Genetic Diversity of *Sclerotinia homoeocarpa* Isolates from Turfgrasses from Various Regions in North America

G. Viji and W. Uddin, Department of Plant Pathology, The Pennsylvania State University, University Park 16802; and N. R. O'Neill, S. Mischke, and J. A. Saunders, United States Department of Agriculture–Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, MD 20705

ABSTRACT

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.

Sixty-seven isolates of *Sclerotinia homoeocarpa*, causing dollar spot disease in creeping bentgrass, annual bluegrass, Bermudagrass, and perennial ryegrass turf, collected from 23 golf courses in various geographical regions of the United States and Canada between 1972 and 2001, were characterized by vegetative compatibility, genetic diversity, and pathogenicity. Eleven vegetative compatibility groups (VCGs A to K) were identified among the isolates tested in this study, and five of them (VCGs G to K) were new. VCG B was the most predominant group, typifying 33 isolates (51%) tested. *S. homoeocarpa* isolates collected from golf courses in Pennsylvania belonged to seven VCGs (A, B, E, F, G, I, and K), whereas three groups were observed in those collected from New York (B, E, and G) and New Jersey (E, H, and I). Two isolates, one each from Pennsylvania and Canada, were incompatible when paired with the tester isolates in all possible combinations, and did not fall into any known VCG. An isolate collected from Canada was compatible with tester isolates from two VCGs (C and D). Genetic analyses using amplified fragment length polymorphism (AFLP) showed the presence of two genetically distinct groups, designated as major group and the minor group. The major group included 36 isolates collected from various golf courses in the United States and Canada. Two isolates collected from bermudagrass in Florida formed a separate cluster, the minor group. Isolates that belonged to the major group were further divided into two subgroups (1 and 2). Subgroup 1 consisted of all the isolates that belonged to VCGs A, E, G, H, and I. Three of the four isolates that belonged to VCG K also were clustered with isolates of subgroup 1. Subgroup 2 consisted of all the isolates from VCG B, and one each from VCGs F and K. Pathogenicity assays on Penncross creeping bentgrass showed significant differences ($P = 0.05$) in virulence among the isolates. Overall, a relationship between virulence and VCGs was observed, in which certain virulence groups corresponded to specific VCGs; however, such a relationship was not observed between virulence and AFLPs. Close similarity among isolates of *S. homoeocarpa* collected from different locations in the United States and Canada suggests that isolates of the same genotype could be involved in outbreaks of dollar spot epidemics at multiple locations.

Dollar spot, caused by *Sclerotinia homoeocarpa* F. T. Bennett, is a destructive disease of cool- and warm-season turfgrass species in North America, Europe, and Australasia (25,35). The disease initially appears as yellowish-green blotches on grass leaf blades which turn into creamy white, light-brown lesions. The lesions have a characteristic tan to reddish-brown margin. Under favorable conditions, circular necrotic spots, 2 to 5 cm in diameter, develop on close-cut turf. When the disease is severe, individual spots coalesce, extending to the entire width of the leaves.

On high-cut turf, affected areas develop into circular patches ranging from a few to several centimeters in diameter. Loss of large areas of turf may result if the disease is not controlled by fungicides (6,12). In the United States, more money is spent in the management of the disease than any other disease on turf (39).

The systematics of *S. homoeocarpa* has been debated since the description of the fungus. Studies based on various anatomical and cultural characteristics, and immunological and rDNA sequencing data, have shown that the pathogen may belong either to the genera *Lanzia*, *Moellerodiscus*, or *Rustroemia*, rather than *Sclerotinia* (2,15,16,18,27). Information on the teleomorph in *S. homoeocarpa* is not sufficient to validate reclassification of the fungus. Some studies have shown that dollar spot may be caused by multiple species (13,36); however, a recent study suggested that the disease in the northern United States is caused by a single species (30).

Characterization of dollar spot isolates into various biotypes based on degree of virulence, resistance to various fungicides, and vegetative compatibility has been reported (8,11,24,30,37). The use of vegetative compatibility groups (VCGs) has been a valuable tool to measure population diversity of fungi and enable appropriate identification and characterization of individual isolates. VCGs correlate to genetically distinct populations; therefore, they have been used to determine genetic relationships among isolates of various fungi (3,5,31). Markers such as auxotrophic mutants (3) and nitrate-nonutilizing mutants (20) also have been used to test compatibility, assess relatedness among strains, and subdivide populations of various fungi. In a recent study, six VCGs were identified among 1,300 isolates of *S. homoeocarpa* isolates collected from eight different locations in the northern United States (30). Additionally, assessment of genetic structure and variation in a pathogen population, and precise characterization of individual isolates, is possible by the use of molecular techniques. Analysis by amplified fragment length polymorphism (AFLP) has been used to assess genetic relatedness in various plant-pathogenic fungi (7,9,22,28). This technique, based on the selective amplification of DNA fragments, is a powerful fingerprinting tool for analyzing population complexity (40).

Characterization of the genetic diversity among isolates of *S. homoeocarpa* using molecular techniques could aid in the development of cultivars that are resistant to dollar spot. Additionally, elucidating the relationship between genetic variability and VCGs also could provide information to help resolve the taxonomy of the fungus. The objectives of the present study were to (i) determine VCGs among the isolates of *S. homoeocarpa* obtained from different geographic locations using standard tester isolates, (ii) determine the genetic diversity and the relationship between VCGs using AFLPs, and (iii) evaluate virulence of the isolates on creeping bentgrass.

MATERIALS AND METHODS

Fungal isolates. In all, 67 isolates of *S. homoeocarpa* collected from creeping bentgrass, annual bluegrass, Bermudagrass, and perennial ryegrass from 23 golf courses in the United States and Canada

Corresponding author: W. Uddin
E-mail: wxu2@psu.edu

Financial support for this study was provided by the Pennsylvania Turfgrass Council.

Accepted for publication 6 July 2004.

Publication no. D-2004-0830-04R
© 2004 The American Phytopathological Society

were used in this study. Of these isolates, 60 were isolated from turfgrass samples received at the turfgrass disease research laboratory at The Pennsylvania State University. These samples were obtained from golf courses in Delaware, Illinois, New Jersey, New York, North Carolina, Pennsylvania, and Virginia from 1972 to 2001. Seven isolates of *S. homoeocarpa* were obtained from Lawrence Datnoff (University of Florida) and Tom Hsiang (University of Guelph). In addition, 11 tester isolates that belonged to six known VCGs were obtained from Joseph Vargas (Michigan State University) (Table 1). In this study, isolates were arbitrarily classified as contemporary or archival. Whereas contemporary isolates refer to those collected from 1993 to 2001, the archival isolates refer to those collected prior to 1993. *S. homoeocarpa* was isolated from turfgrass samples by surface-sterilizing 1-cm-long segments of symptomatic leaves in 0.05% sodium hypochlorite solution for 30 to 60 s, rinsing with sterilized distilled water, and plating the leaf tissues on potato dextrose agar (PDA) amended with lactic acid to 0.1%. The plates were incubated at 22°C for 2 to 4 days, after which hyphal tips of *S. homoeocarpa* were aseptically removed and placed on PDA plates. For long-term storage, sterilized Whatman filter paper disks were placed on PDA plates and the mycelium was allowed to grow over the disks. After the fungus had completely colonized the disks, they were removed from the culture plate and air dried, and the disks were placed in sterilized envelopes and stored at -20°C (38). Two weeks prior to the assays, filter paper disks containing the individual isolates of *S. homoeocarpa* were retrieved from storage, placed on full-strength PDA plates, and incubated at 28°C under 12-h day-and-night cycles of fluorescent light ($76 \mu\text{E}^{-2} \text{s}^{-1}$) for 4 days.

Vegetative compatibility tests. Tests for vegetative compatibility of *S. homoeocarpa* isolates were performed on PDA containing red food color (10 drops per liter of PDA; McCormick and Co., Inc., Hunt Valley, MD) following the procedure proposed by Kohn et al. (17). The accumulation of red color in the interaction zone of incompatible pairings, caused by an accumulation of the red coloration in the cytoplasm of hyphal tips, was useful in classifying vegetative compatibility. Identification of VCGs was determined using 11 tester isolates belonging to groups A, B, C, D, E, and F. After each isolate of *S. homoeocarpa* was grown on PDA plates for 3 days prior to pairing, approximately 3-mm³ blocks of the culture were placed on one side of a PDA plate containing red food color, and paired with same-size agar blocks containing a tester isolate on the opposite side of the plate. The inoculated plates were sealed with parafilm, incubated at 26°C under 12-h day-and-night cycles of

fluorescent light ($76 \mu\text{E}^{-2} \text{s}^{-1}$) for 1 week, then scored for compatibility and classified into VCGs. Isolates were classified as incompatible if a barrage zone and abundant aerial mycelia were formed at the interface of the two isolates when observed from either the top or bottom of the plate. Compatible isolates, which did not form these structures, were classified within a particular VCG (26,30). Isolates that were incompatible with any of the 11 tester isolates were paired against each other and tested for compatibility. These isolates were classified into new VCGs, with one isolate representative of a newly identified VCG tester. Each experiment was conducted twice.

DNA extraction. Cultures (four to five 1-mm³ plugs) of actively growing mycelia of *S. homoeocarpa* were transferred into yeast extract glucose (YEG) medium in a 250-ml conical flask and grown under continuous shaking at 60 rpm at room temperature (25 to 27°C) for 7 days. Mycelial mats were harvested under vacuum, washed with sterilized distilled water, lyophilized and ground to a fine powder in liquid nitrogen, and stored over silica gel at -20°C. Total genomic DNA of the 78 isolates of *S. homoeocarpa* was extracted using the Qiagen DNeasy Plant Mini Kit procedure. DNA samples were checked for quality by running a minigel and stored at 4°C.

AFLP analyses. Genetic variation among the 78 isolates of *S. homoeocarpa* was assessed by AFLP analysis using ³²P-labeling and gel electrophoresis. AFLPs were generated according to manufacturer instructions of the AFLP System II (Life Technologies Inc., Gaithersburg, MD) with modifications (21,28,40). The primary template was prepared by digesting genomic DNA with restriction endonucleases *Eco*RI and *Mse*I to completion and heating at 70°C for 15 min to inactivate the enzymes. The DNA fragments were ligated to the *Eco*RI and *Mse*I adapters for 2 h at 20°C. After termination of the reaction, the ligation mixture was diluted 10-fold with sterilized distilled water, and the fragments were preamplified by 20 polymerase chain reaction (PCR) cycles using primers in pre-amp primer mix-II (Life Technologies; 40). Preselective amplification was performed in a PE-2400 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) using a temperature profile of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. The primers *Eco*RI+AG in combination with *Mse*I+C provided by Life Technologies were used for selective amplification of restriction fragments. Autoradiographs were obtained by exposure of the gels to Kodak BioMax MR-2 film (Eastman Kodak Co., Rochester, NY), scanned onto a computer, and each lane was scored for the presence or absence of bands. Bands were scored between molecular sizes of 80 and 385 bp using Proscore (version 2.36; DNA

Proscan Inc., Nashville, TN) and represented as binary data with "1" representing the presence of a band and "0" representing its absence. The resulting binary matrices were analyzed to obtain simple matching coefficients among the isolates using NTSYS-pc (version 1.60; Exeter Software, Setauket, NY). Simple matching coefficients were clustered to generate similarity trees using the SAHN clustering program, selecting the unweighted pairgroup method with arithmetic average (UPGMA) algorithm in NTSYS-pc.

Fluorescent AFLP with automated capillary electrophoresis (fAFLP-CE) was used to examine a subset of *S. homoeocarpa* isolates, which included the tester isolates from each VCG and representative isolates from each AFLP group identified in the initial study. In this method, DNA fragments were amplified by a multistep procedure, originally proposed by Vos et al. (40), and modified as follows. *S. homoeocarpa* template DNA was digested by *Eco*RI and *Mse*I (New England BioLabs, Beverly, MA) and ligated to commercial *Eco*RI and *Mse*I oligonucleotide adapters (Applied Biosystems, Foster City, CA) in a single step with incubation overnight at room temperature. The procedure described previously (33,34) was followed, with modifications as noted below. Commercial *Eco*RI and *Mse*I AFLP preselective primers and AFLP core mix (Applied Biosystems) were used for the preselective amplification of the restricted and ligated fragments. The mixture was subjected to amplification at 94°C for 3 min, followed by 20 cycles of the following profile: 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, with a final hold of 60°C for 30 min (GeneAmp 9700 PCR system; Applied Biosystems).

For selective amplification, custom primers with a fluorescent WellRED D4 dye-labeled phosphoramidite (D4) added to the 5' end of the *Eco*RI-AG primer (Invitrogen Life Technologies, Carlsbad, CA) were used. No modifications were made to the *Mse*I-C primer (Qiagen, Valencia, CA). The products from the preselective amplification were diluted as described previously (33) and used as template for selective amplification. The labeled *Eco*RI primer at 0.05 μM and the unlabeled *Mse*I primer at 0.10 μM were added. The mixture was subjected to a thermocycling profile of 94°C for 2 min, followed by 10 cycles of 94°C for 20 s, 1°C per cycle stepdown of annealing temperature from 66°C held for 30 s and 72°C for 2 min. This was followed by 25 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, with a final hold of 60°C for 30 min.

Samples were prepared for capillary electrophoresis by diluting the final amplified product 1:30 (vol/vol) in commercial Sample Loading Solution (Beckman-Coulter, Fullerton, CA), which included 1% (vol/vol) Beckman-Coulter 400 BP DNA

Table 1. Isolates of *Sclerotinia homoeocarpa* from various gramineous hosts from North America used in this study

Isolate	Host of origin	Location	Year of collection	VCG ^a	Virulence ^b
S-11	<i>Agrostis palustris</i>	Virginia	1972	K	AV
S-15	<i>Agrostis palustris</i>	Virginia	1972	–	WV
S-18	<i>Agrostis palustris</i>	Pennsylvania	1972	K	WV
S-20	<i>Agrostis palustris</i>	Pennsylvania	1972	K	WV
S-28	<i>Agrostis palustris</i>	Pennsylvania	1972	F	MV
S-30	<i>Agrostis palustris</i>	Pennsylvania	1972	I	WV
S-44cad	<i>Poa annua</i>	New Jersey	1972	H	AV
S-44cd	<i>Poa annua</i>	New Jersey	1972	H	WV
S-45	<i>Poa annua</i>	New Jersey	1972	E	WV
S-68	<i>Poa annua</i>	Delaware	1973	K	WV
S-83	<i>Poa annua</i>	Pennsylvania	1974	B	WV
S-85Ben	<i>Poa annua</i>	New Jersey	1974	I	WV
S-96Ben	<i>Poa annua</i>	Pennsylvania	1974	E	MV
S-107Ben	<i>Agrostis palustris</i>	North Carolina	1975	F	MV
S-108IP	<i>Agrostis palustris</i>	Pennsylvania	1983	B	WV
S-109IP	<i>Agrostis palustris</i>	Pennsylvania	1983	B	WV
S-111	<i>Poa annua</i>	Illinois	1993	A	WV
SH94064	<i>Agrostis palustris</i> , <i>Poa annua</i>	Ontario	1994	C/D	AV
SH94148	<i>Agrostis palustris</i> , <i>Poa annua</i>	Ontario	1994	B	MV
SH94247	<i>Agrostis palustris</i> , <i>Poa annua</i>	Ontario	1994	G	MV
SH94321	<i>Agrostis palustris</i> , <i>Poa annua</i>	Ontario	1994	G	MV
SH94544	<i>Agrostis palustris</i> , <i>Poa annua</i>	Ontario	1994	–	WV
5008A	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008AA	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008B	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008BB	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008C	<i>Poa annua</i>	Pennsylvania	1999	B	AV
5008CC	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008D	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008EE	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5008I	<i>Poa annua</i>	Pennsylvania	1999	B	WV
5012A	<i>Agrostis palustris</i>	Pennsylvania	2000	B	MV
5012B	<i>Agrostis palustris</i>	Pennsylvania	2000	B	HV
5012C	<i>Agrostis palustris</i>	Pennsylvania	2000	B	MV
5012D	<i>Agrostis palustris</i>	Pennsylvania	2000	B	WV
5012F	<i>Agrostis palustris</i>	Pennsylvania	2000	B	MV
5016B	<i>Lolium perenne</i>	Pennsylvania	2000	B	WV
5016C	<i>Lolium perenne</i>	Pennsylvania	2000	B	MV
5016D	<i>Lolium perenne</i>	Pennsylvania	2000	B	WV
5016E	<i>Lolium perenne</i>	Pennsylvania	2000	E	WV
5016G	<i>Lolium perenne</i>	Pennsylvania	2000	G	WV
5021B	<i>Poa annua</i>	New York	2000	B	MV
5021D	<i>Poa annua</i>	New York	2000	B	WV
5021E	<i>Poa annua</i>	New York	2000	B	MV
5021F	<i>Poa annua</i>	New York	2000	B	WV
5023A	<i>Poa annua</i>	Pennsylvania	2000	B	WV
5023B	<i>Poa annua</i>	Pennsylvania	2000	F	MV
5023C	<i>Poa annua</i>	Pennsylvania	2000	F	MV
5030B	<i>Poa annua</i>	New York	2000	E	WV
5030D	<i>Poa annua</i>	New York	2000	G	WV
5030F	<i>Poa annua</i>	New York	2000	G	WV
5038A	<i>Agrostis palustris</i>	New York	2000	B	WV
5038B	<i>Agrostis palustris</i>	New York	2000	B	MV
5044B	<i>Agrostis palustris</i>	Pennsylvania	2000	A	HV
5044D	<i>Agrostis palustris</i>	Pennsylvania	2000	A	HV
5044E	<i>Agrostis palustris</i>	Pennsylvania	2000	A	HV
5059A	<i>Poa annua</i>	Pennsylvania	2000	B	MV
5059D	<i>Poa annua</i>	Pennsylvania	2000	B	MV
5059F	<i>Poa annua</i>	Pennsylvania	2000	B	MV
5059H	<i>Poa annua</i>	Pennsylvania	2000	B	MV
5059J	<i>Poa annua</i>	Pennsylvania	2000	B	MV
5077A	<i>Agrostis palustris</i>	Pennsylvania	2000	E	MV
5077C	<i>Agrostis palustris</i>	Pennsylvania	2000	E	MV
5077D	<i>Agrostis palustris</i>	Pennsylvania	2000	E	WV
5077E	<i>Agrostis palustris</i>	Pennsylvania	2000	E	HV
A1421'A'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	A	MV
A1426'A'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	A	MV
A1438'B'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	B	AV
A1442'B'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	B	WV
A1612'C'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	C	MV
A1619'D'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	D	MV
A1623'F'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	F	MV
A167'C'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	C	MV
ML715'E'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	E	MV
ML729'D'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	D	MV
ML75'E'	<i>Agrostis palustris</i> , <i>Poa annua</i>	Michigan	2000	E	WV
Fl.Sh.A	<i>Cynodon dactylon</i>	Florida	2001	J	WV
Fl.Sh.B	<i>Cynodon dactylon</i> , bermudagrass	Florida	2001	J	WV

^a Vegetative compatibility group.^b Severity index (0 to 10): HV = highly virulent (7.0 to 10), MV = moderately virulent (4.0 to 6.9), WV = weakly virulent (1.0 to 3.9), and AV = avirulent (0; turf asymptomatic).

size standards. Fragment separation and detection was performed by capillary electrophoresis on each sample with a genetic analysis system (CEQ 8000; Beckman-Coulter, Fullerton, CA), beginning with a 30-s electrophoretic injection at 2.0 kV, followed by a 35-min separation at 50°C and 6.0 kV.

Data was analyzed using CEQ 8000 fragment analysis software with parameters that recognized peaks exceeding a height threshold of 10% of the height of the second largest peak and slope threshold of 10. The latter parameter was determined by the minimum rate of signal increase relative to the baseline noise for the sample. A 95% confidence level was imposed upon size estimation. The size standard fit coefficient was 0.38 and used a cubic model. For AFLP analysis, maximum bin width was 1.50 nucleotides. Peaks that were clearly present, but not recognized in one or a few samples by the automated scoring, were hierarchical manually. A few minor peaks, near the analysis threshold for scoring, which appeared in most or all samples but were recognized spuriously by the software, were manually removed from the analysis.

Samples were scored as “1” if a fragment of that size was present and “0” if absent. In rare cases where two fragments were present in one bin, the bin was scored as “1” for that sample. All bins were included in the analysis, even when common to every sample (i.e., nonpolymorphic). Data were imported into the multivariate data analysis program, NTSYSpc (version 2.1; Exeter Software, Setauket, NY). The module SimQual was used to generate a similarity matrix based on simple matching coefficients, and the module SAHN was used to perform hierarchical clustering by UPGMA. The module Tree Plot was used to convert the tree matrix generated by SAHN into a dendrogram.

Pathogenicity. Creeping bentgrass (cv. Penncross) was seeded in plastic pots (10-cm-diameter) that were filled to 1 cm below the rim with granular calcine clay medium (Turface MVP; Allied Industrial Material Corp., Buffalo Grove, IL) at the rate of 0.45 kg/m². Three weeks after

emergence, plants were fertilized weekly with a water-soluble 20-20-20 (N-P-K) fertilizer at the rate of 1.3 g/liter of water. The grasses were maintained at a height of 2 cm.

The 78 isolates of *S. homoeocarpa* were grown on PDA plates at 24°C for 3 days. In all, 10 to 15 agar blocks (approximately 5 by 5 mm) with actively growing mycelia of *S. homoeocarpa* were placed in a 100-ml conical flask containing sterilized rye grain (*Secale cereale* L.). The flasks were maintained at 24°C under fluorescent light (72 $\mu\text{E}^{-2} \text{s}^{-1}$) at 12-h day-and-night cycles for 7 days. The bentgrass was inoculated by placing one *S. homoeocarpa*-infested rye grain on the surface of the turf at the center of each pot. The individual pots were covered with a polyethylene bag and sealed immediately after inoculation. Plants that were not inoculated with the fungus served as controls. Treatments (the isolates) were arranged in a randomized complete block design with three replications. The pots were placed under continuous fluorescent light (155 $\mu\text{E}^{-2} \text{s}^{-1}$) at 28°C and relative humidity of 90% to 95% in a controlled-environment chamber. Seven days after inoculation, the polyethylene bags were removed, and disease assessment (severity index, 0 to 10; 0 = plants asymptomatic and 10 $\geq$ 90% turf area symptomatic) was made based on a method modified from Warren et al. (41). Isolates that did not produce dollar spot symptoms were considered avirulent to creeping bentgrass. The experiment was repeated once. Data from each experiment were subjected to analysis of variance using the SAS General Linear Model procedure (Statistical Analysis System, version 8.1; SAS Institute, Cary, NC).

RESULTS

Vegetative compatibility tests. Vegetative compatibility reaction between isolates was recognized as a line of profuse aerial mycelium between two complementary isolates. Classification of *S. homoeocarpa* isolates into different VCGs was determined by compatibility of the isolates to the standard testers. Isolates were classified into VCGs according to the classifica-

tion system adopted by Powell and Vargas (30). A total of 11 VCGs (A, B, C, D, E, F, G, H, I, J, and K) were detected among the isolates of *S. homoeocarpa* tested. Of the 67 isolates tested, 50 isolates were compatible with 1 or more of the 11 established tester isolates (30), which were grouped into six VCGs (A, B, C, D, E, and F). Although 15 isolates were not compatible with the tester isolates, they were compatible when paired with isolates in our collection and formed five new groups, designated as VCGs G, H, I, J, and K. Two isolates, S-15 and SH94544, were incompatible when paired in all possible combinations with other isolates in our collection, including the testers. These two atypical isolates were not assigned to any VCG.

The distribution of VCGs among isolates obtained from the various geographical locations is shown in Table 2. Isolates collected from Pennsylvania showed the presence of seven VCGs (A, B, E, F, G, I, and K), whereas three VCGs each were observed in New York (B, E, and G) and New Jersey (E, H, and I). Isolates collected from the remaining locations showed the presence of a single VCG. VCG B was the most predominant group among the isolates collected between 1972 and 2002. Of the 65 isolates that were classified into specific VCGs, 33 (51%) belonged to VCG B. Four isolates each were grouped into VCGs A, F, and K (6% each), whereas VCGs E and G had 8 (12%) and 5 (8%) isolates, respectively. VCGs H, I, and J had two representative isolates each (3% each). One isolate, SH94064, was compatible with the tester isolates belonging to both VCGs C and D. All isolates within a VCG produced compatible reactions with the other isolates belonging to a particular group.

The five isolates collected from Ontario, Canada, were variable with regard to VCGs. Two of the five isolates (SH94148 and SH94064) were compatible with the standard tester isolates, whereas isolates SH94247 and SH94321 were compatible with 5030F of our collection and belonged to VCG G. Isolate SH94544 was not compatible with any of the isolates used in the

Table 2. Vegetative compatibility groups (VCGs) among isolates of *Sclerotinia homoeocarpa* from various geographic regions of the United States and Canada

Location	Number of		VCGs ^a									
	Golf courses	Isolates	A	B	C/D	E	F	G	H	I	J	K
Pennsylvania	9	42	3	26	...	6	3	1	...	1	...	2
New York	2	9	...	6	...	1	...	2	...	...	...	...
New Jersey	2	4	...	...	...	1	...	...	2	1	...	...
Illinois	1	1	1	...	...	...	...	...	...	...	...	...
Florida	1	2	...	...	...	...	...	...	...	...	2	...
North Carolina	1	1	...	...	...	...	1	...	...	...	...	...
Delaware	1	1	...	...	...	...	...	...	...	...	...	1
Virginia	1	1	...	...	...	...	...	...	...	...	...	1
Canada	5	4	...	1	1	...	...	2	...	...	...	...
Total	23	65	4	33	1	8	4	5	2	2	2	4

^a VCGs A, B, C, D, E, and F are compatibility groups formed with the tester isolates, and VCGs G, H, I, J, and K are the newly formed groups.

present study. The two Florida isolates were compatible with each other and formed a separate group.

AFLP analysis. AFLP analyses provided a total of 39 scorable bands within the range of 80 to 385 bp. The PCR products of molecular sizes larger than 385 bp were not scored because resolution was insufficient to discriminate bands of different molecular sizes. A similarity dendrogram generated showed polymorphisms among the isolates, indicating that the population consists of multiple genotypes (*data not shown*). In several cases, identical banding patterns were observed in isolates collected from a specific location. Additionally, isolates collected in Canada showed fingerprints that were identical to the isolates from the United States.

Comparison of AFLPs and VCGs. A subset of 38 *S. homoeocarpa* isolates, which included representative isolates from VCGs A, B, C, D, E, F, G, and H and the 11 standard tester isolates, were characterized further using the fAFLP-CE technique. The primer *EcoRI*-AG/*MseI*-C generated 52 categories (bins) of nominal fragment size, of which 16 were common to all samples; thus, 69% of the fragment size bins were polymorphic. The topology of the dendrograms was identical whether based on all fragment bins or on polymorphic bins, but branch length was longer for dendrograms based on polymorphic bins, as expected (*data not shown*).

Effective separation of the *S. homoeocarpa* tester isolates into their known

VCGs was observed using the fAFLP-CE method, with the exception of the isolates from VCG C that separated into different subgroups (Fig. 1). A dendrogram from AFLP data generated from the isolates that were analyzed in the initial study using 32 P-labeling and gel electrophoresis, along with the standard VCG testers, is shown in Figure 2. Representative isolates that belonged to VCGs A, B, D, E, G, and J clustered into groups. A few clusters generated by the fAFLP-CE technique were slightly different from those obtained using 32 P-labeling gel electrophoresis.

In most cases, a close relationship was observed between isolates belonging to specific VCGs and AFLPs. Most isolates that had similar fingerprinting patterns belonged to a specific VCG; however, there were some exceptions. The dendrogram showed the presence of two groups among these isolates, which were designated the major group and minor group. The major group included 36 isolates collected from various geographic locations in the United States and Canada. The remaining two isolates collected from Florida formed a separate cluster, the minor group. Isolates that belonged to the major group were further divided into two subgroups (1 and 2). Subgroup 1 consisted of all the isolates that belonged VCGs A, E, G, H, and I. Three of the four isolates that belonged to VCG K also were clustered together with isolates of subgroup 1. Additionally, all the isolates that belonged to VCG A, the three isolates that belonged to

VCG K (S-18, S-11, and S-20), and one from VCG H (S-44cad) clustered very closely with similarity indices near 1.0 (Fig. 2). Subgroup 2 consisted of all the isolates that belonged to VCG B, and one each from VCGs F (5023B) and K (S-68). Although the tester isolates of VCG C and D belonged to subgroup 1, a single isolate, SH94064, that was compatible with these testers clustered with isolates in subgroup 2. Nine subgroup 1 isolates and six subgroup 2 isolates of *S. homoeocarpa* that were collected from different periods at various locations showed similar fingerprinting profiles (Fig. 2). The two isolates in the minor group, Fl.Sh.A and Fl.Sh.B, collected from bermudagrass in Florida, belonged to VCG J. In contrast to the isolates of the major group, these isolates showed unique AFLP profiles and formed a separate group.

A neighbor-joining (NJ) dendrogram constructed using agglomerative clustering was very similar to that generated by UP-GMA dendrogram. Based on the topology of the NJ tree, the 38 isolates also clustered into two groups, the major group and the minor group as described above (Fig. 3). However, variation in the clustering of isolates that belonged to the major group was observed. Whereas all the five isolates that belonged to VCG E formed a separate cluster, the standard tester isolates that belonged to VCG D clustered with isolates of group B.

Pathogenicity assays. Symptoms of dollar spot developed on creeping bent-

Fig. 1. Dendrogram based on fluorescent amplified fragment length polymorphism with automated capillary electrophoresis showing relationships among the six standard tester isolates of *Sclerotinia homoeocarpa* used in this study. The vegetative compatibility groups are indicated in single quotation marks.

grass inoculated with *S. homoeocarpa* 3 days after inoculation. All but five of the isolates caused disease symptoms. There were significant differences ($P \leq 0.05$) in disease severity among the isolates of *S. homoeocarpa* tested. The disease severity for the 78 isolates, which included the 11 tester isolates, was 0 to 8.7. Based on disease severity, isolates were classified into four groups: (i) highly virulent (disease index 7 to 10), (ii) moderately virulent (4 to 6.9), (iii) weakly virulent (1 to 3.9), and (iv) avirulent (0; plants asymptomatic). Of the 78 isolates, 5 were highly virulent, 30 were moderately virulent, 38 were weakly virulent, and 5 were avirulent (Table 1).

Overall, a relationship between virulence and VCGs was observed (Table 1). All five isolates that belonged to VCG F were moderately virulent, whereas both isolates that belonged to VCG I and VCG J were weakly virulent. Of the five isolates that were highly virulent on creeping bentgrass, three belonged to VCG A. All four virulent groups were observed in isolates that were grouped in VCG B, including 1 highly virulent, 13 moderately virulent, 19 weakly virulent, and 2 avirulent isolates. Two isolates which could not be grouped into any VCG, S-15 and SH94544, were weakly virulent.

Comparison of virulence of the *S. homoeocarpa* isolates and AFLP data showed no relationship between them. A few isolates that were avirulent exhibited finger-

prints similar to those of the moderately and highly virulent isolates, and clustered together. The only exceptions were the isolates from Florida, which formed a separate AFLP group.

DISCUSSION

The use of both gel and capillary electrophoresis methods detected genetic variation among the isolates of *S. homoeocarpa*. Compared with gel electrophoresis, separation of amplified fragments by capillary electrophoresis was superior, with the added benefits of automation, sensitivity, and precision. Less operator input was required, and the laser detection system could detect and score fluorescently labeled fragments that were present in low concentration. A DNA standard was co-electrophoresed with every sample; therefore, the 1-bp separation of fragments was precise and size scoring was accurate (33,34).

AFLP analysis of the *S. homoeocarpa* isolates that belonged to the various VCGs collected from 1972 to 2001 showed the presence of two distinct groups. All the isolates collected from Canada, Delaware, Illinois, New Jersey, New York, North Carolina, Pennsylvania, and Virginia belonged to the major group. The similarity coefficient among isolates of the major group was approximately 0.80. However, the two isolates that were collected in Florida clustered together, forming a separate

group (minor group). These isolates were obtained from bermudagrass and it is likely that host specialization could have been a factor. Further testing of isolates of *S. homoeocarpa* from the southeastern regions of the United States could provide a better understanding of the diversity of these isolates. It is noteworthy that certain archival isolates collected in Pennsylvania from 1972 to 1974 had fingerprints similar to those of the contemporary isolates collected in Pennsylvania from 1993 to 2000, as well as the VCG A tester isolates that were obtained from Michigan in 2000 (Fig. 2). Results of this study are in accordance with previous reports in which isolates of *S. homoeocarpa* evaluated using restriction fragment length polymorphism and random amplified polymorphic DNA markers have shown high genetic similarity (11,32). The close similarity between the isolates collected from different golf courses at various time periods suggests that isolates that belong to the same genetic groups could be involved in simultaneous outbreaks of dollar spot epidemics in different locations. The high degree of genetic similarity between the archival and contemporary isolates of *S. homoeocarpa* also indicate a clonal population. There have been no reports of production of fertile apothecia in *S. homoeocarpa* in nature; therefore, it is unlikely that genetic variation resulted from recombination. Similarity in fingerprinting profiles of nine iso-

Fig. 2. Dendrogram based on fluorescent amplified fragment length polymorphism with automated capillary electrophoresis showing relationships among 38 isolates of *Sclerotinia homoeocarpa* from the 11 vegetative compatibility groups (VCGs) previously defined by manual amplified fragment length polymorphism. VCG designations are indicated on the right side.

lates that belonged to subgroup 1 and six isolates of subgroup 2 collected from various geographic locations also strongly supports this conclusion (Fig. 2).

Vegetative compatibility has been widely used as a natural tool to subdivide fungal pathogen populations (19). Additionally, VCGs have been effective in the interpretation of data based on genetic diversity and isolates have been classified on the basis of VCGs, as demonstrated in *S. sclerotiorum* (10,19). In the present study, some relationship between isolates that belonged to specific AFLP groups and VCGs was observed. Most isolates of *S. homoeocarpa* that had similar fingerprints belonged to a VCG; however, a few exceptions, in which isolates were distributed in either of the two subgroups in the major group, were observed (Fig. 2). It should be noted that the primer set used for the fAFLP-CE technique did not always distinguish between isolates of different VCGs, such as between VCGs A and K (Fig. 2). A Canadian isolate, SH94064, was compatible with tester isolates of two VCGs (VCGs C and D). This isolate showed similarities to both the tester isolates in AFLP analysis.

VCG B was the most frequently represented group among the *S. homoeocarpa* isolates tested in this study. Of the 65 isolates that were compatible with the testers, 33 isolates (51%) belonged to VCG B, of which 26 were from Pennsylvania (Table 1). No definite conclusions on the prevalence of VCGs among isolates collected

from states other than Pennsylvania could be made due to the limited number of isolates tested. Although 65 of the 67 isolates showed compatibility reactions and could be classified into VCGs, two atypical isolates, S-15 and SH94544, were not compatible with any of the 11 tester isolates. Further, these isolates were not compatible with each other or with any isolate in our collection. It would be of interest to further study the biology of these atypical isolates by comparing them with isolates from other countries.

The diversity of VCGs among the *S. homoeocarpa* isolates was computed by dividing the total number of isolates tested in this study (67 isolates) with the number of VCGs identified (11 groups), and the diversity appears to be relatively low (0.17; highest diversity possible is 1.0). It has been demonstrated that pathogenic isolates have low VCG diversity because these VCGs have selected advantage in infecting a specific host. Such results have been reported in studies on VCG diversity in *Fusarium oxysporum* (1,20). On the contrary, a high degree of VCG diversity (0.69) also has been reported, in which 22 VCGs were identified among 32 isolates tested (29).

In several fungi, VCGs also have been correlated with host range, geographic location, production of secondary metabolites, and morphology (1,14,23,31). These studies have shown that isolates within a VCG appear to be more similar to each other than isolates in different VCGs. In

addition to these vegetative characteristics, grouping of isolates on the basis of VCGs also has been used to study variation in virulence (4,31). Although pathogenicity assays did not distinguish clearly among all isolates that we tested, the use of AFLPs and VCGs aided in the definition of distinct relationships between the isolates. All of the five *S. homoeocarpa* isolates in VCG F were moderately virulent on creeping bentgrass, and both isolates in VCG I and VCG J were weakly virulent. Additionally, 5 of the 78 isolates tested were highly virulent, of which 3 belonged to VCG A, indicating additional similarities among the isolates. Studies have shown that virulence assays could help in subdividing populations of fungi into different groups, and that isolates within a VCG group could be similar in virulence (1,20,23). In our study, however, results from the pathogenicity tests did not show a distinct pattern of relationship between virulence and VCGs in all the isolates. It is possible that the low virulence of archival isolates of *S. homoeocarpa* used in our study could have been due to subculturing.

Results of the present study suggest that isolates from a clonal population could be involved in outbreaks of dollar spot epidemics in various regions of the United States. The prevalence of the same genetic lineage among the archival and contemporary isolates of *S. homoeocarpa* also supports this hypothesis. The unique grouping of isolates obtained from Florida indicate that these isolates could be biologically

Fig. 3. Neighbor-joining dendrogram of 38 isolates of *Sclerotinia homoeocarpa* from the 11 vegetative compatibility groups (VCGs) constructed from fluorescent amplified fragment length polymorphism with automated capillary electrophoresis data. VCG designations are indicated on the right side.

distinct, and possibly form a different species. Characterization of additional isolates from warm, temperate regions would help to determine if dollar spot is caused by one or more species in the United States. Additionally, assessment of VCGs over time would provide important information on the stability of a particular VCG of *S. homoeocarpa*.

ACKNOWLEDGMENTS

We thank L. Datnoff, T. Hsiang, and J. Vargas for providing isolates of *Sclerotinia homoeocarpa*.

LITERATURE CITED

- Bosland, P. W., and Williams, P. H. 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. 65:2067-2073.
- Carbone, I., and Kohn, L. M. 1993. Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotiniaceae. Mycologia 85:415-427.
- Clarkson, J. M., and Heale, J. B. 1985. Heterokaryon compatibility and genetic recombination within a hop plant between hop wilt isolates of *Verticillium albo-atrum*. Plant Pathol. 34:129-138.
- Correll, J. C., Puhalla, J. E., and Schneider, R. W. 1986. Identification of *Fusarium oxysporum* f. sp. *apii* on the basis of colony size, virulence and vegetative compatibility. Phytopathology 76:396-400.
- Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. Genetics 114:1111-1129.
- Detweiler, A. R., Vargas, J. M., Jr., and Danneberger, T. K. 1983. Resistance of *Sclerotinia homoeocarpa* to iprodione and benomyl. Plant Dis. 67:627-630.
- Douhan, G. W., Murray, T. D., and Dyer, P. S. 2003. Population genetic structure of *Tapesia acuformis* in Washington State. Phytopathology 93:650-656.
- Golembiewski, R. C., Vargas, J. M., Jr., Jones, A. L., and Detweiler, A. R. 1995. Detection of demethylation inhibitor (DMI) resistance in *Sclerotinia homoeocarpa* populations. Plant Dis. 79:491-493.
- Gonzalez, M., Rodriguez, R., Zavala, M. E., Jacobo, J. L., Hernandez, F., Acosta, J., Martinez, O., and Simpson, J. 1998. Characterization of Mexican isolates of *Colletotrichum lindemuthianum* by using differential cultivars and molecular markers. Phytopathology 88:292-299.
- Hambleton, S., Walker, C., and Kohn, L. M. 2002. Clonal lineages of *Sclerotinia sclerotiorum* previously known from other crops predominate in 1999-2000 samples from Ontario and Quebec soybean. Can. J. Plant Pathol. 24:309-315.
- Hsiang, T., and Mahuku, G. S. 1999. Genetic variation within and between southern Ontario populations of *Sclerotinia homoeocarpa*. Plant Pathol. 48:83-94.
- Hsiang, T., Yang, L., and Barton, W. 1997. Baseline sensitivity and cross-resistance to demethylation-inhibiting fungicides by Ontario isolates of *Sclerotinia homoeocarpa*. Eur. J. Plant Pathol. 103:409-416.
- Jackson, N. 1973. Apothecial production in *Sclerotinia homoeocarpa*. F. T. Bennett. J. Sports Turf Res. Inst. 49:58-63.
- Jacobson, D. J., and Gordon, T. R. 1990. Variability of mitochondrial DNA as an indicator of relationships between populations of *Fusarium oxysporum* f. sp. *melonis*. Mycol. Res. 94:734-744.
- Kohn, L. M. 1979. A monographic revision of the genus *Sclerotinia*. Mycotaxon. 9:365-444.
- Kohn, L. M. 1979. Delimitation of the economically important plant pathogenic *Sclerotinia* species. Phytopathology 69:881-886.
- Kohn, L. M., Carbone, I., and Anderson, J. B. 1990. Mycelial interactions in *Sclerotinia sclerotiorum*. Exp. Mycol. 14:255-267.
- Kohn, L. M., and Grenville, D. J. 1989. Anatomy and histochemistry of stromatal anamorphs in the Sclerotiniaceae. Can. J. Bot. 67:371-393.
- Kohn, L. M., Stasovski, E., Carbone, I., Royer, J., and Anderson, J. B. 1991. Mycelial incompatibility and molecular markers identify genetic variability in field populations of *Sclerotinia sclerotiorum*. Phytopathology 81:480-485.
- Larkin, R. P., Hopkins, D. L., and Martin, F. N. 1990. Vegetative compatibility within *Fusarium oxysporum* f. sp. *niveum* and its relationship to virulence, aggressiveness, and race. Can. J. Microbiol. 36:352-358.
- Lin, J. J., and Kuo, J. 1995. A novel PCR-based assay for plant and bacterial DNA fingerprinting. Focus 17:66-70.
- Majer, D., Mithen, R., Lewis, B. G., Vos, P., and Oliver, R. P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycol. Res. 100:1107-1111.
- Manicom, B. Q., Bar-Joseph, M., Kotze, J. M., and Becker, M. M. 1990. A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*. Phytopathology 80:336-339.
- Massie, L. B., Cole, H., and Duich, J. 1968. Pathogen variation in relation to disease severity and control of *Sclerotinia* dollar spot of turfgrass by fungicides. Phytopathology 58:1616-1619.
- Monteith, J., and Dahl, A. S. 1932. Turf diseases and their control. Bull. U.S. Golf Assoc. Green 12:137.
- Newhouse, J. R., and MacDonald, W. L. 1991. The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. Can. J. Bot. 69:602-614.
- Novak, L. A., and Kohn, L. M. 1991. Electrophoretic and immunological comparisons of developmentally regulated proteins in members of the Sclerotiniaceae and other sclerotial fungi. Appl. Environ. Microbiol. 57:525-534.
- O'Neill, N. R., van Berkum, P., Lin, J. J., Kuo, J., Ude, G. N., Kenworthy, W., and Saunders, J. A. 1997. Application of amplified restriction fragment length polymorphism for genetic characterization of *Colletotrichum* pathogens of alfalfa. Phytopathology 87:745-750.
- Papa, K. E. 1986. Heterokaryon incompatibility in *Aspergillus flavus*. Mycologia 78:98-101.
- Powell, J. F., and Vargas, J. M., Jr. 2001. Vegetative compatibility and seasonal variation among isolates of *Sclerotinia homoeocarpa*. Plant Dis. 85:377-381.
- Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63:179-183.
- Raina, K., Jackson, N., and Chandless, J. M. 1997. Detection of genetic variation in *Sclerotinia homoeocarpa* isolates using RAPD analysis. Mycol. Res. 101:585-590.
- Saunders, J. A., Mischke, S., and Hemeida, A. A. 2001. The use of AFLP techniques for DNA fingerprinting in plants. Beckman Coulter Application Notes A1910A:1-9.
- Saunders, J. A., Mischke, S., and Hemeida, A. A. 2002. Identification of *Theobroma cacao* germplasm by AFLP-generated molecular markers. InCEquence 2:5-8.
- Smiley, R. W. 1992. Compendium of Turfgrass Diseases. American Phytopathological Society, St. Paul, MN.
- Smith, J. D., Jackson, N., and Woolhouse, A. R. 1989. Fungal Diseases of Amenity Turf Grasses. E. and F. N. Spon, New York.
- Sonoda, R. M. 1988. Vegetative compatibility groups among *Sclerotinia homoeocarpa* from leaves of *Paspalum notatum*. Proc. Soil Crop Sci. Soc. Fla. 48:35-36.
- Valent, B., Crawford, M. S., Weaver, C. G., and Chumley, F. G. 1986. Genetic studies of fertility and pathogenicity in *Magnaporthe grisea* (*Pyricularia grisea*). Iowa State J. Res. 60:569-594.
- Vargas, J. M., Jr. 1994. Fungal diseases of turfgrass I: Diseases primarily occurring on golf course turfs. Pages 15-33 in: Management of Turfgrass Diseases. 2nd ed. CRC Press, Boca Raton, FL.
- Vos, P., Hogers, R., Bleeker, M., Reijmans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.
- Warren, C. G., Sanders, P., and Cole, H. 1974. *Sclerotinia homoeocarpa* tolerance to benzimidazole configuration fungicides. Phytopathology 64:1139-1142.