

Validation of a Quick PCR Method Suitable for Direct Sequencing: Identification of *Fusarium* Fungal Species and Chemotypes for Preventive Approaches in Food Safety

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Summary

Species determination by sequencing and PCR genetic chemotyping, used to determine the toxigenic potential of *Fusarium* strains, is fundamental for developing preventive strategies in food safety. Here we propose and statistically validate a quick protocol for standardizing the procedure of species determination by sequencing of the elongation factor 1- α and multiplex genetic chemotyping using the *Tri12* gene, based on fungal growth on Mira cloth tissue coupled with microwave extraction. The test was validated on 75 *Fusarium culmorum* and *Fusarium graminearum* strains.

Key words: *Fusarium culmorum*, *Fusarium graminearum*, microwave DNA extraction, EF1- α

Introduction

Polymerase chain reaction (PCR) is widely used for the identification of fungal species. Specific primers and sequencing procedures are needed for cross-checking morphological properties (1). More often, specific target sequences allow determining features of the strain that can be useful for its characterization. Genes encoding for dangerous fungal toxins found in food items such as aflatoxins, trichothecenes and fumonisins have already been identified (2). For example, by using primers targeting genes involved in the biosynthetic pathway of trichothecenes, it is possible to determine the toxigenic potential of *Fusarium* strains (3,4) by predicting whether a specific strain will produce deoxynivalenol and its acetylated derivatives, or nivalenol, which is of significantly higher toxicity for human cell lines (5). Moreover, fungal species determination can be used as a tool for identifying potential toxigenic risks linked to the presence of species able to produce toxic compounds. Therefore, PCR is essential for preventive approaches in the area of food safety.

For this purpose, a simple, rapid and reproducible preparation of nucleic acids is required. However, as with plants, fungal nucleic acids are often complicated to extract due to the presence of cell walls and secondary metabolites that can inhibit enzymatic reactions, making the DNA extraction process a crucial step. Several protocols for fungal DNA extraction have been reported. Many methods rely on a mechanical disruption of the cell by grinding (6,7), which can be preceded by a freezing step in liquid nitrogen (8–11), with the use of a mortar and pestle (12–15) or a bead milling method (16–18). Other methods for breaking up the mycelium include sonication (16) or thermolysis (16,19,20) to extract DNA.

Chemical extraction such as the one using cetyltrimethylammonium bromide (CTAB), phenol, or chloroform is largely adopted (1,15,16,20–24), as well as enzymatic extraction (25–27) methods. These methods have gradually been incorporated into or replaced by less cumbersome procedures such as the use of kits (1,16,19, 28–35) based on membrane filtration or magnetic separation. In all cited methods, sample preparation, however, is required and even with the use of automatic

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grinders, the time and the possibility of upscaling the extraction procedures are limited and/or expensive.

Other methods have been developed to simplify the extraction, such as techniques allowing a direct extraction into an Eppendorf tube using a pestle or a mortar (36) from fresh or lyophilized mycelium. For example, Cenis (9) cultivated fungi directly in an Eppendorf tube, which can reduce the risk of contamination.

Based on the discoveries of Goodwin and Lee (37), who showed that it is possible to extract DNA from eukaryotic cells with microwave radiation, Ferreira and Glass (38) adapted this technique to fungal spores. Direct spore PCR is performed after irradiation of the spores and buffer solubilization, a stage of vortexing and of centrifugation. The availability of engineered Taq polymerase has improved the efficiency of direct PCR approaches; therefore, the implementation of direct PCR methods would facilitate large screening efforts on fungi. Recently, Fata *et al.* (39) and Borman *et al.* (40) developed a rapid method for the preparation of total genomic DNA using Whatman FTA filter papers (GE Healthcare, Fairfield, CT, USA). The principle is based on the lysis and inactivation of microorganisms, provided by chelators and denaturants present in the Whatman paper, coupled with homogeneous collection of mycelium on the surface of the paper. More recently, Ben Amar *et al.* (41), developed a direct PCR-based procedure for DNA amplification from crude samples or spores in *F. culmorum* by manual sampling of the mycelium.

Fusarium head blight (FHB), mainly caused by *Fusarium* species, is a major disease of small grain cereals. The disease can cause significant problems of yield and quality losses (42). Major concerns arise from the contamination of grains with mycotoxins and their impact on human health and animal development (5,43). The prediction of the presence or absence of *F. graminearum* and *F. culmorum*, the main producers of mycotoxins in wheat, as well as the determination of chemotypes may play an important role in preventive food safety strategies. For this purpose, a cheap, high throughput method is needed.

Here, by combining a homogeneous method of mycelium sampling (simplifying the sample preparation) and a PCR approach, we tested four protocols for their efficacy in multiplexing with four primers and sequencing of PCR products on a large set of *Fusarium* isolates.

Materials and Methods

Samples

Fusarium samples, deposited in the strain collection of the Centre de Recherche Public – Gabriel Lippmann (Belvaux, Luxembourg), were collected from different wheat fields across Luxembourg in 2011 (Table 1). After isolation as described previously (35), strains were stored at -80°C in 15 % of glycerol as spore suspensions.

Samples were prepared according to two protocols. First, a potato dextrose agar medium (PDA, 39 g/L) was prepared, sterilized and poured into Petri dishes. To facilitate the sampling of mycelium, 5-mm diameter pieces of four types of materials were placed on the Petri dish before mycelium inoculation: Miracloth (Merck Millipore, Darmstadt, Germany), Wypall L40 (Kimberly-Clark Professional, Roswell, GA, USA), dialysis membrane (Spectra/Por, cellulose, molecular porous membrane tubing, molecular mass cut-off: 12–14 kDa; Spectrum Laboratories Inc, Rancho Dominguez, CA, USA), and paper (Planet+ plain paper, 80 g/m²; Xerox, Norwalk, CT, USA). Afterwards, Petri dishes were inoculated with 10 μL of spore suspension (approx. 1000 spores) and kept at 22°C in the dark for 5 days (or for 13 days to test the effect of ageing of the mycelium on the extraction method).

In the second procedure used for the Qiagen DNA extraction kit, potato dextrose broth medium (PDB, 24 g/L) was prepared, then 50 mL of it were distributed in Erlenmeyer flasks and sterilized. These were inoculated with 10 μL of spore suspension and incubated on an orbital shaker at 150 rpm at 25°C in the dark. After one week of growth, the mycelium was recovered by filtration and transferred into a 1.5- μL Eppendorf tube. The

Table 1. Strain designation, sampling site, geographic coordinates expressed according to the Luxembourg Reference Frame (LUREF) Datum, sampling date and species identified according to the quick PCR method

Strain designation	Sampling site	LUREF (E/N)	Sampling year	<i>Fusarium</i> species
4000, 4004, 4005, 4006, 4007, 4008, 4009, 4010, 4011, 4012, 4013, 4014, 4015	Bettel	82476 109697	2011	<i>F. culmorum</i>
4016, 4020, 4021, 4022, 4023, 4024, 4025, 4026, 4027, 4028, 4029, 4030, 4031, 4032	Fingig	62534 75512	2011	<i>F. culmorum</i>
4033, 4037, 4038, 4039, 4040, 4041, 4042, 4043, 4044, 4045, 4046, 4047	Hamiville	60812 122138	2011	<i>F. culmorum</i>
4048, 4052, 4053, 4054, 4055, 4056, 4057, 4058, 4059, 4060, 4061, 4062, 4063	Hivange	63997 76842	2011	<i>F. culmorum</i>
4064, 4068, 4069, 4070, 4071, 4072, 4073, 4074, 4075, 4076, 4077	Strassen	73998 75159	2011	<i>F. culmorum</i>
4079	Erpeldange	76367 102557	2011	<i>F. culmorum</i>
4083, 4084, 4085, 4086, 4087, 4088, 4089, 4090, 4091, 4092, 4093	Erpeldange	76367 102557	2011	<i>F. graminearum</i>

mycelium was freeze-dried for 24 h (Christ Alpha 2–4 LSC, Marin Christ and Co., Osterode am Harz, Germany), immersed in liquid nitrogen and crushed with a mixer mill (Mixer mill MM200, Retsch GmbH and Co., Haan, Germany) at a frequency of 25 Hz (3 times, 20 s). The mycelium was stored at -20°C .

DNA extraction methods

The following DNA extraction methods were used (Fig. 1): microwave DNA extraction, extraction by a heating step, direct extraction in PCR tubes, mycelium in-tube grinding extraction coupled with microwave extraction, and, for comparison, the DNeasy plant Mini Kit extraction (Qiagen, Hilden, Germany).

Microwave DNA extraction

Discs of the materials covered with mycelium were removed from the Petri dishes with a sterilized toothpick and placed into an Eppendorf tube with 100 μL of AE buffer (10 mM Tris-HCl, 0.5 mM EDTA; pH=9.0; Qiagen). DNA was extracted using microwave irradiation, with a slightly modified protocol of Ferreira and Glass

(38). Each tube containing the disc with the mycelium on it and the AE buffer was irradiated in a domestic microwave (Easytronic, M571, Whirlpool, Benton Harbor, MI, USA) with full power (750 W) for 5 min.

Extraction by a heating step

The initial steps of the extraction were carried out according to the same protocol as in the microwave DNA extraction. Once the discs were removed from the Petri dishes, Eppendorf tubes were placed in a heating block at 95°C for 10 min.

Direct PCR extraction

Samples were also processed in a direct PCR approach using the Phusion[®] High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Wilmington, DE, USA). One of the discs covered with mycelium was transferred from the Petri dish with a sterilized toothpick and placed into PCR tubes containing 50 μL of the Master Mix (Thermo Fisher Scientific), 2.5 μL of each primer and 45 μL of DNase/RNase-free water. The denaturation was extended to 8 min at 98°C .

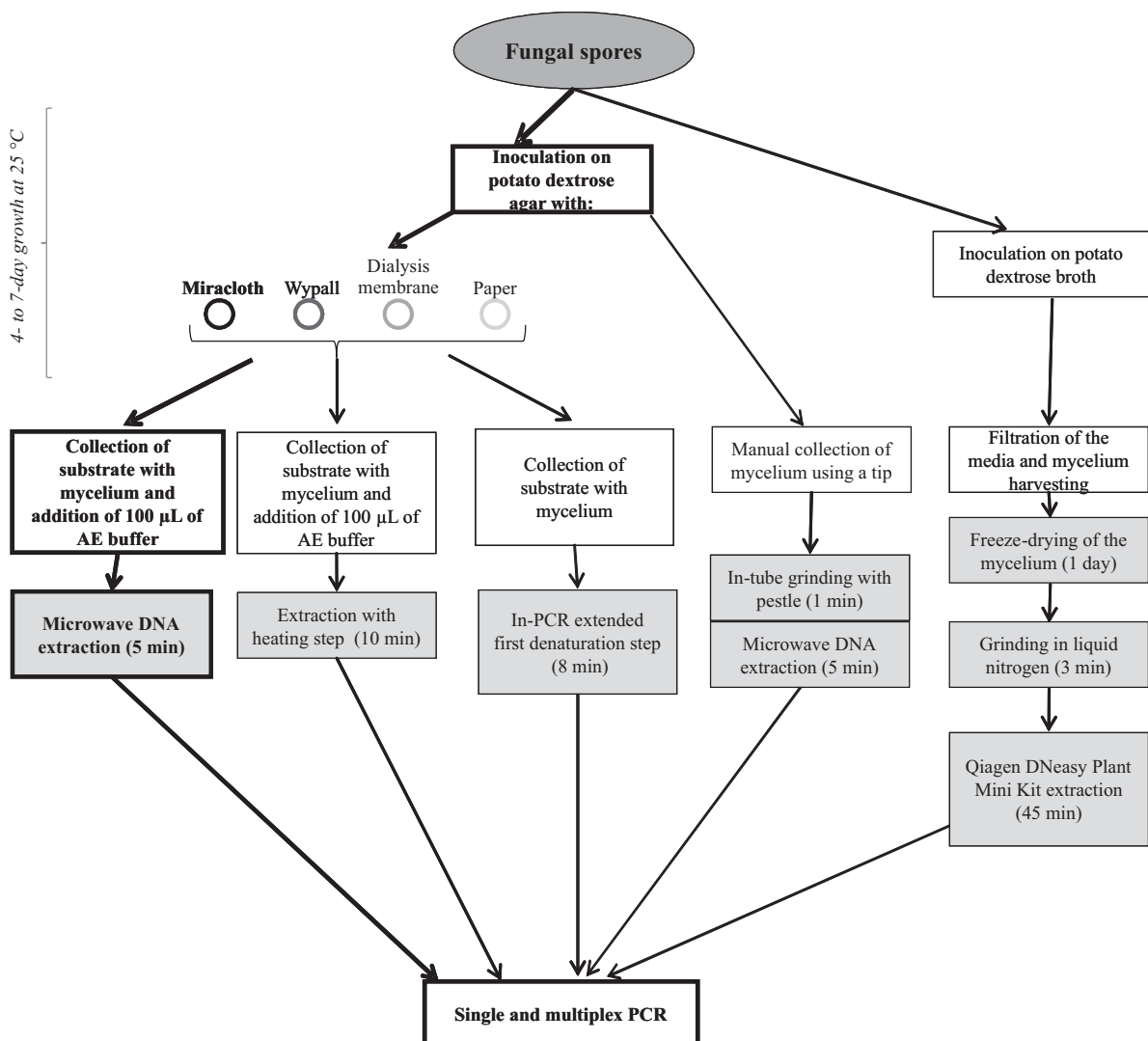


Fig. 1. The scheme represents the different extraction procedures tested in this work. The procedure that is proposed and validated in this work is highlighted in bold letters

Grinding of mycelium and microwave extraction

Aerial mycelium was manually collected from a strain inoculated on PDA, mixed with 180 µL of AE buffer and ground with a pestle for 1 min (4) in the Eppendorf tube. The mixture was placed in a microwave at 750 W for 5 min. A centrifugation for 30 s at 12 000×g allowed the deposition of mycelia. A volume of 3 µL of the upper phase was used for the single and multiplex PCRs carried out as described below.

DNeasy Plant mini kit extraction

Samples were also extracted according to Dubos *et al.* (35) using the Qiagen DNeasy Plant mini kit (Qiagen). After DNA extraction, an evaluation of the DNA quality and quantity was done on precast 1 % agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) using ethidium bromide colouration for staining. The gel was run at 110 V for 5 min, then at 80 V for 90 min. Fluorescent bands, exposed to UV light, were compared relative to a 2-log DNA ladder (0.1–10.0 kb, New England Biolabs, Ipswich, MA, USA). Estimation of purity ratio (260/280 nm) was calculated using the NanoDrop® ND-1000 (Thermo Fisher Scientific).

Single and multiplex PCR

Single and multiplex PCRs were carried out on the materials obtained from the different sampling and extraction procedures. Primers EF1 and EF2 (44) were used to identify the *Fusarium* species (1). The amplification was optimised using 2× Phusion High-Fidelity PCR Master Mix with high fidelity buffer (Thermo Fisher Scientific). This mix contains a Phusion DNA polymerase, 2× Phusion High-Fidelity buffer and 400 µM of each dNTP and 1.5 mM MgCl₂. PCR was performed in a final volume of 50 µL containing 5 µL of the supernatant, 25 µL of the Master Mix, 1.25 µL of each primer (10 µM) and 17.5 µL of DNase/RNase-free water.

Gradient PCR was performed on a Biometra TProfessional cyler (Göttingen, Germany) to define the optimum temperature for annealing, resulting in the final PCR program: 98 °C during 2 min for DNA strand separation; then 40 cycles of denaturation at 98 °C for 15 s, annealing at 55.2 °C for 20 s, elongation at 72 °C for 20 s; 5 min at 72 °C for the final extension followed by 4 °C until gel loading. A precast 3 % agarose gel (1×32 wells, Bio-Rad Laboratories) was used. The gel was exposed to 110 V for 40 min. Gel pictures were captured using an image analyzer (Ingenius Syngene Bio Imaging, Syngene, Cambridge, UK). Fluorescent bands were compared rela-

tive to a MassRuler™ Low Range DNA Ladder, ready-to-use (80–1031 bp, Thermo Fisher Scientific) and to the positive control strain PH1 from the USDA collection. A band between 700 and 800 bp was the evidence of the presence of a *Fusarium* strain.

PCR products were then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Durham, NC, USA) and quantified before PCR labelling. The concentration of each sample was adjusted to 25 ng/µL. Labelling of PCR was carried out according to the manufacturer's procedures using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit method (Applied Biosystems, Thermo Fisher Scientific) with EF1 and EF2 primers. PCR conditions were the following: 96 °C for 1 min, 25 cycles of 10 s at 96 °C and 4 min at 60 °C and to finish the cooling at 4 °C until purification. BigDye® XTerminator® Purification Kit (Applied Biosystems) was used for eliminating all the non-incorporated bases and the primers.

Sequencing was carried out using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Use of the Sequence Scanner v1.0® (Applied Biosystems) and CLC Main Workbench 6® (CLC bio, Mühlthal, Germany) software allowed to complete the sequence translation from the fluorescence signal intensity to nucleotide sequence. Sequencing was performed twice in order to evaluate the technical variability. The sequencing parameters QV20+ and sequence length were investigated.

The chemotyping PCR was performed using the primers 12CON, 12NF, 12-15F and 12-3F (Table 2) (44–46). The amplification was optimised using the Phusion Master Mix (Thermo Fisher Scientific). PCR was performed in a final volume of 50 µL containing 4 µL of the supernatant liquid, 25 µL of the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific), 1 µL of each primer (10 µM) and 19 µL of DNase/RNase-free water. In all comparative tests among the methods, three strains (*Fusarium culmorum* 4000, 4016 and 4033) were used.

To test the long-term storage features of the DNA extracted from Miracloth-growing mycelium, samples were stored for 18 months at –20 °C and then used for EF1-α amplification as described above.

Data analysis

Statistical analyses were performed using SPSS v. 19 software (SPSS Inc., Chicago, IL, USA) and SigmaStat v. 2.03 (Systat Software Inc., Chicago, IL, USA). In order to evaluate the normal distribution, the Kolmogorov-Smir-

Table 2. Gene target, primer sequence, expected product size, reference of the study and chemotype identified (when available)

Gene target	Primer name	Primer sequence	Product size bp	Chemotype identified	References
<i>Tri12</i>	12CON	5'-CATGAGCATGGTGATGTC-3'			
	12NF	5'-TCTCCTCGTTGTATCTGG-3'	840	NIV	(45)
	12-15F	5'-TACAGCGGTGCGCAACTTC-3'	670	15-ADON	
	12-3F	5'-CTTTGGCAAGCCCGTGCA-3'	410	3-ADON	
Elongation factor 1	EF1	5'-ATGGGTAAGGAGGACAAGAC-3'	704		
	EF2	5'-GGAAGTACCAGTGATCATGTT-3'			

NIV=nivalenol, 15-ADON=15-acetylated deoxynivalenol, 3-ADON=3-acetylated deoxynivalenol

nov test was applied. Because comparisons were carried out between methods, different strains processed with the same method were considered as replicates to also take into account the biological variability. To assess the purity of the samples, statistical analysis was done on the absorbance ratio at 260/280 nm using one-way analysis of variance in SigmaStat.

To compare the quality of the sequencing results between each modality, two parameters were chosen: the contiguous read length of the sequence and the QV 20+, which is a score of accuracy of sequencing of 99 % or above. The QV 20+ and the contiguous read length are assessed automatically by the Sequencing Analysis software (Life Technologies, Carlsbad, CA, USA).

First, the effect of the two primers was compared using independent samples for a Mann-Whitney *U* test. As no significant effect was evident, data of both primers were combined for each treatment. Contiguous length and QV20+ parameters were analysed by the Kruskal-Wallis test for comparison of the five independent distributions. A corrected $p < 0.05$ (two-sided) was considered as statistically significant.

Results and Discussion

The quick standardized sampling of the microwave extraction procedure was analysed and compared to the other methods. Five different procedures for species identification by PCR were compared. Three isolates (*Fusarium culmorum* 4000, 4016 and 4033) were used to compare the methods with each other.

Only three of the methods extracted DNA and allowed to consistently amplify the elongation factor (EF) 1 alpha gene from *Fusarium* species as confirmed by agarose gel electrophoresis (Fig. 1): DNeasy Plant Mini Kit, microwave, and mycelium grinding with pestle plus microwave (Fig. 2). Indeed, extraction by a heating step and the use of direct PCR with Phusion Master Mix without further processing were not able to amplify the EF consistently (data not shown). This contrasts with the result of Ben Amar *et al.* (41), who successfully applied direct PCR to characterise a number of *F. culmorum* strains. This may be due to two factors: the Taq polymerase and Master Mix combination differed from the present con-

ditions (41), and the mycelium sampling which could play a role in the efficiency of PCR reaction. We observed that the most difficult aspect of direct PCR is that reproducibility varies according to the amount of mycelium collected and the strain used. For this reason, we developed a quick and cheap method of growing colonies over a standardised defined area of material that can be easily transferred to PCR tubes, and we validated the assay on a large number of samples. We also observed that microwave processing increased the reproducibility of the assay and for this reason we focused on the optimal method able to combine the standardised extraction and the speed in the process.

We therefore compared the different materials used for collecting the mycelium from the plate with a standard kit procedure (Qiagen kit) and with a previously developed method based on manual grinding in Eppendorf tubes (4). No statistically significant difference ($p = 0.333$) among Miracloth, paper, Wypall, dialysis membrane and Qiagen was found with respect to the purity of the sample as tested by the 260/280 absorbance ratio (Table 3).

When the DNA was then used for EF1/EF2 amplification, it was evident that the paper modality was working less efficiently (Fig. 2), suggesting a potential increase of inhibitory effects that could be linked to the more intense colouring of the mycelium on the substrate. The grinding method performed well in PCR amplification

Table 3. Ratio of the absorbance at 260/280 nm obtained using the NanoDrop® ND-1000 (Thermo Fisher Scientific) from two replicates of three biological samples

Extraction method	$A_{260/280\text{ nm}}$
Miracloth	1.730±0.023
Wypall	1.690±0.006
Dialysis membrane	1.700±0.050
Paper	1.680±0.053
Qiagen kit	1.730±0.025

No significant differences were detected among the methods (ANOVA, $p = 0.333$). Values are expressed as average±standard deviation (S.D.)

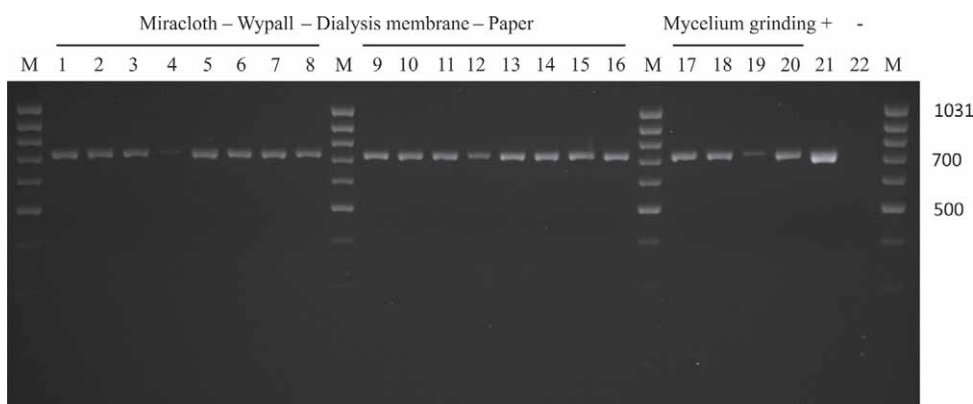


Fig. 2. Agarose gel (3 %) showing PCR products of elongation factor of 4 *Fusarium culmorum* strains. Lanes 1, 5, 9, 13: Miracloth; lanes 2, 6, 10, 14: Wypall L40; lanes 3, 7, 11, 15: dialysis membrane; lanes 4, 8, 12, 16: paper; lanes 17 to 20: mycelia in-tube grinding; lane 21: positive control PH1 obtained using Qiagen DNA extraction kit; lane 22: negative control; lane M: marker MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Fisher Scientific)

with a lower efficiency of amplification of one isolate (Fig. 2). This difference may be explained by the collection method that did not allow a sampling with a homogeneous amount of mycelium, further suggesting the advantage of a defined area of mycelium to obtain consistent results.

In order to compare the efficacy of the method on old colonies, a PCR was performed comparing five- and thirteen-day-old colonies. Strains of *F. culmorum* (4043, 4044, 4056 and 4057) were used and DNA 260/280 values were obtained by using the NanoDrop® ND-1000 (Thermo Fisher Scientific). The purity of the extract of the older colonies was slightly lower (1.73 ± 0.15 at 5 days and 1.44 ± 0.07 at 13 days), suggesting that the amount of pigments may increase with the age of the colony ($p = 0.004$).

Nonetheless, no evident difference of the PCR efficiency of the EF1- α could be observed comparing the extraction of 5- and 13-day-old colonies (Fig. 3a). Storing, similarly, did not influence the ability to efficiently am-

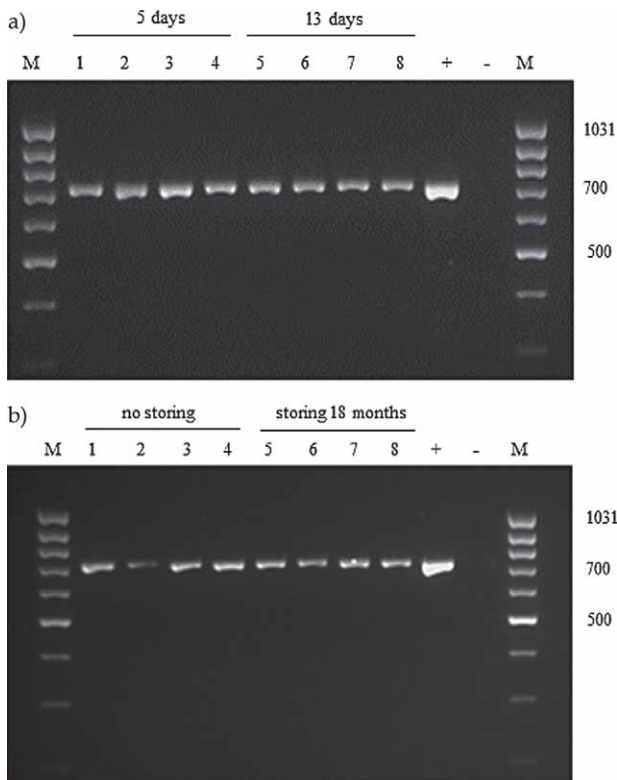


Fig. 3. Agarose gel (3 %) showing: a) five PCR products obtained by quick extraction method of DNA from *F. culmorum* isolates (4043, 4044, 4056, 4057) using Miraclloth filter paper. Lanes 1 to 4: sampling five days after inoculation; lanes 5 to 8: thirteen days after inoculation; lane + is a positive control and lane - is a negative control; lane M is the marker: MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Fisher Scientific); b) four PCR products obtained by quick extraction method of DNA from *F. culmorum* isolates (4021, 4022, 4045, 4046) using Miraclloth filter paper. Lanes 1 to 4: the direct PCR was realized on December 18, 2013 directly after quick DNA extraction; lanes 5 to 8: the PCR after storage was realized on May 22, 2012 after one year and a half of storage at -20°C ; lane + is a positive control and lane - is a negative control; lane M is the marker: MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Fisher Scientific)

plify EF1- α , as 1.5 years of storage of samples allowed good quality amplification (Fig. 3b).

As the method using the Miraclloth filter paper was efficient and allowed to identify all the 75 strains as listed in Table 1, the same extraction was used for determining the chemotype, by using primers developed on *Tri12*. The procedure included multiplexing 4 primers, requiring therefore a good DNA quality to be performed. Indeed, chemotypes could be distinguished by using DNA amplification (Fig. 4).

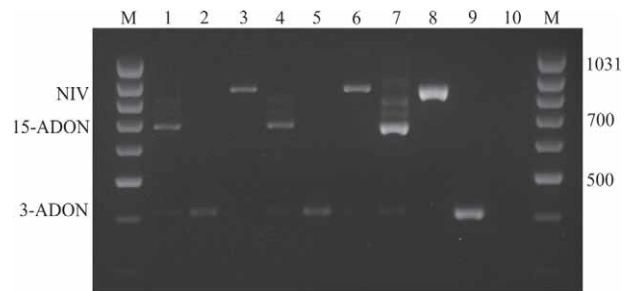


Fig. 4. Agarose gel (3 %) showing PCR amplification products obtained using primers developed by Ward *et al.* (45). Lane M is the marker: MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Fisher Scientific); lanes 1 to 6: Multiplex PCR from *F. culmorum* (2, 3, 5, 6) and *F. graminearum* (1, 4); lanes 7 to 9: positive control of chemotype 15-ADON (7), NIV (8), 3-ADON (9) as from Pasquali *et al.* (46); lane 10 is a negative control

To evaluate the possibility to perform sequencing reactions on the quickly extracted mycelium, sequencing was performed on the different EF products using EF1 and EF2 primers obtained from the strains *Fusarium culmorum* 4000, 4016 and 4033.

A few differences could be detected among the methods; the Qiagen kit had a better QV20+ (Table 4) and was significantly different from the membrane ($p = 0.0001$), the Miraclloth ($p = 0.025$) and the paper ($p = 0.0001$) (Table 5) extraction methods. Concerning the length of the sequence, Wypall was significantly different from Qiagen kit ($p = 0.012$) and paper ($p = 0.024$), but did not differ from the other two methods (Table 5).

Table 4. Sequence read length and QV20+ values obtained with different extraction methods

Extraction method	Contiguous length	QV20+
Miraclloth	639 \pm 4	632 \pm 5
Wypall	640 \pm 5	634 \pm 3
Dialysis membrane	634 \pm 7	608 \pm 42
Paper	577 \pm 88	574 \pm 75
Qiagen kit	630 \pm 8	662 \pm 15

Values are expressed as average \pm S.D., based on $N = 3$ independent biological replicates

Despite the higher intensity of the sequencing signal obtained with the Qiagen kit, sequences obtained using Miraclloth, Wypall and dialysis membrane were readable and allowed the identification of the sequences without

Table 5. Corrected p-values of each pairwise comparison among all methods tested for read length and QV20+ values

Pairwise comparison	Length p-values	QV20+ p-values
Membrane – Paper	1.000	1.000
Paper – Miracloth	0.200	0.499
Paper – Wypall	0.024	0.120
Paper – Qiagen	1.000	0.0001
Membrane – Miracloth	1.000	0.564
Membrane – Wypall	0.284	0.140
Membrane – Qiagen	1.000	0.0001
Miracloth – Wypall	1.000	1.000
Miracloth – Qiagen	0.116	0.025
Qiagen – Wypall	0.012	0.133

Each row tests the null hypothesis that the two sample distributions are the same. Significant differences are marked in grey

mistakes by a BLAST search in the *Fusarium* database (47), according to Geiser *et al.* (1) and the BLAST tool (48). Aligned sequences showed identical results. All sequences are deposited at NCBI database (GenBank IDs: KJ170153- KJ170227).

The efficacy of the paper method for subsequent DNA extraction and sequencing was lower compared to the other methods due to the variability of results obtained with the paper. As paper may induce different colourations in the strains, it probably activates secondary metabolite pathways that may produce molecules that inhibit the PCR. PCR amplification had an effect on the sequence quality afterwards. For this reason the use of the paper is not ideal for our purposes.

In order to study the application of the method to other fungi, the method was also tested on 69 strains of *Zymoseptoria tritici* (data not shown) confirming the effectiveness and speed of this method.

Conclusion

In conclusion, we demonstrated that coupling the growth of mycelium on Miracloth with microwave processing is a valuable, cheap and reliable method for large screenings of *Fusarium* isolates in order to determine the toxigenic potential of the strains. We verified that direct sequencing of the products and a multiplex with 4 primers could be performed with 100 % efficiency on 75 *Fusarium* strains. We could not reproduce recent results reported by Ben Amar *et al.* (41) of direct PCR with our Master Mix. It seems that the method developed by those authors relies on the operator's ability to collect similar amounts of mycelium in all assays. For this reason, the use of a surface with defined area for collecting the mycelium guaranteed better reproducibility for large screenings. Given the easy handling, rapid preparation of samples and the high reproducibility of the results for the identification and characterisation of strains, the method proposed here will be further used in currently running chemotyping studies aiming at the characterisation of *Fusarium* chemotypes over Europe.

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