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Research Article

Phylogenetic Analysis of the Sequences of rDNA Internal Transcribed Spacer (ITS) of *Phytophthora sojae*

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Abstract: The internal transcribed spacer (ITS) region (ITS1, ITS2 and 5.8S rDNA) of the nuclear ribosomal DNA (nrDNA) was amplified via the PCR method in seventeen different isolates of *Phytophthora sojae* using the common primers of the ITS of fungi. Around 800 bp−1,000 bp fragments were obtained based on the DL2000 marker and the sequences of the PCR products were tested. Taking isolate USA as outgroup, the phylogenetic tree was constructed by means of maximum parsimony analysis, and the genetic evolution among isolates was analyzed. The results showed that there is a great difference between the base constitution of ITS1 and ITS2 among various isolates. The seventeen isolates are classified into three groups, and the isolates from the same region belong to the same group, which shows the variation in geography.

Keywords: soybean; *Phytophthora sojae*; rDNA; phylogeny

Phytophthora root rot caused by *Phytophthora sojae* is a serious soil-borne fungi disease endangering soybean production[1]. It was first discovered in Indian state in the United States in 1948, and then in about 20 countries throughout the world[2-4]. Owing to the separation technique, *P. sojae* was not found in China until Shen *et al.*[5] isolated the pathogen in Heilongjiang Province in 1991 for the first time.

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Thereafter, the phytophthora root rot occurred widely and affected the soybean production of Heilongjiang Province[6].

Phytophthora root rot is a pathogen with the characteristics of host specification. By the interaction process with the hosts, the pathogenicity and virulence evolved continually, and at least fifty-five races have been identified worldwide. Analysis of the genetic diversity of *P. sojae* is significant and meaningful for disclosing its virulence variation. It is not only localization but is also difficult to determine the genetic diversity of *P. sojae* at the DNA level, if only the differential hosts with different resistance genes are used in virulence screening and race identification. With the development of molecular biology, ITS, RAPD, SSR, and RFLP have been used widely in studying the genetic background of microbe and the genetic similarity and diversity of interspecies and intraspecies[7-9]; the ITS (Internal Transcribed Spacer) technique is broadly used as a molecular marker for the identification of the interspecies or intraspecies to reveal the phylogenetic evolution relationship among isolates within a species or from different geographic regions and for species identification because of its high diversity. In this study, the pathogen of *P. sojae* from infected soybean plants that originated from various regions of Heilongjiang Province was isolated, identified, and purified. ITS analysis was carried out using the isolates separated in this study and those from the North America to study their genetic variation and phylogenetic relationship.

1 Materials and Methods

1. 1 Collection of infected plant sample and isolation, purification, and identification of pathogen

1.1.1 Infected plant sample collection

The soybean plants with evident PRR symptoms were mainly collected from three locations (i.e., Jixian, Jiansanjiang, Jiamusi County) of Heilongjiang Province, China, from July to August in 2005. Plant stems with lesions of 10 ± 15 cm length were kept in a cool room (4℃) for maximum of 3 d before isolation.

1.1.2 Pathogen isolation

P. sojae was isolated from 26 infected plants with typical PRR symptoms collected from the districts mentioned above by means of infected tissue separation. Four or five cross sections were made at the transition areas between the lesions and the healthy tissues. These sections were thoroughly flushed under tap water for about 1 h and immersed in 70% ethanol for 5 sec, the ethanol was then rinsed off with sterilized distilled water for three times. Following blotting up the surface water with sterilized filter paper, the sections were quickly passed through the outer blaze of alcohol burner twice, and finally put on the CA solid selection medium[10]. The tissues were incubated at 25℃ in an incubator.

1.1.3 Pathogen purification and identification

The pathogen colony was inspected under an inversion microscope of 15×10 amplification after 3 d, and a mark was made when *P. sojae* was observed. The pathogen within the selection part was transited to the CA solid selection medium. The purification process was repeated for 6−7 times. The mycelium was put into the distilled water for 24−36 h at 25℃ to observe the zoospore. The isolate was identified as *P. sojae* if it is according with the following characteristics. i.e., the asexual sporangium could be seen like an inversion pear, and the hypha was almost a right angle and had no separation, and the oospore was almost round on the CA solid selection medium. The zoospore could be seen in water.

1.1.4 Single spore separation and pathogen preservation

The zoospore of optimal concentration was transferred to the cell culture plate based on the method of Zuo *et al*[11]. The single zoospore separation was carried out under the inversion microscope after 2−3 days. The mycelium that originated from

single spore separation was transited to normal CA medium after culturing for 5−6 days. The hypha was preserved at 4℃ when the mycelium was full.

1.2 Sequence analysis of *P. sojae*

1.2.1 Isolates for testing

The isolates for testing are listed in Table 1, which includes 8 isolates separated in this study and 9 North-America isolates provided by the Agriculture and Agri-Food Canada or the Greenhouse and Processing Crops Research Centre and the August First Land Reclamation University.

1.2.2 Primers

Common primers of ITS1 and ITS4 of epiphyte ribosome DNA were used in this study. The sequence of the primers ITS1 and ITS4 were 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′, respectively.

1.2.3 DNA Extraction

P. sojae isolates were initially grown on clarified V8 agar plates at 24℃ for 7 days. Five to ten agar plugs (6 mm) with mycelium were cultured in 150 mL sterile clarified 20% V8 liquid in 250 mL flasks at 24℃ and 100 r/min for 7−14 days. The mycelia were then harvested by vacuum filtration through no.4 filter papers. The harvested mycelia were washed three times with sterile distilled water and then stored at −70℃. DNA extraction was carried out as described by Panabieres *et al*[12].

1.2.4 DNA amplification

The volume of the PCR reaction system was 25 μL, which contained 2.5 μL 10×buffer, 2.5 μL $MgCl_2$ (5 mmol/L), 1 μL dNTP (2.5 mmol/L), 0.25 μL forward and reverse primers (20 μmol/L), 0.25 μL Tag polymerase (10 U/μL), and 3 μL template DNA (15 ng/μL). The PCR program consisted of 30 cycles of denaturing at 95℃ for 1 min, annealing at 55℃ for 30 s, and extension at 72℃ for 1 min. The final cycle had a 10 min extension step at 72℃. The PCR products

Table 1 Taxa of *Phytophthora sojae* for studying nrDNA ITS

No.	Isolate	Locality	Voucher
1	D35Race7	North America	Yu KF
2	R1	North America	Yu KF
3	R4	North America	Yu KF
4	R7	North America	Yu KF
5	XUP_100	North America	Yu KF
6	XUP_101	North America	Yu KF
7	XUP_102	North America	Yu KF
8	XUP_103	North America	Yu KF
9	XUP_104	North America	Yu KF
10	USA	China	Zhang SZ
11	XUP_5	China	Zhang SZ
12	XUP_47	China	Zhang SZ
13	XUP_weizhi	China	Zhang SZ
14	Hs_3	China	Zhang SZ
15	Hs_6	China	Zhang SZ
16	Ps411	China	Zhang SZ
17	W1	China	Zhang SZ

were sequenced after purification using the purification agent box (Promega).

1.2.5 Sequence analysis

Analogue was detected with BLAST on the server on NCBI (httP://www.ncbi.nih.gov). The ITS sequence of all the isolates was saved as phylip format after calibration using the ClustalX software. The maximum parsimony tree was made by dnapars software.

2 Results

2. 1 Pathogen isolation, purification and identification

The infected plants with typical PRR symptoms were picked up from the soybean field (Fig. 1A): the leaf stalks were drooping, the leaves were yellow but were not falling off, and the entire plant was withering. Brown lesions spread to the upper node, and finally the cortex and vascular bundle changed to chocolate color.

Eight isolates were obtained after pathogen culture, purification, and single-spore separation (Fig. 1B). The mycelium was white looking like felt, and the mycelium grew blooming on the CA medium (Fig. 1C). The hypha had many nuclus with right-angle branch and no papillate. The base of the hypha was shrinking, and the width was 3−9 μm, and most of the hypha was curving (Fig. 1D). The sporangium appeared to be an inversion pear. The size of the sporangium was 42−65×32−53 μm (Fig. 1 E). The sporangium germinated to form vesicle: the vesicle was thin, with several zoospores inside. The zoospores were oval, one end or two ends were blunt round, with one flagellum in front and the other at the end. The oospore was round, and the diameter was 30−35 μm (Fig. 1 F). The results showed that these isolates agreed with the characteristics of *P. sojae*.

2. 2 rDNA ITS amplification and sequence analysis

Using primer specific for ITS, a single band 800

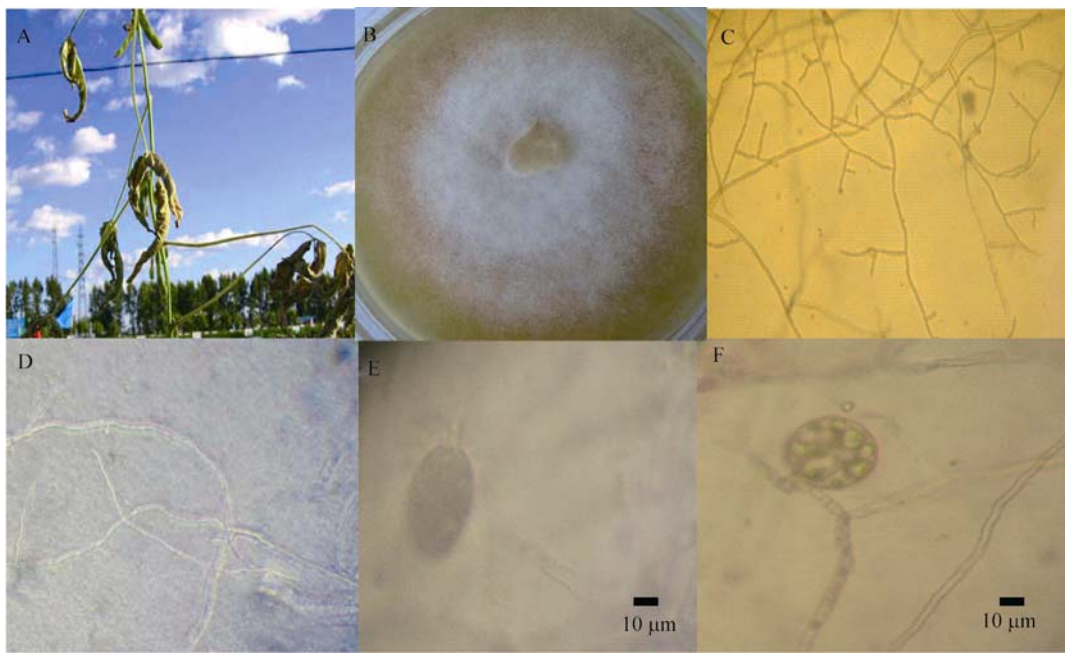


Fig. 1 The isolation, purification and identification of *P. sojae*

A: Typical infected soybean plant; B:The colony of *P. sojae* on the CA solid selection medium; C:The hypha of *P. sojae*(15×10); D:The hypha of *P. sojae* originating from single-spore separation(15×20); E: Asexual sporangium of *P. sojae* like inversion pear (15×40); F: Oospore of *P. sojae* (15×40).

bp to 1,000 bp in length was amplified from 8 Chinese isolates and 9 North American isolates. The analogue of the PCR products of the 17 isolates was detected using the BLAST on NCBI server. The analogue of the sequence is above 94% when compared to that from GenBank, and 83% by means of DNAMAN software.

The length of the ITS region (including ITS-1, 5.8S, and ITS-2) varied greatly, and the concrete sequence length is shown in Table 2. The length of ITS-1 ranged from 208 bp to 329 bp, and the average length was 268.84 bp. The percentage of the (C+G) content of ITS-1 ranged from 48.1% to 56.8% with an average of 52.52%. The length of ITS-2 varied from 396 bp to 748 bp, and the (C+G) content was above 50%. The 5.8S region was very conservative, its length was 134 bp, and the percentage of the (C+G) content was 50%.

A variation occurred in the ITS sequence among the 17 isolates based on the map of ITS and 5.8S rDNA (Fig. 2), and the scope from 1 to 100 bp of the ITS1 region also varied greatly. The ITS1 region from 1−50 bp of D35Race7, Hs_3, Hs_6, Ps411, XUP_104, XUP_100, XUP_101, USA, W1, XUP_5, and XUP_47 was completely deleted. The non-dele- tion sequence of R1, R4, XUP_103, and R7 was the same. The base sequence from 51-100 bp of R1, XUP_102, XUP_103, and R7 was identical, but there was base displacemant and transition in the sites of other isolates. Insertion and deletion occurred frequently in the latter part of the ITS2 region of 17 isolates, which indicates that the sites with phylogeny evolution information are mainly distributed on the ITS-2 region. In addition, base displacement and transition are the main evolution forms for ITS-1, and frequent base insertion and deletion are the main evolution forms for ITS-2.

2. 3 Phylogeny evolution relationship among groups of *P. sojae*

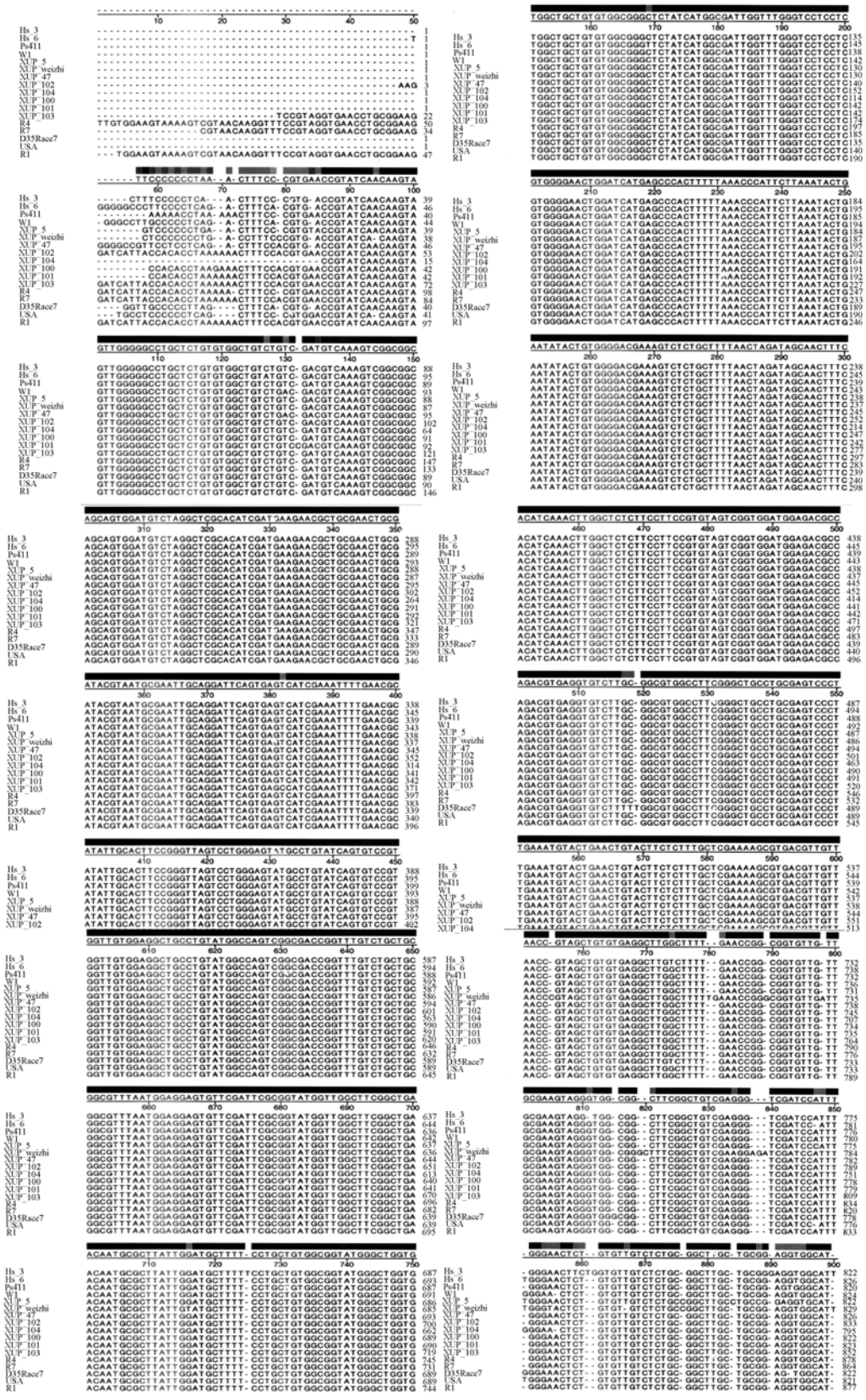
A maximum parsimony phylogeny tree was made based on the ITS sequence including the 5.8S region using Phylip3.5 analysis software (Fig. 3). The value on the branch was 1,000 multiple self-expansion, and these isolates were classified into 4 groups according to the phylogeny evolution results. Four isolates from China were all grouped into the 4th system, and there was a relationship between W1 and Hs 6, which acquires the sustainability of bootstrap (99%). These were also close to XUP_47 (100%). There were 6 isolates in the 3rd pedigree, and all these isolates originated from North America, with the exception of Ps411 from China. The second pedigree consisted of both Chinese and North American isolates. The results indicate that the distribution of Chinese isolates is wide and the genetic variation is complicated. Most of the isolates from the same district are grouped into the same pedigree, which shows that there exists geographic difference among *P. sojae*.

3 Discussion

Crawford *et al.*[8] and Cooke *et al.*[13] tested the sequence of ITS of *P. sojae* and other phytophthora pathogen from some other crops, but the number of pathogen of soybean phytophthora root rot in their study was relatively few. Zhang *et al*.[14] reported that

Table 2 Sequence analysis of nrDNA ITS and 5.8S regions in *Phytophthora sojae* isolates

	ITS-1		5.8S		ITS-2	
	Length	%(G+C)	Length	%(G+C)	Length	%(G+C)
Maximum	329	56.8	134	50	748	57.6
Minimum	208	48.1	134	50	396	52.4
Mean	268.84	52.52	134	50	572.32	55.34



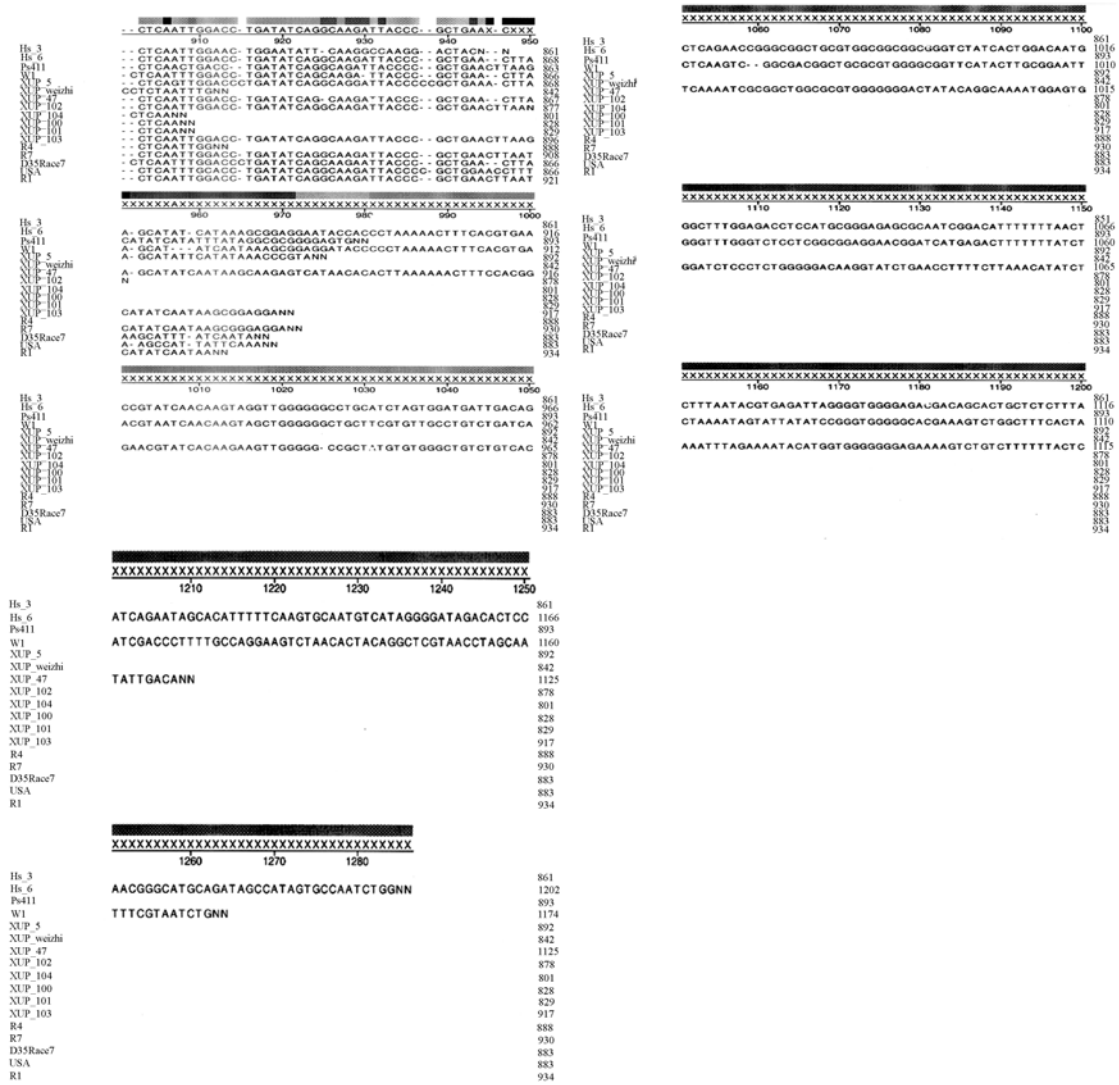


Fig. 2 The map of ITS and 5.8S rDNA sequence of *Phytophthora sojae*

"."means nucleotide deletion.

there was an ITS base sequence variation among different isolates in *P. sojae*. The intraspecies identification of *P. sojae* isolates from North America and China was done in this study. When compared to the former research results, the number of isolates was relatively great and the geography distribution was relatively wide, which could contribute to the origin and evolution of *P. sojae* in China. The phylogenetic relationship of *P. sojae* isolates from North-America and China was examined, and the results showed that there are variation sites on the rDNA ITS sequence in different isolates, and there is genetic differentiation in various degrees.

There exists ITS sequence diversity among *P. sojae* individuals in this study. Cooke *et al*.[13] concluded that the ITS sequence of *P. sojae* was very conservative and had no variation. In this study, after comparation of the ITS sequence of 17 isolates with those registered on the Genbank, there was ITS sequence variation among different *P. sojae* isolates, which showed that there was tiny variation among *P. sojae* individuals of the same intraspecies.

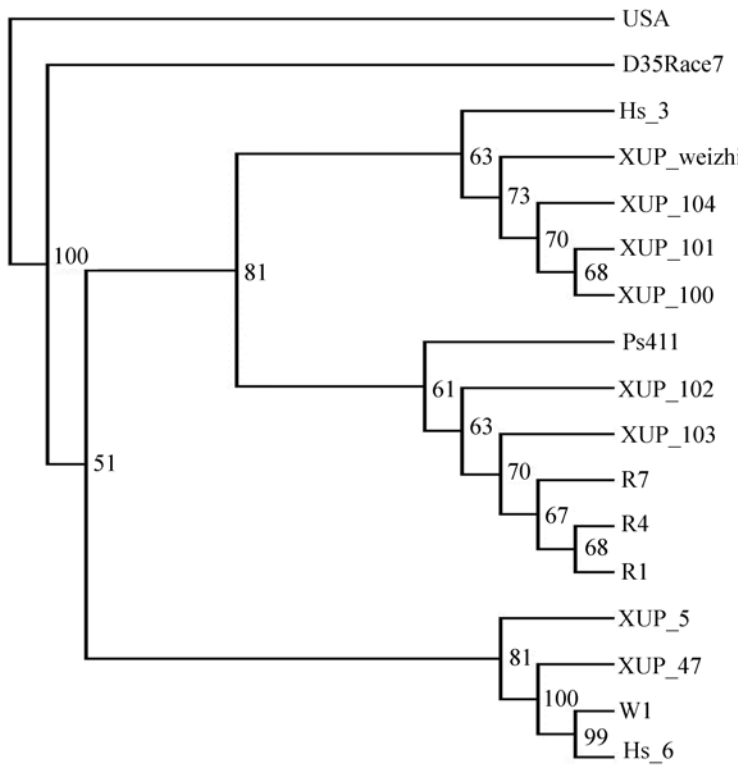


Fig. 3 Phylogeny tree of *Phytophthora sojae* based on the sequence of ITS+5.8S

Whisson *et al*.[7] deduced that *P. sojae* in Australia was probably introduced from America based on the analysis of the genetic relationship between *P. sojae* from Australia and that from America using the RFLP technique. The genetic variation of *P. sojae* in China was more complicated than that in North America, and had a relatively unique genetic background. The diversity and uniqueness of *P.sojae* in heredity in this study were similar to the previous report[15], and it can be detected that *P. sojae* from China probably has independent origination.

The results showed that there is a corresponding variation degree among the ITS sequence of different isolates. The isolates can be carved up different pedigree based on the ITS sequence. Therefore, ITS sequence analysis is an effective measure for studying the phylogenetic evolution and bio-geography of *P. sojae*.

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大豆疫霉根腐病菌的 rDNA ITS 序列分析

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摘 要: 采用真菌核糖体基因转录间隔区(ITS)通用引物, PCR 扩增了大豆疫霉根腐病菌具有差异的 17 个菌株的 ITSI 与 ITS2, 经过与 DL2000 的标准分子量 DNA 进行比较, 得到了大约 800~1000 bp 左右的片段, 并对 PCR 产物进行了序列测定。以 USA 为外类群利用最大简约法构建了大豆疫霉根腐病菌的系统发生树, 并分析了菌株之间的遗传进化关系。结果表明: 不同菌株 ITS1 和 ITS2 在碱基构成上有很大差异, 17 个菌株大致分为 4 个谱系中, 且来自于同一地区的菌株大都分布在同一谱系中, 显示出地理上的差异。

关键词：大豆；疫霉根腐病菌；rDNA；系统发育

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