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Variability and characterization of mycotoxin-producing *Fusarium* spp isolates by PCR-RFLP analysis of the IGS-rDNA region

A. Llorens[1], M.J. Hinojo[1], R. Mateo[2], A. Medina[1], F.M. Valle-Algarra[1] M.T. González-Jaén[3] and M. Jiménez[1,*]
[1]*Departamento de Microbiología y Ecología, Universidad de Valencia, Dr. Moliner 50, E-46100, Burjassot, Valencia, Spain;* [2]*Departamento de Química Analítica, Universidad de Valencia, Dr. Moliner 50, E-46100, Burjassot, Valencia, Spain;* [3]*Departamento de Genética, Universidad Complutense de Madrid, José Antonio Novais 2, E-28040, Madrid, Spain; *Author for correspondence (e-mail: misericordia.jimenez@uv.es; phone: +34-96-3543144; fax: +34-96-3543202)*

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Abstract

In the present report, a total of 75 *Fusarium* spp isolates (35 of the *Gibberella fujikuroi* species complex, 26 of *F. oxysporum*, 7 of *F. graminearum*, 5 of *F. culmorum*, 1 of *F. cerealis*, and 1 of *F. poae*) from different hosts were characterized morphologically, physiologically and genetically. Morphological characterization was performed according to macroscopic and microscopic aspects. Physiological characterization was based on their ability to produce fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), zearalenone (ZEA) and type B trichothecenes (deoxynivalenol, nivalenol and 3-acetyldeoxynivalenol). FB_1, FB_2, and ZEA were determined by liquid chromatography and trichothecenes by gas chromatography. Molecular characterization of isolates was carried out using an optimized and simple method for isolation of DNA from filamentous fungi and polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) of the intergenic spacer region (IGS) of the rDNA. The results indicated that *G. fujikuroi* complex isolates can be divided into low and high fumonisin producers. The haplotypes obtained with *Hha*I, *Eco*RI, *Alu*I, *Pst*I and *Xho*I enzymes provided very characteristic groupings of *G. fujikuroi* isolates as a function of host type and fumonisin producing capacity. *F. graminearum*, *F. culmorum* and *F. cerealis* isolates were high ZEA and type B trichothecene producers, while *F. oxysporum* and the *G. fujikuroi* complex isolates did not show this ability. The haplotypes obtained with *Cfo*I, *Alu*I, *Hap*II, *Xho*I, *Eco*RI and *Pst*I enzymes permitted to discern these five *Fusarium* species and *G. fujikuroi* complex isolates but the restriction patterns of the IGS region did not show any relationship with the geographic origin of isolates.

Introduction

Complex communities of fungal pathogens are involved in foot rot and head blight diseases of small grain cereals and grasses as well as ear and stalk rot of corn. In temperate regions, *Fusarium* species, including *Fusarium culmorum* W.G. Smith, *F. graminearum* Schwabe, *F. oxysporum* Schlechtend and *Gibberella fujikuroi* species complex (Sawada) Wollenw. are the most abundant and aggressive plant pathogens (Nelson et al. 1981; Edel et al. 1995). These fungi occur on a variety of host plants, such as mango, pine, pineapple, wheat, banana fruit or sugarcane (Jiménez et al. 1993; Mirete et al. 2003; Mateo et al. 2004). Devastating epidemics that occurred in Europe,

USA, China, and South America resulted in yield losses due to reduced yields and negative impact on the quality of grain crops (McMullen et al. 1997; Storer et al. 1998). The high stability of these mycotoxins during storage and processing (Widenstrand and Pettersson 2001) and their occurrence in a wide range of agricultural commodities lead to the fact that harmful mycotoxins are regularly found in animal feed and in human foodstuff (IPCS 1990).

Fusarium species produce significant quantities of toxic secondary metabolites such as many mycotoxins. Infection by *F. graminearum* and *F. culmorum* mainly can lead to grain contamination by mycotoxins such as type B trichothecenes nivalenol (NIV), deoxynivalenol (DON), 3- and 15-acetyldeoxynivalenol (3-AcDON, and 15-AcDON) and also zearalenone (ZEA). ZEA has heavy estrogenic activity (Long and Diekman 1986; Lemke et al. 1998). Type B trichothecenes present various toxicological characteristics; they can inhibit protein synthesis and may be immunosuppressive (Desjardins et al. 1993; Cahill et al. 1999). Animal feeding experiments have demonstrated that trichothecenes are teratogenic but have provided no evidence that they are carcinogenic (Desjardins et al. 1993). Although several toxic effects such as esophagitis and esophageal cancer in the People's Republic of China have been reported in humans following consumption of damaged cereals (Luo et al. 1990), data on carcinogenic properties of ZEA and type B trichothecenes are necessary to evaluate whether these metabolites should be considered as potential human carcinogens.

Fumonisins (FBs) are the main mycotoxins produced by the *G. fujikuroi* species complex. These mycotoxins have toxicological properties in both domestic and farm animals. They are responsible for equine encephalomalacia and porcine pulmonary edema (Gelderblom et al. 1991). There is epidemiological evidence that these toxins may be a contributing factor to the high incidence of esophageal cancer in South Africa, China and Northern Italy (Yoshizawa et al. 1994).

Fusarium is one of the most heterogeneous and difficult to classify fungal genera (Bluhm et al. 2002). Studies based on mycotoxin production profiles are very useful to characterize the isolates (Jiménez et al. 2000; Moss and Thrane 2004). Nevertheless, the methodology for carrying out the morphological and physiological characterization of toxigenic fungi is generally very time-consuming. Therefore, a rapid and reliable assay for the routine identification of toxigenic *Fusarium* spp would benefit the food-processing industry.

Molecular techniques are already having a significant impact on mycological research. These methods may be used in fields such as mycotoxicology (Jiménez et al. 2000), plant pathology (Mirete et al. 2003), or fungal systematics (O'Donnell et al. 1998). The most currently used methods are often based on the analysis of ribosomal DNA (rDNA) sequences. The rDNA sequences are universal and contain both conserved and variable regions, allowing discrimination at different taxonomic levels (Edel et al. 1995). Restriction analysis of PCR-amplified rDNA sequences has been shown to be a suitable method for taxonomic studies in *Fusarium* (Nicholson et al. 1993; Appel and Gordon 1996; Mirete et al. 2003). Variations in rDNA among closely related taxa are found in the intergenic spacer (IGS), which separates the repeated ribosomal units (Fernández et al. 1994). IGS sequences might be good candidates for the differentiation of strains at the intraspecific level (Hillis and Dixon 1991; Edel et al. 1995) presumably due to relative lack of selective constrains, at least in a large part of its sequence.

Isolation of DNA from filamentous fungi is difficult because of fungal nucleases, polysaccharides or pigments and this had led to the development of time-consuming methods and expensive extraction kits.

The aim of the present report was the characterization of *Fusarium* spp isolates involved in mycotoxin production in food products using both a simple, low-cost method for DNA isolation from filamentous fungi, and a rapid molecular technique (PCR-RFLP) of the IGS region.

Materials and methods

Fungal isolates

All isolates were identified morphologically according to Nelson et al. (1983). The *Fusarium* isolates used in this study, their sources and geographic origin are listed in Table 1. A total of 5 isolates of *F. culmorum*, 7 isolates of *F. graminearum*, 1 isolate of *F. cerealis*, 1 isolate of *F. poae*, 26 isolates of *F. oxysporum* and 35 isolates of the *G. fujikuroi* species complex were assayed. They

are held lyophilized at the collection of the Department of Microbiology and Ecology of the University of Valencia.

Mycotoxin analysis

Single-spore cultures were made of each isolate. Cultures were performed on Potato–Dextrose Agar (PDA). Pieces of these cultures were used to inoculate 500-ml Erlenmeyer flasks containing 100 g of corn. Before inoculation, corn was moistened with about 40 ml deionized water to obtain water activity (a_w) 0.98 and kept overnight in a controlled atmosphere having suitable equilibrium relative humidity. Then, the media were sterilized (115 °C, 30 min), inoculated and incubated for 3 weeks at different temperatures depending on the particular mycotoxin to be investigated (20 °C for ZEA, NIV and FBs, 28 °C for DON, and 15 °C for 3-AcDON) (Hinojo et al. 2004; Llorens et al. 2004a, b). After incubation, the cultures were dried at 40 °C in an air stove for 48 h and ground to powder with a mill. Corn used as substrate was previously analyzed and found to contain undetectable levels of DON, NIV, 3-AcDON, ZEA and FBs.

Type B trichothecenes analysis

Finely ground cultures were thoroughly homogenized and extracted for analysis of type B trichothecenes, following a previously optimized method developed in our laboratory (Llorens et al. 2004b). Ground cultures were extracted with acetonitrile–water (84:16, v/v). Mycosep 225 columns (Romer, Union, Mo., USA) were used for clean-up following the provided protocol.

Heptafluorobutyric anhydride was chosen as a derivatization reagent. A HP-6890 Plus gas chromatograph (Hewlett-Packard, Avondale, Pa., USA) equipped with a ^{63}Ni electron capture detector was used to analyze the extracts. All experiments were performed in triplicate. The limits of detection of the method were 15 pg for DON and NIV, and 25 pg for 3-AcDON.

ZEA analysis

Ground cultures were thoroughly homogenized and extracted for analysis of ZEA following an optimized method developed by Llorens et al. (2002). ZEA extraction was performed with methanol-1% aqueous NaCl (80:20, v/v), Florisil cartridges were used to clean up the extracts and liquid chromatography (LC) was used to determine ZEA. LC-ion trap mass spectrometry was used to confirm the presence of ZEA in all cultures. All experiments were done in triplicate. The limit of detection of the method was 4 ng ZEA/g of dry culture.

FB_1 and FB_2 analysis

We used an optimized methodology previously developed by Hinojo et al. (2004, 2006). Ground cultures were extracted with 50 ml acetonitrile–water (1:1). The filtrate was cleaned up on a C_{18} cartridge (Waters, Milford, Ma., USA). Extracts were derivatized immediately or dried and stored at −20 °C until LC analysis.

A 50-µl aliquot of the processed extract was mixed with 200 µl of previously prepared *o*-phthaldialdehyde (OPA) reagent. A 20-µl aliquot of the fumonisin/OPA mixture was used for LC analysis. Fumonisin derivatives were analyzed by LC with fluorescence detection using a reversed-phase column. Due to instability of derivatives, injections were made within 2 min after derivatization. All experiments were carried out in triplicate. The limit of detection of the method for each mycotoxin was 10 µg FBs/g of dry culture.

Molecular analysis

For DNA isolation a rapid and suitable method developed in our laboratory was used (Hinojo et al. 2004). Negative controls consisting of only Milli-Q water (Millipore, Billerica, Ma. USA) were used in every experiment to test for the presence of contaminated DNA. DNA concentrations were determined with a DNA fluorometer (Gene Quant, Pharmacia, Sweden).

The IGS region of the rDNA was amplified using the primers IGS1 (Appel and Gordon 1995) and DGS2 (Mirete 1999) and performed in a programmable thermocycler (Eppendorf, Mastercycle personal). Negative controls (no DNA template) were included for each set of experiments to test for the presence of DNA contamination of reagents and reaction mixtures.

Table 1. Fusarium isolates, host, geographic origin and mycotoxin production.

Isolates[a]	Original host	Geographic origin	ZEA[b] (μg/g)	DON[b] (μg/g)	NIV[b] (μg/g)	3-AcDON[b] (μg/g)	FB_1[c] (μg/g)	FB_2[c] (μg/g)
F. culmorum								
Fcu1	Wheat	Valencia	317.0	ND	6.9	ND	ND	ND
Fcu2	Corn	Valencia	22.5	1.8	ND	7.7	ND	ND
Fcu3	Corn	Córdoba	77.4	TR	46.4	0.8	ND	ND
Fcu4	Leek	Valencia	727.0	1.2	701.0	7.2	ND	ND
Fcu5	Leek	La Rioja	98.9	4.9	79.4	ND	ND	ND
F. graminearum								
Fg1	Wheat	Valencia	818.5	TR	27.1	TR	ND	ND
Fg2	Corn	Valencia	30.1	ND	1.5	ND	ND	ND
Fg4	Corn	Tarragona	62.2	ND	16.7	ND	ND	ND
Fg5	Banana	Canary Islands	57.2	ND	18.1	ND	ND	ND
Fg6	Wheat	La Mancha	229.0	ND	20.1	ND	ND	ND
Fg8	Corn	Tarragona	63.3	1.0	124.0	0.6	ND	ND
Fg10	Wheat	La Mancha	14.8	TR	51.8	TR	ND	ND
F. cerealis								
Fce1	Corn	Valencia	245.0	0.8	117.0	TR	ND	ND
F. poae								
Fpo2	Rice	Valencia	ND	ND	0.8	ND	ND	ND
F. oxysporum								
Fo2	Rice	Valencia	ND	ND	ND	ND	ND	ND
Fo4	Rice	Valencia	ND	ND	ND	ND	ND	ND
Fo5	Rice	Valencia	ND	ND	ND	ND	ND	ND
Fo6	Rice	Valencia	ND	ND	ND	ND	ND	ND
Fo8	Saffron	Motilla	ND	ND	ND	ND	ND	ND
Fo9	Saffron	La Mancha	ND	ND	ND	ND	ND	ND
Fo10	Saffron	La Mancha	ND	ND	ND	ND	ND	ND
Fo11	Gladiolus	Valencia	ND	ND	ND	ND	ND	ND
Fo12	Gladiolus	Unknown	ND	ND	ND	ND	ND	ND
Fo13	(CBS: 620-72)		ND	ND	ND	ND	ND	ND
Fo15	Melon	Valencia	ND	ND	ND	ND	ND	ND
Fo17	Tomato	Valencia	ND	ND	ND	ND	ND	ND
Fo19	Gladiolus	Valencia	ND	ND	ND	ND	ND	ND
Fo20	Gladiolus	Valencia	ND	ND	ND	ND	ND	ND
Fo21	Gladiolus	Valencia	ND	ND	ND	ND	ND	ND
Fo22	Linen	Valencia	ND	ND	ND	ND	ND	ND
Fo23	Tomato	Valencia	ND	ND	ND	ND	ND	ND
Fo24	Unknown		ND	ND	ND	ND	ND	ND
Fo25	Tomato	Valencia	ND	ND	ND	ND	ND	ND
Fo26	Unknown		ND	ND	ND	ND	ND	ND
Fo27	Gladiolus	Valencia	ND	ND	ND	ND	ND	ND
Fo29	Unknown		ND	ND	ND	ND	ND	ND
Fo30	Pine	Madrid	ND	ND	ND	ND	ND	ND
Fo31	Tomato	Madrid	ND	ND	ND	ND	ND	ND
Fo32	Tomato	Madrid	ND	ND	ND	ND	ND	ND
Fo33	Tomato	Madrid	ND	ND	ND	ND	ND	ND
G. fujikuroi								
Gf133	Corn	Tarragona	ND	ND	ND	ND	181.2	49.3
Gf134	Barley	La Mancha	ND	ND	ND	ND	1481	422.0
Gf135	Corn	Valencia	ND	ND	ND	ND	342.0	212.5
Gf136	Rice	Valencia	ND	ND	ND	ND	1222	512.0
Gf5	Banana	Panama	ND	ND	ND	ND	3.6	ND
Gf10	Banana	Ecuador	ND	ND	ND	ND	ND	ND
Gf12	Banana	Panama	ND	ND	ND	ND	ND	ND
Gf14	Banana	Canary Islands	ND	ND	ND	ND	1.8	ND
Gf15	Banana	Ecuador	ND	ND	ND	ND	5.7	ND
Gf16	Banana	Ecuador	ND	ND	ND	ND	ND	ND

Table 1. (Contd.)

Isolates[a]	Original host	Geographic origin	ZEA[b] (μg/g)	DON[b] (μg/g)	NIV[b] (μg/g)	3-AcDON[b] (μg/g)	FB_1[c] (μg/g)	FB_2[c] (μg/g)
Gf18	Banana	Canary Islands	ND	ND	ND	ND	ND	ND
Gf19	Banana	Panama	ND	ND	ND	ND	7.0	ND
Gf2	Corn	Valencia	ND	ND	ND	ND	2679	185.2
Gf7	Corn	Lérida	ND	ND	ND	ND	1340	91.9
Gf20	Corn	Lérida	ND	ND	ND	ND	1516	569.2
Gf23	Corn	Valencia	ND	ND	ND	ND	76.0	32.0
Gf24	Corn	Valencia	ND	ND	ND	ND	1471	115.0
Gf55	Corn	U.S.A	ND	ND	ND	ND	2386	410.7
Gf56	Corn	China	ND	ND	ND	ND	1588	532.0
Gf60	Corn	U.S.A	ND	ND	ND	ND	4775	1055
Gf62	Corn	Argentina	ND	ND	ND	ND	62.0	53.0
Gf63	Corn	South Africa	ND	ND	ND	ND	56.0	27.0
Gf64	Corn	China	ND	ND	ND	ND	889.2	273.1
Gf65	Corn	Russia	ND	ND	ND	ND	141.4	61.5
Gf66	Corn	China	ND	ND	ND	ND	979.0	237.1
Gf26	Pine	Madrid	ND	ND	ND	ND	136.6	19.9
Gf28	Pine	Madrid	ND	ND	ND	ND	1725	199.1
Gf29	Pine	Madrid	ND	ND	ND	ND	212.2	54.4
Gf31	Pine	Madrid	ND	ND	ND	ND	468.3	61.0
Gf32	Pine	Madrid	ND	ND	ND	ND	37.4	2.7
Gf34	Pine	Madrid	ND	ND	ND	ND	1186	142.6
Gf25	Pine	Madrid	ND	ND	ND	ND	188.6	36.0
Gf27	Pine	Madrid	ND	ND	ND	ND	20.4	2.7
Gf30	Pine	Madrid	ND	ND	ND	ND	1793	95.7
Gf35	Pine	Madrid	ND	ND	ND	ND	1732	158.9

[a]Isolate reference in the fungal collection of the Department of Microbiology and Ecology (Valencia University, Spain).
[b]ZEA (zearalenone), DON (deoxynivalenol), NIV (nivalenol) and 3-AcDON (3-acetyldeoxynivalenol) levels are averages of measurements made in triplicate (μg/g dry ground corn culture) in cultures grown at the optimal conditions for production of each mycotoxin (15 °C for 3-AcDON, 20 °C for ZEA and NIV, 28 °C for DON, and a_w 0.980).
[c]FB_1 (fumonisin B_2) and FB_1 (fumonisin B_2) levels are averages of measurements made in triplicate (μg/g dry ground culture) in cultures grown at the optimal conditions for production of these mycotoxins (20 °C and a_w 0.980).
TR: Traces (detected but not quantified).
ND: Not detected.

The estimated size of the amplified IGS fragment was approximately between 2.6 and 3.0 kb based on comparison to size marker (100 bp DNA ladder) on 1.5% agarose gel after 1.5 h.

PCR products for each *Fusarium* spp isolate were digested individually with different restriction enzymes. The enzymes *Hha*I, *Mbo*I (both from Roche), *Alu*I, *Hap*II, *Pst*I, *Xho*I and *Eco*RI (all five from Amersham) were used for 31 *G. fujikuroi* species complex isolates (Figure 2). For analysis of the other *Fusarium* isolates we used three 4-bp recognition sequence restriction enzymes *Cfo*I (Roche), *Alu*I and *Hap*II and other three 6-bp recognition sequence restriction enzymes (*Xho*I, *Eco*RI and *Pst*I) (Figure 2).

After overnight incubation at the optimal temperature for each enzyme (37 °C) in a total volume of 40 μl (Sambrook et al. 1989), digested amplification products were separated by electrophoresis on 2.5% agarose gels. Sizes of restriction fragments were determined by comparison to a 100-bp DNA ladder.

Each profile was compared on the basis of the presence (1) vs absence (0) of RFLP products of the same electrophoretic mobility. A cluster analysis of the Jaccard similarity coefficients was performed with the unweighted pair-group method algorithm (UPGMA) and was conducted using the computer program NTSYS-PC 2.0.

Results

Mycotoxin analysis

Seventy-five *Fusarium* isolates from different hosts and geographic origins were investigated for their

ability to produce ZEA, FB_1, FB_2 and type B trichothecenes by LC and GC-ECD, respectively. The morphological characterization of all the isolates was based on the taxonomic system proposed by Nelson et al. (1983). According to this system, the isolates of the species *F. culmorum*, *F. graminearum* and *F. cerealis* belong to section Discolor, *F. poae* to section Sporotrichiela, *F. oxysporum* to section Elegans and *G. fujikuroi* to section Liseola.

Different levels of ZEA and type B trichothecene were observed in cultures of the different isolates at the most favorable incubation conditions for production of the mycotoxins (Llorens et al. 2004a, 2004b, 2006) (Table 1). According to the results, we can divide the isolates into producers and non-producers of these mycotoxins. Representatives of the Discolor section were the main producers. These results agree with those previously reported (FAO/OPS 1992; Jiménez et al. 1996; McMullen et al. 1997, Llorens et al. 2006). The ZEA levels found in the cultures included in this work ranged from 14.8 to 818.5 μg/g dry culture. Fcu4 and Fg1 isolates from leek and wheat, respectively, were very high ZEA producing strains as they provided levels of 727 and 818.5 μg ZEA/g dry culture, respectively. With regard to type B trichothecene production, in most cases, isolates were NIV producers mostly. Their production levels ranged from 1.5 to 701 μg NIV/g of dry culture. Isolates Fcu4 and Fg8 showed the highest NIV production.

Type B trichothecenes and ZEA were detected in cultures of *F. cerealis* (Fce1, Discolor section) and production levels of our isolate (245 μg ZEA/g dry culture and 117 μg NIV/g dry culture) were very high. Identification of type B trichothecenes was achieved by GC-ECD. Their identity was confirmed by GC-mass spectrometry. Species belonging to the Elegans and Liseola sections produced undetectable levels of these mycotoxins (Table 1).

Differences in FB_1 and FB_2 production were observed among isolates of the *G. fujikuroi* species complex (Hinojo et al. 2004) so that they can be divided into low- and high-producing isolates according to Leslie (1991) and Leslie et al. (1992). On this basis, all isolates from bananas were included in the low-fumonisin producing group (FB_1 levels in cultures ranged from undetectable to 7 μg/g dry culture). In contrast, 100% of isolates from corn and 90% of isolates from pine seeds were included in the high-fumonisin producing group. FB_1 levels ranged from 56 to 4775 μg/g dry culture and 37.4 to 1793 μg/g dry culture in cultures of *G. fujikuroi* isolates from corn and pine seeds, respectively. The highest concentrations of FB_1 and FB_2 were produced by isolate Gf60 (4775 and 1055 μg/g dry culture, respectively).

Molecular analysis

Oligonucleotide primers IGS1 and DGS2 permitted the amplification of a single DNA fragment of about 2.6 kb and 3.0 kb for each of the 75 tested *Fusarium* isolates. The PCR products were subsequently digested with each of the six restriction enzymes Restriction fragment sizes lesser than 200 bp were not taken into consideration because they were not clearly resolved by electrophoresis on 2.5% agarose gel. The obtained RFLP patterns revealed substantial variability. Band patterns or haplotypes and their estimated restriction fragment sizes from rDNA IGS digestions are summarized in Tables 2 and 3.

Each of the six restriction enzymes digested the PCR products into two to five fragments (Table 2), with the exception of *Alu*I, which had no site in the amplified fragments of *F. graminearum*, *F. culmorum* and *F. cerealis* isolates. *Xho*I had only one site in the amplified IGS region of seven tested *Fusarium* isolates (six of *F. oxysporum* isolates and one *G. fujikuroi* species complex). Other authors (Edel et al. 1995, 2001) found no site in the amplified IGS region assayed with this enzyme, in most tested strains. *Eco*RI had no site in the amplified IGS region in *G. fujikuroi* isolates and in three *F. oxysporum* isolates. *Pst*I had no site in the amplified IGS region from isolates of *F. oxysporum* and the *G. fujikuroi* species complex.

Depending on the enzyme and the isolates, two to thirteen different restriction patterns were resolved. *Cfo*I showed the highest variability with 13 haplotypes. Only two and three haplotypes were observed with *Xho*I and *Eco*RI, respectively (Table 2). The remaining enzymes showed intermediate variabilities.

Table 4 shows the different haplotypes identified for the endonucleases used in this work. These haplotypes were not correlated to hosts or geographic origin.

As various *Fusarium* species were subdivided in different haplotypes, cluster analysis was performed

Table 2. Band patterns (A-M) and their estimated restriction fragments sizes (base pairs) obtained from digestion of the IGS rDNA region of 44 isolates of *Fusarium* spp.

Band pattern	Enzyme					
	*Cfo*I	*Alu*I	*Hap*II	*Xho*I	*Eco*RI	*Pst*I
A	1500, 900, 300	1200, 850, 600, 400, 250	850, 700, 500, 250	1500, 1200	2000, 450	1200, 600, 450, 350
B	1500, 900, 450, 300	700, 600, 400, 200	550, 500, 400, 300, 200	2000, 700	1800, 1100, 500	1200, 450, 350
C	1200, 900, 300	900, 650, 400, 300, 200	1000, 600, 350	–	1100, 1000, 450	1200, 450, 350
D	1000, 500, 300	1500, 600, 400	500, 300, 200	–	1500, 1100	1000, 600 (double, 450)
E	900 (double), 600, 300,250	600, 500, 400 (double), 200	450, 300, 200	–	–	–
F	700 (double), 400, 300	750, 600, 400, 200	650, 450, 300, 200	–	–	–
G	900, 400, 300 (double)	1000, 800, 750, 400	550, 400, 300, 200	–	–	–
H	900,700,400, 300 (double)	–	550, 300, 200	–	–	–
I	900, 400 (double)	–	700, 300, 200	–	–	–
J	900, 300 (double)	–	700, 500, 300	–	–	–
K	900 (double), 500, 300	–	850, 700, 250	–	–	–
L	1300, 300 (double)	–	600, 500, 300, 200	–	–	–
M	700, 400, 350, 300	–	600, 300	–	–	–

to estimate the levels of intra- and interspecific variability, using data from the six restriction enzymes.

To estimate the level of divergence or similarity among PCR-amplified rDNA fragments, a matrix of genetic distance was calculated for the rDNA haplotypes defined in Table 4. The dendrogram constructed from the distance matrix with the UPGMA algorithm is shown in Figure 1 and it reveals four genetic groups. Each single group corresponds to each of the four different sections used in this study, Sporotrichiella, Discolor, Elegans and Liseola (*G. fujikuroi* species complex).

The Discolor section was divided into three subgroups or branches that included isolates of *F. graminearum*, *F. culmorum* and *F. cerealis*. One branch was formed only by the *F. culmorum* isolates, and was closely related to the *F. graminearum* and *F. cerealis* isolates. All strains belonging to this section were ZEA and type B trichothecene producers.

The Liseola and Elegans sections are closely related. Gf136, an isolate of the *G. fujikuroi* species complex, is included in the Elegans section, because its haplotypes are very similar to those from *F. oxysporum* isolates for most enzymes used

Table 3. Band patterns (A–D) and their estimated restriction fragments sizes (base pairs) obtained from digestion of the IGS rDNA region of 31 isolates of the *G. fujikuroi* species complex.

Band pattern	Enzyme				
	*Hha*I	*Eco*RI	*Xho*I	*Pst*I	*Alu*I
A	1000, 900, 500, 300	2550, 400	3000	3000	800, 460, 370, 200
B	1000, 700, 400, 300, 270	2000, 500, 400	1500 (double)	2200, 700	700, 650, 460, 370, 250
C	1000, 600, 500, 400, 300	–	–	–	–
D	1000, 900, 300, 270, 200	–	–	–	–

Table 4. Haplotypes obtained by restriction analysis of the IGS rDNA region of 44 *Fusarium* spp isolates.

Isolate	Enzyme					
	*Cfo*I	*Alu*I	*Hap*II	*Xho*I	*Eco*RI	*Pst*I
Fcu 1	J	ND	M	ND	D	C
Fcu 2	J	ND	M	ND	D	C
Fcu 3	J	ND	M	ND	D	C
Fcu 4	J	ND	M	ND	D	C
Fcu 5	J	ND	M	ND	D	C
Fg 1	G	ND	K	ND	D	A
Fg 2	G	ND	K	ND	D	A
Fg 4	I	ND	J	ND	D	A
Fg 5	G	ND	J	ND	D	A
Fg 6	I	ND	J	ND	D	A
Fg 8	L	ND	I	ND	D	B
Fg 10	M	ND	I	ND	D	B
Fce 1	G	ND	I	ND	D	A
Fpo 2	D	B	A	ND	C	D
Fo 2	A	A	G	ND	A	ND
Fo 4	A	A	G	B	ND	ND
Fo 5	A	A	G	B	ND	ND
Fo 6	C	D	E	A	A	ND
Fo 8	A	A	G	ND	A	ND
Fo 9	A	A	H	ND	ND	ND
Fo 10	A	A	G	ND	A	ND
Fo 11	A	A	G	ND	A	ND
Fo 12	A	C	G	ND	A	ND
Fo 13	B	B	F	ND	A	ND
Fo 15	B	A	G	A	A	ND
Fo 17	A	C	H	ND	A	ND
Fo 19	A	A	G	ND	A	ND
Fo 20	A	A	H	ND	A	ND
Fo 21	B	B	F	ND	A	ND
Fo 22	E	B	D	ND	A	ND
Fo 23	A	C	H	ND	A	ND
Fo 24	A	C	H	ND	A	ND
Fo 25	A	B	F	ND	A	ND
Fo 26	A	A	G	A	A	ND
Fo 27	A	A	G	ND	A	ND
Fo 29	F	E	C	ND	B	ND
Fo 30	A	A	H	ND	A	ND
Fo 31	A	A	H	ND	A	ND
Fo 32	A	A	H	ND	A	ND
Fo 33	A	A	H	ND	A	ND
Gf 136	C	D	E	A	A	ND
Gf 135	H	F	L	ND	ND	ND
Gf 133	K	B	L	ND	ND	ND
Gf 134	J	G	L	ND	ND	ND

ND = Not detected.
Patterns are defined in Table 2.

in this study. This result indicates a close relatedness between isolates of the two sections. These sections included isolates that were unable to produce ZEA and type B trichothecenes.

Both *Mbo*I and *Hap*II provided irreproducible results in the *G. fujikuroi* complex isolates analysis and were consequently omitted from this study. *Hha*I showed the highest variability with four haplotypes. Only two haplotypes were observed with each of the other enzymes (Table 3). RFLP patterns for the tested isolates are shown in Table 5.

Four groupings were obtained using *Hha*I (Table 5). Haplotype A included all isolates from banana (*Musa sapientum*). They also showed low or undetectable fumonisin production. Haplotype D included isolates from corn (*Zea mays*). All of them belonged to the high-fumonisin producing group. The ten isolates from pine (*Pinus pinea*) were distributed in haplotypes B and C. With the exception of Gf27, the remaining isolates in haplotypes B and C were typified as high-fumonisin producers.

In agreement with these results, the restriction analysis with *Hha*I provides very characteristic band patterns for the *G. fujikuroi* isolates from banana, corn and pine. The remaining restriction enzymes used individually did not show characteristic patterns with regard to the hosts from which the fungi had been isolated.

A dendrogram was obtained by UPGMA using the data from the RFLP analysis (Figure 2). The similarities observed in *G. fujikuroi* isolates vary from approximately 36 to 100%. At 100% similarity level, five clusters (I, II, III, IV and V) can be observed. Cluster I encompasses all *G. fujikuroi* isolates from banana (low-fumonisin producers). Cluster II includes all isolates from corn (high-fumonisin producers).

Discussion

F. graminearum and *F. culmorum* are, among the *Fusarium* species, the most important producers of ZEA and type B trichothecenes (FAO/OPS 1992; Jiménez et al. 1996; McMullen et al. 1997). From our results it is shown that *F. cerealis* is related to *F. graminearum* and *F. culmorum* not only from the morphological viewpoint but also from the chemotaxonomic viewpoint. It is important to emphasize the high capacity of the two *F. culmorum* isolates from leek for producing ZEA and NIV. Therefore, a study on the occurrence of ZEA and type B trichothecenes in leek might be interesting.

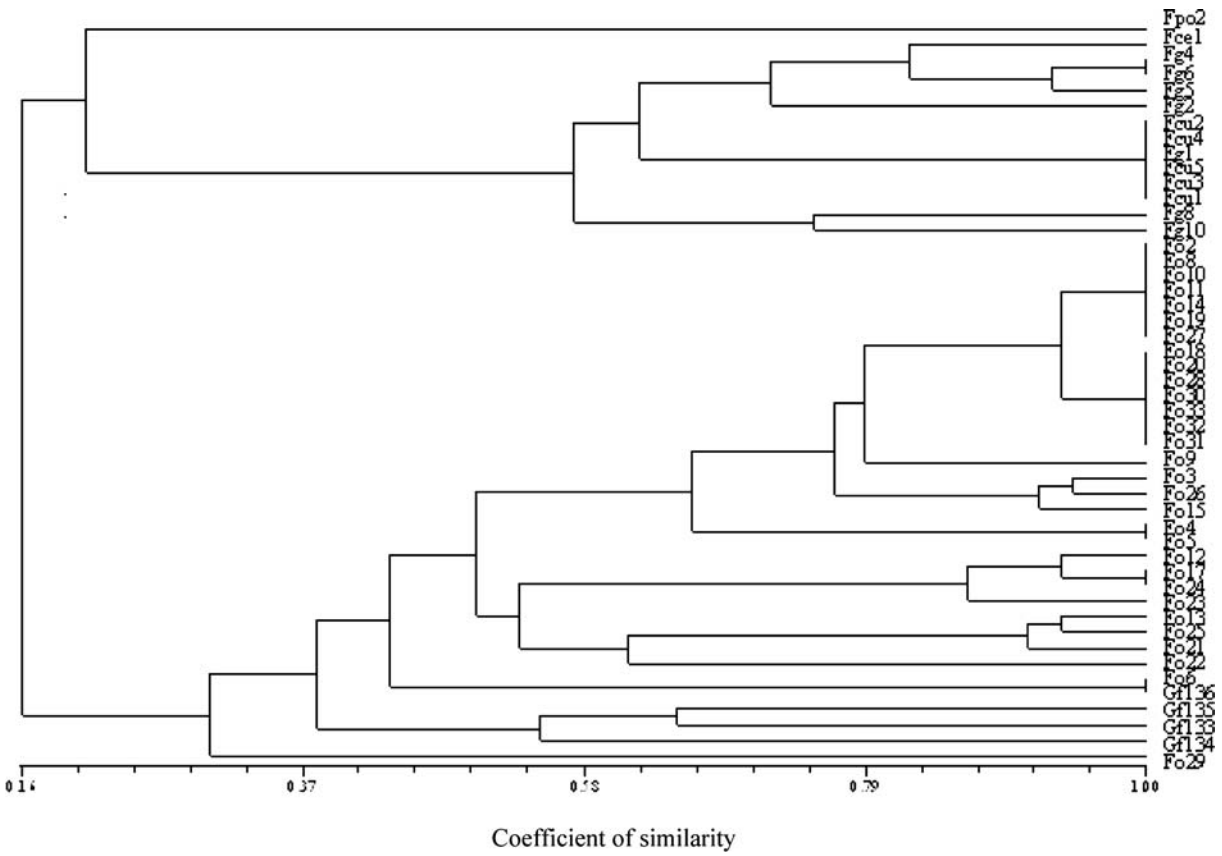

Figure 1. Dendrogram showing relationships among 44 *Fusarium* spp isolates listed in Table 1. Genetic distances were obtained by UPGMA of restriction digest patterns of rDNA IGS products from IGS1 and DGS2 primers digested with six restriction enzymes. Scale value of 1.0 indicates 100% genetic similarity.

Concerning type B trichothecene production, three chemotypes have been described within the B-trichothecene lineage of *Fusarium*. They are the DON/3-AcDON chemotype that can form DON and 3-AcDON, the DON/15-AcDON chemotype that can produce DON and 15-AcDON and the NIV-chemotype that can yield NIV and 4-AcNIV (also known as fusarenon X) (Miller et al. 1991; Perkowski et al. 1997; Chandler et al. 2003; Hammond-Kosack et al. 2004). DON chemotypes are unable to synthesize NIV or 4-AcNIV because they have a nonfunctional *Tri13* gene and *Tri7* gene is deleted or disrupted (Lee et al. 2002). Most of our isolates belonging to *F. graminearum* and *F. culmorum* and the *F. cerealis* isolate studied in this work seem to belong to the NIV chemotype. Nevertheless, one isolate of *F. culmorum* (Fcu2) had ability to synthesize low levels of 3-AcDON and very low levels of DON but no NIV. Thus, it should be included in the DON/3-AcDON chemotype. DON and NIV chemotypes of *F. culmorum* has been reported to occur in some European countries (Jennings et al. 2004 and references cited herein). Regarding the production of both DON and NIV by *F. graminearum*, Sugiura et al. (1990) found that some isolates of *G. zeae* (=*F. graminearum*) belonging to the NIV chemotype produced trace or low levels of DON while NIV was not detected in cultures of DON chemotypes. Other authors have also detected low levels of DON in cultures of a limited number of strains of *F. graminearum* that were high NIV producers (Scézsi and Bartók 1995; Seo et al. 1996; O'Donnell et al. 2000; Nielsen and Thrane 2001; Jurgenson et al. 2002). Our results are compatible with these reports.

F. graminearum, *F. culmorum* and *F. cerealis* are very close, both morphologically and chemotaxo-

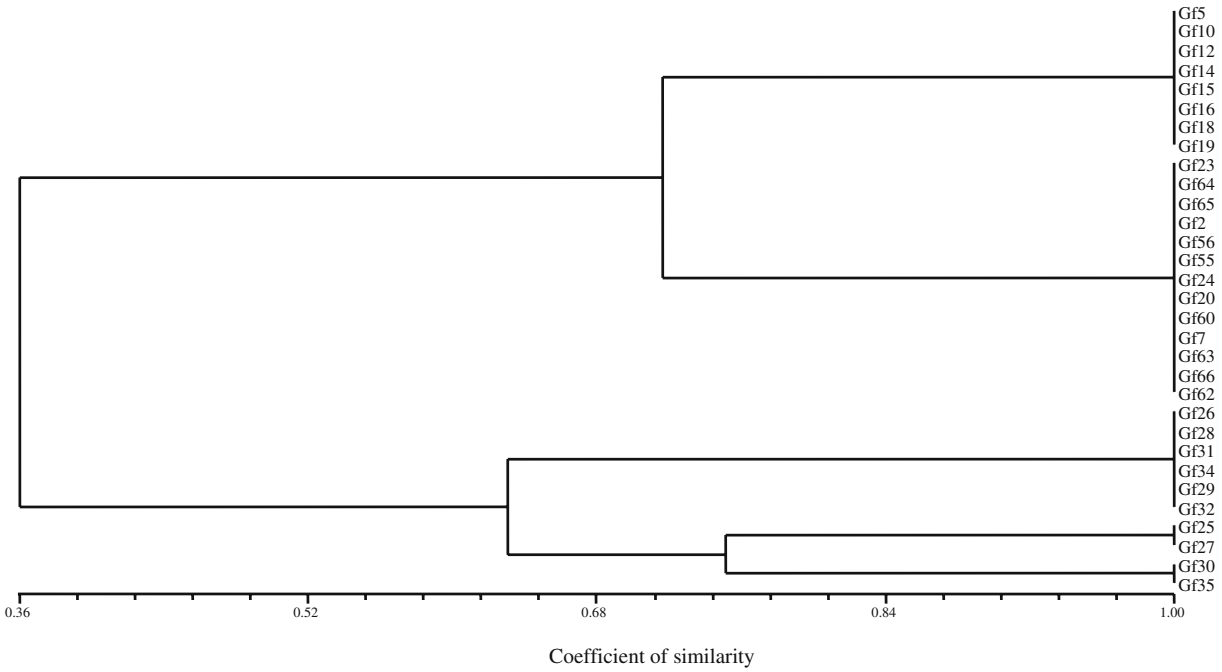

Figure 2. Dendrogram showing relationships among 31 isolates of the *G. fujikuroi* complex listed in Table 1, which were isolated from *Zea mays*, *Musa sapientum* and *Pinus pinea*. Genetic distances were obtained by UPGMA of restriction digest patterns of rDNA IGS products from IGSI and DGS2 primers digested with five restriction enzymes. Scale value of 1.0 indicates 100% genetic similarity.

nomically. These results have been confirmed with molecular analysis data, as the three species are clustered in the same branch. *F. oxysporum* does not produce ZEA and type B Trichothecenes. This species is better known as a major pathogenic fungus of plants and vegetables than as a mycotoxin-producing fungus. This species is a soil pathogen that induces widespread and very destructive vascular wilts, causing severe losses in flax crops (Nelson et al. 1981; Edel et al. 1995; Rafin et al. 2000).

To perform a first molecular characterization, we used restriction fragment analysis of PCR-amplified region of rDNA to characterize 44 isolates belonging to six *Fusarium* species (Table 4). The analysed rDNA region included the IGS sequence. It was amplified and resulted in a PCR product of an appropriate size for RFLP analysis with frequent-cutting restriction enzymes. Enough polymorphism was found to identify species or groups of closely related species. The *Fusarium* spp assayed in this study could be clearly differentiated from their specific rDNA haplotypes. However, we have found levels of intraspecific variability, mainly in *F. oxysporum*. It was the most polymorphic group, with 13 different haplotypes for the six restriction enzymes. However, it has to be taken into account that the number of tested *F. oxysporum* isolates was higher than the number of isolates of the remaining species tested in this study. These results of intraspecific variability within *F. oxysporum* agree with some taxonomic systems that have already recognized several varieties and races within this species (Nelson et al. 1997; Alves-Santos 1999; Vakalounaki and Fragkiadakis 1999; Edel et al. 2001).

The four *G. fujikuroi* complex isolates clustered together (Figure 1), but one of these (Gf136) was more closely related to section Elegans isolates than to the three other isolates of the *G. fujikuroi* complex. Similar results have been previously reported by Donaldson et al. 1995 and Edel et al. 1996. Due to high intraspecific variability in *F. oxysporum*, contradictory results can be found in the literature (Baayen et al. 2000; O'Donnell et al. 1998). However, the Liseola section is known to be related to the Elegans section. Sequence analysis of part of the gene encoding the 28S rRNA from several species of *Fusarium* and related genera has confirmed that Elegans and

Table 5. Haplotypes obtained by restriction analysis of the IGS rDNA region of 31 isolates of the *G. fujikuroi* species complex.

Isolate	Enzyme				
	*Hha*I	*Eco*RI	*Xho*I	*Pst*I	*Alu*I
Gf2	D	B	A	A	B
Gf5	A	A	A	A	B
Gf7	D	B	A	A	B
Gf10	A	A	A	A	B
Gf12	A	A	A	A	B
Gf14	A	A	A	A	B
Gf15	A	A	A	A	B
Gf16	A	A	A	A	B
Gf18	A	A	A	A	B
Gf19	A	A	A	A	B
Gf20	D	B	A	A	B
Gf23	D	B	A	A	B
Gf24	D	B	A	A	B
Gf25	C	B	B	B	A
Gf26	B	A	A	B	A
Gf27	C	B	B	B	A
Gf28	B	A	A	B	A
Gf29	B	A	A	B	A
Gf30	C	A	B	B	A
Gf31	B	A	A	B	A
Gf32	B	A	A	B	A
Gf34	B	A	A	B	A
Gf35	C	A	B	B	A
Gf55	D	B	A	A	B
Gf56	D	B	A	A	B
Gf60	D	B	A	A	B
Gf62	D	B	A	A	B
Gf63	D	B	A	A	B
Gf64	D	B	A	A	B
Gf65	D	B	A	A	B
Gf66	D	B	A	A	B

Patterns are defined in Table 3.

Liseola sections are closely related (Guadet et al. 1989).

F. culmorum isolates were grouped in the same haplotype by the six restriction enzymes, whereas some other *Fusarium* species were subdivided in different haplotypes. The grouping of *F. culmorum*, *F. graminearum* and *F. cerealis* in the same main branch was in agreement with the difficulty to distinguish these species by their morphological characteristics, since they only differ by slight variation in the shape of their macroconidia or microconidia.

F. graminearum and *F. cerealis* isolates were not differentiated regardless of the restriction enzyme used. However, this method has the potential to differentiate closely related species such as *F. graminearum* and *F. culmorum*.

Concerning characterization of *G. fujikuroi* species complex isolates 31 additional isolates (Table 1) from different hosts were used. In general, the results of our study shown the existence of host specificity with regard to *G. fujikuroi* isolates, showing high- and low-fumonisin production capacity. Corn is a preferential host for *G. fujikuroi* isolates with high-fumonisin producing potential and banana is a preferential host for *G. fujikuroi* isolates with low-fumonisin producing potential.

Except for Gf27, all isolates of *G. fujikuroi* species complex from pine can be included in the cluster associated with high-fumonisin production ability, the predominant chemotype in this host. Fumonisin levels are similar to those from corn and other crops (Leslie et al. 1992; Visconti and Dokko 1994).

The results from the present study prove that there are differences at molecular level in *G. fujikuroi* isolates from corn, banana and pine. PCR-RFLP analysis of the IGS region is useful to differentiate these three clusters (Figure 2).

Further investigation such as rDNA sequence analyses would be necessary to compare these similar *Fusarium* species and to clarify their taxonomic position. More strains should also be compared among the closely related species.

The rDNA regions have already been used successfully for the identification of *Aspergillus* (Moody and Tyler 1990), *Entomophaga* (Walsh et al. 1990) and other fungi (Cubeta et al. 1991; Chen 1992). RAPD analysis has been used for many years to distinguish among *G. fujikuroi* species complex isolates (Leslie et al. 1992, 1996; Jiménez et al. 2000), and to design the relationship between *F. culmorum* and *F. graminearum* (Schilling 1996; Nicholson et al. 1998) and other *Fusarium* spp. Intraspecific polymorphism appears within some *Fusarium* species among *Fusarium* spp isolates used in this study. Comparison of more variable DNA regions might allow discrimination of species clustering in the same haplotype. Therefore, the IGS-RFLPs procedure used in this study appears to be a convenient tool for the characterization of *Fusarium* strains at the species level. New strains will be rapidly assigned to a species or a group of closely related species by using this procedure.

As revealed by RFLP analysis, polymorphism in the amplified IGS region was found to be consistent

with the current taxonomy of *Fusarium* species, and sufficient to distinguish taxa at interspecific level. In the case of *F. oxysporum* it can also be used to establish differences at intraspecific level.

In conclusion, IGS-RFLPs markers therefore proved to constitute a rapid and suitable way to group closely related *Fusarium* isolates and to estimate the genetic relationships between the groups, and it is a complement to the morphological and mating studies for delineation of *Fusarium* species.

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