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A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans*

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Abstract

Fusarium proliferatum, *F. subglutinans* and *F. verticillioides* are the most important *Fusarium* species occurring on maize world-wide, capable of producing a wide range of mycotoxins which are a potential health hazard for animals and humans. The ribosomal internal transcribed spacer and a portion of the calmodulin gene were sequenced and analysed in order to design species-specific primers useful for diagnosis. The primer pairs were based on a partial calmodulin gene sequence. Three pairs of primers (PRO1/2, SUB1/2 and VER 1/2) produced PCR products of 585, 631 and 578 bp for *F. proliferatum*, *F. subglutinans* and *F. verticillioides*, respectively. Primer specificity was confirmed by analyzing DNA of 150 strains of these species, mostly isolated from maize in Europe and USA. The sensitivity of primers was 12.5 pg when the pure total genomic DNA of each species was analyzed. The developed PCR assay should provide a powerful tool for the detection of toxigenic fungi in maize kernels.

Introduction

Members of *Fusarium proliferatum*, *F. subglutinans*, and *F. verticillioides* (syn. *F. moniliforme*), belonging to the *Liseola* section of *Fusarium* (Nelson et al., 1983), are well known pathogens of maize, causing stalk and ear rot world-wide (Nelson et al., 1981; Leslie et al., 1990; Logrieco et al., 1993; Bottalico, 1998). Besides yield losses and the reduction of seed quality, these pathogens accumulate mycotoxins in infected tissues that could have dangerous consequences on human and animal health (Bottalico, 1998). Each of these species has a specific toxin profile, including fumonisins (Nelson et al., 1993), fusaric acid (Bacon et al., 1996), moniliformin (Marasas et al., 1984), beauvericin (BEA) and fusaproliferin (FUP) (Moretti et al., 1996) (for a recent review see Summerell et al., 2001). Because of the toxic and carcinogenic potential of these toxins, there is an urgent need to develop accurate detection methods to identify the occurrence of the species that produce them on maize and its by-products. The teleomorphic stages of these species belong to the *Gibberella fujikuroi* complex: specifically, isolates of *F. subglutinans* from maize usually belong to mating population E (*G. subglutinans*), whereas *F. proliferatum* corresponds to mating population D (*G. intermedia*) and *F. verticillioides* corresponds to mating population A (*G. moniliformis*) (Leslie, 1995). Assessing the mating groups by testing the fertility of strains belonging to the *Liseola* section (Leslie, 1995), together with morphological identification are the current methods for detecting the occurrence of *G. fujikuroi* mating groups in maize seeds. Several studies have reported the genetic distinction of the mating populations of *G. fujikuroi* complex, including vegetative compatibility (Leslie, et al., 1993), isozyme analysis (Huss et al., 1996) and electrophoretic

karyotyping (Xu and Leslie, 1996). They are time-consuming, labour-intensive and mostly require considerable ability and training, because these species have small morphological differences and it is difficult to distinguish them from each other. Therefore, there is a need for reliable and simple methods for the detection of such toxigenic species using DNA techniques. Amoah et al. (1995) reported genetic variability among *F. verticillioides* isolates from different hosts in Ghana by DNA fingerprints detected as RFLPs of ribosomal DNA and RAPDs analysis. Successively, they used RAPD analysis to determine the mating populations of several members of *Fusarium* section *Liseola* (Amoah et al., 1996) and in particular they determined differences between A, D, and F mating population members. Moreover, Schlacht et al. (1997) analysed the DNA of members of *Fusarium* species belonging to mating populations A, D, and E, the teleomorphs of *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, respectively, but no specific primers were reported for these species. Moeller et al. (1999) developed specific primers for *F. verticillioides* and *F. subglutinans* based on sequences of RAPD fragments that were successively applied for the analysis of infected maize kernels. However, although RAPDs have been widely used as diagnostic tools in many laboratories for the identification of *Fusarium* species (Blakemore et al., 1994) they are considered not very reliable because of the lack of reproducibility of the technique (Murillo et al., 1998). On the other hand, Murillo et al. (1998) developed a PCR method to detect *F. verticillioides* from infected maize plant tissue by initially screening the Pucf2 genomic clone of this fungus and therefore designing a specific primer for *F. verticillioides*. However, although *F. verticillioides* is considered a severe pathogen of maize being among the most occurring and harmful toxigenic coloniser fungus of maize, other *Fusarium* species are to be regarded as dangerous contaminants.

Sequences of the ribosomal DNA genes, including internal transcribed spacer regions ITS1 and ITS2 surrounding the 5.8S gene and calmodulin gene, have been used to develop specific primers for detection of several phytopathogenic fungi (O'Donnell et al., 1998, 2000). Therefore, we concentrated on the calmodulin gene region in order to obtain reliable DNA variability for distinguishing such species.

The objectives of this work were to

(i) study DNA variability within the calmodulin region of strains of *F. proliferatum*, *F. subglutinans* and *F. verticillioides* isolated from maize;
(ii) develop species-specific primers for these species;
(iii) develop a rapid PCR method for the identification of these pathogens from maize kernels.

Material and methods

Fungal strains

The strains were obtained from ITEM Culture Collection of Institute of Sciences of Food Production, Bari, Italy (http://www.ispa.cnr.it/Collection) (Table 1).

Fertility tests

Tester strains for mating populations tests were received from J.F. Leslie (Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas). Strains for which the mating group was unknown were crossed on carrot agar as male parents with tester strains of mating population A to F (Klittich and Leslie, 1988). Mated cultures were considered fertile when cirri were extruded from perithecia. All strains were crossed twice with both testers from each mating population.

DNA extraction from fungal cultures and maize kernels

Fungal strains were grown in Wikerman liquid medium (40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and water up to 1 l) in shaken cultures (150 rpm). Mycelia were harvested by filtration, frozen and lyophilized. Total genomic DNA was isolated from dried mycelium (~40 mg) using EZNA Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, USA). DNA was recovered and dissolved in sterile water. Concentrations of DNA were determined by gel electrophoresis, by measuring the ultraviolet-induced fluorescence emitted by ethidium bromide molecules intercalated into DNA, and comparing the fluorescent yield of the samples with a standard. Total genomic DNA was extracted from maize kernels according to the protocol used for

Table 1. Strains used in this study

Species	ITEM	Geographic origin
F. verticillioides	2006	Basilicata, Italy
	2007	
	2008	
	2009	
	2010	
	2011	
	2396	Iowa, USA
	3970	
	3985	
	3993	
	3996	
	3998	
	4013	
	4015	
	4024	
	4029	
	4032	
	4034	
	4037	
	4038	
	4040	
F. proliferatum	1724	Sardinia, Italy
	1725	
	1727	
	1748	
	1749	
	1752	
	2401	Iowa, USA
	2402	
	4014	
	2620	Slovakia
	2631	
	2635	
F. subglutinans	2223	Slovakia
	3485	Iowa, USA
	3851	
	3853	
	3922	
	3925	

cereal grains by Simpson et al. (2000). The samples were lyophilized and ground in a small mortar and 4 g of powder were extracted. The PCR assays containing species-specific primers were set up with 1.25 U of Taq Gold DNA polymerase (Applied Biosystems) in 50 µl reaction mixtures, containing 15 pmol of each outside primer, 12.5 µM of each dNTP (Applied Biosystems), and 50 ng of total DNA (from maize). Reactions were performed as above for species-specific primers. Amplification products were assessed on 2% agarose gel stained with ethidium bromide (EtBr).

The total genomic DNA was also extracted from pure cultures of each fungal species grown on maize kernels (Logrieco et al., 1995), in order to confirm the primer specificity and applicability to maize kernels.

Fungal DNA amplification and sequencing

The PCR reaction was set up with 2.5 U of Taq Gold DNA polymerase (Applied Biosystems) in 100 µl reaction mixtures, containing 30 pmol of each outside primer, 12.5 µM deoxynucleoside triphosphates (Applied Biosystems), and 1 µl (approximately 10 ng) of fungal template DNA. The ribosomal internal transcribed spacers and a portion of the calmodulin gene were amplified using the following primer pairs: ITS1 (5′-GAAGTAAAAGTCGTAACAAG-3′) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) (White et al., 1990), CL1 (5′-GARTWCAAGGAGGCCTTCTC-3′) and CL2A (5′-TTTTGCATCATGAGTTGGAC-3′) (O'Donnell et al., 2000). The reactions were performed in a 9700 thermal cycler (Applied Biosystems) set to the following: denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min). Tubes without the DNA template were included in each experiment as a negative control. After amplification, PCR products were purified by agarose gel-electrophoresis: DNA bands were excised from an agarose gel and loaded onto spin columns (Gene Elute Agarose spin columns-SIGMA).

Purified PCR products were directly sequenced in both directions using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). All sequencing reactions were purified by gel filtration through columns containing Sephadex G-50 (Pharmacia) equilibrated in double-distilled water and were analyzed on a 310 Genetic Analyzer (Applied Biosystems). DNA sequences from each loci sequenced were aligned visually with Sequence Navigator software (Applied Biosystems). The resulting sequences of the calmodulin region were aligned by the clustal method with the programme DNAMAN (Lynnon BioSoft).

Primer designing and testing

Species-specific primers were designed using Primer Express software (Applied Biosystems). Primers were chosen from divergent regions, inside

the calmodulin-encoding sequences, resulting from the multiple alignment of sequences obtained in this study. PCR reactions for the three species were performed in different tubes. PCR products were resolved in 2% Tris-acetate-EDTA-agarose gel and were visualized with EtBr and ultraviolet illumination. Images were captured and stored using an EasyShare Kodak DX3215 Zoom Digital Camera. The PCR containing species-specific primers were set up with 1.25 U of Taq Gold DNA polymerase (Applied Biosystems) in 50 µl reaction mixtures, containing 15 pmol of each outside primer, 12.5 µM of each deoxynucleoside triphosphate (Applied Biosystems), and 1 µl (approximately 5 ng) of fungal template DNA. Reactions were performed using the following PCR conditions: denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 56 °C for 50 s, extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples. Amplification products were assessed on 2% agarose gel stained with EtBr. The specificity of the primers was tested against 50 strains of the three species and representative strains of other toxigenic fungi occurring on maize (namely, *F. graminearum* ITEM 4765, *F. poae* ITEM 3726, *Aspergillus flavus* ITEM 4591; *A. niger* ITEM 4855; *Acremonium strictum*. ITEM 3105).

Results

Fertility tests

All strains, crossed with mating tester strains, were fertile. In particular, all strains classified as *F. proliferatum* belonged to mating population D; *F. subglutinans* strains were mating population E and *F. verticillioides* strains were mating population A.

Sequences analyses

The first experiments sought to analyse 39 isolates of *Fusarium* species isolated from maize: 20 of *F. verticillioides*, 13 of *F. proliferatum* and 6 of *F. subglutinans*, from different geographic regions.

In order to select a suitable species-typical variability in nucleotide sequences among the isolates of *F. proliferatum*, *F. verticillioides and F. subglutinans* from maize, the ITS regions of ribosomal genes and a region of the calmodulin-encoding gene (O'Donnell et al., 2000) were sequenced. DNA was amplified by PCR with ITS1 and ITS4 primers resulting in 520–530 bp fragments (data not shown). The amplification products were sequenced in both directions. Some of these ITS sequences were compared with the EMBL database. ITEM 1748, ITEM 1475 and ITEM 2620 showed 100% homology with *F. proliferatum* NRRL 31071, Acc. No. AF291061. ITEM 3922, ITEM 3845 and ITEM 2620 showed 99% homology with *F. subglutinans*, Acc. No. U34559, and with *F. verticillioides*, Acc. No. X94166; ITEM 3970, ITEM 2006 and ITEM 3996 showed 100% homology with *F. subglutinans* NRRL 22016, Acc. No. U34559 and with *F. verticillioides* CBS 218.76, Acc. No. X94166. An ITS homology tree revealed separation between two groups of strains: one consisting of *F. proliferatum* and one consisting of *F. subglutinans* and *F. verticillioides*. Identical ITS rDNA sequences in *F. subglutinans* and *F. verticillioides* species and different ITS rDNA sequences in *F. proliferatum* were observed. Visual inspection of the ITS rDNA sequences showed that they were not suitable for the design of species-specific primers.

The calmodulin gene showed to be highly reliable for phylogenetic analysis on the *G. fujikuroi* complex and *Fusarium* related species (O'Donnell et al., 2000) and a candidate gene for population genetic analysis (Geiser et al., 2000). DNA extracted from all 39 isolates was amplified by PCR with CL1 and CL2A primers resulting in 670–680 bp fragments (data not shown). Some of these calmodulin gene sequences were compared with the EMBL database. ITEM4014 showed 99% homology with *F. proliferatum* NRRL 22944, Acc. No. AF158333. ITEM 3925 showed 99% homology with *Fusarium* sp. NRRL 25622, Acc. No. AF158354. ITEM 2008 showed 100% homology with *G. moniliformis* NRRL 22172, Acc. No. AF158315. A calmodulin gene tree revealed separation of three groups corresponding to the three species (Figure 1). In contrast to the ITS regions, visual inspection of the aligned calmodulin partial gene sequences revealed unique regions that were used to design three species-specific primer pairs (Figure 2). Comparing calmodulin nucleotide sequences of analysed isolates, there was 100% identity among isolates of *F. subglutinans*, 100%

Figure 1. Dendrogram obtained from multiple alignment of calmodulin partial gene sequences from isolates of *F. proliferatum*, *F. subglutinans* and *F. verticillioides*.

identity among isolates of *F. verticillioides* and 99.7% among isolates of *F. proliferatum*. Moreover, 94.2% identity was seen between partial calmodulin sequence of *F. subglutinans* and *F. verticillioides*, 92.8% identity between *F. proliferatum* and *F. verticillioides* and 92.7% identity between *F. subglutinans* and *F. proliferatum*.

Primer design and testing

Three species-specific primers were designed: PRO1/2, SUB1/2 and VER1/2 specific for *F. proliferatum*, *F. subglutinans* and *F. verticillioides*, respectively (Table 2). All primers were designed to operate at high annealing temperatures (56 °C),

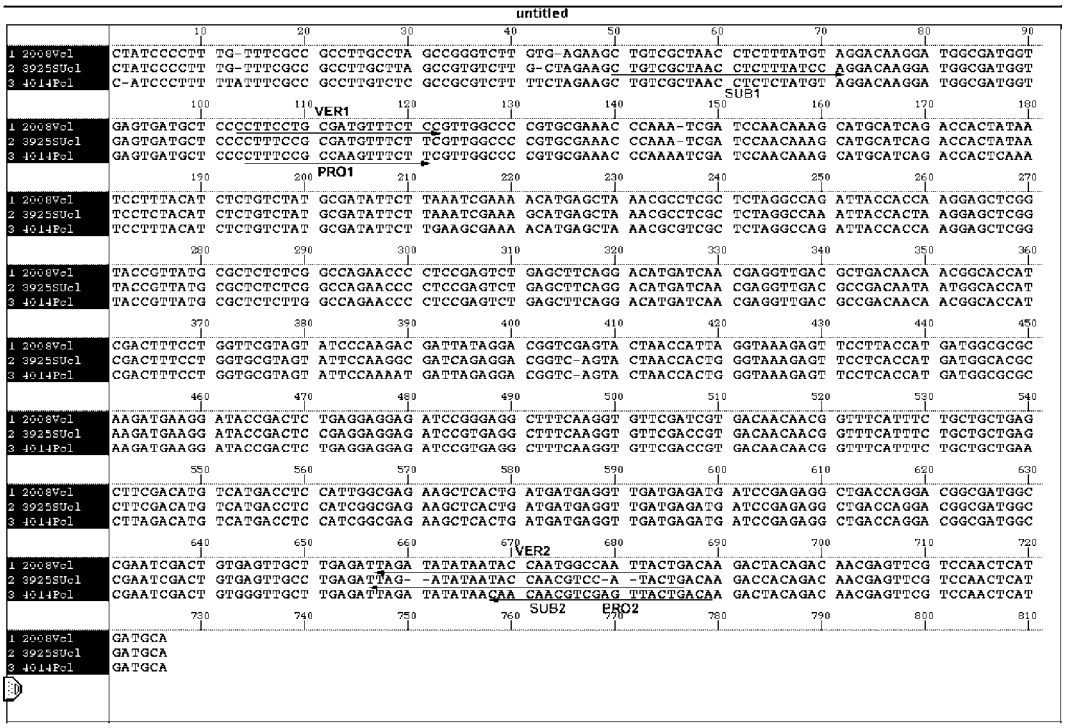

Figure 2. Full alignment of the partial calmodulin gene from *F. proliferatum* strain (4014Pcl), *F. subglutinans* strain (3925SUcl) and *F. verticillioides* (2008Vcl). Arrows indicate species-specific primers: PRO1/2 specific for *F. proliferatum* species, SUB1/2 specific for *F. subglutinans* and VER1/2 specific for *F. verticillioides*.

Table 2. Sequences of oligonucleotide primers designed within the calmodulin gene

Primer name	Primer sequence	Species-specificity
SUB1	5′-CTGTCGCTAACCTCTTTATCCA-3′	*F. subglutinans*
SUB2	5′-CAGTATGGACGTTGGTATTATATCTAA-3′	
PRO1	5′-CTTTCCGCCAAGTTTCTTC-3′	*F. proliferatum*
PRO2	5′-TGTCAGTAACTCGACGTTGTTG-3′	
VER1	5′-CTTCCTGCGATGTTTCTCC-3′	*F. verticillioides*
VER2	5′-AATTGGCCATTGGTATTATATATCTA-3′	

thereby preventing the co-amplification of non-specific DNA targets. Species-specific primers were chosen with mismatches located in 3′. Primer sequences were compared against existing sequence in GenBank, EMBL, DDBJ and PDB and result of BLAST (Basic Local Alignments Search Tool) showed 100% homology of primers with sequences of strains belonging to the species for which primers were designed. Single bands of correct size were obtained with species-specific primers from all strains belonging to the three species. The PCR product from *F. subglutinans* was 631 bp, for *F. proliferatum* it was 585 bp and for *F. verticillioides* it was 578 bp. In order to confirm primer specificity, fragments derived from the species studied were sequenced. Comparison of the sequences obtained previously with calmodulin sequences, used for primer design, confirmed the species-specificity of the primers, since the sequences were 100% homologous.

In order to validate the primers, the specificity of the primer pairs was tested against 50 strains each of the three species. No cross-reactions were observed with the other species tested (Figure 3). In order to avoid false-negative results, some PCR assays were performed at least three times. The

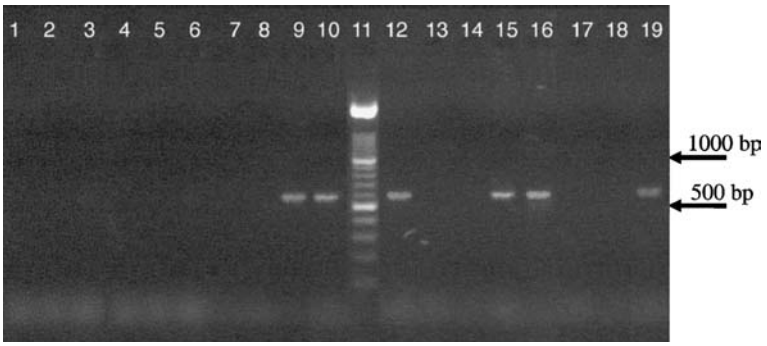

Figure 3. PCR amplifications with the three species-specific primer pairs. Lanes 1–3: PCR amplifications of *F. poae*: PRO1-PRO2 (lane1), primers VER1-VER2 (lane 2), SUB1-SUB2 (lane3). Lanes 4–6: PCR amplifications of *F. graminearum*: primers PRO1-PRO2 (lane 4), VER1-VER2 (lane 5), SUB1-SUB2 (lane 6). Lanes 7–9: PCR amplifications of ITEM 1724 (*F. proliferatum*): primers SUB1-SUB2 (lane 7), VER1-VER2 (lane 8) and PRO1-PRO2 (lane 9). Lane 10: PCR amplification of ITEM 1727 (*F. proliferatum*): primers PRO1-PRO2. Lane 11: DNA size marker (100 bp multiples). Lanes 12–14: PCR amplifications of ITEM 2006 (*F. verticillioides*): primers VER1-VER2 (lane 12), PRO1-PRO2 (lane 13), SUB1-SUB2 (lane 14). Lane 15: PCR amplification of ITEM 2396 (*F. verticillioides*): primers VER1-VER2. Lanes 16–18: PCR amplifications of ITEM 2223 (*F. subglutinans*): primers SUB1-SUB2 (lane 16), PRO1-PRO2 (lane 17) and VER1-VER2 (lane 18). Lane 19: PCR amplifications of ITEM 3925 *F. subglutinans*: primers SUB1-SUB2.

sensitivity of primers was of 12.5 pg when the pure total genomic DNA of each species was analysed. Finally, the sequences of species-specific primers were patented: MI2003A000375, in 03/03/2003.

With a view to using the PCR assay to detect these pathogens in maize tissues, the primers on total genomic DNA extracted from healthy maize kernels were tested to avoid false positive results for cross-reaction with plant DNA. Ten samples from five different companies generated no fragments in any experiment. Finally, the analysis of total genomic DNA extracted from pure cultures of each species grown on maize kernels generated the fragments expected for each of the primer sets tested.

Discussion

Several authors have reported that *F. verticillioides*, *F. subglutinans* and *F. proliferatum* can occur on maize kernels and in maize-based food and feed (Leslie et al., 1990; Logrieco et al., 1995; Munkvold et al., 1998; Srobarova et al., 2002). The correct identification of these species is therefore very important because each of them possess a specific toxigenic profile and it is important to know the potential toxigenic risk of the contaminated plant or food products. In this respect, the primers that we developed can be useful as genetic markers for the identification of population of *F. proliferatum*, *F. subglutinans* and *F. verticillioides* isolated from maize. PCR is an extremely sensitive technique but the value of techniques in diagnostic work depends on designing good specific primers and developing the appropriate experimental conditions for the DNA amplification process. Using this knowledge, it may be possible, in the future, to develop strategies to ensure that all the fungal species involved in a particular disease complex are effectively controlled and that suppression of one fungus does not merely lead to it being replaced by another.

The development of specific primers described here for the to identification of *F. verticillioides*, *F. subglutinans* and *F. proliferatum* should provide tools to prevent health risks caused by mycotoxin contaminated maize based food and feed, and thus have a positive impact on plant, animal and human health.

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