

**“Studies on genome recovery percentage for *opaque2*
introgressed in backcross population for marker
assisted selection of quality protein maize”**

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By

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CERTIFICATE- I

This is to certify that the thesis entitled, “**Studies on Genome Recovery Percentage for *opaque2* Introgressed in Backcross Population for Marker Assisted Selection of Quality Protein Maize**” submitted in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE IN AGRICULTURE (Molecular Biology and Biotechnology)** of Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur is a record of the bonafide research work carried out by **Mr. VISHWA VIJAY THAKUR** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instruction.

No part of the thesis has been submitted for any other degree or diploma (Certificate awarded etc.) or has been published / published part has been fully acknowledged. All the assistance and help received during the course of the investigation has been acknowledged by him.

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CERTIFICATE-II

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List of Abbreviations

<u>S. No.</u>	<u>Abbreviations</u>	<u>Stands for</u>
1	%	Percent
2	μl	Micro Litre
3	μM	Micro Molar
4	AFLP	Amplified Fragment length Polymorphism
5	BC	Backcross
6	bp	Base pair
7	CAPS	Cleavage Amplification Polymorphism
8	CTAB	Cetyl Trimethyl Ammonium Bromide
9	ddW	Double Distilled Water
10	EDTA	Ethylene Diamine Tetra Acetate
11	EMS	Ethyl Methane Sulphonate
12	EST	Expressed Sequence Tag
13	ISSR	Inter Simple Sequence Repeats
14	M	Molar
15	mA	Milli Ampere
16	mg	Milli Gram
17	ml	Milli Litre
18	mM	Milli Molar
19	NARS	National Agricultural Research Systems
20	ng	Nano Gram
21	O.D	Optical density
22	PCR	Polymerase Chain Reaction
23	QTL	Quantitative Trait Loci
24	QPM	Quality Protein Maize
25	RAPD	Random Amplified Polymorphic DNA
26	RFLP	Random Fragment Length Polymorphism
27	rpm	Revolutions per minute
28	SCAR	Sequence Characterized Amplified Region
29	SLS	Sodium Lauryl Sulphate
30	SNP	Single Nucleotide Polymorphism
31	SSR	Simple Sequence Repeats
32	STMS	Sequence Tagged Microsatellite Sites
33	TAE	Tris base Acetic acid Glacial EDTA

INTRODUCTION

Maize (*Zea mays* L.), commonly known as corn, is a grain domesticated by indigenous people in Mesoamerica in prehistoric times. Maize is the world's third most important cereal crop after rice and wheat and used for human consumption, livestock feed as well as a raw material for several industrial uses. The USA has the largest harvested area of corn and contributes one fifth of the world corn harvested area whereas, China, Brazil, Mexico, India and Indonesia contribute to nearly 60 per cent of the world harvested area. USA has 30 percent share in total global maize production, whereas other major maize producers i.e. China, Brazil and India account 15, 4 and 3, per cent respectively.

In India, nearly 90 per cent of maize is produced during *kharif* season, 7-8 per cent during *rabi* season and remaining 1- 2 per cent during summer season. Among the major corn producing states, Karnataka is on the top with 18.94 per cent contribution to the total Indian maize production followed by Andhra Pradesh (17.42%), Maharashtra (10.65%), Rajasthan (7.74%), Tamil Nadu (7.27%), Bihar (7.04%), Uttar Pradesh (6.07%), Madhya Pradesh (5.97%), Gujarat (3.57%) and others (3.80%). In Madhya Pradesh i.e. Neemuch, Mandsaur, Ratlam, Indore, Ujjain, Khandwa and Chhindwara districts are the main maize producing areas.

Maize contributes to 15 – 56 per cent of the total daily calories to people in about 25 developing countries in the world (Prasanna *et al.*, 2001). Maize provides high amount of starch in its various food forms i.e. flour corn, popcorn, flint corn, sweet corn, waxy corn, amylomaize, pod corn and stripped maize. Maize supplies up to 60 per cent of the daily human protein (Musila *et al.*, 2010; Sofi *et al.*, 2009). However protein content of normal maize lacks two important amino acids, namely lysine and tryptophan that are nutritionally essential for humans (Krinavek *et al.*, 2007; Sofi *et al.*, 2009; Azevedo and Arruda, 2010; Mbuya *et al.*, 2011). Normal maize like other cereal proteins contains on an average of only about 2 per centage lysine which is less than one-half of the amount required for human nutrition as recommended by food and Agricultural Organization of the United Nations

(Prasanna *et al.*, 2001; Akande and Lamidi, 2006). The normal maize without supplementation with high protein, lysine and tryptophan sources in diets lead to malnutrition in routine consumers. Therefore, improved maize varieties with high protein content especially rich in lysine and tryptophan are required to cater quality protein to human and animal as food and feed.

There is a great hope from the effort to improve Quality Protein Maize (QPM) as a feedstuff since it was developed as Opapue-2 (*o2*) mutant maize in 1964 which contained protein about twice the levels of lysine and tryptophan as compared to normal maize. Isolation and characterization of *opaque2* gene showed that it is responsible for a transcriptional factor which regulates the expression of *zein* genes and a gene encoding ribosomal inactivating protein (Schmidt *et al.*, 1990; Lohmer *et al.*, 1991; Bass *et al.*, 1992). The *o2* mutation reduces zein content by one-half and enhances the synthesis of a number of non-*zein* proteins including lysine and tryptophan (Damerval and Devienne, 1993; Habben *et al.*, 1993). However, the *o2* maize was unpopular amongst maize breeders because of low yields, soft kernel texture and susceptibility to ear rot and stored grain pest (Crow and Kermicle, 2002). Several research efforts made at different research institutes, all over the world particularly at CIMMYT, Mexico has led to the development of quality protein maize (QPM) through the introgression of *o2* modifiers. India is one of the first countries to release QPM composites and hybrids. Over the years, Vivekananda Institute of Hill Agriculture, Almora, India has developed short duration quality protein maize inbreds (Gupta, 2009). Those inbreed along with other available inbreds needs to be studied for their effective use in breeding program.

The development of modern plant breeding techniques has greatly facilitated the wider use of a wealth of diversity from many sources including landraces. Molecular markers have been applied for quantification of genetic diversity, genotype identification, gene mapping, association mapping, marker assisted selection (MAS) and genome recovery percentage. Assessment of the extent of genetic variation in maize has been carried out based on restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) (Moeller and Schaal, 1999) and simple sequence

repeats (SSR) (Yao *et al.*, 2008; Babu *et al.*, 2009; Kamalesh *et al.*, 2009). SSR markers are preferred due to their potential advantages of reliability, reproducibility, discriminating power, standardization and cost effectiveness over other marker technologies (Smith *et al.*, 1997). The SSR markers have also been found to be useful for mapping of *opaque2* modifiers and introgression of trait of interest using MAS (Dragana *et al.*, 2009).

Breeding work has led the concept of quality protein maize (CIMMYT, 2001; Prasanna *et al.*, 2001; Vasal, 2006). Breeding for QPM genotypes becomes straight forward with the help of *o2* locus-specific simple sequence repeats (SSRs). Three SSR *phi57*, *phi112* and *umc1066* (www.agron.missouri.edu) are located within the *o2* locus, and are useful for the QPM breeders to discard non-QPM genotypes at seedling stage and to select heterozygous and homozygous genotypes for *o2* during marker-assisted breeding (Babu *et al.*, 2005, Gupta *et al.*, 2009). The introgression of *o2* allele reported using gene-specific markers to the parental lines of a released Indian maize hybrid through marker-assisted backcross breeding followed by reconstitution of the original hybrid with enhanced lysine and tryptophan content (Gupta *et al.*, 2013). Recurrent parent genome recovery analysis is necessary for the selection of better lines for QPM development. It is always to complete the selection process at early stages *i.e* BC₁F₁ however to overcome the genetic drag in BC₂F₁. The present study was carried out with the following objectives:

Objective

1. Foreground selection of plants using *opaque2* specific markers.
2. To compare genome recovery percentage between BC₂F₁ and BC₃F₁ using SSR markers.

REVIEW OF LITERATURE

Maize – An important crop

Maize (*Zea mays* L.) ranks third to rice and wheat as the most important cereal crop, mainly used as staple food and animal feeds in most of the developing countries (Akande and Lamidi, 2006; Olakojo *et al.*, 2007; Mboya, 2011). It is cultivated on an area of 147 million hectares with production of 692 million tons in the world. India alone accounts for 7.27 mha area with a production of 15.86 mt where maize follows wheat and rice (DES, 2012). Several million people, particularly in the developing countries, derive their protein and calorie requirements from maize (Mbuya *et al.*, 2011). With its high content of carbohydrates, fats, proteins, some essential minerals and vitamins, maize acquired a well deserved reputation as a nutritive-cereal. Maize grain accounts for about 15 to 56% of the total daily calories in diets of people in about 25 developing countries, particularly in Latin America and Africa, where animal protein is expensive and consequently, unavailable to a vast sector of the population (CIMMYT 2001, Vasal 2000). It is used as human food, chemical and medicines, biofuel and fodder etc. Its various subspecies are flour, pop, dent, sweet flint, waxy, pod, striped and amylo-maize.

Malnutrition- A Major Problem

Hunger and malnutrition are prevalent in the economically deprived areas especially the rural poor. They were also prone to a number of essential amino acid deficiencies and health problems such as kwashiorkor, underweight, weakened resistance to infections and impaired intellectual development (Mbuya *et al.*, 2010). Unfortunately, complementary protein sources such as animal products or grain legumes required are beyond the means of the rural poor. International Maize and Wheat Improvement Center (CIMMYT) in 1964 containing about twice the levels of lysine and tryptophan and 10% higher grain yield than the most modern varieties of tropical maize, brought a great hope in the effort of its improvement as human and animal nutrition (Akande and Lamidi, 2006; Olakojo *et al.*, 2007). High level of these two amino acids not only enhance manufacture of complete proteins in the body, but also offers 90% of the nutritional value of skim milk, thereby

alleviating malnutrition (Olakojo *et al.*, 2007 Upadhyay *et al.*, 2009). Quality Protein Maize (QPM) combining the enhanced levels of lysine and tryptophan with high grain yield and desirable agronomic traits could reduce food insecurity and malnutrition in West and Central Africa (Bello *et al.*, 2012).

Quality protein maize (QPM)

The nutritional value of maize is rather poor, due in part to the low biological value of its protein (Truswell and Brock, 1962). Maize protein is particularly low in the limiting amino acids tryptophan and lysine. To optimize the use of maize protein, it should therefore be consumed with complementary protein sources, high in the limiting amino acids. These complementary sources, usually animal products or legumes, tend to be expensive or particularly for beans, take a lot of time and fuel to cook.

To improve its nutritional value, breeders have been looking for ways to increase lysine and tryptophan content in maize (Vasal *et al.*, 1980). The strategy was to provide this maize to populations with diets of poor protein quality, in regions with high maize production and consumption, to reduce malnutrition and improve growth and health, particularly in young children (Lauderdale, 2000).

Maize is an important cereal crop but has the lacuna of poor nutritional quality. This lacuna was overcome in the mid of 1960s, when mutants with an increased lysine content containing *opaque2* (*o2*) was discovered. The *opaque2* gene was first mentioned by Singleton and Jones in the 1920s as reported by Emerson *et al.* (1935), but the nutritional significance of this mutant was first pointed out 40 years later by Mertz *et al.* (1964) and led to the development of high quality protein maize (QPM).

Quality protein maize (QPM) is controlled by *opaque-2* gene, which is a recessive gene that almost doubles lysine and tryptophan content (Mertz *et al.*, 1964). Unfortunately, the gene also carried negative side effects, in particular reduced yields and susceptible to storage pests (Prasanna *et al.*, 2001). Continued efforts by breeders at the International Maize and Wheat Improvement Centre (CIMMYT), however, yielded varieties with the *opaque-2* gene resulting improvement in protein quality, but without the negative effects

on agronomy and storage (Vasal, 2000). These varieties, called quality protein maize (QPM), have been developed for a wide range of agro-ecological zones and have been released in Latin America, Africa and Asia (Krivanek *et al.*, 2007). Quality Protein Maize (QPM) contains nearly twice as much usable protein as other maize (or corn) grown in the tropics and yields 10 per cent more grain than traditional varieties of maize.

Increase in lysine observed in *o2* mutants results from several phenomena (Moro *et al.*, 1996): (i) the reduction in the synthesis of zein storage proteins, which do not contain lysine, (ii) the enhancement of the synthesis of numerous other non-zein proteins, the most of which contains lysine, a general increase in the level of free amino-acids, including lysine. Studies conducted during the past 30 years revealed that the homozygous *o2* mutation causes a 70 per cent reduction in zein content and affects the content of a number of proteins and enzymes related to nitrogen and sugar metabolism in the maize endosperm (Habben *et al.*, 1993; Giroux *et al.*, 1994; Gallusci *et al.*, 1996).

Maize homozygous for the *o2* (recessive) mutation has substantially higher lysine and tryptophan content than maize that is either homozygous dominant (*O2O2*) or heterozygous (*O2o2*) for the *opaque2* locus (Crow and Kermicle, 2002). The increase of two amino-acids, lysine and tryptophan, in *o2* maize results from an alteration of the relative amounts of different fractions constituting the maize endosperm proteins. The zein or prolamin fractions are reduced substantially, thus increasing the relative part of other fractions. Since zein is practically devoid of lysine, while other fractions are richer in lysine, the overall amino acid profile exhibits an increase in lysine. No new proteins are formed and the composition of the different protein fractions remains unaffected (Vasal, 2001).

The improved nutritive value of QPM both as human food and as an animal feed especially for poultry has been widely demonstrated in many countries (Graham *et al.*, 1989 and 1990). The biological value of QPM protein is about 80% that of milk is about 90% and that of normal maize 45% (FAO 1992). Potential contribution of QPM to human nutrition, especially in

poor countries where maize is staple food, has been well documented (Bressani, 1991; Akuamo-Boateng, 2002; Morales-Guerra, 2002).

Many years of research work at CIMMYT led to the improvement of these varieties, now called Quality Protein Maize (QPM), making its yields competitive with conventional varieties of improved maize (Lauderdale, 2000).

The *o2* mutation also affects several agronomic traits including kernel characteristics and grain yield. The lower yield caused by an increased endosperm size that is soft chalky and dull usually results in more easily damageable kernels, and thus in a higher susceptibility to pests and/or fungal diseases (Vasal, 2001). Incorporation of *opaque2* into high yielding cultivars was not commercially successful, because of its numerous agronomic and processing problems – reduced grain yield, soft endosperm, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests. These drawbacks have been corrected in genetically improved, hard endosperm QPM (Vivek *et al.*, 2008).

Various methods have been explored in order to overcome the ‘defects’ of *o2* maize. The integration of the *o2* gene into a series of maize inbred lines (IL) by repetitive backcrosses resulted in a wide range of different endosperm textures of *o2* genotypes. While some IL produced completely soft kernels, others had particularly hard kernels; these differences appeared to be related to the modifier gene(s) for *opaque2* (Vasal, 1975, 1999).

High quality protein maize, in which the lysine content of the maize endosperm is nearly doubled, is controlled by the recessive allele *o2* of the *Opaque2* gene located on chromosome 7 (Babu *et al.*, 2005; Gibbon and Larkins, 2005; Lopes and Larkins, 1995; Lopes *et al.*, 1995). The high quality protein maize has opaque kernels, less vigorous plants, low yield than wild type maize and therefore was not preferred although its nutritional quality was higher. QPM is an improvement of standard *o2* maize with regard to endosperm and agronomic traits. It has vitreous endosperm, higher yield, is more vigorous than the standard *o2* counterpart (Vasal, 2001).

Conventionally, backcross (BC) and self-pollination are the main ways for breeding maize lines and QPM is until now bred by hybridizing inbred

QPM lines developed at International Maize and Wheat Improvement Center (CIMMYT, Mexico) by recurrent selection. In breeding programs, the development of inbred lines is the stage where the greatest amount of *de novo* genetic variation is present, created through genetic recombinations that give rise to novel alleles and new allelic variation (Lee and Tracy, 2009). The aim of recurrent selection is to recover the recurrent parent, where the new characteristic(s) of interest has been introgressed (Tracy, 2004). In each BC, 50% of the recurrent parent's alleles are recovered, which means that the development of elite QPM inbred lines by this process is very time and labor-intensive. Besides, unwanted alleles of the donor parent may be conserved due to linkage and epistasis phenomena.

The application of molecular markers as selection tools can facilitate the selection of interesting genotypes and so speed up the process. With the advent of DNA marker technology, several types of DNA markers like RAPD, ISSR, RFLP, etc are now available, but SSR markers are widely used in comparison to other types of markers because, co-dominant, species specific and highly polymorphic. Microsatellites (Litt and Luty, 1989) are tandemly arranged repeats of short DNA motifs (1-6 bp in length) that frequently exhibit variation in the number of repeats at a locus. Microsatellite (or simple sequence repeats-SSR), (Akkaya *et al.*, 1992) markers have been proposed for gene/QTL mapping in species. After the development of SSR markers, they have become the first choice for genotyping and genetic purity due to highly reproducible and transferability nature of the marker. SSR markers have been effectively used in cereals and various crops like wheat (Devos *et al.*, 1995), barley (Russell *et al.*, 1997), potato (Provan *et al.*, 1996), maize (Chin *et al.*, 1996), rice (McCouch *et al.*, 1997), sorghum (Taramino *et al.*, 1997) and soybean (Cregan *et al.*, 1999).

The microsatellite markers were also perfectly suited for use in map-based cloning, because identification of marker-anchored clones could be conveniently carried out by PCR using specific flanking primers (Beckmen and Soiler, 1986).

To facilitate the identification of plants that carry the recessive *o2* allele in QPM breeding programs, two specific simple sequence repeat (SSR) markers were developed: the dominant marker phi112, which identifies *O2* alleles in homozygous and heterozygous forms but does not amplify *o2* (Danson *et al.*, 2006; Vivek *et al.*, 2008), and the co-dominant marker phi057 (Vivek *et al.*, 2008), which makes it possible to identify and distinguish the three haplotypes, *o2o2*, *o2O2* and *O2O2*.

Maize breeding – Conventional breeding and application of MAS

For successful maize hybrid breeding programs, the development of elite inbred lines (IL) is crucial. IL development is the stage, where the highest amount of *de novo* genetic variation is present, created through recombination giving rise to novel alleles and new allelic variation (Lee and Tracy, 2009). Conventionally, backcrosses and self-pollinations combined to phenotypic selection are the main ways for developing IL. By conventional backcrossing, the achievement of new IL usually takes at least seven to nine generations. For ensuring global food security in the future, the requirement in new efficient breeding tools is high. Marker assisted selection is a potent strategy to develop QPM.

Maize was one of the major crop species for which a complete molecular map was developed (Helentjaris *et al.*, 1986). Since the first map publication, many other maps have been produced, and are now consolidated into a consensus map using a “bin” allocation to chromosome segments (Gardiner *et al.*, 1993). The Maize Mapping Project (MMP) at the University of Missouri has derived high-precision versions of the genetic and physical maps of maize (Cone *et al.*, 2002). An important component for achieving this was the mapping of more than 2600 simple sequence repeat (SSR) markers onto high-resolution recombinant inbred and F2 maps (Sharopova *et al.*, 2002) and application of them for physical map anchors to maize DNA BAC clones (Yim *et al.*, 2002).

Conventional breeding mainly focuses on phenotypic selection and may not be able to distinguish the influence of the environment or of other factors related to growing conditions from the genetic impact. This can be

improved by implementing molecular marker assisted selection (MAS) as a direct selection process, where the trait of interest is selected by the mean of a molecular marker that is either directly linked to the gene of interest or to the genome fragment impacting the expression of this trait. MAS allows to select according to the genotype, independently of environmental and epistatic effects. Meanwhile, MAS is approved as an applicable tool that does complement and substantially facilitate conventional breeding and selection techniques (Balding *et al.*, 2003; Ribaut and Hoisington, 1998).

Dreher *et al.* (2000) observed that the relative cost effectiveness of conventional breeding methods as compared to MAS for QPM line conversion differs depending on whether breeders can identify segregating materials by visual inspection.

Chin *et al.* (1996) reported that two simple sequence repeat (SSR) markers, phi057 and phi112 (Ku-vector Custom DNA Laboratory, Kasetsart University) were used to detect the *opaque-2* plants. These markers give amplification product of about 140–160 bp. The applicability of these two markers for *o2*, phi112 and phi057, was investigated on inbred lines used for QPM development. According to the Maize Genetics and Genomics Database (Lawrence *et al.*, 2008), three primers pairs, phi022, phi027 and phi061, were available for the selection of opaque gene. The phi057 marker was more closely linked to *opaque-2* gene than phi112 marker. Thus phi057 should be feasible to use as marker-assisted selection to detect the heterozygote line during the conversion of normal elite inbred line to QPM inbred line through backcross method without tryptophan analysis in endosperm of each backcross generation. In case of backcross designed for transferring recessive gene (*o2* gene), identification of heterozygote in the seedling stage prior to pollination aided in the rejection of non-target backcross progenies i.e. dominant homozygote (Ribaut and Hoisington, 1998; Babu *et al.*, 2005).

Jompuk *et al.* (2006) reported that the phi057 would be used as a tool for early indication of three possible genotypes of the *opaque-2* plants, i.e., *O2O2*, *O2o2* and *o2o2*, especially at seedling stage.

Given the high level of polymorphism found even between highly related lines, this consensus map allows one to rapidly identify possible markers for use in further saturating a region of interest, or for developing alternative (e.g., PCR-based) marker systems. Efforts are now under way to add thousands of SSR markers to the map, and maps composed of dense amplified fragment length polymorphisms (AFLP) loci are available for a number of maize populations. CIMMYT, therefore, uses SSR markers to test DNA extracted from leaf tissue of very young plants, allowing QPM plants to be identified early in the breeding cycle. SSR markers distinguish between homozygous recessive and heterozygous plants, reducing the length of period for the breeding process (Mugo and Hoisington, 2001).

Babu *et al.* (2005) reported that foreground selection for *opaque 2* was performed using three SSR markers viz. phi057, phi112 and umc1066. Background marker analysis was conducted with 88 SSR markers spanning the entire 10 chromosome of maize genome. Benchimol *et al.*, 2005 observed that more than 250 microsatellite markers were screened and used to genotype the BC₁ generation but only 53 were shown to be polymorphic and thus used for backcrossing in maize.

Prigge *et al.* (2011) used Marker assisted selection for introgression of one dominant target gene for genome model of maize (*Zea mays* L) with varying marker distances of 5-80 cM and population sizes of 30-250 plants across BC₁ to BC₃ generations.

Dragana *et al.* (2009) used SSR markers for mapping of *opaque2* modifiers and introgression of trait of interests using Marker Assisted Selection and found useful for recurrent parent genome recovery analysis.

Herzog and Frisch (2011) reported that when equally spaced (10cM) background markers were used in BC₂ generation for recurrent parent genome recovery then 92.3% recurrent parent genome were recovered.

Gupta *et al.* (2013) screened more than 250 SSR markers, for genotyping BC₂ generation and found 108 polymorphic markers for CM 212 x CML 180 and 126 for CM 145 x CML 170 and used for background selection in maize.

MATERIALS AND METHODS

The present study entitled “**Studies on genome recovery percentage for *opaque2* introgressed in backcross population for marker assisted selection of quality protein maize**” was conducted at Biotechnology Centre, Jawaharlal Nehru Agricultural University, Jabalpur, India

3.1. Materials

3.1.1. Source of biological materials

Two non QPM lines *i.e.* HKI1126 and HKI287 were selected for conversion to QPM lines using HKI161 and HKI193 as donors under DBT sponsored QPM Network project.

3.1.2. Molecular markers for foreground selection

Among 150 plants from each crosses 20 heterozygous plants from each BC₂F₁ and BC₃F₁ populations were selected by using 3 specific SSR markers namely phi112, phi057 and umc1066. The molecular markers were purchased from Integrated DNA Technology (IDT). The selected heterozygous 20 plants from each population were further used in the study for genome recovery analysis.

3.1.3. Collection of samples

Green young and healthy leaves from plants of each crosses were collected one month after sowing (before flowering) in the morning hours from the field during *Kharif* 2012 for extraction of genomic DNA. The collected samples were placed in cooling pads to transfer and then stored at –20°C.

3.1.4. Chemicals

The chemicals used in the investigation were of molecular biology grade or analytical grade of Sigma Chemicals Co. USA, Merck Pvt. Ltd. India. Molecular weight of bands was estimated using a wide range 100bp ladder (50-10000 bp) obtained from Fermentas Life Sciences.

3.2. Methods

3.2.1. Isolation of DNA

Principle: To isolate nuclear DNA, pure and intact nuclei were isolated from fresh plant material. Leaves are the most preferred material as it is easy to collect at any time, relatively clean compared to other tissues and has fewer amounts of inhibitors and pigments that may hamper the quality of DNA. The primary requirement for DNA isolation is to extract DNA from cells in solution. Physical grinding results in physical breakage of cells. Subsequently, addition of extraction buffer containing detergents like sodium lauryl sulfate (SLS) or cetyl-trimethyl ammonium bromide (CTAB) assists breakage of cell and nuclear membrane made up of lipids, which aids in cell lysis and all the components of cell are dispersed in the buffer. Tris-HCl in the buffer ensures that the pH of the solution is maintained around 8.0. EDTA forms complexes (chelates) with several kinds of metal ions. Divalent metal cations, such as Mg^{2+} and Ca^{2+} are the required cofactor by the majority of DNases. The DNA being extracted is protected from DNase degradation since the enzyme cannot utilize the complexed Mg^{2+} . NaCl being a salt, increases solubility of DNA in the buffer solution and also increases the osmoticity of the buffer and hence facilitates the process of cell lysis. Phenol:Chloroform or chloroform extractions remove proteins by denaturing them and they aggregate in the intermittent phase along with cell debris.

For DNA precipitation, isopropanol (0.6 to 1 volume) or ethanol (2 volumes) is used. The RNA is removed by RNase treatment. After precipitation, the pellet is washed with 70% ethanol for removing any salts retained after precipitation. The DNA is preferably dissolved in a molecular grade nuclease free water.

The DNA extraction buffer was prepared as per details given below. 2-Mercaptoethanol was added just prior to placement of DNA Extraction Buffer in water bath for incubation.

Table 3.2: Composition of DNA extraction buffer

S. No.	Chemicals	Final Concentration	Working Concentration
1	Tris HCl (pH 8.0), 1M	100mM	10ml
2	EDTA (pH 8.0), 0.5M	20 mM	4ml
3	β – Mercaptoethanol	0.2%	200 μ l
4	NaCl, 4M	1.4 M	35ml
5	CTAB	2%	2gm

- 1 M Tris·HCl (pH 8.0): 30.28g of Trizma base (Qualigens, FW = 121.1) was dissolved in 200 ml of distilled water. The pH was adjusted to 8.0 with concentrated HCl (about 10.5 ml). The solution was allowed to cool at room temperature (25°C) before final adjustment of the pH. The volume of the solution was adjusted to a total of 250ml with distilled water before autoclaving.
- 0.5M EDTA (pH 8.0): 46.53g of ethylenediaminetetraacetate (EDTA) (Qualigens, FW = 372.2) was added in to 200ml of distilled water. It was stirred vigorously with a magnetic stirrer. Maintain pH by adding NaOH. The total volume was adjusted to 250ml with distilled water before sterilization.
- 5M NaCl: 73.05g NaCl (Merck, FW = 58.44) was dissolved in 200ml pellets. The total volume was adjusted to 250ml with distilled water before sterilization.
- RNase (10 mg/ml): 100mg of RNase was dissolved in 10 ml of sterile ddW in a sterile 15ml centrifuge tube. The contents were boiled in water bath for 15 minutes to remove traces of DNases, which may be present along with RNase. The solution was allowed to cool down at room temperature and the volume was made up to 10ml using ddW as some volume may be lost in the process of boiling. It was dispensed into sterile 1.5ml microcentrifuge tubes and stored at –20°C.

DNA was isolated following the protocol of Saghai Maroof *et al.* (1984) with some modifications. The steps for DNA isolation are:

1. 0.25g of leaf sample was taken for DNA isolation and was homogenized in liquid nitrogen using a pre-chilled pestle and a mortar.
2. The fine powder was transferred to a 1.5ml eppendorf tube and 750 μ L of DNA extraction buffer (preheated to 65°C) was added and mixed thoroughly.
3. The samples were incubated in a water bath at 65°C for one hour. Meanwhile, the samples were frequently mixed every 10min to ensure complete and even extraction.
4. The samples then removed from water bath were cooled to room temperature.
5. The samples were then centrifuged for about 15min at 10000rpm at room temperature.
6. Supernatant so obtained was transferred to a fresh tube.
7. Then an equal (to supernatant) volume of chloroform : isoamyl alcohol (24:1) v/v was added and mixed thoroughly but gently for not less than 5 min.
8. The mixture was then centrifuged for about 15 min at 10000rpm at room temperature.
9. Supernatant so obtained was transferred to a fresh tube.
10. An equal (to supernatant) volume of isopropanol was added, mixed gently by inverting tubes and kept for 10 min undisturbed.
11. DNA was pelleted by centrifugation at 10000rpm for 15 min
12. The supernatant was now discarded and pellet was washed twice with 70% ethanol by centrifuging for 10 min at 10000rpm
13. The tubes were placed at room temperature and allowed the traces of ethanol to evaporate. Then it was dissolved in 400 μ l of nuclease free water for further use.

3.2.2. DNA purification

The purification of DNA was carried out in order to remove the impurities like RNA, proteins and polysaccharides. These are considered as inhibitors in DNA amplification during PCR. Following method was followed.

1. 5 μ l of RNase (5mg ml⁻¹) was added to DNA extract, mixed well and incubated at 37°C for 30 min.
2. This was followed by the addition of equal volumes of chloroform: isoamyl alcohol (24:1) v/v and mixed vigorously.
3. The above mixture was centrifuged at 14000rpm for 10 min.
4. Supernatant was transferred to a fresh micro centrifuge tube and 1/10 volume of 3M sodium acetate (pH 5.4) was added followed by further addition of two volumes of pre-chilled ethanol mixed gently for DNA precipitation.
5. The precipitated DNA was pelleted by centrifugation at 12000rpm for 5 min.
6. The pellet was dried at room temperature to completely remove ethanol and was then dissolved in 200 μ l of nuclease free water and stored at -20°C for further use.

3.2.3. Quantification of DNA

Isolated DNA was quantified by measuring the absorbance at 260nm and 280nm on a UV-spectrophotometer. 50 μ g/ml concentration of double stranded DNA showed an absorbance of 1 at 260nm. Concentration of DNA samples was calculated using following formula:

$$\frac{\text{O.D.}_{260 \text{ nm}} \times 50 \mu\text{g DNA/ml} \times \text{Dilution factor}}{1000}$$

The quantity, quality and integrity of isolated DNA were also checked by gel electrophoresis. 2 μ l of DNA sample was loaded from each line in 0.8% agarose gel and electrophoresis was done along with the λ Hind-III standard DNA at 60 volts for 90 min. The DNA was stained by ethidium bromide and

observed under UV-Trans-illuminator. The amount of fluorescence was proportional to the total mass of DNA.

3.2.4. Dilution of DNA

The quantified DNA was diluted according to the DNA quantity in each sample for PCR amplification in sterile double distilled water. Dilutions were carried out according to the following formula:-

$$\text{Dilution} = \frac{\text{Required concentration of DNA (ng/}\mu\text{l)} \times \text{Total volume required (}\mu\text{l)}}{\text{Available concentration of DNA (ng/}\mu\text{l)}}$$

3.2.5. Selection of molecular markers for foreground selection

For hybrid testing three specific molecular markers namely, phi057, phi112 and umc1066 were used for hybrid testing these molecular markers were selected from respective work.

Table 3.2.1: SSR markers selected for foreground selection

Markers	Sequences	References
phi112	5'-TGCCCTGCAGGTTACATTGAGT3' (F) 5'-AGGAGTACGCTTGGATGCTCTTC- 3' (R)	Danson <i>et al.</i> , 2006 Vivek <i>et al.</i> , 2008
umc1066	5'-ATGGAGCACGTCATCTCAATGG-3' (F) 5'-AGCAGCAGCAACGTCTATGACACT-3 (R)	Babu <i>et al.</i> , 2005
phi057	5'-CTCATCAGTGCCGTCGTCCAT-3' (F) 5'-CAGTCGCAAGAAACCGTTGCC-3' (R)	Vivek <i>et al.</i> , 2008

3.3. DNA analysis of simple sequence repeat

SSRs are co-dominant markers that target single loci in the genome and can easily and economically assayed by PCR. SSRs are one of the widely used markers for genotypic analysis, molecular mapping, cultivar identification, determination of hybridity, analysis of gene pool variation, and as diagnostic markers for traits of economic value in plants due to their species specificity and high re-productibility. During the present study SSR markers were used in PCR amplification for hybrid testing.

The PCR components required for SSR analysis are given in table 3.3.

Table 3.3: Components with their concentrations used for SSR PCR

S. No.	Components	Concentration
1.	PCR buffer	1 x
2.	MgCl ₂	1.5 mM
3.	dNTPs	100µM
4.	Primer	10pmol
5.	<i>Taq</i> Polymerase	0.5 unit
6.	DD H ₂ O	-
7.	DNA	15 ng

3.3.1. PCR conditions for SSR markers

PCR conditions were standardized with different parameters viz., initial denaturation, denaturation, annealing, extension and final extension using BIO-RAD PCR Machine. PCR thermal and reaction profile was optimized for amplification purpose by using primers of unique sequence with higher GC ratio at high stringency. The optimized conditions are presents in the table.

3.3.1.

Table 3.3.1: Optimum profile of the conditions used in PCR Amplification for SSR

Steps	Temperature	Duration	Cycles	Activity
1.	94 ⁰ C	4 min	1	Denaturation
2.	94 ⁰ C	30 sec	35	Denaturation
3.	60 ⁰ C	30 sec		Annealing
4.	72 ⁰ C	30sec		Elongation
5.	72 ⁰ C	5 min	1	Final elongation
6.	4 ⁰ C	∞		Storage

3.3.2. Electrophoretic analysis of SSR-PCR products

The PCR products for SSR were resolved by electrophoresis on 4% (w/v) agarose gels stained with ethidium bromide (5µg/ml) and photographed

under UV-light in 'Syngene' multigenious bio-imaging system. Molecular weight of bands was estimated using a wide range 100bp ladder (50-10000 bp) obtained from Fermentas Life Sciences.

3.4. Background selection

100 polymorphic SSR primers were selected for analysis of genome recovery of recurrent parent in BC₂F₁ and BC₃F₁ plants.

3.4.1. Sources of SSR markers

Source of all the 100 primers which were used for BC₂F₁ and BC₃F₁ 1126 and 287 obtained from www.maizegdb.org.

3.4.2. PCR amplification of SSR markers

PCR amplification of markers was done as same manner as previously described.

3.4.3. Electrophoretic analysis of SSR-PCR products

The PCR products for SSR were resolved by electrophoresis on 4% (w/v) agarose gels stained with ethidium bromide (5ug/ml) and photographed under UV-light in 'Syngene' multigenious bio-imaging system. Molecular weight of bands was estimated using a wide range 100bp ladder (50-10000 bp) obtained from Fermentas Life Sciences.

3.4.4. Data scoring

For the data scoring P1 was male parent considered as B and P2 was female parent considered as A on the basis of samples having both the allele was considered as heterozygous and other apart from this considered as specific allele.

3.4. List of sequences of SSR markers and their chromosome number

S.No	Primer	Forward Sequences 5'-3'	Reverse Sequences 5'-3'	Ch. No.
1	umc1685	TAGTTTGAGGGATCAAGAACCACC	GCTCAAAGGCAAGGCAGTATTTTA	1
2	umc1292	GAAGTGGGGAACATGGTTAATGTC	TCACGGTTCAGACAGATACAGCTC	1
3	umc1305	CAATTTGATGCTGGGATTTCAGATA	TGCTGTGTGTTTGATGCTTTAGTTT	1
4	umc1363	TGTTTAAGTGTTGGCAGAAAGCAA	TCTCCCTCCCCTGTACATGAATTA	1
5	umc1484	GCGTACAGAACAGAGCAGTTACGA	ACTGAAGCTGCCTGCCTTCTATTT	1
6	umc1566	ATCTCGTCTACCTAACCCACCCTC	CAGGTGAAGAATCTGGTGAGGTC	1
7	umc1106	CATCCAGTCATCGGTAATACGACA	AAGTGTGCTTCTTTTTCCCTTC	1
8	umc1177	CGTGTACCGCTCCTCTATAGTCGT	AAGTGGCCGAATTCATCCTTTATT	1
9	umc1222	CTCAGAACAGAAGCCATCAAAAGC	CGTCTTCGTGAGAGACATCCTGT	1
10	umc1269	TATATTAGAGGCACCTCCCTCCGT	AGCTGCTTCAGCGACTTTGG	1
11	bnlg1083	ACAGTCTGTTGGGGAACAGG	CAACGCTGGTTTGTTCGTTTA	1
12	bnlg1007	GATGCAATAAAGGTTGCCGT	ATGTGCTGTGCCTGCCTC	1
13	bnlg1614	CCAACCCACCCAGAGGAGA	AGCGGGCGAGATCTTCAT	1
14	bnlg1953	CCTCGGAGCTCGATTTACAC	AACATTTAACCGCCGTCATC	1
15	mmc0103	CTGCTGCTGGTACTACTACGT	CTCACCAGTGTGAATGTTTTT	1
16	umc1200	GTTGCGATGCGTCTATTAATCTCC	TAGGAACACAACAGGTGCAGAGAG	1
17	umc1467	GTGCCAACTCAGGTAATCTCCAAT	AAGGCTAGTGGCTATAGTGGAGGG	1
18	umc1568	AAGTCCAGCCAAGTTCATCAAAGA	ACTGTAACTAACTGGGTGTGCCC	1
19	umc1711	TAGGTTACTCCACAAACAATGGCA	TTGTGTGGTGGCAAGTTTATATGC	1
20	umc1976	TGCCGAGGCTTCTAGTAGACCAA	CGCTATATCTATCCCGCAGCAAC	1

21	umc1880	CGCATCCGCCTAGCTCTATACTAA	GCATCGAACAGCTGAGATTGTAGA	
22	umc2145	GTAGTAGAGGCAGGGCTTCCAAC	TAGGAGTTCGTCAGTCAACGAGC	1
23	umc2171	ACATAATCCCTCGGTACAGGACAA	GCCCTTGTGTGTCTATTTAGGGTG	1
24	phi96100	AGGAGGACCCCAACTCCTG	TTGCACGAGCCATCGTAT	2
25	phi098	GAGATCACCGGCTAGTTAGAGGA	GTATGGTTGGGTACCCGTCTTTCTA	2
26	mmc0111	TACTGGGGATTAGAGCAGAAG	AATCTATGTGTGAACAGCAGC	2
27	umc1542	TAAAGCTATGATGGCACTTGCAGA	CATATTTGCCTTTGCCCTTTTGTA	2
28	umc1756	ATCTCAGGTA CTCTGCCTACGGG	AACAGAGGGTAGCTTGTGGCCT	2
29	umc1824	ATCGTGCTTAAGCGGTTATAGGAAT	TGCACATGCTTTGTATAAGATGCC	2
30	umc1934	TCCGGGACTAGTTGCTTGGTAGTA	TAGTGGTATCGTGATTCATGGGC	2
31	umc1961	GGATGAGAATTGAGAAAGAGCCAA	CATGTCCATACACAGTCATACACTGC	2
32	umc1980	CCTAGCATTTACGCGGAGGAG	CTGGTCCGAGGTCTCGTCTTC	2
33	mmc0231	GAGCGACTGCGAGACGG	AGATCGCGCCACCGCTC	2
34	phi109642	CTCTCTTTCCTTCCGACTTTCC	GAGCGAGCGAGAGAGATCG	2
35	umc2007	GTGCATATGCGCACGGAAGT	AGTAGTTACACAACGCAACACGAGG	2
36	bnlg1909	CCTGACCCTGTTCTGAAAA	GTGTGTCTGGAGCTGTTCGA	2
37	mmc0401	CTCAGCTGCACTCCACA	CCTTCCTTCTTCTCCAGC	2
38	umc1581	GGCACGAGATTTGAAACAGTTAAGA	ACTCCATTACCTTTGTCTTGGCAG	2
39	umc1746	ACACGAGCATCCTACATCCTCCTA	ACCTTGCCTGTCCTTCTTTCTCTT	3
40	umc1780	CTGTCCCCAGGTTGCTGTAGTAGT	CATGATGTACCCGCAACAAATG	3

41	umc1793	TGCACACCCTTTATTGAATCATCA	CGTATAAGCTTTTTGGGGTCCTCT	3
42	umc1931	TAGCCTGAGTCGTTACAATGTGCT	TAGGTCCACAAAATAAACAGGCAG	3
43	phi104127	CTTTGCTGCTGCTTCCTACG	AACCAGTGACGTACACAAAGCA	3
44	umc1394	CCCGAGTCAGAAAAACATTCACTT	CCTAACCTGAAGAAGGGAGGTCAT	3
45	bnlg1523	GAGCACAGCTAGGCAAAAGG	CTCGCACGCTCTCTCTTCTT	3
46	bnlg1886	TCTCTCTCACATGCACGCC	TTTGATTTGGGGAACCAGAG	3
47	umc2259	GAGGAGGAGAGGGACAGGGAAG	GGCTCGACTTCGAGGACACC	3
48	bnlg1447	GAGAGGAGAGGCTGAGCTGA	TCCTCCCACTGAATTTCCAC	3
49	umc2369	GATCCTCATCAAGACCAGCAGAGT	TTCGTCTGATGAAAGGTTCAAGAG	3
50	umc2261	ACCAGTCCACCAGGAACATCTTTA	GTCGAAAGACGGAGAGAGGAAAAG	3
51	bnlg2047	CATGCATCCATCCTTTTCCT	ATCCATCGGCAACTACAAGC	3
52	umc1655	ACGCAAGTACAAAATTACTGCGGA	ATTAACGGCATGCTTGTAGCCTAA	3
53	bnlg1022	GTGTTGTGATCCACTCCCT	GCAAAGATCTGTGAGGGGAC	3
54	bnlg1113	GCTCCCAACTTTTCAGAATCG	TTCTCCCTTTTTTTTCGCAGA	3
55	bnlg1904	AGGAGCATGCACTTGGTTCT	ACTCAACTGATGGCCGATCT	3
56	umc2002	TGACCTCAACTCAGAATGCTGTTG	CACAAAATCCTCGAGTTCTTGATTG	3
57	bnlg372	TTCACATGCCATCCTCCTATAT	TATCCCTCTCTGATCACGTTGG	4
58	bnlg1241	ATTCTTGACATCCATCCGGT	TGTTGTTTCACTCAGCGTCC	4
59	umc1228	TCCTCAAGGACCTGCTCCAC	ACCTATACAGACGGAGACGGGG	4
60	umc1757	TTTTCTGCAGGGATAACATTTGTG	ATAGGAGGTGAGGTGAGGAGGAAG	4

61	umc1758	CTTCCTCCTCACCTCACCTCCTAT	GGTAGCCAATCCTTCCTTCCTATG	4
62	umc1759	GTGAGGAGAGGAGACGGAGAGAG	GAAGCTCCTGTGGAACGTGTG	4
63	umc2410	AGCTACTGTGGACTGTGGACTGTG	CAAGATCACCCAAGGTACTGTGC	4
64	bnlg1126	GAGATCGAAGGTCATGGCAC	ATGGTTCCTGGTTCAGATGG	4
65	umc1902	CCTCATCTCTCATGGGATGGATA	TTCAGCATGACATATCATAACAGTAGCA	4
66	umc1926	ATGCCAGCATTCTTCATCCTACAT	TGAGGCTTGGTCCACTAAAGAAAG	4
67	umc2211	CTGACAGCCATGCGCATCTT	TGTATGCTCAGATTATTCTTCGAGGC	4
68	umc1117	AATTCTAGTCCTGGGTTCGGAAGTC	CGTGGCCGTGGAGTCTACTACT	4
69	umc1963	CTCGTTCGAGGGGATGTACAAG	CTTGCACTGGCACAGAGACG	4
70	mmc0471	TTAGCACATTTGAAGAGTTTTG	TTTCCTTCACGTTTCTCTGT	4
71	bnlg1741	TGCCAAATTGCCAACCTAAT	TCAGACGGTGCATCTGAAAC	4
72	bnlg1168	CGATAAGTTAGGGACGGCTG	CGCTCACTCCCTCTCTCTCT	4
73	bnlg1265	GGTTGTCCGTAAAGGCAAGA	TGTGAAGGCCAGACAGTCAG	4
74	umc2061	GTCTGGAGAACTCCCTACCCATTC	TAGCTTGAGAGACCGGAACAGC	4
75	bnlg667	CGTGGATGTAAGGGGGCGCGCT	GGCCGCTGCTCAACACAGGCAG	4
76	bnlg1006	GACCAGCGTGTTGATCCC	GGAGACCCCGACTCTCTCTC	5
77	umc2022	TTAGTCTAACCGAGTCCAACCAAGTG	ACCAGCAGACGGAGAGCTTG	5
78	mmc0151	AAACCATGCATCCAACRAATG	AGACCCAGAGATGATTTAGG	5
79	umc1901	CCTATAATAAGTTCACGCAAGTGGC	AAGTACCATTGCACATTTCTGGTG	5
80	umc1679	CACTGCTAAGCTGCTCCCTGTT	TGCTAACTAACCCTGACCCTCTCA	5

81	umc1097	CTCGTCAACGTCAACCCAAGTAAG	CTGTTAGATGTGCGACAACAGAGC	5
82	bnlg1382	TTTTCTTTCAAAAATATTCAGAAGC	GCAGGATTTTCATCGGTTGTT	5
83	umc2179	TTAACACCTCAAACAGTGATTGCC	GTAGAGCAGCACTCTGGGTTTCTC	5
84	umc1894	TTTCTCATGACATTGCAAGCATCT	TATGCAAAAGGTGAGCCTGCTTAT	5
85	umc1766	ACAAGAAGGAATCGAGAGCAAATG	CTTCGGGATGGAGTCGTAGTTC	5
86	umc1781	CAAAAGGTGCAACTGTAATTGCCT	ATGAGGCGAAGCCAAGAAAAAGT	5
87	bnlg1660	AACCAAGGTTCTTGGAGGCT	ACCATTGTATTTTCCTAGAGAATCG	5
88	umc2400	TTTGGTGAAAGTGAAACCAAAGGT	CTAGCTAGCTTCCTTCCTCCTCG	5
89	mmc0261	TAGTAATGCTTAACGAAAGCAA	CGCTTACGCTTACTACTAGCTT	5
90	umc1373	ATGATGATGACGACGACGAAGAT	CGTCAGGTCTGTTGCATATCTGTT	5
91	umc1935	TGTGCTGTGTGTAATATGGTCGTG	TAATCAGGAGGAGAATTCGGTCAA	5
92	umc1850	ACACATAAAAATGCTCGTTTTGGG	GTGCTGTCGTCTGATGAGGAACTA	5
93	umc1557	CTAGTTCTCTCAAAGCGCCG	ACAATAGGTAGGTCCCCTGCTTT	5
94	bnlg1660	AACCAAGGTTCTTGGAGGCT	ACCATTGTATTTTCCTAGAGAATCG	5
95	umc2068	CCGCTCCTTCTCCTCCTCATC	GGAACCTCTCGAGCGTGAGC	6
96	bnlg1597	GATAATCTCGTCTCGCCAGG	CATAAAAGGATGCCGACGAC	6
97	bnlg1043	TTTGCTCTAAGGTCCCCATG	CATACCCACATCCCGGATAA	6
98	bnlg1600	CGATCAGTGCGTGGAGAGTA	TAGGCATGCATTGTCCATTG	6
99	umc2208	ACCAAATCAAACGAAGAAAAGTGG	CTTGATCGACATTTTCGTTATGCTG	6
100	umc2309	CTGTGTTTTGTGTATTAGCGCCAG	GTCGAAATTCCTGACACAAAAAGG	6

101	bnlg1188	ATTAAGTAAATGACTATCTAGTGTTTGTCTG	AATAGCAAGGCATCAGCCAT	6
102	bnlg1641	ATCGTAACTCGATGGTTCGC	TACGCTATTCAAAGCGGTCC	6
103	umc2312	GCTGATGAGCTCGCCATTCT	ACCCCATCTCAGTCTCACTCACTT	6
104	bnlg1371	TTGCCGATAAGAACCAAACA	ACGACCGGTGTGGTTACATT	6
105	bnlg1047	ATGGAGATGGAGGAGAGAGAGA	GATGCGGCGATGGCTAA	6
106	bnlg1165	CGCTTGCATCATCTCAAGAA	TTCAAGTTTAGCCACCCACC	6
107	umc1444	ACTAGCACACACCCCCTACCACTA	TGTGCTTGTGAGAAGGATTTGTTC	6
108	umc1517	TAACACTCGGAACCTTCCTTCTC	GATGGGAAAATGTGTGGAATTTAT	6
109	phi077	GAGAAGAGGATCAGGTTCGTTCCA	CGCGTTGTACATCTTGCCTGCTT	6
110	umc2327	GATCGATGCTAATGTGAAGAGCCT	CCAGCAGCATATGTACACAAATCA	7
111	umc2057	CCATCCGTAACACTTAAGGACGAC	TCATGAAGCTCCAGCAGTATCTGT	7
112	mmc0162	CGAAATCAAATCAAAACCATA	CCTCCTATCGCTAGCTCGATC	7
113	bnlg1003	GACCCTCAACCGACCAGTAA	ATGGCCTTTTTTAAAGGAGGG	7
114	bnlg1808	CTTTTCTCTTCTAGTAATGAACAGTCA	GCATGATCGAACGAAGGC	7
115	bnlg1094	GTGAAGAACGATGACGCAGA	CAGCAACGCTCTCACATTGT	7
116	bnlg2233	AGGGACAGCGAGATAAAGCA	GTCCCTGACAGGCGGACC	7
117	umc1927	AAGACAAATGGTTCTCTTCTATTGTTG	CGCCTAAAAAGCAAATGAGAGTTA	7
118	umc1929	TCCAAGCATTATGCATCACAACT	GCATGAATTCAACACAGAACCACT	7
119	umc1932	ACTTGCGCATATAAGTCGGGATG	AGGATTAACGATGAGGAGCCTCTG	7
120	umc1787	TGTAGTCCATGGAGCTCTTCTCCT	CTTTTTCACACTCTGCACCTCCTC	7

121	umc1881	TATACACTACTCAAGCTGCGCGTC	GGCACTCAATCTCGGTGAACAT	7
122	umc1585	CGGCCTATGTAACAATCCCTAGC	AAGGGAAAGAATAATCCAACCGTC	7
123	umc1433	TTGTCAGACAGAACCCACACATTT	TTTTTGGCTTCTTTTGTGTGGAT	7
124	bnlg1252	GATTTTGCTTGAAGCCGAAG	GCTTTGCAGCACTGTCGTAG	8
125	phi420701	GATGTTTCAAAACCACCCAGA	ATGGCACGAATAGCAACAGG	8
126	bnlg1194	GCGTTATTAAGGCAAGCTGC	ACGTGAAGCAGAGGATCCAT	8
127	bnlg1073	TCGATCTAAGTATTGTAAACGTACG	GTATTTGGAGGCGCCATAGA	8
128	umc1075	GAGAGATGACAGACACATCCTTGG	ACATTTATGATACCGGGAGTTGGA	8
129	umc1139	TTTGTAATATGGCGCTCGAAACT	GAAGACGCCTCCAAGATGGATAC	8
130	umc1327	AGGGTTTTGCTCTTGAATCTCTC	GAGGAAGGAGGAGGTCGTATCGT	8
131	umc1786	ACCGTGACTTCCTCCTCATAACTG	CATTTTTCGCATTTAGGAAATCCA	8
132	umc1974	ACAAGGAGACCCTCCTCAGCTAGT	GTAAGCTGTGGCCATACTACCACC	8
133	umc1304	CATGCAGCTCTCCAAATTAAATCC	GCCAACTAGAACTACTGCTGCTCC	8
134	umc1360	GCTAGTTGAGTTCGACACCAGGTT	TGACTGTGACTGTGACTATGACCG	8
135	umc1790	GAGAGGGAGGAGCAAGGAAATAGA	GAGAAGAGTTCTCCATGGAACGAG	8
136	umc1913	AAACAACATCCATGTGGCTGACC	CGTTCAGTACAATTTGGCTCAGTG	8
137	umc2075	CCTGGTATCTTGATGAGCTGGATT	AGAAACATGTCCGGTTCTTCAGAC	8
138	umc1121	AAAACGACATGTCATCGTCTTCAA	GGGGTCAGGTACAGGGCTAGTAGT	8
139	umc1933	CGGAAAGACATGAAGCACACTATG	CACGGAGAAAGTGGGTCTATATGC	8
140	umc1916	AGCTGGCCCTGTTCTTCACAG	CAACAATTCTGGCCCACCAAG	8

141	bnlg1272	ACCGAAGATGAGGTGTGACA	TCAGTGCAAGGGCAATTTAG	9
142	bnlg2122	TCATCTGGCAAAACCTAGCC	CTTGCCAACTTGAGGACATG	9
143	umc1170	TGGGTGCTAAAACGTAACAACAAA	GAGGACGAAGCAGAAATCCTACC	9
144	umc2128	CACGGGGAATTCTCATATAGCAAG	GAGTGTGTACGTAGGTATGCCCCG	9
145	umc2130	CGAGTTACCTTTGGCACTAGCACT	ATCATGACGTATCTTTCCGAGAGC	9
146	umc2219	GTCCCAAACAACAAGTGAAGGAAG	GTGTTGTCTGTTGGCTGTTGCT	9
147	umc1191	AAGTCATTGCCCAAAGTGTTGC	ACTCATCACCCCTCCAGAGTGTC	9
148	umc1586	TAGGAGATGAGCTCGTCGGATAAG	GAGGATGAGGAGGATGGTAATGGT	9
149	umc1921	AGATCTCCCTTTTTGTAGATGGGG	AGGGTGCACTTTTATTGAGGAGTG	9
150	mmc0051	ACGACTCTATCCCTGCCAACT	TCTGGTTGTGAAAGCTATCCT	9
151	bnlg1626	TTAAATCCAGAGTGTCCCCG	TTCTGGATGGTTGCACACAT	9
152	umc2120	ACGTCAAGTAGTGAATCCTGCTCC	ATTCTCCGCGGAAGAGACAAG	9
153	umc2121	AAAAGTGGGCGCTAGTACAAGATG	GTGGTGTTCTCCTTTTGATACGCT	9
154	bnlg1714	CATCATGGAGGCATATGTCG	ACACATTTAGACCCACCCCA	9
155	bnlg1012	GAGTGAGCGTGCGGAGTC	AACAGGCCAACTCCTCCTC	9
156	umc2095	GTGCTGGTTGTGCATGGTATATTG	CCATGCATGACGCAAGTCTCT	9
157	umc1789	ACCTCTCCTTTTTCTCGCCTT	GTCAGAGAAGAGGCCGGGTC	9
158	umc2399	TGGTCACCACCATCTCTTTTACA	AGGTACTGACGACGTGCTGATTTT	10
159	umc1380	CTGCTGATGTCTGGAAGAACCCT	AGCATCATGCCAGCAGGTTTT	10
160	umc1318	ACTTCGTCTAGTGTCCCTCCGTT	TGCCAGATTAAAAGCAACACAAGA	10

161	umc1319	TGAGAGCCACCTTCTTGAGCTACT	TTCCTTGAAGGCGAAGGTAGGTAT	10
162	umc2069	ACAACCTCCTCCACGACCAAAC	GTAGAGGTCCCACTTGTTCCCAAT	10
163	umc1239	ATCAACACACCTTTTCGATTTCTGG	CGGTGATTAGTCGATGAAGAGTGA	10
164	umc1739	CAAAAAGCTTTAGTTTTTACAGGCAGA	GCGTGTAATTTGCTCCTTCTTCTT	10
165	umc1336	GTACAAATGATAAGCAAGGGGCAG	CTCTGTTTTTGAAGAAGCTTTTGG	10
166	umc2180	ATCAGCATCGATAGCGAAGAAAGA	ATTGCTACTAGGGTTGTTGTTGCC	10
167	umc2349	TACAACAAGAAACGAAAACGGCTT	CCTATTGCTGCGCATACCTAACTAA	10
168	umc1827	CGAAGTCAGGGAGTCCAAGAGAG	CCACCTCACAGGTGTTCTACGAC	10
169	umc2021	AAACTCAAGCTCGGAATGTACTGC	CGATACTGATCTACTTCACGCTGG	10
170	umc1569	CGTAAGTACAGTACACCAATGGGC	ACACCGACTACAAGCCTCTCAACT	10
171	umc2017	TCCCTCTTGAGTGTTTATCACAAA	GTTTCCATGGGCAGGTGTAT	10
172	umc1381	CTCTAGCTACGAGCCTACACGAGCA	CCGTCGAGTCAACTAGAGAAAGGA	10
173	umc1678	GGCTCGACTTCGAGGACACC	GAGGAGGAGAGGGACAGGGAAG	10

RESULT

Quality protein maize (QPM) is a special type of maize which has exactly the same qualities as normal maize in grain texture, taste and colour but possess almost double the levels of lysine and tryptophan, high yield and tolerance to biotic and abiotic stresses. QPM is a new type of maize genotype in which *opaque2* has been incorporated along with associated modifiers.

The objective of the present investigation was to evaluate genome recovery percentage between BC₂F₁ and BC₃F₁ generations of HKI1126 (Non-QPM) x HKI161 (QPM) and HKI287 (Non-QPM) x HKI193-1 (QPM) and selection of progenies using SSR markers. The result of the study is being presented under the following headings:

4.1. Hybrid testing

Two non QPM inbreeds namely HKI287 and HKI1126, the parents of a promising hybrid identified for Central India were undertaken for conversion to QPM. On the basis of parental polymorphism for markers linked to *o2* gene the HKI287 was crossed with HKI193 (QPM donor) and HKI1126 was crossed with HKI 161 (QPM donor). Hybrid testing was done using three *Opaque2* gene specific markers namely phi057, phi112 and umc1066. Among 100 BC₁F₁ plants, 20 heterozygous plants were sown for raising BC₂F₁ generation and among 120 BC₂F₁ plants 20 heterozygous lines were sown for raising BC₃F₁.

For selection of heterozygous plants, the genomic DNA from each plant was isolated from leaf tissue using CTAB method described by Saghai Maroof *et al.* (1984) with slight modifications. Genomic DNA was dissolved in nuclease free water and final concentration was made to 25 ng/ µl used for further SSR amplification. The PCR products for SSR were resolved by electrophoresis on 4 per cent (w/v) agarose gels stained with ethidium bromide (5µg/ml) and photographed under UV light in “Syngene” Multigenious Bioimaging System.

Table 4.1. Polymorphism between parents of cross combinations

SN	Primer	HKI287 x HKI193-1	HKI1126 x HKI161	Bin no.
1	umc1685	Monomorphic	Polymorphic	1.01
2	umc1292	Polymorphic	Polymorphic	1.01
3	umc1305	Polymorphic	Monomorphic	1.00
4	umc1363	Monomorphic	Polymorphic	1.01
5	umc1484	Polymorphic	Monomorphic	1.01
6	umc1566	Polymorphic	Polymorphic	1.01
7	umc1106	Monomorphic	Monomorphic	1.00
8	umc1177	Monomorphic	Polymorphic	1.01
9	umc1222	Polymorphic	Monomorphic	1.01-1.02
10	umc1269	Polymorphic	Monomorphic	1.01
11	bnlg1083	Polymorphic	Polymorphic	1.02
12	bnlg1007	Polymorphic	Monomorphic	1.02
13	bnlg1614	Polymorphic	Monomorphic	1.02
14	bnlg1953	Polymorphic	Polymorphic	1.02
15	mmc0103	Monomorphic	Monomorphic	1.02-1.04
16	umc1200	Polymorphic	Polymorphic	1.02
17	umc1467	Monomorphic	Monomorphic	1.02
18	umc1568	Monomorphic	Polymorphic	1.02
19	umc1711	Monomorphic	Polymorphic	1.02
20	umc1976	Monomorphic	Polymorphic	1.02
21	umc1880	Monomorphic	Monomorphic	1.03
22	umc2145	Monomorphic	Monomorphic	1.03
23	umc2171	Monomorphic	Polymorphic	1.03-1.04
24	bnlg1886	Polymorphic	Monomorphic	1.05
25	phi96100	Polymorphic	Monomorphic	2.01
26	phi098	Monomorphic	Polymorphic	2.02
27	mmc0111	Polymorphic	Polymorphic	2.02
28	umc1542	Polymorphic	Monomorphic	2.02
29	umc1756	Polymorphic	Polymorphic	2.02
30	umc1824	Polymorphic	Monomorphic	2.02
31	umc1934	Monomorphic	Polymorphic	2.02
32	umc1961	Polymorphic	Monomorphic	2.02
33	umc1980	Polymorphic	Polymorphic	2.02
34	mmc0231	Polymorphic	Monomorphic	2.03
35	phi109642	Monomorphic	Polymorphic	2.03-2.04
36	umc2007	Polymorphic	Polymorphic	2.04
37	bnlg1909	Monomorphic	Polymorphic	2.05
38	mmc0401	Polymorphic	Polymorphic	2.05
39	umc1581	Polymorphic	Monomorphic	2.05
40	umc1746	Monomorphic	Polymorphic	3.00
41	umc1780	Monomorphic	Monomorphic	3.01
42	umc1793	Monomorphic	Monomorphic	3.00
43	umc1931	Monomorphic	Polymorphic	3.00
44	phi104127	Monomorphic	Polymorphic	3.01
45	umc1394	Polymorphic	Monomorphic	3.01

46	bnlg1523	Monomorphic	Polymorphic	3.03
47	umc2259	Polymorphic	Monomorphic	3.02-3.03
48	bnlg1447	Polymorphic	Monomorphic	3.03
49	umc2369	Polymorphic	Polymorphic	3.03
50	umc2261	Polymorphic	Polymorphic	3.04
51	bnlg2047	Polymorphic	Monomorphic	3.04
52	umc1655	Monomorphic	Polymorphic	3.04
53	bnlg1022	Monomorphic	Monomorphic	3.04
54	bnlg1113	Polymorphic	Monomorphic	3.04
55	bnlg1904	Polymorphic	Monomorphic	3.04
56	umc2002	Polymorphic	Polymorphic	3.04
57	bnlg372	Monomorphic	Monomorphic	4.00
58	bnlg1241	Polymorphic	Polymorphic	4.01
59	umc1228	Monomorphic	Monomorphic	4.01
60	umc1757	Monomorphic	Polymorphic	4.01
61	umc1758	Polymorphic	Polymorphic	4.01-4.02
62	umc1759	Monomorphic	Polymorphic	4.01
63	umc2410	Polymorphic	Polymorphic	4.02
64	bnlg1126	Monomorphic	Polymorphic	4.03
65	umc1902	Monomorphic	Polymorphic	4.03
66	umc1926	Monomorphic	Monomorphic	4.03
67	umc2211	Polymorphic	Polymorphic	4.03
68	umc1117	Polymorphic	Monomorphic	4.04
69	umc1963	Monomorphic	Polymorphic	4.04
70	mmc0471	Polymorphic	Monomorphic	4.04
71	bnlg1741	Monomorphic	Monomorphic	4.06
72	bnlg1168	Polymorphic	Monomorphic	4.05
73	bnlg1265	Polymorphic	Monomorphic	4.05
74	umc2061	Polymorphic	Polymorphic	4.05
75	bnlg667	Monomorphic	Monomorphic	4.05
76	bnlg1006	Polymorphic	Polymorphic	5.00
77	umc2022	Monomorphic	Monomorphic	5.00
78	mmc0151	Polymorphic	Monomorphic	5.00
79	umc1901	Polymorphic	Monomorphic	5.00
80	umc1679	Polymorphic	Monomorphic	5.01
81	umc1097	Polymorphic	Polymorphic	5.00
82	bnlg1382	Polymorphic	Polymorphic	5.01
83	umc2179	Polymorphic	Polymorphic	5.01
84	umc1894	Monomorphic	Polymorphic	5.01-5.02
85	umc1766	Polymorphic	Polymorphic	5.01
86	umc1781	Monomorphic	Polymorphic	5.01
87	bnlg1660	Monomorphic	Polymorphic	5.03
88	umc2400	Polymorphic	Polymorphic	5.04
89	mmc0261	Monomorphic	Monomorphic	5.02-5.03
90	umc1373	Monomorphic	Monomorphic	5.03
91	umc1935	Polymorphic	Polymorphic	5.03
92	umc1850	Polymorphic	Monomorphic	5.03
93	umc1557	Polymorphic	Polymorphic	5.03

94	bnlg1660	Polymorphic	Polymorphic	5.03
95	umc2068	Monomorphic	Polymorphic	6.00
96	bnlg1597b	Polymorphic	Polymorphic	6.00-6.01
97	bnlg1043	Polymorphic	Polymorphic	6.00
98	bnlg1600	Polymorphic	Monomorphic	6.00
99	umc2208	Monomorphic	Monomorphic	6.00
100	umc2309	Polymorphic	Monomorphic	6.00
101	bnlg1188	Polymorphic	Monomorphic	6.01
102	bnlg1641	Monomorphic	Polymorphic	6.01
103	umc2312	Polymorphic	Polymorphic	6.01
104	bnlg1371	Monomorphic	Polymorphic	6.01
105	bnlg1047	Monomorphic	Monomorphic	6.01
106	bnlg1165	Polymorphic	Monomorphic	6.01
107	umc1444	Monomorphic	Monomorphic	6.01
108	umc1517	Polymorphic	Polymorphic	6.01
109	phi077	Polymorphic	Polymorphic	6.01
110	umc2327	Polymorphic	Polymorphic	7.02
111	umc2057	Polymorphic	Monomorphic	7.02
112	mmc0162	Monomorphic	Polymorphic	7.02
113	bnlg1003	Polymorphic	Monomorphic	7.02
114	bnlg1808	Polymorphic	Monomorphic	7.02
115	bnlg1094	Polymorphic	Monomorphic	7.02
116	bnlg2233	Polymorphic	Monomorphic	7.02
117	umc1927	Monomorphic	Polymorphic	7.02
118	umc1929	Monomorphic	Polymorphic	7.02
119	umc1932	Polymorphic	Polymorphic	7.02
120	umc1787	Monomorphic	Monomorphic	7.02
121	umc1881	Polymorphic	Monomorphic	7.02
122	umc1585	Polymorphic	Polymorphic	7.02
123	umc1433	Polymorphic	Polymorphic	7.02
124	bnlg1252	Monomorphic	Polymorphic	8.00-8.01
125	phi420701	Monomorphic	Polymorphic	8.00
126	bnlg1194	Polymorphic	Polymorphic	8.02
127	bnlg1073	Polymorphic	Monomorphic	8.01
128	umc1075	Polymorphic	Polymorphic	8.01
129	umc1139	Monomorphic	Polymorphic	8.01
130	umc1327	Monomorphic	Polymorphic	8.01
131	umc1786	Monomorphic	Polymorphic	8.01
132	umc1974	Monomorphic	Polymorphic	8.02
133	umc1304	Monomorphic	Polymorphic	8.02
134	umc1360	Polymorphic	Monomorphic	8.02-8.03
135	umc1790	Polymorphic	Monomorphic	8.02
136	umc1913	Polymorphic	Monomorphic	8.02
137	umc2075	Monomorphic	Polymorphic	8.03
138	umc1121	Polymorphic	Polymorphic	8.21
139	umc1933	Monomorphic	Polymorphic	8.08
140	umc1916	Monomorphic	Polymorphic	8.09
141	bnlg1272	Polymorphic	Monomorphic	9.00

142	bnlg2122	Polymorphic	Polymorphic	9.01
143	umc1170	Monomorphic	Polymorphic	9.02
144	umc2128	Polymorphic	Monomorphic	9.02-9.03
145	umc2130	Polymorphic	Polymorphic	9.02
146	umc2219	Monomorphic	Polymorphic	9.02
147	umc1191	Monomorphic	Polymorphic	9.03
148	umc1586	Polymorphic	Polymorphic	9.03
149	umc1921	Polymorphic	Polymorphic	9.03
150	mmc0051	Polymorphic	Polymorphic	9.03-9.04
151	bnlg1626	Polymorphic	Polymorphic	9.03-9.04
152	umc2120	Monomorphic	Monomorphic	9.04
153	umc2121	Polymorphic	Monomorphic	9.04
154	bnlg1714	Polymorphic	Monomorphic	9.04
155	bnlg1012	Monomorphic	Polymorphic	9.04
156	umc2095	Monomorphic	Polymorphic	9.05
157	umc1789	Polymorphic	Polymorphic	9.06
158	umc2399	Polymorphic	Monomorphic	10.00
159	umc1380	Polymorphic	Polymorphic	10.00
160	umc1318	Monomorphic	Polymorphic	10.01
161	umc1319	Polymorphic	Polymorphic	10.01
162	umc2069	Monomorphic	Polymorphic	10.02
163	umc1239	Polymorphic	Polymorphic	10.03
164	umc1739	Polymorphic	Monomorphic	10.03
165	umc1336	Polymorphic	Monomorphic	10.03
166	umc2180	Monomorphic	Monomorphic	10.03-10.04
167	umc2349	Monomorphic	Monomorphic	10.03-10.04
168	umc1827	Monomorphic	Polymorphic	10.04
169	umc2021	Polymorphic	Polymorphic	10.07
170	umc1569	Monomorphic	Polymorphic	10.07
171	umc2017	Monomorphic	Polymorphic	10.03
172	umc1381	Polymorphic	Monomorphic	10.03
173	umc1678	Polymorphic	Monomorphic	10.04

4.2. Foreground selection in BC₂F₁ and BC₃F₁ generations

In case of BC₂F₁ and BC₃F₁ HKI1126, 100 plants for each were screened for foreground selection out of which 36 plants in case of BC₂F₁ and 42 plants in BC₃F₁ were found heterozygous whereas, in case of BC₂F₁ and BC₃F₁ HKI287, 150 plants for each were screened and 24 plants in case of BC₂F₁ and 32 plants in BC₃F₁ were found heterozygous producing products corresponding to both allele (*o2* and *O2*), while other produced a single band corresponding to other homozygous (*O2O2*) condition. Foreground selection in BC₂F₁ and BC₃F₁ generations were done using 3 specific markers viz. phi057, phi112 and umc1066. umc1066 can easily discriminate between the *opaque2* alleles of backcrossed populations of HKI1126 and the normal (*O2*)

allele. Only locus phi057 exhibited co dominant polymorphism between HKI287 x HKI 193-1 genotypes thus used in BC₂F₁ and BC₃F₁287.

4.3. Background selection in BC₂F₁ and BC₃F₁ generation HKI1126

A total of 173 SSR markers (Source: www.maizegdb.org) were screened for parental polymorphism, 101 markers were found polymorphic between HKI 287 and HKI 193-1 while 102 markers between HKI1126 and HKI161 (Table: 4.3). Polymorphic SSR markers covering the all chromosomes were used for analysis of genome recovery percentage (Table: 4.3). Besides their use for polymorphic profiling, markers were also chosen on basis of their map position (Bin no.) and distribution along the chromosome.

In case of HKI1126 populations, 102 SSR primers were screened and amplified only the parental alleles designated as A and B where A signifies recipient female parent allele and B signify donor male parent allele. In case of HKI287 populations, 101 SSR primers were amplified parental alleles. Twenty plants from each population were selected for background selection using 100 polymorphic SSR markers screened.

4.4 Genome recovery in BC₂F₁ and BC₃F₁ generation

Genome recovery of recurrent parent in BC₂F₁ and BC₃F₁ generation was calculated and dendrograms were generated by polymorphic SSR markers using the software Power Marker.

4.4.1 HKI1126 populations

4.4.1.1 BC₂F₁ HKI1126 Population

Among twenty plants of BC₂F₁ 1126 population the genome recovery percentage was ranged from 0.902-0.972. The highest genome recovery (0.972) shown in plant no. 7 followed by plant no 2 and 12 with genome recovery 0.961, while lowest recovery was in plants no. 16 (0.902) followed by plant no. 17 with genome recovery 0.904.

4.4.1.2 BC₃F₁ HKI1126 Population

From the table 4.4.1 genome recovery of BC₃F₁ 1126 plants can be grouped under three categories viz. High genome recovery (0.987-0.996),

Medium genome recovery (0.980-0.985) and Low genome recovery (0.971-0.979).

Table: 4.4.1 Genome recovery in HKI1126 populations

Plant Number	BC₂F₁ HKI1126 x (HKI161)	BC₃F₁ HKI1126 x (HKI161)
1	0.931	0.978
2	0.961	0.983
3	0.943	0.974
4	0.911	0.989
5	0.935	0.982
6	0.921	0.985
7	0.972	0.991
8	0.921	0.984
9	0.912	0.980
10	0.943	0.991
11	0.931	0.979
12	0.961	0.983
13	0.952	0.992
14	0.941	0.987
15	0.932	0.986
16	0.902	0.988
17	0.904	0.979
18	0.921	0.996
19	0.912	0.983
20	0.914	0.971
Average	0.931	0.984

From the above mentioned categorization, in case of BC₃F₁ 1126, 6 plants come under the range of high genome recovery, 7 plants come under the range of medium recovery and 7 plants come under the range of low recovery. Highest genome recovery was found in plant no. 18 (0.996) and lowest recovery was found in plant no. 20 (0.971). The average genome recovery was 0.984.

4.4.2 HKI287 populations

4.4.2.2 BC₂F₁ HKI287 Population

Recurrent parent genome recovery was calculated among BC₂F₁ HKI287 plants. The highest genome recovery was 0.976 in plants no. 6 followed by 0.971 in plant no. 13 and lowest genome recovery was 0.891 in plant no. 17.

4.4.2.2 BC₃F₁ HKI287 Population:

From the table 4.4.2 in case of BC₃F₁ HKI287 plants genome recovery of recurrent parent can be grouped under three categorization viz. High genome recovery (0.986 – 0.993), Medium genome recovery (0.981 – 0.985) and Low genome recovery (0.968 – 0.980).

Table: 4.4.2 Genome recovery in Crosses F₁ x HKI287

Plant number	BC₂F₁ HKI287 x (HKI193-1)	BC₃F₁ HKI287 x (HKI193-1)
1	0.891	0.972
2	0.911	0.987
3	0.932	0.986
4	0.941	0.968
5	0.933	0.980
6	0.976	0.981
7	0.921	0.976
8	0.942	0.985
9	0.933	0.981
10	0.912	0.979
11	0.902	0.986
12	0.922	0.979
13	0.971	0.991
14	0.942	0.993
15	0.933	0.989
16	0.904	0.985
17	0.891	0.978
18	0.922	0.991
19	0.913	0.979
20	0.902	0.987
Average	0.925	0.983

From the above mentioned categorization, in case of BC₃F₁ HKI287 8 plants come under the range of high genome recovery, 4 plants come under the range of medium recovery and 8 plants come under the range of low recovery.

Highest recovery was found in plant no. 14 (0.993) and lowest recovery was found in plant no. 4 (0.968). The average genome recovery was found 0.983.

DISCUSSION

Maize is one of the most important crops in human and animal nutrition however like most other cereals; its nutritive value is poor due to deficiency of two essential amino acids – tryptophan and lysine. It was found that *opaque 2* mutation can nearly double the lysine content of the endosperm compared with the wild type (Mertz *et al.*, 1964). Isolation and characterization of *opaque 2* gene showed that this gene is a transcriptional factor which regulates expression of zein genes and a gene encoding a ribosomal inactivating protein (Schmidt *et al.*, 1990; Lohmer *et al.*, 1991; Bass *et al.*, 1992). The *opaque 2* mutation reduces zein content by one half and enhances the synthesis of a number of non zein proteins (Damerval and Devienne, 1993, Habben *et al.*, 1993). Attempts to incorporate *opaque 2* trait into high yielding commercial cultivars failed because of numerous associated agronomic and processing problems viz., reduced grain yield, soft endosperm, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests. These drawbacks have been overcome in genetically improved hard endosperm quality protein maize (QPM). QPM is a genotype in which *opaque 2* has been incorporated along with associated modifiers and possesses enhanced level of lysine and tryptophan as compared to non-QPM genotype. Multiple genes have been identified in controlling the levels of protein synthesis factor correlated with lysine level and additional nine implicated in free amino acid content (Wang *et al.*, 2001; Wu *et al.*, 2002).

Within the *opaque 2* gene sequence, three different SSR markers have been identified namely phi057, phi112 and umc1066. These markers work as specific markers for the identification of presence of *opaque 2* gene in maize inbred lines or their progenies. The internal position of these markers enable direct selection for the *opaque 2* gene without false positive or false negative results i.e. enables effective marker assisted selection (Babu *et al.*, 2005, Danson, 2006).

During the present study foreground selection for the presence of desired allele, was carried out using above mentioned *opaque 2* specific primers (phi057, phi112 and umc1066) for identification of polymorphism

between QPM (Donor) and non-QPM (Recipient) parents and progenies of BC₂F₁ and BC₃F₁ generation of cross HKI1126 (Non-QPM) x HKI161(QPM) and HKI287(Non-QPM) x HKI193(QPM). In case of BC₂F₁ and BC₃F₁ derived from cross HKI1126 x HKI161, a total of 100 plants for each population were used for screening and 20 heterozygous plants were selected for background selection in both cases. Whereas in case of BC₂F₁ and BC₃F₁ derived from cross HKI287 x HKI193, a total of 120 plants were screened and 20 heterozygous plants were selected for further background selection in both cases. The plants were morphologically similar to recipient parents producing bands corresponding to both allele (*o2 and O2*), while other produced a single band corresponding to homozygous (*O2O2*) condition. In the present study, phi112 marker could not clearly separate the heterozygous from homozygous plants. Similarly, Babu *et al.* (2005) found same result with phi112 marker during their study with QPM (CML176) and non-QPM (V25) parents.

Marker assisted backcrossing is widely used to introgress one or several target trait(s) from a donor (QPM) into the genome of a recipient parent (Non-QPM). Marker assisted selection for the genetic background of recurrent parent in the backcrossed progenies also known as background selection accelerates recovery of the recurrent parent genome (RPG) (Hospital *et al.*, 1992; Ribaut and Hoisington 1998; Wang *et al.*, 2007,). The effect of marker density on the recovery of the recurrent parent genome in background selection was first investigated by Hospital *et al.* (1992). They studied six generations of backcrossing and concluded that two generations can be saved by conducting marker assisted background selection.

During the present investigation, for background selection the markers used were the ones which are evenly spread throughout the genome and covering all the chromosomes. While, Prigge, *et al.* (2011), used evenly spaced markers situated on hypothetical marker maps of which the first and last were located at the telomeres of chromosome.

A total of 173 SSR markers (Source: www.maizegdb.org) were screened for parental polymorphism to select the markers for background selection. Total 101 markers were found to be polymorphic between HKI287 x HKI193 and 102 markers were found polymorphic between HKI1126 x

HKI161. Similarly, Gupta *et al.* (2013) screened more than 250 SSR markers, for genotyping BC₂ generation and found 108 polymorphic markers for CM212 x CML180 and 126 for CM145 x CML170 and used for background selection in maize.

To screen the BC₂F₁ and BC₃F₁ generations, 100 polymorphic SSR markers were used for each HKI1126 and HKI287 populations. In both cases the parental alleles were designated as A and B where A signifies recipient female parent allele, B signifies donor male parent allele and AB signifies the heterozygous condition. Genome recovery of recurrent parent in both generations was calculated and dendrograms were generated by polymorphic SSR markers using the software 'Power Marker' (Liu and Muse, 2005). In case of BC₂F₁ HKI1126, highest genome recovery ranged from 0.902 to 0.972 with an average of 0.931. Three plants showed genome recovery of more than 96%. In BC₃F₁ HKI1126 genome recovery ranged from 0.971 to 0.996 with an average of 0.984. Out of twenty plants analyzed, only four plants had more than 99% genome recovery. Similarly, in case of BC₂F₁ HKI287, genome recovery ranged from 0.891 to 0.976 with an average of 0.925. Two plants out of twenty, showed genome recovery of more than 97%. In BC₃F₁ HKI287, genome recovery ranged from 0.968 to 0.993 with an average of 0.983. Out of 20 plants analyzed only two plants had more than 99% genome recovery. Similarly, Gupta *et al.* (2013) found the highest recovery of the recipient genome 94.4% for the best lines of BC₂F₁ progenies CM212 x CML180 and 93.7% for CM145 x CML170 when they used 108 and 126 SSR markers, respectively during their investigation. Herzog and Frisch (2011) reported that when equally spaced (10cM) background markers were used in BC₂ generation for recurrent parent genome recovery then 92.3% recurrent parent genome were recovered which is similar to the result found in present investigation.

Prigge *et al.* (2009) examined effects of constant and sequentially increasing marker densities across the first three backcross generations in marker assisted backcrossing using the genome model of maize and the value of genome recovery was 80%. Randhawa *et al.* (2009) also found similar result in wheat using marker assisted backcross selection approach to

transfer “Yr 15” stripe rust resistance gene from Avocet S*6/ Yr 15’ to a susceptible soft white spring wheat cultivar ‘Zak’ using 55 SSR markers. Average RPG (recurrent parent genome) recovery was found to be 74.9%. The RPG recovery for the control simulation with 208 markers ranged from 68.5% at $Q_{\min}$ to 82% $Q_{\max}$.

The objective of the background/whole genome selection is to recover rapidly maximum proportion of recurrent parent genome at non target loci through markers that are distributed evenly throughout the genome (Young and Tanksley 1989; Hospital *et al.* 1992; Visscher *et al.* 1996; Frisch *et al.* 1999a, b). Availability of robust anchored marker maps in maize renders application of marker aided background selection an easy and attractive proposition. However, the cost of employing background selection in each BC generation could be prohibitive to many public sector breeding programs. The earlier simulation studies of Frisch *et al.* (1999a, b) and Ribaut *et al.* (2002) have indicated that application of background selection in one later generation along with foreground selection in each BC generations could be efficient and cost-effective. During the present investigation, we followed a two generation marker based breeding program in which whole genome background selection at non-target loci was applied in the BC₂ and BC₃ generations. As predicted by Ribaut *et al.* (2002), the selection response for background analysis depends on the recombination frequency between the target gene and the flanking markers and on the densities of markers on the carrier and non carrier chromosomes. The critical issue of number and distribution of unlinked target loci to be screened depends upon the required levels of line conversion, the extent of saturation of the base molecular marker map and availability of technical resources at a given point of time. In our study, we selected markers representing each established bin location on all the chromosomes of maize genome as per the SSR consensus map available in public domain (<http://www.maizegdb.org>). Although it has been demonstrated through simulation studies that increasing the number of markers to more than three per non-carrier chromosome was not efficient at early generations (Hospital *et al.* 1992), care needs to be taken in a practical MAS program to avoid sampling error and exaggerated estimates of recurrent parent genome

associated with smaller number of marker data points. The distribution of individuals with low, moderate and high recurrent parent genome content in the BC₂ and BC₃ population in more or less symmetrical fashion in the present study indicated unbiased sampling and sufficient number of marker data points.

It has been proved from our study that even one SSR polymorphism was sufficient for the selection of desirable *o2* genotypes. Because *umc1066* and *phi057* are present within the gene, the selection of genotypes in backcross breeding was very simple and straightforward. Also, identification of heterozygotes in the seedling stage prior to pollination aided in the rejection of non-target backcross progenies (dominant homozygotes) resulting in substantial saving of labour and consumables. In present investigation comparative analysis between BC₂ and BC₃ generations was carried out using 100 polymorphic SSR makers distributed throughout the genome. The recurrent parent genome recovery in BC₃ was high as compared to BC₂ without losing parental characteristics. This study demonstrated that background selection in BC₃ is beneficial as compared to BC₂ because it reduces the time required for release of variety. Whereas, BC₂ plants with more than 87% recurrent parent genome may also be used for selfing. However, BC₃ progenies with 99% genome of recurrent parent may directly used for hybrid preparation of Quality Protein Maize.

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1. Summary

The present study entitled “Studies on genome recovery percentage for *opaque2* introgressed in backcross population for marker assisted selection of quality protein maize” was carried out with objectives of Foreground selection of plants using *opaque2* specific markers and analysis of genome recovery of recurrent parent in BC₂F₁ and BC₃F₁ populations using SSR markers. For introgression of quality protein *opaque 2* gene into maize, steps were followed for foreground selection and background selection.

Two non QPM inbreds namely HKI287 and HKI1126 are the parents of a promising hybrids identified for Central India were undertaken for conversion into QPM. On the basis of parental polymorphism for markers linked to *o2* gene the HKI287 was crossed with HKI193 (QPM donor) and HKI1126 was crossed with HKI 161. Hybrid testing was done using three *Opaque2* gene specific markers namely phi057, phi112 and umc1066. Among 100 BC₁F₁ plants, 20 heterozygous plants were sown for raising BC₂F₁ generation and among 120 BC₂F₁ plants 20 heterozygous were sown for raising BC₃F₁.

For foreground selection, the genomic DNA from each plant was isolated from leaf tissue using CTAB method described by Saghai – Maroof *et al.*, (1984) with slight modifications. The PCR products for SSR were resolved by electrophoresis on 4 per cent (w/v) agarose gels stained with ethidium bromide (5µg/ml) and photographed under UV light in “Syngene” Multigenious Bioimaging System.

A total of 173 SSR markers (Source: www.maizegdb.org) were screened for parental polymorphism, 101 markers were found polymorphic between HKI287 and HKI193-1 while 102 markers between HKI1126 and HKI161. To screen both populations using all polymorphic SSR markers covering the all chromosomes for HKI1126 and HKI287 were selected.

Amplified parental alleles designated as A and B where A signifies recipient female parent allele and B signify donor male parent allele. Genome recovery of recurrent parent in both generations calculated and dendrograms generated separately using polymorphic SSR markers data. The software 'Power Marker' used for dendrogram construction.

Among twenty plants of BC₂F₁ 1126 population the genome recovery percentage was ranged from 0.902-0.972. The highest genome recovery (0.972) shown in plant no. 7 followed by plant no.2 and 12 with genome recovery 0.961, while lowest recovery was in plants no. 16 (0.902) followed by plant no. 17 with genome recovery 0.904. Genome recovery of BC₃F₁ 1126 plants can be grouped under three categories viz. High genome recovery (0.987-0.996), Medium genome recovery (0.980-0.985) and Low genome recovery (0.971-0.979).

Recurrent parent genome recovery was calculated among BC₂F₁ HKI287 plants. The highest genome recovery was 0.976 in plants no.6 followed by 0.971 in plant no.13 and lowest genome recovery was 0.891 in plant no. 17. in case of BC₃F₁ HKI287 plants genome recovery of recurrent parent can be grouped under three categorization viz. High genome recovery (0.986 – 0.993), Medium genome recovery (0.981 – 0.985) and Low genome recovery (0.968 – 0.980). Highest recovery was found in plant no. 14 (0.993) and lowest recovery was found in plant no. 4 (0.968). The average genome recovery was found 0.983.

6.2. Conclusion

- In this study, distinct polymorphism could be observed between the normal and QPM inbred lines with all the three SSR markers. However, the nature of polymorphism was different with respect to phi112, which exhibited dominant (presence-absence) polymorphism, restricting its potential utility in identifying the three forms of genotypes for the *opaque2* gene. Co-dominant nature of the polymorphism exhibited by phi057 and umc1066 enables their potential utility in MAS programs to successfully discriminate between homozygotes and heterozygotes.

- For background selection a total of 173 SSR markers were screened to detect parental polymorphism, 101 markers were found polymorphic between HKI 287 and HKI 193-1 while 102 markers between HKI 1126 and HKI 161.
- To screen both generations 100 polymorphic SSR markers covering all the chromosomes were used.
- Among twenty plants of BC₂F₁ 1126 population the genome recovery percentage was ranged from 0.902-0.972. The highest genome recovery (0.972) shown in plant no. 7 and lowest recovery was in plants no.16 (0.902). Genome recovery of BC₃F₁ 1126 plants can be grouped under three categories viz. High genome recovery (0.987-0.996), Medium genome recovery (0.980-0.985) and Low genome recovery (0.971-0.979).
- Recurrent parent genome recovery was calculated among BC₂F₁ HKI287 plants. The highest genome recovery was 0.976 in plants no. 6 followed by 0.971 in plant no.13 and lowest genome recovery was 0.891 in plant no. 17. In case of BC₃F₁ HKI287 plants the highest recovery was found in plant no. 14 (0.993) and lowest recovery was found in plant no. 4 (0.968). The average genome recovery was found 0.983.
- Under the condition of PCR used, SSR markers phi1066 and phi057 are polymorphic in most of the population and will, therefore, constitute the framework of marker assisted introgression of *opaque2* trait in maize genotypes.
- As previously described in the result plant which possess high genome recovery are found suitable for growing next generation.

6.3. Suggestions for further work

- Large plant population size should be used for growing next generation so that more number of heterozygous plants can be selected.
- More number of markers per chromosome should be used for background selection thus more number of plants will be closer to the recurrent parent.

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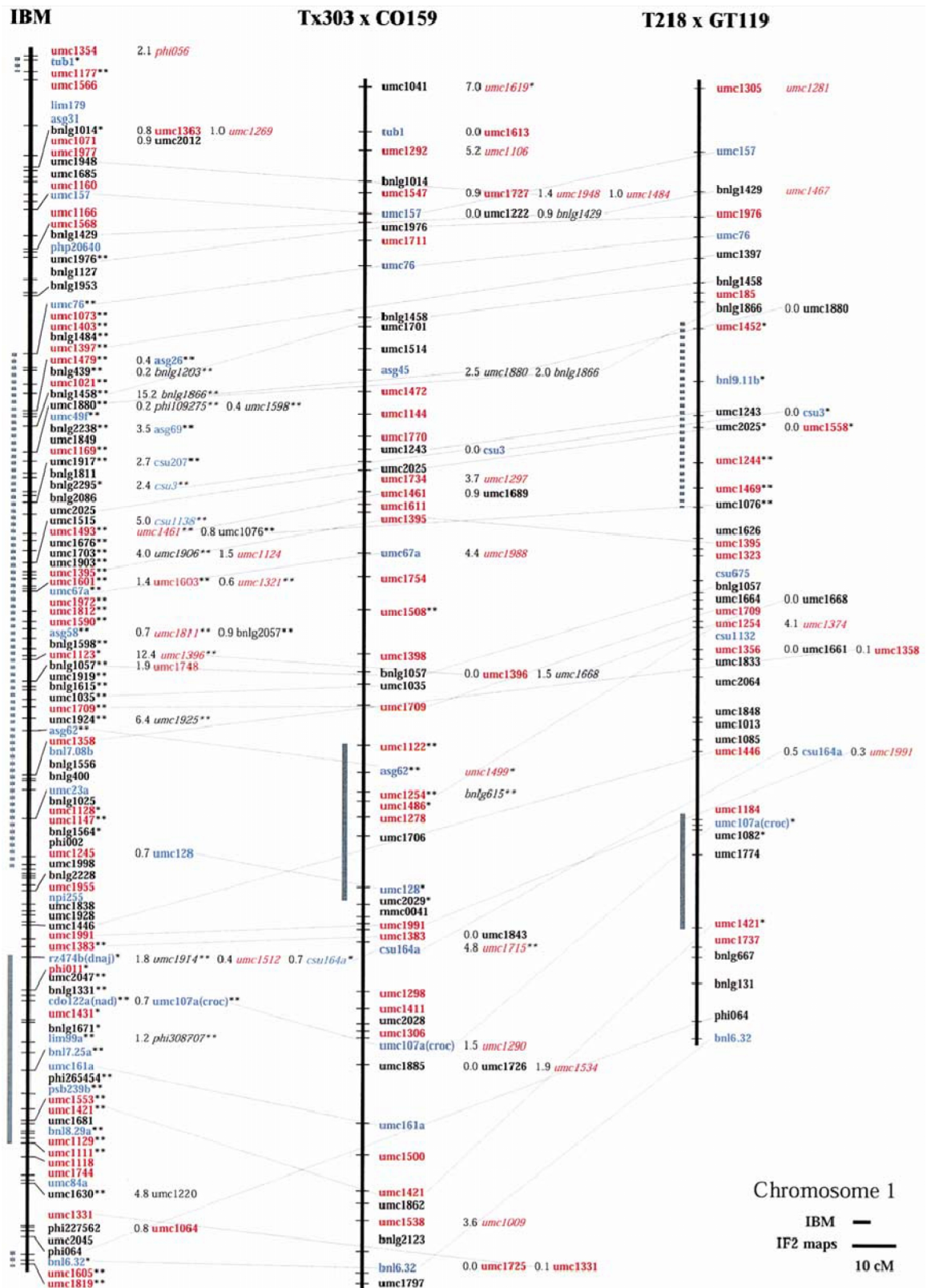


Fig.No.1-Showing different SSR Marker located on chromosome no. 1.

IBM

Tx303 x CO159

T218 x GT119

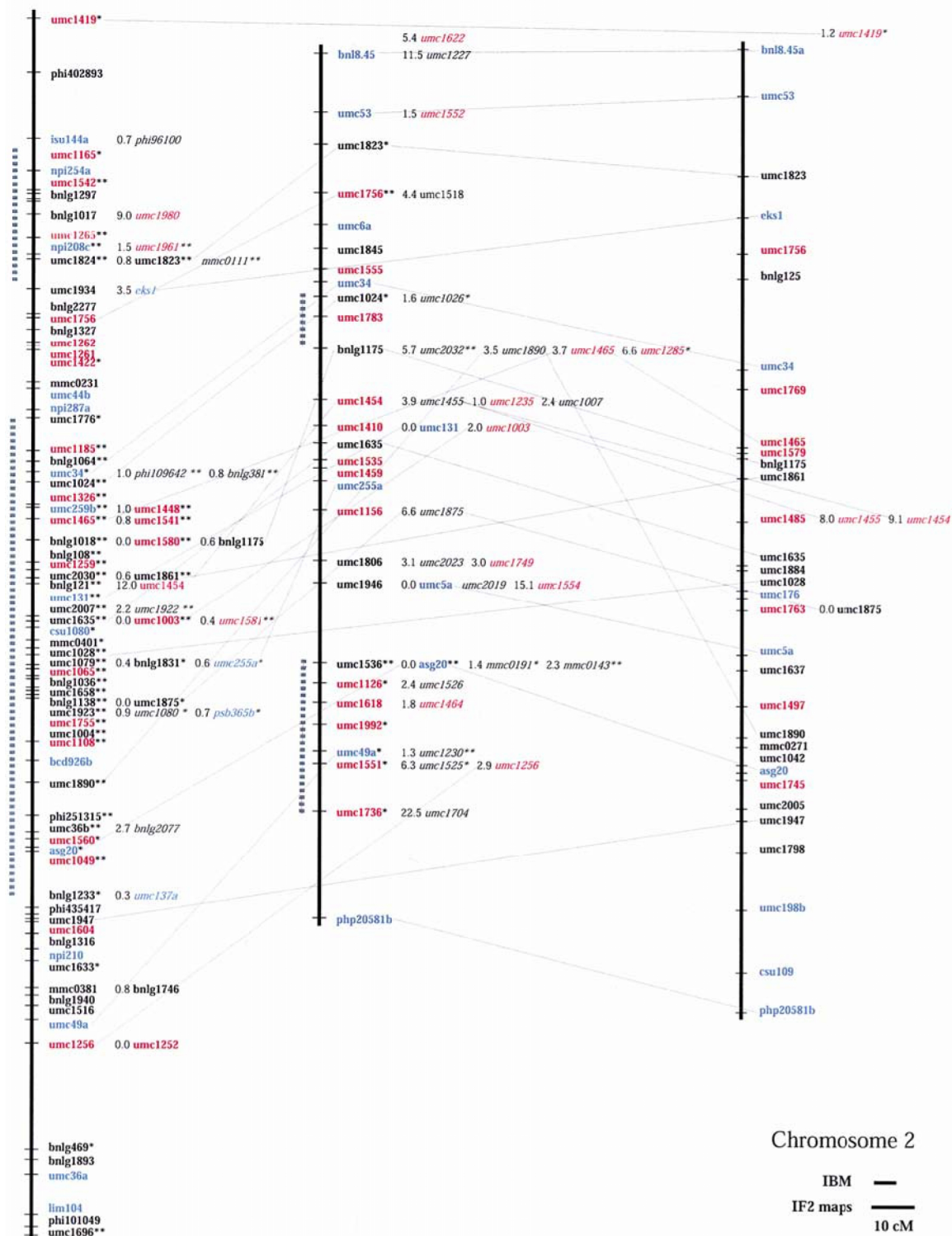


Fig.No.2-Showing different SSR Marker located on chromosome no. 2.

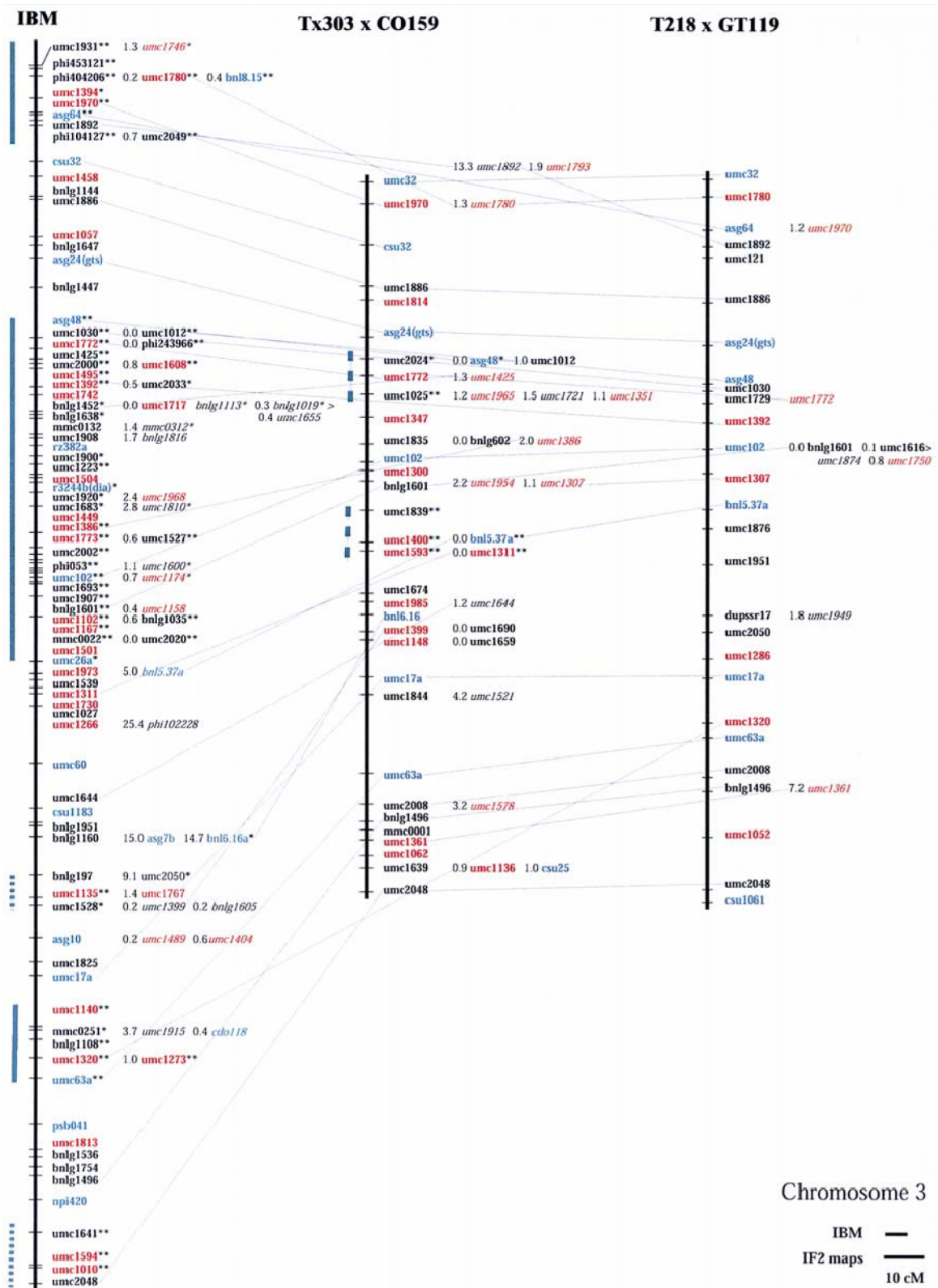


Fig.No.3-Showing different SSR Marker located on chromosome no. 3.

IBM

Tx303 x CO159

T218 x GT119

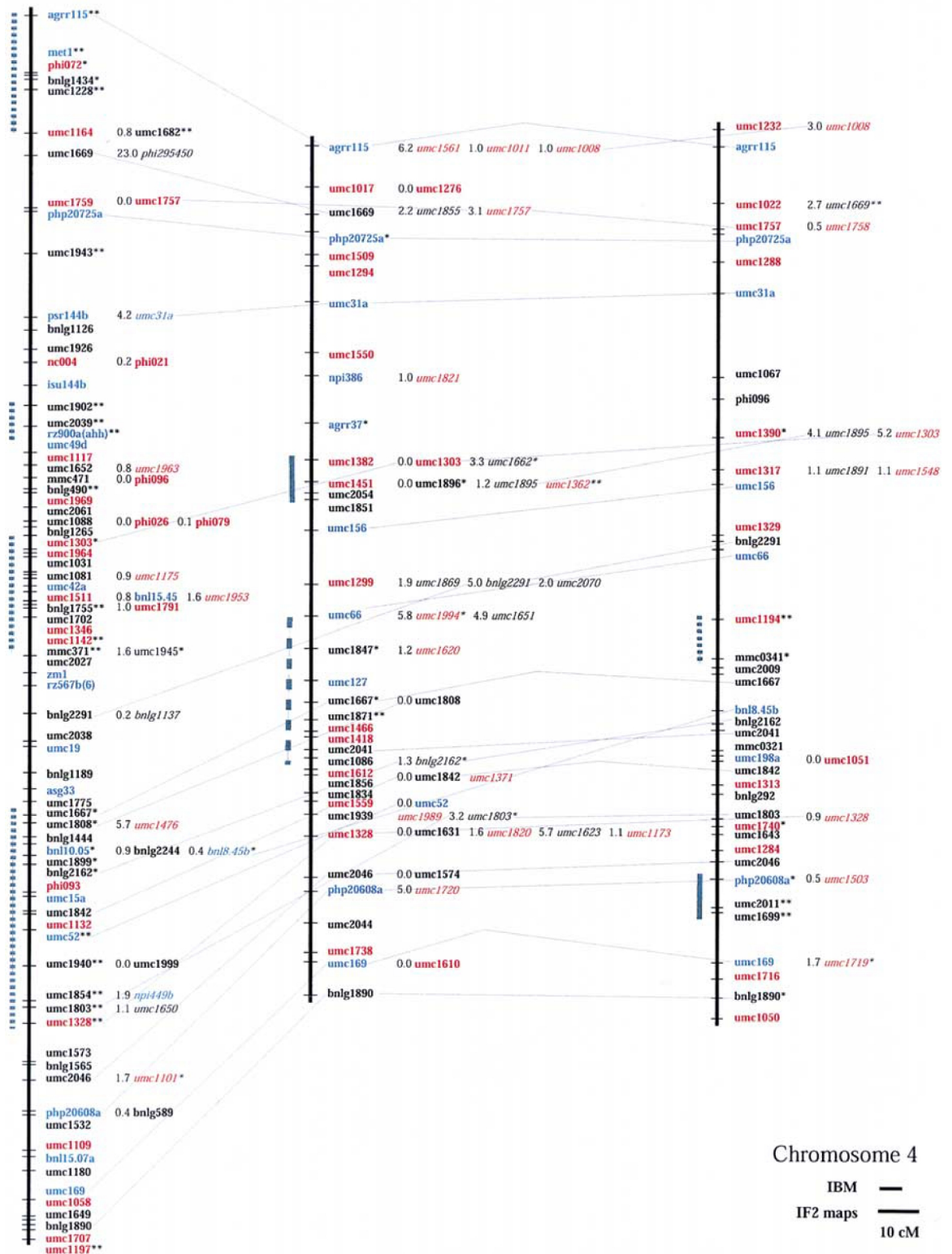


Fig.No.4- Showing different SSR Marker located on chromosome no.4.

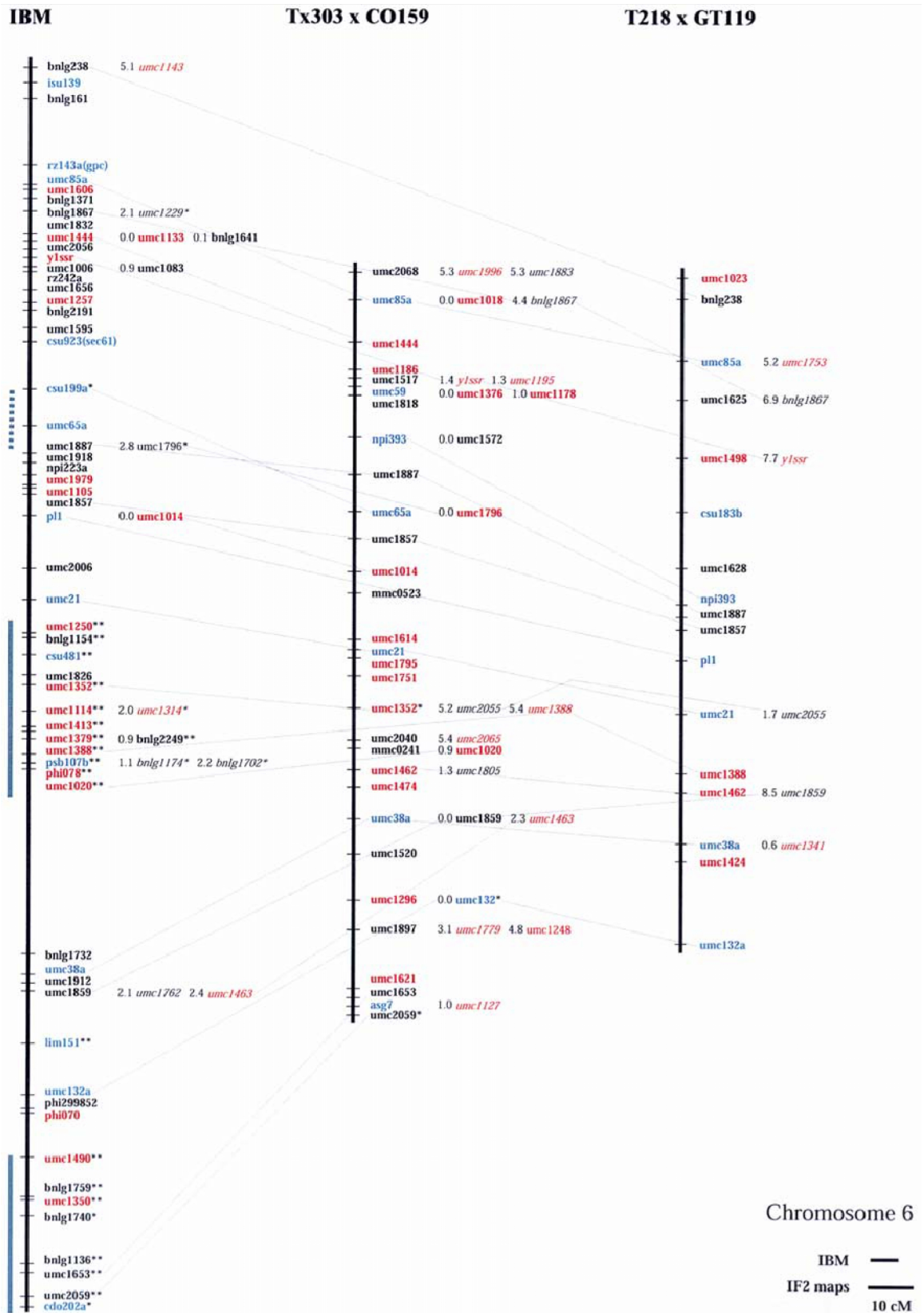


Fig.No.6- Showing different SSR Marker located on chromosome no. 6

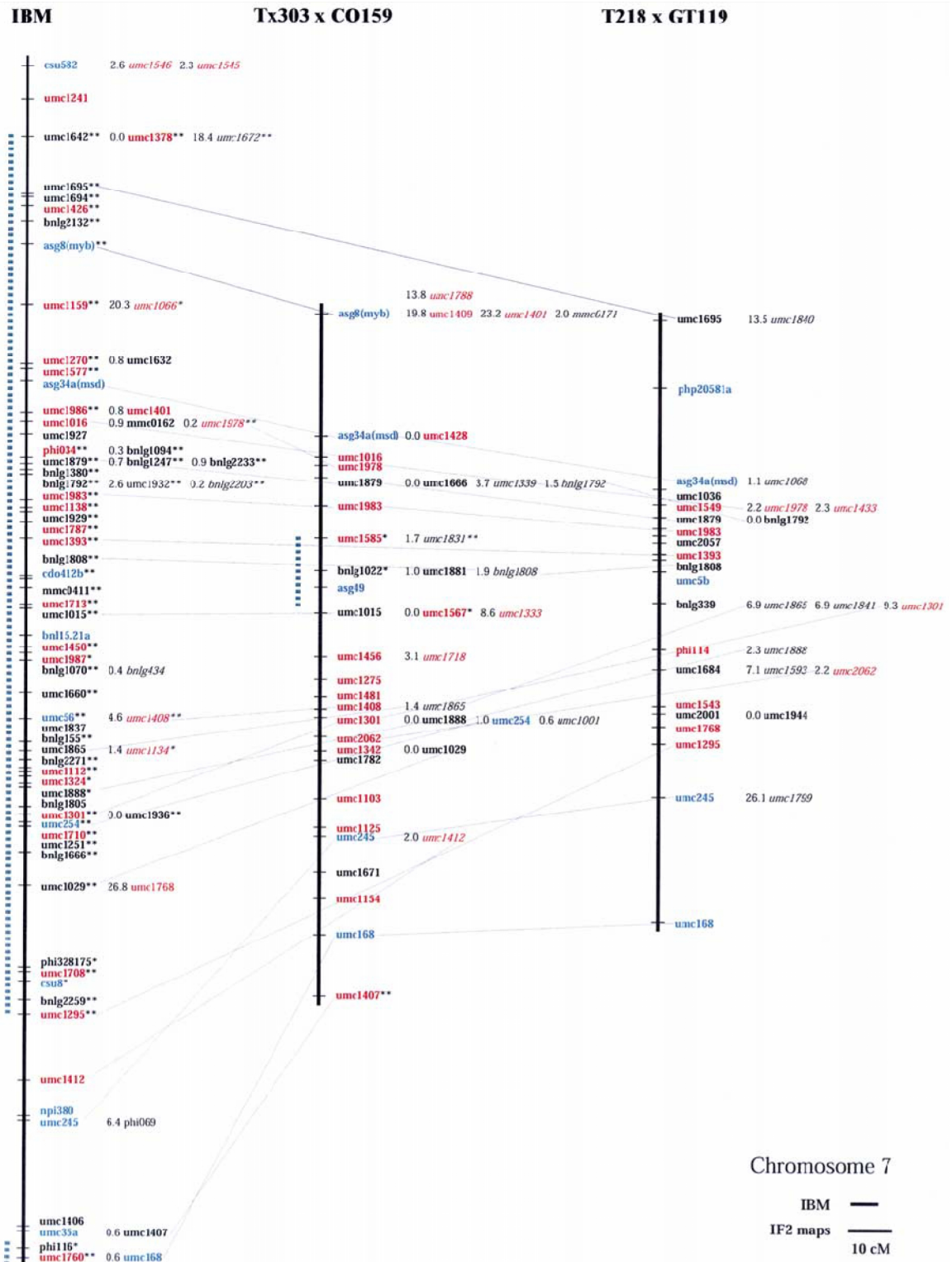


Fig.No.7- Showing different SSR Marker located on chromosome no. 7.

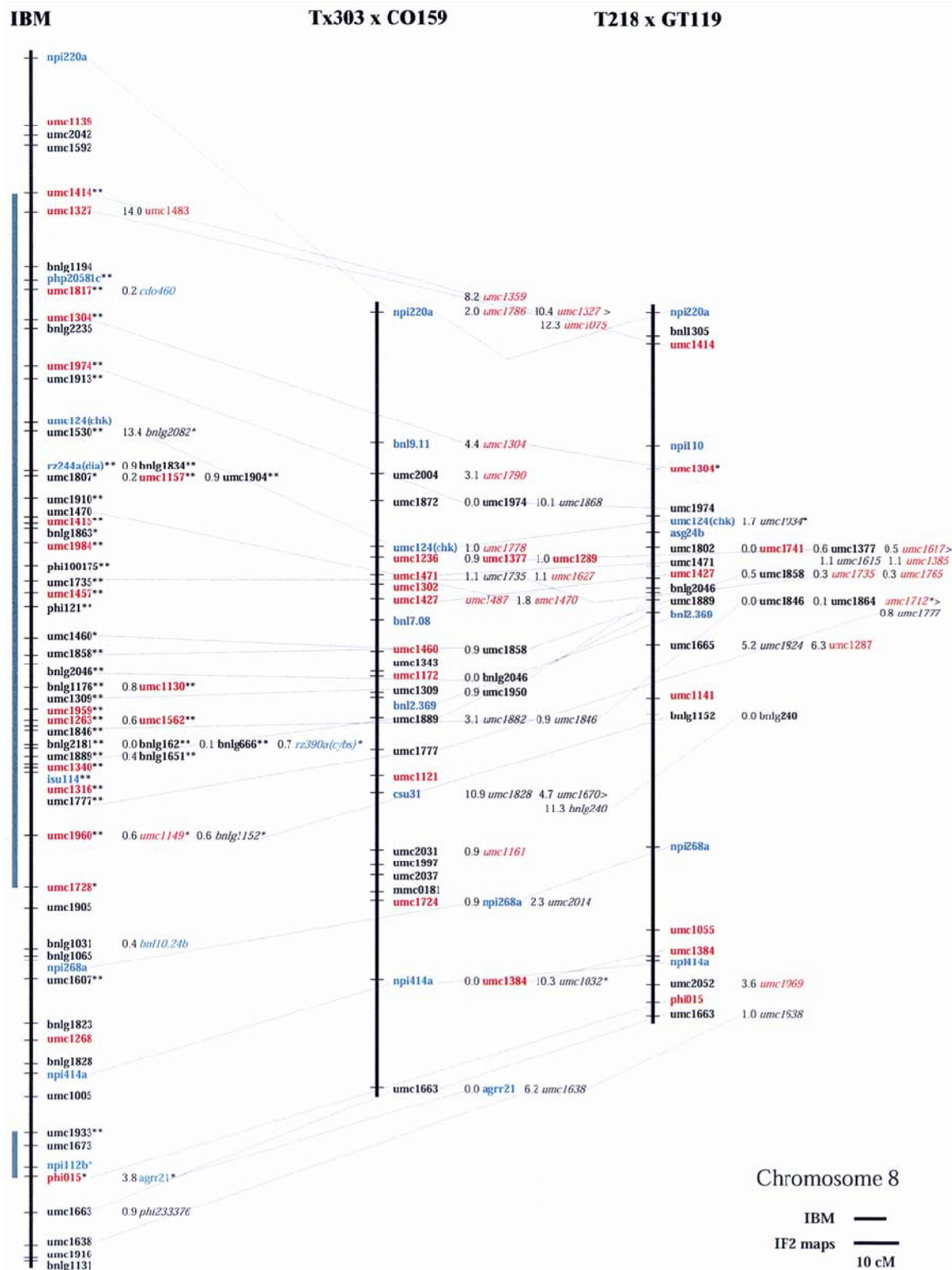


Fig.No-8- Showing different SSR Marker located on chromosome no. 8.

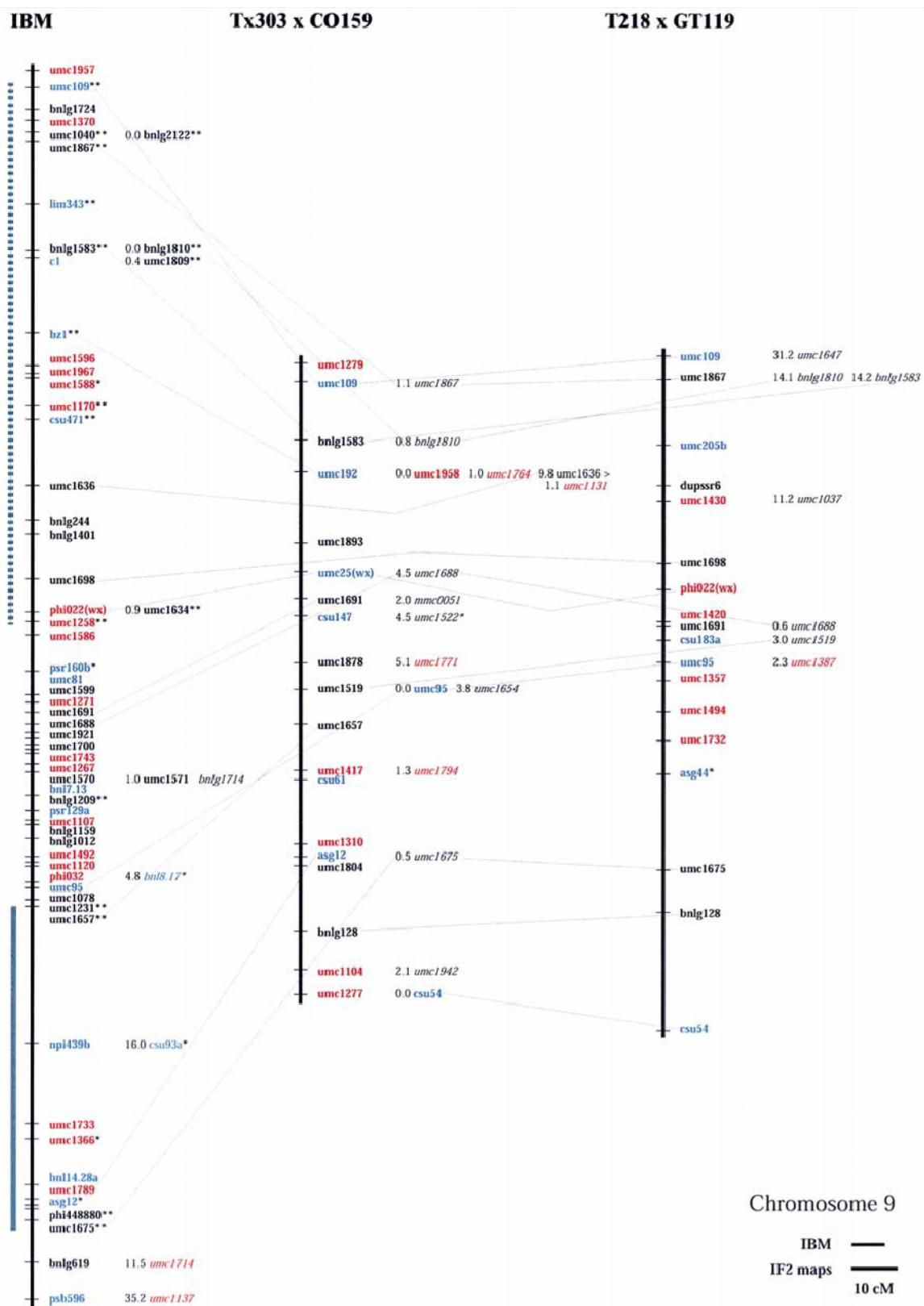


Fig.No.9- Showing different SSR Marker located on chromosome no. 9.

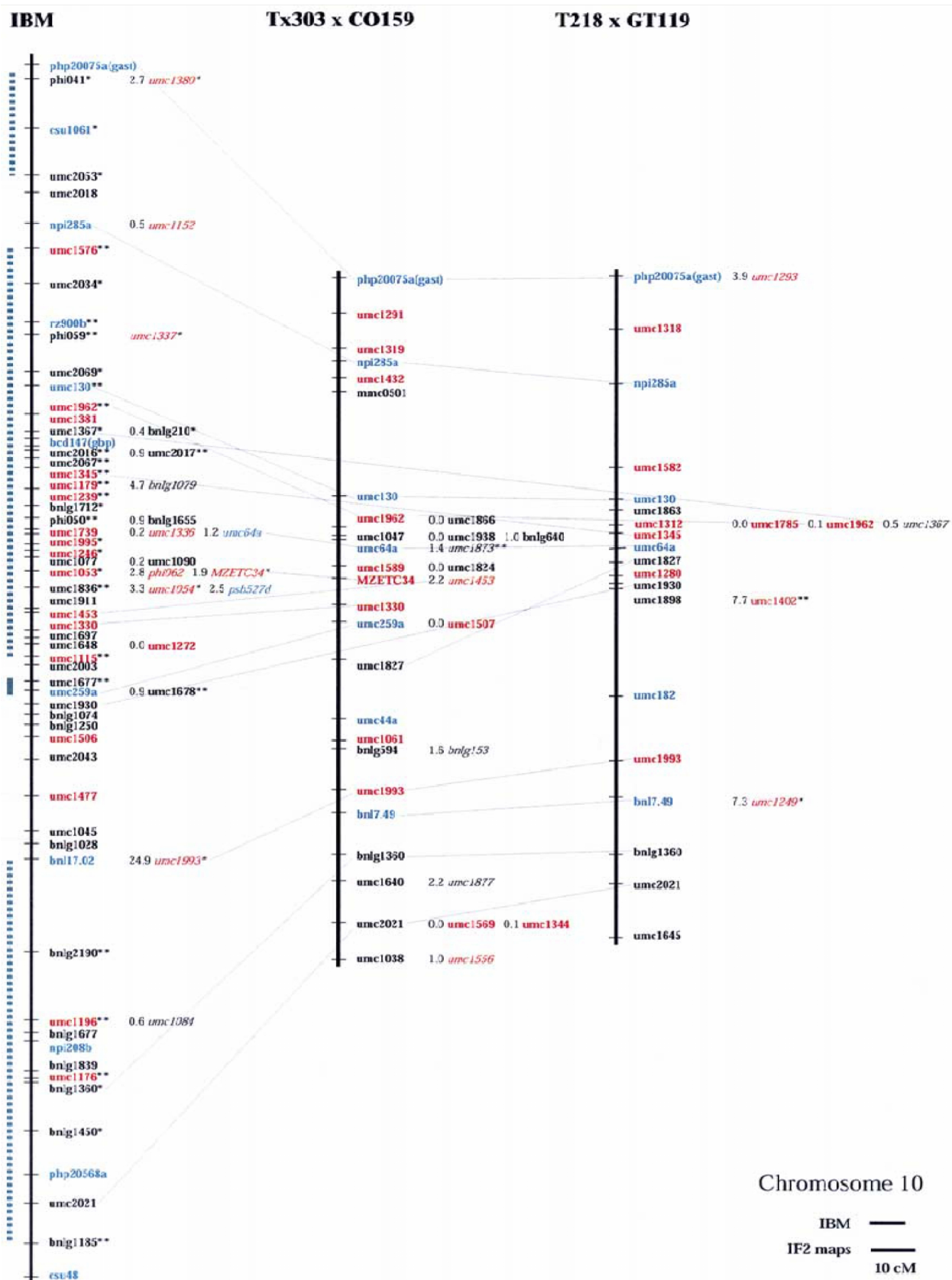


Fig.No.10 -Showing different SSR Marker located on chromosome no. 10

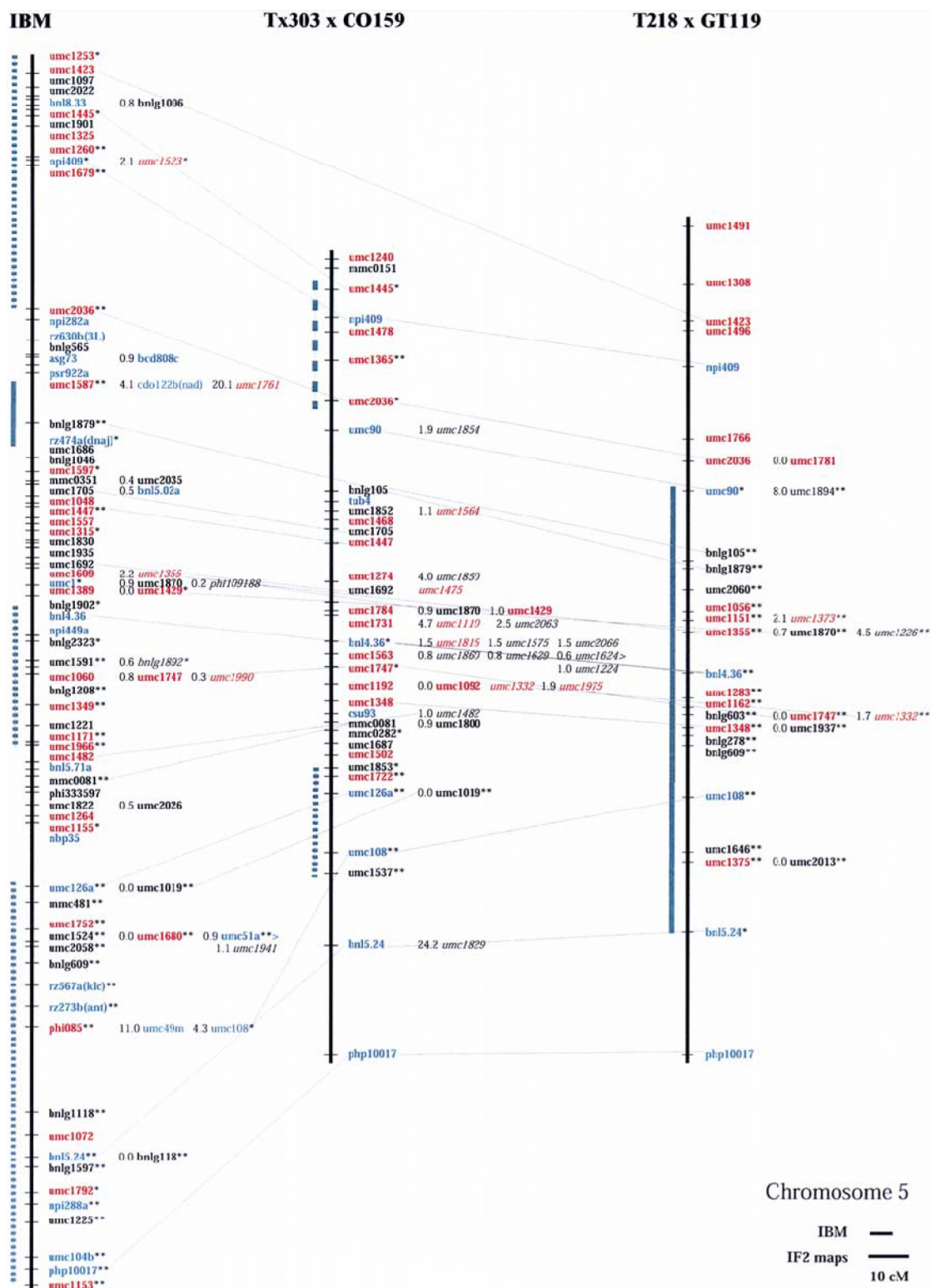


Fig.No.5 -Showing different SSR Marker located on chromosome no. 5.

VITA

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