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Assessment of molecular divergence in sesame (*Sesamum indicum* l.) genotypes using microsatellite (SSR) markers

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Abstract
An investigation was carried out to detect the molecular diversity among 45 genotypes of sesame using 50 microsatellite markers. A considerable amount of genetic diversity was observed among the sesame genotypes under consideration. Out of 50 SSR markers screened, 30 markers (60.0 %) displayed clear and repeatable polymorphic bands for the analysis of 45 sesame genotypes. The polymorphism information content (PIC) value of SSR loci ranged from 0.5209 to 0.9128 with mean of 0.3940. NTsys analysis clustered the 45 genotypes into six main clusters forming the cluster VI as the largest one comprising of 32 genotypes. The genotype, N18- 8316 was clustered separately, which indicates its genetic isolation from other genotypes. Cluster I (IC-41945, IS-469-1-84-A) and cluster IV (FRP-8351-B, DS-37) were with two genotypes each indicating the relative closeness of those genotypes within the cluster. Further, cluster V was with five genotypes and cluster II was having three genotypes respectively. The study indicates that the molecular analysis of sesame using SSRs could aid in future population genetic structure studies and breeding programs of sesame.

Keywords: sesame, SSR markers, molecular diversity, PIC value

Introduction
Sesame is an important source of edible oil widely cultivated over the world for more than 5000 years and it is largely used as one of the ingredients in food products especially in bakery foods and animal feed. Despite being the first oilseed crop known to human, and its wide importance, sesame is an unexploited and neglected crop with a great economic potential. Measuring genetic variation is very useful for selective breeding, conservation of populations and/or rapid domestication of any crop plants. Genetic diversity can be studied by using various methods such as morphological, biochemical and molecular markers.

Several studies based on morphological markers have showed a high genetic diversity in sesame populations (Arriel *et al.* 2007) [2]. However, morphological markers have limitations in their ability to estimate genetic diversity because of strong influence from environmental factors. Tabatabaei *et al*. (2011) [13] reported that diversity analyses based only on morphological characters are prone to environmental bias due to environmental influences and complex genetic structure of different morphological traits. Genetic markers are the essential tools for quick detection and characterization of genetic variation. A number of DNA based markers have been developed to identify genetic variability within species. One of the recent advances in molecular genetics is the introduction of microsatellite markers to identify the genetic diversity of natural and hybrid population of crops. They are very useful for applications in plant breeding because of their reproducibility, high genome coverage, co-dominant inheritance, transferability to close species, multiallelic nature and relative abundance. Therefore, SSR markers may offer a particular advantage to investigate genetic relationships within accessions of sesame. In this context, the present investigation was aimed to find out the molecular diversity among sesame genotypes using microsatellite markers and to analyze their genetic relationships for further breeding programmes.

Material And Methods
Plant Material
The material for the present study consists of 45 genotypes and was sown in the experimental farm at Seed Research and Technology Centre, Rajendranagar, Hyderabad. The lists of genotypes used in the study are given in table 1.

Table 1: List of genotypes used in the study

S. No.	Name of the genotype	S. No.	Name of the genotype	S. No.	Name of the genotype
1	Nirmala	16	IC-205071	31	IS-644-A
2	Smarak	17	KMR-43-A	32	IC-310438-B
3	Krishna	18	CT-60	33	IS-112
4	TMV-7	19	FRP-8351-B	34	JULANG Sesame
5	RT-54	20	IS-476	35	KMS-4-323-B
6	Rama	21	IS-195	36	SI-241
7	Chandana	22	NI8-8316	37	IC-310438-B
8	DS-1	23	CT-40	38	EC-208652
9	PKDS-11	24	IS-54039-B	39	IS-469-1-84-A
10	Savitri	25	NIC-16220	40	FFAT-10-20
11	Guatama	26	DS-37	41	IS-54034-B
12	IC-56196	27	AT-238	42	Gowri
13	IC-205439	28	KMR-38	43	Madhavi
14	IC-41945	29	ES-33477	44	Rajeshwari
15	IC-205311	30	Kanpur local	45	Swetha Til

DNA extraction

Fresh leaves from twenty days old seedlings were used for DNA isolation. Five to ten grams of apical young leaves of each germplasm accession were collected. Approximately 150-200 mg of leaf tissue taken in a pestle and mortar and ground into a fine powder and total genomic DNA was isolated following CTAB-DNA extraction protocol modified according to Fulton *et al.* (1995) [7]. DNA isolated was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20 ˚C until use. DNA concentration was determined using Nanodrop and the purity was confirmed by electrophoresis on 1% agarose gel.

SSR primers and PCR conditions

A total of 50 SSR primer pairs were used for PCR amplification. The sequences of the primers are given in Table 2. The annealing temperature of SSR primer pairs was standardized using temperature gradient PCR (Eppendorf, Mastercycler). PCR was carried out in a reaction volume of 10 µl containing 1 µl PCR buffer (100 mM Tris– HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 100 µM dNTP mix, 0.5 µl of each of forward and reverse primer, 1U Taq Polymerase, and 2 µl of template DNA. The amplification was carried out using the PCR programme: 94 ˚C for 10 min, then 35 cycles each of 94 ˚C for 45 s, 50- 55 ˚C for 45 s, and 72 ˚C for 1 min and a final extension of 72 ˚C for 10 min. The details are given in table number 3.

Table 2: Detail of SSR primers used for the study

S. No.	Primer ID	Primer sequence	Annealing temperature (˚C)
	SEM 32	TTCCAGTACCGATCCTCACC (F) AAAATCTGCCAAATAAACCAAAA (R)	50
	SEM 38	ACAGCACTTACCCCAAAGGA (F) TGGGAGGCAACTTTCATTCT (R)	51
	SEM 44	TGCCTTTACAAATGGCTTCA (F) CCCATGAACCCATATCCTTG (R)	50
	SEM 62	CGAAAGAAGAGGCAGAGGTG (F) TCTCCGACCATCAAAACCAT (R)	52
	SEM 64	TCCATTCCTCTCATCCTCAA (F) CTGTGTCCGATCACCAAAAA (R)	50
	SEM 74	CTAGGAATGTCGGAGGCGTA (F) AATCCGAAACGTTGGCACT (R)	52
	SEM 76	GCTTCTGCGCTTTTACATCC (F) TTCTTACCCGCTGCCCTAAT (R)	52
	SEM 83	TTGCATCAGGAGATCCAACA (F) CACTCAAAGCAAACCAGCAA (R)	50
	SEM 90	AGGACAAGATCCACGGTGAG (F) TCCCTTATTTGCAAGCAACC (R)	52
	SEM 100	CCAACTTTTCTGGGTTGGAA (F) ATGGGCGTATCAGTTTCGAC (R)	51
	SEM 104	CAAACCTCACTGGTCTTCGAT (F) CCCGGATTGTCAAAGTCATT (R)	52
	SEM 138	TCCCCCAAATTTCACAAAAA (F) AGAGTAGGTTGCGCTCCTCA (R)	51
	SEM 146	AGGCTGGAGTCCATTGAGAA (F) TTACTTGGACCACCACAAAAA (R)	51
	SEM 260	AACGACATCACTTCGATCCAT (F) TGCTGACTTTCTTCCCGTTA (R)	50
	SEM 265	GCTCATGGACTACCCTCACG (F) AATTCGTCGACACTGTGGTG (R)	53
	SEM 270	TGAGAGGAATTGGATTGGAAA(F) GTGGGGAATGAGGAAATGTG (R)	51
	SEM 279	GAATTGAGAAAAAGAAAATGTTTGAA (F) AACGTTGAAGGTCCAACCAG (R)	55
	SEM 282	CTGGGGAAGGAAGTGGTGTA (F) TTGCAGAAGCCTTAACAGCA (R)	52
	SEM 285	GGATTCCGACTGTTTCCAGA (F) ATTCACGCAACTCTCCCTCT (R)	52
	SEM 315	CACTTACAGGGCTCCTTGAATC (F) GGAGAGAACAAAGACAGACACG (R)	55
	SEM 326	CCACAGGAATTCCGACACTT (F) CCTTTCCCTCGAAGATCACA (R)	52
	SEM 396	TTCTGTGGCACTCGTAGTCG (F) TAGGCATTGCCAATTTGTGA (R)	52
	SEM 428	CAAGAAAAGGCCACAGAGGA (F) CAACACAAACTCGACAGCACA (R)	52
	SEM 430	CACGGAAGCAGCTCATCAT (F) TCTGGCTGCTCAACAAGAAA (R)	51
	SEM 435	CAACCAAATCAACACCAACG (F) CGTCGCTTGCACATACAAAT (R)	50
	SEM 436	TTCTGTGGCACTCGTAGTCG (F) CTGTCGCCTTTGCTTTTACC (R)	52
	ZM 1	GTTTCTTGGTCTTATCACAGC (F) TACCAACGTCACTCTTCTTTC(R)	52
	ZM 2	CTTCTTGAAGTTCTGGTGTTG(F) ATTCTTGGAGAAAGAGTGAGG(R)	52
	ZM 3	ATCACCACACACTGACACAG (F) CGTGTCTGAGAATCCAATATC(R)	52
	ZM 4	TCCAGAGAGGAGACAATAAGA (F) GAGATAGATTGCGAGTTGTGT(R)	52
	ZM 6	GGTGTGTTCTCTCTCTCACAC (F) GGGCTGCTCAATAAATGTAG(R)	52
	ZM 8	TCTCTCTCTCTCTCGTTCTTG (F) CCCACTGTACCTCTCCATATT(R)	52
	ZM 12	ATTGCTGTGCAATCCTTATC (F) ATCTCTTTCTACCACCACGTT(R)	52
	ZM 13	GCAGAAGGCAATAAAGTCAT(F) GCGTCAGAAGAAAAATACTGG(R)	53
	ZM 14	GGAAGGCGAGTTGATAGATAA (F) CATGGGATGTTCAAAGAACT(R)	53
	ZM 16	AGGTAGAATTACATGCTGTGC (F) GCTTCCTCCTTCATTCATATC(R)	53
	ZM 18	AATACCCTTCAGTATTCAGGTG(F) CAACAACACAAACACTGCTAC(R)	53
	ZM 20	GGGATGTTGATAGAGATGTTG (F) TCTTTCACTCTCACACACACA(R)	53
	ZM 21	CTCTCTCTCTCTGCTGTTTCA (F) GCCATACGATCTCAAAATCAC(R)	53
	ZM 22	ACCACCGATCTACTCACTTTT (F) CCACTGCACACTACAGTTTTT(R)	53
	ZM 23	CGTATGTCAAGATGAAGCAGT (F) ATCAACAATTCCACTCAACC(R)	53
	ZM 24	CCACACTCAAAACCAAGAAA (F) GCGAAGAGATTATATACACACG(R)	53
	ZM 26	ACTTCAACTTCAACCTCAACC (F) TGTGCATAAAAACCCTCTCT(R)	53
	ZM 29	CATTACAATAGCCCGAAAAG (F) TACTGTTCCTCCTCCTCTCTT(R)	53
	ZM 32	CACGAAGAGTGAGAGAGAGAG(F) CTACCAAAAGTCCCTGAATCT(R)	53
	ZM 33	GAGACAGTACACTTGGGACAA (F) CTCTTCTTGGGCATTAACTCT(R)	53

	ZM 34	AAGTCCCTTTTCAAGCAATC (F) GAGAGAGGAAAATGCAGAGAG(R)	53
	ZM 38	CAGCTTCCTGATTTGATTTG (F) AGATTGCAAGAATCGCTTAG(R)	53
	ZM 39	AGAGGCAGAGGAGTTGATAAT (F) CTTAACTGTAACTCCCTTTTCG(R)	53
	ZM 45	GCAAAATCTCTGTTGTCTCAG (F) GTGTTCCTACCACTCAACACA(R)	55

Table 3: Steps of PCR program

Sl. No	Steps	Temperature	Time
1	Initial denaturation	94˚C	10 minutes
2	Cyclic Denaturation	94˚C	45 seconds
3	Primer annealing	50-55 ˚C	45 seconds
4	Cyclic Extension	72 ˚C	1 minute
5	Final extension	72˚C	10 minutes
6	Cooling	4 ˚C	α

Agarose gel electrophoresis for resolving of SSR markers

The PCR products were analyzed by electrophoresis using a 4 per cent agarose gel. The samples were run at 120 V for 1 to 1 ½ hours. During electrophoresis, 50 bp or 100 bp ladder was added in one well along with the samples for each primer pair to determine the size of amplified fragments. The gel was stained with ethidium bromide and viewed under a gel documentation unit.

Data scoring and analysis

To determine genetic diversity among sesame accessions, SSR marker results were transformed into numerical data. For this, results were scored as present (1) and absent (0). NTSYS-pc version 2.2 (Numerical Taxonomy Multivariate Analysis System) software program was used for dendrogram construction. Simple similarity coefficient used for dendrogram construction. PIC estimates the per cent of polymorphism content of the combinations for each sample and compares them. PIC calculation was done according to PICi= 2 fi (1 – fi) formula: where PIC is the polymorphism information content of marker "i", fi is the frequency of band present and 1- fi is the frequency of the band absence (Roldan-Ruiz *et al.*, 2000) [10].

Results

SSR Amplification and Polymorphism Information Content

Out of 50 SSR markers screened, 30 markers (60.0%) displayed clear and repeatable polymorphic bands for the analysis of 45 sesame genotypes. The level of polymorphism was higher in this study compared to earlier report by Ramprasad *et al.* (2017) [9] who reported only 29 % of polymorphism. Rao *et al.* (2012) [15] determined the level of genetic diversity among nine genotypes, by using 207 sesame-specific microsatellite markers. Of these, 46 markers were found to be polymorphic. Dar *et al.* (2016) [4] analysed 47 different sesame accessions and a total of 64 DNA bands were obtained, of which all of were polymorphic. Similarly, Wei *et al.* (2011) [6] randomly selected fifty EST-SSRs to detect the polymorphism and 80% of these primer pairs successfully amplified fragments, revealing abundant polymorphism among the sesame accessions.

The remaining 20 primer pairs were monomorphic and were not contributed to the polymorphism. The total number of alleles amplified was 91 and it ranged from 2 to 3 per locus with a mean of 2.36 alleles per locus which is less compared to the reports by Badri *et al.* (2014) [3] and Lalitha *et al.* (2014) [8]. The highest number of alleles (3) was detected for eleven markers *viz.*, SEM 38, SEM 83, SEM 100, SEM 138, SEM 270, SEM 279, SEM 430, ZM 6, ZM 22, ZM 23 and ZM 34. The lowest number of alleles (2) was detected for nineteen markers *viz.*, SEM 32, SEM 62, SEM 64, SEM 74, SEM 146, SEM 282, SEM 285, SEM 326, SEM 396, SEM 428, ZM 1, ZM 14, ZM 16, ZM 18, ZM 21, ZM 24, ZM 32, ZM 33 and ZM 38. A representative gel picture has been given in figure 1 and 2. Dixit *et al.* (2005) [5] reported a PIC ranged from 0.34 to 0.80 after screening, 10 polymorphic microsatellites were selected to determine their usefulness in diversity analysis among 16 sesame accessions.

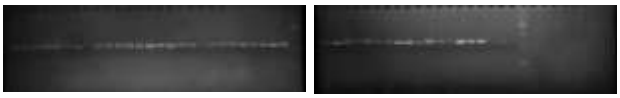

Fig 1: Amplification of SSR marker ZM 22

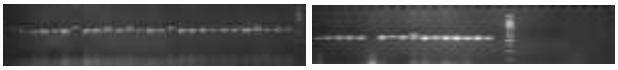

Fig 2: Amplification of SSR marker ZM 33

Polymorphism Information Content

The Polymorphism Information Content (PIC) value is a measure of polymorphism among genotypes for a marker locus used. The PIC value of each marker can be calculated on the basis of its alleles, which varied greatly for all tested SSR loci. The PIC value ranged from 0.5209 to 0.9128 with an average PIC value of 0.3940 (Table no.4). The results indicated the presence of high level of genetic diversity. Anitha *et al.* (2010) [1] reported similar values which ranged between 0.50 and 0.85. Dixit *et al.* (2005) [5] reported a PIC ranged from 0.34 to 0.80 after screening, 10 polymorphic microsatellites were selected to determine their usefulness in diversity analysis among 16 sesame accessions.

The analysis of SSR markers from 6 non-coding regions in sesame by Sehr *et al.* (2015) [12] revealed a mean PIC value of 0.56 whereas the PIC value of eight selected EST-derived SSRs was reported to be 0.26. In the year 2015, Singh *et al.* studied patterns of genetic variation among the 44 commercially cultivated sesame accessions using 12 selected SSR primers generated 41 amplified bands with a PIC ranged from 0.404 to 0.740.

The highest PIC value 0.9128 was obtained for SEM 428 followed by SEM 100 (0.8325), SEM 270 (0.7897), SEM 38 (0.7771), SEM 282 (0.7716), SEM 83 (0.7623), SEM 396 (0.7409), SEM 138 (0.7283) and SEM 32 (0.6987). The lowest PIC value recorded (0.5209) by ZM 38. As many as 20 primers recorded zero PIC value. The PIC values for the SSR markers used in the study clearly indicate that the markers are robust and informative and polymorphic. Thus these markers could be used for molecular characterization of sesame germplasm from various sources.

The markers, SEM 38, SEM 100, SEM 270, SEM 282, SEM 83, SEM 138, SEM 270, SEM 279, SEM 426, ZM 22, ZM 23 and ZM 34 are good markers which showed high PIC values along with highest number of alleles per markers (3.0). Markers such as SEM 32, SEM 62, SEM 64, SEM 74, SEM 146, SEM 282, SEM 285, SEM 396, SEM 326, SEM 423, ZM 1 and ZM 32 recorded

high values of PIC with an average of two alleles per locus. Hence selections of these markers for the measurement of diversity among the genotypes are advisable to get reliable results.

Table 4: Diversity parameters of polymorphic SSR loci

Sl No	Primer ID	No. of alleles	PIC content
1.	SEM 32	2	0.6987
2.	SEM 38	3	0.7771
3.	SEM 62	2	0.6498
4.	SEM 64	2	0.6775
5.	SEM 74	2	0.6372
6.	SEM 83	3	0.7623
7.	SEM 100	3	0.8325
8.	SEM 138	3	0.7283
9.	SEM 146	2	0.5395
10.	SEM 270	3	0.7897
11.	SEM 279	3	0.6949
12.	SEM 282	2	0.7716
13.	SEM 285	2	0.6886
14.	SEM 326	2	0.6775
15.	SEM 396	2	0.7409
16.	SEM 428	2	0.9128
17.	SEM 430	3	0.7022
18.	ZM 1	2	0.6372
19.	ZM 6	3	0.5239
20.	ZM 14	2	0.5987
21.	ZM 16	2	0.5827
22.	ZM 18	2	0.5622
23.	ZM 21	2	0.5412
24.	ZM 22	3	0.7259
25.	ZM 23	3	0.6934
26.	ZM 24	2	0.1288
27.	ZM 32	2	0.6641
28.	ZM 33	2	0.5486
29.	ZM 34	3	0.6925
30.	ZM 38	2	0.5209

NTsys Analysis

The analysis was carried out by using SSR allelic data and a dendrogram was constructed using NTsys version 2.02. The cluster diagram is given in Figure 1. In the present study, 45 sesame genotypes were grouped into six clusters based on the polymorphism of 50 SSR markers. Clustering pattern based on molecular markers analysis is given in table 5.

Cluster III had single genotype, *i.e.*, NI8-8316, indicating that this genotype was highly diverse than the rest of the genotypes. Clusters I and IV had two genotypes each. The genotypes, IC-41945 and IS-469-1-84-A of cluster I were more closely related to each other similarly trend was observed in the genotypes, FRP-8351-B and DS-37 of cluster IV. The cluster II comprised of three genotypes which was in two sub-clusters. TMV- 7 was in the sub cluster I whereas the genotypes SI-241 and Gautama were in the sub cluster II. This suggests that the genotypes SI -241 and Gautama have more closeness in the same cluster.

Similarly, cluster V was composed of five genotypes *viz.*, IS-644-A, IC-310438-B, Kanpur local, ES-33477 and IC-56196 which were divided into two sub clusters. Sub cluster I includes the genotypes IS-644-A and IC-310438-B indicating that these two genotypes were much more similar in comparison with the other genotypes, whereas, the genotypes Kanpur local, ES-33477 and IC-56196 were fallen in sub cluster II, expressing its closeness.

Among the six clusters, cluster VI was largest, as it comprised thirty two genotypes *viz*., Rama, IC-205071, IS-54039-B, IS-112, FFAT-10-20, Rajeshwari, DS-1, IC-205439, CT-40, IS-54034-B, Swetha Til, CT-60, Krishna, RT-54, Chandana, PKDS-11, Savitri, IS-476, IS-195, EC-208652, IS-644-A, IC-205311, Nirmala,CT-27, NIC-16220, Julang Sesame, KMS-4-323-B, Smarak, KMR-43-A, KMR-38, Gowri and Madhavi which fall under two main sub clusters and four secondary sub clusters projecting the presence of less diversity among these genotypes. The genotypes belonging to this cluster showed more similar genetic background while it reveals high distinctiveness with other clusters. Hence the allelic diversity revealed by the polymorphic SSR markers was sufficient enough to distinguish between the genotypes.

Anitha *et al*. (2010) [1] used NTSYS-pc (ver 2.02) software to calculate the Jaccard's similarity coefficients and to categorize 10 sesame genotypes from Tamil Nadu in to five clusters. Similarly Saxena and Bison (2017) attempted to group 28 sesame varieties using RAPD data with the software NTSYS version 2.1 and reported its successful efficiency.

Table 5: Clustering pattern of sesame genotypes based on molecular marker analysis

Cluster number	Number of genotypes	Genotypes
I	2	IC-41945, IS-469-1-84-A
II	3	SI-241, Gautama, TMV-7
III	1	NI8-8316
IV	2	FRP-8351-B, DS-37
V	5	IS-644-A, IC-310438-B, Kanpur local, ES-33477, IC-56196
VI	32	Rama, IC-205071, IS-54039-B, IS-112, FFAT-10-20, Rajeshwari, DS-1, IC-205439, CT-40, IS-54034-B, Swetha Til, CT-60, Krishna, RT-54, Chandana, PKDS-11, Savitri, IS-476, IS-195, EC-208652, IS-644-A, IC-205311, Nirmala,CT-27, NIC-16220, Julang Sesame, KMS-4-323-B, Smarak, KMR-43-A, KMR-38, Gowri, Madhavi

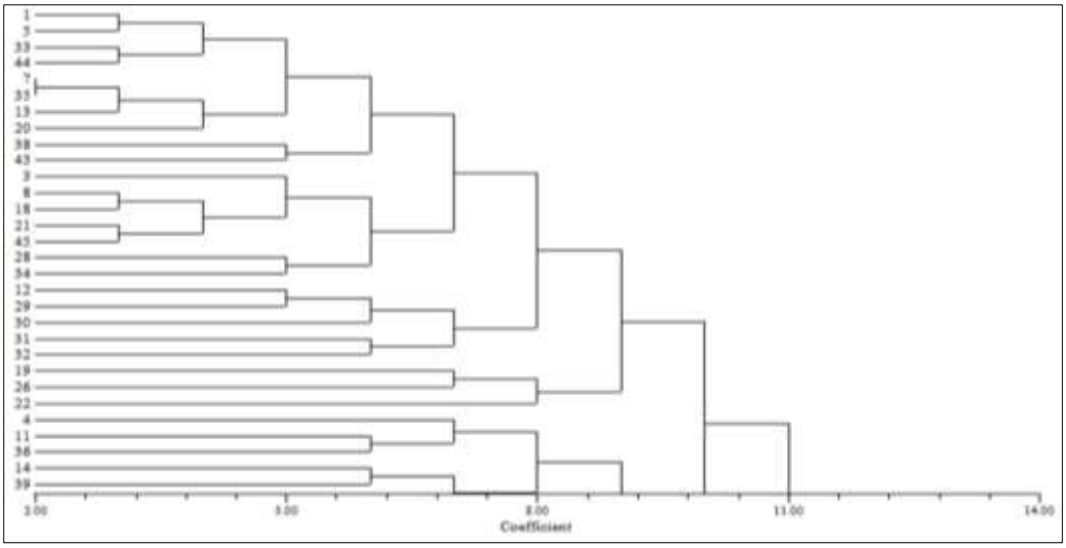

Fig 1: Dendrogram for molecular diversity using SSR markers

Discussion

Molecular markers can be well utilized to analyse the genetic diversity and genetic relationships among sesame genotypes. The present investigation revealed a considerable amount of genetic diversity among 45 sesame lines based on genotyping data from 50 SSR markers. The genotypes were grouped into six main clusters based on the polymorphism observed from 50 SSR markers. The genotypes FRP-8351-B and DS-37 were recorded to be in the same cluster of molecular diversity analysis, suggesting that these two genotypes are closer by descent. Similarly, the genotypes IS-644-A and IC-310438-B which fall under cluster V indicating the presence of more genetic similarity. Molecular clustering pattern further divided the genotypes ES-33477 and IC-56196 in to the same cluster which suggests their closeness as it grouped them under cluster II. NTsys analysis clustered the remaining 32 genotypes in cluster VI, being the largest cluster apparently exhibiting their genetic resemblance.

Yepuri *et al*. (2013) [3] mined about a total of 16,619 ESTs sequences (SSRs) of sesame from gene bank, which was used to construct the dendrogram to group the 49 genotypes into five separate clusters exhibiting a high genetic similarity coefficient from 0.59 to 1.0. Similarly, Dossa *et al*. (2016) [6] collected 96 sesame accessions from 22 countries and were genotyped using 33 polymorphic SSR markers. The analysis of molecular variance revealed that more than 44% of the genetic variance was due to diversity among geographic regions. These works indicate the functionality of SSR markers in genetic dissection of sesame genotypes.

From the study it was concluded that, the molecular analysis of sesame using SSRs could aid in future population genetic structure studies and breeding programs of sesame.

Declaration

The authors do not have any conflict of interest in the research and also regarding the publication of the data as this article.

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