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

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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 were 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

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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-

etative mycelium, and long distance dissemination may occur by infected grass clippings via wind, water, machinery, or human traffic (Baldwin and Newell, 1992; Subbarao et al., 1995). During the vegetative phase, it has been demonstrated that hyphal fusion and heterokaryosis may occur between mycelia within the same compatibility group (Glass et al., 2004; Jo et al., 2008). Vegetative hyphal fusions are vital to genetic exchange, translocation of water and nutrients, and general homeostasis within or between fungus populations in the field (Glass et al., 2000).

Vegetative compatibility refers to the ability of the hyphae of two individual fungal isolates to fuse together and form viable heterokaryons (Leslie, 1993). In fungi lacking sexual stages, vegetative compatibility may serve as an important means of genetic exchange and generating new genetic diversity (Leslie, 1993). Isolates that are vegetatively compatible with each other are said to be members of the same vegetative compatibility group (VCG) (Joaquim and Rowe, 1991; Leslie, 1993). Conversely, vegetatively incompatible isolates are incapable of establishing stable heterokaryosis.

The vegetative compatibility assay has been used to measure population diversity of fungi and to enable appropriate identification and characterization of individual isolates. Vegetative compatibility assays have been used to determine the population structures of many plant-pathogenic fungi, including *Fusarium* spp. (Katan and Katan, 1988; Marlatt et al., 1996), *Verticillium* spp. (Joaquim and Rowe, 1991), and *Colletotrichum* spp. (Brooker et al., 1991; Cecilia De Lima Favaro et al., 2007). The conventional method of determining VCGs involves the detection of a darkly pigmented lytic area, or barrage zone, where mycelium from two incompatible isolates meet. According to the conventional method VCG determination, if the barrage zone is absent, isolates are said to be vegetatively compatible. Another type of VCG assay that has been widely used relies on the complementation between nitrate-nonutilizing (*nit*) mutants (Leslie, 1993). This assay proceeds as follows: mutants deficient in one of several steps of the nitrogen assimilation pathway are made for each isolate tested. A single mutant is chosen from each of two parent isolates to be tested against one another, with each mutant deficient for a different step in the nitrogen assimilation pathway. They each are inoculated onto opposite ends of a petri plate with an agar medium amended with nitrate as the sole nitrogen source. The *nit* mutant exhibits sparse growth on the nitrate medium, but when the mycelium of compatible mutants meets, they fuse and form distinct and prolifically growing heterokaryotic mycelium, since the mutations of the nitrogen assimilation pathway are complemented (Correll et al., 1998; Joaquim and Rowe, 1991; Katan and Katan, 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 determination (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 assigned 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 heterokaryon 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 (*nit*2), 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. homoeocarpa* share significantly more genetic similarity of microsatellite and mitochondrial genotypes groupings, as compared 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. homoeocarpa* used in this study originated from various geographical locations in the US including Arkansas, Illinois, Michigan, Florida, and Minnesota (Table 1). Powell and Vargas and Chakraborty et al. used these isolates previously 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),

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)[a]	Jo et al. (2008)[b]	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
BRS	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	–	–

[a]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).
[b]VCGs were determined using *nit* mutants but specific names were not assigned.

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*

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[a]	Mutant designation	Growth on nitrogen sources[b]		Nitrite excretion[c]
		Nitrite	Hypoxanthine	
Structural gene for nitrate reductase	*nit*1	+	+	NT
Major nitrogen regulatory gene	*nit*2	−	−	NT
Pathway-specific regulatory gene	*nit*3	−	+	−
Genes controlling production of a molybdenum-containing cofactor	NitM	+	−	NT
None	Wild type	+	+	+

[a]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.
[b]Growth on Czapek solution agar (CDA) medium amended with two nitrogen sources. + = typical wild-type growth and − = thin growth with no aerial mycelium.
[c]Nitrite 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: $NaNO_2$ 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 $NaNO_2$ (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 25ºC 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 (*nit*1), a major nitrogen regulatory locus (*nit*2), a pathway-specific regulatory locus (*nit*3), 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 *nit*1. Mutants incapable of using nitrate, nitrite and hypoxanthine were referred to as *nit*2. Mutants that could not use nitrate and nitrite but could use hypoxanthine were designated as *nit*3. Mutants capable of utilizing nitrite but not nitrate and hypoxanthine were designated as NitM (Table 2).

Subsequently, all *nit*3 mutants were further tested for nitrite excretion using the procedures by Correll et al. (1987). Each *nit*3 isolate was grown on urea medium (CDA amended with 0.04% urea) in a 9-cm Petri dish at 25ºC for 3 days. The plate was then flooded with 10 ml of 3 M $NaNO_3$ solution (Sigma-Aldrich). After 24 h incubation at 25ºC, $NaNO_3$ was poured off. By adding 1 ml of a sulfanilamide 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 25ºC 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

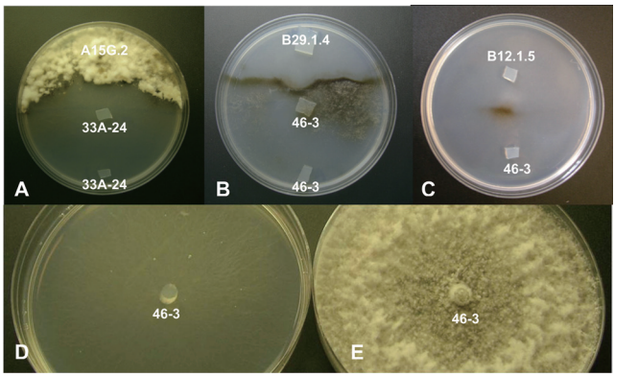

Fig. 1. Vegetative compatibility reactions between *nit* mutants of *Sclerotinia homoeocarpa* on Czapek solution agar medium (CDA) after 30-day incubation at 25ºC. A, Formation of vigorous heterokaryotic mycelium at the contact site between compatible isolates (A15G.2 and 33A-24). B. Formation of a distinct barrage with dark pigmentation between incompatible isolates (B29.1.4 and 46-3). C. Formation of small spot of aerial mycelium 2 weeks after incubation but later a barrage with dark pigmentation between incompatible isolates (B12.1.5 and 46-3). D. Mycelia of isolate 46-3 grown on CDA after 30-day incubation at 25ºC. E. Mycelia of isolate 46-3 grown on potato dextrose agar medium (PDA) after 7-day incubation at 25ºC.

screening.

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 65ºC. 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 nM $MgCl_2$, 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 95ºC for 60 s, followed by 35 cycles of 95ºC for 60 s, annealing at 72ºC for 1 min and decreasing by increments of 1.0 degree per cycle for the first 10 cycles, and 72ºC for 90 s, with a final extension of 72ºC 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[a]	Size (bp)	Left primer	Right primer	No. alleles
SSR02063	$(CTCAC)_{12}$	243	CCTTGGCAGCCTCTGATTAT	TGAGGGTTCATGGAATAGCA	2
SSR05848	$(GTATGA)_{12}$	232	TTGGTGTAGGTGGAGGCTAGA	CTCACGTTCACTCACGCACT	2
SSR06235	$(TG)_{17}$	197	TTCTTCCTTTCGGGTGACAG	CTTTTTCGTCTGCCTTGTGG	3
SSR08045	$(AC)_{13}$	157	GTCGTGGAGAGGAGAGGTGA	AACGCGAGCCAACACTATCT	4
SSR08400	$(ATCT)_{12}$	202	CTATTCTCGCGCATCCTCAT	TCGACGGTATCCTAGCAAGTG	3
SSR08569	$(TATGGGA)_{13}$	227	CCTCTCGTTCCTGGTTTCAC	CAATATCCATCCATCCATCCA	3
SSR09987	$(CA)_{12}$	233	CTCCAACCAATCCTCCTTGA	TGGGCTACCGAGTACTTTGC	2
SSR15983	$(TAG)_{13}$	150	TATAGCTCGCGGATGATGTG	AGACGGACTTACGCAATGCT	4
SSR17616	$(AC)_{16}$	172	TCCGCACTACCGTTACACAC	GTGCGATGGAGATGGAGTCT	2
SSR20589	$(TCA)_{13}$	243	ATCGACCCAAGAATCACCAA	AGGCTGGGTGCCTTAGTTTT	2
SSR20624	$(TTCA)_{16}$	184	AGTTGGGCGAACGAATAAGA	GGCTGAAAGGGAGAAAGAACA	3
SSR21791	$(GAA)_{12}$	215	CCATTCGTTCTATGGGTTCG	GGGACTTCTCCTTCCCATTC	2
SSR22804	$(AT)_{15}$	232	CGGCTAGTTCGTCAATCAGG	AGCGGACGAGGAGGTAAACT	2
SSR23039	$(TC)_{16}$	197	TCTTGCTCTGCTCTGCTCTG	TGGCCTTTTGCTTGCTTACT	3
SSR25827	$(AATC)_{15}$	188	CCTTCCTTTCCAGCCTATCC	CCCGCTTTTTGGTTTTTGT	5
SSR27998	$(GTTAT)_{12}$	165	ATTGATGGGCATCGGTTG	TCCTCTCCTCTCCTCCTCTGTA	4
SSR30326	$(TTGAC)_{14}$	167	GCAATGAGTGAGCGTCTTGT	TCATATCATCAAACGCATCCA	4
SSR30530	$(TC)_{12}$	156	AATCGAGCACAGTCCAGTCC	TCTGTCTACTTGTCCGTCGATTT	3
SSR30647	$(AC)_{14}$	170	GCTGTGGCCCATAATACGAT	GGCTGGATGTGCTGGATAAA	2

[a]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 *atp6-rns* 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 67ºC, optimum melting temperature 70ºC, and maximum melting temperature 73ºC. The forward and reverse primer sequences for mtSSU were 5′-GCTGAACCAGCAACTTGGGGGAAT-3′ and 5′-TCCCTTCCACGAATTCACACTCCA-3′, and amplified an 838 bp amplicon. The forward and reverse primer sequences for the *atp6-rns* spacer were 5′-GCAGAGGACTTTCTCGTGCAAAGCA-3′ and 5′-GGGTAGGGGAGACAAACCTCCCACTT-3′, and amplified a 721 bp amplicon. The forward and reverse primer sequences for the COII locus were 5′-GTCGCCTG-GCGGAGGGAGTA-3′ and 5′-GCGGAGCTAGAGCTT-GCTGTGTC-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 GeneAlEx, 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 (Felsentein, 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 GeneAlEx 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, BRS, 30B-24, 48-54 and 64-14) failed to produce any *nit* mutants in

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

Isolate	No. of colonies ($\times 10^5$ cfu/ml)[a]	No. of mutants[b]	Mutation rate ($\times 10^5$)[c]	*nit* mutant class[d]			
				*nit*1	*nit*2	*nit*3	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	2.5 ± 0.3	4	0	0	1
I16	3.4 ± 3.0	4	4.8 ± 4.2	1	0	0	3
I18	3.0 ± 0.7	6	5.1 ± 1.2	4	0	0	2
L36	3.4 ± 0.5	9	7.5 ± 10.6	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	5.0 ± 7.1	1	0	0	0
TB64D	1.8 ± 1.3	3	5.0 ± 1.8	0	3	0	0
30B-13	1.3 ± 0.8	5	11.3 ± 4.2	2	0	1	2
30B-24	2.2 ± 1.9	0	0	0	0	0	0
30B-48	2.7 ± 1.2	6	8.3 ± 11.8	6	0	0	0
32-47	2.6 ± 0.4	1	1.1 ± 1.6	1	0	0	0
33A-9	1.4 ± 0.1	1	1.9 ± 2.7	1	0	0	0
33A-24	2.1 ± 0.6	42	51.9 ± 14.0	22	0	0	20
46-3	1.7 ± 0.9	5	6.8 ± 2.6	3	0	1	1
48-54	6.4 ± 0.5	0	0	0	0	0	0
64-14	1.6 ± 0.6	0	0	0	0	0	0
64-41	2.4 ± 0.8	3	3.6 ± 2.7	2	0	0	1
64-49	2.0 ± 0.7	5	7.0 ± 4.2	3	0	0	2
Total		101		61	3	2	35

[a]CFU: colony-forming unit, number of colony produced from the shredded mycelial suspension on PDA (Potato Dextrose Agar).
[b]Number of *nit* mutants recovered from water agar media supplemented with 4.0% potassium chlorate.
[c]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.
[d]*Nit* mutant phenotypes determined according to growth on CDA amended with different nitrogen sources. *nit*1: mutation in a nitrate reductase structural locus, *nit*2: mutation in major nitrogen regulatory locus, *nit*3: 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.

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 $2.1 \pm 0.6 \times 10^5$ cfu/ml. In contrast, only one *nit* mutant was isolated from isolate MN1 out of $3.3 \pm 0.7 \times 10^5$ 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, *nit*1 mutants were predominant (60.4%) and NitM mutants were the second most prevalent (34.7%). The frequency of *nit*2 and *nit*3 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 5. Complementation reactions among four types of nitrate nonutilizing (*nit*) mutants of *Sclerotinia homoeocarpa*

nit mutant phenotype[a]	*nit*1	*nit*2	*nit*3	NitM
*nit*1	–	+	–	+
*nit*2		–	+	+ or –[b]
*nit*3			–	+
NitM				+ or –

[a]*nit*1: mutation in a nitrate reductase structural locus, *nit*2: mutation in major nitrogen regulatory locus, *nit*3: 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.

[b]– = prototrophic growth absent or inconspicuous between *nit* mutants of isolates. + = dense prototrophic growth at the mycelial interface between *nit* mutants of isolates.

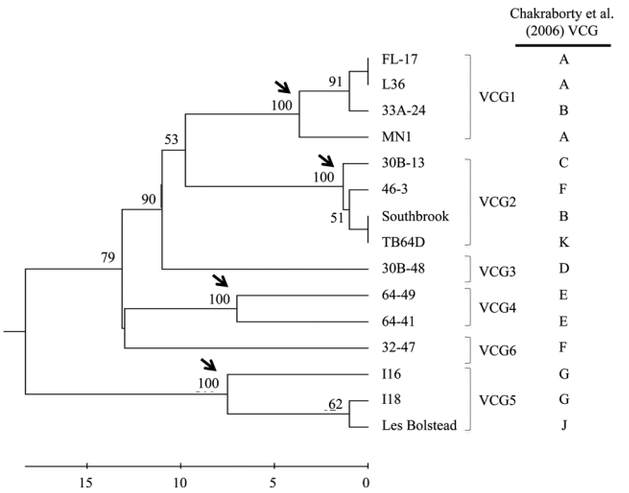

Fig. 2. UPGMA dendrogram of microsatellite genotypes of 15 isolates characterized using the improved *nit* mutant VCG assay. The *nit* mutant VCGs of taxa are indicated by brackets, and the VCGs of Chakraborty et al. (2006) (determined using the conventional method of Powell and Vargas, 2001) are indicated to the right of each taxon (Chakraborty et al., 2006; Powell and Vargas, 2001). Bootstrap proportions are indicated from 10,000 bootstrap replications. Arrows indicate bootstrap support for *nit* mutant VCGs containing multiple isolates.

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 *nit*1 or *nit*3, and *nit*2 with *nit*1 or *nit*3 combinations (Table 5). The complementary reactions between NitM and *nit*2 showed either abundant, profuse mycelium or no aerial mycelium. No clear complementation was observed on pairings between same mutant types of *nit*1, *nit*2 and *nit*3, and between *nit*1 and *nit*3. However, distinct complementation reactions of some pairing combinations between different NitM mutants produced rapid and robust heterokaryons with dense aerial mycelium. NitM or *nit*2 mutants readily complemented with *nit*1 and *nit*3 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 I16, 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).

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[a]	Average % genetic distance within VCG[b]	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	–

[a]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 [*nit*1 (or *nit*3) and *nit*2 (or NitM)].
[b]Average % genetic distance was calculated by averaging all genetic distance values between isolates within VCG.

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 ϕ_{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

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 ϕ_{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 ϕ_{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 ϕ_{PT} value was 0.572, with $P<0.001$ (Table 7) (Powell and Vargas, 2001). Pairwise ϕ_{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 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	ϕ_{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			

gion and COII were all identical among isolates representing each VCG.

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 *nit*3 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 (*nit*1, *nit*3, and NitM) of *nit* mutants were identified. In this current study, the *nit*2 mutant was also included in the VCG analysis, but was only produced in one isolate (Jo et al., 2008). The *nit*2 mutant type has also been previously recovered in *Neurospora crassa* (Marzluf, 1981) and *Fusarium moniliforme* (Klittich and Leslie, 1988). The dominant recovery of *nit*1 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 *nit*2 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.

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References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–3402.

Baldwin, N. A. and Newell, A. J. 1992. Field production of fertile apothecia by *Sclerotinia homoeocarpa* in Festuca turf. *J. Sports Turf Res. Inst.* 68:73–76.

Bennett, F. T. 1937. Dollar spot disease on turf and its causal organism *Sclerotinia homoeocarpa* n. sp. *Ann. Appl. Biol.* 24:236–257.

Berbegal, M., Ortega, A., Jiménez-Gasco, M. M., Olivares-García, C., Jiménez-Díaz, R. M. and Armengol, J. 2010. Genetic diversity and host range of *Verticillium dahliae* isolates from artichoke and other vegetable crops in Spain. *Phytopathology* 94:396–404.

Brooker, N. L., Leslie, J. F. and Dickman, M. B. 1991. Nitrate nonutilizing mutants of *Colletotrichum* and their use in studies of vegetative compatibility and genetic relatedness. *Phytopathology* 81:672–677.

Burpee, L. L. 1997. Control of dollar spot of creeping bentgrass caused by an isolate of *Sclerotinia homoeocarpa* resistant to benzimidazole and demethylation-inhibitor fungicides. *Plant Dis.* 81:1259–1263.

Cai, G. and Schneider, R. W. 2008. Population structure of *Cercospora kikuchii*, the causal agent of *Cercospora* leaf blight and purple seed stain in soybean. *Phytopathology* 98:823–829.

Cecilia De Lima Favaro, L., Luiz Araujo, W., Aparecida De Souza-Paccola, E., Lucio Azevedo, J. and Paccola-Meirelles, L. D. 2007. *Colletotrichum sublineolum* genetic instability assessed by mutants resistant to chlorate. *Mycol. Res.* 111:93–105.

Chakraborty, N., Chang, T., Casler, M. D. and Jung, G. 2006. Response of bentgrass cultivars to *Sclerotinia homoeocarpa* isolates representing 10 vegetative compatibility groups. *Crop Sci.* 46:1237–1244.

Chang, S. W., Chang, T. H., Hong, J. K., Park, J. H. and Jung, S. W. 2011. Vegetative compatibility grouping of *Sclerotinia homoeocarpa* isolates infecting turfgrass in South Korea. *Asian J. Turfgrass Sci.* 25:171–176.

Chang, S. W., Jung, S. W., Kim, S., Park, J. H. and Lee, J. Y. 2012. Synergistic interaction of fungicides in mixtures under different conditions of dollar spot disease caused by *Sclerotinia homoeocarpa*. *Asian J. Turfgrass Sci.* 26:96–101. (in Korean).

Chang, S. W., Jung, S. W., Kim, S., Park, J. H. and Lee, J. Y. 2013. Control effect on dollar spot disease caused by *Sclerotinia homoeocarpa* under different application rates and intervals with two mixed fungicides. *Weed & Turf. Sci.* 2:408–412. (in Korean).

Correll, J. C., Klittich, C. J. R. and Leslie, F. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77:1640–1646.

Correll, J. C., Gordon, T. R. and McCain, A. H. 1988. Vegetative compatibility and pathogenicity of *Verticillium albo-atrum*. *Phytopathology* 78:1017–1021.

Cove, D. J. 1976. Chlorate toxicity in *Aspergillus nidulans*-selection and characterization of chlorate resistant mutants. *Heredity* 36:191–203.

Cox, K. D., Bryson, P. K. and Schnabel, G. 2007. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. *Phytopathology* 97:448–453.

DeVries, R. E., Trigiano, R. N., Windham, M. T., Windham, A. S., Sorochan, J. C., Rinehart, T. A. and Vargas, J. M. 2008. Genetic analysis of fungicide-resistant *Sclerotinia homoeocarpa* isolates from Tennessee and Northern Mississippi. *Plant Dis.* 92:83–90.

Felsentein, N. J. 1989. PHYLIP-phylogeny inference package (version 3.2). *Cladistics* 5:164–166.

Ghikas, D. V., Kouvelis, V. N. and Typas, M. A. 2010. Phylogenetic and biogeographic Implications inferred by mitochondrial intergenic region analyses and ITS1-5.8S-ITS2 of the entomopathogenic fungi *Beauveria bassiana* and *B. brongniartii*. *BMC Microbiol.* 10:1–15.

Grubisha, L. C. and Cotty, P. J. 2009. Twenty-four microsatellite markers for the aflatoxin-producing fungus *Aspergillus flavus*. *Mol. Ecol. Res.* 9:264–267.

Glass, N. L., Jacobson, D. J. and Shiu, P. K. T. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous Ascomycete fungi. *Annu. Rev. Genet.* 34:165–186.

Glass, N. L., Rasmussen, C., Roca, M. G. and Read, N. D. 2004. Hyphal homing, fusion and mycelial interconnectedness. *TRENDS in Microbiol.* 12:135–141.

Jackson, N. 1973. Apothecial production in *Sclerotinia homoeocarpa* F. T. Bennett. *J. Sports Turf Res. Inst.* 49:58–63.

Joaquim, T. R. and Rowe, R. C. 1991. Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. *Phytopathology* 81:552–558.

Jo, Y. K., Chang, S. W., Rees, J. and Jung, G. 2008. Reassessment of vegetative compatibility of *Sclerotinia homoeocarpa* using nitrate-nonutilizing mutants. *Phytopathology* 98:108–114.

Katan, T. and Katan, J. 1988. Vegetative compatibility grouping of *Fusarium oxysporum* f. sp. *vasinfectum* from tissue and rhizosphere of cotton plants. *Phytopathology* 78:852–855.

Klittich, C. J. R. and Leslie, J. F. 1988. Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). *Genetics* 118:417–423.

Korolev, N. and Katan, T. 1997. Improved medium for selecting nitrate non-utilizing (*nit*) mutants of *Verticillium dahlia*. *Phytopathology* 87:1067–1070.

Lamour, K. H., Finley, L., Hurtado-Gonzalez, O., Gobena, D., Tierney, M. and Meijer, H. J. G. 2006. Targeted gene mutation in *Phytophthora* sp. *Mol. Plant Microbe In.* 19:1359–1367.

Leslie, F. J. 1993. Fungal vegetative compatibility. *Annu. Rev. Phytopathol.* 31:127–150.

Ma, Z., Luo, Y. and Michailides, T. J. 2004. Spatiotemporal changes in the population structure of *Botryosphaeria dothidea* from California pistachio orchards. *Phytopathology* 94:326–332.

Marlatt, M. L., Correll, J. C., Kaufmann, P. and Cooper, P. E. 1996. Two genetically distinct populations of *Fusarium oxysporum* f. sp. *lycopersici* race 3 in the United States. *Plant Dis.* 80:1336–1342.

Marzluf, G. A. 1981. Regulation of nitrogen metabolism and gene expression in fungi. *Microbiol. Rev.* 45:437–461.

Mitkowski, N. A. and Colucci, S. 2006. The identification of a limited number of vegetative compatibility groups within isolates of *Sclerotinia homoeocarpa* infecting *Poa* spp. and *Agrostis palustris* from temperate climates. *J. Phytopathology* 154:500–503.

Nitzan, N., Hazanovsky, M., Tal, M. and Tsror (Lahkim), L. 2002. Vegetative compatibility groups in *Colletotrichum coccodes*, the causal agent of black dot on potato. *Phytopathology* 92:827–832.

Peakall, R. and Smouse, P. E. 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mole. Ecol. Notes* 6:288–295.

Powell, J. F. and Vargas, J. M. 2001. Vegetative compatibility and seasonal variation among isolates of *Sclerotinia homoeocarpa*. *Plant Dis.* 85:377–381.

Rozen, S. and Skaletsky, H. 2000. Primer 3 on the WWW for general users and biologist programmers. In Bioinformatics Methods and Protocols: Methods in Molecular Biology (eds

Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

Saitoh, K., Togashi, K., Arie, T. and Teraoka, T. 2006. A simple method for a mini-preparation of fungal DNA. *J. Gen. Plant. Pathol.* 72: 348–350.

Skovgaard, K., Nirenberg, H. I., O'Donnell, K. and Rosendahl, S. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races Inferred from multigene genealogies. *Phytopathology* 91:1231–1237.

Smiley, R. W. Dernoeden, P. H. and Clarke B. B. 2005. Compendium of turfgrass diseases. American Phytopathological Society, St. Paul, MN.

Smith, J. D., Jackson, N. and Woolhouse, A. R. 1989. Fungal diseases of amenity turfgrasses. 3rd. Ed. E. and F. Spon, London.

Subbarao, K. V., Chassot, A., Gordon, T. R., Hubbard, J. C., Bonello, P., Mullin, R., Okamoto, D., Davis, R. M. and Koike, S. T. 1995. Genetic relationships and cross pathogenicities of *Verticillium dahliae* isolates from cauliflower and other crops. *Phytopathology* 85:1105–1112.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596–1599.

Tomsett, A. B. and Garrett, R. H. 1980. The isolation and characterization of mutants defective in nitrate assimilation in *Neurospora crassa*. *Genetics* 95:649–660.

Viji, G., Uddin, W., O'Neill, N. R., Mischke, S. and Saunders, J. A. 2004. Genetic diversity of *Sclerotinia homoeocarpa* isolates from turfgrasses from various regions in North America. *Plant Dis.* 88:1269–1276.

Warnke, S. 2003. Creeping bentgrass (*Agrostis stolonifera* L.). Pages 175–185 in: Turfgrass Biology, Genetics, and Breeding. M. D. Casler and R. R. Duncan, eds. John Wiley & Sons, Hoboken, NJ.