Clustering of Trichothecene-Producing Fusarium Strains Determined from 28S Ribosomal DNA Sequences

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The genus Fusarium includes several species that produce trichothecenes. We analyzed DNA sequences from a variable region at the 5′ end of the large nuclear ribosomal DNA (rDNA) (28S) to determine the genetic relatedness of trichothecene-producing Fusarium species. All trichothecene-producing strains clustered together, and two monophyletic groups were resolved. The first clade included strains of F. acuminatum, F. sambucinum, F. tundi-um, F. compactum, F. campiocteras (red pigment), F. sporotrichioi-de, and F. venenatum, which produced type A trichothecenes (T-2 toxin, HT-2 toxin, neosanolioi, and diacetoxyscirpenol). The second clade consisted of strains of F. crookwellense, F. culmorum, and F. graminearum, which produced type B trichothecenes (fusarenone-X, nivalenol, and deoxynivalenol). The phylogenetic placement of the species based on rDNA correlated better with toxic secondary metabolite data rather than with the current classification system based on morphology.

Trichothecene toxins are produced by several species of Fusarium. Trichothecenes are a closely related group of sesquiterpenes that are potent eukaryotic protein synthesis inhibitors and are often associated with a variety of animal and human mycotoxicoses (18). Chemically, Fusarium trichothe-cenes have been classified based on the substituent functional groups, and they can be characterized by the presence or absence of a keto group at the C-8 position. Consistent differences in toxigenicity exist between type A and B trichothecenes (28).

In spite of their similar biosynthetic abilities, trichothecene-producing Fusarium species have been placed in various sections of the genus. The species reported as major producers of type A trichothecenes (e.g., T-2 toxin [T-2], HT-2 toxin [HT-2], neosanolioi [NEOS], and diacetoxyscirpenol [DAS]) are F. sambucinum Fuekel, F. venenatum Niren. (Discolor Woll. sec- tion) (1, 18), F. acuminatum Ell & Ev., F. compactum (Woll. Gordon (Gibbous Woll. section) (12, 29), and F. sporotri-chioide Sherb. (Sporotrichiella Woll. section) (14, 18). The majority of strains producing type B trichothecenes (e.g., nivalenol [NIV], fusarenone-X [FUS], and deoxynivalenol [DON]) are F. crookwellense Burgess, Nelson & Tousson (6, 8), F. culmorum (W. G. Smith) Sacc., and F. graminearum Sch. (13, 18), all part of the Discolor section. Many Fusarium species are not well described, however, because they have not been intensively studied or because they include only a few atypical strains that can produce trichothecenes. For example, atypical strains of a relatively rare population of F. campiocteras Wollenw. & Reink. (Arthrosporiella Woll. section) with red pigmentation and strains of F. tundi-um Sherb. (Discolor Woll. section), a “not well documented” species (21), were recently reported to produce trichothecenes (2, 15).

Our objective in this study was to examine the genetic relatedness of trichothecene-producing Fusarium species, through sequence analysis of a portion of the ribosomal DNA (rDNA) coding region. A short, highly variable region of the large-subunit rDNA has been used for phylogenetic studies in lower eukaryotes (4), including various fungal genera (9, 19, 23, 26). In a preliminary study, we found a possible correlation between phylogenetic affinities obtained by sequencing the 5′ end of the region coding for the large-subunit rRNA and the production of trichothecenes (16). In this report, we identify a phylogenetic affinity within Fusarium trichothecene-producing species, based on DNA sequences at the 5′ end of the larger nuclear rDNA.

MATERIALS AND METHODS

Fungal strains. The strains used in this study are described in Table 1. Before further study, they were stored on slants of special nutrient agar (22) at 4°C.

Chemical analysis. We tested all of the strains for their ability to produce trichothecenes. The strains were grown on 50 g of maize kernels var. Plata, which had been brought overnight to approximately 45% moisture in 250-ml Erlen-meyer flasks and then autoclaved for 30 min at 120°C. The substrate was inocu- lated with 2 ml of approximately 10⁶ conidia/ml. The cultures were shaken once daily for 3 days to distribute the inoculum and were incubated at 25°C in the dark for 4 weeks. The harvested culture material was dried in a forced-air oven at 50°C for 48 h, finely ground in a Waring blender, and stored at 4°C until use. As a control, uninoculated cornmeal was used.

For extractions, culture material (20 g) was extracted with 100 ml of metha- nol–1% aqueous NaCl (55:45; vol/vol) in a blender for 3 min and filtered through paper (Whatman no. 1) under vacuum at room temperature. The filtrate (50 ml) was defatted with n-hexane (50 ml per extraction for three extractions) and then extracted exhaustively with dichloromethane (30 ml per extraction for three extractions). The organic extracts were collected and evaporated to dryness under reduced pressure at room temperature (24 to 28°C), and the residue was dissolved in 1 ml of methanol. The qualitative analyses of trichothecenes of type A (T-2, HT-2, NEOS, and DAS) and type B (NIV, FUS, and DON) were performed by thin-layer chromatography and high-performance thin-layer chromatography (5). The detection limit for each trichothecene was approximately 1 μg/g of dried corn culture. Toxic reference standards were purchased from Sigma Chemical Co. (St. Louis, Mo.).

rDNA sequences. DNA for PCR was extracted by a modified version of the sodium dodecyl sulfate protocol of Raeder and Broda (25). Fresh mycelium (=50 mg) was scraped from a petri dish culture, resuspended in 600 μl of extraction buffer (200 mM Tris [pH 8.4], 200 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) in an Eppendorf tube, and incubated for 1 h at 37°C. The slurry was homogenized twice with 600 μl of phenol and centrifuged for 10 min in an Eppendorf centrifuge (at 1,300 × g). The upper, aqueous phase was extracted with 600 μl of chloroform and centrifuged as described above. DNA was pre-cipitated by addition of 2.5 volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate. The samples were centrifuged for 20 min at 1,300 × g, and the DNA pellet was recovered and dissolved in 50 μl of sterile water. The PCR was set up with 2.5 U of Top DNA polymerase (Boehringer Mannheim Biochemicals) in 100-μl reaction mixtures containing 50 pmol of each outside primer, F65
**TABLE 1. Fusarium strains used in this study**

<table>
<thead>
<tr>
<th>Fusarium species</th>
<th>Section</th>
<th>Strain no.</th>
<th>Host plant and/or origin</th>
<th>Trichothecene(s) produced</th>
<th>EMBL accession no.</th>
<th>Reference</th>
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<td><strong>Trichothecene producers</strong></td>
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<td></td>
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<tr>
<td><em>F. acuminatum</em></td>
<td>Gibbosum</td>
<td>NRRL-6227&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Festuca sp., United States</td>
<td>T-2, HT-2, NEOS</td>
<td>X80239</td>
<td>18</td>
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<tr>
<td><em>F. acuminatum</em></td>
<td>Gibbosum</td>
<td>KF-359&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Poland</td>
<td>T-2</td>
<td>12</td>
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<td><em>F. acuminatum</em></td>
<td>Gibbosum</td>
<td>MRC-3826&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Avena sp., South Africa</td>
<td>T-2</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>F. camptoceras</em> (R)</td>
<td>Arthrosporiella</td>
<td>ITEM-1138&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Musa sp., Panama</td>
<td>T-2, HT-2</td>
<td>X80803</td>
<td>15</td>
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<tr>
<td><em>F. compactum</em></td>
<td>Gibbosum</td>
<td>R-6784&lt;sup&gt;e&lt;/sup&gt;</td>
<td>River sediments, Japan</td>
<td>T-2, NEOS</td>
<td>X80804</td>
<td>18</td>
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<tr>
<td><em>F. sambucinum</em></td>
<td>Discolor</td>
<td>64935&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Brassica sp., Netherlands</td>
<td>DAS, NEOS</td>
<td>X80805</td>
<td>1</td>
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<td><em>F. sporotrichioides</em></td>
<td>Sporontrichilla</td>
<td>NRRL-3299&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Zea mays, France</td>
<td>T-2, HT-2, DAS</td>
<td>X80806</td>
<td>18</td>
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<tr>
<td><em>F. sporotrichioides</em></td>
<td>Sporontrichilla</td>
<td>ITEM-390&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Zea mays, Italy</td>
<td>T-2</td>
<td>14</td>
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<tr>
<td><em>F. timentum</em></td>
<td>Discolor</td>
<td>NRRL-13394&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Lupinus sp., New Zealand</td>
<td>NEOS</td>
<td>X80807</td>
<td>2</td>
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<tr>
<td><em>F. venenatum</em></td>
<td>Discolor</td>
<td>64935&lt;sup&gt;j&lt;/sup&gt;</td>
<td>Solanum tuberosum, Poland</td>
<td>DAS</td>
<td>L.28694&lt;sup&gt;k&lt;/sup&gt;</td>
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<td><strong>Type B</strong></td>
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<td>Discolor</td>
<td>KF-748&lt;sup&gt;l&lt;/sup&gt;</td>
<td>Solanum tuberosum, Poland</td>
<td>FUS, NIV</td>
<td>X80808</td>
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<td><em>F. crookwellense</em></td>
<td>Discolor</td>
<td>ITEM-610&lt;sup&gt;m&lt;/sup&gt;</td>
<td>Triticum sp., Yugoslavia</td>
<td>FUS</td>
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<tr>
<td><em>F. culmorum</em></td>
<td>Discolor</td>
<td>NRRL-3288&lt;sup&gt;n&lt;/sup&gt;</td>
<td>Unknown</td>
<td>DAS</td>
<td>X80809</td>
<td>18</td>
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<td>Discolor</td>
<td>ITEM-326&lt;sup&gt;o&lt;/sup&gt;</td>
<td>Triticum durum, Italy</td>
<td>DON</td>
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<td>Discolor</td>
<td>ITEM-626&lt;sup&gt;p&lt;/sup&gt;</td>
<td>Triticum sp., Yugoslavia</td>
<td>DON</td>
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<tr>
<td><em>F. camptoceras</em> (B)</td>
<td>Arthrosporiella</td>
<td>ITEM-1128&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Musa sp., Ecuador</td>
<td>ND&lt;sup&gt;r&lt;/sup&gt;</td>
<td>X80811</td>
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<td><em>F. equiseti</em></td>
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<td>NRRL-13405&lt;sup&gt;s&lt;/sup&gt;</td>
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<td>ND</td>
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<td>NRRL-13327&lt;sup&gt;t&lt;/sup&gt;</td>
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<td>Sporontrichilla</td>
<td>T-429&lt;sup&gt;u&lt;/sup&gt;</td>
<td>Hordeum sp., Germany</td>
<td>ND</td>
<td>X80814</td>
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<td><em>F. decemcellulare</em></td>
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<td>NRRL-13411&lt;sup&gt;v&lt;/sup&gt;</td>
<td>Unknown</td>
<td>ND</td>
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<td><em>F. solani</em></td>
<td>Maertilled</td>
<td>NRRL-13417&lt;sup&gt;w&lt;/sup&gt;</td>
<td>Unknown</td>
<td>ND</td>
<td>X80816</td>
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* H. Niremberg, Biologische Bundesanstalt fu ¨r Land-und Forstwirtschaft, Berlin, Germany.
* Sequence obtained from GenBank data.
* ND, not detected.

(5'-GATATCATAAAGCGGAGGAAAAAG) and R635 (5'-GGTCCGTGTTTCAAGAGC), 1.25 mM deoxynucleoside triphosphates (Perkin-Elmer Cetus), and 1 µl (approximately 5 ng) of fungal template DNA. The reactions were cycled (Perkin-Elmer Thermocycler) 30 times (1 min at 94°C, 1 min at 52°C, and 2 min at 72°C). Control tubes without the DNA template were included in each experiment (negative control). After amplification, a 10-µl aliquot from each sample was subjected to electrophoresis.

From the samples containing sufficient DNA, excess primer and deoxynucleoside triphosphates were removed with a Qiaex gel extraction kit (Qiagen, Chatsworth, Calif.) as specified by the manufacturer. Single-stranded DNA templates were sequenced completely on both strands with the chain-terminating dideoxynucleotide Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) and [³²P]dATP. Two sequencing reactions were run on each template, one in which the template was primed with 10 pmol of an external limiting primer (R635) and the second in which the template was primed with 10 pmol of an internal primer (5'-GATGAAAAAGCAGCTTGAAGAG). Sequencing reactions were run on 6% polyacrylamide Wedge gels for approximately 3 h, fixed, vacuum dried, and exposed overnight to Kodak Extomat-G film.

Construction of the phylogenetic tree. The phylogenetic tree was constructed by using a stationary Markov model (27). This method permits the calculation of time-of-divergence ratios (t/τ) for any pair of homologous sequences by analyzing any class of site, which is assumed to be subjected to the same evolutionary dynamics.

**Nucleotide sequence accession numbers.** The sequences at the 5' end of the 28S rDNA have been deposited with the EMBL database. The accession numbers are given in Table 1.

**RESULTS**

A 239-base region at the 5' end of the 28S rDNA was sequenced on both strands for all the strains listed in Table 1. These sequences have been deposited with the EMBL database or may be obtained collectively in an aligned format from the first author. No intraspecific sequence variation was found in *F. acuminatum*, *F. sporotrichioides*, *F. crookwellense*, and *F. culmorum*. Trichothecene-producing strains clustered together, and two principal monophyletic groups were resolved (Fig. 1). The first clade included strains of *F. acuminatum*, *F. sambucinum*, *F. tumidum*, *F. compactum*, *F. camptoceras* (red), *F. sporotrichioides*, and *F. venenatum*, which produced type A trichothecenes. The second clade consisted of strains of *F. crookwellense*, *F. culmorum*, and *F. graminearum*, which produced type B trichothecenes.

The sequences of the six type A trichothecene-producing species differed from each other by no more than 2.5% (1/239 to 6/239 [number of differences/total number of aligned sites]). *F. tumidum* NRRL-13394 and *F. sambucinum* 64995 had the same sequence in this region. Interspecific nucleotide differences among trichothecene type B producers also were very low. *Fusarium crookwellense* and *F. culmorum* had the same sequence, and these species differed from *F. graminearum* group I (R-6710) by 2 bases (0.8%). Not surprisingly, the trichothecene-nonproducing strains are more diverse than is either group of trichothecene-producing strains (2.0 to 10.4%). The two *F. camptoceras* strains (ITEM-1138 and ITEM-1128) had quite different (9/239 bp, 3.7%) sequences, a result that is consistent with their abilities to produce pigments and mycotoxins. *F. camptoceras* ITEM-1138 (T-2 and NEOS producer)
was genetically related to other type A trichothecene-producing strains (sequence divergence of 0.8 and 2.5% from \textit{F. venenatum} 64935 and \textit{F. compactum} R-6784, respectively). On the other hand, \textit{F. camptoceras} ITEM-1128 (trichothecene non-producer) was genetically related to trichothecene nonproducing strains, particularly \textit{F. semitectum} NRRL-13327.

**DISCUSSION**

rRNA coding sequences have been used to establish phylogenetic relationships. The advantages of using these sequences for phylogenetic studies are that this molecule is universally present in all living cells and that its sequence is not correlated with a particular morphology. It is known, however, that not all portions of the molecule are equally suitable for detecting molecular changes, as different portions of the molecule are subject to different selection pressures. The structural features shared by 28S-like molecules in all species provides a versatile and sensitive phylogenetic indicator, since highly conserved sequences are interspersed with much more rapidly evolving domains (7, 10).

In this study, we found that the 5’ region we sequenced is relatively well conserved in all 15 \textit{Fusarium} species examined. The nucleotide changes that have occurred can be used to divide the trichothecene-producing \textit{Fusarium} strains into two distinct clusters.

In a dendrogram (Fig. 1) based on our data, \textit{F. solani} and \textit{F. decemcellulare} are unequivocally separated (bootstrap interval, 95) from the other species analyzed, in agreement with their traditional morphological characters suggest that a major revision of the systematics of this genus is needed if the systematics are to be consistent with the phylogeny. In addition, the DNA sequence data from this region could be used to design a PCR primer pair which specifically amplifies DNA from trichothecene-producing \textit{Fusarium} strains.

To our knowledge, this is the first report of a correlation between the phylogenetic placement of a large number of toxigenic \textit{Fusarium} species belonging to different sections and their trichothecene production. Correlations between genetic data and secondary metabolite profiles are known to occur in other toxigenic genera. For example, studies examining the taxonomic position of some toxigenic \textit{Penicillium} species inferred from the phylogenetic analysis of rDNA sequences, including the region used in this study, found that rDNA sequences and mycotoxin profiles were correlated (11, 17, 24). In conclusion, we found that the variable region of the 5’ end of the 28S large nuclear rDNA was useful for taxonomic purposes, correlated with mycotoxin production potential, and could provide the basis for identification of uncertain and atypical toxigenic \textit{Fusarium} strains.

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**REFERENCES**