

**DEVELOPMENT OF MICROSATELLITE
MARKERS AND MOLECULAR ANALYSIS IN
OKRA (*Abelmoschus esculentus* (L.) Moench)**

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**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE**

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MARKERS AND MOLECULAR ANALYSIS IN
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JULY, 2011

AFFECTIONATELY
DEDICATED TO
FARMING COMMUNITY
MY BELOVED PARENTS,
TEACHERS AND FRIENDS

**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
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CERTIFICATE

This is to certify that the thesis entitled “**DEVELOPMENT OF MICROSATELLITE MARKERS AND MOLECULAR ANALYSIS IN OKRA (*Abelmoschus esculentus* (L.) Moench)**” submitted by **Ms. SUJATA. C. HEBBALLI, ID No. PAK 9237** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** is a record of research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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With regardful memories.....

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(**SUJATA. C. HEBBALLI**)

DEVELOPMENT OF MICROSATELLITE MARKERS AND MOLECULAR ANALYSIS IN OKRA (*Abelmoschus esculentus* (L.) Moench)

SUJATA. C. HEBBALLI

ABSTRACT

Okra (*Abelmoschus esculentus* (L.) Moench) an annual, often cross-pollinated crop belonging to the family *Malvaceae*, is an important crop of the tropics and subtropics of the world. Simple sequence repeats (SSR's) or microsatellites are useful DNA markers in plant genetic research. Microsatellite-enriched library was constructed using DNA from Arka Anamika. A total of 71 clones were sequenced. Fourteen simple sequence repeat (SSR) loci were characterized using 10 okra genotypes. Genetic analysis showed a total of 26 alleles with an average of 1.86 alleles per SSR locus Polymorphic Information Content (PIC) values ranged from 0 to 0.375 and the observed (H_o) and expected (H_E) heterozygosity values varied from 0 to 0.111 and 0 to 0.526, respectively.

Another objective of this study was to investigate the transferability of cotton SSR markers to okra because of the availability of a large number of cotton SSR markers. Ninety cotton SSR primer pairs were used to amplify okra genomic DNA. The results showed that only 26 cotton SSR primer pairs tested in this study could amplify okra genomic DNA. Among these transferable SSR markers, 50 per cent of them detected polymorphism in okra genotypes. We obtained a total of 26 alleles with an average of two alleles per SSR locus and values of polymorphism information content (PIC) varying from 0 to 0.3750 and the observed (H_o) and expected (H_E) heterozygosity values varied from 0 to 0.52 and 0.080 to 1 respectively. Identification of transferable markers would help in okra genome research by providing additional DNA markers and also helps in comparative genome mapping study with cotton.

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IV	EXPERIMENTAL RESULTS	48-62
V	DISCUSSION	63-71
VI	SUMMARY	72-74
VII	REFERENCES	75-86

LIST OF TABLES

Table No.	Title	Page No.
1	Composition of various reagents used in DNA isolation	23
2	List of the genotypes used in SSR analysis	37
3	List of the genotypes used in cross amplification study	42
4	List of the cotton SSR primers used in cross amplification	44-46
5	Microsatellite primers designed from sequence data of DNA fragments from enriched genomic library	52
6	Genetic analysis of okra SSR primers in 10 genotypes	53
7	Distance matrix (Squared Euclidean Distance) for 10 okra genotypes based on SSR data	55
8	Polymorphic bands generated in 25 okra genotypes generated by 26 cotton SSR primers	58
9	Distance matrix (Squared Euclidean Distance) for 25 okra genotypes based on cotton SSR data	60
10	Genetic analysis of cotton SSR's in okra genotypes	61

LIST OF FIGURES

Fig. No.	Title	Between Pages
1	Dendrogram analysis of ten okra accessions using SSR marker data.	54-55
2	Dendrogram analysis of 25 okra accessions using cotton SSR marker data.	59-60

LIST OF PLATES

Plate No.	Title	Between Pages
1	Genomic DNA of okra (Arka Anamika)	48-49
2	Restriction enzyme digestion of total genomic DNA	48-49
3	Gel profile after linker ligation	49-50
4	Gel profile after first PCR enrichment	49-50
5	Gel profile after second PCR enrichment	49-50
6	Gel profile of colony PCR and plasmid isolation	50-51
7	SSR gel profile of 10 okra genotypes using SSR primers	52-53
7a	SSR gel profile of okra genotypes using primer 10K	52-53
7b	SSR gel profile of okra genotypes using primer 30K	52-53
7c	SSR gel profile of okra genotypes using primer 80K	52-53
8	PCR optimization with different annealing temperature	56-57
8a	Gel profile showing PCR products amplified at 50°C annealing temperature	56-57
8b	Gel profile showing PCR products amplified at 53°C annealing temperature	56-57
8c	Gel profile showing PCR products amplified at 57°C annealing temperature	56-57
9	SSR marker gel profile showing 25 okra genotypes using cotton SSR primers	57-58
9a	SSR marker gel profile showing 25 okra genotypes using BNL285 SSR primers	57-58

Plate No.	Title	Between Pages
9b	SSR marker gel profile showing 25 okra genotypes using BNL2544 SSR primers	57-58
9c	SSR marker gel profile showing 25 okra genotypes using BNL358 SSR primers	57-58
9d	SSR marker gel profile showing 25 okra genotypes using BNL226 SSR primers	57-58
9e	SSR marker gel profile showing 25 okra genotypes using BNL827 SSR primers	57-58
9f	SSR marker gel profile showing 25 okra genotypes using BNL2960 SSR primers	57-58

INTRODUCTION

I. INTRODUCTION

Okra (*Abelmoschus esculentus* (L.) Moench) is a tropical summer vegetable, often cross-pollinated crop belongs to the family Malvaceae. It is an important crop of the tropics and subtropics of the world. Okra is also known as Lady's finger in English, *gumbo* in French, *bhendi* in Hindi and *bamiah* in Arabic. The species is annual and related to such species of cotton, cocoa, jute, mesta and hibiscus.

It is traditional vegetable crop commercially cultivated in West Africa, India, South East Asia, the southern United States, Brazil, Turkey and Northern Australia and is also popular home-garden in many areas. In India, it is popular green vegetable commonly grown for its tender fruits in tropics, subtropics and warmer parts of temperate region. India is the largest producer of okra covering an area of 432.0 (in 000' ha) with an annual production of 4528 (in 000' MT) and productivity of 10.5 MT/ha (NHB database, 2009). The world okra production was 4.8 million tonnes, but the most of the production was in India (70 per cent) followed by Nigeria (15 per cent), Pakistan (2 per cent), Ghana (2 per cent), Egypt (1.7 per cent) and Iraq (1.7 per cent) (Gulsen *et al.*, 2007). In India, it is cultivated in almost all the states and major area is covered in states like, Uttar Pradesh, Bihar, West Bengal, Gujarat, Maharashtra, Andhra Pradesh, Tamil Nadu and Karnataka. Where, Karnataka occupies an area of 7700 ha with an annual production of 63, 200 tonnes and productivity of 8.2 tonnes/ha (Anon, 2008).

Africa is the centre of origin of okra. The genus *Abelmoschus* includes about 30 species in the old world, four in the new world and four in Australia. It is having highest chromosome number of $2n=130$, is polyploidy in nature (Joshi *et al.*, 1956). They further observed that *A. esculentus* arose through hybridisation of one species with $n=29$ and

another with $n=36$ followed by doubling of the chromosome number. The progenitor of *A. esculentus* with $n=29$ is reported to be *A. tuberculatus*.

Okra has several uses. Its tender fruits are used as vegetable, eaten boiled or in culinary preparations as sliced and fried pieces. It is also used in thickening of soups and gravies because of its high mucilage content. In addition to fruits, leaves are also consumed in some African countries (Charrier, 1984; Hamon, 1986). Medicinal properties have been reported in some species (Velayudhan and Upadhyay, 1994). It has good nutritional value, as it contains 1.9 g of protein, 1.2 g of fibre, 1.5 mg of Fe, 90 mg of Ca, 88 IU vitamin A and 30 mg of vitamin C as per 100 g of edible part (Pal *et al.*, 1952). The high iodine content of fruits is useful in curing goitre disease.

The development and use of molecular marker technologies have found various applications in the field of biotechnology for improvement of crops (Prince *et al.*, 1993). Various applications include diversity analysis, mapping, gene tagging, QTL analysis, paternity analysis, finger printing and genotype characterization of plants. Markers such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeats) and SNP's (Single Nucleotide Polymorphisms) have been employed in various studies in crops.

Molecular markers have been widely used in genetic analysis and breeding of plant species, with a multiple of applications. Among the various types of molecular markers available, microsatellites or SSR's have received a greater attention recently, especially for breeding purposes. Microsatellite markers (Litt and Luty, 1989), also known as simple sequence repeats or SSRs are clusters of short (usually 2 to 6) tandemly repeated nucleotide bases distributed throughout the genome. Microsatellite markers distinguish themselves as co-dominant,

multiallelic, highly polymorphic genetic markers, requiring small amounts of DNA for straight forward PCR and gel electrophoresis analysis.

These are ranges from one to six nucleotides in length (Van Oppen *et al.*, 2000) and are classified as mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats and are further classified as simple perfect, simple imperfect, compound perfect or compound imperfect (Roy *et al.*, 2004). Simple-perfect repeats are tandem arrays of a single repeat sequence (e.g., [AGG]_n); whereas, simple imperfect arrays consist of one or more repeat units of different lengths (e.g., [AAC]_n [ACT] [AAC]_{n+1}). Compound-perfect arrays are composed of two or more different repeat motifs of the same length (e.g., [AGG]_n[AATC]_n) and compound-imperfect motifs are interrupted by one or more repeats of different length (e.g., [GGAT]_n[ACT][GTAA]_{n+1}). Its main disadvantage is the high cost of the initial investment necessary for marker development. Microsatellites are thought to be generated by the slippage of one DNA strand during replication of double-stranded DNA or by unequal crossing over during meiosis (Ellegren, 2004).

Microsatellites are actually the most efficient markers, although limited in use due to the long and laborious steps of development. There are several methods to develop microsatellite markers. The traditional method is done by screening the genomic libraries using SSR motifs as probes. The use of SSR-enriched libraries with selective hybridization of DNA fragments using streptavidin coated magnetic beads or nylon membrane gives a large number of clones containing microsatellites. The other method for development of SSR marker is based on searching for sequences containing microsatellites deposited in the data bases (EMBL, GenBank) and this method also used in okra related species like, cotton (Qureshi *et al.*, 2004), Hibiscus (Bruna *et al.*, 2009) and other plant

species, including sugarcane (Pinto *et al.*, 2004), rice, wheat and barley (Rota *et al.*, 2005). Although the development of microsatellite marker is time consuming and costly, they have advantages over RFLP, RAPD and AFLP markers, on several aspects, i.e., being PCR-based, multi-allelic, high reproducibility, codominance, fast and easy assaying of genotypes. They have been efficiently applied in genetic diversity studies, population analysis, genotyping and fingerprinting of individuals, cultivar discrimination, genetic mapping and marker-assisted selection (MAS) in several plant species.

Microsatellite DNA loci have become important sources of genetic information for variety of purposes. To amplify microsatellite loci by PCR, primers must be developed from the DNA that flank specific microsatellite repeats. These regions of DNA are among the most variable in the genome, thus primer-binding sites are not well conserved among distantly related species (Moore *et al.*, 1991; Zhu *et al.*, 2000; Primmer *et al.*, 1996).

SSR primers developed for one particular species are normally applicable across wide range of related species. DNA markers could be transferable among related species due to the conserved regions in their genomes. Microsatellites first have to be generated from a species of interest and then their usefulness as a polymorphic genetic marker has to be demonstrated. Some economically important species have enough interest so that the financial resources for marker development are available; whereas, many minor species do not have this luxury. Researchers, however, can test whether the SSR markers developed in other species will work in a related species of their interest. This intention may be the major driving force for testing of transferable SSR markers. This situation is especially true for germplasm characterization and evaluation (Wang *et al.*, 2009).

Recently, comparative genetics revealed that gene content and order are highly conserved among closely related species. Thus, primer pairs designed on the basis of the sequences obtained from one species could be used to detect SSRs in related species. This process depends on the conservation of priming sites within the flanking sequences to enable amplification, and on the maintenance of repeat arrays long enough to promote polymorphism. SSR transferability across related species and genera makes these markers powerful for comparative genetic studies. However, study of the development on SSR markers has not been done.

Keeping this in view, the present work envisaged is the first of its kind with the following objectives.

1. To develop SSR markers in okra.
2. To study cross amplification in okra by using cotton SSR primers.
3. To assess genetic diversity using microsatellite markers.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The use of molecular markers to track loci and genome regions in crop plants is now routinely applied in many breeding programs. The location of major loci is now known for many disease resistance genes, tolerances to abiotic stresses and quality traits. For markers to be effective, they must be closely linked to the target locus and be able to detect polymorphisms in material likely to be used in a breeding program (Langridge and Chalmers, 2004). The use of molecular markers, in plant breeding has advantages over conventional breeding methods in that it can significantly decrease the time required to breed an improved cultivar (Yousef and Juvik, 2001). Marker-assisted selection (MAS) and cultivar identification have been used in plant breeding programs and cultivar commercialization. The potential efficiency of MAS depends upon the heritability of the trait, the proportion of genetic variance explained by the markers, and the selection method.

Some molecular markers are increasingly being used for germplasm diversity, linkage analysis and molecular breeding. Despite these advantages the number of molecular markers reported in okra is considerably less compared to many crop species, signifying the need for expanding the repertoire of these genetically highly informative markers.

A review of relevant research work on okra, its related species and other crops carried out in India and elsewhere is presented under different headings in this chapter as follows.

2.1 Development of molecular markers and estimation of genetic diversity for crop improvement

The concept of genetic marker is not a new one; Gregor Mendel used phenotypic based genetic marker in his experiment in the 19th

century. Later, phenotypic based genetic markers for *Drosophila* led to the establishment of the theory of genetic linkage. The phenotypic based genetic markers led to the development of more general and useful direct DNA based markers that became known as molecular markers. A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. Molecular markers may or may not correlate with phenotypic expression of a trait and it also offers numerous advantages over conventional phenotypic based marker as they are stable and detectable in all tissues regardless of growth, differentiation, development, or defence status of the cell, not confounded by the environment, pleiotropic and epistatic effects.

An ideal molecular marker should have the following criteria: (1) be polymorphic and evenly distributed throughout the genome; (2) provide adequate resolution of genetic differences; (3) generate multiple, independent and reliable markers; (4) simple, quick and inexpensive; (5) need small amount of tissue and DNA samples; (6) have linkage to distinct phenotypes and (7) require no prior information about the genome of an organism (Agarwal *et al.*, 2008).

The information available on different kinds of molecular markers in okra as well as other crops is reviewed here with special emphasis on microsatellite markers.

2.2 Different types of markers

2.2.1 Morphological markers

Morphological traits are the oldest and most widely used markers and they may still be optimal for certain management practices of germplasm and cultivars, where these have been identified on the basis of leaf, panicle, fruit and other physical characteristics. Even though it is

affected by environmental conditions, poor polymorphic and due to dominant nature, these markers are not widely used in diversity studies.

In a study, Gulsen *et al.*, (2007) 33 heritable traits were evaluated in field with ten replications, 28 of them (85per cent) were found polymorphic. The UPGMA (unweighted-pair group method arithmetic average) dendrogram based on the 33 phenotypic markers distinguished all genotypes, but failed to detect any geographic association of okra genotypes.

2.2.2 Biochemical markers

The most commonly used biochemical marker is protein marker isozymes. Functional enzymes that are multimeric in nature normally exist in different molecular forms. It has been recognised that while retaining substrate specificity these forms are distinguishable based on electrophoretic mobility. Isozyme loci are co-dominant, as alleles are identifiable as distinct bands on the gel. The major limitation of isozyme is the small number of loci that can be detected. Isozymes as genetic markers have been proven to be reliable, in crops like avocado (Torres and Bergh, 1980; Baily, 1983), citrus (Torreas *et al.*, 1982), apple (Weeden and lamb, 1985) and cherimoya (Elistrand and Lee, 1987).

Tarkpo *et al.*, (2006) assayed 22 accessions of okra (*Abelmoschus esculantus*) from Ghana, for diversity in esterases, total protein and total storage proteins. Isozyme markers were used for genetic diversity analysis. The phenogram produced by the UPGMA of the Jaccard similarity matrix data revealed three clusters.

2.2.3 DNA based markers

DNA based assays have revolutionised and modernised our ability to characterise genetic variation. The first advantage of molecular

techniques is their capacity to detect genetic diversity at a higher level of resolution than other methods. Furthermore DNA based assays are robust, speedy, information may be obtained from the little amounts of plant material at any stage of development and it is not affected by environmental conditions. A large number of different molecular techniques are at present available and each of them differs in its informational content. Some of the techniques are discussed below.

2.2.3.1 Restriction Fragment Length Polymorphism (RFLP) markers

Restriction fragment length polymorphism is the first DNA marker, and was developed in the late 1970's (Botstein *et al.*, 1980). The development of this technique was facilitated by the discovery of restriction enzymes. It is a co-dominant marker where DNA is digested with restriction enzymes; the fragments are separated based on size using agarose gel electrophoresis. Then transferred in denatured form to a nylon membrane, which is then fragments are incubated in a solution containing a probe previously labelled, either radioactively or chemically for hybridisation. RFLPs were first used by Grodzicker *et al.*, (1974) in retrovirus and later found its applications in many crop improvement programmes. RFLPs have also been used to estimate genetic diversity in several crops like tomato (Peterson *et al.*, 1988), pepper (Tanksley *et al.*, 1989) and lettuce (Landry *et al.*, 1985). However, RFLP analysis is expensive, laborious, time consuming involves the use of hazardous radioactive isotopes and need large amount of good quality DNA.

2.2.3.2 Random Amplified Polymorphic DNA (RAPD) marker

Its simplicity and low cost involvement has made it very handy and widely used techniques by geneticists. It is now being used extensively in place of RFLP (Welsh and Mc Cleland, 1990; Williams *et al.*, 1993). This detects polymorphism by use of a single primer of arbitrary to nucleotide. The amplified products are separated by agarose gel electrophoresis. The

major advantage of this assay over RFLP is that, there is no need for prior information on the genomic DNA sequences, universal set of primers can be used for a variety of species, and the process can be automated.

Aladele *et al.*, (2008) studied the 93 accessions of okra comprising of 50 West African genotypes (*Abelmoschus caillei*) and 43 Asian genotypes (*A. esculentus*) were assessed for genetic distinctiveness and relationships using RAPD. Molecular analysis showed that all 13 primers were used, revealed clear distinction between the two species. Six duplicates were discovered while accession TOT7444 distinguished itself from two okra species.

Samarajeewa and Rathnayaka, (2004) studied genetic variation between the *Abelmoschus* accessions by using RAPD marker. In this study, 10 primers were screened. These primers produced a total number of 130 polymorphic bands and data was used to construct dendrogram for phylogenetic study.

Martinello *et al.* (2001) investigated genetic diversity in 39 *Abelmoschus* spp. accessions at the DNA level with the 31 random amplified polymorphic DNA (RAPD) primers and at the phenotypic level with stable and highly heritable morphological characters. The 103 RAPD fragments were generated. Dendrograms were generated for genetic distance based on RAPD marker.

Prakash *et al.* (2011) studied 44 okra genotypes collected from different parts of India, and we assessed genetic distinctiveness and relatedness using RAPD markers. Total genomic DNA was extracted and subjected to RAPD analysis using 14 arbitrary 10 mer primers. The molecular analysis showed that all the fourteen primers used revealed clear distinction between the genotypes. They generated a total of 104

RAPD bands most of which were polymorphic across accessions (74.03 per cent). The number of bands resolved per amplification was primer dependent and varied from 4 (OPV-07, OPV-08) to 11 (OPD-05) with average of 7.41 number of bands per primer. The RAPD data was used to calculate a Squared Euclidean Distance matrix, and based on this data, cluster analysis was done using minimum variance algorithm. The cluster analysis showed two major groups. Each sub-group was characterized using morphological and genetic characteristics of the respective genotypes.

Overall, so far, the RAPD markers were used only for genetic diversity analysis and also to group *Abelmoschus* accessions.

2.2.3.3 Sequence Related amplified polymorphism (SRAP)

The SRAP technique (Li and Quiros, 2001) consists of preferential amplification of ORFs using PCR. For this purpose, combinations of two types of primers were employed. The first type of primer (forward) is 17 bp long, and contains a fixed sequence of 14 nucleotides rich in C and G, and three selective bases at the 3' end. This primer preferentially amplifies exonic regions, which tend to be rich in these nucleotides. The second type of primer (reverse), with 18 bp, contains a sequence of 15 nucleotides, rich in A and T, and three selective bases at the 3' end. These primers preferentially amplify intronic regions and regions with promoters, rich in these nucleotides

Gulsen *et al.* (2007) described the use of SRAP marker for determining diversity and genetic relationships among 23 okra genotypes. The 39 combination of SRAP primers were used to evaluate the 21 Turkish and 2 USA genotypes as out groups, and produced 97 scorable markers, of which 50 per cent was polymorphic for all 23

genotypes. 17 out of 23 genotypes (74 per cent) were distinguished with each other of mean similarity 0.93.

2.2.3.4 Simple sequence repeats (SSR) markers

Microsatellites are tandem repeats of DNA sequences of only a few base pairs (1-6 base pairs) in length, the most abundant being the dinucleotide repeats. These are most important class of markers which are abundant with long hyper variable region. (CA)_n repeat is one of the most frequently occurring microsatellite in human and many mammalian genomes. In contrast (AT)_n is more abundant in plants. Variation in the number of tandemly repeated core nucleotide sequences at SSR locus among different genotypes provides the basis for polymorphism that can be used in genetic studies. The DNA sequences flanking SSRs are conserved and these conserved sequences have been used in designing suitable primers.

2.2.3.4.1 Development of microsatellite markers

Reddy *et al.* (2001) initiated to streamline the process of microsatellite capture and characterization, development of microsatellites in cotton into informative molecular markers, and dissemination of marker information to the cotton research community. A simple and efficient biotin capture method was optimized and used to capture more than 10,000 fragments. Out of 588 fragments sequenced, nearly all contained a microsatellite repeat structure. Several repeat types were represented, including AGA, GA, CA, and ACA. Primers were designed to amplify 307 unique microsatellite loci (305 nuclear and two chloroplast encoded). One hundred fifty-two microsatellite loci were amplified from *G. hirsutum* L. cv. TM-1 and Tamcot SP37, and *G. barbadense* L. cv. Pima 3-79 and Pima S-7. In this comparison, 74 of the primers (~49 per cent of the subset) showed detectable polymorphism. In a comparison of upland *G. hirsutum* cultivars, ~26 per cent of the

primers exhibited intraspecific polymorphism. Polymorphism was widely distributed among the various repeat types and structures (e.g., imperfect and compound repeats). Redundancy with two other previously derived microsatellite marker sets (BNL, CM) was low, implying that the total pool of microsatellites present in the cotton genome is large enough to satisfy the requirements of extensive genome mapping and marker-assisted selection projects.

Ritschel *et al.* (2004) developed seven hundred clones in cucumber, containing microsatellite sequences from a *Tsp*-AG/TC microsatellite enriched library were identified and one-hundred and forty-four primer pairs designed and synthesized. When 67 microsatellite markers were tested on a panel of melon and other cucurbit accessions, 65 revealed DNA polymorphisms among the melon accessions. For some cucurbit species, such as *Cucumis sativus*, up to 50 per cent of the melon microsatellite markers could be readily used for DNA polymorphism assessment, representing a significant reduction of marker development costs. A random sample of 25 microsatellite markers was extracted from the new microsatellite marker set and characterized on 40 accessions of melon, generating an allelic frequency database for the species. The average expected heterozygosity was 0.52, varying from 0.45 to 0.70, indicating that a small set of selected markers should be sufficient to solve questions regarding genotype identity and variety protection. Mapping analysis was initiated with 55 newly developed markers and most primers showed segregation according to Mendelian expectations. Linkage analysis detected linkage between 56 per cent of the markers, distributed in nine linkage groups.

Wang *et al.* (2006) selected EST-SSRs for cotton gene mapping and characterized functional markers in *Gossypium raimondii*, which consisted of 58,906 non-redundant EST sequences from NCBI. Among

them there were 2620 microsatellite sequences containing 2,818 EST-SSRs, which amounted to 4.45per cent of the non-redundant starting sequence population. This incidence was equivalent to one EST-SSR in every 14.8 kb of *G. raimondii* genetic material. Among the different motifs ranging from 1 to 6 bp, trinucleotide repeats were most abundant (38.31per cent), followed by dinucleotide repeats (24.09per cent) and mononucleotide repeats (23.35per cent). Among all identified motif types, A/T had the highest frequency (18.67per cent), followed by AT/TA (14.83per cent). Among the compound motifs, tandem trinucleotides occurred with the highest frequency (48.65per cent). In all, they identified 1,554 EST-SSRs primer pair sequences. 300 of them were randomly selected to screen the polymorphisms between the mapping parents *G. hirsutum* accession. TM-1 and *G. barbadense* cv. Ha7124, to construct linkage groups in cultivated allotetraploid cotton. Among them, 129 (43per cent) primer pairs were found to have polymorphisms. Using these EST-SSRs they compare EST-SSR distributions among different cotton species and various chromosomal locations.

Xiao *et al.* (2009) reported that SSR markers are very useful for saturation of the large and complex upland cotton (*Gossypium hirsutum* L) genetic linkage map. Genomic clones from microsatellite-enriched cotton DNA libraries were sequenced to identify SSR containing target regions and SSR-containing EST collections were searched. PCR primers pairs were generated for 5,475 target sequences and utilised to amplify SSR marker loci which provide useable levels of polymorphism in interspecific and intraspecific genetic population.

Reyazul *et al.* (2009) developed 2469 simple sequence repeats (SSRs) in tossa jute (*Corchorus olitorius*) JRO 524 using four SSR-enriched genomic libraries. A random subset of 100 SSRs (25 SSRs from each library) was used to detect polymorphism between the parental

genotypes of each of the two recombinant inbred line (RIL) mapping populations. The RILs were being developed from JRO 524 × PPO4 (for fiber fineness) and JRC 321 × CMU 010 (for lignin content) crosses to prepare molecular maps and conduct quantitative trait loci (QTL) analyses. Both SSR length polymorphism and null alleles (presence and absence of specific SSR) were detected; 50 SSRs detected polymorphism between the two genotypes of tossa jute, whereas 45 SSRs detected polymorphism between the two genotypes of white jute (*Corchorus capsularis*). This SSR allelic polymorphism in jute is higher than that reported in other crops and is adequate for construction of genetic maps for QTL analysis.

Arunita *et al.* (2010) screened a total of 177 SSR markers using a set of 47 upland cotton genotypes comprising 14 commercial varieties, 14 germplasm accessions and 19 advanced breeding lines to identify informative markers for genetic diversity assessment and fingerprinting in *Gossypium hirsutum*. Only 21 per cent (38/177) of SSR markers tested showed polymorphism with mean of 2.18 alleles per locus and with average polymorphism information content (PIC) of 0.32. The SSR markers revealed a jaccards similarity coefficient ranging between 0.43 and 0.89, with an average of 0.67 among accessions. Cluster analysis using unweighted pair group method with arithmetic average (UPGMA) and principal component analysis (PCA) indicated that majority of the genotypes were very closely related. All the genotypes showed heterozygosity for at least one of the SSR loci. They discovered 19 rare and 6 unique alleles among the tested genotypes in cotton. Fingerprinting based on all 38 loci revealed a probability of identical match by chance of 3.98×10^{-8} . A set of 10 SSR markers was identified which could distinguish all the 47 genotypes with a moderate probability of identical match by chance.

Pritesh *et al.* (2010) described the use of SRR marker for determining the genetic distance information in tomato; this might be useful to breeder for planning crosses among these cultivars. This analysis will be useful in the selection of parental genotypes for mapping populations and breeding programmes attempting to broaden the genetic base of future tomato cultivars. In particular, this opens up significant opportunities for the development of interspecific mapping populations that will be highly relevant to modern tomato breeding programmes.

Overall the development of microsatellite markers can be based on DNA sequence information deposited in databases, or it could be based on the screening of genomic DNA libraries specifically constructed for the discovery of repeated sequences in the genome. The use of genomic libraries enriched for microsatellite sequences is a strategy devised to decrease the cost of marker development while increasing the opportunity for marker discovery. The development of microsatellite markers will have a major impact on genetic analysis and breeding of several crops, especially on the generation of marker saturated genetic maps and implementation of marker assisted breeding programs. Genomic microsatellite enriched libraries can be an efficient alternative for marker development in such species.

2.2.3.4.2 Cross species transferability of microsatellites for estimation of genetic diversity.

Sawadogo *et al.* (2009) reported that sixteen cross species SSR primers of *Medicago truncatula* were used to amplify genomic DNA samples of 20 different okra accessions collected from the different regions of Burkina Faso. These primers amplified a number of fragments that range from 1-16 with the sizes of 396-506bp. Each data clustered the 20 accessions into 5 different groups. Primer MT-27 amplified a unique 440bp PCR product in two okra accessions. Data indicates cross

species of SSR primer can effectively used for to analyze genetic diversity in unrelated species, like okra.

Ruas *et al.* (2009) isolated and characterized ten microsatellite loci for *Luehea divaricata*, a South American outcrossing pioneer tree species (Malvaceae) that is frequently used in reforestation programs of tropical riparian forests in Brazil. A total of 45 alleles were detected across a sample of 42 individuals, with an average number of 4.5 alleles per locus. The average polymorphic information content (PIC) was 0.546 and the observed (H_o) and expected (H_e) heterozygosity values varied from 0 to 0.929 and 0.194 to 0.821, respectively. Four loci exhibited significant deviation from Hardy-Weinberg equilibrium ($P \leq 0.001$) and 24 pair combinations of the ten loci showed significant linkage disequilibrium ($P \leq 0.01$). The primers were tested for cross amplification in nine species of the Malvaceae family. These preliminary results demonstrate the usefulness of these microsatellite loci for assessing the genetic structure of *L. divaricata* and related genera.

Khar *et al.* (2010) studied diversity assessment of tropical Indian onion and cross amplification of genomic and EST-SSR markers in distantly related native wild species. Out of 60 SSRs, 10per cent genomic SSRs were able to amplify as compared to EST-SSR where 53.3per cent primers were able to reveal amplicons. Clustering revealed five groups and indigenous short day onion formed separate cluster from the exotic short day and long day onions. *A. roylei* accessions exhibited a dissimilarity index of 35.5per cent between themselves whereas 'Pran' and *A. proliferum* grouped together at a genetic distance of 0.4. *A. fistulosum* accessions formed a tight cluster and distantly related wild species *A. carolinianum*, *A. chinense* and *A. hookeri* formed neutral nodes. These results are important to aid in analyzing the diversity present in short day onion and its utilization in breeding programs. This was first

time that three accessions of *A. roylei* Stearn were used and rare alleles amplified in wild species will aid in detecting population structure and molecular marker aided selection for interspecific hybrid development.

Bruna *et al.* (2009) reported the development of 10 microsatellite markers in *Hibiscus rosa-sinensis* (Malvaceae). Three markers were obtained from sequences available in Gen- Bank and seven were isolated using a two-step 'primer extension' procedure, based on the microsatellite-AFLP (M-AFLP) technique. Polymorphism was explored in 21 *Hibiscus rosa-sinensis* genotypes representing the genetic variation within commercial varieties. Inter-specific amplification was assessed on 12 *Hibiscus* wild species. A total of 45 and 56 alleles (ranging from 1 to 10 for each locus) were amplified respectively from the 21 *Hibiscus rosa-sinensis* varieties and among the full *Hibiscus* spp. genotype set. Primers and conditions for polymerase chain reaction (PCR) amplification of the detected loci are reported.

Sim *et al.* (2005) investigated the transferability of 12 lychee (*Litchi chinensis* Sonn.) simple sequence repeat (SSR) loci to pulasan (*Nephelium ramboutan-ake* L.), they are from the same family and closely related, therefore the SSR markers are expected to be highly transferable between these two taxa. Genomic DNA was extracted from 20 accessions of pulasan for the PCR amplification of the SSR loci using 12 pairs of SSR primers derived from lychee. The PCR products were resolved on denaturing polyacrylamide gels. The percentage of SSR transferability from lychee to pulasan was 58.3per cent and the percentage of polymorphic SSR markers was 25per cent. The moderate transferability and low polymorphism rates suggest the possibility of interruptions within the repeat motif and mutations in the flanking sequences of SSR repeat motifs. Their results did not reveal a high rate of transferability between lychee and pulasan. However, this study showed that the SSR

markers developed in lychee are a good source of molecular markers for pulasan.

Akter *et al.* (2008) generated DNA fingerprint using jute specific SSR markers on 10 jute (Malvaceae) cultivars from two *Corchorus* species (*C. olitorius* and *C. capsularis*) and the genetic relatedness among the cultivars was estimated. A total of 106 alleles were identified using 23 primer pairs among the 10 cultivars with an average of 4.61 ± 1.92 alleles per locus, and a mean genetic diversity of 0.68 ± 0.16 . Four *C. olitorius* cultivars could be easily distinguished with 6 markers using 5 primer pairs and six *C. capsularis* cultivars with 7 markers using 6 primer pairs. The UPGMA analysis enabled the grouping of the cultivars into two major clusters, which matched with the known information on jute. This experiment provides proof that in spite of low levels of genetic polymorphisms among jute cultivars, SSR markers can reliably distinguish among them. This finding reinforces the utility of SSR primers for providing unique genetic identities or fingerprints of various jute cultivars.

The transferable SSR markers can be using for genetic and comparative mapping, assessment of genetic diversity and phylogenetic analysis, marker-assisted selection and cultivar identification, and homologous gene identification and cloning. There are several potential drawbacks including the presence of stutter bands (or peaks), null alleles (Wattier *et al.*, 1998; Dakin and Avise, 2004) and heterologous amplicons (Whitton *et al.*, 1997).

Overall the SSRs markers will be useful in studying genetic diversity, population structure, and association mapping. The use of microsatellites, as well as other molecular markers, in plant breeding has advantages over conventional breeding methods in that it can significantly decrease the time required to breed an improved cultivar

(Yousef and Juvik, 2001). Microsatellite markers, for example, can be used at any stage of a plant's development. They do not vary with the environment, and can be used to detect heterozygosity. They are also useful in finding genes that contribute to polygenic traits (Prasad *et al.*, 2003).

2.2.3.5 Single nucleotide polymorphism (SNP) marker

The vast majority of differences between individuals are single nucleotide polymorphisms due to point mutations. As such, there are a vast number of potential SNP markers in all species. Considerable amounts of sequence data required from parental genotypes to develop SNP marker, however, their great advantage lies in the potential to screen them using methods such as microarrays, which do not involve electrophoresis. SNPs can also be used for fingerprinting and the generation of genetic maps, although these techniques frequently employ marker techniques that do not require any knowledge of genetic sequence (Gupta *et al.*, 2001). SNPs can mark functionally important allelic differences. They are employed to genetic mapping in potato and tomato (Joy *et al.*, 2007), male sterility in onion (Alcala *et al.*, 1997) etc.

2.2.3.6 Amplified fragment length polymorphism (AFLP) marker

Amplified fragment length polymorphism involves digestion of DNA with the restriction enzymes as in case of RFLP and PCR amplification. AFLP combines the reliability of RFLP with the power of PCR technique. PCR amplification of restriction fragments is carried out using oligonucleotide adapters (Vos *et al.*, 1995). Fingerprints can be produced without prior knowledge using limited sets of generic primers. This method generates large number of DNA fragments facilitating detection of polymorphism. AFLPs are also dominant and can detect large number of polymorphisms. They are employed to detect genetic diversity in common bean (Adom Blendon *et al.*, 1994; Meletto *et al.*, 1996) and other crops

like tomato (Ohmori *et al.*, 1994) and strawberry (Haymes *et al.*, 2000) etc.

The literature search evaluated that there are no studies on development of SSR markers in okra, and also cross species transferability of SSR from malvaceae species for okra.

MATERIAL AND METHODS

III. MATERIALS AND METHODS

The investigation on development and assessment genetic diversity in okra using microsatellite marker was undertaken during the year 2010-2011. The details of experiments, materials used and techniques adopted in the present investigation are presented in this chapter.

3.1 Experiment I: Development of microsatellite markers in okra

3.1.1 DNA isolation

3.1.1.1 Sample preparation

Plant material:

Young, healthy third to fourth leaf of Okra genotype (Arka Anamika) and also 10 genotypes of IIHR series (Table. 2) were collected from the field in an aluminium foil and washed thoroughly with distilled water and air dried to remove moisture. 100mg of leaf material was taken for DNA extraction.

3.1.1.2 Isolation of genomic DNA by CTAB using “mini-prep” method

Okra leaves are rich in polysaccharides and phenols, which interferes with the isolation and purification of DNA samples (Huang *et al.*, 2008). The main obstacle preventing the extraction of DNA from green leaves of all members of genus is that, even after grinding in liquid nitrogen, the resulting suspension is very sticky and was almost impossible to obtain DNA. This thick consistency is principally due to large amount of polysaccharides produced during photosynthesis. The polysaccharides contamination can also cause problems in downstream processing/application by inhibiting certain enzymes in PCR (Hugo *et al.*, 1998). Hence, different DNA isolation protocol was standardised during the investigation.

3.1.1.3 Reagents used for DNA isolation.

Table 1: Composition of various reagents used in DNA isolation

1. Extraction buffer	1.86g Na EDTA (20mM) and 3.03g Tris HCl (100mM). Dissolve both these in small quantities of water, mix and adjust pH to 8.0, to this add 1.4 mM NaCl (20.45g) and 2per cent w/w CTAB (Cetyl Trimethyl Ammonium Bromide 5g) by heating to 60 ^o C. Make up the volume to 250 ml and stored at 37 ^o C. Add 0.2per cent of β -mercapto ethanol just before use.
2. TBE buffer (10X)	54g Tris base, 27.5g Boric acid, 7.44g EDTA (pH 8.0). Dissolve these in 500ml water and sterilize. Working solution 1X.
3. TE buffer	1.211g Tris HCl (10mM), 0.372g Na EDTA (1mM). Dissolved separately, mixed and made up the volume to 1 litre, pH to 8.0 and autoclaved.
4. Chloroformisoamyl alcohol	24:1 v/v
5. NaCl	5M (146.1g in 500ml and autoclaved)
6. Ammonium acetate	7.5M (adjust the pH 7.7 and autoclaved)
7. Wash solution	76per cent Ethanol (v/v); chilled
8. Alcohol	95per cent Ethanol; stored at -20 ^o C
9. Absolute alcohol	99.9per cent Ethanol; stored at -20 ^o C
10. RNA ase	10mg/ml; dissolve RNA ase in 10mM Tris HCl + 15mM NaCl, pH 5.0, boil for 5 minute and cool to room temperature
11. PVPP	A pinch of required
12 Bromo phenol blue	Stock solution: 0.25per cent in 50per cent glycerol Working solution: 200 μ l stock + 50per cent glycerol
13. Ethidium bromide	0.5mg/ml

Extraction protocol:

1. 1ml extraction buffer with 5 μ l of 0.5per cent β -mercapto ethanol preheated to 60°C.
2. 100mg tissue was grand to fine powder using liquid nitrogen. 50mg PVPP and 20mg sodium metabisulphate added and mixed, the contents transferred into microfuge tube containing 1ml CTAB buffer pre-heated to 60°C and shaken gently.
3. Tubes were incubated for 1hr at 60°C, shaken intermittently for every 10 minutes and cooled to room temperature.
4. 1ml of CHCl₃: Iso-amyl alcohol (24:1) solution was added and mixed gently by inverting tubes about 25 times to form an emulsion.
5. Emulsion was spinned at 10,000 rpm for 10 minutes and transferred aqueous phase to new centrifuge tubes using cut tips. If supernatant was still cloudy equal volume of supernatant with CHCl₃: Iso-amyl alcohol was added and repeated the centrifugation step.
6. Transferred clear aqueous phase to fresh microfuge tubes. Equal volume of potassium acetate mixture (2.5mM) and CHCl₃: Iso-amyl alcohol was added and mixed gently by inverting tubes about 25 times to form an emulsion.
7. Centrifuged at 10,000 rpm for 10 minutes and aqueous phase was transferred to new microfuge tubes using cut tips.
8. Equal volume of cold isoproponol was added and kept at -20⁰ C for one hour.
9. Centrifuged at 10,000 rpm for 5 minutes and supernatant poured off and pellet was washed with 1ml of absolute ethanol (99.9per cent) was added to aqueous phase and kept at -20⁰ C for one hour.

10. Centrifuged at 10,000 rpm for 5 minutes and aqueous phase was transferred to new microfuge tubes.
11. Again pellet was washed with 200 µl of 76per cent ethanol.
12. Centrifuged at 10,000 rpm for 5 minutes.
13. Supernatant drained out; DNA was completely dried to remove ethanol, by leaving tubes uncovered at 37^o C for 20-30 min or vacuum dry at room temperature.
14. The pellet was resuspended in 50µl TE buffer and pooled it by using cut tips.
15. RNAase was added to a final concentration of 10µg/ml (3 µl of con. RNAase or 30 µl of diluted RNAase). Incubated at 37°C for 30 min and stored at -20°C.

3.1.1.4 Purification of DNA (Silica method)

1. 250µl of phenol: chloroform: isoamylalcohol (25:24:1) added to DNA sample and mixed.
2. Centrifuged at 12000rpm for 15 min and transferred aqueous phase to fresh tube containing 500 µl 6M Nacl and mixed by vertexing the tube.
3. 200 µl silica matrix was added and kept the tube at room temperature for 2 min.
4. Centrifuged at 12000rpm for 10 sec and supernatant was removed.
5. The matrix was washed by resuspending 1ml of washing solution (50 mM Nacl, 10mM Tris (pH=7.5), 2.5 mM EDTA and 50per cent ethanol) and vertexed vigoursly.
6. Pelleted the matrix by centrifugation at 12000rpm for 10 sec and supernatant was removed using pipette.

7. Centrifuged for another 10 seconds and pipette out the residual liquid.
8. 40 μ l of sterile water added to resuspend the pellet by brief vortexing and placed the microfuge tube at 70°C for 2 minutes.
9. Centrifuged for another 10 seconds and only 36 μ l of supernatant was taken out from the microfuge tubes.

This purified DNA samples were taken for the development and PCR standardization.

3.1.1.5 Casting of agarose gel

1. 5 μ l of the DNA solution pipetted into a microfuge tube. 2.5 μ l of Bromo-phenol dye, added and mixed for few seconds this solution used for gel electrophoresis.
2. 0.8per cent agarose solution in 1X TAE buffer prepared for 100ml was prepared. Heated in microwave oven to dissolve agarose completely, and later cooled to 40°C, ethidium bromide solution (0.5 μ g/ml) added to the prepared gel, gel was poured into boat.
3. When the gel was set, the comb was removed carefully and gel was kept it in the gel electrophoresis unit.

3.1.1.6 Running the gel

1. The gel electrophoresis tray was filled with 0.5X TBE buffer. After placing the gel, DNA samples were loaded.
2. 75 volts current was applied for 1½ to 2 hours.
3. Gel was observed under UV light. A zigzag pattern of a single band indicates intact plant DNA.

3.1.1.7 DNA quantification

DNA concentration in the sample was estimated by recording absorbance at 260 nm using UV/ VIS spectrophotometer.

1. 10 μ l of DNA sample in a quartz cuvette was taken and the volume made up to 1 ml with distilled water or TE buffer.
2. The absorbance of the solution at 260 and 280 nm were measured.
3. The ratios A₂₆₀/A₂₈₀ were calculated. DNA concentration calculated using the relationship for double stranded DNA (1 O.D. at 260 nm = 50 μ g/ ml).

$$\text{Total quantity of DNA } (\mu\text{g}/ \mu\text{l}) = \frac{\text{O.D.at260nm} \times 50 \times \text{Dilution factor}}{1000}$$

$$\text{Dilution factor} = \frac{\text{Volume made}}{\text{Volume of the aliquot}}$$

$$\begin{aligned} \text{Therefore, Dilution factor} &= \frac{1000 \mu\text{l}}{10\mu\text{l}} \\ &= 100 \end{aligned}$$

3.1.2 Restriction enzyme digestion

In order to fragment the DNA into approximately 500bp fragments restriction enzyme digestion was done. Fragments of this size are small enough to sequence easily while retaining a high probability of having enough DNA flanking the microsatellites that primers can be designed.

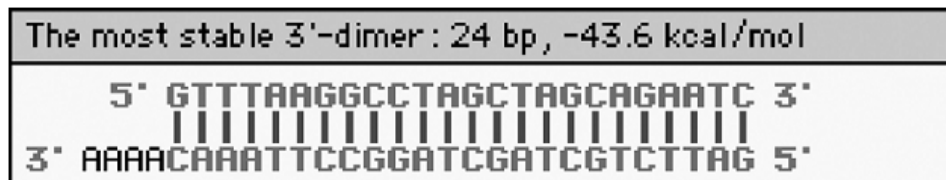
- | | | |
|-----------------------------|--|------|
| 1. 20µl of mixture contain; | PCR water | 7µl |
| | 10X Fast digest buffer | 2µl |
| | Genomic DNA (1100ng/µl) | 10µl |
| | Fast digest enzyme <i>Rsa I</i> (From NEB) | 1µl |
2. Master mix was prepared for restriction enzyme digestion into a new tube 0.2 ml tubes.
 3. Water bath was used for incubation the sample at 37°C for 16 hours.
 4. While the incubating, 1per cent agarose gel, including ethidium bromide (0.5mg/ml) was added.
 5. Small aliquot (4 µl) of the digested DNA was loaded on gel.

3.1.3 Ligating Linkers to DNA Fragments

In order to ligate a double-stranded linker onto both ends of each DNA fragment. The linkers will provide the primer-binding site for subsequent PCR steps. They also provide sites to ease cloning of the fragments into the vectors that will subsequently be used. The linkers are, therefore, compatible with the restriction sites in the vector's multiple cloning sites. The SuperSNX also incorporates a GTTT "pigtail" to facilitate nontemplate A addition by *Taq* DNA polymerase during PCR, which can be used for TA cloning.

SuperSNX24 Forward: 5`GTTTAAGGCCTAGCTAGCAGAATC

**SuperSNX24 p 4P Reverse: 5`pGATTCTGCTAGCTAGGCCTTAA
CAAAA**



1. Preparation of double-stranded (ds) SuperSNX linkers:

The equal volumes of equal molar amounts of SuperSNX24 and SuperSNX24p 4P primers was mixed (e.g., 100 μ l of 10M each). Salt was added to a final concentration of 100mM (i.e., 4 μ l of 5M NaCl for 200 μ l of primers). This mixture was heated to 95°C, and kept it for cool slowly to room temperature to form the double stranded SuperSNX linkers.

1. Linker-ligation recipe

ds SuperSNX linkers	7.0 μ l
10X ligase buffer	1.0 μ l
DNA ligase (NEB #M0202S; 400 U/ μ l)	2.0 μ l
Restriction digested DNA sample	10.0 μ l

2. Incubated in water bath at 37°C for 16 hours.

3. To ensure ligation was successful, PCR was performed on the linker ligation using the following recipe for a 25 μ l reaction:

10X PCR buffer (complete buffer 15mM MgCl ₂)	2.5 μ l
SuperSNX24 (10 μ M ->0.5 μ M final)	1.3 μ l
dNTPs (2.5 mM each->150M final)	1.5 μ l
dH ₂ O	12.5 μ l
Taq DNA polymerase (5 U/ μ l)	0.2 μ l
Linker-ligated DNA fragments	2.0 μ l

4. Cycling: 95°C for 2 min; then 20 cycles at 95°C for 20 s, 60°C for 20 s, 72°C for 1.5 min. Hold at 15°C.

5. 4 μ l of PCR product loaded on 1 per cent mini gel to see the linker ligation.

3.1.4 Dynabead enrichment for microsatellite-containing DNA fragments

In order to capture DNA fragments with microsatellite sequences complementary to the microsatellite oligos (probes) and wash away all other DNA fragments, this following protocol was followed.

- 1. 2X Hyb Solution:** 12X SSC, 0.2per cent SDS (warmed; stock solution 20X SSC: 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0).
- 2. 1X Hyb Solution:** 6X SSC, 0.1per cent SDS (warmed to get everything into solution).

Washing Solutions: 2X SSC, 0.1per cent SDS (warmed to get everything into solution), and 1X SSC, 0.1per cent SDS (warmed to get everything into solution).

- 3. Biotinylated oligos:** Di repeat probes (AG)₁₂, (TG)₁₂
- 4. NaOAc EDTA Solution:** To a 50-ml conical, 20 ml of 3M NaOAc from the dry chemical stock was taken. 20 ml of 500mM EDTA was added. The pH was checked it has to be at 8. The final was 1.5 M NaOAc and 250 mM EDTA. Aliquotes in 1.5-ml microcentrifuge tubes were taken and kept in the freezer.

Protocol:

- In a 0.2-ml PCR tube, add:

2 X Hyb solution (warmed to get everything into solution)	25.0µl
Biotinylated microsatellite probe (mix of oligos at 1µM each)	10.0µl
Linker-ligated DNA	10.0 µl
PCR H ₂ O	5.0 µl
- We have used the thermal cycler program OligoHyb. This program denatures the DNA-probe mixture at 95°C for 5 min. It then quickly ramps to 70°C and steps down 0.2°C every 5 s for 99

cycles (i.e., 70°C for 5 s, 69.8°C for 5 s, 69.6°C for 5 s, .down to 50.2°C), and stays at 50°C for 10 min. It then ramps down 0.5°C every 5 s for 20 cycles (i.e., 50°C for 5 s, 49.5°C for 5 s, 49°C for 5 s, down to 40°C), and finally quickly ramps down to 15°C.

3. While the DNA–probe mixture was in the thermal cycler, 50µl of Dynabeads (Dyna, Oslo, Norway) washed. Resuspend the beads in their original tube, and transferred to a 1.5-ml tube. 250µl of TE added and was shaken properly. Using the Magnetic Particle Concentrator (MPC) (Dyna, Oslo, Norway) the beads were captured. This was repeated with TE and twice with 1X Hyb solution. Finally, beads were resuspended in 150µl of 1X Hyb solution.
4. Pulse-spin: DNA–probe was mixed and added to the 150 µl of washed, resuspended Dynabeads (i.e., to the 1.5-ml tube).
5. The tube was incubated on rotator in slow speed at room temperature for 30 or more min.
6. Beads were captured using the MPC. The supernatant was removed by pipetting with a P200 pipetter.
7. Dynabeads was washed two times with 400µl 2X SSC, 0.1per cent SDS(washing solution I) each time using the MPC to collect the beads and remove the supernatant by pipetting. Beads were resuspended for (i.e., flick or gently vortex) next wash each time.
8. Two more washes using 400 µl 1X SSC (washing solution II), 0.1per cent SDS was done.
9. Two more washes using 400µl 1X SSC, 0.1per cent SDS, and heating the solution to within 5–10°C of the T_m for the oligo mix used (usually 50°C) were done.

10. 200µl TLE added and vortexed, and incubated at 95°C for 5 min. Labelled a new tube while incubating. Captured beads using the MPC. Quickly removed the supernatant by pipetting to the new tube. This supernatant contains the enriched fragments (i.e., “the gold”).
11. 22µl of NaOAc/EDTA solution was added and mixed by pipetting up and down.
12. 444µl of 95per cent EtOH was added and mixed by inverting the tube and placed on ice for 15 min.
13. Centrifuged at 12,000rpm for 10 min.
14. The supernatant was discarded and approximately 0.5 ml of 70per cent ethanol was added. Centrifuged at 12000rpm for 1 min.
15. The supernatant was carefully pippetted out and the sample was air dried until there was no trace (smell) of ethanol.
16. The pellet was resuspended in 25 µl of TLE. That was the “pure “GOLD”. Let the pellet hydrate, kept it in -20°C for overnight rehydration to be sure that the DNA in solution.

3.1.5 PCR Recovery of Enriched DNA

In order to increase the amount of “pure gold” DNA, serial (double) enrichments was done by using the resulting PCR products for the second enrichment. PCR was performed on supernatant to recover the enriched of DNA fragments:

1. PCR recipes

10X PCR buffer (complete)	2.5µl
BSA (250 µg/ml-> 25µg/ml final)	2.5µl
dNTPs (2.5 mM each ->150 µM final)	1.5 µl

SuperSNX-24 (10 μ M \rightarrow 0.5 μ M final)	1.3 μ l
PCR H ₂ O	15.0 μ l
<i>Taq</i> DNA polymerase (3 U/ μ l)	0.2 μ l
Eluted DNA fragments (“pure gold”)	2.0 μ l
Total	25μl

2. Cycling : 95°C for 2 minutes.; then, 25 cycles of 95°C for 20 sec., 60°C for 20 sec., 72°C for 1.5 min.; then 72°C for 30 min.; then hold at 15°C.

3. Loaded 4 μ l of PCR products on a 1.0per cent minigel next to a 100-bp ladder as a standard to verify whether DNA recovery was successful.

3.1.6 Ligating-Enriched DNA into Plasmids (TA cloning kit: #K1213, #K1214)

In order to incorporate (ligate) the enriched/ recovered DNA (amplified pure gold) into a cloning vector.

1. Ligation components	Volume
1. Vector pTZ57R/T	1.5 μ l
2.5X Ligation buffer	3.0 μ l
3. PCR product	1.0 μ l
4. Water (nuclease free)	9.0 μ l
5. T4 DNA ligase	0.5 μ l
Total	15.0μl

2. Ligation was carried out at 4°C for overnight. The ligation mixture was used for transformation.

3.1.7 Transforming Plasmid DNA

In order to incorporate enriched/recovered DNA (amplified pure gold; or insert) cloning vector into a bacterial host. The idea is to place one vector (which, ideally, has one fragment of amplified pure gold

[insert]) into one bacterial host, and do this for as many vectors–inserts as possible.

1. Preparation of competent cells for transformation:

Competent cells of *Escherichia coli* strain DH5 α was prepared by calcium chloride method as described by Sambrook and Russel (2001).

Procedure:

Overnight grown culture of DH5 α cells was inoculated into 30ml of LB media. The cells were grown at 37°C on a rotary shaker at 200rpm till OD₆₀₀ reaches 0.3 to 0.4. The cells were then aseptically transferred to a sterile chilled centrifuge tube and immerse in ice for 15 min. then the cells were pelleted by centrifugation at 5000rpm for 5 min at 4°C. The pelleted cells were resuspended in 15ml ice cold 0.1M CaCl₂ and incubated on ice for 30 min. the cells were again recovered by centrifugation at 5000rpm for 5 min at 4°C. The pellet obtained was resuspended in 6ml of 0.1M CaCl₂ 100per cent of glycerol. The cells were then stored in chilled 1.5ml of microfuge tubes in aliquots of 200 μ l and quick froze by keeping it in liquid nitrogen and stored in -80 freezer.

Transformation:

The tube containing competent cells was removed from -80 freezer and kept briefly in ice for thawing. 10 μ l of ligation mixture was added to 100 μ l of competent cells and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 2.5 min and snap chilled for 2 min on ice. 800 μ l of sterile LB was added to this and cells were grown at 37°C for 2 hour in shaking incubator at 120rpm. After incubation the cells were grown spun down and dissolved in 50 μ l of LB media and plated on LB agar plates containing ampicillin (50mg/ml) and X-gal/IPTG and incubated overnight at 37°C.

3.1.8 Screening of clones:

In order to determine the number and proportion of colonies with vector and vector-insert, to amplify inserts from the bacteria/vectors, and to archive bacteria from each colony of interest (i.e., those with inserts). The T-vector used in cloning was compatible for blue-white screening when plated on X-gal and IPTG. The recombinant clones with a foreign DNA insert ligated at 3' T overhang disrupt the reading frame of lac-Z and produce white colonies. Whereas the self ligated (circularized) vector produces blue colonies due to enzymatic activity of lac-Z on the substrate X-gal. The suspected transformants which appear as white coloured colonies were picked from the plate and colony PCR was carried out.

3.1.9 Colony PCR:

In order to determine PCR product concentration and size and to purify the PCR product for subsequent sequencing.

PCR was performed to directly analyse the positive transformants using the M13 primers as follows:

1. 25µl reaction volume PCR cocktail was prepared consisting of PCR buffer, dNTPs, primers, water and Taq DNA polymerase.
2. White colonies were selected and resuspended individually into 25µl of the PCR cocktail and simultaneously the colonies were patched onto separate plate to preserve it for future.
3. PCR was performed as described earlier.
4. Amplified products were resolved on 1.5per cent agarose gel.

When gel was run for the colony PCR, expected amplification was not found. So couldn't perform the further steps of plasmid isolation.

After colony PCR the colonies which were at around 500bp were selected and plasmid was isolated by using manufacture instruction kit (Bioserve, Catalogue No: 1002-250). About 71 plasmids were sent for sequencing.

3.1.9 Mining the microsatellite loci

Microsatellite loci were identified using SSR Identification Tool (SSRIT, www.gramene.org/db/searches/ssrtool).

3.1.10 Designing primers

Sequences with more than 15bp repeat motifs were selected for designing the primers in the flanking region of the repeat motifs. Primers were designed using Primer3 software (Rozen *et al.*, 2000) in the flanking region of the repeat motifs.

3.1.10.1 Screening of cotton SSR primers

A total of 10 genotypes from were selected from previous study (Prakash *et al.*, 2011) for this experiment (Table 2).

Table 2: List of the genotypes used for SSR analysis

S. No.	Accession No.	<i>Taxonomic name</i>	Source of collection
1	IIHR 239	<i>A. esculentus</i>	Farmers field, India
2	IIHR 240	<i>A. esculentus</i>	Farmers field, India
3	IIHR 241	<i>A. esculentus</i>	IIVR, Varanasi
4	IIHR 242	<i>A. esculentus</i>	Junagharh, Gujarat
5	IIHR 243	<i>A. esculentus</i>	Farmers field, India
6	IIHR 244	<i>A. esculentus</i>	Farmers field, India
7	IIHR 245	<i>A. esculentus</i>	Farmers field, India
8	IIHR 246	<i>A. esculentus</i>	Farmers field, India
9	IIHR 247	<i>A. esculentus</i>	Farmers field, India
10	IIHR 248	<i>A. esculentus</i>	Farmers field, Mandya, KA

3.1.10.2 PCR reagents

1. Reaction buffer (10X in 100ml):
- | | |
|---|-----------|
| a) Tris HCl | 100mM |
| b) KCl | 800mM |
| c) MgCl ₂ .6H ₂ O | 25mM |
| d) TritonX | 1per cent |
| e) Gelatin | 1per cent |

(Adjust the pH to 9.0)

2. Primers : Stock 5pmol
3. Taq DNA polymerase : Stock 3U/ μ l
4. Template DNA : Stock 20ng/ μ l
5. dNTP's : Stock 1mM (Pipette out 100 μ l of each dNTP & dilute to 1000 μ l)
6. Bromo phenol blue : *Stock solution:* 0.25per cent in 50per cent glycerol

(Working solution: 200 μ l stock + 200 μ l of 50 glycerol)

B) PROTOCOL

1. The reagents were pipetted out accurately using appropriate auto pipettes into sterile 0.5ml micro tubes the reagents in the following order master mix was prepared

Reaction buffer 10X	2 μ l
dNTP's (1mM)	2 μ l
Taq DNA polymerase (3u/ μ l)	0.3 μ l
Template DNA (20 ng/ μ l)	3 μ l
Primer (5 pM)	1.5 μ l(forward)+1.5 μ l(reverse)
Water	14.7 μ l
Total reaction volume	25.0 μl

2. Mixed by repeated pipetting, spin down the contents for 15 seconds at 5000 rpm.
3. Placed the tubes firmly in the wells of the thermo cycler and set the following temperature programme.
 - Step 1** 94° C for 4 min
 - Step 2** 94° C for 30 sec
 - Step 3** 55° C for 30 sec
 - Step 4** 72° C for 1 min
 - (Go to 2 for 34 times)**
 - Step 5** 72° C for 2 min
 - Step 6** 4° for hold.
4. At the end of the run take out the tubes, added 1µl of loading dye to 10 µl of PCR product (remaining was used for PAGE analysis) and spinned for 2-5 seconds at top speed in micro centrifuge. Stored at 4°C till electrophoresis.
5. The amplified products of PCR (10 µl) were separated by electrophoresis on 2per cent high resolution agarose gel along containing ethidium bromide (0.5mg/ml) and gel was visualised under UV light.

After standardization the remaining 15 µl of amplified products of PCR of all 10 individual genotypes were separated by 8per cent polyacrylamide gel electrophoresis (PAGE).the components for PAGE analysis is as follows;

Components	Quantity (8per cent)
Acrylamide (30per cent)	16 ml
50X TAE	1.2 ml
TEMED	200 µl
APS, 100mg/ml	250 µl

Double distilled water	42.4 ml
Total volume	60 ml

60ml of 8per cent polyacrylamide gel was prepared and casted in the gel plate. Upon solidification of the gel the comb was removed and the plate was placed in the tank saturated with TAE buffer. The samples were loaded into the wells and ran the gel at 100V. After about half an hour the gel was stained with ethidium bromide (0.5mg/ml) for about 15 minutes, observed under UV trans illuminator and took photographed.

3.1.11 Statistical analysis

Amplification profiles of SSR primers in 10 genotypes of okra were scored as presence (1) or absence (0) in each position. The data obtained was statistically analysed.

3.1.11.1 Genetic analysis

Genotypes showing two allelic bands with equal intensity were considered as heterozygous for the locus. Lack of amplicon for any specific marker (null allele) in a genotype was considered to result from absence of primer binding site, and after reruns with specific check, was designated as 0.

The PIC value refers to the value of the marker for detecting polymorphism based on the number of alleles and their distribution frequency within a population (Botstein *et al.*, 1980). The PIC (Polymorphic information content) value was estimated using the equation of Anderson *et al.* (1993):

$$PIC = 1 - \sum_{j=1}^n P_{ij}^2$$

Utility of markers was estimated using Cervus 3.0 software (Kalinowski *et al.*, 2007) these includes locus-wise total number of alleles detected (n), major allele frequency (A) and PIC values.

3.1.11.2 Cluster analysis and estimating genetic distance

The distance matrix was developed using Squared Euclidean Distances (SED) that estimated all pair wise differences in the amplification product (Sokal and Sneath, 1973). The genetic distance was computed as,

$$\sum^n j = 1 dj^2$$

Where, $dj = (X_{ik} - X_{jk})$

X_{ik} = Binary code of i^{th} plant for allele 'k'

X_{jk} = Binary code of j^{th} plant for allele 'k'

A dendrogram was developed based on Ward's method of clustering using minimum variance algorithm (Ward, 1963).

3.2 Experiment II: Study on cross amplification of cotton SSR primers in okra

3.2.1 DNA isolation

A total of 25 okra genotypes were randomly selected from previous study (Prakash *et al.*, 2011) for this experiment. The total genomic DNA was isolated as described earlier in 3.1.1.

3.2.2 Screening of cotton SSR primers (Table 4)

A total of 90 cotton SSR markers were used for screening okra DNA (Table 3). For standardization PCR condition here each PCR programme was carried out at different annealing temperature.

3.2.2.1 Reagents

The reagents as described earlier in **3.1.10.2**

Table 3: List of genotypes used for cross amplification of cotton SSR markers

Sl. No.	Accession number	Taxonomic name	Source of collection
1	IIHR 15	<i>Abelmoschus esculentus</i>	LAM, Hyderabad
2	IIHR 18	<i>A. esculentus</i>	LAM, Hyderabad
3	IIHR 20	<i>A. esculentus</i>	IIHR, Bangalore, KA
4	IIHR 55	<i>A. esculentus</i>	GAU, Gujarat
5	IIHR 72	<i>A. esculentus</i>	Bentley seeds, New York
6	IIHR 81	<i>A. esculentus</i>	FSI coastal region, New York
7	IIHR 91	<i>A. esculentus</i>	HAU, Hissar
8	IIHR 101	<i>A. esculentus</i>	NBPGR, New Delhi
9	IIHR 108	<i>A. esculentus</i>	NBPGR, New Delhi
10	IIHR 116	<i>A. esculentus</i>	NBPGR, New Delhi
11	IIHR 104	<i>A. esculentus</i>	IIHR, Bangalore, KA
12	IIHR 133	<i>A. esculentus</i>	NBPGR, New Delhi
13	IIHR 134	<i>A. esculentus</i>	NBPGR, New Delhi
14	IIHR 181	<i>A. esculentus</i>	NBPGR, New Delhi
15	IIHR 213	<i>A. esculentus</i>	Farmers field, Varanasi
16	IIHR 219	<i>A. esculentus</i>	Farmers field, Mysore, KA
17	IIHR 226	<i>A. esculentus</i>	Farmers field, DA Karnataka
18	IIHR 227	<i>A. esculentus</i>	Farmers field, Dakshina Kannada
19	IIHR 229	<i>A. esculentus</i>	Farmers field, Udupi, KA
20	IIHR 230	<i>A. esculentus</i>	Farmers field, Udupi, KA
21	IIHR 231	<i>A. esculentus</i>	Farmers field, Udupi, KA
22	IIHR 232	<i>A. esculentus</i>	Farmers field, Udupi, KA
23	IIHR 233	<i>A. esculentus</i>	Nigeria
24	IIHR 237	<i>A. esculentus</i>	Farmers field, India
25	IIHR 238	<i>A. esculentus</i>	Farmers field, India

B) Protocol

1. The reagents were pipetted out accurately using appropriate auto pipettes into sterile 0.2 ml micro tubes the reagents in the following order. Master mix was prepared for 25 μ l reaction.

Reaction buffer 10X	2 μ l
dNTP's (1mM)	2 μ l
Taq DNA polymerase (3u/ μ l)	0.3 μ l
Template DNA (20 ng/ μ l)	3 μ l
Primer (5 pM)	1.5 μ l(forward)+1.5 μ l(reverse)
Water [25 μ l - (2 +2 +0.3+ 3 + 3)]	14.7 μ l
Total reaction volume	25.0μl

- 2 Mixed by repeated pipetting, spin down the contents for 15 sec at 5000 rpm.
- 3 Placed the tubes firmly in the wells of the thermo cycler and set the following temperature programme.

Step 1 94° C for 4 min

Step 2 94° C for 30 sec

Step 3 50°C/53°C/57°C (primer specific)

Step 4 72° C for 1 min

(Go to Step 2 for 34 times)

Step 5 72° C for 2 min

Step 6 4° for hold.

5. At the end of the run take out the tubes, 1 μ l of loading dye was added to 10 μ l of PCR product and spinned for 2-5 seconds at top speed in micro centrifuge. Stored at 4°C till electrophoresis.

Table 4: List of the cotton BNL SSR primers (Brookhaven National Lab, www.cottonmarker.org)

S. NO.	MARKE R NAME	FORWARD PRIMER	REVERSE PRIMER	REPEAT TYPE
1	BNL 113	CCCTCGAGGTCGACGGTATCGATA	CCCGGTTCTTGCTGATCCGGATGT	(AG) ₁₈
2	BNL 116	GCGGCATGCTTTCTTCATCATATA	ATAACCTGTGACATCTTTTTTTGTC	(CT) ₁₆
3	BNL117	GCTTTCGTCATACACACACATTCA	TAGCTAGGCTGCTGCACTACTTGA	(AG) ₁₀
4	BNL118	CTTTATCCGGAGACCGAAC	CTAACTTTTGCTCACCCCC	(TC) ₉
5	BNL119	CGATCCTTCTTATTCTCATCTCTC	GAAACACTTCTTCACAAATCCTAAT	(AG) ₁₀
6	BNL169	TCACAAATAAAAGTGAAATTGCG	GGCTGGTGACCATAAAAGGA	(GA) ₁₅
7	BNL226	TTATTCTCACAGCCGGAACC	TTCACCCTCTCGCTTCTCAT	(GA) ₁₆
8	BNL252	TGAAGAGCTCGTTGTTGCAC	CGAAAGAGACAAGCAATGCA	(CT) ₂₁
9	BNL285	ACGCTGTTGATAGAGAGAAATACC	TCACCGTCCGTTTTAACACA	(GA) ₁₂ , (GA) ₃ +GC+(GA) ₁₂ +A+(AG) ₂
10	BNL341	ACCTGGGGTACTTGTCCACA	CCATCCCATTTGTGATACCC	(GA) ₁₄
11	BNL358	ATTAATTGCACCAGACATCCC	AAATCTTGGTTTTGAGATTTTCA	(AG) ₁₂
12	BNL390	CTTTGGGTTGGAATGTGAGG	CATTCTGGCCACACTCAC	(AG) ₁₁ , (CT) ₁₁ , (AG) ₁₁ +T+(GA) ₅
13	BNL409	AGAAGTCGGACGTGGAAAGA	GCCGTTTTCTCAGTGATGT	(GA) ₁₂
14	BNL448	GCAGCTTGCTTTTCTGCTT	ACGCAAGCTTGGTCAATAC	(CT) ₁₃
15	BNL 530	CGTAGGATGGAACGAAAGC	GCCACACTTTTCCCTCTCAA	(GA) ₁₀
16	BNL 598	TATCTCCTTCACGATTCCATCAT	AAAAGAAAACAGGGTCAAAGAA	(CT) ₁₂ , (AG) ₁₂ +(TG) ₄
17	BNL625	AGAGAGGGGGGAAAAGTTCA	GCCAGGCATGGTTTCTATGT	(AG) ₁₄
18	BNL 827	AAGCTCCACGTGCTCAAGTT	CTCATGTTGTGCGGTGGTGT	(CA) ₁₉
19	BNL 946	GCTGTTGCTCCACATCTCCT	GGCAAACAGATAGGCAGAA	(GA) ₁₄
20	BNL1030	TTTGGAGCCATTTACATGCA	AAACCACTTCTGCATCTGGA	(GT) ₁₆ , (CA) ₁₃
21	BNL 1034	TTGCTTTCAATGGAAAACCC	CGTCGCAAAGTTGAGAATCA	(CT) ₁₆
22	BNL1045	GGCAATCAACTTTAGGCTGC	TGGTGAAGATCCCCATTTTC	(AG) ₁₆ , (CA) ₁₀
23	BNL1047	GCTTGTCTATCTCCATTGCTG	TAGCCCGGTTTCATGTTCTTC	(CA) ₁₂
24	BNL1053	AGGGTCTGTATGGTTGGAG	CATGCATGCGTACGTGTGTA	(AC) ₁₆
25	BNL1066	ACATTTCCACCCAAGTCCAA	ACTCTATGCCGCCTCTCGTA	(GT) ₁₀ +(GA) ₉
26	BNL1079	TCATACTCTTTCATCTAGCGCG	AAAGGAATCCAGGTGAGCCT	(CA) ₁₁ , (GT) ₁₁
27	BNL1151	AAAGTAGCAGCGTTCCAAA	GAGCCGCTTCTGTAGCTTCA	(AG) ₁₅
28	BNL1161	CATCTCCTCTGGAAAGAGCG	ATGAAGCAGCACATTCCATG	(AG) ₂₄

29	BNL1162	GCGCAAGCGTAGGAGTTTAC	GCGCAAGCGTAGGAGTTTAC	(GA) ₁₄
30	BNL 2544	GCCGAAACTAAAACGTCCAA	TCCTTACTCACTAAGC	(AG) ₁₁
31	BNL2571	TCGCTATCGCTCTGAAATCA	ATGCCACGGAATTAGCAAAC	(AG) ₁₃ , (TC) ₁₃
32	BNL2589	CAAGCTTTGGAGTCCCTGAG	ATACATTCAAGTCCAATCAAAACG	(AG) ₁₃
33	BNL2597	AACTGATCAATCTAGACAAACATCC	CGACCCTGTGTATGTGTTGC	(GA) ₁₆
34	BNL2632	CGTGTCTCCAGACCAACAAA	GGGAGTTGAAGCCGACATAA	(GA) ₁₄ , (TC) ₁₄
35	BNL2634	AACAACATTGAAAGTCGGGG	CCCAGCTGCTTATTGGTTTC	(AG) ₁₁
36	BNL2646	CCCCTTTGATAGATACACATTTTTA	AAAATAAACTACGAAAGAGAAAGAGAA	(GA) ₃ +G+A ₂ +(AG) ₄ , (TC) ₄ +(CT) ₁₇
37	BNL2652	TTCATCATTCTAGCCTGAGTCC	GCGATAATCCTTCCAGGGAT	(TC) ₃₂
4+3 8	BNL2656	AACCACAACCAAAATTTACAG	CTTTGGTTTCGTAGGGCTTG	(CT) ₁₀ , (AG) ₁₀
39	BNL2662	TACAACAGACCGCATCACAGTG	TCGAGGTACTCTCTCTCTCTCTC	(CT) ₁₅
40	BNL2805	AGTTTGGAAATTACAATAAATGTACTC G	CCAAGGTCGGTCGGTACTA	(GA) ₁₀
41	BNL2847	AACAATGCCGGAAGCTAGTG	CTTTTCTTGCTGCCTCTGCT	(GA) ₁₇
42	BNL2882	CAACCTTTGGTAATCTTCTTTCG	CGCTAACGCATTTGACATCT	(GA) ₁₂
43	BNL2884	TCAACTCATACCAAATCAATTCC	CCCTGTTTTGTTCAATGGGT	(AG) ₁₁
44	BNL2895	CGATTTTACTGCTTCAGACTTG	TACCATCTCACGGATCCACA	(GA) ₁₀
45	BNL2920	TTCTTGCATTGAATAATACTGGC	CTTAATTCTAAAAATCAATAAATTTAGC C	(AG) ₁₂
46	BNL2921	CGAGAGATTTTAAAGGGAAACA	GGGAGTGGTCTGATGGAAAA	(AG) ₁₀
47	BNL2960	TAAGCTCTGGAGGCCAAAAA	CCATTTCAATTTCAAGCATACG	(GA) ₁₀
48	BNL3034	AAAGGAAATGGTCATTGGCA	AGTACCCGCCATTTCAAGTG	(AG) ₁₂
49	BNL3103	ACTTTGAGATATTGTTATTCTACCCG	TCGAACAATTACGAATCAAATG	(GA) ₁₃ , (TC) ₁₄
50	BNL3147	ATGGCTCTCTCTGAGCGTGT	CGGTTCAAGGGCTTTGTTGT	(AG) ₁₁
51	BNL3255	GACAGTCAAACAGAACAGATATGC	TTACACGACTTGTTCCACG	(GC) ₆ AT(AC) ₁₄
52	BNL3379	AACGGAACAAACCTTGAGGA	GTGCATGTGGTATGTTGGGA	(GA) ₁₄ , (CT) ₁₄
53	BNL3383	GTGTTGTCATCGGCACTGAC	TGCAATGGTTCAGTGGTGAT	(AG) ₁₀ , (CT) ₁₁
54	BNL3408	ATCCAAACCATTGCACCACT	GTGTACGTTGAGAAGTCATCTGC	(GT) ₂ AT(GT) ₁₂
55	BNL3441	CGTCATAAACCGTGCTTGTG	GGCCACTTTAAGGCTGTAC	(AC) ₁₈ , (AT) ₂ (AC) ₁₈ (AT) ₄
56	BNL3442	CATTAGCGGATTTGTCTGTA	AACGAACAAAGCAAAGCGAT	(CA) ₁₄ (TA) ₅

57	BNL3449	AAGCTGTGGCTATGATGCCT	AGAGCAAAAAACAATTACAAAAGC	(CA) ₁₂ , (CT) ₆ TA(CA) ₁₂
58	BNL3452	TGTAAGTACTGAGCAGCCGTACG	GCCAAAGCAGAGTGAGATCC	(CA) ₁₃
59	BNL3479	AGTGGGTTGGACTTTCATGC	CACGGGCTTTTTTTTTTTCA	(AC) ₁₅ , (TC) ₆ T(AC) ₁₅ G(CA) ₂
60	BNL3500	TCATCACCTCCGTACCCTCTAA	CATGTGTGTGTATGTATGTGTGTGTG	(CA) ₁₄
61	BNL3502	AATTTCTAAGATAACACACAAACACA	TACAATCAAATAGCAGTTTAGAGTATCG	(AC) ₁₂ +(AT) ₂
62	BNL3510	GCACCAGTGCTCAGACACACA	ATNTGAGTTGAAATCTGCCGTAA	(AC) ₁₅ +(TC) ₂₀
63	BNL3511	TAGAACATAGGGAGGCGTGG	AATGGAGAGACAATGATTTTTTCG	(AC) ₁₁
64	BNL3512	TCGATGGAAGTGTAAGAATTCG	ACGCCGAAAAGGGAGTTACT	(AC) ₁₃
65	BNL3530	TCCCAGAAAGTTTCAGTTGG	TCCTCAAAGTCATCGTGTCCG	(AC) ₁₂
66	BNL3535	CTGGGATACATACCGTGGCT	ACTTTGCTGAATAAAGGTGAGTG	(CA) ₁₄
67	BNL3537	TGAAATGGACGTGACATGGT	TTGCAGGTTCTGATGAGCAG	(AC) ₁₁
68	BNL3545	AGTCAGTTTTTTGTTAGCAATATGC	AACCATTAATCCCTATTTAACCG	(CA) ₁₀
69	BNL3547	TCGATGGAAGTGTAAGAATTCG	ACGCCGAAAAGGGAGTTACT	(AC) ₁₃
70	BNL3556	CCTTTCATGACCCTGCAAAT	AGATGGGGAATGGATCTGTG	(AC) ₁₂ (AT) ₄
71	BNL3558	AAGCAAATCATGATGAACATACG	TGCGAAGAGTAGCTCTGCTG	(AC) ₁₁
72	BNL3563	AAGCATAAACTTGACACAAGCC	AATGGGCAAGAAAAGGGAAC	(CA) ₁₃ (TA) ₄
73	BNL3566	TGGTGCCATGATTCTGTCTC	TGCTCTAAATGCAAGCCATG	(AC) ₁₂ +(AT) ₄
74	BNL3569	GCCAATCACCGAGAACAATT	CGCTTATTGCCTTGATTGGT	(CA) ₂₁
75	BNL3580	CTTGTTTACATTCCCTTCTTTATACC	CAAAGGCGAACTCTTCCAAA	(AC) ₁₅
76	BNL3582	TGGCAGCTCACCTTCTTTTT	CATGTTGTGTTTATAATGGTGC	(CA) ₂₀ +(TA) ₆
77	BNL3590	TCTTCCCTCTCTTTCTCTTTTCG	ACACGGAAGACCAACCAAGT	(CA) ₂₀
78	BNL3592	GTTCTAGTCTCTTTCTTTTATGGGC	TTGATTGAGATGCCAATGGA	(TC) ₁₇
79	BNL3594	AGGGATTTTGATTGTTGTGC	TGAATTCAAACAAATGTTAGCC	(TC) ₃₇
80	BNL3598	GCCCTCTTCCACCTCTCTCT	AGCCGTGCAATTTAATTTTA	(CT) ₁₆
81	BNL3599	TTAGCCCCAGTAACATGCC	ACTGCAAGCTCTGCCCTAAA	(TC) ₁₅
82	BNL3601	TTCCGTTGATGGAAATTGAA	ACAAGAATGCGTGTGTCTGC	(CT) ₂₀
83	BNL3604	CCAATTTTGTCTCTCTATTGC	CGATCAAATACAATTTTAACCA	(CT) ₃₀
84	BNL3623	TTAATAGAGGGACCAAAAAGTGATAT	TTAGCGTTAATATTGTATGTTCAACTC	(GA) ₁₃ +(G)+(GA) ₂₀
85	BNL3627	TATGGGCTGTCCACCTAAG	CAAAGCAACATGCACACACA	(TC) ₁₇
86	BNL3792	TTCGAGATCCCCTGTTCTGA	CATATCCAGTCAAACCAAACG	(TG) ₂₁
87	BNL3816	GTTAGCCACGTGTTAGTTCTATG	ATCGATCACTTGCTGGTTCC	(TG) ₁₅ , (TG) ₅ TA(TG) ₁₅

88	BNL3835	ATTTGGTCATTTTGGTGTATGG	TGCATAATAAAGGGCATTTACA	(TG) ₁₈
89	BNL3838	GGAATCTGGGTTTATGGGG	CCCCAAAGACTGCACAATT	(GT) ₁₀ , (GT) ₁₀ +A+(TG) ₃ +T ₁₂
90	BNL3848	GATGTGATAAGTGTATGTGAATAGTG C	TGCAAGCATTACAAAACCCA	(TG) ₁₁

6. The amplified products of PCR were separated by electrophoresis on 2per cent high resolution agarose gel along containing Ethidium bromide (0.5mg/ml) and gel was visualised under UV light.

After standardization the amplified products of PCR were separated by 8per cent polyacrylamide gel electrophoresis (PAGE). The components for PAGE as described above in 3.1.10.2.

3.2.3 Statistical analysis

Amplification profile of 92 primers (Table: 4) in 25 genotypes of okra were scored for their presence (1) or absence (0) in each position. The data obtained was genetically analysed as described in 3.1.11.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The present investigation entitled on the “**Development of microsatellite markers and molecular analysis in okra (*Abelmoschus esculentus* (L.) Moench)**” was carried out during the period of 2010-2011 at Division of Biotechnology, Indian Institute of Horticultural Research, Bangalore. The results obtained from the different experiments are presented in this chapter.

4.1. Development of microsatellite marker in okra

Microsatellite development was carried out following Glenn and Schable (2005).

4.1.1 Isolation of genomic DNA and its quantification

Genomic DNA was isolated from okra cultivated species (*Abelmoschus esculentus*) Arka Anamika “using mini-prep” method as described in materials and methods from fresh young leaves. The extraction method yielded good quality and quantity of DNA. The amount of DNA present was determined by UV-Vis spectrophotometer at an emission spectrum of 260 nm. The quantity of DNA sample 1100ng/ μ l and A260/A280 ratio of DNA was 1.87. Further the intensity of isolated DNA samples were tested and found good gel electrophoresis (0.8per cent agarose) to carry out the PCR amplification (Plate 1).

4.1.2 Restriction enzyme digestion

Arka Anamika genomic DNA was digested by *RsaI* (GT ^AC), a blunt end cutter. The digestion resulted DNA fragments of approximately 500bp size (Plate 2).

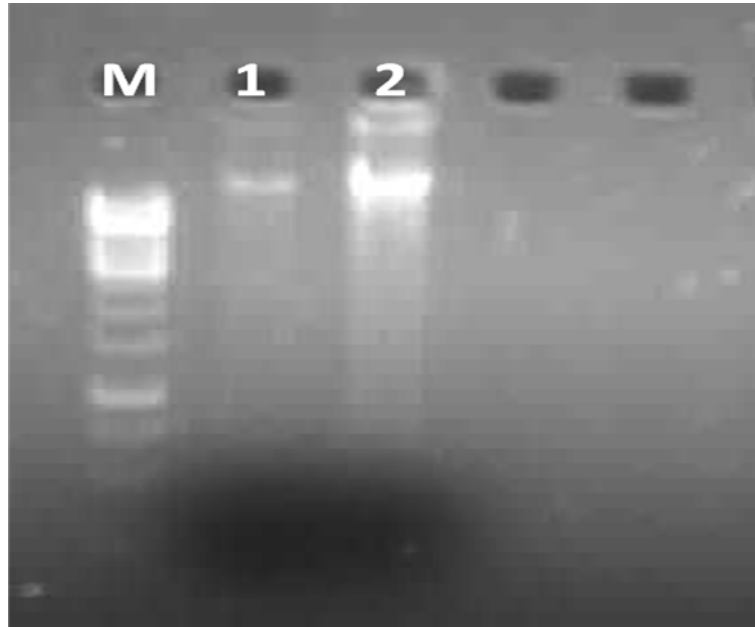


Plate 1: Gel profile of genomic DNA of Arka anamika
(Lane M:1kb ladder; lane 1 and 2= DNA samples)

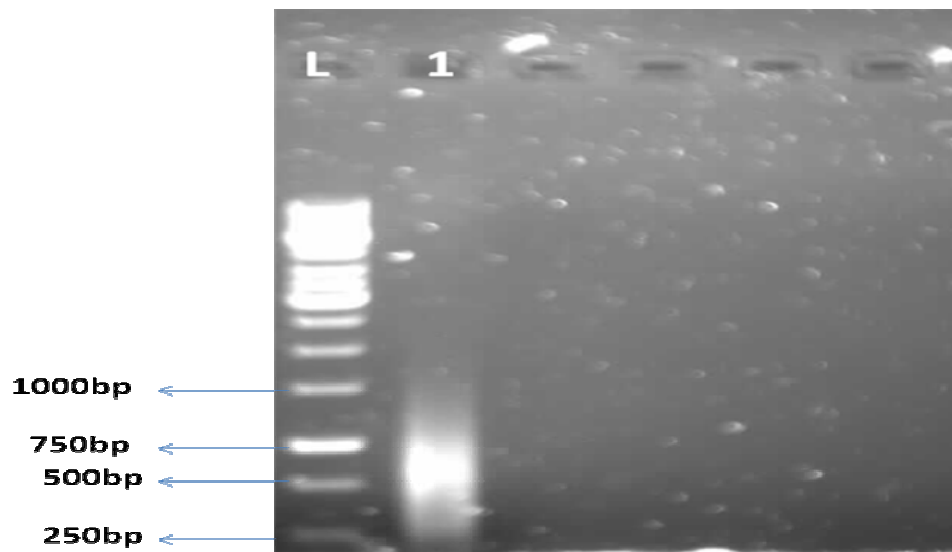


Plate 2: Gel profile of *RsaI* restriction digestion of total genomic DNA
(Lane L=1 kb ladder; Lane 1= Restricted DNA sample)

4.1.3 Ligating Linkers to DNA Fragments

Ligation was done between digested DNA with SuperSNX linkers, so that this linker provides the primer-binding site for the subsequent PCR steps and also provides the sites for cloning of fragment into the vectors. In order to ensure linker ligation and reduce the redundancy of the amplified sequences, an asymmetric PCR was performed using the linker ligated DNA and SNX24 forward primers alone. The PCR product was resolved on 1per cent agarose gel. The smear indicated successful linker ligation. Ligated reaction product yielded a smear of fragments centered at approximately 500bp (Plate 3).

4.1.4 Dynabead enrichment for microsatellite-containing DNA fragments

In order to capture DNA fragments with microsatellite sequences complementary to the microsatellite oligos (probes) and wash away all other DNA fragments. Streptavidin dynabeads was added to the DNA fragments hybridized with biotinylated probes. The streptavidin dynabeads was washed with 1x buffer prior to its use to increase its efficiency. These beads captured the biotinylated probes hybridized to the DNA under magnetic particle collecting (MPC) unit. These beads were again washed with hybridization and washing solutions, to remove the unwanted DNA fragments. The beads were finally incubated with 200 μ l TE (Tris EDTA) buffer at 95°C for 5 minutes and then captured using MPC unit. The supernatant containing enriched fragments of microsatellite repeats pool (called "GOLD") was collected. This enriched product was further precipitated with 95per cent ethanol and resuspended in 25 μ l of TE buffer (pure "GOLD").

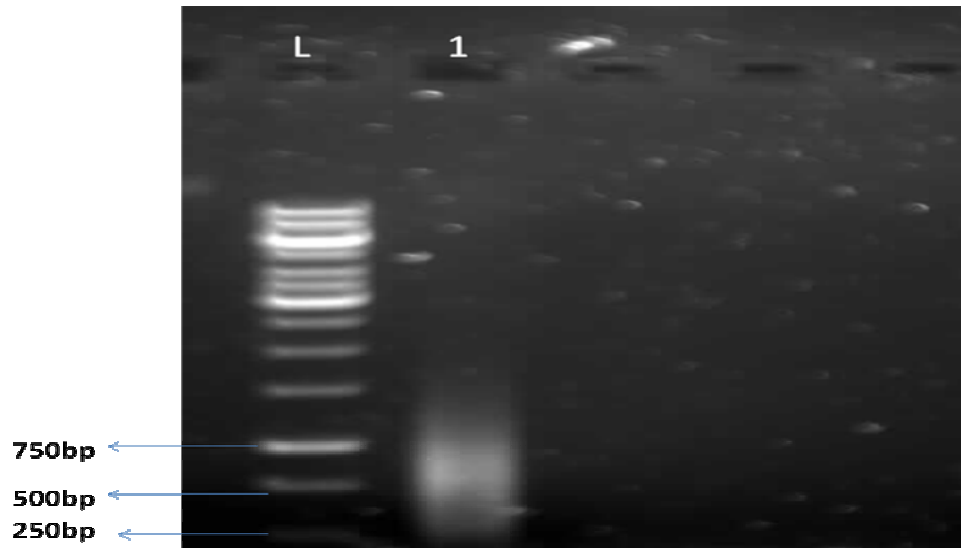


Plate 3: Gel profile of successful Linker ligation

(L=1kb ladder; 1= ligated sample)

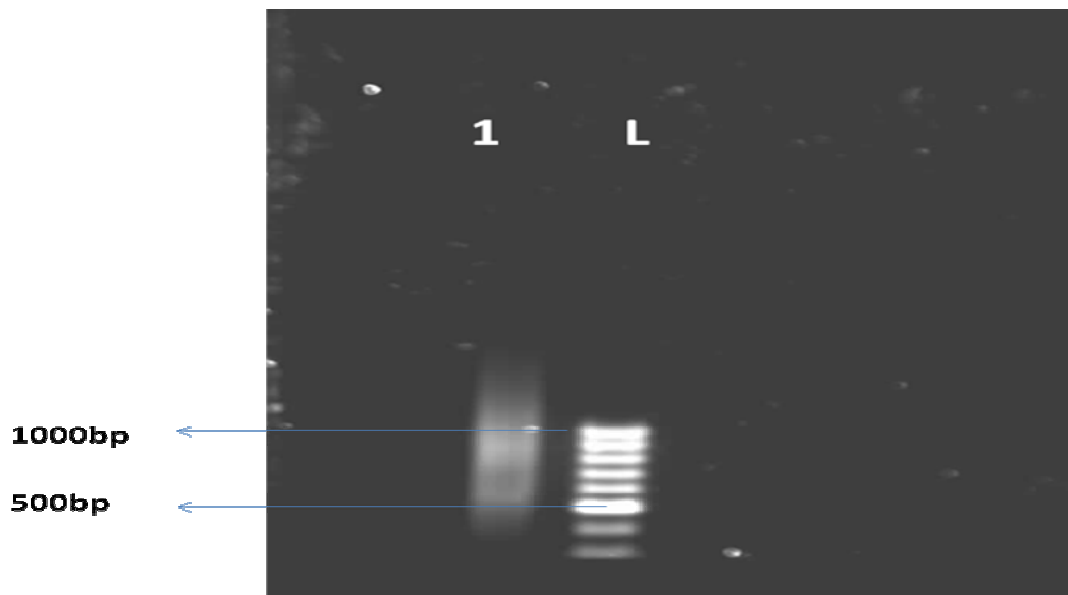


Plate 4: Gel profile for first PCR enrichment

(L=100bp ladder; 1=enriched sample)

4.1.5 PCR recovery of enriched DNA

The amount of enriched microsatellite repeat fragments (pure GOLD DNA) was further increased by PCR amplification using pure gold DNA as template. The smear of fragments visible, centered at approximately 500bp size was visible (Plate 4 and 5).

4.1.6 Cloning and transformation of plasmid DNA

In order to incorporate (ligate) the enrichment/recovered DNA (amplified pure Gold) into a cloning vector. The amplified enriched and recovered pure gold DNA fragments with microsatellite repeat regions were cloned into pTZ57R/T vector and transferred into competent *E. coli* cells.

4.1.7 Screening and sequencing of recombinant clones

Recombinant clones were selected based on blue/white screening. White colonies indicate the transformed cells with insert and blue indicates transformed with only vector.

We have observed around a total of 100 of colonies with only few blue colonies. In order to confirm, that white colonies with insert, colony PCR was performed using M13 primers. PCR products were separated on 1per cent agarose gel with ethidium bromide (0.5mg/ ml). The results showed that amplified bands of different size ranging from 500-1200bp (Plate.6). We have selected colonies with insert size around 500bp for sequencing.

Plasmids were isolated from the selected colonies using plasmid isolation kit according to manufacture instruction (Bioserve, catalogue No: 1002-250). Plasmids were sent for sequencing. (Plate 6)

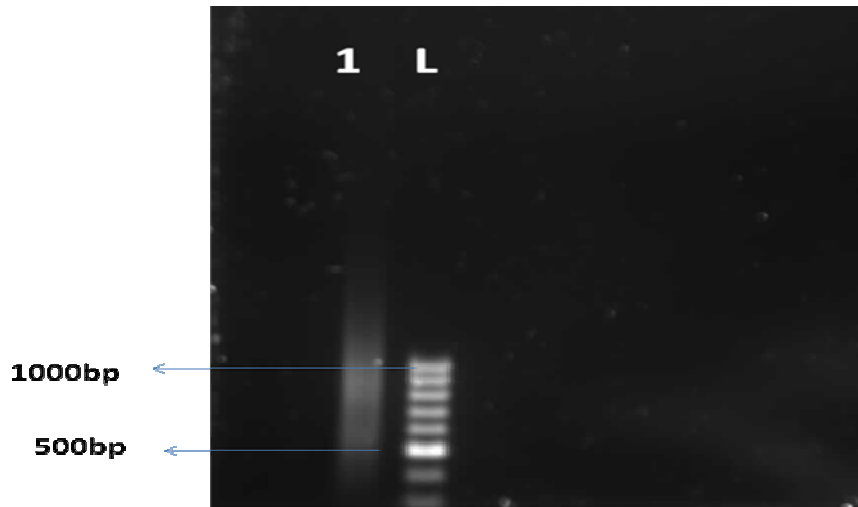


Plate 5: Gel profile of second PCR enrichment (L=100bp ladder; 1=enriched sample)

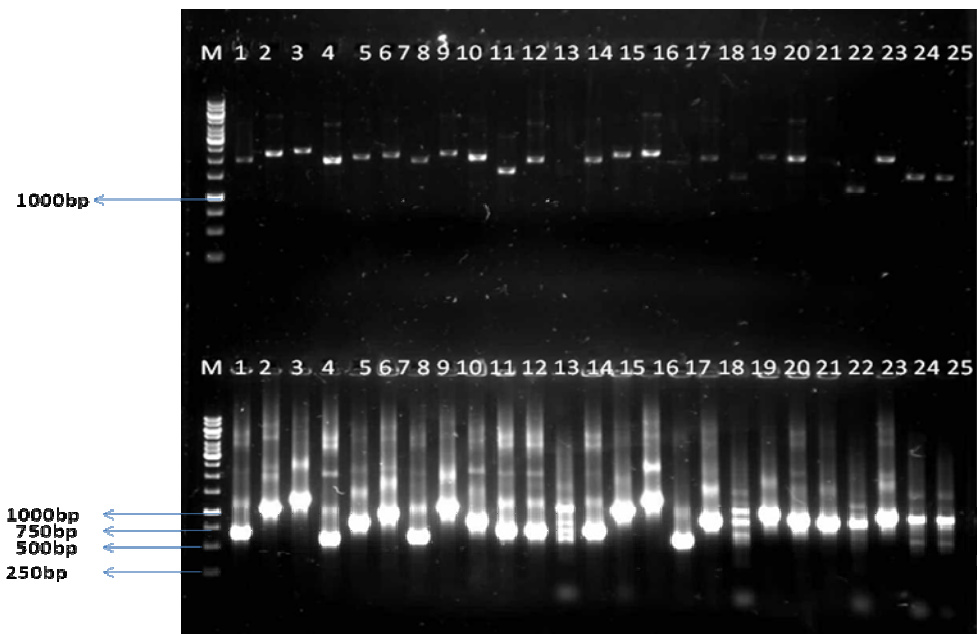


Plate 6: Gel showing colony PCR and plasmids isolation (M= 1 kb ladder; lane 1-25 (above) =plasmid isolation; lane 1-25 (below) = colony PCR)

4.1.8 Mining for microsatellite markers

Around 71 clones were sent for sequencing and out of this 55 were passed. From this sequence data, only 14 microsatellite repeats were identified using SSRIT, microsatellite identification tool (www.gramene.org/db/searches/ssrtool).

Out of fourteen microsatellite repeats eight were di-nucleotide repeats (57.1per cent) followed by two tri-repeats (14.2per cent), similarly two were tetra-repeats (14.2per cent), one was hexa-repeat (7.1per cent) and one compound repeat (7.1per cent).

4.1.9 Primer designing

Sequences with more than 20bp repeat motifs were selected for designing the primers in the flanking region of the repeat motifs. Primers were designed using Primer3 software (Rozen *et al.*, 2000) on the flanking region of the repeat motifs. Primers were designed with GC content of minimum 50 percent and annealing temperature between 50 to 55°C. A total of 14 primer pairs were designed. The length of primer was 20-23 bp and the product size ranged between 142 and 347 bp (Table 5).

4.1.9.1 Primer Standardization

Gradient PCRs (from 50± 5 °C to 60± 5 °C) were performed to standardize the primer annealing temperature by using only two genotypes. All 14 primers did show clear banding pattern at 55°C temperature. These standardized primers were further used for 10 genotypes of okra (Table 2).

Table 5: Microsatellite primers designed from sequence data of DNA fragments from enriched genomic library.

S. NO.	Primers	Primer sequence	Sequence length	Expected size(bp)	Repeat type
1	10K F	ACCATCAATTTGCCGAACTC	20	347	(GT) ₅
	10K R	ATTGGCTCAAGGATTGGTTG	20		
2	20K F	TGCCTAGAGGATCTCCACAGA	21	261	(TC) ₇
	20K R	TTCCGCAAATAAATTTCTCAA	23		
3	30K F	TGCTTTCTCTATCCC GTTCC	20	279	(CT) ₅
	30K R	GCTCATCTCGTCACAGGTCA	20		
4	40K F	CCTTGCCATGAGAGGAGAGA	20	219	(GT) ₉
	40K R	ACTGCTATTGCCTTGGTGCT	20		
5	50K F	TGCGTAAGTTGTTGGGATGA	20	142	(ATAC) ₁₅
	50K R	CCCTTTTCATGGCTTTTCA	20		
6	60K F	TCCCCACGCAAGTAAAAGAC	20	280	(AGAGA) ₄
	60K R	GGAAGTGTCCCCATTTCAGA	20		
7	70K F	GGATCCTTGGTGGATCATTG	20	190	(AGTG) ₃
	70K R	ACATCGAGTGGGGATTGAAA	20		
8	80K F	TGCTCCGTCAAATGTAATGC	20	296	(TTG) ₄
	80K R	GCCTGTGTGCATGTTTGT TT	20		
9	90K F	TTTGCAGAAGGATTCATGGAG	21	200	(GAA) ₄
	90K R	GAGAAGGTTTGGGAGCTGTG	20		
10	100K F	GCTCAAGATTTGGGGTTTGA	20	273	(AC) ₅
	100K R	CTTGTCCTCAAGCAATGCAA	20		
11	110K F	TCTCCGGAGCTATGAAATGAA	21	171	(AC) ₇
	110K R	GGGTGCATAGCTGGATGAGT	20		
12	120K F	GGAAAATTGGAAAGAAAAGGAAA	23	300	(GA) ₃ -X ₆ -(GA) ₅
	120K R	AATGCCCCCTTTGTTATTTT	20		
13	130K F	TGATCTCCCCCATCACAATC	20	213	(GT) ₇
	130K R	AAGCTGCATGGGCCTATATT	20		
14	140K F	CTTGTCCTCAAGCAATGCAA	20	197	(GT) ₅
	140K R	AGCCTTGAATCAGCCTTGTC	20		

Table 6: Genetic analysis of okra SSR primers in 10 genotypes.

Locus	K	N	H_{Obs}	H_{Exp}	PIC
10K	2	9	0.111	0.503	0.362
20K	2	10	0	0.505	0.365
30K	2	8	0	0.5	0.359
40K	2	10	0	0.442	0.332
50K	2	10	0	0.337	0.269
60K	1	9	0	0	0
70K	2	10	0	0.505	0.365
80K	2	9	0	0.471	0.346
90K	2	10	0	0.337	0.269
100K	2	10	0	0.337	0.269
110K	2	10	0	0.189	0.164
120K	1	4	0	0	0
130K	2	10	0	0.505	0.365
140K	2	10	0	0.526	0.375
Mean	1.86	0.9214	0.008	0.3684	0.2742

(K=Number of alleles; N=Number of genotypes; H_{Obs}=Observed heterozygosity; H_{Exp}=Expected heterozygosity; PIC=Polymorphic information content)

**Microsatellite polymorphism, genetic diversity in okra genotypes
revealed by cotton SSR markers**

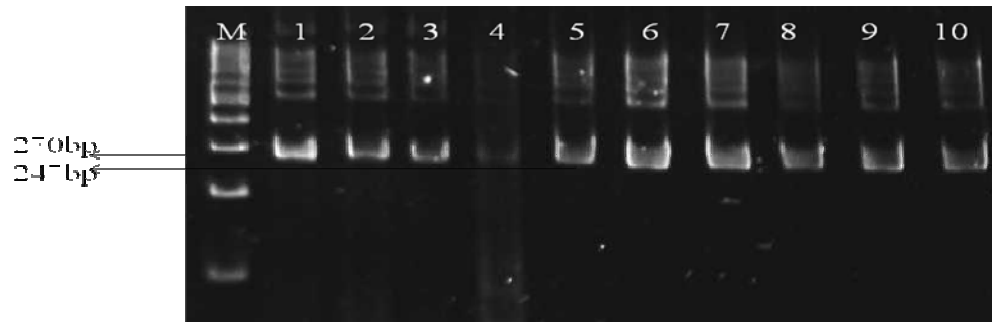


Plate 7a: Gel image showing amplification of 10K primer in 10 okra accessions

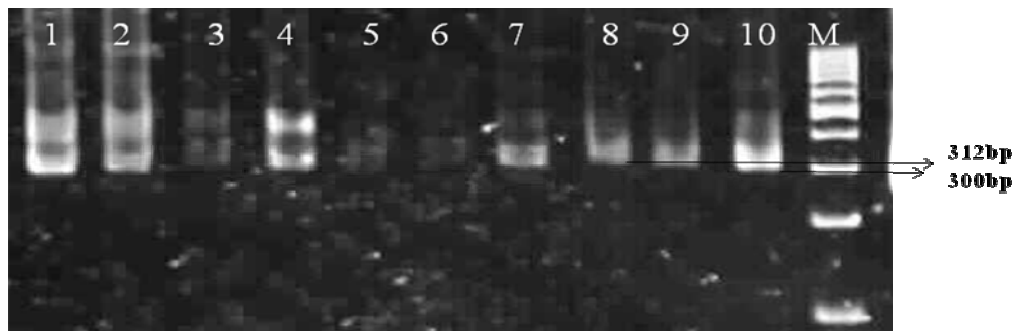


Plate 7b: Gel image showing amplification of 30K primer in 10 okra accessions

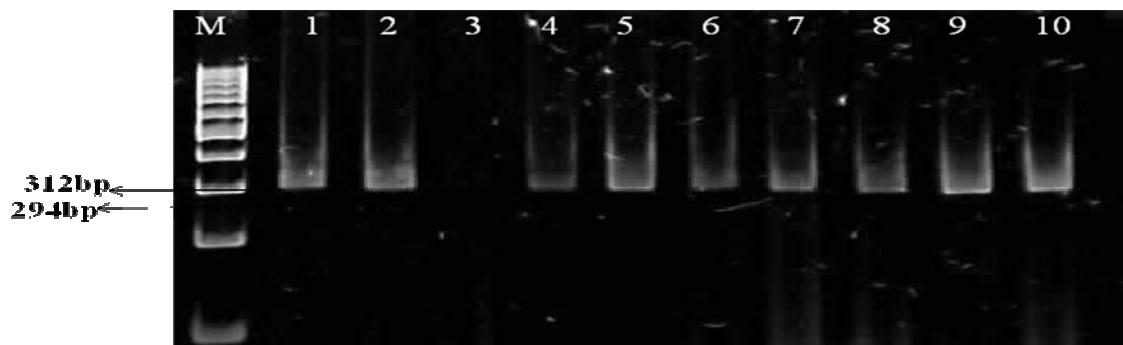


Plate 7c: Gel image for amplification of 80K primer in 10 okra accessions

Plate 7: Gel images for amplification of Okra SSR primers in 10 accessions

(Lane 1-10=okra genotypes, Lane M= 100bp ladder)

4.1.9.2 Screening of the Genomic SSR primers for polymorphism in okra genotypes

The fourteen SSR primers designed for were tested using 10 okra genotypes. These primers amplified in all 10 okra genotypes at 55°C, and showed polymorphism.

4.1.9.2.1 Genetic analysis

Genetic analysis was done by using Cervus 3.0 software (Kalinowski *et al.*, 2007).

PIC values ranged from 0 to 0.375 with mean of 0.2742 and observed heterozygosity (Hobs) ranged from 0 to 0.111 with mean of 0.008. The highest value of PIC and expected heterozygosity observed for 140K marker (HExp= 0.526, PIC= 0.375). The lowest value (0.00) observed in marker 60K and 120K. Number of alleles locus ranged from 1 to 2.

4.1.9.2.2 Cluster analysis

SSR bands were manually scored from the gel profiles (plates) by assigning values “1” for the presence and “0” for the absence at each position. Such a binary data generated from all profiles were used for statistical analysis. Dissimilarity matrix was computed using squared Euclidean distance (SED) to estimate pair-wise differences in amplification product for all varieties (Sokal and Sneath, 1973). The pooled SSR binary data was utilized for cluster analysis using the dataform for the 10 genotypes.

The genetic dissimilarity value in the distance matrix ranged from 0 to 19 (Table 7), suggesting a narrow genetic base within the okra genotypes used here. The highest genetic distance of 19 was observed in IIHR-245 and IIHR-242(19) and IIHR-247 & IIHR-242 (19) followed by

Table 7: Distance matrix (Squared Euclidean Distance) for 10 okra genotypes based on SSR data.

Genotypes	IIHR 239	IIHR 240	IIHR 241	IIHR 242	IIHR 243	IIHR 244	IIHR 245	IIHR 246	IIHR 247	IIHR 248
IIHR 239	0.00	1.00	9.00	6.00	13.00	11.00	13.00	15.00	17.00	15.00
IIHR 240	1.00	0.00	8.00	7.00	12.00	10.00	12.00	14.00	16.00	16.00
IIHR 241	9.00	8.00	0.00	7.00	10.00	14.00	18.00	10.00	12.00	12.00
IIHR 242	6.00	7.00	7.00	0.00	11.00	13.00	19.00	17.00	19.00	17.00
IIHR 243	13.00	12.00	10.00	11.00	0.00	12.00	12.00	10.00	12.00	14.00
IIHR 244	11.00	10.00	14.00	13.00	12.00	0.00	8.00	6.00	8.00	14.00
IIHR 245	13.00	12.00	18.00	19.00	12.00	8.00	0.00	10.00	12.00	14.00
IIHR 246	15.00	14.00	10.00	17.00	10.00	6.00	10.00	0.00	2.00	8.00
IIHR 247	17.00	16.00	12.00	19.00	12.00	8.00	12.00	2.00	0.00	6.00
IIHR 248	15.00	16.00	12.00	17.00	14.00	14.00	14.00	8.00	6.00	0.00

IIHR-245 and IIHR-241 (18) similarly the lowest genetic distance 1 was observed between IIHR-240 & IIHR-239 followed by IIHR-247 & IIHR-246 (2), IIHR-246 & IIHR-244(6).

A dendrogram was constructed by Ward's method of clustering using minimum variance algorithm (Ward, 1963).

It is clearly evident from the dendrogram (Fig. 1) that all the 10 okra accessions are grouped into two major clusters as A and B. Total dissimilarity value across all the accession was 36.5 units. Cluster A differed from cluster B with 18 units. Cluster A comprises total of 4 genotypes, subdivided in to 2 sub cluster A₁ and A₂ based on their relatedness, whereas cluster B comprises 6 genotypes, Further cluster B subdivided into 2 sub clusters namely B₁ to B₂. A phylogenetic tree constructed on the basis of dissimilarity between the genotypes made it possible to classify 10 okra genotypes into a total of 4 distinct sub clusters.

Sub cluster A₁ consisted of two genotypes namely IIHR-239 and IIHR-240; they were closely related to each other. Similarly sub cluster A₂ comprised of two genotypes namely IIHR-241 and IIHR-242. Sub cluster B₁ contains of three genotypes like IIHR-243, IIHR-244 and IIHR-245. Sub cluster B₂ has three genotypes were IIHR-246, IIHR-247 and IIHR-248. The dissimilarity between A₁ and A₂ sub cluster was 14 units and the dissimilarity between B₁ and B₂ was 18 units.

4.2 Cross amplification of cotton SSR primers in okra genotypes.

4.2.1 Isolation of genomic DNA and its quantification

Genomic DNA was isolated from the 25 okra accessions (cultivated) using “mini-prep” method as described in materials and methods from fresh young leaves. The extraction method yielded good quality and quantity of DNA. The amount of DNA present was determined by UV-Vis spectrophotometer at an emission spectrum of 260 nm. The quantity of DNA sample varied 310 to 1100ng/μl with mean of 576.20ng/μl and A260/A280 ratio of DNA ranged from 1.70 to 2.28 with mean of 1.87. Further the intensity of isolated DNA samples were tested and found good gel electrophoresis (0.8per cent agarose) to carry out the PCR amplification.

4.2.2 PCR amplification using cotton SSR markers

Initially, the PCR amplification was carried out, a total of 90 cotton SSR primers (Table 4) with two okra genotypes. The PCR parameters were optimized like, primer concentration at 5 pM, at different annealing temperature 50°C, 53°C and 57°C, DNA concentration at 20 ng/μl per reaction and dNTPs concentration at 1 mM and Taq DNA polymerase at 1U by observing high stringency, clear and reproducible bands. Initially PCR products were separated on 2per cent agarose gel (Plate. 8). Later, the amplified products were separated by 8per cent polyacrylamide gel electrophoresis with ethidium bromide staining, which shows good resolution to score the SSR bands. Annealing temperature 57°C was taken as optimum for all genotypes because at this temperature we have observed good amplification compared to annealing at 50°C and 53°C.

4.2.3 SSR marker analysis

The study aims at determining the genetic diversity among okra genotypes using cotton SSR primers. Ninety cotton primers of microsatellites [Brookhaven National Laboratory (BNL), WWW.cottonmarker.org] were used to amplify DNA from okra samples. Out 90 primers, 26 were successfully showed good amplification with polymorphism. Those giving single or double bands were preferably selected. The size range of amplification products also ranged between 180bp to 350bp (Plate 9).

Out of 90 primers, 26 primers were amplified/transferred (29per cent). In this 14 primers showed 1 or 2 bands and 12 primers showed multiple bands.

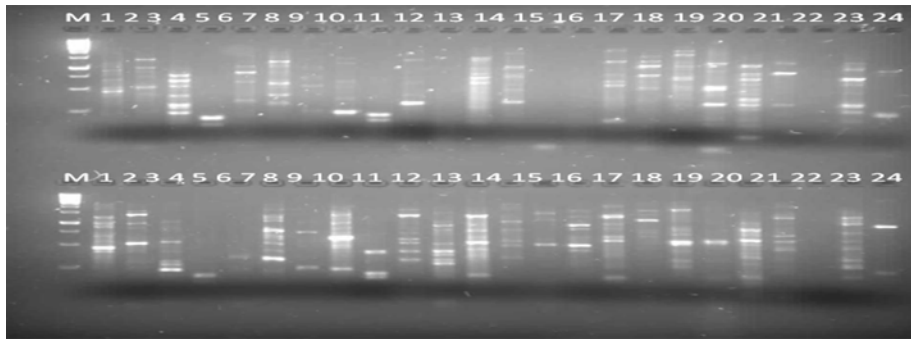


Plate 8a: Gel profile showing bands obtained on 2% agarose gel for standardization of the annealing temperature at 50°C

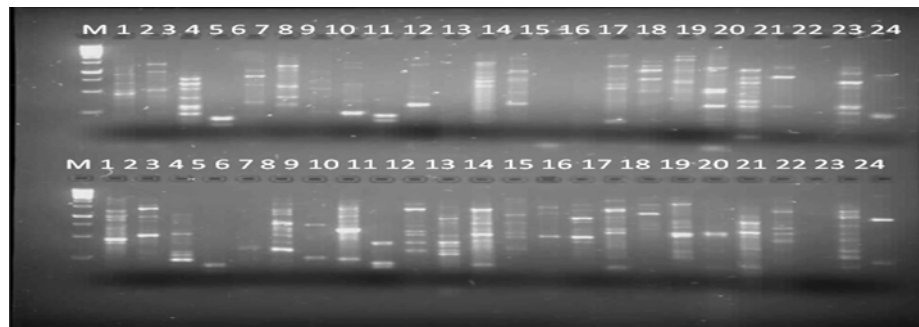


Plate 8b: Gel profile showing bands obtained on 2% agarose gel for standardization of the annealing temperature at 53°C.

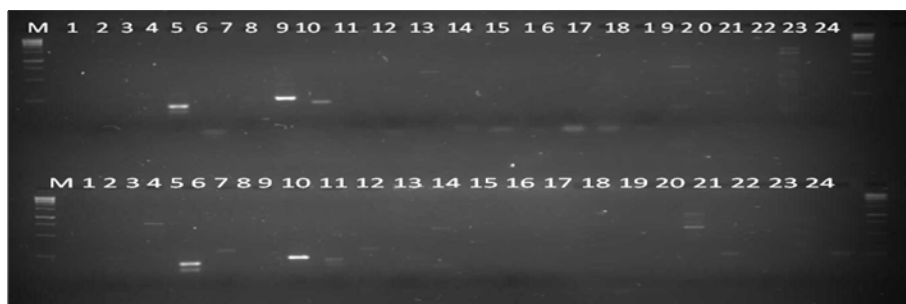


Plate 8c: Gel profile showing bands obtained on 2% agarose gel for standardization of the annealing temperature at 57°C

Plate 8: PCR standardization at different annealing temperature (M=100bp ladder, Lane.1-24 (above) IIHR-15 and Lane 1-24 (below) IIHR-18.

Table 8: Transferability of cotton SSR markers in okra.

Cotton marker (Di-repeats)	Total marker	Amplifiable marker	Polymorphic marker	Transferability (per cent)	Per cent polymorphic primers (per cent)
Simple	59	19	9	32	47
Compound	19	6	3	32	50
Compound imperfect	12	1	1	8	100
Total	90	26	13	29	50

Transferability= Amplified markers/total markers.

Per cent polymorphic markers = Polymorphic markers/amplified markers

4.2.4 Cluster analysis

SSR bands were manually scored from the gel profiles by assigning values “1” for the presence and “0” for the absence at each position. Such a binary data generated from all profiles were used for statistical analysis. Dissimilarity matrix was computed using squared Euclidean distance (SED) to estimate pair-wise differences in amplification product for all varieties (Sokal and Sneath, 1973). The pooled SSR binary data was utilized for cluster analysis using the data form for the 26 genotypes. The genetic dissimilarity value in the distance matrix ranged from 0 to 36 (Table 9), suggesting a narrow genetic base within the okra genotypes used here. The highest genetic distance of 36 was observed between IIHR-226 and IIHR-101 followed by IIHR-219 and IIHR-101(35), IIHR-219

and IIHR-108(33), IIHR-226 and IIHR-108(32), IIHR-227 and **Microsatellite polymorphism, genetic diversity in okra genotypes revealed by cotton SSR markers on PAGE (8%).**



Plate 9a: Lane 1-25: Amplification shown by BNL285 marker in 25 different okra genotypes

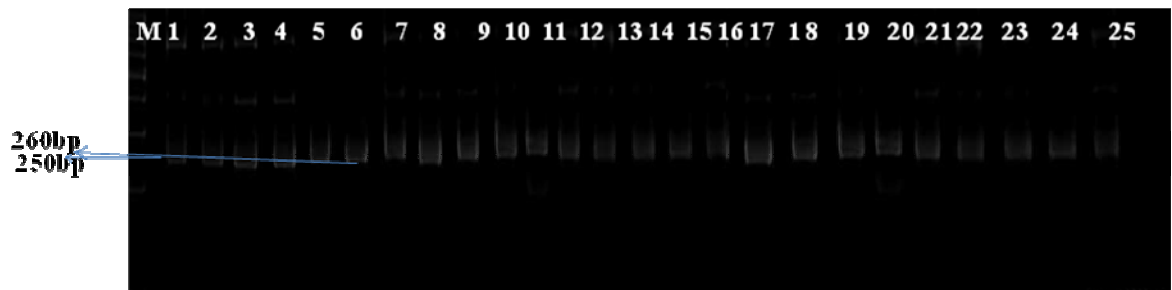


Plate 9b: Lane 1-25: Amplification shown by BNL2544 marker in 25 different okra genotypes

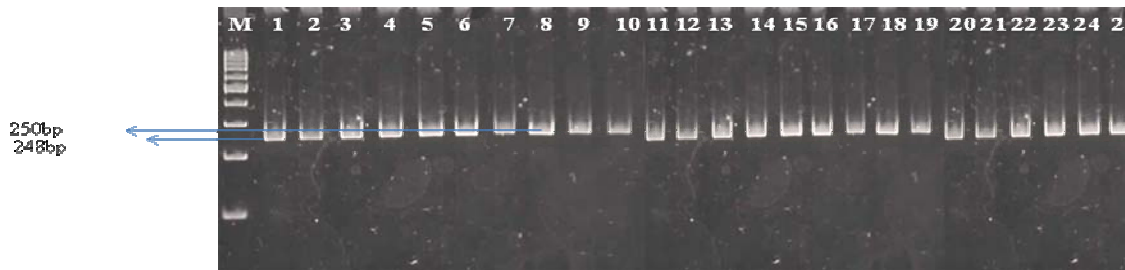


Plate 9c: Lane 1-25: Amplification shown by BNL358 marker in 25 different okra genotypes

Plate 9: Gel profile showing amplification for cotton SSR primers in okra genotypes (M=100bp ladder; Lane 1-25=okra genotypes)

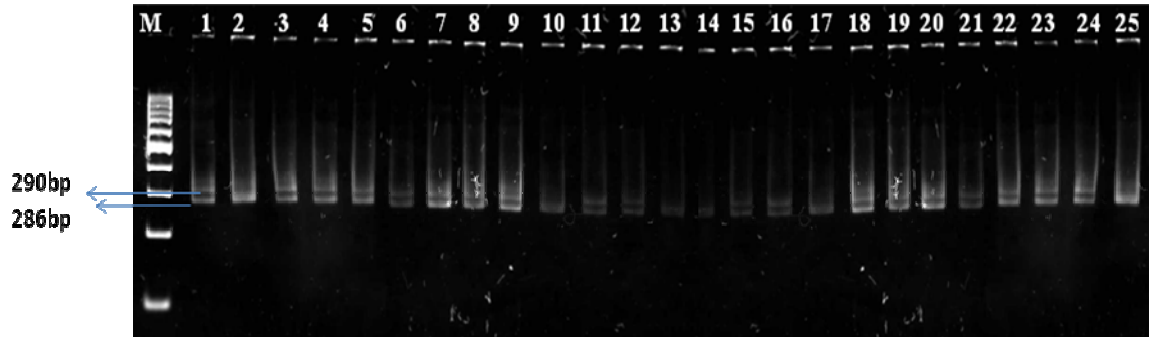


Plate 9d: Lane 1-25: Amplification shown by BNL226 marker in 25 different okra genotypes

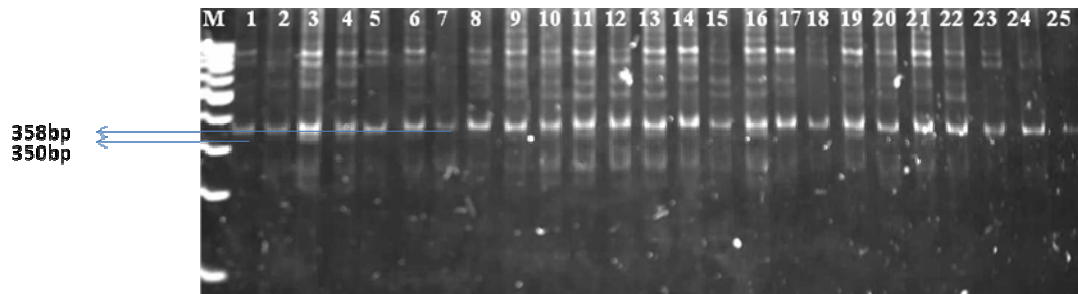


Plate 9e: Lane 1-25: Amplification shown by BNL827 marker in 25 different okra genotypes

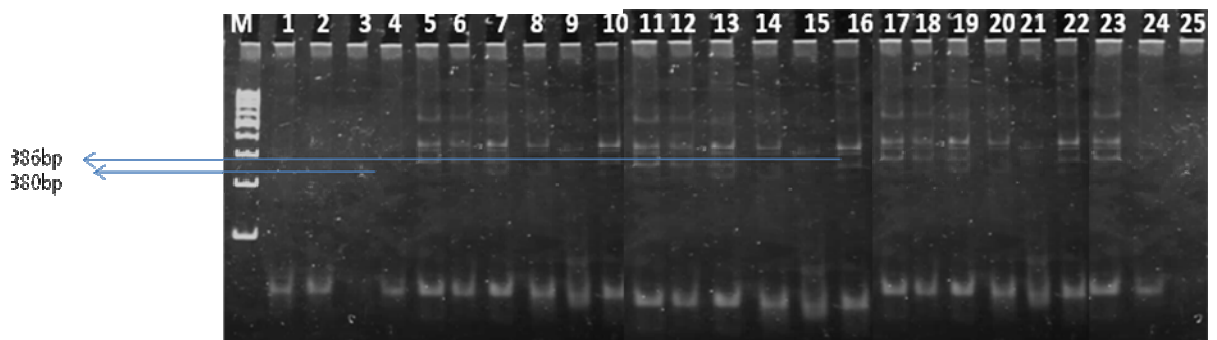


Plate 9f: Lane 1-25: Amplification shown by BNL2960 marker in 25 different okra genotypes

Plate 9: Gel profile showing amplification for cotton SSR primers in okra genotypes (M=100bp ladder; Lane 1-25=okra genotypes)

IIHR-101 and IIHR-227 and IIHR-108. Similarly the lowest genetic distance 3 was observed between IIHR-134 and IIHR-133, IIHR-233 and IIHR-237, IIHR-238 and IIHR-233 (5) and IIHR-229 and IIHR-230, IIHR-232 and IIHR-233(6).

A dendrogram was constructed by Ward's method of clustering using minimum variance algorithm (Ward, 1963).

It is clearly evident from the dendrogram (Fig. 2) that all the 26 okra genotypes were grouped into two major clusters as A and B. Total dissimilarity value across all the accession was 82 units. Cluster A differed from cluster B with 69 units. Cluster A comprises total of 7 genotypes, subdivided in to 2 sub cluster A₁ and A₂, whereas cluster B comprises 19 genotypes, Further cluster B subdivided into 5 sub clusters namely B₁ to B₅. A dendrogram constructed on the basis of dissimilarity values between the genotypes made it possible to classify 26 okra genotypes into a total of 7 distinct clusters.

Sub cluster A₁ consisted of three genotypes namely IIHR-15, IIHR-18 and IIHR-20. Sub cluster A₂ comprised of four genotypes namely IIHR-55, IIHR-72, IIHR-81 and IIHR-91. sub cluster B₁ included two genotypes like IIHR-101 and IIHR-108. Sub cluster B₂ involved five genotypes were IIHR-231, IIHR-232, IIHR-233, IIHR-237 and IIHR-238. Sub cluster B₃ consisted of six genotypes as IIHR-106, IIHR-104, IIHR-133, IIHR-134, IIHR-181 and IIHR-213. Sub cluster B₄ contain three genotypes namely IIHR-219, IIHR-226 and IIHR-227. Sub cluster B₅ comprises only two genotypes namely IIHR-229 and IIHR-230.

The

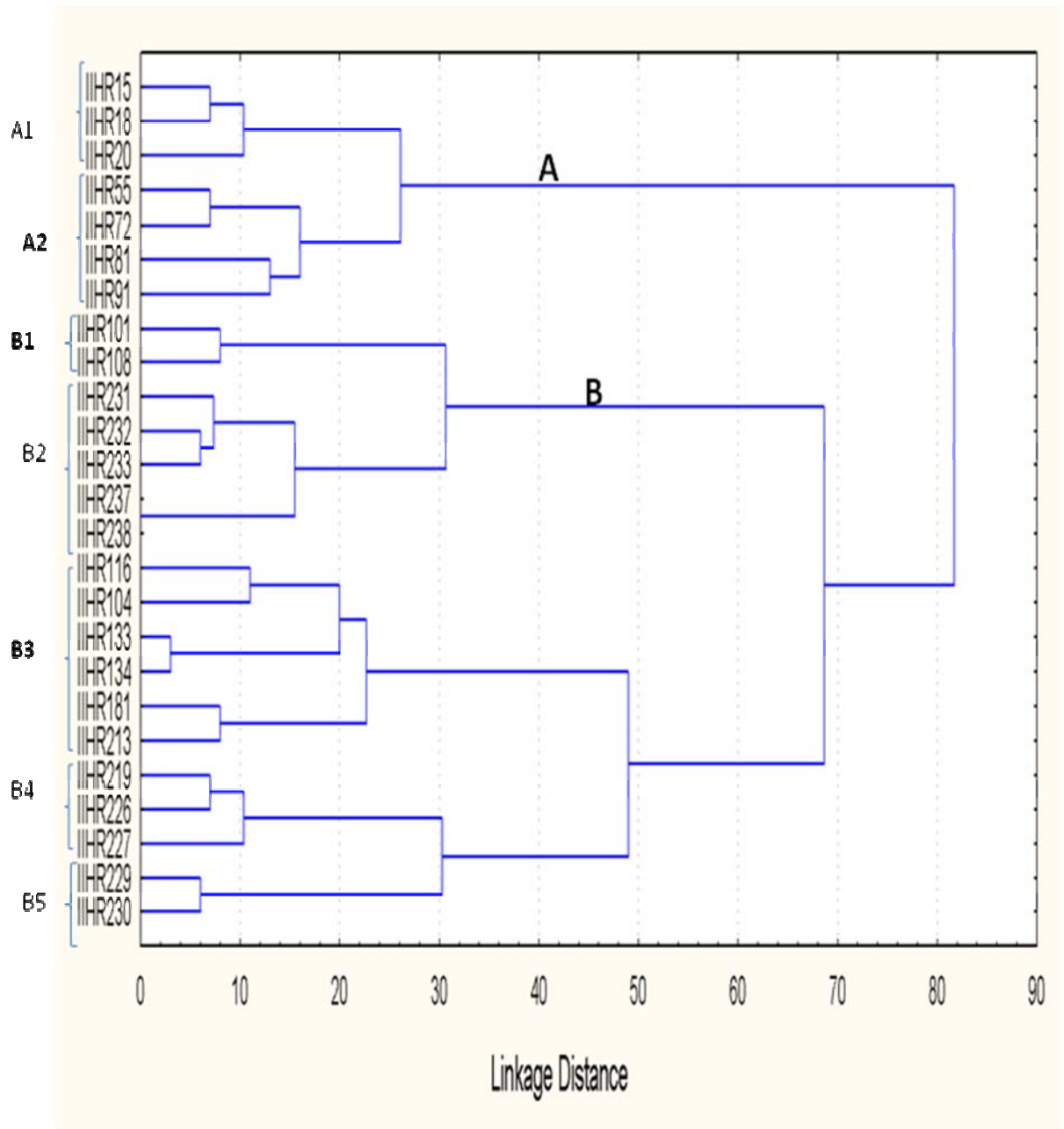


Fig 2: Dendrogram showing the genetic relatedness among 25 okra accession using cotton SSR marker data.

Table 9 Distance matrix (Squared Euclidean Distance) for 25 okra genotypes based on cotton SSR markers data.

	IIHR15	IIHR18	IIHR20	IIHR55	IIHR72	IIHR81	IIHR91	IIHR101	IIHR108	IIHR116	IIHR104	IIHR133	IIHR134	IIHR181	IIHR213	IIHR219	IIHR226	IIHR227	IIHR229	IIHR230	IIHR231	IIHR232	IIHR233	IIHR237	IIHR238
IIHR15	0.00	7.00	10.00	14.00	13.00	14.00	19.00	23.00	19.00	20.00	23.00	19.00	22.00	22.00	24.00	22.00	21.00	25.00	18.00	16.00	16.00	21.00	17.00	20.00	20.00
IIHR18	7.00	0.00	9.00	13.00	12.00	15.00	20.00	26.00	22.00	19.00	22.00	20.00	23.00	21.00	19.00	17.00	18.00	24.00	19.00	17.00	17.00	20.00	20.00	23.00	23.00
IIHR20	10.00	9.00	0.00	10.00	17.00	14.00	19.00	23.00	19.00	20.00	23.00	19.00	22.00	18.00	20.00	22.00	25.00	29.00	24.00	20.00	18.00	23.00	23.00	26.00	26.00
IIHR55	14.00	13.00	10.00	0.00	7.00	14.00	15.00	25.00	19.00	26.00	29.00	23.00	26.00	26.00	24.00	22.00	25.00	27.00	20.00	16.00	18.00	21.00	21.00	24.00	24.00
IIHR72	13.00	12.00	17.00	7.00	0.00	11.00	12.00	22.00	18.00	25.00	26.00	24.00	27.00	29.00	25.00	23.00	24.00	24.00	17.00	13.00	17.00	16.00	18.00	21.00	21.00
IIHR81	14.00	15.00	14.00	14.00	11.00	0.00	13.00	17.00	17.00	24.00	25.00	23.00	26.00	28.00	26.00	28.00	27.00	29.00	22.00	18.00	16.00	17.00	19.00	22.00	22.00
IIHR91	19.00	20.00	19.00	15.00	12.00	13.00	0.00	14.00	18.00	27.00	28.00	24.00	27.00	29.00	29.00	29.00	28.00	28.00	25.00	19.00	23.00	22.00	24.00	27.00	27.00
IIHR101	23.00	26.00	23.00	25.00	22.00	17.00	14.00	0.00	8.00	17.00	16.00	20.00	23.00	23.00	27.00	35.00	36.00	30.00	23.00	21.00	19.00	16.00	16.00	19.00	19.00
IIHR108	19.00	22.00	19.00	19.00	18.00	17.00	18.00	8.00	0.00	13.00	18.00	18.00	21.00	19.00	23.00	33.00	32.00	30.00	23.00	21.00	13.00	12.00	12.00	15.00	15.00
IIHR116	20.00	19.00	20.00	26.00	25.00	24.00	27.00	17.00	13.00	0.00	11.00	17.00	16.00	12.00	18.00	20.00	23.00	21.00	24.00	24.00	16.00	19.00	17.00	18.00	18.00
IIHR104	23.00	22.00	23.00	29.00	26.00	25.00	28.00	16.00	18.00	11.00	0.00	10.00	11.00	13.00	17.00	21.00	20.00	14.00	15.00	19.00	17.00	18.00	16.00	17.00	17.00
IIHR133	19.00	20.00	19.00	23.00	24.00	23.00	24.00	20.00	18.00	17.00	10.00	0.00	3.00	13.00	19.00	23.00	22.00	20.00	19.00	21.00	19.00	18.00	20.00	21.00	21.00
IIHR134	22.00	23.00	22.00	26.00	27.00	26.00	27.00	23.00	21.00	16.00	11.00	3.00	0.00	10.00	16.00	20.00	19.00	17.00	20.00	22.00	20.00	19.00	21.00	22.00	22.00
IIHR181	22.00	21.00	18.00	26.00	29.00	28.00	29.00	23.00	19.00	12.00	13.00	13.00	10.00	0.00	8.00	14.00	17.00	19.00	22.00	26.00	20.00	21.00	21.00	22.00	22.00
IIHR213	24.00	19.00	20.00	24.00	25.00	26.00	29.00	27.00	23.00	18.00	17.00	19.00	16.00	8.00	0.00	10.00	13.00	19.00	22.00	26.00	20.00	17.00	21.00	16.00	16.00
IIHR219	22.00	17.00	22.00	22.00	23.00	28.00	29.00	35.00	33.00	20.00	21.00	23.00	20.00	14.00	10.00	0.00	7.00	11.00	16.00	22.00	22.00	23.00	25.00	22.00	22.00
IIHR226	21.00	18.00	25.00	25.00	24.00	27.00	28.00	36.00	32.00	23.00	20.00	22.00	19.00	17.00	13.00	7.00	0.00	8.00	17.00	23.00	21.00	22.00	24.00	21.00	21.00
IIHR227	25.00	24.00	29.00	27.00	24.00	29.00	28.00	30.00	30.00	21.00	14.00	20.00	17.00	19.00	19.00	11.00	8.00	0.00	9.00	15.00	19.00	20.00	22.00	23.00	23.00
IIHR229	18.00	19.00	24.00	20.00	17.00	22.00	25.00	23.00	23.00	24.00	15.00	19.00	20.00	22.00	22.00	16.00	17.00	9.00	0.00	6.00	10.00	15.00	15.00	20.00	20.00
IIHR230	16.00	17.00	20.00	16.00	13.00	18.00	19.00	21.00	21.00	24.00	19.00	21.00	22.00	26.00	26.00	22.00	23.00	15.00	6.00	0.00	8.00	13.00	13.00	18.00	18.00
IIHR231	16.00	17.00	18.00	18.00	17.00	16.00	23.00	19.00	13.00	16.00	17.00	19.00	20.00	20.00	20.00	22.00	21.00	19.00	10.00	8.00	0.00	7.00	7.00	12.00	12.00
IIHR232	21.00	20.00	23.00	21.00	16.00	17.00	22.00	16.00	12.00	19.00	18.00	18.00	19.00	21.00	17.00	23.00	22.00	20.00	15.00	13.00	7.00	0.00	6.00	9.00	9.00
IIHR233	17.00	20.00	23.00	21.00	18.00	19.00	24.00	16.00	12.00	17.00	16.00	20.00	21.00	21.00	21.00	25.00	24.00	22.00	15.00	13.00	7.00	6.00	0.00	5.00	5.00
IIHR237	20.00	23.00	26.00	24.00	21.00	22.00	27.00	19.00	15.00	18.00	17.00	21.00	22.00	22.00	16.00	22.00	21.00	23.00	20.00	18.00	12.00	9.00	5.00	0.00	0.00
IIHR238	20.00	23.00	26.00	24.00	21.00	22.00	27.00	19.00	15.00	18.00	17.00	21.00	22.00	22.00	16.00	22.00	21.00	23.00	20.00	18.00	12.00	9.00	5.00	0.00	0.00

Table 10: Genetic analysis of cotton SSR markers in okra genotypes

Marker	K	N	H_{Obs}	H_{Exp}	PIC
BNL 285	2.0000	24	0.52	1.0000	0.3750
BNL 3379	2.0000	25	0.00	1.0000	0.3750
BNL 827	2.0000	25	0.00	1.0000	0.3750
BNL 1162	2.0000	25	0.00	1.0000	0.3750
BNL 2960	2.0000	23	0.00	0.8400	0.3685
BNL 2544	2.0000	24	0.00	1.0000	0.3750
BNL 530	2.0000	25	0.00	1.0000	0.3750
BNL 358	2.0000	24	0.00	0.9600	0.3746
BNL 1079	2.0000	25	0.00	1.0000	0.3750
BNL 1030	2.0000	25	0.00	0.0800	0.3546
BNL 625	2.0000	23	0.00	0.1600	0.1364
BNL 226	2.0000	24	0.00	0.0800	0.0739
BNL 1034	2.0000	23	0.00	0.9200	0.3734
Mean	2.000	24.23	0.04	0.7538	0.3312

(K=Number of alleles; N=Number of genotypes; H_{Obs}=Observed heterozygosity; H_{Exp}=Expected heterozygosity; PIC=Polymorphic information content)

dissimilarity between A1 and A2 sub cluster was 26 units and the dissimilarity between B1 and B2 was 31 units, B4 and B5 was 30 units.

4.2.4.2 Genetic analysis

PIC values were assessed for 25 okra genotypes by using 13 cotton primers in order to assess marker diversity (Table.9).

PIC value ranged from 0.3750 TO 0.0739 and heterozygosity (Hex) ranged from 1 to 0.0800, with mean of 0.3312 and 0.7538 respectively. The highest value of PIC and heterozygosity found in 7 markers (BNL285, BNL3379, BNL827, BNL1162, BNL2544, BNL530, BNL1079). The lowest value (0.0739) found in marker BNL226 followed by BNL625 (0.1364). Number of alleles two.

DISCUSSION

V. DISCUSSION

Okra (*Abelmoschus esculentus* (L.) Moench) is an important crop of the tropics and subtropics of the world. It is having chromosome number of $2n=130$, is polyploidy in nature (Joshi *et al.*, 1956). In India, it is popular green vegetable commonly grown for its tender fruits in tropics, subtropics and warmer parts of temperate region.

Molecular markers have been widely used in genetic analysis and breeding of plant species, which has many applications. Molecular markers have emerged as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. Molecular breeding strategy using tightly linked DNA based molecular markers to identify the desirable genotype is expected to significantly complement and accelerate breeding programmes (Varshney *et al.*, 2007).

Among the various types of molecular markers available, microsatellites have received greater attention recently, especially for breeding purposes. Microsatellite markers, also known as simple sequence repeats or SSRs (Litt and Luty, 1989), are clusters of short (usually 2 to 6) tandemly repeated nucleotide bases distributed throughout the genome. Microsatellite markers distinguish themselves as co-dominant, multiallelic, highly polymorphic genetic markers, requiring small amounts of DNA for straightforward PCR and gel electrophoresis analysis. Its main disadvantage is the high cost of the initial investment necessary for marker development.

Molecular markers are increasingly being used for germplasm diversity, linkage analysis and molecular breeding. Despite these advantages the number of molecular markers reported in okra is considerably less compared to many related crop species like cotton,

hibiscus, and jute. Keeping this in view, in this study we have attempted to develop SSR markers in okra and also examined cross species amplification of cotton SSR markers in okra.

5.1 Development of microsatellite marker in okra from microsatellite enriched genomic library

5.1.1 DNA isolation

Okra leaves are rich in polysaccharides and phenols, which interferes with the isolation and purification of DNA samples (Huang *et al.*, 2008). The main obstacle preventing the extraction of DNA from green leaves of all members of genus is that, even after grinding in liquid nitrogen, the resulting suspension is very sticky and it is almost impossible to obtain DNA. This thick consistency is principally due to large amount of polysaccharides produced during photosynthesis. The polysaccharides contamination can also cause problems in downstream processing/ application by inhibiting certain enzymes in PCR (Hugo *et al.*, 1998). Hence, different DNA isolation protocols were standardized during the investigation, thereby DNA purification by silica method was performed using silica matrix that helps to remove some polysaccharides or protein contamination in DNA.

5.1.2 Microsatellite development

The traditional method of microsatellite isolation involves isolation of microsatellite loci from partial genomic libraries (selected for small insert size) from the species of interest after screening several thousands of clones through colony hybridization with repeat containing probes (Rassmann *et al.*, 1991). The method, though simple and useful for microsatellite rich genomes, was inefficient for species with low microsatellite frequencies. Ostrander *et al.* (1992) and Paetkau (1999) proposed isolation method based on primer extension that increased the

efficiency to 40-50 percent and also upto 100 per cent positive clones. However, the protocol involved many steps, which was a limitation for its applicability. Hence the present study employed enrichment or selective hybridization strategy, which has evolved as a best alternative strategy to both traditional and primer extension methods.

The simplest approach for obtaining microsatellite DNA loci is, cloning small genomic fragments and using non-radio labeled oligonucleotide probes of microsatellite repeats to identify clones with microsatellites. Hybridization capture is the predominant strategy because it allows selection prior to cloning, and therefore is faster and easier for library construction (Kandpal *et al.*, 1994).

According to Glenn and Schable (2005), restriction digestion of genomic DNA step is very important, RE`s are an easy way to fragment DNA. The DNA is cut approximately at the size of 500bp, this size would be ideal for sequencing. Incorporation of a GTTT “pig-tail” on the Super SNX linker (Hamilton et al, 1999), ensures efficient A-tailing of each PCR product yielding good results from TA cloning, it also helps in amplification of DNA using the SuperSNX linker/primer which is biased against producing small PCR products. Thus, PCR products obtained from the experiment can be cloned directly without obtaining a large proportion of small DNA fragments.

In present study around 71 clones were isolated and sent for sequencing, out of this 55 sequence data was obtained. A total 14 microsatellite repeats were identified using SSRIT (WWW.gramene.org). Out of fourteen microsatellite repeats, eight were di-nucleotide repeats (57.1 per cent), followed by two tri-repeats (14.2 per cent), similarly two were tetra-repeats (14.2 per cent), one was hexa-repeat (7.1 per cent) and one compound repeat (7.1 per cent). Primers were designed for flanking regions using Primer3 software tool (Rozen *et al.*, 2000). Here di repeats

were higher in number compared to tri-, tetra-, hexa- and compound repeats. 57.1 per cent of the SSR motifs isolated in the present study were di-nucleotide repeats, among them GT (50 per cent), AC (25 per cent), TC (12.5 per cent) and CT (12.5 per cent) (Table 8). The di-repeats were more abundant in plants when SSR's are isolated from genomic library (Lagercrantz *et al.*, 1993).

About 20 per cent of the SSR's isolated were found useful for primer design. In the present study, of the 14 potential primer pairs synthesized, they amplified all 10 okra genotypes at 55°C, and showed polymorphism. The PCR products were from 140 to 300bp sizes.

5.1.3 Genetic analysis

PIC (Polymorphic Information Content) values ranged from 0.000 to 0.375 with a mean of 0.2742 and observed heterozygosity(H_o) ranged from 0.111 to 0, the highest value of PIC and heterozygosity observed for 140K marker, which indicated that 140K marker had a greater potential to reveal allelic variations, and can be preferentially used for assessing genetic diversity within a population. The lowest value of 0.00 was found for markers 60K and 120K. Numbers of alleles were either 1 or 2 for all the markers (Table 6).

The genetic dissimilarity value in the distance matrix ranged from 0 to 19 (Table-5), suggesting a narrow genetic base within the okra genotypes used here. The highest genetic distance (19) was observed between IIHR-245 and IIHR-242, IIHR-247 and IIHR-242 followed by IIHR-245 and IIHR-241 (18). Similarly the lowest genetic distance was observed between IIHR-240 and IIHR-239 followed by IIHR-247 and IIHR-246 (2), IIHR-246 and IIHR-244 (6).

The development of microsatellite markers can be based on DNA sequence information deposited in databases, or it could be based on the

sequence data of genomic DNA libraries specifically constructed for the discovery of repeated sequences in the genome. The use of genomic libraries enriched for microsatellite sequences is a strategy devised to decrease the cost of marker development thereby increasing the probability of marker discovery (Kandpal *et al.*, 1994).

In this study, we have attempted to develop microsatellite markers in okra and have isolated 71 clones from microsatellite enriched genomic library. From these clones, we were able to obtain DNA sequences data for 55 clones. Using this sequence data 14 microsatellites were identified. Fourteen primers were designed that amplified all the 10 genotypes. Genetic analysis showed these SSR markers showed PIC values from 0.000 to 0.375, which indicated their usefulness in genetic characterization and mapping.

5.2 Cross amplification cotton SSR primers in okra

Development of SSR's have been difficult due to the relatively high cost and time required for their development. Therefore possibility of transferring SSR primers developed for one particular species are normally applicable across wide range of related species. DNA markers are transferable among related species due to the conserved regions in their genomes. The chance of successful cross species SSR amplification is inversely related to the evolutionary distance between them (Wang *et al.*, 2009). The polymorphism level detected by EST-SSRs is almost comparable at cross-species and cross-genus level highlighting the fact that genic SSR markers have higher transferability and thus better applicability than genomic SSR markers (Wang *et al.*, 2005). Since EST-SSRs are located within genes, and thus more conserved across species, they may be less polymorphic than anonymous SSRs (Ellis and Burke, 2007).

Cross genus amplification was considered only when the amplicons were generated from other genera but not from their own genus, using which primers were designed (Wang *et al.*, 2004). Transferable SSR markers can be divided into the following three steps: a) collection of SSR markers from related species, b) Estimation of the transfer rate of SSR markers, and c) identification of the polymorphisms (or polymorphism level) of transferable SSR markers (Wang *et al.*, 2004). Only clear and reproducible bands were considered as the amplified products, across species to genera. PCR stringency is also one of the important dependable factors which largely determine the rate of transferability.

In this study a total of 90 primers (BNL, primers) were collected from cotton (*Gossypium hirsutum*) of the same family, Malvaceae. For PCR standardization we have tried at different temperature regimes of 50°C, 53°C and 57°C for annealing temperature. At annealing temperature of 50°C, we observed all primers amplified bands, but showed multiple bands (Plate.8.1), similarly in 53°C also (Plate.8.2) but at 57°C we found 1 or 2 bands (Plate 8.3), therefore we have taken 57°C as optimum temperature for our study. Out of 90 markers 26 markers (29 per cent) showed good amplification with all okra accessions out of which 13 markers (50 per cent) showed polymorphism (Table.8). The amplified products ranged from 180bp-350bp.

SSR bands were manually scored from the gel profiles (Plate.9) by assigning values “1” for the presence and “0” for the absence at each position. Dissimilarity matrix was computed using Squared Euclidean Distance (SED) to estimate pair-wise differences in amplification product for all varieties.

The genetic dissimilarity values in the distance matrix ranged from 0 to 36, suggesting that a narrow genetic base within the okra genotypes

here. The highest genetic distance of 36 was observed in IIHR-226 and IIHR-101, followed by IIHR-219 and IIHR-101(35), IIHR-219 and IIHR-108(33), IIHR-226 and IIHR-108(32), IIHR-227 and IIHR-101(32) and finally IIHR-227 and IIHR-108(32), indicated that these were genetically more distant. The hybrid vigour may be exploited by effective crossing between these genotypes to obtain desirable segregates (Prakash *et al.*, 2011). The primary reason for narrow genetic base in okra genotypes is due to low cross pollination (4-19 per cent). Candida *et al.* (2006) also found a narrow genetic base in cotton.

A dendrogram was constructed by Ward's method of clustering using minimum variance algorithm (Ward, 1963). Cluster analysis was done based on polymorphic bands in 25 okra genotypes using 26 cotton SSR primers. It is clearly evident from the dendrogram that, all the 26 okra accessions are grouped into two major clusters A and B (Fig.2). Cluster A differed from cluster B with 69 units. Cluster A comprised a total of 7 genotypes, subdivided into 2 sub clusters A₁ and A₂, Sub cluster A₁ consisted of three genotypes namely IIHR-15, IIHR-18 and IIHR-20. Sub cluster A₂ comprised of four genotypes namely IIHR-55, IIHR-72, IIHR-81 and IIHR-91, whereas cluster B comprised 19 genotypes, further cluster B subdivided into 5 sub clusters namely B₁ to B₅. A phylogenetic tree constructed on the basis of dissimilarity between the genotypes made it possible to classify 25 okra genotypes into a total of 7 distinct clusters (Fig. 2).

In the present study the okra accessions, IIHR-15 and IIHR-18 were closely related to each other morphologically, and they both shared the same pedigree, so they were grouped onto one cluster. IIHR-237 and IIHR-238 were more genetically related and have similar morphological characters. (Fruit weight, leaf shape, leaf colour). Similarly, both IIHR-

133 and IIHR-134 (pure line) were closely related as they were collected from the same location (Prakash *et al.*, 2011).

PIC values ranged from 0.3750 TO 0.0739 and heterozygosity (H_{ex}) ranged from 0.08 to 1, with a mean of 0.3312 and 0.7538 respectively. The higher values of PIC and heterozygosity were found in 7 markers (BNL285, BNL3379, BNL827, BNL1162, BNL2544, BNL530, and BNL1079). The lower values (0.0739) were found in marker BNL226 followed by BNL625 (0.1364), the number of alleles were found to be two. Also the cotton SSR markers which amplified okra DNA, it was observed that, almost all were di-repeats (100 per cent) and 26 markers showed transferability/amplification in all 25 okra genotypes. Among these di-repeats, on the basis of arrangement, it was found that, out of 26 markers (Table 8), six were simple compound repeats (23.1 per cent) and one (3.84 per cent) was compound imperfect (BNL 285). The marker with higher PIC value has greater potential to reveal allelic variations, and can be preferentially used for choosing appropriate parental line for molecular mapping or accessing genetic diversity within a population (Anderson *et al.*, 1993).

Usefulness of transferability of SSR`s, is mostly restricted to congeners. For studies requiring more extensive cross-species amplification in plants, it may be necessary to target SSR`s anchored to genes. The level of polymorphism revealed by transferrable SSR markers probably depends on the phylogenetic distance among species and the genetic distance within the species, but not on the basis of transferable SSR markers. The level of polymorphism detected may not be related to the transfer rate of SSR markers. In other words, high transfer rate markers do not necessarily reveal a high level of polymorphism. In terms of the degree of outcrossing of the species, SSR markers may not be easily transferred because of their sequence divergence which may occur

rapidly compared to other species. However, once the SSR markers are transferred to the out crossing species, the level of polymorphism detected should be high within the species (Wang *et al.*, 2009).

The results revealed that, the ability of cross amplification is very less between okra and cotton, because there was an equally lower percentage of amplification and polymorphism. Some of the amplifiable markers imply that there are primer binding sites conserved between cotton and okra. So this indicates that transferability of polymorphic markers in plants is likely to be successful mainly within the genus or between species, which are compared within the family or between the genera (Wang *et al.*, 2007).

Both okra SSR's and cotton SSR markers showed the okra germplasm has low genetic variability (narrow genetic base) as revealed by cluster analysis, this was also observed in RAPD analysis in okra (Prakash *et al.*, 2011). The primary reason for narrow genetic base is that okra genotypes are often cross pollinated (only 4-19 per cent crosse pollination) in nature (Charrier, 1984).

SUMMARY

VI. SUMMARY

The present research work on “Development of microsatellite markers and molecular analysis in okra (*Abelmoschus esculentus* (L) Moench)” was conducted at Indian Institute of Horticultural Research (IIHR), Hesaraghatta, Bangalore during 2010-2011 to study the development of SSR markers and transferability of cotton SSR markers in okra. The findings of the research work are summarized here.

One of the possible ways for crop improvement is through breeding by exploiting variation in the germplasm. The major problem in classical plant breeding is that it takes many years to develop and release an improved variety. Molecular biology offers various techniques that can be used to assess genetic diversity in different crops and markers can be effectively used in marker assisted selection.

In the present study, DNA was isolated by CTAB method from young healthy leaves of okra genotypes. Okra contains high mucilaneous substance (polysaccharides), so to get good amount of DNA, purification was done by silica method, which was used for the development of microsatellite markers and template for PCR reaction.

6.1 Microsatellite development:

Development of microsatellite markers by constructing enriched genomic library involves several distinct steps. First, the genomic DNA was isolated from the freshly collected leaves of okra genotype (Arka Anamika) by CTAB method. It was digested using *RsaI* enzyme. Digested fragments were ligated to SNX adaptors. Then the linker ligated DNA was subjected to hybridization using the Biotinylated probes (AG)₁₂ and (TG)₁₂. DNA fragments containing di-repeat enriched microsatellite sequences were captured by Dynabeads (streptavidin) using magnetic particle concentrator and the unbound fragments were washed away.

Enriched fragments were recovered by PCR. The amplified DNA was cloned by using TA cloning method and the partial genomic library was constructed. A total of 14 microsatellite repeats were identified by using SSRIT software, among them eight were di-nucleotide repeats (57.1per cent) followed by two tri-repeats (14.2per cent), similarly two were tetra-repeats (14.2per cent), one was hexa-repeat (7.1per cent) and one compound repeat (7.1per cent). About 20 per cent of the SSR`s isolated were found useful for primer designing. Primers were designed for flanking regions using Primer 3 software tool (Rozen *et al.*, 2000). In the present study, of the 14 potential primer pairs synthesized, which amplified in all the ten okra genotypes at 55°C annealing temperature and generated polymorphism. The product size ranged from 140-300bp.

PIC (Polymorphic Information Content) values ranged from 0.000 to 0.375 with a mean of 0.2742 and observed heterozygosity values ranged from 0 to 0.111. The highest value of PIC and heterozygosity was observed for 140K marker, which indicated that this marker had greater potential to reveal allelic variation, and assessing genetic diversity within a population.

The genetic dissimilarity values in the distance matrix ranged from 0 to 19 (Table-5), suggesting a narrow genetic base within the okra genotypes. The highest genetic distance of 19 was observed between IIHR-245 and IIHR-242, IIHR-247 and IIHR-242 followed by IIHR-245 and IIHR-241 (18).

6.2 Cross amplification cotton SSR primers in okra

In this study the cross species amplification of 90 cotton SSR markers were tested on 25 okra genotypes. Out of 90, only 26 markers amplified (28.9per cent) for okra, among them 23 showed polymorphism (78.89per cent), most of them were di-repeats, and a few were compound repeats based on their arrangement.

The pooled binary data derived from the profile of 25 genotypes was used to develop a distance matrix displaying the pair wise genetic distance between the genotypes. The cluster analysis (Fig.2) drawn out of the distance matrix resulted grouping of the genotypes into two major clusters.. Total dissimilarity value across all the accessions were 82 units. Cluster A differed from cluster B with 69 units. Cluster A comprises a total of 7 genotypes, subdivided into two sub clusters A₁ and A₂, whereas cluster B comprises 18 genotypes, subdivided into five sub clusters namely B₁ to B₅. A phylogenetic tree was constructed on the basis of relatedness between the genotypes, which made it possible to classify the 25 okra genotypes into a total of seven distinct clusters. The highest genetic distance of 36 was observed in IIHR-226 and IIHR-101, indicating that these are genetically more distant and would be useful for increasing high yield and quality of the okra crop.

In summary, the results demonstrated that SSR markers from cotton can be successfully applied for the characterization of okra accessions and this diversity information can be used for future breeding programs. It also shows that there is a large scope for the cross amplification studies in okra using the SSR markers, as okra is poor as for as genome resources are considered.

Future line of work

1. There is a need to develop large number of SSR markers in okra for mapping and MAS (Marker-assisted selection) in crop improvement program.
2. The transferability of DNA markers between the genome of different species not only provides researchers with a large pool of available markers, but also allows the better understanding of the evolution And speciation of the crop through comparative mapping.

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VII. REFERENCES

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