Spring 2024

Investigating the Role of DNA Interval v377 in Spore Killing by Neurospora crassa Sk-3

Carolina Okleiteris
Illinois State University

INVESTIGATING THE ROLE OF DNA INTERVAL *V377* IN SPORE KILLING BY *NEUROSPORA CRASSA SK-3*

Carolina Okleiteris

40 Pages May 2024

Neurospora crassa is a fungus that serves as a model organism for genetic research. *N. crassa Spore killer-3* (*Sk-3*) is a genetic element transmitted to offspring through spore killing. *Sk-3* is located on Chromosome III and it is thought to require two genes for spore killing. These two genes are the poison gene, for killing, and the antidote gene, for resistance to killing. While the *Sk-3* resistance gene has been identified (*rsk*), the *Sk-3* killer gene has not. The primary goal of this study is to help identify the killer gene by investigating the role of a DNA interval called *v377* in spore killing. To determine if this interval is required for spore killing, a DNA deletion vector (Vector v377) was constructed and used to replace the *v377* interval with a hygromycin resistance gene in strain RDGR170.3. My results demonstrate that *v377* is required for spore killing. The possibility that *v377* is within a gene required for spore killing, or a regulatory element that controls spore killing, is discussed.

INVESTIGATING THE ROLE OF DNA INTERVAL *V377* IN SPORE KILLING BY *NEUROSPORA CRASSA SK-3*

CAROLINA OKLEITERIS

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

2024

INVESTIGATING THE ROLE OF DNA INTERVAL *V377* IN SPORE KILLING BY *NEUROSPORA CRASSA SK-3*

CAROLINA OKLEITERIS

COMMITTEE MEMBERS

Dr. Thomas Hammond (Chair)

Dr. Marjorie Jones

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Thomas Hammond for offering incredible mentorship and support during my time at Illinois State University. I was offered the opportunity to join his undergraduate lab and was able to gain research experience throughout the past two years. Dr. Hammond always offered guidance and support and made a huge impact during my academic journey. I would also like to thank Dr. Marjorie Jones for serving on my thesis committee. I would also like to thank the members of the Hammond Laboratory, especially my lab group, Makenna Klann and Paulina Paulikas, for always supporting me. Lastly, I would like to thank my parents, as well as my siblings, for believing in me throughout undergrad.

This work was supported by the National Science Foundation (Award Number 200595, Elucidating the mechanism of meiotic drive by mRNA editing-mediated spore killing in Neurospora fungi).

CONTENTS

TABLES

FIGURES

CHAPTER I

INTRODUCTION

Neurospora crassa is a genus of fungus found in climates that are tropical, subtropical, and temperate (Turner 2001). This wide range of living conditions allows for life nearly all over the world. This fungus consists of five different species, with *N. crassa* being the genetic model. Since *N. crassa* has a simple life cycle and characterized genetic traits, it is an ideal model organism for genetic research. *N. crassa* produces asexual spores called macroconidia in addition to sexual spores called ascospores. These allow *N. crassa* to spread to various ecological environments. Meiotic drive occurs when the meiotic process is manipulated to favor a specific allele. This tends to violate Mendel's law of equal segregation. In *N. crassa,* meiotic drive transmits specific genetic elements through ascospores. Meiotic drive in this fungus has been specifically attributed to genetic elements called Spore killers. There are three known Spore killers in Neurospora fungi: *Sk-1*, *Sk-2*, and *Sk-3* (Turner and Perkins 1979). *Sk-3* is the focus of this research. This selfish genetic element is found on Chromosome III of certain *N. crassa* strains and is thought to require a killing (poison) gene and a resistance (antidote) gene to function properly (Hammond 2012). While the resistance gene, *rsk*, has been discovered, the killer gene remains unknown.

Meiotic drive can affect genetic inheritance. When a spore killer strain crosses with a strain that is sensitive to spore killing, only the spores that contain the genes for spore killing survive, while those that do not contain the spore killing genes are killed. The purpose of this study is to generate knowledge on the location of *Sk-3*'s killer gene. Preliminary research has

suggested that the deletion of a 1.3 kb DNA interval called *v350* eliminates spore killing (Rhoades and Hammond, unpublished data). In this study, I investigate the effect of deleting a subinterval of *v350* called *v377.* At the beginning of this study, I hypothesized that deletion of interval *v377* will not disrupt spore killing. Below, I provide evidence that this hypothesis is incorrect, as deletion of *v377* appears to disrupt spore killing.

CHAPTER I

METHODS

In Figure 1, an overview of the steps used to delete the *v377* interval, a DNA interval on Chromosome III in *N. crassa Sk-3* strains, is shown. DNA intervals can be replaced by constructing a DNA replacement vector, which is the DNA element used to deliver a transgene to an organism, and typically includes a hygromycin resistance gene (*hph*) when used to delete DNA in *N. crassa* (Margolin et al. 1997). The left and right flanks of the DNA replacement vector are identical to the flanks of the DNA interval to be replaced in the genome. First, I amplified the Left Flank and the Right Flank with PCR. For Vector v377 (related replacement vectors and DNA intervals share the same name), the left flank was amplified from genomic DNA of strain RDGR170.3 (Table 1) with forward primer V377-A (Table 2) and reverse primer V377-B (Table 2), while the right flank was amplified with forward primer V350-C (Table 2) and reverse primer V350-D (Table 2). During this procedure, in addition to the described primers and DNA template, DNA polymerase buffer, a dNTP solution, sterile water, and Q5 DNA polymerase (New England Biolabs) were used. Two primer mixes were made, one for amplification of the left fragment, and one for amplification of the right fragment. The standard primer mix protocol uses a 1.5 ml MCT tube to hold a solution containing 6.25 µl of a forward primer (100 pmol / µl), 6.25 µl of a reverse primer (100 pmol / µl), and 487.5 µl of sterile water. A 5 µl aliquot of each primer mix is then transferred to PCR tubes, along with 1 µl of DNA template (approximately 10 ng / µl). An enzyme master mix is prepared by adding 67.0 µl of sterile water, 25 µl of DNA polymerase buffer, 2.5 µl of dNTP solution (10 mM), and 0.5 µl of Q5 enzyme. A

thermal cycler was used to perform PCR according to the manufacturer's recommendations for Q5 DNA polymerase.

Gel purification of the PCR products was then performed. An agarose gel was made by mixing 1.8 grams of agarose with 200 ml of 1x TAE buffer in a 1 L flask. This was heated for 1-minute intervals in a microwave until the agarose completely dissolved. A 10 µl aliquot of an ethidium bromide solution (10 mg/ml) was added to the molten agarose, and the flask was set aside to cool before pouring the mixture into a gel tray. To prepare for gel purification of the Left and Right Flanks, 5 µl of 6x loading buffer was added to the PCR reactions. The PCR reactions were then then loaded into the gel, which was run at 120 V for 90 minutes. The PCR product was visualized with a UV transilluminator, excised from the gel with a clean razor blade, and placed into a MCT tube. An IBI Scientific Gel Extraction Kit was then used to isolate DNA from the gel slice. Specifically, 500 µl of DF buffer was added to the sample and mixed by vortex. This was then incubated at 60°C for 10-15 minutes until the agarose dissolved. Next, 800 µl of the sample mixture was added to a DF Column and centrifuged at 15,000 x g for 30 seconds. The flow-through was captured in a collection tube and discarded. A 400 µl aliquot of W1 Buffer was then added to the DF column and passed through the filter of the column by centrifugation. The flow-through was discarded again. A 600 µl aliquot of Wash Buffer was added to the DF column, the column was centrifuged at 15,000 x g for 30 seconds, and the flow-through was once again discarded. Then, the column was centrifuged for 3 minutes to dry the column’s filter matrix. Next, the dry DF column was transferred to a sterile 1.5 ml microcentrifuge tube, 30 µl of Elution Buffer was added, and the tube was centrifuged for 2 minutes at 15,000 x g to elute the purified DNA. A sample of eluted DNA was then examined by gel electrophoresis by preparing an agarose-TAE gel (as described above). The samples for electrophoresis were prepared by

adding 5 µl of eluted DNA, 5 µl of 6x loading buffer, and 20 µl of water. The 30 µl solution was loaded to a well of the agarose gel and a 10 µl aliquot (500 ng) of Gene Ruler 1 Kb Plus DNA ladder (Thermo Scientific) was included as a size marker. The gel was run at 120 V for 90 minutes and imaged with a gel documentation system. An image of the gel is shown in Figure 2. The lengths of the left fragment and right fragment are predicted to be 900 bp and 813 bp, respectively. The predicted sequences of the left flank and right flank are provided in Figure 3 and Figure 4. These data, along with the gel electrophoresis results, indicate that the lengths of the purified left and right flanks are consistent with predictions.

These steps were repeated for the center fragment for the transformation vector (Figure 5), except that the DNA template was plasmid pTH1256.1 (GenBank MH550659.1) and the primers were HPH-CEN-F (Table 2) and HPH-CEN-R (Table 2).

Next, Step 5 (figure 1) involved fusing the left, center, and right fragments using a PCR technique called double joint PCR (DJ-PCR; Yu et al. 2004). The reagents used during this step were the left flank, the right flank, center fragment, sterile water, DNA polymerase buffer, dNTP mix (10mM), and Q5 enzyme. This reaction was run in the PCR machine. Step 6 (figure 1) involves the amplification of the DNA replacement vector from fusion products with nested primers. The nested primers bind the DNA replacement vector within the left and right flanks. The nested primers were V377-E and V377-F. These primers were then used in PCR with the fusion product as template to amplify the DNA replacement vector. The amplified DNA replacement vector was then column purified with the IBI Scientific Gel Extraction and PCR Clean Up Kit. This process involved adding 5 volumes of DF buffer to 1 volume of the amplified

DNA vector. The mixture was transferred to a DF column, which was centrifuged and the flow-through was discarded. Wash buffer was added, and the sample was centrifuged. Then, the DF column was dried by centrifugation for three minutes and transferred to a new microcentrifuge, after which 50 µl of elution buffer was added to the column matrix. The DNA was eluted from the column matrix with EB buffer by centrifugation. The 8th step (figure 1) was performing the final check of the DNA replacement vector by gel electrophoresis. This was performed similarly to the process depicted for analysis of the purified left and right flanks described above. An image of the gel is shown in Figure 6. The length of the DNA replacement vector on the gel is consistent with its predicted length of 2821 bp. These results suggest that the DNA replacement vector v377 was assembled properly. DNA replacement vector v377 was designed to replace interval v377. The sequence of interval *v377* is shown in Figure 7 and its relationship to interval *v350*, deletion of which initiated this project, is shown in Figure 8.

The next step was *N. crassa* transformation. Transformation was preformed essentially as described by Margolin et al. (1997) and Rhoades et al. (2020). Transformation medium was prepared prior to transformation. Bottom agar was created by adding 172 ml milliQ water, 8 ml of 25x Vogel's Salts, and 3 g agar to a 500 ml glass bottle. This mixture was autoclaved. Then, 150 µl of hygromycin (400 mg/ml) and 20 ml 10x FIGS solution was added to the mixture. This was then poured into 20 ml petri dishes. Top agar was created with 105 ml milliQ water, 27.3 grams sorbitol, 6 ml of 25x Vogel's Salts, and 2 g agar. This was autoclaved until cooled. Then, 15 ml of 10x FIGS solution was added. The top agar was aliquoted to 50 ml conical tubes (40 ml total volume) and stored at 50 °C to keep from solidifying. The transformation began by using the wood applicators to scoop conidia of strain RDGR170.3 into a conical vial containing 30 ml

of ice cold sterile 1M sorbitol, which was placed on ice. A 100-micron cell strainer was then used to remove mycelia. Next, the conidia were centrifuged at 2000 x g for 13 minutes, supernatant was removed, and the pellet of conidia was resuspended in 20 ml of ice cold 1 M sorbitol. The centrifugation step was repeated, and the conidial pellet was suspended in sterile ice cold 1 M sorbitol to a concentration of approximately 1 billion conidia per ml. With the resuspension step complete, approximately 500 ng of the DNA replacement vector was mixed with 100 µl of conidia and electroporated according to the protocol of Margolin et al (1997). Transformants were plated in top agar onto bottom agar and incubated for several days at 32 °C.

Transformants were selected by transferring hygromycin-resistant colonies from the transformation medium to 3 ml of Vogel's minimal agar (Vogel 1956) plus hygromycin and incubating for several days in a 32°C incubator or on a bench top at room temperature. The medium was prepared with 288 ml DI water, 12 ml of 25x Vogel's salts, 6 g sucrose, and 4.5 g of agar. Medium was autoclaved and hygromycin was added to 200 µg / ml. The medium was then transferred to sterile 16 x 125 mm glass tubes with vented caps. The tubes were tilted to allow the medium to form a slanted surface during solidification.

N. crassa transformants typically contain a mixture of transformed and untransformed nuclei (i.e., they are often heterokaryotic). Therefore, the next step was to cross the transformants to a standard mating partner to obtain homokaryotic offspring deleted of interval *v377*. Crossing was performed as essentially described in Samaraweera et al. 2014. It involved making crossing medium containing 470 milliQ water, 7.5 grams sucrose, 25 ml of 20x WG salts (Westergaard and Mitchell 1947), 50 µl of 10,000x Trace elements, 25 µl of 100 µg / ml biotin stock solution

(100 mg/ml), and 10 g agar. The crossing medium was aliquoted to 60 mm petri dishes. After solidification, strain RTH1005.2 was transferred to the crossing plates and cultured for 7 days at room temperature. This strain, RTH1005.2, served as the protoperithecia parent. Protoperithecia are the immature fruiting bodies of *N. crassa*, and the protoperithecial parent of a cross is often called the female parent. Conidia from the male parent were used to fertilize the female parent's protoperithecia. Specifically, conidial suspensions (conidia plus sterile water) were made from several transformants, and 200 µl of each conidial suspension were transferred to the surface of each crossing plate. Crosses were placed in a tray with a clear humidity dome and incubated at room temperature for several weeks (Winter Break 2023-2024).

Ascospores were then collected from the underside of the crossing plate lid. A 200 µl barrier pipette tip and sterile water was used to transfer ascospores from the lid to a microcentrifuge tube. The ascospore suspension was vortexed and placed in the dark at 4°C for storage. Working suspensions of ascospores were made by transferring approximately 500 ascospores from the stock suspension to 500 µl of sterile water in a microcentrifuge tube. The working stock was incubated for 30 minutes at 60 °C and then placed at room temperature once the incubation period had completed. Sterile technique was used to transfer 50 µl, 100 µl and 200 µl aliquots of the suspension to the centers of 100 mm petri dishes containing Vogel's minimal agar plus hygromycin. The cultures were incubated overnight on a bench top. The next day, germlings were isolated from the cultures. Ascospores tend to stick to one another, so care was taken to make sure each isolated germling emanated from a single ascospore and no other ascospores were transferred with the germling. The germlings that were resistant to hygromycin were chosen because these are expected to have the *v377Δ* genotype. Germlings not resistant to

hygromycin are expected to have the *v377+* genotype. A sterile syringe needle was used to cut a square around a single germinating ascospore. The square of medium was then transferred to Vogel's minimal agar plus hygromycin in 16 x 125 mm glass tubes (as described for transformant isolation). Cultures with transferred germlings were stored in an incubator for two days at 32 °C before transfer to room temperature.

The germlings were next used in test crosses for spore killing. The test crosses required the preparation of protoperithecial parents on crossing medium as described for the crossing of heterokaryotic transformants above. However, two protoperithecial parents were used in this procedure: RTH1623.1 and RTH1623.2. After an eight-day incubation at room temperature, conidial suspensions of hygromycin resistant germlings were prepared in sterile water and used to fertilize the RTH1623.1 and RTH1623.2 protoperithecial parents. After a 14-day incubation at room temperature, fruiting bodies (perithecia) were isolated and the perithecial contents were dissected into 25% glycerol with syringe needles and a dissecting microscope. Specifically, perithecia were transferred to a microscope slide containing a drop of 100 µl of 25% glycerol. The surface hyphae and agar from each perithecium were removed, the perithecia sliced open, and the rosettes of asci (spore sacs) containing ascospores were released. After removal of the perithecial shells, a cover slip was placed over the rosettes and sealed with nail polish. The microscope slide was then stored at room temperature in the dark until imaging with a Leica Compound Microscope and Zeiss Imaging System (within 24 hours).

To confirm that the hygromycin-resistant offspring have the *v377Δ* genotype as expected, DNA was isolated from four hygromycin-resistant offspring and used in a PCR-based

genotyping assay. This required culturing the hygromycin-resistant offspring in Vogel's minimal liquid medium and harvesting mycelia for freeze drying under vacuum. Wood inoculating rods were used to harvest the mycelia from the liquid medium culture and transferring it to a sheet of course filter paper over a stack of paper towels. The stack of paper towels was then folded over to squish the liquid out of the mycelia to remove most of the moisture. Clean forceps were then used to transfer the dried mycelia into microcentrifuge tubes. The partially dry mycelial samples were frozen at —80 °C before lyophilization, which is a process where ice is removed after it is frozen and is placed under a vacuum to allow the ice to go from solid to vapor.

DNA was then isolated from lyophilized tissue with IBI Scientific's Mini Genomic DNA Kit for Plants/Fungi. The tissue samples were ground into a fine powder with clean micro spatulas. Then, 400 µl of GP1 buffer and 5 µl of RNase A (10 mg/ml) was added to the ground tissue. The sample was then vortexed before incubating at 65 °C for 10 minutes. Next, a 100 µl aliquot of GP2 buffer was added, the sample was mixed by inversion 5x, and incubated on ice for 3 minutes. The sample was then transferred to a filter column and centrifuged for 1 minute at 1,000 x g. The filter column was then be discarded, and 750 µl of GP3 buffer was added to the flowthrough, which was then gently mixed by pipetting. Approximately 650 µl of the flowthrough was then transferred to a GD column in a 2 ml collection tube. The GD column was centrifuged at 15,000 x g for 1 minute. The flow-through was discarded and the GD column was loaded with 400 ul of W1 buffer before centrifugation at 15,000 x g for 45 seconds. Again, the flowthrough was discarded, and the GD column was loaded with 600 µl of Wash buffer before centrifugation at 15,000 x g for 45 seconds. Then, the flow-through was discarded and the GD

column was centrifuged for 3 minutes at 15,000 x g to dry the column. Finally, the GD column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of 65 °C Elution Buffer was added to the filter matrix of the GD column. This was allowed to sit for one minute at room temperature before centrifugation for 1 minute at 15,000 x g. The eluted DNA was stored at –20 °C.

DNA from the hygromycin-resistant offspring were then used in a PCR-based genotyping assay. The forward and reverse primers were V0377-E (Table 2) and V0377-F (Table 2), respectively. The DNA samples from the hygromycin-resistant transformants and a control strain (RDGR170.3) was thawed at 65 °C, vortexed, centrifuged, and placed on ice. A primer mix containing the forward and reverse primers was made as described above for the construction of DNA replacement Vector v377. Each reaction contained 13.4 µl of sterile water, 5.0 µl of primer mix, and 1.0 µl of DNA template (10 ng/µl), 5.0 µl of 5x DNA polymerase buffer, 0.5 µl of dNTP mix (10 mM), and 0.1 µl of Q5 enzyme. The reactions were placed in a thermal cycler and cycled according to the manufacturer's recommendations for Q5 DNA Polymerase. The PCR products were analyzed by gel electrophoresis and imaged (Figure 9) as described above.

The expected PCR products from *v377+ and v377Δ* genotypes are 1402 bp and 2821 bp, respectively. The predicted sequences of these PCR products are shown in Figure 10 and Figure 11. The genotyping assay was somewhat inconclusive. For example, strain RCO10.6's PCR product is consistent with *v377+* and the PCR product for RCO11.107 is consistent with a *v377Δ* genotype. PCR products were not observed from RCO10.12 and RCO11.2 templates. Taking into account the weak *v377Δ* product observed for RCO11.107, these results suggest that the *v377Δ*

allele is recalcitrant to amplification by PCR. My tentative interpretation of these results is that RCO10.6 is a *v377+/v377Δ* heterokaryon, while RCO10.12, RCO11.2, and RCO11.107 are *v377Δ* homokaryons.

CHAPTER III

RESULTS

As described in the Introduction, preliminary research has suggested that the deletion of a 1.3 kb DNA interval called *v350* eliminates spore killing (Rhoades and Hammond, unpublished data). To build upon this finding, I deleted interval *v377,* which is a subinterval of *v350* (Figures 7 and 8) and examined the effect of *v377* deletion on spore killing. In all, I performed six test crosses (Figure 12). In Figure 13, I show images of asci from each test cross, one image for each test cross. Figures 14 through 19 are each specific for one of the six test crosses. As we can see from these figures, the majority of asci from crosses of the hygromycin-resistant transformants (putative or confirmed *v377Δ* genotypes) possess eight viable ascospores (Figure 14, RCO10.12 x RTH1623.1; Figure 15, RCO10.1 x RTH1623.1; Figure 16, RCO10.2 x RTH1623.1; Figure 17, RCO10.6 x RTH1623.1; Figure 18, RCO11.107 x RTH1623.2; Figure 19, RCO11.2 x RTH1623.2). Overall, these results demonstrate that deletion of interval *v377* disrupts spore killing by *Sk-3*.

CHAPTER IV

DISCUSSION

N. crassa is an ideal model organism to study genetic processes. Although my findings do not support my initial hypothesis, my results are intriguing because they suggest that interval *v377* harbors an important genetic element for spore killing. At this point, it is unknown if the *v377* interval is found within a protein coding gene (such as the killer gene) or a regulatory element (such as a promoter or enhancer) that controls the expression of genes required for the killing process.

Studies of spore killing in Neurospora fungi could contribute to future efforts to control fungal pathogens. Natural gene drivers are genetic elements that exist within organisms and can spread themselves through populations in nature (Zanders and Johannesen, 2022). There are typically known as selfish genetic elements and in *Neurospora crassa* as the Sk-3 spore killing system. *Sk-3* is thought to produce a toxin that kills spores that lack sk-3, which then results in inheritance of this gene within a population. This ensures that the sk-3 can spread throughout generations. Understanding how this element works may allow us to use it to control specific genetic pathways in other fungi that can lead to benefitting society.

Natural gene drivers are important to study in biological systems. Researchers are struggling to understand the mechanisms that occur during genetic inheritance in fungal species. In an article called “Can natural gene drives be part of future fungal pathogen control strategies in plants?” by Donald M. Gardiner et al. 2020, the authors discuss that fungal pathogens can cause enormous crop loss and that being able to effectively manage the genetics of these

populations could solve plant disease problems. Having the ability to manipulate gene drivers like *Sk-3* may allow us to remove pathogenic traits from fungal populations by favoring the specific inheritance of non-pathogenic traits in these species (Gardiner et al., 2020). The article also mentions cloning a spore killer from a model fungus called Podospora (Silar et al., 2012). The Podospora spore killers described by Gardiner have substantial similarities with the *N. crassa Sk-3* spore killer.

Overall, natural gene drivers are important to study because they may offer societal benefits. Recent findings suggest that gene drivers may even function when transferred between fungal genera, as reported by Urquhart et al., (2022). In conclusion, *N. crassa* serves as a model organism to investigate the *Sk-3* spore killing genetic mechanism. The study of natural gene drives allows us to manipulate genetic inheritance in populations of fungal species. Understanding the impacts of the specific genes can also help us understand the various impacts of genetic evolution.

REFERENCES

Freitag, M., Williams, R. L., Kothe, G. O., Selker, E. U. (2002) A cytosine methyltransferase homologue is essential for repeat-induced point mutation in *Neurospora crassa*. PNAS. 99(13):8802-7. doi: 10.1073/pnas.132212899.

Gardiner, D. M., Rusu, A., Barrett, L., Hunter, G. C., & Kazan, K. (2020). Can natural gene drives be part of future fungal pathogen control strategies in plants? New Phytologist. https://doi.org/10.1111/nph.16779

Hammond, T. M., Rehard, D. G., Xiao, H., & Shiu, P. K. T. (2012). Molecular dissection of Neurospora Spore killer meiotic drive elements. Proceedings of the National Academy of Sciences, 109(30), 12093–12098.https://doi.org/10.1073/pnas.1203267109

Margolin B. S., M. Freitag, and E. U. Selker, 1997 Improved plasmids for gene targeting at the his-3 locus of Neurospora crassa by electroporation. Fungal Genet. Newsl. 44: 34–36.

Ninomiya, Y., Suzuki, K., Ishii, C., Inoue, H., 2004. Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining. Proc. Natl. Acad. Sci. U. S. A. 101, 12248–12253. https://doi.org/10.1073/pnas.0402780101

Perkins, D.D., Radford, A., Sachs, M.S., 2000. The Neurospora Compendium: Chromosomal Loci. Academic Press.

Rhoades N. A., E. K. Webber, and T. M. Hammond, 2020 A nonhomologous end-joining mutant for *Neurospora sitophila* research. Fungal Genet. Rep. 64: Article 1. https://doi.org/10.4148/1941-4765.2172

Samarajeewa D. A., P. A. Sauls, K. J. Sharp, Z. J. Smith, H. Xiao, et al., 2014 Efficient detection of unpaired DNA requires a member of the Rad54-like family of homologous recombination proteins. Genetics 198: 895–904. https://doi.org/10.1534/genetics.114.168187

Shiu P. K. T., D. Zickler, N. B. Raju, G. Ruprich-Robert, and R. L. Metzenberg, 2006 SAD-2 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1 RNA-directed RNA polymerase. Proc. Natl. Acad. Sci. U. S. A. 103: 2243–2248. https://doi.org/10.1073/pnas.0508896103

Silar, P., Jean-Marc Dauget, Gautier, V., Grognet, P., Chablat, M., Sylvie Hermann-Le Denmat, Arnaud Couloux, Wincker, P., & Debuchy, R. (2018). A gene graveyard in the genome of the fungus Podospora comata. Molecular Genetics and Genomics, 294(1), 177–190. https://doi.org/10.1007/s00438-018-1497-3

Smith, Z., Bedore, S., Spingler, S., Hammond, T.M., 2016. A *mus-51* RIP allele for transformation of *Neurospora crassa*. Fungal Genet. Rep. 62, 1–7.

Turner, Barbara C, and David D Perkins. “Spore Killer, a Chromosomal Factor in Neurospora That Kills Meiotic Products Not Containing It.” Genetics, vol. 93, no. 3, 1 Nov. 1979, pp. 587–606, https://doi.org/10.1093/genetics/93.3.587. Accessed 24 May 2022.

Turner, Barbara C. “Geographic Distribution of Neurospora Spore Killer Strains and Strains Resistant to Killing.” Fungal Genetics and Biology, vol. 32, no. 2, Mar. 2001, pp. 93– 104, https://doi.org/10.1006/fgbi.2001.1253. Accessed 25 Feb. 2023.

Urquhart, A., & Gardiner, D. M. (2022). A Natural Fungal Gene Drive Enacts Killing via DNA Disruption. 14(1). https://doi.org/10.1128/mbio.03173-22

Vogel H. J., 1956 A convenient growth medium for Neurospora (Medium N). Microb. Genet Bull 13: 42–43.

Westergaard M., and H. K. Mitchell, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34: 573–577. https://doi.org/10.2307/2437339

Yu J.-H., Z. Hamari, K.-H. Han, J.-A. Seo, Y. Reyes-Domínguez, et al., 2004 Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. FG B 41: 973–981. https://doi.org/10.1016/j.fgb.2004.08.001

Zanders, S., & Johannesson, H. (2021). Molecular Mechanisms and Evolutionary Consequences of Spore Killers in Ascomycetes. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/mmbr.00016-21

Table 1 Strains used in this study

Strain name	Genotype
RTH1005.2	*rid; fl; Sk-S^{ChrIII} a+*
RZS27.10	*rid; Sk-S^{ChrIII}; mus-51^{RIP70} a+*
RTH1623.1	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ A+*
RTH1623.2	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ a+*
RDGR170.3	*rid; Sk-3^{ChrIII}; mus-51Δ::bar+ A+*
TSPS7.6	v377-based, hygromycin-resistant transformant of RDGR170.3
TCO2.3	v377-based, hygromycin-resistant transformant of RDGR170.3
RCO10.1	hygromycin-resistant offspring of TCO2.3 × RTH1005.2
RCO10.2	hygromycin-resistant offspring of TCO2.3 × RTH1005.2
RCO10.6	hygromycin-resistant offspring of TCO2.3 × RTH1005.2
RCO10.12	hygromycin-resistant offspring of TCO2.3 × RTH1005.2
RCO11.2 RSPS13.2	hygromycin-resistant offspring of TSPS7.6 × RTH1005.2
RCO11.107	hygromycin-resistant offspring of TSPS7.6 × RTH1005.2

The *Sk-3* genetic element spans most of Chromosome III. *Sk-3^{ChrIII}* indicates that the strain carries the *Sk-3* genetic element. *Sk-S^{ChrIII}* indicates that the strain does not carry the *Sk-3* genetic element. The *mus-51^{RIP70}* and *mus-51Δ::bar+* allele disrupts Non-Homologous End Joining (Ninomiya et al. 2004; Smith et al. 2016). The *rid* allele disrupts Repeat Induced Point Mutation (Freitag et al., 2002). The *fl* allele disrupts macrocondition (Perking et al. 2001). *The sad-2Δ::hph+* allele suppresses Meiotic Silencing by Unpaired DNA (Shiu et al. 2006)

Table 2 Oligonucleotide Primers

Name	Sequence (5′ > 3′)
HPH-CEN-F	AACTGATATTGAAGGAGCATTTTTTGG
HPH-CEN-R	AACTGGTTCCCGGTCGGCAT
V0377-A	AGGAGCATGAACAGGCAACTGCG
V0377-B	AAAAAATGCTCCTTCAATATCAGTTAAGCCGTCGAGGAAATGAGAAGAGA
V0377-E	GCCCCAGTTCTTCCTGCCTGCTG
V0377-F	TCATGGGCATCCTGCATAGCGAC
V350-C	GAGTAGATGCCGACCGGGAACCAGTTCCTTGTCCTCTCGGTCCTCTCTGT
V350-D	GGTAGCGTTCTGGGATGAGTGTGG

Underlined bases indicate bases used for fusion of flanks to the hph center fragment.

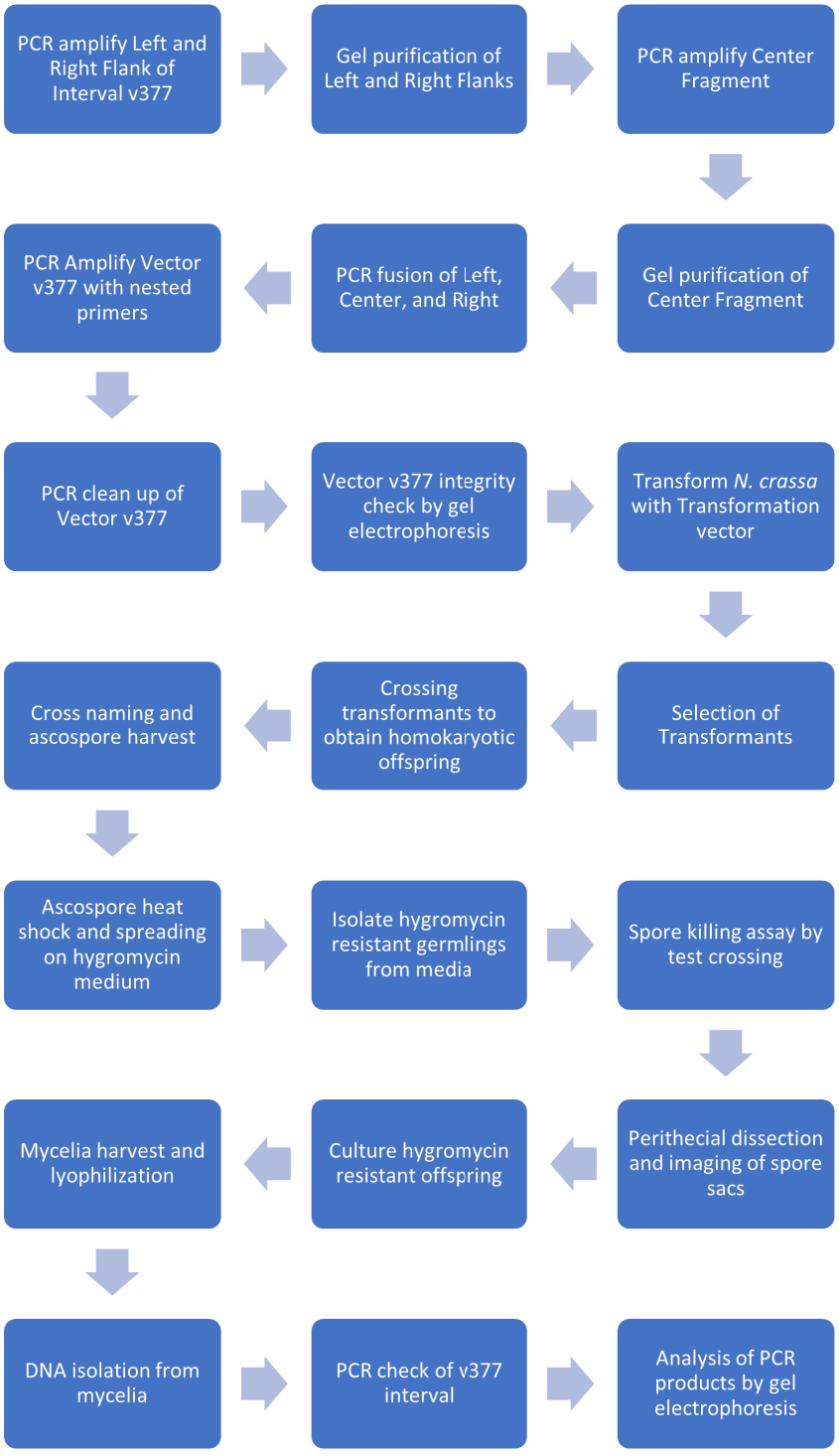

Figure 1 Methods Overview.

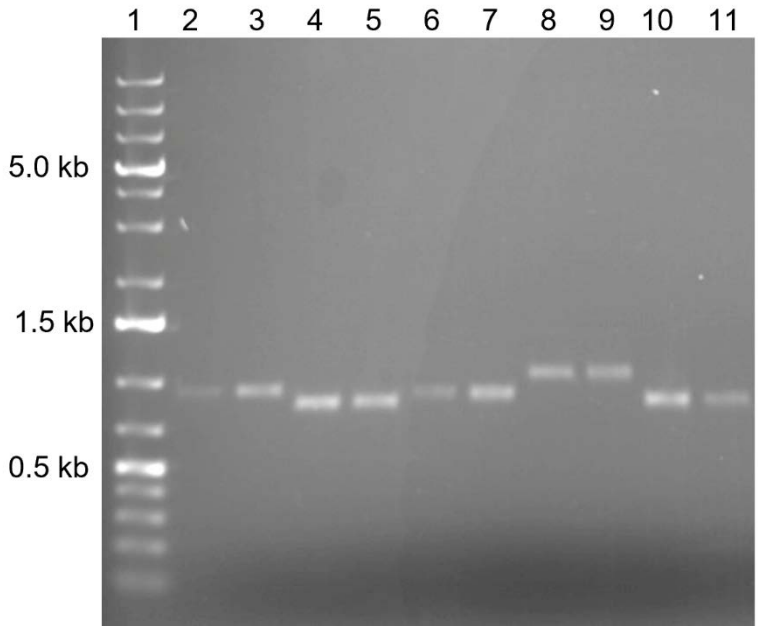

Figure 2 Analysis of the left and right flanks of interval *v377* by gel electrophoresis. The left flank of interval *v377* was amplified from RDGR170.3 genomic DNA with primers V0377-A and V0377-B. The right flank was amplified with primers V0377-C and V0377-D. Products were gel purified as described 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 GeneRule 1 kb Plus DNA Ladder; Lanes 2–7) not applicable; Lane 8) 5 µl purified product (*v377* left flank Trial 1); Lane 9) 5 µl purified product (*v377* left flank Trial 2); Lane 10) 5 µl purified product (*v377* right flank Trial 1); Lane 11) 5 µl purified product (*v377* right flank Trial 2). The expected product lengths for the *v377* left and right flanks are 900 bp and 813 bp, respectively.

>V3077 left flank
AGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCA
AGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGAT
GGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGA
TGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGC
TCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCAT
CTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGG
TCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGC
ACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGG
GAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGG
CACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCT
CCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACA
TGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTT
GGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCT
AGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTC
TCATTTCCTCGACGGCTT

Figure 3 The left flank of interval *v377*. The 900 bp sequence to the left of interval *v377* is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V3077-A and V0377-B from RDGR170.3 genomic DNA.

```
>V3077 right flank
CCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCC
CCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCC
CCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCA
GGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCC
GTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCT
CCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGC
TTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAA
TTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTG
CCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGG
AGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAG
GTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAAC
CCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCC
AACGGCGACGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACC
```

Figure 4 The right flank of interval *v377*. The 813 bp sequence to the right of interval *v377* is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V3077-C and V3077-D from RDGR170.3 genomic DNA.

>V3077 center fragment
AACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGC
CTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTC
TTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTG
CGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCT
TTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACT
CACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCA
GCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCT
GCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATC
GGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTG
CATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGG
GTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGC
GATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACCGTCAGTGCGTCCGT
CGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGT
GCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGA
CTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCC
GTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGG
ATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGT
TGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGG
AGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTG
TGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATA
GAGTAGATGCCGACCGGGAACCAGTT

Figure 5 The center fragment used to replace interval *v377*. The 1412 bp sequence of the DNA molecule used to replace interval *v377* is shown. This sequence was PCR-amplified with primers HPH-CEN-F and HPH-CEN-R from plasmid pTH1256.1. The center fragment sequence contains the *Aspergillus nidulans trpC* and the 1026 bp *hph* coding region. The positions of the *hph*+ start and stop codons are indicated with gray shading.

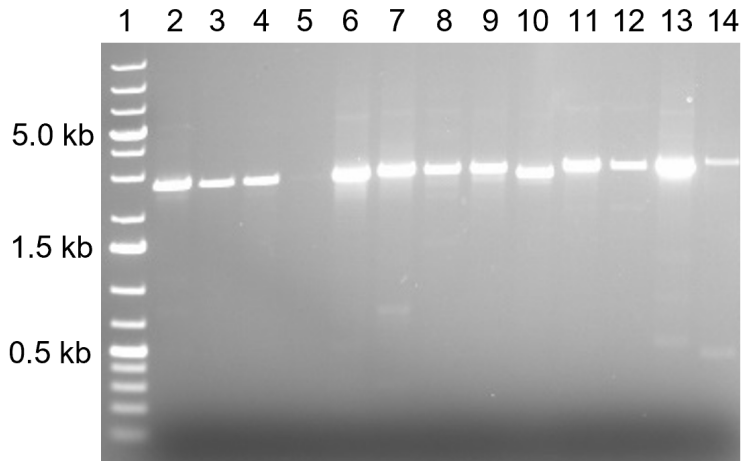

Figure 6 Transformation Vector v377 integrity check by gel electrophoresis. The *v377* flanks and center fragment were fused by DJ-PCR and amplified with primers V0377-E and V0377-F. The amplified product was column purified as described in the methods section. The amplified and purified product was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lane 2) 3 µl of amplified and purified Vector v377. Lanes 3–14) not applicable. The expected length of Vector v377 is 2821 bp.

```
>v377 interval
CGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCAT
TCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCT
TGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCC
ACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGC
```

Figure 7 The v377 interval. The *v377* interval is a single A:T bp.The single base of the interval is highlighted in gray and shown with flanking sequence, 100 bp to the left of the interval and 100 bp to the right.

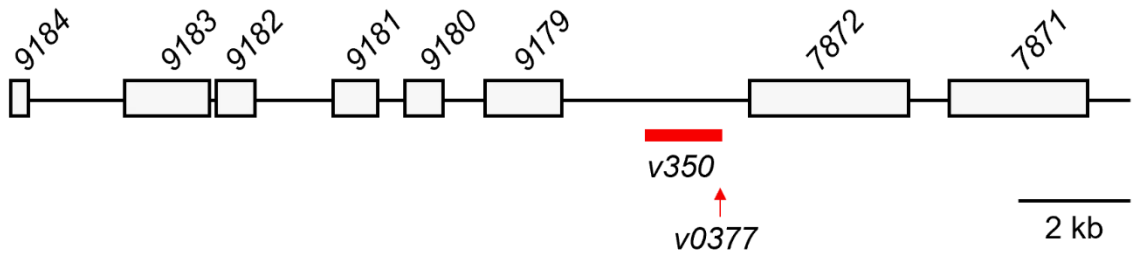

Figure 8 *Sk-3* Chromosome III positions 320,000–340,000. A diagram of Chromosome III, positions 320,000 to 340,000 in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the locations of predicted protein coding genes. Gene numbers were identified with blastx searches of an *N. crassa* protein database. The location of intervals *v350* and *v377* are indicated with a red bar and a red arrow, respectively.

Figure 9 Test strain genotype confirmation. DNA was isolated from test strains and used for genotyping by PCR with primers V377-E and V377-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 *v377+* and *v377Δ* genotypes are 1402 bp and 2821 bp, respectively. Lane 1 contains 0.5 µg of GeneRule 1 Kb Plus DNA ladder DNA; Lanes 2–13) Not applicable; Lane 14) RCO10.6; Lane 15) RCO10.12; Lane 16, RCO11.2, Lane 17) RCO11.107; Lane 18) RDGR170.3. These results suggest that RCO11.107 is homokaryotic for the *v377Δ* genotype. They also suggest that *v377Δ* allele is recalcitrant to amplification by PCR. While the genotypes of RCO10.6, RCO10.12 and RCO11.2 are inconclusive for *v377Δ*, they suggest that RCO10.6 is either heterokaryotic or homokaryotic for *v377+*.

>*v377+* PCR product, predicted sequence, primers V0377-E and V0377-F
GCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAG
TCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGA
AGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTA
TGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTT
GGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAA
CATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCA
TCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATA
CACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATT
CTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCTCGGTCC
TCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTG
TCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGAC
CAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGC
GTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGA
ATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTC
GAAAGCTTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATT
CATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCA
TGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCC
GGCCAAATGTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGC
CGGATACAAGTATGATTCATACCAGGCCGGTCTGAACCCGAGCGCGCAACCACAGTCCTCCTCCATTTCCC
CAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGAC**GTCGCTATGCAGGATGCCCATGA**

Figure 10 PCR product sequence for the *Sk-3 v377+* genotype. The predicted sequence of DNA amplified with primers V0377-E and V0377-F from a template consisting of *Sk-3 v377+* genomic DNA is shown in FASTA format. The binding sites of V0377-E and V0377-F are indicated with bold font. The *v377* interval is indicated with red font. The length of the sequence is 1402 bp.

>*v377Δ::hph+* PCR product, predicted sequence, primers V0377-E and V0377-F
GCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTC
CAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGC
GCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGG
GGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACC
CAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAG
GACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCT
GGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCT
GTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTT
TCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTG
GAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCG
TTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGT
GTGACTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGT
TTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTC
AATCTGACTTACCTATTCTACCCAAGCATCCAA**ATG**AAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGT
TTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAG
CTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTAT
GTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCC
TGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCA
TTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGT
ATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTG
GGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAAT
GGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCC
TCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGC
AGGATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGC
AATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGC
GTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAA
CCGACGAA**TAG**AGTAGATGCCGACCGGGAACCAGTCCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAG
AAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCT
GCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTAT
TCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCA
GGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGC
GAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCC
CTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCATTGCCAAACCAGACCCAGCAACAGCCCCGAG
CCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCC
GCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACGCCAGCA
ACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAACCC
GAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGAC**GTC**
GCTATGCAGGATGCCCATGA

Figure 11 PCR product sequence: *Sk-3 v0377Δ::hph+* genotype. The predicted sequence of DNA amplified with primers V377-E and V377-F from a template consisting of *Sk-3 v0377Δ::hph+* genomic DNA is shown in FASTA format. The binding sites of V0377-E and V0377-F are indicated with bold font. The start and stop codons of the *hph* coding region are shown with black font on grey background. The length of the sequence is 2821 bp.

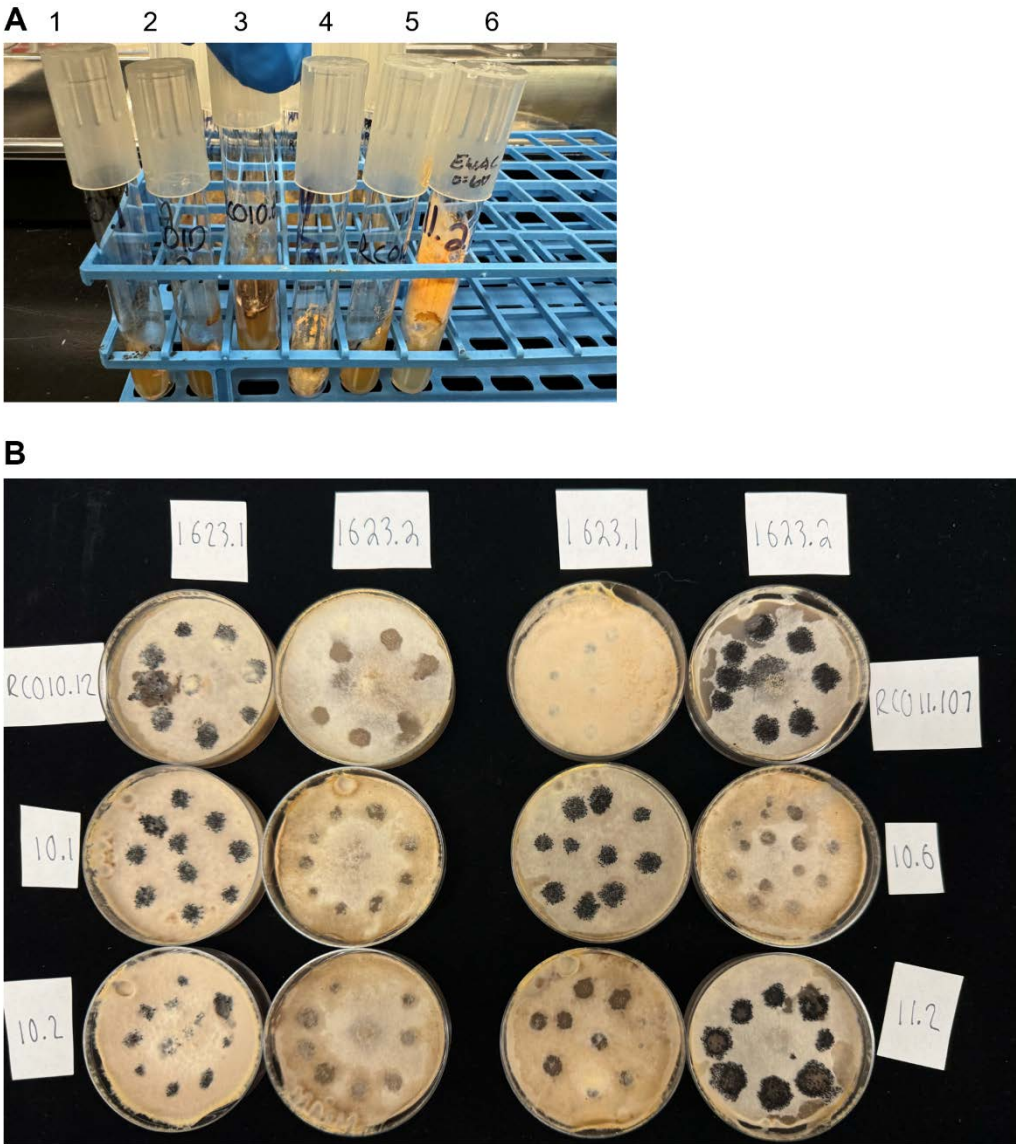

Figure 12 Test strains and crosses. **(A)** Six hygromycin-resistant offspring from cross were selected for test crosses. 1) RCO10.1, 2) RCO10.12, 3) RCO10.6, 4) RCO11.2, 5) RCO11.107, and 6) RCO10.1; l. (B) Test crosses were performed with RTH1623.1 and RTH1623.2. Images are of crossing dishes approximately three weeks post fertilization.

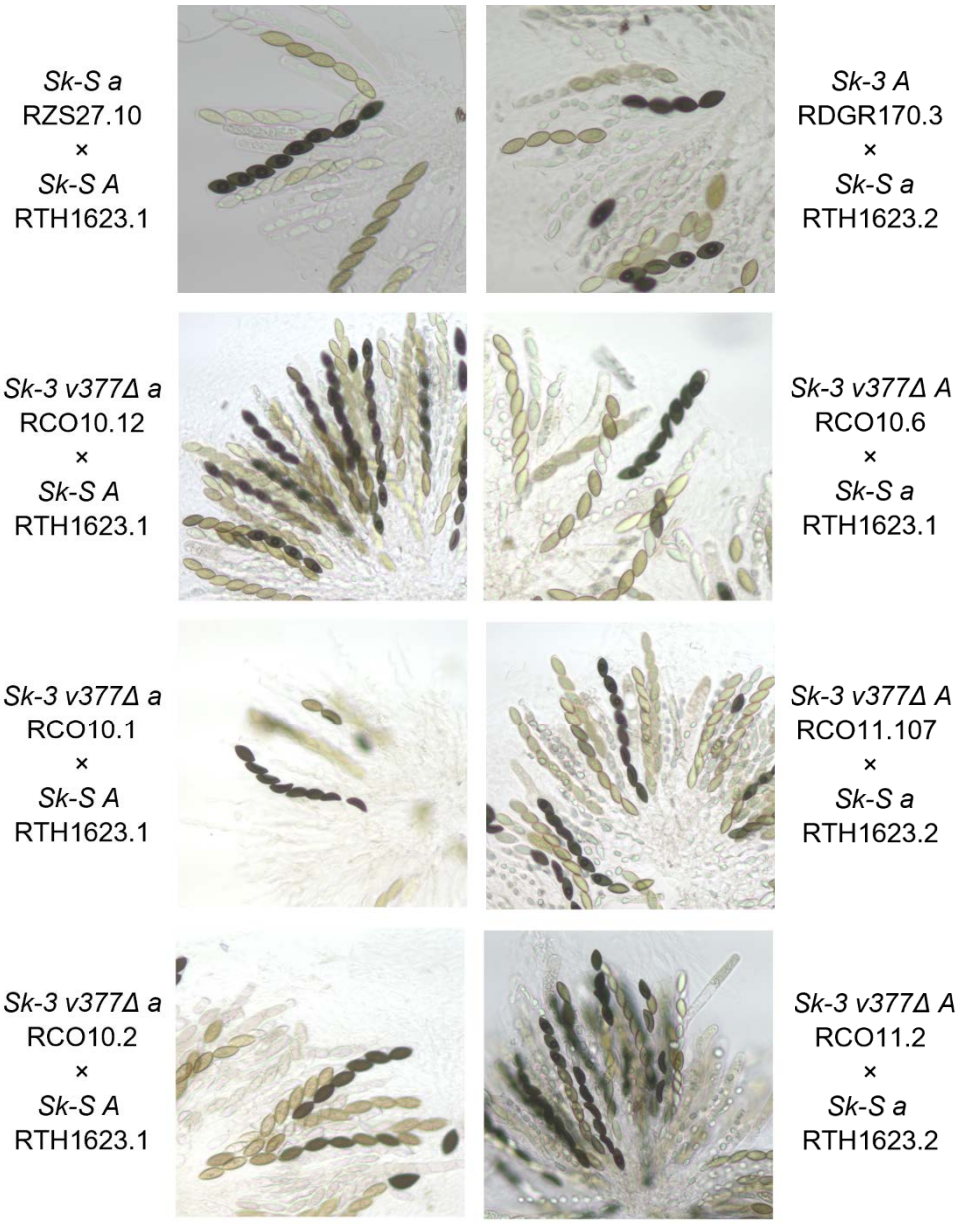

Figure 13 Ascus phenotype summary. Asci were dissected from perithecia of eight crosses on day 14 post fertilization and imaged under magnification. Strain names and putative genotypes are indicated. These results suggest that deletion of interval *v377* disrupts spore killing.

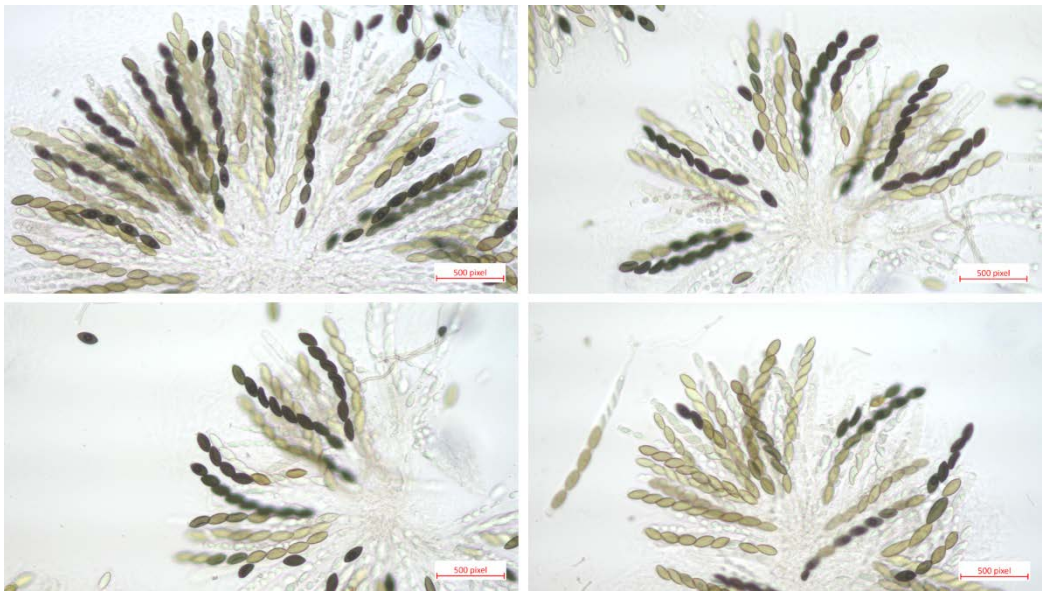

Figure 14 Ascus phenotypes. Asci were dissected from perithecia of RCO10.12 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RCO10.1 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RCO10.2 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

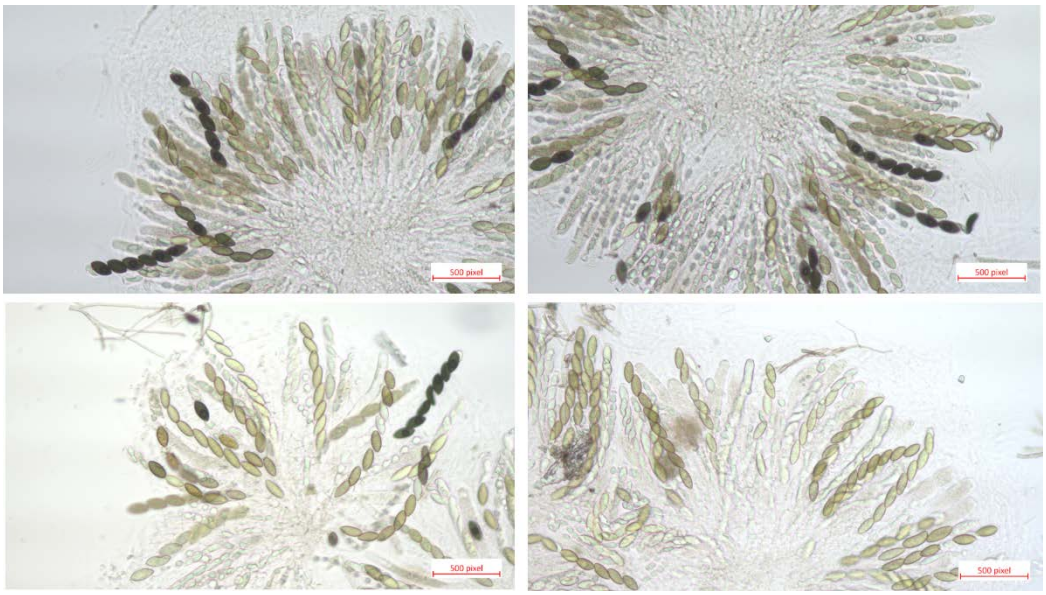

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RCO10.6 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

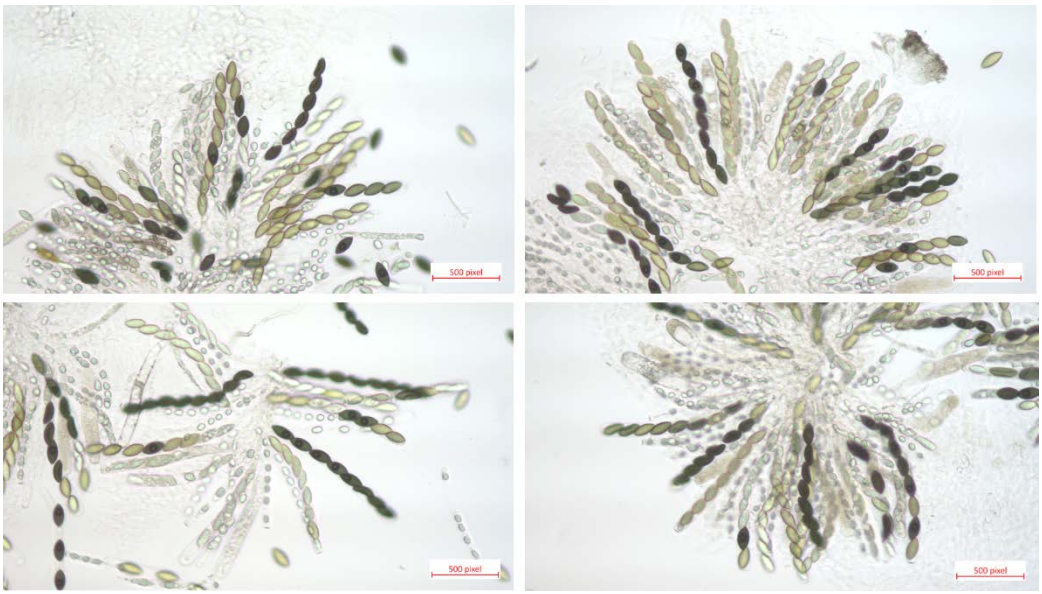

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RCO11.107 × RTH1623.2 on day 14 post fertilization and imaged under magnification.

Figure 19 Ascus phenotypes. Asci were dissected from perithecia of RCO11.2 × RTH1623.2 on day 14 post fertilization and imaged under magnification.