Assessment of genetic purity and diversity among hybrids and varieties of sunflower using morphological and molecular markers

By

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CERTIFICATE

Mr. **Dhammaprakash P. Wankhade** has satisfactorily prosecuted the course of research and that the thesis entitled **"Assessment of genetic purity and diversity among hybrids and varieties of sunflower using morphological and molecular markers "** Submitted is the result of original research work done and is sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

Date : 10-09-2004
Place : Hyderabad

(Dr.M.Ganesh)
Chairman of Advisory Committee

CERTIFICATE

This is to certify that the thesis entitled **" Assessment of genetic purity and diversity among hybrids and varieties of sunflower using morphological and molecular markers "** submitted in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture** of the **Acharya N. G. Ranga Agricultural University, Hyderabad,** is a record of the bonafide research work carried out by **Mr. Dhammaprakash P. Wankhade** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.

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(DHAMMAPRAKASH. P. WANKHADE)

DECLARATION

I, **DHAMMAPRAKASH P. WANKHADE** hereby declare that the thesis entitled **"ASSESSMENT OF GENETIC PURITY AND DIVERSITY AMONG HYBRIDS AND VARIETIES OF SUNFLOWER USING MORPHOLOGICAL AND MOLECULAR MARKERS "** submitted to the **Acharya N. G. Ranga Agricultural University** for the degree of **MASTER OF SCIENCE IN AGRICULTURE** is the result of original work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

(**DHAMMAPRAKASH P. WANKHADE)**

Date:

Place: Hyderabad

LIST OF SYMBOLS AND ABBREVIATIONS

AFLP	:	Amplified Fragment Length Polymorphism
ALP	:	Amplicon Length Polymorphism
ASAP	:	Allele Specific Associated Primers
CAPS	:	Cleaved Amplified Polymorphic Sequences
CMS line	:	Cytoplasmic Male Sterile line
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DAMD-PCR	:	Directed Amplification of Minisatellite DNA-PCR
DNA	:	deoxy Ribo Nucleic Acid
dNTP	:	deoxy Nucleotide Tri-Phosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
g	:	gram
DUS	:	Distinctness, Uniformity and Stability
GOT	:	Grow Out Test
IPGRI	:	International Plant Genetic Resources Institute
ISSRs	:	Inter Simple Sequence Repeats
ISTA	:	International Seed Testing Association
min	:	minutes
ml	:	millilitre
M	:	Molar
mM	:	milli molar
µg	:	micro gram
µl	:	microlitre
ng	:	nano gram
PBR	:	Plant Breeders' Right
PCR	:	Polymerase Chain Reaction
pH	:	pussancea hydrogen (potential hydrogen)
PVP	:	Plant Variety Protection
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RLGS	:	Restriction Landmark Genomic Scanning
RM	:	Rice Microsatellite
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
rpm	:	revolutions per minute

SAHN	:	Sequential Agglomerative Hierarchial Non-overlapping
SAMPL	:	Selective Amplification of Microsatellite Polymorphic Loci
SCAR	:	Sequence Characterized Amplified Regions
SNP	:	Single Nucleotide Polymorphism
SSRs	:	Simple Sequence Repeats
$T_{10}E_1$	:	10 mM of Tris.Cl, 1 mM of EDTA
TAE	:	Tris.Cl, acetic acid, EDTA
TE	:	Tris.Cl, EDTA
TRIPS	:	Trade Related Intellectual Property Rights
Tris.Cl	:	Tris (hydroxy methyl) aminomethane hydro chloride
U	:	units
UBC	:	University of British Columbia
UPGMA	:	Unweighted Pair Group Method with Arithmetic Averages
UPOV	:	Union for Protection of New Plant Varieties
VNTR	:	Variable Number of Tandem Repeats
WTO	:	World Trade Organization
µl	:	micro litre

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ABSTRACT

The present investigation was carried out with the aim of development of molecular markers (RAPD and SSR) for genetic purity testing of hybrids and varieties of sunflower and to study diversity among selected hybrids and varieties.

Morphological characters specified in published descriptors of sunflower were used to select genetically pure genotypes based on visual observations. Of 13 hybrids and 13 varieties included in the study, 12 hybrids and 12 varieties were selected based on their uniformity and similarity for characters specific to that genotypes mentioned in the descriptors for these cultivars. Selected hybrids and varieties were used for development of molecular markers for genetic purity assessment and to study diversity.

RAPD markers were identified for testing genetic purity of the twelve hybrids included in the study (BSH-1, KBSH-1, KBSH-41, KBSH-42, KBSH-44, HSFH-848, LSH-1, SCH-35, PSFH-118, DSH-1, RSFH-1 and TCSH-1). Unique fingerprints (IDs) were identified for two varieties (CO-2 and EC-68415) using RAPD primers. SSR markers were identified for seven hybrids (KBSH-1, KBSH-41, PSFH-118, LSH-1, SCH-35, DSH-1 and TCSH-1) and two varieties (PKVSF-9 and EC-68415).

Genetic diversity among 12 hybrids and 12 varieties was investigated at DNA level using RAPD and SSR markers and at the phenotypic level with eight morphological characters. Seventy random decamer primers were used to amplify DNA via the Polymerase Chain Reaction (PCR); 315 and 335 RAPDs were generated for hybrids and varieties respectively. Twenty-two SSR primer pairs were used for amplification of SSRs via PCR; 69 and 58 SSRs were generated for hybrids and varieties respectively.

Dendrograms were generated of genetic similarity from morphological data, from RAPDs and SSRs. Dendrograms generated from morphological data on hybrids and varieties produced four clusters for hybrids and four for varieties. Dendrograms generated from RAPDs generated four and five clusters respectively of hybrids and varieties. Dendrograms produced from SSRs generated three and four clusters respectively for hybrids and varieties. Classifications of the genotypes using three methods were quite different.

These results indicate the practical usefulness of RAPD and SSR markers in assessing genetic purity testing of hybrids and varieties of sunflower and assessing diversity among them.

CHAPTER I

INTRODUCTION

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'As you sow, so shall you reap' adage succinctly puts the importance of seed in achieving the desired productivity levels. The success of improved variety/ hybrid in the farmer's field depends upon the availability of seed with high genetic purity and seeds of provenance are the most critical input which decide the effect of all other inputs in increasing the productivity (Agarwal, 1980). Therefore, assessing the genetic purity is of utmost importance before the seed reaches the farmers field. Also, in the context of PVP (Plant Variety Protection), identification of the *cultivar* has assumed increased significance (Troyer, 1986). Conventionally, the genetic purity of the hybrids is assessed by the Grow-Out-Test (GOT) (Bellester and de Vincete, 1998). This test requires one full season thus precluding the immediate cultivation of the hybrid seeds produced. The locking up of the capital invested on hybrid seed production and additional expenditure incurred on storage of hybrid seeds ultimately increases the hybrid seed cost. This limitation and the environmental dependence of the entire procedure can be alleviated effectively by employing molecular markers. Biochemical methods such as isozyme analysis and electrophoresis of seed protein have been successfully developed for identification of seed lots (Woods and Thurman, 1976; Graybosch and Morris, 1990; Gupta and Shepherd, 1990; Anisomova et al., 1991). However, these techniques cannot be applied for the discrimination of closely related genotypes due to the limited polymorphism, environmental influence and also, they do not allow sufficiently thorough sampling of the genome to provide estimates of genetic distances (Ainsworth and Sharp, 1989). Thus, more effective methods are required for detecting polymorphism for the identification and purity assessment of the *cultivar*.

DNA based markers hold greater promise with several advantages *viz*., high polymorphism, insensitivity to environment, stability, independent of developmental stages of plants, etc. Several molecular markers have been developed and used successfully for varietal discrimination in various crops. Choosing one method from the plethora of molecular markers available depends on the purpose, availability of the resources, facilities, rapidity, ease, reliability, etc. Among the markers employed, Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) analyses, being Polymerase Chain Reaction (PCR) based, are easy and quite discriminatory. Therefore, they have been employed successfully in several crops (Tateneni et al., 1996; Gupta and Varshney, 2000). Once the specific molecular markers are identified for each variety or hybrid, they could be used successfully to assess the genetic purity and thus could avoid the laborious GOT (Yashitola et al., 2002).

Improvement in yield of a crop species in general is accompanied with a reduction in the variability among the commercial cultivars of that species. Thus the improved varieties of the crop species are becoming increasingly similar to each other due to the commonness of one or more parents in their ancestry. This has led to narrowing down of the genetic base of these cultivars (Singh, 1993). Presently cultivated oilseed sunflower planted around the world has been derived from a limited genetic base, which make the crop vulnerable to pests and diseases that can exploit this genetic weakness (Vranceanu, 1985). The dependency on single source of male sterile cytoplasm derived from wild *Helianthus petiolaris,* and the associated restoration genes for global hybrid sunflower production makes the crop extremely vulnerable to potential disasters (Annual report 2003, USDA). So, it is imperative to estimate genetic diversity among

public sector sunflower hybrids and varieties. Extensive studies have shown that DNA markers provide highly efficient and informative ways of estimating diversity concern with artificial or cultivated populations, including accessions, collections of germplasm and breeding lines (Karp et al, 1997).

Considering the importance of these issues, the present work was formulated and conducted using molecular markers (RAPD and SSR) and morphological markers for assessment of genetic purity and diversity among the public sector sunflower hybrids and varieties with following important objectives.

1. To identify specific molecular markers (RAPD and SSR) for identification of hybrids and varieties of sunflower.
2. To evaluate utility of (identified) genotype specific markers to assess genetic purity of few hybrids.
3. To assess genetic diversity among released sunflower hybrids and varieties using molecular and morphological markers

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

The success of high yielding hybrids and varieties depends upon of the availability of seeds with high genetic purity. High quality seeds with assured genetic purity can be expected to respond fully to all other inputs. (Agarwal, 1980) So it is important to assess the purity of commercial cultivars before it reach to farmer's field. Particularly it is more important to assess genetic purity of F1 hybrids to exploit heterosis.

Variety identification is a prerequisite for the effective provision of Plant Breeders' Rights (PBR) which can be achieved by trade secrets, Plant Variety Protection (PVP) or where available, through utility patents. All three forms of protection require some measure of distinctness. Variety identification for the attainment of PBR is a taxonomic and genetic approach to determine varietal distinctness. The chief goals are to promote the release of fresh genetic diversity into agriculture and to create an environment of continued funding for plant breeding research and genetic resource conservation. At the international level, variety identification and grain commodity usage become linked because seeds are the encapsulated intellectual property, the protection of which forms an integral component of the General Agreement on Trade and Tariff (GATT) (Smith *et al*., 1995).

Various methods are followed for varietal identification depending upon the utility of the method, purpose and cost involved.

2.1 Approaches to Genetic purity assessment (variety identification)

Genetic markers have been observed and used since the dawn of genetics. More recently, as the genetic marker technology progressed from visible (morphological) markers to molecular markers. Markers have been used to investigate a wide range of ecological, agronomical and evolutionary questions. Markers are typically used as tools to characterize varieties and they should be heritable, discriminating individuals between individual examined and be easy to measure and evaluate, provide comparable results, be known to be either neutral or unlinked.

2.1.1 Morphological method

International Plant Genetic Research Institute (IPGRI) promotes a minimal set of morphological characters that entails genetic diversity at particular point of genome corresponding to the observed characters descriptors recognized internationally and thought to be satisfactory for custodial management of crop germplasm collection which cover most of the important crop genera (Erksine and Williams, 1980). The plant breeder who develops the variety or hybrid gives descriptors mentioning the unique characters, mostly the morphological characters to identify that variety or hybrid. Currently they are the main source of description information for plant variety protection and assessing genetic purity. The characters to be determined are either qualitative or quantitative. Qualitative characters can be determined visually *eg* colour of the leaf, panicle type, leaf shape etc, but only a few available of these discontinuous characters are available to extend varietal discrimination (Higgins and Evans, 1983). Quantitative characters are visually determined, measured or counted. All measurement and counts are carried on specific number of plants as provided in IPGRI descriptors. Varieties are distinguished on

the basis of statistical difference in measured characters with further resolution based on field observed differences. Both types of genetic variation (quantitative and qualitative character) have been used to establish distinctness criterion for cultivar identification. The minimum descriptors used may vary from one crop to another (Ashri, 1973).

Morphological characters manifesting in various parts of the plant have traditionally been used to distinguish one cultivar from the other (Harlan and Wet, 1972; Pierce and Warhner, 1990) and grain characters are also valuable in seed certification process (Hervey-Murray, 1980).

The use of seed morphological characters along with laboratory technique has been reported in sunflower for characterization of hybrid (Sahoo *et al.,* 2002).

Although the observation, phenotype undoubtedly represents a very successful means of variety identification it cannot be a reliable system under all situations. The problems with morphological descriptors are: they are subjected to environmental conditions, shows differential expression which depends on ontogeny, not distributed throughout the genome and it necessitates the growth of plants to maturity and recording can be rather subjective.

Morphological characters have traditionally provided signature of varietal genotype and purity. However, molecular characters that reveal genetic differences more quickly and accurately without the obscurance of environment provide significant advantage in term of discrimination, reliability reduced time and reduced cost.

2.1.2 Biochemical approach

The attraction of utilizing biochemical methods in which specific gene product(s) is estimated is that, they enable a closer examination of genotype, as opposed to the phenotype and hence the environmental influence is minimized. The relation between chemical composition and genotype, which occurs at several different levels, has been described by Zuckerkandl and Pauling (1965). Protein and isozyme profiles have been extensively used in the characterization of crop varieties.

2.1.2.1Proteins in genetic purity assessment

Proteins, because of their easy accessibility, are a reasonable compromise between direct study of the genotype and of gene products and thus protein profiles of cultivated varieties provide fingerprints that are stable descriptors of genotypes. Proteins are molecules with net electrical charges that are affected by pH that can be separated by electrophoresis on the basis of their net electrical charge, molecular weight, isoelectric point or a combination of these. Profiles of alcohol soluble proteins are frequently utilized for varietal description and the genetic control of these proteins is well established, at least for some species of cultivated plants (Shewry *et al*., 1983; Soave and Salamini, 1983; Wilson, 1986; Wilson *et al*., 1989; Graybosch and Morris, 1990 and Gupta and Shepherd, 1990).

In sunflower use of seed storage protein globulin and helianthinin for genetic purity assessment of varieties and hybrids has been demonstrated (Anisomova *et al*., 1991).

2.1.2.2 Isozymes in varietal characterization

Isozymes, the multiple molecular forms of an enzyme with similar or identical catalytic activities occurring within the same organism (Markert and Moller, 1959), can be the rapid sensitive tools for cultivar identification provided enough allozyme markers are present in the analyzed species. It has been possible to characterize genetically distinct isozyme variants for many enzyme systems in numerous species of cultivated plants (Tanksley and Orton, 1983; Nielsen, 1985). Arus *et al.,* (1985) suggested that electrophoresis test could be universally applicable to almost all commercially available hybrids. Isozyme data has been submitted as evidence in PVP and patent applications for maize in the United States (Troyer, 1986).

Isozyme in hybrid purity assessment was first used in *Brassica spp* by Nijenhuis (1971). Subsequently many others (Woods and Thurman, 1976; Wills *et al.,* 1979; Wills and Wiseman, 1980; Arus *et al.,* 1982) used this technique for purity analysis in same. Similarly it has been used in maize (Smith and Weissinger, 1984; Smith and Wych, 1986; Brink *et al.*, 1989) and in tomato (Tanksley and Jones, 1981).

Isozyme and protein electrophoresis still fail to differentiate a number of varieties since they measure variation at a very limited number of genetic loci. These results may not reflect overall patterns of genetic variation throughout the genome (Aldrich *et al.,* 1992). Also it has been established that isozyme fail to provide unequivocal identification (Ainsworth and Sharp, 1989).

Biochemical methods continue to be used in routine testing of parentage, monitoring of genetic purity and act as additional descriptors in DUS testing (*Smith et al.*, 1996). However, these methods neither possess sufficient discrimination ability nor they allow sufficient thorough sampling of the genome to provide a reasonably good estimate

of genetic distance that could be used in granting IPP (Intellectual Property Right) and in effective varietal identification.

2.1.3 Markers at DNA level in varietal identification

Biochemical methods using storage protein and isozyme have been widely used since late 70s (Smith *et al.*, 1995). However, the main limitations of the methods are the influence of environment on manifestation of these characters, developmental stage specificity and representation of very limited variability present in the genome. Consequently, technologies that utilize DNA directly and which allow more complete sampling of the genome with greater power of discrimination are becoming the choice of the method.

Markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and proteins based markers and constitute a new generation of genetic markers (Bostein *et al.,* 1980; Tanksley *et al.,* 1989). Hence, varietal profiling methods that directly utilize DNA could potentially address all of the limitations associated with morphological and biochemical data. Cultivar identification using DNA fingerprinting is currently being investigated in a number of laboratories using different methods (Weising *et al.,* 1991).

Wide arrays of molecular techniques that can be used to detect polymorphism at the DNA level have been described in the recent times. Most molecular markers fall into one of three basic categories of techniques that use either hybridization based (non PCR) techniques, arbitrarily primed PCR and other PCR based multi locus profiling techniques and sequence targeted and single locus PCR. Some techniques are also derivatives or combinations of other techniques. Hybridization based techniques includes Restriction

Fragment Length Polymorphism (RFLP) analysis where probes are hybridized to filters containing DNA that has been digested with restriction enzymes. The resultant fragments are separated by gel electrophoresis and transferred onto filters by southern blotting. Hybridization could also be carried out with the probes for minisatellite or microsatellite sequences to give Variable Number of Tandem Repeats (VNTR) and Oligonucleotide Fingerprinting respectively. Arbitrarily primed PCR (Polymerase Chain Reaction) techniques use arbitrary or semi-arbitrary primers for amplification of DNA products through PCR, including those derived from RNA. A common feature of these techniques is the lack of requirement for sequence information from the genome under investigation. The range of different approaches in this category differs in the length and sequence of the primers used, the stringency of the PCR conditions and the method of fragment separation and detection. A limitation of arbitrarily primed PCR is the lack of allelic information, both in terms of dominance and the assignment if alleles to loci. These problems are overcome with sequence targeted and single locus PCR where PCR directed to specific single locus targets for which a necessary prerequisite is that knowledge of the sequence of the target or flanking target regions. Some of the examples of this category are sequence tagged microsatellite site (STMS); Cleaved amplified polymorphic sequence (CAPS). (Karp and Edwards, 1997).

Markers which have been used and those which hold greater promise in varietal identification and diversity studies in near future are discussed here.

2.1.3.1 Restriction Fragment Length Polymorphisms (RFLPs)

RFLP approach relies on the cleavage of genomic DNA by restriction enzymes; length polymorphism between a given pair of sites is then detected by hybridization to a

labeled DNA probe. Use of RFLPs in varietal identification has been discussed in a number of reviews. RFLPs provide a potentially very powerful tool for variety identification and purity screening (Ainsworth and Sharp, 1989).

However RFLP technology is a relatively slow process. Further more it having difficult automation and high running cost.

2.1.3.2Variable Number of Tandem Repeats (VNTR) and Minisatellites

In view of the relatively low proportion of unique DNA probes that can uncover RFLPs in most cultivars, other classes of probes were considered that might have greater resolving power, particularly in cultivar-to-cultivar comparisons. The discovery of hyper variable regions (HVRs) in many species of animals and plants has provided new opportunities for evaluating genetic variability. HVRs contain minisatellite sequences, which consist of a series of tandem repeats of a core consensus sequence usually of 10-60bp (Jeffreys *et al.,* 1985a). As these sequences are dispersed throughout the nuclear genome, they may represent many loci. Variability of such regions arises from differences in the copy number of tandem repeats and thus they are also called as Variable Number of Tandem Repeats (VNTR).

DNA fingerprinting with minisatellite sequences has been widely used in both humans and animals for individual identification (Jeffreys *et al.,* 1985b; Hillel *et al.,* 1989). In plants, this technology has been used for variety identification (Nybom, 1990; Nybom and Hall, 1991).

2.1.3.3 Randomly amplified polymorphic DNA (RAPD)

To overcome limitation of hybridization based technique such as RFLP, procedures that use Polymerase Chain Reaction (PCR) were developed and employed successfully for genetic purity assessment. Several widely used PCR based methods employ arbitrary primers. One of these is RAPD analysis. Amplification of genomic DNA using one oligonucleotide primer under low stringency condition, result in multiple amplification products from loci distributed throughout genome Williams *et al*.,1990; Welsh and McClelland, 1990). This observation led to development of fingerprinting application (Welsh and McClelland, 1990). RAPD method became popular due to its advantages over hybridization based techniques like non requirement of radioactive detection, prior genome sequence information, use of universal primers that work in any genome, sufficiency of very small amount of genomic DNA, detection of high polymorphism, and experimental simplicity.

DNA polymorphisms generated by RAPD markers have been used for identification of germplasm lines and cultivars (Cubero *et al*., 1996).

Reproducibility of RAPD profile has been a subject of considerable discussion. However, it has been documented that if standard parameters like primer quality and concentration, Magnesium concentration, pipetting accuracy, choice of DNA polymerase are followed, satisfactory reproducibility of RAPD profile could be obtained (Rafalski, 1997).

Constant and reproducible RAPD patterns were identified which could be employed to distinguish cultivars and germplasm lines in cauliflower (Boury *et al*., 1992), rice (Hinrchesan *et al*., 1996), carrot (Grzebelus *et al*., 1997).

The genetically pure hybrid is one, which results from the cross of respective female and male parents. Generally, in most of the crop species, hybrid seeds are produced on female plant. If marker specific to male parent is identified and if such male parent specific marker shows it's presence in the hybrid individual gives the proof that the hybrid seeds resulted by the pollination from respective male parent and received a genome complement from male parent. RAPD markers were used in genetic purity assessment of F_1 hybrids (Hashizume *et al*., 1993). This technique was found useful for identification of hybrids from their parents especially those, which have no characteristic morphological and isozyme markers (Paran *et al*., 1995). Hybrid purity in tomato was detected by identifying a male parent specific marker in hybrid enabling clear distinction from maternal parent. It was concluded that despite dominant behavior of RAPD markers they could be used in hybrid purity assessment (Bellster *et al*., 1998).

Use of RAPD in genetic purity testing of cotton was demonstrated by Yadav *et al*., 2001 where, using pollen parent specific RAPD markers the genetic purity of DCH-32 hybrid seed was detected. The result by RAPD for different hybrid seeds of DCH-32 agreed well with field based GOT. It was concluded that the RAPD method could be an alternative to the time consuming GOT. Usefulness of RAPD analysis in seed quality testing programme of chilli has been demonstrated (Ilbi, 2003).

Even though RAPD analysis has been very useful in hybrid purity assessment, its usefulness in varietal discrimination has not well documented. Less polymorphism generated by RAPD markers in varietal discrimination was reported in few fruit trees such as in peach and nectarine where RAPD analysis could not differentiate cultivars

(Vinatzer *et al*., 1999). In these cases morphological analysis of fruits and trees proved more effective than molecular fingerprinting of these varieties.

Rubeena *et al*., (2000) have reported that though no single primer could distinguish all the twenty-two varieties of cotton used in the study. However, a combination of five primers could identify all the cultivars.

Despite limitations, RAPD analysis has been employed in varietal identification and genetic purity studies of hybrids. A comprehensive list of RAPD analysis in genetic purity studies has been presented in table 3.1.

While no one marker system can be considered ideal for all the applications, RAPD method provides a valuable tool in the repertoire of molecular geneticist, allowing easy fingerprinting applications.

2.1.3.4 Simple sequence repeats (SSR)

Simple sequence repeats (SSR) or microsatellites are DNA sequences that consist of two to five nucleotide units such as (AT), (CTT) and (ATGT) which are tandemly repeated. These small repetitive DNA sequences, which are spread throughout the genome of eukaryotes, provide the basis of polymerase chain reaction (PCR) based multi-allelic, co-dominant marker system. The regions flanking the microsatellite are generally conserved among the genotypes of the same species. PCR primers complementary to the flanking region are used to amplify SSR containing DNA fragment. Length polymorphism is created when PCR product from different individuals vary in length as a result of variation in the number of repeat units in the SSR (Cregan *et al*., 1997). SSR have been reported in many plant genomes such as Brassica (Poulsen *et al*., 1996), rice

(Wu and Tanksley, 1993) and sunflower (Tang *et al*., 2002). The first application of microsatellite in plants has been in cultivar identification and now they are markers of choice in genotyping cultivars (Weising *et al*., 1991; and Beyermann *et al*., 1992).

Application of SSR markers in fingerprinting cultivars was reported by Rongwen *et al*., (1995) to develop unique DNA profiles or fingerprints for 96 soybean cultivars. Seven SSR loci clearly differentiated all except two closely related individuals. Similar results are being reported for other crop species *viz.,* grape (Thomas and Scott, 1993; Botta *et al.,* 1995), avocado (Lavi *et al.,* 1994), sorghum (Brown *et al.,* 1996), hops (Brady *et al.,* 1996), cucumbers and melons (Katzir *et al.,* 1996), apple (Szewc-McFadden *et al.,* 1996; and Guilford *et al.,* 1997) and barley (Russell *et al.,* 1997). The screening of microsatellite alleles in varieties would generate a database useful for variety identification and the development of molecular markers for marker assisted selection (Garland *et al.,* 1999). Significantly greater allelic diversity of microsatellites was reported in rice over RFLPs (Mc Couch *et al*., 1997). In the same crop SSRs could classify 71 cytoplasmic genetic male sterile lines of rice into different groups (Subudhi *et al.,* 1998). Use of microsatellite polymorphisms for the identification of Australian breeding lines of rice was investigated (Garland *et al.,* 1999) and most of the cultivars could be uniquely identified by at least one microsatellite marker.

Application of SSR and STS (sequence tagged sites) markers in genetic purity assessment was demonstrated in rice with the objective of replacing GOT with DNA based assay (Yashitola *et al*., 2002). Cytoplasmic male sterile (CMS), restorer lines, and hybrids have been screened by means of microsatellite and sequence tagged site (STS) polymorphisms and a simple procedure was developed for detecting heterozygosity. The

results obtained could be used for detection of off-types in hybrid seed lots. These results indicated that a single, appropriately chosen microsatellite marker should be sufficient for assessing hybrid seed purity.

Given this high level of informativeness, abundance and apparent random distribution in plant genomes combined with reliable amplification via PCR, SSR markers have become an important and widely used DNA marker system in plants.

3.1.2.5 Inter simple sequence repeats (ISSR)

This PCR based technique involves amplification of DNA segments in between two identical microsatellite repeat regions on the complementary strands. The inter simple sequence repeat (ISSR-PCR) (Zietkiewiez *et al.,* 1994) is another newly developed method which relies on one PCR primer and holds promise for variety identification. It involves the amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR) containing primer and can be undertaken for any species that contains a sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required (Gupta *et al.,* 1994; Goodwin *et al.,* 1997). This technique amplifies large numbers of DNA fragments per reaction, representing multiple loci from across the genome; it is an ideal method for fingerprinting varieties and a useful alternative to single-locus PCR or hybridization based methods. This method was found useful in fingerprinting varieties of corn (Kantety *et al.,* 1995), finger millet (Salimath *et al.,* 1995) and rice (Parsons *et al.,* 1997; Blair *et al.,* 1999; Naveen Kumar 2003).

3.1.2.6 Amplified Fragment Length Polymorphism (AFLP)

Recently, a new multiplex PCR based method (AFLP) has been developed in which a subset of restriction fragments are selectively amplified using oligonucleotide primers complementary to sequences that have been ligated to each end (Zabeau and Vos, 1993; and Vos *et al.,* 1995). AFLP analysis allows the reliable identification of over 50 loci in a single reaction (Zabeau and Vos, 1993; and Vos *et al.,* 1995). This technique combines the reliability of the RFLP and ease of the PCR and thus AFLP is a new typing method for DNA of any origin or complexity (Janssen *et al.,* 1996, and Lin *et al.,* 1996a, b). The ability of AFLPs to distinguish among genotypes is not hindered by their bi-allelic nature (presence or absence) and thus polymorphisms can be identified between very closely related genotypes. AFLP as a tool for DNA fingerprinting is used in *Lactuca* species (Hill *et al.,* 1996), barley (Ellis *et al.,* 1997), rice (Fuentes *et al.,* 1999 and Singh *et al*., 1999) and Bermuda grass (Zhang *et al.,* 1999).

Application of AFLP as alternative for the assessment of Distinctness, Uniformity and Stability, was reported in sugarbeet varieties (De Riek 2001). AFLP data were obtained for three consecutive seed deliveries of fifteen sugarbeet varieties. As a first approach, a cluster analysis based on standard genetic distances between varieties and/or seed deliveries was made. Three major groups put together varieties belonging to corresponding breeding programmes. In a second approach, the genetic structure inferred by varieties and seed deliveries was submitted to an AMOVA (Analysis of Molecular Variance). Major genetic variations were attributed to individual plant differences within seed deliveries. Difference among seed deliveries seemed to be as important as difference among varieties or breeding programmes. Varieties were classified according to the degree to which the distribution over the different accessions was mainly allocated to

their appropriate seed deliveries (from the same variety) or cross -allocated to other varieties.

However, drawbacks of using AFLP routinely to test genetic purity of cultivars are DNA of reasonably good quality has to be used which has to be digested with selected restriction enzymes. Further, it needs a relatively high running cost and high level of skill compared to other methods such as RAPD and SSR analysis.

3.1.2.7New generation DNA markers

A non-conventional fingerprinting methodology called, Restriction Landmark Genomic Scanning (RLGS) was developed for analyzing genomic DNA of higher organisms (Hatada *et al.,* 1991) which uses restriction sites as landmarks for genomes. It employs direct labeling of the genomic DNA at the restriction sites and two dimensional electrophoresis to resolve and identify these land marks which gives a 2D pattern with thousand of scattered spots of land marks after autoradiography and this was applied to rice cultivars as a new fingerprinting technique (Kawase, 1994).

The use of unique fragments if available for a variety that is identified by RAPD might simplify future cultivar identification if they could be cloned and the sequence used to develop Sequence Characterized Amplified Regions (SCARs) (Paran and Michelmore, 1993). If primers screened among the varieties do not reveal much polymorphism then Cleaved Amplified Polymorphic Sequences (CAPs) (Akopynaz *et al.,* 1992; Konieczny and Ausbel, 1993) can be used where PCR amplified DNA (STS-EST or SCAR-product) can be digested with restriction endonucleases to reveal polymorphisms in restriction sites.

Microsatellite- AFLP, a method that combines the AFLP technique with simple sequence repeats would be a good candidate for varietal identification studies, where one of the two AFLP amplification primers is replaced by a compound simple sequence repeat in the PCR, so the polymorphisms detected are presumed to be contributed by the simple sequence repeats (Vogel *et al.*, 1994).

Allele Specific Associated Primers (ASAP), a new PCR method, which at high stringent annealing temperatures generates a single DNA fragment only in those individuals possessing the appropriate allele (Gu *et al.,* 1995) that can be identified by the sequence of the decamer oligo derived from normal RAPD.

Directed Amplification of Minisatellite DNA-PCR (DAMD-PCR) is another approach where minisatellite core sequences are used as primers for PCR amplification. It is found to reveal various degrees of polymorphism and generate individual specific DNA fingerprints, which could be used for species differentiation and cultivar identification. It is used as a tool for the isolation of informative probes for DNA fingerprinting (Bebeli *et al.,* 1997).

Use of Single Nucleotide Polymorphism (SNP) in recent years, offered great promise for rapid and highly automated genotyping, which led to rapid advancement in development of human genetic map. SNPs are biallelic markers, which need only a plus/minus assay, permitting easier automation. Further more, the high-density oligonucleotide arrays on DNA chips and the Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) mass spectrometry (Ross *et al.,* 1998) that recently became available; allow genotyping of these diallelic loci in large numbers in

parallel. However, the use of SNPs in plant systems has yet to start, but in near future these markers will certainly be used extensively in plants for finger printing.

2.2 Varietal identification in seed industry

The need to establish a procedure for seed certification and production of the varieties has been pointed out by many authors (Lorenzetti and Falcinelli, 1987; Papni, 1987; Downey, 1988; Rutz, 1990). All these authors underline the necessity for providing appropriate methodology for preserving the genetic purity of the varieties and for facilitating the inspection of field crops or certified seed production. For controlling the genetic purity of seeds during multiplication, a four stages of generation scheme *viz*. breeder seed, foundation seed, registered seed and certified seed is followed and inspected by a seed certifying authority.

Besides the above general seed classes, in India another class of seed called nucleus seed stage is in vogue where the initial seed materials generated by the breeder is used for breeder seed production. It is under the control of the concerned breeder who produces the variety and there is no separate class of registered seed as followed in other countries. Certified seed is the direct progeny of the foundation seed, eliminating the registered seed class stage. If situation warrants additional classes of seed viz., foundation seed stage II (FS-II) and certified seed stage II (CS-II) may be permitted if the seed demand is high and particular class of seed cannot be produced with available seed stock.

The traditional way to assess the genetic purity of seed of established varieties of crops is grow-out test (GOT), where the crop is grown in isolation and rigorous rouging during different phases of crop growth is done with aid of morphological descriptors available for that variety under consideration. The main problem for variety identification

during field inspection of the seed crop is the lack of satisfactory standard characteristics for varietal assessment. The authorities responsible for this task require stable characters for detecting the performance of registered varieties and of new releases. Further characterization such as laboratory tests like electrophoresis pattern and cytology allow the opportunity to improve the characterization of varieties and could provide tools to improve the efficiency of field inspection (Lorenzetti and Falcinelli, 1987; Downey, 1988; Rutz, 1990). Recently, International Seed Testing Association (ISTA) has recommended use of electrophoresis in seed purity testing and possibility of usage of DNA markers is underway.

2.3 Genetic diversity

Improvement in yield of a crop species in general is accompanied with a reduction in the variability among the commercial cultivars of that species, many of the improved varieties have one or more parents (immediate or some what removed in the ancestry) common with each other. Thus the improved varieties of the crop species are becoming increasingly similar to each other due to the commonness of the one or more parents in their ancestry. This has led to narrowing down of the genetic base of these varieties. The narrow genetic base has created genetic vulnerability to diseases, insect pests or some other stress due to a similarity in their genotypes. An example of genetic vulnerability is the out break of *Helimenthosporium* leaf blight of maize in epidemic proportion in 1970s in USA due to the extreme susceptibility of most of the commercial maize hybrids were produced using the Texas male sterile cytoplasm. Susceptibility of the early bajra hybrids in India to Downey mildew and Ergot is other example of using

same genotype in hybrid development, where susceptibility was contributed by male sterile parent Tift 23A (Singh, 1993).

It is generally assumed that commercial sunflower genetic diversity is limited because of its extensive breeding as well as the fact that this crop was introduced in most of the important sunflower producing areas world wide (Eastern and Western Europe, Argentina, South Africa and India) (Paniego *et al*., 2002). It has become essential to assess extent of genetic diversity available among commercials hybrids and varieties of sunflower, which could help in forming strategies to broaden the genetic base of the cultivars.

2.3.1 Approaches to study genetic diversity

Several approaches have been used in studying genetic diversity among genotypes. Conventionally morphological characters (both quantitative and qualitative) have been used for diversity assessment and still it is the widely used approach in plant breeding for studying genetic diversity. Among non-conventional approaches biochemical markers and DNA markers have been used in several crops. Biochemical approaches (isozyme analysis and electrophoresis of seed proteins) still fail to differentiate the cultivars as they measure variation at very limited number of loci (Aldrich *et al*., 1992).

Extensive studies have shown that DNA markers offer highly efficient and informative ways of characterizing diversity. DNA markers can help in determining different genetic classes present and the genetic similarity among them, (how much diversity present in those classes) and their evolutionary relationship with wild relatives (Karp and Edwards, 1997). With respect to DNA markers for diversity, there is also a

trade off between multi-locus and single locus profiles. Techniques that generate single locus profiles *e.g*. VNTR, MAAP (Multiple arbitrary amplicon profile), AFLP, etc provide information on numerous loci, but information content on single loci is low. The data derived from arbitrarily amplified DNA, AFLP and VNTR multilocus fingerprinting approaches have their strength in differentiating the individuals.

2.3.1.1 Morphological characters in diversity assessment

Assessment of diversity using morphological characters is most widely used approach. Morphological characters, which have been used in assessment of diversity, are both quantitative (viz. 100 seed weight, seed yield etc) and qualitative characters (viz. stem or petiole pigmentations). Several morphological traits such as plant height, number of seeds per capitulum, number of leaves on main stem, head diameter, 100 seed weight, seed yield per plant, days to 50 % flowering, days to maturity, oil content etc have been used by many workers for studying diversity among sunflower genotypes (Narsaiah 1995, Sankara Pandaiah *et al*., 1996; Venkateshwara Rao, 1999; Ravi *et al*., 2001; Komuraiah *et al*., 2004; Laxminarayana *et al*., 2004).

2.3.1.2 RAPD markers in diversity assessment

Amplification of genomic DNA using one short oligonucleotide primer, under low stringency conditions result in multiple amplification products from loci distributed through out genome (Williams *et al*., 1990; Welsh and Mc Clelland 1990). These observations led to the development of genome mapping (Williams *et al*., 1990), fingerprinting and diversity assessment (Welsh and Mc Clelland 1990). This method became popular in diversity studies because of its simplicity and applicability to any genome.

Use of RAPD markers for diversity study has been reported in many crops including sunflower (sunflower-Lawson *et al*., 1994; rice- Mackill *et al.,* .1995; Cotton-Rana *et al*., 2002; chickpea-Singh *et al*., 2002).

One of important applications of RAPD is measurement of genetic distances between individuals and phylogenic subsequent studies. RAPD analysis is widely used for the taxonomic studies of various genera (Devos *et al.*, 1992), species (Igbal *et al.*, 1995) and for differentiation of intraspecific units. RAPD markers have been in use in finding genetic relation of different genotypes in hybrid breeding programme (Naghia, 2002; Neeraja *et al*., 2002). RAPD markers were found to be relatively more polymorphic than isozyme analysis (Popov *et al*., 2002). However it was found slightly less polymorphic than ISSR analysis (Souframanien *et al*., 2002) in *Vigna mungo* L. where significant polymorphisms among the *Vigna* mutants were observed using RAPD (25.8 %) and ISSR markers (33.3 %).

Reliability of RAPD markers in assessment of genetic diversity and genetic relationship over morphological characters was studied in cotton (Tateni *et al*., 1996). The correlation between distances estimated from RAPD and the average taxonomic distance estimated from morphological characters was 0.63. This value was greater than that observed in *Avena sterilis* L where a correlation of -0.35 was observed between Jaccards similarity based on RFLP and taxonomic distance based on morphological characters (Beer *et al*., 1993).

Identification of combination of diverse inbred lines with strong yield heterosis is most important step in the developing crop hybrids. Generally parents with a higher combining ability and a long genetic distance can produce a hybrid with better yield

performance (Shamsuddin 1985, Cox and Murphy, 1990; He 1991). However, identification of combining ability is costly, time and labour intensive. Scientists have been trying to predict yield heterosis on the molecular level. Investigation was carried out to determine the relationship between genetic diversity and hybrid performance in wheat (*Triticum aestivaum* L.) where it was found that genetic distance revealed by RAPD markers did not significantly correlate with hybrid performance or heterosis.

2.3.1.3 SSR markers in diversity assessment

Many studies have reported significantly greater diversity of microsatellite over RFLPs (Mc Couch *et al*., 1997) and high number of alleles for microsatellite markers. The similar result was reported in a comparative study conducted using RAPD, RFLP and SSR markers in sunflower regarding extent of polymorphism (Ju-Kyung Yu et al., 2002). Genetic diversity revealed by RAPD was concordant with pattern produced by RFLP markers and it was found that SSR markers (SSRs) were more polymorphic than RFLP markers. SSR markers were used in distinguishing seedlings of *Sangivese* variety of grapevine and mother plants, which were phenotypically similar. SSR markers could distinguish seedlings and mother plants where as morphological characters could not distinguish these genotypes (Tessier *et al*., 1999). Simple sequence repeats are spread through out the eukaryotes genome (Cregan *et al*., 1997). It has been found that genetically mapped microsatellite markers cover the whole rice genome with at least one microsatellite for every 16 to 20 cM (Chen *et al.,* 1997). Rice microsatellites have been

demonstrated to be polymorphic between (Wu and Tanksley, 1993; Yang *et al.,* 1994; Panaud *et al.,* 1996) and within rice varieties (Olufowote *et al.,* 1997).

Subudhi *et al.* (1998) classified 71 cytoplasmic genetic male sterile lines of rice into different groups. Use of microsatellite polymorphisms for the identification of Australian breeding lines of rice was investigated (Garland *et al.,* 1999) and most of the cultivars could be uniquely identified by at least one microsatellite marker. Application of SSR markers in estimation of diversity among cultivars, inbreds and parental lines have been reported rice (Enoki *et al*., 2002; Naveen kumar, 2003)

Relationship between diversity estimated from SSR markers and heterosis was studied by Liu and Wu (1998). It was found that SSRs were able to detect a high level of polymorphism among inbred lines. Using 26 pairs of SSR primers, a total of 106 different alleles were observed among the 20 parental lines although they were similar in the genetic background. The genetic diversity among the 20 lines shows that it is certainly related to heterosis because the male sterile lines were clearly clustered apart from the restorer lines.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present investigation was undertaken at Directorate of Oilseeds Research (DOR), Rajendranagar, Hyderabad with two important objectives. First to identify molecular markers (RAPD and SSR markers) for assessment of genetic purity of sunflower hybrids and varieties so that the loopholes of conventional methods (time consuming, sensitive to environmental conditions, less polymorphic etc), which use morphological characters for purity testing of commercial cultivars can be overcome. Secondly, to study diversity among sunflower hybrids and varieties using three different approaches, that is, by using morphological characters, RAPD and SSR markers.

The details of the materials used and methods adopted in the present investigation are presented in this chapter.

3.1 MATERIALS

The experimental plant material for the present study comprised of thirteen public sector sunflower hybrids along with their parents and thirteen varieties obtained from different sunflower AICRP (All India Coordinated Research Project) centers in India from where those cultivars were released. Among the varieties, morden was obtained from two different sources (from Coimbatore and Akola) and considered as two different entries and were treated as two different varieties and designated as Morden (TN) and Morden (Ak) respectively. The objective behind considering the same variety as two different entries was to check whether any differences existed at molecular level of the same genotype maintained at two different places.

Details of sunflower hybrids and varieties used in the study are presented in table 3.1 and table 3.2 respectively.

3.2 METHODS

3.2.1 Lay out of the experiment

The field experiment was laid out in Randomized Block Design (RBD) with three replications so that observations recorded on morphological characters for hybrids and varieties could be subjected to Mahalanobis D^2 statistics to study genetic diversity The crop was sown during *Rabi* season (August, 2003) at DOR farm Rajendranagar, Hyderabad. All the genotypes included in the study constituted treatments. Each genotype was sown in one row of 5 meters length with a spacing 60 x 30 cm. Two to three seeds were sown per hill to facilitate better emergence and to provide uniform stand. The genotypes, which did not germinate or showed very poor germination, were re-sown. Recommended agronomic package of practices along with plant protection were followed to raise a healthy crop.

3.2.2 Observations recorded on morphological characters

3.2.2.1 Assessment of genetic purity using morphological characters

The main objective behind inclusion of morphological characters in present study was to select genetically pure plants of the genotypes by comparing the observed morphological characters with the published varietal descriptors so that genetically pure material would be used in diversity analysis using morphological characters and RAPD and SSR analysis for purity and diversity studies. To accomplish the said objective, plants of all the genotypes were studied for eleven morphological characters, which were selected from sunflower descriptors published by National Seed Project, IARI, New Delhi. All plants of the hybrids along with their parents and varieties were screened for

all eleven selected characters and observations recorded. The genotypes where majority of the plants were in agreement with characters specific to respective genotypes and sufficiently uniform were selected for studying diversity using morphological characters and for RAPD and SSR analysis. The genotypes, which did not comply with respective descriptors, were not included in the study.

Morphological characters recorded for assessment of genetic purity of hybrid and variety at morphological level are presented here under.

3.2.2.1.1 Leaf size

The Leaf length and width from central third portion of the plant selected at flowering stage was measured in centimeters and leaf size was calculated The genotypes were classified into different categories according to their score as (1) Very small, (2) Small, (3) Medium, (4) large and (5) very large.

3.2.2.1.2 Leaf shape

Leaf shapes for all the genotypes were observed visually and grouped as (1) oblong, (2) lanceolate, (3) triangular, (4) cordate, (5) rounded, and (6) ovate

3.2.2.1.3 Number of leaves on main stem

Number of leaves on main stem were counted at harvest stage and classified into different categories according to their score as (1) Low (<21), (2) medium (21-25) and (3) and high (>25).

3.2.2.1.4 Days to 50% flowering

The number of days taken from the date of sowing to the opening of the ray florets in 50 percent of the plant population in each replication was recorded and accordingly the genotypes were grouped as (1) early, (2) medium and (3) late.

3.2.2.1.5 Ray flower colour

Ray flower colour was visually observed at flowering stage. The different categories included under these characters were (1) Ivory, (2) pale yellow, (3) yellow, (4) orange, (5) purple, (6) red brown and (7) multicoloured.

3.2.2.1.6 Head diameter (cm)

Head diameter was measured in cm by taking the distance between two diametrically opposite extremities of the head (capitulum) at harvest maturity and were classified as per standards as (1) small (<15 cm), (2) medium (15-20 cm) and (3) large (>20 cm).

3.2.2.1.7 Plant height (cm)

Plant height was measured from the base of the plant at ground level to the point of attachment of the capitulum at harvesting maturity in centimeter and were classified as per their score in to (1) very short (<80 cm), (2) short (80-110 cm), (3) medium (111-140 cm), (4) tall (141-170 cm), and (5) very tall (>170).

3.2.2.1.8 Seed weight (g)

One hundred seed each in three replications were weighed separately after harvest and mean weight was measured as (1) low,(2) medium and (3) high.

3.2.2.1.9 Seed stripes

Seed stripes were observed visually after harvest and indicated as (1) absent or (2) present.

3.2.2.1.10 Seed mottling

Seed mottling was observed visually and indicated as (1) absent or (2) present.

3.2.2.2 Morphological characters for genetic diversity analysis

For studying diversity eight morphological characters were selected. Five morphological characters mentioned under assessment of genetic purity using morphological characters (*viz* Days to 50 per cent flowering, Plant height; number of leaves on main stem, head diameter, and 100 seed weight) were recorded for purity and diversity studies. Three characters *viz* days to maturity, seed yield per plant and oil content percent in addition to mentioned characters were recorded for diversity analysis. Observations were recorded on five randomly selected plants from each replication and mean was calculated for each replication to subject for diversity study among hybrids and varieties. Morphological characters used in diversity analysis are discussed here.

3.2.2.2.1 Days to maturity

Days to maturity was recorded as the number of days taken from the date of sowing to the date from which the back of capitulum in 50 percent of the plants in a genotype turned to lemon yellow colour.

3.2.2.2.2 Seed yield per plant (g)

The total weight of filled seed per plant was recorded in grams and considered as seed yield per plant.

3.2.2.2.3 Oil content (percent)

Eight grams of seeds were selected randomly from each replication and subjected to NMR to measure oil content in percent.

3.3 Isolation and quantification of genomic DNA

3.3.1 Strategy adopted for isolation of genomic DNA

Genomic DNA isolation was done by two different protocols for two different purposes. First, Leaf samples were collected and pooled together from all the plants of certain genotype, which were found genetically pure by visual observation. DNA was isolated from pooled leaf samples of each genotype and termed as bulk DNA**.** This was used in screening the primers for identification of markers for genetic purity assessment. After identification of markers validity, reproducibility and ability of identified markers to test genetic purity of individual hybrid plants was checked. To test the purity of individual plants, DNA was isolated from approximately twenty individual plants or if less, from the available number plants of hybrids and varieties. DNA isolated from individual plants was called 'individual plant DNA'. To work on second step, primers, which generated marker(r) for genetic purity assessment, were used in PCR experiment with bulked DNA of hybrids along with both female and restorer parents, and hybrid individual plants DNA in case of hybrids; in case of varieties, bulked DNA and individual plants DNA of varieties. CTAB maxiprep DNA isolation protocol of Doyle and Doyle (1987) was followed for isolation of bulk DNA from leaf samples. CTAB miniprep DNA isolation protocol based on Doyle and Doyle (1987) protocol was followed for isolation of genomic DNA from individual plants. Details of each protocol are given as under.

3.3.2 DNA Isolation from pooled leaf samples

A. Materials:

- Liquid nitrogen
- Isolation buffer:

  2% [W/v] CTAB, 1.4M NaCl,

  20mM EDTA, 100mM Tris HCl pH 8.0,

  0.2% β-mercaptoethanol (added just before use)
- Chloroform isoamylacohol (24:1).
- RNase solution:

  10 mg/ml RNase A in 10mM Tris HCl, 15mM NaCl (pH 7.5); boiled for 15 minutes, cooled to room temperature and stored at -20°C.
- 100% Isopropanol.
- Washing solution: 76% ethanol, 10mM ammonium acetate.
- TE buffer: 10mM Tris HCl, 1mM EDTA (pH 8.0).
- 7.5M ammonium acetate.

B. Method:

- 3 g of fresh leaf sample was ground to a fine powder using liquid nitrogen, mortar and pestle
-

he ground leaf powder was transferred to 15ml of prewarmed (60°C) isolation buffer in a capped polypropylene tube and clump was suspended using spatula.

-

t was incubated for 30-60 minutes at 60°C in a water bath and mixed every 10 minutes.

➢

Volume of chloroform isoamyl alcohol was added; the tubes were capped and extracted for 10 minutes on a rotatory shaker.

➢

t was centrifuged for 10 minutes (5,000g, room temperature) and the aqueous phase was re-extracted with fresh chloroform isoamylalcohol and centrifuged again.

➢

he final solution was transferred to a glass centrifuge tube using large bore pipette. Heat treated RNase A was added to a final concentration of 100µg/ml mixed and incubated at room temperature for 30 minutes.

➢

.6 Volume of ice-cold isoproponal was added and mixed gently but thoroughly by inverting the tube several times. The amorphous precipitate was collected by centrifugation (5000g, 10 minutes at 4°C).

➢

he tubes was inverted and drained on a paper towel for about one hour.

➢

ppropriate volume of TE Buffer was added and allowed the pellet to dissolve over night (4°C) without agitation. 0.5 Volumes of 7.5M-ammonium acetate solution was added, mixed and chilled on ice for 15 min.

➢

t was centrifuged for 30 minutes (10000g, 4°C). Supernatant was transferred to new tube

and 3 volumes of 96% ethanol was added, mixed by inversion and stored for 1 hour at -20°C.

➢

0% ethanol and centrifuged again. The final pellet was drained and dissolved in appropriate volumes of TE buffer

3.3.3 DNA Isolation from individual plants by CTAB miniprep method

A. Materials:

Material used for isolation of genomic DNA from individual plants was same as used in protocol mentioned earlier.

B. Method:

- Approximately 0.1 g of fresh tissue was harvested and freezed in a 1.5 ml centrifuge tube using liquid nitrogen.
- The frozen tissue was ground to a fine powder by mini pestle in micro centrifuge tube and 0.5 ml of extraction buffer was added and vortexed for 10 seconds, the tubes were kept in ice while processing the rest of the samples.
- 35 µl of 20% SDS was added and vortexed briefly followed by incubation for 10 minutes at 65^{o}C to 70^{o}C.
- 130 µl of 5 M potassium acetate was added and mixed thoroughly by shaking back and forth gently and incubated on ice for 5 min and it was spun at 14000 rpm to remove undigested debris.
- The supernatant was transferred to a fresh tube (~700ul), and nucleic acid was precipitated by adding 60 µl of 3M sodium acetate and 640 ul of isoproponal. From this step on samples were handled gently to decrease shearing of the DNA.

- Supernatant was discarded after spinning for 5 minutes at 14,000 rpm to discard insoluble material. Pellet was suspended in 200 µl BTE and spun at 14,000 rpm to discard insoluble material.
- Supernatant was transferred to a fresh tube and reprecipitated again by adding $1/10^{th}$ volume of 3M-sodium acetate and 2 volume of ethanol.
- Pellet was resuspended in 100 µl RNase A and incubated 1 hour at 37 ^{o}C or overnight at 4 ^{o}C.
- DNA was precipitated as in earlier step and centrifuged for 10 minutes at 14,000 rpm in micro centrifuge.
- DNA was washed in 70 percent ethanol and centrifuged for 5 minutes to obtain DNA pellet. The second ethanol precipitation was to improve amplification of DNA in PCR reactions.
- Supernatant was removed carefully. The final pellet was briefly air dried and resuspended in 50µl of TE buffer.

3.3.4 Quantification of genomic DNA:

The isolated genomic DNA was quantified by running on one percent agarose gel. 2µl of DNA was loaded along with lambda uncut DNA with four standards having DNA concentration of 50ng, 100ng, 150ng and 200ng. After running for one hour the DNA concentration was determined by comparing with standard and diluted to required concentration for RAPD and SSR analysis.

3.4 RAPD analysis:

RAPD analysis was done for two different purposes, first was, for genetic purity analysis for hybrids and varieties and second for diversity analysis among hybrids and varieties.

For both the purpose 250 random decamer oligonucleotide primers from 20 different kits from Operon Technologies, USA was used. To select primers that would generate robust PCR banding profile, these 250 random decamer oligonucleotide primers were screened with few genotypes of sunflower and the amplified products were run on 1.5 percent Agarose gel with four genotypes of sunflower. Primers, which generated robust banding profile and gave more number of bands, were selected for genetic purity study and seventy such primers were selected based upon robustness of PCR profile and number of bands amplified.

3.4.1 Genetic purity assessment by RAPD analysis

In sunflower hybrid seed production, three-line system is followed and hybrid seeds are produced by crossing a cytoplasmic male sterile line and restorer line. Since hybrid seeds are produced on female parents, so genetic purity could be assessed if marker specific to restorer parent is identified which is expected to express in hybrids. So the strategy followed for assessment of genetic purity of hybrid was to identify marker specific to restorer parent. However, hybrid with common restorer parent can be distinguished based on female parent specific parent. So such marker was searched along with restorer parent specific marker so that genetic purity could be assessed and hybrids could be distinguished.

For genetic purity assessment of varieties, seventy selected RAPD primers were used in PCR experiment to establish fingerprint or identity tag.

Materials used and experimental protocol followed are as given under

3.4.1.1 Materials:

- Thermocycler

- Horizontal agarose gel apparatus
- Electrphoretic power supply
- Trans illuminator

3.4.1.2 Experimental protocols

RAPD reactions were assembled as described in the following table using the Taq polymerase holoenzyme.

Table 3.3 Components of RAPD reaction

Reagents	**Stock**	**Final concentration**	**Amount used**
DNA		2.5ng/µl	2µl
PCR buffer KCl Tris HCl $MgCl_2$	10X 500mM 100mM 15mM	1X 10mM KCl 10mM Tris HCl 1.5mM	2µl
Primer	4µM	0.4µM	2µl
dNTP mix	2mM each	0.1mM each	0.8µl
Taq polymerase	1 unit		0.3µl
Double distilled water		Complete to 20µl	

3.4.1.3 Cycling conditions

The following conditions were kept for amplified of genomic DNA using random beamer primer.

Initial denaturation at 94°C for 3 minutes followed by 45 cycles of denaturation 92°C for 20 seconds, annealing temperature at 36°C for 45 seconds, initiation 72°C for 2 minutes concluding with final extension for 7 minutes at 72°C and 4°C incubation.

3.4.1.4 Electrophoresis of RAPD-PCR products

After the cycling was finished appropriate volume of 10X Bromophenol blue (BPP) gel-loading dye was added (1ml of 50% glycerol, 100µl Tris HCl 1M, 50µl of 0.5M EDTA and appropriate volume of Bromophenol blue) and mixed well.

1.5% Agarose gel was prepared using 1.5 g agarose (from Bangalore GENEI Pvt. Ltd, Bangalore) in 100ml 1X TAE (Tris Acetate EDTA) and 6µl of ethidium bromide (10mg/ml). Total PCR product with appropriate volume loading dye was loaded on 1.5% agarose gel. The gel was electrophorised under standard conditions (voltage 3- 5 V cm^{-1} length). Accurate sizing of the bands was done was achieved by running the standard size marker lambda DNA cut with Eco-RI and Hind III restriction endonucleases enzymes. The gel was examined and photographed under UV-rays after completion of electrophoresis.

3.5 SSR analysis

Genetic purity of hybrids could be assessed using SSR markers if variants of microsatellite loci are available between female and restorer parent. Hybrid from cross of such parents gives co-dominant banding pattern expressing alleles specific to both the parents. So first step in genetic purity assessment of hybrid was to detect polymorphism between female and restorer parent. Twenty-two SSR primers were used in PCR experiment with bulked DNA of female and restorer parents and their hybrids to detect polymorphism between two parents. Accurate sizing of the alleles was achieved by running the standard size marker DNA such as 50 BP or 100 BP markers on the gel along with PCR product. In second step, SSR primer, which could detect variant of particular microsatellite loci for parental lines of hybrids were tried at individual plant level using individual hybrid plant DNA to check genetic purity of these hybrid plants. In the same PCR experiment, bulked DNA of hybrids and their both the parents were also used with same SSR primer to check repeatability of the markers.

Genetic purity of varieties could be assessed if it could be differentiated from other variety based on its fingerprint. So strategy followed for genetic purity assessment of the varieties or for identification of varieties was to detect their fingerprint using SSR markers. To detect polymorphism among varieties at different microsatellite loci, twenty-two SSR primer pairs were screened with bulked DNA of varieties. PCR products using SSR primers were electrophoresized on 3 % agarose gel.

Information regarding microsatellite loci and SSR primer pairs is available in public domain. Using the same information SSR primers were synthesized by Bioserve Biotechnology Private Ltd. Hyderabad.

3.5.1 Materials and Reagents

- Agarose
- Standard PCR reagents (mentioned for RAPD analysis) including forward and reverse primers.

3.5.2 Experimental protocols

3.5.2.1 PCR reaction mix

Standard PCR reactants were used including 25ng of genomic DNA template, 1X PCR buffer including 1.5mM Mg^{+2}, 100µM of each nucleotide and 0.5 unit of Taq polymerase in a total volume of 10µl.

3.5.2.2 PCR cycling:

Initiate denaturation 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing 50 to 60°C (based upon Tm of primer) for 30 seconds, extension at 72°C for 30 minutes and incubation at 4°C.

3.5.2.3 Agarose gel electrophoresis

Agarose gel of 3% with 10µl of ethidium bromide (10mg/ml) was casted in a standard horizontal gel frame and products were mixed with BPP loading dye solutions and 7µl of the mix loaded on gel with standard DNA ladder of 100bp or 50bp. The products were visualized on a UV transilluminator.

3.6 Data analysis

3.6.1 Scoring

Markers were scored for presence and absence of the corresponding band among the genotypes. Score one was given for the presence and zero for the absence of band. In case

of binary loading, a data matrix comprising of '1' and '0' were formed depending upon the character and this date matrices were subjected to further analysis.

3.6.2 Quantitative data

Means of all three replications were taken for all the characters and subjected to analysis.

3.6.3 Cluster analysis

Three different sets of data gathered (RAPD, SSR and Quantitative data) were subjected to cluster analysis. Sequential Agglomerative Hierarchical Nonoverlapping (SAHN) clustering was performed on similarity matrix using Jaccard's coefficient (RAPD) and Dice coefficient (SSR) for binary data utilizing the Unweighted in Pair Group Method with Arithmetic Averages (UPGMA) method.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

Assessment of genetic purity of F_1 hybrids and varieties is a quality control requirement of critical importance in plant breeding and seed production. Knowledge of these data is essential for control and uniformity of yield, as well as for avoidance of unacceptable impurity level in seed lots prior to market release. Genetic purity test is conventionally conducted by field trials to observe morphological characters of a cultivar, which takes few months to evaluate. However, limited variation observed for morphological characters make it difficult for varietal identification using phenotype of the plants. Also, the longer time requirement to carry out field-based test has created great disadvantage in commercial seed production. DNA based molecular markers have been identified as an alternative method to field based testing for varietal identification and genetic purity assessment. Being nucleic acid based techniques; molecular markers are free of most of the disadvantage encountered with morphological markers. The use of molecular markers for genetic purity assessment or varietal identification has been increasing and has been adopted in several crops. The present investigation was carried out to develop molecular markers useful in assessment of genetic purity of hybrids and varieties of sunflower. One aspect of present study was to identify RAPD and SSR markers, which would help in purity assessment of twelve hybrids and twelve varieties of sunflower. Another aspect of the study was to assess the diversity among the selected hybrids and varieties. Three different approaches were used for diversity study, *viz*. morphological characters, RAPD markers, and SSR markers. The results obtained and

observations recorded during the present investigation are presented here under two broad categories.

4.1 ASSESSMENT OF GENETIC PURITY

4.1.1 Assessment of Genetic purity by morphological characters

The main objective of inclusion of study of morphological characters in present study was to select pure and representative plants of each of the cultivar at field level on the basis of morphological descriptors and homogeneity within the genotype, so that genetically pure plants could be used further for the identification of appropriate RAPD and SSR markers for genetic purity testing at molecular level. Eleven morphological characters were recorded at different plant growth stages as per guidelines given in descriptors published by National Seed Project, IARI, New Delhi. All characters recorded were in agreement with characters mentioned in the descriptors for respective cultivars except hybrid NDSH-1 and variety CO-1. NDSH-1 was not sufficiently uniform and showed presence of many male sterile plants in the hybrid population. Variety CO-1 did not comply with the values for different descriptors like height of the plants. So these two genotypes were excluded from the study. Observations recorded on eleven morphological characters for twelve sunflower hybrids and varieties are presented in Table 4.1 and Table 4.2 respectively.

4.1.2 Assessment of genetic purity by RAPD markers

4.1.2.1 Assessment of genetic purity of hybrids by RAPD markers

In sunflower hybrid breeding, cytoplasmic genetic male sterility system is being exploited through conventional three-line system. Therefore, pure hybrid is one, which results from a cross between respective sterile and restorer lines. In order to know the

genetic purity of a hybrid, it is essential to know whether the hybrid seeds resulted from cross of respective parents or not. In general, sunflower hybrid seeds are contaminated with seeds of maintainer line (B line), seeds resulting from cross of male sterile line and its maintainer line (B line) and rarely with the seeds from a cross between 'A' line and any other pollen parent (if the restorer line are in seed production plot is not pure or if isolation distance is not maintained). So any approach to check purity of sunflower hybrids should clearly be able to distinguish selfed seeds of 'B' line, seeds resulted from cross of 'A' line and 'B' line and seeds of any other cross. Since hybrid seeds are produced on female parent it leaves no doubt about the contribution of fifty percent genes (DNA) from the maternal side to hybrid individual. Another fifty percent of nuclear genetic material is contributed to the hybrid by the male parent. So, a marker specific to male parent (which is not present in female parent) could help in knowing genetic purity of hybrid. If such male parent specific marker shows its presence in hybrid individual, it gives the proof that the hybrid seeds resulted from the cross with respective male parent and also confirms that the hybrid received genome complement from the male parent. However, this strategy would not help in distinguishing hybrids, which have a common male parent because all the hybrids with the common male parent will have same male parent specific marker. Even though as in other hybrids, genetic purity of these hybrids also could be assessed using male parent specific markers, in order to distinguish these hybrids among one another, one needs markers specific to female parents along with the male parent specific markers. Thus with the help of different female specific markers the hybrids having common male parent could be distinguished.

One of the main objectives of the present study therefore was to identify specific molecular markers, which could be used to assess purity of hybrids. The main strategy adopted included

1. Identification of RAPD primers, which could give robust PCR profiles.
2. Screen such primers to identify and select primers, which could distinguish 'A' line and 'R' line (female and male parental lines of the hybrid).
3. Use of identified marker to validate their use for assessing hybrid purity using DNA from individual plants of the hybrid.

To work on foresaid strategy, DNA was isolated from hybrids and their respective female and male parents by two ways. First, leaf samples of each genotype were collected from all representative plants of the genotype and pooled. DNA was isolated from these pooled leaf samples of each genotype and designated as bulk DNA. Second, DNA was isolated from individual plants of each genotype separately and designated as individual plant DNA.

In order to select primers with good and robust profiles, 250 random decamer primers were screened with a few (four) genotypes and 70 primers were selected based on the robustness of PCR profile as judged by higher number of amplicons generated by them. The selected primers were screened with all the hybrids and their respective female and male parents at bulk DNA level for identification of male parent specific markers. Primers, which gave markers specific to male parent were selected, and reproducibility of identified markers were evaluated. These markers were used at individual plant level along with bulk DNA of female, male and hybrid. Presence of identified marker in male parent and hybrid bulk DNA, hybrid individual plants and its absence with female bulk

DNA was the proof for its reproducibility and such markers were called reproducible RAPD markers. Those individual plants, which did not show male specific marker(s) were considered as off type plants (or false hybrid plants). This general strategy was adopted for testing the genetic purity of all the hybrids.

For most of the hybrids, at least two RAPD primers were identified which could generate male parent specific markers in order to assess their genetic purity. Details of identified RAPD markers for each hybrid are given below and tabulated in Table 4.3.

4.1.2.1.1 BSH-1 (CMS 234 A x RHA -274):

Two RAPD markers of size 1.1 kb generated by primer OPJ-1 and 0.9kb generated by primer OPJ-14 were identified specific to male parent RHA-274 (Plate 1). Utility of markers in assessment of purity of individual plants was checked. The identified male parent specific markers were observed in all the 21 hybrid individual plants as well as in bulk hybrid and male parent DNA, claiming that all 21 hybrid individual plants were genetically pure plants.

4.1.2.1.2 KBSH-1 (CMS-234 A x RHA-6-D-1):

Three reproducible RAPD marker of size 0.85 kb, 0.9 kb (Plate 2) and 1.0 kb (picture not shown) specific to male parent RHA-6-D-1 were obtained with primers OPM-2, OPJ-14 and OPG-11 respectively. These markers were present in bulk hybrid and male parent RHA-6-D-1. The identified markers were tested with 21 individual KBSH-1 plants and all the 21 plants showed male specific bands confirming that the plants were genetically pure.

4.1.2.1.3 KBSH-41 (CMS-234 A x RHA-95-C-1), KBSH-42 (CMS-851 A x RHA-95-C-1) and, KBSH-44 (CMS-17 A x RHA-95-C-1):

In present study, of 12 sunflower hybrids studied three hybrids, KBSH-41, KBSH-42 and KBSH-44, have RHA-95-C-1 as the common male parent. In order to distinguish these three hybrids from each other, a search was made for primers which could give female parent specific as well as male parent specific markers. But no such primer was identified which could generate female specific markers along with male specific marker for all three hybrids However, two primers were found, each of which could generate female parent specific marker for two of the three hybrids and thus when used collectively could clearly distinguish all the three hybrids. Primer OPK-15 generated 0.5 kb CMS 234 A (female parent of KBSH-41) specific and 1.8 kb CMS 851 (female parent of KBSH-42) specific marker along with RHA-95-C-1 specific marker of size 0.9 kb (which was observed in all three hybrids). No CMS 17A (female parent of KBSH-44) specific marker was identified by primer OPK-15. Second primer OPJ-16 generated RHA-95-C-1 specific marker of size 1.1kb, a marker of size 1.2 kb specific to CMS 851 and marker of size 1.0 kb specific to CMS 17 A. No marker was found specific to CMS 234 A by primer OPJ-16. Even though OPK-15 and OPJ-16 could not generate markers specific to CMS 17A and CMS-234A respectively, both primers could distinguish all the three hybrids separately as well as in combination. Primer OPK-14 generated 0.5 female specific marker and 0.7 kb male specific marker for hybrid KBSH-42.

In order to merely assess genetic purity of these hybrids, identification of just male specific marker is sufficient.

For hybrid KBSH-41 four primers OPK-15, OPJ-1 (plate 3) and OPJ-16, OPG-11 were identified which generated male specific markers of size 0.9 kb, 2.0 kb, 1.1 kb, and 1.0 kb respectively. Twenty individual hybrid plants were screened with identified

primers and respective male and female specific markers were observed in all twenty plants.

For genetic purity testing of KBSH-42 five primers OPK-15, OPK-14, (Plate 4), OPJ-16, OPJ-1 and OPG-11 were identified which generated male specific markers of size 0.9 kb, 0.7 kb, 1.1 kb, 2.0 kb, and 1.0kb respectively. Twenty-one individual hybrid plants were screened with identified primers, respective male and female specific markers were observed in all the twenty-one plants.

Genetic purity testing of KBSH-44 could be done using four primers OPK-15, OPG-11, (Plate 5), OPJ-16 and OPJ-1, which generated male specific markers of size 0.9kb, 1.0 kb, 1.1 kb, and 2.0 kb respectively. Nineteen individual hybrid plants were screened with the identified primers and respective male and female specific markers were observed in all plants.

4.1.2.1.4 HSFH-848 (CMSH-91 A x RLA-298)

Two markers of size 1.3 kb and 1.4 kb specific to male parent RLA-298 were identified using RAPD primers OPK-9 and OPM-4 respectively (Plate 6). These markers were also observed in bulk hybrid DNA. Efficiency of these markers was validated by using same primers with twelve individual plants along with bulk DNA of hybrid, female and male parent. Identified markers were observed with bulk DNA of hybrids and male parent and all individual plants.

4.1.2.1.5 LSH-1 (CMS 234 A x MRHA-2)

Two RAPD primers were identified which could generate MRHA-2 specific markers. Primer OPJ-14 generated marker of size 0.8 kb and primer OPK-8 generated two markers of size 0.8 kb and 0.5 kb. Twenty-two individual hybrid plants were

screened with same primers to check their genetic purity. Male specific marker generated by primer OPJ-14 was observed in all plants. Out of two male specific markers generated by primer OPK-8, 0.5 kb male specific marker was observed in all plants. However, 0.8kb male specific marker was observed only in sixteen plants. (Plate7).

4.1.2.1.6 SCH-35 (CMS-234-A x RHA-1-1)

Two RHA-1-1 specific markers of size 1.1 kb and 1.4 kb were identified with primers OPJ-1 and OPG-11 respectively. The identified markers were checked for reproducibility and genetic purity at individual plant level using eighteen plants along with bulk DNA of parents and hybrid. Out of eighteen hybrids plants, six plants did not amplify the marker of size 1.1kb generated by primer OPJ-1, and, three plants did not show male specific markers of size 1.4kb with primer OPG-11 (Plate 8). Rest of the plants showed both male specific markers.

4.1.2.1.7 PSFH-118 (CMS-10A x P 61R)

Two P 61R specific markers of size 1.2 kb and 1kb were identified with primers OPI-11 and OPE-14 respectively. Eighteen individual plants of the hybrid were screened with both the primers. All the plants showed the identified male specific marker, which clearly demonstrated that these plants were genetically pure (Plate 9).

4.1.2.1.8 DSH-1 (CMSDSF-15A x RHA 857)

Primer OPI 16 generated two RHA-857 specific markers of size 1.2 kb and 0.4 kb and 0.8 and 0.7 kb female parent specific marker. Other primer OPJ-1 generated 1.1kb RHA-857 specific markers. These identified markers were tested with individual hybrid plants. When OPI–16 primer was used, out of seventeen only seven plants showed

identified marker of size 1.2 kb and fourteen individual plants showed 0.4 kb male parent specific marker, while 0.8kb and 0.7 kb female parent specific markers were present in sixteen and fourteen plants respectively. Using primer OPJ–1, only seven hybrid individual plants showed 1kb male specific marker (Plate 10).

4.1.2.1.9 RSFH –1 (CMS–103A x R-64NB)

Two R-64NB specific markers of size 0.8 kb and 0.9 kb were identified using primer OPK-3 and OPJ-7 respectively. One female parent specific marker of size 1.1 kb was also observed using primer OPJ-7. Both the male parent specific and female parent markers were observed in the bulk of RFSH-1. These markers were tested with twenty-three individual plants. Male and female specific markers of size 0.9kb and 1.1kb generated by OPJ-7 were observed in all the twenty three individual plants, while male specific marker of size 0.8kb generated by OPK-3 was present only in nineteen individual plants (Plate 11).

4.1.2.1.10 TCSH–1 (CMS –234A x RHA 272)

Two primers OPJ-14 and OPH-2 were identified which generated RHA-272 specific marker of size 0.9 kb and 2.0 kb. Twenty individual plants of the hybrid were screened with same primers. Identified male specific markers generated by both the primers were observed in all individual plants (Plate 12).

4.1.2.2Genetic purity assessment of varieties by RAPD markers

Varieties unlike hybrids are developed by deliberate selection for genetic homogeneity and high productivity under scientific crop improvement programmes. Since varieties are homozygous and homogeneous populations, the genetic purity of varieties could be assessed at molecular level only if it is having the unique molecular ID

(identity). In the present study to identify unique molecular ID for twelve varieties, eighty RAPD primers were selected from 250 RAPD primers (from twenty different primer kits)tried, based on robustness of PCR profiles generated by these primers as indicated by higher number of amplicons obtained. Out of the twelve varieties analyzed, molecular IDs were identified for two varieties, CO-2 and EC-68415, using RAPD primers. No single primer could distinguish all the varieties. However, combination of ten primers could differentiate all the twelve varieties included in the study.

RAPD primers which generated specific molecular IDs for varieties and which could differentiate varieties are presented in Table 4.4a and 4.4b respectively.

Results obtained with each variety are presented below

4.1.2.2.1 Surya

No specific molecular identity was established for this variety. However, it could be differentiated from other varieties with a combination of two RAPD primers OPE-14 and OPJ-11. Primer OPE-14 generated five major bands in most of the varieties while Surya, Gujarat Sun-1, S-11 and CO-4 amplified one clear extra band of size 0.4 kb. Variety Surya could be distinguished among these four varieties by using primer OPJ-11 which generated a distinct 1.3 kb band only in Surya (absent in other three varieties).

4.1.2.2.2 PKVSF-9

PKVSF-9 could be distinguished from other varieties using two primers OPJ-5 and OPK-19. Primer OPJ-5 generated about seven major bands in most of the varieties, while 0.9kb distinct band was absent in PKVSF-9. The primer OPK-19 generated five major bands and three minor bands in most of the varieties. Out of the five major bands

1.4kb band and one minor band of size 1.8 kb were absent in PKVSF-9, which made the PCR banding pattern of this variety unique among all the varieties.

4.1.2.2.3 Gujarat sunflower-1

No unique molecular ID was found but the variety could be differentiated from other varieties with a combination of two primers OPE-14 and OPK-15. Primer OPE-14 generated five major bands in most of the varieties. However, Gujarat sunflower-1, Surya, LS-11 and CO-4 amplified one more major band of size 0.4kb. Among these four varieties Gujarat sunflower-1 could be distinguished with the primer OPK-15. Gujarat sunflower-1 amplified five major bands and five minor bands ranging from size 0.5kb to 3kb. Out of these, a 2 kb minor band and 0.5 kb major band could clearly distinguish Gujarat sunflower-1 from other three varieties.

4.1.2.2.4 LS-11

LS-11 could be distinguished from rest of the varieties by using primer OPC–4 which generated five major bands and few minor bands ranging from size 0.65kb to 2.4 kb. However LS-11 amplified only four major bands of size 1.2 kb, 1.3kb, 1.8kb and 2 kb. This pattern made it distinct from other eleven varieties.

4.1.2.2.5 SS-6

RAPD primer OPC-4 could differentiate SS-56 from other varieties. Primer OPC-4 amplified six major bands and one minor band. Unique banding pattern of this variety-included bands of size 0.58 kb, 0.65kb, 1.1kb, 1.2kb, 1.5kb, 1.8kb and 2.0kb. However, the bands, which distinguish SS-56 from rest of the varieties, were faint compared to other robust bands, which were not polymorphic.

4.1.2.2.6 CO-2

Molecular identity of CO-2 was detected using primer OPC-2. This primer amplified unique band of size 1.5 kb, that could be considered as molecular fingerprint of CO-2. (Plate 13 A)

4.1.2.2.7 CO-3

Molecular ID of CO-3 could not be identified. However, primer OPE-14 could differentiate this variety from rest of the varieties. This random primer generated four major bands of 0.65kb, 0.8kb, 1.1kb and 1.8kb in most of the varieties. However, CO-3 did not show a major band of size 0.65 kb and showed one extra band of size 0.4kb, which made the banding pattern of CO-3 unique. Banding profile of CO-3 included bands of size 0.4 kb, 0.8 kb, 1.1 kb and 1.8 kb.

4.1.2.2.8 CO-4

Unique molecular ID was not found using seventy RAPD primers. However, the variety could be differentiated from other eleven varieties using a combination of three primers OPE-14, OPK-15 and OPC-4. Primer OPE-14 could differentiate Surya, Gujarat sunflower-1, LS-11 and CO-4 from rest of the varieties while there was no difference among these four varieties. Primer OPK-15 could differentiate Surya and Gujarat sunflower-1 from LS-11 and CO-4 and primer OPC-4 differentiated CO-4 from LS-11.

4.1.2.2.9 EC-68415

Molecular ID of the variety was identified using primer OPE-14. Molecular marker of size 1.7 kb generated by using primer OPE-14 made the variety unique (Plate 13 B). This marker could be considered as a fingerprint of the variety.

4.1.2.2.10 Morden

Differences were observed among the variety Morden obtained from different sources. Primer OPJ-9, OPJ-7 and OPH-7 could differentiate Morden from two sources.

4.1.2.2.10.1 Morden (TN)

Primer OPJ-9 could differentiate Morden (TN) from Morden (AK) as well as from rest of the varieties. Primer OPJ-9 generated unique banding pattern for the Morden from Coimbatore. Four major bands of size 1.0kb, 1.25 KB 1.30 KB and 1.35 KB were observed in the unique banding profile of Morden (TN).

4.1.2.2.10.2 Morden (AK)

Unique banding pattern was found for Morden (Akola) with primer OPJ-7 which generated four major bands of size 0.6 kb, 0.7 kb 1.1 kb and 1.2 kb and three minor bands. Absence of 1.5 KB major band made the pattern of Morden (AK) unique among all the varieties.

4.1.2.2.11 Dwarf Morden

Dwarf Morden could be differentiated from other varieties using primer OPJ-7. Three major and four minor bands were amplified using same primer. Presence of three major bands of size 1.2 kb, 1.5kb and 2.5 kb and absence of the major band of size 1.1kb could differentiate dwarf Morden from other varieties.

4.1.3 Genetic purity assessment by SSR markers

4.1.3.1 Genetic purity assessment of hybrids by SSR markers

SSR markers were used in genetic purity analysis of hybrids and varieties. In SSR, polymorphism occurs if variants of microsatellite loci occur in the population. Variants of microsatellites occur due to addition, deletion or duplication of few repeats

from respective micro satellite loci. SSR being co-dominant marker shows, in diploids, two bands if the individual is heterozygous and one band if homozygous for that particular microsatellite locus.

Parental lines used in hybrid breeding are inbred lines sufficiently homozygous and so expected to be homozygous also for microsatellite loci. If polymorphism occurs between female and male parents for certain microsatellites, the hybrid resulting from cross of such parents would show both alleles which were found in parental lines. Under such conditions hybrids could be considered as pure hybrid.

In the present study, the strategy adopted in hybrid purity analysis by SSR markers was to screen SSR primers with parental lines of hybrids. Polymorphism was looked for, to confirm the co-dominant nature (presence of bands which were found in both the parents) of hybrids. If variants of the microsatellite were observed among the parents, use of such SSR markers in testing genetic purity of individual hybrid plants was assessed.

22 SSR primers were used to screen twelve sunflower hybrids along with parental lines in PCR. Initial screening of primers with hybrids and parental lines were done using respective bulk DNA and testing of genetic purity of individual hybrid plants was done by using individual hybrid plant DNA. PCR product was electrophoresed on three percent agarose gels.

Polymorphism was observed between female and male parental lines of seven hybrids. Single primer ORS-317 was found useful in assessing genetic purity of five hybrids. All the hybrids, where polymorphism was observed between male and female parental lines were co-dominant for respective microsatellite loci. Details of the markers

identified using SSR primers for genetic purity testing of hybrids are presented in Table 4.5.

Since, variations among different alleles of the microsatellite are only few bases, the agarose gels had to be run for longer time to resolve the differences. However, running the gel for longer time resulted in fainter bands, which could not be photographed clearly (using Polaroid). Results obtained in these studies are presented here.

4.1.3.1.1 KBSH-1 (CMS-234 A x RHA-6-D-1)

SSR primers ORS-317 generated marker specific to female parent CMS-234 A of size 205 bp and male parent RHA-6-D-1 specific marker of size 215 bp. Hybrid KBSH-1 showed presence of both the female and male parents specific bands. Testing of the same primer with individual hybrid plants of KBSH-1 also confirmed their co-dominant nature, showing both parental line specific markers.

4.1.3.1.2 KBSH-41 (CMS-234 A x RHA-95-C-1)

Marker specific to male parent RHA-95-C-1 of size 215 bp and CMS-234 A specific marker generated by primer ORS-317 were found useful in knowing the genetic purity of hybrid KBSH-41. Hybrid with bulk DNA as well as individual plant hybrid showed bands specific to both the parents.

4.1.3.1.3 TCSH-1 (CMS-234 A x RHA-274)

SSR marker of size 205 bp specific to CMS-234 A and marker of size 212 bp specific to male parent RHA-274 generated by primer ORS-317 were found to be useful

in assessing genetic purity of the hybrid. Hybrid TCSH-1 was co-dominant for both the parent specific markers with bulk DNA level as well as with individual plant level.

4.1.3.1.4 LSH-1 (CMS-338 A x MRHA-2)

Female parent CMS-338 A specific marker of size 220 bp and male parent specific marker of size 210 bp were identified with primer ORS-317. The same primer was used for testing genetic purity of 20 individual hybrid plants of LSH-1. All the hybrid plants tested were pure as they showed both parent specific markers.

4.1.3.1.5 PSFH-118 (CMS 10 A x P 61 R)

Two primers ORS-317 and ORS-237 were useful in knowing the purity of hybrid PSFH-118. Primer ORS-317 generated female CMS-10 specific marker of size 200 bp and male parent specific marker of size 212 bp. Second primer ORS-237 generated CMS-10 A specific marker of size 185 bp and P-61R specific marker of size 195 bp. Both the primers were tested in checking genetic purity of the hybrid and all 20 plants were heterozygous with respect to the respective microsatellite loci.

4.1.3.1.6 DSH-1 (CMSDSF -15 A x RHA-857)

Primer ORS-342 generated marker specific to CMSDSF-15 A and RHA-857 of size 335 bp and 342 bp respectively. Both the identified female and male specific markers were observed in all the 20 hybrids individual plants of the hybrid. (Plate14 A)

4.1.3.1.7 SCH-35 (CMS-234 A x RHA-857)

Female parent and male parent of hybrid SCH-35 were heterozygous for microsatellite locus produced by primer ORS-154. Female parent CMS-234 A generated two bands of size 210 bp and 190 bp. Male parent RHA-857 generated three bands of

size 305 bp, 210 bp and 190 bp. In hybrid at bulked DNA level as well as at individual plant level three bands were observed of size 305 bp 210 bp and 190 bp (Plate 14 B).

4.1.3.2 Assessment of genetic purity of varieties by SSR markers

SSR markers were used in differentiating twelve sunflower varieties. Varieties are expected to be homozygous and homogeneous populations at the population level. SSR markers could establish molecular identity of the varieties, if variations occur at microsatellite loci among the varieties. In order to identify unique SSR profiles, twenty SSR primers were screened with twelve varieties. Very less polymorphism was observed among all twelve varieties for twenty-two microsatellite loci. Molecular IDs were identified for only two varieties.

4.1.3.2.1 PKVSF-9

This variety was found to be heterozygous for microsatellite locus amplified by SSR primer ORS-134. Two bands were observed of approximate sizes 400 bp and 700 bp. Both the bands were unique to the variety compared to other eleven varieties.

4.1.3.2.2 EC-68415:

SSR primer ORS-134 generated two bands of sizes 300bp and 400 bp. Presence of 300 bp band made the banding pattern of the EC-68145 unique among all the varieties.

In SSR analysis, two bands were observed in some of the varieties with eleven SSR primer pairs. Amplification of two bands in sunflower varieties and parental lines of hybrids indicate their heterozygosity for those microsatellite loci. In seven of the twenty-

two SSR primer pairs screened in PCR analysis with both hybrids and varieties, stuttering (multiple bands in microsatellite allele) was observed.

4.2 ASSESSMENT OF GENETIC DIVERSITY

4.2.1 Assessment of genetic diversity using morphological characters

In the present investigation twelve sunflower hybrids and twelve sunflower varieties were evaluated during rabi 2003. Observations were recorded on eight different morphological traits *viz* days to 50 % flowering, days to maturity, plant height, number of leaves per plant, head diameter, seed yield per plant, 100 seed weight and oil content (%). Mean performance of twelve hybrids and twelve varieties for eight characters are presented in Table 4.8 and 4.9 respectively.

4.2.1.1 Analysis of variance

The ANOVA (Analysis of Variance) of twelve hybrid and twelve varieties in sunflower is furnished in Table 10 and 1 respectively. The differences in genotypes were less for all the characters under study.

4.2.1.2 Grouping of genotypes into different clusters

Genetic diversity was estimated by using Mahalanobis Euclidean^2 technique for different morphological traits studied. The 12 hybrids (Fig. 1) and 12 varieties (Fig. 2) were grouped into four separate clusters respectively based on Euclidean^2 values such

that the genotypes belonging to same clusters had on an average smaller Euclidean^2 values than those belonging to different clusters. The distribution of different hybrids and varieties into clusters are shown in Table 4.10a and 4.10b respectively.

Out of four clusters formed in hybrids, cluster IV was the largest group comprising four hybrids followed by cluster III and I each with three hybrids and cluster II with two hybrids. While in varieties, out of four clusters, cluster IV and I were largest clusters comprising five varieties each. The remaining clusters III and II comprised one variety each.

4.2.1.3 Intra and inter cluster average distance

The average intra and inter cluster distance values are presented in Table 15 (A) and 15 (B) for hybrids and varieties respectively.

The intra cluster Euclidean^2 values in hybrid ranged from 615.46 (cluster I) to 2468.6 (cluster II) while, in varieties the range was from 1473.29 (cluster I) to 0.00(cluster II and III).

From inter cluster Euclidean^2 values of four clusters of hybrids, it could be noticed that the highest (10218.79) divergence occurred between cluster II and III while, lowest (2368.11) divergence was seen between clusters I and IV. The inter cluster distance between I and II, I and III, II and IV, III and IV indicated that they were

moderately divergent. From Inter cluster Euclidean^2 values among four clusters of varieties showed that highest (9359.52) divergence was between cluster II and III while, lowest (4110.39) was between cluster II and I. The inter cluster values for clusters II and I, II and IV and, III and IV were moderately divergent.

Statistical distance of twelve hybrids and twelve varieties are shown in Fig. 3 and Fig. 4 respectively.

4.2.2 Diversity analysis based on RAPD markers

UPGMA cluster analysis was performed using Jacquard's similarity coefficient matrices calculated from RAPD markers to generate a dendrogram of twelve hybrids and twelve varieties. The similarity coefficient among the hybrids ranged from 0.64 to 1.00 (Fig 5). The dendrogram showed the grouping pattern of twelve hybrids. The twelve hybrids were grouped into four clusters at 78% similarity. The cluster I which included six hybrids (BSH-1, KBSH-1, SCH-35, KBSH-41, KBSH-42 and KBSH-44) was further divided into two sub clusters at 84% similarity. Sub-cluster I contained three hybrids, of them BSH-1 and KBSH-1 formed one group and showed 86% similarity and both these hybrids showed 84% similarity to SCH-35, the third hybrid in sub-cluster I. The sub cluster II comprised three hybrids *viz* KBSH-41, KBSH-42 and KBSH-44 formed a group, which showed higher similarity (90%) to each other and both of these hybrids were similar to KBSH-44 at 83%. The second cluster consisted of only two hybrids, HSFH-848 and LSH-1. They showed 74% similarity and were related to cluster I at 69%. Third cluster comprised hybrid RSFH-1 which showed only 61% similarity to cluster I and cluster II. Cluster four consisted of three hybrids PSFH-1, DSH-1 and TCSH-1. PSFH-1 and DSH-1

formed one sub-cluster while TCSH formed a separate sub-cluster at with 69% similarity between the sub-clusters.

Genetic similarity coefficient among twelve varieties ranged from 0.8 to 1.00 (Fig 6), indicating that the genetic distance among the genotypes tested was very low. In the dendrogram constructed, the varieties formed five clusters. The first cluster was the largest, with five varieties (Surya, Gujarat sunflower-1, SS-56, CO-4 and LS-11). Among them Surya and Gujarat sunflower-1 were 95% similar to each other. The other three varieties SS-56, CO-4 and LS-11 formed a sub-cluster. LS-11 was similar to the rest of the four varieties at 91 percent. Second cluster consisted of only two varieties CO-2 and CO-3, which were 91% similar to each other. The third cluster had only one variety PKVSF-9 which was 88% similar to cluster one and cluster two. Cluster four comprised three varieties Morden (TN), Dwarf Morden and EC-68415. Morden and Dwarf Morden were 95% similar to each other. Modern (Akola) which was the only variety in cluster five, was distinct from all the varieties.

4.2.3 Diversity analysis based on SSR markers

Based on SSR markers, genetic similarity among the sunflower hybrids using the Jaccard's coefficient was estimated. Dendrograms constructed using data generated by SSR primers for hybrids and varieties. The similarity ranged from 0.6 to 1.0, (Fig 7) indicated that the genetic diversity among the hybrids tested was quite wide compared to the genetic diversity revealed by the RAPD markers. Twelve sunflower hybrids formed three major clusters. Cluster one comprised five hybrids BSH-1, KBSH-1, KBSH-41, TCSH-1 and SCH-35. In this cluster BSH-1 and KBSH-1 showed 92% similarity to each other. Cluster two consisted of three hybrids KBSH-44, LSH-1 and HSFH-848. Cluster

two was separated from cluster three at 68% similarity. Cluster three consisted of four hybrids DSH-1, RSFH-1, PSFH-1 and KBSH-42. DSH-1 and RSFH-1 were 94% similar to each other.

Among twelve varieties, Jaccards coefficient ranged from 0.68 to 1.00 (Fig. 8). Twelve varieties formed four major clusters. Cluster I was the largest among all clusters and included eight of the twelve varieties. Major cluster I was divided into two minor clusters, one sub-cluster including seven varieties and the other with only one variety CO-3 which was away from sub-cluster I showing 77 percent similarity. In minor cluster I two sub- clusters were observed. Sub-cluster II included only one variety SS-56, which showed 81% similarity to sub-cluster I. In sub-cluster one, six varieties formed three groups. Gujarat sunflower-1 and LS-11 were in one group with 93% similarity between them. Group two comprised CO-2 and EC-68415, which were 94% similar to each other. CO-4 and Morden (TN) were 92% similar to group two. Major cluster II included only variety Surya, which showed 74% similarity to major cluster I. Major cluster III consisted of Morden (AK) and Dwarf Morden, which was 88% similar to each other. Major cluster IV, which comprised only one variety PKVSF–9, was distinct from all the other varieties.

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

Genetic purity of hybrids and varieties is a quality requirement of critical importance in seed production. Knowledge of these data is essential for maintaining uniformity of the proved yield potential of the cultivars as well as for avoidance of unacceptable impurity level in seed lots prior to market release. This is particularly more important for F_1 hybrids where high level of genetic purity is essential for exploitation of heterosis.

Purity of the cultivars is assessed conventionally by the GOT (Grow-Out-Test) on a representative sample of the seed that is to be marketed (Bellster and de Vicente, 1998). The GOT involves growing of the plants to maturity and assessing several morphological and floral characteristics that distinguish the cultivars. Seeds of cultivars produced for commercial crop production are not used for raising the crop because it is necessary to check genetic purity by GOT in succeeding season. This entails a lot of cost in terms of locked up capital and attendant problems of storage. Moreover, GOT can be subjective; several aspects of crop phenotype (morphology, yield etc.) can be affected by environmental conditions. Further, there is also a possibility that adverse climatic conditions (like, heavy rain or wind) can damage and destroy the crop and make it difficult to collect the requisite data. Thus, there is a need to assess the genetic purity of cultivars using an assay that is both accurate and faster so that the seed produced in one season can be released for commercial cultivation in immediate next season.

Other methods such as electrophoresis of seed storage proteins, isozyme analysis and RFLP (restriction fragment length polymorphism) analysis have been used for genetic purity testing and detection of contamination in hybrid seed lots (Ladizinsky and Hymowits, 1979; Arus *et al.,* 1985; Livneh *et al.,* 1990). Use of isozyme and seed storage proteins is limited due to its inability to detect polymorphism among closely related genotypes. Though RFLP analysis reveals more genetic variability, the technique is time consuming and labour intensive.

The above problems associated with morphological, isozyme and RFLP analysis have necessitated development of alternative tools. The recent developments in molecular biology have resulted in simple, easily assayable PCR based DNA marker techniques such as Random Amplified Polymorphic DNA (RAPD) and microsatellite markers which provide alternative approaches for evaluating genetic diversity (Asemota *et al.,* 1996; Gupta and Varshney, 2000) and genetic purity assessment (Yashitola *et al.,* 2002).Universal acceptance of PCR based markers in genotype specific fingerprinting and diversity analysis provides an opportunity for their deployment in seed purity assay.

One of the objectives of the present study was to identify RAPD and SSR markers for assessment of genetic purity of hybrids and varieties so that such information could be used in genetic purity testing of commercial hybrids and varieties. In the present study thirteen public sector sunflower hybrids and varieties were used to develop RAPD and SSR markers for genetic purity assessment. Among the varieties, Morden which was maintained and obtained from two different locations (Coimbatore and Akola), was included as two different entries. This was done to know whether any changes occurred

at molecular level over time due to random genetic drift (RGD), location or maintenance at different research stations.

Strategy adopted to fulfil the objectives of the study was:

- To select genetically true plants of the genotypes using morphological characters.
- To screen RAPD and SSR primers in PCR experiments with hybrids along with their parents and with varieties.
- To evaluate utility and reproducibility of identified markers to test genetic purity of individual plants of respective genotypes.

To work on the aforementioned strategy, DNA was isolated from thirteen hybrids along with their parents and thirteen varieties by two different protocols and for two different purposes. DNA of each genotype was isolated as bulk (bulk DNA) i.e. leaf samples from all representative plants of that genotype was collected, pooled and this was then used for the DNA isolation. DNA was also isolated from individual plant of each genotype separately (individual plant DNA). Bulk DNAs were used for initial screening of hybrids along with parents and varieties in PCR experiments. This was done to identify markers for genetic purity assessment so that DNA from maximum number of plants of that genotype would be included. DNA was isolated from individual plants, which could be used in validation of identified markers and testing genetic purity of individual plants.

5.1 Genetic purity assessment using morphological characters

Eleven morphological characters were selected from sunflower descriptors. Intension behind inclusion of morphological characters in present investigation was to select genetically pure plants of the cultivars for RAPD and SSR analysis. Inclusion of wrong genotypes in RAPD and SSR analysis would have misguided and led to wrong conclusions. Observations were recorded on eleven morphological characters. Those genotypes, which agreed well to the characters specific to that genotype mentioned as descriptors and were sufficiently uniform, were used for genetic purity assessment by RAPD and SSR markers and diversity analysis by all three approaches *i.e.* by using morphological characters, RAPD and SSR analysis.

One hybrid NDSH-1 and one variety CO-1 were excluded from the study based on their morphological characters. This is due to lack of uniformity in NDSH-1 hybrid population and also due to presence of many male sterile plants. The seeds of variety CO-1 obtained from Coimbtore, Tamilnadu were not included in further studies, as the plants did not show any characters as specified in the descriptors for this variety. Rest of the hybrids (12) and varieties (12) were used for genetic purity study using RAPD and SSR markers and for studying genetic diversity.

5.2 Genetic purity assessment using RAPD markers

In order to identify RAPD markers for genetic purity assessment, seventy RAPD primers were selected from the stock of two hundred and fifty primers of twenty different

primer kits after screening with few sunflower genotypes in a pilot experiment. This was done to select the primers, which could give robust PCR profiles and generate relatively more number of amplicons without any smear.

In sunflower hybrid seed production, three line breeding system is followed, where hybrid seeds are produced on female parent which have been pollinated by the restorer parent. For assessment of genetic purity of hybrids, RAPD markers specific to restorer parents were identified. Since RAPDs are dominant markers, they would be seen in hybrid plants also. If the restorer parent specific RAPD markers are observed in the hybrid plants, then the hybrid could be considered as genetically pure. While in case of hybrids, where restorer parent is common, along with restorer parent specific marker, markers specific to female parent are also necessary in order to differentiate the hybrids among themselves. Many workers (Hashizume *et al.,* 1993; Xiangdong Meng *et al.,* 1998) followed the same strategy for hybrid purity testing by RAPD markers.

In the present investigation RAPD markers were identified for all the twelve hybrids analyzed. For all the hybrids, at least two primers were identified which could generate markers for genetic purity testing of hybrids. These primers were used in PCR reactions with available number of individual plants (approximately twenty) of respective hybrids to test reproducibility and efficiency of identified markers to check the genetic purity at individual plant level.

Genetic purity assessment of individual plants of the hybrids BSH-1, KBSH-1, KBSH-41, KBSH-42, KBSH-44, HSFH-848, PSFH-118 and TCSH-1 with the identified markers showed similar results and clearly indicated that the seeds of the hybrids were genetically pure. These results also confirmed the utility of RAPD markers for hybrid

purity testing. Similar kinds of results have been reported in other crops (tomato, watermelon, chilli) where genetic purity of hybrids could be assessed using RAPD markers (Hashizume *et al.,* 1993; Rom *et al.,* 1995 and Crockett *et al.,* 2000)

However, from RAPD analysis with individual plants of the hybrids LSH-1, SCH-35, DSH-1 and RSFH-1 using primers that generated restorer parent specific markers, some interesting results were observed. In one case, few individual plants of hybrids showed male parent specific marker using one primer and thus could be considered as genetically pure, however with other primer the same plants did not show identified male parent specific marker (Plate 8, 10 and 11) indicating that the plants were not genetically pure. In case of hybrids LSH-1 and DSH-1, when primers that generated two male parent specific markers for the same hybrid were used, few plants showed presence of one marker and absence of the other (Plate 8 and 10).

Reason behind such results, where the individual hybrid plants, which were classified as genetically pure (due to presence of restorer parent specific marker in hybrid) using one primer and impure or false hybrid (due to absence of restorer parent specific marker in hybrid) using another primer may be the heterogeneity and heterozygosity of restorer parent which was used in crossing programme to produce the hybrid under consideration. Two different RAPD primers amplify fragments from different regions of the genome based upon sequence complementarities of the primers. Presence of band specific to restorer parent in the hybrid individual using one primer indicates that the respective RAPD locus was present in that individual hybrid while the absence of the band indicates the absence of the locus. If restorer plants are not sufficiently homozygous (pure) and the population is heterogeneous, then the pollen

grain, which effect hybrid seeds, will not be same genetically and may carry different alleles present in restorer parent. If certain RAPD locus is present in heterozygous condition (due to genetic contamination) in such restorer plants, and due to segregation of those loci, some hybrid individuals may not show restorer parent specific marker, even if hybrid resulted from the pollination from respective restorer parent. Other reason for such a result may be the occurrence of mutations in germinal tissue of restorer plant at the priming site of certain RAPD primers. Hybrid seeds produced from such restorer plant and cytoplasmic male sterile line would not show restorer parent specific marker even though it has received genomic complement from the restorer plant.

Three hybrids KBSH-41, KBSH-42 and KBSH-44 used in the present study have a common restorer parent RHA-95-C-1. Since the restorer parent is common, genetic purity of these hybrids could be determined based on just the restorer parent specific marker. However, this marker cannot be useful to distinguish hybrids among themselves. Under these circumstances, to circumvent the problem, along with the identified common male specific marker, identification of atleast one female specific marker for each of the hybrids would facilitate distinction of these hybrids among themselves as well as determination of their genetic purity. However, no single primer was found which could generate female parent specific markers for all three hybrids along with male parent specific marker. Two primers were found which could generate marker specific to two of the three female parents along with male parent specific marker. Thus with the help of these two primers, KBSH-41, KBSH-42 and KBSH-44 could be distinguished from each other as well as the genetic purity of these hybrids could be assessed. Similar kinds of

results are reported in *Cichorium intybus* L., where RAPD markers were used for purity testing as well as for distinguishing hybrids (Bellamy *et al*., 1996).

In case of varieties, attempts were made to establish genetic fingerprint of twelve sunflower varieties using RAPD markers. All twelve varieties were screened using seventy RAPD primers to establish unique fingerprint or molecular ID for each variety. Varietal specific molecular IDs were identified for two varieties CO-2 and EC-68415. These results are in agreement with that obtained in sunflower (Lawson et al., 1994) and in *Cichorium intybus* L (Bellamy *et al*., 1990) where utility of RAPD markers for fingerprinting varieties were demonstrated.

Reproducibility of RAPD profile has been a subject of discussion. Since the introduction of this technique. Prerequisite for the use of RAPDs as a routine method for genetic purity test in seed industry at commercial scale are efficiency, reproducibility and reliability. In the present study reproducibility of identified RAPD markers was checked and most of them were found to be reproducible. Similar kind of results was observed in *Cichorium intybus* L (Bellamy *et al*., 1990) where reproducibility of RAPD markers was studied and majority of decamer arbitrary primers used in PCR reaction gave reproducible pattern. It has been opined that the strictly following standardized thermal profile and other parameters of RAPD assay, would assure satisfactory reproducibility (Rafalski, 1997). So, identified RAPD markers in the present study could be used for genetic purity testing of respective hybrids and varieties in seed industry.

Reproducibility of RAPD patterns with change in laboratory conditions was questioned in many studies (Jones *et al.*, 1998). Hence for practical purpose and for assurance of sure reproducibility, the RAPD markers identified for genetic purity testing

could be converted easily into more reproducible PCR-type assay based on secondary DNA sequence information, by the use of allele specific PCR (AS-PCR) or a sequence-characterized amplified region (SCAR) assay (Paran and Michelmore, 1993).

5.3 Genetic purity assessment using SSR markers

SSR markers were also used for genetic purity testing of sunflower hybrids and varieties. Universal acceptance of SSR markers in genotype specific fingerprinting and diversity analysis provides an opportunity for their deployment in genetic purity testing of commercial cultivars. Since hybrids are obtained by crossing diverse homozygous parents, they are heterozygous in genetic constitution. Genetic purity of hybrids could be determined using SSR primers, if polymorphism is present for the microsatellite loci between CMS (female parent) and restorer line (male parent). Since SSRs are codominant in nature, hybrid would express both alleles of that locus specific to parents using the same SSR primer pair.

Length polymorphism in the SSR is created when PCR product from different individuals vary in length as a result of variation in the number of repeat units which could be from single to few bases (Cregan *et al.,* 1997). Such differences can be resolved by using polyacrylamide gel electrophoresis (PAGE). PAGE is relatively difficult, costly and requires more skill. Markers identified from genetic purity assay would be deployed in seed industry for genetic purity testing of commercial cultivars on a large scale, so there is need for a method which is relatively simple, fast, cost effective and requires minimum technical skills. Considering this point, in the present study, agarose gel electrophoresis was used rather than PAGE for running PCR products of SSR analysis.

Twenty-two SSR primers were used in genetic purity study of twelve hybrids. Polymorphism was observed between CMS and restorer parents of seven hybrids using different SSR primer pairs. Thus SSR markers were identified for seven hybrids (KBSH-1, KBSH-41, TCSH-1, LSH-1, PSFH-118, SCH-35 and DSH-1) for genetic purity testing. Results obtained in the present study are similar to that obtained by Yashitola *et al.,* 2002 in rice where SSR markers were used for genetic purity testing of hybrid rice in order to replace GOT.

Using SSR analysis genetic purity of varieties could be assessed by identifying distinct microsatellite allele. Twenty-two SSR primers were used for establishing fingerprints for twelve sunflower varieties. Fingerprints of two varieties PKVSF-9 and EC-68415 were identified. SSR markers revealed by primer pair ORS-134 as fingerprints of the varieties were interesting since they showed higher molecular size than expected which could be attributed to duplication of microsatellite's loci within the flanking regions that might have led to amplification of larger fragment. SSR primers revealed less polymorphism among the varieties, which might be due to use of agarose gel which could detect differences of only bigger size and small differences might have gone undetected. Similar results were also observed in apricot (Romero *et al.,* 2003) where limited polymorphism revealed by SSR was attributed to use of agarose gels for separation of amplified products.

Multiple banding patterns were noticed in some varieties or parental lines of hybrids. This kind of amplification profile can be explained by atleast three main reasons (1) heterozygosity or heterogeneity of parental lines and varieties, indicating that they are derived from heterogeneous cultivar selections. These bands might be corresponding to

the microsatellite alleles belonging to the same locus, reflecting that segregation of the loci present in those inbreds and varieties. (2) Replication slippage during amplification process which could lead to the presence of less intense bands of usually one to five repeat unit smaller (and occasionally, greater) than the actual allele which was described by Claudio et al., 1998. (3) The other reason for such amplification pattern has been explained in other species by the occurrence of duplication process within the genome and evolution of families of respective sequences (Akagi *et al.,* 1998). Similar results were reported in sunflower by Paniego et al. (2002).. If this were to be the case in the observed results of the present study, the usefulness of multilocus microsatellite for fingerprinting and calculation of genetic diversity would be the same as those microsatellites revealing just a single locus, because the two (or more) loci are genetically linked, thus representing the same genomic region.

Results from the present study demonstrated that RAPD and SSR provide simple and reliable methods for genetic purity assessment of sunflower hybrids and varieties.

In India, the seed companies contract hybrid seed production to farmers and the produce from the single farmer is being considered as one seed lot for purity purposes. A sample of 400 seeds is collected randomly from each seed lot for conducting GOT (Verma, 1996). A similar sample size can be used for estimating genetic purity of seeds by the DNA assay. It has been estimated in a study (Yashitola *et al.,* 2002) that three technicians working together could complete the assaying of 400 seedlings in approximately 30 work hours. By deploying suitable number of personnel and equipment, it should be possible to complete the DNA test within 15 to 20 days from the time of harvest, so that the seed can be marketed for commercial cultivation in immediate

season. This will result in considerable saving for seed industry, especially in India, where large famounts of capital are locked up in the form of stored seed. The cost of storage for a whole season and cost of acquiring land and growing the crop for the GOT also can be avoided. Besides these, the RAPD and SSR analysis would be much more accurate for assessing genetic purity of cultivars than morphological characteristics as they would be directly assessing the genotype rather than the phenotype.

In varietal identification for attainment of PBR (Plant Breeder's Right), if varietal profiles using molecular data *per se* were to be used for DUS then it would be likely that the genetic control would have to be established, even though this is not a prerequisite for morphological characters. Also, use in DUS would require international standardization in methodologies. These criteria would be challenging to achieve for most arbitrarily primed PCR methods owing to their poor reproducibility. Profiling procedures, which are more sequence specific, such as SSR, may be more suitable to evaluate distinctness (Stephen Smith, 1997). SSRs were found to be more useful in other crops (Rongwen *et al.,* 1995) where they revealed extensive diversity among elite varieties, diversity that has been recalcitrant using other methods. Indeed, the Plant Variety Protection Office of USDA marketing service now accepts microsatellite allele profiles as a supporting evidence for the uniqueness of the new cultivars. Varietal specific IDs obtained in present investigation using SSR analysis could be used for DUS test. Varietal specific IDs obtained using RAPD analysis if converted to SCAR markers could satisfy the principles of distinctness, uniformity, and stability, since it is sequence specific profiling procedure and is considered more reliable and reproducible.

5.4 Genetic diversity assessment

It is generally assumed that genetic diversity among sunflower cultivars is limited because of its extensive breeding as well the fact that the crop was introduced in most of the important producing areas worldwide (Eastern and Western Europe, Argentina, Indonesia, India, and North America). In India most of the cultivars are developed from Russian introductions (Paniego *et al.,* 2002) The narrow genetic base of cultivars has created genetic vulnerability to diseases, insect pests or some other stress due to a similarity in their genotypes (Singh, 1993).

The extent of genetic diversity in the germplasm can be estimated by adopting various methods like morphological, biochemical and/or molecular analyses. Since morphological analyses are generally unreliable and time consuming, isozymes, which are basically biochemical markers are being used widely for characterization of cultivated varieties and their wild relatives in other crops (Glaszmann, 1985).

Though morphological and isozyme markers have been employed in assessing the genetic diversity of a species, the accuracy of the assessment is questionable. The availability of low number of morphological and biochemical markers, their poor or unknown genetic control, environmental influence on the phenotypic expression, stage specific expression and procedural difficulties are known impediments in using these as genetic markers in genetic diversity analysis (Ainsworth and Sharp, 1989; Aldrich *et al.*, 1992).

The above said problems associated with morphological and isozyme markers have necessitated the development of alternative tools. The recent developments in molecular biology have resulted in development of simple, easily assayable PCR based DNA markers.

DNA markers such as microsatellites and RAPD have been used for assessment of genetic diversity due to their relative simplicity and inexpensive assay (Gupta and Varshney, 2000; Tatineni *et al.,* 1996).

Assessment of genetic diversity using different approaches among commercial cultivars of sunflower may lead to better understanding of the pattern of genetic diversity that exists among sunflower cultivars.

In the present investigation, genetic diversity among public sector sunflower hybrids and varieties was assessed using conventional method (using morphological characters), RAPD and SSR analysis independently.

Several measures of genetic distance have been proposed so far, among which Mahalanobis D^2 statistic is most widely used in plant breeding. In the present study also, Mahalanobis D^2 analysis was used to measure genetic distance between the genotypes using morphological characters. Mahalanobis D^2 considers the variations produced by characters and consequent effect that it has on the other characters. The statistical tool has been widely employed to resolve genetic divergence at intervarietal and subspecies level in classifying crop plants (FAO. 1997). This is possible by clustering the entries based on the D^2 value as it represents the index of the genetic diversity among genotypes and clusters.

Based on the D^2 analysis, 12 sunflower hybrids and 12 varieties were grouped into four clusters independently using Euclidean cluster analysis. The magnitude of D^2 suggested that there was limited amount of diversity among the hybrids and varieties of sunflower. This may be due to relatively limited gene exchange or selection practice among the parental lines / population which are used for the development of these hybrids and varieties. Cluster II (2468.6) in hybrids and cluster II and III (0.00) in varieties displayed least intracluster divergence showing the similarity of hybrids. While, in varieties the least score zero was due to the presence of only one genotype in each of the clusters II and III.

Highest inter cluster distance in hybrids was observed between cluster II and III (10218.79) and II and III in varieties (9359.52). This could be due to use of diverse parents or populations involved in the development of these hybrids and varieties. Lowest intercluster distance was observed between I and IV (2368.19), I and II (4110.39) in hybrids and varieties respectively. This could be due to use of relatively less diverse parents and populations for development of hybrids and varieties.

For assessment of genetic diversity using RAPD and SSR markers Jackards IJ similarity coefficient was followed. UPGMA analysis of the RAPD was done using Jackard similarity coefficient. Dendrograms show low genetic diversity in sunflower commercial cultivars.

Dendrogram of hybrids showed four major clusters with cluster I being the largest cluster consisting of six hybrids, of which four hybrids have the same CMS parent. Thus Similarity within this cluster could be due to the common female parent. Second cluster comprised of two hybrids HSFH-848 and LSH-1, which showed 74% similarity to cluster

I. Cluster III comprised only one hybrid RSFH-1. Cluster IV comprised three hybrids PSFH-118, DSH-1 and TCSH-1 which showed 68% similarity to cluster I, II and III. TCSH-1 was the only hybrid with male sterile parent CMS-234 A which was not grouped in Cluster I where rest of the hybrids with CMS–234 A male sterile parents were grouped. This could be due to high divergence of restorer parent RHA-272 of TCSH-1. Dendrogram of varieties showed five clusters. Five varieties, Surya, Gujarat Sunflower-1, SS-56, C0-2 and LS-11 fell into cluster I, which showed 89% similarity to Cluster II. Cluster II comprised CO-2 and CO-3. Cluster III included only one variety PKVSF-9 which shows 87% similarity to cluster IV and V. Cluster IV comprised variety Morden (Tamilnadu), dwarf Morden and EC-68415. Cluster V consisted of only one variety Morden (Akola). Dendrogram formed using RAPD data for varieties presented a different picture regarding varieties. Morden from two different sources (from Tamilnadu and Akola) were found to be different, which was contrary to the results of dendrogram formed using observations on morphological characters. These results indicated that even phenotypically similar genotypes could be distinguished based on molecular markers. Minor changes that occur at molecular level might not be detected at morphological level and such changes may not have detectable effect on the expression of complex traits like yield. However such small changes could be detected with the help DNA markers, which reveal variations at DNA level. Single base pair change in DNA may change the priming site of certain primer or create a new priming site for some other primer, which ultimately changes the banding patterns of two individuals of same genotype. Genetic contamination, which may not be detected with the help of morphological characters but detected by molecular markers, may also be a reason for the foresaid problem. Similar

results have been reported in sunflower where variability was detected within the open pollinated variety (sunfola) (Lawson *et al.,* 1994). This indicates that RAPD analysis may be suitable for detecting intra varietal differences.

Dendrograms were generated using 69 bands in hybrids generated by 22 SSR primers and 58 bands in varieties generated by 21 SSR primers. Mean genetic similarity of 0.75 (range 0.5 to 1.00) and 0.82 (0.65 to1.00) was observed for hybrids and varieties respectively. Dendrogram of hybrids showed three clusters. All the hybrids with CMS-234 A as female parent were grouped into Cluster I. Cluster II consisting of three hybrids KBSH-44, LSH-1, and HSFH-848, showed 69% similarity to Cluster III which included DSH-1, RSFH-1, PSFH-118 and KBSH-42. Dendrogram of varieties showed four clusters. Cluster I was the largest cluster with eight varieties and it showed 75% similarity to Cluster II. Eight varieties in cluster I were Gujarat sunflower-1, SS-56, C0-2 LS-11 CO-4, CO-3, EC-68415 and Morden (TN). Cluster II contained only one variety Surya. Variety Morden and Dwarf Morden were grouped into Cluster III. Cluster IV contained only one variety PKVSF-9, which is in agreement with the dendrogram constructed using RAPD data of varieties. In both dendrograms it was grouped into separate cluster, which indicated that PKVSF-9 is diverse from rest of the varieties.

In the present study, it was observed that, few genotypes, which were found to be more similar and included in one cluster using one approach of studying diversity, were grouped with some other genotypes using the other approach. This could be due to the reason that different approaches target different portions of the genome. Morphological characters result from expression of many genes. Hence studying morphological characters is studying of a portion of the genome, which code for one or the other

characters. RAPD is based on the use of single random decamer primer, which amplifies a few segments of genome between sequences that are complementary to the primer used, are on opposite strands and sufficiently close together for amplification to work. Thus, in RAPD, amplicons are obtained from both coding and non-coding regions of the genome where the primer could find its complementary sequences for binding. SSRs amplify only the non-coding microsatellite DNA that has not been implicated in any biological process and the functioning of microsatellite is still not established. Thus variations for microsatellite loci do not reflect any changes in the genome which might have functionality and thereby these loci may not reflect the useful variability which a breeder looks for, in any crop improvement programme.

Results similar to the present study was reported in cotton by Tatineni *et al.,* (1996), where morphological characters and RAPD analysis were used for studying diversity in elite cotton germplasm. A certain genotype, which was placed in the 'hirsutum' (*Gossypium hirsutum*) cluster based on the morphological data, was within 'barbadense' (*G. barbadense*) cluster when RAPD data were considered.

From the present study, it was concluded that overall genetic diversity observed among sunflower cultivars was low, which confirmed the general assumption of narrow genetic diversity of the commercial cultivars of sunflower (Paniego *et al.,* 2002). The reason for this may be the origin of cultivated sunflower from small number of ancestral germplasm sources (Vranceanu, 1985; Korell *et al.*, 1992). Again less diversity among the cultivars may be due to its extensive breeding in sunflower as well the fact that the crop is an introduced in India and most of the important sunflower producing areas worldwide (Paniego *et al.,* 2002).

The limited genetic diversity among sunflower cultivars makes them vulnerable to biotic and abiotic stresses. This risk could be reduced by introgressing genes for resistance to pests and diseases and for desirable agronomic characteristics from wild species into the cultivated species.

This study has clearly shown that molecular markers could effectively be used to test the genetic purity of sunflower cultivars, and thus they could be effectively used in place of the conventional GOT if the technology is proven to be cost effective.

CHAPTER VI

SUMMARY

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SUMMARY

The present investigation was undertaken with the objective to establish molecular markers (RAPD and SSR) for genetic purity assessment and to study diversity among hybrids and varieties using morphological characters, RAPD and SSR markers. Genetic purity of hybrids and varieties is one of the crucial aspects for exploitation of experimentally demonstrated yield potential of high yielding cultivars. Assessments of genetic purity of cultivars through conventional GOT and biochemical approaches have many limitations owing to their limited polymorphism and environmental influence etc. The limitations of these methods can be alleviated effectively by employing DNA based markers.

All cultivated oilseed sunflower planted around the world has been derived from a limited genetic base, which make the crop vulnerable to the disease and insect pests that can exploit this genetic weakness. It is imperative to study diversity among sunflower cultivars. One of the goals of the study was to assess diversity among hybrids and varieties sunflower using morphological characters, RAPD and SSR markers.

Experimental plant material for present study comprises thirteen hybrids and thirteen varieties of sunflower. A set of ten morphological characters specified in the published descriptors of sunflower was used to select genetically pure genotypes, which were used for development of molecular markers (RAPD and SSR) and to study diversity among selected hybrids and varieties using morphological characters, RAPD and SSR analysis. Out of 13 hybrids and 13 varieties, one hybrid (NDSH-1) and one variety (CO-

1) were non-uniform and genetically impure according to morphological markers and therefore not included in further study.

RAPD markers were identified for testing genetic purity for all twelve hybrids (BSH-1, KBSH-1, KBSH-41, KBSH-42, KBSH-44, HSFH-848, LSH-1, SCH-35, PSFH-118, DSH-1, RSFH-1 and TCSH-1) included in the study. Unique fingerprints (IDs) were identified for two varieties (CO-2 and EC-68415) using RAPD primers. SSR markers were identified for seven hybrids (KBSH-1, KBSH-41, PSFH-118, LSH-1, SCH-35, DSH-1 and TCSH-1) and two varieties (PKVSF-9 and EC-68415).

Genetic diversity among 12 hybrids and 12 varieties was investigated at DNA level with RAPD and SSR procedures and at the phenotypic level with eight morphological characters. Eighty random decamer primers were used to amplify DNA via the polymerase chain reaction (PCR); 315 and 335 RAPDs were generated for hybrids and varieties respectively. Twenty-two SSR primer pairs were used for amplification of SSRs via the PCR; 69 and 58 SSRs were generated for hybrids and varieties respectively. Dendrograms were generated for genetic similarity from morphological data, from RAPDs and SSRs. Dendrograms generated from morphological data on hybrids and varieties produced four clusters for hybrids and four for varieties. Dendrograms generated from RAPDs generated four and five clusters respectively of hybrids and varieties. Dendrograms produced from SSRs generated three and four clusters respectively of hybrids and varieties. Classifications of all the genotypes using three methods were quite different.

This experiment demonstrates that RAPD and SSR markers could be effectively used for assessment of genetic purity of hybrids and varieties of sunflower. The use of

RAPD and SSR markers provides a simple, efficient and cost effective methods for studying diversity in sunflower.

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The pattern of "Literature cited" presented above is in accordance with "Guidelines" for thesis presentation for Acharya N. G. Ranga Agricultural University, Hyderabad.

APPENDICES

Appendix 1. List of RAPD primers used in the study

Serial No.	Primer	Sequence 5'⟶ 3'
1.	OPC-01	**TTCGAGCCAG**
2.	OPC-02	**GTGAGGCGTC**
3.	OPC-04	**CCGCATCTAC**
4.	OPC-07	**GTCCCGACGA**
5.	OPC-10	**TGTCTGGGTG**
6.	OPC-11	**AAAGCTGCGG**
7.	OPC-12	**TGTCATCCCC**
8.	OPE-08	**TCACCACGGT**
9.	OPE-14	**TGCGGCTGAG**
10.	OPF-09	**CCAAGCTTCC**
11.	OPF-10	**GGAAGCTTGG**
12.	OPG-03	**GAGCCCTCCA**
13.	OPG-04	**AGCGTGTCTG**
14.	OPG-08	**TCACGTCCAC**
15.	OPG-11	**TGCCCGTCGT**
16.	OPG-12	**CAGCTCACGA**
17.	OPG-13	**CTCTCCGCCA**
18.	OPG-16	**AGCGTCCTCC**
19.	OPG-17	**ACGACCGACA**
20.	OPH-02	**TCGGACGTGA**
21.	OPH-05	**AGTCGTCCCC**
22.	OPH-07	**CTGCATCGTG**
23.	OPH-12	**ACGCGCATGT**
24.	OPI-11	**ACATGCCGTG**
25.	OPI-16	**TCTCCGCCCT**
26.	OPI-18	**TGCCCAGCCT**
27.	OPJ-01	**CCCGGCATAA**
28.	OPJ-04	**CCGAACACGG**
29.	OPJ-05	**CTCCATGGGG**
30.	OPJ-07	**CCTCTCGACA**
31.	OPJ-09	**TGAGCCTCAC**
32.	OPJ-10	**AAGCCCGAGG**
33.	OPJ-11	**ACTCCTGCGA**
34.	OPJ-12	**GTCCCGTGGT**
35.	OPJ-13	**CCACACTACC**
36.	OPJ-14	**GACCCGGATG**
37.	OPJ-15	**TGTAGCAGGG**
38.	OPJ-16	**CTGCTTAGGG**
39.	OPJ-19	**GGACACCACT**
40.	OPJ-20	**AAGCGGCCTC**

Contd…

41.	OPK-02	**GTCTCCGCAA**
42.	OPK-03	**CCAGCTTAGG**
43.	OPK-04	**CCGCCCAAAC**
44.	OPK-05	**TCTGTCGAGG**
45.	OPK-06	**CACCTTTCCC**
46.	OPK-08	**GAACACTGGG**
47.	OPK-09	**CCCTACCGAC**
48.	OPK-10	**GTGCAACGTG**
49.	OPK-14	**CCCGCTACAC**
50.	OPK-15	**CTCCTGCCAA**
51.	OPK-12	**TGGCCCTCAC**
52.	OPK-19	**CACAGGCGGA**
53.	OPL-01	**GGCACGACCT**
54.	OPL-02	**TGGGCGTCAA**
55.	OPL-03	**CCAGCAGCTT**
56.	OPL-07	**AGGCGGGAAC**
57.	OPL-08	**AGCAGGTGGA**
58.	OPL-09	**TGCGAGAGTC**
59.	OPL-19	**GAGTGGTGAC**
60.	OPM-02	**ACAACGCCTC**
61.	OPM-04	**GGCGGTTGTC**
62.	OPM-05	**GGGAACGTGT**
63.	OPM-09	**GTCTTGCGGA**
64.	OPM-10	**TCTGGCGCAC**
65.	OPM-12	**GGGACGTTGG**
66.	OPM-15	**GACCTACCAC**
67.	OPM-16	**GTAACCAGCC**
68.	OPM-17	**TCAGTCCGGG**
69.	OPM-18	**CACCATCCGT**
70.	OPN-02	**ACCAGGGGCA**

Appendix 2. List of SSR markers used in the study

Marker name	Repeat motif and length	Foreword primer sequence	Reverse primer sequence	Reference allele length (bp)	Annealing temperature
ORS-3	$(ATG)_6$	AGA ACT GGC AGC TTG GAA AA	GTC CAA ATG GTG GAA AACTACC	225	52°C
ORS-7	$(TCA)_4$	CCC TCA CTA CCG TGT GGT G	TTG AAA GAG ACG AAG CGA AA	262	52°C
ORS-8	$(CT)_8$	TTG GAT CGATTGATGATT GTTG	GAA TCC GTC ATG TAT AAA ACG A	195	52°C
ORS-12	$(ATT)_7$	GCT TTG AGA AAC CGC TTC AC	TTGATATGGTAA CTA ACGAACACAA	138	52°C
ORS-31	$(AGG)_{10}$	AAT TCA TGC CCC AAG AGA TG	CAC AAT TCA TGC ATTTCTCTGG	286	52°C
ORS-64	$(T)_{17}$	CCG GTT CGG TTT TGT TCT AA	TTA AGT TAG GGC CGT TTA CCC	126	55°C
ORS-70	$(CTT)_9$	GAC CCT GGT CAC CGA AGT TA	ATC TGA AAT CGG ACA AGA TTC A	126	55°C
ORS-78	$(AAG)_{10}$	GTT CGTCGAGTACATGTT CTG C	TTT CCC TCT GGA AAG TTG TCA	161	55°C
ORS-90	$(AC)_8$	GGA TTT CGC GTG ATT GTT G	AGC AGT TAC GAG TGT GTG TGT G	125	57°C
ORS-124	$(AC)_{14}$	AATCGCCATACCACTCCATC	GATATCACCCCACGATAACATG	252	51°C
ORS-154	$(AT)_5(GT)_7N2(GT)_9$	GCACCTTTGGTGAGGAGATA	TGCATCAGTAGCTATTGTCTAT	203	53°C
ORS-169	$(CA)_8$ N34 $(CA)_8$	TGGAACTGTAAATGGACCCAAG	GCACTGCACCATTTATGAGAAG	198	55°C
ORS-235	$(CAA)_9$	AAGCAACTGCCGCTCCCAC	AGCGACAGCTGTGACAATGC	396	54°C
ORS-237	$(GTT)_{22}$	CAAGGTCTGTCTACATCCCACC	GCTGTAAAGCCTGCATATCCTC	192	53°C
ORS-257	$(TGTA)_{62}$	GTGACTACGTTATGGATGCATG	GCCTTTGCTTGCATATCTACG	650	53°C
ORS-258	$(CT)_4N52$ $(CA)_4$	GGCCCGATTACAAGATAACG	TTGCGTCCGATGCTGTTC	332	53°C
ORS-317	$(AC)_{11}(AT)_8$ $(AG)_{16}$	TTTGGCAGTTTGGTGGCTTA	GGTCGTATGCTTAATTCTTTCTCT	204	58°C
ORS-323	$(GT)_{13}$	CGGGAAACTAGGATCAGAGG	GCCGGAGGATTAGAGGAGTT	405	58°C
ORS-328	$(ACAAC)_{34}$	GACCTGTAGGCCAATATGAGACTT	TTATACCGGTGTTGTATCGTATCC	271	58°C
ORS-342	$(GT)_{10}$	TGTTCATCAGGTTTGTCTCCA	CACCAGCATAGCCATTCAAA	342	58°C
ORS-343	$(AC)_8$	TCCACAAGGACCAACTACGA	TCCAACTGCACACACTCGAT	242	58°C
ORS-344	$(AC)_{13}$	TCCGTATATTGCCTTGTTGAGA	ATTTGCGCCTATCATTTGCT	220	58°C

Tables

Table 2.1 RAPD markers in assessment of genetic purity of hybrids and varieties

Scientist	**Year**	**Crop**
Boury *et al*.	1992	Grapevine
Calo *et al*.	1995	Grapevine
Bellamy *et al*.	1996	Grapevine
Manfredi *et al*.	1996	Apple
Quian *et al*.	1996	Rice
Zhang-Jian Hua *et al*.	1996	Maize and Soybean
Grzebelus *et al*.	1997	Corrot
Zhang-Jian Hua *et al*.	1997	Petunia and Cycleman
Meng-Xiang Dong *et al*.	1999	Chinese cabbage
Chuang-SuJean *et al*.	1999	Grapevine
Liu-Jie *et al*.	2000	Rape (*Brassica napus)*
Song-ShunHua *et al*.	2000	Chinese cabbage

Table 3.1 Details of sunflower hybrids used in the study

Sl.No.	Name of hybrid	CMS 'A' line	Restorer 'R' line	Place of origin
1	BSH-1	CMS-234A	RHA-274	Bangalore
2	KBSH-1	CMS-234A	RHA-6-D-1	Bangalore
3	KBSH-41	CMS-234A	RHA-95-C-1	Bangalore
4	KBSH-42	CMS-851A	RHA-95-C-1	Bangalore
5	KBSH-44	CMS-17A	RHA-95-C-1	Bangalore
6	HSFH-848	CMSH-91A	RLA-298	Hissar
7	LSH-1	CMS-338A	MRHA-2	Latur
8	SCH-35	CMS-234A	RHA-1-1	Latur
9	PSFH-118	CMS-10A	P61R	Ludhiana
10	DSH-1	CMSDSF-15A	RHA-857	Dharwad
11	RSFH-1	CMS-103A	R64NB	Raichur
12	TCSH-1	CMS-234A	RHA-272	Tamilnadu
13	NDSH-1	CMS-234A	RHA-859	Nandyal

Table 3.2 **Details of sunflower varieties used in the study**

Sr. No.	**Name of variety**	**Place of origin**
1	Surya	Akola, Maharashtra
2	PKVSF-9	Akola, Maharashtra
3	Gujarat sunflower-1	Gujarat
4	LS-11	Latur, Maharashtra
5	SS-56	Solapur, Maharashtra
6	CO-1	Coimbatore, Tamilnadu
7	CO-2	Coimbatore, Tamilnadu
8	CO-3	Coimbatore, Tamilnadu
9	CO-4	Coimbatore,Tammilnadu
10	EC-68415	Akola, Maharashtra
11	Morden (Tamilnadu)	Coimbatore,Tammilnadu
12	Morden (Akola)	Akola, Maharashtra
13	Dwarf Morden	Raichur, Karnataka

Table 4.1 Observations recorded on morphological characters for genetic purity assessment of sunflower hybrids

Sr. No	Hybrids	Leaf size	Leaf Shape	No. of leaves on main stem	Time of flowering (50%) (days)	Ray flower colour	Head diameter (cm)	Plant height (cm)	Seed Weight (gm)	Seed stripe	Seed mottling
1	BSH-1	Medium	Rounded	High (26.6)	Early (49.0)	Yellow	Small (14.8)	Medium (140.3)	Medium (5.3)	Absent	Absent
2	KBSH-1	Medium	Cordate	High (28.0)	Medium (53.0)	Yellow	Medium (17.6)	Tall (164.4)	Medium (5.2)	Present	Absent
3	KBSH-41	Large	Cordate	High (31.4)	Medium (53.3)	Yellow	Medium (17.7)	Very Tall (176.9)	Medium (5.5)	Present	Absent
4	KBSH-42	Large	Cordate	High (31.1)	Medium (52.6)	Yellow	Medium (18.4)	Tall (162.96)	Low (4.3)	Present	Absent
5	KBSH-44	Large	Cordate	High (30.5)	Late (58.6)	Yellow	Medium (19.2)	Very Tall (176.6)	Medium (4.9)	Present	Present
6	HSFH-848	Large	Cordate	Medium (23.3)	Early (50.3)	Yellow	Small (14.6)	Medium (115.2)	Medium (4.6)	Absent	Absent
7	LSH-1	Medium	Cordate	Medium (22.6)	Early (48.0)	Yellow	Small (14.2)	Medium (114.3)	Medium (4.8)	Absent	Absent
8	SCH-35	Large	Cordate	High (26.8)	Medium (52.3)	Yellow	Small (14.7)	Medium (135.6)	Medium (5.8)	Absent	Absent
9	PSFH-118	Large	Cordate	High (24.0)	Medium (53.6)	Yellow	Small (14.9)	Medium (139.3)	High (5.8)	Absent	Absent

10	DSH-1	Medium	Cordate	High (28.7)	Early (48.0)	Yellow	Medium (15.2)	Medium (121.4)	Medium (5.4)	Present	Absent
11	RSFH-1	Large	Cordate	High (32.2)	Late (59.6)	Yellow	Medium (18.0)	Tall (147.8)	Medium (5.4)	Absent	Absent
12	TCSH-1	Medium	Cordate	High (30.8)	Medium (51.9)	Yellow	Medium (17.2)	Tall (169.8)	Medium (4.9)	Present	Absents

Table 4.2 Observations recorded on morphological characters for genetic purity assessment of sunflower varities

	Variety	Leaf size	Leaf Shape	No. of leaves on main stem	Time of flowering (50%) (days)	Ray flower colour	Head diameter (cm)	Plant height (cm)	Seed Weight (gm)	Seed stripe	Seed mottling
1	Surya	Medium	Cordate	High (26.0)	Medium (52.0)	Yellow	Medium (17.8)	Tall (143.4)	Medium (4.7)	Present	Absent
2	PKVSF-9	Large	Cordate	Medium (23.6)	Medium (51.5)	Yellow	Medium (16.3)	Medium (118.2)	Medium (4.3)	Present	Absent
3	Gujsun-1	Large	Cordate	High (27.3)	Medium (56.3)	Yellow	Medium (16.0)	Tall (160.8)	Medium (4.3)	Absent	Absent
4	LS-11	Medium	Cordate	Medium (22.1)	Early (48.0)	Yellow	Small (13.2)	Short (109.4)	Medium (4.6)	Absent	Absent
5	SS-56	Large	Cordate	Medium (23.0)	Medium (54.3)	Yellow	Medium (17.2)	Tall (161.3)	Medium (4.1)	Present	Absent
6	CO-2	Large	Cordate	High (25.6)	Medium (57.3)	Yellow	Medium (16.4)	Medium (128.3)	Medium (5.2)	Present	Absent
7	CO-3	Large	Cordate	High (27.0)	Late (58.6)	Yellow	Medium (15.9	Tall (160.8)	Medium (4.2)	Present	Absent
8	CO-4	Large	Cordate	High (26.0)	Late (59.0)	Yellow	Medium (17.8)	Tall (156.3)	Medium (4.4)	Present	Absent
9	EC-68415	Large	Cordate	High (27.7)	Medium (58.6)	Yellow	Medium (18.9)	Tall (152.4)	Medium (4.8)	Absent	Absent
10	Morden (TN)	Large	Cordate	Medium (24.8)	Early (48.0)	Yellow	Medium (16.3)	Short (90.3)	High (5.1)	Absent	Absent
11	Morden (AK)	Large	Cordate	Medium (24.4)	Early (48.0)	Yellow	Medium (16.2)	Short (84.5)	Medium (4.9)	Absent	Absent
12	Dwarf Morden	Large	Cordate	Medium (21.8)	Early (49.2)	Yellow	Medium (16.4)	Short (83.5)	High (5.1)	Absent	Absent

Table 4.3 RAPD markers identified for purity assessment of hybrids

Hybrid	Primer	Female specific marker (kb)	Male specific marker (kb)
BSH-1	OPJ-1	--	1.1
	OPJ-14	--	0.9
KBSH-1	OPM-2	--	0.85
	OPJ-14	--	0.9
	OPG-11		1.0
KBSH-41	OPK-15	0.5	0.9
	OPJ-16	--	1.1
	OPJ-1	--	2.0
	OPG-11	--	1.0
KBSH-42	OPK-15	1.8	0.9
	OPJ-16	1.2	1.1
	OPK-14	0.5	0.7
	OPJ-1	--	2.0
	OPG-11	--	1.0
KBSH-44	OPK-15	--	0.9
	OPJ-16	1.0	1.1
	OPJ-1	--	2.0
	OPG-11	--	1.0
HSFH-848	OPK-9	--	1.3
	OPM-4	--	1.4
	OPK-3	--	0.7
LSH-1	OPJ-14	--	0.8
	OPK-8	--	0.8,0.5
SCH-35	OPJ-1	--	1.1
	OPG-11	--	1.4
PSFH-118	OPI-11	--	1.2
	OPE-14	--	1.0
DSH-1	OPI-16	0.8, 0.7	1.2, 0.4
	OPJ-1	--	1.2
RSFH-1	OPK-3	--	0.8
	OPJ-7	1.1	0.9
TCSH-1	OPJ-14	--	0.9
	OPH-2	--	2.0

Table 4.4a Variety specific molecular IDs using RAPD markers

Varieties	Primer	Marker size (kb)
CO-2	OPC-2	1.4 kb
EC-68415	OPE-14	1.7 KB

Table 4.4b Differentiation of sunflower varieties using RAPD primers

Varieties	Primers
PKVSF-9	OPJ-5 OPK-9
LS-11	OPC-4
SS-56	OPC-4
CO-3	OPE-14
Morden (Coimbatore)	OPJ-9
Morden (Akola)	OPJ-7
Dwarf Morden	OPJ-7
Surya	OPE-14 and OPJ-11
Gujarat Sunflower-1	OPE-14 and OPK-15
CO-4	OPE-14, OPK-15 and OPC-4

Table 4.5: SSR markers identified for genetic purity assessment of sunflower hybrids

Sr. No.	Primers	Hybrids	Female parent specific marker (size in bp)	Male parent specific marker (size in bp)
1	ORS-317	KBSH-1	205	212
2	ORS-317	KBSH-41	205	215
3	ORS-317	TCSH-1	205	213
4	ORS-317	LSH-1	220	210
5	ORS-317	PSFH-118	200	212
6	ORS-237	PSFH-118	185	195
7	ORS-154	SCH-35	210, 190	305,210,190
8	ORS-342	DSH-1	342	335

Table 4.6 Mean performance of hybrids for different character

Hybrid	Days to 50 % flowering	Days to maturity	Plant height (Cm)	Head diameter (Cm)	No. of leaves/ plant	Seed yield /plant (g)	100 seed weight (g)	Oil content (%)
BSH-1	49.0	90.2	140.3	14.8	26.6	54.0	5.3	37.7
KBSH-1	53.0	96.3	164.4	17.6	28.0	66.0	5.2	40.5
KBSH-41	53.3	97.3	176.9	17.7	31.4	68.8	5.5	36.8
KBSH-42	52.6	96.3	162.9	18.4	31.1	62.4	4.3	37.2
KBSH-44	58.6	103.6	170.6	19.2	30.5	71.2	4.9	32.2
HSFH-848	50.3	91.6	115.2	14.6	23.3	53.2	4.6	39.8
LSH-1	48.0	89.0	114.3	14.2	22.6	55.4	4.8	32.8
SCH-35	52.3	94.6	135.6	17.7	26.8	55.6	5.8	38.6
PSFH-118	53.6	92.3	139.3	14.9	24.0	61.2	5.8	36.7
DSH-1	48.0	91.0	121.4	15.2	28.7	58.6	5.4	37.7
RSFH-1	59.6	106.3	147.8	18.0	32.0	68.4	5.4	39.6
TCSH-1	51.9	94.0	169.8	17.2	30.8	58.7	4.9	36.5

Table 4.7. Mean performance of varieties for different characters

Varieties	Days to 50% flowering	Days to maturity	Plant height (cm)	Head diameter (cm)	No. of leaves per plant	Seed yield per plant (g)	100 seed weight (g)	Oil content (percent)
Surya	52.0	97.0	142.7	17.8	26.0	58.4	4.7	32.6
PKVSF-9	51.5	92.3	118.2	16.3	23.6	45.2	4.3	36.5
Gujarat sunflower-1	56.3	102.3	160.6	16.0	27.3	55.0	4.3	39.6
LS-11	48.0	91.0	109.4	13.2	22.1	43.5	4.6	34.3
SS-56	54.3	98.3	163.6	17.2	23.0	56.0	4.1	35.1
CO-2	57.3	98.0	127.4	16.4	25.6	60.0	5.2	32.9
CO-3	58.6	102.0	159.1	15.9	27.0	61.4	4.2	34.3
CO-4	59.0	104.3	156.1	17.8	26.0	63.4	4.4	35.9
EC-68415	58.6	105.3	204.5	18.8	27.7	65.6	4.8	39.5
Morden (TN)	48.0	91.0	89.7	16.3	24.8	57.1	5.1	36.4
Morden (AK)	48.0	90.0	84.5	16.2	24.4	53.4	4.9	36.2
Dwarf Morden	49.0	92.0	83.5	16.4	21.8	55.1	5.1	35.5

Table 4.8: Analysis of variance for eight characters in twelve sunflower hybrids

Characters	Mean sum of squares			General mean	SE Diff	SE Diff from mean	C.V.(%)	C.D. 5 %	C.D. 1 %
	Replication	Treatments	Error						
Days to 50 % flowering	0.03	331.81	0.35	52.5	0.48	0.33	1.05	0.99	1.35
Days to maturity	0.19	383.42	0.28	95.2	0.43	0.29	0.59	0.90	1.22
Plant height (cm)	3.08	1498.77	1.87	146.5	1.11	0.75	0.93	2.31	3.14
Head diameter (cm)	1.16	18.06	0.32	16.6	0.46	0.31	3.58	0.97	1.32
No of leaves Per plant	1.78	36.24	0.85	27.9	0.75	0.51	3.30	1.56	2.13
Seed yield Per plant	2.06	557.32	1.58	61.1	1.09	0.69	2.38	2.13	2.90
100 seed weight (gm)	0.06	2.14	0.01	5.1	0.09	0.06	2.22	0.19	0.26
Oil content (percent)	0.04	68.52	0.12	37.1	0.29	0.19	1.11	0.60	0.82

Table 4.9 : Analysis of variance for eight characters in twelve sunflower varieties

Characters	Mean sum of squares			**General mean**	**SE Diff**	**SE Diff from mean**	**C.V.(%)**	**C.D. 5 %**	**C.D. 1 %**
	Replication	**Treatments**	**Error**						
Days to 50 % flowering	**0.03**	**359.08**	**0.39**	**54.1**	**0.51**	**0.34**	**1.15**	**1.06**	**1.44**
Days to maturity	**0.25**	**346.31**	**0.58**	**97.5**	**0.62**	**0.42**	**0.76**	**1.29**	**1.75**
Plant height cm)	**1.19**	**2994.43**	**1.58**	**129.2**	**1.07**	**0.69**	**0.98**	**2.13**	**2.89**
Head diameter (cm)	**1.25**	**48.99**	**0.50**	**16.8**	**0.57**	**0.39**	**3.85**	**1.2**	**1.63**
No of leaves Per plant	**1.65**	**12.01**	**0.23**	**25.3**	**0.39**	**0.26**	**1.94**	**0.82**	**1.11**
Seed yield Per plant	**7.43**	**1037.69**	**1.49**	**59.4**	**0.99**	**0.67**	**2.02**	**2.06**	**2.81**
100 seed weight (gm)	**0.14**	**3.13**	**0.026**	**4.8**	**0.13**	**0.09**	**3.26**	**0.27**	**0.37**
Oil content (percent)	**0.35**	**24.32**	**0.22**	**35.8**	**0.38**	**0.26**	**1.43**	**0.80**	**1.10**

Table 4.10a Distribution of 12 Sunflower hybrids in different clusters

Cluster	No. of genotypes	Genotypes
I	3	BSH-1, SCH-35, PSFH-1
II	2	HSFH-848, LSH-1
II	3	KBSH-1, KBSH-42, DSH-1
IV	4	RSFH-1, TCSH-1, KBSH-41, KBSH-44

Table 4.10b Distribution of 12 Sunflower varieties in different clusters

Cluster	No. of genotypes	Genotypes
I	5	Surya, SS-56, CO-4, Gujarat sun-1, CO-2
II	1	EC-68415
II	1	CO-3
IV	5	PKVSF-9, LS-11, Morden (AK), Morden (TN), Dwarf Morden

Table 4.11a: Euclidean ^{2} cluster distances for hybrids

	Cluster I	Cluster II	Cluster III	Cluster IV
Cluster I	615.46	2902.35	5519.45	2368.11
Cluster II		1926.72	10218.79	7548.56
Cluster III			2468.60	2847.98
Cluster IV				1148.98

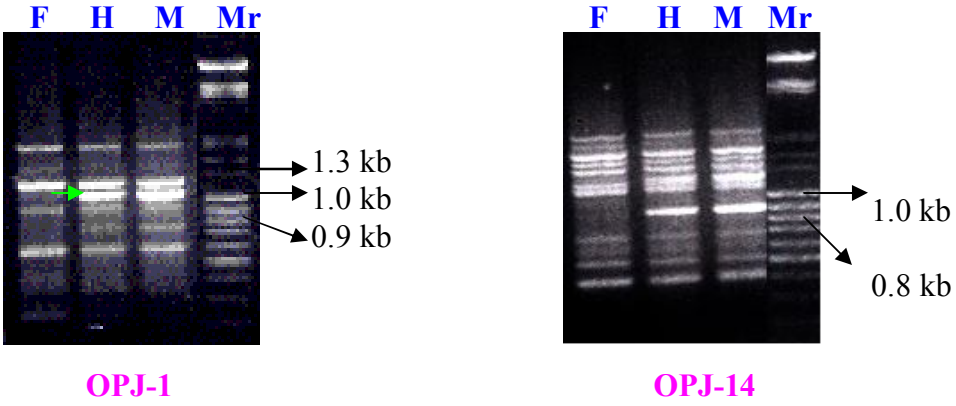
Table 4.11b Euclidean ^{2} cluster distances for varieties

	Cluster I	Cluster II	Cluster III	Cluster IV
Cluster I	1473.29	4110.39	5198.42	8815.15
Cluster II		0.00	9359.52	9309.85
Cluster III			0.00	8138.05
Cluster IV				1396.74

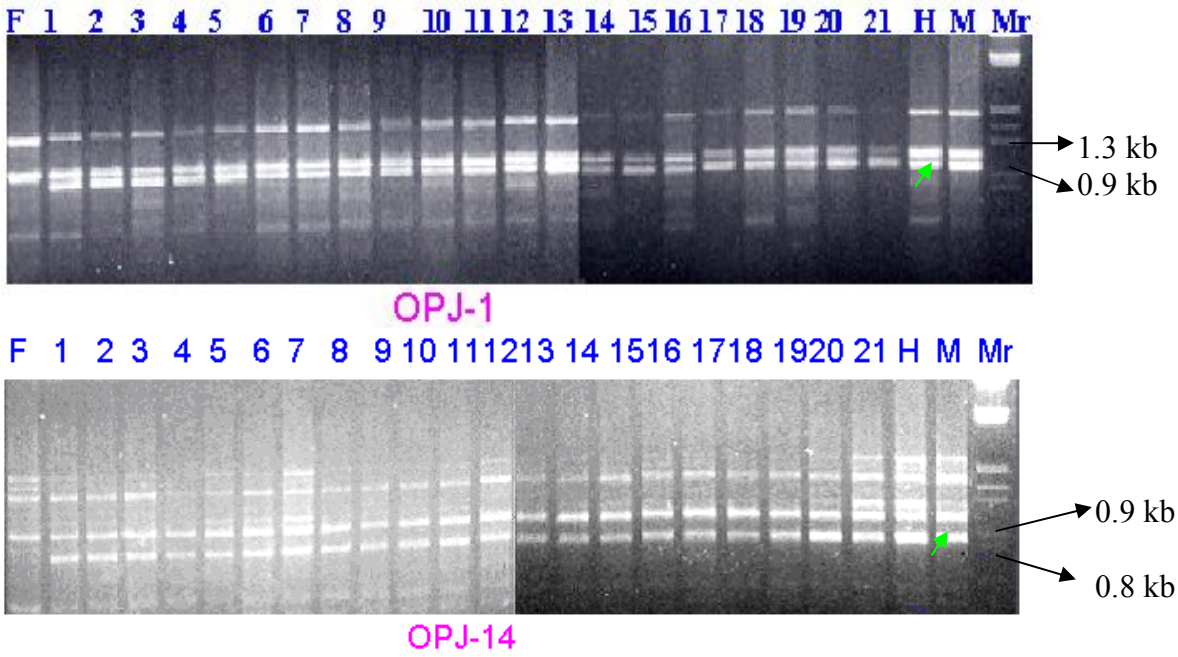
Plates

Plate 1. RAPD markers generated for hybrid BSH-1 using primer OPJ-1 and OPJ-14

A. RAPD with bulk DNA of BSH-1 and parents.



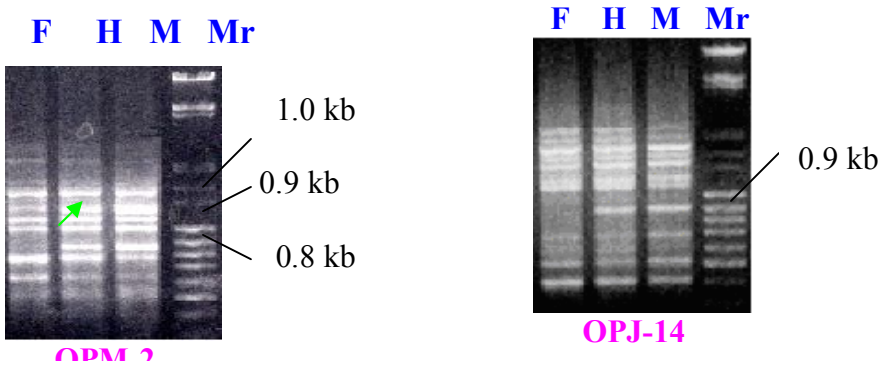
B. RAPD with individual hybrid plants of BSH1



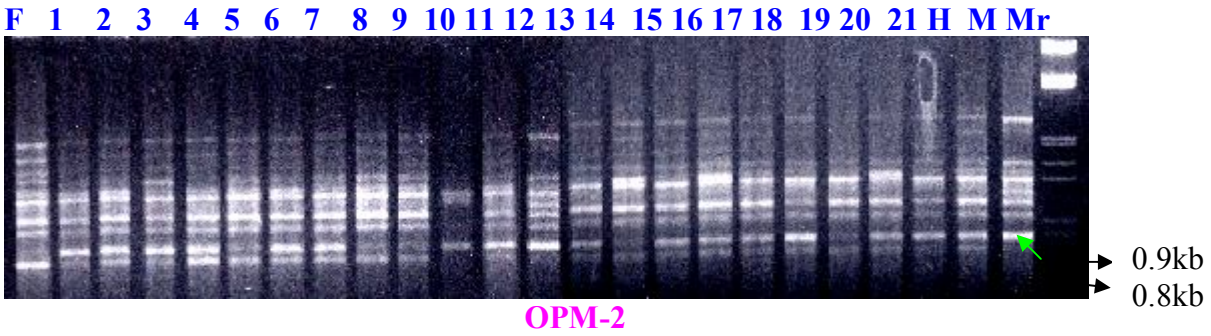
F-Female bulk
H-Hybrid bulk
M- Male bulk
1-21- Individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band

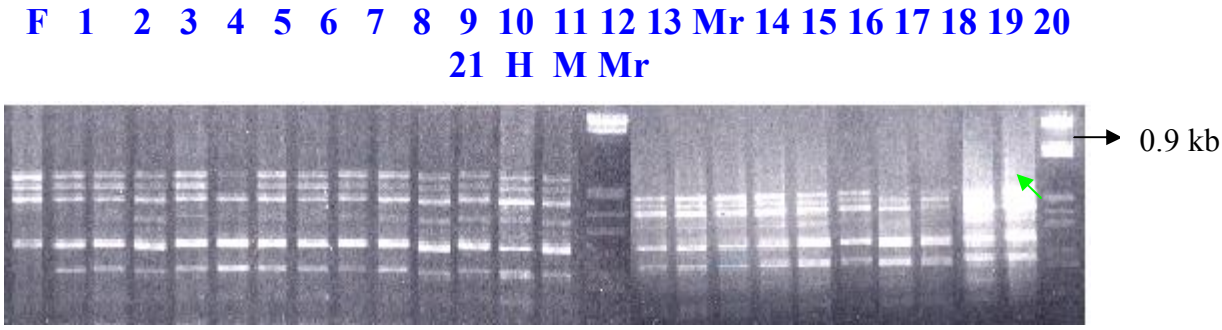
Plate 2. RAPD markers generated for hybrid KBSH-1 using primer OPM-2 and OPJ-14

A. RAPD with bulk DNA of KBSH-1 and parents.



B. RAPD with individual plants of KBSH-1

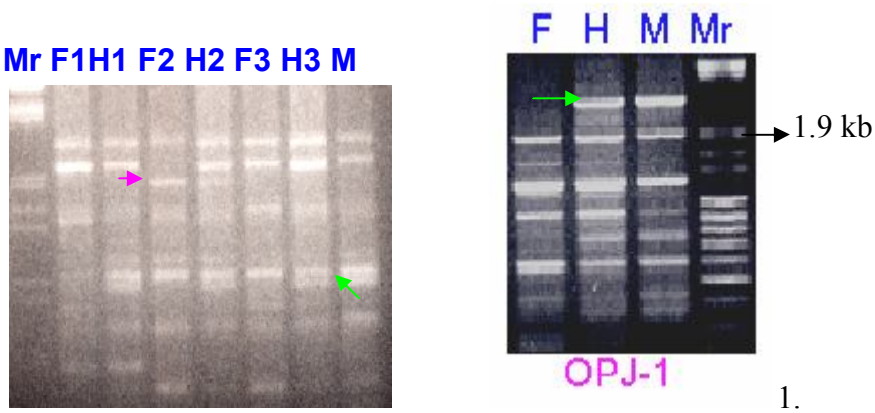




M- Male bulk
H- hybrid bulk
1-21- Individual hybrid plants
Mr-DNA size marker (λ DNA\ *Hind III Eco RI*)
→ -Indicating male specific band

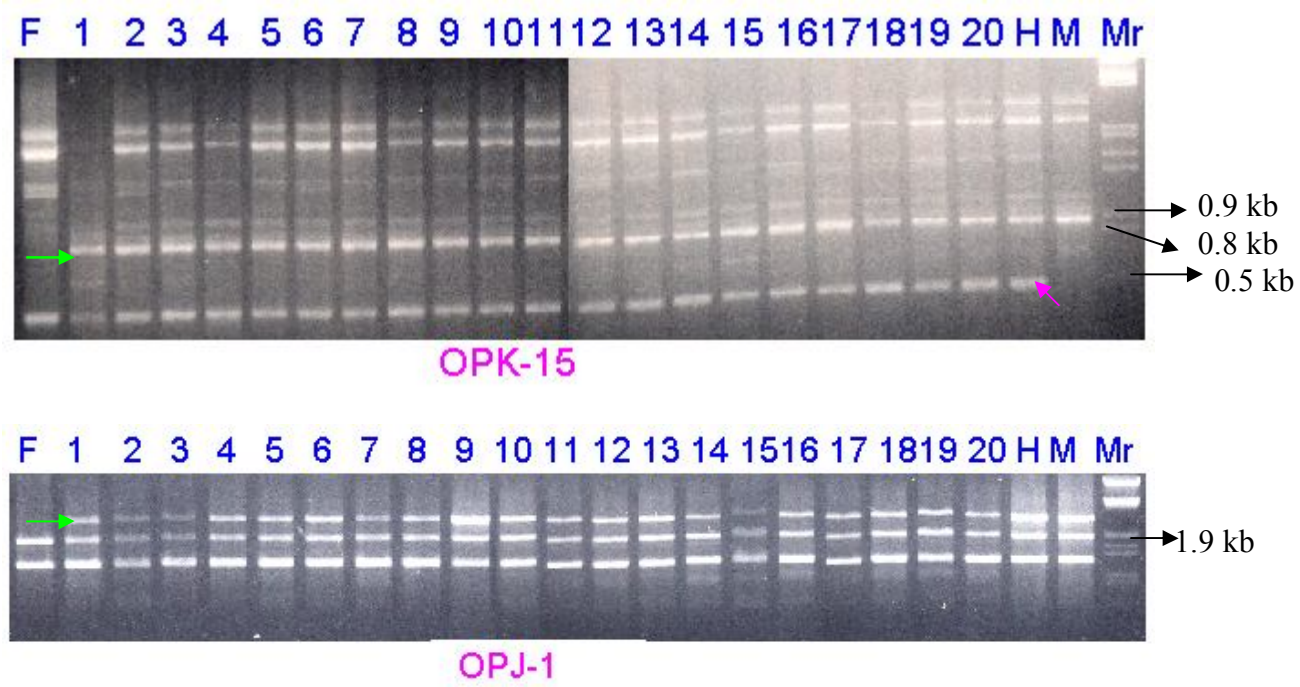
Plate 3. RAPD marker for hybrid KBSH-41 using primer OPK-15 and OPJ-1

A. RAPD with bulk DNA of KBSH-41 and parents.



1.

B. RAPD with individual hybrid plants of KBSH-41

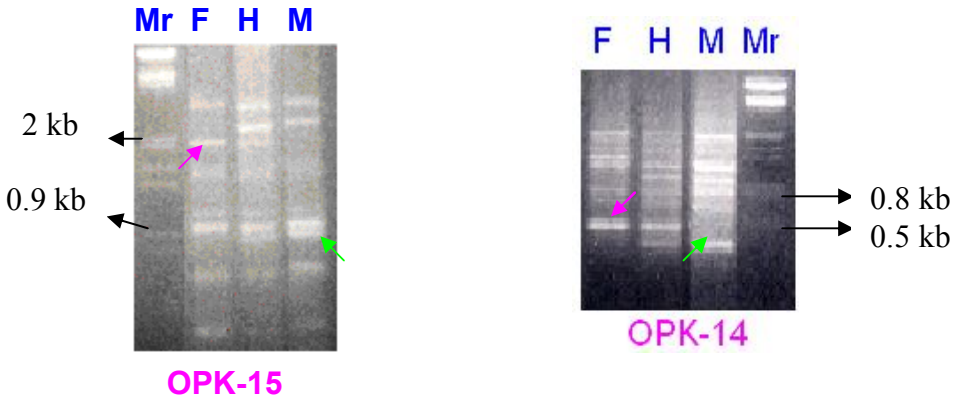


F-Female bulk (KBSH-41)
H-Hybrid bulk (KBSH-41)
M- Male bulk (KBSH-41)
1-20-Iindividual plants of hybrid (KBSH-41)
Mr- DNA size marker λ DNA\ *Hind III Eco RI*+ 100bp DNA ladder
→ -Indicating male specific band
→ -Indicating female specific band

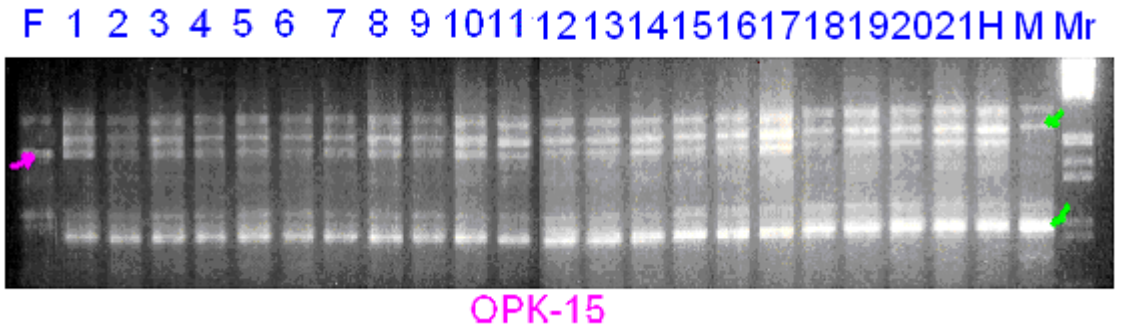
F1-CMS-234A (bulk)
H1-KBSH-41 (bulk)
F2-CMS851A (bulk)
H2-KBSH-42 (bulk)
F3-CMS17A (bulk)
H3-KBSH-44 (bulk)
M-RHA-95-C-1 (bulk)

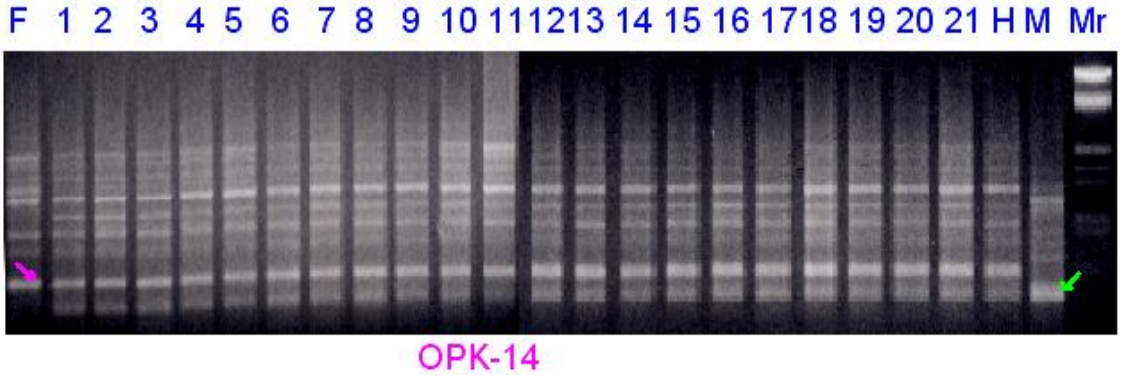
Plate 4. RAPD marker for hybrid KBSH-42 using primer OPK-15 and OPK-14

A. RAPD with bulk DNA of KBSH-42 and parents.



B. RAPD with individual hybrid plants of KBSH-42

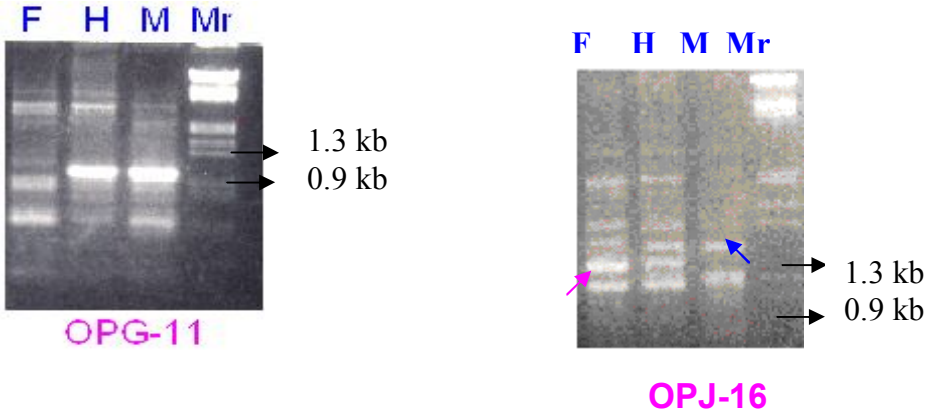




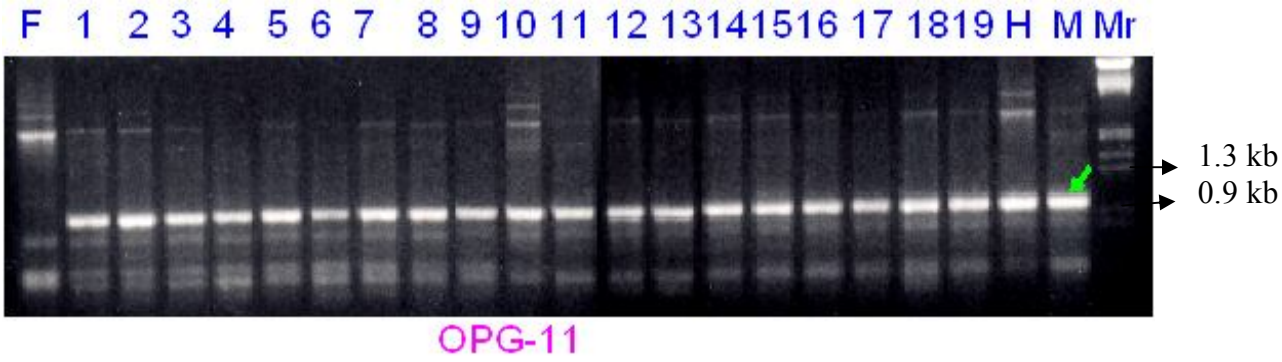
F-Female bulk
H-Hybrid bulk
M- Male bulk
1-21-Individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band
→ -Indicating female specific band

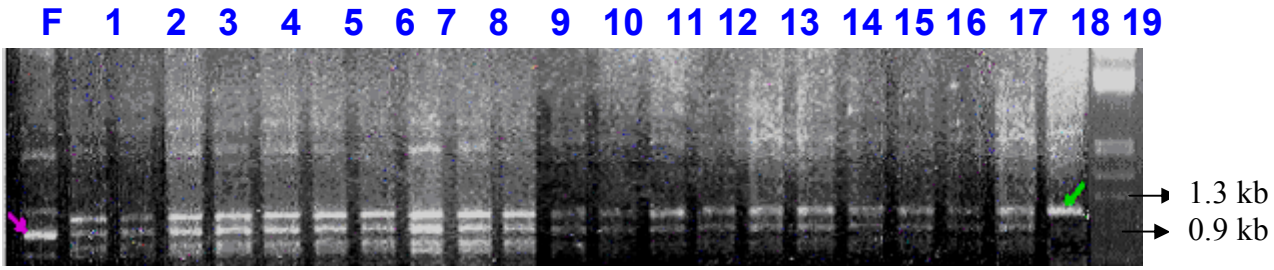
Plate 5. RAPD marker for hybrid KBSH-44 using primer OPG-11 and OPJ-16

A. RAPD with bulk DNA of KBSH-44 and parents.



B. RAPD with individual hybrid plants of KBSH-44

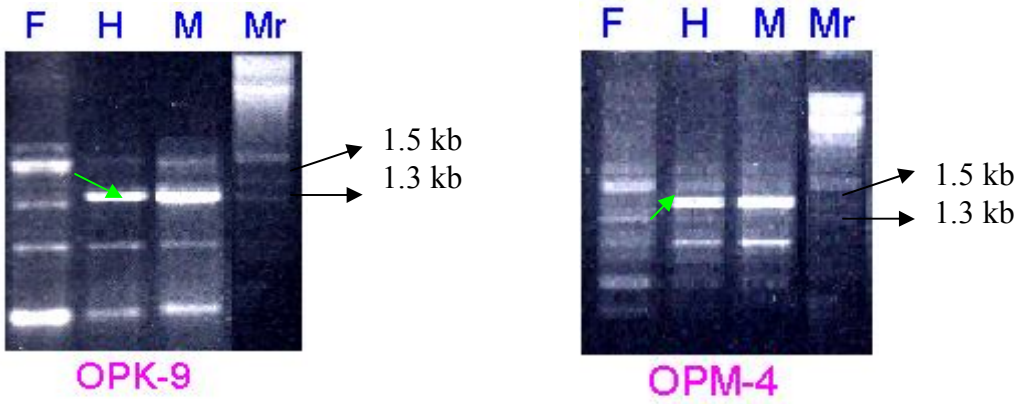




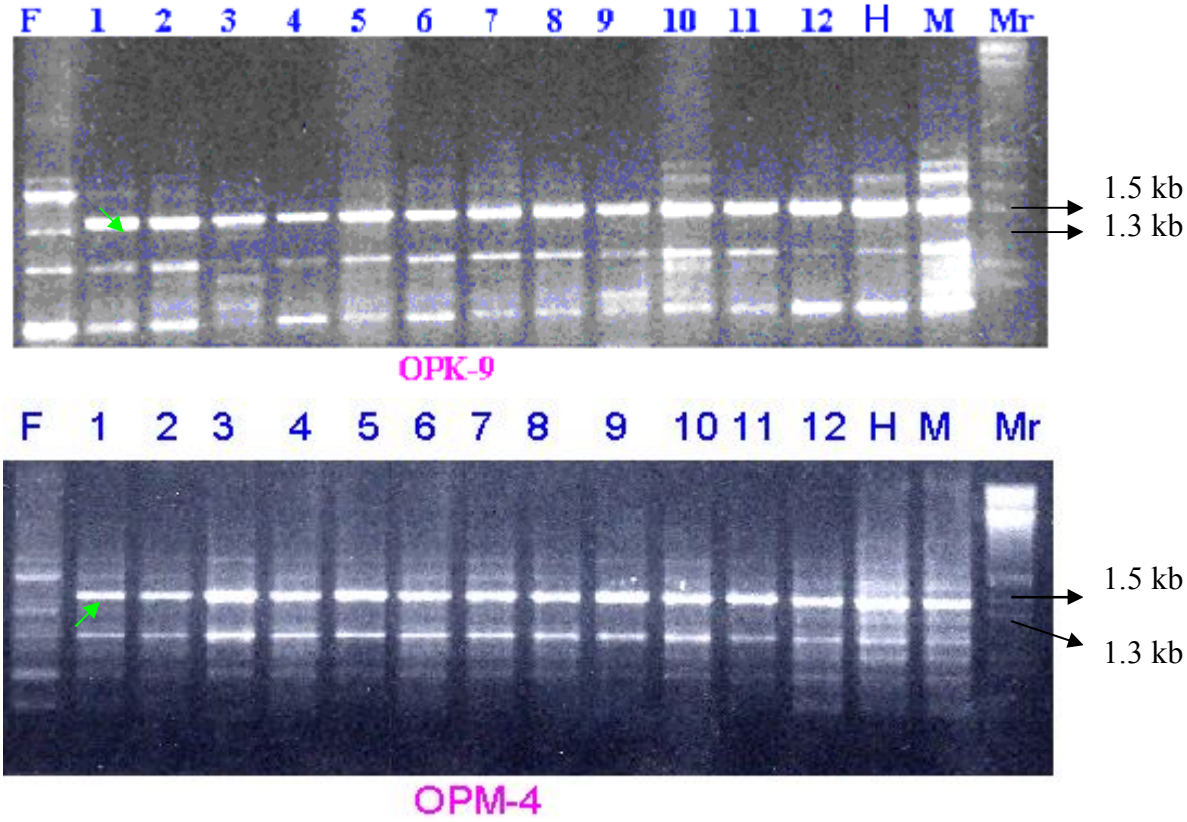
F-Female bulk
H-Hybrid bulk
M- Male bulk
1-19-Individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band
→ -Indicating female specific band

Plate 6. RAPD marker for hybrid HSFH-848 using primer OPK-9 and OPM-4

A. RAPD with bulk DNA of HSFH-848 and parents.



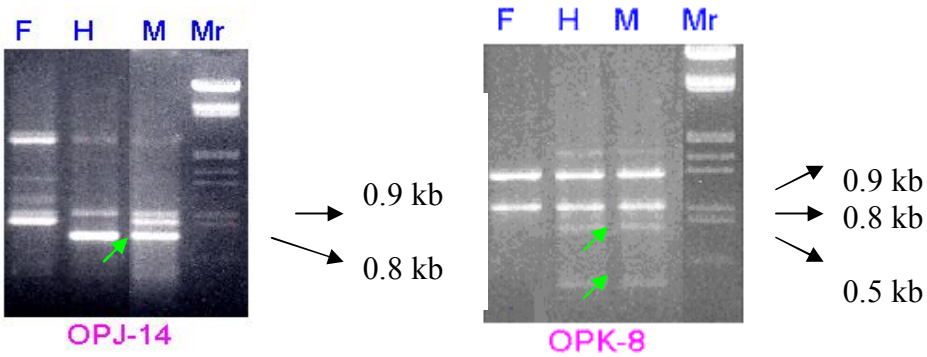
B. RAPD with individual plant of HSFH-848



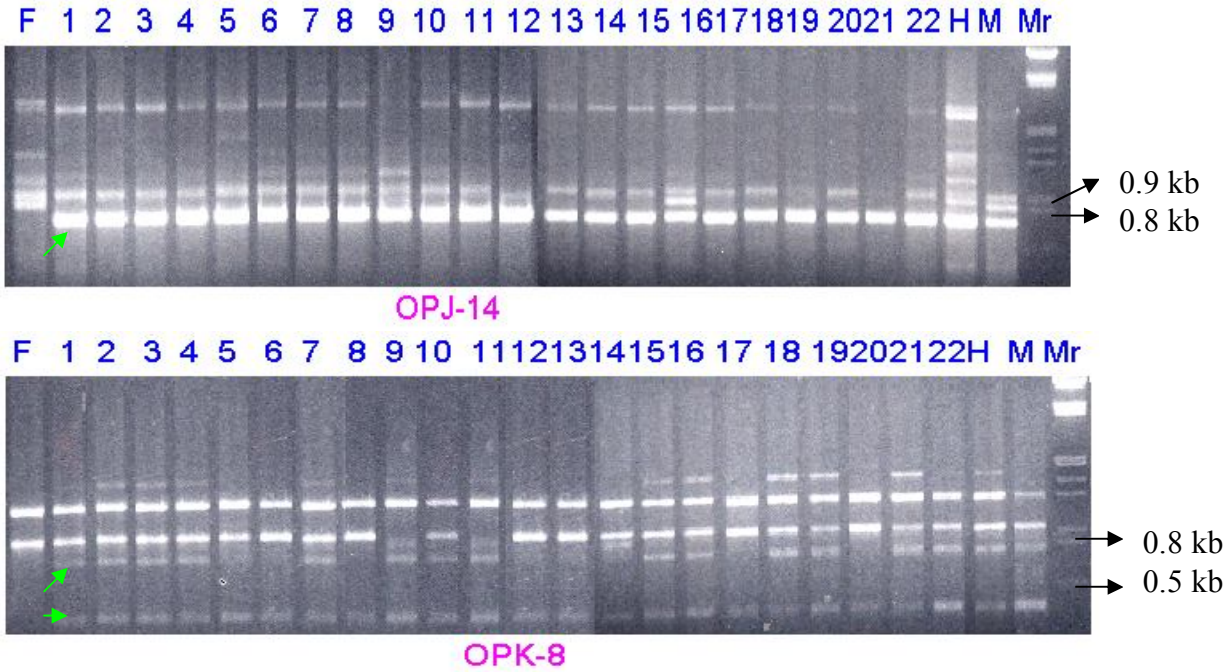
F-Female bulk
H-hybrid bulk
M- male bulk
1-21 individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band

Plate 7. RAPD marker for hybrid LSH-1 using primer OPJ-14 and OPK-8

A. RAPD with bulk DNA of LSH-1 and parents.



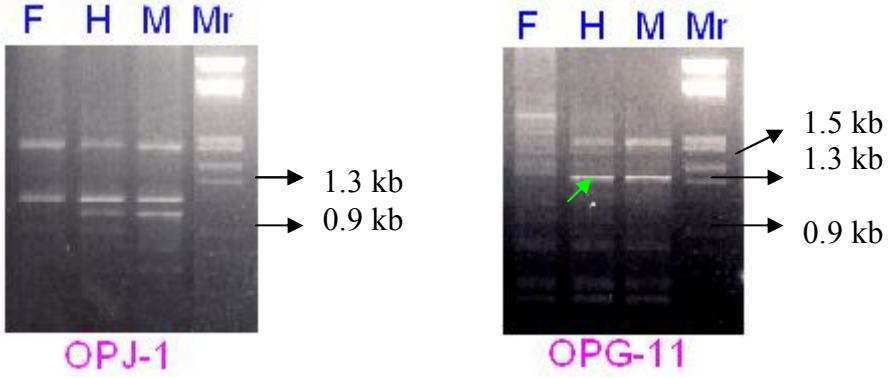
B. RAPD with individual hybrid plants of LSH-1



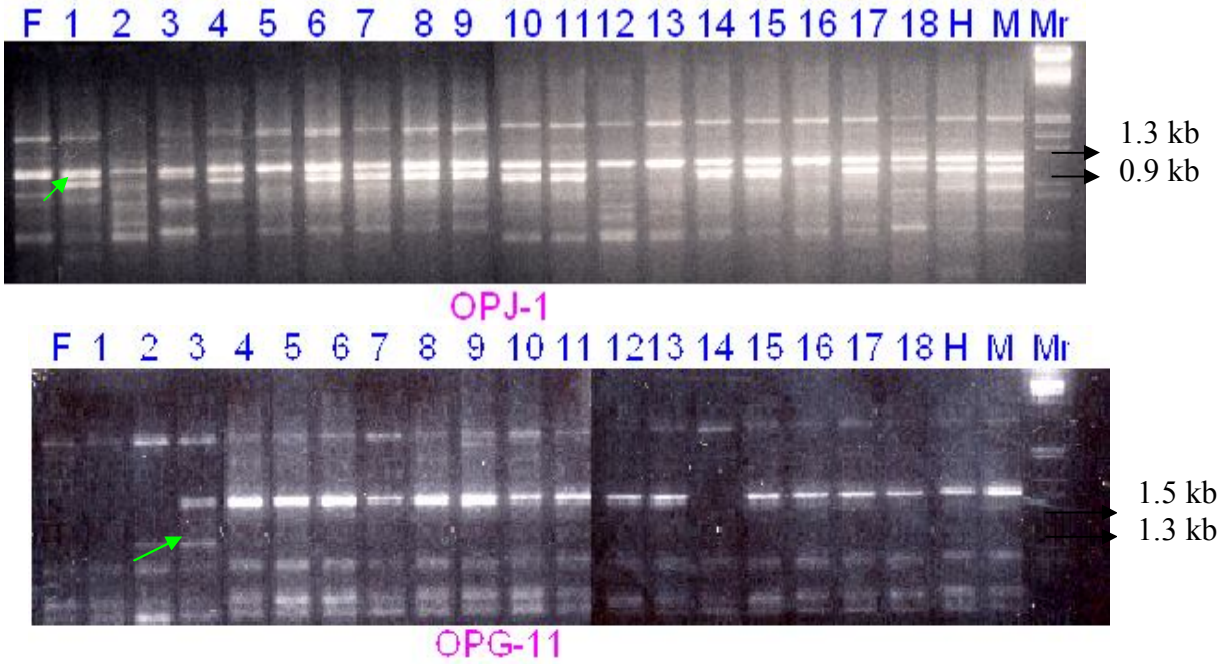
F-Female bulk
H-hybrid bulk
M- male bulk
1-21 individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band

Plate 8. RAPD marker for hybrid SCH-35 using primer OPJ-1 and OPG-11

A. RAPD with bulk DNA of SCH-35 and parents.



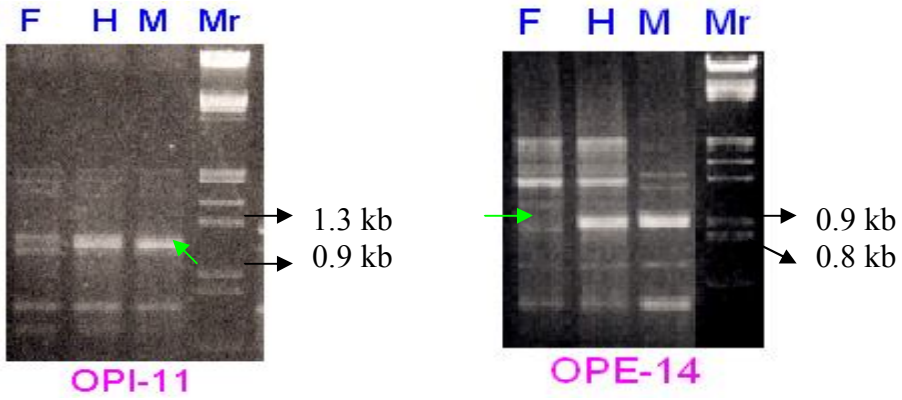
B. RAPD with individual hybrid plants of SCH-35



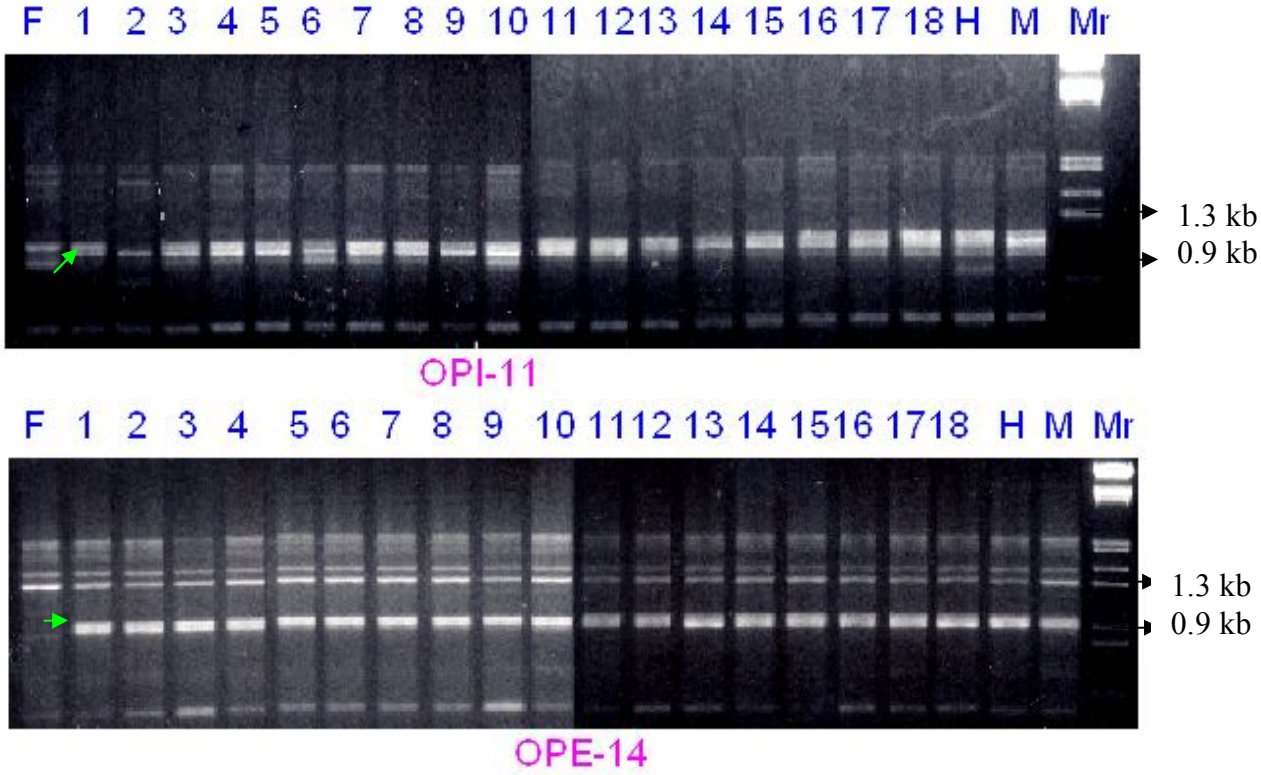
F-Female bulk
H-hybrid bulk
M- male bulk
1-21 individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
-Indicating male specific band

Plate 9. RAPD marker for hybrid PSFH-118 using primer OPI-11 and OPE-14

A. **RAPD with bulk DNA of PSFH-118 and parents.**



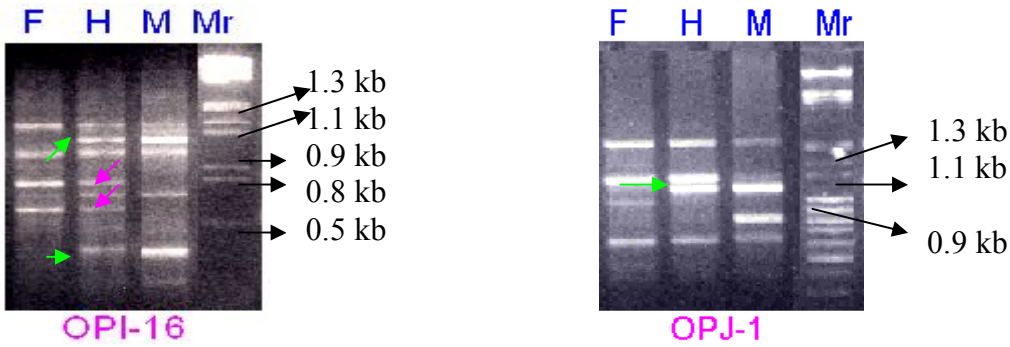
B. RAPD with individual hybrid plants of PSFH-118



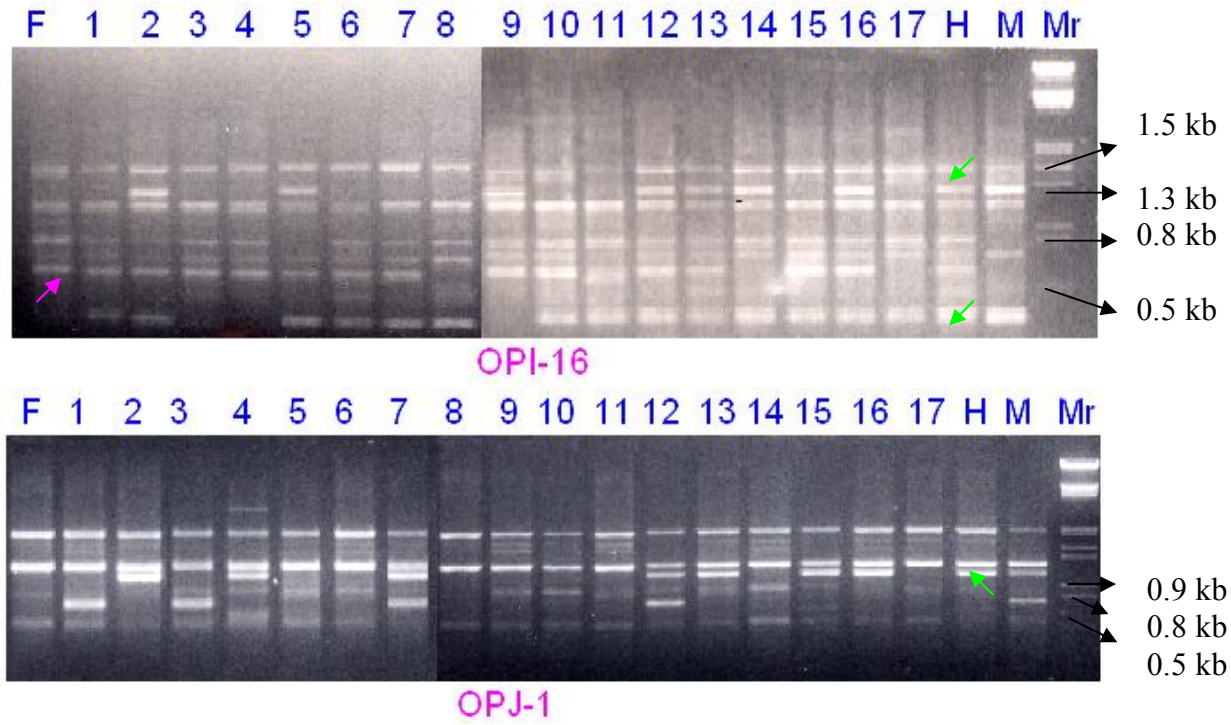
F-Female bulk
H-hybrid bulk
M- male bulk
1-21 individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band

Plate 10. RAPD marker for hybrid DSH-10 using primer OPI-16 and OPJ-1

A. **RAPD with bulk DNA of DSH-1 and parents.**



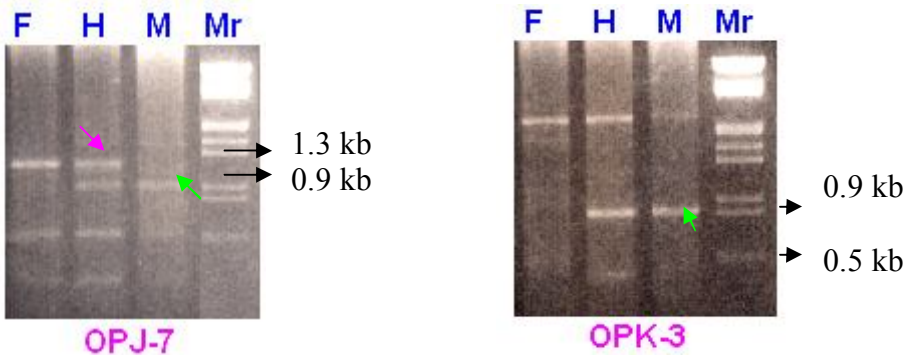
B. RAPD with individual hybrid plants of DSH-1



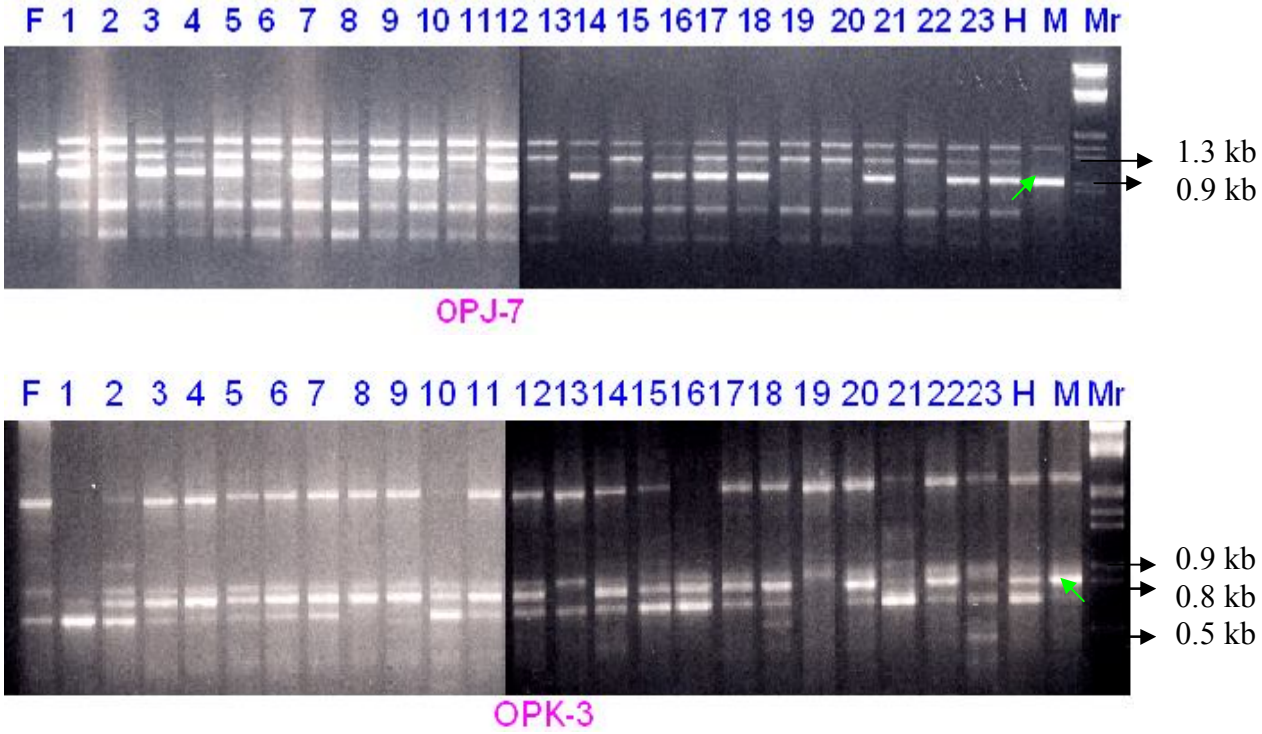
F-Female bulk
H-hybrid bulk
M- male bulk
1-21 individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→-Indicating male specific band
→ -Indicating female specific band

Plate 11. RAPD marker for hybrid RSFH-1 using primer OPJ-7 and OPK-3

A. **RAPD with bulk DNA of RSFH-1 and parents.**



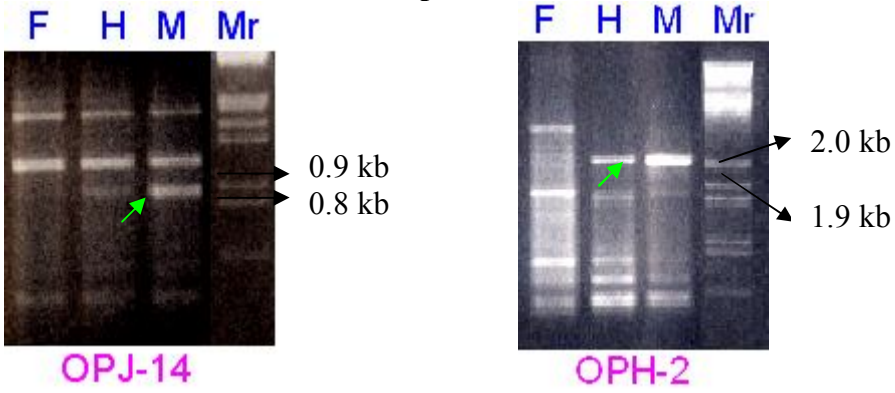
B. RAPD with individual hybrid plants of RSFH-1



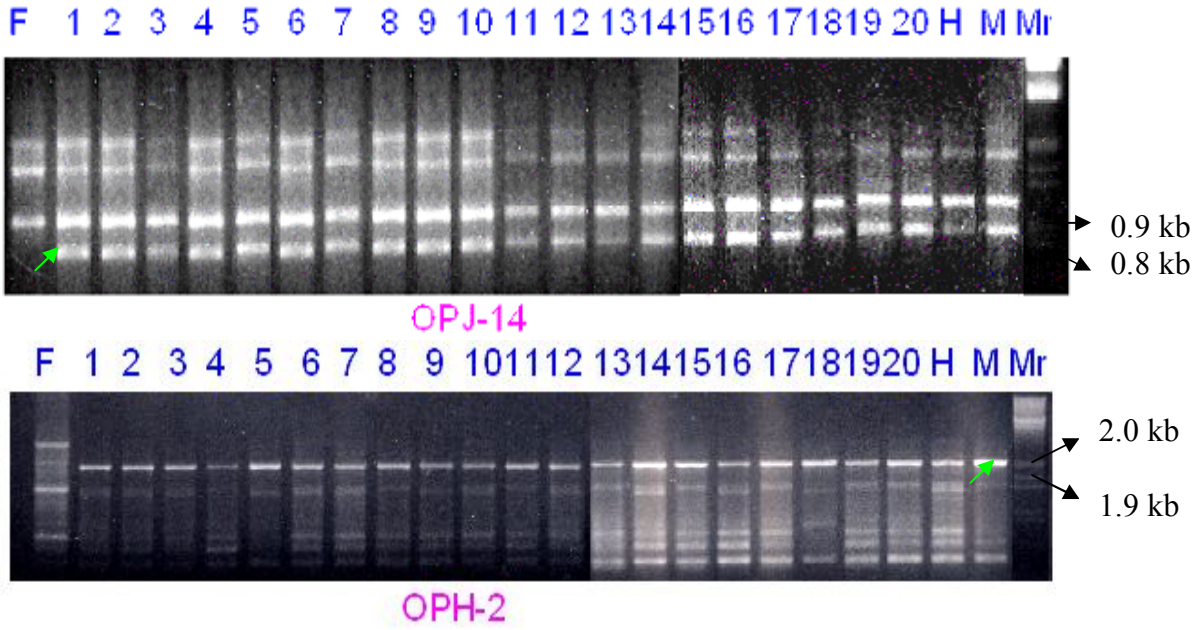
F-Female bulk
H-Hybrid bulk
M- Male bulk
1-23-Individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band
→ -Indicating female specific band

Plate 12. RAPD marker for hybrid TCSH-1 using primer OPJ-14 and OPH-2

A. **RAPD with bulk DNA of KBSH-41 and parents.**



B. RAPD with individual hybrid plants of TCSH-1



F-Female bulk
H-hybrid bulk
M- male bulk
1-21 individual plants of hybrid
Mr- DNA size marker λ DNA\ *Hind III Eco RI*
→ -Indicating male specific band

Plate 13 A. RAPD marker for sunflower variety CO-2

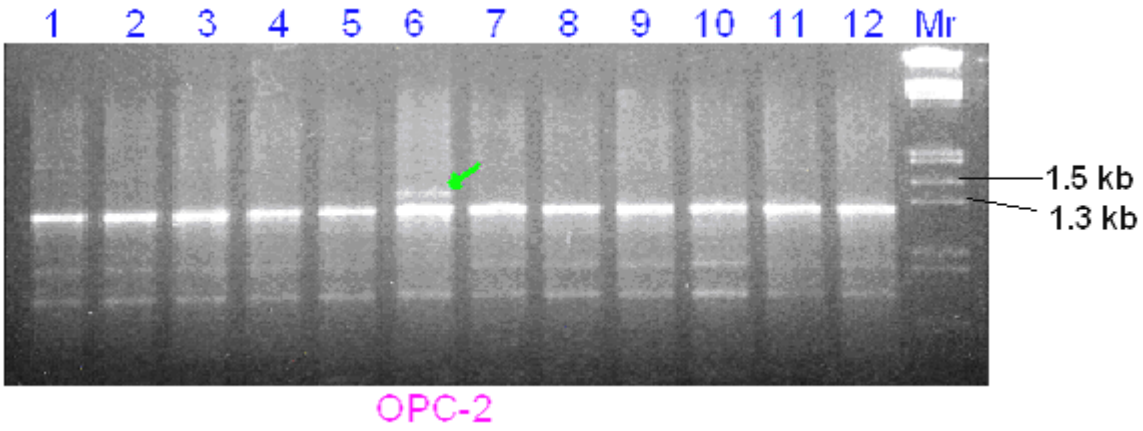
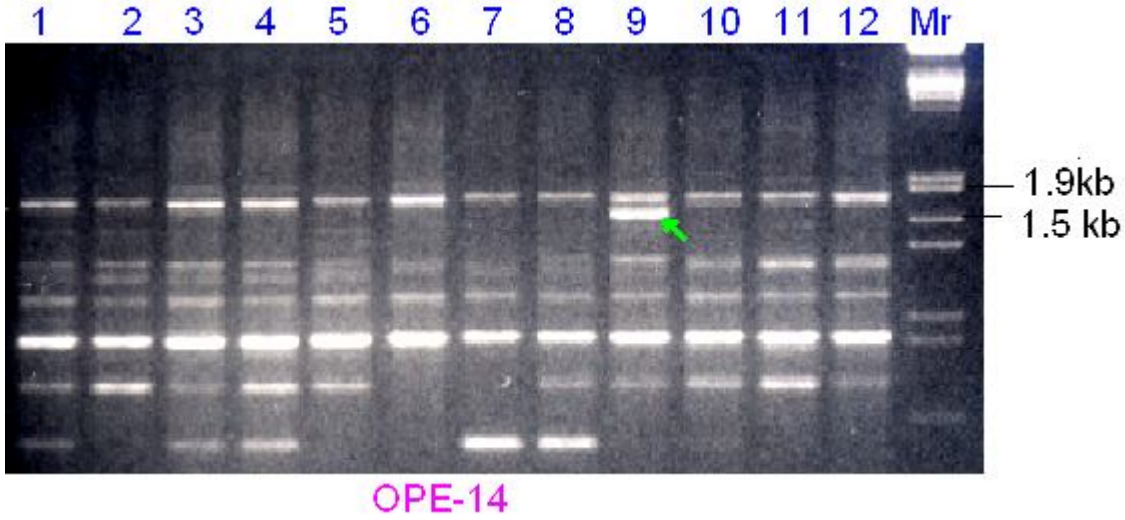


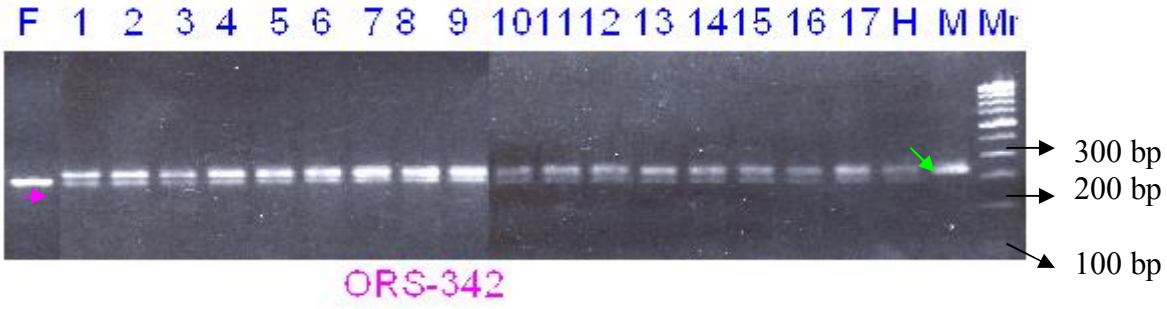
Plate 13 B. RAPD marker for sunflower variety EC-68415



1- Surya
2 -PKVSF-9
3 -Gujarat sunflower-1
4 -LS-11
5 -SS-56
6 -CO-2
7-CO-3
8-CO-4
9-EC-68415
10-Morden (TN)
11-Morden (AK)
12-Dwarf Morden

Mr- DNA size marker
(λ DNA \ *Eco* RI and *Hind* III)

Plate 14 (A) SSR markers for hybrid DSH-1 using primer ORS-342



F- Bulk CMSDSF-15A (Female parent of DSH-1)
H-Bulk DSH-1
M- Bulk RHA-857 (Male parent of DSH-1)
Mr-DNA size marker (100 bp ladder)
1-17-Individual plants of DSH-1

Plate 14 (B) SSR markers for hybrid SCH-35 using primer ORS-154

F- Bulk CMS-234A (Female parent of SCH-35)
H-Bulk SCH-35
M- Bulk RHA-1-1 (Male parent of SCH-35)
Mr-DNA size marker (100 bp ladder)
1-18-Individual plants of SCH-35

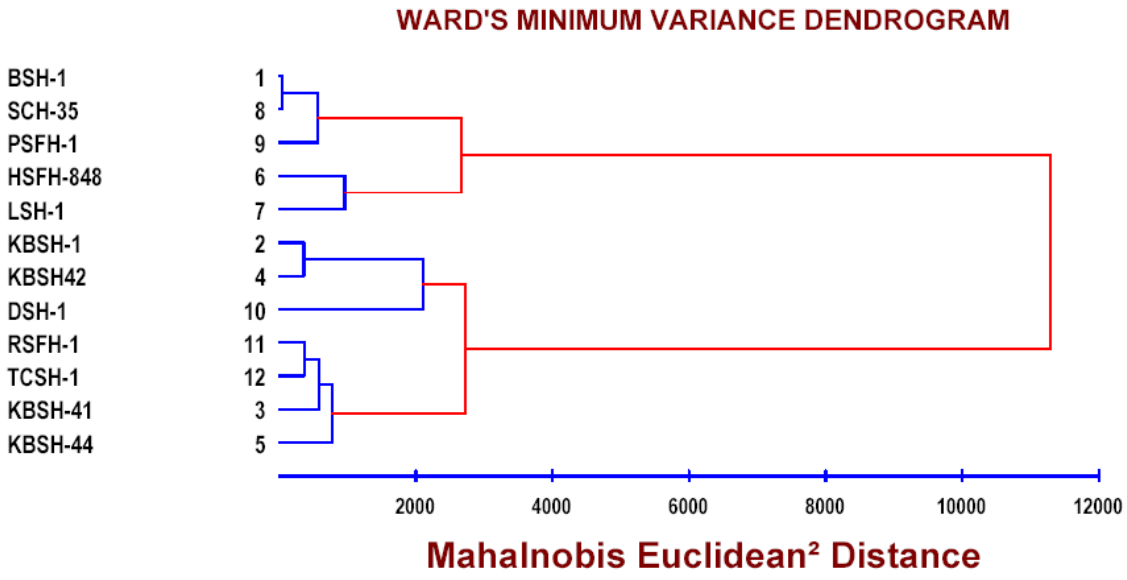


Fig-1: Ward's minimum variance dendrogram of hybrids using morphological characters

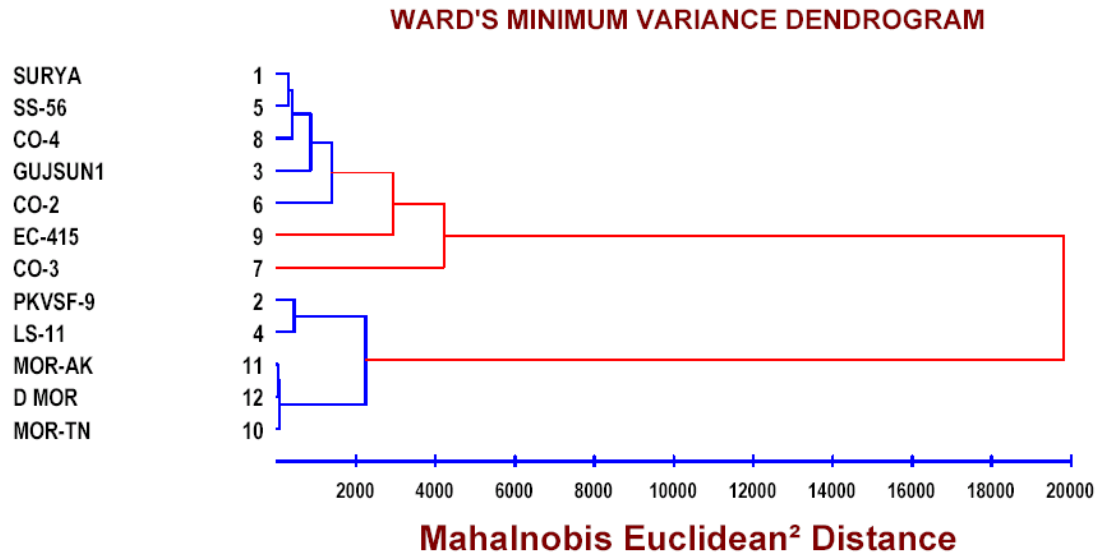


Fig-2: Ward's minimum variance dendrogram of varieties using morphological characters

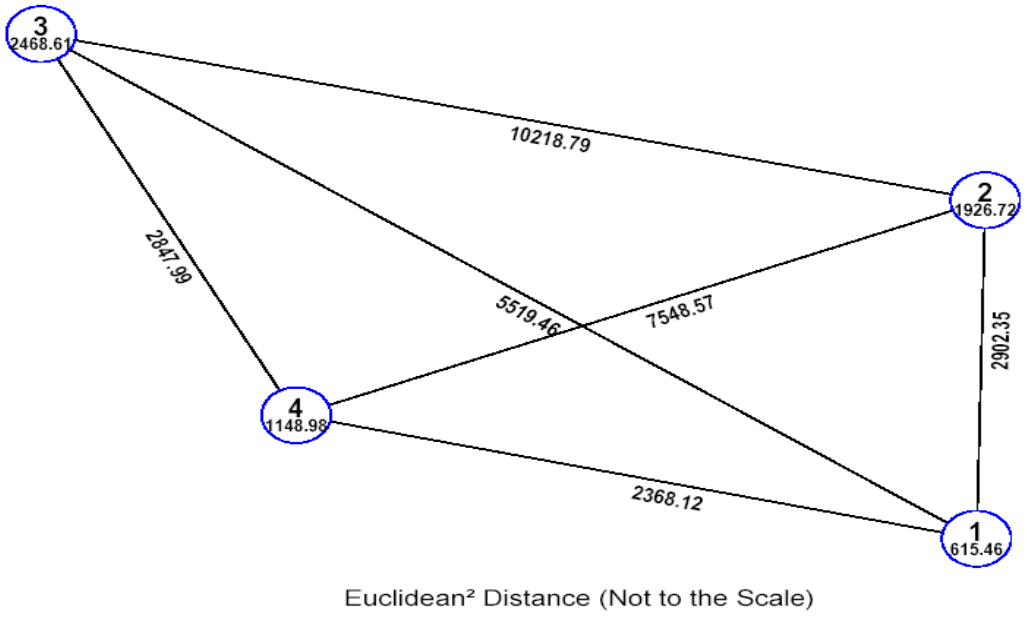


Fig-3 Euclidean^2 distance among hybrids

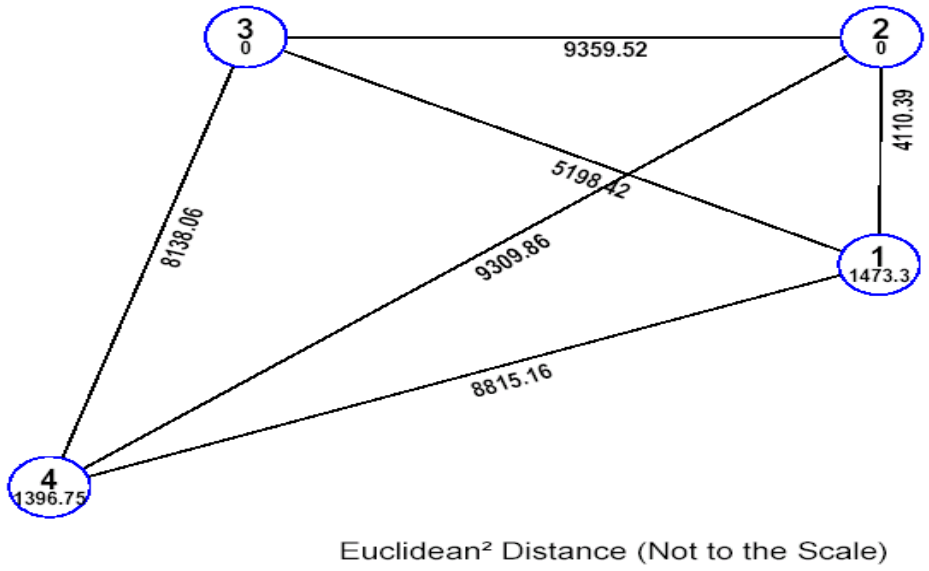


Fig-4 Euclidean^2 distance among varieties

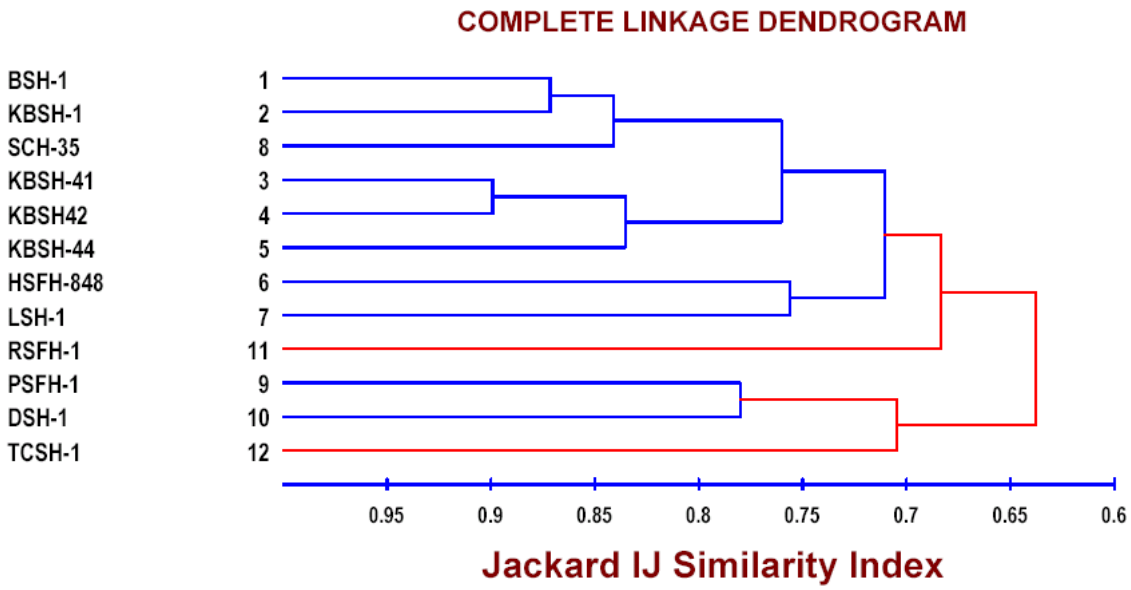


Fig-5 Complete linkage dendrogram of hybrids using RAPD markers

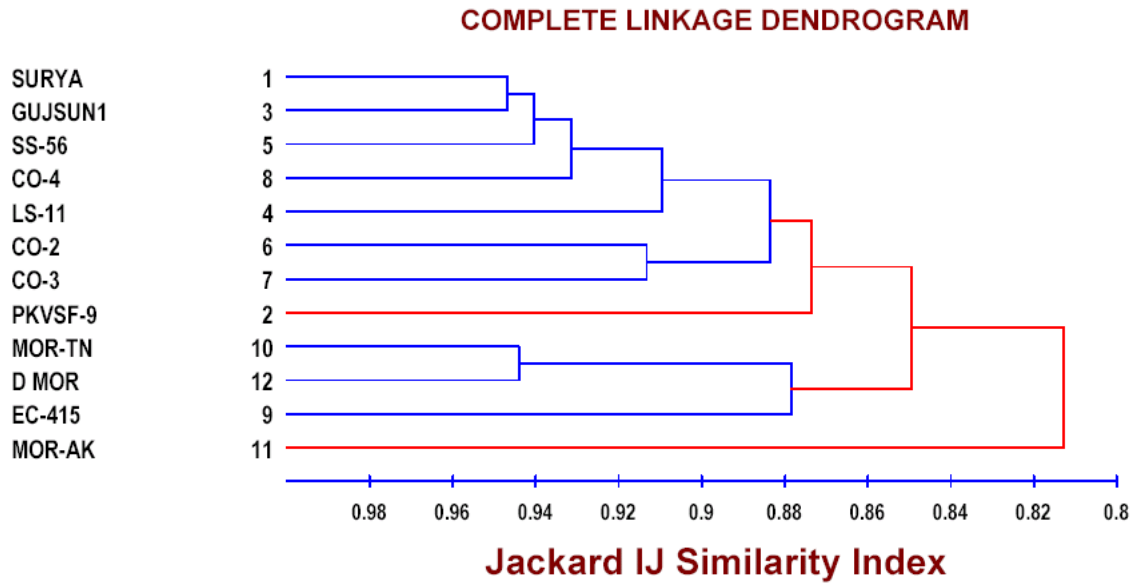


Fig-6 Complete linkage dendrogram of varieties using RAPD markers

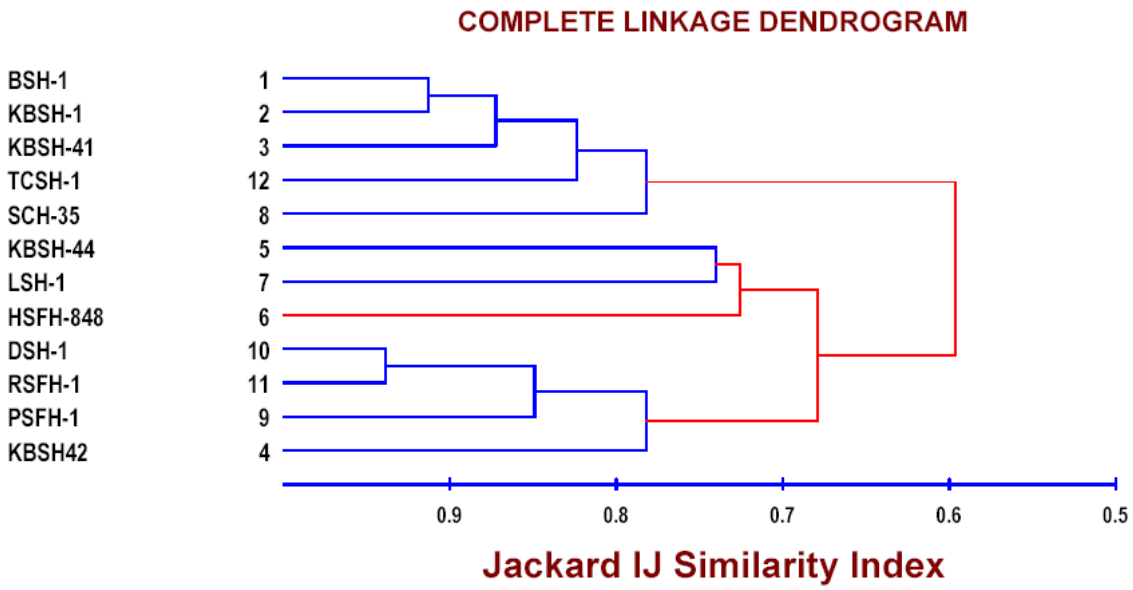


Fig-7 Complete linkage dendrogram of hybrids using SSR markers

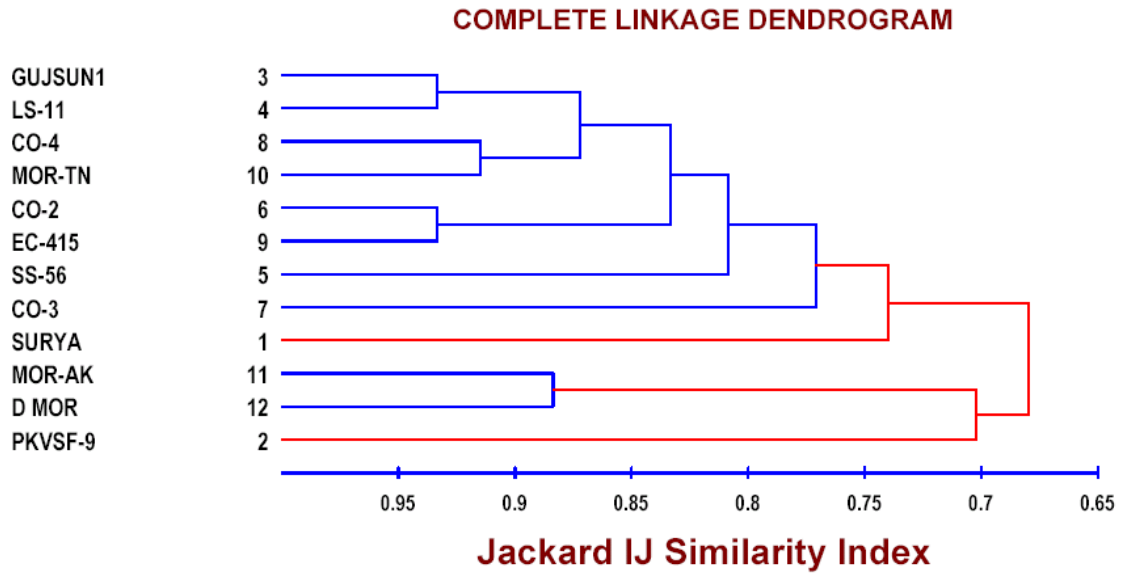


Fig-8 Complete linkage dendrogram of varieties using SSR markers