Evidence for Genetic Similarity of Vegetative Compatibility Groupings in *Sclerotinia homoeocarpa*

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(Received on August 11, 2014; Revised on September 22, 2014; Accepted on September 30, 2014)

Vegetative compatibility groups (VCGs) are determined for many fungi to test for the ability of fungal isolates to undergo heterokaryon formation. In several fungal plant pathogens, isolates belonging to a VCG have been shown to share significantly higher genetic similarity than those of different VCGs. In this study we sought to examine the relationship between VCG and genetic similarity of an important cool season turfgrass pathogen, *Sclerotinia homoeocarpa*. Twenty-two *S. homoeocarpa* isolates from the Midwest and Eastern US, which were previously characterized in several studies, were all evaluated for VCG using an improved nit mutant assay. These isolates were also genotyped using 19 microsatellites developed from partial genome sequence of *S. homoeocarpa*. Additionally, partial sequences of mitochondrial genes cytochrome oxidase II and mitochondrial small subunit (mtSSU) rRNA, and the atp6-rns intergenic spacer, were generated for isolates from each nit mutant VCG to determine if mitochondrial haplotypes differed among VCGs. Of the 22 isolates screened, 15 were amenable to the nit mutant VCG assay and were grouped into six VCGs. The 19 microsatellites gave 57 alleles for this set. Unweighted pair group methods with arithmetic mean (UPGMA) tree of binary microsatellite data was used to produce a dendrogram of the isolate genotypes based on microsatellite alleles, which showed high genetic similarity of nit mutant VCGs. Analysis of molecular variance of microsatellite data demonstrates that the current nit mutant VCGs explain the microsatellite genotypic variation among isolates better than the previous nit mutant VCGs or the conventionally determined VCGs. Mitochondrial sequences were identical among all isolates, suggesting that this marker type may not be informative for US populations of *S. homoeocarpa*.

**Keywords**: nit mutants, *Sclerotinia homoeocarpa*, microsatellites, VCG

The ascomycete fungus *Sclerotinia homoeocarpa* (Order: Pezizales, Family: Sclerotiniaceae) causes dollar spot, one of the most common and destructive fungal diseases on intensively managed turfgrasses in North America (Smith et al., 1989; Subbarao et al., 1995) and Korea (Chang et al., 2011). It is most prevalent on golf courses, and turfgrass damage from dollar spot is most severe during the growing season from spring to fall (Chang et al., 2012 and 2013; Smiley, 2005). Warm and humid daytime conditions coupled with cool nights promote extended periods of leaf wetness, which is particularly conducive to disease development. Demands for high aesthetic quality and playability of golf courses require low mowing heights, which also increases the susceptibility of turfgrasses to dollar spot infection. Accordingly, more money is spent on controlling dollar spot on golf courses in the US than any other disease (Burpee, 1997; Viji et al., 2004).

The conidial or teleomorphic forms of *S. homoeocarpa* have not been observed in the field and have not been produced in the laboratory, with the exception of a few cases of *in vitro* induction of apothecial sterile spores using isolates from the United Kingdom (Bennett, 1937; Jackson, 1973). It is assumed that the fungus spreads locally by veg-
Vegetative Compatibility Grouping in *Sclerotinia homoeocarpa* - nit mutants were optimized and carried out the nitrogen assimilation pathway are complemented (Cor- 
Smith et al., 1998; Joaquim and Rowe, 1991; Katan and Kan-

tan, 1988). This method of determining VCGs is preferred because it demonstrates the ability of isolates to form heterokaryotic mycelium.

Jo et al. (2008) developed the *nit* mutant assay for *S. homoeocarpa* to clear up confusion arising from ambiguous VCG results using the conventional method of VCG deter-
mination (Jo et al., 2008; Powell and Vargas, 2001). This assay revealed four VCGs from a panel of isolates shown to represent eight groups by Chakraborty et al. (2006) using the conventional method of VCG determination of Powell and Vargas (2001). Over half of the isolates tested were as-
signed to one of two dominant *nit* mutant VCGs (Jo et al., 2008), and the authors report that some of the compatible complementation interactions resulted only in weak hetero-
karyon formation. In this current study, the *nit* mutant VCG assay for *S. homoeocarpa* was optimized and carried out with isolates previously used by Powell and Vargas (2001), Chakraborty et al. (2006), and Jo et al. (2008). The new assay requires using a fourth mutant type (*nit2*), a much more robust sampling of mutants, and a longer incubation time for paired mutants than was previously performed. Our improved *nit* mutant assay gave slightly discordant results to the former *nit* mutant VCG assay of Jo et al. and showed that there were two more VCGs than had been described previously with the initial *nit* mutant VCG assay (Jo et al., 2008).

The objective of this work was to determine if the VCGs found with the improved *nit* mutant assay for *S. homoeo-
carpa* share significantly more genetic similarity of micro-
satellite and mitochondrial genotypes groupings, as com-
pared to previous studies using these isolates (Chakraborty et al., 2006; Jo et al., 2008; Powell and Vargas, 2001).

**Materials and Methods**

**Isolation of *nit* mutants.** Twenty-two isolates of *S. h**
omoeocarpa* used in this study originated from various geo-

**graphical locations in the US including Arkansas, Illinois, Michigan, Florida, and Minnesota (Table 1). Powell and Vargas and Chakraborty et al. used these isolates previ-

ously for barrage based vegetative compatibility assays (Chakraborty et al., 2006; Powell and Vargas, 2001). The conventional VCGs of a majority of these isolates (VCGs A-F) were presented by Powell and Vargas (2001), and the remainder of the conventional VCG designations (VCGs G, H, J, K, and L) was made by Powell and Vargas (2001) for Chakraborty et al. (2006). Jo et al. (2008) used this entire panel of isolates for *nit*-based VCG (Jo et al., 2008). In the time between the experiments of Chakraborty et al. (2006),
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Jo et al. (2008), and this study, all isolates were stored in long term cultures of Kentucky bluegrass seed and potato dextrose broth at 4ºC.

The nit mutants of each S. homoeocarpa isolate were isolated using the modified procedure developed by Jo et al. (2008). At first, one 5-mm diameter plug was taken from the colony edge of each S. homoeocarpa isolate grown on potato dextrose agar (PDA), and transferred to 10 ml potato dextrose broth (PDB) in a 9-cm Petri dish. After incubation for 10 days at 25ºC, mycelia were harvested and dried on sterilized paper towels. A 5 g sample of the mycelia was homogenized with 5 ml sterile, distilled water using a blender (Brinkmann Instruments Co, Switzerland) at 15,000 rpm for 30 s. A 200 µl aliquot of shredded mycelial suspension was spread on ten plates of water agar medium amended with chlorate (WAC: 2% agar, 0.2% glucose and 4% potassium chlorate) for screening the nit mutants. After spreading, all plates were air dried in a laminar flow hood for 5 min and incubated at 25ºC. When colonies of nit mutants became distinct, showing an expansive and thin mycelial growth on WAC after 30 days incubation, the hyphal tip of each colony was transferred to Czapek solution agar medium (CDA; Becton, Dickson and Company, Sparks, MD) containing nitrate as a single source of nitrogen. Each colony transferred was examined for the typical nit mutant phenotype: a thin and expansive growth with no aerial mycelium on CDA (Correll et al., 1987). Confirmed nit mutants were sub-cultured on WAC and stored at 4ºC. The screening of nit mutants from the 22 isolates was repeated twice, independently.

Colony-forming units (CFUs) from the shredded mycelial suspension were measured to estimate the mutation rate. Serial dilutions of mycelial suspension of each isolate (wild type) were made at 10⁻¹, 10⁻² and 10⁻³ using sterile, distilled water. A 20 µl aliquot of the shredded mycelial suspension was spread on four PDA plates. After incubation at 25ºC for 3 days, colonies were counted. The frequency of nit mutants was determined as the number of isolated nit mutants became distinct, showing an expansive and thin mycelial growth on WAC after 30 days incubation, the hyphal tip of each colony was transferred to Czapek solution agar medium (CDA; Becton, Dickson and Company, Sparks, MD) containing nitrate as a single source of nitrogen. Each colony transferred was examined for the typical nit mutant phenotype: a thin and expansive growth with no aerial mycelium on CDA (Correll et al., 1987). Confirmed nit mutants were sub-cultured on WAC and stored at 4ºC. The screening of nit mutants from the 22 isolates was repeated twice, independently.

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**Table 1. Sclerotinia homoeocarpa isolates used, and their vegetative compatibility groups (VCGs) determined by pairings of nitrate nonutilizing (nit) mutants in this study and comparison with two previous studies**

| Isolate | Site of collection | Chakraborty et al. (2006) | Jo et al. (2008) | This study |
|---------|-------------------|--------------------------|-----------------|------------|
| FL17    | Unknown golf course, FL | A      | Group 1 | VCG 1  |
| L36     | Unknown golf course, IL | A      | Group 1 | VCG 1  |
| MN1     | Unknown golf course, MN | A      | Group 1 | VCG 1  |
| 33A-24  | Hancock Turf Research Center, East Lansing, MI | B      | –    | VCG 1  |
| ARK     | Unknown golf course, AR | B      | Group 1 | –  |
| Southbrook | Southbrook, MN | B      | Group 1 | VCG 2 |
| A7      | Southbrook, MN | H      | Group 1 | –  |
| TB64D   | Unknown            | K      | Group 1 | VCG 2 |
| 30B-13  | Lakewood Shores, MI | C      | Group 1 | VCG 2 |
| 30B-24  | Lakewood Shores, MI | C      | Group 1 | –  |
| 46-3    | Forest Akers Golf, East Lansing, MI | F      | Group 1 | VCG 2 |
| 30B-48  | Lakewood Shores, MI | D      | Group 1 | VCG 3 |
| 48-54   | North Shore County Club, Chicago, IL | D      | Group 1 | –  |
| 64-41   | Evergreen Golf, Hudson, MI | E      | Group 1 | VCG 4 |
| 64-49   | Evergreen Golf, Hudson, MI | E      | Group 1 | VCG 4 |
| I16     | Les Bolstead, St. Paul, MN | G      | Group 2 | VCG 5 |
| I18     | Les Bolstead, St. Paul, MN | G      | Group 2 | VCG 5 |
| Les Bolstead | Les Bolstead, St. Paul, MN | J      | Group 2 | VCG 5 |
| BR5     | Unknown            | L      | Group 2 | –  |
| 32-47   | Evergreen Golf, Hudson, MI | F      | Group 3 | VCG 6 |
| 33A-9   | Hancock Turf Research Center, East Lansing, MI | C      | Group 4 | –  |
| 64-14   | Evergreen Golf, Hudson, MI | E      | –    | –  |

*Vegetative compatibility groups (VCGs) determined by barrage formation at pairings of wild type isolates on potato dextrose agar medium using the method of Powell and Vargas (2001).*  
*VCGs were determined using nit mutants but specific names were not assigned.*
Vegetative Compatibility Grouping in Sclerotinia homoeocarpa

Table 2. Identification of four types of nitrate nonutilizing (nit) mutants in Sclerotinia homoeocarpa grown in Czapek solution agar medium amended with different nitrogen sources

| Function† | Mutant designation | Growth on nitrogen sourcesa | Nitrite | Hypoxanthine | Nitrite excretionb |
|-----------|--------------------|-----------------------------|---------|--------------|------------------|
| Structural gene for nitrate reductase | nit1 | + | + | NT |
| Major nitrogen regulatory gene | nit2 | − | − | NT |
| Pathway-specific regulatory gene | nit3 | − | + | − |
| Genes controlling production of a molybdenum-containing cofactor | NitM | + | − | NT |
| None | Wild type | + | + | + |

†Compiled from Correll et al. (1987), Marzluf (1981), and Tomsett and Garrett (1980) on the basis of mutant phenotypes used for Fusarium oxysporum, Aspergillus nidulans, and Neurospora crassa, respectively.

aGrowth on Czapek solution agar (CDA) medium amended with two nitrogen sources. + = typical wild-type growth and − = thin growth with no aerial mycelium.

bNitrite excretion test as described by Cove (1976). + = nitrite excretion, − = no nitrite excretion, and NT = not tested.

Mutants divided by the total number of CFUs on PDA per the same amount of mycelial suspension.

Characterization of nit mutant phenotypes. Specific phenotypes of nit mutants were determined by their utilization of two different nitrogen substances: NaNO₃ and hypoxanthine (both obtained from Sigma-Aldrich) (Correll et al., 1987). Agar plugs (5 mm in diameter) were taken from the edge of each nit mutant culture growing on CDA, and transferred to three types of media: CDA, CDA amended with NaNO₃ (0.05% w/v), and CDA amended with hypoxanthine (0.02% w/v). An agar plug of each original wild-type isolate was also transferred to CDA as a wild-type control for each test run. The plates were incubated at 25°C for 10 days and the colony morphology was examined. Nit mutant phenotypes were determined by following the previously published nomenclature system (Correll et al., 1987; Marzluf, 1981). Mutants were divided into four phenotypic classes which are associated with mutated loci encoding members of the nitrogen assimilation pathway. These nit mutants were classified based on mutation of the following loci: a nitrate reductase structural locus (nit1), a major nitrogen regulatory locus (nit2), a pathway-specific regulatory locus (nit3), and the locus encoding the cofactor containing molybdenum necessary for nitrate reductase activity (NitM). Mutants unable to use nitrate but able to utilize both nitrite and hypoxanthine were designated as nit1. Mutants incapable of using nitrate, nitrite and hypoxanthine were referred to as nit2. Mutants that could not use nitrate and nitrite but could use hypoxanthine were designated as nit3. Mutants capable of utilizing nitrite but not nitrate and hypoxanthine were designated as NitM (Table 2).

Subsequently, all nit3 mutants were further tested for nitrite excretion using the procedures by Correll et al. (1987). Each nit3 isolate was grown on urea medium (CDA amended with 0.04% urea) in a 9-cm Petri dish at 25°C for 3 days. The plate was then flooded with 10 ml of 3 M NaNO₃ solution (Sigma-Aldrich). After 24 h incubation at 25°C, NaNO₃ was poured off. By adding 1 ml of a sulfonilamide solution and 1 ml of a color indicator to the plate, the presence of nitrite was indicated by distinct purple color reaction.

Vegetative compatibility assay. Different types of nit mutants selected from each of the isolates tested were paired in all possible combinations. Agar plugs (5 mm in diameter) were cut from the edge of each nit mutant colony actively growing on CDA. Agar plugs from two different isolates were placed 4 cm apart on a 9-cm diameter plate of CDA (Fig. 1). The plates were incubated at 25°C and monitored for 30 days. A complementary reaction was evident by the development of dense aerial mycelial growth. The pairing experiment was conducted twice.

Genome sequencing and development of microsatellite markers. Genomic sequences were generated using 454 Next Generation Sequencing and sequencing to up to 8X depth of the S. homoeocarpa isolate SD-20 from Joseph Troll Turfgrass Research Center, South Deerfield, MA (Macrogen Inc.). The assembled data represented over 34,000 contigs, each over 100 bp in size and totaling over 30 Mb of genome sequence. The contigs generated were searched for microsatellites using the Simple Sequence Repeat Identification Tool (SSRIT) (http://www.gramene.org/db/markers/ssrtool). Search parameters specified octamer as the maximum motif length and the minimum number of repeats at ten. Three-hundred nine SSRs were identified, and 96 of these were selected for further
Forward and reverse PCR primers flanking the repeat sequences were designed using the Primer3 primer design server (Rozen and Skaletsky, 2000). The parameter settings used to generate primer sequences were as follows: primer length between 18 and 25 bases and annealing temperature from 55 to 65°C. Primers were designed to amplify products ranging from 100 to 250 bases.

Microsatellites were amplified in a 10 μl reaction volume with a final concentration of 1× PCR Buffer, 0.2 mM dNTP with 2.5 mM MgCl₂, 0.2 μM of each primer, 1 unit Taq DNA Polymerase (New England Biolabs Inc.) and 50 ng of genomic DNA template. The DNA for PCR was prepared using the method of Saitoh et al. (2006). The PCR regime was as follows: an initial denaturation at 95°C for 60 s, followed by 35 cycles of 95°C for 60 s, annealing at 72°C for 1 min and decreasing by increments of 1.0 degree per cycle for the first 10 cycles, and 72°C for 90 s, with a final extension of 72°C for 5 min. The PCR reactions were performed in 96 well plates on an MJ PTC-200 thermocycler (MJ Research), with one reaction per primer pair without template as a negative control. Eight microliters of the PCR products and 2 μl of 6× loading dye.

**Table 3.** Descriptions of nineteen microsatellite loci mined from the *S. homoeocarpa* genome sequence using SSR-IT and the forward and reverse primer sequences used to amplify these markers. Primer sequences are listed in 5’ to 3’ orientation. The number of alleles among the 22 isolates genotyped is listed.

| Sequence | Repeat motif | Size (bp) | Left Primer | Right Primer | No. alleles |
|----------|-------------|-----------|-------------|--------------|-------------|
| SSR02063 | (CTCAC)₁₂  | 243       | CTTTGGCAGCCTCTGATTAT | TGAGGGTTCATGGAATAGCA | 2           |
| SSR05848 | (GTATGA)₁₂ | 232       | TTTGGTAGTGGTGGAGGCTAGA | CTCAGCTCTACCTACGCAC  | 2           |
| SSR06235 | (TG)₁₇     | 197       | TTCTCTCTCTCCGTCAGAC | CTTTTCCTTGCTTTGGAG | 3           |
| SSR08045 | (AC)₁₃     | 157       | TGGTGGAGAGGGAGAGGTGA | AAGGCGAAGGACACTATCT  | 4           |
| SSR08400 | (ATCT)₁₂   | 202       | CTATTCTGGCCATCTCTCAT | TCGACGATTCATCTAGAAGTG | 3           |
| SSR08569 | (TATGGGA)₁₃| 227       | CTTCTCGTCCTGTGTTCAC | CAATATCCATCCCATACATC | 3           |
| SSR09987 | (CA)₁₂     | 233       | TCCCAACCAATCTCTCTTTGA | TGGGCTACCGGACTACTTTCG | 2           |
| SSR15983 | (TAG)₁₃    | 150       | TATACTCGCAGGATGATGTG | AGACGCAACGTACCACAGTCT | 4           |
| SSR17616 | (AC)₁₆     | 172       | TCCGCACTACCGCTACACAC | GTGCGATGGAGATGGGTGTG | 2           |
| SSR20589 | (TCA)₁₃    | 243       | ATCGACCCGAGAACACTACAA | AGGCTGGGACGCCCTTTTTT | 2           |
| SSR20624 | (TTCA)₁₆   | 184       | AGTGGGCGAAGCAGGACAAGAAGAAGAACA | GGGCGTGAAGGGAAGGAAAGAACA | 3           |
| SSR21791 | (GAA)₁₂    | 215       | CCATTGCGCTCTAAGGTTCG | GGGACACCTCCTTCCATTCC | 2           |
| SSR22304 | (AT)₁₅     | 232       | CGCGACTTGGACCAAGAGG | AGCGGACAGGAGGAAACTC | 2           |
| SSR23039 | (TC)₁₆     | 197       | TCTTGCTCGCTGTGCTGCT | TGGCCTTGGGCTGTCATCT | 3           |
| SSR25827 | (AATC)₁₅   | 188       | CCTTCCCTCTCACAGCTACC | CCCGCTTTTTGGTTTTTTG | 5           |
| SSR27998 | (GTAT)₁₂   | 165       | ATTAGGGGGCATTAGTGGTG | TCCCTCTCTCTCCTCTCTCCTGTA | 4           |
| SSR30326 | (TTGAG)₁₄  | 167       | GCAATGGAGTGAGCTGCTGTTT | TCATACTCATTGAAAGGCATCA | 4           |
| SSR30530 | (TC)₁₂     | 156       | AATCGAGCAGATCGAGTGCTC | TCTGCTCTCTGTCCTGCTTAT | 3           |
| SSR30647 | (AC)₁₄     | 170       | GCTGTTGGCCATATAACGAT | GGCTGGATGTGGCTGATAA | 2           |

*Repeat motif determined from genome of *S. homoeocarpa* isolate SD-20.
per reaction were mixed and amplicons separated by gel electrophoresis on 3% (w/v) MetaPhor agarose (Cambrex Bio Science Rockland, Inc.) gels in 1× TBE buffer (Tris base, Boric acid, and EDTA) at 160 V for 2.5 hours. Gels were post-stained with ethidium bromide (0.5 μl/ml) for 30 minutes, followed by 10 minutes destaining in 1X TBE buffer. Gel bands were visualized under UV light, photographed and gel pictures were captured as TIFF files for further analysis.

Microsatellites were screened initially for polymorphism among a panel of four isolates (FL17, ARK, I18, and 33A-9) (Table 1). Microsatellite primer pairs were selected for further analysis if they were found to amplify single bands and were found polymorphic among the panel of four isolates. Nineteen microsatellite primer pairs were chosen based on this initial screening, and these primers were utilized to genotype the 22 isolates from Table 1 (Table 3).

**Amplification and sequencing of mitochondrial loci.** Primers were designed from partial genome sequence of *S. homoeocarpa*. Two mitochondrial loci, the *mitochondrial small subunit* rRNA (mtSSU) and the *ap6-rus* intergenic spacer region of the mitochondrial genome, were chosen for sequencing based on their utility as intraspecific genetic markers in other studies of pathogenic ascomycete fungi (Ghikas et al., 2010; Skovgaard et al., 2001). The partial gene sequence of *cytochrome oxidase II* (COII) was examined to look for variation, as well, though there are no reports of this gene showing intraspecific variability for ascomycete fungi. These genes were mined from *S. homoeocarpa* genomic scaffolds using a local BLAST search (Altschul et al., 1997). Primers for amplification were designed in Primer3 (Rozen and Skaletsky, 2000) to genomic scaffolds using the following criteria: minimum primer length 20, optimum primer length 24, maximum primer length 27; minimum melting temperature 67°C, optimum melting temperature 70°C, and maximum melting temperature 73°C. The forward and reverse primer sequences for mtSSU were 5′-GCTGAAACCAGCAACTTGGGGAAT-3′ and 5′-TCCCTTCCACGAATTCAACTCCA-3′, and amplified an 838 bp amplicon. The forward and reverse primer sequences for the *ap6-rus* spacer were 5′-GCAGAGGGAGACAAACCTCCCA-3′ and 5′-GGGTAGGGGAGACAAACCTCCCA-3′, and amplified a 721 bp amplicon. The forward and reverse primer sequences for the COII locus were 5′-GTCGCCCTGGCGGAGGAGTAGTA-3′ and 5′-GGCAGCTAGAAGCTTGCTGTC-3′, and amplified a 1091 bp amplicon. The PCR temperature regime followed that of Lamour et al. (2006).

**Microsatellite data analysis.** Microsatellite alleles were coded into a binary data matrix, and this dataset was converted into a genetic distance matrix using the program GeneAIEx, and add in for population genetics analyses in Microsoft Excel (Peakall and Smouse, 2006). The genetic distance matrix was exported and analyzed in the program MEGA v4.0 using UPGMA distance analysis (Tamura et al., 2007). Additionally, the binary data matrix was interrogated with 10,000 bootstrap replicates using Phylip genetic analysis software package (Felsenstein, 1989). The bootstrapped data matrices were converted to distance matrices, and a consensus tree was obtained.

The microsatellite distance matrices were also analyzed to determine genetic distance among and within VCGs found here, those of Jo et al. (2008), those of Chakraborty et al. (2006), which were determined with the conventional method of Powell and Vargas (2001). The isolates for which *nit* mutant VCG was determined in the current study and the previous two studies were analyzed for genetic relationships among and between the VCGs with more than one isolate (Chakraborty et al., 2006; Jo et al., 2008; Powell and Vargas, 2001).

**Analysis of molecular variance (AMOVA) of microsatellite genotypes and VCGs.** Binary matrices of microsatellite allelic data from three data sets were analyzed using GeneAIEx v6.4 for an Analysis of Molecular Variance (AMOVA). An AMOVA was used to determine how much of the microsatellite genetic variation could be explained by the VCG groupings of this current study and previous studies (Jo et al., 2008; Powell and Vargas, 2001). AMOVA was performed for isolates from VCGs with more than one isolate as is required for AMOVA, and only included isolates which were genotyped in this study. Three separate AMOVAs were carried out to determine which VCG designations explained the microsatellite genotypic variation best: our *nit* mutant VCGs, those of Jo et al. (2008), or those of Chakraborty et al. (2006). For each analysis, 9,999 pairwise permutations and total permutations were carried out with calculated distances based on haploid-SSR parameters.

**Results**

**Isolation of nit mutants.** A total of 101 *nit* mutants were isolated from 16 out of 22 *S. homoeocarpa* isolates tested in this current study, and six isolates (A7, ARK, BR5, 30B-24, 48-54 and 64-14) failed to produce any *nit* mutants in...
two additional attempts (Table 4). The nit mutants showed fast growth with thin and sparse colonies on WAC medium, and grew slowly on CDA containing only nitrate as the nitrogen source. The frequency of nit mutants significantly varied among the isolates tested. Over two runs of the experiment, isolate 33A-24 produced the greatest number of mutants: 42 mutants out of 2.1 ± 0.6 × 10^5 cfu/ml. In contrast, only one nit mutant was isolated from isolate MN1 out of 3.3 ± 0.7 × 10^5 cfu/ml (Table 4).

**Characterization of nit mutant phenotypes.** At least one of the four nit mutant types were selected from 16 of the 22 isolates tested (Table 4). Of all mutants isolated, nit1 mutants were predominant (60.4%) and NitM mutants were the second most prevalent (34.7%). The frequency of nit2 and nit3 mutants was relatively rare at 3.0% and 2.0%, respectively.

**Vegetative compatibility assay.** The complementation interaction of mutants exhibited abundant aerial mycelium growth at the junction zone where the two colonies met, and sometime extended to the entire colony (Fig. 1A). Two phenotypic types of incompatible reactions were observed (Figs. 1B-C). Strong incompatibility produced a distinct dark borderline where two colonies met 2–4 weeks after contact between two incompatible isolates (Fig. 1B). Weak incompatibility was characterized as formation of small spots of spontaneous aerial mycelium 2 weeks after contact but later a barrage with dark pigment was formed on the contacted zone after 30-days of incubation (Fig. 1C).

### Table 4. Frequency and phenotype of nitrate nonutilizing (nit) mutants recovered from 22 isolates of Sclerotinia homoeocarpa

| Isolate        | No. of colonies (×10^5 cfu/ml) | No. of mutants | Mutation rate (×10^5) | nit mutant class |
|----------------|--------------------------------|----------------|-----------------------|-----------------|
|                |                                |                |                       | nit1 | nit2 | nit3 | NitM |
| A7             | 2.8 ± 0.8                      | 0              | 0                     | 0    | 0    | 0    | 0    |
| ARK            | 1.4 ± 0.2                      | 0              | 0                     | 0    | 0    | 0    | 0    |
| BRS            | 5.2 ± 2.6                      | 0              | 0                     | 0    | 0    | 0    | 0    |
| FL17           | 4.9 ± 0.8                      | 5              | 25.0 ± 0.3            | 1    | 0    | 0    | 3    |
| I16            | 3.4 ± 1.0                      | 4              | 48.0 ± 4.2            | 1    | 0    | 0    | 3    |
| I18            | 3.0 ± 0.7                      | 6              | 53.0 ± 1.8            | 1    | 0    | 0    | 3    |
| L36            | 3.4 ± 0.5                      | 9              | 75.0 ± 10.8           | 9    | 0    | 0    | 0    |
| Les Bolstead   | 4.4 ± 0.8                      | 4              | 2.3 ± 0.4             | 1    | 0    | 0    | 3    |
| MN1            | 3.3 ± 0.7                      | 1              | 0.9 ± 1.3             | 1    | 0    | 0    | 0    |
| Southbrook     | 0.9 ± 0.5                      | 1              | 1.3 ± 1.3             | 1    | 0    | 0    | 0    |
| TB64D          | 1.8 ± 1.3                      | 3              | 1.3 ± 1.3             | 0    | 3    | 0    | 0    |
| 30B-13         | 2.0 ± 0.6                      | 9              | 1.1 ± 1.6             | 1    | 0    | 0    | 0    |
| 30B-24         | 2.2 ± 1.9                      | 2              | 1.1 ± 1.9             | 2    | 0    | 1    | 2    |
| 30B-48         | 2.7 ± 1.2                      | 6              | 1.3 ± 1.1             | 6    | 0    | 0    | 0    |
| 32-47          | 2.6 ± 0.6                      | 1              | 1.3 ± 1.3             | 1    | 0    | 0    | 0    |
| 33A-9          | 1.4 ± 0.1                      | 1              | 1.3 ± 1.3             | 1    | 0    | 0    | 0    |
| 33A-24         | 2.1 ± 0.6                      | 42             | 51.0 ± 14.0           | 22   | 0    | 0    | 20   |
| 46-3           | 1.7 ± 0.9                      | 5              | 6.8 ± 2.9             | 3    | 0    | 0    | 0    |
| 48-54          | 6.4 ± 0.8                      | 1              | 1.9 ± 1.3             | 1    | 0    | 0    | 0    |
| 64-14          | 1.6 ± 0.6                      | 0              | 1.9 ± 1.3             | 1    | 0    | 0    | 0    |
| 64-41          | 2.4 ± 0.8                      | 3              | 1.9 ± 1.3             | 2    | 0    | 0    | 0    |
| 64-49          | 2.0 ± 0.7                      | 5              | 1.9 ± 1.3             | 3    | 0    | 0    | 0    |
| **Total**      | **101**                        | **61**         | **3**                 | **2** | **35** |

*CFU: colony-forming unit, number of colony produced from the shredded mycelial suspension on PDA (Potato Dextrose Agar).

*Number of nit mutants recovered from water agar medium supplemented with 4.0% potassium chlorate.

*Mutation rate was determined as the number of nit mutants isolated on water agar medium amended with chlorate divided by the total number of CFUs on PDA per the same amount of mycelial suspension.

*Nit mutant phenotypes determined according to growth on CDA amended with different nitrogen sources. nit1: mutation in a nitrate reductase structural locus, nit2: mutation in major nitrogen regulatory locus, nit3: mutation in a nitrate assimilation pathway-specific regulatory locus, NitM: mutation in one of five loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity.*
Table 5. Complementation reactions among four types of nitrate nonutilizing (nit) mutants of Sclerotinia homoeocarpa

| nit mutant phenotype | nit1 | nit2 | nit3 | NitM |
|----------------------|------|------|------|------|
| nit1                 | −    | +    |      | +    |
| nit2                 | −    | +    | + or + | −    |
| nit3                 | −    | +    | +    | +    |
| NitM                 | + or + | −    | +    | +    |

"nit1": mutation in a nitrate reductase structural locus, "nit2": mutation in major nitrogen regulatory locus, "nit3": mutation in a nitrate assimilation pathway-specific regulatory locus, and "NitM": mutation in one of five loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity.

Under the compound microscope at 400X magnification, increased compartmentalization and vacuolization of hyphae were observed on the dark spots and borderline of the incompatible reaction. As a negative control, no reaction on CDA was produced between plugs of the same isolate (Figs. 1A-B) or single plug culture (Fig. 1D), whereas abundant aerial mycelium on PDA, a positive control medium (Fig. 1E).

Functional restoration after mutant complementation was more rapid and distinct in the pairings of NitM with nit1 or nit3, and nit2 with nit1 or nit3 combinations (Table 5). The complementary reactions between NitM and nit2 showed either abundant, profuse mycelium or no aerial mycelium. No clear complementation was observed on pairings between same mutant types of nit1, nit2 and nit3, and between nit1 and nit3. However, distinct complementation reactions of some pairing combinations between different NitM mutants produced rapid and robust heterokaryons with dense aerial mycelium. NitM or nit2 mutants readily complemented with nit1 and nit3 mutants derived from the same VCG, producing distinct prototrophic aerial mycelium.

Out of the sixteen isolates produced nit mutants, 15 were amenable to the VCG assay and grouped into six VCGs based on pairings in all possible combinations between nit mutants (Table 1). Each VCG was designated as VCG 1 to VCG 6. VCGs 1 and 2 were dominant groups containing four isolates originating from different states. The VCGs 3 and 6 contained a single isolate.

Microsatellite marker analysis and concordance of nit mutant VCG and microsatellite genotypes. A total of 309 microsatellite loci were mined from genome sequence data: 148 di-nucleotide (48%), 51 tri- (17%), 47 tetra- (16%), 20 penta- (16%), 26 hexa- (8%), 8 septa- (3%), and 9 octa- (3%). Of these, 96 were screened for efficient amplification and single products. In total, 19 primer sets were selected, and the microsatellites produced a total of 57 alleles from the panel of all 22 S. homoeocarpa isolates. Each marker produced from two to five alleles (Table 3). Sixteen of the microsatellite markers were amplified from the complete set of isolates, while SSR17616 wasn’t amplified in isolates 64-49 and 116, SSR20589 wasn’t amplified in isolate 32-47, and SSR22804 wasn’t amplified in isolate 48-54. Distance analysis of the data set (including missing data) from the 15 isolates showed four groups with genetic similarity of 14% or less (Fig. 2, Table 6), with the exceptions of isolates 30B-48 of VCG3 and 32-47 from VCG6, which have isolated positions on the dendrogram.

The average genetic distances of isolates from within and among the VCGs with more than one isolate from three studies, including this one, are presented in Table 6. Genetic distances indicate that the six nit mutant VCGs found here, though they contained more isolates on average (2.67 ± 1.21) than those of Chakraborty et al. (2006) included in the analysis (1.6 ± 0.70), displayed less average genetic distance (7.72 ± 4.93) than those (13.4 ± 4.7) of Chakraborty et al. (2006). Several VCGs of Chakraborty (2006) are nested within the nit mutant VCGs 1, 2, and 5 found in our current study (Table 6).
Analysis of molecular variance (AMOVA). The percentages of molecular variance within the nit mutant VCGs presented in our current study were 18.4%, while among them the value was 81.6%. The $\phi_{PT}$ value, which is a measure of how much of the genetic variation is explained by the VCG groupings, was 0.795, with $P < 0.001$. Conversely, among the two VCGs identified from Jo et al. which contained more than one isolate, the percentages of molecular variance were 81% within VCGs, and 19% among the two VCGs (Joaquim and Rowe, 1991). The $\phi_{PT}$ value was 0.299, with $P < 0.006$. The percentages of molecular variance observed among and within the seven VCGs of Powell and Vargas (2001) with multiple isolates were 69.7% and 30.3%, respectively (Peakall and Smouse, 2006). The $\phi_{PT}$ value was 0.572, with $P < 0.001$ (Table 7) (Powell and Vargas, 2001). Pairwise $\phi_{PT}$ values and probabilities from the AMOVA of the nit mutant VCGs are presented in Table 8.

Mitochondrial loci sequencing. Mitochondrial loci sequences including mtSSU, atp6-rns intergenic spacer re-

### Table 6. Summary of genetic distances from VCGs of the 14 isolates amenable to all three VCG assays: the current nit mutant VCG assay (1-6), the nit mutant VCG assay of Jo et al. (2008) (J1-J3), and the conventional VCG assay (A-K) (Chakraborty et al., 2006)

| VCG | Average % genetic distance within VCG | Number of isolates | Chakraborty et al. (2006) VCGs nested within |
|-----|--------------------------------------|--------------------|-------------------------------------------|
| 1   | 5.3                                  | 3                  | A, B                                      |
| 2   | 2                                    | 4                  | B, C, F, K                                |
| 3   | –                                    | 1                  | D                                         |
| 4   | 14                                   | 2                  | E                                         |
| 5   | 10.7                                 | 3                  | G, J                                      |
| 6   | –                                    | 1                  | F                                         |
| J1  | 18.7                                 | 10                 | A, B, C, D, E, K                          |
| J2  | 10.7                                 | 3                  | G, J                                      |
| J3  | –                                    | 1                  | F                                         |
| A   | 5.3                                  | 3                  | –                                         |
| B   | –                                    | 1                  | –                                         |
| C   | –                                    | 1                  | –                                         |
| D   | –                                    | 1                  | –                                         |
| E   | 14                                   | 2                  | –                                         |
| F   | 21                                   | 2                  | –                                         |
| G   | 15                                   | 2                  | –                                         |
| J   | –                                    | 1                  | –                                         |
| K   | –                                    | 1                  | –                                         |

*Average % genetic distance was calculated by averaging all genetic distance values between isolates within VCG.

*For the current nit mutant VCG assay (1-6), pairings among mutants of eighteen isolates recovered in Table 2 were made between two different nit mutants in all possible combinations [nit1 (or nit3) and nit2 (or NitM)].

### Table 7. Summary of AMOVA statistics for hierarchical analysis of Sclerotinia homoeocarpa isolates and the VCG designations of three separate studies

| Source of variation | df | Sum of squares | Percentage of variation | $\phi_{PT}$ | P value |
|---------------------|----|----------------|-------------------------|-------------|---------|
| This study          |    |                |                         |             |         |
| Among nit VCG       | 3  | 120.68         | 81.6                    | 0.795       | <0.001  |
| Within nit VCGs     | 9  | 27.17          | 18.4                    | 0.795       | <0.001  |
| Total               | 12 | 147.85         |                         |             |         |
| Jo et al. (2008)    |    |                |                         |             |         |
| Among nit VCG       | 1  | 41.32          | 19.0                    | 0.299       | <0.006  |
| Within nit VCGs     | 16 | 180.68         | 81.0                    | 0.299       | <0.006  |
| Total               | 17 | 222.00         |                         |             |         |
| Chakraborty et al. (2006) Among nit VCG | 6  | 145.83         | 69.7                    | 0.572       | <0.001  |
| Within nit VCGs     | 12 | 63.33          | 30.3                    | 0.572       | <0.001  |
| Total               | 18 | 209.16         |                         |             |         |

### Table 8. Pairwise comparisons of microsatellite genotypes of isolates from four nit mutant VCGs found here using AMOVA. Probability values based on 9999 permutations are located above the diagonal, and pairwise $\phi_{PT}$ values are located below the diagonal

| VCG1 | VCG2 | VCG4 | VCG5 |
|------|------|------|------|
| VCG1 | 0.000| 0.028| 0.066| 0.029|
| VCG2 | 0.838| 0.000| 0.069| 0.029|
| VCG4 | 0.768| 0.760| 0.000| 0.092|
| VCG5 | 0.810| 0.851| 0.689| 0.000|
Vegetative Compatibility Grouping in Sclerotinia homoeocarpa

Discussion

In the VCG assay based on nit-mutant complementation with four mutant types and over 100 mutants, 15 isolates of S. homoeocarpa were grouped into six VCGs (Table 1). These isolates were previously grouped into nine VCGs based on the macroscopic assessment of barrage formations and three VCGs based on nit-mutant complementation of Jo et al. (2008) which included three mutant types, less than half the number of mutants used here, and a much shorter incubation time for isolates being tested for complementation (10 days versus 30 days in this study) (Chakraborty et al., 2006; Jo et al., 2008; Powell and Vargas, 2001). The discordance of the nit mutant VCGs of Jo et al. (2008), and those presented here can be explained by the following caveats of the previous and updated nit mutant VCG assay types (Jo et al., 2008). While the conventional VCG assay relies on barrage formation only, the nit-mutant complementation assay relies on the ability of isolates to complement the nitrate assimilatory mutations of one another. Jo et al. (2008) scored several isolates as being vegetatively compatible, because they produced distinct or weak prototrophic mycelial growth indicative of complementation on nitrate medium with a lack of detectable barrage formation after 10 days of incubation (Jo et al., 2008). These isolates were placed preliminarily in the group 1 of Jo et al., the largest VCG that they found (Jo et al., 2008). This VCG is split into four VCGs using the updated nit mutant VCG assay in the current study, because after 30 days incubation, obvious and barrage zones were detected between the paired isolates and microscopic observations of incompatibility concurred with these results, indicating the heterokaryotic stage in weak spontaneous aerial mycelium is not stable.

The nit mutant VCGs we found were concordant with microsatellite genotype groupings in the UPGMA dendrogram of these 15 isolates, and each clade of isolates representing a VCG showed robust bootstrap support. In our knowledge, this is the first report showing genetic similarity of vegetative compatibility groupings in S. homoeocarpa. The VCGs of several fungi, including Verticillium, Cercospora, Aspergillus, and Botryosphaeria, have been shown concordant with genotype (Berbegal et al., 2010; Cai and Schneider, 2008; Cove, 1976; Grubisha and Cotty, 2009; Ma et al., 2004). In addition, the microsatellite groupings and nit mutant VCG groupings are also congruent with the genotype groupings of Chakraborty et al. (2006), which utilized RAPD markers to delineate the genotypic relationships among 23 isolates, 15 of which were characterized in our current study for nit mutant VCG (Chakraborty et al., 2006). Twenty-two of the 23 isolates of Chakraborty et al. (2006) were analyzed here with microsatellite genotyping and UPGMA analysis, and this tree gave the same overall topology (Chakraborty et al., 2006, unpublished data). Also, the AMOVA analysis results reported by Chakraborty et al. (2006) for the 23 isolates for which Powell and Vargas (2001) determined VCGs for were comparable to those presented in our study (Chakraborty et al., 2006). The convergence of groupings produced from our nit mutant VCGs and analysis of microsatellite genotypes from this study and RAPD genotype analysis of data of Chakraborty et al. (2006) respectively, suggests that isolates with similar genotypes are more likely to undergo nit-mutant complementation, and thus heterokaryon formation in the field (Chakraborty et al., 2006).

Within each of the VCGs B and F of Powell and Vargas (2001), there are isolates we characterized that are both not capable of nit complementation and are not genotypically similar in either of the RAPD or microsatellite analyses (Powell and Vargas, 2001). This raises concern that these isolates are likely not capable of heterokaryon formation, but yet they do not form a distinct barrage zone when they come in contact during the conventional VCG assay. Thus, the lack of a detectable incompatible interaction (barrage zone) was used to place them in the same VCG by Powell and Vargas (2001). However, our results suggest that the lack of a detectable barrage formation during the conventional VCG assay does not indicate the potential for heterokaryon formation between two S. homoeocarpa isolates. On the contrary, isolates from several of the VCGs of Powell and Vargas (2001) are nested within one of the six nit mutant VCGs presented here (Table 6). This suggests that these isolates appeared incompatible using the conventional VCG assay of Powell and Vargas (2001), and were assumed to be incapable of heterokaryon formation. However, we show that these isolates are indeed capable of heterokaryon formation and are genetically similar.

DeVries et al. (2008) and Viji et al. (2004) used amplified fragment length polymorphism (AFLP) genotyping to examine the genetic relatedness of conventionally determined VCGs and genotypic diversity, and they found similar results (DeVries et al., 2008; Warnke, 2003). In summary, both Viji et al. (2004) and DeVries et al. (2008) found representative isolates of VCGs A, B, C, D and F grouping with multiple other VCGs with high genetic similarity in the AFLP genotype analysis, and also
that several isolates were found compatible to testers of multiple VCGs.

Even though this study was carried with a limited number of isolates, these results suggest that the conventional VCG assay may not be a reliable measure of the ability to form heterokaryons, and in keeping with previous studies, may not be a reliable indicator of genetic similarity (Chakraborty et al., 2006; DeVries et al., 2008; Warnke, 2003). However, there are several inherent difficulties in using the nit mutant system in place of the conventional VCG assay, since this system has recently introduced to S. homoeocarpa (Jo et al., 2008). Several isolates examined here were able to yield only a single mutant type per isolate, while others yielded more than two mutant types per isolate (Table 4). Isolate 33A-24 showed a ten-fold greater nit mutation rate than all other isolates screened, whereas six isolates failed to produce any mutants. Although the number of isolates tested was limited, these data suggest an isolate-dependent frequency of nit mutant generation, which is independent of genotype. Conversely, five of these six aforementioned isolates did yield nit mutants in the previous work. Also, even fewer nit3 mutants were selected here than previously reported with many fewer mutants by Jo et al. (2008). The inconsistencies in mutant generation for these isolates between this current study and the previous study may be due to deleterious effects brought on by repeated subculture of the isolates used. Continuous subculture in the present study has been shown to result in spontaneous genetic mutations affecting physiology and phenotype of a number of fungi, and perhaps this phenomenon may explain the differential formation of mutants among S. homoeocarpa isolates (Cox et al., 2007).

In the previous study of nit mutants in S. homoeocarpa by Jo et al. (2008) only three phenotypes (nit1, nit3, and NitM) of nit mutants were identified. In this current study, the nit2 mutant was also included in the VCG analysis, but was only produced in one isolate (Jo et al., 2008). The nit2 mutant type has also been previously recovered in Neurospora crassa (Marzluf, 1981) and Fusarium moniliforme (Klittich and Leslie, 1988). The dominant recovery of nit1 and NitM mutants found in this study was consistent with the phenomenon previously reported in other plant-pathogenic fungi (Cecilia De Lima Favaro et al., 2007; Korolev and Katan, 1997; Nitzan et al., 2002). Obviously, the chance of selecting the rare nit2 mutant type increased as more mutants are isolated and screened. Given the high variability in mutant generation among isolates and mutant types within isolates (Table 4), the inherent difficulty in VCG determination using the conventional and nit-mutant methods can be complemented by microsatellite markers and other genotyping tools for measuring the variability of isolates from populations of S. homoeocarpa.

One putative clonal genotype was identified, and shared by two isolates FL-17 and L36 belonging to the same nit mutant VCG1, which were identical at 19 microsatellite loci despite the isolates FL17 and L36 were collected in Florida and Illinois, respectively. Viji et al. (2004) also document clonality of S. homoeocarpa isolates from geographically disparate locations, and found clonality among isolates collected nearly 30 years apart in Pennsylvania (Warnke, 2003). Given that S. homoeocarpa is thought to be an exclusively sterile fungus, low amounts of genetic diversity are to be expected, as has been suggested for S. homoeocarpa populations in New England (Mitkowski and Colucci, 2006). Lack of nucleotide substitutions in the mitochondrial loci sequenced from this panel of isolates from diverse locations support this hypothesis.

Acknowledgments

We thank K. Campbell-Nelson and J. Popko for editorial review of the manuscript.

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