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Confirming the F_1 Hybridity Using SSR Markers in Sesamum (*Sesamum indicum L*)

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ABSTRACT

A field experiment was conducted during Late summer, 2010-11 at Institute of Biotechnology , Rajendra nagar, involving two parents. In the present study, Swetha til, a high yielding popular white seeded variety and BB3-8 accession of *Sesamum mulayanum*, a wild variety resistant to powdery mildew from RARS, Jagtial were selected as parents for hybridization. F1 was developed by crossing the resistant parent (BB3-8 accession of *Sesamum mulayanum*) and the susceptible parent (Swetha til) during late summer, 2010. Parentage of F_1 hybrids of sesamum was verified using microsattelite (SSR) markers. Out of 300 primers surveyed, 240 showed clear amplification pattern and 24 markers(10%)were found polymorphic between two sesamum parents (Swetha til and *Sesamum mulayanum*). These highly informative primers not only differentiated the parent genotypes but also confirmed the parentage of their true F_1 hybrids. Our findings revealed that SSR procedures are excellent genomic tools for parentage confirmation and hybridity determination, and would also enhance efficiency of our breeding programmes through marker assisted selection.

Key words : F_1 hybrids, Markers, Sesamum.

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Sesame is regarded as the "Queen of oil seeds" because of its high oil content (38-54%), protein (18-25%), calcium, phosphorous, oxalic acid and excellent qualities of the seed oil and meal. India is the world leader in the area and production of sesame. In India sesamum is cultivated in an area of 1.85 million hectares, with a production of 0.64 million tones (CMIE, 2009). It is grown in marginal and sub marginal lands as rain fed crop mainly in the states of Gujarat, West Bengal, Uttar Pradesh, Rajasthan, Madhya Pradesh, Andhra Pradesh, Maharastra, Tamilnadu, Orissa and Karnataka, which account for more than 96% of the total area and production In Andhra Pradesh, it is cultivated in an area of 0.07 million hectares with a production of 0.018 million tones (CMIE, 2009).

The oilseed scenario in India has undergone a dramatic change in the last 25 years with the setting up of Technology Mission on Oilseeds (TMO) in May, 1986. India dominates the world scenario in area, production and export of sesame (*Sesamum indicum L.*). Recently, sesame has up surged as a silver line in agri-export with its highest contribution to the export earnings among the edible oilseeds in India. Therefore, there is an urgent need to increase the sesame production by improving its productivity level which otherwise is lower compared to other oil seeds.. Therefore, breeding efforts are mainly concentrated on development of high yielding varieties. The main goals of sesamum breeding programs worldwide are the genetic enhancement of yield .

For the improvement of agronomically and economically important traits, plant breeding generally recombines traits present in different parental lines of cultivated and wild species. Conventional breeding programmes reach this goal by generating an F1 hybrid and F2 segregating population and then screening the phenotypes of pooled or individual plants for presence of desirable traits, which is followed by a process of repeated backcrossing, selfing and testing. During this process breeder depends on accurate screening methods and availability of lines with clear-cut phenotypic characters, which is time consuming and difficult to achieve with classical methods (Beckmann & Soller, 1986). Use of molecular markers facilitate these breeding processes, since it can provide means of detecting and resolving complications and accelerate the generation of new

varieties and allow association of phenotypic traits with genomic loci (Jiang *et al.,* 2000). Ideal molecular markers are stable, abundant and detectable in plant tissues regardless of growth, differentiation and defense status. These properties make molecular markers indispensable for crop improvement.

A number of DNA fingerprinting techniques are available for detection of polymorphism (Semagn *et al.,* 2006). Restriction fragment length polymorphisms (RFLPs) are very reliable markers in linkage analysis and crop breeding. These are time consuming, expensive and require large amount of DNA for restriction and hybridization analysis (Paterson *et al.,* 1993). Most of the DNA marker assays use polymerase chain reaction (PCR), among them are random amplified polymorphic DNA (RAPD), microsatellites or simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphisms (SNPs). RAPD is much faster and cheaper than RFLP analysis and uses only minute amounts of DNA (Williams *et al.,* 1990). Microsatellites are typically the repeat unit of 1-6 nucleotides and SSR analysis is performed by using pairs of specific primers flanking tandem arrays of microsatellite repeats. SSR markers are codominant and extremely polymorphic (Liu *et al.,* 2002). AFLP is robust and reliable for DNA fingerprinting of different genomes because it combines the use of restriction enzymes and PCR amplification (Vos *et al.,* 1995). The AFLP system is technically intricate and expensive to set up, but it detects a large number of loci (up to 100). SNPs are the single base substitutions or small insertions and deletions (Indel) in homologous genomic regions. SNPs are more frequent and codominant in nature (Lindblad *et al.,* 2000).

Recent developments of molecular techniques and application of molecular markers have brought a new dimension into the traditional area of plant breeding. Molecular markers not only allow the easy and reliable identification of breeding lines, hybrids and cultivars (Bastia *et al.,* 2001; Asif *et al.,* 2005, 2006; Tabbasam *et al.,* 2006) but also facilitate the monitoring of introgression, mapping of QTLs (Jiang *et al.,* 2000), marker assisted selection (MAS) (Ribaut & Hoisington, 1998; Zhang *et al.,* 2003) and estimation of genetic diversity (Mukhtar *et al.,* 2002; Rahman *et al.,* 2002, 2008). High-density genetic linkage maps (Guo *et al.,* 2007; He *et al.,* 2007) established using molecular markers, for economically important crops provide a basis for MAS of agronomically useful traits, for pyramiding of resistance genes and the isolation of important genes by map-based cloning strategies (Ribaut & Hoisington, 1998). The proposed research work was conducted to confirm the parentage of sesamum hybrids with DNA markers. Efficiency of SSR assay was evaluated successfully for hybridity determination which would be a valuable genomic tool for the sesamum breeders.

MATERIAL AND METHODS

Plant Materials

In the present investigation Swetha til, a high yielding popular variety of Andhra Pradesh susceptible to powdery mildew from RARS, Jagitial and BB3-8 accession of *Sesamum mulayanum* line, resistant to powdery mildew were chosen as parents for development of mapping population to achieve the targets proposed.

Crossing Programme

At flowering, plants were selected and tagged for effecting crosses. Emasculation of female parent (Swetha) was done in the previous evening with ease taking advantage of the epipetalous nature of sesame flower. After removal of the stamens/ anther along with corolla, the stigma was covered with a small piece of cotton to prevent dessication. On the next day, anthers along with petals collected from the male parent in a petri plate were pressed gently with finger so that pollen get released. The released pollen was dusted on the stigma of the emasculated flower of the female parent after removing the cotton. After pollination, the straw was placed on the stigma.

The crossed flowers were properly labeled by tying with a piece of colour thread. After three to five days the straw cover was removed to ensure normal development of capsule. Matured capsules were harvested before dehiscence and F_1 seeds collected from them were dried and stored safely. Simultaneously, young unopened leaves were collected from both the parents for parental polymorphism study

Table 1. Basic steps in Polymerase chain reaction (PCR).

Step	Temperature (^{0}C)	time (sec)
Denaturation	94	45 sec
Primer annealing	50-60	45 sec
Extension	72	60 sec

Table 2. List Of Polymorphic SSR Markers.

S.No	Polymorphic Primers	Allele Size
1.	SM 10-102	198
2.	SM 10-103	248
3.	SM 10-106	198
4.	SM 10-115	198
5.	SM 10-116.	146
6.	SM 10-144	163
7.	SM 10- 160	193
8.	SM 10- 176	237
9.	SM 10- 178	175
10.	SM 10- 182	212
11.	SM 10- 183	188
12.	SEM 38	400-450
13.	SEM 76	170
14.	SEM 270	187-224
15.	SEM 249	290-310
16.	SEM 396	175-220
17.	SEM 436	175
18.	SI 2	190
19	SI 9	150
20	SI 14	243
21.	SI 15	236
22.	SI 34	250
23.	SI 42	189
24.	SP15	180

Plant material and DNA exraction:

Two sesamum genotypes (Swetha til and *Sesamum mulayanum*) contrasting for different traits and their F1s were used for the research work. DNA was extracted from these two selected sesamum parents and their F1s by CTAB method proposed by Porebski *et al.* (1997).

The quality and quantity of DNA used for amplification by PCR is the key to reproducible results and success of genotyping. Especially, DNA purity is extremely crucial for obtaining clear and discriminate patterns.Approximately 3-5 g of leaves was taken and ground into powder in liquid nitrogen with the help of pestle and mortar.Using a spatula powder was transferred to a 2 ml eppendorf tube containing 1 ml of extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% PVP, 2% Mercaptoethanol) preheated to 65ºC for 60 minutes with occasional stirring. After bringing back tubes to room temperature, equal amount of

chloroform: isoamyl alcohol (in the ratio of 24:1) added and gently mixed by inverting it for 5 minutes.Then centrifuged it for 10 minutes at 10000 rpm. Upper aqueous layer was transferred into a fresh sterile tube with the help of micropipette. Repeated the steps once again to remove proteins. Add equal volume of isopropanol and mix gently until DNA precipitate is formed. Tubes are kept in -20ºC for 20 minutes and centrifuged at 7500 rpm for 8 minutes, the pellet was washed with 70% ethanol for 2 times and kept for air dry for 1 hr. The pellet was dissolved in 300-400 µl of 0.1x TE buffer or double distilled water. The Nanospectrophotometer is used to determine the concentration of DNA in a sample. All measurements were carried out using double stranded calf thymus DNA (Sigma, USA). Data here represent the entire specified range of 50 – 3700 ng/ìl for 0.2 mm path length and 15- 1000 ng/ ìl for 0.7 mm path length. Samples (0.1or 0.5 ul) were placed on the pedestal using a small volume pipette. Each measurement took 3 seconds. Data analysis was carried out using the built-in software. The formula used was: Sample concentration = dilution factor x (OD260-OD320) x Nucleic acid concentration factor.

PCR amplification

A total of 300 SSR primer pairs developed at ANGRAU were used to study parental polymorphism. Five pico moles of each primer were used to amplify DNA. Template DNA was isolated as described above and then diluted to a working concentration of 50 ng/ìl. PCR amplification was carried out on thermal cycler (AB veriti, USA). PCR amplification was performed in a 10 ìl volume of PCR reaction mix containing the following.

Sterile distil water	: 5.0 µl
10X buffer	: 1.0 µl
dNTPs (2.0 mM)	: 1.0 µl
Forward primer (10 mM)	: 0.4 µl
Reverse primer (10 mM)	: 0.4 µl
Taq DNA Polymerase (5U/µl)	: 0.1 µl
Template (50ng/ µl)	: 2.1 µl
Total Reaction volume	: 10.0µl

PCR condition for SSR analysis included an initial pre denaturation step of 5 min at 94^{0}C and following 35 cycles of amplification:

Final extension was carried out at 72^{0}C for 10 min.The amplified fragments were stored at 4^{0}C for short periods and at -20^{0}C for long duration.

Gel electrophoresis

SSR products were electrophoresed on 3%agarose gels. Horizontal electrophoresis system was used and after electrophoresis, finely resolved PCR products were visualized under UV light and photographed. The polymorphic primers between the two sesamum parents were then used to survey their F1 hybrids for parentage confirmation.

RESULTS AND DISCUSSION

Parental Polymorphism:

300 SSR markers were used to study polymorphism between the parents, of which 240 showed clear amplification pattern out of which 24 were polymorphic.Though the parental genotypes were distinct for various important traits, polymorphism at molecular level was quite low, i.e., (10%).

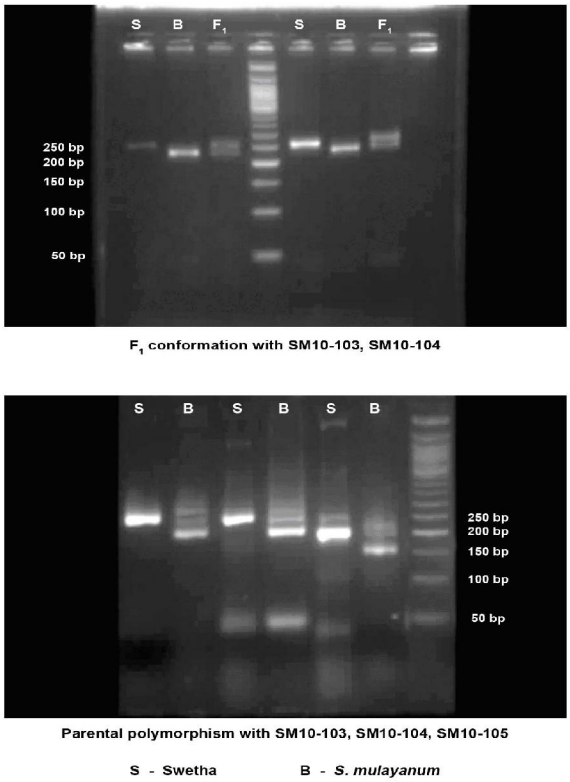
Marri et al. (2005) used two hundred and ten microsatellite markers in rice to screen the parents (O. *rufipogon* and *O. sativa*) for identifying polymorphic markers. Eighty markers (38%) detected polymorphism. The lower percentage of polymorphism may be due to a higher degree of genetic similarity between *O. rufipogon* and *O. sativa* used in this study compared to those used earlier.

Although parents involved in the present study belong to cultivated and wild species, many primers showed monomorphic pattern indicating closeness of the two species. However, some of polymorphic fragments have also been identified which are useful in studies of F1 confirmation, tagging, mapping and genetic linkage studies.

Hybrid nature of F_1

24 polymorphic SSR primers were used to confirm the hybrid nature of sesamum parents. Polymorphic molecular markers produced unique banding and not only discriminated the two sesamum parents but also identified their true

Figure 1.



hybrids. SSRs are more informative and highly polymorphic and their polymorphism is based on differences in number of repeats in amplified regions. Many of the variations are due to the mutations within primer binding regions that may yield null allele, whereas a mutation between the primer regions may result in new alleles.

RAPD and microsatellite analysis have been successfully employed for parentage verification, hybrid identification, cultivars characterization and purity testing in other crop plants (Asif *et al.,* 2006; Bertini *et al.,* 2006; Tabbasam *et al.,* 2006). Yamagishi (1995) developed RAPD markers for *Lilium* species characterization and hybrid identification. In cherokee rose, RAPD analysis identified the erroneous classification of the hybrid 'Silver Moon' (Walker & Werner, 1997). Zhang *et al.,* (2003) characterized and evaluated commercial cotton cultivars with microsatellites and found some specific SSR alleles for discriminating cotton germplasm. RAPDs and SSRs were also surveyed for verification of interspecific hybridization (Benedetti *et al.,* 2000; Mei *et al.,* 2004). Our results confirmed the efficacy of RAPDs and SSR assay for the verification of hybridity and parentage identification. Moreover, it exemplified the importance of increased level of homogeneity and purity at intra-varietal level for better implementation of plant breeder's rights (PBRs).

Molecular markers linked to a gene of interest are the milestones and these tags are useful starters for identification of genes. Once the molecular markers closely linked to desirable traits are detected, MAS can be performed in early segregating populations and at early stages of plant development (Zhang *et al.,* 2003; Francia *et al.,* 2005). Therefore, it is safe to say that molecular markers will gain more and more influence on plant breeding in future and will speed up breeding processes considerably. In view of potential development of new strategies, the future for improvement of polygenic traits through DNA markers appears bright. Moreover, by adopting new and novel marker systems like EST-SSRs, SNPs,

DNA chips and microarrays, indeed, some day it may be possible to select best lines for breeding based on RNA expression profiles as much as marker genotypes.

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