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Development and application of multiplex PCR for the rapid identification of four *Fusarium* spp. associated with *Fusarium* crown rot in wheat

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Fusarium crown rot (FCR), caused by *Fusarium* spp., is a devastating disease in wheat growing areas. Previous studies have shown that FCR is caused by co-infection of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* in Hubei Province, China. In this study, a method was developed to simultaneously detected DNAs of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* that can efficiently differentiate them.Whole genome sequence comparison of these four *Fusarium* spp. was performed and a 20 bp sequence was designed as an universal upstream primer. Specific downstream primers of each pathogen was also designed, which resulted in a 206, 482, 680, and 963 bp amplicon for each pathogen, respectively. Multiplex PCR specifically identified *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* but not from other 46 pathogens, and the detection limit of target pathogens is about 100 pg/μl. Moreover, we accurately determined the FCR pathogen species in wheat samples using optimized multiplex PCR method. These results demonstrate that the multiplex PCR method established in this study can efficiently and rapidly identify *F. graminearum*, *F. pseudograminearum*, *F. proliferatum*, and *F. verticillioides*, which should provide technical support for timely and targeted prevention and control of FCR.

Development and application of multiplex PCR for the rapid identification of four *Fusarium* spp. associated with *Fusarium* crown rot in wheat

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Abstract

Fusarium crown rot (FCR), caused by *Fusarium* spp., is a devastating disease in wheat growing areas. Previous studies have shown that FCR is caused by co-infection of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* in Hubei Province, China. In this study, a method was developed to simultaneously detected DNAs of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* that can efficiently differentiate them.Whole genome sequence comparison of these four *Fusarium* spp. was performed and a 20 bp sequence was designed as an universal upstream primer. Specific downstream primers of each pathogen was also designed, which resulted in a 206, 482, 680, and 963 bp amplicon for each pathogen, respectively. Multiplex PCR specifically identified *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* but not from other 46 pathogens, and the detection limit of target pathogens is about 100 pg/μl. Moreover, we accurately determined the FCR pathogen species in wheat samples using optimized multiplex PCR method. These results demonstrate that the multiplex PCR method established in this study can efficiently and rapidly identify *F. graminearum*, *F. pseudograminearum*, *F. proliferatum*, and *F. verticillioides*, which should provide technical support for timely and targeted prevention and control of FCR.

Keywords: *Fusarium* crown rot; *Fusarium* spp.; multiplex PCR; identification; specificity; whole genome sequence comparison

Introduction

Wheat is a gramineous plant widely grown throughout the world and is one of the most important food crops that contribute significantly to human civilization, providing 19% of the daily caloric and 21% of the protein requirements for humans (Braun et al., 2010; Tadesse et al., 2019). *Fusarium* crown rot (FCR), a soil-borne disease, is one of the most serious cereal diseases that affects the entire plant growth period in cereal crops and causes serious yield and quality losses worldwide (Kazan and Gardiner, 2018; Lin et al., 2022; Xu et al., 2017). FCR has been observed in many arid and semiarid wheat growing regions of the world, including the Americas (Cook, 1968, 1980; Fernandez and Zentner, 2005; Mishra et al., 2006; Smiley et al., 2005), Australia (Akinsanmiet al., 2004; Burgesset al., 1975), Africa (Gargouri et al., 2011; Kammoun et al., 2009), New Zealand (Cromey et al., 2006), the Middle East (Gebremariam et al., 2017; Hameed et al., 2012; Pouzeshimiab et al., 2016), and China (Li et al., 2012; Xu et al., 2015, 2018; Zhang et al., 2015). In recent years, damage caused by FCR has gradually worsened in the Huanghuai wheat region of China. In many wheat growing areas in Henan Province, yield loss caused by FCR is up to 30-50% (Wang et al., 2022). In some high incidence areas, FCR caused yield losses up to more than 70%, with an average annual yield reduction of 9-35% from 2008 to 2019 (Luan et al., 2022).

FCR is commonly caused by several *Fusarium* spp., including *F. pseudograminearum*, *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. verticillioides*, and *F. proliferatum*. (Agustí-Brisach et al., 2018; Li et al., 2012; Meng et al., 2019; Zhang et al., 2015). Previous studies have shown that *F. pseudograminearum* is the predominant species to cause FCR in wheat but often mixed infection with other *Fusarium* spp. (Li et al., 2012; Kazan and Gardiner, 2018; Zhang et al., 2023). However, Zhang et al. (2015) reported that *F. graminearum* is the dominant pathogen of FCR in Anhui, Jiangsu, Henan, Shandong, and Hebei provinces. Thus, as a disease complex, the predominant pathogen of FCR may differ due to sampling location and ecological environment of the field (Saremi et al., 2007). Rapid and accurate identification of FCR pathogen species is of great importance, which may provide timely targeted prevention and control of FCR.

With the rapid development of molecular biology techniques, many molecular detection methods for pathogens have been developed. Compared with traditional detection methods based on isolation, cultivation, and morphological observation as well as biochemical characteristics, molecular identification method can be more accurate and efficient. Previous reports, however, have shown that soil-borne diseases are often caused by pathogen complexes. For example, *Fusarium* spp., *Botryodiplodia theobromae* and *Armillaria* spp. are important fungal groups associated with cassava root rot (Bandyopadhyay et al., 2006). *F. boothii*, *F. graminearum* and *F. meridionale* mixed infection led to maize crown and root rot (Lamprecht et al., 2011). *F. graminearum* species complex (FGSC), which includes at least 16 known species, is the major cause of *Fusarium* head blight (FHB) in many parts of the world (Del Ponte et al., 2014). *F.*

oxysporum f. sp. *melonis* and *Monosporascus cannonballus* co-infection causes melon radicle necrosis and rot (Wu et al., 2021). Therefore, identification of a single pathogen cannot meet the requirements of disease complex identification. Compared with single PCR, multiplex PCR possesses higher detection efficiency and can detect several pathogens simultaneously, which might reduce cost and save time. In order to efficiently and accurately monitor the occurrence of FCR, it is critical to develop a detection method that can detect multiple *Fusarium* spp. simultaneously.

Multiplex PCR amplify multiple target sequences simultaneously and has been used for detecting DNA of pathogen in medicine, environmental and agricultural sciences (Ali et al., 2015; Asano et al., 2010; Rappo et al., 2016). Previous reports have shown that multiplex PCR has enabled simultaneous detection of *F. oxysporum* sp. *lycopersici*, *Clavibacter michiganensis* subsp. *michiganensis*, *Leveillula taurica*, and begomoviruses on tomato plants (Quintero-Vásquez et al., 2012). *F. verticillioides*, *F. subglutinans*, and other species of the *Gibberella fujikuroi* complex were identified by PCR assays (Faria et al., 2012). Multiplex PCR was also reported to specifically identify *F. oxysporum*, *Sclerotium rolfsii*, and *Lasiodiplodia theobromae* in Peanut (Wang et al., 2023) and *Listeria monocytogenes* serovars, *Listeria* spp., and other species based on the target genes *LMxysn_1095*, *lmo1083* and *smcL* (Feng et al., 2020). In these previous reports, a primer pair is designed for each pathogen, resulting in an excessive number of primers in a multiplex PCR system. Too many primers processed simultaneously in a PCR system may lead to primer cross-binding and primer dimer formation, thus reducing amplification efficiency. Whole genome sequence comparison can be used to identify universal primers for multiple pathogens, thus reducing the total number of primers in a multiplex PCR molecular detection system, which is an easier and more efficient choice (Hu et al., 2020; Kim et al., 2015; Liu et al., 2023; Park et al., 2017; Yu et al., 2019).

Our previous investigation showed that FCR mainly consists of *F. graminearum*, *F. pseudograminearum*, *F. proliferatum* and *F. verticillioides* in wheat in Hubei Province, China. To develop a multiplex PCR system for detecting these four *Fusarium* spp., we designed primer sets based on the genome sequence of *F. pseudograminearum* Class 2-1C (GenBank Accession No. CP064755.1), *F. graminearum* PH-1 (GenBank Accession No. HG970332.2), *F. proliferatum* ET1 (GenBank Accession No. NW_022194799.1), and *F. verticillioides* 7600 (GenBank Accession No. CM000579.1). A 20 bp sequence was selected as a universal upstream primer and specific downstream primers of four *Fusarium* spp. with different amplicon size were designed. We then evaluated the specificity and applicability of this method in accurately detecting four *Fusarium* spp. in infected wheats.

Materials & Methods

Fungal Strains, Culture Conditions, and DNA Extraction

A total of 22 strains of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* were collected by Hubei Academy of Agricultural Sciences, and a total of 46

fungal strains were kindly provided by Nanjing Agricultural University, Jiangsu Academy of Agricultural Sciences, Northwest Agriculture and Forestry University, and Yulin Normal University. All strains were routinely cultured on potato dextrose agar (PDA) plates (200 gL^{-1} of potato extracts, 1% glucose, and 2% agar), and incubated at 25℃ culture for 7-10 days. Mycelia of each isolate were collected with a sterile spatula for DNA extraction. Genomic DNA was extracted from mycelia using the Plant DNA kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. DNA samples were measured with spectrophotometry to determine quality and concentration and stored at -20°C until use.

Comparative genomics for identifying multiplex PCR primers

The genome sequences of *F. pseudograminearum* Class 2-1C (GenBank Accession No. CP064755.1), *F. graminearum* PH-1 (GenBank Accession No. HG970332.2), *F. proliferatum* ET1 (GenBank Accession No. NW_022194799.1), *F. verticillioides* 7600 (GenBank Accession No. CM000579.1), *F. equiseti* D25-1(GenBank Accession No. QOHM01000001.1), *F. oxysporum* f. sp. *lycopersici* 4287 (GenBank Accession No. NC_030986.1), *F. solani* JS-169 (GenBank Accession No. NGZQ01000001.1) and *F. incarnatum* MOD1-FUNGI18 (GenBank Accession No. RBBZ01000100.1) were downloaded from the National Center for Biotechnology Information (NCBI) database. The primer sets design method is as described previously (Liu et al., 2023), we performed multiple alignments of the conserved sequences using Mauve software (version 2.3.1) to obtain homologous gene sequence fragments of these genomes. A $\geqslant$ 20 bp genome sequence was selected from homologous fragments in *F. pseudograminearum*, *F. graminearum*, *F. proliferatum*, and *F. verticillioides*,and served as an universal forward primer. A 1000 bp downstream sequence was obtained in each genome for sequence alignment using BioEdit software (version 7.0.9.0). Then, nucleotide sequence of the designed specific downstream primers of each target strain was verified in the Basic Local Alignment Search Tool (BLAST) of the NCBI database. The primers are described in **Table 1**. The primer sets were synthesized by Sangon Biotech (Shanghai, China).

Optimization of multiplex PCR condition for detection of four *Fusarium* spp.

Multiplex PCR assay-related parameters were evaluated and optimized, including primer annealing temperatures, primer dosage, and concentrations of dNTPs and Mg^{2+}. . Multiplex PCR was performed in 50 µl reaction volumes containing 0.25 µl TaKaRa Ex Taq polymerase (5 U/µl), 5 µl 10×Ex Taq buffer (Mg^{2+}-free), 1-8 µl (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 mM)of $MgCl_2$ (25 mM), 2-16 µl (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mM) of dNTPs mixture (2.5 mM each), and 1µl for each of the four fungal DNA templates. To adjust optimal concentration of each primer in the multiplex PCR system, different primer concentration combinations were tested, including four groups of concentration ratios for the universal upstream primers (Fu-4F) and downstream primers (Fgram-R, Fpseu-R, Fprol-R, and Fvert-R) (group I: 1:1 ; group II: 2:1 ; group III: 3:1 ; and group IV: 4:1). The final concentrations of each specific downstream primer were set at 0.05 µmol/L, 0.1 µmol/L, 0.15 µmol/L, and 0.2 µmol/L, respectively **(Table S1)**. Multiplex PCR

amplification was performed with the following program: 95℃ for 5 min, 32 cycles of denaturation at 95℃ for 30 s, annealing at 45-65℃ for 30 s, extension at 72℃ for 1 min and final extension for 10 min at 72℃. Twelve temperature gradients were set, including 45, 46.1, 47.7, 50.5, 53, 55, 57.2, 59.4, 61.6, 63.4, 64.6 and 65℃ to determine the optimal reaction conditions for annealing temperature. PCR products were visualized under UV light after being size-fractionated by electrophoresis through a 2% agarose gel made with TAE buffer and stained with ethidium bromide solution.

Multiplex PCR specificity test

To evaluate the specificity of the multiplex PCR primer set, 1 μl of 22 target pathogen DNA (six *F. graminearum*, eight *F. pseudograminearum*, five *F. proliferatum* and three *F. verticillioides*) from different hosts and other 46 fungal strains were used as templates for multiplex PCR amplification under the optimized multiplex PCR system and conditions. All strains are listed in **Table 2**. PCR products were visualized under UV light after being size-fractionated by electrophoresis through a 2% agarose gel made with TAE buffer and stained with ethidium bromide solution.

Multiplex PCR sensitivity test

To determine the sensitivity of the multiplex PCR assay, genomic DNA from the four target pathogens was serially diluted to 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 100 fg/μl, and 10 fg/μl by a 10-fold gradient with sterile double distilled water. 1 μl of each DNA dilution concentration was used as a single PCR template to test the detection limit of each target pathogen by single PCR. Subsequently, each DNA dilution concentration was mixed, respectively, as a multiplex PCR template to test the detection limit of multiplex PCR for each target pathogen. PCR was performed according to the optimized conditions. Finally, PCR products were visualized under UV light after being size-fractionated by electrophoresis through a 2% agarose gel made with TAE buffer and stained with ethidium bromide solution.

Detection of target pathogen DNA from field wheat samples and artificially inoculated wheat samples

To evaluate the applicability of the multiplex PCR assay for four *Fusarium* pathogens of FCR, we collected 22 wheat samples in a wheat growing area of Xiangyang (32.2015913°N, 110.901005°E) and Suizhou (31.9938899°N, 113.0270585°E) in Hubei Province of China in June 2022. After a small piece of tissue was excised from the stem of the 22 wheat samples using a sterilized scalpel, genomic DNA was extracted from field wheat samples using the Plant DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions.

For the artificial inoculation test, fungal strains were cultured on PDA for three days at 25°C, then mycelium plugs were transferred to mung bean medium and cultured at 25°C for seven days with shaking at 200 rpm. Conidial suspensions were filtered through four layers of cheesecloth to separate conidia from mycelia. Concentration of the conidial spore suspensions was estimated using a hemocytometer and adjusted to 1×10^7 spores/ml. Wheats were inoculated with conidia

suspensions of each fungus (1×10^7 spores/ml) in the stem of each wheat. We inoculated 17 healthy wheats with four *Fusarium* strains in different combinations and 7 wheats with sterile water. Wheat samples inoculated with four *Fusarium* strains served as positive controls, while samples treated with sterile water were used as negative controls. Genomic DNA from all wheat samples was extracted using the Plant DNA Kit according to the manufacturer's instructions. All DNA extracted from the wheat sample used as a template for the multiplex PCR, which was performed using an optimized multiplex PCR system. PCR products were visualized under UV light after being size-fractionated by electrophoresis through a 2% agarose gel made with TAE buffer and stained with ethidium bromide solution. Amplified products of multiplex PCR were verified by sequencing of Sangon Biotech (Shanghai, China).

Results

Specific primers for four *Fusarium* spp. were designed via whole genome sequence comparison

To detect DNA from *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* simultaneously, we screened specific primer combinations and established a multiplex PCR system (**Figure 1A**). First, whole genome sequence comparison analysis identified a 20 bp sequence located within a tRNA-lle gene in the genomes of four *Fusarium* strains. This 20 bp sequence is located at nucleotide positions 1,558,947 to 1,558,966, 1,532,724 to 1,532,743, 2,012,079 to 2,012,098, and 2,435,141 to 2,435,160 in *F. pseudograminearum* Class 2-1C (GenBank Accession No. CP064755.1), *F. graminearum* PH-1 (GenBank Accession No. HG970332.2), *F. proliferatum* ET1 (GenBank Accession No. NW_022194799.1), *F. verticillioides* 7600 (GenBank Accession No. CM000579.1) genome respectively (**Figure 1B**). This sequence was selected as an upstream universal primer (Fu-4F), and specific downstream primers (Fgram-R, Fpseu-R, Fprol-R, and Fvert-R) of four pathogens with different amplicon sizes were designed. The amplicon size of *F. graminearum*, *F. pseudograminearum*, *F. proliferatum* and *F. verticillioides* were 206 bp, 482 bp, 680 bp and 963 bp, respectively (**Figure 1 and Table 1**). In addition, the downstream primers matched only the sequence of the target pathogens.

Standardization of for the multiplex PCR system

We tested the effects of different primer concentrations, dNTPs, Mg^{2+} concentration and annealing temperature combinations on the efficiency of multiplex PCR amplified DNA from the target pathogens. Our results showed that more PCR product was amplified under following primer concentrations (Fu-4F: 0.8 μmol/L, Fgram-R: 0.2 μmol/L, Fpseu-R: 0.2 μmol/L, Fprol-R: 0.2 μmol/L, Fvert-R: 0.2 μmol/L) when 2 mM $MgCl_2$ and 0.2 mM dNTPs were added with 53℃ annealing temperature **(Figure 2)**.

DNA from four target pathogens were specifically and sensitively detected by multiplex PCR

Using the optimal multiplex PCR system, an unambiguous detection result was obtained by multiplex PCR using mixed or individual genomic DNA of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* as templates. This result indicates that the established multiplex PCR method could specifically detect DNA of 22 target strains from different hosts **(Figure 3 and Table 2)**. As expected, DNA from other 46 fungal pathogens had no amplified product **(Table 2 and Figure S1).**

In addition, the PCR detection limit for individual DNA was 10 pg for *F. verticillioides,* 1 pg for *F. proliferatum*, 100 pg for *F. pseudograminearum*, and 100 pg for *F. graminearum* (**Figure 4 B,C,D,E**). However, the detection limit for multiplex PCR was about 100 pg for DNA mixture from *F. verticillioides* , *F. proliferatum*, *F. pseudograminearum*, and *F. graminearum* (**Figure 4A**).

Multiplex PCR was successfully applied to detect pathogen DNA within wheat samples from the field and artificially inoculated samples

To determine the applicability of this multiplex PCR assay, we detected pathogen DNA in 22 wheat samples from the field and 24 artificially inoculated wheat samples. Our results showed that *F. pseudograminearum*, *F. graminearum*, and *F. verticillioides* were identified in 15, 10, and 3, respectively, of the 22 wheat samples from the field (**Figure 5A and Table S2**). Among them, 10 wheat samples were co-infected with *F. pseudograminearum* and *F. graminearum*, 2 wheat samples were co-infected with *F. pseudograminearum* and *F. verticillioides*, and 1 wheat sample was co-infected with *F. pseudograminearum*, *F. graminearum*, and *F. verticillioides* (**Figure 5A**). In addition, the results of the 24 artificially inoculated wheat samples were consistent as expected (**Figure 5B and Table S2**). The amplified products were further verified by sequencing.

Discussion

FCR is a common wheat disease caused by several *Fusarium* spp. (Meng et al., 2019; Scherm et al., 2013; Tunali et al., 2012). Due to different ecological environments in different regions, the composition of FCR-causing pathogens is also different. This makes phenotype-based and single PCR identification methods for FCR pathogen detection tedious and time-consuming. In this study, we designed a primer set via whole genome sequence comparison and developed a multiplex PCR assay to simultaneously detect DNA from four *Fusarium* species. This method will reduce the cost of pathogen analysis.

Multiplex PCR molecular detection methods have been applied to pathogen detection in medicine, environment, agricultural science, and other related fields (Ali et al., 2015; Asano et al., 2010; Rappo et al., 2016). Previously, multiplex PCR was reported to specifically identify *Fusarium* spp. *Rhizoctonia cerealis*, and *Bipolaris sorokiniana* based on ITS and TEF1-α (Sun et al., 2020). Previous reports have showed that multiplex PCR molecular detection method has enabled the simultaneous detection of DNA from *F. oxysporum* sp. *lycopersici*, *C. michiganensis* subsp. *michiganensis*, *L. taurica*, and begomoviruses on tomato plants (Quintero-Vásquez et al.,

2012). *F. verticillioides*, *F. subglutinans*, and other species of the *G. fujikuroi* complex were also identified by PCR assays (Faria et al., 2012). Multiplex PCR was reported to specifically identify *F. oxysporum*, *S. rolfsii*, and *L. theobromae* in Peanut (Wang et al., 2023). A multiplex method RT-PCR based on five primer pairs was developed for differentiation and simultaneous diagnosis of five *Porcine astroviruses* (Liu et al., 2021). Usually, multiplex PCR contains numerous primers, leading to primer cross binding and primer dimer formation. In this study, we designed a single common upstream primer for simultaneous amplification of DNA from four *Fusarium* strains by reducing the number of primers in the PCR system.

Primers are directly related to the specificity and sensitivity of PCR (Henegariu et al., 1997; Zhao et al., 2007). With the rapid development of genome sequencing technology and bioinformatics, comparative genomics can be used to identify new molecular detection targets of pathogens and design universal upstream primers for multiple pathogens to reduce the number of primers (Hu et al., 2020; Kim et al., 2015; Liu et al., 2023; Park et al., 2017; Yu et al., 2019). In this study, we identified a 20 bp sequence as a common forward primer based on comparative genomics to reduce the complexity of primers, and then specific downstream primers of *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides* were designed sequentially with different sequence fragment sizes. However, PCR application of primer combinations is complicated. We found that the length and G+C content of the primers affected the amplification efficiency when designed a multiplex PCR system. Therefore, we continuously adjusted the length and G+C content of the primer combination and finally designed a primer combination that could stably amplify *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides*.

The composition of PCR reagents and PCR conditions are key factors that influence multiplex PCR amplification (Zhao et al., 2007). In the process of multiplex PCR, different primers will compete for other reaction components to amplify target DNA, so it is necessary to optimize the concentration of primer combination in the reaction system to ensure simultaneous amplification of multiple targets (Markoulatos et al., 2002). In this study, we optimized the primer concentration as well as dNTPs, $MgCl_2$ and annealing temperature, which also affect multiplex PCR results (Markoulatos et al., 2002; Zhao et al., 2007). PCR systems with dNTPs at 0.2-0.4 mM are usually the most favorable for amplification, and amplification is rapidly inhibited above this value, while lower dNTP concentration (dNTPs at 0.1 mM) allows PCR amplification with reduced products (Markoulatos et al., 1999, 2002). In addition, optimization of Mg^{2+} is crucial as excessive Mg^{2+} concentration stabilizes DNA double strand and prevents complete denaturation of DNA, thus reducing amplification yield, while insufficient Mg^{2+} concentration would also reduce PCR product (Markoulatos et al., 2002; Deng et al., 2023). In this study, we optimized our PCR system with 2 mM $MgCl_2$, 0.2 mM dNTPs with the annealing temperature of 53°C in a 50 µl reaction.

In addition, we also analyzed the specificity, sensitivity, and detection limit of wheat samples based on the optimized reaction system and conditions. This study showed that multiplex PCR only amplified DNA of the target strains with the expected amplicon size, indicating that the

designed primer sets had high specificity for detection of the target pathogens. In addition, the detection limit of multiplex PCR for *F. graminearum*, *F. pseudograminearum*, and *F. verticillioides* and *F. proliferatum* was 100 pg, which can meet the requirements of low DNA concentration. Moreover, we successfully identified the presence of these *Fusarium* strains in the wheat samples from the field and the artificially inoculated wheat samples using the established multiplex PCR. These results indicated that this multiplex PCR detection method can be used to simultaneously detect *F. graminearum, F. pseudograminearum, F. proliferatum* and *F. verticillioides*.

Conclusion

We developed primer sets for *F. graminearum*, *F. pseudograminearum*, *F. proliferatum*, and *F. verticillioides* via whole genome sequence comparison and established a multiplex PCR method for simultaneous identification of four *Fusarium* spp. in a single PCR. This ability to detect four target pathogens in a single reaction is more cost-effective and saves time. Multiplex PCR system can specifically identify four target pathogens, but not 46 other fungal pathogens, with the detection limit of four target pathogens at 100 pg/μl. In addition, we accurately identified FCR pathogen species in wheat samples using the optimized multiplex PCR method. Therefore, the multiplex PCR method described here is a useful tool for diagnosing FCR pathogen species.

Reference

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Figure legends:

Figure 1. Schematic design and location of primers for multiplex PCR detection of four *Fusarium* strains. (A) The diagram represents the genomics sequences used to design the primers based on comparative genomics. Arrows indicate the positions and directions of the primers. **(B)** The genomic regions of *F. pseudograminearum*, *F. graminearum*, *F. verticillioides* and *F. proliferatum* used to design the universal upstream primer and specific downstream primers. Homologous bases are shaded in black. Each designed primer was marked with red rectangle. Fu-4F: Universal upstream primer. Fgram-R, Fpseu-R, Fprol-R, and Fvert-R: Specific downstream primers. Arrows indicate the positions and directions of the primers.

Figure 2. Multiplex PCR amplification at different PCR reagent composition and conditions. (A) Primer concentration ratio between the common forward primer Fu-4F and the specific reverse primer: 1:1 (group I), 2:1 (group II), 3:1 (group III) and 4:1 (group Ⅳ). Lane M: 2000 bp DNA ladder, Lanes 1-4: concentration of each primer in group I, Lanes 5-8: . concentration of each primer in group II, Lanes 9-12: concentration of each primer in group III, Lanes 13-16: concentration of each primer in group Ⅳ. **(B)** $MgCl_2$ concentrations. Lane M: 2000 bp DNA ladder, lanes 1-8: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 mM, respectively. **(C)** dNTP concentrations. Lane M: 2000 bp DNA ladder, lanes 1-8: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mM, respectively. **(D)** Gradients of annealing temperature. Lane M: 2000 bp DNA ladder, Lanes 1-12: 45, 46.1, 47.7, 50.5, 53, 55, 57.2, 59.4, 61.6, 63.4, 64.6 and 65 °C. Red rectangle indicates the optimal reaction system and conditions of multiplex PCR.

Figure 3. Specificity of multiplex PCR. Multiplex PCR primer sets only amplified the DNAs for *F. graminearum*, *F. pseudograminearum*, *F. proliferatum* and *F. verticillioides*. M: DL2000 marker; Mix: mixed DNA samples from the four *Fusarium* species; Lanes 1-3: *F. verticillioides*. Lanes 4-8: *F. proliferatum*. Lanes 9-16: *F. pseudograminearum*. Lanes 17-22: *F. graminearum*.

Figure 4. Sensitivity of multiplex and single PCR assay. (A) sensitivity of multiplex PCR assay for *F. graminearum*, *F. pseudograminearum*, *F. proliferatum* and *F. verticillioides* at

100pg/μL. **(B)** sensitivity of PCR assay with Fu-4F/Fgram-R primer for *F. graminearum* at 10pg/μL. **(C)** sensitivity of PCR assay with Fu-4F/Fpseu-R primer for *F. pseudograminearum* at 1pg/μL. **(D)** sensitivity of PCR assay with Fu-4F/Fprol-R primer for *F. proliferatum* at 100pg/μL. **(E)** sensitivity of PCR assay with Fu-4F/Fvert-R primer for *F. verticillioides* at 100pg/μL. Lane M: 2000bp DNA ladder, Lanes 1-7: 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 100 fg/μl, and 10fg/μl pure genomic DNA.

Figure 5. Multiplex PCR detection of four *Fusarium* strains in wheat samples from the field and artificially inoculated samples. (A) target pathogen strains were detected in wheat samples from the field at Xiangyang and Suizhou,Hubei province using multiplex PCR assay. Lane M: 2000bp DNA ladder, PC: positive control, NC: negative control, Lane 1-22: wheat samples. (B) target pathogen strains were detected in artificially inoculated wheat samples using multiplex PCR assay. Lane M: 2000 bp DNA ladder, Lanes 1-24: wheat samples. where lanes 10, 11, 14, 15, 16, 20, and 21: wheat samples were inoculated with sterile water.

SUPPORTING MATERIAL

Figure S1. Forty-six fungi pathogens with no amplified product. M: DL2000 marker; PC: positive control; Lanes 1-46: *Fusarium solani, Fusarium incarnatum, Fusarium equiseti, Fusarium oxysporum, Fusarium oxysporum, Fusarium oxysporum, Fusarium oxysporum, Fusarium oxysporum, Fusarium oxysporum, Fusarium humuli, Fusarium brachygibbosum, Fusarium fujikuroi, Alternaria alternata, Alternaria spp, Ascochyta pisi Libert, Botryophaeria dothidea, Botrytis cinerea, Botrytis cinerea, Cercospora kikuchii, Colletorichum lagenerium, Colletotrichum gloeosporioides, Diaporthe phaseolorum, Glomerella cingulata, Leptosphaeria biglobosa , Leptosphaeria maculans , Mycosphaerella melonis, Mycosphaerella melonis, Ophiostoma ulmi, Pestalotiopsis theae, Phellinidium lsulphurascens, Phialophora gregata, Phoma pinodella, Phoma spp, Phomopsis amygdali, Phomopsis fukushii, Phomopsis helianthi, Phomopsis longicolla, Phomopsis truncicola, Rhizoctonia cerealis, Rhizopus oryzae, Sclerotinia sclerotiorum, Sclerotium rolfsii, Stenocarpella maydis, Verticillium albo-atrum, Verticillium dahliae, Verticillium dahliae.*

Table S1. Final concentration of primers in the multiplex PCR.

Table S2. Multiplex PCR detection of target pathogen DNA within wheat samples.

Table 1(on next page)

Primers used in the multiplex PCR.

Table 1. Primers used in the multiplex PCR.

Target organisms	Primer	Primer Sequence(5'-3')	Length of production
Fusarium spp.	Fu-4F	CTTGAACCTGAGACCTTCGC	
Fusarium graminearum	Fgram-R	CTCATAGCGATATTCTCGTATAC	206 bp
Fusarium pseudograminearum	Fpseu-R	CGCACATTGCTTATTGCTTA	482 bp
Fusarium proliferatum	Fprol-R	ATTCACGGATGAGAATCAAG	680 bp
Fusarium verticillioides	Fvert-R	TCAAAGGAATGTCCGGTAGA	963 bp

Table 2(on next page)

List of fungal strains used in study.

Table 2. List of fungal strains used in study.

Serial number	Strains [a]	Host species	Source [b]	Amplification Result [c]
	Target pathogens			
1	*Fusarium graminearum**	Wheat	HBAAS	+
2	*Fusarium graminearum**	Wheat	HBAAS	+
3	*Fusarium graminearum**	Maize	HBAAS	+
4	*Fusarium graminearum**	Maize	HBAAS	+
5	*Fusarium graminearum**	Maize	HBAAS	+
6	*Fusarium graminearum**	Rice	HBAAS	+
7	*Fusarium pseudograminearum**	Wheat	HBAAS	+
8	*Fusarium pseudograminearum**	Wheat	HBAAS	+
9	*Fusarium pseudograminearum**	Wheat	HBAAS	+
10	*Fusarium pseudograminearum**	Wheat	HBAAS	+
11	*Fusarium pseudograminearum**	Wheat	HBAAS	+
12	*Fusarium pseudograminearum**	Maize	HBAAS	+
13	*Fusarium pseudograminearum**	Maize	HBAAS	+
14	*Fusarium pseudograminearum**	Soil	HBAAS	+
15	*Fusarium proliferatum**	Wheat	HBAAS	+
16	*Fusarium proliferatum**	Wheat	HBAAS	+
17	*Fusarium proliferatum**	Maize	HBAAS	+
18	*Fusarium proliferatum**	Soil	HBAAS	+
19	*Fusarium proliferatum**	Soil	HBAAS	+
20	*Fusarium verticillioides**	Wheat	HBAAS	+
21	*Fusarium verticillioides**	Wheat	HBAAS	+
22	*Fusarium verticillioides**	Maize	HBAAS	+
	Other pathogens			
1	*Fusarium solani*	Tomato	HBAAS	−
2	*Fusarium incarnatum*	Tomato	HBAAS	−
3	*Fusarium equiseti*	Pepper	HBAAS	−
4	*Fusarium oxysporum*	Wheat	HBAAS	−
5	*Fusarium oxysporum*	Tomato	YLNU	−
6	*Fusarium oxysporum*	Pepper	HBAAS	−
7	*Fusarium oxysporum*	Watermelon	NJAU	−
8	*Fusarium oxysporum*	Tobacco	NJAU	−
9	*Fusarium oxysporum*	Cucumber	NJAU	−
10	*Fusarium humuli*	Tomato	HBAAS	−
11	*Fusarium brachygibbosum*	Tomato	HBAAS	−
12	*Fusarium fujikuroi*	Rice	HBAAS	−
13	*Alternaria alternata*	Tomato	HBAAS	−
14	*Alternaria spp*	Liriodendron	NJAU	−

		chinese		
15	*Ascochyta pisi Libert*	Pea	NJAU	−
16	*Botryophaeria dothidea*	Peach	NJAU	−
17	*Botrytis cinerea*	Strawberry	NJAU	−
18	*Botrytis cinerea*	Cucumber	NJAU	−
19	*Cercospora kikuchii*	Soybean	NJAU	−
20	*Colletorichum lagenerium*	Watermelon	NJAU	−
21	*Colletotrichum gloeosporioides*	Pear	NJAU	−
22	*Diaporthe phaseolorum*	Soybean	NJAU	−
23	*Glomerella cingulata*	Tea	NJAU	−
24	*Leptosphaeria biglobosa*	Oilseed rape	NJAU	−
25	*Leptosphaeria maculans*	Oilseed rape	NJAU	−
26	*Mycosphaerella melonis*	Watermelon	NJAU	−
27	*Mycosphaerella melonis*	Cucumber	NJAU	−
28	*Ophiostoma ulmi*	Elm	NJAU	−
29	*Pestalotiopsis theae*	Tea	NJAU	−
30	*Phellinidium lsulphurascens*	Pine	NJAU	−
31	*Phialophora gregata*	Soybean	NJAU	−
32	*Phoma pinodella*	Pea	NJAU	−
33	*Phoma spp*	Jujube	NJAU	−
34	*Phomopsis amygdali*	Peach	NJAU	−
35	*Phomopsis fukushii*	Pear	NJAU	−
36	*Phomopsis helianthi*	Sunflower	NJAU	−
37	*Phomopsis longicolla*	Soybean	NJAU	−
38	*Phomopsis truncicola*	Apple	NJAU	−
39	*Rhizoctonia cerealis*	Wheat	JAAS	−
40	*Rhizopus oryzae*	Soil	NJAU	−
41	*Sclerotinia sclerotiorum*	Cauliflower	HBAAS	−
42	*Sclerotium rolfsii*	Pepper	HBAAS	−
43	*Stenocarpella maydis*	Maize	NJAU	−
44	*Verticillium albo-atrum*	Alfalfa	NJAU	−
45	*Verticillium dahliae*	Tomato	NWAFU	−
46	*Verticillium dahliae*	Wheat	NJAU	−

[a] Asterisks (*) indicate the target pathogens

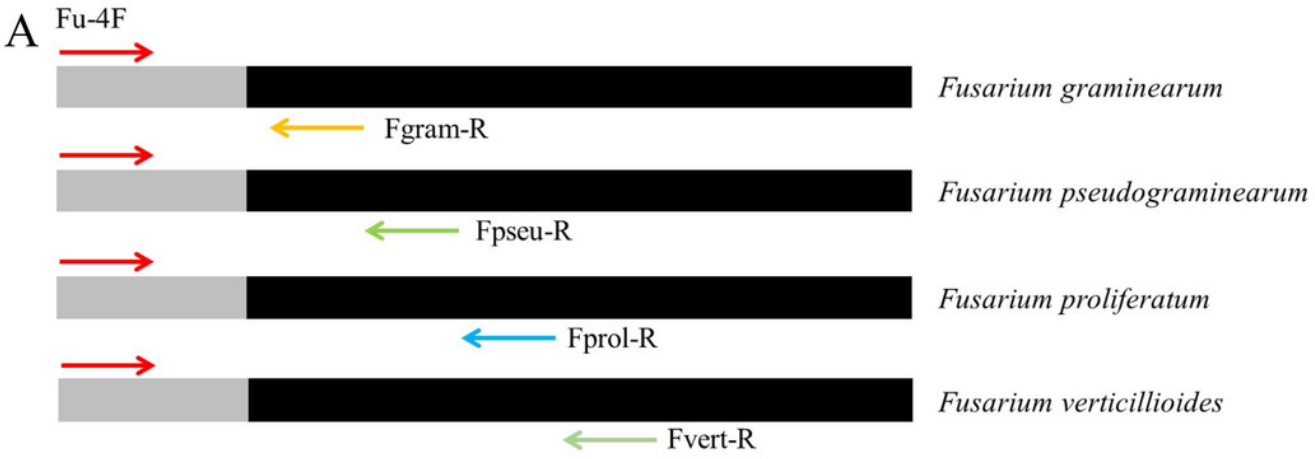
[b]HBBAS=Hubei Academy of Agricultural Sciences; JAAS=Jiangsu Academy of Agricultural Sciences; NWAFU=Northwest Agriculture and Forestry University; NJAU=Nanjing Agricultural University; YLNU=Yulin Normal University; HBAAS=Hubei Academy of Agricultural Sciences

[c]Specificity test results of multiplex PCR are indicated as positive (+) or negative (−).

Figure 1

Schematic design and location of primers for multiplex PCR detection of four *Fusarium* strains.

(A) The diagram represents the genomics sequences used to design the primers based on comparative genomics. Arrows indicate the positions and directions of the primers. **(B)** The genomic regions of *F. pseudograminearum*, *F. graminearum*, *F. verticillioides* and *F. proliferatum* used to design the universal upstream primer and specific downstream primers. Homologous bases are shaded in black. Each designed primer was marked with red rectangle. Fu-4F: Universal upstream primer. Fgram-R, Fpseu-R, Fprol-R, and Fvert-R: Specific downstream primers. Arrows indicate the positions and directions of the primers.


A
Fu-4F
Fusarium graminearum
Fgram-R
Fusarium pseudograminearum
Fpseu-R
Fusarium proliferatum
Fprol-R
Fusarium verticillioides
Fvert-R

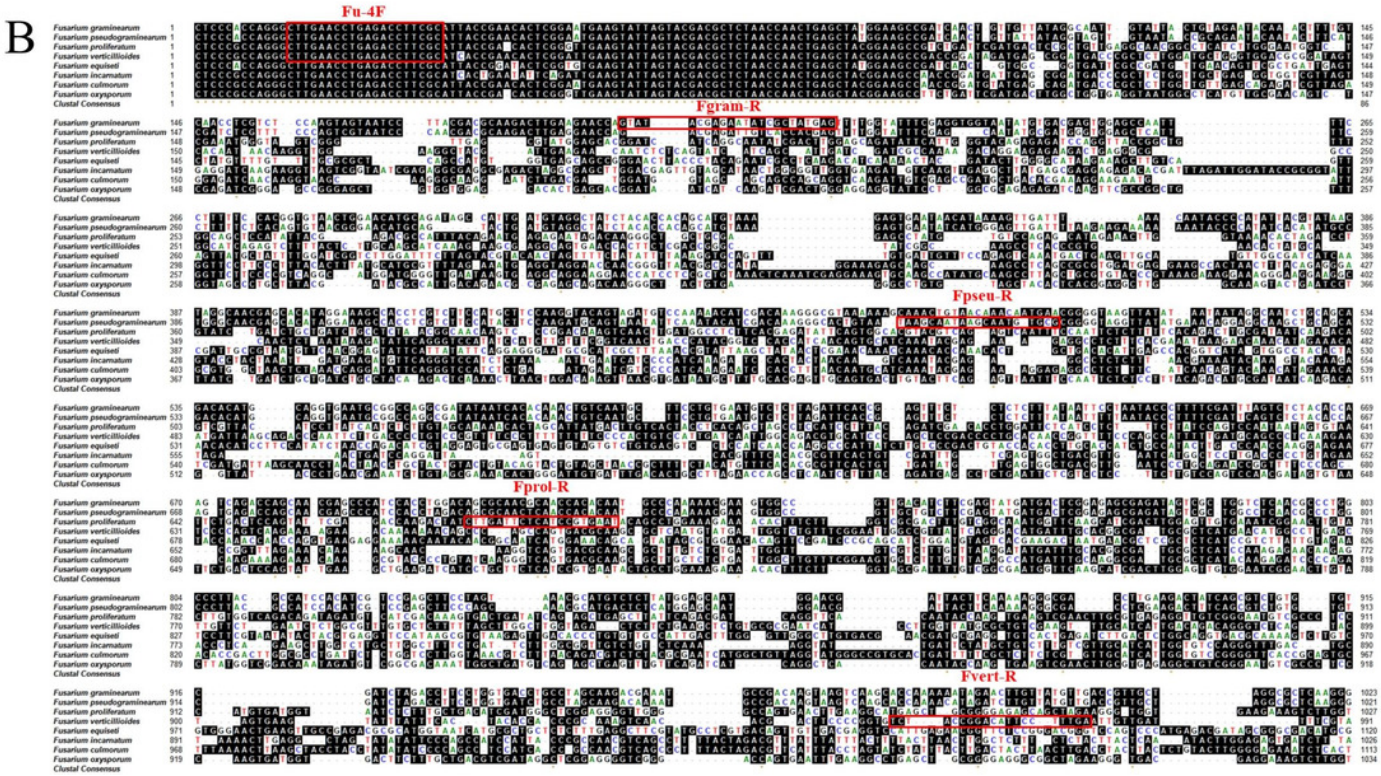

B
Fu-4F
Fgram-R
Fpseu-R
Fprol-R
Fvert-R

Figure 2

Multiplex PCR amplification at different PCR reagent composition and conditions.

(A) Primer concentration ratio between the common forward primer Fu-4F and the specific reverse primer: 1:1 (group I), 2:1 (group II), 3:1 (group III) and 4:1 (group IV). Lane M: 2000 bp DNA ladder, Lanes 1-4: concentration of each primer in group I, Lanes 5-8: . concentration of each primer in group II, Lanes 9-12: concentration of each primer in group III, Lanes 13-16: concentration of each primer in group IV. **(B)** $MgCl_2$ concentrations. Lane M: 2000 bp DNA ladder, lanes 1-8: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 mM, respectively. **(C)** dNTP concentrations. Lane M: 2000 bp DNA ladder, lanes 1-8: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mM, respectively. **(D)** Gradients of annealing temperature. Lane M: 2000 bp DNA ladder, Lanes 1-12: 45, 46.1, 47.7, 50.5, 53, 55, 57.2, 59.4, 61.6, 63.4, 64.6 and 65℃. Red rectangle indicates the optimal reaction system and conditions of multiplex PCR.

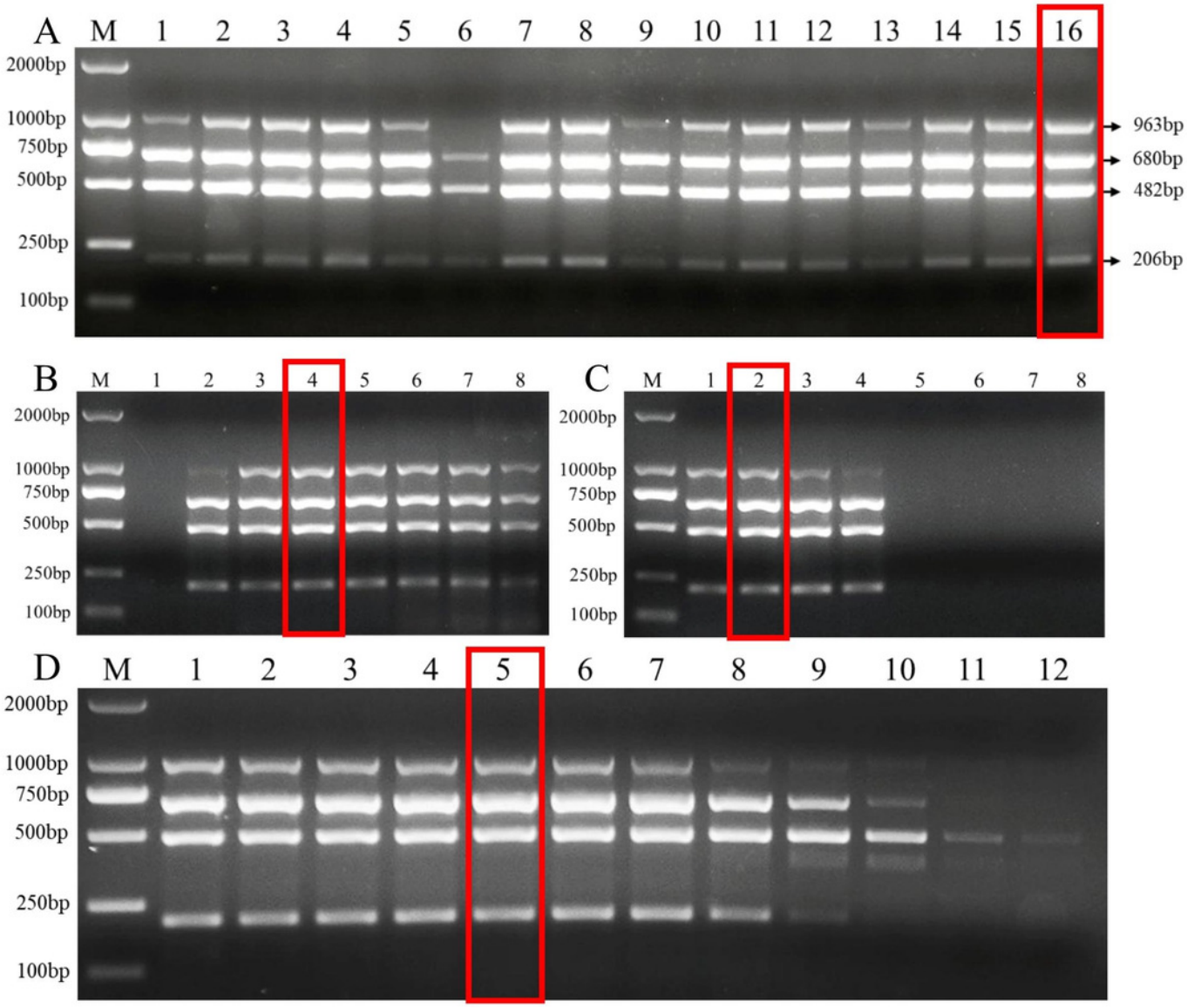

A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2000bp
1000bp
750bp
500bp
250bp
100bp
963bp
680bp
482bp
206bp
B
M 1 2 3 4 5 6 7 8
2000bp
1000bp
750bp
500bp
250bp
100bp
C
M 1 2 3 4 5 6 7 8
2000bp
1000bp
750bp
500bp
250bp
100bp
D
M 1 2 3 4 5 6 7 8 9 10 11 12
2000bp
1000bp
750bp
500bp
250bp
100bp

Figure 3

Specificity of multiplex PCR.

Multiplex PCR primer sets only amplified the DNAs for *F. graminearum*, *F. pseudograminearum*, *F. proliferatum* and *F. verticillioides*. M: DL2000 marker; Mix: mixed DNA samples from the four *Fusarium* species; Lanes 1-3: *F. verticillioides*. Lanes 4-8: *F. proliferatum*. Lanes 9-16: *F. pseudograminearum*. Lanes 17-22: *F. graminearum*.

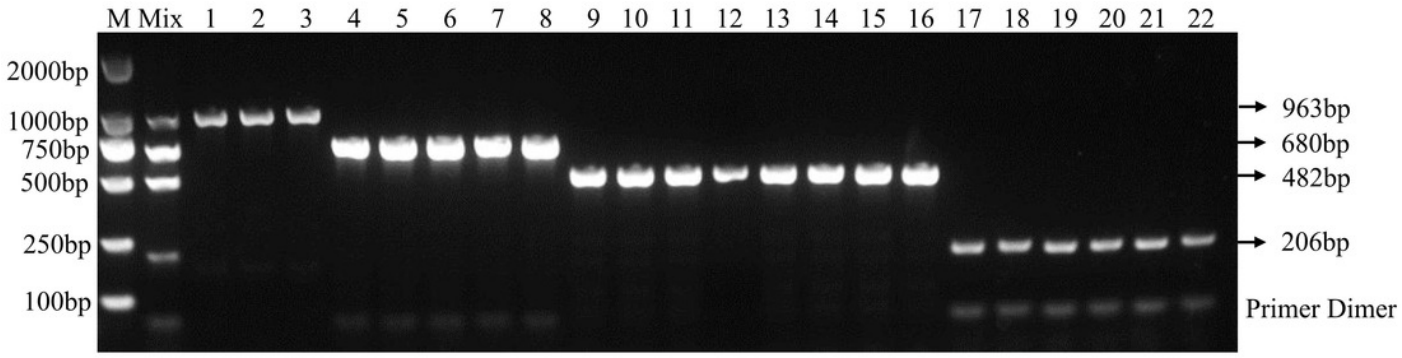


Figure 4

Sensitivity of multiplex and single PCR assay.

(A) sensitivity of multiplex PCR assay for *F. graminearum*, *F. pseudograminearum*, *F. proliferatum* and *F. verticillioides* at 100pg/µL. **(B)** sensitivity of PCR assay with Fu-4F/Fgram-R primer for *F. graminearum* at 10pg/µL. **(C)** sensitivity of PCR assay with Fu-4F/Fpseu-R primer for *F. pseudograminearum* at 1pg/µL. **(D)** sensitivity of PCR assay with Fu-4F/Fprol-R primer for *F. proliferatum* at 100pg/µL. **(E)** sensitivity of PCR assay with Fu-4F/Fvert-R primer for *F. verticillioides* at 100pg/µL. Lane M: 2000bp DNA ladder, Lanes 1-7: 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 100 fg/µl, and 10fg/µl pure genomic DNA.

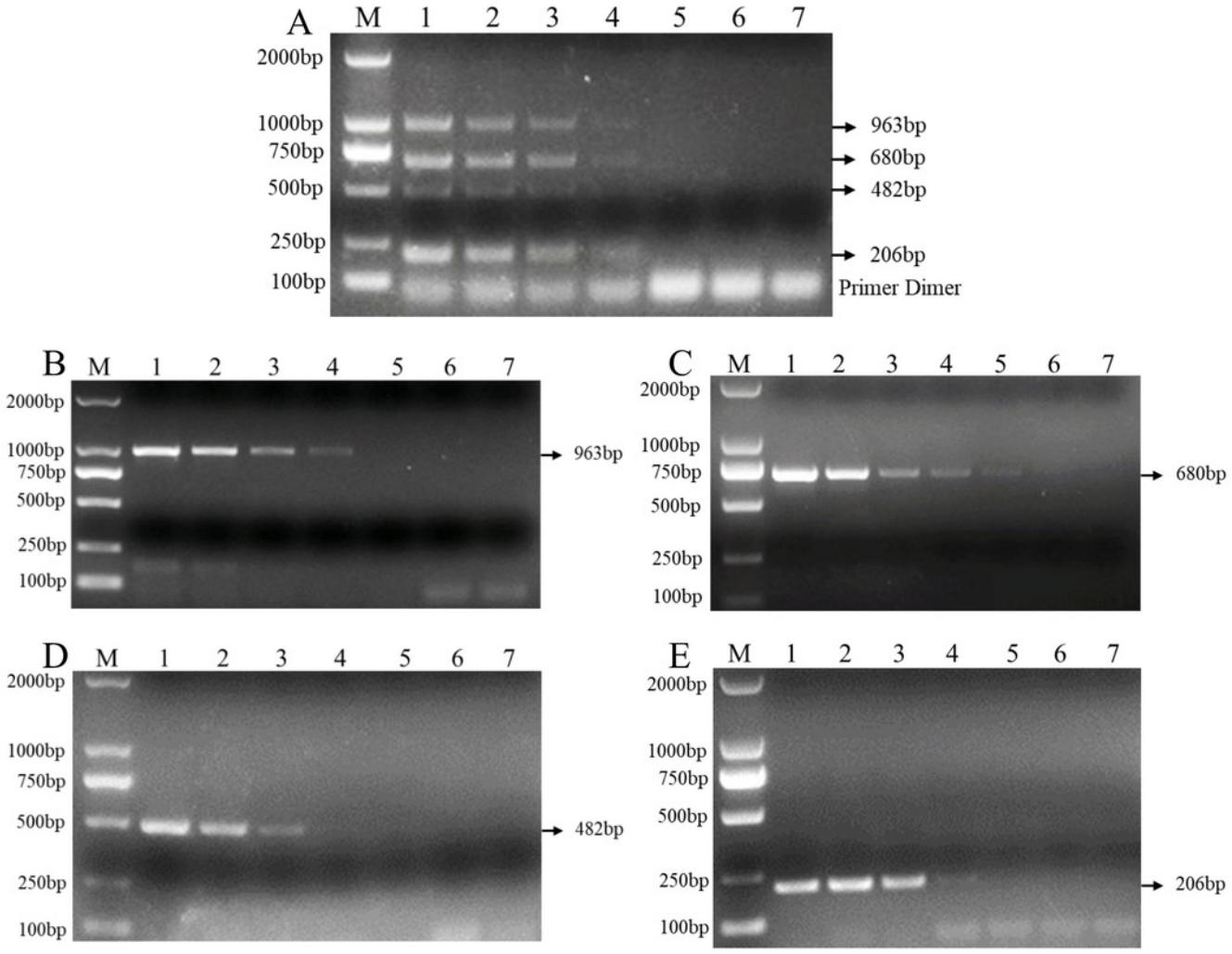

A
M 1 2 3 4 5 6 7
2000bp
1000bp
750bp
500bp
250bp
100bp
963bp
680bp
482bp
206bp
Primer Dimer
B
M 1 2 3 4 5 6 7
2000bp
1000bp
750bp
500bp
250bp
100bp
963bp
C
M 1 2 3 4 5 6 7
2000bp
1000bp
750bp
500bp
250bp
100bp
680bp
D
M 1 2 3 4 5 6 7
2000bp
1000bp
750bp
500bp
250bp
100bp
482bp
E
M 1 2 3 4 5 6 7
2000bp
1000bp
750bp
500bp
250bp
100bp
206bp

Figure 5

Multiplex PCR detection of four *Fusarium* strains in wheat samples from the field and artificially inoculated samples.

(A) target pathogen strains were detected in wheat samples from the field at Xiangyang and Suizhou,Hubei province using multiplex PCR assay. Lane M: 2000bp DNA ladder, PC: positive control, NC: negative control, Lane 1-22: wheat samples. (B) target pathogen strains were detected in artificially inoculated wheat samples using multiplex PCR assay. Lane M: 2000 bp DNA ladder, Lanes 1-24: wheat samples. where lanes 10, 11, 14, 15, 16, 20, and 21: wheat samples were inoculated with sterile water.