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Molecular Polymorphism in Cultivated *Sesamum indicum* L. and its Wild Species using PCR Based Molecular Markers

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ABSTRACT

Sesame (Sesamum indicum L.) is one of the ancient oilseed crop and its oil is highly priced because of its natural antioxidants. Genus Sesamum belongs to family Pedaliaceae, which consists of 20 odd species distributed in inaccessible parts of the world especially in Africa. The only cultivated species Sesamum indicum has large number of varieties. Genetic divergence of S. indicum L. and its wild relatives is poorly understood and even the basic chromosome number in many species is still not known. The present study deals with the use of PCR based DNA markers viz., RAPD decamer primers to assess the genetic diversity of the taxa. Out of the twenty eight primers initially screened, only sixteen primers generated polymorphism among all the species. A total of 370 amplified DNA fragments were recorded and among them 364 DNA fragments were polymorphic with an average of 22.13 bands per primer. The average genetic polymorphism recorded among the species was 98.61%. Polymorphic bands were scored and analyzed using statistical software NTSYS-pc version 2.1 and dendrogram was constructed based on UPGMA for cluster analysis of cultivated taxon and its proposed progenitor along with other wild species.

Keywords: *Sesamum indicum, RAPD,Unweighted Pair Group Method with Arithmetic mean, Progenitor, Genetic diversity*

INTRODUCTION

Oil seeds are group of crop species cultivated in tropical and temperate regions of the world for their seed oil. These plants store part of their food reserves as oil in their seeds as concentrated source of energy used during seed germination. *Sesamum indicum* L. is an important ancient oil seed crop cultivated extensively in India, Burma, China, Pakistan, Thailand, Ceylon, Turkey, Iraq, Syria, Sudan, Uganda, Nigeria, Tanzania, Congo, Egypt, Somaliland, Ethiopia, Greece, Mexico, Nicaragua, Guatemala, Costa Rica, El Salvador, Columbia, Venezuela and in many other countries[1]. It is commonly known as til, gingelly, simsim, benniseed, gergelim etc[2, 3].

Sesame is cultivated in 7.8 million hectare area with a production of 3.83 million tons worldwide[4]. India and China are the world's largest producers of sesame[5].

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India ranks first, both in the total area under cultivation and production of this oilseed crop in the world (27.7% area) but its productivity is quite low (368 kg/ha) as compared to worlds average (489 kg/ha) (www.fao.org). The major sesame growing states in India are Karnataka, Orissa, Uttar Pradesh, Tamil Nadu and Gujarat. However, this crop is also cultivated to a lesser extent in all other parts of India. The low ranking with respect to productivity in sesame among other oilseed crops may be due to its dry dehiscent capsules, indeterminate growth, lack of high yielding and disease resistant varieties, biotic and abiotic factors. Major yield loss is due to a pest *Antigastra catalaunalis*. Sesame oil is considered as highly prized premium cooking oil because of its high quality and stability. It possesses resistance to oxidative rancidity as the oil consists of natural antioxidants viz. sesamin and sesamolin[6].

Genus *Sesamum* L. (Pedaliaceae) is represented by 35 species[7,8] but most of them are synonyms of only 20 odd species[9,10,11,12,13]. Among them, *Sesamum indicum* L., is widely cultivated for its high quality premium oil[6]. Fatty acid analysis of wild and cultivated species of *Sesamum* were analyzed by Hiremath and Patil[14]. The wild relatives of the genus *Sesamum* are distributed widely in inaccessible parts of Tropical Africa, Madagascar, Indian subcontinent and some islands of Malayan Archipelago[3]. Cytogenetics of genus *Sesamum* is poorly understood and in many species basic chromosome number is still unknown. Species of genus *Sesamum* exhibits two basic chromosome numbers n=8 and n=13. Based on n=8 basic chromosome number, only tetraploids and octaploid species exists with no diploid species[15]. However, based on n=13 basic chromosome number only diploid species exists in the natural population. Karyotypic analysis of octaploid species has been compared by Patil and Hiremath[16]. Apart from cultivated *S. indicum* L., *S. mulayanum* Nair, *S. laciniatum* Klein and *S. prostratum* Retz, are the three wild species distributed in Indian subcontinent[17,18]. An exotic wild species *S. alatum* also has been recently collected from Karnataka, India (Patil and Pujar, 2015).

Antigastra catalaunalis (shoot webber) and hairy caterpillar *Diacrisia obliqua* (capsule borer) are the major pests on cultivated sesame, which causes considerable yield loss [1, 15]. Despite the economic importance of *S. indicum* as oil seed crop, genome relations between cultivated sesame and wild species is poorly understood. Wild taxa *S. mulayanum* is the rich source of disease and pest resistant genes[1,19,20,21,13]. Disease resistant genes can be transferred from wild to the cultivated varieties through conventional breeding or through gene transfer technology if the genetic relationship is established[15]. Hence, the present study was undertaken to evaluate the extent of phylogenetic relationships among cultivated *S. indicum* and its wild taxa.

MATERIALS AND METHODS

Plant material

Indian wild species viz., *S. mulayanum, S. laciniatum, S. prostratum, S. orientale* var. *malabaricum*, an exotic species *S. alatum* and cultivated *S. indicum* were personally collected. Another wild species *S. capense* was procured from Silverhill seeds, South Africa. Details of species accession number, place of collection are presented in Table 1. Seeds of the wild species were germinated in the moist chamber by breaking the dormancy through mechanical scarification and later transferred to plastic cups for hardening in the controlled environment and then transferred to the experimental garden. Young leaves of such plants were taken for DNA extraction.

Table 1. List of *Sesamum* species used for RAPD analysis, their accession number and place of collection

S.No	Name of the Species	Accession No	Place of Collection
1	*Sesamum alatum* Thonn.	w1	Tumkur, Karnataka
2	*Sesamum capense* Burm.	w2	Silverhill Seeds, South Africa
3	*Sesamum prostratum* Retz.	w3	Pondicherry
4	*Sesamum laciniatum* Willd.	w4	Badami, Karnataka
5	*Sesamum mulayanum* Nair.	w5	Goa
6	*Sesamum indicum* L.	w6	Dharwad, Karnataka
7	*S. orientale* var. *malabaricum* Burm.	w7	NBPGR, New Delhi

***Genomic DNA extraction* (modified Russell *et al.*)[22]**

The young leaves (2-3gm) were ground to fine powder in liquid nitrogen. To each ground leaf sample, 4 mL extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.35 M Sorbitol, 15 mM EDTA, pH 8.0, 20 mM SDS with 1% PVP-40) was added and centrifuged for 2 min at 10000 rpm. Discarded the supernatant, dissolved the pellet in extraction buffer, and repeated the same step until no visible mucilage layer is present in the sample pellet after centrifugation (usually 3 or 4 times if the tissue is mucilage rich). To each sample, 2 mL lysis buffer with 6 per cent sarcocyl was added and incubated samples for 1 h at 65°C. Mix the samples with this buffer before the samples were placed in the bath. To facilitate mixing, place the tubes in the bath for 2 min at 60°C. Added equal quantity of Chloroform: Isoamyl alcohol (24:1), mixed gently for 20 min, and then centrifuged for 10 min at 13,000 rpm. Transferred the supernatant to a new tube, and 1/10 volume sodium acetate (3 M, pH 5.2) and 2/3 volume cold isopropanol was added. Mixed gently, and incubated at -20°C overnight. Centrifuged for 10 min. Washed the pellet twice with 500 µL 70% ethanol and once with 500 µL 100% ethanol, and centrifuged for 5 min. Dried the DNA, and dissolved the pellet in 10-250 µL TE buffer (10mM Tris, 1mM EDTA pH 8.0) and allowed for complete suspension of DNA.

DNA quality and quantity estimation

The quantification of DNA was done by nanodrop technology. The quality check of DNA was done by recording absorbance at 260 and 280 nm. To counter check the quality of DNA, samples were run on 0.8 per cent agarose gel in 1X TAE buffer and stained with ethidium bromide. DNA samples with a single crisp band were selected for the present investigation and stored at -20ºC until further use.

PCR amplification

The PCR reaction was carried out using master cycler gradient 5331 Eppendorf, Germany and the results were analyzed as described by Williams *et al.*[23]. Each 25 µl of the reaction mixture consisted of 50 ng genomic DNA, 10X *Taq* buffer with 15 mM $MgCl_2$, 10mM each dNTP's (dATP, dGTP, dTTP, dCTP), 5 pM primer, 0.5 units of *Taq* DNA polymerase (GeNei^{TM}, Bangalore, India). The PCR reaction cycle was programmed with initial denaturation temperature of 94 ºC for 4 minutes, 42 cycles of 60 seconds denaturation at 94 ºC, 60 seconds annealing at 38 ºC, 120 seconds for extension at 72 ºC, followed by final extension of 420 seconds at 72 ºC to ensure the completeness of the primer extension and hold at 4ºC. Amplified PCR products were separated on 1.2% agarose gel. Ethidium bromide stained DNA bands were viewed under UV trans-illuminator and photographed for documentation using gel documentation unit (UVtech, Cambridge). The size of PCR amplicon was estimated by comparing it with a 1kb molecular weight standard (Bangalore Genie, Bangalore).

Data analysis

RAPD profiles were scored manually for presence or absence of a band. Presence of a band was indicated as '1' and absence as '0' into binary matrix. Dendrogram was constructed by the Unweighted Pair Group Method with Arithmetic means (UPGMA)[24,25], using statistical software NTSYS-PC Version 2.1[26].

RESULTS AND DISCUSSION

Results

A total of 28 decamer primers were initially screened to amplify the genomic DNA of six wild and one cultivated *S. indicum*. Only 16 primers amplified robust and reproducible bands. These primers differed greatly in their amplifying efficiency for revealing polymorphism (Table 2). Sixteen primers were finally selected and used to analyze all the *Sesamum* species for genetic diversity.

All the reactions were repeated thrice and only highly reproducible PCR amplicons were considered for data analysis. A total of 370 reproducible fragments were obtained, out of which 364 fragments were polymorphic, which were consistently generated from all the primers across seven *Sesamum* species. Each primer produced 5 to 42 polymorphic fragments with an average of 22.75 bands per primer (Fig. 1). Primer OPA18 generated highest number of bands (43), while OPB16 generated least number of bands (05). The approximate size of the largest fragment observed was 3000bp, whereas the smallest recognizable fragment was 150 bp in size.

Certain amplicons were found to be species specific and polymorphic. These amplicons could be used for designing species specific RAPD based SCAR markers.

The similarity matrix was calculated based on Jaccard's similarity coefficient. The interspecific genetic similarity indices ranged from 0.25 to 0.88 (Table 3). Cluster analysis of the genetic similarity was performed using Jaccard's similarity coefficient to generate a UPGMA dendrogram showing over all genetic relatedness among the species (Fig. 2). Further, cluster analyses resulted into two major groups. Cluster-I consisted of four species viz., *S. alatum, S. capense*, *S. prostratum* and *S. laciniatum* while Cluster–II comprised of three species viz., *S. mulayanum, S. indicum* and *S. orientale* var. *malabaricum.*

Table 2. RAPD Primers and their sequences, Total Number of Bands, Number of Polymorphic Bands, Percentage Polymorphism and Amplicon size range

Primer	Sequence	Total Number of Bands	Number of Polymorphic Bands	% Polymorphism	PCR amplicon size
OPM01	GTTGGTGGCT	35	35	100	500 – 2000bp
OPM02	ACAACGCCTC	32	32	100	200 – 2000bp
OPM03	GGGGGATGAG	20	19	95	700 – 1700bp
OPM04	GGCGGTTGTC	35	34	97.14	300 – 2500bp
OPM05	GGGAACGTGT	22	20	90.90	400 – 1800bp
OPA04	AATCGGGCTG	36	36	100	200 – 3000bp
OPA09	GGGTAACGCC	27	27	100	400 – 1900bp
OPA14	TCTGTGCTGG	07	07	100	700 – 2000bp
OPA16	AGCCAGCGAA	34	33	97.05	200 – 2100bp
OPA18	AGGTGACCGT	43	42	97.67	200 – 2000bp
OPA19	CAAACGTCGG	09	09	100	150 – 2500bp
OPA20	GTTGCGATCC	28	28	100	200 – 2000bp
OPB07	GGTGACGCAG	09	09	100	400 – 1900bp
OPB08	GTCCACACGG	09	09	100	300 – 2500bp
OPB16	TTTGCCCGGA	05	05	100	600 – 1800bp
OPB18	CCACAGCAGT	19	19	100	400 – 1900bp
	TOTAL370	**364**	**AVERAGE**	**98.61**	**150 – 3000bp**

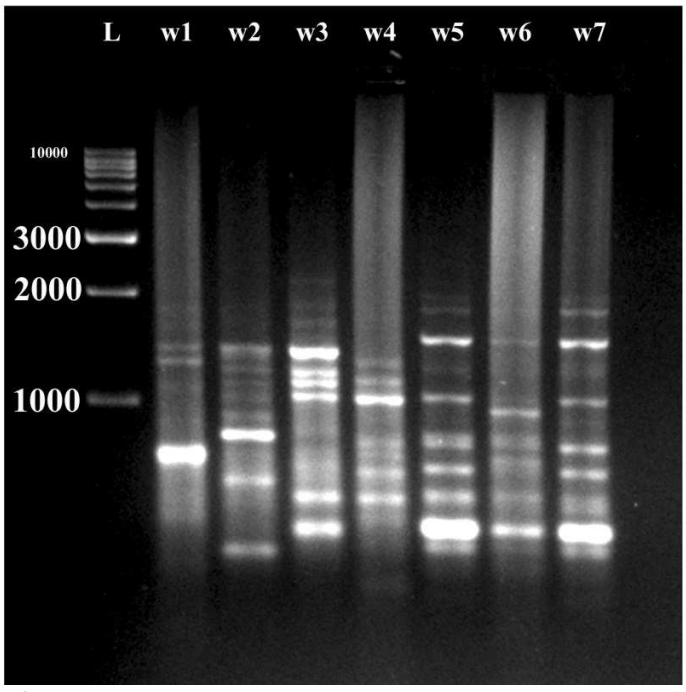

Fig. 1:RAPD profile of seven *Sesamum* species generated by OPA 18. The lanes w1 – w7 correspond to the accession number as listed in Table 1. The lanes L correspond to 1kb standard molecular weight marker

Table 3.Similarity matrix generated by RAPD markers. The w1 – w7 correspond to the accession number as listed in Table 1

	w1	w2	w3	w4	w5	w6	w7
w1	1						
w2	0.54	1					
w3	0.47	0.46	1				
w4	0.34	0.35	0.4	1			
w5	0.25	0.33	0.38	0.31	1		
w6	0.3	0.36	0.33	0.31	0.77	1	
w7	0.26	0.32	0.39	0.28	0.88	0.69	1

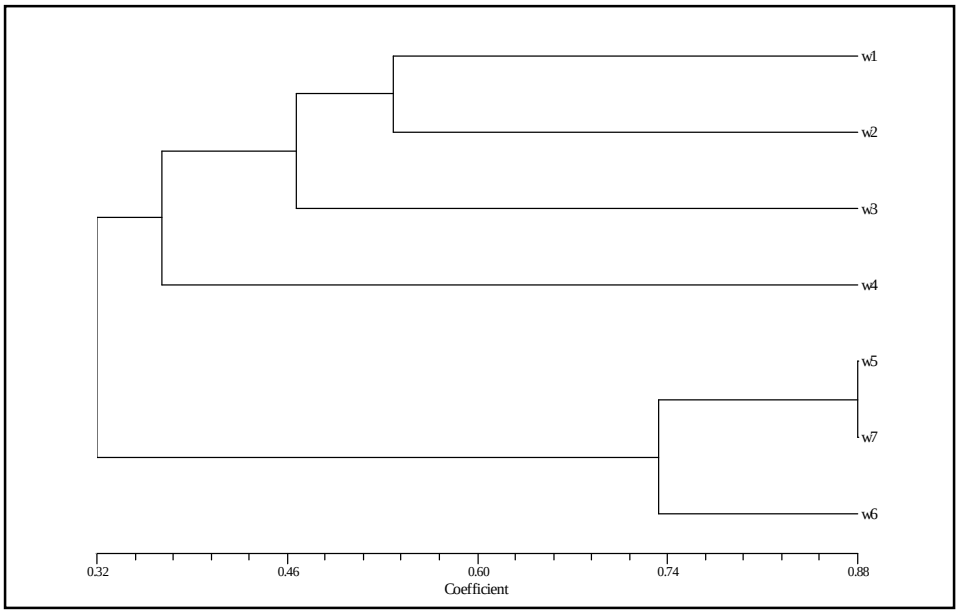

Fig. 2: Dendrogram showing clustering of seven *Sesamum* species based on UPGMA

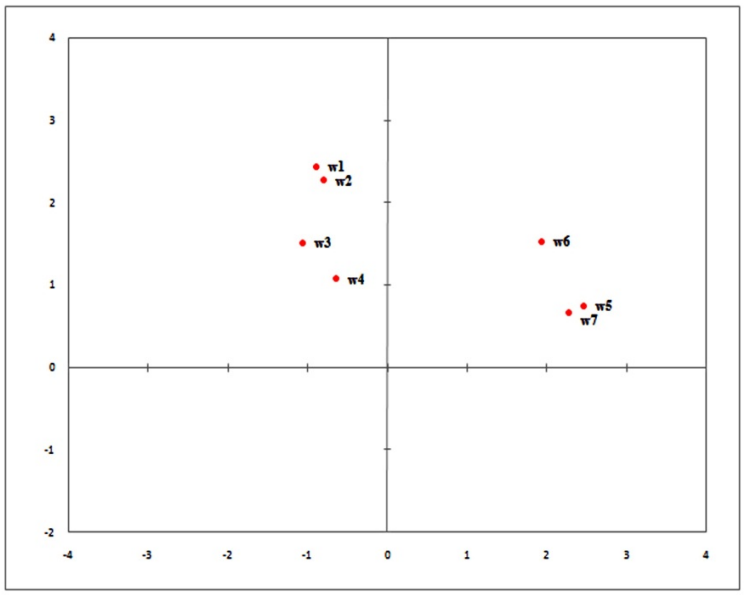

Fig. 3: Principle Components Analysis (PCA) based on RAPD data. The labels refer to accession numbers of *Sesamum* species as in Table 1

DISCUSSION

Random amplified polymorphic DNA[23] technology is a fast, reliable and cost effective

method for characterization of genetic variations among species. The RAPD technology is independent of environmental factors, so these molecular markers are helpful in assessment of genetic purity in a short time. A clear understanding of the genetic relationships among various species is essential for successful and efficient utilization of the genetic makeup present in the wild species[27,28]. Interspecific genetic divergence based on RAPD markers is suitable and evident for studying phylogenetic relationships and is important for conservation strategies[29].

Variation in morphological traits, geographical distribution, cytogenetic relationships, breeding system, cross compatibility and biochemical markers though used extensively to elucidate the relationship among the species are restricted in their resolving power mainly because of small number of variables available and some of them are development specific. In contrast, molecular approaches provide genetically interpretable variability with extensive genomic coverage and are becoming immensely important in studies on population biology and systematic. Among the PCR based marker techniques, randomly amplified polymorphic DNA (RAPD) technology is widely used, as it is easy, simple and requires no prior sequence information[23,30].

Many PCR based molecular markers have been used to study the extent of genetic variability in various crops but only limited information is available in Sesame and its wild species. Genetic diversity studies have been undertaken and reported by many researchers using RAPD technique[31,32,33,34,35,36].

In the present study, the observed average polymorphism was 98.61% and a total of 370 amplicons were obtained, out of which 364 amplicons were polymorphic, which were consistently generated from all the primers across seven *Sesamum* species. Based on the dendrogram, all the species of *Sesamum* included in the present study were grouped into two clusters. The highest similarity index 0.88 was recorded between *S. mulayanum* and *S. orientale* var. *malabaricum* which share similar morphological characters and least similarity (0.25) was observed between *S. mulayanum* and *S. alatum.* Bedigian[37] and Annapurna[38] have the opinion that *S. mulayanum* and *S. orientale* var. *malabaricum* are single taxon from side by side morphological comparisons with different names. PCA also depicts both the species at a very close proximity exhibiting their genetic similarities. The present investigation confirmed the proximity between *S. indicum* and its proposed progenitor species *S. mulayanum* with similarity index 0.77 and our results were in agreement with the previous studies on chromosome number, high crossability, 2C DNA content[15] and successful reciprocal crosses between the species were confirmed that *S. mulayanum* is the wild progenitor of cultivated oil seed crop *S. indicum* with an evolutionary decrease in the DNA content during domestication[13,15,38]. Findings of the present investigation is also supported by palynological studies on cultivated taxon and its wild species[39].

Among the *Sesamum* wild species grouped in Cluster I, high level of genetic similarity (0.54) is evident between *S. alatum* and *S. capense* which are morphologically distinct. *S. alatum* originated in Ethiopia and appeared as an exotic species in Indian subcontinent exhibits leaves with variously lobed and winged seeds. Both species are diploid with n=13 basic chromosome number. The lowest level of genetic similarity (0.34) is apparent between a diploid species *S. alatum* and tetraploid prostrate creeping annual herb *S. laciniatum* (n=8).

The second cluster included three species, of which all are diploid species viz., *S. mulayanum, S. indicum* and *S. orientale* var. *malabaricum.* Among the diploid species, highest level of genetic similarity matrix of 0.88 is evident between *S. orientale* var. *malabaricum* and *S. mulayanum.* However, the lowest similarity of 0.69 was exhibited by *S. indicum* and *S. orientale* var. *malabaricum.* Some reports[40,41,42] suggested that *S. orientale* var. *malabaricum* is a subspecies of *S. indicum* butthe present study revealed that *S. orientale* var. *malabaricum* is an independent taxa exhibiting several differential characters with *S. indicum*.The overall range of genetic similarity between the species ranged from 0.25 to 0.88, which indicated that there is a wider range

variability among all the *Sesamum* species. This cluster pattern studied among *Sesamum* species is also supported by Principle Components Analysis (PCA) (Fig. 3).

Genetic variation is a pre requisite for any crop improvement programmes. DNA based molecular markers acted as versatile tools to study variability and diversity in different plant species. Though a range of plant characters are currently available for distinguishing closely related individuals, their sensitivity to environment and less genome coverage hinders their usage[43].

CONCLUSION

Based on the results of present investigation, it can be concluded that the RAPD technique can be solely used to crackdown the genome relationship among *Sesamum* species, as it is fast, cost effective, simple with better genome coverage and species specific PCR amplicons generated for each species can be cloned to design RAPD-SCAR markers because these designed SCAR markers are more stable and reproducible but in order to resolve controversial taxonomic status of progenitor and its synonym and also to elucidate the origin and evolution of cultivated taxon, a combination of DNA manipulations, use of higher level molecular markers and improved conventional breeding practice is an urgent need for sesame improvement.

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