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Investigating Sk-3 based Spore killing in Neurospora crassa through Deletion Analysis of DNA Intervals i383 and i394

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Neurospora fungi are found around the world. The species *N. crassa* is a popular model for use in genetics research. *N. crassa* produces sexual spores, called ascospores, during mating between strains of opposite mating types. *N. crassa* also produces spore sacs called asci, and each ascus typically contains eight viable ascospores. However, some Neurospora fungi carry selfish genetic elements called Spore killers, and when a strain carrying a Spore killer mates with a spore killing-susceptible strain, asci contain four black viable ascospores and four white inviable ascospores. In this project, I investigated a Spore killer called *Sk-3.* To act as a selfish genetic element, *Sk-3* is thought to require at least two genes, a poison gene and an antidote gene. The *Sk-3* antidote gene (*rsk*) has been identified, but the poison gene has not. The purpose of this study is to help identify the location of the poison gene. To do this, I deleted two DNA intervals (*i383* and *i394*) from a location of the Neurospora genome that may harbor the poison gene. My results indicate that deletion of *i383* eliminates spore killing while deletion of *i394* has no effect on spore killing. The possibility that *i394* overlaps with the poison gene is discussed.

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CONTENTS

TABLES

FIGURES

CHAPTER I

INTRODUCTION

Neurospora is a genus of fungus found in a diverse range of climates, including temperate, subtropic, and tropic regions (Aramayo and Selker 2013). It is an ascomycete commonly found growing on bread. There are at least five species recognized within this genus; including *N. tetrasperma*, *N. discreta*, *N. sitophila*, *N. intermedia*, and the genetic model, *N. crassa*. These fungi all produce spores, which are structures that may produce new life. Spores can be either asexual or sexual. Asexual spores are produced by mitosis, meaning the offspring will be genetically identical as long as no chance events occur, such as mutations. Macroconidia and microconidia are two possible types of asexual spores. Sexual spores are produced during mating by meiosis, meaning the products will be genetically different. The sexual spores produced by *Neurospora* are named ascospores and tend to be larger than asexual conidia.

Asexual conidia exhibit apical growth, absorbing nutrients from their environment and forming hyphae – branching filaments which also grow apically. Hyphae are compartmentalized by septa, which contain pores that allow cytoplasmic continuity, sharing of mitochondria, and dozens of nuclei within the compartments. This organism is primarily haploid, with a genome composed of approximately 40 million base pairs spread throughout its seven chromosomes.

Mating in *N. crassa* occurs only between strains of opposite mating types, designated as "*A*" and "*a*". During mating, the trichogyne of one strain's protoperithecium will sense the conidium of the other strain. Chemical signaling between the trichogyne and conidium leads to cellular fusion. A nucleus from the conidium will migrate through the trichogyne to reach the protoperithecium, triggering its development into a mature perithecium. After this fertilization,

the perithecium will contain dikaryotic hyphae, meaning the haploid nuclei of both mating types will exist within the hyphae. These hyphae produce crozier hook cells. When a crozier hook cell's penultimate compartment contains one haploid nucleus from mating type "*A*" and another from "*a*", the nuclei will fuse to form a diploid meiotic cell. This cell is elongated to form an ascus which contains the diploid nucleus. Meiosis I will transform the meiotic cell into a cell that contains two haploid nuclei. Meiosis II will follow to produce a total of four haploid daughter nuclei. These four nuclei will undergo mitosis to produce an ascus containing eight nuclei. Next, ascospores will develop around each nucleus. As the ascospores develop, they will become pigmented with melanin, which will protect them from harsh environments. A perithecium will contain approximately 200–400 asci in different stages of development. When the ascus reaches maturity, it will be ejected out of the perithecium through the ostiole, which is a pore located at the top of the perithecium.

There are three known spore-killing selfish genetic elements in *Neurospora*, named *Sk-1*, *Sk-2*, and *Sk-3*, all of which are located on Chromosome III (Turner and Perkins 1979). This study focuses on *Sk-3*. Spore killers are selfish genetic elements due to their selective killing of ascospores that do not have the same spore killing genotype. This phenomenon leads to meiotic drive, a process that allows for skewing genetic transmission in a way that deviates from Mendelian expectations (Burt and Trivers 2008). To function successfully, *Sk-3* requires both a killing (poison) gene and a resistance (antidote) gene (Hammond et al. 2012). The *Sk-3* resistance gene (*rsk*) has been discovered, but the killer gene is unknown.

The normal phenotype of an ascus is eight viable, black ascospores, but this does not always occur (Turner and Perkins 1979). When *Sk-3* killer ($Sk\text{-}3^K$) strains are crossed with other $Sk\text{-}3^K$ strains (homozygous crosses), eight viable ascospores are present. However, when $Sk\text{-}3^K$

and *Sk-3* sensitive ($SK\text{-}3^S$) strains are crossed (heterozygous crosses), only four of the ascospores are black and viable, while the other four are hyaline and inviable (Velazquez et al. 2022). Spore-killing resistant ($Sk\text{-}3^R$) isolates have been found in *N. crassa* and *N. intermedia*. These isolates do not engage in spore killing. When $Sk\text{-}3^R$ phenotypes are crossed with $Sk\text{-}3^K$ or $Sk\text{-}3^S$ phenotypes, all eight ascospores are viable.

This study examines a genomic location thought to be associated with the *Sk-3* killer gene. Two DNA sequences, called *i383* and *i394*, were deleted from Chromosome III of an *Sk-3* strain and replaced with a hygromycin resistance gene called *hph+*. It was expected that crosses would not display spore killing if the killer gene had been deleted.

CHAPTER II

METHODS

A process called the double joint polymerase chain reaction (DJ-PCR) was used to construct deletion vectors for two DNA intervals (Yu et al. 2004): *i383* and *i394*. The final DJ-PCR product will help refine the killer gene's location by eliminating spore killing (or not) when the deletion vectors integrate into the genome and delete the target intervals. My primary goal was to cross *Sk-3* strains deleted of the target intervals with an *Sk-3*^{S} strain. A map (**Figure 1**) demonstrates the steps I took to achieve this goal.

2.1 Culturing conidia of *N. crassa* strain RDGR170.3

The strains used in this study and their genotypes are listed in **Table 1**. To begin this experiment, it was essential to first produce macroconidia (conidia) of *N. crassa* strain RDGR170.3. The following procedure was used to produce conidia for transformation. A 50 ml volume of solid Vogel's medium (Vogel 1956; Davis 2000) was made in a 250 ml flask. The flask containing the medium was autoclaved on a liquid cycle, then placed on a culture shelf for one to two days to allow excess moisture to evaporate. When the flask lacked visible condensation, it was used. In a sterilized biosafety hood, a single aliquot of a cryogenic conidial suspension of strain RDGR170.3 was thawed in a gloved hand, and 20 µl was transferred into the culture flask. A sterile wood applicator was positioned between the cap (a 50–150 ml glass beaker) and flask to increase air exchange between the culture and the laboratory environment. After two days of incubation at 32 °C, the flask was moved to clean tray for up to four weeks to allow cultures to produce conidia.

2.2 *N. crassa* DNA deletion vectors

The *N. crassa* DNA deletion vectors were made from three parts: a left flank of 500–1000 bp, a center fragment of 1.4 kb, and a right flank of 500–1000 bp. The left and right flanks are required to match the sequences that flank the DNA interval being replaced with the center fragment (**Figures 2–5**). DNA intervals *i383* and *i394* of Chromosome III in the *N. crassa Sk-3* strain were replaced with a hygromycin resistance gene named *hph+*. These intervals are located within *i350,* an interval that appears to play a role in spore killing (Rhoades and Hammond, preliminary results; **Figure 6**).

First, the left and right flanks were amplified by PCR. Both the left and right flank for each vector have a designated primer set containing a forward and reverse primer (**Table 2**). For Vector v383, Primer Set 1 (left flank) includes forward primer V0375-A and reverse primer V0375-B. Primer Set 2 (right flank) includes forward primer V0383-C and reverse primer V0383-D. Additionally, Primer Set 1 for Vector v394 consists of forward primer V0394-A and reverse primer V0394-B. Primer Set 2 for this vector includes forward primer V0394-C and reverse primer V0394-D. To perform the PCR, primers were obtained in addition to the DNA template of RDGR170.3, dNTP solution, Q5 DNA polymerase (New England Biolabs), Q5 buffer, and sterile autoclaved water. Two primer mixes were made for each DNA deletion vector, one for the left fragment and one for the right (6.25 µl of forward primer at 100 pmol/µl, 6.25 µl of reverse primer at 100 pmol/µl, and 487.5 µl of sterile water). Four PCR tubes were used for each replacement vector, labeled one through four. Aliquots of 5.0 µl of the left fragment primer mix were transferred to tubes one and two, and 5.0 µl of the right fragment primer mix were transferred to tubes three and four. For tubes one through four, 1.0 µl of the DNA template

(RDGR170.3 genomic DNA at approximately 10 ng/µl) was added. An enzyme master mix for Q5 DNA polymerase was then made in a sterile 1.5 ml MCT tube. The master mix contained the following volumes of reagents per reaction: 13.4 µl of sterile water, 5.0 µl of Q5 reaction buffer, 0.5 µl of dNTP (10 mM), and 0.1 µl of Q5 DNA polymerase. Aliquots of 19.0 µl of the master mix were transferred to each PCR tube containing template and primer. The reactions were mixed by pipetting. The PCR tubes were placed in the PCR machine and the reactions were cycled according to manufacturer's recommendations for Q5 DNA polymerase. The samples were then stored at 4 °C for one to several days before gel purification.

An agarose gel was made by pouring molten 1.8% agarose, made in 1× TAE buffer, into a gel tray with a 12-tooth comb. The electrophoresis chamber was filled with 1× TAE buffer and the solidified gel was transferred to the chamber and submerged in the buffer. Additional 1× TAE buffer was added until the gel was completely covered with buffer. The comb was removed. PCR products were prepared for gel purification by adding 5.0 µl of a 6× loading buffer into each of the four PCR tubes and pipetting up and down to gently mix the solution. A total of 10.0 µl of the DNA ladder was loaded into the left-most well of the gel, and each PCR product + loading dye mixture (30 µl) was then added to the remaining wells. The gel was run for 90–120 minutes at 120 V. After gel electrophoresis, the PCR products were excised from the gel. Using a UV transilluminator, a rectangle was cut around each PCR product one at a time from the gel with a clean razor blade. The gel slices were transferred into MCT tubes. A gel extraction kit (IBI Scientific) was then used to isolate DNA from the gel slices according to the manufacturer's recommended protocol. A volume of 50 µl of elution buffer was used to elute the DNA from the gel purification column matrix. Upon completion, purified products were examined by gel electrophoresis.

To prepare DNA samples for analysis, a 1.5 ml MCT tube was used for each purified PCR product. The following volumes were added to an MCT tube: 5.0 µl of the gel purified PCR product, 5.0 µl of 6× loading buffer, and 20.0 µl of sterile water. The solutions were mixed by pipetting up and down gently five times. A 10.0 µl volume of the DNA ladder was then loaded into the left-most well, and all 30 µl of the sample mixes were loaded into the other wells. The gel was run for 90–120 minutes at 120 V and an image of the gel was taken.

The next step was to fuse the left and right fragments to a center fragment using the purified left and right flanks. The highest concentration left flank and right flank were chosen for this next step by analyzing the gel electrophoresis image of the purified DNA. In a PCR tube, I transferred 5.0 µl of the left fragment, center fragment (provided by my research mentor), and right fragment. Then, I added 62.6 µl of sterile autoclaved water, 20.0 µl of Q5 reaction buffer, 2.0 µl of dNTP mix, and 0.4 µl of Q5 enzyme. The reaction was mixed by slow pipetting up and down five to ten times. The PCR tube was transferred to the PCR machine and cycled according to the following parameters: 98 °C for 30 seconds; 98 °C for 5 seconds; 58 °C for 4 minutes, 72 °C for 6 minutes, return to the short denaturation step 10 times, 72 °C for 4 minutes, and 12 °C indefinitely. When the cycles had completed, the fusion products were stored at 4 °C for a few days (or at -20 °C for longer periods).

The fusion products were then used as templates with nested primers to amplify the DNA deletion vectors. For Vector v383, the primer set included the nested forward primer V0375-E and nested reverse primer V0383-F. Vector v394 had a primer set consisting of nested forward primer V0394-E and nested reverse primer V0394-F. Primer mixes were made by adding 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 autoclaved water. In a single PCR tube, for each vector, I added 20.0 µl of the primer mix,

5.0 µl of the fusion PCR reaction, 52.6 µl of autoclaved sterile water, 20.0 µl of Q5 reaction buffer, 2.0 µl of dNTP mix, and 0.4 µl of Q5 enzyme. After carefully pipetting up and down five to ten times to mix each reaction, I transferred them to a PCR machine and cycled the reactions according to the Q5 DNA polymerase manufacturer's recommendations. The reactions were then stored at 4 °C until they were purified by column purification and checked by gel electrophoresis.

For column purification of the 100 µl PCR product, I followed the manufacturer's recommended protocol (IBI Scientific) with 40 µl as the final elution volume. The purified vector was then checked by gel electrophoresis by adding 5.0 µl of column purified DNA sample, 5.0 µl of 6× loading buffer, and 20.0 µl of sterile autoclaved water into a MCT tube. The solution was mixed by pipetting up and down gently five times. I then loaded 10 µl of DNA ladder into the left-most well of a 1.8% agarose TAE gel and 30.0 µl of the sample into a different well (one well per sample). The gel was run at 120 V for 90–120 minutes and an image was taken.

2.3 Transformation of *N. crassa* strain RDGR170.3

Transformation was essentially performed by electroporation as previously described (Margolin et al. 1997; Rhoades et al. 2020). Using a sterile biosafety hood, four to five wood sterile applicators were used to transfer conidia of RDGR170.3 into a 50 ml vial of 30 ml ice cold 1 M sorbitol. After wiping down a laboratory bench with 70–95% ethanol, a 100 µl cell strainer was used to remove mycelia and large fungal tissue from the suspension. This was done by placing a 100 µl cell strainer over a sterile 50 ml conical vial, then slowly pouring the conidial suspension through the strainer. The vial of filtered conidia was then placed on ice. Next, 900 µl

of sterile 1 M sorbitol was added to two disposable spectrophotometry cuvettes. An aliquot of 100 µl of filtered conidia was added to one of the cuvettes, and the cuvette was tapped on a bench to mix the suspension. The second cuvette was used as a blank for spectrometry. The absorbance of the conidia-containing cuvette was measured at 420 nm. Units of conidia were calculated with the following equation: A_{420} × # ml filtered conidia suspension × 100 units/ml = # units.

The conidial suspension was then centrifuged at 2000 × g for 10 minutes at 12 °C. The supernatant was poured into a flask for disposal (as hazardous waste), and the pellet was resuspended in 20 ml of cold 1 M sorbitol. The conidia was centrifuged again under the same conditions. The supernatant was again poured into a flask for disposal, and a microliter volume of cold 1 M sorbitol equal to $1/4^{th}$ the number of units calculated from the previous equation was used to resuspend the pellet. The resuspended conidia were then stored on ice. The transformation vectors were electroporated into the conidia (approximately 10 µl of 50 ng/µl vector and 90 µl conidial suspension) at 1500 V. Electroporated conidia were resuspended in 750 µl of cold 1 M sorbitol and transferred to a 50 ml conical tube containing 5 ml of Rescue Medium (Vogel's minimal medium + 1 M sorbitol). Rescue cultures were placed on their sides (in a tray to capture any possible leakage) and incubated at 32 °C for three and a half hours. The shaker was set at approximately 40–80 rpm, just fast enough to see slight ripples in the medium and to keep electroporated conidia from clumping into single mass.

After the three and a half hour incubation, the rescue cultures were plated onto selective medium. One vial of 40 ml molten top agar was used as temperature check vial so that temperatures of other 40 ml molten top agar vials could be estimated. When the molten top agar vials had reached a temperature of 47 °C to 50 °C, 100 µl of rescue culture was added to the

molten top agar and mixed by inversion. Then, 10 ml of the suspension was poured into a petri dish containing bottom agar. To the same vial of top agar, 500 µl of rescue culture was added, mixed by inversion five times, and 10 ml was poured to a second petri dish containing bottom agar. Next, to the same vial of top agar, 1000 µl of rescue culture was added, mixed by inversion five times, and 10 ml was poured to a third petri dish containing bottom agar. The remaining rescue culture was poured into the same vial of top agar, mixed by inversion, and 10 ml was poured to a fourth petri dish containing bottom agar. These steps were repeated for each batch of electroporated conidia (including a no DNA negative control). All petri dishes were stored right side up at room temperature overnight on a sheet of aluminum foil. After 24 hours, the petri dishes were inverted and placed at 32 °C. After three to five days of incubation, hygromycin resistant colonies were transferred from the transformation plates to Vogel's slants containing hygromycin (200 µg/ml). Hygromycin resistant colonies were transferred by first wiping down the work area with 70–95% ethanol. An ethanol candle was then used to sterilize dissecting syringes and needles. Approximately 10 colonies were transferred to slants, each slant getting a single colony. The slants were placed at 32 °C incubator for two to three days and then room temperature for up to one month.

2.4 Crossing transformants to a standard mating strain to obtain homokaryotic offspring

Unidirectional crosses were performed essentially as previously described (Samarajeewa et al. 2014). Westergaard crossing medium (Westergaard and Mitchell 1947) in 60 mm petri plates was used for crosses. A 5.0–10.0 µl aliquot of a cryogenic suspension of strain RTH1005.2 was transferred to the center of each plate. After inoculation, the petri dishes were organized in a single layer on top of aluminum foil in a 1020 tray. The plates were cultured at room temperature

for 6 to 8 days. The plates were then fertilized with conidial suspensions of the transformant strains. Conidial suspensions were made in 500 µl sterile water for six to eight transformants. A sterile wood applicator was used to transfer a clump of conidia from each transformant to 500 µl sterile water in an MCT. Conidial suspensions were then vortexed and 200 µl of each conidial suspension was transferred in small drops around the plates containing RTH1005.2. The plates were incubated in a single layer right-side up in a tray without a humidity dome for one week. After one week, a humidity dome was placed over the tray.

2.5 Cross naming and ascospore harvest

When ascospores were ready for harvest (at least 24 days post fertilization), the crosses were named (e.g., Cross TPPak1.6 × 1005.2 was named RPPak10 and Cross TPPak2.1 × 1005.2 was named RPPak11). A barrier pipette tip was used to transfer ascospores from the lids of crossing plates to 500 µl aliquots of sterile water. Specifically, a 50 µl volume of sterile water was added to the crossing lid, and ascospores were drawn into the water by pipetting up and down, and the water was transferred to an MCT. The ascospore suspensions were vortexed and placed at 4 °C in the dark for at least 24 hours.

2.6 Isolating hygromycin resistant germlings

Ascospore suspensions that had been cold stored for at least 24 hours were heat shocked and spread on Vogel's medium containing hygromycin at 200 µg/ml. Specifically, three 100 mm plates containing 20–25 ml of solid Vogel's medium with hygromycin were used for each ascospore suspension. A working suspension of ascospores was made at approximately 1–5 ascospores per µl. The working suspension was vortexed for five seconds, gently tapped on the

bench to get liquid away from the cap, and placed in a 60 °C heat block for 30 minutes. Three aliquots (50 µL, 100 µL, and 200 µl) of the heat shocked working suspension were then spread upon the Vogel's hygromycin medium with sterile steel spreaders. The plates were incubated overnight right-side up at room temperature for 16–24 hours. Syringe needles and a dissecting microscope were used to transfer single germinating ascospores to Vogel's slants containing hygromycin. An ethanol candle was used to sterilize needles in between transfers. A total of 12 germlings were selected for each batch of ascospores. The slants were incubated at 32 °C for 1–3 days and room temperature for 1–3 weeks.

2.7 Performing test crosses

Female strains for test crosses were RTH1623.1 and RTH1623.2. A total of 20 petri dishes (60 mm) containing Westergaard medium were prepared. A 5–10 µl aliquot of a cryogenic suspension of RTH1623.1 was transferred to the center of 10 petri dishes, while a 5–10 µl aliquot of a cryogenic suspension of RTH1623.2 was transferred to the center of the other 10 petri dishes. After inoculation, the plates were arranged in a single layer on top of aluminum foil-covered 1020 tray and placed on an incubation shelf. The plates were fertilized with male strains 8–10 days later. Conidial suspensions were prepared for six male strains (RPPak10 or RPPak11 hygromycin resistant offspring) and two control strains (RDGR170.3 and RZS27.10). Conidial suspensions were made, and plates were fertilized as described above. Fertilized plates were placed in a single layer right-side up in a 1020 tray. After one week, a humidity dome was placed over the tray. After 12–16 days post-fertilization, perithecia were dissected as part of the spore killing assay (**Figures 11 and 20**).

2.8 Spore killing assay

The laboratory bench and dissecting microscope was wiped down with 70-95% ethanol. A 100.0 µl volume of 25% glycerol was then transferred to the center of two microscope slides. A sterile 200 µl pipette tip was used to cut a small clump of perithecia from a crossing plate, and the clump was placed in one of the two 100 µl drops of 25% glycerol. Syringe needles were used to separate 10–15 perithecia from the clump. Each perithecium was cleaned of surface hyphae and agar before transfer to the second slide containing 100 µl of 25% glycerol. The needles were used to slice each perithecium and release intact rosettes of asci into the glycerol solution. Enough perithecia were sliced so that approximately ten rosettes could be observed on the microscope slide. Perithecial walls were removed, and a cover slip was placed over the rosettes. A Kimwipe was used to dry the sides of the cover slip. The slides were air dried for an additional one to two minutes, then the cover slips were sealed with clear nail polish to prevent over drying. A Leica compound microscope with Zeiss imaging system was used for rosette imaging.

2.9 Genotyping

Mycelia for genotyping was obtained by culturing each strain in 3–5 ml of liquid Vogel's medium at 32–37 °C and 150–180 rpm. Mycelia was collected from the tube with wooden applicators and placed on a single sheet of filter paper over a stack of approximately 10 paper towels. The filter paper and paper towels were folded over the mycelia and pressed to release liquid from the fungal tissue. Clean forceps or sterile wooden applicators were then used to transfer the tissue to prelabeled MCTs. The process was repeated for all samples while being careful not to cross contaminate the samples. The MCTs were stored at -80 °C until lyophilization. Tissues were then lyophilized for 3–5 hours, after which they were stored in a dry

cabinet at room temperature.

DNA was isolated from lyophilized tissue with a genomic DNA isolation kit (IBI Scientific). Tissue was ground into a powder in an MCT using a fine point spatula (max 80 µg of tissue). The manufacturer's protocol for DNA extraction was then followed and the isolated DNA was eluted into 100 µl of preheated elution buffer (65 °C).

The isolated DNA was used in PCR-based genotyping assays. The nested primer sets for each vector were used in the genotyping assays. The primer mixes contained the forward and reverse primers in a sterile 1.5 ml MCT; specifically, 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. Aliquots of 5.0 µl of primer mix were used in each PCR reaction. Aliquots of 1.0 µl DNA template were used in each PCR reaction. An enzyme master mix (sufficient for five reactions) was prepared in a sterile 1.5 ml MCT by adding 67.0 µl of water, 25.0 µl of Q5 reaction buffer, 2.5 µl of dNTP mix, and 0.5 µl of Q5 enzyme. Aliquots of 19 µl of the master mix were used in each reaction. Reactions were cycled according to the Q5 DNA polymerase manufacturer's recommendations.

PCR products were then examined by gel electrophoresis. Samples were prepared for analysis by adding 5 µl of 6× loading buffer to each of the 25 µl PCR reactions. The solutions were mixed by pipetting and 10 µl of each PCR product/loading dye mix were added to the wells of the gel. DNA ladder was added to the left-most well of the gel. The gel was run at 120 V for 90–120 minutes. A gel documentation system was used to image the gel. The PCR products for *i383Δ::hph*+ and *i383*+ genotypes are predicted to be 2840 bp (**Figure 7**) and 1443 bp (**Figure 8**), respectively. The PCR products for *i394Δ::hph*+ and *i394*+ genotypes are predicted to be 2956 bp (**Figure 9**) and 1581 bp (**Figure 10**), respectively.

CHAPTER III

RESULTS

My initial hypothesis was that deletion of interval *i383* and *i394* would disrupt *Sk-3*-based spore killing. These intervals are located within *i350* (**Figure 6**).

To delete *i383*, I constructed Vector v383 and transformed strain RDGR170.3. I crossed several hygromycin-resistant transformants to RTH1005.2. I selected cross RPPak10 (transformant TPPak1.6 × 1005.2) for isolation of hygromycin resistant offspring. This crossing step was performed because *N. crassa* transformants usually contain a mixture of transformed and untransformed nuclei, while offspring from a cross are usually homokaryotic. The six offspring I selected for use in spore killing assays were named RPPak10.1, RPPak10.6, RPPak10.10, RPPak10.21, RPPak10.23, and RPPak10.26. Images of the crossing plates from the spore killing assays show that #1, #6, and #23 have the *mat A* genotype, while #10, #21, and #26 have the *mat a* genotype (**Figure 11**). Results from the spore killing assays indicate that deletion of *i383* causes a loss of spore killing (most mature asci from each test cross contain eight viable ascospores). A summary of the ascus phenotypes from the *i383* spore killing assays is shown in **Figure 12**, while additional images from the spore killing assays are provided in **Figures 13–18**. A PCR-based genotyping assay was used to confirm that strains RPPak10.1, 6, 10, and 21 have the *i383Δ::hph+* genotype. To conserve resources, not all test strains were genotyped. As expected, the genotyping results show that RPPAK10.1, 6, 10, and 21 are homokaryotic for the *i383Δ::hph+* genotype (**Figure 19**).

To delete *i394*, I constructed Vector v394 and transformed strain RDGR170.3. As with v383, I crossed several hygromycin-resistant transformants to RTH1005.2. I selected cross

RPPak11 (transformant TPPak2.1 × 1005.2) for isolation of hygromycin resistant offspring. The six offspring I selected for use in spore killing assays were named RPPak11.1, RPPak11.2, RPPak11. 3, RPPak11.4, RPPak11.5, and RPPak11.12. Images of the crossing plates from the spore killing assays show that #1, #3, #4, and #12 have the *mat A* genotype, while #2 and #5 have the *mat a* genotype (**Figure 20**). Results from the spore killing assays indicate that deletion of *i394* does not disrupt spore killing because most asci from each test cross show the spore killing phenotype (four viable ascospores per ascus). A summary of the ascus phenotypes from the *i394* spore killing assays is shown in **Figure 21**, while additional images from the spore killing assays are provided in **Figures 21–26**. A PCR-based genotyping assay was used to confirm that strains RPPAK11.1, 2, and 12 have the *i394Δ::hph+* genotype. To conserve resources, not all test strains were genotyped. As expected, the genotyping results indicate that RPPAK11.1, 2, and 12 have the *i394Δ::hph+* genotype (**Figure 28**).

CHAPTER IV

DISCUSSION

Deletion of *i383* did not disrupt spore killing. In contrast, deletion of *i394* did disrupt spore killing. Although I hypothesized that both interval deletions would disrupt spore killing, the results for *i394* deletion reject this hypothesis. Both *i383* and *i394* reside within *i350*, an interval thought to be associated with spore killing. The opposing results for *i383* and *i394* suggest that only some subintervals of *i350* are required for spore killing.

The phenotypic results for *i383* and *i394* are interesting in light of previous findings by Turner and Perkins (1979). Crossing between *Sk-K* and *Sk-S* generally results in only four black viable ascospores, so the removal of the killer gene sequence should produce eight black ascospores. This matches the results from deletion of *i383*. Because *i394* deletion does not disrupt spore killing, my results suggest that the killer gene is associated with *i383* but not *i394*, and that the latter interval can be eliminated as a possible location for the killer gene.

The results from this study have contributed to refining the location of the *Sk-3* killer gene. Data from these experiments may also be used to gain a broader understanding of eukaryotic genetics. Meiotic drive likely occurs in all sexually reproducing eukaryotes and well documented examples exist in organisms such as *Drosophila*, mice, and plants (Burt and Trivers 2008). Obtaining more information on meiotic drive processes in *N. crassa* should help us understand how similar processes work in other eukaryotic organisms.

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Table 1 Strains used in this study

Strain name	Genotype
F2-23 (RTH1005.1)	*rid; fl A+*
F2-26 (RTH1005.2)	*rid; fl a+*
FGSC 10340 (RZS27.10)	*rid; mus-51^{RIP70} a+*
ISU-3036 (RTH1623.1)	*rid; fl; sad-2Δ::hph+ A+*
ISU-3037 (RTH1623.2)	*rid; fl; sad-2Δ::hph+ a+*
ISU-3291 (RDGR170.3)	*rid; Sk-3+; mus-51Δ::bar+ A+*
TPPAK1.6	*v383*-based hygromycin resistant transformant of ISU-3291
TPPAK2.1	*v394*-based hygromycin resistant transformant of ISU-3291
RPPAK10.1	*rid; Sk-3+ i383Δ::hph+; mus-51? A+* (offspring of TPPAK1.6 × F2-26)
RPPAK10.6	*rid; Sk-3+ i383Δ::hph+; mus-51? A+* (offspring of TPPAK1.6 × F2-26)
RPPAK10.10	*rid; Sk-3+ i383Δ::hph+; mus-51? a+* (offspring of TPPAK1.6 × F2-26)
RPPAK10.21	*rid; Sk-3+ i383Δ::hph+; mus-51? a+* (offspring of TPPAK1.6 × F2-26)
RPPAK10.23*	*rid; Sk-3+ i383Δ::hph+; mus-51? A+* (offspring of TPPAK1.6 × F2-26)
RPPAK10.26*	*rid; Sk-3+ i383Δ::hph+; mus-51? a+* (offspring of TPPAK1.6 × F2-26)
RPPAK11.1	*rid; Sk-3+ i394Δ::hph+; mus-51? A+* (offspring of TPPAK2.1 × F2-26)
RPPAK11.2	*rid; Sk-3+ i394Δ::hph+; mus-51? a+* (offspring of TPPAK2.1 × F2-26)
RPPAK11.3*	*rid; Sk-3+ i394Δ::hph+; mus-51? A+* (offspring of TPPAK2.1 × F2-26)
RPPAK11.4*	*rid; Sk-3+ i394Δ::hph+; mus-51? A+* (offspring of TPPAK2.1 × F2-26)
RPPAK11.5*	*rid; Sk-3+ i394Δ::hph+; mus-51? a+* (offspring of TPPAK2.1 × F2-26)
RPPAK11.12	*rid; Sk-3+ i394Δ::hph+; mus-51? A+* (offspring of TPPAK2.1 × F2-26)

*Genotype predicted by lineage (not genotyped by PCR). The *rid*, *fl, mus-51,* and *sad-2* alleles have been described previously (Perkins et al. 2000; Freitag et al. 2002; Ninomiya et al. 2004; Shiu et al. 2006; Smith et al. 2016)

Table 2 Primers used in this study

Name	Sequence (5′ > 3′)	Purpose
Hph-cen-f	AACTGATATTGAAGGAGCATTTTTTGG	Center fragment amplification from Plasmid pTH1256.1 (GenBank: MH550659.1)
Hph-cen-r	AACTGGTTCCCGGTCGGCAT	
V0375-A	ATCACGGGGACTTGGCTCCATGC	Left flank amplification for v383 from RDGR170.3 genomic DNA
V0375-B	AAAAAATGCTCCTTCAATATCAGTTCCCTCTTTCCCTTCCCTTCCCCGT	
V0383-C	GAGTAGATGCCGACCGGGAACCAGTTTGAGCTACAGCACGCTTTTCCAGC	Right flank amplification for v383 from RDGR170.3 genomic DNA
V0383-D	CGATGGACGCGCACTAACCTGGG	
V0375-E	ACCTCATGTCTCGGTGAAGGGCG	Nested amplification of v383 left flank, center fragment, and v383 right flank fusion product; *i383+* and *i383Δ* genotyping
V0383-F	CTGGGTGGGGTGGTGTTTCGCTG	
V0394-A	TCCAAAGGGAAGGACCGGGCACA	Left flank amplification for v394 from RDGR170.3 genomic DNA
V0394-B	AAAAAATGCTCCTTCAATATCAGTTCGTGAGCCGGAGCAGTCGTCGTA	
V0394-C	GAGTAGATGCCGACCGGGAACCAGTTGCCCCAGTTCTTCCTGCCTGCTG	Right flank amplification for v394 from RDGR170.3 genomic DNA
V0394-D	GACGCGCACTAACCTGGGTGGG	
V0394-E	GGGACAGAGAGTGGCGTCTGCCT	Nested amplification of v394 left flank, center fragment, and v394 right flank fusion product; *i394+* and *i394Δ* genotyping
V0394-F	CACTGCCGGAAACGCACTGGTGT	

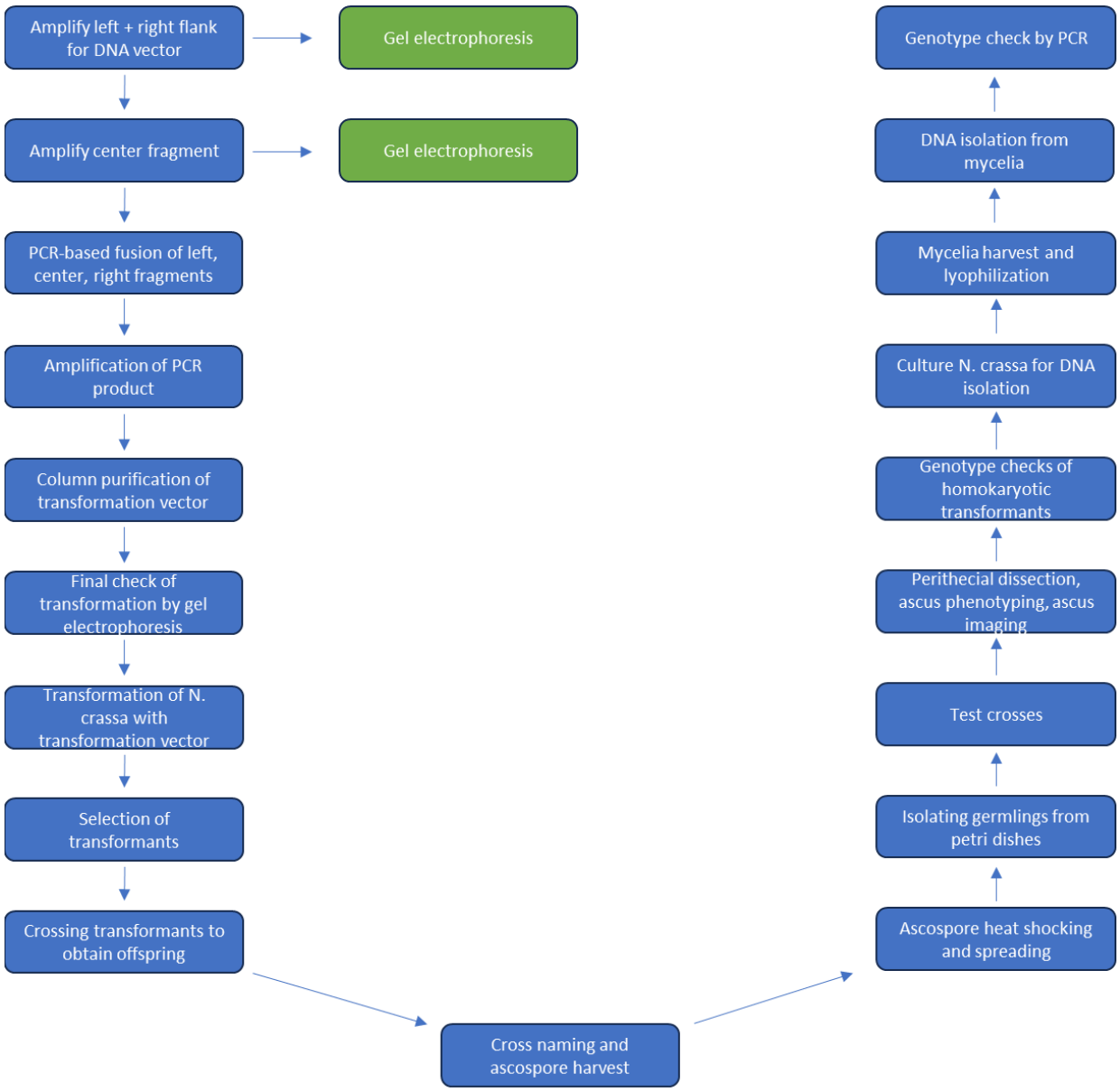

Figure 1 Methods Summary.

>V383 left flank
ATCACGGGGACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTG
CTCGAATTCACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCTGGTAA
TACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGA
CGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAG
AAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTT
CCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTC
CAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAG
AATTTGGATGGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAG
GCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGC
CCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAA
GGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGA
CCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCT
AGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGA
CGGGGAAGGGAAGGGAAAGAGGG

>V394 left flank
TCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACACTAACGAGCGATTT
TGCCACCTAGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCCTACCACGCTTCTTTTTC
CGTCCACCGGCTCTTGGAGTACCGTACATACCTCAAACACTTCATTCCACCCTGTTCTGGAAT
TGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGG
AAGGAGGAGAGGTACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGG
ACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTC
ACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCT
CATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAAC
CCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAG
GCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAA
AGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCA
AGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGAT
GGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGA
TGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG

Figure 2 Transformation vector left flanks. **(Top)** The 842 bp sequence of the v383 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0375-A and V0375-B from RDGR170.3 genomic DNA. **(Bottom)** The 872 bp sequence of the v394 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0394-A and V0394-B from RDGR170.3 genomic DNA.

>V383 right flank
TGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCC
CCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTT
TTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCC
GCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTG
GCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTC
TCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTC
TCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGG
ATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCT
GGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAA
TCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGG
CCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTA
TCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCG

>V394 right flank
GCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGT
AAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTC
ACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTAC
GTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACG
ACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTT
CCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTT
TTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGG
CCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGC
TGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATAT
TCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCC
TCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGG
GGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCC
CTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGA
AATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGC
GGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGAT
TATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTC

Figure 3 Transformation vector right flanks. **(Top)** The 735 bp sequence of the v383 right flank is shown. This sequence was PCR-amplified with primers V0383-C and V0383-D from RDGR170.3 genomic DNA. **(Bottom)** The 984 bp sequence of the v394 right flank is shown. This sequence was PCR-amplified with primers V0394-C and V0394-D from RDGR170.3 genomic DNA.

>v383 and v394 center fragment
AACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGC
CTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTC
TTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTG
CGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCT
TTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACT
CACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCA
GCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCT
GCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATC
GGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTG
CATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGG
GTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGC
GATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGT
CGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGT
GCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGA
CTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCC
GTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGG
ATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGT
TGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGG
AGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTG
TGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATA
GAGTAGATGCCGACCGGGAACCAGTT

Figure 4 The v383 and v394 center fragment. The 1412 bp sequence of the v383 and v394 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 an *Aspergillus nidulans trpC* promoter upstream of a 1023 bp *hph*+ coding region.

>Interval *i383*
GACGGAGAAAACGAC

>Interval *i394*
CCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGAT

Figure 5 Sequences of DNA intervals *i383* and *i394*. **(Top)** Interval *i383* contains 15 base pairs. The sequence is shown. **(Bottom)** Interval *i394* contains 37 base pairs. The sequence is shown. DNA deletion vectors v383 and v394 were designed to replace *i383* and *i394*, respectively, with *hph*+.

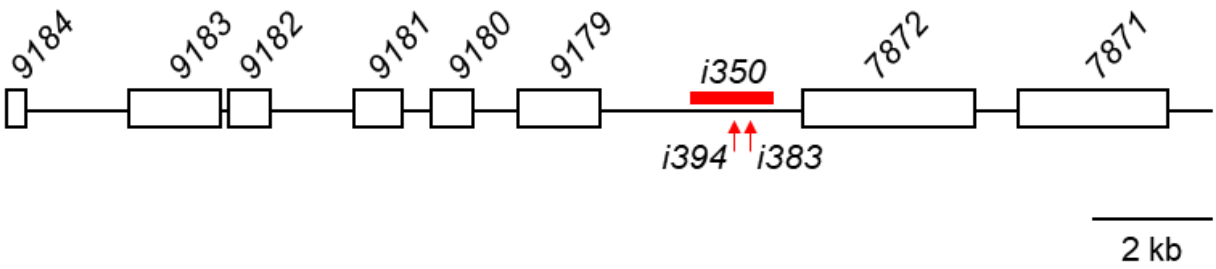

Figure 6 Locations of *i383* and *i394*. A diagram of of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown with white rectangles marking the locations of protein coding genes (*N. crassa* database numbers for each predicted gene are indicated). The location of intervals *i350, i383,* and *i394* are indicated with red bars or arrows.

>*i383Δ::hph+* PCR product, predicted sequence, primers V375-E and V383-F
ACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAACCCTT
TCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAGGCCTTTGAGGAG
GAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAAAGGAGCATGAACAGGCAACT
GCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGG
AGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTCCCATGCGCACTTGCACATCATGATCATGACA
CCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTC
CGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTC
AAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTC
GGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTAC
CAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGG
GAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTT
GGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTC
AGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGA
CATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCG
GTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAG
CATCCAA**ATG**AAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCG
TCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGA
TATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATC
GGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCC
GCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCG
GAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGG
AATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAA
CTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGAC
TGCCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCAT
AACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCT
GGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGA
TCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAA
TTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGC
GTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGA
AACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAA**TAG**AGTAGATGCCGACCGGGAACCAGTTTGAGCTA
CAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCT
CGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTT
TCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCG
TTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGG
GTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGAC
GGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCC
CGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCT
GGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGAT
CGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCA
ATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAG

Figure 7 Predicted PCR product sequence for the *Sk-3 i383Δ::hph+* genotype. The predicted sequence of DNA amplified with primers V0375-E and V0383-F from a template consisting of *Sk-3 i383Δ::hph+* genomic DNA is shown. The start and stop codons of *hph+* are shown with white font on black background. The length of the sequence is 2840 bp.

>*i383+* PCR product, predicted sequence, primers V375-E and V383-F
ACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAACCCTT
TCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAGGCCTTTGAGGAG
GAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAAAGGAGCATGAACAGGCAACT
GCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGG
AGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTCCCATGCGCACTTGCACATCATGATCATGACA
CCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTC
CGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTC
AAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTC
GGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTAC
CAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGG
GGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATT
CCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTT
TTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCC
CTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCC
GCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTT
CTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAAC
AGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGC
TGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTAT
TCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTC
CAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTAT
CAGCGAAACACCACCCCACCCAG

Figure 8 Predicted PCR product sequence for the *Sk-3 i383+* genotype. The predicted sequence of DNA amplified with primers V0375-E and V0383-F from a template consisting of *Sk-3 i383+* genomic DNA is shown. The sequence is 1443 bp. Interval *i383* is indicated with red font.

>*i394Δ::hph+* PCR product, predicted sequence, primers V394-E and V394-F
GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGG
TACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAA
ATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTT
GAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGA
CTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGA
ACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCG
TTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCC
CCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTC
CCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGAACTGATATTGAAGGAGCATTTTTTGGGCTT
GGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATT
TGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGC
GCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATA
ACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCT
TTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAA**ATG**AAAAAGCCTGAACTCACCGCGA
CGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAA
GAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGG
TTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACA
TTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTG
CCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCT
TAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCA
TATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTC
GCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCATGCGGA
TTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGT
TCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAG
ACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGTATATGCTCCGCAT
TGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGAT
GCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTC
TGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAA
GGAA**TAG**AGTAGATGCCGACCGGGAACCAGTTGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTT
CTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGC
CGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGA
GGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACG
ACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGC
CCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCA
TCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCA
TGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGG
GCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCAT
TTCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCA
CCTCCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCC
CACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAA
ATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTG

Figure 9 Predicted PCR product sequence for the *Sk-3 i394Δ::hph+* genotype. The predicted sequence of DNA amplified with primers V0394-E and V0394-F from a template consisting of *Sk-3 i394Δ ::hph+* genomic DNA is shown. The start and stop codons of *hph+* are shown with white font on black background. The length of the sequence is 2956 bp.

>*i394+* PCR product, predicted sequence, primers V394-E and V394-F

GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGG
TACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAA
ATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTT
GAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGA
CTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGA
ACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCG
TTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCC
CCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTC
CCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG**CCCGTCTCGCTCTTCCCGGGCCTTTTGTCAG**
GCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAA
AAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAAT
TGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGG
AGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGC
TCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCT
CTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAG
CATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTC
GGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTT
TATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCT
CGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGACCTTG
CCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTC
CGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACAC
CAGTGCGTTTCCGGCAGTG

Figure 10 Predicted PCR product sequence for the *Sk-3 i394+* genotype. The predicted sequence of DNA amplified with primers V0394-E and V0394-F from a template consisting of *Sk-3 i394+* genomic DNA is shown. The sequence of Interval *i394* is indicated with red font. The length of the sequence is 1581 bp.

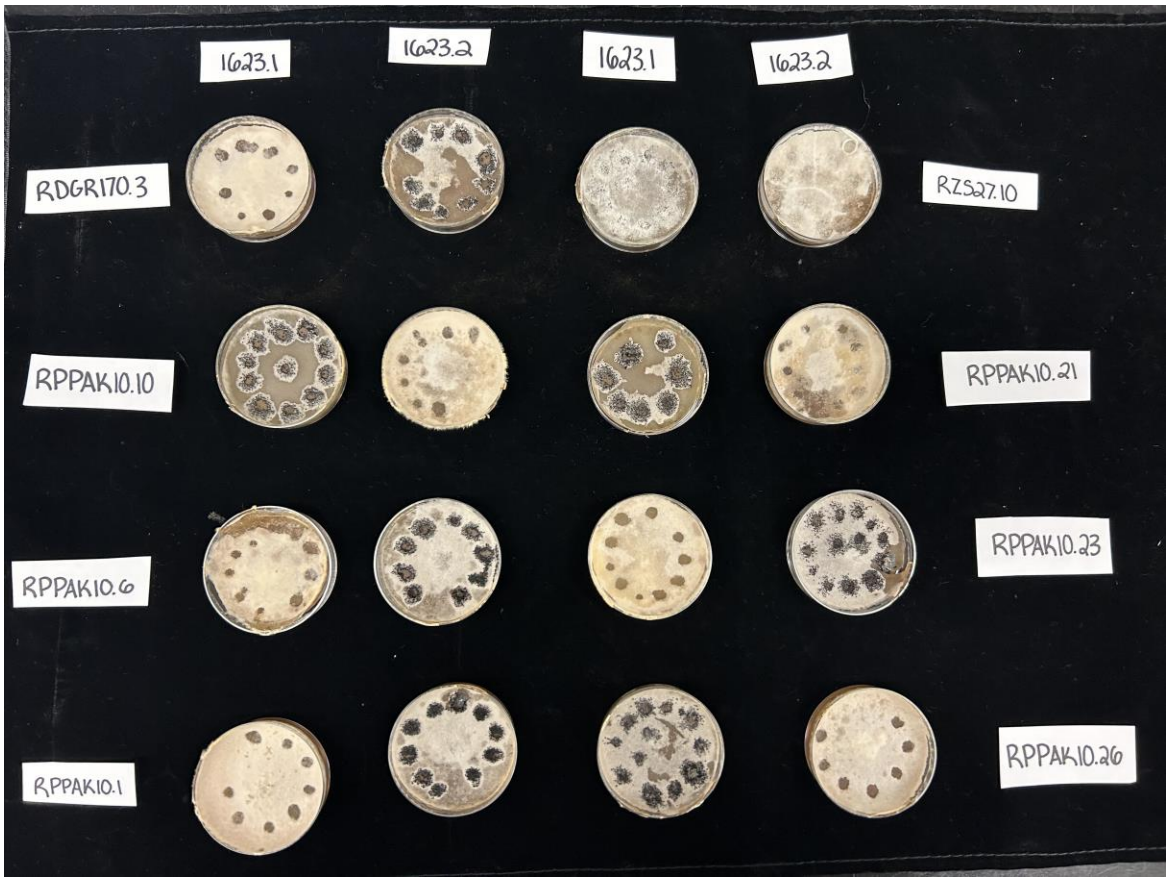

Figure 11 RPPAK10 crossing assays. Hygromycin resistant offspring from cross RPPAK10 (TPPAK1.6 × RTH1005.2) were isolated and examined for spore killing ability by crossing as males to strains RTH1623.1 and RTH1623.2. Strains RZS27.10 (*Sk-S*) and RDGR170.3 (*Sk-3*) were used as control male strains for no spore killing and spore killing, respectively. Images are of crossing plates after fruiting body development, approximately three weeks post fertilization.

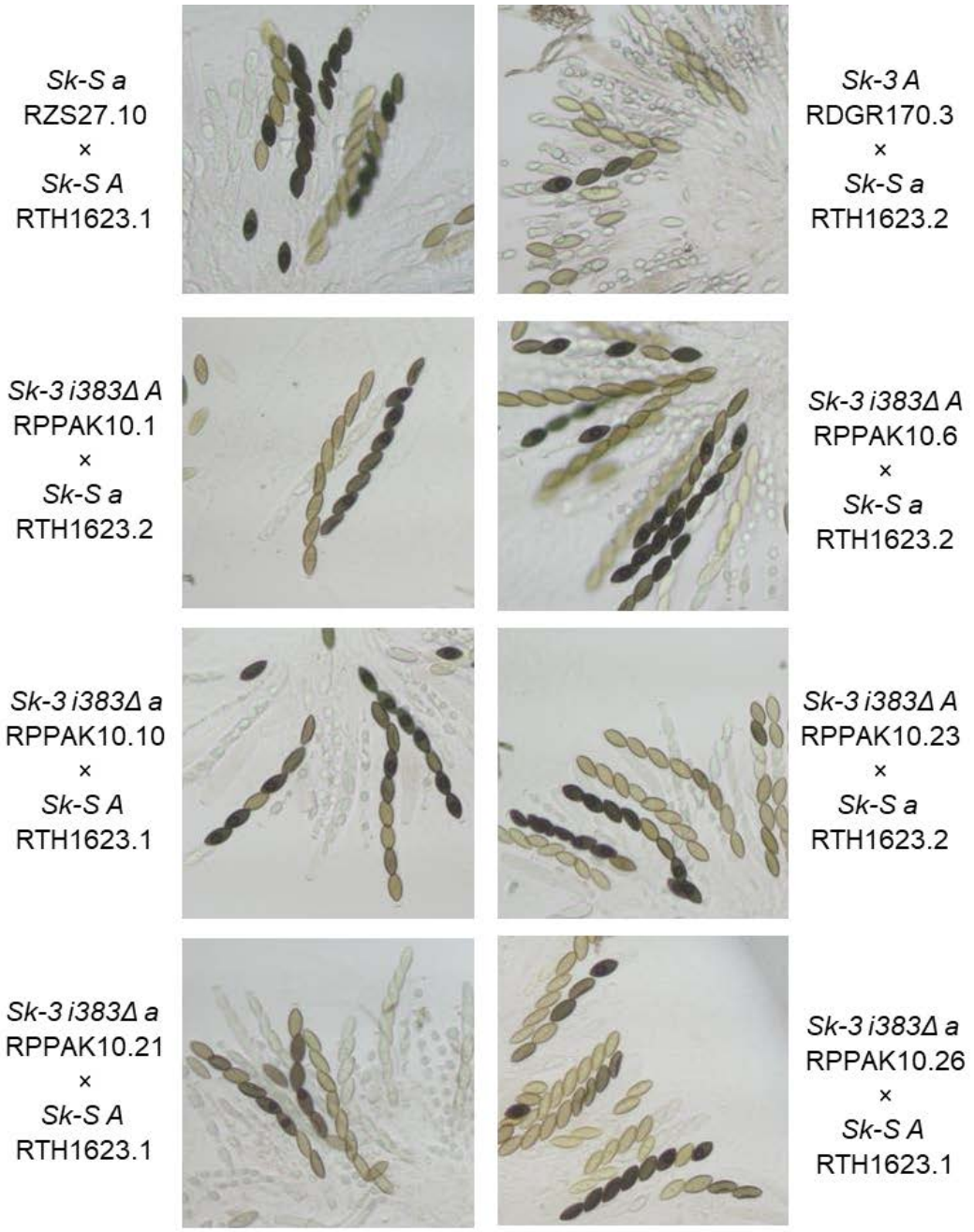

Figure 12 Ascus phenotype summary. Asci were dissected from perithecia of eight crosses 11 to 13 days post fertilization (dpf) and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *i383* disrupts spore killing.

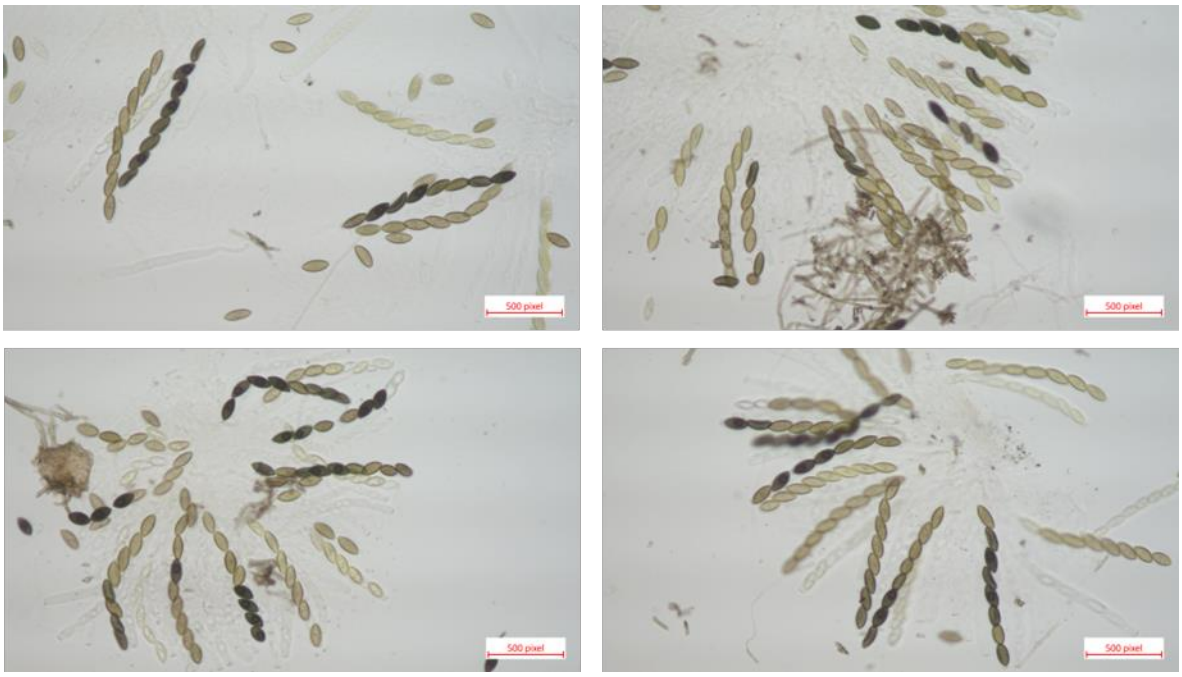

Figure 13 Ascus phenotypes. Asci were dissected from perithecia of RPPAK10.1 × RTH1623.2 on Day 12 post fertilization and imaged under magnification.

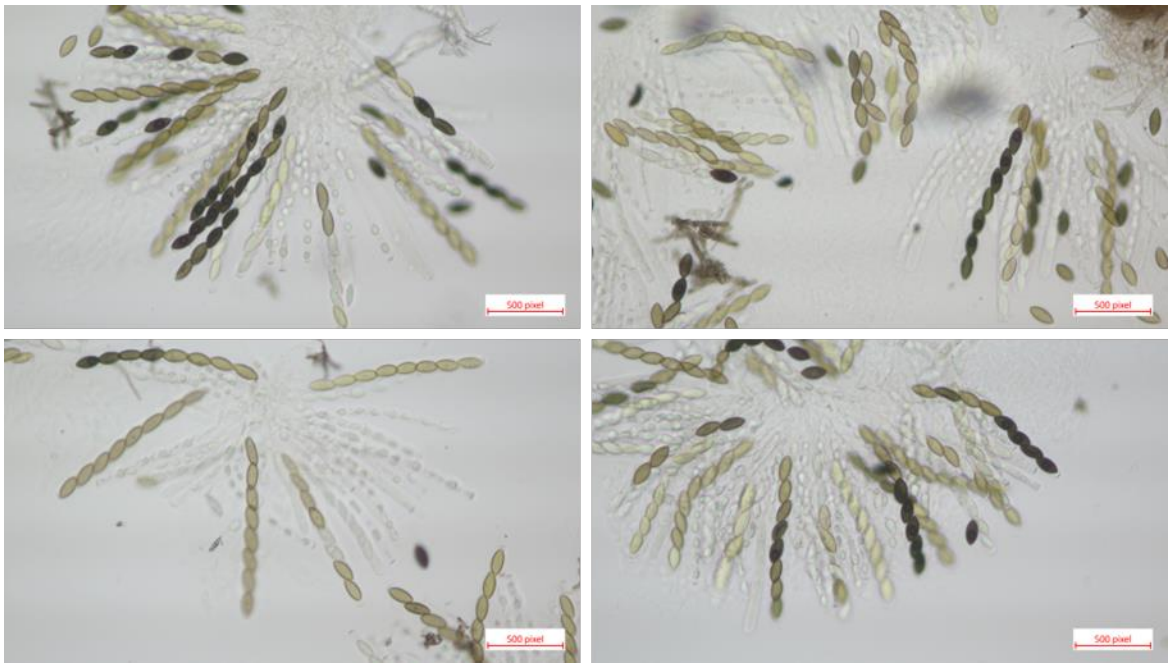

Figure 14 Ascus phenotypes. Asci were dissected from perithecia of RPPAK10.6 × RTH1623.2 on Day 12 post fertilization and imaged under magnification.

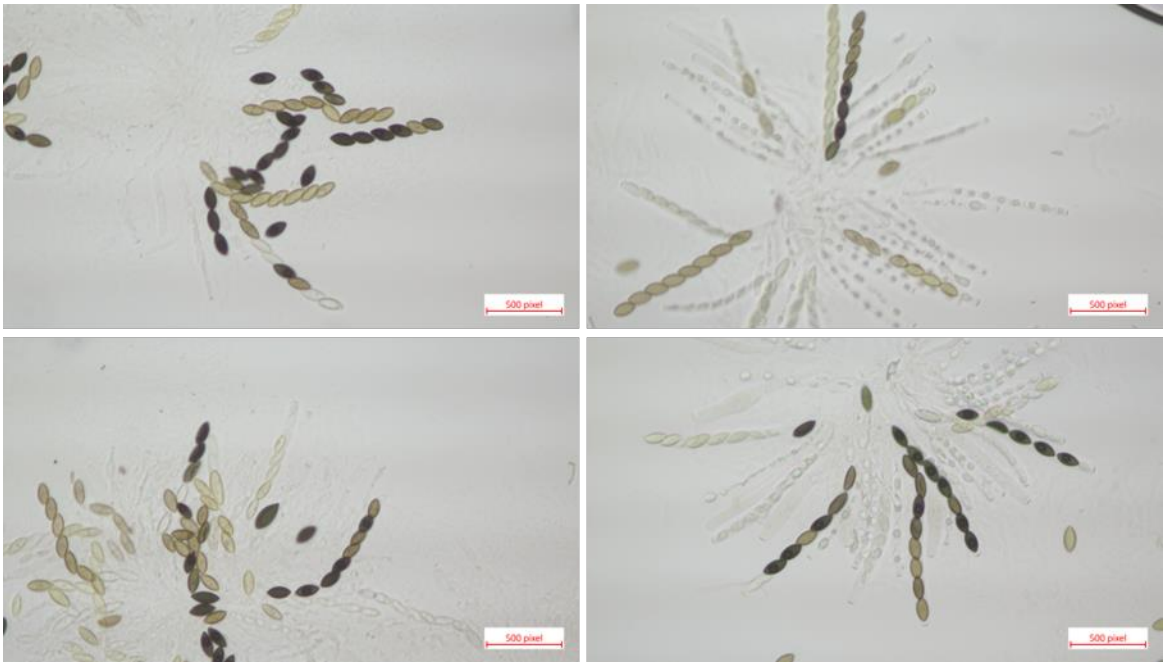

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RPPAK10.10 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

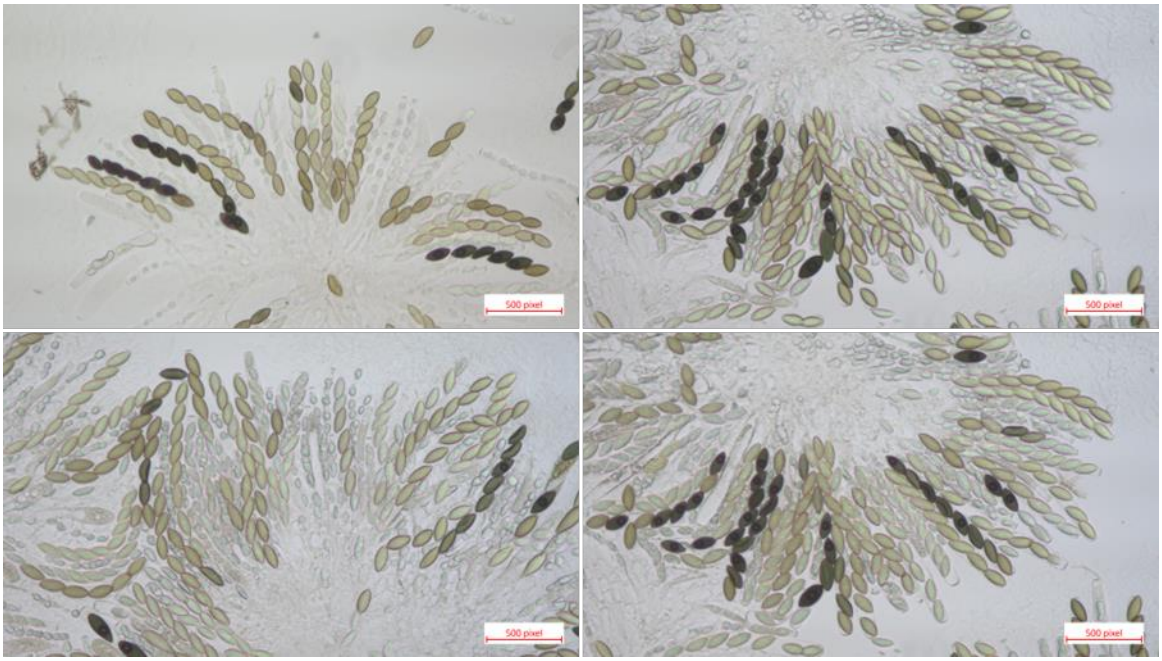

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RPPAK10.23 × RTH1623.2 on Day 12 post fertilization and imaged under magnification.

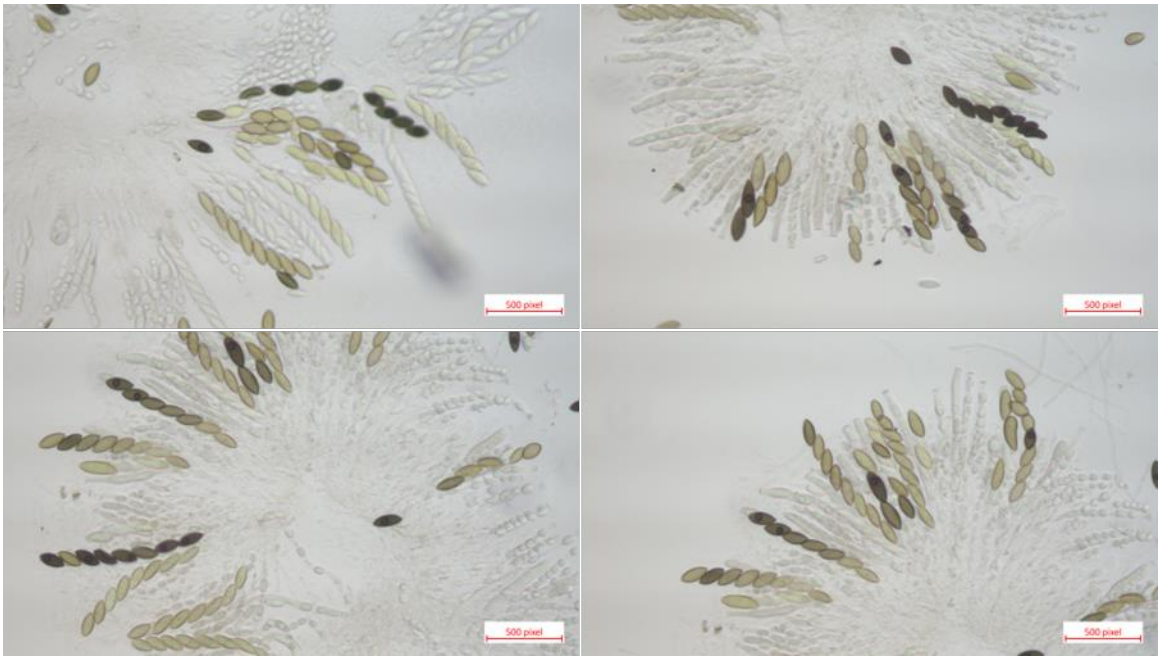

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RPPAK10.21 × RTH1623.1 on Day 13 post fertilization and imaged under magnification.

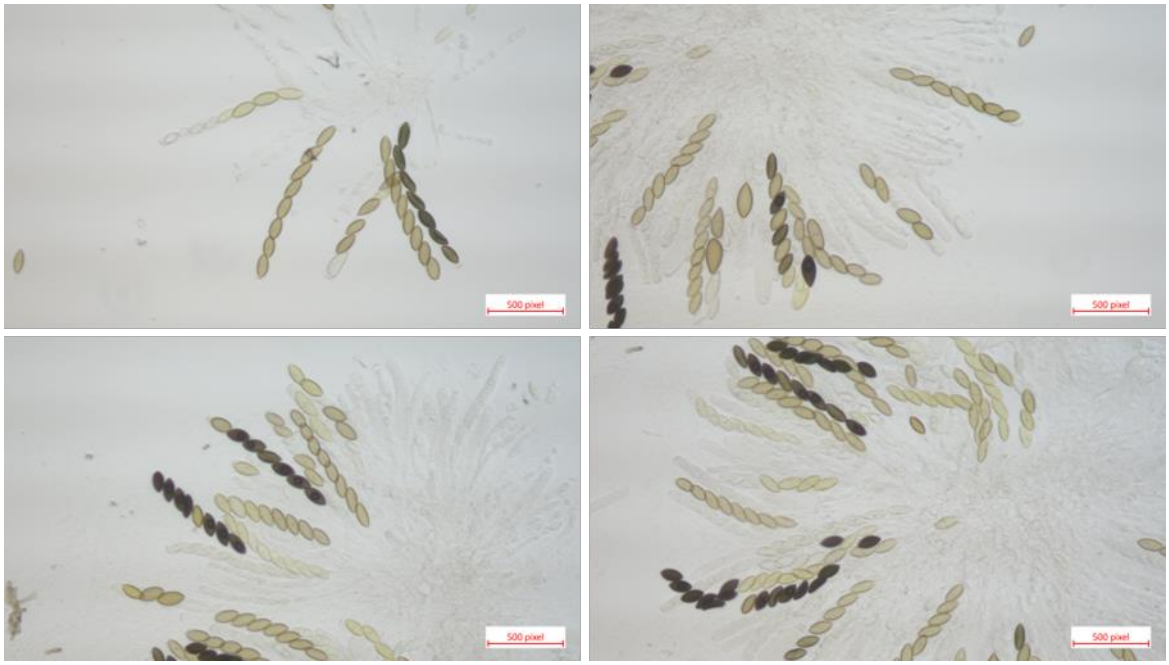

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RPPAK10.26 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

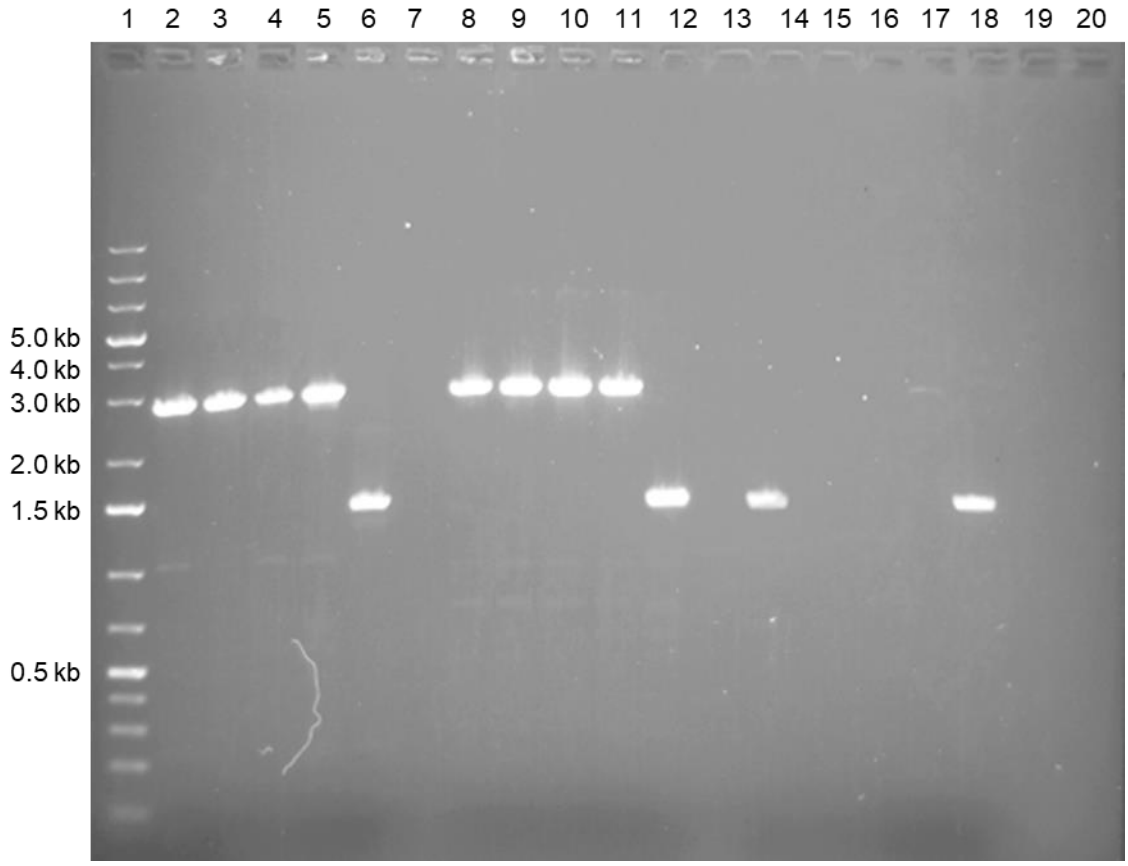

Figure 19 RPPAK10 genotyping. DNA was isolated from test strains and used in a PCR-based genotyping assay with primers V0375-E and V0383-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product lengths for the *i383+* and *i383Δ* genotypes are 1443 bp and 2840 bp, respectively. Lane 1 contains 0.5 µg of GeneRuler 1 Kb Plus DNA ladder (ThermoFisher). DNA templates for each PCR reaction are as follows: Lane 2, RPPAK10.1; Lane 3, RPPAK10.6; Lane 4, RPPAK10.10; Lane 5, RPPAK10.21; and Lane 6, RDGR170.3; Lanes 7–20) Not applicable. These results show that test strains RPPAK10.1, RPPAK10.6, RPPAK10.10, and RPPAK10.21 have the *i383Δ* genotype.

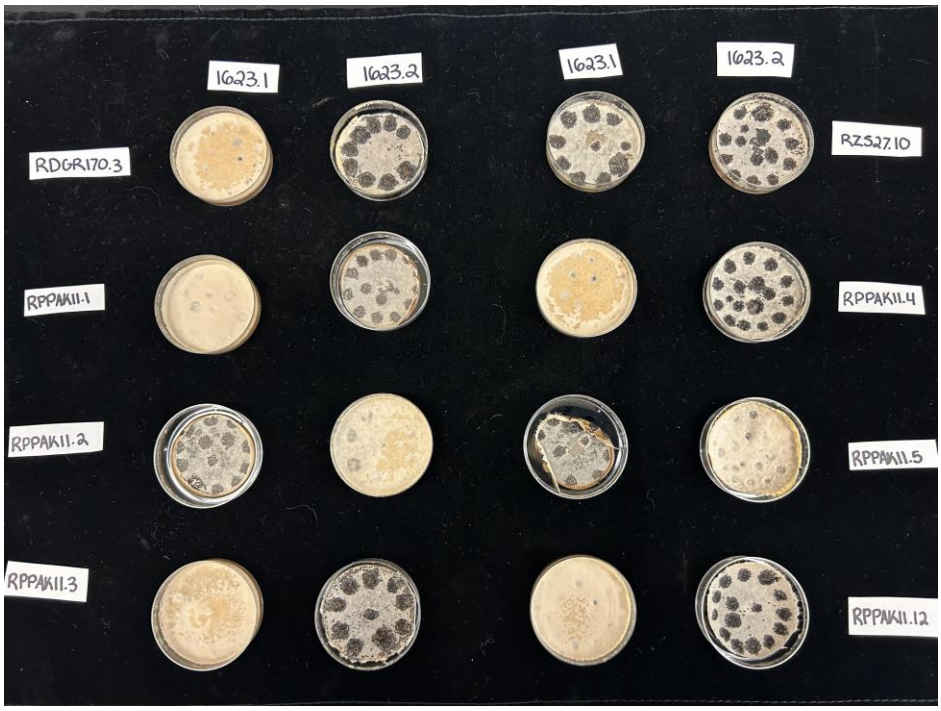

Figure 20 RPPAK11 crossing assay. Hygromycin resistant offspring from cross RPPAK11 (TPPak2.1 × RTH1005.2) were isolated and examined for spore killing ability by crossing as males to strains RTH1623.1 and RTH1623.2. Strains RZS27.10 (*Sk-S*) and RDGR170.3 (*Sk-3*) were used as control male strains for no spore killing and spore killing, respectively. Images are of crossing plates after fruiting body development, approximately three weeks post fertilization.

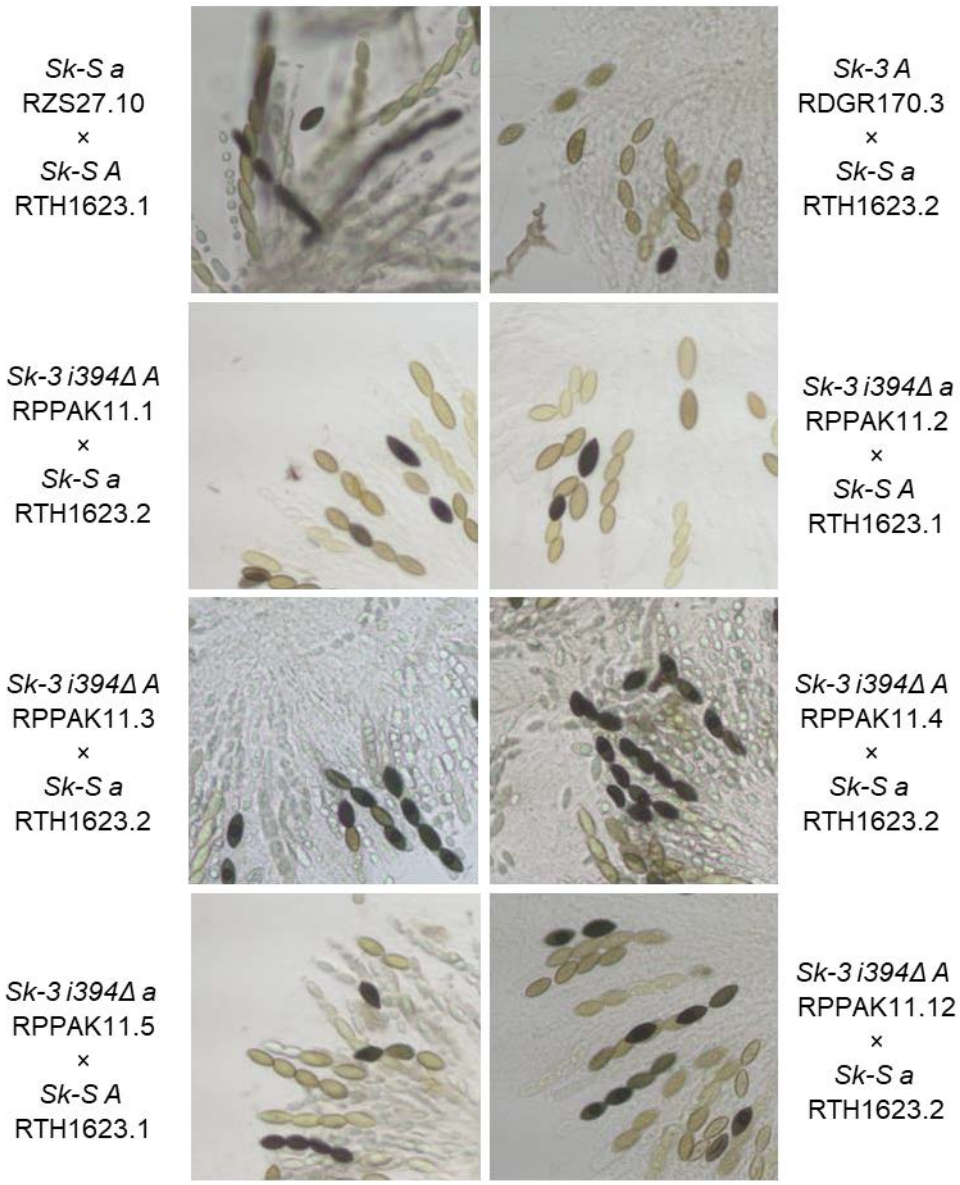

Figure 21 Ascus phenotype summary. Asci were dissected from perithecia of eight crosses 13 dpf and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *i394* does not disrupt spore killing.

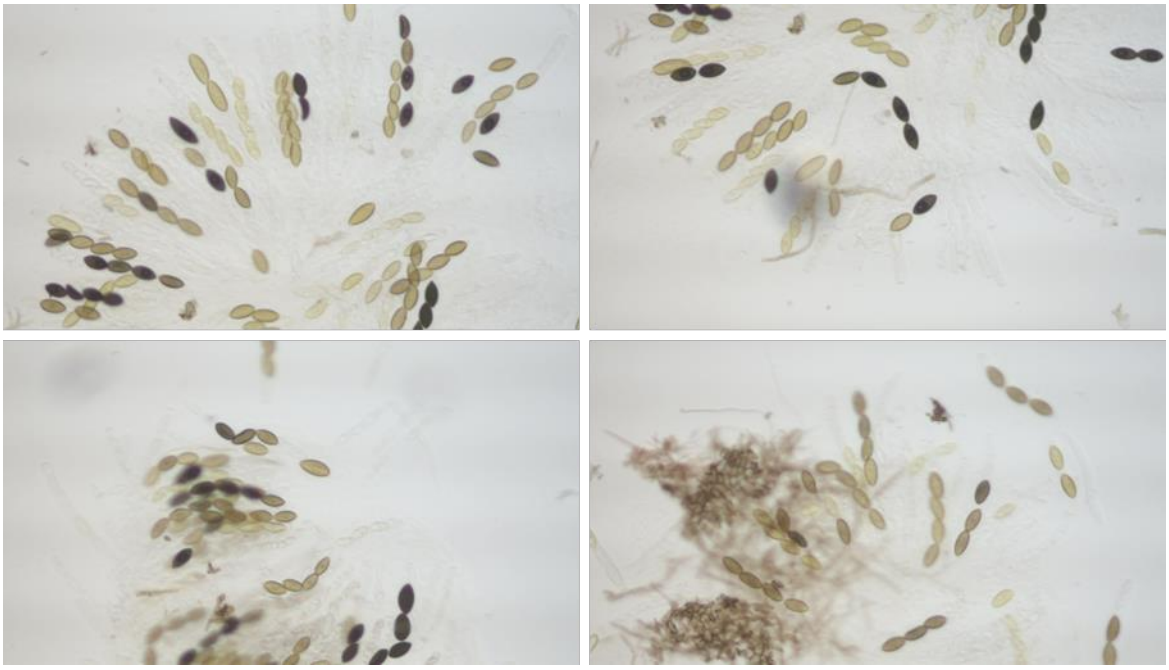

Figure 22 Ascus phenotypes. Asci were dissected from perithecia of RPPAK11.1 × RTH1623.2 on Day 13 post fertilization and imaged under magnification.

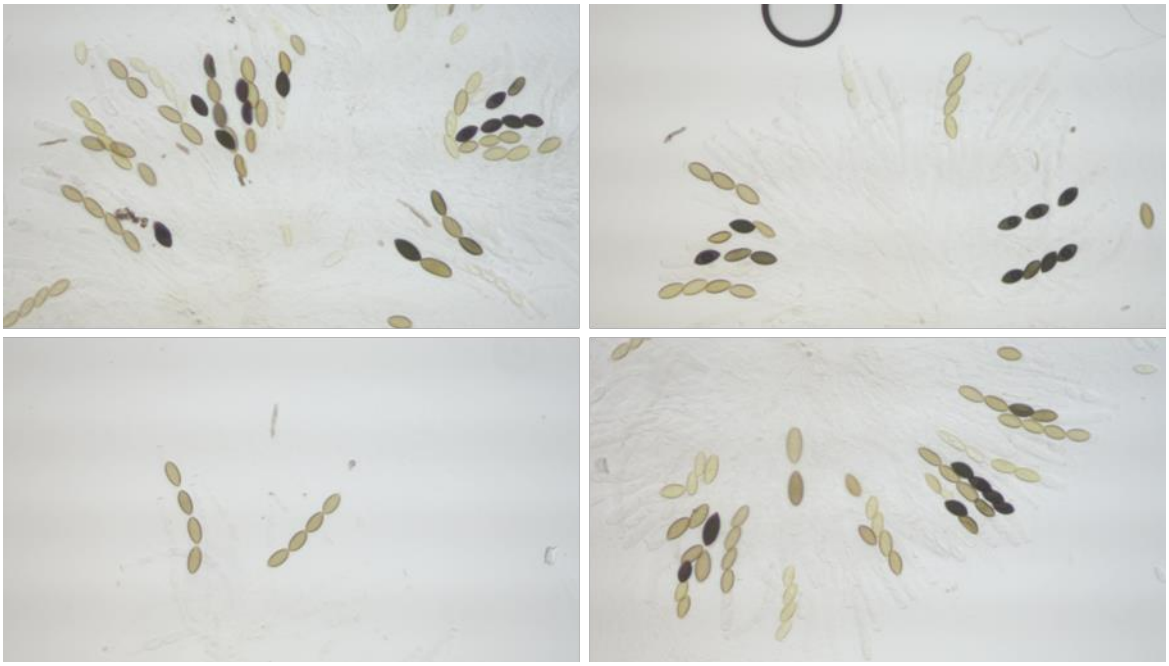

Figure 23 Ascus phenotypes. Asci were dissected from perithecia of RPPAK11.2 × RTH1623.1 on Day 13 post fertilization and imaged under magnification.

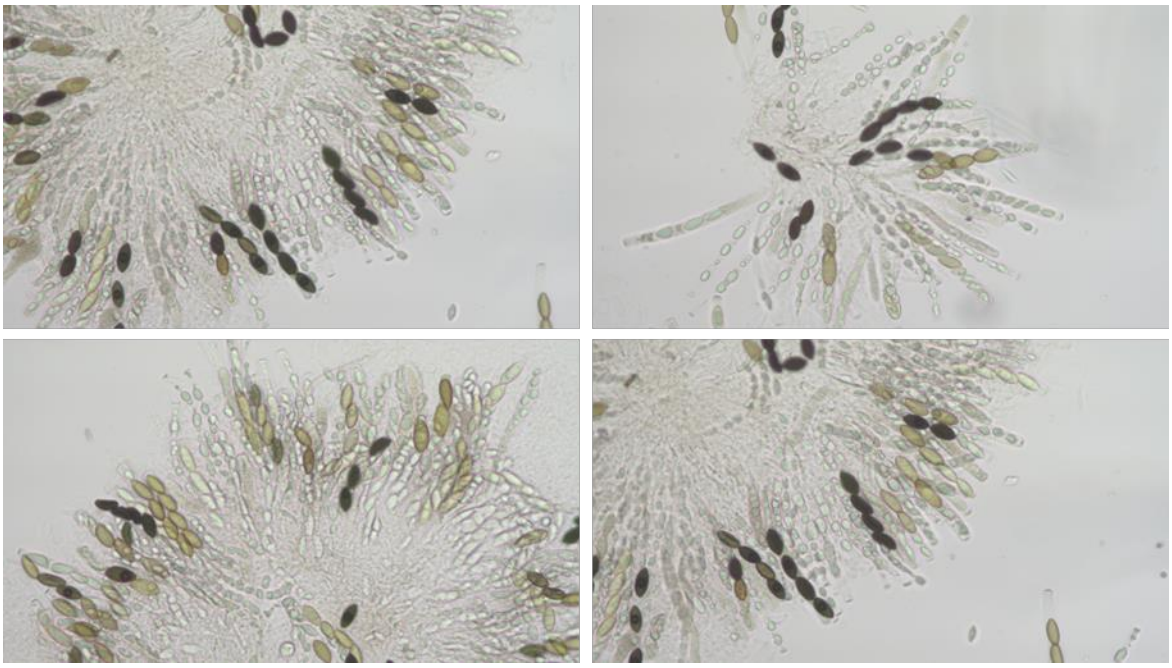

Figure 24 Ascus phenotypes. Asci were dissected from perithecia of RPPAK11.3 × RTH1623.2 on Day 13 post fertilization and imaged under magnification.

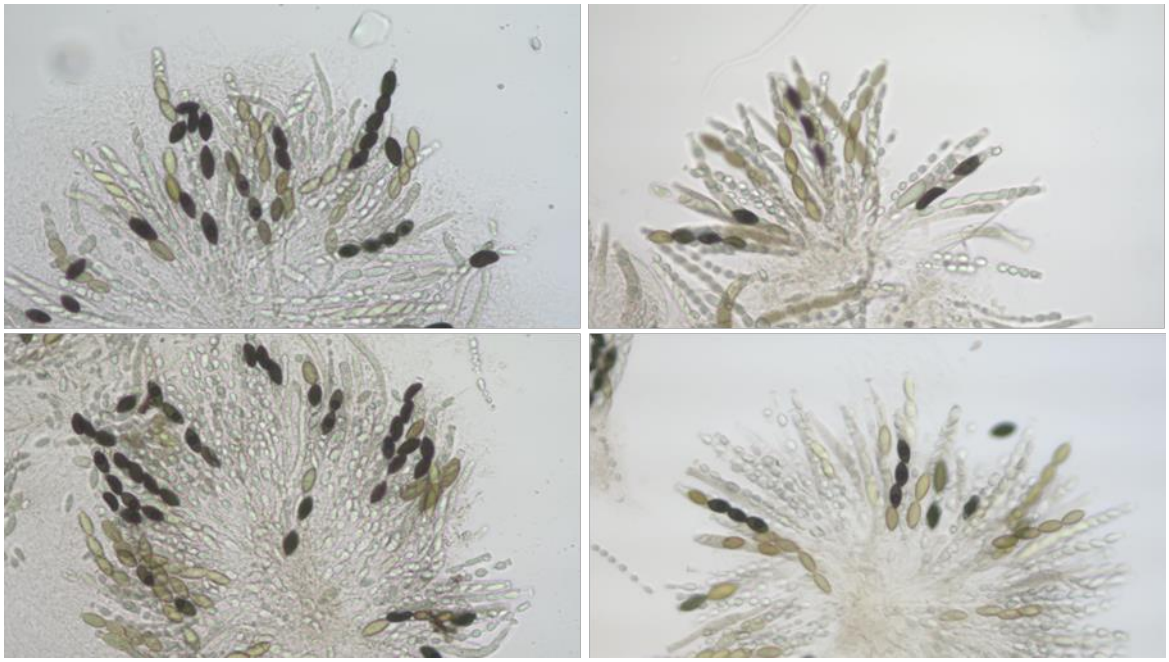

Figure 25 Ascus phenotypes. Asci were dissected from perithecia of RPPAK11.4 × RTH1623.2 on Day 13 post fertilization and imaged under magnification.

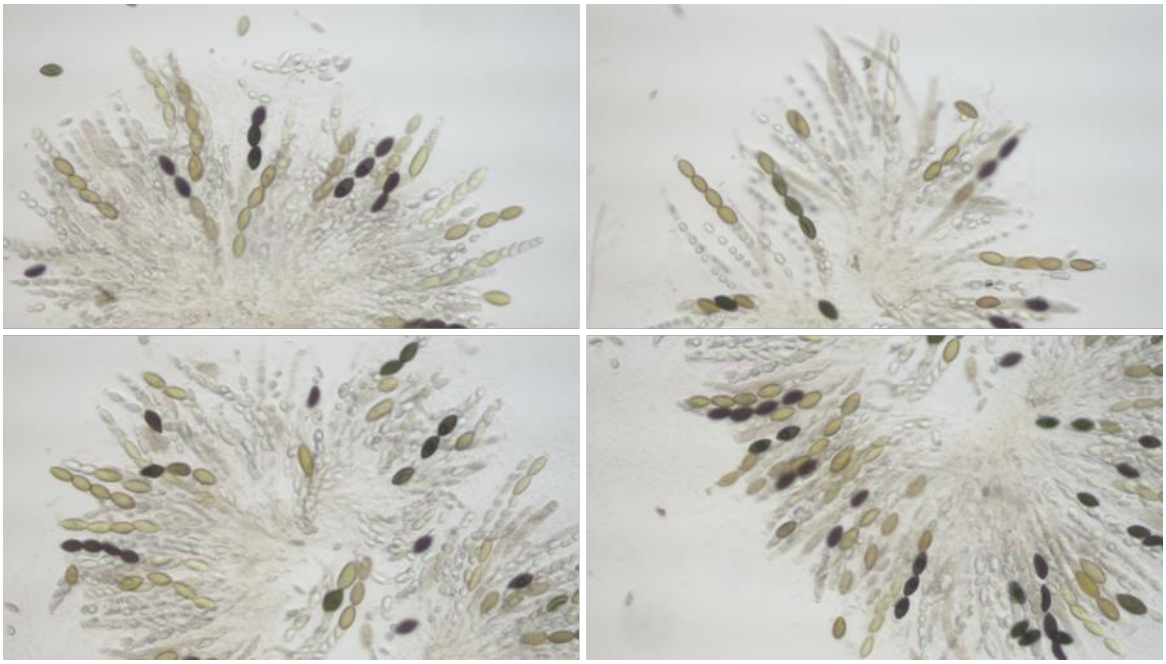

Figure 26 Ascus phenotypes. Asci were dissected from perithecia of RPPAK11.5 × RTH1623.1 on Day 13 post fertilization and imaged under magnification.

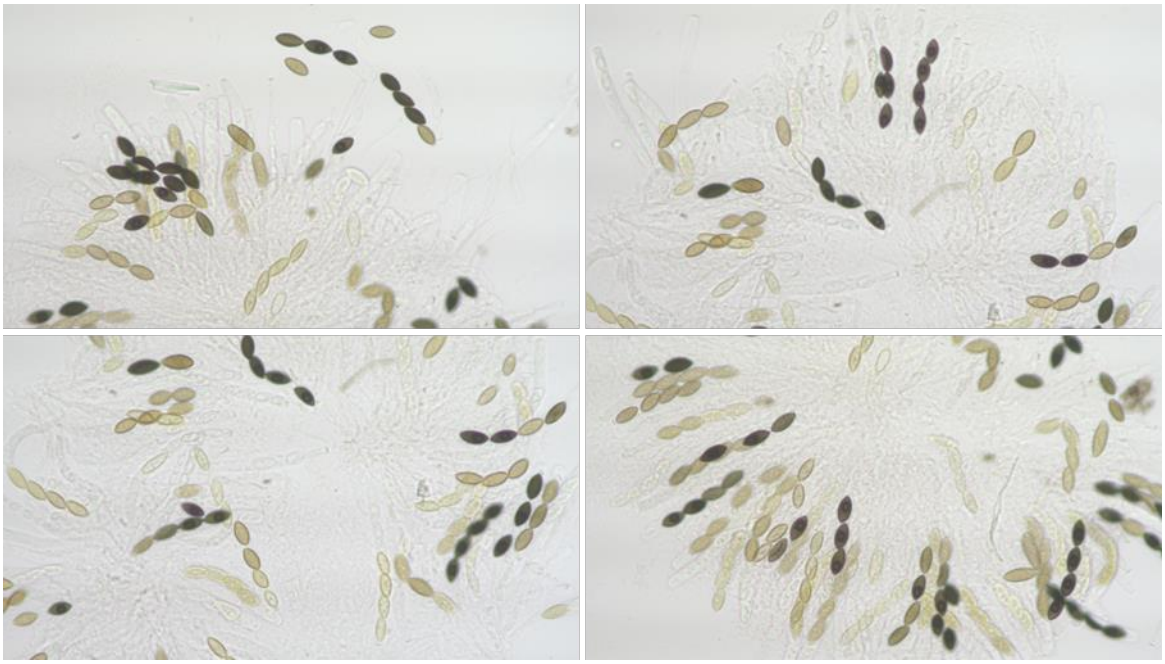

Figure 27 Ascus phenotypes. Asci were dissected from perithecia of RPPAK11.12 × RTH1623.2 on Day 13 post fertilization and imaged under magnification.

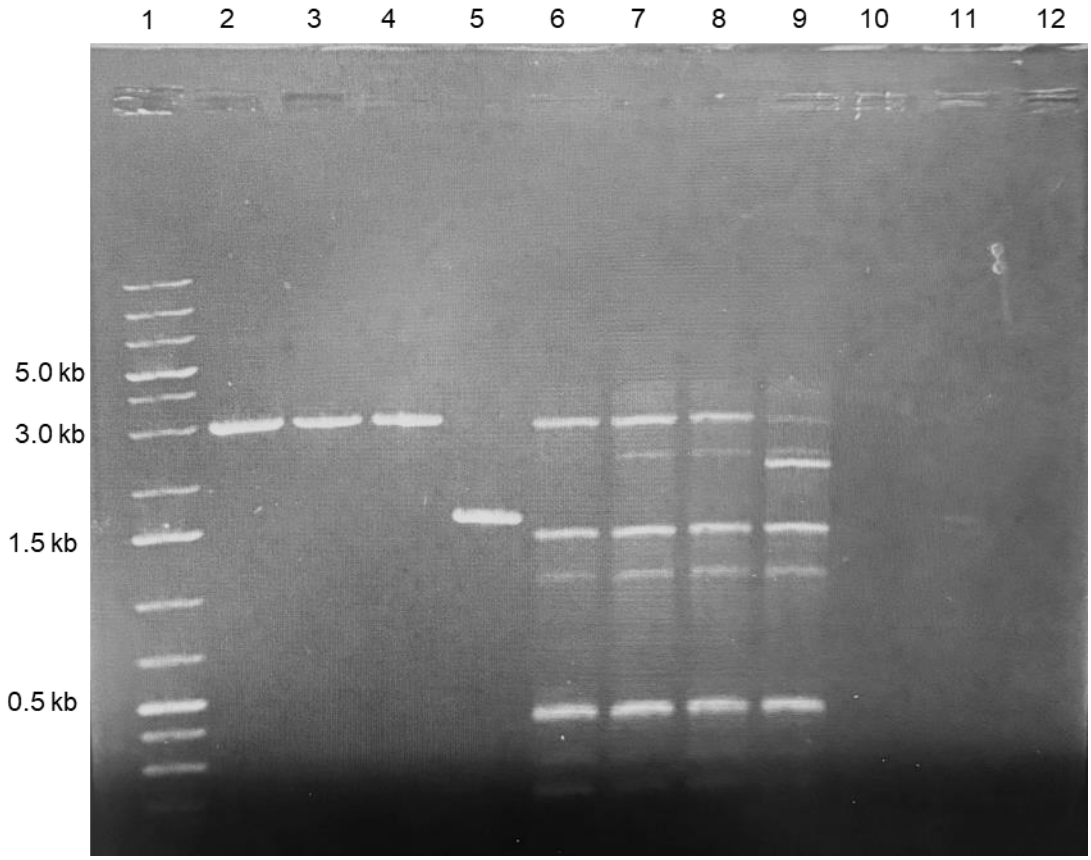

Figure 28 RPPAK11 genotyping. DNA was isolated from test strains and used in a PCR-based genotyping assay with primers V0394-E and V0394-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product length for the *i394+* and *i394Δ* genotypes are 1581 bp and 2956 bp, respectively. Lane 1 contains 0.5 µg of GeneRuler 1 Kb Plus DNA ladder (ThermoFisher). DNA templates for each PCR reaction are as follows: Lane 2, RPPAK11.1; Lane 3, RPPAK11.2; Lane 4, RPPAK11.12; and Lane 5, RDGR170.3; Lanes 6–20) Not applicable. These results show that test strains RPPAK11.1, RPPAK11.2, and RPPAK11.12 have the *i394Δ* genotype.