Techniques

# Comparison of Three Molecular Methods for the Characterization of *Fusarium oxysporum* Strains

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## ABSTRACT

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Sixty strains of *Fusarium oxysporum* were characterized using three DNA-based methods: restriction fragment length polymorphism (RFLP) analysis of total DNA after hybridization with a random DNA probe, polymerase chain reaction (PCR)-based fingerprinting with primers matching enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palindromic (REP) elements, and restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (IGS). All methods yielded intraspecific polymorphisms, but different levels of discrimination were obtained. Good correlation was found between the groupings obtained by the three methods. RFLP analysis of total DNA was the most sensitive method, enabling the detection of 40 variants within the sample of 60 strains. However, this technique is more time-consuming than the PCR-based methods. By ERIC- and REP-PCR fingerprinting and PCR/RFLP analysis of the IGS, the 60 strains were categorized into 27 and 11 genotypes, respectively. Though less discriminant, the PCR/RFLP method allowed estimation of the genetic relationships between the strains. Discrimination of closely related strains within IGS genotypes could be achieved by ERIC- or REP-PCR fingerprinting, which is the most efficient procedure in terms of simplicity and rapidity. Therefore, the two PCR-based procedures described in this paper appear to be rapid tools for the genetic characterization of large populations of *F. oxysporum*.

*Additional keywords*: DNA fingerprinting, repetitive DNA.

The asexual fungus *Fusarium oxysporum* Schlect. emend. Snyd. & Hans. is a common inhabitant of soil and has a worldwide distribution. All strains of *F. oxysporum* are successful as saprophytes and are able to grow and survive for long periods on organic matter in soil. However, some strains are responsible for vascular wilt diseases on many plants of economical importance. These pathogenic strains show a high level of host specificity and are classified on this basis into more than 120 formae speciales and races (3). These pathogenic strains are not identifiable using phenotypic characters. Molecular tools have been used to characterize the diversity among pathogenic strains of *F. oxysporum* with the aim of determining genetic relatedness among formae speciales (19,20,25).

Nonpathogenic strains of *F. oxysporum* have received less attention. However, they play an important role in the ecology of Fusarium diseases. Indeed studies on soils naturally suppressive to Fusarium wilts have established the role of nonpathogenic *F. oxysporum* populations (1). Even if their mode of action is not totally understood, experimental evidence indicates that they compete with the pathogenic *F. oxysporum* population in soil for nutrients (10) and on roots for colonization sites (14). Experimental evidence also indicates that the nonpathogenic strains vary in their ability to compete with pathogenic strains and to limit disease incidence in a bioassay (2). Therefore, it would be interesting to be able to characterize the diversity among populations of nonpathogenic *F. oxysporum*. Moreover, as stated by Correll (8), studies of the diversity of nonpathogenic *F. oxysporum* populations should help in understanding the diversity among pathogenic strains of *F. oxysporum*. The aim of this study was to evaluate the usefulness of different techniques allowing characterization of the diversity within populations of nonpathogenic *F. oxysporum*.

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Genetic variation within nonpathogenic populations of *F. oxysporum* has been assessed by vegetative compatibility analysis (9,12). These studies were based on the selection of nitrate nonutilizing mutants for each isolate and performing complementary pairing of the deficient mutants. This is time-consuming and not always compatible with broad application in ecological studies that require screening of large populations. In addition, this method cannot provide information about genetic relatedness between strains that are not vegetatively compatible.

Direct analysis of DNA polymorphisms is a more general approach to establishing genetic variation in organisms. Restriction fragment length polymorphism (RFLP) analyses of nuclear or mitochondrial DNA have been used to estimate the genetic diversity of *F. oxysporum* within (13,18) and between formae speciales (20,25) and among nonpathogenic strains (15).

Molecular methods involving the use of the polymerase chain reaction (PCR) have been recently described to resolve genetic variation between strains. One strategy is the use of defined PCR-amplified fragments as substrate for RFLP or sequence analysis. Ribosomal DNA (rDNA) regions have often been chosen for taxonomic and phylogenetic studies because sequence data are available and because they contain both variable and conserved regions, allowing discrimination at the genus, species, or intraspecific level. In fungi as well as other organisms, the noncoding regions of rDNA have been used as variable regions. The internal transcribed spacers (ITS) of the rDNA can display variation within genera and are used in the differentiation of species (6,7,32). At the intraspecific level, variability in ITS sequences is generally very low or undetected (22), although a surprising level of divergence was reported for ITS sequences within the species *F. sambucinum* (26). Few differences in ITS sequences were found within *F. oxysporum* (5). The intergenic spacer (IGS) sequence that separates ribosomal repeat units was variable enough to allow discrimination of closely related fungi within the genus *Laccaria* (17). IGS sequences might be good candidates for the differentiation of strains at the intraspecific level.

DNA amplification by PCR with primers directed against repetitive elements, arbitrarily chosen or defined, is another strategy that can be called interrepeat PCR. The analysis of random amplified polymorphic DNA (RAPD) was proposed for discrimination between isolates of *F. oxysporum* within a particular forma specialis (4,16,23). Repetitive DNA sequences are a common feature of all eukaryotic species. Even though their function remains generally unknown, some of these sequences have been exploited in designing PCR primer pairs to amplify interrepeat sequences to produce species- or strain-specific gel electrophoresis patterns. In fungi, this strategy has been used with various primers to differentiate *Aspergillus* species, but only prokaryotic repeat motif primers derived from enterobacterial repetitive intergenic consensus (ERIC) sequences permitted discrimination between isolates that belong to the same species (30). It has also been shown that oligonucleotides matching repetitive extragenic palindromic (REP) elements and ERIC sequences can be used as PCR primers to distinguish between very closely related bacterial strains (11).

In the present study, we evaluated the use of three molecular methods to discriminate among nonpathogenic strains of *F. oxysporum*: RFLP analysis of total DNA after hybridization with a random DNA probe, PCR-fingerprinting with prokaryotic repeat motif primers derived from ERIC and REP sequences, and restriction fragment analysis of PCR-amplified IGS DNA.

## MATERIALS AND METHODS

**Fungal strains.** A collection of nonpathogenic strains of *F. oxysporum* was made by either direct isolation from a clay loam soil from Dijon (France) or isolation from the rhizoplane of four plants (flax, melon, tomato, and wheat) grown on this soil. Several hundred isolates were collected by single spore transfer and were stored by cryopreservation at −80 C. A subsample of 60 strains was chosen at random for this preliminary study. The strains were characterized morphologically as *F. oxysporum*. The absence of symptoms on the four plants grown on this soil indicated that they are nonpathogenic on these hosts, but their potential pathogenicity on other plants was not investigated.

**DNA extraction.** For large-scale preparations of pure DNA, fungal strains were cultivated at 25 C for 2 days without shaking in 200 ml of potato-dextrose broth inoculated with a spore suspension. The mycelium was harvested by filtration, lyophilized, and ground with liquid nitrogen. A 1.5-ml microtube was filled halfway up the conical portion with ground lyophilized mycelium, and 500 μl of extraction buffer (1% cetyltrimethylammonium bromide, 50 mM Tris-HCl [pH 8.0], 0.7 M NaCl, 0.5% polyvinylpyrrolidone molecular weight 40,000 [PVP40], and 10 mM EDTA) was added. The extraction then was performed by following Rogers and Bendich's procedure (27). Finally, DNA was dissolved in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA), with a final concentration of 2 μg/μl, and stored at 4 C until use.

For minipreparations of DNA, *F. oxysporum* strains were cultivated for 5 days on potato-dextrose agar plates. A 1.5-ml microtube was filled one-third of the way up the conical portion with mycelium that was recovered directly from the surface of the plate. DNA was extracted using the method of Lee and Taylor (21). Finally, DNA was resuspended in 100 μl of TE buffer and stored at 4 C until use.

**Southern blot and hybridization.** Aliquots of 2 μg of pure DNA were digested for 2 h with 10 units of *Eco*RI (Boehringer Mannheim, Meylan, France). The restriction fragments were separated by horizontal gel electrophoresis with 0.8% agarose (Type II: medium EEO, Sigma, Saint-Quentin-Fallavier, France) in TAE buffer (40 mM Tris, 4 mM sodium acetate, 1 mM EDTA, pH 7.9). DNA was transferred from the agarose gels to nylon filters (Pharmacia LKB Biotechnologie, Saint-Quentin-Yvelines, France) using the Vacugene XL apparatus (Pharmacia) according to the manufacturer's instructions.

Southern blots were probed with a random DNA fragment from a *F. oxysporum* strain, probe 46 (24). This probe consisted of a 2,330-bp *Eco*RI DNA fragment from total DNA of *F. oxysporum* f. sp. *elaeidis* Foe16 cloned into plasmid pUC19. For the preparation of the probe, the recombinant plasmid was digested by *Eco*RI according to the manufacturer's recommendations, and the mixture was electrophoresed on a 0.8% agarose gel (SeaPlaque GTG agarose, FMC, Rockland, ME) in TAE buffer. After electrophoresis, the gel was stained with ethidium bromide, and the 2,330-bp *Eco*RI insert was located under UV light and recovered from the gel with agarase (Boehringer Mannheim) as described by the manufacturer. Digoxigenin labeling of the probe, DNA hybridization, and probe detection were done with a nonradioactive DNA labeling and detection kit (Boehringer Mannheim) according to the supplier's instructions.

**ERIC- and REP-PCR.** The two oligonucleotide primer pairs used for PCR amplification, ERIC1R/ERIC2 (22 nucleotides in length) and REP1R-1/REP2-1 (18 nucleotides in length), were as described by Versalovic et al (31). The primers were synthesized by Appligene (Illkirch, France). PCR reactions were carried out in a 25-μl volume containing the DNA template (50 ng of purified DNA or 1 μl of DNA from minipreparations); 50 pmol each of primers; 1.25 mM each of dATP, dCTP, dTTP, and dGTP; and 2 units of *Taq* DNA polymerase (Appligene) in the buffer described previously (31). A thin layer of paraffin oil was added to prevent evaporation. DNA amplifications were performed in a thermal cycler (Biometra TRIO-Thermoblock TB1, Göttingen, Germany) with an initial denaturation (ERIC: 7 min at 95 C; REP: 6 min at 95 C) followed by 30 cycles of denaturation (1 min at 94 C), annealing (ERIC: 1 min at 52 C; REP: 1 min at 40 C), and extension (8 min at 65 C) with a final extension (16 min at 65 C). Amplification reactions were performed twice to check the consistency of the method. Aliquots (4–8 μl) of each PCR product were analyzed by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and photographed over a 302-nm UV transilluminator with Polaroid 665 positive/negative film.

**PCR-RFLP analysis of the IGS.** The oligonucleotide primers PNFo (5′-CCCGCCTGGCTGCGTCCGACTC-3′) and PN22 (5′-CAAGCATATGACTACTGGC-3′) were used to amplify a fragment of the IGS region of the rDNA. The forward oligonucleotide PNFo, designated according to the IGS sequence of *F. oxysporum* f. sp. *melonis* Fom24 (5), anneals to nucleotides 636–657 in the IGS sequence. The reverse oligonucleotide PN22 is derived from a conserved region at the 5′ end of the *Saccharomyces cerevisiae* 18S rRNA gene. The amplified fragment included two-thirds of the IGS region and the flanking portion of the 18S rDNA. The primers were synthesized by Appligene. PCR amplifications were performed in a total volume of 100 μl by mixing the template DNA (1 μl of DNA from the minipreparation or 50 ng of purified DNA) with 0.1 μM of each primer; 100 μM each of dATP, dCTP, dGTP, and dTTP; 1 unit of *Taq* DNA polymerase (Appligene); and PCR reaction buffer (10 mM Tris-HCl [pH 9.0] at 25 C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml of bovine serum albumin). A thin layer of paraffin oil was added to prevent evaporation. Amplifications were conducted in the same thermal cycler as above, with 30 cycles of 90 s at 95 C, 60 s at 50 C, and 90 s at 72 C. The specificity and efficacy of amplification were checked by electrophoresis with 5-μl aliquots of PCR products in a 0.8% agarose gel.

Aliquots (5–10 μl) of PCR products were digested for 2 h with 10 units of restriction endonucleases. The following restriction enzymes were used: *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I, *Xho*I (Gibco BRL, Gaithersburg, MD), and *Scr*FI (Boehringer Mannheim). The restriction fragments were separated by electrophoresis in TAE buffer with 1% agarose gels for the 6-base cutting restriction enzyme, *Xho*I, and 4–6% Nusieve 3:1 agarose (FMC) gels for the 4-base cutting restriction enzymes.

Each strain was assigned a composite IGS genotype defined by the combination of the patterns obtained with the nine restriction endonucleases. Map locations of the restriction sites in the IGS region were inferred from the known sequence of the strain *F. oxysporum* f. sp. *melonis* Fom24 (5). First, the restriction sites were compared in all pairwise combinations to produce a

matrix of the relative numbers of restriction site differences. Next, the restriction site differences between the composite IGS genotypes were analyzed by the computer program PAUP (phylogenetic analysis using parsimony; 28). Heuristic, branch-and-bound, and exhaustive searches with Dollo parsimony were employed in the analysis. Dollo parsimony is recommended for restriction site data (28,29) because of the asymmetry in the probabilities of gaining and losing restriction sites.

## RESULTS

**Hybridization patterns with a random probe.** Probe 46 was hybridized to the *Eco*RI-digested DNA from each of the 60 strains of *F. oxysporum*. The resulting patterns consisted of one to 17 bands. The strains that displayed the same hybridization pattern were assigned the same type. At a second level of analysis, hybridization pattern types that shared a common arrangement of at least three bands were grouped into the same class. Examples of hybridization patterns are shown in Figure 1. The 60 strains were categorized into 40 hybridization pattern types and 25 classes (Table 1).

**ERIC- and REP-PCR fingerprinting.** Both ERIC and REP primers were designed to match inverted repeat sequences in bacterial genomes (31). ERIC1R/ERIC2 and REP1R-1/REP2-1 primer pairs were used for PCR amplification of the 60 strains. Both ERIC and REP primer sets generated multiple distinct DNA fragments, ranging in size from approximately 100 to 4,000 bp. The profiles were reproducible from one experiment to the other.

Various patterns were found among the *F. oxysporum* isolates examined. However, some strains that exhibited very similar patterns with only weak differences concerning the intensity of minor bands were assigned the same ERIC or REP pattern type. Examples of REP and ERIC patterns are shown in Figure 2. The 60 strains analyzed were grouped into 19 ERIC pattern types and 21 REP pattern types (Table 1). Some strains that shared the same ERIC pattern type could be distinguished by their REP pattern type and vice versa. For example, strains of ERIC pattern type E1 were distinguished by their REP-pattern type R1, R2, R3, or R4. In the same way, two ERIC pattern types, E4 and E5, occurred within the REP pattern type R4 strains. ERIC- and REP-PCR fingerprinting together allowed the discrimination of 27 types among the 60 strains.

**Restriction fragment analysis of amplified IGS sequences.** Oligonucleotide primers PN22 and PNFo permitted the amplification of a single DNA fragment of about 1,700 bp for each of the 60 *F. oxysporum* strains tested. This size corresponded to the expected size according to the IGS sequence of the strain Fom24. The PCR products were digested with each of nine restriction enzymes. Each of the eight 4-base cutting restriction enzymes digested the PCR products into three to 20 fragments. The 6-base cutting enzyme, *Xho*I, had no site or one site in the amplified IGS region. Depending on the restriction enzyme, two to five distinct restriction patterns were obtained within the sample strains (Table 2). As an example, results of digestion with *Scr*FI and *Rsa*I are shown in Figure 3. The 60 strains analyzed with the nine restriction enzymes gave 11 combinations of patterns representing 11 IGS genotypes (Table 2). Seven of the nine restriction enzymes were sufficient to resolve the 11 composite genotypes among the 60 strains.

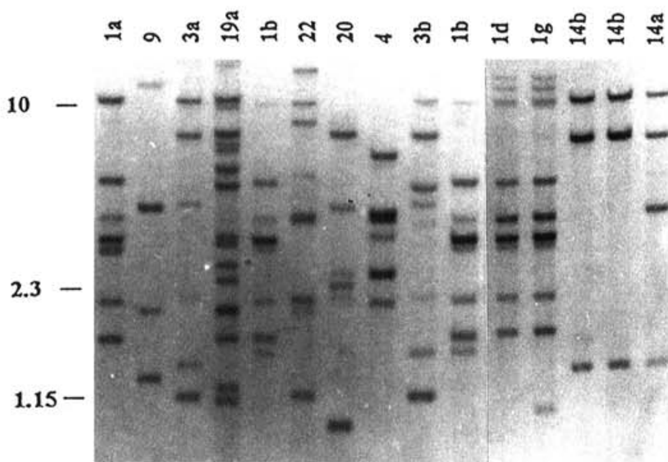



**Fig. 1.** Examples of Southern blots with probe 46 of *Eco*RI-digests from 15 *Fusarium oxysporum* strains. Hybridization pattern classes and types are indicated across the top of the figure. The size (in kilobases) and relative location of some of the molecular weight marker bands are indicated to the left of the figure.

TABLE 1. Hybridization pattern types, enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palidromic (REP) pattern types, and ribosomal intergenic spacer (IGS) genotypes of 60 *Fusarium oxysporum* strains

| Number of strains | Hybridization pattern class and type[a] | ERIC pattern type | REP pattern type | IGS genotype[b] |
|---|---|---|---|---|
| 3 | 1a | E1 | R1 | |
| 8 | 1b | E1 | R2 | |
| 1 | 1c | E1 | R3 | |
| 1 | 1d | E1 | R3 | |
| 1 | 1e | E1 | R4 | 1 |
| 1 | 1f | E2 | R1 | |
| 3 | 1g | E3 | R3 | |
| 1 | 1h | E4 | R4 | |
| 1 | 1i | E5 | R4 | |
| 1 | 2 | E6 | R5 | 2 |
| 1 | 3a | E7 | R6 | |
| 1 | 3b | E7 | R6 | |
| 1 | 3b | E7 | R7 | |
| 1 | 4 | E8 | R8 | 3 |
| 1 | 5 | E9 | R8 | |
| 1 | 6 | E10 | R9 | |
| 1 | 7a | E10 | R9 | |
| 2 | 7b | E10 | R9 | |
| 1 | 8 | E10 | R10 | |
| 1 | 9 | E11 | R11 | |
| 1 | 10 | E11 | R11 | |
| 1 | 11 | E11 | R11 | |
| 1 | 12 | E11 | R11 | 4 |
| 3 | 13a | E11 | R11 | |
| 1 | 13b | E11 | R11 | |
| 1 | 14a | E11 | R11 | |
| 2 | 14b | E11 | R12 | |
| 1 | 15 | E11 | R13 | |
| 1 | 16 | E10 | R10 | 5 |
| 1 | 17 | E12 | R14 | 6 |
| 1 | 18 | E13 | R15 | 7 |
| 1 | 19a | E14 | R16 | 8 |
| 1 | 19b | E14 | R16 | |
| 3 | 20 | E15 | R17 | 9 |
| 1 | 21 | E16 | R18 | 10 |
| 1 | 22 | E17 | R19 | |
| 2 | 23a | E17 | R19 | |
| 1 | 23b | E17 | R20 | 11 |
| 1 | 24a | E17 | R19 | |
| 1 | 24b | E18 | R19 | |
| 1 | 25a | E17 | R19 | |
| 1 | 25a | E19 | R21 | |

[a]The number designates the hybridization pattern class and the following letter designates the type within the class.
[b]The IGS genotypes are defined in Table 2.

The map locations of the restriction sites in the IGS region were inferred from the known IGS sequence of strain Fom24. A total of 81 restriction sites were examined; 30 of them were polymorphic among the *F. oxysporum* strains (Table 3).

Analysis of the matrix of pairwise restriction site differences for the IGS genotypes revealed three distinct groups. The first one included the 45 strains belonging to IGS genotypes 1–7. Within this group, the relative distances between pairs of IGS genotypes varied from 1.2–8.6% base substitutions in the restriction sites, with an arithmetic mean of 4.1%. The seven strains grouped into IGS genotypes 8–10 formed the second cluster. The mean distance between these two clusters was 11.6% base substitutions. Within the second cluster, the relative distances between IGS genotypes ranged from 3.7 to 8.6% base substitutions (mean distance of 5.7%). The eight strains belonging to genotype 11 were distantly related to all others (23.5–28.4% base substitutions).

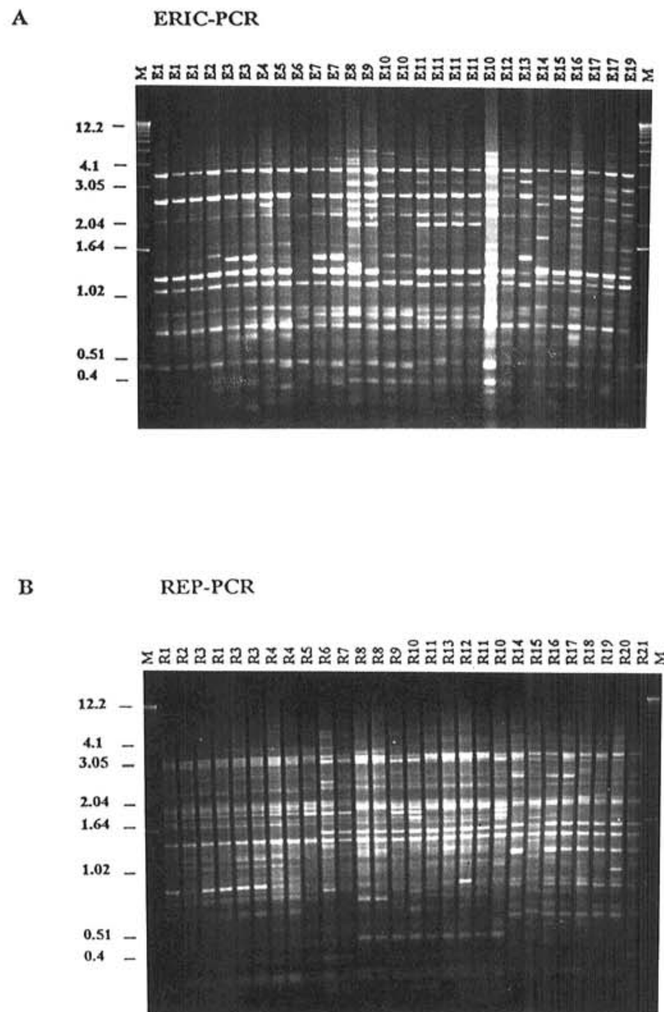



**Fig. 2. A,** Enterobacterial repetitive intergenic consensus (ERIC) sequences and **B,** repetitive extragenic palindromic (REP) polymerase chain reaction (PCR) patterns of 28 *Fusarium oxysporum* strains. ERIC and REP pattern types are indicated across the top of the figure. Lane M, 1-kb ladder (Gibco BRL). The sizes (in kilobases) of the molecular weight marker are indicated to the left of the figure.

The genetic relationships of the *F. oxysporum* strains were estimated from the restriction analysis of the IGS by the PAUP program (28). Heuristic, branch-and-bound, and exhaustive analyses yielded identical results. Four equally parsimonious trees were obtained, with the same branching topology for IGS genotypes 8–11 but some topological differences for IGS genotypes 1–7. The tree resulting from the bootstrap analysis is shown in Figure 4.

**Correspondence between hybridization pattern types, ERIC and REP pattern types, and IGS genotypes.** A decreasing level of discrimination was found for RFLP analysis of Southern-blotted DNA hybridized with probe 46 (40 hybridization pattern types), ERIC- and REP-PCR fingerprinting (27 ERIC-plus-REP pattern types), and restriction fragment analysis of the IGS region (11 IGS genotypes). There were only two cases in which two strains exhibiting the same hybridization pattern type (3b and 25a) could be differentiated by ERIC- or REP-fingerprinting (Table 1). For the others, strains that grouped with the same hybridization pattern type also grouped with the same ERIC or REP pattern type. When results from both methods were combined, 42 types were differentiated within the sample of 60 strains. The grouping obtained by the third method, restriction fragment analysis of the IGS region amplified by PCR, was well correlated with those obtained by the two other methods. In general, the same hybridization pattern type or the same ERIC-plus-REP pattern type never occurred in two different IGS genotypes. One exception was the ERIC-plus-REP pattern type E10-R10, which was shared by one strain of IGS genotype 3 and one strain of IGS genotype 5.

## DISCUSSION

In this study, we have evaluated three molecular methods for their ability to discriminate between strains that belong to the species *Fusarium oxysporum*: RFLP analysis after hybridization with a random DNA probe, PCR amplification with primers matching ERIC and REP repetitive elements, and RFLP analysis of the IGS region.

Hybridization of total DNA of *F. oxysporum* with probe 46 gave multiple banding patterns and yielded useful polymorphisms within the *F. oxysporum* strains. Other random DNA probes corresponding to repetitive sequences have been used successfully to discriminate between *F. oxysporum* strains by RFLP analysis (13,20).

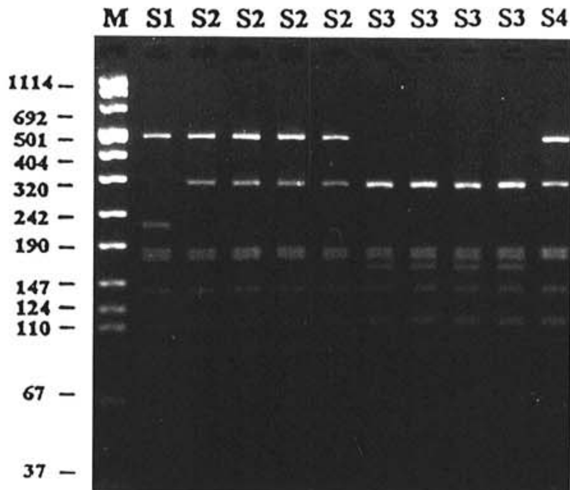
TABLE 2. Ribosomal intergenic spacer (IGS) genotypes and restriction patterns of *Fusarium oxysporum* strains revealed by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified IGS sequences

| IGS genotype[a] | Restriction patterns of amplified IGS fragments digested with enzymes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Alu*I | *Hae*III | *Hha*I | *Hin*fI | *Mbo*I | *Msp*I | *Rsa*I | *Scr*FI | *Xho*I |
| 1 | A1 | Ha1 | Hh1 | Hi1 | Mb1 | Ms1 | R1 | S1 | X1 |
| 2 | A1 | Ha1 | Hh1 | Hi2 | Mb1 | Ms1 | R1 | S1 | X1 |
| 3 | A1 | Ha1 | Hh1 | Hi2 | Mb1 | Ms1 | R1 | S1 | X2 |
| 4 | A2 | Ha1 | Hh1 | Hi2 | Mb1 | Ms1 | R1 | S1 | X2 |
| 5 | A1 | Ha1 | Hh1 | Hi2 | Mb1 | Ms1 | R1 | S2 | X2 |
| 6 | A1 | Ha2 | Hh1 | Hi2 | Mb1 | Ms1 | R1 | S1 | X1 |
| 7 | A1 | Ha3 | Hh1 | Hi2 | Mb2 | Ms1 | R2 | S1 | X1 |
| 8 | A3 | Ha4 | Hh1 | Hi1 | Mb3 | Ms1 | R1 | S2 | X1 |
| 9 | A4 | Ha4 | Hh1 | Hi1 | Mb1 | Ms2 | R1 | S2 | X1 |
| 10 | A4 | Ha4 | Hh1 | Hi1 | Mb1 | Ms3 | R3 | S3 | X1 |
| 11 | A5 | Ha5 | Hh2 | Hi1 | Mb4 | Ms3 | R4 | S4 | X1 |

[a] IGS genotypes represent the combination of patterns obtained with the nine restriction enzymes used.

*Scr*FI *Rsa*I

A B



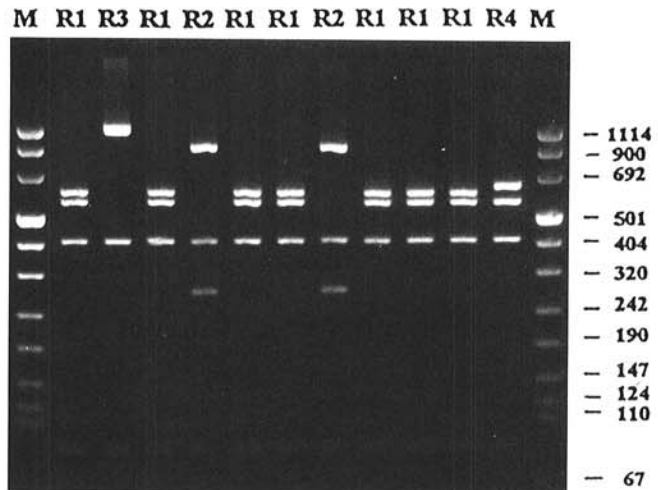




**Fig. 3.** Restriction patterns of polymerase chain reaction-amplified ribosomal intergenic spacer fragments **A,** digested with *Scr*FI and analyzed in a 6% Nusieve agarose gel or **B,** digested with *Rsa*I and analyzed in a 4% Nusieve agarose gel. Similar restriction patterns were grouped and assigned the same number (S1–S4 or R1–R4). Lane M, molecular weight marker VIII (Boehringer Mannheim). The sizes (in basepairs) of the molecular weight marker are indicated to the left (**A**) or right (**B**) of the figure.

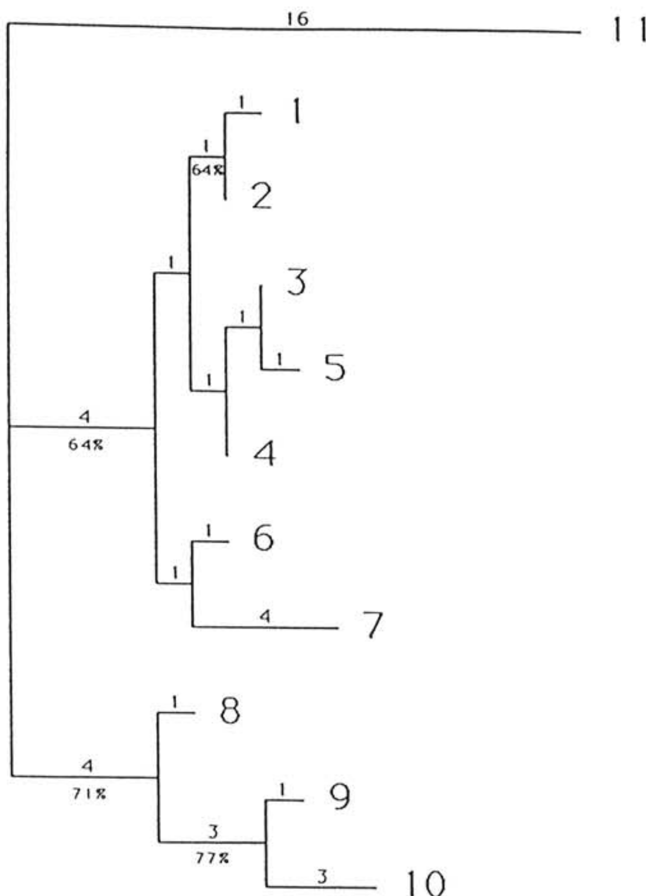



**Fig. 4.** Tree constructed by the PAUP (28) computer program showing the relationships between the ribosomal intergenic spacer genotypes defined in Table 2. The percentages below the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. Bootstrap values below 50% are not displayed. The horizontal branches are drawn proportionally to the number of restriction site changes. The numbers above the branches are the total number of changes assigned to each branch by PAUP. The tree length was 44 steps, with a consistency index of 0.682.

The distribution of ERIC and REP elements has been examined in diverse prokaryotic genomes (31), and interrepeat PCR with primers matching these repetitive sequences has been described for the characterization of bacterial strains (11). Our results indicate that these repetitive elements, which are highly conserved in the bacterial kingdom, also are present in the fungus *F. oxysporum*. The distribution of these elements was variable among *F. oxysporum* strains, which allowed us to differentiate the 60 strains of *F. oxysporum* used in this study into 27 types. Recently, it also was reported that ERIC-like sequences occur in fungal strains from the genus *Aspergillus* (30) and that ERIC-PCR fingerprinting permits differentiation of closely related strains. An alternative method to ERIC- or REP-PCR fingerprinting could be RAPD analysis, which was used recently to differentiate isolates of *F. oxysporum* (4,16,23). However, this method requires arbitrary testing of several oligonucleotides as PCR primers to select those that give useful polymorphisms.

Although the discriminating power of RFLP analysis of Southern-blotted DNA was higher than that of interrepeat PCR, some technical limitations must be taken into consideration: The first method is more time-consuming in analyzing a large number of strains. It requires large quantities of pure DNA samples, probe preparation, and the fastidious procedures of Southern-blotting and -hybridization. In contrast, the interrepeat PCR procedure involves only rapid minipreparation of DNA, PCR amplification, and agarose gel electrophoresis.

The third method developed in our study was RFLP analysis of the amplified IGS region, which allowed us to categorize strains within the species *F. oxysporum*. It also was previously reported that the IGS varied between strains of *Laccaria* spp. (17). We found this third method less discriminate than the other two. However, it provided a way to group closely related strains and to estimate the genetic relationships between the groups, which were easily inferred from the differences in restriction sites in the IGS sequences. In contrast, the complex banding patterns generated by ERIC and REP fingerprinting were difficult to analyze to quantify variations between strains, and with the RFLP method, the number of DNA fragments hybridized with the probe were insufficient to quantify strain variations.

Interrepeat PCR and hybridization of total DNA probed with a repetitive DNA fragment both produced multiple band patterns corresponding to DNA fragments dispersed in the whole genome,

TABLE 3. Data matrix showing presence (1) or absence (0) of restriction sites at 30 polymorphic positions[a] in the ribosomal intergenic spacer (IGS) region for the 11 IGS genotypes

| IGS genotype[b] | *Rsa*I 36 | *Scr*FI 173 | *Msp*I 200 | *Hae*III 215 | *Rsa*I 311 | *Xho*I 356 | *Mbo*I 369 | *Mbo*I 418 | *Alu*I 448 | *Scr*FI 502 | *Alu*I 593 | *Hha*I 594 | *Rsa*I 633 | *Hae*III 667 | *Scr*FI 696 | *Hae*III 720 | *Msp*I 756 | *Alu*I 763 | *Msp*I 776 | *Alu*I 818 | *Hae*III 827 | *Hae*III 867 | *Mbo*I 877 | *Mbo*I 892 | *Hin*fI 910 | *Alu*I 928 | *Alu*I 968 | *Hae*III 982 | *Mbo*I 987 | *Scr*FI 1032 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| 2 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| 4 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| 5 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| 6 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| 7 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| 8 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |

[a]The position of each polymorphic restriction site is given according to the IGS sequence of *Fusarium oxysporum* Fom24 and is numbered from the first nucleotide of the oligonucleotide primer PN22. The position of the restriction sites missing in the known sequence were approximated.

[b]The IGS genotypes are defined in Table 2.

whereas PCR/RFLP analysis provided information about sequence variation in a specific region of the genome. However, a good correlation was found between PCR/RFLP analysis of the IGS region and the two other methods. Interrepeat PCR and the hybridization method both provided a way of subtyping the IGS genotypes defined by PCR/RFLP.

The two PCR-based procedures described in this paper appear to be well adapted for large-scale characterization of *F. oxysporum* strains as required by studies of diversity of natural populations. Interrepeat PCR with ERIC and REP primers, which is the most rapid and simple procedure, may be useful for routine identification of *F. oxysporum* isolates. Since these repetitive sequences have been used successfully for PCR amplification within another fungal genus (30), the procedure may find widespread application for the identification of many fungal strains.

PCR/RFLP analysis of the IGS region appears suitable to categorize groups of closely related strains at the intraspecific level. The method could be developed for study of other fungal genera, provided that sequence data becomes available to design either specific or universal primers. This PCR/RFLP technique will be used to characterize a larger number of strains in our study of the diversity of nonpathogenic *F. oxysporum* populations, with the intention of correlating this intraspecific diversity with ecological traits such as rhizospheric competence and antagonistic ability.

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