Mitochondrial DNA Restriction Patterns of *Phialophora gregata* Isolates from Soybean and Adzuki Bean

L. E. Gray and A. G. Hepburn

First author: United States Department of Agriculture, Agriculture Research Service, Plant Protection Research, and Department of Plant Pathology, University of Illinois, Urbana 61801; and second author: Department of Agronomy, University of Illinois, Urbana 61801.

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ABSTRACT

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Mitochondrial DNA was examined from 20 soybean and seven adzuki bean *Phialophora gregata* isolates. The restriction enzyme fragment patterns of mitochondrial DNA for five restriction enzymes were compared among the soybean fungal isolates to determine if restriction fragment length polymorphisms could be detected from isolates collected from Illinois, Indiana, Wisconsin, Ohio, Minnesota, and Iowa soybean fields. No differences in mitochondrial DNA restriction fragment patterns were detected among the 20 soybean fungus isolates. Restriction fragment length polymorphisms were detected between adzuki bean and soybean isolates. Two groups, based on restriction fragment length polymorphisms, could be distinguished within the adzuki bean fungus isolate collection. One group, composed of three isolates, had 33% of the mitochondrial DNA restriction fragments that comigrated with the soybean reference isolate. The other group, composed of four isolates, had no bands in common with either the other three adzuki bean isolates or the soybean reference isolate. All four of these isolates had identical mitochondrial DNA restriction fragment patterns. These isolates morphologically appeared to be unidentified *Acremonium* species isolates rather than unidentified *Phialophora* species. Restriction fragment patterns of mitochondrial DNA may be a valuable aid in identifying *Phialophora* isolates as well as an aid in separating *Phialophora* isolates from *Acremonium* isolates that are associated with vascular browning of soybean and adzuki bean plants.

Determination of species of *Phialophora* is based on growth habit in culture and morphology of spores and conidiophores (26); however, this can be difficult because of the variation in morphological characteristics that occur on various growth media and also with culture age. *Phialophora gregata* (Allington & D. W. Chamberlain) W. Gams causes a vascular disease of soybeans (*Glycine max* (L.) Merr.) (1) and adzuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi) (15,17). This disease has been found in many soybean-producing areas of the midwestern and southeastern United States (1,8,21,24,29). The disease is characterized by discoloration of the stem vascular tissue and the development of interveinal chlorosis and necrosis of leaves of infected plants. Various yield reductions have been reported for soybeans plants with brown stem rot (1,22,27).

Both the soybean and adzuki bean isolates are morphologically similar and are therefore difficult to separate except by host range (15,17). Additionally, there is very little information about the genetic variability of isolates of the fungus. There is variation in growth rates and culture morphology of *P. gregata* isolates (24), and in the virulence of isolates recovered from different midwestern U.S. soybean production areas (8,29).

To date, determination of intraspecific variation in isolates of *Phialophora* taken from soybean has been conducted by isozyme analysis (21). No differences were found in isozyme patterns for 20 isolates identified as *P. gregata*. Differences in isozyme banding patterns were, however, seen between *Phialophora* and *Acremonium* isolates recovered from diseased soybean plants (21).

Considerable genetic variation can exist within a species designation (26). Therefore, despite the evidence from morphological and isozyme analyses, it is likely that wild isolates of *Phialophora* will show such variation if a higher resolution analytical technique is used. Restriction fragment length polymorphism (RFLP) pattern analysis of organelle DNA has been used extensively in systematics (2–4,11,12,19,20). In fungi, the mitochondrial genome is small 19–176 kb (28) and present in high copy number. It therefore offers a molecular system in which to test for the presence of interisolate variation. The objective of this study was to determine the amount of molecular variation in mitochondrial DNA between isolates of *P. gregata* from midwestern U.S. soybean-producing areas. These isolates were also compared with adzuki bean isolates obtained from Japan.

MATERIALS AND METHODS

Fungal isolates and culture conditions. Isolates of *P. gregata* obtained originally from diseased soybean plants in fields in Illinois, Indiana, Iowa, Minnesota, and Ohio were collected between 1975 and 1990 by the authors and by other workers (Table 1). All *P. gregata* isolates pathogenic to adzuki bean were obtained from K. Kobayashi, Sapporo, Japan (Table 1). The soybean and adzuki bean isolates were grown on soybean stem extract agar at 21 C (8). A single-spore culture of each isolate was obtained and, for long term storage, agar disks containing spores of each isolate were placed in individual vials containing 30% (v/v) sterile glycerol and were maintained at −70 C (29). A liquid medium containing 5 g of sucrose, 5 g of glucose, 2 g of Bacto-peptone, 1 g of yeast-extract, 1/20 dilution (v/v) of Murashige and Skoog basal salts medium (23) per liter distilled water adjusted to pH 6.5 was used to grow all cultures. The medium was dispensed 100 ml per 250-ml Erlenmeyer flask and was autoclaved. Six 7-mm agar plugs from stock stem extract agar cultures were used to inoculate each flask, and the flasks were incubated without shaking in the dark at 21 C for 10–14 days. The yield of mycelium after this period was 0.5–0.75 g wet weight per flask.

DNA isolation. Mycelium was collected on miracloth filters and frozen in powdered dry ice. Next, the mycelium was ground in dry ice to a fine powder with a chilled mortar and pestle (25). A buffer composing 100 mM Tris-HCl, 100 mM EDTA (pH 8.0), 6% 4-aminosalicylic acid, (Sigma Chemical Co., St. Louis, MO) and 2% triisopropyl naphthalene sulfonic acid-sodium salt,

(Kodak Co., Rochester, NY) was added to each tube (1.5 ml of extraction buffer per gram of mycelium), and the tubes were incubated for 30 min at 60 C in a water bath. After centrifugation at 6,500 rpm (International Equipment Co., Needham Hts., MA) for 20 min, the supernatant was extracted with phenol-chloroform, isoamyl alcohol (50:49:1 [v/v]), recentrifuged, and the resulting supernatant was transferred to a new tube. The DNA in the sample was precipitated with 2 vol of 95% ethyl alcohol (18) and recovered by centrifugation. The nucleic acid pellet was resuspended in 400 μl of TE buffer (10mM Tris, pH 8.0, 0.1 mM EDTA, pH 8.0). RNAse A (20 mg/ml) was added (10 μl), and the samples were digested for 2 h at 37 C. At the end of the digestion period, a sodium chloride CTAB (hexadecyltrimethylammonium bromide) solution was added (25) plus chloroform-isoamyl alcohol (24/1 [v/v]), and the tube contents were gently mixed and centrifuged in a Beckman E microcentrifuge (Beckman Instruments, Fullerton, CA) at room temperature (25 C) for 15 min. The upper phase was removed to a new tube, and 2.5 vol of cold ethanol was added to precipitate the DNA. After washing twice in 70% ethanol, the DNA was resuspended in sterile TE buffer. The CTAB extraction step was necessary to remove contaminants that could cause problems either during CsCl gradient fractionation or restriction endonuclease digestion.

Isolation of mitochondrial DNA. Mitochondrial DNA was obtained by banding total DNA isolated from soybean *P. gregata* reference strain G3, or adzuki bean reference strain M35 in a CsCl gradient in the presence of bisbenzimide (7). The upper band that formed in the gradient was removed and rebanded at the same CsCl density. After this final equilibrium centrifugation, the band of DNA was isolated, and the bisbenzimide was removed by five extractions with isopropyl alcohol saturated with CsCl. The DNA solution in CsCl was diluted with water (4 vol) and precipitated by the addition of 2 vol of cold ethanol. DNA was recovered by centrifugation, and the pellet was washed twice with 70% ethanol and after drying resuspended in 100 μl of sterile TE buffer.

A plasmid clone pUF1-38, containing the mitochondrial cytochrome oxidase subunit II gene from *Fusarium oxysporum* (12) was used in preliminary Southern hybridization analysis (18) of the isolated DNA from isolate G3 and M35. The clone pUF1-38 was supplied by H. C. Kistler, University of Florida.

Restriction endonuclease analysis and agarose electrophoresis. Restriction enzymes were purchased from Bethesda Research Labs (BRL, Gaithersberg, MD), and all DNA digestions were carried out according to the manufacturer's protocol. After digestion (1 μg of DNA per sample), fragments were separated by horizontal agarose electrophoresis in 0.8% (w/v) agarose in a glycine buffer (200 mM glycine, 15 mM NaOH, 2.6 mM EDTA, final pH 9.0; 9) at 1.5 vol/cm for 18 h. Bacteriophage λ DNA digested with *Hin*dIII was used as the molecular weight marker. After electrophoresis, the gels were stained in ethidium bromide (0.25 μg/ml) for 30 min, destained in water for 30 min, and photographed under UV illumination (310 nm) on Polaroid type 667 film.

Southern blots and hybridization procedures. After DNA digestion and electrophoresis, DNA fragments were transferred to Hybond-N (Amersham Corp., Arlington Heights, IL) nylon membranes using the manufacturer's protocol. Prehybridization and hybridization reactions were performed at 58 C in 5× SSPE (18), 5× Denhardt's solution (18), and 0.5% sodium dodecyl sulfate (SDS) solution. For hybridization analysis, total purified mitochondrial DNA from soybean isolate G3 or adzuki bean isolate M35 was labeled with Biotin-7-dATP using a nick translation kit (BRL) according to the manufacturer's instructions. Labeled probe DNA (0.5 μg) was purified on a Sephadex G50 column and denatured by boiling for 10 min followed by quick cooling on ice for 10 min. Hybridizations were carried out for 18 h at 58 C and, after hybridization, membranes were washed twice in 1× SSPE, 1% SDS, for 15 min at room temp (23 C), twice in 0.5× SSPE, 1% SDS at 65 C for 15 min, and twice in 0.1× SSPE, 1% SDS for 15 min at 65 C. Individual bands on the membrane were detected using a blue gene detection system (BRL).

Comparison of mitochondrial DNA restriction fragment patterns. Banding patterns of hybridization on nylon membranes were used to compare fragment patterns of fungus isolates. All comparisons were made against a soybean reference isolate G3. Each enzyme digest, fragment separation on agarose gels, Southern blot, and hybridization was done twice. This was done to ensure that no fragments were missed. The size in kilobase pairs of the mitochondrial DNA for the soybean reference isolate G3 and adzuki bean isolates was estimated by summation of the

TABLE 1. Designation and source of *Phialophora gregata* isolates

Designation	Location	Source	Host plant	Symptoms on inoculated plant[a]
G3	Illinois	L. E. Gray	Soybean	Ls,Vb
C3	Illinois	L. E. Gray	Soybean	Ls,Vb
W3	Wisconsin	C. Grau	Soybean	Ls,Vb
W6	Wisconsin	L. E. Gray	Soybean	Ls,Vb
W27	Wisconsin	R. Waller	Soybean	Vb
I16	Iowa	R. Waller	Soybean	Ls,Vb
I59	Iowa	R. Waller	Soybean	Ls,Vb
It	Iowa	H. Tachibana	Soybean	Vb
M7	Minnesota	R. Waller	Soybean	Vb
M9	Minnesota	R. Waller	Soybean	Vb
Oh1	Ohio	L. E. Gray	Soybean	Ls,Vb
Oh2	Ohio	L. E. Gray	Soybean	Ls,Vb
Oh6	Ohio	L. E. Gray	Soybean	Ls,Vb
In1	Indiana	L. E. Gray	Soybean	Ls,Vb
Hob-1	Indiana	L. E. Gray	Soybean	Ls,Vb
Harp-1	Indiana	L. E. Gray	Soybean	Ls,Vb
Rbs1-2	Indiana	L. E. Gray	Soybean	Ls,Vb
Sbn2	Illinois	L. E. Gray	Soybean	Ls,Vb
G1	Illinois	L. E. Gray	Soybean	Vb
L2	Illinois	L. E. Gray	Soybean	Vb
522	Japan	K. Kobayshi	Adzuki bean	Ls,Vb
A8	Japan	K. Kobayshi	Adzuki bean	Vb
19	Japan	K. Kobayshi	Adzuki bean	Vb
M35	Japan	K. Kobayshi	Adzuki bean	Vb
S35	Japan	K. Kobayshi	Adzuki bean	Vb
30-2	Japan	K. Kobayshi	Adzuki bean	Vb
M8	Japan	K. Kobayshi	Adzuki bean	Vb

[a] Pathogenicity of each fungus isolate was determined by inoculating century soybean and adzuki bean (Plant Introduction 273.485) plants (four replications, two plants per pot) by the root-dip method (29). Plants were grown in a greenhouse at 25 C day, 21 C night temperature for 7 wk and then evaluated for both leaf symptoms, Ls; and internal stem vascular browning, Vb (L. E. Gray and A. G. Hepburn, *unpublished data*). None of the soybean isolates caused any symptoms on inoculated adzuki bean, and the adzuki bean isolates did not infect soybean (L. E. Gray and A. G. Hepburn, *unpublished data*).

TABLE 2. Restriction fragment patterns (kilobase pairs) of *Bgl*II, *Eco*RI, *Eco*RV, and *Xho*I digested mitochondrial DNA from *Phialophora gregata* isolates pathogenic on soybean and adzuki bean[a]

Fragment number	522[b]				G3[b]			
	*Bgl*II	*Eco*RI	*Eco*RV	*Xho*I	*Bgl*II	*Eco*RI	*Eco*RV	*Xho*I
1	8.6	11.6	17.2	28.5	7.4	10.6	15.8	29.1
2	7.7	9.5	9.2	8.9	7.0	7.8	8.8	10.4
3	6.5	9.2	8.6	7.4	6.1	6.1	7.8	7.6
4	6.1	4.8	6.0	5.9	5.3	4.8	5.9	
5	6.0	3.6	5.6		4.9	4.0	5.5	
6	4.4	3.1	2.5		4.4	3.9	1.8	
7	3.3	2.8	2.2		3.3	3.3	1.6	
8	2.8	2.3			2.8	2.9		
9	2.7	1.6			2.7	2.8		
10	1.7	1.2			1.7	1.6		
11	1.6	1.0			1.6			

[a] Total *Bgl*II, *Eco*RI, *Eco*RV, and *Xho*I digested mitochondrial DNA was probed with total labeled mitochondrial DNA isolated from soybean *P. gregata* isolate G3.

[b] *P. gregata* isolates 522, A8, and 19 are from adzuki bean and showed identical restriction fragment patterns, and *P. gregata* isolate G3 was the reference strain from soybean (Fig. 1A).

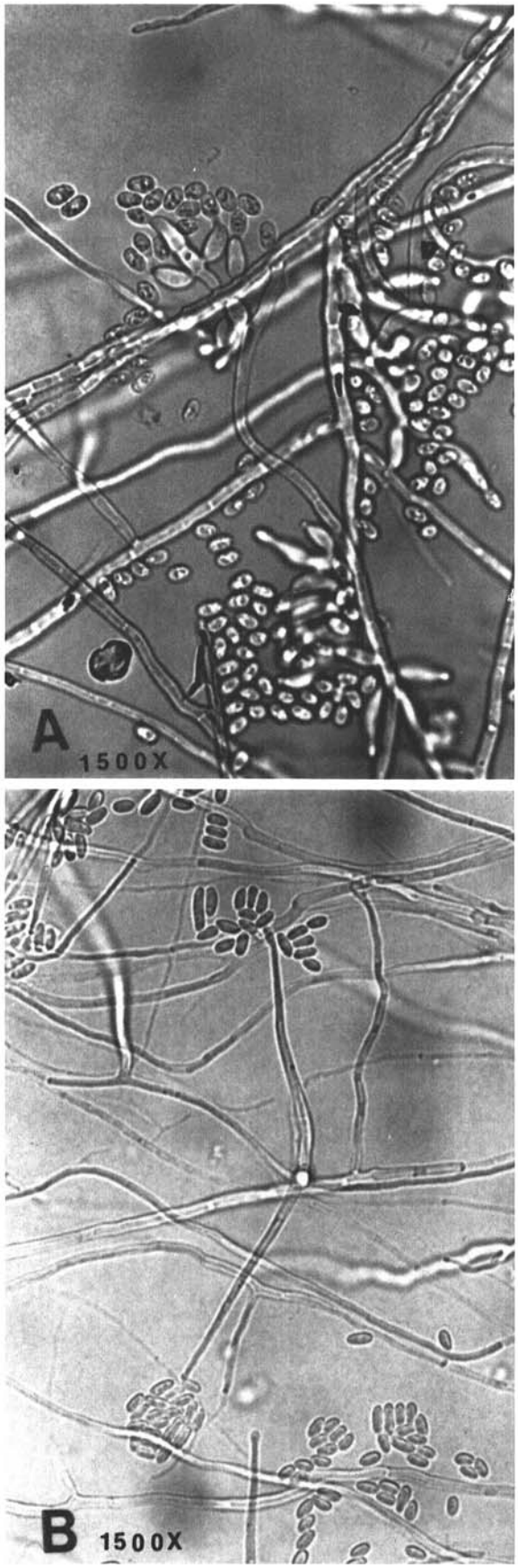

Fig. 1. Photomicrographs of *Phialophora gregata* isolates pathogenic on soybean and adzuki bean. **A,** *P. gregata* isolate G3 isolated from soybean in Illinois; **B,** *P. gregata* isolate M35 pathogenic on adzuki beans in Japan.

sizes of the restriction fragments in duplicate determinations.

Spore nuclear staining. Spore nuclei were stained with mithramycin (Sigma) by previously reported procedures (5). Spores from 21-day cultures were fixed for 30 min in a 60% ethanol, 40% acetic acid (v/v) mixture by flooding the culture plates with the solution. The spores were aspirated from the plate with a syringe and collected on a 13-mm Durapore filter disk (Millipore Inc., Bedford, MA). The disk with the spores was washed with 70% ethanol, 2.5 ml of sterile water, and finally with 2.5 ml of 0.05 M phosphate buffer, pH 6.5. The disks were then transferred to 24-well tissue culture plates in which a mithramycin solution (100 μg/ml) was added. The spores were stained in this solution for 30 min in the dark. The stained spores were transferred to glass slides, and a coverslip was affixed. An Olympus fluorescent microscope was used with exciter filter-B and barrier filter 0-530 to examine the spores (Olympus Corp. of America, New Hyde Park, NY). Two-hundred fifty spores were examined for each fungus isolate.

RESULTS AND DISCUSSION

The number of nuclei in conidia were determined to eliminate the possibility that the fungal isolates were binucleate. All conidia contained mononucleate single cells with the exception of the soybean isolate W27. Isolate W27, in contrast, contained both single- and double-celled conidia in approximately equal proportions but again, each cell was mononucleate. Because all isolates were cultured from single spores, we eliminated the possibility that hyphae used for DNA isolation could contain nuclei from different genetic backgrounds.

When total DNA from soybean reference strain G3 and adzuki bean strain M35 was isolated and fractionated on CsCl-bisbenzimide density gradients, three DNA-containing bands were observed. The DNA from the lower band was digested with *Bam*H1, and the digest was run on an agarose gel. With this

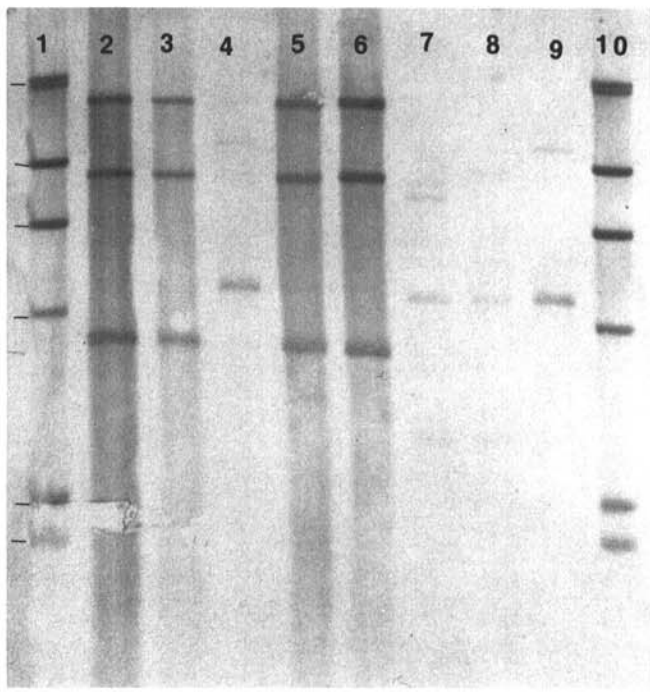

Fig. 2. Agarose gel electrophoresis and Southern blot of *Phialophora gregata* mitochondrial (mt) DNAs digested with *Eco*RI and probed with total mtDNA from adzuki bean isolate M35, lanes 1 and 10. *Hin*dIII-digested λ DNA with the sizes, from top to bottom, 23.1, 9.4, 6.7, 4.4, 2.3, and 2.0 kb. *P. gregata* adzuki bean isolates M35, S35, 30-2, and M8, lanes 2, 3, 5, and 6. Soybean *P. gregata* isolate G3, lane 4. Adzuki bean *P. gregata* isolates 522, A8, 19, lanes 7, 8, and 9.

restriction enzyme-digested DNA, a continuous series of bands was produced. This banding pattern is characteristic of enzyme-digested nuclear DNA (7).

The DNA from isolate G3 and M35 contained in the upper band was digested with *Bgl*II or *Eco*RI and then run on an agarose gel. This enzyme-digested DNA showed discrete fragment patterns and hybridized to the cloned cytochrome oxidase subunit 2 (CO II) gene from *F. oxysporum* (data not shown). These results are similar to other reports in which mitochondrial DNA from other fungi has been separated from nuclear DNA on CsCl-bis-benzimide density gradients (3,7,10).

Aliquots of total DNA from soybean isolates of *P. gregata* (Table 1) were digested with each of five different restriction endonucleases, fractionated, blotted, and probed with biotin-labeled, purified mitochondrial DNA from isolate G3. There was no detectable difference in banding patterns of mitochondrial DNA fragments from 20 different soybean *P. gregata* isolates with any of the enzymes. The isolates chosen covered a wide geographical range within the Midwest soybean-growing area, covering six states with representative isolates from as far apart as 1,600 km. Isolates were also included that had been maintained under laboratory culture conditions for prolonged periods. Isolate IT has been in continuous culture for 15 yr, and isolates G1 and L1 have been in continuous culture for 4 yr. Because total labeled mitochondrial DNA from reference strain G3 was used to probe enzyme-digested mitochondrial DNA from other soybean isolates, unique restriction fragments present in other isolates could be missed with this technique. On the basis of the mitochondrial genome analyses presented here, the soybean isolates of *P. gregata* appear to constitute a highly homologous group with very little variability in the mitochondrial genome. Using the size data listed in Table 2, obtained from duplicate restriction endonuclease digests, we calculated by using the *Eco*RI and *Bgl*II fragments that the size of the mitochondrial genome of the soybean reference isolate G3 is 47.1 kb (+/− 3%). The other digests produced one or more fragments that were too large to measure accurately, and their sizes were therefore predicted using this value for the total mitochondrial DNA size.

When the mitochondrial DNA from soybean isolate G3 was used to probe digests of the seven adzuki bean isolates, two different strain groupings could be seen. The first included isolates 5-22, A8, and 19 (Fig. 1A). These isolates showed homology to the probe with approximately 30% of the mitochondrial DNA in fragments that comigrated with fragments in comparable soybean reference isolate G3 DNA digests. The sizes of these fragments are listed in Table 2. By totaling the *Bgl*II fragment sizes, we calculated that the mitochondrial genome of adzuki bean isolates 522, A8, and 19 was at least 51.1 kb, slightly larger than that of the soybean isolates.

The second group of adzuki bean isolates included M35, S35, M8, and 30-2. These isolates showed no comigrating bands when restriction enzyme-digested DNA of each isolate was probed with total labeled mitochondrial DNA from soybean reference strain G3. To eliminate the possibility that technical problems with the blotting or hybridization processes could be responsible for the negative signals, labeled mitochondrial DNA from the *P. gregata* adzuki bean isolate M35 was used as a probe against total DNA from both the soybean G3 and adzuki bean isolates 522, 19, and A8, M35, M8, S35, and 30-2. Clear, strong hybridization signals were seen with M35, S35, M8, and 30-2 (Fig. 2). In contrast, no homologous hybridization bands were seen when M35 mitochondrial DNA was used as a probe against the other three adzuki bean isolates or the soybean isolate G3 under normal hybridization and washing stringency conditions (Fig. 2). When reduced stringency hybridization and filter washing conditions were used, however, faint signals could sometimes be seen. Isolates M35, S35, M8, and 30-2 clearly represent a distinct group. The sizes of the *Eco*RI and *Bgl*II fragments of the M35 mitochondrial genome are listed in Table 3. From these sizes, we calculated that the size of the mitochondrial genome of the adzuki bean isolates M35, M8, S35, and 30-2 is only 30 kb, considerably smaller than that of the *P. gregata* adzuki bean isolates 522, 19, A8, and soybean isolate G3.

These observations suggest that the second group of presumed *P. gregata* adzuki bean isolates might not in fact be *P. gregata*. It has been noted that at least some of the members of this group do not produce the gregatin toxins that are ubiquitous to *P. gregata* (13,14,16). In addition, morphological examination (Fig. 1B) of these isolates indicated that they resembled more closely the *Acremonium* species description of Gamms (6) than that of *Phialophora* (26). These results are based on our examination of the conidiophore lengths, basal septation of conidiophores, culture growth rates, and spore sizes. Pathogenicity of these isolates was based on the amount of internal stem vascular browning these isolates caused on inoculated adzuki bean plants (13,14,16). It has been shown that *Acremonium* isolates cause vascular browning on inoculated soybean plants (21). In the case of adzuki bean, as in soybean, it may be common for certain *Acremonium* isolates to cause vascular browning and for both *Phialophora* isolates and *Acremonium* isolates to co-infect the host plant vascular system at the same time (21).

We assume from these data that the adzuki bean isolates M35, S35, M8, and 30-2 in our possession are either culture contaminants or related fungi found in the same host plant. Our observations show that they represent a distinct molecular group from the other three adzuki bean isolates tested. On the basis, therefore, of the molecular hybridization data and the morphological observations, it is likely that the out-group isolates are not, in fact, mutant *Phialophora* isolates, but rather *Acremonium* species.

Molecular analysis of the mitochondrial genome has distinguished between true *Phialophora* isolates. The molecular divergence of the mitochondrial genome between *Phialophora* and *Acremonium*-like isolates M35, M8, S-35, and 30-2 was such that cross hybridization was not detectable under the hybridization and washing stringency conditions used in the present study. In contrast, there was enough molecular identity for the mitochondrial genomes of *P. gregata* to cross-react and enough genetic

TABLE 3. Restriction fragment patterns (kilobase pairs) of mitochondrial DNA from adzuki bean *Phialophora gregata* isolates M35, M8, 30-2, S35, 522, and soybean isolate G3 digested with *Bgl*II and *Eco*RI[a]

Fragment number	M35		M8		30-2		S35		522		G3	
	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI
1	6.9	15.7	6.9	15.7	6.9	15.7	6.9	15.7	NCB[b]	NCB	NCB	NCB
2	5.6	9.2	5.6	9.2	5.6	9.2	5.6	9.2				
3	5.1	4.2	5.1	4.2	5.1	4.2	5.1	4.2				
4	4.2		4.2		4.2		4.2					
5	2.8		2.8		2.8		2.8					
6	2.2		2.2		2.2		2.2					
7	2.0		2.0		2.0		2.0					
8	1.9		1.9		1.9		1.9					

[a]Total *Bgl*II and *Eco*RI digested mitochondrial DNA was probed with total labeled mitochondrial DNA isolated from adzuki bean *P. gregata* isolate M35. Adzuki bean isolates M35, M8, S35, and 30-2 are morphologically similar isolates (Fig. 1B).
[b]There were no comigrating bands with adzuki bean *P. gregata* isolates 522, A8, 19, and soybean reference isolate G3.

variation to distinguish between the soybean and adzuki bean isolates on the basis of fragment size. No variation was detected among any of the soybean isolates, although they represented a wide diversity of geographical separation. Clearly, DNA sequences with higher levels of variability must be used to distinguish these. Therefore, we are currently screening nuclear genomic fragments for such sequences.

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