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Detection and quantification of *Uromyces setariae-italicae* in foxtail millet by a real-time PCR assay

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Research Article

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ABSTRACT

Rust is a plant disease that has posed a serious problem to foxtail millet cultivation in Northern China since 1980. In this study, we developed a real-time fluorescence, quantitative PCR assay to accurately quantify genomic DNA of *U. setariae-italicae* based on the ITS region of rDNA gene. The resulting assay was highly species-specific, and was insensitive to DNA of other fungal isolates. The limit of quantification of the assay was 10 fg/µl, and it was able to detect the rust fungus in foxtail millet leaves 6 h post inoculation. This work is mainly about the development of a molecular tool for the detection of *U. setariae-italicae* and will be helpful in diagnosis of rust infections in foxtail millet.

INTRODUCTION

Foxtail millet *(Setaria italica Beauv.)* is a diploid and self-pollination cereal crops [1]. It is reported that foxtail millet originated in China and had a history of more than 7300 years [2]. Rust is a serious plant disease that affects foxtail millet (*Setaria italica* (L.) *P. Beauv*.) around the world. It is caused by fungal pathogens in the order Pucciniales (formerly Uredinales). The important food crop foxtail millet is widely parasitized by *Uromyces setariae-italicae* Yosh; in rust epidemic years, yields are impacted significantly, with susceptible varieties experiencing losses of more than 30%, even to the extreme of complete loss of production in regions that are most affected [3,4].

U. setariae-italicae has a wide range of hosts, including the genera *Brachiaria, Cyrtococcum, Eriochloa, Melinis, Ottochloa, Panicum, Paspalidium, Setaria and Urochloa* [3]. Among species of *Setaria, S. italica, S. viridis, S. verticillata, S. geniculata, S. pallidefusca, S. poiretiana, S. rubiginosa and S. spacelata* have all been recorded as hosts of the rust [5,6]. It is known especially as a pathogen of *S. italica* due to the economic importance of this plant. Yet, despite its prominent role as a plant pathogen, a detailed modern molecular study of *U. setariae-italicae* is still lacking and modern techniques for detection are needed.

Rapid detection and correct identification are important for the prevention of plant diseases. Rust fungal species are difficult to identify in early stages of disease development based on disease symptoms or urediniospore morphology. Molecular methods, such as conventional polymerase chain reaction (PCR) with sequence analysis, can be useful, but are often labor intensive and can require several days to confirm the identity of a sample. Real-time PCR is an established diagnostic technique that facilitates plant disease diagnosis [7,8,9]. Apart from delivering quantitative data, qPCR is often more sensitive and faster than conventional PCR, making it suitable for quantification of pathogen DNA in infected host plant tissues.

The specific objective of this study is to develop a specific and sensitive qPCR assay for the rapid quantification of *U. setariae-italicae* in foxtail millet samples.

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MATERIAL AND METHODS

Collection of fungi

Isolates of the pathogen *U. setariae-italicae*, and other plant pathogens including *Sclerospora graminicola, Pyricularia setariae, Rhizoctonia solani, Ustilago crameri, Bipolaris setariae, Curvularia lunata* and *Cercospora setariae* were collected from foxtail millet plantation located in Shijiazhuang, Hebei Province and deposited at the Millet Institute, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang, China. Other rust species including *Puccinia striiformis, P. graminis, P. recondite, P. polysora, and P. sorghi* were also deposited at the Millet Institute, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang, China.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from the urediniospores as previously described by Wang et al. [10]. Genomic DNA of other pathogens was extracted in a similar way. The rDNA ITS region was amplified using the universal primer pair ITS1 5'-TCCGTAGGTGAACCTGCGG -3' and ITS4 5'- TCCTCCGCTTATTGATATGC-3'. Amplification was carried out in 25-µl volumes that contained 12.5 µl 2 × Taq MasterMix (Beijing ComWin Biotech Co., Ltd., China), 1 µl of each primer (10 µM), 1 µl DNA template and 9.5 µl sterile highly purified water. Thermal cycling condition was an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. PCR amplification products were analyzed in 1.5% agarose gel and gel-purified using a TIANgel Midi Purification Kit (TIANGEN Biotech Co. Ltd, Beijing, China) following the manufacturer's protocol. Purified PCR products were ligated into a pMD19-T vector (TaKaRa Bio Inc, Dalian, China) and transformed into *Escherichia coli* DH5α. Positive clones were selected, and recombinant plasmids were sequenced by Sangon Biotech Co., Ltd (Shanghai, China). The resulting DNA sequence was submitted to GenBank.

Primer design and specificity test of Q-PCR

Primer sequences for Q-PCR analysis of U. *setariae-italicae* were based on the sequenced ITS rDNA region. Sequences for the forward and reverse primer were RT-F (5'- GGTGCACTTAATTGTGGCTCAA -3') and RT-R (5'- CTCCTTTTCTTTTTCCCTCTCAAA -3'). Q-PCR amplification and detection of samples was conducted with an AB StepOne Real-Time PCR System. The Q-PCR system with SYBR Green detection was optimized for 20 µl reactions containing 10 µl 2 × SYBR Premix (TaKaRa, Dalian, China), 0.4 µl ROX Reference Dye, 0.8 µl of each primer (10 µM), 2 µl cDNA template (100 ng/µl), and 6 µl nuclease free water, according to the manufacturer's guidelines. Control reactions contained the same mixtures, with 2 µl of sterile water replacing the DNA template; three technical replicates were performed per sample. Amplification of all samples was based on the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. After each run, a melting curve of the product was acquired to check for amplification specificity by increasing the temperature to 95°C for 15 s, lowering it to 60°C for 1 min, and then increasing it by 0.3°C/s to 95°C for 15 s with continuous measurement of fluorescence. To determine whether the primers were specific to U. *setariae-italicae*, they also were tested against the DNA of other rust species including *Puccinia striiformis, P. graminis, P. recondite, P. polysora, and P. sorghi*, and other fungal isolates on *S. italica* including *Sclerospora graminicola, Pyricularia setariae, Rhizoctonia solani, Ustilago crameri, Bipolaris setariae, Curvularia lunata* and *Cercospora setariae* using conventional PCR and Q-PCR.

Sensitivity test and generation of the standard curve

We used a 10-fold dilution series from 10 ng/µl to 10fg/µl of *U. setariae-italicae* DNA to test the sensitivity of the primer pair. The standard curve for this rust species was generated by plotting the log of the known DNA concentration on the x-axis against the C_t value generated by the second derivative (2^{nd} derivative-C_t) on the y axis. The simple linear regression line, Q-PCR reaction efficiency, coefficient of determination (R^2) and standard deviation of each DNA concentration were calculated using AB Step one software (version 2.0).

Quantitative detection of pathogen in the millet leaves

The foxtail millet variety 'Yugu1' was inoculated with the rust fungus. When the plants were at the growth stage with six or seven leaves, inoculation was performed by spraying rust urediniospores (5×10^6 spores/ml) on leaves. Next, plants were incubated for 48 h at 28°C in darkness at 95% relative humidity, and then incubated at 28°C under a photoperiod of 14 h light and 10 h dark with a light intensity of 6000 lux. Leaf samples were taken at 0, 6, 18, 30, 36, 42 and 72 h post inoculation. Genomic DNA of foxtail millet leaves was extracted as above. Quantity of pathogen in millet leaves was calculated according to the standard curve of genomic DNA from *Uromyces setariae-italicae.*

RESULTS

Amplification, cloning and sequencing

PCR amplification of the rDNA ITS region was successful, and resulted in PCR product of 727 bp. The DNA sequence was submitted to GenBank, with accession codes KJ755671.

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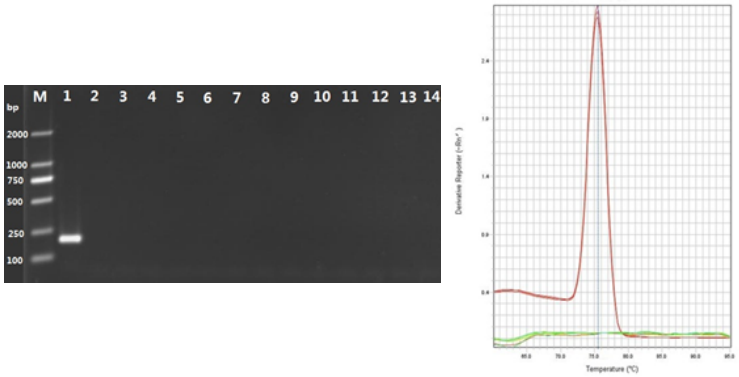
Specificity of Q-PCR primers

The specificity of the primer pair was tested against the DNA of other plant pathogens. The specific PCR fragment was amplified from the DNA of *U. setariae-italicae,* but not from the DNA of other fungal isolates and a negative control **(Figure 1A).** The Q-PCR assays also did not amplify DNA from other non-target plant pathogens, as evidenced by the fact that there was only one peak in the dissociation curve **(Figure 1B).**

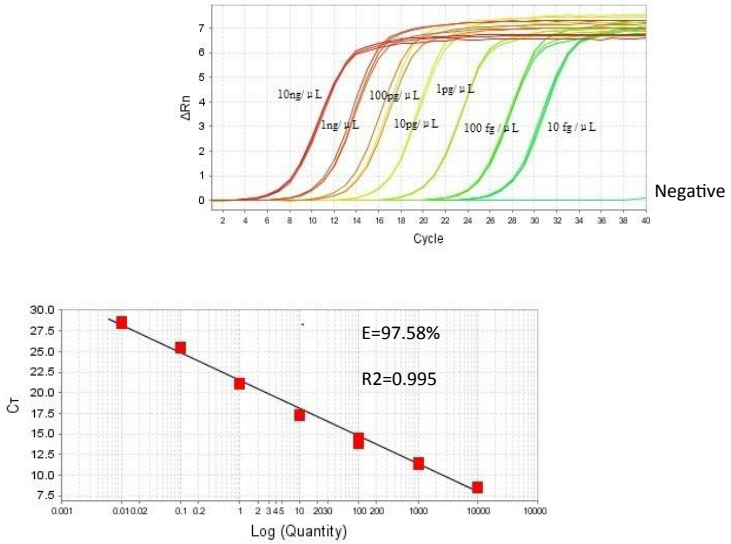


(A) M, DL2000 Marker; lane 1, *U. setariae-italicae*; lane 2, *Puccinia striiformis*; lane 3, *P. graminis*; lane 4, *P. recondite*; lane 5, *P. polysora*; lane 6, *P. sorghi*; lane 7, *Sclerospora graminicola*; lane 8, *Pyricularia setariae*; lane 9, *Rhizoctonia solani*; lane 10, *Ustilago crameri*; lane 11, *Bipolaris setariae*; lane 12, *Curvularia lunata*; lane 13, *Cercospora setariae*; and lane 14, negative control. (B) Dissociation curve analysis of amplicon produced by *U. setariae-italicae* primers; peak=*U. setariae-italicae*, Tm=75.49°C.

Figure 1. Primer specificity test for *Uromyces setariae-italicae*.

Sensitivity of Q-PCR assay and standard curve

The sensitivity of the Q-PCR assay was evaluated using serial dilutions of genomic DNA standards of known concentrations (from 10 ng/µl to10 fg/µl) **(Figure 2A).** A standard curve was generated using a range of genomic DNA standards from 104 pg/µl to 10-2 pg/µl. Quantification showed a linear association (R^2=0.995) between the log of the DNA concentration and the Ct value over the range of DNA concentrations examined. The amplification efficiency with pure template DNA was 97.58% **(Figure 2B).**



(A) Sensitivity detection of *Uromyces setariae-italicae*-specific primers using a series of template DNA dilutions. DNA concentrations from left to right are 10 ng/µl, 1 ng/µl, 100 pg/µl, 10pg/µl, 1 pg/µl, 100 g/µl, and 10 fg/µl. (B) Standard curve for Q-PCR of genomic DNA from *Uromyces setariae-italicae*. A DNA standard series from 104 pg/µl to 10-2 pg/µl was used to generate the graphs. Each DNA concentration was tested in triplicate.

Figure 2. Sensitivity detection of Uromyces setariae-italicae-specific primers and standard curve of genomic DNA from Uromyces setariae-italicae.

Assessment of *Uromyces setariae-italicae* development in inoculated foxtail millet leaves

Concentration of DNA as low as 10 fg/µl could be detected indicating that the assay was highly sensitive **(Figure 2A)** .The standard curve of genomic DNA from *Uromyces setariae-italicae* showed a linear relationship between the log of the DNA concentration and the C_t value **(Figure 2B).** Thus, the quantification system was successfully applied to detect the rust fungus on foxtail millet.

In our study, *U. setariae-italicae* was detectable on foxtail millet 6 hours after inoculation with uredinospores, and the amount of fungal DNA in each gram of millet leaves was 0.03 ng **(Figure 3).** With the increase of inoculation time, the amount of fungal DNA increased in millet leaves. Growth of the pathogen was rapid, and was associated with extensive lesion development.

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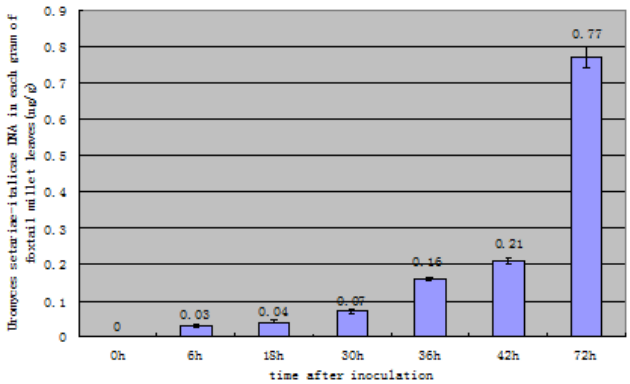

The data are the means, and bars represent standard errors of the means.

Figure 3. Growth dynamics of Uromyces setariae-italicae illustrated by the amount of DNA detected in infected foxtail millet leaves after inoculation.

DISCUSSION

Real-time PCR-based fungal quantification assays of other plant pathogen have been previously published. Four common cereal and grass rust pathogens, *Puccinia graminis, P. recondita f. sp. secalis, P. striiformis,* and *P. triticina*, were identified and detected using Q-PCR [11]. Gachon et al. developed a highly sensitive Q-PCR assay to score disease progression on Arabidopsis thaliana infected with the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* [12]. Our report is the first to successfully detect and quantify the pathogen *U. setariae-italicae* on foxtail millet in China using real-time PCR. The sensitivity of real-time PCR is higher than standard PCR. Previously, Wang et al. used regular PCR to detect *Puccinia striiformis*, achieving a detection limit of 10 pg of DNA [10]. When Pan et al. used real-time PCR to assess the same species; the detection limit was 100 fg, so sensitivity had increased 100-fold [13]. The limit of quantification of the Q-PCR primer pair reported here is 10 fg genomic DNA, providing an excellent sensitivity with this technique. Similarly, Barnes et al. reported the ability to detect 0.053 pg *P. graminis* DNA using real-time PCR assays, and Guo et al. detected *Rhizoctonia cerealisat* quantities as low as 100 fg of purified pathogen DNA using real-time PCR [11,14]. Such improved sensitivity is an important development because it enables detection of small quantities of the target pathogen's DNA, and hence improved the assessment of rust fungal development in foxtail millet tissues from the very first stages of infection. In our study, the rDNA ITS region amplified used the primer pair ITS1 and ITS4, but that primer pair was not suitable for Q-PCR because it produced an amplicon of 727 bp, which is too long for Q-PCR [15]. Hence, we developed a suitable primer pair from the sequenced ITS region, to be specific for *U. setariae-italicae*. The pair was tested against DNA of other rust species and plant fungi, and no amplified DNA was detected from any of these species, indicating that the designed primers were indeed specific to the DNA of our target species.

The method for detection and quantification of *U. setariae-italicae* that we developed will serve as a useful tool for future study of this pathogen species. These Q-PCR methods have potential for use in monitoring the rust infection of foxtail millet tissues, forecasting the development of fungi epidemics, and studying fungi–plant interactions.

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