MOLECULAR ANALYSIS OF WHEAT GENOME USING ISSR AND RAPD MARKERS

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BY

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CERTIFICATE

Certified that the work incorporated in this thesis entitled '**MOLECULAR ANALYSIS OF WHEAT GENOME USING ISSR AND RAPD MARKERS'** submitted by Mrs.Suvarna Tushar Tavale was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

Date:

(Dr. Vidya S. Gupta)

Research Guide

DECLARATION

I hereby declare that the thesis entitled ‘‘**MOLECULAR ANALYSIS OF WHEAT GENOME USING ISSR AND RAPD MARKERS’** submitted for M.Sc. (PPPR) degree to the University of Pune has not been submitted by me to any other university for a degree or diploma.

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TO MY PARENTS

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Suvarna Tavale.

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LIST OF ABBREVIATIONS

AACC	:	American Association of Cereal Chemists
ASAP	:	Allele Specific Associated Primers
AFLP	:	Amplified Fragment Length Polymorphism
bp	:	Base Pair
CAPS	:	Cleaved Amplification Polymorphic Sequence
cm	:	Centimeter
cM	:	Centi Morgan
CTAB	:	Hexadecyltrimethyl Ammonium Bromide
cv	:	Cultivar
DAMD	:	Directed Amplification of Minisatellite Region DNA
DAF	:	DNA Amplification Fingerprinting
DH	:	Double Haploid
DNA	:	Deoxy Ribonucleic Acid
DNTPs	:	Deoxy Ribonucleotide Tri Phosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Expressed Sequence Tag
g/gm	:	gram
GPC	:	Grain Protein Content
h	:	Hour
ha	:	Hectare
HMW	:	High-Molecular Weight
IAA	:	Iso-Amyl Alcohol
ISSR	:	Inter Simple Sequence Repeat Markers
ITMI	:	International Triticecae Mapping Initiative
kb	:	kilobase pair
KCl	:	Potassium Chloride
kg	:	Kilogram
LMW	:	Low-Molecular Weight
Lr	:	Leaf rust
m	:	Minute
M	:	Molar
MAS	:	Marker Assisted Selection
Mb	:	Megabase pair
$MgCl_2$	:	Megnesium Chloride
mm	:	milliitre
mM	:	millimolar
mt	:	million tones
MW	:	Molecular Weight
N_2	:	Nitrogen
NaCl	:	Sodium Chloride
ng	:	nanogram
NIL	:	Near Isogenic Line
nM	:	nanomoles

P	:	Phosphorus
PAGE	:	Poly-Acrylamide Gel Electrophoresis
PCR	:	Polymerase Chain Reaction
pmoles	:	picomoles
QTL	:	Quantitative Trait Loci
RAPD	:	Random Amplified Polymorphic DNA
RAMPO	:	Randomly Amplified Microsatellite Polymorphism
RFLP	:	Restriction Fragment Length Polymorphism
RIL	:	Recombinant Inbred Line
rpm	:	Revolution Per Minute
s	:	Second
SCAR	:	Sequence Characterized Amplified Region
SDS	:	Sodium Dodecyl Sulphate
SSR	:	Simple Sequence Repeat
STMS	:	Sequence Tagged Microsatellite Site
STS	:	Sequence Tagged Site
TAE	:	Tris-acetate EDTA
TE	:	Tris-EDTA
Tm	:	melting temperature
Tris.	:	Tris-hydroxymethyl amino methane
°C	:	degree centigrade
µg	:	microgram
µl	:	microlitre
µM	:	micromolar

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REVIEW OF LITERATURE

CHAPTER 1

1.1 Introduction :

Wheat is a major source of energy, protein and dietary fiber in human nutrition since decades. It has been estimated that about 65 % of wheat grain is directly used up as food for humans, indicating its acceptance as main staple food and 21% as a feed for live stock, 8% as seed material, and remaining 6% for the other uses like industrial raw material. The raw parts of wheat plants, the stem and leaf are generally used as straw or fresh forage. Wheat has the potential to be used as non food raw material in many industries such as shiffening / surface coating agent in the manufacturing of paper and board, as an adhesive in the manufacturing of corrugated boxes, as fermentation substrate, in the production of vitamins, antibodies, etc (Jones *et at* 1987). Wheat straw is used in construction of thatch and panels, in crafts and as fuel for production of heat. Globally wheat trade is a major and impacting issue in political and economic relationships between nations.

India presently holds the second position (after China) in total world wheat production which is grown over 25 million hectors of land. Three types of wheat are grown in India, 1) *Triticum aestivum* (bread wheat), 2) *Triticum durum* (durum wheat) and 3) *Triticum dicoccum* (dicoccum wheat). Wheat as a staple food is consumed in many different forms in India mainly as chapati, bread, noodles, macaroni, sphaghetti, cakes, pizzas, doughnuts, etc. It is also consumed as semolina locally known as rava or sooji, to prepare different food products like kheer, upma, sooji, halwa etc. Vermicelli made from durum wheat is also used for making kheer. Some end products of wheat are summarized in Figure 1.1. Due to changing life style and modernization, bread is rapidly overtaking as daily meal along with other food products, instead of traditional chapati or roti. Though we are self sufficient in producing and fulfilling our requirement for wheat per year, because of population explosion and changing life style, there is an immense need for production of good quality wheat to enhance wheat based industries and wheat export.

Figure 1.1: Different types of wheat end products (http://www.wheatfoods.org).

1.2 Wheat : Classification and evolution

Cytogenetic studies and wheat systematics: In grass family Poaceae and Tribe Triticeae, wheat belongs to the sub-tribe Tritineae, which is of recent origin, and contains about 35 genera including *Triticum, Aegilops, Thinopyrum, Dasypyrum, Lophopyrum and Secale.* The various species of these genera easily hybridize with each other resulting in either a direct exchange of genetic material or polyploidy. Linnaeus (1953) recognized seven species within the genus *Triticum* while the wild relatives were included in the genus *Aegilops.* Taxonomists upheld this dichotomy for over 200 years after which Stebbins (1956) initiated the merger of two genera into a single genus *Triticum*, which was subsequently supported by Bowden (1959) and Kimber and Feldman (1987). The nomenclature suggested by Miller (1987) has been depicted in Table 1.1.

Cytogenetic studies by Sakamura (1918) and Kihara (1924) led to the understanding that various species of *Triticum* formed a polyploid series from a basic haploid set of seven chromosomes. Three ploidy levels, namely, diploids (2x=14), tetraploids (4x=28) and hexaploids (6x=42) were recognized. The polyploid wheats (tetraploid and hexaploid) are amphiploids, composed of two or more different genomes, each derived from a distinct ancestor and behaving like a diploid. The corresponding chromosomes of the different genomes are known to be homeologous. The diploid-like behaviour of the polyploid wheats is due to a `diplodizing gene' or pairing-inhibior gene *Ph,* situated on chromosome 5B, which prevents the pairing of homeologous segregation of chromosomes (Riley and Chapman, 1958). Diploidization ensures regular segregation of chromosomes, genetic stability, heterosis and high fertility, and provides protection to the genome enabling greater tolerance to structural changes in chromosomes. Earlier studies on chromosome pairing (Kihara, 1924, 1954) established that the chromosomes of different diploid species did not pair regularly in interspecific hybrids and hence designated each genome with a distinct genomic formula. For example, the genomic formula of *Triticum urartu* is AA and that of *Aegilops speltoids* is SS. The wild and cultivated

emmer wheat (*T.dicoccoides* and *T.dicoccum*, respectively) and durum wheat (*T.durum*) all share the genomic formula AABB. Table 1.1 shows the genomic formulae of some wild and cultivated species of three ploidy levels of wheat.

Table 1.1 Genomic constitution of wild and cultivated wheat species

Species	Common name	Genomic constitution
Diploid (2n=14)		
Triticum boeoticium	Wild einkorn	AA
Triticum urartu	Two grained einkorn	AA
Triticum monoccoccum	Cultivated einkorn	AA
Triticum speltoides	Wild grass	SS
Triticum tauschii	Wild grass	DD
Tetraploid (2n=28)		
Triticum dicoccoides	Wild emmer	AABB
Triticum dicoccum	Cultivated emmer	AABB
Triticum durum	Macaroni/durum wheat	AABB
Triticum turanicum	Kamul	AABB
Triticum polonicum	Polish wheat	AABB
Hexaploid (2n=42)		
Triticum spelta	Spelt wheat (cultivated)	AABBDD
Triticum aestivum	Bread/common wheat	AABBDD

Early cytogenetic studies (Kihara 1954), recognized the einkorn wheat *Triticum monococcum* as the A genome donor. However, subsequent molecular studies (Dvorak *et al*, 1993; Jiang and Gill, 1994) identified *Triticum urartu,* a biological species of the former, as the A genome ancestor of emmer and durum wheat. The identity of the B genome donor is still elusive, though morphological, geographical and cytological evidences indicate that *Triticum speltoides* (SS) or a closely related species is the B genome donor (Riley *et al*, 1958). Molecular evidences have confirmed that *Triticum speltoides* is the closest extant relative of the species that has contributed to the B genome (Dvorak and Appels, 1982, Dvorak and Zhang, 1990, 1992). However, studies on chromosome banding, *in situ* hybridization, meiotic pairing and isozymes suggested that the genome of *Triticum speltoides* is not identical to the genome of present day tetraploid and hexaploid wheat (Waines and Barnhart, 1992).

The origin of genome D is almost related to *Aegilops squarrosa L.* Its addition to AABB tetraploid gave rise to hexaploid AABBDD genome. It has major impact on global wheat distribution. Genes accounting the 'D' genome have main influence on the bread making characteristics and dough rheology of wheat flour. Therefore, from various such angles, the most important wheat is the hexaploid bread wheat or common wheat (*Triticum aestivum L*.Syn.T.vulgare Host). It is further grouped on the basis of various factors such as milling property (hard or soft), dough rheology (strong or weak), and bran colour (red or white).

1.3 Wheat seed storage proteins

Wheat grain precisely known as caryopsis, consists of the pericarp or fruit and the true seed. In the endosperm of the seed, about 72% of the protein is stored which forms 8-15% of total protein per grain weight. Wheat grains are also rich in pantothenic acid, riboflavin and some minerals, sugars etc. (Orth and Shellenberg, *et al* 1988). The barn which consists of pericarp testa and aleurone, is also a dietary source for fiber, potassium, phosphorus, magnesium, calcium, and niacin in small quantities. The composition of wheat grain and flour is given in Table 1.2.

Table 1.2 Composition of wheat grain and flour(Gooding and Davis,1997)

Parameter	**Grain %**	**Flour %**
Moisture	9-18	13-15.5
Starch	60-68	65-70
Protein	8-15	8-13
Cellulose	2-2.5	Trace
Fat	1.5-2	0.8-1.5
Sugars	2-3	1.5-2
Mineral matters	1.5-2	3-6

After milling of grains or caryopsis to the flour, it is further used in preparation of different wheat end products. The quantity and quality of endosperm proteins are the major factors responsible for baking quality, and nutritional value of wheat. (Wringly, and Bietz, 1988, Orth and Bushuk, 1972). Wheat flour also needs to have relatively high protein content of the right quality.

1.3.1 Wheat: Albumins, Globulins, Gliadins and Glutenins

Various seed storage proteins in wheat, are classified upon their solubility in different solvents, such as water, saline solution, 70% aqueous ethanol and diluted acid or alkali solutions and are named as albumins, globulins, gliadins, and glutenins, respectively, (Tatham and Shewry;1985, Byers, *et al* 1983, Wall, 1979). Later, Miffin *et al* (1983) and Shewry *et al* (1983) classified both gliadin and glutenin fractions belonging to the prolamin group based on the similar amino acid composition as well as chromosomal localization of genes encoding them.

Albumins and globulins are referred as soluble or cytoplasmic proteins and are represented by enzymes involved in metabolic activity. They are mainly located in germ layer and aleurone layers. They constitute about 20% of the total proteins in wheat caryopsis wherein albumins with 12,000 – 16,000 molecular weight occur in more amount than globulins with 20,000 to 2,00,000 molecular weight.

The major wheat endosperm proteins, the gluten proteins, comprise two prolamin groups, gliadins and glutenins. Both of these high molecular weight proteins, play vital role in dough rheology and bread making quality. The unique elastic and cohesive properties of wheat dough which determine the bread making quality are mainly due to its water insoluble gluten proteins (Wall, 1979). For many years wheat breeders have selected cultivars with high gluten content measured by using mixographs for quality improvement (Cox *et al*, 1989).

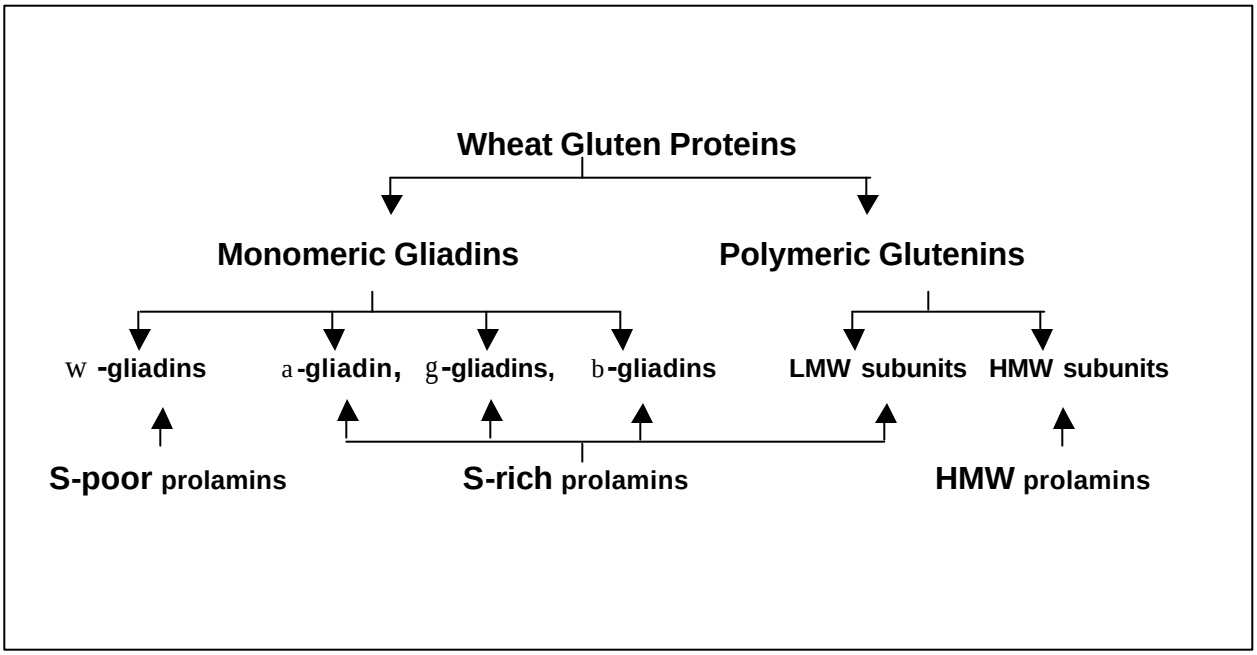
The gliadins constitute about 40% of the total endosperm protein, and are a heterogeneous mixture of single polypeptide chains with interchain disulfide binding. They include glutamine, proline and low amount of lysine. Gliadins are

further classified as α, β, γ, and ω gliadins on the basis of N-terminal amino acid sequence homologies (Kasarda, *et al* 1982). However, ω gliadins differ in all aspects such as amino acid composition, molecular weight, and N-terminal amino acid sequences. They have high molecular weight ranging from 65,000 to 80,000. The low lysine content (0.5mol %) in the gliadins is the main negative factor affecting the nutritional quality of wheat. Gliadins have also low levels of histidine, arginine and free carboxyl groups of glutamic acid.

Glutenins, are high molecular weight complex proteins, with number of polypeptide subunits, which are joined by covalent and non-covalent linkages. Glutenins, ranging from 40,000 to several millions exhibit a broad spectrum of molecular weights and are extremely dispersed proteins. They constitute about 30-40% of flour protein and are the major contributors to visco-elastic property of wheat dough. High molecular weight of glutenins has an importance in bread making quality and rheology of dough. The quality of glutenin reduces on exposure to excessive heat. Furthermore, two major classes of glutenin subunits have been identified in wheat endosperm, the high molecular weight (HMW) glutenins and the low molecular weight (LMW) glutenins. Both types of these subunits are present in the flour as cross-linked proteins resulting from inter polypeptide disulphide linkages.

On the basis of structural and genetical relationships among the different polypeptides in the gliadin and glutenin fractions, Shewry *et al* (1983,1986) and Miflin *et al* (1983,1986) have proposed a different scheme for classifying gluten proteins as shown in Figure 1.2. In this classification, all wheat gluten proteins are considered as prolamins, and are subdivided into three groups namely, a) sulphur rich prolamins (they correspond to α-, β-, γ- gliadins and the low molecular weight subunits of glutenin, in the older version of classification), b) sulphur- poor prolamins, (corresponding to the ω-gliadins), and c) high molecular weight (HMW) prolamins, (which correspond to the high molecular weight (HMW) subunits of glutenin).

Fig 1.2. Classification of wheat gluten proteins (Shewry and Miflin1983)



1.3.2 Genetics of HMW and LMW gluten subunits

HMW subunits : The HMW glutenin subunits form a minor component in terms of quantity but is a major determinant of elasticity of gluten (Payne, *et al* 1980). Each common wheat cultivar possesses three to five HMW subunits. These subunits differ from other subunits and the gliadins in molecular weight (90 – 150 kDa by SDS-PAGE) and possess high glycine content. The genes coding for the HMW glutenin subunits are located on the long arm of chromosomes 1A, 1B, and 1D (Payne, 1987). Each of the three loci, collectively named *Glu*-1, contains only two genes namely *Glu*-1-1 and *Glu*-1-2, which code for x-type (slower mobility) and y-type subunits (faster mobility), respectively, based on their relative mobility in SDS-PAGE (Payne, 1981). Individual cultivars of *T.aestivum* have 0 or 1 subunits controlled by *Glu*-A1 (chromosome 1A), 1 or 2 subunits controlled by *Glu*-B1 (chromosome 1B) and 2 subunits controlled by *Glu*-D1 (chromosome 1D). Intra locus recombination is very rare and the map distance between the two genes of the *Glu*-B1 locus has been estimated as 0.1-

0.2cM (Payne, 1987). The HMW glutenin subunit genes exhibit extensive allelic variation and many alleles have been identified in both common and durum wheats and in diploid species (McIntosh, 1990).

LMW subunits :- The LMW glutenin subunits exist as polymers stabilized by interchain disulphide bonds and are called as alcohol soluble reduced glutenins. After the reduction of the disulphide bond, LMW subuints divide into two main groups; a major group of basic proteins (B subunits) with molecular weights of 42-51 kDa and a minor group (C subunits) with molecular weights of 30-40 kDa. The LMW subuints are difficult to separate from α / β gliadins and ω- gliadins by SDS-PAGE; because of their similar molecular size (Jackson, *et al* 1983). They are easily fractionated and purified combining differential solubility, ion exchange chromatography and SDS-PAGE (Autran, *et al* 1987). The LMW glutenin subunits have proved to be much more difficult to analyze by 1D-SDS-PAGE because of their overlap with the gliadins. The introduction of a two-step 1D-SDS-PAGE or Acid-PAGE/SDS-PAGE procedure provides a rapid method for analyzing a large number of samples in a gliadin free background.

Genetic evidence has indicated that the LMW glutenin subunits are controlled by genes at *Glu*-A3, *Glu*-B3 and *Glu*-D3 loci on the chromosome arms 1AS, 1BS and 1DS, respectively. Genes coding for LMW subunits are closely linked and have also been found to be linked to genes coding for γ and ω - gliadins. The estimated map distance between Glu-B3 and Gli-B1 on the short arm of chromosome 1B is 2cM in both bread and durum wheat. (Gupta, and Shepherd, 1988, Ponga, and Mellini 1988). Their results also suggest that *Glu*-B3 is located between *Glu*-B1 and *Gli*-B1. Allelic variations in the LMW subunits have been shown to be primarily responsible for differences in gluten visco-elastic properties in both bread and durum wheats (Ponga, *et al* 1990, Gupta, *et al* 1991).

1.3.3 Role of gluten proteins in bread making quality in wheat

Common wheats cover a wide range of technological characteristics and hence end uses. The main products from these wheats can be classified into relatively distinct groups based on grain hardness and protein content. The term ' quality' means the performance of a cultivar at a specific protein level, in a test that reflects a specific end product. When the cultivars are selected on similar protein quality, their breadmaking potential is directly related to the protein content. In such cases, protein content becomes an accurate measure of breadmaking potential, and thus, it is used to market wheat by several wheat exporting countries. However, functional properties of flour, such as ability of hard wheat flour to make bread, depends on other constituents of the flour, the ingredients and process used. Work done on wheats with similar hardness have shown that the variation in protein quality for breadmaking depends almost on variation in gluten components of flour protein (Orth and Bushuk, 1972).

Moreover, breadmaking performance has also been related to the glutenin polymer size distribution, cultivars of higher bread making quality having higher proportions of glutenin polymers of greater molecular size (Huebner,1976, Bottomely, 1982). The intrinsic viscosities of glutenins have shown to be related with breadmaking quality (Ewart, 1980). For the last 10-15 years, much of the emphasis has been given on defining the molecular basis of breadmaking quality in relation with the effects of specific polypeptide of the gluten protein complex, especially HMW subunits of glutenin (MacRitchie, F du Cros, *et al* 1990, Payne *et al* 1984). In order to correlate the breadmaking potential with HMW subunit composition, two statistical approaches have been used. Based on the SDS-sedimentation volume, Payne (1987) assigned quality score to each subunit and then individual values were summed to calculate the total quality score for a wheat cultivar, with maximum score of 10, In another approach, Ng and Bushuk (1988) developed an equation for predicting the unit loaf volume, based on HMW subunit composition of a bread wheat cultivar. Further work in this area has suggested the role of specific HMW subunit in contribution of

glutenin to bread making quality. Payne *et al* (1979) observed a direct relationship between breadmaking and proportion of subunit Glu-1Ax1. Several studies have shown that amount and relative proportion of HMW subunit is an important factor in functional performance for breadmaking quality (Kolster *et al* 1992; Kolster and Vereijken 1993; Marchylo *et al*, 1992). Khelifi and Branlard (1992) and Menifesto *et al*, (1998) have shown that allelic variations in composition of LMW glutenin subunits also contribute to breadmaking quality, however, determination of the best combination for LMW subunits is in progress. It is generally accepted that glutenins are mainly responsible for viscoelastic properties, but gliadins are important in conferring extensibility to dough. Three loci, *Gli*-A1, *Gli*-B1 and *Gli*-D1, that are located on distal end of chromosome arms 1AS, 1BS, and 1DS, respectively, encode the ω-gliadins, most of γ-gliadins and some β-gliadins. Separate complex loci on group-6 chromosome (Gli-A2, Gli-B2 and Gli-D2) encode other gliadins (Skerritt,1998) are important in conferring extensibility to dough. Dal Belin Peruffo *et al* (1985) have identified individual gliadins encoded by *Gli*-B1 as markers of dough strength in bread wheat. Some gliadin alleles have also been shown to be positively associated to dough extensibility and dough strength (Branlard and Felix, 1994, Metakovsky *et al*, 1997).

Several tests have been developed for end use quality characteristics of bread wheat, which can be performed with small amount (~50g) of wheat grains. These include, grain hardness (PSI) and protein content, which can be measured by NIR spectroscopy and protein quality (gluten strength), which can be predicted by different tests such as SDS-sedimentation, micromixograph, SDS-PAGE of HMW glutenins and SDS-gel protein test (Bushuk, 1998). In order to assess the dough strength for breadmaking performance of a wheat variety, several tests based on dough rheology (farinograph, extensigraph, mixograph and alveograph) are available. However, ultimate and best screening test for bread making potential is the baking test, which reflects the product that will eventually be made from a wheat variety. For the soft common wheats, which are generally used for breads like French bread, Arabic flat bread, and other noodles,

both starch and protein quality are important factors. Wheats that are very soft, with the lowest protein content and the weakest dough strength are more suitable for cookies, cakes and some type of noodles. The early generation test for such wheat grains include grain hardness (PSI), protein content and Pelshenke test or SDS-sedimentation test. At later stages as more grains are available, quality testing can be performed with Falling number value (for prediction of sprouting), ash content, flour color etc, while the rheological tests include amylograph, farinograph and alveograph for this class of softer wheats.

1.4 Molecular markers for wheat genome analysis

Large genome size 16 x 19^9 bp / 1C (Bennett *et al* 1976) and wide range of uses have imparted wheat agronomically and nutritionally important status among the several other cereal crops. This has reflected in the development of a large number of cultivars by the wheat breeders. However, enormously growing population (about 1 billion by the end of last century with present growth rate of 1.8 %), and the changing scenario of modern life style have posed challenges to the wheat breeders to develop newer wheat varieties with high yielding performance, high quality seed, resistance to pests and pathogen etc. Therefore, during the last few decades wheat biotechnology has gained importance and is looked upon as the potential tool in wheat breeding program. DNA marker technology is one such important area of biotechnology which can definitely enhance the efficiency of plant breeding practices and can be effectively used as positive addition in crop improvement programs.

1.4.1 Various types of DNA markers

There are various types of DNA markers presently available to evaluate DNA polymorphism in sample genomes. Selection of a correct marker system depends upon the type of study to be undertaken and whether that marker system would fulfill at least a few of the mentioned characteristics such as easy availability, highly polymorphic nature, Mendelian inheritance, frequent occurrence in genome, selective neutral behavior, easy and fast assay, high

reproducibility, free of epistasis and pleiotropy etc, (Weising *et al*, 1995). These markers are generally classified as hybridization based markers and polymerase chain reaction (PCR) based markers. In the hybridization-based markers, the DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to a labeled probe which is a DNA fragment of known / unknown sequence. In case of PCR based markers, the primers of known sequence and length are used to amplify genomic and cDNA sequences which are visualised by gel electrophoresis technique. The invention of PCR which is a very versatile and extremely sensitive technique, (Saiki and Scharf *et al*, 1985) uses a thermostable DNA polymerase (Saiki *et al*, 1988) and has changed the total scenario of molecular biology and has also brought about a multitude of new possibilities in molecular marker research. Some of the hybridization as well as PCR based marker systems have been detailed below:

[A] RFLP - Restriction Fragment Length Polymorphism :

In RFLP analysis, restriction endonuclease digested genomic DNA is resolved by gel electrophoresis and then blotted on to a nitrocellulase membrane (Southern 1975). Specific banding patterns are then visualized by hybridization with a labeled probe. RFLPs are simply inherited Mendelian characters. These are co-dominant in nature and are very reliable markers in linkage analysis and breeding. Botstein *et al* (1980) used RFLPs for the first time to construct a genetic map. For RFLP analysis large amount of DNA is required for restriction digestion and there is also a requirement of radioactive isotope or non-radioactive kit which makes the analysis relatively hazardous and expensive, respectively. The assay is time consuming and labour intensive and less informative i.e. many markers need to be attempted using one at a time to get polymorphism. Because of these drawbacks, the utility of RFLP markers has been limited. Further modifications of RFLP marker system have been detailed below.

1. Sequence Tagged Sites (STS) - In this, RFLP probes specifically linked to a desired trait can be converted into PCR based STS oligonucleotide primers based

on nucleotide sequence of the probe giving a polymorphic band pattern. This is extremely useful for studying the relationship among several species at a specific locus (Bustos *et al*, 1999).

2.Allele Specific Associated Primers (ASAP) - In this case, specific allele is sequenced and based on the sequence, specific primers are designed for DNA template amplification to obtain a single fragment at stringent annealing temperature condition. These are used to tag given plant and the gene of interest. (Gu *et al,* 1995).

3. Expressed Sequence Tag Markers (EST) - These are introduced by Adams *et al*, (1991) and are obtained by partial sequencing of random cDNA clones. They are also useful in genome sequencing and mapping programs.

4. Single Strand Conformation Polymorphism (SSCP) - This is a powerful and popularly used technique for detection of point mutations. It can identify heterozygosity of DNA fragments of the same molecular weight. (Orita *et al* ,1989).

[B] Microsatellites and Minisatellites :

It is known that about 30-90% of the genome of virtually all eukaryotic species is constituted of repetitive DNA, which is highly polymorphic in nature. One major form of repetitive DNA is microsatellites and minisatellites. The term microsatellite was introduced by Litt and Lutty (1989) while the term minisatellite was coined by Jeffrey (1985). Minisatellites are tandem repeats with a monomer repeat length of about 11-60bp while microsatellites are short tandem repeats or simple sequence repeats of 1-6bp length, repeated several times. Micro and minisatellites form an ideal marker system creating complex banding pattern and detecting multiple DNA loci simultaneously. These are dominant fingerprinting markers, exhibit high level of heterozygosity and follow Mendelian inheritance. Various Minisatellite and microsatellite sequence based markers have been detailed below.

1.Sequence Tagged Microsatellites sites (STMS) - In this marker system, DNA polymorphism is detected using specific primers designed from sequence

data of a specific locus. Primers complementary to the flanking regions of the simple sequence repeat loci yield high polymorphism. Di-, tri- and tetra-nucleotide microsatellites are more popular for STMS analysis as they give a clear banding pattern (Hearne *et al,* 1992). However, dinucleotides are generally abundant in the genome and have been used for diversity analysis (Rafalski *et al* ,1993).

2. Directed Amplification of Minisatellite - Region DNA (DAMD) - In this case, minisatellites are used as primers for DNA amplification. It is introduced by Heath *et al* (1993) for the first time and is found to be useful for species differentiation and cultivar identification (Somers *et al*, 1996).

3. Inter Simple Sequence Repeat Markers (ISSR) - Zietkiewicz *et al* (1994) reported this technique for the first time, where microsatellites anchored at the 3' end are used for amplifying genomic DNA. They are mostly dominant markers. Number of primers can be synthesized for various combinations of di-, tri-, tetra- and penta- nucleotides [e.g. 3^3 =27, 4^4=256] with a few based anchor.

[C] Randomly Amplified Polymorphic DNA Markers (RAPDs)

Here, a single species of primer anneals to the genomic DNA at two different sites on complimentary strands of the DNA template. A discrete DNA product is obtained after PCR amplification, if these priming sites are within the amplification range of each other. This system was introduced by Welsh and McClelland (1990) which produces amplification of several discrete loci. With slight variation in primer synthesis or reaction assay or the stringency of the PCR technique other types of RAPD markers are developed such as - DAF, AP-PCR, SCAR, CAPS, RAMPO, and AFLP.

1. DNA Amplification Fingerprinting (DAF) -This technique was introduced by Caetano-Anolles *et al*, (1991). In this a single arbitrary primer of only 5 bases is used to amplify the DNA by PCR. Very optimized reaction conditions are required for this marker assay which gives simple banding pattern, useful for DNA fingerprinting. Such banding patterns are analyzed by polyacrylamide gel electrophoresis.

2. Arbitrary Primed Polymerase Chain Reaction (AP-PCR) - DNA amplification patterns are obtained using single primer of 10-50 bases long in PCR and annealing is carried out under nonstringent conditions (Welsh and McClelland 1991).

3. Sequence Characterized Amplified Regions (SCAR) - These are similar to STS markers and are more reproducible as compared to RAPD. These were introduced by Michelmore (1991) and Martin *et al* (1991). SCARs are mostly dominant markers but also behave as co-dominant markers by digesting them with tetra cutting restriction enzymes. From our laboratory sex identification of papaya has been carried out using SCAR marker (Parasnis *et al* ,2000).

4.Cleaved Amplification Polymorphic Sequence (CAPS) - PCR primers for this process can be synthesized based on sequence information in databank and the electrophoretic patterns are obtained by using restriction enzyme digestion of the PCR products (Koniecyzn and Ausubel 1993; Jarvis *et al* 1994).

5. Randomly Amplified Microsatellite Polymorphism (RAMPO) - In this PCR based markers, genomic DNA is first amplified using arbitrary (RAPD) primers. The amplified products are then electrophoretically separated and the dried gel is hybridized with microsatellite oligonucleotide probes. Several advantages of oligonucleotide fingerprinting (Epplen 1992), RAPD (Williams *et al,* 1990) and Microsatellite primed –PCR (Weising *et al*, 1995, Gupta *et al,* 1994a) are thus combined in RAMPO. Some of the advantages include speed of the assay, high sensitivity, high level of variability detected and no requirement of prior DNA sequence information (Richerdson *et al* 1995).

6.Amplified Fragment Length Polymorphism (AFLP) - Zabeau *et al* (1993) developed AFLP, in which fingerprinting patterns are obtained by detection of genomic restriction fragments by PCR amplification. AFLP is an ingenious combination of RFLP and PCR and is extremely useful in detection of polymorphism between closely related genotypes (Saiki *et al*, 1988, Ehrlich *et al*, 1991). AFLP is based on selective amplification of restriction enzyme digested DNA fragments with specific primers. Multiple bands are generated in each amplification reaction that contains DNA markers of random origin. In this

technique, the DNA is digested with one or two restriction enzymes, and double stranded adapters are ligated to the fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. These amplified samples are analysed on denaturing polyacrylamide gel which results in the production of 50 to 100 bands per individual sample. Polymorphism detected in DNA fingerprints, obtained by restriction cleavage, can result from alterations in the DNA sequence including mutations abolishing or creating a restriction site, and insertions, deletions or inversions between two restriction sites. Similar to RAPD analysis, AFLP assay also dose not require prior sequence knowledge, but detects a 10 fold greater number of loci than those detected by RAPD analysis. Thus, the AFLPs have the capacity to rapidly screen thousands of independent genetic loci. The AFLP markers are typically inherited in Mendelian fashion and therefore, can be used for identification, typing, and mapping of genetic loci. The AFLP markers have gained more importance in developing saturated genetic linkage maps in various crops, plants.

1.4.2 Applications of DNA markers in genetic diversity studies

In simple terms, genetic diversity is a statistical concept referring to the variations within the individual gene loci / among alleles of a gene, or gene combinations, between individual plants or between plant populations. Genetic diversity has several 'indicators', which are measured using various tools such as classical or Mendelian genetic analysis, that can be employed to evaluate variation in single known gene (qualitative traits), such as resistance to disease (Smale and McBride,1996). On the other hand, multivariate analysis can be used to analyze variation in quantitative traits. Also, pairwise coefficients of parentage are calculated from pedigree information and used as indicators of genetic diversity (Cox *et al*, 1986). The classical methods of diversity studies are based on morphological characters which are influenced by various environmental factors. However, the molecular markers, which are unrestricted

in number and not influenced by the environment, have the ability of sampling diversity directly at the genome level. The molecular biology tools provide a detailed information about the genetic structure of natural population which was not available in the past (Statkin, 1987).

During domestication, the genetic variation in crop plants has narrowed due to continuous selection pressure for particular traits like high yield or disease resistance. It is extremely important to study the genetic composition of the germplasm of existing cultivars with their ancestors and related species for comparison. Such studies help to find new and useful genes, and also provide information about the phylogenetic relationship. Secondly, in case of the large size germplasm it becomes a critical step for selection of parental genotypes from the germplasm. Maintenance and exploitation of germplasm and understanding the genetic relationships among the genotypes is also possible with such analysis. In addition, molecular markers are being widely used to classify breeding lines, populations and landraces and to establish genetic linkages with traits of agronomic and economic interest.

1.4.2.1 Applications of DNA markers in wheat genomics

Winkler (1920) coined the term `genome' and Kihara (1930) defined the term as a set of chromosomes that forms a fundamental and physiological unit which is indispensable for normal development and growth of the plant. Later on the field of `genomic analysis' steadily progressed. Constitutive genomes in an organism and the various genomes of related genera and species are being analysed using various tools such as chromosome pairing, karyotype analysis, DNA-DNA hybridization, chromosome staining, immunochemical reactions and electrophoresis.

Kihara (1930) developed methods for genome analysis in wheat, which consisted of crossing diploid species with each other and with polyploides and drawing conclusions based on amount of chromosome pairing and fertility in hybrids. Later studies in characterization of germplasm diversity in wheat were based on morphological characters such as variation in heading time, earliness,

winter habit, and coleoptile length (Scarascia *et al*, 1974; Qualset and Puri, 1974a, 1974b, 1975; Jain *et al*, 1975; Porceddu,, 1975). Two main seed-storage proteins in wheat, glutenins and gliadins also served as valuable tools to assess genetic diversity. These proteins display allelic variation at various loci which is partly responsible for the differences observed in the technological properties of wheat. The variation in high molecular weight (HMW) glutenin subunits of *T.dicoccoides* from Isreal (Nevo and Payne, 1987, Levy and Feldman, 1988; Levy *et al*, 1988), Jordan and Turkey (Ciaffi *et al*, 1993,) and that of durum wheat (Branlard *et al*, 1989) have been extensively studied. These studies revealed the presence of wide allelic variation at *Glu*A1 and *Glu*B1 loci and indicated (in case of *T.dicoccoides*) that glutenin polymorphism could be at least partly accounted for by ecogeographical factors. However, protein based markers suffer from some drawbacks such as instability across environments and low levels of polymorphism (especially in case of isozymes).

Schlegal and Mettin (1979) and Kimber (1971) carried out studies on relative chromosome lengths and arm-ratios, respectively, in wheat and studied its phylogenetic relationships with other related species. Differential staining (chromosome banding) and DNA hybridization methods gave improved understanding about the content and distribution of repeated DNA in wheat (Flavell and Smith, 1976, Ranjekar *et al*, 1978). More recently the techniques such as genomic in situ hybridization (GISH) and fluorescent in-situ hybridization (FISH) revolutionized plant cytogenetics and were used in genome mapping project (Werner *et al*,1992;Jian and Gill, 1994), in studies of species relationships and evolution, DNA sequence organization and nuclear architecture (Heslop-Herrison *et al*, 1990) and in detection of alien chromosome segments (Mukai and Gil,l, 1991; Schwarzacher *et al*, 1992; Schwarzacher, 1996). Molecular map of wheat is now available which includes not only RFLP markers but various other subsequently developed markers such as RAPD, microsatellites and AFLPs (Anderson *et al*,1992; Xie *et al*,1993; Hohmann *et al*, 1994; Nelson *et al*,1995a,1995b,1995c,VanDeynze *et al*, 1995; Roder *et al*, 1996). The various

DNA marker systems used for wheat genetic diversity analysis are detailed below.

Hybridization-based DNA markers : RFLP analysis of the two wild tetraploid wheat species *T.dicoccoides* and *T.araraticum* by Mori *et al*, (1995) indicated greater variation in the former, suggesting a recent origin of *T.araraticum* and diphyletic origin of the two wild species. In another study, Mori *et al* (1997) indicated that *T.dicoccum* was the earliest domesticated tetraploid wheat, while Li *et al*, (1999) suggested that the domestication of emmer wheat was gradual and involved long term introgression between cultivated emmer and its wild progenitor. The first attempt to use RFLPs for mapping the chromosomes of wheat was reported by Chao *et al*, 1989 who constructed a linkage map of the homoelogous group 7 of wheat.

PCR-based DNA markers : Because of the built-in disadvantages of the RFLP procedure and the low levels of the RFLP-associated polymorphism in wheat due to high proportion of repetitive DNA (Ranjekar *et al*, 1976; Flavell and Smith 1975), polymerase chain reaction based markers such as RAPD emerged as an alternative. RAPD markers have been successfully used for effective germplasm management involving estimation of diversity, monitoring genetic erosion and removal of duplicates form germplasm collections of crop species (review by Virk *et al*, 1995). Being rapid, efficient and amenable to automation, RAPDs have proved to be an effective method to detect polymorphism in wheat (Vierling and Nguyen, 1992; Joshi and Nguyen, 1993a,1993b, Sun *et al* 1998). Wang *et al*, (1995) assigned 11 RAPD markers to specific chromosomes and chromosome-arms using Langdon durum wheat disomic substitution lines, suggesting the possible role of these markers in genome mapping. D'Ovidio *et al* (1990) demonstrated PCR-amplification of gamma-gliadin gene as an efficient method to detect genetic polymorphism among wheat species and cultivars. PCR amplification of low molecular weight (LMW) glutenin genes was used by D'Ovidio (1993) to distinguish between durum wheat cultivars with good and poor technological properties. Using PCR-based microsatellite divergence of *T.dicoccoides* accessions highly resistant to yellow rust Fahima *et al* (1998)

demonstrated that a relatively small number of microsatellites could be used to detect diversity in the wild species. Recently Li *et al* (2000) studied genetic diversity in accessions of *T.dicoccoides* from a microsite in Isreal by amplifying microsatellite loci and observed that natural selection, probably through aridity stress, influenced microsatellite divergence. In similar studies, Dograr *et al* (2000) used wheat microsatellite markers to distinguish between Turkish durum wheat genotypes, while Hammer *et al* (2000) used them to distinguish between diploid species.

Inter-simple sequence repeats : ISSRs have been used for detection of polymorphism (Nagaoka and Ogihara,1997) and in genetic mapping of wheat (Kojima *et al*, 1998). They have been used to study diversity among the Indian tetraploid wheat genotypes in our laboratory (Pujar *et al*, 2001). ISSRs have also been used to identify markers associated with seed size (AmmiRaju, *et al*, 2001), grain protein content (Dholakia *et al* 2001) and yellow berry tolerance in wheat (AmmiRaju *et al*, 2001) in our laboratory.

AFLP markers : Recently Maccaferi *et al*, (2000) used 14 AFLP primer combinations, alongwith 38 simple sequence repeat (SSR) markers, to assess genetic diversity in 58 durum wheat accessions. Of these 43 were composed of old and modern Italian durum cultivars, while 15 were exotic (5 French, 4 from US, 3 Turisian and 3 cultivars from CIMMYT, Mexico). The SSR and AFLP similarity matrices were highly correlated (r=0.74) in this study, suggesting good genome coverage and accurate representation of genetic diversity by both the marker systems.

1.5 Genesis of my thesis

Several HMW subunits of glutenin have been shown to be associated with breadmaking quality. Payne (1987) analysed numerous unselected progeny of crosses between common wheat cultivars for both SDS sedimentation value (correlated with loaf volume) and subunit composition and showed that certain allelic subunits impart differential effects on gluten quality. Since the x- and y-type of HMW subunits encoded by the *Glu*-B1and *Glu*- D1 loci are always

inherited as pairs, it is not possible to determine the effects of individual subunit on gluten quality. Further, Branlard and Dardevet, (1985) have shown that the alveograph parameters, W (gluten strength) and P (tenacity) and the Zeleny sedimentation value are correlated positively with subunits 5 +10 and 7+9 and negatively with 2+ 12 , whereas subunits 2* and 17+ 18 and 1 are correlated with W (swelling). Thus allelic variation in LMW and HMW glutenin subunits has cumulative effect on dough properties. Presence or absence of glutenin subunit alleles which are associated (positively or negatively) with bread making quality in wheats can be easily estimated using molecular markers. The effect of individual allele on bread making quality can be assessed if specific Near Isogenic Lines for individual subunit allele are available. Such an effort is ongoing with the help of funding from the Department of Biotechnology, New Delhi, at G. B. Pant Agricultural and Technology, Pantnagar, by Dr. N.K.Singh in a network programme in the country. In the present work, I have selected 12 genotypes used in NIL development and have studied genetic diversity among these bread wheat genotypes using ISSR and RAPD markers. I have also included HD2329, a genotype with no such alleles in the analysis.

1.5.1 Organization of my thesis

Chapter 1 Introduction, wheat seed storage proteins, genetics of LMW, HMW gluten subunits, molecular markers and their application in wheat

Chapter 2 Materials and Methods

Chapter 3 Results

- 3.1. Identification and evaluation of ISSR primers
- 3.2. Genetic diversity and clustering pattern of ISSR markers
- 3.3. Identification and evaluation of RAPD primers
- 3.4. Genetic diversity and clustering pattern of RAPD primers
- 3.5. Combined dendrogram and correlation between ISSR and RAPD similarity matrices of RAPD primers

Chapter 4 Discussion

Chapter 5 Summary

References

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2
MATERIALS AND METHODS

2.1. Plant material :

The seeds of 13 different wheat genotypes were received from Dr. N. K. Singh, G.B. Pant University, Patanagar, (Table2.1). All these genotypes except HD2329 were selected on the basis of presence HMW gluten alleles in their seed storage protein profiles.

About 25-30 seeds of each of the genotype were sown in plant growth chamber at Agharkar Research Institute, Pune under controlled conditions of temperature, humidity and light. Young leaf tissue was harvested from all the genotypes for DNA extraction.

2.2. DNA extraction :

DNA extraction was carried out as per the procedure described by Anderson for monocot plant DNA extraction (USDA-ARS, Wheat Genetics Protocols, 1998). Essentially, 15g of leaf tissue was ground to a fine powder in liquid nitrogen using a morter and pestle. 25ml of hot extraction buffer was added (For 1lit. stock; 5M NaCL 100ml, 1M Tris.HCl 100ml, 25 mM EDTA 200ml, 20% SDS 42ml, 0.38g/100ml of sodium bisulphite) to each tube. The contents were mixed to form an emulsion, which was incubated at 65°C for 30 min. Following incubation, the emulsion was allowed to cool to RT and an equal amount of chloroform: isoamyl alcohol (24:1) mixture was added to each tube which was then capped and vigorously swirled to mix the contents. The tubes were centrifuged at 10,000 rpm for 20 min at RT in a Sorvall RC5B+ centrifuge (Du Pont, USA). Then the aqueous layer was recovered and pipetted out into fresh SS34 tube. To this, an equal volume of chloroform : isoamyl alcohol (24:1) mixture was added and after proper mixing, the tubes were centrifuged for 10 min, at 8000 rpm. The aqueous layer was separated into fresh SS34 tube and an equal volume of chilled propanol was added for DNA precipitation and the tubes were kept for 45 min at 4°C. The pellet of the precipitated DNA obtained by centrifuging the tubes for 10 min, at 8000

Table 2.1 Details of hexaploid wheat genotypes used for RAPD and ISSR analysis

Sr.No	Genotype	HMW glutenin allele	Pedigree	Organization	Year of the variety release
1.	HD 2329		SLS113/NP852/4/PJSIB/P14//KT 54B/3/K65/5/SKA/6/UP262	IARI,DL	1985
2	Kalyansona	*Glu*A3 / *Glu* B1 (17+18) / C sub unit	PJ SIB/GB55	JT	1967
3.	K-68	*Glu* D5(b) / *Glu*D1 (5+10)	NP773/K13	DUP,KP	1963
4.	UP1109	*Glu* A3 (d) / *Glu* B1 (7+8)	UP262/UP368	GBPUA&T,PN	1989
5.	UP319	*Glu* A3	CNOSIB/Sn 64/KLRE/3/8156	GBPUA&T,PN	1973
6.	UP2121	*Glu* A1/*Glu* B1(13+16)	NA 160/KAL/UP301/3/SAM 68	GBPUA&T,PN	1984
7.	UP115	*Glu* A3 (e)	RiDLEU/Np7 10/3/PJ SIB/P14//KT54B/4/PJ SIB/GB56/T2PP/NA160	GBPUA&T,PN	1979
8.	Sunkota	B *Glu* D3	-	-	-
9.	HUW 55	*Glu* B4 (d) / *Glu* D3	MALAVIYA 55	BHU, VN	-
10.	UP 301	Alb	LR 64/SN 64	GBPUA&T,PN	1970
11.	WH 147	*Gli*A1(C)	PJ SIB/P14/KT54B/3/C286/C273/4/S339/PV18	HAU, HR	1977
12.	Chinese spring	*Glu* B1 (7+8) / *Glu* (null)	-	-	-
13.	CS1BL	-	-	-	-

rpm at RT was dried and dissolved in high salt TE buffer (1M NaCl, 10mM Tris-HCl pH 8.0 1mM EDTA pH 8.0). Then 100µg / ml of RNase A (DNase free) was added to remove any RNA if present and incubated for one hour at RT. The dissolved DNA was reprecipitated by centrifuging the tubes for 10 min, at 8000 rpm at RT. The DNA pellet was washed with 70% ethanol followed by a short spin of 8000 rpm for 10 min at RT. After removing the supernatant, the DNA pellet was dried, dissolved in an appropriate volume of TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) and stored at -20°C until further use.

2.3. DNA quantification :

Genomic DNA (1µl) was loaded on 0.8% agarose gel in 0.5 x TAE buffer (0.02M Tris-acetate, 0.5M EDTA pH 8.0) containing ethidium bromide (10µg/ml) alongwith known amount of standard λ bacteriophage DNA such as 50ng, 100ng, 150ng, 200ng, and 250ng each in separate lane. The concentration of the extracted DNA was estimated by visual comparison of the band with λ bacteriophage DNA. The purity and concentration of the extracted DNA was also checked by measuring absorbance on UV-VIS spectrophotometer(V-530,Jasco, USA) at 230, 260, 280 and 300nm. Purity was analysed by the absorbance ratios; 230/260 and 280/260 while concentration was calculated assuming 1 O.D. at 260nm corresponds to 50ng/ml DNA.

2.4. ISSR-PCR analysis :

A set of 100 ISSR primers (set #9) was procured from UBC (University of British Columbia, Biotechnology Laboratory, Vancouver, Canada). Initially, 3 genotypes namely HD2329, Kalyansona and K-68 were used for PCR amplification using all the 100 primers. The primers which gave clear and polymorphic patterns were used for further analysis with all the 13 genotypes. For each primer, 25µl amplification reaction contained 10mM Tris-HCl pH 9.0, 50mM KCl,0.1% TritonX-100, 1.5mM $MgCl_2$, 1mM dNTP, 0.2µM of primer, 15ng of genomic DNA and 0.8 units of Taq DNA polymerase (Perkin Elmer, USA). PCR amplifications were performed in

a Perkin Elmer Cetus 9700 thermal cycler (P.E.Biosystems,USA) with initial denaturation at 94°C for 5 min followed by 45 cycles of 94°C for 30 s, annealing at 50°C for 45 s, extension at 72°C for 2 min, with final extension at 72^0C for 5 min. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualised on UV transilluminator (U.V.P,USA). The gel was photographed using 36mm SLR camera (K1000,Pentax) and was also documented on gel documentation system(Amersham Pharmacia, USA) for digital storing.

2.5. RAPD-PCR analysis :

RAPD analysis was carried out with 100 decamer random primers from set I obtained from University of British Columbia, Canada. Initially, PCR amplifications were carried out with HD-2329,Kalyansona and K-68 as representative genotypes. The primers that gave clear and polymorphic amplification patterns were used for further analysis with all the 13 genotypes. For each primer, a 25µl amplification reaction contained 2.5µl of 10 x PCR buffer with $MgCl_2$, 2.5µl of 1mM dNTP,1.0µl of 0.2µM of primer, 1.0µl of 1mM Spermidine, 15ng of genomic DNA and 0.8 units of Taq DNA polymerase (Perkin Elmer,USA). PCR amplifications were performed in a Perkin Elmer Cetus 9700 thermal cycler (P.E. Biosystems, USA. The PCR conditions included initial denaturation at 94^0C for 4 min, followed by 5 cycles at 92^0C for 30s , 2min at 35^0C, 90s at 72^0C; 35 cycles of denaturation at 92^0C for 5s, annealing at 40^0C for 20s, extension at 92^0C for 90s with final extension at 72^0C for 5 min.

2.6.Reproducibility of amplification patterns :

DNA amplifications with each ISSR and RAPD primer were repeated at least thrice to ensure reproducibility. The bands were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer. Clear and intense bands were scored while faint bands against background smear were not considered for the further analysis.

2.7. Scoring and data analysis :

For each genotype, each fragment / band that was amplified using ISSR and RAPD primers was treated as an unit character. Unequivocally scorable and consistently reproducible amplified DNA fragments were transformed into binary character matrices (1 for presence, 0 for absence). The commercial software package NTSYS-PC (Rohlf 1989) was used to develop similarity matrices based on the Dice coefficient which is defined as 2a/2a+*u*, where a is the number of positive matches and *u* is the number of nonmatches. These data were then used to construct dendrogram for cluster analysis based on the unweighted pair group method with arithmatic mean (UPGMA) using WINDIST (Yap and Nelson 1996). Confidence limit of the dendrogram was estimated using the software WINBOOT (Yap and Nelson 1996) using PHYLIP format (Felsenstein 1985) in which 2000 replications were carried out. Two separate dendrograms for ISSR and RAPD data and a combined dendrogram by pooling ISSR and RAPD data were generated. Molecular weight of each of the potential specific bands was calculated using the software program Seqid (Rhoads and Roufa, 1989).

The distance matrices obtained in RAPD and ISSR analysis were compared using correlation analysis. Band informativeness (Ib) and Resolving power (Rp) were calculated as given by Prevost and Wilkinson (1999). Marker index (MI) was calculated as described by Powell *et al*, (1996). The formulae used for the above mentioned parameters are :

1. Band informativeness of a given band : $\mathbf{I_b = 1 - (2 x \left| 0.5- p \right|)}$ where p is the proportion of the total genotypes containing the band.
2. Resolving power of a primer**:** $\mathbf{Rp = S\ I_b}$
3. Average heterozygosity **:** $\boldsymbol{H_{av} = S\ H_n / n}$ where $H_n = 1 - \Sigma$ pi, pi is the allele frequency for the ith allele and n is the number of markers (loci) analyzed.
4. Marker index**:** $\mathbf{MI = n\ H_{av}}$
5. Average heterozygosity using polymorphic markers only $\boldsymbol{H_{av}(p) = MI / n\ b}$, where β is the fraction of polymorphic markers out of total number of markers analyzed.

CHAPTER 3

RESULTS

ASSESSMENT OF GENETIC DIVERSITY IN INDIAN HEXAPLOID WHEAT GENOTYPES USING ISSR AND RAPD MARKERS

CHAPTER 3

Results

3.1 Identification and evaluation of ISSR primers for diversity estimates in 13 wheat genotypes

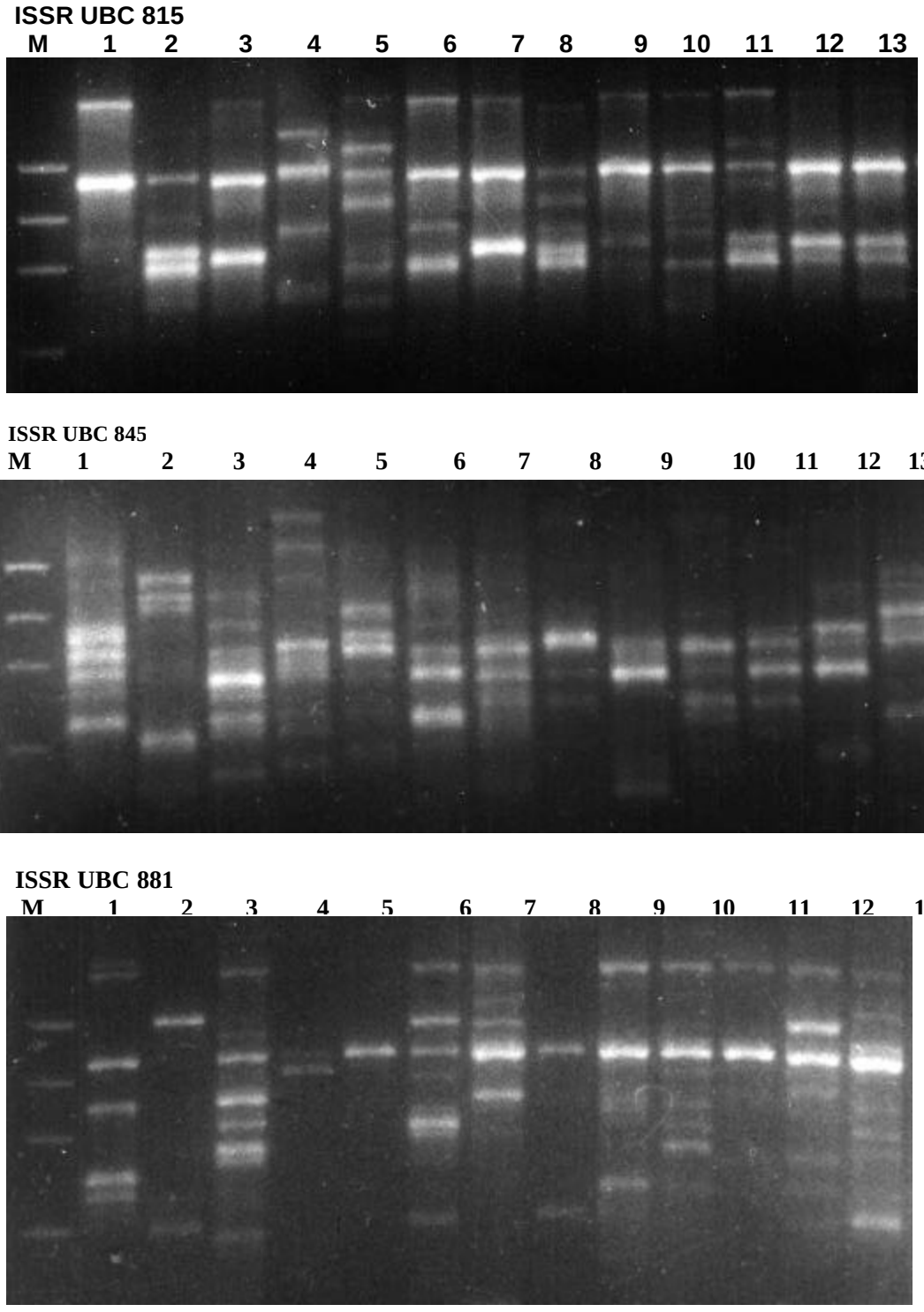
A total of 100 primers consisting of di-,tri-,tetra- and penta-nucleotide repeat motifs were used for initial screening with 3 genotypes, namely HD-2329, Kalyansona and K-68. Out of these, 18 primers gave no amplification at all, while only 11 primers were found to give clear and polymorphic patterns, and were subsequently used to analyze the entire set of 13 genotypes. The oligonucleotide sequences of these polymorphic primers are given in Table 3.1. Out of these, 11 primers one contained $(AG)_n$ repeat motif, three primers contained $(CT)_n$ motif, two primers contained $(TC)_n$ repeat motif and three primers contained $(GA)_n$ repeat motif. Apart from these, one primer contained $(GATA)_2$ $(GACA)_2$ repeat motif while one primer contained (GGGTG)n repeat motif. These 11 ISSR primers amplified a total of 87 bands in size ranging from 400bp to 2000bp out of which 47 bands were polymorphic. These primers showed variation in the percentage of polymorphism, band informativeness (I_b) and resolving power (Rp). Average band informativeness (AvI_b), is a measure of closeness of a band to be present in 50% of the genotypes under study and resolving power (Rp) is the sum of I_b values of all the bands amplified by a primer. The percentage of polymorphism ranged from 37.50% to 66.66%, average I_b ranged from o.182 to 0.918 while Rp ranged from 1.638 to 7.986 (Table 3.1). The primer UBC 822 showed the highest values of percentage of polymorphism (66.66%) and average I_b (0.918) while UBC 881 revealed the highest value of Rp (7.986).

Table3.1 Polymorphism exhibited by ISSR primers in wheat

Sr.NO	Primer UBC	Sequence	No. of bands Amplified	No of polymorphic bands	% of polymorphism	Av/I_b	Rp
1.	810	GAGAGAGAGAGAGAGAT	5	3	60.00	0.908	4.540
2.	812	GAGAGAGAGAGAGAGAA	6	3	50.00	0.364	2.184
3.	814	CTCTCTCTCTCTCTCTA	7	4	57.14	0.546	3.822
4.	815	CTCTCTCTCTCTCTCTG	8	3	37.50	0.728	5.824
5.	822	TCTCTCTCTCTCTCTCA	3	2	66.66	0.918	2.724
6.	834	AGAGAGAGAGAGAGAGYT	9	4	44.44	0.182	1.638
7.	845	CTCTCTCTCTCTCTCTRG	10	6	60.00	0.364	3.640
8.	840	GAGAGAGAGAGAGAGAYT	11	6	54.54	0.545	5.995
9.	852	TCTCTCTCTCTCTCTCRA	7	3	42.85	0.378	2.646
10.	876	GATAGATAGACAGACA	10	6	60.00	0.190	1.900
11.	881	GGGTGGGGTGGGGTG	11	7	63.63	0.726	7.986

Out of 11 ISSR primers used for the present analysis, gel electrophoresis pattern obtained using primers UBC-815, UBC-845 and UBC-881 are depicted in Figure 3.1 as a representative picture. Figure 3.1.a shows agarose gel electrophoresis pattern obtained using primer UBC-815. This primer has amplified a total number of 8 bands in the size range ~850bp to ~1300bp. Wheat genotype UP-319 (lane 5) has shown the highest number of (6) amplified bands. A common band of size ~1250bp is present in all 13 genotypes. A band of size ~850bp is present in K-68 (lane 3) alone and is absent in all the other genotypes. Genotypes namely Kalyansona (lane 2),K-68(lane3), WH147 (lane11), Chinese Spring(lane12) and CS1BL (lane13) exhibit a monomorphic amplification pattern. Figure 3.1.b shows the amplification pattern using the primer UBC-845, where it has amplified a total number of 10 scorable bands, out of which 6 are polymorphic. One band of ~800bp is present in a few genotypes namely K-68 (lane 3), UP2121(lane 6), HUW55(lane 9), WH147(lane 11), and Chinese spring (lane 12) and absent in the remaining genotypes. A single band of size ~1100bp is unique to Kalyansona (lane 2) and is absent in all the remaining genotypes. On the other hand, a band of size ~900bp is common to all the other genotypes except Kalyansona (lane 2).Gel electrophoresis picture obtained for the primer UBC-881 has been depicted in Figure 3.1.c. and gives a total number of 11 scorable bands. A band of ~600bp is unique to CSIBL(lane 13) and absent in all the other genotypes. Similarly, a band of ~1000bp is present only in genotype UP1109 (lane 4) and absent in other genotypes. Genotypes namely HD2329 (lane 1), K-68(lane 3),UP 310(lane 5), UP2121(lane 6), UP115 (lane7), Sunkota (lane8), HUW55 (lane 9), UP301(lane 10), WH147(lane 11), Chinese Spring (lane 12), and CS1BL(lane13) have amplified a common band of size ~1100bp which is absent in Kalyansona (lane 2) and UP1109 (lane 4). Genotypes namely Kalyansona (lane2), UP2121 (lane 6), UP115 (lane 7) and Chinese Spring (lane 12) have a common band of ~1200bp which is absent in all the other genotypes.

Figure 3.1 ISSR amplification pattern using the primers a) UBC 815, b) UBC 845, c) UBC881. M= molecular size marker λ DNA/HindIII digest and φX174 DNA /HaeIII digest. Lane numbers correspond to serial numbers in the Table 3.1.



3.2 Genetic diversity and clustering patterns of 13 wheat genotypes based on ISSR polymorphism data

As described in Materials and Methods (Chapter 2.2) based on the polymorphism data generated using ISSR markers, a similarity matrix (Figure 3.2) and a dendrogram (Figure 3.3) were developed for 13 genotypes under present study. The dendrogram clearly indicates two main clusters. The first cluster includes HD-2329 and WH-147 with the highest bootstrap value of 99.9%, indicating that WH-147 is genetically closer to HD-2329. The second cluster includes all the remaining genotypes namely K-68, UP-115, CS1BL, CS, UP2121, HUW55, UP301, UP319, UP1109 and Sunkota also with very high bootstrap value of 99.8% except for Kalyansona. Kalyansona separates from all these remaining genotypes and remains out grouped (35.1%). The second cluster has three sub clusters where K-68, UP115, CSIBL and Chinese spring form the first subcluster with bootstrap of 25.9%. UP2121 and HUW55 form the second subcluster with bootstrap value of 33.8%, while UP1109 and Sunkota form the third subcluster with bootstrap value of 68.3%. Genotypes UP301 and UP319 do not form a cluster and remain ungrouped. The bootstrap values for various groups in this dendrogram range from 16.6 to 99.9 which is a very wide range. The genotype with bootstrap values below 50% indicate that the positions of these genotypes may change if other marker systems are applied or other genotypes are included in the analysis.

Figure 3.2 ISSR similarity matrices

1	2	3	4	5	6	7	8	9	10	11	12	13
0.0												
0.578947	0.0											
0.309524	0.513514	0.0										
0.552239	0.543860	0.600000	0.0									
0.470588	0.551724	0.575758	0.551020	0.0								
0.400000	0.571429	0.410256	0.573771	0.483871	0.0							
0.341463	0.555556	0.350000	0.619048	0.468750	0.315789	0.0						
0.542857	0.633333	0.529412	0.607843	0.538462	0.562500	0.575758	0.0					
0.307692	0.558824	0.421053	0.593220	0.466667	0.361111	0.243243	0.612903	0.0				
0.317073	0.638889	0.300000	0.555556	0.437500	0.289474	0.256410	0.484848	0.324324	0.0			
0.452055	0.650794	0.549296	0.703704	0.490909	0.432836	0.449275	0.508772	0.446154	0.391304	0.0		
0.392405	0.507246	0.376623	0.533333	0.442623	0.424658	0.253333	0.523810	0.352113	0.333333	0.454545	0.0	
0.418605	0.473684	0.309524	0.611940	0.500000	0.400000	0.390244	0.542857	0.461538	0.317073	0.479452	0.265823	0.0

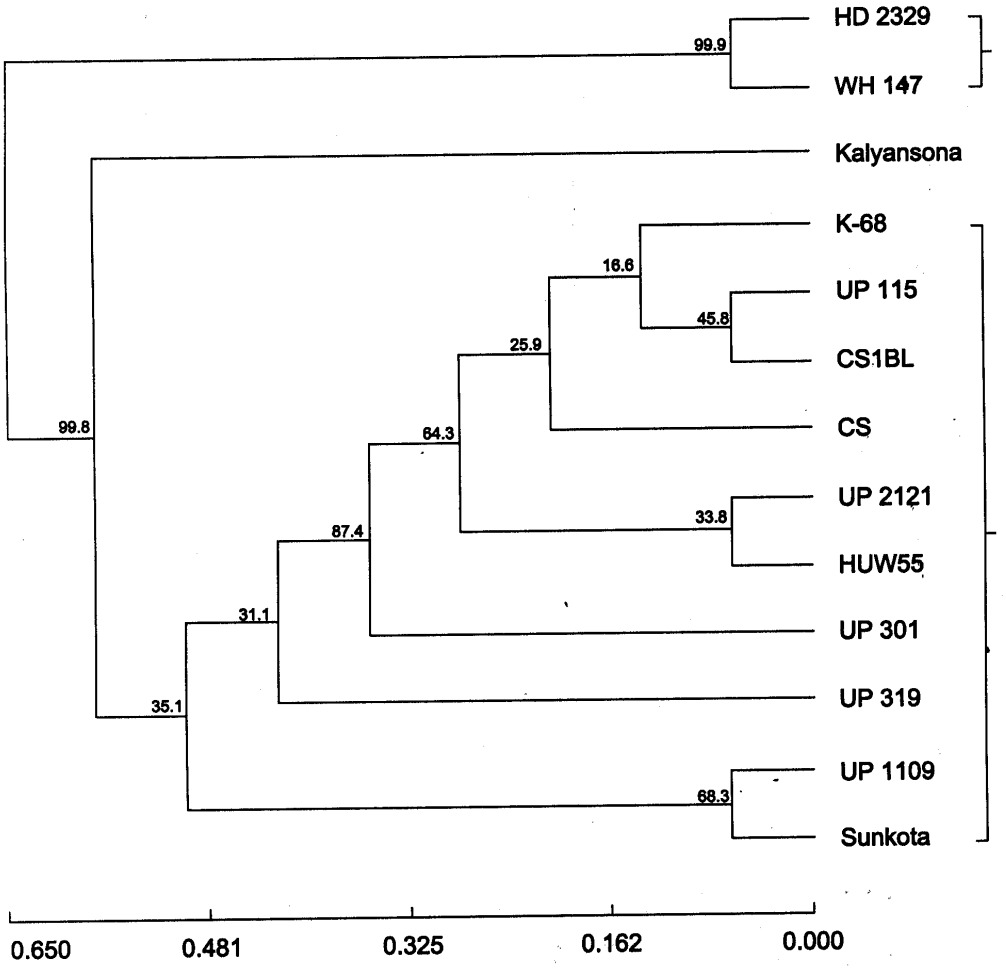


Figure 3.3 : UPGMA clustering pattern revealed by the 13 wheat genotypes usingISSR data.

3.3. Identification and evaluation of RAPD markers for diversity estimates in 13 wheat genotypes

Out of 100, decamer random primers used for initial screening with three representative genotypes namely HD-2329, Kalyansona and K-68, 17 primers gave no amplification at all, while only 12 primers amplified polymorphic patterns. These primers were then used for RAPD analysis of all the 13 genotypes. The oligonucleotide sequences of these polymorphic primers are given in Table 3.2. Amplification products of the 13 genotypes with these 12 primers yielded a total of 87 scorable bands, out of which 67 were polymorphic (Table 3.2). The size of the amplification products ranged from 450bp to 1800bp. The highest number of bands (10) were obtained with primers UBC-28, UBC-29, UBC-31 and UBC-51, while the lowest number (3) was obtained with primer UBC-52. Different primers showed variation in their ability to detect polymorphism. The percentage of polymorphism ranged from 60.00% to 90.00%. Primer UBC-51, revealed the highest polymorphism (90.00%) while primer UBC-75 exhibited the lowest polymorphism (60%). The 12 polymorphic primers exhibited variation with regard to average band informativeness (AvI_b) and resolving power (Rp). The AvI_b and Rp values of these polymorphic primers have been depicted in Table 3.2. The primer UBC-28 showed the lowest AvI_b (0.182) while the highest AvI_b of 0.182 was exhibited by the primers UBC 18, UBC23 and UBC 75. The primer UBC-52 showed the lowest Rp (1.011) and the primer UBC-18 showed the highest Rp (6.656) values.

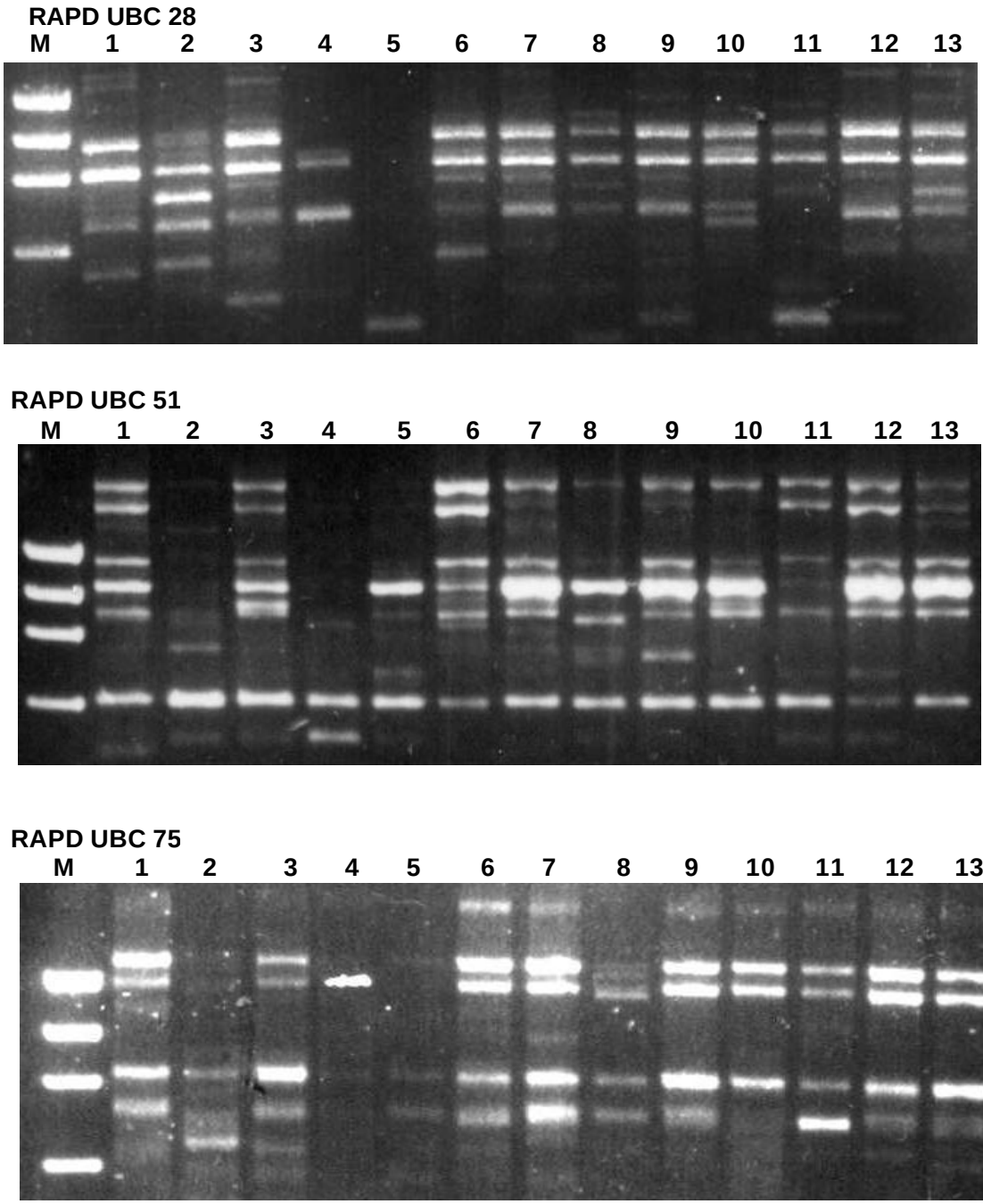
Out of 12 RAPD primers used for present analysis, gel electrophoresis patterns obtained using primers UBC-28, UBC-51, and UBC-75 are depicted in Figure 3.4 as a representative picture. Figure 3.4.a. shows agarose gel electrophoresis pattern obtained using the primer UBC-28. This primer has amplified a total number of 10 scorable bands in the size range from 300bp to 1300bp. Genotypes namely HD-2329 (lane 1), K-68 (lane 3), UP2121 (lane 6), UP-115(lane 7), Sunkota (lane 8), HUW-55 (lane 9), UP-301 (lane 10), WH-147(lane 11), Chinese Spring (lane 12) and CS1BL (lane 13) exhibit a

Table 3.2. Polymorphism exhibited by RAPD primers in wheat

Sr.No	Primer	Sequences	No of bands amplified	No of polymorphic bands	% of polymorphism	Av/Ib	Rp
1.	1	CCTGGGCTTC	8	5	62.50	0.668	5.344
2.	9	CCTGCGCTTA	6	5	83.33	0.534	3.204
3.	18	GGGCCGTTTA	8	7	87.50	0.832	6.656
4.	23	CCCGCCTTCC	6	4	66.66	0.832	4.992
5.	28	CCGGCCTTAA	10	8	80.00	0.182	1.82
6.	29	CCGGCCTTAC	10	7	70.00	0.234	2.34
7.	31	CCGGCCTTCC	10	8	80.00	0.664	6.64
8.	51	CTACCCGTGC	10	9	90.00	0.510	5.10
9.	52	TTCCCGGAGC	3	2	66.66	0.337	1.011
10	75	GAGGTCCAGA	5	3	60.00	0.832	4.160
11.	82	GGGCCCGAGG	4	3	75.00	0.334	1.336
12.	89	GGGGGCTTGG	7	6	85.71	0.538	3.766

monomorphic amplification with a common band of ~1020bp except the genotypes Kalyansona (lane 2) UP-1109 (lane 4) and UP-319 (lane 5). One band of ~750bp is present only in Kalyansona (lane 2) and CS1BL (lane 13) and is absent in remaining 11 genotypes. Similarly ~850bp band is present in two genotypes namely, UP-2121 (lane 6) and UP-115 (lane 7) and is absent in all the other genotypes. A ~550bp band is unique to Kalyansona (lane 2) which is absent in all the remaining genotypes. Figure 3.4.b shows the amplification pattern using the primer UBC-51, where it has amplified a total number of 10 scorable bands. A band of ~450bp is unique to UP-1109 (lane 4), which is absent in all the remaining genotypes. All the 13 genotypes reveal a monomorphic band of ~600bp size. A band of size ~780bp is present only in HUW-55 (lane 9). Genotypes HD-2329 (lane 1), K-68 (lane 3), UP-2121 (lane 6), UP-115 (lane 7), Sunkota(lane 8), HUW-55 (lane 9), UP-301 (lane 10), WH-147 (lane 11), Chinese Spring (lane 12) and CS1BL (lane 13) possess ~950bp band which is absent in the genotypes Kalyansona (lane 2), UP-1109 (lane 4) and UP-319 (lane 5). Similarly, genotypes HD-2329, K-68, UP-319, UP-2121, UP-115, Sunkota, HUW-55, UP-301, Chinese Spring and CS1BL (lane numbers 1,3,5,6,7,8,9,10,12,13, respectively) possess ~1100bp band which is not seen in Kalyansona, UP-1109 and WH-147 (lane numbers 2,4, 11 respectively). Gel electrophoresis pattern obtained for the primer UBC-75 has been depicted in Figure 3.4.c and gives a total number of 5 scorable bands. A band of size ~650bp is unique to genotype Kalyansona (lane 2) which is absent in all the other remaining genotypes. Genotypes HD-2329 (lane 1), K-68 (lane 3), UP-2121 (lane 6), UP-115 (lane 7), Sunkota (lane 8), HUW-55 (lane 9), UP-301 (lane 10), Chinese Spring (lane 12), and CS1BL (lane 13) reveal ~1100bp band which is absent in other four genotypes ie Kalyansona (lane 2), UP-1109 (lane 4), UP-319 (lane5), and WH-147 (lane 11). Similarly a band ~1250bp size is present in HD-2329 (lane 1), K-68 (lane 3), UP-2121 (lane 6), UP-115 (lane 7), HUW-55 (lane 9), UP-301 (lane 10), WH-147 (lane 11), Chinese Spring (lane 12) and CS1BL (lane 13) which is absent in Kalyansona (lane 2), UP-1109 (lane 4), UP-319 (lane 5), and Sunkota (lane 8). Genotypes HD-2329 (lane 1), K-68(lane 3), UP2121(lane 6), UP115(lane 7),HUW55 (lane 9) and Chinese Spring (lane12) reveal a similar amplification / banding pattern with this primer.

Figure 3.4 RAPD amplification pattern using the primers a) UBC 28, b) UBC 51, c) UBC 75. M= molecular size markers λ DNA/HindIII digest and φX174 DNA/HaeIII digest. Lane numbers correspond to serial numbers in Table 3.2.



3.4 Genetic diversity and clustering pattern of 13 wheat genotypes, basedon RAPD data

The RAPD amplification data were used to obtain a similarity matrix (Figure 3.5) and for generation of dendrogram (Figure.3.6). The dendrogram obtained clearly indicates two main clusters. The first cluster includes HD-2329 and WH-147 with the highest boot strap value of 99.8%, indicating that WH-147 is genetically closer to HD-2329.The position of these two genotypes remains the same as in the ISSR dendrogram (Figure 3.3). The second cluster includes other genotypes, namely K-68, UP-2121, Chinse Spring, UP-115, CS1BL, HUW-55, UP-301, UP-319, UP-1109 and Sunkota except the genotype Kalyansona which separates from all these remaining genotypes and stands out grouped (94.4%). The second cluster has two subclusters where the first subcluster has K-68, UP-2121, Chinese Spring, UP-115 and CS1BL (40.8%), while UP-1109 and Sunkota form the second cluster (44.3%). The position of these two genotypes remains same as that in ISSR dendrogram. HUW-55, UP-301, and UP-319 do not group and remain separated. The bootstrap values for this dendrogram ranges from 17.9% to 99.8%. The genotype with bootstrap values below 50% indicate that the positions of these genotypes may change if other marker systems are applied or other genotypes are involved in the analysis.

Figure 3.5 RAPD similarity matrices

1	2	3	4	5	6	7	8	9	10	11	12	13
0.0												
0.400000	0.0											
0.142857	0.397849	0.0										
0.407407	0.449275	0.404762	0.0									
0.391304	0.425000	0.347368	0.380282	0.0								
0.153846	0.304348	0.140187	0.421687	0.319149	0.0							
0.113208	0.382979	0.100917	0.435294	0.354167	0.111111	0.0						
0.311111	0.461538	0.397849	0.391304	0.400000	0.347826	0.361702	0.0					
0.207921	0.393258	0.192308	0.450000	0.318681	0.126214	0.142857	0.348315	0.0				
0.312500	0.404762	0.272727	0.413333	0.372093	0.244898	0.280000	0.333333	0.221053	0.0			
0.290323	0.432099	0.291667	0.416667	0.373494	0.242105	0.278351	0.358025	0.260870	0.310345	0.0		
0.174312	0.402062	0.089286	0.431818	0.313131	0.117117	0.097345	0.402062	0.203704	0.242718	0.240000	0.0	
0.140187	0.347368	0.109091	0.418605	0.340206	0.119266	0.063063	0.368421	0.150943	0.247525	0.265306	0.105263	0.0

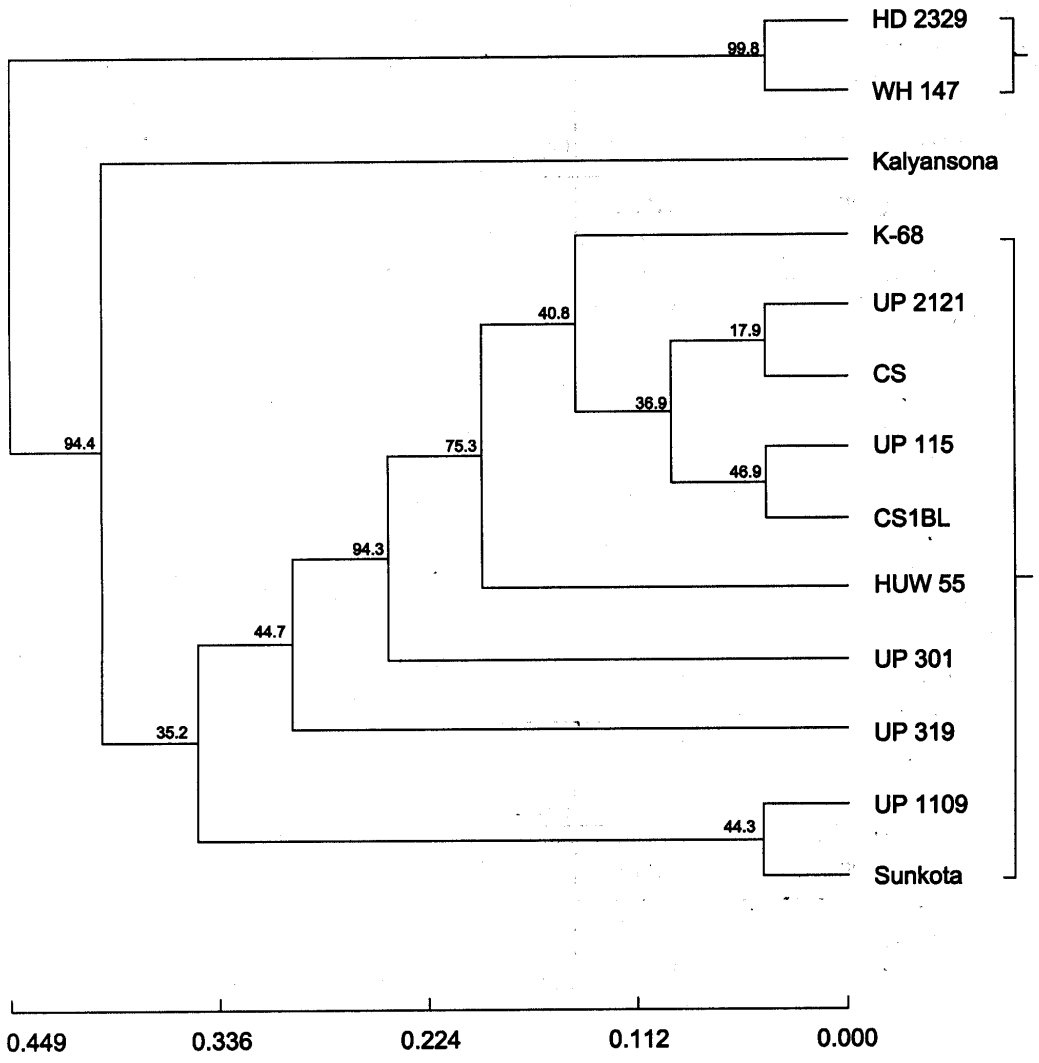


Figure 3.6: UPGMA dendrogram obtained by using RAPD data for 13 wheat genotypes.

3.5 Combined dendrogram obtained using RAPD and ISSR data

The amplification data obtained from ISSR and RAPD primers were used for a similarity matrix (Figure3.7)and to generate a dendrogram (Figure 3.8). The bootstrap values for this dendrogram ranges from 28.8% to 90.3% which is a wide range. This dendrogram has most of the features similar to those of RAPD (Figure 3.6) and ISSR (Figure 3.3) dendrograms and has two main clusters. The first cluster has HD2329 and WH147 with the bootstrap value 90.3%, indicating the close genetic relationship between these two genotypes. All the remaining genotypes form second cluster which in turn has three subclusters. The first subcluster includes three genotypes, namely K-68, Chinese Spring and CS1BL(49.0%). UP2121, HUW55 and UP301 form the second subcluster (33.8%), while UP1109 and Sunkota form the third subcluster (48.8%). However, Kalyansona, UP115 and UP319 do not cluster and remain separate. The clusters formed by HD2329, WH147, Kalyansona, UP1109 and Sunkota (48.8%) are similar in all the three dendrograms ISSR, RAPD and combined dendrograms (Figures 3.3, 3.6 and 3.8 respectively).

3.6 Correlation between distances matrices:

In order to study the correlation between the two marker systems used under present investigation the distance matrices obtained using Dice's coefficient in the RAPD and ISSR analysis were compared using correlation analysis. The two matrices showed a positive correlation (r= 0.89) as shown in Fig 3. 9.

Figure 3.7 Similarity matrix for combined dendrogram

1	2	3	4	5	6	7	8	9	10	11	12	13
1.000												
0.530	1.000											
0.750	0.542	1.000										
0.571	0.601	0.524	1.000									
0.589	0.595	0.589	0.649	1.000								
0.708	0.583	0.708	0.565	0.571	1.000							
0.732	0.548	0.756	0.542	0.583	0.786	1.000						
0.595	0.601	0.524	0.679	0.661	0.601	0.565	1.000					
0.702	0.565	0.702	0.524	0.625	0.744	0.792	0.583	1.000				
0.714	0.554	0.726	0.583	0.601	0.732	0.744	0.571	0.750	1.000			
0.643	0.601	0.643	0.619	0.625	0.696	0.685	0.690	0.726	0.679	1.000		
0.685	0.571	0.720	0.577	0.619	0.714	0.798	0.577	0.744	0.685	0.720	1.000	
0.673	0.571	0.732	0.542	0.548	0.726	0.726	0.565	0.673	0.708	0.696	0.798	1.000

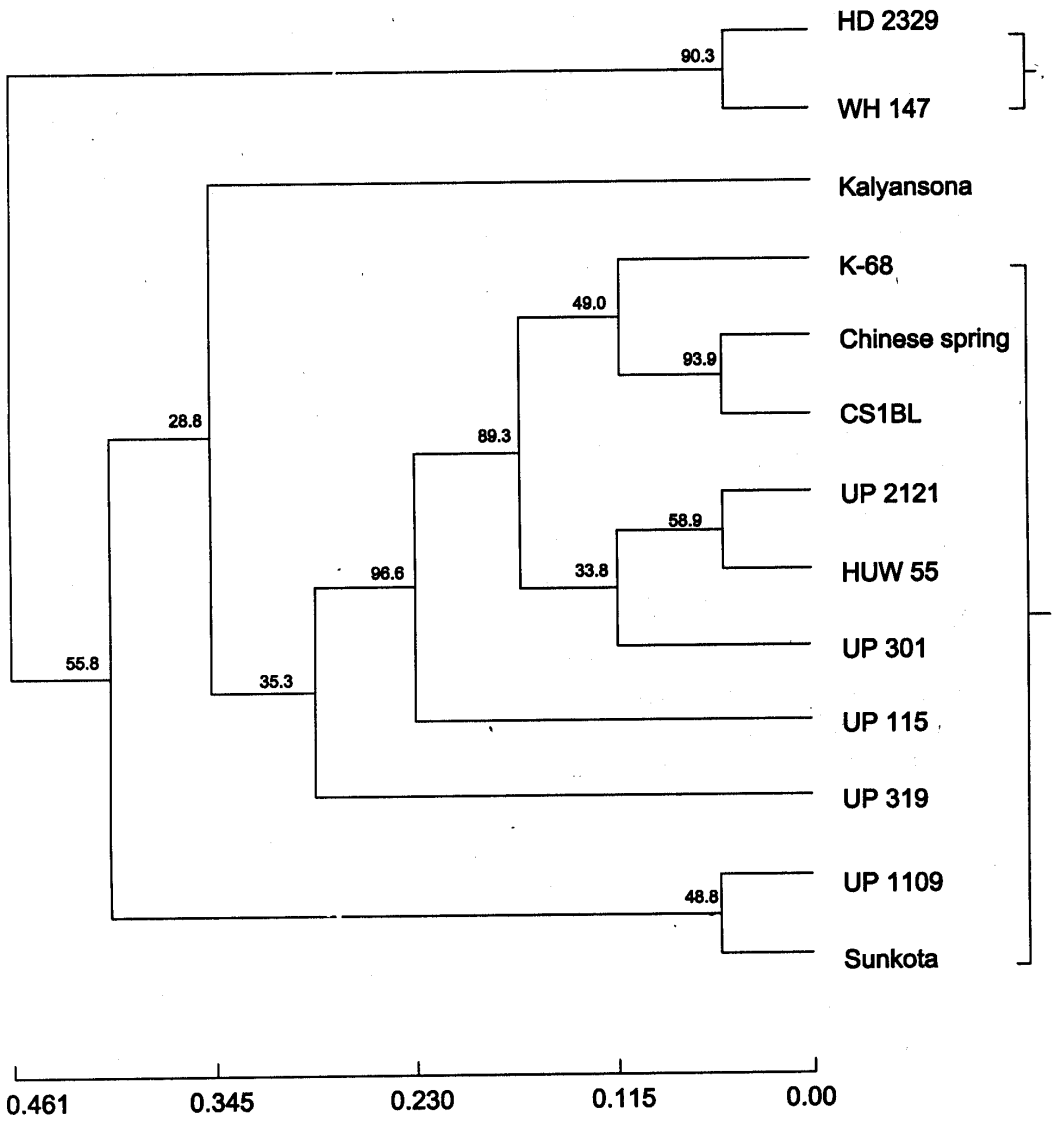
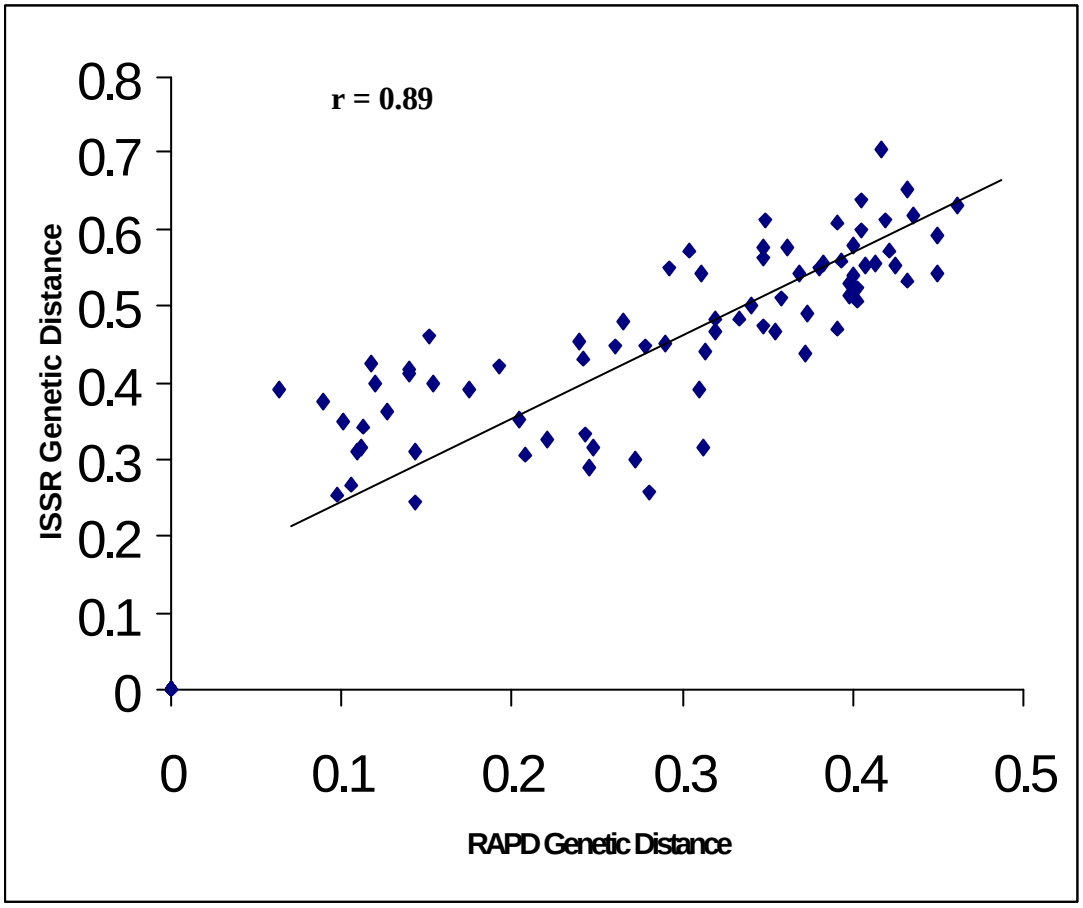


Figure 3.8 : Combined dendrogram obtained using ISSR data and RAPD data for 13 wheat genotypes.

Figure3.9 : Positive correlation matrices between distance matrices of RAPD and ISSR markers.



CHAPTER 4

DISCUSSION

COMPARISON OF RAPD AND ISSR MARKERS IN DIVERSITY ASSESSMENT OF 13 HEXAPLOID WHEAT GENOTYPES

CHAPTER 4

DISCUSSION

Comparison of RAPD and ISSR markers in diversity assessment of 13 wheat genotypes

Indian wheats have optimum characters needed for chapati-making but they are not ideal for industrial based food products like bread, biscuits, cakes, noodles and also lack international standards for export purposes. The end use quality of wheat is greatly influenced by grain protein quality and protein content of wheat which also plays a major role in human nutrition. In order to get a good quality bread, high loaf volume, uniform and small grains in the crumb, smooth crust and hardness of the grain, are some of the essential characteristics that should be present in a wheat variety. Therefore, the selection of a good genotype which can confer above characteristics is a recent breeding objective in India. In recent years, molecular markers have received considerable attention and have been extensively used for genetic diversity analysis, phylogenetic and evolutionary studies and mapping and tagging genes of agronomic importance in different plant species. In my thesis, I have made an attempt to compare the utility of two marker systems viz. ISSRs and RAPDs in studying genetic diversity of 13 hexaploid Indian wheat varieties, having either the presence of 5+10 gluten subunit alleles (supposed to be correlated with good bread making) or of 17+18 subunit alleles (supposed to be associated with poor bread making quality) and their pedigree information.

The performance of these markers was evaluated using various parameters such as percentage of polymorphism, average band informativeness, resolving power, marker index, average heterozygosity and clusters formed in the dendrogram. The comparison of these parameters done using two marker systems is summarized in Table 4.1.

1. **Percentage of polymorphic markers :** The 11 ISSR primers yielded average 7.91 bands per primer while the 12 RAPD primers amplified average 7.23 bands per primer. The average number of polymorphic bands per primer was higher in case of RAPDs (5.5) as compared to that in ISSRs (4.26).

2. **Average band informativeness :** The range of I_b values of both marker systems as seen in Table 4.1, shows that though the lowest value (0.182) for a specific primer is same for both the marker systems, the highest value (0.908) displayed by ISSR markers is higher than the RAPD markers (0.832). The average value of band informativeness considering all RAPD primers together and ISSR markers together, was higher (0.541) for RAPD than that for (0.531) ISSR.

3. **Resolving powers :** It is a characteristic of a primer which reflects overall suitability of a marker system for the purpose of identification, as it is related to the number of accessions distinguished by that primer (Prevost *et al*, 1999; Gilbert *et al*, 1999). Rp value for both RAPD and ISSR polymorphic primers was calculated and it was observed that ISSR primers had greater (2.78) Rp than that of RAPD primers (2.30).

4. **Marker Index (MI) :** MI is the parameter specifically used in comparing the utility of two or more marker systems (Powell *et al*, 1996). ISSR markers obtained a higher value (3.5) of MI than RAPD primers (2.8), indicating greater potential of ISSR markers vis a vis RAPD markers in the evaluation of 13 hexaploid wheat varieties.

5. **Average heterozygosity (Hav) :** In the present study, ISSRs could detect greater heterozygosity (0.78) than RAPDs (0.51), taking into consideration all the scored bands. On the other hand, when only polymorphic bands are considered, the values obtained for ISSR and RAPD primers are 0.81 and 0.62 respectively. The average heterozygosity of a marker system indicates its ability to detect heterozygosity in the germplasm.

6. Dendrogram analysis : The composition of clusters obtained using ISSR markers alone (Fig.3.3), RAPD markers alone (Fig 3.6), and using both ISSR and RAPD markers together (Fig 3.8) has revealed similar groupings in many cases with few variations. I also attempted to correlate the pedigree information of the 13 wheat genotypes under study with the dendrograms generated using molecular markers. Following are some of such examples.

A cluster has been formed with HD2329 and WH147 genotypes in all the three dendrograms with very high bootstrap values (90.3% - 99.9%). HD 2329 and WH147 have five parents common in them namely, PJ SIB/ P14/ KT 54B/3/4 (Table 2.1.), thus suggesting pedigree as the basis of clustering of these two genotypes. Among the remaining genotypes, Kalyansona has out grouped from rest of the genotypes based on ISSR and RAPD data alone with high bootstrap values. However, surprisingly in the combined dendrogram it has not shown clustering with high bootstrap values, Kalyansona has only one parental genotype PJ SIB common to HD 2329 and WH147.

Another cluster is formed by UP1109 and Sunkota in all the three dendrograms, but with a low bootstrap values (44.3% to 68.3%). These two genotypes do not contain any common parent between them. UP1109 has one parent UP262 common with HD2329 but has not clustered in any of the three dendrograms. Another wheat genotype UP115 though has five common parents (PJ SIB/P14/ KT 54B /4/3) with WH147 and HD2329 has not grouped with them in any of the dendrogram. The genotype UP2121 has two parents Kalyansona and UP301 which have also been included in the analysis, however they have not shown clustering in all the three dendrograms. Two other genotypes UP301 and UP 319 have one parent in common i.e. SN64 but they also do not cluster together. Interestingly, genotypes Chinese Spring and CS1BL have not clustered based on ISSR or RAPD data alone, however, in the combined dendrogram, they have clustered together. Thus, it is difficult to carry out comparison in the ISSR and RAPD marker systems based on the dendrograms obtained.

Table 4.1: Comparison of polymorphism detected by RPAD and ISSR markers in 13 wheat varieties.

Marker Type and polymorphic primers	Average no of bands /primer	Average no of polymorphic bands/primer	Av/I_b range	Rp	Hav	Hav(p)	MI	AV/I_b
RAPD (12)	7.23	5.56	0.182 to 0.832	2.30	0.51	0.62	2.8	0.541
ISSR (11)	7.91	4.26	0.182 to 0.908	2.78	0.78	0.81	3.5	0.531

Further exploitation of ISSR and RAPD markers in wheat

The microsatellites or inter simple sequence repeat (ISSR) markers and randomly amplified polymorphic DNA (RAPD) markers have proved to be the most polymorphic markers in wheat and hence are highly useful markers for various applications in wheat (Roder *et al*, 1998; Nagaoka and Ogihara 1997). Apart from using them in diversity analysis ISSR markers have been shown to be associated with various agronomically important traits namely, dwarfing and vernalization response (Korzan *et al*, 1997); leaf rust resistance (Feuillet *et al*, 1995, 1997;), kernel hardness (Sourdille *t et al*, 1997), cadmium uptake (Penner *et al*, 1995) preharvest sprouting tolerance (Roy *et al*, 1998), protein content (Prasad *et al*, 1999; Mesfin*t al*, 1999; Blanco *et al*, 1996), resistance to common bunt (Demeke *et al*, 1996), powdery mildew resistance (Qi *et ai*, 1996), kernel traits (Campbell *et al*, 1999) flour viscosity (Udall *et al*, 1999) and seed size in wheat (AmmiRaju *et al*, 2001). RAPD markers have also shown to be associated with various traits such as the *Aegilops speltoides* leaf rust resistance gene *Lr* 28 in wheat (Naik *et al,* 1998), various traits contributing to kernel hardness in bread wheat (Galande *et al* 2001) and cadmium intake in durum wheat (Penner *et al* 1995). These markers can, thus be used for selection of linked traits of agronomic importance which would increase the efficiency and precision of breeding. Molecular breeding will, thus soon become a common agricultural practice in coming year.

CHAPTER 5

SUMMARY

CHAPTER 5

Summary Wheat (*Triticum spp)* has a large genome size and is one of the most important cereals in the world. It is cultivated in many countries and harvested throughout the year. India presently holds the second position in the total world wheat production and contributes around 12%(1995-1996 year data) to the world's wheat basket. The area under wheat cultivation in India is around 24.5mha and production is of the order of 68.7 mt in 1997(Anonymous 1997). The projected demand for wheat by the year 2020 A.D. is 95 to 109 mt (depending upon the projection levels) to provide food security to the increasing population of India which by the year 2020 A.D will be 1.25 billion. Within the country, growing urbanization and changing food habits have led to the growth of baking industries and consequent increase in the demand for good breadmaking quality wheat. As a result, wheat cultivars with better processing characteristics and good breadmaking quality are the major end use requirements for the baking industry. Thus, apart from food security, the end use quality is also a major concern of wheat breeding in India. With the development of highly informative and technically simple PCR based markers like RAPDs, ISSRs, AFLPs and microsatellites, the diversity studies and marker based analysis of agronomically important traits in wheat have gained momentum. In the present study, I have included 12 genotypes which are being used in NIL development program supported by the Department of Biotechnology, New Delhi, India and possess gluten subunits 5+10 and 17+18 and a genotype HD2329 with no such alleles to understand the genetic diversity among these 13 hexaploid wheat genotypes using the PCR based ISSR and RAPD markers.

Review of literature In this chapter, an overall view about the wheat as a crop, world wheat production, classification and evolution is incorporated. Wheat seed storage proteins, their classification and importance in wheat

end product industries is also summerized. The second part in the review comprises different types of DNA markers with various applications in relation to plant breeding, especially their utility in wheat for various traits.

Materials and methods All the protocols used in the thesis have been described in detail in this chapter.

Results

Identification and evaluation of ISSR and RAPD primers for diversity estimates in 13 wheat genotypes The analysis was carried out using ISSR and RAPD primers with the 13 wheat genotypes selected for the present study. In order to compare utility of these two markers in the diversity study, the performance of these markers was evaluated using various parameters such as percentage polymorphism, average band informativeness , resolving power, marker index, average heterozygosity and clusters formed in the dendrogram. In order to study the correlation between the two marker systems, the distance matrices were compared using correlation analysis. The two matrices showed a positive correlation.

Dendrogram analysis ISSR and RAPD markers were individually and together used to cluster the 13 genotypes in different groups using UPGMA algorithm. In ISSR analysis, two main clusters were obtained with two subclusters. Similarly, in RAPD dendrogram and in combined dendrogram two main clusters with two and three subcluters respectively were obtained. In all the three dendrograms, genotypes HD2329 and WH147 grouped together sharing five parents common in them. Although, genotypes UP1109 and Sunkota formed the cluster in all the three dendrograms, these two genotypes do not contain any common parent between them. The genotype UP115 has five common parents with WH147 and HD2329, but did not group with them in any of the dendrogram. Kalyansona with one parent common to HD2329 and

WH147, out grouped in the dendrograms based on ISSR and RAPD data individually but surprisingly in the combined dendrogram it formed a cluster with them with low boot strap values.

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