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Development of *Penicillium italicum*-Specific Primers for Rapid Detection among Fungal Isolates in Citrus [S]

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Blue mold in citrus is caused by *Penicillium italicum*. In this study, the *P. italicum*-specific primers were developed for rapid detection based on the conserved genes *RPB1* and *RPB2* among *Penicillium* genomes. The two primer pairs RPB1-a and RPB1-b proved to be specific to detect *P. italicum*. The PCR assay among 39 fungal isolates and the colonial, pathogenic morphologies and molecular methods validated the specificity and reliability of these two primer pairs. This report provided a method and *P. italicum*-specific primers, which might greatly contribute to citrus postharvest industry.

Keywords: *Penicillium italicum*, specific primers, conserved gene, citrus

For species identification, it was a popular strategy to identify species by means of conserved genes (CG) and homologous alignments, and universal primers were used to perform PCR [1]. This method has been applied widely in animal, plant and microorganism identification [2–4]. However, species-level discrimination usually encountered highly homologous genes in closely-related species [5, 6]. Universal primers might not be able to distinguish close species effectively, which often happened with the same genus [7]. With the environment becoming more complex, the variety and number of species increase more, especially in the microbial world. In citrus, a great variety of fungal pathogens cause postharvest diseases, which come from different genera, such as *Penicillium*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Alternaria*, and *Botrytis* [8]. Some citrus *Penicillium* pathogens had high ITS (internal transcribed spacers) sequence identity. These all brought difficulty for species discrimination [9, 10]. Therefore, a variety of different methods are encouraged and required to be developed for species identification.

Species-specific PCR was a good strategy to identify species [11]. This method was usually based on conserved genes for designing specific primers [12]. The genes *RPB1* (RNA polymerase II largest subunit), *RPB2* (RNA polymerase II second largest subunit), *BTUB* (beta tubulin), the mitochondrial cytochrome *c* oxidase subunit I (*COI*) [13] and *TEF* (translation elongation factor 1 alpha) were often used [14]. The specific primers were generally designed based on the variable regions of the conserved genes among several inter-genus species [15]. In our previous study, the specific primers were developed to detect *Penicillium digitatum* rapidly among isolates in citrus [16].

Blue mold, caused by *P. italicum*, is a very serious postharvest disease in citrus [17]. This study designed *P. italicum*-specific primers targeting the *RPB1* and *RPB2* genes based on the genomes of *P. italicum*, *P. digitatum*, *P. chrysogenum* and *P. expansum.* Two primer pairs, RPB1-a and RPB1-b, were successfully developed by detecting *P. italicum* among citrus-related fungal isolates. *P. italicum*

(B3), *P. digitatum* (N1), *P. chrysogenum* (Q) and *P. expansum* (L), which were deposited in Huazhong Agricultural University, China, were used as the sources of genomic DNA to develop the *P. italicum*-specific primers (Table S1). The gene sequences of *RPB1* (Accession no. JN121649.1) and *RPB2* (Accession no. KJ527377.1) of *P. italicum* were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/) and subjected to BLAST analysis against the genomes of *P. digitatum* (Accession no. AKCU01000000), *P. chrysogenum* (JMSF01000000) and *P. expansum* (JQFZ01000000). The homology search produced the *RPB1* and *RPB2* sequences of each strain, which were used to design *P. italicum*-specific primers by using ClustalX 1.81 and Primer premier5. The cores of the primers were from the mutational sites in the homologous regions (Figs. 1, 2, and Table S2).

All the isolates used in this study were cultured using potato dextrose broth media (PDB) at 28°C for three days, respectively. The total DNA was extracted with a fungal genomic DNA extraction kit (Aidlab Biotech, China) and the concentrations were examined by using an ultra-micro UV spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). The DNA samples were diluted 20- and 100-fold to reduce the amount of impurities (Table S1). PCR was performed in a 25 μl reaction system (1 μl 10 μM of each primer, 1 μl DNA template, 12.5 μl 2×Es Taq MasterMix (CW Biotech, China) and 9.5 μl ddH$_2$O). The PCR condition was programed as follows: pre-heating at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 7 min [18].

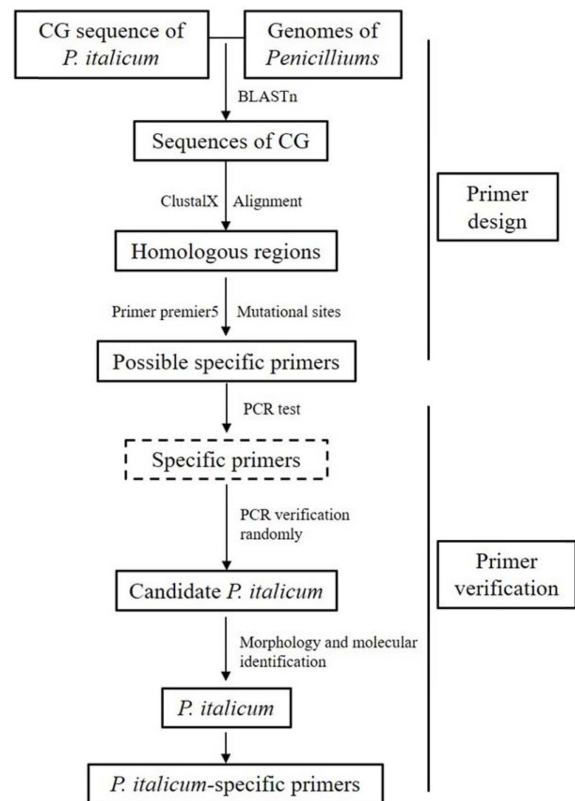The primers were tested for specificity in *P. italicum* (B3),

Fig. 1. The schema of the method for developing *P. italicum*-specific primers.
The image shows the whole process of the method, from primer designing to verifying. CG represents conserved genes.

P. digitatum (N1), *P. chrysogenum* (Q) and *P. expansum* (L) (Table S2). The results indicated that diluting the DNA templates 20-fold was not sufficient to exclude the
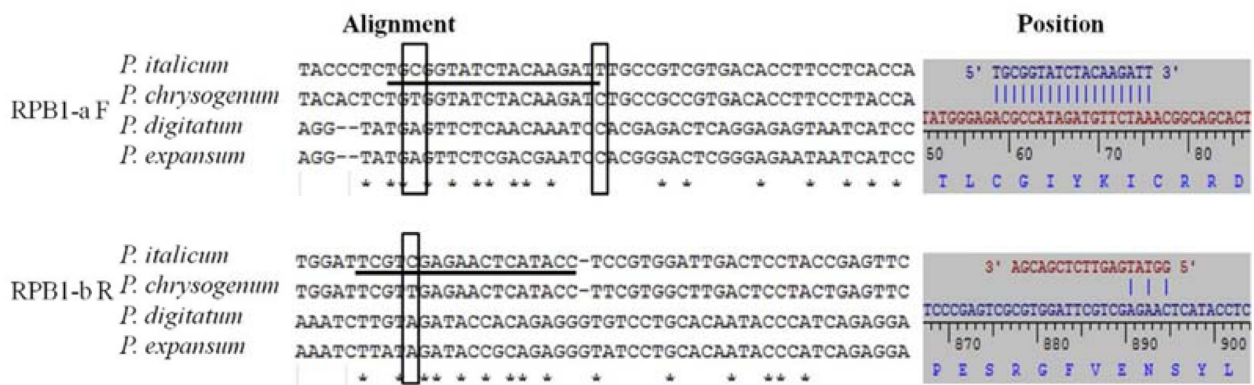

Fig. 2. Profile for designing the specific primers of *P. italicum*.
Sequences of the *RPB1* genes of *P. italicum*, *P. digitatum*, *P. chrysogenum* and *P. expansum* were aligned using ClustalX, producing homologous regions. The mutational sites that showed that the nucleotide of *P. italicum* was different from the other three *Penicilliums'* were considered as primer positions.
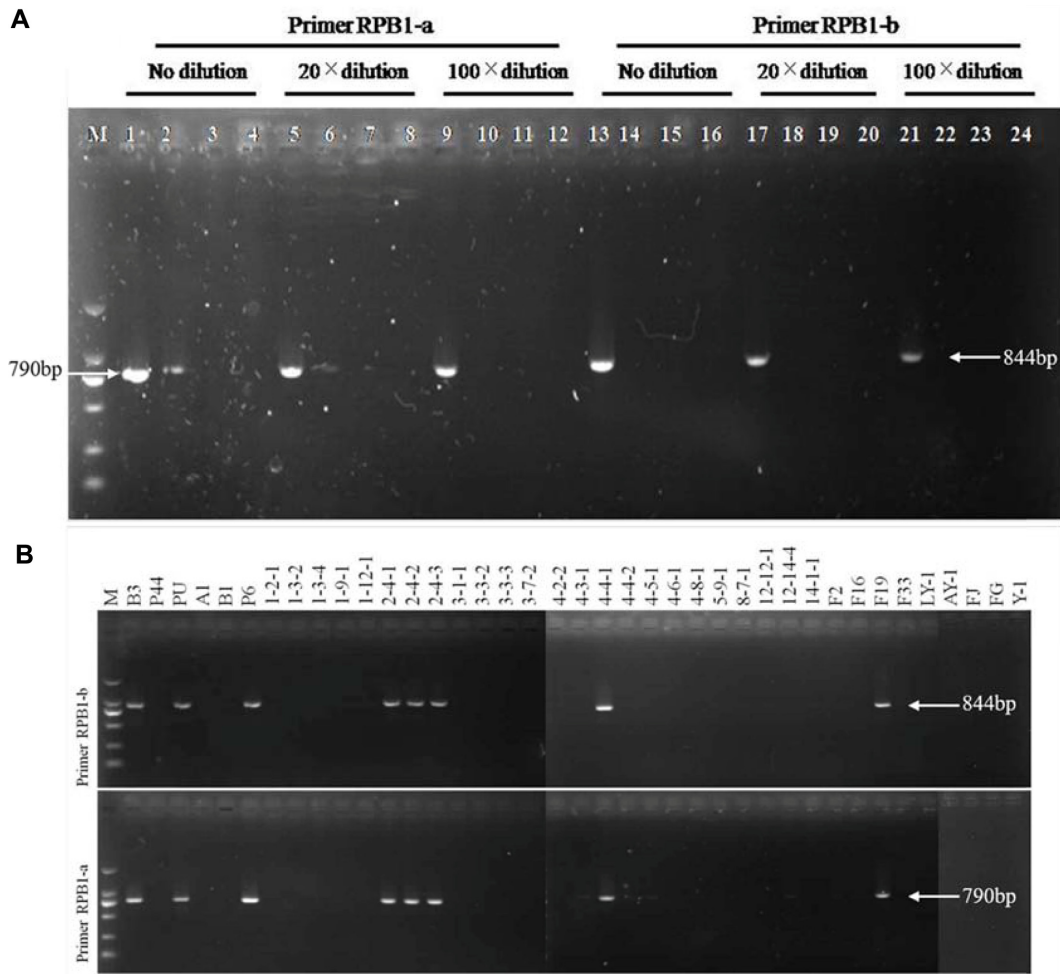
Fig. 3. Primer testing and candidate specific primer verification.
(**A**) Primer testing. Lane M, 2 kb molecular weight marker (Takara); lanes 1-4, *P. italicum*, *P. digitatum*, *P. chrysogenum*, *P. expansum*; lanes 5-8, 9-12, 13-16, 17-20, and 21-24, the same PCR templates as lanes 1-4. (**B**) Candidate specific primer verification by detecting *P. italicum* from 39 fungal isolates using the candidate primers RPB1-a and RPB1-b. Lane M, 2 kb molecular weight marker (Takara). The positions of the DNA bands were corresponding with their expected sizes.

interference of the impurities in the PCR (Fig. 3A). When diluting 100-fold, the effect of the impurity was absolutely excluded, and the blurry PCR band did not appear. Referring to the DNA concentrations in Table S1, diluting the DNA samples to 0.5−2 ng/μl might be suitable. The primer testing revealed that the primer pairs RBP1-a and RPB1-b were specific to detect *P. italicum* (Fig. 3A). The sequences of the PCR products also have high identity of 99% with the organism *P. italicum* strain CBS 339.48 (*RPB1*, Accession No. JN121649.1) [19]. Therefore, these two primer pairs were regarded as candidate specific primers. To verify the accuracy and specificity of RPB1-a and RPB1-b, they were used to detect *P. italicum* among 39 fungal isolates (randomly isolated from citrus fruits), including

some typical pathogens, such as *Penicillium polonicum* (A1), *Penicillium crustosum* (B1), *Geotrichum citri-aurantii* (AY-1), *Alternaria solani* (FJ), *Rhizopus nigricans* (FG) and *Botrytis cinerea* (Y-1) (Table S1). The DNA templates were directly diluted 100-fold. After PCR reaction, six unknown strains PU, 2-4-1, 2-4-2, 2-4-3, 4-4-1 and F19 were found out by the two primer pairs from the 39 fungal isolates. *P. italicum* (B3, P6) were recognized, *P. digitatum* (P44), *P. polonicum* (A1), *P. crustosum* (B1), *G. citri-aurantii* (AY-1), *A. solani* (FJ), *R. nigricans* (FG), *B. cinerea* (Y-1) and the other strains could not be detected. In addition, RPB1-a and RPB1-b could offer the same detecting result and the PCR bands were all clear (Fig. 3B). This indicated that RPB1-a and RPB1-b were accurate and specific for detection of *P. italicum*. The six
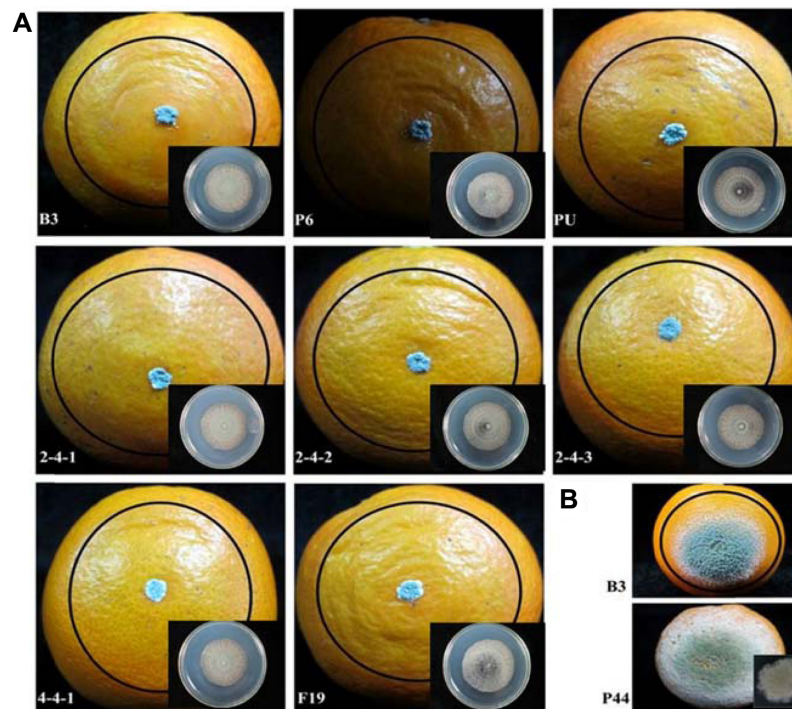
Fig. 4. Colonial and pathogenic morphologies of the candidate *P. italicum* strains.
(**A**) The colonial and pathogenic morphologies of the six candidate *P. italicum* strains and the control strains (*P. italicum* B3, P6) (cultivating on the fifth day). (**B**) The control groups *P. italicum* (B3) and *P. digitatum* (P44) on the sixth day.

strains could be taken as candidate *P. italicum*.

To determine if the candidate *P. italicum* strains were *P. italicum*, the six candidate *P. italicum* strains were observed for colonial morphology (inoculating 2 μl 10^5 CFU/ml spore suspension of each strain on the center of PDA media plates at 28°C for 6 days) and pathogenic morphology in citrus fruits. We pricked two wounds around the waist of "Fuben" navel orange fruits and inoculated 10 μl 10^6 CFU/ml spore suspension of each strain in the wounds. All of the in vitro or in vivo experiments, as well as the morphologies of the six candidate *P. italicum* strains were basically consistent with *P. italicum* (B3, P6) (Fig. 4). The navel orange fruits turned seriously watery around the wounds on the fifth day, with a mass of spores germinating in the wounds (Fig. 4A). On the sixth day, the mycelia and spores spread around fast (Fig. 4B) [20], and the pathogenic morphology was greatly different from *P. digitatum*. In addition, the six candidate *P. italicum* strains were examined by molecular identification with the primer pair ITS1 5′-TCCG TAGG TGAA CCTG CGG-3′, ITS4 5′-TCCT CCGC TTAT TGAT ATGC-3′. The result also indicated the ITS sequences of the six candidate *P. italicum* strains had high identity of 99% with *P. italicum* (Table S3) [21]. These

results revealed that the candidate *P. italicum* strains PU, 2-4-1, 2-4-2, 2-4-3, 4-4-1, and F19 were exactly *P. italicum*, which demonstrated that the two pairs of primers, RPB1-a and RPB1-b, were accurate and specific for *P. italicum* detection. They could be used to detect *P. italicum* rapidly among a large number of unknown citrus postharvest pathogens. This research may significantly facilitate the fungal detection in the citrus industry, and this method may also be utilized as a reference for species detection in other fields.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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