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Original Article

Genetic Diversity of *Phytophthora Sojae* in Iran

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ABSTRACT

Objective: The aim of this study was to estimate genetic diversity and morphological relationships of *Phytophthora sojae* from Iran. **Methods:** During 2005–2007, 142 isolates of *P. sojae* were collected from soil samples and diseased soybean plants from Lorestan, Mazandaran, Golestan and Ardabil provinces. Races of the isolates were determined. DNA of fifty isolates of *P. sojae* was amplified with 20 RAPD and 5 ISSR primers. **Results:** Five primers amplified 77 fragments, of which 20 were polymorphic, indicating low variability. RAPD and ISSR primers clustered isolates into three distinct groups at 85% similarity. Genetic diversity among isolates detected intra specific variability even among isolates of the same geographic origin. There is no correlation between races, geographical distribution, and molecular markers.

1.INTRODUCTION

Soybeans were first grown in Iran about 1939 (Amirshahi, 1975), but in about 1970 interests in soybean cultivation began to increase sharply. Soybean production rose steadily from 6,000 tons in 1970 to 162,000 tons in 2010 (Average yield per hectare, production costs, and farm-gate prices of selected crops, 2010). *Phytophthora* root rot of soybean caused by *Phytophthora sojae*, first was noted in Iran in Lorestan province (Rezaee and Alizadeh, 1998). *P. sojae* is a homothallic oomycete with more than 50 races described worldwide (Gally et al., 2007). The knowledge of pathogenic composition of populations is essential for addressing local breeding programs but demands significant amounts of time and space. Molecular technologies have been applied to study genetic variation in plant pathogen populations. Random amplified polymorphic DNA (RAPD) is a powerful tool for detection direct analysis of DNA polymorphisms to detect genetic variation (Whisson et al., 1994). This method uses primers of arbitrary sequence, which allows an extensive random sampling of the genome. For a reason RAPD has been used to detect genetic variation at the species level (Whisson et al., 1994) .RAPD has been used to estimate genetic diversity of other *Phytophthora* species (Cooke et al., 1999; NyassÉ et al., 1999; Purwantara et al., 2001). Whisson *et al*. (1995) used RAPD and other molecular techniques to establish the genetic relation among species of the *P. megasperma* complex. RAPD technology also has been used to confirm sexual recombination of *P. sojae* in vitro and to study the segregation of avirulence genes (Rezaee and Alizadeh, 1998). Meng *et al*. (1999) used RAPD markers to study populations of *P. sojae* from Indiana, Iowa, and Minnesota and found no correlation of populations with geographic origin. Wang *et al*. (2006)

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analyzed genetic diversity of isolates from China and the United States through RAPD and distinguished 12 genetic groups (Wang et al., 2006). RAPD markers also were used to detect interspecific hybrids between *P. sojae* and *P. vignae* (Drenth et al., 1996). So far, the biology and pathogenic strains have been studied in Iran, but this is the first study of genetic variation in *P. sojae* populations by molecular markers. The aim of this study was to estimate genetic variability and phonetic relationships of 50 isolates of *P. sojae* from Iran.

2. MATERIALS AND METHODS

Isolation and race identification of *P. sojae* from stem with symptoms and infested soils from Iran was made using the methods of Schmitthenner *et al* (Schmitthenner et al., 1994).

2.1. Growth in liquid media

Erlenmeyer flasks containing 25 mL of the liquid lima bean medium (LB) were inoculated by transferring a 5 mm disk cut from the margin of a 5 d old colony growing on corn meal agar (CMA) medium at 23 C. The mycelium was harvested on the fifth day, vacuum filtered in a Buchner funnel through filter paper, rinsed three times with bi distilled water, fractionated and kept at –70 C (Gally et al., 2007).

2.2. Extraction and purification of DNA samples

Mycelium grown in liquid LB medium was ground to a fine powder in liquid N_2. The powder was transferred to 2–3 ml of DNA extraction buffer (MacGregor et al., 2002), debris was removed by centrifugation at 20,000x *g* for 10 min, and the supernatant was transferred to clean tubes. After treatment with RNase A (1 µl of 10 µg/ml), the DNA was isolated by precipitation with isopropanol and washed once with 70% ethanol. Samples were resuspended in 50 µl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 and stored at 4 (Gally et al., 2007; Whisson et al., 1995; MacGregor et al., 2002).

2.3. RAPD and ISSR analysis

RAPD reactions were performed as Kasuga *et al.* (1997), except that reactions contained 1.0 ng of DNA and 1.0 unit*Taq* DNA polymerase. PCR was carried out in 50 µL final volume with 1.0 ng genomic DNA, 5 µL L dNTP mix (100 mM), 6 µL L $MgCl_2$ (25 mM), 10 µL L primer (3 ng/mL), 5 µL L 10x Taq DNA polymerase buffer (10 mM tris-HCl, pH 9.0 at 25 C, 50 mM KCl, 0.1% Triton X-100) and 1.0 unit Taq DNA polymerase. The mixture was amplified in a thermal cycler (Eppendorf). The thermal cycler was programmed for one cycle of 94 C for 6 min; 45 cycles of 94 C for 1 min, 36 C (60-65 C for ISSR primers) for 1 min and 72 C for 2 min, followed by a final extension step of 72 C for 6 min. Two PCR amplifications per isolate were carried out to ensure reproducibility of banding patterns (Kasuga et al., 1997; Gally et al., 2007). Twenty-seven arbitrary primers were screened for suitability in a small number of individuals. Only those primers that produced clear and reproducible RAPD or ISSR bands were chosen (NyassÉ et al., 1999; Kasuga et al., 1997).Amplification products were resolved on 1.4mlagarose gels stained with EtBr (0.5 µg/mL). Fragments were observed and photographed on a UV transilluminator (312 nm). Initially, a matrix of 1 and 0 was used from Photo-Capt (ver3-0) software. Using this software, the generated band's weights were determinate on the gels based on applied molecular marker weight(1 kb DNA Ladder).

2.4. Statistical methods

RAPD and ISSR bands were scored as present (1) or absent (0) across all genotypes to create a binary matrix, which was analyzed with the program NTSYS-PC version 1.8. The unweighted pair-group arithmetic mean method (UPGMA) cluster analysis was performed based on the simple matching (SM) and Jaccard (J) association coefficients (Gally et al., 2007; Meng et al., 1999).

3. RESULTS

During 2005-2007, we made an extensive survey on *P. sojae* in Iran. The field survey indicated that *Phytophthora* root and stem rot of soybean mainly occurred in Lorestan, Mazandaran, Golestan and Ardabil provinces. Of 142 *P. sojae* isolates tested in this study, 110 were identified as race 1 (killed differentials containing *rps 7)* and 32 as race 3 (killed differentials containing *reps 1a and 7)*. To assess genetic variability within the *P. sojae* isolates, including 50 isolates from races 1 and 3 were analyzed. Among tested Operon decanucleotide primers, only 5 primers identified 23 polymorphic fragments. Other primers were used to amplify DNA of all isolates, no polymorphic fragments were discovered. RAPD359 (AGGCAGACCT), OPA20 (GTTGCGATCC), OPE07 (AGATGCAGCC), RAPD77 (GAGCACCAGG) and ISSR ((AG) 8YG Y=c or t) primers were used for the analysis.Among the three geographic populations, a total of 77 reproducible RAPD and ISSR fragments were scored, of which 20 (28%) were polymorphic. The band size ranged from 100 to 2000 bp, with an average of 15.4 bands per primer. Based on the results of combining four primers, the isolates showed more than 85% similarity (Fig. 1). ISSR primer replicated 15 bands and divided isolates into two groups in 70% similarity. Each group contained both races 1 and 3. Combination of RAPD and ISSR primers showed more than 83% similarity between isolated race 1 and 3 from different geographical regions of Iran. These primers separated all isolates from each other at 99% similarity. Seventy-seven bands were amplified with primers that 20 bands showed polymorphism and separated isolates in 99% similarity. There was not any correlation between (Fig. 2)

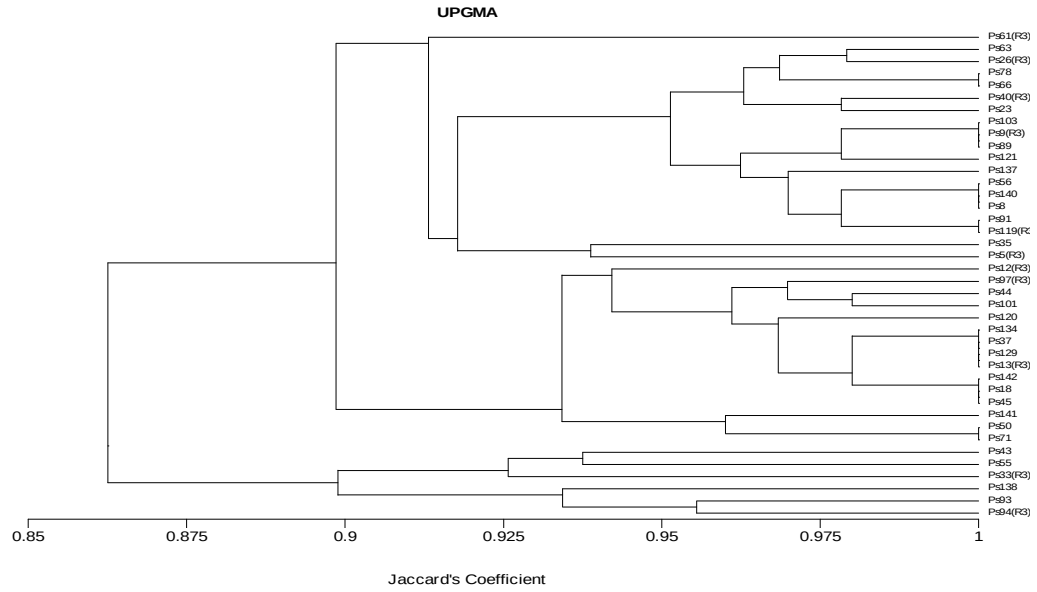

Fig. 1 UPGMA dendrogram of genetic relationships based on genetic similarity among the *Phytophthora sojae* isolates from Iran using RAPDs markers.

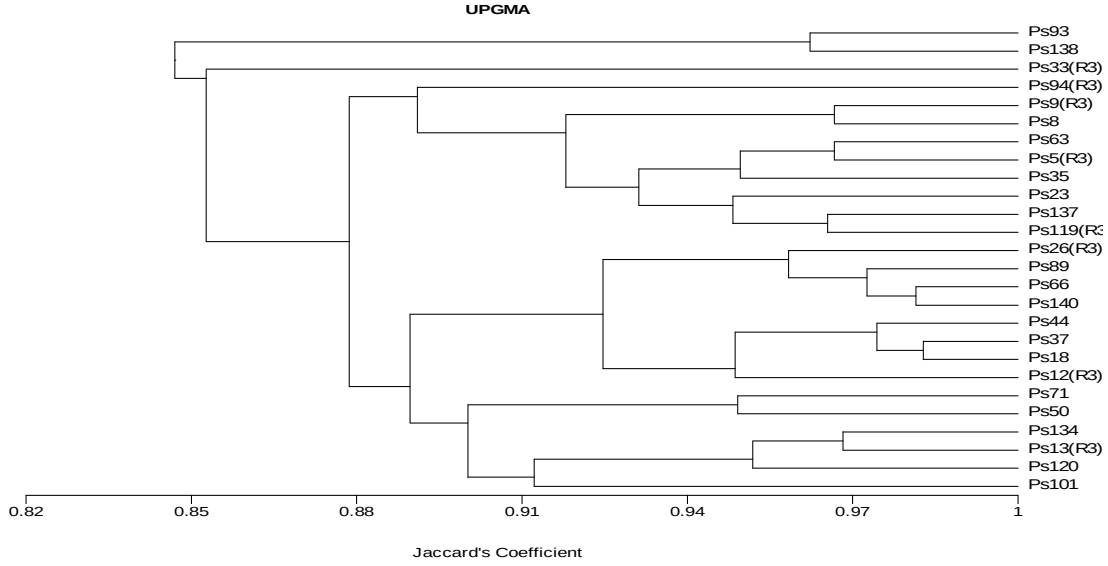

Fig. 2. UPGMA dendrogram of genetic relationships based on genetic similarity among the *Phytophthora sojae* isolates from Iran using RAPDs and ISSR markers

4. DISCUSSION

The genetic variation among isolates of *P. sojae* from four geographically isolated populations (Loresatan, Golestan, Mazandaran and Ardabil) was explored in this investigation. RAPD markers were used to estimate genetic variation and to determine whether any population structure occurred with regard to physiological race, host or geographical origin. The range of amplification products obtained with the five primers in this study demonstrated low intraspecific variation among *P. sojae* isolates. The UPGMA cluster analysis revealed that *P. sojae* populations of Iran are clearly separated into seven clusters (genotypes) at the 88% similarity level (Fig. 2). This arrangement confirmed that with the primers used in this study, we did not find any relation between the grouping of isolates and their geographic origin. Our study indicates that *P. sojae* is a lowly variable pathogen in Iran because low variability was found among isolates from the different locality. These observations indicate a low level of initial genetic variation in the founding population of *P. sojae* in Iran. It

is likely that only one or at most a few genetically closely related race 1 genotypes of *P. sojae* were introduced to Iran. This introduced race spread to all soybean-growing areas of Iran. The main factors that contribute to genetic changes within fungi populations are mutation, mating system, gene flow, migration, population size and selection (Stewart et al., 2011). However, in agricultural ecosystems pathogen populations evolve adapting to constant changes in environment conditions, such as the use of resistant varieties, applications of fungicides and fertilizers, irrigation and crop rotation (Dorrance and Grünwald, 2008). These changes impose a strong directional selection, which may be the main force acting in these systems (McDonald and Cahill, 1999). The evolution of populations of *P. sojae* in USA and Canada has responded mainly to the resistance genes included in the commercial genotypes of soybean cultivated (Dorrance and Grünwald, 2008). Although populations of *P. sojae* in soil usually present high variability, the origin of that variability has not been elucidated fully(Forster et al., 1994) . A high degree of homozigosis of populations of *P. sojae* could be presumed due to its homothallic character. However a high level of heterozygosity was reported for virulence genes(Bhat and Schmitthenner, 1993). Sexual recombination between pathotypes has been demonstrated in vitro and is presumed to play an important roll in the generation and maintainance of genetic diversity in the field(Bhat and Schmitthenner, 1993; Whisson et al., 1994). Parasexual recombination, may be a less important factor because of its low frequency in field conditions and the adaptative advantage of sexual populations, considering the longer viability of oospores compared to zoospores(Goodwin, 1997). In Iran, Williams was common variety in soybean fields. This variety is susceptible to all races of *P. sojae* and did not produce any selection pressure for production of new races. Recently Williams was replaced by other varieties and in the future, new strains of pathogens may occur on farms. This paper constitutes the first report of genetic variability in *P.sojae* populations from Iran. Previous analyses of low-copy RFLP markers in *P. sojae* have revealed much smaller diversity in Australia than in the United States (Drenth et al., 1996). In fact, most of the diversity in Australia occurred within a single clonal lineage. Based on these results, *P. sojae* in Australia may have originated from a single introduction, and possibly was introduced from Papua New Guinea and the United States, but it is unknown whether these were primary or secondary centers of diversity (Drenth et al., 1996). Wang *et al*. (*2006*) used RAPD markers to analyze 75 isolates from China and 11 from USA. They found two genetic groups but most of the isolates (44 Chinese and 10 American) were included in one group and no relationship between clustering and geographic origin was found (Wang et al., 2006). Meng *et al* (1999) also found genetic diversity among isolates of *P. sojae*, with four distinct groups obtained by RAPD, with no relationships detected with geographic origin of isolates.

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