Molecular Differentiation of Fungi Associated with Brown Stem Rot and Detection of *Phialophora gregata* in Resistant and Susceptible Soybean Cultivars

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the names by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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ABSTRACT

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A collection of 79 isolates of *Phialophora gregata* from soybean, mung bean, and adzuki bean obtained from several midwestern states, Brazil, and Japan was studied for intraspecific genetic variation in the nuclear ribosomal DNA (rDNA). The *Phialophora* isolates also were compared with 16 isolates of *Acremonium* spp. isolated from soybean. All the isolates of *P. gregata* shared one unique banding pattern after restriction enzyme digestion of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS) and the 5′ end of the large subunit rDNA. Isolates of *Acremonium* spp. from soybean were clearly differentiated from *P. gregata* isolates. The ITS region of isolates representing various DNA groups, based on restriction digestion, were sequenced completely on both strands. The isolates of *P. gregata* from soybean from the United States and Brazil had identical ITS sequences. The ITS sequence of *P. gregata* isolated from adzuki bean from Japan was 98% similar to that of *P. gregata* from soybean. At least two groups of *Acremonium* spp. were associated with soybean brown stem rot, and one of the groups could be a *Plectosporium* sp., based on ITS sequence comparisons. Two PCR primers, BSR1 and BSR2, based on the ITS sequence, were designed specifically for *P. gregata* from soybean to detect the pathogen in infected plants. The specific primers were used in PCR to amplify a 483-bp DNA fragment in isolates of *P. gregata* from soybean and mung bean but not from *P. gregata* from adzuki bean at a specified annealing temperature. PCR with the specific primers did not detect the DNA fragment in *Acremonium* spp. or any other fungi tested, nor in soybean DNA. PCR experiments with mixed DNAs of *P. gregata* and *Acremonium* sp. showed that the specific primers were necessary to detect *P. gregata* in its natural habitats. PCR with the specific primers and the traditional isolation technique were used to detect *P. gregata* in artificially inoculated soybean cvs. BSR101 and Century, which are resistant and susceptible to brown stem rot, respectively. No differences were found in the infection and movement of the pathogen between the two soybean cultivars. The specific DNA fragment also was detected in naturally infected stems of soybean cvs. Bell, BSR101, Newton, and Sturdy collected from fields, and sequence analyses verified that these amplified fragments were from *P. gregata*. Results of PCR with specific primers confirmed field observations that cv. BSR101 may not be resistant to brown stem rot under certain conditions.

Additional keywords: DNA isolation, molecular ecology, PCR primers, *Plectoshaerella*.

Brown stem rot, an important vascular disease of soybean in the midwestern United States, is caused by a Deutomycete, *Phialophora gregata* (Allington & D.W. Chamberlain) W. Gams. Extensive studies have been carried out on the pathogenicity of *P. gregata* on soybean and mung bean (9,11,22,33), soybean resistance to brown stem rot (23,30,31), and, to a lesser extent, the close ecological association of *P. gregata* with *Acremonium* sp. (21). The disease is favored by cool weather during the soybean seed development stage and is associated with various yield losses of soybean. The most prominent symptom of the disease is browning of vascular and pithy tissue of soybean, as the common name implies. However, significant yield losses have been associated only with expression of foliar symptoms (1,22). Foliar symptoms include yellowing and interveinal necrosis.

Although not all isolates of *P. gregata* are equally able to induce foliar symptoms, foliar symptoms have been associated only with infection by *P. gregata*, not with *Acremonium* sp. Mengistu and Grau (21) found that *P. gregata* and *Acremonium* sp. from soybean are clearly different in morphological, cultural, and pathogenic characteristics and in isozyme banding patterns. The soybean and adzuki bean isolates of *P. gregata* are host specific (14–16). Gray and Hepburn (10) were able to differentiate cultures of *P. gregata* isolated from soybean from those isolated from adzuki bean based on restriction banding patterns of mitochondrial DNA. Knowledge of genetic variation among *P. gregata* isolates from a wide range of geographic locations is lacking, and the genetic difference between *P. gregata* and its frequent cohabitant, *Acremonium* sp., is unknown

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(10). The most effective means of controlling this disease is through the use of resistant cultivars. Some soybean cultivars resistant to brown stem rot are commercially available, but the performance of these cultivars has not been consistent under various field conditions. This could be due to variation in the pathogen in different locations or to the environmental conditions affecting disease expression.

Molecular techniques, such as polymerase chain reaction (PCR) and DNA sequencing, have significantly advanced our understanding of fungi, including plant pathogens (4,5,17) and plant disease diagnosis (12). Nuclear ribosomal DNA (rDNA), particularly the internal transcribed spacer (ITS) region, is very useful in differentiating fungi at the species level (6–8,18,25). In this study, our objectives were: (i) to investigate the genetic variation within the nuclear rDNA among worldwide isolates of *P. gregata* isolated from soybean, mung bean, and adzuki bean; (ii) to differentiate *P. gregata* from its frequent cohabitant, *Acremonium* spp., and from other pathogens; and (iii) to develop specific PCR primers for *P. gregata* and use the primers to detect *P. gregata* in artificially and naturally infected soybean plants of resistant and susceptible cultivars.

MATERIALS AND METHODS

Isolation and maintenance of fungal strains. Isolates of *P. gregata* and *Acremonium* spp. were originally obtained from diseased soybean and mung bean plants in fields in Illinois, Indiana, Iowa, Ohio, and Wisconsin and from Brazil by the authors and other investigators (Table 1). All isolates of *P. gregata* isolated from adzuki bean were obtained from K. Kobayashi, Sapporo, Japan. Isolations were achieved by placing surface-sterilized soybean stems on a selective medium (24) and incubating at 18 to 20°C. Single-spore cultures were obtained at the time of isolation. For long-term storage, agar disks containing spores of each isolate were placed in individual cryovials containing 30% sterile glycerol and were maintained at –70°C (10) as part of the culture collections at the University of Illinois, Urbana, and the University of Wisconsin, Madison.

Culture conditions and DNA isolation from mycelium. All fungal cultures were grown on soybean-stem agar plates by previously reported methods (10). For DNA isolation, colonized agar plugs were transferred to a sucrose-peptone broth and incubated for 14 days at 18 to 20°C. Mycelia were harvested by filtration

TABLE 1. List of fungal species and isolates used in the study

Species	Isolate	Host[a]	Geographic origin	Year of isolation	DNA grouping[b]
Phialophora gregata	B1	SB	Ill.	1992	I
	B2	SB	Ill.	1992	I
	B3	SB	Ill.	1992	I
	G3	SB	Ill.	1990	I
	LM1	SB	Ill.	1994	I
	LM2	SB	Ill.	1994	I
	LM3	SB	Ill.	1994	I
	LM4	SB	Ill.	1994	I
	AR1	SB	Ill.	1994	I
	AR2	SB	Ill.	1994	I
	AR3	SB	Ill.	1994	I
	K1	MB	Ill.	1992	I
	K3U	MB	Ill.	1992	I
	K4	MB	Ill.	1992	I
	K5	MB	Ill.	1992	I
	MBU9	MB	Ill.	1992	I
	MBU10	MB	Ill.	1992	I
	MBU11	MB	Ill.	1992	I
	Hob2	SB	Ind.	1991	I
	RBSI-1	SB	Ind.	1991	I
	IN1	SB	Ind.	1994	I
	IN10	SB	Ind.	1994	I
	IN2	SB	Ind.	1994	I
	IN3	SB	Ind.	1994	I
	IN4	SB	Ind.	1994	I
	IN5	SB	Ind.	1994	I
	IN6	SB	Ind.	1994	I
	IN8	SB	Ind.	1994	I
	I16	SB	Iowa	1990	I
	IT	SB	Iowa	1985	I
	K93-1	SB	Iowa	1995	I
	OH2	SB	Ohio	1991	I
	BSR101	SB	Wis.	1994	I
	WI2	SB	Wis.	1993	I
	WI3	SB	Wis.	1993	I
	WI6	SB	Wis.	1993	I
	WI8	SB	Wis.	1993	I
	A101-2	SB	Wis.	1995	I
	A101-3	SB	Wis.	1995	I
	AB-1	SB	Wis.	1995	I
	AB-2	SB	Wis.	1995	I
	AB-3	SB	Wis.	1995	I
	AN-2	SB	Wis.	1995	I
	AS-1	SB	Wis.	1995	I
	AS-2	SB	Wis.	1995	I
	C101-2	SB	Wis.	1995	I
	C101-3	SB	Wis.	1995	I
	CB-1	SB	Wis.	1995	I
	CB-2	SB	Wis.	1995	I
	CB-3	SB	Wis.	1995	I
	CN-1	SB	Wis.	1995	I
	CN-2	SB	Wis.	1995	I
	CS-1	SB	Wis.	1995	I
	CS-2	SB	Wis.	1995	I
	H101-1	SB	Wis.	1995	I
	H101-2	SB	Wis.	1995	I
	H101-3	SB	Wis.	1995	I
	H101-4	SB	Wis.	1995	I
	HB-1A	SB	Wis.	1995	I
	HB-1B	SB	Wis.	1995	I
	HB-3	SB	Wis.	1995	I
	HN-1	SB	Wis.	1995	I
	HN-2	SB	Wis.	1995	I
	HS-1	SB	Wis.	1995	I
	HS-2	SB	Wis.	1995	I
	HS-3	SB	Wis.	1995	I
	HS-4	SB	Wis.	1995	I
	Pg10	SB	Wis.	...	I
	Pg11	SB	Wis.	...	I
	Pg12	SB	Wis.	...	I
	Pg13	SB	Wis.	...	I
	Pg18	SB	Ill.	...	I
	Pg19	SB	Ill.	...	I
	SSA1	SB	Brazil	1993	I
	SSA2	SB	Brazil	1993	I
	SSA3	SB	Brazil	1993	I
	46906 (K5)	AB	Japan	1981	I
	A8	AB	Japan	1981	I
	5-22	AB	Japan	1981	I
Acremonium sp.	Arc2	SB	Ill.	1994	II
	SBFW	SB	Iowa	1994	II
	SBBSR	SB	Iowa	1994	II
	SBF1	SB	Iowa	1994	II
	Pg14	SB	Wis.	1995	II
	Acr1	SB	Wis.	1995	II
	Acr2	SB	Wis.	1995	IV
	Acr4	SB	Wis.	1995	II
	Acr4-3	SB	Wis.	1995	II
	Acr4-4	SB	Wis.	1995	II
	Acr5	Alfalfa	Wis.	1994	II
	Acr6	Corn	Wis.	1995	II
	Acr7	RW	Wis.	1995	II
	Acr8	LQ	Wis.	1995	II
	YS08	SB	Wis.	1993	III
	wc7994	Alfalfa	Wis.	1994	III
A. dichromosporum	32182	...	ATCC	...	V
A. nepalense	32181	Soil	ATCC	...	VI
Plectosporium tabacinum	Pt1		M. E. Palm	...	VII

[a] SB = soybean; MB = mung bean; AB = adzuki bean; RW = ragweed; LQ = lambsquarter; and ... = unknown.

[b] DNA grouping was based on the restriction banding patterns of the polymerase chain reaction-amplified internal transcribed spacer and the 5′ end of large subunit nuclear ribosomal DNA regions. The banding patterns of each DNA group are shown in Figure 1.

through Miracloth (Chicopee Mills, Inc., Milltown, NJ) and frozen to –70°C until DNA isolation. Total genomic DNA from the mycelium was isolated by a procedure described previously (8).

PCR and restriction enzyme digestion. PCR was used to amplify the ITS of the nuclear rDNA and the 5′ end of the large subunit rDNA. Primers ITS1 and ITS4 were used to amplify ITS-1 and ITS-2, including the 5.8 rDNA (32). Primers F63 and R635 were used to amplify the 5′ end of the large subunit rDNA (7,19). PCR amplifications generally were carried out in 50-µl reactions, with PCR buffer and *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD) under the following temperature regimes: 95°C for 3 min, 50°C for 2 min, and 72°C for 2 min for the first cycle, and 1 min each at 94, 50, and 72°C for the next 30 cycles, with a final extension at 72°C for 10 min. The temperature parameters were controlled using a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT). Aerosol-resistant pipette tips were always used in handling PCR reagents and in setting up PCR to prevent cross-contamination. A negative control without DNA template was included in each set of PCR to monitor possible contaminations. The amplification efficacy was checked by agarose gel electrophoresis of 5-µl PCR products. The PCR products were used in restriction enzyme digestion without further purification. Four 4-base recognition restriction enzymes (*Hin*fI, *Hae*III, *Mbo*I, and *Taq*I) (Gibco-BRL) were used in digesting the PCR products. The restricted fragments were separated on a composite agarose gel (2% NuSeive GTG agarose from FMC BioProducts, Rockland, ME, and 1% regular agarose), as described previously (8). Agarose gels were stained with ethidium bromide and photographed under UV light (29). Isolates were grouped based on restriction banding patterns.

To analyze the relationships of the various DNA groups of the isolates, the PCR product sizes and the sizes of the restriction bands were considered as discrete characters and coded as 1 (presence) or 0 (absence) to form a data matrix. The data matrix was subjected to cluster analyses based on a simple matching coefficient by the program NTSYS-pc (28), as described previously (8).

DNA sequencing and sequence analyses. Representative isolates of the various DNA groups, based on the restriction banding patterns of the PCR products, were used for DNA sequencing. Specifically, the ITS region of isolates H101-3, LM1, G3, and SAA1 of *P. gregata* from soybean; isolate 46906 of *P. gregata* from adzuki bean; and isolates Arc2 and YS08 of *Acremonium* spp. from soybean was sequenced. PCR products amplified with primers ITS1 and ITS4 were purified with 2% NuSeive GTG agarose gel electrophoresis in Tris-acetate-EDTA buffer (29). DNA was purified from agarose blocks by the GeneClean II kit (Bio 101, Inc., La Jolla, CA). The purified DNA was ligated into the pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM109 highly competent cells by the heat shock method following the manufacturer's instructions. Five white colonies were selected randomly. After confirming the insertion size, the plasmid DNA from the five clones was combined and the insert sequence was determined with cycle sequencing with fluorescent dye-labeled terminators by the ABI 373 automated DNA sequencer (Perkin-Elmer) at the Biotechnology Center of the University of Illinois, Urbana. Each sequence reaction can generate 600 to 700 bases. The insert sequences for each isolate were completely sequenced on both strands. The contiguous sequences from the two opposite reactions were assembled by GeneJockey II (BIOSOFT, Cambridge). Searches for the most similar sequences in the sequence databanks were performed by the BLAST search algorithm (2). Sequence alignment was done with the CLUSTAL V program (13).

Design and specificity of primers specific for *P. gregata*. Two PCR primers, BSR1 and BSR2, were designed specifically for *P. gregata* from soybean based on the ITS sequences. The design of the primers was aided by the computer program Amplify (version 1.2, Dr. W. Engels, University of Wisconsin, Madison) to check for potential nonspecific primering sites and primer dimer formations. The primers were designed so they had sufficient GC contents and enough length to withstand highly stringent PCR conditions. PCR amplifications with the specific primers were carried out in conditions similar to those described above, except that various annealing temperatures, from 50 to 60°C, were used with a 25-µl volume. PCR was done with DNA preparations from all the isolates of *P. gregata* and *Acremonium* sp. listed in Table 1; some additional fungal pathogens, such as *Cercospora* sp., *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, *Septoria* sp., and *Verticillium dahliae*; and DNA of healthy soybean plants. When no PCR products were observed in amplifications with the specific primers, the DNAs were used in PCR with primers ITS1 and ITS4 to check the quality of the DNA.

Amplifications from mixed DNAs. To test the applicability of PCR primer pairs ITS1 and ITS4 and BSR1 and BSR2 in amplifying the desired DNA fragments in mixed DNAs, DNAs of isolate Pg11 of *P. gregata* and isolate Acr1 of *Acremonium* sp. were mixed in various proportions from 0 to 100%. The percents were based on the volumes of the working DNA solutions; therefore, they may not reflect the absolute concentrations. The mixed DNAs were used in PCR with primers ITS1 and ITS4 at an annealing temperature of 50°C and primers BSR1 and BSR2 at an annealing temperature of 60°C. The PCR products were checked by agarose gel electrophoresis and digested by restriction enzymes, as described above, to verify the banding patterns.

Detection of *P. gregata* in soybean plants. Both artificially inoculated soybean plants and naturally infected field stem samples were used in the experiments. A variety of methods were used to isolate DNA from soybean plant tissue. The methods included grinding in liquid nitrogen, grinding in type A-5 alumina (Sigma Chemical Co., St. Louis), homogenization with a Polytron homogenizer (Brinkman Instruments, Westbury, NY), followed by phenol-chloroform extraction. Methods of isolating DNA from soybean plant also included boiling ground soybean tissue in Tris-EDTA buffer, as described for wheat roots (20). These methods generated DNA that contained substances that interfered with PCR. The presence of inhibitory substances in the DNA extracts from infected soybean plants was tested by mixing the extracts with pure DNA of fungal isolates in PCR. The *Fast*DNA kit (Bio 101) provided a reliable method of isolating DNA from infected soybean plants suitable for PCR. The procedure involved placing chopped soybean stem or leaf vein tissue (about 70 mg of tissue for stems and all the vein tissue available for leaves) into a homogenization tube with DNA binding buffer. After the tube was tightly capped, the tissue was homogenized for two 12-s periods at an intensity setting of 4.5 in the FastPrep FP120 (Bio 101). The DNA in the homogenate was purified and eluted with 80 µl of sterile distilled water. The DNA was usually diluted 10- to 100-fold before being used in PCR.

For greenhouse experiments, two soybean cultivars, Century (susceptible to brown stem rot) and BSR101 (resistant to brown stem rot), were used. Two-week-old seedlings were inoculated with isolate BSR101 of *P. gregata*, as described previously (11). Noninoculated plants were used as controls. Plants were kept in the shade to promote development of the disease. In the first test, November through December 1995, two plants from each treatment were sampled at 21 and 35 days after inoculation. DNA was isolated from the stem and the combined vascular vein tissue of leaves of each plant and was used in PCR with specific primers BSR1 and BSR2. In the second test, February through March 1996, two plants were taken from each treatment 17 and 31 days after inoculation. For each inoculated plant, total DNAs were isolated from the first and second internodes of the stem and the vascular vein tissue of the first unifoliar and first trifoliate leaves. For each noninoculated control plant, DNA was isolated from the stem and combined leaf veins. The presence of *P. gregata* in the plant tissue of various plant sections was confirmed by plating tissue pieces from chopped samples used in DNA isolation on selective medium (24). Five cross-sections of each stem section or five pieces of

leaf vein tissue were plated on isolation agar plate and incubated at 18 to 20°C. The number of tissue pieces out of five that showed fungal growth conforming to the morphology of *P. gregata* was recorded with the aid of a microscope.

Field samples of soybean stems that showed brown stem rot symptoms were collected at the end of the 1995 growing season from experimental plots located near Arlington, Cochrane, Hancock, and Racine, WI. In the first test, six diseased stems each of cvs. Newton and Sturdy were used. Each stem was first ground into a fine powder with a Wiley Mill (Curtin Matheson Scientific, Inc., Houston, TX). About 75 mg of the powder from each stem was used in DNA isolation by the *fast*DNA kit, as described above. Other methods, as described above for greenhouse inoculated plants, to isolate DNA from the woody soybean stem were unsuccessful. The stem powder was spread on selective medium (24) for observation of the growth of *P. gregata*, as described above. In the

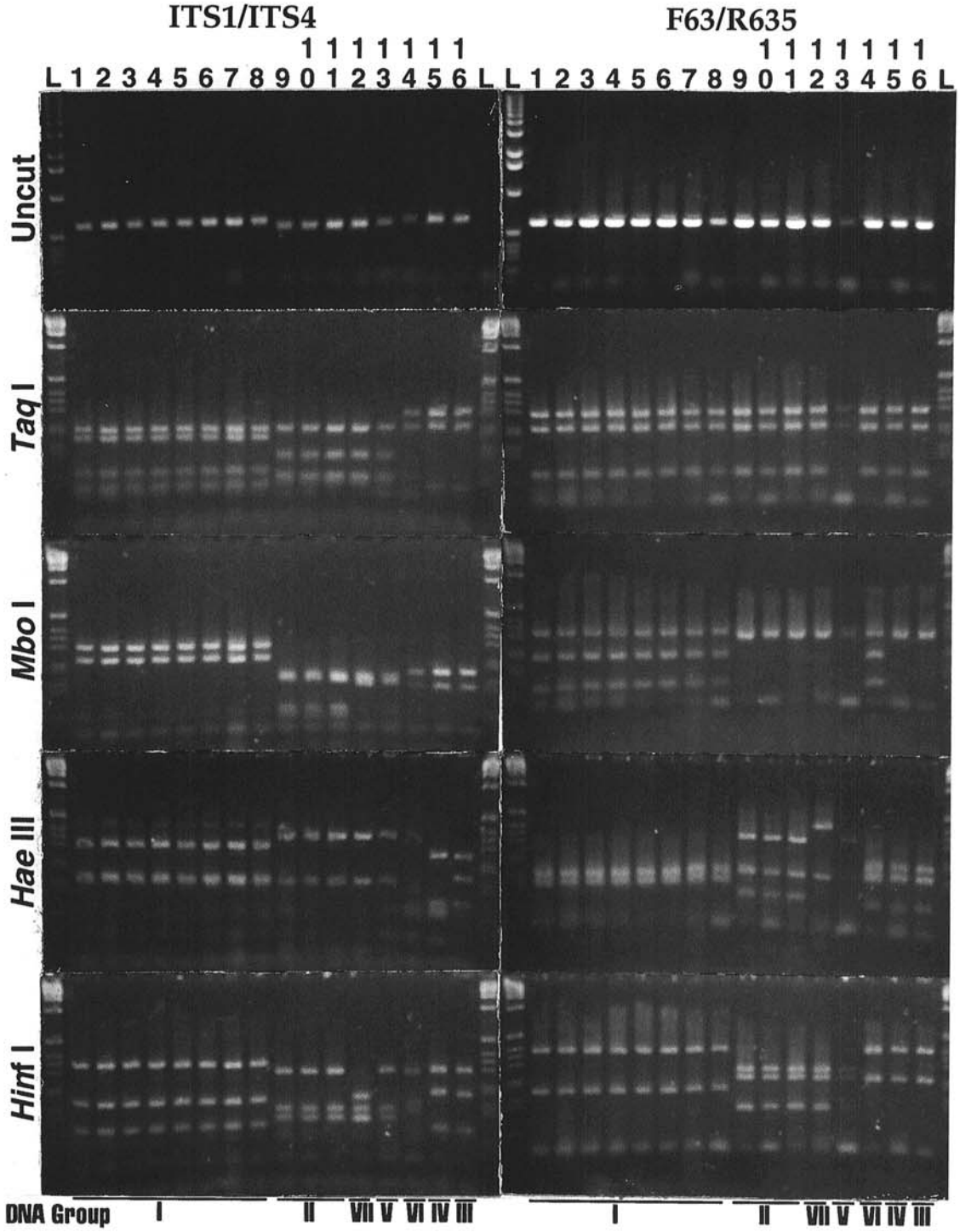

Fig. 1. Agarose gels showing the sizes of polymerase chain reaction (PCR) products and representative banding patterns after restriction enzyme digestion. The amplified products were DNA regions of the internal transcribed spacer (ITS1/ITS4) and the 5′ end of the large subunit nuclear ribosomal DNA (F63/R635) and were electrophoresed on 1% agarose gels. Restriction enzyme digestions were electrophoresed on 3% composite agarose gels (2% GTG agarose and 1% regular agarose). Lanes 1 through 8: isolates B1, G3, LM2, H101-2, IT, WI8, A101-2, and PG11 of *Phialophora gregata*, respectively; lanes 9 through 11: isolates Arc2, Acr4, and Acr5 of *Acremonium* sp. from soybean, respectively; lane 12: isolate Pt1 of *Plectosporium tabacinum*; lane 13: ATCC32182 of *A. nepalense*; lane 14: ATCC 32181 of *A. dichromosporum*; lanes 15 and 16: isolates Acr2 and YS08 of *Acremonium* sp. from soybean, respectively; lane L: Gibco-BRL 1-kb DNA ladder, except in the uncut PCR product gels, in which the last lane is the negative control without template DNA.

second test, six diseased stems each of cvs. Bell and BSR101 were used, and the procedure for DNA isolation and PCR was the same as for the first test.

PCR conditions for detecting *P. gregata* in soybean plants were the same as described above, except the annealing temperature was either 50 or 60°C. The presence of PCR products and the product size were determined with agarose gel electrophoresis. The PCR products with the specific primers from the greenhouse inoculated plants and the field samples were digested with restriction enzymes, and the banding patterns were compared with those from DNAs of *P. gregata* isolates. When no PCR products were detected in DNA samples with the specific primers, such as in non-inoculated plants, the DNAs were used in PCR with "universal" primers ITS1 and ITS4 to check the suitability of the DNA for PCR and the presence of the ITS region.

The isolation of DNA from each fungal isolate, each section of inoculated plants, and each field soybean stem was done only once, but each DNA preparation was used in PCR or restriction digestion of the same DNA fragment at least twice at different times. All experiments (except DNA isolation and sequencing reactions) were replicated in time, and the same results were obtained unless otherwise indicated.

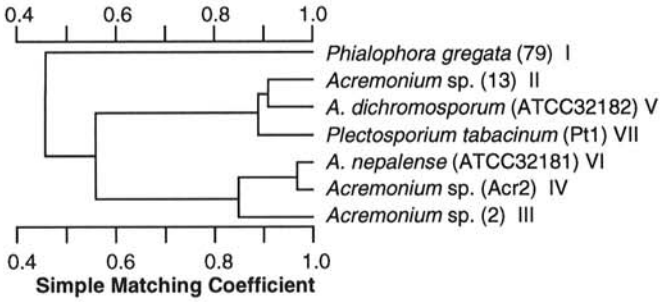

Fig. 2. Phenogram showing the relationships of the various isolate groups based on the DNA bands of the polymerase chain reaction products of the internal transcribed spacer and the 5′ end of the large subunit nuclear DNA and of the restriction banding patterns. The isolate number or the number of isolates, if more than one isolate shared the same banding pattern, is indicated in parenthesis. Roman numerals indicate the isolate groups corresponding to the DNA groups in Figure 1 and Table 1.

RESULTS

PCR and restriction enzyme digestion. PCR with primers ITS1 and ITS4 was successful in amplifying a single specific product from each isolate. Three different sizes of products were observed among the isolates listed in Table 1. The isolates of *P. gregata* showed one size, about 610 bp, and the isolates of *Acremonium* sp. showed two sizes, 550 and 580 bp (Fig. 1). After restriction enzyme digestion, each length variant showed different banding patterns (Fig. 1). All the isolates from *P. gregata* showed the same banding pattern. The *Acremonium* isolates with 550 bp and the *Plectosporium tabacinum* isolate showed two banding patterns after

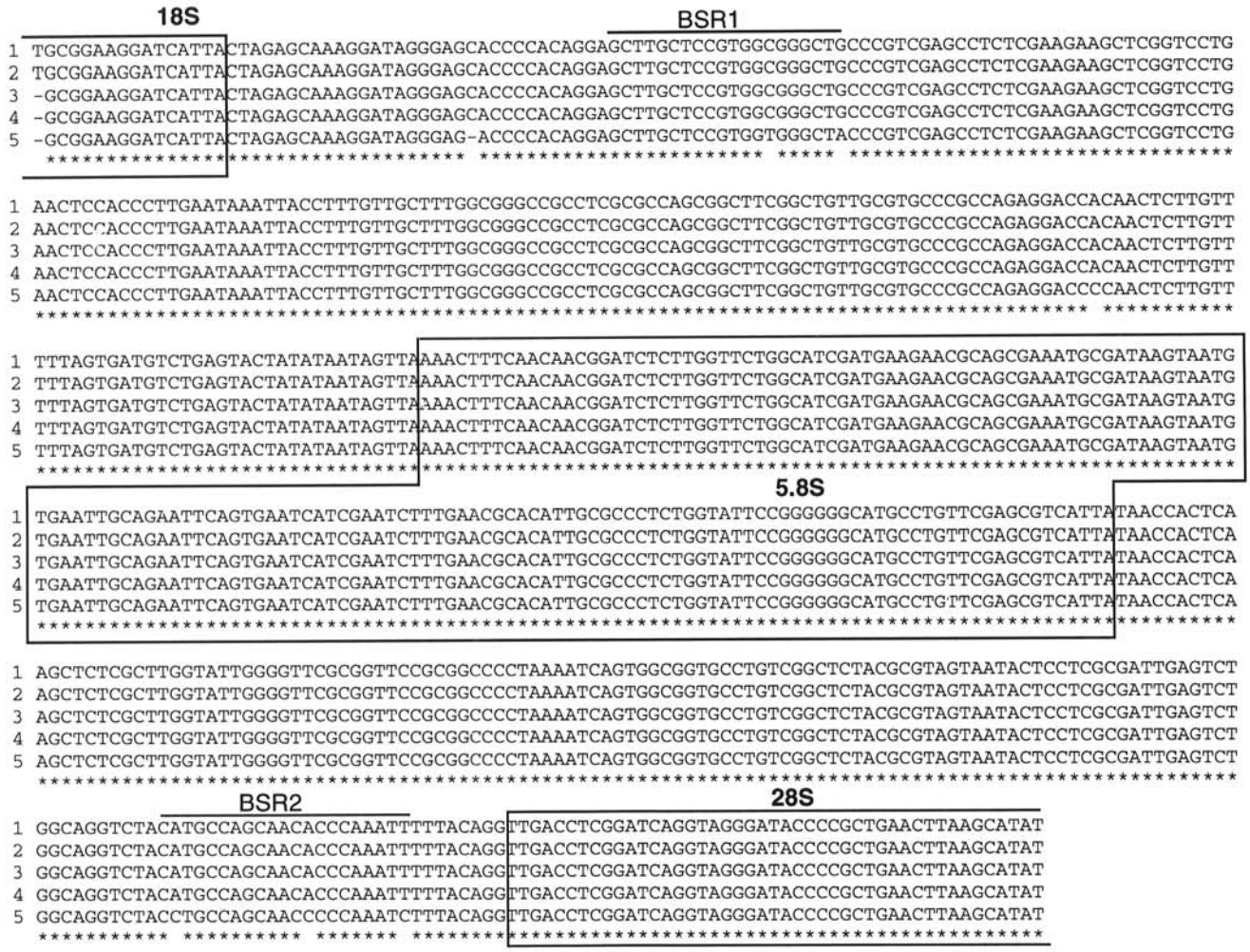

Fig. 3. Alignment of DNA sequences of the internal transcribed spacer region of five isolates of *Phialophora gregata*. The coding regions of 18S, 5.8S, and 28S are boxed, and the locations of specific primers BSR1 and BSR2 are indicated. An asterisk indicates that the nucleotide is identical among the five sequences. Sequence numbers 1 through 4: isolates H101-2, LM3, G3, and SAA1 of *P. gregata* isolated from soybean, respectively; and sequence number 5: isolate 46906 of *P. gregata* isolated from adzuki bean.

digestion with enzymes *Mbo*I and *Hin*fIII (Fig. 1). The two isolates (YS08 and Acr2) of *Acremonium* sp. showed the same banding pattern after digestion with three enzymes but showed different banding patterns after digestion with *Hae*III. *A. dichromosporum* (ATCC 32181) also showed similar banding patterns with these two *Acremonium* isolates.

The PCR products with primers F63 and R635, which amplify the 5′ end of the nuclear large subunit rDNA (7,19), were about the same size for all the isolates included in this study. Digestion with enzyme *Taq*I did not detect variation among the isolates studied. Digestion with enzyme *Mbo*I showed two banding patterns. All isolates of *P. gregata* and *A. dichromosporum* shared one banding pattern, and the *Acremonium* isolates from soybean, the *Plectosporium* isolate, and the *A. nepalense* isolate shared another banding pattern. Digestion with enzyme *Hin*fI produced three banding patterns that corresponded to the three ITS product sizes. The digestion banding patterns with enzyme *Hae*III also corresponded to the three ITS product sizes, except the *Plectosporium* isolate showed a banding pattern that differed from the *Acremonium* species. The isolates were separated into different groups based on a combination of the banding patterns of the ITS and 5′ end of large subunit rDNA after digestion with the four enzymes (Fig. 1). The isolates belonging to perspective DNA groups are presented in Table 1.

Cluster analysis, based on the DNA banding patterns, showed that *P. gregata* was distinct from the *Acremonium* isolates from soybean (Fig. 2). The DNA group II isolates of *Acremonium* sp. were more closely related to *A. dichromosporum* and *Plectosporium tabacinum,* whereas DNA groups III and IV isolates of *Acremonium* sp. from soybean clustered with *A. nepalense* (Fig. 2).

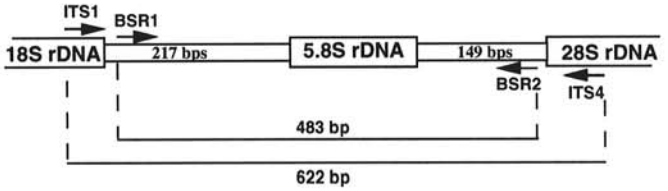

Fig. 4. Schematic drawing showing the relative locations of the polymerase chain reaction (PCR) primers and the expected fragment sizes (inclusive of the primers) of PCR products from *Phialophora gregata*. Wide bars indicate coding regions, as labeled; narrow bars indicate internal transcribed spacers with their sizes (base pairs) indicated.

Fig. 5. Agarose gel showing polymerase chain reaction amplification with specific primers BSR1 and BSR2 at an annealing temperature of 60°C. Lane L: Gibco-BRL 1-kb DNA ladder; lane 1: DNA of soybean cv. Century; lanes 2 and 3: isolates G3 and WI6 of *Phialophora gregata* isolated from soybean; lane 4: isolate 46906 of *P. gregata* isolated from adzuki bean; lanes 5 through 9: isolates Arc2, Acr1, Acr4, Pg14, and YS08 of *Acremonium* sp. isolated from soybean, respectively; lane 10: isolate 32182 of *A. dichromosporum*; lane 11: isolate 32181 of *A. nepalense*; lane 12: *Cercospora* sp.; lane 13: *Septoria* sp.; lane 14: *Pythium ultimum*; lane 15: *Verticillium dahliae*; lane 16: *Gaeumannomyces graminis* var. *tritici*; and lane N: negative control without template DNA.

DNA sequencing and sequence comparisons. The ITS sequences of isolates H101-2, LM1, SAA1, G3, 46906, and Arc2 were deposited in the GenBank and assigned accession numbers U66727, U66728, U66729, U66730, U66731, and U66732, respectively. The ITS sequences of four isolates of *P. gregata* from soybean from Illinois, Wisconsin, and Brazil were identical and were very similar to the ITS sequence of isolate 46906 of *P. gregata* from adzuki bean (Fig. 3). The ITS-1 region between the small subunit and the 5.8S rDNA was 217 bases long in soybean isolates and 216 bases long in the adzuki bean isolate. In addition to the 1-base difference in length, there are three 1-base mutations in this DNA region. There were 149 bases in the ITS-2 region between the 5.8S and the large subunit rDNA in both the soybean and adzuki bean isolates, and there were three 1-base mutations (Fig. 3). The ITS sequences of *P. gregata* were compared with published ITS sequences of other *Phialophora* species, including *P. americana* (U31837), *P. parasitica* (U31841), *P. richardsiae* (U31844), *P. verrucosa* (U31846), and *P. graminicola* (U17217). The ITS sequences of the *P. gregata* isolates were so different from other ITS sequences that they could not be aligned (data not shown). The ITS sequences of *P. gregata* also were very different from the ITS sequences of *Acremonium* spp., except in the coding regions.

A BLAST database search with the ITS-1 sequence (217 bases) of *P. gregata* as the query showed that the most similar sequence in the databases was a *Hymenoscyphus ericae* sequence (L06325) that showed 88% similarity in a 62-base stretch. A BLAST search with the ITS-2 sequence (149 bases) as the query showed that the most similar sequences from the databases were sequences of a *Phialophora*-like species (X62991) and two unidentified Deuteromycetes (X62979 and X62980) (all three were symbionts of *Festuca arizonica*) (3) showing 92% similarity over the entire ITS-2 sequence. The entire ITS sequence, including ITS-1, 5.8S, and ITS-2 of isolate Arc2 (DNA group II) of *Acremonium* sp., was identical to that of *Plectosphaerella cucumerina* (L36640) (26), the teleomorph of *Plectosporium* (27). A BLAST search with the ITS-1 sequence (164 bases) of isolate YS08 (DNA group III) of *Acre-*

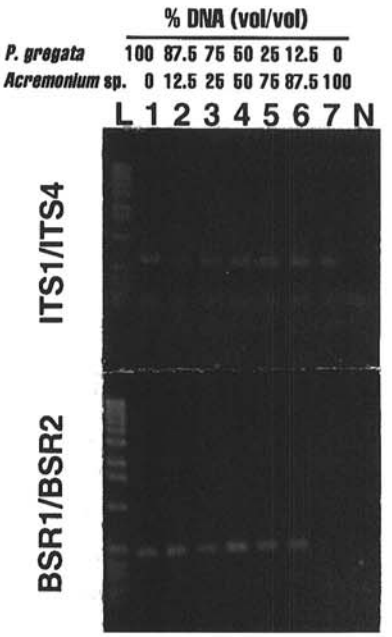

Fig. 6. Agarose gels showing polymerase chain reaction amplification with primers ITS1 and ITS4 at an annealing temperature of 50°C and with primers BSR1 and BSR2 at an annealing temperature of 60°C with DNA from isolates Pg11 (*Phialophora gregata*) and Acr1 (*Acremonium* sp.) and mixtures of these two DNA solutions in various proportions, as indicated at the top of the figure (lanes 1 through 7). The percentages of the mixed DNAs were based on the volume of the working DNA solutions and, therefore, may not reflect the absolute DNA concentrations. Lane L: Gibco-BRL 1-kb DNA ladder; and lane N: negative control without template DNA.

monium sp. as the query found no similar sequences in the databanks, and a BLAST search with the ITS-2 sequence (175 bases) of the same isolate as the query revealed that the most similar sequences in the databanks were *Fusarium ventricosum* (L36657) and *Beauveria* spp. (U19040 to U19042) sequences, which showed 73% similarity in a 127-base stretch and 90% similarity in a 75-base stretch, respectively. The complete ITS sequence of isolate YS08 will be presented elsewhere.

Specificity of PCR primers for *P. gregata*. After comparing the *P. gregata* sequences with sequences of other *Phialophora* spp., *Acremonium* spp., and soybean, two PCR primers, BSR1 (5′-GCTTGCTCCGTGGCGGGCTG-3′) and BSR2 (5′-AATTTGGGTGTTGCTGGCATG-3′), were designed specifically for *P. gregata* from soybean (Fig. 3). The relationship of the specific primers with primers ITS1 and ITS4 and the expected DNA fragments are schematically represented in Figure 4. In PCR with primers BSR1 and BSR2 at an annealing temperature of 50°C, the expected PCR product (about 480 bp) was observed in all isolates of *P. gregata*, including isolates from adzuki bean, but not from the *Acremonium* spp. listed in Table 1, five other fungal DNAs, nor soybean DNA. Occasionally, a nontargeted fragment (about 150 bp) was observed in a *Acremonium* sp.; however, at an annealing temperature of 60°C, this nonspecific band was not observed. In addition, at an annealing temperature of 60°C, no PCR product was observed in isolates of *P. gregata* from adzuki bean, whereas PCR still efficiently amplified the specific product from all isolates of *P. gregata* from soybean and mung bean (Fig. 5).

Amplification in mixed DNAs. Using primers ITS1 and ITS4, PCR showed that only the product size of *Acremonium* sp. was detectable from mixed DNAs of *P. gregata* and *Acremonium* sp. (Fig. 6A, lanes 2 through 6). Although the working DNA concentrations were not adjusted to the same level, the amplification of the expected fragment size from pure *P. gregata* DNA (Fig. 6A, lane 1) and from pure *Acremonium* sp. DNA (Fig. 6A, lane 7) indicated that sufficient DNA was present for amplification. Using primers BSR1 and BSR2 in PCR at 60°C as the annealing temperature, the specific DNA fragment was detected in all reactions that contained DNA of *P. gregata*, even in the reaction with DNA templates containing only 12.5% *P. gregata* DNA (Fig. 6B). As expected, no amplification product was detected in the reaction containing only *Acremonium* sp. DNA. Restriction digestion showed that the products amplified from mixed DNAs with primers ITS1 and ITS4 were the same as that from *Acremonium* sp., whereas the products amplified with primers BSR1 and BSR2 were the same as that amplified from *P. gregata* (digestion gels not shown).

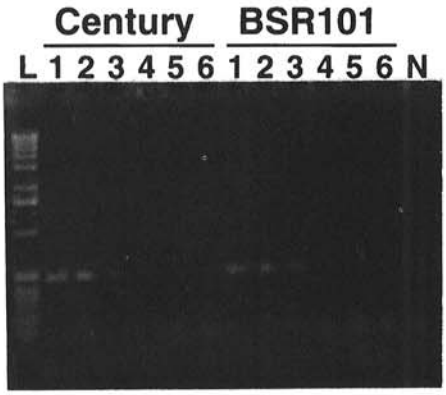

Fig. 7. Agarose gel showing polymerase chain reaction amplification with specific primers BSR1 ad BSR2 from inoculated cvs. Century and BSR101 soybean plants. Lanes 1 through 4: the first and second internodes of the stem and the first unifoliate and first trifoliate leaves, respectively, of an inoculated plant; lanes 5 and 6: stem and leaves of a control (noninoculated) plant, respectively; and lane N: negative control without template DNA.

Detection of *P. gregata* in soybean plants. A variety of methods for isolating DNA from infected soybean plants were studied. The greatest problem we experienced was the presence of a brown substance(s) in the DNA, especially in infected plants, which interfered with PCR. The only method by which DNA suitable for PCR was obtained was with the *Fast*DNA kit. All the experiments with artificially inoculated plants or naturally infected field samples described hereafter were done with the *Fast*DNA isolation kit. PCR with the specific primers did not amplify any DNA fragments from noninoculated soybean plants, although the DNA was suitable for PCR with primers ITS1 and ITS4. In both of the greenhouse tests comparing soybean cvs. BSR101 and Century, BSR101 showed foliar symptoms 3 to 6 days after Century did. In the first test, *P. gregata* was detected in the stems of both cultivars at 21 days after inoculation. At the second sampling (35 days after inoculation), *P. gregata* was detected in stems and leaves of both cultivars. Isolation by plating also showed colonization of soybean tissue by *P. gregata*. In the second test, 17 days after inoculation, *P. gregata* was detected in the first and second internodes of stems of both cvs. Century and BSR101, but only in the leaves of inoculated Century plants not in the leaves of BSR101 plants. However, *P. gregata* was detected in both the stems and leaves in both cultivars at day 31 (Fig. 7). The presence of *P. gregata* in the various sections of the soybean plants also was confirmed by plating on selective medium. The number of tissue pieces out of a total of 10 (5 from each plant) that were colonized by *P. gregata* from each section of the plants as well as with PCR detection with the specific primers are shown in Table 2.

P. gregata was detected in DNA isolated from soybean stems collected from fields during 1995. In the first test, with stems of soybean cvs. Newton and Sturdy, the expected DNA products were observed from all stems by PCR with primers BSR1 and BSR2 at both 50 and 60°C as the annealing temperatures (gels not shown). In the second test, with diseased stems of soybean cvs. Bell and BSR101, a nonspecific band of about 150 bp also was observed in some stems in PCR at an annealing temperature of 50°C. However, when the annealing temperature was raised to 60°C, the nonspecific band was absent, but the specific 483-bp

TABLE 2. Detection by isolation and polymerase chain reaction (PCR) of *Phialophora gregata* in artificially inoculated soybean cvs. Century and BSR101 at 17 and 31 days after inoculation

	17 days[a]				31 days[a]			
	Century		BSR101		Century		BSR101	
Plant section	Isolation[b]	PCR[c]	Isolation	PCR	Isolation	PCR	Isolation	PCR
First internode	10	+	9	+	10	+	9	+
Second internode	8	+	5	+	9	+	7	+
First leaf[d]	7	+	0	–	5	+	2	+
Second leaf[d]	7	±	0	–	4	±	1	–

[a] At each sampling date, *P. gregata* was not detected in noninoculated plants by either isolation or PCR.
[b] Number of tissue pieces out of a total of 10 (5 from each plant) that were colonized by *P. gregata*, identified under a microscope.
[c] Presence (+) or absence (–) in both sampled plants of a 483-bp DNA fragment detected by PCR with specific primers BSR1 and BSR2. ± indicates the DNA was detected in one plant but not in the other.
[d] The first leaf was the unifoliate leaf, and the second leaf was the first trifoliate leaf.

fragment was still present and was the only PCR product from all the stems (Fig. 8). As expected, the product intensities varied from stem to stem. The PCR products amplified from field stems with the specific primers were compared with the products amplified from pure DNA of *P. gregata* isolates by restriction enzyme digestion. Identical restriction banding patterns were observed (Fig. 9). The PCR product from a field stem of cv. BSR101, shown in Figure 8, lane 5, was purified and sequenced. The nucleotide sequence was the same as those of the *P. gregata* isolates (sequence not shown).

DISCUSSION

The results, based on a large number of isolates from a wide range of geographic locations, showed that the restriction banding patterns of the PCR-amplified ITS and the 5′ end of the large subunit rDNA could be used to unambiguously differentiate *P. gregata* from *Acremonium* spp. isolated from soybean. Previous reports have shown that these two species have different morphologies and isozyme banding patterns and that they vary in pathogenicity on soybean (21). Isolates of *P. gregata* from soybean from various locations had identical ITS sequences and were 98% similar to the sequence in *P. gregata* isolated from adzuki bean. Restriction maps based on the sequences showed that the recognition sites of the four enzymes did not fall in the variable nucleotide region, explaining the identical restriction banding patterns between the soybean and adzuki bean isolates. This finding is consistent with pathogenicity studies.

Isolates obtained from soybean and mung bean are pathogenic to both crops and not pathogenic on adzuki bean and vice versa (14–16). Isolates from soybean have different restriction banding patterns of mitochondrial DNA (10), different isozyme banding patterns (34), and smaller genome sizes (35) than those of isolates from adzuki bean. The adzuki bean isolates have an intron located upstream of the primer ITS1 location in the small subunit rDNA, whereas the intron is absent in the *P. gregata* isolates from soybean and mung bean (W. Chen and L. E. Gray, *unpublished data*).

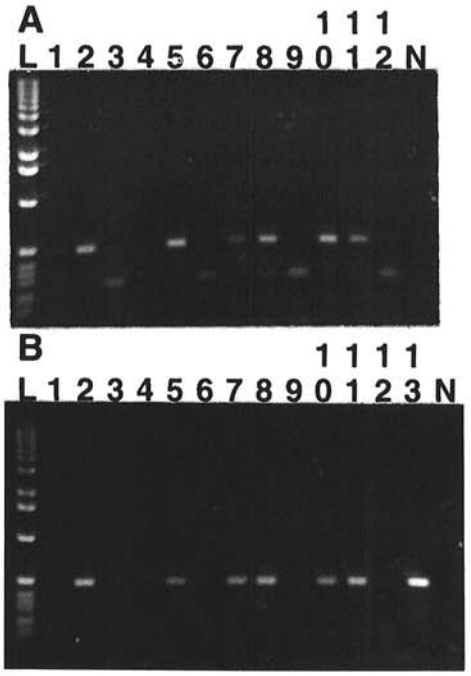

Fig. 8. Polymerase chain reaction (PCR) amplification with specific primers BSR1 and BSR2 from field-collected soybean stems. **A,** PCR with an annealing temperature of 50°C; and **B,** PCR with an annealing temperature of 60°C. Lanes 1 through 6: stems of cv. BSR101; lanes 7 through 12: stems of cv. Bell; lane 13 (**B**): isolate LM3 of *Phialophora gregata*; and lane N: negative control without template DNA.

Different formae specialis of *P. gregata* have been proposed based on host specificity (16).

At least two groups of *Acremonium* spp. were associated with soybean brown stem rot. One group (DNA group II) was clustered with *A. dichromosporium* and *Plectosporium tabacinum* (Fig. 2), and its entire ITS sequence (isolate Acr2) was identical to that of *Plectosphaerella cucumerina*, the teleomorph of *P. tabacinum*. This isolate group could presumably be a *Plectosporium* sp., whereas the identity of the other group (DNA group III) of *Acremonium* sp. from soybean remains elusive.

Specific PCR primers were designed for *P. gregata* isolates from soybean. The specific primers have three applications: (i) to identify and differentiate cultures of *P. gregata* from *Acremonium* sp. isolated from soybean; (ii) to detect *P. gregata* in soybean plants and, hence, diagnose brown stem rot disease; and (iii) to monitor the movement of the pathogen in soybean plants to study the interactions of *P. gregata* with different soybean genotypes. Although the presence of *P. gregata* in a soybean plant can be checked by plating and microscopic examinations, the isolation process takes three or more weeks to complete. The detection of *P. gregata* with specific PCR primers can be accomplished within 1 day, which speeds up the process considerably. Caution should be used with the specific primers in identifying *P. gregata* cultures. We had three isolates that contained both *P. gregata* and *Acremonium* sp. Amplification with primers ITS1 and ITS4 produced the DNA band of *Acremonium* sp., but amplification with primers BSR1 and BSR2 showed they were *P. gregata*. After sequencing both fragments from one of the three isolates, we concluded that they were mixed cultures. After single-sporing the three cultures, we amplified the DNA fragment of *Acremonium* sp. with primers ITS1 and ITS4 but did not detect any DNA with primers BSR1 and BSR2, confirming that the original isolates were mixed cultures.

The PCR experiment with mixed DNAs demonstrated two facts: (i) "universal" primers ITS1 and ITS4 cannot be used to detect mixed

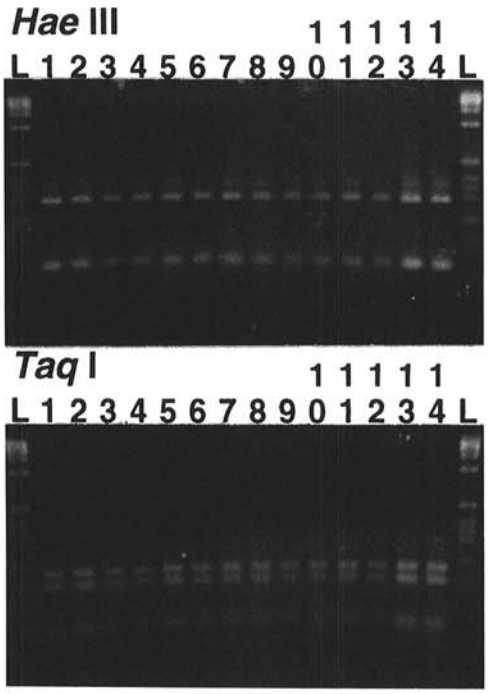

Fig. 9. Restriction enzyme digestion (*Hae*III and *Taq*I) of polymerase chain reaction products amplified with specific primers BSR1 and BSR2 from field-collected soybean stems and purified DNA of *Phialophora gregata* isolates. Lane L: Gibco-BRL 1-kb DNA ladder; lanes 1 through 3: stems of cv. Newton; lanes 4 through 6: stems of cv. Bell; lanes 7 through 9: stems of cv. BSR101; lanes 10 through 12: stems of cv. Sturdy; and lanes 13 and 14: isolates IN1 and WI3 of *P. gregata*.

DNAs for *P. gregata* and *Acremonium* sp., as in the case of the three mixed cultures mentioned above, and (ii) specific primers BSR1 and BSR2 can be used to detect *P. gregata* in the presence of *Acremonium* sp. Theoretically, primers ITS1 and ITS4 could amplify both DNAs and allow us to detect the presence of both DNAs of *P. gregata* and *Acremonium* sp. after restriction digestion. This approach would not work in the case of *P. gregata* and *Acremonium* sp., which might be due to preferential primering and extension of one species over the other. This experiment showed that specific primers were necessary to detect *P. gregata* in its natural habitats.

Consistent results were obtained in detecting *P. gregata* in soybean stems, and detection with PCR is consistent with detection by traditional isolation techniques. However, improvements are needed to increase consistency in detecting *P. gregata* in vein tissue of soybean leaves. The amount of vascular vein tissue of a leaf varies considerably depending on the size of the leaf, and it is usually in small quantities. The isolation technique should be modified to accommodate the small amount of tissue, which should improve consistency in PCR detection. Isolation of DNA from lignified soybean stems presented a challenge. The woody stems were hard to break, and infected tissue usually contained substances that inhibited amplification in PCR. The *fast*DNA kit proved to be an effective alternative. This procedure is efficient and can accommodate a large number of samples. Additionally, the homogenization process was completed within closed centrifuge tubes; therefore, the possibility of cross-contamination between samples was minimized.

Isolate BSR101 of *P. gregata* is highly virulent on soybean cv. BSR101, and *P. gregata* can be isolated readily from other resistant cultivars that do not show foliar symptoms (24). Cv. BSR101 is considered resistant to brown stem rot (31). *P. gregata* was detected in artificially infected plants of soybean cv. BSR101. The specific PCR fragment also was detected in naturally infected field stems of cv. BSR101 as well as in other cultivars, and sequence analyses confirmed they were from *P. gregata*. These experimental results, in addition to field observations, suggest that cv. BSR101 may not be resistant to specific isolates of *P. gregata*.

PCR with specific primers is an efficient alternative to identify *P. gregata* cultures and study interactions of *P. gregata* with soybean plants. Although both *Acremonium* sp. and *Phialophora*-like sp. are endophytes of grasses (3), the potential interaction of *Acremonium* sp. with *P. gregata* in soybean and the role of *Acremonium* spp. in brown stem rot disease are unknown. Development of new primers specific for the *Acremonium* sp. from soybean in combination with the specific primers for *P. gregata* provide a new approach to studying the interactions among *Acremonium* sp., *P. gregata*, and soybean plants.

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