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Original Article

Development and characterization of microsatellite markers from expressed sequence tags for analysing genetic diversity among *Phytophthora* isolates from black pepper

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ABSTRACT

Bioinformatic mining of 56,457 publicly available Expressed Sequence Tags of *Phytophthora capsici* in dbEST Genbank was performed to identify SSRs. A total of 223 SSRs was identified from 9831 UniGene sequences. The most and least frequent repeats were triniculeotides (69.50%) and dinucleotides (1.3%) respectively. Thirty six SSR primers designed from ESTs were used to analyse the diversity of 114 *Phytophthora* isolates from black pepper and twenty nine could be successfully amplified and of these 3 were polymorphic among the *Phytophthora* isolates. A total of 48 alleles were detected of which only 33.3% were polymorphic. The similarity coefficient values of the dendrogram ranged from 0.79 to 1.00. The isolates were clustered into two major groups. Clustering did not follow the pattern of geographical origin of the isolates or plant part from where the isolates were obtained.

Keywords: Black pepper, Expressed Sequence Tag, Foot rot, Genetic diversity, Microsatellites, *Phytophthora*.

INTRODUCTION

Black pepper is the dried berry of perennial climber *Piper nigrum* L. of the family Piperaceae and is a major spice with various applications in processing industries. Black pepper products include black pepper powder, pepper oil and pepper oleoresin. Black pepper as a climbing vine is well adapted to grow in mixed cropping system along with plantation crops. Crop losses due to foot rot caused by *Phytophthora capsici* are one of the major production constraints in black pepper cultivation. All parts of black pepper are prone to infection as such foliar, collar and root. The pathogen exhibited varying responses to different strategies of management employed. Genotypic diversity analysis of pathogen aids in understanding disease epidemiology, host-pathogen interaction and identification of races if any.

Microsatellites are molecular markers that consist of tandem repeats of one to six DNA base pairs which vary due to slippage of the DNA polymerase during replication or unequal crossing over resulting in differences in the copy number of the core nucleotide sequence (Yu and Kohel, 1999). Historically SSR markers were developed by generating a small-insert genomic library, performing hybridization with SSR oligonucleotides and sequencing candidate clones. However, this is quite time-consuming, costly, and labour intensive. Alternatively, SSRs can be identified in sequence databases and used for marker development (Morgante and Olivieri, 2003). With the availability of large numbers of expressed sequence tags (ESTs) and other DNA sequence data, development of SSR markers through data mining has become fast, efficient, and economical. Transcript sequences are a valuable source of informative molecular markers as they represent genes that are expressed in an organism. Coding sequences are generally more informative than anonymous markers because they allow for a more direct association between the molecular marker and the phenotype. This characteristic makes EST-SSRs easily transferable among related organisms (Herron *et al.*, 1998). Therefore, SSRs from transcript sequences have considerable potential for comparative mapping studies, as well as for analyses of genetic diversity within the expressed portions of the genome in which they are located. One hundred and eleven novel EST-SSR markers developed were useful for genetic variation study of *P. sojae* and its related species (Lin *et al.*, 2008). Eleven pairs of primers were designed and analysed on 63 isolates of *P. infestans* (Wang *et al.*, 2013).

The objective of the current study was to develop SSR markers from *P. capsici* EST database to analyse genetic diversity of *Phytophthora* isolates from black pepper.

MATERIALS AND METHODS

Origin of *Phytophthora* Isolates

One hundred and fourteen *Phytophthora* isolates from black pepper 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 that are maintained in the National Repository of Phytophthora at ICAR-IISR, Kozhikode, were used for genetic diversity analysis.

Primer Designing

A total of 56,457 ESTs of *P. capsici* was downloaded from dbEST of NCBI (National Centre for Biotechnology Information). EST assembly was used to cluster the ESTs into putative genes. Contig Assembly Program 3 (CAP3) (Huang and Madan 1999) with default parameters, was used to cluster the overlapping ESTs and a total of 9831 unigenes was identified. These unigenes were further explored for SSR sequences using MISA software (Thiel *et al.*, 2003). The criterion set for identification of minimum SSR length was determined by the number of repeats, which were (2/8) (3/5) (4/4) (5/3) (6/3) (the first digit refers to the SSR repeat type and the second to the minimum number of repeat units). Primers were designed using Primer3. Oligonucleotides of 18-22 nucleotides in length were designed to yield amplification products of 129-300 bp based on GC content of 40%-70%, optimum annealing temperature of 60°C (57.0°C-63.0°C), and primers' Tm difference (<2°C). Primers were synthesized by Sigma Genosys Bangalore, India.

DNA Isolation and Diversity Analysis

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 filtered through sterile Whatman No.1 filter paper. Genomic DNA was extracted according to the protocol (Cooke and Duncan, 1997). Mycelia were ground in a mortar with extraction buffer. Extraction with phenol: chloroform: isoamyl alcohol (25:24:1v/v) was then carried out. Precipitation was carried out with ice-cold isopropanol, and the pellet was washed with 70% ethanol and re-suspended in sterile double-distilled water. The purified DNA was quantified using Eppendorf biophotometer.

Twenty nine EST SSR primers developed were used to analyse the diversity of 114 *Phytophthora* isolates from black pepper. Amplification reactions were carried out in a 20 µl reaction containing 20 ng of genomic DNA, 10 picomol of each primer, 100 µM of dNTPs (Thermoscientific, USA), 1 unit of *Taq* polymerase (Promega, USA) in 1X PCR reaction buffer and 15mM $MgCl_2$. Amplification was performed in a programmable thermal cycler (Eppendorf Master Cycler Gradient S) with initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 45s, primer annealing at 55°C- 60°C for 45s, extension of annealed primer at 72°C for 1 min and a final extension at 72°C for 10 min. The amplification products were resolved on 3% metaphor agarose gel. The polymorphic bands obtained with each primer pair were scored for their presence (1) or absence (0). From the binary data matrix, a dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) with Dice's similarity coefficient (Nei and Li, 1979) using the software NTSYS-pc2.1 (Rohlf, 2000). To evaluate the reliability of the dendrogram bootstrap analysis was performed on the binary data set using 1000 permutations in Past 3 software (Hammer *et al.*, 2001).

RESULTS

A total of 223 SSRs were detected from 9831 Unigene sequences of *P. capsici* suggesting that only 2.3% of sequences contained SSRs. The SSRs were defined as <=8 bp dinucleotide repeats; <=5 bp tri- nucleotide repeats; <=4 bp tetra-nucleotide repeats; <=3 bp penta-nucleotide repeats and <=3 bp hexa-nucleotide repeats. The most frequent repeat type found within the sequence of *P. capsici* were trinucleotide repeats (69.5%) followed by hexa-nucleotide (13%), penta-nucleotide (11.2%), tetra-nucleotide (4.9%) and di-nucleotide repeats (1.3%) (Fig.1).

Observed frequency of different repeat types comprising the SSRs is given in Fig. 2. Among tri- nucleotide repeats,

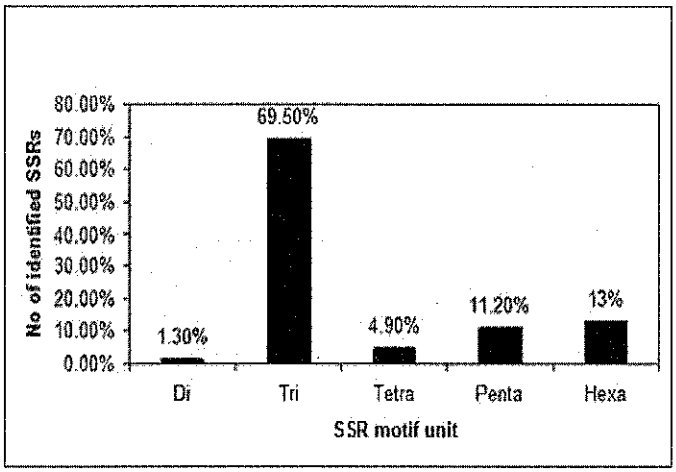

Fig. 1: Frequency distribution of repeat types identified in UniGene sequences of *P. capsici*

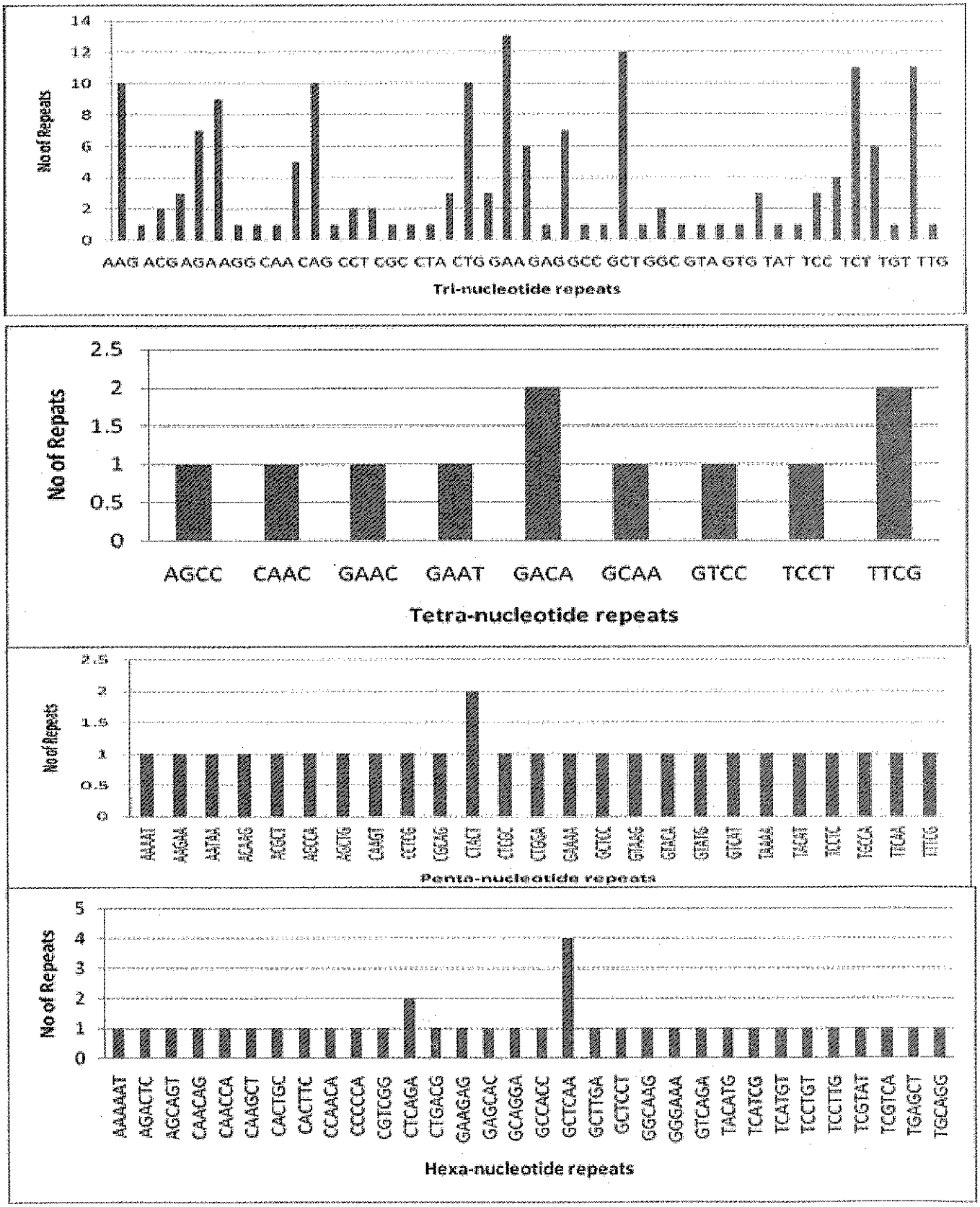

Fig. 2: Frequency distribution of tri-, tetra-, penta- and hexa-nucleotide repeat motifs

$(GAA)_n$ were more frequent whereas among tetra nucleotide repeats, $(GACA)_n$ and $(TTCG)_n$ showed relatively higher frequency, $(CTACT)_n$ were the most frequent repeat in penta- nucleotide repeat and $(GCTCAA)_n$ showed higher frequency in hexa nucleotide repeats. Out of 223 SSRs, primers could be designed only for 194 SSRs and remaining sequences did not produce any acceptable primers. Thirty six pairs of SSR primers were selected for SSR analysis.

Twenty nine EST primer pairs (Table 1) had successful PCR amplifications. Only three EST-SSR markers *viz,* PC32, PC33 and PC34 were polymorphic among the *Phytophthora* isolates. The primer PC 33 was the most polymorphic (Fig. 3). The 29 EST-SSRs generated 48 bands, of which sixteen (33.33%) were polymorphic. The sizes of the amplicons ranged from 150 to 350 bp. More than one loci was amplified with the three polymorphic primers. Genotyping data that were obtained for all polymorphic alleles were used to estimate pair wise similarity comparisons among these isolates. The similarity coefficient values of the dendrogram ranged from 0.79 to 1.0 indicating low level of genetic diversity (Fig.4). The isolates were clustered into two major groups with 92 isolates belonging to Group1. Clustering pattern was not found to be associated with geographical origin or plant part from where the isolates were obtained. EST SSRs could not detect the diversity existing among the *Phytophthora* isolates.

Table 1: Details of SSR primers designed from ESTs of *P. capsici* including flanking primer sequences, repeat motif, working annealing temperature (Ta) and expected amplicon size.

Locus	RepeatMotif	Forward Sequence	Reverse Sequence	Ta	Expected Product Size
PC1	(GCA)6	ATCATCTTGGCTGCACACAG	CGGTCTCTTACTCCCTGCAC	55	282
PC2	(TCT)6	CAGCAGCAGCAGTTTGAGTC	AGTCTCCGTTGGTGGAAAGA	55	277
PC3	(TCATGT)4	GTCTTGGCGATGTAGCCAGT	GGTGTTGGATCCTCTTCTCG	55	282
PC4	(CAC)6	CATCATGCCCACCAAGAAG	CTTCCACCTTCTCTGTCATCG	55	268
PC5	(TCG)6	GGCGAGAGTGACAAAGCAAT	CGGATCACACCATGACAGAA	55	298
PC10	(CGT)6	TAGGCGTGGAGCTCTTGAAC	GGAGACGCCTAGCAATTCTG	55	251
PC11	(CAG)6	GGAGGACGATCTGCTACTGG	CCACTGACTGCTGTTGCTGT	55	299
PC13	(TGC)9	CGCACAACATGGAACTACGA	GGCTCTTATGGCCAGCACTA	55	272
PC14	(GAA)6	CGTCGAGAAGCAAGACCTTC	CGTGCAGCTTAGCCATGATA	55	251
PC15	(GCT)7	GGTCGCGACACAGATGAGTA	GGACTTTAGGGGCAGCACTT	55	250
PC16	(GAA)6	GTGGCTGCATTACCATCCTT	AGTGCTGGTGGAACTGACCT	55	253
PC18	(AGC)8	CCGAGATTGCCAAGAAGAAG	CCGAGATTGCCAAGAAGAAG	55	263
PC19	(CTCAGA)4	GCGAGCATTTGACGTCTGTA	CTCTGTGAACACTCGCTTGC	55	262
PC20	(AAG)7	AAGAAGGCTGAGGAGGAGGA	GCACTGCACGTAGTCCTTCA	55	300
PC21	(CAG)6	CCTGAACGATGCCAGAAGAT	GACGCTACTGAGGCTGCACT	55	255
PC22	(GTG)8	CACACCGATGCTCTGTTGTC	GGAAGGATGCTCCGACTATG	55	250
PC23	(GCTCAA)4	AAGTGCTGGAAAGCGAGAAC	GCTTGTGCGTAGGCTTCC	55	259
PC24	(CTG)7	GGCTTCTCCTGCTGACTCTG	CGTCGCTACGAAGACTCGAC	55	260
PC25	(CTT)6	GCCGTTCCGACTGATGTATT	GGCGAGATTGAAGAGGAAGA	55	275
PC26	(GAA)10	GAGCTGATCGTTGAGAGCTG	GGTTCGGGTACTTGTCCTTG	55	274
PC27	(AGA)7	CAGCACCGAAGGAGAAAAAG	AGGTCTTCGTCGTCCATCAC	55	267
PC28	(AGC)6	TGAAGGAAAGGGACATCCAC	AGTGGCAGCTGCGTTCTTAG	55	299
PC29	(GCG)7	CCACACATCGACAAATGCTC	ACCGTCCCAAGTGAAGACAC	55	261
PC30	(ACT)8	TCGTAGAACTCAAGGGAACT	CTTGGCAGTCACACTCTCA	53	211
PC31	(AGC)8	CCGAGATTGCCAAGAAGAAG	TCAGTTCCGATGACAGGTT	54	247
PC32	(CT)10	AACGAGGCAACGTCCATA	TCGTCTCAATCCCTGCTGT	55	194
PC33	(CTG)6	CTTGCGTTACTGCCGTCCAT	GTGCAGCACAGAAGAAGACCAT	55	196
PC34	(GAA)8	GTCAAGTTTATGGATGGCAGTG	TCGACCTTAGAAGCTGGTGC	55	194
PC35	(GCA)11	TACCGAGTATTTGAACGC	TCGACCTTAGAAGCTGGTGC	50	129

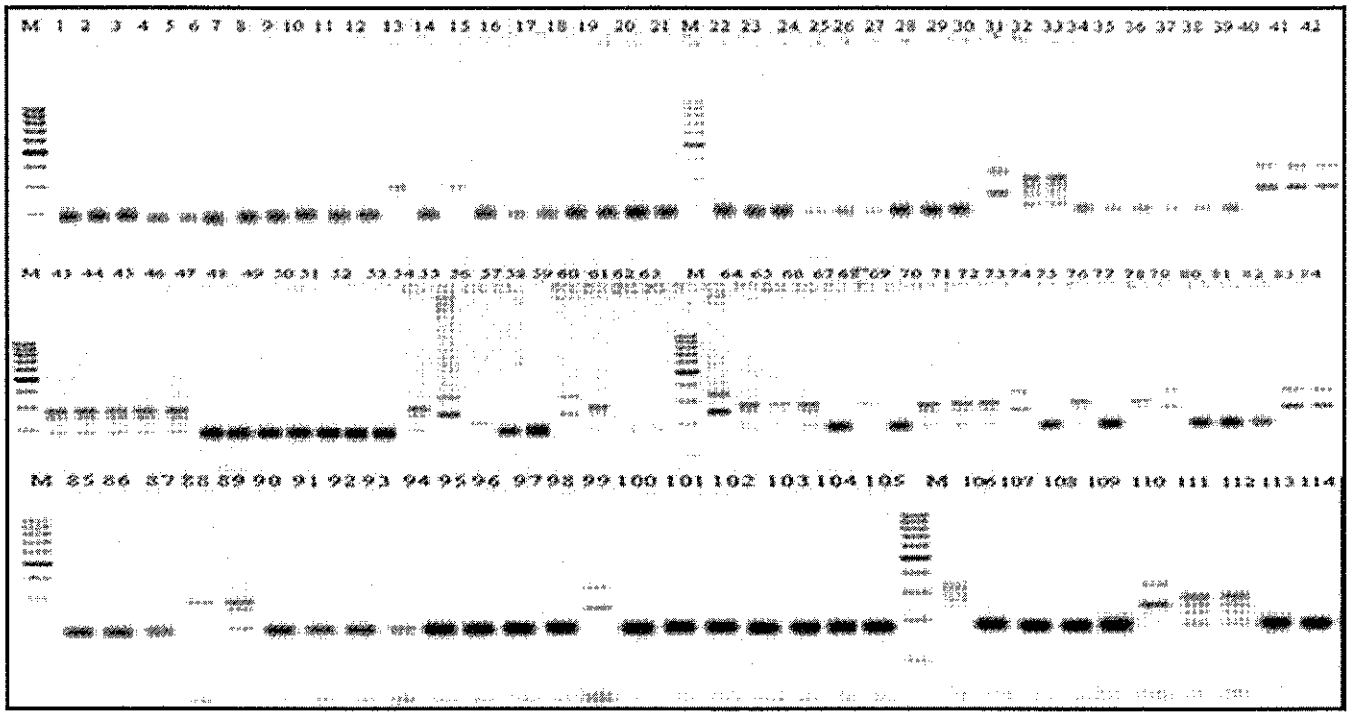

Fig. 3: SSR analysis with EST SSR primer PC33F-PC33R M: 100 bp ladder; 1-114: *Phytophthora* isolates from black pepper.

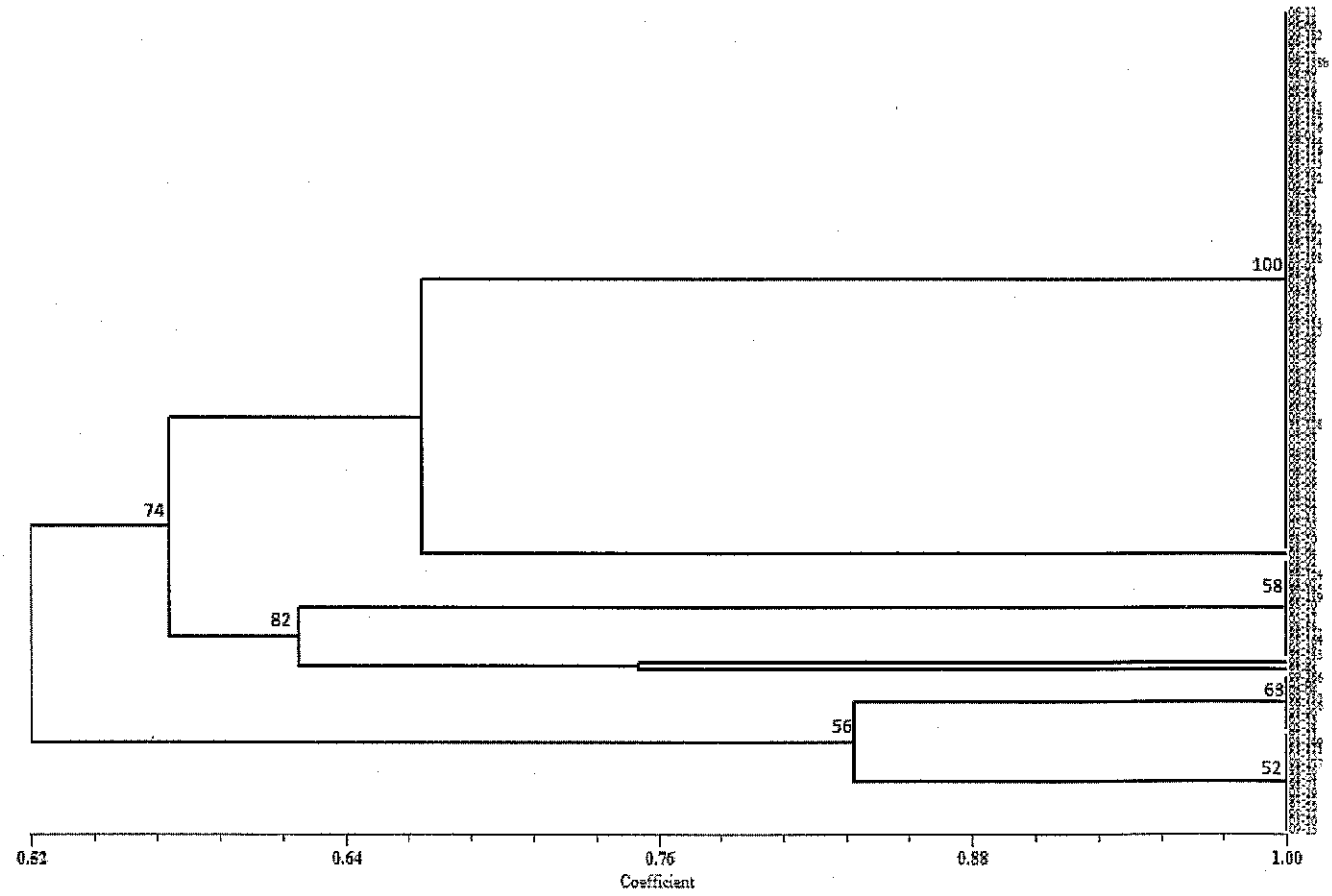

Fig. 4: Dendrogram depicting diversity and interrelationships among the *Phytophthora* isolates from black pepper with EST SSR markers.

DISCUSSION

The current study represents the first intensive study of genetic diversity analysis of *Phytophthora* isolates associated with black pepper collected from various black pepper growing regions in South India with EST-SSR markers. Nine thousand eight hundred and thirty one unigene sequences from *P. capsici* EST database were searched and 223 of them (2.3%) were found to contain SSRs. In case of *P. sojae,* out of five thousand and eight hundred ESTs 369 (6.4%) ESTs were found to contain SSRs (Zhu *et al.,* 2004). The frequency of ESTs containing SSRs in *P. capsici* ESTs was lower than that of *P. sojae.* The trinucleotide motifs were the most abundant type of SSRs found in the database (70.0%). This is in agreement with previous reports on various plants (Cardle *et al.,* 2000; Kantety *et al.,* 2002; Gao *et al.,* 2003). This dominance of trimeric SSRs over di-, tetra-, and pentameric SSRs may be due to the suppression of non-trimeric SSRs in coding regions owing to the risk of frameshift mutations that may occur when those microsatellites change in length in one unit (Rungis *et al.,* 2004). The fact that repeat numbers of trinucleotide microsatellites can alter without changing the reading frame of the messenger RNA can also be a reason for the dominance. Higher number of possible trinucleotide combinations than for dinucleotide repeats can also contribute to its dominance. Mutation pressure and positive selection for specific single amino acid stretches could account for the doubled frequency of tri-nucleotide repeats relative to mono- and dinucleotide repeats in the genes of plant species (Morgante *et al.,* 2002).

The lack of amplification of a few primers may be due to the primer pair encompassing a long intron producing a PCR product that could not be visualized on the electrophoretic profile or sequence errors or problems during primer synthesis (Nicot *et al.,* 2004). Multiple loci amplification may be attributed to the presence of multiple priming sites in the genome.

The EST SSRs showed lower level of polymorphism. This result is in agreement with the statement of other workers that EST SSR markers exhibit lower polymorphism and is less efficient in distinguishing closely related individuals when compared to that of genomic SSR markers (Cho *et al.,* 2000; Scott *et al.,* 2000; Chabane *et al.,* 2005). This may be due to sequence conservation of the coding regions and narrow coverage of genic-SSRs within the genome. Lower mutation rate of coding sequences could also contribute to low level of polymorphism.

Low genotypic diversity was observed among the *P. ramorum* isolates in North America with microsatellite markers (Prospero *et al.,* 2005). In the present study the there was no significant relationship between genetic pattern of the *Phytophthora* population and location. The lack of geographic clustering implies a relatively recent expansion of a single diverse population. The genetic diversity of *Phytophthora capsici* isolates from black pepper was low 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 did not reveal any clear correlation between DNA pattern and geographic origin (Forster *et al.,* 1990; Hwang *et al.,* 1991). The SSR markers derived from expressed sequences could not reveal the diversity among the population studied.

Twenty nine functional microsatellite markers were developed and three polymorphic markers could be added to the available repository of microsatellite markers. The markers developed in the present study may be useful for cross species amplification and transferability to other *Phytophthora* species.

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