Chapter 1

VARIATION OF *SCLEROTINIA HOMOEOCARPA* WITHIN AND AMONG GOLF COURSES IN MICHIGAN

Introduction

Dollar spot disease of turfgrasses is caused by the pathogen, *Sclerotinia homoeocarpa* F.T. Bennett (3). The disease is common throughout the world and is destructive to both cool and warm season grasses (20, 21, 23). In North America, with the exception of the Pacific Northwest, dollar spot is the most important pathogen of most cultivated fine turfgrass species (20, 21, 23). In Michigan, the disease is a major problem for most golf courses where the epidemic begins in June and can continue into late September causing extensive damage if left untreated. The disease can blight large areas of turf as a result of coalescing disease foci. Diseased turf has a poor aesthetic appearance, impairs the playing surface by creating depressions that affect ball roll, and leaves areas of bare soil where weed species can encroach on the area (20, 23). The pathogen is not known to produce conidia or undergo sexual reproduction in North America (1, 10, 11). Jackson found that while United Kingdom isolates of *S. homoeocarpa* will undergo sexual reproduction, no isolate from the US has been known to develop fertile apothecia (11). However, Hsiang and Mahuku (10) reported that some populations in Southern Ontario had random amplified polymorphic DNA (RAPD) patterns consistent with recombination events within a local population. It is commonly assumed that *S. homoeocarpa* is disseminated via direct transfer of mycelium from infected leaves (7, 20, 21, 23).

Control of dollar spot via fungicide application is generally accomplished using the contact fungicide, chlorothalonil. However, the EPA is expected to restrict the use of this fungicide on golf courses. In the event that a limited amount of fungicide is available to a golf course it is critical that superintendents be able to apply chlorothalonil judiciously. Other single-site mode of action fungicides are available to control dollar spot, but fungicide resistanceis a problem in many dollar spot populations (6, 9, 25).

Vegetative compatibility is the ability of the pathogen to form a stable heterokaryon as a result of a self/nonself genetic recognition event when two individual strains fuse (8, 16). The systems can be allelic or non-allelic in nature. Fungi that have an allelic compatibility system determine whether two strains are compatible via identity of alleles at a particular compatibility locus. In contrast, a non-allelic system usually involves alleles at multiple loci interacting to determine compatibility (16). Studies of compatibility are useful for studying diversity in populations, detecting new lineages in a local area, and observing population dynamics. *Aspergillis flavus* was examined in a cotton field using vegetative compatibility groups (VCGs) as a measure of genetic diversity (2). Large numbers of VCGs were identified and the distribution changed from year to year over the three-year study. The large number of VCGs suggests a large change in the genetic makeup of the population each season. The authors suggested the observed diversity could be a result of the migration of conidia from other locations and/or a seasonal change in the number of strains making up each VCG. Kohn *et al.* (15) studied mycelial compatibility, a specific component of

vegetative compatibility, in *Sclerotinia sclerotiorum*. Using DNA fingerprinting techniques, they found that the mycelial compatibility group (MCG) diversity was high and that MCGs made up genotypically distinct lineages. The observed diversity was attributed to be due to the occasional outbreeding event and the migration of new strains into populations. Powell and Vargas (18) identified 6 VCGs from isolates sampled from creeping bentgrass and annual bluegrass from 8 locations in Michigan and the Midwest. They found that the VCG distributions at a location change over a season. They also reported that isolates from the same VCG could be isolated from both creeping bentgrass and annual bluegrass indicating that host specificity is not associated with particular VCGs. Using the sequence of the nuclear internal transcribed spacer region 1 (ITS1) they also found that all sampled isolates shared the same sequence and were from the same species. Raina *et al.* (19) studied the genetic variability of *S. homoeocarpa* using RAPDs and found that isolates of dollar spot from the midwest and northeastern United States were very similar. Both of these studies support the empirical evidence that *S. homoeocarpa* is a clonal pathogen.

In contrast, Sonoda (22) identified more than 54 VCGs of *S. homoeocarpa* isolated from bermudagrass (*Cynodon dactylon*). One hundred nineteen isolates were collected from three locations; nearly 50% represented VCGs, indicating a significant amount of genetic exchange or migration. Hsiang and Mahuku's (10) study using RAPDs of dollar spot isolates from eight populations in Southern Ontario supported random mating in three of the eight populations sampled.

Many different molecular tools are available for the study of plant pathogens (5, 10, 12, 15, 17, 19). Isozymes are relatively inexpensive, but problems often occur in generating enough polymorphic markers to be of use. RFLPs (restriction fragment length polymorphisms) can often be very informative, however suitable DNA probes must be available. The widely used RAPDs (random amplified polymorphic DNA) suffer most generating reproducible results because of sensitivity to running conditions. AFLPs (amplified fragment length polymorphisms) are noted for their ability to rapidly generate large numbers of reproducible and neutral (not under independent selection) markers at independent loci (17, 24). AFLPs avoid the problems inherent in most other tools used for fungal genetic analysis. The primary drawback to AFLPs is the relatively high startup cost. Cilliers *et al.* (5) used AFLP analysis to differentiate isolates and MCGs of *Sclerotium rolfsii* from South Africa. They identified 9 MCGs in a collection of 73 isolates from 10 locations in South Africa. Isolates were identified with a specific MCG using AFLPs.

The objective of this study was to determine if isolates of *S. homoeocarpa* from golf courses in Michigan could be differentiated using AFLP markers and VCGs.

Materials and Methods

Isolation and Culture. Isolates were collected from symptomatic plants infected with *S. homoeocarpa*. Three different locations in Michigan were sampled in July 2000 and 2001 (Fig. 1). Four fairways at each location were

selected for sampling from which symptomatic leaves of 50 infection centers along a transect running the length of the fairway were individually collected in paper coin envelopes. Two to three small segments of leaf tissue displaying lesions were placed on acidified water agar plates (10mL lactic acid/L) and allowed to grow for 2-3 days at 25°C. Hyphae growing out of the leaf tissue were then isolated onto potato dextrose agar (PDA) plates and allowed to grow for about 5 days at 25°C. Using a modified method of Boesewinkel (4), ten agar plugs were removed from the PDA plates using a sterile coffee stirrer and placed in 1.5 mL microfuge tubes containing 1 mL sterile H_2O for long term storage at room temperature. David Gilstrap provided additional isolates from 2 golf courses (Fig. 1) for the AFLP evaluation of the genetic diversity of *S. homoeocarpa*. They were stored in the same manner as the other isolates.

VCGs. All isolates were paired with six tester isolates representing the six known VCGs in Michigan (18) using a method modified from Kohn (15). Sets of four isolates were paired against all six tester isolates in 24 well culture plates. Each well contained 1 mL of PDA amended with 5 drops/L of McCormick's Red Food Coloring to highlight antagonistic zones. Each isolate was also paired with itself as a control. Isolates that were not classified in the first screening were then paired with all six tester isolates on 100 X 15 mm petri dishes to clarify the interactions between the isolate and tester strains. Chi-square (X^2) analyses were performed on the observed frequency distributions of the three most frequent VCGs (A, B, C) using the null hypothesis that there were

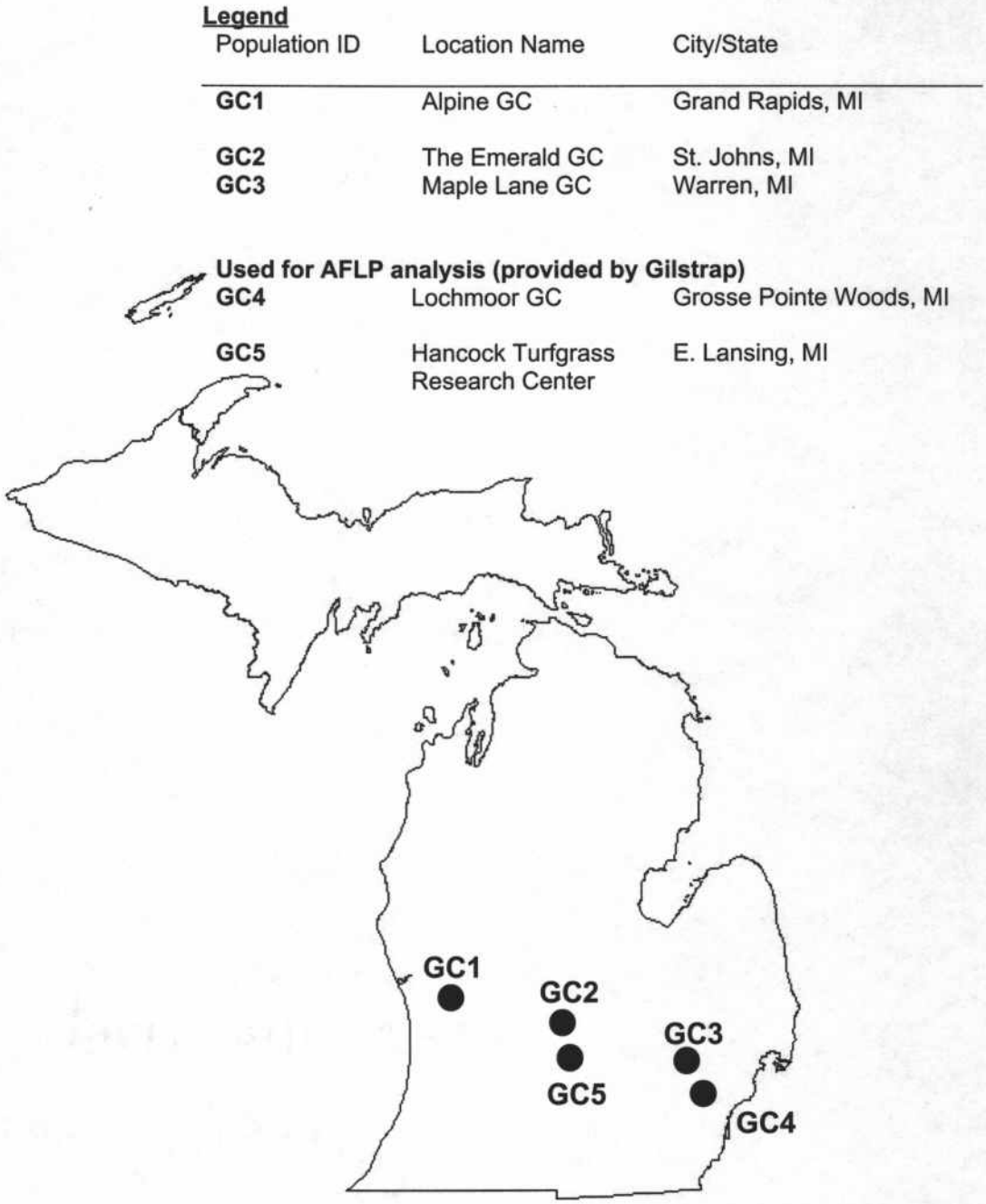

Figure 1. Map depicting geographical location of 5 *S. homoeocarpa* populations sampled for VCG and AFLP analysis. Legend indicates population ID, name of location, and city/state.

no differences in the frequency distributions of these VCGs between fairways within golf courses, within golf courses, or between years. Isolates provided by Gilstrap were also classified into VCGs using the same techniques described above for the other isolates. However, these isolates were not subjected to chi-square analysis due to a different sampling scheme.

DNA extraction and AFLP fingerprinting. A subset of isolates from the three populations sampled for this study and the additional isolates provided by Gilstrap were fingerprinted using the AFLP technique (Table 1). Isolates were grown in approximately 20 mL of potato dextrose broth in 100 x 15 mm petri dishes for seven days at 23 to 25°C. Mycelial mats were washed with distilled water and dried briefly under vacuum before being frozen to -20°C and lyophilized.

Lyophilized mats were ground with a sterile mortar and pestle. Whole genomic DNA from approximately 50 mg of ground mycelium was extracted using a QIAGEN Dneasy Plant Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's directions. DNA was quantified by comparing the intensity of illumination of a 1 uL drop on 1.5% agarose gels amended with ethidium bromide and viewed under UV light to known standards ranging from 10 to 250 ng/uL. Approximately 100 ng of DNA was then subjected to a restriction/ligation reaction, pre-selective amplification, and selective amplifications using the PCR core mix, adaptor sequences, core primer sequences and fluorescence labeled primers provided in the AFLP™ Microbial Fingerprinting Kit (Perkin-Elmer Corp.,

Isolate ID	Year Isolated	Population-VCG ID[a]
A14-8-00	2000	GC1-D
A16-1-01	2001	GC1-D
ML7-29-00	2000	GC3-D
ML8-46-00	2000	GC3-E
ML11-19-01	2001	GC3-D
ML12-40-00	2000	GC3-F
E17-14-01	2001	GC2-A
ML7-7-00	2000	GC3-C
A16-16-01	2001	GC1-E
1-7003-SH-R	1994	GC4-E
7039-SH-S	1998	GC5-E
1-7016-SH-R	1994	GC4-E
ML7-11-01	2001	GC3-A
1-7024-SH-R	1994	GC4-C
1-7018-SH-R	1994	GC4-C
1-7021-SH-R	1994	GC4-C
1-7008-SH-R	1994	GC4-C
1-7005-SH-R	1994	GC4-E
1-7013-SH-R	1994	GC4-E
1-7004-SH-R	1994	GC4-C
7041-SH-S	1998	GC5-C
A9-36-01	2001	GC1-B
7034-SH-S	1998	GC5-A
E4-3-01	2001	GC2-C
E4-1-00	2000	GC2-B
1-7015-SH-R	1994	GC4-E
A9-10-01	2001	GC1-C
ML7-2-01	2001	GC3-B
7043-SH-S	1998	GC5-B
7033-SH-S	1998	GC5-B
7040-SH-S	1998	GC5-B
7036-SH-S	1998	GC5-B

[a] Designation of isolate in Fig. 3 listed by population ID and VCG.

Table 1. Sampled isolates for AFLP fingerprinting. Isolates are listed in the order from top-bottom as they appear in Fig. 3 .

Foster City, CA) and performed exactly as described in the PE/ABI AFLP Microbial Fingerprinting protocol part# 402977 Rev A. All PCR reactions were performed using an MJ Research Minicycler (MJ Research Inc., Waltham, MA) in 0.2 mL tubes according to the cycling parameters outlined in the microbial fingerprinting protocol.

An initial optimization set of reactions was performed using pre-selective products from two randomly chosen isolates. Amplifications with the selective primers EcoRI-AA, AC, AG and AT were performed in all 16 combinations with the MseI-CA, CC, CG and CT selective primers. EcoRI selective primers were labeled at the 5' end with either carboxyfluorescein (FAM), carboxytetramethyrhodamine (TAMRA), or carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) fluorescent dyes. The fluorescent dyes were excited by laser radiation and visualized by their characteristic absorption-emission frequencies. Only the fragments containing an EcoRI restriction site were resolved.

Selective amplification AFLP products and a carboxy-X-rhodamine (ROX) size standard were loaded into each lane on a denaturing polyacrylamide gel and the fragments resolved in an ABI 3700 DNA Sequencer. Results were prepared for analysis in the form of electropherograms using GeneScan Analysis software (PE/ABI). AFLP fragments were scored manually as present = 1 or absent = 0 using Genotyper software (PE/ABI). Only DNA bands that consistently exhibited unambiguous presence/absence profiles were scored.

Using the program NTSYS-pc (Rohlf, F. J. 1993. NTSYS-pc - Numerical Taxonomy and Multivariate Analysis System, Version 2.02k. Applied Biostatistics Inc.), the combined 0/1 data matrix for isolates was used to construct a genetic similarity matrix of all possible pairwise comparisons of individuals using Jaccard's similarity coefficient: $GS(ij) = a/(a + b + c)$. $GS(ij)$ is the measure of genetic similarity between individuals *i* and *j*, where *a* is the number of polymorphic bands shared by *i* and *j*, *b* is the number of bands present in *i* and absent in *j*, and *c* is the number of bands present in *j* but absent in *i*. Trees were constructed using unweighted pair group with mathematical averaging (UPGMA) cluster analysis to provide a graphical representation of the relationships among isolates.

Results

Isolation and Culture. A total of 1200 samples of *S. homoeocarpa* were collected from three golf courses in Michigan. Of the 1200 samples, 889 isolates were placed in pure culture and stored. The collection efficiency (% success in obtaining an isolate from a sampled spot) was 74.1%. Isolates that were stored in H_2O at room temperature have been routinely recovered over the entire course of our study.

VCGs. 860 isolates were placed into one of the six VCGs (Table 2) described by Powell and Vargas (18). Isolates were scored as compatible when

			VCG Group							
Location	**Year**	**Fairway**	**A**	**B**	**C**	**D**	**E**	**F**	**OTHER**	**Grand Total**
Alpine	2000	9	1	21	25	0	0	0	1	48
		12	0	35	11	0	0	0	0	46
		14	6	26	11	1	0	0	0	44
		16	1	33	12	1	0	1	0	48
	2000 Total		*8*	*115*	*59*	*2*	*0*	*1*	*1*	*186*
	2001	9	14	12	8	0	2	0	0	36
		12	16	20	4	0	2	0	0	42
		14	24	19	0	0	1	0	0	44
		16	19	20	0	2	1	0	0	42
	2001 Total		*73*	*71*	*12*	*2*	*6*	*0*	*0*	*164*
Alpine Total			**81**	**186**	**71**	**4**	**6**	**1**	**1**	**350**
Emerald	2000	4	0	36	5	0	2	0	1	44
		5	0	36	10	0	0	0	0	46
		11	0	37	3	0	0	0	0	40
		17	0	32	13	0	0	0	1	46
	2000 Total		*0*	*141*	*31*	*0*	*2*	*0*	*2*	*176*
	2001	4	3	15	16	0	0	2	6	42
		5	2	33	5	0	2	0	2	44
		11	2	21	7	0	1	1	1	33
		17	4	22	11	0	4	0	0	41
	2001 Total		*11*	*91*	*39*	*0*	*7*	*3*	*9*	*160*
Emerald Total			**11**	**232**	**70**	**0**	**9**	**3**	**11**	**336**
Maple Lane	2000	7	1	11	28	1	7	0	0	48
		8	0	10	3	1	3	1	0	18
		11	0	16	11	1	5	0	0	33
		12	0	21	16	0	3	2	0	42
	2000 Total		*1*	*58*	*58*	*3*	*18*	*3*	*0*	*141*
	2001	7	3	6	0	1	0	0	0	10
		8	3	0	0	2	0	0	4	9
		11	7	5	0	1	0	2	11	26
		12	11	1	2	0	0	1	2	17
	2001 Total		*24*	*12*	*2*	*4*	*0*	*3*	*17*	*62*
Maple Lane Total			**25**	**70**	**60**	**7**	**18**	**6**	**17**	**203**
Grand Total			**117**	**488**	**201**	**11**	**33**	**10**	**29**	**889**

Table 2. Vegetative compatibility group (VCG) distributions of *Sclerotinia homoeocarpa* isolated in 2000 & 2001 from four fairways at each location.

there was no noticeable barrage zone between an isolate and a tester strain. Isolates were scored as incompatible when a barrage zone was formed between an isolate and a tester strain (Fig. 2). Twenty-nine isolates either did not fit in one of these six groups or the incompatibility reaction was indistinguishable, and were classified as "other" (Fig. 2). These isolates were ignored when isolates were sampled for the AFLP analysis and they were not subjected to chi-square analysis. Over the entire course of the study isolates from VCGs A, B, and C were found in all fairways of each golf course. VCGs D, E, and F were either absent completely or present at very low frequencies in each population. Of the three major VCGs, group B was present in the highest frequency over both years, followed by group C, and group A. Over all locations in 2000, group A was much less prevalent than in 2001. The reverse was true for group C where it was more frequent in 2000 than in 2001. Overall, fewer isolates were collected in 2001. Chi-square analysis showed there were significant differences in VCG frequency distributions between fairways within a golf course at the Maple Lane and Alpine locations (Table 3). The analysis also showed there were significant differences between locations and between years.

AFLP Genotyping. The EcoRI + AC/ MseI + CA primer combination resolved the greatest number of clear fragments of the selective primers tested and resulted in more than 80 clearly resolved AFLP fragments in each of the 32 isolates analyzed. In total, 100 AFLP fragments were resolved with 15 being

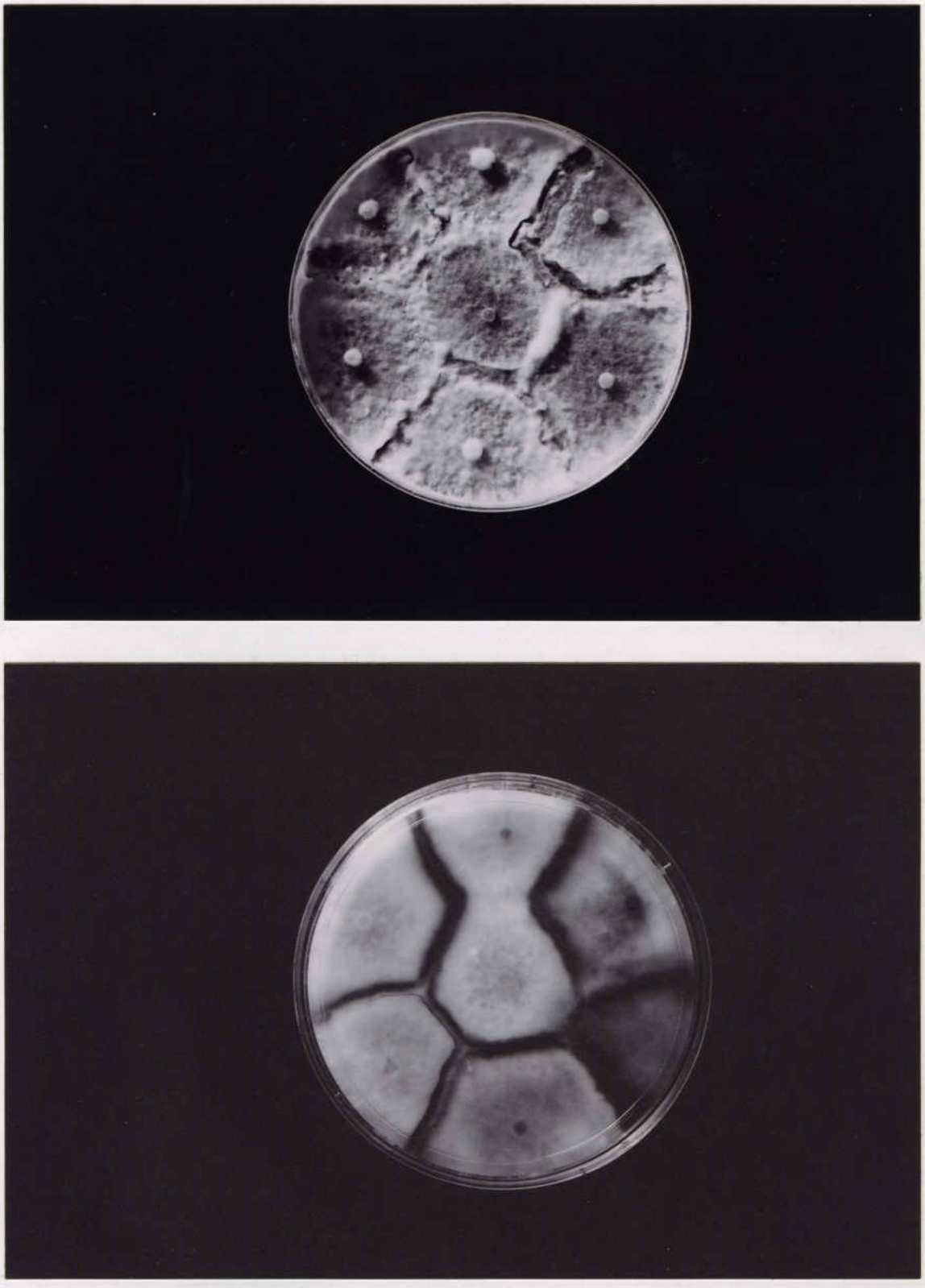

Figure 2. Images showing surface (top) and reverse (bottom) views of mycelial interactions of a *S. homoeocarpa* isolate (center of image) against tester isolates (surrounding center) in a petri dish.

Comparison	Chi-square	d.f.	P-value
Within Maple Lane GC fairways	14.29	6	.0266
Within Emerald GC fairways	8.99	6	.1741
Within Alpine GC fairways	31.73	6	<.0001
Between Locations	79.16	4	<.0001
Between 2000 and 2001	150.16	2	<.0001

Table 3. Results of chi-square analyses of VCG distributions of *Sclerotinia homoeocarpa* from 3 populations in Michigan.

present in some isolates and absent in others (polymorphic). Isolates were from 55 to 100% similar (Fig. 3). Isolates with identical AFLP profiles did not necessarily come from the same location or have the same VCG. Overall, isolates from the same location or with the same VCG were not more similar.

Discussion

Several Our evaluation of as many as 185 isolates from a single sampling at one location represents the largest sample of isolates of *S. homoeocarpa* ever examined for VCG diversity at a location. We found clear evidence that VCG distributions can vary within a golf course, among golf courses, and over time. These data adds to the findings of Powell and Vargas (18) who found that there were differences in the distribution of VCGs over time and location. Other studies have attempted to understand the structure of *S. homoeocarpa* populations (10, 18, 19, 22). It thus appears plausible that VCG distributions on each fairway within a golf course operate as independent populations, each with a unique distribution of VCGs.

One exception in this study was the Emerald location in St. John's, MI. Chi-square analysis for isolates within a fairway at this location revealed no significant differences in the VCG distributions between fairways. This golf course was completely redesigned and renovated in 1996 making it much younger than both Alpine and Maple Lane golf courses that are well established and have been in play for at least 25+ years. The predominance of the VCGs A, B, and C in the populations sampled are similar to the results found

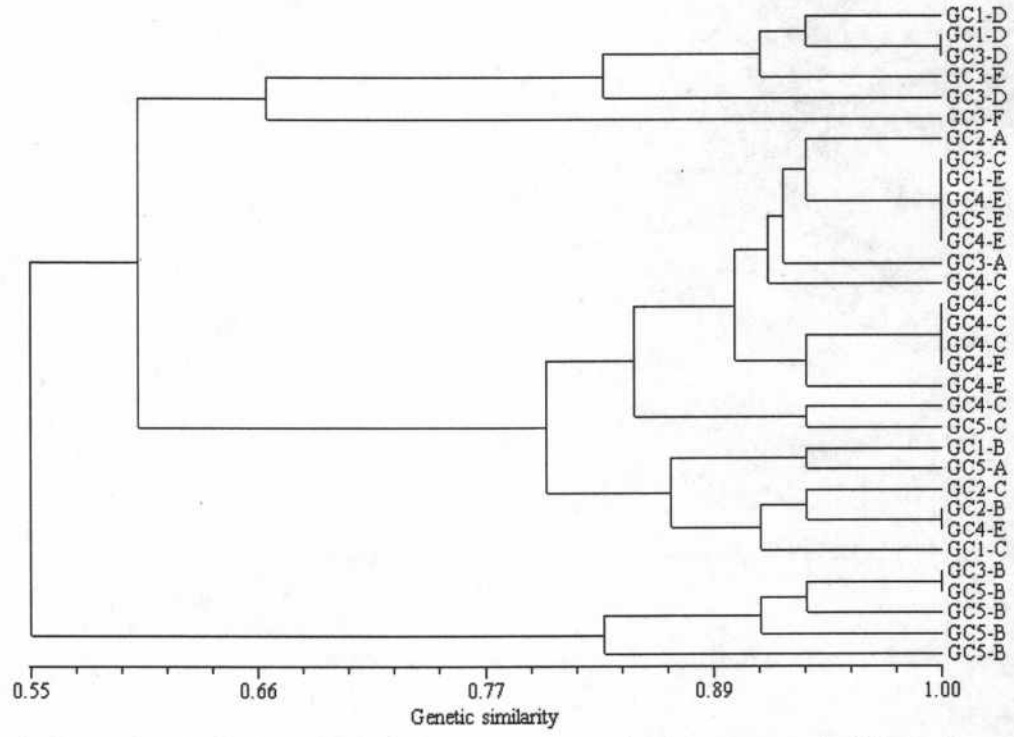

Figure 3. Genetic similarity of 32 *S. homoeocarpa* isolates sampled from five populations in Michigan based on 15 polymorphic AFLP markers. Populations are designated GC1-GC5 followed by the VCG.

for one of the fields sampled by Kohn *et al.*(15) for MCG diversity in *S. sclerotiorum* where they hypothesize that the relative lack of diversity in the field was indicative of the diversity that was initially introduced into the area or as a result of selection of strains from an initially diverse population. The evolutionary forces of drift and migration as well as the putative lack of sexual recombination in *S. homoeocarpa* can limit the number of VCGs found in a population (16, 18). Also, age of the golf courses, cultural practices, fungicide management regimes, and environmental conditions may all be potential factors in the development and distribution of VCGs of *S. homoeocarpa*.

Further research should focus on developing testable theoretical models that seek to explain the variation in VCG distribution that has been observed within sampling locations, between sampling locations, and over time. It would also be worthwhile to investigate the possibility of bias in the sampling scheme used by both this study and Powell and Vargas (18) that is based on selecting a single isolate from a few infected lesions that were cultured from a single dollar spot. An exhaustive sampling scheme that characterizes the presence of all strains of *S. homoeocarpa* growing in a single dollar spot would serve to close this question. Finally, examining the VCG diversity that is present in the less highly maintained areas of a golf course may also aid in our understanding of the factors responsible for the distribution of VCGs present in different populations.

The use of molecular markers for the study of fungal plant pathogen populations is well documented (2, 5, 10, 13, 14, 15, 17, 19). Recently, these techniques have been used with greater frequency for examinations of turfgrass

pathogens. RAPDs were used to examine genetic variation present in a collection of 26 isolates from the northeastern and midwestern areas of the U.S (19). Raina *et al.* found a very high level of genetic similarity between isolates regardless of location, indicating a strong clonal population structure. However, a limitation of their study was the small number of isolates from a single location, making inferences about population structure difficult. Hsiang and Mahuku (10) also used RAPDs to assess variation in *S. homoeocarpa* populations in Southern Ontario. They sampled populations of *S. homoeocarpa* more intensely than Raina *et al.* (19), collecting over 20 isolates per population. They found that 5 of the 8 populations exhibited significant linkage disequilibrium indicating a clonal population structure. The remaining 3 populations had linkage disequilibria consistent with a random mating system. In the populations that they studied, they did not perform any VCG comparisons to corroborate their results of random mating. This could have provided crucial information about the disease cycle of *Sclerotinia homoeocarpa*. Most of the genetic variation was found between populations and very little variation was found within populations. Corroborating the findings of Raina *et al.* (19), our results support a clonal population structure in the *S. homoeocarpa* populations sampled because of the low amount of genetic diversity present.

AFLP fingerprints were not able to resolve isolates based on VCG or geographic location. An isolate from Grand Rapids (Alpine GC) had the same AFLP fingerprint as an isolate from one of the Detroit locations (Maple Lane). Also, isolates from Maple Lane were present in all of the major branches of the

tree. This indicates a significant amount of the genetic variation observed in this study is present within a population. The lack of a pattern between AFLP genotypes and independent measures such as geographic location and VCG is interesting because these results point to a fairly recent introduction of the pathogen into Michigan. The construction and development of golf courses in Michigan is an activity that has taken place over the last century and so the introduction of the pathogen on golf course turf presumably would have occurred at some point over this period. Another possibility that could explain these data is regular migration between populations so that there is no differentiation of the populations. Migration seems to be a less likely scenario because of the large distances between the populations sampled and the lack of any evidence for a spore forming stage that could be aerially disseminated. One other possibility is that selection could be a factor involved in the lack of diversity present at the sampled populations. Kohn *et al.* (15) suggested that diversifying selection (26) was an important driver of diversity because it predicts that a mosaic of pathogen genotypes that are specialized for differing conditions are favored in the absence of other selective factors. Certainly the presence of diverse microclimates, different management practices, and cultivar selections on today's golf courses would provide a similar disturbed environment compared to the environments discussed by Kohn *et al.*(15). This type of selection would also fit well with the data generated by Powell and Vargas (18) who found that the VCGs at a location change over time and hypothesized that the change could be the result of environmental conditions.

Future research should use both molecular as well as VCG characters to test the hypotheses generated by this research. How does dollar spot first appear on a golf course? This question is important to understanding and elucidating the population structure of this pathogen. The question could be approached by monitoring a population over time on a newly established golf course using the techniques applied in this study. Also, research determining the mode and survival of overwintering inoculum of *S. homoeocarpa* would also help to shed light on the recalcitrant population structure of this pathogen.

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