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Current Approaches to Identification of *Fusarium* Fungi Infecting Wheat

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Abstract—Fungi of the genus *Fusarium* are especially dangerous phytopathogens affecting common wheat (*Triticum aestivum* L.) among other crops, as they may cause not only crop losses but poisoning of humans and livestock. The review highlights current approaches to identify fungi of the genus *Fusarium* infecting common wheat. Microbiological techniques for identification of *Fusarium* species are still among laboratory protocols and recommendations, therefore some of the most popular genus- and species-specific media are mentioned in the review. However, in the modern literature, much more attention is paid to identification of *Fusarium* fungi with the use of the polymerase chain reaction (PCR). Therefore, conventional PCR assays for identification of representatives of the genus *Fusarium* in general or only species producing especially dangerous metabolites (nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 4-acetylnivalenol, and enniatin) are highlighted in the review. The primer pairs to identify the presence of certain *Fusarium* species or their combinations in samples are described. For real-time PCR assays, which may be used for more precise quantitative and qualitative genus- and species-specific identification of *Fusarium* fungi, protocol details, primer and probe sequences are described, as well as recommended dyes are mentioned for the probes. For some primer pairs, additional details regarding their validation and assay sensitivity are mentioned. Thus, the techniques described in the review are precise and comprehensive enough and may be used in combination and separately for genus- and species-specific quantitative or qualitative identification of fungi of the genus *Fusarium*.

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Necrotrophic fungal phytopathogens feed on cell remnants after their forced destruction (so called "hypersensitive death") (Gazenbrook, 2005). Such destruction is often elicited by one or several toxins produced by fungi (Miller et al., 1991; Desmond et al., 2008). For example, in Ukraine necrotrophic phytopathogenic fungi, among others, are represented by fungi of the genus *Fusarium,* in particular, causative agents of Fusarium head blight *Gibberella zeae* (Schwein.) Petch. (anamorph – *F. graminearum*), *F. culmorum* Wm. G. Sm., *F. sporotrichioides*, *F. oxysporum*, *F. avenaceum*, *F. verticillioides* (*F. moniliforme*), *F. langsethiae*, *F. poae*, *F. tricinictum*, *F. cerealis* (*F. crookwellense*) and causative agents of pink snow mold *Microdochium* (*Fusarium*) *nivale* (Kovalyshyna et al., 2008; Hrytsev et al., 2018). Probability, severety levels, and crop losses of wheat caused by *Fusarium* fungi are influenced by weather conditions, physical parameters and flowering time for plants of each cultivar (Snijders and Perkowski, 1990; Ward et al., 2008). In China, Fusarium head blight every year affects on average 5.4 Mha of the crops (23% of the total area under wheat) and causes yield losses of up to 2 Mt in the case of epiphytotics (Ma et al., 2019). In the USA, epiphytotics of Fusarium head blight cause average direct losses of $1.3 and $4.8 bln of losses as accumulated economic effect (Johnson et al., 2003), and, according to the calculations of Salgado et al. (2015), Fusarium head blight damage of 19% reduces yield by 1 t/ha. In the Forrest-Steppe zone of Ukraine, the reduction in grain weight caused by *Fusarium* fungi may amount up to 70% (Kyslukh, Shevchuk, 2006). In addition, mycotoxins produced by *Fusarium* fungi may cause intoxication of humans and livestock (Miller et al., 1991; Desmond

et al., 2008). Therefore, detection of fungi of the genus *Fusarium* infecting wheat is a necessary measure to control these pathogens.

Detection of *Fusarium* fungi using selective media. According to numerous recommendations, detection of *Fusarium* fungi is divided into two stages: detection of the presence of any of *Fusarium* fungi and their species-specific detection by phenotypic analysis or additional cultivation on a selective medium and species- or even race-specific detection by polymerase chain reaction (PCR) (Leslie et al, 2006). For cultivation of fungi of the genus *Fusarium*, Carnation Leaf-piece Agar (CLA), Spezieller Nährstoffarmer Agar (SNA), and Potato Dextrose Agar (PDA) are often used (Anderson and Atkinson, 1974; Fisher et al., 1982; Leslie et al., 2006); in other sources, the use of minimal media (MM) and derivatives is also described (Puhall, 1985). Methods of phenotypic detection are fast and cheap enough but each paper or laboratory handbook suggests changes to the above media or their own media and cultivation conditions. It is believed that only in the case of strict compliance with the recommendations the morphology of the resulting colonies may be compared with those given in a particular source. However, even in that case the result might be different from the expected one (Leslie et al., 2006).

Selective media for *Fusarium* fungi offer a much more convenient and accurate tool for species-specific detection. They are used for isolation of fungi of this genus in general: the most popular medium is peptone-pentachloronitrobenzene (PCNB) (Papavizas, 1967); besides there are studies recommending the use of the Czapek-Dox Iprodione Dichloran agar (CZID) (Abildgren et al., 1987; Thrane, 1996). Moreover, there is Komada's medium, which is proposed for selective isolation of some *Fusarium* species from soil with further distinguishing them by colony color (Komada, 1976). Such media have been developed for some *Fusarium* species infecting wheat. For instance, the Segalin and Reis agar (SRA-FG) based on PDA supplemented with iprodione (0.05 g/L), nystatin (0.025 g/L), triadimenol (0.015 g/L), neomycin sulfate (0.05 g/L), and streptomycin sulfate (0.3 g/L) was proposed as a semi-selective medium for detection of *F. graminearum* (Segalin and Reis, 2010). Media based on MM or PDA supplemented with toxoflavin from *Burkholderia glumae* in the final concentration of 80 mg/L may be used for detection of *F. graminearum* and *F. oxysporum* (Jung et al., 2013). A number of effective selective media for detection of *F. oxysporum* were developed: Fo-G1 and Fo-G2 for detection of wild-type strains in regular soils; Fo-W1 and Fo-W2 for the wild types in the soils where it is possible to encounter mutants that do not utilize nitrates, and Fo-N1 and Fo-N2 for such mutants (Nishimura, 2007). As for the causative agent of pink snow mold, a selective medium based on Komada's medium (Komada, 1976) supplemented with 10 mcg/L thiophanate-methyl to inhibit growth of other *Fusarium* fungi was recommended (Hayashi et al., 2014).

Thus, there are media that are selective for particular *Fusarium* species, but they are not numerous: development of a species-specific medium is rather time-consuming, and such a medium might require many sometimes exotic components; for some species, it might be impossible to find such a component for the medium on which related *Fusarium* species would not form colonies. Therefore in publications on detection of *Fusarium* species much more attention is paid to techniques based on PCR (Leslie et al., 2006).

Genus-specific primers for detection of *Fusarium* fungi by PCR. One may use genus-specific primers for detection of the presence of *Fusarium* fungi in a sample to avoid using a larger amount of chemicals for species-specific identification (Leslie et al., 2006). The details for some of them are listed in Table 1. These are commonly pairs of primers that are complementary to DNA fragments controlling the mating type (MAT) (Leslie et al., 2006; Steenkamp et al., 2000; Kerenyi et al., 2004). For instance, the pairs of primers GFmat1a/b and GFmat1c/d in a multiplex reaction amplify fragments of 200 and 800 bp, respectively; the authors recommend to use the multiplex, as not all *Fusarium* species carry one or another target DNA sequence; amplicon length may vary from species to species (Steenkamp et al., 2000). Based on sequencing the respective genes, other primer pairs were designed: fusALPHAfor/fusALPHArev producing 200 bp amplicons and fusHMGfor/fusHMGrev producing 260-bp fragments (Kerenyi et al., 2004; Leslie et al., 2006). Other authors developed a genus-specific marker for the fragment of the internal transcribed spacer (ITS) of rDNA with primers IrsF/R amplifying 431-bp fragments, as well as the *TRI6* marker flanked by the primers tri6f/tri6r amplifying 596 bp fragments in the case of trichothecene-producing *Fusarium* species and the *FUM5* marker flanked by the primers fum5f/fum5r yielding 845-bp fragments in the case of fumonisin-producing *Fusarium* species (Bluhm et al., 2002).

Arif et al. (2012) developed genus-specific primers based on ITS (ITS-Fu1f/ITS-Fu1r) and those based on the gene for translation elongation factor TEF-1α (TEF-Fu3f/TEF-Fu3r) producing amplicons of approximately 466 and 420 bp, respectively, in the case of the presence of *Fusarium* fungi. Genus-specific primers based on rDNA intergenic spacer (IGS) sequences were designed by Jurada et al. (2006): in the presence of *Fusarium* species, PCR yields amplified fragments of approximately 200 bp. The majority of *Fusarium* species produce trichothecene toxins. For their detection, the pairs of primers Tri5F/Tri5R or Tri6Fsp/T6EndR may be used, the former produces 545 bp amplicons, the latter yields 550-bp fragments (Nicholson et al., 2004). Primers for detection of trichothecene-producing *Fusarium* fungi developed by

Table 1. Primers and PCR details for identification of fungi of the genus *Fusarium*

Primer	Sequence (5'-3')	Annealing temperature, °C	Target region	Amplicon length, bp	Reference
GFmat1a	gttcatcaaagggcaagcg	67	MAT	200	Steenkamp et al., 2000
GFmat1b	taagcgccctcttaacgccttc				
GFmat1c	agcgtcattattcgatcaag	67	MAT	800	Steenkamp et al., 2000
GFmat1d	ctacgttgagagctgtacag				
fusALPHAfor	cgccctctkaaygscttcatg	60	MAT box	200	Kerenyi et al., 2004
fusALPHArev	Ggartaracyttagcaatyagggc				
fusHMGfor	cgacctcccaaygcytacat	60	MAT HMG	260	Kerenyi et al., 2004
fusHMGrev	tgggcggtactggtartcrgg		box		
ItsF	Aactcccaaacccctgtgaacata	62	rDNA	431	Bluhm et al., 2002
ItsR	tttaacggcgtggccgc	68	ITS	~466	Arif et al., 2012
ITS-Fu1f	acaactcataaccctgtgaacat		rDNA		
ITS-Fu1r	cagaagttgggtgttttacgg	58	ITS	~466	Arif et al., 2012
TEF-Fu3f	ggtatcgacaagcgaaccat		TEF-1α		
TEF-Fu3r	tagtagcggggagtctcgaa				
tri6f	ctctttgatcgtgttgcgtc	62	TRI6	596	Bluhm et al., 2002
tri6r	cttgtgtatccgcctatagtgatc				
fum5f	gtcgagttgttgaccactgcg	62	FUM5	845	Bluhm et al., 2002
fum5r	cgtatcgtcagcatgatgtagc				
Tri5F	agcgactacaggcttccctc	60	Tri5	545	Nicholson et al., 2004
Tri5R	aaaccatccagttctccatctg				
Tri6Fsp	catgccaaggactttgtccc	56	Tri6	550	Nicholson et al., 2004
T6EndR	gtgtatccgcctatagtgat				
Fus-R	ggcgaaggacggcttac	67	IGS	~200	Jurado et al., 2006
Fps-F	cgcacgtatagatggacaag				
Tct-F	cactgcgtgctgattcactgg	62	IGS	~500	Jurado et al., 2006
Tct-R	gagacaagcatatgactactggcag				

Jurada et al. (2006), Tct-F and Tct-R, produce amplicons of approximately 500 bp.

A number of qualitative conventional PCR assays with the use of species-specific primers, as well as primers that are complementary to DNA sequences controlling virulence factors which are common for several species, were developed to identify *Fusarium* species infecting wheat (Demeke et al., 2005; Kuzdraliński et al., 2017a; Villafana et al., 2020). For instance, primers that are complementary to the genes *Tri3*, *Tri5*, *Tri7*, *Tri13*, and *Ensyn* involved in the production of nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3A-DON), 4-acetyl-nivalenol (4A-NIL), and enniatin according to the European chemotypes of *F. graminearum*, *F. culmorum* and *F. cerealis* were developed and validated (Nicholson et al., 2004; Quarta et al., 2005, 2006) (Table 2).

Validation of primers with the use of multiplex PCR revealed that for all samples of *F. cerealis* studied with the primers 3551H and 4056H only fragments that are complementary to the *Tri7* gene were amplified; European samples of *F. culmorum* showed either the similar profile to *F. cerealis* or the combination *Tri3*-3A-DON/*Tri5* with the primers Tri3F1325/Tri3R1679 and 3551H/4056H; as for all the samples of *F. graminearum*, amplicons corresponding to the fragment *Tri3*-3A-DON with the primers 3551H/4056H, along with fragments amplified with any of the primer pairs Tri7F340/Tri7R965, Tri3F1325/Tri3R1679 or Tri3F1325/Tri3R1679, were observed (Quarta et al., 2006). The pair of primers 22F/122R flanking the marker sequence based on the partial transcript of the *Tri5*/*Tri6* genes controlling DON biosynthesis was also reported; at the annealing temperature of 60°C they produce 100-bp amplicons and might be used in real-time PCR with SYBR Green (then it is 40 cycles with denaturation at 95°C for 20 s and annealing/denaturation at 60°C for 1 min) as well as in conventional PCR (the annealing time should be reduced up to 30–40 s and the elongation phase at 72°C for 20–30 s is added) (Terzi et al., 2007). This PCR assay

Table 2. Primers and PCR details for genes associated with biosynthesis of certain biotoxins

Primer	Sequence (5'–3')	Annealing temperature, °C	Target region	Amplicon length, bp	Chemotype	Reference
3551H	actttcccaccgagtatttt	53	*Tri5*	525	DON	Quarta et al., 2006
4056H	caaaaactgttgttccactgcc					
Tri7F	tgcgtggcaatatcttcttcta	60	*Tri7*	>380		Nicholson et al.,2004
Tri7DON	gtgctaatattgtgctaatattgtgc					
MinusTri7F	tggatgaatgacttgagttgaca	58		483		
MinusTri7R	aaagccttcattcacagcc					
Tri13F	catcatgagacttgtkcragtttggg	58	*Tri13*	282		
Tri13DON	gctagatcgattgttgcattgag					
Tri3F971	catcatactcgctctgctg	53	*Tri3*	708	15A-DON	Quarta et al., 2005
Tri3R1679	tt(ag)tagtttgcatcatt(ag)tag					
Tri3F1325	gcattggctaacacatga	53	*Tri3*	354	3A-DON	
Tri3R1679	tt(ag)tagtttgcatcatt(ag)tag					
Tri7F340	atcgtgtacaaggtttacg	50	*Tri7*	625	NIV	
Tri7R965	ttcaagtaacgttcgacaat					
Tri7F	tgcgtggcaatatcttcttcta	60		465		Nicholson et al., 2004
Tri7NIV	ggttcaagtaacgttcgacaatag					
Tri13NIVF	ccaaatccgaaaaccgcag	58	*Tri13*			
Tri13R	ttgaaagctccaatgtcgtg					
Ensyn6065F	gctggcaggaccatttcg	58	*Ensyn*	1164	Enniatin	
Ensyn7229R	ggatggaaagtggtggaagac					

was validated with DON-producing *F. culmorum* and *F. graminearum* (Rossi et al., 2007).

Species-specific primers for detection of *Fusarium* fungi using PCR. The first reliable PCR assay for species-specific detection of *F. graminearum* was considered to be the pair of SCAR primers Fg16F/R producing amplicons of 400–500 bp (Nicholson et al., 1998; Kuzdraliński et al., 2017a) (Table 3). In particular, it was used for species-specific identification of this pathogen in wheat grains of different origin (for instance, see Martinez et al., 2014; Khaledi et al., 2017; Kuzdraliński et al., 2017b; Wang and Cheng, 2017; Krnjaja et al., 2018). Another primer pair, Fg16NF/Fg16NR (Table 3), also developed in 1998, has not received wide recognition, although at the same annealing temperature it produced 280-bp amplicons (Nicholson et al., 1998). The touchdown protocol, in particular, 66°C in cycles 1–5, 64°C in cycles 6–10, and 62°C in the following cycles 11–30 is recommended for these pairs of primers (Nicholson et al., 2004). The primers Fg16F/R were used in the early studies on quantitative detection of this pathogen in real-time PCR with SYBR Green (Brandfass and Karlovsky, 2006). However, amplicons of different lengths were obtained in later studies with DNA samples from other species analyzed with the primer pair Fg16F/R (Covarelli et al., 2011; Castañares et al., 2014; Kuzdraliński et al., 2017a). Further, on the basis of the IGS region of *F. graminearum*, more precise primers Fgr-F/Fgc-R producing 500-bp amplicons were developed (Table 3) (Jurado et al., 2005; Kuzdraliński et al., 2017a).

For detection of *F. culmorum,* the SCAR primers Fc01F/R with expected 570-bp amplicons were also developed (Nicholson et al., 1998; Kuzdraliński et al., 2017a). The reaction with the touchdown protocol, like for *F. graminearum*, is recommended for these primers (Nicholson et al., 2004). The primers Fc01F/R were validated with Canadian strains of *F. culmorum* (Demeke et al., 2005). Other PCR-based assays were developed for fungi of this species. Mishra et al. (2003) developed the assay based on the ITS region. The corresponding marker is flanked by the primers 175F/430R producing 245-bp amplicons (Table 3) (Mishra et al., 2003; Kuzdraliński et al., 2017a). The primer pair FcuF/Fgc-R producing amplicons of approximately 200 bp was designed based on the IGS region (Table 3) (Jurado et al., 2005; Kuzdraliński et al., 2017a). The assay with the primer pair Fc03/Fc02 producing 140-bp amplicons was developed to detect *F. culmorum* in real-time PCR, but the amplified products may be also resolved in agarose gels (Table 3) (Sanoubar et al., 2015).

One of the first assays for detection of *F. sporotrichioides* was developed via analysis of the IGS region sequence. As a result, the primer pair CNL12/PulvIGSr produced 610-bp amplicons with DNA samples of *F. sporotrichioide*s and 750-bp fragments with DNA of *F. langsethiae* (Table 3) (in the primer pair, only PulvIGSr is complementary to DNA of the target fungal species and CNL12 is common for many eukary-

Table 3. Primers and PCR details for species-specific identification of fungi of the genus *Fusarium*

Species	Primer pair	Sequence (5'-3')	Annealing temperature, °C	Amplicon length, bp	Reference
F. graminearum	Fg16F	ctccggatatgttgcgtcaa	62	400–500	Nicholson et al., 1998
	Fg16R	ggtaggtatccgacatggcaa			
F. graminearum	Fg16NF	acagatgacaagattcaggcaca	62	280	Nicholson et al.,1998
	Fg16NR	ttctttgacatctgttcaaccca			
F. graminearum	Fgr-F	gttgatgggtaaaagtgtg	53	500	Jurado et al., 2005
	Fgc-R	ctctcatataccctccg			
*F. graminearum**	FgramB379 fwd	ccattccctgggcgct			Nicolaisen et al., 2009
	FgramB411 rev	cctattgacaggtggttagtgactgg			
F. culmorum	Fc01F	atggtgaactcgtcgtggc	62	570	Nicholson et al., 1998
	Fc01R	cccttcttacgccaatctcg			
F. culmorum	175F	ttttagtggaacttctgagtat	58	245	Mishra et al., 2003
	430R	agtgcagcaggactgcagc			
F. culmorum	Fcu-F	gactatcattatgcttgcgagag	54	200	Jurado et al., 2005
	Fgc-R	ctctcatataccctccg			
F. culmorum	Fc03	ttcttgctagggttgaggatg	62	140	Sanoubar et al., 2015
	Fc02	gaccttgactttgagcttcttg			
*F. culmorum**	FculC561 fwd	caccgtcattggtatgttgtcact			Nicolaisen et al., 2009
	FculC614 rev	cgggagcgtctgatagtcg			
F. sporotrichioides/ *F. langsethiae*	CNL12	ctgaacgcctctaagtcag	58	610/750	Konstantinova and Yli-Mattila, 2004
	PulvIGSr	gaaccgtccggcacccatcc	55	400	
F. sporotrichioides/ *F. langsethiae*	SporoITSf	tcagcccgcgccccgtaa			Konstantinova and Yli-Mattila,2004
	SporoITSr	caatttgggactgtgtttgc			
F. sporotrichioides	FspITS2K	cttggtgttgggatctgtgtgcaa	68	288	Kulik et al., 2004
	P28SL	acaaattacaactcgggcccgaga			
F. sporotrichioides	Tox5-1	gctgctcatcactttgctcag	56	400	Niessen et al., 2004
	Tox5-sporo-R2	tcaacttcgggatgtggagg			
F. sporotrichioides	FsporF1	cgcacaacgcaaactcatc	62	332	Wilson et al., 2004
	LanspoR1	tacaagaagacgtggcgatat			
*F. sporotrichioides**	FspoA18 fwd	gcaagtcgaccactgtgagtaca			Nicolaisen et al., 2009
	FspoA85 rev	ctgtcaaagcatgtcagtaaaaatgat			
F. avenaceum	FA-ITSF	ccagaggacccaaactctaa	59	272	Schilling et al., 1996
	FA-ITSR	accgcagaagcagagccaat			
F. avenaceum/ *F. tricinictum*	Fa-U17f	caagcattgtcgccactctc	62	345	Turner et al., 1998
	Fa-U17r	gtttggctctaccgggactg			
F. avenaceum	JIAf	gctaattcttaacttactaggggcc	58	220	Turner et al., 1998
	JIAr	ctgtaataggttatttacatgggcg			
F. avenaceum	FAF1	aacataccttaatgttgcctcgg	52	314	Mishra et al., 2003
	FAR	atccccaacaccaaacccgag			
F. avenaceum	Fa-8f	cacgactcgctccctcattcgccagt	63	188	Pollard and Okubara 2019
	Fa-13R	ggttagtgactgcaagacatag			
*F. avenaceum**	Fave574 fwd	tatgttgtcactgtctcacaccacc			Nicolaisen et al., 2009
	Fave627 rev	agagggatgttagcatgatgaag			
F. poae	Fp82F	caagcaaacaggctcttcacc	62	220	Parry and Nicholson, 1996
	Fp82R	tgttccacctcagtgacaggtt			
F. poae	CNL12	ctgaacgcctctaagtcag	58	306	Konstantinova and Yli-Mattila, 2004
	PoaeIGSr	caagctctcctcggagagtcgaa			
F. poae	Tox5-1	gctgctcatcactttgctcag	57	400	Niessen et al., 2004
	Tox5-poae-R	tcgtggtgaaacaatgtat			

Table 3. (Contd.)

Species	Primer pair	Sequence (5'-3')	Annealing temperature, °C	Amplicon length, bp	Reference
F. poae	Fps-F	cgcacgtatagatggacaag	61	400	Jurado et al., 2005
	Fpo-R	cagcgcacccctcagagc			
*F. poae**	FpoaeA51 fwd	accgaatctcaactccgcttt			
	FpoaeA98 rev	gtctgtcaagcatgttagcacaagt			
F. proliferatum	Fp3-F	cggccaccagaggatgtg	69	~230	Jurado et al., 2006
	Fp4-R	caacacgaatcgcttcctgac			
F. proliferatum	PRO1	ctttccgccaagtttcttc	56	585	Mulè et al., 2004
	PRO2	tgtcagtaactcgacgttgttg			
*F. proliferatum**	Fpro220 fwd	cttcgatcgcgcgtcct			Nicolaisen et al., 2009
	Fpro270 rev	cacgtttcgaatcgcaagtg			
F. verticillioides	VER1	cttcctgcgatgtttctcc	56	578	Mulè et al., 2004
	VER2	aattggccattggtattatatatcta			
F. verticillioides	VERT-1	gtcagaatccatgccagaacg	64	~800	Patiño et al., 2004
	VERT-2	cacccgcagcaatccatcag			
F. verticillioides (only those that produce fuminosin)	VERTF-1	gcgggaattcaaaagtggcc	64	~400	Patiño et al., 2004
	VERTF-2	gagggcgcgaaacggatcgg			
F. verticillioides	FV-F1	gtacaatccccctgttaagg	64	649	Faria et al., 2012
	FV-R	caccctgagtgcccttggtg			
F. verticillioides	FV-F2	actggtggtaacgatgcg	64	370	Faria et al., 2012
	FV-R	caccctgagtgcccttggtg			
*F. verticillioides**	Fver356 fwd	cgtttctgccctctccca			Nicolaisen et al., 2009
	Fver412 rev	tgcttgacacgtgacgatga			
F. tricinctum	tri1	cgtgtccctctgtacagctttga	65	215	Kulik, 2008
	tri2	gtggttacctcccgatactcta			
*F. tricinctum**	Ftri573 fwd	ttggtatgttgtcactgtctcacactat			Nicolaisen et al., 2009
	Ftri630 rev	tgacagagatgttagcatgatgca			
F. pseudograminearum	Fp1-1	cggggtagtttcacatttc(c/t)g	55	523	Aoki and O'Donnell, 1999
	Fp1-2	gagaatgtgatga(c/g)gacaata			
F. oxysporum	FOF1	acataccacttgttgcctcg	58	340	Mishra et al., 2003
	FOR1	cgccaatcaatttgaggaacg			
F. equiseti	FEF1	catacctatacgttgcctcg	58	389	Mishra et al., 2003
	FER1	ttaccagtaacgaggtgtatg			
*F. equiseti**	FequiB569 fwd	caccgtcattggtatgttgtcatc			Nicolaisen et al., 2009
	FequiB598 rev	tgttagcatgagaaggtcatgagtg			
F. equiseti	Feq-F	ggcctgccgatgcgtc	66	~990	Jurado et al., 2005
	Feq-R	cgatactgaaaccgacctc			
F. solani	ITS-Fu2f	ccagaggacccccctaactct	63,5	595	Arif et al., 2012
	ITS-Fu2r	ctctccagttgcgaggtgtt			
F. solani	ITS-Fs5f	cgtcccccaaatacagtgg	61	485	Arif et al., 2012
	ITS-Fs5r	tcctccgcttattgatatgctt			
F. solani	TEF-Fs4f	atcggccacgtcgactct	58	658	Arif et al., 2012
	TEF-Fs4r	ggcgtctgttgattgttagc			
F. solani	FS1	gcaggtatggctttttggaa	57	175	Casasnovas et al., 2013
	FS2	gtaaactccgacaggtgcaa			
F. cerealis	TCRO-A-f	ctcagtgtccaccgcgttgcgtag	60	842	Yoder and

Table 3. (Contd.)

Species	Primer pair	Sequence (5'-3')	Annealing temperature, °C	Amplicon length, bp	Reference
	CRO-A-r	ctcagtgtcccaatcaaatagtcc			Christianson, 1998
F. langsethiae	FlangF3	caaagttcagggcgaaaact	62	310	Wilson et al., 2004
	LanspoR1	caagtcgaccactgtgagtacctct			
*F. langsethiae**	FlangA29 fwd	caagtcgaccactgtgagtacctc			Nicolaisen et al., 2009
	FlangA95 rev	tgtcaaagcatgtcagtaaagatgac			
F. subglutinans	61-2 F	ggccactcaagaggcgaaag	64	445	Möller et al., 1999
	61-2 R	gtcagaccagagcaatgggc			
F. subglutinans	SUB1	ctgtcgctaacctctttatcca	56	631	Mulè et al., 2004
	SUB2	cagtatggacgttggtattatatctaa			
F. subglutinans	FS-F1	gtacaacccgcctgctaagg	62	649	Faria et al., 2012
	FS-R	taccctgagtacccctatcg			
F. subglutinans	FS-F2	tactggcggcaacgacgct	62	370	Faria et al., 2012
	FS-R	taccctgagtacccctatcg			
*F. subglutinans**	Fsub565 fwd	tcattggtatgttgtcgctcatg			Nicolaisen et al., 2009
	Fsub622A rev	gtgatatgttagtacgaataaagggagaac			
M. nivale var. *majus*	Mnm2F	tgcaacgtgccagaagct	61	750	Nicholson et al., 1996
	Mnm2R	aatcggcgctgtctactaaaagc			
M. nivale var. *nivale*	Y13NF	accagccgatttgtggttatg	61	310	Nicholson and Parry, 1996
	Y13NR	ggtcacgaggcagagttcg			

* Primers for quantitative detection by real-time PCR with SYBR Green.

otes); those primer pair also produced amplicons with DNA from other plants but not other fungi (Konstantinova and Yli-Mattila, 2004). SporoITSf/SporoITSr was another ITS-based primer pair developed by the same authors, which was supposed to produce 400-bp amplicons with DNA of *F. sporotrichioides* and *F. langsethiae* (Table 3), but the authors obtained more non-specific fragments with DNA from other species (Konstantinova and Yli-Mattila, 2004; Kuzdraliński et al., 2017a). Other assay based on the ITS2 fragment with the primer pair FspITS2K/P28SL resulted in 288-bp amplicons (Table 3) and was sufficiently species-specific (Kulik et al., 2004; Kuzdraliński et al., 2017a). In another PCR assay based on the *Tox5* gene, a universal primer Tox5-1 was used, which, in combination with the species-specific primer Tox5-sporo-R2, produced 400-bp amplicons with DNA extracted from the majority of *F. sporotrichioides* samples employed for validation of the primers (Table 3) (Niessen et al., 2004). Based on the ITS region, Wilson et al. (2004) developed species-specific pairs of primers for identification of *F. sporotrichioides* (FsporF1/LanspoR1) and *F. langsethiae* (FlangF3/LanspoR1) sharing the reverse primer (Table 3).

The first PCR assay for detection of *F. avenaceum* was developed based on the ITS region. It involved the primer pair FAITSF/FA-ITSR producing 272-bp amplicons (Table 3). That primer pair was validated with DNA of fungal isolates from different regions, as well as plant DNA, and no false-positive results were reported by the authors (Schilling et al., 1996). Other assays were also based on that region. For instance, with a recommended touchdown PCR protocol (5 cycles at the annealing temperature of 66°C, 5 cycles at 64°C followed by 30 cycles at 62°C), the primer pair FaU17f/Fa-U17r produced 345-bp amplicons, whereas the pair JIAf/JIAr yielded 220-bp amplicons (Table 3) (Turner et al., 1998). However, the primer pair Fa-U17/fFa-U17r was specific not only for *F. avenaceum* but also for *F. tricinictum* (Turner et al., 1998; Kuzdraliński et al., 2017a). The primer pair JIAf/JIAr turned out to be more specific and was validated in later studies (Doohan et al., 1998; Demeke et al., 2005). Another primer pair, FAF1/FAR, with expected amplified fragments of 314 bp (Table 3) was developed for possible detection *of F. avenaceum* in PCR with labeled primers, but obviously ethidium bromide added to agarose gel may be used as a dye (Mishra et al., 2003). In addition, the primer pair Fa-8f/Fa-13r was developed: it produces 188-bp amplicons and can also be used in real-time PCR with SYBR Green (Table 3) (Pollard and Okubara, 2019).

The primer pair Fp82F/R is most commonly used for detection of *F. poae* (Table 3) (Parry and Nicholson, 1996; Kuzdraliński et al., 2017a). This primer pair yielded 220-bp amplicons and did not produce any nonspecific fragments with other DNA samples that its authors used for validation (Parry and Nicholson, 1996). The primer pair CNL12/PoaeIGSr producing 306-bp amplicons with DNA samples of *F. poae* was also

described (Table 3), but the authors also obtained amplified fragments with DNA samples of *F. kyushuense* and *F. langsethiae* used for validation (Konstantinova and Yli-Mattila, 2004). Similarly, the primer pair Tox5-1/Tox5-poae-R produced 400-bp amplicons with DNA of 45 isolates of *F. poae* and non-specific fragments with one isolate of *F. langsethiae* (Niessen et al., 2004; Kuzdraliński et al., 2017a). Jurado et al. (2005) developed a test system based on the partial IGS sequence involving the primers Fps-F/Fpo-R. They produced 400 bp amplicons (Table 3). This test system was further validated with a wide range of DNA samples from different *Fusarium* species (Jurado et al., 2006).

To detect *F. tricinctum,* a PCR assay based on partial IGS sequence involving the primers tri1/tri2 was proposed. With those primers, the author obtained 215-bp amplicons for all DNA samples of the target phytopathogen used in the study and did not observe any nonspecific fragments (Table 3) (Kulik, 2008).

For detection of *F. oxysporum*, Mishra et al. (2003) developed species-specific primers FEF1/FER1 based on the ITS sequence of nuclear rDNA producing 340 bp amplicons. Similarly, the same authors proposed primers for detection of *F. equiseti*: FEF1/FER1 yielding species-specific amplicons of 389-bp. For *F. equiseti*, the IGS-based primers Feq-F/Feq-R were also designed (Table 3) (Jurado et al., 2005).

As for *F. cerealis* (*F. crookwellense*), back in 1998, Yoder and Christianson (1998), based on analysis of RAPD patterns, developed species-specific primers CRO-A producing 842-bp amplicons (Table 3). In particular, those primers were validated by Demeke et al. (2005).

Different asays are used for different subspecies of *M. nivale*. For instance, for detection of *M. nivale* var. *majus,* the PCR assay with the primers Mnm2F/R producing 750-bp amplicons was proposed (Nicholson et al., 1996). For detection of *M. nivale* var. *nivale*, the assay based on primers Y13NF/R with expected 310 bp amplicons was developed (Table 3) (Nicholson and Parry, 1996).

F. pseudograminearum, a causative agent of *Fusarium* crown rot of wheat, was earlier classified as *F. graminearum* of group 1 (Aoki and O'Donnell, 1999; Kazan and Gardiner, 2018). Primers for detection of *F. pseudograminearum* were developed by Aoki and O'Donnell (1999) (Table 3). Using those primers it was shown that *F. pseudograminearum* is a prevalent causative agent of Fusarium crown rot of wheat in Western Australia (Khudhair et al., 2019) and Eastern China (Deng et al., 2020).

Primers Fp3-F/Fp4-R yielding amplified products of approximately 200 bp were developed for detection of fuminosin-producing *F. proliferatum* (Table 3) (Jurado et al., 2006). Those primers were also used for quantitative detection of the pathogen by real-time PCR with SYBR Green I (Amato et al., 2015). For *F. proliferatum* and *F. verticillioides,* species-specific primers were also designed based on the calmodulin gene: *F. proliferatum*-specific PRO1/PRO2 produce 585-bp amplicons, and *F. verticillioides*-specific VER1/VER2 produce 578-bp amplicons (Table 3) (Mulè et al., 2004). Based on the 28S rDNA (IGS) sequence, Patiño et al. (2004) developed primers for identification of *F. verticillioides* (VERT-1/VERT-2 producing 800-bp amplicons) or only its fuminosin-producing isolates (the primers VERTF-1/VERTF-2 producing 400-bp fragments) (Table 3). For identification of *F. verticillioides,* Jurado et al. (2006) proposed to combine the above primer VERT-2 (Patiño et al., 2004) with the genus-specific primer Fps-F (Table 1), producing amplicons of about 700-bp. For *F. verticillioides*, Faria et al. (2012) also developed species-specific primers based on the sequence of the gaoA gene of galactose oxidase: FV-F1/FV-R producing 649-bp amplicons and FV-F2/FV-R with a marker fragment of 370 bp (Table 3).

Möller et al. (1999) were the first to develop species-specific primers for *F. subglutinans* based on analysis of RAPD fragments, 61-2 F/R producing a specific fragment of 445 bp at the annealing temperature of 64°C (Table 3). However, at the annealing temperature of 62°C and lower, these primers also produced amplified fragments of low intensity and other length in *F. nygamai* and *F. oxysporum*. The sequence of the calmodulin gene was also used to design the species-specific primers SUB1/SUB2 for F. *subglutinans*, giving amplified PCR products of 631 bp (Table 3) (Mulè et al., 2004). Species-specific primers for detection of *F. subglutinans,* FS-F1/FS-R and FS-F2/FS-R, based on the sequence of the gaoA gene of galactose oxidase with respective marker products of 649 and 370 bp were proposed by Faria et al. (2012) (Table 3).

Sequences of rDNA (ITS) and the gene for translation elongation factor EF1α were used by Arif et al. (2012) to design primers for detection of *F. solani*: ITS-Fu2f/ITS-Fu2r, ITS-Fs5f/ITS-Fs5r, and TEF-Fs4f/TEFFs4 producing amplicons of 595, 485, and 658 bp, respectively (Table 3). Primers FS1/FS2 developed by Casasnovas et al. (2013) based of AFLP (amplified fragment length polymorphism) product analysis (with a specific amplicon of 175 bp, see Table 3) are also used for detection of *F. solani*. Although those primers had been initially developed for detection of Fusarium crown rot of peanut, they were also used for detection of *F. solani* in wheat (Sadhasivam et al., 2017).

Based on sequences of translation elongation factor EF1α, Nicolaisen et al. (2009) developed species-specific primers for quantitative detection of *F. graminearum, F. culmorum, F. poae, F. langsethiae, F. sporotrichioides, F. equiseti, F. tricinctum, F. avenaceum, F. verticillioides, F. subglutinans*, and *F. proliferatum* by real-time PCR with SYBR Green. The primers are actively used for detection of quantitative and qualitative composition of *Fusarium* species in grain samples or qualitative detection of a particular species in many studies (for instance, Birr et al., 2020; Góral et al., 2018).

Table 4. Real-time PCR assays for detection of *Fusarium* species using a labeled probe

Species	Primer pair and probe	Sequence (5'-3')	Reference
F. avenaceum	avenaceum_MGB-F	ccatcgccgtggctttc	Waalwijk et al., 2004
	avenaceum_MGB-R	caagcccacagacacgttgt	
	avenaceum_MGB-probe	acgcaattgactattgc	
F. avenaceum	EF1-FA_F2	catcttgctaactcttgacagaccg	Elbelt et al., 2018
	EF1-FA_R3	gggtaatgaatgcgtttcgaa	
	Ef1-FA	agcgagtcgtgggaatcgatggg	
F. culmorum	culmorum_MGB-F	tcacccaagacgggaatga	Waalwijk et al., 2004
	culmorum_MGB-R	tggctaaacagcacgaatgc	
	culmorum_MGB-probe	cacttggatatatttcc	
F. culmorum	EF1-FC_F2	cgaatcgccctcacacg	Elbelt et al., 2018
	EF1-FC-R2	gtgatggtgcgcgcctag	
	culmo2-EF1-R2	atgagccccaccagaaaaattacgacaa	
F. graminearum	graminearum_MGB-F	ggcgcttctcgtgaacaca	Waalwijk et al., 2004
	graminearum_MGB-R	tggctaaacagcacgaatgc	
	graminearum_MGB- probe	agatatgtctcttcaagtct	
F. graminearum	EF1-FCFG_F	tcgatacgcgcctgttacc	Elbelt et al., 2018
	EF1-FG_R	atgagcgcccagggaatg	
	grami2-EF1_rev	agccccaccgggaaaaaaattacgaca	
F. graminearum	FGtubf/	gtctcgacagcaatggtgtt	Reischer et al., 2004
	FGtubr	gcttgtgtttttcgtggcagt	
	FGtubTM	acaacggaacggcacctctgagctccagc	
F. graminearum	TMFg12f	ctccggatatgttgcgtcaa	Yli-Mattila et al., 2008
	TMFg12r	cgaagcatatccagatcatcca	
	TMFg12p	tgagaatgtcttgaggcaatgcgaacttt	
F. poae	poae_1-F	aaatcggcgtatagggttgagata	Waalwijk et al., 2004
	poae_1-R	gctcacacagagtaaccgaaacct	
	poae_1-probe	caaaatcacccaaccgacccttc	
F. poae	EF1-FP2_F	ctcgagcgattgcatttcttt	Elbelt et al., 2018
	EF1_FP2_R	ggcttcctattgacaggtggtt	
	EF1-FP	cgcgaatcgtcacgtgtcaatcagtt	
F. poae	TMpoaef	gctgagggtaagccgtcctt	Yli-Mattila et al., 2008
	TMpoaer	tctgtcccccctaccaagct	
	TMpoae-probe	atttccccaacttcgactctccgagga	
F. sporotrichioides	EF1-FS_F3	tcgatacgcgcctgttacc	Elbelt et al., 2018
	EF1-FS_R2	tcgatacgcgcctgttacc	
	EF1-FS	tcgatacgcgcctgttacc	
F. langsethiae	EF1-FL-F3	gccgtgtcgtaatttttttgtg	Elbelt et al., 2018
	EF1-FL-R3	aaatggctatgtgggaaggaag	
	EF1_FL	gggctcataccccgccactcga	
M. nivale var. *majus*	nivale_2-F	cgccaaggactcctccagtag	Waalwijk et al., 2004
	nivale_2-R	gccgacgaatggatattaagaact	
	nivale_2-probe	tcccgccttcacggtggaaagc	
M. nivale	Mniv_Btub_F	tctacttcaacgaggtatgtcaccat	Elbelt et al., 2018
	Mniv_Btub_R	cctaagttatgtgggtggtcagttag	
	Mniv_Btub	ttcgggcttcacacattcggcc	

Table 4. (Contd.)

Species	Primer pair and probe	Sequence (5'-3')	Reference
F. langsethiae/F. sporotrichioides	TMLANf	gagcgtcatttcaaccctcaa	Halstensen et al., 2006
	TMLANr	gaccgccaatcaatttggg	
	TMLANp	agcttggtgttgggatctgtccttaccg	
F. avenaceum/F. arthrosporoides	TMAVf	agatcggacaatggtgcattataa	Halstensen et al., 2006
	TMAVr	ggccctactatttactcttgcttttg	
	TMAVp	ctcctgagaggtcccagagatgaacata acttc	
All trichothecene-producing *Fusarium* species	TMTrif	cagcagmtrctcaaggtagaccc	Halstensen et al., 2006
	TMTrir	aactgtayacraccatgccaac	
	TMTrip	agcgactacaggcttccctccaaacaat	

For different species of the genus *Fusarium*, real-time PCR assays with the use of a labeled probe were proposed (Table 4). In particular, the first test systems for detection of F. *graminearum, F. culmorum, F. avenaceum, M. nivale* var. *majus,* and *F. poae* using TaqMan probe were based on sequences that had been previously employed for conventional PCR (Nicholson et al., 1996, 1998; Waalwijk et al., 2003). For their design, so called Minor Groove Binder or MGB ligands were used (Table 4) (Waalwijk et al., 2004). The primer pair and the probe that are complementary to DNA of Potato leaf roll virus (the primers PLRV-F and PLRV-R and the probe PLRV) were recommended as a positive internal control (Table 4). The PCR mix contained 83 nM of each probe and 333 nM of each primer (Waalwijk et al., 2004). FAM (6-carboxyfluorescein, emission at 518 nM) was attached at the 5'-end of specific probes for certain *Fusarium* species; the probe PLRV, which was used as an inner standard, was VIC-labelled; the TAMRA (5-carboxy-tetramethyl rhodamine with emission wave length of 582 nm) dye was attached to all the probes at the 3'-end.

Particularly for *F. graminearum,* a specific assay was developed based on the tubulin gene with the use of the TaqMan probe: the primer pair FGtubf/FGtubr and the probe FgtubTM labeled with FAM at the 5'-end and TAMRA at the 3'-end (Table 4) (Reischer et al., 2004; Sanoubar et al., 2015). Another assay for detection of *F. graminearum* was based on PCR products with the primer pair Fg11f and Fg11r amplifying 382-bp fragments of the IGS region (Table 4) (the sequence in GeneBank is AY937106) according to Yli-Mattila et al. (2004). The authors proposed the primers TMFg12f/TMFg12r and the probe TMFg12p (Table 4). The probe was also labeled with FAM at the 5'-end and TAMRA at the 3'-end; the PCR protocol was similar to that proposed by Waalwijk et al. (2004) (Yli-Mattila et al., 2008). For *F. poae*, a highly specific assay was developed based on the primers TMpoaef/TMpoaer and the probe TMpoaep (Table 4), which are complementary to the IGS fragment (Yli-Mattila et al., 2008). The probe was labeled with TET (tetrachloro-6-carboxyfluorescein) at the 5'-end and the 3' Eclipse Dark dye at the 3'-end as a quencher. The PCR protocol was identical to that proposed by Waalwijk et al. (2004). The authors (Yli-Mattila et al., 2008) suggested that the assay was the most sensitive among analogues not only for *F. poae* but also for other *Fusarium* fungi at the time of its development, in particular, as compared to the assays published by Waalwijk et al. (2004).

Based on the sequence of the EF1α gene of *Fusarium* fungi collected in different localities in France, a number of assays listed in Table 4 were developed (Elbelt et al., 2018). In the case of *M. nivale*, the β-tubulin gene was chosen. All the probes in the proposed assays were labeled with FAM and TAMRA at the 5'- and 3'-ends, respectively (Elbelt et al., 2018).

In cases when species-specificity of *Fusarium* fungi is not essential, the assays based on the *Tri5* gene controlling trichothecene biosynthesis or sequences that are common for several species may be used (Halstensen et al., 2006) (Table 4). The probes in such assays are labeled with FAM (TMLANp, TMAVp) or VIC (TMTrip) at the 5'-end and TAMRA at the 3'-end; all the primers are added to the final concentration of 300 nM, probes – 100 nM; the PCR protocol is identical to that proposed by Waalwijk et al. (2004) (Halstensen et al., 2006).

Another modern approach to evaluation of species identity for *Fusarium* fungi is sequencing of amplified DNA fragments and their comparison with sequences from DNA database (for instance, see Shikur Gebremariam et al., 2018; Minati and Mohammed-Ameen, 2019).

Thus, as one may see, a lot of attention is paid to detection of fungi of the genus *Fusarium*. Although the majority of current studies are devoted to PCR analysis, methods of microbiological detection of these pathogens are still important (for instance, see Abass et al., 2021). Not all PCR techniques listed in this review are perfect: some of the assays that had been

developed as species-specific turned out not to be so specific. However, even they may be used in the research where detection of any *Fusarium* combination rather than a particular species is important. Similarly, assays for producers of particular mycotoxins irrespective of their species identity may be used.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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