"ASSESSMENT OF HYBRID PURITY IN SUNFLOWER BY USING MOLECULAR MARKERS"

Submitted By

BHOSLE SHEETAL MADHUKARRAO

B.Sc. (Agril. Biotechnology)

DISSERTATION

Submitted to the Marathwada Krishi Vidyapeeth in Partial Fulfilment of the Requirements For the Degree of

MASTER OF SCIENCE

(AGRICULTURE)

IN

AGRICULTURAL BIOTECHNOLOGY

COLLEGE OF AGRICULTURAL BIOTECHNOLOGY, LATUR.

MARATHWADA KRISHI VIDYAPEETH

PARBHANI- 431 402 (M.S.) INDIA

2011

Dedicated

With

Profound Gratitude

To

My Parents and my research guides

CANDIDATES DECLARATION

I hereby declare that the entire work embodied in this thesis or a part thereof has not been previously submitted by me for a Degree or Diploma of any University or Institute

PLACE: LATUR
DATE: 16 /8/2011.

(Bhosale S.M.)

Dr. M. K. GHODKE
M.Sc (Agri.), Ph. D.
Sunflower Breeder
Oilseed Research Station, Latur.
MKV, Parbhani.

CERTIFICATE - I

This is to certify that Ms. Sheetal Madhukarrao Bhosale has satisfactory prosecuted her course and research for a period of not less than four semester and that the dissertation entitled "**ASSESSMENT OF HYBRID PURITY IN SUNFLOWER BY USING MOLECULAR MARKERS**" submitted by her is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination.

I also certify that, the thesis or a part thereof has not been previously submitted by her for a degree of any university.

PLACE: LATUR
DATE: 16 /8 /2011

(M. K. GHODKE)
Research Guide

CERTIFICATE - II

This is to certify that the dissertation entitled "**ASSESSMENT OF HYBRID PURITY IN SUNFLOWER BY USING MOLECULAR MARKERS**" submitted by Ms. Sheetal Madhukarrao Bhosale to the Marathwada Krishi Vidyapeeth, Parbhani in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **AGRICULTURAL BIOTECHNOLOGY** has been approved by the student's advisory committee after oral examination in collaboration with the external examiner.

(C.D. R. Deosarkar)
External Examiner

(Dr. M. K. GHODKE)
Research Guide

Members of Advisory Committee:

(Dr. R. L. CHAVHAN)

(Prof. A. A. BHAROSE)

(Dr. S. P. SHIRSHIKAR)

Associate Dean and Principal,
College of Agricultural Biotechnology,
Marathwada Krishi Vidyapeeth,
Parbhani.

ACKNOWLEDGEMENT

In the few pages I take the opportunity to acknowledge the kindness shown by various people I had the pleasure of being associated with during the conduct of experiment.

First of all, my sincere, humble indebtedness and whole-hearted sense of gratitude to my honourable guide Dr. M. K. Ghodke, Sunflower Breeder, Oilseed Research station, Latur, MAU, Parbhani, for his inspiring guidance and valuable suggestion, which helped me to complete the research, work in time. It's my privilege to be the student of Dr. M.K.Ghodke whose sympathetic behaviour and co-operative nature during the course of present investigation have encouraged me and will remain encouraging forever in my life.

Really fortunate I am, for I got the opportunity to work under the versatile guidance of my co guide Dr. R. L. Chavhan Assistant professor, College of Agricultural Biotechnology, Latur. I wish to place on record my indebtedness for his constant perspiration, talented and well-versed advice, constructive criticism, kind civility and sustained interest in completion of my dissertation. I am greatly thankful to him for his support and providing all technical help to accomplish this goal and also for his help in preparing this manuscript and going critically through this dissertation. It is because of his guidance and encouragement, that I could complete this work successfully.

I sincerely express my gratitude to Dr. H. B. Patil, Associate Dean and Principal, College of Agricultural Biotechnology, Latur for able guidance, valuable counselling, kind inspiration and supervision of my research work from initiation until its completion. I am hard of words to express my gratitude for his kind co-operation and affection that he extends throughout the course of this investigation.

I am also thankful to the members of advisory committee Prof. A. A. Bharose, Associate Professor, College of Agricultural Biotechnology, Latur and Dr.

S. P. Shirshikar, Sunflower pathologist, Oilseed Research station, Latur, MKV, Parbhani for their help and encouragement. I remain grateful to Dr. Kishanrao Gore, Vice Chancellor, MAU, Parbhani for providing the facilities during the course of my studies.

I sincerely thanks to Dr. S. S. Shende, Associate Professor and Prof B. N. Aglave, Associate Professor, College of Agricultural Biotechnology for providing technical help and guidance during my dissertation. I owe high esteemed respect to Prof. R. N. Dhawale, Prof. K. M. Sharma, Mr. Damse, Prof. Surve, Dr. A. M. Dethe, College of Agricultural Biotechnology, Latur and Mr. Satish Sutar, Central Institute of Cotton Research, Nagpur. I also thanks to non-teaching staff of College of Agricultural Biotechnology for their kind co-operation during completion of my PG education.

I sincerely express my heart-felt gratitude and indebtedness to Mrs. Vidya Chavan, Assistant Professor, COCSIT, Latur for her guidance, whole hearted co-operation to bring out this document.

I also wish to express my sincere thanks to Dr. Mangesh Dudhe, Senior Scientist, DOR, Hyderabad for their valuable and kind guidance during the course of this investigation.

One needs sincere friends at all major junctures in life to bear strains and fatigue cheerfully. It is my great pleasure to acknowledge a friend and colleague Mr. Mahesh Verma for the help, encouragement and kindly co-operation throughout this academic programme. I wish to record my cardiac sense of gratitude towards my colleagues and friends Priyanka, Leeshan, Indrani, Sanket, Swapnil, Datta, Vivek, Pavan and Avinash.

I wish to record my sense of gratitude towards my dear friends Poonam, Aanand, Amol, Sangharsh, and GV. for their kindness, inspiration and moral support throughout my studies. I also like to thanks my friends Harshraj, Ravi and Abhijeet for their help and valuable suggestion.

I also like to express my sincere thanks to my seniors Kishor, Shyam, Deepti and my loving juniors Rajendra, Dev, Dhruv, Ashwin and Rupali for their loving inspiration and great caring during the completion of my research work.

I wish to express my sincere gratitude to Vishnu mama, Swati, Rukmini maushi, Anusaya maushi for their valuable support and co-operation.

I felted honour to dedicate this work to my beloved parents for their bondless love, constant inspiration, encouragement and moral support to achieve my goal. I am also thankful to my elder Di, Ram Bhauji and brother Vishal and my dear sweet nephew 'Shourya' for their love, affection and good wishes.

Last but not the least; I thank almighty for blessing me throughout my life. I wish to record my sincere gratitude to all those who helped me directly or indirectly in my endeavour.

Bhosale

Sheetal M. Bhosale

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

^{O}C	- Degree Celsius
AFLP	- Amplified Fragment Length Polymorphism
bp	- Base Pairs
CMS	- Cytoplasmic Male Sterile
CTAB	- Centyl trimethyl ammonium bromide
DI	- Diversity Index
DNA	- Deoxyribose Nucleic Acid
dNTP	- Deoxyribose Nucleotide Triose Phosphate
EDTA	- Ethylene Diamine Tetra Acetic Acid
et al.	- et alia (and other)
FPS	- Female Parent Specific
GOT	- Grow Out Test
H–PAGE	- Horizontal Polyacrylamide Gel Electrophoresis
hr	- Hour
ISSR	- Inter Simple Sequence Repeat
kb	- Kilo base pair
MPS	- Male Parent Specific
$MgCl_2$	- Magnesium chloride
min	- Minutes
ml	- milliliter
mM	- Milli Molar
PCR	- Polymerase Chain Reaction
pH	- log H ion concentration

RAPD	- Random Amplified Polymorphic DNA
RFLP	- Restriction Fragment Length Polymorphism
RNAse	- Ribonuclease
rpm	- revolutions per minute
SCAR	- Sequence Characterised Amplified Region
SPARS	- Single Primer Amplification Reaction
SSR	- Simple Sequence Repeat
STR	- Short Tandem Repeat
STMS	- Sequence Tagged Microsatellite Sites
STS	- Sequence Tagged Sites
Taq	- *Thermus aqaticus* DNA polymerase
TBE	- Tris Borate EDTA
Tris	- Tri-(Hydroxy Methyl) - amino methane
Tris-HCL	- Tris –Hydrochloride
U	- Units
UV	- Ultra Violet
µg	- Microgram
µl	- Microliter
µM	- Micro Molar
pM	- Pico Molar
ng	- Nano gram
AGE	- Agarose Gel Electrophoresis
NTSYS	- Numerical Taxonomic System
PVP	- Polyvinyl Pyrolidone

Introduction

CHAPTER-I

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an annual, diploid (2n = 34) species of crop plants. It belongs to the family *Compositae* and the genus *Helianthus*. Sunflower is day neutral and salt tolerant crop. It is a cross-pollinated plant, contemplating varying degrees of self-incompatibility.

The genus name associates the characteristic heliotropism exhibited during the flowering period. Sunflower is native to North America. It is cultivated as an oilseed crop in Argentina, Russia, Uruguay, Turkey, Yugoslavia, South Africa China, USA, Italy and France. Beside this, it is being cultivated in small scale in England, South Africa, parts of Asia and Australia.

The sunflower was grown as ornamental crop in India until 1920. Its cultivation has been popularized under oilseed mission and now it is cultivated as one of the major oilseed crops beside peanut, soybean & mustard.

Among the four major oilseed crops in the world *viz.* soybean, Brassica, sunflower and groundnut, sunflower ranks third in area under cultivation and fourth in total production in the year 2010. The present cultivating area of sunflower in the world is 23.7 million ha with a production of 33.33 million tones and 1322 kg/ha productivity. Among the world Europe and America account far nearly 70% of total area and 80% of total production. Asian countries shares 20-22% of the total cultivating area of sunflower and about 18% of production in the world. In Asia, India is the largest grower of sunflower since year 2006-07. In India sunflower is cultivated as an area of 14.83 lakh ha with production of 9.00 lakh tones. The average yield of 607 kg/ha in India is one of the lowest in the world (Anonymous, 2010).

The important sunflower growing states of India are Karnataka, Andhra Pradesh, Maharashtra and Tamil Nadu. Almost 50% of the area and production is accounted by Karnataka followed by Andhra Pradesh, Maharashtra and Tamil Nadu.

Maharashtra state ranks third position by cultivating this crop on area of 2.03 lakh ha with 1.27 Lakh tones production and 625 kg/ha productivity in the year 2009-10 (Anonymous, 2010).

The genus *Helianthus* comprised of 49 species consists of 13 annual and 36 perennial with different ploidy level. Only two of species viz. *Helianthus tuberosus* and *Helianthus annuus* L. being cultivated as an oilseed confectionary or ornamental crop. In India sunflower becomes more popular oilseed crop due to high quality nutritional value, short duration crop, photo insensitivity, wide adoptability, drought and salt tolerance. Also it is a being cultivated as cash crop at any season in India.

Sunflower seed is highly nutritious containing 20% protein and 40-45% vegetable oil with a very high calorific values, cholesterol lowering factor constitute around 80-95% of total fatty acid, 60-70% linoleic acid with sufficient amount of calcium, iron, vitamin A, D, E, and B complex, manganese, magnesium, copper, selenium, phosphorous etc.

In addition to these sunflower seed have industrial importance. The advancement in Agriculture research and development helpful to develop high yielding cultivars, hybrids are becoming more popular and replacing the traditional open pollinated varieties because of their maturity, production stability and high yield.

Being the important oilseed crop heterosis development in sunflower could be exploited for better seed and oil yield. Hybrids are more vigorous, uniform, self fertile and resistant against important foliar diseases. In a systematic hybrid breeding programme, it is essential to identify superior parents to exploit the genetic variability for better heterosis also the combining abilities, general and specific, promise could be exploited for the development of better heterosis.

Commercial sunflower hybrids are produced by utilizing the cytoplasmic genetic male sterility (CGMS) system present in cytoplasmic male sterile (A line), maintainer lines (B line) and restorer line (R lines).

Non-availability of adequate quantity of good quality seed, particularly of hybrids, non-availability of insect pollinator, partial development of axial flowers, in-breeding depression, self incompatibility, heavy rains hampering crops-pollination and non/partial adoption of recommended package of practices lead to low productivity and yield (Anonymous, 2010-11).

Sunflower necrosis disease (SND) is emerging as a major disease of sunflower recently. Within the span of 10 years the disease has spread in almost all sunflower

growing states in India. Yield loss ranged from 30 to 100 % (Chander Rao *et al.*, 2000). The disease caused by tobacco streak virus and found to be transmitted by thrips. Being a new disease reliable resistance hybrids need to screen for commercial cultivation. Sunflower is a cross-pollinated crop in which genetic contamination is often observed that lead to loss of purity and vigor in hybrids. Hence, the present study was under taken to study the assessment of genetic purity of Sunflower hybrids which were essential to ascertain the purity of genotype and development of hybrids.

For the last few years, the yield improvement in sunflower varieties has not been substantial; due to narrow genetic base of the germplasm was used in breeding programme. DNA based molecular markers are the effective and rapid tool being used to discriminate genotypes among each other. Knowledge of the diversity pattern of cultivars and hybrids enables the plant breeders to better understand the evolutionary relationships among selected cultivars/accessions.

Analysis of genetic purity and relatedness between individuals within a specific or between different species of populations by using phylogenetic studies were initially conducted based on qualitative and quantitative traits. Conventionally hybrid purity assessment is established by using grow out test (GOT). This test is based on morphological and agronomical trait or characters which are highly influenced by environmental effects. These tests require tedious statistical procedures and it shows a high degree of plasticity.

To tackle, the problems raised in GOT test, biochemical and molecular techniques have been used to monitor genetic purity and also used to solve taxonomic and phylogenetic problems. Unambiguous identification of élite crop varieties and hybrid by using molecular marker is reliable and rapid. It is essential for their protection and prevention of unauthorized commercial use. In India it is highly relevant because the hybrid seed production and marketing of public sector bred hybrids is largely covered by the private sector.

As biochemical marker like isozyme and storage protein do not provide sufficient discrimination and effective varietals identification. The molecular marker viz., non PCR based molecular marker Restriction Fragment Length polymorphism (RFLP) is highly discriminative and co-dominant marker useful in hybrid purity

assessment but their use remained limited as it is a relatively slow in process and requires skill and expertise also. However, the PCR based DNA a marker technique seems to provide the means for generating useful information on genetic purity and varietal identification. The PCR based RAPD markers is dominant marker and extensively used in purity assessment of F_1 hybrids. The technical simplicity and speed of RAPD methodology has been used for seed purity testing in seed technologies

The molecular marker, Simple Sequence Repeat (SSR) or Microsatellite is reliable, reproducible and authentic marker described by several researches (Dechmer and Fried, 1998). Microsatellites are short DNA sequence harboring motifs of 1-6 bases that are tandemly repeated. The characteristic feature of this marker such as ubiquity, abundance and wide distribution within almost all the genome of eukaryote organism made SSR a powerful genetic marker. This marker has exploited for genetic purity assessment, gene tagging, and cultivar identification and becoming a markers of choice in genotyping cultivars and hybrid purity assessment. The SSR a promising molecular marker was exploited for hybrid purity assessment. This marker is based on variants of microsatellite loci of parental lines of hybrids, and limited to the particular species only. Beside this Inter Simple Sequence Repeat (ISSR) is also reliable and semi arbitary marker. This marker was designed based on complementary sequence of targeted microsatellite sequence could help to assess genetic purity analysis (Li-Wang *et al.,* 2007).

Similarly, Targeted Region Amplified Polymorphism (TRAP) simple, rapid and effective molecular marker was recently developed. This marker is based on EST sequence information and bioinformatics tool to generate polymorphic markers around targeted candidate gene fixed sequences. TRAP marker system utilizes a combination of pair of primers, one primer designed from EST sequence and second from an arbitary sequence except for AT or GC rich cores that anneal with introns and exon respectively. TRAP marker is being used in genotyping germplasm collection and tagging of genes governing desirable agronomic trait of crop plants.

In present context to fulfill the need of increasing population, it is prerequisite to develop high yielding hybrids with higher percentage of oil. The sunflower breeders are

engaged to vigours breed hybrids in open pollinated crop like sunflower with less or negligible contamination from other sources.

The advancement in agriculture made breeder and biotechnologist easier to select genetically pure parent by using DNA based molecular marker (viz, RAPD, ISSR and SSR) in order to develop pure hybrid. Also, DNA fingerprint profile of sunflower hybrids by using molecular marker not only showed their genetic purity but also provide legal protection of these hybrids.

Present study was conducted to assess hybrid purity among 10 hybrids of sunflower with following objectives:

1) DNA fingerprinting analysis of hybrids by using molecular markers like RAPD and SSR.
2) Screening of sunflower hybrids against sunflower necrosis virus *in vivo* condition.

Review of Literature

CHAPTER-II

REVIEW OF LITERATURE

2.1. Importance of sunflower crop

Sunflower is the most important oilseed crop. Cultivated sunflower (*Helianthus annuus* L.) is the main source of edible oil in many countries of the world. In vegetable oil production it ranks second next to soybean in world. For the last 30 years, the production of sunflower has been increased many folds due to the expansion of its cultivation area in several parts of the world (Qureshi *et al.,* 1992). Consumption of high levels of saturated fats is associated with increasing risk of coronary heart disease. The oil derived from sunflower cultivar has more level of saturated fatty acid (SFA) content, which is present in fewer amounts in other vegetables oils. It creates a great scope to breed sunflower cultivars with minimum level of saturated fat content. Sunflower Research Unit has taken effort and developed genetic stocks with reduced palmitic, stearic acids, compound of saturated fatty acid of sunflower oil (Miller and Fick., 1997).

2.2 DNA extraction

Murray and Thompson (1980) had described the method of rapid extraction of high molecular weight plant DNA which is free of contaminates. This method yields total cellular DNA comprising nuclear, chloroplast and mitochondrial DNA.

Doyle *et al.,* (1987) modified the DNA isolation protocol of CTAB method described by Saghai-Maroof (1984) by doubling the concentration of components involved in extraction buffer to compensate for the greater water content of fresh tissue.

Li *et al.,* (2007) described modified CTAB, DNA extraction protocol which was efficient and gave high yield of pure DNA from mature leaves of mutant sunflower, containing large fragments of polyphenols, tannins and polysaccharide. The use of PVP and β-mercaptoethanol plays important role in removing polyphenols which yielded high quantity pure genomic DNA, which is applicable for molecular biology studies.

2.3 Hybrid purity assessment by using molecular markers

2.3.1 RAPD markers in varietal identification and assessment of genetic purity of hybrids

Hashizume *et al.,* (1993) used RAPD as a molecular marker for purity assessment of hybrids seeds (f1) in watermelon and tomato crops. He has screened 59 oligonucleotide primers to assess purity of inbred parental lines and showed 3% polymorphism, revealed that there was a close relationship among parents used in hybridization programme. This study could reveal RAPD is effective methods to determine genetic purity of hybrid seeds in comparison to morphological method (GOT).

McDonald *et al.,* (1995) also utilized RAPD markers in genetic purity assessment technologies.

Paran *et al.,* (1995) described that RAPD marker was useful for identification of hybrids from their parents especially those which were difficult to differentiate morphologically.

The PCR based markers viz., RAPD & Single primer amplification Reaction (SPARS) were used as a tool for hybrid purity assessment by Bellester *et al.,* (1998). Five hybrids of peppers (*Capsicum annum* L.) & their parents were analyzed with 100 decamer primers & 10 nucleotide repeat primer.

Vinatzer *et al.,* (1999) have studied RAPD markers in varietal identification of fruit trees such as in peach and nectarine where RAPD analysis could not differentiate these cultivars. However, morphological analysis of fruit and trees proved more effective than RAPD primed fingerprinting of these varieties.

Crockett *et al.,* (1999) were used RAPD markers to evaluate seed purity in commercial hybrid of cabbage (*Brassicca oleracea* var. capitata). Two RAPD primers were selected and assessed for purity of f_1 hybrid seeds. The comparative study of RAPD analysis with grow out trial and isozyme analysis showed RAPD analysis could work efficiently for seed purity testing of commercial hybrid cabbage seeds.

Rubeena *et al.,* (2000) used RAPD markers in varietal identification of 22 cotton genotypes where single random primer could discriminate all cotton varieties at

molecular level. However, combination of five primers could enable identification of all cultivars.

The potential of RAPD markers in varietal identification and genetic purity test of hybrids were exploited by Ilbi *et al.,* (2003). They have screened five Japanese hybrids and their corresponding parents with 12 arbitary primers in which four RAPD markers were found to be cultivar specific for three hybrids and other RAPD markers were determined seed purity of hybrid varieties.

Jang *et al.,* (2004) standardized simple and efficient method for purity testing of hybrid pepper in which two RAPD markers identifying male and female parent's specific amplicons were cloned, sequenced and designed SCAR marker. This marker was used to assess purity of hybrid.

Nandini *et al.,* (2005) have been used sunflower hybrids, their parental lines and open pollinated varieties to identify and establish phylogenetic relationship among genotypes but, they were failed to correlate morphological trait with molecular markers. The total number of 25 scorable bands was generated with five arbitrary primers and the numbers of amplicons were ranged between three to nine. A male specific third band fragment of size (2027 bp) generated by the primer OPI-16 was inherited in KBSH 1 hybrid variety.

Akhare *et al.,* (2008) studied the suitability of RAPD marker for characterization of sorghum hybrids and their parents. Out of 15 decamer primers, six primers yielded good and scorable amplicons among four sorghum hybrids namely CHS-14, CSH-9, CSH-19 R, CSH-15 R and their respective parental lines.

Hybrid identification in *Gossypium hirsutum* L. through Random amplified polymorphic DNA (RAPD) molecular marker among three genotypes (CIM-511, SLS1 and Paymaster) and their hybrids (SLS1 × CIM-511, Paymaster × CIM-511, Paymaster × SLS1) was studied by Ali *et al.,*(2008). They have used 16 primers and generated 518 fragments in parents and hybrids and out of which 76 amplicons were polymorphic. The comparative study of RAPD banding pattern of the parents with the respective hybrids enabled identification of genuine hybrids. The dendrogram analysis on similarity matrix obtained by unweighted pair group method using arithmetic average (UPGMA) also

revealed the same results. Based on this RAPD analysis they have suggested that RAPD marker was reliable, less time consuming and efficient in hybrid identification.

Srinivasan *et al.,* (2009) developed SCAR markers for assessing hybrid purity of four sunflower hybrids HSFH 848, KBSH 1, KBSH 44 AND DSFH 118 and reported that RAPD markers have inherent disadvantage of less reproducibility. This limitation, of RAPD marker has been solved by using SCAR markers that significantly improve the reproducibility and reliability of PCR assays. The developed SCAR markers were dominant and showed the expected size of band in male and female parent during amplification.

Pranavi *et al.,* (2009) identified RAPD markers which could be used for genetic purity assessment of DCH 32 and DCH 519 hybrids of castor released from DOR, Hyderabad. OPE 16 gave a male specific and female specific band with the hybrid DCH 32. Based on this analysis it indicated that RAPD marker system could be used for seed purity testing of hybrids.

Kumar *et al.,* (2009) identified rapid method and assessed genetic purity of newly developed hybrid DRSH 1 in sunflower. They have screened 58 arbitrary primers with hybrid and their respective parents. The primer OPK 14 primer was generated male and female specific banding pattern. These unique amplicons were utilized and developed SCAR marker to asses the purity of hybrid of sunflower.

2.3.2 SSR markers used in hybrid purity assessment

Microsatellite also known as short tandem repeats (STR) or SSR consist of tandemly repeated units, each lies between 1 to 10 base pair in length such as (TG)n or (AAT)n and these repetitive DNA sequence which are spread throughout genome of eukaryotes, are often highly polymorphic due to variation in no. of repeat units. Thus, these sequences could serve as molecular signature to discriminate parental lines and their hybrids and help to assess hybrid purity.

Weising *et al.,* (1991) and Beymer *et al.,* (1992) determined application of microsatellite in plant for cultivar identification and now they are being the markers of choice in genotyping of cultivars.

Cregan *et al.,* (1997) reported that 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 microsatellite sequences and used to detect fragment length polymorphism among PCR product generated from different individuals based on variation in length. It results due to variation in the number of repeat units in the SSR. The cytoplasmic male sterile, Restorer line and hybrids can be screened by using microsatellite variation and STS polymorphism generated by SSR markers.

Yashitola *et al.,* (2002) demonstrated application of SSR and STS markers in genetic purity assessment in rice with objectives of replacing GOT with DNA based assays. Cytoplasmic male sterile (CMS), Restorer and hybrid lines have been screened by means of microsatellite and sequence tagged sites (STS) polymorphism. A simple procedure for detecting hetrozygosity and purity has been investigated which could be used for detection of hybrids from seed lots. Extent of hetrozygosity within parental lines of rice hybrids was assessed and the result could suggest that a single, appropriately chosen microsatellite marker enable to assess hybrid seed purity.

Nandkumar *et al.,* (2004) were used microsatellite markers for fingerprint analysis of hybrids to assess variation within parental lines & to test genetic purity of hybrid seed lot of rice. Ten sequence tagged microsatellite sites nine (STMS) markers were employed for fingerprinting of 11 rice hybrids & their parental lines, nine STMS markers were found polymorphic across the hybrids and produced unique fingerprint pattern for 11 hybrids. A set of STMS markers viz; RM 206, RM 216, RM258, & RM263 were differentiated all these hybrids from each other.

Li *et al.,* (2005) were reported the purity test method for identification of four maize hybrids using SSR markers. Twenty SSR markers were used to detect hybrid purity in maize hybrids. The polymorphic SSR loci have been selected and applied for purity testing of hybrids.

Gomez *et al.,* (2008) differentiated self lines and hybrid lines of peanut by using microsatellite marker and amplified SSR amplicons were separated by novel submarine horizontal polyacrylamide gel electrophoresis (H-PAGE). The 70% of putative hybrids were considered to be true hybrid on the basis of possessing allele specific marker from the male parent. H–PAGE gels gave better separation of bands and quick assay to

distinguish hybrids from in adherent self's and could result in greater efficiency and more effective use of resources in peanut breeding programmes.

Meng *et al.,* (2009) assessed purity of maize hybrid Suyu by using SSR markers. Maize hybrid Suyu 20 and its parents were subjected to SSR fingerprint by using 80 pairs of SSR. The four pairs of primers i.e. bnlg 1306, phi 065, bnlg 2291 and umc 1590 could detect polymorphism between parents and considered as candidates for purity assessment of Suyu 20.

Naresh *et al.,* (2009) used simple, rapid unbiased and cost effective DNA based assay and detected genetic purity of hybrid by replacing conventional, land and labor intensive, time consuming Grow Out Test (GOT). He has screened the parental lines of three commercial safflower hybrids of India viz; NH-1, NH-15 & DSH 129 by using 74 safflower EST–SSR markers. Five markers were shown polymorphic amplicons. A PCR based assay of three SSR markers showed the alleles from both parental lines in pure hybrids proving the hetrozygosity, while off types were also identified by the presence of either of the parental alleles.

Iqbql *et al.,* (2010) studied hybrid identification test of 16 sunflower hybrids by using simple sequence repeat (SSR) marker. He has screened 20 specific SSR primers out of which 18 markers showed purity of hybrids while remaining two primers gave ambiguous DNA fragments. They concluded that simple sequence repeat (SSR) marker could help in identification of hybrids derived from the crossing of different inbred sunflower lines. It also improved accuracy of selection, saved time and reduced cost of the experiment.

Sexena *et al.,* (2010) has recently developed a hybrid breeding technology based on elements of the cytoplasmic nuclear male sterility system with an objective of achieving breakthrough in productivity of pigeon pea. They were used 148 SSR markers including 32 novel markers reported for the first time on 159 A (CMS), B (maintainer) and R (fertility restorer line). The total of 41 (27.7%) markers showed polymorphism with two to six alleles. Among these polymorphic markers, 22 SSR markers showed polymorphism between A (ICPA 2039) & R (ICPR 2438) lines of commercial hybrid (ICPH 2438). Two SSR markers viz; CCB4 & CCttC 006 were found most suitable for purity assessment of hybrid seeds of the ICPH 2438.

2.4 Screening of sunflower hybrid against sunflower necrosis virus (SNV) *in vivo* condition

Screening test of hybrids or cultivars against diseases is required to pass pathogenicity test *in vivo* under controlled environment condition. Several plant breeder and pant biotechnologist have followed this test.

Jain (2000) has screened cowpea cultivars against TSV/SNV through pathogenicity test. The cultivar viz. *Vigna unguiculata* cvs. C 152 and Pusa Komal were infected by ELISA positive SNV infected sunflower samples from Dharwad and Hyderabad by using 0.1 M phosphate buffer (pH 7.2).

Sunflower necrosis virus was isolated from SNV infected sunflower plants (cv. Morden) collected from Tamilnadu Agricultural University by Bhat *et al.*, (2001). The SNV isolates were propagated on cowpea (*Vigna unguiculata* cv. Pusa Kiran) in a glasshouse condition through mechanical sap inoculations by using 0.1 M Phosphate buffer (pH 7.2) containing 0.1% mercapto ethanol.

Arvind (2002) reported that, the disease prevailed on most of the parental lines/hybrids/checks entries examined in experimental plots at Hebbal in kharif sown crop. The incidence of disease was more in hybrids, ranging from (0 to 13.04%) followed by parental lines, (0 to 9.4%) and least in check entries (0 to 5.12%). Disease incidence was also recorded in sunflower cv. Morden at GKVK campus in all the seasons. The maximum incidence was observed during summer months (April sown 26-27%), whereas it was moderate in kharif (12-13%) and moderately low during rabi (7-12%).

Materials & Methods

CHAPTER-III

MATERIALS AND METHODS

3.1 Place of research work

The present study entitled, "**Assessment of hybrid purity in sunflower by using molecular markers**" was carried out at college of Agricultural Biotechnology, Latur MKV, Parbhani during the year 2010-11. The materials and laboratory procedures followed during this research works are described in this chapter.

Material:

In the present investigation the source of material comprising of 10 hybrids and their male parent (Restorer line) and female parent (male sterile line A) were obtained from Oilseed Research Station, Latur (Table 1-3).

Table 1: Details of CMS lines used during course of investigation

Sr. No.	CMS Lines	Feature	Source
1	7-1A & B	Susceptible to downy mildew and necrosis, high seed yield, big head diameter	DOR, Hyderabad
2	17 A & B	High seed yield, susceptible to downy mildew and necrosis, low oil content	AICRP, Bangalore
3	10 A & B	Early, high seed yield potential	PAU, Ludhiana
4	234 A & B	Early, high oil content, susceptible to downy mildew, necrosis and *Alternaria.*	AICRP, Bangalore
5	343 A & B	Downy mildew tolerant, high seed yield potential	ORS, Latur.

Table 2: Details of Restorer lines (R lines)

Sr. No.	Restorer	Feature	Source
1	AK 345	Multihead, high seed yield potential	ORS, Latur
2	RHA-1-1	Multihead, downy mildew resistant, low hull content	ORS, Latur
3	LTR-08-08	Monohead, bold seed	ORS, Latur
4	NDLR 06	Monohead.	ORS, Latur
5	NDR-7-1B	Monohead, low hull content, high oil content	ORS, Latur
6	J-6	Monohead, Downy mildew, Necrosis and *Alternaria* resistant (multiple resistance), High seed yield and high oil content	ORS, Latur
7	LTR 07	Monohead, bold seed size and high seed yield potential	ORS, Latur

Table 3: Hybrids and their parental line used in this study:

Sr. No	Name of hybrid	Parents	source	Features
H1	LSFH-35	234A X RHA-1-1	ORS, Latur	Downy mildew resistant
H2	LSHT-16	7-1A X NDLR-06	ORS, Latur	High oil content
H3	LSHT-11	10A X NDLR-06	ORS, Latur	Earliness
H4	LSFH-10126	343A X NDR7-1B	ORS, Latur	High seed yield
H5	LSFH-07-03	234A X NDR7-1B	ORS, Latur	Earliness, high seed yield
H6	LSFH-10128	343A X J/6	ORS, Latur	high seed yield
H7	LSFH-9124	17A X RHA-1-1	ORS, Latur	High seed yield, Downy mildew resistant
H8	LSFH-10129	234A X LTR07	ORS, Latur	seed yield
H9	LSFH-1706	17A X NDLR-06	ORS, Latur	seed yield
H10	LSFH-7345	7-1A X AK345	ORS, Latur	seed yield

3.2 Grow Out Trials:

Conventionally genetic purity of hybrids is ensured by grow out test (GOT), which involves growing plants to the maturity and assessing several morphological and floral characteristics that distinguish the hybrids.

Ten different hybrids and their respective male and female parent were grown in the greenhouse. Regular irrigation, fertilization and crop protection measures were adopted and purity through visual evaluation was conducted on the main important morphological characters throughout the growth period.

3.3 Hybrid purity assessment by using molecular markers

3.3.1 Plant material

Ten hybrids and their respective parents were individually planted in pots at green house. After 15-20 days, fresh and primordial leaves were collected for bulk DNA extraction.

Reagents:

- CTAB buffer
- 0.17% 2-β Mercaptoethanol
- Liquid nitrogen
- Phenol/ Chloroform/ iso- amyl alcohol (25:24:1)
- Chilled iso-propanol
- TE buffer
- *RNase*
- 3M sodium acetate .(pH 5.2)
- 70% Ethanol

3.3.2 DNA Extraction

The plant genomic DNA was extracted by following the modified CTAB method described by Doyle and Doyle (1987). DNA was further purified by *RNase* treatment followed by extraction with phenol/chloroform/ iso- amyl alcohol (25:24:1) and ethanol precipitation as described by Mace *et al.*, (2003).

A. Preparation

CTAB buffer was preheated at 65°C in a water bath before the start of DNA extraction. Two-three young leaves were collected (final weight approximately 30 mg) from 15-20 days old plants.

B. Grinding and extraction

Grinding was carried out using liquid N_2 with the help of mortar and pestle and crushed leaf sample were transferred in microfuge tube containing 800 µl of preheated 2% CTAB buffer was added and incubated at 65°C in water bath for 45 min with gentle swirling.

C. Solvent extraction

Phenol: chloroform: iso-amyl alcohol (25: 24: 1) mixture of 450 µl was added to each tube, inverted twice and the sample was centrifuged (Eppendorf 5415R, Rotor model-F45-24-11) at 10,000 rpm for 10 min. The aqueous layer (approximately 300 µl) was transferred to a fresh microfuge tube. This step was repeated 3- 4 times.

D. Initial DNA precipitation

To each tube containing aqueous layer, 0.7 volumes (approximately 210 µl) of chilled iso-propanol was added, the solution was carefully mixed. The samples were centrifuged at 10,000 rpm for 10 min. Supernatant was carefully decanted and pellets were allowed to air dry.

E. *RNase* treatment

In order to remove co-isolated RNA, pellets were dissolved into 50 µl of low salt TE buffer and 1 µl of *RNase* (stock 10 mg/ml) was added to each tube. Tubes were incubated at 37°C for one hr. in water bath or overnight at room temperature.

F. Solvent extraction

After incubation 200 µl of phenol/chloroform/iso-amyl alcohol (25:24:1) was carefully added to each tube, mixed and centrifuged at 10,000 rpm for 10 min. This step was repeated 2-3 times.

G. DNA precipitation

The volume of 15 µl (approximately $1/10^{th}$ of volume) 3M sodium acetate and 300 µl (2 volumes) of 100% ethanol (kept at -20°C) were added to the supernant and the mixture was subsequently incubated in a freezer for 5 minute at -20°C. Following incubation at -20°C the tubes were centrifuged at 10,000 rpm for 15 minute.

H. Ethanol wash

After centrifugation the supernatant was carefully decanted in order to ensure that the pellet remains inside the tube and 200 µl of 70% ethanol was added to the tube followed by centrifugation at 10,000 rpm for 5 min.

I. Final suspension

The supernatant was carefully decanted and the pellet was allowed to air dry. Air dried pellets were resuspened in 100 µl of TE buffer and kept overnight at room temperature to dissolve completely. Finally DNA samples were kept at 4°C and after that at -20°C for long term storage.

J. Technical notes

The β-Mercaptoethanol (2%) and PVP should be added to the washing buffer just prior to use. The β-Mercaptoethanol inhibits the oxidation of polyphenolic

substances and PVP absorbs polyphenolic, thereby preventing their interaction with DNA (Loomis, 1974).

3.3.3. Determination of Quantity and Quality of Isolated DNA:

Quantitative and qualitative analysis of DNA was done by photometer (Eppendorf, *Bio*photometer). The blank reading was taken with 50 µl of distilled water. In 49 µl sterile distilled water 1 µl of DNA sample was added in Eppendorf Cuvette and absorbance was measured at 280 nm wavelength, also the purity of DNA was checked by measuring the OD ratio of A260/A280 nm. If the ratio is higher than 2.0 indicate the impurity of proteins and less than 1.8 indicates RNA impurity in sample. The amount of DNA was calculated by using the formula,

$$\text{DNA } (\mu g/\mu l) = \frac{\text{OD at 260 nm X dilution factor}}{1000}$$

3.3.4 Agarose Gel Electrophoresis

Agarose gel (0.8%) was prepared by dissolving 0.8 g of agarose in 100 ml 1X TBE buffer and ethidium bromide (10 mg/ml) was added. After solidification 5 µl of DNA was mixed with 1µl of 6X gel loading dye and loaded on 0.8 % agarose gel. The electrophoresis was carried out at 100 V for 1.5 hr using 1X TBE buffer.

3.3.5. Dilution of DNA samples

DNA sample was diluted with appropriate quantity of sterilized distilled water to yield a working concentration of 30 ng /µl for RAPD and SSR markers analysis.

3.3.6 Selection of markers

3.3.6.1 RAPD Primers

Table 4: List of RAPD primers used for purity assessment study

No.	Primers	Sequences (5'- 3')	No. of nucleotides bases
1.	OP-03	GGCACTGAGG	10
2.	OPA 02	TGCCGAGCTG	10
3.	OPA 03	AGTCAGCCAC	10
4.	OPA 07	GAAACGGGTG	10
5.	OPA 08	GTGACGTAGG	10
6.	OPA 09	GGGTAACGCC	10
7.	OPA 11	CAATCGCCGT	10
8.	OPA 13	CAGCACCCAG	10
9.	OPA 20	GTTGCGATCC	10
10.	OPE 16	GGTGACTGTG	10
11.	OPL 18	ACCACCCACG	10
12.	OPC 16	GGTGACTGTG	10

3.3.7. RAPD Analysis

Table 5: Reagents and stock solutions used for RAPD analysis

Sr. No.	Reagents	Stock solutions
1.	dNTPs	10 mM
2.	$MgCl_2$	50 mM
3.	*Taq* DNA polymerase	5 U/µl
4.	PCR buffer	10X
5.	Primer	10 pm/µl
6.	TBE	10X
7.	DNA	30 ng/µl

3.3.7.1. PCR Reaction

Random primers derived from Imperial life science were used for RAPD analysis of 10 hybrids and their respective parents. For these, 12 decamer primers were used. The PCR reactions of 25 µl volumes were set (Table 6).

Table 6: Components, volume and concentration of PCR reaction mixture were used in RAPD fingerprint analysis

Sr. No.	Components	Final concentration	Volume for one reaction (µl)
1.	Sample DNA (25 ng/µl)	100 ng	2.0
2.	10X PCR buffer	1X	2.5
3.	dNTPs	0.2 mM	0.5
4.	*Taq* DNA polymerase	1 U	0.2
5.	$MgCl_2$	1.5 mM	1.5
6.	Primer	10 pmol	2.0
7.	Sterile dH_2O	---	16.3
8.	**Total**	---	**25.00**

3.3.7.2 Optimization of Polymerase Chain Reaction (PCR) condition for RAPD markers

PCR reaction mixture was prepared with the above mentioned components and equally distributed (23 µl) into 30 PCR tubes. Genomic DNA (2 µl) derived from 30 different samples of sunflower hybrids and their parental lines were added. PCR tubes were placed in thermal cycler (Eppendorf) for amplification of the genomic DNA as per the standardized protocol, which is enumerated below

3.3.7.3 Temperature profile

Table7: PCR Program used for RAPD DNA fingerprint profile

Sr. No.	Steps	Temperature (^{0}C)	Time requirement
1	Initial denaturation	94	5 min
2	Denaturation	94	45 sec
3	Annealing temp.	36	1 min
4	Extension	72	1 min
5	No. of cycles	45 Cycles	
6	Final extension	72	10 min
7	Hold	4	Forever

3.3.7.4 Resolution of amplified product

The PCR amplified products was resolved on 1.5 % agarose gel at 100 V for 2.5 hr. The gel was stained with ethidium bromide (5mg/ml) and image was captured by Gel Documentation System (Alpha imager, TM-2200).

3.3.8 SSR markers analysis

The SSR marker DNA fingerprint technique was used to assess genetic purity of 10 hybrids with their respective parents through genotyping. PCR reaction was performed with parental DNA by using 5 pair of Sunflower SSR markers. The source of these primers was cited in Table 8.

3.3.8.1 SSR Primers

Table 8: List of SSR Primers selected for hybrid purity test in sunflower

Sr. No.	Primers		Sequences (5'- 3')	References
1	ORS 13	F	GAA TAA CCT TGT GGA GTT TGC C	Liu *et al.*, (2007)
		R	CCT CAT TCT CAT TCT CTC CAC C	
2	ORS 1114	F	AGATGGTGGCAGGAGAGTTAAAG	
		R	GCAGAAACAGATCAGGAGGGTAT	
3	ORS 536	F	GAAATAGGAGGGGATCTTACCG	
		R	GCGGAGAGAAAGACGAAGAG	
4	ORS 243	F	GGGATGACGTGCGTTTGG	
		R	ACCACCATTTCTACCGTTTCTC	
5	ORS 662	F	CGGGTTGGATATGGAGTCAA	
		R	CCTTTACAAACGAAGCACAATTC	
6	Ha 432	F	GT CTT TAT CCC CCA CCC CCT CC	Antonova *et al.*,(2006)
		R	GGG TTT AGT GGC CAG TAG TTG TC	
7	Ha 514	F	GA GGT CAA CGG ATT TAG AGT C	
		R	GTA TTG ATT CCA ACA TCC AG	
8	Ha 1327	F	ATT CCG TTA GGT AGT TTA CTT GCGAC	
		R	GGT GGG GGG AAT ATT CTG AGG TG	
9	Ha 1442	F	ATT GCT TAT GTG CTT ACG TGT TCC TG	
		R	CTA AAC AGT TCG GCG AGT GTA GG	
10	IUB 3	F	TTTTTTTGGCATTAGGTAGATAGCCCCAG	
		R	GTG GTA CCC TCA CTA GTC CTC T	
11	ORS 6	F	AGG GTG GAG AGA GGT GTA GAG AGC	
		R	CAC CCC TCA CCC TGA CAC	
12	ORS 5	F	AAC ATC TGG AGC AGC AAA TTC AG	
		R	CTG CTG CCC ACC ATA CTG	
13	IUB 6	F	GT TCG GTA TCG TTT GCT AAT GG	
		R	GGT AAC TCT AAA GCT CTG TC	
14	HNCA 2	F	GT TGA GAC AAG CAT AAG CAC	
		R	TAG ACA AGA CAA GGG ACT	
15	CRT 162	F	TAACCACCGTTCACCACCACAC	Stephen Smith *et al.*, (2009)
		R	GTTTCTTTCCGGTCTTTTTCCGATGATGT	
16	ORS 317	F	TTTGGCAGTTTGGTGGCTTA	Dinesh Kumar *et al.*, (2009)
		R	GGTCGTATGCTTAATTCTTTC TCT	
17	ORS 391	F	AGACTGGAGGGTATGGAGAGC	
		R	GCTCGGTAAGGAGGGAGAAA	
18	ORS 323	F	CGGGAAACTAGGATCAGAGG	
		R	GCCGGAGGATTAGAGGAGTT	

3.3.8.2 Optimization of Polymerase Chain Reaction (PCR) condition

The PCR conditions were optimized using a modified Taguchi method which is designed to reveal the effect and interaction of PCR components simultaneously on PCR product, (Cobb and Clarkson, 1994 and Ferguson *et al.*, 2004). In Taguchi method, reaction components that are likely to affects the PCR product are arranged in a orthogonal array, each of the components occur at one of three predetermined level (A, B and C), each occur in an equal number of times within the orthogonal array.

PCR amplification reaction was carried out for 5 µl reaction mixture in thermal cycler (Eppendorf) containing 10 ng of genomic DNA, 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 10 pmol of each forward and reverse primer and 5 U/µl of *Taq* DNA polymerase (MBT, Fermentas, U.K.).

The touchdown PCR reaction conditions were laid for initial 5 cycles containing cyclic parameters of initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 61°C (-0.5°C/ cycle) for 45 seconds and extension at 72°C for 1 minute similarly that cyclic parameter for next 30 cycles was also set and contains denaturation at 94°C for 45 seconds, annealing at 56°C for 45 sec and extension at 72°C for 1 min at the end final extension was set at 74°C for 7 min.

Table 9: Orthogonal arrays used for reaction components for SSR

Reaction number	DNA* (ng)	$MgCl_2$†(mM)	dNTPs‡(mM)
1	A	A	A
2	B	B	B
3	C	C	C
4	A	B	C
5	B	C	A
6	C	A	B
7	A	C	B
8	B	A	C
9	C	B	A

* DNA concentration: A = 5, B = 10, C = 15

† $MgCl_2$ concentration: A = 1.0, B = 1.5, C = 2.0

‡ dNTPs concentration: A = 0.10, B = 0.15, C = 0.20

3.3.9. Data scoring and Analysis

3.3.9.1 Data scoring and analysis of RAPD markers

The amplified products generated from RAPD PCR reaction was resolved on agarose gel. The RAPD amplicons which distinguish female and male parents from hybrid lines were scored. The amplicon size was determined by comparison with 100 bp and 1 kb DNA ladder (MBT, Fermentas, U.K.).

Data analysis was performed using PASTA software 2.07 (Hammer *et al.*, 2001) The DICE program was also used to calculate the dissimilarity coefficient among genotypes. Similarly dendrogram was constructed using Neighbor-Joining weighted analysis.

3.3.9.2 Data scoring and analysis of SSR markers

For purity assessment experiments, the allelic data obtained in bp were analyzed as allele sizes. For understanding relationships among parental lines of hybrids, allelic data thus obtained were used to prepare a dissimilarity matrix and to construct a two dimensional (2D) plot using the factorial analysis method with PASTA software.

3.4 Screening of Sunflower hybrids against Sunflower necrosis disease (SND):

3.4.1 Isolation and inoculation of SNV isolates

Materials:

Ten sunflower hybrids listed in Table no. 3 were grown in earthen pots filled with sterilized soil, sand and compost mixture in the proportion of 2:1:1 at greenhouse.

Reagents:

i) Potassium phosphate buffer (KH_2PO_4)

ii) Corborundum powder (400 mesh)

iii) Absorbent cotton

iv) Muslin cloth

Procedure:

1. The sunflower necrosis virus infected leaf samples were collected from Oilseed Research Station, Latur.
2. The infected leaf sample (100 gm) was ground in cold mortar & pestle with potassium phosphate buffer (KH_2PO_4).
3. The homogenized sap was filtered through muslin cloth.
4. The small amount of corborundum powder (400 mesh) was dusted on leaf of old healthy sunflower plant.
5. Inoculations were made by conventional leaf rub method by using prewetted cotton swab to the viral inoculation.
6. The inoculated plants were washed immediately with sterile water and maintained in an insect free green house.

3.4.2 Grading of symptoms:

Symptoms were graded as per following:

1. No symptoms
2. Necrosis on inoculated leaves only (no systemic spread)
3. Systemic chlorotic symptoms
4. Systemic chlorotic and necrotic symptoms
5. Severe chlorosis and necrosis and premature death of plants.

Using this disease scale from 1-5 rating, percent disease severity for each of the event worked out separately by using individual seedling using disease scale. On the basis of disease scale SND disease severity was measured.

Based on visual external symptoms percent disease incidence for ten hybrids were laid for screening against SND.

$$PDI = \frac{\text{No. of plats infected}}{\text{Total No. of tested plants}} X\ 100$$

PDI: Percent Disease incidence

For calculation of percent disease incidence, disease severity (SND) score was retrieved from manual of Annual Research worker's group meeting on sunflower, DOR Hyderabad.

Table 10: SND disease severity scoring (0-5 scale)

Sr.No.	Diseases severity	Percent disease incidence
1	Immune	No infection or "0" incidence
2	Resistant	1 to 10% incidence
3	Moderately Resistant	11 to 25% incidence
4	Moderately susceptible	26 to 50% incidence
5	Susceptible	51 to 75% incidence
6	Highly susceptible	>75% incidence

Results

CHAPTER-IV

RESULTS

Full potential of any hybrid can be realized only by using good quality seeds and hence determination of genetic purity is an essential requirement for its commercial success. Conventionally genetic purity test is being done through GOT, which requires one full season. Due to this, the hybrid seeds are not available for immediate cultivation leading to additional expenditure in storage and hence increased the cost of hybrid seed lot. Considering the inherent disadvantage of GOT for genetic purity testing, molecular-based seed purity assay could be a better alternative and it is currently receiving more attention. Several researches have used RAPD and SSR markers for assessing the hybrid purity in crop plants (Pendse *et al.,* 2001, Ilbi, 2003, Liua *et al.,* 2007, Sundaram *et al.,* 2008).

4.1 Genetic purity assessment of sunflower hybrids by using molecular markers (RAPD and SSR)

4.1.1 DNA isolation and quality analysis

The high molecular weight genomic DNA was extracted from young leaves of ten hybrids and their respective parents of sunflower by modified CTAB method described by Doyle and Doyle (1987). This method yielded qualitatively as well as quantitatively pure genomic DNA. The quantification of extracted DNA was done by measuring absorbance of 260 nm wavelength. Purity of DNA was checked by reading absorbance ratio of A260/280 for protein contamination and A260/A230 for the presence of polyphenolic / polysaccharide compound. Also the quantitative and qualitative analysis was done by resolving DNA on 0.8 % agarose gel. The concentrations of all samples were ranged between 500-1000 ng /µl. Working samples were prepared by diluting with nuclease free sterile water of concentration 25 ng /µl.

4.1.2 Optimizations of PCR components for RAPD reaction

4.1.2.1 DNA

The quantity of template DNA has a great influence on the generation and resolution of amplified products. The quantity of DNA in PCR reaction mixture was optimized with different concentration viz; 20 ng /µl, 25 ng /µl, 30 ng /µl and 35 ng /µl.

The 2 µl volume of DNA having concentration of 25 ng /µl was found good for PCR amplification.

4.1.2.2 Primer

All primers used in this study were synthesized from Imperial life science. Primers were dissolved in nuclease free sterile water as per manufacture's instruction. The 2 µl volume of 10 pmol primer were found best for amplification of DNA.

4.1.2.3 $MgCl_2$

Different concentration (0.5 mM, 1.0 mM, 1.5 mM, 1.75 mM) of $MgCl_2$ were tried to obtain good amplification of DNA. The low concentration of $MgCl_2$ gave poor amplification while excess concentration results in smearing of bands. Therefore among these concentrations 1.5 mM concentration of $MgCl_2$ was found good for amplification of DNA.

4.1.2.4 dNTPs

The four types of deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) react with Mg^{2+} ions and influence precise amplification of product. Lower concentration of $dNTP_S$ minimizes the chances of generation of amplicons. Four different concentrations of $dNTP_S$ (0.05 mM/µl, 0.1 mM/µl, 0.15 mM/µl, 0.2 mM/µl) were used and the concentration 0.2 mM/ µl gave good amplification of PCR product.

4.1.2.5 *Taq* DNA polymerase

The *Taq* DNA polymerase was used at various concentrations (0.5 U, 0.8 U, 1.0U, 1.5U), from which 1 U of *Taq* DNA polymerase was found good for PCR amplification.

Table 11: Optimization of PCR component for RAPD analysis

PCR component	Tested quantity	Optimum condition	Result
$MgCl_2$ (mM)	0.5, 1.0, **1.5**, 1.75	1.5 mM	Low concentration of $MgCl_2$ gives poor amplification while excess concentration results in smearing of bands or non specific amplification
Primers (pmol)	5, **10**, 15, 20	10 pmol	Lower concentration of primers fails to amplify DNA
DNA (ng/µl)	20, **25**, 30, 35	25 ng/ µl	Higher concentration gives high background effect
dNTP (mM)	0.05, 0.1, 0.15, **0.2**	0.2 mM	Lower concentration of dNTPs showed lack of reproducibility.
***Taq* DNA polymerse** (U/ µl)	0.5, 0.8, **1.0**, 1.5	1.0 U/ µl	Higher concentration high background (smearing) and decreased specificity in gel electrophoresis.

4.1.2.6 PCR cyclic parameters for RAPD reaction

Annealing temperature is the critical parameter of PCR cyclic conditions of RAPD fingerprint reaction. Four different levels of annealing temperature (35-38^0 C) of RAPD-PCR was set in RAPD fingerprint reaction. The annealing temperature 36^0 C could produce more distinct and clear RAPD fingerprint pattern. Thus annealing temperature 36^{0}C was further used in purity assessment of sunflower hybrid through RAPD fingerprint reaction.

4.1.2.7 Optimization of PCR components and cyclic condition for SSR marker

PCR conditions for SSR fingerprint reactions were optimized as per method described by Taguchi.

After optimization PCR amplification was carried out in 25 µl of reaction mixture containing 25 ng of genomic DNA, 1X PCR buffer and 0.2 Mm each of four

dNTP, 2 mM $MgCl_2$, 0.5 µM of each forward and reverse primer (Imperial Life Science) and 0.5 U of *Taq* DNA polymerase.

Eighteen SSR primers were used for PCR amplification of 10 hybrids and their respective parents. The annealing temperatures for eighteen SSR primers were optimized by using different levels of temperature gradients. The list of primers and their optimized annealing temperature is listed in Table 12.

Table 12: Optimized annealing temperature of SSR markers

Sr. No.	SSR primers	Annealing temperature (^{0}C)
1	ORS 243	55(for initial 5 Cycles) and 53 (for remaining 30 Cycles)
2	ORS 536	57 (for initial 5 Cycles) and 55 (for remaining 30 Cycles)
3	ORS 1114	57 (for initial 5 Cycles) and 55 (for remaining 30 Cycles)
4	ORS 317	53.6
5	ORS 323	55
6	ORS 391	55.2
7	ORS 662	55
8	ORS 13	54.7
9	CRT 662	53.2
10	Ha 432	59.2
11	Ha 1327	59.2
12	Ha 1442	61.2
13	ORS 6	60.9
14	ORS 5	56.9
15	IUB 6	50.8
16	HNCA 2	47.3

4.1.3 Genetic relationship among hybrid and their parental lines based on RAPD fingerprint profile analysis

Sunflower parental lines (13) were used in different combination for the production of ten hybrids of sunflower at ORS, Latur. Therefore RAPD marker genotyping data were used for understanding the diverse nature and similarity among parental lines used in different combinations.

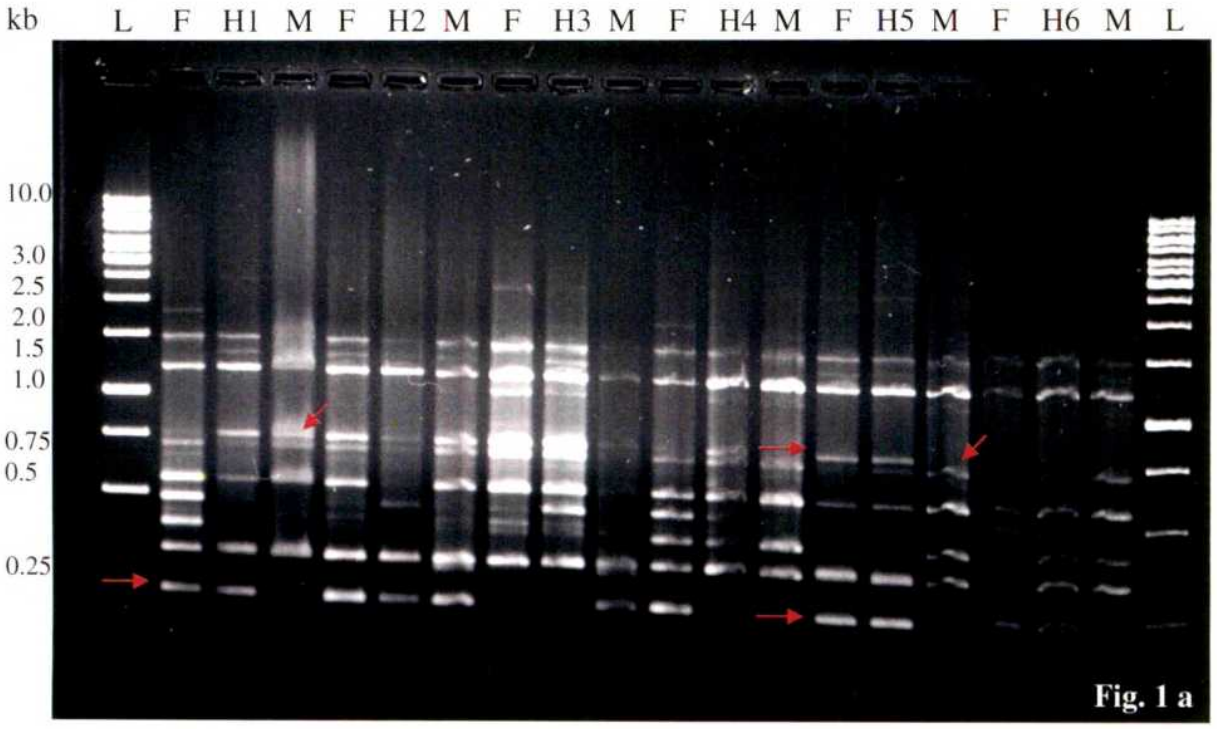
For this study twelve number of arbitary primers were used and generated similarity matrix of parental lines and hybrids of sunflower. The total of 12 primers, nine were found polymorphic and generated total of 189 polymorphic RAPD amplicons. The primer OPA 11 could produce maximum of 60 % polymorphism while primer OPL 18 showed minimum 12.5 % polymorphism (Table 13).

Table 13: Polymorphism among hybrids and their parents on the basis of RAPD analysis (RAPD data)

S. No.	RAPD primers / Hybrids	No. of polymorphic bands										Total no. of polymorp-hic amplicons	Total no. of bands	Mean	Polymorphism (%)
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10				
1	OP 03	7	5	7	4	4	3	8	3	5	2	48	10	4.8	48
2	OPA 07	3	1	3	4	2	2	4	4	6	3	32	8	3.2	40
3	OPA 08	1	1	1	2	1	2	1	1	1	1	12	2	1.2	60
4	OPA 09	2	0	2	1	1	2	4	2	1	0	15	5	1.5	30
5	OPA 11	2	2	1	3	1	3	4	3	3	2	24	4	2.4	**60**
6	OPC 16	0	0	2	3	3	4	4	2	4	1	23	4	2.3	57.5
7	OPE 16	2	2	3	4	2	1	4	1	4	2	25	8	2.5	31.25
8	OPL 18	0	0	0	0	1	0	2	0	1	1	5	4	0.5	**12.5**
9	OPA 20	0	0	0	0	1	1	0	1	1	1	5	3	0.5	16.66

Where **H1 to H10**: Ten sunflower hybrids as listed in Table 3 respectively.

Similarity matrix was constructed from RAPD data by using dice similarity coefficient. Based on RAPD data similarity coefficient was ranged from 61 % common



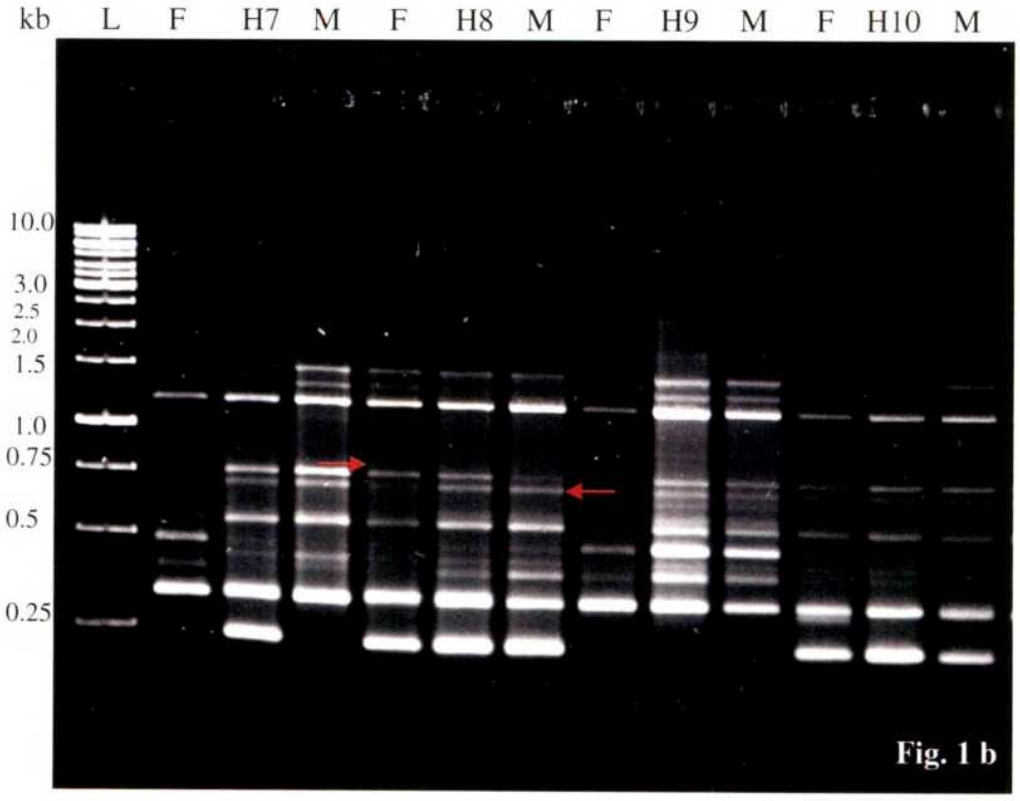
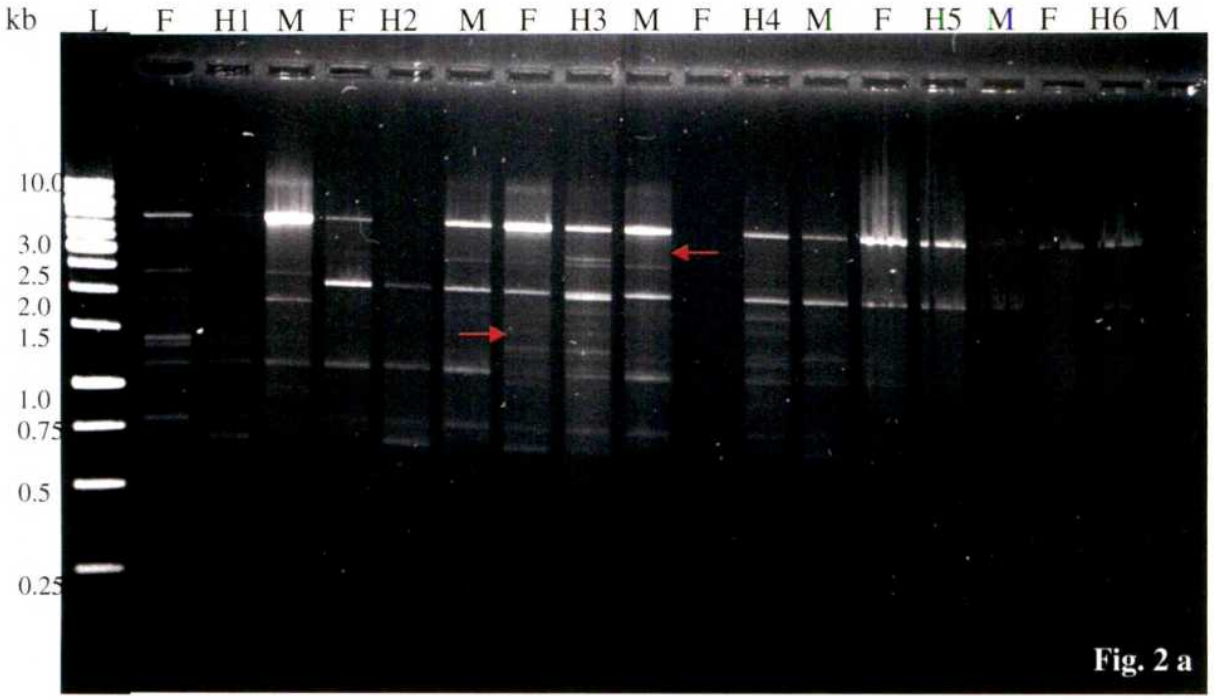


Fig. 1(a & b):RAPD profile of 10 hybrids, generated with primer OP-03; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.



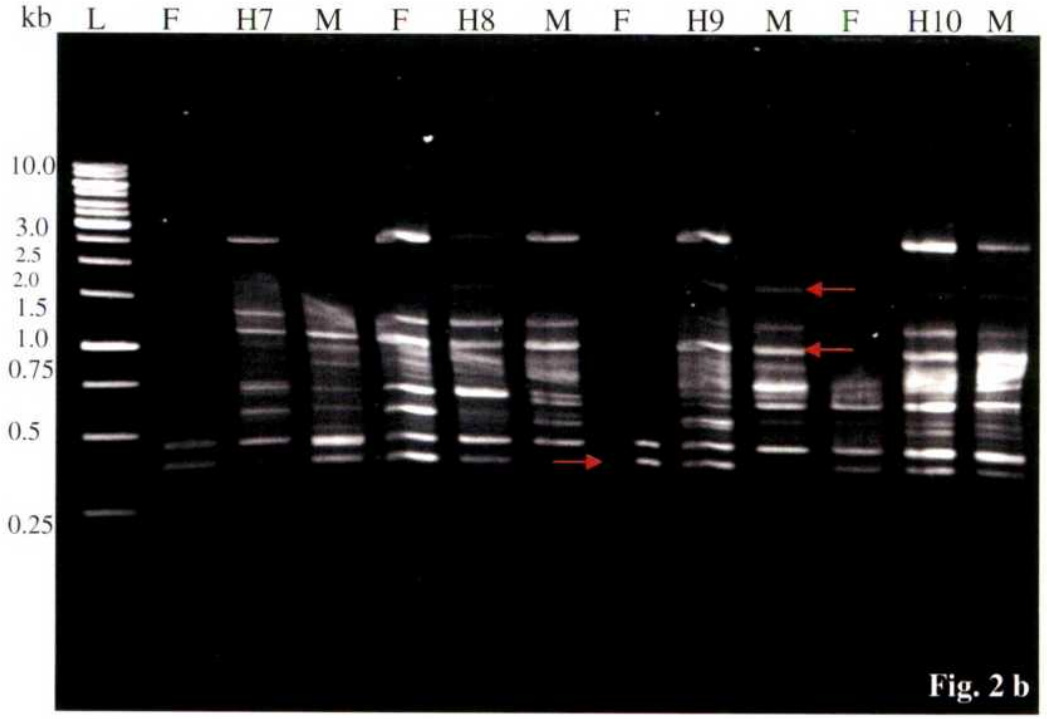
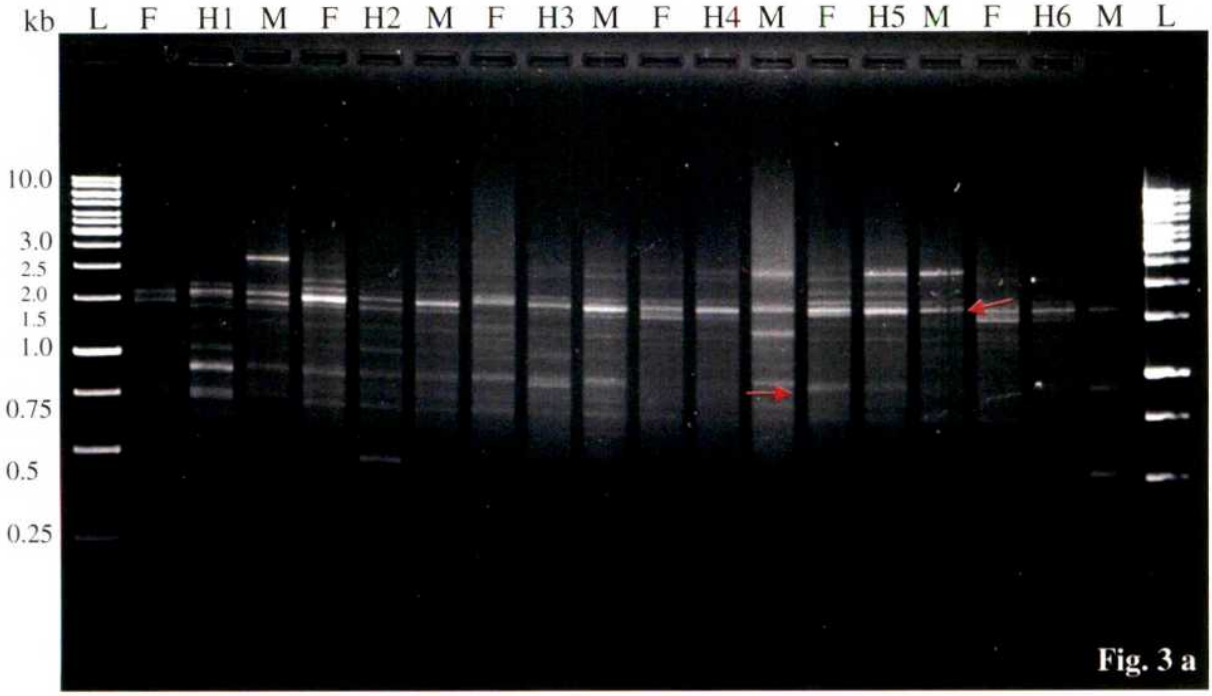


Fig. 2 (a & b):RAPD profile of 10 hybrids generated with primer OPA 02; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.



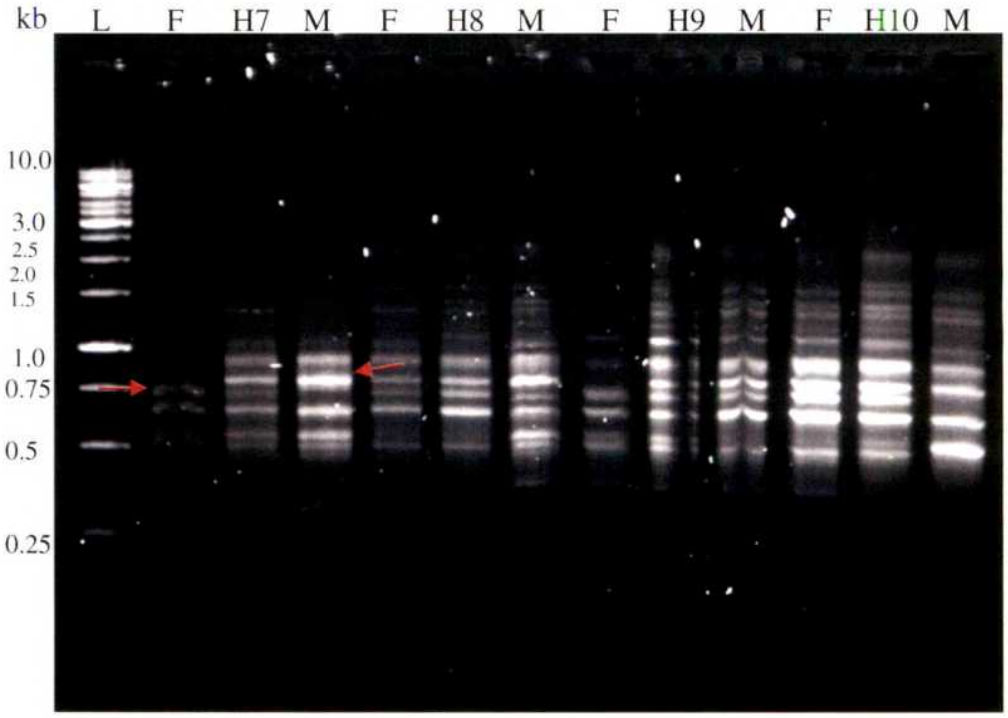
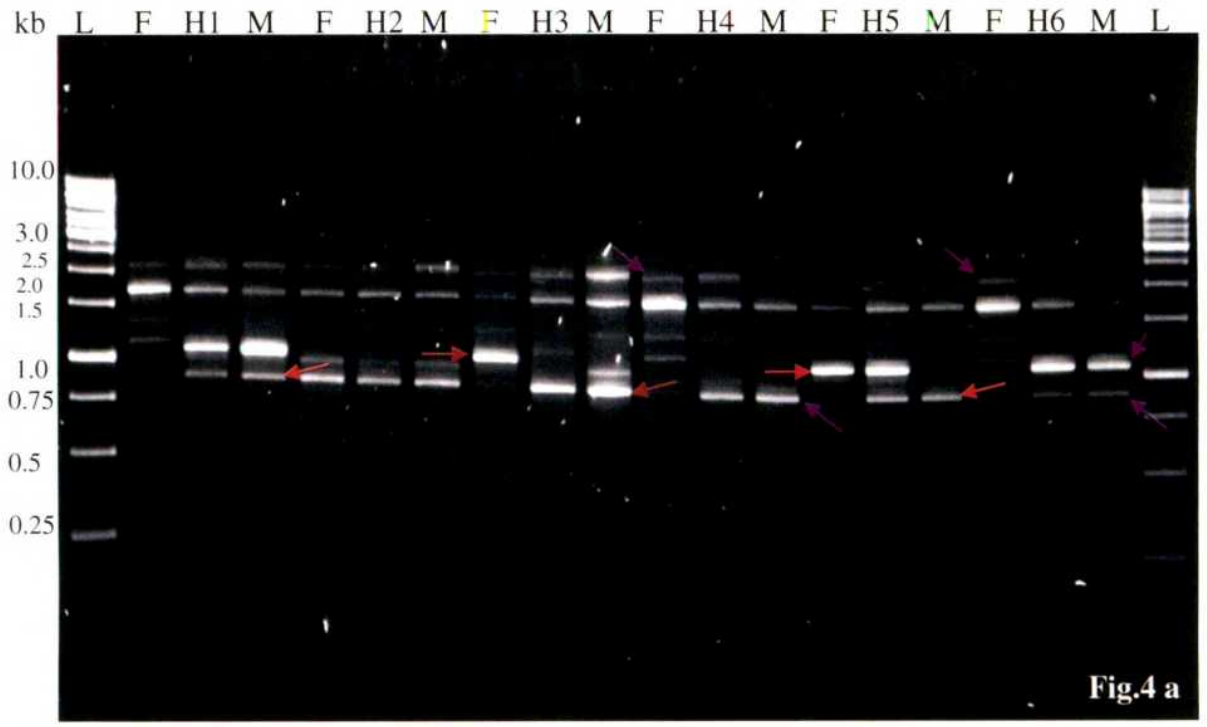


Fig. 3 (a & b): RAPD profile of 10 hybrids generated with primer OPA 03; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



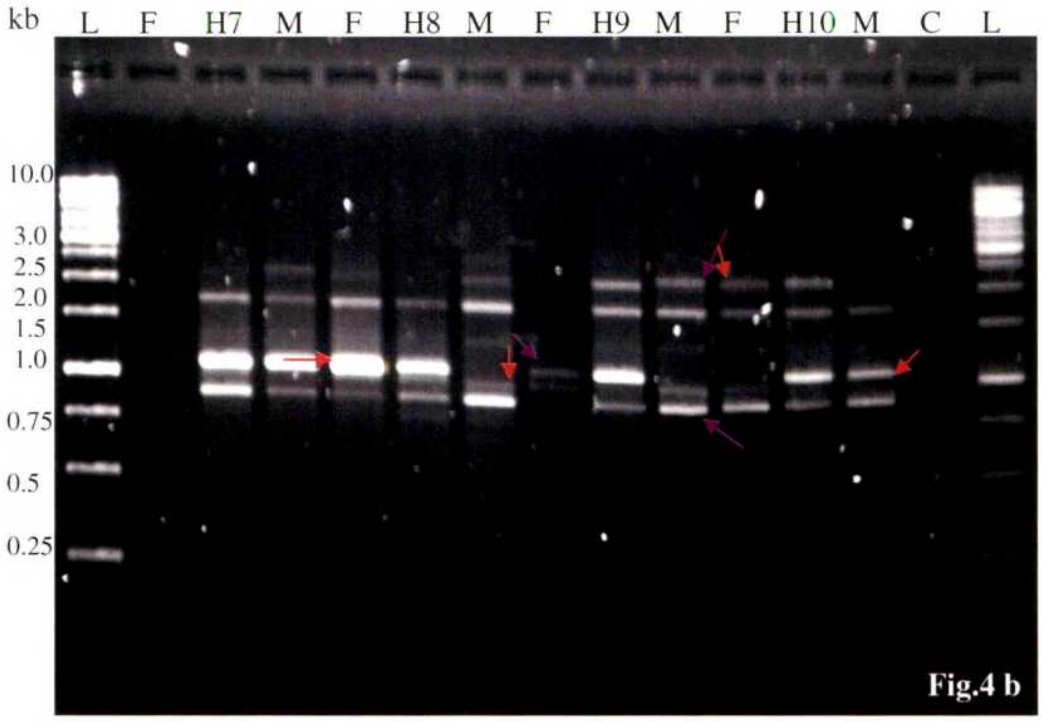
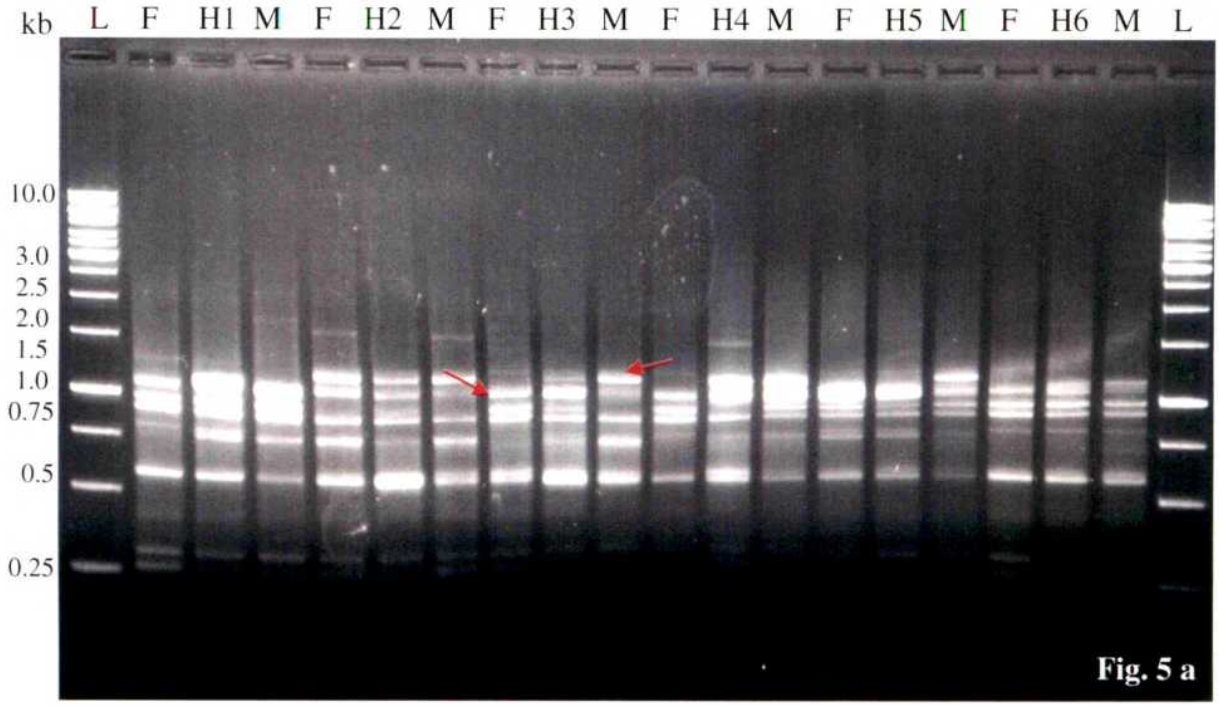


Fig. 4(a & b): RAPD profile of 10 hybrids generated with primer OPC 16; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids, C- control.



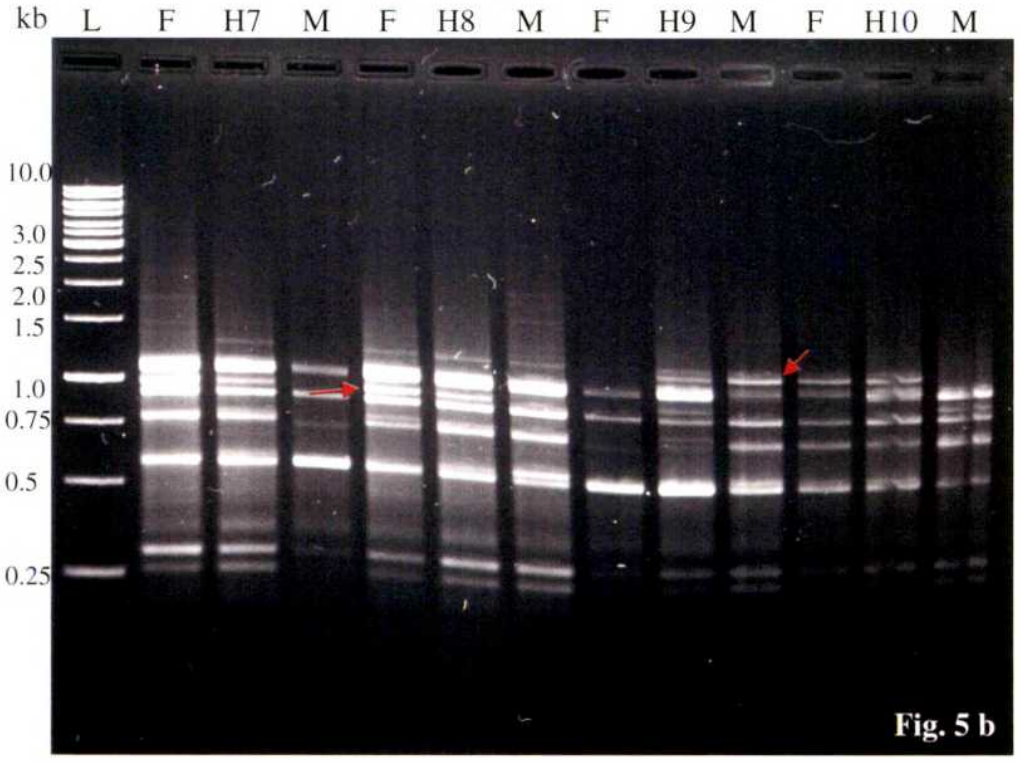
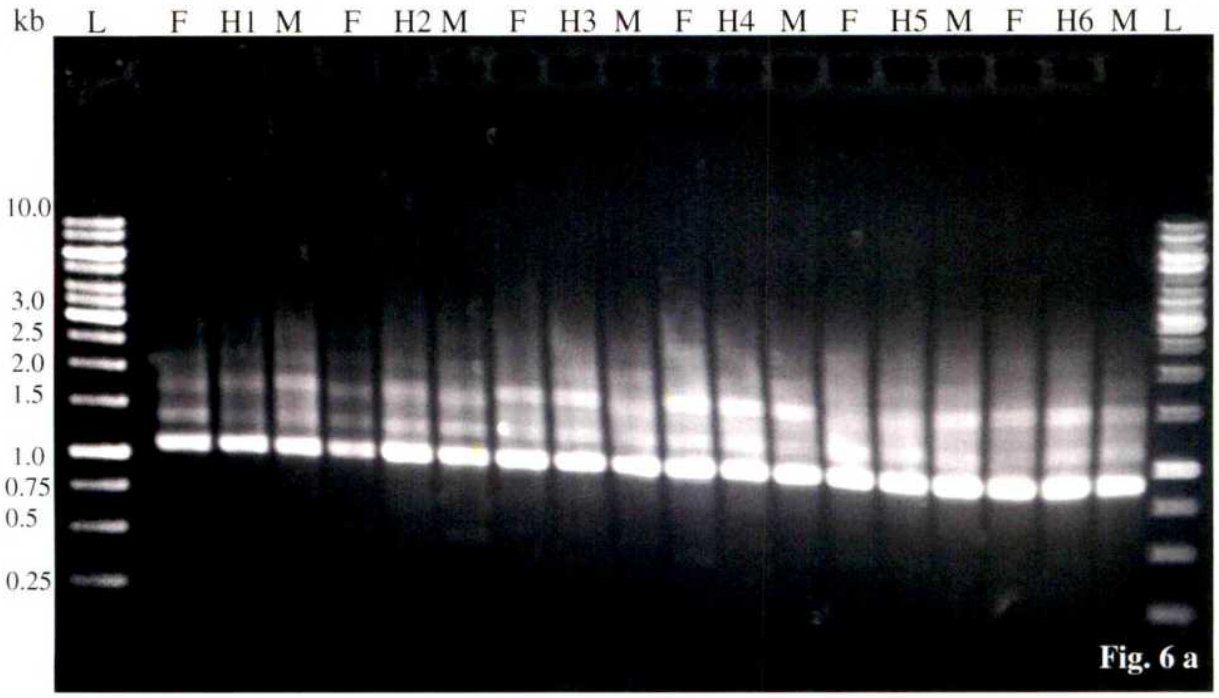


Fig. 5 (a & b): RAPD profile of 10 hybrids generated with primer OPE 16; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.



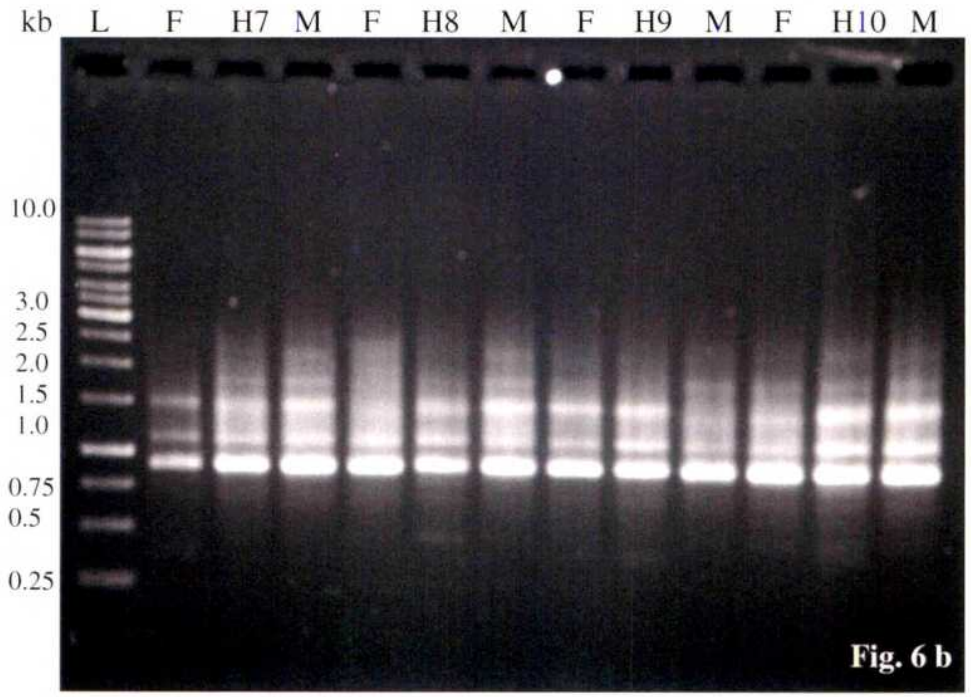
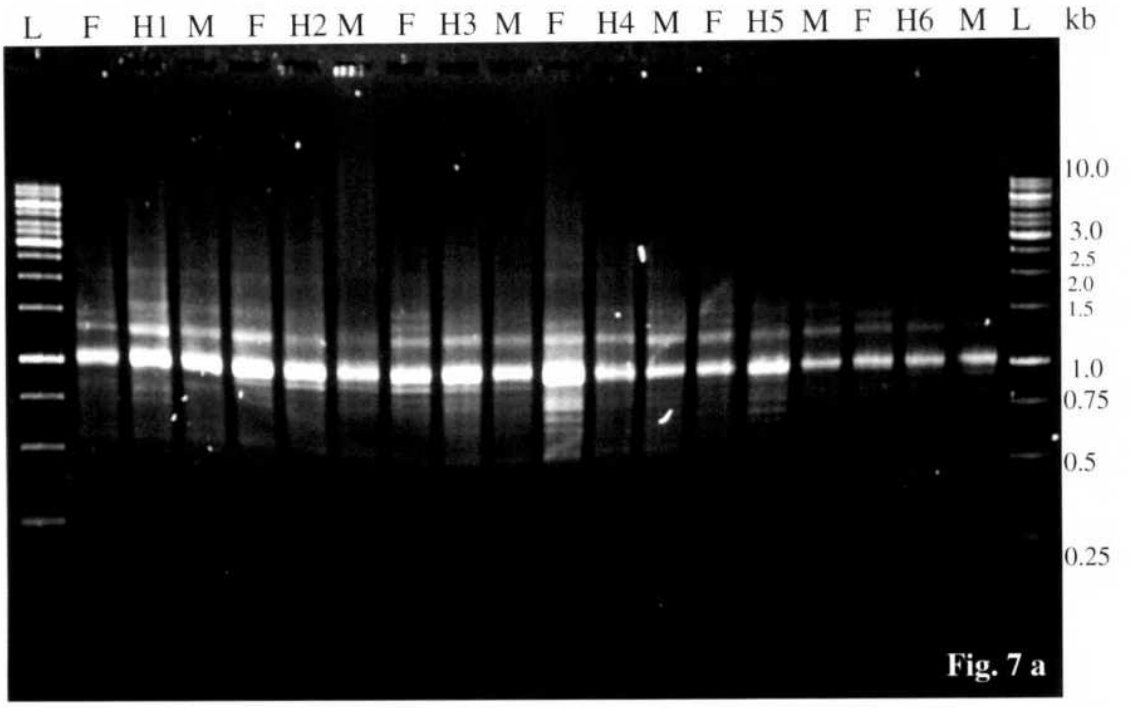


Fig.6(a & b): RAPD profile of 10 hybrids generated with primer OPA 20; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.



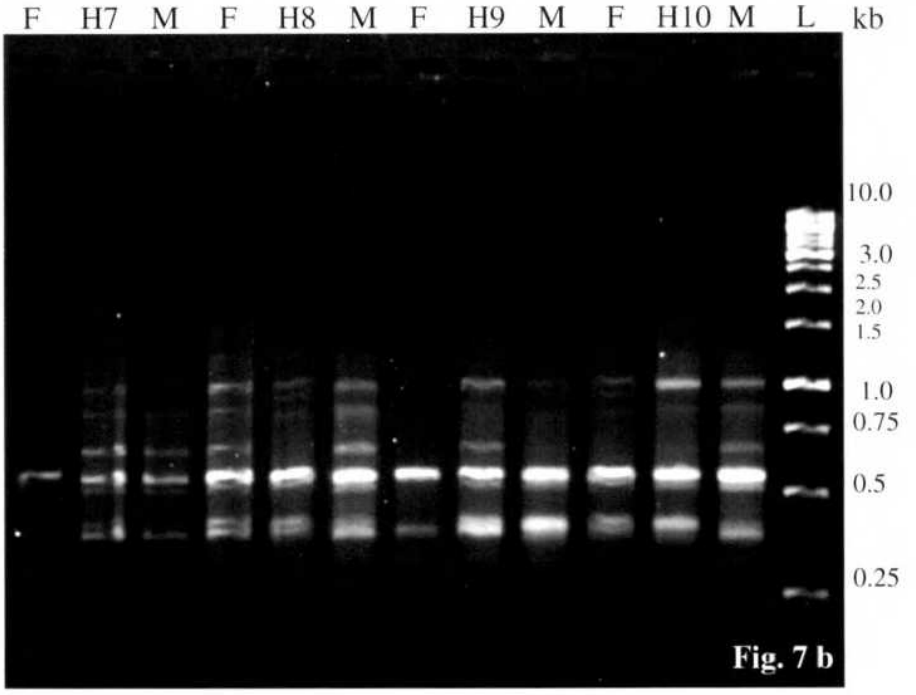
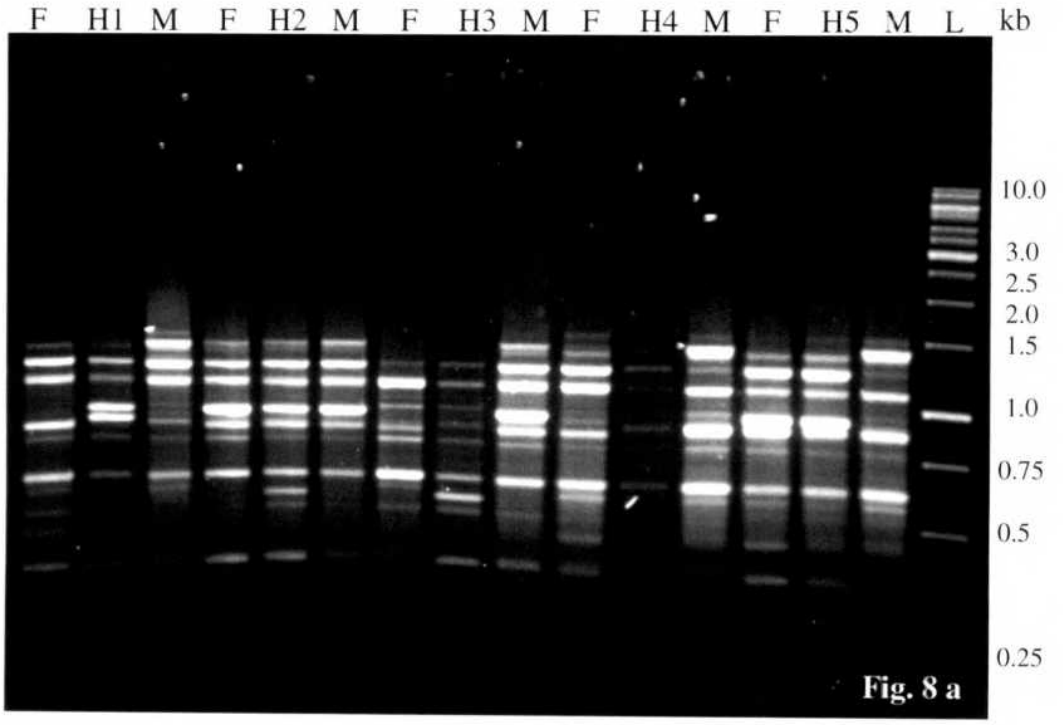


Fig. 7 (a & b): RAPD profile of 10 hybrids generated with primer OPA 13; L-1kb DNA ladder, F- Female parent, M- male parent and H1-H10: 10 hybrids.



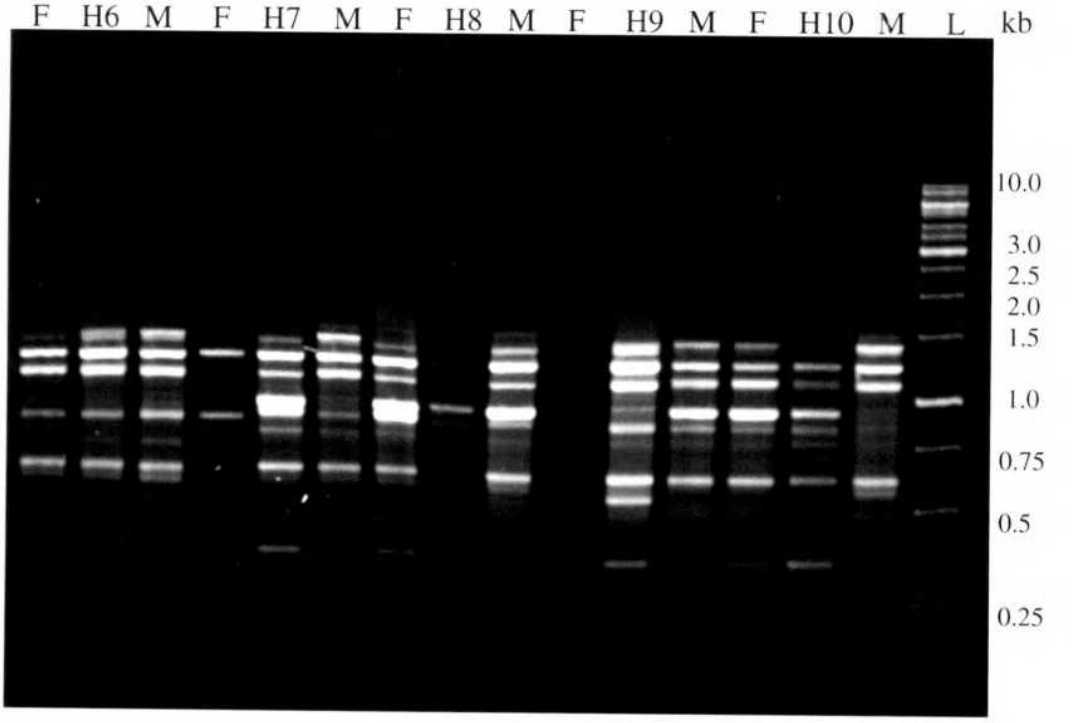
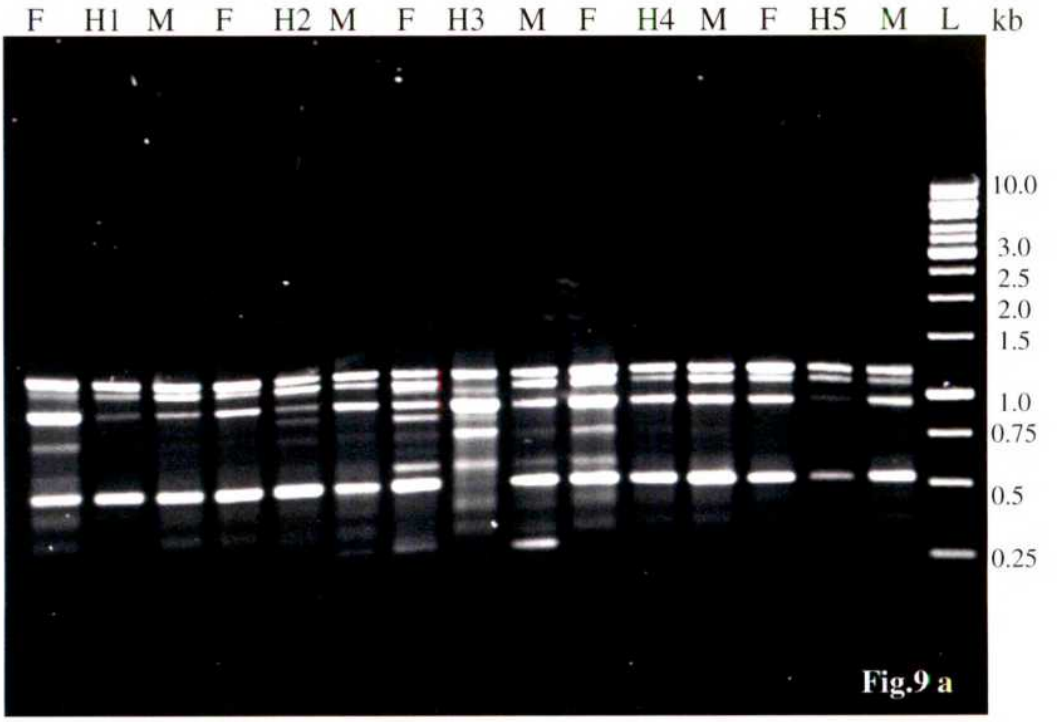


Fig. 8 (a & b): RAPD profile of 10 hybrids generated with primer OPA 07; L-1kb DNA ladder, F- Female parent, M- male parent and H1-H10: 10 hybrids.



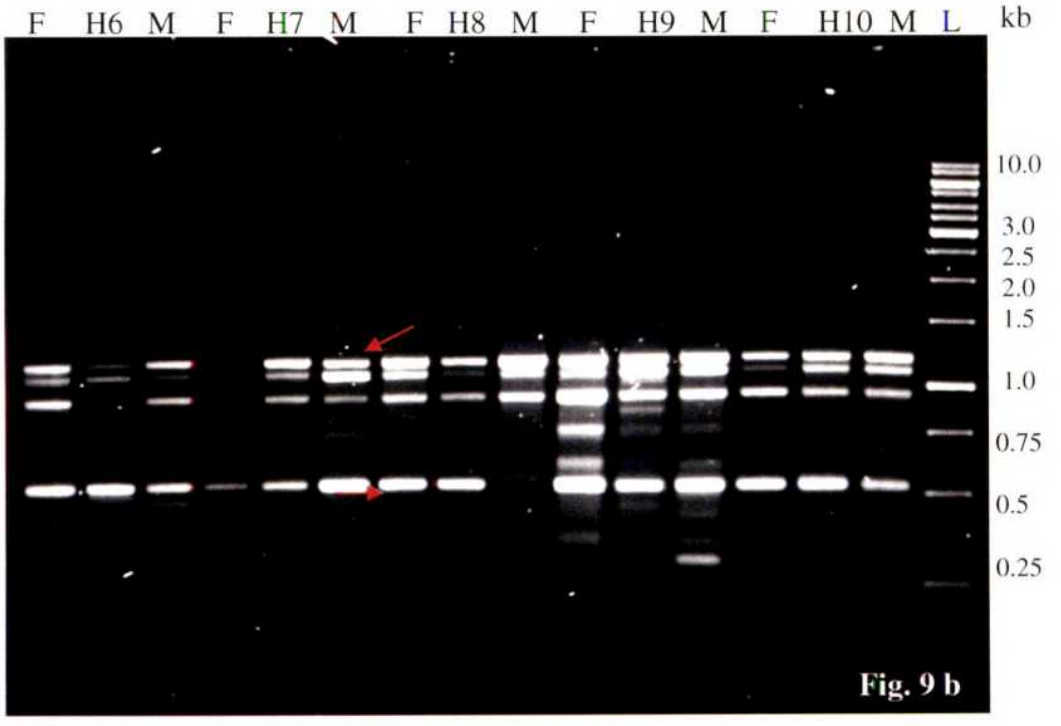


Fig.9 (a & b):RAPD profile of 10 hybrids generated with primer OPA 09; L- 1kb DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.

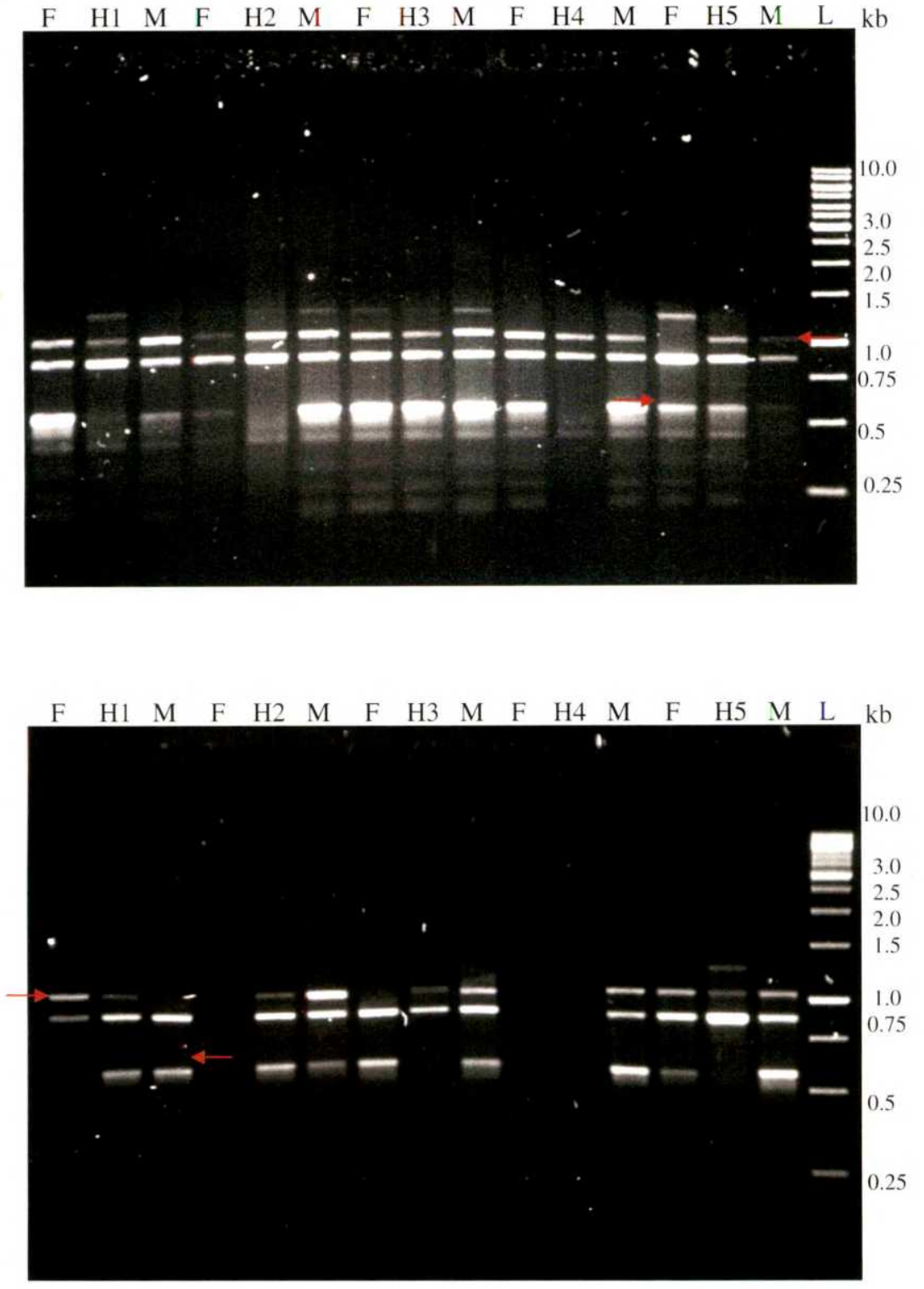


Fig. 10 (a & b): RAPD profile of 10 hybrids generated with primer OPA 11; L-1kb DNA ladder, F- Female parent, M- male parent and H1-H10: 10 hybrids.

in parent 17 A and hybrid 17 A X RHA-1-1 and 17 A and AK 345 to 96 % between parent 7-1 A and NDLR 06 (Table 14). Thus diversity among these parental lines and hybrids were ranged between 4 % to 39 %.

The Dendrogram was depicted through UPGMA cluster analysis by using software PASTA with bootstrap value. Based on Dendrogram analysis of RAPD fingerprint profile eleven parental lines were categorized into three major clusters (Fig. 25). The major cluster I comprised six parental lines (LTR 07, 7-1 A, NDLR 06, 10 A, RHA-1-1 and AK 345) showing 86% similarity. The cluster I included nine members comprising six subcluster. The parental line 7-1 A and NDLR 06 showed maximum genetic similarity of 96% and indicated these two parents are genetically very close. Similarly subcluster of major cluster I comprised two hybrids 17 A X RHA-1-1 and 7-1 A X AK 345 were 92% genetically similar and only 8% dissimilar to each other. The hybrid 10 A X NDLR 06 categorized into separate cluster II, showed 84% genetic similarity with members of cluster I. cluster III included each of parental line (LTR 07) and hybrid (234 A X LTR 07) in together showing 86% genetic similarity. The cluster IV comprised five member included four parental line and lone hybrid distributed into three subclusters. The parent 343 A and 234 A of subcluster of major cluster IV showed 94% similarity. Cluster V comprised two hybrids (343 A X NDR7-1B and 17A X NDLR 06) showed 84% similarity. While cluster VI and VII were formed by a lone member of hybrid (7-1 A X NDLR 06) and parent (17 A) respectively. The parent 17 A was most divergent parent showed 66 % similarity with hybrid 7-1 A X NDLR 06.

Also the Principal Co-ordinate analysis (PCoA) was used to find out multidimensional relationship among parent and hybrid lines on the basis of their genetic variance. Based on PCoA analysis all parents and hybrids used in this study were categorized into seven major groups corresponding to the same VII subcluster were formed in RAPD analysis. The parental line 17 A was also out grouped in PCoA analysis and designated as most divergent parental line (Fig. 26).

4.1.4 Genetic relationship among hybrid and parental lines by using microsatellite (SSR) polymorphism

The SSR marker genotyping data were used for understanding diverse nature of parental lines and association among themselves. Fifty four polymorphic amplicons

Table 14: Similarity matrix based on Jaccard's similarity coefficient value obtained from RAPD analysis of ten Hybrid with their parents

	234 A	234A X RHA-1-1	RHA-1-1	7-1 A	7-1A X NDLR-06	NDLR-06	10A	10A X NDLR-06	343 A	343A X NDR7-1B	NDR7-1 B	234A X NDR7-1B	343A X J/6	J/6	17 A	17A X RHA-1-1	234A X LTR-07	LTR -07	17A X NDLR-06	7-1A X AK 345	AK 345
234 A	1.00																				
234A X RHA-1-1	0.82	1.00																			
RHA-1-1	0.85	0.87	1.00																		
7-1 A	0.83	0.90	0.86	1.00																	
7-1A X NDLR-06	0.79	0.84	0.79	0.89	1.00																
NDLR-06	0.83	0.89	0.85	0.96	0.85	1.00															
10A	0.87	0.89	0.90	0.85	0.75	0.87	1.00														
10A X NDLR-06	0.85	0.81	0.88	0.85	0.82	0.85	0.86	1.00													
343 A	0.94	0.82	0.86	0.81	0.80	0.81	0.88	0.83	1.00												
343A X NDR7-1B	0.81	0.82	0.84	0.90	0.80	0.86	0.85	0.83	0.78	1.00											
NDR7-1 B	0.85	0.79	0.83	0.83	0.79	0.85	0.85	0.82	0.83	0.84	1.00										
234A X NDR7-1B	0.86	0.90	0.92	0.89	0.80	0.88	0.88	0.83	0.86	0.78	0.81	1.00									
343A X J/6	0.92	0.83	0.85	0.82	0.81	0.82	0.86	0.81	0.90	0.79	0.87	0.88	1.00								
J/6	0.89	0.78	0.85	0.79	0.72	0.82	0.86	0.81	0.90	0.79	0.90	0.82	0.86	1.00							
17 A	0.70	0.68	0.66	0.67	0.68	0.63	0.68	0.69	0.67	0.69	0.69	0.63	0.71	0.68	1.00						
17A X RHA-1-1	0.84	0.85	0.86	0.87	0.78	0.89	0.85	0.83	0.79	0.76	0.81	0.89	0.80	0.80	0.61	1.00					
234A X LTR-07	0.84	0.83	0.84	0.82	0.72	0.78	0.80	0.78	0.76	0.82	0.78	0.82	0.77	0.77	0.70	0.85	1.00				
LTR -07	0.87	0.81	0.87	0.88	0.81	0.87	0.81	0.84	0.82	0.82	0.82	0.85	0.83	0.81	0.64	0.85	0.86	1.00			
17A X NDLR-06	0.86	0.77	0.81	0.84	0.80	0.81	0.82	0.83	0.81	0.84	0.81	0.81	0.82	0.82	0.67	0.82	0.76	0.79	1.00		
7-1A X AK 345	0.84	0.91	0.86	0.92	0.83	0.91	0.88	0.86	0.79	0.85	0.78	0.87	0.80	0.77	0.65	0.92	0.88	0.88	0.84	1.00	
AK 345	0.88	0.88	0.92	0.86	0.77	0.86	0.88	0.83	0.84	0.81	0.83	0.92	0.88	0.82	0.63	0.89	0.85	0.88	0.84	0.87	1.00

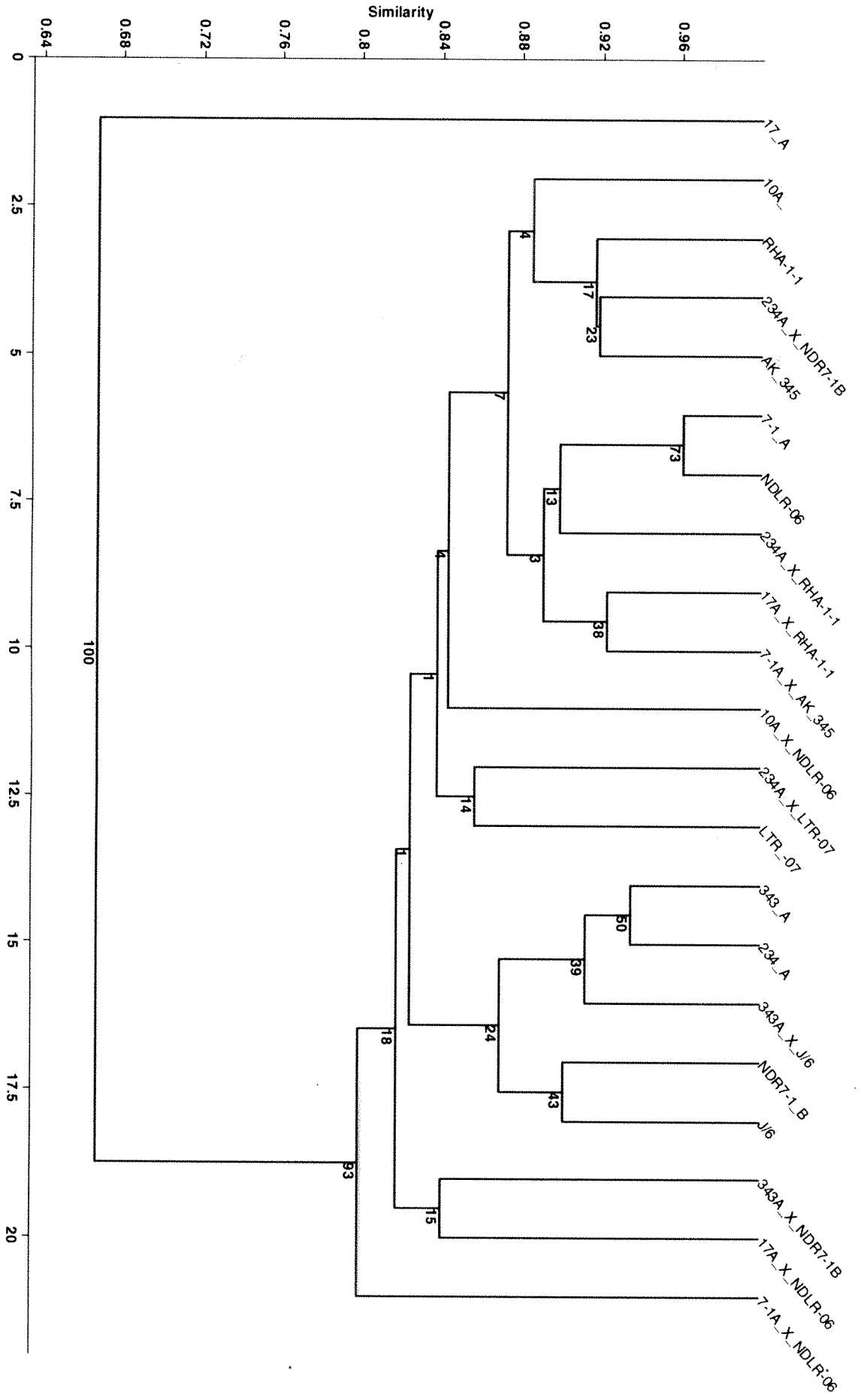


Fig. 25 Dendrogram generated using UPGMA analysis demonstrating relationship among ten hybrids with their parents based on RAPD data.

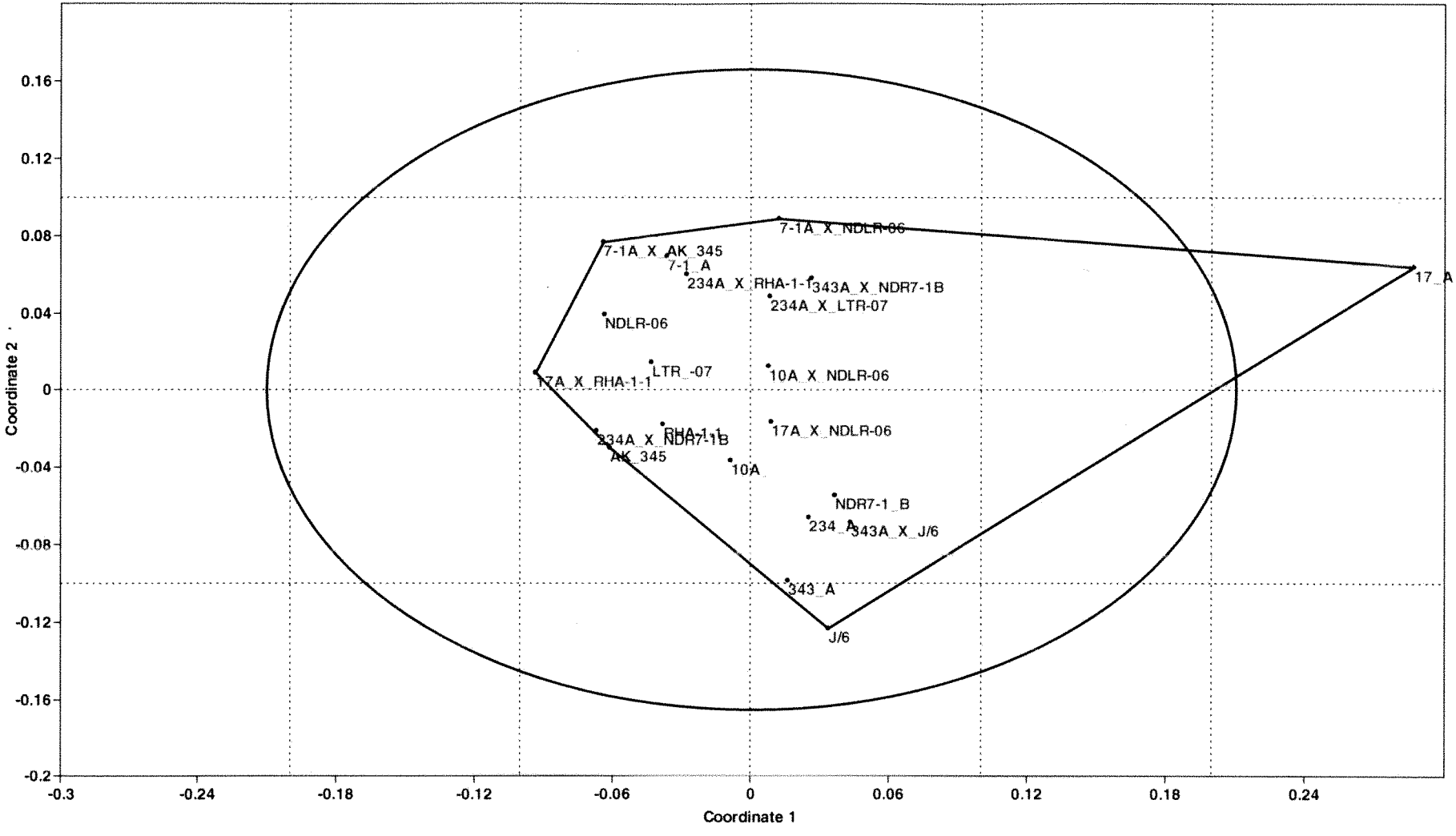


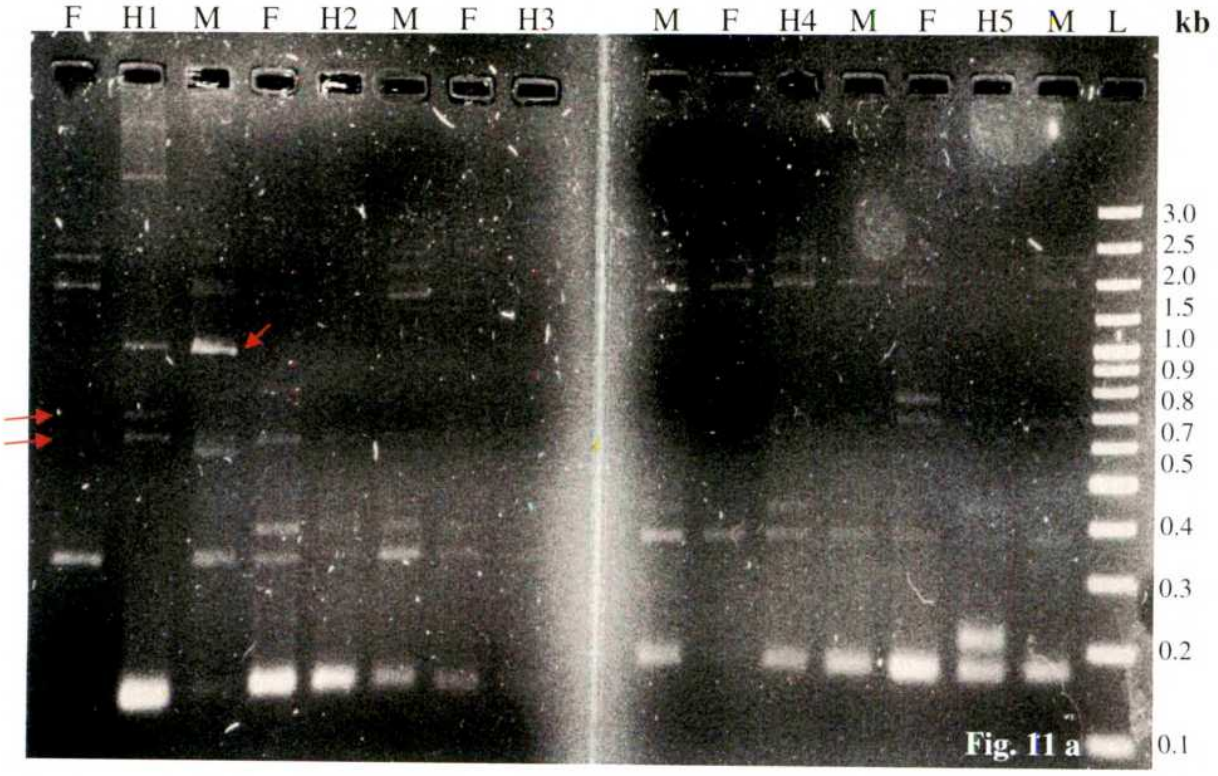
Fig.26 Principal Co-ordinate analysis (PC0) analysis of RAPD data of hybrids and their parents

were generated by 13 SSR primers among parents and hybrids. The primer IUB 6 significantly determined genetic relatedness among parental lines by producing 58% polymorphism (Table 15) followed by primer ORS 13 showed 47% polymorphism. The total seven polymorphic SSR markers have been identified and showed polymorphism among hybrid and their parents (Table 19). Out of seven markers four markers showed polymorphism among hybrid and their respective parental lines, while two were efficiently determined purity of hybrids of sunflower.

The genetic similarity was retrieved from microsatellite (SSR) data using Dice coefficient (Table 16) the parent similarities ranged from 75% among parental line 17 A and 234 A to the 98% between parental line 343 A and 10 A and parental line NDR7-1B and hybrid 343 A X NDR7-1B. Thus, diversity percentage among hybrids and parental lines were ranged between 2% to 25% (Table 16).

The Dendrogram was generated by using UPGMA cluster analysis based on SSR data of parental lines. Dendrogram could categorize 11 parental lines of sunflower into three distinct clusters. The major cluster I comprised eight parental lines in one group showing 90% similarity to each other. While cluster I comprised two parental lines (J/6 and AK 345) in together showing 88% similarity. Similarly parental line 17 A formed a separate cluster showing 81% similarity with other parental lines (Fig. 27).

Also the common Dendrogram analysis was done for parental lines and hybrids for identification of their genetic relatedness. Based on UPGMA clustering method 11 parental lines and ten hybrids were grouped into IV major clusters. The cluster I comprised six members pertaining parental lines and hybrids showed > 90 % similarity. The members present within cluster II comprised three hybrids (7-1 A X NDLR 06, 234 A X NDR7-1B, 2354 A X RHA-1-1) and one parental line NDLR 06 and showed 71.88% genetic similarity. The major cluster III included ten members of seven parental lines and three hybrids (Fig. 27). Cluster IV comprised a lone parental line J/6 and showed 84 % genetic similarity with members of cluster I, II, and III. This Dendrogram revealed that the parental lines 10 A, 343 A were found very close to each other while parent J/6 was very diverse parent.



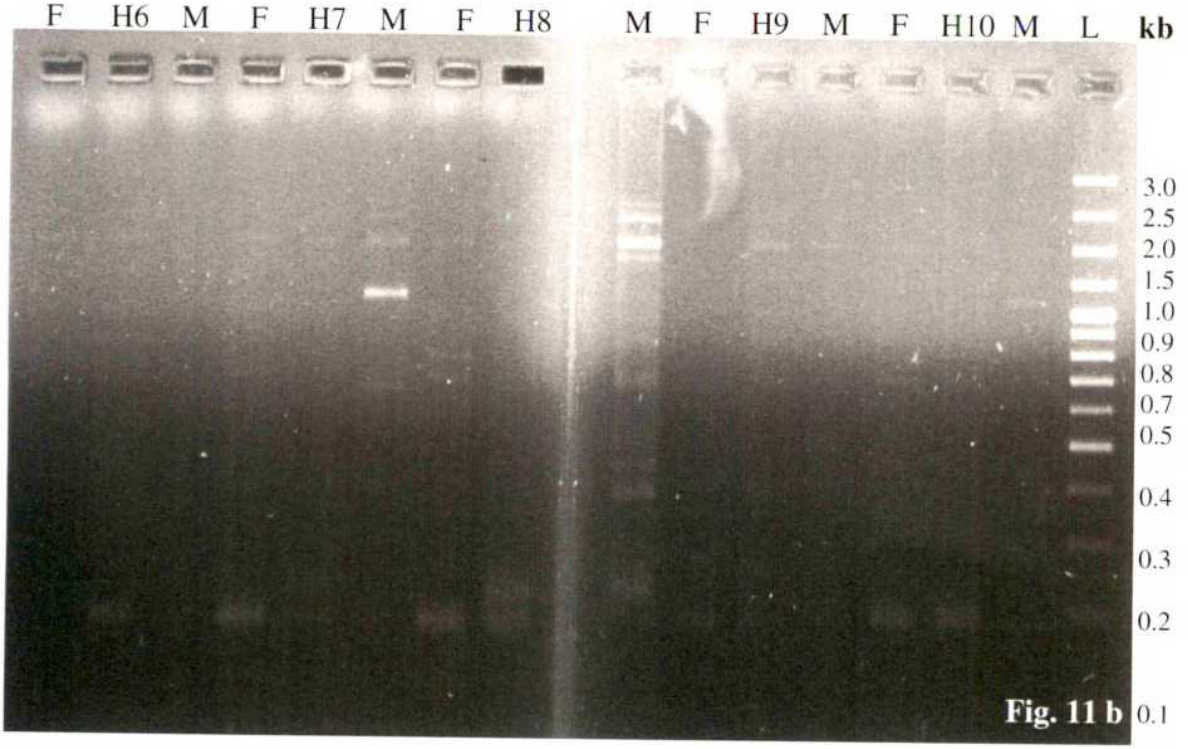
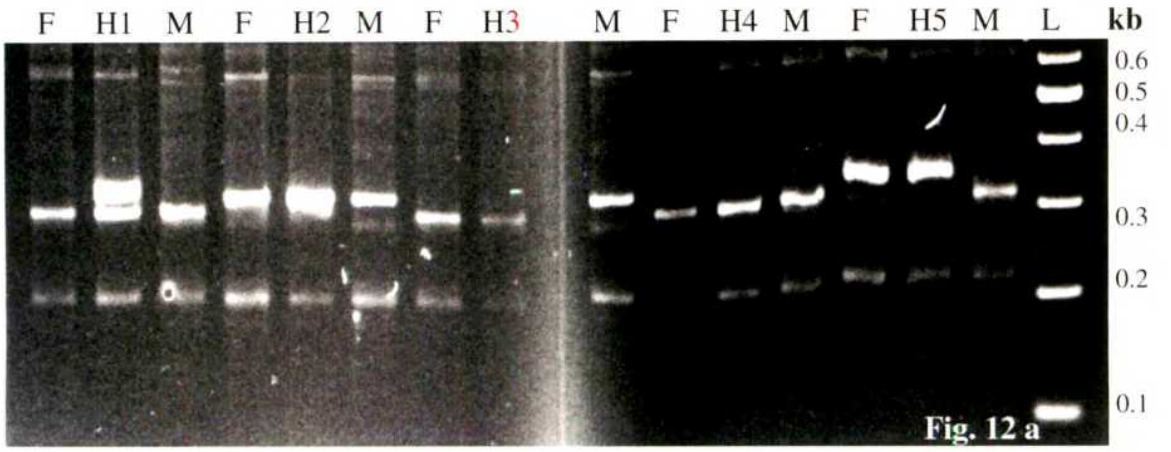


Fig. 11 (a & b): SSR profile of 10 hybrids generated with primer Ha 1327; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



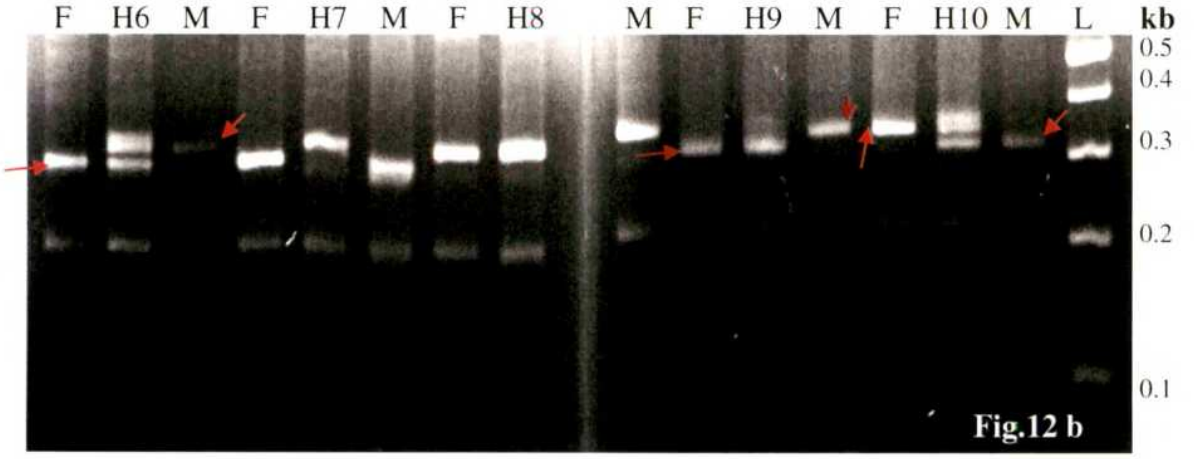


Fig. 12 (a & b): SSR profile of 10 hybrids generated with primer ORS 5; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.

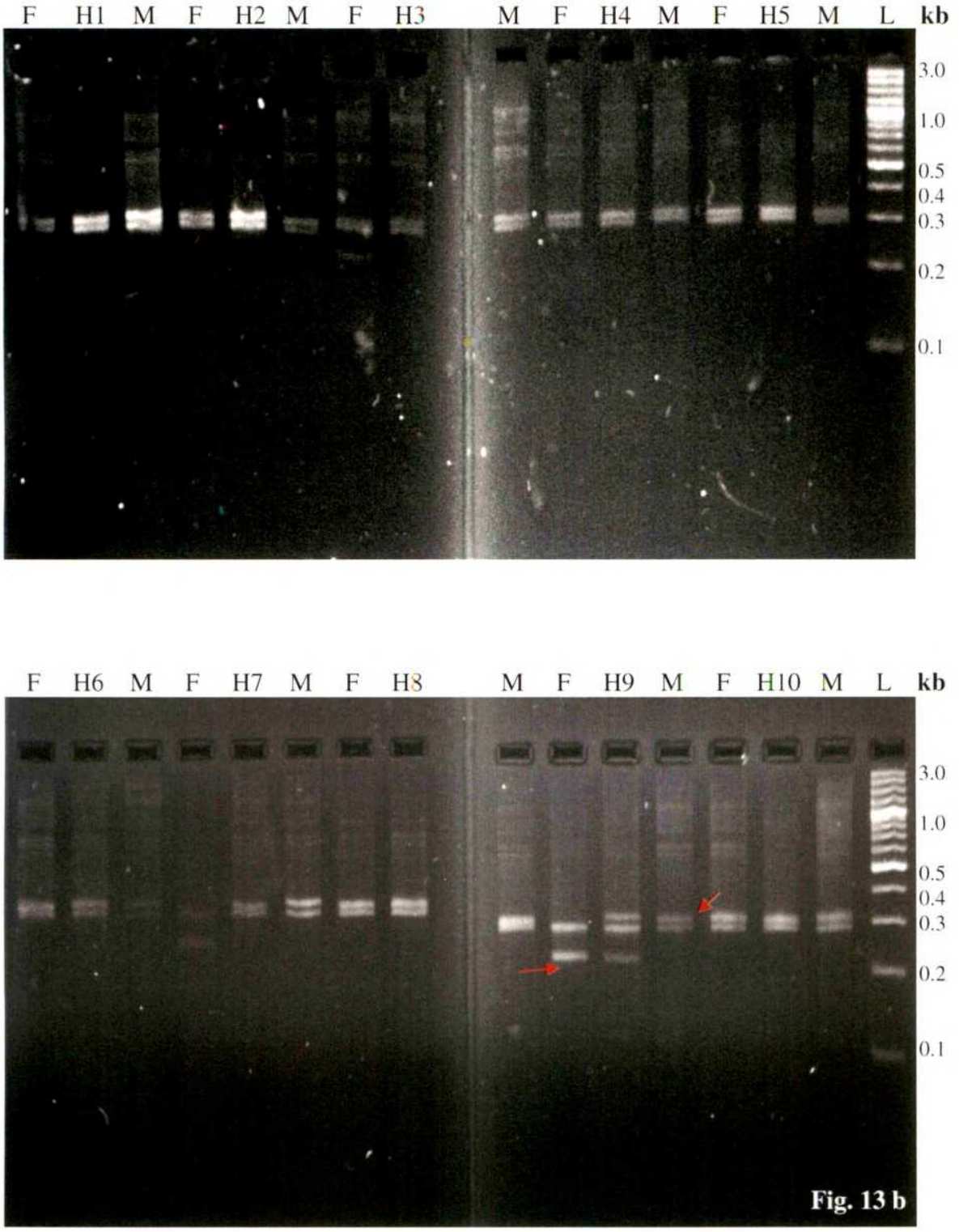
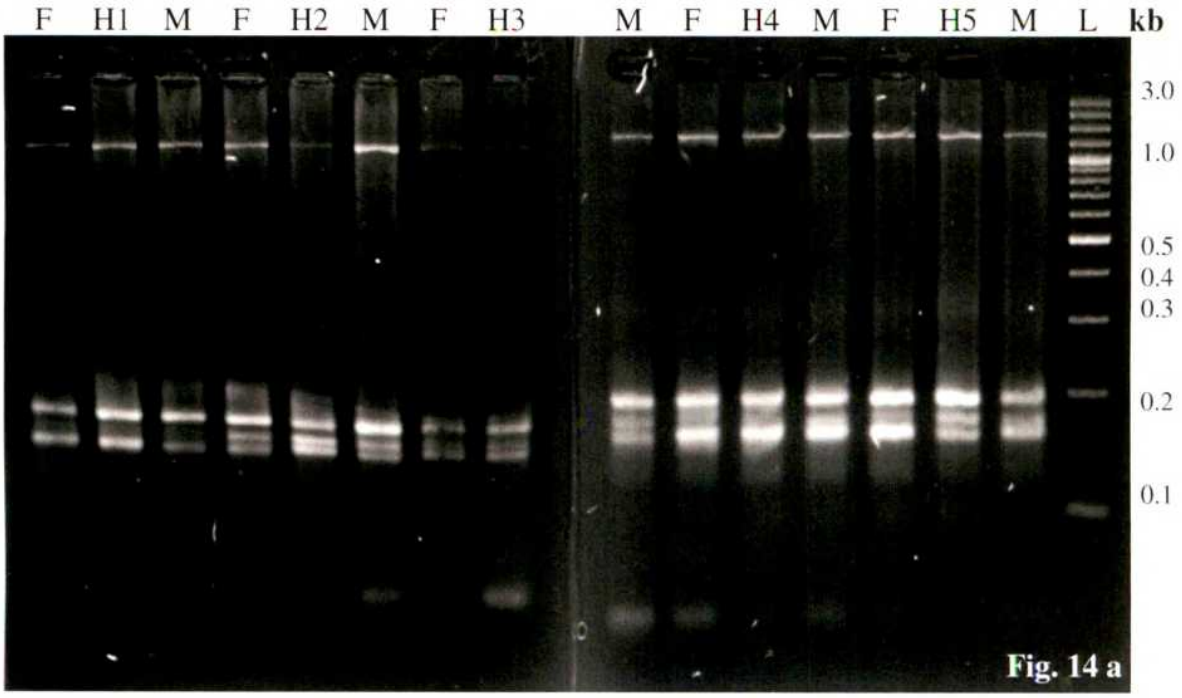


Fig. 13(a & b): SSR profile of 10 hybrids generated with primer ORS 662; L-100 bp DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



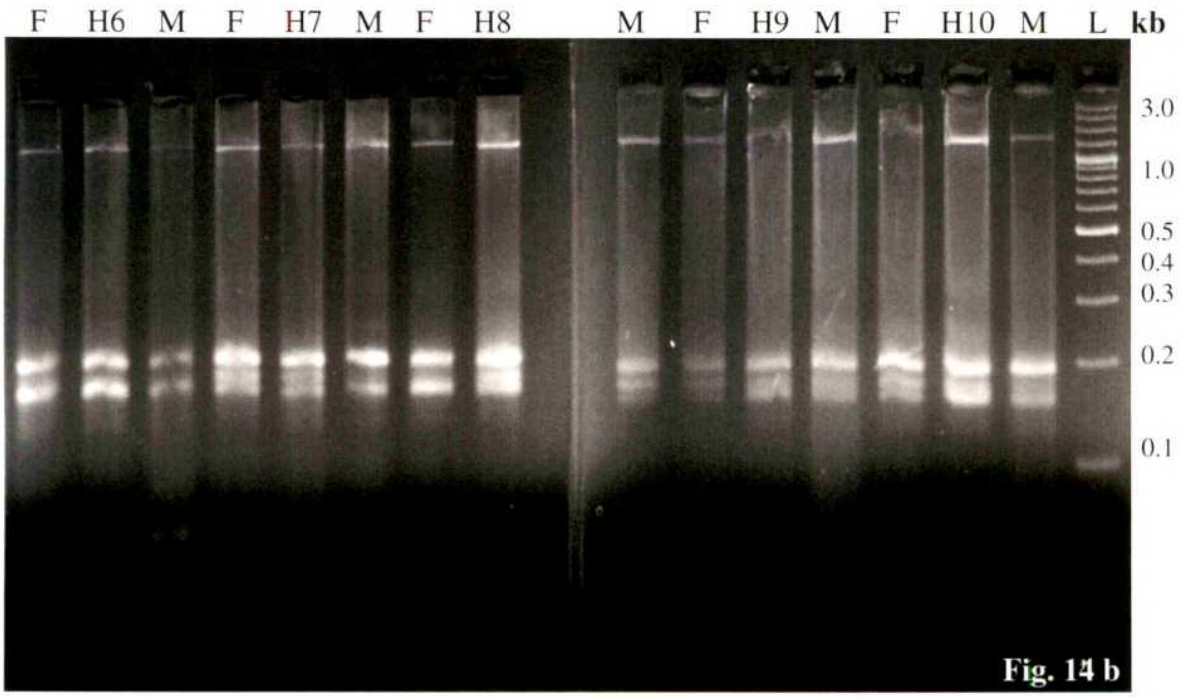
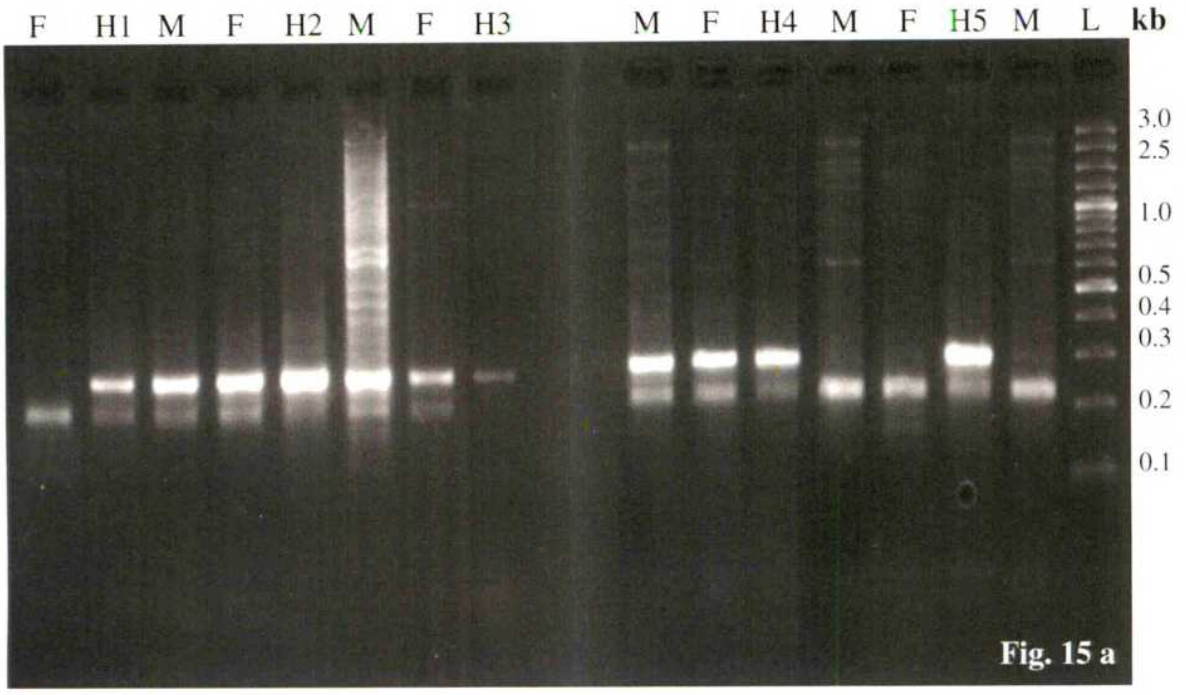


Fig. 14 (a & b): SSR profile of 10 hybrids generated with primer Ha 1442; L-100 bp DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



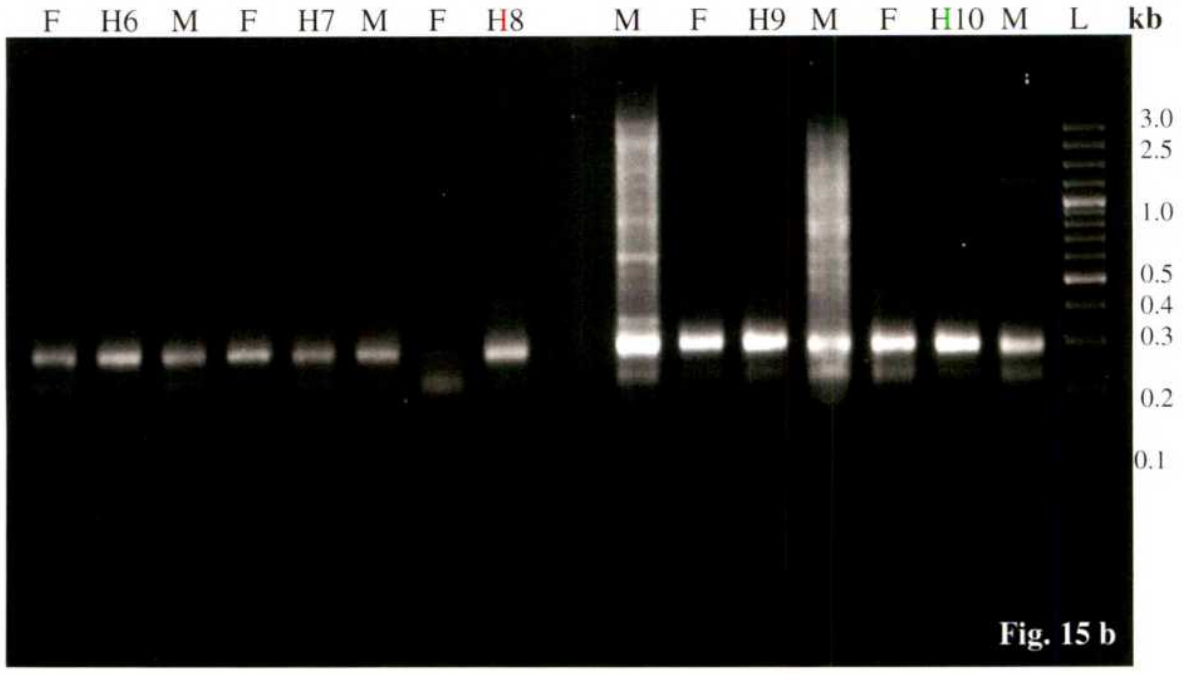


Fig.15 (a & b): SSR profile of 10 hybrids generated with primer ORS 536; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.

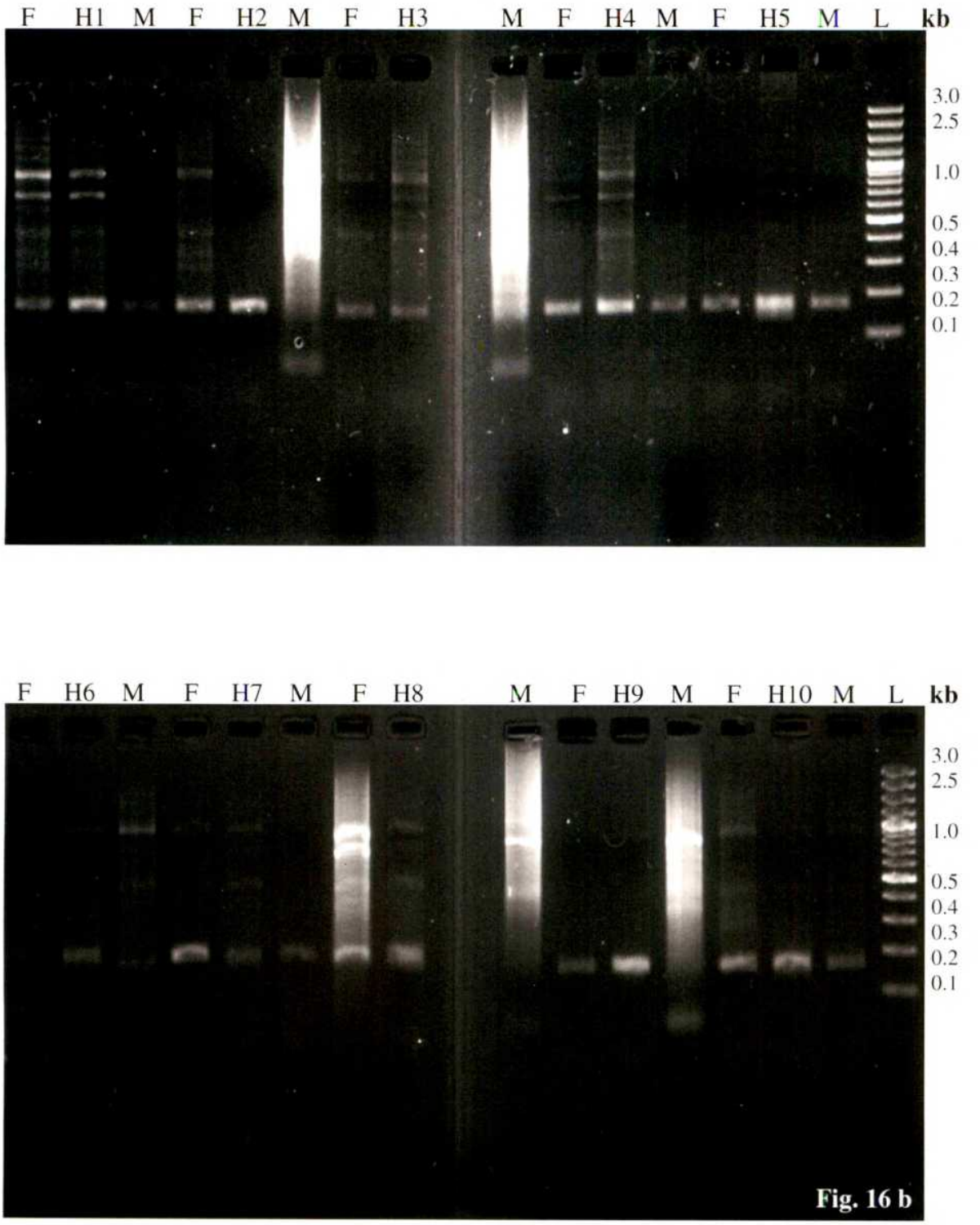
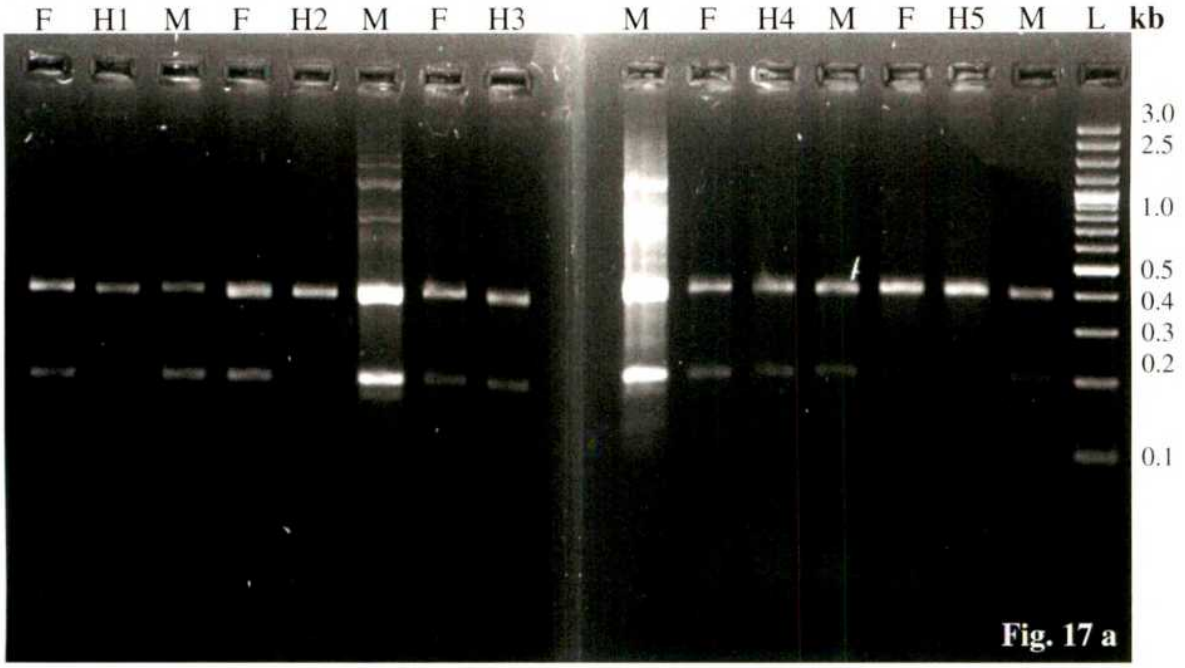


Fig.16(a & b): SSR profile of 10 hybrids generated with primer ORS 243; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids



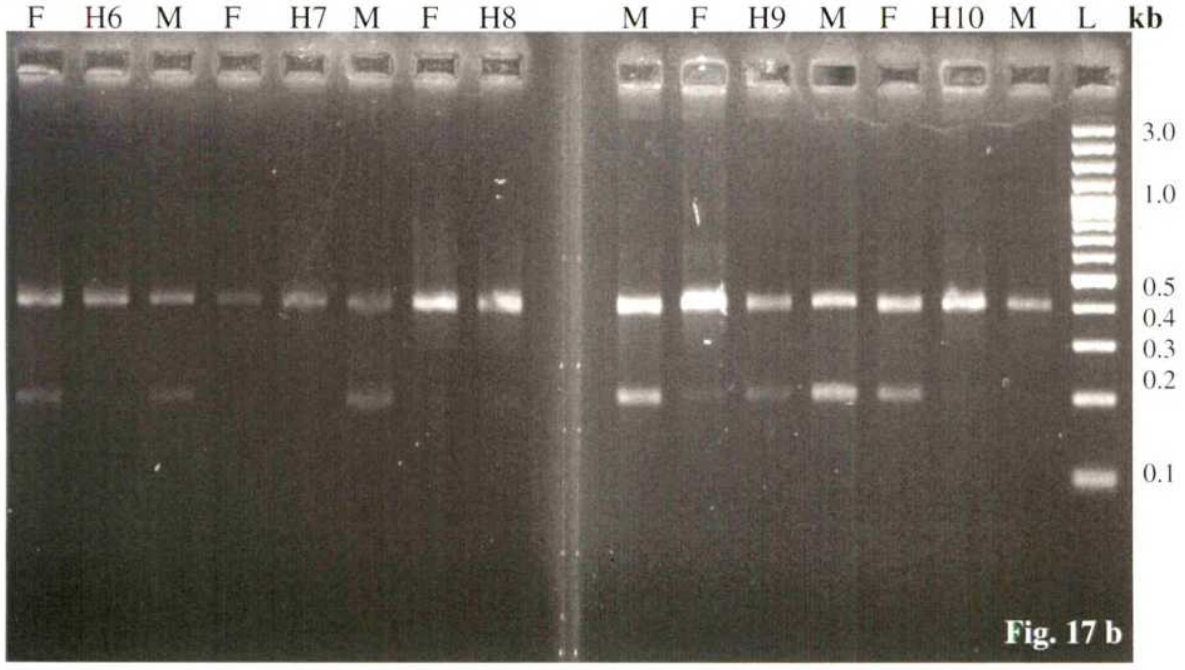
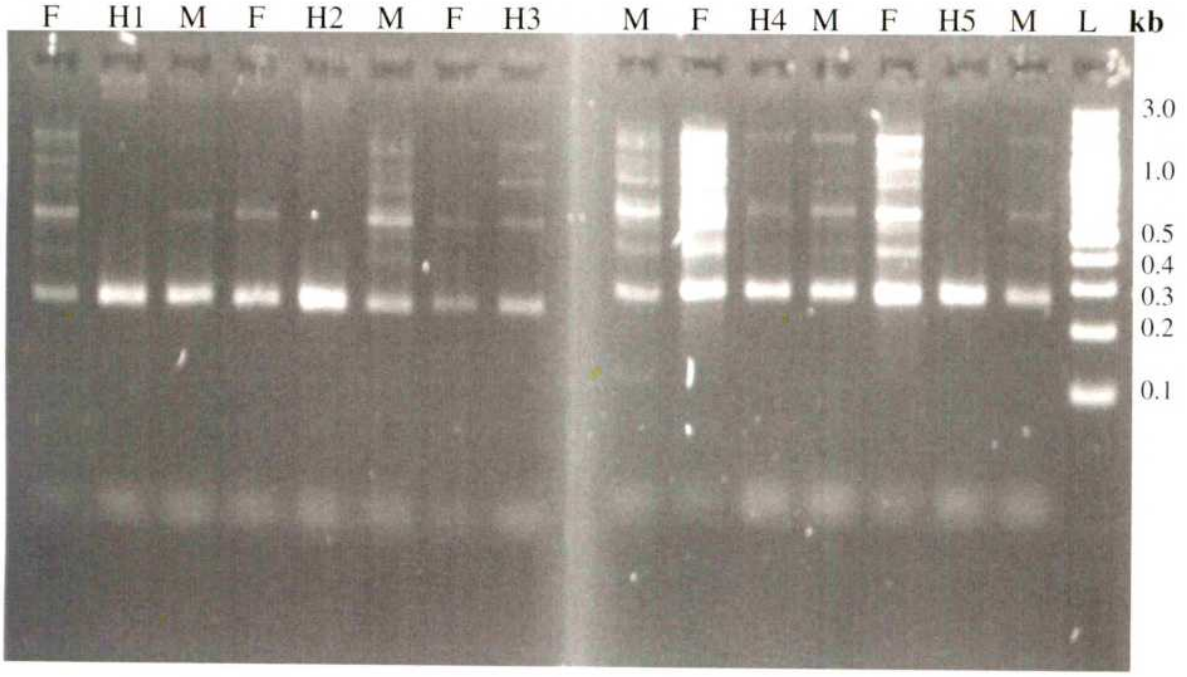


Fig. 17(a & b): SSR profile of 10 hybrids generated with primer ORS 323; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



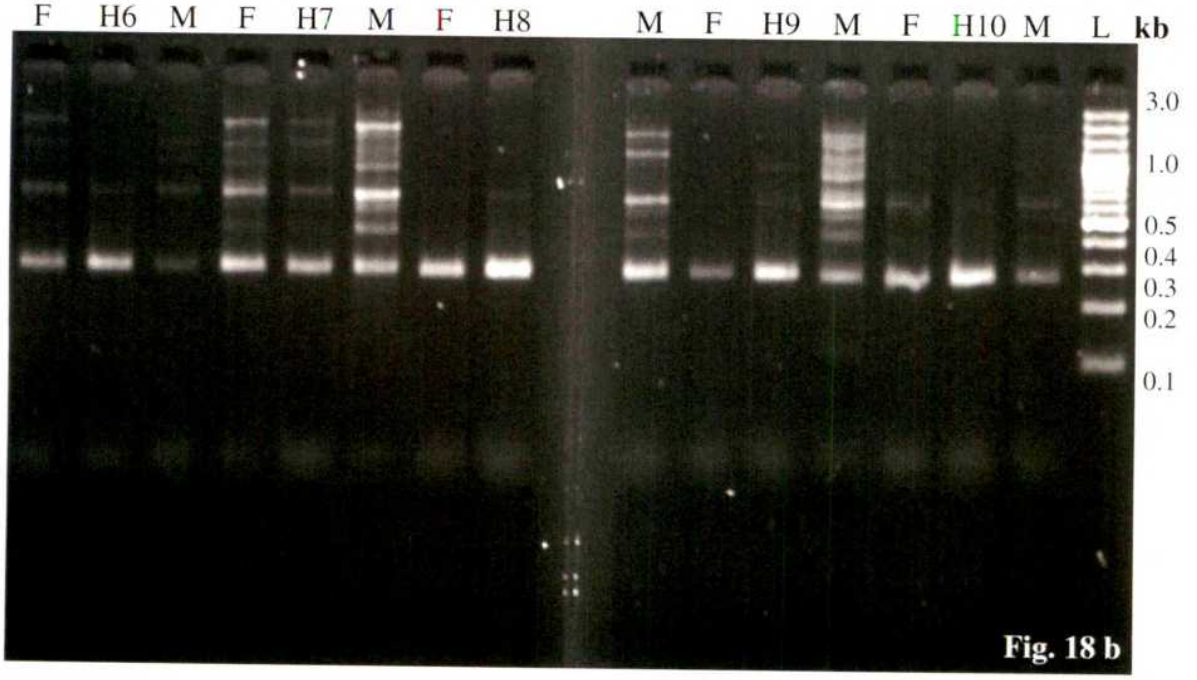
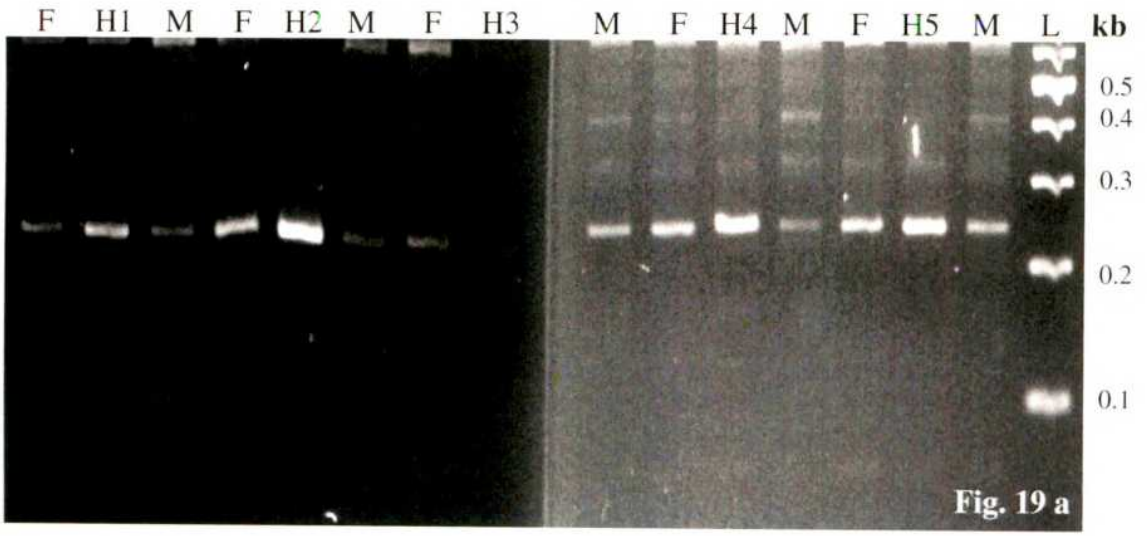


Fig. 18 (a & b): SSR profile of 10 hybrids generated with primer ORS 391; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



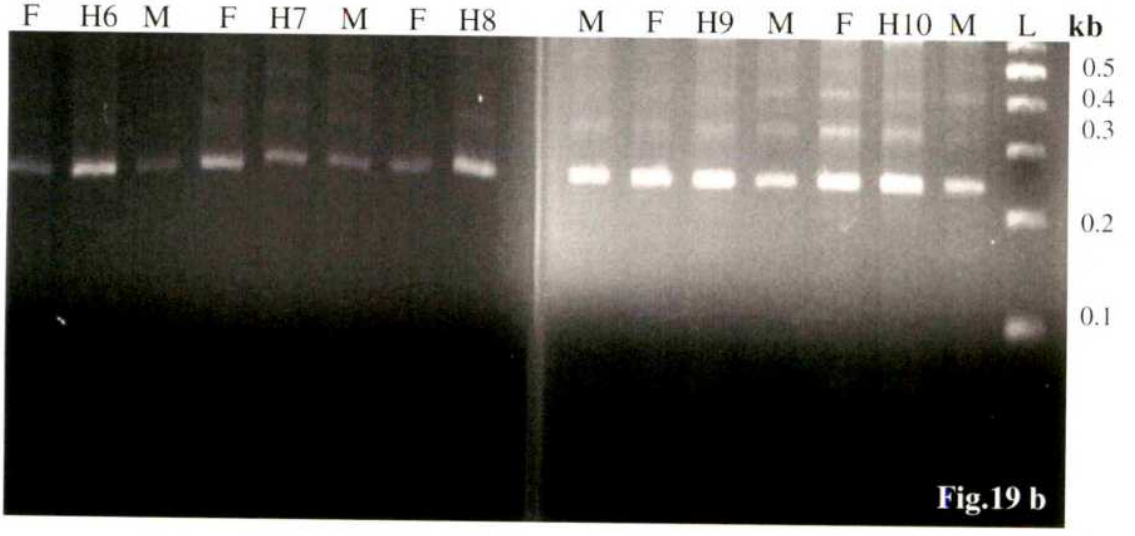
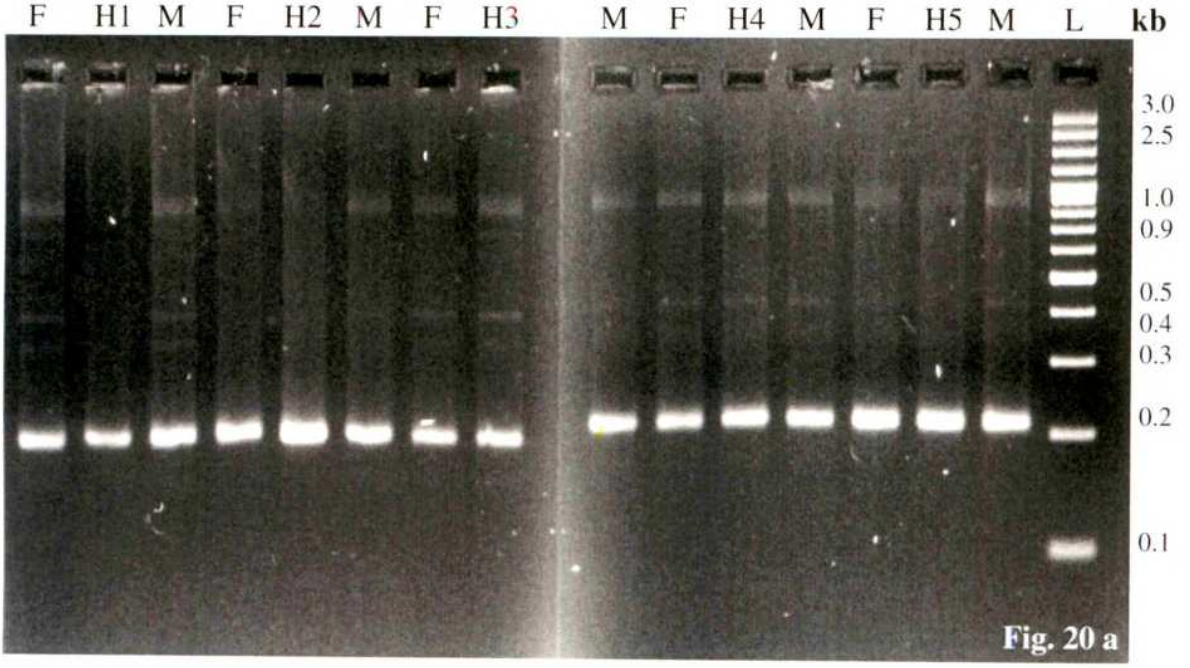


Fig. 19 (a & b): SSR profile of 10 hybrids generated with primer ORS 6; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.



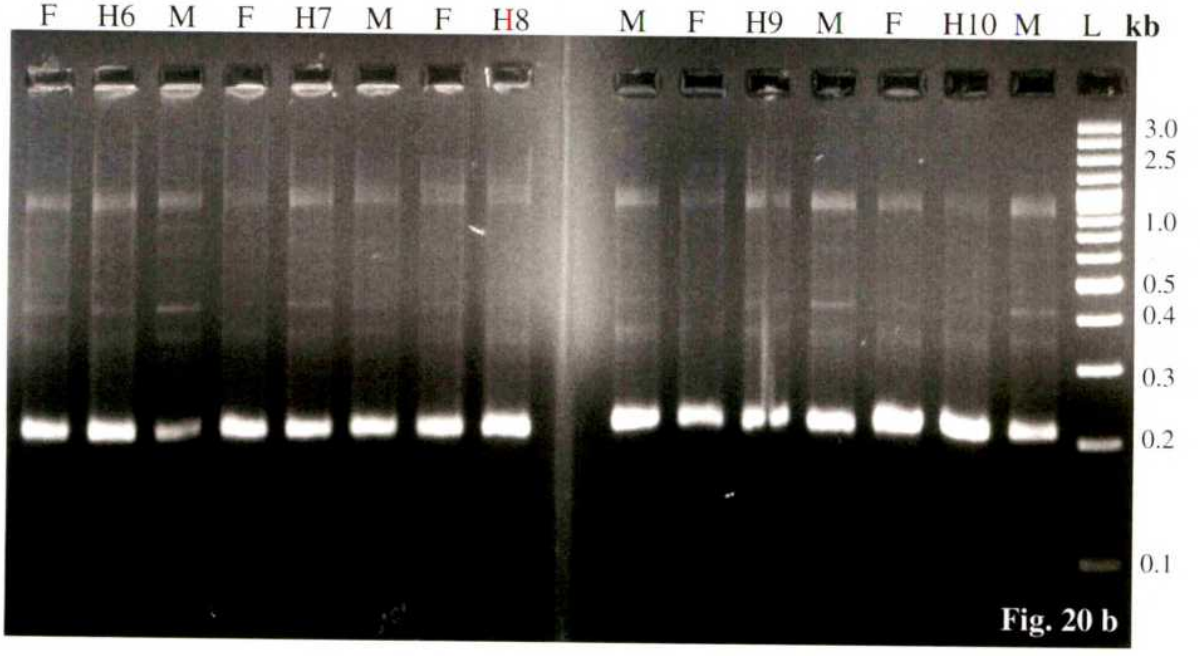
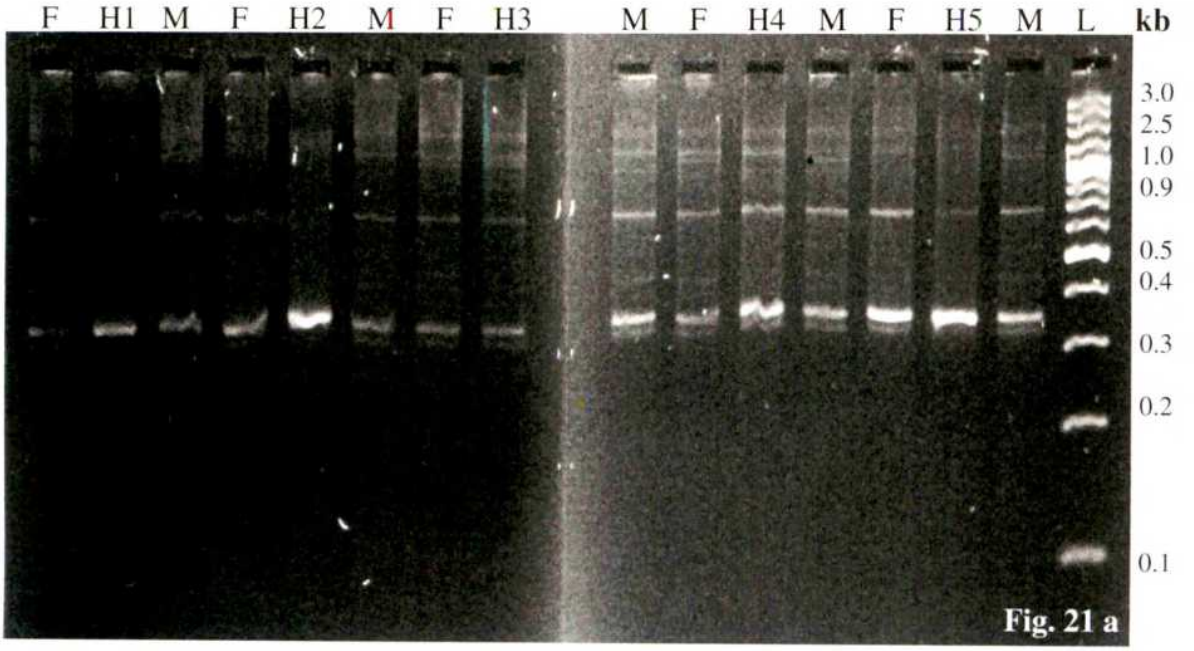


Fig. 20 (a & b): SSR profile of 10 hybrids generated with primer HNCA 2; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10:10 hybrids.



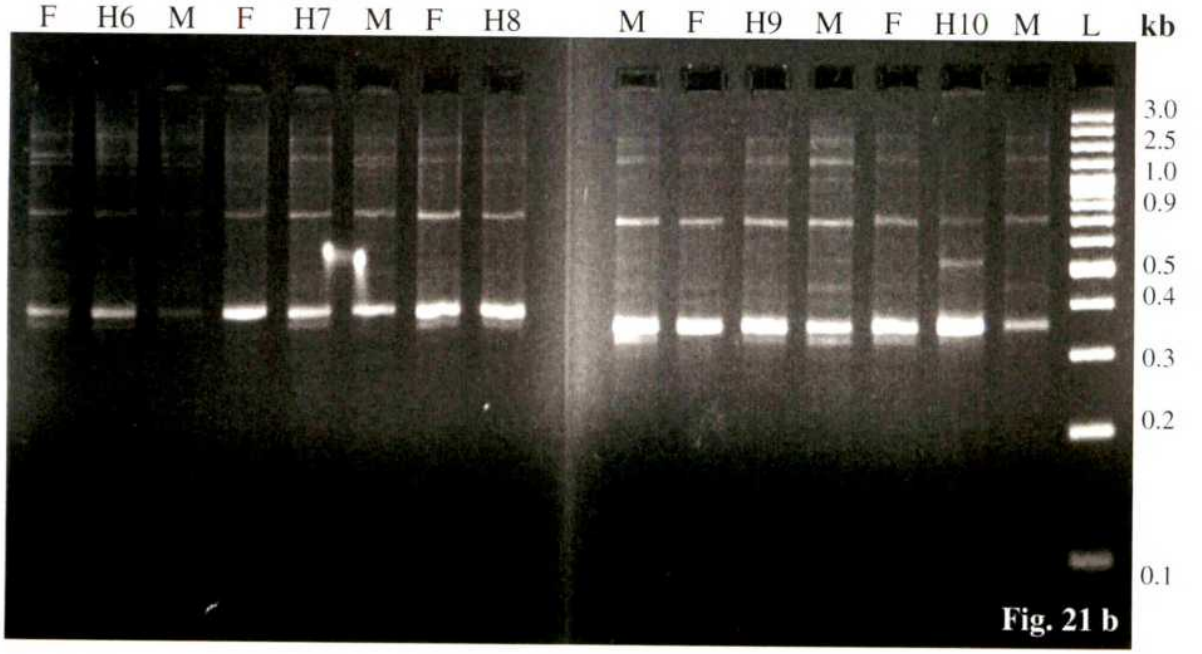
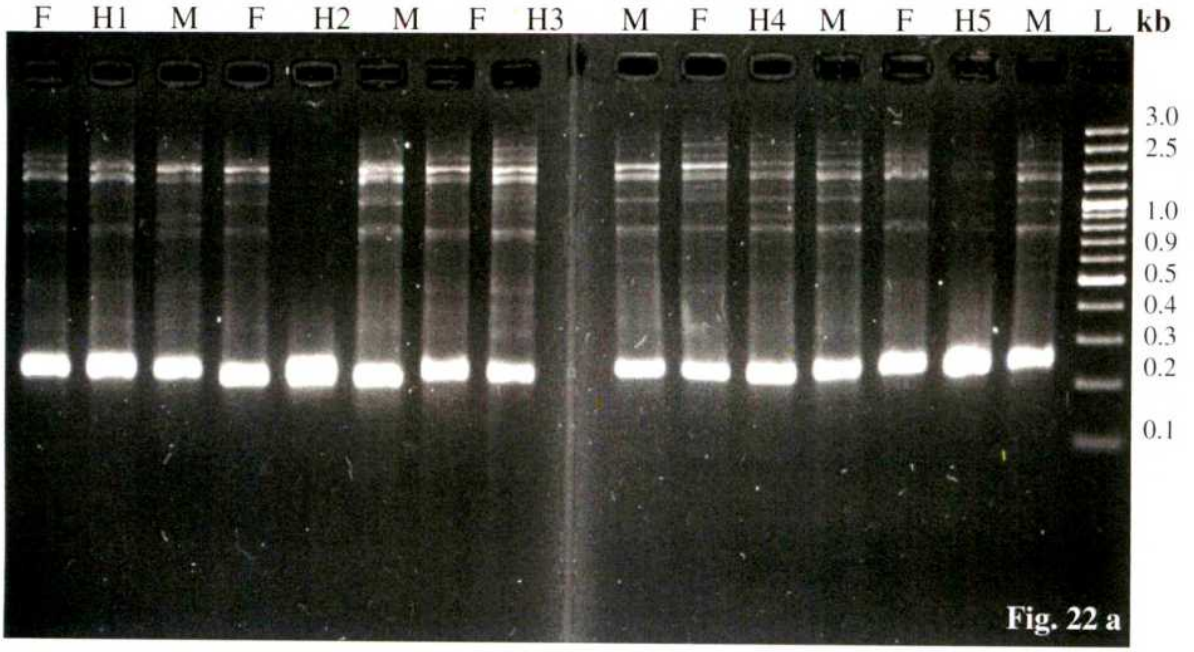


Fig. 21(a & b): SSR profile of 10 hybrids generated with primer IUB 6; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.



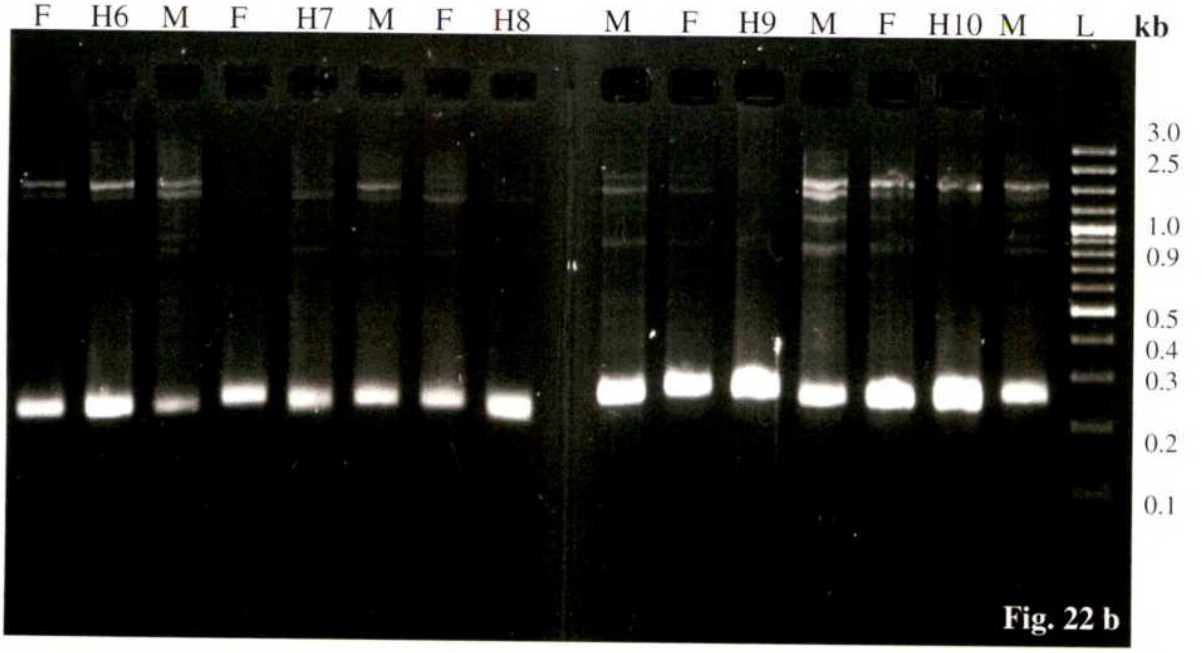


Fig. 22(a & b): SSR profile of 10 hybrids generated with primer ORS 1114; L- 100 bp DNA ladder, F- Female parent, M- male parent and H1- H10: 10 hybrids.

Table 15: Polymorphism among hybrids and their parents on the basis of Microsatellite (data) SSR analysis

S. No.	SSR primers / Hybrids	No. of polymorphic bands										Total no. of polymorphic bands	Total no. of bands	Mean	Polymorphism (%)
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10				
1	Ha 1327	3	1	1	1	2	1	1	4	0	2	16	4	1.6	40
2	Ha 1442	0	0	1	0	1	0	1	1	0	1	5	3	0.5	16.66
3	ORS 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	ORS 13	0	0	0	0	0	2	0	1	0	1	4	2	0.4	47
5	ORS 243	3	3	2	2	0	3	3	1	0	0	17	4	1.7	42.5
6	IUB 6	1	1	0	0	0	0	0	0	0	0	2	2	0.2	58
7	ORS 323	0	1	0	0	0	0	1	0	0	1	3	2	0.3	15
8	ORS 391	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	ORS 5	1	2	1	0	2	2	1	0	2	2	13	3	1.3	43
10	HNCA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	ORS 536	1	0	1	1	1	0	0	2	0	0	6	2	0.6	30
12	ORS 662	0	0	1	0	0	0	2	0	2	0	5	3	0.5	16
13	ORS 114	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Where **H1 to H10**: Ten sunflower hybrids as listed in Table 3 respectively.

divergent parent showed maximum genetic distance (16%). Three major clusters further divided into several subclusters. Cluster I was divided into four subclusters, while cluster II and III subdivided into three and six subclusters respectively.

Principal co-ordinate analysis (PCoA) was used to identify multidimensional relationship that describes portions of the genetic variance in a data set. Comparable to UPGMA cluster analysis PCoA separated the parental lines and hybrids into four main groups corresponding to cluster I, II, III, and IV (Fig. 28). However cluster III separated into subcluster a and subcluster b comprising hybrid 234 A X LTR 07 and parent 7-1 A respectively. However parental line J/6 was considered nearer to parent AK 345.

Table 16: Similarity matrix based on Jaccard's similarity coefficient value obtained from SSR analysis of ten Hybrid with their parents

	234 A	234A X RHA-1-1	RHA-1-1	7-1 A	7-1A X NDLR-06	NDLR 06	10A	10A X NDLR-06	343 A	343A X NDR7-1B	NDR7-1 B	234A X NDR7-1B	343A X J/6	J/6	17 A	17A X RHA-1-1	234A X LTR-07	LTR 07	17A X NDLR-06	7-1A X AK 345	AK 345
234 A	1.00																				
234A X RHA-1-1	0.86	1.00																			
RHA-1-1	0.90	0.91	1.00																		
7-1 A	0.88	0.89	0.93	1.00																	
7-1A X NDLR-06	0.78	0.88	0.88	0.91	1.00																
NDLR 06	0.86	0.86	0.91	0.93	0.93	1.00															
10A	0.93	0.84	0.93	0.91	0.81	0.88	1.00														
10A X NDLR-06	0.88	0.84	0.93	0.91	0.86	0.93	0.90	1.00													
343 A	0.95	0.86	0.95	0.93	0.83	0.90	0.98	0.93	1.00												
343A X NDR7-1B	0.93	0.88	0.93	0.95	0.86	0.93	0.95	0.90	0.98	1.00											
NDR7-1 B	0.95	0.86	0.90	0.93	0.83	0.90	0.93	0.88	0.95	0.98	1.00										
234A X NDR7-1B	0.81	0.91	0.91	0.93	0.93	0.95	0.84	0.88	0.96	0.88	0.86	1.00									
343A X J/6	0.85	0.90	0.90	0.88	0.88	0.90	0.88	0.88	0.90	0.93	0.90	0.90	1.00								
J/6	0.81	0.82	0.87	0.80	0.79	0.87	0.84	0.84	0.86	0.84	0.81	0.87	0.86	1.00							
17 A	0.75	0.81	0.81	0.84	0.83	0.86	0.83	0.83	0.80	0.83	0.80	0.86	0.90	0.81	1.00						
17A X RHA-1-1	0.80	0.86	0.86	0.88	0.88	0.90	0.83	0.88	0.85	0.88	0.85	0.90	0.95	0.86	0.95	1.00					
234A X LTR-07	0.88	0.84	0.88	0.95	0.86	0.93	0.90	0.95	0.93	0.95	0.93	0.88	0.88	0.79	0.83	0.88	1.00				
LTR 07	0.90	0.86	0.91	0.93	0.84	0.86	0.88	0.88	0.90	0.88	0.86	0.86	0.81	0.82	0.81	0.86	0.88	1.00			
17A X NDLR-06	0.86	0.86	0.91	0.93	0.88	0.95	0.88	0.93	0.90	0.93	0.90	0.91	0.90	0.87	0.90	0.95	0.93	0.91	1.00		
7-1A X AK 345	0.80	0.90	0.90	0.88	0.88	0.86	0.83	0.83	0.85	0.88	0.85	0.90	0.95	0.86	0.90	0.95	0.83	0.86	0.90	1.00	
AK 345	0.86	0.82	0.87	0.85	0.79	0.82	0.89	0.84	0.92	0.89	0.86	0.82	0.92	0.88	0.86	0.92	0.84	0.87	0.87	0.92	1.00

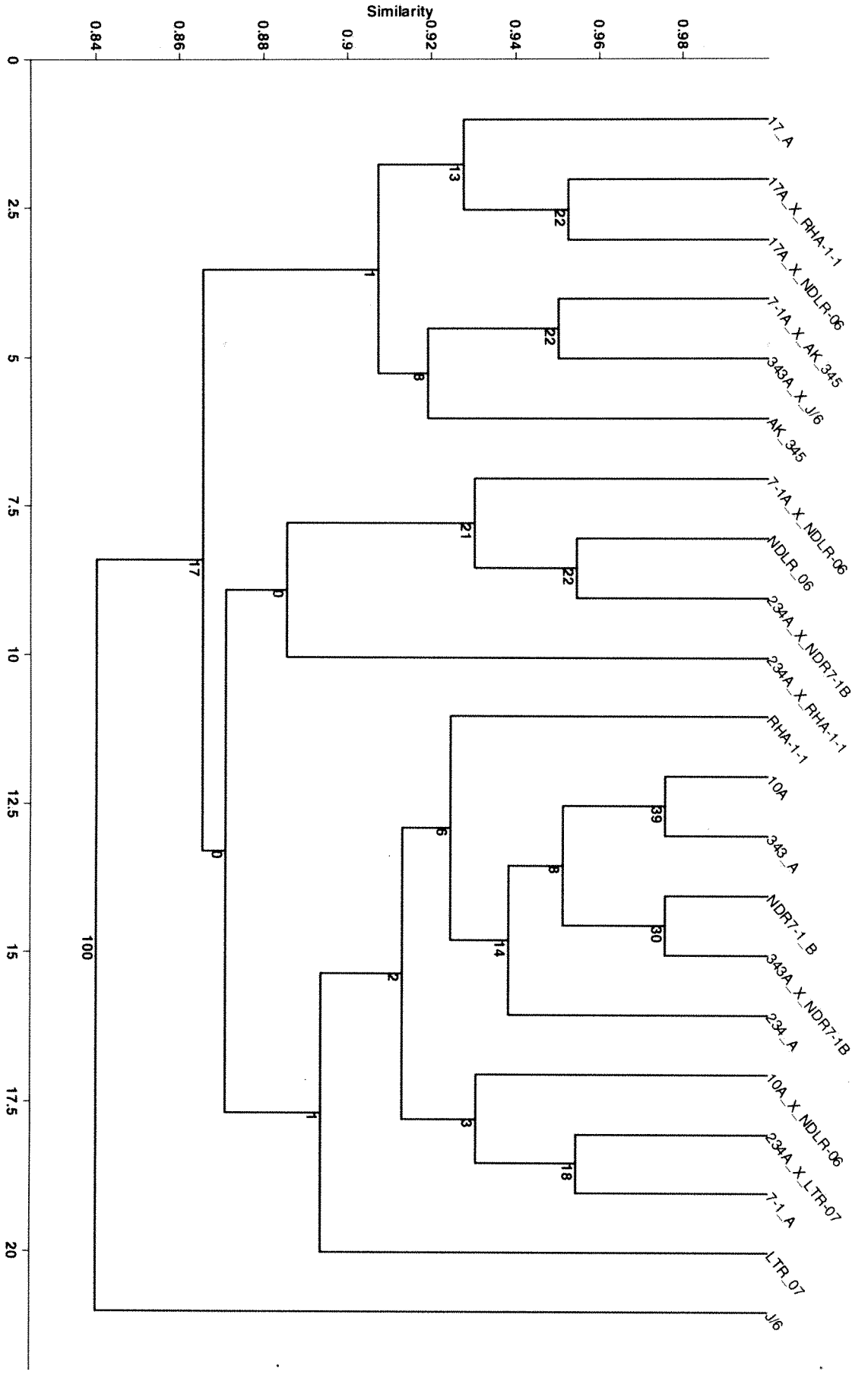


Fig. 27 Dendrogram generated using UPGMA analysis demonstrating relationship among ten hybrids with their parents based on SSR data.

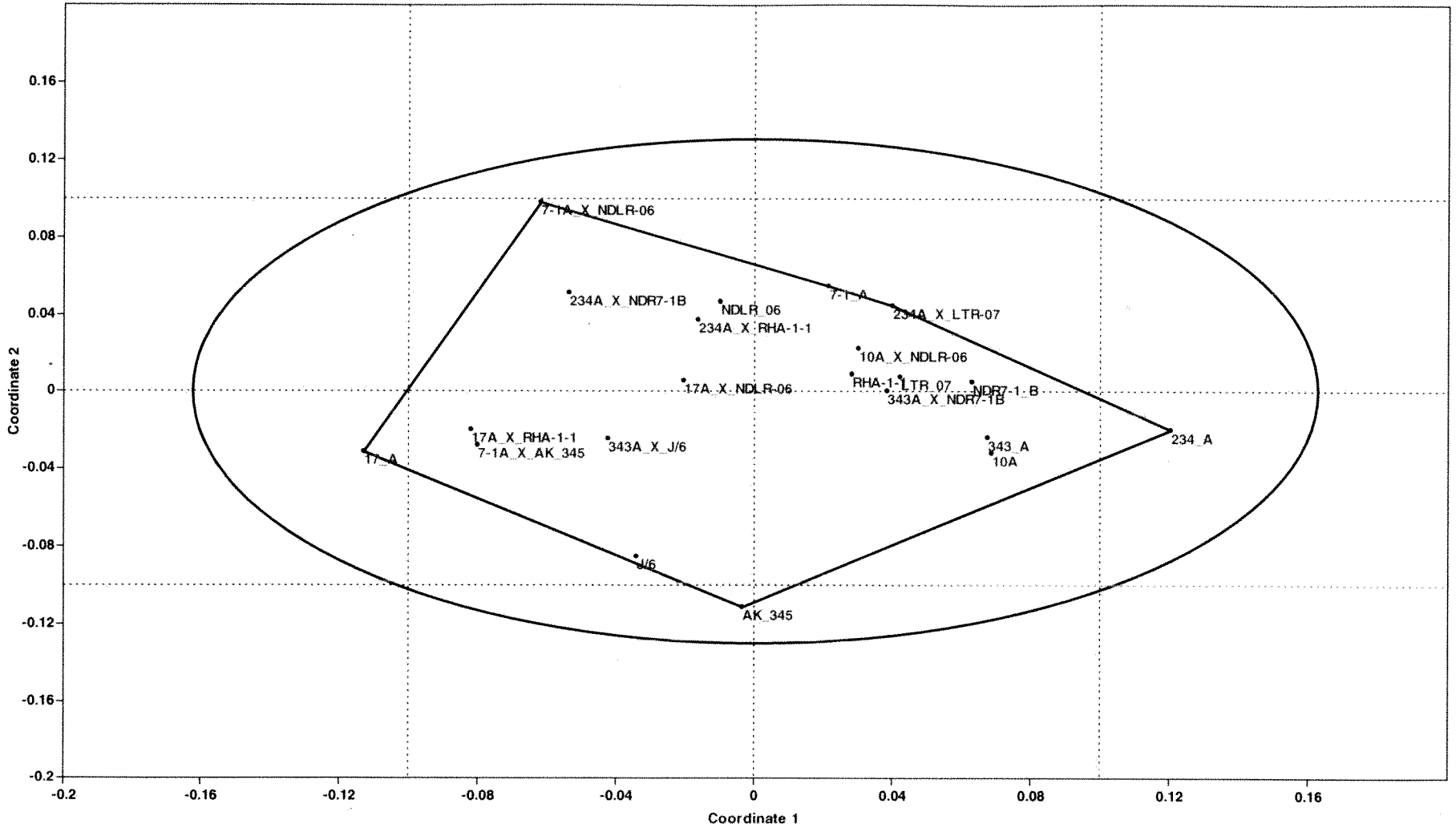


Fig.28 Principal Co-ordinate analysis (PC0) analysis of SSR data with hybrids and their parents

4.1.5 Genetic relationship analysis among hybrids and parents by using combines RAPD and SSR polymorphism

Nine RAPD primer and thirteen microsatellite (SSR) primers fingerprint data was combinely analyzed and generated Dice Similarity Matrix. The Similarity Matrix could effectively identify close association of parental lines and their respective hybrids. While few of them were genetically distant could form a separate clade. The percentage similarity was ranged from 71% to 95% (Table 17).

The similarity matrix was used to plot dendrogram and based on similarity percentage; sunflower hybrid and parental line were categorized into 7 major clusters. The cluster I comprised six members included five parental lines and one hybrid (343 A X J/6). The major cluster II included six members comprising four hybrids and two parental lines. While major cluster III comprised 3 members including two parental line (7-1 A and NDLR 06) and one hybrid line (343 A X NDR7-1B). Similarly cluster IV and V comprised individually two members in one cluster. While parental line 17 A and hybrid 7-1 A X NDLR 06 were found more distant and grouped individually into separate cluster VI and VII (Fig. 29).

Principle co-ordinate analysis further finely categorized all these members into nine groups based on their genetic variance (Fig. 30). The PCoA analysis also revealed similar results obtained during RAPD and SSR Dendrogram analysis. The parental line 17 A was outgrouped and genetically found more distant compare to other parental line and respective hybrids.

4.1.6 Hybrid purity assessment by using RAPD markers

Two hybrids and their parental DNA were initially checked with RAPD primers for amplification. Twelve different random decamer primers were used for RAPD fingerprint pattern of hybrids and their parental lines. The RPAD profile of parents and their hybrids were correlated to find out direct introduction of character / genes in to the hybrids through male or female parent by possessing of male and female parent specific bands among RAPD profiles of 10 hybrids.

Based on RAPD fingerprint profile generated by using 12 random primers, polymorphism and relationship between hybrids and parents were documented. Nine RAPD primers showed good polymorphism with all 10 hybrids and their parental lines.

Table 17: Similarity matrix based on Jaccard's similarity coefficient value obtained from RAPD and SSR combine analysis of ten hybrids with their parents

	234 A	234A X RHA-1-1	RHA-1-1	7-1 A	7-1A X NDLR-06	NDLR-06	10A	10A X NDLR-06	343 A	343A X NDR7-1B	NDR7-1 B	234A X NDR7-1B	343A X J/6	J/6	17 A	17A X RHA-1-1	234A X LTR-07	LTR -07	17A X NDLR-06	7-1A X AK 345	AK 345
234 A	1.00																				
234A X RHA-1-1	0.83	1.00																			
RHA-1-1	0.87	0.89	1.00																		
7-1 A	0.85	0.90	0.89	1.00																	
7-1A X NDLR-06	0.79	0.86	0.83	0.89	1.00																
NDLR-06	0.84	0.88	0.87	0.95	0.88	1.00															
10A	0.89	0.87	0.91	0.87	0.77	0.87	1.00														
10A X NDLR-06	0.86	0.82	0.90	0.87	0.83	0.88	0.88	1.00													
343 A	0.94	0.83	0.89	0.85	0.81	0.84	0.91	0.86	1.00												
343A X NDR7-1B	0.85	0.85	0.87	0.92	0.82	0.89	0.89	0.86	0.85	1.00											
NDR7-1 B	0.89	0.81	0.86	0.87	0.81	0.87	0.88	0.84	0.88	0.89	1.00										
234A X NDR7-1B	0.84	0.91	0.91	0.91	0.85	0.91	0.86	0.85	0.86	0.82	0.82	1.00									
343A X J/6	0.90	0.86	0.87	0.84	0.84	0.85	0.87	0.83	0.90	0.84	0.88	0.89	1.00								
J/6	0.87	0.79	0.85	0.80	0.75	0.83	0.85	0.82	0.89	0.81	0.87	0.84	0.86	1.00							
17 A	0.72	0.73	0.72	0.74	0.74	0.72	0.74	0.75	0.72	0.75	0.73	0.73	0.79	0.73	1.00						
17A X RHA-1-1	0.82	0.85	0.86	0.87	0.81	0.89	0.84	0.85	0.81	0.80	0.82	0.90	0.85	0.82	0.75	1.00					
234A X LTR-07	0.85	0.83	0.86	0.87	0.77	0.84	0.84	0.84	0.82	0.87	0.84	0.84	0.81	0.78	0.76	0.86	1.00				
LTR -07	0.88	0.83	0.89	0.90	0.82	0.87	0.83	0.85	0.85	0.85	0.83	0.85	0.82	0.81	0.71	0.85	0.87	1.00			
17A X NDLR-06	0.86	0.80	0.84	0.87	0.83	0.86	0.84	0.86	0.84	0.88	0.84	0.85	0.85	0.84	0.76	0.86	0.82	0.84	1.00		
7-1A X AK 345	0.82	0.91	0.88	0.91	0.85	0.89	0.86	0.85	0.81	0.86	0.81	0.88	0.85	0.80	0.75	0.93	0.86	0.87	0.86	1.00	
AK 345	0.88	0.86	0.90	0.86	0.78	0.84	0.88	0.83	0.86	0.84	0.84	0.88	0.89	0.84	0.72	0.90	0.84	0.88	0.85	0.88	1.00

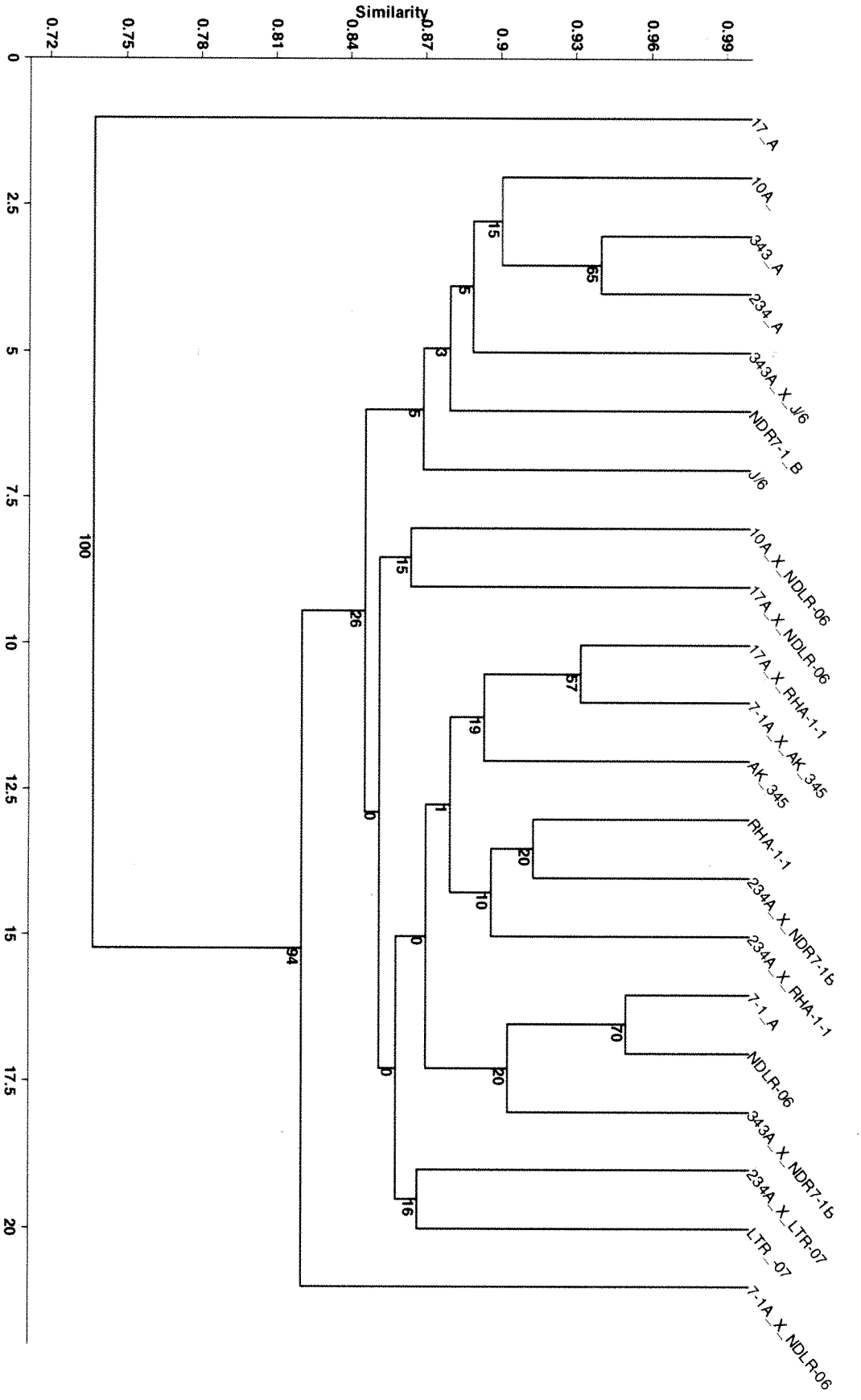


Fig. 29 Dendrogram generated using UPGMA analysis demonstrating relationship among ten hybrid with their parents based on RAPD and SSR combine data.

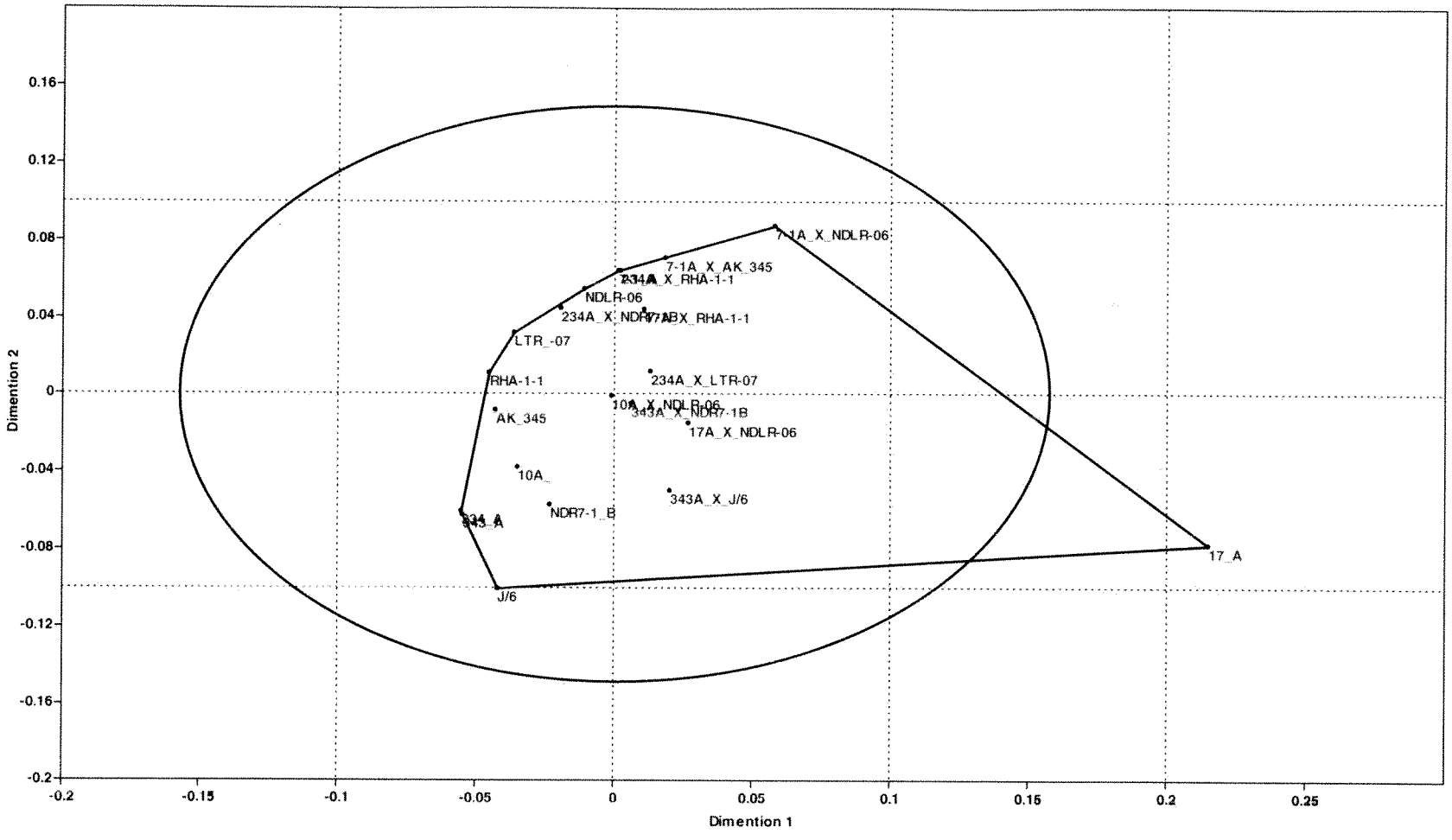


Fig. 30 Principal Co-ordinate analysis (PC0) analysis of RAPD and SSR combine data of hybrids and their parents

The RAPD fingerprint pattern of sunflower hybrid LSFH-35 generated by primer OP 03 could produce one female parent specific amplicon of size OP-03 $_{250}$ (250 bp) and one male parent specific amplicon OP-03_{750} (750 bp), similarly primer OPA 02 produced three female parent specific amplicons of size OPA 02_{400}; OPA 02_{750} and OPA 02_{1000}. (Fig. 1 & 2). While remaining RAPD primers have generated male parent specific amplicons except primer OPA 08, OPA 11 and OPA 13, where they shared monomorphic amplicons present in both parents. The primer OPA 03 amplified two male parent specific amplicons of size 750 and 900 bp. Similarly the primer OPA 07, OPA 09, OPE 16 and OPL 18 could generate a unique male parent specific amplicon of size 850 bp, 1200 bp, 750 bp and 250 bp respectively. The primer OPC 16 has generated two male parent specific amplicons of size 1000 bp and 900 bp respectively.

The RAPD fingerprint data generated for hybrid LSHT-16 by 11 different arbitary primers also produced 3 female parent specific fragments commonly shared by hybrid LSHT-16. The primer OPA 03, OPE 16 and OPA 13 have generated female parent specific amplicons of size 750 bp, 1000 bp and 1300 bp respectively commonly shared by hybrid LSHT-16 (Plate 3, 5 & 7).

Six arbitary primers were found more efficient to assess the purity of sunflower hybrid LSHT-11. The primer OPA 02, OPC 16 (Plate 4) and OPE 16 amplified a unique amplicon of size 2000 bp, 750 bp and 1200 bp respectively from male parent and hybrid lines. This male specific amplicons could add the signature of assessment of purity of hybrid indicating direct introduction of character from male parent. The primer OP-03, OPA 02, OPA 08, OPA 09 and OPC 16 generated female parent specific amplicons

The hybrid line LSFH-10126 showed four male specific amplicons and a one female specific amplicon (Table 18). The maximum number of male specific amplicons present in hybrid LSFH-10126 confirms the purity of this hybrid.

The genetic purity of hybrid line LSFH-07-03 was also assessed by using 11 arbitary primers. The primers OP-03, OPA 03, OPA 11 and OPC 16 have generated both male parent and female parent specific amplicons (band size shown in Table 18). Similarly the purity of hybrid LSFH-10128 was assessed by using random primers OPA 11, OPC 16 and OPL 18 produced male parent specific amplicons. While primer OP-03, OPA 02, OPA 11 and OPC 16 could amplify a common fragment between hybrid line

and female parent, while remaining primers could not produce polymorphic amplicons and failed in test of hybrid purity assessment.

The purity of hybrid LSFH-9124 was significantly assessed by primer OP-03, OPA 02, OPA 03, OPA 07, OPA 08, OPA 09, OPA 11, OPA 13, OPC 16 and OPE 16 amplifying 19 male parent specific amplicons (as shown in Table 18).

The purity of hybrid LSFH-10129 was assessed by generating polymorphic male parent specific amplicons by primer OP-03, OPA 11 and OPC 16. Similarly purity of hybrid LSFH-10129 was also assessed by generating polymorphic female parent specific amplicons by primer OP-03, OPA 02, OPA 09, OPC 16 and OPE 16. Also the purity of hybrid LSFH-1706 was assessed through polymorphic data generated by 11 arbitary primers producing male parent specific and female parent specific amplicons. The primer OP-03, OPA 02, OPA 03, OPC 16, OPE 16 and OPL 18 could generate 15 male parent specific polymorphic amplicons and significantly assessed purity of hybrid LSFH-1706.

The genetic purity of hybrid LSFH-7345 was assessed by decamer primers. The primer OPA 02 was found significant to find out genetic purity of hybrid LSFH-7345 by generating 5 male parent specific polymorphic amplicons. Similarly each of primer OPA 03, OPA 08, OPA 13, OPC 16 and OPL 18 has amplified a single polymorphic amplicons of varying sizes for purity analysis of hybrid LSFH-7345 (Table 18).

Table 18: Female and Male parent specific bands generated by RAPD markers

RAPD primers	Name of Hybrid	OP-03		OPA02		OPA03		OPA07		OPA 08		OPA 09		OPA11		OPA 13		OPC16		OPE 16		OPL18		OPA 20	
Sr.No.		FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)
1	LSFH-35	0.25	0.75	0.4 0.75 1.0	---	---	0.75 0.9	---	0.85	---	---	---	1.2	---	---	---	---	---	0.9 1.0	---	0.75	---	0.25	---	---
2	LSHT-16	---	---	---	---	0.75	---	---	---	---	---	---	---	---	---	1.3	---	---	---	1.0	---	---	---	---	---
3 →	LSHT-11	0.5 1.0	---	0.8	2.0	---	---	0.6	---	0.85	---	0.5	---	---	---	---	---	1.0	0.75	0.9	1.2	---	---	---	---
4 ↓	LSFH-10126	0.5	---	---	---	---	2.7	---	0.9	---	---	---	---	---	---	---	---	2.0	0.9	---	1.2	---	1.3	---	---
5	LSFH-07-03	0.25 0.75	0.68	---	---	0.8	2.0	0.9	---	---	---	---	---	0.6	1.0	---	---	1.05	0.9 2.0	.85	---	---	---	---	---
6	LSFH 10128	0.25 0.75	---	0.25	---	---	0.5	---	---	---	---	---	---	1.0	0.6	---	---	2.0	1.05 0.95	---	---	---	1.3	---	---
7	LSFH-9124	---	0.3 0.7 0.75	---	0.5 0.75 1.2 2.5	0.75	0.8	---	1.5 0.6	---	0.85	---	1.2 1.3 0.95	---	0.6 0.9 1.0	---	0.6	---	0.85	1.0 0.75 0.3	---	---	---	---	---
8	LSFH-10129	0.75	0.7	0.4	---	---	---	0.9	---	---	---	0.55	---	---	1.0	---	---	1.0	0.85	1.0	---	---	---	---	---
9	LSFH-1706	---	0.5 0.75 1.25 1.5	0.4	1.0 1.5 2.0	---	1.3	---	---	---	---	---	---	---	---	---	---	1.0	0.85 1.5 2.0	1.1	0.25 0.3 1.1	---	0.5	---	---
10	LSFH-7345	---	---	---		0.7 1.0	0.5	0.35 0.75 0.9	---	---	0.85	---	---	---	---	---	1.0	2.0	1.0	1.0	---	---	1.3	---	---

Where **FPS**- Female parent specific, **MPS** - Male Parent Specific, **Kb**-kilo base pair

4.1.7 Hybrid purity assessment by using microsatellite (SSR) marker

Growing popularity of commercial hybrids and the active involvement of public and private sector in large scale hybrid seed production demands quality control in terms of monitoring seed genetic purity at both parental and hybrid seed production stages for success of hybrid technology. In this situation molecular marker based genetic purity assay will be highly useful in rapid and large scale screening of hybrid seed lots. To demonstrate the utility of these polymorphic SSR markers for hybrid purity testing, these hybrids were screened to detect female specific and male specific marker to assess true hybrid or pure hybrid.

In present investigation 18 SSR primers (Table 5) have been exploited for assessment of purity of ten hybrids of sunflower. Out of 18 SSR primers, seven primers were found polymorphic while five primers have generated monomorphic amplicons and remaining six have shown non specific amplification so these 11 primers were not able to assess purity of hybrid. The SSR primer IUB 6 has generated 58% polymorphism followed by primer ORS 13 and ORS 5 produced 47% and 48% polymorphism respectively. Among 13 microsatellite primers seven different types of SSR polymorphic marker pattern have been identified which could help to identify purity of hybrids (Table 19).

Table.19 Six types of SSR markers observed in hybrids and their parents

Type of markers	Male (M)	Hybrid (H)	Female (F)	No. of polymorph -ic bands	Polymorphi sm (%)	Remark
1	+	+	-	26	43.33	* Hybrid confirm
2	+	-	+	8	13.33	Polymorphic parent
3	-	+	+	16	26.66	* Hybrid confirm
4	+	-	-	06	10	Polymorphic parent
5	-	+	-	02	3.3	Polymorphic parent
6	-	-	+	02	3.3	Polymorphic parent
Total				**60**	**99.9**	

Seven SSR primers viz, Ha 1327, Ha 1442, ORS 13, ORS 5, ORS 536, ORS 243 and ORS 662 enabled to assess the purity of hybrid by generating 27 female parent specific polymorphic band and 16 male parent specific polymorphic bands (Table 20). The SSR primer ORS 5 was identified as superior marker could assessed purity of nine hybrids while not able to identify purity of hybrid LSFH-10129 (Plate 14). The primer Ha 1327 rank in second position for confirmation of purity of seven hybrids out of ten hybrids. While primer Ha 1442 could assessed purity of four hybrids (LSHT-11, LSFH-07-03, LSFH-10129 and LSFH-10129), similarly SSR markers ORS 13 and ORS 536 (Plate 14,15) have been individually assessed purity of three hybrids and primer ORS 662 determined purity of two hybrids LSFH-9124 and LSFH-1706 (Plate 13).

Primer IUB 6, ORS 323, ORS 391, HNCA 2 and ORS 1114 have generated maximum number of monomorphic amplicons. Hence they were failed to assess purity of sunflower hybrid. The maximum number of 4 alleles generated by primer Ha 1327 comprising female specific (Ha 1327_{600}, Ha 1327_{700}) and one male specific Ha 1327_{900} marker (Plate 11). The male parent specific SSR marker suggested direct introduction of character from male parent and confirm the identity of respective hybrid. Several male and female parent specific polymorphic SSR alleles were reported (Table 20) and potentially helped to asses purity of hybrid of sunflower. The primer ORS 5 could produce maximum number of male specific alleles in five hybrids and confirm the direct introduction of character and identity of the hybrid (Table 20).

Among ten hybrids five hybrids LSFH-35, LSFH-10128, LSFH- LSFH-1706, and LSFH-7345 were heterozygous with three primers (Ha 1327, ORS 5, ORS 662) showing the presence of both female and male parent specific marker to hybrid individual (Plate 11,12,13). These five hybrids were designated as pure hybrid while other five hybrids could show the presence of either female or male parent specific marker into which transfer of character either male or female to that hybrid individual, which can also be confirmed five as hybrid (Table 21).

Table 20: Female and Male parent specific bands generated by SSR markers

SSR primer →		Ha 1327		Ha 1442		ORS 13		ORS 5		ORS 536		ORS 243		ORS 662	
Sr.No ↓	Name of Hybrid	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb)	MPS (kb)	FPS (kb	MPS (kb)	FPS (kb	MPS (kb)	FPS (kb)	MPS (kb)
1	LSFH-35	0.7	0.6 1.0	—	—	—	—	—	0.33	—	0.3	0.8 1.0	—	—	—
2	LSHT-16	0.6	—	—	—	—	—	—	0.3	—	—	0.19	—	—	—
3	LSHT-11	—	—	—	—	—	—	0.3	—	—	—	0.19	—	—	—
4	LSFH-10126	—	0.2	—	—	—	—	0.28	—	0.3	—	0.8 1.0	—	—	—
5	LSFH-07-03	—	—	—	0.18	—	—	0.35	—	—	—	—	—	—	—
6	LSFH 10128	—	—	—	—	0.15 0.18	—	0.3	0.33	—	—	1.0	0.6	—	—
7	LSFH-9124	0.2	—	—	—	—	—	0.3	—	—	0.25	0.6	—	—	0.33
8	LSFH-10129	0.2	0.25	—	—	0.3	—	—	—	—	0.3	0.19	—	—	—
9	LSFH-1706	—	—	—	—	0.18	—	0.3	0.31	—	—	—	—	0.22	0.32
10	LSFH-7345	0.2	—	—	—	—	—	0.33	0.3	—	—	—	—	—	—

Where FPS- Female parent specific, MPS - Male Parent Specific, Kb-kilo base pair

Table 21: Confirmation of sunflower hybrids by using different SSR primers

Primer	Hybrids									
	H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**
Ha 1327	C*	C	N	C	N	N	C	C*	NC	C
Ha 1442	N	N	C	N	C	N	C	C	N	N
ORS 6	N	N	N	N	N	N	N	N	N	N
ORS 13	N	N	N	N	N	C	N	C	N	C
ORS243	C	C	C	C	N	C*	C	C	N	N
IUB 6	N	N	N	N	N	N	N	N	N	N
ORS 323	N	N	N	N	N	N	N	N	N	N
ORS 391	N	N	N	N	N	N	N	N	N	N
ORS 5	C	C	C	N	C	C*	C	N	C*	C*
HNCA 2	N	N	N	N	N	N	N	N	N	N
ORS 536	C	N	N	C	N	N	N	C	N	N
ORS 662	N	N	N	N	N	N	C	N	C*	N
ORS 1114	N	N	N	N	N	N	N	N	N	N

Where **H1 to H10** - Ten sunflower hybrids as listed in Table 3 respectively.

C*- Confirmed hybrid having presence of both male and female specific amplicons/alleles

C - Confirmed hybrid with either presence of male or female amplicon/alleles

N - Not confirmed

4.2 In vivo screening of sunflower hybrids for tolerance of Sunflower necrosis disease (SND)

Sunflower hybrids used in this investigation were mechanically sap inoculated with sunflower necrosis virus. Plants were maintained in green house under controlled condition and monitored for appearance of symptoms of SNV (Plate 23).

On the basis of disease grade and symptoms (Plate 24) and percent disease incidence of SND, ten sunflower hybrids were evaluated into four different categories (Table 22).

The hybrid LSHT-16, LSFH-10126 and LSFH-10128 were designated as highly tolerant by scoring percent disease incidence o.1%, 0.5%, 0.5% respectively. While

Fig.23 Screening of ten sunflower hybrids for tolerance against Sunflower Necrosis Virus (SNV) in greenhouse

Plate No. 23

Fig. 24 (a) 1. Necrosis on inoculated leaves

Fig. 24 (b) 2. Chlorotic spots on leaves

Fig.24(c) 3. Systemic chlorotic and necrotic symptoms

Fig. 24 (d) 4. Severe chlorosis and necrosis and premature death of plants

Fig.24: (a, b, c, d) Grades used for measuring intensity of Sunflower necrosis disease (SND)

hybrid LSFH-35 and LSHT-11 were found moderately tolerant by scoring individually percent disease incidence of 20%, similarly the hybrid LSFH-1706 and LSFH-7345 were categorized as moderately susceptible and their percent disease incidence was 60%. Hybrids LSFH 07-03 and LSFH-9124 were found highly susceptible, showed high percent disease incidence of 80%.

Table 22: SND disease severity of 10 sunflower hybrids

Sr. No.	Name of hybrids	Percent disease incidence (PDI)	SND disease severity
1	LSFH-35	20%	Moderately tolerant
2	LSHT-16	0.1%	Highly tolerant
3	LSHT-11	20%	Moderately tolerant
4	LSFH-10126	0.5%	Highly tolerant
5	LSFH-07-03	80%	Highly susceptible
6	LSFH-10128	0.5%	Highly tolerant
7	LSFH-9124	80%	Highly susceptible
8	LSFH-10129	1%	Highly tolerant
9	LSFH-1706	60%	Moderately susceptible
10	LSFH-7345	60%	Moderately susceptible

Discussion

CHAPTER-V

DISCUSSION

Sunflower is an important source of oil, feed and widely grown ornamental crop. Sunflower breeding programme are focused basically on yield, oil content, disease resistance, use of germplasm of *Helianthus* species for hybrid development required detailed genotype control. Narrow genetic base are the major problem in sunflower breeding for development of genetically diverse & vigorous hybrids from their parents i.e. male sterile & restorer lines. The impurity of pollen source and mixture of parental seeds or other cultivar seeds with the hybrids will also lower the genetic purity of the produced hybrids. Low purity seed would cause great loss in yield, quality & productivity. Therefore it is crucial to elucidate the diversity among parental line used in hybrid development programme and assessment of genetic purity of developed hybrids.

Grow out trial (GOT)

The GOT a conventional method of assessment of hybrids is based on set of morphological characters including qualitative as well as quantitative characters studied from growing stage to maturity of plant. In present investigation 10 hybrid lines were studied in respect to the comparative study of hybrid vigour and morphology with their respective parents. Similar types of test have been followed by several researchers in different crops including tomato (Liu, *et al.,* 2007), cotton (Ali *et al.,* 2008) and sunflower (Nandini and Chikkadevaiah, 2005).

5.1.1 Assessment of genetic relationship among hybrid and parental line by using RAPD fingerprint profile

The molecular marker could overcome the problems that are associated with phenotype based marker. RAPD (Williams *et al.,* 1990) an important molecular marker has advantages of its simplicity, rapidity, requirement for only a small quantity of DNA and ability to generate numerous polymorphisms (Akhare *et al.,* 2008). Therefore, it has been utilized as powerful tool for genetic analysis (Chapco *et al.,* 1992, Kiss *et al.,* 1993, Hashizume *et al.,* 1993, Nandini and Chikkadevaih, 2005 and Ali *et al.,* 2008). RAPD is best marker could help to identify genetic relationship among parents and

hybrids without knowing their sequence. Thus, 11 RAPD primers have been utilized and assessed genetic dissimilarity and closeness among parents and hybrids. Out of total 11 primers nine were found polymorphic and generated 189 polymorphic amplicons. The primer OPA 11 could produce maximum of 60% polymorphism thus, primer OPA 11 was significantly marked as informative primer. While primer OPL 18 showed minimum of 12.5% polymorphism level. The dendrogram analysis revealed seven major clusters and several subcluster. The genetic distance among parental line 7-1A and NDLR -06 was 4% and indicated their close association. Similarly, parent 17 A was found most divergent parent showed 34% dissimilarity. Therefore such divergent source of parental line would be useful in hybrid breeding programme.

5.1.2 Assessment of genetic relationship among hybrid and parental line by using SSR fingerprint profile

To the exploitation of full potential of heterosis and enhancement of hybrid development, it is essential to characterize parental lines at genetic as well as morphological level. The genetic divergence study helps breeder to concentrate on possible promising parent combinations (Saxena *et al.,* 2010). Molecular marker SSR is codominant marker efficiently used in identification of genetic relationship among parental lines and their hybrids of peanut (Kottapalli *et al.,* 2007), Tomato (Smith & Register, 1998), Maize (Salgado *et al.,* 2006), rice (Yashitola *et al.,* 2002) and cotton (Dongre & Parkhi, 2005). In present study 18 SSR markers have been exploited and identified genetic relationship among 10 sunflower hybrids and their parents. All 18 SSR primers were screened with 10 hybrids with respective parents and identified seven polymorphic SSR markers could able to distinguish hybrids and parents at allelic level. The primer IUB 06 was determined genetic relation among hybrids and parents by generating 58% polymorphism. The diversity percentage among parents and hybrids based on SSR marker analysis were ranged between 2% to 25%. The dendrogram analysis could categories 10 hybrids and their parents into IV major clusters. The parental line 10 A and 343A were found closer. Similarly, parent J/6 was found divergent parent showed 16% dissimilarity. Therefore, adequate genetic diversity information in parental lines should be useful for selecting crossing parental genotypes that may enhance the hybrid vigour.

5.2 Hybrid purity assessment by using molecular markers:

The traditional GOT method, which is based on morphological character, require large plot of land & take several method to evaluate & it may limited by environmental condition (Liu *et al.,* 2007). The hybrids having less purity causes big loss in terms of yield and prices therefore it is important to critically evaluate the genetic purity in seed production and trade (Wang et al, 2005, Garg *et al.,* 2006 and Dongre & Parkhi, 2005). Therefore to overcome these problems a molecular markers viz., RAPD and SSR could exploit and assessed purity of 10 sunflower hybrids.

5.2.1 Hybrid purity assessment using RAPD markers:

PCR based marker technologies including RAPD, SCAR have been developed as simple, safe & cost effective method for molecular analysis (Bellester & Levinch *et al.,* 1998). Recently these marker systems have been extensively employed in varietal identification & diverse plant breeding programme (Kim *et al.,* 2004 and Lee *et al.,* 2002).

Also RAPD marker was significantly utilized in purity assessment of hybrids of various crops including Tomato (Liu *et al.,* 2007), watermelon (Hashizume *et al.,* 1993), cotton (Ali *et al.,* 2008), chilli (Ilbi, 2003) and Sorghum (Akhare *et al.,* 2008).

RAPD marker was able to confirm hybrids based on the polymorphic band which were polymorphic either of male or female line. In present investigation 10 hybrids were screened with nine RAPD primers and identified their purity. The primer OPC16 and OPA11 were designated as informative marker showed 57.5% and 60% polymorphism respectively. Out of nine RAPD primers six RAPD primers were generated female parent specific & male parent specific RAPD marker present to the corresponding hybrid individuals.

The RAPD primers OPC 16 was found most useful in assessment of purity of most of the hybrids tested along with their parents. In the pair-wise comparison of marker band the primer OPC 16 generated 1 to 6 bands aiding in purity assessment of hybrids. Similar report was reported by Nandini *et al.,* (2005). The primer OPC 16 generated RAPD amplicons that were specific to male and female parents. A RAPD fragment of size ~2.0 kb was identified specific to parent 343 A, CMS A (female) used

for 2 hybrids i.e. LSFH-10126 & LSFH-10128. An amplicons of size 850 bp was found specific to restorer line NDLR-06, LSHT-16 & LSHT-11. While the same fragment was also specific to the restorer line RHA-1-1. LSFH-35 & LSFH-9124 served as a marker for differentiation of hybrids from their parental lines and assessment of purity of hybrids. The primer OPA 02 and OPA 03 generated amplicons of size 2500 bp, 1500 bp, 1000 bp and 800 bp, 1300 bp, 500 bp respectively, were specific for male parents could assess purity of hybrids LSFH-9124, LSFH-1706 and LSFH-7345. The RAPD amplicons commonly shared by hybrid and male parent as well as female parent and hybrid in different RAPD primers (OP-03, OPA 02, OPA 03 OPA 11, OPC 16, and OPE 16) would be exploited to generate SCAR marker for identification of hybrid lines. Similar study of development of SCAR marker by using polymorphic RAPD fingerprint data has done in sunflower hybrid DRSH1 (Dinesh Kumar *et al.,* 2009) and in chilli pepper (Jang *et al.,* 2004).

5.2.2 Hybrid purity assessment using SSR markers

DNA markers provide an opportunity for assessing the purity of hybrid or genotype precisely even at the seed stage. PCR based and locus specific co-dominant marker SSR is the most suitable marker being used for hybrid purity assessment. Also the hetrozygosity of hybrids can be easily determined by the presence of both parental alleles (Naresh *et al.,* 2009). A few SSR primers were developed in sunflower and readily used for purity testing of hybrids (Antonova *et al.,* 2006, Li *et al.,* 2007, Dinesh kumar *et al.,* 2009). The use of SSR markers for assessing seed purity of hybrids is almost routine for several crops example Rice (Yashitola *et al.,* 2002, Nandakumar *et al.,* 2004 and Sundaram *et al.,* 2008) , Maize (Mingsheng *et al.,* 2006), Safflower (Naresh *et al.,*2009), cotton (Ali *et al.,* 2001) and horticultural crops like tomato (Smith and Register, 1998), Cabbage (Liu *et al.,* 2007), tomato (Paran *et al.,* 1995) and melon (Jinali *et al.,* 2006). Although some studies reported the suitability of even single marker for hybrid purity assessment tests (Yashitola *et al.,* 2002, Nandakumar *et al.,* 2004). The present study reports suitability of seven SSR markers out of 18 markers for hybrid purity test of sunflower. The SSR marker Ha1327, Ha1442, ORS13, ORS 55, ORS536, ORS 243 and ORS662 could generate both FPS and MPS SSR marker in ten sunflower hybrids.

Two SSR markers were assessed for the hybrid purity testing of LSFH-1706. Both of these marker amplified distinct alleles in A (ORS662-220 bp, ORS 5-310bp) and R lines (ORS 662- 320 bp, ORS 5-340 bp) which suggest their use in multiplex manner for hybrid purity assessment. This will reduce costs and time of PCR assays and also will increase accuracy in determining purity in parental lines as well as in hybrid seeds.

The SSR marker ORS 5 enabled to assess purity of most of the hybrids except hybrid LSFH 10126 and LSFH 10129. Similarly, the primer Ha 1327 also assessed purity of seven hybrids based on FPS and MPS SSR markers.

Thus, seven SSR markers (Ha 1327, Ha 1442, ORS 13, ORS 5, ORS 536, ORS 243 and ORS 662) were enabled to assess purity of 10 hybrids of Sunflower separately.

5.3 *In vivo* screening of sunflower hybrids against SND infection:

5.3.1 Importance of disease

Sunflower necrosis disease (SND) becoming severe problem for cultivation of sunflower. Previously SND was identified as a Tospovirus related groundnut bud necrosis virus and water melon silver mottle virus (Venkat subbaiah *et al.*, 2000 and Jain *et al.*, 2000). Subsequently the casual agent was identified as TSV belonging to the ilarvirus genus (Prasad Rao *et al.*, 2000, Ravi *et al.*, 2001, Ramiah *et al.*, 2001 and Bhat *et al.*, 2002). This virus has a wide range of alternate host such as cotton, mungbean, soybean, sunhemp, okra, cucumber, gherkin, safflower, chilli and several other cereal and ornamental crops. The wide host range of SND enabled rapid spread and perpetuation of this disease and could result into major outbreak. It is an epidemic disease and cause losses to the extent of 100% (Anon., 2000) in terms of yield loss, the disease intensity does not have 1:1 relation but SND incidence could directly translated to yield loss. The SND symptoms reported are necrosis of leaf lamina, petiole, stem and floral calyx, lodging of taller plants, stunted growth and death of plant (Sharman *et al.*, 2008, Jain *et al.*, 2000 and Bestar, 2004)

5.3.2. Symptomatology:

The symptom of sunflower necrosis (black burning) begins from the margin of the leaf and extends to the growing tip. The mild mosaics to mottling symptoms were observed (Nagaraju *et al.,* 1998). The symptoms like mosaic, chlorotic rings/ streaks, marginal necrosis, stunting of plant distortion of young leaves in early infected plant were reported (Halakeri, 1999). Symptoms developed through systemic infection are various types of mosaic mottling, puckering, twisting of leaves, narrowing and yellowing (Anilkumar, 1999 and ShivaSharanayya, 2000).

5.3.3. Disease severity and disease score of 10 hybrids:

The screening of germplasm was conducted in several crops to find out resistant genotypes against targeted pathogen viz., Sunflower chlorotic mottle virus in sunflower, (Lenardon *et al.,* 2005). Complete resistance to SNV infections does not find in crop cultivars & has not been reported till. A germplasm screening helps to identify a wide genetic base comprising hybrid lines with improved resistance/ tolerance to SNV.

The hybrid LSFH-10126, LSFH-10128 and LSFH-10129 exhibited highest level of tolerance under artificial sap inoculation experiment. These hybrids were characterized on the basis of development of necrosis symptoms on inoculated leaves only & no systemic spread, which resembles hypersensitivity reactions. While systemic chlorotic symptoms were detected in hybrid LSHT-35 & LSHT-11. Similarly LSFH-07-03 & LSFH-9124 which exhibited highest level of incidence is characterized by severe chlorotic, necrosis and premature death of plant.

The detected level of resistance against SNV could be useful for agronomic purpose & may contribute to reducing losses caused by this disease.

Summary And Conclusion

CHAPTER-VI

SUMMARY AND CONCLUSION

Sunflower (*Helianthus annuus* L.) is an annual diploid, cross pollinated crop belongs to family compositae, sunflower seed is highly nutritious containing 40-45% vegetable oil with a very high calorific value. Being the important oilseed crop heterosis development in sunflower could be exploited for better seed and oil yield. Hybrids are more vigorous and resistant against important foliar diseases. In a systematic hybrid breeding programme it is essential to identify superior parent to exploit the genetic variability for better heterosis development. Being cross pollinated crop, genetic contamination is often observed in sunflower that lead to loss of purity and hybrid vigor. In consideration of limitation of GOT, there is need of rapid and accurate assay for assessment of genetic purity of hybrid seed. Thus, present study was implemented to exploit the utility of molecular marker for assessment of purity of ten hybrids of sunflower.

Sunflower necrosis disease (SND) is emerging as a major disease of sunflower recently. Within the span of 10 years the disease has spread in almost all sunflower growing states in India. Yield loss is ranging from 30 to 100% (Chandar Rao *et al.*, 2000). Being an important disease of sunflower, it is essential to identify resistant hybrids through screening procedure.

In present investigation ten hybrids of sunflower were mechanically sap inoculated. Based on disease scale and severity ten hybrids were categorized into highly tolerant (LSHT-16, LSFH-10126, LSFH-10128 and LSFH-10129), moderately tolerant (LSFH-35 and LSHT-11) and highly susceptible (LSFH 07-03, LSFH-9124).

Genetic relationship among hybrids and their parental line was carried out by using 29 primers comprising 12 RAPD and 18 SSR markers.

Based on RAPD fingerprint profile the percent similarity among hybrid and parental line was ranged between 61-96%. The Dendrogram analysis categorized parental lines and hybrids into VII distinct clusters. The PCoA analysis based on variance level also categorized hybrids and their parental line into VII major groups.

Similarity genetic relationship among hybrid and parental line was also predicted by using microsatellite (SSR) marker. Among 18 SSR markers, eight SSR markers could identify polymorphism among hybrids and respective parental lines. The primer IUB 06 was found most informative by producing 58% polymorphism. The percent similarity among hybrid and their parental line was range between 75 to 98%. Similarly, diversity percentage among hybrids and parental lines were ranged between 2 to 25%. The Dendrogram analysis could categorize hybrid and their parental line of sunflower into four distinct groups and several subcluster.

The data generated by RAPD and SSR fingerprint profile was exploited and assessed the purity of ten hybrids of sunflower. Among 12 RAPD primers nine primers were abled to assess purity of hybrids by generating polymorphism. The primer OPA 11 could produce 60% polymorphism while OPL 18 showed 12.5% polymorphism. The RAPD primer OPC 16 was found significant in discrimination of seven hybrids with their parental lines, also primer OPC 16 could generate bands that were specific to male as well as female genotype among seven hybrids except LSFH-35, LSHT-16 and LSFH-9124. Similarly primer OP-03, OPA 02, OPA 03, OPA 11 and OPE 16 could significantly assess purity of ten hybrids by generating MPS and FPS RAPD markers. In comparison with RAPD, the marker SSR found significant for assessment of purity of sunflower hybrids.

Thirteen SSR markers have been exploited for purity assessment of ten hybrids. Out of which eight primers found significant and abled to detect hetrozygosity among hybrids. The primer Ha 1327 and ORS 5 was found most informative in discrimination and assessment purity of hybrids in sunflower. The primer ORS 5, ORS 662 and Ha 1327 produced both male and female parent specific marker in hybrids, LSFH-7345, LSFH-1706 and LSFH-35 respectively. While eight SSR markers could produce either male or female parent specific marker and found informative in assessment of hybrid purity.

Thus, present investigation was concluded with hybrid LSHT-16, LSFH-10126, LSFH-10128 and LSFH-10129 could able to tolerate SND compare to remaining moderately susceptible (LSFH-1706 and LSFH-7345) and highly susceptible (LSFH

07-03, LSFH-9124) hybrids. The genetic relationship study among hybrid and their parental lines revealed their close relationship by using RAPD and SSR fingerprint polymorphism. The parental line 10 A and 343 A was found very close to each other. While parent J/6 designated as divergent parent and could be useful in hybrid breeding programme. The SSR was found comparatively more significant over RAPD marker in hybrid purity assessment test.

All of ten hybrids used in this investigation were initially found genetically pure and further need to screen with large number of polymorphic marker. Similarly generated polymorphic male parent and female parent specific RAPD and SSR markers would be useful in development of hybrid specific SCAR marker.

Literature cited

CHAPTER-VII

LITERATURE CITED

Akhare, A., Sakhare, S. B., Kulwal, P.L., Dhumale D. B. and Abhilasha, K. (2008). RAPD profile studies in Sorghum for identification of hybrids and their parents. *International Journal of Integrative Biology* 3(1):18-24.

Anilkumar, H. R. (1999) Studies on sunflower necrosis virus disease. *M. Sc.(Agri.) Thesis*, University of Agricultural Sciences, Bangalore.

Anonymous 2010.Directorate of Oilseed Development.

Anonymous 2010-11.Directorate of Oilseed Development.

Antonova, T.S., S.Z. Gucheti, T.A. Ramasanova and S.A. Ramasanova. 2006. Development of markers system for identification and certification of sunflower lines and hybrids on the basis of SSR-analysis. *Heli*a, 29, Nr. 45: 63-72.

Ali, M. A., Muhammad, T.S., Awan, S., Shahid, N., Shiraz, A. and Abbas. A. (2008). Hybrid authentication in upland cotton through RAPD analysis. *Australian Journal of Crop Science* 2(3):141-149.

Arvind, S.M. (2002).Transmission, vector relationship weed harts of Sunflower Necrosis virus disease M.Sc. (Agri.) thesis, University of Agricultural Sciences, Banglore.

Bhat, A.I., Jain, R.K., Chaudhry, V., krishna Reddy M., Ramiah M., Chattannavar S.N. and Verma S. (2002). Sequence conservation in the coat protein gene of Tobacco streak virus causing necrosis in cotton, mungbean, sunflower and sun-hemp in India. *Indian J. Biotechnolo.* 1:350-356.

Bellester, J., and de Vicente, M.C. (1998). Determination of F1 hybrid seed purity in pepper using PCR-based markers. *Euphytica,* 103: 223–226.

Bestar, G., 2004, Epidemiology and management of sunflower necrosis disease. *M. Sc.(Agri.) Thesis,* University of Agricultural Sciences, Dharwad

Beymer, N. B., Nurenberg, P., Wiehe, A., Meixner, M., Epplen, J.T. and Bonier, T. (1992). Fingerprinting plant genome with oligonucleotide probes specific for repetitive DNA sequences. *Theoretical and Applied Genetics* 83:691-694.

Chander Rao, S., Raoof, M.A. and Singh, H.(2000). Sunflower necrosis disease a preliminary study on transmission. In proceedings of extended summaries 2000. National seminar on oilseed and oil research development needs in millennium, *Indian Society of Oilseed Research*, Hyderabad. Feb 2-4-2000:285-286.

Chapco, W. and Ashton, N.W.(1992). A feasibility study of the use of random amplified polymorphic DNA in the population of genetics and systematics of grasshoopers. *Genome* 35:241-244.

Cobb, B. D. and Clarkson, J.M. (1994). A simple procedure for optimizing the Polymerse Chain Reaction (PCR) using modified Taguchi method. *Nucl. Acids Res.*, 22: 3801-3805.

Cregan, P. B. and Quigly C. V.(1997). Simple sequence repeats DNA marker analysis. In: DNA markers: *Protocols, Application and Overviews*: 173-185. Wiley-VCH publishing co. New York.

Crockett, P. A., Bhalla, P. L., Lee, C.K. and Singh, M.B.(1999). RAPD analysis of seed purity in a commercial hybrid cabbage (*Brassica oleracea* var. *capitata*) cultivar. *Genome* 43: 317–321

Dehmer, K.J. and Fried, W. (1998). Evaluation of different microsatellite motifs for analyzing genetic relationship in cultivated sunflower. *Pl. Breeding* 117: 45-48.

Dongre, A. and Pakhi, V. (2005). Identification of cotton hybrid through the combination of PCR based RAPD, ISSR and microsatellite markers. *Jr. Plant Biochem. Biotechnol.*14, 53-55.

Doyle, J. J. and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus,* 12:13- 15.

Ferguson, M.E., Burow, M.D., Schulze, S. R., Bramel, P. J., Paterson, A.H., Kresovich, S. and Michell, S.(2004). Microsatellite identification and characterization in peanut (*Arachis hypogea* L.). *Theor. Appl. Genet.*108: 1064-1070.

Garg, A., Singh, A.K., Parbhu, K.V., Mohapatra, T., Tyagi, N.K., Nandkumar, N., Singh, R., Zaman, F.U.,(2006).Utility of a fertility restorer gene linked marker

for testing genetic purity of hybrid seeds in rice (Oryza sativa L.) *Seed Sci. technol* .34,9-18.

Gomez, S.M., Denwar, N.N., Ramasubramanian, T., Simpson, C. E., Burow, G., Burke, J.J., Puppala, N. and Burow1, M.D.(2008). Identification of Peanut Hybrids Using Microsatellite Markers and Horizontal Polyacrylamide Gel Electrophoresis. *Peanut Science* 35:123–129.

Halakeri, A. V. (1999), Studies on sunflower necrosis disease. *M. Sc. (Agri.) Thesis,* University of Agricultural Sciences, Dharwad.

Hammer, Q., Harper, D.A.T. and Ryan, P.D.(2001). PAST: Paleontological Statistic Version 2.07 software package for education and data analysis. *Palaeontolia Electronica* 4(1):9.

Hashizume, T., Sato, P., Hirai, M. (1993).Determination of genetic purity of hybrid seed in watermelon (*Citrullus lanatus*) and tomato (*Lycopersicon esculentum*) using random amplified polymorphic DNA (RAPD). *Japanese Journal of Breeding*, 43(3):367-375.

Thein, H. X., Bhat, A.I. and Jain R. K. (2003). Mungbean necrosis diseased caused by a strain of Ground nut bud necrosis virus. *Omanrice* 11: 110-116.

Ilbi, H. (2003).RAPD marker assisted varietal identification and genetic purity test in pepper (*Capsicum annum* L.).97(3:4):211-218.

Iqbql, M. J., Paden, D. and Ryburn, A.L.,(1995).Assessment of relationship among *Rhododendron* species, varieties and hybrids by RAPD analysis. *Scientia horticulture*63:215-223.

Iqbal, H.A., Sadaqat, A.S. and Amjad M. (2010). Identification of sunflower (Helianthus annuus, *Asteraceae) hybrids using simple-sequence repeat markers. Genet. Mol. Res. 10 (1): 102-106.*

Jain, R.K., Bhat, A.I., Byadgi, A.S., Nagarju, Harvindra Singh, Halkeri, A.V., Anahosur, K.H. and Verma, A. (2000). Association of tospovirus with sunflower necrosis disease. *Current Science*.29 (12):1703-1705.

Jang, I., Moon, J., Yoon, J., Jae-Heung Yoo, Yang, T., Yong, J.K., and Park, H. (2004). Application of RAPD and SCAR Markers for Purity Testing of F*1* Hybrid Seed in Chili Pepper (Capsicum annuum). *Mol. Cells,* 18(3):295-299.

Jianli, L., Xiaohua, S., Shouchunn, Z., Jianshe, W. (2006). Purity test of melon hybrid with SSR molecular markers. *Mol Plant Breed* 4:23-26.

Kim, K.M., Kwon, Y.S., Lee, J.J., Eun, M.Y. and Sohn, J.K.(2004).QTL mapping and molecular marker analysis for the resistance of rice to ozone. *Mol Cells* 17:151-155.

Kiss, G.B. and Csanadi, G. (1993). Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. *Mol Gen Genet.*238: 129-137.

Kottapalli, K.R., Burow, M.D., Burow, G., Burke J. and Puppala N. (2007). Molecular characterization of the U.S. peanut mini core collection using microsattelite markers. *Crop Sci.*47:1718-1727.

Kumar, D., Haritha, V. B., Anusha, S. and Ashfaq, M.A. (2009). SCAR and RAPD markers for genetic purity assessment of sunflower hybrid DRSH1. *J. Oilseed Res.*, 26:192-194.

Lee, S. J., Jeung, U., Cho, S.K., Um, B.Y., Chung, W.I., Bae, J.M. and Shin, J.S.(2002). Diversity and varietal classification of Hibiscus syriacus L.with the heterogeneity wihin retrotransposon-like elements. *Mol. Cells.*13, 362-368.

Lenardon, S. L., Bazzalo, M. E., Abratti, G., Cimmino, C. J., Galella, M. T., Grondona, M., Giolitti, F. and Leon, A. J.(2005). Screening Sunflower for Resistance to Sunflower chlorotic mottle virus and Mapping the Rcmo-1 Resistance Gene. *Crop Sci* 45:735-739.

Li, J.T., Yang, J., Chen, D.C., Zhang, X.L., and Tang, Z.S. (2007). An optimized mini-preparation method to obtain high-quality genomic DNA from mature leaves of sunflower. *Genet. Mol. Res.*6 (4): 1064-1071.

Li, R., Li, Q., Tan Zhen-xin, (2005). Purity Testing Method for Maize Hybrids Using SSR Markers. *A Journal maize Science*, 3.

Liua, LW., Wanga Y., Gonga YQ., Zhaob TM., Liua G., Lia X.Y., YUC, F.M. (2007). Assessment of genetic purity of tomato (*Lycopersicon Esculentum* L.) hybrid using molecular markers. *Sci Hortic* (Amsterdam).115:7-12.

Li-Wang Liu, Wang, Y., Gong, Y., Zhao, T., Liu, G., Li, X. and Yu, F. (2007).Assessment of genetic purity of tomato (*Lycopersicon esculentum* L.). *Scientia Horticulturae*, 115:7-12.

Loomis, W.D. (1974). Overcoming problems of phenolics and quinines in the isolation of plant enzymes and organelles. *Methods Enzymol.* 31:528-544.

Mace, E.S., Buhariwalla H.K. and Crouch J.C. (2003). A high-throughput DNA extraction protocol for tropical molecular breeding programs. *PMBR.*21:459a-459h.

Maroof, S. and Saliman, M.A. (1984).Ribosomal spacer length polymorphism in Barley: Mendelian Inheritance, chromosomal location and population dynamics. *Proc.Natnl.Acad.Science* (USA) 81:8014-8018.

McDonald, M.B., Elliot, L.J. and Sweeney, P.M. (1994). DNA extraction from dry seeds for RAPD analyses in varietal identification studies. *Seed Sci. Technol.* 22: 171–176.

Meng, Q., Chen, Y., Yang, X., Zhao, W., Zhang, Y. and Yuan J. (2009). Seed Purity Test for Maize Hybrid Suyu 20 Using SSR Markers. *Jiangsu Journal Agricultural Sciences*, 3.

Miller, J.F. and Fick, G.N. (1997). Sunflower Genetics. In: A.A. Schneiter (ed.) Sunflower Technology and Production. Agron. Monogr. 35. ASA. CSSA and SSSA. Madison, WI, USA: 441- 495.

Mingsheng, W., Xihai, J., Lei, T. and Baochun, L. (2006). Rapid and reliable purity identification of F1 hybrids of Maize (*Zea mays* L.) using SSR markers. *Mol. Plant Breed.* 4:381-384.

Murray, M.G. and Thompson, W.F.(1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8 (19):4321-4325.

Nagaraju, V., Muniyappa, V., Singh, S. J. and Virupahshappa, K. (1997). Occurrence of a mosaic virus disease on sunflower in Karnataka. *Indian Phytopathol.* 50:277-281.

Nandakumar, N., Singh, A.K., Sharma, R.K., Mohapatra, T., Prabhu, K.V. & Zaman F.U. (2004). Molecular fingerprinting of hybrids and assessment of genetic

purity of hybrid seeds in rice using microsatellite markers. *Euphytica* 136: 257–264.

Nandini, R. and Chikkadevaiah, (2005). DNA fingerprinting of sunflower genotypes (Helanthus *annus* L.). *HELIA* 28(42):9-18.

Naresh , V., Yamini, K. N., Rajendrakumar, P. and Kumar, V. (2009). EST-SSR marker-based assay for the genetic purity assessment of safflower hybrids. *Euphytica* 170:347–353.

Paran, I., Horowitz, M., Zamir, D. and Wolf, S. (1995). Random Amplified Polymorphic DNA marker is useful for purity determination of tomato hybrids. *Hortscience.*30(2):377.

Pendse, R., Malhotra, S., Pawar, S.E., Krishna, T.G. (2001) use of DNA markers for identifying inbreds and hybrid seed in cotton (*Gossypium hirusutum* L.). *Seed Sci Technol* 29:503-508.

Pranavi, P., Anusha, S., Bhavani D. and Kumar V. (2009). RAPD markers for genetic purity assessment of castor hybrids. *J. Oilseed Res.*, 26: 195-197.

Prasad Rao, R.D., Reddy, V.J., Chander Rao, A.S., Varaprasad, S., Thirumala Devi, K.S., Nagaraju, K., Muniappa, V. and Reddy, D.V.R. (2000). Tobacco strak ilar virus as a causal agent of sunflower necrosis disease in India. *Journal of Oilseed Research,*17(2):400-401.

Qureshi, Z., Sabir H. and Ahmad S. (1992). Annual report of NODP, Islamabad. 2: 20-21.

Ramiah, M. Bhat, A. I., Jain, R. K., Pnt, R. P. and Ahalwat, Y, S. (2001). Partial characterization of an isometric virus causing sunflower necrosis disease. *Indian Phyto pathol.* 54:246-250.

Ravi, K.S., Buttgereitt, A., Kitkaru, A.S., Deshmukh, S., Lesemann D.E. and Winter S. (2001). Sunflower necrosis disease from India is caused by an ilarvirus related to Tobacco streak virus. *Pl. Path*,, 50:800.

Ravi Kumar, B. (2009), Molecular characterization of sunflower necrosis virus in weeds and its vector thrips prevalent in sunflower ecosystem. *Ph.D.Thesis*, Univ.Agric.Sci.., Bangalore (India).

Rubeena, Randhava, L., Singh Harjit, S. and Singh, H. (2000). Varietals identification in cotton by RAPD markers. *Crop improvement* 27(2):145-151.

Salgado, K.C.P.C., Vieira, M.G.G.C., Pinho, E.V.R.V, Guimaraes, C.T., Pinho, R.G.V. and Souza L.V.(2006). Genetic purity certificate in seeds of hybrid maize using molecular markers. *Revista Brasileria de Sementes* 28:169-175.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York.

Saxena, R.K. and Saxena, K. (2010). Application of SSR markers for molecular characterization of hybrid parents and purity assessment of ICPH 2438 hybrid of pigeonpea. *Mol Breeding* 26:371–380.

Sharman, M., Thomas, J.E., and Persley, D.M. (2008). First reoort of Tobacco streak virus in sunflower (*Helianthus annuus*), cotton (*Gossypium hirusutum*), chickpea (*Cicer arietinium*) and mungbean (*Vigna radiate*) in Australia. *Australian Plant Pathology Disease Notes*. 3: 27-29.

Shivasharanayya (2000).Transmission, screening for resistance and epidemiology of sunflower necrosis virus disease. *M. Sc. (Agri.) Thesis,* Univ. Agric. Sci.,Bangalore, (India).

Srinivasan, A., Wankhede, D. and Kumar V. (2009). Genetic purity assessment of sunflower hybrids by using SCAR markers. *J. Oilseed Res*., 26:202-205.

Smith JDC, Register JC III (1998). Genetic purity and testing technologies for seed quality: a company perspective. *Seed Sci Res* 8:285-293.

Smith, S. J. C., Hoeft, E., Cole, G., Lu, H., Elizabeth, S., Jones, Wall, S. J. and Berry, D. A. (2009). Genetic diversity among U.S. sunflower Inbreds and Hybrids: assessing probability of ancestry and potential for use in plant variety protection. *Crop Sci.* 49:1295–1303.

Sundaram, R.M., Naveenkumar, B., Biradar, S.K., Balchandran, S.M., Mishra, B., IlyasAhmad, M., Viraktamath, B.C., Ramesha M.S. and Sarma N.P. (2008). Identification of informative SSR markers capable of distinguishing hybrid rice parental lines and their utilization In seed purity assessment. *Euphytica* 163:215-224.

Vinatzar, B., Pancaldi, M. and Sansavini, S. (1999).potential and limits of varietals identification of peach. *Rivista-di-fruticultura-e-di-ortojilouricultura* 61 (4):97-101.

Wang, D., Shi, J., Carlson, S.R., Cregan, P.B., Ward R. W. and Diers B.W. (2003). A low –cost, high –throughput polyacrylamide gel electrophoresis system for genotyping with microsatellite DNA markers. *Crop Sci.* 431:1828-1832.

Weising, J., Beyermann, B., Ramser, J. and Kahi, G. (1991).plant DNA fingerprinting with radioactive and digoxygenated oligonucleotide probes complementary to simple repetitive DNA sequences. *Electrophoresis* 12:159-169.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified polymorphic DNA markers. *Nucl. Acids Res.*, 18:6531-6535.

Yashitola, J., Thirumurugan, T., Sundaram, R.M., Naseerullah, M. K., Ramesha, M. S., Sarma N. P. and Ramesh V. S. (2002). Assessment of purity of rice hybrids using microsatellite and STS markers. *Crop Science*, 42(4): 1369-1373.

Appendices

CHAPTER-VIII

APPENDIX

Buffers and solutions used

Buffer used in virus inoculation

- Homogenization buffer (0.05 M phosphate buffer, pH 8.0)

KH_2PO_4	0.68 g
$K_2HPO_4, 3H_2O$	20.52 g
Sterile Distilled water	1.0 L

Reagents for DNA isolation

1. **Extraction buffer:** To prepare extraction buffer, 10 X stocks of reagents were prepared and then subsequent extraction buffer was prepared.

   a) CTAB (10%):

   10 gm CTAB was added to make 100 ml final volume in sterile double distilled water.

   b) 4 M NaCl:

   23.4 gm of NaCl was added to sterile double distilled water and made final volume up to 100 ml.

   c) 1 M Tris Cl (pH 8.0):

   15.76 gm of Tris-Cl was added in sterile double distilled water to make final volume up to 100 ml.

   d) 0.5 M EDTA (pH 8.0):

   It was prepared by adding 14.612 gm of EDTA in 100 ml double distilled water and pH was adjusted by adding pellets of NaOH.

   From these stocks, 2% extraction buffer was prepared by taking following volumes.

Components of 2 % extraction buffer

Sr. No.	Components	Quantity
1.	10 % CTAB	20 ml
2.	4 M NaCl	35 ml
3.	1M Tris HCl (pH 8.0)	10 ml
4.	0.5M EDTA (pH 8.0)	4 ml
5.	Double distilled sterile water	31 ml
	Total	**100 ml**

2. 0.2 % β-mercapto ethanol was also prepared.

3. Chloroform-isoamyl alcohol mixture was prepared in the ratio of 24: 1 (v/v).

Chloroform/ Isoamyl alcohol Solution (100 ml)

Chloroform 96 ml

Isoamyl Alcohol 4 ml

4. 3 M sodium acetate (pH 5.2) was prepared by adding 40.81 gm of sodium acetate in sterile double distilled water and pH was maintained by adding NaOH pellets. Final volume was made up to 100 ml.

5. *RNase* A stock (10 mg/ml solution)

10 mM Tris HCl (pH 7.5) - 10 µl

1.5 mM NaCl - 3 µl

RNase A - 10 mg

Final volume was made up to 1 ml with sterile double distilled water and heated at 100^{o}C for 15 minutes in water bath. The solution was allowed to cool slowly at room temperature and aliquot was taken and stored at – 20^{o}C.

6. Other reagents

Ethanol 70 % and 100 % chilled isopropanol etc. were also prepared and used in appropriate quantities.

- T.E Buffer (pH 8.0)

Components	**Quantity**
10 mM Tris – HCl	0.121 gm
1 mM EDTA	0.03 gm
Double distilled water	80 ml
Final volume	100 ml

- **Tris Borate EDTA (TBE) 10X buffer** (1000 ml)

(Sambrook *et al.,* 1989)

Tris base	:	108 gm
Boric acid	:	55 gm
0.5 M EDTA	:	40 ml (pH 8.0)

Make the volume of the solution up to 1000 ml with double distil water Autoclave and store at room temperature.

"ASSESSMENT OF HYBRID PURITY IN SUNFLOWER BY USING MOLECULAR MARKERS"

ABSTRACT

Sunflower (*Helianthus annuus* L.) being a cross pollinated crop, genetic adulteration is vested problem and varietal description is cumbersome. For screening genetically pure hybrids RAPD and SSR marker were used for fingerprinting of hybrids, assessing variation within parental lines and testing the genetic purity of ten hybrids. Among 29 primers nine RAPD and seven SSR primers found polymorphic across the hybrids and produced unique fragment for ten hybrids. Four set of RAPD and SSR markers OPC 16, OPA 11, OP-03, OPA 02 and Ha 1327, ORS 5, ORS 662, ORS 243 respectively differentiated most of the hybrids. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped the hybrids into five clusters. Within the clusters most of the hybrids showed a common cytoplasmic male sterile line as female parent and restorer line as male parent.

According to presence and absence of bands that RAPD and SSR markers were classified into five groups. Bands commonly showed by in parents and hybrids included in marker pattern I and III found significant in respect to the hybrid confirmation test. The bands from parents does not appear in hybrids include in II, IV and V marker pattern. The parental bands expressed uniquely in hybrid and individual in marker type so it has direct implication in identification of hybrids and can be useful in protecting the rights of plant breeders. SSR marker was found more significant over RAPD in hybrid purity assessment

Keywords: Hybrid, Genetic Purity, fingerprinting

S.M. Bhosale
Ref. No. 09/BT/01/M

Course Co-ordinator
College of Agril. Biotechnology
MAU, Parbhani

Dr. M.K. Ghodke
Research Guide