Techniques

Potential Applications of Random DNA Probes and Restriction Fragment Length Polymorphisms in the Taxonomy of the Fusaria

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ABSTRACT

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A range of *Fusarium* species was screened for DNA restriction fragment length polymorphisms in ethidium-stained agarose gels and by hybridization to random probes generated from total DNA of an isolate of *Fusarium oxysporum* f. sp. *dianthi*. DNA was digested with Hind III and ligated into the Hind III site of pBR322, and the recombinant plasmids were used to transform *Escherichia coli* MM294. Plasmid DNA from four clones harboring inserts ranging in size from 760 to 3,400 base pairs were ^{32}P-labeled and hybridized to Southern blots of total DNA from two isolates of *F. o.* f. sp. *dianthi* and isolates of *F. o.* f. spp. *lycopersici* and *gladioli*. Three plasmids gave hybridization patterns that indicated that their inserts were present in one or a few copies per genome. A fourth plasmid hybridized to multiple fragment lengths in Hind III and Eco R1 digests of the *Fusarium* DNA. The restriction fragment length polymorphism patterns differed among the three forma speciales tested, but there were constant bands within *F. o.* f. sp. *dianthi* and patterns were conserved over time despite phenotypic variation. The fourth plasmid hybridized only to a subset of the species *oxysporum* and not to isolates of seven other *Fusarium* spp. Combinations of probes and restriction enzymes enabled differentiation at species, forma speciales, and isolate levels. The technique holds promise for addressing problems in the taxonomy and identification of *Fusarium*.

The genus *Fusarium* comprises a wide and heterogeneous group of fungi, many of considerable importance in industry, as food contaminants, and as pathogens in agriculture. Taxonomically, the species are notorious for their variability, especially in culture, to the extent that different species are morphologically identical (16). Furthermore, within a species, sensu Snyder and Hansen (18), forma speciales are only identifiable in terms of host specificity.

Attempts have been made to classify *Fusarium* on the basis of soluble protein electrophoretic patterns (8), zymograms (17), immunoelectrograms (1), and monoclonal antibody reactions (10), with partial success. Restriction fragment length polymorphisms (RFLPs) provide a powerful technique for the determination of molecular variation at the DNA level. Polymorphic markers have applications, among others, in taxonomy and in identifying marker loci of biological significance (2), and they are becoming widely used for genetic analysis and linkage mapping (3). This study essays to evaluate the application of RFLP methodology as a tool for addressing some of the problems of *Fusarium* taxonomy.

MATERIALS AND METHODS

Fungal cultures. *Fusarium* isolates were either newly isolated or cultures were obtained from the collection of Dr. H. Vigodsky-

Haas, Volcani Centre, Israel. Original sources and maintenance media are listed in Table 1. All cultures were single spored and stocks were maintained on carnation leaf agar (CLA) (5).

Preparation of DNA. Spore suspensions from CLA plates were used to inoculate cellophane-covered potato-dextrose agar (PDA) plates. Mycelium was collected after 7–10 days' growth at room temperature, lyophilized, and stored at −20 C. Various methods of DNA preparation were tested (6,7,15,19), and the following modification of the method of Murray and Thompson (15) was selected. Lyophilized mycelium (0.5 g) was ground with a mortar and pestle in the presence of sand and added to 15 ml of hot extraction buffer (1% cetyltrimethylammonium bromide [CTAB], 50 mM tris-HCl [pH 8.0], 0.7 M NaCl, and 10 mM EDTA) and kept at 65 C for 10 min. The suspension was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 12,000 g for 10 min at room temperature. The upper aqueous phase was transferred to a clean tube, 0.1 volume of extraction buffer containing 10% CTAB was added, and the extraction was repeated.

The upper phase was mixed with an equal volume of 1% CTAB, 50 mM tris-HCl (pH 8.0), and 10 mM EDTA to precipitate nucleic acids and then incubated at room temperature for 30 min. The DNA was pelleted at 3,000 g for 5 min, washed twice by centrifugation with a large volume of cold 70% ethanol/0.3 M sodium acetate (pH 5.5), and taken up in 700 μl of TE buffer (10 mM tris-HCl [pH 8.0] and 1 mM EDTA). The preparation was digested with 100 μg of DNAse-free RNAse for an hour at 37 C and then with 10 μg self-digested proteinase K for an hour at 37 C. Proteinaceous material was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 0.1 volume 3 M sodium acetate and 0.6 volume isopropanol, taken up in TE, adjusted spectrophotometrically to 1 μg/ml, and stored at −20 C.

Construction of a DNA library. Standard protocols were used throughout (13). Total DNA of isolate 1 of *Fusarium oxysporum* f.

TABLE 1. List of *Fusarium* isolates and their sources

Species	Code	Source[a]
F. episphaeria	E15	1
F. lateritium	L112	1
F. moniliforme	M125	1
	MRC3235	5
	MRC2293	5
F. nivale	N13	1
F. oxysporum	MRC3239	5
f. sp. dianthi isol. 1	SS1	2
f. sp. dianthi isol. 2	SS2	2
f. sp. dianthi isol. 3	J	3
f. sp. gladioli isol. 1	S3	3
f. sp. gladioli isol. 2	S6	3
f. sp. lycopersici race 1	TR1	4
f. sp. lycopersici race 2	TR2	4
f. sp. cubense race 1	NJ	6
f. sp. cubense race 4	TM	6
F. rigidiusculum	R37	1
	MRC3224	5
F. roseum var. graminearum	GR132	1
	MRC2556	5
	MRC3234	5
var. culmorum	MRC2626	5
var. gibbosum	MRC3278	5
	MRC3312	5
F. solani	S133	1
	MRC3237	5
F. tricinctum	T415	1
	MRC3295	5

[a] 1 = Fusarium Workshop held at University of Minnesota, St. Paul, 1981, via H. Vigodsky-Haas; 2 = field isolates, Israel, 1984; 3 = H. Vigodsky-Haas, Volcani Centre, Israel, 1978; 4 = J. Katan, Hebrew University, Israel; 5 = W. O. Marasas, NRIND, South Africa; 6 = field isolates, South Africa, 1982. Isolates J, S3, S6, TR1, and TR2 had been stored on potato-dextrose agar; all others were on carnation leaf agar (see Materials and Methods).
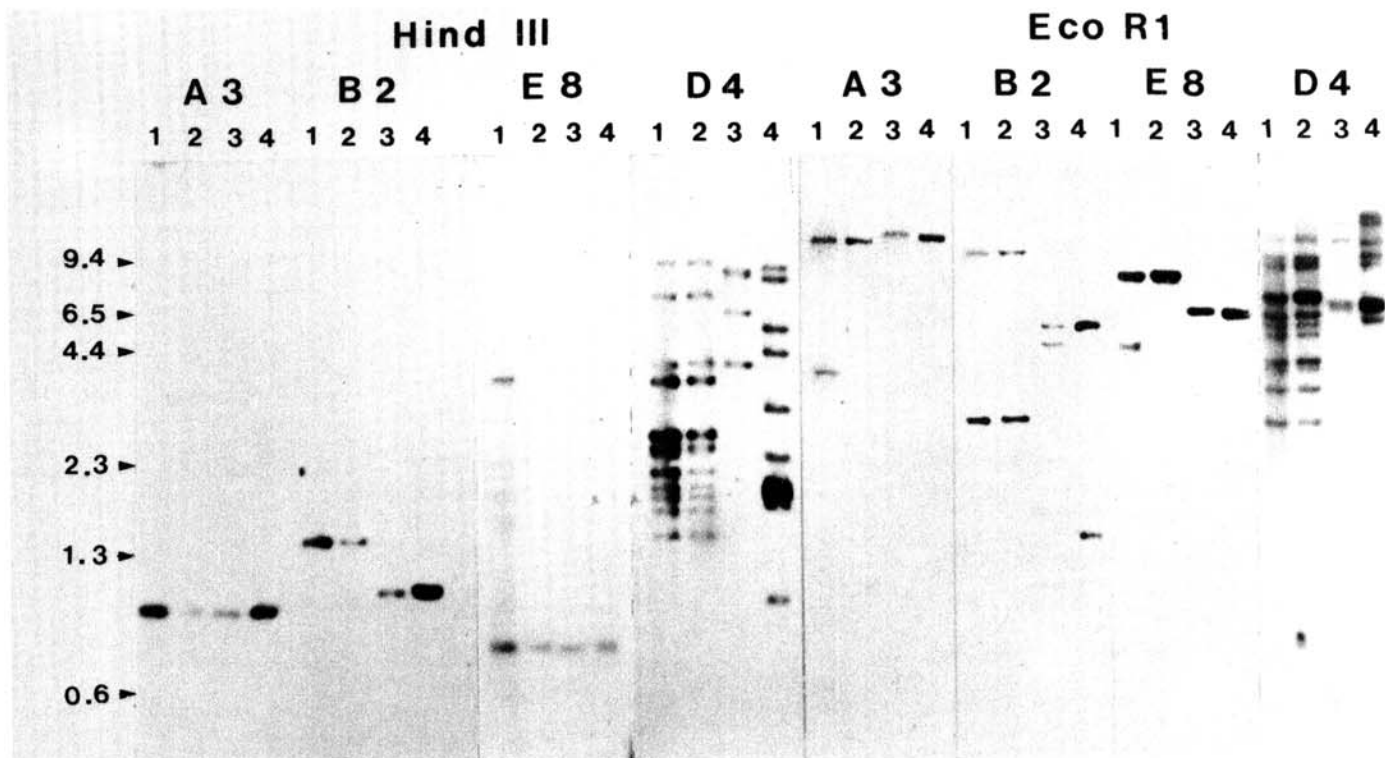
sp. *dianthi* (Prill. & Del.) was digested with Hind III and ligated into the Hind III site of pBR322 that was used to transform *Escherichia coli* MM294. Plasmid DNA containing *Fusarium* DNA inserts was prepared from ampicillin-resistant, tetracycline-susceptible clones by the alkaline lysis method (13).

Southern blots and hybridization. Approximately 8 μg per lane of total DNA was digested overnight with 40 units of restriction enzyme according to manufacturer's (Boehringer) recommendations, and the DNA was separated on 1% agarose gels at 0.75 V/cm until the bromophenol blue dye front had moved 14 cm. The methods of Meinkoth and Wahl (14) were used for blotting and hybridization. Plasmid probes were labeled with dCT ^{32}P to a specific activity of approximately 10^8 dpm/μg by nick translation as recommended for the Amersham kit N 5000. The hybridization mix contained 50% formamide and hybridization was at 42 C overnight. Blots were washed for 2 × 15 min in 1 × SSPE (0.15 M NaCl, 0.01 M NaH$_2$PO$_4$, and 0.001 M EDTA [pH 7.4]), 0.1% SDS at room temperature and 2 × 15 min in 0.1 × SSPE, 0.1% SDS at 42 C. Autoradiography was for 18 hr to several days at −70 C with intensifying screens.

RESULTS

DNA preparations. The alternative methods of DNA preparation tested (7,19) resulted in *Fusarium* DNA preparations with heavy polysaccharide contamination. The method as described is rapid and yields in excess of 1 mg of DNA per gram of dried mycelium, of a size greater than the 23-kilobase-pair (kbp) lambda marker with an $A_{260/280\ nm}$ of 1.8–2.0.

Ethidium-stained gels. Bands of repetitive DNA were faintly visible with several hexanucleotide-recognizing restriction enzymes. Eco R1 produced two distinct bands, at 3.2 and 4.2 kb, respectively, with 10 isolates of *F. oxysporum* of widely different cultural morphology and comprising four different forma speciales. The 3.2-kb band was also common to an isolate of each of the eight other species of the Snyder and Hansen classification of the fusaria, each of which had a different pattern of fainter bands (data not shown).

Hybridization patterns with selected probes. In initial tests, plasmid minipreparations (13) of 60 random recombinant clones were screened by electrophoresis and eight arbitrary choices with inserts ranging from 600 to 4,000 base pairs (bp) were further analyzed for restriction patterns with seven restriction enzymes recognizing hexanucleotide sites. Four of these plasmids containing inserts with multiple restriction sites were selected, labeled by nick translation, and hybridized to blots of four *F. oxysporum* isolates (f. sp. *dianthi*, isolates 1 and 2; f. sp. *lycopersici* race 1 and f. sp. *gladioli*, isolate 2).

As shown in the Southern blots depicted in Figure 1, each of the four cloned probes hybridized to fragments of the expected size in Hind III digests of the source isolate. This fragment was identical over the four isolates for probe A3 (950 bp) but polymorphic for probe B2 (1,550 bp), enabling a differentiation between *F. o.* f. sp. *dianthi* and the other two forma speciales. Probe E8 (760 bp) separated the two isolates of *F. o.* f. sp. *dianthi* by means of an additional band in isolate 1. Probe D4 (3,400 bp) gave multiple bands that were highly polymorphic among the three forma speciales but identical for the two *F. o.* f. sp. *dianthi* isolates (Fig. 1; a band at 3.1 kb has not reproduced well—cf. Fig. 2).

When the fungal DNA was digested with Eco R1, further polymorphisms were evident. Probe A3 now distinguished between the two *F. o.* f. sp. *dianthi* isolates on the basis of an extra band, as did probe E8, which in addition separated *F. o.* f. sp. *dianthi* from f. spp. *lycopersici* and *gladioli*. Probe B2 also distinguished between the latter two. Probe D4 gave the same results as before, although the band patterns differed.

When tested against Hind III digestion blots of isolates from each of the eight *Fusarium* species of the Snyder and Hansen classification, probe D4 reacted only with a subset of members of the species *oxysporum* (Fig. 2). The RFLP pattern obtained for isolate 3 of *F. o.* f. sp. *dianthi* (Fig. 2, lane j) was identical to that obtained for isolates 1 and 2, although it had been isolated from the

Fig. 1. Composite autoradiogram of restriction fragment length polymorphisms of Southern blots of four isolates of *Fusarium oxysporum* digested with Hind III or Eco R1, run for 14 cm on 1% agarose gels, and hybridized to four random recombinant DNA probes derived from *F. o.* f. sp. *dianthi*. 1 = f. sp. *dianthi* isolate 1, 2 = f. sp. *dianthi* isolate 2, 3 = f. sp. *lycopersici* race 1, and 4 = f. sp. *gladioli* isolate 2.

same field more than 7 yr previously and had mutated in culture to a highly pigmented pionnotal form (22). Results were consistent over three batches of *Fusarium* DNA preparations and digestions. A Bam Hl digestion of the set of isolates shown in Figure 2 probed with D4 also gave useful polymorphisms within the *F. oxysporum* species shown while giving a single band for the other species (data not shown).

DISCUSSION

Ethidium staining of restriction digests offers the possibility of a rapid confirmation of species identification, although a large collection of isolates would have to be surveyed to establish this. It is, however, difficult to visualize repetitive bands on the smear obtained with a digest of total DNA. For this methodology it would be better to use the less complex mitochondrial DNA, as has been done with other fungi (4,7). Hybridization patterns, on the other hand, were highly effective, and by using a limited set of probes and restriction enzymes we were able to distinguish isolates within a species, forma speciales, races, and isolates (Figs. 1 and 2). The RFLP patterns were also conserved over time and despite morphological differences in culture.

The hybridization patterns and signal strengths of probes A3, B2, and E8 are indicative of sequences with one or a few copies per genome, whereas the multiple banding of probe D4 is of the type associated with satellite DNA, repetitive DNA, transposable elements, or gene family hybridization patterns. Previous DNA reassociation experiments on various fungi have shown that repetitive DNA ranges from 2–3% in *Aspergillus nidulans* (Eidam) Wint. (21) to 21–23% in *Fusarium graminearum* Schwabe (20). The sequence organization of repetitive DNA in the fungi has been found to be of a very long interspersion pattern (9,11), and most is believed to code for ribosomal RNA (21).

If probe D4 is recognizing repetitive ribosomal coding DNA of nuclear or mitochondrial origin, one would expect these sequences to be conserved throughout the fusaria. A similar argument would apply to conserved gene families such as that for tubulin (12). However, when hybridized to a range of *Fusarium* species, probe D4 reacted only with a subset of the species *oxysporum*. It is
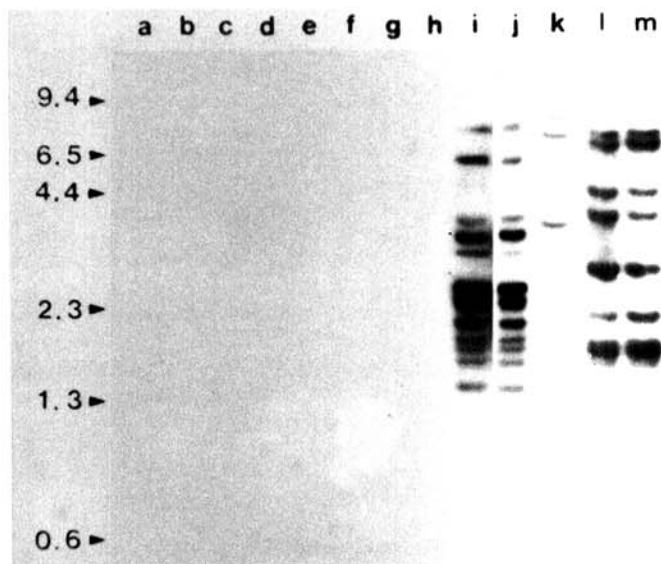

Fig. 2. Composite autoradiogram of restriction fragment length polymorphisms of Hind III digest of *Fusarium* species probed with clone D4. a = T415, b = M125, c = R37, d = S133, e = E15, f = N13, g = L112, h = GR132, i = SS2, j = J, k = TR2, l = S6, and m = S3. Also tested and found negative were MRC3295, MRC3235, MRC2293, MRC3224, MRC3237, MRC2556, MRC3234, MRC2626, MRC3278, MRC3312, MRC3239, NJ, and TM.

suggested that D4 is recognizing a component of a gene family particular to a group within the species *oxysporum*, with obvious implications for the taxonomy of this species.

Based on the evidence presented here, RFLPs show the potential of being a useful diagnostic tool in the study of *Fusarium* taxonomy. If anything, short probes such as those used here are too specific, and although this in itself has applications in race typing, for example, we would have preferred to find a probe that recognizes a species in its entirety, a not insurmountable problem given the very few probes herein tested. Many isolates must still be

screened to establish constant restriction patterns within a group before the method can be applied practically. Probes such as D4, with its multiple-banding pattern and specificity for a group, are seen as being particularly useful in that particular bands could indicate membership in a group and polymorphisms enable finer distinctions to be made within the group.

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