# Analysis of pathogenicity and genetic variation among *Phytophthora sojae* isolates using RAPD

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Fifty-five *Phytophthora sojae* isolates were collected from soil samples and diseased soybean plants from Illinois, Indiana, Iowa, and Minnesota in 1994 and 1995. Races for the isolates were determined. DNA of *P. sojae* isolates was amplified with 16 Operon decanucleotide primers. Twenty-three of 75 amplified fragments were polymorphic. Based on the 23 RAPD markers, a dendrogram depicting the relatedness of the isolates was constructed using UPGMA. The *P. sojae* isolates clustered into four distinct groups. The isolates of races 3, 4 and 25 clustered into group I. The isolates of races 1, 8 and 13 clustered into group II. The isolates of race 5 clustered into group III, and the isolates of race 7 clustered into group IV. Genetic diversity was detected among isolates of races 1, 3, 4, 5, 7 and 25 but not among isolates of races 8 and 13.

Phytophthora root rot (PRR) of soybean (*Glycine* max (L.) Merr.), caused by *Phytophthora sojae* Kaufm. & Gerd. was first noted as a soilborne disease of unknown etiology in northeast Indiana in 1948 (Schmitthenner, 1989). *P. sojae* is host-specific to soybean. This disease now has been identified in all soybean-growing regions in the United States and is also found in Argentina, Australia, Canada, China, Hungary, Italy, Japan, and Russia. PRR is of great importance to soybean production of the United States. The disease can cause pre- or post-emergence damping-off, and root and stem rot. During wet springs about 25% of damping-off of soybeans in Iowa is caused by PRR (Rizvi & Yang, 1996); and yield can be reduced by $>$ 50% (Sinclair, 1982).

Natural resistance, as well as cultural, chemical, and biological means can be used to control this disease. Use of PRR-resistant cvs, however, is the most effective and economic method. Thirteen single dominant resistance alleles have been identified at seven loci in soybean (Ward, 1990). Five alleles clustered at the *Rps1* locus (Athow, 1987; Buzzel & Anderson, 1992), three alleles clustered at the *Rps3* locus (Mueller, Athow & Laviolette, 1978), and one allele was identified at each of the *Rps2, 4, 5, 6, 7* loci (Anderson & Buzzel, 1992; Athow *et al.*, 1980; Athow & Laviolette, 1982; Buzzel & Anderson, 1981; Kilen, Hartwig & Keeling, 1974). Resistance can be overcome by the development of new races of the pathogen. Thirty-nine races now have been reported (Henry & Kirkpatrick, 1995). Race is a genetically and geographically different mating group within a species or a group of plant pathogens that infect a given set of plant varieties (Agrios, 1988). Different races within a plant pathogen species are alike in morphology but unlike in certain cultural, physiological, pathological, or other characters (Hawksworth *et al.*, 1995). New races could develop by mutation or outcrossing between different races (Whisson *et al.*, 1994). Although *P. sojae* is homothallic, a low frequency of outcrossing can occur *in vitro* (Bhat & Schmitthenner, 1993). It has also been suggested that a new race might have been produced by outcrossing between two different races in Australia (Irwin, Cahill & Drenth, 1995).

Pathogenic variability in *P. sojae* has been assessed traditionally through virulence tests using a set of host differentials containing different resistance (*Rps*) genes. Differentials are inoculated with *P. sojae* isolates and races are distinguished by the reaction types on the differentials. There are several disadvantages to using pathogenicity as a marker for genetic variation studies: it is labour-intensive and time-consuming, requires a large greenhouse space, and often generates variable results which are influenced by temperature, moisture, and inoculation techniques. The end result, however, is that the classification of resistance or susceptibility sometimes may be subjective. More effective genetic markers are needed to study genetic variation in *P. sojae*.

Molecular technologies have been successfully used to study genetic variation in fungal populations. Molecular markers have advantages over other types of markers such as codominance and no have deleterious or strong epistatic effects (Michelmore & Hulbert, 1987). These techniques include isozyme analysis (Nygaard *et al.*, 1989), ribosomal DNA internal transcribed spacer sequence analysis (Lee & Taylor, 1992), repetitive DNA polymorphism analysis (Panabieres *et al.*, 1989), RFLP analysis (Liew *et al.*, 1991; Whisson *et al.*, 1992), restriction analysis of mitochondrial DNA (Hoeben, 1986), and RAPD analysis (Crowhurst *et al.*, 1991; Tyler, Förster & Coffey, 1995; Whisson *et al.*, 1995). In

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**Table 1.** Code, origin, and races of 55 *Phytophthora sojae* isolates used in the study

| Isolates* | Origin (source) | Race or ITM† |
|---|---|---|
| 2906IA01M | Plants | 1 |
| 2912IA13M | Plants | 13 |
| 1914IA01M | Plants | 1 |
| 3605IA03M | Plants | 3 |
| 3603IA08M | Plants | 8 |
| 2403IA03M | Plants | 3 |
| 2408IA03M | Plants | 3 |
| 2103IA04M | Plants | 4 |
| 3503IA08M | Plants | 8 |
| 3014IA25M | Plants | 25 |
| 2513IA04M | Plants | 4 |
| 7001IA25M | Plants | 25 |
| 7009IAITM | Plants | ITM |
| 7011IAITM | Plants | ITM |
| 4502IA04M | Soils | 4 |
| 4506IA01M | Soils | 1 |
| 4901IA03M | Soils | 3 |
| 4902IA03M | Soils | 3 |
| 4803IA01M | Soils | 1 |
| 5101IA04M | Soils | 4 |
| 4201IL04M | Soils | 4 |
| 4202IL01M | Soils | 1 |
| 4701IL04M | Soils | 4 |
| 4704IL01M | Soils | 1 |
| 4002IN25M | Soils | 25 |
| 4601IN01M | Soils | 1 |
| 4605IN07M | Soils | 7 |
| 4603IN01M | Soils | 1 |
| 4606INITM | Soils | ITM |
| 4609INITM | Soils | ITM |
| 4610IN01M | Soils | 1 |
| 4301MN01M | Soils | 1 |
| 4302MN03M | Soils | 3 |
| 4406MNITM | Soils | ITM |
| 4407MN03M | Soils | 3 |
| 4412MN04M | Soils | 4 |
| 4402MN01M | Soils | 1 |
| 4403MN04M | Soils | 4 |
| 4404MN05M | Soils | 5 |
| 4405MN25M | Soils | 25 |
| 4409MN03M | Soils | 3 |
| 4411MN01M | Soils | 1 |
| 4413MN03M | Soils | 3 |
| 4414MN04M | Soils | 4 |
| 4417MN03M | Soils | 3 |
| 4418MN03M | Soils | 3 |
| 4419MN03M | Soils | 3 |
| PSOJAE01R | Iowa, plants | 1 |
| PSOJAE03R | Iowa, plants | 3 |
| PSOJAE04R | Iowa, plants | 4 |
| PSOJAE05R | Ohio, plants | 5 |
| PSOJAE07R | Ohio, plants | 7 |
| PSOJAE08R | Iowa, plants | 8 |
| PSOJAE13R | Iowa, plants | 13 |
| PSOJAE25R | Iowa, plants | 25 |

* The name of the isolate was composed of nine characters, the first four represented the county of origin, the fifth and six letters represented the state of origin (IA = Iowa; IL = Illinois; IN = Indiana; MN = Minnesota), the seventh and eighth numbers represented the race type, and the last letter M or R is for data formatting. Eight *P. sojae* isolates, from PSOJAE1R to PSOJAE 25R, were identified as races 1, 3, 4, 5, 7, 8, 13 and 25, respectively.

† ITM = isolate was unclassified.

this study, we collected 55 *P. sojae* isolates from diseased plants on Iowa soybean fields or from soils from Illinois, Indiana, Iowa, and Minnesota and classified them into races. Objectives of the research were to detect genetic variation among these isolates from different geographic regions and to determine the relatedness of these isolates using RAPD data.

## MATERIALS AND METHODS

### P. sojae *isolates*

Plants with PRR symptoms were collected from Iowa soybean fields in 1994. A portion of the basal stem 10–15 cm long with lesions was placed in a paper bag and kept in a cool room (4 °C) no more than 3 d before isolation. Four or five cross-sections were made at the transition areas between lesions and healthy tissues. Sections were aseptically transferred to a selective medium containing dilute V8 juice agar (1000 ml distilled water, 40 ml V8 juice, 0·6 g $CaCO_3$, 1 g sucrose, 0·01 g cholesterol), a combination of antibiotics (PARP: pimaricin 10 ppm, ampicillin 250 ppm, rifampicin 10 ppm, PCNB 100 ppm, and hymexazol (25 ppm) in Petri plates (Payne, 1994). Cultures were examined microscopically for the presence of oospores 10 d after inoculation. Pure cultures were obtained from hyphal tip sections if an isolate was contaminated by bacteria. One isolate from each sample was transferred and maintained on V8 juice medium at 25° for future use.

In 1995, *P. sojae* isolates were obtained from soil samples of soybean fields in Illinois, Indiana, Iowa, and Minnesota by using a modified leaf–disc–bioassay method (Canaday & Schmitthenner, 1982; Schmitthenner, Hobe & Bhat, 1994; Yang *et al*., 1996). Soil (60 $mm^3$) was spread over an 8-cm column of sterile soil in 500 ml perforated cups, wetted to saturation, and drained for 4 d in greenhouse benches. Ten leaf discs (6 mm) of the susceptible cv. Sloan were floated on flooded cups for 24 h. The leaf discs were then surface sterilized with 0·05% NaOCl for 30 sec, and plated (four discs per plate) onto the selective medium mentioned above. The plates were incubated in the dark at 25° for one week before examination. Tips of hyphae with growth characteristics of *P. sojae* were transferred to selective medium for further purification. Eight isolates (PSOJAE1R, PSOJAE3R, PSOJAE4R, PSOJAE5R, PSOJAE7R, PSOJAE8R, PSOJAE13R, and PSOJAE25R) previously stored at Iowa State University and identified as races 1, 3, 4, 5, 7, 8, 13 and 25, respectively, were used as controls. The 55 *P. sojae* isolates used in this study are listed in Table 1.

### *Virulence testing*

Virulence testing was conducted with the hypocotyl inoculation method described previously (Yang *et al*., 1996) Nine differentials were used for inoculation: L75-6141 (*Rps1*), L77-1863 (*Rps1b*), Williams 79 (*Rps1c*), PI 103091 (*Rps1d*), Williams 82 (*Rps1k*), L83-570 (*Rps3*), Altona (*Rps6*), Harosoy (*Rps7*), and Williams (*rps*). Seed of each differential was separately planted into pots (10 cm diam.) at a rate of 11 per pot and placed in a greenhouse at 19–27° for 10 d. Pure

cultures of *P. sojae* were transferred onto oatmeal agar plates (40 g oatmeal agar in 1000 ml distilled $H_2O$) from V8 medium and incubated for about 10 d. A small piece of mycelium (about 1 $mm^2$) with medium was inserted into a small slit in the hypocotyl of 10-d-old seedlings using a scalpel. Each isolate was used to inoculate each of the nine differentials. The inoculated seedlings were placed in mist chambers in a greenhouse at 19–25° and then taken out after 24 h.

The reaction types of individual isolates were evaluated 4–5 d after inoculation. For each of the above nine differentials, the reaction type was classified as resistant if 0, 1 or 2 were killed, or as susceptible if nine or more seedlings were killed. If 3–8 seedlings were killed, the reaction was classified as intermediate (ITM). The tests were repeated two to four times.

### *DNA extraction*

*P. sojae* isolates were initially grown on clarified V8 agar plates. Five to ten agar plugs (5 $mm^3$) with mycelium were cultured in 50 ml sterile clarified 20% V8 liquid in 250 ml flasks for 4–5 d. The liquid culture was blended aseptically to inoculate 250 ml of sterile clarified 20% V8 liquid media in 500 ml flasks. Cultures were incubated without shaking for 10–12 d at about 21°. The mycelia were then harvested by vacuum filtration through no. 4 filter papers. The harvested mycelia were either used immediately for DNA extraction or stored at −70°. The procedure for DNA extraction was based on the method of Panabieres *et al.* (1989). DNA samples were quantified and evaluated by electrophoresis on 1·5% agrose gel and Beckman DU7400 Spectrophotometer.

### *RAPD PCR*

DNA concentration (from 10 to 300 ng in each reaction), $MgCl_2$ concentration (from 1·5 to 3 mM in each reaction), and different batches of DNA were tested for RAPD optimization. We found they had no effects on RAPD patterns. Eight *P. sojae* isolates representing races 1, 3, 4, 5, 7, 8, 13 and 25, respectively, were initially tested with 10 specially designed primers and 220 decanucleotide primers (Operon Technologies, Alameda, CA). The designed primers were based on the conserved sequence of an oligopeptide from *P. megasperma* f. sp. *glycinea* (Pep-13) (Nurnberger *et al.*, 1995) and they failed to yield polymorphic fragments. The 16 primers which detected polymorphisms were then used to amplify the DNA of 47 other isolates. Each 235 µl reaction mixture contained 20 ng DNA, 0·2 µM primer, 200 µM dATP, dGTP, dCPT, and dTTP, 1 × PCR Buffer (GIBGOBRL), 1·5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase (GIBCOBRL).

PCR amplification was performed in a Perkin Elmer Cetus DNA thermal cycler. The thermal cycling parameters for amplification were 45 cycles of denaturation at 94° for 1 min, annealing at 34° for 1 min, and extension at 72° for 2 min. Amplified DNA fragments were size-fractionated on a 1·5% agrose gel in 1 × TE buffer at 70 V for 4 h using Lambda DNA digested with *Hin*d III as the standard size marker. The amplified fragments were stained with ethidium bromide, visualized over uv light, and photographed.

### *Data analysis*

Polymorphic fragments were scored as presence (1) or absence (0) and a binary presence (1) and absence (0) data matrix for the RAPD markers of 55 *P. sojae* isolates was constructed. The data matrix was used to generate dissimilarity proportional coefficients using PAUP Version 3.1.1. The proportional coefficients of dissimilarity were calculated by the formula $E = 2mn/(m+n)$, where E is the proportional coefficient of dissimilarity, $m$ and $n$ are total number of fragments in each isolate, and $mn$ is number of fragments that are not common to both isolates (0–1 or 1–0). A dendrogram depicting the relatedness among the 55 *P. sojae* isolates was constructed using the unweighted pair-group arithmetic average method (UPGMA).

## RESULTS

Of 55 *P. sojae* isolates tested in this study (22 from diseased plants of Iowa and Ohio and 33 from soil samples of Illinois, Indiana, Iowa, and Minnesota), 13 were identified as race 1 or 3 (23·6%), 10 as race 4 (18·2%), five as race 25 (9·1%), three as race 8 (5·5%), two as races 5, 7, or 13 (3·6%); five isolates were unclassified. Three unclassified isolates (4609INITM from Illinois and 7009IAITM and 7011IAITM from Iowa) showed the same reaction types. These isolates killed differentials containing *Rps 1a, 1b, 1k, 7,* and partially killed differentials containing *Rps 5* (54·6%).

Among the 220 Operon decanucleotide primers, 16 primers identified 23 polymorphic fragments (Fig. 1). When the 16 primers were further used to amplify DNA of 47 other isolates, no additional polymorphic fragments were discovered.

The 55 isolates clustered into four distinct groups (Fig. 2). Group I included 30 isolates, of which 13, 10, and five isolates were identified as races 3, 4, and 25, respectively, and two were unclassified (7009IAITM and 7011IAITM). Eleven isolates identified as races 3 and 4 showed the same RAPD banding patterns. Group II included 19 isolates, of which 13, 3, and 2 were identified as races 1, 8, and 13, respectively, and one unclassified (4406MNITM). Regardless of geographic origin, nine isolates of race 1 shared the same banding patterns as three race 8 isolates and two of as race 13. These nine isolates of race 1, however, had different RAPD patterns from

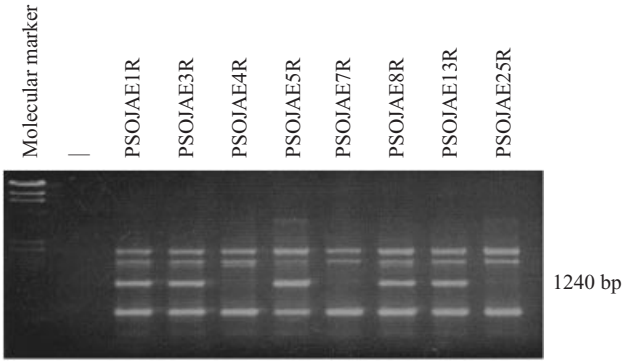


**Fig. 1.** RAPD patterns of genomic DNA of eight *Phytophthora sojae* races amplified with primer OPP17 (Molecular marker = Lambda DNA digested with *Hin*d III; — = negative control).

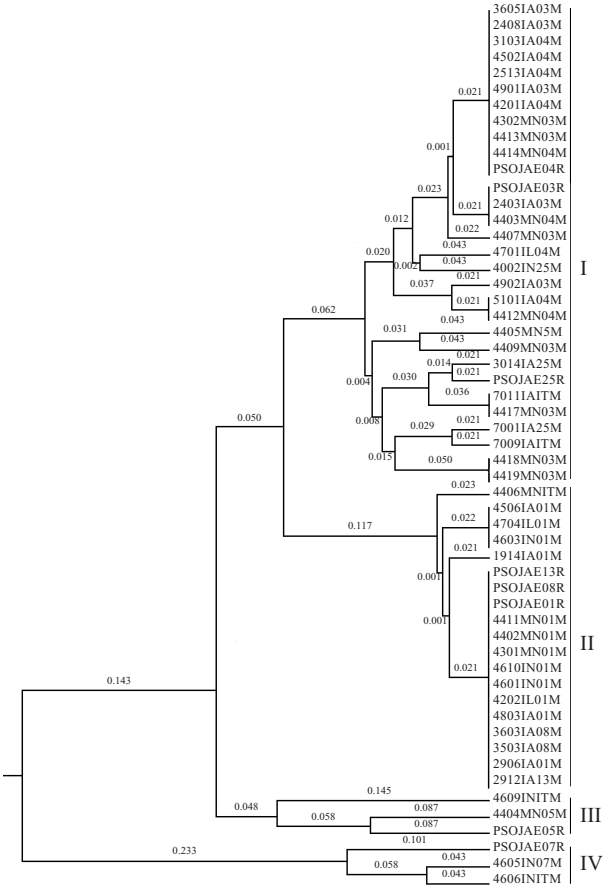


**Fig. 2.** Dendrogram depicting the relatedness among *Phytophthora sojae* isolates based on 23 RAPD markers. A dissimilarity matrix was calculated using proportional coefficient and the tree was generated from the dissimilarity matrix by UPGMA.

those of four other race 1 isolates (4506IA01M, 4704IL01M, 4603IN01M, and 1914IA01M). Group III included two isolates of race 5 (from Minnesota and Ohio) and one unclassified isolate (4609INIT). Group IV included two isolates (from Indiana and Ohio) of race 7 and one unclassified, 4606INITM. The three unclassified isolates, 4609INITM, 7009IAITM, and 7011IAITM were differentiated into two groups although they had the same reaction types when they were inoculated on the nine differentials. 7009IAITM and 7011IAITM in Group I from Hamilton County of Iowa were more closely related to each other than to 4609INITM (in Group III) from Indiana.

Although the 55 *P. sojae* isolates used in this research were collected from different regions, they could not be clearly differentiated geographically. Our results suggested that the divergence existed among isolates of the same race regardless of where they originated. No variation was detected among the isolates identified as races 8 and 13 in this research.

## DISCUSSION

Of 55 *P. sojae* isolates used in this study from different geographic areas in the North Central region of the United States, 50 gave consistent reaction types when they were inoculated onto the differentials, and were successfully classified. Races 1 and 3 were more common than other races among the collected isolates. Five isolates were unclassified as races and gave intermediate reactions which were probably caused by environmental conditions (temperature and moisture), impure seeds of differentials, or impure fungal isolates. Of the five unclassified isolates, two from Iowa (7009IAITM and 7011IAITM) killed differentials containing *Rps 1a*, *1b*, *1k*, *7*, and partially killed differentials containing *Rps 5* (54·6%). They may represent new races in Iowa and independent cultivars containing the *Rps 5* gene may be needed in future tests to determine the race types of these isolates.

We selected RAPD markers to detect genetic variation among *P. sojae* isolates from different regions because this method is technically simple and fast. Our results demonstrated that RAPD analysis is useful for detection of genetic variation among *P. sojae* isolates. Based on the 23 RAPD markers, the 55 isolates clustered into four distinct groups. Although the molecular markers we used were different from those of Förster, Tyler & Cofey (1994), we still can compare our clustering patterns with theirs. Förster (1994) analysed the relationships among 48 *P. sojae* isolates which were identified as 25 different races using nuclear RFLPs data. Their 48 isolates also clustered into four groups. All isolates of race 1 differentiated into group IA. The eight race 1 isolates showed identical nuclear RFLP patterns. These eight race 1 isolates were different from three other race 1 isolates which shared the same nuclear RFLPs patterns. Once race 13 isolate had the same RFLPs patterns as the above eight race 1 isolates. Three race 3 isolates and one race 25 isolate clustered into group IB. There were differences among three race 3 isolates although they clustered into the same group. We obtained similar results to those of Förster *et al*. (1994). In our results, 13 race 1 isolates clustered into group II. The nine race 1 isolates and the two race 13 isolates showed the same RAPD patterns but they were different from the other four race 1 isolates. Although all thirteen race 1 isolates clustered into group II, they are divergent.

Differences, however, existed between our results and those of Förster *et al*. (1994). In the nuclear RFLPs dendrogram of Förster *et al*. (1994), one race 8 isolate in group III was different from isolates of races 1 and 13 in group IA, but in our results the three race 8 isolates showed the same RAPD patterns as the two isolates of race 13 and the nine isolates of race 1. Of five race 4 isolates used by Förster *et al*. (1994), four race 4 isolates which had the same RFLP patterns clustered into group II and once race 4 isolates clustered into the same group (group IA) with the isolates of races 1 and 13. In our results all race 4 isolates clustered into the same group with the isolates of races 3 and 25. In our study two race 5 isolates and two race 7 isolates were differentiated into groups III and IV. Isolates of races 5 and 7, however, clustered into a single group IV in their results. Two reasons may explain the differences. First, we used isolates of different geographic regions from those of Förster *et al*. (1994). Secondly, RFLPs can detect more polymorphism than RAPD analysis. It was reported that RFLPs were more effective than RAPD analysis in detection of genetic variation in plants (Godin, Aitken & Smith, 1997).

Our results also can be compared with those of Whisson *et al*. (1992) in which multiclone DNA probes were used to

detect genetic variation among 10 *P. sojae* isolates (five from North America and five from Australia). Their results showed that one race 7 isolate from the U.S. was clearly different from nine other isolates. We obtained similar results in that the two race 7 isolates in group IV were also clearly different from all isolates of the other races. The remaining nine isolates of Whisson *et al*. (1992) clustered into two groups. The four U.S. isolates formed one discrete cluster and the five Australia isolates formed another separate cluster, but the two clusters were closer to each other than to the race 7 isolate from America. Five race 1 isolates (one from U.S. and four from Australia) split into two clusters. The U.S. isolate of race 1 was more closely related to the other U.S. isolates of races 4, 5 and 9 than to four race 1 isolates from Australia. In our research, thirteen race 1 isolates from Illinois, Indiana, Iowa, and Minnesota clustered into the same group. This is probably because the four states are not geographically isolated.

We did not find any race specific RAPD fragments in this study. The isolates tested as different races may have the same RAPD patterns. Three race 8 isolates, two race 13 isolates, and nine race 1 isolates in group 2 shared the same RAPD patterns. The isolates tested as the same races may have different RAPD patterns. There are differences in RAPD patterns within the isolates of races 1, 3, 4, 5, 7 and 25 respectively. Thus it may be impossible to identify *P. sojae* races using RAPD analysis unless RAPD markers which are linked to avirulence genes were found in the future. This supports the results of Förster *et al*. (1994) that RFLPs may not be used in *P. sojae* race identification.

Although RAPD analysis has some advantages over other molecular markers, RAPD analysis sometimes showed unstable amplification. This problem can be resolved by use of longer primers and high annealing temperature. More effective molecular markers, such as AFLP, ISSR, and SSR, have been used in genetic variation studies. AFLP (Amplified Fragment Length Polymorphism), a PCR-based technique, has been used with fungi, bacteria, nematodes, and plants (Lin & Kuo, 1995; Vos *et al*., 1995; Folkertsma *et al*., 1996; Mueller, Lipari & Milgroom, 1996). SSR (Simple Sequence Repeat) (Queller, Strassmann & Hughes, 1993), also known as microsatellite markers, are present in a high levels in soybean (Akkaya, Bhagwat & Cregan, 1992; Cregan *et al*., 1994) and can be up to tenfold higher in variability than other molecular markers in plants (Zhao, 1996). A SSR is composed of tandemly repeated 2 to 5 nucleotide DNA core sequences such as (AT)n, (TAC)n, or (CTAG)n, etc. The DNA sequence flanking these repeats are conserved within individuals of the same species, and the intervening SSR can be PCR amplified by the primers designed on the basis of DNA sequences of flanking regions. Size variations in PCR result from difference in number of tandem repeats. SSR have been successfully used in rice (Wu & Tanksley, 1993), soybean (Rongwen *et al*., 1995), and wheat (Roeder *et al*., 1995). One disadvantage of SSR markers is that the flanking sequence must be known to design primers for amplification. Knowledge of flanking sequence is not necessary for ISSR markers. The primer of simple sequence repeats will amplify the region between two SSR. ISSR markers have been used in maize (Kantety *et al*., 1995) and sorghum (Yang *et al*., 1996). Compared with other molecular markers, ISSR has all the advantages of RAPD, but is more effective (Godwin *et al*., 1997) and has higher levels of polymorphism than RFLP and RAPD (Kantety *et al*., 1995). AFLP, ISSR, and $SSR_s$ may have a great potential to reduce new genetic markers for population studies of *P. sojae*.

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