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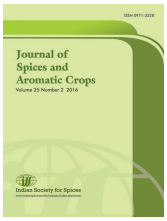

Cross species amplification of microsatellite loci from *Phytophthora* spp to assess genetic diversity among the *Phytophthora* isolates from black pepper

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Abstract

Phytophthora capsici is one of the most destructive pathogens of black pepper in India and the world over. Diversity of 114 *Phytophthora* isolates obtained from leaf, stem, root and soil of different agro climatic areas was studied using 25 SSR primers that were previously described. twenty primers were successfully amplified. Of these, 15 were polymorphic among the *Phytophthora* isolates and altogether 37 loci were detected. Of the 37 bands generated from 20 primers, 75.67% were polymorphic. Cluster analysis using the unweighted pair-group method with arithmetic averages divided the isolates into seven major groups. The dendrogram generated showed that all the isolates were separated at similarity coefficient between 57-95%. Group 7 with a single isolate stood as an out group. Clustering did not follow the pattern of geographical origin or plant part from where the isolates were obtained.

Keywords: black pepper, cross species transferability, diversity, *Phytophthora*, SSR

Introduction

Black pepper (*Piper nigrum* L.) (Family: *Piperaceae*) is a perennial climbing vine grown for its berries that are extensively used as a spice and in medicine. In India, black pepper is being cultivated in Kerala (96%), Karnataka (3%) and to a lesser extent, in Maharashtra, Andhra Pradesh, Tamil Nadu and north eastern regions. The crop is cultivated as a monocrop in Brazil, Indonesia and Vietnam, while in India, it is mostly grown as an intercrop along with cardamom, coffee, coconut, arecanut and others. The drastic drop in the black pepper production in all growing countries has been attributed to pronounced death of vines by the dreaded disease caused by *Phytophthora capsici* Tsao. Though a number of plant protection measures are available including chemical and biological, identification of resistant cultivars is the most effective and economic method. Morphological characterization using biological markers showed wide variability among *Phytophthora* isolates from black pepper. The pathogen also showed varying responses to different strategies of management. Hence, population diversity analysis is valuable to investigate the pathogenic, phenotypic and genotypic diversity existing among *Phytophthora* isolates of black pepper collected from different locations. The genetic analysis of pathogen populations is

essential for understanding disease epidemiology, host-pathogen interactions and resistance breeding (McDonald *et al*. 1989; McDonald & McDermott 1993; Milgroom & Fry 1997; McDonald & Linde 2002; Milgroom & Peever 2003). Molecular markers have taken over other tools to study and detect genetic variation in plant pathogen populations (Chen *et al*. 2004). Microsatellites or simple sequence repeats (SSRs) are short, tandemly repeated motifs of DNA ubiquitous in all analyzed eukaryotic genomes (Tautz & Renz 1984; Toth *et al*. 2000; McDonald & Linde 2002). They are highly polymorphic, multiallelic and co-dominant and are more powerful marker system than RFLP and RAPD. Microsatellites are excellent markers in the study of *Phytophthora* population biology, ecology, genetics and evolution.

Genome sequence data of *P. sojae*, *P. ramorum* and *P. infestans* was used to design SSR markers and was analysed on 16 different species (Schena *et al*., 2008). SSR markers were developed from EST database of *P. sojae* and were tested on five strains (Zhu *et al.* 2004). Novel EST-SSR markers developed in *P. sojae* were found to be useful for genetic variation study of *P. sojae* and its related species (Lin *et al*. 2008). Thirty-one pairs of SSR primers were designed from expressed sequence tags of *P. capsici* available in dbEST GenBank and SSR analysis indicated that the 61 *P. capsici* isolates from irrigation ponds in Georgia were genetically distinct (Wang *et al*. 2009). The present study focuses on diversity analysis of *Phytophthora* isolates from black pepper collected from different regions of India using SSR markers.

Materials and methods

Origin of Phytophthora *isolates*

One hundred and fourteen *Phytophthora* isolates from black pepper, maintained in the National Repository of *Phytophthora* at ICAR-IISR, Kozhikode, were used for diversity analysis (Table 1). These isolates were collected in a span of 12 years from 1997–2009 from different pepper growing tracts of Kerala, Karnataka, Tamil Nadu and Andhra Pradesh in India.

Table 1. Details of black pepper *Phytophthora* isolates analysed in the study

Isolate	Locality	District	Plant part	Latitude	Longitude
		A. KERALA STATE			
06-12	Irikkur	Kannur	Leaf	11º98′N	75º55′E
06-13	Irikkur	Kannur	Stem	11º98′N	7º55′E
06-09	Iritti	Kannur	Leaf	11°98′N	75°68′E
06-10	Iritti	Kannur	Stem	11°98′N	75°68′E
03-10	Thikkodi	Kozhikode	Root	11°49′N	75°63′E
08-07	Koorachundu	Kozhikode	Stem	11º54′N	75º84′E
06-01	Koothali	Kozhikode	Stem	11°59′N	75°76′E
06-02	Koothali	Kozhikode	Stem	11°59′N	75°76′E
07-08	Koothali	Kozhikode	Leaf	11°59′N	75°76′E
98-02	Peruvannamuzhi	Kozhikode	Soil	11°60′N	75°85′E
98-50	Peruvannamuzhi	Kozhikode	Soil	11°60′N	75°85′E
99-166	Peruvannamuzhi	Kozhikode	Leaf	11°60′N	75°85′E
05-05	Peruvannamuzhi	Kozhikode	Leaf	11°60′N	75°85′E
05-06	Peruvannamuzhi	Kozhikode	Spike	11°60′N	75°85′E
07-01	Peruvannamuzhi	Kozhikode	Soil	11°60′N	75°85′E
07-02	Peruvannamuzhi	Kozhikode	Stem	11°60′N	75°85′E
08-01	Peruvannamuzhi	Kozhikode	Root	11°60′N	75°85′E
08-03	Peruvannamuzhi	Kozhikode	Stem	11°60′N	75°85′E

Isolate	Locality	District	Plant part	Latitude	Longitude
08-06	Peruvannamuzhi	Kozhikode	Soil	11°60′N	75°85′E
09-01	Peruvannamuzhi	Kozhikode	Stem	11°60′N	75°85′E
09-02	Peruvannamuzhi	Kozhikode	Collar	11°60′N	75°85′E
09-03	Peruvannamuzhi	Kozhikode	Soil	11°60′N	75°85′E
06-03	Chelavoor	Kozhikode	TC plants	11°30′N	75°84′E
07-05	Chelavoor	Kozhikode	Leaf	11°30′N	75°84′E
07-03	Chelavoor	Kozhikode	Leaf	11°30′N	75°84′E
08-04	Chelavoor	Kozhikode	Soil	11°30′N	75°84′E
98-128	Kunnamangalam	Kozhikode	Stem	11°31′N	75°87′E
06-08	Thiruvampadi	Kozhikode	Leaf	11°36′N	76°02′E
98-01	Thamarassery	Kozhikode	Soil	11°77′N	75°50′E
99-91	Thamarassery	Kozhikode	Leaf	11°77′N	75°50′E
08-02	Thamaraserry	Kozhikode	Leaf	11°77′N	75°50′E
06-04	Puthupadi	Kozhikode	Leaf	11°48′N	76°00′E
98-95	Adivaram	Kozhikode	Stem	11°49′N	76°02′E
01-04	Adivaram	Kozhikode	Stem	11°49′N	76°02′E
03-07	Adivaram	Kozhikode	Leaf	11°49′N	76°02′E
03-02	Vythiri	Wayanad	Root	11º54′N	76º04′E
03-03	Vythiri	Wayanad	Leaf	11º54′N	76º04′E
03-08	Vythiri	Wayanad	Stem	11º54′N	76º04′E
05-09	Vaduvanchal	Wayanad	Collar	11°56′N	76°22′E
99-145	Sugandhagiri	Wayanad	Berry	11°62′N	76°06′E
99-139	Meenangadi	Wayanad	Leaf	11°66′N	76°17′E
98-70	Vageri	Wayanad	Leaf	11º70′N	76º09′E
97-11	Pulpally	Wayanad	Leaf	11°80′N	76° 17′E
97-52	Pulpally	Wayanad	Leaf	11°80′N	76°17′E
97-53	Pulpally	Wayanad	Leaf	11°80′N	76°17′E
98-17	Pulpally	Wayanad	Leaf	11°80′N	76°17′E
09-13	Pulpally	Wayanad	Soil	11°80′N	76°17′E
98-75	Wayanad	Wayanad	Foot	11°80′N	76°17′E
07-06	Wayanad	Wayanad	Leaf	11°80′N	76°17′E
07-07	Wayanad	Wayanad	Berry	11°80′N	76°17′E
09-16	Padamala	Wayanad	Leaf & Soil	11º70′N	76º09′E
98-198	Manjachola	Malappuram	Soil	10º94′N	75º99′E
98-81	Kalpakanjeri	Malappuram	Leaf	10º94′N	75º97′E
97-19	Kottakkal	Malappuram	Stem	11º00′N	76º00′E
01-20	Silent valley	Palghat	Soil	11º13′N	76º42′E
97-54	Ayoor	Kollam	Stem	11º13′N	76º42′E
		B. KARNATAKA STATE			
98-48	Madikeri	Kodagu	Leaf	12°43′N	75°76′E
98-49	Madikeri	Kodagu	Berry	12°43′N	75°76′E
98-59	Chettali	Kodagu	Leaf	12°37′N	75°84′E
05-17	Chettali	Kodagu	Leaf	12°37′N	75°84′E
05-19	Chettali	Kodagu	Soil	12°37′N	75°84′E
05-20	Chettalli	Kodagu	Root	12°37′N	75°84′E
98-60	Valnoor	Kodagu	Leaf	12°40′N	75°90′E
98-66	Valnoor	Kodagu	Leaf	12°40′N	75°90′E
98-71	Sundicopa	Kodagu	Leaf	12°45′N	75°89′E
98-74	Sundicopa	Kodagu	Leaf & Stem	12°45′N	75°89′E

Isolate	Locality	District	Plant part	Latitude	Longitude
98-76	Sundicopa	Kodagu	Leaf	12°45′N	75°89′E
98-87	Chettali	Kodagu	Leaf	12°37′N	75°84′E
98-90	Chettali	Kodagu	Stem	12°37′N	75°84′E
98-92	Sundicopa	Kodagu	Leaf	12°45′N	75°89′E
05-13	Appangala	Kodagu	Leaf	12°42′N	77°35′E
05-14	Appangala	Kodagu	Leaf	12°42′N	77°35′E
05-15	Appangala	Kodagu	Leaf	12°42′N	77°35′E
05-16	Appangala	Kodagu	Leaf	12°42′N	77°35′E
98-93	Adyanadka	Dakshina Kannada	Leaf	12°45′N	75°89′E
98-171	Putur	Dakshina Kannada	Leaf	12°45′N	75°89′E
98-142	Nukkal	Dharward	Root	12°45′N	75°89′E
98-149	Mukkal	Dharward	Stem	11º70′N	76º09′E
98-143	Yadally	Uttara Kannada	Root & Soil	14°62′N	74°83′E
98-155	Isloor	Uttara Kannada	Soil	14°62′N	74°83′E
98-156	Sirsi	Uttara Kannada	Soil	14°62′N	74°83′E
98-157	Vergup	Uttara Kannada	Root	14°62′N	74°83′E
98-164	Sirsi	Uttara Kannada	Leaf	14°62′N	74°83′E
98-172	Sirsi	Uttara Kannada	Leaf	14°62′N	74°83′E
98-192	Sirsi	Uttara Kannada	Root/Soil	14°62′N	74°83′E
96-08	Bairumba	Uttara Kannada	Leaf	12°43′N	75°76′E
98-174	Isloor	Uttara Kananda	Leaf	14°62′N	74°83′E
98-176	Isloor	Uttara Kananda	Leaf	14°62′N	74°83′E
98-177	Rayarpet	Uttara Kananda	Root	14°62′N	74°83′E
98-182	Isloor	Uttara Kananda	Leaf	14º67′N	74º88′E
98-183	Yadally	Uttara Kananda	Leaf	14°62′N	74°83′E
98-184	Shelloor	Uttara Kannada	Leaf	14°62′N	74°83′E
98-185	Puttannamana	Uttara Kannada	Root, Stem	14°62′N	74°83′E
99-144	Pollibetta	Kodagu	Leaf	12°32′N	75°83′E
98-135	Sirsi	Uttara Kannada	Root & Soil	14°62′N	74°83′E
00-38	Siddapur	Uttara Kannada		14°20′N	74°55′E
00-42	Sirsi	Uttara Kannada		14°62′N	74°83′E
08-05	Koppa	Uttara Kannada	Collar	12°42′N	77°35′E
09-26	Aathur cross	Kodagu	Soil	12°42′N	77°35′E
09-37	Mudigere	Chikmangalur	Soil	13°12′N	75°63′E
09-38	Mudigere	Chikmangalur	Soil	13°12′N	75°63′E
09-39	Mudigere	Chikmangalur	Soil	13°12′N	75°63′E
09-40	Mudigere	Chikmangalur	Soil	13°12′N	75°63′E
98-07	Sakleshpur	Hassan	Root	12°95′N	75°79′E
09-41	Sakhleshpur	Hassan	Soil	12°95′N	75°79′E
09-42	Sakhleshpur	Hassan	Soil	12°95′N	75°79′E
09-43	Sakhleshpur	Hassan	Soil	12°95′N	75°79′E
99-188b	Gundanka	Hassan	Leaf	12°95′N	75°79′E
		C. TAMIL NADU STATE			
06-17	Yercaud	Salem	Leaf	11°48′N	78°13′E
97-55	Valparai	Coimbatore	Root	10°34′N	76°95′E
99-124	Gudallur	Nilgiris	Stem	11º49′N	76º49′E
99-132	Gudallur	Nilgiris	Stem	11º49′N	76º49′E
99-136	Gudallur	Nilgiris	Leaf	11º49′N	76º49′E
99-162	Pedavalana	Nilgiris	Leaf	11º49′N	76º49′E

DNA extraction

Phytophthora isolates were grown as stationary cultures in Ribeiro's liquid medium (Ribeiro 1978) for four days at room temperature for DNA extraction. Mycelia were harvested by filtration through sterile Whatman No.1 filter paper. Genomic DNA was extracted according to the Cooke and Duncan protocol (Cooke & Duncan 1997). Mycelia were ground in a mortar with extraction buffer. Extraction with phenol: chloroform: isoamyl alcohol (25:24:1, v/v) was then carried out. Precipitation of DNA was carried out with ice-cold isopropanol, and the pellet was washed with 70% ethanol and re-suspended in TE buffer. The purified DNA was quantified using biophotometer plus (Eppendorf, Germany).

SSR analysis

Twenty SSR primers designed by Schena *et al.* (2008) from whole genome of *P. sojae, P. ramorum and P. infestans* were used in the present study (Table 2). Amplification reactions were carried out in a 25 μL reaction containing 40ng of genomic DNA, 10 picomol of each primer (Sigma Genosys, Bangalore), 100 μM of dNTPs (Thermoscientific, USA), 1 unit of *Taq* polymerase (Promega Corporation, USA) in 1X PCR reaction buffer (Promega Corporation, USA) and 15mM $MgCl_2$. Amplification was performed in a programmable thermal cycler (Master Cycler Gradient S, Eppendorf, Germany) with the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 45 sec, primer annealing

Table 2. Set of microsatellite primers used in the present study

Locus	Repeat motif	Forward sequence	Reverse sequence	Ta
S1-S3	$(CCG)_7$; $(AGC)_4$; $(AGC)_{14}$	ACGACGTGTCCAAGAACCAC	ATGTTGACCGTGTTCTGCTG	55
S6-S7	(AAG)14	GGAGTTCGCCATCAACAACT	TCAGCTTCTGTCGRTCGAC	55
S16-S17	(AAG)15	TCTACGTGAATGCCATGAGG	CGTTCAGCTTCTGTCGATCR	55
S18-S19	(AGC)7; (AGG)13	YACCATCTCCAACCTGCTG	CACCACCTCGAGTAGCTCCC	58
S19-S20	(AGC)6	GGGAGCTACTCGAGGTGGG	TCGTCTCAATCTCKGACTGA	55
S23-S25	(AGG)7; (AAG)10; (GAG)4; (AAG)12; (GAG)5; (AGA)6	GACTCGGACTCGGACGAC	GACTCGGACTCGGACGAC	58
S27-S31	(AAG)4; (AGG)4; (ACG)4; (AGG)11	GAAGCGCGGGCGWGT	TCCTCCTCTTCTTCTTCGTCW	55
S31-S28	(GAG)4	WGACGAAGAAGAAGAGGAGGA	TCATTCATCAGCGTGTCRAT	57
S29-S30	(AGG)4; (AAG)11	MGCAAGAAGGCGTCGTA	CCTTCATCATGAGCTTCTGG	56
R1-R3	(ACC)4;) (ACC)5; (ACC)4	GYGGCGGTGGCTACAGYG	CTGCTGYTGCTGGTTGAAAG	58
R3-R4	(AGC)8	CTTTCAACCAGCARCAGCAG	GTTCATCATGCCWCCCATR	55
R4-R5	(AGC)4; (AGC)12; (AGC)4	YATGGGWGGCATGATGAAC	AGGACCAGGAGATGGAGGAC	55
R7-R9	(ACG)9; (AAC)5; (AGC)10	TGTTCCARACCCGCTTCC	GGAACGCACCAAAGACGC	55
R13-R14	(AAG)10	AAGTCGAAGCTCGTGGTSAC	GTATCCGCTGRAAGAGCGTC	55
R16-R17	(ATC)4; (AGG)8	CAAGAAGCCGCTCAACTACC	TAACGGATCAGCTCTTGCTG	55
I3-I4	(AAG)8	GCCTGTGGAYGAGAATGGYS	CAGATCCACGACACCRGGY	58
I5-I6	(AGC)5; (AGC)8	CATCAACAAGTGCTCGTWCS	TAGTCRAYGTTCTTGTTGTTCA	57
I7-I8	(AG)9	GHGTGGGCGAGTACTCCAAG	AAGCTGGCTATRWACACTGCCG	56
I11-I12	(ACC)8	TCGTCBGTGTCCTCBACGTC	ACCAGCATCTTRTTCTGRGCAG	55
I13-I14	(AAG)7; (AAG)4	GTCTGCGCTGTCGGAACT	TRATGATGCGGTTCATCTCG	55

at 55°C or 58°C for 45 sec, extension of annealed primer at 72°C for 1 min and a final extension at 72°C for 8 min. The amplification products were resolved on 3% metaphor agarose gel.

Data analysis

The polymorphic bands obtained with each primer pair was scored for their presence (1) or absence (0). Polymorphism information content (PIC) of each individual SSR allele was calculated according to the formula described by Tehrani *et al*. (2008): PIC = 2PiQi where Pi is the frequency of presence and Qi is the frequency of absence of a particular band. PIC values for all the polymorphic bands produced by a primer pair were averaged to calculate PIC value for a primer pair. From the data scoring matrix, a dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) using the software NTSYS-pc2.1 (Rohlf 2002). The genetic similarity matrix that was obtained from Dice similarity coefficient was subjected to principal coordinate analysis (PCA) to investigate the structure of the population. To determine support for the various clusters of the tree, bootstrapping was performed with 1,000 replications using the program PAST 3 (Hammer *et al*. 2001).

Results and discussion

The current study represents the first intensive analysis of genetic diversity of *Phytophthora* isolates associated with black pepper collected from various black pepper growing regions in South India. Twenty SSR primers were used to estimate the genetic diversity of 114 *Phytophthora* isolates from black pepper. The SSRs developed from *P. sojae*, *P. ramorum* and *P. infestans* genome could readily be transferred to *P. capsici*. The same sets of primers were tested on 16 different species of *Phytophthora* and successful cross species amplification could be obtained (Schena *et al*. 2008). Out of 25 genomic SSRs, twenty had successful PCR amplifications. Successful cross species amplification could be obtained with 9 out of 12 primers (75%) from *P. sojae*, 6 out of 7 primers (85.7%) from *P. ramorum* and 5 out of 6 primers (83.3%) from *P. infestans*. Among the 20 genomic SSR primers, five primers amplified more than three fragments. More than one loci could be amplified with most of the primers used. This may be due to the presence of multiple priming sites in the genome. Out of 20 primers, 15 generated polymorphic bands and 5 were monomorphic among the isolates tested. A total of 37 alleles were identified, of which 28 (75.67%) were polymorphic. The number of alleles ranged from 2 to 6 with an average of 3.6. The average PIC value ranged from 0.17 to 0.45. The highest average PIC value was exhibited by primers namely S27F-S31R and R16F and R17R. The primer I13F- I14R showed least value for PIC.

Table 3. Polymorphism information content (PIC) values for 15 polymorphic primers

Primer	PIC value range	Average PIC
S1F-S3R	0.23 – 0.47	0.35
S6F-S7R	0.25 – 0.33	0.29
S16F-S17R	0.32 – 0.47	0.39
S18F-S19R	0.03 – 0.49	0.33
S23F-S25R	0.03 – 0.49	0.30
S27F-S31R	0.45 – 0.46	0.45
S31F-S28R	0.22 – 0.49	0.41
S29F-S30R	0.02 – 0.48	0.22
R1F-R3R	0.24 – 0.5	0.43
R3F-R4R	0.13 – 0.39	0.26
R4F-R5R	0.19 – 0.41	0.30
R13F-R14R	0.05 – 0.49	0.36
R16F-R17R	0.43 – 0.47	0.45
I11F-I12R	0.29 – 0.49	0.38
I13F-I14R	0.08 – 0.25	0.17

Genotyping data that was obtained for all polymorphic alleles were used to estimate pair wise similarity comparisons among these isolates. The dendrogram generated showed that the similarity coefficient ranged from 0.57 to 0.95 and all the isolates could be separated (Fig. 1). The isolates were clustered into 7 groups. The 7th group comprised of a single isolate namely 07-01, a soil isolate from Kerala. In the present study, the genetic pattern of the *Phytophthora* population was not found to be associated with geographic origin. The lack of geographic clustering suggested a relatively recent expansion of a single diverse population.

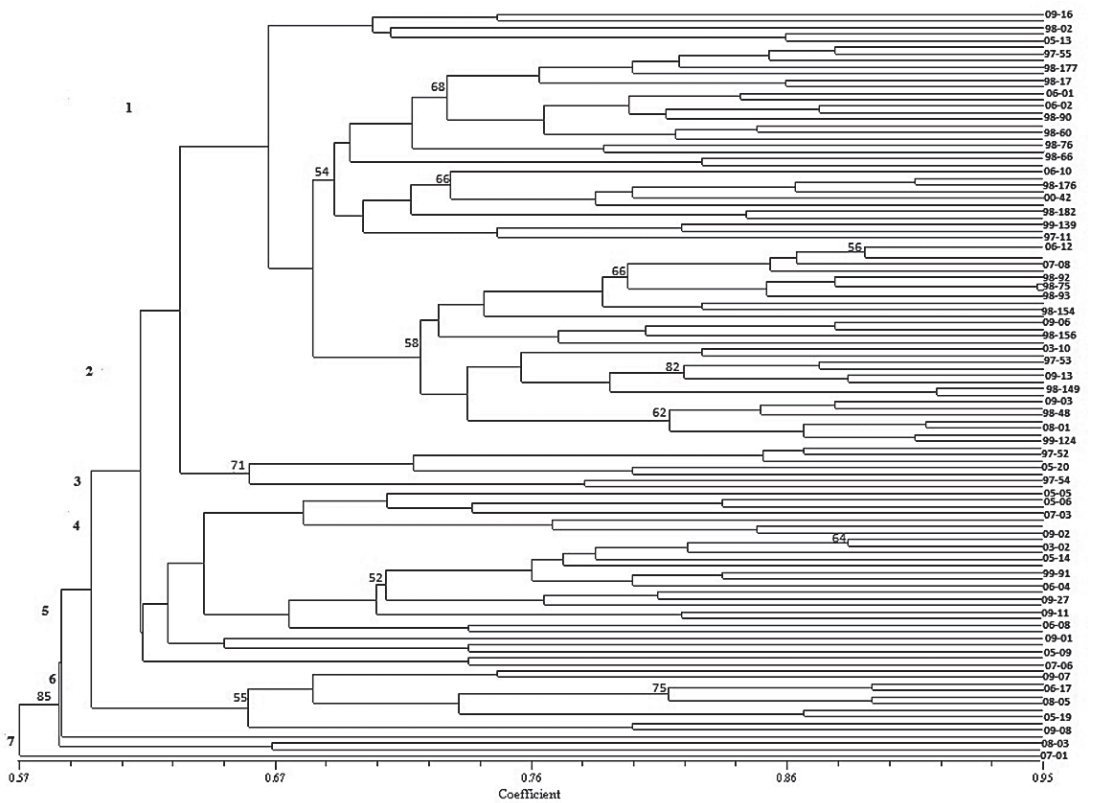

Fig. 1. Dendrogram depicting diversity and interrelationships among the *Phytophthora* isolates from black pepper with SSR markers. The X-axis shows percent similarity. The numbers at the nodes are the percentage of the trees above 50% that were supported by bootstrap analysis (1,000 replications).

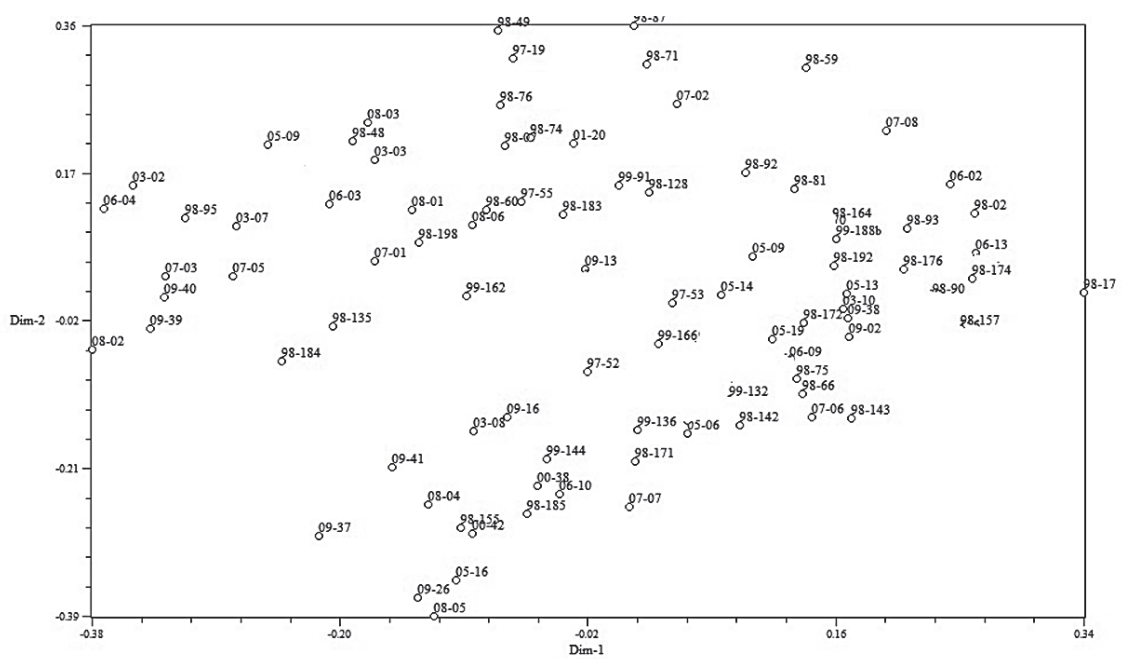

Fig. 2. Principal co-ordinate scatter plot of 114 *Phytophthora* isolates from black pepper

Note : Only representative isolates have been denoted both in dendrogram and scatter plot

In Vietnam, *Phytophthora capsici* isolates from black pepper were characterized by RAMS and REP fingerprinting and the overall genetic diversity was found to be low with most of the isolates belonging to one clonal group and isolates from different climatic regions could not be genetically differentiated (Truong *et al*. 2010). Previous studies on genetic diversity among isolates of *P. capsici* from capsicum found a high level of diversity but did not reveal any clear link between DNA pattern and geographic origin (Förster *et al*. 1990; Hwang *et al*. 1991). On the other hand, SSR markers could differentiate four *P. sojae* isolates from China based on geographical origin (Zhu *et al*. 2004). The SSR markers could detect the diversity existing among the *Phytophthora* isolates of black pepper. But these markers were not sufficiently powerful to discriminate the isolates on the basis of geographical origin. The three dimensional principal co-ordinate scatter plot (Fig. 2) constructed was in agreement with the UPGMA dendrogram.

This study of *Phytophthora* isolates from black pepper in India generated important data that will have implications for the management of the foot rot disease. The high level of genotypic diversity indicates that the pathogen has a great evolutionary potential that will enable it to adapt and overcome management strategies over time (McDonald & Linde 2002). The potential sources of genetic variation could be mitotic recombination and mutation.

It throws light on an important aspect for resistance breeding programs that multiple isolates should be chosen for screening for *Phytophthora* resistance in black pepper. The present study could contribute to a more comprehensive understanding of the genetic variation of *Phytophthora* isolates from black pepper. The study reveals the potential of microsatellite markers for the analysis of genetic diversity within *Phytophthora* populations.

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