CHAPTER 1
INTRODUCTION

"Basmati" is long grain aromatic rice grown for many centuries in the specific geographical area; at the Himalayan foot hills of Indian sub-continent, blessed with characteristics extra-long slender grains that elongate at least twice of their original size with a characteristics soft and fluffy texture upon cooking, delicious taste, superior aroma and distinct flavor. Basmati rice is unique among other aromatic long grain rice varieties. Agro-climatic conditions of the specific geographical area as well as method of harvesting, processing and aging attribute these characteristic features to Basmati rice. Owing to its unique characteristics the "scented Pearl" lends a touch of class that can transform even the most ordinary meal into a goumet's delight. Basmati is also available in white or brown versions, depending on the extent of the milling process.

1.1 AREAS OF CULTIVATION IN INDIA

The areas of Basmati Rice production in India are in the states of Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, Delhi, Uttarkhand and Western Uttar Pradesh.

1.2 EXPORTS

India is the leading exporter of the Basmati Rice to the global market. The country has exported 40,00,471.56 MT of Basmati Rice to the world for the worth of Rs.21,604.58 crores during the year 2016-2017.

1.3 MAJOR EXPORT DESTINATIONS (2016-2107)

Saudi Arabia, Iran, United Arab Emirates, Iraq and Kuwait (apeda.gov.in)

1.4 CHARACTERISTICS OF BASMATI RICE

Some of the major characteristics of Basmati rice which has captivated the world with its irresistible qualities are:

Colour - Basmati rice has a distinct translucent, almost pearly creamy white color. Although brown Basmati rice is also produced, the white polished variety is better preferred over it.

Grain - Basmati rice is known to have long and slender grains. This forms one of the key characteristics for its recognition.

Shape - Another factor for identification of basmati rice is its unique shape. The length to breadth ratio of basmati rice grain is equal to or more than three is to one and has a delicate curvature.

Texture - Basmati rice grains have a silky texture and the grains are firm, separate and dry. Upon cooking the grains remain separate and become fluffy.

Elongation – Basmati rice grains upon cooking elongate to nearly or more than double the length of the uncooked grain with minimal or no fattening.

Fragrance – Basmati rice is famous for its unique and enticingly supreme aroma even in the uncooked state.

Flavour - The grains of basmati rice boast of a rich appetising flavor. The delectable taste of the basmati rice is an extremely magnificently delicate blend of nutty and sweet flavours.

Yield - Yield of basmati rice from the agricultural land is almost half the non-basmati varieties. This is also one of the reasons for its higher costs. Basmati is grown only in the Himalayan region of India or few parts of Pakistan whereas non-basmati varieties could be grown anywhere in the world.

1.5 NUTRITIONAL HIGHLIGHTS OF BASMATI RICE

Basmati contains complex carbohydrates with low fat content. The fat content is extremely low and is of polyunsaturated nature. These polyunsaturated fats reduce the cholesterol levels in the blood and provide essential fatty acids called as Omega -3 and Omega -6 .Basmati is also an important source of proteins and complex carbohydrates along with being a good source of Iron, Selenium Thiamine and niacin. (It must be noted that according to International standards foods that are a "good source" of a particular nutrient provide between 10 and 20% of the Recommended Daily Value). Glycemic Index is a measure of the effect of carbohydrates on blood glucose levels. Foods having low GI help control the level of blood glucose level. Basmati is proven for its lower Glycemic Index options and thus does not adversely affect blood glucose levels. A cup of Basmati Rice (200g, cooked) includes:

- Calories:205
- Protein:4.2g
- Carbohydrate:44.5g
- Total: 0.44g (very less)
- Fibre: 0.63g.

Good source of:

- Iron (1.9mg)
- Selenium (11.8mcg)
- Thiamine (0.26mg)

1.6 APEDA:

Agricultural & Processed Food Products Export Development Authority(APEDA) is a government organization established in 1985 through an act for the development and promotion of export of scheduled products.It provides financial assistance, information, guidelines towards the development

of scheduled products. The products specified under the APEDA ACT are called schedule products and exporters of such scheduled products are register under APEDA.

1.7 OBJECTIVES OF APEDA

The objective of APEDA is to promote schedule products export and to achieve this various functions has been undertaken by this body under the regulation of central government.Central government lays down the rules and regulation and implements through this body for efficient administration of APEDA Act.

1.8 BEDF

Basmati Export Development Foundation (BEDF) has been founded by Agricultural and Processed Food Products Export Development Authority (APEDA) and registered under Societies Registration Act, 1860. State Government of UP has provided about 10 acres of land at long-term lease for 70 years to APEDA to be used for activities of BEDF. India is the leading exporter of the Basmati Rice to the global market. The country has exported 40,00,471.56 MT of Basmati Rice to the world for the worth of Rs.21,604.58 crores during the year 2016-2017.

Major Export Destinations (2016-2107): Saudi Arabia, Iran, United Arab Emirates, Iraq and Kuwait.

1.9 BASMATI ADULTERATION

Adulteration in food has become a major burning problem these days. The adulterants chosen for mixing are so similar to the naturally existing food items that it becomes nearly impossible to detect these by employing traditional methods. Basmati rice is often adulterated with look-alike long-grain non-Basmati varieties affecting the export market. Difficulty in differentiating genuine basmati from other types of rice and the significant price difference between them has led fraudulent traders to adulterate basmati rice with

crossbred basmati varieties and long-grain non-basmati varieties. (Priyankaran tanck, 2014)

1.10 RECENT ADVANCEMENT

With recent advances in biotechnology, DNA based techniques evolved rapidly and proved successful over conventional non-DNA based methods to purge the problem of adulteration.

1.10.1 CDFD RELEASED STANDARD PROTOCOL

A PCR based assay similar to DNA fingerprinting in humans which allows adulterated and non-basmati rice to be detected ,with detection limit from 1% adulteration upwards with an error rate of ±1.5% was first developed in the Centre for DNA Fingerprinting and Diagnostics, the Indian Company Labindia in Hyderabad. Exporters of basmati rice also use "purity certificates" based on DNA tests for their basmati rice consignments from CDFD. (Telegraphaindia.com) Microsatellite markers are more preferred DNA-based markers due to their easiness and reproducibility and for their co-dominant inheritance and high polymorphism. Multiplex approach with eight SSR markers using capillary electrophoresis is widely used for detection of adulteration in Basmati rice. (cdfd.apeda.org.in) However recent development of many Basmati varieties necessitates identification of more markers for accurate detection of adulteration.

1.11 AIM AND OBJECTIVES

Aim

To identify new SSR Markers and SNP markers for detecting adulterations in Basmati rice.

Objectives

1. To isolate the DNA from 30 notified Basmati varieties and non-Basmati variety Sharbati to amplify the DNA using different gene markers.
2. To Genotype all the Basmati and non-Basmati Varieties with SSR markers.
3. Genotyping all the varieties with single nucleotide polymorphisms (SNPs) present in the genes conferring quality traits to Basmati rice.
4. To make a profile for all Basmati varieties and non-Basmati variety based on their SSR and SNPs.

CHAPTER 2

REVIEW OF LITERATURE

Rice (Oryza sativa L.), a staple food, is the world's most important cereal crop. Grain weight, number of grains per panicle and number of panicles per plant are the most important components of grain yield. Based on a number of morphological, physiological, biochemical and molecular traits, Asian cultivated rices are organized in two major subspecies, i.e. Oryza sativa japonica and Oryza sativa indica (Second 1982; Glaszmann 1987; Oka 1988). These two subspecies are commonly associated with differences in growth habitat (Khush 1997) and are the products of independent domestication events from ancestral Oryza rufapogon populations in different locations and at different times (Vitte et al. 2004; Ma and Bennetzen 2004; Sang and Ge 2007a).

In addition to this major genetic organization, several other minor groups of varieties, usually based on more limited geographical distribution or special adaptation and characteristics, have been identified with genetic markers (Second 1982; Glaszmann 1987) and confirmed more recently (Bautista et al. 2001; Garris et al. 2005; Londo et al. 2006). For instance, these minor groups include Aus cultivars of India and Bangladesh, Ashinas varieties of Bangladesh, and aromatic Basmati rice of India, among others. Nevertheless, the identification of these minor secondary groups does not contradict the fundamental genetic organization of the Asian rice in japonica and indica subspecies and Basmati rices. This latter forms a small group of closely related varieties with strong affinity to the japonica group, but which is genetically distinct (Garris et al. 2005; McCouch et al. 2007), as confirmed by specific marker studies and rare allelic associations. Therefore, it has been proposed that Basmati rices, may have been independently domesticated (Garris et al. 2005; McCouch et al. 2007).

2.1 FRAGRANCE IN BASMATI RICE:

Interestingly aromatic rices varieties are also appreciated to an increasing extent in Western societies and thus the world market for these scented rices is in continuous full expansion. Unfortunately, aromatic rice cultivars, particularly Basmati rices from India, often produce poor yields because of their low resistance to rice diseases and limited adaptation outside their original geographical distribution. Basmati rice types also have a poor combining ability when crossed with other rice genotypes. Among the more than 100 volatile flavor compounds which constitute rice aroma, 2-acetyl-1-pyrroline (2-ACP) has been identified as the main agent in Basmati and Jasmine-style fragrant rices (Buttery et al. 1983; Paul and Powers 1989; Petrov et al. 1996). 2-ACP is actually detected in all parts of the rice plant, except in the roots (Lorieux et al. 1996). The detailed biosynthesis pathway of this compound has not yet been completely elucidated (Lorieux et al. 1996; Bradbury et al. 2005a). However, it was demonstrated, using the aromatic variety Thai Hom Mali, that the osmoprotectant proline was its precursor and the nitrogen source of 2-ACP (Yoshihashi et al. 2002).

In higher plants, proline is synthesized from glutamate or ornithine and highly accumulated under osmotic stress conditions (Verbruggen et al. 1996; Tuteja 2007). Initial genetic studies, performed by Tanksley's group (Ahn et al. 1992), localized a gene controlling aroma or fragrance (frg gene) in Della (Jasmine-derived aromatic variety) on the long arm of chromosome 8. Later, in our group Lorieux et al. (1996) tagged this gene as a major and recessive quantitative trait locus (QTL) in the same region, but limited to a 12 cm genetic interval, and in a (IR64 £ Azucena) DH population where the traditional upland variety Azucena was the donor of aroma. For the first time, this study demonstrated that 2-ACP detected by sensitive methods and gas chromatography (GC) was perfectly correlated and that large quantitative

variations were observed among aromatic DH lines. Moreover, in the same study, two other possible minor QTLs were identified on chromosome 4 and 12, which may affect the strength of aroma. Since then, several authors have reported mainly identifying markers (SSR, PCR-based markers) associated with the frg locus that could be used to help in distinguishing aromatic and non-aromatic rice varieties for marker-assisted selection (MAS) (Garland et al. 2000; Cordeiro et al. 2002; Jin et al. 2003). More recently, in traditional Basmati and Jasmine like rices, Bradbury et al. (2005) further restricted the aroma region and identified a single recessive gene responsible for aroma. This gene is a defective allele of a gene encoding betaine aldehyde dehydrogenase BADH2. The deletion observed in exon 7 of this (BADH2) gene generates a premature stop codon and presumably results in loss of activity. It was hypothesized that loss of BADH2 activity causes 2-ACP accumulation (Bradbury et al. 2005a).

Up to now, most studies on aroma gene in rice have mainly concerned traditional aromatic rices to assist breeders in the development of new cultivated fragrant rice varieties better adapted to particular environmental constraints (Bradbury et al. 2005b; Wanchana et al. 2005). Here we report the identification and characterization of the fragrance aroma gene in the Azucena cultivar, one of the few japonica rices referred as aromatic. This allowed us to analyse the diversity of the region surrounding this major aroma gene.

2.2 GRAIN SIZE OF BASMATI RICE

Grain size is one of the key quality traits of Basmati rice as far as traders and consumers are concerned. Preference for the grain size varies among different geographical regions of the world. For instance, Americans and Europeans prefer long grain varieties while Asians prefer medium slender grain varieties. Hence, the development of rice varieties suitable to various consumer preferences is the challenge that lies with rice breeders. Grain size is largely determined by its grain length, grain breadth and grain length-breadth ratio.

Elucidation of the molecular basis of the grain size is necessary for breeding consumer-preferred rice varieties. Currently available evidence suggests that the grain size is a complexly inherited quantitative trait governed by many genes with small genetic effects-known as quantitative traits loci (QTLs). As of now more than 400 QTLs governing grain size have been identified using different mapping populations (www.gramene.org). Some of them have been molecularly characterized and exploited for advances in molecular marker technology and high density linkage maps. Among the cloned genes, GS3 (Fan et al. 2006), qSW5 (Shomura et al. 2008), GW2 (Song et al. 2007), qGL3.1 (Qi et al. 2012), GIF1 (Wang et al. 2008) and TGW6 (Ishimaru et al. 2013), are positive regulators while GS5 (Li et al. 2011) and GW8 (Wang et al. 2012) are negative regulators of the grain size (Zheng et al. 2015). Increasing evidence suggests that the grain size is mainly influenced by multiple signaling pathways such as ubiquitin mediated proteosomal degradation, phytohormones and G-protein signaling pathways. Moreover, the genes GS3, GW5/qSW5, GS5, GW8 and GIF1 have been strongly selected during domestication and rice breeding (Zuo and Li 2014). However, the GW2 gene has been reported to be selected in japonica varieties only (Lu et al. 2013). This raises the possibility that some of the genes/QTLs might have been selected exclusively in Basmati varieties over a period of time. Recently, a major QTL on chromosome 8, GW8 (OsSPL16) encoding SQUAMOSA promoter binding protein has been identified in a mapping population derived from Basmati385 (Wang et al. 2012). However, systematic studies are lacking for complete understanding of the grain size controlling mechanism, especially in Basmati rice.

2.3 SEED SHAPE IN BASMATI RICE

Seed shape is determined mainly by its dimensions: seed length (SL), seed width (SW) and the ratio of length to width (RLW). The seed or grain shape is generally recognized as a quantitative trait that is controlled by multiple

genes and affected by environmental factors. QTLs elsewhere, frequently accounted for large proportions of the genetic variation in seed dimensions. So far, most of the loci and their functions remain to be examined, except a small number of genes (e.g., GS3, GW2, qSW5/GW5, GS5, SRS3) associated with grain size or shape that has been identified. The qSS7 on the long arm of chromosome 7 described here acts as a major QTL affecting seed shape parameters. A consistent QTL for grain length has been mapped to an approximately 300-kb or larger interval (Amarawathi et al. 2008; Shao et al. 2010) or adjacent region but with only a small amount of the SL variation explained in the populations (Bai et al. 2010). qSS7 is simultaneously associated with SL, SW and RLW, but has little or no effect on TSW . The effect of qSS7 is different from previously reported QTLs and/or cloned genes for seed shape which affect TSW to some extent. For example, GS3 and SRS3 had major effects on both grain length and weight (Fan et al. 2006; Kitagawa et al. 2010), and GW2 and qSW5 act as major QTLs for both grain width and weight (Song et al. 2007; Shomura et al. 2008). Notably, qSS7 has an opposite effect on SL and SW: the Cypress allele contributes to an increase in SL and decrease in SW but does not alter seed thickness, and consequently does not change the seed volume or weight. This finding suggests that qSS7 might regulate seed length and width independent of seed weight. The role of this gene in seed shape needs to be explored in rice. It is also noteworthy that the sub-fragment GL285–DGS220 of the qSS7 region that harbors LOC_Os07g41210 seemed to have a relatively large effect on seed shape. Moreover, this predicted gene has a premature termination causing a truncated protein in ZS97. Therefore, LOC_Os07g41210 cannot be ruled out as a candidate underlying the QTL. Further, transgenic complementation experiments and RNA interference of the potential candidate genes and association analysis of the sequence variations of these genes in a large panel of rice germplasm are necessary to test the definitive role either or both of these genes play in determining seed shape.

2.4 SSR MARKERS:

Microsatellites, otherwise called Simple sequence repeats (Ssrs) or Short Tandem Repeats (Strs), are rehashing sequences of 2-5 base sets of Dna.it is a sort of Variable Number Tandem Repeat (VNTR). Microsatellites are commonly co-prevailing. They are utilized as atomic markers as a part of STR dissection, for connection, populace and different studies. They can additionally be utilized for investigations of gene duplication, marker aided determination, and fingerprinting. SSR markers are co-dominant, multi-allelic and can be reliably used to analyze both indica and japonica germplasm, as well as groups of AA genome Oryza species15–19 makes them attractive as genetic markers and facilitates the integration of results from independent studies. In addition, the highly polymorphic nature of many microsatellites is of particular value when analyzing closely related genotypes, as is often the case in breeding programs working within narrowly adapted gene pools. Thus, the availability of a high-density SSR map is valuable as a public resource for studies aiming to interpret the functional significance of the rapidly emerging rice genome sequence information.In this present work, SSR Markers were used for detecting adulteration in Basmati rice varieties and designing a SingleNucleotide Polymorphism based markers associated with genes governing quality traits of Basmati .

CHAPTER 3
METHODS AND MATERIALS

3.1 ISOLATION OF DNA FROM BASMATI RICE

The DNA from 30 Basmati varieties were isolated using the following conventional method of DNA isolation. Conventional genomic DNA extraction protocols need expensive and hazardous reagents for decontamination of phenolic compounds from the extracts and are only suited for certain types of tissue. We developed a simple, time-saving and cost-efficient method for genomic DNA extraction from various types of organisms, using relatively innocuous reagents. The protocol employs a single purification step to remove contaminating compounds, using a silica column and a non-hazardous buffer, and a chaotropic-detergent lysing solution that hydrolyzes RNA and allows the selective precipitation of DNA from cell lysates.

SINGLE GRAIN DNA ISOLATION (Qiagen kit)

- Grind the sample by using homogenizer .Keep in aluminium foil and transfer it in the 2ml centrifuge tube.
- Soak the grain in 600µl of lysis buffer (Qiagen AP1) and add 6 µl of RNAse. Vortex thoroughly.
- Incubate at 65˚C in water bath for 10 to 15 minutes.
- Add AP2 solution (Neutralizing Buffer) and keep it in the ice for 20 to 30 minutes.
- Centrifuge at 13,000rpm for three minutes and transfer the supernatant to a fresh tube (1.5ml).
- To this add 600µl Chloroform and invert it gently for few seconds.
- Centrifuge at 14,000rpm for 15 minutes and transfer the upper phase to a fresh tube.

- Add 600µl or equal volume of Isopropanol to the tube and gently invert the tubes for 10 times.
- Centrifuge at 14,000rpm for 15minutes and wash the pellet with 70% ethanol.
- Centrifuge at 14,000rpm for 5 minutes.
- Decant the supernatant and centrifuge for a further washing of impurities.
- Pipette of all ethanol and air dry the pellet (not more than 10 minutes).
- Resuspend DNA in 50 µl TE Buffer.

3.2 POLYMERASE CHAIN REACTION

PCR **is** a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

3.2.1 COMPONENTS OF PCR

DNA template

The sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

DNA polymerase

A type of enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is*Taq*DNA polymerase (from*Thermis aquaticus*), whereas*Pfu*DNA polymerase

(from*Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA. Although these enzymes are subtly different, they both have two capabilities that make them suitable for PCR: 1) they can generate new strands of DNA using a DNA template and primers, and 2) they are heat resistant.

Primers

Short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

Nucleotides (dNTPs or deoxynucleotide triphosphates)

Single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.

RT-PCR

Reverse Transcription PCR is PCR proceeded with conversion of sample RNA into cDNA with enzyme reverse transcriptase.

3.2.2 Limitations of PCR and RT-PCR

The PCR reaction starts to generate copies of the target sequence exponentially. Only during the exponential phase of the PCR reaction is it possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Because of inhibitors of the polymerase reaction found in the sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs, making the end point quantification of PCR products unreliable. This is the attribute of PCR that makes Real-Time Quantitative RT-PCR so necessary.

3.2.3 PCR MIX

PCR MIX FOR SSR MARKERS

Table 1: Compositions used for making 28X Reaction Mix for 28 Basmati varieties.

NAME OF THE REAGENT	AMOUNT (in µl)
M.Q Water	120.4
25mM Taq Buffer	28
15Mm $MgCl_2$	16.8
1Mm dNTP	28
Forward Primer	28
Reverse Primer	28
Enzyme	2.8
Template DNA	28

3.2.4 GRADIENT PCR

Problems with the amplification of a specific DNA fragment using the PCR are an everyday occurrence in the lab. Non-specific secondary bands may form after the PCR reaction, which hinder, or even prevent, further analysis (cycle sequencing, mutation detection, etc.) or an unequivocal assessment of the PCR result. In such cases, PCR conditions must be optimized. This is normally achieved by titrating the magnesium-, template-, primer-, dNTP- and Taq-Polymerase concentration, "Hot Start PCR," "Touch-down PCR," adding detergents, reducing the PCR cycles or by gradually increasing the annealing temperature. The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. In most cases, this temperature must be empirically tested. The PCR is normally started

at 5°C below the calculated temperature of the primer melting point (T_m). However, the possible formation of non-specific secondary bands shows that the optimum temperature is often much higher than the calculated temperature (>12°C).

To avoid non-specific bonds, Gradient PCR is used. Gradient PCR is a technique that allows the empirical determination of an optimal annealing temperature using the least number of steps. This optimization can often be achieved in one experiment. The Gradient provides a gradient function that in one single run evaluates up to 12 different annealing, elongation, or denaturation temperatures. During the same run a number of possible concentration parameters can also be tested, row by row. Gradient PCR is used for optimization of annealing temperature for standardizing.

3.2.5 PCR CONDITIONS

TABLE 2: PCR CONDITIONS

PCR STEPS	TEMPERATUE(˚C)	TIME
INITIAL	94	5minutes
DENATURATION	94	30 seconds
ANEALING	55	30 seconds
INITIAL EXTENSION	72	1 minute
FINAL EXTENSION	72	7 minutes

3.2.6 ALLELE SPECIFIC PCR

Allele-specific polymerase chain reaction (ASPCR) is an application of the polymerase chain reaction (PCR) that permits the direct detection of any point mutation in human DNA by analyzing the PCR products in an ethidium

bromide-stained agarose or polyacrylamide gel. ASPCR works because an oligonucleotide primer that forms a 3′ mismatch with the DNA template will be refractory to primer extension by *Thermus aquaticus* DNA polymerase. Therefore, oligonucleotide primers specific for all known alleles can be synthesized and used to detect the alleles in DNAs of unknown genotype. ASPCR has already been used in DNA-based diagnostic techniques involving the diagnosis of genetic and infectious diseases. Primer design and well-optimized PCR methodology are the crucial aspects in creating a working AS-PCR-based genotyping system. Once the optimized protocol has been achieved, the execution of AS-PCR is relatively simple, analogous to the conventional PCR.

3.2.7 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNAand RNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix. Agarose gel is easy to cast, has relatively fewer charged groups, and is particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease. Most agarose gels used are between 0.7–2% dissolved in a suitable electrophoresis buffer.

Agarose gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass. The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state. The melting temperature is different from the gelling temperature, depending on the sources, agarose gel has a gelling temperature of 35–42 °C and a melting temperature of 85–95 °C. Low-melting and low-gelling agaroses made through chemical modifications are also available. The separated DNA bands are often used for further procedures, and a DNA band may be cut out of the gel as a slice, dissolved and purified. Contaminants however may affect some downstream procedures such as PCR, and low melting point agarose may be preferred in some cases as it contains fewer of the sulphates that can affect some enzymatic reactions. The gels may also be used for blotting techniques.

3.2.8 PCR PURIFICATION

PCR Purification - QIAquick Kit Protocol

This protocol is designed to purify single- or double-stranded DNA fragments from PCR. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a micro centrifuge.

Procedure:

1. Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and mix. For example, add 500 µl of Buffer PBI to 100 µl PCR sample.
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30– 60 s.
4. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.

5. To wash, add 750 µl Buffer PE to the QIAquick column and centrifuge for 30– 60 s.

6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

7. Place QIAquick column in a clean 1.5 ml micro centrifuge tube. 8. To elute DNA, add 30 µl of water to the center of the QIAquick membrane, let the column stand for 1 min and centrifuge the column for 1 min. Materials required Fresh PCR product QIAquick PCR purification kit 200 and 1000 µl pipette tips Sterilized DI H2O 1.5 ml micro centrifuge tubes.

Materials Required

Fresh PCR product QIAquick PCR purification kit 200 and 1000 µl pipette tips, Sterilized DI H2O and 1.5 ml micro centrifuge tubes.

3.2.9 DNA SEQUENCING

DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery.

Knowledge of **DNA sequences** has become indispensable for basic biological research, and in numerous applied fields such as medical diagnosis, biotechnology, forensic biology, virology and biological systematics. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes, of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species.DNA Sequencing is done for detecting the nucleotide order for finding SNPs and Deletions.

3.2.10 PRIMERS USED FOR ARM PCR AND SSR MARKERS

S.NO	PRIMER NAME	PRIMER SEQUENCES
1	KA717600-1-F	GGAATGA0AGTGTCTACGTGAACA
2	KA717600-1-G	GGAGTATCTGACATCATTTCTGTAC
3	KA717600-1-A	GGAGTATCTGACATCATTTCTGTAAC
4	KABadA-1R	CTCCGGTGTGAACAAAACA
5	KABadh1-1-T	CCAATCTCTCTACCCATGGAAGAT
6	KABadh1-1-A	CCAATCTCTCTACCCATGGAAGAA
7	KA717600-1-T	CTTCTCAATGTCTTGCAATGGTTCTGTTT
8	KA717600-1-2-T	GCGAGTTTGAAGAACAGTGGCT
9	KA717600-2-C	CGAGTTTGAAGAACAGTGGCC
10	DGS220F	AAATGAGTTTAAGTCGGCTGGA
11	DGS220R	GAGAACGGTTTTGTTGGTTG

CHAPTER 4
RESULTS AND DISCUSSIONS

Basmati is a variety of long, slender-grained aromatic rice which is traditionally from the Indian subcontinent. India exported 65% of the overseas Basmati rice market, while Pakistan accounted for the remainder, according to the Indian state-run Agricultural and Processed Food Products Export Development Authority. However, many countries use domestically grown Basmati rice crops. Difficulty in differentiating genuine Basmati from other types of rice and the significant price difference between them has led fraudulent traders to adulterate Basmati rice with crossbred Basmati varieties and long-grain non-Basmati varieties. In Britain, the Food Standards Agency found in 2005 that about half of all Basmati rice sold was adulterated with other strains of long-grain rice, prompting rice importers to agree to a code of practice. A 2010 UK test on rice supplied by wholesalers found 4 out of 15 samples had cheaper rice mixed with Basmati, and one had no Basmati at all. There is a need to separate Basmati varieties from Non-Basmati varieties. The project work of mine is to find markers for detecting adulterations in Basmati rice .It has two major objectives to discuss the results.

- To find SNPs differing between basmati varieties and Non Basmati varieties in the genes conferring quality traits
- To find new SSR markers that can differentiate Basmati from Non-Basmati varieties.

4.1 SNPS

The main aim is to detect the adulteration in Basmati varieties by finding variations in the nucleotide bases in genes conferring quality traits. Analysis of single nucleotide polymorphism (SNP) and small insertion/deletion (InDel), which are the basis of most differences between alleles, has been simplified by recent developments in sequencing technology. Due to the ability of SNPs to

generate numerous markers within a target region and the availability of high-throughput SNP assays, SNP genotyping is becoming a valuable tool for gene mapping and marker-assisted selection in crops (Rafalski 2002; Belo et al.2008) In comparison to individual SNPs, haplotype analysis of a group of linked SNPs is more informative in determining association with the phenotypes. My present work is a continuation of a project which had already started by my guide to detect the SNP differences in QTLs of rice chromosomes. An 8-bp deletion in the exon 7 of badh2 gene is responsible for fragrance (Sakthivel et al., 2009) and C to A mutation in the exon 2 of GS3 imparts long grain phenotype (Takano-Kai et al., 2009).Similarly SNPs in *waxy* and *alk* genes responsible for differences in amylose content and gelatinization temperatures are known. These SNPs were genotypes in all Basmati varieties and few non-Basmati varieties. However as shown in **Table 4** few Basmati varieties cannot be differentiated from non-Basmati varieties due to presence of same allele in both Basmati and non-Basmati varieties. For example all Traditional Basmati varieties cannot be differentiated from Pusa Sugandh 3 and VB21, PB1121, HB, PUSA1718 cannot be differentiated from Pusa Sugandh 2 and Pusa Sugandh 5.

BASMATI	VARIETY NAME	FRAGRANCE	GELATANI-ZATION TEMP (^{O}C)		AMYLOSE CONTENT		
		DEL	4198	4330	Tn1	Ex1	Ex6
TRADITIONAL	B370,TYPE 3,RANBIR BASMATI,TAROARI BASMATI,B386	8 bp	G	GC	G	C	C
EVOLVED	PUNJAB B2,VB22,B564,P1121,PB1509,VB21,PB1718	8 bp	G	TT	G	A	T
NON	TERRICOT	8 bp	C	TT	G	A	C
	SHARBATI	8 bp	G	GC	G	A	-
	PEPSI	8 bp	G	TT	G	A	-
	RH10	8 bp	G	TT	G	A	-
	PS2	8 bp	G	TT	G	A	C
	PS3	8 bp	G	TT	G	A	C
	PS5	8 bp	G	TT	G	C	C
	PS3	8 bp	G	TT	G	C	C

4.2 SNPS IN GENES:

Mutations in some other genes like in Os03g0717600, Os03g0802600, Os03g02050 (wang et al., 2017). Mutations in *Badh1* gene (Singh et al., 2010) are also shown to confer fragrance in rice along with the mutations in Badh2 gene. Similarly mutations in Os07g41200 (Xianjin Qiu ,2012) are shown to cause grain size differences. In this study (**TABLE 5)**it was tested whether these SNPs will help in differentiating the Basmati varieties mentioned above from non-Basmati varieties.

S.NO	GENE	CHROMOSOME NUMBER	RAP ID	Mutation
1	*GT3*	3	Os03g0717600	G/A C/T
			Os03g0802600	T/A
			Os03g02050	C/A
2	*Badh1*	4	Os04g0464200	T/A
3	*qSS7*	7	Os07g41200	G/A

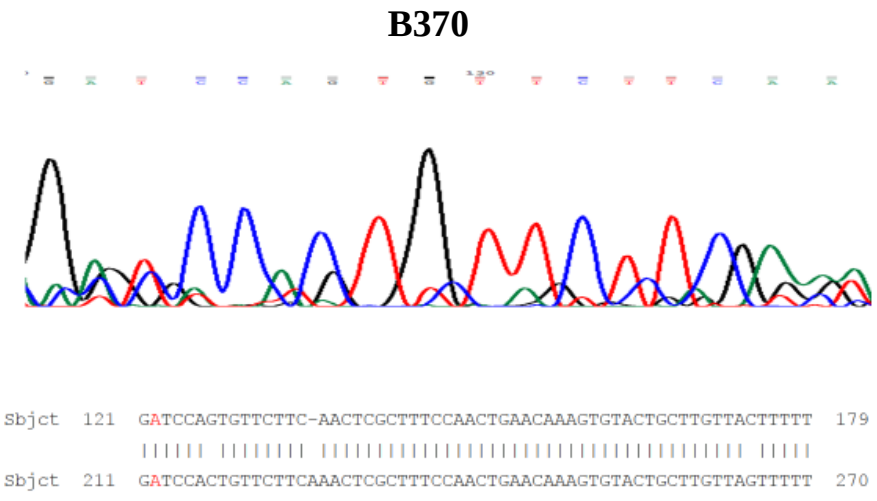
OsO3G0717600

G/A and C/T: To analyze the mutation in os03g0717600,the target gene from the template DNA is amplified using primers KA-GT-717600F and KA-GT-717600F the purified PCR product is sequenced to know the allelic status in Basmati 370, Haryana Basmati, Pusa Sugandh 2, Pusa Sugandh 3, Pusa Sugandh 5 and Vallabh Basmati 21 and the results are as presented in Table 3. Basmati 370 and Vallabh Basmati 21 has allele A and Pusa Sugandh 3, has allele G and allelic status at SNP2 in Vallabh Basmati 21 (T) is different from that of all tested varieties (C).

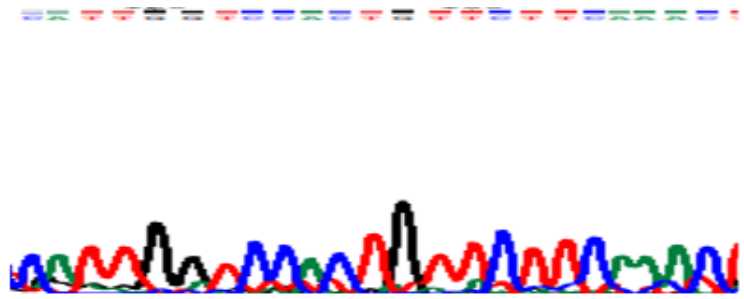
Table 6: The sequencing results of six rice varieties

S.NO	VARIETY NAME	SNP 1	SNP2
		G/A	C/T
1	Basmati 370	A	C
	Vallabh Basmati 21	A	T
2	Haryana Basmati	G	C
3	Pusa Sugandh 2	G	C
4	Pusa Sugandh 3	G	C
5	Pusa Sugandh 5	G	C

Fig: 1 Sequence of Basmati 370 and Pusa Sugandh 3 at SNP1 region along with the sequence alignment to reference sequence.

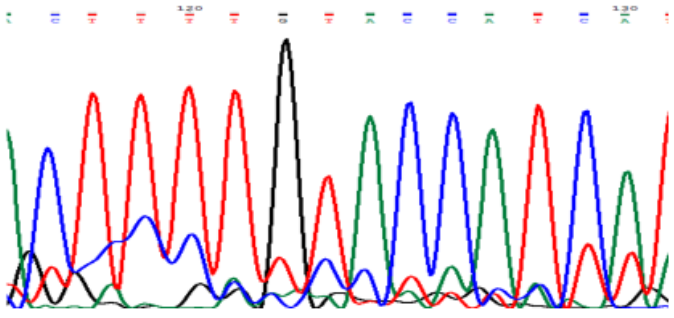


PS3



```
Query  80   CTGTCTCTGGGTC-TTGCTCTCCC-GCC-GGCACTTCACCATTGGTCCACTGTTCTTCAA  136
           || |||||||||| |||||||||| || |||||||||||||||| ||||||||||||||||
Sbjct  168  CTTTCTCTGGGTCCTTGCTCTCCCATCCAGGCACTTCACCATTGATCCACTGTTCTTCAA  227
```

VB21



```
Query  85   CATTATTTATTTATTTTGGATGAAATATAAAACTTTTGTACCATCATAATTTAGCATACA  144
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  CATTATTTATTTATTTTGGATGAAATATAAAACTTTTGTACCATCATAATTTAGCATACA  457
```

FIG 2: Nucleotide order of VB21 which varies from other Basmati varieties

ARM PCR FOR SNP2:

ARM PCR was designed to differentiate B370 and PS3 ,but the SNP shows same allele(C),hence it needs more screening .

OS03G0802600

The SNP T/A in this gene is found to be associated with differences in amylose content in rice. SNP carrying region is amplified usingKA-AC-802600F and KA-AC-802600R primers in the same 6 varieites and given for sequencing with the KA-AC-802600F primer. The results presented in Table 7 shows that there is no sequence variations in all these varieties.

S.N0	**BASMATI VARIETY**	T/A
1	Basmati 370	T
2	Haryana Basmati	T
3	Pusa Sugandh 2	T
4	Pusa Sugandh 3	T
5	Pusa Sugandh 5	T
6	Vallabh Basmati 21	T

VB21

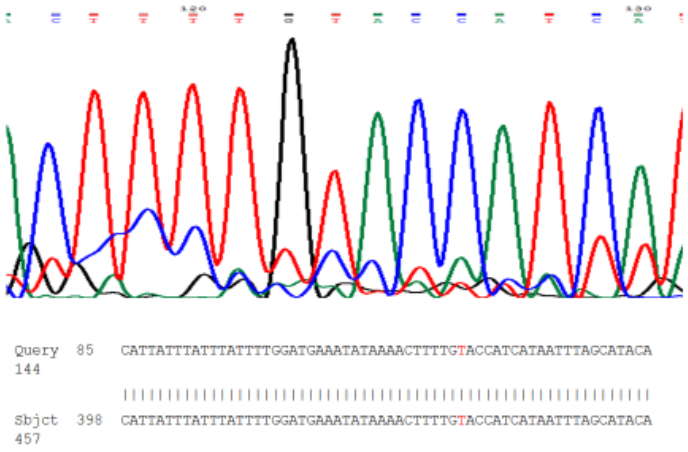


FIG : 4 The SNP variant in this VB21 is T and it is same in all other types .

0S03G0802050

The SNP reported in this gene C/A is studied in the same varieties using KA-AC-802050F and KA-AC-802050R primers and the PCR product was sequenced. The results are presented in Table 7 and all the varieties have the same base ‘C’ at this position.

S.NO	BASMATI VARIETY	C/A
1	Basmati 370	C
2	Haryana Basmati	C
3	Pusa Sugandh 2	C
4	Pusa Sugandh 3	C
5	Pusa Sugandh 5	C
6	Vallabh Basmati 21	C

VB21

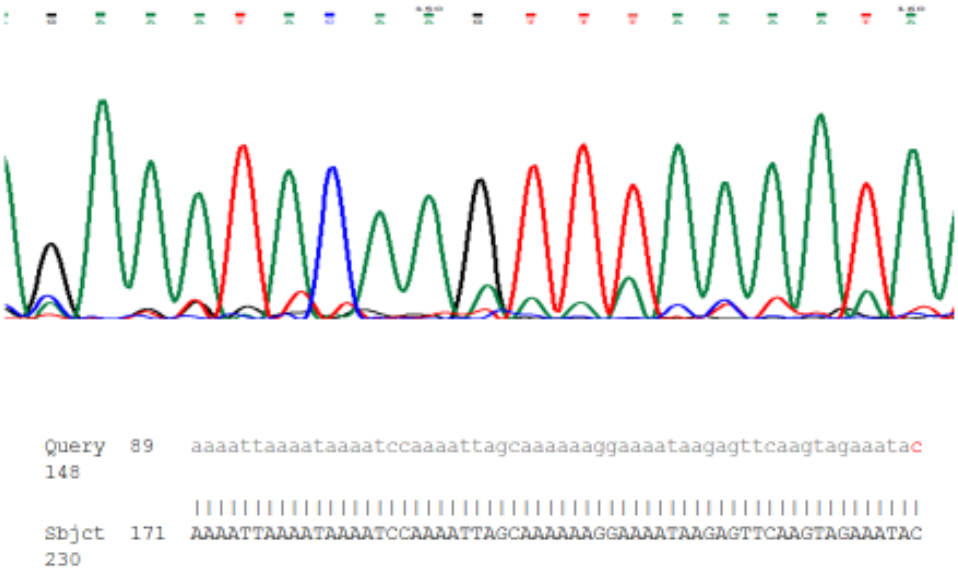


FIG : 5 In all the six varieties, the SNP was found to be C.

4.3 *Badh1*

Betaine aldehyde dehydrogenase (*BADH*) is a key enzyme involved in the synthesis of glycinebetaine a powerful osmoprotectant against salt and drought stress in a large number of species. Rice is not known to accumulate glycinebetaine but it has two functional genes coding for the *BADH* enzyme.(Anuradha Singh et al. 2010) A minor quantitative trait locus (QTL) for aroma has been mapped on rice chromosome 4 that is co-localized with the *BADH1* gene A nonfunctional allele of the *BADH*2 gene located on chromosome 8 is a major factor associated with rice aroma.

Rice has two functional genes coding for the *BADH* enzyme: *BADH1* gene located on chromosome 4 and *BADH2* gene on chromosome 8. Both the genes have 15 exons that show high sequence homology to their orthologs in other species. Rice *BADH*1 is induced by salt and water stresses whereas the

BADH2 gene is expressed constitutively at low levels. Expression of both genes also appears to be regulated by post-translational processing directed by paired short direct repeat s in response to stress (Niu et al. 2007).

One SNP (T/A) present in the exons of Badh1 gene were selected to analyze the SNP variations in 30 varieties of Basmati rice. Allele specific PCR approach was used to genotype the varieties for this SNP (Table 8).

S.NO	**BASMATI VARIETY**	**T/A**
1	BASMATI 370	T
2	TYPE 3 BASMATI	T
3	RANBIR BASMATI	T
4	TAROARI BASMATI	T
5	BASMATI 386	T
6	BASMATI CSR 30	T
7	HARYANA BASMATI	T
8	PUNJAB BASMATI 2	T
9	KASTURI	T
10	PUNJAB BASMATI 3	T
11	MAHI SUGANDHA	T,A
12	VALLABH BASAMTI 21	T
13	BASMATI 564	A
14	PUSA BASMATI 1121	A
15	PUSA BASMATI 1509	A
16	PUSA BASMATI 1718	A
17	PUSA BASMATI 1401	A
18	PUSA BASMATI 1460	A
19	PUSA BASMATI 1609	A

20	PUSA BASMATI 1	A
21	PUSA SUGANDH 2	A
22	PUSA SUGANDH 3	A
23	PUSA SUGANDH 5	A
24	PANT BASMATI 1	A
25	PANT BASMATI 2	A
26	SHARBATHI	T,A
27	MALAVIYA BASMATI	T,A
28	VALLABH BASMATI 22	A
29	VALLABH BASMATI 23	A,T
30	VALLABH BASMATI 24	A

All the Traditional Basmati varieties have T while most of evolved varieties have A. Allelic status of Pusa Basmati 1121, Pusa Basmati 1718 is same as that in Pusa Sugangh2 and Pusa sugaadh 5.

Allele-specific PCR for SNP (A/T) in BAdh1 gene in Basmati and non-Basmati varieties.

Figure:6 Lane 1,2 – Basmati 370, Lane 3,4 – PB1121, Lane 5,6 - PB1509, 7,8-PS2, Lane 9,10 – PS3, Lane 11,12 - PB1718, Lane 13,14-Katuri, Lane 15,16 – Sharbati, Lane 17,18 – VB21, Lane 19,20 – PS5, Lane 21,22 - Type3, Lane 23,24- Ranbir Basmati,Lane 25,26-Taroari Basmati,Lane 27,28 – Basmati

CSR30,Lane 29,30- Punjab Basmati 3,Lane 31,32-PB1609,Lane 33,34-Haryana Basmati,Lane 35,36- Punjab Basmati 2,Lane 37,38- Pusa Basmati 1,Lane 39,40 –PB1401,Lane 41,42- PB1460,Lane 43,44- Mahi Sugandha,Lane 45,46- VB24,Lane 47,48- VB23,Lane 49,50 – VB22,Lane 51,52 – Pant Basmati 1,Lane 53,54- Basmti 386,Lane 55,56- Basmti 564,Lane 57,58- Pant Basmati 2 ,Lane 59,60 – Malaviya Basmati.

Primers

KA-BADH1-R(reverse primer for both SNP)

KA-BADH1-T

1,3,5,7,9,11,13,15,17,19,21,23,25,27,29,31,33,35,37,39,41,43,45,47,49,51,53,55,57,59.

KA-BADH1-A-

2,4,6,8,10,12,14,16,18,20,22,24,26,28,30,32,34,36,38,40,42,44,46,48,50,52,54,56,58,60

4.4 qSS7 – OS07G41200

Seed shape in rice (Oryza sativa) is an important factor that determines grain appearance, cooking quality and grain yield. A major quantitative trait locus qSS7 on the long arm of chromosome 7 for seed length, seed width and the ratio of seed length to width are reported in a rearch paper (Xianjin Qiu et al.2012)and it is used to segregate population derived from an indica variety and japonica variety.There are two nucleotide substitutions in the coding region of LOC_Os07g41200 which changes the predicted amino acid and function of protein. In LOC_Os07g41210, there are four nucleotide substitutions in the coding region which changes the predicted amino acids, one of which, a single nucleotide transition (G-to-A), results in a Tryptophan codon (TGG).

To find the different nucleotide substitutions in qss7 gene,DGS220 INDEL Marker was used to amplify the DNA samples.They showed different alleles in their chromosome 7 .

The following 20 samples(TABLE 9) are used to screen the qss7 gene by using DGS220 marker.DGS220 Marker was already reported by Umakanta Ngangkham as a good marker to fiind out the qss7 gene mutation.(Umakanta Ngangkham et al.2018)

S.N0	**BASMATI VARIETY**	**ALLELE SIZE (bp)**
1	BASMATI 370	350
2	PUSA BASMATI 1121	300, 485
3	PUSA BASMATI 1509	485
4	PUSA SUGANDH 2	485,750
5	PUSA SUGANDH 3	485,750
6	PUSA BASMATI 1718	485
7	KASTURI	474
8	SHARBATI	474
9	VALLABH BASMATI	485
10	PUSA SUGANDH 5	485,750
11	TYPE 3 BASMATI	474
12	RANBIR BASMATI	474
13	TAROARI BASMATI	474

14	BASMATI 386	474
15	BASMATI 564	474,485
16	BASMATI CSR 30	474
17	PUNJAB BASMATI 3	485
18	PUSA BASMATI 1609	485
19	HARYANA BASMATI	474
20	PUNJAB BASMATI 2	474

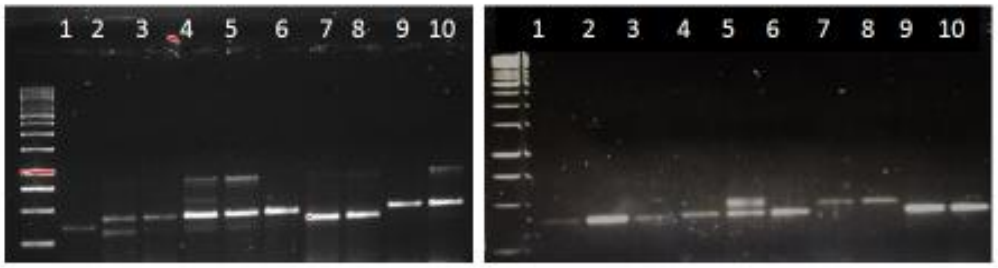


Figure7 Image of ten rice varieties along with 1kb molecular weight size standard.

Lane 1,2: B370,Lane 3,4:PB1121,Lane 5,6 : PS2 ,Lane 7,8: PS3, Lane 9,10: PS5, lane 11,12: Type 3,Lane 13,14m: RB, Lane 15,16 :PB1718,Lane 17,18: VB21,Lane 19,20 : HB.

Primers :

DGS220F ,DGS220R.

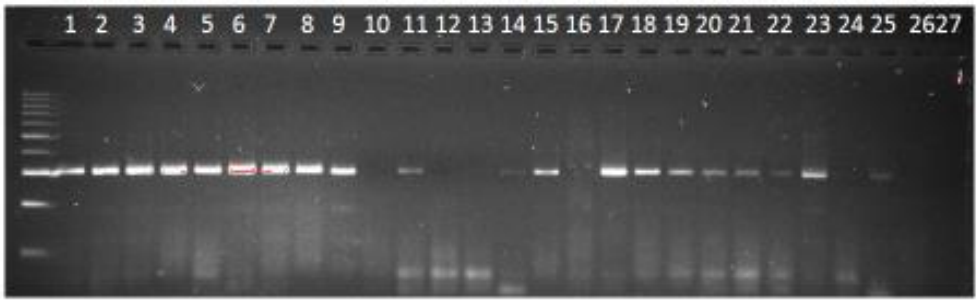
The base pair differences between the varieties are identified and they will be screened for detecting SNPs by designing allele specific PCR in my future work.

4.5 SECOND OBJECTIVE

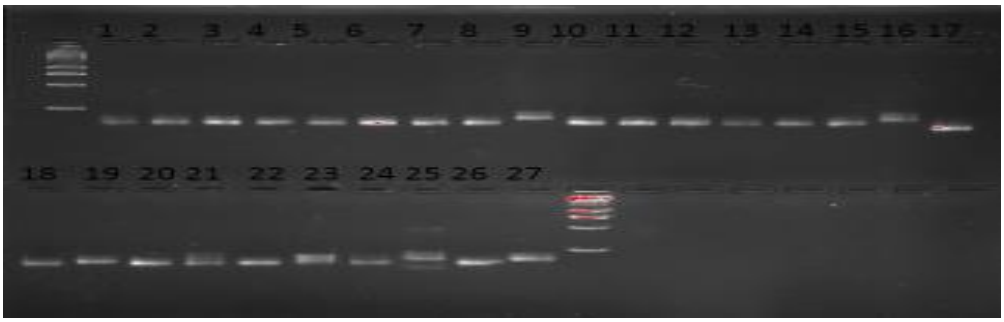
The second objective of my present work is to find new SSR Markers for detecting adulterations in Basmati varieties. SSR markers are co-dominant, multi-allelic and can be reliably used to analyze both indica and japonica germplasm and facilitates the integration of results from independent studies. In addition, the highly polymorphic nature of many microsatellites is of particular value when analyzing closely related genotypes, as is often the case in breeding programs working within narrowly adapted gene pools. Thus, the availability of a high-density SSR map is valuable as a public resource for studies aiming to interpret the functional significance of the rapidly emerging rice genome sequence information.(Rajeev.K et al.2004). SSR markers RM28067, RM1739, RM1340, RM1350 are used to screen 27 Basmati varieties along with one non-Basmati varieties.

Fig8 Screening of Basmati and non-Basmati varieties with RM28067, RM1739, RM1340, RM1350

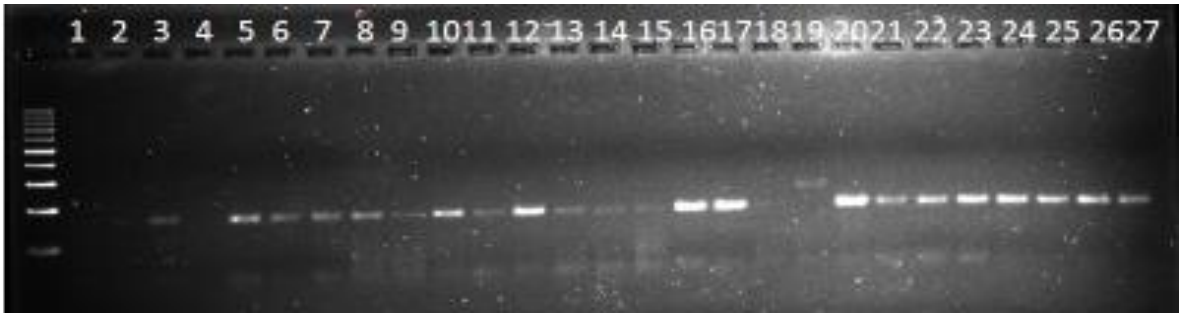
RM28067



RM1739



RM1350



RM1340

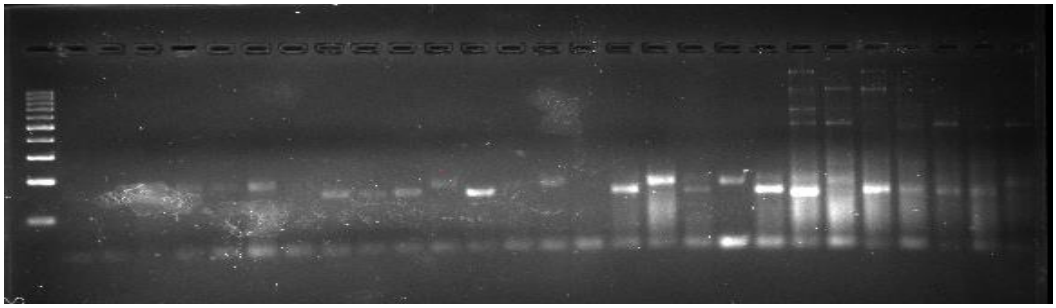


TABLE 10- SSR MARKER PROFILE

BASMATI VARIETIES	RM28067(1)	RM1379	RM1340 (3)	RM1350(2)
B370	1	4	3	2
TYPE 3	1	1	1	1
RANBIR BASMATI	1	1	1	1
TARAORI BASMATI	1	1	1	1
B386	1	1	1	1
BCSR30	1	1	1	1
HARYANA BASMATI	1	1	1	1
PUNJAB BASMATI 2	1	1	2	1
KASTURI	1	1	1	1
MAHI SUGANDHA	1	1	1	1
PUNJAB BASMATI 2 ASMATI 3	1	1	1	1
B564	1	1	1	1
MALAVIYA BASMATI	1	2	1	1
VB23	1	1	2	1
VB21	1	1	2	2
PB1121	1	1	2	1
PB1509	1	2	1	1
PS2	1	4	1	1
PS3	1	2	1	1
PB1718	1	1	1	1
PS5	1	4	1	1
PB1401	1	2	1	1
PB15460	1	1	2	1
PB1609	1	2	1	1

VB22	1	1	1	1
VB24	1	4	1	1

RM28067 is monomorphic in all rice varieties while other three markers are polymorphic. However these three markers are monomorphic in Traditional rice varieties aand polymorphic in Evolved varieties.

Based on the SSR Marker profile, it is easy to analyze the alleles in each different variety. But still it requires a very large screening to differentiate basmati from non basmati variety. These SSR Markers are chosen based on their high index of polymorphic content. None of the markers gave a unique allele in non-Basmati variety. This result suggests these varieties need to be screened with large number of SSR markers to get profiles which are different for Basmati and non-Basmati varieties.

5.CONCLUSION

- To search in literature if SNPs are available in some more genes this can test for differentiating PB 1121 and Pusa Sugandh2/5.
- To work on more SSR Markers to find suitable makers for detecting adulteration in Basmati Rice.

S.NO	VARIETY NAME	717600		802600	802050	*Badh1*
		G/A	C/T	T/C	C/A	T/A
1	BASMATI370 VB21	A A	C	T	C	T T,A
2	PS2	G	C	T	C	A
3	PS3	G	C	T	C	A
4	PS5	G	C	T	C	A
5	HARYANA	G	C	T	C	T

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