



An Oligonucleotide Array for Differentiation of *Fusarium* Species in Cereal Grain

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INTRODUCTION

Fusarium head blight

Many species belonging to the genus *Fusarium* are known to cause *Fusarium* head blight (FHB) of small grain cereals. *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, *F. sporotrichoides*, *F. equiseti*, *F. langsethiae* and *F. tricinctum* are frequently identified in cereal grain samples (Kosiak et al., 2003). In addition to causing disease symptoms, the majority of the causal organisms of FHB are producers of mycotoxins such as the highly toxic trichothecenes (Bottalico and Perrone, 2002). The most common trichothecenes in cereals are deoxynivalenol (DON) produced by certain isolates of *F. graminearum*, *F. pseudograminearum* and *F. culmorum*. Another closely related trichothecene, nivalenol (NIV) is also produced by isolates of *F. poae* and *F. equiseti* and *F. poae*. T-2 toxin and HT-2 toxin are produced by *F. sporotrichoides* and *F. langsethiae*, whereas many isolates of *F. poae* and *F. equiseti* produce diacetoxyscirpenol (DAS) (Thrane et al. 2004). *F. avenaceum* and *F. tricinctum* do not produce trichothecenes, but do produce other mycotoxins such as enniatins and moniliformin.

Identification

Because *Fusarium* species involved in FHB differ in e.g. pathogenicity, fungicide sensitivity, and in their ability to produce mycotoxins, correct identification is of paramount importance. As a consequence, numerous techniques based on PCR have been developed for identification and differentiation of *Fusarium* species. However, microarray technology may be a useful alternative for identification and differentiation of a high number of species of *Fusarium* in parallel. We are currently developing arrays for identification of FHB related species based on the ITS region and the elongation factor 1 α . This poster presents data on the identification of *Fusarium* spp. in Lithuanian organic cereals using a newly developed *Fusarium* identification array based on the ITS region.



MATERIALS AND METHODS

Field samples

Different varieties of winter wheat, winter barley, spring wheat, spring barley and oat were raised in an organic field experiment at the Lithuanian Institute of Agriculture during 2005 – 2006 in the three field replications. Samples (0.2 kg) were stored in plastic boxes at -20 °C before analysis.

Plating method

Surface - sterilised grains (200 for each sample) were plated on Potato Dextrose Agar (PDA). The infection level of grain was evaluated in percent (0 = all grain healthy, 100 % = all grain infected). Microscopic studies of *Fusarium* fungi were carried out after 7-8 days. The purified single spore cultures were identified on the basis of their cultural and morphological characteristics according to Nelson et al. (1983).

Selection of capture oligonucleotides (CO)

ITS sequences of relevant *Fusarium* species, and other relevant non-*Fusarium* species, were retrieved from GenBank or obtained in this study. From sequence alignments, COs were identified manually with preferably at least 4 mismatches to non-target sequences. The length of oligonucleotides was adjusted to give COs with a Tm of 59°C +/- 2°C calculated using the nearest neighbour method. The COs were synthesised with a 5' NH₂ group followed by a T₁₀ spacer.

Printing and post processing of oligonucleotide arrays

Ten μ l of 20 μ M oligonucleotide solutions in 50% DMSO, 0.5 X SSC and 0.01 % SDS were prepared in trays. Samples were spotted with a Qarray Mini (Genetix, UK) onto Nexterion E slides (Schott) using Genetix tungsten split pins at 45 % humidity. Arrays were ordered in identical 'subarrays' and overlaid with a Fast Frame to allow simultaneous hybridisation to 12 arrays (see example of a 'subarray' hybridisation).

Sample preparation

DNA was extracted from pure cultures or field samples using DNeasy (QIAGEN) for wheat or NucleoSpin Food (Machery-Nagel) for barley and oat according to the manufacturer's instructions. PCR products for hybridisation were produced using primer sets specific for the ITS region. PCR products were random primed labeled with Cy3 using BioPrime CGH (Invitrogen). PCR products were purified using MinElute PCR Purification Kit (QIAGEN) according to manufacturer's instructions.

Microarray hybridisation, washing and scanning.

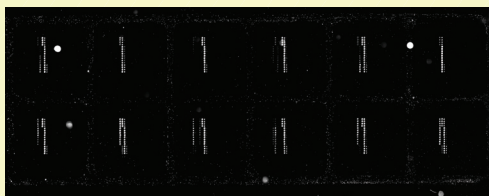
Arrays were hybridised and washed as recommended by the manufacturer (Schott). Briefly, after prehybridisation, approximately one μ g of labelled probe in 100 μ l 5 X SSC, 0.1 μ g sheared salmon sperm DNA and 0.1 % SDS was heated to 95°C for 3 min before the probe mix was applied to multichamber arrays and the arrays were placed in a moist chamber and incubated at 42°C overnight. The hybridised and washed arrays were scanned using an ArrayWoRx[®] Biochip Reader (Applied Precision).

Real-time PCR primers.

DNA sequences of the EF1 α (Elongation factor) gene were retrieved from GenBank. Primers were designed by sequence alignment analysis and the *F. poae* specific primers, FpoaeA51 fwd, 5'ACCGAATCTCAACTCCGCTTT 3' and FpoaeA98 rev, 5'GTCTGTCAAGCATGTAGCAAGT 3' and *F. sporotrichoides* specific primers, FspoA18 fwd, 5'GCAAGTCCAGCACTGTGAGTACA 3' and FspoA85 rev 5'CTGTCAAAGCATGTGAGTAAATGAT 3' were used in real-time PCR.

Real-time PCR reaction conditions.

Real-time PCR were carried out in 12.5 μ l consisting of 6.25 μ l 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 0.375 μ l (10 μ M) forward and reverse primers, 3 μ l water and 2.5 μ l template DNA. Genomic DNA from grain template were diluted 1:10 or 1:20 and pure cultures 1:100 before PCR. PCR was run in a 7900HT Sequence Detection System using the following cycling protocol: 2 min at 50 °C; 95 °C 10 min, 40 cycles of 95 °C for 0.15 min and 62 °C for 1.0 min, 0.15 min at 95 °C; 0.15 min at 60 °C. Dilution series of *F. poae* and *F. sporotrichoides* DNA were used for standard curves.



Example of an array with 'subarrays' showing hybridization with labeled PCR products from different *Fusarium* species.

RESULTS

Species-specific array

COs with specificity for a range of *Fusarium* species were designed and spotted onto arrays. DNA from isolates representing each species was used as template in PCR using ITS specific primers. Products were labeled using random primed labeling and hybridised to the array. Table 1 shows the results obtained for each ITS CO and each of the species or sequence groups.

Field sample analyses

Classical mycological analysis showed that *Fusarium poae* and *Fusarium sporotrichoides* infection levels in grain samples varied from 0 % to 16.7 % in 2005 (Figure 1a,b). Preliminary microarray results indicated that *F. poae* was present in 20 grain samples (from 1342 to 17088 in relative signal intensity) and weak in 1 grain samples (Figure 1c), whereas *F. sporotrichoides* was present in 7 grain samples (1182 – 3554 in relative signal intensity) and weak or negative in 14 samples (Figure 1d). For comparison, *F. poae* and *F. sporotrichoides* were quantified using real-time PCR in the same samples (Figure 1e,f).

Table 1. Results for individual COs hybridised with Cy3 labelled PCR probes from selected *Fusarium* isolates. Signals arbitrarily considered high (from 1000 to 25000 in relative signal intensity) are dark-grey shaded; signals considered weak (200 – 1000) are light-grey shaded and signals below 200 were regarded as negative and are not shaded. The last three digits in the CO code refers to the 3' position of the CO in the following GenBank accession: for Fcul COs, AY147355; Fpse, FFS491294; Fecu, AY147365; Fspo, AF111053; FpoaA, AF414972; FpoaB, AF414967; FpoaC, AY053440; Fave, AY147281; FaveB, AF009187; FtriA, AF111054; FtriB, AF111066; Ffusi, AF111056; Ffucul, AY147355; G. zeae tub, AY225893S1.

Intended specificity of CO	Sequence of CO (5' - 3')	CO code	<i>F. culmorum</i>	<i>F. graminearum</i>	<i>F. pseudograminearum</i>	<i>F. avenaceum</i>	<i>F. poae</i>	<i>F. sporotrichoides</i>	<i>F. equiseti</i>	<i>F. langsethiae</i>	<i>F. tricinctum</i>
<i>F. culmorum</i> / <i>F. graminearum</i>	GGGAGCTGCGATCTCTCTGCG	Fcul-368									
	CAGTCTCTCTCTCTCTCTCTCT	Fcul-377									
<i>F. pseudograminearum</i>	GAGCTCTCTCTCTCTCTCTCT	Fpse-377									
<i>F. equiseti</i>	GTGGAGCTCTCTCTCTCTCTCT	Fecu-395a									
	GTACCCCGCTCTCTCTCTCTCT	Fecu-409									
<i>F. sporotrichoides</i>	GGATCTCTCTCTCTCTCTCTCT	Fspo-357									
	TCTTGGATCTCTCTCTCTCTCT	Fspo-352a ny									
<i>F. langsethiae</i>	GGAGCTCTCTCTCTCTCTCTCT	FspoA-388									
	TCTCTTACCCAGCTCTCTCTCT	FspoA-391									
<i>F. poae</i>	TCTTGGATCTCTCTCTCTCTCT	FpoaA-370									
(FpoaA sequence type)	CTCTCTCTCTCTCTCTCTCTCT	FpoaA-378									
<i>F. poae</i>	GTCTTGGATCTCTCTCTCTCTCT	FpoaB-371									
(FpoaB sequence type)	GAACTCTCTCTCTCTCTCTCTCT	FpoaB-380									
<i>F. avenaceum</i>	GGAGCTCTCTCTCTCTCTCTCT	Ffusti-420									
<i>F. tricinctum</i>	GGATCTCTCTCTCTCTCTCTCT	Ffusti-433									
<i>F. culmorum</i>	GTCTCTCTCTCTCTCTCTCTCT	Fcul-422									
<i>F. graminearum</i>	GTCTCTCTCTCTCTCTCTCTCT	Fcul-448									
<i>F. sporotrichoides</i>	GTCTCTCTCTCTCTCTCTCTCT	Ffucul-408									
<i>F. poae</i>	GTCTCTCTCTCTCTCTCTCTCT	Ffucul-417									
<i>F. pseudograminearum</i>	GTCTCTCTCTCTCTCTCTCTCT	G. zeae tub1									
<i>F. langsethiae</i>	GTCTCTCTCTCTCTCTCTCTCT	G. zeae tub2									
Negative control	GTCTCTCTCTCTCTCTCTCTCT	G. zeae tub3									



Figure 1. *Fusarium poae* and *F. sporotrichoides* infection level measured by morphological data (a and b); microarray data (c and d) and real-time PCR data (e and f) in 21 Lithuanian cereal samples from an organic field experiment in 2005. Units are relative units for the microarray experiment and relative amount of *Fusarium* DNA for the real-time PCR experiment (as calculated from a standard curve).

DISCUSSION

An oligonucleotide array was designed for the detection and differentiation of the most important *Fusarium* species occurring on cereal grain. We tested these oligonucleotide arrays using Cy3 labeled PCR products from the ITS region obtained from pure cultures of a range of *Fusarium* species. The results show that the analysis is capable of differentiating such pure cultures (table 1).

Field samples from a Lithuanian field experiment under organic conditions were analysed using the developed microarray and real-time PCR together with classical mycological methods. This analysis showed that there was a good correspondence between microarray and real-time PCR results for *F. poae* (Figure 1 c, e). These microarray and real-time PCR results did also show quite good correspondence with morphological data (Figure 1 a). For *F. sporotrichoides* correspondence between microarray and morphological (Figure 1 b, d) data were good, however the correspondence between microarray and real-time PCR (Figure 1 d, f) was poor, as the PCR method did not detect *F. sporotrichoides* in all samples that were positive in the microarray analysis. These results are unexpected and will be further investigated. These preliminary microarray results indicate that microarray method is usable for *Fusarium* detection.

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