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Short communication

Novel EST-SSR marker development and validation in black pepper cultivars and varieties

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Abstract

Expressed Sequence Tags-Simple Sequence Repeat (EST-SSR) markers are more preferred because it is more efficient, fast and low cost. The main objective of the study was to develop new EST-SSR markers in black pepper. Sixty eight unigene sequences containing 70 SSRs were detected from 1048 unigenes using MIcroSAtellite (MISA) identification tool. Sixty two mononucleotides, two dinucleotides and six trinucleotides were the repeat motifs obtained. Nine primer pairs were designed for eight unigene sequences. Primers were screened using DNA isolated from Panniyur-1 variety at different annealing temperature. Primers which are clear and unambiguous were selected for validation in 35 black pepper varieties and cultivars. In total, 18 alleles were obtained from five EST-SSR markers with an average of 3.6.

Keywords: Black pepper, Cultivars, EST-SSR markers, Genotypes

Black pepper (*Piper nigrum* L), the king of spices belonging to the family Piperaceae is a perennial climbing vine and a native of the tropical forests of Western Ghats, South India. It is economically valuable because of the berries which are extensively used as a spice and has medicinal value. In black pepper, most of the genomic analysis reports are based on RAPD markers (Pradeepkumar et al., 2003; George et al., 2005; Sen et al., 2010), AFLP markers (Joy et al., 2007) and ISSR markers (Jiang and Liu, 2011; Sheeja et al., 2013).

Among the array of molecular markers Simple Sequence Repeats (SSRs) are considered as the favourite marker due to their hypervariability, co-dominance, mendelian inheritance, multiallelism, reproducibility, good genome coverage, chromosome specific location, high throughput genotyping and amenability to automation (Parida et al., 2009). SSRs, referred to as microsatellites, are a class of DNA sequences consisting of simple motifs of one to six nucleotides that are repeated in tandem up to a few dozen times per site (Litt and Luty, 1989). The variation in the repeating units results in the polymorphic bands which are detected by Polymerase Chain Reaction (PCR) with the help of locus specific flanking region primers (Farooq and Azam, 2002). The major limitation of SSR markers is that its development depends on sequence information. However, after the advent of Next Generation Sequencing (NGS) methods, genomic or Expressed sequence tags (EST) data are available for many crops.

Nine genomic SSRs were used for characterizing twenty clonal varieties of black pepper and four species of *Piper* (Menezes et al., 2009). Genetic diversity among 40 genotypes of black pepper and four species of *Piper* was analyzed using seven genomic SSR markers (Joy et al., 2011). Eleven SSR markers were used to distinguish *P. colubrinum*, *P. nigrum* and their corresponding hybrids (Jagtap et

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al., 2016). The reason for the limited number of SSR marker is the lack of sufficient sequence information in black pepper. This can be tackled by EST databases as it is easy to be obtained than the complete genome sequence. The aim of the present study was to develop new SSR markers from EST sequences and validation of the developed EST-SSR markers in black pepper varieties and cultivars for genetic relationship among genotypes and variety identification.

Data mining for SSR markers

SSRs were detected from 1048 unigenes using microsatellite motif identification program, MISA tool (http://pgrc.ipk-gatersleben.de/misa/). Specification given for selecting the microsatellite includes minimum repeat length of ten for mononucleotide, six for dinucleotide, five for trinucleotide, tetranuleotide, pentanucleotide and hexanucleotide. The maximum number of bases interrupting two SSRs in a compound microsatellite was given as 100.

Sequence specific forward and reverse primers were designed from 5'end and 3' end of the flanking region of repeat region of eight unigene sequences. Primers were designed using Oligo Calc: Oligonucleotide Properties Calculator (basic.northwestern.edu/biotools/ Oligo Calc. html) based on the following criteria: (1) primer length ranging from 18-23 bp with 20 base pair as the optimum (2) product size ranging from 100-400 bp (3) melting temperature (Tm) between 53ºC and 63ºC; (4) a GC content between 40% - 70%. Eight forward primers and nine reverse primers were designed for eight unigene sequences (Table 1).

Plant materials, Genomic DNA extraction and PCR

A total of 35 *P. nigrum* genotypes including cultivars and varieties were included in the study. The plant genomic DNA was isolated from young leaf tissue (first or second leaf from the tip) from all the genotypes using modified Cetyl trimethyl ammonium bromide (CTAB) extraction method (Doyle, 1987). The PCR reaction mix was set up with the components: 1X PCR Buffer with $MgCl_2$, 200 µM dNTPs, 0.05 µM each forward and reverse primers, 1U Taq polymerase and 50 ng μl^{-1} DNA template. The final volume of the PCR mix was 25 µl. The PCR conditions were as follows: initial

Table 1. EST-SSR primer combinations designed from selected unigene sequences of black pepper

Primer name	F/R	Primer sequence(5'—3')	Length (bp)	GC %	Tm ^{O}C	Amplicon size (bp)	Sequence details
PNS1	F1	TATTTGCATCCCGGAGCGCAT	21	52	61.2	212	$(TGG)_8$
	R1	GAGCTTCAAGAGACAACAATGG	22	45	60.1		
PNS2	F2	CCATCCAAGGTCAATGCAGAATC	23	48	62.9	200	$(TCA)_5$
	R2	CCACATCCAACTTTATTCTTCC	22	41	58.4		
PNS3	F3	GGTGAAGAAGGAGGAAGTAGT	21	48	59.5	201	$(AGG)_6$
	R3	GATCATGCGTTTTACACAGGG	21	48	59.5		
PNS4a	F4	GGGCACGGTACCAGAGGA	18	67	60.8	146	$(GCG)_5$
	R4a	GCAGACACAACATAGATCCC	20	50	58.4		
PNS4b	R4b	CAGATCACTATTCCACAACC	20	45	56.4	252	
PNS5	F5	TCGCGGCCAGTACACTACAA	20	55	60.5	188	$(CAC)_5$
	R5	CATCGTTTAGGGAAGCTAGGC	21	52	61.2		
PNS6	F6	CGACAATGCGACGAAACAAATA	22	41	58.4	224	$(T)_{10}$CCCG AGCC
	R6	CAAATGGCTTGATCGAGATGA	21	43	57.5		$(AT)_7$
PNS7	F7	ATTCCTTCAGGCCAATCCTTCA	22	45	60.1	182	$(TA)_9$
	R7	GTATAAACGGTCCAATGTAGTC	22	41	58.4		
PNS8	F8	AACTGACTGTCCACGGCTTTCT	22	50	62.1	201	$(TCT)_5$
	R8	TCAGTCCTATGTGATCGCAAC	21	48	59.5		

denaturation at 94º C for 2 minutes, 40 cycles of denaturation at 92º C for 1 minute, annealing at 52º C-56º C for 1 minute, and extension at 72º C for 1 minute and with a final elongation step at 72º C for 10 minutes. PCR products were resolved on 4 per cent agarose gel and were run at 80 V. Allele sizes were compared with 50 bp ladder.

Characteristics of SSRs from EST in P. nigrum

Totally 1048 unigene sequences having a total length of 518179 bp (approximately 0.5 Mb) were examined for detecting the SSRs. Seventy SSRs were detected from sixty eight unigene sequences, which accounts for 6.49 per cent of all the sequences. Analysis of the nucleotide sequences containing SSR revealed that mononucleotides were most abundant, represented by 88.6 per cent among all the identified SSRs. Other types of SSR including dinucleotides and trinucleotides were accounted for 2.8 per cent and 8.6 per cent respectively. Similar results were also found in castor bean in which occurrence rate of mononucleotides was high (37.51 per cent) followed by trinucleotide repeats (34.63 per cent) and dinucleotide repeats (25.61 per cent) (Zhou et al., 2012). In a recent study on rubber also, mononucleotides were the dominant (38.89%) repeat found, among the entire repeat types (Hou et al., 2017). Totally 12 types of repeat motifs were observed in EST-SSR sequences including four types of mononucleotide repeats, two types of dinucleotide repeats and six types of trinucleotide repeats. $(T)_{10}$ CCCGAGCC$(AT)_7$ and $(C)_{10}$ G$(C)_{10}$ were the two compound SSRs found in the sequence. The number of SSR repeat motif was in the range of 5 to 12. The trinucleotide repeats AGG, CAC, GCG, TCA and TCT were repeated for five times. TGG was repeated for eight times and dinucleotide repeats, AT and TA was repeated for seven and nine times respectively. Highest repeat motif was for the mononucleotide $(A)_{12}$ (Table 2)

Screening of EST-SSR primers

Nine sets of primers designed from eight unigene sequences that contain dinucleotide repeat, trinucleotide repeat and compound repeat were screened using DNA of Panniyur-1 at different annealing temperature. Amplification was strong at 56.1º C for PNS1, PNS4a and PNS8, 54.8º C for PNS3 and 53.4º C for PNS6. Based on the clarity and specificity of amplification, five primers *viz*., PNS1, PNS3, PNS4a, PNS6 and PNS8 were selected for further validation. The amplicons produced by PNS2 and PNS5 were faint and not of the expected size in PNS2. Amplification was not

Table 2. Features and frequencies of identified SSR motifs

Type of SSR	Repeat motif	Number of repeats	Total	Grand total	Frequency (%)
Mononucleotide	$(A)_{10}$	21	28	62	88.6
	$(A)_{11}$	6			
	$(A)_{12}$	1			
	$(C)_{11}$	3	7		
	$(C)_{10}$	4			
	$(G)_{10}$	1	1		
	$(T)_{11}$	6	26		
	$(T)_{10}$	20			
Dinucleotide	$(TA)_9$	1	1	2	2.8
	$(AT)_7$	1	1		
Trinucleotide	$(TGG)_8$	1	1	6	8.6
	$(TCA)_5$	1	1		
	$(AGG)_6$	1	1		
	$(GCG)_5$	1	1		
	$(CAC)_5$	1	1		
	$(TCT)_5$	1	1		

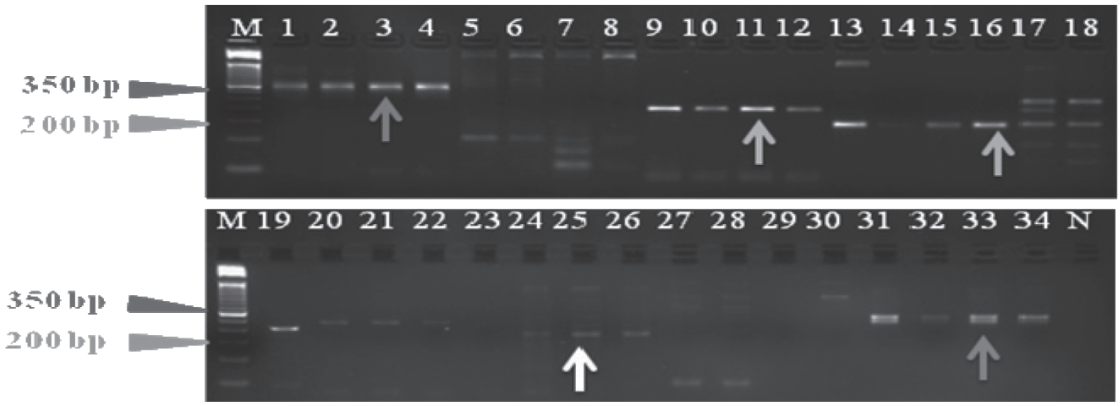

Plate 1: Screening of EST-SSR primers

M- 50 bp ladder N- Negative control

PNS1 at	(1) 53.4ºC	(2) 54.8ºC	(3) 56.1ºC	(4) 57.1ºC
PNS2 at	(5) 49.6ºC	(6) 50.7ºC	(7) 53.4ºC	(8) 54.8ºC
PNS3 at	(9) 52.0ºC	(10) 53.4ºC	(11) 54.8ºC	(12) 56.1ºC
PNS4a at	(13) 52.0ºC	(14) 53.4ºC	(15) 54.8ºC	(16) 56.1ºC
PNS4b at	(17) 49.6ºC	(18) 50.7ºC	(19) 53.4ºC	
PNS5 at	(20) 53.4ºC	(21) 54.8ºC	(22) 56.1ºC	(23) 57.1ºC
PNS6 at	(24) 52.0ºC	(25) 53.4ºC	(26) 54.8ºC	
PNS7 at	(27) 49.6ºC	(28) 50.7ºC	(29) 52.0ºC	(30) 54.8ºC
PNS8 at	(31) 53.4ºC	(32) 54.8ºC	(33) 56.1ºC	(34) 58ºC

observed in the primer PNS7. PNS4a and PNS4b are the primer sets for the same region and good amplification was given by PNS4a. The annealing temperature of each primer was standardized based on the intensity of bands (Plate 1)

Validation of EST-SSR primers

Five primers among nine designed primers were used for validation in thirty five genotypes comprising of black pepper varieties and cultivars (Plate 2). The PCR products were run on 4 per cent agarose gel to view the amplification and to detect polymorphism, if any. All the five primers successfully amplified the target region in different genotypes. Polymorphic as well as monomorphic bands were obtained and in most of varieties and cultivars, monomorphism was observed. Two amplicons in Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-5, Panniyur-9, Arka Coorg Excel, Malamundi and TMB-4 showed heterozygosity at that particular locus (Plate 2). Totally, 18 alleles were obtained from five EST-SSR markers. The average number of alleles per locus was 3.6.

The present study was undertaken with the objective of developing EST-SSR markers in black pepper and validation of those markers in different genotypes. These markers can be used for studying the genetic relationship among the genotypes. Traits associated with markers can be identified and use it for marker assisted selection. These markers can be used for developing genetic maps in black pepper.

Acknowledgements

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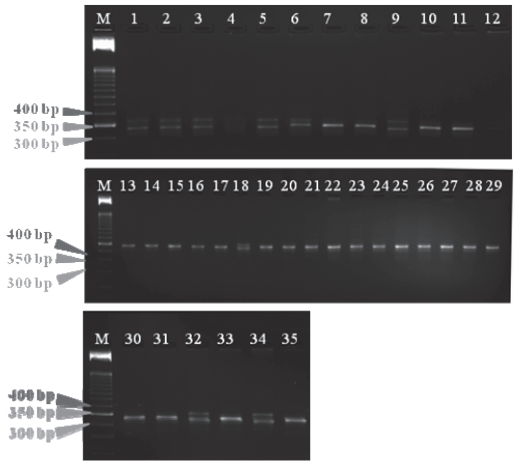

M- 50 bp ladder, 1- Panniyur-1, 2- Panniyur-2, 3- Panniyur-3, 4- Panniyur-4, 5- Panniyur-5, 6- Panniyur-6, 7- Panniyur-7, 8- Panniyur-8, 9- Vijay, 10- Panchami, 11- Pournami, 12- Sreekara, 13- Subhakara, 14- IISR Thevam, 15- IISR Sakthi, 16- IISR Girimunda, 17- IISR Malabar Excel, 18- Arka Coorg Excel, 19- PLD-2, 20- Karimunda, 21- Paalikodi, 22- Aimpiriyan, 23- Narayakodi, 24- Vadakkan, 25- Thevanmundi, 26- Arakulam munda, 27- Kottanadan, 28- Arivally, 29- Balankotta, 30- Kalluvally, 31- Kuthiravally, 32- Malamundi, 33- Karivilanchi, 34- TMB-2, 35- TMB-4

Plate 2. Validation of PNS1 marker in 35 genotypes

References

Doyle, J.J. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.

Farooq, S. and Azam, F. 2002. Molecular markers in plant breeding-I: Concepts and characterization. Pak. J. Biol. Sci., 5(10): 1135-1140.

George, K.J., Ganga, G., Varam, R.S., Sasikumar, B. and Saji, K.V. 2005. Identification of hybrids in black pepper (*Piper nigrum* L.) using male parent specific RAPD markers. Curr. Sci., 88(2): 216-218.

Hou, B., Feng, S. and Wu, Y. 2017. Systemic identification of *Hevea brasiliensis* EST-SSR markers and primer screening. J. Nucleic acids 2017. Available: http://dx.doi.org/ 10.1155/2017/6590902 [16 May 2017].

Jagtap, A.B., Sujatha, R., Nazeem, P.A., Meena, O.P. and Pathania, S. 2016. Morpho-molecular characterization of putative interspecific crosses in black pepper (*Piper nigrum* L. and *Piper colubrinum*). Plant Omics. J., 9(1): 73-80.

Jiang, Y. and Liu, J.P. 2011. Analysis of genetic diversity of *Piper* spp. in Hainan Island (China) using inter-simple sequence repeat ISSR markers. Afr. J. Biotechnol., 10(66): 14731-14737.

Joy, N., Abraham, Z. and Soniya, E.V. 2007. A preliminary assessment of genetic relationship among agronomically important cultivars of black pepper. BMC Genet., 8:42-48.

Joy, N., Prasanth, V.P., and Soniya, E.V. 2011. Microsatellite based analysis of genetic diversity of popular black pepper genotypes in South India. Genetica., 139(8): 1033-1044.

Litt, M. and Luty, J.A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet., 44: 397- 401.

Menezes, I.C., Cidade, F.W., Souza, A.P. and Sampaio, I.C. 2009. Isolation and characterization of microsatellite loci in the black pepper, *Piper nigrum* L. (piperaceae). Conserv. Genet. Resour., 1(1): 209-212.

Parida, S.K., Kalia, S.K., Sunita, K., Dalal, V., Hemaprabha, G., Selvi, A., Pandit, A., Singh, A., Gaikwad, K., Sharma, T.R., Srivastava, P.S., Singh, N.K. and Mohapatra, T. 2009. Informative genomic microsatellite markers for efficient genotyping applications in sugarcane. Theor. Appl. Genet., 118: 327-338.

Pradeepkumar, T., Karihaloo, J.L., Archak, S. and Baldev, A. 2003. Analysis of genetic diversity in *Piper nigrum* L. using RAPD markers. Genetic Resources Crop Evol., 50(5): 469-475.

Sen, S., Skaria, R. and Muneer, P.M.A. 2010. Genetic diversity analysis in *Piper* species (Piperaceae) using RAPD markers. Mol. Biotechnol., 46: 72-79.

Sheeja, T.E., Uma, G., Sasikumar, B., Saji, K.V. and Rahul, P. R. 2013. Genetic diversity study in *Piper* spp. using inters simple sequence repeat (ISSR) markers. J. Spices aromat. Crop, 22(2): 111-119.

Zhou, M. L., Xia, Z. Q., Chen, J. R., and Wang, W.Q. 2012. Analysis of SSR information and annotation in EST resource of Castor Bean (*Ricinus communis*). Chinese J. Trop. Crop, 33(12): 2138–2143.