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Development of an FgMito assay: A highly sensitive mitochondrial based qPCR assay for quantification of *Fusarium graminearum* sensu stricto

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ABSTRACT

An ascomycete fungus, *Fusarium graminearum* sensu stricto (s.s.), is the major cause of *Fusarium* head blight (FHB), a devastating disease of cereals worldwide. The fungus contaminates crops with mycotoxins, which pose a serious threat to food and feed safety. In this study, we developed a highly sensitive mitochondrial based qPCR assay (FgMito qPCR) for quantification of *F. graminearum* s.s. To ensure high sensitivity of the assay, primers and a Minor-groove binding (MGB) probe were designed based on multi-copy mitochondrial DNA. The FgMito assay was successfully validated against a range of geographically diverse *F. graminearum* s.s. strains to ensure uniformity of the assay at an intraspecific level, as well as with other fungal species to ensure specificity. The assay was further evaluated in terms of efficiency and sensitivity against a test panel of different *F. graminearum* s.s. strains with various levels of pure fungal DNA and in the presence of wheat background DNA. The results showed a high efficiency of the assay developed, ranging from 93% to 101% with r^2-values of >0.99. We further showed that three low concentrations of fungal template 2 pg, 0.6 pg and 0.2 pg could be reliably quantified in the presence of wheat background DNA. The FgMito assay was used to quantify *F. graminearum* s.s. DNA on 65 field samples from a range of hosts with defined levels of trichothecenes. We revealed a significant positive correlation between fungal DNA quantity and the sum of trichothecenes. Lastly, we showed a higher sensitivity of the FgMito assay than the nuclear based qPCR assay for *F. graminearum* s.s. by comparing Ct-values from both assays.

1. Introduction

An ascomycete fungus, *Fusarium graminearum* sensu stricto (s.s.), is the major cause of *Fusarium* head blight (FHB), a devastating disease of small grain cereals worldwide. Besides reducing yield, the fungus contaminates crops with mycotoxins, such as trichothecenes and zearalenone, which pose a serious threat to food and feed safety (Desjardins, 2006). *F. graminearum* s.s. belongs to the monophyletic fungal complex referred to as *F. graminearum* species complex (FGSC), which consists of at least 16 phylogenetic species (Sarver et al., 2011). Surveys from diverse geographic areas such as Asia, Africa, America, Europe and Oceania revealed panglobal distribution of *F. graminearum* s.s., while the other phylogenetic species within FGSC appeared to be endemic. Prevention and control of foodborne diseases are international public health goals. For this reason, numerous diagnostic tests have been developed for quantification of foodborne viruses, bacteria and fungi (Rodriguez-Lázaro, 2013). Unlike bacteria and yeasts, fungi do not grow as single cells, but as hyphal filaments that cannot be quantified reliably by the usual enumeration techniques (Pitt, 1984; Taniwaki, 2006). Other methods, such as biochemical (Taniwaki, 2006) and immunological methods (Narayanasamy, 2011) are not quantitative and lack specificity. Barcoding approaches coupled with next-generation sequencing (NGS) are effective for detecting and characterizing fungi, but they are relatively expensive, require complex analyses and are not quantitative (Liu et al., 2012). The application of qPCR (quantitative polymerase chain reaction) has been found to be the most promising alternative in fungal diagnostics and has been promoted as a standard method in many food aspects, e.g. bacteria or quantification of genetically modified organisms (Rodriguez-Lázaro, 2013). The advantages of qPCR over other diagnostic techniques are its high specificity, increased sensitivity and rapid turnaround time. At least

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Table 1
List of fungal strains used for specificity testing of FgMito qPCR assay.

Fungal species	Strain code	Geographical origin, host/habitat of origin	FgMito qPCR assay
F. graminearum	CBS 139513	Argentina, Tandil, barley	+
	33–30		+
	87–7		+
	6–2	Argentina, Azul, barley	+
	29–5		+
	CBS 139514	Argentina, Tapalqué, barley	+
	23–4	Argentina, T. Lauquen, barley	+
	114–2	Argentina, Loberia, barley	+
	119–12	Argentina, San Cayetano, unknown	+
	35–4	Argentina, La Madrid, barley	+
	CBS 128539	Belgium, wheat	+
	MUCL 53455	Belgium, corn	+
	MUCL 53451		+
	CBS 110263	Iran, maize	+
	CBS 110271	Netherlands, leatherleaf fern	+
	CBS 389.62	Netherlands, wheat	+
	CBS 138561	Poland, Tywęzy, wheat	+
	CBS 138562		+
	CBS 138563	Poland, wheat	+
	CBS 119800	South Africa, corn	+
	CBS 119799	South Africa, wheat	+
	CBS 123688	Sweden, oats	+
	CBS 110266	USA, Kansas, wheat	+
	CBS 119173	USA, Louisiana, wheat	+
	CBS 110265	USA, Ohio, wheat	+
	CBS 110264	USA, Michigan, maize	+
	CBS 110261	USA, Ohio, maize	+
	CBS 182.30	Unknown	+
	CBS 104.09		+
F. aethiopicum	CBS 123667	Ethiopia, Gugsa womberma district, wheat	−
F. acaciae-mearnsii	CBS 110253	South Africa, *Acaciae mearnsii* (Leguminosae-mimosoideae)	−
	CBS 123662	Australia, soil	−
F. asiaticum	CBS 110258	China, Shanghai Province, wheat	−
	CBS 110257	Japan, barley	−
	CBS 110256	Barley	−
F. austroamericanum	CBS 110246	Brazil, maize	−
	CBS 110245	Venezuela, herbaceous vine	−
	CBS 110244	Brazil, polypore	−
F. boothii	CBS 110270	South Africa	−
	CBS 110250	South Africa, corn	−
F. brasilicum	CBS 119180	Brazil, oats	−
F. cortaderiae	CBS 123655	New Zealand, corn	−
F. gerlachii	CBS 123666	USA, North Dakota, wheat	−
F. louisianense	CBS 127524	USA, Louisiana, wheat	−
F. meridionale	CBS 110247	New Caledonia, orange twig	−
	CBS 110248	Nepal, maize	−
	CBS 110249	South Africa, soil	−
F. mesoamericanum	CBS 415.86	Honduras, Musa (Musaceae)	−
F. nepalense	CBS 127503	Nepal, rice	−
F. ussurianum	CBS 123754	Russian Federation, near Ussuriysk	−
	CBS 123751	Russian Federation, Jewish Autonomous Region, wheat	−
F. vorosii	CBS 123664	Japan, wheat	−
Unknown	CBS 123663	South Africa, soil	−
F. cerealis	CBS 195.80	Colombia, burnt páramo soil	−
	CBS 623.85	Netherlands, potato tuber buried in soil	−
	CBS 314.73	New Zeland, *Azalea*	−
F. culmorum	CBS 110568	France, wheat	−
	CBS 139512	Poland, wheat	−
	CBS 129.73	Portugal, *Populus nigra*	−
	CBS 110269	Canada	−
	CBS 251.52	*Triticum aestivum*, grain, cv. Koga	−
	CBS 122.73	UK, *Triticum*	−
	CBS 256.51	Netherlands, soil	−
	CBS 171.28	Unknown	−
	CBS 579.97	Denmark, chopped stems, buried in soil	−
	CBS 173.31	Canada, *Avena sativa*	−
F. pseudograminearum	CBS 109956	Australia, barley	−
	CBS 109953	New South Wales, soil	−
F. avenaceum	DBNP 0404	Poland, wheat	−
	DDPP 03162	Hungary, wheat	−
F. aywerte	CBS 395.96	Australia soil under *Plectrachne* sp.	−
F. chlamydosporum	CBS 445.67	Australia, wheat	−
F. graminum	CBS 119845	Australia, *Claviceps paspali sclerotia* on *Paspalum* sp.	−
F. oxysporum	CBS 620. 87	Denmark, barley	−

(continued on next page)

Table 1 (*continued*)

Fungal species	Strain code	Geographical origin, host/habitat of origin	FgMito qPCR assay
F. oxysporum f. sp. *pisi*	CBS 127.73	UK, *Pisum sativum*	−
F. poae	CBS 180.96	Norway, wheat	−
F. sambucinum var. *sambucinum*	CBS 135.73	Egypt, *Lycopersicon esculentum*	−
F. venenatum	CBS 458.93	Austria, Rohrau, wheat	−
F. tricinctum	CBS 410.86	Denmark, mouldy grain	−
	DBNP 168bm	Poland, wheat	−
F. vericillioides	CBS 734.97	Germany, maize	−
F. nurragi	CBS 393.96	Australia, soil	−
F. reticulatum	CBS 618.87	Denmark, soil from fruit plantation	−

(+) — Positive result, (−) — negative result.

nine specific real time PCR-based assays have been developed for *F. graminearum* alone, utilizing either SybrGreen or TaqMan chemistries (Morcia et al., 2013); however, only one was found to be specific to *F. graminearum* s.s. (Demeke et al., 2010). One of the greatest advantages of qPCR techniques over conventional or immunological methods of pathogen diagnostics is their extremely high sensitivity, although it should be underlined that quantification of trace amounts of fungal DNA is often a challenge. This is mainly caused by relatively low fungal load in environmental samples and the structure of the fungal cell wall, which makes its disruption for nucleic acid extraction difficult (Leite et al., 2012). The sensitivity of detection of fungi can be improved by the use of diagnostic assays targeting multi-copy DNA regions, such as mitochondrial DNA. Recently, such assays have been developed for quantification of fungi, including *Aspergillus fumigatus* (Oliveira et al., 2014), species of the genus *Phytophthora* and the *Phytophthora citricola* species complex (Bilodeau et al., 2014). The purpose of this study was to develop a highly sensitive FgMito qPCR assay for quantification of *F. graminearum* s.s. To ensure high sensitivity of the assay, primers and MGB probe (Minor-groove binding) were designed based on multi-copy mitochondrial DNA. Specificity of the assay was evaluated against all known phylogenetic species of the FGSC complex, as well as other *Fusarium* species. The assay was further evaluated in terms of efficiency and sensitivity against a test panel of different *F. graminearum* s.s. strains with various levels of pure fungal DNA and in the presence of wheat background DNA. The FgMito assay was finally used to quantify *F. graminearum* s.s. from 65 field samples from a range of hosts with defined levels of trichothecenes.

2. Materials and methods

2.1. Fungal strains

Eighty-four fungal strains used for the specificity testing are listed in Table 1. The CBS strains of *Fusarium* tested are held in the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands. The MUCL strains tested are held in MUCL Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium, and were kindly provided by Dr. Jonathan Scauflaire. The Argentinian strains tested are held in the fungal collection of BIOLAB Azul-INBIOTEC-Fac. Agronomía de Azul (UNCPBA), Buenos Aires, Argentina. The DBNP strains are held in the Department of Botany and Nature Protection, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland. The *Fusarium* isolates were maintained on potato dextrose agar (PDA) at 25 °C before DNA extraction.

2.2. Grain samples

Grain samples from Luxembourg (n = 16) and Poland (n = 49) were analyzed in this study. Wheat samples from Luxembourg were harvested in 2007 and 2008 (Giraud et al., 2010) and were obtained from the Luxembourg Institute of Science and Technology. Polish samples of barley, rye, wheat and crop mixture were harvested in 2011 and 2012. 100 g of grain from each sample were initially ground to a fine powder using an IKA A10 analytical grinding mill (IKA Laboratory, Germany) after arrival at the laboratory and were stored at −25 °C.

2.3. DNA extraction

0.1 g of mycelium scraped from the surface of PDA plates was used for DNA extraction from fungi. In the case of DNA extraction from grains, 0.25 g of ground grain per sample was used. The material was homogenized twice (30 s at a speed of 6.0 m/s) on a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA) in tubes with 1 mm silica spheres (Lysing matrix C, MP Biomedicals). Genomic DNA from fungal cultures, as well as from grains, was carried out using a ChargeSwitch® gDNA Plant Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Fungal DNA concentrations were determined on a Qubit® 2.0 Fluorometer with Qubit®dsDNA BR Assay (Invitrogen, USA) following the manufacturer's recommendations.

2.4. Sequencing of mitochondrial cytochrome b (COB) intron 2

Complete COB intron 2 sequences of *Fusarium aethiopicum* (CBS123667), *Fusarium acaciae-mearnsii* (CBS 110253), *Fusarium asiaticum* (CBS 110258), *Fusarium austroamericanum* (CBS 110244), *Fusarium boothii* (CBS 119170), *Fusarium brasilicum* (CBS 119180), *Fusarium cortaderiae* (CBS 123655), *Fusarium culmorum* (CBS 139512), *Fusarium louisianense* (CBS 127524), *Fusarium mesoamericanum* (CBS 110252), *Fusarium meridionale* (CBS 110247), *Fusarium nepalense* (CBS 127503), *Fusarium pseudograminearum* (CBS 109956), *Fusarium ussurianum* (CBS 123754) and *Fusarium vorosii* (CBS123664) were obtained using the Illumina Miseq platform with the 250 bp paired-end read, version 2. De-multiplexed and trimmed reads were aligned to the COB intron 2 sequence of *F. graminearum* (PH-1 strain) with Geneious (v.6.1.6 created by Biomatters, available from http://www.geneious.com). A complete and annotated mt genome of the PH-1 strain is available in NCBI under accession number NC009493. Gene annotation was carried out using Geneious software. COB intron 2 sequences of fungal strains have been deposited in the NCBI database under GenBank accession numbers KM408154–KM408169 and KR381808–KR381810.

2.5. Design of a primer/probe set specific for F. graminearum s.s.

To reveal the polymorphic site for the design of a primer/probe set specific for *F. graminearum* s.s., we first aligned complete mitogenomes of *F. graminearum* s.s. (GenBank: NC009493) and *F. gerlachii* (GenBank: KM486533). The revealed polymorphic sites (data not shown) were then tested for allelic discrimination using recommended parameter values of the software package PRIMER EXPRESS 3.0 (Applied Biosystems, Foster City, USA). After testing a dozen polymorphic sites, a SNP (T/C, 49.696) was revealed within COB intron 2, fulfilling the criteria for TaqMan allelic discrimination. The specificity of this SNP has been verified by alignment of COB intron 2 sequences of 13 *F. graminearum* s.s. strains (GenBank: KP966550–KP966561 and KR011238) and the other closely related species obtained in this study. To improve specificity, COB 2 primer was designed manually

Table 2
Primers and MGB probe developed for quantification of *F. graminearum*.

Primer/probe name	Primer/TaqMan probe sequence	Melting temperature (Tm) °C	GC %
COB1 primer	TGGCCTGAATGAAGGATTTCTAG	58.4	43
COB2 primer[a]	CATCGTTGTTAACTTATTGGAG**A**TG	55.8	36
COB probe	FAM-TTAAACACTCAAACACTACA-MGB	65.0	30

[a] The additional mismatch (A/G, 49.752) is indicated in bold.

based on the other SNP (A/G, 49.752), with additional mismatch by replacing C by A in the 3rd nucleotide from the 3′ end of COB 2 primer (Table 2). The COB probe, conjugated with an MGB group, was labeled at the 5′-end with 6-carboxyfluorescein (FAM). All primers were synthesized by Sigma-Aldrich (Germany), while probes were ordered from ABI PRISM Primers and TaqMan Probe Synthesis Service.

2.6. Optimization of a TaqMan assay specific for F. graminearum s.s.

The optimized reagents of the TaqMan reaction included: 2 μL gDNA (5000 to 100 pg), 14.3 μL H_2O, 6.7 μM of primer each, 1.7 μM of probe and 3.6 μL TaqMan Fast Advanced Master Mix (Applied Biosystems, USA). TaqMan reaction conditions were optimized as follows: 95 °C for 20 s (95 °C for 3 s, 60 °C for 30 s) × 40 in a ViiA 7 Real-Time PCR System (Applied Biosystems, USA) with a final volume of 20 μL. The reaction setup was done with the epMotion® 5070 automated pipetting system (Eppendorf, Germany). The threshold for the Ct analysis was manually adjusted to 0.04. The specificity of the optimized TaqMan assay was verified by testing the fungal strains listed in Table 1. To determine the efficiency and sensitivity of the assay, various amounts of gDNA (in picograms) of different *F. graminearum* s.s. strains were serially diluted by a factor of 10 with water and used as a template (Table 3). To determine the impact of background DNA, the efficiency and sensitivity of the assay was calculated in the presence of 30 ng of background wheat DNA. For each DNA dilution, a TaqMan reaction was prepared in six replicates, including two no template controls (NTCs): water-only and background DNA-only samples. The Ct-values obtained were used to calculate the reaction efficiency and correlation coefficient. The false positive rate was determined by using 30 ng of wheat DNA in the TaqMan reaction in 96 replicates using conditions as described above. To determine the false negative rate, four low concentrations of *F. graminearum* s.s. DNA: 2 pg, 0.6 pg, 0.2 pg and 0.06 pg were studied in the presence of 30 ng of wheat DNA. Each template concentration was analyzed in 96 replicates using conditions as described above.

2.7. Quantification of F. graminearum s.s. DNA from naturally contaminated grain samples

DNA samples extracted from grains were first normalized using plant Internal Positive Control (pIPC) as previously described in Kulik et al. (2014a). Samples with normalized Ct-values were subjected to quantification of *F. graminearum* s.s. using the FgMito assay. The amount of input DNA per reaction was 36 ± 12 ng. The amount of *F. graminearum* s.s. DNA (pg) was calculated from Ct values using the standard curve. Each template concentration was analyzed in 3 replicates using conditions as described above. In addition, grain samples were analyzed with the nuclear based assay for *F. graminearum* s.s. (Demeke et al., 2010) and *F. culmorum* (Waalwijk et al., 2004). The Ct values of both the FgMito assay and nuclear based assay (Demeke et al., 2010) were compared to reveal difference in sensitivity between both assays.

2.8. Trichothecene determination from grain samples

Grain samples from Poland were analyzed for the presence of trichothecenes according to Perkowski et al. (2003). Briefly, subsamples (10 g of ground grain) were extracted with acetonitrile/water (82:18) and cleaned-up on a charcoal column. The trichothecenes of group B (DON, 3AcDON, 15AcDON, NIV, FUS-X) were analyzed as trimethylsilylsilyl ether derivatives. After silylation samples were extracted with isooctane, and 1 μL of the sample was injected on a GC/MS system. The analyses were run on a gas chromatograph (Varian 450-GC, The Netherlands), hyphenated to a mass spectrometer (Varian 320-MS, Walnut Creek CA, USA), using an HP-5MS, 0.25 mm × 30 m capillary column. The injection port temperature was 280 °C, the transfer line temperature was 280 °C, and the analyses were performed with a programmed temperature. The initial temperature was 80 °C held for 1 min, from 80 °C to 200 °C at 15 °C min^{-1} held for 6 min and from 200 °C to 280 °C at 10 °C min^{-1}, the final temperature being maintained for 3 min. The helium flow rate was held constant at 0.7 mL min^{-1}. Quantitative analysis was performed in single ion monitored mode, and qualitative analysis was performed in SCAN mode (100–700 amu). Recovery for analyzed toxins was as follows: DON 84 ± 3.8%; 3AcDON 78 ± 4.8%; 15AcDON 74 ± 2.2%; FUS × 87% ± 5.9% NIV 81 ± 3.8%. The limit of detection was 0.01 mg kg^{-1}. Grain samples from Luxembourg were analyzed for the presence of trichothecenes according to the protocol described by Giraud et al. (2010). Briefly, 500 g of wheat grains per field were dried at 30 °C for 48 h, and aliquots (200 g) were milled (CyclotecTM 1093, Foss, Belgium). 5 g of the flour were transferred into 15 ml of acetonitrile/water (80:20, v/v), sonicated

Table 3
Validation results of FgMito assay based on 12 PCR runs using pure strain standards and templates mixed with 30 ng of wheat DNA.

Strain	Assay quantitative dynamic range (pg)[a]	Ct range	R[b]	Efficiency (%)
MUCL 53451	15.520 ± 2000–0.16	16.86 ± 0.12–33.68 ± 0.35	0.998	95.95
MUCL 53451[b]		16.9 ± 0.27–33.8 ± 0.36	0.999	97.37
CBS 104.09	14.600 ± 2160–0.15	16.27 ± 0.15–32.96 ± 0.44	0.999	98.96
MUCL 53455	12.660 ± 2000–0.12	16.43 ± 0.01–33.28 ± 0.09	0.999	96.5
M-62:3	9860 ± 1600–0.1	17.09 ± 0.22–33.12 ± 0.34	0.998	101.7
CBS 128539	8700 ± 2700–0.09	17.3 ± 0.17–34.0 ± 0.3	0.999	98.35
CBS 110266	6720 ± 160–0.067	18.76 ± 0.09–36.18 ± 0.35	0.998	93.0
IBT 1590	5680 ± 660–0.06	18.82 ± 0.23–35.46 ± 0.53	0.997	96.36
IBT 1590[b]		19.18 ± 0.1–35.54 ± 0.53	0.998	100.58
CBS 119173	5240 ± 520–0.05	18.0 ± 0.21–33.76 ± 0.3	0.996	101.0
CBS 119173[b]		17.29 ± 0.23–34.46 ± 0.72	0.996	95.96
CBS 110271	3060 ± 40–0.031	19.3 ± 0.36–36.32 ± 0.48	0.997	96.82

[a] Fungal DNA was quantified by Qubit fluorometer in three independent measurements.
[b] Diluted in the presence of 30 ng of background wheat DNA.

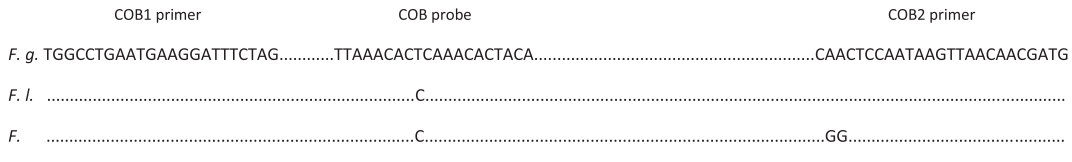

Fig. 1. The position of the primers and MGB probe specific for *F. graminearum* s.s.

for 15 min (Elma Transonic TS540) and centrifuged (720 *g*, 20 °C; Sigma 3K12 Bioblock Scientific). An aliquot of the supernatant was filtered (0.20-mm GHP membrane filter, PAL, MI, USA) and diluted 10 folds with water. Mycotoxin contents were determined by LC–MS/MS (Dionex Ultimate 3000, Applied Biosystems API 3200) using an Altima HP RP-C18 column (150 × 2.1 mm, 3 mm, Grace Davison, Deerfield, IL, USA). The mobile phase consisted of a methanol/water mix (80:20, v/v) containing 2.5 mM ammonium acetate. Detection and quantification limits were 35 and 70 ng/g, respectively. External standards were used for the quantification of mycotoxins plus measurement of the following fragment ions of the parent molecules: 371 → 311 for NIV, 355 → 295 for DON, 397 → 337 for AcDONs, 442 → 323 for HT2, 484 → 215 for T2 and 317 → 131 for ZON.

2.9. Statistical analyses

Spearman's rank correlation was used to determine whether there was a relationship in the DNA quantity and sum of trichothecenes in grain samples ($P < 0.05$). Quantity mean (pg) and quantity median (IQR) (pg) of four low concentrations of the template (2, 0.6, 0.2, 0.06 pg) analyzed in the presence of background wheat DNA were calculated. All the above statistical analyses were performed using the software package STATISTICA (Data Analysis Software System, ver. 12; StatSoft Inc., 2014).

3. Results

3.1. Design of a primer/probe set for specific quantification of *F. graminearum* s.s. based on the mitochondrial COB gene

To design a primer/probe set specific for *F. graminearum* s.s., we aligned complete mitogenomes of *F. graminearum* s.s. and *F. gerlachii* (Kulik et al., 2014b). We found one candidate SNP (T/C, 49.696) within COB intron 2 fulfilling the criteria for TaqMan allelic discrimination. The specificity of this SNP site has been confirmed by alignment of complete COB intron 2 sequences of 13 *F. graminearum* s.s. strains and 16 other closely related species found in NCBI. *In silico* specificity of this SNP site has been further confirmed by BLAST searches. Three approaches were used to improve assay specificity. First, we used an MGB probe, which offers increased specificity for single base mismatches (Kutyavin et al., 2000). Second, we designed a reverse COB 2 primer manually based on the other SNP (A/G, 49.752). This SNP site distinguishes *F. graminearum* s.s. from the other non target fungi except for *F. louisianense*. Third, to decrease the possibility of amplification of non target DNA, we generated an additional mismatch by replacing C with A in the 3rd nucleotide from the 3′ end of the COB 2 primer (Table 2). The position of the designed primers and MGB probe is presented in Fig. 1.

3.2. Optimization of a TaqMan assay specific for *F. graminearum* s.s.

After testing multiple primer and probe concentrations (data not shown), we optimized conditions for a 20 μl reaction volume (shown in the Materials and methods section) that was used to test the specificity of the assay against 82 fungal strains. All 29 *F. graminearum* s.s. strains tested presented a fluorescent signal with the FgMito assay, while no fluorescent signals were recorded on the other non target *Fusarium* species tested (Table 1). We evaluated the assay efficiency and correlation coefficients on a test panel of different pure strain standards, as well as on standards mixed with 30 ng of wheat background DNA. The assay developed had excellent amplification profiles against different standards, with a quantitative dynamic range of around 15.500–0.03 pg (Table 3). We further determined that the FgMito assay amplification profile and assay dynamic range were not affected by the presence of 30 ng of wheat background DNA (Table 3). In addition, we calculated the false negative rate of the FgMito assay by analyzing four low concentrations of *F. graminearum* s.s. DNA in the presence of background wheat DNA. No false negative results were reported for 2 pg, 0.6 pg and 0.2 pg of input DNA; however, a 24% false negative rate was calculated for 0.06 pg of the template. We found no false positive results when 30 ng of wheat DNA were used as the template. The medians and means of quantified *F. graminearum* s.s. DNA are shown in Table 4.

3.3. Quantification of *F. graminearum* s.s. DNA from naturally contaminated grain samples

The FgMito assay was used to quantify *F. graminearum* s.s. DNA from a total of 65 grain samples with defined levels of trichothecenes (Table 5). In addition, grain samples were subjected to analysis with the TaqMan assay targeting the nuclear genome of *F. graminearum* s.s. (Demeke et al., 2010). As shown in Table 4, qPCR analysis revealed the presence of *F. graminearum* s.s. in all samples analyzed; however, the amounts of template DNA between the samples differed greatly, ranging from around 506 pg to 0.04 pg. Notably, 11 of 65 grain samples gave negative results with nuclear based assay. These 11 samples contained low amounts of *F. graminearum* s.s., ranging from 2 to 0.04 pg, as revealed by FgMito assay.

Table 4
Results of the quantity median (IQR) and quantity mean of four low concentrations of *F. graminearum* s.s. DNA (in picograms) analyzed in the presence of 30 ng of wheat DNA.

Amount of input template (pg)	No. of positive amplifications	Quantity mean (pg)[a]	Quantity median (IQR) (pg)
2	96/96	2.240 (1.417–4.162)	1.972
0.6	96/96	0.872 (0.497–1.252)	0.863
0.2	96/96	0.134 (0.057–0.307)	0.120
0.06	70/96	0.077 (0.007–0.147)	0.065
30 ng of wheat background DNA only	0/96	–	–
No template	0/96	–	–

[a] Values in brackets indicate the minimum and maximum values of quantified template.

In total, 17 of 65 samples contained low (≤1 pg) quantity of target template, as revealed by FgMito assay. Thus, it could be calculated that approximately 60% of samples with low contamination levels gave false negative results with nuclear based assay. This is in contrast to results obtained by Demeke et al. (2010) who showed that as little as 0.64 pg of template DNA resulted in consistent Ct values. Nevertheless, comparing the above value to the limit of quantification (LOQ) of the FgMito assay (0.2 pg) it is evident that the latter is approximately

Table 5
Results of quantification of *F. graminearum* s.s. and *F. culmorum* DNA from cereals with defined levels of trichothecenes using three different TaqMan assays.

Sample no.	Cereal crop	Nuclear based assay specific for *F. graminearum* s.s. (Demeke et al., 2010)		FgMito assay		Nuclear based assay specific for *Fusarium culmorum* (Waalwijk et al., 2004)		DON	3AcDON	15AcDON	NIV
		Ct mean ± Ct SD	Mean quantity ± quantity SD pg	Ct mean ± Ct SD	Mean quantity ± quantity SD pg	Ct mean ± Ct SD	Mean quantity ± quantity SD pg	μg/kg			
1	Rye	36.0 ± 0.1	<0.6 ± <0.6	33.0 ± 0.1	0.1 ± <0.01	n.d.	–	n.d.	n.d.	n.d.	13.6
2	Wheat	33.5 ± 0.3	3.0 ± 1.0	26.0 ± 0.1	13.4 ± 1.0	30.8 ± 0.2	12.5 ± 2.0	7.1	n.d.	n.d.	2.2
3	Wheat	n.d.	–	30.8 ± 0.2	0.5 ± 0.06	n.d.	–	n.d.	n.d.	n.d.	n.d.
4	Rye	n.d.	–	32.6 ± 0.4	0.1 ± 0.04	33.6 ± 0.3	2.0 ± 0.4	2.8	n.d.	n.d.	n.d.
5	Wheat	32.7 ± 0.1	5.0 ± <0.6	27.8 ± 0.2	4.0 ± 0.6	n.d.	–	n.d.	n.d.	n.d.	20.1
6	Wheat	32.7 ± 0.1	5.0 ± <0.6	27.8 ± 0.5	4.1 ± 1.3	30.5 ± <0.1	15.5 ± 0.8	82.0	n.d.	n.d.	2.8
7	Wheat	n.d.	–	29.9 ± 0.1	0.9 ± 0.1	n.d.	–	n.d.	n.d.	n.d.	n.d.
8	Rye	33.1 ± 0.5	4.0 ± 1.0	27.9 ± 1.3	4.7 ± 3.5	32.2 ± 0.1	4.9 ± 0.4	7.8	n.d.	n.d.	8.8
9	Crop mixtures	29.1 ± <0.1	65.0 ± 1.0	24.1 ± 1.4	71.7 ± 56.8	n.d.	–	5.0	n.d.	n.d.	3.0
10	Rye	32.7 ± 0.1	5.0 ± <0.6	29.0 ± 0.4	1.7 ± 0.4	30.5 ± <0.1	15.1 ± <0.6	18.7	n.d.	n.d.	6.2
12	Crop mixtures	30.4 ± <0.1	27.0 ± <0.6	23.2 ± <0.1	94.4 ± 2.2	n.d.	–	4.6	2.0	1.0	1.1
13	Wheat	28.8 ± 0.1	82.0 ± 4.0	23.7 ± 0.2	66.6 ± 8.4	25.4 ± 0.2	485.0 ± 52.0	n.d.	n.d.	n.d.	n.d.
14	Spelt	n.d.	–	32.9 ± 0.3	0.1 ± 0.03	n.d.	–	22.6	n.d.	n.d.	n.d.
15	Wheat	n.d.	–	28.7 ± <0.1	2.0 ± 0.03	31.1 ± 0.1	10.1 ± 1.0	10.5	10.7	5.9	11.2
16	Wheat	36.0 ± 0.1	<0.6 ± <0.6	31.5 ± 1.8	0.5 ± 0.5	n.d.	–	n.d.	n.d.	n.d.	n.d.
17	Wheat	27.7 ± 0.2	178.0 ± 18.0	22.2 ± 0.1	192.3 ± 11.6	n.d.	–	222.6	n.d.	n.d.	7.7
18	Emmer wheat	n.d.	–	29.1 ± 0.2	1.5 ± 0.3	31.9 ± 0.2	6.1 ± 0.9	n.d.	n.d.	n.d.	6.7
19	Wheat	28.9 ± 0.2	75.0 ± 8.0	21.6 ± 0.1	275.7 ± 22.3	30.6 ± 0.1	14.6 ± 1.0	163.9	n.d.	n.d.	n.d.
20	Wheat	27.7 ± 0.2	178.0 ± 18.0	21.7 ± 0.2	276.0 ± 34.7	25.2 ± 0.2	553.7 ± 91.0	94.4	n.d.	n.d.	13.5
21	Rye	25.8 ± 0.1	355.0 ± 22.0	21.6 ± 0.1	287.1 ± 28.3	30.5 ± <0.1	15.5 ± 0.8	9.0	n.d.	n.d.	n.d.
22	Wheat	36.6 ± 0.1	<0.6 ± <0.6	30.4 ± 0.2	0.6 ± 0.07	n.d.	–	n.d.	n.d.	n.d.	n.d.
23	Barley	30.5 ± <0.1	16.1 ± 1.7	27.7 ± 0.2	4.2 ± 0.6	29.9 ± <0.1	42.9 ± 1.6	4.4	8.4	n.d.	n.d.
24	Wheat	28.5 ± 0.2	100.0 ± 12.0	21.5 ± 0.4	318.9 ± 90.5	n.d.	–	332.5	n.d.	n.d.	n.d.
25	Wheat	32.9 ± 0.2	5.0 ± 1.0	26.1 ± 0.3	12.9 ± 2.9	31.1 ± 0.1	16.2 ± 1.2	24.1	n.d.	n.d.	n.d.
26	Wheat	n.d.	–	31.4 ± 0.2	0.3 ± 0.05	n.d.	–	n.d.	n.d.	n.d.	n.d.
27	Wheat	n.d.	–	31.5 ± 0.7	0.3 ± 0.6	32.6 ± 0.3	3.7 ± 0.9	54.1	n.d.	n.d.	n.d.
28	Wheat	30.9 ± 0.4	19.0 ± 5.0	27.0 ± 2.2	14.0 ± 14.1	n.d.	–	10.8	n.d.	4.1	39.0
29	Wheat	29.8 ± 0.3	40.0 ± 7.0	21.9 ± 0.1	226.9 ± 13.4	n.d.	–	34.4	0.7	0.5	0.5
30	Wheat	35.3 ± 0.1	<0.6 ± <0.6	31.8 ± 0.4	0.3 ± 0.1	n.d.	–	1.3	n.d.	0.3	0.7
31	Wheat	n.d.	–	33.6 ± 0.5	0.07 ± 0.02	n.d.	–	0.6	n.d.	n.d.	n.d.
32	Wheat	27.7 ± 0.2	178.0 ± 18.0	23.0 ± 1.2	143.5 ± 117.1	n.d.	–	6.1	0.7	n.d.	n.d.
33	Wheat	n.d.	–	34.5 ± 0.9	0.04 ± 0.02	n.d.	–	1029.5	9.5	4.1	n.d.
34	Barley	33.9 ± 0.5	2.0 ± <0.6	27.6 ± 0.2	4.5 ± 0.6	30.6 ± <0.1	14.8 ± 0.9	27.1	6.8	n.d.	n.d.
35	Wheat	32.3 ± 0.1	7.0 ± <0.6	24.2 ± 0.2	48.1 ± 5.1	n.d.	–	7.5	n.d.	n.d.	n.d.
36	Wheat	31.4 ± 0.4	14.0 ± <0.6	25.7 ± 0.2	16.8 ± 2.0	31.3 ± 0.2	8.9 ± 1.4	27.9	n.d.	n.d.	n.d.
37	Wheat	32.3 ± 0.1	7.0 ± <0.6	28.6 ± 0.3	2.2 ± 0.4	n.d.	–	4.5	6.4	n.d.	n.d.
38	Wheat	33.9 ± 0.5	2.0 ± <0.6	28.7 ± 0.2	2.2 ± 0.4	33.9 ± 0.3	1.6 ± 0.3	38.7	n.d.	n.d.	n.d.
39	Wheat	33.1 ± 0.4	4.7 ± 1.1	27.6 ± 0.4	4.6 ± 1.3	34.2 ± 0.3	1.3 ± 0.2	21.1	n.d.	n.d.	2.2
40	Barley	31.7 ± 0.2	11.0 ± 1.0	27.7 ± 0.1	4.2 ± 0.4	33.3 ± 0.7	2.4 ± 2.4	44.9	6.6	16.4	n.d.
41	Crop mixtures	35.3 ± 0.1	<0.6 ± <0.6	30.9 ± 0.1	0.4 ± 0.04	n.d.	–	35.8	n.d.	n.d.	n.d.
42	Barley	34.2 ± 0.2	2.0 ± <0.6	30.2 ± 0.2	0.8 ± 0.1	31.6 ± 0.2	7.2 ± 0.8	3.4	n.d.	n.d.	1.7
43	Wheat	31.2 ± 0.2	14.3 ± 0.7	26.0 ± 0.1	13.1 ± 1.0	29.2 ± 0.1	38.4 ± 2.3	22.4	n.d.	3.4	13
44	Wheat	34.8 ± 0.2	<0.6 ± <0.6	31.5 ± <0.1	0.3 ± <0.01	31.3 ± 0.1	8.8 ± 0.4	31.0	8.8	16.4	27.5
45	Wheat	35.2 ± 0.4	<0.6 ± <0.6	30.1 ± 0.1	0.8 ± 0.03	n.d.	–	14.9	n.d.	n.d.	n.d.
46	Crop mixtures	30.9 ± 0.4	19.0 ± 5.0	25.9 ± 0.2	14.7 ± 1.6	29.2 ± 0.1	36.3 ± 2.3	37.6	n.d.	n.d.	n.d.
47	Triticale	28.2 ± 0.3	128.0 ± 14.0	20.8 ± 0.2	506.0 ± 56.7	n.d.	–	183.5	n.d.	n.d.	n.d.
48	Wheat	32.8 ± 0.5	5.0 ± 2.0	28.7 ± 0.3	2.1 ± 0.4	n.d.	–	42.1	10.7	19.9	n.d.
49	Crop mixtures	33.9 ± 0.5	2.0 ± <0.6	28.6 ± 0.4	2.3 ± 0.6	n.d.	–	n.d.	n.d.	n.d.	n.d.
50	Wheat	33.1 ± 0.4	4.7 ± 1.1	27.1 ± 0.2	6.2 ± 0.8	34.3 ± 0.1	<0.6 ± 1.0	220.0	n.d.	n.d.	n.d.
51	Wheat	33.9 ± 0.5	2.0 ± <0.6	29.4 ± 0.1	1.3 ± 0.1	n.d.	–	n.d.	n.d.	n.d.	n.d.
52	Wheat	27.7 ± <0.1	166.0 ± <0.5	22.3 ± <0.1	177.7 ± 10.4	32.2 ± 0.1	5.0 ± 1.0	928.0	n.d.	n.d.	n.d.
53	Wheat	27.7 ± 0.1	178.0 ± 18.0	22.0 ± 0.1	211.6 ± 14.6	35.5 ± 0.2	<0.6 ± 1.0	1014.0	n.d.	n.d.	n.d.
54	Wheat	24.2 ± 0.3	1 290.0 ± 227.0	21.1 ± <0.1	393.6 ± 8.7	n.d.	–	8.1	n.d.	n.d.	n.d.
55	Wheat	31.3 ± 0.1	14.0 ± 2.0	27.8 ± 0.2	4.0 ± 0.5	n.d.	–	101.0	n.d.	n.d.	n.d.
56	Wheat	n.d.	–	34.4 ± 0.1	0.04 ± <0.01	n.d.	–	n.d.	n.d.	n.d.	n.d.
57	Wheat	30.9 ± 0.1	19.0 ± 1.0	26.1 ± 0.1	12.6 ± 0.5	34.2 ± 0.3	1.3 ± 0.2	463.0	n.d.	n.d.	n.d.
58	Wheat	28.1 ± <0.1	133.0 ± 3.0	22.0 ± 0.1	210.4 ± 16.4	n.d.	–	487.0	n.d.	n.d.	n.d.
59	Wheat	28.3 ± 0.1	114.0 ± <0.5	25.4 ± 0.2	20.6 ± 2.7	29.9 ± 0.2	23.0 ± 3.0	681.0	n.d.	n.d.	n.d.
60	Wheat	29.6 ± 0.2	46.0 ± 7.0	25.5 ± 0.1	19.2 ± 0.7	28.7 ± 0.4	55.0 ± 15.0	268.0	n.d.	n.d.	341
61	Wheat	32.3 ± 0.1	7.0 ± <0.6	27.9 ± 0.1	3.7 ± 0.1	n.d.	–	1903.0	n.d.	n.d.	n.d.
62	Wheat	31.2 ± 0.2	16.0 ± 2.0	27.3 ± 0.1	5.6 ± 0.2	29.2 ± 0.1	38.0 ± 2.0	842.0	n.d.	n.d.	n.d.
63	Wheat	31.3 ± 0.3	14.0 ± 3.0	28.0 ± <0.1	3.3 ± <0.01	32.6 ± 0.3	<0.6 ± 1.0	968.0	n.d.	n.d.	532
64	Wheat	31.1 ± 0.3	16.0 ± 3.0	27.8 ± 0.1	4.0 ± 0.2	31.3 ± 0.2	9.0 ± 1.0	489.0	n.d.	n.d.	202
65	Wheat	27.9 ± <0.1	145.0 ± <0.5	22.5 ± 0.1	152.4 ± 8.0	35.4 ± 0.4	<0.6 ± <0.6	1215.0	n.d.	n.d.	462

n.d. — not detected.

three times more sensitive. However, comparison of the Ct values of both assays showed approximately 3–6 cycles of earlier amplifications in the case of the FgMito assay, indicating its higher than predicted difference in sensitivity (Table 5). To establish a correlation between *F. graminearum* s.s. DNA and total trichothecenes, DNA quantities (pg), were plotted against the respective sums of trichothecenes in all grain samples. Spearman's rank-order correlation showed a positive correlation ($R = 0.34$) between the sum of trichothecenes and *F. graminearum* s.s. DNA. We hypothesized that the accumulation of trichothecenes in grains could result not only from the presence of *F. graminearum* s.s. but also *F. culmorum* which is the other important trichothecene producer in Europe (Logrieco and Visconti, 2004). To confirm this, we performed additional qPCR analysis using a species specific TaqMan assay by Waalwijk et al. (2004). Indeed, the presence of *F. culmorum* was revealed in 33 of 65 samples, ranging from around 554 pg to <0.6 pg. However, additional statistical analysis between the sum of *F. graminearum* s.s./*F. culmorum* DNA and the sum of trichothecenes had no impact on Spearman's rank-order correlation.

4. Discussion

QPCR has been recently recognized as the most promising alternative in fungal diagnostics, including for toxigenic fungi of the genus *Fusarium* (Morcia et al., 2013). Although not considered a replacement for chemical analytical methods of mycotoxin determination, qPCR can become a risk prediction tool of contamination of grains with these metabolites (Pasquali and Migheli, 2014). It is worth noting that chemical methods of mycotoxin determination have limitations related to their inability to identify masked mycotoxins such as deoxynivalenol-3-β-D-glucoside (D3G) or DON-sulfates (Warth et al., 2015). In addition, strains of *F. graminearum* s.s. can produce novel trichothecene compounds such as NX-2 toxin (Varga et al., 2015), which, together with masked mycotoxins, are currently neither routinely screened for in foodstuffs nor regulated by legislation (Berthiller et al., 2013). In the present paper, we have developed a mitochondrial based FgMito assay for specific quantification of *F. graminearum* s.s. To achieve this, we used mitochondrial sequence data in two respects. Firstly, it is well known that the mitochondria of filamentous fungi have uniparental inheritance, and their genomes evolve faster than the corresponding nuclear DNA, a fact that makes them more suitable to differentiate closely related organisms (Bullerwell and Lang, 2005). Secondly, the high copy number of mitochondria in cells makes them a suitable target for development of an assay with improved sensitivity. With respect to sensitivity, the haploid cell of *F. graminearum* s.s. contains 36 Mb (Ma et al., 2013), which equals 0.04 pg. Thus, the limit of quantification (LOQ) of the FgMito assay (0.2 pg) is the equivalent of approximately five haploid cells of *F. graminearum* s.s. The limit of detection (LOD) corresponds to the lowest amount in a sample that can be detected with a false-negative rate below a given threshold (Nutz et al., 2011). We showed that 0.06 pg of input template could be detected with the FgMito assay, but with a 24% false negative rate only. Thus, the LOD of the assay developed could be determined between 0.2 and 0.06 pg. The mean of these two concentrations of input template (0.13 pg) equals approximately three haploid fungal cells. Similar to previous studies (Demeke et al., 2010), we showed a positive correlation between the quantity of *F. graminearum* s.s. DNA and the sum of trichothecenes in naturally contaminated grains, although Spearman's rank correlation coefficient ($R = 0.34$) was not high, but significant. The above outcome may have resulted from masking the major trichothecene compound DON by plant conjugates as discussed earlier and/or lack of trichothecene production that may occur under unfavorable environmental conditions. Finally, trichothecene production by other producers could not be neglected. We showed that the other important trichothecene producer *F. culmorum* was present in more than 50% of grain samples, which supports previous reports indicating that both *F. culmorum* and *F. graminearum* s.s. are responsible for the total trichothecene content in European grains (Logrieco and Visconti, 2004). Our next study will aim to develop mitochondrial-based TaqMan assay for quantification of *F. culmorum*, which, together with the assay presented in this paper, would offer a more sensitive diagnostic tool for quantification of these two major trichothecene contaminants. Finally, such tools would be valuable in the study of the distributions of geographically diverse fungal populations and could be used in plant breeding programs, as well as an initial step in quality assessment of food. Fungicides against *Fusarium* head blight pathogens are most effective when they are applied during the period of infection (Scheider et al., 2009). At this point in time, the amount of fungal material is very low, and the assay developed here could be particularly valuable for detecting infections at very early stages of epidemics, when control actions reducing mycotoxin formation can still be taken.

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