Contrasting Genetic Structure Between *Magnaporthe grisea* Populations Associated with the Golf Course Turfgrasses *Lolium perenne* (Perennial Ryegrass) and *Pennisetum clandestinum* (Kikuyugrass)

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Accepted for publication 21 August 2010.

ABSTRACT

Douhan, G. W., de la Cerda, K. A., Huryn, K. L., Greer, C. A., and Wong, F. P. 2011. Contrasting genetic structure between *Magnaporthe grisea* populations associated with the golf course turfgrasses *Lolium perenne* (perennial ryegrass) and *Pennisetum clandestinum* (kikuyugrass). *Phytopathology* 101:85-91.

Gray leaf spot (GLS) disease of perennial ryegrass (*Lolium perenne*) and kikuyugrass (*Pennisetum clandestinum*) in golf courses in California was first noted in 2001 and 2003, respectively, and within 5 years had become well established. The causal agent of the disease is the fungus *Magnaporthe grisea*, which is known to consist primarily of clonal lineages that are highly host specific. Therefore, our objective was to investigate host specificity and population dynamics among isolates associated primarily from perennial ryegrass and kikuyugrass since the disease emerged at similar times in California. We also obtained isolates from additional hosts (tall fescue, St. Augustinegrass, weeping lovegrass, and rice) and from the eastern United States for comparative purposes. A total of 38 polymorphic amplified fragment length polymorphism makers were scored from 450 isolates which clustered by host with high

bootstrap support (71 to 100%). Genetic structure between kikuyugrass and perennial ryegrass isolates differed significantly. Isolates from kikuyugrass were genotypically diverse ($n = 34$), possessed both mating types, and some tests for random mating could not be rejected, whereas isolates from perennial ryegrass were less genotypically diverse ($n = 10$) and only consisted of a single mating type. Low genotypic diversity was also found among the other host specific isolates which also only consisted of a single mating type. This is the first study to document evidence for the potential of sexual reproduction to occur in *M. grisea* isolates not associated with rice (*Oryza sativa*). Moreover, given the significant host specificity and contrasting genetic structures between turfgrass-associated isolates, the recent emergence of GLS on various grass hosts in California suggests that potential cultural practices or environmental changes have become conducive for the disease and that the primary inoculum may have already been present in the state, despite the fact that two genotypes associated with perennial ryegrass and St. Augustinegrass in California were the same as isolates collected from the eastern United States.

Gray leaf spot (GLS) is a major fungal disease of turfgrass that appeared in epidemic proportions in 2003 in California on both perennial ryegrass (*Lolium perenne*) and kikuyugrass (*Pennisetum clandestinum*). The disease is caused by the fungus *Magnaporthe grisea* (anamorph = *Pyricularia grisea*) but has also been suggested to constitute a new species (*M. oryzae*) based on molecular data (7). However, no formal nomenclatural name change has occurred; therefore, *M. grisea* will be used throughout the text. GLS is a devastating disease that can kill turf in as little as 3 to 7 days at temperatures of 26 to 29°C (33). Although GLS can infect numerous turf species in the urban environment, the impact is most severe on golf courses that utilize perennial ryegrass and kikuyugrass.

M. grisea can infect a range of grasses and has been known to occur on cultivated turfgrasses in the United States on St. Augustinegrass since 1957 (21) and on annual ryegrass since the 1970s (2,6). It was only identified as causing disease on perennial ryegrass since 1991 (19) and since then has caused severe losses on perennial ryegrass fairways in the Mid-Atlantic States and has been observed in the Midwestern and New England states. Although known to be present in California on St. Augustinegrass

since the 1970s (24), the disease had not been reported on perennial ryegrass in the state until 2001 (32) and only reported on kikuyugrass in 2003 (36).

Very little is known about the origin and spread of *M. grisea* populations associated with turfgrass hosts compared with *M. grisea* populations associated with rice and grass weeds associated with rice production areas. For example, Couch et al. (7) determined the genetic relationship between rice isolates and isolates of other rice associated grass hosts and suggested a single ancient invasion of rice followed by shifts to other grass hosts that grew in association with rice. However, there was no indication of subsequent shifts back to rice hosts. This inference was made based on the occurrence of both mating types and a high copy number of a repetitive element (Pot3) in the rice pathogen population that appeared to have occurred after the host shift from rice to grasses and that grass-infecting isolates had few copies of Pot3 and only one mating type. No such studies have been conducted with turf host populations of *M. grisea* but Viji et al. (34) found significantly fewer copies of Pot3 (25 to 35) in perennial ryegrass hosts compared with rice which often contains more than 50 copies of the repetitive element, which was also consistent with the findings of Couch et al. (7) and other studies (11,12).

Based on many studies utilizing various types of molecular markers (8), it appears that fungi considered *M. grisea* are actually composed of a complex of species and that host selection is one of the major driving forces in the speciation process. With respect to turf-associated isolates, Tredway et al. (31) used amplified fragment length polymorphism (AFLP) to study the

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doi:10.1094/PHYTO-08-10-0205

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genetic diversity of isolates collected from 13 grass genera, collected from a broad geographic distribution. They identified 14 lineages and determined that host species was the primary factor determining population structure based on analysis of molecular variance. However, the vast majority of their isolate collections were from St. Augustinegrass (*Stenotaphrum secundatum*) and tall fescue (*Festuca arundinacea*) collected over eight locations in Georgia. Five AFLP lineages were isolated from tall fescue but one lineage comprised 90% of the isolates. Two AFLP lineages were isolated from St. Augustinegrass with one lineage comprising 99.8% of the isolates. Thus demonstrating the high level of host specificity of *M. grisea* AFLP lineages for these two turf hosts. Moreover, this study also demonstrates that low levels of infection from several lineages on one host may give the pathogen the ability to interact, which could be a possible mechanism for species divergence.

Two such interactions that could potentially occur would be genetic exchange via heterokaryosis and the parasexual cycle or the ability to sexually reproduce. To the best of our knowledge, studies on heterokaryosis and asexuality have only been conducted on rice isolates. Auxotrophic mutants have been used to demonstrate segregation of morphological and nutritional markers (9,16) and Zeigler et al. (37) used molecular markers to demonstrate the detection of parasexual DNA exchanges in unselected wild-type strains. More recently, Noguchi et al. (25) provided the first direct evidence of genetic exchange through parasexual recombination using two antibiotic resistance genes. However, the role of heterokaryosis and the parasexual cycle in generating diversity under field conditions has not been determined for any fungal pathogen to date but it may be important. For example, Forgan et al. (13) recently demonstrated that parasexual process can occur in the plant pathogen *Rhynchosporium secalis* in planta under experimental conditions for the first time for any fungal pathogen.

In order for sexual reproduction to occur in *M. grisea*, both mating types must be present within the same field or population since *M. grisea* is a heterothallic ascomycete. An important aspect of sexual reproduction in outcrossing species is that unlinked genes will segregate independently during meiosis and result in offspring with recombined genotypes (23). Increased genotypic diversity resulting from recombination may allow sexual populations to more rapidly respond to selection imposed by fungicides or resistant cultivars than asexual populations and ascospores could also serve as a long-distance source of inoculum (23). However, the presence of both mating types and analyses suggestive of sexual reproduction have only been found from rice isolates. For example, Kumar et al. (18) failed to reject random

mating based on multilocus disequilibrium tests in rice populations of the pathogen collected in the Himalayan region of India. *M. grisea* is thought to primarily reproduce asexually in nature but Kumar et al. (18) hypothesized that sexual reproduction may occur in this region because it is the proposed center of origin for the pathogen. Additional evidence supporting this was the frequent recovery of sexually fertile hermaphroditic isolates. Moreover, they found that over 3 years of sampling that one population shifted from putatively recombining and genetically diverse to almost completely dominated by a single clonal lineage, demonstrating the possible effects of clonal selection (18). With respect to *M. grisea* isolates associated with turf, both mating types have been found from several turf hosts but no genetic analyses have been conducted in any study to determine reproductive mode (30). However, it has been shown that only a single mating type is usually associated with any given host and in instances where both mating types occurred, either Mat1-1 or Mat1-2 accounted for over 98% of the isolates, suggesting that sexual reproduction is rare or does not occur (30).

The determination of the diversity of *M. grisea* populations in California is a key component to managing the disease and understanding the origin and method of introduction and spread of the pathogen in and throughout the state. Therefore, our objectives were to study the population structure of turf isolates associated primarily with perennial ryegrass and kikuyugrass, which are the dominant turf types used in the golf course industry in California. However, we also sampled one golf course in Nevada and several rice fields in northern California for comparative purposes. Isolates were also obtained from other grass hosts from the eastern United States to explore the possibility that some introductions may have occurred since the disease was first reported to be severe on golf turf in the eastern United States in the early 1990s (31) and was not considered important in California until the early 2000s as described above.

MATERIALS AND METHODS

Sampling, isolation, culturing, and DNA extraction. Isolates included in this study were collected from kikuyugrass, perennial ryegrass, and or St. Augustinegrass in 2006 and 2007 from 14 different locations in California and Nevada (Table 1). Isolates were also collected from rice located in northern California from three fields in 2006. Turfgrass isolates were obtained either from field collections or diagnostic samples sent to the laboratory by golf course superintendents or turf managers. For field collections of turfgrass isolates, golf course fairways were scouted in the mid-

TABLE 1. *Magnaporthe grisea* isolates collected for this study

Population	Location	Number of isolates	Host origin	Collection method ^a
BPGCSD	San Diego, CA	65	Kikuyugrass	Field collection
CCCCRI	Riverside, CA	23	Kikuyugrass	Field collection
SCCCHB	Huntington Beach, CA	143	Kikuyugrass	Field collection
ALCCSJ	San Jose, CA	13	Perennial ryegrass	Diagnostic sample
HAGCLH	La Habra, CA	16	Perennial ryegrass	Field collection
MPCCMP	Menlo Park, CA	2	Perennial ryegrass	Diagnostic sample
NVCCNV	Napa, CA	44	Perennial ryegrass	Field collection
SHCCMP	Menlo Park, CA	10	Perennial ryegrass	Field collection
SSGCLV	Las Vegas, NV	4	Perennial ryegrass	Diagnostic sample
TIPIOS	Oceanside, CA	36	Perennial ryegrass	Field collection
TOSTNA	Napa, CA	8	Perennial ryegrass	Diagnostic sample
VCOMSB	Santa Barbara, CA	5	Perennial ryegrass	Diagnostic sample
Colusa	Colusa, CA	14	Rice	Field collection
Maxwell	Maxwell, CA	12	Rice	Field collection
Wally	Williams, CA	3	Rice	Field collection
HDMVND	Moreno Valley, CA	20	St. Augustine	Field collection
SDSALS	San Diego, CA	2	St. Augustine	Diagnostic sample
Total		420		

^a Field collections were made by sampling symptomatic fairways or fields in a zigzag pattern with samples taken 1 to 3 m or more apart. Diagnostic samples were 20 cm plugs of turf sent in for disease identification. One single conidia isolate was obtained per field sample or diagnostic turf plug.

summer to fall for the presence of symptomatic plants, and 60 plants were typically collected from affected fairways. Sampling was performed in a zigzag pattern across fairways with each sample taken no less than 3 m apart. For some locations, the disease was limited to only a few fairways or locations on the course. Generally, fairways were dominated by a single host species (kikuyugrass or perennial ryegrass), and sampling at any location focused on the collection of a single host species. Population HDMVND, was collected from a nursery in which one sample was taken from individual plastic nursery trays (0.6 m × 0.6 m) containing symptomatic St. Augustinegrass. The samples were placed into paper bags and taken to the laboratory for isolate collection.

For collections from diagnostic samples, a single plant was taken from each 20-cm-diameter sample sent into the lab for disease identification. In most cases, multiple samples were sent in by turf managers, with samples collected from different parts of turfgrass areas.

For rice isolates, fields showing symptoms in early September of 2006 were identified and then sampled in middle September. Samples were also taken in a zigzag pattern through the rice fields, taking 60 samples in each field of plants showing symptoms of neck blast, e.g., lesions on the rice plant stem. Samples were taken approximately 1 to 3 m apart, placed into paper bags, and taken to the laboratory for fungal isolations.

For turfgrass samples, isolates were obtained by adhering a single, symptomatic leaf piece (5 to 7 cm long) to the inside of a 100-mm-diameter petri dish lid with a small amount of petroleum jelly. The lid was then placed on top of the bottom portion of the dish filled with acidified 1.5% water agar (7.5 g of granulated agar and 300 µl of 85% lactic acid [Fisher Scientific, Pittsburgh, PA] per 500 ml of water) and incubated at room temperature (25 to 28°C) in the dark. The next day, the dish was tapped gently to dislodge any conidia formed on the leaf, and then 1 day later, the dish was checked for the presence of germinating conidia on the agar surface using a stereomicroscope. Five germinating conidia were separately transferred to a petri dish containing acidified 1/4-strength potato dextrose agar (1/4-APDA; 4.95 g of potato dextrose agar, 5.63 g of granulated agar, and 300 µl of 85% lactic acid [Fisher Scientific]), and incubated at room temperature for a week. A single isolate was obtained from one of the growing colonies by transferring a small piece of mycelia to fresh 1/4-APDA in a petri dish. A total of 393 turfgrass isolates were obtained in this way.

For rice isolates, 3- to 5-cm-long stem pieces were first cleaned in 10% commercial bleach solution for 1 min, rinsed three times in sterile water, and blotted dry. Stem pieces were adhered to the inside of petri plate lids, and isolates were obtained as described above. A total of 29 isolates were obtained from rice samples in this way.

Thirty-five additional isolates collected from the eastern United States were also used for comparison. Nineteen were from perennial ryegrass (courtesy of Wakar Uddin, Pennsylvania State University), 2 from weeping lovegrass (*Eragrostis curvula*), 10 from tall fescue, and 3 from St. Augustine (courtesy of Lane Tredway, North Carolina State University).

For this study, each isolate was stored by transferring the isolate to 1/4-APDA overlaid with a piece of autoclaved filter paper (9 cm diameter [Fisher Scientific]). After 7 days at room temperature, the filter was lifted from the agar surface, placed on small envelopes, dried, and stored at -20°C. Additionally, isolates from both rice and turfgrass were transferred to 15-mm screw top vial PDA slants (19.5 g of PDA per liter of water), incubated at room temperature for 14 days, and subsequently stored at -4°C.

For DNA extraction, isolates were grown on 1/4-APDA for 7 days at room temperature. Mycelia was scrapped off of the plates and total DNA was extracted using C-TAB lysis buffer (14) followed by precipitation using ethanol and sodium acetate (20).

After the last step, DNA was recovered as a pellet and resuspended on 100 µl of TE buffer (10 ml of Tris, pH 8.0, 1 Mm EDTA) for storage at -4°C.

Mating-type designation and AFLP. Mating type for each isolate was determined for all isolates following the molecular protocol of Tredway et al. (30). For AFLP fingerprints, a slightly modified method of Vos et al. (35) was used. A total of 12 primer combinations with the addition of two selective nucleotides at the 3' end were screened using a subset of isolates using a single polymerase chain reaction (PCR) from the diluted restriction-ligation reaction. A single primer pair was chosen and used to obtain a genetic fingerprint of all of the isolates. Only one primer was used because in the preliminary screening, isolates that had polymorphic loci in one primer pair were usually the only isolates that would also show polymorphisms with additional primer pairs. Moreover, isolates associated with all of the hosts were highly clonal with the exception of kikuyugrass isolates in which the final primer pair chosen yielded 10 polymorphic loci.

Each 20-µl reaction contained 1× PCR buffer (Invitrogen, Carlsbad, CA), 2.5 mM MgCl₂, 2.5 mM each dNTP (Invitrogen), 0.375 µM of each primer, 0.5 U of *Taq* polymerase (Invitrogen), and 10 µl of template. Thermocycling conditions consisted of an initial hold at 72°C and 94°C for 1 and 4 min, respectively, followed by 44 cycles with an annealing temperature of 65°C for 30 s and an extension temperature of 72°C for 1 min with the annealing temperature reduced by 1°C for the first nine cycles. All amplifications were performed in a MyCycler (Bio-Rad Laboratories Inc., Hercules, CA). Several isolates were used as controls by conducting independent DNA extractions, restriction-ligation reactions, and PCR amplifications.

Following amplification, 5 µl of loading dye (98% formamide, 10 mM EDTA, pH 8.0, 0.25% bromphenol blue, 0.25% xylene cyanole) was added to each reaction and the reaction mix was denatured for 4 min at 94°C and cooled on ice. Three to four microliters of each sample was loaded and separated on 4% acrylamide gels buffered in TBE (1.35 M Tris, pH 8.0, 0.45 M boric acid, and 25 mM EDTA) for approximately 2 h at 1800 to 1900 volts using a Bio-Rad Sequi-Gen GT Sequencing Cell (Bio-Rad Laboratories Inc.). The gels were then silver-stained according to the method of Bassam et al. (3) and dried overnight before scoring bands visually. Putative alleles at each polymorphic AFLP locus were scored using a binary code (1 or 0) corresponding to positive and null alleles, respectively. For the selection of AFLP markers, only polymorphic loci that were reproducible and easily identifiable were scored.

Data analysis. Cluster analysis on genetic distances using the mean character difference option and unweighted pair-group method with arithmetic average (UPMGA) were conducted using PAUP* (version 4.0b10) (28) for the entire data set. Once putative clonal genotypes were identified, the number of clones was noted and the data set was reduced to represent one clonal genotype for fungi that were isolated from this study. All genotypes from cultures obtained from other researchers were retained in the final analysis where the UPMGA analysis was repeated. Confidence in tree topology was examined using bootstrap with 10,000 replicates.

The hypothesis of random mating was tested using three approaches for isolates associated with kikuyugrass only. First, a chi-square test was used to test the segregation of mating type, which should be 1:1 for a randomly mating haploid fungus (23). Second, multilocus gametic disequilibrium was tested using the index of association (I_A) statistic as implemented in the program MULTILOCUS (1). I_A has an expected value of zero if there is no association of alleles at unlinked loci as expected in a randomly mating population (4,22). The significance of I_A was tested by randomization (1,000 times) procedures by comparing the observed value of I_A to that expected under the null hypothesis of random mating (1,5). Third, the parsimony tree length permu-

tation test (PTLPT) was used to determine reproductive mode (5,29). PTLPT was analyzed using PAUP version 4.0b10 (28) and is a phylogenetic approach based on the concept that a clonal population should evolve like a parsimonious tree with little homoplasy whereas recombining populations will have little phylogenetic single due to the reshuffling of alleles during meiosis (29). The significance of PTLPT was determined using randomization procedures by comparing the observed tree lengths to the lengths of 1,000 artificially recombined trees in PAUP from an input file generated in MULTILOCUS.

RESULTS

Cluster analysis and mating type distribution. A total of 38 polymorphic AFLP makers were scored. For the cluster analysis, 450 isolates were analyzed; 420 were isolated for this study and 34 were from cultures that were obtained from other researchers. For isolates obtained for this study, 231 were from kikuyugrass, 138 from perennial ryegrass, 22 from St. Augustinegrass, and 29 from rice consisting of 32, 10, 22, and 2 unique genotypes, respectively (Table 2). For the additional isolates, 19 were from perennial ryegrass, 2 from weeping lovegrass, and 10 from tall fescue, and 3 from St. Augustine consisting of 1, 2, 5, and 2 unique genotypes, respectively (Fig. 1). All isolates clustered by host with high bootstrap support (71 to 100%) (Fig. 1).

For kikuyugrass, only three genotypes (G1, 18, and 31) were found in more than one population of the three populations

sampled and both mating types were found (Table 2). Four genotypes were found in population CCCCRI, 13 in BPGCSD, and 18 in SCCCHB and each genotype was found from a singleton to up to 38 times on any given golf course (Table 2). In contrast, for perennial ryegrass, three genotypes (G1, 2, and 8) were also found but genotype G1 was found in eight populations whereas genotypes 2 and 8 were only found in two populations. Genotype 1 was the most dominant genotype collected, accounting for 92.5% of the isolates collected (124 of 134), and all isolates from perennial ryegrass consisted of the single mating type MAT1-2 (Table 2). Genotype 1 was also the same genotype as the isolates obtained from a broad sampling outside of California (Fig. 1). Only one or two genotypes were found per population for perennial ryegrass isolates except for population TIPIOS in which seven genotypes were found and each genotype was found from a singleton to up to 44 times on any given golf course (Table 2). For St. Augustine and rice, only two genotypes were found and all isolates were MAT1-1 (Table 1). Genotype 1 from St. Augustine was also the same as two isolates obtained from Georgia (Fig. 1). No isolates from California were isolated and analyzed from tall fescue. Five unique genotypes were identified from the tall fescue isolates that were obtained from Georgia.

Reproductive mode. The reproductive mode analyses were only conducted on isolates from kikuyugrass since both mating types were found in contrast to isolates from the other hosts and only two populations were analyzed since one of the three populations sampled only consisted of four genotypes (Table 2). Only

TABLE 2. Amplified fragment length polymorphism phenotypes and mating-type designations from isolates of *Magnaporthe grisea* collected for this study

Kikuyugrass				Perennial ryegrass			
Genotype	Location	No. of isolates	Mating type	Genotype	Location	No. of isolates	Mating type
1	BPGCSD	5	MAT1-2	1	NVCCNV	44	MAT1-2
"	CCCCRI	8	MAT1-2	"	ALCCJS	13	MAT1-2
2	BPGCSD	4	MAT1-1	"	TIPIOS	27	MAT1-2
3	BPGCSD	2	MAT1-2	"	TOSTNA	8	MAT1-2
4	CCCCRI	6	MAT1-1	"	VCOMSB	5	MAT1-2
5	CCCCRI	8	MAT1-1	"	SSGCLV	3	MAT1-2
6	SCCCHB	38	MAT1-1	"	SHCCMP	9	MAT1-2
7	SCCCHB	12	MAT1-1	"	HAGCLHR	15	MAT1-2
8	SCCCHB	11	MAT1-2	2	TIPIOS	3	MAT1-2
9	SCCCHB	17	MAT1-2	"	MPCCMP	1	MAT1-2
10	SCCCHB	7	MAT1-2	3	TIPIOS	1	MAT1-2
11	SCCCHB	2	MAT1-1	4	SSGCLV	1	MAT1-2
12	SCCCHB	1	MAT1-2	5	SHCCMP	1	MAT1-2
13	BPGCSD	20	MAT1-1	6	HAGCLHR	1	MAT1-2
14	BPGCSD	1	MAT1-1	7	TIPIOS	1	MAT1-2
15	BPGCSD	26	MAT1-1	8	TIPIOS	1	MAT1-2
16	BPGCSD	1	MAT1-1	"	MPCCMP	1	MAT1-2
17	BPGCSD	1	MAT1-1	9	TIPIOS	2	MAT1-2
18	BPGCSD	1	MAT1-1	10	TIPIOS	1	MAT1-2
"	SCCCHB	7	MAT1-1	Total		138	
19	SCCCHB	7	MAT1-2	St. Augustine			
20	SCCCHB	2	MAT1-1	Genotype	Location	No. of isolates	Mating-type
21	SCCCHB	8	MAT1-1	1	HDMVND	20	MAT1-1
22	SCCCHB	6	MAT1-1	"	SDSALS	2	MAT1-1
23	BPGCSD	1	MAT1-1	Total		22	
24	SCCCHB	1	MAT1-1	Rice			
25	SCCCHB	12	MAT1-1	Genotype	Location	No. of isolates	Mating-type
26	SCCCHB	8	MAT1-1	1	Collusa	13	MAT1-1
27	SCCCHB	1	MAT1-2	"	Maxwell	12	MAT1-1
28	SCCCHB	2	MAT1-1	"	Wally	3	MAT1-1
29	SCCCHB	1	MAT1-1	2	Collusa	1	MAT1-1
30	BPGCSD	1	MAT1-2	Total		29	
31	BPGCSD	1	MAT1-1				
"	CCCCRI	1	MAT1-2				
32	BPGCSD	1	MAT1-1				
Total		231					

polymorphic loci that were unique to this host were used and we also created a data set where loci that were nearly fixed or rare were excluded because this can sometime influence phylogenetic and multilocus disequilibrium approaches to infer if recombination is occurring (26). The two populations were also analyzed by combining the data to increase the sample size. However, we realize that this assumes that these isolates would then represent one single population, which may not be a proper assumption. The mating-type ratios for SCCCHB and the combined data were

significantly different from 1:1 ($P = 0.052$ and 0.028 , respectively). In contrast, the mating-type ratio from BPGCSD was not significantly different from 1:1 ($P = 0.157$) (Table 3). The null hypothesis of random mating could not be rejected for SCCCHB and the combined data in the full analysis and the reduced data set analyses based on the I_A ($0.443 < P < 0.949$) and PTLPT ($0.248 < P < 0.968$) tests (Table 3). The null hypothesis of random mating was rejected for BPGCSD in the full analysis and the reduced data set analysis based on the I_A ($P = 0.003$ and 0.001 , respec-

Fig. 1. Dendrogram of *Magnaporthe grisea* isolates based on unweighted pair-group method with arithmetic average cluster analysis using PAUP with genetic distances calculated from 38 amplified fragment length polymorphism (AFLP) loci. Bootstrap values are based on 10,000 replicates with values over 50% shown. A single representative California isolate from perennial ryegrass and kikuyugrass are shown followed by their location collected and the AFLP genotype number (Table 2 provides details). All other isolates are named followed by the state abbreviation where they were originally collected.

tively). However, for PTLPT analyses the *P* values were just outside of the range of rejection at the $\alpha = 0.05$ level (*P* = 0.060 and 0.069, respectively) (Table 3).

DISCUSSION

As with previous studies, we found that the *M. grisea* isolates were genetically structured based on host. However, this is the first study to document the potential for sexual reproduction to occur in *M. grisea* populations not associated with *Oryza sativa*. Isolates from kikuyugrass were genotypically diverse, possessed both mating types, and some tests for random mating could not be rejected. We also determined that genotype G10, associated with perennial ryegrass, was identical to isolates broadly distributed from the eastern United States. This was also true for genotype G1 from St. Augustine in that it was identical to some isolates from Georgia. These results support a potential introduction of these genotypes into California from the eastern United States. However, we do not feel that this is the most likely explanation as discussed below.

Introduced plant pathogens usually exhibit reduced genetic and genotypic diversity compared with their native residual populations due to genetic bottlenecks that often occur when a subset of the pathogen population, or in some cases only a single genotype, is introduced into a new area (23). The classic plant pathogen example is *Phytophthora infestans* when introductions into the United States and Europe from either Mexico or South America, led to a series of crop failures; the most notable resulting in the Irish potato famine. The population genetics of *P. infestans* has been extensively studied which has documented various waves of clonal lineages through time based on mating type, metalaxyl resistance, mtDNA and allozyme genotypes which has greatly aided in the understanding the movement, spread, and reproductive capacity of this significant pathogen (27).

In our system, do genotypes G10 and G1 from perennial ryegrass and St. Augustine, respectively, represent recent introductions into California? These genotypes are based only on a single AFLP fingerprint but we also sequenced eight microsatellite loci (seven unlinked) developed by Kaye et al. (17) from representative isolates from California and non-California collections which also demonstrated they were identical genotypes (data not shown). This provides strong evidence that they are identical but given the contrasting genetic structures observed between kikuyugrass (diverse) and perennial ryegrass (highly clonal) isolates, it is possible that these genotypes from perennial ryegrass and St. Augustine were already present in California. A more likely explanation is that environmental or management conditions became more conducive for disease development under California conditions when GLS emerged as a new disease and became epidemic on both kikuyugrass and perennial ryegrass. More evidence in favor of this hypothesis is that kikuyugrass is only grown as golf turf in California, and the GLS epidemics on

both grass hosts occurred at similar times making it unlikely that host specific isolates were introduced into California at the same time. Additional evidence contradictory to the hypothesis of an introduction into California is that we detected more genotypic diversity in isolates associated with perennial ryegrass from California compared with the eastern United States (31). However, the sample size of perennial ryegrass isolates from Treday et al. (31) was small (*n* = 11). Therefore, we cannot fully rule out that an introduction did occur and additional detailed studies would have to be conducted to more adequately address this.

We were also interested in understanding the reproductive biology of *M. grisea* populations associated with turf in California. The most definitive way to determine if sexual reproduction is occurring in a fungal pathogen population is to find fertile sexual reproductive structures, which has not been documented for *M. grisea* in nature. An alternative approach, as taken here, is to use molecular markers and mating-type ratios to make inference on reproductive biology. We found contradictory results based on mating-type ratios and tests for random mating (*I*_A and PTLPT) for *M. grisea* isolates associated with kikuyugrass. For example, for population SCCCHB, the mating type ratio was not in the 1:1 ratio expected in a sexually reproducing ascomycete but we could not reject random mating based on *I*_A and PTLPT. In contrast, for population BPGCSD, mating type ratio was 1:1 as expected in a sexually reproducing ascomycete but random mating was rejected based on *I*_A but was not rejected based on PTLPT. However, an important caveat to these analyses is that the statistical tests used in this and other studies appear to be very sensitive and statistical power of these tests are not known. For example, *I*_A measures the association among alleles in a population and it takes only a few individuals reproducing sexually per generation to create enough recombination to reject the null hypothesis (5). This makes it impossible to determine how often sexual reproduction is occurring or if the underlying recombining genetic structure is due to present, past, or parasexual recombination events (15,29).

Inconsistencies between tests can also occur as demonstrated in this study and has also been found in population genetic analyses of *Coccidioides immitis* (5) and *Tapesia yallundae* (10). Such discrepancies with these analyses warrant theoretical investigations to better understand how parameters such as the marker system used, number of loci, number of individuals, linkage and allele frequency distribution affect the outcome of these tests, especially if inferences about sexual reproduction are going to be made without additional evidence. However, to the best of our knowledge, this is the first study to document any evidence of potential sexual reproduction in natural populations of *M. grisea* not associated with *Oryza sativa* and warrants further studies on populations from kikuyugrass given the amount of diversity found and the fact that both mating types are present. It should also be noted that attempts were made to make crosses between isolates from each host and within hosts as well as with known mating

TABLE 3. Segregation of mating type and tests for random mating among populations of *Magnaporthe grisea* associated with kikuyugrass

Population	Mating-type segregation					Index of association (<i>I</i> _A)		Parsimony tree length (L) permutation test	
	No. of loci	<i>n</i>	MAT1-1:MAT1-2	<i>X</i> ²	<i>P</i>	<i>I</i> _A	<i>P</i>	L	<i>P</i>
SCCCHB	10	13	10:3	3.77	0.052	0.0954	0.792	21	0.796
BPGCSD	10	18	12:6	2.00	0.157	0.5715	0.003	17	0.060
Combined	10	30	24:11	4.83	0.028	0.0198	0.585	36	0.248
SCCCHB ^a	7	13	***	***	***	-0.1610	0.949	21	0.968
BPGCSD ^b	8	18	***	***	***	0.5710	0.001	17	0.069
Combined ^c	8	30	***	***	***	0.0087	0.443	32	0.898

^a Three loci were removed; one locus was fixed, one was absent, and one was rare in this population (frequency of 0.0769). The remaining frequencies ranged between 0.2301 and 0.6923.

^b Two loci were removed; both were absent in this population. The remaining allele frequencies ranged between 0.7692 and 0.384.

^c Two loci were removed; both were rare in this population (0.030 and 0.1667). The remaining allele frequencies ranged between 0.700 and 0.2330.

type tester strains but all attempts failed (data not shown). Therefore, it will take more intensive sampling within and between golf courses over multiple years to more definitively investigate the reproductive biology of isolates associated with kikuyugrass. For example, if sexual reproduction is occurring and ascospore inoculum is involved, then allele frequencies between years should remain the same whereas new genotypes should be found between years in the same sampled populations.

Kikuyugrass has been adopted for use in fairways and roughs in a number of subtropical areas including southern California, Mexico, Australia, and South Africa (F. P. Wong, *personal communication*). In 2003, a foliar disease of kikuyugrass was reported from a number of golf courses in southern California and it was determined that the causal agent was indeed *M. grisea* (36). Outside of California and in parts of Mexico (Patrick Gross, United States Golf Association, *personal communication*) the disease has not been reported even though the grass is widely distributed and is considered an invasive grass in many locations. In this study, we found that isolates associated with kikuyugrass were clearly differentiated from isolates from all other hosts and that the population was diverse compared to perennial ryegrass isolates. To our knowledge, only one population genetic study has included isolates from the genus *Pennisetum*. Tredway et al. (31) determined that two different divergent AFLP lineages were associated with the two *Pennisetum* species they studied and they were not very closely related to isolates from rice. In contrast, we found that the California kikuyugrass isolates clustered into a clade with the California rice isolates with high bootstrap support (88%) but they were still considerably divergent from one another. Therefore, this likely does not represent a recent host jump from rice to kikuyugrass so the origin of this population remains unclear. However, given the high diversity, existence of both mating types, and some evidence of random mating, it is possible that this host population is actually endemic or local to California. Perhaps with additional sampling of weedy grass hosts that are found in and around golf courses in California, we could identify any putative native or introduced weedy grass species as a possible reservoir for the kikuyugrass population. Moreover, more population genetic analyses from additional field sampling of isolates associated with this host and actually looking for perithecia in the field will further our understanding of the population biology of this pathogen.

LITERATURE CITED

- Agapow, P. M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* 1:101-102.
- Bain, D. C., Patel, B. M., and Patel, M. V. 1972. Blast of ryegrass in Mississippi. *Plant Dis. Rep.* 56:210.
- Bassam, B. J., Caetano-anolles, G., and Gresshoff, P. M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196:80-83.
- Brown, A. H. D., Feldman, M. W., and Nevo, E. 1980. Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics* 96:523-536.
- Burt, A., Carter, D. A., Koenig, G., White, T. J., and Taylor, J. W. 1996. Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proc. Natl. Acad. Sci. USA* 93:770-773.
- Carver, R. B., Rush, M., and Lindberg, G. D. 1972. An epiphytotic of ryegrass blast in Louisiana. *Plant Dis. Rep.* 56:157-159.
- Couch, B. C., Fudal, I., Lebrun, M. H., Tharreau, D., Valent, B., van Kim, P., Nottéghem, J., and Kohn, L. 2005. Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. *Genetics* 170:613-630.
- Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94:683-693.
- 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.
- Douhan, G. W., Peever, T. L., and Murray, T. D. 2002. Multilocus population structure of *Tapesia yallundae* in Washington State. *Mol. Ecol.* 11:2229-2239.
- Eto, Y., Ikeda, K., Chuma, I., Kataoka, T., Kuroda, S., Kikuchi, N., Don, L. D., Kusabe, M., Nakayashiki, H., Tosa, Y., and Mayama, S. 2001. Comparative analyses of the distribution of various transposable elements in *Pyricularia* and their activity during and after the sexual cycle. *Mol. Gen. Genet.* 264:565-577.
- Farman, M. L. 2002. *Pyricularia grisea* isolates causing gray leaf spot on perennial ryegrass (*Lolium perenne*) in the United States: Relationship to *P. grisea* isolates from other host plants. *Phytopathology* 92:245-254.
- Forgan, A. H., Knogge, W., and Anderson, P. A. 2007. Asexual genetic exchange in the barley pathogen *Rhynchosporium secalis*. *Phytopathology* 97:650-654.
- Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for Basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2:113-118.
- Geiser, D. M., Pitt, J. I., and Taylor, J. W. 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proc. Natl. Acad. Sci. USA* 95:388-393.
- Genovesi, A. D., and Magill, D. W. 1976. Heterokaryosis and parasexuality in *Pyricularia oryzae* Cavara. *Can. J. Microbiol.* 22:531-536.
- Kaye, C., Milazzo, J., Rozenfeld, S., Lebrun, M., and Tharreau, D. 2003. The development of simple sequence repeat markers for *Magnaporthe grisea* and their integration into an established genetic linkage map. *Fungal Gen. Biol.* 40:207-214.
- Kumar, J., Nelson, R. J., and Zeigler, R. S. 1999. Population structure and dynamics of *Magnaporthe grisea* in the Indian Himalayas. *Genetics* 152:971-984.
- Landschoot, P. J., and Hoyland, B. F. 1992. Gray leaf spot of perennial ryegrass turf in Pennsylvania. *Plant Dis.* 76:1280-1282.
- Lee, S. B., and Taylor, J. W. 1990. Isolation of DNA from fungal mycelia and single spores. Pages 282-287 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego.
- Malca, I., and Owen, J. H. 1957. The gray leaf spot disease of St. Augustinegrass. *Plant Dis.* 41:871-875.
- Maynard Smith, J., Smith, N. H., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? *Proc. Natl. Acad. Sci. USA* 90:4384-4388.
- Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. *Ann. Rev. Phytopathol.* 34:457-477.
- Mueller, K. E. 1972. Gray leaf spot of St. Augustinegrass. *Calif. Turfgrass Cul.* 22:19-20.
- Noguchi, M. T., Yasuda, N., and Fujita, Y. 2006. Evidence of genetic exchange by parasexual recombination and genetic analysis of pathogenicity and mating type of parasexual recombinants in rice blast fungus, *Magnaporthe oryzae*. *Phytopathology* 96:746-750.
- Saville, B. J., Kohli, Y., and Anderson, J. B. 1993. mtDNA recombination in a natural population. *Proc. Natl. Acad. Sci. USA* 95:1331-1335.
- Smart, C. D., and Fry, W. E. 2001. Invasions by the late blight pathogen: Renewed sex and enhanced fitness. *Biol. Inv.* 3:235-243.
- Swofford, D. L. 2002. *PAUP* Phylogenetic Analysis Using Parsimony (* and other methods)*. Version 4.0b10: Sinauer Associates, Inc. Sunderland, MA.
- Taylor, J. W., Jacobson, D. J., and Fisher, M. C. 1999. The evolution of asexual fungi: Reproduction, speciation and classification. *Annu. Rev. Phytopathol.* 37:197-246.
- Tredway, L. P., Stevenson, K. L., and Burpee, L. L. 2003. Mating type distribution and fertility status in *Magnaporthe grisea* populations from turfgrasses in Georgia. *Plant Dis.* 87:435-441.
- Tredway, L. P., Stevenson, K. L., and Burpee, L. L. 2005. Genetic structure of *Magnaporthe grisea* populations associated with St. Augustinegrass and tall fescue in Georgia. *Phytopathology* 95:463-471.
- Uddin, W., Viji, G., and Stowell, L. 2002. First report of gray leaf spot on perennial ryegrass turf in California. *Plant Dis.* 86:75.
- Uddin, W., Viji, G., and Vincelli, P. 2003. Gray leaf spot (blast) of perennial ryegrass turf: An emerging problem for the turfgrass industry. *Plant Dis.* 87:880-889.
- Viji, G., Wu, B., Kang, S., Uddin, W., and Huff, D. R. 2001. *Pyricularia grisea* causing gray leaf spot of perennial ryegrass turf: Population structure and host specificity. *Plant Dis.* 85:817-826.
- Vos, P., Hogers, R., Bleeker, M., Reijmans, M., Vandelee, 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.
- Wong, F. P., Gelernter, W., and Stowell, L. 2005. First report of *Pyricularia grisea* causing gray leaf spot on Kikuyugrass (*Pennisetum clandestinum*) in the United States. *Plant Dis.* 89:433.
- Zeigler, R. S., Scott, R. P., Leung, H., Bordeos, A. A., Kumar, J., and Nelson, R. J. 1997. Evidence of parasexual exchange of DNA in the rice blast fungus challenges its exclusive clonality. *Phytopathology* 87:284-294.