Genetic Characterization of *Fusarium graminearum* Strains Using RAPD and PCR Amplification

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ABSTRACT

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Strains of *Fusarium graminearum* were characterized using random amplified polymorphic DNA (RAPD) and restriction analysis of amplified fragments from the polymerase chain reaction (PCR). For RAPD analysis, three of 40 oligonucleotide primers were selected after testing with two *F. graminearum* strains and used to characterize an additional 17 strains of *F. graminearum*. For PCR amplifications, two sets of primers were designed from the sequence of cloned DNA fragments specific to *F. graminearum*. Most RAPD primers or PCR primer pairs yielded one of two common patterns, but the strains could be identified by the combined profile of patterns. The RAPD and PCR primers generally did not react with other *Fusarium* species or gave distinctly different patterns. The results suggest a relatively low amount of genetic diversity among the *F. graminearum* strains tested. The RAPD and specific PCR profiles are now being used for tracking strains of *F. graminearum* in field experiments.

Additional keywords: corn ear rot, DNA fingerprinting, *Gibberella zeae*, head blight.

Fusarium graminearum Schwabe (teleomorph = *Gibberella zeae* (Schwein.) Petch) causes Fusarium head blight of wheat and ear rot of corn in Canada, the United States, and other temperate regions. In Canada, periodic epidemics of these diseases result in economic losses resulting from reduced grain quality and the toxic effects of the mycotoxins deoxynivalenol and zearalenone on farm animals (26). Other species of *Fusarium*, such as *F. culmorum* (Wm.G. Sm.) Sacc., *F. crookwellense* L.W. Burgess, P.E. Nelson & T.A. Toussoun, *F. moniliforme* Sheld. (= *F. verticillioides* (Saccardo) Nirenberg), and *F. proliferatum* (T. Matsushima) Nirenberg, cause similar diseases and produce additional toxins (15,27).

Fusarium species are currently identified using combinations of microscopic and colony characters (20). However, the differences between some species are rather subtle, and identifications require an experienced eye. Formae speciales and races within a species are defined by nonmorphological characters, and no morphological correlations have been discovered. Characterization usually requires time-consuming and/or expensive procedures, such as pathogenicity tests on a range of hosts, the determination of a vegetative compatibility group (VCG) by pairing experiments, or examination of the mycotoxin profile. The latter is the most sensitive method for differentiating *F. graminearum* strains that has been reported to date (17). Francis and Burgess (5) described two groups within *F. graminearum* that have different pathological characteristics. The two groups are correlated with the inability of group 1 and the ability of group 2 to produce perithecia in culture.

Precise identification of laboratory-reared strains is a prerequisite for field studies of pathogen dispersal, spread, and host infection. Previously, this has been done by generating mutants with specific markers that do not occur in wild-type strains (1). Unfortunately, deleterious mutations may be introduced along with marker mutations. DNA-fingerprinting techniques avoid the necessity of generating mutants and allow the tracking of unaltered wild-type strains.

Random amplified polymorphic DNA (RAPD; 29,30) and analysis of DNA amplified by the polymerase chain reaction (PCR; 19,23,24) are two relatively new techniques that are sensitive, are quick to perform, and can be applied to a large number of samples. These techniques amplify minute amounts of DNA with the help of either a pair of specific oligonucleotide primers that recognize a specific DNA region in the genome (PCR) or a single, short, arbitrarily-designed primer to produce a complex amplification pattern (RAPD). Both methods have become popular for identifying or differentiating strains of plant pathogens such as *F. solani* (Mart.) Sacc. (3), *Colletotrichum graminicola* (Ces.) G.W. Wils. (7), *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (6), and *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier (8).

In this paper, we assess both of these molecular techniques for their utility in characterizing strains of *F. graminearum* and for differentiating *F. graminearum* from other *Fusarium* species.

MATERIALS AND METHODS

Fungal strains. The *Fusarium* strains used in this study and their sources are listed in Tables 1 and 2. DAOM numbers refer to cultures maintained in the Canadian Collection of Fungal Cultures (Centre for Land and Biological Resources Research, Agriculture Canada, Ottawa, Ontario). The strains in Table 2 from Ottawa were obtained from a single field. Cultures were identified and single spored using the methods of Nelson et al (20). For most strains of *F. graminearum*, perithecial production was tested by growing single-conidium isolates on 2% water agar with segments of autoclaved wheat straw in polystyrene petri dishes. Dishes were examined for perithecia and ascospores after 3- to 5-wk incubation under near-UV and fluorescent lights. Colony phenotypes were scored as red or yellow according to pigment production on synthetic nutrient agar slants (SNA; 21) after 4–8 wk.

TABLE 1. Molecular characterization of *Fusarium graminearum* strains using random amplified polymorphic DNA (RAPD) and polymerase chain reaction (PCR) primers

Code	DAOM no.[s]	Origin	RAPD[t]			PCR[u]	
			A11	B10	B12	B1	H1
T	170785	Corn, Ottawa, Ontario, Canada	A[v]	T	A	A	B
J	180376	Corn, Ottawa, ON	A	A	A	A	A
N	180377	Corn, Ottawa, ON	A	A	A	A	A
A[w]	180378	Corn, Ottawa, ON	A	A	A	A	A
U	180379	Corn, Ottawa, ON	B	B	A	B	B
O	177406	Wheat, Chatham, ON	B	B	B	B	B
M	177408	Wheat, Chatham, ON	B	B	B	B	B
S	177409	Wheat, Chatham, ON	A	B	A	A	B
B[w]	178148	Wheat, Chatham, ON	B	B	B	B	B
D	215629	Wheat, Harrow, ON	B	B	B	B	A
Q	178149	Barley, Petrolia, ON	B	B	B	B	A
P2	215630	Wheat, SAB[x], Quebec, Canada	A	B	A	A	B
E[y]	215631	Wheat, SAB, PQ	E	E	A	ND[z]	E
R	192130	Wheat, Manitoba, Canada	B	B	B	B	B
P	192131	Wheat, MB	A	B	A	A	B
K	192132	Wheat, MB	A	B	B	A	A
V	213384	Weeds, Saskatchewan, Canada	A	B	B	A	A
W	213295	Wheat, Alberta, Canada	A	B	A	A	B
C	212262	Soil, England	C	C	C	C	C

[s] Cultures maintained in the Canadian Collection of Fungal Cultures.
[t] RAPD amplification using primers A11, B10, or B12.
[u] PCR amplification using primer pairs A11a + A11b, followed by restriction analysis with the enzyme *Bam*HI (B1), or B10a + B10b, followed by restriction analysis with the enzyme *Hin*fI (H1).
[v] A, B, C, E, or T: banding pattern characteristic of strain A, B, C, E or T.
[w] Strains used for the initial screening of the 40 RAPD primers.
[x] SAB: Ste.-Anne de Bellevue.
[y] Strain subsequently reidentified as *F. crookwellense*.
[z] ND: Nothing detected.

TABLE 2. Cultures of other *Fusarium* species used for random amplified polymorphic DNA (RAPD) and polymerase chain reaction (PCR) analyses

Species	DAOM no.[z]	Origin
F. culmorum	213294	Soil, Ottawa, Ontario, Canada
F. crookwellense	213291	Soil, Ottawa, ON
F. sambucinum	213274	Potato, Germany
F. equiseti	215633	Corn, Ottawa, ON
F. moniliforme	195167	Vankleek Hill, ON
F. proliferatum	215632	Corn, Ottawa, ON
F. avenaceum	196490	Fir needles, New Brunswick, Canada
F. sporotrichioides	215944	Soybean, Ottawa, ON
F. oxysporum	213293	Soil, Ottawa, ON
F. solani	213292	Soil, Ottawa, ON

[z] Cultures maintained in the Canadian Collection of Fungal Cultures.

For molecular analysis, single-conidium strains were incubated for 1–2 wk under near-UV and fluorescent light on SNA in 6-cm polystyrene petri dishes. A spore suspension was made in 5 ml of sterile distilled water, and 2 ml was inoculated into 50 ml of the medium A of Miller et al (17) in a 250-ml Erlenmeyer flask. Cultures were grown for 3 days at room temperature on a rotary shaker set at 200 rpm. Mycelium was harvested by filtration through autoclaved cheesecloth and either used immediately for DNA extraction, kept at −70 C, or lyophilized and kept at −20 C until needed. All three methods gave similar results.

DNA extraction. DNA was extracted from mycelium using a modification of the method of Edwards et al (4). Approximately 50 mg of mycelium was ground and suspended in extraction buffer. After grinding, samples were centrifuged at 12,000 *g* for 10 min. After precipitation with cold isopropanol, the pellets were vacuum-dried and dissolved in 100 μl of TE (10 mM Tris-HCl, pH 8; 0.1 mM EDTA). The samples were allowed to sit overnight at 4 C and were spun at 12,000 *g* for 5 min to clear the extracts. The supernatants, containing the DNA, were stored at −20 C. The DNA was quantified on 0.7% agarose gels containing ethidium bromide by visual comparisons with known quantities of lambda DNA (Promega Co., Madison, WI).

Amplification conditions. RAPD and PCR assays were performed using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT). The total reaction volume was 20 μl, and the conditions were 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 3 mM $MgCl_2$; 0.001% gelatin; 200 μM each dNTP; 0.5 U of native *Taq* DNA polymerase. For RAPD assays, the primer was used at 1 μM; for PCR assays, each primer was used at 0.5 μM. No more than 2 ng of target DNA was used in the assays; routinely, a fivefold dilution series starting with 2 ng of DNA was assayed with one RAPD primer, and the quantity of DNA showing the best results was used for the remaining RAPD and PCR assays. For each amplification round, a master mix containing everything except the polymerase and the target DNA was prepared in a 0.5-ml microcentrifuge tube (Perkin-Elmer Cetus) and immediately treated for 20 min by placing the tube on its side under a short-wavelength UV germicidal lamp. This step irreversibly damaged contaminant DNA that may have been present in any of the reagents (25). After UV treatment and addition of the polymerase, the master mix was aliquoted into the assay tubes. The target DNA was added last.

For RAPD amplifications, the thermocycler was programmed for one cycle of 2 min at 94 C, followed by 35 cycles of 1 min at 94 C, 1 min at 35 C, ramping at 18 C/min to 72 C, 2.5 min at 72 C, and one cycle of 5 min at 72 C. The primers used for RAPD were from Operon Technologies Inc. (Alameda, CA), and the supplier's nomenclature is used here. For PCR amplifications, the cycler was programmed for one cycle of 2 min at 94 C, followed by 30 cycles of 1 min at 94 C, 30 s at 56 C, ramping at 18 C/min to 72 C, 1 min at 72 C, and one cycle of 5 min at 72 C.

For PCR, new primers were designed according to the method of Rychlik and Rhoads (22). Selected amplified fragments from RAPD assays were cloned into the vector pCR1000, which is specifically designed to clone amplified fragments (TA cloning system, Invitrogen Co., San Diego, CA). The cloned fragments were sequenced using the T7 DNA Polymerase Sequencing System

(Promega Co.). The sequences of the PCR primers were A11a-CCTATCCAAGACCACGAAG; A11b-GAGGGATTCAGCA-AGAGG; B10a-AGTCCAAAATGTCCCGATGC; and B10b-GCTGGGACCTGAGAAGTA.

RAPD- and PCR-amplified products were analyzed by electrophoresis on 1.5% agarose gels and detected by staining with ethidium bromide.

Restriction analysis of PCR products. After PCR amplification, half the 20-μl reaction was loaded on a gel for analysis. To the other half, 1.0 μl of *Eco*RI digestion buffer (Promega Co.) and approximately 2 U of the restriction endonucleases *Bam*HI or *Hin*fI were added. After a 2-h incubation at 37 C, the digested products were separated on a 2% agarose gel.

RESULTS

Perithecial production. All the *F. graminearum* strains tested produced perithecia after 3–5 wk, except DAOM 170785 and 215629. Three strains, DAOM 213384, 213295, and 212262, were not tested. These results suggest that most, and probably all, of the strains used in this study are from *F. graminearum* group 2 as defined by Francis and Burgess (5).

RAPD amplification. Two *F. graminearum* strains were selected (A, DAOM 180378, and B, DAOM 178148, listed in Table 1) to screen a set of 40 RAPD primers and identify those that might give strain-specific patterns. Most of the primers produced a similar banding pattern with both strains (data not shown). However, five of the 40 primers revealed significant differences between the two strains (Fig. 1). The patterns were complex, showing between two and 12 bands of various intensities, most of which were common to both strains. However, with each of these five primers, the presence or absence of a distinctive band could be observed reproducibly (dots in Fig. 1). Three of these primers (A11, B10, and B12) were selected for analysis of additional *F. graminearum* strains because they produced consistently reproducible patterns that were less sensitive to variations in assay conditions, such as concentrations of $MgCl_2$ or DNA (data not shown).

Primers A11, B10, and B12 were used to compare a collection of 19 *F. graminearum* strains, 18 from Canada and one from England, in a blind test (Table 1). Figure 2 shows examples of the different patterns observed with each primer. For each primer used, the patterns of most strains were identical or similar to the patterns for either A or B (Table 1). The three exceptions were strains C (DAOM 212262, from England), E (DAOM 215631, from Montreal, Canada, subsequently reidentified as *F. crookwellense*), and T (DAOM 170785, from Ottawa, Canada), which had banding patterns that were different from A and B for one or more of the primers.

Primers A11, B10, and B12 also were used to compare the RAPD patterns of 11 common *Fusarium* species that also may be expected to occur in corn or wheat fields. Figure 3 shows the results obtained with primer B12. A different pattern is produced for each species. The other two primers showed different patterns for most, but not all, species.

PCR amplification. PCR primers specific to *F. graminearum* were designed to produce less complex patterns than RAPD primers produced. The high-intensity bands (Fig. 1) produced by RAPD with primers A11 and B10, which were unique to strains A and B, respectively, were purified from an agarose gel and cloned into vector pCR1000. The ends of the cloned fragments were sequenced, and the information obtained was used to design 18- to 20-mer primer pairs that would specifically amplify the

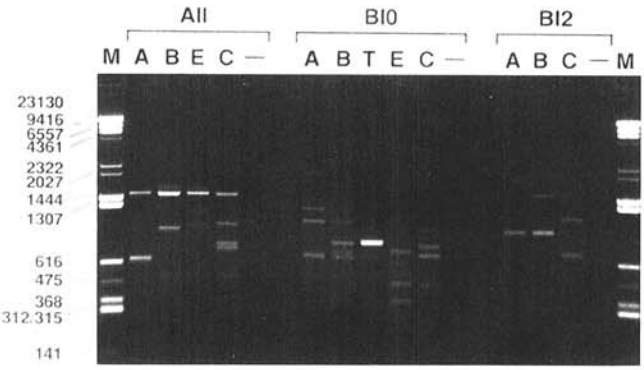

Fig. 2. Examples of patterns observed with *Fusarium graminearum* strains. Primers A11, B10, and B12 were used in random amplified polymorphic DNA assays with the DNA of *F. graminearum* strains DAOM (Canadian Collection of Fungal Cultures) 180378 (A), 178148 (B), 2122262 (C), 215631, (E, subsequently reidentified as *F. crookwellense*), and 170785 (T). A negative control (−) containing water instead of DNA also was included for each primer. A mixture of lambda DNA digested with *Hin*dIII- and *Taq*I-digested pBR322 was used as the molecular weight marker (M); the sizes (in base pairs) of the fragments are indicated on the left.

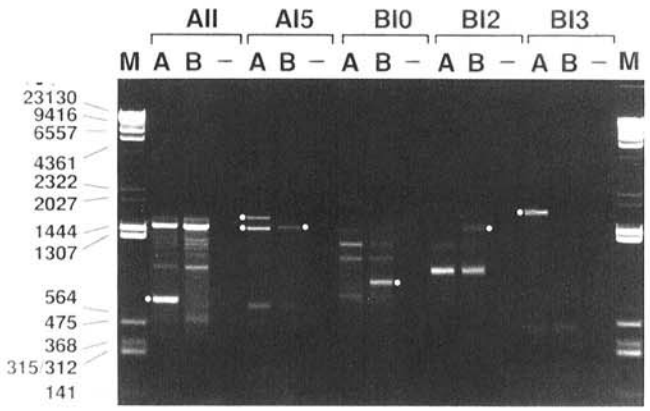

Fig. 1. Random amplification patterns obtained using *Fusarium graminearum* strains. Commercially available primers A11, A15, B10, B12, and B13 were used in random amplified polymorphic DNA assays with the DNA of *F. graminearum* strains DAOM (Canadian Collection of Fungal Cultures) 180378 (A) and 178148 (B). A negative control (−) containing water instead of DNA also was included for each primer. With each primer used, dot(s) indicate the distinct band(s) specific to one of the two strains used. A mixture of lambda DNA digested with *Hin*dIII- and *Taq*I-digested pBR322 was used as the molecular weight marker (M); the sizes (in base pairs) of the fragments are indicated on the left.

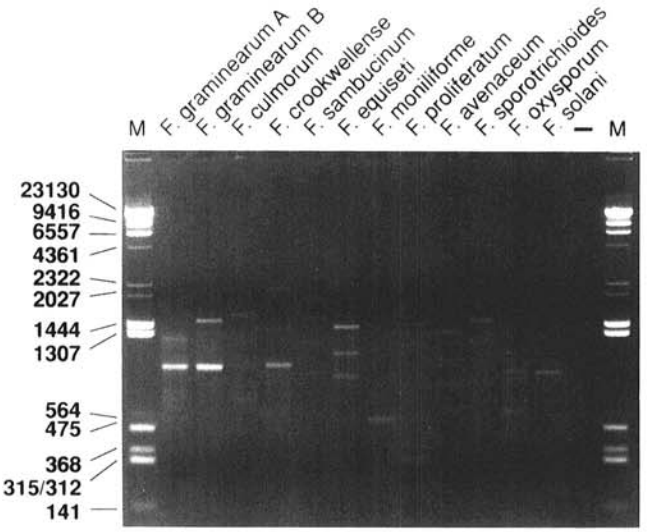

Fig. 3. Random amplified polymorphic DNA patterns obtained when using the primer B12 with DNA from 12 *Fusarium* species. A negative control (−) containing water instead of DNA also was included for the primer. A mixture of lambda DNA digested with *Hin*dIII- and *Taq*I-digested pBR322 was used as the molecular weight marker (M); the sizes (in base pairs) of the fragments are indicated on the left.

DNA corresponding to the cloned fragments. A comparison of the primers with sequences in GeneBank revealed no significant homology to known genes.

The primer pairs were used to perform PCR assays on the *F. graminearum* strains listed in Table 1. All the Canadian strains were amplified, but the English strain was only poorly amplified. Figure 4A shows examples of the results obtained. Surprisingly, strains A and B had similar results, although the corresponding RAPD bands were specific to either A or B. This suggests that the difference in sequence responsible for the presence or absence of the specific RAPD band did not cover a large area, because the regions flanking the RAPD priming sites, homologous to the PCR primers, were identical or very similar. In an attempt to detect sequence differences between strains A and B, the PCR-amplified fragments were digested with a number of restriction enzymes. Most of them revealed no differences between the two strains (data not shown). However, *Bam*HI and *Hin*fI revealed polymorphisms in the fragments amplified by the primer pairs A11a + A11b and B10a + B10b, respectively (Fig. 4B).

PCR amplification followed by restriction analysis was used to further characterize the original 19 *F. graminearum* strains. As for the RAPD assays, most of the *F. graminearum* strains showed a pattern similar to A or B (Table 1), whereas strains C and E had a very different pattern or did not amplify at all (Fig. 4A and B). Blotting of the gel shown in Figure 4A and probing with the appropriate amplified fragment from strains A or B revealed no sequence homology between the fragments amplified from strains A and B and those from strains C and E (data not shown). Because of these anomalous results, the identification of strain E was checked and redetermined to be *F. crookwellense*.

The specificity of the PCR primer pairs also was assessed by trying to amplify fragments from a range of *Fusarium* species.

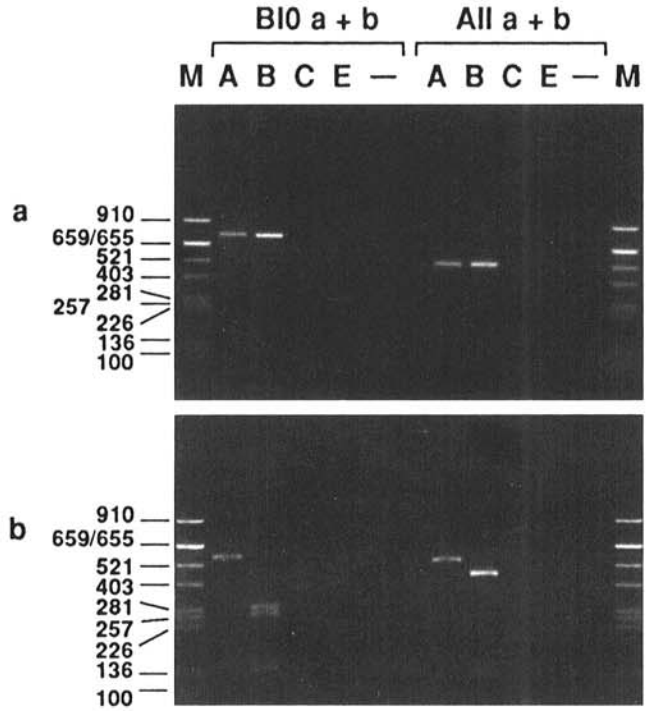

Fig. 4. Polymerase chain reaction (PCR) assay and restriction of the amplified fragment in *Fusarium graminearum*. Primer pairs B10a + B10b (left side of **a and b**) and A11a + A11b (right side of **a and b**) were used in PCR assays with the DNA of the *F. graminearum* strains DAOM (Canadian Collection of Fungal Cultures) 180378 (A), 178148 (B), 212262 (C), and 215631 (E, subsequently reidentified as *F. crookwellense*). A negative control (—) containing water instead of DNA also was performed with each primer pair. **a,** Half of each PCR assay was directly analyzed on agarose gel, and **b,** the other half was digested with either the restriction enzyme *Hin*fI (left side **a and b**) or *Bam*HI (right side **a and b**). The sizes (in base pairs) of the fragments of pBR322 digested by *Alu*I (M) are indicated on the left.

Figure 5 shows the results obtained when using pair B10a + B10b. Most of the *Fusarium* species gave no amplification at all, irrespective of the amount of DNA used. *F. culmorum* and *F. crookwellense*, both classified in section *Discolor* with *F. graminearum* (20), showed some small amplified fragments. However, hybridization experiments showed that these fragments were not homologous to those amplified in *F. graminearum*. PCR amplifications using the primer pair A11a + A11b (data not shown) also reacted with only a few species other than *F. graminearum*, yielding small amounts of amplified fragments of various sizes with no sequence homology with the amplified products present in *F. graminearum*.

DISCUSSION

Different procedures are used to differentiate strains of fungal species. A common method is the identification of restriction fragment length polymorphisms (RFLPs) by probing restricted DNA with randomly selected DNA clones or fragments of known genes. For example, RFLPs have been useful in separating strains of *F. oxysporum* into groups correlating with forma speciales (12,16) or VCGs (10,13). Although RAPD assays are more convenient than RFLPs because there is no need for radioactive probes, RFLPs reveal genetic variation in some cases in which RAPD does not (14). Very few polymorphisms were detected in preliminary attempts to characterize the genomic DNA of *F. graminearum* strains A and B (S. Denes, *personal communication*).

The *F. graminearum* strains in this study, which all appear to represent the group 2 of Francis and Burgess (5), could be distinguished by the combined profiles using the five RAPD and PCR primers or primer pairs. The patterns observed were specific to *F. graminearum* and did not occur in other *Fusarium* species. Strains of *F. graminearum* from the same location often had the same set of amplification patterns, suggesting that they represent the same genotype. However, some strains from the same location had different patterns. Except for strain C from England, which was very different from the Canadian strains of *F. graminearum*, the strains could not be grouped according to host or geographic origin. Strains with similar patterns were scattered across the country. Similar analyses of *F. oxysporum* populations also showed that polymorphisms could not be correlated with geographic region of origin of the strains (11,12). Similarly, no correlations were detected between the banding patterns produced

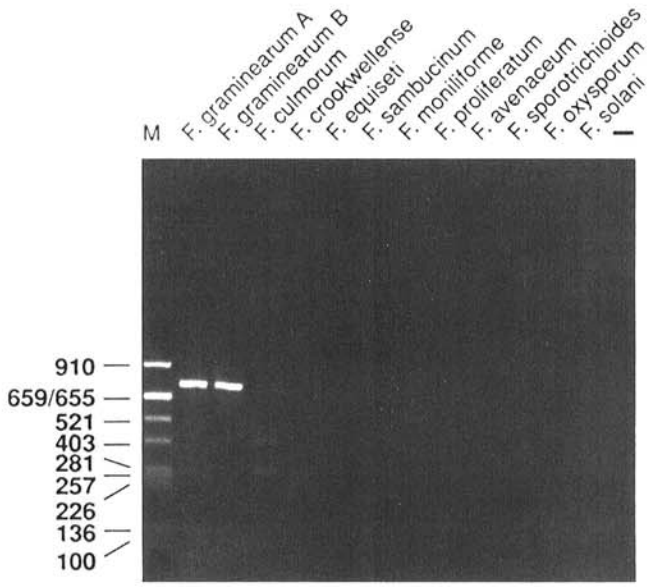

Fig. 5. Polymerase chain reaction assay using primer pair B10a + B10b and the DNA from 12 *Fusarium* species. A negative control (—) containing water instead of DNA also was performed with each primer pair. The sizes (in base pairs) of the fragments of pBR322 digested by *Alu*I (M) are indicated on the left.

in this study and the mycotoxin profiles or titer of the Ontario strains reported by Miller et al (18).

Our results with RAPD and PCR revealed a limited amount of genetic diversity among the different strains of *F. graminearum* we have studied. Strains of *F. graminearum* could not be identified with single RAPD primers or PCR primer pairs because only a limited number of patterns were produced. A similar situation was noted for *C. graminicola*, in which the patterns of different strains were similar for any one RAPD primer, but the strains could be differentiated by the combined profile from three primers (7). In contrast, in *F. solani* most of the RAPD primers used yielded different patterns between the two mating populations but yielded very little or no difference among the strains of the same mating population (3). The limited amount of genetic variability we observed in *F. graminearum* is comparable to that observed within the mating populations of *F. solani*. Bowden and Leslie (2) reported that all strains of *F. graminearum* from 23 locations in Kansas represented genetically distinct VCGs, suggesting that more variation may occur in parts of the genome that we did not sample.

For *F. graminearum*, PCR amplifications followed by restriction digestion provided additional markers for differentiating strains. This technique has not been used previously for other *Fusarium* species. However, it has been used to differentiate *Cryptococcus* (28) and *Lentinus* species (9). Patterns unique to each species and to each strain for some of the species were observed after restriction digestion of amplified rDNA fragments.

A comparison of the results we obtained with the RAPD and the PCR-restriction assays suggests that neither method is superior for comparing *F. graminearum* strains. It is the number of primers or primer pairs employed that is critical for reliable strain identification. Screening of a set of RAPD primers is much more rapid than cloning DNA fragments to design PCR primers. However, the PCR assay is less sensitive to assay conditions and is more easily reproduced and interpreted than the RAPD assay. Furthermore, the RAPD assay requires axenic cultures of the fungus, whereas the PCR assay can be done on samples containing plant material.

Our results demonstrate that PCR and RAPD assays can be used to identify *F. graminearum* strains. The method described in this paper is now being used to analyze strains of *F. graminearum* isolated in a 3-yr field experiment. Strains A and B were inoculated into the field in the summer of 1990. Using PCR and RAPD, isolates from crop plants and debris, weeds, soil, insects, and the air are being compared with the introduced strains. The results obtained will be used to confirm the establishment and follow the persistence of strains A and B and to study inoculum input from crop debris and other sources.

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