CHAPTER 3

PCR-Based Diagnosis and Quantification of Mycotoxin-Producing Fungi

Ludwig Niessen*

Contents

*Technische Universität München, Lehrstuhl für Technische Mikrobiologie, Weihenstephaner Steig 16, D-85350 Freising, Germany

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Abstract

Mycotoxins are secondary metabolites produced by filamentous fungi which have toxicologically relevant effects on vertebrates if administered in small doses via a natural route. In order to improve food safety and to protect consumers from harmful contaminants, the presence of fungi with the potential to produce such compounds must be checked at critical control points during the production of agricultural commodities as well as during the process of food and feed preparation. Polymerase chain reaction (PCR)-based diagnosis has been applied as an alternative assay replacing cumbersome and time-consuming microbiological and chemical methods for the detection and identification of the most serious toxin producers in the fungal genera *Fusarium*, *Aspergillus*, and *Penicillium*. The current chapter covers the numerous PCR-based assays which have been published since the first description of the use of this technology to detect *Aspergillus flavus* biosynthesis genes in 1996.

I. INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi that in small concentrations can evoke an acute or chronic disease in vertebrate animals when introduced via a natural route (Gravesen *et al.*, 1994). Some authors have added attributes such as missing immunogenicity, thermostability, or low molecular weight. Some mycotoxins have an additional effect on bacteria or plants, for example, they act as antibiotics or phytotoxins. Ustiloxins, cyclic peptide mycotoxins produced by *Ustilaginoidea virens* in false smut balls on rice panicles, are highly active phytotoxins (Koiso *et al.*, 1994). Also the mycotoxin, tenuazonic acid from *Alternaria* spp., acts as a phytotoxin in solanaceous plants and has also antibiotic activity (Janardhanan and Hussain, 1983). Some compounds, which are usually classified among the mycotoxins, do not exactly fit the above definition, for example, zearalenone, which acts as an estrogen analogue (Mirocha *et al.*, 1978). The substance is regularly produced together with other *Fusarium* mycotoxins so that the toxicological effects observed in animals after consumption of *Fusarium*-contaminated feed were attributed to zearalenone in early studies. The majority of the >400 mycotoxins currently known (Bauer and Gareis, 1987) can be categorized in 8 groups according to their chemical characteristics, that is, ergot alkaloids (example: ergovalin), chinones (citrinin), coumarins (ochratoxin), cyclopeptides (enniatins), diketopiperazines (roquefortine C), sesquiterpenes (deoxynivalenol), furofuranes (aflatoxins), and lactones (patulin, zearalonone) (Turner and Aldridge, 1983). Substances can

also be grouped according to their toxic activity under chronic conditions as mutagenic, carcinogenic, or teratogenic. Grouping according to their site of action results in hemo-, hepato-, nephro-, dermato-, neuro-, or immunotoxins. Exposure may occur through ingestion, inhalation, and dermal contact (Hendry and Cole, 1993; Pitt, 2000).

Mycotoxins are among the oldest environmental toxicants menacing human existence since ancient times (Kampelmacher, 1973; Schöntal, 1984). To date, massive outbreaks of human mycotoxicoses occur but are more or less restricted to developing countries, that is, an outbreak of acute aflatoxicosis in the Makueni district and neighboring districts in Kenya in 2004 with 125 fatalities as one of the most recent cases (Muture and Ogana, 2005). In developed countries and in threshold countries, the major concerns are chronic effects of ingesting small concentrations of mycotoxins over a long period of time (Etzel, 2002). The majority of mycotoxin producers can be found in the fungal genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* which concomitantly happen to be the most abundant contaminants of food and feed. All genera belong to the ascomycotina but the most potent toxin producers among them are the species which regularly occur in the anamorphic state or of which no teleomorph is known. All four genera have in common the presence of high numbers of species, distinction of which is very complicated and requires a high degree of specialization. Moreover, constant changes in the taxonomy of these genera may lead to misidentification of an isolate and false evaluation of its toxigenic potential.

Aimed at both overcoming the obstacles of identification and developing more rapid tools for detection, nucleic acid-based methods have been developed and used over the last 10 years as a tool for the analysis of mycotoxigenic fungi. The polymerase chain reaction (PCR) (Saiki *et al*., 1988) has replaced cumbersome and time-consuming microbiological analysis by amplification of specific genomic markers rather than growing the living organism under study. Reviewing the literature shows that within the last 10 years from the first report to the present, PCR-based detection systems have been set up for the major species and groups of mycotoxigenic fungi. Systems were developed in three major ''waves'' of innovation which very much reflect the three periods in which different groups of toxins came into focus of scientific interest and public awareness. Figure 3.1 gives a graphic representation of publications over time from 1996 until April 2007. It must be noted here that only the first publication of a detection system for the depicted species and groups of species are shown. In many cases, alternative PCR-based systems have been subsequently described. An exhaustive list with full appreciation of the systems and application published until November 2006 can be found in Table 3.1. The list gives fungal species and groups of fungal organisms detected with the mycotoxins typically produced

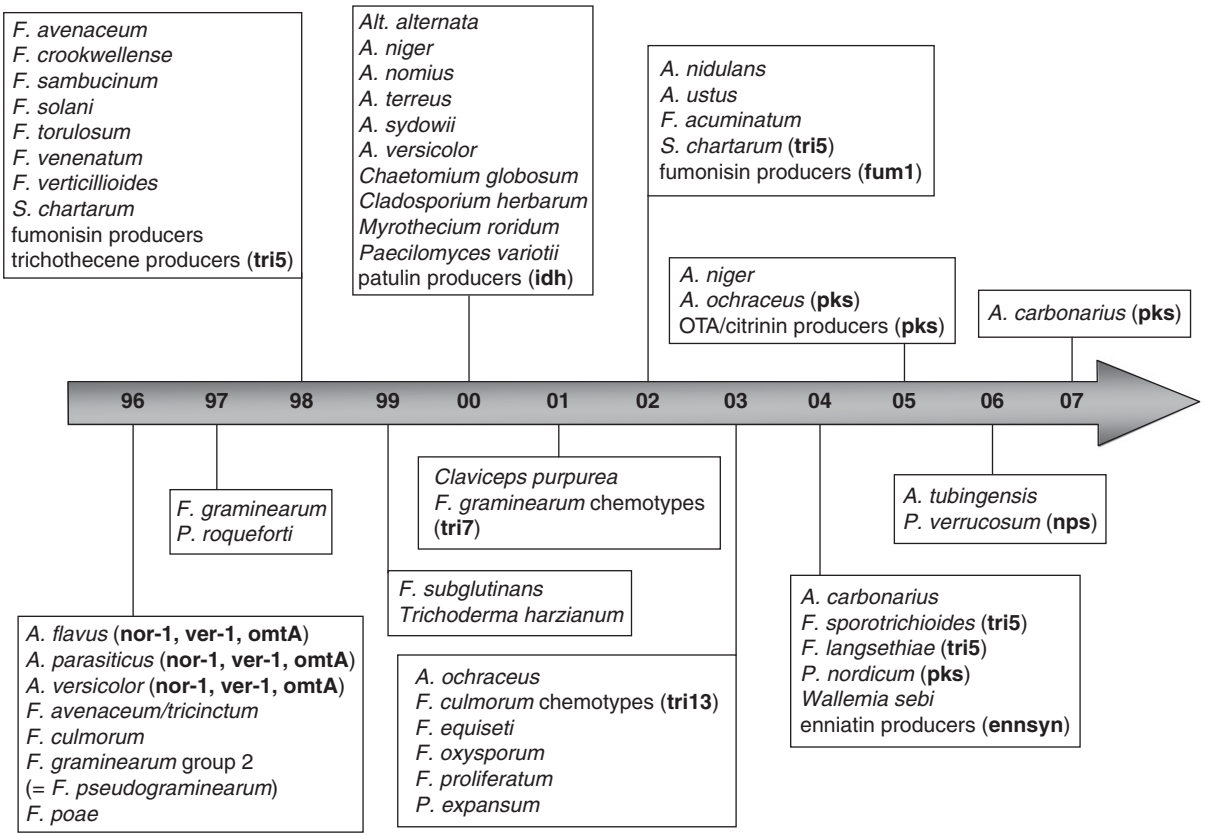

FIGURE 3.1 Development of PCR-based detection systems for mycotoxin producing-fungi from 1996 until April 2007. First publication of a diagnostic primer pair was taken as the time mark for each species depicted. In many cases, more systems were published later for the same fungus. Mycotoxin biosynthesis genes used as sequence source are given in bold.

TABLE 3.1 Overview of PCR assays for the detection of mycotoxin-producing fungi and toxins produced by the target organisms

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
Alternaria alternata	see *A. alternata*	rRNA gene, ITS-region	PCR, quant. real-time PCR (TaqMan)	n.s.	*AaltrF1 *AaltrR1 probe *AaltrP1	GGCGGGCTGGAACCTC GCAATTACAAAAGGTTTATGTTTGTCGTA FAM-TTACAGCCTTGCTGAA TTATTCACCCTTGTCTTT-TAMRA	Haugland and Vesper, 2000
A. alternata	see *A. alternata*	*rRNA gene, ITS1-5.8S-ITS2 region*	PCR	450	Aalt-F Aalt-R	GGCGGGCTGGAACCTCTCGG AATGGATGCTAGACCTTTGC	Haugland and Vesper, 2000 Zur *et al.*, 2002
A. alternata	*alternariol, alternariol monomethylether, altertoxins*	rRNA gene, ITS1–5.8S-ITS2 region	PCR	340	*AAF2* *AAR3*	TGCAATCAGCGTCAGTAACAAAT ATGGATTGCTAGACCTTTGCTGAT	Konstantinova *et al.*, 2002
Aspergillus carbonarius	see *A. carbonarius*	RAPD fragment	PCR	809	$OPX7F_{809}$ $OPX7R_{809}$	AGGCTAATGTTGATAACGGATGAT GCTGTCAGTATTGGACCTTAGAG	Fungaro *et al.*, 2004
A. carbonarius	see *A. carbonarius*	Calmodulin gene	PCR	371	CARBO1 CARBO2	AAGCGAATCGATAGTCCACAAGAATAC TCTGGCAGAAGTTAATATCCGGTT	Perrone *et al.*, 2004
A. carbonarius	see *A. carbonarius*	AFLP marker	PCR	189	A1B_fw A1B_rv	GAATTCACCACACATCATAGC TTAACTAGGATTTGGCATTGAAC	Schmidt *et al.*, 2004a
A. carbonarius	see *A. carbonarius*	rRNA gene, ITS1–5.8S-ITS2 region	PCR	420	CAR1 CAR2	GCATCTCTGCCCCTCGG GGTTGGAGTTGTCGGCAG	Patino *et al.*, 2005
A. carbonarius	naphto-4-pyrones, ochratoxin A	Calmodulin gene	quant. real-time PCR (TaqMan)	167	CARBO_q1 CARBO_q2 probe CARBO_probe	CCGATGGAGGTCATGACATGA AATGCGAACCGGATATTAACTTCTG FAM-CAGCGGCGGAGATA-MGB	Mulè *et al.*, 2006
A. carbonarius	see *A. carbonarius*	otapks gene, AT domain	quant. real-time PCR (SYBR green 1)	141	Ac12RL-OTAF Ac12RL-OTAR	AATATATCGACTATCTGGACGAGCG CCCTCTAGCGTCTCCCGAAG	Atoui *et al.*, 2007

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
A. flavus *A. parasiticus*	kojic acid, 3-nitropropionic acid, cyclopiazonic acid, aflatoxin B_1, B_2, G_1, G_2, aspergillic acid	*nor-1* gene (=*aflD*)	PCR, multiplex with omt-A and ver-1	400	nor-1 nor-2	ACCGCTACGCCGGCACTCTCGGCAC GTTGGCCGCCAGCTTCGACACTCCG	Geisen, 1996 Färber *et al.*, 1997; Geisen *et al.*, 1998; Criseo *et al.*, 2001; Chen *et al.*, 2002
A. flavus *A. parasiticus*	see *A. flavus*/ *A. parasiticus*	*ver-1* gene (=*aflM*)	PCR, multiplex with nor-1 and omt-A	537	ver-1 ver-2	GCCGCAGGCCGCGGAGAAAGTGGT GGGGATATACTCCCGCGACACAGCC	Geisen, 1996 Färber *et al.*, 1997; Geisen *et al.*, 1998; Criseo *et al.*, 2001; Chen *et al.*, 2002
A. flavus *A. parasiticus*	see *A. flavus*/ *A. parasiticus*	*omt-A* gene (=*aflP*)	PCR, multiplex with nor-1 and ver-1	797	omt-1 omt-2	GTGGACGGACCTAGTCCGACATCAC GTCGGCGCCACGCACTGGGTTGGGG	Geisen, 1996 Färber *et al.*, 1997; Geisen *et al.*, 1998; Criseo *et al.*, 2001; Chen *et al.*, 2002
A. flavus *A. parasiticus*	kojic acid, 3-nitropropionic acid, cyclopiazonic acid, aflatoxin B_1, B_2, G_1, G_2, aspergillic acid	*apa-2* gene (=*nor*-1) (=*aflD*)	PCR	1032	APA-450 APA-1482	TATCTCCCCCCGGGCATCTCCCGG CCGTCAGACAGCCACTGGACACGG	Shapira *et al.*, 1996 Zachova *et al.*, 2003
A. flavus *A. parasiticus*	see *A. flavus*/ *A. parasiticus*	*ver-1* gene (=*aflM*)	PCR	895	VER-496 VER-1391	ATGTCGGATAATCACCGTTTAGATGGC CGAAAAGCGCCACCATCCACCCCAATG	Shapira *et al.*, 1996 Zachova *et al.*, 2003
A. flavus *A. parasiticus*	see *A. flavus*/ *A. parasiticus*	*omt-1* gene (=*omt-A*) (=*aflP*)	PCR	1024	OMT-208 OMT-1232	GGCCCGGTTCCTTGGCTCCTAAGC CGCCCCAGTGAGACCCTTCCTCG	Shapira *et al.*, 1996
A. flavus *A. parasiticus*	see *A. flavus*/ *A. parasiticus*	Various genes in aflatoxin biosynthesis cluster	PCR, RT-PCR			Various primers, see reference	Scherm *et al.*, 2005

A. flavus *A. parasiticus*	see *A. flavus*/ *A. parasiticus*	*aflR* gene	Nested PCR	798 400	*aflR*1 forward *aflR*1 reverse nested *aflR*2 forward *aflR*2 reverse	AACCGCATCCACAATCTCAT AGTGCAGTTCGCTCAGAACA GCACCCTGTCTTCCCTAACA ACGACCATGCTCAGCAAGTA	Manonmani *et al.*, 2005
A. flavus	see *A. flavus*/ *A. parasiticus*	*nor-1* gene (=*aflD*)	quant. real-time PCR (TaqMan)	66	nortaq-1 nottaq-2 Probe norprobe	GTCCAAGCAACAGGCCAAGT TCGTGCATGTTGGTGATGGT 6-FAM-TGTCTTGATCGGCGCCCG-TAMRA	Mayer *et al.*, 2003
A. flavus	see *A. flavus*/ *A. parasiticus*	rRNA gene, ITS1 region	PCR	420	ASPU Fl2r	ACTACCGATTGAATGGCTCG TTCACTAGATCAGACAGAGT	Sugita *et al.*, 2004 Bu *et al.*, 2005
A. flavus	see *A. flavus*/ *A. parasiticus*	rRNA gene, ITS1–5.8S region	quant. real-time PCR (LightCycler)	199	Forward Reverse	CTCCCACCCGTGTTTACTGT GCTTTCTTCATCGATGCCT	
A. flavus	see *A. flavus*/ *A. parasiticus*	Various genes in aflatoxin biosynthesis cluster	Multiplex RT-PCR			Various primers, see reference	Degola *et al.*, 2007
A. flavus, *A. fumigatus*	Kojic acid, 3-nitropropionid acid, cyclopiazonid acid, aspergillic acid, aflatoxin B_1, gliotoxin, verrucologen, fumitremorgin A & B, fumitoxins, fumigaclavines, tryptoquivalins	Alkaline protease gene	PCR	690 747 527	alp11 alp12 nested alp13 alp14	AGCACCGACTACATCTAC GAGATGGTGTTGGTGGC CTGGCATACAACGCCGCTG TTGTTGATCGCAACC	Tang *et al.*, 1993 Hayette *et al.*, 2001
A. niger	naphto-4-pyrones, malformins, ochratoxin A (few isolates)	DNA topoisomerase II gene	PCR	600	ANGF79 ANGR139	CACGTTCAAGCCGGACTACGC CAAGATGTTGTCCATCACCGCT	Kanbe *et al.*, 2002

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
A. niger	see *A. niger*	rRNA gene, ITS1 region	PCR	n.s.	ASPU Ni1r	ACTACCGATTGAATGGCTCG ACGCTTTCAGACAGTGTTCG	Sugita *et al.*, 2004
A. niger	see *A. niger*	rRNA gene, ITS1–5.8S-ITS2 region	PCR	420	ITS1 NIG	TCCGTAGGTGAACCTGCG CCGGAGAGAGGGGACGGC	Gonzalez-Salgado *et al.*, 2005
A. niger	see *A. niger*	RAPD marker	PCR	372	$OPXF_{372}$ $OPXR_{372}$	CAGTCGTCCAGTACCCTAAC GAGCGAGGCTGATCTAAGTG	Sartori *et al.*, 2006
A. nomius	Kojic acid, tenuazonic acid, aflatoxin B_1, B_2, G_1, G_2, aspergillic acid	rRNA gene, ITS-region	PCR, quantitative real-time PCR (TaqMan)	n.s.	*AflavF1 *nomiR1probe *AflavP1	CGAGTGTAGGGTTCCTAG CGA CCGGCGGCCTTGC 6-FAM-TCCCACCCGTGTTTA CTGTACCTTAGT TGCT T-TAMRA	Haugland and Vesper, 2000
A. ochraceus	Penicillic acid, ochratoxin A, xanthomegnin, viomellin, vioxanthin	AFLP marker (AJ511647)	PCR, quant. real-time PCR (LightCycler)	260	OCA-V OCA-R	ATACCACCGGGTCTAATGCA TGCCGACAGACCGAGTGGATT	Schmidt *et al.*, 2003
A. ochraceus	see *A. ochraceus*	rRNA gene, ITS1–5.8S-ITS2 region	PCR	400	OCRA1 OCRA2	CTT CCT TAG GGG TGG CAC AGC GTT GCT TTT CAG CGT CGG CC	Patino *et al.*, 2005
A. ochraceus	see A. ochraceus	polyketide synthase gene, KS domain	PCR	690	AoOTA-L AoOTA-R	CAT CCT GCC GCA ACG CTC TAT CTT TC CAA TCA CCC GAG GTC CAA GAG CCT CG	Dao *et al.*, 2005
A. parasiticus	kojic acid, aspergillic acid, aflatoxin B_1, B_2, G_1, G_2	*apa-2* gene	PCR	1032	APA-450 APA-1482	TAT CTC CCC CCG GGC ATC TCC CGG CCG TCA GAC AGC CAC TGG ACA CGG	Shapira *et al.*, 1996
A. parasiticus	see *A. parasiticus*	*ver-1* gene	PCR	895	VER-496 VER-1391	ATG TCG GAT AAT CAC CGT TTA GAT GGC CGA AAA GCG CCA CCA TCC ACC CCA ATG	Shapira *et al.*, 1996
A. parasiticus *A. sojae*	Kojic acid, aspergillic acid, aflatoxin B_1, B_2, G_1, G_2	rRNA gene, ITS-region	PCR, quant. real-time PCR (TaqMan)	n.s.	*AflavF1 *AparaR3 probe *AflavP1	CGA GTG TAG GGT TCC TAG CGA GCC CGG GGC TGA CG TCC CAC CCG TGT TTA CTG TAC CTT AGT TGC T T	Haugland and Vesper, 2000

A. terreus	Terrein, patulin, citrinin, citreoviridin, territrem	rRNA gene, ITS region	quant. real-time PCR (TaqMan)	n.s.	*AterrF1 AterrR1 probe *AterrP1	TTA CCG AGT GCG GGT CTT TA CGG CGG CCA GCA AC FAM-AAC CTC CCA CCC GTG ACT ATT GTA CCT TG-TAMRA	Haugland and Vesper, 2000
A. terreus	see *A. terreus*	DNA toposiomerase II gene	PCR	386	ATRF81 ATRR120	TAC CTT CAA GCC TGA CTA CG ACC TGC TCG GCC AGT TTG CTG	Kanbe *et al.*, 2002
A. ustus	austamide, austdiol, austins, austocystins	rRNA gene, ITS-region	quant. real-time PCR (TaqMan)	n.s.	*AustsF1 *AustsR probe *AustsP1	GAT CAT TAC CGA GTG CAG GTC T GCC GAA GCA ACG TTG GTC FAM-CCC CCG GGC AGG CCT AAC C-TAMRA	Haugland and Vesper, 2000
A. versicolor	see. *A. versicolor*	rRNA gene, ITS-region	quant. real-time PCR (TaqMan)	n.s.	*AversF2 *AversR1 probe *AversP1	CGGCGGGGAGCCCT CCATTGTTGAAAGTTTTGACTGATTTTA FAM-AGACTGCATCACTCT CAGGCATGAAGTTCAG-TAMRA	Haugland and Vesper, 2000
A. versicolor	sterigmatocystin, nidulotoxin	β-tubulin gene	PCR	127	AspBTF AspBTR	CAT CCA TTT CAG ATG GTA TTT CCT TGT TTT GAT CGA GTC TTG GAC G	Dean *et al.*, 2005
Chaetomium globosum	chaetoglobosins, chetomin	rRNA gene, ITS region	quant. real-time PCR (TaqMan)	n.s.	*CglobF1 *CglobR1 probe *CglobP1	CCGCAGGCCCTGAAAAG CGCGGCGCGACCA FAM-AGATGTATGCTACTAC GCTCGGTGCGACAG-TAMRA	Haugland and Vesper, 2000
Cladosporium herbarum	epi- and fagi-cladosporic acid	rRNA gene, ITS region	quant. real-time PCR (TaqMan)	n.s.	*CherbF1 *CherbR1 probe *CherbP1	AAGAACGCCCGGGCTT CGCAAGAGTTTGAAGTGTCCAC FAM-CTGGTTATTCATAACCCTT TGTTGTCCGACTCT G-TAMRA	Haugland and Vesper, 2000
Claviceps purpurea	Ergot alkaloids	β-tubulin gene, intron 3	PCR	275	BT3 BTPUR2	TCTAGA(G/T)GT (A/G)CCCATACCGGCA GGCTGGAGAATGTCCCACAA	Tooley *et al.*, 2001
Fusarium acuminatum	Antibiotic Y, chlamydosporol, trichothecenes type A, enniatins, moniliformin	600-bp RAP D marker	PCR	602	FAC-F FAC-R	GGGATATCGGGCCTCA GGGATATCGGCAAGATCG	Williams *et al.*, 2002
F. avenaceum	antibiotic Y, chlamydosporol, fusarin C, moniliformin, enniatins	RAPD marker	PCR	920	FaF FaR	CAAGCATTGTCGCCACTCTC GTTTGGCTCTACCGGGACTG	Lees, 1995 Doohan *et al.*, 1998

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
F. avenaceum	see *F. avenaceum*	RAPD marker	PCR	220	JIAf JIAr	GCTAATTCTTAACTTACTAGGGGCC GTGTAATAGGTTATTTACATGGGCG	Turner *et al.*, 1998 Straumfors Halstensen *et al.*, 2006b
F. avenaceum	see *F. avenaceum*	RNA gene, ITS region	PCR	314	FAF1 FAR	AACATACCTTAATGTTGCCTCGG-ROX ATCCCCAACACCAAACCCGAG	Mishra *et al.*, 2003
F. avenaceum	see *F. avenaceum*	RAPD marker of Lees, 1995 in Doohan *et al.*, 1998	quant. real-time PCR (TaqMan)	n.s.	avenaceum MGB-F avenaceum MGB-R avenaceum MGB probe	CCATCGCCGTGGCTTTC CAAGCCCACAGACACGTTGT ACGCAATTGACTATTGC	Waalwijk *et al.*, 2004b
F. avenaceum, *F. arthrosporioides*	see *F. avenaceum*	RAPD marker	quant. real-time PCR (TaqMan)	85	TMAVf TMAVr TMAVp	AGATCGGACAATGGTGCATTATAA GGCCCTACTATTTACTCTTGCTTTTG FAM-CTCCTGAGAGGTC CCAGAGATGAACATAACTTC-TAMRA	Straumfors Halstensen *et al.*, 2006a
F. avenaceum, *F. tricinctum*	antibiotic Y, chlamydosporol, fusarin C, moniliformin, enniatins, butenolide, chrysogine, visoltricin	RAPD marker	PCR	345	Fa-U17f Fa-U17r	CAAGCATTGTCGCCACTCTC GTTTGGCTCTACCGGGACTG	Schilling *et al.*, 1996 Turner *et al.*, 1998; Simpson *et al.*, 2001
F crookwellense	culmorin, fusarin C, trichothececenes type B, zearalenone, butenolide, chrysogine	RAPD marker	PCR	842	CRO-Af CRO-Ar	CTCAGTGTCCACCGCGTTGCGTAG CTCAGTGTCCCAATCAAATAGTCC	Yoder and Christianson, 1998

F. crookwellense, *F. culmorum*	see *F. crookwellense*	RAPD marker	PCR	897	CRO-Cf CRO-Cr	TATTGGGATCTATCCAAGTCTTGT AAGCAGGAAACAGAAACCCTTTCC	Yoder and Christianson, 1998
F. culmorum	culmorin, fusarin C, trichothececenes type B, zearalenone, butenolide, chrysogine	RAPD marker	PCR	472	OPT18F OPT18R	GATGCCAGACCAAGACGAAG GATGCCAGACGCACTAAGAT	Schilling *et al.*, 1996 Chelkowski *et al.*, 1999; Knoll *et al.*, 2000; Williams *et al.*, 2002; Tan and Niessen, 2003; Agodi *et al.*, 2005; Brandfass and Karlovsky, 2006
F. culmorum	see *F. culmorum*	RAPD marker	quant. PCR (competitive)	570	Fc01F Fc01R	ATGGTGAACTCGTCGTGGC CCCTTCTTACGGCAATCTCG	Nicholson *et al.*, 1998; Simpson *et al.*, 2001
F. culmorum	see *F. culmorum*	rRNA gene, ITS region	PCR	245	175F 430R	TTTTAGTGGAACTTCTGAGTA T-FAM AGTGCAGCAGGACTGCAGC	Mishra *et al.*, 2003
F. culmorum	see *F. culmorum*	RAPD marker of Nicholson *et al.*, 1998	quant. real-time PCR (TaqMan)	n.s.	culmorum-MGB-F culmorum MGB-R culmorum MGB probe	TCACCCAAGACGGGAATGA GAACGCTGCCCTCAAGCTT FAM-CACTTGGATATATTTCC-TAMRA	Waalwijk *et al.*, 2004b
F. culmorum	see *F. culmorum*	RAPD marker of Schilling *et al.*, 1996	nested PCR	368 255	FculAF FculAR nested FculBF FculBR	TGCCAGACCAAGACGAAGTGAGAG TGAACTGCCACTCCGTTGCAAGTG TTGATCAAACCATCATCATC AGAAAGGGTTAGAATCATGC	Klemsdal and Elen, 2006 Straumfors Halstensen *et al.*, 2006b
F. culmorum	see *F. culmorum*	RAPD marker of Lees, 1995 in Doohan *et al.*, 1998	Quant. Real-time PCR (TaqMan)	131	Fc131s2 forw. Fc131s2 rev. Fc131s2-MGB probe	GTACCAGAGCACGGCGTTG TCGCTCTCGATCAAAAGAAGG FAM-ACAAGTCCCCTCGACGTG-TAMRA	Leisova *et al.*, 2006
F. culmorum	see *F. culmorum*	β-tubulin gene	PCR	296	βt1 βt2	GGTAACCAAATCGGTGCTGCTTTC GATTGACCGAAAACGAAGTTG	Pinson-Gadais *et al.*, 2007
F. culmorum high DON producing isolates	see *F. culmorum*	*tri5-tri6* intergenic region	PCR	200	N1–2 N1–2R	CTTGTTAAGCTAAGCGTTTT AACCCCTTTCCTATGTGTTA	Bakan *et al.*, 2002

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
F. culmorum low DON producing isolates	see *F. culmorum*	*tri5-tri6* intergenic region	PCR	650	4056 3551	ATCCCTCAAAAACTGCCGCT ACTTTCCCACCGAGTATTTC	Bakan *et al.*, 2002
F. graminearum group 2 isolates (*F. pseudograminearum*)	culmorin, fusarin C, trichothecenes type B, zearalenone, butenolide, chrysogine	RAPD marker	PCR	332	UBC85F UBC85R	GCAGGGTTTGAATCCGAGAC AGAATGGAGCTACCAACGGC	Schilling *et al.*, 1996 Chelkowski *et al.*, 1999; Gargouri *et al.*, 2001; Obradovic *et al.*, 2001; Williams *et al.*, 2002; Straumfors Halstensen *et al.*, 2006b
F. graminearum	culmorin, fusarin C, trichothecenes type B, zearalenone, butenolide, chrysogine	*gaoA* gene	PCR	898	GaoA-V2 GaoA-R2	AGGGACAATAAGTGCAGA ACTGTGCACTGTCGCAAGTG	Niessen and Vogel, 1997
F. graminearum	see *F. graminearum*	RAPD marker	quant. PCR (competitive)	400–500	Fg16F Fg16R	CTCGGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	Nicholson *et al.*, 1998 Simpson *et al.*, 2001
F. graminearum	see *F. graminearum*	β-tubulin gene	quant. real-time PCR (TaqMan)	111	Fgtubf Fgtubr probe FGtubTM	GGTCTCGACAGCAATGGTGTT GCTTGTGTTTTTCGTGGCAGT FAM-ACAACGGAACGGCACCTCTGA GCTCCAGC-TAMRA	Reischer *et al.*, 2004
F. graminearum	see *F. graminearum*	RAPD marker of Nicholson *et al.*, 1998	quant. real-time PCR (TaqMan)	n.s.	graminearum MGB-F graminearum MGB-R graminearum MGB probe	GGCGCTTCTCGTGAACACA TGGCTAAACAGCACGAATGC AGATATGTCTCTTCAAGTCT	Waalwijk *et al.*, 2004b
	see *F. graminearum*		PCR	173–327	GzTri7f1 GzTri7r1	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG(C/T)ACTGGTGC	Lee *et al.*, 2001

F. graminearum DON producing isolates		*tri7* gene, inserted region					
F. graminearum DON producing isolates	see *F. graminearum*	*tri13* gene	PCR	243	Tri13F Tri13R	TACGTGAAACATTGTTGGC GGTGTCCCAGGATCTGCG	Waalwijk *et al.*, 2003
F. graminearum NIV producing isolates	see *F. graminearum*	*tri7* gene, inserted region	PCR	161	GzTri7f1 GzTri7r1	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG(C/T)ACTGGTGC	Lee *et al.*, 2001
F. graminearum NIV producing isolates	see *F. graminearum*	*tri13* gene	PCR	415	Tri13F Tri13R	TACGTGAAACATTGTTGGC GGTGTCCCAGGATCTGCG	Waalwijk *et al.*, 2003
F. graminearum *F. culmorum*	see *F. graminearum*	RAPD marker	quant. PCR (competitive)	340	Fgc17F Fgc17R	TCGATATACCGTGCGATTTCC TACAGACACCGTCAGGGGG	Nicholson *et al.*, 1998
F. graminearum *F. culmorum*	see *F. graminearum*	RAPD marker	PCR	380	CUL-Af CUL-Ar	TTTCAGCGGGCAACTTTGGGTAGA AAGCTGAAATACGCGGTTGATAGG	Yoder and Christianson, 1998
F. equiseti	equisetin, fusarochromanone, trichothecenes type A & B, zearalenone, chrysogine	rRNA gene, ITS region	PCR	389	FEF1 FER1	CATACCTATACGTTGCCTCG-Fluorescine TTACCAGTAACGAGGTGTATG	Mishra *et al.*, 2003
F. equiseti	see *F. equiseti*	RAPD marker	PCR	96	198F2 198R1	GACAGCAAGATTGACCTTTTGG GACATACTCTACAAGTGCCAA	Nicholson *et al.*, 2004
F. langsethiae *F. sporotrichioides*	see *F. sporotrichioides*	*tri5* gene	PCR	400	Tox5–1 Tox5-powd R	GCTGCTCATCACTTTGCTCAG GGGATGTGGGGAATAACAAGGCC	Niessen *et al.*, 2004
F. langsethiae *F. sporotrichioides*	see *F. sporotrichioides*	rRNA gene, ITS1–5.8S-ITS2 region	PCR	300	Pfusf Flanr	CCGCGCCCCGTAAAACG CTGGCGTGTRAAGGACAGATC	Yli-Mattila *et al.*, 2004
F. langsethiae *F. sporotrichioides*	see *F. sporotrichioides*	rRNA gene	quant. real-time PCR (TaqMan)	77		GAGCGTCATTTCAACCCTCAA GACCGCCAATCAATTTGGG	

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
					TMLANf TMLANr TMLANp	FAM-AGCTTGGTGTTGGGATCTGT CCTTACCG-TAMRA	Straumfors Halstensen *et al.*, 2006a
F. oxysporum	Fusaric acid, naphthoquinone pigments, nectriafurone, monilifomin, gibepyrones	rRNA gene, ITS region	PCR	340	OF1 FOR1	ACATACCACTTGTTGCCTCG-HEX CGCCAATCAATTTGAGGAACG	Mishra *et al.*, 2003
F. poae	Fusarinc, trichothecenes type B & B, butenolide, gamma-lactones	RAPD marker	PCR	220	Fp82F Fp82R	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	Parry and Nicholson, 1996
F. poae	see *F. poae*	*tri5* gene	PCR	400	Tox5–1 Tox5-poae R	GCTGCTCATCACTTTGCTCAG TCGTGGTGAAACAAT GTA T	Niessen *et al.*, 2004
F. poae	see *F. poae*	RAPD marker of Parry and Nicholson, 1996	quant. real-time PCR (TaqMan)	n.s.	poae 1-F poae 1-R poae probe	AAATCGGCGTATAGGGTTGAGATA GCTCACACAGAGTAACCGAAACCT FAM-CAAAATCACCCAACCGACCCTTTC-TAMRA	Waalwijk *et al.*, 2004b
F. poae	see *F. poae*	rRNA gene, ITS1–5.8S-ITS2 region	PCR		FpoF ITS4	CGGATCAGCCCGTCCTTC TCCTCCGCTTATTGATATGC	Straumfors Halstensen *et al.*, 2006b
F. proliferatum	fumonisins, fusaric acid, fusarin C, moniliformin, naphthoquinone pigments, beauvericins, fusaproliferin, fusapyrone	mitochondrial rRNA gene, small subunit region	PCR	n.s.	*FCORN2 *FPRO1	AAGTCTTCCAGTATGGGGGAG TAAACTAACTCAACTAGACGAG	Beck and Barnett, 2003
F. proliferatum	see *F. proliferatum*		PCR	585			Mulé *et al.*, 2004

		calmodulin gene			PRO1 PRO2	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG	
F. pseudograminearum	Culmorin, fusarin C, trichothecenes type B, zearalenone, butenolide, chrysogine	EF-1α gene	PCR	532	Fp1–1 Fp1–2	CGGGGTAGTTTCACATTC(C/T)G GAGAATGTGATGA(C/G)GACAATA	Aoki and O'Donnell, 1999 Gargouri *et al.*, 2001
F. pseudograminearum	see *F. pseudograminearum*	RAPD marker	PCR	779	FPG-F FPG-R	GTCGCCGTCACTATC CACTTTTATCTCTGGTTGCAG	Williams *et al.*, 2002
F. sambucinum	trichothecenes type A, butenolide, enniatins	RAPD marker	PCR	312	SAM-Ef SAM-Er	CAGAAGCGGAGCAAGTTCACAATC CAGAAGCGGATGGAGATGTAAAGT	Yoder and Christianson, 1998
F. sambucinum	see *F. sambucinum*	RNA gene, ITS region	PCR	315	FSF1 FSR1	ACATACCTTTATGTTGCCTCG-TAMRA GGAGTGTCAGACGACAGCT	Mishra *et al.*, 2003
F. solani	Fusaric acid, naphthoquinone pigments	cutinase gene	PCR	189	forward reverse probe	ATCGAGGACCTCGACTCG GCAGCAACGATCAAGCTA biotin-AGATCGCCGGAACTGTTCT GTTCGGCTACA	Alexandrakis *et al.*, 1998
F. solani	see *F. solani*	rRNA gene, 18S region	nested PCR	744	first roundPffor Pfrev2 second round	AGGGATGTATTTATTAGATAAAAAATCAA CGCAGTAGTTAGTCTTCAGTAAATC	Jaeger *et al.*, 2000
					Fusofor	CCAATGCCCTCCGGGGCTAAC	
				565	Fusorev	GCATAGGCCTGCCTGGCG	
F. sporotrichioides	Trichothecenes type A, butenolide, fusarin C	rRNA gene, ITS2–28S region	PCR	288	FspITS2K P28SL	CTTGGTGTTGGGATCTGTGTGCAA ACAAATTACAACTCGGGCCCGAGA	Kulik *et al.*, 2004
F. sporotrichioides	see *F. sporotrichioides*	*tri5* gene	PCR	400	Tox5–1 Tox5-sporo R2	GCTGCTCATCACTTTGCTCAG TCAACTTCGGGATGTGGAGG	Niessen *et al.*, 2004
F. sporotrichioides	see *F. sporotrichioides*	rRNA gene, ITS1–5.8S-ITS2 region	PCR	300	Pfusf Fspor	CCGCGCCCCGTAAAACG ACTGTGTTTGCACACAGATC	Yli-Mattila *et al.*, 2004

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
F. sporotrichioides	see *F. sporotrichioides*	*tri13* gene	PCR	332	AF330109CF AF330109CR	AAAAGCCCAAATTGCTGATG TGGCATGTTCATTGTCACCT	Demeke *et al.*, 2005
F. subglutinans	Fumonisins, fusaric acid, fusarin C, moniliformin, naphthoquinone pigments, beauvericin	RAPD marker	PCR	445	61–2F 61–2R	GGCCACTCAAGAGGCGGAAAG GTCAGACCAGAGCAATGGGC	Möller *et al.*, 1999
F. subglutinans	see *F. subglutinans*	calmodulin gene	PCR	631	SUB1 SUB2	CTGTCGCTAACCTCTTTATCCA CAGTATGGACGTTGGTATTATATCTAA	Mulé *et al.*, 2004
F. subglutinans, *F. nygamai*	see *F. subglutinans*	RAPD marker	PCR	608 550	1–3F 1–3R	TGCAGATAATGAGGGTCTGC GGAACATTGGGCAAAACTAC	Zheng and Ploetz, 2002
F. torulosum	see *F. sambucinum*	RAPD marker	PCR	664	TOR-Bf TOR-Br	CAAAGCGCTCCCTCAATCTCGTAC CAAAGCGCTCATCAACTCCATATA	Yoder and Christianson, 1998
F. venenatum	see *F. sambucinum*	RAPD marker	PCR	276	VEN-Bf VEN-Br	GGCGGATAAGGATAGTGGTAGAAG GGCGGATAAGCAAATAAGATGCTT	Yoder and Christianson, 1998
F. verticillioides	Fumonisins, fusaric acid, fusarin C, moniliformin, naphthoquinone pigments, gibepyrones	fragment from shotgun cloning	PCR	1600	FUS1 FUS2	CTTGGTCATGGGCCAGTCAAGAC CACAGTCACATAGCATTGCTAGCC	Murillo *et al.*, 1998
F. verticillioides	see *F. verticillioides*	RAPD marker	PCR	561	53–6F 53–6R	TTTACGAGGCGGCGATGGGT GGCCGTTTACCTGGCTTCTT	Möller *et al.*, 1999
F. verticillioides	see *F. verticillioides*	mitochondrial rRNA gene, small subunit region	PCR	n.s.	*FCORN2 *FVERT1	AAGTCTTCCAGTATGGGAAG TGGTGGACTAGTCTGAATCC	Beck and Barnett, 2003

F. verticillioides	see *F. verticillioides*	calmodulin gene	PCR	587	VER1 VER2	CTTCCTGCGATGTTTCTCC AATTGGCCATTGGTATTATATATCTA	Mulé *et al.*, 2004
F. verticillioides	see *F. verticillioides*	*fum1* gene	quant. real-time RT-PCR (SYBR green 1)	69	PQF1-F PQF1-R	GAGCCGAGTCAGCAAGGATT AGGGTTCGTGAGCCAAGGA	López-Errasquín *et al.*, 2007
F. verticillioides	see *F. verticillioides*	*Fum19* gene	quant. real-time RT-PCR (SYBR green 1)	68	PQF19-F PQF19-R	ATCAGCATCGGTAACGCTTATGA ACTGTAAGTTGAGGAAGCCCTTGT	López-Errasquín *et al.*, 2007
F. verticillioides, fumonisin producing isolates	see *F. verticillioides*	*fum1* gene	PCR	250	Fum5–5F Fum5–6R	GAAATGGATCT(A/T)TTCGAGGC CCTTTCGATACATGCAGAAAG	Gonzalez-Jaen *et al.*, 2004
F. verticillioides, fumonisin producing isolates	see *F. verticillioides*	rRNA gene, IGS region	PCR	n.s.	VERTF-1 VERTF-2	GCGGGAATTCAAAAGTGGCC GAGGGCGCGGAAACGGATCGG	Patino *et al.*, 2004
F. verticillioides fumonisin producing isolates	see *F. verticillioides*	*fum1* gene	PCR	354	FUM35F FUM35R	CTTGAACGCGGAGCTAGATTAT ATCCGTGTATGCATATGTCGAG	Sanchez-Rangel *et al.*, 2005
Fusarium spp. potential fumonisin producers	Fumonisins, beauvericin, moniliformin, naphthoquinone pigments, fusaric acid, fusarin C, gibebyrones	rRNA gene, ITS1 region	PCR PCR-ELISA	108	int1 int2 probe	CCGAGTTTACAACTCCCAA ACAGAGTTTAGGGGTCCTCT biotin-ATCAGCCCGCTCCCGGTAA	Grimm and Geisen, 1998
Fusarium spp. potential fumonisin producers	see fumonisin producers	*fum1* gene	PCR	845	Fum5F Fum5R	GTCGAGTTGTTGACCACTGCG CGTATCGTCAGCATGATGTAGC	Bluhm *et al.*, 2002

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
Fusarium spp. potential trichothecene producers	Trichothecenes type A & B, zearalenone, culmorin, fusarin C, butenolide, enniatin, beauvericin, chrysogine, gamma-lactones, chlamydosporol, antibiotic Y, equisetin, fusarochro manone, chrysogine	*tri5* gene	PCR	658	Tox5–1 Tox5–2	GCTGCTCATCACTTTGCTCAG CTGATCTGGTCACGCTCATC	Niessen and Vogel, 1998; Schnerr *et al.*, 2001; Tan and Niessen, 2003; Demeke *et al.*, 2005; Agodi *et al.*, 2005; Strausbaugh *et al.*, 2005; Pinson-Gadais *et al.*, 2007
Fusarium spp. potential trichothecene producers	see trichothecene producers	*tri5* gene	PCR		tr5F tr5R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCTG	Doohan *et al.*, 1999
Fusarium spp. potential trichothecene producers	see trichothecene producers	*tri6* gene	PCR	596	Tri6F Tri6R	CTCTTTGATCGTGTTGCGTC CTTGTGTATCCGCCTATAGTGATC	Bluhm *et al.*, 2002
Fusarium spp. potential trichothecene producers	see trichothecene producers	*tri5* gene	quant. real-time PCR (TaqMan)	76	TMTrif TMTrir TMTrip	CAGCAG(A/C)T(A/G)CTCAAGGTAGACCC AACTGTA(C/T)AC(A/G)ACCATGCCAAC VIC-AGCGACTACAGGCTTCCC TCCAAACAAT-TAMRA	Straumfors Halstensen *et al.*, 2006a
Myrothecium roridum, *M. verrucaria*	verrucarins, roridins, satratoxins, mycotoxins, roritoxins	rRNA gene, ITS region	PCR, quant. real-time PCR (TaqMan)	n.s.	*MyroF1 *MyroR1 *MyroP1	AGTTTACAAACTCCCAAACCCTTT GTGTCACTCAGAGGAGAAAACCA FAM-CGC CTG GTT CCG GGC CC-TAMRA	Haugland and Vesper, 2000

ochratoxin A/ citrinin producers	ochratoxin A, citrinin and others	polyketide synthase gene, KS domain	PCR	520	AoLC35- 12L AoLC35–12R	GCCAGACCATCGACACTGCATGCTC CGACTGGCGTTCCAGTACCATGAGCC	Dao *et al.*, 2005
Paecilomyces variotii	patulin, viridoxin	rRNA gene, ITS region	PCR, quant. real-time PCR (TaqMan)	n.s.	*PvariF1 *PvariR1probe *PvariP1	CCCGCCGTGGTTCAC GTTGTTGAAAGTTTTAATTGATTGATTGT FAM-CTCAGACGGCAACCTTCCAGGCA-TAMRA	Haugland and Vesper, 2000
Patulin producers	patulin, roquefortine C, citrinin, communesins, chaetoglobosin C	*idh* gene	PCR	600	IDH1 IDH2	CAATGTGTCGTACTGTGCCC ACCTTCAGTCGCTGTTCCTC	Paterson *et al.*, 2000 Paterson, 2004, 2006
P. brevicompactum, *P. alberechii*	botryodiploidin, mycophenolic acid, Raistrick phenols, brevianamide A	rRNA gene, ITS region	PCR, quant. real-time PCR (TaqMan)	n.s.	*PbrevF1 *PbrevP1 probe *Pbrev P1	CCTTGTTGCTTCGGCGA TCAGACTACAATCTTCAGACAGAGTTCTAA FAM-CCTGCCTTTTGGCTGCCGGG-TAMRA	Haugland and Vesper, 2000
P. citrinum, *P. westlingi*	citrinin, tanzawaic acid A	rRNA gene, ITS-region	PCR, quant. real-time PCR (TaqMan)	n.s.	*PcitrF1 *PcitrR1probe PcitrP2	CCGTGTTGCCCGAACCTA TTGTTGAAAGTTTTAACTAATTTCGTTATAG FAM-CCCCTGAACGCTGTCTGAAGTTGCA-TAMRA	Haugland and Vesper, 2000
P. digitatum, DMI resistant isolates	tryptoquivalins	*cyp51* gene	PCR	750	Pri-207 Pri-38c	TAGCTCCAAAACAAATCGTCTGGC CACTTGATCTGCCCTGTTAACA	Hamamoto *et al.*, 2001
P. expansum	roquefortine C, patulin, citrinin, communensins, chaetoglobosin C	polygalact uronase gene	PCR	404	PEF PER	ATCGGCTGCGGATTGAAAG AGTCACGGGTTTGGAGGGA	Marek *et al.*, 2003
P. nordicum	ochratoxin A, anacine, verrucolone	*otapks*PN gene	quant. real-time PCR (TaqMan)	n.s.	otapkstaq1 otapkstaq2 probe otapks PNprobe	CACGGTTTGGAACACCACAAT TGAAGATCTCCCCCGCCT FAM-CGTACCAATCCCCATCCAGGGCTC-TAMRA	Geisen *et al.*, 2004

(*continued*)

TABLE 3.1 (*continued*)

Target species	Toxins produced[a]	DNA target	Assay type	Size (bp)	Primer name[b]	Primer sequence 5′→ 3′	References
P. verrucosum	ochrtoxin A, citrinin, verrulolone, verrucins	*otanps*PN gene	PCR	800	otanps-for otanps-rev	AGTCTTCGCTGGGTGCTTCC CAGCACTTTTCCCTCCATCTATCC	Bogs *et al.*, 2006
P. roqueforti	roquefortin C, isofumigaclavine A & B, PR-toxin, mycophenolic acid	rRNA gene, ITS region	PCR, quant. real-time PCR (TaqMan)	n.s.	*PchryF1 *Pchry R2probe *PenP2	CGGGCCCGCCTTAAC TTAAATAATTTATATTTGT TCTCAGACTGCAT FAM-CGCGCCCGCCGAAGACA-TAMRA	Haugland and Vesper, 2000
P. roqueforti, *P. carneum*	roquefortin C, isofumigaclavine A & B, PR-toxin, mycophenolic acid, patulin, penitrem A	rRNA gene, ITS1- 5.8S-ITS2 regions	PCR	300	ITS183 ITS401	CTGTCTGAAGAATGCAGTCTGAGAAC CCATACGCTCGAGGACCGGAC	Pedersen *et al.*, 1997 Esberg Boysen, 1999; Williams *et al.*, 2001
Stachybotrys chartarum	satratoxin G & H	rRNA gene, 18S and ITS1 region	PCR	210	IT51 StacR3	GATATGCTTAAGTTCAGCGGGTA TGCCACTCAGAGAATACTGAAA	Haugland and Heckman, 1998 Li *et al.*, 2001
S. chartarum	satratoxin G & H	rRNA gene, ITS1 region	quant. real-time PCR (TaqMan)	107	STAF1 STAR1 probe	GTTGCTTCGGGCGGGAAC TTTGCGTTTGCCACTCAGAG FAM-CTGCGCCCGGATCCAGGC-TAMRA	Cruz-Perez *et al.*, 2001

S. chartarum	satratoxin G & H	*tri5* gene	PCR	445	ScTox5–1 ScTox5–4	GTCTATACTCGACAATAGTCC GTCCTTCTGAGAGAACACTA	Peltola *et al.*, 2002
S. chartarum	Satratoxin G & H	*tri5* gene	PCR	n.s.	tri5S1 tri5S2	CCTCACCCTCAGATGTTGACATAC TCCTTGTAGAAGGACATGAGGTCA	Koster *et al.*, 2003
S. chartarum	Satratoxin G & H	*tri5* gene	PCR	165	ST5F ST5R	GTGGCAACCCGCAAAAGC TTGCTCTTTCTTGGAATATTTTGG	Dean *et al.*, 2005
Trichoderma harzianum biotypes 2 und 4	chrysophanol, koninginin A, trichorzianines A & B	RAPD marker	PCR	444	Th-F Th-R	CGGTGACATCTGAAAAGTCGTG TGTCACCCGTTCGGATCATCCG	Chen *et al.*, 1999
Wallemia sebi	wallemiol A & B	rRNA gene, 18S region	PCR, quant. real-time PCR	328	Wall-SYB7 Wall-SYB8	GATTGGATGACGTTATATTAT ACAACAAAATGTCGTACCG	Zeng *et al.*, 2004

AGE = agarose gel electrophoresis, BAL = bronchoalveolar fluid, FAM = 6-carboxyfluorescein label, MGB = minor groove binder, n.s. = not specified, RAPD = randomly amplified polymorphic DNA, RT-PCR = reverse transcription PCR, TAMRA = 6-carboxytetramethylrhodamine label.

[a] According to Frisvad and Thrane, 2004.

[b] Primers marked with * are subject to patent.

(see Frisvad and Thrane, 2004), the source of the sequence used for primer design, type of assay applied, primer names and sequences, and the references of first publication as well as follow-up studies in which the respective primers were used.

The following chapters provide an overview over the use of PCR-based systems available to date for molecular identification and detection of mycotoxin producing fungi.

A. Detection of aflatoxin producers

Accordingly, the first systems were developed for producers of aflatoxins as the most potent compounds among the mycotoxins. The first PCR-based detection system for a mycotoxin-producing fungus was published by Tang *et al*. (1993) who described detection of *Aspergillus flavus* by nested PCR in human bronchoalveolar lavages. Their primers were based on the gene coding for alkaline protease, an enzyme with elastolytic activity which was suggested to function as a virulence factor during induction of allergic bronchopulmonary aspergillosis in *A. fumigatus* and other pathogenic *Aspergillus* species (Moser *et al*., 1994). However, this early publication aimed at *A. flavus* as a lung pathogen rather than as a toxin producer and can therefore not be regarded as the starting point of developments in PCR-based detection systems for mycotoxigenic fungi. Clearly focused at evaluation of toxigenic properties of *Aspergillus* species from section Flavi were two PCR assays published by Geisen (1996) and in a parallel publication by Shapira *et al*. (1996), which are therefore regarded as the first PCR-based assays for a mycotoxigenic fungus. Both authors used sequences of the same three genes involved in the biosynthesis of aflatoxins in *A. flavus*, *A. parasiticus*, and *A. versicolor* to design gene-specific primers. The assay published by Geisen (1996) made use of the three primer pairs in a multiplex PCR in which it was demonstrated that *A. sojae* and *A. oryzae*, both essentially identical with *A. flavus* but typically not producing aflatoxins, lack the *nor-1* gene and that other non-aflatoxin producing *A. flavus* strains gave no rise to a PCR product with one or all of the primer pairs used. The feasibility of both copublished assays for detection of aflatoxin producers in contaminated corn (Shapira *et al*., 1996), cereals (Geisen *et al*., 1998), and figs (Färber *et al*., 1997) was demonstrated in follow-up studies. Mayer *et al*. (2003) used sequences of the *nor-1* gene to set up primers and a probe for a TaqMan™ real-time PCR assay with which *A. flavus* was quantified in contaminated food samples and cereals. Using a different concept for primer design and SYBR-Green I as a fluorescent dye, Bu *et al*. (2005) described a quantitative real-time PCR assay for *A. flavus*, among other medically important fungi, in pure cultures and medical specimens. Primers used were based on sequences from the ITS1–5.8S region of the

ribosomal RNA (rRNA) gene of *A. flavus*. Similar to the formerly mentioned work, the conventional PCR assay described by Sugita *et al.* (2004) made use of specific primers designed from the sequence of another part of the ITS1 region of the *A. flavus* rRNA gene to analyze medical samples. Besides the *Aspergillus* spp. mentioned above, *A. nomius* is another species in section Flavi which is known as a producer of aflatoxins but also of tenuazonic acid (Frisvad and Thrane, 2004), a compound typically produced by *Alternaria tenuissima*. Haugland and Vesper (2000) published primers for the diagnosis of a wide range of fungal species in a patent application. The system described for specific detection of *A. nomius* was based on sequences from the ITS region of the rRNA gene and was described together with a fluorescently labeled probe to be applied in a TaqMan assay for quantitative real-time PCR. No PCR-based detection assays have been described until presently for the rarely encountered aflatoxin producers *A. bombycis*, *A. ochraceoroseus*, and *A. pseudotamari* (Bennett and Klich, 2003).

B. Detection of trichothecene producers

Trichothecenes are sesquiterpenoid mycotoxins which share the 12,13-epoxytrichothecene skeleton as the common structural feature. The presence or absence of an 8-keto moiety leads to differentiation of group B and group A trichothecenes, respectively, the latter of which have a valerianyl-, acetyl-, or hydroxyl moiety in that position. Furthermore, group C trichothecenes (macrocyclic trichothecenes) are differentiated by the presence of a macrolide ring system attached at position 4β and 15 of the trichothecene verrucarol (Grove, 1993). Trichothecenes are produced by species in the fungal genera *Cryptomela*, *Fusarium*, *Myrothecium*, *Stachybotrys*, *Trichoderma/Hypocrea*, *Trichothecium*, and *Verticimonosporium* (Davis and Diener, 1987; Frisvad and Thrane, 2004; Turner and Aldridge, 1983). Toxins of the trichothecene type were also found to be produced by a hypocrealean epibiont of the plant species *Baccharis cordifolia* (Jarvis *et al.*, 1991; Rosso *et al.*, 2000). *Fusarium* spp., however, produce the widest variety of different trichothecene compounds among which the B-trichothecenes deoxynivalenol (DON) and nivalenol (NIV) as well as the A-trichothecenes T-2 toxin, HT-2 toxin, neosolaniol (NEOS), and diacetoxyscirpenol (DAS) are the most widespread and/or toxic compounds isolated from natural sources.

C. Trichothecene biosynthesis cluster genes as sequence source for primer design

The genetics and regulation of trichothecene biosynthesis have been elucidated in detail in *F. sporotrichioides* (Hohn *et al.*, 1993), *Myrothecium roridum* (Trapp *et al.*, 1998), and *F. graminearum* (Gibberella zeae; Kimura *et al.*, 2003).

Sequencing of parts of the trichothecene gene cluster was done for newly described species in the *F. graminearum* group, *F. crookwellense*, *F. culmorum*, *F. lunulosporum*, and *F. pseudograminearum*. The *tri5* gene, which codes for trichodiene synthase catalyzing the first specific step in the biosynthesis of all known trichothecenes in all producing fungi, was particularly well characterized in many *Fusarium* spp., but also in *Stachybotrys chartarum* (Koster *et al.*, 2003 direct submission to GenBank), *M. roridum* (Trapp *et al.*, 1998), and in *Trichoderma harzianum* (Gallo, 2004). Niessen and Vogel (1998) aligned *tri5* sequences of *F. sporotrichioides*, *F. poae*, *F. sambucinum*, and *F. graminearum*, which at that time were the only available data, to find two highly conserved regions in the gene. Primers designed to hybridize to the gene amplified a 658-bp product from 20 different species and varieties within *Fusarium*, including 1 odd strain of *F. dlamini* and *Cladobotryum dendroides*, the industrial producer of galactose oxidase which had previously been demonstrated to be a non-sporulating isolate of *F. graminearum* (Niessen and Vogel, 1997). Based on the same gene, Schnerr *et al.* (2001) designed primers which resulted in a smaller PCR product detecting essentially the same set of species as in Niessen and Vogel (1998) but with a better fit to application in quantitative real-time PCR. SYBR green I and a calibration curve produced with known concentrations of *F. graminearum* DSM 4527 DNA as a template was used to quantify DNA concentrations of trichothecene producers. The method was applied to analyze DNA in 300 naturally infected samples of wheat. Comparison of DNA concentrations with DON concentrations in the samples revealed a positive correlation between both parameters. Primers developed by Niessen and Vogel (1998) were applied in follow-up studies by various authors to detect the *tri5* gene in pure cultures and in sample materials by conventional PCR (Agodi *et al.*, 2005; Demeke *et al.*, 2005; Tan and Niessen, 2003). Sequence information was also used to set up species-specific assays for identification, detection, and quantification of typical producers of trichothecene mycotoxins. During a project in which a polyphasic approach was applied to study the taxonomy of species within section *Sporotrichiella* of *Fusarium*, Niessen *et al.* (2004) developed *tri5* gene-based primers to set up species-specific detection assays for *F. langsethiae*, which was described as a new species a result of that study (Torp and Nirenberg, 2004), *F. kyushuense*, *F. poae*, and *F. sporotrichioides*. The former and the latter species were identified as the major producers of A-type trichothecenes in cereals in Scandinavian countries (Torp and Langseth, 1999). The detection systems made use of a forward primer described in Niessen and Vogel (1998), which were combined to reverse primers binding specifically to the intron region of the *tri5* gene in the respective species. Primer pairs were highly specific for identification of *F. kyushuense*, *F. poae*, and *F. sporotrichioides*. Due to the close taxonomical relation of *F. langsethiae* to the latter species, that fungus

could only be identified by using a combination of two separate PCR reactions. Primers were demonstrated to be useful for the detection of all the four species in inoculated barley and in naturally contaminated oats. Strausbaugh *et al*. (2005) set up a pair of *tri5*-directed primers for identification and quantification of *F. culmorum* with a TaqMan quantitative real-time PCR assay in the roots of wheat and barley plants. The test was highly sensitive in pure cultures and root materials. However, the authors stated that the designed primers fully cross-reacted with *F. graminearum* and *F. pseudograminearum* genomic DNA. *S. chartarum* (syn. *S. atra*) has been identified as a producer of satratoxins, a group of highly potent mycotoxins belonging to the macrocyclic trichothecenes. The fungus was identified as the source of mycotoxicosis in the so called ''sick building syndrome,'' a condition observed in individuals living or working in buildings with elevated aerial concentrations of fungal propagules which leads to severe health problems and has caused the death of children in some cases (Etzel *et al*., 1998). Straus and Wong published the DNA sequence of the *tri5* gene of the fungus [GenBank, direct submission (1998), accession no. AF053926] which was subsequently used by Peltola *et al*. (2002) to design a species-specific pair of PCR primers for the identification of the toxigenic subgroup 1 of *S. chartarum*. Nontoxic strains and strains with low toxicity in a boar spermatozoa motility test were combined in subgroup 2 and did not give a product with the primer pair which thus was useful to distinguish the two taxonomical groups found in *S. chartarum*. The presence of two lineages in this fungus were later also found by Koster *et al*. (2003), who found two phylogenetically distinct lineages when testing genotypic variation of tri5, ITS, and two housekeeping genes in a geographically diverse distinct set of isolates. Also Dean *et al*. (2005) developed *tri5*-based primers for the identification of *S. chartarum*. The primers were combined with oligonucleotides specific for *A. versicolor*, *P. purpurogenum*, and *Cladosporium* spp., respectively, in a multiplex PCR assay. The authors state that the system might be useful to alert building occupants and remediators to the potential presence of mycotoxin-producing fungi in their indoor environment. However, the system was not demonstrated to work with drawn air samples.

Besides *tri5*, other genes from the trichothecene biosynthesis cluster have also been used as the source of sequences to design species- and group-specific PCR primers. A group-specific PCR assay for the detection of trichothecene-producing *Fusarium* spp. involving primers binding to the *tri6* gene, which codes for a transcription factor in the biosynthetic pathway of that group of toxins, was set up by Bluhm *et al*. (2002). The authors used the system together with primers for detection of fumonisin producers in pure cultures and artificially infected cornmeal with a sensitivity comparable to enzyme-linked immunosorbent assays. Bakan *et al*. (2002) applied intergenic sequences between the *tri5* and

tri6 genes (transcription factor) to distinguish between high and low DON-producing isolates of *F. culmorum* through amplification of DNA fragments differing substantially in size in a duplex PCR. Testing of 17 and 13 isolates of high and low DON producers, respectively, revealed 100% correlation between producer type and the size of the amplicon produced in this assay. In order to differentiate DON- and NIV-producing chemotypes in *F. graminearum*, Lee *et al.* (2001) designed a pair of primers hybridizing adjacent to an inserted region present in the *tri7* gene of the fungus. It was demonstrated that a 161-bp PCR product was produced from DNA of isolates producing NIV, whereas the product resulting with DNA isolated from DON-producing isolates was between 173 and 327 bp in size resulting from the presence of 2–16 copies of an 11-bp tandem repeat in that portion of the gene. Primers were used by Waalwijk *et al.* (2003) to study the incidence of both genotypes in wheat isolations of *F. graminearum* from the Netherlands. The PCR assay described by Lee *et al.* (2001) might prove very useful in differentiating both chemotypes in epidemiological studies in Asian countries, where both chemotypes occur but also could it be used in plant quarantine in the United States and Canada, where no NIV-producing isolates of *F. graminearum* have been observed (Mirocha *et al.*, 1989). A primer pair hybridizing to sequences within the *tri13* gene of the trichothecene gene cluster was published by Demeke *et al.* (2005). The gene codes for a cytochrome *P450* monooxygenase for C-4 hydroxylation of trichothecenes. The screening of 85 samples of wheat, barley, oats, and corn from Canada revealed detection of *F. sporotrichioides* in 56% of samples analyzed. The comparison with results of a kernel-plating technique showed good correlations between both methods. However, most samples positive for *F. sporotrichioides* with PCR did not contain detectable concentrations of T-2 toxin or HT-2 toxin, the principal mycotoxins produced by the fungus. A much better correlation between PCR detection of fungal biomass and presence of detectable concentrations of a mycotoxin was found in this study for *F. graminearum* and DON. Also based on the nucleotide sequence of the *tri13* gene, Waalwijk *et al.* (2003) published a pair of primers which gave rise to PCR products of different sizes in producers of DON and NIV, respectively, in the latter fungus and in *F. culmorum*. The authors noted a slight increase in the number of NIV genotypes in a comparative study of wheat isolations of both species from the years 2000 and 2001.

D. Other sequence sources for detection of trichothecene producers

Various sequence sources other than genes from the trichothecene biosynthesis pathway were used to set up PCR-based systems for identification and detection of trichothecene producers. Highly specific

identification of *F. graminearum* was demonstrated by Niessen and Vogel (1997) who used the gaoA gene published for *C. dendroides* NRRL 2903, which is the source of industrial production of galactose oxidase. The authors demonstrated that *F. graminearum* strains not only contained a 100% homologous gene but most strains tested showed galactose oxidase activity in a microplate assay. Using the gaoA-specific primer pair in a triplex PCR assay, Knoll *et al.* (2000) demonstrated the usefulness of the method for detection of trichothecene producers in contaminated wheat. The same gaoA-specific primers were used by Knoll *et al.* (2002) in an assay involving DNA Detection Test Strips™ for rapid detection of the PCR product without agarose gel electrophoresis. Another alternative detection and quantification method was used by Niessen *et al.* (1998), who employed the gaoA-specific primers in a solid phase PCR assay using the DIAPOPS technology (Chevrier *et al.*, 1993). One of the primers was covalently bound to microwell material, thereby fixing the product during PCR. The detection of solid phase-bound product was done in a colorimetric assay using a sequence-specific biotinylated hybridization probe and streptavidin-coupled alkaline phosphatase with 4-nitrophenylphosphate as the substrate. The system was sensitive enough to detect an equivalent of 300 copies of the target gene even in a 100-fold excess of wheat DNA as background. The authors did not demonstrate usefulness of their system to detect *F.* graminearum DNA in contaminated sample material.

Two different housekeeping genes were used as the sequence sources for the development of PCR identification and detection systems for trichothecene-producing *Fusarium* spp. The gene coding for elongation factor 1α (EF-1α) was used by Aoki and O'Donnell (1999) as the sequence source for a PCR assay detecting *F. pseudograminearum*, a species formerly recognized as the heterothallic group 1 population of *F. graminearum*. The primers were shown to be highly specific for the identification of the target species. Gargouri *et al.* (2001) applied the assay to screen *Gibberella zeae* isolates obtained from plants with wheat foot rot collected at different climatic regions in Tunisia. *F. pseudograminearum* (*F. graminearum* group 1) was mostly detected in samples grown under semiarid low land conditions. Reischer *et al.* (2004) aligned sequences of the beta tubulin gene from several isolates of *F. graminearum* and of closely related species as well as *Fusarium* spp. frequently found as part of the Fusarium Head Blight complex and extracted a primer pair for the specific identification of the former species. TaqMan quantitative real-time PCR technology was used to quantify *F. graminearum* in field inoculated wheat plants with high sensitivity and a dynamic range of six orders of magnitude of target DNA concentrations.

As in aflatoxin producers, also producers of *Fusarium* toxins have been detected by PCR assay which were based on primers hybridizing to genes

coding for rRNA. Primer design is specifically facilitated by the availability of complete or partial rRNA sequences from public databases so that much work can be done *in silico* without the necessity of sequencing ones own DNA. Most authors used variable regions present within the internal transcribed spacers separating the genes coding for 18S rRNA and 5.8S rRNA (ITS1) and between the latter gene and the gene coding for 28S rRNA. Haugland and Vesper (2000) filed a patent with the US Patent and Trademark Office in which detection of a wide variety of fungal organisms based on rRNA sequences was claimed. All systems described in the text make use of group- or species-specific primer pairs derived from rRNA genes in combination with a specific detection probe. Probes were tagged with 6-FAM as the fluorochrome and TAMRA as a specific quencher to be used in TaqMan quantitative real-time PCR assays. Mishra *et al*. (2003) used the ITS region in the rRNA genes of *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. oxysporum*, and *F. sambucinum* to identify the target organisms in a fluorescent assay. Each forward primer used was marked with a different fluorescent dye in order to evaluate the presence of a PCR product by visual inspection of the PCR tubes. Kulik *et al*. (2004) designed a forward primer for identification of *F. sporotrichioides* which included a 3′mismatch in the ITS2 region of the rRNA gene which could be used to differentiate isolates of that species from closely related isolates. Combination of the forward primer with a reverse primer published earlier by Hue *et al*. (1999) resulted in a PCR assay, which was useful for identification of *F. sporotrichioides* pure cultures but also for detection of the toxin producer in plant tissue.

As an alternative to the use of sequence information from defined genes, otherwise functionally defined DNA also sources of undefined origin can be used to set up specific PCR primers. Such undefined sequence sources can be generated by amplification of genomic DNA with short (decamer) oligonucleotide primers under quite unspecific conditions (Williams *et al*., 1990). The analysis results in production of a polymorphic pattern of few to several PCR fragments which are separated on an agarose gel. Since the pattern may differ between the taxonomic groups analyzed, single fragments can be used as species-specific markers. These markers are subsequently extracted from the gel and sequenced after cloning into an appropriate vector. Sequence information can be used to design specific PCR primers either by simple elongation of the decamer primers with the specific sequence or by designing new primers according to the internal sequence of the fragment. Assays using primers based on randomly amplified polymorphic DNA (RAPD) have been developed for the most toxic and widespread toxigenic species within *Fusarium*. Most of the assays were set up quite early during the development of the use of PCR as a diagnostic method in toxigenic fungi because little specific sequence information was available then.

Schilling *et al*. (1996) were among the first authors to report on the use of a PCR-based detection system for *Fusarium* species. They set up primers for the identification of *F. culmorum*, *F. graminearum*, and *F. avenaceum* which they used to detect the organisms in contaminated cereals. The primers for *F. culmorum* and *F. graminearum* proved to be highly specific for their targets. However, the system developed for *F. avenaceum* showed cross-reaction with *F. tricinctum*, two species now known to be closely related taxonomically. Primers developed by Schilling *et al*. (1996) were used in various studies by other authors who found further proof for the usefulness of the system (Chelkowski *et al*., 1999; Gargouri *et al*., 2001; Obradovic *et al*., 2001; Williams *et al*., 2002). Only recently, Klemsdal and Elen (2006) used the complete sequence of the *F. culmorum*-specific 472-bp RAPD fragment described by Schilling *et al*. (1996) to design two new sets of primers which they used in a nested PCR to detect *F. culmorum* in a highly sensitive assay. This assay was applied to identification of pure cultures but also to the detection of the target species in inoculated field samples.

Quite early during the development of the use of PCR for toxigenic *Fusarium* species, Nicholson *et al*. (1998) used internal competitors in defined concentrations to set up a quantitative PCR for *F. graminearum* and also for *F. culmorum*, the major producer of DON in cereals. The system made use of a competitor fragment which was constructed to have binding sites for the forward and reverse primer but resulted in amplification of a fragment of different length and sequence so that it could be distinguished from the specific product by agarose gel chromatography. The intensity of the competitor band was used to calculate the amount of target DNA present in an unknown sample. Aimed at resolving the identity of the fungus used in the production of mycoprotein (Quorn), Yoder and Christianson (1998) applied RAPD to the taxonomic study of species within section Discolor of *Fusarium*. Several taxon-specific RAPD fragments were obtained and PCR identification systems were set up for *F. crookwellense*, *F. culmorum*, *F. graminearum*, *F. sambucinum*, *F. torulosum*, and *F. venenatum*, to which species the Quorn-producing strain was finally assigned.

E. Detection of ochratoxin A producers

The following paragraphs of this chapter deal with PCR-based methods which were developed during recent years in order to provide analytical tools for detection and identification of those fungal organisms which were described as producers of ochratoxin A. Most of the systems were developed to analyze pure cultures or contaminated commodities to estimate toxicological hazards associated with a fungal strain. However, it is worthwhile to note that some of the diagnostic tools described in the following chapters were rather aimed at the

organisms in their role as human or animal pathogens than as sources of toxicosis. This is especially true for *A. niger*, a generally regard as safe (GRAS) fungus nowadays widely used in the industrial production of food enzymes and organic acids. Recent findings which show that between 3% and 10% of isolates in *A. niger* are able to produce ochratoxin A under favorable conditions might put some constraints to safety considerations when using that organism. However, Schuster *et al.* (2002) concluded that isolates can still be used in safe biotechnological applications provided that nontoxigenic properties have been properly tested. The PCR-based methods now available may provide useful tools to analyze either the taxonomic classification or even the mycotoxigenic potential of a given isolate. They will thus make both products and processes safer.

F. Primers targeted to anonymous genomic markers

Anonymous genomic markers are sections within the genome, which are specifically linked to a taxonomical unit, a certain phenotype, or physiological property without being assigned to a defined genetic function. Typically they are generated either by treatment of the genomic DNA with restriction endonucleases, amplification of DNA regions flanked by microsatellites or other repetitive sequence elements, or amplification of DNA using short randomly priming oligonucleotides under low stringency conditions. All the different techniques available have in common that polymorphic banding patterns are generated which may be employed to distinguish taxonomical groups. Sequence information obtained from anonymous markers have widely been used to set up PCR primers for specific detection and identification of fungal organisms.

The following sections provide information on how these techniques have been employed to set up species-specific detection systems for potentially OTA-producing fungi.

G. AFLP marker-based primers for *A. ochraceus* and *A. carbonarius*

In analogy to the detection of trichothecene-producing *Fusarium* spp., anonymous genomic markers have also been applied as sequence source for primer design in PCR assays for producers of ochratoxin A. Amplified fragment length polymorphism (AFLP) is a molecular biological fingerprinting technique introduced by Vos *et al.* (1995) for genetic mapping of plants. Majer *et al.* (1996) appear to be the first who used the technique to detect genetic variation in fungi (*Cladosporium fulvum* and *Pyrenopaziza brassicae*). For a recent review of the AFLP technique, see Bensch

and Akesson (2005). In AFLP, genomic DNA of the organism under study is fragmented by restriction endonucleases (mostly *Eco*RI together with *Mse*I) as a first step. In a second step, adapter oligonucleotides are ligated to the sticky ends of the resulting restriction fragments. Oligonucleotide primers incorporating the adapter sequence and one of either restriction sites are used to amplify all restriction fragments in a preamplification step. Preamplified product is used as the template in a second round PCR using oligonucleotides similar to the first round primers but with a 3′-end extension of one to three nucleotides which lead to selective amplification of only a fraction of the total preamplified fragments. Typically, about 100 fragments are amplified which are size separated by polyacrylamide gel electrophoresis (PAGE) and detected by either silver staining or a fluorescent dye attached to one of the primers. The resulting banding patterns can be normalized using internal and external size markers and compared using appropriate software packages. Data are used to set up phylogenetic trees, for taxonomical identification down to the level of strains or individuals, to study population structures, gene mapping, and linkage studies or marker analysis in all groups of organisms. Since AFLP fingerprints are highly reproducible, they can be stored in reference databases and used for species identification of unknown samples circumventing cumbersome multilocus sequencing.

Schmidt *et al*. (2003, 2004a) used AFLP to detect specific markers for *A. ochraceus* and *A. carbonarius*. Both fungal species were found to be the predominant producers of OTA in green coffee (Martins *et al*., 2003; Pardo *et al*., 2004; Taniwaki *et al*., 2003). Moreover, the latter species has been identified as the major producer of that toxin in grapes (Battilani *et al*., 2003; Serra *et al*., 2003, 2006) and raisins (Abarca *et al*., 2003; Tjamos *et al*., 2004). Schmidt and coworkers compared strains of both species isolated from coffee-related sources as well as closely related taxa and demonstrated that the resulting AFLP fingerprints were quite distinct at the species level in both taxa. Several of the amplified fragments were found to be characteristic to either of the species and could be used as specific AFLP markers for *A. ochraceus* (Schmidt *et al*., 2003) and *A. carbonarius* (Schmidt *et al*., 2004a), respectively. The marker fragments were cloned and sequenced and primers designed from the sequenced markers enabled detection of both species. Specificity of the primers was tested with DNA of several different target strains as well as closely related *Aspergillus* and *Penicillium* spp. and DNA isolated from noninfected green coffee. Based on the *A. ochraceus*-specific primer pair, Schmidt *et al*. (2004b) set up a real-time PCR assay for quantitative estimation of *A. ochraceus* DNA. The authors used a LightCycler™ (Roche diagnostics) system with SYBR Green I as the intercalating fluorescent dye to quantify the concentration of DNA of the fungus in 30 samples of naturally infected green coffee from various regions. The assay had a

detection limit of 4.7 pg per reaction compared to a detection limit of ~400 pg per reaction in conventional PCR with detection in ethidium bromide-stained agarose gels. Correlation between the DNA concentration found with the assay and the OTA content of the respective samples was low ($r = 0.55$) but statistically significant. Perrone *et al*. (2006) used AFLP to distinguish isolates of closely related *Aspergillus* section *Nigri* species derived from grapes and to compare the results with the OTA-producing capacities of the isolates under study. They found that isolates assigned to *A. carbonarius*, *A. niger*, and the morphologically indistinct *A. tubingensis* were clearly distinct by their AFLP fingerprint. Moreover, the authors demonstrated that 25% of the *A. tubingensis* strains analyzed were able to produce ochratoxin A in pure cultures with a proportion of toxin producers similar to that found in *A. niger*. From that result it was concluded that *A. tubingensis* strains may well account for OTA concentrations found in samples of Italian wine (Brera *et al*., 2005; Otteneder and Majerus, 2000; Pietri *et al*., 2001). Similar observations were made by Medina *et al*. (2005) who analyzed *Aspergillus* section *Nigri* isolates from Spanish grapes for their ability to produce OTA. In their study, 14% of the *A. tubingensis* isolates produced the toxin.

H. Detection of *A. ochraceus* and *A. carbonarius* with cDNA AFLP-based primers

cDNA AFLP is a variation of the original protocol of Vos *et al*. (1995) in which expressed genes are analyzed rather than the complete genome of an organism (Bachem *et al*., 1996). Messenger RNA is isolated and used as a template for the preparation of cDNA by reverse transcription which then undergoes the regular AFLP procedure. In an attempt to spot genes differentially expressed under conditions promoting OTA production in *A. ochraceus*, Mühlencoert (2004) used this technique to find expressed markers for genes involved in biosynthesis of the toxin. Most interestingly, Mühlencoert *et al*. (2004) reported that OTA production in *A. ochraceus* NRRL 3174 depended on the initial pH of the culture broth (AM medium; Adye and Mateles, 1964) rather than on a specific combination of carbon and nitrogen source as is the case in *P. nordicum* (Färber and Geisen, 2004). OTA production in *A. ochraceus* NRRL 3174 grown at starting pH 5.0 was induced by shifting the medium to pH 6.5 after 80 h of cultivation. AFLP of cDNA produced from mycelia at different growth stages of cultures grown under the respective starting pH conditions resulted in four fragments which were present only under OTA permissive conditions. Two of the fragments were cloned successfully and sequenced. Pairs of PCR primers were designed from the nucleotide sequences of the 550-bp fragment 3 and the 470-bp fragment 4. PCR primers designed from the longer fragment 3 showed high specificity to

DNA isolated from various strains of *A. ochraceus*, whereas primers specific to fragment 4 revealed two products of different sizes with DNA of *A. ochraceus* (410 bp) and *A. carbonarius* (350 bp), respectively. Although Mühlencoert (2004) demonstrated by reverse transcription quantitative real-time PCR analysis that both primer pairs amplified a specific product only from cDNA present under OTA-permissive conditions, neither fragment could be clearly assigned to a gene potentially involved in the biosynthesis of the toxin. The 3′-end of the sequence of fragment 3 shared limited similarity with some zinc finger proteins in *Mus musculus*. The only similarity to a fungal sequence was with the *Nectria haematococca* kinesin-related protein 1, which is involved in chromosome movement during the mitotic cell cycle.

I. RAPD marker-based primers for *A. carbonarius* and *A. niger*

Fungaro *et al*. (2004) described the development of primers with high specificity to *A. carbonarius*, one of the major OTA producers in coffee (Buecheli and Taniwaki, 2002; Joosten *et al*., 2001; Magnani *et al*., 2005; Taniwaki *et al*., 2003). Fungaro *et al*. (2004) subjected strains of *A. carbonarius*, *A. tubingensis*, and *A. niger* to RAPD analysis. Primer OPX7 revealed informative patterns which resolved the three taxa. An 809-bp fragment differentiated *A. carbonarius* from the other species. Primers designed from the sequence of fragment $OPX7_{809}$ resulted in amplification of an 809-bp PCR product exclusively from *A. carbonarius* DNA (see Table 3.1). However, primers could not distinguish between toxigenic and nontoxigenic isolates of that species.

Based on the same methodology, Sartori *et al*. (2006) developed a pair of primers that lead to amplification of a 372-bp PCR product specifically with isolates of *A. niger* (see Table 3.1). In a multiplex PCR application, the authors combined three primer pairs for the simultaneous detection of *A. ochraceus* (primers OCA-V/R; Schmidt *et al*., 2003), *A. carbonarius* (primers $OPX7F_{809}$/$OPX7R_{809}$; Fungaro *et al*., 2004), and *A. niger* (Sartori *et al*., 2006) in DNA mixtures as well as in artificially and naturally infected green coffee beans using a CTAB extraction protocol originally described for leaf tissue (Doyle and Doyle, 1987). Sartori *et al*. (2006) state that their assay was the first to detect *A. niger* in a species-specific assay. However, reviewing the literature revealed that three different pairs of primers had already been described for that purpose in earlier publications. The earliest was by Kanbe *et al*. (2002) who used the topoisomerase II gene as their sequence source for primer design. Sugita *et al*. (2004) described successful use of a primer pair designed upon the ITS1 sequence of *A. niger* to amplify a PCR product of nonspecified length in a species-specific assay. The former two primer pairs were developed for medical applications, whereas Gonzalez-Salgado *et al*. (2005) and Sartori *et al*. (2006)

had their focus on detection of *A. niger* in vine berries. However, none of the earlier publications gave proof of applicability of the respective assays to detect the target fungus in contaminated sample materials.

J. Primers targeted to genetically defined sequences

Nucleotide sequences of a great variety of fungal genes can be readily retrieved from sequence databases, for example, GenBank, RefSeq, or PDB, opening the opportunity to develop and even test PCR primers *in silico* by searching for sequence similarities with the 130 billion bases (as of April 2006) accessible with the Entrez Nucleotide database and the BLAST tool (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). The hits produced by this *in silico* hybridization already provide a good estimate of the range of taxa covered by the primers under development. Of course, this kind of approach does not prevent from testing the newly developed primers for specificity with genomic DNA isolated from several strains of the taxon under study as well as from closely related taxa. Also testing of the primers with DNA isolated from the matrix should be exercised when the assay will subsequently be applied to contaminated sample materials.

In the following section of this chapter, several PCR assays are reviewed which use the nucleotide sequence of well-defined fungal genes in order to set up primers for detection and identification of potential OTA-producing fungi.

K. rRNA gene-based approaches for the PCR diagnosis of OTA-producing fungi

rRNA genes are multicopy genes, varying in copy number from 50 to 300 between species; however, there is also considerable variation between isolates in certain fungal species (Howlett *et al.*, 1997). Because of the higher copy numbers, assays based on rRNA genes tend to be more sensitive as compared to single copy genes. The presence of two distinct types of ITS2 sequences was described in isolates belonging to the *Gibberella fujikuroi* species complex (O'Donnell *et al.*, 1998). Similar observations were made for *Ascochyta* spp., where Fatehi and Bridge (1998) demonstrated the presence of multiple PCR fragments of identical length but with significant sequence differences. This might pose problems when analyzing the results of a PCR in which one or both primers bind to that part of the rDNA. However, it is unknown to date if this is a general phenomenon or only restricted to the group where it was first described.

The first PCR-based assay for detection of *A. niger* used sequences from the 18S rRNA gene as the target for primer binding (Jimenez *et al.*, 1999). The authors used primers originally published by Melchers *et al.* (1994),

which they applied to analyze the presence of the fungus in artificially contaminated cosmetic and pharmaceutical raw materials and products after an enrichment step. It has to be noted that the primer pair used in the studies was not designed for specific detection of *A. niger* but rather is genus specific for *Aspergillus*. These primers were not included in Table 3.1 of the current chapter since their application to naturally contaminated samples will show a high degree of false positive results from the presence of fungi other than *A. niger*.

Specific variation within the ITS region of the rRNA gene was recently used to set up species-specific detection systems for species in *Aspergillus* section *Nigri*. In an attempt to develop a generic PCR assay for the recognition of the seven most frequently encountered opportunistic pathogens among *Aspergillus* spp., Sugita *et al*. (2004) successfully used ITS sequences as the source for primer development. During the study, species-specific assays were developed for detection of *A. fumigatus*, *A. flavus*, and *A. niger* as the most frequently occurring fungi in human pulmonary infections. In order to optimize the sensitivity of the assays developed, primers were used in a nested PCR with a pair of *Aspergillus*-specific primers for the first round and respective species-specific oligonucleotides as nested primers (see Table 3.1). However, the authors clearly did not intend to detect the latter fungus because of its toxigenic potential, rather they were interested in its role as inducer of pulmonary infections in humans. Aiming at the development of molecular biological tools to facilitate the discrimination of species within the *Aspergillus* section *Nigri* (black aspergilli), Gonzalez-Salgado *et al*. (2005) combined a generic forward primer hybridizing in the ITS1 region of the rRNA gene (ITS1; White *et al*., 1990) with various reverse primers to design PCR assays detecting and identifying *A. niger*, *A. japonicus*, *A. heteromorphus*, and *A. ellipticus*, respectively. Testing of the primers with a wide range of black aspergilli from different hosts and regions revealed specific reaction with strains of the respective species. However, the assay could not distinguish strains of *A. niger* from *A. tubingensis*, a feature which would be a great advantage because, according to the authors, only the former species produces OTA but is morphologically indiscernible from the latter. Digestion of the resulting PCR product with the restriction endonuclease *Rsa*I, however, revealed that the 420-bp *A. niger* fragment was restricted into two portions of 345 and 76 bp, while the *A. tubingensis* product remained uncut.

Two ITS-based assays specific for *A. carbonarius* and *A. ochraceus*, respectively, were developed by Patino *et al*. (2005). Both species are considered the most important producers of OTA in coffee (Taniwaki *et al*., 2003) and grapes (Cabenes *et al*., 2002) in warmer climates. Primer pairs were designed to bind to the ITS1 and ITS2 regions flanking the 5.8S rRNA gene in fungal genomes. The authors selected those parts of the

aligned sequences for primer design that displayed the maximum degree of bias between *A. carbonarius* and *A. ochraceus*. Although this strategy promises a high degree of specificity of the primers selected, it has the disadvantage of producing products of equal length in both PCR assays, which renders impossible their use in a multiplex assay for both species. However, multiplexing several primer pairs in one assay is highly recommended if PCR is going to be used as a screening tool in quality control because the number of tests run and the time needed for their performance must be kept as low as possible. The assays developed proved to be highly specific when tested with DNA of a wide range of nontarget organisms. However, no test was run using DNA isolated from noninfected matrix material, for example, coffee beans and grapes. The detection limit of 1–10 pg of purified DNA per assay demonstrated the high sensitivity of the method developed.

L. Calmodulin gene-targeted primers for detection of *A. carbonarius* and *A. japonicus*

Calmodulin is a CALcium MODULated proteIN (Cheung, 1970) abundantly present in the cytoplasm of all eukaryotic cells. Its structure has been highly conserved during evolution. It confers calcium sensitivity on a number of different enzymes including Ca^{2+}/calmodulin-dependent protein kinases involved in the regulation of the mycotoxin, aflatoxin, in *A. parasiticus* (Jayashree *et al.*, 2000). Similar to rRNA genes, *β*-tubulin (*ben*A) or elongation factor 1α (EF-1α), the calmodulin (*cmd*A) gene provides highly conserved as well as variable sequence regions suitable for development of species-specific PCR primers. The positions of the variable regions within the gene are highly stable in eukaryotes. Based on the alignment of a partial sequence of representative strains of *A. carbonarius*, *A. japonicus*, and *A. aculeatus*, Perrone *et al.* (2004) detected a high degree of homology between strains assigned to *A. carbonarius* (99.98%) and *A. japonicus*/*A. aculeatus* (99.40%), respectively. This fact provides further evidence for identity of the latter two species. Apart from highly conserved regions, the authors identified three variable regions suitable for design of specific PCR primers. Sequences of *cmd*A within the *A. niger* group isolates showed only 89% homology between isolates and no sequences were found which distinguished all *A. niger* group isolates from other OTA producers analyzed during the study. Primer pairs designed for detection of *A. carbonarius* (CARBO1/CARBO2, see Table 3.1) and for *A. japonicus*/*A. aculeatus* were found to be highly specific for their respective target when tested with DNA from closely related *Aspergillus* spp. of sections *Nigri* and *Circumdati*. The detection of *A. carbonarius* DNA was demonstrated to be highly sensitive in an optimized PCR assay with a detection limit of 12.5 pg of DNA per reaction.

The assay was demonstrated to be useful in screening vast numbers of isolates of black aspergilli from grapes in order to evaluate the toxigenic potential connected with this commodity. Based on the same gene, Mulè *et al*. (2006) recently published a quantitative real-time PCR using the TaqMan chemistry for quantification of *A. carbonarius*. Primers were chosen to amplify a shorter fragment of the gene as compared to Perrone *et al*. (2004) and a specific probe was designed to detect the PCR product in a fluorescence assay. Primers and probe were demonstrated to be highly specific for detection of *A. carbonarius* DNA and the system was applied in the detection and quantification of the fungus in samples of grapes. Quantification of the *A. carbonarius* DNA content and the concentrations of OTA were shown to be highly correlated ($R^2 = 0.918$) in 15 grape samples analyzed. Therefore, the system might prove to be useful for future quality control and HACCP testing during growth of grapes and production of wine and grape products.

M. Primers for OTA biosynthetic pathway genes

Following the biosynthetic pathway proposed by Huff and Hamilton (1979), the presence of various enzymes catalyzing key reactions in the formation of OTA can be anticipated (see Niessen *et al*., 2005). A polyketide synthase can be postulated to link together the building block C2-bodies of the isocumarine portion of the toxin. Also some kind of cyclase will be necessary to build a closed ring system from a polyketide chain. An enzymatic activity can be anticipated to be necessary for chlorination of the isocumarine body to result in OTα, the actual precursor of OTA, and finally an enzyme with peptide synthase activity must be postulated to link together OTα and phenylalanine to result in OTA as the toxic end product. As with several other mycotoxins, for example, trichothecenes, aflatoxins, and fumonisins, it might further be anticipated that the genes coding for these enzymes as well as genes coding for possible regulators and transporters will form a cluster of genes in close physical vicinity within the fungal genome (for review see Keller and Hohn, 1997).

Based on the aforementioned assumptions, Geisen *et al*. (2004) applied a reverse genetical approach to detect and characterize a polyketide synthase gene from OTA-producing *Penicillium* spp. They used generic primers LC3 and LC5 to amplify a 750-bp portion of a fungal polyketide synthase of the MSAS type (Bingle *et al*., 1999) from *P. nordicum* BFE487. The primers gave rise to a 750-bp fragment with DNA isolated from *P. nordicum* strains but not with the closely related *P. verrucosum*. The fragment was sequenced (GenBank accession no. AY196315) and compared to published sequences. It displayed homology to the polyketide synthase gene *pksL2* from *A. flavus* (Feng and Leonard, 1998). After transferring the nucleotide sequence (the fragment contained one continuous

open reading frame) to an amino acid sequence, an even more stringent homology with different fungal polyketide synthases over the whole fragment length was observed. A nested specific primer pair (otapks 1 and otapks 2, see Table 3.1) was deduced from the identified nucleotide sequence and tested for specificity with DNA isolated from several food borne fungi as well as closely related *P. verrucosum* strains. The primers yielded a 500-bp PCR fragment exclusively with DNA from *P. nordicum* strains, whereas all other analyzed food-related fungi yielded negative results. TaqMan® quantitative real-time PCR with a specific primer pair and a fluorogenic probe (see Table 3.1) was applied to monitor the growth kinetics of *P. nordicum* BFE487 in artificially inoculated wheat grains (Geisen *et al.*, 2004). The results of that experiment showed congruence between growth kinetics established by counts of colony forming units (cfu) and quantitative real-time PCR (qRT-PCR). The same primers were applied by the authors to quantify the level of expression of the *otapks*PN gene during growth of *P. nordicum* BFE487 in artificially inoculated wheat (Geisen *et al.*, 2004). Data were compared to concentrations of OTA found in the respective samples. It was demonstrated that OTA concentrations became detectable at day 4 of the experiment, when expression of the *otapks*PN gene was first detected by qPCR with cDNA. The levels of gene expression declined after day 8, when OTA concentrations were found to remain at a constantly high level. Using the qRT-PCR system described above, Geisen (2004) demonstrated that environmental parameters prevailing under food production conditions had a substantial influence on the expression of the *otapks*PN gene in *P. nordicum* and concluded that expression analysis of OTA biosynthetic key genes might be useful as a critical control point in HACCP concepts for the food industry.

In order to isolate a genomic clone containing the complete *otapks*PN gene and additional adjacent genomic regions, a lambda phage genomic DNA gene bank was constructed (Karolewiez and Geisen, 2005). It was anticipated that the genes of the ochratoxin A biosynthetic pathway display similarly clustered organization as can be found in other secondary metabolite biosynthetic pathway genes, for example, aflatoxins (Yu *et al.*, 2004). The 500-bp PCR product generated with primers otapks 1/otapks 2 was digoxigenin-labeled and used as a probe to screen the library. Five different phage clones were found to yield a hybridization signal with the probe, indicating that the *otapks*PN gene or at least a part of it was present in the fungal insert DNA. Excision of the inserted fragment from the multiple cloning site revealed a 10-kbp genomic DNA fragment of *P. nordicum* BFE487, which hybridized with the *otapks*PN gene probe. The fragment was completely sequenced. Karolewiez and Geisen (2005) detected four genes located on the fragment: *asph*PN (alkaline serine protease homologue), *asp*PN (alkaline serine protease), *nps*PN (nonribosomal peptide synthase), and *otapks*PN

(ochratoxin polyketide synthase). Two of these genes had homologies to genes which are to be anticipated to be present in the ochratoxin biosynthetic pathway as described above. The involvement of the *otapks*PN gene in the biosynthesis of OTA was demonstrated by knocking the gene out. The resulting transformant was unable to produce the toxin under conditions under which the parent wild type produced the metabolite.

PCR primers were designed from each of the three open reading frames present on the 10-kbp genomic fragment. The primers designed from the sequence of a putative *nps*PN gene (GeneBank accession no. AY534879, coding for a non ribosomal peptide synthase) yielded a 750-bp PCR product from strains of both *P. nordicum* and *P. verrucosum*, whereas the primer pair derived from the *otapks*PN gene (GeneBank accession no. AY199315) yielded a 500-bp product exclusively with DNA isolated from strains of *P. nordicum* (Bogs *et al.*, 2006). The latter two primer pairs were useful in differentiating *P. verrucosum* from *P. nordicum* by analyzing isolates with both PCR assays. The specificity of the designed primers was fully verified when target DNA isolated from various *Penicillium* spp. and other fungal contaminants typically occurring in food and feed-related sources were used as template in a PCR with *otapks*PN- and the *otanps*PN-specific primer pairs. When the OTA-producing capabilities of *P. nordicum* and *P. verrucosum* strains isolated from cured meat and from meat production environments were compared to the results obtained with the *otapks*PN- and *otanps*PN-specific primers, the latter PCR displayed positive reaction exclusively with strains capable of producing detectable amounts of OTA in pure culture (HPLC analysis).

It must be noted here that none of the sequences described for the OTA biosynthetic pathway genes in *P. nordicum* have homologies with genes found in OTA-producing *Aspergillus* spp. In particular, no homologies were found between the sequences of the *otapks*PN gene and a polyketide synthase potentially involved in OTA biosynthesis in *A. ochraceus* as demonstrated by knockout experiments (O'Callaghan *et al.*, 2003). Also, neither of the gene-specific primers described above for *P. nordicum* showed any cross-reactivity when tested with *A. ochraceus* or other OTA-producing *Aspergillus* spp., even under low stringency conditions (R. Geisen, personal communication). These results indicate that OTA biosynthesis in *A. ochraceus*, but also in other OTA-producing *Aspergillus* spp., might follow a different route as compared to *Penicillium*.

During their studies on OTA biosynthetic pathway genes, O'Callaghan *et al.* (2003) did not only clone a polyketide synthase from *A. ochraceus* but cloned and sequenced several more differentially expressed cDNA fragments of yet unassigned origin from typical producers of OTA. Based on that work, University College Cork (Ireland) filed worldwide (WO 2004/072224 A2) as well as European (EP 1 592 705 A2)

patent applications based on an Irish priority application dated 12.03.2003 (IE 20030095). Claims cover the use of those sequences for the purpose of detecting OTA producers in pure cultures and sample materials as well as the use of those sequences for primer walking to uncover sequences beyond those already cloned.

Finally, Dao *et al*. (2005) used a strategy similar to that described by Geisen (2004) to sequence the ketosynthase domain of a polyketide synthase gene in *A. ochraceus* NRRL 3174. According to the authors, part of their sequence (not accessible in GenBank at the time of writing this chapter) was identical with the sequence published by O'Callaghan *et al*. (2003) (accession no. AY272043). From the sequences obtained during their study, Dao *et al*. (2005) designed a PCR assay which specifically detected *A. ochraceus* (primers AoOTA-L/AoOTA-R, see Table 3.1) and another one for detection of fungi potentially able to produce OTA and the mycotoxin, citrinin, in a group-specific manner (primers AoLC35–12L/AoLC35–12R, see Table 3.1). The spectrum of species detected by the latter assay comprised *A. carbonarius*, *A. melleus*, *A. ochraceus*, *P. citrinum*, and *P. verrucosum*, but also *Monascus ruber* as a typical producer of citrinin in food and feed, among other *Monascus* spp. (Wang *et al*., 2005). Detecting producers of both toxins may be an advantage because they can be found to be co-occurring in contaminated cereals (Vrabcheva *et al*., 2000). Primer pairs designed from the sequences of two unassigned DNA fragments cloned from *A. ochraceus* (presumably strain NRRL 3174 or M333) were patented by Evialis, a French-based company specialized in animal nutrition products. Patent application EP 1 329 521 A1 from 20.01.2003 was based on a French priority application (FR 0200682) of 21.01.2002 (Librihi, 2003). The claim covers the use of unassigned DNA sequences cloned from *A. ochraceus* and from *P. viridicatum* for the detection of fungi producing OTA or citrinin. Nucleotide–nucleotide BLAST search against the EMBL nucleotide data base revealed weak similarities of the sequences with fungal polyketide synthases. The specificity of the primer pairs S3/S4 and S5/S6 were identical with the primers described by Dao *et al*. (2005), with the former primers being specific for *A. ochraceus* and the latter group specific for producers of OTA and citrinin. However, primers described in the two publications are neither identical nor were the primers of Dao *et al*. (2005) based on the patented sequences, although the senior author of that publication is listed as inventor in EP 1 329 521 A1 (Librihi, 2003).

N. Detection of fumonisin producers

Fumonisins are a group of mycotoxins produced by species within the *G. fujikuroi* complex. The major producing species are *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, and *F. nygamai* (Bennett and Klich, 2003).

They affect animals by interfering with their sphingolipid metabolism (Merrill *et al*., 2001). Besides various animal diseases, they are suspected to be responsible for certain forms of human esophageal cancer observed in Transkai (South Africa), China, and Northeast Italy (Peraica *et al*., 1999; Sydenham *et al*., 1991). Moreover, fumonisin B_1 has been associated with neural tube defects in experimental animals and may therefore be involved in cases of spina bifida in humans (Hendricks, 1999). The toxin has been assigned a 2B carcinogen status (probably carcinogenic to humans) by the International Research Agency for Cancer (IARC, 1993; Rheeder *et al*., 2002). As in other mycotoxin producers, genes involved in the biosynthesis and regulation of fumonisins are organized in clusters within the genome. Waalwijk *et al*. (2004a) studied the fumonisin gene cluster of *F. proliferatum* and found 19 genes to be involved in biosynthesis and regulation of the toxin.

F. proliferatum (mating population D of *G. fujikuroi* Leslie, 1995) was isolated from rice, corn, and other cereals in tropical and subtropical countries (Samson *et al*., 2004). Beck and Barnett (2003) filed a patent to the US Patent and Trademark Office, in which primer pairs for the specific identification of *F. proliferatum* and *F. verticillioides* as the major fumonisin producers in corn were described. The system was based on the sequence of the small subunit of the mitochondrial rRNA gene of the fungus. No follow-up studies have been published in which the system was used for detection of *F. proliferatum* or *F. verticillioides* in contaminated sample materials. Using a partial sequence of the calmodulin gene from *F. proliferatum*, *F. subglutinans*, and *F. verticillioides*, Mulé *et al*. (2004) designed specific primer pairs for the identification of the three species. The authors found their primers to be highly specific for the sensitive detection of the respective species in a screening of DNA from 150 strains, mainly isolated from maize in Europe and the United States. However, application of the primers to detection of the target species in sample materials was not reported. RAPD markers were used as the sequence source for development of PCR-based detection systems for *F. verticillioides* and *F. subglutinans*, both of which species produce fumonisins besides various other mycotoxins. Möller *et al*. (1999) set up a multiplex PCR in which primers for the detection of *F. subglutinans* and *F. verticillioides* were combined to analyze both species in contaminated maize samples. Decamer primers were applied in RAPD analysis of species within the *G. fujikuroi* complex. A 600-bp amplification fragment was found to be specifically produced with primer UBC18 and the sequence of the fragment was used as source for the design of a species-specific primer pair for *F. verticillioides* (assigned to *F. moniliforme* by the authors). PCR did not amplify a specific fragment with DNA from *F. nygamai* despite its high degree of similarity with the UBC18 RAPD fragment of both species. Zheng and Ploetz (2002) developed another

RAPD-based PCR which they applied in the screening of *F. subglutinans* isolates from the mango plant, *Mangifera indica*, where the fungus causes a destructive disease called mango malformation. Primers derived from an RAPD fragment generated with the arbitrary decamer primer OPZ5 were specific for the mango malformation isolates, which were not picked up by the primers published by Möller *et al.* (1999). Testing various isolates of different species within the *G. fujikuroi* complex showed that the system was specific for *F. subglutinans* and *F. nygamai*. DNA from the latter species resulted in amplification of a significantly shorter product with the primer pair developed by Zheng and Ploetz (2002). No application of the primers in contaminated sample material was demonstrated. Genomic markers of unknown function were used by Murillo *et al.* (1998) who used the sequence of cloned shotgun fragments of genomic *F. verticillioides* DNA to set up a primer pair which lead to amplification of a 1600-bp PCR product with DNA of that species. The assay was applied for identification of pure fungal cultures grown from infected maize tissues but was not applied directly for detection of the fungus in sample material. Möller *et al.* (1999) tested the primers developed by Murillo *et al.* (1998) for cross-reaction with DNA isolated from representatives of other species from the *G. fujikuroi* complex and found that the expected 1600-bp product was also amplified with DNA from isolates of mating populations B, C, D, E, F, and also with *F. nygamai* and *F. oxysporum* DNA. It would therefore be of advantage to apply the primers described in Murillo *et al.* (1998) in screening for potential fumonisin producers rather than to screen for *F. verticillioides* alone.

Attempts were made to use PCR in the analysis of the mycotoxigenic potential of fumonisin producers because it is well established that only a certain percentage of isolates are able to produce the toxin *in vitro* and *in planta*. Based on sequence differences within the intergenic region (IGS) of the rRNA gene between fumonisin-producing and nonproducing isolates of *F. verticillioides*, Patino *et al.* (2004) set up a PCR system which resulted in amplification of a fragment only with DNA from toxigenic isolates among 54 strains of *F. verticillioides* tested from different geographical regions and hosts. Since rRNA genes are multicopy genes, the assay developed with the primers was highly sensitive. Application of the primers for detection of fumonisin producing *F. verticillioides* in sample material was not demonstrated.

Recently, two PCR-based assays were developed for the selective identification of fumonisin-producing isolates of *F. verticillioides* which used sequence information from genes involved in the biosynthesis of the toxin. Gonzalez-Jaen *et al.* (2004) analyzed the occurrence of the genes *fum1* (=*fum5*), *fum6*, and *fum8* in species within the *G. fujikuroi* complex by Southern blot hybridization and found that the genes were only present in *F. verticillioides*, *F. proliferatum*, *F. fujikuroi*, and *F. nygamai*, which represent

the principal producers of fumonisins. The authors used the alignment of a partial genomic sequence of the *fum1* genes and found similar phylogenetic relations among the four species as obtained with genes not related to fumonisin biosynthesis. Primers were derived from the sequence representing the β-ketoacyl reductase domain within the *fum1* gene and tested for specificity with DNA of *Fusarium* species and *Fusarium*-related species including fumonisin-producing and nonproducing isolates of *F. verticillioides*. Primers appeared to be highly specific for *F. verticillioides* isolates which produced fumonisin *in vitro*, and it was assumed that the nonproducing isolates must have lost the *fum1* gene or at least the part of it where PCR primers hybridize in fumonisin producers. No application of the primers for detection of fumonisin producers in sample materials was published. Recently, also Sanchez-Rangel *et al.* (2005) set up a PCR in which primers hybridized to the β-ketoacyl reductase domain of the *fum1* gene in *F. verticillioides*. Similar to the results obtained by Gonzalez-Jaen *et al.* (2004), they were able to distinguish between fumonisin-producing isolates and nonproducing isolates of *F. verticillioides* with the primer pair designed. No amplification of a 354-bp PCR product occurred with DNA from other *Fusarium* spp. tested, even if closely related to the target species. The correlation of PCR results to *in vitro* fumonisin production of *F. verticillioides* revealed a number of cases in which a *fum1* gene was detected but toxin concentrations produced were negligible or very low. A similar effect was not described by Gonzalez-Jaen *et al.* (2004); however, the number of isolates tested by these authors was rather small compared to what Sanchez-Rangel *et al.* (2005) published. The latter authors speculated that the principal ability of a *F. verticillioides* isolate to produce fumonisin will depend on the presence or absence of the *fum1* gene but there might be other factors which are responsible for the concentrations finally produced. These concentrations might be too low to be detected by the analytical system they used.

Besides PCR-based detection of single species of toxicologically relevant taxa within species, primers and assays were developed for the identification and detection of groups of species sharing fumonisin production as a common feature. Based on sequences of the *fum1* gene, Bluhm *et al.* (2002) published a pair of primers with specificity for the identification of *F. proliferatum* together with *F. verticillioides* which they found to be useful for detection of these fungi in artificially contaminated cornmeal. The primers were applied in a multiplex PCR assay in which potential producers of fumonisins (*fum1* gene) were detected together with potential trichothecene producers (*tri6* gene). In this assay, also a primer pair detecting *Fusarium* spp. at the genus level based on rRNA sequences (ITS) was integrated. No attempts were made, however, to apply the system in naturally contaminated sample material. Grimm and Geisen (1998) compared nucleotide sequences of the ITS1 region

within the rRNA genes of typical fumonisin-producing *Fusarium* species with those of species which do not produce that toxin. They found a high degree of homology but also some differences between the two groups of species analyzed. A pair of PCR primers and a biotinylated probe were designed which made use of those differences to specifically amplify a 108-bp fragment from typical fumonisin producers. For detection of the PCR fragment, Grimm and Geisen (1998) used a microplate-based method, enzyme-linked oligosorbent assay (ELOSA), in which a specific biotinylated capture probe was fixed to a microwell plate previously coated with streptavidin. PCR was done with a mastermix containing DIG-11-dUTP which was incorporated into the PCR product instead of dTTP to label it with digoxigenin. Labeled PCR product was then trapped specifically by binding to the immobilized probe and was subsequently detected by binding of a peroxidase-conjugated anti-DIG antibody. Enzyme activity leads to measurable changes in OD at 405 nm when a specific DIG-labeled PCR product had been amplified with the primers used and was bound to the specific capture probe. With this double specificity assay, *F. verticillioides*, *F. proliferatum*, *F. nygamai*, and *F. napiforme* were detected, whereas other *Fusarium* spp. and fungi from other genera tested did not give a signal. The assay was not used to quantify the biomass of fumonisin producers and it was not applied to detect target organisms in sample material.

O. Detection of patulin producers

Patulin is a tetraketide mycotoxin which has been found to be produced by a variety of different fungi, most of which are referable to the ascomycete genera *Byssochlamys* and *Eupenicillium*. Also *Aspergillus* species (*A. clavatus*, *A. giganteus*, and *A. terreus*) have been found to be effective producers of the toxin. Frisvad and Filtenborg (1989) revised the taxonomy of terverticillate penicillia based on secondary metabolite profiles and found that two ecological groups of fungal species produce patulin: *P. carneum*, *Paecilomyces varioti*, and *P. glandicola* are producers mainly found in silage, *P. coprobium*, *P. glandicola*, *P. vulpinum*, *P. clavigerum*, and *P. concentricum* are producers among the coprophilic fungi. In foods, however, *P. expansum* and *P. griseofulvum* are the major producers of patulin with apples and unfermented apple juice being the main source of the toxin in human consumption. To prevent consumers from patulin contamination, the Joint Food and Agriculture Organisation-World Health Organisation Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for the compound of 0.4 mg/kg bw per day (WHO, 1995). Patulin is regulated in the European Union at levels of 50, 25, and 10 µg/kg, respectively, in fruit juices and fruit nectar, solid apple products, and apple-based products for

infants and young children [Regulation (EC) No. 466/2001, amended by Regulation (EC) No. 455/2004].

The *idh* gene codes for an isoepoxidon dehydrogenase which catalyzes conversion of isoepoxydon to phylostine as a specific step in the biosynthesis of patulin (Sekiguchi and Gaucher, 1979). The sequence of a complete *idh* gene was published by Fedeshko (1992) and partial sequences are available on GenBank from *B. nivea*, *P. griseofulvum*, and *P. expansum*. Using the sequence of Fedeshko (1992), Paterson *et al.* (2000) set up a gene-specific PCR for producers of patulin. Testing of various isolates of *P. expansum* revealed the presence of the *idh* gene and all isolates which produced patulin *in vitro*. Few of the *P. brevicompactum* isolates tested during the study obviously had the *idh* gene present, but these did not produce patulin under *in vitro* conditions. The authors demonstrated the usefulness of their primer pair to detect the gene in contaminated twigs, bark, soil, and fallen apples but failed to have a positive reaction in healthy apples picked just prior to analysis. No PCR product and patulin production was observed in several other *Penicillium* species tested. In a follow-up study, Paterson (2004) demonstrated that the *idh* gene is a quite widespread feature in the genus *Penicillium* but they also showed the gene to be present in *Aspergillus*, *Paecillomyces*, and *Byssochlamys*. The detection of the gene was in most cases improved if DNA extracts were diluted prior to PCR in order to reduce inhibitor effects and to enable proper product formation. Only recently, Paterson (2006) introduced results from a study in which the *idh* gene-specific primers were applied to the analysis of orchard soil as critical control point in an HACCP concept for the prevention of patulin contamination of apple products.

Based on the nucleotide sequence of the polygalacturonase gene of *P. expansum*, Marek *et al.* (2003) designed a pair of primers which lead to amplification of a 404-bp PCR product specifically with DNA isolated from strains of this fungus. No product was amplified from various *Penicillium* spp. tested. The assay was sensitive to detecting DNA a concentration equivalent to 25 cfu of *P. expansum*. However, the detection of the fungus in contaminated food was not demonstrated by the authors.

P. carneum and *Pae. variotii* are producers of patulin which frequently occur in silage and may therefore be dangerous as a contaminant of animal feed. The former species was taxonomically separated from *P. roqueforti* because of genotypical and chemotypical differences, with patulin produced by the new species *P. carneum* and *P. paneum* and not by *P. roqueforti*. The three species were not tested by Patterson (2004) for presence of an *idh* gene but it can be anticipated to be present in both new species. Pedersen *et al.* (1997) designed primers binding to the ITS region of isolates of both *P. roqueforti* and *P. carneum* so that both species were detected in complex food samples (cereals, cheese). The problem of detection a patulin nonproducer together with a patulin producer with the PCR

described was reduced by the fact that *P. roqueforti* produces other mycotoxins, for example, PR toxin and mycophenolic acid, so this species is also a hazardous fungus in foods were it can produce its toxins. A PCR-based detection system for *A. terreus* as another producer of patulin was published by Haugland and Vesper (2000) as a patent application. Primers were designed to bind to the ITS region of the rRNA gene of the fungus and were applied in quantitative real-time PCR using the TaqMan system with a species-specific probe.

II. CONCLUSIONS AND FUTURE PERSPECTIVES

Reviewing the literature on PCR-based diagnosis of mycotoxin-producing fungi has shown that a tremendous toolbox of oligonucleotide primers has been developed during the last decade, which allows amplification of an appropriate DNA fragment from all of the relevant toxigenic fungi, and from many of the less relevant species, too. In many publications, specificity has been tested with a more or less extensive set of fungal species. However, some of the publications reviewed do not offer much detail regarding the spectrum of species picked up by a primer pair. It should therefore become standard procedure in future developments to test the cross-reactivity of a primer pair at least with the most closely related species. Building up a new assay should start with an *in silico* analysis of possible cross-reactions with the primers designed. This first check should give a gross overview over the spectrum of species which are to be tested for cross-reaction. Testing of a wide taxonomical spectrum becomes even more demanding if the target sequence for primer design was taken from genes coding for universal proteins, for example, TEF1α, β-tubulin, calmodulin, or genes coding for rRNA. The spectrum of species tested should at least take in account the fungi potentially occurring in sample materials which might be planned to be analyzed with the future assay and the DNA isolated from the sample itself if food or feed material is involved. Various PCR-based assays developed during the last decade used genes as sequence sources for primer design which were either present only in the target species but unrelated to mycotoxin biosynthesis, for example, gaoA in *F. graminearum* (Niessen and Vogel, 1997), or which were involved in the biosynthesis and regulation of the mycotoxins produced by the target species or group of species. Examples for assays developed along that line may be found for all of the mycotoxins of major concern, except for producers of zearalenone. Biosynthesis of that toxin is currently under investigation and various genes involved have recently been characterized (Gaffoor and Trail, 2006; Lysoe *et al.*, 2006). The development of PCR-based detection systems based on those genes can therefore be anticipated in due course.

One of the major motivations for the development of PCR-based detection systems in many publications is the prospect of using the analysis to forecast the presence and concentrations of mycotoxins in sample material. One might therefore anticipate that assays based on mycotoxin biosynthetic genes might better fit that purpose as systems based on genes unrelated to their biosynthesis. However, in only a few cases has correlation between the presence of a PCR signal or its intensity and the presence or concentrations of certain mycotoxins been analyzed and in even fewer cases have positive correlations been established. At that point, the general question has to be raised, if PCR with genomic target DNA as a template is the technology of choice to estimate mycotoxin concentrations in sample materials. Given the fact that biosynthesis is a highly complex process with poorly understood regulation at the transcriptional level (other mechanisms as well?) and that it is highly influenced by environmental factors, it seems unlikely to find a highly positive correlation between the amplification signal and mycotoxin concentrations. A study of the literature reviewed here shows that in some cases such a correlation has been established with quantitative real-time PCR based on mycotoxin biosynthesis genes (Sarlin *et al.*, 2006; Schnerr *et al.*, 2002). However, similarly good correlations between results of quantitative real-time PCR and mycotoxin concentrations were found when primers were targeted to anonymous sequences (Leisova *et al.*, 2006; Waalwijk *et al.*, 2004b) or to sequences of a housekeeping gene (Mulè *et al.*, 2006). Other authors found very poor correlation between the parameters (Schmidt *et al.*, 2004b). Overall, this means that the prediction of mycotoxin concentrations using a PCR-based system is unlikely to work in a way that would allow for this technique to replace analysis of mycotoxins unless assays are developed which are based on the expression of genes involved in mycotoxin biosynthesis, that is, systems using cDNA as the target for amplification. Such a system has been developed by R. Geisens group at the Federal Institute for Nutrition in Karlsruhe, Germany. The system makes use of a microarray to which cDNA of genes involved in the production of OTA can be immobilized and visualized by fluorogenic detection probes. Good correlations of signals were found to fungal biomass but also to OTA produced under various environmental conditions.

With the currently available systems for PCR-based detection and identification, however, qualitative information about the presence or absence of a certain fungus can be obtained and this should be used to advantage in food and feed quality control because the technology has the power to provide insights into the mycotoxigenic potential of analyzed samples. This information can then be used in order to decide whether samples should proceed down the process of production or should be retained for further analysis of mycotoxins. PCR-based multiplex systems

could be used to determine which mycotoxins to analyze, as recently introduced by Kristensen *et al*. (2006a,b) who used microarray technology and the SnaPshot technology to detect and differentiate 16 different mycotoxin-producing *Fusarium* spp. in multiplex assays. The latter platform offers the possibility of detecting very high numbers of different fungal species and groups of species in a short time and with limited work.

The systems described above might show one possible way in which molecular detection of mycotoxigenic fungi may be utilized in order to optimize food and feed production processes for minimized risk of mycotoxin production. In analogy to computer aided manufacturing (CAM), this would be addressed as genome-aided processing (GAP) in food production. Still, enormous work has to be done in order to accomplish that state. However, keeping in mind that most of the developments described in this chapter were done in very recent years, GAP-based systems may yet become reality and be applied for the welfare of consumers.

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