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Refining the rfk-2 Locus of Sk-3 using the DNA Interval v373 In Neurospora crassa

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Meiotic drive is a non-Mendelian inheritance phenomenon where selfish genetic elements change gene transmission in their own favor. This phenomenon occurs in the fungus *Neurospora crassa* during spore killing. When a strain carrying a spore killer genetic element is crossed with a non-spore killing wild type strain, the cross will produce half viable and half inviable offspring. The *N. crassa Sk-3* spore killer is found on Chromosome III. *Sk-3* is one of the most studied meiotic drive elements in Neurospora fungi and it is thought to require a killer gene and a resistance gene for spore killing. While the killer gene has not been identified, recent work has isolated a mutation (*rfk-2*^{UV}) that disrupts spore killing. Although this mutation has been mapped to Chromosome III, its exact location is not known. In this work, I investigate the role of one DNA interval in *Sk-3*-based spore killing. This DNA interval, referred to as V373, is thought to reside within or near *rfk-2*^{UV}. My results will contribute to future efforts to identify the *Sk-3* killer gene.

REFINING THE *RFK-2* LOCUS OF *SK-3* USING THE DNA INTERVAL *V373* IN *NEUROSPORA CRASSA*

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CHAPTER I

INTRODUCTION

Meiotic drive is a non-Mendelian inheritance phenomenon where selfish genetic elements change gene transmission in their own favor. This can be observed in the fungi with fungal spore killing in *Neurospora crassa*. Sexual spores in *N. crassa* can carry a spore killer element to survive over others. This was originally discovered in a study done by Turner and Perkins on *N. intermedia*, but scientists have since backcrossed *N. intermedia* to the closely related *N. crassa,* which is a better model organism (Turner and Perkins 1979). When a strain carrying a spore killer genetic element is crossed with wild type, the cross will produce half viable and half inviable offspring. Researchers are studying the spore killer *(Sk)* loci, *Sk-1*, *Sk-2*, and *Sk-3*. The latter two, *Sk-2* and *Sk-3*, have been mapped to the same region on Chromosome III. *Sk-2* and *Sk-3* are the most studied meiotic drive elements in Neurospora, each containing a killer element and a resistance gene. Crosses between *Sk-2* and *Sk-3* have shown that the spore killers do not encode resistance to each other, and all eight offspring are inviable (Hammond et al 2012).

A gene called *rsk* (*resistant to spore killer*) was found in a *Spore killer-2* (*Sk-2*) strain of *N. crassa. rsk* is a fungal-specific gene and deletion of the gene in a killer strain leads to self-killing. This gene was narrowed down to a region with four genes. All four genes were individually deleted from an *r(Sk-2)* strain (a strain resistant to *Sk-2*, but not a killer itself) and the data showed that only deletion of gene *09151* resulted in loss of resistance to spore killing.

This helped identify specifically that *09151* was the resistance gene, and it was renamed *rsk*. Once this gene was identified, it allowed scientists to further explore how spore killing works. A model called the Killer Neutralization model was proposed. In *N. crassa* the killer element targets one or multiple molecules that are important for ascospore formation and maturation. When *rsk* is absent, the killer targets all developing ascospores, resulting in the production of empty asci. When *rsk* is present, it somehow neutralizes the killer's activity and allows ascospore development (Hammond et al 2012).

Both *Sk-2* and *Sk-3* have the *rsk* gene and use their own alleles to resist spore killing. There are three different alleles of the *09151* gene. Class I is found in *Sk-2* and *Sk-3* sensitive strains, meaning they have no resistance to either spore killer. Class II is found in *r(Sk-2)* and *Sk-2* strains, which both resist *Sk-2*-based spore killing. Class III is found in *r(Sk-3)* and *Sk-3* strains, they both resist *Sk-3*-based spore killing (Hammond et al 2012).

To find a killing gene, scientists looked more at *Sk-2*. A region required for killing (*rfk-1*) has been identified in *Sk-2*, but the *Sk-3* ortholog of this gene is not required for spore killing (Svedberg et al. 2018). Scientists have thus started looking for the *Sk-3* killer, which appears to be unrelated to *rfk-1*. Velazquez et al. (2022) performed a genetic screen for the *required for killing (rfk)* mutations in *Sk-3*. Their genetic screen resulted in the identification of *rfk-2*^{UV}, which disrupts spore killing. They then located the approximate position of the *rfk-2*^{UV} with three-point crosses and found that it was located on Chromosome III, centromere-proximal of markers called *hphA*, *hphB*, and *mus-52Δ* (Velazquez et a. 2022). This gives a general region of where the *required for killing* gene is on *Sk-3*. For my senior thesis, I am refining the locus to help identify where the *rfk* gene is located within the *Sk-3* genetic element. During this work, I deleted and replaced an interval of DNA from the *Sk-3* genetic element to determine the effect on

spore killing. My original hypothesis was that the deletion of this interval, called *v373*, will disrupt *Sk-3*-based spore killing

CHAPTER II

METHODS

STRAINS AND MEDIA

The strains used in this study are listed in Table 1. Vogel's Minimal Medium (VMM) or Vogel's Minimal Agar (VMA: VMM + 2% agar) (Vogel 1956) was used for vegetative propagation of all Neurospora strains unless otherwise indicated. Hygromycin B (Gold Biotechnology) was used at 200 μg per ml when selecting for hygromycin resistance. Westergaard and Mitchell's synthetic crossing agar (SCA) (Westergaard and Mitchell 1947) with 1.5% sucrose and a pH of 6.5 was used for *N. crassa* crosses.

VECTOR CONSTRUCTION

A brief overview of the materials and methods can be seen as a flow chart in Figure 1. Transformation vector v373 was constructed with Q5® High-Fidelity DNA Polymerase (New England Biolabs) via DJ-PCR as described by Yu *et al.* (2004). Table 2 lists all of the primers used. The left and right flanks of vector v373 were amplified from RDGR170.3 genomic DNA with primer sets v0373-A/v0373-B and v350-C /v350-D, respectively. The left flank is predicted to be an 865 bp interval (Fig. 2). The right flank is predicted to be an 813 bp interval (Fig. 3). During the process of constructing the flanks, a standard primer mix protocol was followed. A primer mix of 6.3 μl of forward primer (all primer stocks were at 100 pmol / μl), 6.3 μl of reverse primer, and 487.5 μl of H_2O were added to a sterile 1.5 ml microcentrifuge tube (MCT),

vortexed, spun down, and placed on ice. This was done for both the left and right flanks using their respective primers. Then, a PCR tube rack was placed on ice along with four PCR tubes labeled 1, 2, 3, and 4. Next 5.0 µl of the left flank primer mix was added to tubes 1 and 2, while 5.0 µl of the right flank primer mix was added to tubes 3 and 4. A 1.0 µl aliquot of the DNA template was added to each PCR tube. The enzyme master mix was prepared next for five reactions. The mix had 67.0 µl of H_2O, 25.0 µl of Q5 reaction buffer, 2.5 µl of dNTP mix (10 mM), and 0.5 µl of Q5 enzyme. The PCR cycle was programmed as follows: step 1 lasting 30 seconds at 98 °C, step 2 lasting 5 seconds at 98 °C, step 3 lasting 10 seconds at 62.5 °C, step 4 lasting 1 minute and 30 seconds at 72 °C, then it cycles back to step number 2, 25 times before moving to step 5 for 10 minutes at 72 °C, lastly, a forever hold at 12 °C. Then 19.0 µl of the enzyme master mix was added to each PCR tube and mixed by pipetting up and down slowly 5 to 8 times. The PCR tubes were closed and transferred to the thermocycler to start the cycle. When the cycle was complete, the samples were moved to -20 °C until processing by gel purification. The center fragment for DJ-PCR was amplified from plasmid pTH1256.1 (GenBank MH550659.1) with primer set (Hph-cen-f) (Hph-cen-r). Flank and center fragment PCR products were gel purified with the Gel Extraction & PCR Cleanup Kit (IBI Scientific). The center fragment is a 1412 bp sequence (Fig. 4). The center fragment was designed to replace the *v373* interval with *hph+*. The *v373* DNA interval is 869 bp (Fig. 5). The location of the *v373* interval can be seen in Figure 6.

A gel was made by combining 1.8 g agarose and 200 ml of 1X TAE buffer into a 1-liter flask. After being heated for 1 minute in a microwave the mixture slowly swirled and returned to

the microwave for 1 minute. Then 10 µl of 10 mg/ml ethidium bromide was added and swirled to mix. The mix cooled on a bench top and a gel tray and comb were set up. A thin layer of the mix was poured onto the gel tray and let cool for 20 seconds until the rest of the mix was poured into the gel tray. While the gel solidified, the buffer in the electrophoresis chamber was changed. The electrophoresis chamber was rinsed for 1 minute with tap water before being refilled with new 1X TAE buffer. The gel was moved into the clean electrophoresis chamber and the comb was removed. Samples were prepared by thawing the PCR products in gloved hands until they were liquid. Then 5 µl of 6x loading buffer was added to the 25 µl PCR reaction and mixed by slowly pipetting up and down 5 times and stored on ice. Then 10 µl of the DNA ladder was loaded into the leftmost well and all 30 µl of the PCR reaction was added to each of the remaining wells. This was run at 120 V for 90 minutes, then PCR products were excised from the gel using a clean razor blade and UV light. Aluminum foil was used to shield PCR products in other lanes from UV light while cutting out one product. Each PCR product was cut out and added to an MCT to be purified.

Gel purification was performed with the IBI PCR gel purification kit (IBI Scientific). For purification, 500 µl of DF buffer was added to the each MCT and vortexed. Then the tubes were incubated at 60 °C for 10-15 minutes while being inverted every 2-3 minutes to dissolve the gel. The mix was cooled to room temperature and DF columns were placed in 2 ml collection tubes and labeled. An 800 µl aliquot of the sample mixture from the previous MCT was transferred to the new DF columns and centrifuged at 12-16,000 x g for 30 seconds. The flow through into the collection tube was discarded and the DF columns were placed back into the collection tubes.

Then 400 µl of W1 buffer was added into the DF column and centrifuged at 14-16,000 x g for 30 seconds and the flow through was discarded. The DF column was placed back into the collection tube and 600 µl of wash buffer was added to the DF column and let stand for 1 minute. Then, the tubes were centrifuged at 14-16,000 x g for 30 seconds and the flow through was discarded. The DF column was placed back into the collection tube and centrifuged at 14-16,000 x g for 3 minutes to dry the column matrix. The flow through was discarded and the DF column was placed into a new 1.5 ml MCT. Next, 50 µl of elution buffer were added to the column matrix and the column was left to stand for 2 minutes. The tubes were centrifuged for 2 minutes at 14-16,000 x g to elute the purified DNA.

The above products were examined by gel electrophoresis. The gel was made the same way as described earlier and gel electrophoresis was run using the prepared samples. Samples were prepared by adding 5 µl of the gel purified PCR product, 5 µl of 6x loading buffer, and 20 µl of sterile water and mixed slowly by pipetting and then stored on ice. This was done for each of the reactions for both the right and the left flanks. For loading, 10 µl of the Gene Ruler 1 kb Plus DNA ladder (Thermo Scientific) was placed into the first well and 30 µl of the sample mix was added into other wells. The gel was run at 120 V for 90 minutes and then imaged (Fig. 7).

The flank and center fragment products were then fused by DJ-PCR. The fusion product was amplified with primer set v0373-E/v0372-F and column purified with IBI Scientific's Gel Extraction & PCR Cleanup Kit before use in *N. crassa* transformation. To start the fusion both of the flanks and the center fragment were placed on ice. A PCR tube rack was placed on ice with a

single PCR tube. Then 5.0 µl of the left fragment, 5.0 µl of the center fragment, and 5.0 µl of the right fragment were added into the PCR tube. The thermocycler was turned on and the fusion protocol was chosen which was step 1 lasting 30 seconds at 98 °C, step 2 lasting 5 seconds at 98 °C, step 3 lasting 4 minutes at 58 °C, step 4 lasting 6 minutes at 72 °C, then it cycles back to step 2, 10 times before moving onto step 5 lasting 4 minutes at 72 °C and step 6 lasting forever at 12 °C. Then 62.6 µl of sterile H2O, 20 µl of Q5 reaction buffer, 2.0 µl of dNTP mix, and 0.4 ul of Q5 enzyme were added to the PCR tube and mixed gently by pipetting 5 times. This reaction was transferred to the thermocycler, cycled, and then stored at -20 °C until nested amplification. For nested amplification, a primer mix was made with 6.3 µl of forward primer v373-E, 6.3 µl of reverse primer V372-F, and 487.5 µl of H2O being added to a sterile 1.5 ml MCT. This was vortexed, spun down, and placed on ice. A PCR tube rack was placed on ice along with a single PCR tube. Then 20.0 µl of the primer mix was added to the PCR tube along with 5.0 µl of the fusion PCR reaction. The thermocycler was set to the Q5 protocol using the same parameters as mentioned above except step 4 lasted 3 minutes and 30 seconds and then it was cycled back to step 2, 30 times before moving to step 5. The PCR reaction was finished being set up by adding 52.6 µl of sterile H2O, 20.0 µl of Q5 reaction buffer, 2.0 µl of dNTP mix, and 0.4 µl of Q5 enzyme. This was mixed gently by pipetting 5 times and transferred into the thermocycler. This reaction was stored at -20 °C until column purification and gel electrophoresis.

For column purification with the IBI scientific PCR cleanup kit, 100 µl of the reaction product from PCR nested amplification was transferred to a 1.5 ml MCT. Then 500 µl of DF buffer was added and mixed by vortexing. A DF column was placed in a 2 ml collection tube and

the sample mix from the MCT was transferred into the DF column and centrifuged at 14-16,000 g for 30 seconds. The flow through was discarded and 600 µl of Wash buffer was added into the center of the DF column and let stand for 1 minute. The tube was centrifuged at 14-16,000 x g for 30 seconds and the flow through discarded. The DF column was placed back into the 2 ml collection tube and centrifuged at 14-16,000 x g for 3 minutes to dry the column. The flow through was discarded and the DF column transferred into a new 1.5 ml MCT. Then 40 µl of elution buffer was added into the DF column and let stand for 2 minutes. Then it was centrifuged for 2 minutes at 14-16,000 x g to elute the purified DNA. The tube was placed at -20 °C until gel electrophoresis.

The gel was made according to the protocol mentioned above. The sample was prepared by putting 5 µl of column purified DNA, 5 µl of 6x loading buffer, and 20 µl of sterile water into a new MCT and mixed by pipetting up and down 5 times. Next 10 µl of DNA ladder was loaded into the first well and the samples were loaded into the others. The gel was run at 120V for 90 minutes and imaged (Fig.8).

TRANSFORMATION

Transformation of *N. crassa* was performed by the electroporation method of Margolin *et al.* (1997) with modifications as described by Rhoades *et al.* (2020). Transformants were selected and transferred to Vogel's slants containing hygromycin. Everything was wiped down with ethanol and an ethanol candle, sterile beaker, two syringes, two needles, an empty culture tube rack, a sharpie, and Vogels slants with antibiotic were all placed on the bench. The syringes and

needles were sterilized by passing them through the flame. The syringe and needles were cooled and used to cut around a single colony and transfer that colony from the transformant to a Vogels slant containing Hygromycin B. The syringe and needle were sterilized and left to cool while the other syringe and needle were used for the next transformant. This was repeated for about 12 transformants and their slants were placed in the 32 °C incubator for 1-2 days and then placed at room temperature until they were ready to use. These transformants were labeled TTKB1.13, TTKB1.14, etc.

TRANSFORMANT SCREENING

Transformants TTKB1.13, TTKB1.14, and TTKB1.15 were analyzed by DNA isolation and PCR. This was started by culturing *N. crassa.* The lab bench was cleaned with ethanol and an ethanol candle was lit. Next four sterile 15 ml snap cap polystyrene tubes were placed in a rack and 2–4 ml of liquid Vogel's medium was placed into each tube. Then using sterile technique, a wooden inoculating rod was used to transfer a small amount of conidia from the transformant into the tube. This was repeated for each transformant, and the negative control had a sterile inoculating rod inserted into the media to help test that the conditions were sterile. The tubes were placed in a shaking incubator at 150 rpm, 32 °C, for 24-48 hours. The mycelia were harvested and lyophilized. Paper towels, filter paper, and forceps were laid out for each strain and new MCTs were labeled for each strain. The wooden inoculating rods were used to transfer mycelia from the tubes in the incubator onto filter paper and paper towels. The mycelia were dried and transferred into MCTs before lyophilization.

The IBI Scientific genomic DNA mini kit protocol was used to isolate DNA from lyophilized mycelia. Each MCT of lyophilized tissue was then ground into a fine powder and 400 µl of GP1 buffer was added. Then 5.0 µl of RNase A (20 mg/ml) was added before vortexing for five seconds, inverting, and placing at 60-65 °C for 10 minutes. During incubation, each sample was inverted five times every few minutes. Then 100 µl of GP2 buffer was added and the tube was mixed and inverted. The tubes were then incubated on ice for three minutes. The suspension was transferred into a filter column that was placed in a 2.0 ml collection tube. The filter columns were centrifuged for one minute at 1,000 x g and then the filter column was discarded. Then 750 µl of GP3 buffer was added to each collection tube and mixed by pipetting. A GD column was then placed in a new 2.0 ml collection tube. A 650 µl aliquot of the fungal tissue mixture was transferred to the new GD column tubes. These were centrifuged at 14-16,000 x g for 45 seconds and the flow through was discarded. Next 600 µl of wash buffer was added to the GD column and centrifuged at 14-16,000 x g for 45 seconds. The flow through was discarded and then it was centrifuged again at 14-16,000 x g for three minutes to dry the column matrix. The GD column was transferred to a clean 1.5 ml MCT that was labeled. Then 100 µl of elution buffer was added to the column and let stand for one minute. The tubes were centrifuged at 14-16,000 x g for one minute to elute purified DNA and the columns were tossed. The MCTs were then placed at -20 °C until PCR. For PCR forward primer V0373-E and nested reverse primer V0372-F were used. A primer mix was made in a 1.5 ml MCT using 6.3 µl nested forward primer, 6.3 µl nested reverse primer, and 487.5 µl of water. This was vortexed, spun down, and placed on ice. A PCR rack was placed on ice with four PCR tubes. Next 5.0 µl of the primer mix was added to each tube and 1.0 µl of the transformation DNA was added to each tube and

labeled. An enzyme master mix was made with 67 µl of water, 25 µl of Q5 reaction buffer, 2.5 µl of dNTP mix, and 0.5 µl of Q5 enzyme then mixed gently by pipetting and stored on ice. The PCR cycle was programmed as follows: step 1 lasting 30 seconds at 98 °C, step 2 lasting 5 seconds at 98 °C, step 3 lasting 10 seconds at 62.5 °C, step 4 lasting 3 minutes and 30 seconds at 72 °C, then it cycles back to step number 2, 30 times before moving to step 5 for 10 minutes at 72 °C, lastly a forever hold at 12 °C. Next 19 µl of the enzyme master mix was added to each PCR tube and mixed by pipetting before putting them in the thermocycler. When PCR was finished the samples were stored at -20 °C before gel electrophoresis and imaging.

The above reactions were examined by gel electrophoresis. A gel was made using the same protocol mentioned previously. The PCR products were prepared by adding 5.0 µl of 6x loading dye directly into the reaction and mixing. Then 5.0 µl of DNA ladder was loaded into the wells and the other wells were loaded with 5.0 µl of the PCR reactions. This gel ran at 120 V for 90 minutes before imaging (Fig. 9).

CROSSING FOR HOMOKARYONS

Transformants are often heterokaryotic. Homokaryons can be obtained by crossing transformants and collecting the homokaryotic offspring. Crosses were performed with unidirectional crosses on SCA as previously described (Samarajeewa *et al.* 2014). Specifically, Westergaard crossing media was made and inoculated. 60 plates were placed into the biosafety hood and sanitized with UV light along with a pipettor and tips. A single use aliquot of cryogenically frozen RTH1005.2 was obtained from the -80 °C freezer and a drop was transferred to the center of each plate. Once

all of the plates were inoculated, they were stored on a tray at room temperature until they were ready to use 6-8 days later.

The next step was fertilization of the inoculated Westergaard crossing plates. Conidial suspensions were made by placing one 1.5 ml MCT in the rack for each suspension. Then 500 µl of sterile water was transferred into each tube and the tubes were labeled for each of the transformants. A sterile wood applicator was used to transfer a clump of conidia into the appropriately labeled MCT and repeated for each tube. The tubes were placed on ice and then used to fertilize the Westergaard media inoculated with RTH1005.2. The suspensions were vortexed and 200 µl of each suspension was dropped onto the plates in 10 µl drops around the plate. These were labeled and placed on a tray and let sit at room temperature for 24 days.

After 24 days post fertilization the crosses were named and ascospores harvested by first sterilizing the working area with ethanol and placing 500 µl of water into a labeled MCT. The lid of the crossing plate was removed and placed upside down on the lab bench. A barrier pipette tip was used to transfer 50 µl of water from the MCT to some of the dried ascospores on the crossing lid. The water was pipetted up and down slowly to uptake ascospores from 3 different spots on the plate and then they were dispensed into the labeled MCT, and the crossing plate was placed back on the lab shelf. The suspensions were vortexed and placed in the dark at 4 °C for at least 16 hours.

The stock suspension of ascospores labeled RTKB10 was used to create a working suspension of ascospores. To a sterile MCT, 500 µl of sterile water was transferred and so was approximately 75 µl of the stock suspension to make a working suspension of approximately 1 ascospore per µl. The stock suspension was returned to 4 °C. The working stock was vortexed and gently tapped to collect the suspension on the bottom. The working stock was placed in a 60 °C heat block for 30 minutes to incubate while the biosafety hood was prepared with Vogel's hygromycin medium plates, sterile spreaders, and a sterile beaker. After 30 minutes the working suspension was removed from the heat block and placed at room temperature. The working suspension was inverted several times to resuspend the ascospores before transferring. Using sterile technique 50 ul, 100 ul, and 200 µl of the working suspension were transferred into the center of the petri dishes in the biosafety hood. Each was spread with a sterile spreader in a figure-eight motion while rotating the petri dish until the suspension was absorbed. The plates were then incubated at room temperature overnight.

The next day germlings were isolated from the petri dishes. A microscope was sterilized, and an ethanol candle was lit. Two new sterile syringes and needles were placed on sterile petri dishes. Twelve Vogels hygromycin slants were obtained from the fridge. Using a sterile needle, a square was cut around a single germinating ascospore through the microscope and transferred to the Vogels hygromycin slant. After transferring the germling the syringe and needle were heat sterilized and this procedure was repeated to put a germling in each of the 12 slants. Each were labeled RTKB10.1–RTKB10.12 and placed in a 32 °C incubator for 2 days and then moved to room temperature to culture on a shelf.

TEST CROSSES

Test crosses were performed with unidirectional crosses on SCA as described previously (Samarajeewa *et al.* 2014). Specifically, female strains were then prepared for the test crosses as follows: 20 Westergaard crossing medium plates were placed in the biosafety hood along with single use aliquots of cryogenically frozen RTH1623.1 and RTH1623.2 and pipette tips. Everything was sterilized and 5.0 µl of RTH1623.1 was transferred to the center of 10 petri plates while 5.0 µl of RTH1623.2 was transferred to the center of 10 petri plates. Each plate was labeled and placed at room temperature. A new set of test crosses was performed with the germlings on the Vogel's hygromycin slant and the petri dishes of RTH1623.1 and RTH1623.2. The *Sk-3* control strain (RDGR170.3), the wild type control strain (RZS27.10), and the six best growing germlings on the Vogel's hygromycin slants were all used in these crosses (Fig. 10A). Conidial suspensions were made for each of the controls and the test strains. Using sterile technique, 500 µl of water was transferred into each of the nine labeled MCT. A sterile wood applicator was used to transfer a clump of conidia into their respective MCT, leaving one MCT with only water as a negative control. These suspensions were then vortexed and the cultures of the RTH1623.1 and RTH1623.2 were placed on the lab bench to be fertilized. A 100 µl aliquot of the suspension was used to fertilize an RTH1623.1 and an RTH1623.2 plate. Once each plate was done, they were labeled with the name of the fertilizing strain and the date. This was repeated for each MCT suspension. The plates were then incubated at room temperature for 14 days before dissection of the fruiting bodies (Fig.10B).

Asci (ascospore sacs) were dissected from fruiting bodies into 25% glycerol. A dissecting microscope, 2 syringes and several new needles, microscope slides, Kim wipes, and 25% glycerol were all placed on a lab bench for the dissection. A 100 µl aliquot of 25% glycerol was placed on the center of two microscope slides and a small clump of perithecia was cut out of the crossing plate and placed onto one of the microscope slides. Using the dissecting microscope perithecia were separated and cleaned off then transferred to the second microscope slide. Using the needles the perithecia were cut open and a rosette of asci was pushed out. After releasing around 10 rosettes they were pushed to the center of the glycerol solution and the perithecial wall debris were removed. Then a coverslip was carefully placed, and the extra solution was wicked out with Kim wipes. The plates were air dried for a couple minutes and labeled with the cross, name, and days post fertilization. The cover slip was sealed with clear nail polish and placed in a drawer in the dark until imaging. This was completed for all 8 crosses. Digital images of asci (ascospore sacs) were obtained with a Leica DMBRE microscope and Zeiss imaging system.

Genotyping of test strains

The IBI genomic DNA mini kit protocol previously mentioned was used to isolate DNA from test strains RDGR170.3, RZS27.10, RTKB10.4, RTKB10.5, RTKB10.6 and RTKB10.7. For PCR the nested forward primer V0373-E and nested reverse primer V0372-F (2428) were used. A primer mix was made in a 1.5 ml MCT using 6.3 µl nested forward primer, 6.3 µl nested reverse primer, and 487.5 µl of water. This was vortexed, spun down, and placed on ice. A PCR rack was placed on ice with 4 PCR tubes. Next 5 µl of the primer mix was added to each tube and 1 µl of the transformation DNA was added to each tube and labeled. An enzyme master mix was made

with 67 µl of water, 25 µl of Q5 reaction buffer, 2.5 µl of dNTP mix, and 0.5 µl of Q5 enzyme then mixed gently by pipetting and stored on ice. The thermocycler was set to the Q5 protocol which was step 1 lasting 30 seconds at 98 °C, step 2 lasting 5 seconds at 98 °C, step 3 lasting 10 seconds at 62.5 °C, step 4 lasting 3 minutes and 30 seconds at 72 °C, then it cycles back to step number 2, 30 times before moving to step 5 for 10 minutes at 72 °C, lastly a forever hold at 12 °C. Next 19 µl of the enzyme master mix was added to each PCR tube and mixed by pipetting before putting them in the thermocycler. Once PCR was finished the samples were stored at -20 °C before being imaged. The gel was made the same way as previously mentioned. The samples were prepared by adding 5 µl of 6x loading buffer into the 25 µl reaction and mixed by pipetting. Then 5 µl of the DNA ladder was loaded and 30 µl of the PCR samples were loaded and ran at 120 V for 120 minutes and imaged (Fig. 11). This was done to confirm the genotype. The length of the *v373Δ* genotype is 2708 bp (Fig. 12). The length of the regular *v373+* genotype is 2165 bp (Fig. 13).

CHAPTER III

RESULTS

My original hypothesis was that the deletion of the *v373* interval in *N. crassa* will disrupt *Sk-3* based spore killing. This hypothesis was based on preliminary results that suggested a related interval, called *v350*, is required for spore killing by *Sk-3*. The interval *v350* and interval *v373* are located on the *Sk-3* Chromosome III, between positions 320,000 to 340,000, and the relationship between the two intervals is shown in Fig. 6. A normal perithecium produces asci, with each typically containing eight viable ascospores while asci that demonstrate spore killing typically have four mature viable ascospores and four shriveled inviable ascospores. Imaging the rosettes provided an ascus phenotype summary of each test cross (Fig.14). The control cross, RZS27.10 x RTH1623.1, was dissected from perithecia on day 14 post fertilization showed multiple asci with eight mature ascospores and only a few asci with four mature ascospores and four shriveled ascospores as expected (Fig. 15). The other control cross, RDGR170.3 x RTH1623.2, was dissected from perithecia on day 14 post fertilization showed all asci with four mature ascospores and four shriveled ascospores, demonstrating the spore killing in effect as expected (Fig. 16). The test cross RTKB10.4 x RTH1623.1, dissected from perithecia on day 14 post fertilization, showed most asci with eight mature ascospores (Fig.17). The test cross RTKB10.5 x RTH1623.2, dissected from perithecia on day 14 post fertilization, showed most asci with eight mature ascospores (Fig.18). The test cross RTKB10.6 x

RTH1623.1, dissected from perithecia on day 14 post fertilization, showed most asci with 8 mature ascospores (Fig. 19). The test cross RTKB10.7 x RTH1623.2, dissected from perithecia on day 14 post fertilization, showed most asci with eight mature ascospores (Fig. 20). The test cross RTKB10.11 x RTH1623.1, dissected from perithecia on day 14 post fertilization, showed most asci with eight mature ascospores (Fig.21). The test cross RTKB10.12 x RTH1623.2, dissected from perithecia on day 14 post fertilization, showed most asci with eight mature ascospores (Fig.22).

CHAPTER IV

DISCUSSION

In this study, we looked at refining the *rfk-2* locus of *Sk-3* in *Neurospora crassa.* The data showed that deleting interval *v373* eliminates sport killing, which leads us to believe that interval *v373* may contain *rfk-2.* In summary, my results support my initial hypothesis and indicate that interval *v373* is required for spore killing

There may have been some technical errors in the study. In Figures 15-22, there are some asci of 8 ascospores while there are a couple asci with 4 ascospores, this could be because the dissection could have disrupted some spores or that some are naturally driven to kill ascospores. Some ascospores were white which means they needed more time to mature. In Figure 11, 30 µl of PCR product was put into the wells instead of 10 µl, which led to too much product and the possibility the multiple bands were present but missed.

This study can give us further knowledge and insight into meiotic drive. If we can locate specifically the region that controls spore killing, then we can learn more about how meiotic drive works. Understanding this form of spore killing could lead to scientists learning how to control other fungal species as well. Once we understand gene drive in fungi, we may be able to use gene drive to control plant pathogens to help farming and agriculture (Gardiner et al. 2020).

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Table 1 Strains used in this study

Strain name	Genotype
RTH1005.1	*rid; fl A+*
RTH1005.2	*rid; fl a+*
RZS27.10	*rid; mus-51^{RIP70} a+*
RTH1623.1	*rid; fl; sad-2Δ::hph+ A+*
RTH1623.2	*rid; fl; sad-2Δ::hph+ a+*
RDGR170.3	*rid; Sk-3+; mus-51Δ::bar+ A+*
TTKB1.15	V373-based hygromycin resistant transformant of ISU-3291
TTKB1.14	V373--based hygromycin resistant transformant of ISU-3291
TTKB1.13	V373--based hygromycin resistant transformant of ISU-3291
RTKB10.4	*rid; Sk-3+* v373Δ*::hph+-; mus-51?::bar+ a+* (offspring of TTKB1.15 × F2-26)
RTKB10.5	*rid; Sk-3+* v373Δ*::hph+-; mus-51?::bar+ A+* (offspring of TTKB1.15 × F2-26)
RTKB10.6	*rid; Sk-3+* v373Δ*::hph+-; mus-51?::bar+ a+* (offspring of TTKB1.15 × F2-26)
RTKB10.7	*rid; Sk-3+* v373Δ*::hph+-; mus-51?::bar+ A+* (offspring of TTKB1.15 × F2-26)
RTKB10.11*	*rid; Sk-3+* v373Δ*::hph+-; mus-51?::bar+ a+* (offspring of TTKB1.15 × F2-26)
RTKB10.12*	*rid; Sk-3+* v373Δ*::hph+-; mus-51?::bar+ A+* (offspring of TTKB1.15 × F2-26)

*Predicted genotype based of lineage of strain and hygromycin resistance (not genotyped by PCR). The *rid* allele disrupts Repeat Induced Point Mutation. The *fl* allele disrupts macroconidiation. *The sad-2Δ::hph+* allele suppresses Meiotic Silencing by Unpaired DNA

Table 2 Primers used in this study

Name (Alias)	Sequence (5′ > 3′)
Hph-cen-f	AACTGATATTGAAGGAGCATTTTTTGG
Hph-cen-r	AACTGGTTCCCGGTCGGCAT
V0373-A	AGAGAACCAAGGGAAGGCAGGGC
V0373-B	AAAAAATGCTCCTTCAATATCAGTTTGGCCCTAACGCAGTTGCCTGTT
V350-C	GAGTAGATGCCGACCGGGAACCAGTTCCTTGTCCTCTCGGTCCTCTCTGT
V350-D	GGTAGCGTTCTGGGATGAGTGTGG
V0373-E	TCCAAAGGGAAGGACCGGGCACA
V0372-F	GGACCTGGCTGTTGCTGGCGTAG

Figure 1 Methods overview flow chart

>V373 left flank
AGAGAACCAAGGGAAGGCAGGGCCCTTGTCGGAGAAGCTAACCTGAAGGCACCCATAGTTTTC
TTTCCCTTGATGATGCCTGACATATTATTGTAGAGTTCCTTCTTCAACGTACCGGATCGTGTT
CTCACTCTAAGATAGAAAGGGCGAGGAAGGACTTGGTGTCAATTGGTGACTGAACTCCCAACA
ACACAAAGCTGTCGTCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACA
CTAACGAGCGATTTTGCCACCTAGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCCTAC
CACGCTTCTTTTTCCGTCCACCGGCTCTTGGAGTACCGTACATACCTCAAACACTTCATTCCA
CCCTGTTCTGGAATTGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCAT
GTTGGTACTTCAGGAAGGAGGAGAGGTACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAA
GGGCAATCACGGGGACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCG
TTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCT
GGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGA
AGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAG
CCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGA
GCGTTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCA

Figure 2 The V373 left flank. The predicted 865 bp sequence of the V373 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V373-A and V373-B from RDGR170.3 genomic DNA.

>V373 right flank
CCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCC
CCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCC
CCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCA
GGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCC
GTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCT
CCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGC
TTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAA
TTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTG
CCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGG
AGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAG
GTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAAC
CCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCC
AACGGCGACGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACC

Figure 3 The V373 right flank. The predicted 813 bp sequence of the V373 right flank is shown. This sequence was PCR-amplified with primers V350-C and V350-D from RDGR170.3 genomic DNA.

>V373 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 V373 center fragment. The 1412 bp sequence of the V373 center fragment is shown. This sequence was PCR-amplified with primers HPH-CEN-F and HPH-CEN-R from plasmid pTH1256.1. The sequence contains *Aspergillus nidulans trpC* promoter sequences upstream of the 1026 bp *hph+* coding region. The positions of the *hph+* start codon and stop codon are indicated with gray shading.

>V373 DNA interval
TGAAACATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAATGATAGACGGATGAGTAAGGAGTG
TCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTCCCATGCGCACTTGCACATCATGATCAT
GACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATA
CGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCC
AGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAA
AAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTT
TGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACC
ACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGA
GCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCC
GGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTT
TTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCA
GAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCG
CTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCA
TTCGTT

Figure 5 The V373 DNA interval. The DNA sequence of the 1320 bp V373 DNA interval is shown. DNA replacement vector V373 was designed to delete this interval by replacing it with *hph*+.

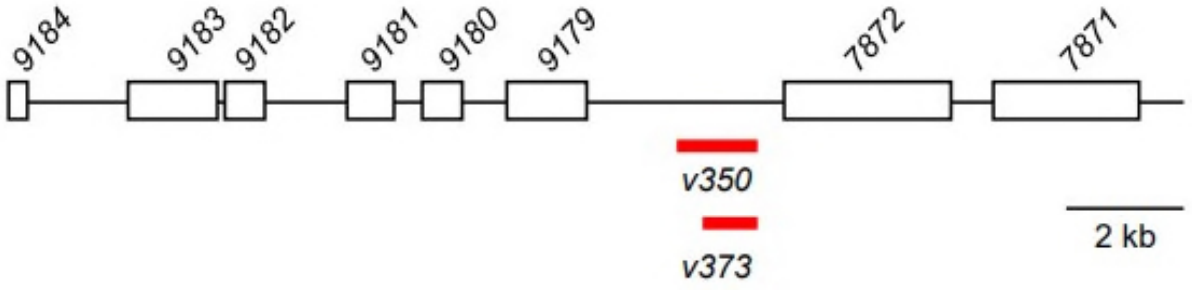

Figure 6 *Sk-3* Chromosome III positions 320,000–340,000. A diagram of of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the locations of protein coding genes. Gene numbers were identified with blastx searches of an *N. crassa* protein database. The location of intervals v350 and v373 are indicated with red bars, respectively.

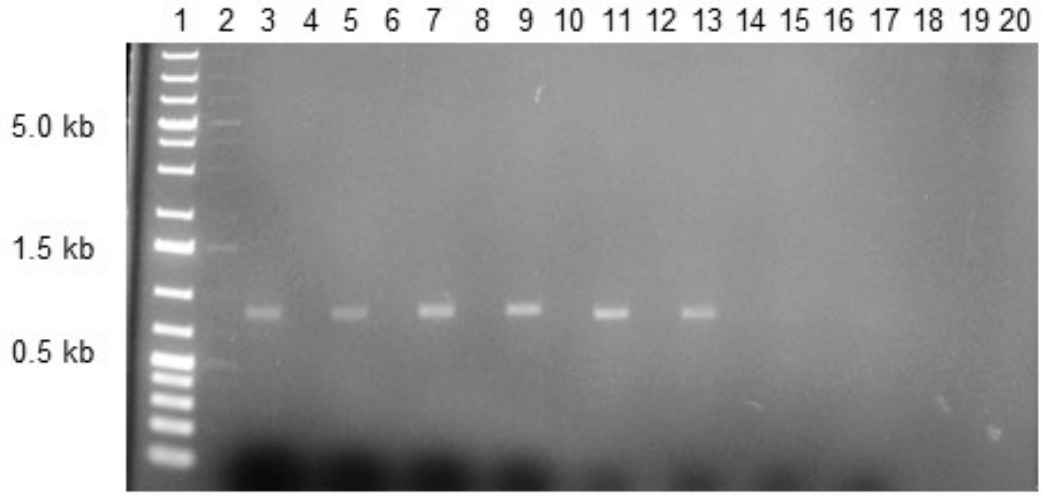

Figure 7 v373 construction: left and right flanks. PCR products for v373 left and right flanks. The left flank for v373 was amplified from RDGR170.3 genomic DNA with primers V0373-A and V0373-B. The right flank for v373 was amplified from RDGR170.3 genomic DNA with primers V350-C and V350-D. Products were gel purified as described in the methods section. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lanes 2,4,6,8,10,12,14,18,19,20) not applicable, Lane 3) 5 µl purified product (v373 left flank trial 1); Lane 5) 5 µl purified product (v373 left flank trial 2); Lane 7) 5 µl purified product (v373 left flank trial 3); Lane 9) 5 µl purified product (v373 left flank trial 4); Lane 11) 5 µl purified product (v373 right flank trial 1); Lane 13) 5 µl purified product (v373 right flank trial 2); Lane 15) 5 µl purified product (v373 right flank trial 3); Lane 17) 5 µl purified product (v373 right flank trial 4); The expected product lengths for the v373 left and right flanks are 865 bp and 813 bp, respectively.

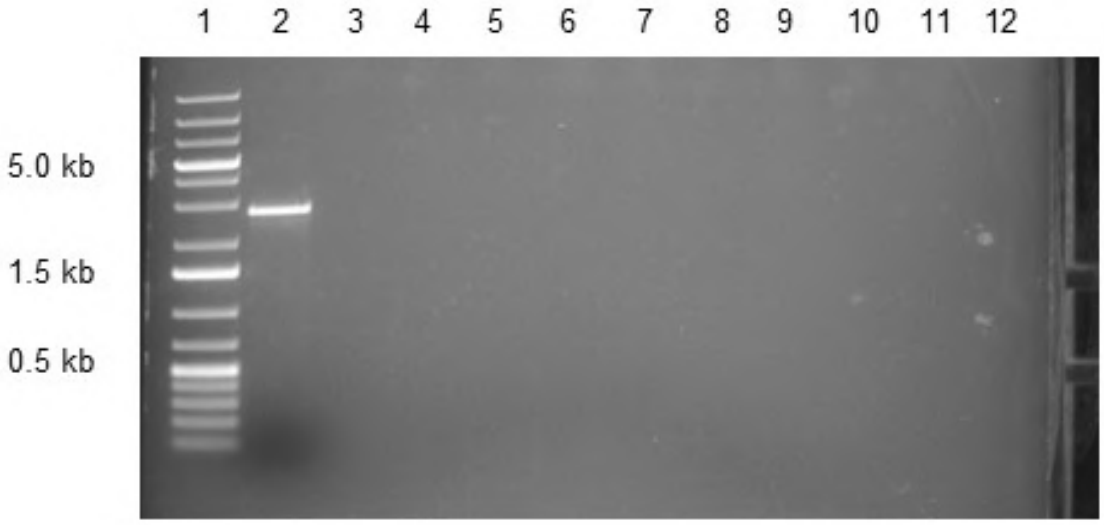

Figure 8 v373 construction: fusion and nested amplification. The v373 left flank, v373 center fragment, and v373 right flank were fused by DJ-PCR. The fusion product was amplified with primers V0373-E and V0372-F. The amplified product was column purified as described in the methods section. The amplified and purified product was examined by gel electrophoresis with ethidium bromide staining. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lane 2) 3 µl of amplified and purified v373. Lanes 3–12) not applicable. The expected length of v373 is 3090 bp after fusion and 2708 bp after nested amplification.

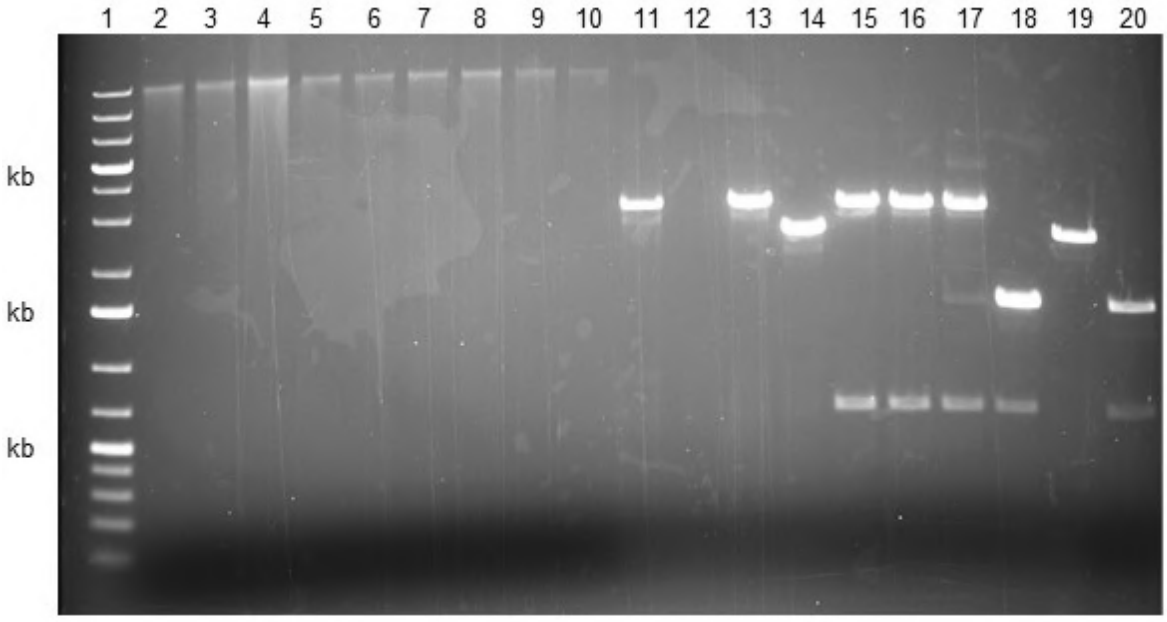

Figure 9 Transformant screening. Genomic DNA samples from candidate transformants TTKB1.13, TTKB1.14, and TTKB1.15, and transformation host RDGR170.3 were used as templates in PCR reactions with primers V0373-E and V0372-F. PCR products were examine by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lanes 2-5) 5 µl of DNA from templates TTKB1.13, TTKB1.14, TTKB1.15, respectively; Lanes 6-10) Not applicable; Lanes 11–14) 5 µl PCR products from templates TTKB1.13, TTKB1.14, TTKB1.15, and RDGR170.3, respectively; Lanes 15-18) Not applicable; Lane 19) 5 µl PCR product of RDGR170.3 with primer set A V0373-E and V0372-F; Lane 20) Not applicable. The expected PCR product lengths are 2165 bp for *v373+* and 2708 bp for *v373Δ::hph+*.

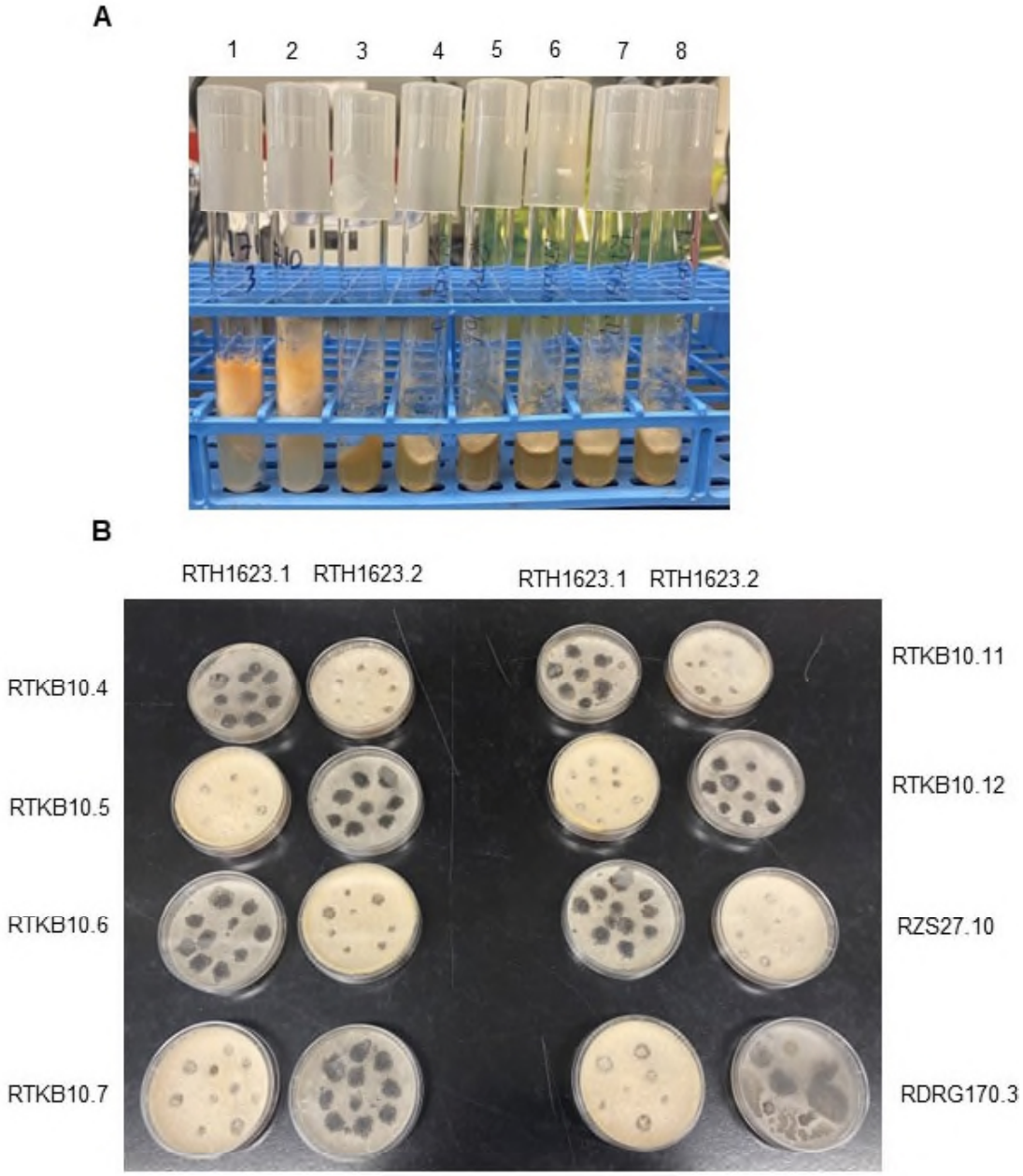

Figure 10 Test strains and crosses. **(A)** 1) RDGR170.3, Sk-3 control; 2) RZ27.10, Sk-S control. Six hygromycin-resistant offspring from cross RTKB10 (TTKB1.15 × RTH1005.2) were selected for test crosses; 3) RTKB10.4, 4) RTKB10.5, 5) RTKB10.6, 6) RTKB10.7, 7) RTKB10.11, and 8) RTKB10.12. (B) Test crosses were performed with RTH1623.1 and RTH1623.2. Images are of crossing dishes approximately nineteen days post fertilization. Perithecial dissection and imaging was done fourteen days post fertilization.

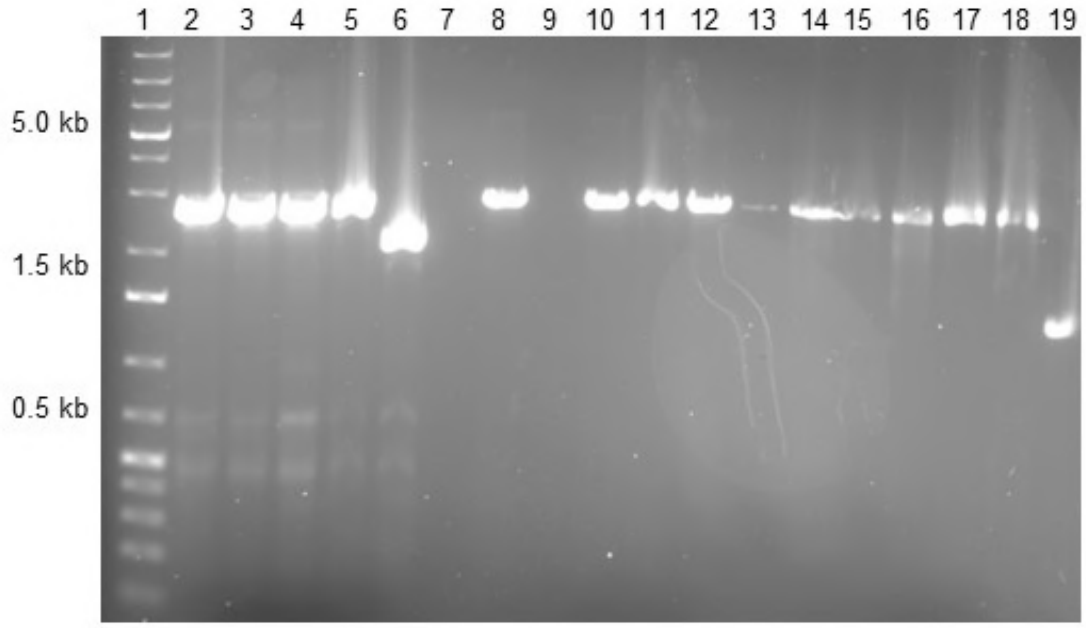

Figure 11 Test strain genotype confirmation. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0373-E and V0-372-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product length for the *v373+* and *v373Δ* genotypes are 2165 bp and 2708 bp, respectively. Lane 1 contains 0.5 µg of GeneRule 1 Kb Plus DNA ladder (ThemoFisher). DNA templates for each PCR reaction are as follows: Lane 2, 30 µl RTKB10.4; Lane 3, 30 µl RTKB10.5; Lane 4, 30 µl RTKB10.6; Lane 5, 30 µl RTKB10.7; and Lane 6, 30 µl RDGR170.3; Lanes 7–19) Not appliable. These results show that test strains RTKB10.4, RTKB10.5, RTKB10.6, and RTKB10.7 have the *v373Δ* genotype.

>*v373Δ::hph* PCR product, predicted sequence, primers V0373-E and V0372-F

TCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACACTAACGAGCGATTTTGCCACCTAGA
AGTATACCTCTATGCTCAACAGTAGGTAGACATCCTACCACGCTTCTTTTTCCGTCCACCGGCTCTTGGAGTAC
CGTACATACCTCAAACACTTCATTCCACCCTGTTCTGGAATTGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTT
GAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGGTACGTTGGGTGCGTTAGTGTATCTTGATCATG
ATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGC
TCGAATTCACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCAT
GTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTT
TGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCT
TGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCAAACT
GATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTTAGT
CGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATCAGCCCC
ACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCT
GCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGT
TCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAA**ATG**AAAAAGCCTG
AACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCG
GAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGC
CGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTG
ACATTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTG
CCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAG
CCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCG
CGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCT
CTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAA
CAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAAT
ACGAGGTCGCCAACATCCTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGG
AGGCATCCGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCA
GAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAG
CCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTC
GCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAA**TAG**AGTAGATGCCGACCGGGAACCAG
TTCCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGG
GATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTT
GTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGC
ACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTC
TAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGT
CTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAAT
TCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATG
GAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCA
AATGTCCATGTCCCCTCGAG**ACTACGCCAGCAACAGCCAGGTCC**

Figure 12 PCR product sequence: *Sk-3 v373Δ::hph+* genotype. The predicted sequence of DNA amplified with primers V0373-E and V0372-F from a template consisting of *Sk-3 v373Δ::hph+* genomic DNA is shown in FASTA format. The binding sites of V0373-E and V0372-F are indicated with bold font. The start and stop codons of the *hph* coding region are shown with white font on black background. The length of the sequence is 2708 bp.

>v373+ PCR product, predicted sequence, primers V0373-E and V0372-F

TCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACACTAACGAGCGATTTTGCCACCT
AGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCCTACCACGCTTCTTTTTCCGTCCACCGGCTCTTG
GAGTACCGTACATACCTCAAACACTTCATTCCACCCTGTTCTGGAATTGTTGGGACAGAGAGTGGCGTCTG
CCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGGTACGTTGGGTGCGTTAGTGT
ATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCAC
GAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCC
TGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACG
GTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAGG
CCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAAAGGAGCATG
AACAGGCAACTGCGTTAGGGCCA**TGAAACATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAATGATAGACG**
GATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTCCCATGCGCACTTGCACATCA
TGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATAC
GACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCC
TGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGA
ATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGT
TTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGA
AGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAA
GCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACT
TTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGG
GCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCT
GTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGG
TTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGC
TAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGG
CAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCC
ATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGG
CGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCT
CCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACC
CTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCATTGCCAAACCAG
ACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGG
CAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCAT
GTCCCCTCGAG**ACTACGCCAGCAACAGCCAGGTCC**

Figure 13 PCR product sequence: *Sk-3 v373+* genotype. The predicted sequence of DNA amplified with primers V0373-E and V0372-F from a template consisting of *Sk-3 v373+* genomic DNA is shown in FASTA format. The binding sites of V0373-E and V0372-F are indicated with bold font. The V373 interval is indicated with red font. The length of the sequence is 2165 bp.

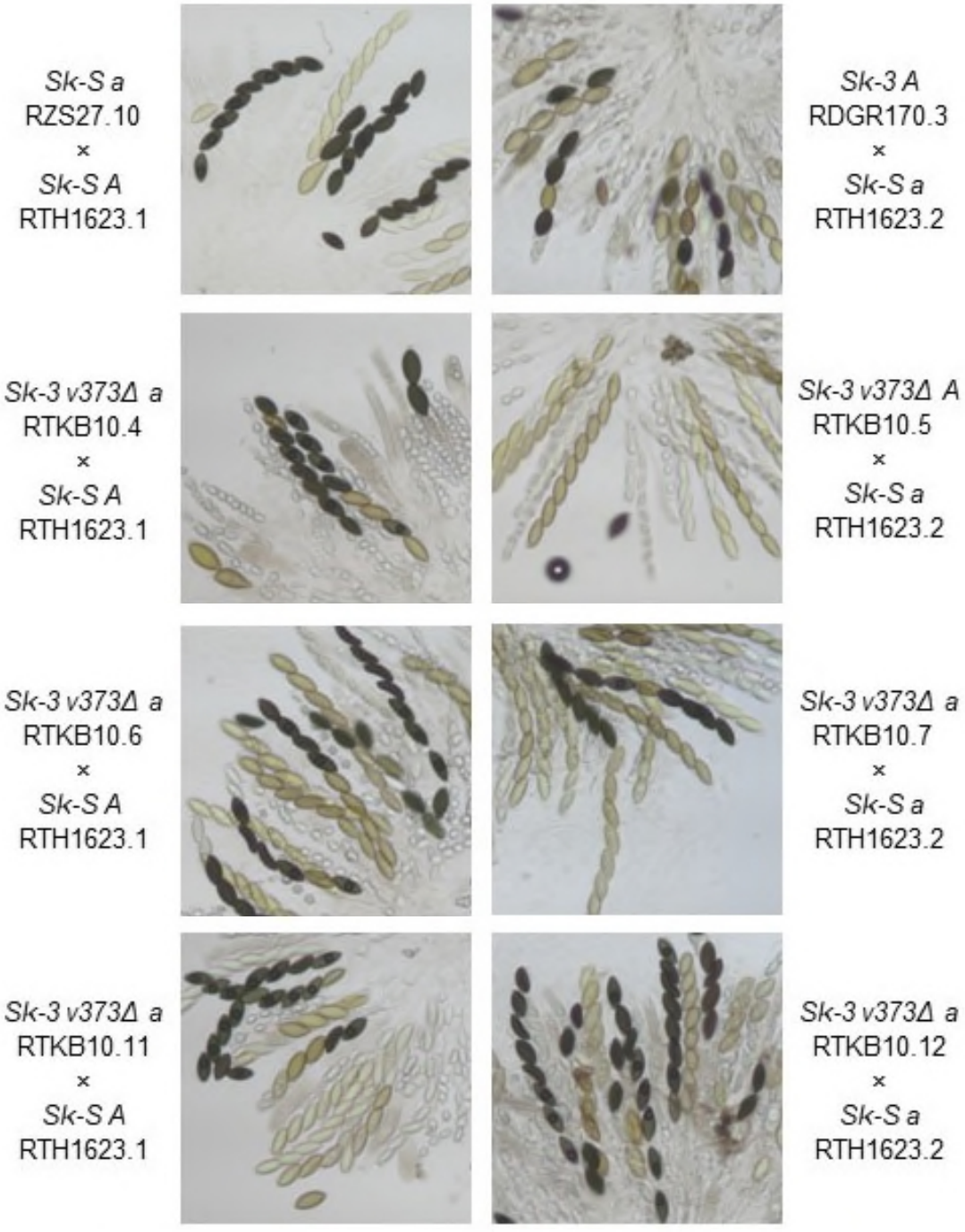

Figure 14 Ascus phenotype summary. Asci were dissected from perithecia of eight crosses on day 14 post fertilization and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *v373* disrupts spore killing.

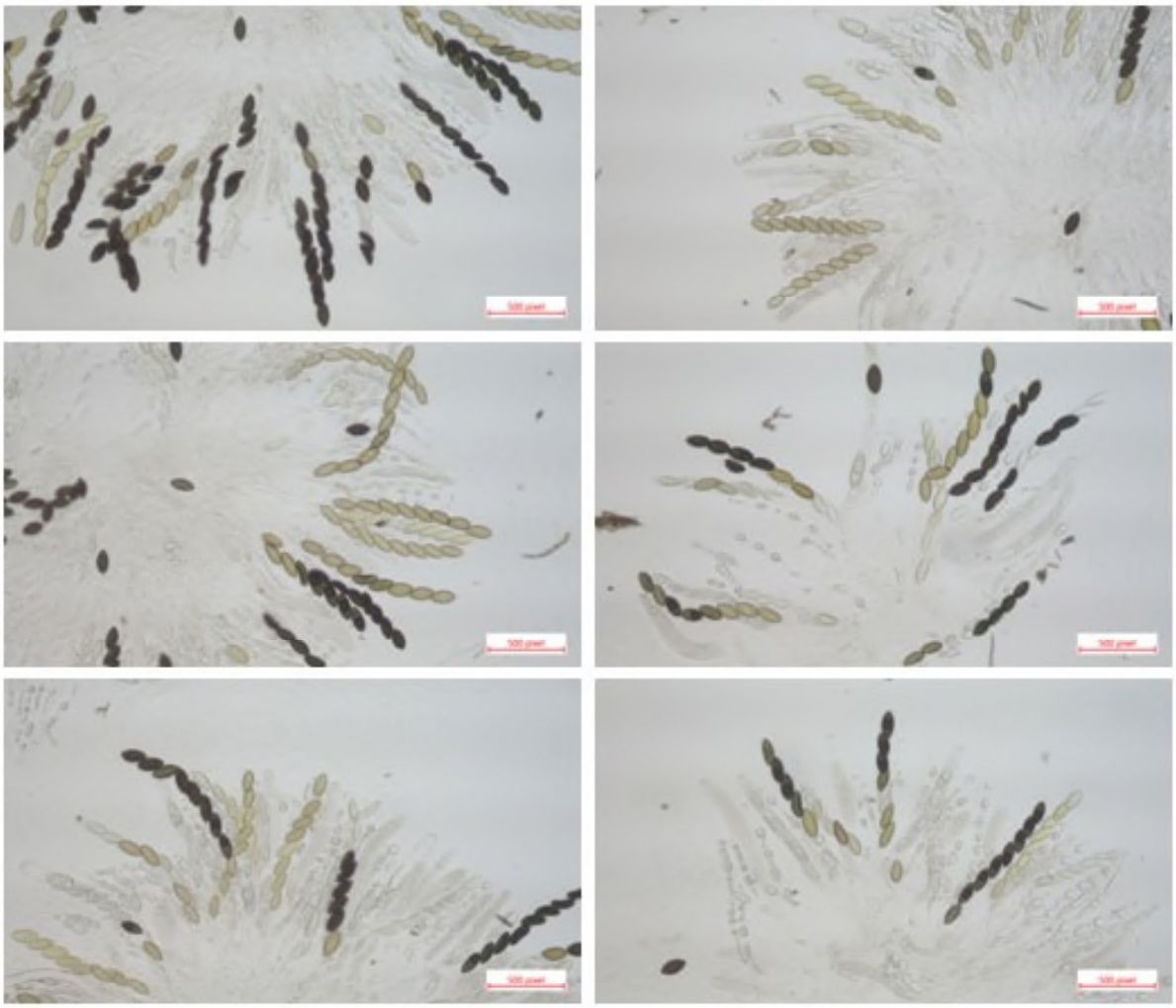

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RZS27.10 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

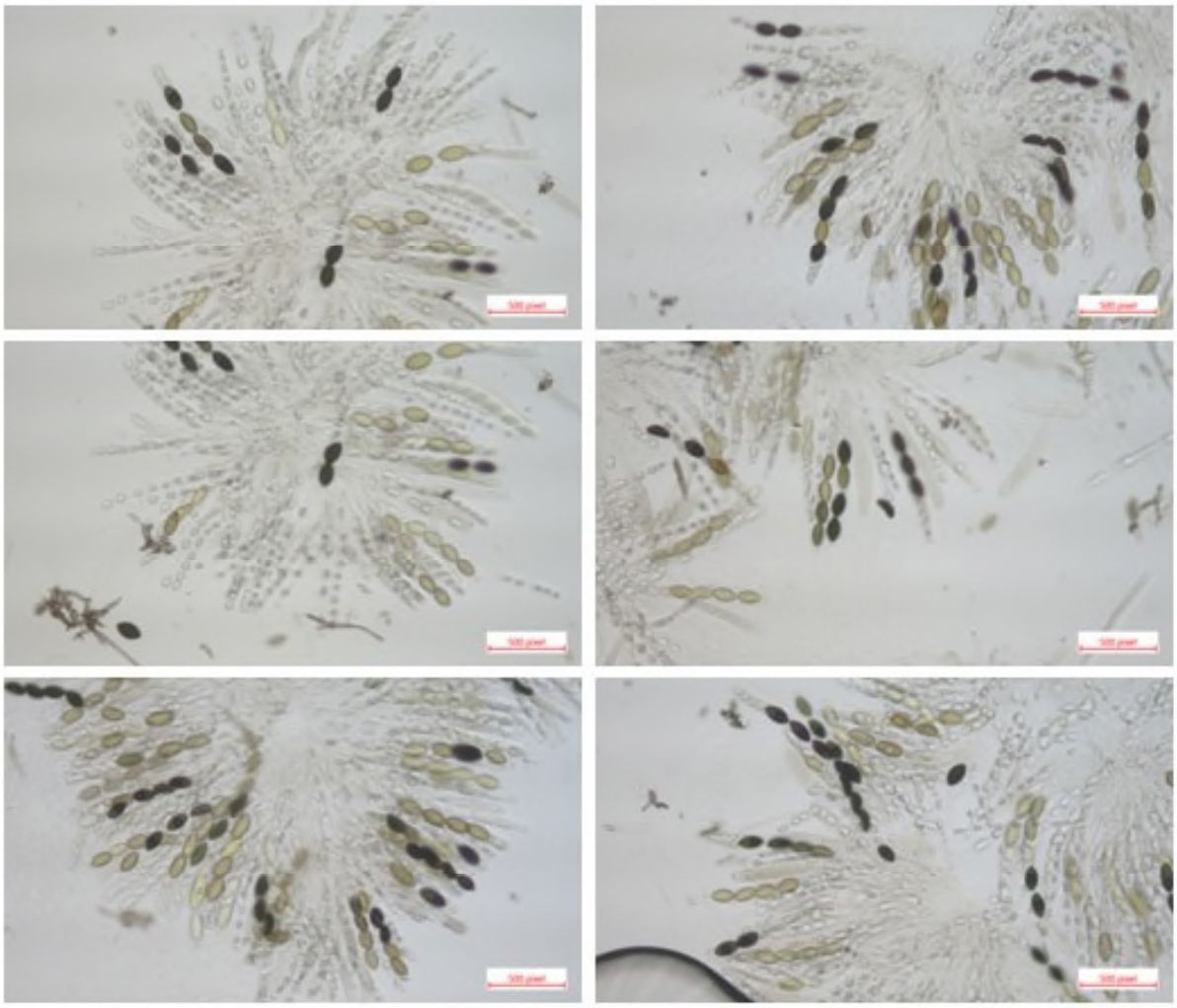

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RDGR170.3 × RTH1623.2 on day 14 post fertilization and imaged under magnification.

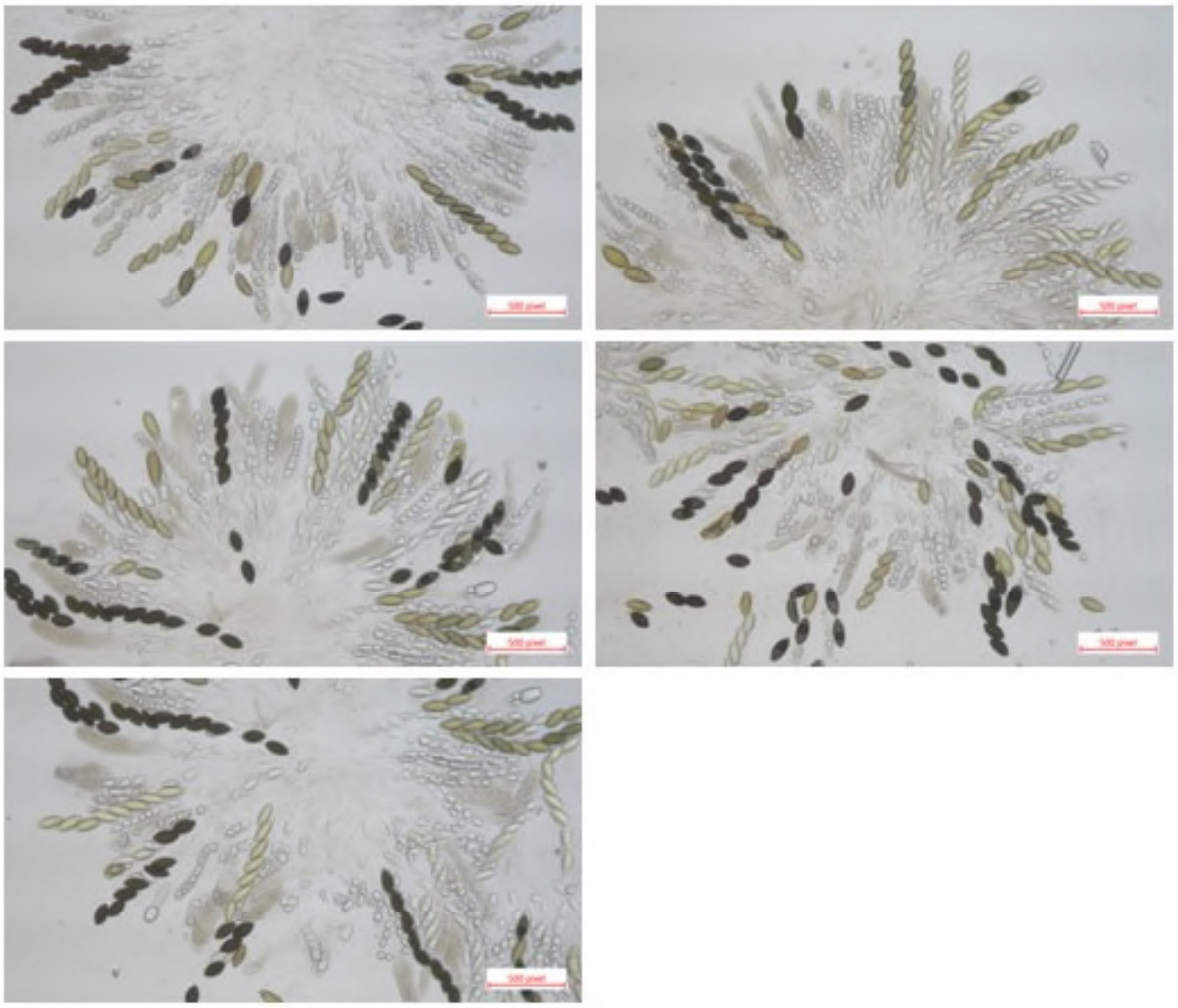

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RTKB10.4 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

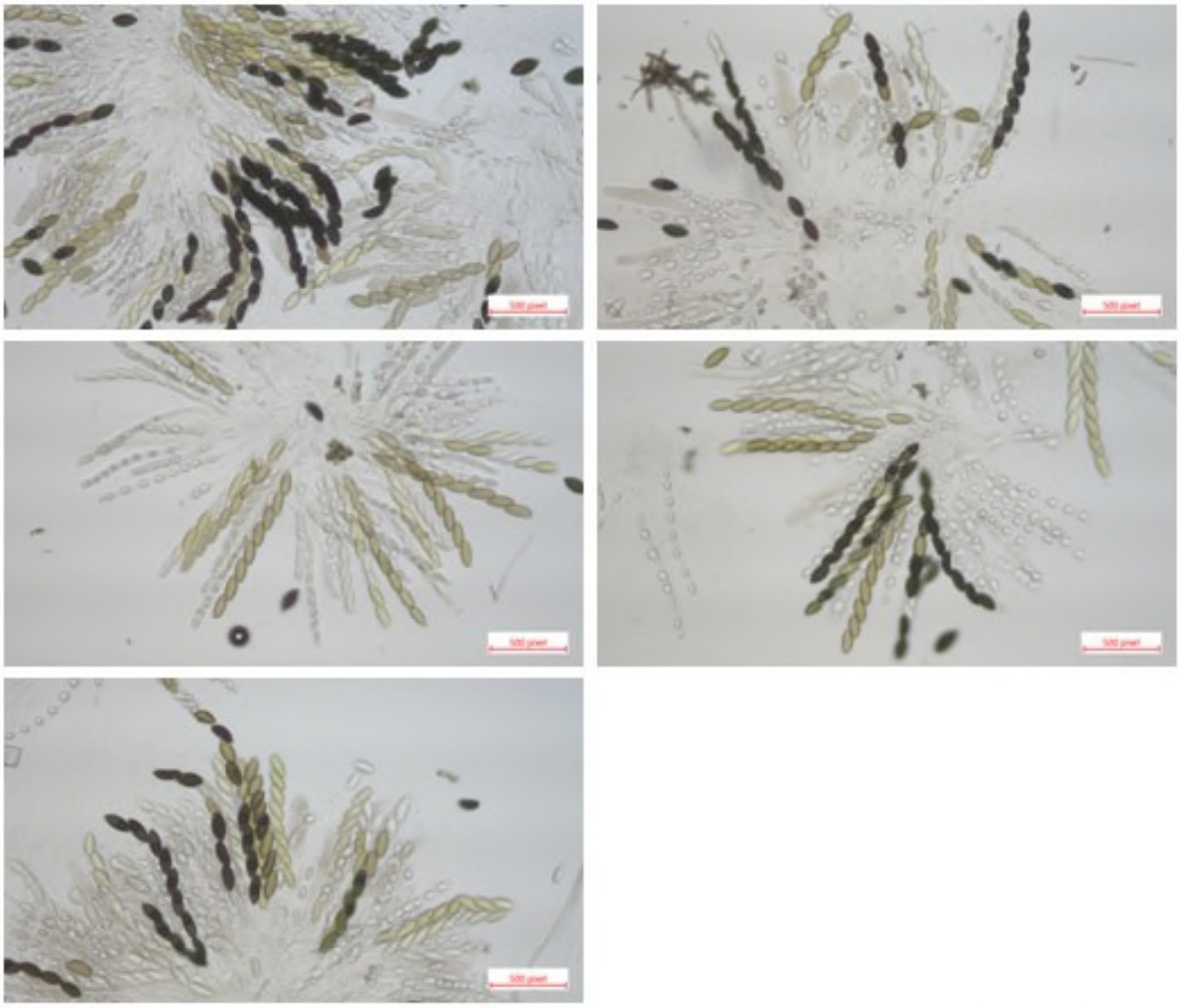

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RTKB10.5 × RTH1623.2 on day 14 post fertilization and imaged under magnification.

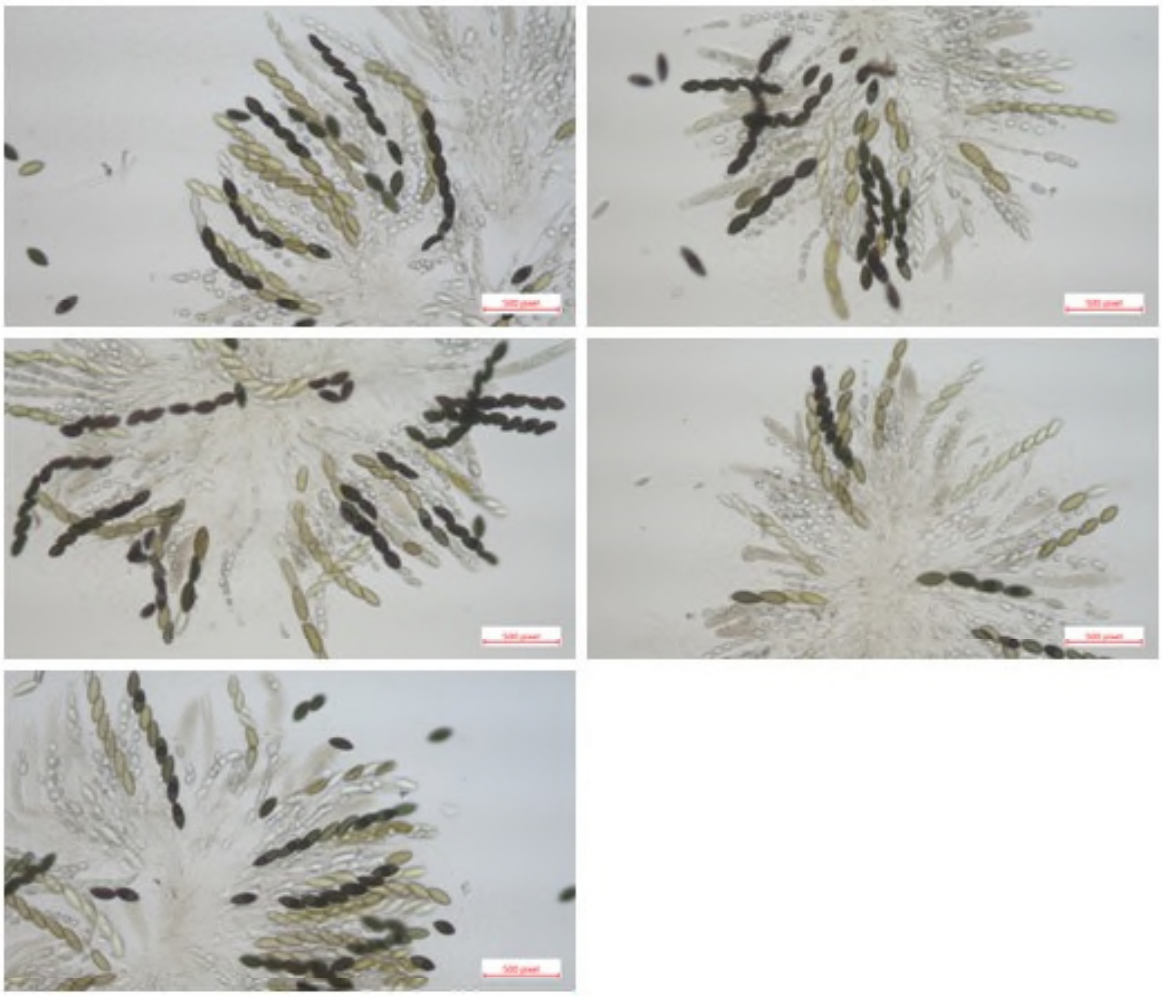

Figure 19 Ascus phenotypes. Asci were dissected from perithecia of RTKB10.6 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

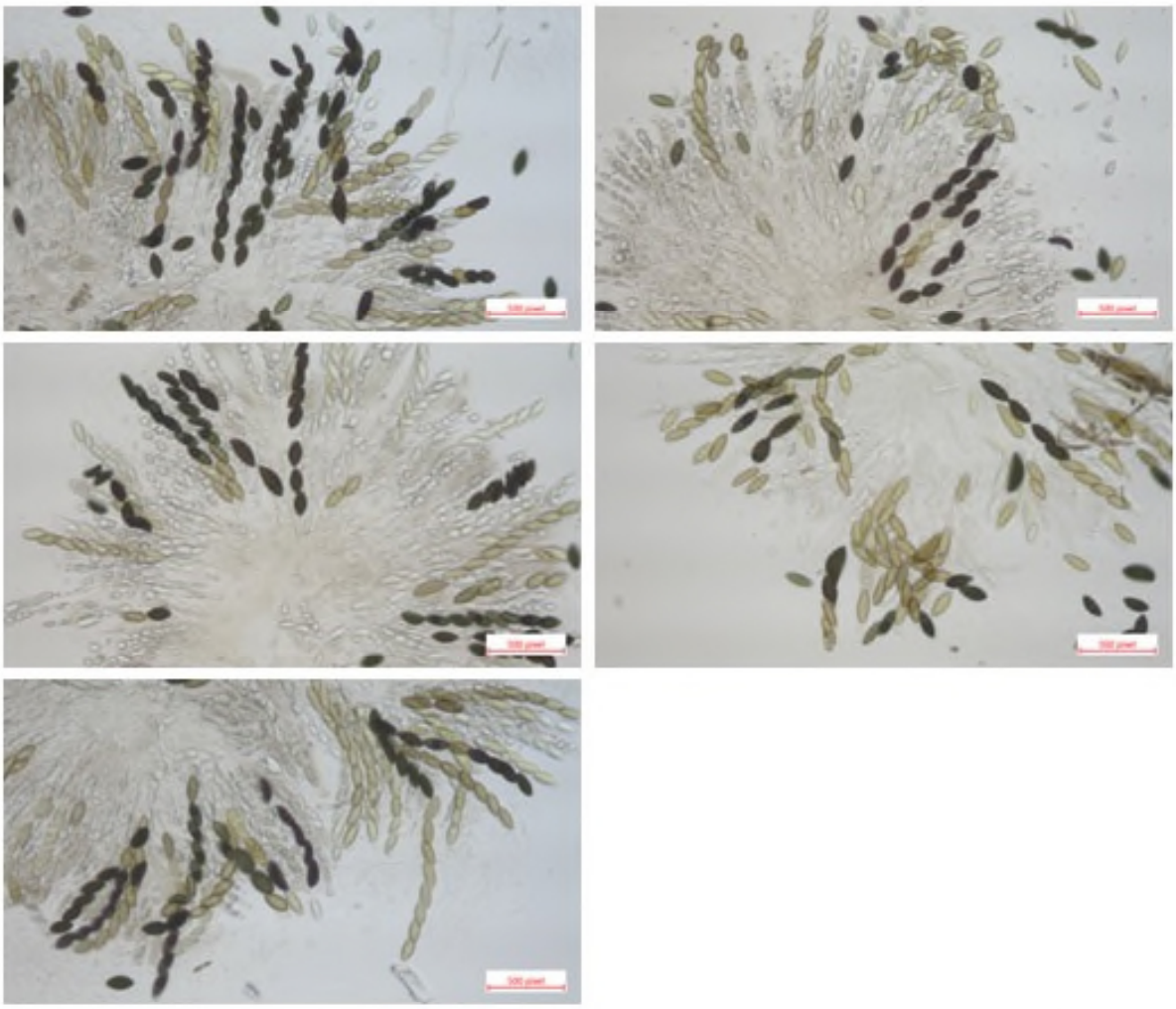

Figure 20 Ascus phenotypes. Asci were dissected from perithecia of RTKB10.7 × RTH1623.2 on day 14 post fertilization and imaged under magnification.

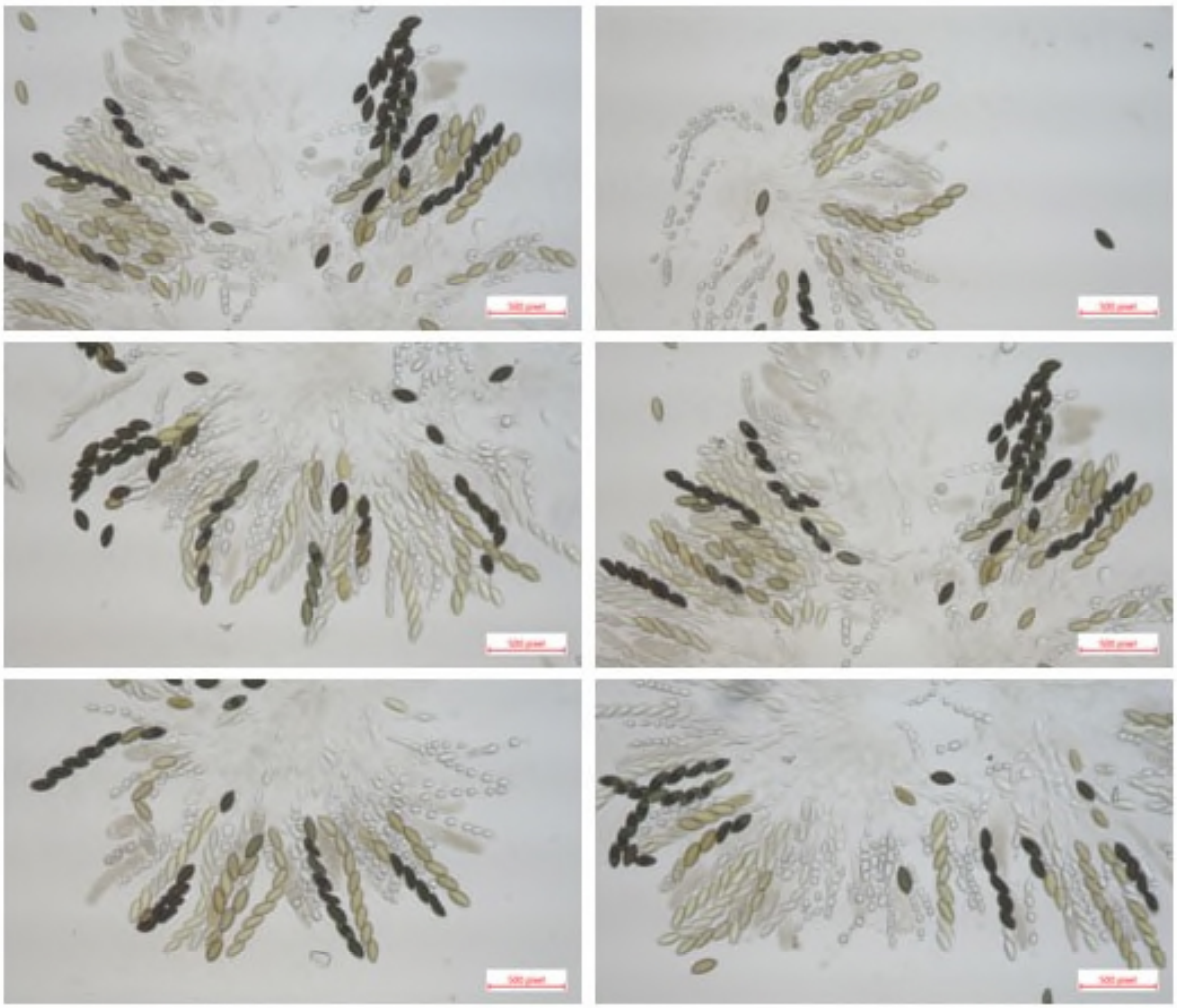

Figure 21 Ascus phenotypes. Asci were dissected from perithecia of RTKB10.11 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

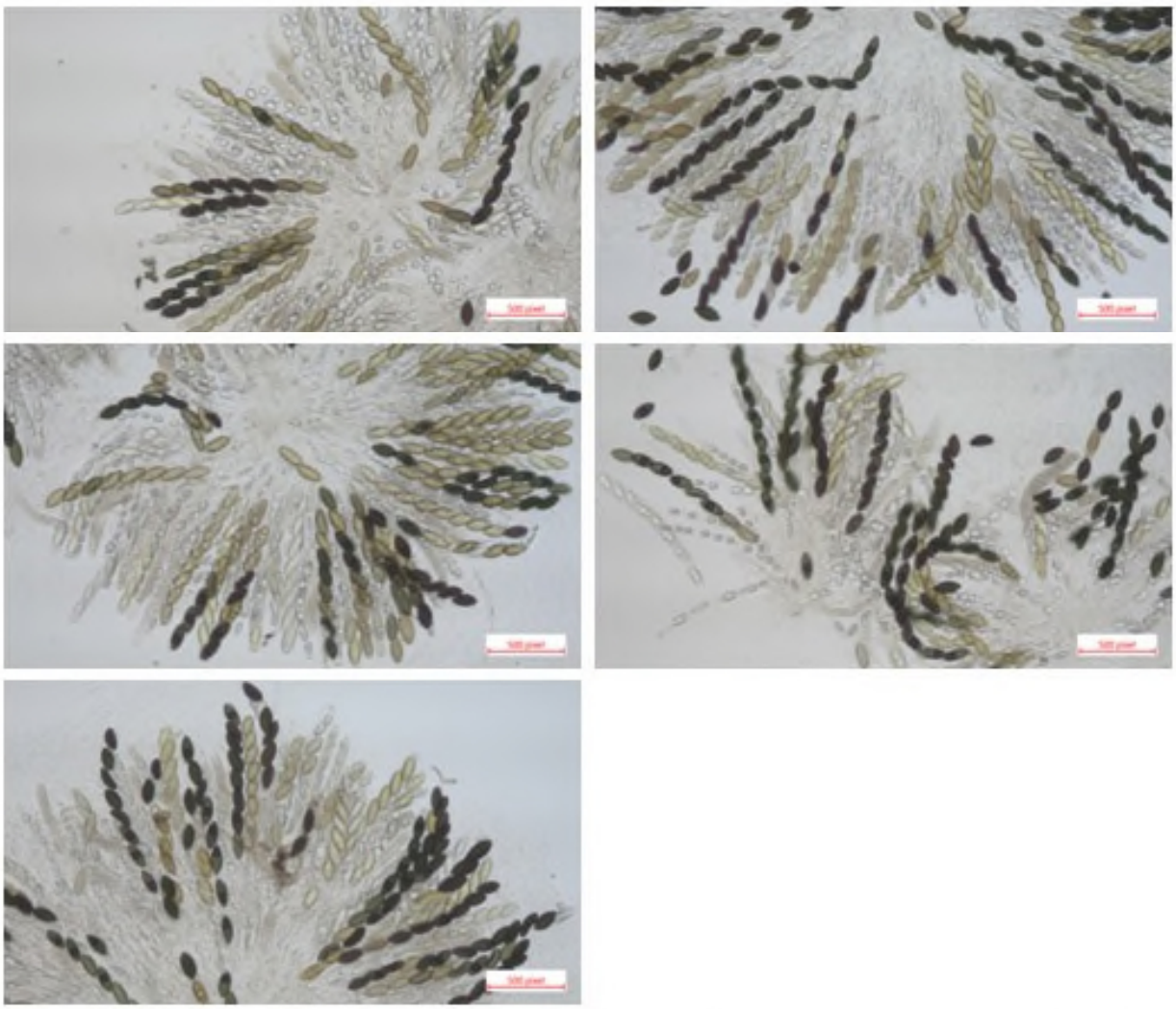

Figure 22 Ascus phenotypes. Asci were dissected from perithecia of RTKB10.12 × RTH1623.2 on day 14 post fertilization and imaged under magnification.