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Development of seven novel specific SCAR markers for rapid identification of *Phytophthora sojae*: the cause of root- and stem-rot disease of soybean

Qin Xiong · Jing Xu · Xinyue Zheng · Yu Zhu · Chen Zhang · Xiaoli Wang · Xiaobo Zheng · Yuanchao Wang

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Abstract *Phytophthora sojae* is a devastating pathogen that causes soybean Phytophthora root and stem rot. In this study, we developed seven pairs of polymerase chain reaction primers derived from sequence-characterized amplified regions (SCAR). These seven SCAR markers allowed discrimination of *P. sojae* from 17 different *Phytophthora* species and three other soilborne pathogens (*Pythium ultimum, Fusarium solani* and *Rhizoctonia* sp.) which also induce root rot in soybean. Among those 17 *Phytophthora* species, *P. melonis* has approximately 98% similarity in ITS sequences; *P. drechsleri* requires an annealing temperature up to 66 °C with an ITS-targeting diagnostic marker (PS primers) developed by Wang et al. (2006) for *P. sojae*; and *P. sansomeana* is a newly described soybean-infecting *Phytophthora* species. These three *Phytophthora* species could be specifically distinguished against *P. sojae* by these seven SCAR markers. After screening 100 random amplified polymorphic DNA (RAPD) primers, eight primers clearly produced specific bands only for *P. sojae* rather than other *Phytophthora* species tested. Subsequently, seven of eight *P. sojae*-specific RAPD markers were successfully converted into SCAR markers, namely, Scar276, Scar304, Scar333, Scar37, Scar519, Scar57 and Scar78. These SCAR markers were used to detect 75 isolates of *P. sojae* specifically, while no products were obtained for 29 additional isolates representing 17 other *Phytophthora* species and three other soilborne pathogens. Furthermore, Scar333 successfully allowed the detection with a sensitivity of 100 pg from genomic DNA of *P. sojae*, Scar276 had a higher sensitivity of 10 pg, and four specific SCAR primers (Scar304, Scar37, Scar519 and Scar78) had a sensitivity of 100 fg, which is the highest for detecting *P. sojae* until now. Six of the seven SCAR markers, with the exception of Scar57, were also used to detected *P. sojae* in artificial or naturally infected soybean seedlings and infested soil. Our findings demonstrate that SCAR markers provide a rapid and sensitive molecular diagnostic tool for the detection of *P. sojae* in plants, and will play a key role in effective management of the disease.

Keywords *Phytophthora sojae* · Molecular detection · RAPD · Root rot · SCAR marker

Qin Xiong and Jing Xu contributed equally to this work.

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Q. Xiong · X. Wang · X. Zheng · Y. Wang (✉)
Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China
e-mail: wangyc@njau.edu.cn

Q. Xiong · J. Xu · X. Zheng · Y. Zhu · C. Zhang
Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, Nanjing 210037, China

J. Xu
Municipal Bureau of Agriculture, Foshan 528000, China

Introduction

Many species of *Phytophthora* are plant pathogens, classified in the kingdom Stramenopiles, which cause

destructive diseases to a huge range of agriculturally and ornamentally important plants, including those in forests and other natural ecosystems (Erwin and Ribeiro 1996). As one of the most predominant soybean diseases, Phytophthora root and stem rot (PRSR), caused by *Phytophthora sojae* (Kaufman & Gerdman), is one of the most devastating globally distributed pathogens of soybean (*Glycine max* (L.) Merr.), primarily affects seeds and seedlings (Tyler 2007).

In China, PRSR has been identified as an important quarantine issue since 1986 (Su and Shen 1993). PRSR was first observed in 1989 in Heilongjiang and Jilin provinces, in the Northeast part of China, and has subsequently been observed in Inner Mongolia Autonomous Region, Fujian Province, Xinjiang Uygur Autonomous Region, Huanghe-Huaihe River Basin and Yangtse River Basin (Chen and Wang 2017).

In wet conditions, *P. sojae* produces motile zoospores from infected tissues that initiate disease outbreak (Tyler 2007; Gijzen and Qutob 2009). Diseased roots develop lesions that may spread up the stem and eventually kill the entire plants. Large numbers of oospores can remain dormant in the soil for many years, and germinate when conditions are favourable (Schmitthenner 1999). In addition, plants infected with *P. sojae* may become more vulnerable to infection by other soilborne pathogens. The complexity of the stem and root rot caused by this pathogen makes it essential to develop effective disease management strategies. The main strategies for reducing damage due to PRSR include growing resistant soybean cultivars and fungicide applications (Sugimoto et al. 2012). Increasing public concern about environmental and health consequences of the widespread use of fungicide has encouraged researchers to explore new potential disease control strategies. Therefore, the ability to sensitively detect and accurately diagnose the organism(s) that cause plant diseases is a crucial step towards effectively managing it.

Traditional methods for detecting *P. sojae* involve direct isolation from diseased plant tissue or baiting from soil (Erwin and Ribeiro 1996). Diagnosis of PRSR by visual inspection requires a skilled and specialized person, is confounded by the similarity of symptoms caused by *P. sojae* with those of other soilborne pathogens, including *Pythium* spp., *Fusarium* spp., *Rhizoctonia* sp. (Wrather and Koenning 2006), and *Phytophthora sansomeana,* a newly described *Phytophthora* species with similar morphological features as *P. sojae* (Hansen et al. 2009; Zelaya-Molina et al. 2010; Tang et al. 2010). *P. sansomeana* and *P. sojae* differ in their host ranges, evolutionary taxonomy and cultivar-specific resistance. *P. sansomeana* had a broad host range including soybean, corn, alfalfa and some weed species, while *P. sojae* had an extremely limited host range, only infecting soybean in the field (Hansen et al. 2009). In addition, *P. sansomeana* is classified in clade 8a along with *P. cryptogea*, *P. drechsleri*, and *P. medicaningis*, which is different from *P. sojae* which is in clade 7b grouping with *P. cinnamomi*, *P. vignae*, and *P. niederhauserii* (Martin et al. 2014). Moreover, *P. sansomeana* has not been associated with cultivar specificity. On the contrary, many race-specific resistance genes (*Rps*) in soybean towards *P. sojae* have been identified (Hansen et al. 2009). Due to the difficulties in the detection and quantification of *Phytophthora* species in plant tissues, in the presence of other morphologically and genetically similar organisms and limitations of traditional detection methods, there is a need to develop highly efficient and sensitive assays for early detection of *P. sojae* in plants.

Molecular techniques have been widely used to lessen or overcome the above difficulties. Reactions such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) have been successfully used to diagnose and quantify viral, bacterial, fungal, and oomycete plant pathogens (Elnifro et al. 2000; Ahonsi et al. 2010; Sangdee et al. 2013; Bilodeau et al. 2014; Hansen et al. 2016; Yadav and Singh 2017). The advantages of PCR are its high reliability, specificity, sensitivity, and rapidity compared with the traditional methods. The priority for nucleic acid-based pathogen detection and identification is to design the specific PCR primers or probes targeting specific gene sequences. For most cases, target sequences are usually obtained by sequence comparison of conserved genes or by random exploration of chromosomal sequences (Gao et al. 2016). In most fungi, conserved genome regions, such as the internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) (Barnes and Szabo 2007; Pryce et al. 2003; Vu et al. 2016; Turenne et al. 1999), along with β-tubulin (Liu et al. 2014), actin-coding sequences (Vu et al. 2016) and elongation factors (Scauflaire et al. 2012), have all been commonly chosen for diagnostics purposes to the species level (Schena et al. 2013).

An ITS-targeting diagnostic marker (PS primers set) for *P. sojae* was first developed by Wang et al. (2006).

However, the sequences of the PS1-PS2 primer set only vary from *P. drechsleri* sequences at one nucleotide, requiring an increase in the annealing temperature up to 66 °C for the primers to remain specific to *P. sojae* (Wang et al. 2006). Bienapfl et al. (2011) found that the PS primers amplified DNA of *P. sojae* and of four other *Phytophthora* species, indicating they are not specific for *P. sojae*. This suggests that the PS primers may be similar to other regions within the genome of other *Phytophthora* species (Bienapfl et al. 2011). Subsequently, based on ITS, Bienapfl et al. (2011) designed the new PSOJ primers that exclusively amplified DNA *P. sojae* in plant tissue and soil using conventional and real-time PCR. However, after preliminary alignment performed in our laboratory, it was observed that a closely related *Phytophthora* species, *P. melonis*, which is not included in the lists of isolates used by Wang et al. (2006) and Bienapfl et al. (2011), showed approximately 98% sequence homology with *P. sojae*, and the new PSOJ primers varied between *P. sojae* and *P. melonis* only for three nucleotides (Fig. S1). To date, other genetic targets described for *P. sojae* detection include a *Ras*-related protein coding gene for loop-mediated isothermal amplification (Zhao et al. 2015) and a *A3aPro* transposon-like element for standard PCR and nested PCR (Dai et al. 2012) offer some advantages relative to the ITS-based methods. Nevertheless, when a morphologically and genetically similar *Phytophthora* species appears in the soybean field, such as *P. sansomeana*, these markers often no longer show specificity to *P. sojae*. In the case of the genus *Phytophthora*, several studies have showed that using the ITS region for diagnostic purposes is not always reliable when there is a need for differentiating among closely related species that share extremely high amounts of similarity in the ITS sequence. For example, *P. infestans*, *P. phaseoli*, *P. ipomoeae*, *P. andina*, and *P. mirabilis* have a 99.9% homology in ITS sequence as reported by Gómez-Alpizar et al. (2007) and Kroon et al. (2004). Also, *P. katsurae* and *P. heveae*, which belong to the phylogenetic clade 5, have up to 98.8% similarity in ITS sequence as shown by Robideau et al. (2011). Therefore, exploring alternative targets from the entire genome is a robust approach to help meet this challenge. Moreover, to the best of our knowledge, no assays have been developed to discriminate *P. sojae* from *P. sansomeana*, *P. melonis* and *P. drechsleri* at the same time.

The development and application of sequence-characterized amplified regions (SCAR) markers, obtained through a genome-wide scan, offer a good opportunity for diagnostics of plant pathogens and allow for simple specific detection by PCR. The most important and significant advantage of SCAR markers is that they do not require any prior knowledge of the strain genome. Also, large numbers of samples can be processed in a short time to differentiate closely related species. PCR-based SCAR markers have been widely used for *in planta* detection of several plant pathogens. Kaluzna et al. (2017) developed the SCAR marker for rapid and specific detection of *Pseudomonas syringae* pv. *morsprunorum* races 1 and 2, using conventional and real-time PCR. Gotor-Vila et al. (2016) developed a PCR protocol using SCAR markers for identification of the biocontrol agent strain CPA-8 *Bacillus amyloliquefaciens* (formerly *B. subtilis*). SCAR markers were developed for identification of *Magnaporthe grisea* isolates infecting finger millets by using RAPD PCR techniques and thus provided a powerful tool for easy detection and differentiation of blast pathogens infecting finger millets and other pathogens (Gnanasing Jesumaharaja et al. 2016). Also, SCAR markers have been successfully used to produce species-specific probes for accurate identification of *Colletotrichum falcatum* in sugarcane planting materials. Nithya et al. (2012) evaluated specificity of the SCAR primers and results indicated that the SCAR markers were highly specific to *C. falcatum* since the 442 bp fragment was amplified solely from DNA of isolates and races of *C. falcatum,* and not from any other *Colletotrichum* spp. tested. Aggarwal et al. (2011) showed that a single 600 bp DNA amplification product was consistently obtained from primers SCRABS600 that was specific for 40 isolates of *Bipolaris sorokiniana,* the cause of spot blotch in wheat, but not for seven other isolates of *Bipolaris* species, and 27 isolates of other pathogens infecting various crops.

The main objectives of the present study were to: (i) generate specific, sensitive and reproducible SCAR markers for distinguishing *P. sojae* from other *Phytophthora* species and soybean soilborne pathogens by RAPD PCR techniques; (ii) evaluate the sensitivity of SCAR markers using PCR; (iii) use the assay to detect the pathogen in infected soybean tissues; and (iv) determine whether SCAR markers can be used to detect the pathogen in soil samples.

Materials and methods

Sources of isolates

A total of 75 isolates of *P. sojae*, 29 isolates of 17 other *Phytophthora* spp., and three isolates of other soilborne pathogens (*Pythium ultimum*, *Fusarium solani* and *Rhizoctonia* sp.) used in this study are listed in Table 1. Twelve known races (R2, R3, R6, R7, R8, R12, R14, R17, R19, R20, R28 and R31) of *P. sojae* were kindly provided by B. Tyler and J. H. Peng. Using a leaf-disc baiting method (Canaday and Schmitthenner 1982), 63 isolates of *P. sojae* were obtained from naturally diseased soybean plots in different provinces of China from 2002 to 2011 and soil carried together with soybeans imported from United States, Brazil, Canada and Argentina. The race types of these isolates have not been tested. *Phytophthora sansomeana* was obtained from China General Microbiological Culture Collection Center. The *P. sojae* isolates, as well as the isolates of other *Phytophthora* spp., *Pythium ultimum*, *Fusarium solani* and *Rhizoctonia* sp. used in this study, are maintained in a collection at Department of Plant Pathology, Nanjing Agricultural University, China (Wang et al. 2006).

Culture conditions and preparation of mycelia

Phytophthora isolates were cultured in clarified V8 juice medium (100 ml V8 tomato juice, 1 g $CaCO_3$, and 15 g agar per 1000 ml of distilled water, sterilized at 120 °C for 20 min) (Zheng 1995). Mycelia of each *Phytophthora* and *Pythium* isolates were obtained by growing the isolates in liquid 10% V8-juice medium at temperatures ranging from 18 °C to 25 °C (according to the optimum temperature of each isolate) with shaking for at least 3 days. Mycelia of other fungi were grown in potato dextrose broth (Erwin and Ribeiro 1996). The mycelia were harvested by filtration, then lyophilized at −80 °C for storage.

DNA extraction

The DNA extraction of freeze-dried mycelium of *P. sojae* as well as artificially or naturally infected seedling materials were completed using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instruction. For soil DNA extraction, about 5 g of sampled soil was divided in 12 tubes using a FastDNA® SPIN Kit for Soil (Q-Biogene Ltd., Morgan Irvine, CA, USA) and DNA was extracted according to the kit's instructions. Soil samples were collected from diseased soybean roots in the field. The extracted DNA was treated with 5 μl of RNase A (10 mg/ml) to remove any contaminating RNA. The amount and purity of DNA samples were determined by a NanoDrop ND-3300 fluorospectrometer (Thermo-Fisher Scientific, Wilmington, DE, USA) and DNA integrity was also analyzed by comparison with standard molecular markers separated by electrophoresis on a 1% (*w*/*v*) agarose gel. Extracted DNA was stored at −20 °C for further use.

RAPD marker screening

A total of 100 RAPD PCR primers containing 10-mer primers from Sangon Biotech (Shanghai, China) were used for screening (Table S1). RAPD analyses were carried out in 25 μl reaction mixtures containing 10× PCR buffer 2.5 μl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 units Taq DNA polymerase (TaKaRa Bio, Dalian, China), 0.8 μM RAPD primers (Sangon Biotech) and 10 ng genomic DNA. All reagents used for PCR amplification were purchased from TaKaRa Bio Company. All reactions were performed in a PTC2000 PCR instrument (MJ Research, Watertown, MA, USA) with an initial denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation, annealing and elongation for 30 s at 94 °C, 30 s at 36 °C and 30 s at 72 °C, respectively, with a final extension step at 72 °C for 10 min. Following amplification, 10 μl of each RAPD product were verified by electrophoresis on a 1.5% agarose gel at a constant voltage of 3.5 V cm^{-1} for 90 min. Negative controls lacking template DNA were included in each experiment to check for possible contamination in reagents. Amplified products were then visualized under transmitted ultraviolet light after staining with ethidium bromide (0.1 mg/l) and images were recorded with a Gel Doc™ 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA). *Trans* 2 K Plus DNA Markers (Biosky, Nanjing, China) and DL 5000 DNA Markers (TaKaRa) covering the molecular weight range 100 bp to 5000 bp were used as size standards.

DNA cloning and sequencing of RAPD fragments

For SCAR marker isolation, polymorphic RAPD bands specific to *P. sojae* were excised from the RAPD agarose gel, purified by the Agarose Gel DNA extraction

Table 1 Fungi and oomycete isolates used in the RAPD-SCAR assay

Species[a]	Isolation/origin		No. of isolates*	Lane order in Fig. S2
	Host/substrate	Source		
P. sojae	*Glycine max*	NJAU	63	1–63
P. sojae (R2)	*Glycine max*	NJAU	1	64
P. sojae (R3)	*Glycine max*	NJAU	1	65
P. sojae (R6)	*Glycine max*	NJAU	1	66
P. sojae (R7)	*Glycine max*	NJAU	1	67
P. sojae (R8)	*Glycine max*	NJAU	1	68
P. sojae (R12)	*Glycine max*	NJAU	1	69
P. sojae (R14)	*Glycine max*	NJAU	1	70
P. sojae (R17)	*Glycine max*	NJAU	1	71
P. sojae (R19)	*Glycine max*	NJAU	1	72
P. sojae (R20)	*Glycine max*	NJAU	1	73
P. sojae (R28)	*Glycine max*	NJAU	1	74
P. sojae (R31)	*Glycine max*	NJAU	1	75
P. sansomeana	*Glycine max*	CGMCC	1	76
P. cactorum	*Malus pumila*	W. H. Ko	1	77
	Rosa chinensis	NJAU	2	78,79
P. melonis	*Amomum xanthoides*	NJAU	2	80,81
	Cucumis sativus	NJAU	2	82,83
P. drechsleri	*Cucumis sativus*	NJAU	1	84
P. vignae	*Vigna sinensis*	Michael D. Coffey	1	85
P. parasitica	*Nicotiana tabacum*	NJAU	1	86
	Salvia splendens	NJAU	1	87
P. capsici	*Capsicum annuum*	NJAU	2	88,89
P. infestans	*Solanum tuberosum*	NJAU	1	90
	Lycopersicon esculentum	NJAU	1	91
P. cinnamomi	*Cedrus deodara*	NJAU	1	92
P. cryptogea	*Gerbera jamesonii*	NJAU	2	93,94
P. palmivora	*Citrus* sp.	NJAU	2	95,96
P. medicaginis	*Medicago sativa*	NJAU	1	97
P. cambivora	*Castanea sativa*	CBS 248.60	1	98
P. boehmeriae	*Gossypium* sp.	NJAU	2	99,100
	Boehmeria nivea	NJAU	1	101
P. fragariae var. *rubi*	*Raspberry*	CBS 967.95	1	102
P. megasperma	*Matthiola incana*	CBS 305.36	1	103
P. hibernalis	*Citrus sinensis*	CBS 270.31	1	104
Pythium ultimum	*Irrigation water*	NJAU	1	105
Fusarium solani	*Glycine max*	NJAU	1	106
Rhizoctonia sp.	*Glycine max*	NJAU	1	107

a All *P. sojae* isolates, other *Phytophthora* species and other fungi genera were maintained in the collection of Nanjing Agricultural University (China).

NJAU, Nanjing Agricultural University. CGMCC, China General Microbiological Culture Collection Center. *number of isolates

Kit from TaKaRa, and subsequently ligated into the pMDTM-19 T vector (TaKaRa) at 4 °C overnight. Ligated DNA was transformed into *Escherichia coli* JM109 cells as described previously (Sambrook et al. 1989). Positive clones were selected by PCR amplification of inserts using M13 sequencing primers. The PCRs were carried out directly with a suspension of transformed bacteria in ultrapure water. Clones containing the RAPD bands of interest were selected according to the expected PCR product sizes. Plasmid DNA was isolated from the positive clones using the plasmid DNA purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The inserts were sequenced bi-directionally using universal primer M13. Sequencing was performed by Invitrogen (Shanghai, China), and the nucleotide sequences of the inserted fragments were checked for homology to other available sequences deposited in GenBank database (www.ncbi.nlm.nih.gov) and *P. sojae* genome V3.0 database (http://genome.jgi.doe.gov/pages/blast-query.jsf?db=Physo3) from Joint Genome Institute (JGI) (http://genome.jgi.doe.gov/pages/blast-query.jsf?db=Physo3) using the BLASTn program.

Design of SCAR primers

Forward and reverse sequences were edited with SeqMan software package Lasergene (DNAStar, Madison, USA). The presence of the RAPD primers was checked at both ends of the sequences and seven sets of primers were designed with the help of Primer 3 software (http://www.frodo.wi.mit.edu/primer3/). Based on sequences of cloned RAPD fragments, new SCAR primers consisted mainly of the 10 bases of the RAPD primer completed by the following 3′ base sequence to design a 19–21 mer primer by Primer 5 and Lasergene software (Fig. 2). Primers were custom synthesized by Invitrogen in salt free status.

Analysis of SCAR primers

PCR conditions for SCAR amplification consisted of 1.0 μl 10× PCR buffer, 0.2 μl 10 mmol/l dNTP, 0.8 μl 25 mmol/l $MgCl_2$, 1 μl of each primer (5 μmol/L), 0.4 μl *Taq* DNA polymerase (2.5 units/μl), 1 μl template DNA (20 ng/μl), and sterile distilled H_2O to a final volume of 10 μl were added. All reactions were performed in a PTC2000 PCR instrument (TaKaRa). The thermal cycling settings used were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60–65 °C for 30 s and extension at 72 °C for 30 s - 2 min (Table 2), with a final extension at 72 °C for 10 min. All of the reagents used for PCR amplification were purchased from TaKaRa. Negative controls lacking template DNA were included in each experiment to test for reagent contamination. Amplified products were all separated on 1%

Table 2 SCAR Primers designed in this study showing sequence, size range of amplified products, optimal annealing temperature, extension time and location of the specific fragments in genome of *P. sojae*

Primer name	Sequence 5′- 3′	Amplication size (bp)	Annealing Temperature (°C)	Extension Time (s)	Location
Scar276-F	AGCCTACCAACCAGCAGGAC	726	62	45	scaffold_3: 5129322–5,130,047
Scar276-R	GGGAAACCTCGACAACAGTG				
Scar304-F	ACCGATGATTAGACGAGTAG	597	60	40	scaffold_8: 2385261–2,385,857
Scar304-R	GCTACCGATTGGTCCAATAAC				
Scar333-F	AAGCCCCAGCCACTTGTTG	693	62	45	scaffold_5: 1885045–1,884,353
Scar333-R	CTAAGCCCTTGTCCTATCG				
Scar37-F	GACCGCTTGTTCAACTTCAG	1092	60	70	scaffold_1: 11621320–11,622,411
Scar37-R	ACCGCTTGTCCTTGGTGTTC				
Scar519-F	CTCCTCATCTGGGTCTCAAC	924	60	60	scaffold_1: 9064440–9,064,439
Scar519-R	TGCTCCGTGCTCACAGCTC				
Scar57-F	ATATGTGGCGGTAACTGACG	1814	60	120	scaffold_7: 2615671–2,617,484
Scar57-R	TCCCACGGGTGATATCGGTC				
Scar78-F	TGAGTGGGTGGCGGTACAGAA	1309	60	90	scaffold_6: 3693266–3,694,574
Scar78-R	GGGTAGTTGCCGCAAATTGAG				

F forward primer, *R* reverse primer

agarose gels through electrophoresis. The DNA fragments in the gels were visualized by staining with ethidium bromide. The initial testing of the specificity of SCAR primers for *P. sojae* was performed using DNA samples from four representative *P. sojae* isolates and other *Phytophthora* species.

Specificity and sensitivity of designed primers

DNA purified from mycelia of a total of 75 *P. sojae* isolates, 29 isolates from different *Phytophthora* species and three other soilborne pathogens (*Pythium ultimum*, *Fusarium solani* and *Rhizoctonia* sp.), were used to determine the specificity of SCAR-F and SCAR-R primers. In the negative controls, the DNA template was replaced by sterile distilled water. To evaluate the sensitivity of the PCR assay, the concentration of purified DNA extracted from pure culture of *P. sojae* (isolate P6497) was quantified with a spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Ten-fold serial dilutions of DNA from mycelia of *P. sojae* (ranging from 1 ng - 10 ag) were prepared and amplified with SCAR primers as described. Genomic DNA (100 ng) from mycelia of *P. sojae* was used as a template of positive control. Experiments were repeated independently at least three times.

Detection of *P. sojae* in soybean seedlings

Seeds of the susceptible soybean (cultivar Hefeng47) were grown in plastic pots containing sterile vermiculite at 25 °C in the dark. The etiolated soybean seedlings were formed after 4 days. Then, after rinsing thoroughly with autoclaved water five to six times, 4-day-old soybean seedlings were placed in a plastic tray (28.4 cm × 20.8 cm × 3.8 cm) in such a way that only their roots and young cotyledons were covered with four layers of wet filter paper. Only the hypocotyl parts of the seedlings were wound-inoculated with three 2-mm-diameter agar plugs derived from the growing edges of 4-day-old V8 agar culture of *P. sojae* P6497. The top of plastic tray was sealed with cling film to prevent excessive evaporation, and then maintained in the dark at 25 °C and at high humidity. After 3 days, visible disease symptoms began to develop. Segments (5 mm length) of symptomatic hypocotyls (15–100 mg wet weight) were excised at 1 cm above the point of inoculation, placed in a tube, and then ground in liquid nitrogen for DNA extraction. Healthy hypocotyls inoculated with water agar plugs were used as a negative control. PCR detection of *P. sojae* in the inoculated soybean tissue was peformed with the SCAR primers using the previously described protocol. Genomic DNA (10 ng) extracted from *P. sojae* mycelia was used as a positive control.

Naturally infected soybean tissues by *P. sojae* were collected from fields in Heilongjiang province in 2015, and DNA extracted from it was then stored at −70 °C. DNA extracted from healthy soybean seedling tissue was used as a negative control, and 100 ng genomic DNA extracted from *P. sojae* mycelia as a positive control. The PCR amplifications above were repeated at least three times per sample.

Detection of *P. sojae* in infested field soil

Three soil samples were collected from soybean fields in Heilongjiang province in 2015 where plants exhibited symptoms of damping-off and root rot, as well as from one field where plants lacked visible symptoms. Five grams of each sample was used to extract DNA. A sample of uninfested soybean field soil that has been autoclaved served as nagative control, and 10 ng purified DNA from *P. sojae* Race 2 was used as positive control.

Results

Identifying, cloning and sequencing of RAPD markers for *P. sojae*

We firstly performed a RAPD analysis on four representative *P. sojae* races (Race 2 P6497, Race 19 P7076, Race 17 P7074 and Race 7 P7064) and 17 other *Phytophthora* species to screen potential DNA markers (Fig. 1). Among the 100 RAPD primer pairs used in this first screening (Table S1), eight RAPD primers (called S254, S276, S304, S333, S37, S519, S57 and S78) provided band patterns specific to the four representative races of *P. sojae* (Fig. 1). RAPD bands were unique for *P. sojae*, with sizes of approximately 600 bp (S254), 750 bp (S276), 600 bp (S304), 700 bp (S333), 1000 bp (S37), 1000 bp (S519), 2000 bp (S57) and 1500 bp (S78). Reproducibility of the RAPD primers was confirmed by repeating the PCR reactions. Given that RAPD primer-based PCR is associated with poor

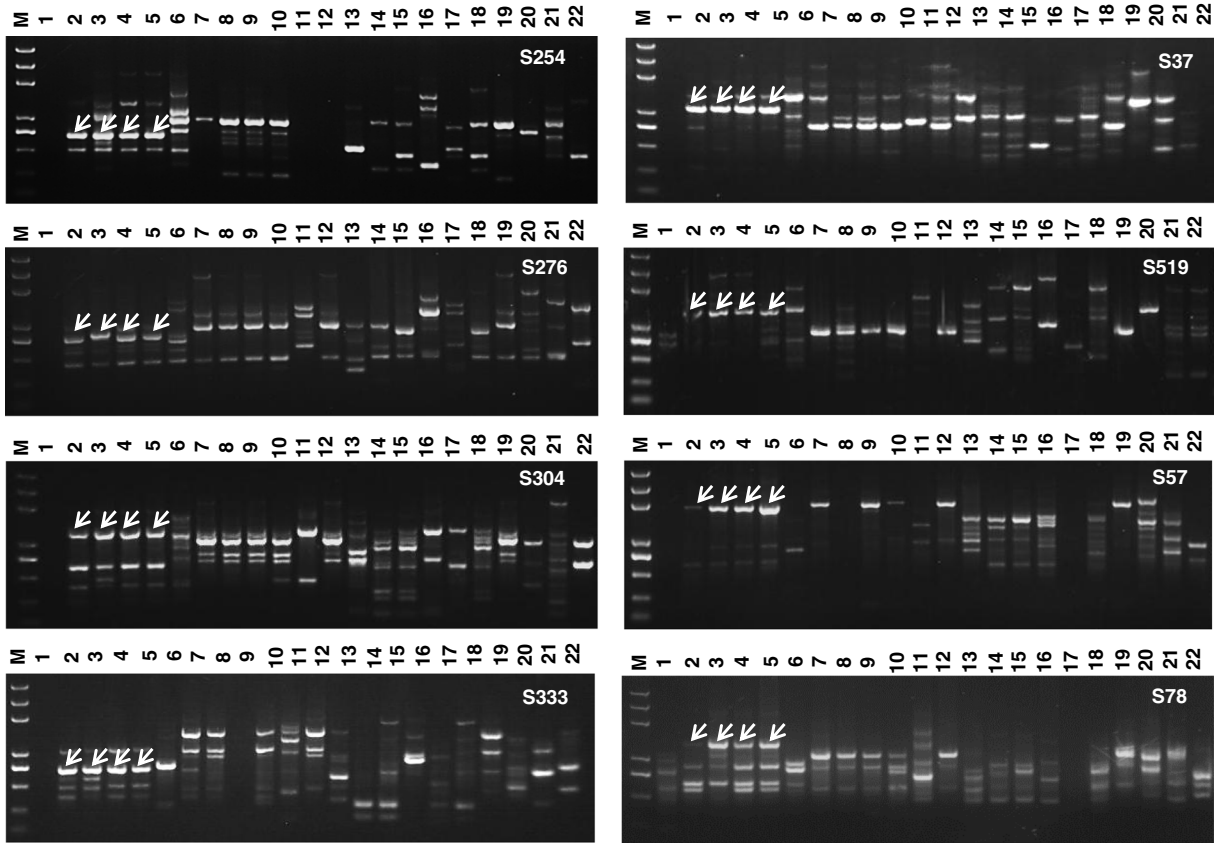

Fig. 1 Agarose gel electrophoresis of PCR-amplified products from genomic DNA of *Phytophthora sojae* and other *Phytophthora* species using 10 RAPD primers. White arrowhead signs indicate the specific fragments of *P. sojae* sequenced to design SCAR primers. M, *Trans*2K Plus DNA Marker (Biosky, Nanjing, China); Lane 1, negative control, no template control; Lanes 2–5, Race 2, Race 7, Race 17, Race 19 of *P. sojae*; Lane 6, *P. sansomeana*; Lane 7, *P. cactorum*; Lane 8, *P. melonis*; Lane 9, *P. drechsleri*; Lane 10, *P. vignae*; Lane 11, *P. parasitica*; Lane 12, *P. capsici*; Lane 13, *P. infestans*; Lane 14, *P. cinnamomi*; Lane 15, *P. crypotogea*; Lane 16, *P. palmivora*; Lane 17, *P. medicaginis*; Lane 18, *P. cambivora*; Lane 19, *P. boehmeriae*; Lane 20, *P. fragariae* var. *rubi*; Lane 21, *P. megasperma*; Lane 22, *P. hibernalis*

reproducibility, seven of eight amplified RAPD markers were subsequently purified, cloned and sequenced to design the SCAR markers. Despite numerous trials, S254–600 bp was unable to get positive results with colony PCR. Seven sequences from the *P. sojae* genome V3.0 database in the following position of scaffold_3:5129321–5,130,057, scaffold_8:2385256–2,385,857, scaffold_5:1884353–1,885,049, scaffold_1:11621320–11,622,411, scaffold_1:9063516–9,064,440, scaffold_7:2615650–2,617,484 and scaffold_6:3693266–3,694,590 were extremely similar to the corresponding RAPD bands amplified by S276, S304, S333, S37, S519, S57 and S78, respectively (data not shown). There was no similarity with known sequences in the Genbank database, indicating the specificity of these RAPD markers for *P. sojae*.

Design and amplification using SCAR primers

The seven identified RAPD markers were redesigned as SCAR primers. The SCAR primers were generated by the addition of 8–15 nucleotide bases to the 3′ and 5′ ends of the original 10-mer RAPD primers (Table S1), and primer sequences for Scar276-R, Scar57-F and Scar78-R were paired to regions distinct from the RAPD priming sequences (Fig. 2, Table 2). The expected sizes of the SCAR markers, the annealing temperature and extension time during amplification were calculated and optimized to enhance amplification efficiency and reproducibility (Table 2). A summary of bulk segregant analysis (BSA) with the RAPD-SCAR technology was shown in Table S2. The seven RAPD markers were identified and successfully converted into SCAR bands.

Fig. 2 Nucleotide sequences of RAPD amplicons specific to *P. sojae*. Arrows represent forward primers (Scar276-F/Scar333-F/Scar519-F/Scar304-F/Scar37-F/Scar57-F/Scar78-F) and reverse primers (Scar276-R/Scar304-R/Scar37-R/Scar57-R/Scar78-R), the sequences in red represent the sequences of the RAPD primers (S276/S333/S519/S304/S37/S57/S78), the sequences in red represent the original random decamer primer sequence (S276/S333/S519/S304/S37/S57/S78) used in the random amplified polymorphic DNA reaction

SCAR primers for specific detection of *P. sojae*

We first tested the specificity of seven SCAR primer sets against four representative *P. sojae* races and 17 other *Phytophthora* species. All SCAR primers resulted in unique and bright bands specific to the four *P. sojae* races with sizes of 726 bp (Scar276-F/R), 597 bp (Scar304-F/R), 693 bp (Scar333-F/R), 1092 bp (Scar37-F/R), 924 bp (Scar519-F/R), 1814 bp (Scar57-F/R) and 1309 bp (Scar78-F/R) (Fig. 3). The specificity of the SCAR primer sets were then tested on a large scale against 75 *P. sojae* isolates, 29 *Phytopthora* spp. isolates and additional soilborne pathogens (*Pythium ultimum*, *Fusarium solani* and *Rhizoctonia* sp.) (Table 1). The SCAR markers were only successfully amplified from genomic DNA from 63 *P. sojae* isolates and 12 known *P. sojae* races, but not from 32 isolates of the other *Phytophthora* species and soilborne pathogens (Fig. S3). No amplification was detected in the negative controls.

SCAR primers for sensitive detection of *P. sojae*

The sensitivity of the seven SCAR markers using total genomic DNA extracted from *P. sojae* revealed that as little as 10 ng of template is sufficient for PCR diagnostics (Fig. 5a). Detection of low levels of target organisms

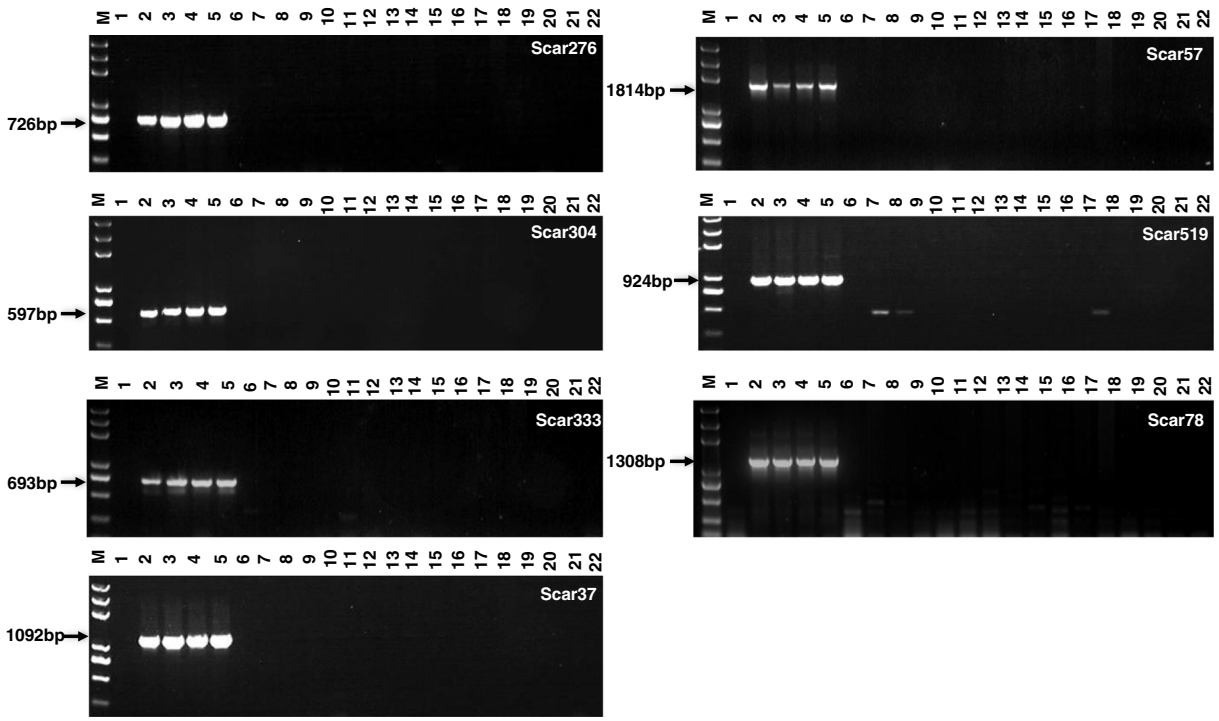

Fig. 3 Agarose gel electrophoresis of PCR-amplified products from genomic DNA of *Phytophthora sojae* and other *Phytophthora* species using seven SCAR primers. Arrows indicates the sizes of seven specific bands in *P. sojae*. M, *Trans*2K Plus DNA Marker (Biosky, Nanjing, China); Lane 1, negative control, no template control; Lanes 2–5, Race 2, Race 7, Race 17, Race 19 of *P. sojae*; Lane 6, *P. sansomeana*; Lane 7, *P. cactorum*; Lane 8, *P. melonis*; Lane 9, *P. drechsleri*; Lane 10, *P. vignae*; Lane 11, *P. parasitica*; Lane 12, *P. capsici*; Lane 13, *P. infestans*; Lane 14, *P. cinnamomi*; Lane 15, *P. crypotogea*; Lane 16, *P. palmivora*; Lane 17, *P. medicaginis*; Lane 18, *P. cambivora*; Lane 19, *P. boehmeriae*; Lane 20, *P. fragariae* var. *rubi*; Lane 21, *P. megasperma*; Lane 22, *P. hibernalis*

in an environment requires a high level of sensitivity (Bulat et al. 2000), therefore analysis of serial 10-fold dilutions (from 1 ng - 10 ag) of purified DNA from *P. sojae* mycelia was performed to gauge the sensitivity of the *P. sojae* SCAR primers in a diagnostic assay designed to simulate natural field sample conditions. As shown in Fig. 4, the minimum detection concentration was found to be 100 fg genomic *P. sojae* P6497 DNA using Scar304, Scar333, Scar519 and Scar78 primer sets, 10 pg using Scar276 primer sets, and 100 pg using Scar333 primer sets. The best result was obtained using a PCR assay with four SCAR primer sets (Scar304-F/R, Scar333-F/R, Scar519-F/R and Scar78-F/R), suggesting that these four SCAR primer sets may be a reliable diagnostic tool for the detection of *P. sojae* in the field.

Detection of *P. sojae* in plants with SCAR primers

Artificially inoculated soybean seedlings exhibiting symptoms of water rot in the greenhouse, as well as soybean naturally infected in the field, were evaluated using the seven SCAR primers. Six of the seven SCAR primers successfully amplified single band from DNA extracted from both inoculated and naturally infected seedlings with *P. sojae*, with the Scar57 primer set being the exception. By contrast, no band was shown in healthy soybean seedling tissue (Fig. 5a and b).

Detection of *P. sojae* in soil with SCAR primers

DNA extracted from infested soils produced clear and single bands by PCR using seven SCAR primers, with the exception of Scar57. The positive control (10 ng purified DNA from *P. sojae* Race 2) also yielded an amplification product with Scar 276 primers (Fig. 6). The results showed that *P. sojae* could be successfully detected by six SCAR primers (Scar276, Scar304, Scar333, Scar37, Scar519 and Scar78) in soil from a field containing plants with symptoms of Phytophthora rot.

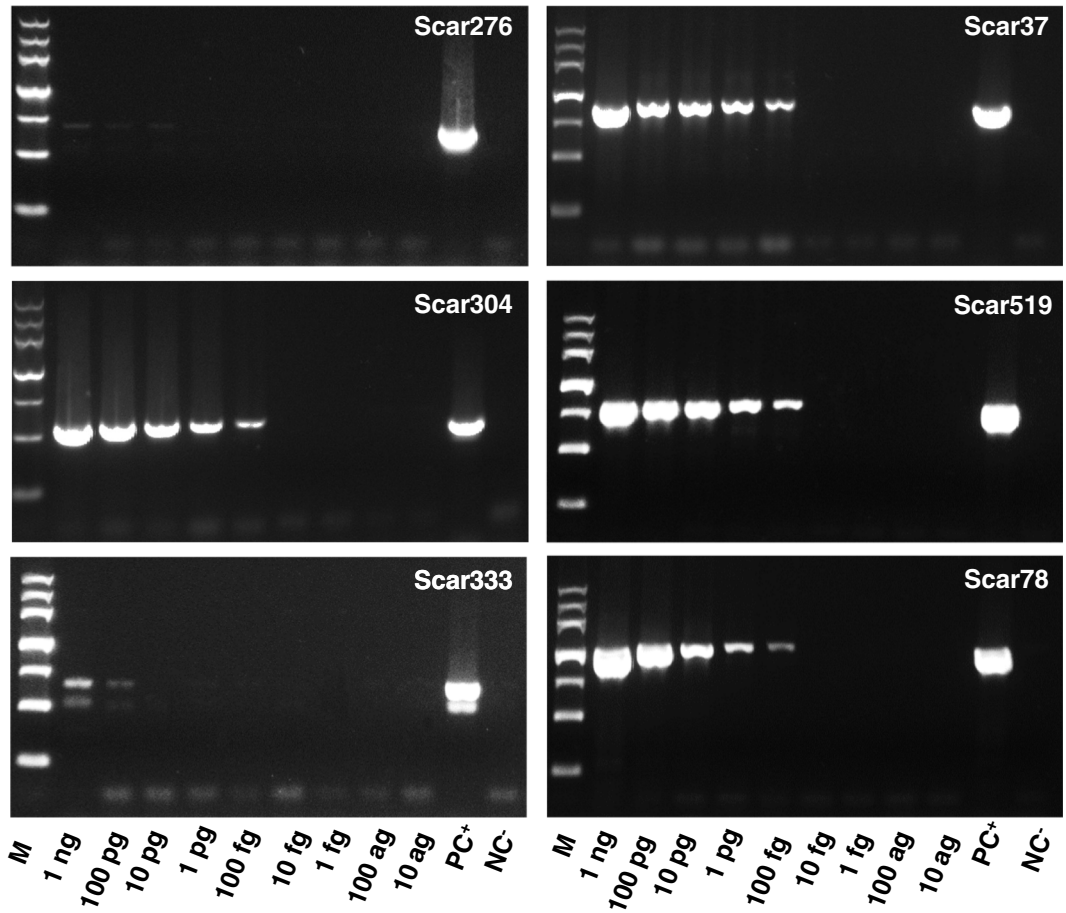

Fig. 4 Sensitivity of PCR assay using six SCAR primers for detection of *Phytophthora sojae*. Sensitivity of detection by SCAR primer sets was tested by using serially diluted genomic DNA (from 1 ng to 10 ag) of *Phytophthora sojae* isolate P6497 as template. Lane M, Marker III (Tiangen, China); Lane PC^+, positive control, 100 ng purified DNA from *P. sojae* Race 2; Lane NC^-, negative control, no template control; Other lanes, 10-fold serial dilutions ranging from 1 ng to 10 ag DNA extracted from mycelia of *P. sojae*

Discussion

We performed experiments to check specificity of PS1/PS2 and PSOJ-F1/R1 primer sets among isolates of *P. sojae*, *P. melonis* and *P. sansomeana*. The results showed that PS1/PS2 could amplify single PCR band not only from *P. sojae* but also *P. melonis*, and a weak band from *P. sansomeana*. PSOJ-F1/R1 also failed to distinguish *P. sojae* against *P. melonis* as well as *P. sansomeana* (Fig. S2), suggesting that PS1/PS2 and PSOJ-F1/R1 primer sets are truly unspecific to *P. sojae*. In this instance, having a species-specific marker used for sequences with insufficient variation will create a bottleneck if there is only one target gene.

In this study, SCAR markers from unique regions of genomic DNA of *P. sojae* were identified after wide screening of different species of *Phytophthora* and other pathogens using RAPD primers. Eight RAPD primers (S254, S276, S304, S333, S37, S519, S57 and S78) yielded single band DNA products with sizes of ~600 bp, ~750 bp, ~600 bp, ~750 bp, ~1000 bp, ~1000 bp, ~2000 bp and ~1500 bp, making them suitable candidates as RAPD markers for detection of *P. sojae*. The specific fragments obtained from seven of eight RAPD primers were successfully converted into SCAR markers, with the exception of S254, the fragments did not show any significant similarity hits within the NCBI database. For Scar304, 19% of the partial sequence showed 99% similarity with transposon *A3aPro* (JX118829.1), which was designed by Dai et al. (2012) for *P. sojae* detection. This shows that the SCAR method has a wide search scope and is very useful for developing

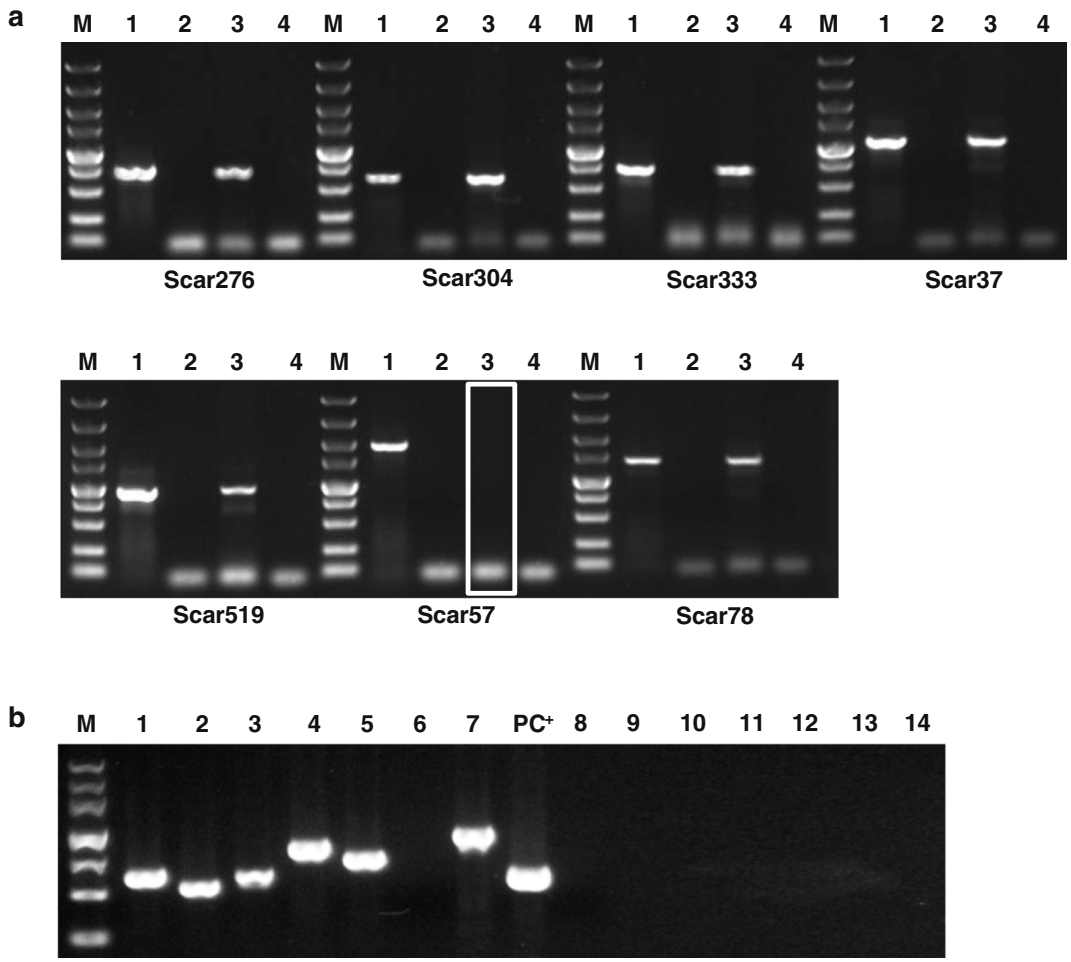

Fig. 5 Detection of *Phytophthora sojae* from naturally and artificially inoculated soybean seedlings by PCR using seven SCAR primers. a Detection of *P. sojae* from the artificially inoculated soybean seedlings by PCR using seven SCAR primers. 10 ng purified DNA extracted from mycelia of *P. sojae* or the artificially inoculated soybean seedlings by *P. sojae* were added and amplified as template in 25 μl PCR reaction system. M, DL 5000 DNA Marker (Takara, Japan); Lane 1, positive control, 10 ng purified DNA from mycelia of *P. sojae* Race 2; Lane 2, no template control; Lanes 3, 10 ng DNA extracted from artificial soybean seedlings infected by *P. sojae*; Lanes 4, negative control, DNA from healthy soybean seedling tissue. **b** Detection of *P. sojae* from naturally infected soybean seedlings by PCR using seven SCAR primers. 100 ng purified DNA extracted from naturally infected soybean seedlings by *P. sojae* were added and amplified as template in 25 μl PCR reaction system. M, Marker III (Tiangen, China); Lane 1, Scar276; Lane 2, Scar304; Lane 3, Scar333; Lane 4, Scar37; Lane 5, Scar519; Lane 6, Scar57; Lane 7, Scar78; Lane PC^+, positive control, 100 ng purified DNA from *P. sojae* Race 2 as template, amplify with Scar276; Lane 8–14, negative control, DNA from healthy soybean seedling tissue as template, amplify with Scar276, Scar304, Scar333, Scar37, Scar519, Scar57 and Scar78 in order

markers for diagnostics. Seven SCAR primer sets were generated and then evaluated for specificity by PCR, with four representative *P. sojae* races and 17 other *Phytophthora* species. The seven SCAR primer sets yielded specific PCR products solely from the four representative *P. sojae* races (Fig. 3), and similar results were obtained using a broader scale PCR assay of 75 *P. sojae* strains from different provinces in China, 29 other *Phytophthora* strains including *P. sansomeana*, and three other soilborne pathogens (*Pythium ultimum*, *Fusarium solani* and *Rhizoctonia* sp.) (Fig. S3). These results suggest that the seven SCAR primers are highly specific to *P. sojae* as the assay was able to detect pathogenic *P. sojae* strains from highly similar species such as *P. melonis* and newly described soybean infecting *Phytophthora* species, *P. sansomeana.*

Analysis of the sensitivity of the SCAR primers in this study revealed that Scar304, Scar333, Scar519 and

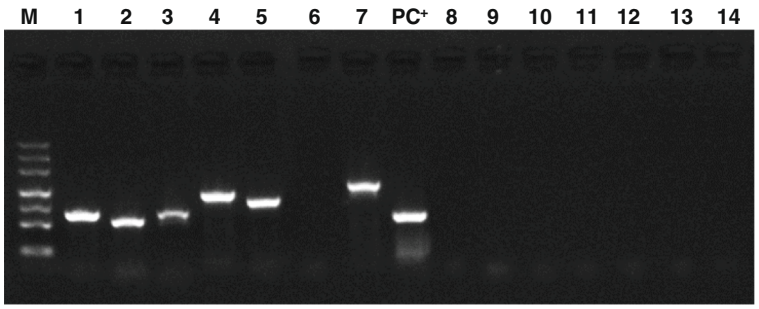

Fig. 6 Detection of *Phytophthora sojae* from infested field soils by PCR using seven SCAR primers. 100 ng purified DNA extracted from soybean field soils were added and amplified as template in 25 μl PCR reaction system. M, Marker III (Tiangen, China); Lane 1, Scar276; Lane 2, Scar304; Lane 3, Scar333; Lane 4, Scar37; Lane 5, Scar519; Lane 6, Scar57; Lane 7, Scar78; Lane PC^+, positive control, 10 ng purified DNA from *P. sojae* Race 2 as template, amplify with Scar276; Lane 8–14, negative control, DNA extracted from uninfested soybean field soil sample (autoclaved) as template, amplify with Scar276, Scar304, Scar333, Scar37, Scar519, Scar57 and Scar78 in order. Three independent experiments were carried out with one soil sample each time, and similar results were obtained. Representative results from one of these experiments were shown

Scar78 had the greatest sensitivity using target DNA extracted from *P. sojae* isolate P6497, in the range of 1 ng to 100 fg, while Scar 276 had a lower sensitivity range between 1 ng - 10 pg, and finally Scar 333, which had the lowest sensitivity range, being 1 ng - 100 pg (Fig. 4). However, even though the sensitivity of these two primer sets, Scar276-F/R and Scar333-F/R, was lower than that of four SCAR primer sets, these two primer sets should also be useful for the identification of *P. sojae* using genomic DNA extracted from pure mycelium in vitro. Moreover, it should be noted that 100 fg sensitivity is the smallest recorded amount for detecting *P. sojae* by the conventional PCR so far. Wang et al. (2006) utilized primers PS1 and PS2 to target the ITS region for the detection of *P. sojae*, with an apparent 1 fg diagnostic limitation by conventional PCR. Subsequently, Bienapfl et al. (2011) developed SYBR-green based assay, using primers PSOJF1 and PSOJR1 to produce a 127 bp product, with a diagnostic limit of detection of 1 pg DNA. The detection limit of the *A3aPro*-specific or *Ypt1*-specific LAMP assay for *P. sojae*, was both 10 pg of genomic DNA per reaction, reported by Dai et al. (2012) and Zhao et al. (2015). Haudenshield et al. (2017) were able to detect *P. sojae* from 34 fg total DNA, which was achieved through serial dilutions by a novel, multiplexed probe-based Q-PCR assay. High sensitivity is especially important in the case of naturally infected materials that are in the presence of a small amounts of pathogen DNA, which could be detected using the SCAR markers (Scar304, Scar333, Scar519 and Scar78).

Moreover, six of the seven SCAR primer sets, with the exception of Scar57, were capable to detecting *P. sojae* from infected soybean seedlings and infested soil sample (Fig. 5 and Fig. 6). However, Scar57 still could successfully detect 10 ng genomic DNA isolated from *P. sojae* pure culture. It is likely that Scar57 has a lower sensitivity to its target compared to the other SCARs, probably due to the relatively lower copy number of the Scar57 hypothetical protein gene. Overall, the observed specificity and sensitivity of our SCAR probes for the detection of *P. sojae* from infected tissues and infested soil samples confirms the potential of these primers to be used as rapid diagnostic tools in specifically identifying *P. sojae* from soybean fields worldwide. Further, the sensitivity of these specific primers also can be significantly improved by dye-binding quantitative PCR (Q-PCR) or biosensor assays, making the tool even more efficient at monitoring the pathogen in soybean.

To our best knowledge, this is the first report of SCAR marker-based molecular detection of *P. sojae* in infected soybean. Compared to other identification methods, SCAR markers represent a useful and effective molecular technique for developing the *P. sojae*-specific primers and detecting *P. sojae* rapidly and sensitively. PCR-based SCAR markers are widely used for molecular identification of several plant varieties (Das et al. 2005; Gao et al. 2010; Vidal et al. 2000), animals (Bardakci and Skibinski 1999; Yao et al. 2012) and detection of plant pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the submission of this manuscript;

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study. All authors in this manuscript have read and approved current version of this article; The manuscript has not been submitted to more than one journal for simultaneous consideration.

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