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Original Research Paper

SSR marker development in *Abelmoschus esculentus* (L.) Moench using transcriptome sequencing and genetic diversity studies

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ABSTRACT

Okra [*Abelmoschus esculentus* (L.) Moench] also known as bhindi or lady's finger is an important vegetable crop in India, West Africa, South Africa, Brazil, USA and Turkey. It belongs to the family Malvaceae. Okra is mainly grown in tropics and subtropics of the world. The studies regarding the molecular marker development are very limited; still there is no SSR marker development from comprehensive transcriptome data in this crop. This study presents the first comprehensive transcriptome data, using RNA from different parts of okra such as root, stem, leaf, bud, flower, different stages of developing pod and from twenty days old plantlets of heat, drought and salt stressed. A total of 10,492 SSRs were identified in this study. Among these tri repeats (2112) were found to be predominant followed by di (1285), tetra (149), penta (24) and hexa repeats. Thirty-four SSRs were standardized for PCR and screened in 36 okra genotypes and accessions. Among these, 18 SSR primers were found to be highly polymorphic with the PIC values more than 0.5. And the overall results of analysis showed that expected heterozygosity ranged from 0.125 to 0.971 with a mean of 0.593; the values for observed heterozygosity ranged from 0.000 to 0.839 with the mean of 0.203; the number of allele per locus ranged from 1 to 30 and the polymorphic information content (PIC) ranged from 0.119 to 0.955 with the mean value of 0.554. The genic SSR markers developed will help in germplasm characterization mapping, genetic diversity studies, molecular assisted breeding and also in gene discovery.

Key words: *Abelmoschus esculentus,* microsatellite markers, next generation sequencing, RNA sequencing and transcriptome

INTRODUCTION

Okra [*Abelmoschus esculentus* (L.) Moench] also known as bhendi or lady's finger is an important vegetable crop in India, West Africa, South Africa, Brazil, USA and Turkey. It belongs to the family Malvaceae and is mainly grown in tropics and subtropics of the world (Priyavathi *et al*. 2018). The total okra production in the world was found to be 9.8 Million-ton pods with an area of around 2.0 million ha and in India it is 6.1 Million ton with an area of around 5.14 lakhs ha followed by Nigeria (FAOSTAT, 2018).

The chromosome number is reported variously for this species as 2n=130 and also 2n=72, invariably the chromosome number was found to be 2n=130 with the genome size of 1.6 Gb (Joshi and Hardas 1956). It was reported that there are two kinds of *A. esculentus* L. as diploid 2n=60-70 and as a tetraploids 2n=120-130, this could be due to irregularities in the chromosome movement during the cell division of mitotic phase (Nwangburuka *et al*. 2011). Further this polyploidy level was assessed through the chloroplast DNA (cpDNA) intronic spacer and revealed that *A. esculentus* are the closest relatives of two wild species that is *A. ficulneus* and *A. moschatus* (Ramya and Bhat 2012).

Molecular markers have paved way for the assessment of genetic variations and genetic relationships among and within the species (Chakravarthi and Naravaneni 2006; Yuan *et al*. 2014, 2015). Molecular marker techniques like RFLP, RAPD, AFLP and SSR are

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widely used for genetic characterization and crop improvement (Sawadogo *et al*. 2009). Especially in the less researched species, transcriptome analysis plays a vital role for the development of molecular markers (Strickler *et al*. 2012). Recently, the first report on genomic SSR marker in okra were developed using Next-Generation Sequencing technology (NGS) which was used for the assessment of genetic relatedness and cross species transferability (Ravishankar *et al*. 2018). SSR markers play a key role in many applications of plant genetics and breeding due to its codominant inheritance, multi-allelic nature, high reproducibility and good genome coverage (Bertini *et al*. 2006). There are some studies reported on SSR developed using transcriptome through NGS in okra (Schafleitner *et al*. 2013; Zhang *et al*. 2017) and transcriptome data on *M. balbisiana* and *M. acuminate* ssp. using illumina GA II X technology (Ravishankar *et al*. 2015). With the advent of sequencing technology, RNA sequencing has become an efficient and convenient technique for the SSR detection (Ronoh *et al*. 2018; Xu *et al*. 2017). However, these studies used transcriptome from one or very few tissues, which may not completely cover genic SSRs in the okra genome. Keeping this in view in this study, we present the first comprehensive characterization of combined okra transcriptome from root, stem, leaf, bud and flower, different stages of developing pods and from the abiotic stressed plantlets (drought, heat and salt). Here we also report SSR markers which would greatly help in mapping genes and linkage map development.

Table 1. Genotypes and the accessions used in the study

Genotypes /Accessions	
1. Pule Vimukha	19. IC-0282266
2. Azad bendi	20. IC-0128903
3. Punjab 7	21. IC-0128885
4. Kashi Kranthi	22. IC-0085595
5. Varsha Upahar	23. IC-0397980
6. Parbhani Kranthi	24. IC-0282296
7. Kashi Leela	25. IC-0282232
8. Pusa Sawani	26. IC-0128891
9. Shakthi	27. IC-0069242
10. Punjab Padmini	28. IC-0433743
11. Azad Bendi 3	29. IC-0069302
12. Kashi Vibhuthi	30. IC-0433628
13. IC-0600808	31. IC-0043750
14. IC-0602363	32. IC-0600832
15. IC-0128888	33. IC-0397271
16. IC-0282274	34. IC-0560493
17. IC-0469655	35. IC-0282233
18. IC-0043752	36. IC-0600256

MATERIALS AND METHODS

Plant material and DNA isolation

Thirty-six okra genotypes including, a few varieties from germplasm collection were used in this study (Table 1). Young leaves were collected from the okra plants which were maintained at Indian Council Of Agricultural Research- Indian Institute of Horticultural Research Bengaluru India (ICAR-IIHR), and the total genomic DNA was isolated by using the modified CTAB method (Ravishankar *et al*. 2000) with the repetition of chloroform: isoamylalcohol (24:1) for three to five times till the mucilage was removed. Finally. sDNA concentration was determined using Nano drop (NABI micro digital) by taking the absorption at 260 and 280nm.

RNA isolation and Sequencing

For the transcriptome sequencing we isolated RNA from tissues of root, stem, leaf, bud, flower, different stages of developing pod and from twenty days old seedlings were stressed for heat (40^0C for 4h), salt (200mM NaCl) for two days and drought (five days of dehydration) of accession IIHR-299 using by Trizol method where 30 mg of the sample were ground into fine powder using liquid nitrogen and 1ml of Trizol (TAKARA BIO INC. Japan) was added to it and centrifuged at 12,500 rpm for 20 min, to the supernatant equal amount of chloroform was added and centrifuged at 12,500 rpm for 15 min and equal amount of isopropanol was added to the supernatant and precipitated at -80^0C for 1hr followed by centrifugation at 12,500 rpm for 15 min and the pellet was washed using 75% ethanol and dried pellet was dissolved using DEPC water and the RNA integrity was examined by gel electrophoresis. RNA purity was examined using Nano drop (NABI micro digital) and the equal amount of RNA from each samples were pooled and sent for RNA sequencing.

Sequencing, quality control and *de novo* assembly

RNAseq was done at Sandoor Speciality Diagnostics Pvt. Ltd. Hyderabad facility using Illumina Hiseq

platform following manufactures instructions. Paired end cDNA library are from the pooled sample (root, stem, leaf, bud, flower, different parts of developing pods, drought stress, heat stress and salt stress plantlets) to get comprehensive okra transcriptome. Then Quality control were carried out to filter out the adaptors low quality reads >20% of bases and the unknown nucleotides with >5% reads. The clean reads was used for calculating the proportion of nucleotides with quality value larger than 20 (Q20). *De-novo* assembly was done using Trinity software assembly with the default parameters for generating contigs and transcripts (Grabherr *et al*. 2011). The NGS data was submitted to NCBI (SRR 13451946).

Mining of SSR primer and designing

The assembled unigenes were further examined for the presence of microsatellites using MISA software (Suping *et al.* 2013). A total of 10492 SSR Primers were identified and 2532 SSR primers were designed using Primer 3.0 software (Untergasser *et al*. 2012). A total of 51 SSR primers were randomly selected and these were used for PCR standardization and amplification of 36 okra genotypes.

PCR conditions and genotyping

For the amplification of mined SSRs, fluorescent based M13 tailed PCR assay was performed (Oetting *et al.* 1995). And all the primers at 5' end were labelled with standard M13 tail (Schuelke 2000). A total of 51 SSR markers were initially synthesised and screened with pooled okra DNA. Further the primers which amplified, clear bands were screened over 36 okra genotypes. The PCR conditions employed are as follows initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation, annealing and polymerization steps (94°C for 30s, 50-60°C and 72°C for 1 min) and a final extension of 72°C for 8 min. PCR amplification was carried out in 20 μL volume containing 75-100 ng of okra DNA, 2 μL of 10x Taq Buffer (Tris pH with 15mM $MgCl_2$), 1.5 μL of $MgCl_2$ (25mM of $MgCl_2$), 0.5μL of dNTPs (10mM), 0.5 μL of forward primer M13 tail (5 pM), 1 μL of reverse primer M13 tail (5 pM), 0.5 μL of probes FAM,VIC, NED and PET (5 pM), 0.2 μL of *Taq* polymerase (5 units per μL) (Genei. Pvt. Ltd Bengaluru) and 9.8 μL of nuclease free water. All the PCR reactions were carried out using Bio-RAD Thermal cycler (Bio-RAD, US). The amplified PCR products were separated on ABI3730 Genetic Analyzer (Applied Biosystem, USA), at M/S Eurofins facility Bengaluru. The obtained data were further analysed using Peak Scanner software (Applied Biosystems, USA) for determining the exact fragment size in base pair.

Statistical analysis

The fragment size in base pair of the PCR products were used for calculating the expected heterozygosity (He), observed heterozygosity (Ho), polymorphic information content (PIC) and number of alleles per locus employing Cervus 3.0 software (Kalinowski *et al*. 2007). And the dendrogram analysis was performed using neighbour-joining method (NJ) employing Darwin Software (Perrier *et al*. 2003; http://darwin.cirad.fr/darwin).

RESULTS

Sequencing and *de novo* assembly

A total of 3.8Gb raw data were obtained using Illumina-Hiseq platform from comprehensive okra transcriptome analysis (root, stem, leaf, bud, flower, different parts of developing pods, drought stress, heat stress and salt stress plantlets). Quality control analysis was performed in order to filter out the reads containing adaptors, low quality reads and the unknown nucleotides. The total number of generated transcripts was 112597 with maximum transcripts length of 20701bp and minimum transcripts length of 201bp and total length of transcripts generated was 72314062bp. The size distributions of the transcripts are given in the Table 2. And the sequencing analysis of GC content was found to be 47.1% and AT content as 53.9%.

Table 2. *De novo* assembly statistics

Transcriptome assembly		
Transcripts Generated	:	112597
Maximum Transcript Length	:	20701bp
Minimum Transcript Length	:	201bp
Average Transcript Length	:	642.2bp
Total Transcripts Length	:	72314062bp
Transcripts > 100 bp	:	112597
Transcripts > 500 bp	:	47905
Transcripts > 1 Kbp	:	21670
Transcripts > 10 Kbp	:	722369251
Number of reads used		
Total number of reads	:	24885138
Percentage of reads used	:	89.9%

Assembly statistics and designing primers

All the obtained unigenes were screened for identification of SSRs using MISA software. The total number of examined sequence was 112597 with a total of 72314062bp and the total number of identified SSRs was found to be 10492 and the number of SSR designed using Primer 3.0 software were 2532 (Untergasser *et al*. 2012). Number of SSR containing sequence were 9849 and the number of sequence containing more than one SSR is 568 and the number of SSR present in compound formation is found to be 783 (Table 3). Further the identified SSRs were screened for di, tri, tetra, penta and hexa nucleotide repeat motifs (a total of 1285 di-repeats, 2112 tri-repeats, 149 tetra-repeats, 24 penta-repeats, 9 hexa-repeats and 783 complex repeats were observed). Tri-repeats were found to be more predominant class of microsatellite than any other classes like di, tetra, penta and hex- repeats (Fig 1). The kind of repeats observed in high frequency among tri was found to be (AGT)10 and (CCA)10 and di-repeats as (TA)22 and tetra as (TTTC)18 and among hexa all repeats were found to present once.

Table 3. Assembly statistics of SSRs

Total number of sequences examined	112597
Total size of examined sequences (bp)	72314062
Total number of identified SSRs	10492
Number of SSR containing sequences	9849
Number of sequences containing more than 1 SSR	568
Number of SSRs present in compound formation	783

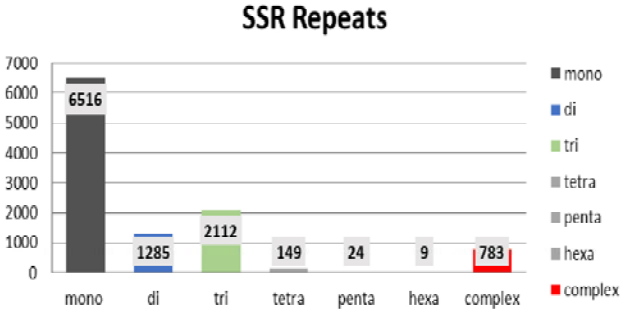

Fig. 1. Distribution to different repeat type classes of SSR repeats

Genetic analysis

The allelic data regarding the expected heterozygosity, observed heterozygosity and number of alleles per locus were examined using Cervus 3.0 software. And the values for expected heterozygosity ranged from 0.125 to 0.971 with the mean value of 0.593; the values for observed heterozygosity ranged from 0.000 to 0.839 with a mean value of 0.203; the number of allele per locus ranged from 1 to 30 and the polymorphic information content (PIC) ranged from 0.119 to 0.955 with the mean PIC value of 0.554 and PI (Probability of identity) values ranged from 0.0036 to 1.0000 with a mean value of 0.263 (Table 4). Dendrogram analysis showed that the genotypes used in the study were classified into three major clusters (Fig 2).

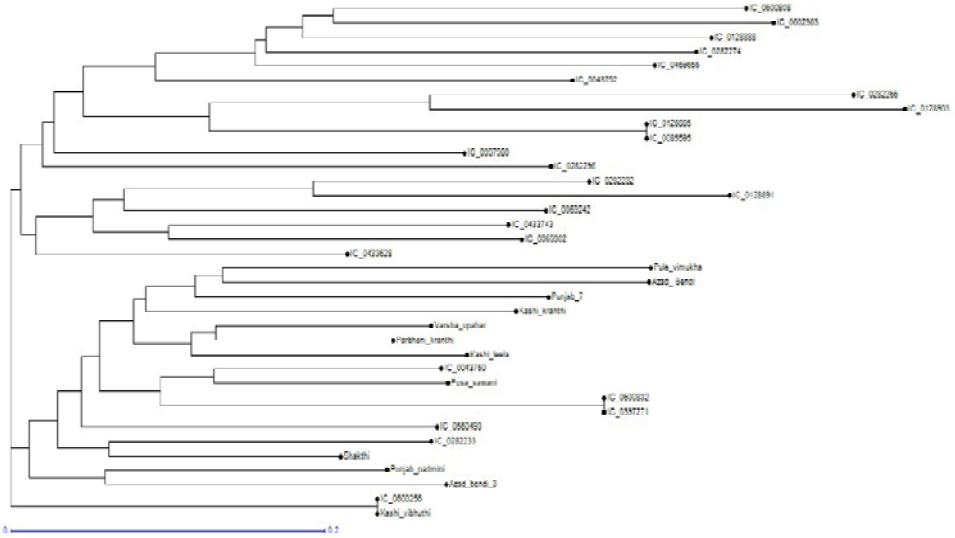

Fig. 2. Dendrogram analysis showing the genetic relationship among *Abelmoschus esculentus* L. accessions using transcriptome SSR marker data

Table 4. Genetic analysis of microsatellite loci using 34 SSRs

Sl. No.	Primer name	Primers	*T*m	Allele size	No. of Allele/locus	Ho	He	PIC value	PI
1	IIHR-2434	F: AGCTTCCGTATATTTTGGATT R: CCAAACTATCCAACTATGCTT	55	160	18	0.156	0.884	0.861	0.0265
2	IIHR-1877	F: TGAGATTCGTTTGATCGTTTA R: CTTTGGGTCAAAGCTGTC	55	151	4	0.200	0.444	0.408	0.3464
3	IIHR-817	F: TAAATATGCTTCTCAGGCATT R: CGTCTTGTTACGATTTATATGC	55	163	1	0.000	0.324	0.307	1.0000
4	IIHR-518	F: TCCCTCGTACTAGATCATTCA R: GTAACAAGGATGAGCAAAAGA	55	150	5	0.143	0.508	0.457	0.2935
5	IIHR-205	F: GGGAAGATTTTGCTAAACTTATT R: CCAATAGGATGTCTCAGTCAA	57	151	5	0.200	0.414	0.386	0.3727
6	IIHR-91	F: TGATCTTCGATTCATCCTTAT R: AGAATGGCAGCGCCAAAAG	55	151	3	0.030	0.287	0.250	0.5472
7	IIHR-30	F: TAAAATTTTCCCATCAATCC R: GGTGTTTGTTTTGTGGTGATA	60	172	4	0.094	0.424	0.371	0.3861
8	IIHR-18	F: TCTCTTTAAAATCACCGCTAA R: TTTAGCAAGGAAGGGAGAA	57	152	19	0.152	0.908	0.887	0.0187
9	IIHR-353	F: TAAAAATCAGAGCCTTCCTTT R: CAGATTTCTGAGAGCAAAGAG	55	174	6	0.457	0.701	0.644	0.1429
10	IIHR-343	F: GATATGGGATGGTTGAAATC R: GAGAAAACCAACGGATGAT	57	152	5	0.171	0.472	0.439	0.3122
11	IIHR-328	F: TAGGAAAACTACAGCAAGGATT R: GGACTTGGTTCTGCAATCT	60	150	3	0.000	0.125	0.119	0.7731
12	IIHR-319	F: GCACTTGATATTGCATTACATT R: CCAAATCATTATCAGGGAGT	55	150	3	0.156	0.347	0.311	0.4641
13	IIHR-303	F: TAGGAGGACAATCACAGAAAA R: GGTAACCCAAGTGTTGTTCTT	57	151	22	0.176	0.921	0.902	0.0139
14	IIHR-277	F: GCTCAAGTAAGCATTAAAACAG R: GTCGTGCAAAACTTGTCTAAG	55	162	11	0.000	0.869	0.839	0.0370
15	IIHR-267	F: TAAGGAGTCCAAACTCCAACT R: TGGTTGTTTAGGTTCCAATTT	55	160	11	0.313	0.760	0.724	0.0881
16	IIHR-254	F: TGTCTGTAGTCTCGCAACTTT R: ATACATTGACGGTACAAGTGG	57	152	13	0.794	0.679	0.620	0.1590
17	IIHR-244	F: TGGGGCCTAAGTAAATACAAT R: AAAGTTAGTTCAATGCAGTTTTC	57	180	11	0.030	0.759	0.732	0.0792
18	IIHR-221	F: ACAGGTCCATAAATGCTATGA R: CCCTAATATTATTGTTTTTACCC	58	161	7	0.000	0.673	0.605	0.1713
19	IIHR-195	F: TCACTTAACCCCATGAAAAAT R: GTTTCTGAGAATCCTTGCTG	55	158	28	0.324	0.957	0.940	0.0060
20	IIHR-165	F: GGATGACCAAAACGAAGTG R: CTGTCATTTTCTTTCCTTCTG	57	151	2	0.000	0.507	0.375	0.3752
21	IIHR-154	F: CGCCGTAGTACCTCAATCTT R: GCAATTAACGGTGACGAC	55	153	30	0.333	0.971	0.955	0.0036
22	IIHR-99	F: TGAAAAGAACATGAAAGCCTA R: CCTTCCTTCCTAGTCATCATC	57	160	15	0.156	0.742	0.707	0.0958
23	IIHR-94	F: TATATTTGCAGCATTTGTCTGT R: AACAGTCGGTACTTAGACAGC	57	151	18	0.545	0.891	0.870	0.0228
24	IIHR-68	F: GAACTTTTGGAATTTGTGTCA R: TTCTTGGAGTAGGAGCTTGAT	60	153	11	0.061	0.822	0.791	0.0543
25	IIHR-50	F: GTTCAGGATCAGAGTCGAG R: GCGGCCTCAATATTCACT	55	150	8	0.032	0.589	0.544	0.2118
26	IIHR-36	F: GGGACAGAGTTGAAAATGAC R: GGATCAGGAATGTTATCGACT	55	150	7	0.065	0.396	0.377	0.3856
27	IIHR-27	F: GGAACTCCGGTGGAGAAG R: AAGCTTTATCTCAAAAATCC	57	150	7	0.188	0.624	0.589	0.1738

28	IIHR-11	F: TGGAAGAGAAGAAGAACAACA R: TTCACGATGAACTGACC	55	151	6	0.645	0.556	0.478	0.2741
29	IIHR-02	F: AACAACAACAACAACAGTCG R: CATAAAAAGTGTTTGCGTCTC	55	158	18	0.147	0.879	0.855	0.0295
30	IIHR-1463	F: TGACGATCTTCACAGGCTAGTA R: AAGTGAACCAGGTAGCATGT	57	153	4	0.219	0.584	0.521	0.2342
31	IIHR-1506	F: TTGAAACTCCCACTATCAAAA R: TAATTATGGAGGTGGAGGTG	55	150	4	0.839	0.543	0.447	0.3042
32	IIHR-1896	F: CAATGCCAGATTTCTTTGTAG R: TTCCTTGCTTTAGTTTTCCTT	55	163	3	0.029	0.140	0.132	0.7494
33	IIHR-1835	F: CCATTATATCTTATCCGTTCG R: CATACACGTCAAAAACATCAA	55	214	5	0.286	0.505	0.467	0.2825
34	IIHR-1680	F: GGTGGCAACATTATCCAT R: GGAGGTGGCTATAACAGAAAT	55	168	3	0.031	0.294	0.256	0.5386
	Mean			**9.412**		**0.2037**	**0.5933**	**0.5546**	**0.2639**

DISCUSSION

Okra is an important vegetable crop in India, Africa and other Asian countries and is considered as a minor crop at the genome studies until recently, very little attention was paid towards its genetic improvement and generation of genomic resources. The studies regarding the molecular marker development are very limited, and there is a still no comprehensive transcriptome data for this crop. This study presents the first comprehensive transcriptome data from different parts of okra such as from root, stem, leaf, bud, flower, different stages of developing pod and from twenty days old plantlets of heat, drought and salt stressed by RNA sequencing. RNA sequencing is considered as an effective way for obtaining the gene sequences of a non-model crop (Strickler *et al*. 2012) and for developing the SSR markers (Zhang *et al*., 2010; Guo *et al*., 2016& Ravishankar *et al*. 2015). The application and development of molecular marker technology as it detects the genetic differences at the DNA level and is commonly used in the evaluation of genetic diversity and mapping (Yoder *et al*. 2018; Niemandt *et al*. 2018 & Pan *et al*. 2017). SSRs are considered as an important marker for application in plant genetics and breeding studies, because of its high reproducibility, codominant, multi allelic nature and good genome coverage. Genic SSRs developed from transcriptome data are highly useful as they reflect functional variability.

On an average 112,597 unigenes were with an maximum length of 20,701 were obtained through comprehensive okra transcriptome which was little lesser than a study on combined leaf and pod transcriptome of okra which yielded a total of 150,000 unigenes (Schafleitner *et al*. 2013) and higher than studies on okra by NGS using RNA sequencing from the leaf samples which yielded a total 66,382 assembled unigenes (Priyavathi *et al*. 2018); 94,769 unigenes with an length of 1921bp were obtained through NGS of transcriptome sequencing in okra to drought stress (Shi *et al*. 2020) and 293971 unigenes with okra transcriptome sequencing of five organs (roots, stem, leaves, flower and fruits) (Zhang *et al*. 2018). In our present study though a large number of unigenes have been produced compound than a study by (Schafleitner *et al*. 2013) and higher than the other studies which indicates that the sequencing depth was not sufficient to represent the whole transcriptome. Deeper and increased sequencing would have reduced the redundancy of unigenes annotation. However, redundancy at certain level is also due to the allopolyploid of *Abelmoschus*, where the transcripts from different genomes with slightly different sequences are present in the transcriptome (Schafleitner *et al*. 2013).

The number of SSRs identified in this study are 10, 492 which is high compared to earlier studies on *Abelmoschus esculentus* 9,574 (Priyavathi *et al*. 2018) and *Clerodendrum trichotomum* which is 6,444 (Chen *et al*. 2019) and among the mined SSRs the tri repeats (2112) are more predominant followed by di (1285), tetra (149), penta (24) and hexa (9) this pattern is similar in earlier studies in *Abelmoschus esculentus* (Shi *et al*. 2020; Priyavathi *et al*. 2018 & Schafleitner *et al*. 2013). The frequency of tri repeats are higher in transcriptome sequencing (Schafleitner *et al*. 2013) because of the shortening or the extension of amino acid in proteins may not cause much alteration in

functions and other type of repeats cause frame shift mutation. But the mechanisms behind the evolution and origin of microsatellite repeats are not very clear. So, the relative dominant occurrence of repeats motifs may be due to their evolution through various selection pressures. It was assumed that replication slippage and unequal crossing over are the few common mutation mechanisms which might come addition or removal of motifs leading to the variation in length (Buschiazzo and Gemmell 2006; Sonah *et al*. 2011).

In the present study, we successfully identified 10,492 SSRs and 34 SSRs were standardized for PCR and screened over 36 okra genotypes and accessions. Among these 18 SSR primers were found to be highly polymorphic with the PIC values more than 0.5. And the overall statistical analysis revealed that expected heterozygosity ranged from 0.125 to 0.971 with the mean 0.593; the values for observed heterozygosity ranged from 0.000 to 0.839 with the mean of 0.203; the number of allele per locus ranged from 1 to 30 and the Polymorphic Information Content (PIC) ranged from 0.119 to 0.955 with the mean value of 0.554. Similar kind of work were performed on okra reported Polymorphic information content (PIC) across all 50 loci values ranged from 0.000 to 0.865 with a mean value of 0.519. The observed and expected heterozygosity ranged from 0.000 to 0.750, and 0.000 to 0.972, respectively. Alleles per locus ranged from 1 to 27 (Ravishankar *et al*. 2018). A study for the development and characterization of SSR in cotton with the mean PIC of 0.65 (John *et al*., 2012) and a genetic diversity in cotton with the mean PIC of 0.8 (Muhammad *et al*. 2013). Dendrogram analysis depicted that all the genotypes used in the study were classified into three major clusters with most of the accessions grouped to cluster I, and the Cluster II & III with the mixture of genotypes and accessions. The SSR markers developed here will help in genetic diversity studies, mapping, marker assisted breeding and helpful in gene discovery. Being gene based SSR markers, these markers would be great help in tagging genes for various traits.

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