Utilization of DNA microarrays for detection and identification of selected *Fusarium* species from the Czech Republic

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Abstract

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Fusarium is a serious phytopathogenic fungal genus with producting of many kinds of highly toxic secondary metabolites – mycotoxins. The consumption of *Fusarium* contaminated food and feed can cause dangerous mycotoxicoses both in humans and animals, therefore the detection of a wider range of *Fusarium* species in the samples of crops is very important. The aim of our work was to test the reliability of detection and identification of three *Fusarium* species in infected wheat grains by DNA microarrays versus classical mycological methods and by specific PCR. The *in-house* DNA microarrays for the detection and identification of the selected *Fusarium* species by using oligonucleotides probes were prepared. For hybridisation on DNA microarrays fluorescent labelled PCR products were used of part of the translation elongation factor 1 alpha. The conditions of hybridisation were optimised on fungal template DNA. The method of DNA microarrays was verified on artificially infected samples of wheat and tested on unknown infected wheat samples with simultaneous analysis by classical mycological methods and by specific PCR.

Keywords: DNA microarrays; fluorescent labelling; *Fusarium*; oligonucleotide probe; PCR

The members of the genus *Fusarium* are well-known phytopathogenic and mycotoxigenic filamentous fungi (Ascomycota) which infect many plant species and are spread almost worldwide. The disease of wheat, barley, and other small grain cereals, called Fusarium head blight (FHB) (= head scab = Fusarium ear blight = ear scab), is a serious problem in the Czech Republic as well as in other parts of the world, especially because of considerable yield losses and a lower quality of crops (Parry *et al.* 1995). The probability of the FHB epidemic is high in warm and wet weather during anthesis of host plants (McMullen *et al.* 1997). The aggressiveness of Fusarium head blight is dependent on the virulence of the *Fusarium* species strains which is connected with the mycotoxins production (Bai *et al.* 2001). There are many types of *Fusarium* mycotoxins, the most common being trichothecenes, fumonisins and zearalenone (D'Mello *et al.* 1999). Trichothecenes are strong inhibitors of eukaryotic protein synthesis (Bennett & Klich 2003). Fumonisins are probable

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carcinogens for humans as published by International Agency for Research on Cancer (1993), and many of them inhibit ceramide synthase, thus influencing sphingolipid metabolism (Merrill *et al.* 2001). Zearalenone is also called mycoestrogen or phytoestrogen (Bennett & Klich 2003), and it causes reproductive problems of pigs, cattle and sheep (Chang *et al.* 1979; Adams 1995).

The identification of *Fusarium* species is useful for the prediction of the mycotoxins contaminations of food and feed. The classical mycological methods based on the cultivation of the fungus under *in vitro* conditions and the identification based on macro- and micromorphological features are time-consuming and difficult because the morphological characters of the *Fusarium* species are variable. On the other hand, the methods based on DNA analysis are fast, sensitive and accurate. Many types of molecular methods were developed for the detection and identification of *Fusarium* species, based mainly on PCR and multiplex PCR techniques (Edwards *et al.* 2002; Bluhm *et al.* 2004; Jurado *et al.* 2006; Kulik 2008a,b; Sampietro *et al.* 2010) and on real-time PCR for quantification (Reischer *et al.* 2004; Waalwijk *et al.* 2004; Dyer *et al.* 2006; Leišová *et al.* 2006; Fredlund *et al.* 2008). The Amplified fragment length polymorphisms (AFLP) and Random amplified polymorphic DNA (RAPD) were used for identification of different pathotypes of *Gibberella zeae* (Cumagun *et al.* 2007). Other methods used for the identification of *Fusarium* species are microsatellite markers (Saharan & Naef 2008) and multiplex SNP analysis which was published by Kristensen *et al.* (2007a).

In several studies detection techniques were used combining PCR with DNA microarrays (Nicolaisen *et al.* 2005; Kristensen *et al.* 2007b). The PCR amplifies the target DNA sequences with universal primer pair while DNA microarrays with attached specific DNA probes are used for *Fusarium* spp. distinguishing. So the DNA microarray could be used as an effective tool for the detection and identification of *Fusarium* species on the basis of sequence polymorphism.

Nicolaisen *et al.* (2005) prepared sample DNA in PCR with Cy3 dye labelled reverse primer. They amplified part of *Fusarium* ITS2 rDNA sequences. On DNA microarray they had oligonucletide probes. The selected DNA sequences lack polymorphisms with some *Fusarium* species, so that their DNA microarray could detect only nine *Fusarium* species and the distinction between some related *Fusarium* species such as *F. culmorum*/*F. graminearum* and *F. tricinctum*/*F. avenaceum* was not possible.

Kristensen *et al.* (2007b) prepared DNA microarrays with oligonucleotide probes targeted at the phylogenetically variable part of the translation elongation factor 1 alpha (TEF-1α). That allows detection of 16 *Fusarium* species but does not contain specific probes for *F. culmorum* and *F. lunulosporum*, so it was possible to identify only 14 *Fusarium* species. Their sample DNA was prepared with biotin labelled PCR and subsequently detected by Silverquant® colorimetric detection method.

In our work, we focused on DNA microarrays because it is a method which enables rapid identification of all *Fusarium* species infected wheat and barley in the Czech Republic within few hours.

The aim of our work was to test the reliability of the detection and identification of the three selected *Fusarium* species by DNA microarrays versus classical mycological methods and by specific PCR in cereal grains in the Czech Republic.

MATERIAL AND METHODS

Fungal isolates. For optimisation of the hybridisation conditions of labelled PCR product on DNA microarray, fungal strains were used from the Collections of Phytopathogenic Microorganisms (CPPF) in the Crop Research Institute, Division of Plant Medicine, Department of Mycology, Prague-Ruzyně, Czech Republic; Culture Collection of Fungi (CCF), Department of Botany, Faculty of Science, Charles University in Prague, Czech Republic; Czech Collection of Microorganisms (CCM), Faculty of Science, Masaryk University, Brno, Czech Republic, and Collection of Phytopathogenic Microorganisms (fungi, cyanobacteria, algae, phytoplasms) (UPC-FUN), Department of Botany, Faculty of Science, Palacký University, Olomouc, Czech Republic.

The fungal strains used in this study and their sources are listed below. Six strains of *Fusarium culmorum* (W.G. Sm.) Sacc. (CPPF 206, CPPF 207, CPPF 221, CPPF 353, CCF 1745, UPC-FUN-149), four strains of *F. graminearum* Schwabe (CPPF 363, CPPF 366, CPPF 369, CCF 1626), five strains of *F. poae* (Peck) Wollenw (CPPF 51, CPPF 63, CPPF 171, CPPF 208, CCM 8313), one strain of *Alter-*

naria alternata (Fr.) Keissl. (CPPF 58), one strain of *Ascochyta* sp. (CPPF 341), one strain of *Botrytis cinerea* (CPPF 184), one strain of *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (CPPF 79), one strain of *C. herbarum* (CPPF 78), one strain of *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur (CPPF 70), one strain of *Epicoccum nigrum* Link (CPPF 57), one strain of *Mycosphaerella graminicola* (Fuckel) J. Schröt. (CPPF 386), one strain of *Oculimacula acuformis* (Boerema, R. Pieters & Hamers) Crous & W. Gams (CPPF 301), one strain of *O. yallundae* (Wallwork & Spooner) Crous & W. Gams (CPPF 300), one strain of *Penicillium corylophilum* Dierckx (CPPF 225), one strain of *Phaeosphaeria nodorum* (E. Müll.) Hedjar. (CPPF 372), one strain of *Pyrenophora tritici-repentis* (Died.) Drechsler (CPPF 60) and one strain of *Ramularia collo-cygni* B. Sutton & J.M. Waller (CPPF 382). For genomic DNA preparation, the cultures were inoculated on 2% malt agar overlaid with sterile cellophane sheets and grown at 25°C for 7–14 days.

Infection of wheat. The infection of wheat (cvs Arina, Ebi, Saskia, Siria) was carried out during flowering by spraying the wheat flowers with a spore suspension in the concentration of 0.8 × 10^7 per ml. The infectious suspension contained in mixture of one isolate of *F. culmorum* (strain CPPF 425) and four isolates of *F. graminearum* (strains CPPF 354, CPPF 356, CPPF 357, CPPF 368) (Šíp *et al.* 2008). The inoculated spikes were then kept for 24 h in polythene bags. Ripening wheat was harvested and the grains were used for analysis.

Samples of wheat obtained from State Phytosanitary Administration of the Czech Republic. Head blight symptoms on wheat spikes were evaluated by visual inspection on a 1–9 scale, where 1 < 5%, 2 = 5–17%, 3 = 18–30%, 4 = 31–43%, 5 = 44–56%, 6 = 57–69%, 7 = 70–82%, 8 = 83–95% and 9 > 95% of the spikelets with FHB symptoms (Chrpová *et al.* 2007) (Table 1).

Isolation of Fusarium DNA. *Fusarium* DNAs from cell cultures were extracted using a modified CTAB method published by Leisova *et al.* (2005) and purified by ethanol precipitation. DNAs from the infected wheat grains were isolated by DNeasy Plant Mini Kit (Qiagen, Germany). DNAs quality and quantity were checked both electrophoretically and spectrophotometrically.

DNA microarray preparation. For DNA microarrays, glass slides were used, covered with an aldehyde group on the surface – SuperAldehyde 2 Substrates (Arrayit Corporation, Sunnyvale, USA). Oligonucleotide probes were modified on the 5' end by primary amino group, so that the covalent bond between the aldehyde group on the glass surface and amino group of the probe could be developed. DNA sequences of the oligonucleotide probes (Table 2) were taken from the literature (Kristensen *et al.* 2007b). The probes were diluted to the final concentration of 10 µmol/l with Micro Spotting Solution Plus 2X (Arrayit Corporation, Sunnyvale, USA). Several positive controls of hybridisation (PCR products of part of the translation elongation factor 1 alpha in the concentration 500 ng/µl) were used for a better orientation in the position of the hybridisation signal. For the negative control of hybridisation, nuclease free water for PCR was used. All probes, positive and negative controls, were spotted in triplicate on glass slides by BioRobotics MicroGrid Compact (Genomic Solution, Huntingdon, UK) at the relative humidity of 70%. The printed arrays were let to dry overnight in the spotting robot at room temperature and stored in the dark at room temperature.

Table 1. The list of samples obtained from State Phytosanitary Administration of the Czech Republic (year of harvest 2009)

Sample No. (wheat grains)	Location	Cultivar	Level of infection 0–9 (9 the highes)t
1	Hodonín	Axis	0
2	Kutná Hora	Sulamit	9
3	Kutná Hora	Munk	9
4	Rychnov nad Kněžnou	Ludwig	9
5	Jihlava	Floret	0
6	Beroun	Alibaba	9
7	Beroun	Ebi	8
8	Prostějov	Ilias	1
9	Beroun	Sakura	9
10	Beroun	Meritto	4

Prehybridisation of DNA microarray. Free aldehyde groups on the glass surfaces of DNA microarray were blocked and unbound oligonucleotide probes were washed out according to manufacturer's protocol.

PCR amplification and labeling. For PCR amplification, primers were used (O'Donnell *et al.*

Table 2. The list of oligonucleotide probes for detection *Fusarium* species

Fusarium species	Probe	Sequence (5'→3')
F. pseudograminearum	FPpse3R	CGAATCGCTCGACGACTC
F. cerealis	FTcer1	CTACTGCTGTCATGACCTTCT
F. graminearum	FTgra2	GTCAACCAGTCACTAAC
F. graminearum	FTgra3	CGTGTCAACCAGTCACTAAC
F. graminearum, F. cerealis, F. culmorum, F. lunulosporum	FTglcc1	GTTTCAAATTTCCAATGTGCTGA
F. graminearum, F. cerealis, F. culmorum, F. lunulosporum	FTglcc2	TCGCMCTCACACGACGAC
F. venenatum	FTven1	GCCCTCTTCCTCAAAGCCA
F. sambucinum	FTsam1	TGACCCAAATCTAAGCTCGC
F. kyushuense	FTkyu1	CCTCCCCAATCCATCTGG
F. poae	FTpoa1	TTGCATTTCTTTGGGCGCGA
F. langsethiae	FTlan2	CGGCCGTGTCGTAATTTTTTT
F. sporotrichioides	FTspo1	GCTTTTGCCCTTCCCACAC
F. langsethiae, F. sporotrichioides	FTcls2	TTTACTGACATGCTTTGACAGAC
F. equiseti	FTequ1	ATCACYTCTTGCGCGTCAC
F. equiseti	FT4eq1	AAAATCACCTCCAGGGCATC
F. equiseti	FTceq	CACCGATCCATCACTCGAAT
F. torulosum	FTtor3	TTACCTGCACTCGGAGYCT
F. flocciferum	FTflo1	TTATCTGCACTCAAAGCCTG
F. tricinctum	FTtri2	TCGCGCACTACATGTCTTG
F. avenaceum	FTcaa1	CGCACTATGTCTTGCAGTCA
F. tricinctum, F. torulosum, F. flocciferum	FTttf1	CACAACATTTTGCTAACTTTTGA

The sequences of probes were designed by Kristensen *et al.* (2007b)

1998) specific for the amplification of the phylogenetic variable part of the translation elongation factor 1 alpha (TEF-1α). Forward primer was bond to the first exon and reverse primer to the fourth exon. The lenght of PCR product was ~700 bp (two exons and three introns).

The PCR amplification was carried out in a final volume of 25 µl containing: 12.5 µl of 2× GC Buffer I, 0.4 mmol/l dNTP, 1.25 U TaKaRa La Taq™ DNA polymerase (all from Takara Bio Inc, Tokyo, Japan), 1 µmol/l of TEF-1α primers (O'Donnell *et al.* 1998), and Mg^{2+} in the final concentration 3 mmol/l. The template DNAs from the cell cultures were diluted to the concentration of 20 ng/µl and 5 µl (100 ng) was used in the reaction. The DNAs from the infected wheat grains were used 5 µl per reaction undiluted, 5 times diluted, and 10 times diluted. The amplification was performed with the following profile: initial heating for 1 min at 94°C, followed by 30 cycles (30 s at 94°C, 30 s at 60°C and 2 min at 72°C) and finally 5 min at 72°C before cooling to 4°C.

The PCR products were purified by using High Pure PCR Product Purification Kit (Roche, Mannheim, USA), the concentrations of the PCR products were controlled by NanoPhotometer Implen (Implen GmbH, Munich, Germany), and afterwards the PCR products were fluorescent labelled by BioPrime Total Genomic Labeling System (Invitrogen, USA). The effectiveness of fluorescent labelling was also controlled on NanoPhotometer Implen (Implen GmbH, Germany) as well.

DNA microarrays hybridisation and detection of fluorescence signal. The conditions of hybridisation of the fluorescent labelled PCR products on DNA microarray were optimised using pure collections cultures of *F. culmorum*, *F. graminearum* and *F. poae*. 10 µl of the labelled PCR product together with 10 µl of hybridisation buffer SlideHyb #2 (Ambion, Grand Island, USA) were placed onto array and covered with glass screen which was fixed by Rubber cement. Hybridisation took place in Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) for 2 h at 65°C. After

hybridisation, the rubber cement was taken out by tweezers and the slides were submerged into the first washing solution (2× SSC, 0.2% SDS) in 50 ml tube with the array face of the slide tilted down so that the cover slip dropped off without damage to the array surface. Washing out of the unbound PCR product in the first solution took 15 min under careful horizontal swinging of the tube. Then the microarray was transferred into the second solution (2× SSC) with swinging for 5 minutes. Finally, the third solution was used with 0.2× SSC for 5 minutes. Thereafter the glass slide was dried by centrifugation.

DNA Microarrays were scanned in GenePi 400a Microarray scanner (MDS Inc., Downingtown, USA). The fluorescent signal was evaluated using TIGR Spotfinder freeware programme (Saeed *et al.* 2003).

Cloning and sequencing of PCR products. The samples of pure *Fusarium* cultures from the culture collections of fungi which had being analysed by DNA microarray were in the case of conflicting results, subsequently analysed by sequencing analysis. PCR products of TEF-1α were cloned into plasmid vector pCR®2.1-TOPO® (Invitrogen, Carlsbad, USA) and transferred into *E. coli*. Part of plasmid DNAs (cloned inserts) from at least four independent colonies were sequenced using ABI PRISM 3130 Genetic Analyzer and M13 primers as the sequencing primers. Sequencing data were analysed using ABI DNA Sequencing Analysis Software Version 5.2.

Sensitivity of DNA microarray. The limiting step in the detection of *Fusarium* spp. by DNA microarray is the sensitivity of the PCR. The detection limits of the PCR were determined by using three template DNA (CPPF 51 – *F. poae*, CPPF 353 – *F. culmorum* and CPPF 369 – *F. graminearum*). The PCR reaction was accomplished in optimised conditions (see above). As a template DNA, the set of dilutions was used starting with 100 ng and was ending with 24 pg.

Detection of *Fusarium* species in samples of wheat grains by microbiological methods

Grains were surface sterilised and laid at five on PCNB agar medium (Leslie & Summerell 2006) in 90 mm Petri dishes. They were incubated at 20°C for 7 days and then inoculated on diagnostic agar media. The identification on the species level was carried out according to the following literature: Samson *et al.* (2004), Leslie and Summerell (2006), and Domsch *et al.* (2007).

Specific PCR of *Fusarium* spp. All samples of the infected wheat were analysed by DNA microarray and the positive detection was subsequently verified by specific PCR for other control.

The specific PCR amplification was carried out in the same conditions as the optimised PCR with only one difference – as the forward primer the same oligo probe was used as for DNA microarray preparation.

RESULTS AND DISCUSSION

In our DNA microarrays, oligonucleotide probes were used as designed by Kristensen *et al.* (2007b) and the sample DNA was labelled by Bioprime Total Genomic Labeling System (Invitrogen, Carlbad, USA) based on the whole DNA amplification primered by random hexamers, the incorporated nucleotides having been fluorescent labelled by Alexa Fluor®3 or Alexa Fluor®5 dye. By reason of using oligonucleotide probes designed by Kristensen *et al.* (2007b), who proved specificity of all probes, the present study was focused on three *Fusarium* species detected by different method which frequently occur on cereals in the Czech Republic.

The first limiting step (after DNA isolation) of the analysis is the detection limit of the PCR. Kristensen *et al.* (2007b) tested the detection limit of the PCR on DNA from *F. sambucinum* spiked with 200 ng of wheat DNA. The stringency condition of the PCR was very low – annealing temperature 54°C and 50 cycles, that is the reason for the very low detection limit of PCR published by Kristensen *et al.* (2007b) – 0.624 pg of the template DNA from *F. sambucinum.* The conditions of our PCR were optimised to get a single band on agarose gel, thus if there was no PCR product, the analysis by DNA microarray was not done. The detection limit of the PCR in our conditions was determined for three *Fusarium* species: *F. graminearum* (CPPF 369) – 3.13 ng of the template DNA in reaction, *F. culmorum* (CPPF 353) – 0.39 ng of the template DNA in reaction and *F. poae* (CPPF 51) – 0.2 ng of the template DNA in reaction. These different values were obtained because of the presence of PCR inhibitors. The same problem was found by Fredlund *et al.* (2008) who tested different types of

Fusarium DNA extraction, but inhibitors were always present. Another factor which can affect the detection limit of the PCR is the different effectivity of the binding of degenerate primers in various *Fusarium* template DNAs.

To prove the specificity of DNA microarray for the genus *Fusarium*, the DNAs from other 14 fungal species (*Alternaria alternata, Ascochyta* sp., *Botrytis cinerea*, *Cladosporium cladosporioides*, *C. herbarum*, *Cochliobolus sativus*, *Epicoccum nigrum*, *Mycosphaerella graminicola*, *Oculimacula acuformis*, *O. yallundae*, *Penicillium corylophilum*, *Phaeosphaeria nodorum, Pyrenophora tritici-repentis*, *Ramularia collo-cygni*) which frequently occurring on cereals (Mathre 1997; Bockus *et al.* 2010) were isolated and tested in amplifiable

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221  AAGACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTG 60
353  AAGACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTG 60
366  AAGACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTG 60
     ************************************************************

221  AGTACCACTGCATCCCAACCCCGCCGATACTTGGCGGGGTAGTTTCAAATTTCCAATGTG 120
353  AGTACCACTGCATCCCAACCCCGCCGATACTTGGCGGGGTAGTTTCAAATTTCCAATGTG 120
366  AGTACCACCGCATCCCAACCCCGCCGACACTTGGCGGGGTAGTTTCAAATTTCCAATGTG 120
     ******** ****************** ********************************

221  CTGACATACTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAA 180
353  CTGACATACTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAA 180
366  CTGACATACTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAA 180
     ************************************************************

221  CCATCGAGAAGTTCGAGAAGGTTGGTCTCATTTTCCTCGATCGCGCGCCCTTTTCCCTTT 240
353  CCATCGAGAAGTTCGAGAAGGTTGGTCTCATTTTCCTCGATCGCGCGCCCTTTTCCCTTT 240
366  CCATCGAGAAGTTCGAGAAGGTTGGTCTCATTTTCCTCGATCGCGCGCCCTTTTCCCTTT 240
     ************************************************************

221  CGAAACATCATTCGAATCGCCCTCACACGACGACTCGATACGCGCCTGTTACCCCGCTCG 300
353  CGAAACATCATTCGAATCGCCCTCACACGACGACTCGATACGCGCCTGTTACCCCGCTCG 300
366  CGAAATATCATTCGAATCGCCCTCACACGACGACTCGATACGCGCCTGTTACCCCGCTCG 300
     ***** ********************************************************

221  AGGTCAAAAATTTTGCGGCTTTGTCGTAATTTTTCT---GGTGGGGCTCATACCCCGCCA 357
353  AGGTCAAAAATTTTGCGGCTTTGTCGTAATTTTTCT---GGTGGGGCTCATACCCCGCCA 357
366  AGGTCAAAAATTTTGCGGCTTTGTCGTAATTTTTTTCCCGGTGGGGCTCATACCCCGCCA 360
     ********************************** *   ********************

221  CTCGAGCGACAGGCGCTTGCCCTCTTCCCACAAACCATTCCCTAGGCGCGCACCATCACG 417
353  CTCGAGCGACAGGCGCTTGCCCTCTTCCCACAAACCATTCCCTAGGCGCGCACCATCACG 417
366  CTCGAGCGACAGGCGTCTGCCCTCTTCCCACAAACCATTCCCTGGGCGCTCATCATCACG 420
     ***************  *************************** ***** ** *******

221  TGTCAATCAGTTACTAACCACCTGTCAATAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTT 477
353  TGTCAATCAGTTACTAACCACCTGTCAATAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTT 477
366  TGTCAACCAGTCACTAACCACCTGTCAATAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTT 480
     ****** **** ************************************************

221  CAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGA 537
353  CAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGA 537
366  CAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGA 540
     ************************************************************

221  TATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTC 597
353  TATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTC 597
366  TATCGCCCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTC 600
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221  ACTACTGCTGTCATCACATTCTCATACTAACACGACTATCAGACGCTCCCGGTCACCGTG 657
353  ACTACTGCTGTCATCACATTCTCATACTAACACGACTATCAGACGCTCCCGGTCACCGTG 657
366  ACCACTGCTGTCATCACATTCTCATACTAACATGGCTATCAGACGCTCCCGGTCACCGTG 660
     ** ***************************** * ************************

221  ATTTCATCAAGAACATGATCACTGG 682
353  ATTTCATCAAGAACATGATCACTGG 682
366  ATTTCATCAAGAACATGATCACTGG 685
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Figure 1. The alignment of nucleotide sequences of the part of translation elongation factor 1 alpha of strains CPPF 221, CPPF 353, and CPPF 366

by PCR under optimised conditions. Contrary to Nicolaisen *et al.* (2005) who noticed the amplification of a few *Fusarium* related species none of the species tested provided PCR product, so they could not interfere with DNA microarray.

The time sufficient for hybridisation of the labelled PCR on DNA microarray was established of 2 h at 65°C. The same condition was also used by Kristensen *et al.* (2007b) for hybridisation of DNA microarray with biotin-labelled PCR products. The detection limit of the DNA microarray hybridisation step for biotin labelling was estimated by Kristensen *et al.* (2007b) to be 7 ng, in contrast with our detection limit for fluorescent labelling which was established at 50 ng.

The analysis of pure cultures of *Fusarium* species by DNA microarrays provided the expected results except the strain CPPF 221 which was considered to be *F. graminearum*, however it did not show positive hybridisation signals of specific probes for *F. graminearum*. For further verification of the result, sequencing analysis of part of TEF-1α was done and it proved that strain 221 is *F. culmorum* (Figure 1).

The DNA microarray was also tested on wheat artificially infected with *F. graminearum* and *F. culmorum*. *F. culmorum,* and *F. lunulosporum* are the only two species which can be detected just by the consensus probes (consensus probes for *F. graminearum*, *F. culmorum*, *F. cerealis*, and *F. lunulosporum;* Table 2). The occurrence of *F. lunulosporum* in wheat is highly improbable, because this species causes post-harvest diseases of citrus (Naqvi 2004). In contrast, the presence of *F. culmorum* in wheat is not unusual. Therefore, if the *F. graminearum* was present, we could not reject the presence of *F. culmorum* either. Such situation was observed in eight of ten wheat samples examined (Table 3). The analysis of the fluorescence signal intensity could not solve this problem because the PCR does not amplify different *Fusarium* template DNA with the same effectiveness.

Ten unknown samples of wheat obtained from the State Phytosanitary Administration (Prague, Czech Republic) were analysed by DNA microarrays and by reisolation of *Fusarium* species (Table 3). Unlike the reisolation, the DNA microarrays proved the presence of *F. langsethiae* in five samples out of ten. This *Fusarium* is a slowly growing species which prefers lower temperatures, thus it is often undetectable by classical mycological methods. The other differences between the results can be explained by the use of destructive analytical methods. It is impossible to analyse the same sample both by DNA microarrays and by classical reisolation and identification by mycologists. The other issue is it the sensitivity of the assessment, in the case of reisolation is necessary to have living cell which is able to grow, but for DNA microarrays is it sufficient to isolate amplifiable DNA.

Specific PCR was used for other checks of the results obtained by DNA microarray. As the template DNA for PCR the same DNA was used as for DNA microarrays analysis. The identification of

Table 3. The identification of *Fusarium* species in wheat samples (grains) obtained from State Phytosanitary Administration of the Czech Republic by DNA microarrays and by classical mycological method

Sample No.	Reisolation – *Fusarium* species	DNA microarrays – *Fusarium* species
1	0	*F. graminearum, "F. culmorum"*
2	*F. graminearum, F. poae*	*F. graminearum, "F. culmorum", F. poae, F. langsethiae*
3	*F. graminearum, F. culmorum*	*F. graminearum, "F. culmorum", F. langsethiae*
4	*F. graminearum, F. poae*	*F. graminearum, "F. culmorum", F. poae, F. langsethiae*
5	*F. poae*	*F. culmorum*
6	*F. graminearum, F. culmorum*	*F. graminearum, "F. culmorum", F. poae*
7	*F. graminearum, F. culmorum*	*F. graminearum, "F. culmorum", F. langsethiae*
8	*F. graminearum, F. tricinctum*	*F. graminearum, "F. culmorum", F. sambucinum*
9	*F. graminearum, F. tricinctum, F. avenaceum*	*F. graminearum, "F. culmorum", F. langsethiae*
10	*F. graminearum, F. culmorum*	0

"F. culmorum" – uncertain occurrence of *F. culmorum*

Fusarium species by specific PCR provided the same results as DNA microarrays analysis.

CONCLUSION

Our *in-house* DNA microarrays could be used as a fast and sensitive method for the identification and detection of three *Fusarium* species (*F. culmorum*, *F. graminearum*, and *F. poae*). It is necessary, however, to design a specific probe for the identification of *F. culmorum* as well.

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