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FLASH-PCR Diagnostics of Toxigenic Fungi of the Genus *Fusarium*

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Abstract—A test system for the diagnostics and identification of seven toxigenic fungi causing Fusarium head blight (*Fusarium graminearum*, *F. culmorum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. avenaceum*, and *F. tricinctum*) was developed using PCR. The identification of pathogen is based on the specific amplification of a DNA fragment of the gene of translation elongation factor 1 alpha (*tef-1*α) and subsequent detection of the results by the fluorescent amplification–based specific hybridization method. The system was tested on 38 isolates of different fungi of the genus *Fusarium*.

Key words: polymerase chain reaction, diagnosis, mycotoxin, fusariosis, phytopathogen

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INTRODUCTION

Fungi of the genus *Fusarium* are widely distributed pathogens that damage a great variety of plants and cause severe economic losses to agricultural production[2]. The most serious disease caused by these fungi is fusariosis of cereals [1-4]. The harmfulness of this disease consists not only in direct crop losses but also in the impairment of crop quality, namely, the accumulation of dangerous mycotoxins, the products of fungal activity. The urgency to medicine and agricultural industry of studying and identifying the toxigenic fungi and mycotoxins produced by these fungi has been recognized throughout the world and is related to the severe hazard to the health of humans and agricultural animals [5-7]. A confirmation of this is the regulations specifying the methods of sampling and assays for the obligatory control of mycotoxins in food products and forages, adopted by the European community [8]. The maximum admissible concentrations for the major fusariotoxins in the Russian Federation, which were specified in 1996 [9], are (ppm): 1 for deoxynivalenol, 0.1 for T-2 toxin, and 1.0 for zearalenone.

The main factors that determine the contamination of grain by mycotoxins are the degree of infection and the species composition of fungi developing on grain. A complex of pathogens that most often cause grain fusariose on the territory of the Russian Federation involves *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, and *F. sporotrichioides* [4, 10]. Besides, in the last few years, a new fungus *F. langsethiae* has been detected in Europe, which has attracted the particular attention of investigators due to its toxigenicity [11-13]. The production of toxins is strictly species-specific [5, 12]. Therefore, based on the analysis of a complex of pathogens in a grain batch, it is possible to predict which toxins may be present or accumulated in this grain.

The mycological analysis of grain based on the morphological characteristics of fungi and their growth rate is time consuming and requires a high qualification and good skills of the staff. In many laboratories, an intensive search for alternative methods of rapid and precise identification of pathogenic fungi is being conducted. Recent publications indicate that, in principle, the grain infection can be assessed using the innovative DNA technologies [14-17]. By sequencing the DNA from different *Fusarium* species, several specific primers have been constructed, which enable the detection of these pathogens in both pure culture and plant material using the PCR. However, the PCR methods by which it

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2 Abbreviations: FLASH, fluorescent amplification-based specific hybridization; PCR, polymerase chain reaction; IC, internal control; *tef*-1α the gene of the translation elongation factor.

is possible to diagnose diseases and identify some fungal species in grain samples are still unavailable on the market [18–23].

One of the simplest approaches to rapid and sensitive diagnosis and identification of pathogens, including plant pathogens, using DNA technologies is a modification of PCR with the detection of fluorescence during the amplification (real-time PCR) or after its termination (PCR–FLASH) [24, 25]. With both methods, the results are obtained within a few hours. As opposed to the "classical" PCR in which the results are analyzed by gel electrophoresis, the analysis in the modified method is carried out without the use of electrophoresis, which almost completely eliminates the contamination with amplicons and hence rules out the acquisition of false-positive results. It should be noted that the PCR–FLASH method requires no expensive equipment, as in the case of the real-time PCR. The principle of the PCR–FLASH method has been described in detail previously in papers devoted to diagnostic tests systems for *Septoria tritici* and *Stangospora nodorum* [25] and some most widely occurring potato viruses [24].

The goal of the present study was the development and optimization of a system for PCR diagnosis and identification in the FLASH format for the most dangerous species of toxigenic *Fusarium* fungi affecting cereals (*F. graminearum*, *F. culmorum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. avenaceum*, and *F. tricinctum*). The strains of these fungi belong to three sections of the *Fusarium* genus: *Discolor* (*F. graminearum* and *F. culmorum*), *Sporotrichiella* (*F. poae*, *F. sporotrichioides*, *F. langsethiae*, and *F. tricinctum*), and *Roseum* (*F. avenaceum*). The species within one section are similar in morphological criteria and profiles of toxins produced and hence are often erroneously identified by microbiological methods [5, 12, 21]. The method of DNA identification detects rapidly and with a high accuracy toxigenic fungi, the producers of hazardous toxins, which makes it possible to considerably reduce the time of assessing the grain quality, and reveal and sort out lots with a high content of dangerous components. On the other hand, the use of these technologies is of considerable promise for the development of molecular mycology, in particular in studies of the variability of the biological properties of pathogenic fungi and in the practice of production and storage of cereals and the products of their reprocessing.

RESULTS AND DISCUSSION

The fungal single spore isolates of *Fusarium* fungi used in the study are given in Table 1.

The DNA sequences of the internal transcribed spacer (ITS) of the gene encoding the ribosomal RNA, which we have previously used as genetic markers in the diagnosis of the causing agents of septorioses [25], have a limited divergence and are therefore not always suited for the development of PCR test systems. Phylogenetic investigations based on the analysis of nucleotide sequences of *Fusarium* fungi available in the database showed that the gene encoding the *tef-1*α factor [23, 26] is best suited as a genetic marker to distinguish between the species of the causative agents of fusarioses. In addition, because *tef-1*α is represented in the genome by a single copy, the test system based on the amplification of its site can be used for the semiquantitative analysis [23, 26].

The optimized structures of primers and probes for the *tef-1*α fragment of the fungi examined are given in Table 2.

After the optimization of temperature parameters of the reaction, the following conditions for conducting the PCR were chosen: heating 93°C, 90 s; then 93°C, 20 s; 64°C, 5 s; 67°C, 5 s (o 5 cycles); 93°C, 1 s; 64°C, 5 s; 67°C, 5 s (× 40 cycles). During the development of PCR diagnosis systems, it is necessary to provide constant control of the adequacy of the reaction course and possible presence of DNA polymerase inhibitors in the sample. For this purpose, an IC system based on the principle of multiplex PCR was used in which a simultaneous amplification of the fragments of the genome of the pathogen DNA and DNA of IC proceeds [24, 25]. IC represents a plasmid and its specific primers. The concentrations of the plasmid and the appropriate primers were chosen such that the PCR with the DNA of IC did not inhibit the amplification of the specific DNA primers [24, 25]. That is why, at a high concentration of specific DNA in the sample, the level of IC amplification can be very low. The size of an IC amplicon is 560 bp, which enables one to clearly distinguish it in the gel from the amplicons from the DNA of *Fusarium* fungi whose length was chosen in the range of 200–300 bp.

A gel electrophoretic analysis of the test system for *F. sporotrichioides* (Fig. 1) showed that, with the use of primers strictly specific for this pathogen, only the *F. sporotrichioides* fungus is clearly identified (lanes 11–13), whereas the amplification with the DNAs of all other fungi gives no amplicons. At the same time, the band of the 560 bp amplicon corresponding to the IC is clearly recognized in all cases. Testing the other six fungus species using the appropriate pairs of primers given in Table 2 gave similar results (data not shown).

As it was mentioned above, the visualization of the PCR products by electrophoresis is a laborious and time-consuming process, which inevitably leads upon large-scale screenings to the contamination by amplification products. In order to simplify and accelerate the procedure and avoid the problems associated with possible contamination, we modified the analysis of the results of PCR amplification and transferred it into the FLASH format [24]. For this purpose, two regions were chosen in the region of the *tef-1*α flanked by specific

Table 1. Strains of *Fusarium* fungi used in studies

Number			*Fusarium* spp.	Origin	Host plant	Year of harvest
Index number	Working number	Collection number				
1	g.29	G.8-8	*F. graminearum*	Germany	Wheat	1998
2	g.48	41807	″	North Ossetia	″	2004
3	g.51	41806	″	North Ossetia	″	2004
4	g.91	48900	″	Tula region	″	2004
5	g.293	2903/1	″	FE, Primorye Territory	Rye	2002
6	c.8	37031	*F. culmorum*	China	Wheat	2003
7	c.9	61916	″	Bashkiria	″	2005
8	c.10	46504	″	Moscow region	″	2004
9	c.217	g.255	″	Rostov region	Thistle	2004
10	c.715	70517	″	Leningrad region	Barley	2006
11	s.2	33100	*F. sporotrichioides*	FE, Primorye Territory	Wheat	2003
12	s.8	2y-1	″	Leningrad region	Barley	2002
13	s.11	47000	″	Moscow region	Oats	2004
14	s.22	61501	″	Saratov region	Wheat	2005
15	s.66	143201	″	FE, Khabarovsk Territory	Thistle	2006
16	T50202/1	T50202/1	*F. tricinctum*	Leningrad region	Barley	2004
17	T48301	T48301	*F. avenaceum*	Ryazan region	Wheat	2004
18	Tfin2/1	Fin-2/1	*F. tricinctum*	Finland	Barley	2004
19	T60602/1	60602/1	″	Orlov region	Wheat	2005
20	T52304	T52304	″	FE, Primorye Territory	″	2004
21	T60602/2	60602/2	″	Orlov region	″	2005
22	T50202	50202	″	Leningrad region	Barley	2004
23	T30141	30141	″	Pskov region	Rye	2003
24	Tfin2/2	Fin–2/2	″	Finland	Barley	2004
25	p.7	33	*F. poae*	FE, Primorye Territory	Wheat	2001
26	p.11	40101	″	Krasnodar Territory	Barley	2003
27	p.10	55202	″	Kaliningrad region	Oats	2004
28	p.24	47401	″	Moscow region	Wheat	2004
29	p.32	61701	″	Saratov region	″	2005
30	A7194	A7194	*F. avenaceum*	Moscow region	″	2004
31	A7225	A7225	″	Kaliningrad region	Oats	2004
32	A7244	A7244	″	Krasnodar	Wheat	1997
33	A7192	A7192	″	Tambov region	″	2004
34	A7196	A7196	″	Moscow region	″	2004
35	l.6	1280/9822–219–1F	*F. langsethiae*	Norway	Oats	2002
36	l.9	1406/TMW1091	″	Germany	Barley	2003
37	l.11	54	″	Finland	Barley	2003
38	l.31	55201	″	Kaliningrad region	Oats	2005

Note: * FE, Far East.

Table 2. Sequences of oligonucleotides and probes used in the study

Species	Oligonucleotide sequence (5'⟶3')		Amplicon size, bp
	Primer	Probe*	
F. culmorum	CCCAACCCCGCCGATACA		310
	GGTTTGTGGGAAGAGGGCAAG		310
F. graminearum	CCCAACCCCGCCGACACT		250
	GGTTTGTGGGAAGAGGGCAGA		200
F. avenaceum	ATCCATTACCCCGCTCGAGC	FAM-CGCGCCCAGTGCGGTGG-TATCGACAAGCGCG-BHQ2	
	CCAGTGGTTAGTGACTGCAAGACATA		
F. langsethiae	GCTCTCATACGACGACTCGACAA		250
	GAAGGAACCCTTACCGAGCTCA		
F. tricinctum	ATCCATTACCCCGCTCGAGC		
	TCCAGTGGTTAGTGACTACAAGACATG		
F. sporotrichioides	AGTCGACCACTGTGAGTACATCTGC		300
	GTATGAGCCCCACTATCAAAAAAAA	FAM-CGGCGCTCGGTAAGGGT-TCTTTCAAGTACGCCG-BHQ2	
F. poae	AGTCGACCACTGTAAGTACAACCGA		300
	GGTATAAGCCCCACCGAAAATTT		

Note: * CF, carboxyfluorescein; BHQ2, black hole quencher 2.

primers, and two probes of the molecular "beacon" were synthesized, one specific for *F. poae* and *F. sporotrichioides*, and the other, for *F. graminearum*, *F. culmorum*, *F. langsethiae*, *F. tricinctum*, and *F. avenaceum* (Table 2). A probe for IC was chosen by a similar scheme, but using another fluorophore. All the details important for the development of the test systems in the FLASH format have been described previously [24, 25].

The identification of *F. sporotrichioides* using the FLASH format (Fig. 2) gave clear and unambiguous results similar to those obtained by the electrophoretic analysis in the determination of this fungus (Fig. 1). Similar results in this format were obtained for the other fungi.

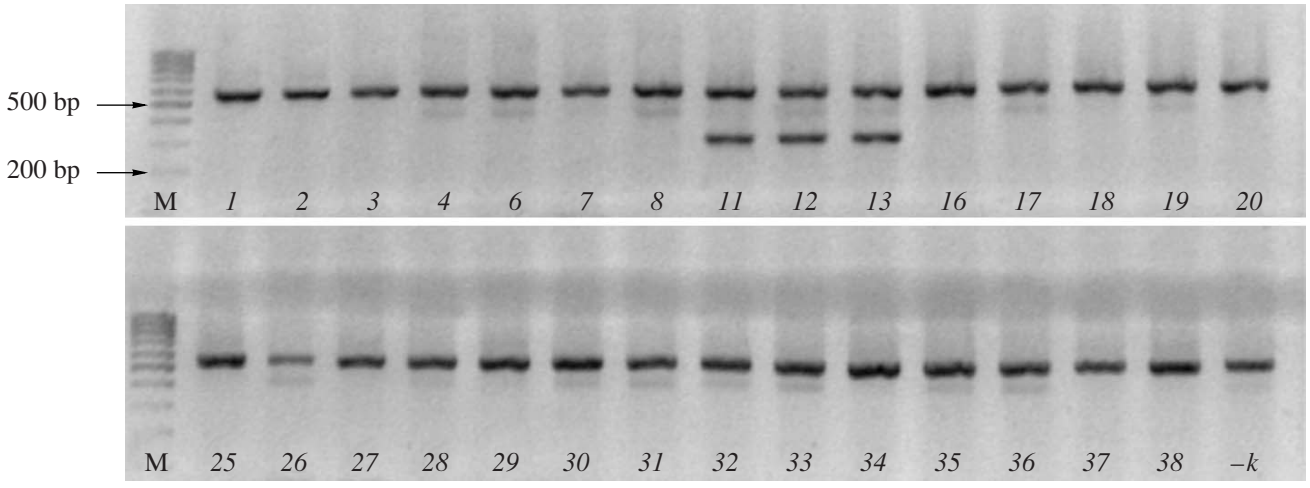

Fig. 1. Diagnosis of *Fusarium* fungi by the PCR-FLASH method by an example of *F. sporotrichioides* (primers and a probe specific for this species were used). Numbers of lanes correspond to the numbers in Table 1. On the abscissa, relative fluorescence intensity; black columns designate specific signal, and grey columns are for the IC.

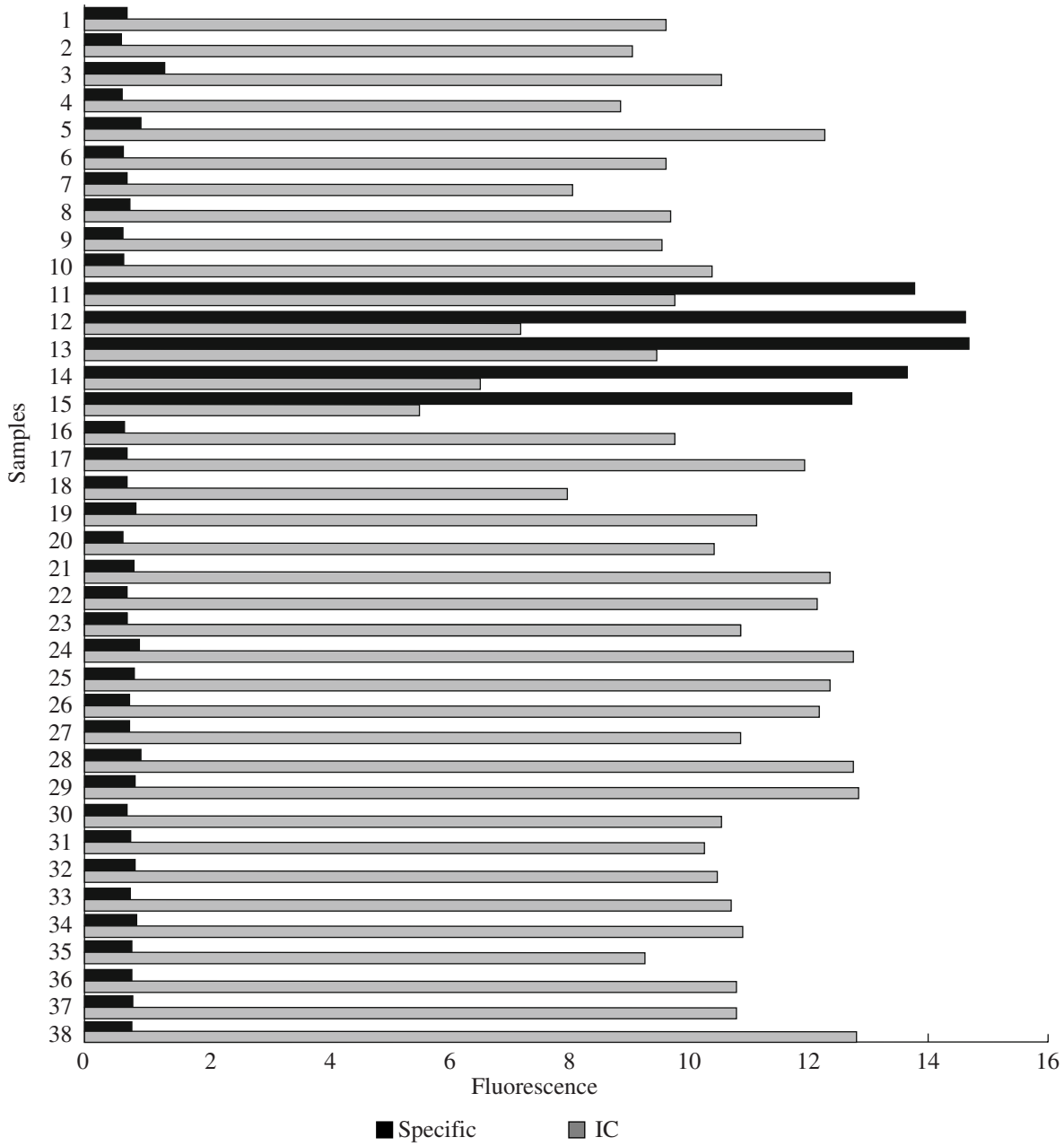

Fig. 2. Electrophoresis in 2.5% agarose gel of the products of the amplification of *Fusarium* fungi DNA using the primers specific for *F. sporotrichioides*. M, DNA marker. The numbers of lanes correspond to the numbers in Table 1.

The results of amplification of DNA preparations from the strains of seven *Fusarium* species of a wide geographical origin, which were obtained under optimized reaction conditions using specific primers in the region of the *tef-1α* gene, are summarized in Table 3. It follows from the table that all diagnostic systems are strictly species-specific, and the results of identifying the *Fusarium* strains by the FLASG-PCR method agree well with the results obtained by the mycological method. It should be noted that only one of the samples, isolate no. 17, did not correspond to the initial identification as *F. tricinctum* species and gave a positive result with a pair of primers specific for *F. avenaceum*. However, subsequent mycological assay confirmed its belonging to *F. avenaceum*, which is shown in Table 3. Although, according to the available classification of fungi, *F. avenaceum* and *F. tricinctum* belong to different sections, they produce similar secondary metabolites [27, 28], and some strains are often morphologically similar. Besides, a cross-reaction with species-specific primers of DNA from *F. tricinctum* and *F. avenaceum* was reported [29, 30]. Therefore, it is believed that these fungus species are closely related, and it is erroneous to assign *F. tricinctum* to the section

Table 3. Results of identification by the PCR-FLASH method of different *Fusarium* strains using the appropriate primers and probes presented in Table 2

Number	Strain	*F. graminearum*	*F. culmorum*	*F. poae*	*F. sporotrichioides*	*F. tricinctum*	*F. avenaceum*	*F. langsethiae*
1	g.29	+						
2	g.48	+						
3	g.51	+						
4	g.91	+						
5	g.293	+						
6	c.8		+					
7	c.9		+					
8	c.10		+					
9	c.217		+					
10	c.715		+					
11	s.2				+			
12	s.8				+			
13	s.11				+			
14	s.22				+			
15	s.66				+			
16	T50202/1					+		
17	T48301						+	
18	Tfin2/1					+		
19	T60602/1					+		
20	T52304					+		
21	T60602/2					+		
22	T50202					+		
23	T30141					?*		
24	Tfin2/2					+		
25	p.7			+				
26	p.11			+				
27	p.10			+				
28	p.24			+				
29	p.32			+				
30	A7194						+	
31	A7225						+	
32	A7244						+	
33	A7192						+	
34	A7196						+	
35	l.6							+
36	l.9							+
37	l.11							+
38	L.31							+

Note: * Sample gave a positive result with none pair of primers.

Table 4. Numbers of DNA sequences deposited in GenBank that were used in the choice of primers and probes

Species	Number under which the sequence is deposited in the GenBank
F. culmorum	AJ543556, AJ543555, AJ543554, AJ543553, AJ543552, AJ543551, AJ543550, AJ543549, AJ543548, AJ543547, AJ543546, AJ543545, AJ543544, AJ543543, AJ543542, AJ543541, AJ543540, AF212463
F. graminearum	DQ382170, DQ382168, DQ382167, DQ382166, DQ382165, DQ382164, AJ543600, AJ543599, AJ543598, AJ543597, AJ543596, AJ543595, AJ543594, AJ543593, AJ543592, AJ543591, AJ543590, AJ543589, AJ543588, AJ543587, AJ543586, AJ543585, AJ543584, AJ543583, AJ543582, AJ543581, AJ543580, AJ543579, AJ543578, AJ543577, AJ543576, AJ543575, AJ601394, XM_382131, XM_382127, XM_382124, AF212461, AF212458, AF212455, AF212453, AF212451, AF212447, AF212445, AF212444, AF212441, AF212439, AF212437, AF212436, AF212435, AY452958, AY452957
F. sporotrichioides	EU128238, EU128196, EU128195, EU128194, EU128193, EU128192, EU128191, EU128190, EU128189, EU128188, EU128187, EU128186, EU128185, EU128184, AJ420819, AJ420817, AJ420844, AJ420843, AJ420842, AJ420841, AJ420840, AJ420821, AJ420820, AJ420818, AY337442, AY337430
F. poae	AJ420833, AJ420838, AJ420839, AJ420837, AJ420836, AJ420835, AJ420834, AJ420832, AJ420816, AJ420815, AJ420814, AJ420813, AY337446
F. avenaceum	EU128237, EU128236, EF512021, EF512020, EF512019, EF512018, EF512017, EF512016, EF512015, EF512014, EF105293, AJ543535, AJ543534, AJ543533, AJ543532, AJ543531, AJ543530, AJ543529, AJ543528, AJ543527, AJ543526, AJ543525, AJ543524, AJ543523, AJ543522, AJ543521, AJ543520, AJ543519, AJ543518, AJ543517, AJ543516, AJ543515, AJ543514, AJ543513, AY337423
F. langsethiae	EF512028, EF512027, EF512026, AJ420827, AJ427273, AJ427272, AJ420831, AJ420830, AJ420829, AJ420828, AJ420826, AJ420825, AJ420824, AJ420823, AJ420822
F. tricinctum	AJ543630, AJ543629, AJ543628, AJ543627, AJ543626, AJ543625, AJ543624, AJ543623, AJ543622, AJ543621, AJ543620, AJ543619, AJ543618

Sporotrichiella [5, 29]. It should also be noted that sample no. 23, which was also assigned to the *F. tricinctum* species, gave positive signals with none of the pairs of primers, indicating probably that it belongs to another species.

Taken together, these results indicate that the system developed enables a simple, rapid, and reliable identification of species of toxigenic *Fusarium* fungi, which damage cereals. This system can serve as the basis for designing standard sets for both the routine diagnosis of these pathogens and research. We would like to emphasize that this system substantially reduces the time of analysis, is strictly specific, and enables one to eliminate contamination. It should be noted that PCR-FLASH systems are readily transformed into the online PCR format, which makes it possible to quantitatively estimate the presence of pathogens in the material being tested.

In subsequent studies, we are going to adapt these test systems for the detection of *Fusarium* fungi in cereals. Preliminary results with the analysis of grain infected with different strains of *F. graminearum*, performed using a test system for this fungus, gave obviously positive results. It is hoped that the technologies described here can be adapted and used to test both grain, first of all grain deposited for long-term storage, and the products of its reprocessing (food and feed) for the presence of toxigenic *Fusarium* fungi.

EXPERIMENTAL

Fungal strains and grain samples. All isolates of *Fusarium* were taken from the collection of the laboratory of Mycology and Phytopathology of the All-Russia Institute of Phytopathology, Russian Academy of Agricultural Sciences. *Fusarium* species were identified using standard parameters given in [31, 32].

Isolation of DNA. A small amount of fungus mycelium (~25 mg) was taken under sterile conditions from a Petri dish by means of a microbiological loop and placed in test tubes containing water (50 μl) after which DNA was isolated using a "Proba-GS" kit (DNK-Tekhnologiya, Russia) according to the manufacturer's protocol. Prior to the addition to the reaction mixture, the DNA preparation was diluted tenfold.

Specific primers were chosen by analyzing the *tef1*α nucleotide sequences deposited in GenBank [33] under numbers given in Table 4. The alignment of the corresponding nucleotide sequences was carried out using the program AlignX of the package Vector NTI Suite 8.0 (algorithm Clustal W for alignment and Neighbor Joining for constructing the phylogenetic trees) [34–36]; primers and beacon probes [37] were chosen using the program Oligo6 [38] and mfold 3.2 [39]. In addition, the program BLAST on the server National Center for Biotechnology Information (NCBI) was used [33].

Oligonucleotides and probes were synthesized by ZAO NPF DNK-Tekhnologiya (Russia). PCR was carried out on a Tertsik amplificator (DNK-Tekhnologiya, Russia) in a reaction mixture (35 μl) containing 3.5 μl of tenfold buffer (750 mM Tris-HCl, pH 8.8, 200 mM ammonium sulfate, and 0.1% Tween-20), dNTP (1 mM each), primers (12.5 pmol each), probes (3.5 pmol), 2.5 units of *Taq* polymerase (ZAO NPF DNK-Tekhnologiya, Russia), and a DNA solution (5 μl). The results of PCR amplification were analyzed either by electrophoresis in 2.5% agarose gel using a 100 bp DNA marker (DIALAT Ltd., Russia) by visualization on an ANO-15-I transilluminator (Vilber-Lourmat, France) or in the FLASH format by measuring the fluorescence on a Dzhin fluorescence detector (ZAO NPF DNK-Tekhnologiya, Russia) [25].

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