European Journal of Plant Pathology **110:** 503–514, 2004.

Detection and differentiation of trichothecene and enniatin-producing *Fusarium* species on small-grain cereals

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Key words: *Gibberella*, head blight, toxins, wheat

Abstract

A large number of *Fusarium* species are associated with Fusarium head blight of wheat and other small-grain cereals as well as seedling blight and brown foot rot. Different *Fusarium* species tend to predominate under different environmental conditions and in different regions. In addition to causing disease, these fungi are of particular significance because they produce a number of mycotoxins including the trichothecenes and enniatins that contaminate infected grain. The nature and amount of the mycotoxins that accumulate will alter according to the species or even the particular isolates involved in the infection. It is highly desirable to be able to analyse such complex infections to determine which species and, preferably, which chemotypes are present, in order to understand the factors that affect the pathogenicity of each species and to evaluate the potential risk for contamination of grain with mycotoxins. This paper reports the development of molecular methods, based upon the polymerase chain reaction (PCR), for the detection of mycotoxigenic fungi. Several of the *Fusarium* species involved are closely related, making the development of specific assays problematic. We describe the development of primers specific to individual species and discuss how this work provides insight into fungal populations and relates to taxonomic studies. In some instances, it is desirable to detect the presence of potential mycotoxin producers rather than individual fungal species. Generic assays have been produced for several genes involved in trichothecene biosynthesis and for enniatin synthetase in order to permit the detection of species able to produce the associated mycotoxins. Additional work is under way to refine assays to enable detection related to the class of trichothecene and chemotype of isolate because of the potential risk posed to human and animal consumers by different trichothecenes.

Introduction

Small-grain cereals including wheat, barley, rye, oats and triticale suffer from several diseases caused by *Fusarium* species. Fusarium head blight (FHB), (also known as Fusarium ear blight or scab), has been associated with up to 17 causal organisms that induce broadly similar symptoms (Parry et al., 1995). This disease is linked to seedling blight and brown foot rot and forms part of a cycle of Fusarium-related diseases on cereals. Although a large number of *Fusarium* species have been isolated from blighted cereals, relatively few are of overall significance (Parry et al., 1995). *Fusarium graminearum* is the major pathogen worldwide while *Fusarium culmorum*, *F. avenaceum* and *F. poae* are more often associated with FHB in cooler regions, such as northern Europe. While these *Fusarium* species are of greatest overall significance, it should be emphasised that other species may be of significance in particular situations (Sugiura et al., 1993; Miller, 1994; Tekauz et al., 2000). For example, in recent studies, the common species present in the temperate regions of northwest Europe included *F. sporotrichioides* (Chelkowski et al., 1989; Langseth et al., 1999) and *F. equiseti* (Langseth et al., 1999). In addition to the true *Fusarium* species, two varieties of

Microdochium nivale (var. *nivale* and var. *majus*), (formerly classified as *F. nivale* (Mueller, 1977)), also cause FHB and are particularly prevalent where cooler, wetter conditions prevail. The relative contribution of each of the above species to the cause of disease will depend upon a range of variables, possibly the most important of which is the environment, particularly temperature and humidity.

In addition to causing disease which leads to reduced yield and quality of grain, FHB is of particular concern because of the ability of the majority of the causal organisms to produce mycotoxins in the grain that are harmful to human and animal consumers (Joffe, 1986). Among these are the trichothecenes, zearalenone, moniliformin, the enniatins, beauvericin and the fumonisins. Studies using isolates carrying gene disruptions indicate that trichothecenes and enniatins are virulence factors, increasing the ability of isolates to colonise their host plants (Desjardins et al., 1996; Herrmann et al., 1996a; Harris et al., 1999). In contrast, similar studies have not indicated that fumonisins play any discernable role in the infection or colonisation of plant hosts (Proctor et al., 2002). The predominant mycotoxins produced within cereals are the trichothecenes, a group of sesquiterpenoid secondary metabolites. Four basic classes have been designated within the trichothecenes, with types A and B being of most relevance to the contamination of cereals. The difference between these types is based upon the presence (type-B) or absence (type-A) of a keto group at C-8 of the trichothecene skeleton.

The most common trichothecene in blighted grain is deoxynivalenol (DON) a type-B trichothecene produced by *Fusarium graminearum* and *F. culmorum*. This compound often occurs along with acetylated forms such as 3-acetyldeoxynivalenol (3-ADON) or 15-acetyldeoxynivalenol (15-ADON) (Placinta et al., 1999). Another closely related trichothecene, produced by certain isolates of these species, is nivalenol (NIV). This compound is believed to be more toxic than DON or its acetylated derivatives and hence is of importance with respect to food safety (Ryu et al., 1988). Nivalenol and acetylated derivatives are also reported to be produced by *Fusarium cerealis*, *F. poae* and *F. equiseti* (Sugiura et al., 1993; Liu et al., 1998; Langseth et al., 1999). Type-A trichothecenes are significantly more toxic to human and animal consumers than those of type-B (Krska et al., 2001). Type-A trichothecenes commonly found in infected wheat grain are diacetoxyscirpenol (DAS), T-2 and HT-2 (Bottalico and Perrone, 2002). *Fusarium sporotrichioides* and *F. armeniacum* are known to produce T-2 and HT-2 (Chelkowski et al., 1989; Burgess and Summerell, 2000). Recently another T-2 producer, resembling *F. poae*, was isolated from Norwegian wheat grain (Torp and Langseth, 1999). The majority of isolates of *F. poae* and *F. equiseti* are reported to produce DAS (type-A) and/or NIV (type-B) (Liu et al., 1998; Morrison et al., 2002). *Fusarium avenaceum* does not produce trichothecenes but does produce other mycotoxins including moniliformin, beauvericin and enniatins (Golinski et al., 1996; Herrmann et al., 1996b). Neither variety of *Microdochium nivale* is known to produce mycotoxins (Logrieco et al., 1991).

The relative contribution of particular *Fusarium* species in causing disease in a given situation will depend upon a range of variables, as will the amount and type of toxin that accumulates. Only by identifying and understanding the nature of the interactions between the environment and the causal organisms will it be possible to understand the consequences for mycotoxin accumulation in grain and the subsequent threat posed to human and animal consumers. In order to evaluate the role of individual species, it is necessary to be able to differentiate between closely related species. The sensitivity and potential specificity of the polymerase chain reaction (PCR) provides a means to dissect disease complexes such as those involving *Fusarium* species.

In some instances, it is of more interest to determine whether toxigenic species are present than to identify all the species present. The gene *Tri5* that encodes trichodiene synthase is present in all known trichothecene-producing *Fusarium* species. Several PCR assays have been developed for this gene to enable detection of fungi with the potential to produce trichothecenes (Doohan et al., 1999; Schnerr et al., 2001; Edwards et al., 2002). Additional assays have also been developed for other genes involved in trichothecene biosynthesis, such as *Tri6* (Bluhm et al., 2002), which encodes a transcriptional regulator of *Tri5*.

Recent work by Brown et al. (2001, 2002) and Lee et al. (2001, 2002) has shown that the genes

Tri13 and *Tri7* from the trichothecene biosynthetic cluster are responsible for conversion of DON to NIV (*Tri13*) and acetylation of NIV to 4-acetyl nivalenol (*Tri7*). Sequencing of these genes from DON-producing isolates of *F. graminearum* revealed a repeated 11 nucleotide insertion within a putative intron of *Tri7* (Lee et al., 2001), and three deletions within the *Tri13* gene sequence (Brown et al., 2002). Both alterations disrupted gene function resulting in the production of DON.

Although most attention is paid to the trichothecene mycotoxins, a number of other mycotoxins, including enniatins, are of significance in diseases of small-grain cereals. To date, no PCR assays specifically designed to detect producers of enniatins have been reported.

Our laboratory has developed a large number of PCR assays for the detection and identification of individual *Fusarium* species associated with disease of small-grain cereals. Generic assays have also been developed to detect trichothecene and enniatin-producing species. In addition, we have developed a number of PCR assays in order to differentiate between producers of type-A and type-B trichothecenes and to characterise isolates of *F. graminearum*, *F. culmorum* and *F. cerealis* in terms of their NIV and DON chemotype. These distinctions are of significance because of the greater toxicity of type-A trichothecenes relative to type-B and the greater toxicity of NIV relative to DON (Ryu et al., 1988; Krska et al., 2001). This paper brings these assays together, along with those reported by other researchers, to provide a reference for the detection and differentiation of trichothecene and enniatin-producing *Fusarium* species in small-grain cereals.

Materials and methods

Origin and maintenance of fungal isolates

The isolates of *Fusarium* and other fungi used in this study are held in the John Innes Centre 'facultative pathogen' fungal collection. For long-term preservation, cultures are stored in vapour-phase liquid nitrogen vessels. Cultures were maintained at 20 °C on potato dextrose agar (PDA) (Difco) containing streptomycin sulphate (50 mg l^{-1}) and penicillin-G (50 mg l^{-1}).

DNA extraction, random amplified polymorphic DNA (RAPD) and PCR assays

DNA was extracted from fungal and plant material according to the CTAB method (Nicholson et al., 1996) and purified and quantified (Simpson et al., 2000). DNA was re-dissolved in TE buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA) to a concentration of ~10 ng μl^{-1} and stored at −20 °C. RAPD assays were performed using a number of isolates of the target species along with isolates from closely related species. Amplification reactions were carried out in volumes of 50 µl containing 10–20 ng of fungal DNA with conditions similar to those of Nicholson et al. (1998). The oligonucleotide primers used to produce RAPD profiles were obtained from Operon Technologies, USA. Amplification products were separated by electrophoresis through 2% agarose gels. Gels were viewed under UV light on a 'Gel Doc 1000' system (Bio-Rad, UK) and analysed using Molecular Analyst software (Bio-Rad, UK). RAPD amplification products common to all the isolates of the target species and absent from all of the isolates of related species were selected for further analysis. Amplification products were excised from gels, cloned into pGEM-T (Promega) and transformed into electro-competent *E. coli* (strain JS5 or DH10β), according to the supplier's instructions (Bio-Rad).

Development of species-specific PCR

Sequence reactions were performed using the BigDye Terminator version 3.0 (Applied Biosystems) system and sequence data were assembled using the Staden software package and analysed with Wisconsin Package Version 10.1 (GCG) (Genetics Computer Group, Madison, WI). Primers were designed using the Primer Design programme in GCG and NetPrimer (1998 Premier Biosoft International, Paolo Alto, CA), and primers selected with a theoretical melting temperature of 62 °C for amplification using 'Touchdown' PCR (Don et al., 1991) as described below. Species-specific PCR amplifications were carried out in volumes of 50 µl containing 10–20 ng of fungal DNA or DNA from 0.2 mg dry weight of plant material on a Perkin Elmer GeneAmp PCR System 9700. PCR reactions and amplification conditions were as per Nicholson et al. (1998),

using 'Touchdown' cycling conditions. In this process the annealing temperature was 66 °C for the first 5 cycles, and 64 °C for the next 5 cycles. For amplification of DNA from fungal cultures this was followed by 15 cycles at 62 °C while, for infected plant material, 25 cycles were carried out at this temperature. The temperature cycle used consisted of denaturation (95 °C) for 30 s, annealing (as described above) for 20 s and extension (72 °C) for 45 s with maximal ramping rates between temperatures. A final extension step of 5 min was incorporated followed by cooling to 10 °C until recovery of samples. Amplification products were separated by electrophoresis through 2% agarose gels and analysed as above. The specificity of the primer pairs was tested against isolates of the target species and a range of other *Fusarium* species and other fungi associated with ear blight and stem-base diseases of cereals.

Generic PCR assays for trichothecene and enniatin biosynthetic genes

A large amount of sequence data for genes involved in trichothecene biosynthesis is present in publicly available databases. This information has been used to amplify homologous regions of a range of *Fusarium* species in order to identify highly conserved regions in which to design PCR primers. Generic primer sets have been designed for conserved sequences within *Tri5* and *Tri6*. It was noted that the sequence of *Tri4* was less conserved across species than that of the two other genes examined. This observation was exploited in order to develop assays to differentiate between type-A and type-B-producing *Fusarium* species. The DON chemotype of *F. graminearum* has recently been shown to be due to mutation in two genes *Tri13* and *Tri7*, responsible for the conversion of DON to NIV and NIV to acetyl-NIV respectively (Lee et al., 2002). We have carried out extensive characterisation of these genes in *F. culmorum, F. graminearum* and *F. cerealis* and have developed PCR assays to discriminate between NIV and DON chemotypes within these species (Chandler et al., 2003). Enniatins are synthesised by the multifunctional enzyme, enniatin synthetase (Haese et al., 1993). A PCR assay has been developed for the *esyn1* gene that encodes enniatin synthetase to permit detection of *Fusarium* species with the potential to produce enniatins.

Results

Species-specific assays

A list of the PCR primers developed to detect specific *Fusarium* species is shown in Table 1. Several of the primer sets have been published previously but a number are shown here for the first time. Also listed are primer sets published by other research groups for species for which we have not developed primer sets of our own. The primers developed in our studies have been designed with similar melting temperatures. This enables all the target species to be assayed using a single PCR protocol, 62 °C 'touchdown', as detailed above. Where possible, the size of the PCR product has been selected to permit the detection of two or more species in a single reaction. It should be noted that some isolates of *F. graminearum* produce a product of similar size with primer pair Fg16F/R to that amplified from *F. culmorum* with primer pair C51F/R. The use of a combination of Fg16NF/NR and C51F/R primers can be used to overcome this potential problem.

Generic assays for trichothecene-producing species

The gene *Tri6* encodes a zinc finger containing protein that is involved in regulation of trichothecene biosynthesis while *Tri5* encodes trichodiene synthase which catalyses the first step in trichothecene biosynthesis. Primer pairs Tri5F/Tri5R and Tri6FSP/T6ENDR have been designed to detect both type-A and type-B trichothecene-producing *Fusarium* species (Tables 2 and 3). The Tri5F/Tri5R primer set amplified a product of 545 nt from all the isolates of known trichothecene-producing species tested to date, including species not associated with diseases of small-grain cereals and not shown in Table 3. The *Tri6* assay was somewhat less robust. While the Tri6FSP/T6ENDR primer set amplified a product of 550 nt from the majority of isolates tested, no product was amplified from *F. chlamydosporum* (Table 3).

The product of *Tri4* catalyses the second step in trichothecene biosynthesis. Sequence divergence in *Tri4* between producers of type-A and type-B trichothecenes has been exploited to develop an assay to detect producers of type-A trichothecenes (primer pair T4F1502/T4ENDR2). This primer set amplified a product of 550 nt from all type-A

Table 1. Sequences and names of PCR primers for selected Fusarium species along with the size of product generated and the reaction conditions for their use

Primers	Sequence	*Fusarium* species detected	PCR Conditions (anneal/extend)	Product size (bp)
C51F[a]	ATGGTGAACTCGTCGTGGC	*F. culmorum*	62 °C Touchdown[b]	570
C51R	CCCTTCTTACGCCAATCTCG			
Fg16F[a]	CTCCGGATATGTTGCGTCAA	*F. graminearum*	62 °C Touchdown	400–500
Fg16R	GGTAGGTATCCGACATGGCAA			
Fg16NF[a]	ACAGATGACAAGATTCAGGCACA	*F. graminearum*	62 °C Touchdown	280
Fg16NR	TTCTTTGACATCTGTTCAACCCA			
JIAF[c]	GCTAATTCTTAACTTACTAGGGGCC	*F. avenaceum*	58 °C/30 s	220
JIAR	CTGTAATAGGTTATTTACATGGGCG			
FA-U17F	CAAGCATTGTCGCCACTCTC	*F. avenaceum*/*F. tricinctum*	62 °C Touchdown	345
FA-U17-R	GTTTGGCTCTACCGGGACTG			
FP82F[d]	CAAGCAAACAGGCTCTTCACC	*F. poae*	62 °C Touchdown	220
FP82R	TGTTCCACCTCAGTGACAGGTT			
FSpoF1[e]	CGCACAACGCAAACTCATC	*F. sporotrichioides*	62 °C Touchdown	310
LanSpoR1	TACAAGAAGAGCGTGGCGATAT			
FlangF3[e]	CAAAGTTCAGGGCGAAAACT	*F. langsethiae*	62 °C Touchdown	332
LanSpoR1	TACAAGAAGAGCGTGGCGATAT			
198F2[e]	GACAGCAAGATTGACCTTTTGG	*F. equiseti*	62 °C Touchdown	96
198R1	GACATACTCTACAAGTGCCAA			
CRO-AF[f]	CTCAGTGTCCACCGCGTTGCGTAG	*F. cerealis*	60 °C/45 s	842
CRO-AR	CTCAGTGTCCCATCAAATAGTCC			
VEN-BF[f]	GGCGGATAAGGATAGTGGTAGAAG	*F. venenatum*	60 °C/35 s	276
VEN-BR	GGCGGATAAGCAAATAAGATGCTT			
53-6F[g]	TTTACGAGGCGGCGATGGGT	*F. moniliforme*	62 °C/40 s	561
53-6R	GGCCGTTTACCTGGCTTCTT			
61-2F[g]	GGCCACTCAAGCGGCGAAAG	*F. subglutinans*	64 °C/30 s	445
61-2R	GTCAGACCAGAGCAATGGGC			
FPG-F[h]	GTCGCCGTCACTATC	*F. pseudograminearum*	65-56 °C[i]/45 s	779
FPG-R	CACTTTTATCTCTGGTTGCAG			

[a]Nicholson et al. (1998); [b]66 °C (cycle 1–5), 64 °C (cycle 6–10), 62 °C (cycle 11–30); [c]Turner et al. (1998); [d]Parry and Nicholson (1996); [e]Wilson et al. 2004; [f]Yoder and Christianson (1998); [g]Möller et al. (1999); [h]Williams et al. (2002); [i]Annealing temperature reduces by 1 °C per cycle from 65 to 56 °C.

Table 2. Sequences and names of PCR primers to detect trichothecene and enniatin-producing *Fusarium* species, type-A and type-B trichothocene-producing species and nivalenol and deoxynivalenol chemotypes of *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium cerealis* (also shown is the size of product generated and the reaction conditions for their use)

Primer	Sequence	Mycotoxin or chemotype detected	PCR conditions (anneal/extend)	Product size (bp)
T4F1506 T4EndR2	CCCCTGGCTACTCTCGAGA AAGCTTTGAGAACCTTCAC	Type-A trichothecenes	54 °C/30 s	550
Tri4BF Tri4BR	CCCGTCAGCCATGTTGC CATGATCGACAGTGGCGG	Type-B trichothecenes	62 °C/30 s	450
Tri5F Tri5R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCTG	All trichothecenes	60 °C/30 s	545
Tri6Fsp T6EndR	CATGCCAAGGACTTTGTCCC GTGTATCCGCCTATAGTGAT	All trichothecenes	56 °C/30 s	550
Tri7F Tri7NIV	TGCGTGGCAATATCTTCTTCTA GGTTCAAGTAACGTTCGACAATAG	Nivalenol	60 °C/30 s	465
Tri7F Tri7DON	TGCGTGGCAATATCTTCTTCTA GTGCTAATATTGTGCTAATATTGTGC	Deoxynivalenol	60 °C/30 s	>380
MinusTri7F MinusTri7R	TGGATGAATGACTTGAGTTGACA AAAGCCTTCATTCACAGCC	Deoxynivalenol	58 °C/30 s	483
Tri13NIVF Tri13R	CCAAATCCGAAAACCGCAG TTGAAAGCTCCAATGTCGTG	Nivalenol	58 °C/45 s	312
Tri13F TRI13DON	CATCATGAGACTTGTKCRAGTTTGGG GCTAGATCGATTGTTGCATTGAG	Deoxynivalenol	58 °C/45 s	282
Ensyn6065F Ensyn7229R	GCTGGCAGGACCATTTCG GGATGGAAAGTGGTGGAAGAC	Enniatin	58 °C/90 s	1164

Table 3. Amplification from selected Fusarium species in PCR assays designed to detect trichothecene-producing *Fusarium* species based upon the biosynthetic genes *Tri5* (Tri5F/R) and *Tri6* (Tri6Fsp/T6EndR) and type-A (T4F1506/T4EndR2) and type-B (Tri4BF/R) trichothecene-producing species based upon *Tri4*

Fusarium species	Trichothecene production	Tri5F/R	Tri6Fsp/T6EndR	T4F1506/T4EndR2	Tri4BF/R
F. armeniacum	Type-A	+	+	+	–
F. sporotrichioides	Type-A	+	+	+	–
F. venenatum	Type-A	+	+	+	–
F. langsethiae	Type-A	+	n.t.	+	–
F. poae	Type-A	+	n.t.	+	–
F. sambucinum	Type-A	+	n.t.	+	+
F. equiseti	Type-A	+	n.t.	n.t.	n.t.
F. cerealis	Type-B	+	+	–	+
F. chlamydosporum	Type-B	+	–	–	+
F. culmorum	Type-B	+	+	–	+
F. graminearum	Type-B	+	+	–	+
F. heterosporum	Type-B	+	n.t.	–	+
F. lateritium	Type-B	+	n.t.	–	+
F. avenaceum	None	–	–	–	–
F. heterosporum	None	–	–	–	–

+: PCR product of expected size; – no product amplified; n.t.: not tested.

trichothecene-producing species tested to date (Tables 2 and 3). This included *F. poae* which is reported to produce both type-A (DAS) and type-B (NIV) trichothecenes (Liu et al., 1998). This primer pair did not amplify a product from any of the type-B trichothecene-producing species tested. The second assay (primer pair TRI4BF/TRI4BR) developed to detect producers of type-B trichothecenes amplified a product of 450 nt from all the type-B trichothecene-producing species. However, this primer pair also amplified a product of the expected size from an isolate of *F. sambucinum* that amplified with the primer pair to detect type-A trichothecene-producing species. Thus this assay requires further refinement for use where this species is of significance.

Differentiation of NIV and DON-producing isolates of F. graminearum, F. culmorum *and* F. cerealis

Two genes (*Tri7* and *Tri13*) have been identified in all NIV-producing isolates of *F. graminearum* tested to date that are non-functional in DON-producing isolates (Lee et al., 2001, 2002). A PCR assay for the identification of DON and NIV chemotypes of *F. graminearum* by size polymorphism based on the disruption of *Tri*7 by a repeated 11 bp insertion has been described (Lee et al., 2001). We have developed a number of positive–negative PCR assays to *Tri*7 and *Tri*13 sequences in order to characterise isolates of *F. graminearum*, *F. culmorum* and *F. cerealis* in terms of their NIV and DON chemotype. The PCR results obtained from *F. graminearum* and *F. culmorum* isolates concurred with known toxin production in all cases (Chandler et al., 2003). All NIV-producing isolates of *F. culmorum* and *F. graminearum* amplified only with primers Tri7F/Tri7NIV and Tri13NIVF/Tri13R specific to the functional versions of these genes, producing PCR products of 465 and 312 nt respectively (Table 2). All isolates of *F. cerealis* amplified only with Tri7F/Tri7NIV and Tri13NIVF/Tri13R primers specific to the functional versions present in NIV-producing isolates. The *Tri7* gene was found to be absent in all DON-producing isolates of *F. culmorum* and some DON-producing isolates of *F. graminearum* (Chandler et al., 2003). Such isolates would not be detected by the size polymorphism assay of Lee et al., (2001). Two primer sets were required to characterise the *Tri7* gene region of DON-producing isolates, Tri7F/Tri7DON and MinusTri7F/MinusTri7R for isolates containing the disrupted and deleted versions respectively (Table 2). All DON-producing isolates of *F. graminearum* and *F. culmorum* were found to have mutations in both *Tri*7 and *Tri*13 genes and no isolate was observed in which only one or other gene was non-functional.

Detection of enniatin-producing Fusarium *species*

Primers to detect producers of enniatins were designed on the basis of regions of homology between the enniatin synthetase genes of *F. scirpi* and *F. sambucinum* (accessions Z18755 and Z48743 respectively). The sequences of primer set EnSyn6065F/7229R are shown in Table 2. A broad range of *Fusarium* species, including a number reported to produce enniatins, were assayed with this primer set using the conditions outlined in Table 2. A product of 1164 nt was amplified from isolates of several species that have been associated with diseases of small-grain cereals, including *F. avenaceum, F. tricinctum*, *F. sambucinum*, *F. lateritium*, *F. chlamydosporum* and *F. acuminatum* (Table 4). A product of the expected size was also amplified from several *formae specialis* of *F. oxysporum* and *F. antophilum* (Table 4). No product was amplified from any isolate of *F. equiseti* although the original DNA sequence from which the primers were designed was termed *F. equiseti* in the database (*F. scirpi* accession Z18755).

Discussion

A prerequisite for the development of assays to detect and differentiate between *Fusarium* species is an appreciation of the overall relationship between species within the genus and the recognition that the power of morphologically based taxonomies may not be sufficient to discriminate between all species. In addition, while 'sections' may be a useful guide to relationships within the genus, this is not always the case (Bateman et al., 1996; Turner et al., 1998).

RAPD assays enable the estimation of similarity between isolates based upon markers distributed across the genome. While difficulties are recognised in transferring the RAPD profile markers between laboratories, this technique en-

Table 4. Amplification from selected *Fusarium* species using PCR primers (Ensyn6065F/Ensyn7229R) designed for the biosynthetic gene enniatin synthetase to detect enniatin-producing *Fusarium* species

Fusarium species	Ensyn6065F/Ensyn7229R
F. acuminatum	+
F. antophilum	+
F. avenaceum	+
F. chlamydosporum	+
F. lateritium	+
F. sambucinum	+
F. tricinctum	+
F. oxysporum f.sp. *lili*	+
F. oxysporum f.sp. *melonis*	+
F. oxysporum f.sp. *opunticrum*	−
F. cerealis	−
F. culmorum	−
F. equiseti	−
F. graminearum	−
F. poae	−
F. proliferatum	−
F. venenatum	−

+: PCR product of expected size; − no product amplified.

ables a detailed comparison of isolates within a laboratory, through the accumulation of high numbers of markers in a relatively short period. The sequence characterised amplified region (SCAR) markers derived from RAPD products are robust and transferable between laboratories as has been demonstrated within ongoing EU-funded projects: DeTox Fungi (QLK1-CT-1999-01380) and RAMFIC (QLRT-1999-31517). In our experience, SCAR provides an excellent tool to highlight potential subgroups within target species. Confirmation of results has been achieved through the complementary approach of sequencing selected DNA regions, in particular the internal transcribed spacer (ITS) region of rDNA.

RAPD fragments produced by all isolates of the target species or subgroup have been isolated, sequenced and used to produce primers for PCR assays. Many other authors have developed assays based upon differences in the ITS region, but for closely related species such as *F. culmorum* and *F. graminearum*, there may be insufficient polymorphism to allow the design of robust assays (Schilling et al., 1996). This characteristic limits the selection of primers and so also hinders the development of uniform PCR protocols to detect several pathogens in a single reaction or using similar amplification conditions. We have noted that many anonymous RAPD fragments do not hybridise even to closely related species in Southern blots of fungal genomic DNA. Such fragments enable the development of a single assay format to detect a large number of closely related species because there is no restriction on the location of primers of desired melting temperature within the fragment. Assays have been produced specifically to amplify a wide range of *Fusarium* species and other fungi associated with diseases of small-grain cereals including *F. culmorum* and *F. graminearum* (Nicholson et al., 1998), *F. poae* (Parry and Nicholson, 1996), *F. avenaceum* (Turner et al., 1998), *M. nivale* var. *majus* and *nivale* (Nicholson et al., 1996) as well as *F. sporotrichioides*, *F. langsethiae*, and *F. equiseti* reported herein. This approach has provided flexibility to enable primers to be designed with closely matching melting temperatures allowing a common PCR annealing temperature (62 °C) to be used. Where the amplification fragments differ in size, multiplex PCR can be used to detect several species within a single reaction sample. Competitive PCR assays have been developed for the majority of these species and used to determine their relative levels in a wide range of plant tissues (Harris et al., 1999; Simpson et al., 2000, 2001).

During the course of these studies, we have identified distinct groups within some species. In other instances, insight has been gained about the

relationships between different *Fusarium* species. For example, *F. avenaceum* and *F. tricinctum* belong to different sections, *Roseum* and *Sporotrichiella* respectively. It was, therefore, unexpected when primer sets designed for *F. avenaceum* were found to cross-react with *F. tricinctum* (Turner et al., 1998). Analysis of the ITS region, however, supports the close relationship between these two species and questions the value of the *Sporitrichiella* section (Bateman et al., 1996; Turner et al., 1998). This unexpected observation also highlights the importance of evaluating the specificity of PCR assays against as broad a range of species as possible and not just those considered to be closely related on the basis of morphological criteria.

It has recently been reported that *F. graminearum* consists of a number of lineages or groupings (Carter et al., 2000, 2002; O'Donnell et al., 2000). Analysis of a global collection of *F. graminearum* isolates from cereal hosts with primer set (Fg16F/R) was found to produce one of six different PCR products from each isolate (Carter et al., 2002). Sequence analysis has revealed that the product of the Fg16F/R primer pair is diagnostic of the lineage/group and hence this primer pair may be used to detect *F. graminearum* and simultaneously determine lineage/group. Furthermore, because particular lineages/groups appear to be associated with geographic regions and mycotoxin chemotypes, the Fg16F/R assay can aid the detection of migrants and monitor pathogen movement (P. Nicholson et al., unpublished).

Initial PCR assays to *F. sporotrichioides*, were found to cross-react to isolates termed 'powdery' *F. poae* isolated from Norwegian wheat grain (Torp and Langseth, 1999). *F. sporotrichioides* is known to produce T-2 (Chelkowski et al., 1989), and 'powdery' *F. poae* also produces T-2 (Torp and Langseth, 1999). Analysis of the ITS sequence confirmed that they represented two distinct, but closely related groups (Wilson et al., 2004). An ITS sequence identical to that of 'powdery' *F. poae* was identified in the NCBI database as *F. langsethiae*. Phylogenetic analysis of ITS sequence data from a range of species also grouped species according to toxin production. HT-2 and T-2 type-A producers, including *F. sporotrichioides* and *F. langsethiae*, grouped together and type-B trichothecene producers, *F. culmorum* and *F. cerealis*, also grouped together. Furthermore, those *Fusarium* species, including *F. poae*, that produce diacetoxyscirpenol (DAS), another type-A trichothocene, also tended to group together, but were distinct from T-2 and HT-2-producing species (Wilson et al., 2004).

RAPD analysis and initial attempts to develop diagnostic PCR assays revealed the close relationship between *F. avenaceum* and *F. tricinctum* and also between *F. sporotrichioides* and *F. langsethiae*. The polymorphic Fg16F/R assay to *F. graminearum* serendipitously aligns with lineages derived from DNA sequence data and from RAPD groupings. Such findings highlight the importance of understanding the population structure of target species and the relationship of the target to other species when developing specific PCR assays. However, considerable insight into these aspects may be gained as part of the process of developing diagnostic assays.

A number of generic PCR assays, including those described above, have been developed to detect trichothecene-producing *Fusarium* species, based either upon the *Tri5* or the *Tri6* genes (Doohan et al., 1999; Schnerr et al., 2001; Bluhm et al., 2002; Edwards et al., 2002). The risk to consumers is, however, not similar for all trichothecenes as type-A (e.g. DAS, T-2, HT-2), are considered to be significantly more toxic than type-B (e.g., DON and NIV). The pair of assays for *Tri4* described herein represent the first attempt to detect and differentiate between producers of the two trichothecene types. The production of both types of trichothecene, by species such as *F. poae*, complicates such efforts. It is desirable that any assay should detect the ability of the target to produce the toxin type of greater potency. The assay to detect type-A producers (primer set T4f1506/T4EndR2) does detect *F. poae*; hence the greater risk posed by the trichothecene mycotoxins produced by this species. The current assay to detect only producers of type-B trichothecenes requires further refinement. A combination of the Tri5F/R assay to detect trichothecene-producing species with the assay to detect type-A producing species may provide useful information in epidemiological studies and preliminary assessment of grain and cereal products.

We have used sequence data available for *F. scirpi* and *F. sambucinum* to design an assay to detect potential enniatin-producing *Fusarium* species. Enniatins are structurally related to beauvericin and some species may produce a mixture of

these cyclodepsipeptides (Bottalico and Perrone, 2002; Logrieco et al., 2002). While the assay described above produced a product of the expected size from the majority of isolates of species that have been reported to produce enniatins, there were some notable exceptions. No amplification was observed from any isolate of *F. poae* examined although isolates of this species have been reported to produce enniatins and/or beauvericin (Logrieco et al., 1998, 2002). However, the isolates that we examined were from the UK and we have yet to test isolates from mainland Europe. The assay also did not amplify a product from any isolate of *F. equiseti* examined. The original enniatin synthetase sequence from which the primers were designed was termed *F. equiseti* in the database (*F. scirpi* accession Z18755). It is possible that this isolate is not *F. equiseti* as, in our experience, identification of this species appears difficult and many isolates received as *F. equiseti* have ITS and other sequences similar to different species. *Fusarium proliferatum* has been reported to produce high levels of beauvericin (Moretti et al., 1997), but the enniatin PCR assay did not amplify any product from the isolates tested, indicating that this assay may be specific to enniatin synthetase and not cross-react to the gene responsible for production of beauvericin. Alternatively, sequence divergence between the enniatin synthetase genes of different *Fusarium* species may render this assay useful only for the detection of some of those species with the capacity to produce enniatins.

The diseases of small-grain cereals caused by *Fusarium* species are often disease complexes. The relative contribution of each of the species to causing disease in a particular situation will depend upon a range of variables, as will the amount of toxin produced by that species. Tools are required to dissect such complexes to determine the role of each species in a given situation and to enable potential risks to consumers to be evaluated. As an example, the importance of identifying which species predominate in an infection was highlighted by studies that showed the differential efficacy of fungicides against FHB species and the consequences for accumulation of mycotoxins in grain (Simpson et al., 2001). These workers used species-specific PCR assays to show that, in some instances, the level of DON in grain might be greater following treatment with certain fungicides than that in untreated samples. The PCR assays developed by us, and others, may overcome many of the problems associated with the study of disease complexes. These assays may be of great benefit for epidemiological studies and may reveal the interaction of different *Fusarium* species with each other and with other micro-organisms. The generic assays for trichothecenes and enniatins may contribute to disease surveys, risk assessment studies and to the development of disease forecasting models.

Acknowledgements

This work was supported by the European Union projects QLK1-CT-1998-001380 and QLK5-CT-2000-01517. P. N. would also like to acknowledge the support of the Department for Environment, Food and Rural Affairs. The authors would like to extend thanks to all those colleagues in the UK and elsewhere who have contributed significantly to this work through the provision of isolates and accompanying information. Without their knowledge and consideration this work would not have been possible.

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