# The Molecular Detection of *Corynespora Cassiicola* on Cucumber by PCR Assay Using DNAman Software and NCBI

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**Abstract.** Objective: to establish a quick molecular detection method in Beijing, which can prevent the occurrence of cucumber target spot disease from the source. Methods and results: The DNA band of *Corynespora cassiicola* had been obtained by PCR and sequenced. Using DNAman software and NCBI database to analyze the sequence, the results showed that the obtained DNA was that of *Corynespora cassiicola* on Cucumber. A specific primers CCC1/CCC2 were obtained by DNAman software and NCBI database. It was also proved to can be used to distinguish *Corynespora cassiicola* from other pathogenic fungi using DNAman software and NCBI database.

Keywords: Molecular detection · Corynespora cassiicola · DNAman · NCBI

### 1 Introduction

A cucumber target spot disease caused by *Corynespora cassiicola* (Berk. & Curt.) occurs serious damage. The leaves are the main victims and in severe cases the pathogenic fungi can spread to the petioles and even to the vine. Both on the top and back of leaves, large and small necrotic lesions can be formed and there is one white bull's eye on the center and even cause leaves dry by the humidity lesion. With the development of modern agriculture, in 2012 the agricultural facilities are expected to develop 35 acres. Facilities agriculture has been positioned as a main direction in the development of agriculture of Beijing. Cucumber, as an important vegetable in Beijing has been more attention in pest and disease control work. Spot disease caused by *Corynespora cassiicola* in cucumber is very serious and rapid progression in recent years and has caused more and more damages in China. Its occurrence was the growing trend and is causing serious economic losses to farmers from 2005 to 2010.

The current study shows that seed infection is an important spread way of cucumber target spot disease (*Corynespora cassiicola*) and in seeds markets the parameters related to purity, germination rate, moisture content are often detected. But the seed infection is often ignored, which often results in the occurrence of target leaf spot disease caused by *Corynespora cassiicola* in cucumber and results in late serious impact on growth and yield.

Traditional detection methods are time-consuming, low sensitivity, susceptible to the interference of man-made and environmental factors, etc. so it is necessary to

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establish a fast, easy and accurate detection method, provide the basis for early diagnosis and prevention of disease, which can prevent the occurrence of cucumber *Corynespora cassiicola* from the source. And if the seed can be resolved through physical measures, it can be effective technical measures to the comprehensive prevention and control of the target leaf spot in cucumber. This study aims to establish a molecular detection method using DNAMAN software and NCBI to detect the spot disease caused by *Corynespora cassiicola* in cucumber. The rDNA-ITS sequences of fungi which is the moderately repetitive sequence widely distributed in the genome. It has been reported to use the diversity of the rDNA-ITS sequences of fungi in the level of family, genus, and species to design specific primers to detect fungi.

# 2 Methodology

DNAMAN is the application of molecular biology in all software packages. The software package provides an integrated system with multiple features for efficient sequence analysis. This software can be used to do multiple sequence alignment for a restriction analysis, design primer, protein sequence analysis or graph. DNAMAN's speed, precision, and high quality versatility makes it a fundamental tool for each molecular biologist to rely on. It is also a sequence analysis software package to each university, research institution, laboratory and research scientist with affordable price. DNAMAN can be used in Microsoft Windows, MacOS and Linux. All three platforms of the DNAMAN file share the same format. DNAMAN common format of the system will help the communication between the PC, Macintosh and Linux, and make your work platform independent.

## 2.1 Materials and Methods

Procedures for the use of DNAman: we have got scar bacteria gene has been got, using DNAman software of sequence specific primers were designed, and the sequence structure analysis, to expect to get the sequence related biological information data, such as: open reading frame, amino acid sequence and protein translation simulation map, and he and other fungal homologous of distance analysis.

## 2.2 DNAMAN Software Sequence Alignment Method

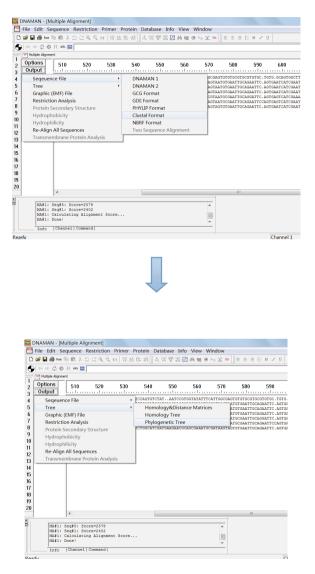
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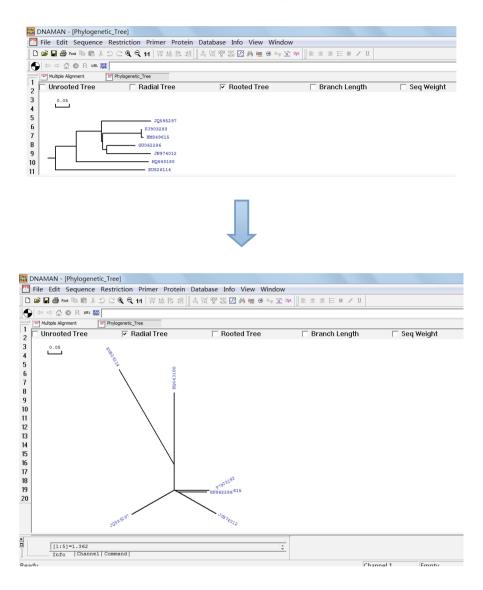
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After analysis of the phylogenetic tree, you can export to Clustal format, you can use bioedit to

view





#### 2.3 NCBI Database

Blast is a search programs based on sequence similarity developed by the U.S. Biotechnology Information (NCBI) database. Blast is the abbreviation of "partial similarity query tool" (Basic Local the Alignment Search Tool).

NCBI BLAST is a sequence similarity search program development, but also as to identify genes and genetic characteristics means. BLAST can be less than 15 s of time for the entire DNA sequence databases to search. NCBI provides additional software tools are: Finder open reading frame (ORF Finder), Electronic PCR, and sequencing

submission tool, Sequin and BankIt. All of the NCBI database and software tools can be obtained from the WWW or FTP. NCBI and E-mail server, providing a text search or sequence similarity search an alternative way to access the database.

The blast software of NCBI Sequence alignment method Input the sequence

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The results showed that the highest gene sequence was obtained.

Descriptions

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		Description		Max score	Total score	Query cover	E value	Ident	Accessio
0 1	Contraspora casalicola isolale CV1 180 ribosomal RNA per	s, partial sequence, internal transcribed spacer 1, 5,85 ribosomal RN	A gene, and infernal	1077	1077	100%	0.0	100%	30595296
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8,	Uncaffured Tunqua clone ABP 45 190 ribosomal RNA gene.	internal transcribed spacer 1, 5.8S ribosomal RNA	ene, and internal tra	1066	1066	100%	0.0	99%	JF 497148
5	Constreagona casalicola laotale 4549 185 stosomal RNA per	gene, and internal transcribed spacer 2, complete seguence; and 285 ribosomal RNA gene, partial	A pane, and inferral	1064	1064	100%	0.0	99%	F.1852574
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8	Contrespora cassificita isolate A565 180 ribosomal RNA per	sequence; internal transcribed spacer 1, 5.85 ribosomal RNA gene, and internal transcribed	A pane, and interna	1059	1059	100%	0.0	99%	EJ852578
0	Connespora cassilicita isolate GU120 185 ribosomal RNA p	ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 285 ribosomal	TVA game, and inter	1053	1053	100%	0.0	99%	EJ852631
0,	Convespora cassicota lastate IFT-221011-28 180 ribosom	RNA gene, partial sequence	somal Rtsk cene, i	1040	1040	100%	0.0	99%	87288850
0.	Conversional cassificate installe JY 180 ribosomal RNA pane	partial sequence, internal transcribed spacer 1, 5,80 ribosomal RNA	pane, and internal tr	1040	1040	50%	0.0	99%	E.8548217
8	Connespora cassitoria strain IHE internal transcribed space	r. 1. partial sequence, 1.80 ribosomal Rhik pane, complete sequence	Land, Informal Rando	1033	1033	95%	0.0	100%	20287447
8	Conversional cassificate strain CATABOOD Informal transcribed	Ispacer 1, partial sequence, 5.85 ribosomal RNA gene, complete sec	avence, and internal	1833	1033	95%	0.0	100%	EF199117
0.5	Connespora cassilicola attain BG internal transcribed apacer	1. partial sequence, 5.85 nbosomal RNA cens, complete sequence.	and internal transport	1029	1029	90%	0.0	99%	2017445
	Connespora cassilicata isolate CATASSR1 eternal transcribe	d spacer 1, partial sequence, 5.85 stopportal RNA pene, complete se	quence, and interna	1027	1027	95%	0.0	99%	EU822317

By blast sequence analysis method of main program is: by gene sequence has received the scar bacteria directly in NCBI nucleic acid libraries for comparison, direct comparisons have been obtained and nucleic acid sequences base sequence homology. The highest number of genes that have been registered with the highest homology sequence homology was obtained by comparison. If the similarity reached 99 %, the obtained sequence should be the gene.

# 2.4 The Tested Strains Were Provided by the Plant Protection Station of Beijing

The tested strains were provided by the plant protection station of Beijing. The tested strains culture: the strains were inoculated into potato dextrose liquid medium, 28 °C, 145 r/m oscillation culture between 4 and 7 d, filtration collection mycelium, and then placed in dry heat sterilization box at the temperature of 60 °C drying – 20 °C frozen preservation reserve. The strain was inoculated onto the agar medium, 28 °C, and 4–7d.

#### 2.5 Cucumber Seed Source

Tested cucumber seed source: Beijing 10 suburban counties (Fangshan, Miyun, Daxing, Changping, Pinggu, Yanqing, etc.); Cucumber (Zhongnong 16, Beijing 203, Beijing 204), fruit cucumber (Mini 2, Dai Duoxing).

#### 2.6 DNA Extraction and rDNA-ITS Amplification and Sequencing

The method of the genomic DNA of the tested strains and the total DNA extraction was use by White T;: Total DNA was extracted from Trout C L's methods: the 0.25 g soil samples were milled into powder, and 0.5 mL 0.4 % of the milk powder solution vortex suspension was added. 12 000 R/m in centrifugal m 3, with 2 ml of supernatant was 0. 3 % SDS extraction buffer vortex mixed, then add other volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, V/V) solution upside down mixing, 12000 r/m in centrifugal 25 min. In the supernatant phase to another centrifugal tube, the addition of 0.6 times the volume of cold ISO alcohol, 4 °C in 20 min, Centrifuge for 10 min in 12000r/m. In the supernatant, wash 2 times with 70 % alcohol. After drying, the precipitation was dissolved by TE with RNase A, and the reserve was kept at 20 °C.

rDNA-ITS region of cucumber scar blotch were amplified using ribosomal DNA in eukaryotes universal primers ITS1 and its4 [11] and the sequence as follows: ITS1: 5 '-TCCGTAGGTGAACCT2GCGG-3', ITS4: 5 '-TCCTCCGCTTATTGATATGC-3'. Reaction system (25  $\mu$ L): ddH<sub>2</sub>O17. 2  $\mu$ L, 10 x buffer of 2.5 M $\mu$ L, MgCl<sub>2</sub> 1. 5  $\mu$ L, dNTP (10 mmol/L) of 0.5  $\mu$ L, 10 mol/L of universal primers ITS1/ITS4 1  $\mu$ L, 1  $\mu$ L of template DNA, Taq polymerase 0. 3  $\mu$ L. PCR reaction program was as follows: 94 °C 3 min as pre degeneration, 94 °C 1 min, 56 °C1 min, 72 °C 50 s as a cycle, and repeats 32 cycles; then 72 °C 10 min as a extension. The PCR products by 1.0 % agarose gel electrophoresis detection and recovery of connection and transformation, sequence by Beijing Shanghai Biological Engineering Co., Ltd.

#### 2.7 Design of Specific Primers

The rDNA-ITS sequence of the *Cladosporium tenuissimum* (NO. FJ603350, FR822778, FR822800, FR822816, FR822843<sup>A</sup>DFR822848) and the *Botryotinia fuckeliana* (NO. GU062311.1), *Trichoderma atroviride* (NO. JP665257), *Corynespora cassiicola* (NO. JQ595296.1) of cucumber were download by GenBank. Specific primers (CCC1/CCC2) designed and CCC1/CCC2 primers and the comparison between specific primers (CCC1/CCC2 and sequencing of the ITS sequence of the scars were got by using DNAMAN, DNAStar and Premer5 softwares.

### 2.8 Primer Specificity Verification

PCR was amplified by CCC1/CCC2 with the specific primers and the genomic DNA of all the strains tested as the template. Reaction system and procedure 1.5. PCR products were detected by 1 % agarose gel electrophoresis (Fig. 1).

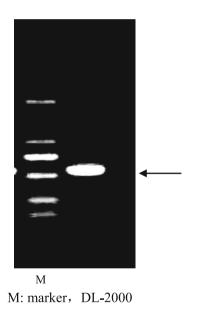


Fig. 1. Gene PCR electrophoresis of scar plaque

# **3** Results and Analysis

## 3.1 Obtaining the Sequence of Corynespora Cassiicola

According to GeneBank query using fungal ribosomal rDNA universal primers ITS1 and ITS4, then got the DNA of pathogenic fungi by PCR. After sequencing, the sequence was compared using the blast software of NCBI. The alignment results were: the similar rate of the DNA sequence of using ITS1 and ITS4 as primers and that of

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internal transc		mal RNA gene, partial sequence; NA gene, and internal transcribed nal RNA gene, partial sequence
GenBank: JQ595297.1		
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partial sequence: int transcribed spacer 2, GGAAGTAAAAGTCGTAACAAG CCCTTCGAGATAGCACCCTT CCCACCACAAACCCATTGTAG CAACCACGGATCTCTGGTC GAATCAGGGATCATCGAAT	5297.11 Corpresport cassicola isolate ZCI 185 ernal transcribed spacer 1, 5.85 ribosomal RNA complete sequence; and 285 ribosomal RNA gene, CTITCOGFAGETGAACCHCGGFAGGCAGCATTATCOTAGGGCCTCC GTITATGAGCACCTCTCGTTCCTGGCAGGCCACCCTCCCACACGG TACAAGAAGTACACGTCTGAACAAACAAACCAAAC	gene, and internal partial sequence CC IGA ITI CA ITC ICC ICC

Fig. 2. The DNA sequence of GenBank accession number JQ595297

	Sequences producing significant alignments:									
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		Description	Max score	Total score	Query cover	E value	Ident			
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		Convnespora cassilicola isolate CM2000-1 internal transcribed spacer 1, 5.85 ribosomal RNA gene, and internal transcribed spacer 2, complete segue	1077	1077	100%	0.0	100%			
		Convnespora cassilicola isolate AS54 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and interna	1070	1070	100%	0.0	99%			
		Uncultured fungus clone ABP 45 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	1066	1066	100%	0.0	99%			
		Convnespora cassilicola isolate AS49 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and interna	1064	1064	100%	0.0	99%			
		Convnespora cassilicola isolate AS119 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	1061	1061	100%	0.0	99%			
		Connespora cassilicola isolate RWB321 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 1, 5.8S ribosomal	1059	1059	100%	0.0	99%			

Fig. 3. Sequence producing significant alignments

scar fungus *Corynespora cassiicola* (GenBank accession number JQ595297 (Fig. 2)) can be reached 100 % (Fig. 3) in NCBI database. The results showed that DNA band obtained from PCR is the DNA fragment from fungus *Corynespora cassiicola*.

#### 3.2 Homology Analysis

The homology analysis of the DNA sequences of *Corynespora cassiicola* (GenBank accession number JQ595297) and *Botryotinia fuckeliana* (GenBank accession number FJ903283, HM849615), *Cladosporium sp.* (GenBank accession number GU062286, JN974012), *Phytophthora capsici* (GenBank accession number HQ643180), *Pseudoperonospora cubensis* (GenBank accession number EU826114.1) were done by DNAman. The results showed that they had homology relationship between them (Fig. 4). The closest relationship with *Corynespora cassiicola* (GenBank accession number JQ595297) was *Botryotinia fuckeliana* (GenBank accession number FJ903283, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286, HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession number GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession humber GU062286), HM849615), followed by *Cladosporium sp.* (GenBank accession humber GU062286), HM

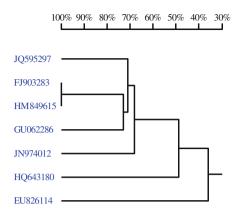


Fig. 4. The homology tree of Corynespora cassiicola and other Fungus pathogenic fungi

JN974012), and the most distant relative to a was *Pseudoperonospora cubensis* (GenBank accession number EU826114.1).

#### 3.3 Obtaining Specific Primers

The DNA of *Corynespora cassiicola* was analyzed by the DNAman software (Fig. 4) and the specific primers were obtained from the Fig. 5.

CCC1(TCGTAGGGGCCTCGCCCCTTCGAGATAGCAC, CCC2(GAAGTGGCTGCGGGTCGGCGCACCATGAGC).

#### 3.4 The Results of Primer Specificity Verification

The results showed that there was an band at about 600 bp in the PCR products that was product of genome DNA as the templates and the ITS1/ITS4 as primers, but there was no band in CK. This result showed that the extracted genomic DNA template was in accordance with the requirements of the amplification;

Using designed specific primers of ccc1/CCC2 as the primers and the genomic DNA extracted from all strains (*Trichoderma*, *Cladosporium sp.*, *Botryotinia fuckeliana* and *Corynespora cassiicola*) as the templates for PCR amplification, electrophoresis results (Fig. 6) found only in *Corynespora cassiicola* genomic DNA as the template amplified specific bands of about 600 bp, and expected results are consistent, other cucumber pathogenic fungi didn't amplify specific band. The results showed that the designed primers were relatively specific, and could be distinguished *Corynespora cassiicola* from other pathogenic fungi.

M:Marker, DL-2000; CK: ddH<sub>2</sub>0;  $1 \sim 2$ : Trichoderma, Cladosporium sp.  $3 \sim 4$ Botryotinia fuckeliana;  $5 \sim 6$ : Cladosporium sp.;  $7 \sim 10$  Corynespora cassiicola

JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	GGAAGTAAAAGTCGTA TTTTGTATGGCGAATTGTAGTCTATAGAGGCGTGGTCAGC TCTTGGTCAATTTAGAGGAAGTAAAAGTCGTA TCTTGGTCCATTTAGAGGAAGTAAAAGTCGTA GGAAGTAAAAGTCGTA	16 40 32 32 0 0
JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATC GTGGCGCGTTGGGGTAAGTTCCTTGGAAGAGGACAGCATG ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAC. ACCAAGGTCTCCGTAGGTGAACCTGCGGAGGATCATTAC. TCCGTAGGTGAACCTGCGGAAGGATCATTAC. CCACACCTAAAAACTTTCCACG ACAAGGTTTCCGTAGGTGAACCTGGCGAAGGATCATTAT. c a a a	56 80 71 72 31 23 55
JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	G.TAGGGGCCTCGCCCCCTTCGAGATAGCACCCTTT GAGGGTGATACTCCCGTTCATCCCTGAGTGGCTCGTGCGT AGAGTTCATGCCGGAAAGGGTAGACCTCCC.ACCCTT AGTGACCCCGGTCTAACCACCGGGATGTTC.ATAACC AGAGTTCATGCCCGAAAGGGTAGACCTCCC.ACCCTT T.GAACCGTATCAACCCTTTTAGTTGGGGGCTCTGTACCC CGAGTTAGGGTCCCCAGGGCCCGAATCTCCCAACCCT	91 120 107 108 67 62 92
JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	GTTTATGAGCACCTCTC.GTTTCCTC. ACGACCCGTTTTCTTGAGTCGCGTTGTTTGGGAATGCAG GTGTATTATTACTTT.GTTGCTTT CTTTGTTGTCCGACTCT.GTTGCTCCGG GTGTATTATTACTTT.GTTGCTTT. TATCATGGCGAATGTT.GG.ACTTCGGT TTTTTTTTCCAACCTCT.GTTGCTTCGGGGGGGCCCGTCCT g c t	116 160 130 136 90 89 131
JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	GCCAGCTCGCCTGCCA CGCAAAGTAGGTGGTAAATTCCATCTAAAGCTAAATATTG GCCGAGCTGCCTTCGG GCCGAGCTGCCTTCGG GCCGAGCTGCCTTCGG CCGGCCGAGTAGCTTTTG TGATGGACCGCCGGGGACCCCCCTTGCGGTGTCCTCTG g	133 200 146 153 106 108 171
JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	ACGGGGACCCACCACAAACCCATTGTAGTAGTACAAGAAGTAC GTGCGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGAT GCCTTGTATGGTCGCCAGAGAATACCAAAACTCTTTTAT GCGGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAA GCCTTGTATGCTCGCCAGAGAATACCAAAACTCTTTTAT TTTTAAACCCATTTCACAATTCTGGTTATACTGTGGGGAC GCCCGTGCCCGTCGATAGCCCACGTCTAAACTCTTGCTTA	173 240 186 191 146 148 211
JQ595297 EU826114 FJ903283 GU062286 HM849615 HQ643180 JN974012 Consensus	ACGTCTGAACAAAACAAAACAAACTATT GAAAAGAACTTTGAAAAGAGAGTTAAAGAGTA.C TAATGTCGTCTGAGTACTATATAATAGT CTTTGCAGTCTGAGTAAACTTAATTAATAAA TAATGTCGTCTGAGTAAACTTAATTAATAAG GAAGTCGTCTGCTTTAACTAGATAGC AAACGTGTTTTTTTGCCTAAATTCATAACTAAAAAAAAAC a	201 273 214 222 174 175 251

Fig. 5. The alignment of sequence of *Corynespora cassiicola* and DNA sequence of other fungus.

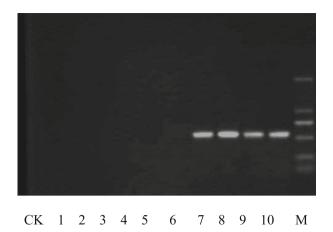


Fig. 6. CCC1/CCC2 primer specific primers were used to detect PCR using different templates.

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