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Determining the Effects that Deletion of i386 and i408 have on Sk-3-Type Spore Killing

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Neurospora crassa is a genus of fungus that exhibits a phenomenon called *Sk-3* spore killing. *Sk-3* spore killing occurs when an *Sk-3* killer strain mates with an *Sk-3* sensitive strain, and it results in the death of half of the offspring. A DNA interval called *i350*, located on *N. crassa* Chromosome III, has previously been identified as critical for spore killing. Here, to obtain a more detailed understanding of this DNA interval, the effects of the deletion of related DNA intervals *i386* and *i408* on spore killing has been studied. Deletion of *i386* resulted in no disruption of spore killing while deletion of *i408* disrupted spore killing. These results provide a better understanding of the DNA sequences required for the spore killing process.

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CHAPTER I

INTRODUCTION

The genus Neurospora, a group of ascomycete fungi, is found all over the world in nearly all climates except subtropic and tropical environments (Davis 2000). Species *N. crassa* presents itself as an excellent model for eukaryotic genetics. *N. crassa* produces spores either sexually or asexually. Asexual spores, also called conidia, are genetically identical and grow to form hyphae. These hyphae are branches that undergo apical growth from the conidium and are divided into compartments called septa. Sexual spores (ascospores) are produced when hyphae transform to produce protoperithecia. Conidia from a strain of a different mating type sense this and undergo cellular fusion with the extensions (trichogyne) of the protoperithecium. After this occurs the nuclei then move to the perithecium (previously protoperithecium) and end up in a cell type known as a crozier hook. This hook contains one nucleus from each parent. The nuclei then fuse and go under a meiotic split to form two haploid nuclei containing dyad chromosomes. These nuclei undergo another meiotic split to form 4 haploid nuclei containing monad chromosomes. Mitosis then occurs to form 8 total nuclei with half of their genetic content coming from each parent. Ascosporogenesis then occurs where cells walls and membranes are formed around each nucleus to produce 8 ascospores within each ascus (Raju 1994).

A phenomenon called *Sk-3* type spore killing has been discovered within *N. crassa* (Turner and Perkins 1979). *N. crassa Sk-3* strains carry a genetic element that causes crosses between *Sk-3* and *Sk-S* strains (*Sk-3* susceptible) to produce asci with 4 viable ascospores and 4 inviable ascospores. When this cross occurs, nearly all viable ascospores have the *Sk-3* genotype, meaning that *Sk-3* is a selfish genetic element.

Gene *ncu09151* (*rsk*) on Chromosome III has been found to confer resistance to spore killing (Hammond et al. 2012). It is present within the *Sk*-genetic element and in non-killer *Sk-3* resistant strains (*Sk-R*). If this gene is deleted from an *Sk-3* strain, and a cross is performed between the *Sk-3 rskΔ* strain and an *Sk-S* strain, no viable ascospores are produced (Hammond et al. 2012). This finding has led to development of the *Killer Neutralization Model of Spore Killing* (Hammond et al. 2012; Zanders and Johannesson 2021). This model states the *Sk-3* strains produce both a poison and antidote to the poison. Both the poison and antidote are produced through the entire sexual reproduction process, but it is not until ascosporogenesis, when the antidote is confined to the ascospores that produce it, that spore killing can occur.

While the gene that produces the antidote (*rsk*) has been identified, the gene that produces the poison and is required for killing has not. However, a recent work has identified a mutation that disrupts *Sk-3*-type spore killing (Velazquez et al. 2022). This mutation, called $rfk\text{-}2^{UV}$ has been mapped to Chromosome III, within the *Sk-3* genetic element. Efforts to further refine the position of $rfk\text{-}2^{UV}$ led to the discovery of a 1.3 kb DNA interval on Chromosome III called *i350*. Preliminary results suggest that deletion of this interval disrupts spore killing (Rhoades and Hammond, unpublished). This could indicate that *i350* spans or partially overlaps the poison production gene or is a regulatory element that controls the expression of that gene. It is also possible that the genetic element connected to *i350* is independent of *rfk-2*. This latter possibility would suggest that there are at least two genes that control poison production.

To gain more insight into the role of *i350* in spore killing, I aimed to delete other intervals within and around *i350,* to see what effect these intervals have on spore killing. Specifically, I examined intervals *i386* and *i408*. Based on the locations of *i386* and *i408* relative to *i350*, I hypothesized that deletion of *i386* would have no effect on spore killing while deletion of *i408* would disrupt spore killing.

CHAPTER II

METHODS

An overview of the methods that I used to examine *i386* and *i408* is provided in **Figure 1**. The first step was to create, amplify, and purify transformation vectors to replace target DNA sequences. The transformation vectors were constructed using Q5 High-Fidelity DNA Polymerase (New England Biolabs). This was done using the DJ-PCR method (Yu et al. 2004). To begin, the left flank (**Figure 2**) and right flank (**Figure 3**) for Vectors v386 and v408 were amplified. This was done using RDGR170.3 (**Table 1**) genomic DNA as the template for amplification. Left flank primers for v386 were V0359-A and V0359-B, and for v408 they were V0394-A and V0394-B (**Figure 2**). Right flank primers for v386 were V0386-C and V0386-D, and for v408 they were V0358-C and V0358-D (**Figure 3**). Flanks were purified with a gel extraction kit (IBI Scientific). The center fragment (**Figure 4**) for v386 and v408 was amplified from plasmid pTH1256.1 (GenBank MH550659.1) with Primer Set HPH-CEN-F/HPH-CEN-R before gel purification. Fusion via DJ-PCR was then completed as previously described (Yu et al. 2004), resulting in the left flanks and right flanks on either side of a hygromycin resistance gene (*hph+*). The fusion products for both v386 and v408 were then amplified with nested primers. Transformation vector v386 was created to replace *i386* (**Figure 5**) and transformation vector v408 was created to replace *i408* (**Figure 6**). These intervals are shown relative to *i350* in **Figure 7**. The nested primers V0359-E and V0386-F were used to amplify v386, resulting in the PCR product shown in **Figure 8**, and column purified with a PCR cleanup kit (IBI Scientific). The nested primers V0394-E and V0358-F were used to amplify v408, resulting in the PCR product shown in **Figure 10,** and column purified with a PCR cleanup kit.

The purified products were analyzed by gel electrophoresis. A total of 1.8 g of agarose was added to a 1 L flask with 200 ml of 1× TAE buffer. This was heated at 100% power in a microwave for 1 minute. The mix was swirled and then reheated for a minute. This process was repeated until the agarose had completely dissolved. Next, 10 µl of 10 mg/ml ethidium bromide was added to the flask and mixed. The flask rested until the heat level was not painful to the touch. A thin layer was poured into a gel tray (13×15 cm) with a 12-tooth comb set in it and allowed to sit for 20 seconds. The rest of the mix was then poured into the tray and allowed to solidify. The comb was then removed and the gel moved to an electrophoresis chamber containing 1× TAE, with more 1× TAE added until the buffer level rose above the gel. The purified PCR products were then prepared for the gel. A total of 5 µl of the column purified DNA for v386 was added to an MCT tube with 5 µl of 6× loading buffer and 20 µl of sterile water, and the contents of the tube were mixed slowly up and down by pipetting. A 10 µl aliquot of a DNA ladder was then loaded into well #1, and 30 µl of the DNA sample mix was loaded into another well. The gel was then run at 120 V for 90 minutes before imaging over UV light. This process was repeated for the column purified v408 PCR product. The lengths of the predicted PCR amplification products for v386 (**Figure 8**) and v408 (**Figure 10**) were used to accurately assess if vector construction was successful. The next step was to use the purified vectors to transform *N. crassa.*

To begin, conidia of strain RDGR170.3 (containing the *Sk-3* genotype) were generated. This was done by preparing a 50 ml Vogel's Minimal Medium (VMM; Vogel 1956) in a 250 ml volumetric flask for each transformation (2 in total). Strain RDGR170.3 was obtained from cryogenic storage and thawed between gloved fingers. Using sterile technique under a biosafety hood, 20 µl of RDGR170.3 was transferred to the flasks. These flasks were labeled and capped with a glass beaker, using a wood applicator between the flask and cap to prevent a tight seal from forming. The flasks were then incubated at 32 °C for 2 days before being moved to a clean 1020 tray for later use in the transformation step.

N. crassa was then transformed through the process of electroporation. The method used followed the protocol recommended by Margolin et al. (1997) with modification as suggested by Rhoades et al. (2020). Two transformations were performed, one with v386 and one with v408. The next step was to select hygromycin-resistant transformants.

An ethanol candle and two syringes with needles were placed on a sterilized lab bench. The needles were passed through the ethanol produced flame then cooled before being used to isolate and cut hygromycin-resistant colonies from the transformation plates. A total of 10 colonies were selected for each transformation (20 total) and transferred to Vogel's slants containing VMM and 200 µg/ml hygromycin. The slants were then incubated at 30 °C for 1–2 days, then placed at room temperature, and used for the next step within a month of the incubation. The next step was to cross transformants to obtain homokaryotic offspring.

A total of 40 (20 for v386 transformants and 20 for v408 transformants) 60 mm petri dishes were filled with 18 ml of Westergaard and Mitchell’s synthetic crossing agar (SCA) (Westergaard and Mitchell 1947) with 1.5% sucrose and a pH of 6.5. This process was performed with a 25 ml serological pipette and an automatic pipettor under a biosafety cabinet that had been sterilized with UV light for approximately 5 minutes.

Unidirectional crosses, as described by Samarajeewa *et al.* (2014), were performed on these plates. Strain RTH1005.2 (**Table 1**) was inoculated onto SCA and the plates were incubated at room temperature for 6–8 days. Conidial suspensions were produced for 6 of each transformant type (v386 and v408). Using sterile technique, 500 µl of sterile water was transferred to MCT tubes totaling the number of conidial suspensions to be produced. Sterile wood applicators were used to transfer conidia from transformants to MCT tubes. This process was repeated for each transformant. These conidial suspensions were then used as the male in unidirectional crosses by fertilizing the female RTH1005.2 strains as previously described (Samarajeewa et al. 2014).

Ascospores were harvested from two crosses: RKD10 (v386; TDMS1.1 × RTH1005.2) and RKDam11 (v408; TKDam1.1 × RTH1005.2). Ascospore harvesting involves transferring ascospores from the lids of the crossing plates to sterile water and storing for at least 16 hours at 4 °C. Three 100 mm plates with Vogel's Minimal Agar (VMA) plus hygromycin (200 μg/ul) were used for each ascospore solution (three for RKD10 ascospores and three for RKDam11 ascospores). A working suspension of the ascospores was created by adding 500 μl of sterile water to a sterile MCT tube. Estimations of the amount of ascospores in the stock suspension were made by dropping 10 μl of stock ascospore suspension to a microscope slide. After estimating how many ascospores were present on the slide, the approximate concentration was determined. For the working suspension, stock suspension was added to create a working suspension of 1–5 ascospores per μl of working suspension. The stock suspension was then placed back into cold storage at 4 °C. The working suspension was vortexed for 5 seconds. The working suspension was then placed on a heat block at 60 °C for 30 minutes. After 30 minutes, the tubes were inverted multiple times. Under a sterilized fume hood, 50 μl of the RKD10 working suspension was transferred to a 100 mm plate, followed by 100 μl to another plate, and 200 μl to a final plate. This was repeated for three different plates with the RKDam11 working suspension. Autoclaved spreaders were used to spread ascospores across the plates. The plates were incubated overnight in a sterilized 1020 flat right-side up.

After approximately 16 hours, syringe needles and a dissecting microscope were used to transfer germinating ascospores to 125 mm culture tubes containing VMA with hygromycin (200 µg/ml). If germlings with abundant hyphal growth were identified, they were picked using a syringe whose needle had been flame sterilized and cooled, then carefully placed into one of the culture tubes. This process was completed for 12 RKD10 germlings and 12 RKDam11 germlings, with the goal of isolating at least three hygromycin resistant offspring (not all germlings are expected to survive the transfer process). The culture tubes were incubated at 30–32 °C for two days and at room temperature for at least one week to allow conidia to develop.

A total of 40 (20 for RKD10 offspring and 20 for RKDam11 offspring) 60 mm petri dishes were obtained. Each was filled with 18 ml of SCA with 1.5% sucrose and a pH of 6.5 as described above. Half the plates were inoculated with strain RTH1623.1 and the other half were inoculated with RTH1623.2. The plates were incubated for 6–8 days at room temperature to allow protoperithecia production. A male control strain of RDGR170.3 was used to fertilize two plates of 1623.1 and two plates of 1623.2 (one of each for the RKD10 control and one of each for the RKDam11 control). Additionally, a second male control strain of RZS27.10 was used on two plates of 1623.1 and two plates of 1623.2 (one of each for the RKD10 control and one of each for RKDam11 control). The male test strains for RKD10 included RKD10.2, RKD10.3, RKD10.4, RKD10.5, RKD10.10, and RKD10.X. The male test strains for RKDam11 included RKDam11.101, RKDam11.102, RKDam11.103, RKDam11.104, and RKDam11.105. The remaining plates were used as no fertilization controls.

On Day 17 post fertilization, the RKD10 test crosses and associated control crosses were dissected and imaged. On Day 12 post fertilization, the RKDam11 test crosses and associated control crosses were dissected and imaged. Two 1 ml syringes with 23-gauge 1-inch needles were placed on a petri dish lid. The plates were analyzed to determine which crosses produced fruiting bodies. Each cross that produced fruiting bodies was selected for dissection. To do this 100 µl of 25% glycerol was pipetted onto a microscope slide. A perithecial clump from the cross was then transferred to the slide. Another slide with 100 µl of 25% glycerol was prepared. Surface hyphae and agar were removed from the perithecia with the syringe needs while working under a dissecting microscope. Approximately ten cleaned perithecia were transferred from this slide to the glycerol solution on the second slide. The cleaned perithecia were sliced opened and the rosettes of asci were pushed out using the needles. After the rosettes were clearly visible, they were pushed to the center of the slide and the perithecial debris was removed. A coverslip was then placed over the rosettes. A Kimwipe was used to wick excess liquid from beneath coverslip. The coverslip was then sealed to the slide along its edges with clear nail polish. Digital images of the rosettes were obtained with a Leica DMBRE microscope and Zeiss imaging system. This process was performed for all crosses that produced fruiting bodies (**Figures 12–18 and 20–25**).

The final step was to confirm the genotypes of the RKD10 and RKDam11 strains. To conserve resources, only a few of the RKD10 and RKDam11 strains were genotyped (RKD10.2, RKD10.3, RKD10.4, RKD10.5, RKDam11.101, RKDam11.102, and RKDam11.105). To do this, small liquid cultures of each strain were incubated at 150–180 rpm, 32 °C, for 24–48 hours. Mycelia were harvested with 6-inch wood applicators, placed on a sheet of filter paper, and partially dried with a stack of clean paper towels. The mycelium was then stored in MCTs at -80 °C before drying by lyophilization. The tissue was lyophilized for 3–5 hours and then the tissue was stored in a dry cabinet at room temperature for later use in DNA isolation. Genomic DNA was then isolated from each mycelial sample with a plant/fungi genomic DNA isolation kit (IBI Scientific) according to the manufacturer's recommendations.

The genomic DNA was then used in a PCR-based genotyping assay. Specifically, for RKD10 strains and the control strain RDGR170.3, primers V0359-E (nested forward) and V0386-F (nested reverse) were used. For the RKDam11 strains and the control strain RDGR170.3, Primers V0394-E (nested forward) and V0358-F (nested reverse) were used. These primers were thawed at 60 °C, vortexed, briefly centrifuged, and placed on ice. Genomic DNA templates that had been isolated previously were thawed at 60 °C, vortexed, briefly centrifuged, and placed on ice. Q5 DNA polymerase buffer was thawed at 60 °C, vortexed, briefly centrifuged, and placed on ice. Next, the dNTP solution (10 mM) was thawed between gloved fingers, vortexed, briefly centrifuged, and placed on ice. Finally, the Q5 DNA polymerase enzyme was briefly centrifuged and placed on ice. Sterile water was poured into a 50 ml conical tube and placed on ice. For each vector, a primer mix was made in a 1.5 ml MCT tube where 6.25 µl of the nested forward primer (100 pmol/µl) , 6.25 µl of the nested reverse primer (100 pmol/µl), and 487.5 µl sterile water was added. The mix was vortexed, centrifuged, and placed on ice. Next, a PCR tube rack was placed on ice with a PCR tube in the rack for each test strain and an additional tube for the RDGR170.3 control template. A 5 µl aliquot of primer mix was added to each PCR tube. A 1 µl aliquot of each genomic DNA sample (approximately 10 ng/µl) was added to each tube. An enzyme master mix was created in a 1.5 ml MCT tube by adding 67.0 µl of sterile water, 25.0 µl of Q5 reaction buffer solution, 2.5 µl dNTP mix, and 0.5 µl Q5 enzyme which was mixed by gently pipetting (this master mix is sufficient for five reactions). A 19 µl aliquot of the master mix was then transferred to each PCR tube and the reactions were mixed by careful pipetting. The PCR reactions were cycled according to the manufacturer's recommendations for Q5 DNA polymerase. The PCR tubes were stored at -20 °C for later analysis by gel electrophoresis.

The next step was to check the products by gel electrophoresis. A 1.8% agarose gel was prepared as described above. The PCR products were prepared for the gel by adding 5 µl of 6× loading buffer to each PCR sample, and 10 µl of each PCR sample/loading dye mix was loaded into a well. Additionally, a 10 µl aliquot of a DNA ladder was loaded into the leftmost well. The gel was then run at 120 V for 90 minutes, then imaged over UV light. This process was completed separately for PCR products from RKD10 strains (v386; **Figure 20**) and RKDam11 strains (v408; **Figure 26**)

CHAPTER III

RESULTS

I predicted that deletion of *i386* would not disrupt *Sk-3*-type spore killing while deletion of *i408* would disrupt *Sk-3* type spore killing. My prediction was based on the location of the intervals relative to *i350* (**Figure 7**).

For deletion of *i386* with v386, spore killing was not disrupted (**Figure 12**). The RKD10.2 × RTH1623.2 cross displayed asci with 4 viable and 4 inviable ascospores indicating spore killing was not disrupted (**Figure 13**). The RKD10.3 × RTH1623.1 cross displayed asci with 4 viable and 4 inviable ascospores indicating spore killing was not disrupted (**Figure 14**). The RKD10.4 × RTH1623.1 cross displayed asci with 4 viable and 4 inviable ascospores indicating spore killing was not disrupted (**Figure 15**). The RKD10.5 × RTH1623.1 cross displayed asci with 4 viable and 4 inviable ascospores indicating spore killing was not disrupted (**Figure 16**). The RKD10.10 × RTH1623.1 cross displayed asci with 4 viable and 4 inviable ascospores indicating spore killing was not disrupted (**Figure 17**). The RKD10.X × RTH1623.2 cross displayed asci with 4 viable and 4 inviable ascospores indicating spore killing was not disrupted (**Figure 18**). The genotype of each transformed *N. crassa* used in these crosses was confirmed to carry the transformation vector (**Figure 19**).

In contrast to the results obtains for *i386*, deletion of *i408* appears to disrupt spore killing (**Figure 20**). For example, the RKDam11.101 × RTH1623.2 cross displayed asci with 8 viable ascospores indicating spore killing was disrupted (**Figure 21**). The RKDam11.102 × RTH1623.2 cross displayed asci with 8 viable ascospores indicating spore killing was disrupted (**Figure 22**). The RKDam11.103 × RTH1623.1 cross displayed asci with 8 viable ascospores indicating spore killing was disrupted (**Figure 23**). The RKDam11.104 × RTH1623.2 cross displayed asci with 8 viable ascospores indicating spore killing was disrupted (**Figure 24**). The RKDam11.105 × RTH1623.2 cross displayed asci with 8 viable ascospores indicating that spore killing was disrupted (**Figure 25**). The genotype of each transformed *N. crassa* used in these crosses was confirmed to carry the transformation vector (**Figure 26**).

CHAPTER IV

DISCUSSION

Two major results were obtained in this study. Deletion of *i386* does not disrupt *Sk-3* type spore killing, and deletion of *i408* does disrupt *Sk-3* type spore killing. These results offer insight into a broader effort being conducted in the investigation of the importance of *i350.* Disruption of spore killing by *i408* likely reaffirms the importance of *i350* in the *Sk-3* spore killing process as this DNA crosses over much of *i350.* These results are also able to be used in the context of other DNA interval deletions. As the effects that deletion of more intervals of DNA within and around *i350* have on spore killing are determined, the picture of how *Sk-3* spore killing is genetically driven will become clearer. It seems that *i350* either contains the poison production gene for spore killing or operates as a regulator for this gene. Deletion of additional intervals around *i350* should help us understand if other locations are involved. The lack of disruption to spore killing due to deletion of *i386* is a valuable result as it eliminates this region as a possible location for the killer gene or regulatory element. Intervals within *i350* can be analyzed to determine if the key portion of DNA to spore killing is more precise than all *i350.* Ultimately, as the picture for the intervals of DNA that effect *Sk-3* spore killing become clearer, so will the understanding of what drives *Sk-3* spore killing.

Because *N. crassa* is an excellent model for eukaryotic genetics, understanding the complexities behind the genetic components that drive phenomena exhibited by it, may offer a greater understanding of eukaryotic genetics as a whole.

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Table 1 Strains used in this study

Strain name	Genotype
F2-23 (RTH1005.1)	*rid; fl A+*
F2-26 (RTH1005.2)	*rid; fl a+*
FGSC 10340 (RZS27.10)	*rid; mus-51*^{RIP70} *a+*
ISU-3036 (RTH1623.1)	*rid; fl; sad-2Δ::hph+ A+*
ISU-3037 (RTH1623.2)	*rid; fl; sad-2Δ::hph+ a+*
ISU-3291 (RDGR170.3)	*rid; Sk-3+; mus-51Δ::bar+ A+*
TDMS1.2	*v386*-based hygromycin resistant transformant of ISU-3291
TKDam1.1	*v408*-based hygromycin resistant transformant of ISU-3291
RKD10.2	*rid; Sk-3+ i386Δ::hph+; mus-51? A+* (offspring of TDMS1.2 × F2-26)
RKD10.3	*rid; Sk-3+ i386Δ::hph+; mus-51? a+* (offspring of TDMS1.2 × F2-26)
RKD10.4	*rid; Sk-3+ i386Δ::hph+; mus-51? a+* (offspring of TDMS1.2 × F2-26)
RKD10.5	*rid; Sk-3+ i386Δ::hph+; mus-51? a+* (offspring of TDMS1.2 × F2-26)
RKD10.10*	*rid; Sk-3+ i386Δ::hph+; mus-51? a+* (offspring of TDMS1.2 × F2-26)
RKD10.X*	*rid; Sk-3+ i386Δ::hph+; mus-51? A+* (offspring of TDMS1.2 × F2-26)
RKDam11.101*	*rid; Sk-3+ i408Δ::hph+; mus-51? A+* (offspring of TKDam1.1 × F2-26)
RKDam11.102*	*rid; Sk-3+ i408Δ::hph+; mus-51? A+* (offspring of TKDam1.1 × F2-26)
RKDam11.103	*rid; Sk-3+ i408Δ::hph+; mus-51? a+* (offspring of TKDam1.1 × F2-26)
RKDam11.104	*rid; Sk-3+ i408Δ::hph+; mus-51? A+* (offspring of TKDam1.1 × F2-26)
RKDam11.105*	*rid; Sk-3+ i408Δ::hph+; mus-51? A+* (offspring of TKDam1.1 × F2-26)

*Predicted genotype (Interval deletion allele was not checked by PCR). The *rid, fl, mus-51.* and *sad-2* alleles have been described by others (Freitag et al. 2002; Ninomiya et al. 2004; Perkins et al. 2002; Shiu et al. 2006; Smith et al. 2016).

Table 2 Primers used in this study.

Name (Alias)	Sequence (5′ > 3′)	Purpose
HPH-CEN-F	AACTGATATTGAAGGAGCATTTTTGG	Center fragment amplification from Plasmid pTH1256.1 (GenBank: MH550659.1)
HPH-CEN-R	AACTGGTTCCCGGTCGGCAT	
V0359-A	ATCGCCGCAAACAGGACAATAGA	Left flank amplification for v386 from RDGR170.3 genomic DNA
V0359-B	AAAAAATGCTCCTTCAATATCAGTTGAACGACTTCCC CAGAACCAGAA	
V0386-C	GAGTAGATGCCGACCGGGAACCAGTTGGGCTGGGCT CAAGCAAGGAACT	Right flank amplification for v386 from RDGR170.3 genomic DNA
V386-D	TCAACACGAGGCAGACGCCACTC	
V0359-E	CGCTGGCTCCGTTCTCAGCTC	Nested amplification of v386 left flank, center fragment, and v386 right flank fusion product; *i386+* and *i386Δ* genotyping
V0386-F	TGTGCCCGGTCCTTCCCTTTGGA	
V0394-A	TCCAAAGGGAAGGACCGGGCACA	Left flank amplification for v408 from RDGR170.3 genomic DNA
V0394-B	AAAAAATGCTCCTTCAATATCAGTTCGTGAGCCGGAG CAGTCGTCGTA	
V0358-C	GAGTAGATGCCGACCGGGAACCAGTTATAAATTGCTG GGCTAGGGAAGGTG	Right flank amplification for v408 from RDGR170.3 genomic DNA
V0358-D	GGTGATGAATGGCGGATAGGTTCTT	
V0394-E	GGGACAGAGAGTGGCGTCTGCCT	Nested amplification of v408 left flank, center fragment, and v408 right flank fusion product; *i408+* and *i408Δ* genotyping
V0358-F	AGTTAGTTTGGCTCTGGATGACTGC	

Figure 1 Research Progression

A

```
>v386 left flank
ATCGCCGCAAACAGGACAATAGATTGTGTTATCAATTGGGTCCTCCCTCGCAAGTGGAACTTC
TGGCGCTGGCTCCGTTCTCAGCTCGCTGGCAGGTGTCGCGATTCCGGCGTTCAAGTTGTGAAG
GGTGATAGTGTAGTCGTGGACCAGATCCTCGATGCGCGTCTTCAGATTGTCGTCACCCTCGGC
AAAGAGAGTGAATAGTACGATTTGGATGCTCATGCAAGGAGCGTTGTGGATCGCATCAAGCAT
TGTCCTGTTGCGTTCGTCCACTAGTGGAACTGGCATGCTCTGTTCGTTCGCCATTTTGGGCGC
TTGATTGCTGGGGCTGGTGGCGGGGTGTGGAAGGGAATTGATGAGGTACACCAGATGGATAAT
TGTTTGGTGTTTGCGCAAGAAATTCAGGGTACGGACTATCGAGAGACAAATTCCATTTTGTTG
GATGTCCGTGGACCGTGCCCTGCCCTGGAATCCTTCGCTCGACTTGTCATTCACTGGTACTCC
ATGGCGAGCGCGAAAGGGCCAGAGGGGGGGCGGCTCTCTTCTTGGGCATCTTGGACAACTGC
GCCTTAGGCGAACAAATGATTGATCAAGAGTTCATGATATCAAACAGCCAGCTTACGTTGATT
GCGTGGCCTTCCTACTGAGATGGGTGACCAAGGAAAAGATATTGCGACCATAAGGTGTCGGAA
AGGAGGAGTGCGAAGGAACAAAAGCTGCAGTAAAGTGCACACTACTAGGGAACATTAGGGCAC
AATGCCTTGCGCGAAAACGAGTCACAAAGGGAAGGCACATTCCTGAGGTCGGGACATTTGATG
AGAAATTTGGTTGTGGTAATCTTCTGGTTCTGGGGAAGTCGTTC
```

B

```
>v408 left flank
TCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGAACACTAACGAGCGATTT
TGCCACCTAGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCCTACCACGCTTCTTTTTC
CGTCCACCGGCTCTTGGAGTACCGTACATACCTCAAACACTTCATTCCACCCTGTTCTGGAAT
TGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGG
AAGGAGGAGAGGTACGTTGGGTGCGTTAGTGTATCTTGATCATGATAAAGGGCAATCACGGGG
ACTTGGCTCCATGCCCAAAAATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTC
ACGATGACGAAGTGCCTCACAGCAACTTGAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCT
CATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGACTTTTTCAGTGGAAGTGACGGTTTAAC
CCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGAACAAGCCAAGAAAGCTGAG
GCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCGTTCCCCGATAA
AGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCA
AGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGAT
GGCTCCCATGCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGA
TGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACG
```

Figure 2 Transformation vector left flanks. **(A)** The 863 bp sequence of the v386 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0359-A and V0359-B from RDGR170.3 genomic DNA. **(B)** The 872 bp sequence of the v408 left flank is shown in the 5′ to 3′ direction. This sequence was amplified with primers V0394-A and V0394-B from RDGR170.3 genomic DNA.

A >v386 right flank

```
GGGCTGGGCTCAAGCAAGGAACTCCTTGGCCCCATATCGTAACCTTCGATACTCGCATCTTGG
GTGCAGCTTCTTCCACGTCGATAGCAACTGTATGCCAAAGGTGTATTGTACTTGACCGCGTAA
CTGAAGGTGTCTGGTTCATTTTTTGACTTAGATGAGCCCTTATCGAAGCTGACGCCGGTTTAG
CGTTGTAAGTGTTAGTGGTGTAACGCCCTCGCCGCCCGACCACAGATCCCGAGTCCCGCTTCT
TCTCCGCTGCCTGCAATTGCGGGCGTTGCACACTGCATTCAGTCGTTGGTCAATGGTCATTGA
TCAATCATTAGATTGACAGTAACTGTTCCCGCCCGATCGAGTGAAGGCTTTAATTCAGGTTCC
CACGGTCCCACCGGGTCTAGGGGGAGACTTCTGGTAGGGGTATGGAACATGACTTCCGGCTTC
ATGAGAGAACCAAGGGAAGGCAGGGCCCTTGTCGGAGAAGCTAACCTGAAGGCACCCATAGTT
TTCTTTCCCTTGATGATGCCTGACATATTATTGTAGAGTTCCTTCTTCAACGTACCGGATCGT
GTTCTCACTCTAAGATAGAAAGGGCGAGGAAGGACTTGGTGTCAATTGGTGACTGAACTCCCA
ACAACACAAAGCTGTCGTCCAAAGGGAAGGACCGGGCACATACCTCTAGCCTTACCAGACGGA
ACACTAACGAGCGATTTTGCCACCTAGAAGTATACCTCTATGCTCAACAGTAGGTAGACATCC
TACCACGCTTCTTTTTCCGTCCACCGGCTCTTGGAGTACCGTACATACCTCAAACACTTCATT
CCACCCTGTTCTGGAATTGTTGGGACAGAGAGTGGCGTCTGCCTCGTGTTGA
```

B >v408 right flank

```
ATAAATTGCTGGGCTAGGGAAGGTGGATTGTGGTTGTTAGGGAAGCGGGCTTTTCGTTGTAGT
TGTTGTACCATTCAGTTTGTACTCACGGATACGATACCTCTTCTTCTTTTTCTCTTTACCGAA
AAATTGTCATTACGCATCGTCACTTTTGTTGGGAGGACTGATTTTGTTGTCGAGCGGAATCGA
ATGGAGAATGGGGGATGAGGAATGGGGGAAGAGGAAGAAAGTCAGGGAGCAGGCAGAGGGCAG
CAGAGCAGGATTTATAGAAAGAAGTAACTGATATGATATGCTGCGCGAAGGGATTCTTCTATT
GATGCTGATATGTGCTTCATATGCATTTGTCCGAAAGGGAATCTCTAAGGGAACGAGTTCAGG
GGGTAAGCATCCTGCCTGCGTAATCATTGAACTCTCAAGTACCAACTATTGAAGTTATTATCC
TACCGTTACGCGGTTCAGTGTATTTTTGCCGTCAAGGCTCTCTCTTCTTGTAGCCTAGTTATC
TATCACTGGTCCCATAGGGCTCGGACAGATACTGCCACGAGAAAAGGTGTAAAGGGGGAGGAT
ATGAAGAACATACTGTATTCAGCCATATAGACACACATATGTCCAACAAGACCAATTGAGGTA
TCAGCAAAAATATATATATACAGTAAATGATCATCTGCCAGGCAGTCATCCAGAGCCAAACTA
ACTTACAACCTATCTAACAAGAACCTATCCGCCATTCATCACC
```

Figure 3 Transformation vector right flanks. **(A)** The 871 bp sequence of the v386 right flank is shown. This sequence was PCR-amplified with primers V0386-C and V0386-D from RDGR170.3 genomic DNA. **(B)** The 736 bp sequence of the v408 right flank is shown. This sequence was amplified with primers V0358-C and V0358-D from RDGR170.3 genomic DNA.

```
>v386 and v408 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 Transformation vector center fragment. The 1412 bp sequence of the v386 and v408 center fragment is shown. This sequence was amplified with primers HPH-CEN-F and HPH-CEN-R from plasmid pTH1256.1 (GenBank: MH550659.1). The positions of the *hph* start and stop codons are underlined.

```
>Interval i386
CTTTCTTGTAGATAACAAAACGAAACATTCTCCTCTTCGTGCAGTGTGACCTAACGCCCGAAA
ACCACTGCGCCAACTGAAGCTGTCTTGCTTTTCTCCAAACTATCGCTTCGGGAAACTGATTAC
CCGCTTGGGAGGGGAGTCATTATTTCCCTTGCTGAATCATGCCTGTCCTCAATCAGTCCTTTT
GCTCCGTCTTCCCTCTCTTTCCGGGCTTTCTTCTCGCTTTCGCTTGAGACTGTTTGAGCCCAG
ACCACTGGTATACGATACTATCGAGTGTACCTCCACCGTCAAGGTCTGATTCAGGAGAGGTCC
ACAATGGGATTCATAGGCCTCTCTTGTAGGAAAGGCCTCCCGCAAAGCGATCTCTTGAGCCCT
CAACAAGGCGCGCCTCTTTTTTCTAGGCAGTTGCGTAATGTCAGGAAAGTCCCGGCCAGCTTT
GTCGCACTCATTTACCCAGCTGGGTCCCTCGTCTTCATTGAAGCGTGCTTCAACTATGCAGAG
AAGGTGGATGACTTTCACAGTCTGGGGGTTCCTGAACCCACATCCACCGGAGCGGCCTGTTGA
AGGACGGTTCGTCTACAGCCAGCTTTCTTTGTTGAAGTTTCTTGCGCTCTCATTGGCTCAGAG
CGTGGCACGGCCCTACAGCTAGTCAGGGCGTTTTGCCAAGCCACTTCTCATTGGTCAACTTGC
GAACAGTGTTTCTCACAGTCCGCGCTAGAATGCACTGGGTCCTTCCGTTTCTGAAGTCCTTCA
CTAAATCTCTCGATATCGCCTATCGATAAAGCTATCGTCGTCCTCCTTGCTATGAAGCAATTC
CAATGAAGCAATTCCAATGAAGGAATGAACCAATGAAGCAAGGAAGTAATGAAGTAAGGAAGC
AAGGAAGTTAGGGAGTAAGGGAGTAATACGGTATAGGAGTAGAGTGGAAAGAAAGAACAGCGA
AGCAATTGGGTGAGGACGGCTGTAGCCGTCCGAATAAGAGATGCATCTAGATATAGGTAGTAG
TCTCGGTAGTATCCACGCTTCAACAATACAGCGGGCGTTGCTTGAACTAGGGATCCAGCATCG
CATCGATGTCTGAAAACTAATTTAATTATCTTGCCCATCGGTATTGGCGACGTCTGGGATCTG
GGTGTCGTGTTTGGCGGTTGGTTCCTCCGTAACGGGGGTAGACCTGTCCGAATGTCTTGTGGC
TGGAGTGGAGTCCGACTCCTCCGCATCGGAATCAGAATCTACGGCCATTGAGGCTTGCTTGCG
GAGAGCGATGCAATGAGCCTCCCATGACAACATGTCCTCTTTTCTTGGTACACCCATAGCCTC
CTGAAAAGCCTCGTAGCTTTTCCAGCCATCGAGGTCTTTCTCCCGCACTCCATCAGCGGCTCT
ATGCGGACCTAGCCTGCACCCGAGCTTGTCGTGCGTTCCCCACTGTTGACAGCAGGGGAACCA
GTATCCTGGCTTGTGTACTTAATGGAAGAAGAAGAAAGAAGAGGAAAATGAAGGAAGGGGGGG
GGGGGGGGGGGGGGGCCTCTGTAACTGTACATTGAGACGCGAGTACCTCTAAGAATCCATG
TGTGGATCGAAAATCGAGCAGTACCTCCTCAACAACGGCCTGGTTACATTCATTGCCAGCGAA
AATCACATTTCGTTCAGTTTTGCTTCTCAGAGCTGGCGCAAGTTGAAGTGGAAGTTACGATTC
AGACTGCTGGGTCTTTACCGTGCAATACCGGGGCAATCTGGACCTGCTATGATTCACCCCACA
CCCGGAATACGTCATCTGTCAACTCCATTCGCACTTGCTACTCGCTGACCAGGCACAGGTCGA
GGTACATAGAGTAATGCGATGGACACCCGCAGGTCTCAACAAGTGCCTTCCATCTCACAGATG
GCAATGCTCGGCTATTGGACACAACCGTTTACTACCTAGGTACCTAGACTGCGGCCTGTTTAC
AAAAGACAAGAACGGTAAGTAATGGCATCTCTCGGCTGATCGGATGGGGGAAAGGCAAGTGAA
TCGGTCACGACAGATCCTCCGCCAAGATGAGTTTCTTTTGTCGACAGAAGGTGTCAGGCAAGA
GGCAGGAAGAGACAGCATGCCCGGACCGAACAATCGGAATGACTCCATTGAAACGCTGAGAA
CGGCGCCCCGGCCTACTACTAGGTACCATCGAGCTGAGTGGTCCTCCCTCCCAAGCTTGA
```

Figure 5 Sequence of DNA interval *i386.* The 2202 bp sequence of *i386+* is shown.

>Interval *i408*

CCCGTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGT
GGCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCG
GGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCAC
TACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGG
AAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAA
GCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAG
GACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCATTCATCCTAA
TGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTGTATTCGGAATACA
CATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTGTAAGTCTACTTTATA
TTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGACGGCTTACCTTGTCCTCT
CGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCTCCCCCCCGACGGGGATGAC
CTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGT
TGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTG
ACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGTTCAA
TTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGAAACACCACCCCACC
CAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACCCTCCCTCTCCTTCCCCCCTCTCGCGCT
GACGACACCACCGGCCACCGCAACAGAATTCATTGCCAAACCAGACCCAGCAACAGCCCCGAGCCCA
TCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCAATGGCAACCGTACTCTGACTCTG
CCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTA
CGCCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACAAGTATGATTCATACCAG
GCCGGTCTGAACCCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGC
GCGACGCCAACGGCGACGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAACGCTACCAT
CAAGTACCCCCTGAGGCCGCATCACTCGCTCTCCGGTGGTCGCCCGGCCAGCCGTCAAGCATCCCAA
CAGCTTCCCCCCATCAACCCATACGCACCCGCTCAACACGGCCACAGCTATCCAAACTCGGCAATCA
GCTCTACCATGGACGGCTCTTATATGGACCCCAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCA
ACAGATGCCCATGCCCGAGAGGACGCCAGTTCCCGAATTTAGGAAAATGCGAGGACCCCAGGACCTT
CGACCAAAGATTAACAAGCAGCCGGCTCATCGACGAGCTAACCCGGAAGGCGGCTTTATCAGTGTAT
GTACATGTTCGCGAGCCATAGCTTTGGGCAAGCCTGCTGACGAACCACGATAGCCCCTCCAAGCGCT
AACAGTTCACCTCCCCGCCACCTACCGAATATGCAACCCCGGCTTCAAGTACGAGTCGTCTAGGAAT
CCTCGGCGCGTTCTTACCAAGCCTAGCAAGGGAGTGAAGAATGATGGCTATGACAACGAGGACAGCG
ATTATATCCTCTATGTGAATGATATCCTGGGCTCAGAGGAGGCTGGTCATAAGTAAGTTGCTGCCCA
CCACGAGTCGAGAAGCAGTCACTTACATGTTTGTACCAGGAACCGCTACCTGATTCTCGATGTCCTT
GGCCAGGGTACCTTCGGCCAGGTCGTAAAGTGCCAAAACTTGAAGACGCAAGAGGTCGTTGCGGTCA
AGGTCATCAAGAACCGAACAGCTTACTTCAACCAAAGCATGATGGAAGTGTCTGTTTTGGATTTGGT
TCGTACACCAGCATTGTTGACTTGATTGTTTGCGACACGCGCTAACCTCCCGTTCAGCTCAATACAA
AGCTCGACAAAAACGACGATCACCATCTGTTGCGACTAAAGGACACGTTCATCCATCGCCAACACTT
GTGCTTGGTATTCGAGTTGCTTAGTGTCAACCTATACGAGCTGATCAAGCAAAACCAGTTCCGAGGC
TTGAGCACGACACTGGTTCGCGTCTTTGCGCAGCAGCTGCTGAATGGGCTTTCTCTGCTCAACAAGG
CGAGACTGATCCATTGCGACCTGAAACCCGAGAACATTCTCCTGAAGAACCTCGAGAGCCCGATCAT
CAAAATTATCGATTTCGGATCCGCTTGCGACGAACGGCAGACTGTCTATACGTACATCCAGTCCAGA
TTCTACCGATCCCCTGAAGTGTTGCTTGGCTTGCCTTATTCCTCGGCTATTGATATGTGGTCTTTGG
GATGCATTGTGGTTGAGCTTTTCTTGGGTCTTCCCCTCTTCCCCGGTTCTTCCGAGTACAACCAGGT

```
GTCACGAATCGTCGAGATGCTGGGCAATCCTCCAAACTGGATGATCGAGATGGGCAAGCAGGCAGGA
GAATTCTTCGAGAAGAGGCAAGATGAGTTCGGCAGAAAAACCTACCACCTGAAGTCTATGGAGCAAT
ACTCTCGGGAGCATGGCACGAAGGAACAACCTAGCAAGAAATACTTCCAAGCCAACACACTGCCCGA
GATTATCAAGACGTACCCGATGCCGAGGAAGAACATGAAGCAGTCAGAGATTGACAGAGGTGAGTCG
AGCGCATTCGGTGTGTTTGTGCTCTGAAAGCTGACCTTGTCCTAGAAATGAACAACCGTATCGCTTT
CATCGATTTTGTCAGGGGTCTGTTGACGATCAATCCCTTGGAACGATGGTCGCCTCAACAAGCCAAG
CTACATCCTTTCATCACCCAATCGAAGTTTACTGGACCGTTTGTACCGCCCATGAACCTCAAGTCAA
GTTCGCTCAACAGATCACCAGCCCCGGGAACTCAACAGCAGATACAGGCCGAGGCATTCAGCAAGCA
AAAGGCGCAACAAGCGCAAGCCAACGCCATTGCGGCAAACCAGGCCCAAAACCCTTACGGGTCGATG
GCCACTGGGCAGCAATATCCCCAGCAGACCCACACGCAACCTCCGCCCTTGTATTCCAACAACAACA
TTTACGCTCCTGGTGGCAGCAGCAGTCACGCTAGCGCGCCTCCACCGTACGGCTCTCAGCAGGGCGC
ATACCCTCAACAAGGTATGCCCCAACAACAGCAGCCGCAGGTACCGCAAGTACAGATGCCTCCAGCG
AACTACGCGGGCGTGTCCCAGTCAAATCTGTACGCCCAGCAACAGGCGGCAGCGGCGGCGCGCCAGA
GGCAACGGTCCTCGACAATGGAGCAGCAGCAAAGTGGTATTCCCGTGTCCATCCAGCGCGTCGCGAG
CCATCTTGATCCCACCCAGCCAATTCGTCTGCAACCGAGCCCCGCCTACTACCCGCCGCCACCAGAC
GGCCTCATGGGAATGGACTCGCAGCCCAGCCAAAGGATGCCGAGGAGGGGAAGCCGTGCTCAGGCGT
CTGGACGGGGTCAGGGCAACAACCGTGACTTCATCAGGAACTTGGAGGAGAGGACGTTGGAGGAAGG
GTTTATGGGCGGGAACGGTGGAGGTCAGGGTCAAAGTCAATGGCATTGAGC
```

Figure 6 Sequence of DNA interval *i408.* The 4004 bp sequence of *i408+* is shown.

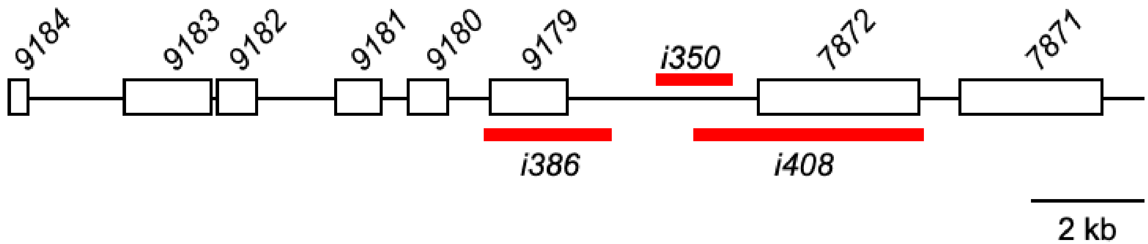

Figure 7 *i386* and *i408* locations**.** A diagram of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the positions of likely protein coding genes. *N. crassa* gene database numbers are shown for each predicted gene. The locations of *i350, i386,* and *i408* are indicated with red bars.

```
>i386Δ::hph+ PCR product, predicted sequence, primers V359-E and V386-F
CGCTGGCTCCGTTCTCAGCTCGCTGGCAGGTGTCGCGATTCCGGCGTTCAAGTTGTGAAGGGTGATAGTGT
AGTCGTGGACCAGATCCTCGATGCGCGTCTTCAGATTGTCGTCACCCTCGGCAAAGAGAGTGAATAGTACG
ATTTGGATGCTCATGCAAGGAGCGTTGTGGATCGCATCAAGCATTGTCCTGTTGCGTTCGTCCACTAGTGG
AACTGGCATGCTCTGTTCGTTCGCCATTTTGGGCGCTTGATTGCTGGGGCTGGTGGCGGGGTGTGGAAGGG
AATTGATGAGGTACACCAGATGGATAATTGTTTGGTGTTTGCGCAAGAAATTCAGGGTACGGACTATCGAG
AGACAAATTCCATTTTGTTGGATGTCCGTGGACCGTGCCCTGCCCTGGAATCCTTCGCTCGACTTGTCATT
CACTGGTACTCCATGGCGAGCGCGAAAGGGCCAGAGGGGGGGGCGGCTCTCTTCTTGGGCATCTTGGACAA
CTGCGCCTTAGGCGAACAAATGATTGATCAAGAGTTCATGATATCAAACAGCCAGCTTACGTTGATTGCGT
GGCCTTCCTACTGAGATGGGTGACCAAGGAAAAGATATTGCGACCATAAGGTGTCGGAAAGGAGGAGTGCG
AAGGAACAAAAGCTGCAGTAAAGTGCACACTACTAGGGAACATTAGGGCACAATGCCTTGCGCGAAAACGA
GTCACAAAGGGAAGGCACATTCCTGAGGTCGGGACATTTGATGAGAAATTTGGTTGTGGTAATCTTCTGGT
TCTGGGGAAGTCGTTCAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACA
ATGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGC
AGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGG
TAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTA
CCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCG
AAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGAT
GTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTA
TCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGA
CCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTT
CTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCC
ATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATG
TGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATG
CTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGAC
GGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCG
CCAACATCCTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCAT
CCGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAG
CTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAG
CCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTA
CTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAGAGTAGATGCCGACCGGG
AACCAGTTGGGCTGGGCTCAAGCAAGGAACTCCTTGGCCCCATATCGTAACCTTCGATACTCGCATCTTGG
GTGCAGCTTCTTCCACGTCGATAGCAACTGTATGCCAAAGGTGTATTGTACTTGACCGCGTAACTGAAGGT
GTCTGGTTCATTTTTTGACTTAGATGAGCCCTTATCGAAGCTGACGCCGGTTTAGCGTTGTAAGTGTTAGT
GGTGTAACGCCCTCGCCGCCCGACCACAGATCCCGAGTCCCGCTTCTTCTCCGCTGCCTGCAATTGCGGGC
GTTGCACACTGCATTCAGTCGTTGGTCAATGGTCATTGATCAATCATTAGATTGACAGTAACTGTTCCCGC
CCGATCGAGTGAAGGCTTTAATTCAGGTTCCCACGGTCCCACCGGGTCTAGGGGGAGACTTCTGGTAGGGG
TATGGAACATGACTTCCGGCTTCATGAGAGAACCAAGGGAAGGCAGGGCCCTTGTCGGAGAAGCTAACCTG
AAGGCACCCATAGTTTTCTTTCCCTTGATGATGCCTGACATATTATTGTAGAGTTCCTTCTTCAACGTACC
GGATCGTGTTCTCACTCTAAGATAGAAAGGGCGAGGAAGGACTTGGTGTCAATTGGTGACTGAACTCCCAA
CAACACAAAGCTGTCGTCCAAAGGGAAGGACCGGGCACA
```

Figure 8 Genotyping assay PCR product: *Sk-3 i386Δ::hph+* genotype. The predicted sequence of a DNA molecule amplified with primers V0359-E and V0386-F from a template consisting of genomic DNA from a strain with the *Sk-3 i386Δ::hph+* genotype is shown. The start and stop codons of *hph+* are shown with white font on black background. The length of the sequence is 2879 bp.

>*i386+* PCR product, predicted sequence, primers V359-E and V386-F

```
CGCTGGCTCCGTTCTCAGCTCGCTGGCAGGTGTCGCGATTCCGGCGTTCAAGTTGTGAAGGGTGATAGTGT
AGTCGTGGACCAGATCCTCGATGCGCGTCTTCAGATTGTCGTCACCCTCGGCAAAGAGAGTGAATAGTACG
ATTTGGATGCTCATGCAAGGAGCGTTGTGGATCGCATCAAGCATTGTCCTGTTGCGTTCGTCCACTAGTGG
AACTGGCATGCTCTGTTCGTTCGCCATTTTGGGCGCTTGATTGCTGGGGCTGGTGGCGGGGTGTGGAAGGG
AATTGATGAGGTACACCAGATGGATAATTGTTTGGTGTTTGCGCAAGAAATTCAGGGTACGGACTATCGAG
AGACAAATTCCATTTTGTTGGATGTCCGTGGACCGTGCCCTGCCCTGGAATCCTTCGCTCGACTTGTCATT
CACTGGTACTCCATGGCGAGCGCGAAAGGGCCAGAGGGGGGGGCGGCTCTCTTCTTGGGCATCTTGGACAA
CTGCGCCTTAGGCGAACAAATGATTGATCAAGAGTTCATGATATCAAACAGCCAGCTTACGTTGATTGCGT
GGCCTTCCTACTGAGATGGGTGACCAAGGAAAAGATATTGCGACCATAAGGTGTCGGAAAGGAGGAGTGCG
AAGGAACAAAAGCTGCAGTAAAGTGCACACTACTAGGGAACATTAGGGCACAATGCCTTGCGCGAAAACGA
GTCACAAAGGGAAGGCACATTCCTGAGGTCGGGACATTTGATGAGAAATTTGGTTGTGGTAATCTTCTGGT
TCTGGGGAAGTCGTTCCTTTCTTGTAGATAACAAAACGAAACATTCTCCTCTTCGTGCAGTGTGACCTAAC
GCCCGAAAACCACTGCGCCAACTGAAGCTGTCTTGCTTTTCTCCAAACTATCGCTTCGGGAAACTGATTAC
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TGTGGCTGGAGTGGAGTCCGACTCCTCCGCATCGGAATCAGAATCTACGGCCATTGAGGCTTGCTTGCGGA
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CCACACCCGGAATACGTCATCTGTCAACTCCATTCGCACTTGCTACTCGCTGACCAGGCACAGGTCGAGGT
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GCATCTTGGGTGCAGCTTCTTCCACGTCGATAGCAACTGTATGCCAAAGGTGTATTGTACTTGACCGCGTA
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AGTGTTAGTGGTGTAACGCCCTCGCCGCCCGACCACAGATCCCGAGTCCCGCTTCTTCTCCGCTGCCTGCA
ATTGCGGGCGTTGCACACTGCATTCAGTCGTTGGTCAATGGTCATTGATCAATCATTAGATTGACAGTAAC
TGTTCCCGCCCGATCGAGTGAAGGCTTTAATTCAGGTTCCCACGGTCCCACCGGGTCTAGGGGGAGACTTC
TGGTAGGGGTATGGAACATGACTTCCGGCTTCATGAGAGAACCAAGGGAAGGCAGGGCCCTTGTCGGAGAA
GCTAACCTGAAGGCACCCATAGTTTTCTTTCCCTTGATGATGCCTGACATATTATTGTAGAGTTCCTTCTT
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AACTCCCAACAACACAAAGCTGTCGTCCAAAGGGAAGGACCGGGCACA
```

Figure 9 Genotyping assay PCR product: *Sk-3 i386+* genotype. The predicted sequence of DNA amplified with primers V0359-E and V0386-F from a template consisting of *Sk-3 i386+* is shown in FASTA format. The sequence is 3669 bp long. Interval *i386* is indicated with red font.

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>i408Δ::hph+ PCR product, predicted sequence, primers V394-E and V358-F
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ATGAAAGGGTCACCAGTCACGAAAGGCCGTTTTGCTCGAATTCACGATGACGAAGTGCCTCACAGCAACTT
GAGGTTGGTTAGGCTGCCCCTGGTAATACCAACCTCATGTCTCGGTGAAGGGCGCCCTCTTCGATGAAAGA
CTTTTTCAGTGGAAGTGACGGTTTAACCCTTTCCTTTCTTTTGCGGATTACGTCCCCAACTCACGTCATGA
ACAAGCCAAGAAAGCTGAGGCCTTTGAGGAGGAACCTCCGTCCTTGTGTCTTTTGAATGTGGAAATGAGCG
TTCCCCGATAAAGGAGCATGAACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCC
CCAAGCCCGAATGATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTC
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TCCAGAGCCAAACTAACT
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Figure 10 Genotyping assay PCR product: *Sk-3 i408Δ::hph+* genotype. The predicted sequence of a DNA molecule amplified with primers V0394-E and V0358-F from a template consisting of genomic DNA from a strain with the *Sk-3 i408Δ::hph+* genotype is shown. The start and stop codons of *hph+* are shown with white font on black background. The length of the sequence is 2787 bp.

>*i408+* PCR product, predicted sequence, primers V394-E and V358-F
GGGACAGAGAGTGGCGTCTGCCTCGTGTTGAATCAAGACCGGCATGTTGGTACTTCAGGAAGGAGGAGAGG
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TATATATACAGTAAATGATCATCTGCCAGGCAGTCATCCAGAGCCAAACTAACT
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Figure 11 Genotyping assay PCR product: *Sk-3 i408+* genotype. The predicted sequence of DNA amplified with primers V0394-E and V0358-F from a template consisting of *Sk-3 i386+* is shown in FASTA format. The sequence is 5379 bp. Interval *i408* is indicated with red font.

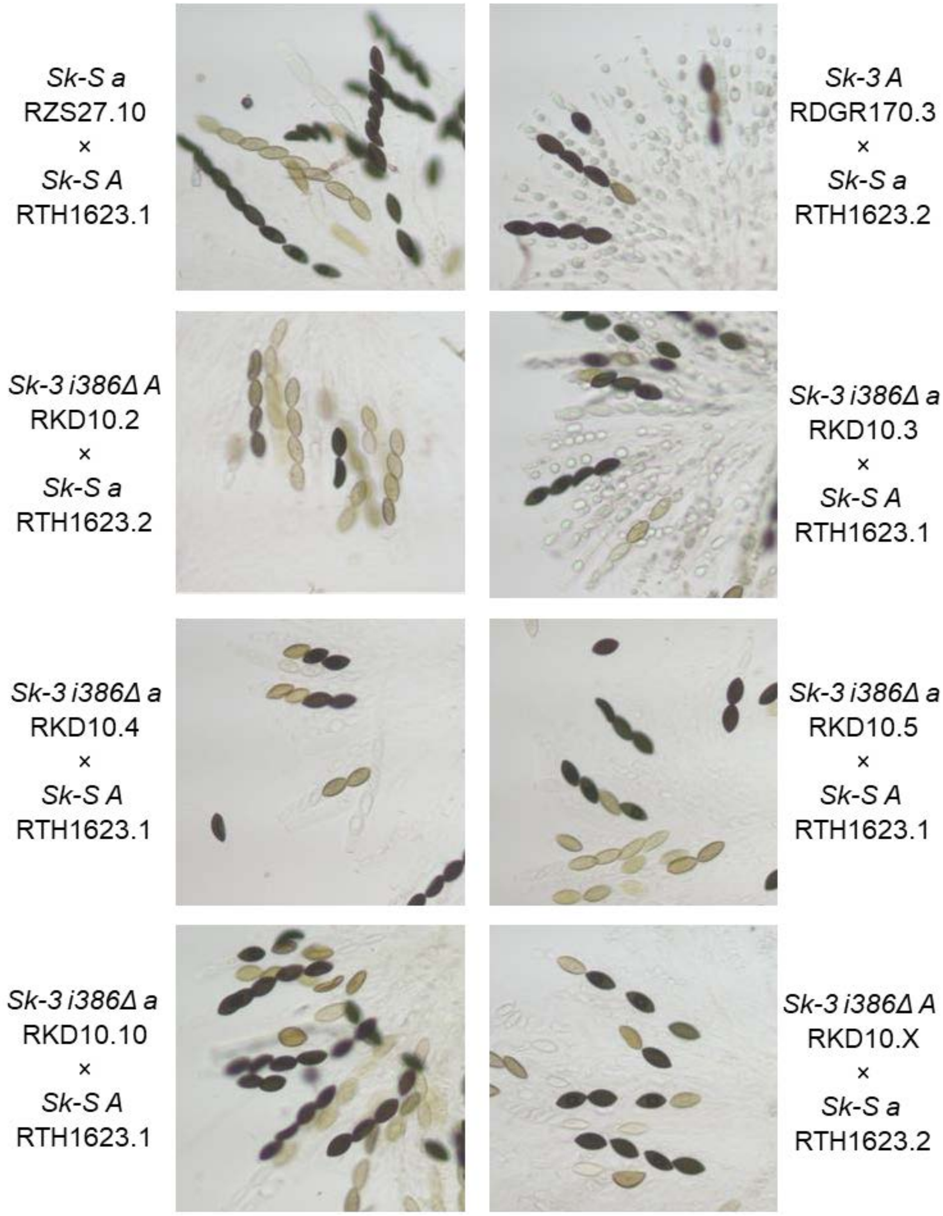

Figure 12 Ascus phenotype summary. Asci were dissected from perithecia of eight crosses 17 days post fertilization (dpf) and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *i386* does not disrupt spore killing.

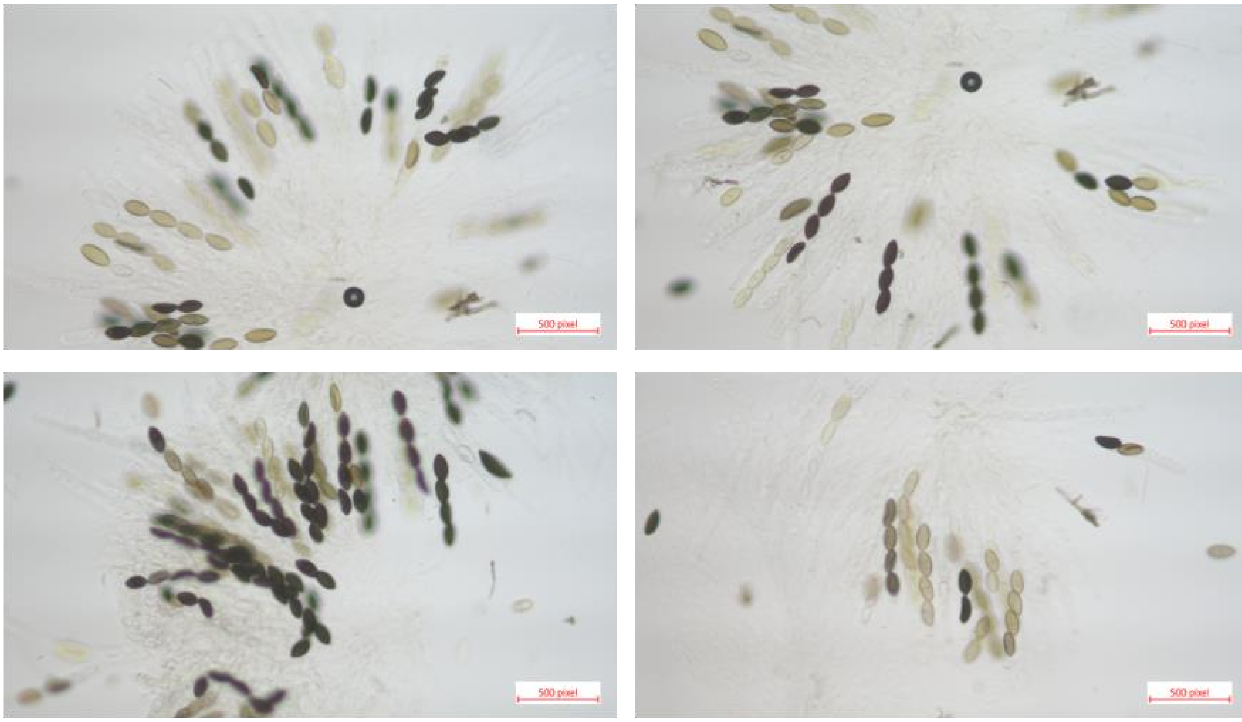

Figure 13 Ascus phenotypes. Asci were dissected from perithecia of RKD10.2 × RTH1623.2 on Day 17 post fertilization and imaged under magnification.

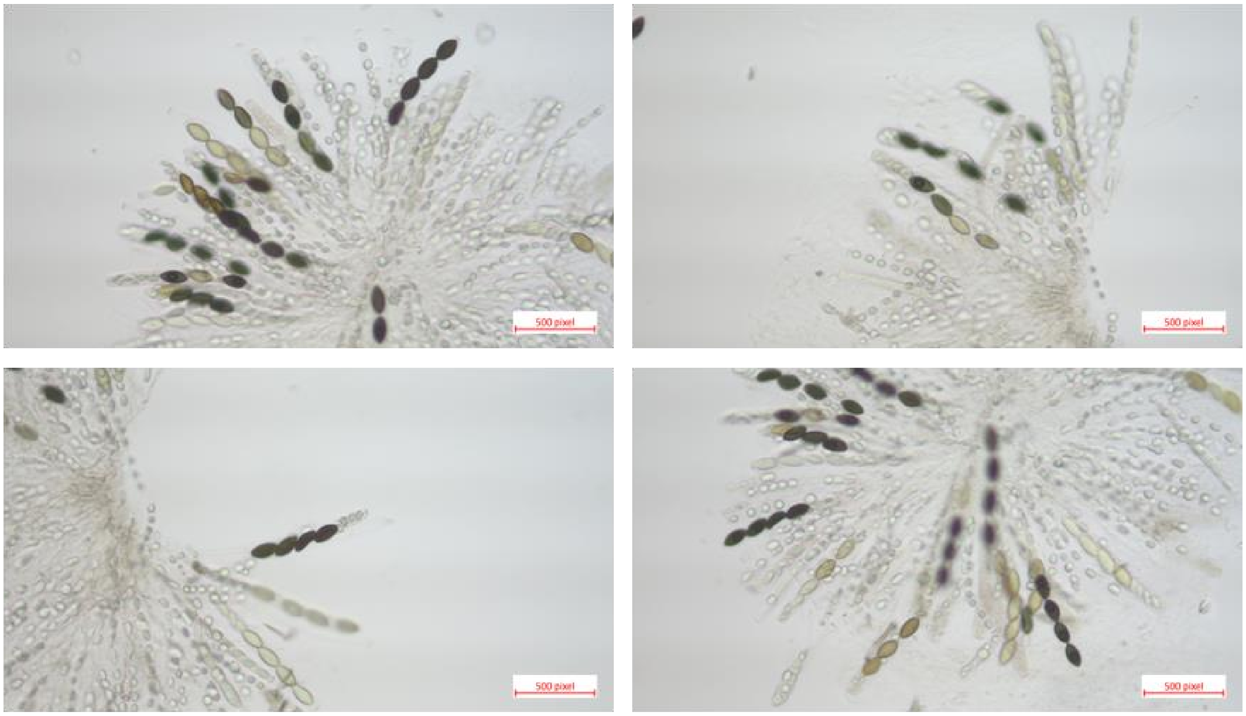

Figure 14 Ascus phenotypes. Asci were dissected from perithecia of RKD10.3 × RTH1623.1 on Day 17 post fertilization and imaged under magnification.

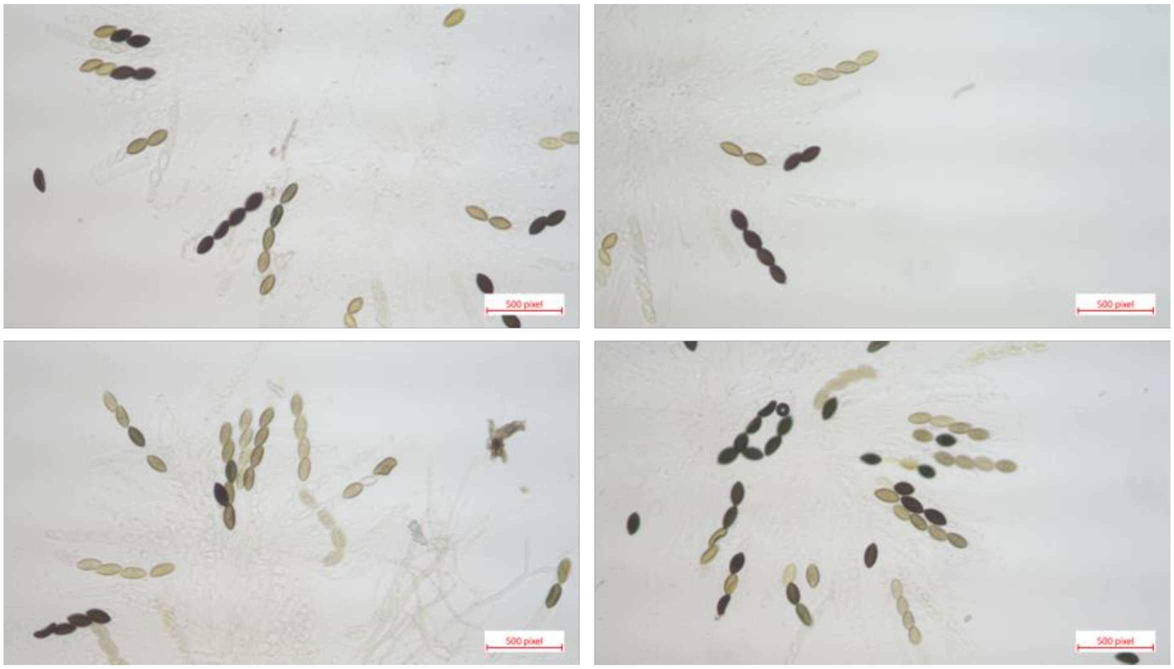

Figure 15 Ascus phenotypes. Asci were dissected from perithecia of RKD10.4 × RTH1623.1 on Day 17 post fertilization and imaged under magnification.

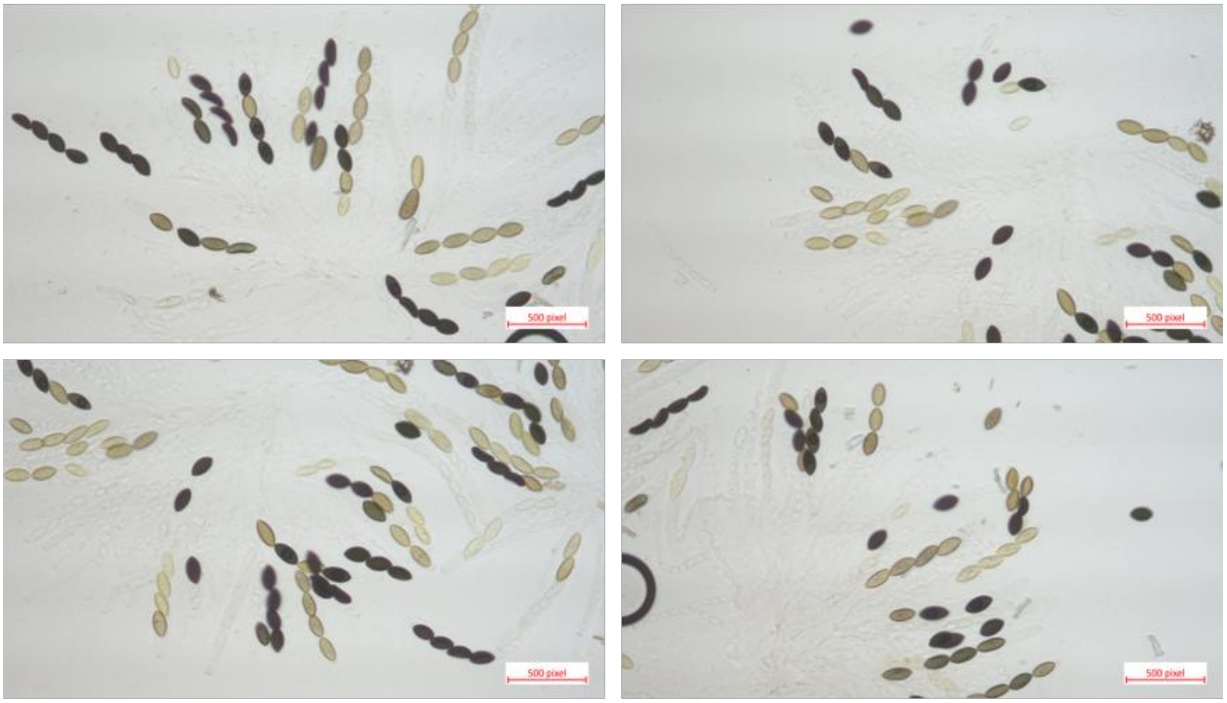

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RKD10.5 × RTH1623.1 on Day 17 post fertilization and imaged under magnification.

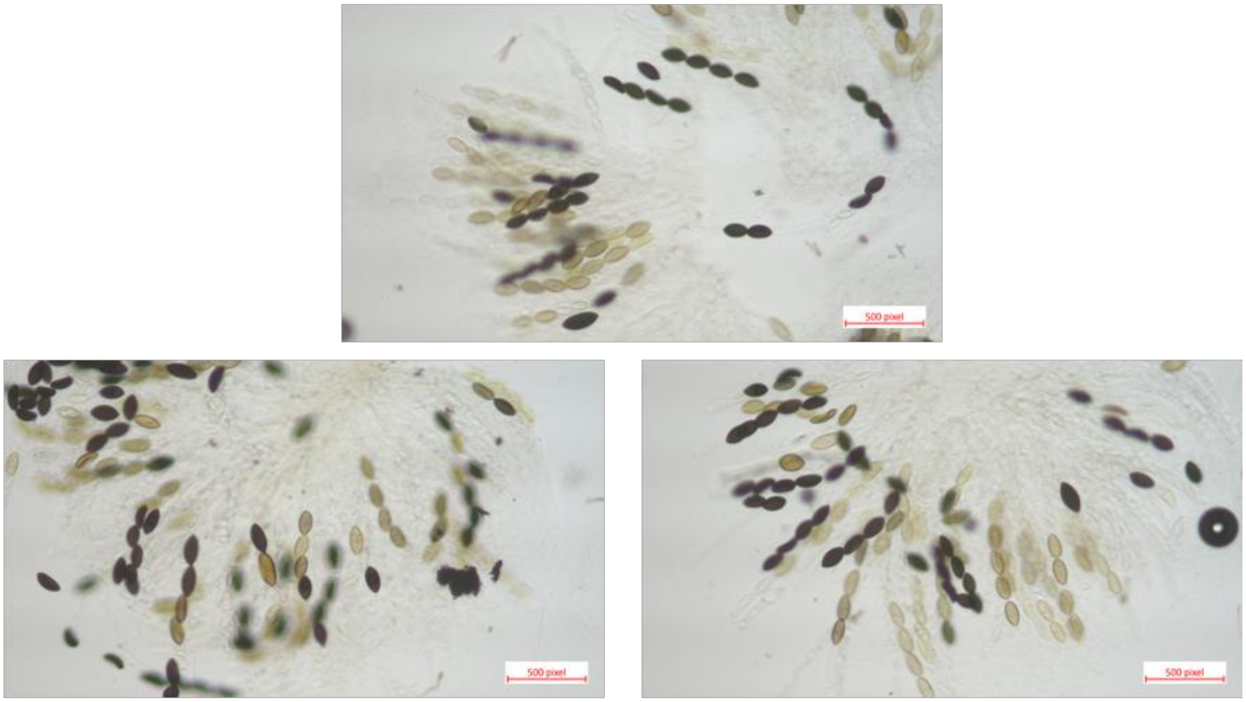

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RKD10.10 × RTH1623.1 on Day 17 post fertilization and imaged under magnification.

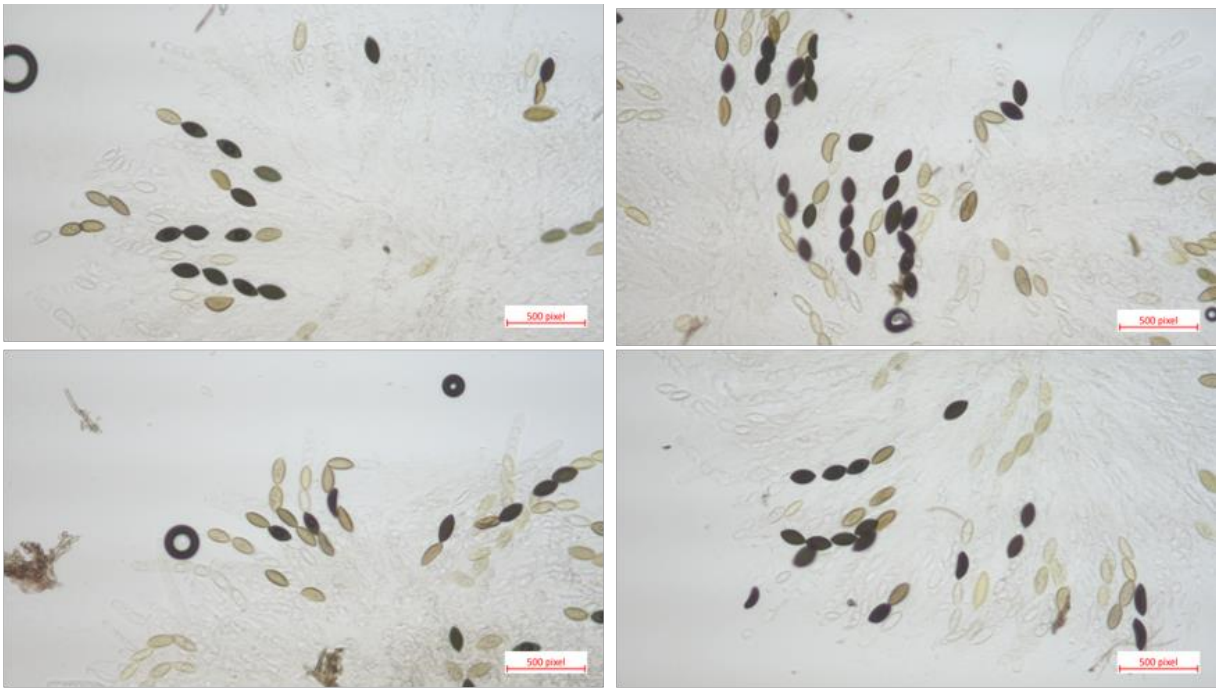

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RKD10.X × RTH1623.2 on Day 17 post fertilization and imaged under magnification.

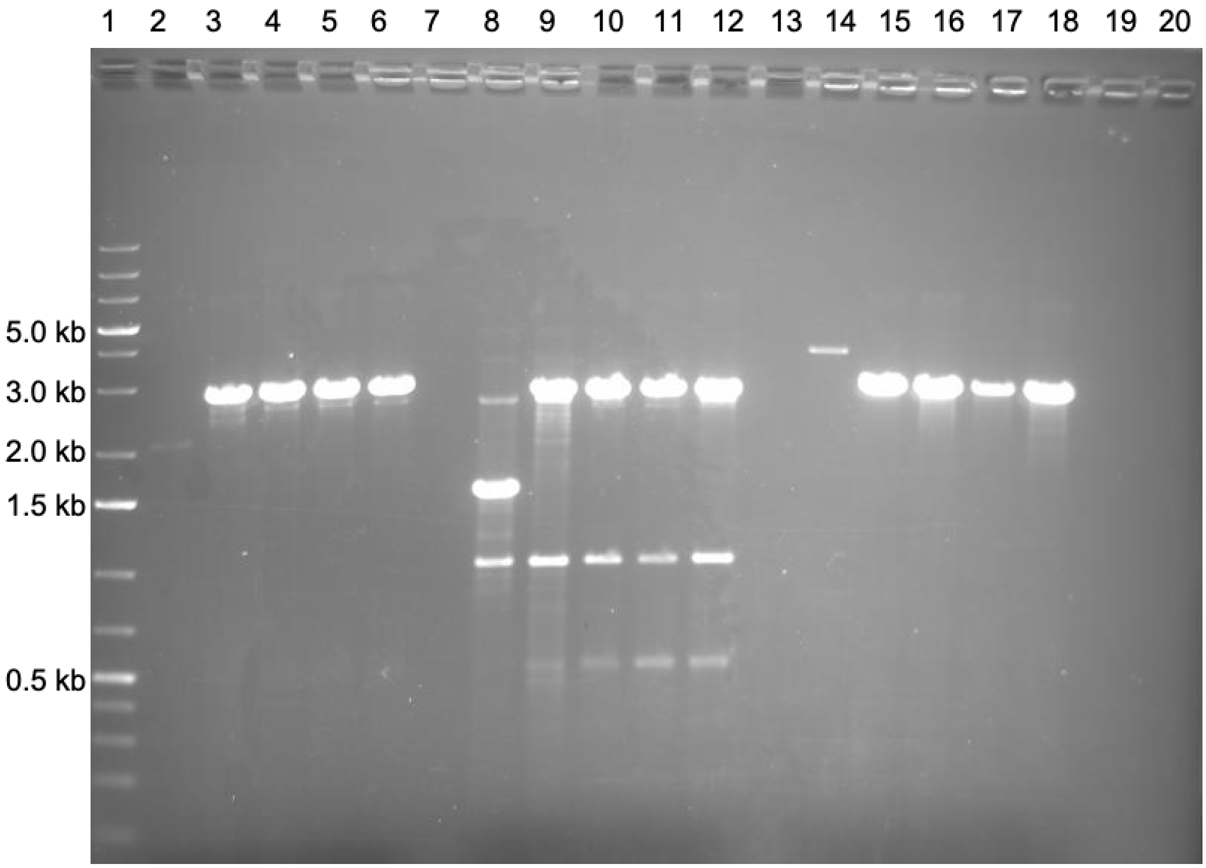

Figure 19 RKD10 genotyping. DNA was isolated from test strains and used in a PCR-based genotyping assay with primers V0359-E and V0386-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product lengths for the *i386+* and *i386Δ* genotypes are 3669 bp and 2879 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 2–13, not applicable; Lane 14, RDGR170.3; Lane 15, RKD10.2; Lane 16, RKD10.3; Lane 17, RKD10.4; Lane 18, RKD10.5. These results indicate that RKD10.2, RKD10.3, RKD10.4, and RKD10.5 have the *i386Δ::hph+* genotype.

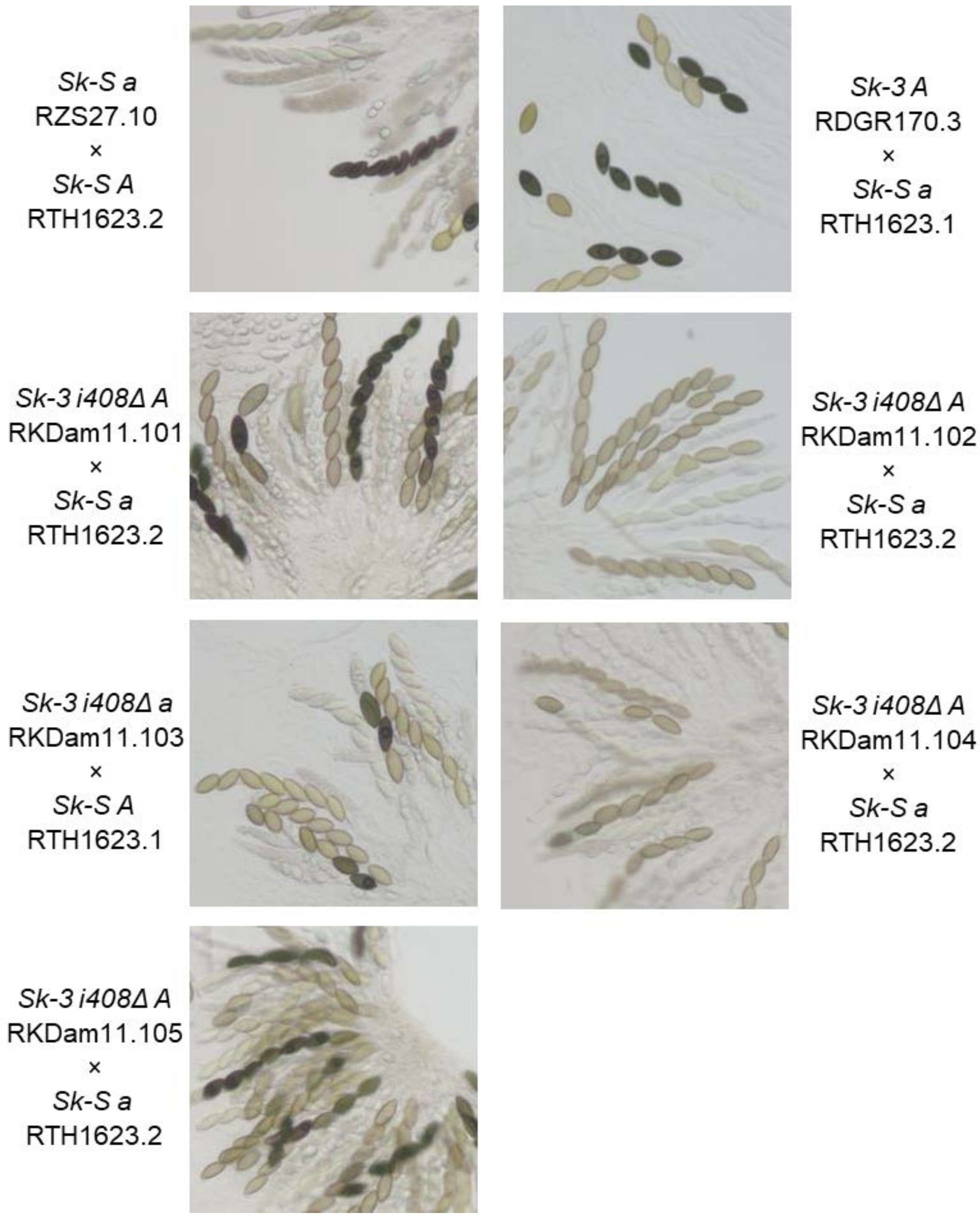

Figure 20 Ascus phenotype summary. Asci were dissected from perithecia and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of *i408* disrupts spore killing.

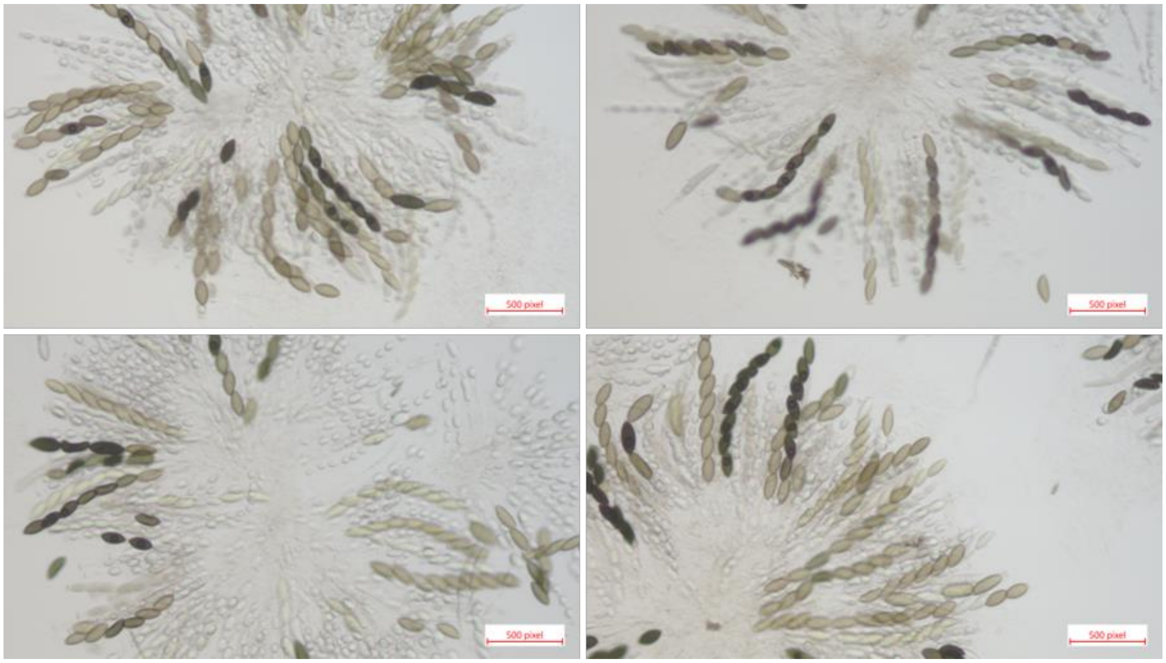

Figure 21 Ascus phenotypes. Asci were dissected from perithecia of RKDam11.101 × RTH1623.2 and imaged under magnification.

Figure 22 Ascus phenotypes. Asci were dissected from perithecia of RKDam11.102 × RTH1623.2 and imaged under magnification.

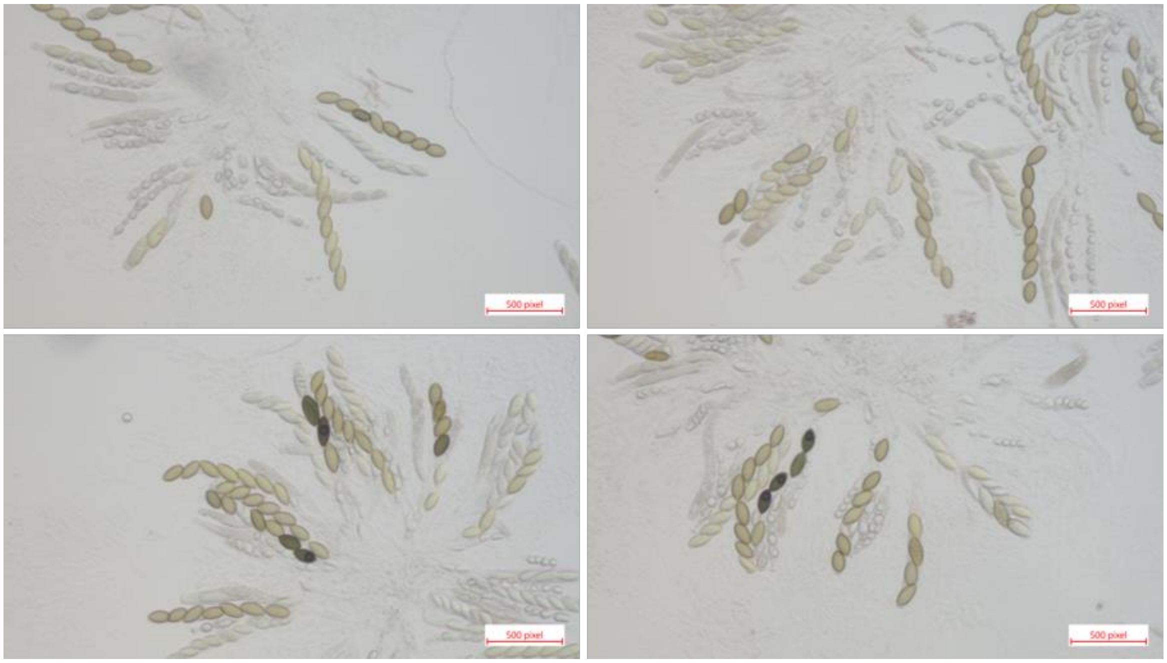

Figure 23 Ascus phenotypes. Asci were dissected from perithecia of RKDam11.103 × RTH1623.1 and imaged under magnification.

Figure 24 Ascus phenotypes. Asci were dissected from perithecia of RKDam11.104 × RTH1623.2 and imaged under magnification.

Figure 25 Ascus phenotypes. Asci were dissected from perithecia of RKDam11.105 × RTH1623.2 and imaged under magnification.

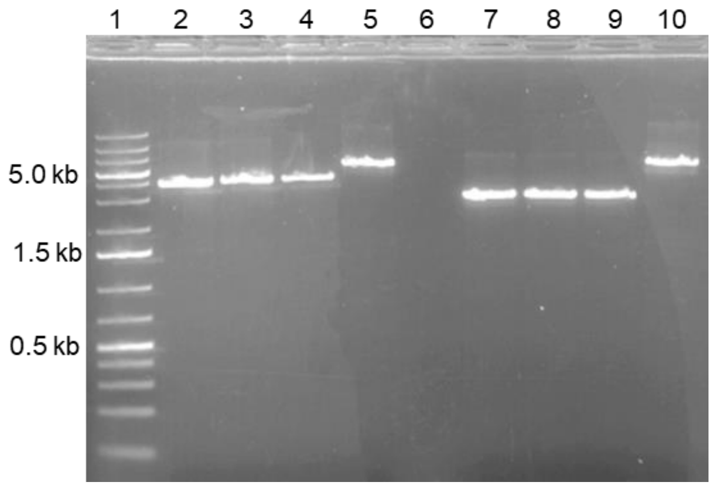

Figure 26 RKD11 genotyping. DNA was isolated from test strains and used in a PCR-based genotyping assay with primers V0394-E and V0358-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel wth UV transillumination is shown. The predicted PCR product lengths for the *i408+* and *i408Δ* genotypes are 5379 bp and 2787 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 2–6, not applicable; Lane 7, RKDam11.105; Lane 8, RKDam11.101; Lane 9, RKDam11.102; and Lane 10, RDGR170.3. These results show that RKDam11.101, RKDam11.102, and RKDam11.105 have the *i408Δ* genotype.