A Molecular Marker Identifying Subspecific Populations of the Soybean Brown Stem Rot Pathogen, *Phialophora gregata*

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Accepted for publication 27 April 2000.

ABSTRACT

Chen, W., Grau, C. R., Adee, E. A., and Meng, X.-Q. 2000. A molecular marker identifying subspecific populations of the soybean brown stem rot pathogen, *Phialophora gregata*. *Phytopathology* 90:875-883.

A molecular marker was developed to separate and identify subspecific populations of *Phialophora gregata*, the causal agent of soybean brown stem rot. A variable DNA region in the intergenic spacer of the nuclear rDNA was identified. Two specific primers flanking the variable region were developed for easy identification of the genotypes using polymerase chain reaction (PCR). These two specific primers amplified three DNA products. The three PCR products were used to separate isolates of *P. gregata* into distinct genotypes: A (1,020 bp), B (830 bp), and C (660 bp). Genotype C was found in isolates obtained from Adzuki beans from Japan, whereas all 292 isolates obtained from

soybean and the 8 isolates from mung bean belonged to either genotype A or B. The original nondefoliating (type II) strain ATCC 11073 (type culture of *P. gregata*) belonged to genotype B. The difference between genotypes A and B was due only to an 188-bp insertion or deletion; genotype C, however, differs from genotypes A and B at 58 point mutations, in addition to the length difference. Isolates of both genotypes A and B were widespread in seven Midwestern states. Genotype A was found mostly in certain susceptible soybean cultivars like Sturdy and Pioneer 9305, whereas genotype B was found predominately in brown stem rot-resistant soybean cvs. Bell, IA 3003, and Seiben SS282N. The specific primers were also used to directly detect cultivar-preferential infection by the two genotypes in infected soybean stems growing in the same field. Data from direct detection in soybean stems showed that cultivar-preferential infection by the two genotypes of *P. gregata* was significant.

The fungal pathogen *Phialophora gregata* (Allington & D.W. Chamberlain) W. Gams causes soybean brown stem rot (BSR). The disease has been reported in the United States (3), Canada (22), Egypt (17), Japan (24), Mexico (31), and Yugoslavia (41). In the United States, it occurs mainly in the north central states (16,29,46), although it also has been reported in some eastern and southern states (12,17). The fungus is soilborne, infects soybean vascular tissue, and causes discoloration of the pith and vascular tissue of the stem and interveinal necrosis. The disease may cause up to 30% yield loss under conditions favorable for disease development (1,29,36). Resistant cultivars and crop rotation are the only practical methods for controlling BSR. Significant progress has been made in breeding soybeans for BSR resistance and in understanding the soybean genetics of BSR resistance (9,26,33,44) and the effect of cultural practices on development of the disease (2,19,29,36). However, limited information is available on the genetics of the pathogen, particularly on the genetic structure of *P. gregata* that infects soybean.

Isolation and identification of the pathogen is problematic due to the presence of other morphologically similar fungi in soybean stems and the slow growth of *P. gregata* (28). The pathogen can be differentiated from other fungi associated with soybean stems based on isozyme banding patterns (28), DNA sequences of internal transcribed spacer (ITS) regions of rDNA (10,12,20), and total cellular DNA content determined with a flow cytometer (15). Based on ITS sequences, Chen et al. (10,12) identified one of the

fungi ecologically associated with BSR, *Plectosporium tabacinum*, which was later confirmed by Harrington et al. (20) also using ITS sequences. Specific primers were developed for detection of *P. gregata* in infected stems of resistant and susceptible soybean cultivars (10).

Variation in virulence among *P. gregata* isolates has been reported. Gray (16) reported two pathogenicity types of isolates in *P. gregata*, which were also observed in other independent studies (28,45). Defoliating (type I) isolates caused vascular discoloration and wilting symptoms, whereas nondefoliating (type II) isolates, which include the type culture of *P. gregata*, cause only vascular discoloration in stems (16). A high level of variability in virulence was found using a set of four differential soybean cultivars (45). Mengistu and Grau (28) found a wide range of pathogenicity among 24 isolates of *P. gregata* on a susceptible soybean cultivar. Isolates of *P. gregata* obtained from the same field also exhibited variation in pathogenicity against soybean genotypes (36).

Isolates of *P. gregata* from soybean and Adzuki bean have different pathogenicity spectra and are recognized as different formae specialis (24). Genetic differences between the formae speciales are well documented (10,11,18,47,48). However, isolates from soybean appear monomorphic based on isozyme banding patterns (28), restriction banding patterns of mitochondrial DNA (18), and ITS sequences of rDNA (10).

rDNA repeat units have coding and noncoding spacer regions that evolve at different rates (7). The spacer regions evolve rapidly and are useful in studying closely related taxa. The ITS region is useful for taxonomic studies at the species level (13). The intergenic spacer region (IGS) between rDNA transcription units is more variable and has been used to discriminate subspecific populations of plant-pathogenic fungi (5,6,32,38,39). Henrion et al. (21) found that the IGS region was variable within species of truffles.

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Publication no. P-2000-0619-02R

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The IGS region was also used to differentiate strains in clinically important fungi (23,34).

This study was aimed at discovering genetic structure within *P. gregata* isolated from soybean by exploring the variation in the IGS region of nuclear rDNA. We found that the IGS region can be used to subdivide *P. gregata* into distinct genotypes and that the genotypes of *P. gregata* preferentially infect certain soybean cultivars under field conditions.

MATERIALS AND METHODS

Fungal strains and DNA isolation. Strains of *P. gregata* were isolated from infected soybean plants with a semiselective medium (30) or water agar (16 g of agar per liter of water) amended with 100 ppm of streptomycin. Each isolate was obtained from a different plant. Isolates of *P. gregata* were collected from seven states in the United States and from Brazil, Canada, and Japan (Table 1). Strains were further purified either from the hyphal tip or by isolation of single conidia. Strains were grown in green bean agar (25 g of frozen green beans blended and filtered through four layers of cheesecloth with 15 g of agar per 1 liter) for 2 weeks. Agar plugs containing mycelium and conidia were stored in sterile water at 4°C.

Strains were grown in green bean broth for 2 weeks, and the mycelium was harvested by filtration. The harvested mycelium was used either immediately in DNA isolations or frozen at -80°C until used. The mycelium was ground, using a mortar and pestle, to a fine powder in liquid nitrogen, and DNA was isolated from

the mycelium powder as previously described (10). DNA was also isolated from mycelium grown in potato dextrose agar using the FastDNA kit (Bio 101, Vista, CA) following the manufacturer's instructions.

Polymerase chain reaction and DNA cloning. Polymerase chain reaction (PCR) primers 28SF and CNS1R, based on the conserved 28S and 18S coding regions, were used to amplify the IGS region between transcription units of rDNA (Fig. 1) in four soybean isolates: H96-5, Pg11, Pg13, and Pg19 of *P. gregata*. Primers 28SF (5'CTG AAC GCC TCT AAG TCA GAA3') and CNS1R (5'GAG ACA AGC ATA TGA CTA C3') were the reverse complements of primers LR12 (42) and NS1 (43), respectively. PCR reactions consisted of 0.3 µM of each primer, 10 to 50 ng of DNA, 200 µM dNTPs, 2 mM MgSO₄ and 2.5 units of Platinum *Taq* DNA polymerase high fidelity (GIBCO BRL, Rockville, MD) in 1× PCR buffer in a total volume of 50 µl. All PCR reactions were set up using pipette tips with aerosol barriers to prevent contamination. Temperature cycles were 94°C for 75 s, 55°C for 30 s, and 70°C for 6 min for the first cycle, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 70°C for 6 min. The temperature was then reduced to and kept at 4°C. The efficacy of the PCR was monitored by running 5 µl of the PCR product through 1% agarose gel electrophoresis.

Restriction enzyme digestion was used to detect variation in the PCR amplified IGS region. Eight six-base recognition enzymes, *AccI*, *Bam*HI, *Hinc*II, *Hind*III, *Eco*RI, *Eco*RV, *Nco*I and *Sst*I

TABLE 1. Geographic distribution of isolates of *Phialophora gregata* used in this study

State/Country	Location	Number of isolates of <i>P. gregata</i>		
		Genotype A	Genotype B	Total
Illinois	Belvidere	8	6	14
	DeKalb	6	0	6
	Monmouth	52	71	123
	Urbana	7	2	9
	Unknown	4	0	4
Indiana	Unknown	8	2	10
Iowa	Unknown	7	2	9
Michigan	Unknown	3	2	5
Minnesota	Unknown	3	3	6
Ohio	Unknown	2	1	3
Wisconsin	Arlington	20 ^a	15	35
	Dairyland	2	0	2
	Hancock	30	16	46
	Lancaster	1	0	1
	Janesville	1	0	1
	West	2	0	2
	Madison			
	Unknown	15 ^a	2	17
Brazil	Unknown	3	0	3
Canada (Ontario)	Unknown	4	0	4
Japan	Unknown	...	...	2 ^b
Total		178	122	302

^a Includes one genotype A' isolate.

^b Two genotype C isolates were obtained from Adzuki beans, all other isolates were from either soybean (292 isolates) or mung bean (8 isolates).

Fig. 2. Agarose gels showing **A**, polymerase chain reaction amplification of the intergenic spacer region (IGS) of *Phialophora gregata* using primers 28SF and CNS1R, and **B**, *Hind*III restriction enzyme digestion of the amplified IGS region. Lane L, BRL 1-kb DNA ladder with approximate DNA sizes indicated; lanes 1 to 4, isolates H96-5, Pg11, Pg13, and Pg19, respectively; lane N, negative control without DNA template.

Fig. 1. Schematic showing the nuclear ribosomal DNA (rDNA) repeat unit, locations of coding regions (boxed) and spacer regions (single lines), of the two conserved primers (28SF and CNS1R) used to amplify the intergenic spacer (IGS) region and of the two specific primers (BSRIGS1 and BSRIGS2) designed for the brown stem rot pathogen *Phialophora gregata*. The transcription unit of rDNA, the single *Hind*III restriction site (arrow) in IGS, and the amplified product size of the IGS region are indicated.

(GIBCO BRL) were used according to the manufacturer's instructions. Restriction fragments were separated by agarose (1%) gel electrophoresis and photographed under ultraviolet light after staining with ethidium bromide.

To facilitate cloning PCR products, an *EcoRI* site was added to the 5' end of both PCR primers, resulting in E28SF (5'GCG AAT TCC TGA ACG CCT CTA AGT CAG AA3') and ECNS1R (5'GCG AAT TCG AGA CAA GCA TAT GAC TAC3'). The *EcoRI* site was chosen because the enzyme *EcoRI* does not have a restriction site in the amplified IGS region (discussed below). The two primers were used in PCR as described above. The resulting PCR products were digested with restriction enzyme *HindIII* which is known to cut once in the amplified IGS region (Fig. 2B) and with *EcoRI* to cut the added restriction site in the PCR primers. This digestion process resulted in two DNA fragments, each with an *EcoRI* and a *HindIII* cohesive end, so that only one direction of insertion is possible during cloning. The two restriction fragments from each PCR product were separated by agarose gel electrophoresis. Bands were excised from the gel, purified with GeneClean (BIO 101, Inc., Vista, CA), and ligated into the *EcoRI* and *HindIII* sites of pGEM 3ZF(+) plasmid vector (Promega Corporation, Madison, WI). The resulting recombinant plasmids were used to transform *Escherichia coli* strain JM 109 high competent cells prepared according to standard protocols (35). Recombinant colonies were identified by color selection after overnight growth at 37°C on Luria-Bertani agar plates containing ampicillin at 150 µg/ml and X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) at 27 µg/ml and IPTG (isopropyl-thio-β-D-galactoside) at 27 µg/ml. Two to eleven white colonies from each insert were selected, and plasmid DNAs were isolated from each clone with the Wizard Plus SV Miniprep kit (Promega). After confirming the fragment size of the insert by digestion with enzymes *EcoRI* and *HindIII* and electrophoresis, the plasmid DNAs from each DNA insert of a given isolate were combined before sequencing.

DNA sequencing and specific PCR primers. A primer walking strategy was used to sequence the cloned DNA fragments. The two promoter sequences (T7 and SP6) located near each end of the insert on the vector were first used in cycle sequencing with fluorescent dye terminators in either the Thermal Sequenase kit (Amersham Life Sciences, Cleveland, OH) or the

ABI Prism BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA). DNA sequences were determined with the ABI 377 automated DNA sequencer at the Biotechnology Center of the University of Illinois, Urbana-Champaign. During the design of additional sequencing primers, DNA sequences of the same sequencing primer from all four isolates were compared using the program Sequencher (Gene Codes Corporation, Ann Arbor, MI) to ensure that the new primers would work for all four isolates.

The Sequencher program was also used to determine the DNA region that was variable among the four isolates. Two specific primers were designed to flank the variable region. The two primers were BSRIGS1 (5'GGG GTT CCG GGA TTC ACA GG3') and BSRIGS2 (5'GAG TGG TAA ATG GGG TAA TCA AC3'). The composition of PCR reactions was the same as those described above except that regular *Taq* DNA polymerase (GIBCO BRL) and a total volume of 10 µl were used. PCR conditions for these primers were 94°C for 3 min, 55°C for 1 min, and 72°C for 2 min for the first cycle followed by 40 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 1 min with a final extension of 3 min at 72°C. The two primers were tested for specificity on DNAs of *P. gregata*, a wide range of other fungi, and soybean plants. DNA from the following fungi were included in the test, *Acremonium dichromosporum*, *A. nepalense*, *Cercospora* sp., *Gaeumannomyces graminis*, *Plectosporium tabacinum*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, *Septoria* sp., and *Verticillium dahliae*. *Plectosporium tabacinum* is a frequent inhabitant of soybean stem (10,12). The two *Acremonium* spp. were tested because they are morphologically similar to *Plectosporium*, and, in fact, *Plectosporium tabacinum* from soybean has been referred to previously as *Acremonium* sp. (18,28). *G. graminis* has a *Phialophora* anamorph (asexual state). *Cercospora* sp., *Pythium ultimum*, *S. sclerotiorum*, and *Septoria* sp. are known soybean pathogens (37) and are likely to occur in soybean plants.

BLAST searches (4) were conducted using the amplified sequences as queries to search for the most similar sequences in

Fig. 3. Agarose gel showing polymerase chain reaction (PCR) amplification using specific primers BSRIGS1 and BSRIGS2. The three PCR product sizes of ≈ 1,020, 830 and 660 bp were designated as genotypes A, B, and C, respectively. Lane L, BRL 1-kb DNA ladder with approximate DNA sizes indicated; lanes 1 to 3, isolates 98A1-1-2, 98A2-3, and 98B4-6 of genotype A, respectively; lanes 4 to 6, isolates 98A5-3, 98A5-4, and 98A5-5 of genotype B, respectively; lanes 7 and 8, isolates ATCC 46906 and 5-22 of genotype C, respectively; lane N, negative control without DNA template.

Fig. 4. Agarose gel for genotyping *Phialophora gregata* isolates by polymerase chain reaction amplification, using the specific primers BSRIGS1 and BSRIGS2. Lane L, BRL 1-kb DNA ladder with approximate DNA sizes indicated; lanes 1 to 17, upper panel: isolates 98A1-1-2, 98A2-3, 98A5-3, 98A5-4, 98A5-5, 98A5-6, 98B3-1, 98B3-2, 98B3-3, 98B4-1, 98B4-2, 98B4-3, 98B4-4, 98B4-5, 98B4-6, S1-1a, and S1-1b, respectively; lower panel: S1-2a, S1-4, S2-1, S2-2a, S2-2b, S2-3, S2-5, S3-2a, S3-2b, S3-3, S3-4, S4-1, S4-2, S4-3, S4-4, S4-5, and S5-1, respectively.

Fig. 5. Agarose (2% NuSeive GTG and 1% regular agarose) gel showing restriction enzyme digestion of polymerase chain reaction products amplified with specific primers BSRIGS1 and BSRIGS2. Restriction digestion were carried out with enzymes of **A**, *AccI*; **B**, *HinfI*; **C**, *BglII*; or **D**, undigested. Lane L, BRL 1-kb DNA ladder with approximate DNA sizes (in kilobases indicated); lanes 1 to 8, isolates 98A1-1-2, 98B4-6, 98C1-5, Fulton-OH, M2, MN1, P63, and S1-4 of genotype A, respectively; lanes 9 to 16, isolates 98A4-2, 98A5-3, 98B4-1, IN4, M3, MN4, OH39, and S2-1 of genotype B, respectively.

GenBank. Sequence alignments were performed using the program Clustal W (40). Minor adjustments were made manually to the alignment outputs.

Genotype determination of isolates. The length variation in the region defined by the two specific primers was used to determine isolate genotypes. The size of the PCR product amplified with the two specific primers was determined by comparing them with DNA standards in 1% agarose gel electrophoresis. The three PCR product sizes were used to assign genotypes to isolates. Each set of PCR reactions included at least one isolate of a known genotype and a negative control without a DNA template.

To determine if there was any variation in the amplified products within each genotype, eight isolates representing various geographic origins were selected from each of the two genotypes, and the PCR products were subjected to restriction digestion with eight restriction enzymes: *AccI*, *BglII*, *HinfI*, *MboI*, *MspI*, *RsaI*, *TaqI*, and *XbaI* (GIBCO BRL). The digested fragments were separated on 3% agarose gels (1% regular agarose and 2% NuSeive GTG agarose from FMC BioProducts, Rockland, MD) as previously described (13). In addition, the PCR products from two isolates (one from each genotype) were purified and sequenced on both strands to confirm sequence identity.

Genotype detection in infected soybean plants. The genotype data of isolates indicated that the pathogen genotypes had cultivar preferences. Therefore, this experiment was designed to verify that the cultivar preference occurred under the same field conditions. The specific primers were used to detect the presence of the pathogen and the pathogen genotype in infected soybean plants. A total of 50 plants of cvs. AP1991, Bell, BSR101, and Sturdy growing in the same field near Arlington, WI were randomly collected regardless of BSR symptoms at the end of the growing season. Plants of the same cultivars from different plots in the same field were pooled before DNA isolation. DNAs from individual soybean stems were isolated with the FastDNA kit (Bio 101) as previously described (12) and were amplified with the specific primers as described above. PCR product size was used to indicate the pathogen genotype in the particular plant. The number of plants of each cultivar that were infected by each genotype was recorded, and χ^2 analyses were used to test the significance of the cultivar preference of the pathogen genotypes.

RESULTS

Amplification of the IGS region and DNA sequencing. PCR with primers 28SF and CNS1 produced a DNA band of approximately 7 kb in the four isolates tested (Fig. 2A). Due to its length, this IGS region can be amplified only with the high fidelity DNA polymerase. Numerous attempts with regular *Taq* DNA polymerase were unsuccessful. Among the eight enzymes used in digestion, four enzymes, *AccI*, *BamHI*, *HindIII* and *NcoI*, cut this DNA one to three times. The other four enzymes did not have restriction sites in this region (gel not shown). Digestion with *HindIII* produced two fragments of $\approx$ 4 kb (top band) and 3 kb (bottom band). The prominent difference among the four isolates is evident in the bottom band (Fig. 2B).

After *HindIII* digestion, the top and bottom bands (Fig. 2B) from each of the four isolates were cloned into a pGEM 3Zf(+) vector and transformed into JM 109 competent cells. When the bottom bands were used as inserts, seven clones were selected from isolate Pg13, and five clones were selected from each of the other three isolates. When the top bands were used as inserts, three clones were selected from isolate Pg13, and two clones were chosen from each of the other three isolates. The clones from each insert from the same isolate were combined before sequencing. Although both the top and bottom bands from each of the four isolates were cloned, only the clones containing the bottom bands were sequenced for this study.

A

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Type A  ggggttcocgggattcacagggtag-tcagatgtgagactggtgggagaggcttcagggcaccgctacaggcttctacacactgtaaggacacctagaagt
Type B  ggggttcocgggattcacagggtag-tcagatgtgagactggtgggagaggcttcagggcaccgctacaggcttctacacactgtaaggacacctagaagt
Type A' ggggttcocgggattcacagggtagtcagatgtgagactggtgggagaggcttcagggcaccgctacaggcttctacacactgtaaggacacctagaagt
*****

Type A  cccttggcacctccctacagcgtcggaggccctgtagccgaccctgaaggagtgccctgaggtgcgacgtgcaacaaaaccagatctgccacgtgatt
Type B  cccttggcacctccctacagcgtcggaggccctgtagccgaccctgaaggagtgccctgaggtgcgacgtgcaacaaaaccagatctgccacgtgatt
Type A' cccttggcacctccctacagcgtcggaggccctgtagccgaccctgaaggagtgccctgaggtgcgacgtgcaacaaaaccagatctgccacgtgatt
*****

Type A  ccctcagggttaggtcgagtggtgaattggtgggagaggcttcagggcactgctacaggcttctacacactgtagggacaccctagaagtcoccttggcagt
Type B  ccctcagggttaggtcgagtggtgaattggtgggagaggcttcagggcactgctacaggcttctacacactgtagggacaccctagaagtcoccttggcagt
Type A' ccct-----
****

Type A  gaccctacagggtgaggacgctgtagcctaccctgtaggagtgccctgaagtgcgacgtgcaacaaaaccagatttggccacgtgaccagcgacggcga
Type B  gaccctacagggtgaggacgctgtagcctaccctgtaggagtgccctgaagtgcgacgtgcaacaaaaccagatttggccacgtgaccagcgacggcga
Type A' -----

Type A  gatcgctgggggaggtccagggtgctccgacaggcttctacacactgtagggacaccctagaagtcoccttggcacctccctacagcgtcggaggccctg
Type B  gatcgctgggggaggtccagggtgctccgacaggcttctacacactgta-----
Type A' -----

Type A  tagccggccctgaaggagtgccctgaggtgcgacgtgcaacaaaaccagatctgccacgtgattccctcagggttaggtcgagtggtgaattggtgggag
Type B  -----
Type A' -----cagggttaggtcgagtggtgaattggtgggag

Type A  aggtccagggtactcttacagggtctctgtacactgtagacataccctagaagtcoccttggcacctctctacagcgtcggaggccctgtagctggccct
Type B  -----gacataccctagaagtcoccttggcacctctctacagcgtcggaggccctgtagctggccct
Type A' aggtccagggtactcttacagggtctctgtacactgtagacataccctagaagtcoccttggcacctctctacagcgtcggaggccctgtagctggccct
*****

Type A  gaaggaatgcctgaactgcgacgtgcaacaaaactcggaaacgccaaggcccttggagggttaaccaagtgtgacccttagccactccacctacccaac
Type B  gaaggaatgcctgaactgcgacgtgcaacaaaactcggaaacgccaaggcccttggagggttaaccaagtgtgacccttagccactccacctacccaac
Type A' gaaggaatgcctgaactgcgacgtgcaacaaaactcggaaacgccaaggcccttggagggttaaccaagtgtgacccttagccactccacctacccaac
*****

Type A  tccccaaaaccaggccgatcaggacctcccgatggtttctagagccattttttacgctgaatccgatggtgccattgactgctctatcaggggctgct
Type B  tccccaaaaccaggccgatcaggacctcccgatggtttctagagccattttttacgctgaatccgatggtgccattgactgctctatcaggggctgct
Type A' tccccaaaaccaggccgatcaggacctcccgatggtttctagagccattttttacgctgaatccgatggtgccattgactgctctatcaggggctgct
*****

Type A  aatgtggagtttaccocccoccttgagggtataaagtgtcaagtataccgccaaagtgccgaatcggagccagccagccttttcatcgtgttgcocggg
Type B  aatgtggagtttaccocccoccttgagggtataaagtgtcaagtataccgccaaagtgccgaatcggagccagccagccttttcatcgtgttgcocggg
Type A' aatgtggagtttaccocccoccttgagggtataaagtgtcaagtataccgccaaagtgccgaatcggagccagccagccttttcatcgtgttgcocggg
*****

Type A  ttgattaccocatttaccactc
Type B  ttgattaccocatttaccactc
Type A' ttgattaccocatttaccactc
*****

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B

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Type A' ggggttcocgggattcacagggtagtcagatgtgagactggtgggagaggcttcagggcaccgctacaggcttctacacactgtaaggacacc-tagaag
Type C  ggggttcocgggattcacagggtagtcagatgtgagactggtgggagaggcttcagggcaccgctacaggcttctacacactgtagacagaccctagcag
*****

Type A' tcccttggcacctccctacagcgtcggaggccctgtagccgaccctgaaggagtgccctgaggtgcgacgtgcaacaaaaccagatctgccacgtgac
Type C  tgccttggcacctccctcagggcctcgaagcctgtagccgaccctgaaggagtgccctgaggtgcgacgtgcaacaaaaccagatctgccacgtgac
* *****

Type A' tccctcagggttaggtcgagtggtgaattggtgggagaggctccagggtactcttacagggtctcgtacactgtagacataccctagaagtcoccttggcac
Type C  tctcccagggttaggtcgagtggtgaattggtgggagaggctccagggtaccgctacagggtgtgacacgtgtagacagaccctagcagtcoccttggcac
* * *****

Type A' ctctctacagcgtcggaggccctgtagctggccctgaaggaatgcctgaactgcgacgtgcaacaaaactcggaaacgccaaggcccttggagggttaac
Type C  ctccccctcagggtcgaagcctgtagccgaccctgtaggagtgccctgaggtgcgacgtgcaacaaaactcagaccgccaagggttttcggagggttaac
*** ** *

Type A' ccaagtgtgaccgtagccactccacctaccocaaactcccccacccagggccgatcaggacctcccgatggtttctagagccattttttacgctgaat
Type C  ccaagtgtgaccgtagccactccacctaccocaaactcccccacccagggccgatcaggacctcccgatggtttctagagccattttttacgctgaat
*****

Type A' ccgatggtgccattgactgctctatcaggggtgctaatgtggagtttaccocccoccttgagggtataaagtgtcaagtataccgccaaagtgccgaat
Type C  ccgatggtgctattgactgctctatcaggggtgctaatgtggagtttaccocccoccttgagggtataaagtgtcaagtataccgccaaagtgccgaat
*****

Type A' cggagccagccagccttttcatcgtgttgcocgggttgattaccocatttaccactc
Type C  cggagccagccagccttttcatcgtgttgcocgggttgattaccocatttaccactc
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Fig. 6. Alignment of the sequences of polymerase chain reaction products amplified using specific primers BSRIGS1 and BSRIGS2 from *Phialophora gregata*. **A**, Genotypes A (AF249311), B (AF249312) and A' (AF249313) show identical sequences except the insertion or deletion. **B**, Genotypes A' and C (AF249314) show 58 nucleotide differences. An asterisk indicates that the column is a constant, and a – indicates an insertion or deletion. Primer sequences are underlined.

Primer design and specificity. The two specific PCR primers flanking the variable region were tested for their specificity in *P. gregata*, a range of other fungal DNAs, and soybean DNA. Three distinct products of 1,020, 830, and 660 bp, were detected in isolates of *P. gregata* (Fig. 3). These bands were designated as genotypes A (1,020 bp), B (830 bp), and C (660 bp). No PCR products were detected when DNAs of the other fungi and soybean plant were used in PCR with the specific primers (gels not shown). In BLAST searches using the amplified sequences as queries, only four sequences of human and *Drosophila* from GenBank showed 20-bp stretches of similarity (search results not shown), suggesting that the sequences of this region are unique to *P. gregata*.

Genotyping of strains. Isolates of *P. gregata* were easily assigned to respective genotypes based on the PCR product sizes amplified with the specific primers, as shown in Figure 4. All 292 isolates from soybean and 8 isolates from mung bean belonged to either genotype A or B (Table 1), except two soybean isolates (WI2 and AS-1; described below). Genotype B also included the isolate ATCC 11073, the type culture of *P. gregata* (14) from the American Type Culture Collection (Manassas, VA). The two genotype C isolates were from Adzuki bean from Japan. Of the eight restriction enzymes used in digestion, enzymes *RsaI* and *XbaI* did not have restriction sites in the amplified region. Based on digestion with the other six enzymes, genotypes A and B always showed different restriction banding patterns, but no variation within each genotype was detected among isolates representing a wide geographic area (Fig. 5). Sequences of genotypes A and B in the amplified region were identical except for an 188-bp insertion or deletion at a discrete location (Fig. 6A).

Two isolates, WI2 and AS-1, showed the genotype A band but also showed a lower band similar in size to the genotype C band. The purity of these two isolates was confirmed by reisolating six single-spore subcultures. Each of the subcultures produced the

same two bands. The two bands from each of the two isolates were purified after separation by agarose gel electrophoresis. Restriction enzyme digestion showed that the upper band had the same restriction banding pattern as the genotype A band from other soybean isolates, whereas the lower band had a completely different restriction banding pattern from that of the genotype C band from the two Adzuki bean isolates (digestion gels not shown). The lower band was then completely sequenced. Its sequence is identical to the genotype A band except two (one single base and one 365 base) insertion or deletion (Fig. 6A), and it differed from the genotype C band from the Adzuki bean in point mutations at 58 locations (Fig. 6B). Because the lower band in isolates WI2 and AS-1 was a derived form of, and always occurred with, the genotype A band and appeared in low frequency (2 of 300 isolates), these two isolates were designated as genotype A' and were tentatively considered genotype A in general discussions here. The four sequences of genotypes A, B, A', and C were deposited in GenBank and assigned Accession nos. AF249311, AF249312, AF249313, and AF249314, respectively.

Both genotype A and B isolates were found in the seven Midwestern states that we sampled, indicating that the two genotypes are widespread (Table 1). Genotype B was not detected in Brazil and Canada from a limited sample size of isolates. When the isolates were grouped based on cultivars or breeding lines from which the isolates were obtained, interesting trends in cultivar preference were observed (Table 2). For example, there were 17 isolates from the cv. Sturdy, 13 of them are of genotype A. Similarly, 34 of the 38 isolates from Pioneer 9305 are also genotype A. On the other hand, there were 23 isolates from cv. SS282N, 17 of them were of genotype B. Seven of the nine isolates from cv. Bell were of genotype B. All 12 isolates from cv. IA 3003 were of genotype B. Interestingly, both cvs. Sturdy and Pioneer 9305 were susceptible to BSR, whereas the cvs. Bell, IA 3003, and SS282N were resistant to BSR (27, Grau, unpublished

TABLE 2. Relationship of two genotypes of *Phialophora gregata* with soybean cultivar or breeding lines under field conditions as well as the host genotypes for resistance to brown stem rot and soybean cyst nematode (SCN)

Cultivar/ breeding lines ^a	Proposed <i>Rbs</i> gene	SCN resistance source	Number of isolates of <i>Phialophora gregata</i>		
			Genotype A	Genotype B	Total
Archer	<i>Rbs1</i> and <i>Rbs3</i>	None	4	0	4
A2247	Resistant ^b	None	4	0	4
Bell	Resistant ^b	PI 88788	2	7	9
BSR 101	<i>Rbs1</i> & <i>Rbs3</i>	None	29	8	37
Century 84	<i>rbs</i>	None	3	5	8
Corsoy	<i>rbs</i>	None	1	0	1
CX313	<i>rbs</i>	None	4	1	5
H 1128	<i>rbs</i>	None	2	0	2
Hardin	<i>rbs</i>	None	0	1	1
IA 3003	Resistant ^b	None	0	12	12
KB 241	<i>rbs</i>	None	4	0	4
L78-4094	<i>Rbs 1</i>	None	1	0	1
LN92-12033	<i>Rbs 2</i>	None	2	21	23
LN92-12054	<i>rbs</i>	None	3	15	18
Newton	<i>rbs</i>	Peking	5	0	5
S 19-90	<i>rbs</i>	None	0	1	1
PI 437833	<i>Rbs 2</i>	None	0	3	3
PI 437970	<i>Rbs 3</i>	None	1	6	7
9305	<i>rbs</i>	None	34	4	38
SS24-92	<i>rbs</i>	None	4	2	6
SS282N	Resistant ^b	PI 88788	6	17	23
Sturdy	<i>rbs</i>	None	13 ^c	4	17
Unknown	N/A ^d	N/A	49 ^c	14	63
Total			171	121	292 ^e

^a Sources of soybean germ plasm used: A2247 (Asgrow), CX313 (DeKalb), H1128 (Golden Harvest), KB241 (Kaltenberg), S19-90 (Novartis), 9305 (Pioneer), SS24-92 (Seiben), and SS282N (Seiben). All other germ plasm is from public sources.

^b Cultivar expressed low levels of symptom severity of brown stem rot but without known *Rbs* genes.

^c Includes one genotype A' isolate.

^d Not applicable.

^e Two isolates from Adzuki bean from Japan and eight isolates from mung bean were not included.

data). Similar trends were also observed in the susceptible cv. Newton (five of five isolates were of genotype A), and resistant PI 437970 (six of seven isolates were of genotype B). Most of the isolates, 21 of 23, and 15 of 18, obtained from the *Rbs2* near-isogenic lines (33), LN92-12033 (resistant), and LN92-12054 (susceptible), respectively, belonged to genotype B (Table 2). However, not all resistant cultivars were preferentially infected by genotype B. For example, 29 of 37 isolates from resistant cv. BSR101 were genotype A (Table 2).

Genotype detection in infected soybean plants. The purpose of this experiment was to verify that preferential infection by the genotypes occurred in cultivars growing in the same field. The specific primers were used successfully to detect the pathogen genotypes in infected soybean plants. Among 50 randomly collected (infected as well as noninfected) plants, 33 of them were positive for *P. gregata* based on the presence of a PCR product using the specific primers (Table 3). Cultivar preference by the two genotypes of *P. gregata* was also observed. For example, 11 plants of resistant cv. Bell were infected by *P. gregata* and 10 of them were infected by genotype B, whereas among 9 infected plants of the susceptible cultivar Sturdy, 7 of them were infected by genotype A. A similar trend was also seen in the susceptible cv. AP1991 (Table 3). Cultivar-preferential infection was significant in cvs. AP1991 and Bell and Sturdy (Table 3), but no significant difference was detected in cv. BSR 101. Two plants (one each in cvs. AP1991 and BSR 101) showed two PCR bands, a major band of genotype A and a minor band of genotype B (Table 3), probably the result of infection by both genotypes.

DISCUSSION

It is significant that soybean isolates of *P. gregata* can be separated into two subspecific populations (genotypes A and B) based on variation in the IGS region of the nuclear rDNA. It is the first experiment to show a genetic subdivision in soybean isolates of *P. gregata*. Furthermore, the two genotypes preferentially infect certain soybean cultivars under field conditions. Gray (16) reported two pathogenicity types of *P. gregata* on soybean, defoliating (type I) and nondefoliating (type II) (described in introduction). The original nondefoliating (type II) strain, ATCC 11073, the type culture on which the description of *P. gregata* was based (14), belonged to genotype B. A recent claim by Harrington et al. (20) that *Plectosphaerella cucumerina* and *Gliocladium roseum* were the non-defoliating (type II) form is contrary to the fact in the original report (16). Unfortunately, the strains of the original defoliating (type I) form were no longer available for genotyping in this study. In Japan, Kondo et al. (25) reported two races of *P. gregata* f. sp. *adzukicola* on Adzuki bean. It seems that the two forma speciales (*adzukicola* and *sojae*) of *P. gregata* have evolved in parallel on their respective hosts. The relationship of the two genotypes of soybean isolates with pathogenicity under controlled conditions remains to be assessed.

TABLE 3. Polymerase chain reaction detection of genotypes of *Phialophora gregata* in infected soybean plants of four cultivars growing in the same field near Arlington, WI

Cultivar	Number of plants tested	Number of plants harboring <i>P. gregata</i>			χ^2 ^a
		Genotype A	Genotype B	Total	
AP1991	12	7 ^b	1	8	4.5**
Bell	16	1	10	11	7.36***
BSR101	10	2 ^b	3	5	0.2
Sturdy	12	7	2	9	2.78*
Total	50	17	16	33	

^a *, **, and *** indicate significance at $P < 0.1$, 0.5 , and 0.01 , respectively.

^b Includes one plant showing a faint genotype B band in addition to a major genotype A band.

In general, most isolates obtained from BSR-susceptible cultivars are of genotype A, and most isolates from resistant cultivars are of genotype B (Table 4). The susceptible cultivars could be arbitrarily subdivided into two groups. Susceptible group I was mostly infected by genotype A isolates, whereas susceptible group II, which includes Century 84 and LN92-12054, a near-isogenic line of BSR-resistant *Rbs2* (33), was mostly infected by genotype B (Table 4). Resistant cultivars could be divided based on their resistance genes. BSR 101-derived resistant cultivars were mostly infected by genotype A, whereas all other resistant cultivars, including those with alleles *Rbs* 1, 2, and 3 breeding lines, were infected predominately by genotype B (Table 4). Genotype B also preferentially infected cvs. Bell and SS282N, both of which are resistant to both BSR and the soybean cyst nematode (27; C. R. Grau, unpublished data) with derived resistance from PI 88788, whereas genotype A infected cv. Newton, which is resistant only to the soybean cyst nematode with derived resistance from Peking (Table 4). It appears that genotype B is adapted to some yet unidentified traits associated with certain resistant soybean cultivars.

The plants used in isolations of *P. gregata* were from different fields and different geographic locations (Table 1). The comparison of cultivar preference (Tables 2 and 4) is confounded by the fact that the distribution and relative abundance of the two genotypes in those fields are not known. However, the soybean cultivars used for DNA isolation and direct genotype detection were from the same field, and cultivar-preferential infection by the two genotypes is also evident (Table 3). Preferential infection of cvs. Bell and Sturdy by genotypes A and B, respectively, observed under the same field conditions (Table 3) is consistent with the data based on genotyping of isolates obtained from the same two cultivars (Table 2). This fact provides credence for concluding cultivar preference of the two genotypes based on genotyping of the isolates.

We previously showed that the ITS region of the rDNA contains no variation among soybean isolates of *P. gregata* and was used to separate *P. gregata* from other fungi, including *Plectosporium tabacinum*, that are ecologically associated with BSR (10,12). Variation in the ITS region and an intron in the small subunit rDNA differentiate soybean isolates of *P. gregata* from Adzuki bean isolates (10,11). Here we report that genetic variation in the IGS region can subdivide soybean isolates into two distinct populations (genotypes A and B), in addition to separating Adzuki bean isolates from soybean isolates. These studies demonstrate that the various regions of the rDNA evolve at different rates and

TABLE 4. Relationship between brown stem rot (BSR) disease reactions of soybean genotype groups and genotypes of *Phialophora gregata*

Soybean reaction group	Number of isolates of <i>P. gregata</i>	
	Genotype A	Genotype B
All BSR susceptible	73	33
Susceptible group I ^a	67	13
Susceptible group II ^b	6	20
All BSR resistant	49	74
BSR 101-derived resistance ^c	37	8
Other resistance sources ^d	12	66
<i>Rbs</i> 1, 2, and 3 breeding lines ^e	4	30
SCN resistance, PI 88788 ^f	8	24
SCN resistance, Peking ^g	5	0

^a Includes Corsoy, DeKalb CX313, GH 1128, Hardin, KB241, Newton, NK19-90, Pioneer 9305, Seiben SS24-92, and Sturdy.

^b Includes Century 84 and LN92-12054 (a near isogenic line of *Rbs* 2).

^c Includes Archer, ASGR A2247, and BSR 101.

^d Includes Bell, IA 3003, L78-4094, LN92-12033, PI 437-833, PI 437-970, and Seiben SS282N.

^e Includes L78-4094, LN92-12033, PI 437-833, and PI 437-970.

^f Soybean cyst nematode (SCN) resistance derived from PI 88788 includes Bell and Seiben SS282N.

^g Soybean cyst nematode resistance derived from Peking includes Newton.

provide different levels of resolution for addressing specific questions at appropriate taxonomic levels.

The IGS region of *P. gregata* is unusually large (≈ 7 kb) compared with other fungi (6,21,32,38,39), making it difficult to amplify the whole region. In basidiomycetes and some morel fungi, 5S rDNA repeat units reside in the IGS region, and primers based on the conserved sequences in the large subunit rDNA and the 5S rDNA were used to amplify a portion of the IGS region. The IGS region is approximately 2 to 2.5 kb in *Verticillium* spp. (32,38) and approximately 2.6 kb in *Fusarium oxysporum* (6). In the studies of *Verticillium* and *Fusarium* spp. (6,38), the restriction banding patterns of PCR-amplified IGS regions were used. However, the IGS region of *P. gregata* is too long for routine amplification. We sequenced the IGS region and designed specific primers flanking the variable location for easy PCR amplification and identification of the genotypes.

Repeated-gene families like rDNA genes including spacer regions are subject to concerted evolution (8,49), which means variation in the rDNA family is homogenized among the repeating units within an individual and among members of a population. The two distinct genotypes of the IGS region suggest that there are at least two separate evolving populations within soybean isolates of *P. gregata*. The IGS region of nuclear rDNA is unlikely to have a function in the infection process. The correlation of the genotypes with cultivar preference provides an example of nonrandom association of genetic traits. Furthermore, we have identified six additional DNA loci generated by microsatellite-primed PCR that are strictly correlated with the two genotypes (X. Q. Meng and W. Chen, unpublished data), providing evidence that the genetic difference between genotypes A and B extends far beyond the rDNA and that there is limited gene flow between the two genotypes of *P. gregata*.

ACKNOWLEDGMENTS

We dedicate this publication to L. E. Gray of USDA-ARS for his many contributions to the understanding of, and insightful discussions about, brown stem rot, his encouraging and facilitating cooperative research efforts, and his enthusiastic support of this research. We thank A. Dorrance and X. B. Yang for providing *P. gregata* isolates for this study, and B. Diers for providing infected soybean plants used in isolation and for reviewing the manuscript. We thank Y.-C. Chen for technical assistance. This research was funded, in part, by grants from the CSREES North Central IPM Program, the Illinois Soybean Program Operating Board, the Illinois Council on Food and Agricultural Research (C-FAR), and the Wisconsin Soybean Marketing Board.

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