MOLECULAR CHARACTERIZATION OF MULTICUT FORAGE SORGHUM [*Sorghum bicolor* (L.) Moench] USING SSR MARKERS

BY

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CERTIFICATE-I

This is to certify that this thesis entitled, **"Molecular characterization of multicut forage sorghum [*Sorghum bicolor (L.*) Moench] using SSR markers"**, submitted for the degree of **Master of Science,** in the subject of **Molecular Biology and Biotechnology** to the CCS Haryana Agricultural University, is a bonafide research work carried out by **Anita Yadav (2010BS127M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that this thesis entitled, **"Molecular characterization of multicut forage sorghum** [***Sorghum bicolor*** **(L.) Moench] using SSR markers"**, submitted by **Anita Yadav (2010BS127M)** to the CCS Haryana Agricultural University in partial fulfillment of the requirements for the degree of **Master of Science,** in the subject of **Molecular Biology and Biotechnology** has been approved by the Student's Advisory committee after an oral examination on the same.

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
bp	Base pair
CTAB	Cetyl trimethyl ammonium bromide
cm	Centimeter
mm	Millimeter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
EDTA	Ethylene diamine tetra acetic acid
G	Gram
m	Meter
MAS	Marker assisted selection
NTSYS-PC	Numerical taxonomy and multivariate analysis system programme
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
SSR	Simple sequence repeat
STS	Sequence tagged site
Taq	*Thermus aquaticus*
TBE	Tris Boric acid EDTA
TE	Tris EDTA
Tris	2 amino-2 (hydroxymethyl)-1, 3-propanediol
UPGMA	Unweighted pair group methods with arithmetic average
v/v	Volume by volume
w/v	Weight by volume

CHAPTER-I

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench), a tropical plant belonging to the tribe Andropogonaceae and family *Poaceae* , is ranked fifth major cereal crops in the world after rice, wheat, maize and barley. It is well adapted to the areas with too little rainfall and high temperature. Doggett (1988) suggested that sorghum was domesticated and originated in the Northeast quadrant of Africa, most likely in the Ethiopian-Sudan border regions. The presence of wild and cultivated sorghums in Ethiopia reveal that Ethiopia is the primary centre of origin and centre of diversity (Mekibeb, 2009).

It is primarily a crop of hot, semi- arid environment with 400-600 mm rainfall and can tolerate a soil pH 5.0-8.5. Sorghum has a high yield potential, comparable to those of rice, wheat and maize because sorghum often proven versatility, hardiness, dependability and stability of yield under very adverse conditions. Because it has demonstrated its adaptability over such a wide range of culture and climates, it offers great potential for supplementing the world's feed and fodder resource. Sorghum may offer the best opportunity with input from biotechnological and molecular approaches as well as from management in the developing world to become the 'Global Grain of the Future". In recent years, following the introduction of molecular marker in plant genetic research, considerable effort has been made to gain a better understanding of sorghum genetics and evolution.

The genus sorghum has been divided into three main species, namely *bicolor, verticelliflorum, drumondii.* Among these, *Sorghum bicolor* is the most important cereal crop. It is a diploid (2n=20), self pollinated and possesses considerable diversity both morphologically and agronomically (Kong *et al.* 2000). It consists of five main races *viz.,* bicolor, caudatum, durra, guinea, kafir, and several hybrid races on the basis of morphological traits especially panicle and grain traits. Numerous sub- races have been identified. Sorghum was first described by Linnaeus in 1753 and was referred to as Holcus. In 1794, Moench distinguished the genus *Sorghum* from the genus *Holcus* (Clayton, 1961). Sorghum is indigenous to Africa, and many of today's varieties originated in this continent. Sorghum was also grown in India before recorded history as early as 700 BC.

India grows the largest acreage of sorghum in the world followed by Nigeria and Sudan, and produces the second largest tonnage after the US having a total area of about 7.65 million hectares with an output of 7.33 million tons (Department of Agriculture and Cooperation, Ministry of Agriculture, 2010). Major sorghum producing states in India are

Maharashtra, Karnataka, Madhya Pradesh, Andhra Pradesh, Rajasthan, Gujarat, Tamil Nadu and Uttar Pradesh where it is cultivated in rainy (kharif), post rainy (Rabi) and summer seasons. More than 90% of India's sorghum occurs in these states. In Haryana, about 80% population is engaged in agriculture, directly or indirectly. In Haryana, the total area under sorghum cultivation has been estimated as 0.09 million hectares with the production of 0.045 million tons during 2010-2012 having an average productivity of 500g/ha. (Anonymous, 2010).

More than 35% of sorghum is utilized as a food grain and rest is used primarily for animal feed, alcohol production and industrial products (FAO, 1995; Awika and Rooney, 2004; Dicko *et al.* 2006.; Mehmood *et al.* 2008). Sorghum is used as fodder for livestock because of its wide adaptability, rapid growth, high green, dry fodder and drought tolerance. Forage sorghum grows rapidly when temperatures rise in July and August. The crop residue (stover) after grain harvest is valuable source of fodder. Sorghum is cultivated for forage mostly in North India and West Africa. Forage sorghums are fed to animals as a green chop or hay. Sorghum plants, particularly young plants contain an alkaloid which releases hydrocyanic, or prussic acid, when hydrolyzed, this can be toxic to livestock. During periods of drought or other plant stress, sorghums tend to accumulate nitrates, which can poison livestock. If retarded crop growth is observed, analyze the forage for excessive nitrates before feeding it. In the case of high nitrate levels, the forage should be ensiled or combined with other feeds low in nitrate to reduce daily nitrate intake. Being a C_4 plant, it has high water use efficiency. For the same reason, sorghum is grown in regions where most other crops fail to grow. Traditional foods made from sorghum include unfermented and fermented breads, boiled rice resembling foods, snacks, as well as alcoholic beverages. Hard endosperm sorghum is used extensively in South East Asia for noodles and related products. It can be used for sugar, alcohol, syrup, jaggery, fodder, fuel, bedding and roofing or fencing. It has been used for many years to produce concentrated syrup with a distinctive flavor.

Multicut sorghum helps to supply green fodder spread over a long period which mitigates the problem of frequent field preparations and reseedings. The quality of fodder in terms of protein content and digestibility has also been reported to be improved because of frequent cuts (Satripanan *et al.* 1991). Population of livestock during the last decade was 1020.5 million which will increase in future. The availability of green and dry fodder remains in deficit on an average to the extent of 40% which may further increase to extent of 45% by 2025. To narrow down the gap between demand and supply of fodder for the increasing livestock population the concept of breeding multicut varieties of sorghum has been conceived. Varieties with quick regeneration lead to early vigor of crop which not only increases the fodder yield per unit area/time but also escapes the attack of insects and pests at initial stages (Grewal, 2005).

Biotechnological and genetical approaches hold great potential for improving the quality of various crop plants. Molecular markers are recognized as significant tools to orient plant genetic resource conservation management, providing a means to accurately estimate the genetic diversity and genetic structure for species of interest. With the use of molecular marker, it would now be possible to hasten the transfer of desirable gene among varieties and to introgress novel genes from related wide species. Molecular genetic markers such as RFLP, RAPD and SSRs have been used to characterize genetic diversity represented by elite inbred genotypes and cultivated races of sorghum (Menkir *et al*. 1997). Molecular markers are advantageous over traditional phenotypic markers as they offer greater scope for improving the efficiency of conventional breeding by carrying out selection of markers linked to the trait of interest. Besides this, these markers are not environmentally regulated and therefore unaffected by the condition in which the plants are grown and are detectable in all stages of growth. Of the various kinds of DNA-based markers, restriction fragment length polymorphism (RFLP) was in use earlier but it is not suitable for large number of samples and is more laborious. The second group is based on polymerase chain reaction (PCR) based DNA amplification techniques. PCR utilizes primer sequence which is either arbitrary or specific to the flanking ends of the target DNA. Simple sequence repeats (SSRs) also known as microsatellites, are based on tandem repeats of short (2-6) base pairs DNA sequences (Litt and Lutty, 1989).

Germplasm analysis to study genetic diversity is an important area in which lots of efforts have been put in. Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. SSR markers are the markers of choice in most area of molecular genetics as they are highly polymorphic even between closely related lines and provide many different alleles for each marker screened even among closely related individuals (Saghai-Maroof *et al.* 1984). They require low amount of DNA, can be easily automated for high throughput screening, and can be exchanged between laboratories. SSR markers can be analyzed by rapid, technically simple and inexpensive PCR-based assay that require only small quantity of DNA. SSR markers are co-dominant molecular markers. Several efforts have been made to utilize SSR markers to study genetic diversity, characterize germplasm and evaluate population dynamics in various cereal crops like rice (Zhang *et al.* 1997; Liu and Wu, 1998), maize (Senior *et al.* 1998), barley (Struss and Plieske, 1998) and sorghum (Agrama and Tuinstra, 2003).

In view of these, the present study was undertaken with following objective:

- **To study polymorphism in forage sorghum varieties *viz*., HJ 541, SSG 59-3 and their F_2 genotypes using SSR Markers.**

CHAPTER-II

REVIEW OF LITERATURE

Sorghum bicolor (L.) Moench is a diploid (2n=20), self pollinated crop belonging the genus *Sorghum* of the tribe Andropogonaceae and family *Poaceae*. The genus has been divided into three main species: namely *bicolor, verticelliflorum, and drummondii* (Doggett, 1988), among which *bicolor* and *verticelliflorum* are important and commonly grown in different parts of the India. In India, Sorghum is grown where rainfall ranges from 500 to 1000 mm and temperature from 26 to 32^0 C. Harlen and de Wet (1972) classified cultivated sorghum on the basis of agronomic and morphological characteristics. Cultivated sorghums are divided into five basic races: bicolor, guinea, caudatum, kafir and durra; and ten hybrid races that combine the characteristics of any two or more basic races. There is an extremely large amount of variation between the cultivated taxa in the species of *Sorghum bicolor*. In this definitive classification of the cultivated races of sorghum availability of diverse sources of morphological and multicut traits, forage yield and its quality, resistance and nature of genetic component for every trait, There is an urgent need to re-orient our forage sorghum improvement strategies to give more emphasis to develop multicut forage sorghum varieties in order to obtain a quantum jump in per unit area per unit time production of quality fodder. Sorghum is used as fodder for livestock because of its wide adaptability, rapid growth, high green and dry fodder, rationability, drought tolerance. The crop residue (stover) after grain harvest is valuable source of fodder. Sorghum is cultivated for forage mostly in North India and West Africa.

Pahuja *et al.* (1999) observed 24 genotypes of multicut sorghum from different geographical area for green and dry fodder yield along with the stability parameters. They recorded that genotype FSH 16 for green fodder yield and FSH 13 for dry matter yield was found to be stable across the environments.

Selvaraju and Sivasubramaniam (2000) studied varietal identification keys in breeder seeds of 19 sorghum varieties. The keys were made based on seed and seedling morphological characters and chemical tests. The varieties were identified based on the variations in the morphological characters of seeds, such as length, breadth, length/breadth ratio and color. The coleoptiles color of seedlings can also be combined with the seed characters for variety identification. In addition, the reaction of seed coat color to chemicals was also employed for varietal identification.

Pahuja *et al.* (2002) studied evaluation of forage sorghum hybrids for yield and morphological traits. Eighteen hybrids and two standard controls (PHC 106 and FSH 92079) were grown in randomized block design during the rainy season 2000. Information on green and dry fodder yields were recorded on a whole plot basis. Data on leaf length and breadth, stem girth, percentage total soluble sugar (TSS) , early vigour, plant height, Leaves/ plant and tillers/plant were also recorded. Superior green and dry fodder yield were shown, respectively, by hybrids HH2 (69.0 and18.63t/ha), HH88 (70.33 and 18.29 t/ha) and HH85 (67.67 and 18.27 t/ha).

Mohan *et al.* (2007) studied combining ability effects for multicut traits in forage sorghum in seven male sterile lines, ICS4A, ICS15A, ICS79A, ICS95A, ICS 242A, 2219A, ICS 88020A and six pollinator parents IS 699, IS 720, SSG59-3, IS 3230, IS 3274, IS 3289 and their 42 cross. They observed that male sterile lines ICS 4A, ICS79A and tester IS3289 were good combiners for most of the multicut traits

Borad and Gangani (2007) observed the character association in 49 forage sorghum types and revealed high positive and significant association of stem girth, stem weight, leaf weight and crude protein yield with green fodder as well as dry matter yields both at phenotypic and genotypic levels.

Joshi *et al.* (2009) studied morphological characterization of 26 forage sorghum [*Sorghum bicolor* (L.) Moench] varieties which included 20 released and notified and 6 indigenous local varieties for DUS testing. These were characterized using 40 morphological descriptors adopted from the DUS guidelines of PVP and FR Authority and ICAR and subsequently examined for their Distinctiveness, Uniformity and Stability. No intra-varietal variation was observed for any of the visual characteristics and expression of characters in different varieties remained same for the two consecutive years confirming the uniformity and stability of the varieties.

Reddy *et al*. (2009) studied genetic variability and divergence in 29 sorghum (*Sorghum bicolor*) cultivars for 39 agro-morphological traits used in DUS testing. Large variation among cultivars was found for the traits, time of panicle emergence (65- 87days), plant height (120-272cm), leaf length (57-87 cm), panicle length (22-35cm) and 1000-grain weight (23-46 g).

Vittal *et al*. (2010) analyzed 23 genotypes, including both local and exotic cultivars, based on their water-soluble protein and prolamine-protein profiles and they observed that there were no differences in the banding patterns of water-soluble proteins, indicating that it is highly conserved among the genotypes. The prolamine profiles showed differences in their banding patterns among the genotypes. Microsatellites detected a higher degree of genetic variation among the sorghum genotypes compared to the prolamines. All the 20 SSR loci

were polymorphic with a total of 94 alleles. The number of alleles per locus ranged from 2 to 8, with an average of 4.7.

Nguni *et al.* (2012) studied fourteen sorghum accessions, six from Malawi (MW), four each from Tanzania (TZ) and Zambia (ZMB) considered most common and widely grown varieties in those countries were assessed for genetic diversity based on ten SSR loci and grain-Fe, Zn, total protein and starch contents. The lowest within accessions genetic diversity was exhibited by Tanzanian accessions. MW734, TZ4031 and TZ3966 were identified as a potential resource material for grain-Fe and Zn variety enrichment programme.

MOLECULAR MARKERS

Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Creation, detection and exploitation of polymorphism in plants represents one of the most significant developments in biology. The genetic diversity of plants has been classified over the years based on morphological characters, chemical composition as well as cytological characters. However, these have certain limitations as they are influenced by environmental and developmental factors. Currently many molecular marker based techniques are being used for precise and speedy characterization of crop varieties. Molecular markers are rapidly being adopted by crop improvement researchers globally as an effective and appropriate tool for basic and applied studies addressing biological components in agricultural production system (Jones *et al*. 1997; Prioul *et al*. 1997). Assessment of genetic relationships using molecular markers provides polymorphism information about a germplasm pool, which is useful for developing, mapping and breeding populations or lines (Beer *et al*. 1997).

With the development of modern molecular technique and PCR based markers, tremendous progress has been made in mapping and tagging of many agriculturally important genes. A review by Bennetzen (1995) provides detail information on the roles and uses of modern molecular technique for genetic improvement of sorghum. Several kind of molecular markers are available namely, amplified fragment length polymorphism (AFLP), amplicon length polymorphism (ALP), arbitrarily primed PCR (AP-PCR), allele specific PCR (AS-PCR), DNA amplification finger printing (DAF), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), specific amplicon polymorphism (SAP), sequence characterized amplified region (SCAR), single strand conformation polymorphism (SSCP), simple sequence length polymorphism (SSP), simple sequence repeat (SSR) and sequence tagged sites (STS).

A number of reviews have been published on different aspects of molecular markers and their application in crop improvement (Tanksley, 1983; Paterson *et al.* 1990; Beckman and Osborne, 1992; Staub *et al.* 1996). Out of all these markers RFLP, RAPD, ISSR, AFLP

and SSRs are the important classes of markers. The greater utility of molecular markers arises from the five inherent properties that distinguish them from morphological markers.

i) The phenotype of most morphological markers can only be determined at the whole plant level; whereas molecular loci can be assayed at the whole plant, tissue and cellular levels.

ii) Allele frequency tends to be much higher at molecular loci compared with morphological markers.

iii) Morphological markers tend to be associated with undesirable phenotypic effect.

iv) Alleles at morphological loci interact in a dominant recessive manner that limits the identification of heterozygous genotypes. Molecular loci exhibit a co-dominant mode of inheritance that allows the genetic identification of individuals in segregating populations.

v) Fewer epistatic or pleiotropic effects are observed with molecular markers than with morphological markers. Hence a large number of polymorphic markers can be generated and monitored in a single cross.

Gene tagging in turn permits marker assisted selection (MAS) in backcross, pedigree and population improvement programmes. This is especially useful for crop traits that are otherwise difficult or impossible to deal with by conventional mean.

Genetic diversity studies in sorghum using DNA markers

Each cell of a living individual contains DNA as genetic material, and the DNA determines the individual characteristics via the control of protein synthesis in the cell. These marker have the general advantage that the DNA content of a cell is independent of environmental conditions, organ specificity or growth stage. These markers show Mendelian inheritance, get stably inherited and remain unaffected by environmental and developmental stages (Brar and Dhaliwal. 1997). DNA markers are numerous in number and represent a milestone in genetics by providing the capacity for complete coverage of nuclear, mitochondrial and chloroplast genome. DNA markers provide a possibility due to a favorable combination of circumstances to detect, monitor and manipulate genetic variation more precisely compared to morphological and biochemical markers. (Yamamoto *et al*. 1994).

Many studies have been devoted to assesss the pattern of sorghum genetic variation based on morphology (Appa-Rao *et al. 1*996; Dje *et al.* 1998) or pedigree. The isozymes have been utilized by Morden *et al*., (1989), Aldrich and Doebley (1992) to study genetic diversity analysis in sorghum germplasm. Dje *et al.* (1999) assessed the level of genetic diversity of sorghum landacres from north-western Morocco using allozymes marker. They scored 14 allozyme loci and observed a total 20 alleles. Four loci were polymorphic in the overall samples with two alleles dectected. The remaining nine were monomorphic.

DNA based genetic markers are being increasingly utilized in cultivar development, quality control of seed production, measurement of genetic diversity for conservation

management, varietal identification and intellectual property protection. Previously reported methods to characterize diverse lines of sorghum include RFLPs, RAPD, ISSR and SSRs or microsatellites. Each of these classes of molecular markers has characteristics affecting comprehensivity of genome coverage, discrimination ability reproducibility, speed and cost of data generation and scoring that impact application of the indivisual technologies by plant breeders and conservators. Ribisomal ITS (inter-terminal repeats) regions have been used to determine phylogenetic relationships within sorghum. Combined analysis of ITS 1 regions of all twenty five sorghum species has been undertaken by Dillon and Henry (2004).

Ahnert *et al*. (1996) reported a study of 105 sorghum inbreds that used 104 RFLP probes which showed higher level of polymorphism and associations of lines that were congruent with pedigree information and breeder classification of germplasm.

Arya *et al.* (2001) performed RAPD analysis using selected decamer random primers in three hybrids of sorghum CSH 6, CSH 16 and CSH 13R along with their parents for understanding their usefulness in the identification of hybrids.

Basu. (2001) used eighty four random decamer primers and 30 SSR primers pairs to characterize anthracnose resistance gene in sorghum out of which 24 primers produced polymorphic RAPD bands. Bulked segregant analysis was done and found that I kb RAPD fragment produced by marker OPJ 01 and SSR primers Xtxp 61 and Xtxp 212 were tightly linked to anthracnose resistance gene.

Pandey *et al.* (2002) found RAPD primers OPI 16 and OPD 12 closely linked to locus for anthracnose resistance by bulked segregant analysis. The locus for disease resistance in sorghum accession G73 was found to segregate as a simple recessive trait in a cross to susceptible cultivar HC136.

Prakash *et al* (2006) analyzed thirty-two sorghum genotypes by using 64 random amplified polymorphic DNA (RAPD) markers. Out of the total 814 loci generated, 794 loci (97.4%) were polymorphic and informative to differentiate the accessions. Cluster analysis grouped the 32 sorghum accessions into two major clusters. Among the 32 sorghum genotypes, AS 376 and K 2 were distantly related with a low similarity index of 0.28.

Singh *et al.* (2006) studied the inheritance of anthracnose resistance using the parental cultivars of *Sorghum bicolor* (L.) Moench, HC 136 (susceptible to anthracnose) and G 73 (anthracnose resistant). RAPD (random amplified polymorphic DNA) marker OPJ 01_{1437} was identified as marker closely linked to anthracnose resistance gene in sorghum by bulked segregant analysis of HC 136 x G 73 derived recombinant inbred lines (RILs) of sorghum. A total of 84 random decamer primers were used to screen polymorphism among the parental genotypes. Among these, only 24 primers were polymorphic. On bulked segregant analysis, primer OPJ 01 amplified a 1437 bp fragment only in resistant parent G 73 and resistant bulk.

Sivaramakrishnan *et al.* (2007) compared three sorghum cytoplasmic male sterile lines CSV4 A (V), CSV4 A (G_1) and CSV4 A (M), grouped as A_4, with a milo (A_1) and two

other non-milo (A_2 and A_3) cytoplasms for their RFLP patterns of mitochondrial DNA (mtDNA). A 9.7 kb clone from pearl millet mtDNA discriminated each of the three A_4 entries whereas other maize and pearl millet mtDNA clones used could not distinguish this group completely. The molecular differences within the A_4 cytoplasmic group offer some explanation for the inconsistency in the fertility restoration behaviour of these A_4 lines obtained with a definite set of testers in the field.

Akbar *et al* .(2011) examined genetic diversity among 20 sesame (*Sesamum indicum* L.) accessions was examined at DNA level by means of random amplified polymorphic DNA (RAPD) analysis. Ten primers used produced a total of 93 RAPD fragments, of which 70 (75%) were polymorphic. Each primer generated 5 to 17 amplified fragments with an average of 9.3 bands per primer.

Simple sequence repeats (SSRs)

Microsatellites are important genetic markers in identification and characterization of plant species. They are co-dominant markers and are a PCR-based technique, amenable to automation and thus permit analysis of large populations/lines in a short period of time. Microsatellites are highly polymorphic and evenly spread throughout a genome (Areshchenkova and Ganal. 1999). Microsatellite markers are multi allelic and detect a much higher level of DNA polymorphism than any other known marker system (Rafalski and Tingey. 1993). The products generated have been found to be highly reproducible and cost effective (Jones *et al*. 1997) and the polymorphisms can easily be detected both by southern hybridization and by PCR (Arens *et al*. 1995).

Boora et al. (1998) found resistance to anthracnose in sorghum [Sorghum bicolor (L.) Moench] accession SC326-6 segregate as a simple recessive trait in a cross to the susceptible cultivar BTx623. F_3 progeny tests following self pollination of 115 F_2 individuals identified homozygous resistant and susceptible F_2 plants for use in bulked-segregant analysis. DNA from the parental cultivars and the bulks was screened by PCR amplification with 300 RAPD (random amplified polymorphic DNA) primers. Two RAPD primers OPF 07 and OPL 04 amplified a sequence that co-segregated with the recessive allele, while another primer OPK 16 amplified a band linked to the susceptible allele. Markers OPF 07 and OPL 04 showed no recombination and hence were found to be very closely linked to disease resistance locus whereas marker OPK 16 was located 10 cM from anthracnose resistance gene.

Boora et al. (1999) identified a RAPD marker linked to oval leaf spot resistance gene in sorghum parental cultivars and homozygous resistant and susceptible bulks were analyzed by 56 random oligomers. Primer OPH 07amplifoed a RAPD fragment which was found to be linked with oval leaf spot resistance gene.

Dean *et al.* (1999) used SSRs to access genetic variation in sorghum and were able to detect genetic redundancy among sorghum accessions. They showed that a limited number of SSRs could be sufficient to draw conclusions on genetic relationship among sorghum genotypes. Dje *et al.* (1999) reported genetic diversity among and within germplasm accessions in cultivated sorghum with microsatellite markers. They showed that Microsatellite data are useful in identifying individual accessions with high relative contribution to the overall allelic diversity of the collection.

Smith *et al*. (2000) compared the discrimination abilities of 15 SSR primers with 104 RFLPs using 50 genetically diverse elite sorghum [*Sorghum bicolor* (L.) Moench] inbreds. They also compared associations among these lines revealed by these molecular data and by pedigrees. RFLP data allowed all lines to be uniquely identified; two lines could not be distinguished by the SSR data. The mean polymorphism information content (PIC) values were 0.62 (RFLPs) and 0.58 (SSRs). Correlations for pair wise molecular distances with pedigree distances among the maintainer female lines were 0.52 and 0.53 for RFLP and SSR data, respectively; data for the male parental restorer lines were 0.41 and 0.47.

Agrama *et al.* (2003) assayed 22 sorghum genotypes, representing an array of germplasm sources with important agronomic traits, for polymorphism using 32 RAPD primers and 28 sets of sorghum SSR primers. The results indicated that SSR markers were highly polymorphic with an average of 4.5 alleles per primer. The RAPD primers were less polymorphic with nearly 40% of the fragments being monomorphic.

Boora. (2003) used SSR markers to tag resistance genes to the pathogens in sorghum. SSR markers Xtxp 212, Xtxp 274 and Xtxp 105 were found to be closely linked to the resistance gene for anthracnose, leaf blight and oval leaf spot, respectively.

Anas *et al*. (2004) collected data among 22 inbred lines obtained using SSR marker significantly correlated with those of phenotypic performance and grouping of inbred lines based on the combination of performance of six phenotypes was similar to that based on SSR markers.

Menz *et al*. (2004) utilized microsatellites and pedigree data to determine the genetic diversity in a group of sterility maintainer and fertility restorer public inbreds and compared the estimates of genetic diversity to a group of exotic lines from the world collection. Marker haplotypes were also constructed for the chromosomes of BTX 623 and RTX 430, two inbreds of sorghum with excellent combining ability.

Dillon *et al.* (2005) used twelve *Sorghum bicolor*-derived simple sequence repeat (SSR) markers for cross-species amplification in all 25 Sorghum species. The SSR markers were highly polymorphic, with diversity indices ranging from 0.59 to 0.99 with mean of 0.91. Five markers combined were able to differentiate 24 of the 25 *Sorghum* species, with intra-species polymorphism apparent. *Sorghum bicolor* derived SSRs have proven to be an efficient

source of markers for genetic diversity studies of the relatively poorly characterized Australian indigenous *Sorghum* species.

Assar *et al.* (2005). studied genetic diversity genetic relationship among 96 sorghum [*Sorghum bicolor* (L.) Moench] accessions from Sudan, ICRISAT, and Nebraska, USA, using 16 simple sequence repeats (SSRs). In total, 117 polymorphic bands were detected with a mean of 7.3 alleles per SSR locus. Genetic similarity estimates ranged from 0 to 0.91, with a mean of 0.30. The polymorphic information content (PIC) for SSRs ranged from 0.46 (SB4-72) to 0.87 (SBAGF06).

Dhillon *et al.* (2006) studied simple sequence repeat (SSR) markers linked to quantitative trait loci (QTL) associated with resistance to sorghum shoot fly, *Atherigona soccata* resistance were used to characterize the genetic and phenotypic diversity of 12 cytoplasmic male-steriles (CMS) and maintainers, 12 restorer lines, and 144 F_1 hybrids. The genetic diversity was quite high among the shoot fly-susceptible parents and the hybrids based on them, as indicated by high polymorphic information content (PIC) values.

Perumal *et al.* (2007) fingerprinted forty-six converted exotic sorghum lines representing all five races and nine intermediate races of sorghum using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers. A total of 453 scored AFLP and SSR loci were used to calculate genetic similarities between the lines. The dendrogram constructed using UPGMA grouped 31 lines into three major clusters with Jaccard coefficients greater than 0.75. The remaining 15 lines were grouped into four small sub-clusters each with two lines and seven single accession nodes.

Yadav. (2008) studied regeneration capacity and genetic variability among F_3 genotypes of a cross between HC308 (single cut variety) and SSG59-3 (multicut variety) by using SSR markers. On the basis of regeneration in different cuts, F_3 lines were categorized into 4 groups. 41 F_3 lines were non-regenerated (no regeneration after first cut), Three F_3 lines moderately non-regenerated (two cuts), six F_3 lines were moderately regenerated (three cuts) and 69 F_3 lines were highly regenerated (four cuts). Average polymorphism across the 72 genotypes was 3.62 by using 21 SSR primers.

Singh *et al.* (2008) studied the genetic diversity among 22 forage sorghum accessions using 40 SSR markers procured from Texas A & M University, USA. 38 SSR primer pairs produced amplification. All of these markers were highly polymorphic with an average of 8.58 alleles per primer. The polymorphic information content (PIC) values ranged from 0.24 to 0.91. Pair wise similarity indices ranged from 0.49 to 0.87 indicating high genetic variability among the accessions tested.

Li *et al.* (2010) searched the complete chloroplast genome sequence of sorghum line Tx623B for simple sequence repeats (SSRs). 31 SSR loci with at least 10 mononucleotide repeats or five dinucleotide repeats were identified, and primer pairs for 27 loci were designed. Chloroplast DNA variation in cultivated sorghum was investigated by using these

primer pairs on 185 Chinese sorghum landraces and 70 cultivated sorghum accessions from other countries. Among the 27 loci, 14 were polymorphic. The number of alleles per polymorphic locus ranged from 2 to 5 with an average of 2.79. Allelic data at 14 polymorphic loci were combined to give 12 haplotypes.

Ji *et al.* (2011) analyzed thirty sorghum accessions including 19 sweet, 10 grain and 1 wild sorghum accessions by using 63 pairs of simple sequence repeats (SSRs). Results showed that SSR markers were highly polymorphic among the sorghum collections and the average alleles per locus were 2.76 with the average of 0.487 PIC (polymorphism information content) values.

Rajarajan *et al.* (2011) conducted genetic diversity analysis with 100 sorghum genotypes for drought tolerance using 13 stay-green specific polymorphic SSR markers and observed that the genotypes B 35, IS 22212, IS22335, IS 22697, IS 29323, IS 22243, IS 23418, IS 22794, IS 21756 and IS 22339 exhibited drought tolerance phenomenon and were grouped as drought tolerant under molecular level of genetic diversity

Sorghum F_2 population grown in field to study genetic diversity

CHAPTER-III

MATERIAL AND METHODS

3.1 MATERIAL

3.1.1 Plant material

In the present investigation, a total of 32 F_2 progenies of a cross HJ 541 (single cut variety) X SSG 59-3 (multicut variety) along with the parents were used for studying their genetic variability. These 32 F_2 progeny along with parents were grown in Farm Area of Forage Section, Department of Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

3.1.2 Reagents and chemicals

All the chemicals and reagents used in the present investigation for DNA extraction and PCR amplification (Taq DNA polymerase, PCR buffer and dNTPs) were of high purity analytical grade and purchased from Geno-Biosciences Pvt. Ltd., New England Biolabs (NEB)., Life technologies Pvt. Ltd and Sigma Chemicals Co.

3.1.3 SSR primers

Fifty simple sequences repeat (SSR) primers of sorghum were used. These primers were synthesized by Imperial Life Sciences Pvt. Ltd. The list of SSR primers used in present study is given in table .4 2.

3.2 METHODS

3.2.1 Genomic DNA isolation

Genomic DNA was isolated from young leaves of 32 F_2 progenies with their parents following modified CTAB (Cetyl trimethyl ammonium bromide) extraction method as reported by Murray and Thompson (1980) and modified by Saghai-Maroof *et al*., (1984) and Xu *et al*., (1994).

Reagents

CTAB extraction buffer

Tris-HCl (pH 8.0)	0.2 M
EDTA (disodium, pH 8.0)	0.02 M
NaCl	1.4 M
CTAB	2%
β-mercaptoethanol	2%
(Added just before use)	

Wash-I buffer

Ethanol	76%
Sodium acetate	0.2 M

Wash-II buffer

Ethanol	76%
Ammonium acetate	10 mM

TE buffer

Tris (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Procedure

After removing the midrib, leaves (5 g) were cut into very fine pieces and hand homogenized to a fine powder in liquid nitrogen using sterilized pestle and mortar. The ground leaf powder was immediately transferred to sterilized 50 ml polypropylene tubes. Fifteen ml of pre-warmed CTAB extraction buffer (65ºC) was added to each tube. The powdered leaf tissue was mixed thoroughly with the extraction buffer by inverting the tubes gently several times and incubated in water bath at 65ºC for three hour. Contents were mixed at an interval of 15-20 minutes by inverting the tubes several times.

After incubation the samples were cooled for 5 minutes at room temperature, followed by addition of 15 ml chloroform: Isoamyl- alcohol (24:1). Samples were again mixed by inverting the tubes several times. The mixture was centrifuged for 10 minutes at 10000 rpm. The upper aqueous phase was transferred in a pre-sterilized clean centrifuge tube and again extracted with 10 ml of chloroform: Isoamyl- alcohol (24:1) solution.

The upper aqueous phase was collected after centrifugation and DNA was precipitated with equal volume of ice-cold isopropanol. DNA was spooled out using sterile glass hooks and washed with wash buffer-I solution for 20 minutes to remove last traces of prior solutions followed by 2 minute washing in wash buffer-II solution. DNA was then air dried at room temperature for overnight and subsequently dissolved in appropriate volume of T.E buffer and stored at -20ºC till further use.

RNase treatment

DNA samples were treated with 2µl of RNase A (10mg/ml) and incubated at 37ºC for 2-4 hour to insure complete removal of RNA contamination from DNA samples.

Purification of DNA

DNA was again extracted by the addition of equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1v/v). The contents were mixed thoroughly until an emulsion was formed. Samples were centrifuged at 8000 rpm for 10 minutes at room temperature. Aqueous phase was transferred to fresh sterilized eppendorf tube and was again extracted with chloroform: isoamyl-alcohol (24:1v/v). The aqueous was transferred in a new sterile tube

DNA was precipitated by adding 1/10th volume of 3M sodium acetate (pH 5.2) and two volumes of ice-cold ethanol and incubated at -40ºC for one hour. DNA was pelleted down by centrifugation at 8000 rpm for 10 minutes at 4ºC. The supernatant was carefully removed and pellet was washed with 70 percent ethanol. Sample tubes were kept open to remove last traces of ethanol. The DNA pellet was dissolved in appropriate volume of TE buffer and stored at -20ºC till further use.

3.2.2. Quantity and Quality of DNA

Quantity and quality of DNA was estimated by UV spectrophotometer and agarose gel electrophoresis.

UV-spectrophotometric estimation

For UV spectrophotometeric estimation, an aliquot of DNA sample was suitably diluted and absorbance (A) was determined at 260 nm and 280 nm wavelength in spectrophotometer. Using the relationship of 1.0 O.D. at 260 nm equivalent to 50 µg DNA per ml, the quantity of DNA was estimated from the following formula:

$$Concentration\ of\ DNA\ (\mu g/ml) = A_{260} \times 50 \times dilution\ factor$$

Quality *of DNA*

Quality of DNA sample was checked both by UV-spectrophotometer and on agarose gel electrophoresis. Using spectrophotometer the ratio of the absorbance at 260 nm and 280 nm was noted. Samples with a ratio of 1.8 were considered of good quality.

$$A_{260}/A_{280} = 1.8$$

Quality of DNA was also tested by submerged horizontal agarose (0.8%) gel electrophoresis intact DNA with high molecular weight was observed and no smear on gel indicated a fairly good quality DNA. DNA quantity was also determined on the basis of band intensity as compared with the lambda DNA marker (used to determine the concentration) on agarose gel using gel documentation.

3.2.3. Polymerase chain reaction (PCR)

DNA extracted from 32 different F_2 progenies of sorghum along with the parents was amplified using SSR primers. PCR reaction was carried out in 20 µl reaction mixture containing 100ng genomic DNA, 1.5 units of Taq DNA polymerase, IX PCR Buffer (10mM Tris HCl, 1.5mM $MgCl_2$), 100µM each dNTPs and 10 µM of primer.

The reactions were carried out in PTC- 100 programmable thermal cycler from MJ research and Biometra Personal. Following PCR conditions were used:

1.	Initial denaturation	94ºC for 3 min.	
2.	Denaturation	94ºC for 1 min.	35 cycles
3.	Annealing	55-60ºC for 1 min.	
4.	Extension	72ºC for 1 min.	
5.	Final extension	72ºC for 15 min.	

Cycles were set by repeating steps 2-4, 35 times. Amplified products were stored at -20^{0}C till further use.

3.2.4 Agarose gel electrophoresis

Amplified DNA fragments were resolved in 3.0 per cent (w/v) agarose gel and visualized by staining with ethidium bromide.

Reagents

10X TBE buffer

Tris	108.0g
Boric Acid	55.0g
EDTA	4.60g
Final Volume	1L

6X loading dye

Sucrose	4.0g
Bromophenol blue	0.025g
Xylene cyanol	0.025g
Final volume	10ml

Loading dye solution was stored at 4ºC in the refrigerator.

Procedure

Gel casting tray was washed, air dried and its ends were sealed with cello tapes or rubber stopper. Agarose (3.0%) was melted by boiling in 1X TBE buffer, cooled to 50-55ºC. Ethidium bromide at a concentration of (5 mg/ml) was added after cooling the gel to 50-55ºC. Gel solution was poured into gel casting plate with an appropriate comb with required number of wells and size inserted. Gel was allowed to solidify for 30 min. After solidification, rubber stopper or sealing tapes were removed to allow conduction. Plate was submerged in IX TBE buffer and comb was removed gently. Samples were prepared by adding 1.2 µl of 6x loading dye and were spin briefly in a micro-centrifuge for proper mixing. DNA samples were loaded in the wells and electrophoresis was carried out at a constant voltage (3V/cm of gel) till bromophenol blue (loading dye) migrated 2-3 cm to other end of the gel. PCR amplified products were visualized under UV trans-illuminator and photographed using Chemilager^{TM} 440 chemiluminescence gel documentation system (Alpha Innotech Corporation).

3.2.5 Allele scoring

Bands for SSR analysis were scored based on the presence (taken as 1) or absence (taken as 0) of band for each primer. Banding pattern for each primer was scored by visual observations, where only clear and unambiguous bands were scored. The size (in nucleotide base pairs) of the amplified bands was determined based on its migration relative to molecular size marker (100bp DNA ladder from New England Biolabs.).

3.2.6. Data analysis

The banding patterns obtained from SSR analysis for each primer were scored by visual observation. This 0/1 matrix was used to calculate the genetic similarity to estimate all pair-wise differences in the amplification products for all genotypes. The genetic similarity between varieties was evaluated by calculating the Jaccard similarity coefficient. Similarity coefficients were used for cluster analysis for varieties performed using sub program of NTSYS-PC (Rohlf, 1990). The dendrogram was constructed by unweighted pair group method with arithmetic averages (UPGMA) sub programme of NTSYS-PC. The data generated from amplified fragments were analyzed according to Nei and Li (1979) formula given below:

$$\text{Similarity (f)} = \frac{2Mx}{My + Mz}$$

Dissimilarity = 1-f

where,

Mx = Number of shared fragments between genotypes y and z

My = Number of scored fragments of genotypes y

Mz = Number of scored fragments of genotypes z.

3.2.7. 2D and 3D principal component analysis

Principal Component Analysis (PCA) was done using the 'EIGEN' sub-programme of NTSYS-PC software. Diagrams in both 2 and 3, dimensions were constructed.

CHAPTER-IV

RESULTS

Multicut varieties are capable of producing fodder at frequent intervals because of their high yielding ability. This concept of better regeneration is important in non-traditional multicut forage crops like sorghum in order to obtain increased green fodder yield and to regulate the fodder supply for a longer period. Multicut varieties generally provide more dry matter (25-30%) than single cut varieties under the same management system. Therefore, development of varieties suitable for multicut system should find high priority in forage sorghum breeding programmes. An ideal multicut variety should combine quick regeneration, high tillering and faster growth in order to obtain more number of cuttings and high fodder yield. For this, there is a need to study variability for regeneration in order to utilize this information in developing an ideal multicut variety of forage sorghum.

Correct identification of genotypes is important for germplasm management and conservation. PCR based molecular markers are highly efficient tools that can be used for: (i) indirect selection of tagged loci affecting qualitative or quantitative traits (ii) to identify and discriminate closely related cultivars (iii) for pedigree analysis (iv) to assess taxonomic and phylogenetic relationships (v) linkage mapping etc.

Accordingly, the present study was undertaken to study molecular characterization in sorghum. For studying these 32 F_2 progenies of cross HJ 541 x SSG 59-3 along with their parents were used. HJ 541 is a single cut variety while SSG 59-3 is a multicut variety. The data was analyzed for genetic variability among F_2 progenies of cross between HJ 541 (single cut variety) and SSG 59-3 (multicut variety) by using SSR markers.,

4.1 DNA quality and quantification

Genomic DNA was isolated from healthy and young leaves of 32 F_2 progenies along with parents (Table 4.1) using modified CTAB extraction method of Murray and Thompson (1980), and Saghai-Maroof *et al*. (1984). Quality and quantity of various DNA samples were determined by UV-spectrophotometer and gel electrophoresis. For testing the purity of the DNA, the ratio of absorbance of DNA from each genotype at 260/280 nm was determined by UV spectrophotometer. The data showed that some of the DNA samples were contaminated. The DNA of the genotypes having RNA was treated with RNase. The DNA of the samples having high protein and gelatinous were again extracted with phenol, chloroform and isoamyl alcohol. The DNA was then checked on 0.8% gel. It was found free from contaminants, high molecular weight and intact.

The quantity of DNA in different progenies ranged from 450 to 1278 µg/ml. Maximum quantity of DNA was recorded in progeny G31 (1278 µg/ml) followed by progenies G21, G1, G2 (1254, 1236, 1212 µg/ml) while minimum quantity of DNA was recorded in HJ 541 (450 µg/ml) followed by SSG59-3, G5, G20, G24 (550, 562, 578, 586, µg/ml) by U.V spectrophotometer and gel electrophoresis.

Table 4.1: Quantity and quality of genomic DNA of sorghum varieties SSG 59-3 and HJ 541 along with their 32 F_2 progenies extracted following CTAB method.

Progenies	Quantity of DNA (ug/ml)	Ratio of A_{260}/A_{280}
G1	1236	1.77
G2	1212	1.83
G3	1044	1.80
G4	652	1.79
G5	562	1.84
G6	860	1.69
G7	1052	1.84
G8	1062	1.81
G9	650	1.78
G10	892	1.75
G11	845	1.85
G12	433	1.84
G13	645	1.79
G14	765	1.77
G15	1208	1.80
G16	596	1.80
G17	730	1.79
G18	944	1.76
G19	1112	1.78
G20	578	1.81
G21	1254	1.78
G22	1051	1.75
G23	600	1.85
G24	586	1.84
G25	900	1.79
G26	1000	1.84
G27	1192	1.82
G28	644	1.78
G29	700	1.85
G30	696	1.80
G31	1278	1.83
G32	1144	1.82
SSG59-3	550	1.79
HJ541	450	1.80

4.2. SSR markers analysis

To identify the polymorphism, a total of 50 SSR markers, nearly having 50% G+C content were selected from published literature. The primers used in this study, with their sequences are given in Table 4.2. Out of 50 primers used 9 primers (Xtxp 10, Xtxp 149, Xtxp177, Xtxp 197, Xtxp 205, Xtxp 215, Xtxp 225, Xtxp 227 and Xtxp 309 did not exhibit

any amplification and 20 primers were monomorphic for any of the progenies under study. This has been rechecked and the same results were obtained.

Table: 4.2.. List of the SSR primers used

Sr. No.	Locus	Sequence of forward primer	Sequence of reverse primer
1.	Pep C	TGG GAA GCA GCT CAG G	AGG GTG GTG AGT TAG GGA
2	Cba f	AAA GCT CGG CGT TAG AAA TA	CGC TTA ACA ACT CCT ACC ATC
3	Kaf 2	TCG GCG AGC ATC TTA CA	TAC GTA GGC GGT TGG ATT
4	Xtxp6	ATC GGA TCC GTC AGA TC	TCT AGG GAG GTT GCC AC
5	Xtxp10	ATA CTA TCA AGA GGG GAG C	AGT ACT AGC CAC ACG TCA C
6	Xtxp21	AAC CTT GCC CTA TCC ACC TC	TAT GAT GAA TCA AGG GAG AGG
7	Xtxp40	CAG CAA CTT GCA CTT GTC	CAG CAA CTT GCA CTT GTC
8	Xtxp47	CAA TGG CTT GCA CAT GTC CTA	GGT GCG AGC TAG TTA AGT GGG
9	Xtxp56	CAC GTC GTC ACC AAC CAA	GTT AAA CGA AAG GGA AAT GGC
10	Xtxp65	CAC GTC GTC ACC AAC CAA	GTT AAA CGA AAG GGA AAT GGC
11	Xtxp92	TTT CAC AGT CTG CTC TCT G	AGG AGA GTT GTT CGT TA
12	Xtxp94	TCT CCG TTT GCC CGC CAG	TCT CCG TTT GCC CGC CAG
13	Xtxp105	CGT CTT CTA CCG CGT CCT	CAT AAT CCC ACT CAA CAA TCC
14	Xtxp114	TTG TTT CGG TGA CCA C	TAT CTT TAA ATT GCC TTT GTT
15	Xtxp115	TGT ATG GCC TAG CTT ATC T	CAA CAA GCC AAC CTA AA
16.	Xtxp141	GTT CCT CCT GCC ATT ACT	CTT CCG CAC ATC CAC
17	Xtxp145	AGC CTT GCA TGA TGT TCC	GCT ATG CTT GGT GTG GG
18.	Xtxp149	ACC CAA AGC CCA AAT CAG	GGG GGA GAA ACG GTG AG
19	Xtxp159	AGT CAA AAC CGC CAC AT	GAG AAG GGG AGA GGA GAA
20.	Xtxp168	TGG CGG ACA TCC TAT T	GGA GAG CCC GTC ACT T
21	Xtxp176	GCC GGT TGT GAC TTG	TTA AAG CGA TGG GTG TAG
22	Xtxp177	TAA GCT GAT TTG GGG GAC AT	GGT GAT CGA GTG CGG AGT A
23	Xtxp179	GCG TCA ATT AAT CCA AAC AGC CTC	GAG TTC CTA TTC CCG TTC ATG GTG AT
24	Xtxp197	GCG TTT ATG GAA GCA AAA T	CTC ATA AGG CAG GAC CAA
25	Xtxp 201	CCT GCC GTG TCT TCC	TAT ATG CAT GCC GTA GAT TT
26	Xtxp205	ACA CAT CTA CTA CCC TCT CAC CCT	TGA TAG ACT TGT GAG CAG CTC C
27	Xtxp 208	CGC TTT TCT GAA AAT ATT AAGGAC	GAT GAG CGA TGG AGG AGA G
28	Xtxp210	TCA ACG GCC AAT GAT TTC TAA C	AGG TTG CGA ATA AAA GGT AAT GTG
29	Xtxp211	TTT CCC CTC TTT CTT GTG TC	CTC GGC GTC GTC GTA
30.	Xtx 215	GGC CTC GAC TAC GGA GTT	TCG GCA TAT TGA TTT GGT TT
31	Xtxp217	AAC CTA TGC GGA TAA AAC AGA	GGA TCG GTG CCA AAT AAA
32	Xtxp219	TTG TTG CAT GTT GGT TAT AG	CAA ACA AGT TCA GAA GCT C
33	Xtxp225	TGA AAG TTT TGG CAT TGA	TGT AGG ATA GCC CAG GTT
34	Xtxp227	ACA GGT TGG CGA TGT TTC TCT	TTC TTT TTC GAA TTC ATT CCT TTT
35	Xtxp228	TGC CCA AGA GGA TAA AAG GT	AGC GAC GGC ACA TCA AT
36	Xtxp248	CAC CAA GTG TCG CGA ACT GAA	GCT TAG TGT GAG CGC TGA CCA G
37	Xtxp258	GTA CCC ATT TAA ATT GTT TGC AGT AG	CAG AGG AGG AGG AAG AGA AGG
38	Xtxp273	GAA ATT ACA ATG CTA CCC CTA AAA GT	ACT CTA CTC CTT CCG TCC ACA T
39	Xtxp274	GGG TTT CAA CTC TAG CCT ACC GAA CTT CCT	ATG CCT CAT CAT GGT TCG TTT TGC TT
40	Xtxp278	CCA GAT TGG CTG ATG CAT ACA CAC T	AAG GGT AAT TTA TGC ACT CCA AGG TAG GAC
41	Xtxp284	ATT TGA TTC TTC TTG CTT TGC CTT GT	TTG TCA TTT CCC CCT TCT TTC TTT T
42	Xtxp289	AAG TGG GGT GAA GAG ATA	CTG CCT TTC CGA CTC
43	Xtxp295	AAT GAG GAA AAT ATG AAA CAA GTA CCA A	AAT AAC AAG CGC AAC TAT ATG AAC AAT AAA
44	Xtxp309	TGC CCT TCA GGA ATG ATT CGA CTA CTA C	TGC AAA ATG CCA CAA ATA GGA GAA AAA
45	Xtxp335	CGA TTG GAC ATA AGT GTT C	TAT AAA CAT CAG CAG AGG TG
46	Xtxp339	CCG CAC TCT CCA CTC T	CGG AAC ACA GGG AAG G
47	Xtxp343	CGA CAT CAG CGT TGT CTT TCT A	GCT TAC GAA TAG GGC AAA AGA ACT
48	Xtxp348	TGG GCA GGG TAT CTA ACT GA	GCC TTT TTC TGA GCC TTG A
49	Xtxp354	TGG GCA GGG TAT CTA ACT GA	GCC TTT TTC TGA GCC TTG A
50	Xtxp 358	CAA GGA CAA GAT TCA TTT TAA GGG	CAA GGA CAA GAT TCA TTT TAA GGG

4.2.1 Genetic diversity analysis

The amplification patterns of DNA obtained with different primers in all the genotypes of sorghum under study were scored on the basis of presence (1) or absence (0) of amplified products on agarose gels (3.0 %). Only clear and unambiguous bands were

considered. The amplified band patterns for all genotypes were obtained in a similar manner for all the primers.

Table 4.2.1: SSR primers used for screening polymorphism among 32 F_2 progenies of sorghum along with their parents.

Primers	Number
Total primers used	50
Primers which showed amplification	41
Primers which produced polymorphism	21
Primers which produced monomorphism	20
Total amplified fragments	78
Total number of monomorphic bands	26
Total number of polymorphic bands	52

A total of 78 sharp and reproducible bands were obtained from 41 primers, Out of which 52 bands were polymorphic and 26 bands monomorphic which showed 46.2% polymorphism among the progenies. The number of amplified DNA bands per primer ranged from 2 to 7. Primer PepC amplified highest number of DNA bands that were 7 while primers Xtxp 56, Xtxp 92, Xtxp 105, Xtxp145, Xtxp 159, Xtxp 258, Xtxp 273, Xtxp 339, Xtxp 343, Xtxp 348, Xtxp 358 and Cbaf amplified with lowest number of bands that was 2 with an average of 1.81 bands per primer. The number of bands amplified by all other primers was in between this range. The minimum level of polymorphism 50% was produced by primer Xtxp 295, Xtxp 339 while 100% polymorphism obtained by all remaining primers except primer Xtxp 210, Xtxp 228, and Xtxp 335 which gave 66% polymorphism, Hence the percent polymorphism across all the 32 sorghum progenies ranged from 50-100%.

4.2.2 Molecular Size Range

The size of the bands obtained as a result of the present study on the sorghum genotypes varied from 110-1000. (Table 4.2.2). The maximum size of the band, 1000 bp was amplified by primer Pepc, while primer Xtxp 217 amplified 110 bp bands which was the minimum size of the bands obtained. The rest of the primers used in the present studies gave bands having size in between this range.

4.2.3. Similarity matrices among all the genotypes

The similarity matrices revealed the relationships among the 32 sorghum progenies along with their parents. The similarity matrices among the progenies ranged from 0.45 to 0.81. The average similarity across all the progenies was found out to be 0.66, indicating a high level of genetic divergence among the genotypes. The maximum similarity value of 0.81 obtained between progenies G12 and G13, G9 and G11, G9 and G30, G29 and G30 while minimum was observed between progenies G7 and G18. (0.45).

Table 4.2.2: DNA amplification bands, their molecular weights and percentage polymorphism generated in 32 F_2 sorghum progenies using SSR primers.

Sr. No.	Locus Code	Total no. of bands	Polymorphic alleles	Monomorphic alleles	Percent polymorphism	Range of molecular Size (bp)
1	Pep C	7	7	0	100%	150-1000
2	Kaf 2	3	3	0	100%	150 -500
3	Cba f	2	2	0	100%	200-300
4	Xtxp 6	1	0	1	0%	170
5	Xtxp 21	1	0	1	0%	250
6	Xtxp 40	1	0	1	0%	150
7	Xtxp 47	4	4	0	100%	150-800
8	Xtxp 56	2	2	0	100%	120-180
9	Xtxp 65	3	3	0	100%	190-650
10	Xtxp 92	2	2	0	100%	200-210
11	Xtxp 94	1	0	1	0%	120
12	Xtxp 105	2	2	0	100%	220-230
13	Xtxp 114	1	0	1	0%	140
14	Xtxp 115	4	4	0	100%	120-500
15	Xtxp 141	1	0	1	0%	190
16	Xtxp 145	2	2	0	100%	180-190
17	Xtxp 159	2	2	0	100%	170-190
18	Xtxp 168	1	0	1	0%	210
19	Xtxp176	1	0	1	0%	250
20	Xtxp 179	1	0	1	0%	200
21	Xtxp 201	1	0	1	0%	220
22	Xtxp 208	1	0	1	0%	230
23	Xtxp 210	3	2	1	66%	200-400
24	Xtxp 211	1	0	1	0%	170
25	Xtxp 217	1	0	1	0%	110
26	Xtxp 219	1	0	1	0%	250
27	Xtxp 228	3	2	1	66%	190-400
28	Xtxp 248	1	0	1	0%	300
29	Xtxp 258	2	2	0	100%	180-190
30	Xtxp 273	2	2	0	100%	280-300
31	Xtxp 274	1	0	1	0%	320
32	Xtxp 278	1	0	1	0%	290
33	Xtxp 284	1	0	1	0%	310
34	Xtxp 289	1	0	1	0%	150
35	Xtxp 295	4	2	2	50%	150-500
36	Xtxp 335	3	2	1	66%	140-400
37	Xtxp 339	2	1	1	50%	250-600
38	Xtxp 343	2	2	0	100%	300-350
39s	Xtxp 348	2	2	0	100%	130-180
40	Xtxp 354	1	0	1	0%	210
41	Xtxp 358	2	2	0	100%	200-210
Total		**78**	**52**	**26**		
Mean		**1.81**	**1.26**	**0.63**	**46.2%**	

Detection of new alleles in parental genotypes and their F_2 progenies

After analyzing all the bands amplified by the 41 primes across 32 progenies, we had been able to see some new alleles in most of the progenies under study. Primer Xtxp 210 gave a unique recombinant allele of size 400bp in progeny G12. Primer Xtxp 210 gave an allele of size 200 bp in parental genotype HJ 541 and also in F_2 progenies G2, G3, G4, G9, G10, G11, G12, G13, G14, G19, G20. This primer Xtxp 210 also gave an allele of size 280 bp in parental genotype SSG 59-3 and in F_2 progenies G1, G15, G16, G17, G18, and G29. Remaining progenies shows heterozygous alleles. Similarily Primer Xtxp 343 amplified an

allele of appox, 300 bp in parental genotype SSG 59-3 and in G12, G14, G15, G16, G19, G20, G21, G22, G24, G25, G27, G28, G29, G30 F_2 progenies. The same primer amplified an allele of size 290 bp in parental genotype HJ 541 and in F_2 progenies G11, G13, G17, G18, G24, G26, and G27. Thus primer Xtxp 343 gave heterozygous alleles in progeny G24, G27. Primer Pep C gave an allele of size 250 bp in parental genotype HJ 541 and also in F_2 progenies G1, G2, G3, G4, G6, G7, G8, G9, G10, G11, G12, G13, G14, G15, G16, G17, G18, G19, G20, G21, G22, G23, G24, G25, G26, G28, G29, G30, G31. This primer Pep C also gave an allele of size 300 bp in parental genotype SSG 59-3 and in F_2 progenies G1, G5, G6, G14, G15, G16, G17 and G27. F_2 progenies G6, G7, G14, G15, G16 and G17 showed heterozygous alleles. Primer Pep C gave unique alleles of size 400bp, 500bp, 700bp and 900bp in F_2 progenies G8, G10, G21, G23, and G24 respectively.

4.2.4. Cluster Tree Analysis (Dendrogram)

The average linkage between 32 F_2 progenies along with the parents in sorghum was used for constructing a phylogenetic tree depicting the relationship among 32 F_2 progenies along with the parents in sorghum. The hierarchical cluster tree analysis depicted the same results as outlined by the similarity matrices of the 32 F_2 progenies along with the parents in sorghum. The association amongst different progenies has been presented in the form of a dendrogram.

The cluster tree was broadly grouped into two major groups at a similarity coefficient of 0.62. Group I consisted of twenty nine progenies along with one parent SSG 59-3 which is a multicut variety. While remaining 3 progenies were grouped with the other parent HJ 541 which is a single cut variety in group II. Group I constituted of two subgroups at a similarity coefficient of 0.63. Out of two subgroups one is again divided at similarity coefficient 0.64. Among them one sub-sub group consisted of progenies G1, G2, G22, G23, G31, and G32. Other sub-sub group is grouped at a similarity coefficient of 0.68 forming a small cluster of 7 progenies (SSG 59-3 parent, G3, G9, G11, G20, G21, G29 and G30) and other subgroup consisted of four progenies G14, G15, G16 and G17. Subgroup II is also divided into 2 sub-subgroups at a similarity coefficient 0.655 in which one sub-subgroup consisted of progenies G4, G26, G24, G27, G28 while other sub-subgroup consisting of G8, G10, G12, G13, G25, G18, G19. while Group II constituted two subgroups at a similarity coefficient of 0.679 with one subgroup consisting of only one parent HJ 541 and other subgroup dividing into two sub-sub groups at a similarity coefficient of 0.732. One sub-sub group consisted of 2 progenies (G6, G7). The other sub-sub group consisting of only one progeny G5. Progenies G12 and G13, G9 and G11, G9 and G30, G29 and G30 show maximum similarity.

4.2.5. Two dimensional (2D) Principle Component Analysis (PCA)

Similar clustering of the 32 F_2 progenies along with the parental genotypes was also evident from the two dimension principle component analysis (PCA) (Fig.2). The progenies

tended to grouped in two clusters. The progenies which were lying nearer to each other in the dendrogram were more similar than those lying apart. Similar observations were made in two dimension PCA analysis as well. First cluster comprised of twenty nine progenies and SSG 59-3 (parent). The second cluster had rest of the three progenies with HJ 541 (parent). As the dendrogram shows that progenies G12 and G13, G9 and G11, G9 and G30, G29 and G30 were closest with the similarity of 81% whereas G7 and G18 were farthest with the genetic distance of 45%.

4.2.6. Three dimensional (3D) Principle Component Analysis (PCA)

The three dimensional PCA revealed similar clustering of the 32 F_2 progenies along with the parents sorghum genotypes as represented in the dendrogram. The progenies which were lying nearer to each other in three dimensional PCA were more similar to one another than those lying apart. Progenies G12 and G13, G9 and G11, G9 and G30, G29 and G30 were the closest. Progenies G7 and G18 were farthest or most diverse and dissimilar to all others progenies.

Table 4.2.3: Similarity matrix data of 32 F_2 progenies of sorghum obtained using database generated by 41 SSR primers.

	SSG59-3	HJ541	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25	G26	G27	G28	G29	G30	G31	G32
SSG59-3	1.00																																	
HJ541	0.39	1.00																																
G1	0.59	0.64	1.00																															
G2	0.73	0.55	0.78	1.00																														
G3	0.62	0.64	0.72	0.73	1.00																													
G4	0.60	0.70	0.68	0.64	0.73	1.00																												
G5	0.59	0.67	0.59	0.63	0.62	0.68	1.00																											
G6	0.65	0.68	0.70	0.67	0.70	0.72	0.73	1.00																										
G7	0.54	0.67	0.72	0.65	0.64	0.60	0.72	0.75	1.00																									
G8	0.51	0.64	0.51	0.53	0.59	0.73	0.56	0.60	0.56	1.00																								
G9	0.63	0.63	0.65	0.69	0.70	0.69	0.63	0.62	0.63	0.70	1.00																							
G10	0.54	0.59	0.59	0.60	0.62	0.65	0.64	0.63	0.59	0.74	0.78	1.00																						
G11	0.69	0.59	0.67	0.65	0.74	0.73	0.59	0.68	0.64	0.59	0.81	0.67	1.00																					
G12	0.60	0.65	0.63	0.67	0.68	0.69	0.68	0.62	0.60	0.65	0.74	0.75	0.73	1.00																				
G13	0.63	0.67	0.64	0.65	0.74	0.73	0.62	0.65	0.59	0.67	0.68	0.67	0.72	0.81	1.00																			
G14	0.60	0.58	0.60	0.62	0.73	0.74	0.53	0.64	0.63	0.65	0.72	0.65	0.70	0.67	0.7	1.00																		
G15	0.69	0.49	0.67	0.65	0.64	0.55	0.56	0.65	0.59	0.67	0.63	0.62	0.67	0.70	0.59	0.65	1.00																	
G16	0.69	0.54	0.62	0.60	0.59	0.68	0.59	0.60	0.54	0.65	0.65	0.59	0.64	0.68	0.62	0.73	0.74	1.00																
G17	0.55	0.65	0.65	0.67	0.73	0.64	0.68	0.69	0.63	0.49	0.69	0.63	0.65	0.74	0.65	0.74	0.75	0.78	1.00															
G18	0.73	0.60	0.60	0.69	0.68	0.74	0.60	0.64	0.45	0.54	0.64	0.63	0.68	0.69	0.65	0.62	0.65	0.68	0.64	1.00														
G19	0.64	0.64	0.59	0.68	0.56	0.70	0.59	0.63	0.54	0.58	0.65	0.64	0.59	0.73	0.65	0.60	0.67	0.59	0.65	0.75	1.00													
G20	0.73	0.55	0.70	0.64	0.65	0.67	0.60	0.67	0.63	0.60	0.69	0.56	0.70	0.59	0.64	0.62	0.65	0.70	0.54	0.69	0.63	1.00												
G21	0.65	0.55	0.68	0.67	0.65	0.64	0.50	0.74	0.65	0.67	0.67	0.63	0.65	0.51	0.60	0.67	0.65	0.63	0.59	0.56	0.65	0.72	1.00											
G22	0.65	0.58	0.75	0.72	0.68	0.54	0.63	0.69	0.73	0.55	0.69	0.63	0.63	0.62	0.60	0.59	0.65	0.60	0.72	0.62	0.63	0.72	0.68	1.00										
G23	0.65	0.50	0.65	0.59	0.50	0.64	0.69	0.67	0.60	0.58	0.59	0.65	0.58	0.51	0.63	0.54	0.53	0.56	0.54	0.59	0.60	0.67	0.64	0.69	1.00									
G24	0.56	0.62	0.67	0.55	0.69	0.70	0.58	0.70	0.62	0.50	0.63	0.67	0.62	0.60	0.55	0.53	0.59	0.58	0.65	0.58	0.59	0.60	0.56	0.60	0.69	1.00								
G25	0.60	0.63	0.58	0.56	0.63	0.64	0.69	0.56	0.53	0.68	0.59	0.62	0.60	0.69	0.56	0.59	0.55	0.64	0.54	0.64	0.68	0.59	0.56	0.59	0.60	0.65	1.00							
G26	0.67	0.67	0.64	0.63	0.59	0.78	0.67	0.65	0.67	0.67	0.60	0.62	0.62	0.70	0.78	0.60	0.56	0.67	0.58	0.68	0.74	0.65	0.53	0.60	0.59	0.62	0.65	1.00						
G27	0.74	0.56	0.59	0.58	0.74	0.70	0.64	0.60	0.59	0.70	0.60	0.65	0.72	0.65	0.69	0.68	0.64	0.65	0.60	0.70	0.59	0.63	0.57	0.60	0.60	0.65	0.72	0.78	1.00					
G28	0.69	0.59	0.67	0.58	0.69	0.73	0.65	0.65	0.62	0.67	0.60	0.69	0.67	0.63	0.69	0.60	0.59	0.59	0.63	0.60	0.56	0.68	0.63	0.65	0.60	0.55	0.64	0.68	0.72	1.00				
G29	0.75	0.58	0.58	0.74	0.75	0.62	0.59	0.64	0.65	0.54	0.77	0.65	0.65	0.67	0.63	0.69	0.70	0.60	0.67	0.67	0.70	0.73	0.69	0.69	0.65	0.60	0.74	0.60	0.77	0.77	1.00			
G30	0.69	0.59	0.64	0.78	0.72	0.65	0.63	0.60	0.67	0.51	0.81	0.64	0.72	0.73	0.67	0.65	0.59	0.72	0.60	0.63	0.64	0.65	0.63	0.68	0.69	0.51	0.60	0.64	0.65	0.73	0.81	1.00		
G31	0.73	0.55	0.73	0.69	0.55	0.67	0.69	0.72	0.70	0.60	0.62	0.59	0.65	0.59	0.60	0.62	0.70	0.60	0.54	0.72	0.65	0.67	0.62	0.74	0.68	0.50	0.56	0.60	0.64	0.64	0.64	0.79	1.00	
G32	0.69	0.59	0.67	0.68	0.64	0.65	0.65	0.60	0.59	0.62	0.70	0.65	0.54	0.63	0.59	0.65	0.59	0.59	0.68	0.73	0.64	0.68	0.60	0.75	0.74	0.74	0.60	0.64	0.78	0.65	0.63	0.62	0.65	1.00

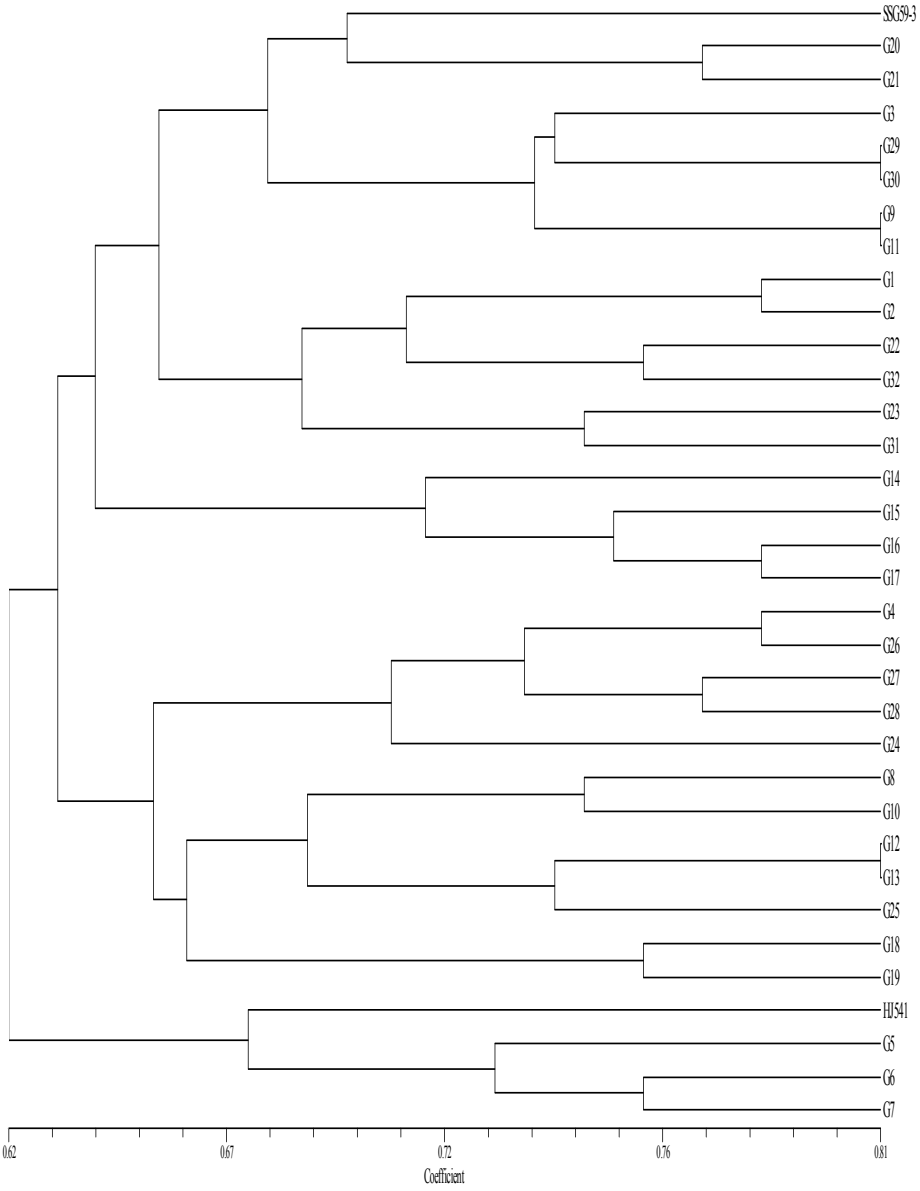

Figure 1: Dendrogram showing genetic relationship between sorghum genotypes *viz.*, SSG59-3, HJ 541 along with their 32 F_2 progenies of sorghum based on SSR marker analysis.

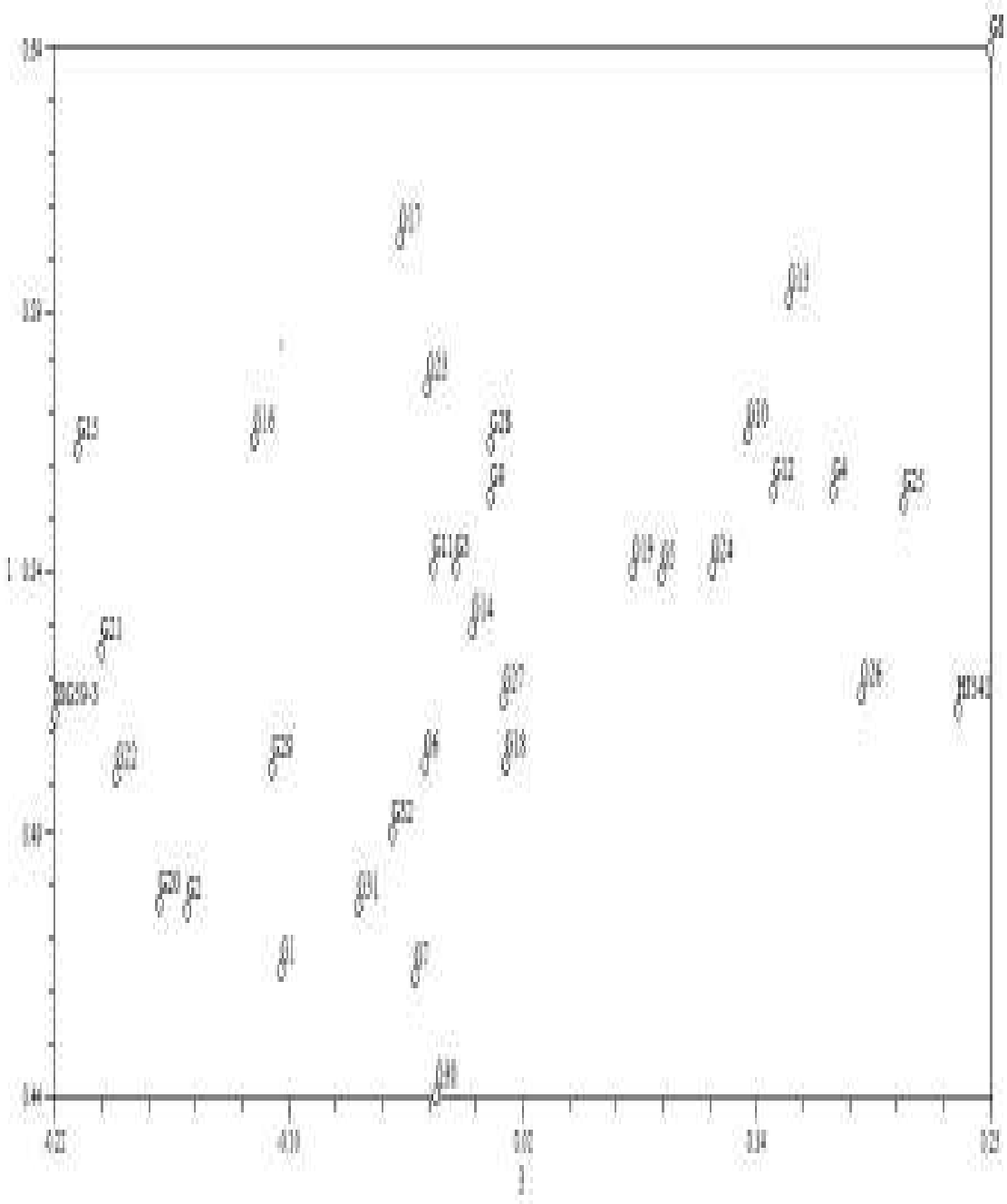

Figure 2: Two-dimensional PCA (Principal Component Analysis) scaling of sorghum genotypes *viz.*, SSG59-3, HJ 541 along with their 32 progenies of sorghum using SSR markers.

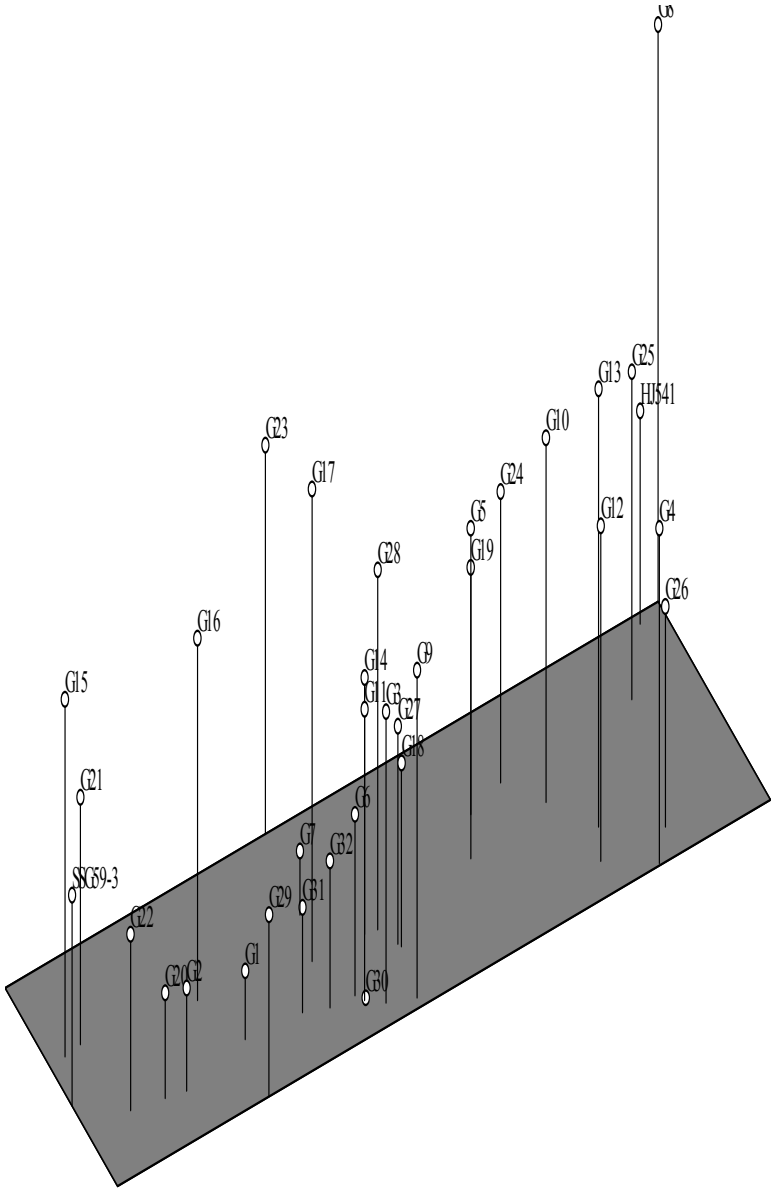

Figure 3: Three-dimensional PCA (Principal Component Analysis) scaling of sorghum genotypes *viz.*, SSG59-3, HJ 541 along with their 32 F_2progenies of sorghum using SSR markers.

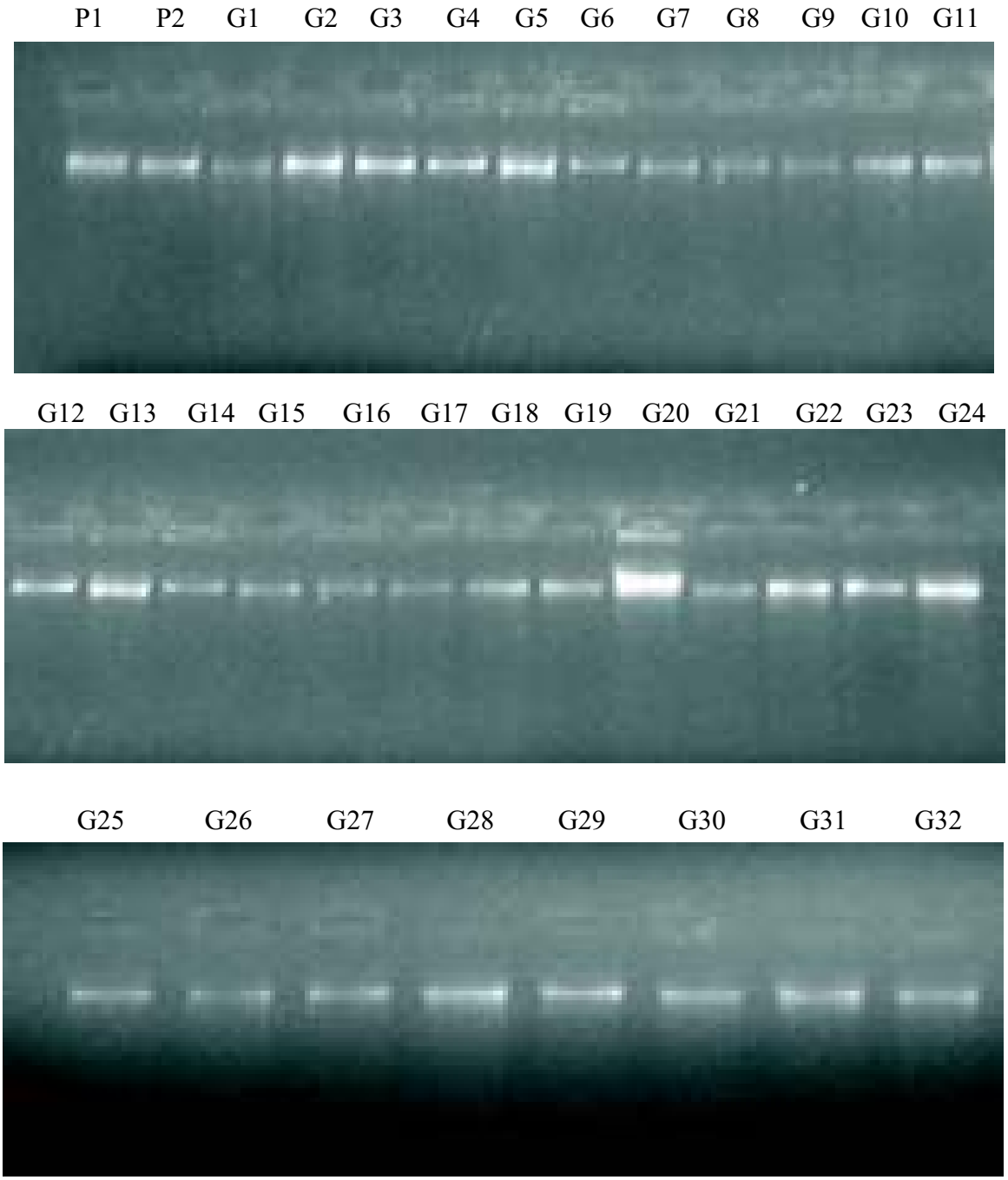

Plate-1

Electrophoretic pattern of highly purified genomic DNA of sorghum genotypes *viz.*, SSG 59-3, HJ 541 along with their 32 F_2 progenies used for genetic diversity analysis

P1 = SSG 59-3
P2 = HJ 541
G1 - G32 = Different F_2 progenies

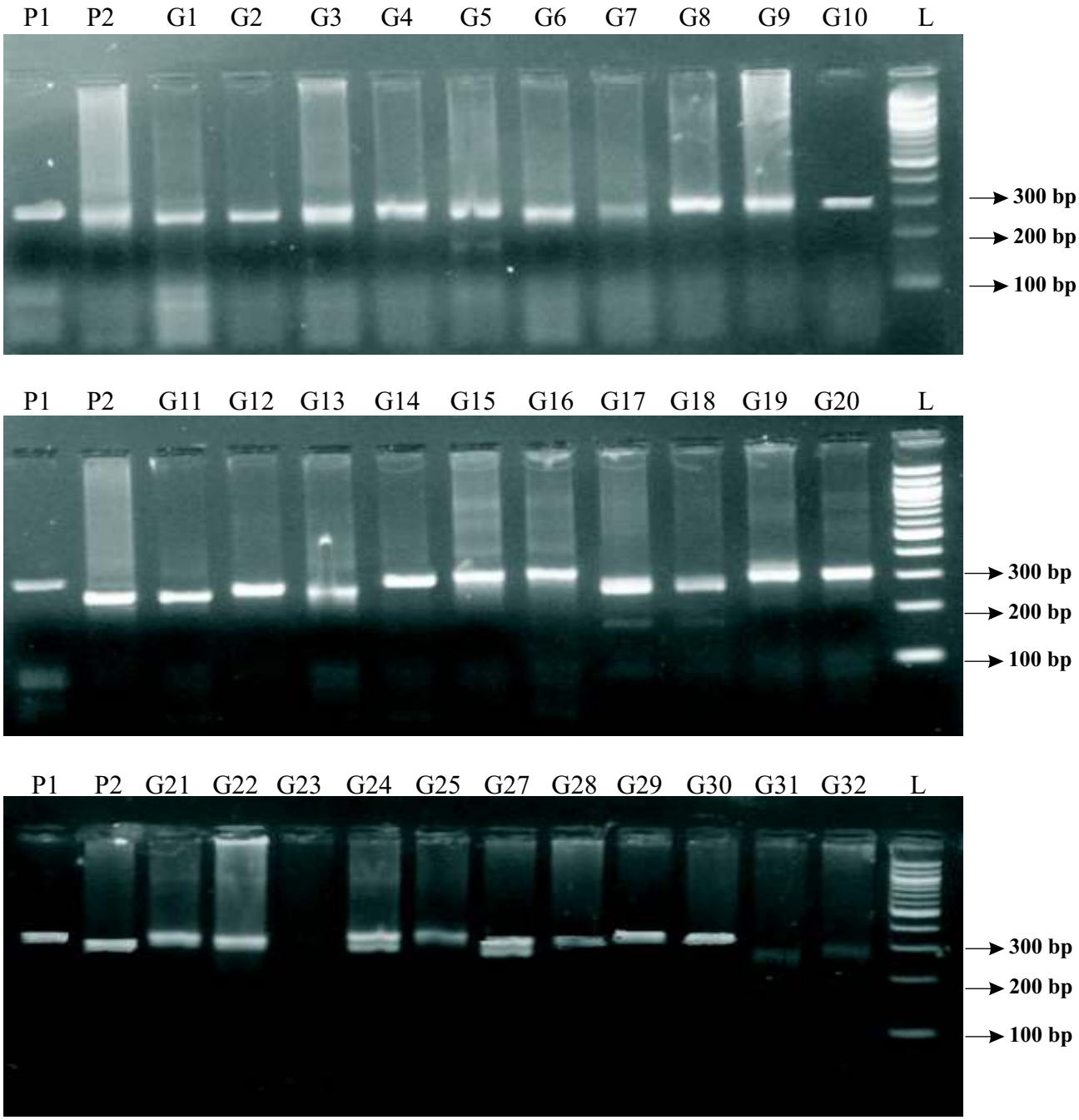

Plate -2

Electrophoretic pattern of PCR amplified fragment of F_2 progenies of sorghum along with their parental genotypes using Xtxp 343 SSR primer

L = 100 bp ladder
P1 = SSG 59-3
P2 = HJ 541
G1 - G32 = Different F_2 progenies

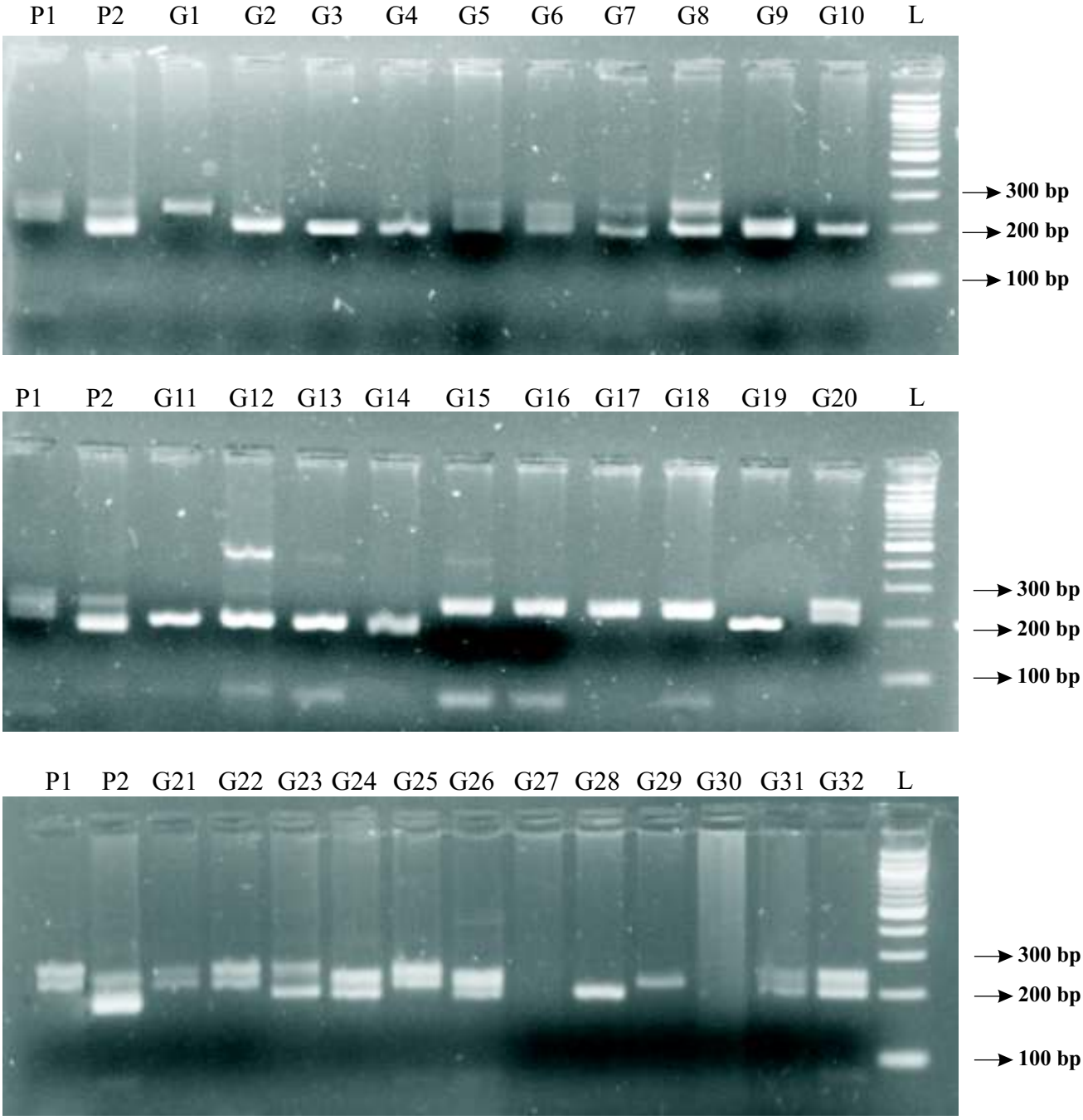

Plate-3

Electrophoretic pattern of PCR amplified fragment of F_2 progenies of sorghum along with their parental genotypes using Xtxp 210 SSR primer

L = 100 bp ladder
P1 = SSG 59-3
P2 = HJ 541
G1 - G32 = Different F_2 progenies

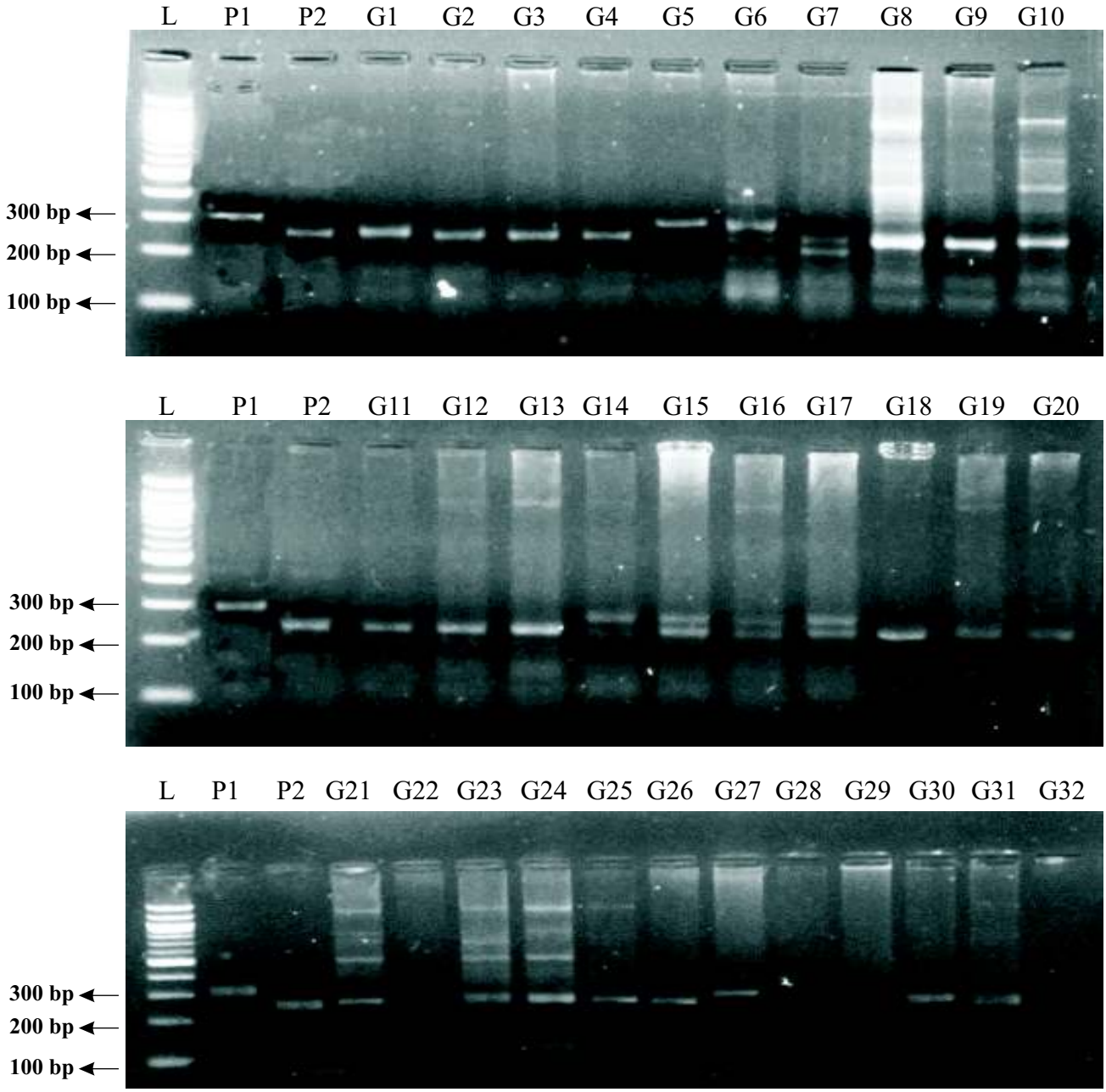

Plate-4

Electrophoretic pattern of PCR amplified fragment of F_2 progenies of sorghum along with their parental genotypes using Pep C SSR primer

L = 100 bp ladder
P1 = SSG 59-3
P2 = HJ 541
G1 - G32 = Different F_2 progenies

CHAPTER-V

DISCUSSION

Sorghum bicolor is a highly diverse species ranking fifth among major cereals in the world after paddy, wheat, maize, barley. Multiple origins for domesticated sorghum, cross-pollination between domestic cultivars and highly variable wild species are considered to be factors contributing to the extensive genetic diversity observed in sorghum (Doggett, 1988). Many plant breeders are concerned that genetic diversity within such primary gene pool has been decreasing at an alarming rate as a consequence of modern agricultural practices. Genetic engineering and biotechnological approaches hold great potential for improving the quality of various crop plants. Molecular markers are recognized as significant tools to orient plant genetic resource conservation management, providing a means to accurately estimate the genetic diversity and genetic structure for species of interest. Over the past one and a half decade, DNA-based genetic markers have been extensively utilized as genetic markers for assessment of genetic diversity, cultivar identification and gene tagging. Introducing novel genes for agronomic traits such as yield, quality traits, disease resistance and abiotic stress tolerance facillates the improvement of any crop species. These new genes are most successfully cloned from the primary gene pool of the crop of interest.

Sorghum is mainly used for fodder purpose in Northern India for livestock because of its wide adaptability, rapid growth, high green and dry fodder, rationability and drought tolerance. So development of multicut forage sorghum varieties/hybrids is the only way or alternate to meet the fodder requirements of ever increasing livestock population all over the world. Multicut varieties are capable of producing fodder at frequent intervals because of their high yielding ability, These Concept of better regeneration is important in non-traditional multicut forage crops like sorghum in order to obtain increased green fodder yield and to regulate the fodder supply for a longer period. Multicut varieties generally provide more dry matter (25-30%) than single cut varieties under the same management system. Therefore, development of varieties suitable for multicut system should find high priority in forage sorghum breeding programmes. An ideal multicut variety should combine quick regeneration, high tillering and faster growth in order to obtain more number of cuttings and high fodder yield. For this, there is a need to study variability for regeneration in order to utilize this information in developing an ideal multicut variety of forage sorghum.

SSR markers offer a potentially attractive combination of features that are useful as molecular markers. SSR markers are co-dominant molecular markers. SSRs are highly

polymorphic and provide many different alleles for each marker screened even among closely related individuals (Saghai-Maroof *et al*. 1984). SSRs can be analyzed by rapid, technically simple and inexpensive PCR-based assay that require only small quantity of DNA.

In view of these, the present study was undertaken to study polymorphism in forage sorghum varieties *viz*., HJ 541, SSG 59-3 and their F_2 genotypes using SSR Markers.

Genetic diversity in sorghum

Simple sequence repeats (SSRs) or microsatellites are tandem repeats of di, tri, tetra, penta or hexa number of nucleotide units in the DNA of plants and animals. SSRs are abundantly distributed throughout the nuclear genomes of plant species. SSRs have several applications including uniform genome coverage, high levels of polymorphism, co dominance, and specific PCR-based assay (Pejic *et al*. 1998). As a result, simple sequence repeat primer has been used as successful tool in genotyping and studying the genetic diversity of many plant species. The analysis of SSRs has been automated, thereby facilitating data exchange among researchers (Saghai-Maroof *et al*. 1994, Powell *et al*. 1996). SSR markers have been utilized for assessment of genetic diversity in sorghum (Dean *et al*. 1999, Dje *et al*. 2000, Smith *et al*. 2000, Dillon *et al.* 2005, Ji *et al*. 2011, Rajarajan *et al.* 2011).

In present study total genomic DNA was extracted from the fresh young leaves by using the modified CTAB method. DNA concentration and quality was estimated by UV spectrophotometer. The quantity of DNA in different progenies ranged from 450 to 1278 µg/ml. Maximum quantity of DNA was recorded in progeny G31 (1278 µg/ml) while minimum quantity of DNA was recorded in one parent variety *i.e.,* HJ 541 (450 µg/ml) followed by SSG 59-3. The DNA quality was also checked by gel electrophoresis. SSR markers were used to screen polymorphisms among 32 progenies of F_2 generation of cross HJ 541 x SSG 59-3. Out of these 41 primers produced amplification and 21 of them were polymorphic. A total of 78 alleles were amplified out of which 52 were polymorphic and 26 were monomorphic. An allelic size varied from 110-1000 base pairs and was comparable with some of the previous studies on sorghum (Smith *et al*. 2000, Dje *et al*. 1999, Menz *et al* 2004, Assar et al. 2005, Yadav 2008, Singh *et al*. 2006, Ji *et al*. 2011).

The number of amplified DNA bands per primer ranged from 2 to7. Primer PepC amplified with the highest number of DNA bands that was 7 while primers Xtxp 56, Xtxp 92, Xtxp 105, Xtxp145, Xtxp159, Xtxp258, Xtxp273, Xtxp 339, Xtxp 343, Xtxp348, Xtxp358 and Cbaf amplified with lowest number of bands that was 2. An average number of 1.81 bands per primer were found out. Smith *et al*. (2000) also studied the discrimination abilities of 15 SSR primers using 50 genetically diverse elite sorghum inbreds. Eighty-nine alleles were revealed by 15 SSR loci number of alleles per locus ranged from 1 to 10 with the mean value of 5.9 allele per locus. Dje *et al*. (2000) analyzed the five microsatellite loci with an

average of 19.2 alleles per locus in overall samples of 25 sorghum accessions belonging to different 5 races.

Agrama and Tuinstra. (2003) assayed 22 sorghum genotypes for polymorphism using 28 sets of sorghum SSR primers, SSR ma rkers were found to be highly polymorphic with an average of 4.5 alleles per primer. . Assar *et al.* (2005) also studied genetic diversity and genetic relationship among 96 sorghum [*Sorghum bicolor* (L.) Moench] accessions from Sudan, ICRISAT, and Nebraska, USA, using 16 simple sequence repeats (SSRs). In total, 117 polymorphic bands were detected with a mean of 7.3 alleles per SSR locus. Singh and Boora. (2006) used 40 SSR primers to screen the polymorphism among the different sorghum genotypes. The number of alleles ranged from 2 to 15 with the mean value of 8.6 alleles per locus Yadav. (2008) studied genetic variability among F_3 genotypes of a cross between HJ 541 (single cut variety) and SSG 59-3 (multicut variety) by using SSR markers. Average polymorphism across the 72 genotypes was 3.62 by using 21 SSR primers Ji *et al.* (2011) analyzed genetic diversity by taking 30 sorghum accessions including 19 sweet, 10 grain and one wild sorghum accessions. They used 63 pairs of simple sequence repeats (SSRs). Results showed that SSR markers were highly polymorphic among the sorghum collections and the average alleles per locus were 2.76 with the average of 0.487 PIC (polymorphism information content) values. The mean number of alleles per locus obtained in present study was lower compared to that reported by Dje *et al*. (1999) but comparable with Smith *et al*. (2000), Agrama and Tuinistra (2003), Assar *et al*. (2005) and Yadav, P. (2008) and Ji *et al*. (2011).

Primer Xtxp 210 gave a unique recombinant allele of size 400bp in progeny G12. Primer Xtxp 210 gave an allele of size 200 bp in parental genotype HJ 541 and also in F_2 progenies G2, G3, G4, G9, G10, G11, G12, G13, G14, G19, and G20. This primer Xtxp 210 also gave an allele of size 280 bp in parental genotype SSG 59-3 and in F_2 progenies G1, G15, G16, G17, G18, and G29. Remaining progenies shows heterozygous alleles. Similarily Primer Xtxp 343 amplified an allele of appox. 300 bp in parental genotype SSG 59-3 and in G12, G14, G15, G16, G19, G20, G21, G22, G24, G25, G27, G28, G29, and G30 F_2 progenies. The same primer amplified an allele of size 290 bp in parental genotype HJ 541 and in F_2 progenies G11, G13, G17, G18, G24, G26, and G27. Thus primer Xtxp 343 gave heterozygous alleles in progeny G24, and G27. Primer Pep C gave an allele of size 250 bp in parental genotype HJ 541 and also in F_2 progenies G1, G2, G3, G4, G6, G7, G8, G9, G10, G11, G12, G13, G14, G15, G16, G17, G18, G19, G20, G21, G22, G23, G24, G25, G26, G28, G29, G30, G31. This primer Pep C also gave an allele of size 300 bp in parental genotype SSG 59-3 and in F_2 progenies G1, G5, G6, G14, G15, G16, G17 and G27. F_2 progenies G6, G7, G14, G15, G16, and G17 showed heterozygous alleles. Primer Pep C gave unique alleles of size 400bp, 500bp, 700bp, and 900bp in F_2 progenies G8, G10, G21, G23, and G24 respectively.

The similarity matrices revealed the maximum similarity between the progenies G12 and G13, G9 and G11, G9 and G30, G29 and G30. (81%). While minimum similarity was observed between progeny number G7 and G18 (45%). Most of the progenies were close to the multicut parent *i.e* near to SSG 59-3 and only three progenies were near to HJ 541 Singh and Boora. (2008) also studied the 40 SSR primer pairs in sorghum. The similarity between the two lines G84 and S247 was found to be maximum with 87% of similarity while minimum similarity was found to be 65% between G48 and G110. Dillon *et al.* (2005) also used 12 *Sorghum bicolor* derived simple sequence repeat (SSR) markers for cross-species amplification in all 25 sorghum species. The SSR markers were highly polymorphic, with diversity indices ranging from 0.59 to 0.99**.** Genetic similarity estimates ranged from 0 to 0.91, with a mean of 0.30. .The results of present study were comparable with Singh *et al*. (2008) and Dillon *et al.* (2005).

The cluster analysis grouped all the whole 32 F_2 progenies in two major clusters and revealed that twenty nine progenies grouped with parent SSG59-3 which is a multicut variety in group I while remaining three progenies were grouped in group II with the other parent HJ 541 which is a single cut variety.

Two and three dimensional principle component analysis (PCA) also gave similar clustering of 32 F_2 progenies. The progenies tended to cluster mainly in two major clusters. The progenies that were closer were more similar than those that were farther. Progeny G12 and G13, G9 and G11, G9 and G30, G29 and G30 showed maximum genetic similarity while progeny number G7 and G18 gave minimum similarity

Perumal *et al.* (2007) fingerprinted forty-six converted exotic sorghum lines representing all five races and nine intermediate races of sorghum using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers. A total of 453 scored AFLP and SSR loci were used to calculate genetic similarities between the lines. The dendrogram constructed using UPGMA grouped 31 lines into three major clusters with Jaccard coefficients greater than 0.75. The remaining 15 lines were grouped into four small sub-clusters each with two lines and seven single accession nodes. Li *et al.* (2010) searched the complete chloroplast genome sequence of sorghum line Tx623B for simple sequence repeats (SSRs). 31 SSR loci with at least 10 mononucleotide repeats or five dinucleotide repeats were identified, and primer pairs for 27 loci were designed. Chloroplast DNA variation in cultivated sorghum was investigated by using these primer pairs on 185 Chinese sorghum landraces and 70 cultivated sorghum accessions from other countries. Among the 27 loci, 14 were polymorphic. The number of alleles per polymorphic locus ranged from 2 to 5 with an average of 2.79. Allelic data at 14 polymorphic loci were combined to give 12 haplotypes.

This information thus obtained can be used for breeding of multicut varieties of sorghum in order to meet ever increasing demand of green fodder for the livestock. SSR markers would provide adequate coverage of genome for germplasm identification, pedigree validation and diversity studies. The markers could be used for identification of genotypes.

CHAPTER-VI

SUMMARY AND CONCLUSION

The present study was undertaken to study polymorphism in forage sorghum varieties *viz.*, HJ 541, SSG 59-3 and their F_2 progenies using SSR Markers.

Molecular characterization of sorghum:

32 F_2 progenies along with their parental genotypes were used for the molecular characterization using SSR markers. Total genomic DNA was extracted from the fresh young leaves by using the modified CTAB method. DNA concentration and quality was estimated by UV spectrophotometer. The DNA quality was also checked by agarose gel electrophoresis (0.8 %). The data revealed that the quantity of DNA in different progenies ranged from 450 to 1278 µg/ml. Maximum quantity of DNA was recorded in progeny G31 (1278µg/ml) while minimum quantity of DNA was recorded in one parent variety HJ 541.(450 µg/ml).

A total of 50 SSR (simple sequence repeat) markers were used to screen polymorphism among the 32 F_2 sorghum progenies along with their parents. Out of these 41 primer pairs produced amplification and 21 of them were polymorphic. The binary coding for the alleles was done as 1 or 0 for their presence or absence. These 41 primers revealed 78 alleles out of which 52 were polymorphic and 26 were monomorphic. Number of alleles ranged from 2-7 with an average value of 1.81 alleles per primer. The size of the bands varied from 110 to 1000 bp. The maximum size of the band, 1000 bp, was amplified by primer PepC while primer Xtxp 217 amplified 110 bp bands which was the minimum size of the bands obtained in these investigations. Primer Xtxp 210 gave a unique recombinant allele of size 400bp in progeny G12. Primer Pep C gave unique allele of size 400bp, 500bp, 700bp,and 900bp in F_2 progenies G8, G10, G21, G23, and G24 respectively All the data recorded was analyzed using software package NTSYS-PC. Similarity indices for pair wise combinations among all the progenies ranged from 0.45 to 0.81 as depicted by Simqual sub-programme, indicating high variability among all the genotypes. Progeny number G12 and G13, G9 and G30, G9 and G11, G29 and G30 showed maximum similarity of 0.81 whereas progeny number G7 and G18 showed minimum similarity of 0.45.

The cluster tree was broadly grouped all the F_2 progenies into 2 major groups. First cluster comprised of 29 F_2 progenies along with the parent SSG 59-3 while remaining was in second group along with the parent HJ 541. Two and three dimensional principle component analysis (PCA) also gave similar clustering of 32 F_2 progenies.

Development of multicut forage sorghum varieties/hybrids is the only way or alternate to meet the fodder requirements of ever increasing livestock population all over world. The utility of PCR-based markers such as SSRs for molecular characterization would be very valuable tools. SSR markers would provide adequate coverage of genome for germplasm identification, pedigree validation and diversity studies. So these markers could be used for identification of genotypes.

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ABSTRACT

Title of Thesis	:	**Molecular Characterization of multicut forage sorghum [S*orghum bicolor* (L) Moench] using SSR markers**
Name of degree holder	:	**ANITA YADAV**
Admission No.	:	2010BS127M
Title of degree	:	Master of Science
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Year of award of degree	:	2012
Major Subject	:	Molecular Biology and Biotechnology
Total No. of Pages in the Thesis	:	35 + vi
No. of words in the abstract	:	289

Keywords: Sorghum, SSR and genetic diversity.

Sorghum is a diverse genus belonging to the tribe Andropogoneae and family *Poaceae*. (2n=20). The present study was undertaken to analyze genetic variability among F_2 progenies of a cross between HJ541 (single cut variety) and SSG59-3 (multicut variety) by using SSR markers. Fifty SSR primers were used to study genetic variability among 32 F_2 progenies along with the parents. A total of 78 amplified DNA products were observed out of which 26 were monomorphic and 52 were polymorphic. Average percentage polymorphism across the 32 progenies was 46.2%. The number of amplified DNA bands varied from 2 to 7 with an average of 1.81 bands per primer. The size of amplified alleles ranged from 110- 1000 bp respectively. Analysis of data grouped the 32 progenies into two major clusters. Cluster I constitutes of 29 F_2 progenies and the parent SSG 59-3 whereas Cluster II had the remaining F_2 progenies and parent HJ 541. Genetic similarity based on 'Simqual' l sub programme among various progenies ranged from 0.45 to 0.81 indicating moderate genetic variability among F_2 population along with their parents. The average similarity between 32 progenies was found to be 0.66. Parental varieties were found to be most diverse followed by F_2 progenies G18 and G7 with a coefficient of 0.45. F_2 progeny G12 and G13, G9 and G11, G9 and G30, G29 and G30 were most similar with 0.81 coefficient of similarity. Two and three dimensional principle component analysis (PCA) also gives similar clustering of 32 F_2 progenies.

The highly diverse F_2 progenies may be used for breeding of multicut varieties of sorghum in order to meet ever increasing demand of green fodder for the livestock. SSR would provide adequate coverage of genome for germplasm identification and pedigree validation.

MAJOR ADVISOR **DEGREE HOLDER**

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Degree	University/Board	Year of passing	Percentage of marks	Subjects
Matriculation	HBSE, Bhiwani	2003	80%	Hindi, Eng., Math, Sci., S.S.
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B.Sc.(H)	Banasthali University, Rajasthan	2010	74%	Biotechnology (Hons.)
M.Sc. Molecular Biology and Biotechnology	CCSHAU, Hisar	2012	77.5%	Major : Molecular Biology and Biotechnology Minor : microbiology

Co-curricular activities :

Medals/ Honours received :

List of publications :

Signature of student

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I, **ANITA YADAV** Admn. No.**2010BS127M** undertakes that I give copy right to the CCS HAU, Hisar of my thesis entitled **Molecular characterization of multicut forage sorghum [*Sorghum bicolor* (L.) Moench] using SSR markers.**

I also undertake that, patent, if any, arising out of the research work conducted during the programme shall be filed by me only with due permission of the competent authority of CCS HAU, Hisar.

(ANITA YADAV)