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Identification by PCR of *Fusarium culmorum* Strains Producing Large and Small Amounts of Deoxynivalenol

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Thirty deoxynivalenol-producing *F. culmorum* strains, isolated from wheat grains, were incubated in vitro and analyzed for trichothecene production. Seventeen strains produced more than 1 ppm of deoxynivalenol and acetyldeoxynivalenol and were considered high-deoxynivalenol-producing strains, whereas 13 *F. culmorum* strains produced less than 0.07 ppm of trichothecenes and were considered low-deoxynivalenol-producing strains. For all strains, a 550-base portion of the trichodiene synthase gene (*tri5*) was amplified and sequenced. According to the *tri5* data, the *F. culmorum* strains tested clustered into two groups that correlated with in vitro deoxynivalenol production. For three high-producing and three low-producing *F. culmorum* strains, the *tri5-tri6* intergenic region was then sequenced, which confirmed the two separate clusters within the *F. culmorum* strains. According to the *tri5-tri6* sequence data, specific PCR primers were designed to allow differentiation of high-producing from low-producing *F. culmorum* strains.

Trichothecenes, including deoxynivalenol, acetyldeoxynivalenol, nivalenol, and fusarenone X, are sesquiterpene toxins produced by *Fusarium* species, including *Fusarium culmorum*, which are common fungal contaminants of cereals. Trichothecenes can be found naturally worldwide on cereals (1, 9, 18, 27, 38, 45, 48, 54, 55, 59), and the consumption of these toxins is a potential problem for humans and farm animals (14, 47).

It has been established that some *Fusarium* species, including *F. graminearum* and *F culmorum*, are able to produce B trichothecenes, such as deoxynivalenol and acetyldeoxynivalenol, while other species are not (31). These two types of *Fusarium* strains (producers and nonproducers) can be distinguished on the basis of DNA polymorphism in the β–tubulin gene (43) as well as in the large ribosomal subunit or the internal transcribed spacer (21, 37, 43).

According to their trichothecene production, some *Fusarium* species, such as *F graminearum*, have been divided into two chemotypes: (i) the nivalenol chemotype, which includes isolates producing nivalenol and fusarenone X, and (ii) the deoxynivalenol chemotype, which includes isolates producing deoxynivalenol and acetyldeoxynivalenol (26, 53). Similar observations have been made for *F. culmorum* strains (28, 39).

In addition, it has been demonstrated that, within the same species and in the same culture conditions, toxin production by *Fusarium* strain may vary sharply; some strains produce large amounts of trichothecenes, whereas others produce small or undetectable amounts of trichothecenes (3, 5, 19, 28, 30, 35, 36, 39, 53, 57). Until now, no method except in vitro culture has been available to distinguish high-producing from low-producing *Fusarium* strains.

Several genes involved in the biosynthesis of trichothecenes have been described, most of them localized in a gene cluster. The *tri5* gene encodes the trichodiene synthase, which catalyzes the first step in the biosynthesis of trichothecenes. The nucleotide sequence of the *tri5* gene has been characterized in several *Fusarium* species (16, 22, 23). The *tri6* gene encodes a protein that regulates the trichothecene biosynthesis genes (46) and has been sequenced in *F. sporotrichioides* (32, 46), *Giberella zeae* (*F. graminearum*) (6, 29, 32), and *F. cerealis* (32). For several *Fusarium* species, it has been shown that the *tri5* (22, 23) and *tri6* (32) genes were present in single copy.

In the present work, we focused on *Fusarium culmorum* strains producing large and small amounts of deoxynivalenol (high-producing and low-producing strains, respectively). The objectives of this study were (i) to study the genetic relationship between strains through analysis of the *tri5* gene and the *tri5-tri6* intergenic region and (ii) to design a PCR test to differentiate these two phenotypes.

MATERIALS AND METHODS

Isolates of *Fusarium* strains. Thirty deoxynivalenol-producing *F. culmorum* strains isolated from cereals from different areas in France were used in this study, as presented in Table 1. *Fusarium* strains may also be obtained from the first author. Isolates were identified as *F. culmorum* by conidial morphology as described by Nelson et al. (40). *Fusarium* identification was confirmed by species-specific PCR-based DNA analysis with the OPT18R and OPT18F primers by the method of Schilling et al. (49). These primers were designed by sequence-characterizing randomly amplified polymorphic DNA fragments. Single-spore strains were maintained on potato dextrose agar slants at 4°C.

Toxin production. Toxin production by the *Fusarium* strains was conducted on autoclaved wheat grains. Wheat grains (Soissons) were moistened with sterile distilled water for 4 days at 4°C until thermodynamic water activity was maximal. Then 100 g of grain was distributed into a 500-ml Erlenmeyer flask and sterilized twice for 25 min at 110°C. Each flask was inoculated with a suspension of 2×10^5 conidia. Incubations were conducted in duplicate for each strain. Flasks were incubated at 25°C for 25 days with daily manual shaking for the first 5 days of inoculation in order to homogenize the inoculum.

Trichothecene analysis. Wheat grains (25 g) were analyzed by gas chromatography-electron capture detection and gas chromatography-mass spectrometry as previously described (4). Standard solutions of 1 μg of deoxynivalenol, nivalenol, fusarenone X, 3-acetyldeoxynivalenol, or 15A-deoxynivalenol (provided by

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TABLE 1. Trichothecene production and biomass formation by *F. culmorum* strains isolated from wheat

Strain	Origin	Host	Trichothecene production[a] (ppm)			Ergosterol production (ppm)
			DON	3-ADON	15-ADON	
A2	Calvados	Wheat	1.20	ND	ND	898
A4	Calvados	Wheat	1.40	ND	ND	990
B3	Loire Atlantique	Wheat	24.10	1, 68	ND	1,237
B5	Loire Atlantique	Wheat	1.41	ND	ND	1,457
B11	Loire Atlantique	Wheat	ND	ND	ND	1,562
C1	Marne	Wheat	ND	ND	ND	1,005
C6	Marne	Wheat	3.80	ND	ND	916
C8	Marne	Wheat	7.20	0.96	ND	1,124
C11	Marne	Wheat	ND	ND	ND	1,237
E1	Seine Maritime	Wheat	6.20	0.64	ND	1,210
E2	Seine Maritime	Wheat	10.10	0.12	ND	1,082
F11	Somme	Wheat	ND	ND	ND	1,151
G1	Gard	Wheat	25.60	2.40	ND	1,654
H1	Gers	Wheat	24.30	1.90	ND	1,594
I1	Vienne	Wheat	47.50	5.60	0.30	962
J11	Loir et Cher	Wheat	ND	ND	ND	1,202
K2	Loir et Cher	Wheat	ND	ND	ND	1,056
K11	Loir et Cher	Wheat	ND	ND	ND	1,094
L2	Loir et Cher	Wheat	21.10	ND	17.80	1,353
L3	Loir et Cher	Wheat	7.29	0.18	ND	1,268
L4	Loir et Cher	Wheat	14.20	ND	ND	1,517
L5	Loir et Cher	Wheat	3.20	0.08	ND	1,166
M11	Charente Maritime	Durum wheat	ND	ND	ND	1,279
N1	Gard	Durum wheat	41.02	4.10	22.34	938
P2	Gers	Durum wheat	ND	ND	ND	1,805
Fcbar	Haute Garonne	Wheat	0.05	ND	ND	942
131O94	Haute Garonne	Wheat	0.06	ND	ND	907
R964	Haute Garonne	Wheat	42.45	38.36	ND	1,021
O594	Haute Garonne	Wheat	0.05	ND	ND	1,285
Fché2	Haute Garonne	Wheat	0.07	ND	ND	1,424

[a] DON, deoxynivalenol; 3-ADON and 15-ADON, 3-acetyl- and 15-acetyldeoxynivalenol, respectively. ND, not detectable.

Sigma, St. Louis, Mo.) were also analyzed. Detection limits were 20 ng/g for nivalenol and 10 ng/g for other trichothecenes. The results were expressed in milligrams of toxin per kilogram of dry matter or as parts per million.

Ergosterol determination. Ergosterol analysis was carried out by high-pressure liquid chromatography as previously described (7). The results were expressed as milligrams of ergosterol per kilogram of dry matter or as parts per million.

DNA preparation. For preparation of DNA, *Fusarium* strains were grown in Roux flasks for 2 days at 25°C in 150 ml of malt medium. For each strain, the mycelium from four flasks was harvested, washed with sterile water, lyophilized, and stored at −20°C. DNA for PCR was extracted as described by Dellaporta et al. (10) with slight modifications. Lyophilized mycelium (≈20 mg) was extracted with 1 ml of extraction buffer (50 mM Tris [pH 8], 50 mM EDTA [pH 8], 2% Sarcosyl, 150 mM NaCl) and 1 ml of phenol for 1 h on ice. After centrifugation (10 min at 13,000 × *g*), the DNA in the upper aqueous phase was precipitated by addition of 1 volume of isopropanol. After centrifugation (15 min, 13,000 × *g*), the DNA pellets were recovered and dissolved in 300 μl of TE (10 mM Tris [pH 8], 1 mM EDTA [pH 8]) and incubated at 37°C for 1 h with 10 μl of RNase (10 mg/ml). The extracts were purified by addition of 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged as described above. The DNA was precipitated by addition of 1 volume of isopropanol and centrifuged. The DNA pellets were dissolved in 300 μl of sterile water.

Primers. The primers used in this study are listed in Table 2. tox5-1 and tox5-2 allowed the amplification of a 650-bp region of the *tri5* gene, including the intron (41). T1 and T2 amplified a 600-bp portion of the β-tubulin gene (42). tri6-54 was designed according to the nucleotide sequence of the *tri6* gene (46), whereas N1-2, N1-2R, 4056, and 3551 were designed according to the aligned *tri6-tri5* sequences of strains C1, K2, L2, and N1. Primers designed in this study were checked with Oligo4 software with the following criteria: melting temperature, limited dimer formation, and self-complementarity.

PCR assays. All PCRs were conducted in 50-μl reaction mixtures containing 25 pmol of each primer (Table 2), 1.25 mM deoxynucleoside triphosphate, 2 U of Sylverstar DNA polymerase (Eurogentec, Seraing, Belgium), and 1 μl (50 to 80 ng) of fungal template. The optimized PCR temperature program (Perkin Elmer Cetus) consisted of 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. Control tubes without DNA template were included in each experiment. After amplification, a 7-μl aliquot was checked by gel electrophoresis. Duplex PCRs were conducted in the conditions described above.

Sequencing of PCR products. The PCR products were purified with a GFX gel band kit (Pharmacia Amersham Biotech) as indicated by the manufacturer.

TABLE 2. PCR primers used in this study

Primer	Nucleotide sequence (5′ to 3′)	Target	Reference
tox5-1	GCTGCTCATCACTTTGCTCAG	*tri5*	41
tox5-2	CTGATCTGGTCACGCTCATC	*tri5*	41
T1	AACATGCGTGAGATTGTAAGT	β-Tubulin	42
T2	TAGTGACCCTTGGCCCAGTTG	β-Tubulin	42
tri6-54	CTTTGATCGTGTTGCGTCTC	*tri5-tri6* intergenic region	This study
N1-2	CTTGTTAAGCTAAGCGTTTT	*tri5-tri6* intergenic region	This study
N1-2R	AACCCCTTTCCTATGTGTTA	*tri5-tri6* intergenic region	This study
4056	ATCCCTCAAAAACTGCCGCT	*tri5-tri6* intergenic region	This study
3551	ACTTTCCCACCGAGTATTTC	*tri5-tri6* intergenic region	This study

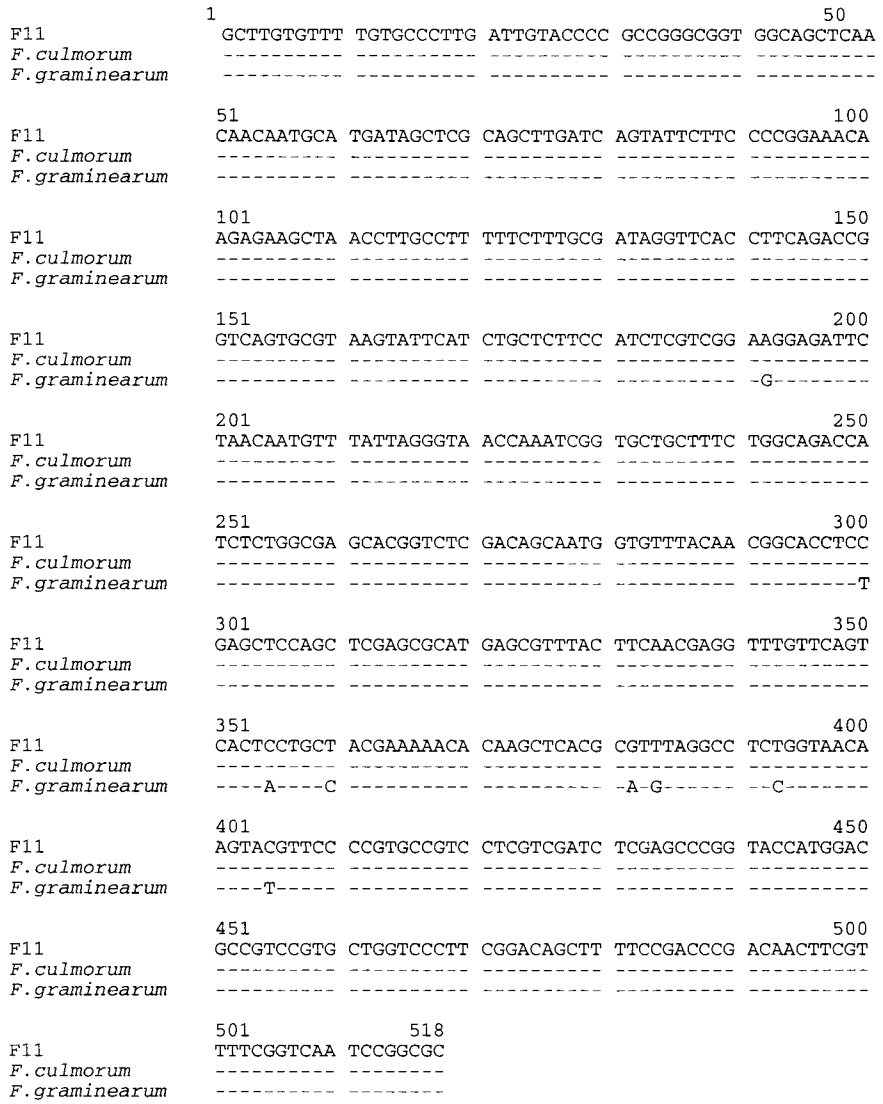

FIG. 1. Sequences of β-tubulin gene region of *F. culmorum* strains and comparison with published data. Matches are indicated by a dash. The nucleotide sequences of the β-tubulin genes of *F. culmorum* strains F11, L2, H1, J1, P2, G1, K2, and M11 were identical. *F. culmorum* strains used for alignment were *F. culmorum* strains NRRL 3288 (GenBank accession number FCU85569) and *F. culmorum* NRRL 25475 (GenBank accession number AF006362). For these strains, the nucleotide sequences of the β-tubulin gene region studied were identical. The *F. graminearum* strains used for alignment were NRRL 29169 (GenBank accession number AF212751), NRRL 28434 (AF212750), NRRL 6394 (AF212748), NRRL 28720 (AF212746), NRRL 13818 (AF212745), NRRL 6101 (AF212744), NRRL 28305 (AF107876), NRRL 28302 (AF107874), NRRL 26895 (AF107860), NRRL 26158 (AF107858), NRRL 26157 (AF107857), NRRL 26156 (AF107856), NRRL 26755 (AF212743), NRRL 26754 (AF212742), NRRL 26752 (AF212741). For these strains, the nucleotide sequences of the β-tubulin gene region studied were identical.

DNA templates were sequenced with unlabeled primers and the *Taq* dideoxy terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions on a 310 DNA sequencer (Applied Biosystems). Both strands were sequenced for each PCR product (tox5-1 and tox5-2 for *tri5* and T1 and T2 for β-tubulin).

Amplification, cloning, and sequencing of *tri6-tri5* region. The *tri6-tri5* region from *F. culmorum* strains C1, K2, N1, L2, G1, and M11 was amplified by with the tri6-54 and tox5-2 primers. The PCR conditions were the same as described above except the temperature program consisted of a denaturation step (4 min, 94°C), 35 cycles of 1 min at 94°C, 1 min at 55°C, and 7 min at 72°C, and an extension step at 72°C for 5 min. The PCR product was checked by agarose gel electrophoresis, purified with a GFX gel band kit (Pharmacia Amersham Biotech), and cloned into pGEM-T with the pGEM-T A 3600 cloning kit (Promega) according to the manufacturer's instructions. Sequencing of the insert in pGEM-T was initiated by using PU and PR primers and extended, as described above, with primers corresponding to the newly sequenced regions.

DNA sequence analysis. *tri5* sequence alignments were performed with the Multalin software (http://www.toulouse.inra.fr/multalin.html) designed by Corpet (8). Unweighted maximum parsimony analyses were performed on this data set with PAUP version 4.0 (52), with heuristic search mode and 1,000 parsimony bootstrap replications.

Nucleotide sequence accession numbers. The sequences of the *tri5-tri6* intergenic regions of *F. culmorum* strains C1, K2, L2, N1, G1, and M11 have been deposited in GenBank under accession numbers AF480834, AF480835, AF480836, AF480837, AY134892, and AY134893, respectively.

RESULTS

***F. culmorum* identification.** The *Fusarium* strains studied were isolated from commercial wheat kernels. Morphological identification of *F. culmorum* strains was confirmed by PCR

FIG. 2. Alignment of the *tri5* gene region of high and low deoxynivalenol producers. –, same sequence; *, deletion; bold letter, *tri5* intron. The nucleotide sequences of the *tri5* genes of *F. culmorum* strains A2, A4, B3, B5, C6, C8, E1, E2, G1, H1, I1, L2, L3, L4, L5, N1, and R964 were identical. The nucleotide sequences of the *tri5* genes of *F. culmorum* strains B11, C1, C11, F11, J11, K2, K11, M11, P2, Fc bar, 131094, 0594, and Fché2 were identical.

analysis with *F. culmorum*-specific Scars primers: all samples had a common band of a 450-bp amplified DNA, as described by Schilling et al. (49). In addition, a portion of the β-tubulin gene of strains K2, F11, L2, J11, H1, P2, G1, and M11 was sequenced (Fig. 1). The β-tubulin region sequences were identical and were also identical to the published β-tubulin gene of *F. culmorum* strains NRRL 3288 and NRRL 25475 (GenBank accession numbers FCU85569 and AF006362, respectively). These data and the morphological examination confirmed that the *Fusarium* strains studied belonged to the same species, *F. culmorum*.

Trichothecene production. Trichothecene production by the 30 *F. culmorum* strains studied is presented in Table 1. Seventeen strains produced more than 1 ppm of deoxynivalenol and were considered high-producing strains, whereas 13 *F. culmorum* strains produced less than 0.07 ppm of deoxynivalenol and were considered low-producing strains. The ergosterol content was used as a measure of the fungal biomass and ranged from 898 to 1,805 mg/kg, which indicates significant growth and is in accordance with previous data (2, 33).

The lack of correlation between toxin production and ergosterol level shows that the variation in the toxigenic potential of *F. culmorum* strains is not explained simply by growth differences.

No detectable differences in morphological characteristics could be observed between the two types of deoxynivalenol-producing strains.

Comparison of *tri5* gene sequences. For all strains, a 650-bp PCR product was obtained with the tox5-1 and tox5-2 primers as described by Niessen and Vogel (41). A 550-base portion of the *tri5* gene, which represent 55% of he *tri5* gene coding region, was sequenced on both strands (Fig. 2). This sequence showed high homology (93 to 99%) to the published *tri5* gene sequences of *G. zeae*, a *Fusarium* species closely related to *F. culmorum* (GenBank accession numbers AF336365, AF336366, AF359361, and GZU22464). As previously described (22, 41), a 60-nucleotide intron region ranging from nucleotide 288 to nucleotide 347 was observed.

According to the *tri5* data, the *F. culmorum* strains clustered into two groups that correlated with in vitro deoxynivalenol production. The sequence variations between the two types of *tri5* gene (35 of 550 bp, 6.36%) were concentrated in the intron region, which carried 40% of the sequence differences. In particular, an 8-nucleotide deletion was observed for the low-producing *F. culmorum* strains. Besides the intron region, differences among strains were mainly restricted to isolated nucleotide substitutions.

TABLE 3. Comparison of *tri5-tri6* intergenic sequences of six *F. culmorum* strains

Strain	Difference (%) vs *F. culmorum* strain:				
	C1	M11	L2	N1	G1
K2	0.42	0.56	13.19	13.44	13.33
C1		0.24	13.15	13.21	13.11
M11			13.59	13.86	13.57
L2				0.22	0.07
N1					0.19

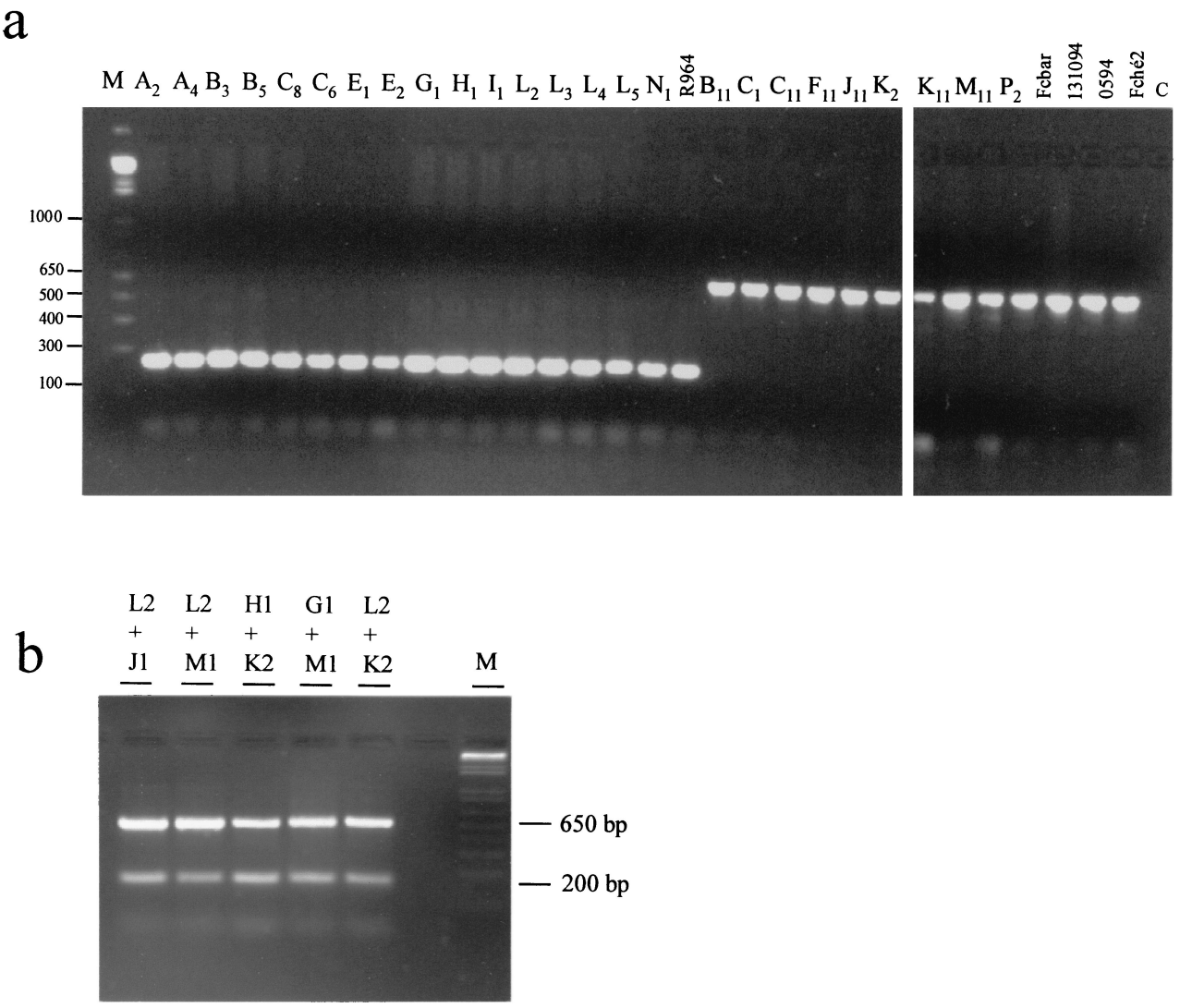

FIG. 3. Duplex PCR amplification patterns of high- and low-deoxynivalenol-producing strains of *F. culmorum* (a) with one *Fusarium* strain per PCR and (b) with two *Fusarium* strains per PCR. Lane M, size markers; lane C, control (without DNA). Amplification with primer pair N1-2 and N1-2R yielded a ≈200-bp fragment; amplification with primer pair 4056 and 3551 yielded a ≈650-bp fragment. Sizes are shown on the left of panel a in base pairs.

Comparison of *tri5-tri6* intergenic region. In order to confirm the clustering of high-producing and low-producing *F. culmorum* strains, the *tri5-tri6* intergenic region was further sequenced for three high-deoxynivalenol-producing strains (G1, L2, and N1) and three low-deoxynivalenol-producing strains (C1, K2, and M11). Sequence analysis clearly confirmed the two separate clusters within the *F. culmorum* strains corresponding exactly to in vitro deoxynivalenol production (Table 3).

Amplification with specific primers. According to the *tri5-tri6* sequences, PCR primers were designed in order to differentiate the high-producing from the low-producing *F. culmorum* strains. Amplification with N1-2 and N1-2R yielded a 200-bp fragment for the high-producing strains, whereas no amplification was observed for the low-producing strains. Conversely, with the 4056 and 3551 primers, amplification yielded a 650-bp fragment for the low-producing strains, whereas no amplification was observed for the high-producing strains (Table 1). A duplex PCR with the N1-2 and N1-2R and the 4056 and 3551 primer pairs was also conducted, which resulted in differentiation of the high-producing from the low-producing *F. culmorum* strains (Fig. 3).

DISCUSSION

Trichothecene biosynthesis may be regulated by environmental conditions such as temperature (20, 56), water activity (12, 20), substrate composition (13, 44), etc. Nevertheless, it is also well established that, within the same species and under optimal conditions of growth, some strains produce large amounts of trichothecenes, whereas other strains produce small or undetectable amounts of trichothecenes (15, 19, 24, 30, 35, 36, 57). Our results are consistent with these observations.

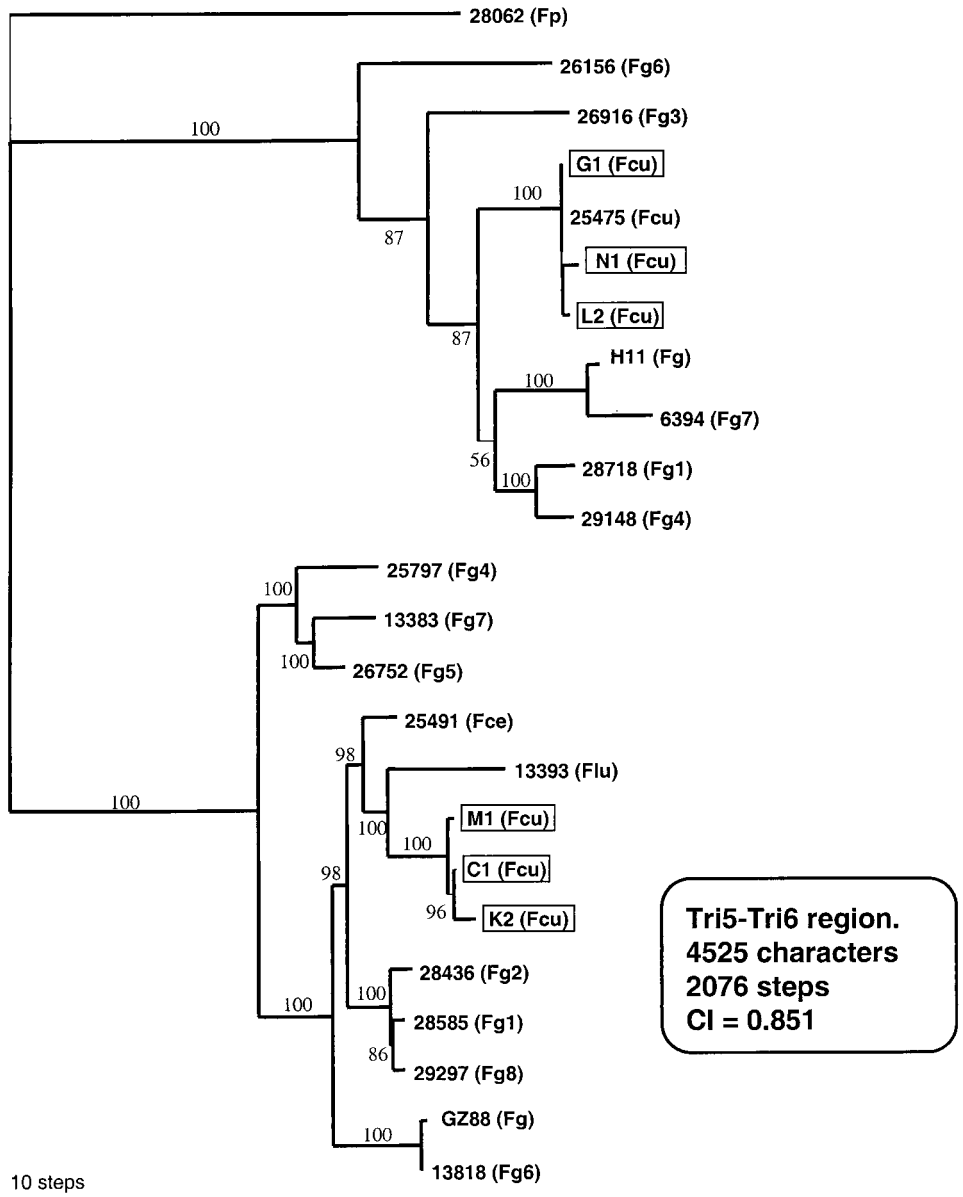

FIG. 4. Comparison of *tri5-tri6* region of the six *F. Culmorum* strains studied with published sequences. One of the nine most parsimonious unrooted trees (differing only in the position of strains in the Fcu clade) inferred from the analysis of the *tri5-tri6* region. Framed blocks indicate the strains sequenced in the present study; *F. graminearum* complex (Fg 1 to 8), *F. lunulosporum* (Flu), *F. cerealis* (Fce), *F. culmorum* (Fcu), and *F. pseudograminearum* (Fp) were compared. Strains 28062, 26156, 26916, 25475, H11,6394, 28718, and 29148 are described as deoxynivalenol-producing strains, whereas strains 25797, 13383, 25491, 13393, 28436, 28585, 29297, and Gz88 are nivalenol-producing strains. Bootstrap values were calculated by using 1,000 replicates. CI, confidence interval.

All the *F. culmorum* strains tested possessed the *tri5* gene, which is in good accordance with the literature, which reports *F. culmorum* as a potential trichothecene-producing species (11, 41). However, the *tri5* sequence data for the 30 *F. culmorum* deoxynivalenol-producing strains revealed low but significant intraspecific variations that strictly correlated with the in vitro deoxynivalenol production. This correlation was confirmed through the *tri5-tri6* intergenic sequence analysis. To our knowledge, this is the first report of such a correlation between in vitro deoxynivalenol production and *tri* gene sequences.

Differences in the sequence of the *tri5* gene region have been reported previously in other species of *Fusarium*. In the case of *G. pulicaris* (*F. sambucinum*), producing diacetoxyscirpenol, a type A trichothecene, Hohn and coworkers (24) observed a 42-nucleotide tandem repeated sequence upstream of the *tri5* gene that was only present in the strains producing large amounts of diacetoxyscirpenol. However, this tandem repeat has not been observed in either the low-producing or high-producing strains.

In addition, Hohn et al. (25) identified a nucleotide motif (TNAGGCCT) in the *tri5* promoter region of *F. sporotrichioides*

that is necessary for the binding of Tri6 protein, which is involved in regulation of the *tri* genes, including the *tri5* gene. The authors indicated that nucleotide changes in the TNAGGCCT sequences dramatically reduced Tri6 binding and consequently the expression of the *tri* genes. For the *F. culmorum* strains studied, three possible *tri6*-binding motifs were located upstream of the *tri5* gene, with identical sequences for both high-producing and low-producing strains. These nucleotide sequences therefore could not account for the variation in deoxynivalenol production.

Recently, the *tri* gene cluster has been sequenced for an *F. sporotrichioides* strain (6), *G. zeae* (*F. graminearum*), and *F. culmorum* strains, including deoxynivalenol-producing strains and nivalenol-producing strains (6, 29, 58). According to the chemotype definition, it can be assumed that the nivalenol-producing *G. zeae* strains produced small or nondetectable amounts of deoxynivalenol. We compared these data with ours through a parsimony analysis of the *tri6-tri5* region of these *F. graminearum* strains and the six *F. culmorum* strains used in our study (Fig. 4). Whatever the *Fusarium* species considered, the tree obtained formed two groups, corresponding to the high-producing and low-producing strains. Further studies are in progress to validate the PCR primers developed in our study with different deoxynivalenol-producing *Fusarium* species, such as *F. graminearum*.

Deoxynivalenol-producing *Fusarium* strains are commonly isolated from cereals (34). It has been demonstrated by using naturally infected barleys that de novo *Fusarium* growth and deoxynivalenol production is a potential problem during malting (17, 50). In addition, several studies indicate that deoxynivalenol is not destroyed during brewing (50, 51). As a consequence, development of a rapid detection method for strains producing large amounts of deoxynivalenol would be of great practical importance.

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