Genetics

Restriction Fragment Length Polymorphisms in the Wheat Glume Blotch Fungus, *Phaeosphaeria nodorum*

P. P. Ueng, G. C. Bergstrom, R. M. Slay, E. A. Geiger, G. Shaner, and A. L. Scharen

First, third, and fourth authors, USDA-ARS, Plant Molecular Biology Laboratory, Beltsville, MD 20705; second author, Department of Plant Pathology, Cornell University, Ithaca, NY 14853; fifth author, Department of Botany and Plant Pathology, Purdue University, W. Lafayette, IN 47907; sixth author, USDA-ARS, Department of Plant Pathology, Montana State University, Bozeman 59717.

ABSTRACT

Ueng, P. P., Bergstrom, G. C., Slay, R. M., Geiger, E. A., Shaner, G., and Scharen, A. L. 1992. Restriction fragment length polymorphism in the wheat glume blotch fungus, *Phaeosphaeria nodorum*. Phytopathology 82:1302-1305.

To develop genetic markers in the fungus *Phaeosphaeria nodorum*, incitant of leaf and glume blotch of cereals and grasses, genomic DNA from 11 geographically diverse isolates from wheat was used to compare restriction fragment length polymorphisms (RFLPs). An isolate from winter wheat in Cayuga County, New York, was used to construct genomic clones, of which 56 were randomly chosen as probes. Twenty-two of the probes produced unique hybridization patterns with the 11 isolates. RFLP loci exhibited one (13 probes), two (seven probes), three (one

probe), or four (one probe) fragment length variants. The isolates could be differentiated by hybridization with a combination of as few as two probes. Our results suggest that DNA fingerprinting may be a useful method for assessing the amount and distribution over space and time of genetic variation in populations of *P. nodorum* as well as for following the fate of introduced 'marked' isolates in epidemiological studies. RFLPs may also have utility in developing genetic maps of *P. nodorum* for virulence and other traits.

Additional keywords: Leptosphaeria nodorum, Septoria nodorum, Stagonospora nodorum, Triticum aestivum.

In recent years, restriction fragment length polymorphism (RFLP) analysis has become a common tool to study the genetics of variation and inheritance in plants (20). This technique offers a relatively easy method of assaying for genetic variation and can provide an unlimited number of genetic markers with or without knowledge of the physiological or morphological characteristics of the markers. It has similar use in the study of plant pathogenic fungi. For example, RFLP analysis was used recently to locate the putative virulence loci in the genome of the barley powdery mildew fungus (3). It has also been shown that an RFLP probe from the rice blast fungus could reveal repeated DNA sequences that specifically identified only those pathotypes of the fungus that were able to infect rice plants (7,10). A set of probes prepared from *Mycosphaerella graminicola* genomic DNA has been used to measure genetic variation among and within fungal populations (11,12).

The fungus *Phaeosphaeria nodorum* (E. Müller) Hedjaroude. = *Leptosphaeria nodorum* E. Müller (anamorph: *Stagonospora nodorum* (Berk.) Castellani & E.G. Germano. = *Septoria nodorum* (Berk.) Berk.) is a foliar and glume pathogen of wheat (*Triticum aestivum* L.) as well as of other cereals and grasses and is of worldwide economic importance (8,17). Isolates of the fungus vary in pathogenicity on different plant species (2,4,8,14) as well as on differential cultivars of the same host species (1,8,14,16-18). The pathogenicity of different isolates of *P. nodorum* is highly variable and is not correlated with their geographic origin (16) nor their morphology (4,14). The process of genetic segregation during ascospore development in the fungus has recently been described (18). Further advances in understanding the genetic basis for pathogenicity in *P. nodorum* and the epidemiology of glume blotch, especially sources and dispersal of inoculum, would be aided greatly by the availability of genetic markers in the fungus.

The goal of this study was to develop a set of genetic markers, based on RFLPs, that could be used to study genetic variation

in populations of *P. nodorum*, to monitor the fate of 'marked' isolates in the environment, and to develop genetic maps of the fungus. A preliminary report of these findings has been published (21).

MATERIALS AND METHODS

Isolation of fungal genomic DNA. Nine single conidium isolates of *P. nodorum* collected from wheat leaves or glumes in western counties of New York between 1986 and 1989 were used in this study (Table 1). One isolate each from Montana and Indiana, SN213MT-89 and SN91-X, respectively, were included for comparison. Total genomic DNA was isolated from *P. nodorum* following the procedure of Lee et al (9) with modifications. The fungus was maintained on yeast malt sucrose (YMS) agar (0.5% yeast extract, 0.5% malt extract, 2% sucrose, and 1.5% agar) and was inoculated to a liter of YMS liquid medium by mycelial agar block. The culture was grown for 7–10 days at room temperature (25–28 C) with constant shaking (150 rpm). After that time, the mycelial mass reached maximum growth and was harvested by filtration through Miracloth (Calbiochem, La Jolla, CA) and centrifugation. Before DNA extraction, the mycelial pellet was

TABLE 1. *Phaeosphaeria nodorum* isolates used for restriction fragment length polymorphism analysis

Isolate	Origin	Year of isolation	Wheat cultivar
SN015NY-86	Tompkins Co., NY	1986	Frankenmuth
SN038NY-89	Orleans Co., NY	1989	Geneva
SN039NY-89	Schuyler Co., NY	1989	Fredrick
SN043NY-89	Genesee Co., NY	1989	Geneva
SN046NY-89	Seneca Co., NY	1989	Houser
SN052NY-89	Ontario Co., NY	1989	Geneva
SN058NY-89	Monroe Co., NY	1989	Geneva
SN167NY-87	Steuben Co., NY	1987	Geneva
SN209NY-88[a]	Cayuga Co., NY	1988	Geneva
SN213MT-89	Montana	1989	Not known
SN91-X	Indiana	1990	Not known

[a] Isolate used to develop 56 probes used in RFLP analysis.

resuspended in 150 ml of 0.1 M ethylenediamentetaacetic acid (EDTA) (pH 8.0) solution and stirred for 30 min at room temperature. The fungal mycelia were recovered by centrifugation, and ground with a mortar and pestle in liquid nitrogen. After thawing, the fungal tissue was further ground with 100 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 3% sodium dodecyl sulfate [SDS], and 1% 2-mercaptoethanol) and heated in a 68 C water bath for 60 min. A 100-ml volume of a phenol/chloroform mixture (1:1, v/v) was added to the sample and stirred for 30 min at room temperature. The solution was centrifuged at 16,500 relative centrifugal force (RCF) for 10 min, and the upper aqueous phase was retained and re-extracted with phenol/chloroform once. The DNA was precipitated by adding 0.1 vol of 3.0 M sodium acetate (pH 7.0) and 2.5 vol of absolute alcohol. The DNA thread was spooled with a sterile glass pipet and transferred to a centrifuge tube. The pellet was washed once with 70% ethanol and drained to dry briefly. Fifteen milliliters of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to the pellet. The pellet was dissolved by gentle shaking and intermittent heating at 68 C, and more 1× TE buffer was added if necessary. Cesium chloride (CsCl, 8.6 g) and 0.3 ml of ethidium bromide (EtBr, 5 mg/ml) were added per 7.5 ml of DNA solution. The mixture was ultracentrifuged at 157,516 RCF for 15 h in a vertical rotor (VTi65.2, Beckman). The DNA band, distinct from a brownish polysaccharide band, was collected and extracted with CsCl-saturated 1-butanol. The genomic DNA was precipitated with 3 vol of water and 4 vol of isopropanol and washed with 70% ethanol. After vacuum drying, the DNA was resuspended in 1× TE buffer.

Construction of a genomic library. A size-fractionated genomic library was constructed from the SN209NY-88 isolate of *P. nodorum*. The genomic DNA was restricted with endonuclease enzyme *Eco*RI (Promega, Madison, WI) and resolved with gel electrophoresis using 1% agarose (w/v) in 1× TBE (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.0) (15). The DNA fragments, ranging from about 1.0 to 4.0 kb, were isolated from sliced agarose blocks by the unidirectional electroelution technique using an electroelutor (Model UEA, IBI, New Haven, CT) and cloned into the *Eco*RI site of the plasmid pGEM 3Z (Promega, Madison, WI). Competent cells of JM83 were transformed, and recombinants appeared as white colonies on LB (Luria-Bertani) agar (10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl per liter with 10 mM MgSO₄ and 1.5% [w/v] agar) containing ampicillin (50 μg/ml), isopropyl β-D-thio-galacto-pyranoside (IPTG) (70 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (70 μg/ml). The plasmid DNA was purified preparatively by alkali lysis and CsCl-EtBr density gradient ultracentrifugation (15).

DNA hybridization. The genomic DNA prepared from 11 *P. nodorum* isolates was cleaved with *Eco*RI restriction enzyme. DNA (5 μg) was restricted with 15 units of enzyme at 37 C overnight. The restricted DNA was resolved by 1% agarose gel electrophoresis at constant voltage (25 V). The gel was pretreated with 0.25 N HCl for 15 min, 1.5 M NaCl/0.5 N NaOH for 25 min, and 1.5 M NaCl/0.5 M Tris-HCl (pH 7.4) for 25 min, successively. The DNA fragments were transferred to Nytran membranes (pore size 0.45 μm, Schleicher & Schuell, Keene, NH) using 10× SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) (19). The blots were then vacuum-baked at 80 C for 2 h. Fifty-six genomic DNA clones constructed from the SN209NY-88 isolate were randomly chosen as the probes for DNA hybridization.

The DNA fragments were digested with *Eco*RI enzyme, electrophoresed, isolated from agarose gel blocks by the unidirectional electroelution technique, and labeled with α-P³²-dATP using the random-priming technique (5,6). The specific activity of the DNA probe was about 1 × 10⁸ dpm/μg. Probes with 2 × 10⁶ dpm of activity were used for each membrane (10.5 × 14 cm in size) in DNA hybridization. Each membrane was prehybridized with 20 ml of solution containing 6× SSC, 0.5% SDS, and 1× Denhardt's solution (50× Denhardt's reagent contains 5 g of Ficoll [Type 400, Pharmacia, Piscataway, NJ], 5 g of polyvinyl-pyrrolidone, and 5 g of bovine serum albumin [Fraction V, Sigma,

St. Louis, MO] per 500 ml) at 68 C, and hybridized in 10 ml of the same solution plus denatured, P³²-labeled probe and calf thymus DNA (10 μg/ml) (Sigma, St. Louis, MO) at 68 C. After DNA hybridization, the membranes were washed twice with hot (68 C) 3× SSC/0.1% SDS solution and blotted dry. X-ray film (X-OMAT, Kodak, Rochester, NY) was exposed to the membranes overnight at −70 C and developed.

RESULTS AND DISCUSSION

The size range of the genomic DNA fragments cloned into the plasmid vector in the library was 0.9–2.5 kb, with the majority about 1.0–1.3 kb. Most of the probes detected only a single discrete
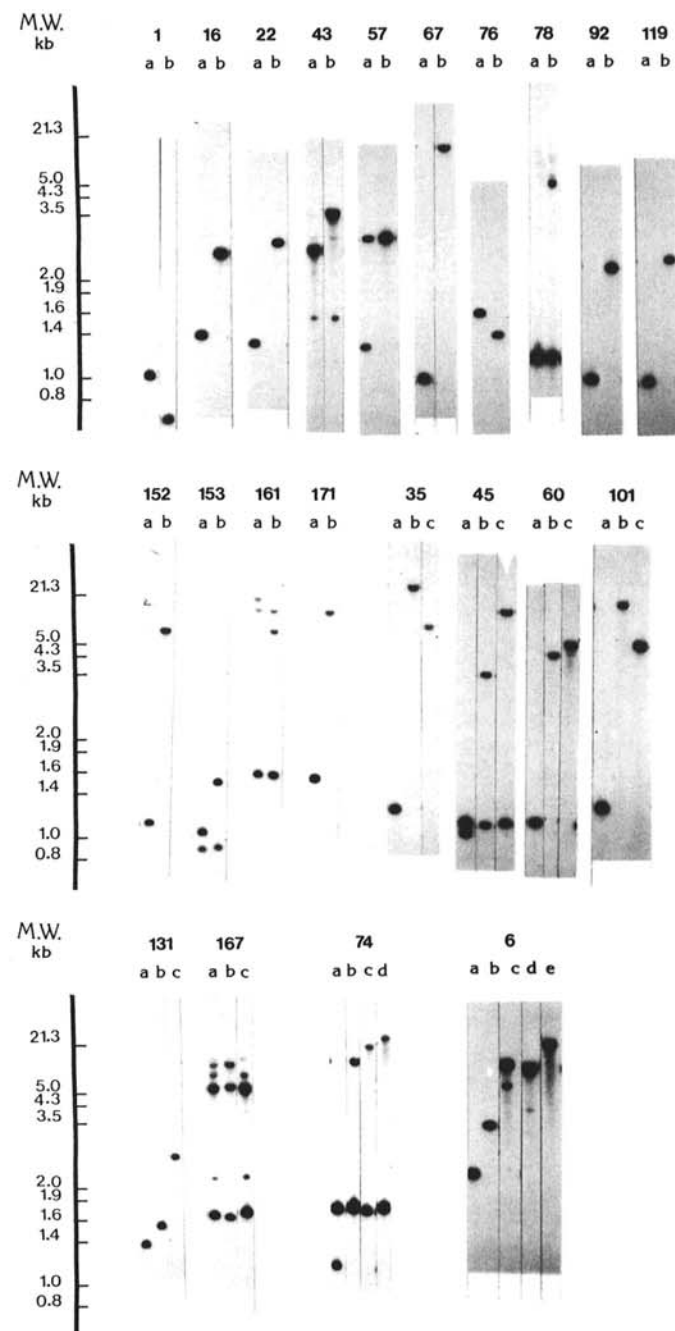

Fig. 1. Restriction fragment length polymorphisms of 22 different probes with 11 *Phaeosphaeria nodorum* isolates. The numbers at the top of each column are the genomic DNA clones used as probes in the Southern hybridizations. In each group, "a" represents the restriction pattern of isolate SN209NY-88 from which all clones were prepared. Letters "b–e" indicate the restriction patterns of other isolates used in this study (see Table 2 for isolates represented by a–e). Size markers were prepared from lambda DNA doubly restricted with *Hin*dIII and *Eco*RI enzymes.

band in Southern hybridization analysis (Fig. 1). A few of the probes did, however, hybridize to two or more bands in the RFLP profiles. When the genomic DNA of one of the isolates, SN209NY-88, was digested with *Eco*RI endonuclease enzyme and electrophoresed on an agarose gel, three distinct bands, 3.0, 1.4, and 1.2 kb in size, could be visualized within the smear with ethidium bromide staining (unpublished data). This is different from what has been reported in the barley powdery mildew fungus genomic DNA where a large number of bands was shown (3). In another experiment, P^{32}-labeled, *Eco*RI-restricted genomic DNA, from the isolate SN209NY-88, was hybridized to a lambda-gt10 library constructed strictly from 3.0–10.0 kb size *Eco*RI-restricted DNA fragments. Very few of the bacteriophage clones hybridized strongly (about 120 out of 250,000 plaques) (unpublished data). Of the lambda phage clones that hybridized, all could detect a single strong band, but not multiple discrete bands in RFLP profiles (unpublished data). It is likely that each of these clones have part of the rDNA sequence.

The results demonstrate that there is a low frequency of repeated sequences in *P. nodorum* genomic DNA, which is different from a recent report for the wheat fungal pathogen *Mycosphaerella graminicola* (11,12). The small amount of repetitive DNA in the *P. nodorum* genome corresponds to the results found with other necrotrophic fungi, but is contrary to what has been observed in biotrophic parasites (3). Multiple copy sequences are very useful because the presence of such sequences indicates the occurrence of frequent polymorphism in fungal populations. Furthermore, some specific repeated DNA sequences from *Magnaporthe grisea* reportedly can be used to distinguish among pathotypes of the rice pathogen (10), as well to distinguish isolates that do not infect rice plants. However, it is not clear if these multiple sequences are responsible for the pathogenicity of this fungus on rice (7). Nevertheless, for the purpose of identifying virulence loci and their possible linkages in the genome, nonrepetitive DNA sequences were more useful (3). The genetic similarity among isolates in a fungal population could not be simply determined for *M. graminicola* by the DNA fingerprint patterns detected by either single copy or multiple sequence probes (13).

Of fifty-six clones randomly chosen for RFLP analysis, 22 displayed unique hybridization patterns with the *P. nodorum* isolates that were studied (Fig. 1, Table 2). RFLP loci exhibited one (13 probes), two (seven probes), three (one probe), or four (one probe) fragment length variants. Several individual probes could specifically identify certain isolates, e.g., probe no. 60 distinguished SN015NY-86 and SN058NY-89 from the rest of the isolates, and probe no. 74 distinguished SN052NY-89 and SN213MY-89 from each other and from all other isolates (Table 2). In addition, isolate SN91-X from Indiana was uniquely identified by probes nos. 35 and 131 and isolate SN213MT-89 from Montana was uniquely identified by probes nos. 45, 74, and 167. Isolate SN052NY-89 was the only isolate to show the presence of a high molecular weight band that hybridized with probe no. 78 (Fig. 1, no. 78-[b]). All 11 isolates could be differentiated by hybridization with a combination of as few as two probes.

Our results suggest that DNA fingerprinting may be a useful method for detecting genetic variation in populations of *P. nodorum*. DNA fingerprints may also advance epidemiological studies that require 'marked' isolates that can be traced in the environment. For example, in New York, winter wheat seed lots have been infected with fingerprinted, clonal isolates of *P. nodorum* to investigate the role of seedborne inoculum in the initiation of glume blotch epidemics (D. Shah, G. C. Bergstrom, and P. P. Ueng, *unpublished*). In addition, RFLPs may have utility in development of genetic maps of *P. nodorum* for pathogenicity and other traits.

TABLE 2. Variations of restriction fragment length polymorphism patterns in *Phaeosphaeria nodorum*

Probes[a]	*Phaeosphaeria nodorum* isolates										
	15	38	39	43	46	52	58	167	209	213	91-X
1	a	a	a	b	b	b	b	a	a	a	b
6	c	d	b	e	c	e	d	c	a	b	b
16	b	b	b	b	a	b	b	b	a	b	b
22	a	a	b	a	a	a	b	b	a	b	a
35	b	b	a	a	a	a	b	a	a	a	c
43	b	a	a	b	a	a	b	a	a	a	b
45	a	a	a	a	a	c	a	a	a	b	c
57	a	a	b	a	a	b	a	a	a	a	a
60	b	a	a	a	a	a	c	a	a	a	a
67	b	b	b	b	a	b	b	b	a	a	b
74	b	a	a	a	a	d	b	b	a	c	a
76	a	a	a	a	b	b	b	b	a	a	a
78	a	a	a	a	a	b	a	a	a	a	a
92	a	a	a	a	a	a	a	b	a	b	b
101	c	b	a	b	c	a	b	a	a	b	c
119	b	b	a	b	a	a	a	b	a	b	a
131	a	b	b	a	b	a	b	b	a	b	c
152	b	a	a	a	a	a	b	a	a	b	b
153	a	a	a	a	a	a	a	b	a	b	a
161	a	a	a	b	a	a	b	a	a	a	a
167	a	a	a	c	a	a	c	a	a	b	a
171	b	a	b	b	b	b	a	b	a	b	b

[a] Derived from cloned fragments of genomic DNA from isolate SN209NY-88 after digestion with *Eco*RI restriction enzyme. Banding profile in isolate 209 labeled with each probe is designated as pattern "a". Different letters within each row indicate different banding patterns labeled with that probe (see Fig. 1).

LITERATURE CITED

1. Allingham, E. A., and Jackson, L. F. 1981. Variation in pathogenicity, virulence, and aggressiveness of *Septoria nodorum* in Florida. Phytopathology 71:1080-1085.
2. Berecket, T., Hebert, T. T., and Leonard, K. J. 1990. Virulence of *Septoria nodorum* as affected by passage through detached leaves of wheat and barley cultivars. J. Phytopathol. 128:125-136.
3. Christiansen, S. K., and Giese, H. 1990. Genetic analysis of the obligate parasitic barley powdery mildew fungus based on RFLP and virulence loci. Theor. Appl. Genet. 79:705-712.
4. Cunfer, B. M., and Youmans, J. 1983. *Septoria nodorum* on barley and relationships among isolates from several hosts. Phytopathology 73:911-914.
5. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
6. Feinberg, A. P., and Vogelstein, B. 1984. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266.
7. Hamer, J. E., Farrall, L., Orbach, M. J., Valent, B., and Chumley, F. G. 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc. Natl. Acad. Sci. USA 86:9981-9985.
8. King, J. E., Cook, R. J., and Melville, S. C. 1983. A review of Septoria diseases of wheat and barley. Ann. Appl. Biol. 103:345-373.
9. Lee, S. B., Milgroom, M. G., and Taylor, J. W. 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Genet. Newslett. 35:23-24.
10. Levy, M., Romao, J., Marchetti, M. A., and Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95-102.
11. McDonald, B. A., and Martinez, J. P. 1990. Restriction fragment length polymorphisms in *Septoria tritici* occur at a high frequency. Curr. Genet. 17:133-138.
12. McDonald, B. A., and Martinez, J. P. 1990. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. Phytopathology 80:1368-1373.
13. McDonald, B. A., and Martinez, J. P. 1991. DNA fingerprinting of the plant pathogenic fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*). Exp. Mycol. 15:146-158.
14. Rufty, R. C., Hebert, T. T., and Murphy, C. F. 1981. Variation in virulence in isolates of *Septoria nodorum*. Phytopathology 71:593-596.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Pages 36-39 in: Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring

Harbor Laboratory, Cold Spring Harbor, New York.

16. Scharen, A. L., and Eyal, Z. 1983. Analysis of symptoms on spring and winter wheat cultivars inoculated with different isolates of *Septoria nodorum.* Phytopathology 73:143-147.

17. Scharen, A. L., Eyal, Z., Huffman, M. D., and Prescott, J. M. 1985. The distribution and frequency of virulence genes in geographically separated populations of *Leptosphaeria nodorum.* Phytopathology 75:1463-1468.

18. Skajennikoff, M., and Rapilly, F. 1989. "Variability of pathogenicity in *Septoria nodorum* Berk. (= *Leptosphaeria nodorum* Mull.)."

Agronomie 9:693-70.

19. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

20. Tanksley, S. D., Young, N. D., Paterson, A. H., and Bonierbale, M. W. 1989. RFLP mapping in plant breeding: New tools for an old science. Biotechnology 7:257-264.

21. Ueng, P. P., Slay, R. M., Geiger, E. S., Shaner, G., Sharen, A. L., and Bergstrom, G. 1991. RFLP maps in fungus *Stagonospora nodorum,* a causal agent of wheat glume blotch disease. Phytopathology 81:1229.