Genetics

Use of Random Amplified Polymorphic DNA Markers for the Detection of Genetic Variation in *Magnaporthe poae*

D. R. Huff, T. E. Bunting, and K. A. Plumley

First author, Department of Plant Science, and second and third authors, Department of Plant Pathology, Cook College, Rutgers University, New Brunswick, NJ 08903-0231.

Current address of first author: Department of Agronomy, Pennsylvania State University, University Park 16802-3504.

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ABSTRACT

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Random amplified polymorphic DNA (RAPD) markers were used to survey genetic variability among 35 *Magnaporthe poae* isolates. Amplification patterns for 23 of the 35 isolates were phenotypically unique and readily distinguishable from an outgroup of six additional ectotrophic, dematiaceous fungi. These results indicate the utility of RAPDs as an accurate and reproducible means of identifying individuals within the species. Analysis of molecular variance demonstrated that isolates of *M. poae* were significantly different ($P = 0.001$) among 12 sampling locations. This finding may have important implications for research programs concerned with obtaining genetically representative isolates of *M. poae*. Moreover, genetic variation was found among isolates within all but one location and within both mating types, suggesting that genetic variation is present among naturally occurring populations, even though natural sporulation has never been observed in *M. poae*. From a subset of 12 isolates, pathogenicity was found to decrease as genetic distance from the most pathogenic isolate increased. The use of RAPD markers to obtain measurements of genetic relatedness and genetic variation within and between fungal populations should aid in our understanding of the disease pathology and reproductive biology of this species.

Magnaporthe poae Landschoot & Jackson, the causal agent of summer patch disease, is a soilborne, ectotrophic, root-infecting fungus. Summer patch is a devastating disease of cool-season turfgrasses that affects Kentucky bluegrass (*Poa pratensis* L.), annual bluegrass (*P. annua* L.), and fine fescues (*Festuca* spp.) (10,15,25). *M. poae* represents a newly described species of *Magnaporthe* R. Kraus & R. E. Webster, and little is currently known about its ecology or biology. Sporulation of its teleomorph

and *Phialophora* anamorph has been induced in culture, but neither reproductive stage has been observed in nature (15). The apparent lack of natural sporulation makes the relative importance of sexual reproduction in this species unclear, although it has been speculated that this pathogen is primarily disseminated in the vegetative state through the movement of infected host tissue (13,26). The fungus is known to display considerable variation in colony morphology and coloration (14). However, estimates of genetic variability among naturally occurring populations have not been reported.

Information regarding the variability within fungal populations is important to better understand disease outbreaks and predict future disease development (27). Molecular markers are useful tools for examining genetic variation within populations of phytopathogenic fungi (20). A number of molecular techniques are currently available for studying the genetic relationships of fungal populations. Isozymes (16), restriction fragment length polymorphisms (19), DNA sequence divergence (23), and DNA fingerprinting (17) have all been successfully applied to the study of fungal populations. The development of random amplified polymorphic DNA (RAPD) markers (29,30) provides another powerful method for investigating intraspecific genetic variation within an ecological context (5). In comparison with other techniques, however, RAPD markers do not require previous molecular genetic information, they often yield large numbers of discriminating markers, and they are technically simple to perform. RAPD markers have been successfully used to differentiate among isolates of *Colletotrichum graminicola* (Ces.) G. W. Wils. (4) and pathogenic races of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *pisi* (J. C. Hall) W. C. Snyder & Hanna (3) and to separate species within the genus *Hypoxylon* (31).

The standard method of visualizing RAPD markers has been to separate amplified fragments in agarose gels and stain the fragments with ethidium bromide (30). An alternative method involves separating the amplified fragments in polyacrylamide gels and uses a silver stain (6–8). We believe the increased resolution of polyacrylamide gels coupled with the increased sensitivity of silver staining improves the precision of RAPD data.

A recently developed method for analyzing molecular marker variation is known as the analysis of molecular variance (AMOVA) (2). The objectives of our study were to use AMOVA to detect genetic marker variation among isolates of *M. poae* and to analyze marker variation within and between collection locations from a narrow geographic region.

MATERIALS AND METHODS

Thirty-five isolates of *M. poae* were examined for RAPD patterns (Table 1). Thirty-three of these isolates were recovered from annual bluegrass exhibiting symptoms of summer patch. The samples were collected from 15 locations throughout New Jersey, New York, and Pennsylvania between 1988 and 1992. At least two isolates were obtained from 12 of the locations, thus allowing for statistical analysis both within and between sampling sites. The remaining two *M. poae* isolates (ATCC 64411 and 64412) were acquired from the American Type Culture Collection (Rockville, MD). An outgroup comprised of six additional ectotrophic, dematiaceous fungi (Table 2) was selected for their taxonomic (12) and molecular genetic similarity (21) to *M. poae*.

Isolates of *M. poae* were stored on potato-dextrose agar at 24 C and cycled through wheat roots once each year to maintain pathogenicity (11). Four agar plugs (4 mm in diameter) of each isolate were cut from actively growing margins of fungal colonies and transferred to petri plates containing potato-dextrose agar overlaid with 8-cm-diameter cellophane disks (Flexel Inc., Covington, IN). After the plates had been incubated for 5 days at 24 C, the agar plugs were removed, and the remaining mycelium was harvested from the surfaces of the cellophane disks. Genomic DNA was extracted (1), and final DNA concentrations were standardized to 10 ng μl^{-1} with a TKO-100 fluorometer (Hoeffer, San Francisco, CA). Polymerase chain reaction (PCR) amplifications of DNA sequences were performed with each of four oligonucleotide primers (OPA-3, OPA-4, OPA-5, and OPA-12), 10 bases in length, obtained from Operon Technologies, Inc. (Alameda, CA). Each 12-μl PCR reaction contained 1.2 units of Stoffel fragment polymerase (Perkin-Elmer, Norwalk, CT) and 1× buffer (Perkin-Elmer) adjusted to 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Perkin-Elmer), 1.3 pg of primer, and 12 ng of template DNA. A Perkin-Elmer thermocycler (model TC-1) was programmed for an initial denaturation of 7 min at 94 C followed by 45 cycles. Each cycle consisted of a denaturation step at 94 C for 1 min, a primer annealing step at 36 C for 1 min, and a primer extension step at 72 C for 2 min. The primer extension step of the final cycle was extended to 5 min. The ramp rate for heating between

TABLE 1. Isolates of *Magnaporthe poae* used in random amplified polymorphic DNA marker analysis

Isolate number[a]	Mating type	Collection location
MP A	A	ATCC[b] 64411
MP a	a	ATCC 64412
MP 1-1[c]	A	Ambler, PA
MP 1-2[c]	A	Ambler, PA
MP 1-3[c,d]	A	Ambler, PA
MP 1-4[c]	A	Ambler, PA
MP 1-5[c]	A	Ambler, PA
MP 1-6[c]	A	Ambler, PA
MP 2-1[c]	A	Morristown, NJ
MP 2-2[c,d]	A	Morristown, NJ
MP 2-3[c]	A	Morristown, NJ
MP 3-1[c]	n/p[e]	New Brunswick, NJ
MP 3-2[c]	n/p	New Brunswick, NJ
MP 3-3[c]	n/p	New Brunswick, NJ
MP 4-1[c]	A	Pitman, NJ
MP 4-2[c]	A	Pitman, NJ
MP 5-1[c]	n/p	Marlton, NJ
MP 5-2[c]	n/p	Marlton, NJ
MP 6-1[c,d]	A	Middletown, NJ
MP 6-2[c,d]	A	Middletown, NJ
MP 7-1	n/p	Pittsburg, PA
MP 8-1[c,d]	A	Wayne, PA
MP 8-2[c]	A	Wayne, PA
MP 9-1[c,d]	A	Oreland, PA
MP 9-2[c]	A	Oreland, PA
MP 10-1[c,d]	a	Spring Lake, NJ
MP 10-2[c,d]	a	Spring Lake, NJ
MP 11-1[c,d]	A	Sparkill, NY
MP 11-2[c]	A	Sparkill, NY
MP 12-1[c]	a	Medford Lakes, NJ
MP 13-1[c]	a	Ridgewood, NJ
MP 13-2[c,d]	a	Ridgewood, NJ
MP 14-1[c]	A	Springfield, NJ
MP 14-2[c,d]	A	Springfield, NJ
MP 15-1	a	Union, NJ

[a] All *M. poae* were isolated from host *Poa annua*.
[b] American Type Culture Collection.
[c] Used for analysis of molecular variance.
[d] Used for pathogenicity correlation.
[e] Did not produce perithecia.

TABLE 2. Outgroup isolates of ectotrophic, dematiaceous fungi used in random amplified polymorphic DNA marker analysis

Isolate number	Genus and species	Host	Source
LK 1	*Leptosphaeria korrae*	*Poa pratensis*	G. Worf[a]
MG 1	*Magnaporthe grisea*	*Oryza sativa*	M. A. Marchetti[b]
MG 2	*M. grisea*	*O. sativa*	P. Landschoot[c]
CG 1	*Colletotrichum graminicola*	*P. annua*	Agri-Diagnostics Associates
CG 2	*C. graminicola*	*P. annua*	R. Kane[a]
CG 3	*C. graminicola*	*P. annua*	J. Vargas[a]

[a] Via Agri-Diagnostics Associates, Cinnaminson, NJ.
[b] Via I. Raskin, Rutgers University, New Brunswick, NJ.
[c] Via J. Correll, University of Arkansas, Fayetteville.

36 and 72 C was 0.3 C s^{-1}; otherwise, all heating and cooling ramp rates were 1 C s^{-1}.

Amplified fragments were resolved in a 7.5% acrylamide-bis gel (37.5:1; Fisher, Fair Lawn, NJ) in 0.375 M Tris buffer (pH 8.8) at 200 V for 40 min with a Mini-Protean II (Bio-Rad, Richmond, CA). A modified protocol of the Bio-Rad silver stain kit (fixation step: 10% acetic acid for 30 min) was used to visualize the amplification products. RAPD patterns were produced for at least two replicate PCR amplifications for each individual isolate. Data were collected only from replicated RAPD bands between fragment sizes 0.1 and 2.6 kb. The genetic data of each RAPD fragment was based on two possible character states: a value of 1 for band presence and 0 for its absence. The character state of each RAPD band was scored for each individual isolate within a species.

Genetic distance, calculated as a Euclidean metric distance, was computed between all pairs of isolates within a species (8). The Euclidean metric distance between two individuals is equivalent to their total number of observed band differences. A dendrogram (Fig. 1) was constructed from the Euclidean distance matrix by the unweighted paired group method of arithmetic averages within the SAHN program of NTSYS-pc software (version 1.5) (22). AMOVA was used to partition and estimate variance components and to compute levels of significance by nonparametric permutational procedures within the WINAMOVA program (2) provided by L. Excoffier (Department of Anthropology and Ecology, University of Geneva, Geneva, Switzerland). Spearman's rank correlation coefficients (r_{sp}) were calculated following the procedure of Steel and Torrie (28).

RESULTS AND DISCUSSION

The four oligonucleotide primers produced a total of 22 RAPD markers among the 35 *M. poae* isolates, resulting in 23 unique RAPD phenotypes. In a previous study, 18 isolates of *M. poae*, from a subset of the 35 isolates included in the present study, were examined for RAPD patterns (7). RAPD patterns of individual isolates from both studies were found to be identical, even though separate teams of researchers performed the PCR reactions and different methods of DNA extraction were utilized. This fact is noteworthy because it demonstrates the repeatability of the RAPD method and its value as an accurate means of isolate identification.

An underlying assumption of RAPD marker analysis is that marker bands of the same size originate from the same genomic locus (Fig. 2). While this assumption is reasonable for intraspecific comparisons (6,29,30), it is most likely not true for interspecific comparisons. However, each primer was capable of distinguishing *M. poae* isolates from an outgroup of six ectotrophic, dematiaceous fungi that are genetically similar on a taxonomic (12) and molecular (21) basis. Isolates of *M. poae* contained both fixed and polymorphic markers not present in one isolate of *Leptosphaeria korrae* J. C. Walker & A. M. Sm., two isolates of *M. grisea* (T. T. Hebert) Barr, or three isolates of *C. graminicola* (Table 3). This result suggests that RAPDs have the potential to aid in distinguishing *M. poae* from other closely related fungi. Traditionally, positive diagnosis of *M. poae* required the pairing of unknown isolates with opposite mating types in culture to produce perithecia, a tedious and time-consuming process (24). In the present study, six of the 35 suspected *M. poae* isolates

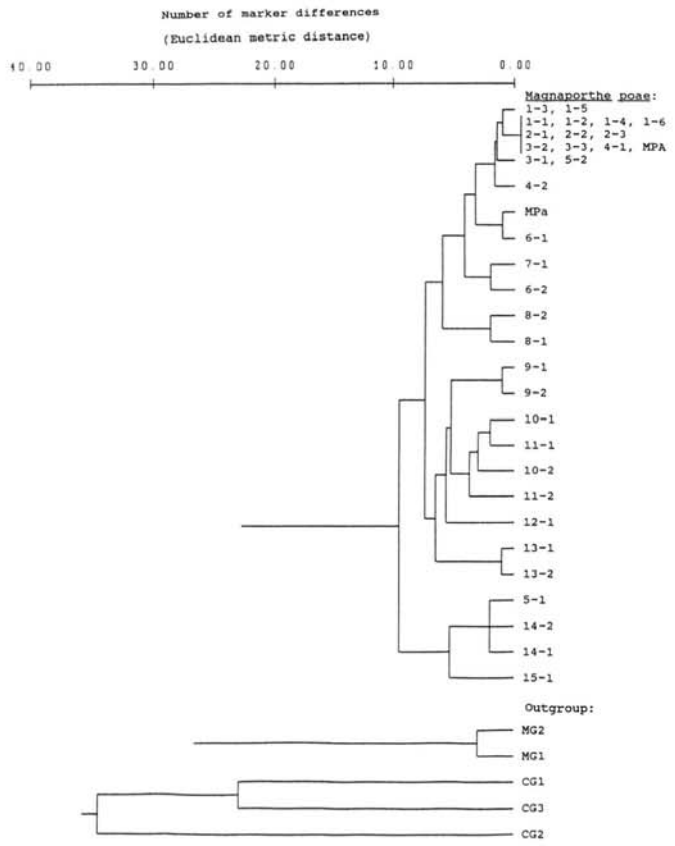

Fig. 1. Dendrographic relationships among phenotypically unique random amplified polymorphic DNA patterns of 35 isolates of *Magnaporthe poae*, two isolates of *M. grisea*, or three isolates of *Colletotrichum graminicola*. Branch length is proportional to the Euclidean metric distance (number of marker differences) among isolates within a species.

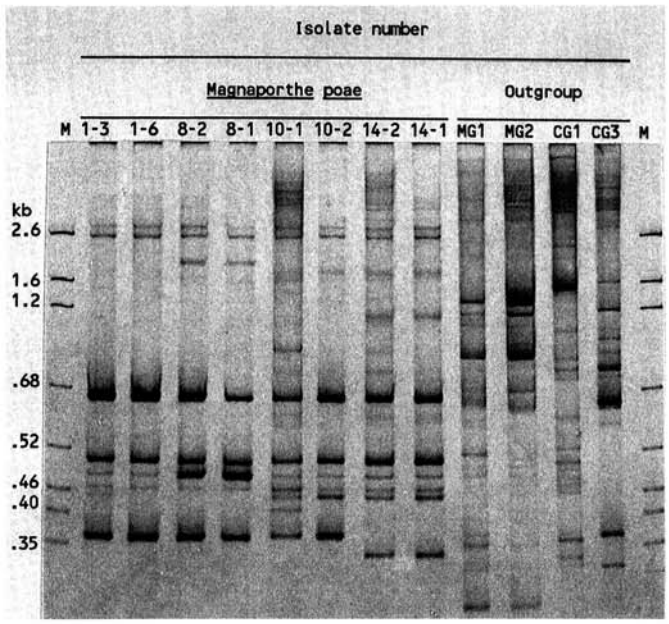

Fig. 2. Polyacrylamide gel of silver-stained random amplified polymorphic DNA patterns generated with primer OPA-3 for eight isolates of *Magnaporthe poae* (lanes 2–9), two isolates of *M. grisea* (lanes 10 and 11), and two isolates of *Colletotrichum graminicola* (lanes 12 and 13). PGEM *Hin*dIII size markers (labeled M, lanes 1 and 14) were measured in kilobases.

TABLE 3. Attributes of oligonucleotide primers used for generating random amplified polymorphic DNA patterns from 35 isolates of *Magnaporthe poae*

Primer	Nucleotide sequence 5′ to 3′	Polymorphic markers	Unique phenotypes	Fixed markers absent in outgroup	Polymorphic markers absent in outgroup
OPA-3	AGTCAGCCAC	12	10	2	9
OPA-4	AATCGGGCTG	2	3	0	2
OPA-5	AGGGGTCTTG	2	4	1	2
OPA-12	TCGGCGATAG	6	17	0	4
Total		22	23	3	17

did not produce perithecia with this mating procedure (Table 1). Even so, analysis of RAPD patterns showed that the genetic distances among these otherwise unidentifiable isolates were comparable to the remaining isolates within the species (Fig. 1). The identity of these six isolates was also confirmed by using a DNA probe specific for *M. poae* (21).

The intraspecific Euclidean distances among the 23 unique phenotypes of *M. poae,* the two isolates of *M. grisea,* or the three isolates of *C. graminicola* are presented in Figure 1. Within *M. poae,* the genetic distance between any two isolates ranged from a 4% difference (1 band difference out of 25 total bands scored) to a 60% difference (15 band differences out of 25 total bands scored). The two isolates of *M. grisea* showed a 13% difference (3 out of 23), while isolates of *C. graminicola* were the most variable, showing an intraspecific distance of 47% (23 out of 49) to 78% (38 out of 49). Similarly, a high degree of variation between three isolates of *C. graminicola* collected from the United States was reported by Guthrie et al (4). For comparison purposes, we converted the RAPD marker data presented by Guthrie et al (4) to Euclidean distances and revealed that the differences between their isolates of *C. graminicola* (TX430BB85, ARKB1, and GA53.90) ranged from 30% (6 out of 20) to 70% (14 out of 20). These results indicate that natural populations of *M. poae* may possess levels of genetic variation comparable to those of other closely related sexual species.

AMOVA was used to examine genetic variation of *M. poae* within and between 12 collection locations (Tables 1 and 4). AMOVA demonstrated that isolate differences among the 12 locations were highly significant ($P = 0.001$) when compared with the distribution of differences generated through random permutations of the data set. The observed data showed that 61% of the total genetic variation was attributable to location differences. Although these results are based on a limited sample, they nonetheless suggest that a unique set of individuals may exist within most locations. If sample sizes were to increase, however, we expect the relative relationships between populations would likely change because of corresponding fluctuations in genetic variance (18). Research programs concerned with obtaining genetically representative isolates of *M. poae* would be advised to examine isolates from as many locations as possible. Even so, the substantial isolates-within-location variance component (39% of the total variance) illustrates the extent to which RAPDs can be used to detect genetic variation among isolates. Locations that display a particularly high degree of variability (i.e., Marlton, NJ, isolates MP 5-1 and MP 5-2) (Fig. 1) might be useful study sites for evaluating summer patch control methods or for investigating disease dissemination patterns.

M. poae is known to occur throughout southern New England, the mid-Atlantic states, and the Midwest. It has also been identified in southern California and Washington state (9). Because neither the perfect nor imperfect state of *M. poae* has ever been observed in nature, the pathogen has been presumed to be disseminated in the vegetative state (13). Inferences concerning the observed pattern of genetic variation are limited because of the small sample sizes involved. The fact that disjunct locations were found to possess isolates of indistinguishable RAPD patterns suggests that some isolates may have migrated recently between locations. Such movement could have occurred by people having utilized or maintained turfgrass at different sites (13), the transportation of contaminated maintenance equipment, or the transplanting of infected sod (26). However, genetic variation was observed among isolates within all but one location and within each mating type (Fig. 1 and Table 1). The extent and source (sexual reproduction, mutation, mitotic recombination, or some combination of these forces) of this genetic variation is unknown. In the future, the use of RAPD markers along with AMOVA on larger sampling frames should allow us to make stronger inferences regarding reproductive modes and patterns of dispersal, thereby increasing our understanding of the ecology and biology of *M. poae.*

TABLE 4. Analysis of molecular variance for 35 isolates of *Magnaporthe poae* within and among 12 collection locations containing two or more isolates

Source of variation	df	Sum of squares deviation	Mean square deviation	Variance component[a]	*P* value[b]
Between locations	11	49.17	4.47	1.44 (61.2)	<0.001
Isolates within locations	18	16.50	0.92	0.92 (38.8)	

[a]Number in parentheses is percentage of the total.
[b]Probability computed by nonparametric procedures from 1,000 data permutations.

In greenhouse trials, Landschoot et al (14) evaluated the pathogenicity of 47 isolates of *M. poae,* 12 of which were also utilized in our study (Table 1). Although they found that the pathogenicity of the 47 isolates was quite variable, our study showed that pathogenicity and genetic distance were significantly correlated ($r_{sp} = 0.593$; $n = 12$; and $P = 0.05$). This limited relationship suggests that pathogenicity of *M. poae* decreased as genetic distance from the most pathogenic isolate increased. Among these 12 isolates, 35% of the variation in pathogenicity was attributable to genetic distance.

In summary, our results demonstrate that RAPD markers have potential as a means of identifying *M. poae* isolates and increasing our understanding of the ecology and biology of this fungus by providing measurements of genetic relatedness and variation within and between fungal populations. Furthermore, the potential ability to correlate specific traits, such as pathogenicity, by using RAPDs may further enhance efforts to monitor and predict disease outbreaks in the future.

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