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杧果露水斑病病原菌枝状枝孢霉的巢氏PCR检测方法的建立

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摘 要:【目的】杧果露水斑病是当前生产上影响杧果产业的重要病害之一,因潜伏期长常在发病初期不易被察觉而错过防治适期,建立其主要致病菌 *Cladosporium cladosporioides* 的快速准确检测方法,对于该病的早期诊断和及时防控尤为重要。【方法】将该病菌的ITS序列与NCBI中的数据大量比对后,在差异位点大的区域设计了8对引物,通过筛选获得两对特异性良好的引物及其扩增条件。【结果】两对特异性引物分别是ML-SF9/ML-SR5和ML-SF10/ML-SR10,扩增条件:94 ℃ 4 min;94 ℃ 45 s,65 ℃ 45 s,72 ℃ 1 min,36周;72 ℃ 10 min,特异条带大小分别为408 bp和424 bp。为了提高检测的灵敏度,又与真菌通用引物ITS1/ITS4结合后,建立了杧果露水斑病病原菌的两套巢式PCR快速检测体系,可检测病菌DNA的含量最低为3.55×10^{-9} ng·μL^{-1},比常规PCR灵敏度提高了1万倍,且能实现对潜伏期果实的特异性检测。【结论】此技术操作简单、特异性强、灵敏度高,为杧果露水斑病的早期诊断提供了新方法。

关键词:杧果;露水斑病菌;常规PCR;巢式PCR;分子检测

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Development of a nested PCR detection method for *Cladosporium cladosporioides* causing mango sooty blotch disease

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Abstract:【Objective】Mango sooty blotch disease, a new disease on mango, has occurred in Hainan, Sichuan, Guangxi and Fujian provinces that are major planting areas of mango in China. Mango varieties like Guifei and Jinhuang were easy to be infected in mango orchards. Generally, the young fruit can be infected, but a lot of spots appeared during the harvest time for its slow expansion and long incubation period. Water-soaked and irregular dark lesion with dark green mould appeared on those infected mango peels, which seriously affected the appearance of mango and caused great loss of commodity value. The incidence of some poorly managed orchards was as high as 100%. Sofar mango sooty blotch disease has been one of the important diseases affecting mango industry. It is often difficult to be detected at the early stage for the long incubation period and cause to miss optimum period for control. To timely prevent this disease, it is important to establish the rapid and accurate method for *Cladosporium cladosporioides* detection, which is the main causal agent of mango sooty blotch disease.【Methods】The Genomic DNA of pathogens was extracted from mycelium using Fungal DNA Kit (OMEGA). Field samples of genomic DNA were extracted from mangoes by the CTAB method. Based on nucleotide differences in the internal transcribed spacer (ITS) sequences of *Cl. cladosporioides* and ITS of other pathogens from the data in NC-

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BI, eight sets of primer pairs were designed and screened for use in PCR assay in the present work. Eleven *Cl. cladosporioides* strains from leaf, branch and fruit samples were collected from diseased symptomatic mango plants from orchards in the mango growing areas in Hainan and Sichuan regions. Eleven pathogenic strains of other mango diseases such as *Botryodiplodia theobromae*, *Colletotrichuma cutatum*, *C. gloeoporioides*, *B. theobromae*, *Xanthomonas campestris* pv. *mangiferae*, *Sphaceloma mangiferae*, *Trichothecium roseum*, *Pestalotiopsis mangiferae*, *Fusarium mangiferae*, *F. proliferatum* and *F. decemcellμlare* were used to determine the specificity of the primers. Each PCR reaction mixture contained 12.5 μL 2×*Taq* PCR Master Mixture (TaKaRa), 1 μL of genomic DNA, 1 μL of 10 μmol·L^{-1} primers 1/2, and ddH_2O 9.5 μL in a total volume of 25 μL. The PCR thermal cyclingreaction was started by denaturation at 94 ℃ for 4 min, followed by 36 cycles of 94 ℃ for 45 s, T_m ℃ for 45 s, and 72 ℃ for 1 min, and then a final extension at 72 ℃ for 10 min, and annealing temperature Tm included 51 ℃, 54 ℃, 57 ℃, 60 ℃, 63 ℃, 65 ℃ and 67 ℃ for screening the optimizing PCR amplifying program. To increase the sensitivity, two nested-PCR protocols were further established. The first round PCR amplification was performed by using the universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') with the similar PCR reaction mixture. PCR was performed using the following parameters: one cycle at 94 ℃ for 3 min, 30 cycles at 94 ℃ for 45 s, 56 ℃ for 45 s, and 72 ℃ for 1 min, followed by one cycle at 72 ℃ for 10 min. Then, 1 μL PCR product was used as templates for the second round of PCR amplification with the primers ML-SF9/ML-SR5 and ML-SF10/ML-SR10 using the same conditions previously screened. To determine the sensitivity of the PCR and nested-PCR protocols, the genomic DNA of *Cl. cladosporioides* was diluted from 0 to 10^{10} with ddH_2O by preparing 10-fold serial dilutions, respectively. To test the feasibility of early detection from the *Cl. cladosporioides*, the mango peel after 8 d inoculation with *Cl. cladosporioides* and six young mango fruits showing the typical, atypical and no visible disease symptoms of sooty blotch disease from Tainong and Jidan mangoes were collected from the field, and total DNA samples were extracted for PCR and nested-PCR amplifications. The amplified PCR products were separated on 1.5% agarose containing goldview, visualized and documented in a Bio-rad UV Trans illuminator. 【Results】A total of 21 fungal and one bacterial DNA samples were subjected to PCR amplification with 8 pairs of primers. The results showed that only two pairs of primers ML-SF9 / ML-SR5 and ML-SF10 / ML-SR10(ML-SF9: 5'-TAGCCTCCCGAGCACCCTT-3'/ ML-SR5: 5'-GTTCATAACCCTTTGTTGTCC-3' and ML-SF10: 5'-TAGCCTCCCGAGCACCCTT-3' /ML-SR10: 5'-GTTTACCACCGGGATGTTCATAAC-3') were highly specific for *Cl. cladosporioides* when the annealing temperature was 65 ℃. A 408 bp and 424 bp unique bands were respectively obtained from each of the 11 *Cl. cladosporioides* strains collected from leaf, branch and fruit samples in various areas in China, but not from other fungal and bacterial species. The results showed that the minimum amount of the fungal DNA could be detected by the PCR using the primer combination ML-SF9 / ML-SR5 and ML-SF10 / ML-SR10 of about 3.55×10^{-4} ng·μL^{-1} and 3.55×10^{-5} ng·μL^{-1}, respectively, while nested-PCR could detect as low as 3.55×10^{-9} ng·μL^{-1} of the fungal genomic DNA, which indicated at least 10 000-fold higher sensitivity than the conventional PCR method. Using PCR, the fungal DNA molecules were not detected from the mango peel after 8d inoculation with *Cl. cladosporioides*, while nested-PCR could amplify target band, which indicated nested-PCR could sensitively detect target pathogen. The nested-PCR could amplify the PCR band from six young mango fruits showing the typical, atypical disease symptoms of sooty blotch disease from Tainong and Jidan mangoes, but could not amplify the band from no visible disease symptoms of fruits, which did not appeared symptoms of sooty blotch disease all the time.【Conclusion】This study has developed two conventional PCR methods and two nested-PCR method for the detection of *Cl. cladosporioides* with higher sensitivity and specificity. The findings would be a good tool for vari-

ous applications, such as mango quarantine, early diagnostic applications and surveillance of mango sooty blotch disease, which would be useful for timely preventing this disease.

Key words: Mango; *Cladosporium cladosporioides*; Conventional PCR; Nested-PCR; Molecular detection

杧果露水斑病最早于2007年在海南昌江发生，目前已经蔓延到海南三亚、乐东、东方及四川攀枝花、广西、福建等杧果主产区，受害较为严重的品种有‘贵妃芒’‘金煌芒’，管理差的果园发病率高达100%[1-2]。该病主要危害果实，幼果期即可受害，但多在采收期表现出明显的症状，病斑呈不规则形水渍状，湿度大时，病斑上产生墨绿色霉层，且在3 d左右出现大量病斑，严重影响果实的外观价值，导致价格大幅下跌甚至没有商家愿意收购，给海南杧果种植户造成了严重的经济损失[2]。引起该病害的病原菌为半知菌类真菌枝状枝孢霉[*Cladosporium cladosporioides*（Fr.）De Varies]和球孢枝孢霉（*Cl. sphaerospermum*）[3]，其中枝状枝孢霉是主要致病菌，其喜好20~25 ℃、弱酸至弱碱、高湿、富含有机营养的环境[4]，20 ℃左右的适温、通风透气差、荫蔽潮湿、管理水平低的老果园发病重，目前较为有效的防治措施是农业防治，其次是化学防治[1, 5]，已报道的抑菌效果较好的药剂有吡唑醚菌酯、多抗霉素B、多菌灵、苯醚甲环唑、咪酰胺、苯甲丙环唑、溴菌腈[6]，其中吡唑醚菌酯和丙环唑以1∶4、咪酰胺和多抗霉素B以2∶3（*m*∶*m*）混配有明显的增效作用[7]。一般幼果期即可被侵染，但因其扩展缓慢、潜伏期长，所以常常到采收期才大量显症，而此时单靠喷药防治很难明显奏效，因此早期诊断该病害显得尤为重要。

巢式PCR技术在杧果病害检测方面的应用亦有报道[8-9]，但迄今国内外还未见利用该技术检测由*Cl. cladosporioides*引起病害方面的快速检测报道。笔者根据杧果露水斑病菌*Cl. cladosporioides*的ITS序列（HM856622.1），与NCBI中数据大量比对后，设计了8对引物，通过特异性检测及扩增条件筛选，获得了2对特异性良好的检测引物，并与ITS结合，建立了杧果露水斑病菌的巢式PCR检测体系，为杧果露水斑病菌的快速诊断和及时防治提供参考。

1　材料和方法

1.1　供试菌株

杧果露水斑病菌（*Cl. cladosporioides*）及杧果其他病害菌株（表1），均由中国热带农业科学院环植

表1　引物特异性检测所用菌株

Table 1　Strains used for specific detection of primers

病害名称 Disease name	病原菌 Pathogen	分离部位 Separated organs	采集地 Collecting location
杧果露水斑病 Mango sooty blotch disease	*Cl. cladosporioides*	叶 Leaf	海南省三亚市千家镇 Qianjia town, Sanya, Hainan
		茎 Stem	
		叶 Leaf	海南省三亚市藤桥镇 Tengqiao town, Sanya, Hainan
		茎 Stem	
		杂草 Weed	海南省三亚市崖城镇 Yacheng town, Sanya, Hainan
		叶 Leaf	
		枝条 Branch	海南省三亚市 Sanya, Hainan
		花 Flower	
		叶 Leaf	
		果 Fruit	四川省攀枝花市 Panzhihua, Sichuan
杧果蒂腐病 Mango stem end rot	*Botryodiplodia theobromae*	果 Fruit	海南省三亚市 Sanya, Hainan
杧果炭疽病 Mango anthracnose	*Colletotrichum cutatum*	果 Fruit	海南省儋州市 Danzhou, Hainan
杧果炭疽病 Mango anthracnose	*C. gloeoporioides*	叶 Leaf	海南省三亚市 Sanya, Hainan
杧果枝枯病 Mango branch wilt	*B. theobromae*	叶 Leaf	海南省三亚市 Sanya, Hainan
杧果细菌性黑斑病 Mango bacterial black spot	*Xanthomonas campestris* pv. *mangiferae*	果 Fruit	广西百色市 Baise, Guangxi
杧果疮痂病 Mango scab	*Sphaceloma mangiferae*	叶 Leaf	海南省昌江县 Changjiang, Hainan
杧果粉红聚端孢叶斑病 Mango *Trichothecium roseum* leaf spot	*Trichothecium roseum*	叶 Leaf	海南省儋州市 Danzhou, Hainan
杧果拟盘多毛孢叶斑病 Mango *Pestalotiopsis* grey leaf spot	*Pestalotiopsis mangiferae*	叶 Leaf	海南省东方市 Dongfang, Hainan
杧果畸形病 Mango malformation disease	*Fusarium mangiferae*	叶 Leaf	四川省攀枝花市 Panzhihua, Sichuan
杧果畸形病 Mango malformation disease	*F. proliferatum*	叶 Leaf	四川省攀枝花市 Panzhihua, Sichuan
杧果镰刀菌顶枯病 Mango *Fusarium* dieback	*F. decemcellμlare*	茎 Stem	四川省攀枝花市 Panzhihua, Sichuan

所热带果树课题组鉴定提供，用于杧果露水斑病菌引物特异性筛选。

1.2 待测杧果病菌gDNA和待测杧果样品gDNA提取

将各供试杧果病原真菌在PDA培养基培养5 d后，刮取菌丝，采用Fungal DNA Kit（OMEGA BIOTEK公司）提取总DNA；杧果细菌性黑斑病菌经NA培养液摇菌培养后，离心获得菌体，用Biomiga细菌DNA提取试剂盒提取总DNA。所得总DNA保存于−20 ℃备用。

杧果果皮样品基因组总DNA提取：将待测杧果样品冲洗凉干后，取果皮组织采用改良的CTAB法[10]提取基因组DNA。

1.3 杧果露水斑病病原菌巢式PCR检测体系建立

1.3.1 *引物设计* 通过将杧果露水斑病病菌的ITS序列与NCBI中的数据大量比对后，在差异位点区域设计8对引物（表2），用于筛选特异性引物。

1.3.2 *特异性引物及最佳退火温度筛选* PCR体系（25 μL）：2×*Taq* PCR Master Mix 12.5 μL，上下游引物（10 nmol · μL^{-1}）各1.0 μL，gDNA 1.0 μL，灭菌ddH_2O 9.5 μL。

表2 供试引物信息表

Table 2 Information of primers

引物名称 Primer name	序列 Sequence (5’－3’)	预期长度 Length/bp	引物名称 Primer name	序列 Sequence (5’－3’)	预期长度 Length/bp
ML-SF1	ccgagcaccctttagcgaata	316	ML-SF7	gcctcccgagcacccttta	406
ML-SR1	ttgcgtaactttgcagtctgagta		ML-SR5	gttcataaccctttgttgtcc	
ML-SF2	tagcctcccgagcacccttta	400	ML-SF8	gacagaagacccagccggt	290
ML-SR2	ccctttgttgtccgactctgtt		ML-SF5	gacacttcaaactcttgcgtaactt	
ML-SF3	gtgaaatgacgctcgaacagg	223	ML-SF9	tagcctcccgagcaccctt	408
ML-SR3	cgggtggacacttcaaactctt		ML-SR5	gttcataaccctttgttgtcc	
ML-SF4	caacgcttaggggacagaaga	287	ML-SF10	tagcctcccgagcaccctttt	424
ML-SR4	tgcgtaactttgcagtctgagta		ML-SR10	gtttaccaccgggatgttcataac	

PCR程序：94 ℃ 4 min；94 ℃ 45 s，T_m ℃ 45 s，72 ℃ 1 min，循环36周；72 ℃延伸10 min。退火温度T_m设置：51、54、57、60、63、65、67 ℃。PCR产物均用1.5%（*w*）琼脂糖凝胶电泳检测。选择只能在杧果露水斑病病菌gDNA中扩增出预期的单一条带且亮度最强的，作为筛选特异性强的引物和最佳退火温度T_m的依据，并对该条带切胶回收、克隆测序。

1.3.3 *巢式PCR检测体系建立及检测灵敏度测定* 巢式PCR用真菌通用引物ITS1（5’-TCCGTAGGTGAACCTGCGG-3’）和ITS4（5’-TCCTCCGCTTATTGATATGC-3’）作外侧引物，第一轮PCR体系同1.3.2，第二轮体系用第一轮扩增产物做模版。第一轮PCR程序为94 ℃ 3 min；94 ℃ 45 s，56 ℃ 45 s，72 ℃ 1 min，循环30周；72 ℃ 10 min。第二轮PCR程序参照1.3.2筛选所得结果。将杧果露水斑病菌gDNA按照0到10^{10}倍稀释后再作模板，分别使用常规PCR和巢式PCR扩增，比较分析2种检测方法的灵敏度。

1.4 杧果样品检测

1.4.1 *接种杧果的检测* 用接种杧果露水斑病菌8 d后的杧果果皮提取gDNA，进行巢式PCR检测，3次重复。

1.4.2 *田间采集样品的检测* 从田间采集杧果露水斑病典型症状、非典型症状和未显症的‘台农芒’‘鸡蛋芒’幼果为检测材料，进行巢式PCR检测。

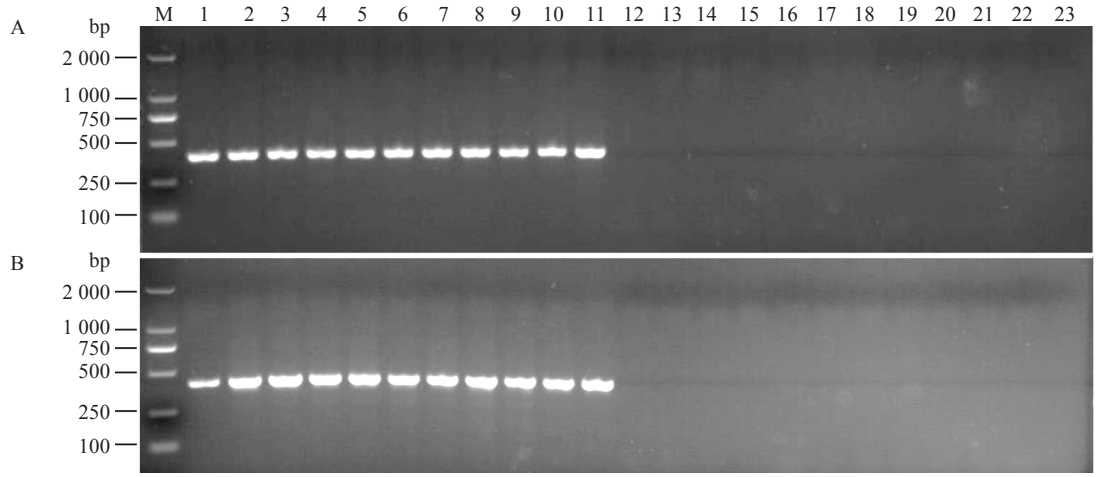
2 结果与分析

2.1 引物特异性分析

分别以供试的11株杧果露水斑病菌及11株杧果其他病害的病原菌gDNA作模板，完成常规的PCR扩增，从8对引物中筛选特异性引物，结果表明，在退火温度为65 ℃时，只有ML-SF9/ML-SR5和ML-SF10/ML-SR10这2对引物分别能从供试的11株杧果露水斑病菌中扩到408 bp和424 bp的单一特异性条带，而在11株杧果其他病原菌中扩增不到条带（图1），测序结果表明，该条带序列符合预期，说明这2对引物特异性很强。

2.2 引物灵敏度测定

将杧果露水斑病菌起始质量浓度为35.5 ng·μL^{-1}的gDNA，经10倍梯度稀释后作模板，分别用引物ML-SF9/ML-SR5和ML-SF10/ML-SR10进行常规PCR和巢式PCR。结果（图2）表明，采用常规PCR时，用引物ML-SF9/ML-SR5、ML-SF10/ML-SR10分

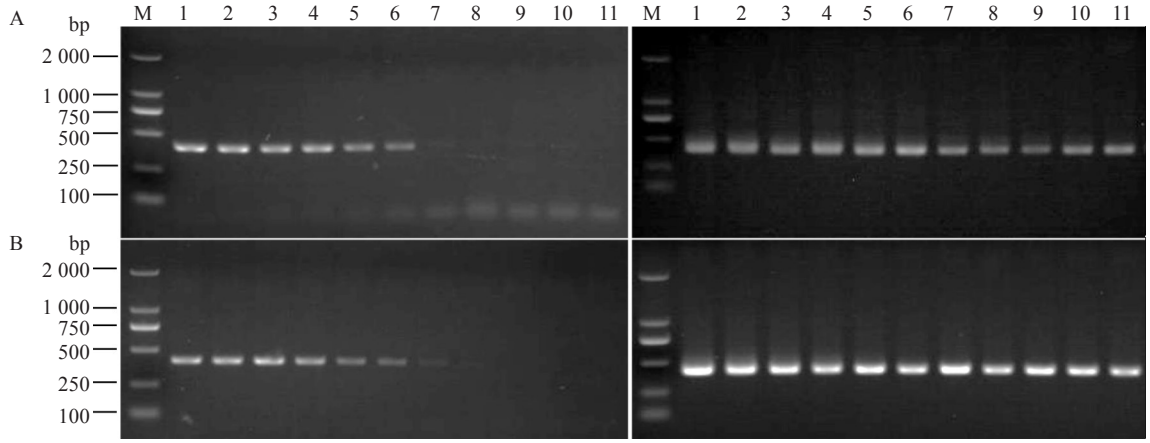


A. 引物 ML-SF9/ML-SR5 检测；B. 引物 ML-SF10/ML-SR10 检测。M. DL2000 Marker；1. 阳性对照；2~11. 不同采集地和不同部位分离的杧果露水斑病菌；12. 杧果蒂腐病菌；13. 杧果尖孢炭疽病菌；14. 杧果胶孢炭疽病菌；15. 杧果枝枯病菌；16. 杧果细菌性黑斑病菌；17. 杧果疮痂病菌；18. 杧果粉红聚端孢叶斑病菌；19. 杧果拟盘多毛孢叶斑病菌；20. 杧果畸形病菌；21. 杧果畸形病菌；22. 杧果镰刀菌顶枯病菌；23. ddH_2O 阴性对照。

A. Detection of ML-SF9/ML-SR5; B. Detection of ML-SF10/ML-SR10. M. DL 2000 Marker; 1. Positive control; 2-11. *Cl. cladosporioides* from different places and organs of mango; 12. *B. theobromae*; 13. *C. acutatum*; 14. *C. gloeoporioides*; 15. *B. theobromae*; 16. *Xanthomonas campestris* pv. *mangiferae*; 17. *Sphaceloma mangiferae*; 18. *Trichothecium roseum*; 19. *Pestalotiopsis mangiferae*; 20. *F. mangiferae*; 21. *F. proliferatum*; 22. *F. decemcellμlare*; 23. Negative control ddH_2O.

图 1　引物 ML-SF9/ML-SR5 和 ML-SF10/ML-SR10 特异性分析

Fig. 1 The specificity analysis of primers ML-SF9/SR5 and ML-SF10/SR10



A. 引物 ML-SF9/ML-SR5；B. 引物 ML-MF10/ML-SR10。M. DL 2000 Marker；1~11. DNA 质量浓度从 35.5 $ng\cdot\mu L^{-1}$ 至 3.55×10^{-9} $ng\cdot\mu L^{-1}$.

A. Primers ML-SF9/ML-SR5; B. Primers ML-MF10/ML-SR10. M. DL 2000 Marker; 1-11. DNA concentration from 35.5 $ng\cdot\mu L^{-1}$ to 3.55×10^{-9} $ng\cdot\mu L^{-1}$.

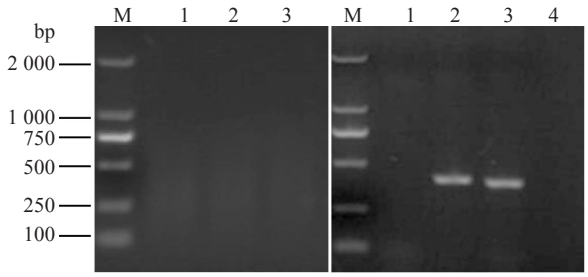
图 2　常规 PCR（左）和巢式 PCR（右）的灵敏度比较

Fig. 2 The sensitivity of conventional PCR （left） and nested-PCR （right）

别可检测的最低DNA质量浓度为3.55×10^{-4} $ng\cdot\mu L^{-1}$和3.55×10^{-5} $ng\cdot\mu L^{-1}$，后一对引物的灵敏度比前一对高10倍；采用巢式PCR时，当gDNA被稀释至3.55×10^{-9} $ng\cdot\mu L^{-1}$时，用上述2对引物也均可扩增出目标条带，但用引物ML-SF10/SR10扩增的条带更亮，同样说明引物ML-SF10/SR10的灵敏度更高。两种方法相比，巢式PCR的检测灵敏度比常规的PCR至少高出1万倍。

2.3　杧果样品的检测

2.3.1　接种样品检测　图3表明，用常规PCR中未



左. 常规 PCR。M. DL 2000 Marker；1. 引物 ML-SF10/ML-SR10；2. 引物 ML-SF9/ML-SR5；3. ddH_2O 阴性对照。右. 巢式 PCR。1. 第一轮 ITS1/ITS4 扩增；2~4. 第二轮扩增，其中 2. 引物 ML-SF10/ ML-SR10；3. 引物 ML-SF9/ ML-SR5；4. 阴性对照 ddH_2O。

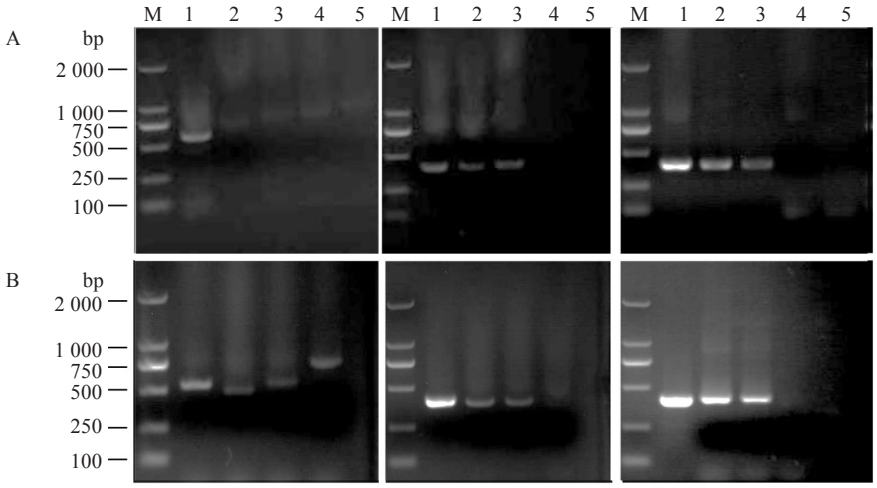
Left. Conventional PCR. M. DL 2000 Marker; 1. Primers ML-SF10/ ML-SR10; 2. Primer ML-SF9/ML-SR5; 3. Negative control ddH_2O. Right. Nested-PCR. 1. First amplification of primers ITS1/ITS4; 2-4. Second amplification，2. Primers ML-SF10/SR10；3. Primers ML-SF9/ ML-SR5；4. Negative control ddH_2O.

图 3 接种杧果的常规 PCR 和巢式 PCR 检测

Fig. 3 The detection results of conventional PCR and nested- PCR in inoculated mango

扩增到清晰的目标条带；用巢式PCR扩增获得的目标条带亮度高，可以很灵敏地检测到目标病原菌。这可能由组织中病原菌的含量少，杧果果肉中酚类物质多，导致所提取的gDNA浓度较低而引起的。

2.3.2 田间样品检测 如图4所示，通过巢式PCR的第一轮扩增检测，除阳性对照的条带位置符合预期且亮度较强外，待检测杧果样品均没有符合预期的目的条带，但经过第二轮扩增，均能从‘台农芒’‘鸡蛋芒’两个品种的杧果典型症状样品、非典型症状样品中扩增到目标条带，从无症状样品（始终未显症）和阴性对照中未扩增到条带，引物ML-SF10/ ML-SR10比ML-SF9/ML-SR5扩增的条带更亮，再次证明ML-SF10/ ML-SR10更灵敏。可见两套巢式PCR检测体系均可用于诊断田间杧果露水斑病。



A. 台农芒；B. 鸡蛋芒。左 . 第一轮 ITS1/ ITS 4 扩增；中 . 第二轮引物 ML-SF9/ ML-SR5 扩增；右 . 第二轮引物 ML-SF10/ML-SR10 扩增。M. DL2000 Marker；1. 阳性对照；2. 典型症状样品；3. 非典型症状样品；4. 未显症样品；5. 阴性对照 ddH_2O。

A. Tainong mango; B. Jidan mango. Left. First amplification of primer ITS1/4; Mid. Second amplification of primer ML-SF9/ML-SR5; Right. Second amplification of primer ML-SF10/ML-SR10. M. DL 2000 Marker; 1. Postive control (*Cl. cladosporioides*); 2. Sample with typical symptoms; 3. Sample without typical symptoms; 4. Sample of no symptoms; 5. Negative control ddH_2O.

图 4 杧果样品巢式 PCR 检测

Fig. 4 The nested PCR detection of mango samples

3 讨 论

虽然rDNA在病原菌的基因组中较为保守，但在不同生物间依然存在差别且为高拷贝基因，因此它依然是生物学研究中进行分子诊断的首选基因[11-12]。先后有研究者参考16S rDNA基因、核糖体蛋白基因rplJ/rplL、转录内间隔区（internal transcribed spacer，ITS）、基因内间隔区（intergenic spacer，IGS）、基因外部转录间隔区（external transcribed spacer，ETS）等设计出了一系列特异性引物用于检测不同微生物。此外，重复序列间的不编码区、存在序列差异的致病相关基因序列、β-operon、β-微管蛋白基因、钙调蛋白基因等也可以用来设计特异性引物。其中ITS序列被更为普遍地用于植物病原真菌

特异性引物设计。

目前我国记载的杧果病害约有88种[3]，已建立了PCR快速检测体系的杧果病害有：杧果炭疽病[9,13-14]、杧果畸形病[8,15-17]、杧果细菌性黑斑病[18]，但由枝状枝孢霉（*Cl. cladosporioides*）引起的杧果露水斑病的检测尚未见报道。笔者大量比对rDNA-ITS序列后，在差异位点设计和筛选引物，获得了2对特异性高的引物ML-SF9/ML-SR5和ML-SF10/ML-SR10及其扩增条件，实现了对*Cl. cladosporioides*的常规检测，同时参考杧果炭疽病菌巢式PCR的检测思路[9]，以ITS1/ITS4为外侧引物，以ML-SF9/ML-SR5和ML-SF10/ML-SR10分别为内侧引物，成功创建对*Cl. cladosporioides*的巢式PCR快速检测体系，其灵敏度可达3.55×10^{-9} ng·μL^{-1}，比常规PCR检测灵敏性高1万倍，更适合田间露水斑病的早期检测。两个检测体系虽只能实现定性检测，尚不能实现定量检测，但为后续研究荧光实时定量检测打下了基础，也为由*Cl. cladosporioides*引起的其他病害如水稻污点病[19]、柑橘腐败菌[20]、红花洋蹄甲叶霉病[21]、番木瓜黑星病[22]、草莓花枯病[23]等的分子检测提供了参考。此外，本试验也比对分析了β-微管蛋白基因，但未筛选到特异性引物。

杧果露水斑病菌虽然在田间只在果实上危害并显症，造成商品外观价值严重下降，但从前期的致病性测定和本研究检测的结果来看，该病菌的寄生部位不只限于果实，也可以寄生在杧果叶、茎、枝条、花和果园的禾草上，所以今后防治时，这些部位应列入防治范围，以有效减少果园菌源量。

4　结　论

本试验建立杧果露水斑病主要致病菌*Cladosporium cladosporioides*的两套常规PCR和两套巢式PCR快速检测体系，能实现对潜伏期果实的特异性检测，可检测病菌DNA的质量浓度最低为3.55×10^{-5} ng·μL^{-1}和3.55×10^{-9} ng·μL^{-1}，为杧果露水斑病的早期诊断提供了新方法。

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