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Investigating the Role of the DNA Interval v376 on Sk-3 Spore Killing in Neurospora crassa

John C. Munn IV
Illinois State University, jmunn@ilstu.edu

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In *Neurospora* fungi, the ascospores formed during reproduction will most often be black and viable. Occasionally, these ascospores will end up inviable and white or yellow. The discovery of a selfish genetic element called *Spore killer* (*Sk*) in 1979 gave researchers insight into a mechanism that causes some *Neurospora* crosses to produce a consistent ratio of 4 black, viable ascospores and 4 inviable, white ascospores. In these 4:4 splits, the *Spore killer* genetic element causes the death of exactly half of the ascospores. There are now three known spore killers in *Neurospora*: *Sk-1*, *Sk-2*, and *Sk-3*. This thesis examines the role of a DNA element within *Sk-3*. In an *Sk-3* × *Sk-3*-sensitive (*Sk-S*) cross, *Sk-3* genes are transmitted to the four black, viable ascospores, and, through a poorly understood mechanism, the *Sk-3* genes kill ascospores that fail to inherit these genes. The *Sk-3* genes reside on Chromosome III, but the exact locations of all critical genes are unknown. Preliminary results suggest that a DNA interval called *v350* may harbor a critical *Sk-3* gene. For example, deletion of the *v350* interval eliminates *Sk-3* spore killing. Here, I explore the deletion of an additional DNA interval located within *v350*. Specifically, I tested the role of DNA interval *v376* on *Sk-3* spore killing. The research presented here should help determine why *v350*, and perhaps *v376,* are required for spore killing by *Neurospora Sk-3*.

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JOHN C. MUNN IV

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JOHN C. MUNN IV

COMMITTEE MEMBERS:

Thomas Hammond (Chair)

Pirmin Nietlisbach

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Chapter I: Introduction

1.1: *Neurospora* Spore Killer

The *Neurospora* genus contains multiple species of fungi found all over the world, with groupings found in the United States, parts of the Caribbean, the Malay Archipelago and more, including all major continents except Antarctica (Turner 2001). These species can sometimes be crossed with one another through laboratory processes such as introgression to investigate aspects of their genomes. In normal *Neurospora* crosses, the ascospores formed during reproduction will, more often than not, be black and viable. Occasionally, these spores are inviable. Inviable ascospores are typically white (hyaline) or yellow. The discovery of a selfish genetic element called *Sk-1* in 1979 gave researchers insight into a mechanism that causes some Neurospora crosses to produce 4 black, viable ascospores and 4 inviable, white ascospores per spore sac (ascus). In Neurospora fungi, the black color of ascospores is due to melanin, and the presence of melanin is a good indicator of viability: the more melanin, the more likely the ascospore is to survive. In *Sk-1* crosses, *Sk-1* directly causes the death of half the ascospores. Other spore killers were identified in Neurospora around the same time as *Sk-1.* These were called *Sk-2* and *Sk-3* (Turner and Perkins 1979), the latter of which is the focus of this thesis.

1.2: *Sk-3* and resistance to spore killing

There are three known Spore killers (Sk) in Neurospora fungi: *Sk-1*, *Sk-2*, and *Sk-3*. Each Sk can be associated with three possible phenotypes: S for sensitive, K for killer, and R for resistant. When different combinations are crossed, such as K × S, almost 95% of the time, the resulting ascospores are 4:4 of black to white (Turner and Perkins 1979). This pattern of 4 viable, 4

inviable is a consistent pattern in these crosses. The action of making ascospores inviable is referred to as spore killing. While K × S crosses show reliable 4:4 patterns, when *Sk-2* is crossed with *Sk-3,* resistance becomes particularly important to survival. Resistance in all Sk crosses is decided by a resistance gene. This resistance protects ascospores from specific killer elements. *Sk-3* for example, protects from *Sk-3* spore killing. Resistance to one Sk element, say *Sk-3*, does not mean the ascospores will be resistant to any other type of spore killing, such as *Sk-2* or others. However, when *Sk-2* is crossed with *Sk-3*, only inviable ascospores are produced (Turner and Perkins, 1979) because none of the ascospores have resistance to both spore killers, and therefore all are killed by one or the other killer. The resistance genes and their specificity were identified by Hammond et al. (2012). Deletion of the resistance gene (called *rsk*, for *resistant to spore killing*) in *Sk-2* isolates and *Sk-3* isolates led to killing of all ascospores in the cross. This shows the importance of resistance. Resistance is also important in homozygous *Sk-K* crosses. For example, in tests done by Turner and Perkins (1979), it was found that *Sk-2* homozygous crosses and *Sk-3* homozygous crosses produce 95% black, viable ascospores. Therefore, *Sk-2* isolates are resistant to spore killing by other *Sk-2* isolates, while *Sk-3* isolates are resistant to spore killing by other *Sk-3* isolates.

1.3: Meiotic Drive

Meiotic Drive, an inheritance pattern, causes irregularity in inherited gene levels. This is often referred to as selfish behavior due to its tendency to cause offspring to inherit the same selfish trait. Examples of meiotic drive have been found in organisms that reproduce sexually such as flies and maize (Harvey et al. 2014). While *Neurospora* fungi are predominately asexual organisms, under certain conditions, they are capable of sexual reproduction. When a “male”

conidium donates a nucleus to “female” protoperithecium, meiosis and meiotic drive can occur (Harvey et al. 2014). In the case of the Neurospora Spore killers, the Sk-killer phenotype is the selfish trait. In K × S crosses, the selfish genes are transmitted to the four surviving ascospores. These selfish genes cause the death of the other four. Found by Hammond et al. (2012) and diagrammed by Zanders and Johannesson (2022), the act of spore killing can be described as a “poison-antidote” system. Some of the selfish genes create a “poison”, and this poison is the key to allowing spore killing to occur. Other selfish genes create an "antidote", and this is the key to resisting the poison and surviving. *Sk-S* ascospores, however, have neither poison nor antidote genes, and thus these ascospores succumb to poison when it is present during development.

The poison and antidote genes must be encoded in the same genome for meiotic drive to occur. For *Sk-3*, it appears that the genes are very close together on Chromosome III. This may help keep the poison and antidote coding regions from being separated by recombination. If that were to happen, a suicide genotype would be created. A suicide genotype would result in death of the ascospores inheriting the poison gene without the antidote gene, eliminating the poison gene's transmission advantage.

1.4: Chromosome III

The selfish genes controlling *Sk-3*-based spore killing reside on Chromosome III. Turner and Perkins (1979) specifically identified a 30-centimorgan region of Chromosome III to be the location of interest. In 2012, Hammond et al. identified *rsk* within this 30 cM region. The *rsk* gene confers resistance to *Sk-3*-based spore killing, and this likely encodes the antidote to the *Sk-3* poison. In 2022, Velazquez et al. isolated a mutation called *rfk-2*^{UV} that disrupts *Sk-3*-based

spore killing. This mutation was mapped to the 30 cM region of interest originally identified by Turner and Perkins, but the exact location of *rfk-2*^{UV} remains undetermined.

While seeking to refine the location of *rfk-2*^{UV} on Chromosome III, Rhoades and Hammond (unpublished data) deleted a DNA interval called *v350*. This interval is found on Chromosome III in *Sk-3* strains, and preliminary analysis of the deletion indicated that it disrupts *Sk-3*-based spore killing. The reason why deletion of interval *v350* causes loss of spore killing is unknown. In this thesis, I examine the role of a related DNA interval, called *v376*, in *Sk-3*-based spore killing.

Chapter II: Methods

2.1: Overview

First, the interval, *v376*, was selected as the interval of interest for this study. An overview of my methodology is diagrammed in **Figure 1**. Left, center, and right flanks of a deletion vector were amplified by PCR and confirmed by gel electrophoresis. Then the PCR products were fused, again using PCR, and amplified with nested primers to create the deletion vector, called Vector v376. This product was then column purified and checked by gel electrophoresis before use in transformation. Next, *N. crassa* was transformed with Vector v376. Heterokaryotic transformants were selected and genotyped by PCR to make sure transformation was successful. Heterokaryotic transformants were then crossed to obtain hygromycin-resistant homokaryotic offspring. These offspring were then used in spore killing assays by crossing to test strains. At the same time, the homokaryotic offspring were cultured to obtain mycelia and isolate DNA. The homokaryotic offspring were genotyped by PCR to confirm that they were homokaryotic and interval *v376* had been deleted.

2.2: Strains, Primers, and PCR

The strains and primers used in this thesis are described in **Table 1** and **Table 2**, respectively. The first PCR products obtained were the left and right flanks of Vector v376, the predicted sequences of which are shown in **Figure 2** and **Figure 3,** respectively. The left flank was constructed using primers V0376-A and V0376-B, described in **Table 2**. At the same time, the right flank was created using primers V350-C and V350-D (**Table 2)**. These primers, and the resulting flanks, were chosen due to the necessity for matching the DNA sequences around our gene of interest. The primers were ordered from Integrated DNA Technologies. After thawing at

65 °C, the primers were vortexed and spun in a microcentrifuge to accumulate the primer solutions at the bottom of the tubes. The Q5 DNA Polymerase Buffer (New England Biolabs), and the DNA template RDGR170.3 (10 ng / µL) were treated the same way. A dNTP solution (10 mM) was vortexed but not heated, and Q5 DNA Polymerase (New England Biolabs), which was not vortexed or heated, were spun in a microcentrifuge to collect contents at the bottom of their respective tubes. Next, primer mixes were produced by mixing 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. Two solutions were created, one for the left flank, and one for the right flank. Next, an Enzyme Master mix was created using sterile water at 13.4 µL per reaction, 5 µL of Q5 DNA polymerase reaction buffer per reaction, 0.5 µL of dNTP per reaction, and 0.1 µL of the Q5 enzyme per reaction. Two 5 µL aliquots of the Left Flank Primer mix were placed into two PCR reaction tubes, and two 5 µL aliquots of the Right Flank Primer mix were placed in two more PCR tubes. A 1 µL aliquot of the DNA template was placed into all four PCR tubes. Finally, 19 µL aliquots of the Enzyme Master mix were placed into each of the four tubes and reactions were cycled in a thermal cycler following manufacturer guidelines for Q5 DNA polymerase. PCR products were purified by gel extraction using IBI Scientific's GEL Extraction and PCR Cleanup Kit.

2.3: Standard Gel Electrophoresis Protocol

To a 1 Liter flask, 1.8 g of agarose and 200 mL of 1x TAE buffer were added and microwaved for 1 minute at 100% power. This mixture was swirled and then heated for another minute, or until all agarose was dissolved. Once dissolved, 10 µL of 10 mg / mL ethidium bromide was added to the flask and swirled again. This mix was allowed to cool until it could be safely

handled but was still slightly warm. A small amount of this mix was poured into a 13 x 15 cm gel try with a 20-tooth comb and sat for approximately 30 seconds. Then the rest of the mixture was poured into the tray and left to solidify for about 30 minutes. After solidification, the gel tray was placed into the electrophoresis chamber, which was subsequently filled with 1× TAE buffer. A 5 µL aliquot (0.5 µg) of a DNA ladder (GeneRuler 1Kb Plus, Thermo Scientific) was placed into the first well of the gel. The rest of the wells were filled with 30 µL of a DNA solution in 1× loading buffer. The gels were run at 120 volts for 90 minutes. All gels were imaged using an Analytikjena imaging machine.

2.4: Analysis of Gel Purified Left and Right v376 flanks.

The gel purified left and right flanks for Vector v376 were analyzed by gel electrophoresis (**Figure 4**). The predicted lengths of the left and right flanks, 935 base pairs (bp) and 813 bp respectively, are consistent with the DNA molecules in the gel.

2.5: Double-Joint PCR

The center fragment for Vector v376 was amplified from plasmid pTH1256.1 (GenBank MH550659.1) with primers HPH-CEN-F and HPH-CEN-R (**Table 2**). The center fragment (**Figure 5**) contains a hygromycin resistance gene (*hph*), which serves as the selectable marker for *N. crassa* transformation. Double-Joint (DJ) PCR (Yu et al. 2004) was then used to fuse the left flank, center fragment, and right flank. Specifically, the same solutions used to create the Enzyme Master mix above were utilized here, but in differing amounts. In this protocol, 62.6 µL of sterile water, 20 µL of Q5 reaction buffer, 2 µL of dNTP mix, and 0.4 µL of Q5 enzyme were used. This mixture was placed directly into a PCR tube, along with 5 µL of the left fragment,

right fragment, and *hph* center fragment and cycled according to Yu et al. 2004. The resulting fusion product was amplified with nested primers V0376-E and V0372-F (**Table 2)**. The Standard Primer Mix Protocol was used again, with 20.0 µL of Primer Mix placed into a PCR tube with 5 µL of the fusion product. Then, 52.6 µL of sterile water, 20.0 µL of Q5 reaction buffer, 2.0 µL of dNTP mix, and 0.4 µL of Q5 enzyme were added to the same PCR reaction and cycled according to manufacturer's recommendations. An IBI Scientific gel extraction and column purification kit was then used to column purify the PCR product. The purified PCR product was examined by gel electrophoresis (5 µL of purified PCR product, 5 µL of 6× loading buffer, and 20 µL of water).

The predicted 2740 bp sequence of Vector v376 is shown in **Figure 6.** The length of the amplified and purified PCR product from Vector v376 construction by DJ-PCR is consistent with this prediction (**Figure 7**), suggesting that v376 was created successfully. Vector v376 was designed to replace interval *v376* (deletion vectors and their target intervals share names). The 159 bp DNA sequence of interval *v376* is shown in **Figure 8**, and its relationship to *v350* is shown in **Figure 9**.

2.6: Transformation

To complete the transformation protocol, special media must be prepared to ensure that only the fungi that integrated the hygromycin resistance gene of the transformation vector into their genomes are able to grow. Bottom Agar plates were created to allow for transformation of strain RDGR170.3 (the 159 bp DNA) with Vector v376. Bottom Agar plates were created by mixing 172 mL of sterile water, 8 mL of Vogel's salts (Vogel 1956), and 3 g of agar in a flask and

autoclaved to ensure stability. Once autoclaved and cooled enough to handle without gloves, 150 µL of hygromycin with 20 mL of 10× FIGS (20% sorbose, 0.5% D-Fructose, 0.2% Inositol, 0.5% D-Glucose) solution were added to the mixture. This mix was then poured into 10 petri dishes and set aside to cool, and the plates were used as the bottom agar for transformation after being allowed to set for approximately 3 days. To create the Top Agar, 105 mL of sterile water, 27.3 grams of sorbitol, 6 mL of 25× Vogel's Salts, and 2 g of agar were placed in a glass bottle and autoclaved to ensure sterility. Once sterile and cool, 15 mL of 10× FIGS solution was added. Three 40 mL aliquots were poured to sterile 50 mL conical tubes and stored at 60 °C.

Conidia for transformation were collected from a culture of *N. crassa* that was grown in a 250 mL flask. The flask was prepared using 48 mL of sterile water, 2 mL of 25× Vogel salts, 1.0 g of sucrose, and 0.75 g of agar. The flask was capped with a glass beaker then autoclaved and placed on a shelf for 1–2 days, after which it was inoculated with 20 µL of RDGR170.3 from a cryogenic stock suspension. The flask was then placed into a 32 °C incubator for 2 days. After 2 days, the flask was moved to a shelf and allowed to incubate at room temperature for 1–4 weeks.

Conidia were scooped from culture flasks using wood applicators and placed into 30 ml of ice cold 1M sorbitol solution in a 50 mL conical tube, then strained using a 100-micron cell strainer to remove mycelia and other fungal tissue. After filtration, the conidial suspension was centrifuged to form a pellet of conidia. After centrifugation at 2000× g for 10 minutes, the supernatant was disposed of, and the conidia in the pellet were suspended to a concentration of about 1 billion conidia per ml in 1 M sorbitol. A 100 µL aliquot of the conidial suspension was then placed into a microcentrifuge tube (MCT) and mixed with 10 µL of the DNA replacement

vector (@500 ng of DNA). This solution was then electroporated following the methods described by Margolin et al. (1997) and Rhoades et al. (2020). A 750 µL aliquot of ice cold 1 M sorbitol was quickly added to the conidial suspension after electroporation and then transferred to rescue medium (Vogel's minimal medium + 1M sorbitol). The conidial suspension in the rescue medium was shaken at 40 rpm for 3.5 hours to prevent conidial clumping. Finally, 100 µL of the rescue culture was added to a 40 ml aliquot of molten Top Agar (after cooling to 50 °C), inverted 5×, and 10 mL were poured to a plate of Bottom Agar. Then, 500 µL of the rescue culture was added to the same vial of Top Agar, inverted 5×, and 10 mL were poured to a different plate of Bottom Agar. This was repeated for 1000 µL of the rescue culture and the remaining volume of rescue culture. The Bottom Agar plates were then left to dry for 24 hours, before inverting and placing them in a 32 °C incubator.

Vogel's hygromycin slants were then prepared. The media, Vogel's Minimal Agar (VMA) is prepared by mixing Vogel's Minimal Media (VMM; Vogel 1956) and 2% agar and autoclaving at 121 °C and 15 psi, for 30 minutes. Once sterilized and out of the autoclave, Hygromycin B (Gold Biotechnology) was added to a final concentration of 200 µg / ml. Next a serological pipette was used to put 2–3 mL of the media into 16 × 125 mm culture tubes and placed at an angle to create slanted Vogel's media. Once the media was cooled and set, they were covered with vented caps and placed at 4 °C for long term storage.

Putative hygromycin resistant transformants were selected from the transformation medium with sterile syringe needles and placed into individual Vogel's slants. This was repeated to produce a total of 12 Vogel's slants with transformed colonies.

A few of these transformants were genotyped by PCR to determine if the transformation was successful. Specifically, DNA was isolated from three transformants: TJCM1.1, TJCM1.2, and TCJM1.3. Mycelia for DNA isolation was generated as follows: a small amount of conidia was transferred from each strain to a 15 ml tube with 2–4 ml of liquid Vogel's minimal medium. These cultures were incubated at 150 rpm, 32°C, for 24–48 hours. The mycelia in each tube was collected with a disposable wooden applicator onto paper towels, blotted dry, and stored at -80°C freezer. The mycelia were then freeze dried under vacuum (lyophilization) for at least three hours, after which it was stored in a dry cabinet until DNA isolation was performed with IBI Scientific's Genomic DNA Mini Kit for Plants and Fungi.

DNA from each strain was used as template with primers V0376-E and V0372-F in a PCR-based genotyping assay. The predicted PCR product from a *v376+* genotype is 1487 bp (**Figure 6)**. The predicted PCR product from a *v376*Δ genotype is 2740 bp (**Figure 10).** DNA from TJCM1.1 and TJCM1.3 produced a PCR product consistent with the *v376*Δ genotype (**Figure 11**).

2.7: Crossing transformants

Westergaard and Mitchell (1947) designed synthetic crossing agar (SCA) with 1.5% sucrose and a pH of 6.5, which promotes the production of protoperithecia in *N. crassa*. SCA was made for crossing transformants, which are typically heterokaryotic, with standard *Sk-S* mating partners, to collect homokaryotic offspring. SCA was made and autoclaved. Once cooled, 18 mL of solution was transferred to 60 mm petri dishes, and these plates were allowed to dry for a couple of days.

Aliquots of a cryogenic suspension of strain RTH1005.2 was added to the plates in 5–10 µL drops at the center of each plate. The RTH1005.2 strain was used as the “female” parent in the crosses with transformants, which served as the "male" parents. These plates were then left to grow and incubate for 6–8 days. Conidial suspensions were then prepared from each transformant as follows: a large clump of conidia was added to an MCT tube along with 500 µL of sterile water and the suspensions were vortexed. Drops (10 µL) of the conidial suspensions were then placed onto the SCA plates of RTH1005.2. Approximately 20 drops were placed onto each culture of RTH1005.2 to initiate crossing. The crossing plates were incubated for at least 24 days. Ascospores were harvested from the lids into 500 µL of sterile water. Ascospore suspensions were stored for 16 or more hours in the dark at 4 °C.

2.8: Isolation of Homokaryotic Offspring

Approximately 500–1000 ascospores were removed from the stock and placed into an MCT tube with 500 µL of sterile water, and this suspension was vortexed and heated for 30 minutes at 60 °C. The heat shocked ascospores were then placed in the center of three plates. One plate had only 50 µL of the ascospore solution, the next had 100 µL, and the last had 200 µL. All three of these solutions were gently spread on the plate using a sterile metal spreader. These plates were left to grow for 24 hours to identify the hygromycin resistant ascospores that had grown.

After 24 hours, germinating ascospores were selected and transferred to Vogel's hygromycin slants. In this procedure, it was essential to harvest those with the most hyphal growth, and keep those hyphae intact, while making sure that the hyphae do not intersect with hyphae from a nearby germling. Some of the ascospores seen growing on these plates were not

resistant to hygromycin, but still grew some before becoming inviable. Sterilized needles were used to transfer isolated germlings which were then moved to the slants. These slants were then labeled RJCM10.1, RJCM10.2, …, to RJCM 10.12. The germlings transferred to these slants were grown at 32 °C for two days before transfer to room temperature.

2.9: Spore killing assays

A total of 20, 60 mm, SCA plates (described above) were inoculated with cryogenic suspensions of strains RTH1623.1 or RTH1623.2. Each plate was inoculated with approximately 5 µL of RTH1623.1 or RTH1623.2 suspension, but never both. These were incubated for 8–10 days at room temperature. Conidial suspensions were made using 500 µL of sterile water and conidia from the RDGR170.3, RZS27.10, and RJCM strains 10.2, 10.4, 10.6, 10.7, 10.8, and 10.10. For each conidial suspension, aliquots of 200 µL were transferred to each of the RTH1623.1 and RTH1623.2 plates in 10 µL drops. The plates were then allowed to sit for approximately 15 days to allow the "male" and "female" strains to mate and produce perithecia.

On day 15 post fertilization, syringe needles were used to dissect perithecial contents into 25% glycerol solution with the aid of a dissecting microscope as previously described (Samarajeewa et al., 2014). Specifically, 100 µL of the glycerol solution was placed onto a microscope slide and a clump of perithecia was removed from a crossing plate and placed into the glycerol suspension of the slide. Most of the hyphae and agar was removed from the perithecial clump to leave only the perithecia in the glycerol. The individual perithecia were then cut open and squeezed to push out the rosettes of asci (spore sacs, each of which contains up to eight ascospores at maturity). After approximately 10 rosettes were teased from perithecia,

perithecial debris were removed from the slide, and the rosettes were pushed to the center of the slide with the tips of syringe needles. A coverslip was then placed on the slides and excess moisture was removed with paper towels. The slides were then sealed with clear nail polish. Imaging was performed within 48 hours with a Leica compound microscope and Zeiss Imaging system. The crossing plates were imaged with a standard smart phone camera (**Figure 12)**.

2.10: Final Genotyping Assay

DNA was isolated from four RJCM10 strains (RJCM10.4, RJCM10.6, RJCM10.7, and RJCM10.10) for genotyping by PCR with primers V0376-E and V0372-F as described above (**Figure 13).**

Chapter III: Results

3.1: Deletion of *v376*

The interval of interest, *v376,* is located on Chromosome III of *N. crassa*. This interval is found within interval *v350*. Preliminary results suggested that deletion of *v350* eliminates *Sk-3*-based spore killing. The relationship between *v376* and *v350* is shown in (**Figure 9).** The rosettes in **Figure 14** are examples of asci from each cross, along with the control crosses involving RDGR170.3 (*Sk-3*) and RZS27.10 (*Sk-S*) as the conidial parents. The control cross images can be used as references for spore killing (RDGR170.3) and no spore killing (RZS27.10). I initially hypothesized that deletion of interval *v376* would disrupt spore killing. Disruption of spore killing is indicated by the presence of eight viable ascospores per ascus in most mature asci (i.e., >90%). My results support my initial hypothesis. For example: spore killing does not appear to be present in any of my six test crosses (**Figure 14**); including RJCM10.2 × RTH1623.2 (**Figure 15)**, RJCM10.4 × RTH1623.1 **(Figure 16)**, RJCM10.6 × RTH1623.2 (**Figure 17)**, RJCM10.7 × RTH1623.1 (**Figure 18),** RJCM10.8 × RTH1623.2 (**Figure 19)** and RJCM10.10 × RTH1623.1 (**Figure 20)**. Additionally, my genotyping assays of RJCM10.4, RJCM10.6, RJCM10.7, and RJCM10.10 confirmed each to be homokaryotic for the *v376*Δ genotype (**Figure 13**). These results demonstrate that deletion of *v376* disrupts spore killing.

Chapter IV: Discussion

4.1: Overview

Previous studies (Turner and Perkins 1979, Hammond et al. 2012, and Velazquez et al. 2022) led to the identification of *rfk-2*^{UV}, a mutation that disrupts *Sk-3*-based spore killing. Studies to refine the location of *rfk-2*^{UV} suggest that an interval called *v350* may be important for *Sk-3*-based spore killing. The exact relationship between *rfk-2*^{UV} and interval *v350* is unclear.

In this thesis, I examined the role of *v376* in *Sk-3*-based spore killing. Crosses were conducted between strains containing a hygromycin resistance gene-based deletion of interval *v376* and a parent strain sensitive to spore killing, allowing for any strains in which killing still functioned to produce the 4 viable: 4 inviable ascospore phenotype. The appearance of a 4:4 ratio is a good indicator of spore killing. **Figure 14** shows a representative image from each of the crosses. In each of these images, there is limited, if any, evidence of the spore killing 4:4 ratio. The genotypes of the four putative *v376Δ* strains examined by genotyping are all consistent with the *v376Δ* genotype, suggesting that deletion of the *v376* interval eliminates spore killing.

4.2: Hypothesis

I hypothesized that the deletion of *v376* would result in disruption of *Sk-3* spore killing. Based on the images I collected for this study (**Figures 14**–**20)**, this hypothesis has support. The asci in my test crosses appear to consistently follow an 8:0 viable/inviable ratio. The meiotic drive discussed before is likely no longer functioning, and the traditional Mendelian genetic patterns are most likely restored in each test cross because of the loss of spore killing.

4.3: Functional uses for Meiotic Drive

These results indicate that the 159 bp *v376* interval could be a region of interest in further investigation into the effects of spore killing in *N. crassa.* This region may not be the only one necessary for spore killing to occur, but my data indicate that it is associated with at least one critical component of spore killing. These results give insight into how this process works not only in *N. crassa*, but also to other organisms and experiments around the world. Other studies are being conducted currently to understand meiotic drive, as well as discovering how this process can be used to improve farming practices, among other uses. Gardiner et al. (2020) discusses meiotic drive, in reference to the fungal pathogen, *Fusarium graminearum*. This fungal pathogen is of particular concern to grain farmers. It is responsible for damaging wheat harvest and other crop yields, affecting not only the quality of wheat produced and harvested, but also contaminating the wheat with a toxin. This is made worse by the fact that wheat is not the only plant affected. In situations like this, meiotic drive can be an option toward controlling these fungi. In most cases, fungicide is not an ideal solution to killing these organisms, mostly due to the adverse effects these substances can have on humans and other animals. Additionally, Gardiner et al. state that working conditions for these fungicides are extremely limited and usually require use in non-ideal weather conditions making it impractical for use. Therefore, with very limited uses, it seems another solution is necessary. This is where meiotic drive and the results of this study are important. Like *N. crassa*, *F. graminearum* is capable of sexual reproduction, and therefore it is an ideal plant pathogen for meiotic drive-based control methods. This is an excellent option for controlling these fungi, as well as the diseases they cause. Instead of needing to apply pesticides when environmental conditions are not ideal for fungicide use, and to reduce the risk to humans who consume these crops, Gardiner et al were able to show that

spore killing was 92% efficient in their test crosses, providing more evidence that it is possible to implement meiotic drive in other organisms. These researchers were also able to show fixation of the Spore killer genes in only a few generations, further showing the efficiency of this method. While *F. graminearum* and *N. crassa* are different species, the concept of meiotic drive follows the same principles in both. By locating the specific location of spore killing genes in *Neurospora crassa*, researchers will have a significantly easier time reproducing and relocating meiotic drive into other organisms. Successful transformation of the meiotic drive genes into other organisms could open up many possibilities in which disease prevention, in humans, animals, plants, and more, can be done in an ecofriendly way. These processes have the potential to help farmers produce higher quality, safer products as well.

4.4: Limitations

The transformation and deletion of the Spore killer gene appears to have been successful in this experiment. Vector v376 contains a hygromycin resistance gene as the center fragment. Throughout the experiments, hygromycin was utilized to ensure any transformants and transformant-derived offspring had the hygromycin resistance gene. While the transformation appeared successful, there are some limitations with ascus analysis. For example, the ascospores gathered for imaging do not represent all ascospores. There are hundreds of ascospores made via these crosses, and only a small sample was taken. It is possible that selections were made that unintentionally excluded evidence that the spore killer gene is still functional. Additionally, these crosses were checked for genotype simply based on the length of PCR products, not by the genetic sequence. While unlikely, it is possible that the transformation was successful, and the genotyping assay results matched expectations for other reasons.

4.5: Current and Future Work

The results of this study are not limited to just the interval *v376*. Additional intervals, , at some within and some surrounding *v350,* were studied by other individuals during 2023–2024 in the Hammond Laboratory at Illinois State University. Combined with my results, these studies should help refine the exact location of the element controlling spore killing. Further studies can then be conducted, including transferring the spore killing element to other organisms, such as *F. graminearum,* to determine if the eliminate can induce spore killing in a different fungal genus.

As mentioned earlier, the uses of meiotic drive can vary greatly. The investigation into the Spore killer gene of *Neurospora crassa* presents an opportunity to greatly influence the risk factors associated with many types of fungi, as well as any other organism that reproduces using meiosis. While researchers may not know how to produce these results in all organisms, understanding how meiotic drive works and where in the genome the critical elements are located presents an opportunity for incredible advances in the near future.

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Table 1 Strains used in this study

Strain name (alias)	Genotype
F2-23 (RTH1005.1)	*rid; fl; Sk-S^{ChrIII}A+*
F2-26 (RTH1005.2)	*rid; fl; Sk-S^{ChrIII} a+*
FGSC 10340 (RZS27.10)	*rid; Sk-S^{ChrIII}; mus-51^{RIP70} a+*
ISU-3036 (RTH1623.1)	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ A+*
ISU-3037 (RTH1623.2)	*rid; fl; ; Sk-S^{ChrIII}; sad-2Δ::hph+ a+*
ISU-3291 (RDGR170.3)	*rid; Sk-3^{ChrIII}; mus-51Δ::bar+ A+*
TJCM10.2.4	v376-based hygromycin resistant transformant of ISU-3291
TJCM10.2.4	v376--based hygromycin resistant transformant of ISU-3291
TJCM10.2.4	v376--based hygromycin resistant transformant of ISU-3291
RJCM10.2	*rid; Sk-3^{ChrIII} v0376Δ::hph+-; mus-51? A+* (offspring of TJCM10.2 × F2-26)
RJCM10.4	*rid; Sk-3^{ChrIII} v0376Δ::hph+-; mus-51? a+* (offspring of TJCM10.4 × F2-26)
RJCM10.6	*rid; Sk-3^{ChrIII} v0376Δ::hph+-; mus-51? A+* (offspring of TJCM10.6 × F2-26)
RJCM10.7	*rid; Sk-3^{ChrIII} v0376Δ::hph+-; mus-51? a+* (offspring of TJCM10.7 × F2-26)
RJCM10.8*	*rid; Sk-3^{ChrIII} v0376Δ::hph+-; mus-51? A+* (offspring of TJCM10.8 × F2-26)
RJCM10.10*	*rid; Sk-3^{ChrIII} v0376Δ::hph+-; mus-51? a+* (offspring of TJCM10.10 × F2-26)

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	Sequence (5′ > 3′)
HPH-CEN-F	AACTGATATTGAAGGAGCATTTTTTGG
HPH-CEN-R	AACTGGTTCCCGGTCGGCAT
V0376-A	ACCTCATGTCTCGGTGAAGGGCG
V0376-B	AAAAAATGCTCCTTCAATATCAGTTGCCACCTTGTAGGGCTCTGCGG
V350-C	GAGTAGATGCCGACCGGGAACCAGTTCCTTGTCCTCTCGGTCCTCTCTGT
V350-D	GGTAGCGTTCTGGGATGAGTGTGG
V376-E	TCGTTCCAGTCCCCAAGCCCGAA
V0372-F	GGACCTGGCTGTTGCTGGCGTAG

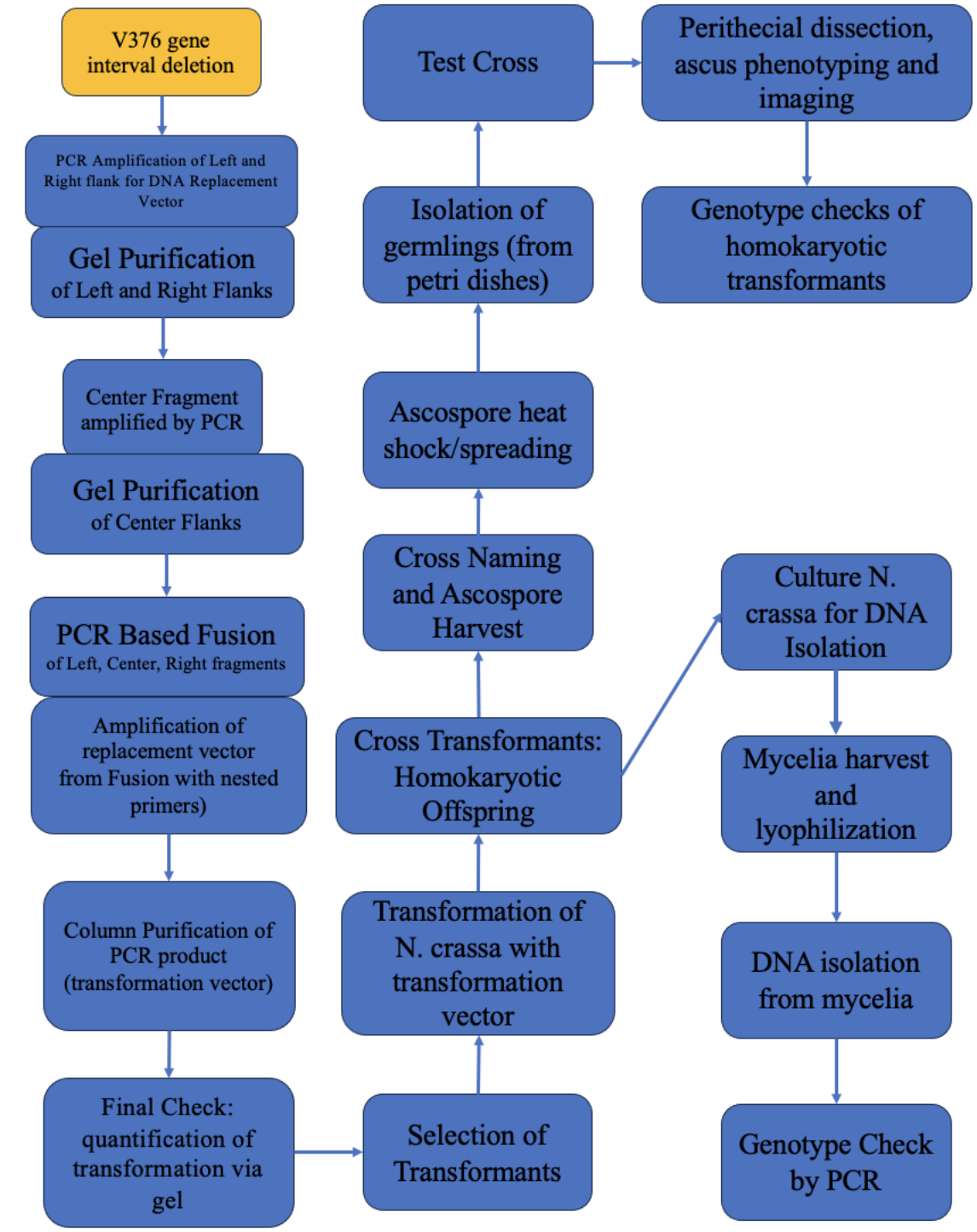

Figure 1 Methods Overview

>V376 left flank
ACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTT
CTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCC
TTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAAC
ATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAG
AGAATTTGGATGGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGT
CCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTG
TCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAA
AAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAG
GCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGG
AAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAA
GCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTT
CTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCC
TACAAGGTGGC

Figure 2 The v376 left flank. The 935 bp sequence of the v376 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0376-A and V0376-B from RDGR170.3 genomic DNA.

>V376 right flank
CCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGA
CCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCC
GCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCG
TTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCA
CCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAAC
CCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCATTGCCAAACCAGACCCA
GCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACCGTACTC
TGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACG
CCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAAC
CCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGACGTCGC
TATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACC

Figure 3 The v376 right flank. The 813 bp sequence of the v376 right flank is shown. This sequence was PCR-amplified with primers V350-C and V350-D from RDGR170.3 genomic DNA.

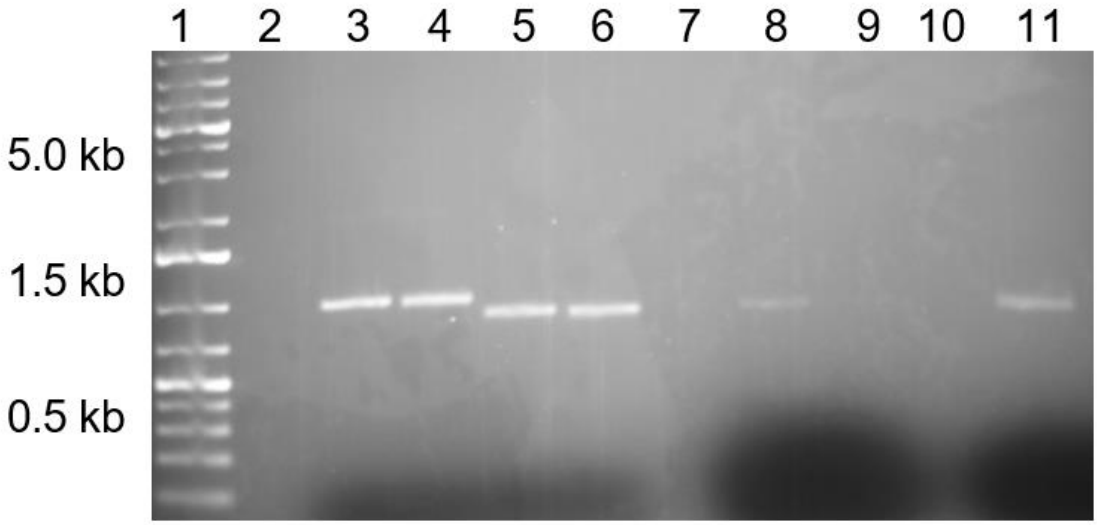

Figure 4 Vector v376 construction: left and right flanks. PCR products for v376 left and right flanks. The left flank for v376 was amplified from RDGR170.3 genomic DNA with primers V0376-A and V0376-B. The right flank for v376 was amplified from RDGR170.3 genomic DNA with primers V350-C and V350-D. Products 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 GeneRuler 1 kb Plus (Thermo Fisher); Lane 2) space, Lane 3) 5 µL purified product (v376 left flank trial 1), Lane 4) 5 µL purified product (v376 left flank trial 2), Lane 5) 5 µL purified product (v376 right flank trial 1), Lane 6) 5 µL purified product (v376 right flank trial 2), Lane 7–11) not applicable.
The expected product lengths for the v376 left and right flanks are 935 bp and 813 bp, respectively.

>v376 center fragment
AACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTTAG
TCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATCAGCCCCAC
TTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGC
TTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTT
CCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACTCACCGCG
ACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATC
TCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAG
ATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAG
AGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCG
GACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGG
CAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTG
CCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGG
TCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCGTGGTTG
GCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGTA
TATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGG
GTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTC
TGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATA
GAGTAGATGCCGACCGGGAACCAGTT

Figure 5 The v376 center fragment. The 1412 bp sequence of the V376 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 is colored green. The stop codon is colored red.

>*v376+* PCR product, predicted sequence, primers V376-E and V372-F

TCGTTCCAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGA
TGGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGAT
GCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGG
ACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTA
GTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGG
AAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGAT
TCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTT
GGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGG
CCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCC
TAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGAC
GGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGG
GGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGT
GGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACC
AGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAA
TTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCT
TCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCATTGCCAAACCA
GACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACC
GTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAG
A**CTACGCCAGCAACAGCCAGGTCC**

Figure 6 PCR product sequence: *Sk-3 v376+* genotype. The 1472 bp predicted sequence of DNA amplified with primers V376-E and V372-F from a template consisting of *Sk-3 v376+* genomic DNA is shown in FASTA format. The binding sites of V376-E and V372-F are indicated with bold font. The *v376* interval is indicated with red font.

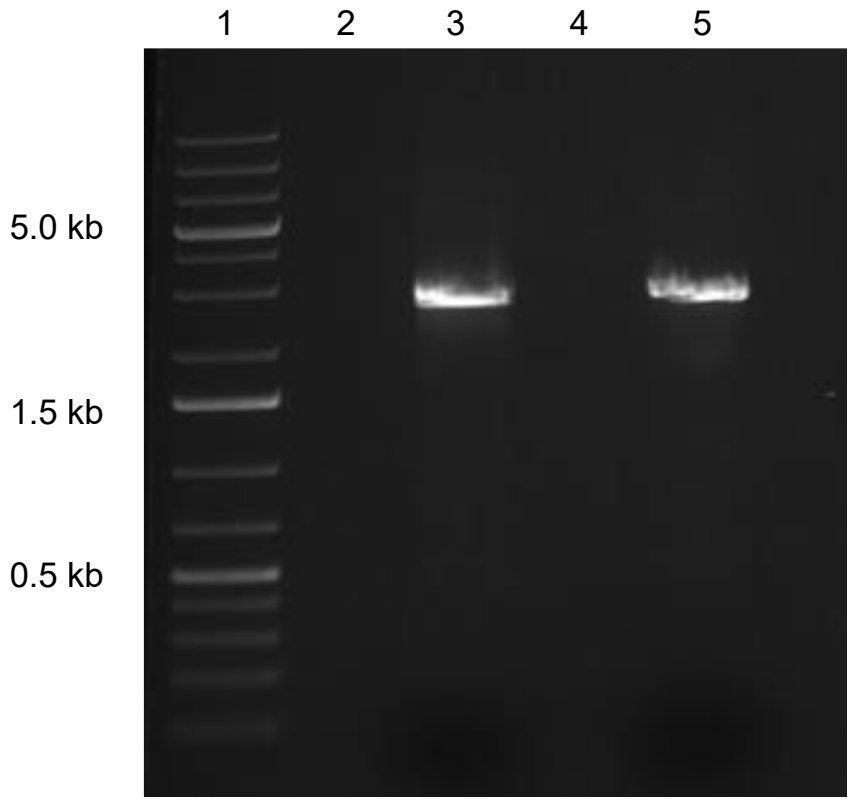

Figure 7 Vector v376 construction: fusion and nested amplification. The v376 left flank, v376 center fragment, and v376 right flank were fused by DJ-PCR. The fusion product was amplified with primers V350-E and V350-F. The amplified product was column purified as described in the methods section. The 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 (ThermoFisher); Lanes 2) not applicable. Lane 3) 3µL of amplified and purified v376. Lanes 4 and 5) not applicable. The expected length of v376 is 2740 bp.

>V376 DNA interval
CATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCT
AGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACG
GCTTA

Figure 8 The *v376* interval. The DNA sequence of the 159 bp *v376 i*nterval is shown. Vector v376 was designed to delete this interval by replacing it with *hph*+.

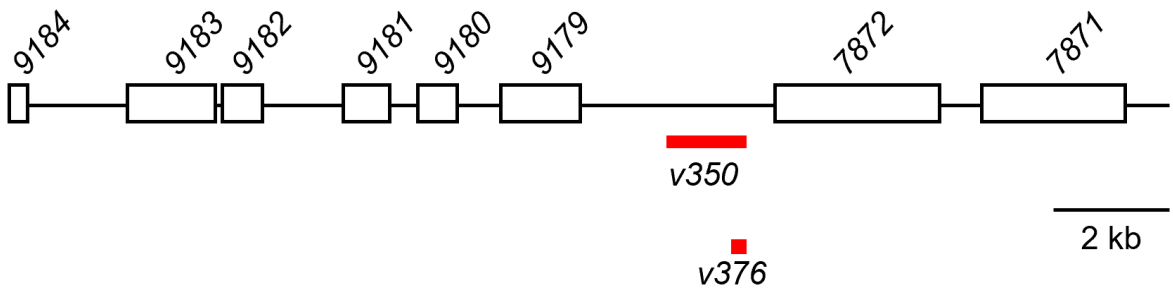

Figure 9 *Sk-3* Chromosome III positions 320,000–340,000. A diagram of of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the locations of protein-coding genes. Gene numbers were identified with blastx searches of an *N. crassa* protein database. The location of intervals *v350* and *v376* are indicated with red bars.

>*v376Δ::hph* PCR product, predicted sequence, primers V376-E and V372-F
TCGTTCCAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGA
TGGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGAT
GCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGG
ACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTA
GTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGG
AAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGAT
TCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTT
GGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGG
CAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTTA
GTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATCAGCCCCA
CTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTG
CTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCT
TCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACTCACCGC
GACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAAT
CTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAA
GATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGA
GAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTG
TTCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTC
GGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTG
GCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACT
GCCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCG
GTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCGTGGTT
GGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGT
ATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAG
GGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGT
CTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAAT
AGAGTAGATGCCGACCGGGAACCAGTTCCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTG
GCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCC
CACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTAC
GATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATT
AATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCG
CGTCCATCGTCTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCA
ACAGAATTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGC
CATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCC
AAATGTCCATGTCCCCTCGAGA**CTACGCCAGCAACAGCCAGGTCC**

Figure 10 PCR product sequence: *Sk-3 v376Δ::hph*+ genotype. The predicted sequence of DNA amplified with primers V376-F and V372-F from a template consisting of *Sk-3 v376Δ::hph*+ genomic DNA is shown in FASTA format. The binding sites of V376-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 2740 bp.

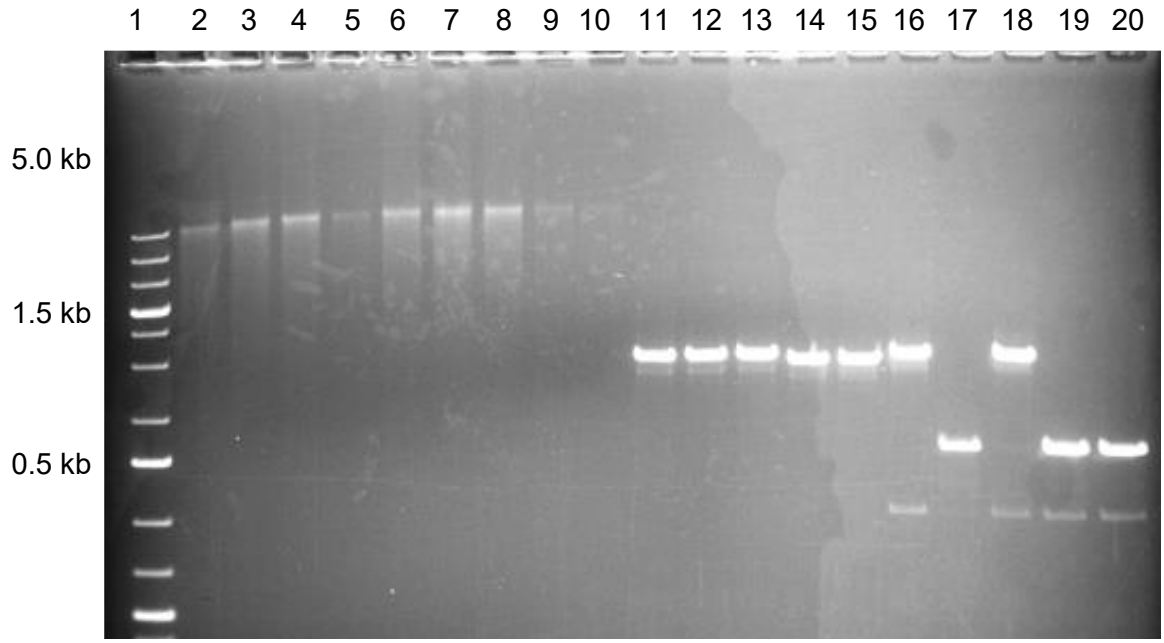

Figure 11 Transformant screening. Genomic DNA samples from candidate transformants TJCM1.1, TJCM1.2, and TJCM1.3, and transformation host RDGR170.3 were used as templates in PCR reactions with primers V350-E and V350-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThermoFisher); Lanes 2–15) not applicable. Lanes 16-20) PCR products from templates TJCM1.1, TJCM1.2, TJCM1.3, and RDGR170.3, respectively. The expected PCR product lengths are 1472 bp for *v376+* and 2740 bp for *v376Δ::hph+*.

Figure 12 Test strains and crosses. (A) Six hygromycin-resistant offspring from cross RJCM10 (TJCM1.3 × RTH1005.2) were selected for test crosses: 1) RJCM10.2, 2) RJCM10.4, 3) RJCM10.6, 4) RJCM10.7, 5) RJCM10.8, and 6) RJCM10,10. (B) Test crosses were performed

with RTH1623.1 and RTH1623.2. Images are of crossing dishes approximately three weeks post fertilization.

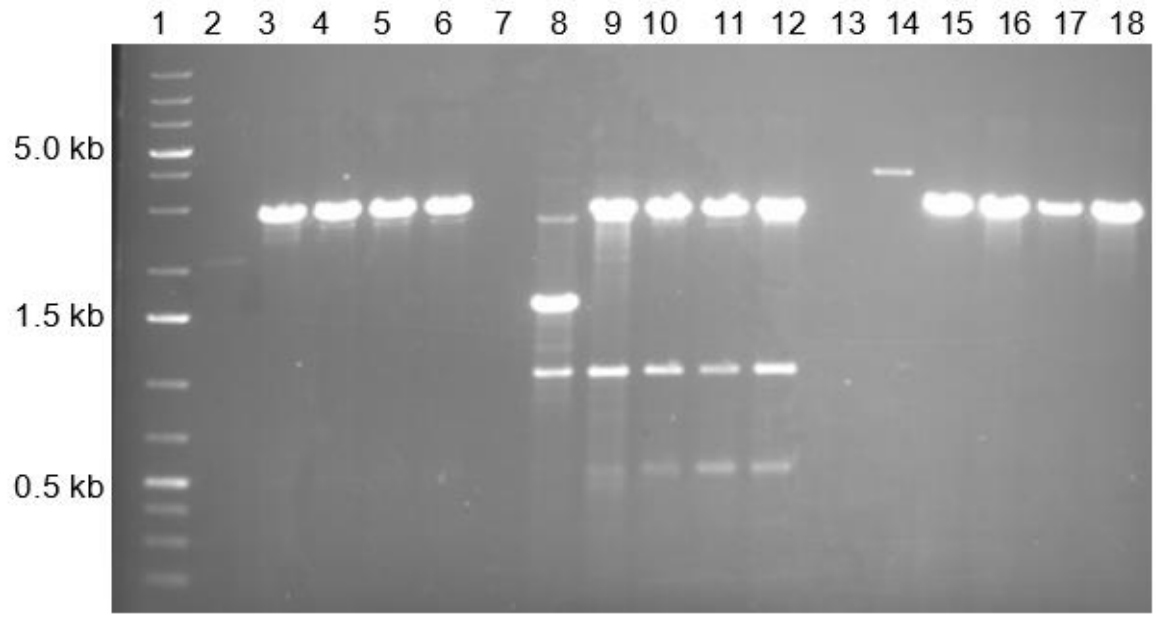

Figure 13 Test strain genotype confirmation. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0376-E and V0372-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel wth UV transillumination is shown. The expected PCR product lengths are 1472 bp for *v376+* and 2740 bp for *v376Δ::hph+*. Lane 1 contains 0.5 µg of GeneRule 1 Kb Plus DNA ladder (ThermoFisher). DNA templates for each PCR reaction are as follows: Lane 2-7 Not appliable. Lane 8, Control (RDGR170.3); Lane 9, RJCM10.2; Lane 10, RJCM10.4, Lane 11, RJCM10.7; Lane 12, RJCM10.10. Lanes 13-18 not appliable. These results show that test strains RJCM10.4, RJCM10.6, and RJCM10.7, and RJCM10.10 have the *v376Δ* genotype.

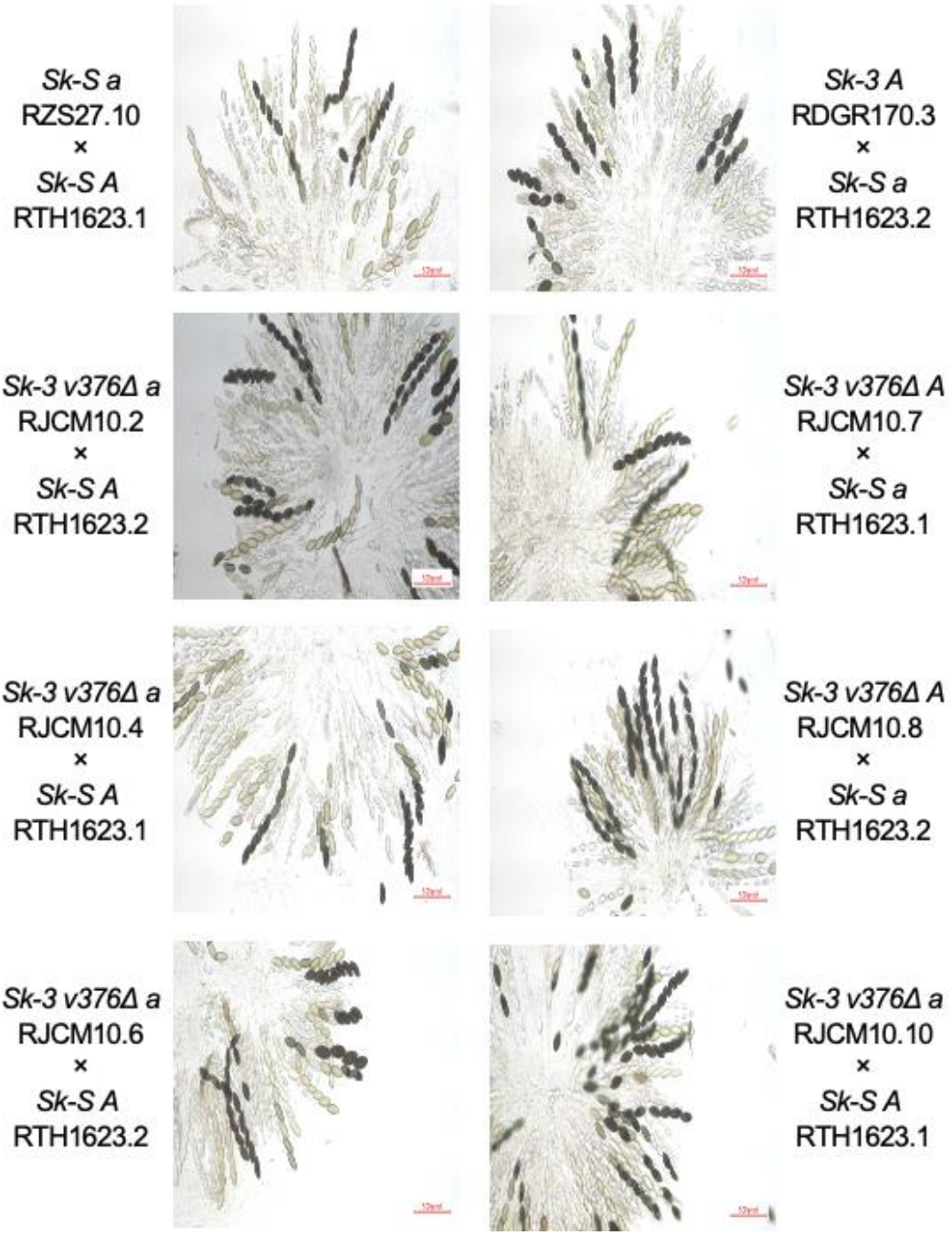

Figure 14 Ascus phenotype summary. Asci were dissected from perithecia of eight crosses on day 15 post-fertilization and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that the deletion of interval *v376* disrupts spore killing.

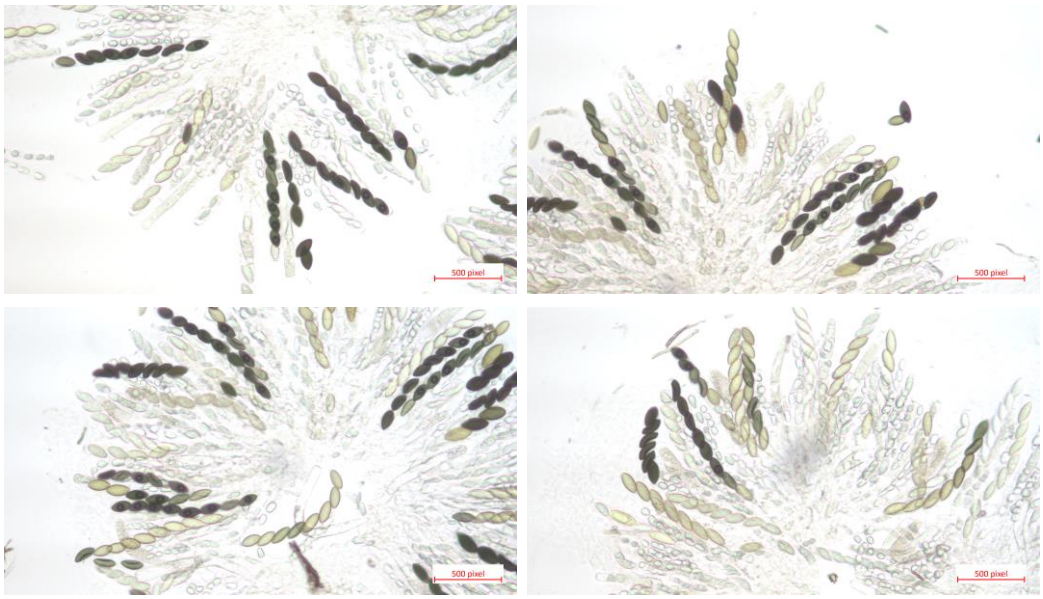

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RJCM10.2 × RTH1623.2 on day 15 post fertilization and imaged under magnification.

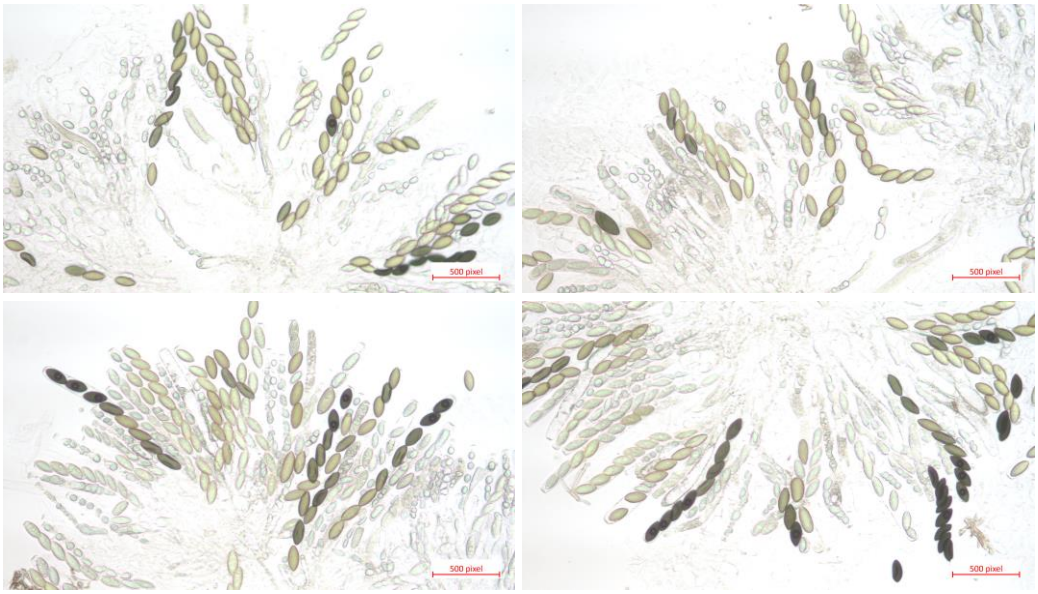

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RJCM10.4 × RTH1623.1 on day 15 post fertilization and imaged under magnification.

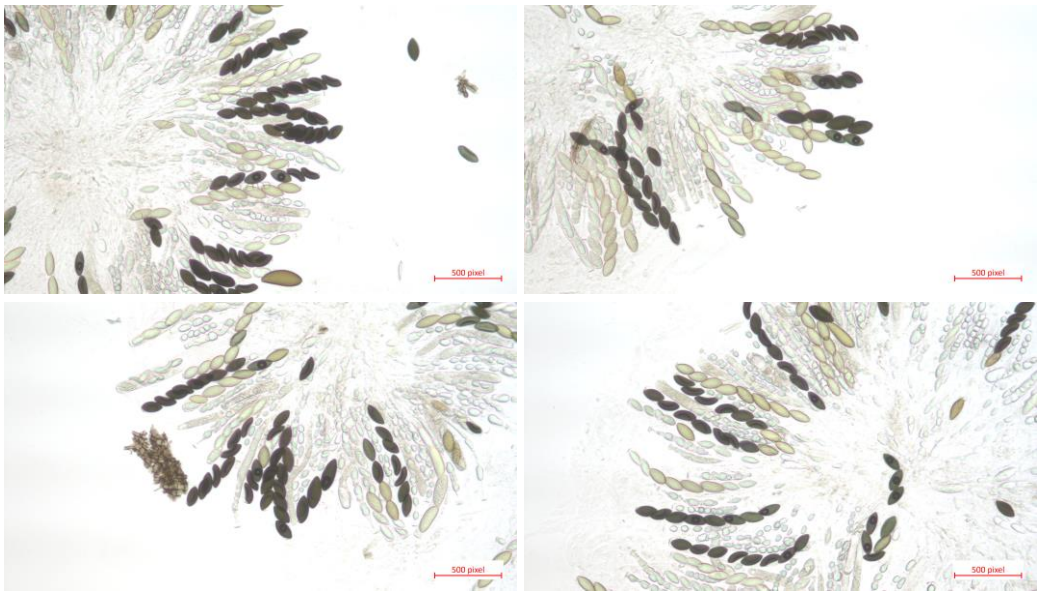

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RJCM10.6 × RTH1623.2 on day 15 post fertilization and imaged under magnification.

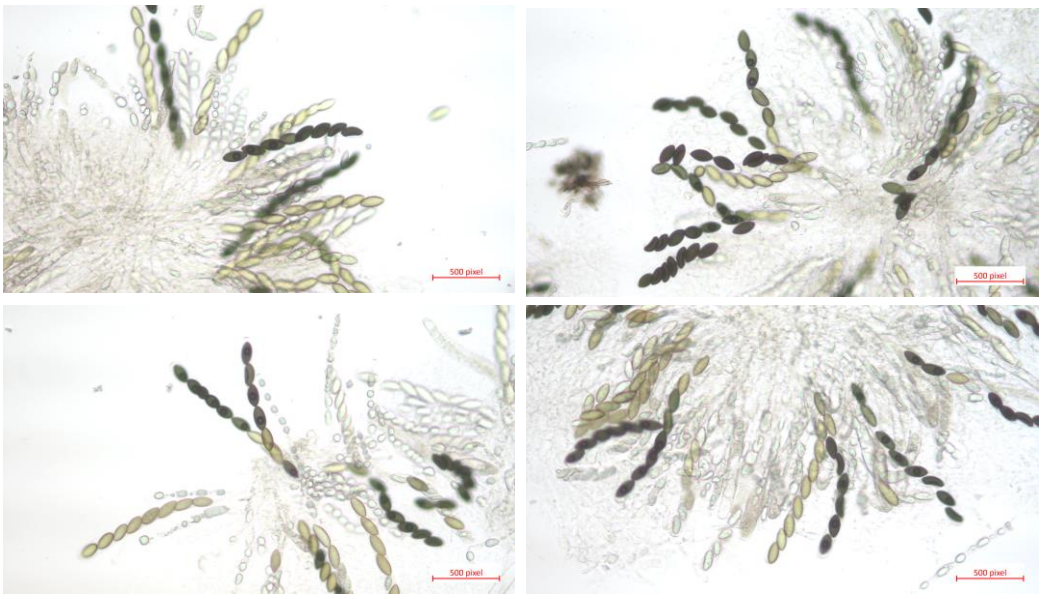

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RJCM10.7 × RTH1623.1 on day 15 post fertilization and imaged under magnification.

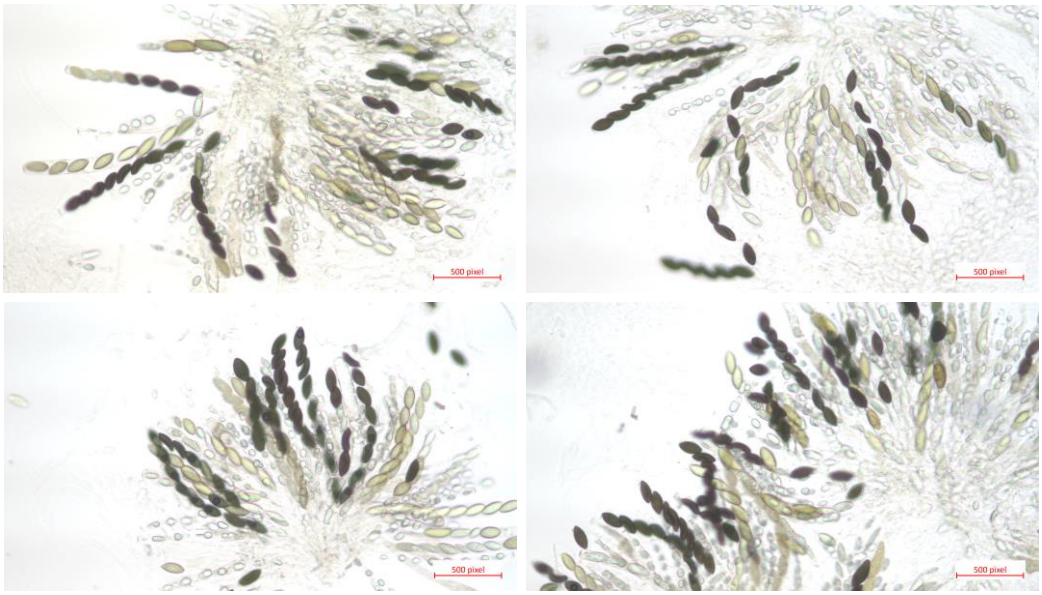

Figure 19 Ascus phenotypes. Asci were dissected from perithecia of RJCM10.8 × RTH1623.2 on day 15 post fertilization and imaged under magnification.

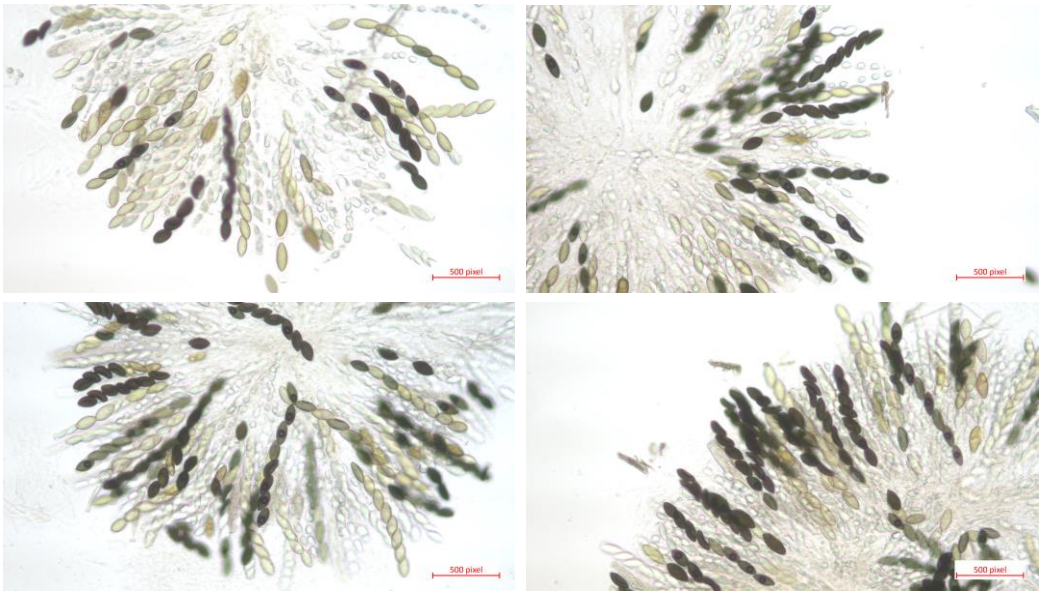

Figure 20 Ascus phenotypes. Asci were dissected from perithecia of RJCM10.10 × RTH1623.1 on day 15 post fertilization and imaged under magnification.