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Examining the Mechanism of Spore Sacs Undergoing Sk-3-based Spore Killing after Deletion of Neurospora crassa DNA Intervals i382 and i400

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EXAMINING THE MECHANISM OF SPORE SACS UNDERGOING *SK-3*-BASED SPORE KILLING AFTER DELETION OF *NEUROSPORA CRASSA* DNA INTERVALS *i382* AND *i400*

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Neurospora crassa is a well-known model organism for studying eukaryotic genetics, particularly non-Mendelian inheritance mechanisms such as meiotic drive. In *N. crassa*, meiotic drive can be observed in fungal spore killing, where *Spore killer-3 (Sk-3)* is a selfish genetic element transmitted to offspring through spore killing. *Sk-3* is thought to contain two principal components: a killer (poison) gene and a resistance (antidote) gene. While the resistance gene (*rsk*) has been identified, the killer gene remains unknown. Building on previous research that identified a 1.3 kb DNA interval (*i350*) essential for *Sk-3*-based spore killing, I analyzed two subintervals, *i382* and *i400*, to narrow down the functional components of the *Sk-3* locus. Deletion of *i382* does not disrupt spore killing and deletion of interval *i400* partially disrupts spore killing but does not eliminate it. Future work should retest the *i400* strains in spore killing assays, to determine if the partial spore killing phenotype can be detected in all crosses when larger numbers of rosettes are examined. The findings presented here help narrow down the search for the unknown poison gene involved in spore killing, which is a critical step towards understanding processes that allow for the evolution of *Sk-3*-type selfish genetic elements.

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INTRODUCTION

1.1 *Neurospora crassa*

Neurospora are a group of fungi found in climates that consist of tropical, subtropical, and temperate environments. These extensive conditions of living empowers the growth of *Neurospora* fungi nearly all over the world. This group of fungi consists of at least five different species including *N. crassa, N. intermedia, N. sitophila, N. discreta,* and *N. tetrasperma*. *N. crassa* is the best known species because of its status as a model for eukaryotic genetics.

The five species of *Neurospora*, previously mentioned, are all capable of producing conidia, known as asexual spores. These asexual spores are produced by mitosis and asexual reproduction, and thus each spore is genetically identical to the parent fungus. Conidia are produced in two types, macroconidia and microconidia. Macroconidia are multinucleate and they are easily dispersed through air after production of conidiophores by repetitive apical budding (Maheshwari 1999). In addition to *N. crassa* producing macroconidia (asexual spores), *N. crassa* also produces sexual spores, called ascospores, during mating. Ascospores are produced by sexual reproduction through a process that requires two haploid nuclei of opposite mating types to fuse together to produce a diploid zygote, which undergoes meiosis and a post meiotic mitosis. The nuclei resulting from this process each provide the genetic information for a single ascospore (Springer 1993). As a result, spore sacs (called asci) typically contain eight haploid ascospores. This process occurs within fruiting bodies called perithecia.

1.2 Meiotic Drive & *Neurospora crassa* Spore Killers

Meiotic drive, a non-Mendelian inheritance phenomenon, is where selfish genetic elements skew sexual transmission (Burt and Trivers 2008). Not all genes follow the Mendelian pattern of inheritance in fungi, plants, and animals. This meiotic drive can affect genetic inheritance. In *Neurospora crassa*, meiotic drive can be observed in fungal spore killing (Turner and Perkins 1979). Previous research has shown that in a cross of Spore killer (*Sk*) × WT (also called *Sk-S* for *Sk*-sensitive), the ascospores containing the Spore killer survive and those containing the sensitive genotype deteriorate (Turner and Perkins 1979; Hammond et al. 2012).

There are currently three known spore killers in *Neurospora*, named *Sk-1, Sk-2*, and *Sk-3* (Turner and Perkins 1979). *Sk-1* was discovered in *Neurospora sitophila* while *Sk-2* and *Sk-3* were discovered in *Neurospora intermedia* (Turner and Perkins 1979). Spore killers have not been discovered in other *Neurospora* species, but it is known that resistance to *Sk-2* and *Sk-3* naturally occurs in *N. crassa* (Turner and Perkins 1979; Turner 2001).

1.3 *Sk-3* & Resistance to Spore Killing

Spore killer-3 (Sk-3) is a selfish genetic element transmitted to offspring through spore killing in the filamentous fungus *N. crassa*. The meiotic drive element of *Sk-3* in *Neurospora* is thought to contain two principal components: a killer element and a resistance gene (Hammond et al. 2012; Harvey et al. 2014). The *Sk* resistance gene is a fungal-specific gene and its deletion in a killer strain leads to self-killing (Hammond et al. 2012). The *rsk* gene is required for resistance to spore killing, and it is important to note that *rsk* is not the cause of spore killing (Hammond et al. 2012). The poison gene for *Sk-3* has not yet been identified. Spore killing takes place within spore sacs, and spore sacs that have undergone spore killing contain four black, viable spores and four white, inviable spores (Turner and Perkins 1979; Velazquez et al. 2022). The black spores almost

always have the *Sk* genotype (99.9%; Turner and Perkins 1979). Spore killing does not occur until late ascospore development at the step of spore delimitation, after the post meiotic mitosis step (Raju 1979).

The *Sk-3* locus, defined by its killing ability and self-resistance, has been mapped to Chromosome III (Turner and Perkins 1979). The spore-killing phenomenon in *N. crassa* allows genetic conflict, evolutionary biology, and environments pertaining to agriculture to be studied.

1.4 Chromosome III/*i350* Interval

The *Sk-3* locus has been mapped to a 30-centimorgan, recombination-suppressed region of Chromosome III (Turner and Perkins 1979). Recombination suppression of this region occurs in crosses between *Sk-3* and WT, but there is no recombination suppression in homozygous crosses. A *Sk-3* resistance gene in an *N. crassa* WT strain was identified through map-based cloning (Hammond et al. 2012). Named *rsk*, it was then found that *Sk-3* also uses *rsk* for resistance to killing (i.e., as the counteragent to the *Sk-3* poison).

In an effort to identify the *Sk-3* poison, researchers have isolated a mutation called *rfk-2*^{UV}. This mutation disrupts *Sk-3*-based spore killing and it has been mapped to the *Sk-3* locus. The *rfk-2*^{UV} mutation disrupts spore killing and gene drive, but its exact location is not known (Velazquez et al. 2022).

To identify the location of *rfk-2*^{UV} on Chromosome III, Rhoades and Hammond deleted a 1.3 kb DNA interval called *i350*. DNA interval *i350* is found on Chromosome III in *Sk-3* strains. A transformation vector of similar name (v350) was designed to replace the interval with a hygromycin selectable marker (*hph*+), and when *i350* was replaced with the hygromycin marker, the resulting strain lost the ability to kill spores (Rhoades and Hammond, unpublished).

1.5 Hypothesis & Focus of Research

Previous research still leaves us in the unknown with respect to why deletion of *i350* eliminates spore killing. It is possible that somewhere within *i350* exists a hidden gene, and this hidden gene is the poison production gene. Another possibility is that the interval contains a regulatory element that controls expression of the poison gene, perhaps in the vicinity of *i350* or at a distant location. In this thesis, I present my research on two DNA intervals, *i382* and *i400*, and I show that replacement of *i382* with *hph*+ does not disrupt spore killing while replacement of *i400* with *hph*+ partially disrupts spore killing.

CHAPTER II

METHODS

2.1 Overview

To start, intervals *i382* and *i400*, DNA intervals on Chromosome III in *N. crassa Sk-3* strains, were chosen as the intervals of interest in this study. An overview of the methods is provided in **Figure 1**. These steps began with a PCR amplification of the left, center, and right flanks for the DNA replacement vectors and all flanks were isolated by gel purification. Next, the left, center, and right fragments were fused by PCR, where the amplification of the DNA replacement vectors, v382 and v400, were amplified from the fusion product with nested primers. These products, the transformation vectors, were then checked and quantified by gel electrophoresis. Then, transformation of *N. crassa* was performed with the transformation vectors, followed by selection of transformants. Following this, the transformants were crossed to obtain hygromycin-resistant homokaryotic offspring. Hygromycin resistant germlings were isolated from petri dishes and test crosses were performed with the offspring by fertilizing female strains. Finally, perithecial dissection, ascus phenotyping, and ascus imaging were performed. Genotypes of transformants and offspring were checked by PCR to confirm that *i382* and *i400* had been successfully replaced with *hph*+. Genotype information of all strains used in this study is provided in **Table 1**.

2.2 Strains, Primers, and PCR

DNA intervals can be replaced by constructing a DNA replacement vector, where the DNA replacement vector is the DNA element used to deliver a transgene to an organism (the

DNA replacement vector often 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, amplification of the left and right flank was completed by PCR. The predicted sequences of the left and right flanks of Vectors v382 and v400 are shown in **Figure 2** and **Figure 3**. Primers (**Table 2**) were used to construct the vectors. The left flanks were constructed with primers V0374-A and V0374-B (for Vector v382) and V0400-A and V0400-B (for Vector v400). The right flanks were constructed with primers V0382-C and V0382-D (for Vector v382) and V0400-C and V0400-D (for Vector v400). For Vectors v382 and v400, the left flank was amplified from DNA of strain RDGR170.3 with the forward primers and reverse primers. The primers were thawed at 65 °C, vortexed, and spun down in a microcentrifuge to accumulate the solution at the bottom of the tubes. A Q5 DNA Polymerase Buffer (New England Biolabs) and the DNA template RDGR170.3 (10 ng/µl) were also thawed at 65 °C, vortexed, and spun down in a microcentrifuge. A dNTP solution (10 mM) was not heated, but thawed between gloved fingers, vortexed and spun down to accumulate the solution at the bottom of the tube. A tube containing Q5 DNA Polymerase (New England Biolabs) was not heated, thawed, or vortexed, but it was spun in a centrifuge to accumulate the enzyme solution at the bottom of the tube. Sterile autoclaved water was transferred to a 50 ml conical tube and stored on ice for use in the PCR reactions. Next, two primer mixes were produced for each vector, where one mix was for the left fragment and one mix was for the right fragment. Primer mixes were produced by adding 6.25 µl of the forward primer (100 pmol/µl), 6.25 µl of the reverse primer (100 pmol/µl), and 487.5 µl sterile water in a sterile 1.5 ml microcentrifuge tube (MCT). A 5 µl aliquot of each primer mix was then transferred to labeled PCR tubes, along with 1 µl of the DNA template. Next, an

enzyme master mix was produced in a sterile 1.5 ml MCT tube by adding 67.0 µl of sterile water, 25.0 µl of Q5 reaction buffer, 2.5 µl dNTP mix, and 0.5 µl of Q5 enzyme for each PCR reaction to be performed. Of this enzyme master mix, 19 µl aliquots were placed in the labeled PCR tubes and the reactions were cycled in a thermal cycler to perform PCR following the manufacturer's recommendations for Q5 DNA polymerase.

2.3 Gel Electrophoresis of PCR Products

Gel purification of the PCR products was performed with IBI Scientific's GEL Extraction and PCR Cleanup Kit. Gels, specifically agarose gels, were produced in a 1 L flask by mixing 1.8 g of agarose and 200 ml of 1× Tris-Acetate-EDTA (TAE) buffer. Once mixed, this was heated for 1 minute time increments in a microwave until the agarose had dissolved. After the agarose had dissolved, 10 µl of ethidium bromide solution (10 mg/ml) was added to the molten agarose and swirled to mix, and then the flask was set to the side to cool before pouring into a gel tray. A thin layer of this mixture was poured into a gel tray (13 × 15 cm), set with a 12-tooth comb, and after a wait time of 20 seconds, the rest of the mixture was poured into the tray. After solidification, the gel tray was placed into an electrophoresis chamber and filled with 1× TAE buffer. To prepare the PCR products for gel purification, 5 µl of a 6× loading buffer was added to each of the 25 µl PCR reactions. After mixing slowly with a pipettor, the PCR reactions were loaded into the wells of the gel. A 10 µl aliquot (0.5 µg) of DNA ladder (GeneRuler 1 kb Plus, Thermo Scientific) was typically loaded into the first well, while the rest of the wells were filled with 30 µl of each PCR sample in loading buffer. Gels were typically run at 120 volts for 90 minutes.

DNA products were then excised from the gel with the aid of a UV transilluminator and a

clean razor blade. The gel slices were placed into MCTs. Following excision, a gel extraction kit (IBI Scientific) was used to isolate DNA from the gel slices. The first step was gel dissociation, where 500 µl of DF Buffer was added to the samples and mixed by vortex, followed by incubation at 60 °C for 10–15 minutes until the agarose gel slice dissolved. The DNA binding step came next, where 800 µl of the sample mixture was added to a DF column and centrifuged at 15,000 × g for 30 seconds. The flow-through, captured in a 2 ml collection tube, was discarded. After the DNA binding step was the wash step, where 400 µl of W1 Buffer was added to the DF column and the column was centrifuged at 15,000 × g for 30 seconds. The flowthrough was discarded again. A 600 µl aliquot of Wash Buffer was then added to the DF column, centrifuged at 15,000 × g for 30 seconds, and the flowthrough was discarded. The column was then centrifuged for 3 minutes to ensure that its filter matrix was dry. Next, the DNA was eluted by transferring the column to a clean MCT, adding 30 µl of Elution Buffer to the center of the column matrix, and centrifuging for 2 minutes at 15,000 × g to elute the DNA. The eluted DNA was then examined by gel electrophoresis by preparing an agarose TAE gel, as described above. The samples for gel electrophoresis were prepared by adding 5 µl of the gel purified PCR product (eluted DNA), 5 µl of 6× loading buffer, and 20 µl of sterile water, all mixed by pipetting up and down 5 times. A 10 µl aliquot of DNA ladder was added to the first well and included as a size marker. The rest of the wells were filled with 10 to 30 µl of solution. The gel was run at 120 V for 90 minutes and imaged using an Analytik Jena imaging machine or a smartphone camera with UV transilluminator. An image of an example gel is shown in **Figure 9 (a)** and **Figure 9 (b)**. The lengths of the left fragment and right fragment of v382 are predicted to be 872 bp and 1006 bp (predicted sequences shown in **Figure 2 (a)** and **Figure 2 (b)**). The lengths of the left fragment and right fragment of v400 are predicted to be 1111 bp and 1075 bp

(predicted sequences of the left and right flank are shown in **Figure 3 (a)** and **Figure 3 (b)**). The predicted lengths of the left and right flanks of each vector are consistent with the DNA molecules observed in the gels.

All above steps were repeated for the center fragment used in construction of v382 and v400 (**Figure 4**). The only difference in the center fragment methods is that the DNA template was plasmid pTH1256.1 (GenBank MH550659) and the primers were Hph-cen-f and Hph-cen-r.

2.4 Double-Joint PCR

Fusion of the left and right flanks to the center fragment was performed with Double-Joint PCR (DJ-PCR; Yu et al. 2004). The center fragment (**Figure 4**) contains a hygromycin resistance gene (*hph*+), which is a selectable marker that can be used for transformation of *N. crassa* (Margolin et al. 1997). Similar to the protocol above, solutions were used to create an enzyme master mix consisting of the following: 62.6 µl of sterile water, 20.0 µl of Q5 reaction buffer, 2.0 µl of dNTP mix, and 0.4 µl of Q5 enzyme. This mixture was placed in a PCR tube with 5 µl of the left fragment, 5 µl of the right fragment, and 5 µl of the center fragment. This reaction was then run in the PCR machine, using the parameters for fusion of the DNA molecules (Yu et al. 2004). The resulting fusion product was then used as template with nested primers to amplify the DNA replacement vector. The nested primers bind the DNA replacement vector within the left and right flanks. The nested primers were V0374-E and V0382-F for v382, and V0400-E and V0400-F for v400. The Standard Primer Mix Protocol was further used again for this process, where 20 µl of the Primer Mix was placed into a PCR tube with 5 µl of the fusion product from the step before. Added to the same PCR reaction was 52.6 µl of sterile water, 20.0 µl of Q5 reaction Buffer, 2.0 µl of dNTP mix, and 0.4 µl of Q5 Enzyme. This was

cycled according to the manufacturer's recommendations. The amplified DNA replacement vector was then column purified with a PCR purification kit (IBI Scientific). These steps consisted of sample preparation, where 5 volumes of DF Buffer to 1 volume of the amplified DNA vector was added. Next, the DNA binding step involved the mixture being transferred to a DF Column in a 2 ml collection tube, which was centrifuged at 15,000 × g for 30 seconds and the flowthrough was discarded. The wash step then consisted of 600 µl of the Wash Buffer added to the center of the DF Column, which was centrifuged at 15,000 × g for 30 seconds and the flowthrough discarded, followed by the DF column being dried again by centrifugation for 3 minutes at 15,000 × g and transferred to a new 1.5 ml microcentrifuge tube. After, 50 µl of Elution Buffer was added to the column matrix, and the DNA was eluted from the column matrix by centrifugation.

The purified PCR product was then examined by gel electrophoresis. This process was performed similarly to the purified left and right flanks described above (5 µl of purified PCR product, 5 µl of 6× loading buffer, and 19 µl of sterile water). The predicted 2831 bp sequence of Vector v382 and predicted 3389 bp sequence of Vector v400 are shown in **Figure 8**. The lengths of the amplified and purified vectors are consistent with their predicted lengths (**Figure 10**), suggesting that v382 and v400 were constructed successfully. Further, Vector v382 was designed to replace interval *i382* and Vector v400 was designed to replace interval *i400* (deletion vectors and their target intervals share similar names). The sequences of Interval *i382* and *i400* are shown in **Figure 5**, and their locations relative to Interval *i350*, which is the interval that initiated this project, is shown in **Figure 6**.

2.5 Transformation of *N. crassa* with Transformation Vector

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

For the transformation to be completed, I followed almost identical protocols described in Margolin et al. (1997) and Rhoades et al. (2020). Specific mediums were made to select for fungi that incorporated has the hygromycin resistance gene from the transformation vectors into their genomes. The transformation medium was prepared before the transformation began. The bottom agar plates allowed for transformation of strain RDGR170.3 with Vectors v382 and v400. The bottom agar plates were created by adding 172 ml milliQ water, 8 ml of 25× Vogel's Salts (Vogel 1956), and 3 g of agar to a 500 ml glass bottle, which was autoclaved to ensure sterility. Then, once autoclaved and cooled, 150 µl of hygromycin (400 mg/ml) and 20 ml of 10× FIGS solution (20% sorbose, 0.5% D-Fructose, 0.2% Inositol, 0.5% D-Glucose) were added to the mixture. This mixture was then poured into 10 petri dishes (20 ml per petri dish), and the plates were used as bottom agar after setting for about 3 days at room temperature.

The top agar was created with 105 ml of milliQ water, 27.3 g of sorbitol, 6 ml of 25× Vogel's Salts, and 2 g of agar, all placed in a glass bottle and autoclaved to ensure sterility. After the top agar had slightly cooled after autoclaving, 15 ml of 10× FIGS solution was added and the top agar was poured in 40 ml aliquots to sterile 50 ml conical tubes and stored at 50 °C.

Conidia of strain RDGR170.3 was scooped from a culture flask using sterile wood applicators and placed into a 50 ml conical vial containing 30 ml of ice cold 1 M sorbitol. This

suspension was then strained using a 100-micron cell strainer to remove mycelia and other large tissues. The conidia were centrifuged at 2000 × g for 10 minutes, the supernatant was removed, and the pellet of conidia was resuspended in 20 ml of ice cold 1 M sorbitol. The centrifugation step was then repeated. The resulting pellet was then suspended to a concentration of about 1 billion conidia per ml in 1 M sorbitol. After the resuspension step was complete, a 100 µl aliquot of conidial suspension was placed into a sterile microcentrifuge tube (MCT) and mixed with 10 µl of the DNA replacement vector (approximately 500 ng). The DNA+conidia suspension was then electroporated at 1500 V. A 750 µl aliquot of ice cold 1 M sorbitol was added to the conidial suspension after electroporation and the entire volume was then transferred to rescue medium (Vogel's minimal medium in 1 M sorbitol). The conidial suspension, which was in the rescue medium, was shaken for 3.5 hours at 32 °C and 40 rpm to prevent conidial clumping. After, 100 µl of the rescue culture was added to a 40 ml aliquot of molten Top Agar that had been cooled just to below 50 °C. The suspension was inverted 5 times, and approximately 10 ml was poured to a plate of Bottom Agar. Then, 500 µl of the rescue culture was added to the same vial of Top Agar, inverted 5 times, and 10 ml was poured to a plate of Bottom Agar. These steps were then repeated for 1000 µl of the rescue culture and the remaining volume of rescue culture. For 24 hours, the Bottom Agar plates were left to dry at room temperature, and then inverted and incubated for several days at 32 °C.

The transformants were selected by transferring hygromycin-resistant colonies from the transformation medium to 3 ml of Vogel's minimal agar (VMA) plus hygromycin (Vogel 1956). The Vogel's Minimal Agar (VMA) was prepared with 288 ml DI water, 12 ml of 25× Vogel's Salts, 6 g sucrose, and 4.5 g of agar, which was then autoclaved for 30 minutes at 121 °C and 15 psi. Then, hygromycin was added to a final concentration of 200 µg/ml. A serological pipette was

used to transfer 3 ml of media to sterile 16 × 125 mm glass culture tubes with vented caps. The tubes were tilted to allow the medium to solidify with a slanted surface. The tubes were covered with vented caps and placed at 4 °C for long term storage.

2.6 Crossing Transformants

N. crassa transformants usually contain transformed and untransformed nuclei, meaning they are heterokaryotic. The next step was thus to cross transformants to a standard mating partner to obtain homokaryotic offspring deleted of intervals *i382* or *i400* (crossing was performed as described in Samaraweera et al. (2014)). A crossing medium was used to promote protoperithecia production in *N. crassa* and transformants were crossed with an *Sk-S* mating partner. Westergaard Crossing Medium (60 mm petri dishes) was made with 470 ml milliQ water, 7.5 g of sucrose (dissolve by swirling), 25 ml of 20× WG salts (Westergaard and Mitchell 1947), 50 µl of 10,000× Trace Elements, 25 µl of 100 µg/ml biotin stock solution (100 mg/ml), and 10 g of agar. This solution was autoclaved for 30 minutes, cooled, and then 18 ml of the crossing medium was aliquoted to 60 mm Petri dishes, and the plates were dried for 1–3 days.

A cryogenic suspension of strain RTH1005.2 was then thawed and transferred to the crossing plates in 5–10 µl drops at the center of each plate, and the plates were then incubated for 6–8 days at room temperature. Strain RTH1005.2 was used as the protoperithecial (immature fruiting body) parent. The protoperithecial parent of a cross is usually called the female parent. Conidia from the male parent was used to fertilize the female parent's protoperithecia and the conidial suspensions were made from the transformants. Conidial suspensions were prepared by transferring a visible clump of conidia to an MCT tube with 500 µl of sterile water followed by vortexing. Then 200 µl of each conidial suspension (about 20 drops) were placed onto each

culture of RTH1005.2. The crossing plates were placed in a tray with a clear humidity dome and incubated for at least 24 days at room temperature.

2.7 Isolation of Homokaryotic Offspring

At this point in the procedure, the crossing plates had many ascospores on the undersides of the crossing plate lids. The ascospores were harvested with a 200 µl barrier pipette tip and 500 µl of sterile water was used to transfer the ascospores from the lid to an MCT tube. After the lid was returned to the crossing plate, the ascospore suspension was vortexed and placed at 4 °C in the dark.

Next, the process of heat shocking ascospores from crosses between the transformants and strain RTH1005.2 was completed and the ascospores were spread on Vogels hygromycin medium. This was done by making working suspensions of ascospores with about 500–1,000 ascospores in 500 µl of sterile water. The working stock was vortexed, incubated at 60 °C for 30 minutes, and then placed at room temperature once the incubation period passed. With sterile technique, the heat shocked spores were then placed in the center of three separate plates (100 mm petri dishes), where 50 µl, 100 µl, and 200 µl aliquots of the suspension were spread on individual plates using a sterile metal spreader. The 100 mm petri dishes contained Vogel's minimal agar plus hygromycin. The plates were then incubated for approximately 16 hours at room temperature for growth and identification of hygromycin resistant ascospores.

After the incubation period, the hygromycin resistant germlings were transferred to Vogels hygromycin slants. The germinating ascospores were isolated from the cultures. Germlings that had the most hyphal growth, had hyphae intact, and were clearly associated with a single ascospore, were selected for transfer. Germlings near other germlings or ungerminated

ascospores were avoided. The germlings that were resistant to hygromycin were chosen because they were expected to have the desired *i382*Δ or *i400*Δ genotype. The germlings not resistant to hygromycin were expected to have *i382*+ and *i400*+ genotype. Sterilized needles were used to dissect the isolated germlings and transfer them from the petri dishes to the Vogel's minimal agar plus hygromycin slants in 16 × 125 mm glass tubes. The slants were then labeled as RMK10.1-10.12 (v382) and RMK11.1-11.12 (v400). The cultures with the transferred germlings were then stored in an incubator at 30–32 °C for two days before transferring to room temperature on a culture shelf for about one week.

2.8 Spore Killing Assays

The transferred germlings that were resistant to hygromycin were next used in test crosses for spore killing. A total of 20, 60 mm petri dishes, prepared with crossing medium as described above, were used to begin this assay (performed two times, once in 2024 and once in 2025). The test crosses required preparation of protoperithecial parents on crossing medium, and the two protoperithecial parents were RTH1623.1 and RTH1623.2. Each plate was inoculated with 5 µl of cryogenic stock suspensions of RTH1623.1 or RTH1623.2 and then incubated for 8–10 days at room temperature. Conidial suspensions of hygromycin resistant germlings were produced in 500 µl of sterile water for the following strains: RDGR170.3 (*Sk-3* control strain), RZS27.10 (WT control strain), and RMK strains 10.1, 10.2, 10.5, 10.6, 10.7, 10.8, 11.5, 11.6, 11.7, 11.9, 11.10, and 11.12. For each conidial suspension, 200 µl aliquots were transferred to each of the RTH1623.1 and RTH1623.2 plates in 10 µl drops. The plates were incubated at room temperature to allow the strains to mate and produce perithecia.

2.9 Dissection & Imaging of Perithecia

On Day 12 post-fertilization, the fruiting bodies, otherwise known as perithecia, were isolated. Syringe needles (1 ml syringe with 23-gauge, 1-inch needles) and a dissecting microscope were used to dissect the perithecial contents into 25 % glycerol. More specifically, 100 µl of the glycerol solution was placed on a microscope slide and the perithecia were transferred to this drop of 100 µl of 25% glycerol. A 200 µl pipette tip was used to transfer a clump (10–20 perithecia) of perithecia from the crossing plate to the glycerol solution. After the clump of perithecia was transferred from the crossing plate, most of the surface hyphae and agar from each perithecium was removed to leave clean perithecia in the drop of glycerol. After, each perithecium was sliced open with a needle and the rosettes of asci (spore sacs, each containing up to eight ascospores at maturity) were released. After the rosettes were pressed out of approximately 10 perithecia, the non-rosette debris was removed from the slide and the rosettes were moved to the center of the slide. A cover slip was then placed over the rosettes. After the cover slip was placed on the microscope slide, Kimwipe tissues were laid on the left and right sides of the cover slip to wick away excess solution. After a minute or two of wicking, the Kimwipes were removed, and the cover slips were sealed to the slide by applying clear nail polish along their edges. Each microscope slide was labeled and stored at room temperature in the dark until imaging was performed within 24–36 hours. Imaging was performed with a Leica Compound Microscope and Zeiss Imaging System (**Figures 13–25**). The crossing plates that remained were imaged with an iPhone 15 Pro Max camera (**Figure 12a** and **Figure 12b**).

2.10 Final Genotyping Assay

To verify that the hygromycin-resistant offspring have either the *i382*Δ genotype or the

*i400*Δ genotype, DNA was isolated from four or more hygromycin-resistant offspring and used in PCR-based genotyping assay.

Mycelial samples for DNA isolation were created by adding a small amount of conidia from each strain to a 15 ml tube with 2–4 ml of liquid Vogel's minimal medium, and the cultures were incubated at 32 °C at 150 rpm for 24–48 hours. Mycelia from the hygromycin-resistant offspring was isolated from the Vogel's minimal liquid medium. To isolate the mycelia, wood inoculating rods were used to harvest it from the liquid medium culture to a stack of approximately 10 paper towels and a single sheet of filter paper. The fungal tissue was placed on filter paper on the paper towels to remove the liquid out of the mycelia by folding the paper towels over the tissue and pressing down to remove most of the moisture. Using clean forceps or disposable wood applicators, the dried mycelia was transferred to MCT tubes. The mycelial samples in the MCT tubes were then put in the -80 °C freezer before lyophilization. Lyophilization, also known as freeze drying, removes water from frozen substances under a vacuum. This allows the ice to transition from solid to vapor without passing through the liquid phase. The sample was left in the -80 °C freezer for at least 24 hours. The mycelia samples were then lyophilized for approximately three hours. After lyophilization, the samples were stored in a dry cabinet for later use in DNA isolation.

DNA isolation from lyophilized mycelia was performed with IBI Scientific's Genomic DNA Kit for Plants/Fungi. First, the dried tissue samples were ground to a fine powder with clean micro spatulas, followed by the lysis step. The lysis step was performed by adding 400 µl of GP1 Buffer and 5 µl of RNase A (10 mg/ml) to the ground tissue. The suspension was then vortexed and incubated for 10 minutes at 65 °C, with inversion of the tubes every 5 minutes. Next, 100 µl of GP2 Buffer was added, mixed by vortex, and incubated on ice for 3 minutes. The

samples were then transferred to a Filter Column in a 2 ml Collection tube and centrifuged for 1 minute at 1000 × g. The Filter Column was discarded, 750 µl GP3 Buffer was added to the flowthrough, and then 650 µl of the flowthrough was transferred to a GD Column in a 2 ml collection tube. This GD column was then centrifuged for 2 minutes at 15,000 × g, the flowthrough was discarded, and 400 µl of W1 Buffer was added to the GD Column. The GD Column was then centrifuged for 45 seconds at 15,000 × g, the flowthrough was discarded, and 600 µl of Wash Buffer was added to the GD Column. The GD Column was centrifuged for another 45 seconds at 15,000 × g, the flowthrough was discarded, and the GD Column was centrifuged for 3 minutes at 15,000 × g to ensure that the column matrix was dry. The dried GD Columns were then transferred to clean 1.5 ml MCT tubes and 100 µl of preheated (65 °C) Elution Buffer was added to the center of the column matrix. The columns sat for 1 minute. They were then centrifuged for 1 minute at 15,000 × g to elute the purified DNA, and the eluted DNA was stored at -20 °C.

The DNA from the hygromycin-resistant offspring was then used in PCR-based genotyping assays. Each transformation vector was designed to delete an interval of DNA in *Sk-3* strain RDGR170.3. Due to the fact that the lengths of deleted intervals are different than the length of the hygromycin resistance marker used in the deletions, PCR can be used to check if the interval has been deleted. Primers V0374-E and V0382-F were used to distinguish between *i382+* and *i382Δ::hph+* genotypes, while primers V0400-E and V0400-F were used to distinguish between *i400+* and *i400Δ::hph+* genotypes. The RMK10 strains (v382) included RMK10.5, RMK10.6, RMK10.7, and RMK10.8 and the RMK11 strains (v400) included RMK11.5, RMK11.6, RMK11.7, RMK11.9, RMK11.10, and RMK11.12. The DNA samples from the hygromycin-resistant transformants and the control strain, RDGR170.3, were thawed at

60 °C, vortexed, and placed on ice. The Q5 DNA polymerase buffer was thawed with the same procedure. A dNTP solution was thawed between gloved fingers, vortexed, and placed on ice, while the Q5 DNA polymerase enzyme was just centrifuged quickly and placed on ice. Primer mixes were made by adding 6.25 µl of the forward primer (100 pmol/µl), 6.25 µl of the reverse primer (100 pmol/µl), and 487.5 µl sterile water to a sterile 1.5 ml MCT tube, which was vortexed, and placed on ice. Each reaction contained 5.0 µl of primer mix and 1.0 µl of DNA template (approximately 10 ng/µl). An enzyme master mix was made in a sterile 1.5 ml MCT tube, containing 13.4 µl of sterile water, 5.0 µl of Q5 reaction Buffer, 0.5 µl of dNTP mix (10 mM), and 0.1 µl of Q5 enzyme per reaction. After the reactions were mixed by gentle pipetting, the reactions were placed in a thermal cycler and cycled according to the manufacturer's protocol for Q5 DNA Polymerase. The PCR products were then analyzed by gel electrophoresis and imaged (**Figure 11**).

The predicted PCR products from *i382+* and *i382*Δ genotypes are 1435 bp (**Figure 7a**) and 2831 bp (**Figure 8a**). The predicted PCR products from *i400+* and *i400*Δ genotypes are 2152 bp (**Figure 7b**) and 3389 bp (**Figure 8b**). The predicted sequences of these PCR products are shown in **Figure 7** and **Figure 8**. The genotyping assays confirm that all offspring have the expected genotypes (**Figure 11**).

CHAPTER III

RESULTS

3.1 Deletion of *i382*

Preliminary research has suggested that deletion of a 1.3 kb DNA interval, *i350*, eliminates *Sk-3*-based spore killing. Interval *i382* is a subinterval of *i350* (**Figure 6**). I deleted *i382* and examined the effect of the deletion on spore killing. Rosette images from crosses of several *i382Δ* strains, as well as control strains, are shown in **Figure 13a.** Control strains were used as references. RDGR170.3 represents spore killing and RZS27.10 represents no spore killing. **Figures 14–19** are each specific for one of the six tested *i382Δ* strains. I hypothesized that the deletion of interval *i382* would not disrupt *Sk-3*-based spore killing. Consistent with my hypothesis, spore killing was observed in each of the *i382*Δ test crosses (**Figure 13a**), which include RMK10.1 × RTH1623.1 (**Figure 14**), RMK10.2 × RTH1623.1 (**Figure 15**), RMK10.5 × RTH1623.1 (**Figure 16**), RMK10.6 × RTH1623.2 (**Figure 17**), RMK10.7 × RTH1623.1 (**Figure 18**), and RMK10.8 × RTH1623.1 (**Figure 19**). In addition to these findings, my genotyping assays of RMK10.5, RMK10.6, RMK10.7, and RMK10.8, confirmed each to be homokaryotic for the *i382*Δ genotype (**Figure 11a**). Together, my results demonstrate that deletion of interval *i382* does not disrupt spore killing.

3.2 Deletion of *i400*

Interval *i400* is also a subinterval of *i350* (**Figure 6**). **Figures 20–25** are each specific for one of the six tested *i400Δ* strains. My hypothesis was that deletion of interval *i400* would disrupt *Sk-3*-based spore killing. My results partially support this hypothesis because spore killing does not appear to be present in all test crosses (**Figure 13b**). For example, spore killing

appears to be present in crosses RMK11.6 × RTH1623.2 (**Figure 21**), RMK11.7 × RTH1623.1 (**Figure 22**), and RMK11.9 × RTH1623.2 (**Figure 23**). However, the rest of my results show a mixed phenotype RMK11.5 × RTH1623.1 (**Figure 20**), RMK11.10 × RTH1623.1 (**Figure 24**), and RMK11.12 × RTH1623.1 (**Figure 25**), with rosettes containing both 8B:0W asci and 4B:4W asci. Interestingly, the mixed phenotype does not appear to be due to a heterokaryotic genotype because the genotyping assays of RMK11.5, RMK11.6, RMK11.7, RMK11.9, RMK11.10, and RMK11.12 show that each is homokaryotic for the *i400*Δ genotype (**Figure 11b**). Overall, these results suggest that deletion of interval *i400* partially disrupts spore killing but does not eliminate it.

CHAPTER IV

DISCUSSION

4.1 Overview

N. crassa is an important genetic model for researching molecular eukaryotic genetics. My project focused on *Sk-3* spore killer and I aimed to help characterize a genetic locus thought to be required for spore killing. *Sk-3* is a selfish genetic element transmitted to offspring through spore killing. I examined the role of DNA intervals *i382* and *i400* in *Sk-3*-based spore killing.

4.2 Hypotheses

My findings in this thesis partially support my initial hypotheses due to the findings that *i382* deletion does not disrupt spore killing and *i400* deletion partially disrupts spore killing (**Figures 14–25**). Deletion of *i382* appears to not eliminate spore killing (4 viable : 4 inviable ascospores per ascus). So, *i382* is not required for spore killing. However, deletion of *i400* results in a mix of 4 viable : 4 inviable ascospores per ascus, and 8 viable : 0 inviable ascospores per ascus, in many observed rosettes. This suggests that *i400* may contain a regulatory element, like a promoter or terminator, of the killer gene. One possibility is that deletion of *i400* decreases expression of the killer gene, allowing an unusually high number of non-*Sk-3* ascospores to escape the killing process. However, an unexplained observation from my work is that the mixed phenotype was not detected in all *i400Δ* test crosses. Future work could retest the *i400Δ* strains to determine if the partial spore killing phenotype can be detected in all crosses when larger numbers of rosettes are examined.

4.3 Meiotic Drive—Practical Applications

While my research focuses on *N. crassa*, meiotic drive is associated with other organisms in regard to infertility and reproductive isolation (Arora and Dumont 2022). It is also important to note that meiotic drive is being studied in many other organisms, including pathogenic fungi. For example, one study has examined the meiotic drive of female-inherited supernumerary chromosomes in the pathogenic fungus *Zymoseptoria tritici*. Supernumerary chromosomes are extra chromosomes lacking homologous pairs (Habig et al. 2018). It has been shown that the genomic composition of *Z. tritici* provides a model to evaluate chromosomes dynamics during mitosis, meiosis, and recombination (Habig et al. 2018). Studying meiotic drive in fungal plant pathogens can also allow one better understand how different strains with certain chromosomes can produce differing amounts of asexual spores. For example, *Z. tritici* isogenic strains that lack supernumerary chromosomes produce higher amounts of asexual spores (Habig et al. 2018). In *N. crassa*, spore killing is the phenomenon where crosses of different strains result in ascospores being inviable, while in *Z. tritici*, the fungal pathogen has the ability to cause a disease in wheat, focusing on the genetic regulation of asexual sporulation (Habig et al. 20182). Habig et al. were able to compare *Z. tritici* and *N. crassa* by noticing that spore selection can be disregarded as a background mechanism of meiotic drive compared to *N. crassa*. This is due to Habig et al. successfully isolating complete tetrads, where the supernumerary chromosomes behave in a different manner during miosis, through female-biased meiotic drive. In *N. crassa*, meiotic drive is exhibited through selfish genetic elements that change segregation during meiosis while in *Z. tritici*, meiotic drive is connected to supernumerary chromosomes that amplify and inherit through the female gamete. Altogether, researchers have found that both systems promote non-Mendelian inheritance and have different mechanisms and biological consequences. *Z. tritici*

focuses on amplification of entire chromosomes while *N. crassa* focuses on specific alleles/genetic elements with less of a focus on the entire chromosomes. While these are different species, meiotic drive still follows, in principle, the same steps. Through our research, locating spore killing genes of *N. crassa* should be easier to do, and relocating meiotic drive in other organisms should be possible. While both meiotic drive and spore killing are examples of non-Mendelian inheritance, they are different mechanisms, in which both have significant evolutionary consequences by increasing selfish genetic elements in a population.

4.4 Current & Future Research

It is evident that *N. crassa* is a model organism that is necessary to examine the mechanism of *Sk-3*-based spore killing. With this, intervals *i382* and *i400* were the only intervals mentioned in this thesis. However, there are more intervals within and surrounding *i350* that have been studied by other researchers in the Hammond Laboratory. With the contributions of other researchers testing different intervals, these results can be combined to refine the exact location of the element that controls this spore killing mechanism.

N. crassa is only one of the many types of fungi that can be studied, so investigating other organisms that undergo a similar mechanism of meiotic drive can help us understand the relationship between meiotic drive and spore killing alleles that impact the construction of genomes and genetic evolution.

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Table 1 Strains used in this study

Strain name	Genotype
F2-23 (RTH1005.1)	*rid; fl; Sk-S^{ChrIII} A+*
F2-26 (RTH1005.2)	*rid; fl; Sk-S^{ChrIII} a+*
FGSC 10340 (RZS27.10)	*rid; Sk-S^{ChrIII}; mus-51^{RIP70} a+*
ISU-3036 (RTH1623.1)	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ A+*
ISU-3037 (RTH1623.2)	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ a+*
ISU-3291 (RDGR170.3)	*rid; Sk-3^{ChrIII}; mus-51Δ::bar+ A+*
TMSK1.1	v382-based hygromycin resistant transformant of ISU-3291
TMSKla2.4	v400-based hygromycin resistant transformant of ISU-3291
RMK10.1	*rid; Sk-3^{ChrIII} i382Δ::hph+; mus-51? mat?* (offspring of TMSK1.1 × F2-26)
RMK10.2	*rid; Sk-3^{ChrIII} i382Δ::hph+; mus-51? mat?* (offspring of TMSK1.1 × F2-26)
RMK10.5	*rid; Sk-3^{ChrIII} i382Δ::hph+; mus-51? a+* (offspring of TMSK1.1 × F2-26)
RMK10.6	*rid; Sk-3^{ChrIII} i382Δ::hph+; mus-51? A+* (offspring of TMSK1.1 × F2-26)
RMK10.7*	*rid; Sk-3^{ChrIII} i382Δ::hph+; mus-51? a+* (offspring of TMSK1.1 × F2-26)
RMK10.8*	*rid; Sk-3^{ChrIII} i382Δ::hph+; mus-51? a+* (offspring of TMSK1.1 × F2-26)
RMK11.5	*rid; Sk-3^{ChrIII} i400Δ::hph+; mus-51? a+* (offspring of TMSKla2.4 × F2-26)
RMK11.6	*rid; Sk-3^{ChrIII} i400Δ::hph+; mus-51? A+* (offspring of TMSKla2.4 × F2-26)
RMK11.7	*rid; Sk-3^{ChrIII} i400Δ::hph+; mus-51? a+* (offspring of TMSKla2.4 × F2-26)
RMK11.9	*rid; Sk-3^{ChrIII} i400Δ::hph+; mus-51? A+* (offspring of TMSKla2.4 × F2-26)
RMK11.10	*rid; Sk-3^{ChrIII} i400Δ::hph+; mus-51? a+* (offspring of TMSKla2.4 × F2-26)
RMK11.12	*rid; Sk-3^{ChrIII} i400Δ::hph+; mus-51? a+* (offspring of TMSKla2.4 × F2-26)

The *rid* allele and the *fl* allele disrupt Repeat Induced Point Mutation and macroconidiation, respectively (Freitag et al. 2002; Perkins et al. 2000). The *mus-51*^{RIP70} and *mus-51?* (could be *mus-51+* or *mus-51Δ*) disrupt Non-Homologous End Joining (Ninomiya et al. 2004; Smith et al. 2016). The *sad-2Δ::hph+* allele suppresses Meiotic Silencing by Unpaired DNA (Shiu et al. 2006). *Predicted genotype based of lineage of strain and hygromycin resistance (not genotyped by PCR).

Table 2 Primers used in this study

Name	Sequence (5′ > 3′)
HPH-CEN-F	AACTGATATTGAAGGAGCATTTTTTGG
HPH-CEN-R	AACTGGTTCCCGGTCGGCAT
V0374-A	TCCAAAGGGAAGGACCGGGCACA
V0374-B	AAAAAATGCTCCTTCAATATCAGTTCGTGAGCCGGAGCAGTCGTCGTA
V0382-C	GAGTAGATGCCGACCGGGAACCAGTTCCGGGCCTTTTGTCAGGCAGAT
V0382-D	GACGCGCACTAACCTGGGTGGG
V0374-E	GGGACAGAGAGTGGCGTCTGCCT
V0382-F	GGTACGGGACAGGGGCAAGGTCA
V0400-A	TCGATACGACGACTGCTCCGGCT
V0400-B	AAAAAATGCTCCTTCAATATCAGTTGGTGGTGTCGTCAGCGCGAG
V0400-C	GAGTAGATGCCGACCGGGAACCAGTTGTCCCCTCGAGACTACGCCAGCA
V0400-D	GAACGGGAGGTTAGCGCGTGTCG
V0400-E	GCCATCTTCTGCCCCGCCATCG
V0400-F	TGATGACCTTGACCGCAACGACC

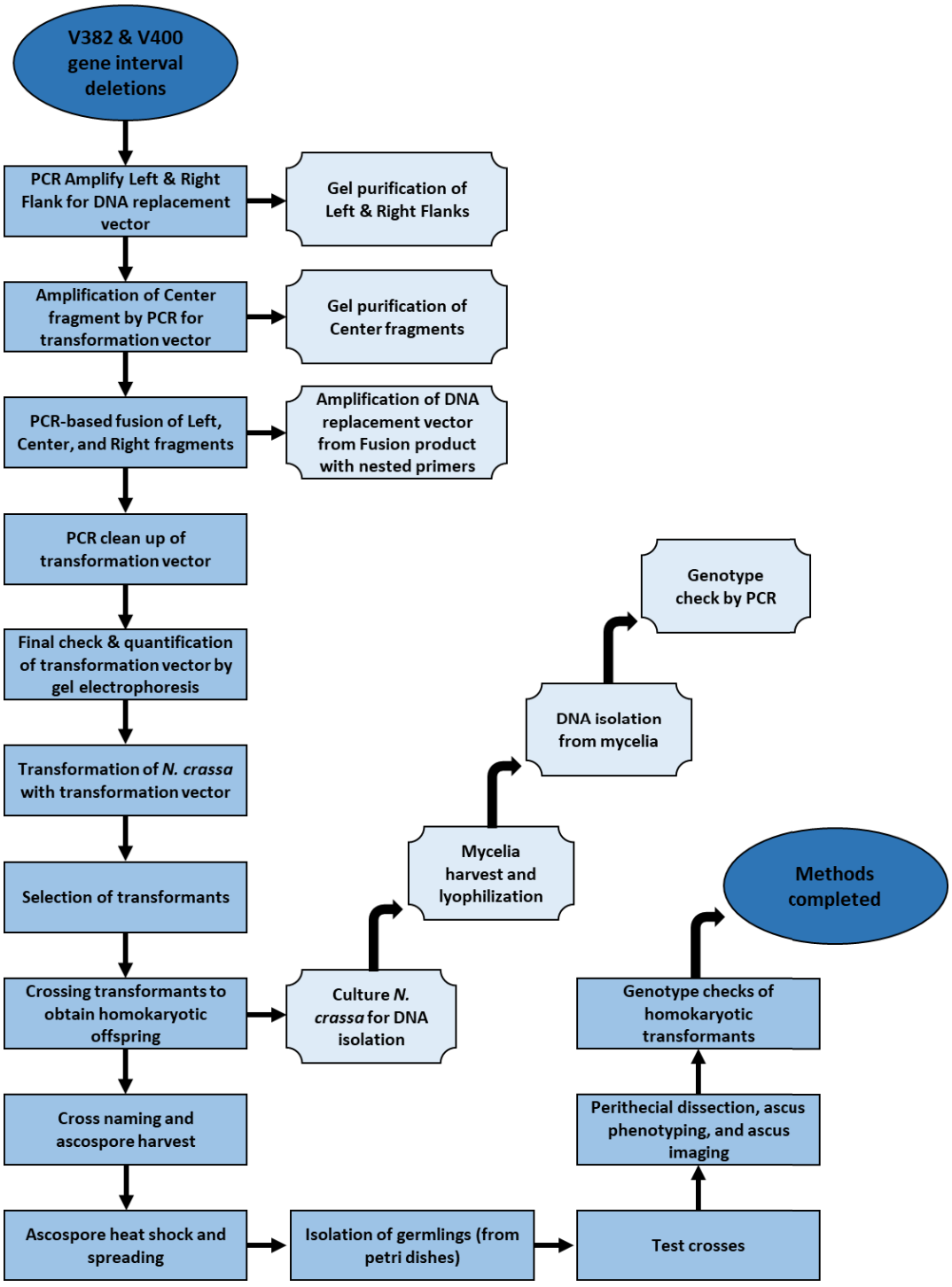

Figure 1 Overview of Methods.

>*i382* left flank
TCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACACTAACGAGCGATTT
TGCCACCTAGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCCTACCACGCTTCTTTTTC
CGTCCACCGGCTCTTGGAGTACCGTACATACCTCAAACACTTCATTCCACCCTGTTCTGGAAT
TGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGG
AAGGAGGAGAGGTACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGG
ACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTC
ACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCT
CATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAAC
CCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAG
GCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAA
AGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCA
AGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGAT
GGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGA
TGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG

>*i382* right flank
CCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCT
GCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGA
GAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTAC
CAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGG
GAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCC
AGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAAC
ATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTA
GCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTT
GTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGG
GTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATT
TCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGT
GGCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTG
CTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCAT
GCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGG
CAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGA
ATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTC

Figure 2 The left flank and right flank of interval *i382*. **(Top)** The *i382* left flank. The 872 bp sequence of the *i382* left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0374-A and V0374-B from RDGR170.3 genomic DNA (primers highlighted in green). **(Bottom)** The *i382* right flank. The 1006 bp sequence of the *i382* right flank is shown. This sequence was PCR-amplified with primers V0382-C and V0382-D from RDGR170.3 genomic DNA (primers highlighted in green).

>*i400* left flank

TCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGA
TGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAG
TAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCT
CACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTA
CGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAAC
GACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCT
TCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCT
TTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGG
GCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATG
CTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATA
TTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTC
CTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACG
GGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGC
CCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATG
AAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGG
CGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGA
TTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAAC
CCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACC

>*i400* right flank

GTCCCCTCGAGACTACGCCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATA
CAAGTATGATTCATACCAGGCCGGTCTGAACCCGAGCGCGCAACCACAGTCCTCCTCCATTTC
CCCAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGACGTCGCTATGCAGGATGCCCATGA
TCCACACTCATCCCAGAACGCTACCATCAAGTACCCCCTGAGGCCGCATCACTCGCTCTCCGG
TGGTCGCCCGGCCAGCCGTCAAGCATCCCAACAGCTTCCCCCCATCAACCCATACGCACCCGC
TCAACACGGCCACAGCTATCCAAACTCGGCAATCAGCTCTACCATGGACGGCTCTTATATGGA
CCCCAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCAACAGATGCCCATGCCCGAGAGGAC
GCCAGTTCCCGAATTTAGGAAAATGCGAGGACCCCAGGACCTTCGACCAAAGATTAACAAGCA
GCCGGCTCATCGACGAGCTAACCCGGAAGGCGGCTTTATCAGTGTATGTACATGTTCGCGAGC
CATAGCTTTGGGCAAGCCTGCTGACGAACCACGATAGCCCCTCCAAGCGCTAACAGTTCACCT
CCCCGCCACCTACCGAATATGCAACCCCGGCTTCAAGTACGAGTCGTCTAGGAATCCTCGGCG
CGTTCTTACCAAGCCTAGCAAGGGAGTGAAGAATGATGGCTATGACAACGAGGACAGCGATTA
TATCCTCTATGTGAATGATATCCTGGGCTCAGAGGAGGCTGGTCATAAGTAAGTTGCTGCCCA
CCACGAGTCGAGAAGCAGTCACTTACATGTTTGTACCAGGAACCGCTACCTGATTCTCGATGT
CCTTGGCCAGGGTACCTTCGGCCAGGTCGTAAAGTGCCAAAACTTGAAGACGCAAGAGGTCGT
TGCGGTCAAGGTCATCAAGAACCGAACAGCTTACTTCAACCAAAGCATGATGGAAGTGTCTGT
TTTGGATTTGGTTCGTACACCAGCATTGTTGACTTGATTGTTTGCGACACGCGCTAACCTCCC
GTTC

Figure 3 The left flank and right flank of interval *i400*. **(Top)** The *i400* left flank. The 1111 bp sequence of the *i400* left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0400-A and V0400-B from RDGR170.3 genomic DNA (primers highlighted in green). **(Bottom)** The *i400* right flank. The 1075 bp sequence of the *i400* right flank is shown. This sequence was PCR-amplified with primers V0400-C and V0400-D from RDGR170.3 genomic DNA (primers highlighted in green).

>V382 & V400 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 v382 & v400 center fragment. The 1412 bp sequence of the v382 & v400 center fragment is shown. This sequence was PCR-amplified with primers HPH-CEN-F and HPH-CEN-R from plasmid pTH1256.1 (GenBank MH550659.1; primers highlighted in green). The sequence contains *Aspergillus nidulans trpC* promoter sequences upstream of the 1026 bp *hph* coding region (indicated with pink highlight). The positions of the *hph* start codon and stop codon are indicated with red highlights and bold text.

>*i382* DNA interval
TTTGGATGGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGC
AATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCC
GTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGG
TGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACC
CGTCGGGT

>*i400* DNA interval
GGCCACCGCAACAGAATTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGA
GCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGC
CAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCAT

Figure 5 The *i382* & *i400* DNA intervals. **(Top)** The *i382* DNA interval. The DNA sequence of the 15 bp *i382* DNA interval is shown (highlighted in blue). DNA replacement vector v382 was designed to delete this interval by replacing it with *hph*+. The 100 nucleotides located to the left and right side of the interval are shown. **(Bottom)** The *i400* DNA interval. The DNA sequence of the 174 bp *i400* DNA interval is shown (highlighted in blue). DNA replacement vector v400 was designed to delete this interval by replacing it with *hph*+.

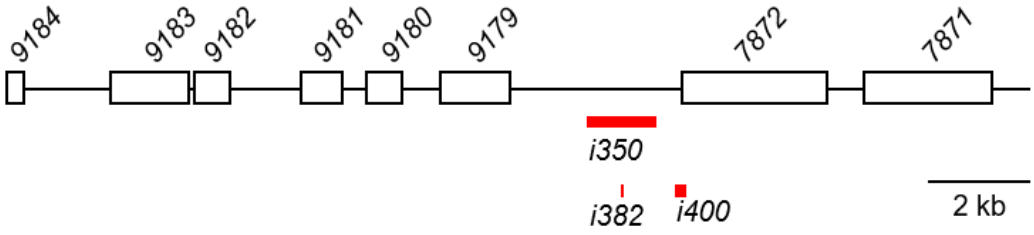

Figure 6 The *i382* & *i400* DNA interval locations. A diagram of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the locations of protein coding genes. Gene database numbers are provided. The location of intervals *i350*, *i382*, and *i400* are indicated with red bars.

>*i382*+ PCR product, predicted sequence, primers V0374-E and V0382-F

GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGG
TACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAA
ATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTT
GAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGA
CTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGA
ACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCG
TTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCC
CCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTC
CCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGG
GCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG**CCCGTCTCGCTCTTC**CCGGGCCTTTTGTCAG
GCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAA
AAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAAT
TGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGG
AGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGC
TCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCT
CTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAG
CATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTC
GGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTT
TATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCT
CGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGA**TGACCTTG**
CCCCTGTCCCGTACC

Figure 7a PCR product sequence: *Sk-3 i382*+ genotype. The predicted sequence of DNA amplified with primers V0374-E and V0382-F from a template consisting of *Sk-3 i382*+ genomic DNA is shown. The binding sites of V0374-E and V0382-F are indicated with bold font. The *i382* interval is indicated with red font. The length of the sequence is 1435 bp.

>*i400*+ PCR product, predicted sequence, primers V400-E and V400-F

GCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCC
GAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGAT
ACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGG
AGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTT
CCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTG
GAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAA
GGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGC
CATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCT
CTTCTCATTTCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGG
TGGCCCCACCTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGC
CCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAG
GTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCG
GCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGA
AACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCC
CTCTCGCGCTGACGACACCACC**GGCCACCGCAACAGAATTCATTGCCAAACCAGACCCAGCAACAGCCCCG**
AGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTC
TGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACG
CCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGT
CTGAACCCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCCAA
CGGCGACGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACCATCAAGTACCCCCTGA
GGCCGCATCACTCGCTCTCCGGTGGTCGCCCGGCCAGCCGTCAAGCATCCCAACAGCTTCCCCCCATCAAC
CCATACGCACCCGCTCAACACGGCCACAGCTATCCAAACTCGGCAATCAGCTCTACCATGGACGGCTCTTA
TATGGACCCCAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCAACAGATGCCCATGCCCGAGAGGACGC
CAGTTCCCGAATTTAGGAAAATGCGAGGACCCCAGGACCTTCGACCAAAGATTAACAAGCAGCCGGCTCAT
CGACGAGCTAACCCGGAAGGCGGCTTTATCAGTGTATGTACATGTTCGCGAGCCATAGCTTTGGGCAAGCC
TGCTGACGAACCACGATAGCCCCTCCAAGCGCTAACAGTTCACCTCCCCGCCACCTACCGAATATGCAACC
CCGGCTTCAAGTACGAGTCGTCTAGGAATCCTCGGCGCGTTCTTACCAAGCCTAGCAAGGGAGTGAAGAAT
GATGGCTATGACAACGAGGACAGCGATTATATCCTCTATGTGAATGATATCCTGGGCTCAGAGGAGGCTGG
TCATAAGTAAGTTGCTGCCCACCACGAGTCGAGAAGCAGTCACTTACATGTTTGTACCAGGAACCGCTACC
TGATTCTCGATGTCCTTGGCCAGGGTACCTTCGGCCAGGTCGTAAAGTGCCAAAACTTGAAGACGCAAGA**G**
GTCGTTGCGGTCAAGGTCATCA

Figure 7b PCR product sequence: *Sk-3 i400*+ genotype. The predicted sequence of DNA amplified with primers V0400-E and V0400-F from a template consisting of *Sk-3 i400*+ genomic DNA is shown. The binding sites of V0400-E and V0400-F are indicated with bold font. The *i400* interval is indicated with red font. The length of the sequence is 2152 bp.

>*i382Δ::hph* PCR product, predicted sequence, primers V0374-E and V0382-F

GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGGTA
CGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGGACTTGGCTCCATGCCCAAAAATGA
AAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTTGAGGTT
GGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCA
GTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAG
AAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAAA
GGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAATGA
TAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTCCCATGCGCACTTGCAC
ATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGAT
ACGACGACTGCTCCGGCTCACGAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTC
AACAATGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTT
CATTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGC
AGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTA
AGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTACCTA
TTCTACCCAAGCATCCAA**ATG**AAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGT
TCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGG
GCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTT
GCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCT
CCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGC
GGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGA
ATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTG
TGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCC
CGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCG
GTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCGT
GGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGCCT
CCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCA
GCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCC
GCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCAC
TCGTCCGAGGGCAAAGGAA**TAG**AGTAGATGCCGACCGGGAACCAGTCCGGGCCTTTTGTCAGGCAGATGCCCC
AGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGA
CAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGG
CTAGTTTCCGCACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAG
GGAAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAA
AGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTT
TGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCC
GCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCG
CGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTT
CTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAA
ACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGA**TGACCTTGCCCCTGTCCCGTACC**

Figure 8a PCR product sequence: *Sk-3 i382*Δ::*hph*+. The predicted sequence of DNA amplified with primers V0374-E and V0382-F from a template consisting of *Sk-3 i382Δ::hph*+ genomic DNA is shown. The binding sites of V0374-E and V0382-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 2831 bp.

>*i400Δ::hph* PCR product, predicted sequence, primers V0400-E and V0400-F
GCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCG
AGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATAC
ATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGA
AAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCC
CGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATA
TCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGC
CATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCG
GGCCTAGTGGGTGCTGTAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCAT
TTCCTCGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCAC
CTCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCA
CCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATC
TACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTC
GTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCAC
CCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACG
ACACCACCAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCC
TATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCATTTAGGCAAC
TGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCAGACAGGAACG
AGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGAC
CCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCA
AGCATCCAA**ATG**AAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGC
GTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGA
TATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCG
GCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCCGC
CGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCGGAG
GCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATC
GGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTG
ATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCC
GAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCG
GTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCG
TGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGC
CTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGAT
GCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATC
GCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCC
AGCACTCGTCCGAGGGCAAAGGAA**TAG**AGTAGATGCCGACCGGGAACCAGTGTCCCCTCGAGACTACGCCAG
CAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAA
CCCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGA
CGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACCATCAAGTACCCCCTGAGGCCGCA
TCACTCGCTCTCCGGTGGTCGCCCGGCCAGCCGTCAAGCATCCCAACAGCTTCCCCCCATCAACCCATACGC
ACCCGCTCAACACGGCCACAGCTATCCAAACTCGGCAATCAGCTCTACCATGGACGGCTCTTATATGGACCC
CAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCAACAGATGCCCATGCCCGAGAGGACGCCAGTTCCCGA
ATTTAGGAAAATGCGAGGACCCCAGGACCTTCGACCAAAGATTAACAAGCAGCCGGCTCATCGACGAGCTAA
CCCGGAAGGCGGCTTTATCAGTGTATGTACATGTTCGCGAGCCATAGCTTTGGGCAAGCCTGCTGACGAACC
ACGATAGCCCCTCCAAGCGCTAACAGTTCACCTCCCCGCCACCTACCGAATATGCAACCCCGGCTTCAAGTA
CGAGTCGTCTAGGAATCCTCGGCGCGTTCTTACCAAGCCTAGCAAGGGAGTGAAGAATGATGGCTATGACAA
CGAGGACAGCGATTATATCCTCTATGTGAATGATATCCTGGGCTCAGAGGAGGCTGGTCATAAGTAAGTTGC
TGCCCACCACGAGTCGAGAAGCAGTCACTTACATGTTTGTACCAGGAACCGCTACCTGATTCTCGATGTCCT
TGGCCAGGGTACCTTCGGCCAGGTCGTAAAGTGCCAAAACTTGAAGACGCAAGA**GGTCGTTGCGGTCAAGGT**
CATCA

Figure 8b PCR product sequence: *i400*Δ::*hph*+. The predicted sequence of DNA amplified with primers V0400-E and V0400-F from a template consisting of *Sk-3 i400Δ::hph*+ genomic DNA is shown. The binding sites of V0400-E and V0400-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 3389 bp.

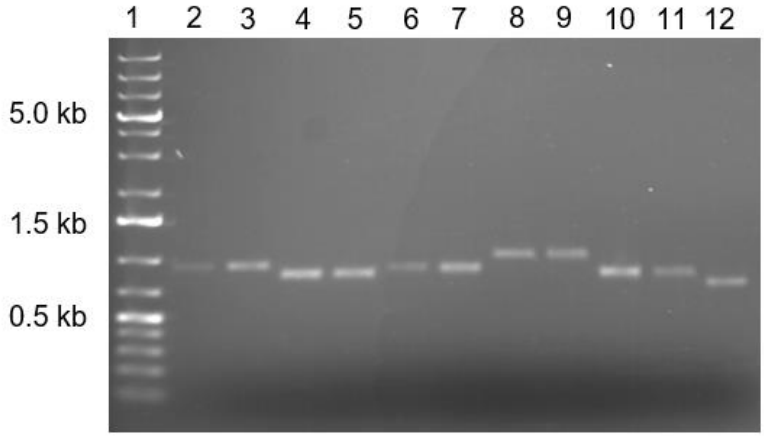

Figure 9a v382 construction: left and right flanks. PCR products for v382 left and right flanks. The left flank for v382 was amplified from RDGR170.3 genomic DNA with primers V0374-A and V0374-B. The right flank for v382 was amplified from RDGR170.3 genomic DNA with primers V0382-C and V0382-D. Products were gel purified as described in the methods section. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1, 0.5 µg GeneRuler 1 kb Plus (ThermoFisher); Lanes 2–5, not applicable; Lane 6, 5 µl purified product (v382 left flank trial 1); Lane 7, 5 µl purified product (v382 left flank trial 2); Lane 8, 5 µl purified product (v382 right flank trial 1); Lane 9, 5 µl purified product (v382 right flank trial 2); Lanes 10–12, not applicable. The expected product lengths for the v382 left and right flanks are 872 bp and 1006 bp, respectively.

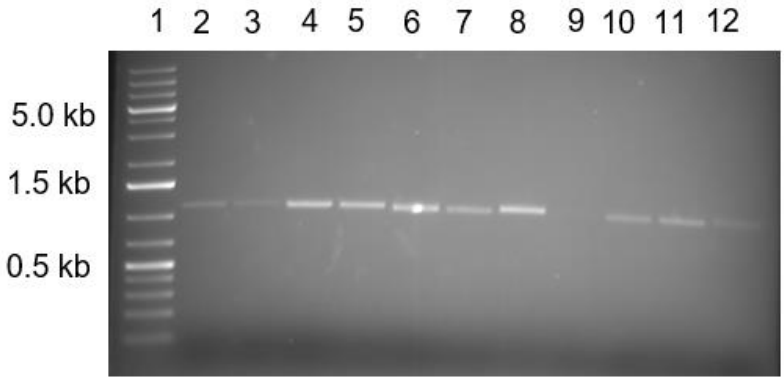

Figure 9b v400 construction: left and right flanks. PCR products for v400 left and right flanks. The left flank for v400 was amplified from RDGR170.3 genomic DNA with primers V0400-A and V0400-B. The right flank for v400 was amplified from RDGR170.3 genomic DNA with primers V0400-C and V0400-D. Products were gel purified as described in the methods section. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1, 0.5 µg GeneRuler 1 kb Plus (ThermoFisher); Lane 2, 5 µl purified product (v400 left flank trial 1); Lane 3, 5 µl purified product (v400 left flank trial 2); Lane 4, 5 µl purified product (v400 right flank trial 1); Lane 5, 5 µl purified product (v400 right flank trial 2); Lanes 6–12, not applicable. The expected product lengths for the v400 left and right flanks are 1111 bp and 1075 bp, respectively.

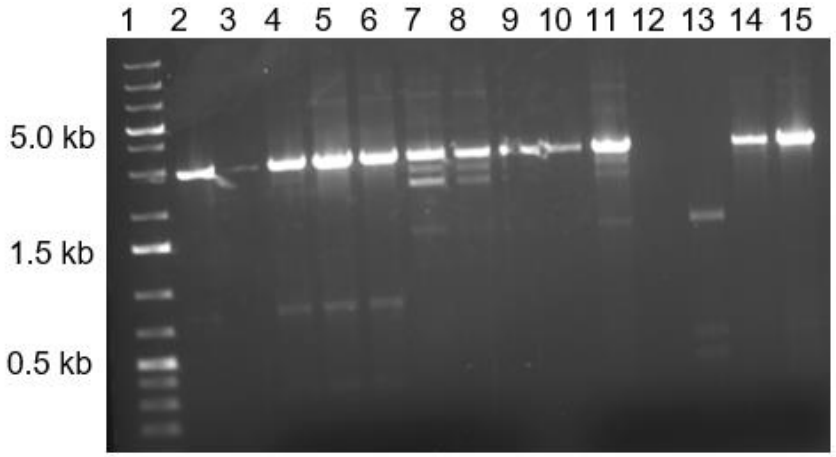

Figure 10a v382 construction: fusion and nested amplification. The v382 left flank, v382 center fragment, and v382 right flank were fused by DJ-PCR. The fusion product was amplified with primers V0374-E and V0382-F. The amplified product was column purified as described in the methods section. The amplified 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 GeneRuler 1 kb Plus (ThermoFisher); Lanes 2–4, not applicable; Lane 5, 3 µl of amplified and purified v382 (trial 1); Lane 6, 3 µl of amplified and purified v382 (trial 2); Lanes 7–15, not applicable. The expected length of v382 is 2831 bp.

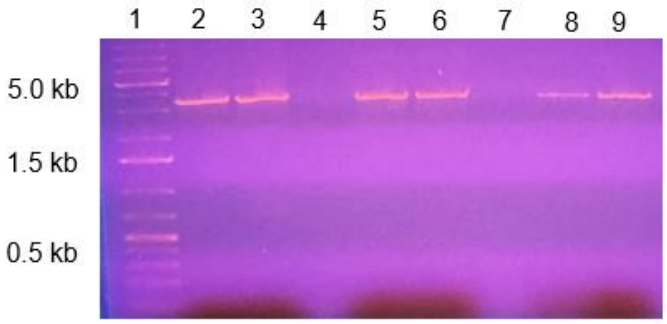

Figure 10b v400 construction: fusion and nested amplification. The v400 left flank, v400 center fragment, and v400 right flank were fused by DJ-PCR. The fusion product was amplified with primers V0400-E and V0400-F. The amplified product was column purified as described in the methods section. The amplified 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 GeneRuler1 kb Plus (ThermoFisher); Lane 2, 3 µl of amplified and purified v400 (trial 1); Lane 3, 3 µl of amplified and purified v400 (trial 2); Lanes 4–9, not applicable. The expected length of v400 is 3389 bp.

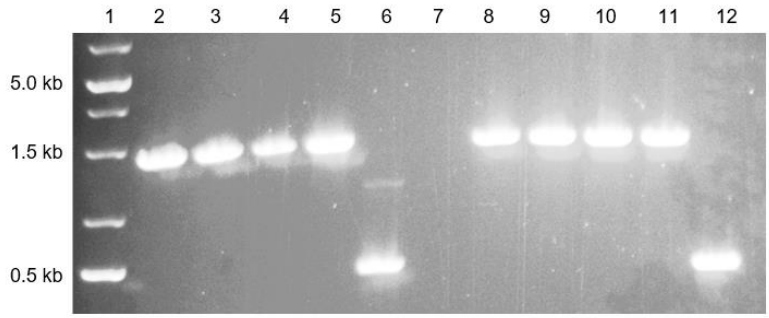

Figure 11a *i382Δ* genotype confirmation. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0374-E and V0382-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product length for the *v382+* and *v382Δ* genotypes are 1435 bp and 2831 bp, respectively. Lane 1 contains 0.5 µg of GeneRuler 1 Kb Plus DNA ladder (ThermoFisher). DNA templates for each PCR reaction are as follows: Lanes 2–7, Not applicable; Lane 8, RMK10.5; Lane 9, RMK10.6; Lane 10, RMK10.7; Lane 11, RMK10.8; Lane 12, RDGR170.3. These results show that test strains RMK10.5, RMK10.6, RMK10.7, and RMK10.8 have the *v382Δ* genotype.

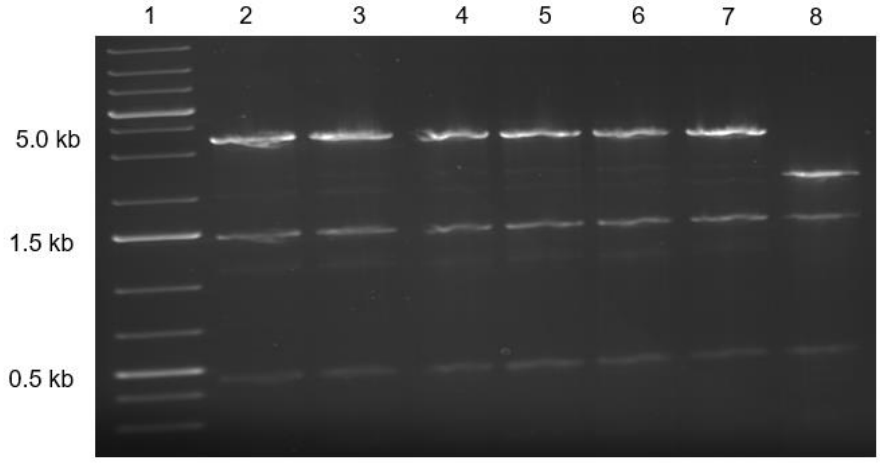

Figure 11b *i400Δ* genotype confirmation.. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0400-E and V0400-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product length for the *v400+* and *v400Δ* genotypes are 2152 bp and 3389 bp, respectively. Lane 1 contains 0.5 µg of GeneRuler 1 kb Plus DNA ladder (ThermoFisher). DNA templates for each PCR reaction are as follows: Lane 2, RMK11.5; Lane 3, RMK11.6; Lane 4, RMK11.7; Lane 5, RMK11.9; Lane 6, RMK11.10; Lane 7, RMK11.12; Lane 8, RDGR170.3. These results show that test strains RMK11.5, RMK11.6, RMK11.7, RMK11.10, and RMK11.12 have the *v400Δ* genotype.

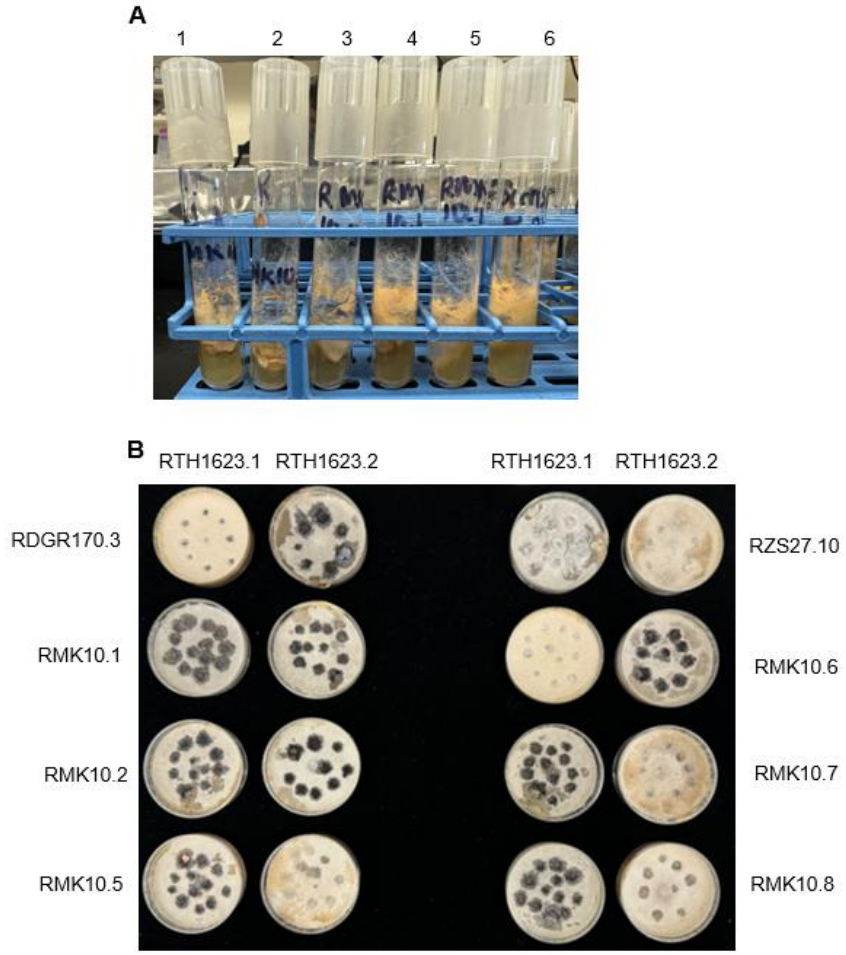

Figure 12a Test strains and crosses for *i382Δ*. **(A)** Six hygromycin-resistant offspring from cross RMK10 were selected for test crosses: RMK10.1, RMK10.2, RMK10.5, RMK10.6, RMK10.7, and RMK10.8. **(B)** Test crosses were performed with RTH1623.1 and RTH1623.2. RMK10.1 and RMK10.2 mated with both RTH1623.1 and RTH1623.2, suggesting that RTH1623.1 and RTH1623.2 are the same strain (one or the other). Images are of crossing dishes approximately three weeks post fertilization.

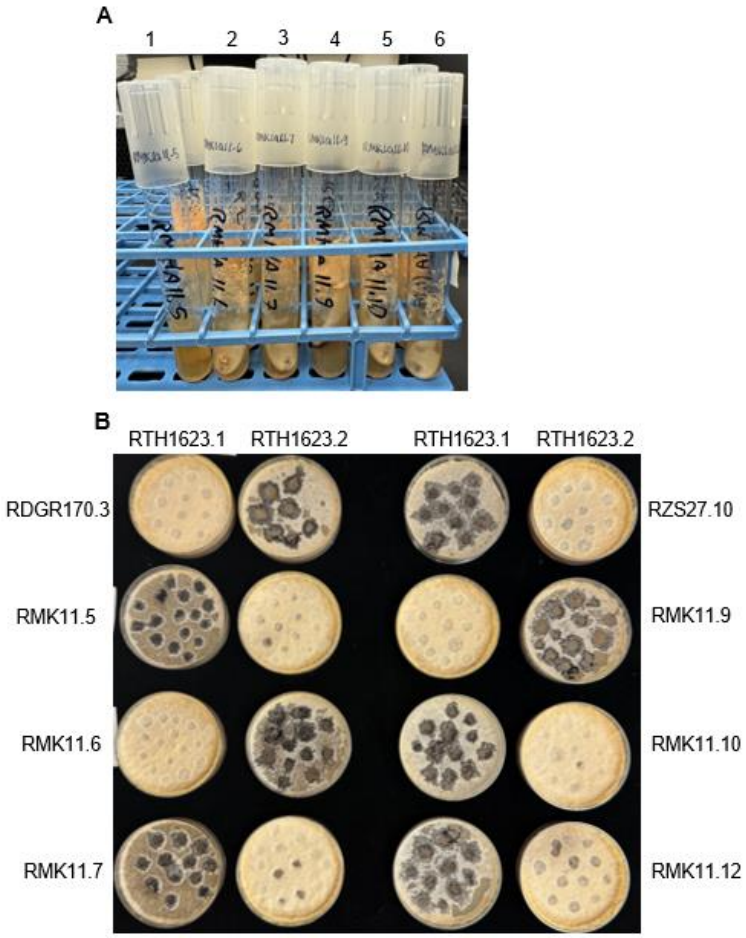

Figure 12b Test strains and crosses for *i400Δ*. **(A)** Six hygromycin-resistant offspring from cross RMK11 were selected for test crosses: RMK11.5, RMK11.6, RMK11.7, RMK11.9, RMK11.10, and RMK11.12 **(B)** Test crosses were performed with RTH1623.1 and RTH1623.2. Images are of crossing dishes approximately three weeks post fertilization.

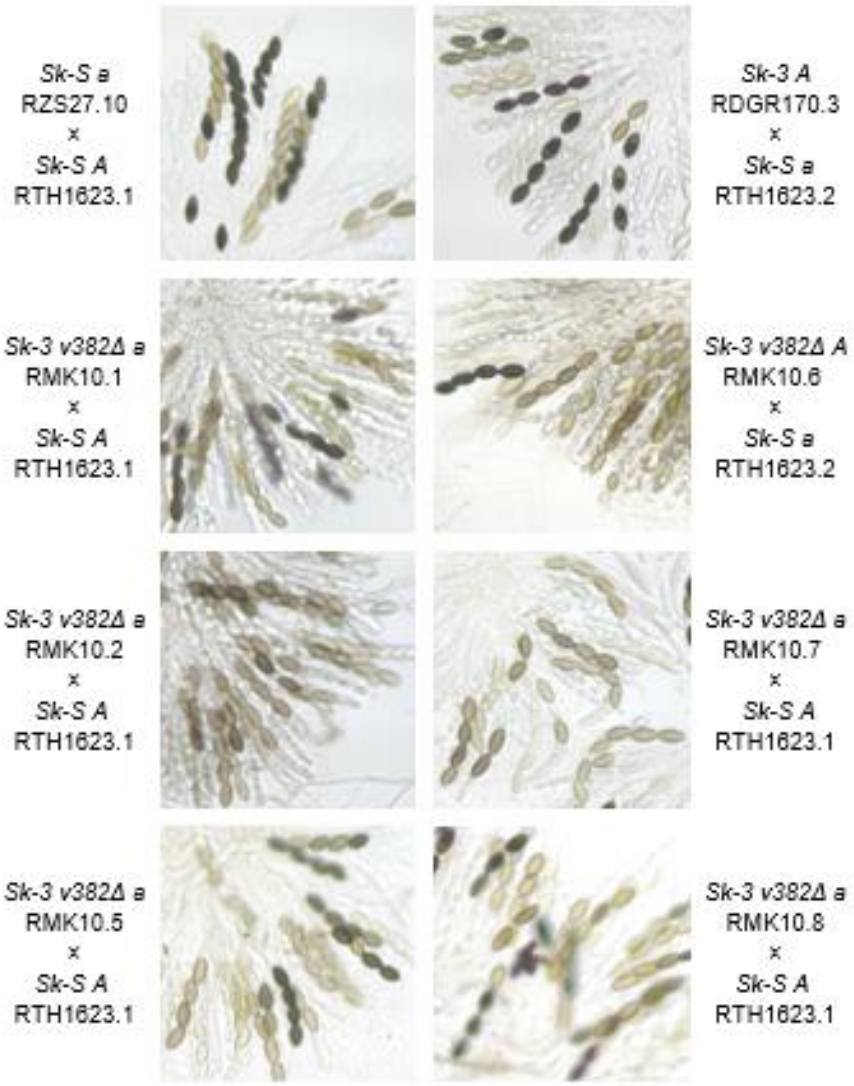

Figure 13a Ascus phenotype summary and results for *i382Δ*. Asci were dissected from perithecia of eight crosses on Day 12 post fertilization and imaged under magnification. Strain names and genotypes are indicated. Controls consist of RZS27.10 and RDGR170.3. These results demonstrate that deletion of interval *i382* does not disrupt spore killing.

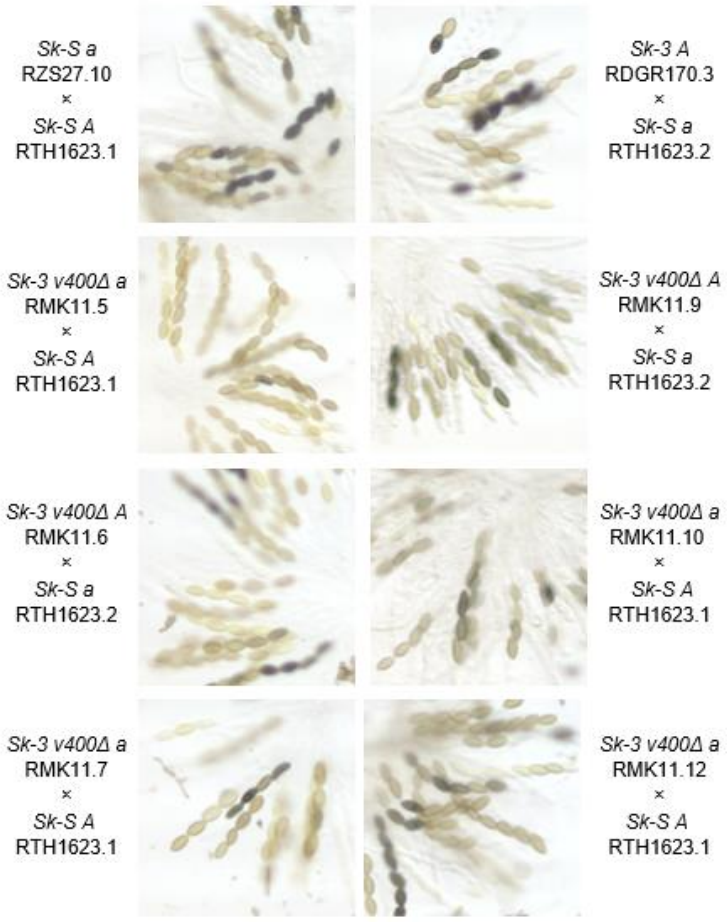

Figure 13b Ascus phenotype summary and results for *i400Δ*. Asci were dissected from perithecia of eight crosses on Day 12 post fertilization and imaged under magnification. Strain names and genotypes are indicated. Controls consist of RZS27.10 and RDGR170.3. These results suggest that deletion of interval *i400* partially disrupts spore killing. For example, spore killing appears absent in the RMK11.5 image and present in RMK11.9 image.

Figure 14 Ascus phenotypes. Asci were dissected from perithecia of RMK10.1 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RMK10.2 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RMK10.5 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

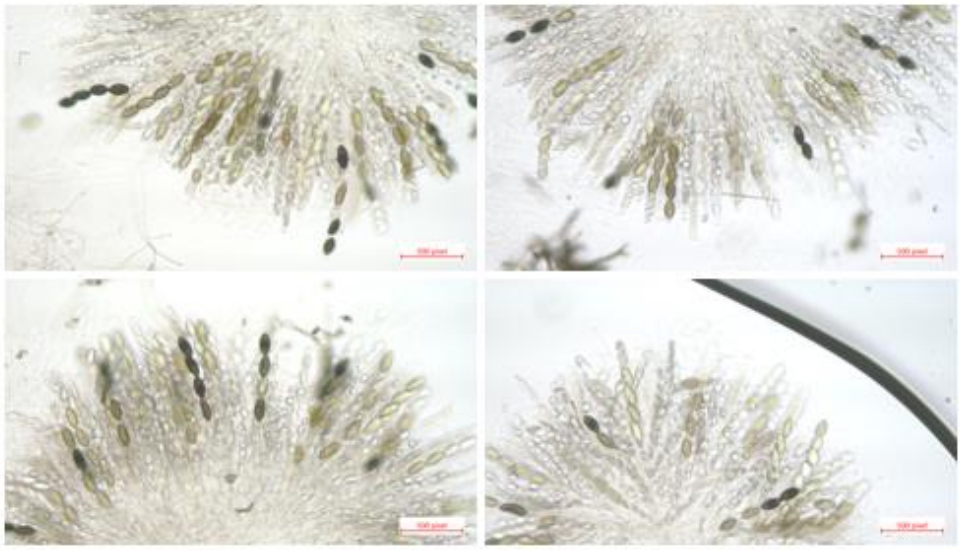

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RMK10.6 × RTH1623.2 on Day 12 post fertilization and imaged under magnification.

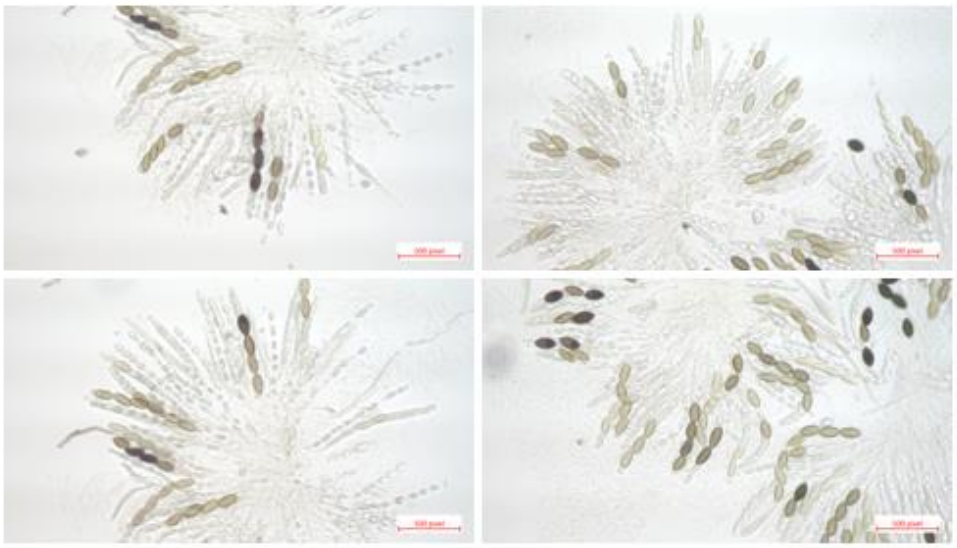

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RMK10.7 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

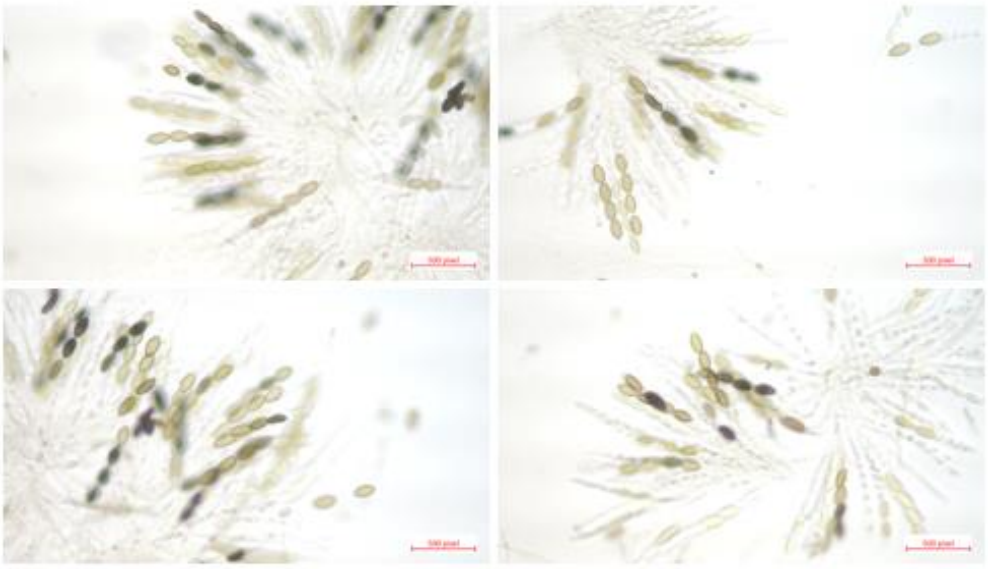

Figure 19 Ascus phenotypes. Asci were dissected from perithecia of RMK10.8 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

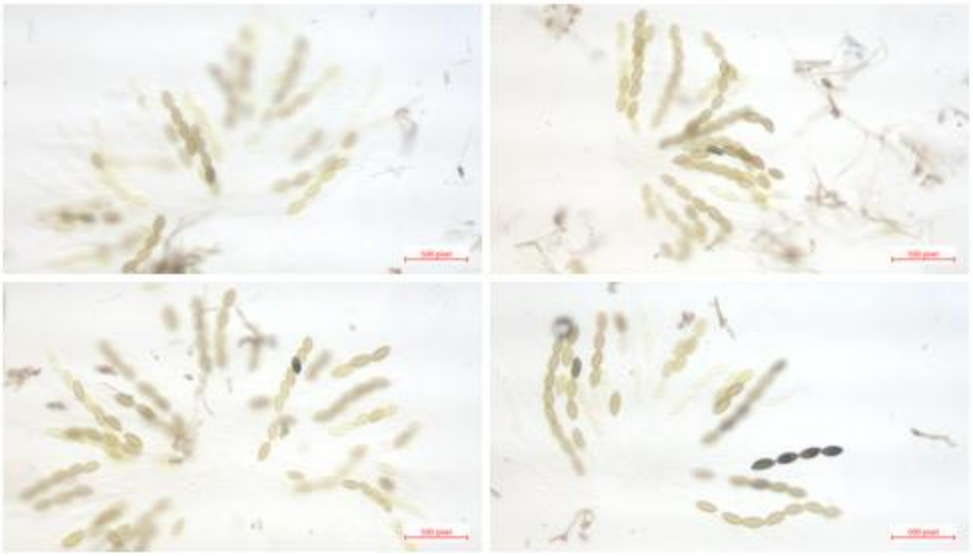

Figure 20 Ascus phenotypes. Asci were dissected from perithecia of RMK11.5 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

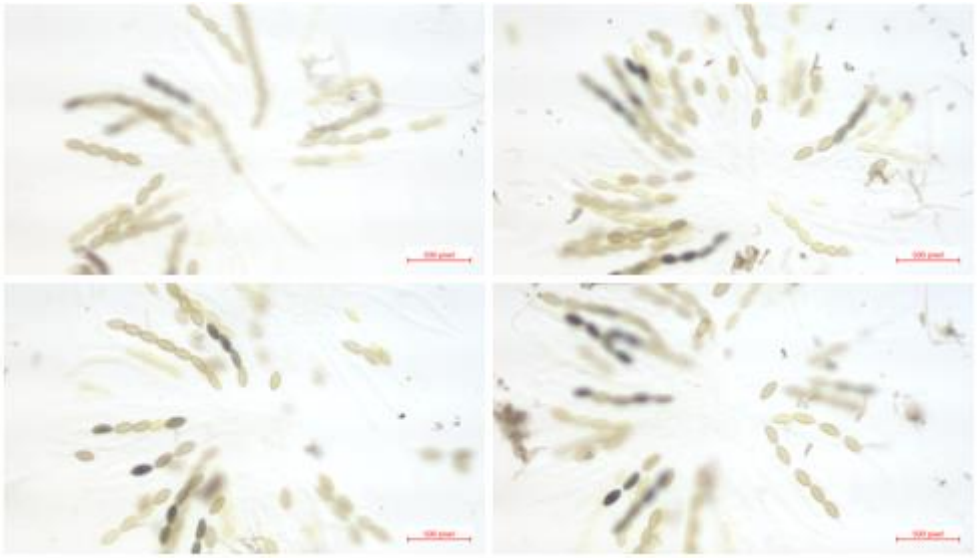

Figure 21 Ascus phenotypes. Asci were dissected from perithecia of RMK11.6 × RTH1623.2 on Day 12 post fertilization and imaged under magnification.

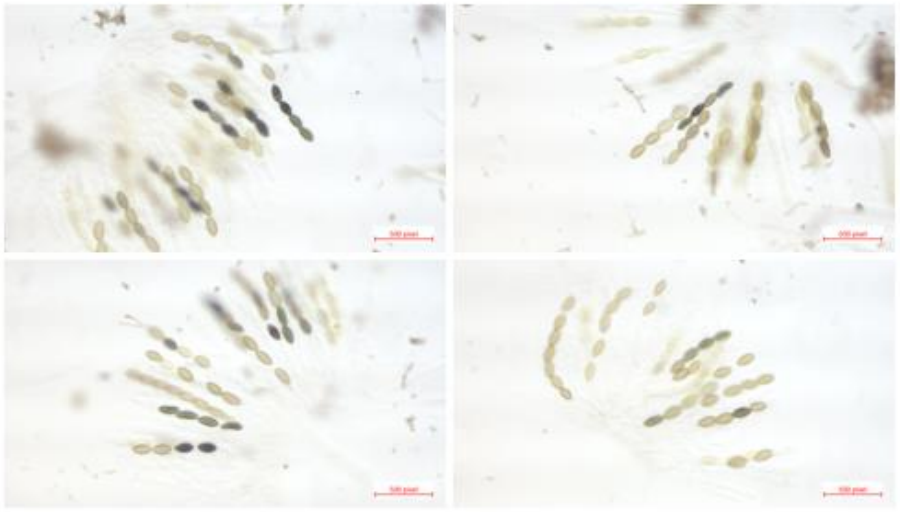

Figure 22 Ascus phenotypes. Asci were dissected from perithecia of RMK11.7 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

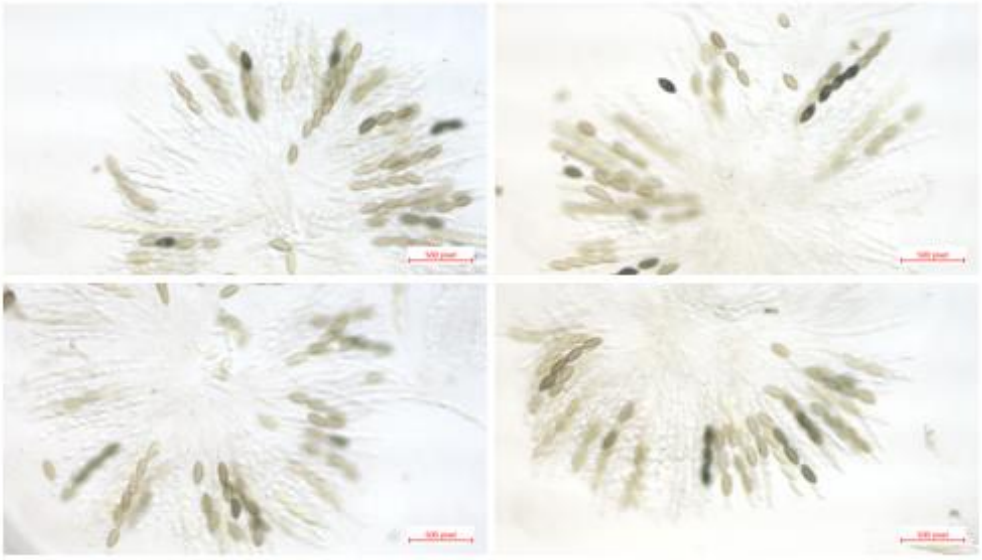

Figure 23 Ascus phenotypes. Asci were dissected from perithecia of RMK11.9 × RTH1623.2 on Day 12 post fertilization and imaged under magnification.

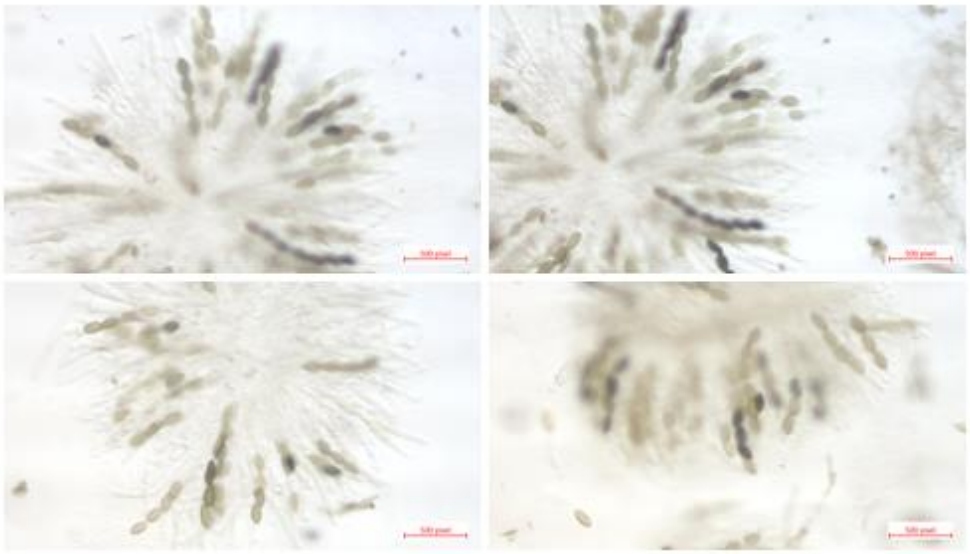

Figure 24 Ascus phenotypes. Asci were dissected from perithecia of RMK11.10 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.

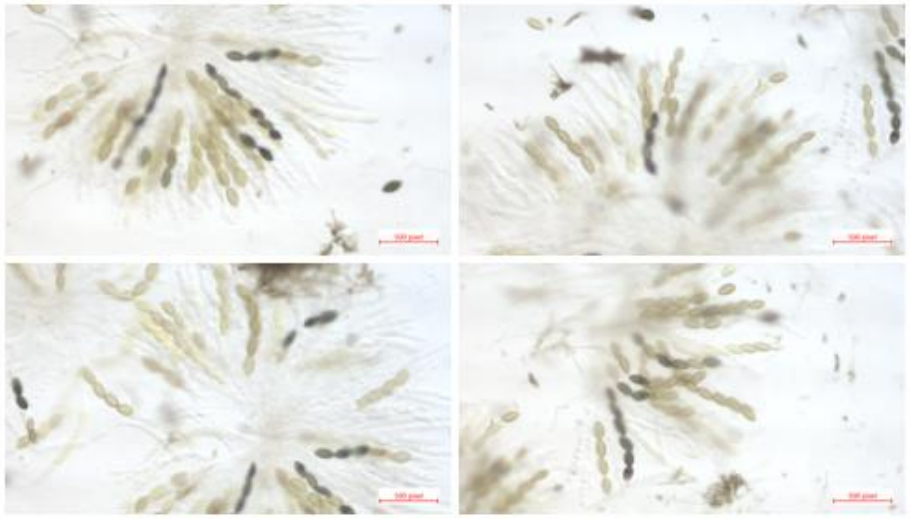

Figure 24 Ascus phenotypes. Asci were dissected from perithecia of RMK11.12 × RTH1623.1 on Day 12 post fertilization and imaged under magnification.