# Identification of the Difference in Genetic Molecules from Different Sources of *Plasmodiophora Brassicae* Worm

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**Abstract:** Clubroot is a serious disease in Brassicaceae species, caused by an obligate biotrophic pathogen, *Plasmodiophora brassicae* Worm. It may result in great damages to many economically important Brassica crops. *P. brassicae* cannot be cultured when it is isolated from the host plant tissues. So the direct investigations in this organism are very difficult and time-consuming. The molecular techniques can be useful tools to investigate and monitor this organism. In present study, the variation in this organism was observed based on the difference in genetic molecules from different sources of *P. brassicae* races. Spores of the pathogen were isolated from the infected clubroot collected from different regions in Sichuan Province and adjacent areas, and also from different cruciferous crops. A pair of specific primers (Forward primer: 5'-CAGCGACAGGGCGAAGA-3', Reverse primer: 5'-GAGGCATCTGGAGTTGGAGTTT-3') were designed to investigate the differences in genomic DNA in *P. brassicae*. Polymerase Chain Reaction (PCR) was conducted to amplify the specific DNA fragment from the different sources of *P. brassicae*, The specific DNA fragments amplified from different sources of *P. brassicae* were cloned into the vector pMD19-T (TaKaRa, Dalian, China) and were sequenced for analysis of the variations in the different pathogen races. The results showed that the different sources of *P. brassicae* possessed a common special DNA fragment, 1031bp in length. This fragment was identical in length among different pathogen races. However, variations were observed by further investigations into the nucleotide sequences in the fragments from different pathogen sources. The sequence analysis showed that the different sources of *P. brassicae* could be classified into two main groups: one including the races from Chengdu city, Mianyang city, and Yaan city; another was from the Kangding Autonomous Region and Chongqing city. It was suggested that the special pair of primers used in present study could be applied for the detection of presence and the early diagnosis of *P. Brassicae* in cruciferous crops. They may also be used to identify the genetic variations in this organism.

**Key words：** *Plasmodiophora brassicae* Worm; Specific DNA fragment; Sequence analysis

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## 1. Introduction

Clubroot is a serious disease in Brassicaceae species, caused by an obligate biotrophic pathogen, *Plasmodiophora brassicae* Worm, which may result in great damages to many economically important Brassica crops. This organism is very difficult to study with traditional methods because it only grows in the infected living tissues of plants, but does not survive in

artificial culture media. The development in modern molecular biological technologies has provided new methods to investigate this pathogen. A widely applied molecular biotechnology is the polymerase chain reaction (PCR) which is fast, sensitive, precise and simple and can be used to identify genetic variations at the DNA molecular level in all living organisms. Hee et al [1] and Faggian et al [2] designed a pair of specific primers according to the internal transcribed spacer (ITS) region in *P. brassicae* genome. They obtained a special DNA sequence using this pair of primers to detect *P. brassicae* in the soil and the plant roots. Yang et al [3] obtained a 629 bp special DNA sequence in the host plants infected by *P. brassicae* in different *Brassica* species, including *B. rapa*, *B. chinensis, B. oleracea, B. alboglabra* and cauliflower, from different regions, This DNA sequence is released on the GenBank database. Additionally, Yang et al [4] analyzed the amplified DNA fragment using the universal primers ITS1 and ITS4 in the recombinant DNA (rDNA) from *P. brassicae*, and designed a pairs of specific primers to detect the pathogenic bacteria. In this study, several *P. brassicae* races were collected from different regions in Sichuan Province and a region in Chongqing City to detect their difference in genetically molecules. A special fragment of DNA molecule was amplified with a pair of specific primers designed based on a conserved genomic sequence of *P. brassicae* released on GenBank database.

**2. Materials and methods**

***Materials and primers*** A total of 9 sources of *P. brassicae* and plant tissues were collected from different crops and different regions in Sichuan Province and in Chongqing City (see Table 1). Clubroot galls and young leaves were taken from the infected plants in field at the 7 to 10 leaf stage. The plant materials were cleaned with tap water and stored at -20℃ for use. Based on the genomic sequence of *P. brassicae* (D85819) released on GenBank database, a pair of specific primers (Forward primer, CR-F, 5'-CAGCGACAGGGCGAAGA-3' and Reverse primer, CR-R, 5'-GAGGCATCTGGAGTTGGAGTTT-3') were designed with the software Primer Premier 5.0(http://www.PremierBiosoft.com), and were synthesized by the Invitrogen Biotechnology Co., Ltd (Shanghai, China).

***Isolation of total DNA*** Samples of total DNA were extracted from the infected clubroot and the young leaves, respectively, and were purified as described by Jia Li [5]. The DNA samples were stored at -20℃for use.

***Isolation of genomic DNA from the clubroot spores*** Clubroot spore suspensions were prepared as described by Williams [6]. The clubroot galls were ground in a blender and filtered through gauze. The spore suspension was centrifuged at 10000g for 5 min. Then, the spore pellet was resuspended and centrifuged. The purified spore pellets were transferred to the SDS extraction buffer [50mM Tris HCl, 50mM ethylenediaminetetraacetic acid, 3%(w/v) sodium dodecyl sulphate (SDS), 1%(v/v) 2-mercaptoethanol, Ph 7.4], containing 100 ul Proteinase K(10mg/ml) and 1ml lysozyme (60mg/ml), and mixed with glass beads. The mixture was roughly shaken for 10 min. Genomic DNA of *P. brassicae* was extracted following the method by Manzanares [7]. The DNA samples were finally dissolved in TE buffer.

Table 1 The pathogen sources of *P. Brassicae* in this study

| Numbers | Sources | Species | Sites |
|---|---|---|---|
| NO.1 | Clubroot-infected roots | *Brassica napus* | Nanjiao, Yaan, Sichuan, China |
| NO.2 | Clubroot-infected roots | *Brassica chinensis* | Nanjiao, Yaan, Sichuan, China |
| NO.3 | Clubroot-infected roots | *Brassica parachinensis* | Nanjiao, Yaan, Sichuan, China |
| NO.4 | Clubroot-infected roots | *Brassica napus* | Kangding, Sichuan, China |
| NO.5 | Clubroot spore suspension | *Plasnmdiophora brassicae* | Kangding, Sichuan, China |
| NO.6 | Clubroot-infected roots | *Brassica napus* | Wenjiang, Chendu, Sichuan, China |
| NO.7 | Clubroot-infected roots | *Brassica juncea* | Fuling, Chongqing, China |
| NO.8 | Clubroot-infected roots | *Brassica napus* | Tianquan, Yaan, Sichuan, China |
| NO.9 | Clubroot-infected roots | *Raphanus sativus* | Fucheng, Mianyang, Sichuan, China |
| NO.10 | Leafs of clubroot-infected plant | *Brassica napus* | Nanjiao,Yaan,Sichuan, China |
| NO.11 | Leafs of health plant | *Brassica napus* | Nanjiao,Yaan,Sichuan, China |
| NO.12 | Roots of health plant | *Brassica napus* | Nanjiao,Yaan,Sichuan, China |

***Cloning and sequencing of the specific DNA fragment of P. Brassicae*** Polymerase chain reaction (PCR) was performed with approximately 200ng genomic DNA as template in a 50 ul reaction volume, using the designed specific primers. The reaction of amplification was programmed at 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 sec, 58℃ for 30 sec and 72℃ for 1 min. The amplified products were separated on 2% agarose gel and stained with Glod-View (a substitute of ethidium bromide). The band with the expected size was excised out and the corresponding DNA fragment was recovered and purified using the AxyPrep DNA Gel Extraction Kit (AxyGEN, USA). The recovered fragment was cloned into the pMD®19-T Vector (TaKaRa, Dalian, China) and transformed into the *E. coli* strain DH5α competent cells (Tiagen, Beijing, China). The inserts in the recombinant clones were amplified by PCR using the same specific primers described above with the sequencing primers (M13). Then the amplified products were sequenced in both directions by the Invitrogen Life Technologies, Co. LTD (Shanghai, China).

***Sequence analysis*** The nucleotide sequence of the amplified DNA fragment was determined by a ABI-PRISM 3730 sequencer (USA). The sequence was spliced, aligned and analyze using the softwares ContigExpress 4.1.1 and DNAMAN 6.0.

**3. Results**

***Identification of amplified specific fragment from P. Brassicae*** DNA samples of the clubroot-infected roots and leafs were extracted and detected for their qualities. The OD values ($OD_{260}/OD_{280}$) of all of the DNA samples were between 1.8 and 2.0, showing good quality and high purity. The PCR was performed with the specific primer pair (CR-F and CR-R) and the amplified products were detected on 2% agarose gel (Figure 1). The results showed that a special 1031 bp DNA fragment was amplified from the genomic DNA samples of the

clubroot-infected roots and the pathogen spore suspensions. No fragment was amplified from the DNA samples of the leaves from clubroot-infected plants, the leaves from healthy plants and the roots from healthy plants. It was shown that this special DNA fragment was specifically amplified from the *P. Brassicae* pathogens, instead of the host plants. In addition, the amplified DNA fragment showed no observable difference in length among the different sources of *P. brassicae*. The designed specific primers can, therefore, be used for the detection of *P. Brassicae* and the early diagnosis of clubroot.

***Alignment of amplified specific fragment from P. Brassicae*** The amplified special DNA fragment was recovered, cloned and sequenced for a further investigation into the difference in nucleotide sequences. The alignment of nucleotide sequence indicated that variations existed in single nucleotides in some of the regions of the special DNA fragment from different sources of *P. Brassicae*. For an example, the sequences of *P. Brassicae* from Nanjiao, Kangding and Fuling, were different from that of Wenjiang and Tianquan. The different hosts infected by *P. Brassicae* in the same region had the same special sequence. This may be due to the fact that different hosts in the same region were infected by the same *P. Brassicae* race.

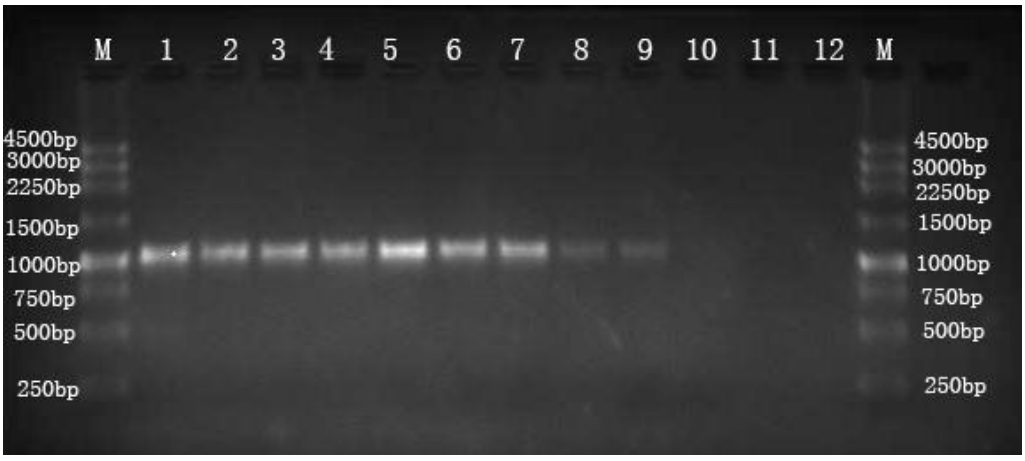


Figure 1 The results of PCR amplification with different sources of *P. brassicae* and plant materials

M,250bp Ladder Marker；lane 1-12, different sources of *P. brassicae* and plant materials (see Table 1)

## 4. Discussion

In this study, the directly extracted DNA from infected roots may contain DNA of the host plants and the other unknown organisms. In order to assure the special DNA fragment from the *P. brassicae*, the genomic DNA was extracted from the purified pathogen spores and used as a reference. This may probably eliminate the influence of DNA fragments from organisms other than *P. brassicae*. We used the same conditions to amplify the special DNA fragment from different tissues including the spore suspension, the clubroot-infected roots, the leaves and the roots of healthy plants, with the specific primers CR-F and CR-R. A common fragment was obtained from the spore suspensions and the clubroot-infected roots, while no fragment was amplified from the leaves and roots of healthy plants. It was suggested that the special DNA fragment was amplified from the genomic DNA of *P. brassicae* rather than of the host plants. This special fragment of DNA amplified by the specific primer pair (CR-F and CR-R) could be used to detect the presence of *P. brassicae* and used for the early diagnosis of clubroot disease.

It was indicated that the special DNA fragments from the different sources of *P. brassicae* were same in length, so it seemed not applicable to use this fragment to distinguish the different races in *P. brassicae* from different regions. However, a further investigation at the nucleotide level of this DNA fragment revealed variations among the different races of *P. brassicae* from different regions. This variation may be used to differentiate different races in *P. brassicae*. This type of variation at the molecule sequence level might be related to the variations in pathogenicity of races in *P. brassicae*. However, further studies are necessary to investigate the relationships between the variations in pathogenicity and in nucleotide sequence of this special DNA fragment.

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