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Investigating Sk-3-based spore killing through DNA deletion in Neurospora crassa

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INVESTIGATING *SK-3*-BASED SPORE KILLING THROUGH DNA DELETION IN *NEUROSPORA CRASSA*

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Meiotic drive describes a process in which selfish alleles are recovered in more than half of a progeny generation. It is a type of gene drive and it has been discovered in strains of *Neurospora*, a filamentous fungus, through its spore killing mechanism. One of the most studied meiotic drive elements within *N. crassa* is *Spore-killer 3* (*Sk-3*). Previous studies have indicated that there is a genomic region within *Sk-3* that encodes resistance to spore killing and another that encodes an element that is required for spore killing. *Sk-3*'s resistance gene, *rsk*, has been identified. However, the exact region that mediates *Sk-3*'s spore killing mechanism is currently unknown. In a previous study, it was found that a mutation called *rfk-2*^{UV} disrupts spore killing by *Sk-3*. To better understand the region of Chromosome III in which *rfk-2*^{UV} is located (its exact location is unknown), I constructed a deletion vector to replace a DNA interval (*v374*) with a hygromycin resistance gene marker (*hph*). Transformants were crossed to produce offspring, and offspring were tested to determine if they possess the ability to kill ascospores. These findings will contribute to future efforts to determine the molecular nature of *rfk-2*^{UV} and why this mutation disrupts the ability of *Sk-3* to kill spores.

INVESTIGATING *SK-3*-BASED SPORE KILLING THROUGH DNA DELETION IN *NEUROSPORA CRASSA*

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CHAPTER I

INTRODUCTION

1.1 Meiotic Drive through Spore Killing

For many there has been an assumption that inheritance is "fair" and always follows the Mendelian pattern of inheritance. For example, Mendel's law of segregation states each allele comprising a heterozygous locus has a 50% chance of being passed to offspring (Abbott and Fairbanks 2016). However, not all genes follow this pattern. Meiotic drive, also known as segregation distortion, describes the phenomenon in which "cheating" alleles are recovered in more than half of the progeny (Burt and Trivers 2006). Segregation Distorter in Drosophila, Pollen killer in wheat, and the Gamete eliminator in tomato are common examples of meiotic drive (Turner and Perkins 1979).

Additionally, meiotic drive can be observed in fungi through spore killing, in which ascospores must contain the "spore killer" element to survive (Raju 2002). Spore killer, also known as *Sk,* has been identified specifically in *Neurospora sitophila* and *Neurospora intermedia*. In a cross that is heterozygous for *Sk*, survivors contain the killer allele Sk^{K}. Each ascus in the cross would contain four viable black ascospores, with the killer allele, and four clear ascospores that are inviable (Turner and Perkins 1979).

1.2 Mating in *Neurospora*

Most of *Neurospora*'s life cycle is spent in a vegetative haploid phase. However, a brief diploid phase can be entered that allows meiotic drive to occur when triggered by environmental cues. Sexual reproduction in *Neurospora* can occur when asexual spores, known as conidia,

donate a nucleus to an immature fruiting body, known as a protoperithecium. Individuals of different mating types must meet for mating and meiosis to take place (Zanders and Johannesson 2021). Crosses within the same species, however, are usually fertile (Turner and Perkins 1979). Conidia serve as the male parent, while the immature fruiting bodies are provided by the female parent.

A diploid nucleus is formed when pairs of nuclei, one from each parent, fuse within meiotic cells, or asci, of the fertilized fruiting body called the perithecium. The diploid nucleus passes through the standard stages of meiosis. Four haploid meiotic products produce eight nuclei through a post-meiotic mitosis. These eight nuclei become incorporated into eight ascospores, one nucleus per ascospore (Raju 1980). As ascospores mature, they darken in color because they accumulate a dark brown pigment. Once mature, ascospores will appear black in color. Viable black ascospores typically appear in asci alongside asci containing various proportions of brown, yellow, white, and clear shrunken ascospores (Turner and Perkins 1979). This is partially due to asci developing asynchronously in *Neurospora*. Mature ascospores are then shot through a small hole in the perithecium (Harvey et al. 2014).

1.3 *Sk-2* and *Sk-3*

Spore killer-1 or *Sk-1* is the element responsible for killing in *N. sitophila*. Additional killers were discovered only in *N. intermedia* (Turner et al. 2001). Two of the most studied distortion elements in *Neurospora* are *Spore killer-2* (*Sk-2*) and *Spore-killer 3* (*Sk-3*). *Sk-2* can be traced back to an *N. intermedia* ancestor, but it was first discovered in crosses with *N. crassa* stocks after a *nit-4* mutant had been introgressed from *N. intermedia* (Turner and Perkins 1979). *Sk-3*^{K} was discovered in *N. intermedia* (Turner and Perkins 1979).

Both the *Sk-2* and *Sk-3* loci have been mapped to a similar region of Chromosome III (Campbell and Turner 1987). Specifically, *Sk-3* has been mapped to a 30 cM interval of Chromosome III (Turner and Perkins 1979). The *Sk-2* and *Sk-3* loci are known to both have a killing ability and self-resistance (Campbell and Turner 1987). Despite their similarity in function, both elements do not have the same effect on one another. $Sk\text{-}2^{K}$ and $Sk\text{-}2^{R}$ both confer resistance to $Sk\text{-}2^{K}$, but are not resistant to $Sk\text{-}3^{K}$ (Turner and Perkins 1979). Additionally, crosses between $Sk\text{-}2^{K}$ and $Sk\text{-}3^{K}$ do not result in viable ascospores. To be specific, about 99.9% of ascospores abort and most rare survivors are aneuploid products of crossing-over (Turner et al. 1988).

1.4 Meiotic Silencing by Unpaired DNA (MSUD)

Studies have indicated that there is a genomic region encoding resistance and another for the spore-killing effect. In *Sk-2* and *Sk-3*, the gene *rsk* is required for resistance to spore killing, but not for spore killing itself (Hammond et al. 2012). Findings on *Sk-2* have indicated that the *Sk-2* killer is encoded by *rfk-1* with spore killing being absent in a cross of *Sk2 rfk-1*Δ × Sk^{S}, where Sk^{S} denotes a genotype that is sensitive to *Sk-2*-based spore killing (Rhoades et al. 2019). Spore killing will only occur, however, if *rfk-1* transcripts avoid silencing caused by a genome defense process known as meiotic silencing by unpaired DNA (MSUD) (Rhoades et al. 2019). MSUD is a process in which unpaired DNA in meiosis silences expression of DNA homologous to it (Shiu et al. 2001). In *Sk-2* and *Sk-3*, the MSUD suppressors do not suppress silencing of all unpaired loci (Raju et al. 2007).

1.5 Discovering *rfk-2*

The exact region that leads to *Sk-3*'s spore killing is currently unknown. The deletion of the most likely *rfk-1* ortholog in *Sk-3* had no effect on spore killing (Svedberg et al. 2018). In a previous study, it was found that a mutation called *rfk-2*^{UV} disrupts *Sk-3*'s spore killing effect. *rfk-2*^{UV} was mapped relative to three markers (*hphA*, *hphB*, and *mus-52*Δ), but its exact location on Chromosome III is unknown (Velasquez et al. 2022).

Previous work to identify the location of *rfk-2*^{UV} has identified a 1.3 kb DNA interval (called *v350*) that appears to be required for *Sk-3* spore killing. These findings suggest that an *Sk-3* killer gene, perhaps *rfk-2,* is located within the *v350* interval. However, not much is known about the *v350* interval. To better understand the region in which *rfk-2*^{UV} may be located, I constructed a deletion vector to replace a DNA interval called *v374* with a hygromycin resistance gene (*hph*). The relationship between *v350* and *v374* is described below. My hypothesis is that deletion of the *v374* interval will not disrupt *Sk-3*-based spore killing. My work will further our understanding of the genetic elements controlling spore killing by *Sk-3*.

CHAPTER II

METHODS

2.1 Overview

A DNA replacement vector was constructed to replace interval *v374* with a hygromycin resistance gene (*hph*) through transformation. Transformants were selected and crossed to produce ascospores. Ascospores were collected and used to isolate homokaryotic deletion strains, which were subsequently examined in test crosses. Perithecia were then collected from the test crosses and dissected to obtain rosette images and score for the presence or absence of spore killing. An overview of the methods is depicted in **Figure 1.**

2.2 DNA Replacement Vector Construction

Transformation vector v374 (named similarly to the DNA interval it is designed to replace with *hph*) was constructed with Q5® High-Fidelity DNA Polymerase (New England Biolabs) via DJ-PCR essentially as described by Yu *et al.* (2004). First, left and right flanks of DNA were amplified from RDGR170.3 genomic DNA. The left flank was amplified using forward primer V374-A and reverse primer V374-B with an expected sequence length of 872 base pairs (**Figure 2**). The right flank was amplified using forward primer V350-C and reverse primer V350-D with an expected sequence length of 813 base pairs (**Figure 3**).

The v374 center fragment for DJ-PCR shown in **Figure 4** was amplified from plasmid pTH1256.1 (GenBank MH550659.1) with forward primer HPH-CEN-F and reverse primer HPH-CEN-R. The *v374* DNA interval designed to be replaced with the center fragment is shown in **Figure 5**. The position of the *v374* deletion on Chromosome III is mapped in **Figure 6.** The predicted sequence of PCR products obtained from strains with primer set V374-E/V372-F and

DNA templates derived from strains with a *v374+* genotype is shown in **Figure 7.** For comparison, the predicted sequence of PCR products obtained with the same primer set for DNA templates from strains with a *v374Δ* genotype is shown in **Figure 8**.

The following methods were used to amplify the left and right flank. Two standard primer mixes were made by adding 6.25 µl of the forward primer solution (100 pmol/µl), 6.25 µl of the reverse primer (100 pmol/µl), and 487.5 µl of sterile water for both the left flank and the right flank. A PCR rack was placed on ice and tubes were labeled 1-4. Next, 5 µl of the left fragment primer mix was added to tubes 1 and 2, and 5 µl of the right fragment primer mix was added to tubes 3 and 4. Then 1.0 µl of DNA template RDGR170.3 (10 ng/µl) was transferred to each tube. An enzyme master mix was prepared for 5 reactions in a sterile 1.5 ml microcentrifuge tube (MCT) using 67.0 µl sterile water, 25.0 µl Q5 reaction buffer, 2.5 ml dNTP (10 mM), and 0.5 µl Q5® High-Fidelity DNA Polymerase (New England Biolabs). An enzyme master mix for 5 reactions was prepared to mitigate for possible pipetting error. To PCR tubes on ice, 19 µl of the enzyme master mix was added to each reaction. Reactions were mixed by slowly pipetting up and down. The PCR machine was set with manufacturer's recommendations for the Q5 DNA polymerase: Step 1 for 30 seconds at 98 °C, Step 2 for 5 seconds at 98 °C, Step 3 for 10 seconds at 62.5 °C, Step 4 for 1 minute and 30 seconds at 72 °C, (Cycle to #2, 25 x), Step 5 for 10 minutes at 72 °C, and Step 6 indefinitely at 12 °C.

The flank and center fragment PCR products were gel purified using the Gel Extraction & PCR Cleanup Kit (IBI Scientific) before DJ-PCR was performed (Yu et al. 2004). Purified left and right flanks (**Figure 9**) and the purified vector (**Figure 10**) were examined by gel electrophoresis. The fusion product was amplified with primer set V374-E/V372-F and column

purified with the Gel Extraction & PCR Cleanup Kit (IBI Scientific) before use in *N. crassa* transformation.

The following methods were used to fuse the left and right flanks to the center fragment. To a PCR tube, 5.0 µl of the amplified left fragment, 5.0 µl of the amplified right fragment, and 5.0 µl of the amplified center fragment were added. Also added to the same PCR tube were 62.6 µl of autoclaved sterile water, 20.0 µl of Q5 Reaction Buffer, 2.0 µl of 10 mM dNTP mix, and 0.4 µl of Q5 enzyme. The reaction was mixed gently by pipetting up and down. The reaction was then transferred to the PCR machine and the following parameters were set: Step 1 for 30 seconds at 98 °C, Step 2 for 5 seconds at 98 °C, Step 3 for 4 minutes at 58 °C, Step 4 for 6 minutes at 72 °C, (Cycle to #2, 10 x), Step 5 for 4 minutes at 72 °C, and Step 6 at 12 °C indefinitely.

The following methods were used to amplify the DNA replacement vector fusion product with nested primers. A primer mix was made using 6.25 µl of the forward primer (100 pmol/µl), 6.25 µl of the reverse primer (100 pmol/µl), and 487.5 µl of sterile water. The mix was vortexed, spun down, and placed on ice. Next, 20.0 µl of the primer mix was added to a PCR tube. To the same PCR tube, 5.0 µl of the fusion product PCR reaction, 52.6 µl of autoclaved sterile water, 20.0 µl of Q5 reaction buffer, 2.0 µl 10 mM dNTP mix, and 0.4 µl Q5 enzyme were added. The reaction was then transferred to the PCR machine and the following parameters were set: Step 1 for 30 seconds at 98 °C, Step 2 for 5 seconds at 98 °C, Step 3 for 10 seconds at 62.5 °C, Step 4 for 3 minutes and 30 seconds at 72 °C, (Cycle to #2, 10x), Step 5 for 10 minutes at 72 °C, and Step 6 at 12 °C indefinitely.

2.3 Transformation of *N. crassa* with DNA Replacement Vector

Conidia used for transformation were produced in a 250 ml flask prepared with 50 ml solid Vogel's Minimal Medium (VMM). For vegetative propagation of all *Neurospora* strains, VMM or Vogel's Minimal Agar (VMA: VMM + 2% agar) (Vogel 1956) was used unless otherwise stated. The medium was prepared using 48 ml miliQ water, 2 ml 25x Vogel's salts, 1.0 g sucrose, and 0.75 g agar. The flank of medium was capped with a glass beaker, autoclaved on liquid cycle, and then placed on a clean 1020 tray (10 x 20 cm tray typically used for plants) on a culture shelf for 1-2 days to allow excess moisture to evaporate. When the flask had no visible condensation, the medium was inoculated with 20 µl of a RDGR170.3 cryogenic conidial suspension in a biosafety hood. The flask was placed in a 32 °C incubator for 2 days, and then transferred to a clean 1020 tray on a culture shelf for 4 weeks.

Top agar used to plate transformants was made prior to transformation by adding 105 ml milliQ water, 27.3 g sorbitol, 6 ml of 25x Vogels Salts, and 2 g agar to a 500 ml glass bottle. The medium was autoclaved on liquid cycle and cooled to a safe handling temperature. Once cooled, 15 ml of 10x FIGS (20% sorbose, 0.5% D-Fructose, 0.2% Inositol, 0.5% D-Glucose) was added. The medium was stored on a laboratory shelf until transformation. On the day of transformation, the medium was liquified by heating in a microwave and poured into conical vials. Bottom agar was made by adding 172 ml milliQ water, 8 ml of 25x Vogels Salts, 3 g agar to a 500 ml glass bottle. The medium was autoclaved on liquid cycle and cooled to a safe handling temperature. Once cooled, 150 µl of hygromycin (400 mg/ml) and 20 ml 10x FIGS solution was added. The medium was then poured into petri dishes and stored at room temperature for 24 hours, followed by 4 °C storage until needed for transformation.

For transformation of *N. crassa*, the electroporation method of Margolin *et al.* (1997) was performed with modifications as described by Rhoades *et al.* (2020). Using a sterile wooden

applicator, conidia grown in the 250 ml flask was collected and placed in 30 ml ice-cold 1 M sorbitol in a 50 ml conical tube. A 100 µm cell strainer was used to remove mycelia and large fungal tissues. The filtered conidia were placed on ice. Concentration of conidia was estimated in unit/ml by placing 100 µl of the filtered conidia and 900 µl sterile 1 M ice-cold sorbitol in a spectrophotometry cuvette. Absorbance was measured at 420 nm. Units were calculated as volume undiluted suspension x A_{420} x 100.

Next, the filtered conidia was centrifuged at 2000 x g for 10 minutes. After centrifugation, the supernatant was poured into a flask for disposal. A 20 ml aliquot of ice-cold 1 M sorbitol was then added to the conidial pellet. The pellet was resuspended by gentle agitation and placed in the centrifuge again with the aforementioned settings. After the second centrifugation, the supernatant was disposed and a volume of ice-cold 1 M sorbitol (no more than ¼ the number of units previously calculated) was added to the pellet. The pellet was resuspended by gentle agitation and the conidial suspension was stored on ice.

Two electroporation cuvettes were placed on ice with one labeled “DNA” and the other labeled “CON”. MCT tubes were also placed on ice with corresponding labels. Next, 100 µl of the conidial suspension was transferred to each of the MCT tubes on ice. To the MCT tube labeled “DNA”, 10 µl of the v374 DNA replacement vector was added. The tube was mixed by vigorously tapping approximately 5 to 10 times and returned to ice. To the MCT tube labeled “CON”, 10 µl of elution buffer (from the IBI Scientific Gel Extraction & PCR Cleanup Kit) was added. The tube was mixed by vigorously tapping approximately 5 to 10 times before returning it to ice. Two sterile 50 ml conical vials were then prepared as rescue vials. Conical tubes were labeled with either “DNA” or “CON” and approximately 5 ml of rescue medium (Vogel’s

minimal medium with 1M sorbitol) was added to each vial. Conical vials containing rescue medium were stored on ice.

Next, electroporation was performed. To the electroporation cuvette labeled, “DNA”, 100 µl of the conidial suspension from the “DNA” MCT was added. The solution was transferred directly between the electrodes and placed back on ice. The aforementioned methods were repeated with the “CON” conidial suspension. Samples were inserted into the electroporator (one at a time) and the voltage was set to 1500 V. Before the machine was started, 750 µl of ice cold 1 M sorbitol was loaded into a 1 ml pipettor tip. Once samples were removed from the electroporator, 750 µl of was transferred directly into the conidial suspension between the electrodes. Using the same tip, the conidial suspension with added 1M sorbitol was transferred to the “DNA” rescue medium. The same procedure was repeated with “CON” sample. Caps on the conical tubes were sealed and the tubes were placed in a shaker at 32°C for 3.5 hours. The shaker was set to 80 RPM.

After rescue cultures incubated for 3.5 hours, bottom agar plates were obtained and labeled “DNA” or “CON”. Top agar vials were also obtained, and one was designated as a “temperature check vial” and the cap was replaced with a “thermometer cap” (a cap with a hole pierced for a thermometer). Once the top agar was estimated to be below 50 °C, 100 µl of the “DNA” rescue culture was added to the top agar and mixed by inverting the tube 5x, then 10 ml was poured onto a bottom agar plate labeled “DNA”. 500 µl of the rescue culture was then added to the same vial of top agar, mixed by inversion 5x, and approximately 10 ml was poured onto a second bottom agar plate labeled “DNA”. 1000 µl of the rescue culture was then added to the same vial of top agar, mixed by inversion 5x, and approximately 10 ml was poured onto a third bottom agar plate labeled “DNA”. The remaining rescue culture was then added to the same vial

of top agar, mixed by inversion 5x, and the remaining top agar was poured onto a fourth bottom agar plate labeled “DNA”. This process was repeated for the control rescue culture and the “CON” bottom agar plates. The petri dishes were placed on a clean 1020 tray overnight “right side up” at room temperature. After 24-72 hours, the petri dishes were inverted and placed at 32 °C.

2.4 Selecting Transformants

Transformants were selected and transferred to Vogel’s slants containing 200 µg/ml Hygromycin B (Gold Biotechnology). The slants were placed in a 32 °C incubator for 1-2 days, and then taken out to grow at room temperature. After the transformants had grown on the slants, the first *N. crassa* cross was performed using Westergaard and Mitchell’s synthetic crossing agar (SCA) (Westergaard and Mitchell 1947) with 1.5% sucrose and a pH of 6.5 ion 60 mm culture plates. To obtain homokaryotic offspring from heterokaryotic transformants, crosses were performed. Each 60 mm SCA plate was inoculated with strain RTH1005.2 which served as the protoperithecial or “female” parent. The plates were left to incubate on a clean 1020 tray on an incubation shelf for 6-8 days. Conidial suspensions were made from the transformants, which served as the “male” parents. A 500 µl aliquot of sterile water was placed into a microcentrifuge tube for each transformant. Using a sterile wooden applicator, conidia was collected from the slants containing transformants over a 10% bleach moat, and the conidia samples were transferred to the microcentrifuge tubes. The suspensions were placed on ice. Next, the suspensions were vortexed, and 200 µl of each suspension was transferred dropwise onto separate RTH1005.2 culture plates. Crosses were named with the transformant name and RTH1005.2. The petri dishes were incubated on a single layer right side up in a 1020 tray

without a humidity dome for one week. After one week, the humidity dome was placed on the tray and the cultures were left to incubate for 24 days at room temperature.

At 24 days post fertilization, ascospores were harvested from the lids of the crossing plates. Sterile water (500 µl) was transferred to two microcentrifuge tubes. A 200 µl barrier tip was used to transfer 50 µl of water from the microcentrifuge tube to the dried ascospores on the crossing lid. By pipetting up and down gently, ascospores were collected and transferred into one of the microcentrifuge tubes. The ascospore suspensions were then vortexed and placed at 4°C in the dark for at least 16 hours.

The following day, these ascospores suspensions, called the stock suspensions, were removed from the dark and used to make working stocks. Using a dissecting microscope, the concentration of ascospores was estimated and working stocks of 1-5 ascospores per µl were made with 500 µl of sterile water in a microcentrifuge tube. The working stocks were then placed on a heat block at 60°C for 30 minutes. After heat shocking, the tubes were inverted multiple times to mix the ascospore suspension. Using a sterile technique in the biosafety hood, 50 µl, 100 µl, and 200 µl aliquots of the working suspensions were spread onto 100 mm petri dishes containing VMA plus hygromcyin (200 µg/ml). The plates were incubated “right side up” overnight at room temperature in a clean 1020 tray. The following day, at the approximate 24-hour timepoint, single hygromycin-resistant germlings were transferred to VMA plus hygromcyin (200 µg/ml) slants in 16 x 125 mm culture tubes using needles and flame sterilization.

2.5 Test Crosses

Unidirectional crosses were performed for spore killing assays using SCA as previously described (Samarajeewa *et al.* 2014). Twenty 60 mm SCA plates were prepared for test crosses. Ten plates were inoculated with strain RTH1623.1 and 9 plates with strain RTH1623.2, both of which served as protoperithecial parents. Once inoculated, the plates were incubated at room temperature on a clean 1020 tray for approximately one week. Conidial suspensions, made from the germling cultures described above) served as the conidial "male" parents. Conidial suspensions were used to inoculate the RTH1623.1 and RTH1623.2 SCA cultures using methods described previously. Additionally, conidial suspensions were made from strains RDRG170.3 (*Sk-3*) and RZS27.10 (*Sk-S*) and used to initiate control crosses with RTH1623.1 and RTH1623.2. Test crosses were performed twice. Crossing plates were incubated right side up in a clean 1020 tray with a humidity dome for 13-14 days. On day 14 post fertilization of the first test cross, asci (ascospore sacs) were dissected from fruiting bodies in 25% glycerol. On day 13 post fertilization of the second cross, asci (ascospore sacs) were dissected from fruiting bodies in 25% glycerol. Asci were imaged with a Leica DMBRE microscope and Zeiss imaging system. Images of conidial parents and second test cross plates are shown in **Figure 11**.

2.6 DNA Isolation

For DNA isolation from *N. crassa,* strains were cultured in 2-4 ml of sterile VMM. Conidia was transferred into tubes with VMM with sterile wooden applicators over a 10% bleach moat. Tubes were sterilized with an ethanol candle, and then capped (with a two position polypropylene caps) in a manner that allows air exchange. Tubes were then placed in a shaking incubator at 150 rpm, 30-37 °C, for 24-48 hours. This procedure was repeated for four transformants. Next, sterile wood applicators were used to transfer mycelia from the cultures to

filter paper on paper towels. Liquid was removed from tissue by folding the filter paper between the paper towels and applying pressure. Dried mycelia were transferred to microcentrifuge tubes with clean forceps. Mycelia was then lyophilized and stored at room temperature in a dry cabinet before DNA isolation.

DNA from strains used in the first test crosses RSAK12.14, RSAK12.7, and RSAK12.12 was isolated from lyophilized tissue. DNA from strains used in the second test crosses RSAK12.101, RSAK12.105, RSAK12.106, RSAK12.107 was also isolated from lyophilized tissue. Mycelial tissue was ground in microcentrifuge tubes with clean microspatulas. Each sample was ground with a separate microspatula to avoid cross contamination. DNA was isolated with IBI Scientific's Mini Genomic DNA Kit (Plant/Fungi). Isolated DNA from test strains and a control strain (RDGR170.3, the transformation host) was used as the template in PCR-based genotyping assays. Gel electrophoresis was used to examine the PCR products (**Figure 12 and Figure 13**).

CHAPTER III

RESULTS

3.1 Test Cross Genotypes

Previous work identified that a 1.3 kb V350 DNA as possibly required for *Sk-3* spore killing (Rhoades and Hammond, unpublished results). DNA interval *v374* is within the *v350* interval on Chromosome III as shown in **Figure 6**. My initial hypothesis was that deletion of the *v374* interval will not disrupt *Sk-3*-based spore killing. In **Figure 12**, genotype results with DNA from test strains RSAK12.14, RSAK12.7, and RSAK12.12 are shown. Strain RSAK12.14 produces a PCR product of approximately 3000 bp, consistent with the *v374Δ* genotype (Figure 8). In contrast, strains RSAK12.7 and RSAK12.12 show two PCR products, one at approximately 3000 bp, which is consistent with the *v374Δ* genotype, and a second at approximately 2000 bp, which is consistent with the *v374+* genotype (Figure 7). These results suggest that RSAK12.7 and RSAK12.12 are heterokaryotic strains, containing a mixture of transformed and non-transformed nuclei. The control strain, RDGR170.3, produced a single PCR product of about 2000 bp, which is consistent with the *v374+* genotype as expected.

In **Figure 13**, genotyping results with DNA from test strains RSAK12.101, RSAK12.105, RSAK12.106, and RSAK12.107 are shown. Each of the test strains RSAK12.101, RSAK12.105, RSAK12.106, RSAK12.107 produces a PCR product consistent with the *v374Δ* genotype. However, RSAK12.106 and RSAK12.107 each also produce a PCR product consistent with *v374+* genotype. These results suggest that RSAK12.101 are RSAK12.105 are homokaryotic *v374Δ* strains, while RSAK12.106 and RSAK12.107 are heterokaryotic *v374Δ* / *v374Δ+* strains.

3.2 Test Cross Phenotypes

Phenotypes can be identified by rosette images. Spore killing was not detected in the first group of test crosses, which consisted of crosses of RSAK12.7, RSAK12.10, RSAK12.12, RSAK12.13, RSAK12.14, and RSAK12.15 to the RTH1623.1 and RTH1623.2 protopertithecial parents (**Figures 14-20**). Images of asci from crosses between control strains RDGR170.3 and RZS27.10 the same protoperithecial parents are shown in **Figure 21 and 22**, respectively. Crosses with RDGR170.3, RZS27.10, RSAK12.12, RSAK12.15 were imaged on day 13 post fertilization, while crosses with RSAK12.7, RSAK12.10, RSAK12.13, and RSAK12.14 were imaged on day 14 post fertilization. Spore killing was also not detected in the second group of test crosses, which consisted of RSAK12.101, RSAK12.105, RSAK12.106, RSAK12.107, and RSAK12.108 as male strains and RTH1623.1 and RTH1623.2 as female strains (**Figures 23-28**). Images of asci from the control crosses for the second group are shown in **Figures 29 and 30**. All crosses were imaged on day 13 post fertilization for the second group. The presence of absence of spore killing is determined by the number of viable ascospores in most mature asci, with 8 viable ascospores indicative of no spore killing. The RDGR170.3 and RZS27.10 control crosses are used as references for spore killing and no spore killing, respectively.

CHAPTER IV

DISCUSSION

All offspring from each of my test crosses show an absence of spore killing. This is demonstrated by the presence of eight viable ascospores in the majority of mature asci and the scarcity of asci with four viable and four inviable ascospores in the first group of test crosses (**Figures 14-20**) and the second group of test crosses (**Figures 23-28**). Thus, based on the spore killing assays, it appears that interval *v374* is needed for *Sk-3*'s spore killing function.

Although interval *v374* is needed for *Sk-3*'s spore killing function, it is still unknown if the coding region for *rfk-2* is within the *v374* interval, or if *rfk-2* overlaps the *v374* interval. It also remains possible that *rfk-2* is an independent locus required for *Sk-3*-based spore killing. The relationship between *rfk-2*^{UV} and the *v374* could be examined by comparing the DNA sequence of the *v374* interval in *rfk-2*+ and *rfk-2*^{UV} strains. Either way, the findings of my work increase our knowledge with respect to the genetic elements controlling *Sk-3*-based spore killing.

Discovering the region that encodes for *rfk-2* can aid in understanding of gene drivers. With proper understanding of how gene drive functions, there are numerous practical applications through synthetic gene drive. Due to a gene drivers' tendency to spread through a population, they can be used to control pest and vector populations. If synthetic gene drivers are formed, it is possible for them to modify or eliminate the targeted population (Wedell et al. 2019). In a recent study, it was shown that a gene driver spore killer element, Spok1, could eliminate virulence traits from populations of *F. graminearum*. Spok1 was cloned from *Podospora spp* (Vogan et al. 2019). Spok1 could also be used to distort inheritance at two genomic loci (Gardiner et al. 2020). This indicates a natural gene drive element can function in a distantly related fungus from than the one it originated in.

New discoveries regarding the role of gene drive in distorting inheritance of virulence loci demonstrates the importance of future research on gene drive mechanisms within fungi. The location of *rfk-2* on Chromosome III will continue to be mapped through further studies.

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TABLES

Table 1 Strains used in this study

Strain name (alias)	Genotype
RTH1005.2	*rid; fl; Sk-S^{ChrIII} a+*
RZS27.10	*rid; Sk-S^{ChrIII}; mus-51^{RIP70} a+*
RTH1623.1	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ A+*
RTH1623.2	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ a+*
RDGR170.3	*rid; Sk-3^{ChrIII}; mus-51Δ::bar+ A+*
TSAK1.12	v374-based, hygromycin-resistant transformant of RDGR170.3
RSAK12.7	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.10	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.12	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.13	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.14	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.15	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.101	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.105	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.106	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.107	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2
RSAK12.108	hygromycin-resistant offspring of TSAK1.12 × RTH1005.2

Sk-3^{ChrIII} indicates the strain carries the *Sk-3* genetic element, while *Sk-S^{ChrIII}* indicates the strain does not carry the *Sk-3* genetic element. The *rid* allele disrupts Repeat Induced Point Mutation (Freitag et al. 2002). The *fl* allele disrupts macroconidiation (Perking et al. 2001). The *mus-*

51^{RIP70} and *mus-51Δ::bar+* allele disrupts Non-Homologous End Joining (Ninomiya et al. 2004; Smith et al. 2016). The *sad-2Δ::hph+* allele suppresses Meiotic Silencing by Unpaired DNA (Shiu et al. 2006).

Table 2 Primers used in this study

Name (Alias)	Sequence (5′ > 3′)
HPH-CEN-F	AACTGATATTGAAGGAGCATTTTTTGG
HPH-CEN-R	AACTGGTTCCCGGTCGGCAT
V0374-A	TCCAAAGGGAAGGACCGGGCACA
V0374-B	AAAAAATGCTCCTTCAATATCAGTTCGTGAGCCGGAGCAGTCGTCGTA
V350-C	GAGTAGATGCCGACCGGGAACCAGTTCCTTGTCCTCTCGGTCCTCTCTGT
V350-D	GGTAGCGTTCTGGGATGAGTGTGG
V0374-E	GGGACAGAGAGTGGCGTCTGCCT
V0372-F	GGACCTGGCTGTTGCTGGCGTAG

FIGURES

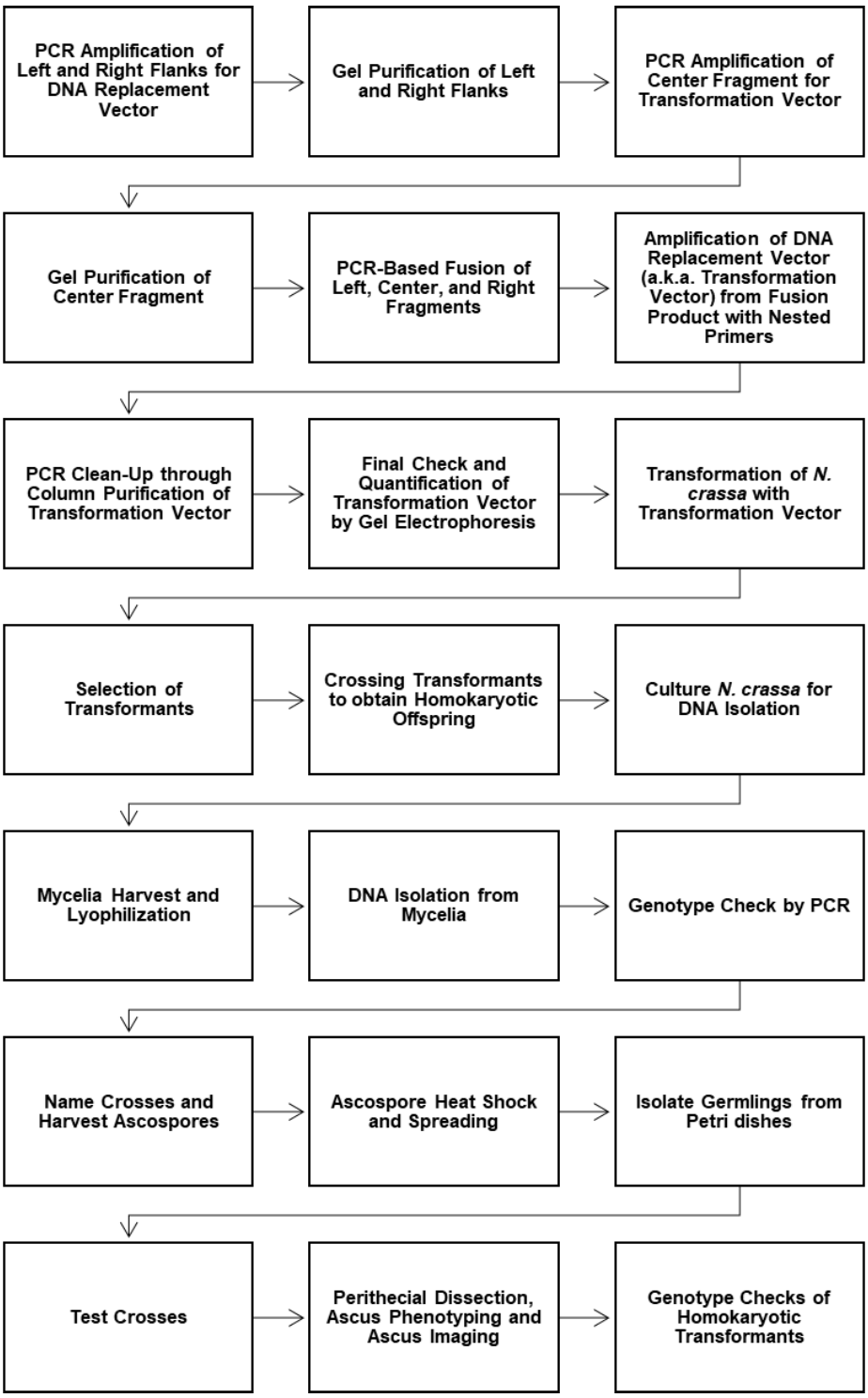

Figure 1 Methods overview.

```
>v374 left flank
TCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACACTAACGAGCGATTT
TGCCACCTAGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCCTACCACGCTTCTTTTTC
CGTCCACCGGCTCTTGGAGTACCGTACATACCTCAAACACTTCATTCCACCCTGTTCTGGAAT
TGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGG
AAGGAGGAGAGGTACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGG
ACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTC
ACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCT
CATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAAC
CCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAG
GCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAA
AGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCA
AGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGAT
GGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGA
TGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG
```

Figure 2 The v374 left flank. The 872 bp sequence of the v374 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0374-A and V0374-B from RDGR170.3 genomic DNA.

```
>v374 right flank
CCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCC
CCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCC
CCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCA
GGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCC
GTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCT
CCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGC
TTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAA
TTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTG
CCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGG
AGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAG
GTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAAC
CCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCC
AACGGCGACGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACC
```

Figure 3 The v374 right flank. The 813 bp sequence of the v374 right flank is shown. This sequence was PCR-amplified with primers V350-C and V350-D from RDGR170.3 genomic DNA.

>v374 center fragment
AACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGC
CTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTC
TTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTG
CGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCT
TTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACT
CACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCA
GCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCT
GCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATC
GGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTG
CATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGG
GTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGC
GATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGT
CGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGT
GCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGA
CTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCC
GTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGG
ATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGT
TGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGG
AGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTG
TGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATA
GAGTAGATGCCGACCGGGAACCAGT

Figure 4 The v374 center fragment. The 1411 bp sequence of the v374 center fragment is shown. This sequence was PCR-amplified with primers HPH-CEN-F and HPH-CEN-R from plasmid pTH1256.1. The sequence contains *Aspergillus nidulans trpC* promoter sequences upstream of the 1026 bp *hph* coding region. The positions of the *hph* start codon and stop codon are indicated with gray shading.

>*v374* DNA interval
CCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCA
AGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTG
ACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGC
TAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATG
ACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGC
TCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATT
TTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTT
CATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATG
TGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCA
TGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTT
CTTTCTCTTCTCATTTCCTCGACGGCTTA

Figure 5 The *v374* DNA interval. The DNA sequence of the 659 bp *v374* DNA interval is shown. DNA replacement Vector v374 was designed to delete this interval by replacing it with *hph*+.

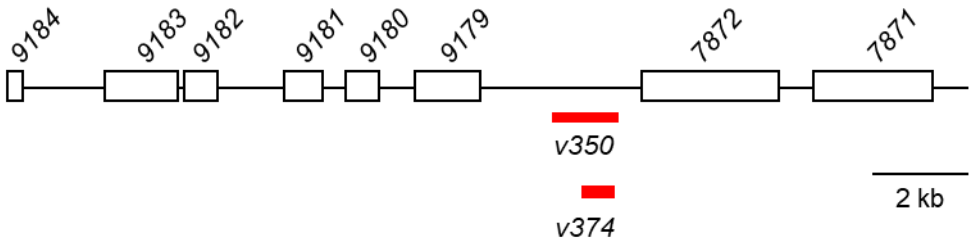

Figure 6 *Sk-3* Chromosome III positions 320,000–340,000. A diagram of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the locations of protein coding genes. Gene numbers were identified with blastx searches of an *N. crassa* protein database. The location of intervals *v350* and *v374* are indicated with red bars.

>*v374+* PCR product, predicted sequence, primers V374-E and V372-F

GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGG
TACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAA
ATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTT
GAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGA
CTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGA
ACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCG
TTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCC
CCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTC
CCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAG
GCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAA
AAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAAT
TGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGG
AGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGC
TCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCT
CTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAG
CATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTC
GGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTT
TATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCT
CGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGACCTTG
CCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTC
CGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACAC
CAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCT
AAACGAATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATC
GTCTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAAC
AGAATTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAG
CAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAAC
GGTTCCGGCCAAATGTCCATGTCCCCTCGAGA**CTACGCCAGCAACAGCCAGGTCC**

Figure 7 PCR product sequence: *Sk-3 v374+* genotype. The predicted sequence of DNA amplified with primers V374-E and V372-F from a template consisting of *Sk-3 v374+* genomic DNA is shown in FASTA format. The binding sites of V374-E and V372-F are indicated with bold font. The *v374* interval is indicated with red font. The length of the sequence is 1972 bp.

>*v374Δ::hph+* PCR product, predicted sequence, primers V374-E and V372-F
GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGG
TACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAA
ATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTT
GAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGA
CTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGA
ACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCG
TTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCC
CCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTC
CCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG*AACTGATATTGAAGGAGCATTTTTTGGGCTT*
GGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATT
TGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGC
GCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATA
ACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCT
*TTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAA**ATC**AAAAAGCCTGAACTCACCGCGA*
CGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAA
GAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGG
TTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACA
TTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTG
CCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCT
TAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCA
TATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTC
GCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCATGCGGA
TTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGT
TCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAG
ACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGTATATGCTCCGCAT
TGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGAT
GCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTC
TGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAA
*GGAA**TAC**AGTAGATGCCGACCGGGAACCAGT*CCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAA
ACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCT
GCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGT
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TCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATT
ATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACCCTCCCTCTC
CTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCATTGCCAAACCAGACCCAGCAA
CAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACCGTAC
TCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCG
AGA**CTACGCCAGCAACAGCCAGGTCC**

Figure 8 PCR product sequence: *Sk-3 v374Δ::hph+* genotype. The predicted sequence of DNA amplified with primers V374-E and V372-F from a template consisting of *Sk-3 v374Δ::hph+* genomic DNA is shown in FASTA format. The binding sites of V374-E and V372-F are

indicated with bold font. The start and stop codons of the *hph* coding region are shown with white font on black background. The length of the sequence is 2724 bp.

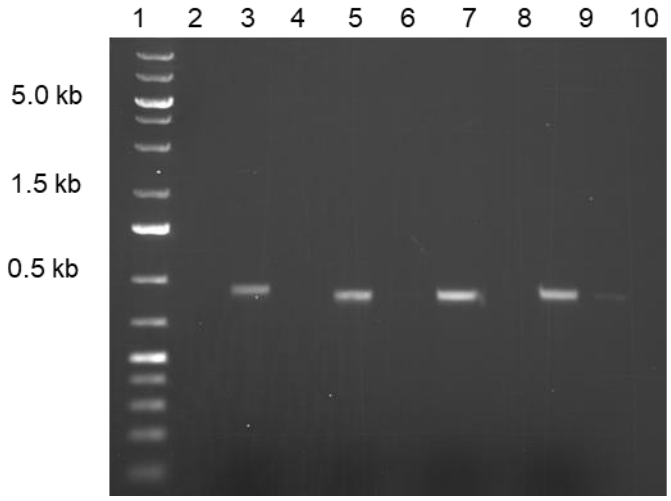

Figure 9 v374 construction: left and right flanks. PCR products for v374 left and right flanks. The left flank for v374 was amplified from RDGR170.3 genomic DNA with primers V374-A and V374-B. The right flank for v374 was amplified from RDGR170.3 genomic DNA with primers V350-C and V350-D. Products were gel purified as described in the methods section. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lane 2) not applicable, Lane 3) 5 µl purified product (v374 left flank); Lane 4) not applicable; Lane 5) 5 µl purified product (v374 right flank); Lanes 6-10) not applicable. The expected product lengths for the v374 left and right flanks are 872 bp and 813 bp, respectively.

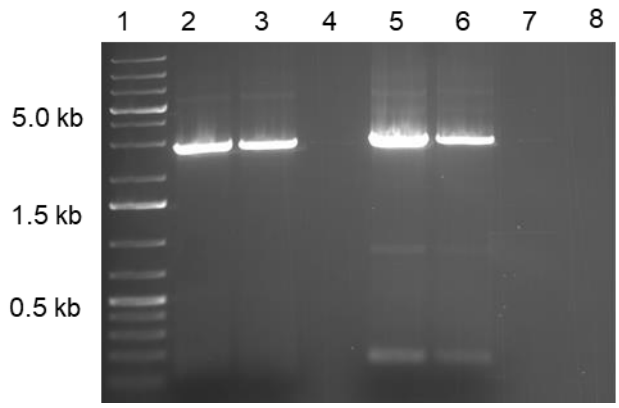

Figure 10 v374 construction: fusion and nested amplification. The v374 left flank, v374 center fragment, and v374 right flank were fused by DJ-PCR. The fusion product was amplified with primers V374-E and V372-F. The amplified product was column purified as described in the methods section. The amplified and purified product was examined by gel electrophoresis with ethidium bromide staining. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lane 2) 5 µl of amplified and purified v374. Lanes 3–8) not applicable. The expected length of v374 is 2724 bp.

Figure 11 Test strains and crosses. **(A)** Six hygromycin-resistant offspring from cross RSAK12 (TSAK1.12 × RTH1005.2) were selected for test crosses. 1) RSAK12.101, 2) RSAK12.105, 3) RSAK12.106, 4) RSAK12.107, 5) RSAK12.108, and 6) RSAK12.109; 7) RDGR170.3, *Sk-3* control; and 8) RZS27.10, *Sk-S* control. (B) Test crosses were performed with RTH1623.1 and RTH1623.2. Images are of crossing dishes approximately three weeks post fertilization.

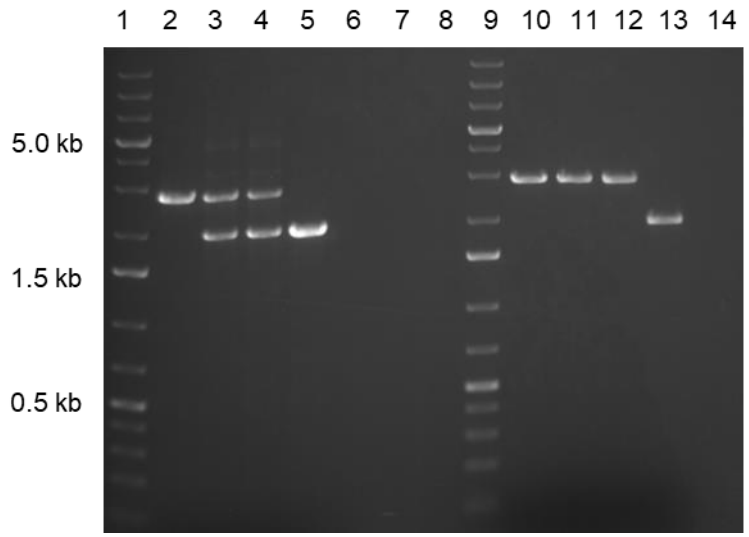

Figure 12 Test strain genotype confirmation. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0374-E and V0372-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lanes 2–5) PCR products from templates RSAK12.14, RSAK12.7, and RSAK12.12, and RDGR170.3, respectively. Lanes 6-14) not applicable. The expected PCR product lengths are 1972 bp for *v374+* and 2724 bp for *v374Δ::hph+*.

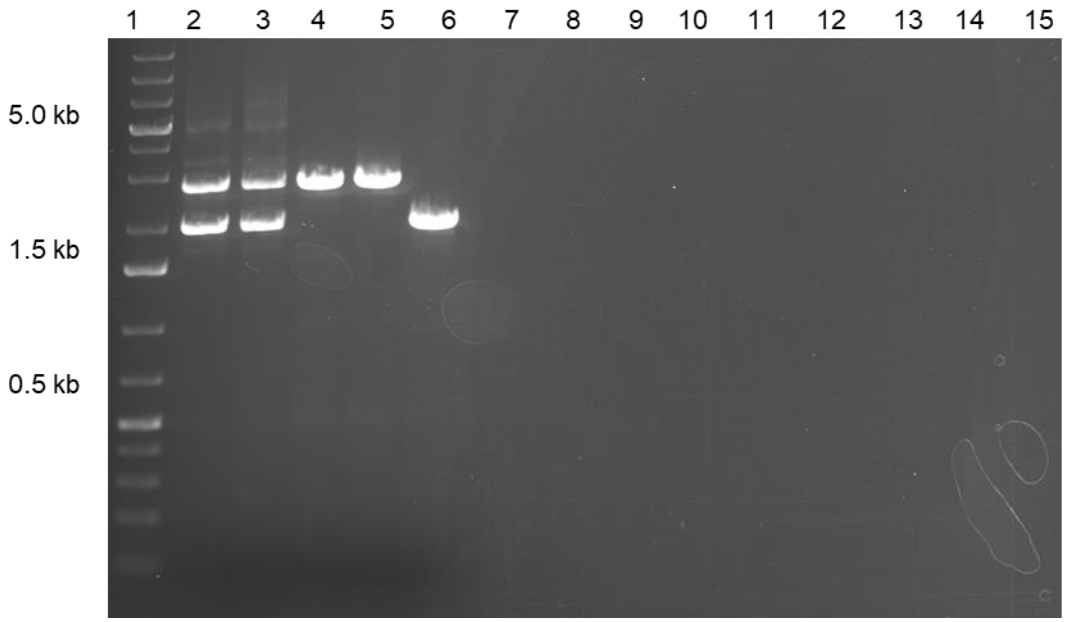

Figure 13 Test strain genotype confirmation. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0374-E and V0372-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product length for the *v374+* and *v374Δ* genotypes are 1972 bp and 2724 bp, respectively. Lane 1 contains 0.5 µg of GeneRule 1 Kb Plus DNA ladder (ThemoFisher). DNA templates for each PCR reaction are as follows: Lane 2, RSAK12.101; Lane 3, RSAK12.105; Lane 4, RSAK12.106; Lane 5, RSAK12.107; and Lane 6, RDGR170.3; Lanes 7–15) Not appliable. These results show that test strains RSAK12.101, RSAK12.105, RSAK12.106, and RSAK12.107 have the *v374Δ* genotype, while RSAK12.101 and RSAK12.105 are heterokaryons (nuclei genotypes are mixed between and *v374+* and *v374Δ*).

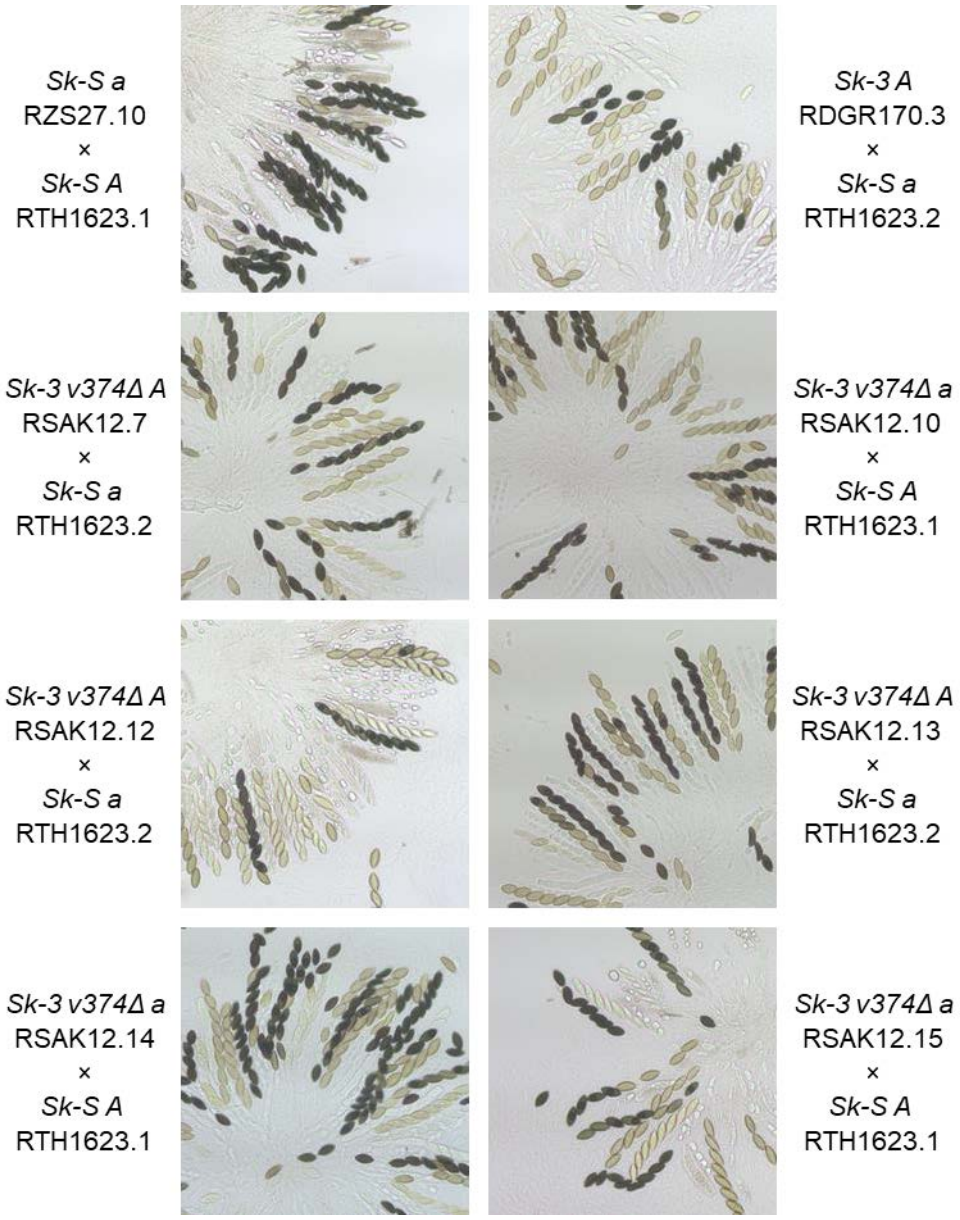

Figure 14 Ascus phenotype summary. Asci were dissected from perithecia of four crosses (RZS27.10 × RTH1623.1, RDGR170.3 × RTH1623.2, RSAK12.12 × RTH1623.2, RSAK12.15 × RTH1623.1) on day 13 post fertilization and imaged under magnification. Asci were dissected from perithecia of remaining four crosses (RSAK12.7 × RTH1623.2, RSAK12.10 × RTH1623.1, RSAK12.13 × RTH1623.2, RSAK12.14 × RTH1623.1) on day 14 post fertilization and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *v374* disrupts spore killing.

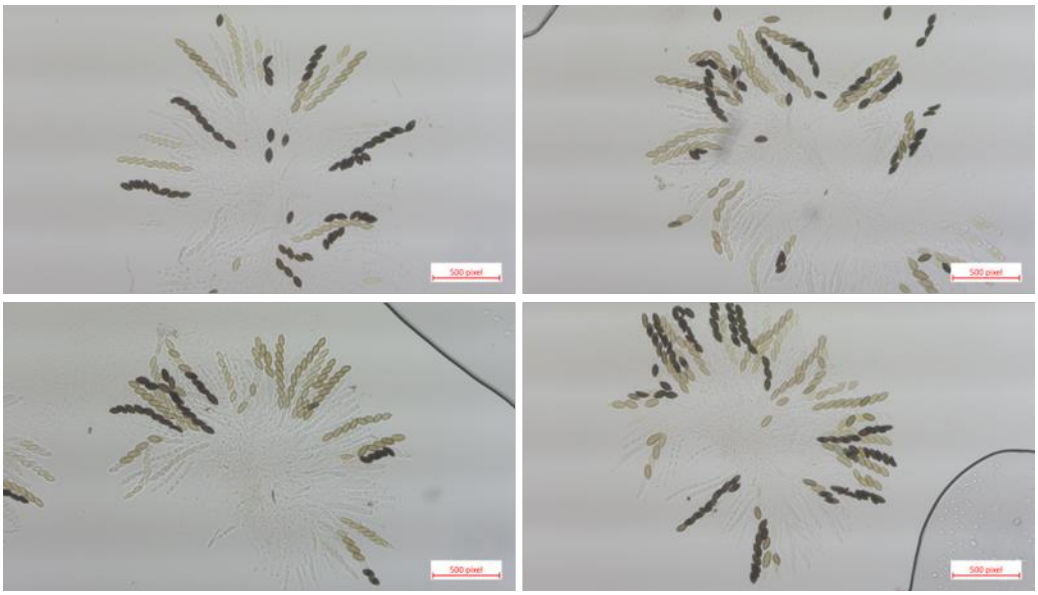

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.10 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

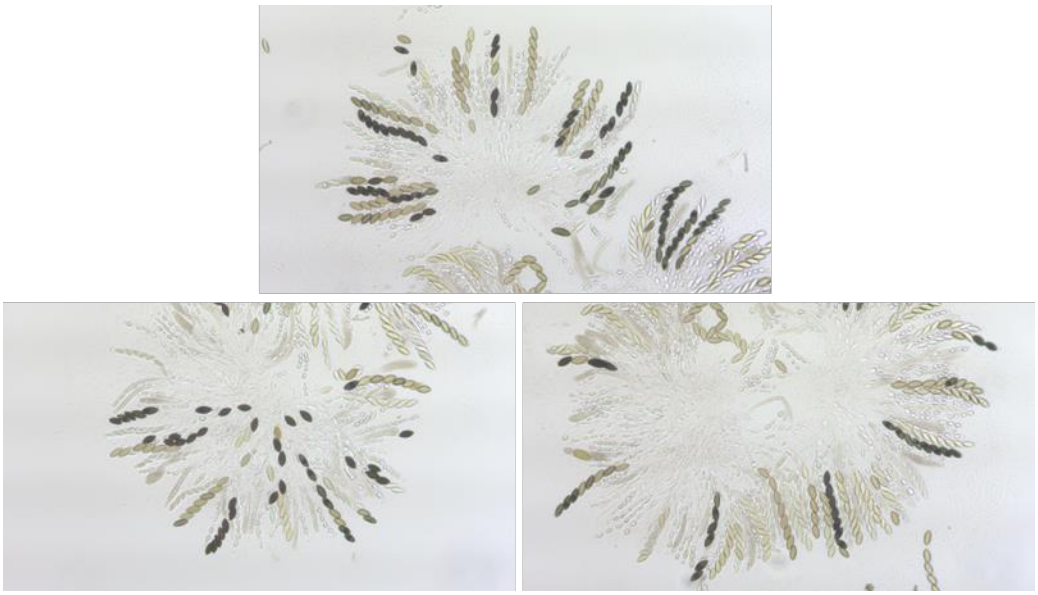

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.12 × RTH1623.2 on day 13 post fertilization and imaged under magnification.

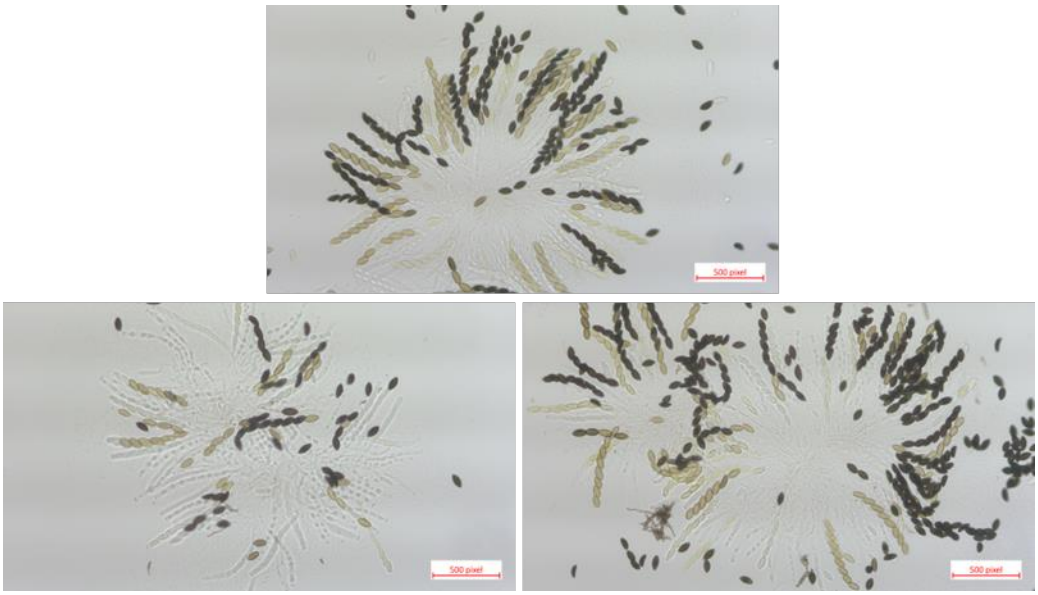

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.14 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

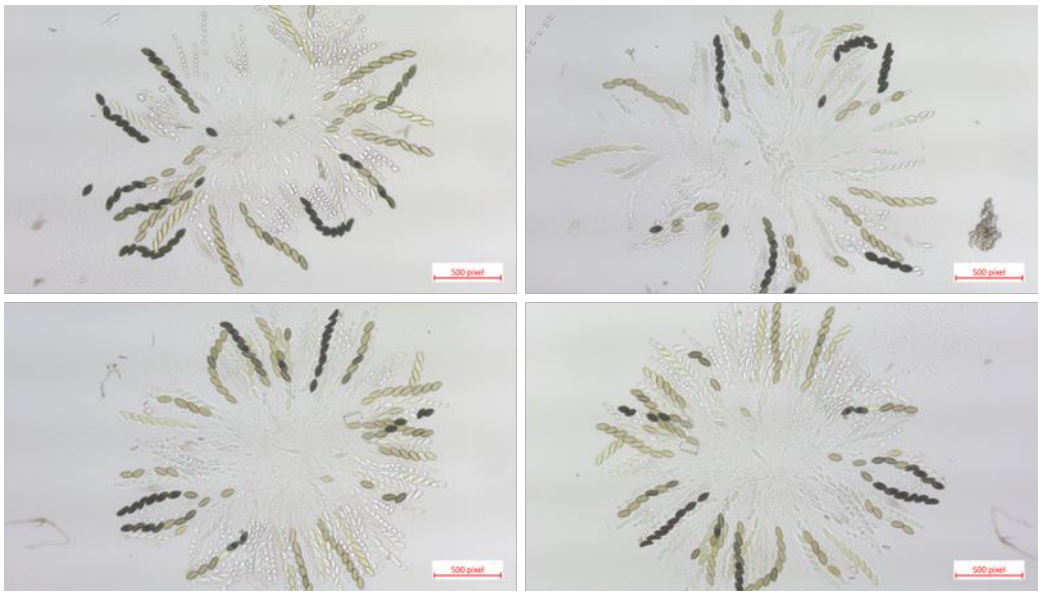

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.15 × RTH1623.1 on day 13 post fertilization and imaged under magnification.

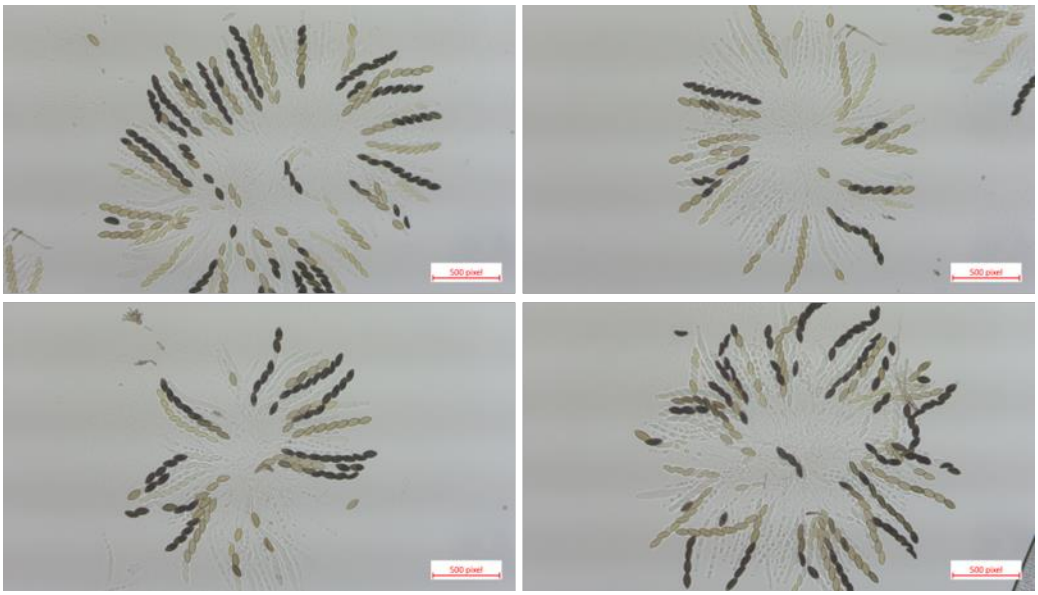

Figure 19 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.13 × RTH1623.2 on day 14 post fertilization and imaged under magnification.

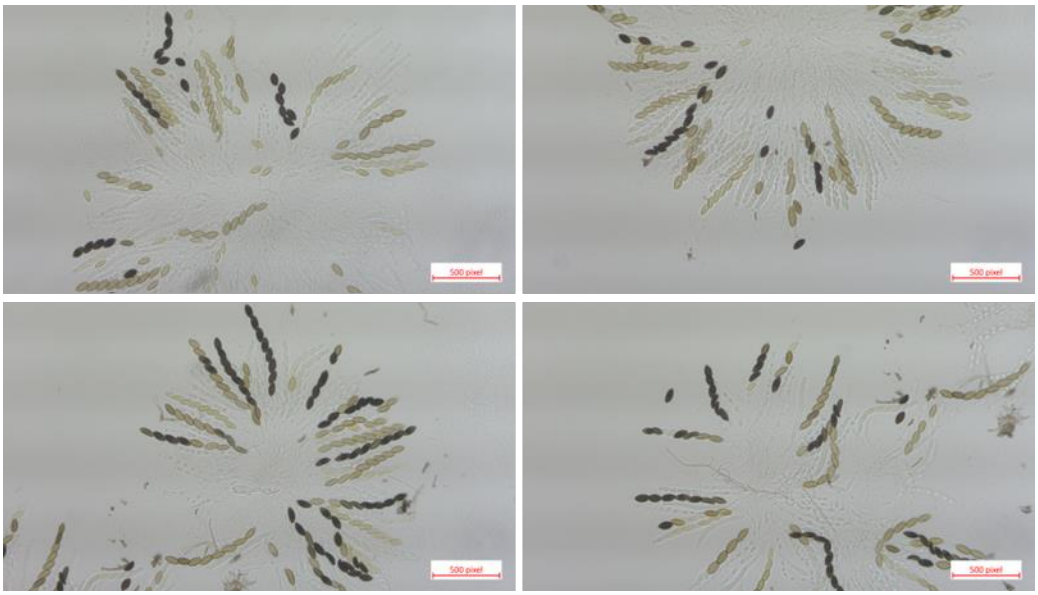

Figure 20 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.7 × RTH1623.2 on day 14 post fertilization and imaged under magnification.

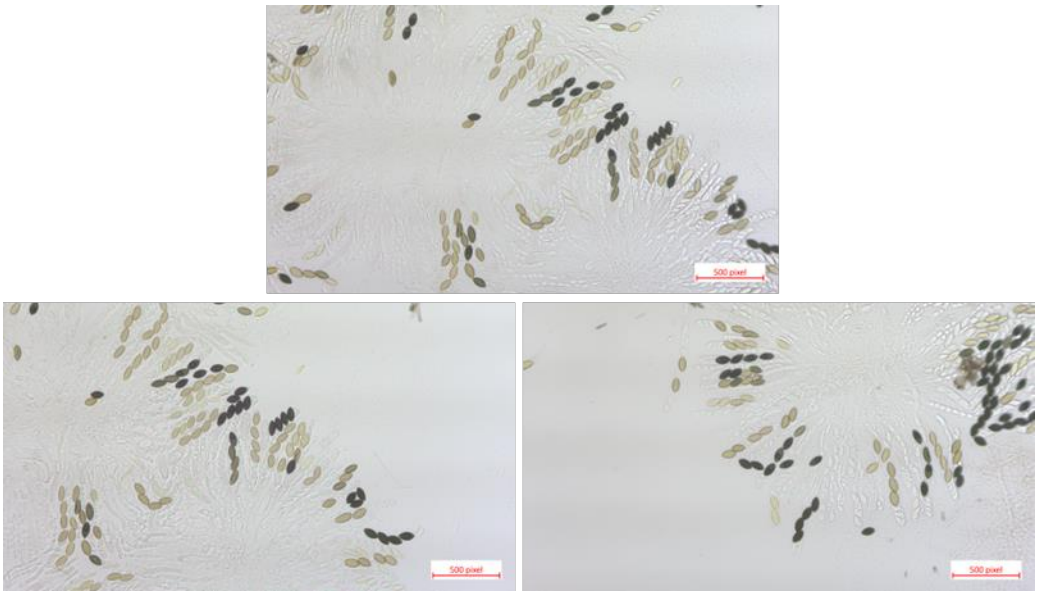

Figure 21 Ascus phenotypes. Asci were dissected from perithecia of RDGR170.3 × RTH1623.2 on day 13 post fertilization and imaged under magnification.

Figure 22 Ascus phenotypes. Asci were dissected from perithecia of RZS27.10 × RTH1623.1 on day 13 post fertilization and imaged under magnification.

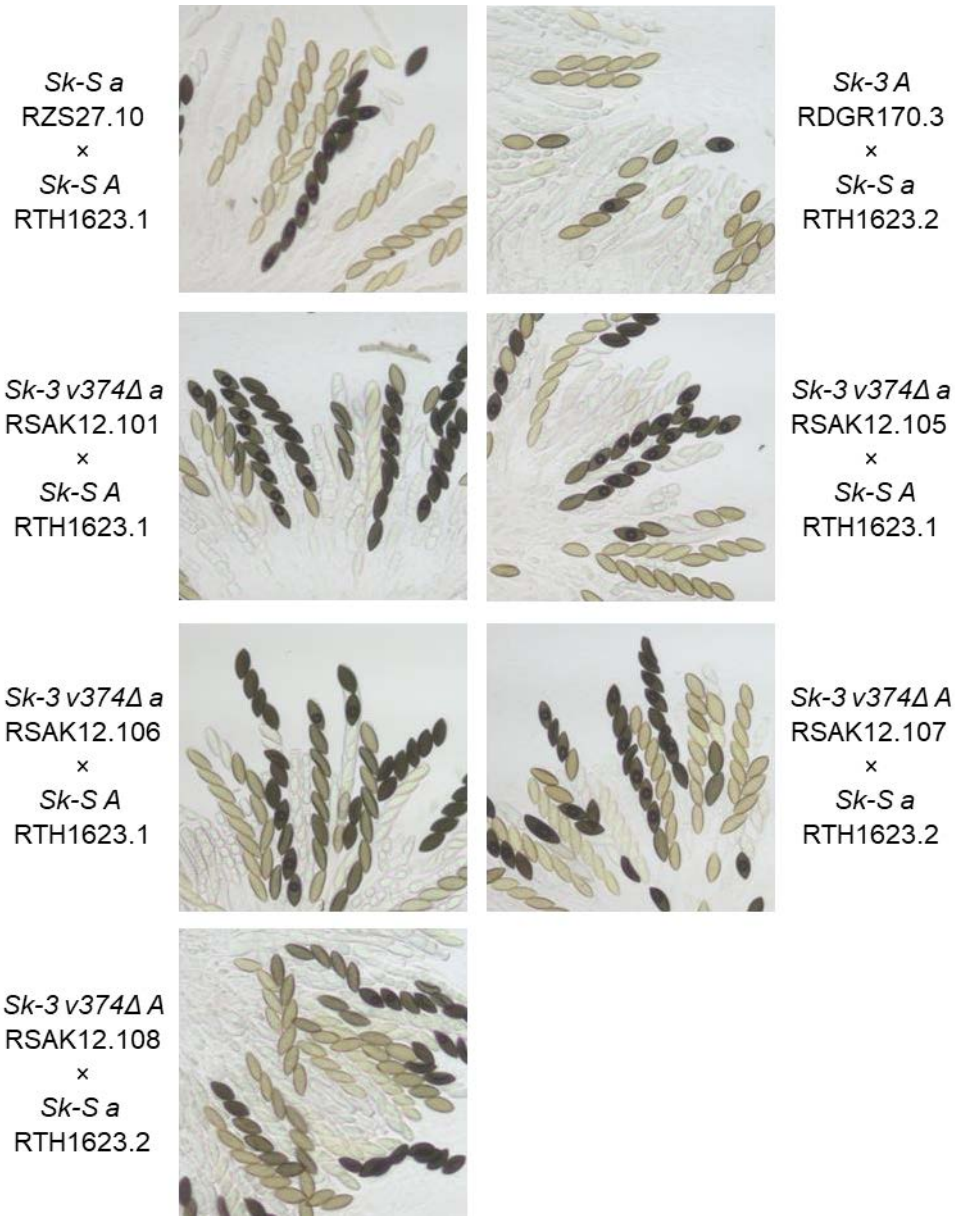

Figure 23 Ascus phenotype summary. Asci were dissected from perithecia of seven crosses on day 13 post fertilization and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *v374* disrupts spore killing.

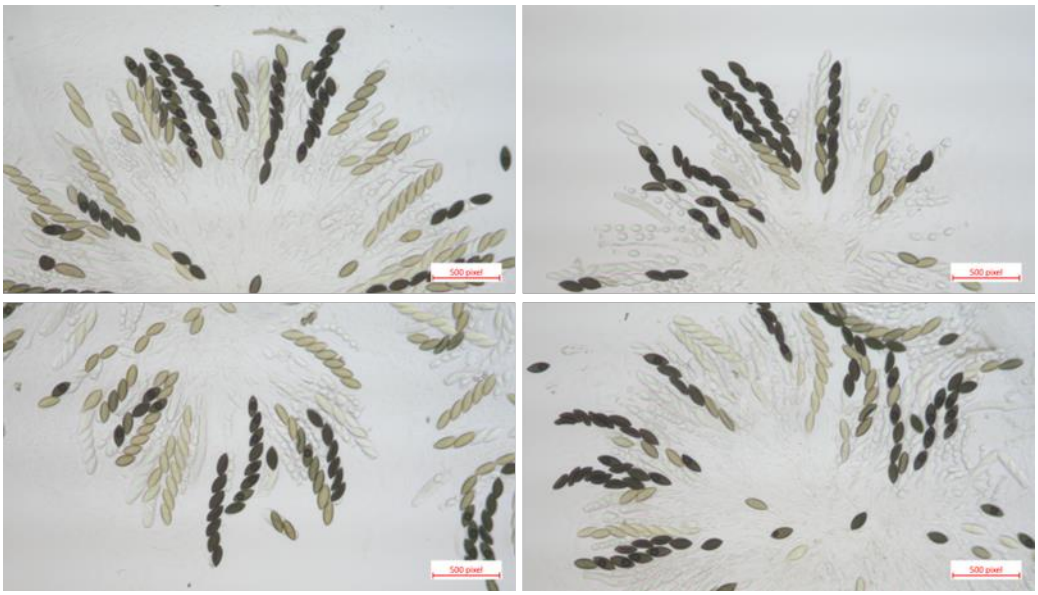

Figure 24 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.101 × RTH1623.1 on day 13 post fertilization and imaged under magnification.

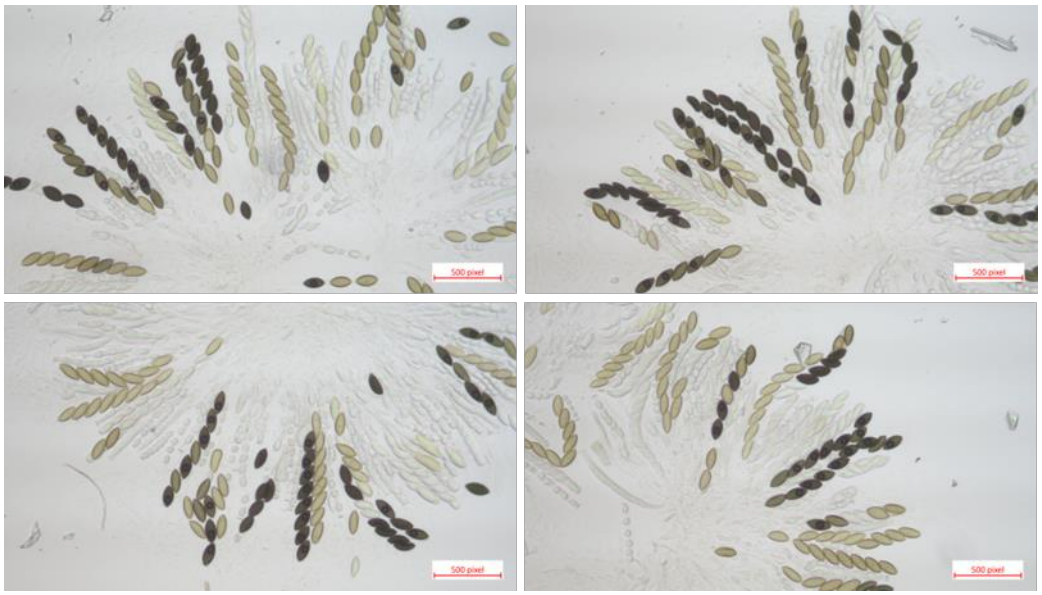

Figure 25 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.105 × RTH1623.1 on day 13 post fertilization and imaged under magnification.

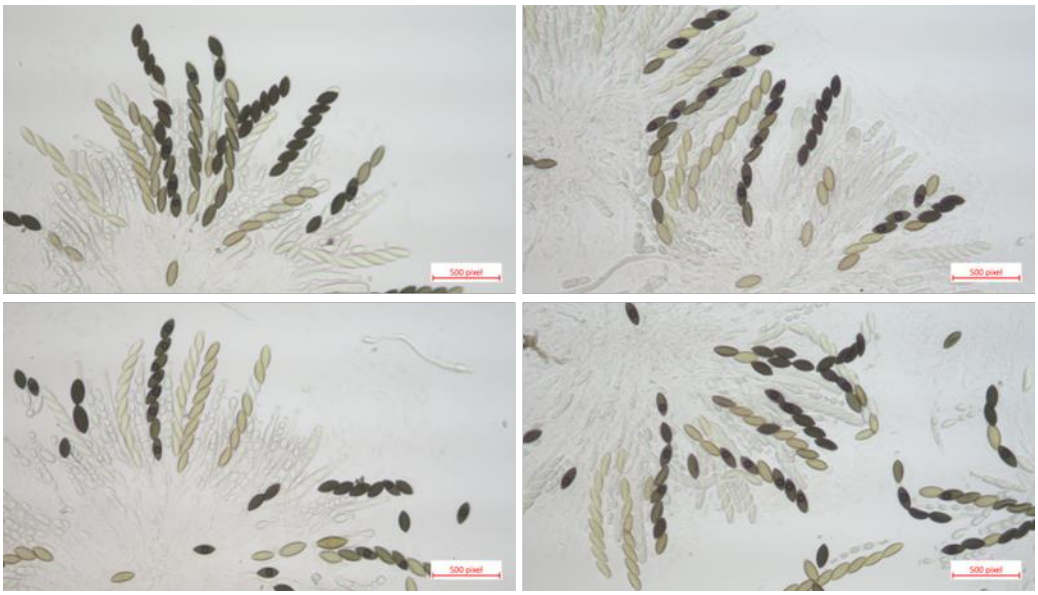

Figure 26 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.106 × RTH1623.1 on day 13 post fertilization and imaged under magnification.

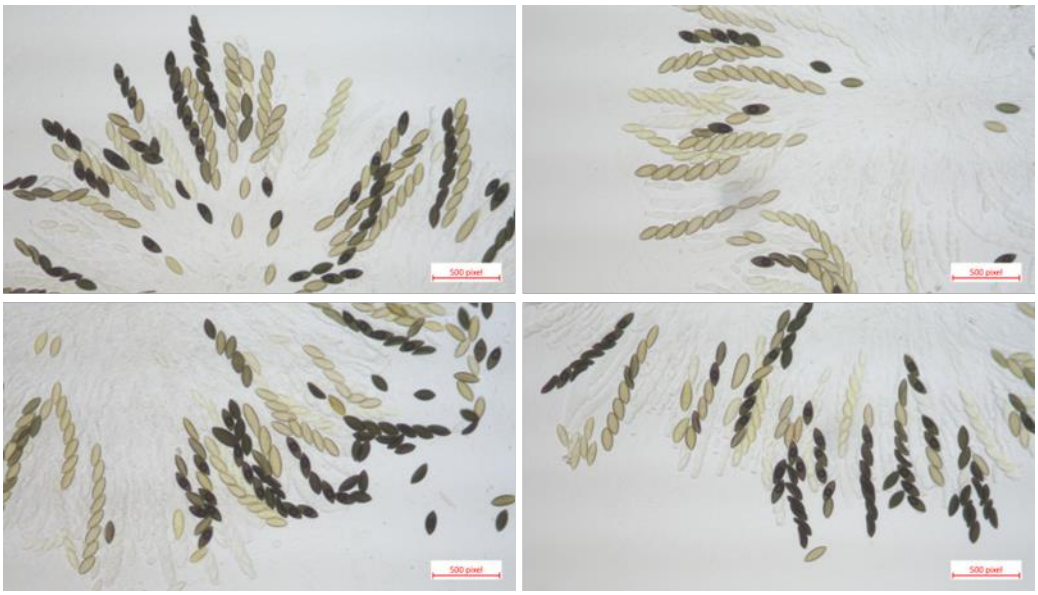

Figure 27 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.107 × RTH1623.2 on day 13 post fertilization and imaged under magnification.

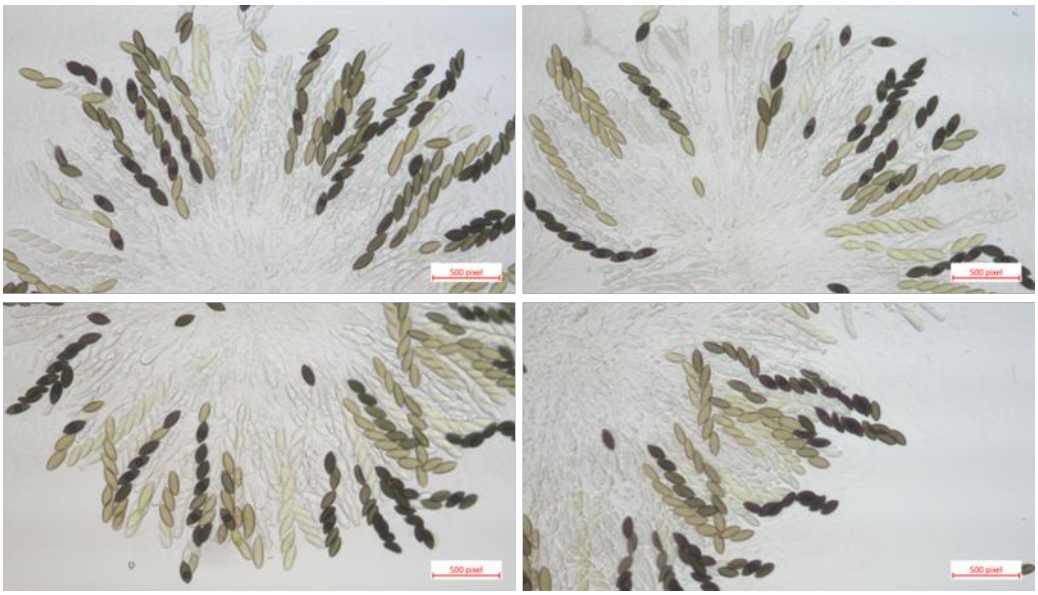

Figure 28 Ascus phenotypes. Asci were dissected from perithecia of RSAK12.108 × RTH1623.2 on day 13 post fertilization and imaged under magnification.

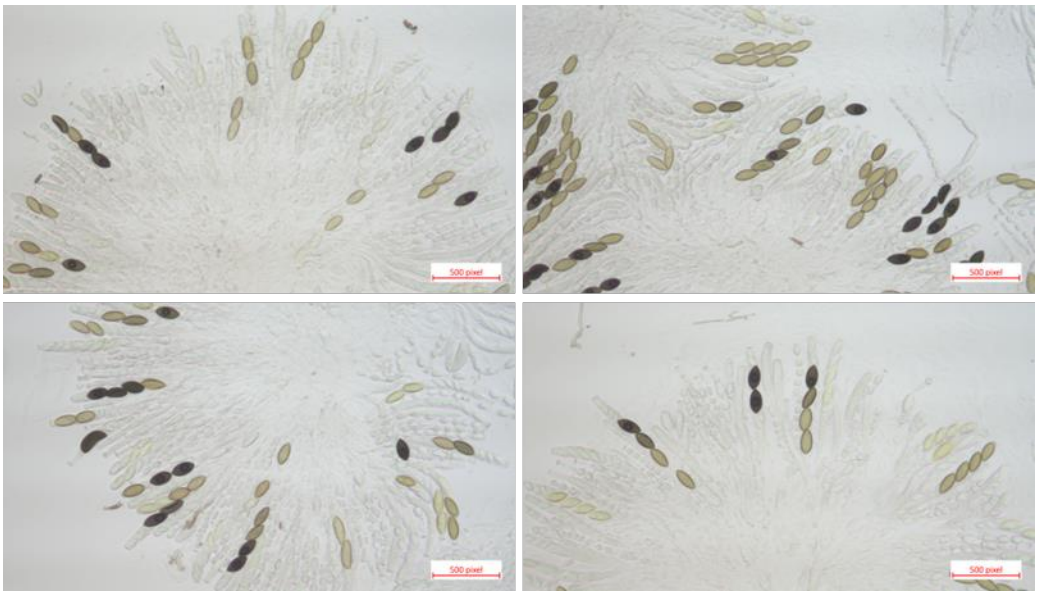

Figure 29 Ascus phenotypes. Asci were dissected from perithecia of RDGR170.3 × RTH1623.2 on day 13 post fertilization and imaged under magnification.

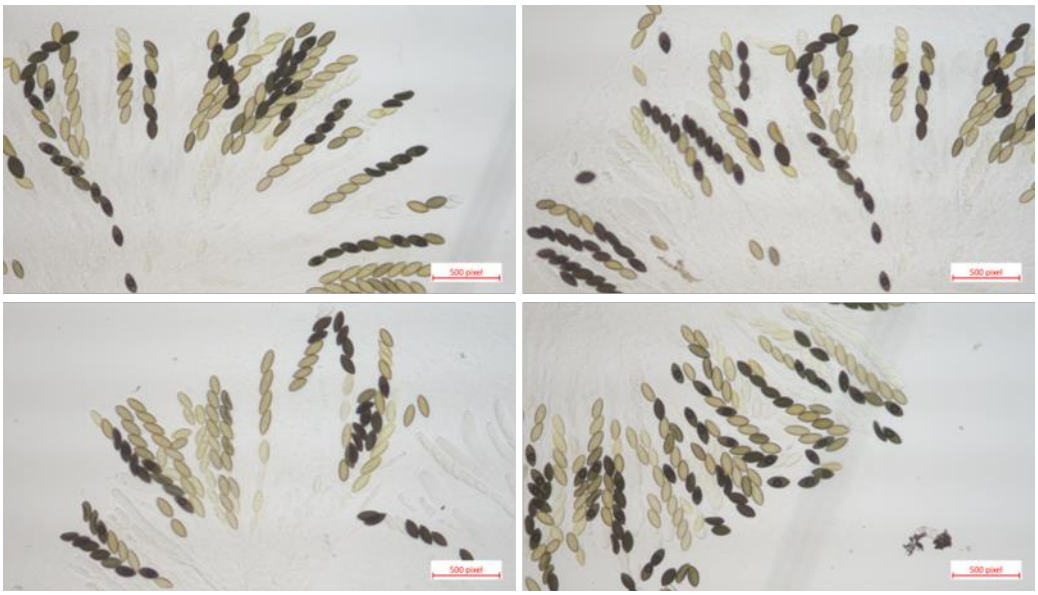

Figure 30 Ascus phenotypes. Asci were dissected from perithecia of RZS27.10 × RTH1623.1 on day 13 post fertilization and imaged under magnification.