Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Molecular diagnostics on the toxigenic potential of *Fusarium* spp. plant pathogens

A. Dawidziuk[1], G. Koczyk[1], D. Popiel[1], J. Kaczmarek[2] and M. Buśko[3]

1 Functional Evolution of Biological Systems Team, Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland
2 Molecular Plant Pathology Team, Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland
3 Department of Chemistry, Poznan University of Life Sciences, Poznan, Poland

Keywords
fungi, markers, mycotoxins, plant pathology.

Correspondence
Grzegorz Koczyk, Functional Evolution of Biological Systems Team, Institute of Plant Genetics, Polish Academy of Sciences, 60-479 Poznan, Strzeszynska 34, Poland.
E-mail: gkoc@igr.poznan.pl

2013/2370: received 27 November 2013, revised 5 February 2014 and accepted 24 February 2014

doi:10.1111/jam.12488

Abstract

Aims: We propose and test an efficient and rapid protocol for the detection of toxigenic *Fusarium* isolates producing three main types of *Fusarium*-associated mycotoxins (fumonisins, trichothecenes and zearelanone).

Methods and Results: The novel approach utilizes partially multiplexed markers based on genes essential for mycotoxin biosynthesis (fumonisin—*fum6*, *fum8*; trichothecenes—*tri5*, *tri6*; zearalenone, *zea2*) in *Fusarium* spp. The protocol has been verified by screening a collection of 96 isolates representing diverse species of filamentous fungi. Each *Fusarium* isolate was taxonomically identified through both molecular and morphological techniques. The results demonstrate a reliable detection of toxigenic potential for trichothecenes (sensitivity 100%, specificity 95%), zearalenone (sensitivity 100%, specificity 100%) and fumonisins (sensitivity 94%, specificity 88%). Both presence and identity of toxin biosynthetic genes were further confirmed by direct sequencing of amplification products.

Conclusions: The cross-species-specific PCR markers for key biosynthetic genes provide a sensitive detection of toxigenic fungal isolates, contaminating biological material derived from agricultural fields.

Significance and Impact of the Study: The conducted study shows that a PCR-based assay of biosynthetic genes is a reliable, cost-effective, early warning system against *Fusarium* contamination. Its future use as a high-throughput detection strategy complementing chemical assays enables effective targeted application of crop protection products.

Introduction

The numerous plant pathogens of the genus *Fusarium* are responsible for significant losses in crop yield due to both loss of biomass and accumulation of mycotoxins in infiltrated parts. The major toxic compounds synthesized by divergent *Fusarium* isolates include the following: zearalenone, fumonisins, trichothecenes and their derivatives (D'Mello *et al.* 1999). While there is a growing body of work documenting biological significance of additional, emergent toxins (e.g. butenolide, fusarins, equisetin, beauvericin and enniatins), their estimated economic and biomedical importance is considerably lower (Desjardins and Proctor 2007).

Notably, the above-mentioned major toxins (fumonisins, trichothecenes, zearalenone and derivative compounds) are frequently not inactivated during food/feed processing and can be present in a masked form (plant-formed conjugates, i.e. glucosides, which can be activated by mammal gut microbiota—e.g. Berthiller *et al.* 2013; Dall'Erta *et al.* 2013), increasing health risks to farm animals and humans (Creppy *et al.* 2002). As more research results are collected, the estimates of health and economical risks (associated with long-term masked mycotoxin

exposure) are revised upwards. The updated estimates lead to increasingly restrictive norms for toxin content for food and feed (e.g. European Commission Recommendation 2006/576/EC proposing norms for ochratoxin A, T-2 and HT-2 toxins as well as deoxynivalenol and zearalenone). This only serves to increase a need for efficient and quick methods of assessing possible sources of contamination, preferably by preventing losses in crop yield, via good farming practices including effective fungicide treatments.

The genetic determinants of fumonisin, trichothecene and zearalenone biosynthesis have been characterized in multiple plant pathogenic taxa. Characterization of both core biosynthetic genes (polyketide synthases, trichodiene synthase) and key accessory genes (such as transcription factors or key processing enzymes) enables construction of toxigenicity assays directly targeting the genetic basis of toxin production and accumulation. At the same time (Stepien *et al.* 2011), the biosynthetic gene alleles exhibit significant interspecific differences, which makes them useful for precise identification of infectious species/populations.

The zearalenone biosynthetic cluster spanning 25 kb of the genomic sequence has been characterized in *Fusarium graminearum* (Kim *et al.* 2005), with four principal genes required for toxin biosynthesis (*zea1*, *zea2*, *zeb1*, *zeb2*) and 3 other genes regulated in conjunction with *zeb2* expression patterns (FG02394, FG02399 and FG012015—uncovered by qRT-PCR experiments described by Lysøe *et al.* (2009)).

Conversely, trichothecene biosynthesis constitutes a multistage process, controlled by at least 12 essential genes, forming a 25-kb-long cluster in *F. graminearum* (Brown *et al.* 2001; Kimura *et al.* 2003). The trichothecene cluster is linked to a key *tri5* gene encoding trichodiene synthase, however, four genes segregate at separate loci (notably *tri13* and *tri14* controlled by a transcription factor encoded by *tri10*—Tag *et al.* (2001)). To date, the main cluster has been extensively characterized with numerous studies targeted especially at *F. graminearum* and *F. sporotrichioides* species (Kimura *et al.* 2007), as well as some members of the genus *Trichoderma* (Cardoza *et al.* 2011). There is considerable evidence for complex gene relocation scenarios underlying chemotype diversification leading to extant trichothecene type-A- and type-B-producing species (Proctor *et al.* 2009).

In the past decade, the fumonisin cluster structure (16 gene cluster spanning 42 kb length) has been determined for three toxigenic *Fusarium* species: *F. verticillioides*, *F. oxysporum* (FRC O-1890 strain) and *F. proliferatum* (Proctor *et al.* 2008). The interspecies differences between individual biosynthesis-related sequences encompass up to 20% of constituent residues. Notably larger differences are found in gene-flanking regions, an observation which suggests divergent evolutionary paths for cluster copies in different species. Here, the difference in species history and gene phylogeny has been attributed to complex birth/death evolution of the cluster (with independent sorting of copies) and/or horizontal gene transfer events (Proctor *et al.* 2013). During fumonisin biosynthesis, substitutions of polyketide synthase and/or termination factor can lead to significant changes in the specificity of polyketide condensation for fumonisin analogs (Zhu *et al.* 2008; Li *et al.* 2009).

As the broad, genetic basis of the biosynthetic pathway for three major *Fusarium* mycotoxins is known and multiple exemplar sequences are readily available, it is now possible to develop targeted diagnostic solutions. Through utilizing knowledge about disparate species for the design of degenerate cross-species-specific primers, it is possible to target well-conserved parts of coding sequence (corresponding to conserved parts of protein sequence). Especially for core, secondary metabolite biosynthetic genes, these regions of the coding sequence are unlikely to change in toxin-producing isolates (corroborated by recent evidence for purifying selection in secondary metabolism genes—e.g. Baker *et al.* 2012).

Current studies on the variability and diversity of the fungal populations make use of various genetic markers, such as the translation elongation factor (*tef-1α*) and internal transcribed spacer (ITS1/2), employed in assays of the genus *Trichoderma* (Chaverri *et al.* 2003; Blaszczyk *et al.* 2011) and conservative fragments of the genome such as a calmodulin gene (CaM) in *Trichoderma* and *Fusarium* populations (Chaverri *et al.* 2003; Mulè *et al.* 2004). Also, mitochondrial DNA (mtDNA) is used as a marker of genetic variation. Its relatively short length and the presence of conserved and variable regions allow the identification of closely related species (Ma and Michailides 2007). The sequence of the large subunit of the RNA polymerase II (Hibbett *et al.* 2007) can also be used to distinguish between divergent phytopathogenic species. Among so many molecular markers, the translation elongation factor (*tef-1α*) appears to be the most useful in taxonomic studies of fungi, especially in the genus *Fusarium* (Geiser *et al.* 2004; Kristensen *et al.* 2005). Recently, more attention is devoted to markers directly involved in the secondary metabolism (Proctor *et al.* 2009). Many researchers use genes from the FUM cluster as a good additional marker for phylogenetic and taxonomic studies of the fumonisin-producing *Fusarium* species (González-Jaén *et al.* 2004; Baird *et al.* 2008; Stepien *et al.* 2011).

The current line of research for the detection of toxigenic species involves simultaneous use of multiple genes belonging to different clusters responsible for toxin production, for example mPCR assays detecting aflatoxigenic,

trichothecene- and fumonisin-producing and ochratoxigenic fungal isolates (Rashmi *et al.* 2013). The recent studies also aim to combine qualitative and quantitative methods for detecting the toxigenic potential. One of the approaches, based on multiplex real-time PCR, is able to detect and quantify mycotoxigenic species in cereal grains with the use of markers targeting the trichothecene synthase (*tri5*) gene in trichothecene-producing *Fusarium* sp. isolates, the rRNA gene in *Penicillium verrucosum* and the polyketide synthase gene (Pks) in *Aspergillus ochraceus* (Vegi and Wolf-Hall 2013).

The problem addressed in the proposed work was to design and standardize a diagnostic tool allowing the identification of toxigenic *Fusarium* isolates producing fumonisin B_1, trichothecenes and zearalenone. The new protocol is applicable for both *in vitro* and field samples, with resolution sufficient for direct sequencing of amplified sequences.

Materials and methods

Fungal isolates and field samples

Fungal isolates originated from the culture collections of the Institute of Plant Genetics (Polish Academy of Sciences, Poznan, Poland). The isolates originated from soil, infected cereal grain samples and buildings infested by fungal pathogens. To avoid contamination of fungal cultures with cryptic species, which are hard to distinguish with traditional morphological methods, isolates were purified using single-spore culturing (Leslie and Summerell 2006). Scabby kernels were plated on small nutrient agar (SNA) medium in Petri dishes, and taxa were morphologically identified using an optical microscope (Olympus, Center Valley, PA) at 400–500 × magnification, according to the manual of Leslie and Summerell (2006). Mycelia of isolates cultivated on potato dextrose agar (PDA) were used for DNA isolation. All 96 isolates were identified with at least one molecular marker (ITS 1/2 and/or *tef-1α* marker), and species assignment was carried out through comparison with reference sequences in NCBI/GenBank and Fusarium-ID (Geiser *et al.* 2004). Assignment of species to monophyletic complexes was based on the recent taxonomic and phylogenetic research conducted by O'Donnell *et al.* (2013).

DNA extraction from fungal cultures and field samples

Fungal cultures

Mycelium used for DNA extraction was grown in Czapek-Dox broth (Sigma-Aldrich, St Louis, MO) with yeast extract (Oxoid, Waltham, MA) and streptomycin sulphate (50 mg l^{-1}; AppliChem, Darmstadt, Germany) and after incubation at 25°C for 21 days on a rotary shaker (100 g). Mycelium was collected on filter paper in a Büchner funnel and freeze-dried. Total DNA was extracted using the CTAB method (Doohan *et al.* 1998). The quality of DNA was estimated by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, NC) and via Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA).

Field samples

Infected wheat chaffs and kernels (2012, Parabola cultivar) were ground to fine powder, and DNA was obtained using the DNase kit (Qiagen, Hilden, Germany).

Primer design

The degenerate, cross-species-specific primers were designed on the basis of backtranslated codon alignments created from protein sequence alignments of homologous genes from NCBI/RefSeq release ver. 56 (Pruitt *et al.* 2012) and NCBI/GenBank release ver. 194 (Benson *et al.* 2013) and Ensembl/Fungi (Flicek *et al.* 2012) release 18. Protein alignments for *fum8*, *fum6*, *zea2*, *tri5* and *tri6* genes were obtained with MAFFT-LINSI (Katoh and Toh 2010), subsequently backtranslated and screened for primers with Python scripts. Primer sequences were screened against propensity for homodimer and heterodimer formation on the basis of nearest-neighbour energy/melting temperature calculations with both IDT OligoAnalyzer and in-house Python scripts implementing nearest-neighbour enthalpy/entropy calculations described by SantaLucia (1998) with corrections based on Owczarzy *et al.* 2008.

PCR amplification

The PCR was carried out in a 25 μl reaction mixture containing the following: 1 μl of DNA (50 ng μl^{-1}), 12·5 μl PCR buffer (50 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris-HCl, pH 8·8, 0·1% TritonX-100), 1U polymerase (Sigma-Aldrich), l0 mmol l^{-1} dNTP (Invitrogen, Carlsbad, CA), 0·5 μl 100 mmol l^{-1} of each primer and 11·5 μl H_2O. Amplifications were performed in C1000 Touch™ Thermal Cycler (Bio-Rad) under the following conditions: initial denaturation 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 53–56°C (Table 1), 1 min at 72°C and for the final extension 10 min at 72°C. Amplification products were separated on a 1·5% agarose gel (Invitrogen) in 1 × TBE buffer (0·178 mol l^{-1} Tris-borate, 0·178 mol l^{-1} boric acid, 0·004 mmol l^{-1} EDTA) and stained with ethidium bromide. The 10 μl PCR products were combined with 2 μl of loading buffer (0·25% bromophenol blue, 30% glycerol). A 100-bp DNA LadderPlus (Fermentas, St. Leon-Rot, Germany) was used

Table 1 The sequences of the primers used for amplification and sequencing

Gene targeted	Primer name	Sequences (5′–3′)	Estimated product length (base pairs)
Trichodiene synthase (*tri5*)	T5_am_fA1	CTY MRR ACM ATY GTN GGC ATG	468
	T5_am_rA1	AVA CCA TCC AGT TYT CCA TYT G	
Zinc finger transcription factor (*tri6*)	TRI6_dm_fA2	TAT GAA TCA CCA ACW TTC GA	526
	TRI6_dm_rA1	CGC CTR TAR TGA TCY CKC AT	
Zearalenone polyketide synthase (*zea2*)	ZEA2_dm_fA1	ACM TCA CCA TCM AAR TTC TG	340
	ZEA2_dm_rA1	GCR TCY CKG TAR TCR CTC AT	
Oxygenase (*fum6*)	FUM6_dm_fA2	CRA CMG AGA TCA TGG TGA C	672
	FUM6_dm_rA1	GTY TCR TGT CCK GCA ATG AG	
Oxoamine synthase (*fum8*)	F8_am_fA1	GGY TCK TTT GAG TGG TGG C	350
	F8_am_rA1	CRA CWG GAA ARC AKA YRA YGG	

as a size standard. PCR products were electrophoresed at 3 Vcm^{-1} for about 2 h, visualized under UV light and photographed (Gel DOC EZ Imager; Bio-Rad).

Sequencing

The 3-μl PCR products were purified with exonuclease I and shrimp alkaline phosphatase according to Chelkowski *et al.* (2003). Sequencing reactions were prepared using the ABI Prism BigDye Terminator Cycle Sequencing ReadyReaction Kit in 5 μl volume (Applied Biosystems, Grand Island, NY). DNA sequencing was performed on an ABI PRISM3100 GeneticAnalyzer (Applied Biosystems).

Sequences were edited and assembled using Chromasv.1.43 (Applied Biosystems). CLUSTAL W (Thompson *et al.* 1994) and MUSCLE (Edgar 2004) were used to align the sequences; the resulting alignments were inspected and refined manually. All positions containing gaps and missing data were eliminated from the data set.

Multiplex PCR

The multilplex PCR was carried out in a 25 μl reaction mixture containing the following: 1 μl 50 ng μl^{-1} of DNA, 4 μl PCR buffer (20 mmol l^{-1} Tris-HCl, 0·1 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, 100 mmol l^{-1} KCl, stabilizers, 200 μg/ml BSA and 50% glycerol), 1U polymerase (Thermo Scientific), l0 mmol l^{-1} dNTP (Invitrogen), 0·5 μl 100 mmol l^{-1} of each primer and 14·5 μl H_2O. To each reaction mixture, 3 μl of Q-Solution was added to avoid primer dimerization. Amplifications were performed in Touch™ Thermal Cycler (Bio-Rad) under the following conditions: initial denaturation 30 s at 98°C, 35 cycles of 5 s at 98°C, 5 s at 55°C, 15 s at 72°C, with the final extension of 1 min at 72°C. Amplification products were separated on 2% agarose gel (Invitrogen) in 1 × TBE buffer (0·178 mol l^{-1} Tris-borate, 0·178 mol l^{-1} boric acid, 0·004 mol l^{-1} EDTA) and stained with Midori Green (Nippon Genetics, Dueren, Germany). A 100-bp DNA LadderPlus (Fermentas) was used as a size standard. PCR products were electrophoresed at 3 Vcm^{-1} for about 2 h, visualized under ultraviolet (UV) light and photographed (Gel DOC EZ Imager; Bio-Rad).

Determination of trichothecenes concentration

Determination of trichothecenes was performed in solid PDA culture. Briefly, subsamples (1 g of mycelium with medium) were extracted with acetonitrile/water (82 : 18) and cleaned-up on a Myco Sep 227 Trich + column. The group B trichothecenes (DON, NIV, 3AcDON, 15AcDON, FUS-X) were analysed as trimethylsilylsilyl ethers derivatives. After sililation, samples were extracted with isooctane and 1 μl of sample was injected on a GC/MS system. The analyses were run on a gas chromatograph (Hewlett Packard GC 6890, Waldbronn, Germany) hyphenated to a mass spectrometer (Hewlett Packard 5972 A, Waldbronn, Germany), using an HP-5MS, 0·25 mm × 30 m capillary column. The injection port temperature was 280°C, the transfer line temperature was 280°C, and the analyses were performed with programmed temperature. Initial temperature was 80°C held for 1 min, from 80 to 200°C at 15°C min^{-1} held 6 min and from 200 to 280°C at 10°C min^{-1}, the final temperature being maintained for 3 min. The helium flow rate was held constant at 0·7 ml min^{-1}. Quantitative analysis was performed in single ion monitored mode, and qualitative analysis was performed in SCAN mode (100–700 amu). Recoveries for analysed toxins were as follows: DON 84 ± 3·8%; 3AcDON 78 ± 4·8%; 15AcDON 74 ± 2·2%; FUS X 87%±5·9%; NIV 81 ± 3·8%. Limit of detection was 0·01 mg kg^{-1}.

Determination of zearalenone concentration

Determination of zearalenone was performed in solid PDA medium. Subsamples (1 g of mycelium with med-

ium) were extracted with acetonitrile/water (82 : 18) and cleaned-up on Zearala test affinity columns. Prepared samples were analysed by HPLC consisting of a Waters HPLC 2695 apparatus with a Waters 2475 Multi λ Fluorescence Detector and a Waters 2996 Array Detector (Waters, Milford, MA). Separation was achieved on a 150 mm length × 3·9 mm diameter Nova Pak C-18, 4-μm particle size column and eluted with acetonitrile–water–methanol (46 : 46 : 8, v/v/v) at a flow rate of 0·5 ml min^{-1}. ZEA was detected with a Waters 2475 Multi λ Fluorescence Detector, and the excitation and emission wavelengths were 274 and 440 nm, respectively. Estimation of ZEA was performed by a comparison of peak areas with those of an external standard (>95%; Sigma-Aldrich) or by co-injection with the standard. The detection limit of ZEA was 3 ng g^{-1}. The similar process was used to determine zearalenone concentration in a wheat bioassay (Gromadzka *et al.* 2009).

Determination of fumonisin B_1 concentration

The samples (5 ml of liquid culture) were filtered through Whatman No. 5 (Whatman, Piscataway , NJ) filter paper and dried under nitrogen stream. The residues were dissolved into methanol water (3 : 1, v/v), adjusted to the pH value of 5·8–6·5 by 0·1 mol l^{-1} KOH water solution and cleaned using a SAX cartridge. The cartridge was conditioned with 5 ml of methanol followed by 5 ml of methanol–water (3 : 1, v/v). FB1 was eluted from the column to a glass collection vial with 10 ml of 1% acetic acid in methanol. The eluate was evaporated to dryness at 40°C under a stream of nitrogen. The cleaned sample was derivatized with OPA reagent (20 mg 0·5 ml^{-1} methanol diluted with 2·5 ml 0·1 mol l^{-1} disodium tetraborate ($Na_2B_4O_7 \times 10H_2O$), then combined with 25 μl 2-mercaptoethanol) immediately before HPLC analysis by mixing the OPA reagent and the sample in ratio 4 : 1 v/v. After 2 min, the reaction mixture (10 μl) was injected in a HPLC C18 Nova Pak column (3·9 × 150 mm). Methanol–sodium dihydrogen phosphate (0·1 mol l^{-1} in water) solution (77 : 23; v/v) was adjusted to pH 3·35 with *o*-phosphoric acid and used as the mobile phase with the flow rate of 0·6 ml min^{-1}. A Waters 2695 HPLC with a fluorescence detector (λ_{Ex} = 335 nm and λ_{Em} = 440 nm, Waters 2475; Waters) was used for analysis.

Benchmarking

Diagnostic quality parameters (sensitivity, specificity and comparison with a naive predictor) were assessed with the open-source software R (ver. 2.15.2) using the caret package (Kuhn 2008). Results of chemotype identification and marker testing were visualized using in-house R scripts dependent on ggplot2 (Wickham 2009).

Results

Sensitivity and specificity of diagnostic markers

Sensitivity and specificity of designed markers (Table 1) were tested on a collection of 96 fungal isolates. The tests took into account divergent *Fusarium* species (72 isolates) as well as multiple non-*Fusarium* filamentous fungi (24 isolates) (Fig. 2). The individual performance of markers was as follows: trichothecene biosynthesis (*tri5* + *tri6*, sensitivity 100%, specificity 95%, *P*-value *vs* naive classifier: 1·42e-09), zearalenone (*zea2*, sensitivity 100%, specificity 100%, *P*-value *vs* naive classifier: 7·48e-09) and fumonisin (*fum6* + *fum8*, sensitivity 89%, specificity 89%, *P*-value *vs* naive classifier: 3·53e-07). The final results show that the protocol can reliably identify the toxigenic potential for all three toxin groups (trichothecenes, zearalenone and fumonisins) with a sensitivity and specificity of over 90%, excepting fumonisin production within the *F. oxysporum* complex (see also Discussion).

Confirmation of results via chemical analysis and sequencing

Taxonomic identification of all isolates was confirmed by sequencing and analysis of rDNA internal transcribed spacer (ITS; 95 isolates) and/or translation elongation factor 1 α (*tef-1*α; 41 isolates) partial sequences (Table 2). The identified *Fusarium* chemotypes are consistent with the recent knowledge on species-related compounds (Moretti *et al.* 2013), and in case of all positive markers, those results were confirmed by direct sequencing of the PCR product (Fig. 1; Table S4).

Chemical analyses have shown that the analysed isolates produce highly varying amounts of toxin. Additionally, in case of *F. sporotrichioides*, no chemotype (zearalenone/trichothecene biosynthesis) was detected on potato dextrose agar (PDA medium); however, we were able to qualitatively observe accumulation of toxins in a wheat bioassay (Tables S1–S3). This is consistent with reported influence of different carbon sources on toxin production (Jiao *et al.* 2008) and suggests differentially regulated expression in *F. sporotrichioides* compared to *F. graminearum*.

Although no quantitative assay of the type-A trichothecene accumulation was conducted, the genes present in *F. sporotrichioides* isolates are highly similar to the model *F. sporotrichioides* counterparts. Notably, polymorphisms in both *tri5* and *tri6* partial sequences, obtained from direct sequencing, can unambiguously differentiate

Table 2 Fungal isolates from the collection of the Institute of Plant Genetics PAS (Functional Evolution of Biological Systems Team) used to develop a multiplex PCR. The naming of monophyletic complexes within *Fusarium* sp. derived from (O'Donnell *et al.* 2013)

					Chemotype				Molecular identification	
Complex	Species	Collection number	Source	Year of isolation	Fumonisin B_1	Trichothecene A	Trichothecene B	Zearalenone	ITS1/2	tef-1α
F. fujikuroi	*F. proliferatum*	1	Italy	1984	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	3	Canada	1982	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	7	Poland	1986	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	18	Norway	2006	+	–	–	–	+	–
F. fujikuroi	*F. proliferatum*	21	Italy	1986	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	44	Poland	1993	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	58	USA	1993	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	59	Poland	1999	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	66	Poland	1999	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	82	Poland	2006	+	–	–	–	+	–
F. fujikuroi	*F. proliferatum*	84	Poland	2006	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	85	Norway	2006	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	99	Italy	1993	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	111	Poland	2008	+	–	–	–	+	–
F. fujikuroi	*F. proliferatum*	113	Poland	2008	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	141	Poland	2010	+	–	–	–	+	+
F. fujikuroi	*F. proliferatum*	142	Poland	2011	+	–	–	–	+	+
F. fujikuroi	*F. subglutinans*	60	Poland	1984	+	–	–	–	+	–
F. fujikuroi	*F. succisae*	4	Poland	1996	–	–	–	–	+	+
F. fujikuroi	*F. temperatum*	151	Poland	1987	+	–	–	–	+	–
F. fujikuroi	*F. proliferatum*	13	Poland	1988	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	16	Poland	1987	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	17	Poland	1987	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	23	Poland	1982	+	–	–	–	+	+
F. fujikuroi	*F. verticillioides*	29	Poland	1985	+	–	–	–	+	+
F. fujikuroi	*F. verticillioides*	43	Poland	1986	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	45	Poland	1982	+	–	–	–	+	+
F. fujikuroi	*F. verticillioides*	71	Poland	1986	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	75	Poland	2010	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	79	Poland	2010	+	–	–	–	+	–
F. fujikuroi	*F. verticillioides*	88	Poland	1982	+	–	–	–	+	–
F. fujikuroi	*F. xyllarioides*	67	Guinea	1985	–	–	–	–	+	+
F. incarnatum-equiseti	*F. equiseti*	72	Poland	2010	–	+	–	+	+	–
F. oxysporum	*F. oxysporum*	19	Poland	2010	+*	–	–	–	+	+
F. oxysporum	*F. oxysporum*	55	Poland	1997	+*	–	–	–	+	–

(Continued)

Table 2 (*Continued*)

Complex	Species	Collection number	Source	Year of isolation	Chemotype				Molecular identification	
					Fumonisin B_1	Trichothecene A	Trichothecene B	Zearalenone	ITS1/2	tef-1α
F. oxysporum	*F. oxysporum*	57	Poland	2010	+*	–	–	–	+	+
F. oxysporum	*F. oxysporum*	62	Poland	2010	+*	–	–	–	+	–
F. oxysporum	*F. oxysporum*	65	Poland	1984	+*	–	–	–	+	–
F. oxysporum	*F. oxysporum*	69	Poland	2010	+*	–	–	–	+	–
F. oxysporum	*F. oxysporum*	115	Poland	2010	+*	–	–	–	+	–
F. oxysporum	*F. oxysporum*	131	Poland	2010	+*	–	–	–	+	–
F. sambucinum	*F. cerealis*	33	Poland	1998	–	–	+	+	+	–
F. sambucinum	*F. cerealis*	41	Poland	1986	–	–	+	+	+	–
F. sambucinum	*F. cerealis*	87	Poland	1987	–	–	+	+	+	–
F. sambucinum	*F. culmorum*	48	Poland	1984	–	–	+	+	+	–
F. sambucinum	*F. culmorum*	49	Poland	1997	–	–	+	+	+	–
F. sambucinum	*F. culmorum*	70	Poland	2010	–	–	+	+	+	–
F. sambucinum	*F. culmorum*	90	Poland	1982	–	–	+	+	+	–
F. sambucinum	*F. culmorum*	93	Poland	1986	–	–	+	+	+	–
F. sambucinum	*F. graminearum*	52	Poland	1986	–	–	+	+	+	–
F. sambucinum	*F. graminearum*	76	Poland	1986	–	–	+	+	+	–
F. sambucinum	*F. graminearum*	144	Poland	2011	–	–	+	+	+	–
F. sambucinum	*F. graminearum*	149	Poland	1986	–	–	+	+	+	–
F. sambucinum	*F. poae*	12	Poland	1987	–	–	+	–	+	–
F. sambucinum	*F. sporotrichioides*	8	Poland	1999	–	+	–	+	+	–
F. sambucinum	*F. sporotrichioides*	32	Poland	2010	–	+	–	+	+	–
F. sambucinum	*F. sporotrichioides*	39	Poland	2010	–	+	–	+	+	–
F. sambucinum	*F. sporotrichioides*	54	Poland	1993	–	+	–	+	+	–
F. sambucinum	*F. sporotrichioides*	106	Poland	2010	–	+	–	+	+	–
F. sambucinum	*F. sporotrichioides*	116	Poland	2010	–	+	–	+	+	–
F. sambucinum	*F. sporotrichioides*	119	Poland	2010	–	+	–	+	+	–
F. tricinctum	*F. avenaceum*	68	Poland	2010	–	–	–	–	+	+
F. tricinctum	*F. avenaceum*	105	Poland	2010	–	–	–	–	+	–
F. tricinctum	*F. avenaceum*	108	Poland	2010	–	–	–	–	+	+
F. tricinctum	*F. avenaceum*	114	Poland	2010	–	–	–	–	+	+
F. tricinctum	*F. avenaceum*	117	Poland	2010	–	–	–	–	+	–
F. tricinctum	*F. avenaceum*	120	Poland	2011	–	–	–	–	+	+
F. tricinctum	*F. avenaceum*	132	Poland	2011	–	–	–	–	+	–
F. tricinctum	*F. tricinctum*	14	Poland	2011	–	–	–	–	+	–
F. tricinctum	*F. tricinctum*	31	Poland	1986	–	–	–	–	+	–

(*Continued*)

Table 2 (*Continued*)

Complex	Species	Collection number	Source	Year of isolation	Chemotype				Molecular identification	
					Fumonisin B_1	Trichothecene A	Trichothecene B	Zearalenone	ITS1/2	tef-1α
F. tricinctum	*F. tricinctum*	109	Poland	2010	–	–	–	–	+	–
F. solani	*F. solani*	6	Poland	1997	–	–	–	–	+	–
NA	*Alternaria alternata*	129	Poland	2010	–	–	–	–	+	+
NA	*A. alternata*	139	Poland	2010	–	–	–	–	+	+
NA	*Alternaria brassicicola*	128	Poland	2010	–	–	–	–	+	+
NA	*Alternaria* sp.	97	Poland	2010	–	–	–	–	+	+
NA	*Aspergillus niger*	148	Poland	2010	+	–	–	–	+	–
NA	*Clonostachys rosea*	20	Poland	2010	–	–	–	–	+	–
NA	*Clonostachys* sp.	104	Poland	2010	–	–	–	–	+	–
NA	*Penicillium commune*	136	Poland	2010	–	–	–	–	+	–
NA	*P. commune*	138	Poland	2010	–	–	–	–	+	–
NA	*P. herbarum*	137	Poland	2010	–	–	–	–	+	–
NA	*Trichoderma aggressivum*	100	Poland	2009	–	–	–	–	+	+
NA	*Trichoderma atroviride*	98	Poland	2009	–	–	–	–	+	+
NA	*T. atroviride*	158	Poland	2010	–	–	–	–	–	+
NA	*Trichoderma hamatum*	95	Poland	2010	–	–	–	–	+	–
NA	*T. hamatum*	133	Poland	2010	–	–	–	–	+	+
NA	*T. harzianum*	5	Poland	2010	–	–	–	–	+	+
NA	*T. harzianum*	25	Poland	2010	–	–	–	–	+	+
NA	*T. harzianum*	123	Poland	2010	–	–	–	–	+	+
NA	*T. harzianum*	125	Poland	2010	–	–	–	–	+	+
NA	*T. harzianum*	153	Poland	2010	–	–	–	–	+	+
NA	*T. harzianum*	154	Poland	2010	–	–	–	–	+	+
NA	*Trichoderma longibrachiatum*	124	Poland	2010	–	–	–	–	+	+
NA	*Trichoderma viridescens*	24	Poland	2010	–	–	–	–	+	+
NA	*T. viridescens*	96	Poland	2009	–	–	–	–	+	+

*Some of *F. oxysporum* isolates have the capacity to produce small amounts of fumonisin B_1.

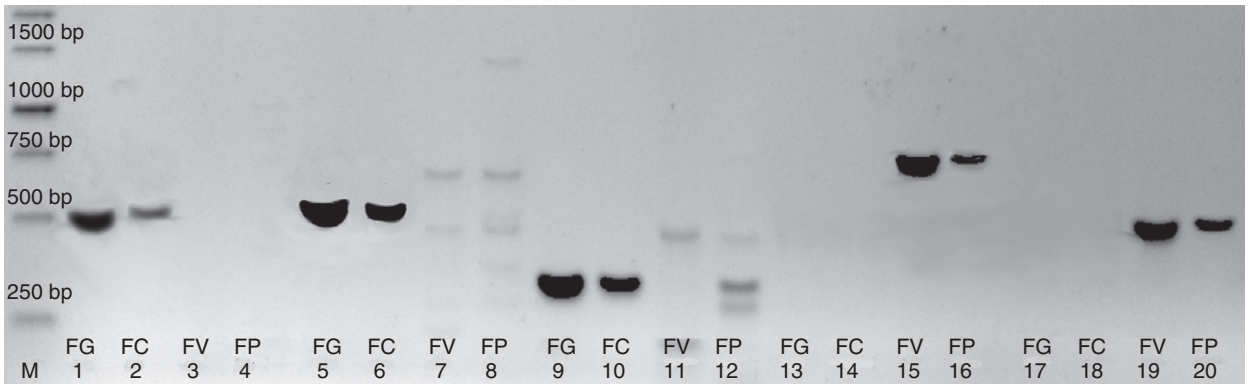

Figure 1 Markers used for diagnostics of the toxigenic potential. (Lane M—DNA marker, line 1–4 *tri5* marker; line 5–8 *tri6* marker; line 9–12 *zea2* marker; line 13–16 *fum6* marker; line 17–20 *fum8* marker; FC—*Fusarium culmorum*, FG—*F. graminearum*, FV—*F. verticilioides*, FP—*F. proliferatum*).

between trichothecene type-A and type-B producers (*F. graminearum*, *F. culmorum*, *F. cerealis*, *F. poae*) within the *F. sambucinum* complex (Fig. 2).

False-positive results observed in *Fusarium xyllarioides* and *F. succisae* were identified as coding sequences corresponding to unrelated genes. In practice, the resulting product band was also visibly different (very weak and of different height) and easily told apart from specific product. In case of *zea2*, the product of amplification in a single *Phoma herbarum* isolate was identified as a related gene (reducing polyketide synthase, highly similar to hypothemycin-reducing polyketide synthase from *Hypomyces subiculosus*; Reeves *et al.* (2008)). Again, the band was of visibly different height; however, the strength and quality of amplification suggests that *zea2* marker could be adapted towards the recognition of different reducing polyketide synthases involved in resorcyclic acid biosynthesis.

Multiplex PCR

Following the assessment of the individual marker performance, the assay was tested and optimized towards multiplexing the PCR. Multiplexing attempts have shown best results for assays over two separate sets of multiplexed markers: *zea2* + *tri5* + *tri6* for the trichothecene/zearalenone and *fum6* + *fum8* for the fumonisine toxigenic potential (Fig. 3). In case of both detection sets, the amplification of at least one product was taken to confirm the toxigenic potential of pathogens infecting the tested sample.

Field samples

In addition to the standard PCRs conducted on DNA obtained from cultivated isolates, the toxigenic potential was also examined on genetic material obtained directly from infected tissue samples. This resulted in the positive identification of infected wheat kernel samples (Fig. 4); however, this was not successful in samples of the wheat chaff. Multiplex PCRs conducted on diluted samples (500, 50, 5 ng, 500 pg) gave distinct, specific signatures even at the lowest DNA concentration level of 500 pg.

Discussion

In this study, we present a novel approach to detect the toxigenic potential of various phytopathogenic fungi by partially multiplexed, degenerate primers based on the genes essential for biosynthesis of major *Fusarium* sp. mycotoxins (fumonisins, trichothecenes and zearalenone). Such tools are especially valuable when updated risk assessments concerning fungal toxin contamination lead to more restrictive norms regulating their acceptable levels in food and/or feed. These trends result in an increase in demand for efficient and rapid methods for the detection and assessment of potential sources of contamination which can be used also as a part of decision support systems (DSS). At the moment, DSS are primarily focused on the observation of the occurrence of pathogens on host plants (Evans *et al.* 2008), spores in the air (Kaczmarek *et al.* 2009) or the impact of weather conditions on the life cycles of pathogens (Dawidziuk *et al.* 2012).

The isolates of the *F. oxysporum* complex constitute a remarkable outlier in the results obtained for fumonisin-producing species. In this case, the trace amounts of fumonisin were found in cultures of several isolates, but mPCR markers were consistently absent. Previous works by Proctor *et al.* (2008, 2013) demonstrate possible divergent origins of the fumonisin clusters in distinct member species of *F. oxysporum* and *F. fujikuroi* complexes. Past research also shows that synthesis of the long reduced polyketide mycotoxins is controlled by accessory

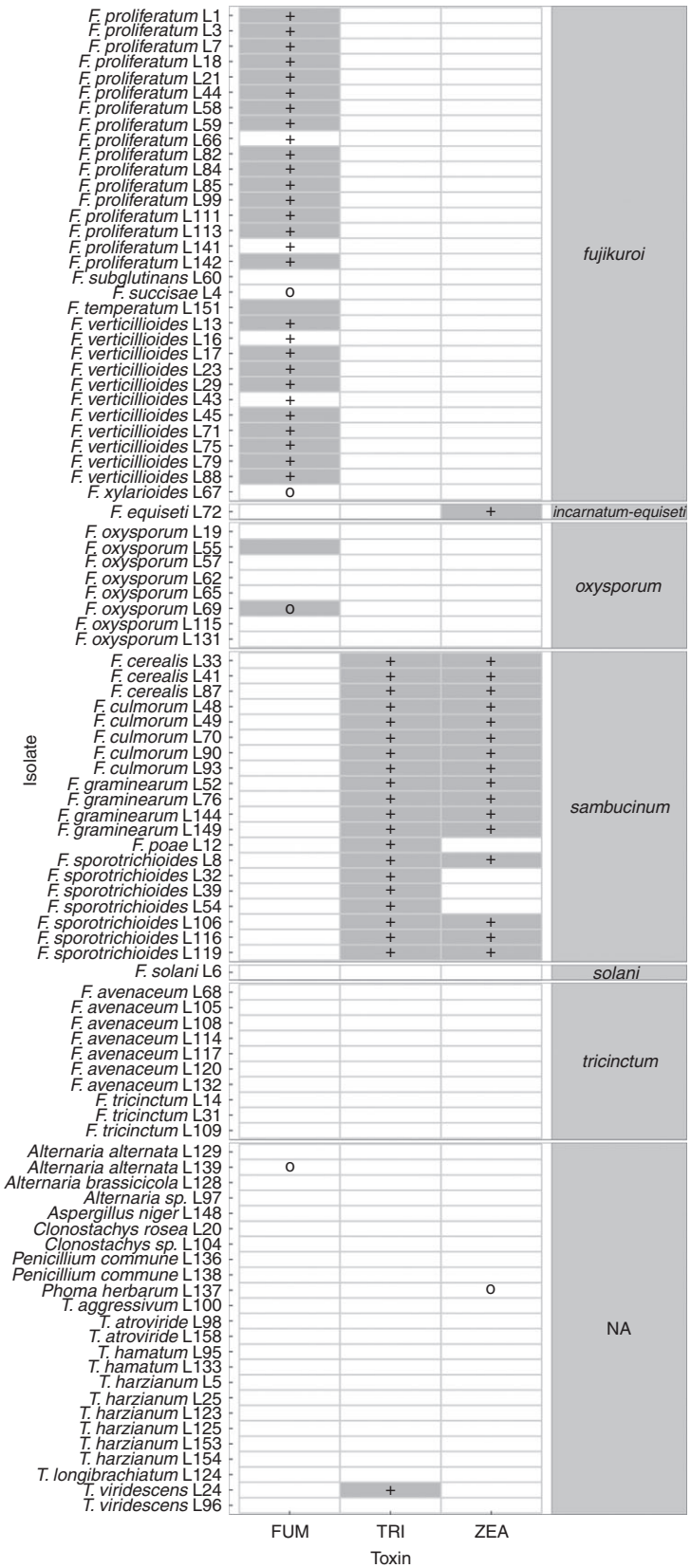

Figure 2 Results of chemical analyses and molecular diagnostics tested for 96 isolates. The results are grouped by monophyletic complexes within the genus *Fusarium* reported by O'Donnell *et al.* (2013). For trichothecene type-A producers (*Fusarium sporotrichioides*), zearalenone and trichothecene-associated chemotype was qualitatively assayed on rice kernels. Non-specific marker amplification denotes a non-specific band at different height. Marker: (□) n/a; (+) present; (o) non-specific. Toxin: (▨) n/a; (▨) present.

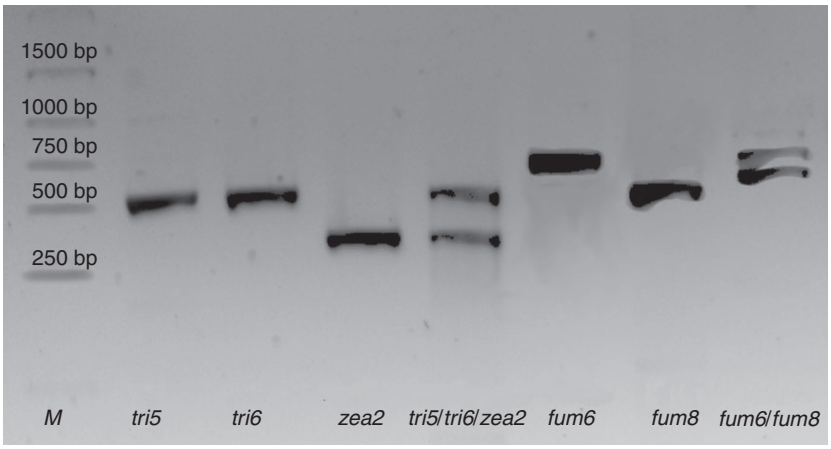

Figure 3 Multiplexed PCRs detecting the toxigenic potential of isolates (Lane M—DNA marker, *Fusarium graminearum tri5*, *tri6*, *zea2*, *Fusarium verticilioides fum6*, *fum8*).

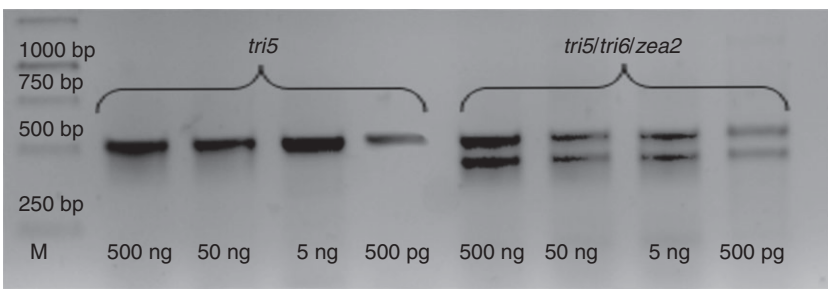

Figure 4 PCRs detecting the toxigenic potential of diluted environmental samples (*tri5*—trichothecene marker; *tri5/tri6/zea2*—trichothecene and zearalenone markers). DNA concentration 500, 50, 5 ng, 500 pg (Lane M—DNA marker).

genes (i.e. *fum8*) under a scheme which permits complementation by different core/accessory genes (Zhu *et al.* 2008; —*fum8* complementation for control of biosynthesis). As *F. oxysporum* is a species with high supernumerary chromosome content (*c.* 25%; Ma *et al.* 2010) likely stemming from past horizontal transfers, there is a possibility of different/highly divergent genetic basis complementing biosynthesis of low amounts of fumonisins and/or fumonisin-like compounds in the *F. oxysporum* complex. Notably, the molecular and morphological identification of isolates can be a grey area in some cases (e.g. newly characterized cryptic species like *F. temperatum*—Scauflaire *et al.* 2011; low resolution of broad barcode markers in complexes of related species—Blaszczyk *et al.* 2011). Current and future research is poised to demonstrate finer splits in the complexes of closely related species, previously characterized as monophyletic species (O'Donnell *et al.* 2013). The taxonomic identification is supplemented and supported by differences in chemotype and sequence of biosynthesis-related genes from closely related taxa—a process made easier by markers designed for direct sequencing of amplification products. Nevertheless, the problematic results do not apply to the most important economic, toxigenic *Fusarium* species occurring in cultivated high-yield crops (e.g. maize—*F. verticillioides*, wheat—*F. graminearum*, *F. culmorum*).

In related research, previously carried out by Rashmi *et al.* (2013), the researchers focused on diverse isolates (mainly toxigenic and non-toxigenic *Fusarium*, *Aspergillus* and *Penicillium*), demonstrating the applicability of multiplex PCR to detect ochratoxin-, fumonisin- and trichothecene-synthesizing isolates. However, Rashmi and co-workers did not attempt to provide a more detailed taxonomic identification of their cultures. In our approach, each pathogenic isolate was obtained by single-spore technique and its species assigned by both morphological and molecular methods. The test can efficiently detect the presence of the marker gene in five hundred picograms of template and about one infected kernel among hundred uninfected seeds and each obtained product can be validated by direct sequencing. Sensitivity on this level can significantly support the farmers for instance in the appropriate and rational use of fungicide treatments in the field. The developed diagnostic approach can directly be used in biological material obtained from the field (infected kernels) without the need for prior cultivation on artificial media. Unfortunately, such analysis is only possible in infected kernels. The DNA isolated from chaffs is not of sufficient quality to give reliable results, likely due to the presence of PCR inhibitors, such as polysaccharides (e.g. dextran sulphate, alginic acid—Demeke and Jenkins 2010). This could be

alleviated by improvement in preparation procedures. There is a possibility of further extending the approach to direct quantitative studies of the mycotoxin-producing pathogens which (up to date) are typically focused on detection of specific fungal producers (*F. graminearum*, *P. verrucosum*, *A. ochraceus*) and not on assessing the toxigenic potential grounded in common genetic basis among related but distinct species (Vegi and Wolf-Hall 2013).

The multiplexed PCR assay used in the protocol allows for the detection of toxigenic potential in many species simultaneously and in a standardized way. The resulting quality of optimized PCRs allows for direct sequencing of amplification products. Additionally, the low cost (relative to HPLC analysis) of the assay allows easy coupling with simple, targeted techniques (e.g. ELISA) to quickly confirm presence of a specific toxin. Thus, the method can be easily adapted as early warning against mycotoxin contamination allowing much more effective application of fungicides and can serve as supplement conventional mycotoxin detection techniques. What is also very important is that, through the usage of the direct sequencing of the PCR products, the results from individually cultivated isolates should allow easy characterization of variability and phylogeny of infecting pathogen populations.

Acknowledgements

Research funded by LIDER/19/113/L-1/09/NCBiR/2010 'Modelling, prediction and verification of fungal toxin accumulation' applied research grant.

Conflict of Interest

The authors declare no conflict of interest.

References

Baird, R., Abbas, H.K., Windham, G., Williams, P., Baird, S., Ma, P., Kelley, R., Hawkins, L. *et al.* (2008) Identification of select fumonisin forming *Fusarium* species using PCR applications of the polyketide synthase gene and its relationship to fumonisin production in vitro. *Int J Mol Sci* **9**, 554–570.

Baker, S.E., Perrone, G., Richardson, N.M., Gallo, A. and Kubicek, C.P. (2012) Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*. *Microbiology* **158**, 147–154.

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Sayers, E.W. (2013) GenBank. *Nucleic Acids Res* **41**, D36–D42.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S.D., Haesaert, G., Karlovsky, P., Oswald, I.P., Seefelder, W. *et al.* (2013) Masked mycotoxins: a review. *Mol Nutr Food Res* **57**, 165–186.

Blaszczyk, L., Popiel, D., Chelkowski, J., Koczyk, G., Samuels, G.J., Sobieralski, K. and Siwulski, M. (2011) Species diversity of *Trichoderma* in Poland. *J Appl Genet* **52**, 233–243.

Brown, D., McCormick, S.P., Alexander, N.J., Proctor, R.H. and Desjardins, A.E. (2001) A genetic and biochemical approach to study trichothecene diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*. *Fungal Genet Biol* **32**, 121–133.

Cardoza, R.E., Malmierca, M.G., Hermosa, M.R., Alexander, N.J., McCormick, S.P., Proctor, R.H., Tijerino, A.M., Rumbero, A. *et al.* (2011) Identification of loci and functional characterization of trichothecene biosynthesis genes in filamentous fungi of the genus *Trichoderma*. *Appl Environ Microbiol* **77**, 4867–4877.

Chaverri, P., Castlebury, L.A., Samuels, G.J. and Gaiser, D.M. (2003) Multilocus phylogenetic structure within the *Trichoderma harzianum/Hypocrea lixii* complex. *Mol Phylogenet Evol* **27**, 302–313.

Chelkowski, J., Golka, L. and Stepien, L. (2003) Application of STS markers for leaf rust resistance genes in near-isogenic lines of spring wheat cv. Thatcher. *J Appl Genet* **44**, 323–338.

Creppy, E.E., Traoré, A., Baudrimont, I., Cascante, M. and Carratú, M.R. (2002) Recent advances in the study of epigenetic effects induced by the phycotoxin okadaic acid. *Toxicology* **181–182**, 433–439.

Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G. and Dall'Asta, C. (2013) Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. *Chem Res Toxicol* **26**, 305–312.

Dawidziuk, A., Kaczmarek, J. and Jedryczka, M. (2012) The effect of winter weather conditions on the ability of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* to release ascospores. *Eur J Plant Pathol* **134**, 329–343.

Demeke, T. and Jenkins, G.R. (2010) Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal Bioanal Chem* **396**, 1977–1990.

Desjardins, A.E. and Proctor, R.H. (2007) Molecular biology of *Fusarium* mycotoxins. *Int J Food Microbiol* **119**, 47–50.

D'Mello, D., Mehta, D., Pereira, J. and Rao, C.V. (1999) A toxicity study of simultaneous administration of Tamoxifen and Diazepam to female Wistar rats. *Exp Toxicol Pathol* **51**, 549–553.

Doohan, F.M., Parry, D.W., Jenkinson, P. and Nicholson, P. (1998) The use of species-specific PCR-based assays to analyze Fusarium ear blight of wheat. *Plant Pathol* **47**, 197–205.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797.

Evans, N., Baier, A., Semenov, M.A., Gladders, P. and Fitt, B.D.L. (2008) Range and severity of a plant disease increased by global warming. *J R Soc Interface* **5**, 525–531.

Flicek, P., Amode, M.R., Barrell, D., Beal, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G. *et al.* (2012) Ensembl. *Nucleic Acids Res* **40**, D84–D90.

Geiser, D.M., del Mar Jiménez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A. *et al.* (2004) FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. *Eur J Plant Pathol* **110**, 473–479.

González-Jaén, M.T., Mirete, S., Patiño, B., López-Errasquín, E. and Vázquez, C. (2004) Genetic markers for the analysis of variability and for production of specific diagnostic sequences in fumonisin-producing strains of *Fusarium verticillioides*. *Eur J Plant Pathol* **110**, 525–532.

Gromadzka, K., Chelkowski, J., Popiel, D., Kachlicki, P., Kostecki, M. and Golinski, P. (2009) Solid substrate bioassay to evaluate the effect of *Trichoderma* and *Clonostachys* on the production of zearalenone by *Fusarium* species. *World Mycotoxin J* **2**, 45–52.

Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T. *et al.* (2007) A higher-level phylogenetic classification of the Fungi. *Mycol Res* **111**, 509–547.

Jiao, F., Kawakami, A. and Nakajima, T. (2008) Effects of different carbon sources on trichothecene production and Tri gene expression by *Fusarium graminearum* in liquid culture. *FEMS Microbiol Lett* **285**, 212–219.

Kaczmarek, J., Jedryczka, M., Fitt, B.D.L., Lucas, J.A. and Latunde-Dada, A.O. (2009) Analyses of air samples for ascospores of *Leptosphaeria maculans* and *L. biglobosa* with light microscopic and molecular techniques. *J Appl Genet* **50**, 411–419.

Katoh, K. and Toh, H. (2010) Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics* **26**, 1899–1900.

Kim, Y., Hutmacher, R.B. and Davis, R.M. (2005) Characterization of California isolates of *Fusarium oxysporum* f. sp. vasinfectum. *Plant Dis* **4**, 366–372.

Kimura, M., Tokai, T., O'Donnell, K., Ward, T.J., Fujimura, M., Hamamoto, H., Shibata, T. and Yamaguchi, I. (2003) The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. *FEBS Lett* **539**, 105–110.

Kimura, M., Tokai, T., Takahashi-Ando, N., Ohsato, S. and Fujimura, M. (2007) Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. *Biosci Biotechnol Biochem* **71**, 2105–2123.

Kristensen, R., Torp, M., Kosiak, B. and Holst-Jensen, A. (2005) Phylogeny and toxigenic potential is correlated in *Fusarium* species as revealed by partial translation elongation factor 1 alpha gene sequences. *Mycol Res* **109**, 173–186.

Kuhn, M. (2008) Building predictive models in R using the caret package. *J Stat Softw* **28**, 1–26.

Leslie, J.F. and Summerell, B.A. (2006) *The Fusarium Laboratory Manual*. Ames, IA: Blackwell Publishing.

Li, Y., Shen, Y., Zhu, X. and Du, L. (2009) Introduction of the AAL-toxin polyketide synthase gene ALT1 into FUM1-disrupted *Fusarium verticillioides* produces metabolites with the fumonisin methylation pattern. *J Nat Prod* **72**, 1328–1330.

Lysøe, E., Bone, K.R. and Klemsdal, S.S. (2009) Real-time quantitative expression studies of the zearalenone biosynthetic gene cluster in *Fusarium graminearum*. *Phytopathology* **99**, 176–184.

Ma, Z. and Michailides, T.J. (2007) Approaches for eliminating PCR inhibitors and designing PCR primers for the detection of phytopathogenic fungi. *Crop Prot* **26**, 145–161.

Ma, L.J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., Di Pietro, A., Dufresne, M., Freitag, M. *et al.* (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* **464**, 367–373.

Moretti, A., Susca, A., Mulé, G., Logrieco, A.F. and Proctor, R.H. (2013) Molecular biodiversity of mycotoxigenic fungi that threaten food safety. *Int J Food Microbiol* **167**, 57–66.

Mulè, G., Susca, A., Stea, G. and Moretti, A. (2004) A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans*. *Eur J Plant Pathol* **110**, 495–502.

O'Donnell, K., Rooney, A.P., Proctor, R.H., Brown, D.W., McCormick, S.P., Ward, T.J., Frandsen, R.J.N., Lysøe, E. *et al.* (2013) Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genet Biol* **52**, 20–31.

Owczarzy, R., Moreira, B.G., You, Y., Behlke, M.A. and Walder, J.A. (2008) Predicting stability of DNA duplexes in solutions containing magnesium and monovalent cations. *Biochemistry* **47**, 5336–5353.

Proctor, R.H., Busman, M., Seo, J.A., Lee, Y.W. and Plattner, R.D.A. (2008) Fumonisin biosynthetic gene cluster in *Fusarium oxysporum* strain O-1890 and the genetic basis for B versus C fumonisin production. *Fungal Genet Biol* **45**, 1016–1022.

Proctor, R.H., McCormick, S.P., Alexander, N.J. and Desjardins, A.E. (2009) Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. *Mol Microbiol* **74**, 1128–1142.

Proctor, R.H., Van Hove, F., Susca, A., Stea, G., Busman, M., van der Lee, T., Waalwijk, C., Moretti, A. *et al.* (2013) Birth, death and horizontal transfer of the fumonisin biosynthetic gene cluster during the

evolutionary diversification of *Fusarium*. *Mol Microbiol* **90**, 290–306.

Pruitt, K.D., Tatusova, T., Brown, G.R. and Maglott, D.R. (2012) NCBI Reference Sequences (RefSeq): current status, new features and genome annotation policy. *Nucleic Acids Res* **40**, D130–D135.

Rashmi, R., Ramana, M.V., Shylaja, R., Uppalapati, S.R., Murali, H.S. and Batra, H.V. (2013) Evaluation of a multiplex PCR assay for concurrent detection of four major mycotoxigenic fungi from foods. *J Appl Microbiol* **114**, 819–827.

Reeves, C.D., Hu, Z., Reid, R. and Kealey, J.T. (2008) Genes for the biosynthesis of the fungal polyketides hypothemycin from *Hypomyces subiculosus* and radicicol from Pochonia chlamydosporia. *Appl Environ Microbiol* **74**, 5121–5129.

SantaLucia, J. Jr (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci USA* **95**, 1460–1465.

Scauflaire, J., Mahieu, O., Louvieaux, J., Foucart, G., Renard, F. and Munaut, F. (2011) Biodiversity of *Fusarium* species in ears and stalks of maize plants in Belgium. *Eur J Plant Pathol* **131**, 59–66.

Stepien, L., Koczyk, G. and Waskiewicz, A. (2011) FUM cluster divergence in fumonisins-producing *Fusarium* species. *Fungal Biol* **115**, 112–123.

Tag, A.G., Garifullina, G.F., Peplow, A.W., Ake, C. Jr, Phillips, T.D., Hohn, T.M. and Beremand, M.N. (2001) A novel regulatory gene, Tri10, controls trichothecene toxin production and gene expression. *Appl Environ Microbiol* **67**, 5294–5302.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Vegi, A. and Wolf-Hall, C.E. (2013) Multiplex real-time PCR method for detection and quantification of mycotoxigenic fungi belonging to three different genera. *J Food Sci* **78**, M70–M76.

Wickham, H. (2009) *Ggplot2: Elegant Graphics for Data Analysis*. New York: Springer Publishing Company, Incorporated.

Zhu, X., Vogeler, C. and Du, L. (2008) Functional complementation of fumonisin biosynthesis in FUM1-disrupted *Fusarium verticillioides* by the AAL-toxin polyketide synthase gene ALT1 from *Alternaria alternata* f. sp. lycopersici. *J Nat Prod* **71**, 957–960.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Fumonisin concentration and molecular detection of the toxigenicity in tested fungal isolates (fumonisin B chemotype).

Table S2 Zearalenone concentration and molecular detection of the toxigenicity in tested fungal isolates (zearalenone chemotype).

Table S3 Trichothecene concentration and molecular detection of the toxigenicity in tested fungal isolates (trichothecene B chemotype).

Table S4 GenBank accessions of obtained PCR products.