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Major changes in *Fusarium* spp. in wheat in the Netherlands

Cees Waalwijk[1], Pieter Kastelein[2], Ineke de Vries[1], Zoltan Kerényi[3], Theo van der Lee[1], Thamara Hesselink[1], Jürgen Köhl[2] and Gert Kema[1]
Plant Research International BV, Business Units [1]Biointeractions and Plant Health and [2]Crop and Production Ecology, Droevendaalsesteeg 1, P.O. Box 16, 6700 AA Wageningen, The Netherlands (Fax: +31317418094; E-mail: c.waalwijk@plant.wag-ur.nl); [3]Agricultural Biotechnology Center, Gödöllo, Hungary

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Abstract

The re-emergence of fusarium head blight throughout the world and especially in Western Europe prompted a survey of the situation in the Netherlands. To allow for a high throughput screening of large numbers of samples, a diagnostic PCR method was developed to detect the most common species of *Fusarium* occurring on wheat. Seven primer pairs were tested for their ability to identify isolates of *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum* and *Microdochium nivale* var. *majus* and *M. nivale* var. *nivale*. Each primer pair only generated a PCR product with the corresponding *Fusarium* species and all PCR fragments had different molecular sizes. This allowed the generation of these amplicons using a mixture of all seven primer pairs. The robustness of this multiplex PCR encouraged us to screen a large series of isolates collected in 2000 and 2001. In both years 40 fields were sampled leading to a collection of 209 isolates from 2000 and 145 isolates from 2001. The results of the multiplex PCR demonstrated that *F. graminearum* was the most abundant species in the *Fusarium* complex on wheat in both years. This is in sharp contrast to reports from the 1980s and early 1990s, which found *F. culmorum* as the predominant species. Primers derived from the *tri7* and *tri13* genes, which are implicated in the acetylation and oxygenation of the C-4 atom of the backbone of the trichothecene molecule, were used to discriminate between deoxynivalenol and nivalenol (NIV) producers. The populations of *F. culmorum* and *F. graminearum* both showed a slight increase in NIV-producers in 2001.

Introduction

Fusarium head blight (FHB) or fusarium scab of small-grain cereals is caused by a complex of species. Although up to 17 species have been associated with the disease (Parry et al., 1995), the predominant species are *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale*. FHB can reduce yield by 30–70% (Bai and Shaner, 1994) but currently the major concern regarding FHB arises from the ability of the majority of species to produce mycotoxins. It is well documented that *F. culmorum*, *F. graminearum* and *F. poae* can produce different trichothecenes. Moreover, *F. avenaceum* produces moniliformin (Schütt et al., 1998). The most predominant mycotoxins found in small-grain cereals are deoxynivalenol (DON) (also known as vomitoxin) and nivalenol (NIV) and their derivatives.

Reports from all continents demonstrate the re-emergence of this devastating disease and its high economic impact. In the mid 1990s, *Fusarium* epidemics caused large losses in wheat production in the US and Canada. Economic damage was estimated at US $2500 million in wheat and US $400 million in barley in the US alone and US $220 million and US $300 million in Canada for wheat and barley respectively (Windels, 2000). The epidemics in the US were primarily caused by *F. graminearum*, which has been shown to be a complex in its own right, with seven or even eight lineages with different geographic and phytopathological characteristics (O'Donnell et al., 2000). In China and New Zealand, *F. graminearum* also seems prominent,

but large variations were observed between years (Carter et al., 2000; Cromey et al., 2002). Studies in many countries in Europe, on the other hand showed that other species of the complex are more dominant. Surveys performed in Scandinavia, Hungary and other mid-European countries identified *F. avenaceum* and *F. poae* as the most important species (Lukanowski and Sadowski, 2002). In Germany, particularly in the Bavarian region, *F. graminearum* was frequently encountered. This might be a special situation since *F. graminearum* is also found on maize. Crop rotations of wheat and maize generate conditions that are very conducive for *F. graminearum*. This may be aggravated by the fact that ascospores can contribute substantially to the inoculum pressure, with two or even more cycles per growing season (Obst et al., 2002). Studies performed in the UK, on the other hand, identified *F. culmorum* as the most important cause of head blight (reviewed by Parry et al., 1995). In more recent years *M. nivale* and *F. poae* were also frequently encountered (http://www.csl.gov.uk/resdev/AH/PDCP/epid/fusarium/inc2.cfm).

In the Netherlands, studies in the 1980s by Daamen et al. (1991) and in the early 1990s by De Nijs et al. (1996) identified *F. culmorum* as the predominant agent for FHB. The majority of the older studies were based on the morphological characterization of the isolated individuals, but more recent work relied on the application of DNA fingerprinting methodologies. Using RAPD analyses, De Nijs et al. (1997) confirmed that the predominant species in the Netherlands was indeed *F. culmorum*. The isolates used had been sampled in the early 1990s (1991 and 1993). Reports from neighbouring countries, where an increased importance of *F. graminearum* was observed, stimulated a new survey in 2000 (Waalwijk et al., 2001). The results of this survey, presented in this paper, demonstrate a drift in the *Fusarium* populations from *F. culmorum* in the early 1990s to *F. graminearum* in 2000 and 2001.

The aim was to develop a molecular screen for field isolates, that in principle belong to any of the 17 species within the Fusarium head scab complex (Parry et al., 1995). To enable screening of large numbers of samples, a multiplex PCR was developed that detects the major species within the complex.

The major mycotoxins that contaminate small-grain cereals are the type A trichothecenes T-2 and HT-2, primarily produced by *F. poae* and *F. sporotrichioides*, and the type B trichothecenes DON (or vomitoxin) and NIV produced mainly by *F. graminearum* and *F. culmorum*. Although the synthesis and regulation of expression of these mycotoxins are largely unknown, some major steps in the biosynthesis have been elucidated and the genes involved appear to be clustered. Comparative analyses of these gene clusters in DON-producers and NIV-producers have identified the genes *tri13* and *tri7* as vital in the synthesis of either NIV or DON. Both genes appear intact in NIV-producing isolates but in DON-producers they contain multiple mutations. Alignment of the *tri13* gene from a NIV-producer with its homologue in a DON-producer reveals many alterations including several deletions (Lee et al., 2002; Brown et al., 2002). Disruption of the *tri13* gene in a NIV producer converts it into a DON producer. Moreover, heterologous expression of an intact *tri13* gene isolate in a DON producer conveys the ability to produce NIV. (Lee et al., 2002; Brown et al., 2002). The *tri7* gene, in turn, appears to be involved in the acetylation of NIV leading to 4-acetylnivalenol (4-ANIV). The most striking feature of the *tri7* gene in DON-producers is the presence of a multiple repetition of 11 bp that occurs only once in NIV-producers. Moreover, this gene apparently misses a functional starting codon (Lee et al., 2001). Gene sequences from both genes were used to develop primers that were utilized to screen our 2000 and 2001 populations for frequencies of DON- and NIV-producers. Part of this work has been presented at the 7th European Fusarium Seminar (Waalwijk, 2002).

Materials and methods

Fungal isolates and sampling strategy

Tester isolates from the collection at Plant Research International B.V. (PRI) were used to obtain DNA samples from *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *M. nivale* (see Table 1). Species-specific primers from *F. proliferatum*, developed in our laboratory, were also included. Isolates representing the seven lineages of *F. graminearum* (O'Donnell et al., 2000) were included for comparison.

In 2000 and 2001, wheat ears with visual symptoms of FHB were sampled from 40 different fields throughout the Netherlands. Isolates were obtained by hierarchical sampling: on five different places in each field a visible diseased ear was collected and from each ear two kernels were taken for isolation of *Fusarium* spp. These kernels were surface disinfected by washing them sequentially with 70% ethanol and 2% sodium hypochlorite. Subsequently, the kernels

Table 1. Isolates from the PRI collection used to develop multiplex PCR, primer designations, anticipated sizes of the PCR fragments and literature sources

Species	Isolate	Primer name	Sequence	Sizes (bp)	Ref[b]
F. avenaceum	IPO 92-3	FaF	CAAGCATTGTCGCCACTCTC	920	A
		FaR	GTTTGGCTCTACCGGGACTG		
F. culmorum	PD90-283	Fc01F	ATGGTGAACTCGTCGTGGC	570	B
		Fc01R	CCCTTCTTACGCCAATCTCG		
F. graminearum	PD88-790	Fg11F	CTCCGGATATGTTGCGTCAA	450	B
		Fg11R	GGTAGGTATCCGACATGGCAA		
F. poae	PD93-1780	Fp8F	ACGACGAAGGTGGTTATG	1600	C
		Fp8R	GGTGAAGAGCCTGTTTGCTTG		
F. proliferatum	ITEM2287	TH5-F	GATAACGTCCAAGGCTACG	330	D
		TH6-R	GGGGTCGTTCAGCTCAAGG		
M. nivale var. *nivale*	—	Y13NF	ACCAGCCGATTTGTGGTTATG	310	A
		Y13NR	GGTCACGAGGCAGAGTTCG		
M. nivale var. *majus*[a]	IPO 1.21	Mnm2F	TGCAACGTGCCAGAAGCT	750	A
		Mnm2R	AATCGGCGCTGTCTACTAAAAGC		
		GzTri7/F	GGCTTTACGACTCCTCAACAATGG	$162 + [11]_n^c$	E
		GzTri7/R	AGAGCCCTGCGAAAGYACTGGTGC		
		Tri13F	TACGTGAAACATTGTTGGC	234 or 415	F
		Tri13R	GGTGTCCCAGGATCTGCG		

[a]The original isolate in the PRI collection was not determined to the variety level, but an amplified product of 750 bp with primer pair Mnm2F/Mnm2R and absence of a product with primer pair Y13MF/Y13MR identified it as *M. nivale* var. *majus*.

[b]References used: (A) Doohan et al. (1998); (B) Nicholson et al. (1998); (C) Parry and Nicholson, (1996); (D) unpublished; (E) Lee et al. (2001) and (F) this study.

[c]$162 + [11]_n$, PCR fragments were obtained of 162 bp in the case of NIV-producers or 162 bp plus a multitude of 11 bp, e.g. 173, 184, 195 etc. in DON-producers (Lee et al., 2001).

were washed extensively with sterile distilled water and placed on PDA plates containing 10 mg l^{-1} tetracycline and 100 mg l^{-1} streptomycin. Plates were incubated for 5 days at 20 °C and *Fusarium*-like mycelium was transferred to new PDA plates containing the antibiotics. In this way, 209 and 145 isolates were obtained in 2000 and 2001, respectively.

DNA manipulations

Isolates were grown on PDA for 3–5 days and mycelium was harvested by filtration though cheesecloth. After lyophilization and grinding, DNA was obtained using the Puregene extraction protocol (Kema et al., 2002). High throughput DNA isolations from field isolates were done as follows: isolates were grown at 20 °C for 4–5 days in 96 well format blocks with individual tubes containing 0.7 ml of potato dextrose medium per well, with daily inversions to mix the content of the tubes. After addition of a tungsten bead and lyophilization, mycelium was ground by vigorous shaking of the blocks in a MM300 mixer mill (Retch, Ochten, the Netherlands), followed by the DNA extraction using the Puregene kit. Gel electrophoresis was used to check the quality of the DNA as well as to estimate the concentration of each sample.

Standard PCRs with a single primer set were performed for 40 cycles (1 min denaturation at 94 °C, 30 s annealing at 60 °C and 1 min extension at 72 °C) followed by a final extension of 5 min at 72 °C and storage at 4 °C until harvest of the samples. Template DNA was used at 20 ng, species-specific primers (Table 1) were added at 6 μM and amplicons were separated on 1.0% agarose. *Tri7* PCRs, using the primers Gztri7F and Gztri7R (Lee et al., 2001) and *tri13* PCRs with the primers tri13F and tri13R (Table 1) were also performed under these conditions.

Multiplex PCRs were performed under identical conditions, except that all seven species-specific primer pairs, i.e. 14 primers, were combined in the same reaction at 6 μM each. PCRs with the ITS primers

ITS1 and ITS4 were done for 30 cycles at 57 °C annealing temperature (Waalwijk et al., 1996). Mating type specific primers for the *Mat1-1* and *Mat1-2* loci in *Fusarium* spp. were used to confirm the homothallic nature of the isolates identified as *F. graminearum*. These reactions were performed as described before (Moretti et al., 2002).

Sequencing of fragments was done using the Big Dye technology of Applied Biosystems and sequence reactions were run on ABI 3700 equipment of the Greenomics® facility at PRI. DNA sequence analyses were performed using the multiple sequence alignment programme MegAlign (DNASTAR).

Results

To test whether a multiplex PCR approach would be feasible for the molecular identification of the field isolates obtained in 2000 and 2001, primers documented in literature were tested on laboratory isolates under regular PCR conditions. DNA from isolates of the major pathogens from the FHB complex, i.e. *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum* and *M. nivale* was subjected to PCRs with their corresponding species-specific primers (Table 1). As the isolate from *M. nivale* in our collection, IPO 1.21, was not identified to the variety level, it was unclear which PCR fragment to expect. The fragment obtained was 750 bp in size thereby identifying IPO 1.21 as *M. nivale* var. *majus* (Doohan et al., 1998). At a temperature of 60 °C, each of these DNAs generated amplicons with sizes that were expected (Table 1). Since these reactions were performed under the same conditions and each species-specific fragment has its unique size, the number of species-specific primers within the same PCR reaction was increased to a combination of all 14 primers for five *Fusarium* and two *Microdochium* species. As with the singular PCR, each of the isolates generated only a single PCR fragment and the sizes of these fragments were in accordance with those expected for the respective species (Figure 1A).

Subsequently, 209 isolates from 2000 and 145 isolates from 2001 were analysed using this multiplex PCR. The majority of isolates (>90%) from these fields generated PCR fragments that allowed classification into the major pathogens of the FHB complex (e.g. Figure 1B). Only a small subset of isolates from each year could not be classified, because (i) no PCR product was obtained (Figure 1B) (ii) a combination

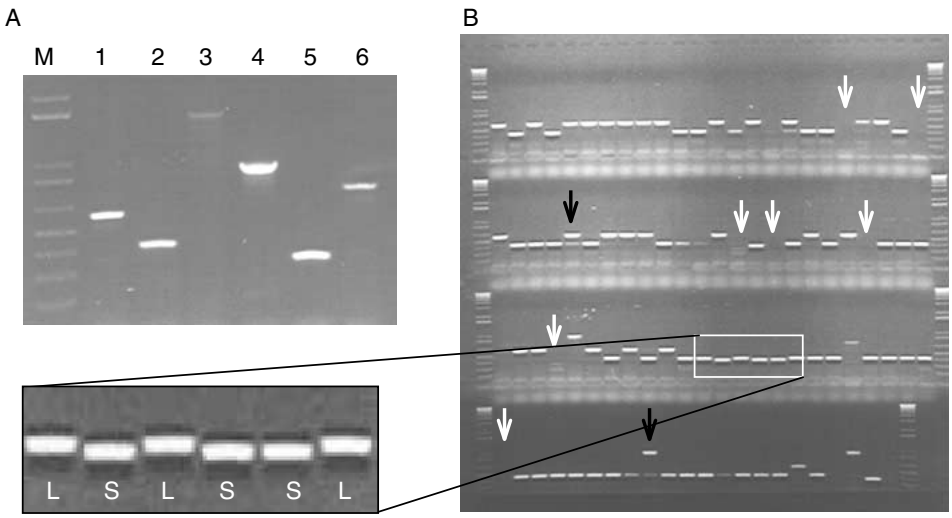

Figure 1. (A) Multiplex PCR with mixture of 14 primers on DNA purified from tester isolates. M, 1 kb molecular markers; 1, *F. culmorum* isolate PD90-283; 2, *F. graminearum* PD88-790; 3, *F. poae* PD93-1780; 4, *F. avenaceum* IPO 92-3; 5, *F. proliferatum* ITEM 2287 and 6, *M. nivale* var. *majus* IPO1.21. (B) Multiplex PCR on a series of field isolates ($n = 88$). On the bottom row the tester isolates were also run for comparison. White arrows indicate lanes without clear fragments and black arrows indicate lanes with dual fragments. The boxed area was zoomed in to illustrate the presence of size variation within the *F. graminearum* isolates (designated L and S).

of two PCR fragments was observed, each corresponding to a different species of the FHB complex (Figure 1B), or (iii) fragments of aberrant size were obtained (Figure 1B). Individuals that did not produce a PCR product, were tested with the generic primers ITS1 and ITS4, which amplify the ITS1 and ITS2 region of the ribosomal RNA genes. Some isolates from this category again failed to generate a PCR product, suggesting that the quality and/or the quantity of DNA was insufficient for proper amplification. However, the majority of these strains generated a clear ITS fragment, indicating that the quality of the DNAs was good. To resolve the inconsistency of a robust ITS-PCR but no species-specific fragment, the amplified ITS regions from these isolates were sequenced. The sequences obtained were very distinct from the *Fusarium* ITS sequences deposited in the publicly accessible databases. Six out of nine ITS sequences obtained were identical to *Epicoccum* spp. and three showed substantial deviation from any ITS sequence in the databases. Further morphological identification at the Fungal Biodiversity Center, CBS, Utrecht, the Netherlands identified isolates 1H2 and 6H1 as *Ascochyta* spp. and isolate 6G1 as *Ascochyta skagwayensis*. The sequences of these isolates have been deposited at Genbank under AF520640–AF520642.

To clarify the category containing dual fragments, monospore cultures were produced which generated only a single fragment indicating that the sample was a mixture of two different species for the FHB complex. Fragments of aberrant size were occasionally obtained, as can be seen in the boxed area in Figure 1B. In PCR reactions with mating type primers these isolates gave both *Mat 1-1* and *Mat 1-2* fragments indicating that these isolates were homothallic, i.e. *F. graminearum*. This was confirmed by sequencing the fragments from the multiplex reactions which identified them as being generated by the *F. graminearum* specific primers Fg11F/R. The sequences from long and short fragments were identical except for 30 nucleotides that were absent in the aberrant fragments (Figure 2A). When isolates representing the seven lineages of *F. graminearum* (O'Donnell et al., 2000) were tested, only Fg11F/R products were obtained in lineages 1, 2, 6 and 7 and no products were found in lineages 3–5. Comparison of the sequences of the Fg11R/F amplicons from the Dutch isolates with those from lineages 1, 2, 6 and 7 identified the presence of one, two or three copies of this 30 bp fragment (Figure 2A). Interestingly all isolates from lineage 6 contained an additional insertion of 86 bp in the Fg11F/R amplicon. The presence of this latter insertion is causing the size increase between isolate PD88-790 and isolate NRRL6101 (Figure 2B). PD88-790 was isolated in 1988 in the Netherlands and therefore likely to belong to lineage 7. Isolate NRRL6101 was identified as a lineage 6 isolate (O'Donnell et al., 2000). Multiple sequence alignment including isolates from the lineages 1, 2, 6 and 7 clearly showed that the Dutch isolates cluster with isolates of lineage 7 (Figure 2C).

The sampled fields were located in different parts of the country. For comparative analyses they were grouped according to the regional structure used by De Nijs et al. (1996). Irrespective of the region of isolation the most common species of the FHB complex observed in 2000 was *F. graminearum*. The percentage of isolates identified as *F. graminearum* ranged from 47.9% in the south-west to 80.0% in the south-east, with an average of 58.4% (Table 2). *Fusarium culmorum* ranged from 8.2% in the south-east to 36.7% in the south-west and *M. nivale* var. *majus* from 0.0% in the south-east to 27.4% in the polders in the centre of the Netherlands (Figure 3). In 2001, the same regions, but different fields, were sampled and again *F. graminearum* was the dominant species, with an average frequency of 58.6%, with *F. culmorum* at 29.7% and *M. nivale* var. *majus* at 6.9% (Table 3). Interestingly, only *M. nivale* var. *majus* was observed and *M. nivale* var. *nivale* was never encountered. The identification of other species in the complex, i.e. *F. avenaceum* and *F. poae*, was very rare in both years and *F. proliferatum* was not found (Table 2). The frequencies of isolation of the different species from various cultivars did not show any interaction with the sole exception of *cv*. Renan in 2000 where 7 out of 10 isolates were *M. nivale var. majus* (Table 4). In total, 81 pairs of isolates from the same ear were obtained, that contained two *F. graminearum* ($n = 26$), two *F. culmorum* ($n = 9$) or two *M. nivale* var. *majus* ($n = 2$) isolates. The remainder ($n = 44$) contained two different species.

The genes *tri13* and *tri7*, involved in oxygenation and acetylation of the C-4 residue of the trichothecene backbone respectively, were used to identify the putative chemotype of each of the isolates. With the primer pair Gztri7F/R, NIV-producers generated a 162 bp fragment and DON-producers generated a fragment of 162 bp plus a multiple of 11 bp, i.e., 173, 184, 195 bp, in size (Lee et al., 2001). Several of these differently sized amplicons were obtained

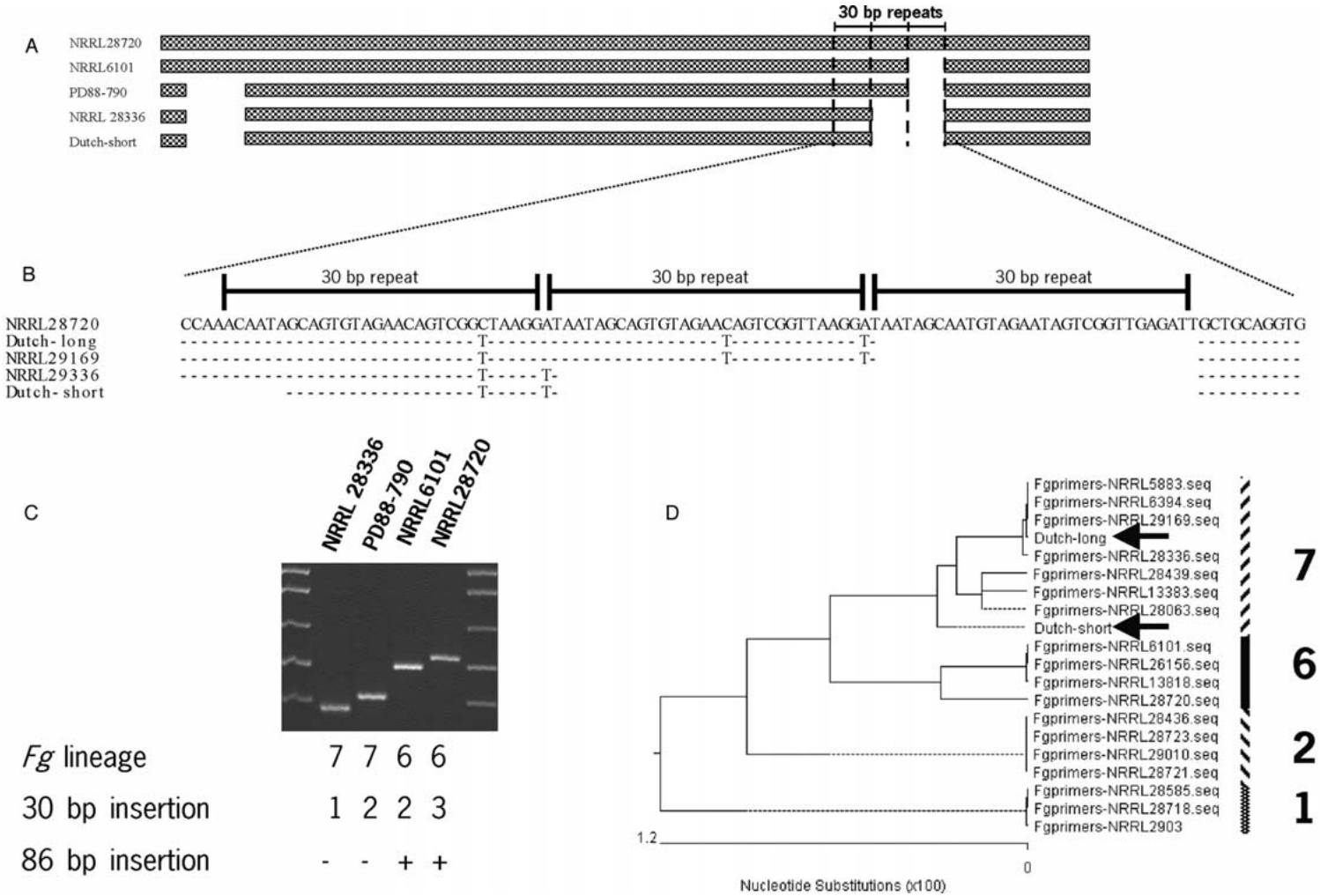

Figure 2. (A) Schematic representation of the alignment of the sequences obtained from *F. graminearum* isolates generating size variation with the primers Fg11F and Fg11R. NRRL28720, isolate of *F. graminearum* from lineage 6 containing three 30 bp repeats and the 86 bp insertion; NRRL6101, isolate of *F. graminearum* from lineage 6 containing two 30 bp repeats and the 86 bp insertion; PD88-790, *F. graminearum* tester isolate from The Netherlands, with two 30 bp repeats but missing the 86 bp insertion; NRRL28336, isolate of *F. graminearum* from lineage 7 containing one 30 bp repeat and missing the 86 bp insertion; Dutch-short, isolate 47E1, from the 2001 survey containing one 30 bp repeat and missing the 86 bp insertion. (B) Multiple sequence alignment of part of the Fg11F/R fragments from different isolates of *F. graminearum*, illustrating the presence of a (several) copies of a 30 bp direct repeat. NRRL28720, isolate of *F. graminearum* from lineage 6 containing three 30 bp repeats: Dutch-long, sequence of isolates from this survey (designated L in Figure 1B) and NRRL29169, isolate of *F. graminearum* from lineage 7 both containing two repeats; NRRL28336, isolate of *F. graminearum* from lineage 7 and Dutch-short (designated S in Figure 1B) containing one repeat. (C) Size variation in the Fg11 F/R amplicon among isolates from different lineages. (D) Multiple alignment using MegAlign (DNASTAR) of the FG11F/R fragments generated from representatives of the lineages of *F. graminearum*. No amplicon was obtained with lineages 3–5. Clustering of the remaining lineages was according to that observed by O'Donnell et al. (2000).

Table 2. Numbers of *Fusarium* spp. in different regions (2000/2001)

Region	*F. avenaceum*	*F. culmorum*	*F. graminearum*	*M. nivale* var. *majus*	*F. poae*	Others	Total
South-west	0/0	18/9	25/10	5/7	0/0	1/1	49/27
East	1/0	4/1	14/6	3/0	0/1	1/0	23/8
Central	1/0	14/15	35/27	20/3	0/1	3/1	73/47
North-east	1/1	4/12	36/29	6/0	0/0	2/1	49/43
South-east	0/0	2/6	12/13	0/0	0/1	1/0	15/20
Total	3/1	42/43	122/85	34/10	0/3	8/3	209/145

when the populations of 2000 and 2001 were screened with these primers (Figure 4A). The primer pair *tri13F/R* was designed by comparing the published sequences for this gene from known NIV- and DON-producers (Accession# AF336365, AF366366 and AY057841–AY057844). They generated a 415 bp fragment in NIV-producers and a fragment of 234 bp in DON-producers (Figure 4B). In general, the *tri13* primers were more robust with 78.8% of the *F. culmorum* and 87.9% of the *F. graminearum* isolates giving a fragment. With the *tri7* primers these values were 74.1% and 86.0%, respectively. A comparison between both primer sets showed a correlation (DON or NIV with both the *tri7* and *tri13* primers) of 76.5% for *F. culmorum* and 91.8% for *F. graminearum*. Table 5 shows the distribution of NIV- and DON-producers for both *F. culmorum* and *F. graminearum*. In general, *F. culmorum* comprised primarily NIV-producers and *F. graminearum* isolates were mainly DON-producers. Nevertheless, both populations showed a small shift towards a higher percentage of NIV producers in 2001 (Table 5).

Discussion

The re-emergence of FHB on many continents has caused much concern related to food and feed production and safety. This prompted a survey of the situation in the Netherlands. To allow for high throughput screening of large numbers of samples, a multiplex PCR was developed to simultaneously detect the most frequently encountered species of the FHB complex. This diagnostic PCR appeared to be very robust, since only a small percentage of isolates were not readily identified. Most of these isolates appeared to belong to other fungal genera, like *Epicoccum* and *Ascochyta* and were apparently erroneously considered to be *Fusarium* spp. during isolation from the surface-sterilized kernels. Although these remote genera can be easily recognized using morphological criteria, the regular emergence of new *Fusarium* spp. in the disease complex is a taxonomic challenge that is appropriate only to experts. The multiplex PCR, contained a combination of primers that allowed simultaneous detection of seven species, but we anticipate that this number can be increased further. The only prerequisite is that the species-specific amplicons can be identified unambiguously.

The results clearly underline the reports from other European surveys that point to an increase in the importance of *F. graminearum* as a major pathogen of wheat in temperate climates. In the Netherlands, *F. culmorum* was the major component of the FHB complex in the 1980s and 1990s (Daamen et al., 1991; De Nijs et al., 1996). However, *F. graminearum* was the primary species in our surveys for two consecutive years which suggests a dramatic shift in the composition of the FHB complex. Although there is a gap of 7 years between the 1990s samplings and the present surveys, this trend seems to have occurred in the mid-1990s, since *F. graminearum* was also predominant in 1997 (S. Edwards, pers. commun.). The causal factors for this shift have not been elucidated, but an increase in maize production has been suggested to play an important role. *F. graminearum*, in contrast to *F. culmorum,* is well recognized as a major pathogen on maize and, more importantly, has the capacity to survive on maize stubble. Nevertheless, other factors can be put forward to explain the increased occurrence of *F. graminearum*. Climatic changes might favour the propagation of *F. graminearum* over *F. culmorum,* as the former species has a higher temperature optimum. The homothallic nature of *F. graminearum* allows the production of large masses of ascospores that can play a role in the epidemiology. In a recent study in Germany the important contribution of ascospores to inoculum

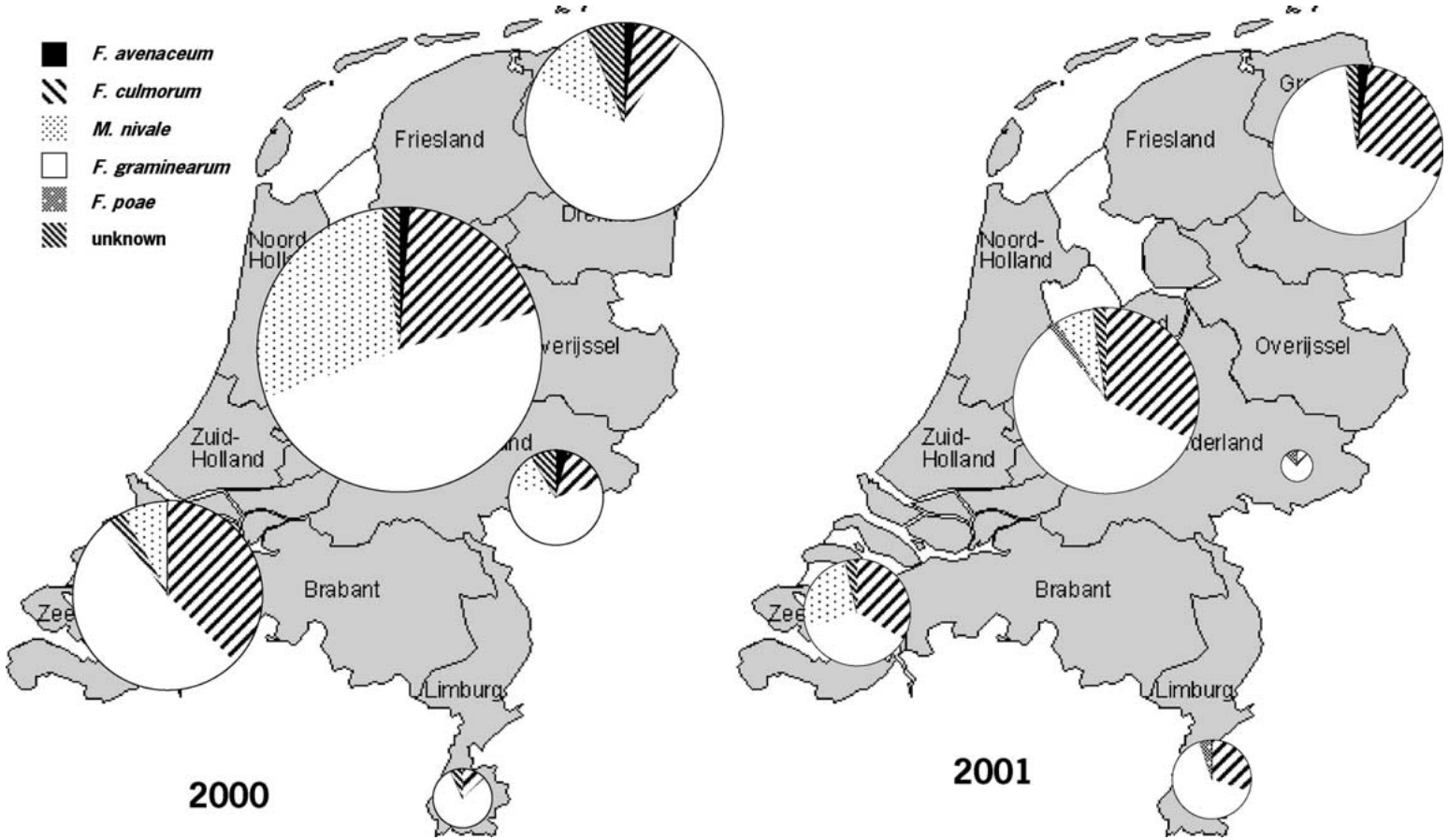

Figure 3. Numbers of *Fusarium* spp. in the various regions of the Netherlands in 2000 (a) and 2001 (b). The numbers of isolates obtained from the five regions are indicated. The sizes of the circles correspond to these numbers.

pressure was emphasized (Obst et al., 2002). Finally, the resistance of cultivars may influence the composition of the FHB complex. It has generally been accepted that resistance to *F. culmorum* and to *F. graminearum* are indistinguishable, but since selection is usually performed under natural disease pressure, selection efficiency towards the individual species in the complex is hardly possible. The distribution of the *Fusarium* species over the different regions and over the different cultivars did not reveal any clear interactions. The sole exception to this was *M. nivale* var. *majus* on Renan (7 out of 10) in 2000. Due to the absence of this cultivar in 2001, we could not substantiate this finding. The frequency of *F. culmorum* on cv. Kampa in 2001 (80% of the isolates) and on a mixture of cultivars in 2000 (80% of the isolates) also showed a strong bias, but samples sizes were very small ($n = 5$). Moreover, in both cases the isolates originated from a single field and were indistinguishable with PCR, suggesting potential clonality. Only a minority of the isolate pairs originating from the same ear were identified as the same species (37 out of 81 pairs) and several of these pairs could be resolved on the basis of the *tri7*, *tri13* and/or mating type PCRs. Only accurate fingerprinting, e.g. with AFLP, will identify clones within ears, field or regions, but when populations were considered at field level, both NIV-producers and DON-producers were frequently encountered in the same field. The mating type PCR was not capable of differentiating *F. graminearum* genotypes because its homothallic nature leads to both *Mat1-1* and *Mat1-2* specific fragments. The *F. culmorum* populations, on the other hand, belonged to either *Mat1-1* or *Mat1-2* and within a single field both mating types were recovered. This suggests that at least some of the *F. culmorum* populations were not clonal, as suggested by De Nijs et al. (1997) and Schilling et al. (1996), but contained at least two different genotypes. Within the scope of the two surveys described here, isolates of *M. nivale* var. *nivale* were not encountered. This is in agreement with results from surveys in the UK (P. Nicholson, pers. comm.) and could be attributed to the postulated difference in pathogenicity between the two fungal varieties (Diamond and Cooke, 1997).

Careful examination of the size variation obtained with the Fg11F and Fg11R primers led to the identification of two groups of isolates having either one or two copies of the 30 bp repeat. When these data were compared with the isolates from the seven lineages described by O'Donnell et al. (2000) isolate NRRL 28720 was found to carry three copies of the repeat. Carter et al. (2000; 2002) encountered an isolate

Table 3. Occurrence of *Fusarium* spp. in the Netherlands (percentages are given in parentheses)

Species	2000	2001
F. avenaceum	3 (1.4)	1 (0.7)
F. culmorum	42 (20.1)	43 (29.7)
F. graminearum	122 (58.4)	85 (58.6)
F. poae	0	3 (2.1)
F. proliferatum	0	0
M. nivale var. *majus*	34 (16.3)	10 (6.9)
Others[a]	8 (3.4)	3 (2.1)
Total	209	145

[a]Others: these isolates did not react with any combination of primers in the multiplex PCR primer mix. A subset did not generate a PCR fragments with the generic primers ITS1 and ITS4, suggesting insufficient extraction from these isolates. The remainder was subjected to morphological and molecular identification that confirmed that these isolates had been falsely identified as for *Fusarium* spp. Morphological characterization and molecular identification through sequencing of the ITS region identified these strains as *Epicoccum* spp. or *Ascochyta* spp.

Table 4. Numbers of *Fusarium* spp. on different cultivars (2000/2001)

Cultivar	*F. avenaceum*	*F. culmorum*	*F. graminearum*	*M. nivale* var. *majus*	*F. poae*	Others	Total
Drifter	0/0	7/15	18/28	7/4	0/1	0/1	32/49
Kampa	0/0	0/4	0/1	0/0	0/0	0/0	0/5
Renan	0/0	1/1	2/0	7/0	0/0	0/0	10/1
Residence	0/0	9/5	32/13	10/1	0/0	1/0	52/19
Ritmo	1/0	7/4	16/6	0/0	0/0	2/0	26/10
Tower	0/0	1/1	6/0	0/0	0/1	2/0	9/2
Vivant	2/1	13/13	47/37	9/5	0/1	2/2	73/59
Mixture	0/0	4/0	1/0	1/0	0/0	1/0	7/0
Total	3/1	42/43	122/85	34/10	0/3	8/3	209/145

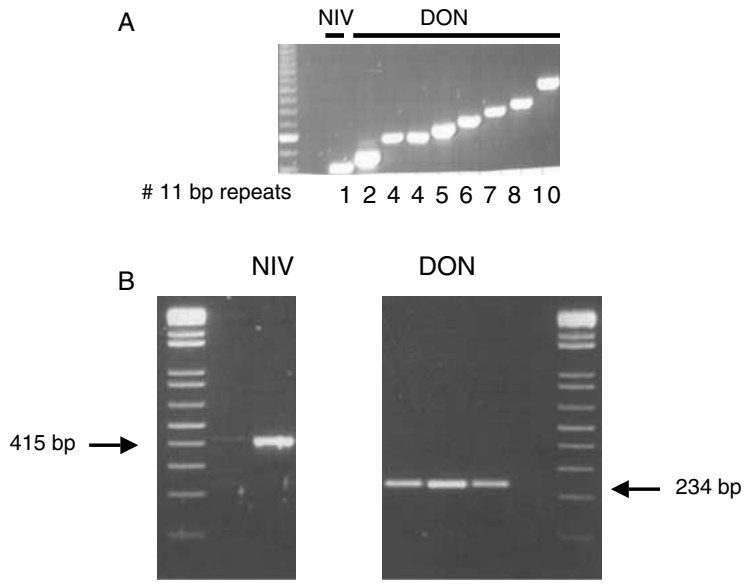

Figure 4. Amplification products generated by primer set Gztri7F/R(A) or tri13F/R (B). (A) Amplification products generated by primer set Gztri7F/R from field isolates of *F. graminearum* from 2000 and 2001 sorted by amplicon size. From left to right: water control, NIV-producing isolate 43B2, DON-producing isolates 45A1, 64C2, 42B1, 44A1, 38D1, 42C1 and 26G1. All isolates originated from 2001 except isolates 38D1 and 26 G1. At the far right lane isolate NRRL 28063 was included which contains 10 copies of the 11 bp repeat. (B) Amplification products of NIV- or DON-producing isolates amplified with primer set tri13F/R. From left to right: 1 kb markers, water control, NIV-producer NRRL28439, DON-producers NRRL5883, NRRL28063 and NRRL6394, water control and 1 kb markers.

Table 5. Frequency of NIV- and DON-producers of *F. culmorum* and *F. graminearum* in 2000 and 2000

	2000		2001	
	DON (%)	NIV (%)	DON (%)	NIV (%)
F. culmorum	11 (26.2)	23 (54.8)	8 (18.6)	31 (72.1)
F. graminearum	93 (76.2)	16 (13.1)	58 (68.2)	18 (21.2)

carrying four copies of the repeat in their studies on Nepalese isolates of *F. graminearum*. Comparison of the sequences from isolates of all lineages revealed that within Fg11F/R amplicons an additional insertion/deletion occurred involving an 86 bp fragment. This fragment was present in isolates from lineages 2 and 6, but was missing in lineages 1 and 7. Interestingly, when a series of Chinese *F. graminearum* isolates from wheat was analysed all ($n = 137$) generated an amplicon that contained the 86 bp insertion (Waalwijk, unpublished). This suggests that this insertion is specific for lineage 6 (and lineage 2) and may provide a tool for the discrimination of these groups of lineages. This could be valuable in Asia where lineages 2, 6 and 7 occur (Carter et al., 2000; O'Donnell et al., 2000). For the European and American situation, such a tool could be used to monitor the introduction of new lineages, since in these regions only lineage 7 has so far been reported (O'Donnell et al., 2000; Ward et al., 2002).

The recent publication of sequences from the *tri7* and *tri13* genes from the trichothecene cluster has allowed the screening of the populations for the production of NIV or DON. Lee et al. (2001; 2002) and Brown et al. (2001; 2002) demonstrated the role of these genes in the biosynthesis of trichothecenes in general and the coordination of either NIV or DON production in particular. Using primers derived from these genes it was possible to discriminate NIV-producers from DON-producers in both *F. graminearum* and *F. culmorum*. Amplification using the *tri7* primers in DON-producing isolates from *F. culmorum* was found to be unsuccessful, since the entire gene appeared to be missing (P. Nicholson, pers. commun.). In general, the *F. culmorum* populations consisted primarily of NIV-producers whereas the *F. graminearum* populations contained mainly DON producers. When the surveys of both years where compared, a slight but consistent increase of NIV-producers in 2001 was observed. Sample sizes were not very

large and the shift was not dramatic, but when populations were separated over their geographic origin the same tendency was observed. As NIV-producers were reported to be more pathogenic on maize than DON-producers (Carter et al., 2002), such a shift might be caused by the inclusion of maize in the crop rotation. Clearly, additional studies are required to confirm this preliminary observation. Studies on the toxicological characteristics of NIV and DON towards animals have led to more stringent recommendations for NIV. If the shifts observed in our studies prove to be consistent, monitoring the chemotypes will form an essential part of future surveys of FHB in wheat and other cereals.

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