Genetic Structure and Temporal Dynamics of a *Colletotrichum graminicola* Population in a Sorghum Disease Nursery

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ABSTRACT

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Restriction fragment length polymorphisms (RFLPs) were used to study the population genetics of *Colletotrichum graminicola* (= *C. sublineolum*), the causal agent of sorghum anthracnose. Screening of 80 anonymous probes from a genomic library detected polymorphisms in 81% of 299 probe-enzyme combinations among nine international isolates. Seven single- or low-copy probes were used to study a collection of 411 isolates

sampled during 1991 to 1993 from a sorghum disease nursery in Georgia. Nei's gene diversity was moderately high, with $H = 0.215$ on average, while genotypic diversity was extremely low with an average genotypic diversity value of $\bar{G} = 1.513$. Only nine multilocus haplotypes were identified, with one haplotype being present at a frequency of approximately 80% each year. Two other haplotypes were found at significant frequencies (4 to 10%). Allele an effect.

The causal agent of sorghum anthracnose, *Colletotrichum graminicola* (Ces.) G.W. Wils. (= *C. sublineola* Henn. in Kab. & Bubák), is generally regarded a "hypervariable pathogen" with respect to virulence (10,14,28). Previous work focused on variation in isolates from sorghum (*Sorghum bicolor* (L.) Moench), mainly using virulence on differential sorghum cultivars as a marker. Several isolates were pathotyped in greenhouse and field experiments (1,6,8,9,13, 15,28). Variation for virulence was observed in all studies, and these data were used to group isolates into different virulence phenotypes.

Despite these efforts, generalizations regarding the extent of variability within the species or on the degree of differentiation between populations have not been possible or should be considered theoretical at best (7), for several reasons. A standard set of differentials and a uniform rating scheme have not been agreed on, making it impossible to compare data sets. Also, the genetic basis of resistance toward *C. graminicola* in most of the differentials is unknown, and a gene-for-gene interaction between sorghum cultivars and fungal races has not been demonstrated. Additionally, since only six to eight differentials are commonly used, the entire spectrum of virulence genotypes is unlikely to be completely resolved in current virulence testing. These limitations make virulence an ambiguous marker to determine the extent of variability in *C. graminicola*.

To generate unbiased information on the degree of genetic variation within *C. graminicola* and to provide a more complete framework for consideration of virulence characteristics, selectively neutral markers were developed. Recent studies used random amplified polymorphic DNAs (RAPDs) as genetic markers (8,11,15,16). These studies demonstrated that variation within *C. graminicola* also was present at the molecular level. However, because of limitations in the use of RAPD data for population genetic analysis (19,22), we decided to develop restriction fragment length poly-

morphisms (RFLPs) as genetic markers, which have been useful to elucidate the population structure of several plant-pathogenic fungi (3,17,18,24,25). We also utilized a more intensive sampling methodology compared with previous studies addressing variability in *C. graminicola*. In the past, a macrogeographical sampling strategy was utilized, in which one or a few isolates from a location were collected and evaluated (1,6,8,9,13,15,28). We sampled a larger number of isolates to assess the genetic structure and population dynamics within this fungal species.

As part of an effort to obtain a global perspective on the population genetic structure of *C. graminicola* in different ecological settings (commercial fields, disease nurseries, and wild populations), the experimental sorghum anthracnose nursery near Griffin, GA, was chosen to establish a reference population. Previous studies of isolates collected from this site indicated that variation occurs for both virulence and RAPDs (4,8,15). This nursery is also of practical importance, because many seed companies test their breeding lines for resistance to anthracnose at this location. Knowledge of the population composition in the nursery will also enable us in the future to determine whether resistance breakdown, which has been observed previously in the nursery (5), is associated with corresponding changes in the genetic structure of the pathogen population.

The specific objectives of this study were to (i) develop robust and efficient genetic markers to measure genetic diversity in *C. graminicola*, (ii) examine the genetic structure of a field population, and (iii) assess the changes in allele and genotype frequencies over time.

MATERIALS AND METHODS

Fungal collections. For 3 consecutive years (1991 to 1993), diseased plant material was collected from the sorghum anthracnose nursery in Price County near Griffin, GA, at the end of the growing season (mid-October). Established in 1978, the nursery had been in the same field every year with only one exception, when it was planted in an adjacent field. Initially inoculated twice

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a year with local isolates derived from diseased material collected at random, the nursery has not been inoculated since 1985. Leaf samples in all years originated from the Texas A&M University part of the nursery, which covers one-third of the nursery (approximately 2,700 m²). In 900 single-row plots, the site contains several individual disease nurseries, in addition to experimental breeding material. An arbitrary sampling strategy was adopted because of the distribution of resistant and susceptible plant material within the nursery. From single plots, two diseased leaf pieces from different plants were collected, with the goal of establishing one single-spored isolate per leaf. For the inbred line SC326-6, a slightly different approach was taken. Because SC326-6 is a frequent parent in sorghum breeding programs, it is one of the few sorghum genotypes that was planted in several locations throughout the nursery each year. Two leaf samples from two different plants were collected in each plot planted with SC326-6, and an attempt was made to obtain two isolates per leaf, one from a sporulating midrib lesion and one from mostly hypersensitive lesions on the blade. In 1991, 84 leaf samples were collected from the nursery, in 1992, 106 leaf samples, and in 1993, 190 leaf samples. The collections are referred to as G91, G92, and G93, respectively.

Diseased plant material was stored in labeled envelopes until the isolation of fungal material. From each sample, several small symptomatic leaf pieces were rehydrated in distilled water for 10 min, surface-sterilized in 0.5% sodium hypochlorite for 3 to 5 min, and rinsed briefly in sterile distilled water before being placed onto water agar. After 4 to 7 days, sporulation occurred and conidial masses from several acervuli were dislodged with a needle and streaked onto petri dishes containing half-strength potato dextrose agar (PDA; 19.5 g of PDA per liter and 7.5 g of agar per liter), amended with 50 ppm of ampicillin. For germination to occur, plates were kept at room temperature for 5 to 7 h. From each plate, two germinated conidia were selected under a dissecting microscope and transferred to separate PDA plates. After 4 to 5 days, a mass of mycelium had formed, and one of the cultures was randomly selected for RFLP analysis. The isolation procedure was repeated for material that did not yield usable fungal material.

Fungal cultures were stored for short periods on PDA at 4°C. A small portion of the stock culture was transferred onto oatmeal agar and grown for a minimum of 3 days. The resulting mycelium was scraped off and transferred to a 250-ml flask containing 100 ml of sucrose-yeast-casein hydrolysate broth (10 g sucrose per liter and 6 g of each of yeast extract and casein hydrolysate per liter of distilled water). The cultures were grown for 4 days on a rotary shaker at 110 rpm, after which they were harvested by filtration through cheesecloth, rinsed with sterile distilled water, blotted dry, and stored at -70°C in 15-ml polypropylene tubes before lyophilization and DNA extraction.

The cultures on oatmeal agar were kept under fluorescent light until sporulation occurred. A 100- μ l spore suspension was prepared for each isolate and transferred to a 2-ml o-ringed cryogenic vial containing silica gel. Vials were kept at -20°C for long-term storage.

Development of RFLP probes. Two genomic DNA libraries from *C. graminicola* isolates originating from the sorghum disease nursery near Griffin, GA, were made using standard methodology (29). Dephosphorylation with calf intestinal alkaline phosphatase was omitted. To compensate, the amount of pUC18 was increased to 150 ng in the ligation reaction, and the ratio between fungal DNA and linearized pUC18 was adjusted to 10:1. Recombinant plasmids were used to transform *Escherichia coli* DH5 α MCR (Gibco BRL, Gaithersburg, MD). Plasmid preparations of resulting clones were obtained by the lysis-by-boiling method (29). Plasmid-bearing *E. coli* were kept in glycerol at -80°C for long-term storage (29).

The screening process to identify useful RFLP probes included nine sorghum isolates of *C. graminicola*, originating from College Station (Texas), Weslaco (Texas), Brazil, Kollo (Niger), Damazin (Sudan), Watsandi (Botswana), Golden Valley (Zambia), Mansa (Zambia), and Venezuela. The DNA of each isolate was digested

with six different restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, and *Xho*I; Promega Corp., Madison, WI), and three replicate blots were established. All probe-enzyme combinations were evaluated for RFLPs and for the number of sites to which the probes hybridized. Probes were defined as repetitive when 10 or more bands were visible and as single- or low-copy when less than 10 bands were seen. Clones eventually selected for generating RFLP data were cut with *Eco*RI and *Bam*HI in two separate digestions to determine insert sizes.

Generation of RFLP data. The DNA from each isolate was extracted using a modified version of the cetyltrimethylammonium bromide (CTAB) method described by McDonald and Martinez (23), whereby up to 56 DNA samples were prepared during one session. Lyophilized mycelium was pulverized with a glass rod in a 15-ml polypropylene tube before adding 5 ml of CTAB extraction buffer consisting of 700 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol (vol/vol), and 1% CTAB (wt/vol) in distilled, deionized water. Tubes were kept in a 65°C water bath for 60 to 90 min and agitated at least once. Chloroform/isoamyl alcohol (10 ml, 24:1, vol/vol) was added to each tube. The mixture was emulsified and spun at 12,900 $\times$ g in a refrigerated Beckman J2-21M centrifuge (Beckman Coulter, Fullerton, CA) for 25 min. The aqueous phase was transferred to another 15-ml tube, and the chloroform extraction step was repeated once. Isopropanol (5 ml) was then mixed into each tube, and the tubes were left at room temperature for 10 min before centrifugation at 10,400 $\times$ g for 6 min. The supernatant was poured off, and the tube was inverted and drained on paper towels before the pellet was resuspended in 2 ml of Tris-EDTA (TE; 10 mM Tris and 1 mM EDTA, pH 8.0). The DNA was precipitated by adding 0.5 volume of 7.5 M NH₄OAc and 2 volumes of 95% ethanol. Tubes were gently inverted 15 to 20 times and kept at room temperature for 10 min before being centrifuged at 10,400 $\times$ g for 6 min. The pellet was resuspended in 400 μ l of TE on a rotator overnight. The DNA solution was then pipetted into a 1.5-ml microfuge tube, and the DNA precipitated with ethanol as described above. Centrifugation was at approximately 11,000 $\times$ g in a microfuge for 10 min. The resulting pellet was resuspended in 50 to 200 μ l of TE, depending on pellet size. The DNA was allowed to go into solution at 4°C for several days before DNA concentrations were determined using a Hoefer TKO 100 fluorometer (Amersham Pharmacia Biotech, Uppsala, Sweden).

A total of 5 μ g of DNA for each isolate was digested overnight with the restriction enzyme *Hind*III. Test gels using 500 ng of DNA revealed partially digested DNA, which was further purified by button dialysis. Microfuge tubes (1.5-ml volume) were cut 1 cm below the rim and autoclaved. For each DNA sample, 2.5 cm of dialysis tubing (Spectra/Por *2 dialysis membrane tubing, molecular weight cut-off: 12,000 to 14,000; Spectrum Medical Industries, Los Angeles) was added to boiling 0.1 mM EDTA (pH 8) for 10 min. The tubing was allowed to cool to room temperature while soaking in the EDTA solution, and thereafter rinsed in sterile, deionized water by slowly stirring for 4 h in a beaker at room temperature. The dialysis tubing was slit open, and up to 150 μ l of a DNA solution was added to the cap of the microfuge tube. The DNA solution was then covered with the membrane and the top closed to secure the membrane. The assembly was floated with the membrane down in a beaker containing 1 $\times$ TE and stirred slowly overnight at 4°C. The membranes were slit open, and the dialyzed DNA solution was pipetted into a microfuge tube.

DNA samples that digested to completion were loaded onto 0.8% agarose gels together with two outer lanes each containing 500 ng of lambda-*Hind*III size marker. The digested DNA was size-separated at 50 V for approximately 20 h, using 1 $\times$ Tris-borate-EDTA (TBE) as running buffer. After staining with ethidium bromide, the gel was photographed and blotted. Alkaline blotting using Zeta-Probe GT nylon membrane (Bio-Rad Laboratories, Hercules, CA) was done according to the manufacturer's recommendations.

Membranes were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), surface-dried, and baked at 80°C for 1 h. Between 1 and 12 blots were rolled together and placed into a hybridization tube. A total of 65 ml of a prehybridization solution was added to the membranes. The prehybridization solution was the same as the hybridization solution and consisted of 0.25 M disodium phosphate and 7% sodium dodecyl sulfate (SDS), adjusted to pH 7.2. The blots were prehybridized in an hybridization oven at 60°C for 1 to 3 h. Probes used for RFLP analysis were hybridized to the DNA of all available isolates. The probes were labeled with (³²P) dCTP (NEN Life Science Products, Boston, MA) using a nick-translation kit (Gibco BRL). Hybridization occurred overnight at 60°C. Membranes were washed twice for 1.5 h each at 60°C with the first wash solution (20 mM Na₂HPO₄ and 5% SDS, adjusted to pH 7.2). Two further wash cycles of the same duration were performed in 20 mM disodium phosphate and 1% SDS, adjusted to pH 7.2. Single membranes were wrapped in plastic wrap and exposed to X-ray film (Kodak X-OMAT AR; Kodak, Rochester, NY) at -80°C using an intensifying screen. On average, the film was exposed for approximately 4 days, but in some cases had to be continued for up to 2 weeks. The X-ray films were developed using an automated film developer.

Probes were stripped from the membranes by placing up to 12 blots in a plastic container containing 1 liter of a boiling stripping solution (0.1× SSC and 0.5% SDS). The blots were boiled in a microwave oven for 30 min. The nylon membranes were surface-dried on paper towels and probed again immediately; or, if this was delayed, membranes were wrapped in plastic wrap and stored at room temperature.

After all single- or low-copy data were analyzed, a final blot was prepared containing several members (if applicable) of all identified haplotypes to confirm and measure allele sizes. This blot was also hybridized to the repetitive probe pSCg7 to identify clonal lineages.

RFLP data analysis. In general, restriction size variants detected by each probe were treated as alleles at a single RFLP locus and assigned numbers. Alleles at different loci were analyzed individually and then combined to form multilocus haplotypes. Gene diversity of single loci was determined using Nei's gene diversity (26):

$$h = 1 - \sum_{i=1}^k x_i^2$$

in which x_i refers to the frequency of the i th allele and k to the number of alleles at a single locus. Gene diversity (h) was calculated for each locus and across all years. The average gene diversity (H) (27) across all loci was also determined for each year and for the combined 3-year data:

$$H = \sum_{l=1}^r h_l / r$$

in which l refers to the l th locus and r to the number of loci under investigation.

Different multilocus haplotypes were assumed to represent different genotypes. Genotypic diversity ($\hat{G}$) and its variance were quantified according to Stoddart and Taylor (30):

$$\hat{G} = 1 / \sum_{i=1}^k \hat{p}_i^2$$

$$\text{Var}(\hat{G}) \cong \frac{4}{N} \hat{G}^2 \left(\hat{G}^2 \sum_{i=1}^k \hat{p}_i^3 - 1 \right)$$

in which p_i is the frequency of the i th genotype, k is the number of distinct genotypes in the population, and N is the sample size. Ninety-five percent confidence intervals for $\hat{G}$ were determined by

$$\hat{G} \pm 1.96 \sqrt{\text{Var}(\hat{G})}$$

Significance testing for differences in allele and haplotype frequencies over the 3 years was performed according to a chi-square test developed by Workman and Niswander (31):

$$\chi^2 = N \sum_{j=1}^k \left(\sigma_{pj}^2 / \bar{p}_j \right)$$

For k alleles at a single locus or k haplotypes, p_i and σ_{pj}^2 denote the mean and variance of the frequency of the j th allele or haplotype, respectively. The genic contingency χ^2 is a function of the total sample size N and the means and the variances of the allele or haplotype frequencies.

RESULTS

Screening for useful RFLP probes. Two total genomic libraries of two sorghum isolates of *C. graminicola* yielded 181 anonymous random clones. Three replicate blots were established, and 54 probes were evaluated using six restriction enzymes. DNA digested with *Bam*HI, *Hind*III, or *Pst*I hybridized to probes at more discernable size ranges and displayed polymorphisms in larger numbers than was the case with DNA digested with *Xho*I, *Kpn*I, or *Eco*RI. For a second set of screening blots, the DNA of each isolate was therefore digested only with *Bam*HI, *Hind*III, and *Pst*I.

Only 80 of the 93 clones tested could be evaluated. The remainder either did not show detectable hybridization or hybridized too weakly for evaluation. Among these 80 probes, 299 probe-enzyme combinations could be scored, with 241 (81%) probe-enzyme combinations showing at least one polymorphism among the nine isolates. However, only a small percentage of screened probes was considered suitable for RFLP analysis. Probes were classified as potentially useful if they hybridized to one or a few DNA fragments in size ranges where polymorphisms were easily differentiated (0.5 to 14 kb). Preference was also given to probes that displayed three or more polymorphisms among the nine international isolates. DNA cut with *Hind*III and *Pst*I most frequently generated RFLPs with these characteristics. Before making a final decision to use a single enzyme, an additional set of blots was prepared to evaluate potential probes for their ability to differentiate among *C. graminicola* isolates within populations. The DNA of nine isolates from each of three different populations, one collected in Georgia from sorghum (G91) and two collected in Texas from johnsongrass, was hybridized to the most promising probes. The final decision to use *Hind*III to analyze all isolates of *C. graminicola* was based on characteristics mentioned above.

Even though 21% of all evaluated probes of *C. graminicola* were classified as repetitive, a probe suitable for DNA fingerprinting was difficult to identify. Most repetitive probes either hybridized to too many loci, resulting in a smear, or did not display enough polymorphisms to qualify them as fingerprinting probes. Some repetitive probes did not hybridize at all or showed a reduced number of bands in some of the isolates used in the screening pro-

TABLE 1. Sizes of anonymous *Colletotrichum graminicola* fragments used to generate restriction fragment length polymorphism data

pSCg ^a	No. of inserts ^a	Insert size ^a	Inferred no. of loci ^b
1	1	0.3	1
3	1	7.5	1
7	1	1.5	Multiple
10	1	3.2	1
17	1	6.5	1
23	1	4.5	2
28	1	1.7	2
31	1	3.2	2

^a Insert sizes in kilobases (kb) and numbers were determined by digesting plasmid clones (pSCg) with *Hind*III and separately with *Eco*RI; a 1-kb ladder was used to approximate sizes.

^b Number of loci inferred from the minimum number of bands observed on autoradiograms for any of the *Bam*HI-digested DNA of nine international isolates used during the screening process.

cess. Isolates that did not show a complex hybridization pattern for some of the repetitive probes originated from Golden Valley (Zambia), Watsandi (Botswana), and Kollo (Niger).

Seven single- or low-copy probes were used in conjunction with the restriction enzyme *Hind*III to generate RFLP data for all isolates of *C. graminicola*. In addition, one repetitive probe (pSCg7) was useful to confirm the genetic identity of isolates grouped into the various haplotypes and to discern clonal lineages. The eight probes were digested with *Hind*III and separately with *Eco*RI to determine insert sizes and numbers using a 1-kb ladder to approximate sizes (Table 1).

The composition and temporal dynamics of a *C. graminicola* population. Plant samples mainly consisted of green leaf material with sporulating lesions, but some samples were also included that only contained hypersensitive lesions or were necrotic. All samples went through the same process of isolation, culturing, DNA extraction, alkaline blotting, and probing. Some of the initial material was

TABLE 2. Sizes of hybridizing DNA fragments representing specific alleles present at individual restriction fragment length polymorphism (RFLP) loci in a *Colletotrichum graminicola* population from Griffin, GA

RFLP locus	Allele ^a	Size (bp)
pSCg1	1	6,400
	2	6,300
	3	6,500
pSCg3	1	8,100/6,000/2,700
	2	6,700/6,600/2,700
	3	9,600/6,000/2,700
	4	7,700/3,700/2,700
pSCg10	1	7,000
	2	5,200
pSCg17	1	9,100/2,900
	2	10,800/2,900
	3	8,100/3,400
pSCg23	1	4,800/3,200
	2	Null
	3	4,800/2,600
	4	7,000/4,800
pSCg28	1	3,800/1,700
	2	5,100/3,800
pSCg31	1	8,700/4,700
	2	10,600/4,700

^a All alleles are based on the digestion of DNA with *Hind*III.

TABLE 3. Allele frequencies and gene diversities (*h*) for seven restriction fragment length polymorphism loci in a *Colletotrichum graminicola* population from a disease nursery near Griffin, GA, from 1991 to 1993

pSCg	Allele ^a	G91 (N = 105) ^b		G92 (N = 98)		G93 (N = 208)		χ^2	G91 to G93 (N = 411)	
		Frequency	<i>h</i>	Frequency	<i>h</i>	Frequency	<i>h</i>		Frequency	<i>h</i>
1	1	0.848		0.786		0.793			0.805	
	2	0.143		0.214		0.188			0.182	
	3	0.010	0.260	0.000	0.336	0.019	0.335	4.89 (2) ^c	0.012	0.319
3	1	0.848		0.786		0.793			0.805	
	2	0.114		0.112		0.101			0.107	
	3	0.038		0.102		0.101			0.085	
	4	0.000	0.266	0.000	0.359	0.005	0.351	6.47 (3)	0.002	0.333
10	1	0.848		0.786		0.793			0.805	
	2	0.152	0.258	0.214	0.336	0.207	0.328	2.05 (1)	0.195	0.314
17	1	0.990		0.990		0.986			0.988	
	2	0.010		0.010		0.010			0.010	
23	3	0.000	0.020	0.000	0.020	0.005	0.028	1.37 (2)	0.002	0.024
	1	0.886		0.878		0.899			0.891	
	2	0.076		0.112		0.063			0.078	
	3	0.029		0.000		0.024			0.019	
28	4	0.010	0.208	0.010	0.216	0.014	0.187	6.00 (3)	0.012	0.200
	1	0.848		0.786		0.793			0.805	
	2	0.152	0.258	0.214	0.336	0.207	0.328	2.05 (1)	0.195	0.314
	3	1.000		1.000		0.995			0.998	
31	1	1.000		1.000		0.995			0.998	
	2	0.000		0.000		0.005	0.010	1.37 (1)	0.002	0.004
<i>H</i>			0.181		0.229		0.224			0.215

^a All alleles are based on digestion of DNA with *Hind*III.

^b N = sample size. Average gene diversities across all loci (*H*) and chi-square tests for differences in allele frequencies are also presented.

^c Values in parentheses are the degrees of freedom.

lost, mainly during the isolation procedure. Necrotic plant samples often did not yield viable cultures despite repeated attempts to isolate fungal material. *C. graminicola* apparently does not survive well in necrotic plant tissues, which may indicate limited saprophytic capability. On the other hand, *C. graminicola* was easily isolated from hypersensitive lesions. This observation is consistent with experiments conducted in the *C. lindemuthianum*-bean system, in which the fungus evidently was able to survive within plant cells undergoing the hypersensitive response (2).

Using the seven single- or low-copy probes, the average number of alleles for G91 and G92 was determined to be 2.43 and 2.29, respectively. The G93 sample displayed a slightly increased average number of alleles with a value of 2.57. The characteristics of all

Fig. 1. Examples of restriction fragment length polymorphisms (RFLPs) for single- or low-copy probes among *Colletotrichum graminicola* isolates from the sorghum anthracnose nursery near Griffin, GA. DNA of isolates was digested with *Hind*III. **A**, RFLPs using pSCg31. **B**, RFLPs using pSCg23 for the same isolates as in **A**. Note the deletion in one of the isolates at the locus homologous to plasmid probe pSCg23. Size standards (λ -*Hind*III) are given in kilobases to the left of both autoradiograms.

identified alleles are presented in Table 2, and examples of observed RFLPs are shown in Figure 1.

Nei's gene diversity (*h*) was calculated for all loci and for all 3 years (Table 3). Average gene diversity (*H*) ranged from 0.181 to 0.229 for the 3 years. When data were combined for the 3 years, *H* was 0.215. Gene diversities were greatly influenced by the predominance of allele "1" at all loci examined, which was always found at frequencies ≥ 0.8 . Alternative alleles were therefore relatively rare.

As expected from observed allele frequencies and the clonal nature of the pathogen, the most frequent multilocus haplotype carried the most frequent allele at all loci and was of haplotype "111111" ("A") (Table 4). This haplotype dominated the population of *C. graminicola* in Georgia and was found at frequencies nearing 80% for all 3 years. A total of nine multilocus haplotypes could be discerned using the seven single- or low-copy probes. Genetic homogeneity of isolates within each multilocus haplotype was independently confirmed using the repetitive probe pSCg7 (Fig. 2). In addition to haplotype "A", three other haplotypes were identified in all 3 years: "222121" ("B") and "232121" ("C"), with frequencies between 4 and 10%; and "222421" ("F"). Haplotype "F" was recovered in all 3 years at a low frequency (approximately 1%). The six rare haplotypes ("D" to "I") represented only 3.65% of the entire collection (15 isolates out of 411). Consequently, genotypic diversity was very low (Table 5). In all 3 years, genotypic diversities barely reached values higher than the minimum value of 1. Confidence intervals for the genotypic diversity values overlapped for all 3 years and for the combined data.

Chi-square analysis was used to compare allele and haplotype frequencies over the 3-year period. The population of *C. graminicola*

was stable with regard to allele frequencies. None of the chi-square values were significant for any of the seven loci (Table 3). The same test was performed for haplotype frequencies whereby data was pooled for the six rare haplotypes. This test also was not significant ($\chi^2 = 7.034$, *df* = 3). Therefore, data sets from the 3 years were pooled into a single data set for further analysis.

Multiple samples were obtained from the cultivar SC326-6, which was planted in several locations throughout the nursery. In a subsequent chi-square test, haplotype data was arranged according to whether samples were collected from SC326-6 or from other cultivars. All isolates originating from SC326-6 belonged to haplotypes "A", "B", or "C" (Table 6). The chi-square value, significant at the 1% level, indicated that composition of the population on this cultivar was different from that of the general population in the nursery.

DISCUSSION

C. graminicola is highly variable for pathogenicity traits (10,14, 28). The screening for RFLP variation indicated a high level of variation at the DNA level also. At least one polymorphism was present for 81% of probe-enzyme combinations among the nine international isolates. This variability was similar to that found by McDonald and Martinez (23), who found RFLPs for 74% of probe-enzyme combinations among six international isolates of the wheat pathogen *Mycosphaerella graminicola*. Though the total number of isolates considered was small, this result suggests that the overall amount of genetic variation within a fungal species does not depend on its reproductive strategy, because

TABLE 4. Frequencies of multilocus haplotypes of *Colletotrichum graminicola* from a disease nursery near Griffin, GA, collected from 1991 to 1993

Multilocus haplotype ^a	G91 (N = 105) ^b	G92 (N = 98)	G93 (N = 208)	G91 to G93 (N = 411)
A 1 1 1 1 1 1 1	0.848	0.796	0.793	0.805
B 2 2 2 1 2 2 1	0.076	0.102	0.063	0.075
C 2 3 2 1 1 2 1	0.038	0.051	0.101	0.083
D 2 2 2 1 3 2 1	0.019	nf ^c	0.010	0.010
E 3 2 2 1 3 2 1	0.010	nf	0.010	0.010
F 2 2 2 2 4 2 1	0.010	0.010	0.010	0.010
G 2 3 2 1 2 2 1	nf	0.010	nf	0.002
H 2 4 2 1 1 2 2	nf	nf	0.005	0.002
I 3 2 2 3 4 2 1	nf	nf	0.005	0.002

^a Multilocus haplotypes were constructed using restriction fragment length polymorphism alleles presented in the following order: pSCg 1, 3, 10, 17, 23, 28, and 31.
^b N = sample size.
^c nf = not found

TABLE 5. Genotypic diversities (*G*), variances (*Var*), and 95% confidence intervals of a *Colletotrichum graminicola* population from Griffin, GA, sampled from 1991 to 1993

Population	<i>G</i>	<i>Var</i> (<i>G</i>)	95% confidence intervals
G91	1.376	0.0112	1.169 to 1.583
G92	1.570	0.0248	1.261 to 1.879
G93	1.554	0.00963	1.362 to 1.746
G91 to G93	1.513	0.00437	1.383 to 1.643

TABLE 6. Frequency and chi-square analysis of multilocus haplotype data of a *Colletotrichum graminicola* population from Griffin, GA, arranged according to sorghum cultivar sampled (SC326-6 versus other cultivars)

Multilocus haplotype	SC326-6 (N = 140) ^a		Other cultivars (N = 271)		χ^2
	No. of isolates	Frequency	No. of isolates	Frequency	
A	116	0.829	215	0.793	15.232** (3) ^b
B	8	0.057	23	0.085	
C	16	0.114	18	0.066	
D to I	0	0.000	15	0.055	

^a N = sample size.
^b ** = *P* < 0.01. The number in parentheses is the degrees of freedom.

Fig. 2. Hybridization patterns using the repetitive probe pSCg7 of multilocus haplotypes of *Colletotrichum graminicola* from the sorghum anthracnose nursery near Griffin, GA. The first lane is a λ -HindIII standard and the size fragments are indicated in kilobases to the left.

the RFLP gene diversity was similar for the asexual fungus *C. graminicola* and the sexual fungus *M. graminicola*. To further illustrate this point, a similar screening process with the oak wilt pathogen *Ceratocystis fagacearum*, which depends on the sexual stage for dispersal, found that only 8% of 437 probe-enzyme combinations detected RFLPs among nine isolates representing its known geographical range (18).

Prior assays for virulence and RAPD variation among *C. graminicola* isolates from the sorghum disease nursery in Georgia (8,11,15) suggested that this population of *C. graminicola* was variable. The extent of variability was measured more precisely in this study. All RFLP loci examined were polymorphic, whereby the number of alleles detected increased with sample size. The probability of detecting rare alleles was higher in the third year, because the sample size was twice as large. The effect of sample size on allele detection is given by the equation $N = \log(1 - P)/\log(1 - F)$, in which N represents the sample size, F the frequency of an allele, and P the probability of detecting alleles of frequency F (24). Setting P at 0.95, this translates into detecting alleles at frequencies of 0.028 and 0.030 at $N = 98$ (G92) and 105 (G91), respectively. The G93 sample with $N = 208$ could detect alleles present at a frequency of 0.014 in the population. Though the average number of alleles increased, Nei's gene diversity did not increase for the larger sample sizes (G93 and combined data), indicating that H was insensitive to the range of sample sizes used in this study.

In general, gene diversities were moderately high and comparable to those of other plant pathogens (20). Direct comparisons with other organisms are not possible, because gene diversity estimates will vary within and among species depending on the type of marker used and the scale of sampling (20). Also, to obtain an unbiased estimate of gene diversity (H), a large number of loci representing a random sample of the genome should be examined (27). This study only considered loci that were polymorphic in the international group of isolates originally screened. Therefore, the results presented here overestimate the actual values of H . Some skewing may also have occurred due to the possibility that some probes may hybridize to more than one locus (Table 1).

In this study, a total of nine haplotypes were differentiated using seven single- or low-copy probes. In addition, the repetitive probe pSCg7 was used to confirm the genetic homogeneity of representatives of the nine haplotypes. Although the banding patterns confirmed the genetic homogeneity within haplotypes, we have so far not succeeded in adequate resolution of the complex hybridization pattern to allow a quantitative treatment of these data. Nevertheless, together with the multilocus haplotype data, the presence of distinct clones or clonal lineages could be validated. Haplotypes "B", "D", "E", and "F" share most alleles at the RFLP loci and also show very similar hybridization patterns with pSCg7. Haplotypes "C" and "G" may represent a separate clonal lineage, because the banding pattern with pSCg7 was found to be very different from the former group. Also, haplotypes "A", "H", and "I" were found to be separate clones, each exhibiting both unique multilocus haplotypes and unique hybridization patterns with pSCg7.

Genotypic diversity was extremely low for this organism. Only nine haplotypes were found among 411 isolates over a 3-year period. Low genotypic diversities are not unusual for an organism that reproduces asexually, but the low genotypic diversity values reported in this study resulted also from the predominance of haplotype "A", which was isolated at frequencies ≥ 0.80 in all 3 years. Initially, the intention was to adjust genotypic diversity data for sample size (genotypic diversity divided by sample size) in order to better compare data across years. This method was previously applied for haplotype data of *M. graminicola*, whose population structure is dominated by sexual reproduction (12). Adjusting for sample size revealed that only 1.3% of the maximum value of G was achieved in 1991. For 1992 and 1993, this value was 1.6 and 0.8%, respectively. Combining the data for all 3 years dropped this value even further, to 0.4%. Adjusting genotypic diversity for

sample size cannot be considered a valid population descriptor if the population is clonal (J. Taylor, Texas A&M University, and J. Harrison, University of Florida, *personal communication*). Instead, confidence intervals were developed based on $\hat{G}$ and its standard error. Though this value should not be used to compare genotypic diversity values for independent populations, it is appropriate for testing whether genotypic diversity takes on specific values within a population (J. Taylor, *personal communication*). Confidence intervals overlapped for all 3 years and also for the combined data across years, indicating the stability of this population over time.

Contingency chi-square tests (30) showed that differences in allele and haplotype frequencies over the 3 years were statistically nonsignificant, again indicating that the population of *C. graminicola* at this site was stable. This suggests that genetic drift did not have a significant impact on the population structure over these 3 years. This is probably due to agronomic practices that were implemented to maintain a large population: delayed planting was practiced to maximize environmental conditions conducive for anthracnose development, a tall border was installed using a silage hybrid to enhance humidity and to lengthen dew periods, highly susceptible cultivars (Martin, SC599) served as spreader rows, and sprinkler irrigation was applied from anthesis to grain maturity during late afternoon two to three times per week. Between growing seasons, no-tillage was practiced and plant debris was maintained on the soil surface for optimum survival.

Stable populations, which are not influenced by genetic drift, significant immigration, or major selection processes, lend themselves to the study of more subtle population genetic processes (e.g., long-term changes in haplotype composition or the evolution of clonal lineages). They also give us the chance to study the effect of differential host selection.

In this study, we sampled diseased plant material from the partially resistant SC326-6, which is used frequently as a parent in anthracnose resistance breeding programs. SC326-6 has lost part of its resistance in some areas (including Griffin) during the last 10 years. Anthracnose is now commonly observed on this cultivar in the form of sporulating midrib lesions. In general, the leaf blade still maintains a hypersensitive reaction, with small sporulating lesions detected on some leaves. RFLP data were collected from 140 isolates originating from this cultivar. When haplotype data were pooled for all 3 years and organized according to whether they were originally derived from SC326-6 or from other cultivars in the nursery, it became obvious that only haplotypes "A", "B", and "C" were isolated from SC326-6. Subsequent chi-square analysis was performed to test whether particular fungal haplotypes were present at a higher-than-expected frequency on SC326-6 host genotypes. Significant departures from expected frequencies were found at the 1% level, indicating that differential host selection could occur, though this result must be interpreted with caution. Removing the pooled group (haplotypes "D" to "I") from the analysis rendered the chi-square value nonsignificant ($\chi^2 = 3.56$, $df = 2$), indicating that the frequency of the common haplotypes ("A", "B", and "C") was similar on SC326-6 and on other cultivars. This shows that significance was due to the absence of rare haplotypes on SC326-6 (the pooled group). Even though pooling of rare occurrences is standard procedure in chi-square analysis, the interpretation of this result is ambiguous, because the rare haplotypes in the pooled group represent independent events. Larger sample sizes will be needed to verify that indeed differential host selection occurs.

Selection processes, other than for specific interaction with certain host cultivars, may also take place in this population, as evidenced by the large predominance of haplotype "A". This haplotype may have a substantial fitness advantage over other haplotypes, the nature of which should be further examined.

We have developed robust and efficient genetic markers useful to studying the population structure of *C. graminicola*. To learn more about the dynamics within this fungal species, the necessary next step is to study how the genetic structure of the population

relates to the prevalence and distribution of selected markers, especially virulence characteristics. Future research strategies should also include what has been learned from *Magnaporthe grisea*, the rice blast pathogen. Similarities between *Magnaporthe grisea* and *C. graminicola* have been noted before (11). Both are considered to be hypervariable pathogens of grasses, with asexual reproduction dominant in host populations. Lineages have been identified in *Magnaporthe grisea*, and the data generated here suggests that discrete clonal lineages may also be present in *C. graminicola*. In detailed studies of virulence characteristics of specific clonal lineages of *Magnaporthe grisea*, each lineage comprised diverse virulence characteristics and pathotypes, suggesting that the mutation rate for specific virulence genes was greater than for RFLP markers (21,32). Pathotypes were assumed to evolve under limits defined by the genetic background of the lineage (11,21), resulting in hierarchical lineage-cultivar-pathotype associations (11). Different lineages of *Magnaporthe grisea* were also observed to have different cultivar ranges. The most frequent lineage in Santa Rosa, which constituted 50% of the population, also possessed the broadest cultivar range. If *C. graminicola* follows similar evolutionary principles, haplotypes of *C. graminicola* with diverse virulence characteristics may be identified, and the more-frequent haplotypes ("A", "B", and "C") may have broader ranges than the less-frequent haplotypes. The observation of nonoccurrence of the rare haplotypes on SC326-6 supports this hypothesis.

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