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Mycotoxin production and molecular variability of European and American isolates of *Fusarium culmorum*

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Abstract

The main causative agents of *Fusarium* head blight are *Fusarium graminearum* and *Fusarium culmorum*. We examined the mycotoxin-producing abilities and molecular variability of 37 *Fusarium culmorum* isolates collected from the Pan-Northern Hemisphere, together with isolates representing related species. Mycotoxin-producing abilities of the isolates were tested by thin layer chromatography and by PCR using primer pairs specific for the *Tri7* and *Tri13* genes. Thirty isolates belonged to chemotype I (producing deoxynivalenol and 3-acetyl-deoxynivalenol), while seven represented chemotype II (producing nivalenol and/or fusarenone X). The presence of a functional *Tri7* gene correlated well with nivalenol production. Isolates belonging to chemotype I were in general more pathogenic in *in vitro* tests than those belonging to chemotype II. Phylogenetic analysis of the random amplified polymorphic DNA profiles (RAPD) of the isolates enabled the isolates to be clustered into different groups. Most isolates from Hungary exhibited identical RAPD profiles. A similar clustering was found on the tree based on restriction analysis of the intergenic spacer region data. Sequence analysis of a putative reductase gene fragment of the isolates was also carried out. A correlation was detected between the geographic origin of the isolates and their position on the cladogram produced based on sequence data. The presence of mating type gene homologues was also tested with primer pairs specific for *MAT1-1* and *MAT1-2*. The isolates carried either *MAT1-1* or *MAT1-2* homologues. No correlation was observed between clustering of the isolates based on RAPD, restriction analysis of the intergenic spacer region or sequence data and the distribution of MAT idiomorphs. Similarly, no correlation was detected between mycotoxin-producing abilities or aggressiveness and molecular characteristics of the isolates. Statistical analysis of RAPD data and lack of strict correlation between trees based on different data sets supported the view that *Fusarium culmorum* has a recombining population structure. The presence of mating type gene homologues in the isolates indicates that the recombining population structure is caused by ongoing or past meiotic exchanges.

Introduction

The main causative agents of *Fusarium* head blight (FHB) are members of section *Discolor* of the *Fusarium* genus. Aggressiveness tests has shown that isolates of *Fusarium graminearum*, *F. culmorum* and *F. avenaceum* are the most aggressive to wheat among Fusaria in Hungary (Mesterházy, 1985). Contamination of wheat by the mycotoxins produced by these and other species is the most serious effect of FHB, since the mycotoxins produced are harmful to both humans and animals (Rotter et al., 1996). *Fusarium graminearum* and *F. culmorum* produce zearalenone, an oestrogenic compound, and a range of trichothecenes including deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), nivalenol (NIV) and 4-acetyl-nivalenol (fusarenone X, FusX). Besides, several lines of evidence suggest that, although DON is possibly not necessary for

initial infection (Bai et al., 2001), trichothecenes are involved in plant pathogenesis (Bai et al., 2001; Eudes et al., 2001; Mesterházy, 2002; Proctor et al., 2002; Schnerr et al., 2002).

Fusarium culmorum is a widespread species with no known sexual cycle, in contrast to the closely related homothallic species *F. graminearum* (*Gibberella zeae*). *Fusarium culmorum* has a worldwide distribution and is more abundant than *F. graminearum* on cereals in northern Europe and in some parts of Germany (Mills, 1989; Birzele et al., 2002). This species has also been suggested as the main causative agent of FHB in several European countries including Denmark, Romania, Bulgaria, The Netherlands and Belgium (Bottalico and Perrone, 2002). Despite its importance in plant pathology and mycotoxicology, there is a lack of appropriate information regarding the genetic diversity of this fungus as most research on causative agents of FHB has been devoted to *F. graminearum* (O'Donnell et al., 2000). The aim was to examine the intraspecific variability of *F. culmorum* using phenotypic approaches, including examination of mycotoxin production and aggressiveness tests, and genotypic methods such as random amplified polymorphic DNA (RAPD) and restriction analysis of the intergenic spacer region (IGS-RFLP) studies, examination of the presence of mating type gene homologues and trichothecene biosynthetic genes by PCR, and sequence analysis of a putative reductase gene.

Materials and methods

Isolates

Forty-four single-spore *Fusarium* isolates (37 *F. culmorum*, 4 *F. graminearum*, 2 *F. cerealis* and 1 *F. pseudograminearum*) from different host and geographic origins were examined (Table 1). The isolates were maintained on potato dextrose agar slants. *Fusarium culmorum*-specific PCR tests were performed using primer pair OPT 18F/R_{470} (Schilling et al., 1996).

Phenotypic methods

The isolates were grown on sterilized rice medium (Szécsi and Bartók, 1995) for 21 days at 25 °C. Mycotoxin-producing abilities of the isolates were determined by high-performance thin layer chromatography (Swanson et al., 1984; Trucksess et al., 1987).

In vitro aggressiveness tests were carried out according to Mesterházy (1985). A double layer of filter paper was placed in Petri-dishes carrying the inoculum, and 25 healthy seeds per dish were sown. The number of germinated seeds was counted on the 2nd day, and the number of healthy seedlings was then scored daily. The wheat cultivars used were Várkony (moderately resistant) and 1933 (Ttj-RC103; susceptible).

Genotypic methods

The *Fusarium* isolates were grown in potato dextrose broth (Sigma), and incubated on a rotary shaker at 200 rpm for 5 days at 28 °C. Total nucleic acids were isolated from the lyophilized mycelia of the strains according to standard protocols (Leach et al., 1986).

RAPD analyses were carried out with Operon (Operon Technologies, Inc., Alameda, CA) and UBC (University of British Columbia, Vancouver, Canada) arbitrary decamer primers according to standard procedures (Rinyu et al., 1995). The primers used were OPC-05 (5′-GATGACCGCC-3′), OPC-06 (5′-GAACGGACTC-3′), OPC-07 (5′-GTCCCGACGA-3′), OPC-08 (5′-TGGACCGGTG-3′), OPD-08 (5′-GTGTGCCCCA-3′), OPD-16 (5′-AGGGCGTAAG-3′), OPE-16 (5′-GGTGACTGTG-3′), OPF-10 (5′-GGAAGCTTGG-3′), OPG-06 (5′-GTGCCTAACC-3′), OPH-07 (5′-CTGCATCGTG-3′), OPH-10 (5′-CCTACGTCAG-3′), OPH-15 (5′-AATGGCGCAG-3′), OPL-11 (5′-ACGATGAGCC-3′), OPP-05 (5′-CCCCGGTAAC-3′), OPR-10 (5′-CCATTCCCCA-3′), OPT-18 (5′-GATGCCAGAC-3′), OPV-01 (5′-TGACGCATGG-3′), OPW-01 (5′-CTCAGTGTCC-3′), OPW-02 (5′-ACCCCGCCAA-3′), OPW-03 (5′-GTCCGGAGTG-3′), OPW-17 (5′-GTCCTGGGTT-3′), OPY-05 (5′-GGCTGCGACA-3′), OPY-07 (5′-AGAGCCGTCA-3′), OPZ-02 (5′-CCTACGGGGA-3′), OPZ-19 (5′-GTGCGAGCAA-3′), UBC-08 (5′-CCTGGCGGTA-3′), UBC-18 (5′-GGGCCGTTTA-3′), UBC-66 (5′-GAGGGCGTGA-3′), UBC-77 (5′-GAGCACCAGG-3′), and UBC-85 (5′-GTGCTCGTGC-3′). All RAPD analyses were repeated at least three times, and only those bands that appeared in all tests were considered.

Table 1. Mycotoxin production and aggressiveness of the isolates

Isolate code	Source	Origin	Trichothecene toxins and oestrogens[1]						Mating type genes	Aggressiveness[1]
			DON	3-ADON	15-ADON	NIV	FusX	ZEA		
Taxon: *F. culmorum*										
Fc 12375[2]	Wheat root	H	+	+	–	–	–	++	MAT1-2	10.44
Fc 12551	Wheat stalk base	H	+	+	–	–	–	+	MAT1-2	60.63
Fc 9	*Avena sativa*	H	+	+	–	–	–	–	MAT1-2	72.00
Fc II	Wheat seed	H	+	+	–	–	–	+	MAT1-2	69.69
Fc III	Wheat seed	H	+	+	–	–	–	–	MAT1-2	74.69
Fc 115	Wheat root	H	+++	+	–	–	–	+	MAT1-2	88.19
Fc 190	Wheat leaf	H	+	+	–	–	–	++	MAT1-2	73.19
Fc 7	Wheat	H	+	+	–	–	–	+	MAT1-2	45.94
Fc 12	Wheat	H	+	+	–	–	–	+	MAT1-2	40.94
Fc 13	Wheat leaf	H	+	+	–	–	–	++	MAT1-2	69.00
Fc 78	Wheat ear	H	+	+	–	–	–	++	MAT1-2	59.19
Fc 207/1	Wheat	H	+	+	–	–	–	++	MAT1-2	68.19
Fc 40	Wheat	H	+	+	–	–	–	+	MAT1-2	85.31
223[3]	Wheat	D	+	+	–	–	–	++	MAT1-2	85.69
NRRL 25745[4]	?	DK	–	n.a.	n.a.	–	–	+	MAT1-2	n.a.
89.4[5]	Wheat	F	–	–	–	++	–	++	MAT1-2	96.38
CBS 251.52[6]	Wheat	NL	–	–	–	+	+	–	MAT1-1	97.56
NRRL 29141[7]	Soil	NL	–	–	–	+	+	+	MAT1-1	89.56
72186[8]	Barley	SF	+++	+	–	–	–	+	MAT1-1	87.69
72305[8]	Wheat	SF	+	–	–	–	–	++	MAT1-2	97.81
NRRL 29368	Wheat	SF	+++	+	–	–	–	++	MAT1-2	83.69
R-5145[9]	Wheat	SF	+	+	–	–	–	++	MAT1-2	57.55
Fc 9.2	*Avena sativa*	CAN	+	+	–	–	–	++	MAT1-2	70.94
CBS 173.31	*Avena sativa*	CAN	+	–	–	–	–	++	MAT1-2	98.00
NRRL 29379	Barley	CAN	+++	+	–	–	–	++	MAT1-2	78.63
R-7042	Barley internode	CAN	+	+	–	–	–	+	MAT1-2	51.63
NRRL 29140	?	USA, MN	–	–	–	+	+	++	MAT1-1	97.75
SUF 995[10]	Wheat root	USA	++	–	–	–	–	++	MAT1-1	93.25
NRRL 29354	Corn	USA, ID	+++	–	–	–	–	+++	MAT1-1	95.69
NRRL 29364	Soil	USA, MI	+++	+	–	–	–	+++	MAT1-2	85.06
R-5216	Rye	USA, ND	–	–	–	+	++	++	MAT1-1	87.63
R-2268	Corn	USA, PA	+++	+	–	–	–	++	MAT1-1	60.75
NRRL 29365	Soil	USA, MI	–	–	–	+	+	++	MAT1-2	94.75
NRRL 29371	Wheat stem	AUS	+++	+	–	–	–	++	MAT1-2	31.88
R-6724	Wheat crown	AUS	+++	+	–	–	–	++	MAT1-1	78.00
NRRL 29388	Soil	MA	+++	+	–	–	–	++	MAT1-1	66.25
NRRL 29393	Corn seed	ISR	+++	+	–	–	–	++	MAT1-1	86.81
NRRL 29138	?	?	–	–	–	+	+	+	MAT1-1	96.38
NRRL 3288[4]	?	?	–	n.a.	n.a.	–	–	–	n.a.	n.a.
Taxon: *F. graminearum*										
Fgr 1	Wheat ear	H	+++	–	+	–	–	+++	both	1.13
Fgr 11	Wheat ear	H	+++	+	–	–	–	++	both	79.31
Fgr 15	Wheat stalk base	H	++	–	+	–	–	++	both	31.00
Fgr 25	Wheat stalk base	H	++	–	+	–	–	++	both	74.19
Taxon: *F. cerealis*										
NRRL 28442	*Eucalyptus nitens* roots	RCH	–	–	–	++	+	++	MAT1-1	94.38
R-4054	Wheat straw	ZA	–	–	–	+	+	++	MAT1-1	76.63

Table 1. (Continued)

Isolate code	Source	Origin	Trichothecene toxins and oestrogens[1]						Mating type genes	Aggressiveness[1]
Yaxon: *F. pseudograminearum* NRRL 28069	Wheat root	MA	–	–	–	–	–	+ + +	MAT1-2	51.25
									LSD 5%	4.76

[1] DON, deoxynivalenol; 3-ADON, 3-acetyl-deoxynivalenol; 15-ADON, 15-acetyl-deoxynivalenol; NIV, nivalenol; FusX, fusarenone X; ZEA zearalenone; n.a., not analysed; –, <0.1 mg kg^{-1}; +, 0.1–0.5 mg kg^{-1}; + +, 0.5–2 mg kg^{-1}; + + +, >2 mg kg^{-1}. % of the plants surviving the treatment (average of eight measurements).
[2] Fc: Collection of Ákos Mesterházy, Szeged, Hungary.
[3] From Dr. C. Kling, Univ. of Hohenheim, Germany.
[4] Sequence from O'Donnell et al. 2000.
[5] From Dr. L. Saur, Le Rheu, France.
[6] CBS: Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.
[7] NRRL: Agricultural Research Service Culture Collection, Peoria, IL.
[8] Agriculture Research Centre, Institute of Plant Pathology, Vantaa, Finland.
[9] FRC, Fusarium Research Centre, Pennsylvania State Univ., University Park, PA.
[10] Shinshu University, Ueda, Nagano-ken, Japan.
[11] H – Hungary, D – Germany, DK – Denmark, F – France, NL – The Netherlands, SF – Finland, CAN – Canada, USA – United States of America, AUS – Australia, MA – Morocco, ISR – Israel, RCH – Chile, ZA – South Africa.

The intergenic spacer region (IGS) of the rRNA gene cluster was amplified (Mishra et al., 2002), and digested with *Hae*III, *Eco*RI, *Hpa*II, *Rsa*I, *Sau*3AI and *Alu*I restriction enzymes (Carter et al., 2002).

A putative reductase gene fragment was amplified and sequenced (O'Donnell et al., 2000). DNA fragments were purified from the excised agarose blocks using Genelute spin columns (Supelco). Direct sequencing of the fragments was performed on ABI 373A DNA sequencer (Applied Biosystems Inc.) using dye dideoxy terminator reaction chemistry. Sequences were determined from both strands. Sequences of part of the putative reductase gene of the examined isolates were deposited into the GenBank database under accession numbers AY315718-AY315754. Two previously published sequences (those of *F. culmorum* isolates NRRL 3288 and NRRL 25745, GenBank accession nos. AF212573 and AF212574) were included in the analysis.

The primer pairs developed by Chandler et al. (2003) were used to analyse the presence of *Tri7* and *Tri13* genes in the isolates. Primer pairs Tri13NIVF/R (5′-CCAAATCCGA-AAACCG-CAG; 5′-TTGAAAGCTCCAATGT-CGTG) and Tri7F/NIV (5′-TGCGTGGCAATATCTTCTTC-TA; 5′-GGTTCAAGTAACGTT-CGACAAT-AG) were used to identify NIV-producing isolates, while primer pairs Tri13F/DONR (5′-CAT-CATGAGACTTGTKCRAGTTTGGG; 5′-GCT-AGATCGATTGTTGCATTGAG) and Minus-Tri7F/R (5′-TGGATGAATGACTTGAGTTGA-CA; 5′-AAAGCCTTCATTCACAGCC) were applied for the identification of DON-producing isolates. In the case of Tri7F/NIV, the PCR conditions used were: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s followed by a final extension step at 72 °C for 5 min. When using other primers, the annealing temperature and extension time were altered. The annealing temperature was 58 °C for the other three primer pairs, while the extension time was 45 s for Tri13NIVF/R and Tri13F/DONR, and 30 s for MinusTri7F/R.

In order to identify the mating type of different *Fusarium* strains, diagnostic PCR primers designed by Kerényi et al. (in press) were used: fusAL-PHAfor (5′-CGCCCTCTKAAYGSCTTCATG), fusALPHArev (5′-GGARTARACYTTAGCAA-TYAGGGC), fusHMGfor (5′-CGACCTCCCAA-YGCYTACAT), and fusHMGrev (5′-TGGGCG-GTACTGGTARTCRGG). The amplification conditions were: 20 ng of fungal DNA, 1 mM of dNTPs, 1.5 mM $MgCl_2$, 0.25 μM of each of the primers, 1 × PCR buffer and 1 unit of Taq polymerase (MBI Fermentas, Lithuania). The PCR

cycle included an initial denaturation at 95°C for 2 min, followed by 30 cycles consisting of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C and a final elongation step at 72 °C for 5 min. Amplification products were separated by electrophoresis in 2.0% (w/v) agarose gel, stained with ethidium bromide and visualized with UV light.

Data analysis

Phylogenetic analyses of RAPD, IGS-RFLP and sequence data were carried out using the PHYLIP software package (Felsenstein, 1995). An isolate of *F. pseudograminearum* was used as an outgroup in these analyses. The binary matrices of RAPD and IGS-RFLP data were converted to distance matrices using PhylTools (Buntjer, 1997). Sequence alignments were performed using CLUSTAL-X (Thompson et al., 1997) and improved manually. Evolutionary distances between the sequences were calculated by Kimura's formula (Kimura, 1980) using the program DNADIST. Phylogenetic trees were prepared by the neighbor-joining method (Saitou and Nei, 1987) using the program NEIGHBOR of the PHYLIP package. Bootstrap values were calculated from 1000 replications of the bootstrap procedure using programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the package (Felsenstein, 1985).

Index of association tests (I_A) and parsimony tree length permutation tests (PTLPTs) were performed using the MULTILOCUS 1.2 software with 1000 randomizations (Agapow and Burt, 2001). For the I_A tests, the observed data were used to simulate recombination by shuffling (resampling without replacement) the alleles at each locus of the observed data. For PTLPTs, the null hypothesis was recombination, and significance was determined by the fraction of tree lengths based on resampled data that are at least as long as those based on the observed data (Burt et al., 1996). The PAUP software package was used for calculating the tree lengths from 1000 randomizations (Swofford, 2000).

Results

The phenotypic and genotypic variability of *F. culmorum* isolates was examined. Altogether thirteen Hungarian and 24 other *F. culmorum* isolates were involved in this study, together with some isolates of *F. graminearum*, *F. cerealis* and *F. pseudograminearum*.

Mycotoxin production

Two chemotypes have been described in *F. culmorum* (Miller et al., 1991; Bakan et al., 2001). Isolates of chemotype I produce DON and/or its acetylated derivatives, while chemotype II isolates produced NIV and/or fusarenone X (Gang et al., 1998). The mycotoxin-producing abilities of the isolates by thin layer chromatography were examined. The mycotoxins tested involved DON, 3-acetyl-DON, 15-acetyl-DON, NIV, zearalenone and fusarenone X (Table 1). Thirty-four isolates produced zearalenone. Thirty isolates belonged to chemotype I, while seven represented chemotype II according to Miller et al. (1991). Twenty-six of the *F. culmorum* isolates produced 3-acetyl-DON, while six of the seven NIV producers also produced FusX (Table 1). All of the Hungarian isolates belonged to chemotype I. All *F. cerealis* isolates produced NIV and FusX, while the *F. pseudograminearum* isolate produced none of the trichothecenes (Table 1). 15-acetyl-DON was produced only by *F. graminearum*. Our aim was to determine the chemotypes of the isolates, not to give precise quantitative amounts of the mycotoxins produced. Accordingly, more sophisticated methods like HPLC or GC/MS tests were not used.

The presence of functional *Tri7* and *Tri13* genes in the isolates was examined. A strict correlation was observed between NIV-producing abilities and the presence of *Tri7* and *Tri13* gene fragments of expected size in the isolates (data not shown).

Aggressiveness tests

The aggressiveness of chemotype I isolates was in general higher than that of isolates belonging to chemotype II. On average 94.3% of plants survived treatment with a NIV-producing isolate, whilst 70.9% of the plants survived treatment with DON-producing isolates (Table 1).

RAPD analysis

Although only a limited degree of variability was detected with most primers, the application of

some, including OPC-06, OPD-16 and OPG-06, resulted in variable RAPD profiles (Figure 1). Using primer OPC-06, a 1.4 kb RAPD fragment was amplified in most Hungarian isolates and in none of the other strains examined (data not shown). For preparing the distance matrix, the presence or absence of 162 RAPD fragments was scored. Statistical analysis was carried out using the neighbor-joining technique (Figure 2). This phylogenetic analysis clustered the *F. culmorum* isolates into 20 haplotypes (Figure 2). *Fusarium cerealis* and *F. graminearum* isolates formed distinct clusters. Most Hungarian *F. culmorum* isolates formed a well-separated clade (Hungarian isolates are set in bold type in Figure 2).

IGS-RFLP analysis

The IGS-specific primer pair amplified a 2.3 kb DNA fragment in each *F. culmorum* isolate. The size of the amplified fragments was identical in all *F. culmorum* isolates examined in contrast to previous observations (Mishra et al., 2002). During IGS-RFLP studies, six *F. culmorum* haplotypes were identified (Figure 3). Among the restriction enzymes used, *Rsa*I, *Hae*III, *Sau*3AI and *Hpa*II resulted in variable profiles for the *F. culmorum* isolates, while *Eco*RI and *Alu*I were used to distinguish *F. culmorum* isolates from those of *F. graminearum*, *F. cerealis* and *F. pseudograminearum* (data not shown). Most Hungarian isolates exhibited identical IGS-RFLP profiles. Another large group of isolates consisted of *F. culmorum* isolates of various origins (including European, Australian and American isolates). Some American and Dutch isolates formed distinct branches on the tree (Figure 3).

Sequence analysis

Two hundred and sixty six nucleotides of a putative reductase gene fragment were involved in the phylogenetic analysis (other parts of the sequenced region aligned ambiguously and were omitted from the analysis). Within this region, 16 parsimony informative sites were found. Hungarian isolates formed a distinct clade together with an Australian and some American isolates. Other European isolates and most American strains belonged to two other clades, respectively. One American and one Danish isolate were not closely related to any of the clades observed (Figure 4).

Mating type gene homologues

All *F. culmorum* isolates carried one or the other MAT gene (Table 1). *Fusarium graminearum* isolates were found to carry both *MAT1-1* and *MAT1-2* homologues. The *F. cerealis* isolates carried *MAT1-1* while *F. pseudograminearum* carried *MAT1-2* (Table 1).

Analysis of reproductive mode of F. culmorum

The reproductive mode of the *F. culmorum* population was assessed by applying index of association tests and tree length tests on RAPD and IGS-RFLP data sets (Table 2). While both tests detected low amounts of homoplasies in the IGS-RFLP data set, they indicated the presence of high levels of ho-

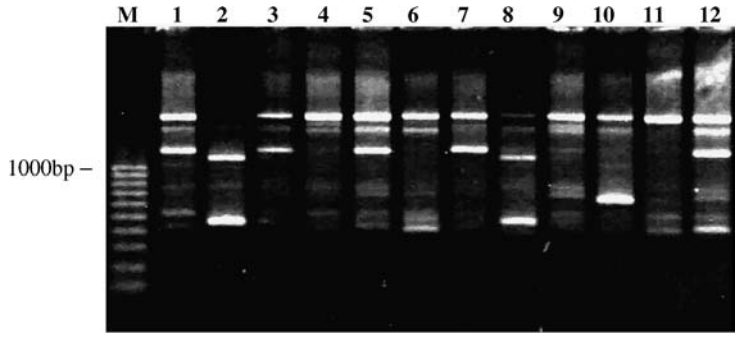

Figure 1. RAPD profiles of *F. culmorum* isolates using OPG-06 as primer. M: DNA size marker (PCR 100 bp Low Ladder, Sigma) Lane1: NRRL 29368. Lane 2: 89.4. Lane 3: 223. Lane 4: NRRL 29138. Lane 5: Fc9.2. Lane 6: NRRL 29140. Lane 7: NRRL 29141. Lane 8: 72186. Lane 9: CBS 173.31. Lane 10: 72305. Lane 11: Fc207/1. Lane 12: SUF995.

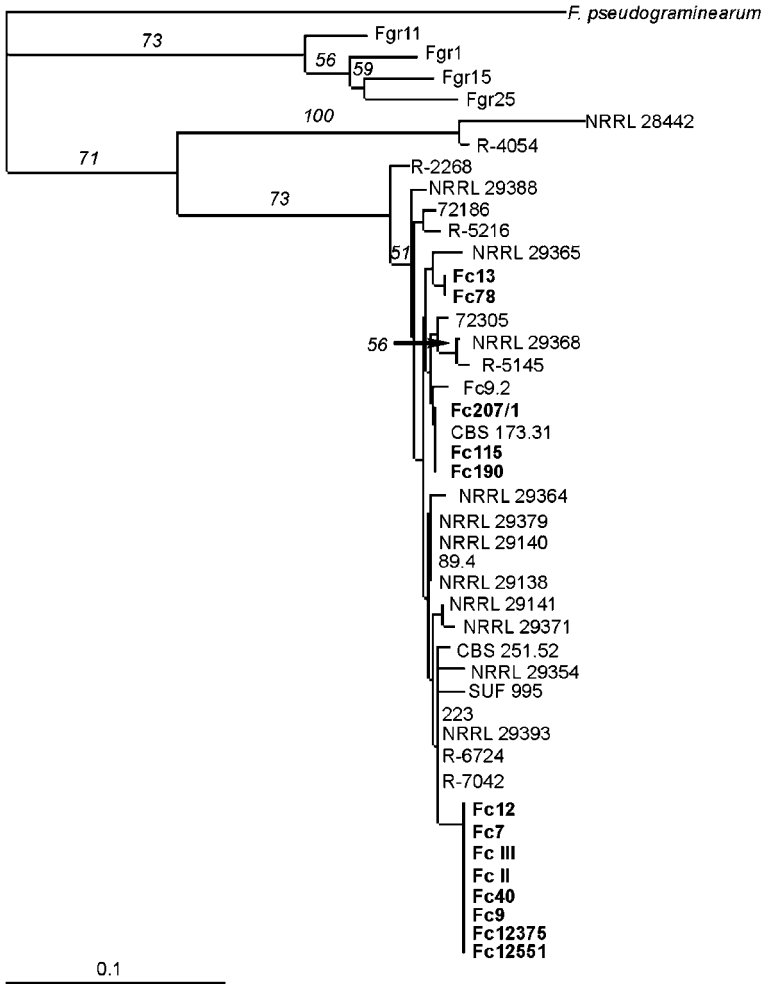

Figure 2. Neighbor-joining tree of *F. culmorum* isolates based on RAPD data. Hungarian isolates are set in bold type in the tree. Bootstrap values > 50% are shown.

moplasies in the RAPD data set which are possibly due to recombination. In the case of RAPD data, the observed index of association values and tree lengths fell within the distribution for randomized data sets. Accordingly, both index of association tests and tree length tests carried out on RAPD data indicated a recombining structure of the examined *F. culmorum* population (Table 2).

Discussion

Phenotypic variability of F. culmorum

The genetic variability of *Fusarium culmorum* isolates was examined using both phenotypic and genotypic methods. Of the 37 *F. culmorum* isolates, 30 belonged to chemotype I and 7 to chemotype II. The ratio of chemotype I and II isolates was close to that observed in a worldwide population of *F. culmorum* (34/42 and 7/42, respectively) (Gang et al., 1998). Hungarian isolates belonged to chemotype I, agreeing with the results of most surveys involving European or North American *F. culmorum* isolates. For example, Hestbjerg et al. (2002b) and Langseth et al. (2000) found that most *F. culmorum* isolates from Denmark and Norway belonged to chemotype I. Canadian *F. culmorum* isolates were of chemotype I (Abramson et al., 2001). In other European surveys, most of the examined *F. culmorum* isolates belonged to chemotype II (Muthomi et al., 2000; Bakan et al., 2002).

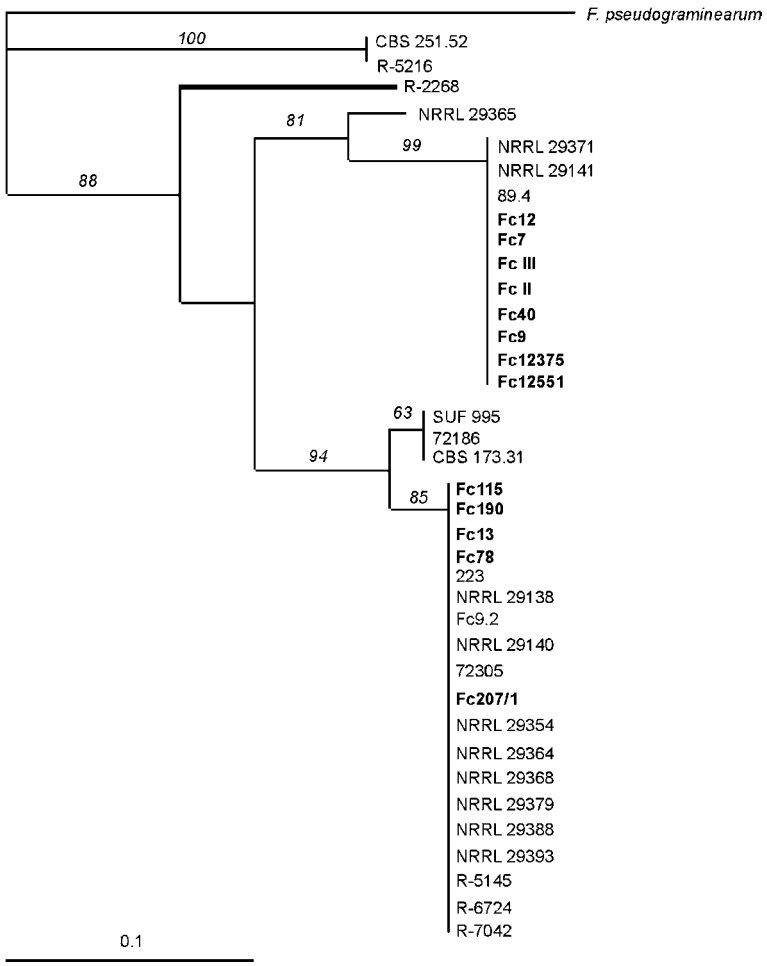

Figure 3. Evolutionary relationships among *F. culmorum* isolates based on IGS-RFLP data. Hungarian isolates are set in bold type in the tree. Bootstrap values $>50\%$ are shown.

Similarly, chemotype II isolates were predominant in isolates collected in New Zealand, Korea and Japan (Lauren et al., 1992; Kim et al., 1993; Lee et al., 2002). In general, both chemotypes were reported in Africa, Asia and Europe, while only the DON chemotype was found in the USA (Miller et al.,1999; Lee et al., 2002). None of the isolates examined in this study produced both NIV and DON in detectable amounts, and none of the *F. culmorum* isolates produced 15-acetyl-DON in agreement with previous findings (Miller et al., 1991; Bakan et al., 2002). DON differs from NIV only in the absence of a hydroxyl group at C-4. However, this difference may have important consequences in the fitness of the producing organism as it alters the bioactivity and toxicity of trichothecenes. For example, the toxicity of NIV is several times higher than that of DON (Ryu et al., 1988; Mirocha et al., 1994; Alexander et al., 2000). The molecular basis of NIV or DON-producing abilities of the isolates lies in the functionality of the *Tri13* gene coding for a cytochrome P-450 enzyme which is functional in NIV-producing isolates, while non-functional in DON-producing strains (Brown et al., 2002; Lee et al., 2002). These chemotypes are maintained by balancing selection acting on the trichothecene gene cluster in *F. graminearum* (Ward et al., 2002).

The presence of functional *Tri7* and *Tri13* genes in *F. culmorum* isolates was studied. Genes *Tri13* and *Tri7* from the trichothecene biosynthetic gene cluster convert DON to NIV (*Tri13*) and NIV to

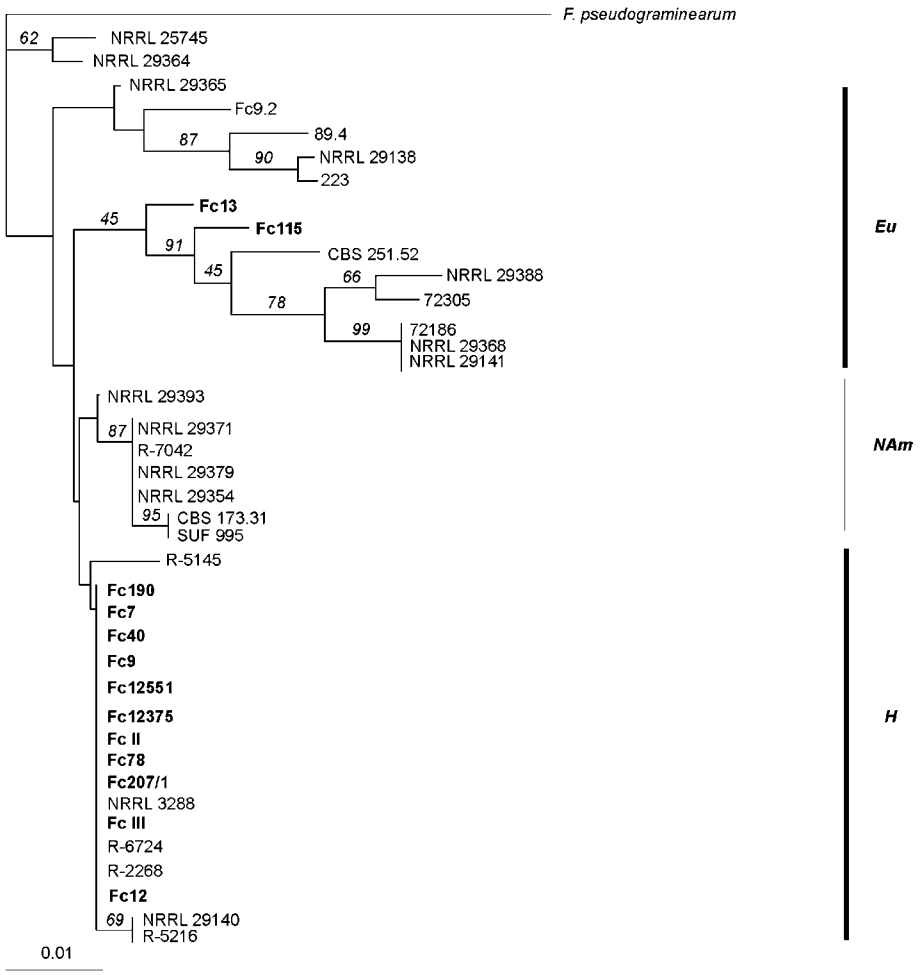

Figure 4. Evolutionary relationships among *F. culmorum* isolates based on sequence data. Hungarian isolates are set in bold type in the tree. Bootstrap values > 50% are shown. Eu: European clade, H: Hungarian clade, NAm: North American clade.

4-Acetyl-NIV (*Tri7*). We used positive–negative PCR assays based on these two genes, which accurately indicate a DON or NIV chemotype in *F. graminearum*, *F. culmorum* and *F. cerealis* (Chandler et al., 2003). The presence of functional *Tri7* and *Tri13* genes was in strict correlation with NIV-producing abilities of the isolates (Table 1). DON-producing *F. culmorum* isolates did not carry the *Tri7* gene, and carried a *Tri13* pseudogene with two deletions, in common with Chinese lineage 6 *F. graminearum* isolates (data not shown).

In these studies, the aggressiveness of chemotype I isolates was in general higher than that of isolates belonging to chemotype II. This observation can be explained by the earlier suggestion that DON is a more effective virulence factor on plants than NIV (Eudes et al., 1997; Gang et al., 1998; Miedaner and Reinbrecht, 2001; Hestbjerg et al., 2002a). The role of NIV in pathogenesis has not yet been examined in detail, although Carter et al. (2002) found that, in a Nepalese *F. graminearum* population, NIV-producing isolates were more pathogenic to maize than DON-producing isolates.

Genotypic variability of F. culmorum

The RAPD technique and IGS-RFLP studies revealed intraspecific variability within *Fusarium* species (Appel and Gordon, 1995; De Nijs et al., 1997; Miller et al., 1999; Edel et al., 2001; Mishra et al., 2002). Using these techniques, the genetic

Table 2. Evaluation of the reproductive mode of *F. culmorum* by index of association tests and tree length tests of RAPD and IGS-RFLP data sets

	RAPD data	IGS-RFLP data
Number of isolates	37	37
Electrophoretic types (ETs)	20	6
Most frequent ET	8	19
Genotypic diversity[1]	0.9984	0.9898
Index of association[2]	0.2317 (NS)	6.8476 ($P < 0.001$)
Index of association (ETs)[3]	0.1959 (NS)	2.6805 ($P < 0.001$)
Length of most parsimonious trees	30 (NS)	29 ($P < 0.001$)
Minimum tree length	17	26
Consistency index	0.5667	0.8966
Average tree length of resampled data	37	81

[1] Genotypic diversity: the probability that two individuals taken at random have different genotypes; $D = n/n{-}1(1{-}\Sigma p_i^2)$, where p_i is the frequency of the ith genotype and n is the number of individuals sampled (Agapow and Burt, 2001).
[2] I_A for the whole data set as calculated by MULTILOCUS.
[3] I_A for unique genotypes as calculated by MULTILOCUS; NS: the differences between index of association values or tree lengths based on resampled data and those based on observed data are not significant.

variability of *F. culmorum* was relatively high compared to previous studies. Mishra et al. (2000) found that *F. culmorum* isolates had very similar ITS sequences. Isoenzyme profiles of all but one of the *F. culmorum* isolates were identical (Láday and Szécsi, 2001). However, Miedaner et al. (2001) detected high genotypic diversity within a Russian *F. culmorum* population by RAPD analysis, and Mishra et al. (2002) divided the 75 *F. culmorum* isolates into 29 unique IGS types using IGS-RFLP. We were able to group the examined *F. culmorum* isolates into 20 haplotypes based on RAPD analysis, and into six haplotypes based on IGS-RFLP data. The genetic diversity observed was close to that detected by Miedaner et al. (2001), who observed 23 haplotypes among the examined 41 Russian *F. culmorum* isolates. Most Hungarian isolates had indistinguishable RAPD and IGS-RFLP profiles and the sequenced region of their putative reductase genes was identical, indicating that these isolates can be identified by molecular methods, thus providing a tool for the identification of the source of contamination in cereal samples. The amount of mycotoxins produced and aggressiveness of the isolates varied greatly indicating that these isolates are really different 'entities' and are not due to resampling of the same strain. Further geographic structuring, as observed by Mishra et al. (2002), was not evident from the IGS-RFLP data. On the contrary, correlation was observed between the geographic origin of the isolates, and their position on the cladogram produced based on sequence data. Three main clades were identified, corresponding to the origin of isolates; one European, one American and one Hungarian clade (Figure 4). Since there is only a limited potential for long-distance dispersal of the splash-dispersed macroconidia of *F. culmorum*, the geographically separated populations evolved separately. The lack of strict correlation between sequence data and geographic origin of the isolates could be due to man-made dispersal of different genotypes as suggested earlier (O'Donnell et al., 2000; Mishra et al., 2002).

Mating type gene homologues were identified in all tested *F. culmorum* isolates. Most heterothallic filamentous ascomycetes have a dimictic mating system with two alleles (called idiomorphs as they do not share any significant sequence similarity) located in a single locus (Turgeon and Yoder, 2000). One idiomorph (*MAT1-2*) contains a single open reading frame (ORF) encoding a regulatory protein with a DNA-binding domain of the high mobility group (HMG) type, while the other (*MAT1-1*) contains an ORF encoding a protein with a motif called alpha box also present in the *MATα1* protein of *Saccharomyces cerevisiae* (Turgeon and Yoder, 2000). Among the tested *F. culmorum* isolates, most European isolates carry *MAT1-2* (with the exception of one Finnish and two Dutch isolates), while five and six American isolates carry *MAT1-1* and *MAT1-2* respectively (Table 1). Correlation between clustering of the isolates based on RAPD,

IGS-RFLP or sequence data and the distribution of MAT idiomorphs was not observed. We also could not detect correlation between mycotoxin-producing abilities or aggressiveness and molecular characteristics of the isolates (data not shown).

Reproductive mode of F. culmorum

Molecular data can serve as raw material for examining the reproductive mode of fungi (Taylor et al., 1999; Varga and Tóth, 2003). There are two fundamental means by which fungi and other organisms transmit genes to the next generation: through clonal reproduction or by mating and recombination. In the case of clonal reproduction, each progeny has only one parent and its genome is an exact mitotic copy of its parent. Recombining populations develop through meiotic recombination following mating, or through a parasexual cycle (mitotic recombination). Two frequently used methods for examining the reproductive mode of fungi are the index of association test and parsimony tree length permutation test (Taylor et al., 1999; Agapow and Burt, 2001). The RAPD and IGS-RFLP data sets of *F. culmorum* isolates were subjected to both the index of association tests and tree length tests (Table 2). Sequence data were not analysed because of the very short region examined in this study. IGS-RFLP data were analysed because this region was shown to be subject to concerted evolution possibly due to suppression of recombination (Dover, 1982; James et al., 2001; Mishra et al., 2002), so the tests applied should indicate a clonal population structure. In accordance with the expectations, both tests carried out on IGS-RFLP data sets indicated a clonal structure of the analysed *F. culmorum* population (Table 2). On the contrary, both tests supported the presence of some recombination in the population based on the RAPD data set (both the observed index of association and tree length fell within the distribution for randomized data sets; Table 2). This observation is also supported by the low bootstrap values and low consistency index of the tree based on RAPD data (Figure 2, Table 2). The absence of a strict correlation between trees based on different data sets also indicates that recombination took place in the examined populations (data not shown) (Tibayrenc, 1998, 1999). The data indicate that while the local Hungarian *F. culmorum* population reproduces clonally as exemplified by its identical RAPD and IGS-RFLP profiles and population genetic analysis (data not shown), the overall *F. culmorum* population has a recombining structure. It was not possible to distinguish between the alternative hypotheses of whether past meiotic exchanges, parasexuality or a cryptic sexual stage were responsible for the recombining population structure of *F. culmorum*. However, the presence of mating type gene homologues in *F. culmorum* (Kerényi and Hornok, 2002; Mishra et al., 2002) indicates that this species lost its sexual cycle relatively recently, in a way similar to that observed in *A. fumigatus* (Varga and Tóth, 2003). In this case, past meiotic processes might be responsible for the observed population structure.

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