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Analysis of simple sequence repeats markers derived from *Phytophthora sojae* expressed sequence tags

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Abstract **Five thousand and eight hundred publicly available expressed sequence tags (ESTs) of *Phytophthora sojae* were electronically searched and 415 simple sequence repeats (SSRs) were identified in 369 ESTs. The average density of SSRs was one SSR per 8.9 kb of EST sequence screened. The most frequent repeats were trinucleotide repeats (50.1%) and the least frequent were tetranucleotide repeats (8.2%). Forty primer pairs were designed and tested on 5 strains of *P. sojae*. Thirty-three primer pairs had successful PCR amplifications. Of the 33 functional primer pairs, 28 primer pairs produced characteristic SSR bands of the expected size, and 15 primer pairs (45.5%) detected polymorphism among 5 tested strains of *P. sojae*. Based on the polymorphisms detected with 20 EST-SSR markers, the 5 tested strains of *P. sojae* were clustered into 3 groups. In this study, the SSR markers of *P. sojae* were developed for the first time. These markers could be useful for identification, genetic variation study, and molecular mapping of *P. sojae* and its relative species.**

Keywords: expressed sequence tag, SSR marker, *Phytophthora sojae*, soybean.

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Phytophthora root rot of soybean (*Glycine* max (L) Merr.), caused by *Phytophthora sojae* Kaufmann & Gerdemann, is a devastating disease of soybean that seriously affects soybean production[1,2]. Use of resistant cultivars is the most effective and economic method of controlling the disease. However, its high variability and resistant cultivars selective pressure have led to complex virulence composition of *P. sojae* population in the field[3—5]. Pathogenic variability in *P. sojae* has been assessed traditionally through virulence tests using a set of host differentials containing different resistance genes (*Rps*). There are several disadvantages in using pathogenicity as an indicator for genetic variation studies: it is labor-intensive and time-consuming, requires a large greenhouse space, often generates variable results, and is unstable due to the influences of temperature, moisture, and inoculation techniques. Besides, the classification of resistance or susceptibility may be sometimes subjective. Therefore, more effective genetic markers are needed in the study of genetic variation in *P. sojae*.

Molecular markers have become important tools to study and detect genetic variation in plant pathogen populations. Several DNA-based molecular markers such as rDNA-ITS, RFLP, and RAPD have been successfully used for identifying and studying genetic variation and diversity, and mapping avirulence genes of *P. sojae*[6—10]. Simple sequence repeats (SSRs), a more efficient marker system than RFLPs and RAPDs, have been widely developed for many plants and animals, however, they are rarely developed for fungi[11—14]. SSRs or microsatellites are tandemly repeated DNA sequence units of 1—6 bp. They have abundant and random distribution throughout eukaryotic genomes. Variation arises in the number of tandem repeats, which can be detected by PCR with primers designed from the conserved flanking sequence. Because they are highly polymorphic, multi-allelic, co-dominant, PCR based, and highly reproducible, SSRs provide an ideal molecular marker system for a variety of purposes. However, the development of SSR markers from genomic libraries is expensive and inefficient. With the availability of large numbers of expressed sequence tags (ESTs) and other DNA sequence data, development of SSR markers through data mining has become a fast, efficient, and low-cost way. EST-SSR markers for some plants such as grape and wheat have been developed and utilized[15—18]. For plant fungal pathogens, a few EST-SSR markers for *Magnaporthe grisea* have also been developed and integrated into an established genetic linkage map[11].

Members of the oomycete genus *Phytophthora* are among the most devastating plant pathogens. In-depth molecular studies of *Phytophthora* pathogenicity will be critical to its eventual control. Recently, the *Phytophthora* genome initiative (PGI) has been carried out in USA[19]. *P. sojae* is a major research object in the PGI, for which expressed sequence tags have been developed[20]. Up to now, there are about 30000 ESTs for *P. sojae* in Genbank, but no SSRs for *P. sojae* have been developed.

The current study was to develop SSR markers from *P. sojae* EST database, aiming to provide a more effective molecular marker system for identification, investigation of genetic variation and evolution, and molecular mapping of *P. sojae* and its relative species.

1 Materials and methods

(i) Source of EST sequence data. *P. sojae* ESTs were extracted from Phytopathogenic Fungi and Oomycete EST Database (Version 1.4) available at http://cogeme.ex.ac.uk/. A total of 5849 unique gene sequences of *P. sojae* are maintained at the database, each consisting of either a single EST or a contig sequence made from a

group of ESTs.

(ⅱ) Detection of SSRs. After downloading, 49 sequences belonging to soybean or contaminated bacteria, or having poor quality were eliminated. A total of 5800 ESTs were searched to identify SSRs using Simple Sequence Repeat Identification Tool (SSRIT), which is available at GRAMENE web site http://www.gramene.org/db/searches/ssrtool. The program was run online and the parameters were set for detection of perfect di-, tri-, tetra-, penta-, and hexanucleotide motifs with a minimum of 8, 5, 4, 3, and 3 repeats, respectively.

(ⅲ) Design of primers and PCR amplification. Thirty-eight ESTs containing trinucleotide motif SSRs, one containing $(TA)_{36}$ SSR, and one containing $(GA)_{31}$ SSR were selected in the design of PCR primers. Primers were designed using Editseq and Primerselect software in DNAstar software package. Oligonucleotides of 17—24 nucleotides in length were designed to yield amplification products of 90—350 bp in size based on criteria of GC content (40%—70%), annealing temperature (45℃—60℃), primers Tm difference (≤2℃), and absence of secondary structure. Primers were synthesized by Saibaisheng Gene Techniques Co. Ltd. (Beijing, China).

The *P. sojae* strains used to screen these primers were PS96-41-1, B77R1.55, PS96-40-2, PS96-16-1 and PS99-15 (Table 1). The genomic DNA of the five strains was maintained in our laboratory. Conditions for PCR reactions were the same as described by Scott et al.[15]. PCR products were analyzed using 6% urea-denatured and silver-stained polyacrylamide gels.

2 Results

(ⅰ) Characteristics of *P. sojae* EST-SSRs. Four hundred and fifteen SSRs were detected in 369 of the 5800 ESTs (about 3700 kb) screened, suggesting that 6.4% of ESTs contained SSRs, which represents an average density of one SSR per 8.9 kb of EST sequence screened. Of the 369 SSR-containing ESTs, 328 ESTs contained one SSR each, 37, 3, and 1 EST contained 2, 3, and 4 SSRs each. The trinucleotide motifs were the most abundant type of SSRs found in the database (50.1%), followed by hexa-(16.6%), di-(12.8%), penta-(12.3%) and tetranucleotide repeats (8.2%) (Table 2). Totally, 185 SSR motifs were found, and the GA motif was the most frequent class of EST-SSRs ever found (Fig. 1).

(ⅱ) Development and polymorphism of SSR markers. Forty EST-SSR primer pairs were designed and tested in PCR reactions with genomic DNA of *P. sojae* under the given conditions. Out of the 40 primer pairs, 7 had no DNA amplification, while 33 (82.5%) amplified characteristic SSR bands. Summary information for the 33 functional primer pairs is shown in Table 3. The PCR amplification profile of some primer pairs is shown in Fig. 2. Out of the 33 functional primer pairs, 4 (12.1%) amplified one fragment each, in which 3 amplified one fragment of the expected size, 1 amplified one fragment smaller than expected; 29 (87.9%) amplified two or more fragments, 25 out of which amplified fragments in the expected size range. Totally, 28 (84.8%) primer pairs amplified fragments in the expected size range. In addition, out of the 29 primer pairs that amplified two or more fragments, 9 amplified fragments larger than expected, while 26 amplified fragments smaller than expected.

Of 33 functional primer pairs, 15 (45.5%) amplified polymorphic markers among *P. sojae* strains PS96-41-1, B77R1.55, PS96-40-2, PS96-16-1, and PSJ99-15. A total of 20 polymorphic EST-SSR markers were produced among the 5 strains by the 15 primer pairs. The dendrogram of the 5 strains was generated using the UPGMA clustering methods, and they were clustered into 3 groups according to the 20 polymorphic EST-SSR markers (Fig. 3).

Among the 4 tested Chinese strains, PS96-41-1, PS96-40-2 and PS96-16-1 were isolated in 1996, PSJ99-15 was isolated in 1999; PS96-41-1, PS96-40-2 and PSJ99-15 were isolated in Jiamusi, Heilongjiang Province, in which PS96-41-1 and PS96-40-2 were isolated from the same soybean cultivar in adjacent fields; PS96-16-1 was isolated in Mulin, Heilongjiang Province. By SSR analysis, PS96-41-1 and PS96-40-2 were clustered together, while PS96-16-1 and PSJ99-15 were differentiated into different groups, suggesting that genetic diversity of *P. sojae* was different in terms of time and space. B77R1.55, an America strain, and PS96-16-1 were clustered in the same group, suggesting that they had a close genetic relationship.

3 Discussion

Five thousand and eight hundred *P. sojae* ESTs were searched and 369 (6.4%) ESTs were found to contain

Table 1 The *Phytophthora sojae* strains used for primers screening

Code	Location	Source	Virulence type[a)]	Time
PS96-16-1	Mulin, Heilongjiang Province	plant	1a, 1d, 3a, 3b, 3c, 4, 5, 7	1996
PS96-40-2	Jiamusi, Heilongjiang Province	plant	1a, 3b, 7	1996
PS96-41-1	Jiamusi, Heilongjiang Province	plant	7	1996
PSJ99-15	Jiamusi, Heilongjiang Province	plant	1a, 3c, 7	1999
B77R1.55	USA	plant	3c, 7	-

a) defeated resistance gene (*Rps* gene)

Table 2 The number and percentage of different SSR motifs discovered in 5800 *Phytophthora sojae* ESTs

Repeat type	Total number	Percent of total
Dinucleotide	53	12.8
Trinucleotide	208	50.1
Tetranucleotide	34	8.2
Pentanucleotide	51	12.3
Hexanucleotide	69	16.6
Total number	415	100

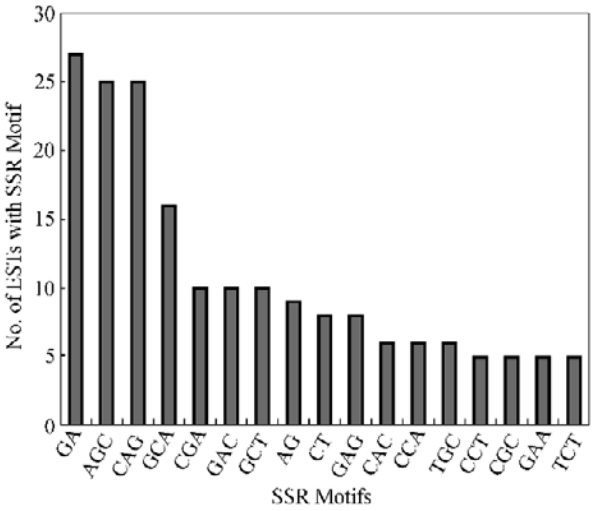

Fig. 1. The distribution of the 17 most abundant SSR motifs in 415 EST-SSRs discovered in this study.

SSRs. The average density of SSRs was one SSR per 8.9 kb of EST sequence screened. With the same criteria, 1414 *Phytophthora infestans* ESTs in the same database were also searched, only 1.8% of these ESTs contained SSRs, showing that the average density of SSRs was one per 30 kb of EST sequence screened. The frequency of ESTs containing SSRs in *P. sojae* ESTs was higher, and SSRs were more abundant than that of *P. infestans*. The haploid genome of *P. sojae* was about 62 Mb in size[21], while the haploid genome of *P. infestans* was about 250 Mb in size[22]. The SSRs frequency in the two *Phytophthora* species seemed to have negative correlation with their genome size. This result was similar to that of Morgant et al.[23]. Morgante et al. assessed microsatellite frequency in plant species exhibiting a 50-fold difference in genome size and found that among species, the overall frequency of microsatellites was inversely related to genome size and to the proportion of repetitive DNA, but remained constant in the transcribed portion of the genome and significantly associated with the low copy fraction of plant genomes.

Trinucleotide motifs in *P. sojae* EST-SSRs were found to be most abundant, amounting to 50.1% of the identified EST-SSRs in *P. sojae*. Also, trinucleotide motifs in *P. infestans* EST-SSRs were found to be most abundant, amounting to 39.3% of the identified EST-SSRs in *P. infestans*. Our result is in agreement with previous reports on several crop plants [17,24,25]. The high frequency of trinucleotide repeats in coding regions is attributed to the absence of frameshift mutations due to variation in the number of trinucleotide repeats. Gao et al. found that many trinucleotides were relevant to genes with important functions; for example, CCG repeats are involved in many genes functions including stress resistance, transcription regulation, metabolic enzyme biosynthesis, signal transduction, and so on[17].

SSRs are believed to be locus specific, therefore, it is generally expected that a genomic SSR primer pair might amplify a single fragment or co-migrating twin fragments. However, in this study, 29 out of the 33 functional EST-SSR primer pairs amplified multiple fragments. This phenomenon has also been reported in literature. Occurrence of multiple EST-SSR loci in hexaploid wheat was presumed to be due to amplification of more than one homoeolocus by single EST-SSR[16]. In some pasture grass endophytes (*Neotyphodium* spp.), it was attributed to heteroploidy owing to a hypothetical hybrid origin[26]. Outcrossing might cause multiple EST-SSR loci in diploid alfalfa genotypes[18]. *P. sojae* is diploid, but it is highly heterogeneous and clonally mutable. Although *P. sojae* is homothallic, new virulence types of *P. sojae* can arise by outcrosses[5,9]. Therefore, a possible reason why a single EST-SSR primer pair amplified more than one fragments in *P. sojae* is that there exist heterokaryons, clone mutants or heterozygotes in *P. sojae*. In addition, gene amplification might be a reason for multiple EST-SSR loci in *P. sojae*. Mao et al. found that *P. sojae* genome contained tandem repeat sequences which varied from strain to strain, suggesting that gene amplification could contribute to the generation of genetic diversity in *P. sojae*[27].

The EST-SSR markers could detect high levels of polymorphism in *P. sojae*. Out of 33 functional primer pairs, 15 amplified 20 polymorphic markers among 5 tested strains. Wang et al. used 13 polymorphic RAPD markers to analyze genetic diversity of 75 Chinese strains and 11 American strains of *P. sojae*. 86 strains were clustered into 4 groups[8]. Five *P. sojae* strains in 4 different RAPD groups were used in the present study (Fig. 4) and clustered into 3 groups according to 20 EST-SSR markers (Fig. 3), which was different from the result of Wang et

Table 3 Description of 33 *Phytophthora sojae* EST-SSR primer pairs

Code	EST	Upper primers 5'→ 3'	Lower primers 5'→ 3'	SSR motif	Anneal temperature (℃)	Expected size/bp
PSE3	Ps30495710	CCCCGCTTCAGTTGGATGGTTC	GTGGTCGGTTTCGGTGTAGCAGTG	$(CGA)_5$	60	234
PSE4	Ps30495782	GACAACTTGCTGGGGACGGACTAC	GATGTTCTCGAGCGGCAGGTTC	$(GAG)_5$	60	192
PSE6	Ps30496662	GGAAACGGGCCACCACAT	CGAAGAGCGAGAGGAAGCACT	$(CCT)_6$	60	269
PSE7	Ps30501163	GGTGGCAACGACTACGCTGATGTG	CGCTCCTCCGCCTGACTCGTTAG	$(GAC)_6$	60	269
PSE8	Ps30502679	AGAATTACTACCGGCGGCACAACC	GCAAAAAGAACCCCGTCAGTCCTC	$(AGG)_5$	60	217
PSE9	Ps30502757	AATCCCACCCTTTACCATCTA	ACAACACCACCTCGCTCAT	$(TA)_{36}$	45	225
PSE10	Ps31048433	TTGGCTGGTTGGTCGGTTTG	TGGCGGCATCTTCATCTCGT	$(AGA)_5$	55	196
PSE11	Ps31047276	CACGATGGCCACCGAAGACTC	GAAGCATAGGACCAGCCCCAACAC	$(TGC)_5$	60	234
PSE12	Ps31050290	GCACGAGGCGAGAACCAACC	GCTGCTGAACGAGTAGGACGAGAA	$(CAG)_5$	60	293
PSE13	Ps31052673	TCCCGAAGCGAAGAAAACAACC	GCGGCGCTTCTTCTCCTTCTCT	$(CGC)_5$	60	192
PSE14	Ps9834059	ATGGGGTCGCTGTCAATCCTA	CGAGTCGAGCGGGTGTTCAG	$(AGC)_5$	60	251
PSE15	Ps9835792	ACGCCCCAGGGAGAGTCATTC	CTCCAACAGGCATCCCATTCC	$(AGC)_5$	60	154
PSE16	Ps9835913	GTTGGAGACGCTGGATGACG	CGCGCTTCTTGGGCTTTTTA	$(GAG)_5$	60	241
PSE20	PsCon[0403]	GGAAACCACGGCAACCA	GGCACGGCTTACCACCAG	$(CAA)_6$	55	185
PSE21	PsCon[0587]	GCCTACTTCGAGCCCGTGGTGA	ACTTCTTGCCCGTGCCCTTGTCC	$(GAG)_5$	60	139
PSE22	PsCon[1286]	GCCATGCGCCTCTCCTC	CCGGTGGGCTGCTTGTT	$(CAG)_6$	60	227
PSE23	PsCon[2080]	AGGTGGCTTCGGCAACA	GTAGCGCTAATTAACAACCAAAGT	$(CAG)_5$	55	132
PSE24	PsCon[2565]	GGCACCGAGCGCAACCTGTC	TGCCTCCCATGTCCATTCCACTCA	$(GAC)_6$	60	154
PSE26	PsCon[2843]	AGCGTTCCCGTCTTGTGT	CAGTCGTCTTGCTCATTTTTC	$(CTT)_9$	55	242
PSE27	PsCon[3330]	CGCTACGAAGTCGCCTGCTCTGA	CATCGGTGGGGACGGGGTATTTCT	$(GAA)_6$	60	159
PSE28	PsCon[3377]	CAAGGGTGGAGACGACGATGATGA	TCCGTCCGCCGCTTCTTCTTCTTA	$(GAC)_5$	60	128
PSE29	PsCon[3515]	CGGAATCGCATCAGCAAAGGAG	CCACGAGGGACGGGAAGATG	$(GCA)_5$	60	158
PSE30	PsCon[3530]	TTCGCAATAACCCCATCAC	TCCGCCAGGTACAAAGAAGTT	$(TCT)_5$	55	216
PSE31	PsCon[3595]	CCACTGGCCTCGCTAATGACC	CTTCGAGCAATGTGGCAGTGTG	$(AGC)_5$	60	255
PSE32	PsCon[3763]	ACCTCGGGCAGCGTCTTCT	CTGTTGCTGTTGCTGCTGTTGTAG	$(CAG)_6$	60	155
PSE33	PsCon[3798]	TGAGAAGGAAGCCAGCACCAC	CACCCTTGATCTTCTTGTCGTTC	$(GAG)_5$	60	221
PSE34	PsCon[4296]	GAGGAGACTGGGGAGGACGAGACG	TCATTCCTGCCGCACAACTACACG	$(AGG)_5$	60	208
PSE35	PsCon[4317]	GCACTTCAAGGCTCACTCGT	GATCGGCTGTCGTCTTCTTC	$(GAG)_5$	55	169
PSE36	PsCon[4543]	TTCGCCAACCGCATCCAC	CACGCTTAGTCCACCTCCTCCATC	$(GCA)_5$	60	146
PSE37	PsCon[4697]	GCCGACTGCATTACCTGGAC	CTTGGGGCCGTTGAGCA	$(AGC)_5$	55	150
PSE38	PsCon[5099]	CCCAAGGCCTACTCGGACTAC	TGCTCTTCTTGCTGCTCTTCTTAC	$(ACC)_5$	55	154
PSE39	PsCon[5734]	CGGTTTAGCGGGAATAGTG	CAAAAGGCGCAGGTGAC	$(GAA)_6$	55	288
PSE40	PsCon[6044]	GGGCATCTACTTGGCTCCTGTCG	GGTGATATCCCGGCCCTGAACG	$(GTG)_5$	60	157

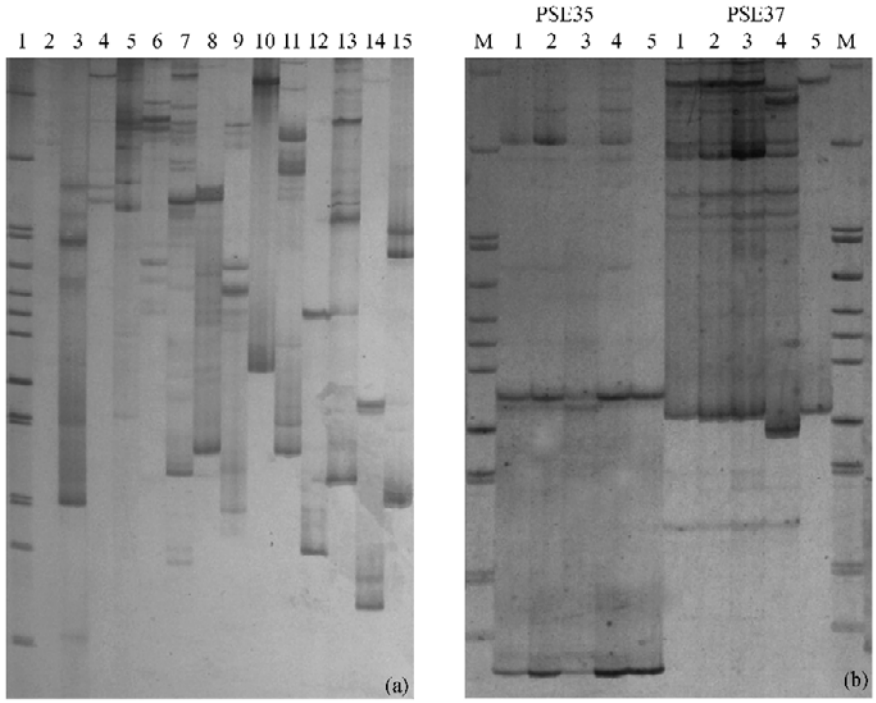

Fig. 2. SSR PCR products amplified from genomic DNA of the tested strains of *Phytophthora sojae* with some designed EST-SSR primer pairs. (a) SSR amplification profiles from strain PS96-41-1 with 14 primer pairs. Lane l, DNA ladder pBR322 DNA/Msp I; lane 2, PES1; lane 3, PSE3; lane 4, PSE2; lines 5—15, PSE4, PSE5, PSE6, PSE7, PSE8, PSE11, PSE12, PSE13, PSE14, PSE15, PSE16. (b) patterns of polymorphism detected by primer pairs PES35 and PES37 in 5 tested strains. M, DNA ladder pBR322 DNA/Msp I. Lanes 1—5, strains PS96-41-1, B77R1.55, PS96-40-2, PS96-16-1 and PSJ99-15.

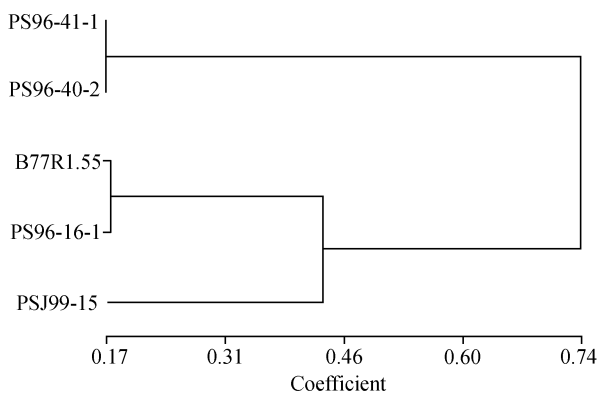

Fig. 3. The UPGMA tree of the five *Phytophthora sojae* strains based on 20 EST-SSR markers.

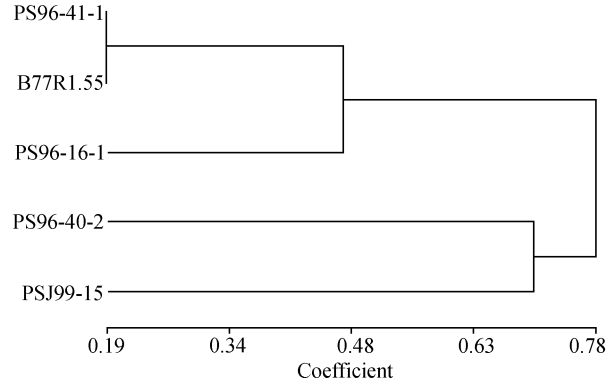

Fig. 4. The UPGMA tree of the five *Phytophthora sojae* strains based on 50 RAPD markers.

al.[8]. The reason lies in the fact that the 13 RAPD primers used by Wang et al. were screened from 200 RAPD primers, and these primers were highly polymorphic and amplified 50 polymorphic markers among the 5 strains. However, only a few of EST-SSR primer pairs were used in the present study. Pervious studies have shown that different molecular genetic marker systems may detect different levels of polymorphism, in some cases, leading to conflicting conclusions[7,9,28]. RAPD has higher levels of polymorphism than SSRs, but it is impossible to analyze geographical distribution and virulence of *P. sojae* population using RAPD markers[8]. In the present study, EST-SSR markers could differentiate 4 Chinese strains of *P. sojae* in terms of time and space. However, this result should be verified by analyzing a great deal of *P. sojae* strains in the future.

With availability of ESTs and other DNA sequence data for plant pathogens, the discovery of SSRs through data mining will provide useful approach for development of SSR markers for plant pathogens. Development of

EST-SSR markers of *P. sojae* has demonstrated that it is fast, efficient and low-cost. Although EST-SSRs may show lower levels of intraspecific polymorphism than those isolated from non-coding regions, they could detect variation in the expressed portion of the genome, so that gene tagging could give "perfect" marker-trait associations and provide direct identification of alleles conditioning economic importance traits. Since EST-SSR markers are derived from transcribed regions of the DNA, and have the potential of identifying avirulence genes in *P. sojae*, it is possible to identify *P. sojae* race using these markers. In addition, as EST-SSR markers have a higher rate of transferability than SSR markers derived from non-transcribed regions, once developed, these markers may be used across a number of related species. Therefore, EST-SSR markers for *P. sojae* provide ideal tools for studying the origin, systemic evolution and genetic relationship of *P. sojae* and its related species.

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