

## ORIGINAL ARTICLE

# SCAR markers linked to *Phytophthora capsici* and *Meloidogyne incognita* resistance in *Piper* sp.

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**Abstract**

Black pepper (*Piper nigrum*) cultivation suffers from pathogenic *Phytophthora capsici* and *Meloidogyne incognita*, causing tremendous yield loss. To solve this problem, genetically sustainable disease-resistant accessions need to be selected and bred. However, the lack of genomic DNA data for *Piper* sp. could be a major obstacle. In this study, we created R-pools and S-pools from resistant and susceptible accessions in bulked segregant analysis (BSA), respectively, selected DNA fragments associated with resistance or susceptibility to *P. capsici* and *M. incognita* from RAPD markers and then converted them into SCAR markers. This is the first publication showing the potential of molecular markers, specifically the SCAR marker, to select *Piper* sp. accessions resistant to both *P. capsici* and *M. incognita*. Based on BSA, four RAPD primers were selected: UBC#377, UBC#359, UBC#360 and UBC#408. Five resistance and susceptibility-specific fragments were isolated, cloned and sequenced to synthesize 16 SCAR primers. We converted the UBC#377 marker into a 46-377F2R1 marker and the UBC#360 marker into a 30-360F1R2 marker. While the 46-377F2R1 marker has the potential to identify *P. capsici*-resistant accessions with a 350bp resistance-associated band, the 30-360F1R2 marker showed the potential to find *M. incognita*-resistant accessions with a 230bp resistance-linked band.

**KEYWORDS***Meloidogyne incognita*, *Phytophthora capsici*, *Piper*, RAPD, resistance, SCAR

## 1 | INTRODUCTION

Black pepper (*Piper nigrum*) is a widely used spice and has high economic value in many regions of the world. In Vietnam, black pepper is currently grown in many provinces, including Dak Lak, Dak Nong, Dong Nai, Binh Phuoc, Ba Ria-Vung Tau and Gia Lai. The Vietnam Pepper and Spice Association (VPSA) reported that Vietnam exported 236,148 tonnes of peppercorns and the total export turnover reached \$770.6 million in 2023 (VPSA, 2024). However, according to the Ministry of Agriculture and Rural Development, the total pepper plantation was 115,000ha in 2023, down 4.2% compared to 2022 and 24.3% compared to 2017 (151,900ha) (Thang, 2024). The main reason for the decline is the influence of pathogenic diseases such as 'slow decline' (or foot rot) caused by root-knot nematode

*Meloidogyne incognita* and 'quick wilt' caused by *Phytophthora capsici* (Bhai et al., 2017; Nguyen et al., 2021; Verma et al., 2023). *P. capsici* is an oomycete plant pathogen that seriously affects the growth and development of black pepper, causing infected black pepper plants to die within 2-3 weeks in rainy conditions and infecting adjacent plants within 1 or 2 months (Nguyen, 2015; Toh et al., 2016). *P. capsici* moves into the stems, roots and root collar, causing destruction of the phloem and xylem, preventing the transfer of nutrients and water. Therefore, plants collapse suddenly with symptoms of leaf wilting and die very quickly (Nguyen, 2015). On the other hand, the slow-decline disease in *P. nigrum* is caused by the pathogenic nematode *M. incognita* (Naik et al., 2017). Invading nematodes combine with secondary pathogenic organisms such as fungi and bacteria to cause damage to plants (Abd-Elgawad & Askary, 2015; Mitiku, 2018). They cause root necrosis,

yellowing of leaves and death of the pepper tree canopy, thereby reducing pepper productivity (Saad et al., 2022; Usman et al., 2020).

Chemical fungicides and nematicides are common methods used by farmers to manage diseases on black pepper in Vietnam, but their application has been gradually banned due to high cost, environmental pollution and toxicity to humans and other organisms (Desaeger et al., 2020; Hassanin et al., 2020; Youssef & El-Nagdi, 2021). Therefore, controlling black pepper pathogens effectively and economically is currently a big challenge in many pepper-growing regions of Vietnam (Nguyen et al., 2021; Subedi et al., 2020). Studies on black pepper cultivation show that it is possible to manage pathogens right from the first growth stage of black pepper by choosing disease-resistant varieties, sowing disease-free seeds, changing farming methods and using biological control measures (Bhat et al., 2023; Burns et al., 2023; Ngo et al., 2020; Nguyen et al., 2021; Toh et al., 2016). However, the most effective method is to employ sustainable disease-resistant pepper varieties (Eapen & Pandey, 2018). Current breeding programmes prioritize the employment of wild *Piper* varieties due to their resistance to plant diseases and pests, while also providing genetic diversity (Nas et al., 2023). While selecting and creating new varieties using traditional methods takes much time and effort, molecular markers allow for quick and accurate selection of varieties with desired traits and correct genetic characteristics, shortening the time taken and increasing productivity (Meena et al., 2023).

Bulked segregant analysis (BSA) is a simple and cost-saving method for identifying markers linked to a trait of interest and mapping that trait by using bulks (pools) of the most extreme phenotypes (Chu et al., 2016; Goettelmann et al., 2024). This method was first described by Michelmore et al. (1991). In disease resistance studies, BSA was efficiently used for developing markers linked to resistance of bacterial wilt (Goettelmann et al., 2024; Truong et al., 2015), bean common mosaic virus (Bello et al., 2014), clu-brood (Dakouri et al., 2018), charcoal rot (Silva et al., 2020), powdery mildew (Tantasawat et al., 2022) and cucumber blight (Wang et al., 2024). Sequence-characterized amplified regions (SCAR) is one of the PCR-based monolocus and co-dominant genetic markers, with high specificity and reproducibility (Bhagyawant, 2016; Paran & Michelmore, 1993). These markers are DNA fragments generated from the PCR process using specific random amplified polymorphic DNA (RAPD) primers (15–30 nucleotides) with known sequences via cloning and sequencing (Kerry et al., 2022). Based on the SCAR technique, it is possible to quickly create molecular markers linked to genes of interest, especially genes related to disease resistance in plants (Bhagyawant, 2016; Sidiq et al., 2020). Srivastava et al. (2012) successfully developed the SCAR marker ScOPX 04<sub>880</sub> closely linked to the *er1* gene to help accurately distinguish pea (*Pisum sativum*) resistant to powdery mildew. In 2014, a SCAR marker (SCAR<sub>ISSR 863</sub>) was created to screen for downy mildew resistance in breeding programmes of pearl millet (*Pennisetum glaucum*; Jogaiah et al., 2014). Black-rot resistant or susceptible cauliflower (*Brassica oleracea* var. *botrytis*) was identified using two SCAR markers, ScOPO-04<sub>833</sub> and ScPKPS-11<sub>635</sub>, linked to the *Xca1b* locus (Kalia et al., 2017). In a study by Rekha et al. (2020), two SCAR primers, JSCAR1 F1

and BJSCAR1 R1, were recommended to screen *Brassica juncea* for aphid tolerance. In addition, SCAR markers are also used to screen many other crops for resistance to blast disease (*Pyricularia oryzae*), *Phytophthora infestans*, bacterial wilt, watermelon mosaic virus, zucchini yellow mosaic virus, begomoviruses and Fusarium wilt (Jaber et al., 2020; Kim et al., 2016; Quoc et al., 2021; Sidiq et al., 2020; Sobir et al., 2005; Truong et al., 2015; Truong, Tran, et al., 2013). In terms of black pepper, there is currently no research on molecular markers related to disease resistance.

Our previous study found three *Piper* accessions resistant to *P. capsici* and two accessions resistant to *M. incognita* (Truong et al., 2023). From this base, we developed SCAR markers associated with the above resistances based on BSA and RAPD markers. This study aims to find a molecular marker that can select *Piper* accessions resistant to *P. capsici* and *M. incognita* and provide important genetic data for pathogen control.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials and genomic DNA

Thirty-nine *Piper* accessions were collected from eight provinces throughout Vietnam (Kien Giang, Dong Nai, Dak Lak, Binh Phuoc, Gia Lai, Quang Ngai, Quang Nam and Quang Tri) and grown in an open-net house at the Institute of Biotechnology, Hue University, Hue City, Vietnam (Rasphone, Dang, et al., 2022; Truong et al., 2023). While three accessions, HUIB\_PH30 (*P. hancei*), HUIB\_PD36 (*P. divaricatum*) and HUIB\_PH46 (*P. hancei*), showed a high level of *P. capsici* resistance, only HUIB\_PH30 and HUIB\_PD36 were resistant to *M. incognita* invasion (Truong et al., 2023). The remaining accessions were susceptible to *P. capsici* and *M. incognita*. The genomic DNA of each accession was extracted from young leaves and purified with a ratio  $A_{260}:A_{280}$  from 1.8 to 2.0 based on the protocol of Rasphone, Ho, et al. (2022). The species name and GenBank code were identified in a previous study by Rasphone, Dang, et al. (2022).

### 2.2 | Bulk segregant analysis

For experiments on *P. capsici* resistance, equal volumes of genomic DNA (concentration 10ng/ $\mu$ L) from three resistant accessions (HUIB\_PH30, HUIB\_PD36 and HUIB\_PH46) and eight susceptible accessions (HUIB\_PN84, HUIB\_PN87, HUIB\_PN114, HUIB\_PN21, HUIB\_PN27, HUIB\_PN29, HUIB\_PN34 and HUIB\_PN45) were pooled into an R-pool and an S-pool, respectively (Michelmore et al., 1991; Truong, Kim, et al., 2013). For *M. incognita* resistance experiments, the R-pool only included the genomic DNA of HUIB\_PH30 and HUIB\_PD36. In addition, HUIB\_PH46 was added to the S-pool along with eight susceptible accessions (HUIB\_PN84, HUIB\_PN87, HUIB\_PN114, HUIB\_PN21, HUIB\_PN27, HUIB\_PN29, HUIB\_PN34 and HUIB\_PN45). These pools and their parents were

then screened with 200 UBC-RAPD primers (University of British Columbia, Bioneer, Korea) to find specific bands that were only observed in the resistant parents and R-pool or in the susceptible parents and S-pool. These bands were purified, cloned and sequenced to design SCAR primers.

### 2.3 | RAPD analysis

A total of 200 UBC-RAPD primers were used in the BSA method. RAPD reaction components and thermal cycling were performed according to the protocol of Rasphone, Ho, et al. (2022). Subsequently, PCR products with RAPD primers were electrophoresed on 1% agarose gel to identify specific bands that linked to *P. capsici* or *M. incognita* resistance.

### 2.4 | Conversion of RAPD markers into SCAR markers

#### 2.4.1 | Cloning and sequencing RAPD fragments

The RAPD fragments that linked to *P. capsici* or *M. incognita* resistance were cut from gel under UV light, transferred into an Eppendorf tube and purified by a TopPURE PCR/Gel DNA purification kit (HI-412; ABT). The purified products were cloned into the pJET1.2 cloning vector and then transformed into *Escherichia coli* TOP10 cells before being sequenced by Apical Scientific Sdn, Selangor, Malaysia. The obtained DNA sequences were analysed using BioEdit v. 7.2.5.0 software (Sofi et al., 2022).

#### 2.4.2 | Primer design

SCAR primers were designed based on the sequencing results of RAPD fragments by Primer3 v. 0.4.0 (Rozen & Skaletsky, 2000), with a 45%–65% GC ratio (Browne et al., 2020). Subsequently, primers were synthesized by Phu Sa Genomics Joint Stock Company, Can Tho Province, Vietnam. The designed primers were then used to amplify the DNA of resistant and susceptible accessions to test their specificity. Genomic DNA of two *P. capsici*-resistant accessions (HUIB\_PD36 and HUIB\_PH46) and a susceptible accession (HUIB\_PN27) were used in PCR with SCAR primers related to *P. capsici* resistance. Testing of SCAR primers for *M. incognita* resistance used DNA from two *M. incognita*-resistant accessions (HUIB\_PH30 and HUIB\_PD36) and two susceptible accessions (HUIB\_PH46 and HUIB\_PN70).

#### 2.4.3 | SCAR analysis

In the experiments to identify resistance to *P. capsici*, each 15  $\mu$ L PCR mixture contained 5–10 ng/ $\mu$ L of genomic DNA, 1 mM

deoxyribonucleotide triphosphate mix, 1.75 mM MgCl<sub>2</sub>, 5 $\times$  PCR buffer, 1 U Taq DNA polymerase (Bioline-Meridian) and 0.01  $\mu$ M of each SCAR primer. The PCR programme was performed as follows: initial strand separation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min; and a final incubation at 72°C for 10 min.

In the experiments for *M. incognita* resistance, each PCR process was carried out in a 15  $\mu$ L reaction volume containing 5–10 ng/ $\mu$ L of genomic DNA, 0.33 mM deoxyribonucleotide triphosphate mix, 2 $\times$  MyTaq Mix (Meridian Bioscience) and 0.01  $\mu$ M of each SCAR primer. The PCR amplification started with an initial strand separation at 95°C for 3 min; then 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and elongation at 72°C for 30 s; and a final extension for 10 min at 72°C.

All amplification reactions occurred in 0.2 mL tubes using a SimpliAmp thermal cycler (Thermo Fisher Scientific). PCR products were stained for 20 min with a 1:10,000 dilution of SYBR Green I (Invitrogen) and separated by 1% agarose gel electrophoresis in 0.5 $\times$  TBE for 3 h at 120 V. PCR results were observed under UV light and photographed using a gel documentation system (Vilber). A 100 bp DNA Ladder (New England Biolabs) was used as standard for estimation of DNA fragment size. In addition, genotyping analysis was conducted and compared with phenotype results to check the appearance of *P. capsici* and *M. incognita* resistance-associated fragments of all accessions.

## 3 | RESULTS

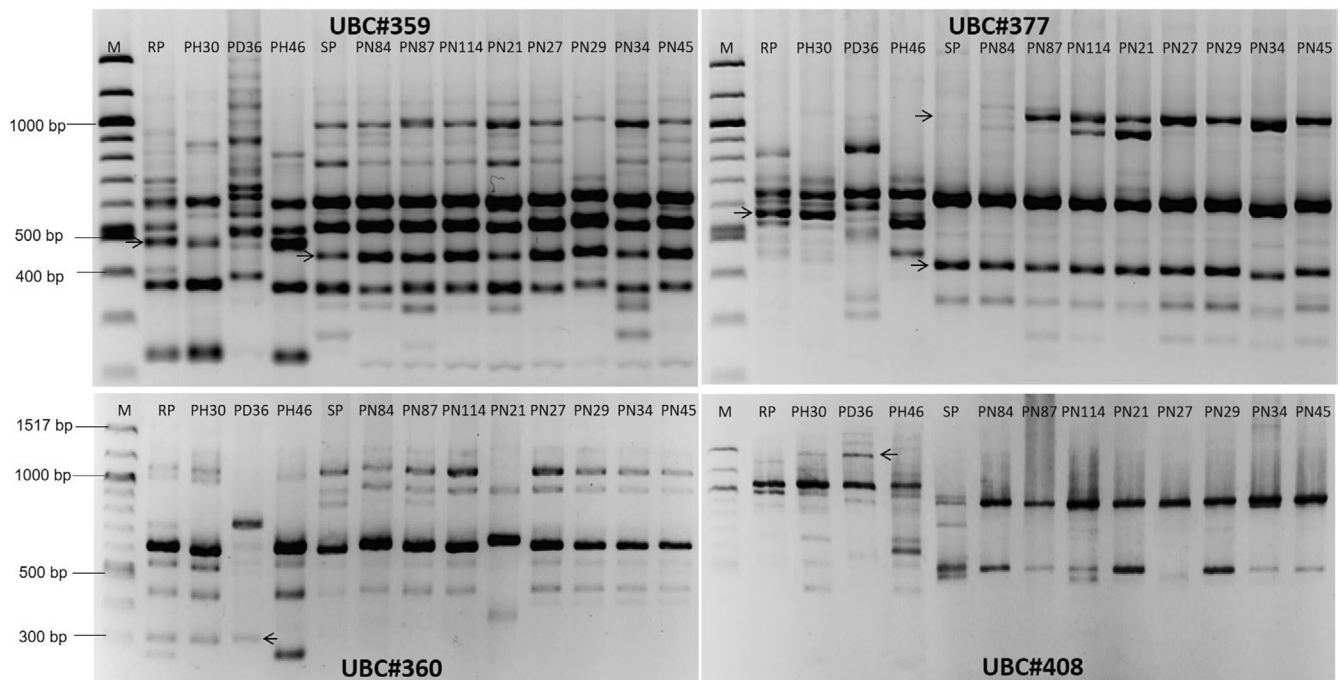
### 3.1 | RAPD marker linked to resistance and susceptibility to *P. capsici* or *M. incognita*

A total of 200 RAPD primers were successfully screened for R-pool and S-pool accessions and their parents using the BSA method. However, only four primers, UBC#359, UBC#377, UBC#360 and UBC#408, showed polymorphism between the R-pool and S-pool. While UBC#359 and UBC#377 showed polymorphic bands between resistant and susceptible accessions with *P. capsici*, UBC#360 and UBC#408 presented polymorphic bands between resistant and susceptible accessions with *M. incognita*. These polymorphic fragments revealed a 100% linkage in the resistant or susceptible accessions. The DNA bands for UBS#359 of approximately 480 bp and for UBC#377 of approximately 550 bp may indicate *P. capsici* resistance because they appear only in the R-pool, HUIB\_PH30, HUIB\_PD36 and HUIB\_PH46. In addition, one fragment (450 bp) of primer UBC#359 and two (400 bp or 1100 bp) of primer UBC#377 were only found in the S-pool and the remaining accessions. These fragments may be related to *P. capsici* susceptibility. When considering the amplification results of primers UBC#360 and UBC#408, only two fragments of 300 and 1450 bp, respectively, characterize *M. incognita* resistance of some accessions (HUIB\_PH30, HUIB\_PD36 and R-pool) (Figure 1).

### 3.2 | Sequence of designed primers

Among the DNA fragments indicating resistance and susceptibility, only the bold, clear fragments including the 300 bp band of

UBC#360, the 550 and 1100bp bands of UBC#377 and the 450 and 480bp bands of UBC#359 were purified, cloned and sent for sequencing. However, the 1450bp fragment of primer UBC#408 had a low DNA concentration after purification, and the mixing of



**FIGURE 1** PCR products of RAPD primers (UBC#359, UBC#377, UBC#360 and UBC#408) show polymorphisms between R-pool (RP), S-pool (SP) and parent accessions: HUIB\_PH30 (PH30), HUIB\_PD36 (PD36), HUIB\_PH46 (PH46), HUIB\_PN84 (PN84), HUIB\_PN87 (PN87), HUIB\_PN114 (PN114), HUIB\_PN21 (PN21), HUIB\_PN27 (PN27), HUIB\_PN29 (PN29), HUIB\_PN34 (PN34) and HUIB\_PN45 (PN45). Arrows represent polymorphic bands expressing association with resistance or susceptibility. M: 100 bp DNA ladder.

Primer	Sequence (5'-3')	Tm (°C)	GC (%)	Resources
27-359F	ACTCGAATCACGGGTTTCAC	60.0	50.0	450bp fragments of UBC#359
27-359R	GGTTCAGAGGGACATGGGTA	59.8	55.0	
46-359-1F	AGCAATGCATTTCCTTGTTGG	61.0	45.0	480bp fragments of UBC#359
46-359-1R	CCAATTTGTTGCAGTCCCTCA	59.7	45.0	
27-377F1	AGGTCAAATGGGTGGAGAT	60.8	50.0	1100bp fragments of UBC#377
27-377R1	GTGGCACGAGTACCCCTTAG	59.6	60.0	
27-377F2	GTTGCGTCCGAACTAAAG	59.9	50.0	
27-377R2	CGAACCGTATGCAATGTGAC	60.0	50.0	
46-377F1	GCATTTTCATATGCCGTGTC	59.0	45.0	550bp fragments of UBC#377
46-377R1	GTCGGAGTGCTACGGTGGT	61.2	63.2	
46-377F2	TTCGGTGCCTTAGGATTGAC	60.1	50.0	
46-377R2	CGCAAGACTCGCTCATTCTA	59.3	50.0	
30-360F1	CTCTCCAGGCCTTCCCCATC	64.6	65.0	300bp fragments of UBC#360
30-360R1	CTCTCCAGGCAAACAGTT	58.4	50.0	
30-360F2	GCCCTCCTCATCTTGCCAAT	60.5	55.0	
30-360R2	TCGGTCTACAGCTTCTTCCA	59.4	47.6	

**TABLE 1** Sequences of SCAR primers.

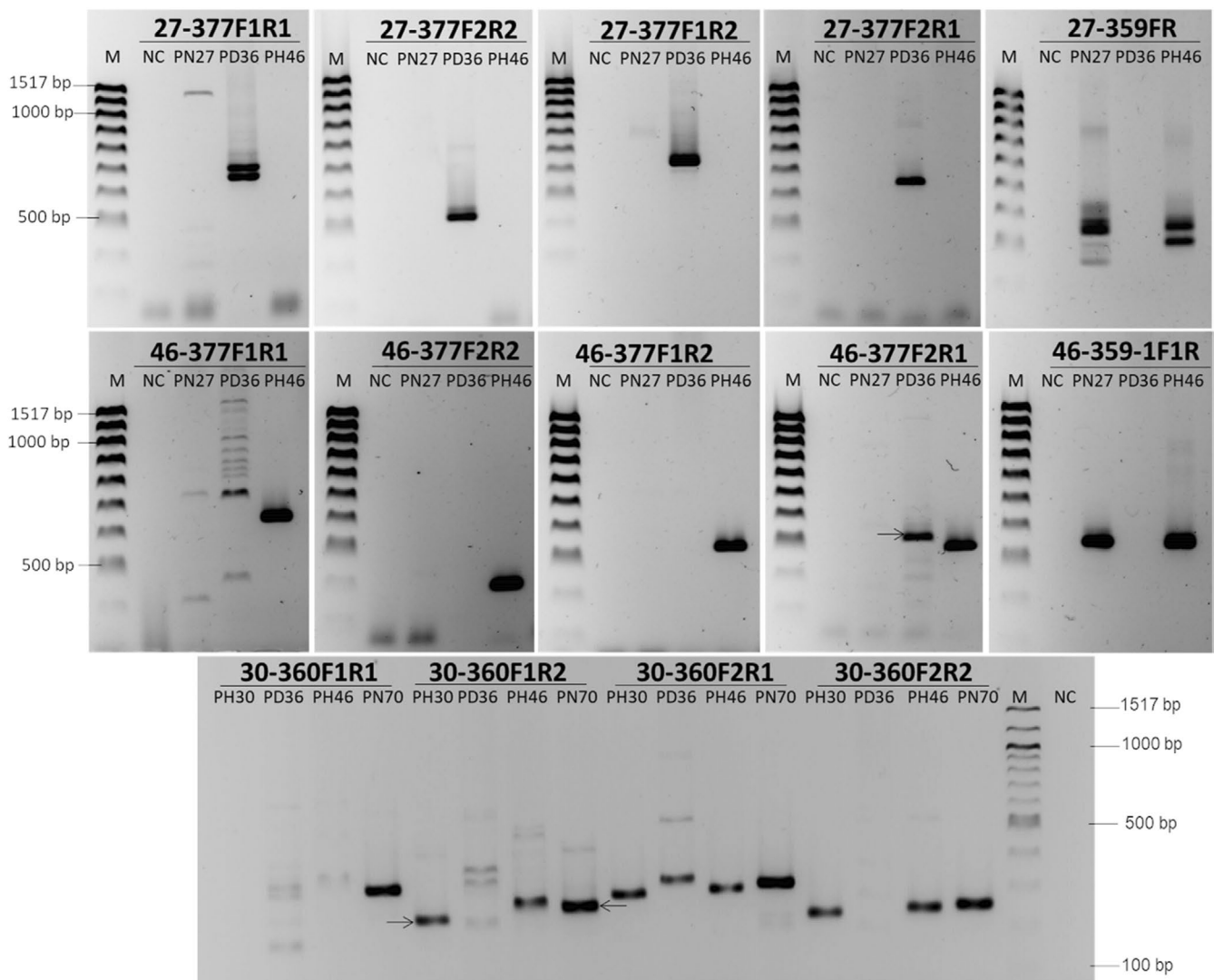
Note: Primer names starting with numbers 27, 30 and 46 mean that the genomic DNA used to generate fragments for primer design are HUIB\_PN27, HUIB\_PH30 and HUIB\_PH46, respectively.

another band was detected after electrophoresis of the purified product of the 400bp fragment of UBC#377, so these fragments were not sent for cloning.

From the results of sequencing polymorphic fragments, the first and last 10 nucleotides of these sequences were removed (sequences of the parent primer pairs) before SCAR primers were designed by Primer3 and synthesized by Phu Sa Genomics Joint Stock Company. A total of 16 synthesized primers with GC ratio above 45% and primer melt temperature above 59°C are given in Table 1.

The results of testing the specificity of 14 pairs of designed primers showed that only primer pair 46-377F2/R1 showed differences between *P. capsici*-resistant and -susceptible accessions and only primer

pair 30-360F1/R2 showed polymorphism between *M. incognita*-resistant and -susceptible accessions. Therefore, only these two primer pairs were used to analyse SCAR for all accessions. Although primer pairs such as 27-277F1/R1, 27-277F2/R2, 27-277F1/R2 and 27-277F2/R1 were created based on the DNA sequence of HUIB\_PN27 accessions, electrophoresis results of PCR products illustrated that these primer pairs were not specific for HUIB\_PN27. No or faint bands appeared at the position of HUIB\_PN27, while there were 1–2 DNA bands at the HUIB\_PH30 position. In addition, polymorphic bands appeared for primer pairs 46-377F1/R1, 46-377F2/R2, 46-377F1/R2, 46-359-1F/1R, 30-360F1/R1, 30-360F2/R2 and 30-360F2/R1, but there was no difference between resistant and susceptible accessions (Figure 2).



**FIGURE 2** Electrophoresis results of PCR products using synthesized primer pairs to amplify accessions including HUIB\_PH30 (PH30), HUIB\_PD36 (PD36), HUIB\_PH46 (PH46), HUIB\_PN27 (PN27) and HUIB\_PN70 (PN70). The small images above depict SCAR markers related to *Phytophthora capsici* resistance (27-377F1/R1, 27-377F2/R2, 27-377F1/R2, 27-377F2/R1, 27-359F/R, 46-377F1/R1, 46-377F2/R2, 46-377F1/R2, 46-377F2/R1 and 46-359-1F/1R), and the large one below represents *Meloidogyne incognita* resistance (30-360F1/R1, 30-360F1/R2, 30-360F2/R1 and 30-360F2/R2). Arrows represent polymorphic bands between resistant and susceptible accessions. M: 100bp DNA ladder. NC, negative control (distilled water).

### 3.3 | Development of SCAR marker

#### 3.3.1 | SCAR marker linked to *P. capsici* resistance

The result of sequencing the 550bp fragment generated by primer UBC#377 was a 726 bp sequence (Table S1). In particular, the sequence length used to design the SCAR primer was 706bp (after removing the first and last 10 nucleotides). Primer pair 46-377F2/R1 amplified a sequence of about 400bp (Figure S1). This was demonstrated because the results of the SCAR marker showed a 350bp band in resistant accessions and a 400bp band in susceptible accessions with *P. capsici* (Figure 3). This result demonstrates the potential of converting the RAPD marker into a SCAR marker. Among the 39 accessions genotyped using RAPD and SCAR markers, only three accessions (HUIB\_PH30, HUIB\_PD36 and HUIB\_PH46) carried fragments associated with *P. capsici* resistance and displayed mild levels of infection (Table 2).

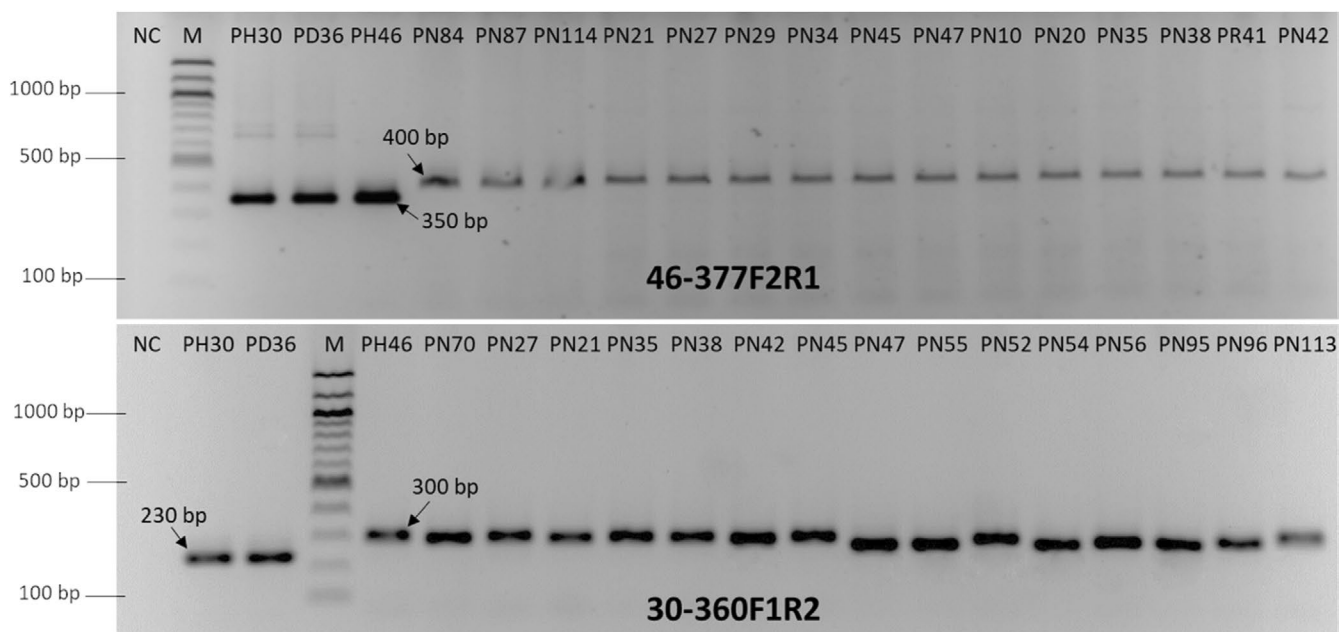
#### 3.3.2 | SCAR marker linked to *M. incognita* resistance

The 403bp sequence was obtained from sequencing the 300bp fragment from primer UBC#360 (Table S1). This sequence was then used to synthesize the primer pair 30-360F1/R2, consisting of two primers, 30-360F1 and 30-360R2, which had the potential to amplify a sequence of about 310bp (Figure S2). SCAR marker results using primer pair 30-360F1/R2 showed the presence of a characteristic band for *M. incognita*-resistant accessions (nearly 230bp) and a band characteristic for susceptible accessions (approximately 300bp) (Figure 3). At the same time, the appearance of the fragment

in HUIB\_PH30 and HUIB\_PD36 associated with *M. incognita* resistance was proven via genotyping analysis. These two accessions did not show yellow leaves and root galls when infected with *M. incognita* (Table 2).

## 4 | DISCUSSION

There are two superior techniques used in this study, BSA and SCAR markers. BSA is a technique for identifying genomic regions containing loci affecting a trait of interest (Shen & Messer, 2022). The advantage of BSA is that it is not necessary to determine the genotype of each individual in a large mapping population (Kurlovs et al., 2019). Furthermore, the basis of BSA is that the alleles must appear when the DNA is composed of the genomic DNA of a group of individuals with the same phenotype (Chu et al., 2016; Michelmore et al., 1991). Therefore, grouping resistant pepper accessions into one group (R-pool) and susceptible accessions into another group (S-pool) can help identify and classify genotypes more easily. SCAR is a molecular marker that is fast, reliable, less sensitive to reaction conditions and easy to perform for genetic characterization, identification and plant authentication. In particular, it can be developed with unknown genomic DNA and used to identify individual disease-resistant plants (Bhagyawant, 2016; Kim et al., 2016; Kiran et al., 2010). SCAR primers can be converted from molecular markers such as amplified fragment length polymorphisms (AFLPs), simple-sequence repeats (SSRs) and inter simple-sequence repeats (ISSRs). However, this is very expensive, difficult and time-consuming and requires information about the genome sequence (Kiran et al., 2010). These limitations can be overcome by developing the SCAR marker based on



**FIGURE 3** Validation of developed SCAR markers in *Piper* sp. accessions. Arrows indicate bands associated with resistance and susceptibility to *Phytophthora capsici* (46-377F2R1) and *Meloidogyne incognita* (30-360F1R2). M: 100bp DNA ladder. NC, negative control (distilled water).

TABLE 2 Genotyping results of 39 black pepper accessions using RAPD and SCAR markers.

Accession	Genotype in <i>P. capsici</i> resistance testing			Genotype in <i>M. incognita</i> resistance testing		Plants with yellow leaf symptoms (%) <sup>b</sup>	Plants with roots with galls (%) <sup>b</sup>
	UBC#377 (550bp fragment)	46-377F2R1	<i>P. capsici</i> infection level 6 days after inoculation <sup>a</sup>	UBC#360 (300bp fragment)	30-360F1R2		
HUIB_PN10	S	S	Rotted	S	S	20–33	50–80
HUIB_PN20	S	S	Rotted	S	S	20–33	50–80
HUIB_PN21	S	S	Rotted	S	S	20–33	50–80
HUIB_PN52	S	S	Severe	S	S	20–33	50–80
HUIB_PN27	S	S	Rotted	S	S	20–33	50–80
HUIB_PN29	S	S	Rotted	S	S	20–33	50–80
HUIB_PH30	R	R	Mild	R	R	0	0
HUIB_PN34	S	S	Severe	S	S	20–33	50–80
HUIB_PD36	R	R	Mild	R	R	0	0
HUIB_PN45	S	S	Severe	S	S	20–33	50–80
HUIB_PH46	R	R	Mild	S	S	20–33	50–80
HUIB_PN47	S	S	Rotted	S	S	20–33	50–80
HUIB_PN55	S	S	Rotted	S	S	20–33	50–80
HUIB_PN56	S	S	Rotted	S	S	20–33	50–80
HUIB_PN69	S	S	Moderate	S	S	20–33	50–80
HUIB_PN70	S	S	Severe	S	S	20–33	50–80
HUIB_PN84	S	S	Rotted	S	S	20–33	50–80
HUIB_PN87	S	S	Severe	S	S	20–33	50–80
HUIB_PN89	S	S	Severe	S	S	20–33	50–80
HUIB_PN91	S	S	Rotted	S	S	20–33	50–80
HUIB_PN93	S	S	Rotted	S	S	20–33	50–80
HUIB_PN95	S	S	Rotted	S	S	20–33	50–80
HUIB_PN96	S	S	Rotted	S	S	20–33	50–80
HUIB_PN97	S	S	Severe	S	S	20–33	50–80
HUIB_PN101	S	S	Severe	S	S	20–33	50–80
HUIB_PN102	S	S	Moderate	S	S	20–33	50–80
HUIB_PN105	S	S	Rotted	S	S	20–33	50–80
HUIB_PN113	S	S	Severe	S	S	20–33	50–80
HUIB_PN114	S	S	Moderate	S	S	20–33	50–80
HUIB_PN115	S	S	Moderate	S	S	20–33	50–80
HUIB_PN116	S	S	Rotted	S	S	20–33	50–80
HUIB_PN38	S	S	Rotted	S	S	20–33	50–80
HUIB_PN42	S	S	Rotted	S	S	20–33	50–80
HUIB_PN43	S	S	Rotted	S	S	20–33	50–80
HUIB_PN35	S	S	Moderate	S	S	20–33	50–80
HUIB_PN54	S	S	Rotted	S	S	20–33	50–80
HUIB_PR48	S	S	Severe	S	S	20–33	50–80
HUIB_PR41	S	S	Rotted	S	S	20–33	50–80
HUIB_PN50	S	S	Severe	S	S	20–33	50–80

Note: R, genotypes are the same with resistance test results; S, genotypes are the same with susceptibility test results.

<sup>a</sup>Phenotype in *Phytophthora capsici* resistance testing (Truong et al., 2023).

<sup>b</sup>Phenotype in *Meloidogyne incognita* resistance testing (Truong et al., 2023).

the RAPD marker with specificity, low cost, ease, fast use, reproducibility, abundance and high polymorphism (Sairkar et al., 2016; Sidiq et al., 2020). These are important grounds for choosing the RAPD-SCAR marker to identify individuals resistant to *P. capsici* or *M. incognita* without data on the black pepper genome.

From the results of BSA analysis of 200 RAPD primers, seven DNA fragments showed associations with resistance or susceptibility to two plant pathogens, *P. capsici* and *M. incognita*. These segments may represent genes linked to resistance or susceptibility. Except for a fragment with low DNA concentration (1450bp fragment of UBC#408) and an unsuccessfully purified fragment (400bp fragment of UBC#377), the remaining five fragments were cloned, sequenced and used to design 16 primers (corresponding to 14 primer pairs). However, the results of studying the specificity of primer pairs for resistance or susceptibility phenotypes showed that the majority of primer pairs were nonspecific, except for two primer pairs 46-377F2/R1 and 30-366F1/R2. This is because the heterogeneous nature of polymorphic fragments is indistinguishable and successful cloning of polymorphic fragments is difficult to achieve. Specifically, heterogeneously amplified fragments can appear as one band on the gel of RAPD results with a similar size and specificity. Therefore, choosing these heterogeneous fragments to convert to SCAR markers will cause non-targeted sequences to be frequently generated (Ardiel et al., 2002; Kim et al., 2016; Truong, Tran, et al., 2013).

*Phytophthora capsici* and *M. incognita* are two common pathogens of black pepper, especially *P. nigrum* (Nguyen, 2015; Verma et al., 2023). Some recent studies suggest the existence of genes that are resistant to *P. capsici* (Kattupalli et al., 2021; Malik & Kokkat, 2018; Suraby et al., 2020) and *M. incognita* (Premachandra et al., 2014; Wiratno et al., 2009) in *Piper* species. Additionally, our previous research showed that among 39 *Piper* sp. accessions, only three accessions were resistant to *P. capsici*, namely HUIB\_PH30 (*P. hancei*), HUIB\_PD36 (*P. divaricatum*) and HUIB\_PH46 (*P. hancei*), and two accessions were strongly resistant to *M. incognita*, HUIB\_PH30 and HUIB\_PD36 (Truong et al., 2023). This result is similar to the results of evaluating the SCAR marker on all 39 black pepper accessions. Specifically, a fragment with 350bp in size associated with *P. capsici* resistance was detected in three accessions, HUIB\_PH30, HUIB\_PD36 and HUIB\_PH46, when using the 46-377F2R1 marker. In addition, during the development of the 30-360F1R2 marker, a 230bp fragment associated with *M. incognita* resistance was discovered in two accessions, HUIB\_PH30 and HUIB\_PD36. The remaining accessions had a band with a different size than the resistant accessions. This study also showed the relationship between phenotype and genotype of accessions in *P. capsici* and *M. incognita* resistance testing, namely, accessions carrying the *P. capsici* resistance band had a mild infection and accessions carrying *M. incognita* resistance band were free of leaf yellowing and root galls when infected with the respective pathogens. Hence, the two SCAR markers are 100% accurate in diagnosing accessions that carry the resistance-associated fragment (i.e., a perfect match between the number of individuals carrying the resistance-associated

fragment in the SCAR marker and the number of resistant individuals in field testing). This shows the potential of two SCAR markers, 46-377F2R1 and 30-360F1R2, in correctly identifying black pepper accessions resistant to *P. capsici* or *M. incognita*. However, the lack of genomic DNA sequence information on *Piper* species limited the search for specific locations of resistance alleles on chromosomes. Consequently, the genetic stability of this resistance trait across generations cannot be assessed.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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