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Research Article

High Sensitive Method for the Simultaneous Detection of Pathogens in *Chrysanthemum morifolium*: A Nested Multiplex PCR Assay

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Chrysanthemum morifolium, a kind of medicine and food homology product, is threatened by plant pathogens. It is crucial to establish a sensitive and accurate method for diagnosing its pathogens. Therefore, the internal transcribed spacer regions of the ribosomal DNA (rDNA) sequences of three common pathogens in *C. morifolium* (*Chaetomium globosum*, *Fusarium incarnatum*, and *Alternaria alternata*) were compared, and the three pairs of specific primers were achieved. A two-step nested multiplex PCR system which consists of two sequential amplification steps was successfully developed to detect those three pathogens in *C. morifolium*. This method was sensitive due to its low detection limits, which were 10 pg/μL, 1 pg/μL, and 10 fg/μL for *C. globosum*, *F. incarnatum*, and *A. alternata*, respectively. The entire detection process of this method only took 5–6 h, demonstrating its high efficiency. The nested multiplex PCR system could be successfully applied to detect actual samples, and a 90% detection rate for those three pathogens in selected *C. morifolium* was achieved. This suggests that our assay could be an operator-friendly tool for diagnosing *C. morifolium* diseases.

Keywords: *Chrysanthemum morifolium*; disease diagnosis; medicine and food homology products; nested multiplex PCR; pathogens

1. Introduction

Chrysanthemum morifolium Ramat. cv. Hangbaiju (Compositae) (HJ) is a traditional plant homologous to food and medicine in China [1], and the dry capitulum of *C. morifolium* is a medicine known as "Ju Hua," which is included in the Chinese Pharmacopoeia as *Chrysanthemi Flos* [2–4]. Modern pharmacological studies have shown that *C. morifolium* possesses the effects of protecting liver, improving eyesight, counteracting blood pressure, and improving intestinal microenvironment [5–8]. In China, the primary production area for *C. morifolium* is Tongxiang City, located in Zhejiang Province, where the perennial planting area spans 46.67 square meters. The annual output value exceeds 100 million Yuan, accounting for half of the total sales of *Chrysanthemum* in the country. However, with the rising demand and large-scale cultivation of *C. morifolium*, continuous cultivation practices and

prolonged soil cropping have become increasingly prevalent. This trend has led to a heightened incidence of various diseases and insect pests. To effectively prevent and control these issues and ensure the safe production of *C. morifolium*, timely diagnosis of diseases is essential [9–11].

Fungi are some of the most crucial pathogens, causing 70%–80% of plant diseases [12–14]. According to previous investigations, more than 40 kinds of diseases exist in *C. morifolium*, and the more serious ones are leaf blight, fusarium wilt, and black spot [15–18]. *Chaetomium globosum*, *Fusarium incarnatum*, and *Alternaria alternata* are three widely disseminated fungal pathogens resulting in these three diseases [19, 20]. Fungal diseases cause several losses in *C. morifolium*, so efficient management is required to avoid economic damage and depreciation at their early stages.

The traditional methods for detecting and identifying phytopathogenic fungi primarily rely on the symptoms exhibited by the host plant. These methods are time-consuming and necessitate the use of microscopic techniques, along with a thorough understanding of fungal taxonomy [21]. The process of fungal isolation from plants is difficult due to the low growth rate in isolation culture media and the presence of other fungi in plant tissues, which means that the traditional detection technology has its limitations [22–24]. Moreover, different pathogens may produce similar symptoms, intraspecific variation, and interweaving characters, so it is even difficult for experts and specialists to correctly identify fungal colonies [25]. Thus, it is necessary to establish a quick and cost-effective detection system for plant samples at early-stage infection in the field.

Novel molecular techniques serve as promising substitutes for traditional culture-based methods. Among these, polymerase chain reaction (PCR) has been developed and is frequently utilized in agricultural production to detect pathogens such as *Acidovorax avenae* subsp. *avenae*, *Candidatus liberibacter asiaticus*, and *Neofusicoccum parvum* due to its rapidity and feasibility, indicating considerable application value [26–30]. However, the traditional nested PCR technology can only detect a single target, while the multiple PCR that can detect multiple targets at the same time is prone to mismatching in the process of specific primer amplification, resulting in the failure of primer synthesis. Nested multiplex PCR technology combines the advantages of both and can detect multiple targets at the same time on the premise of reducing primer mismatch. Previous studies have shown the application of the combination of multiplex PCR and nested PCR to diagnose many plant diseases, such as potato, cassava, and mango [31–33]. Under natural conditions, *C. morifolium* is prone to the same plant infected with multiple pathogens at the same time, or the situation that multiple pathogens infected plants produce similar traits. Nested multiplex PCR can detect multiple pathogens simultaneously while reducing mismatching of primers. Moreover, it has high application potential in diagnostic laboratories and offers more advantages than traditional microbiological techniques, including lower detection limits, shorter test times, and higher specificity [34, 35]. The efficiency and specificity of amplification in this method are improved by two successive rounds of PCR, in which the products from the initial PCR reaction are used as the template for the subsequent PCR reaction.

This study mainly aims to design and optimize a nested multiplex PCR assay capable of identifying three major pathogens affecting *C. morifolium* (*C. globosum*, *F. incarnatum*, and *A. alternata*). Specifically, the sensitivity and specificity of this assay were evaluated, and then it was applied to actual *C. morifolium* plants for diagnosing diseases caused by the mentioned pathogens in a practical application. This study offers a valuable tool for the rapid and accurate diagnosis of diseases in *C. morifolium* caused by these pathogens.

2. Materials and Methods

2.1. Fungal Strains and Culture Conditions. The fungus strains *C. globosum* (ACCC39707), *F. incarnatum* (ACCC39706), *A. alternata* (ACCC39708), *F. oxysporum*, *and S. delphinii* were supplied by Zhejiang Provincial Key Laboratory of Biometrics and Inspection & Quarantine and stored in the Agricultural Culture Collection of China. Diseased plants were collected from Tongxiang City, Jiaxing City, Zhejiang Province, the main producing area of *C. morifolium*. These pathogenic fungi were isolated from suspected *C. morifolium* samples using the conventional plate-culture method with potato-dextrose agar (PDA) medium, which consists of 15 g of agar, 20 g of glucose, 200 g of potatoes, and 1000 mL of ddH_2O. Kochs' postulates were used to confirm the pathogenicity of pathogenic fungi, and then the internal transcribed spacer (ITS) sequence of ribosomal DNA (rDNA) was analyzed to identify the taxonomic status of pathogens. This sequence is conserved in fungi with abundant interspecific and intraspecific variation sites, which can distinguish strains with close kinship [36, 37]. The isolates were stored in a PDA medium at 28°C for short term and maintained at 4°C for a long time.

2.2. DNA Extraction of Fungi and Plants. The cetyltrimethylammonium bromide (CTAB) method was utilized to isolate the total DNA of pathogenic fungi and *C. morifolium* plants. *C. morifolium* is a nutritious plant whose tissue is rich in polyphenols and polysaccharides, so it is difficult to extract its DNA. The improved CTAB method is used to extract the DNA of plant tissues: First, 2 g of frozen plant tissue (both diseased and healthy plant) using liquid nitrogen was broken by a grinder (MP, US) at 30 f/min for 20 min, and the pellet was resuspended with an extraction buffer composed of 500 μL of CTAB and 0.5% β-mercaptoethanol, and then the mixture was incubated with continuous agitation for 30 min at a temperature of 65°C. Second, the mixture was inverted with the 500 μL of extraction solution (phenol/chloroform/isoamyl alcohol, 25: 24:1) and subsequently centrifuged at 12,000 r/min for 10 min. Following the collection of the supernatant (400 μL), the process was repeated for a second time. Finally, the supernatant was mixed with 800 μL of ice ethanol and 200 μL

of 5 mol/L NaCl solution at 4°C for 1 h to precipitate nucleic acids and dissolve polysaccharides. Then, the mixture was centrifuged at 12,000 r/min for 10 min, followed by removing of the supernatant. The precipitate was washed with 70% ethanol, twice resuspended in 50 μL of sterile water, and maintained at −20°C for storage.

Extraction of pathogenic fungal DNA: 2-g mycelium and steel beads and 500-μL CTAB were put into a 2.0-mL flat-bottomed centrifuge tube, placed on a homogenizer, and shaken at 4.0 m/s for 10 min to grind hyphae. The shaken solution was pipetted into a new test tube, frozen and thawed repeatedly in liquid nitrogen and a 75°C water bath three times, and incubated in the water bath for 30 min for the last time. Then, 500 μL of DNA extraction solution was added, mixed upside down, and centrifuged at 12,000 r/min for 10 min; the supernatant was collected and added with 2 times the volume of ice-cold absolute ethanol to precipitate for 1 h and centrifuged at 12,000 r/min for 10 min, and the precipitate was collected. The precipitate was washed twice with 70% ethanol and air-dried, and the DNA was dissolved in 50 μL of ddH_2O and stored at −20°C for later use.

2.3. Primer Design. The field samples of diseased *C. morifolium* with typical symptoms were collected and isolated and then purified and identified the pathogenic bacteria they carried. The modified CTAB method was used to extract the DNA of pathogenic fungi, and then the fungal universal primers ITS 1(TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) were used to amplify the fungal rDNA ITS fragment. The experiment was carried out in a combined volume of 25 μL, containing 2 μL of 10 × buffer (with Mg^{2+}), 0.5 μL of dNTP (10 mol/L), 0.3 μL of both primers (ITS 1/ITS 4) (10 μM/L), 0.4 μL of Taq polymerase (2 U/μL), 2 μL of DNA template (the fungal rDNA ITS fragment, 80–100 ng/μL), and 19 μL of ddH_2O. The amplification conditions were as follows: denatured at 94°C for 45s, annealed at 55°C for 30 s, and extended at 72°C for 45 s, for a total of 35 cycles.

In this study, three pairs of species-specific primers (*C. globosum*, *F. incarnatum*, and *A. alternata*) were designed. The ITS sequences of each pathogen obtained from the NCBI database were taken for reference, and the Clustal X (Version 1.83) and Primer Premier 5.0 were used to design the primers. The dimer and hairpin loop structures were also analyzed. After that, the primers were screened by PCR until the best set of specific and suitable fragment sizes of each primer was obtained. The target sequences were amplified by the designed specific primers, sequenced by Shanghai Sunny Biotechnology Company (Shanghai, China), and compared with the expected sequences using Clustal X.

2.4. Specificity Testing. To accurately identify all three pathogens simultaneously, it is crucial that the primers are specific to each species. Therefore, in order to verify the specificity of a single pathogenic bacteria, the rDNA ITS fragments amplified from two other pathogenic bacteria that can cause *C. morifolium* disease (*F. oxysporum* and *S. delphinii*), the other two pathogenic bacteria used in this nested multiplex PCR system, and a healthy plant were used as no-template controls in each PCR assay for a single target fungus.

2.5. Optimized Nested Multiplex PCR Assay. The amplified rDNA ITS fragment by the method as described in Section 2.3 was used to serve as the template for the subsequent PCR reaction. Simultaneously, the PCR amplification conditions were collected and summarized from a previous study [38]. The experiment took place in a combined volume of 25 μL, containing 2.5 μL of 10 × buffer (without Mg^{2+}), 1.5 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTP mix (2.5 mM each), 0.2 μL of Taq polymerase (5 U/μL, from Yesen, Shanghai, China), 1 μL of first-round PCR products, and 19.3 μL of ddH_2O. The PCR products were electrophoresed by 2% agarose gel using 1 × TAE buffer at 130 V for 45 min and visualized through staining with ethidium bromide (EB). The concentration of the 3 pairs of specific primers and the annealing temperature were optimized by evaluating the brightness of the amplified bands.

2.6. Detection Limit Assays. In order to confirm the detection limit of each pathogen (*C. globosum*, *F. incarnatum*, and *A. alternata*), ddH_2O was used to dilute the DNA concentration of each pathogen from 100 ng/μL to 10 ag/μL. After that, different concentrations of DNA from the three pathogenic bacteria were mixed to test the sensitivity of nested multiplex PCR. The detection limits of the pathogen were assessed according to the amplified band of DNA in the agarose gel electrophoresis (AGE; loading volume: 10 μL). The detection limit is the lowest concentration of DNA amplified band that can be detected by visual and gray value.

2.7. Verified Nested Multiplex PCR. Fifteen actual samples with typical symptoms were randomly selected from the *C. morifolium* planting base in Tongxiang City, Zhejiang Province. The basis for selecting plants infected by leaf blight is as follows: the leaf ends turn yellow and continue to spread to the base of the leaves, the diseased part curls and turns dark brown, and there are obvious bright yellow lines at the junction of the disease and the healthy part (A). The basis for selecting plants infected by fusarium wilt is as follows: the roots rot and turn black, the leaves become yellow and wilted and droop, and the base of the stem appears light brown (B). The basis for selecting plants infected by black spots is that there are irregular black spots on the leaves, which continue to spread to the base of the leaves, and the lower leaves of the plant are the first to become infected (C). According to the typical infection characteristics of the three major fungal diseases of *C. morifolium*, diseased plants that meet the requirements were randomly selected in the field, with 5 plants for each disease (Table 1). Then, healthy plants under the same growth conditions were selected as controls. The objective was to evaluate the practicality and efficiency of early diagnosis using a nested multiplex PCR system for detecting the three primary pathogens of *C. morifolium*

Table 1: Collected samples showing typical symptoms for leaf blight (A), fusarium wilt (B), and black spots (C).

Serial number	Typical character		
	A	B	C
1	+	+	−
2	−	+	−
3	−	+	−
4	−	−	+
5	−	−	+
6	−	−	+
7	−	−	+
8	+	−	−
9	+	−	−
10	+	−	−
11	+	+	−
12	+	+	−
13	−	+	+
14	−	+	+
15	−	+	−

simultaneously and swiftly. The gray value of the amplified bands in the electropherogram was measured by using Image J.

2.8. Statistical Analysis. The experimental data were processed using Origin 2021 and GraphPad Prism 8.0 to draw graphs. Significance analysis was performed by the one-way analysis of variance Tukey test using IBM SPSS 27.0 software, with three replicates set up.

3. Results

3.1. Designed and Screened Species-Specific Primers. ITS amplicon (Table S1) of the three different pathogens (*C. globosum*, *F. incarnatum*, and *A. alternata*) were used with their respective species-specific primers. The primers with suitable fragments used in nested multiplex PCR systems are listed in Table 2, and the specificity of the designed primers is illustrated in Figure 1. In Figure 1(a), the designed primer successfully amplified 409-bp DNA fragment of *C. globosum* but did not amplify DNA fragment in healthy plants, *F. incarnatum*, *A. alternata*, and two other pathogens. Similarly, in Figures 1(b) and 1(c), only 311-bp DNA fragments of *F. incarnatum* and 249-bp DNA fragments of *A. alternata* were amplified. In addition, the primer-generated sequences displayed a similarity of over 97% to the expected sequences. These findings proved that all the designed primers have good specificity and can be successfully applied to nested multiplex PCR.

3.2. Optimized Nested Multiplex PCR Assay. To ensure efficiently amplifying the target sequences, the proportion of each primer and the annealing temperature were optimized. After performing a series of optimizations, the final nested multiplex PCR amplification conditions were established as follows: initial denaturation at 94°C for 2 min, subsequently followed by 20 cycles consisting of 45 s at 94°C, 30 s at 55°C, and 45 s at 72°C. The procedure concluded with a final extension step of 10 min at 72°C. The PCR showed high amplification efficiency when the ratio of HG1:QM5:Al was 1:0.6:1.6. In addition, the cycle number and amount of primer mixture, which considerably affected the amplification efficiency, were optimized. The ideal reagent amounts for a 25-μL PCR reaction system was as follows: 0.6-μL of QM5-F/QM5-R, 1 μL of HG1-F/HG1-R, 1.6 μL of Al–F/Al-R, 2.5 μL of 10 × buffer with Mg^{2+}, 0.5 μL of dNTP (10 mol/L), 0.4 μL of Taq polymerase (2 U/μL), 1 μL of DNA template, and 17.4 μL of ddH_2O. The amplification effect is presented in Figure 2.

3.3. Sensitivity Assays. The DNA concentrations of three pathogens were diluted from the initial 100 ng/μL to 100 ag/μL, and the sensitivity of the optimized nested multiplex PCR technique in this study was evaluated by visual electrophoresis strip analysis and Image J gray value analysis. The detection limit of 10 pg/μL for *C. globosum*, 1 pg/μL for *F. incarnatum*, and 10 fg/μL for *A. alternata* (Figures 3(a), 3(b), and 3(c)) was obtained by using DNA samples from each pathogen. Furthermore, a mixture of the three pathogenic DNA samples with different concentrations was employed to assess the sensitivity of the nested multiplex PCR protocol. The findings indicated that the detection limit was similar to that observed in single DNA detection. *C. globosum*, *F. incarnatum*, and *A. alternata* could be simultaneously tested with 1 ng DNA in a tube, as illustrated in Figure 3(d).

3.4. Verified Nested Multiplex PCR. Experimental results (Figure 4) demonstrated that the detection rate of pathogenic bacteria of *C. morifolium* (15 samples of infected *C. morifolium* from Tongxiang city, Zhejiang Province) by the optimized nested multiplex PCR technique in this study could reach more than 90%. Among the samples, numbers 1, 8, 9, 10, 11, and 12 were amplified with 409-bp bands, which proved that *C. globosum* was detected. Numbers 1, 2, 3, 11, 12, 13, 14 were amplified with 311-bp bands, confirming the detection of *F. incarnatum*. In addition, numbers 4, 5, 6, 7, 13, and 14 were amplified with 249-bp bands, which proved that *A. alternata* was detected. However, samples 1, 11, 12, 13, and 14 all showed

Table 2: Primers used for nested multiplex PCR.

Target	Primers	Primer sequence (5′–3′)	Amplicons (bp)	Reference
C. globosum	QM5-F QM5-R	CGTTACCTATACCGTTGC AGAGCGAGATGTATGCTACT	409	Current study
F. incarnatum	HG1-F HG1-R	GAGGACCCTAAACTCTGTT ATGATTACTACGCTATGGAA	311	Current study
A. alternata	Al-F Al-R	GTCTTTTGCGTACTTCTTGTTT GAACAGGCATGCCCTTTGGA	249	Current study

Note: All the primers were synthesized by Shanghai Sunny Biotechnology Company.

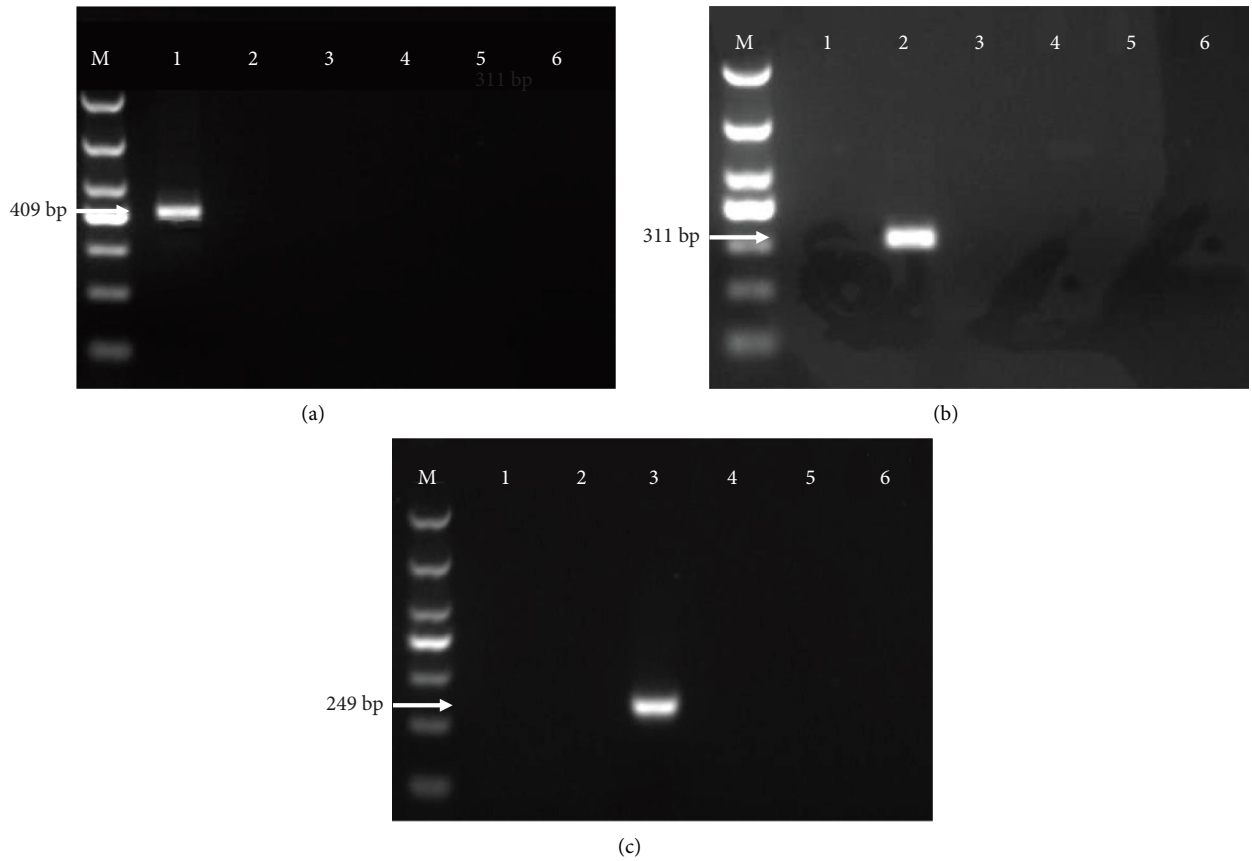


Figure 1: Specificity of the designed species-specific primer sets: 1: *C. globosum*; 2: *F. incarnatum*; 3: *A. alternata*; 4: healthy plant; 5: *F. oxysporum;* 6: *S. delphinii*. (a) QM5-F/QM5-R (primer of *C. globosum*); (b) HG1-F/HG1-R (primer of *F. incarnatum*); (c) Al–F/Al-R (primer of *A. alternata*). M, 1000-bp DNA marker.

two amplified bands, which proved that a single plant could be infected with multiple pathogens at the same time, while sample 15 did not show amplified bands, which proved that different pathogens may cause similar traits in infected plants. In addition, no amplified bands were found in healthy plants (No. 16). The results above proved that the nested multiplex PCR could be successfully applied to the detection of main pathogens of *C. morifolium*.

4. Discussion

C. morifolium is widely recognized as both an ornamental plant and a traditional Chinese medicinal herb. As cultivation areas have expanded, plant diseases have become common and thus significantly affected the economic values. Furthermore, due to the substantial nutrition and economic values of *C. morifolium*, the detection and diagnosis of the economically important plant pathogens are of paramount significance. To meet the demands of early disease diagnosis, a rapid and reliable technique was developed to identify each of the three pathogens (*C. globosum*, *F. incarnatum*, and *A. alternata*). In particular, the concentrations of pathogens' DNA might not be sufficient for detection with conventional methods [39].

Nested multiplex PCR is a high-throughput, specific, and sensitive technique. Several pathogens were simultaneously identified and hold considerable detection sensitivity. According to the preliminary assays, several primers and

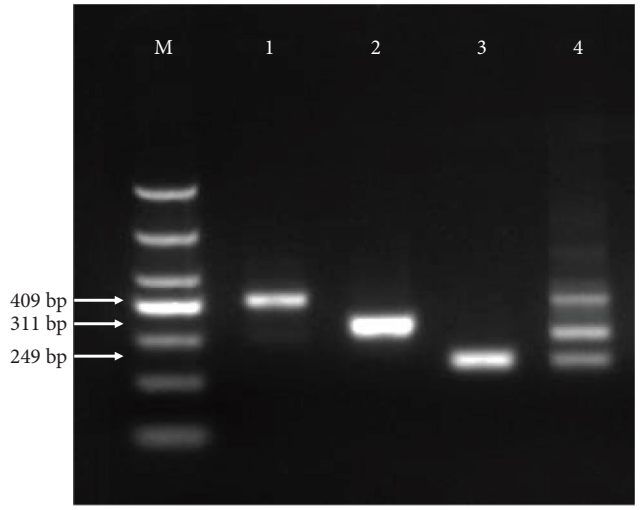


Figure 2: Optimized nested multiplex PCR electrophoretic pattern of the three pathogens. 1: *C. globosum*; 2: *F. incarnatum*; 3: *A. alternata*; 4–6: DNA mixture of the three pathogens. M: 1000-bp DNA marker.

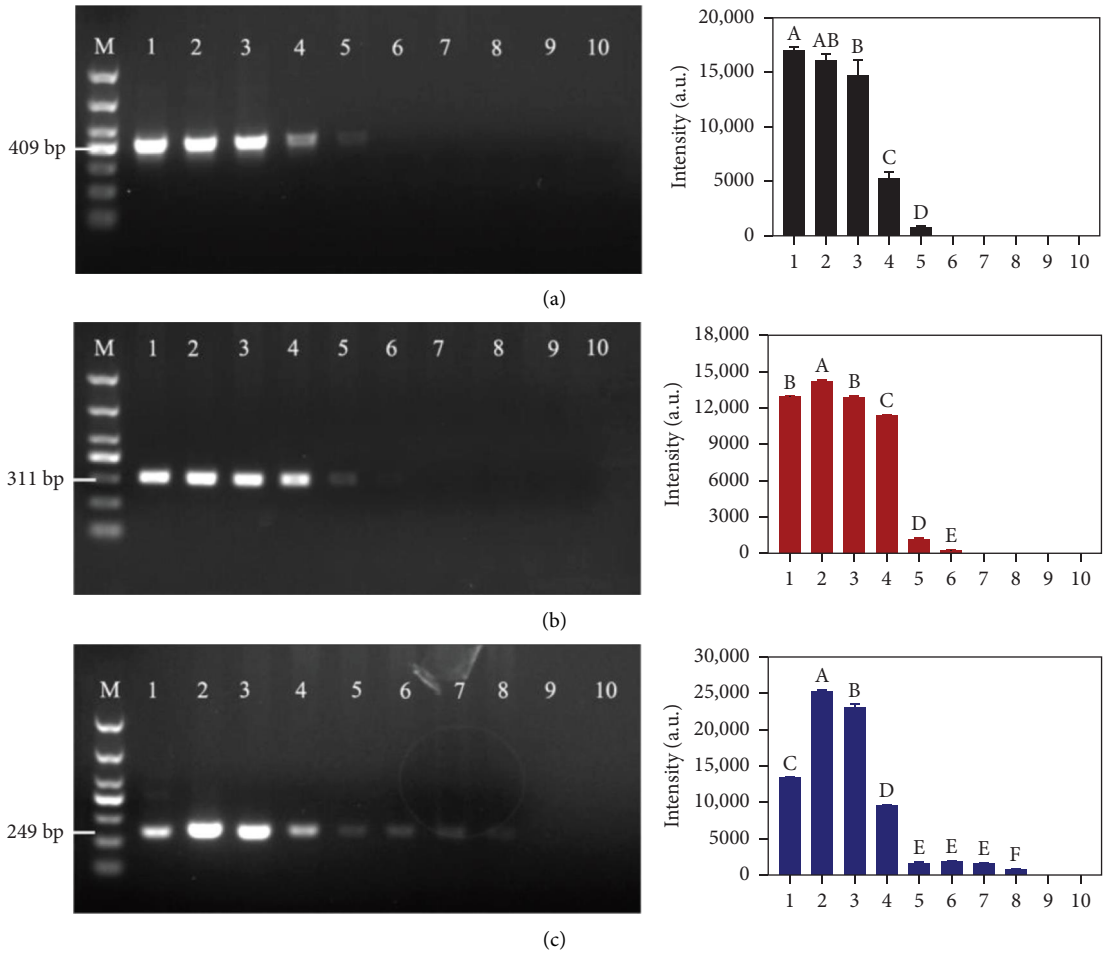
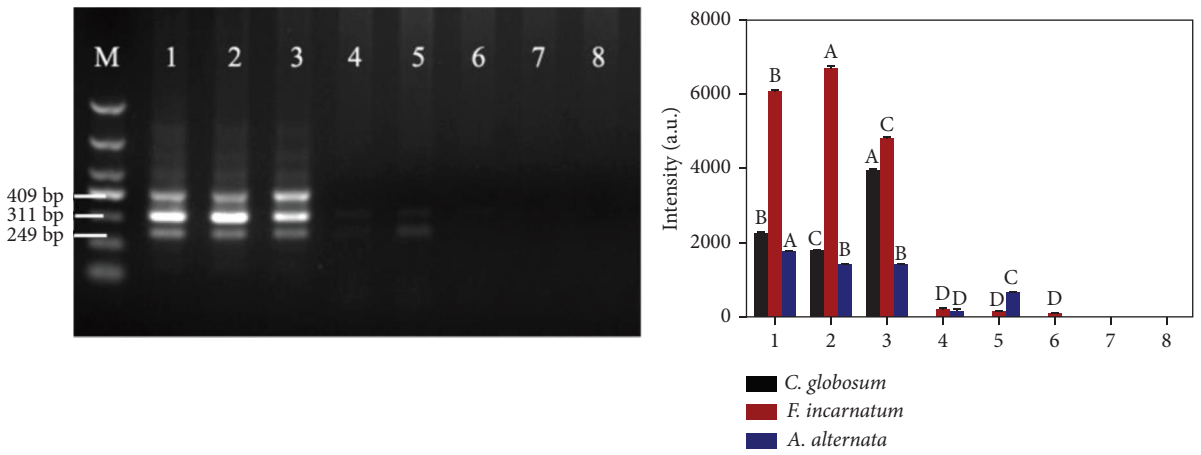


Figure 3: Continued.



(d)

Figure 3: Sensitivity of nested multiplex PCR assay. (a) *C. globosum* (the detection limit: 10 pg/μL), (b) *F. incarnatum* (the detection limit: 1 pg/μL), (c) *A. alternata* (the detection limit: 10 fg/μL), and (d) the DNA template mixture of three pathogens. 1–8: Different concentrations of DNA template (100 ng/μL–100 ag/μL). M: 1000-bp DNA marker. The same pathogen with different letters represented significant difference ($p < 0.05$).

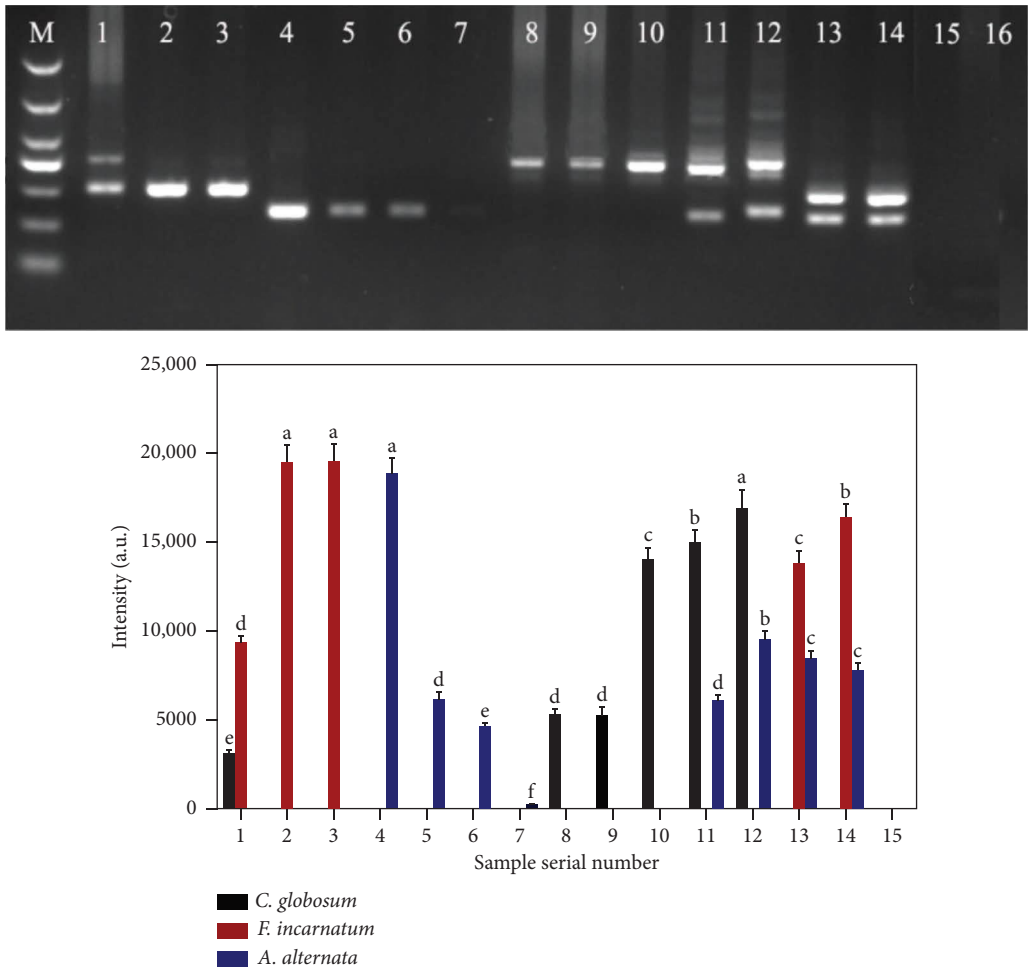


Figure 4: Application of nested multiplex PCR assay for *C. morifolium* field samples. 1–15: Diseased *C. morifolium* with different symptoms correspond to samples 1–15 in Table 1; 16: symptomless *C. morifolium* plants. M: 1000-bp DNA marker. The same pathogen with different letters represented significant difference ($p < 0.05$).

templates were allowed to be used to improve the amplification efficiency. Nevertheless, it may lead to the mismatch of some primers with their templates and nonspecific amplification, affecting the accuracy of the assay [40]. Therefore, in order to avoid the primer mismatch and nonspecific amplification and improve the detection accuracy of the nested multiplex PCR technique, the experimental parameters such as primer concentration, primer specificity, cycle times, and annealing temperature were optimized. Finally, an efficient amplification and specific detection technology was successfully developed, and the entire detection process only needs 5–6 h, saving time and cost.

Nested multiplex PCR was successfully performed with three primer pairs (QM5-F/QM5-R, HG1-F/HG1-R, and Al-F/Al-R) according to the ITS sequence of rDNA, which were the conserved regions of fungus gene sequences and widely applied in the identification of fungi, displaying a higher polymorphism [38]. The primers were designed for *C. globosum*, *F. incarnatum*, and *A. alternata*, with suitable fragment sizes of 409, 311, and 249 bp, respectively. In addition, the detection limit of a single PCR assay was 10 pg of DNA/μL for *C. globosum*, 1 pg/μL for *F. incarnatum*, and 10 fg/μL for *A. alternata*. The sensitivity of simultaneous detection for the three pathogens was 1 ng of DNA/μL. This finding meant that even if plants were symptomless, we could detect these fungi through this nested multiplex PCR system.

Nested multiplex PCR system was used to detect the actual disease samples, and the detection rate of pathogenic bacteria reached 90%. Among the 15 samples selected, the leaves of the plants detected *C. globosum* (1, 8, 9, 10, 11, and 12) were yellow, the disease sites were curled and dark brown, and there was a bright yellow boundary between the sick and healthy strains. Plants detected for *F. incarnatum* (1, 2, 3, 11, 12, 13, and 14) had withered, wilted, and drooped leaves, light brown at the base of the stem, and rotted and blackened roots. Plants infected with *A. alternata* (4, 5, 6, 7, 13, and 14) showed irregular dark spots on the leaves and continued to spread to the base of the leaves. In the controlled experimental conditions, the detection results of pathogenic bacteria were basically consistent with the disease traits displayed by the plants, and only 1 sample (15 samples) did not amplify the corresponding band. The leaves of sample 15 have irregular black spots and began to spread to the base of the leaves, which indicates that the plant does have black spot disease, but the infection is not a common black spot pathogen, which also indicates diverse pathogenic fungi may induce plants to exhibit analogous infectious traits in the natural environment. Therefore, we may also conjectured that some diseases were caused by other pathogens in the field with symptoms similar to leaf blight, root rot, and southern blight. Therefore, the method's effectiveness may be influenced by some factors such as environmental conditions and mixed infections in real-world settings. In future studies, with the continuous expansion of the numbers of actual samples, the applicability of the method will be verified, and more environmental factors that may affect the experimental results will be considered in the experiment.

In this study, compared with nested PCR alone, nested PCR combined with multiple PCR can detect multiple pathogens at the same time, which simplifies the experimental operation and saves time (R. et al., 2023). In comparison to conventional multiplex PCR, the nested multiple PCR that is developed can effectively reduce the errors generated during the amplification of specific primers and enhances detection sensitivity on the basis of simultaneously detecting multiple targets [41, 42]. Therefore, this method demonstrates higher sensitivity and a reduced likelihood of error and saves detection time to a certain extent when compared with the single PCR technique and traditional trait analysis method. Furthermore, the multiplex nested PCR system can also be employed to identify and verify plant diseases caused by other pathogenic bacteria exhibiting similar symptoms.

The detection system can be used to effectively and rapidly diagnose early diseases of *C. morifolium* to control the occurrence and spread of diseases, reduce misuse and abuse of pesticides, and eventually improve the quality and yield of *C. morifolium*. In this study, the infected plants in the breeding base of *C. morifolium* were collected, and the three most common pathogens that can cause *C. morifolium* disease in Zhejiang Province were identified and detected. In future studies, the scope of screening and detection of pathogen in the actual samples will be expanded for practical application. Our aspiration is that this nested multiplex PCR can provide technical support for efficient, sensitive, and accurate diagnosis of *C. morifolium* diseases.

5. Conclusion

In this study, a simple, efficient, accurate, and reliable nested multiplex PCR technology was developed for the early disease diagnosis of *C.morifolium*. By optimizing various parameters of this detection system, the performance of the nested multiplex PCR technology was improved, and the specificity, sensitivity, and flux of detection were also increased. Compared with other methods, this detection method was more efficient and convenient. The detection limits of this method for three pathogenic fungi (*C. globosum*, *F. incarnatum*, and *A. alternata*) were significantly lower than those of traditional methods, and it has been successfully applied for the detection of actual samples. Therefore, the developed detection system in this study provides a simple and efficient idea for rapid diagnosis of early *C. morifolium* diseases and has broad application prospects in controlling the occurrence and spread of diseases, reducing pesticide abuse, and improving the quality and yield of agricultural products.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

Table S1: The results of ITS sequence sequencing of pathogenic bacteria DNA.

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