padieu and Bernet (1967)** and **Hamann and Osiewacz (2004)** as per the interactions of the *Spok* genes.

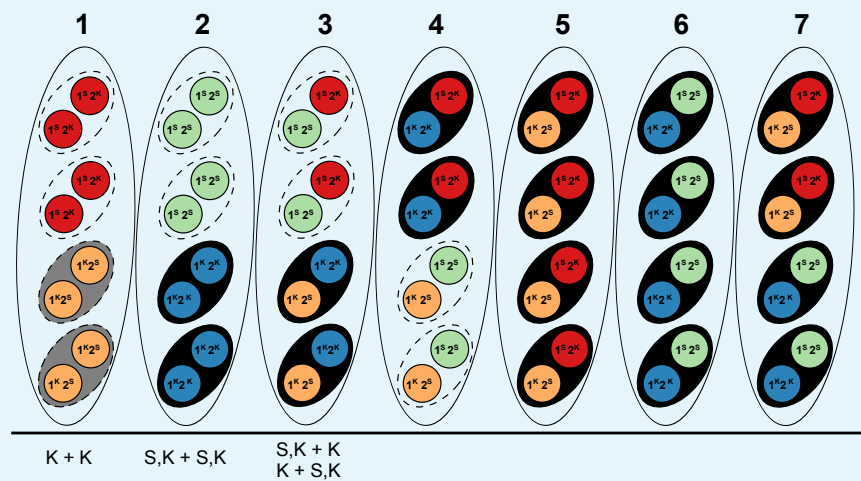
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In **Padieu and Bernet (1967)**, they describe a cross between two strains: T and T'. They identify two genes (one present in T and the other in T') which cause spore killing and interact as mutual killers. The gene from T has a killing percentage of 90%, while the one from T' has a killing percentage of 40% and occasionally produces 3-spored asci. This fits well with a cross of *Psk-5* and *Psk-S* where *Psk-5* kills at 90% and *Spok2* of the *Psk-S* strain kills at 40%, but has incomplete penetrance resulting in 3-spored asci. Unfortunately strain T' has to our knowledge not been maintained in any collections, so this cannot be confirmed experimentally. However, *Psk-S* strains are the most abundant phenotype from French, German, and Dutch populations (T' was isolated in France along with T) (**van der Gaag et al., 2000; Grognet et al., 2014; Hamann and Osiewacz, 2004**).

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In **Hamann and Osiewacz (2004)** they present a number of interesting observations. They report a new spore killer type, identify progeny that appear to demonstrate gene conversion of the killer locus, and observe apparent recombinant spore killer types. The study mostly centres around strain O, which they report to be of the same spore killer type as T<sub>G</sub> and should thus be *Psk-5* given our results. As such, we suspect that their focal cross between O and Us5, a *Psk-S* strain, is the same as the Padieu and Bernet paper. We have independently confirmed that Us5 (kindly provided by A. Hamann and H. Osiewacz) is *Psk-S*, however strain O has not been maintained in any collection. They also state that strain He represents a new type of spore killer. However, with O classified as *Psk-5*, the interactions of He match that of a *Psk-1* strain. Furthermore, strain He exhibited no spore killing with a *Psk-1* strain from Wageningen. From the cross of O and Us5 they identify a number of progeny with unexpected genotypes. They interpret these genotypes as evidence for both gene conversion and recombinant spore killer types. However, under a two locus model of mutual killing, both effects can be explained by incomplete penetrance of *Spok2* (**Figure 2**). As the cross with Us5 showed a particularly high degree of anomalous results, it is possible that Us5 contains a unique allele of *Spok2* that is a particularly weak killer.

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1479 **Appendix 2 Figure 2.** Explanation of results from *Hamann and Osiewacz (2004)* with information  
1480 about *Spok* genes as described in the text. The seven asci represent the possible genotype  
1481 combinations of a cross between a *Psk-5* strain and a *Psk-S* as illustrated in *Turner and Perkins (1991)*.  
1482 Black ovals represent the ascospores, dashed ovals represent killed spores, and coloured circles  
1483 represent the individual nuclei, where each colour corresponds to a given genotype. Genotypes are  
1484 annotated as per *Turner and Perkins (1991)*, wherein locus 1 corresponds to a killer locus with 90% FDS,  
1485 the *Psk-5 Spok* block, and locus 2 represents a killer locus with 40% FDS, *Spok2*. Red nuclei represent the  
1486 *Psk-S* parental genotype with *Spok2*, orange nuclei represent the *Psk-5* parental genotype with *Spok3* and  
1487 *Spok4*, green nuclei represent the recombinant genotype with no *Spok* genes, and blue nuclei represent  
1488 the recombinant genotype with *Spok2*, *Spok3*, and *Spok4*. Note that *Spok3* and *Spok4* are linked and do  
1489 not segregate independently. Below the asci are our interpretations of the annotations from *Hamann*  
1490 *and Osiewacz (2004)*. K + K strains would correspond to a strain with the *Psk-5* parental genotype of  
1491 ascus type 1. These should experience mutual killing and produce empty asci, so the fact that they are  
1492 observed from 4-spored asci suggests that when mutual killing occurs, 4-spores may be observed.  
1493 However as no S + S strains were reported we can infer that only the *Psk-5* type (grey) may be viable. S,K  
1494 +S,K strains are not indicative of a recombinant killer locus as suggested in the original work, but  
1495 represent strains with all three *Spok* genes as produced in ascus type 2. The FDS frequencies reported  
1496 suggest that the isolated strains are indicative of the blue nuclear genotype and not the green nuclear  
1497 genotype. The S,K + K and K + S,K strains are indistinguishable from each other and are indicative of the  
1498 surviving spores of a type 3 ascus. These strains should exhibit spore killing when selfed due to the  
1499 distribution of *Spok2*. Spore killing may not have been observed due to the incomplete penetrance of  
1500 *Spok2*. In all cases, these strains should not have been isolated from 4-spored asci, indicating that either  
1501 methodological issues occurred or that spore-killing may still produced 4-spored asci, but where the  
1502 spores which should be absent are instead inviable.