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

Weiqing Wang

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

[1]Weiqing Wang *

[1]Beijing Vocational College of Agriculture, China

weiqingfine@163.com

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 sequcence, the results showed that the obtained DNA was that of *Corynespora cassiicola* on Cucumber. A specific primers CCC1/CCC2 were obtained by DNAman softeware 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.

Wang Weiqing: Laiyang Town, Shandong Province,China

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

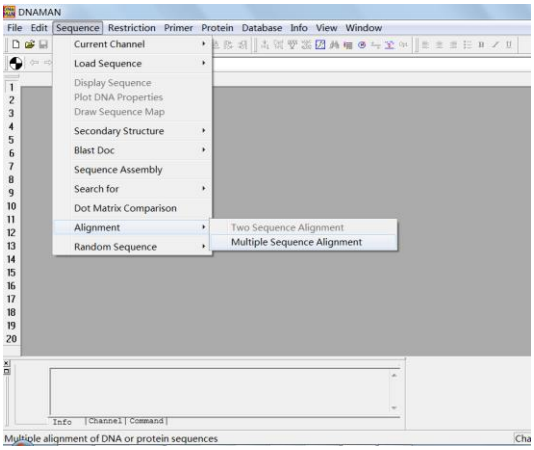
DNAMAN is the application of molecular biology in all software packages. The software package provides an integrated system with multiple features for efficient sequence analysis. This software can be used to do multiple sequence alignment for a restriction analysis, design primer, protein sequence analysis 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.

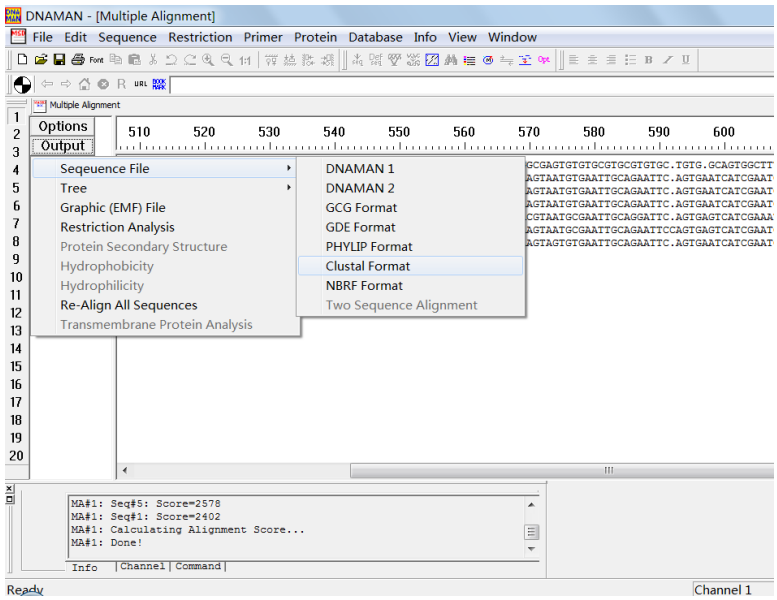
Procedures for the use of DNAman: we have got scar bacteria gene has been got, using DNAman software of sequence specific primers were designed, and the sequence structure analysis, to expect to get the sequence related biological information data, such as: open reading frame, amino acid sequence and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

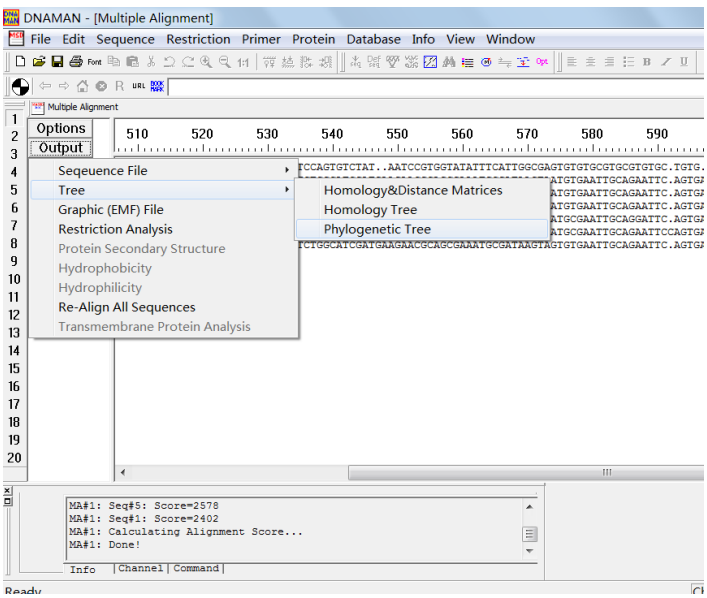
2.2 DNAMAN software Sequence alignment method

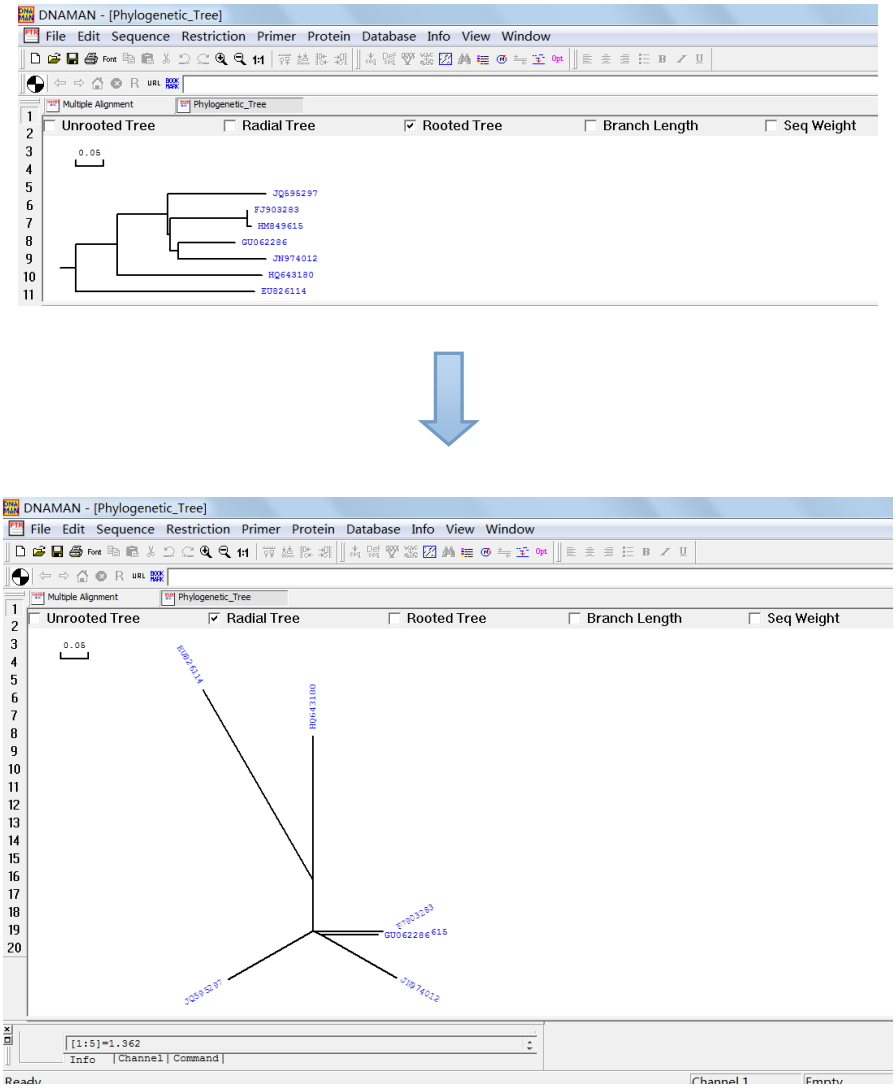
Insertion sequence, multiple sequence alignment:



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







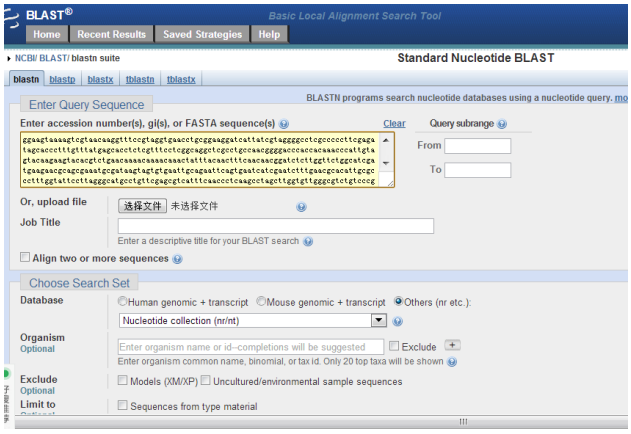
2.3 NCBI database

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).

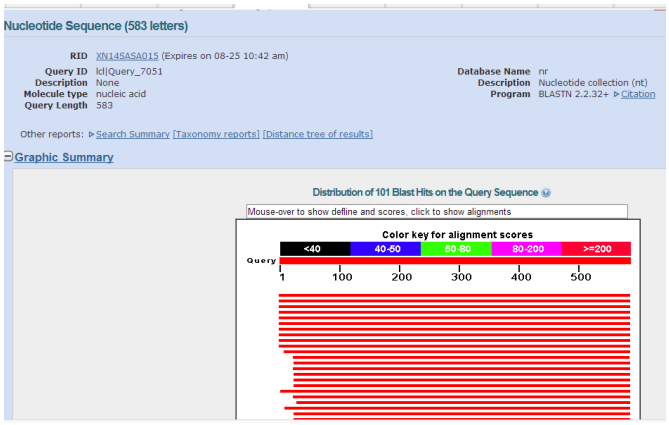
NCBI BLAST is a sequence similarity search program development, but also as to identify genes and genetic characteristics means. BLAST can be less than 15 seconds of time for the entire DNA sequence databases to search. NCBI provides additional software tools are: Finder open reading frame (ORF Finder),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

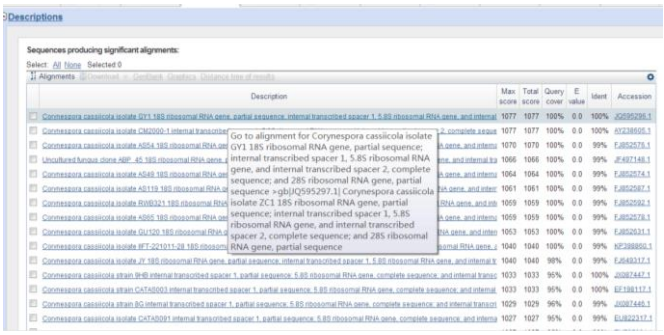
Input the sequence



Blast



The results showed that the highest gene sequence was obtained.



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℃, 145r/m oscillation culture between 4 and 7 d, filtration collection mycelium, and then placed in dry heat sterilization box at the temperature of 60℃ drying - 20℃ frozen preservation reserve. The strain was inoculated onto the agar medium, 28℃, 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, 12 000 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℃ in 20min, Centrifuge for 10 minutes 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℃.

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 (25μL): ddH_2O17. 2μL, 10 x buffer of 2.5 MμL, $MgCl_2$ 1. 5μL, dNTP (10 mmol / L) of 0.5μL, 10 mol / L of universal primers ITS1/ ITS4 1μL, 1μL of template DNA, Taq polymerase 0. 3μL. PCR reaction program was as follows: 94℃ 3 min as pre degeneration, 94℃ 1min，56℃1min，72℃50s as a cycle, and repeats 32 cycles; then 72℃10min 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

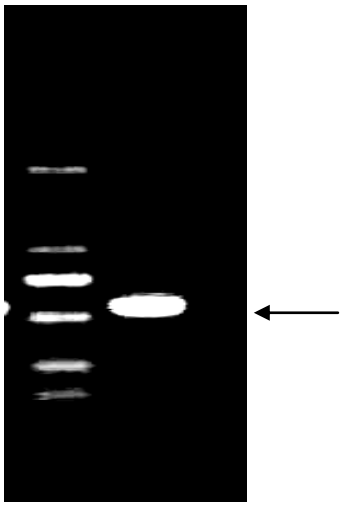
The rDNA-ITS seuence of the *Cladosporium tenuissimum* (NO. FJ603350、FR822778、FR822800、FR822816、FR822843 和 FR822848) and the *Botryotinia fuckeliana* (NO. GU062311.1) 、*Trichoderma atroviride* (NO. JP665257) 、*Corynespora cassiicola* (NO. JQ595296.1) of cucumber were download by GenBank. Specific primers (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.

3 Results and analysis

3. 1 Obtaining the sequence of *Corynespora cassiicola*

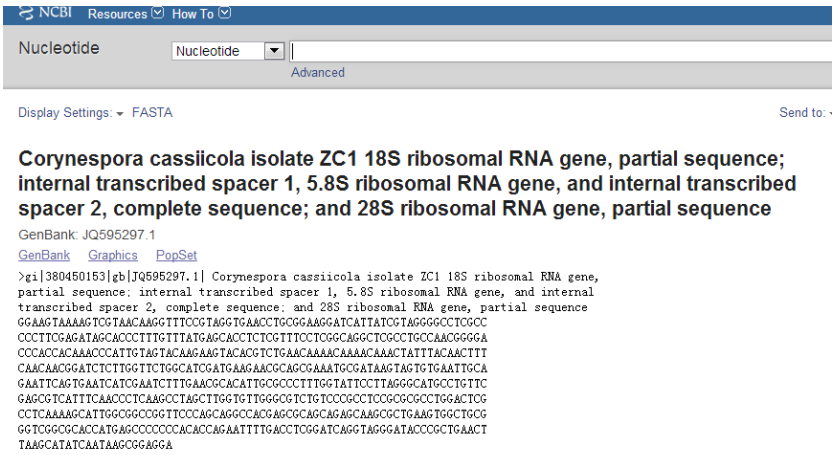


M

M: marker， DL-2000

Fig.1 Gene PCR electrophoresis of scar plaque

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 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*.


NCBI Resources How To

Nucleotide Nucleotide

Advanced

Display Settings: FASTA Send to:

Corynespora cassiicola isolate ZC1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: JQ595297.1

GenBank Graphics PopSet

>gi|380450153|gb|JQ595297.1| Corynespora cassiicola isolate ZC1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTAGGGGCCTCGCC
CCCTTCGAGATAGCACCCTTTGTTTATGAGCACCTCTCGTTTCCTCGGCAGGCTCGCCTGCCAACGGGGA
CCCACCACAAACCCATTGTAGTACAAGAAGTACACGTCTGAACAAAACAAAACAAACTATTTACAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCTTAGGGCATGCCTGTTC
GAGCGTCATTTCAACCCTCAAGCCTAGCTTGGTGTTGGGCGTCTGTCCCGCCTCCGCGCGCCTGGACTCG
CCTCAAAAGCATTGGCGGCCGGTTCCCAGCAGGCCACGAGCGCAGCAGAGCAAGCGCTGAAGTGGCTGCG
GGTCGGCGCACCATGAGCCCCCCCACACCAGAATTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACT
TAAGCATATCAATAAGCGGAGGA

Fig.2 The DNA sequence of GenBank accession number JQ595297

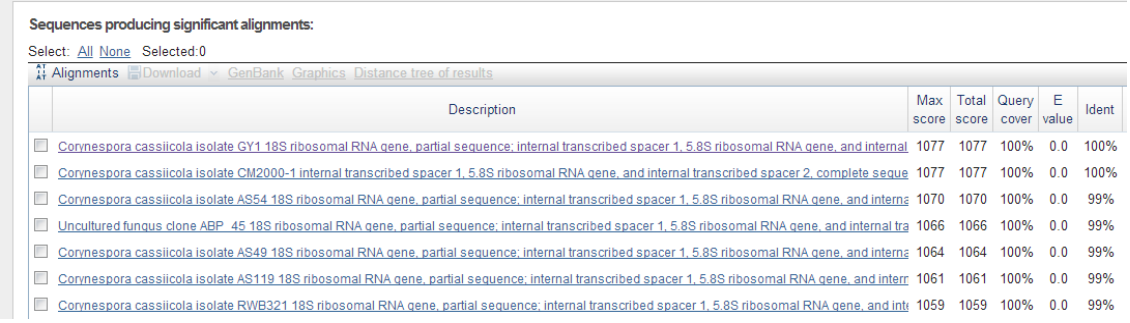


Fig.3 Sequence producing significant alignments

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 capsic*i (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.2). 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, JN974012), and the most distant relative to a was *Pseudoperonospora cubensis* (GenBank accession number EU826114.1).

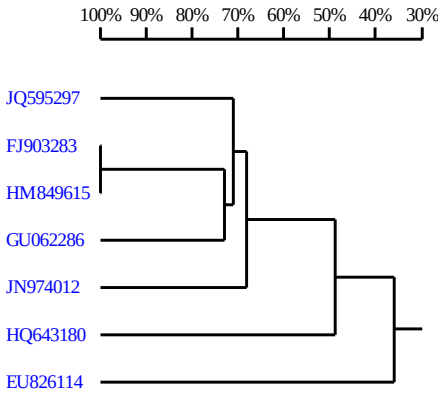


Fig.2 The homology tree of *Corynespora cassiicola* and other Fungus pathogenic fungi

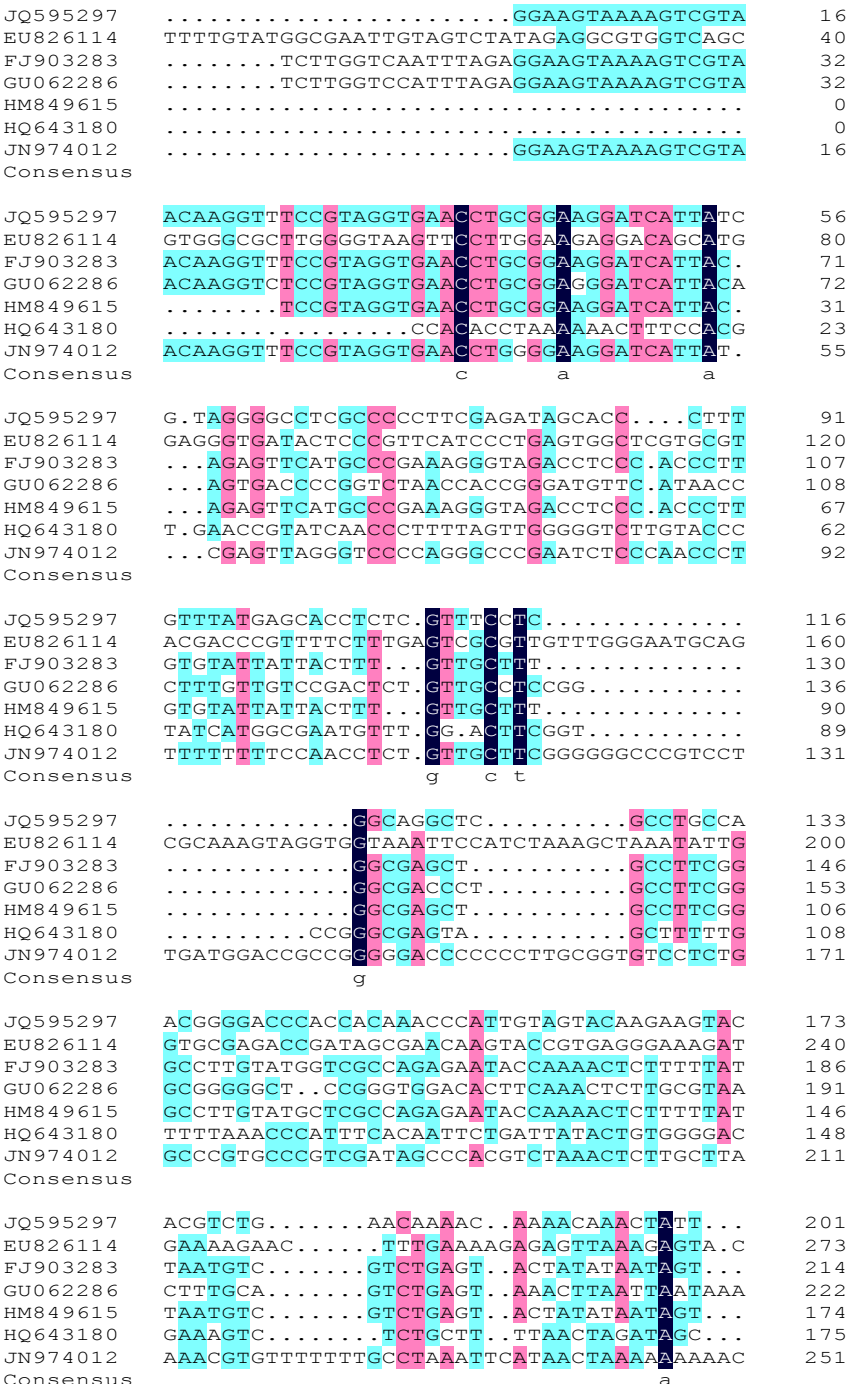


Fig.3 The alignment of sequence of *Corynespora cassiicola* and DNA sequence of other fungus.

3.3 Obtaining specific primers

The DNA of *Corynespora cassiicola* was analyzed by the DNAman software(Fig.2) .and the specific prmers were obtained from the Fig.3.

CCC1（TCGTAGGGGCCTCGCCCCCTTCGAGATAGCAC，

CCC2(GAAGTGGCTGCGGGTCGGCGCACCATGAGC)。

3.4 The results of primer specificity verification

The results showed that there was an band at about 600bp 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.4) found only in *Corynespora cassiicola* genomic DNA as the template amplified specific bands of about 600bp, 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.

CK 1 2 3 4 5 6 7 8 9 10 M

Fig.4 CCC1/CCC2 primer specific primers were used to detect PCR using different templates.

M：Marker,DL-2000; CK: ddH_20； 1~2：*Trichoderma*, *Cladosporium sp.*3~4 *Botryotinia fuckeliana*; 5~6: *Cladosporium sp.*； 7~10 *Corynespora cassiicola*

References

1. Dixon LJ1, Schlub RL, Pernezny K, Datnoff LE. Host specialization and phylogenetic diversity of Corynespora cassiicola. Phytopathology. 2009 Sep;99(9):1015-27

2. L i S， Hartm an G L. Molecula r detection of Fusa riumsolani f. sp. glycines in soybean roots and soil [J]. Plant Pathology， 2003， 52: 74 - 83.

3. White T， B runs J， Lee S， et al. Amplification and direct sequencing of fungal ribosomal RNA genes

forphylogenetics [A]. PCR Protocools: A guide to methods and application [M]. San Diego: Academic Press，1990. 315 – 322

4. Frederick R D， Snyder C L， Peterson G L， Bonde M R. Polymerase chain reaction assay for the detection and discrimination of the soybean rust pathogens Phakopsora pachyrhizi and P. meibomiae.Phytopathology，2002， 92: 217-227.

5. Trout C L， Ristaino J B， Madritch M， Wangsomboondee T. Rapid detection of phytophthora infestans in late blight-infected potato and tomato using PCR. Plant Disease， 1997， 81: 1042-1048.

6. Bonants P J M， Hagenaar De Veerdt M， Van Gent-Pelzer M P，Lacourt I， Cooke D E L， Duncan J M. Detection and identification of Phytophthora fragariae Hickman by the polymerase chain reaction.European Journal of Plant Pathology， 1997， 103: 345-355.

7. Grote D， Olmos A， Kofoet A， Tuset J.J， Bertolini E， Cambra M.Specific and sensitive detection of phytophthora nicotianan by simple and nested-PCR. European Journal of Plant Pathology，2002，108: 1997-2007.

8. Zhang Z G， Zhang J Y， Wang Y C， Zheng X B. Molecular detectionof fusarium oxysporum f.sp. niveum and Mycosphaerella melonis ininfected plant tissues and soil. FEMS Microbiology Letters， 2005，249: 39-47.