INTROGRESSION OF *Saltol* GENE INTO RICE VARIETY SREYAS

ANJU MARIAM JOSEPH

(2018-21-024)

DEPARTMENT OF PLANT BREEDING AND GENETICS

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2023

DECLARATION

I, hereby declare that this thesis entitled **"INTROGRESSION OF *Saltol* GENE INTO RICE VARIETY SREYAS"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

Anju Mariam Joseph

(2018-21-024)

Vellayani,

Date: 09/02/2023

CERTIFICATE

Certified that this thesis entitled **"INTROGRESSION OF *Saltol* GENE INTO RICE VARIETY SREYAS"** is a record of research work done independently by Mrs. Anju Mariam Josph (2018-21-024) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellayani
Date: 09/02/2023

Dr. Arya K
(Major advisor, Advisory Committee)
Professor and Head
Department of Plant Breeding and Genetics
College of Agriculture,
Vellayani, Thiruvananthapuram

CERTIFICATE

We, the undersigned members of the advisory committee of Mrs. Anju Mariam Joseph (2018-21-024), a candidate for the degree of **Doctor of Philosophy in Agriculture** with major in Plant Breeding and Genetics, agree that the thesis entitled **"INTROGRESSION OF *Saltol* GENE INTO RICE VARIETY SREYAS"** may be submitted by Mrs. Anju Mariam Joseph (2018-21-024), in partial fulfilment of the requirement for the degree.

Dr. Arya K.
Professor and Head
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

Dr. Veena Vighneswaran
Assitant Professor
Plant Breeding & Genetics
Rice Research Station, Vyttila,
Ernakulam- 682 019

Dr. Beena Thomas
Assistant Professor
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani.
Thiruvananthapuram-695 522

Dr. Roy Stephen
Professor
Department of Plant Physiology
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

Dr. Swapna Alex
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

EXTERNAL EXAMINER

INTROGRESSION OF *Saltol* GENE INTO RICE VARIETY SREYAS

by

ANJU MARIAM JOSEPH

(2018-21-021)

THESIS

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COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2023

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid
AFLP	Amplified Fragment Length Polymorphism
AgNO3	Silver Nitrate
APS	Ammonium Per Sulphate
BC_1	First Backcross generation
BC_2	Second Backcross generation
bp	Basepair
cM	Centi Morgan
cm	Centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribose Nucleic Acid
^oC	Degree Celsius
dSm^{-1}	Deci Seimens per metre
DP	Donor Parent
EC	Electrical Conductivity
dNTPs	Deoxy Nucleoside Triphosphates
EDTA	Ethylene Diamine Tetra Acetic Acid
et al	And others
FAO	Food and Agriculture Organization
Fig.	Figure
F_1	First filial generation
F_2	Second filial generation
GGT	Graphical Geno Types
g	Gram
h	Hours
Ha	Hectare

i.e.	That is
IRRI	International Rice Research Institute
KAU	Kerala Agricultural University
kg ha-1	Kilogram Per Hectare
M ha	Million ha
MAB	Marker Assisted Breeding
MABC	Marker Assisted Backcrossing
MABB	Marker Assisted Backcross Breeding
Mb	Mega Base
µg	Microgram
µM	Micromolar
mm	Millimetre
mM	Millimolar
ml	Milli litre
Na+/H+	Sodium–Hydrogen Antiporter
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nanometer
No.	Number
%	Per cent
pH	Potential of Hydrogen
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random-Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RG	Recurrent genome

RIL	Recombinant Inbred Line
RP	Recurrent Parent
SES	Standard Evaluation Score
Sl.	Serial
SNP	Single Nucleotide Polymorphism
SOS	Salt Overly Sensitive Regulation Pathway
SSR	Simple Sequence Repeats
STR	Short Tandem Repeat
Ta^0	Annealing Temperature
TBE	Tris-Borate-EDTA
TE	Tris EDTA
TEMED	N,N,N'N'-Tetramethylethylenediamine
Temp	Temperature
Tm^0	Melting Temperature
Tris	Tris (hydroxymethyl) Amino Methane
v/v	Volume/Volume
viz.	Namely
via	Through

Introduction

1. INTRODUCTION

The phenomenon of soil salinity affecting agricultural lands and human civilization has records dating back to Mesopotamian civilization. Flooding, over-irrigation, seepage, silting, and a rising water table are considered to be the main reasons for increased salinization (Gelburd, 1985)

Soil salinization has been identified as a primary contributor to land degradation, rendering fields unfit for crop development. According to the FAO (2011), over 800 million ha of global land has been seriously contaminated with salt and over 20% of irrigated areas (approx. 45 million ha) are reported to be affected by varying degrees of salinization. This is more important because one-third of the world's food production is in irrigated areas. If soil salinization continues at its current rate, 50% of cultivable land will be destroyed by 2050 (Hasanuzzaman *et al*., 2014). Due to soil salinization, India has lost 16.84 million tonnes of farm products including cereals, oilseeds, pulses and cash crops annually valued at INR 230.20 billion (Mandal *et al*., 2018).

Rice (*Oryza sativa* L.), the staple food for over one-third of the world's population is considered a typical glycophyte, sensitive to salinity, particularly during seedling and reproductive stages. It is also one of the few crops that can thrive on salt-affected soils because of its ability to grow well in standing water that can help leach salts from topsoil and is, therefore, recommended as an entry crop for desalinization of salt-affected lands (Singh and Flowers, 2010). However, the productivity of rice is greatly affected due to soil salinity.

Rice is cultivated on 43.86 million hectares in India, with a production of 104.80 million tons and productivity of around 2390 kg ha^{-1} (Department of Agriculture, Cooperation and Farmers Welfare, 2020). About 8.4 million ha of rice cultivated area in India is salinity-affected. The state of Kerala produces 5.87 lakh tonnes of rice from 1.98 lakh ha with a productivity of 2964 kg ha^{-1} (Farm Guide, 2022). In Kerala, 23

agro-ecological units (AEU's) have been delineated for the state based on climatic variability, landforms and soils. Among these 7 AEU's *viz.,* Southern coastal plain (AEU 1), Northern Coastal plain (AEU 2), Onattukara Sandy plain (AEU 3), Kuttanad (AEU 4), Pokkali (AEU 5), Kole lands (AEU 6) and Kaipad lands (AEU 7) are low lying areas prone to sea water inundation or capillarity and resultant salinity. These AEU's account for the major rice tract of Kerala revealing the importance of saline tolerant rice varieties.

In Kuttanad region, often known as the Rice Bowl of Kerala, the additional crop season generates an important share of farmers' income. The intrusion of seawater forces the farmers to delay sowing. Due to delayed sowing, harvesting will coincide with the commencement of the south west monsoon. This leads to an increase in the moisture content of the grains resulting in viviparous germination of the grains. Most of the farmers of this region are leased land cultivators and cannot afford crop loss or yield reduction, especially in this season. Hence it is imperative to develop varieties tolerant to salt stress, suited to Kuttanad for the benefit of the farmers. MO-22 [MO-13 (Pavithra x Triguna,)] commercially released as Sreyas from Rice Research Station, Moncombu is a high yielding (7-7.5 t ha^{-1}) rice variety on par with Uma, the most popular variety cultivated in the state of Kerala. It is non-lodging, medium duration with resistance to BPH, gall midge, moderate resistance to BLB, sheath blight and sheath rot. Sreyas is a variety that is gaining acceptance among farmers. Introgressing the QTL for salt resistance in the background of the variety Sreyas will improve its suitability for cultivation in the Kuttanad region.

Salinity is a soil condition characterised by a high concentration of soluble salts. Soils are classified as saline when EC is 4 dSm^{-1} or more (USDA, ARS, 2008), equivalent to approximately 40 mM sodium chloride. For rice, when the EC is approximately 4 dSm^{-1} the soil is moderately saline; when EC is 8 dSm^{-1} the soil is highly saline. There are two types of salinity: inland salinity, which is due to irrigation

practices with sloppy water, and coastal salinity, mainly due to high ocean tides in the coastal region.

Susceptibility or tolerance of rice plants to high salinity is a co-ordinated action of multiple stress responsive genes which also interact with other components of stress signal transduction pathways. Reproducible differential manifestation in plants with respect to their morphological, physiological or molecular parameters in response to salt stress qualifies for a reliable screening criterion. An ideal high yielding salinity tolerant variety should possess the ability to withstand high amount of sodium, tissue tolerance, minimum per day uptake of sodium, high uptake of potassium per day, good initial vigour, agronomic superiority with high yield potential.

Many salinity tolerant QTLs having identified in rice. A major QTL responsible for salinity tolerance was identified from the land race of Kerala, Pokkali. Pokkali is salt tolerant and grows naturally in the saline tracts of Alappuzha, Ernakulam and Thrissur districts of Kerala. The rice cultivation practice of this tract is organic and is called Pokkali cultivation. The QTL identified from Pokkali, *Saltol* was mapped on chromosome 1 of the Recombinant Inbred Line (RIL) obtained from a cross between IR-29 and Pokkali at IRRI, Philippines. This RIL tagged FL478 is used as a donor for *Saltol* introgression in popular rice varieties. This QTL is responsible for maintaining low Na^+, high K^+ and Na^+ / K^+ homeostasis in shoots of rice (Waziri *et al*, 2016). *Saltol* QTL has been mapped between 10.7 and -12.2 Mb on short arm of chromosome 1.

In this context the present study entitled “Introgression of *Saltol* gene into rice variety Sreyas” was envisaged with the following objectives:

- Introgression of Saltol QTL from the donor parent FL478 to the recurrent parent Sreyas through Marker Assisted Backcross Breeding.
- Phenotypic screening of introgressed lines for abiotic stress tolerance.
- Genotypic screening of introgressed lines for abiotic stress tolerance.

Review of Literature

2. REVIEW OF LITERATURE

2.1. SOIL SALINITY

Soil is considered saline (Szabolcs, 1989) when it contains large quantities of NaCl, as well as soluble SO_4^{2-}, CO_3^{2-} or halide compounds of other minerals such as Ca, Mg, K, Fe, B etc. Soil salinity is frequently described in phrases of electric conductivity (EC) based on the concept that a salt solution causes more electricity to flow through it.

Soil salinity may result from surface water, groundwater, the interaction between them, and the levels of salt they carry. It may be affected by changes in land use, seasonal variations and climate changes. Human-induced salinization may result from land use, particularly the use of high-salt irrigation water or poor drainage. Primary salinity is found in arid and semi-arid areas in the form of ancient salt deposits, fossil salts, and saline groundwater, where salts are drawn to the surface of the soil by capillary action (Carter, 1975; Flowers, 1999). Secondary sources, such as irrigation, might cause soil to become saline in a specific location. Residual salts from water and soil amendments, animal wastes, chemical fertilizers, applied sewage sludge, and the disposal of gas and oil field brines are some of the other causes of secondary salinization.

Tidal estuaries in coastal regions may be a source of salts due to seawater inundation in low-lying areas near the coast. The intrusion of seawater into rivers and aquifers, as well as tidal overflow, cyclones, and tsunamis, makes the area in close proximity salinity-prone (Flowers, 1999). Natural processes such as weathering soil of parent material, deposition of sea salt transported by wind and rain, and inundation of coastal land by tidal water are the main causes of coastal salinity. In Kerala, coastal salinity accounts for the major share of salt stress induced crop loss.

2.2. SALT STRESS ON PLANT GROWTH AND DEVELOPMENT

Plants can respond to various stress as individual cells which interacts synergistically as the whole organism. Salt stress has a variety of effects on plant development, including ion toxicity, nutritional imbalances, and osmotic activities. The extent of inhibition generated by different pathways varies depending on species, plant stage, salinity ionic strength, and organs in question. The plant cell (Munns, 2002) shrinks and dehydrates immediately after being exposed to salt stress, but it recovers within a few hours. Despite this improvement, cell elongation and, to a lesser extent, cell division is impacted, resulting in a slower pace of root and leaf growth. A week after salinity stress, lateral shoot enlargement is impacted, and a month later, substantial variations in overall growth and injury between salt-stressed plants and their non-stressed controls can be observed.

2.2.1. Osmotic phase of salinity stress

Changes in the cell-water relationship results from osmotic changes outside the root cells which is the initial impact on plant known as the osmotic phase. The osmotic effect of salinity is similar to water stress (Munns, 2005). The osmotic pressure in the soil solution surpasses that of plant cells during salt stress, decreasing the plant's ability to absorb water and minerals vital to maintain cellular homeostasis such as K^+ and Ca^{2+} (Glenn *et al.*,1997). The soil solution may become hyper-osmotic in extreme circumstances, leading the root cells to lose water rather than absorb it. Osmotic stress disrupts the water equilibrium in the cell, causing water loss that slows cell elongation, division, stomatal closure, and leaf area, as well as photosynthesis and growth (Hasanuzzaman *et al.,* 2014). The decrease in growth could be related to a decrease in cell elongation. Plant height, survivability, and biomass can be affected by salt stress. The ability of a plant to harness light, water and nutrients is affected by such morphological alterations (Locy *et al.,* 1996).

2.2.2. Ionic phase of salinity stress

The presence of a high salt concentration (NaCl) near the root zone also inhibits plant growth due to ionic toxicity caused by the accumulation of Na^+ and Cl^-. In response to the extended salinity phase, the inhibition of growth over a period of time and premature senescence of those older leaves results, which was termed the ionic phase (Munns and Tester, 2008). Furthermore, Na^+ inflow causes chlorosis, necrosis, and premature senescence of adult leaves, limiting the photosynthetic surface available to sustain salt-affected plants' continuing growth (Hasegawa *et al.,* 2000). Maintenance of plant and shoot water status, as well as mechanisms like Na^+ exclusion or maintenance of K^+ in developing tissues and rapidly growing leaves, contribute to salt tolerance in rice varieties (Yeo *et al.,* 1990).

2.3. EFFECT OF SALINITY ON GROWTH STAGES OF RICE

Rice perceives soil with 4 dS m^{-1} EC to be moderately saline and 8 dS m^{-1} to be highly saline. Depending on the severity and duration of the stress, salinity stress causes alterations in numerous physiological and metabolic systems in rice, which eventually restrict crop output (James *et al.,* 2011). Ologundudu *et al.,* (2014) examined eight rice cultivars at varying salt levels (0–15 dS m^{-1}) and observed that root and shoot length, root and shoot dry weight, and total dry matter production declined with increasing levels of salt.

Among the 4 categories of plants *viz.* tolerant, moderately tolerant, moderately sensitive and sensitive to salinity, rice is categorized as sensitive with a threshold of 3 dS m^{-1} (Mass and Hoffman, 1977). Rice plants respond differently to salt stress at different growth stages (Moradi and Ismail, 2007). In the lifecycle of rice, relative tolerance is exhibited at germination. As growth progresses to the early seedling stage (1-3 weeks) the tendency leans to sensitivity. During active tillering stage, the crop

shows comparative tolerance. The reproductive phase extending from panicle initiation to flowering and fertilization exhibits sensitivity with a severe reduction in yield and quality parameters. The plants show relative tolerance during maturity (Sajid *et al.,* 2019).

2.3.1. Effects of salinity on Germination

Rice plants are anticipated to show relative tolerance at the germination stage. Reportedly germination which can last for 2-3 days is not significantly affected up to 16 dSm^{-1} (Khan *et al.,* 1997). Ologundudu *et al.,* (2014) observed that at 5dSm^{-1}, both seedling-stage tolerant and sensitive genotypes recorded up to 90 per cent germination while at 10 dSm^{-1} tolerant rice genotypes showed 80% germination as against susceptible genotypes which showed a 50% reduction in germination.

2.3.2. Effects of salinity at seedling and early vegetative phase

Seedling and early vegetative stages are considered the most significant salt-sensitive stages of rice along with the reproductive phase (Singh and Flowers, 2010). Seedling stage tolerance is targeted in genetic improvement programmes to develop salt-tolerant lines. Salinity stress (Reddy *et al*., 2017) affects seed germination, seedling growth, leaf size, shoot growth, shoot and root length, shoot dry weight, shoot fresh weight, number of tillers per plant, flowering stage, spikelet number, per cent of sterile florets and productivity.

2.3.3. Effects of salinity at the reproductive phase

Salinity affects yield components such as panicle length, spikelet number per panicle, grain yield and also delays panicle emergence and flowering (Zeng and Shannon, 2000). The mean shoot dry weight at 45 DAS, plant height, number of tillers per plant, panicle length, no. of grains per panicle, grain yield per plant, 1000 grain weight, shoot dry weight at maturity and grain straw ratio were observed to be significantly reduced by salt stress (Mahmood *et al.,* 2009). Increased spikelet sterility

and reduced thousand-grain weight are major impacts of salt-induced stress (Clermont-Dauphin *et al.,* 2010).

However, there are some traditional cultivars and landraces which are naturally tolerant to salt stress due to their adaptation to thrive on saline soil. They generally have poor agronomic characteristics such as tall plant stature, poor grain quality, low yield, and photosensitivity (Ismail *et al.,* 2007). The search for salt-tolerant lines has led to the identification of large variability and numerous such accessions in various germplasm collections (Singh *et al.,* 2021). One of the traditional cultivars, Pokkali has been recognized as a high-potential salt tolerance donor.

2.4. QTLs FOR SALINITY TOLERANCE

Salinity tolerance is the physiological manifestation of a combination of stress-responsive genes. Several attempts have been made to find QTLs connected to salinity tolerance traits. Mapping of QTLs has elaborated the genetic control of the salt tolerance mechanism with possibilities to develop salt-tolerant varieties by precisely transferring QTL into elite varieties. Gregorio *et al.* (2002) mapped a major QTL designated *Saltol* on short arm of chromosome 1 (flanked by SSR markers RM23 and RM140) using a RIL population generated from a cross between IR29 and Pokkali. Niones (2004) fine mapped the common QTL region of *Saltol* in BC_3F_4 Near-isogenic lines (NIL) of IR29/Pokkali. In addition to the major QTL, 7 QTLs including three for Na^+ uptake, two for K^+ uptake and two for Na^+/K^+ ratio were detected on chromosomes 3,4,10 and 12. Two other QTLs with relatively large effects flanked by RM25 and RM210 on chromosome 8, and RM25092 and RM25519 on chromosome 10 were also identified (Islam *et al.,* 2011).

Several QTLs for salinity tolerance have been mapped on different chromosomes in rice in various studies. A few such QTLs along with their major functions are listed in Table 1.

Table 1: QTLs associated with salinity tolerance

Name of QTL	Chr. number	Function
qSKC1	1	OsHKT1-5, a HKT family ionic antiporter that mediates Na+ reabsorption (Rus *et al.,* 2006) 40.1 per cent of the phenotypic variance in shoot K+ concentration (Jena and Mackill, 2008)
qSNC7	7	Regulation of shoot Na+ concentration (Thomson et al., 2010)
QKr1, QKr2	1,2	K+ content appearing to be the most promising, accounting for 30% variance (Ahmadi and Fotokian, 2011)
qSDS1, qSDS6, qSDS7	1, 6,7	Regulates the survival days of the seedlings (Cheng *et al.,* 2012)
qRKC4, qRKC7	4,7	Regulation of K^+ concentration in roots (Zheng *et al.,* 2015)
qST1, qST3	1, 3	Salinity tolerance (Zheng *et al.,* 2015)
qRNC9	9	Regulation of Na+ concentration in roots (Zheng *et al.,* 2015)

2.5. *Saltol* – A MAJOR QTL FOR SALINITY TOLERANCE

2.5.1. Origin of *Saltol*

The major locus providing seedling stage salinity tolerance, *Saltol* was mapped on chromosome 1 in an F8 Recombinant Inbred Line (RIL) population obtained by a cross between salt-tolerant Pokkali and salt-sensitive IR29 at the International Rice

Research Institute (IRRI) in their salt stress tolerance breeding program (Gregorio *et al.,* 1997). One of the lines identified from the RIL population, IR 66946-3R-178-1-1 also known as FL478, showed salt tolerance higher than or comparable to the tolerant parent, Pokkali. FL478 is used as a donor of seedling stage salinity tolerance, more precisely *Saltol* QTL in various breeding programmes.

2.5.2. Structure of *Saltol*

The *Saltol* region in FL478 has a 1Mb DNA fragment from Pokkali at 10.6–11.5 Mb on chromosome 1, flanked by IR29 alleles, as per the analysis of single feature polymorphisms in the *Saltol* locus (Kim *et al.,* 2009). The genomic region where the QTL is located contains a major gene found to possess three common QTLs for maintaining low Na^+ uptake, high K^+ uptake and Na^+/ K^+ homeostasis in shoots conferring seedling stage salinity tolerance. Its location was confirmed on chromosome 1 after the analysis that has been done for 100 Simple Sequence Repeat (SSR) markers on 140 IR29/Pokkali Recombinant Inbred Lines (RILs). The *Saltol* QTL relates 10.8 – 12.3 Mb on chromosome 1 of Pokkali explaining about 64.3-80.2 % of the variability in the shoot Na^+/K^+ ratio (Thomson *et al.,* 2010). In an expression profiling study of genes localized within the Saltol QTL, SSR markers between RM 1287 and RM 6711(10.8 Mb to 16.4Mb) had been carried out in the two contrasting genotypes (Soda *et al.,* 2013).

2.5.3. Annotation of loci within *Saltol*

The *Saltol* QTL is found to house 783 loci (Soda *et al.,* 2013) and the genes retrieved were categorized into 14 groups. These genes were found to be involved in diverse cellular activities such as metabolism, development, DNA processing etc (Table 2). The genes present within Saltol QTL control versatile aspects of cell survival not only under salt stress but also under non-stress conditions.

Table 2: Functional classification of annotated genes within *Saltol* QTL

Sl. No.	Functional categories	Percentage
1.	Retrotransposons and transposons	31%
2.	Proteins of unknown function	25%
3.	Development	12%
4.	Metabolism	7%
5.	Cellular transport related genes	5%
6.	Cell rescue, defence and virulence related genes	4%
7.	Protein with binding function	3%
8.	Cell cycle and DNA processing	2%
9.	Protein fate	2%
10.	Signalling	2%
11.	Interaction with environment	2%
12.	Transcription factors	2%
13.	Protein synthesis	1%
14.	Energy	1%

2.5.4. Gene expression in *Saltol* region

The Saltol QTL was found to be associated with Na+/K+ ratio and seedling stage salinity tolerance (Bonilla *et al.,* 2002) and accounted for low Na+ absorption, high K+ absorption, and low Na+-to-K+ ratio in rice shoots under salinity stress (Gregorio *et al.,* 2002). Two broad categories of stress-related genes include genes that encode (i) proteins involved in cellular homoestasis and protection from stress, (ii) kinases, transcription factors regulating stress signal transduction and stress-responsive gene expression (Lata and Prasad, 2011).

In genome-scale gene expression analysis between IR29 and FL478, the two genotypes expressed striking differences at the transcriptional perspective under

salinity stress. The transcript level of the gene present at locus Os01g20160 (11.46 Mb) coding cation transporter was induced during salt stress but the expression was higher in FL478 (Walia *et al.* 2005). The functional classification of the annotated genes within *Saltol* QTL revealed that signalling-related protein expression is differentially regulated. CaMbP, LEA, OsAP1 zinc finger protein and transcription factor (HBP1b) which are salinity inducible were found in the *Saltol* region. These are known to play an important role in the ABA signalling pathway of plants (Soda *et al.* 2013). Transcripts of various salt stress-induced proteins were upregulated while many constitutive proteins were downregulated. Certain transcripts such as Magnesium protoporphyrin IX monomethyl esterase (EF576502) mapped at a 9.87-Mb showed the presence of high constitutive levels under unstressed conditions in sensitive cultivar IR64 with expression level falling gradually, while Pokkali tends to show an increase in transcript level until 24 hours of salt stress, thereafter reduced sharply. Genes encoding Proteins of Unknown Function (PUFs) also known as SIFs (Salinity Induced Factors) found within the QTL were also studied for their role in providing salinity tolerance due to their differential regulation.

2.6. SALINITY STRESS RESPONSIVE MECHANISM IN RICE

It is critical to understand the basic molecular mechanisms of salt tolerance in order to impart the same in any genotype. Salt tolerance is a complicated quantitative feature that is influenced by a number of genes (Chinnusamy *et al.,* 2005). Salinity tolerance is essentially achieved by Na^+ exclusion and ion homeostasis resulting from the activation of signalling pathways. The first line of defence involves roots, the sites of perception of excess salts, which minimizes Na^+ entry into cells. The second phase of effective salt tolerance is tissue or organ protection against excessive Na^+ by exclusion from photosynthetic tissues and compartmentation in vacuoles (Ji *et al*, 2013). The ability of a plant to resist the drought component of salinity stress while maintaining leaf expansion and stomatal conductance is referred to as osmotic tolerance (Rajendran *et al.,* 2009). Sequestration of Na^+ in the vacuole, synthesis of

suitable solutes, and creation of enzymes that catalyze the detoxification of reactive oxygen species are termed as tissue tolerance.

Some important gene classes and their functions are given in Table. 3.

Table. 3. Important genes involved in salinity tolerance mechanism

Gene	Function	Reference
OsSOS1	$Na+/H^+$ antiporters	Kumar *et al.*, 2013
OsNHX1	$Na+/H^+$ antiporters	Amin *et al.*, 2016
OsHKT1, OsHKT2	$Na+/K^+$ symporter	Mishra *et al.*, 2016
OsCAX1	H^+/Ca^+ antiporter	Kumar *et al.*, 2013
OsAKT1	K^+ inward- rectifying channel	Yang *et al.*, 2014
OsKCO1	K^+ outward-rectifying channel	Kumar *et al.*, 2013
OsTPC1	Ca_2^+ permeable channel	Kurusu *et al.*, 2012
OsCLC1	Cl^- channel	Diedhiou and Golldack, 2006
OsNRT1, OsNRT2	Nitrate transporter	Wang *et al.*, 2012

The mechanisms of salt tolerance can be classified into the following groups.

1. Avoidance of stress
2. Salt transport and compartmentation at the plant level
3. Sequential adaptation to stress

2.6.1. Avoidance of stress

A. Early seedling vigour

Tolerance to salt stress at the germination stage and at seedling emergence determines better plant establishment in saline soils. Early seedling vigour is desirable

due to the high sensitivity of this stage (IRRI, 2006). It is a trait that ensures the survival of plants by avoiding stress at rather sensitive stages.

B. Restricting initial entry of salts to roots

The majority of sodium ions transported to the shoot parts in rice is through the apoplastic pathway (Krishnamurthy *et al.*, 2009). Casparian strips form a barrier to apoplastic flux thereby forcing ions to pass through the selectively permeable plasma membrane into the cytoplasm in the case of rice roots (Watanabe *et al.*, 2006). Roots protect the plant from excessive uptake of salts and filter out most of the salt in the soil while taking up water (Munns and Tester, 2008).

2.6.2. Salt transport and compartmentation at the plant level

The most important mechanism giving salt resistance is compartmentation at the plant level.

A. Partitioning in older leaves and structural tissues

The plant transports the harmful ions to the older leaves and leaf sheaths, which are willing to be sacrificed for early senescence and/or death in exchange for the preservation of young growing meristematic tissues. In stress conditions, old leaves accumulate much higher concentrations of Na^+, Cl^- and NO_3^- compared to young leaves. Under salinity, up-regulation of OsHKT1;1, OsHAK10 and OsHAK16 leads to the accumulation of Na^+ in old leaves, and increased OsNHX1 expression contributes to Na^+ compartmentalization in old leaves (Wang *et al.*, 2012).

B. Differential uptake into reproductive organs and flag leaves:

Most crop plants are susceptible to salt stress, particularly during the reproductive stage. Salt-tolerant cultivars maintain a significantly lower salt concentration in the panicle, in comparison with husks and rachis, with the lowest concentration in grains. Tolerant lines have a lower salt concentration in their flag leaf (Ahmad *et al.*, 2013).

2.6.3. Sequential adaptation to stress

Sequential adaptation to stress includes the following mechanisms.

1. Ion Homeostasis pathway
2. Synthesis of osmoprotectants or compatible solutes (sugar, alcohol, proline, quaternary ammonium compounds)
3. Upregulation of the antioxidant system during stress.
4. Signalling pathways and transcription factors
5. Stress-activated protein pathways

2.6.3.1. Ion Homeostasis Pathway

Ion homeostasis in cells is maintained by ion pumps on membranes such as antiporters, symporters, and carrier proteins (plasma membrane or tonoplast membrane). Ion homeostasis is shown by the Salt Overly Sensitive (SOS) regulation pathway which alters protein activity and gene transcription. SOS3 is a calcium-binding protein that interacts with SOS2, a serine/threonine protein kinase, and activates it (Ji *et al.,* 2013). SOS3 recruits SOS2 to the plasma membrane, where the SOS3-SOS2 complex protein kinase complex phosphorylates SOS1 to increase Na^+/H^+ antiporter function. SOS pathway is conserved in rice and a Na^+ /H^+ antiporter, OsSOS1, a functional homologue of SOS1 reduced total Na^+ content in the cell. Other SOS2 and SOS3 homologs in rice were also identified as OsCIPK24 and OsCBL4, respectively (Atienza *et al.,* 2007).

2.6.3.2. Synthesis of Osmoprotectants

Rice has been examined for the role of osmoprotectants also known as compatible solutes as they do not interfere with enzymatic activities even at high concentrations like proline, glycine betaine, mannitol, and trehalose. Proline is an amino acid required for plant primary metabolism, such as to regulate the pH of the

cell's cytosolic redox and act as an antioxidant and a singlet oxygen quencher (Lehmann *et al.,* 2010).

2.6.3.3. Upregulation of the anti-oxidant system during stress

Due to disrupted election transport systems in chloroplasts, mitochondria, and the photorespiration pathway, salt stress in plants causes greater concentrations of Reactive Oxygen Species (ROS) / intermediates such as superoxide, H_2O_2, and hydroxy-radicals. Plants use detoxifying enzymes including SOD peroxidases, catalases, and antioxidants such as ascorbate and reduced glutathione to scavenge ROS in a variety of ways (Schmidt *et al.,* 2013).

2.6.3.4. Signalling Pathway and Transcription factors

Transcription factors are integral in linking salt sensory pathways to several tolerant responses, regulating the expression levels of various genes that may ultimately influence the salt tolerance level of plants (Zhou *et al.,* 2016). High salinity increases ABA biosynthesis (Kumar *et al*., 2013). The ABA-dependent pathway includes mitogen-activated protein kinase (MAPK) cascades, calcium-dependent protein kinases (CDPK), receptor-like kinases (RLK), sucrose non-fermenting-1 (SNF1) related protein kinases, transcription factors and microRNAs (miRNAs). ABA-independent ROS scavenging system is also involved in salinity tolerance. An alternative oxidase gene in rice, OsIM1, was identified as salt responsive gene by using a differential display method, indicating the role of the alternative oxidase pathway under salinity (Kong *et al.,* 2003). ABA-independent kinases are also involved in salt stress tolerance.

2.6.3.5. Stress Activated protein pathway

Plants produce a variety of stress-responsive proteins in response to various stimuli such as heat, cold, salt, or drought. LEA and dehydrins, for example, are two

of the most important (Bray, 1997). In rice plants, they are said to serve a crucial defensive role during desiccation/salt stress (Munns *et al.,* 2005).

2.7. MARKER-ASSISTED BACKCROSS BREEDING

Conventional breeding is time-consuming and highly dependent on conditions of the climate, availability of land and takes between eight and twelve years to breed a new variety. The use of DNA markers for genotypic selection in plant breeding is referred to as marker-assisted selection (MAS). Molecular breeding (MB) can be generally defined as the use of genetic modification at the molecular DNA level to enhance plant and animal characteristics of interest, including genetic engineering or modification of genes, molecular marker-assisted selection, marker-assisted backcrossing (Akhtar *et al.,* 2010), genomic selection, etc.

The identification of *Saltol* QTL has become a major breakthrough for salinity tolerance breeding program. In terms of salinity tolerance, there is a lot of variation across rice genotypes. This opens up possibilities for crop enhancement. Rice cultivars with built-in tolerance to stresses provide an economically viable and long-term solution for increasing rice productivity (Ali *et al.,* 2013).

Marker-assisted backcross breeding comprises repeated backcrossing to introgress the QTL of interest from a selected donor into the genetic background of the recurrent parent. This is achieved by the phenotypic screening of the backcrossed lines for salinity tolerance at high levels of NaCl (9-12 dS m^{-1}) and genotypic screening using SSR markers. Since the QTL *Saltol* imparts seedling stage salinity tolerance the lines are screened phenotypically at the seedling stage. Such screened lines are subjected to molecular screening to ensure the presence of the locus, reduce linkage drag and reduce the population to a manageable size.

Thomson *et al.,* (2010) stated that to develop a complete MABC package for *Saltol*, markers across the region, the first test for polymorphism and robustness across

several donors and potential recurrent parents. The best markers within the *Saltol* QTL region were AP3206, RM8094, and RM3412, the most useful markers flanking the *Saltol* region were RM1287 and RM10694 (telomeric to *Saltol*) and RM493 and RM10793 (centromeric to *Saltol*), while nearby markers that can be used for negative selection are RM490 above *Saltol* and RM562 and RM7075 below. The initial MABC lines for *Saltol* were developed using FL478 as the donor, due to its high level of tolerance, but without the tallness, photoperiod sensitivity, and late flowering of the original Pokkali landrace.

2.8. INTROGRESSION OF *Saltol* QTL IN RICE

Several salt-tolerant rice lines have been developed by incorporating *Saltol* QTL into modern high-yielding, but salt-sensitive rice varieties through marker-assisted introgression. Sexicon *et al.,* (2009) observed that the morpho-physiological traits are associated with salinity tolerance in rice varieties and classified Pokkali, Chetivirippu, FL478 and IR 651 as salt tolerant varieties due to consistent expression of high vigour, low standard evaluation score, high shoot/root biomass, lower shoot $Na+/K^+$ ratio compared to sensitive genotypes.

Chattopadhyay *et al.,* (2014) reported that the FL478 haplotype for six important *Saltol* marker loci, namely RM493, RM10772, RM10720, RM10745, RM8094, and RM3412, was detected only in Pokkali (AC39416); that for RM3412 marker allele at 190 bp was detected only in the tolerant and moderately tolerant genotypes such as Pokkali (AC41585, AC39416) and Chettivirippu (AC39388, AC39389) and that for RM10745 marker at 200 bp was detected only in the tolerant and moderately tolerant genotypes Pokkali (AC39416) and Chettivirippu (AC39388). Therefore, the two most important *Saltol* primers for the FL478 haplotype, namely RM3412 and RM10745, were found in Pokkali (AC39416) and Chettivirippu (AC39388).

Using marker-assisted backcross, salt tolerance of the BT7 variety was enhanced, which was controlled by a significant *Saltol* QTL (Linh *et al.,* 2012). ADT45, CR1009,

Gayatri, MTU1010, PR114, Pusa 44, and Sarjoo 52 are seven prominent regionally adapted rice varieties in India that have been introgressed with *Saltol* (Singh *et al.,* 2016). Using the SSR markers, Babu *et al.,* (2016) introduced the *Saltol* QTL into the popular Indian Basmati variety Pusa Basmati 1121. Krishna (2016) used marker-assisted backcrossing to increase the salt tolerance of the Karjat 6 rice variety by introgressing *Saltol* QTL from donor parent FL478. The *Saltol* QTL was introgressed into the most popular rice variety of Kerala, Jyothi with FL478 as the donor (Rohini and Shylaraj, 2017). Aiswarya, a rice variety of Kerala was introgressed with *Saltol* QTL using marker-assisted backcross breeding (Nair and Shylaraj, 2021).

2.8.1. Phenotyping for tolerance at the seedling stage

Growth reduced by salinity can be distinguished by measuring effects immediately upon the addition of salt or after several days to weeks (Roy *et al*, 2014). Standardized and repeatable phenotyping protocols are available for tolerance screening at seedling and early vegetative stages. Hydroponics is the best culture method which ensures uniform stress under uniform ambient and nutrient conditions attributing the observed variation to inherent differences of tolerance. An extensively used rapid *in-vitro* screening method was devised by Gregorio *et al.,* (1997) based on the Yoshida culture solution (Yoshida *et al.,* 1976). Na^+ showed adverse effects on other nutrients in the Yoshida culture solution. To counter such effects the original culture solution was modified by preparing the micronutrients in a neutral rather than acid solution. This helps in avoiding high concentrations of Na^+, K^+ or NH_4^+ required to adjust the pH.

Four days old, germinated seeds are grown on floats for three days in distilled water. The distilled water is then replaced with a nutrient solution under stress (usually NaCl of 10-12 dSm^1) before scoring. The scoring of seedling injury (SES Score) is recorded after 2 weeks based on damage to the test entry (Gregorio, 1997). Germination percentage hence forms an important criterion for screening. Depending upon the

concentration of salt and duration of exposure, increased mortality of leaves was observed in plants (Shereen *et al.,* 2005). According to Vibhuti *et al.* (2015) days taken to initiate and complete the germination processes varied in different rice varieties. At the control level, the minimum time taken by all three varieties for initiation was 6 days and for completion was 12 days.

2.8.2. Molecular screening for salinity tolerance

Genetic markers are biological features that can be transferred from one generation to another and can be used as experimental probes or tags to keep track of an organism, a tissue, a cell, a nucleus, a chromosome or a gene, determined by allelic types of genes or genetic loci. It is possible to classify the genetic markers used in genetics and plant breeding into two categories: classical markers and DNA markers (Xu, 2010). Morphological markers, cytological markers, and biochemical markers comprise classical markers. In certain instances, DNA markers are often called molecular markers and play a major role in molecular breeding (Jiang, 2013).

The application of DNA markers includes a small region of the DNA sequence showing polymorphism (base deletion, insertion and replacement) between different genotypes as a DNA marker. Using PCR and/or molecular hybridization followed by electrophoresis (e.g., PAGE polyacrylamide gel electrophoresis, AGE agarose gel electrophoresis, CE capillary electrophoresis), depending on the product characteristics, such as band size and mobility, the variation in DNA samples or polymorphism for a particular region of DNA sequence may be identified.

DNA markers have evolved into several systems based on various techniques or methods of polymorphism detection such as RFLP using Southern blotting (Southern, 1975), a nucleic acid hybridization technique, RAPD, SSR, SNP etc. PCR, polymerase chain reaction (Mullis, 1990) based amplification technique, AFLP (combination of hybridization and PCR based techniques), and DNA sequencing (Collard *et al.,* 2005).

2.8.2.1. Simple Sequence Repeats (SSR)

Simple sequence repeats (SSRs), also known as microsatellites, short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS) are random tandem repeats (2-6 bp/nucleotides long) of short nucleotide motifs. Repeats of di-, tri- and tetra-nucleotides, e.g. (GT)n, (AAT)n and (GATA)n, are commonly distributed across the plant and animal genomes (Vieira *et al.,* 2016). The copy number of these repeats varies between genotypes which is the cause of polymorphism. The primers unique to these regions are optimized for use in the PCR reaction because the DNA sequences flanking microsatellite regions are normally conserved. Their high degree of allelic variation is one of the most important attributes of microsatellite loci, thereby making them useful genetic markers. In most cases, SSR markers are distinguished by their hyper-variability, reproducibility, co-dominant nature, locus specificity, and random distribution across the genome. SSR markers have the advantage of the requirement of a very small quantity of DNA (~100 ng per genotype). However, for primer design, a labour-intensive marker creation process and high start-up costs for automated detections are required. In this study, the PCR-based SSR markers were chosen considering the advantages like species specificity, genomic abundance, high reproducibility and co-dominant nature.

2.8.2.2. Parental Polymorphism Assay

The study of parental polymorphism is a prerequisite to begin genotypic screening in the Marker Assisted Breeding program. Polymorphism is the occurrence of two or more distinct morphs or shapes, often referred to as alternate phenotypes in the population of a species i.e., polymorphism is when there are two or more possibilities for the expression of a gene trait. Unless the parents are polymorphic for the trait of interest, the further selection of progenies carrying the target gene is not possible.

Foreground markers for direct selection for the trait of interest (*Saltol* QTL) are essentially based on the polymorphism between the donor and recurrent parents. The recombinant markers that flank a target gene are used for eliminating the undesirable gene as quickly as possible and linkage drag can be minimized. Further, the recurrent parent genome recovery is an essential factor in the breeding program which also necessitates the parental polymorphism analysis of background markers genome-wide. These polymorphic markers were used for genotypic screening of further generations to identify the introgression of the target locus in the progenies and to assess the recurrent parent genome recovery.

2.8.2.3. Genotyping for seedling stage tolerance

To amplify the SSR alleles via PCR the specific sequences bordering the SSR motifs provide templates for particular primers. Using pairs of oligonucleotide primers specific to particular DNA sequences flanking the SSR sequence, SSR loci are independently amplified by PCR. In high-resolution electrophoresis systems (PAGE), the PCR-amplified items can be isolated, and the bands can be visually recorded by fluorescent labelling or silver staining (www.uwyo.edu).

Hundreds to thousands of plants/individuals are generally screened for desired marker patterns for most plant breeding programmes. In addition, to make selections in a timely manner, the breeders need the results instantly. Therefore, to handle a large number of tissue samples and large-scale screening of multiple markers in breeding programmes, a fast DNA extraction technique and a high throughput marker detection system are highly needed. Therefore, the present study includes an assay for parental polymorphism between the donor parent FL478 and the recurrent parent Sreyas using SSR markers which are specific for the target locus *Saltol* for the donor and background genome for the recurrent parent. The selected polymorphic markers can be used for screening the backcross generation till the desired genotype with the *Saltol* QTL and more than 80% recurrent parent genome recovery is identified.

Materials and Methods

3. MATERIALS AND METHODS

The research work entitled "Introgression of *Saltol* gene into rice variety Sreyas" was conducted under the Department of Plant Breeding and Genetics at Rice Research Station, Vytilla with an aim to introgress *Saltol* QTL from FL-478 to Sreyas variety through marker-assisted backcross breeding method.

3.1 PARENTS USED

3.1.1. FL478 (Donor parent for salinity tolerance):

FL478 is one of the highly tolerant recombinant inbred lines (RIL) from a cross between the *indica* varieties IR29 and Pokkali developed at IRRI. FL478 has been promoted as an improved donor for breeding programs, as it has a high level of seedling stage salinity tolerance and is photoperiod insensitive, semi tall, non-lodging and flowers earlier than the original Pokkali landrace.

3.1.2. Sreyas (Recurrent parent):

MO-22, commercially released as Sreyas, parentage MO-13 (Pavithra) x Triguna, is a high yielding (7-7.5 t/ha) rice variety on par with Uma, the most popular variety. It is non-lodging, medium duration with resistance to BPH, gall midge and moderate resistance to BLB, sheath blight and sheath rot. Sreyas is a variety which has gained acceptance among farmers.

3.2 BREEDING SCHEME

The technique followed is Marker Assisted Backcross Breeding. The procedure involves backcrossing supported by phenotypic and genotypic screening.

3.2.1 F_1 generation

Initial hybridization involves recurrent parent (Sreyas) as the female plant and donor parent (FL478) as the male parent.

3.2.2 Backcross generations

BC_1F_1: The selected F_1 plants based on genotyping were backcrossed with the recurrent parent using F_1 plant as the female parent and Sreyas as the male parent.

BC_2F_1: The selected BC_1F_1 plants based on phenotyping and genotyping were backcrossed with the recurrent parent using BC_1F_1 plant as the female plant and Sreyas as the male parent.

BC_2F_2: The selected BC_2F_1 plants based on phenotyping and genotyping were allowed to self-pollinate to produce BC_2F_2 seeds. The panicles were covered with butter paper before anthesis to ensure 100% self-pollination.

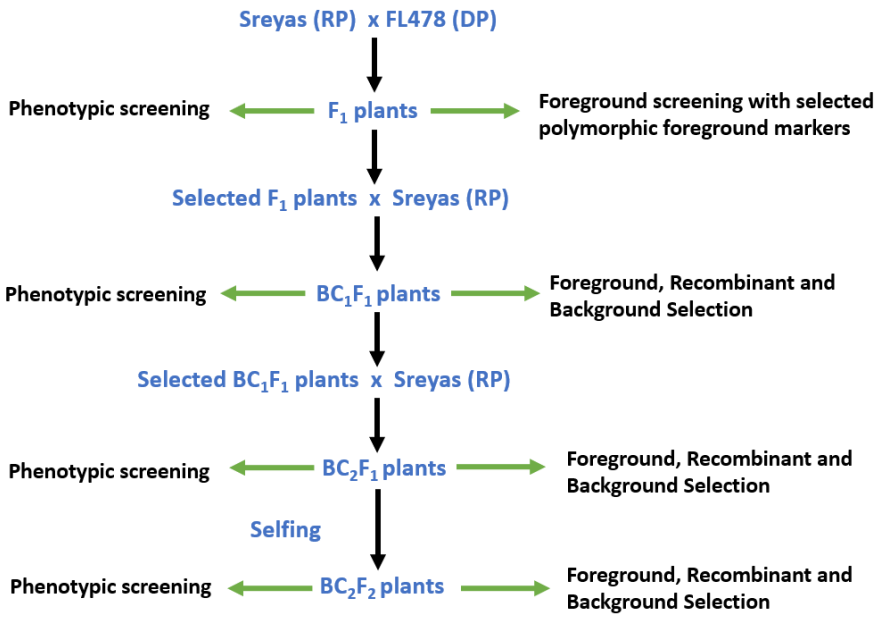


Fig.1. Breeding scheme for *Saltol* introgression

3.3 PHENOTYPIC SCREENING

3.3.1 Screening for salinity tolerance at seedling stage

The screening technique used is based on the ability of seedlings to grow in salinized nutrient solution (Yoshida *et al*. 1976). Seedling floats are prepared, and seedlings are grown in nutrient solution. The solution is maintained at 12 $dSm^{-1}$for screening the seedlings and observation at the 16^{th} days of salinization are recorded.

3.3.2 Preparation of seedling floats

The floats are assembled by sandwiching plastic mesh between acrylic sheets and perforated trays which supports the seedlings the roots of which are immersed in the nutrient solution.

3.3.2.1. *Preparation of stock solutions.*

Proper preparation of stock solutions is essential to avoid nutrient deficiencies and mineral toxicities not attributed to salinity stress. The composition of the medium are given in Table. 4 and Table. 5.

Table 4: Composition of Micronutrient Stock Solution

Element	Reagent	Preparation (g/L solution)
Micronutrients		
Mn	Manganous chloride tetrahydrate ($MnCl_3.4H_2O$)	1.5g
Mo	Ammonium molybdate tetrahydrate [$(NH4)_6Mo_7O_{24}.4H_2O$]	0.074g
Zn	Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	0.035g
B	Boric acid (H_3BO_3)	0.934g
Cu	Cupric sulfate pentahydrate ($CuSO_4.5H_2O$)	0.031g
Fe	Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	4.62g
	Citric acid monohydrate ($C_6H_8O_7.H_2O$)	11.9g

**Dissolve each reagent separately and mix in 50 ml distilled water and then add 50 ml H_2SO_4 and make up the volume up to 1000 ml.

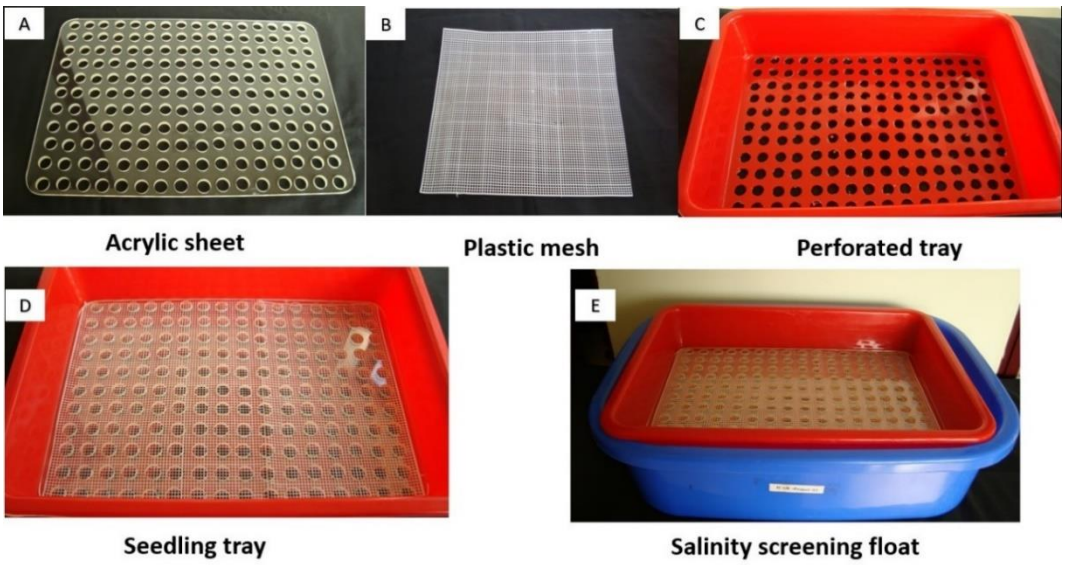


Plate. 1. Salinity Screening Assembly

Plate. 2. Salinity Screening Control

Table 5: Composition of Macronutrients for Nutrient Solution

Element	Reagent	Preparation (g/30 L solution)
Macronutrients		
N	Ammonium nitrate (NH_4NO_3)	3.42g
P	Monosodium phosphate monohydrate ($NaH_2PO_4.H_2O$)	1.51g
K	Potassium sulphate (K_2SO_4)	2.68g
Ca	Calcium chloride dihydrate ($CaCl_2.2\ H_2O$)	4.4g
Mg	Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	12.15g

3.3.2.2. *Management of the nutrient solution*

Monitoring and maintaining the pH of the culture solution is very critical because this checks the balance of available nutrients. Significant deviation (±1.0) of culture solution from pH 5.0 will make some nutrients toxic and others deficient. Monitor the pH every day and adjust to pH 5.0. Replace with fresh nutrient solution every 8 days.

3.3.3 Handling of seedlings and salinization.

Test seeds were heat-treated for 5 days in a convection oven set at 50°C to break seed dormancy. After breaking dormancy, surface sterilized the seeds with fungicide and rinsed well in distilled water. Soaked the seeds overnight in sterile water. Placed sterilized soaked seeds in petridishes with moistened filter papers and incubated at 30°C for germination. Usually, 3-4 day old seedlings were transferred to the seedling floats. The transfer is done when the radicle is long enough to be inserted through the mesh. The seedling float was suspended on the tray filled with filtered water. The

endosperm provides nutrients for the seedlings to normal growth in the initial days. After 3 days, when the seedlings were well established, the distilled water was replaced with salinized nutrient solution of EC 6dSm^{-1}. After 3 days, salinity was increased to 9 dSm^{-1} by adding NaCl to the nutrient solution and in another 3 days to 12 dSm^{-1}. The depleted nutrient solution was replaced at an interval of eight days. The pH was maintained at 5.0 – 6.0 and EC at 12 dSm^{-1} throughout the test. Test entries were rated at 16th day after salinization. The screening was conducted in a polyhouse maintained at 29°/21°C day/night temperature and minimum relative humidity of 50% during the day. to minimize environmental interactions.

3.3.4. Evaluation of salt stress symptoms

Modified Standard evaluation score (Gregorio *et al.,* 1997) was used in rating the visual symptoms of salt toxicity. This scoring discriminates the susceptible from the tolerant and moderately tolerant genotypes based on leaf rolling and cessation of growth. The scoring was recorded on the 16th day after salinization.

Table 6: Modified Standard evaluation (SES) score of visual salt injury at seedling stage

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

3.4. GENOTYPIC / MOLECULAR SCREENING

Marker-assisted selection increases the efficiency of developing improved varieties by allowing selection at early stage and reducing the number of breeding cycles.

3.4.1. Genomic DNA extraction from leaf samples

In this study pure and intact genomic DNA was isolated from the tissues of plants using CTAB method (Doyle and Doyle, 1987).

3.4.1.1 *Reagents*

Table. 7. Stock solution for CTAB Buffer

Reagents	**Mol. Wt.**	**Quantity (g/L)**
5M NaCl	58.44g/mol	292.2g
0.5M EDTA (pH 8.0)	372.2g/mol	186.1g
1M Tris HCl	121.1g/mol	121.1g
10% Cetyl trimethyl ammonium bromide (CTAB)	-	10g/100ml

Table. 8. Working stock for CTAB Buffer

Reagents	**Quantity (ml/100 ml)**
5 M NaCl	28ml
0.5M EDTA	4ml
Tris HCl	10ml
10% CTAB	20ml
Millipore Water	38ml

Table. 9. Chloroform isoamyl alcohol (24:1 v/v)

Reagents	**Quantity (ml/100 ml)**
Chloroform	96 ml
Iso amyl alcohol	4 ml

Table. 10. 70 % ethanol

Reagents	Quantity (ml/100 ml)
Absolute ethanol	70 ml
Millipore water	30 ml

Table. 11. 3M Sodium acetate (pH: 5.2)

Reagents	Mol. Wt.	Quantity (g/250 ml)
Sodium acetate	82.03 g	61.522 g
Millipore water	-	250 ml

Table. 12. RNase (10mg/ml)

Mix the following in a sterile microcentrifuge tube.

Reagents	Quantity (per ml)
Bovine Pancreatic	10 mg
Millipore water	975 µl
1M Tris HCl (pH: 7.5)	10 µl
1M NaCl	15 µl

Puncture the lid of the tube with a needle to allow steam to escape. Place a beaker with sufficient water on a hot plate and allowed to boil. Place the tube in a floater rack and drop it into the boiling water and continue boiling for 15 minutes. Remove from boiling water and allow it to cool to room temperature.

Table. 13. TE buffer (1X)

Reagents	Quantity (ml/100 ml)
1M Tris HCl	1.0 ml
0.5 M EDTA	0.2 ml
Millipore water	98.80 ml

3.4.1.2 *Protocol*

Rice leaf sample from 21 – 30 days old healthy seedlings were collected in polyethene cover, sealed and labelled and maintained in ice. In the laboratory, 1 g of tissue was weighed and transferred into prechilled sterile mortar. The samples were ground ta o fine powder using liquid N_2. 500 µl of prewarmed extraction buffer and 1.6 µl/1ml β- mercaptoethanol was added to the sample and transferred to labelled 1.5ml microtubes. The sample was incubated at 65°C for 1 hour with occasional stirring to homogenize the sample. The centrifuge tubes were removed from the water bath and an equivalent volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed for 15mins by inversion for emulsification. The tubes were centrifuged in a refrigerated centrifuge for 10 minutes at 12,000 rpm at 4^0C. The clear aqueous phase was decanted to new sterile centrifuge tube without disturbing the debris or pipetted out using a micropipette and transferred. Equal volume of ice-cold absolute isopropanol was added and mixed gently by inversion. The mixture was incubated at -20°C for 1hour for DNA to be precipitated. The microtubes were centrifuged in a refrigerated centrifuge at 4^0C for 10 minutes at 10,000 rpm to pellet down the precipitated DNA. The supernatant was discarded without disturbing the DNA pellet and the pellet was washed with 70% and 100% ethanol. The alcohol was discarded and the pellet was air dried in a laminar air flow chamber. The dried DNA pellet was dissolved in 500µl of TE buffer and stored at - 20°C.

3.4.1.3. *DNA purification*

The isolated DNA was treated with RNAse A for purification of DNA from RNA contamination. RNAse A (1µl /100µl DNA sample) was added to DNA sample and incubated at 37^0C for half an hour. Equal volume of Phenol : Chloroform : Isoamyl alcohol (24:24:1 v/v) was added to the incubated samples and mixed thoroughly. The treated samples were spun at 10,000 rpm for five minutes at room temperature. The resultant aqueous phase was collected equal volume of Chloroform : Isoamyl alcohol

(24 : 1 v/v) was added and spun at 10,000 rpm for five minutes at room temperature. The aqueous phase was collected in a fresh microtube and 0.1 v/v of 3 M sodium acetate was added and mixed well. Two volumes of ice cold ethanol were added to the mixture and incubated at -20^{0}C for 30 minutes. The mixture was spun at 10,000 rpm for five minutes at room temperature for pelleting out the genomic DNA. Pellets were washed with 70% ethanol and air dried in a laminar air flow chamber. The pellets were dissolved in 500µl of TE buffer and stored at - 20°C.

3.4.1.4. *Measurement of DNA Quantity and Quality*

The concentration and purity of a DNA sample can be measured spectrophotometrically using (ThermoScientific) NanoDrop2000c. 1 µl of 1 X TE buffer was used as blank. 1 µl of isolated DNA sample was loaded between the probes and measured at a wavelength of 260 nm. Dilution of the sample was done using 1 X TE buffer in order to ensure a resultant concentration of 16 ng/µl and stored at -20^{0}C.

$$\text{Dilution factor} = \frac{\text{Quantity of DNA at 260 nm}}{15}$$

The ratio of the absorbance at 260nm/ absorbance at 280nm is a measure of the purity of a DNA sample. Good-quality DNA would have a 1.7-2.0 A260 / A280 ratio.

3.4.1.5 *Dilution of Extracted DNA Samples*

The amount of DNA in each sample differed. After quantification, DNA was diluted in 1X TE buffer to 25-50 ng/l and used in PCR experiments.

3.4.2. Primer Designing and Optimization

3.4.2.1. *Primer Designing*

For the parental polymorphism assay, the foreground, recombinant, and background SSR markers were either collected from previously published research articles or from databases such as http://www.gramene.org. The nucleotide sequences

of the gene of interest were also collected or designed using Primer 3 software from the NCBI Gene Bank database. Several aspects should be addressed while designing a specific primer, such as oligonucleotide melting point, primer length, Guanine Cytosine content, 3' stability, primer dimer formation, and so on.

3.4.2.2. *Primer Optimization*

Primers are used as starting sites for Taq DNA Polymerase to add bases. Primers must be added in a molar excess over the amount of target DNA for effective PCR. Primers were provided as lyophilized powder, and we optimized the primer volume as a 10µM working stock from which 1µl is used each 20µl PCR reaction, i.e., 0.1µM forward and reverse primers are used in each PCR reaction. The annealing temperature of each primer was optimized based on the melting temperature (Tm^o) of the primers of interest. The annealing temperature (Ta^o) for a specific primer/template combination is usually 5oC higher or lower than the primer Tm^o. Gradient PCR protocols are used to standardize the results.

3.4.2.3. *Primer Resuspension*

Specific sequence of SSR markers was obtained in a lyophilized form which was obtained from Sigma-Aldrich. The lyophilized markers need to resuspend in an aqueous solution using 1X TE buffer to prepare the Master stock. All dry DNA oligonucleotides supplied are ready for use upon resuspension. The guidelines for resuspension and the quantity of buffer to be used for stock solution preparation were given in their technical datasheet. In a frost-free freezer, store the stock solution at -20°C and prevent several freeze-thaw cycles. The concentration of each primer was determined by the formula:

$$100\ \mu M \text{ concentration} = \frac{\text{Concentration of ssDNA X } 1000}{\text{Molecular weight of Primer}}$$

Depending on the application specifications, the stock solution can then be further diluted if required. The primers are diluted to 10 µM concentration and stored at -20^{0}C as the Working stock.

3.4.3. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a scientific technique in molecular biology developed in 1983 by Kary Mullis, to amplify a single or a few copies of apiece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

3.4.3.1. *Procedure*

PCR consists of a series of 30-35 cycles with each cycle consisting of 3 discrete steps.

1. **Initial Denaturation:** This step consists of heating the reaction mixture to a temperature of 94°C, which is held for 5 minutes.
2. **Denaturation:** This is the first regular cycling event and consists of heating the reaction to 94°C for 40 seconds. It causes melting of the DNA template yielding single stranded DNA molecules.
3. **Annealing:** The reaction temperature is lowered to 50-65°C for 40 seconds allowing annealing of the primers to the single-stranded DNA template.
4. **Extension/elongation:** The temperature for this step is fixed at 72°C for 40 seconds. At this step the Taq polymerase synthesizes a complementary strand to the DNA template.
5. **Final elongation:** This single step is performed at a temperature of 72°C for about 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
6. **Final hold:** This step at 4°C for an indefinite time may be employed for short-term storage of the amplified product.

Table 14: List of *Saltol* linked foreground primers

Sl. No.	Primer Name	P	T	Forward sequence	Reverse sequence	EPP
1	RM490	6.6	57	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG	101
2	RM10655	10.37	55	AGTACCGTTGAATCCGATATGC	TGGTTGAGGTGCTGAATTGG	281
3	RM1287	10.83	58	GGAAGCATCATGCAATAGCC	GGCCGTAGTTTTGCTACTGC	162
4	RM10696	10.98	62	CCTTCGACTCCATGAAACAAACG	CTCTTTGCCCTAACCCTATGTCC	294
5	RM10701	11.02	62	GAGACACGGCACAATATACAACG	TTCTATCTCCGACCTCTTCTCAAGG	69
6	RM10711	11.16	62	GCTTCGATCGATGAGAAAGTAGAGG	GAATCTCCCATCCTTCCCTTCC	172
7	RM8094	11.23	55	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA	209
8	RM10713	11.23	65	ATGAACCCGGCGAACTGAAAGG	CTGGCTCCCTCAAGGTGATTGC	144
9	RM10720	11.39	55	GCAAACGTCTACGTGAGAAACAAGC	GCATGTGGTGCCTTAACATTTGG	204
10	RM3412	11.58	55	TCATGATGGATCTCTGAGGTG	GGAGGATGCACTAATCTTTC	211
11	RM10748	11.77	55	CATCGGTGACCACCTTCTCC	CCTGTCATCTATCTCCCTCAAGC	95
12	RM10764	12.09	60	AGATGTCGCCTGATCTTGCATCG	GATCGACCAGGTTGCATTAACAGC	237
13	RM10772	12.16	60	GCACACCATGCAAATCAATGC	CAGAAACCTCATCTCCACCTTCC	395
14	RM493	12.28	55	GTACGTAAACGCGGAAGGTGACG	CGACGTACGAGATGCCGATCC	211
15	RM10793	12.57	62	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC	123
16	RM8115	12.68	55	TATATAGTAAATTTGTTTGGTGTAGG	ACAGATGGATATTATAAGAAGTAACA	112
17	RM10825	13.32	60	GGACACAAGTCCATGATCCTATCC	GTTTCCTTTCCATCCTTGTTGC	97
18	RM10829	13.34	60	TCATCCGTGGAGCAAGGAGAGG	CCTAGCTAATTGGAGTCCGGGTTGG	122
19	RM10843	13.79	65	CACCTCTTCTGCCTCCTATCATGC	GTTTCTTCGCGAAATCGTGTGG	162

20	RM10864	14.25	60	GAGGTGAGTGAGACTTGACAGTGC	GCTCATCATCCAACCACAGTCC	239
21	RM10871	14.38	59	TGAGGCTGTAACGTAGACGATGAACC	AAGCCTGCTAGAGAGGCCCAACC	234
22	RM10890	14.75	63	GCTTCGGCTCTTCATTCACTGG	GCGATTATAGGAGCGCTATGTGG	240
23	RM7075	15.11	57	TATGGACTGGAGCAAACCTC	GGCACAGCACCAATGTCTC	155
24	RM10927	15.4	60	TGGATCCCACTAATCCAAATGC	GAAAGACTCCTTCCAATGTTAGGC	152
25	RM6711	16.11	61	TAGTGATAGGGGTGGTGTGG	TAGTGATAGGGGTGGTGTGG	118

T – Annealing temperature (^{0}C), P- Position (Mbp), EPP – Expected PCR Product (bp)

Table. 15. Composition of a 20 µl PCR reaction mixture:

Reagents	**Volume**
Sterile nanopure water	11 µl
10X PCR Buffer	2 µl
dNTP mix	2µl
Primer forward	1 µl
Primer reverse	1 µl
Taq DNA polymerase	1 µl (1U)
DNA	2 µl
Total	**20 µl**

Table. 16. Composition of a 20 µl PCR using PCR Master Mix:

Reagents	**Volume**
PCR Master Mix	10 µl
Sterile nanopure water	6 µl
Primer forward	1 µl
Primer reverse	1 µl
DNA	2 µl
Total	**20 µl**

3.4.4 Polyacrylamide Gel Electrophoresis

The technique used to distinguish PCR products according to their electrophoretic mobility is polyacrylamide gel electrophoresis (PAGE). Electrophoretic mobility is a function of the molecule's length, conformation and charge. The PCR amplified products were isolated by 8% polyacrylamide gel electrophoresis.

3.4.4.1. *Reagents for 8% polyacrylamide gel*

Table. 17. 30% Acrylamide solution

Acrylamide	29.2g
Bisacrylamide	0.8g

Make up the volume to 100 ml with Millipore water and store in amber coloured bottle at 4^0C.

Table. 18. 10X TBE Buffer

Tris HCl	121.1g
Boric acid	61.82g
0.5 M EDTA	40 ml

Make up the volume to 1000 ml with Millipore water and store in room temperature.

c. 10% Ammonium persulfate (APS)

0.1g APS is dissolved in 1 ml Millipore water (Freshly prepared)

Table. 19. 8% PAGE Composition

Reagents	**10 ml**	**40 ml**	**80 ml**
Millipore water	6.8 ml	27.2 ml	54.4 ml
30% acrylamide	2.7 ml	10.8 ml	21.6 ml
10X TBE buffer	0.5 ml	2 ml	4 ml
10% APS	50 µl	200 µl	400 µl
TEMED	5 µl	20 µl	40 µl

3.4.4.2. *Gel casting*

The gel casting unit includes a short plate and a spacer plate held in place by the clamps of the gel casting assembly. The gel is poured smoothly and continuously starting from one corner until it reaches the top edge of the short plate. The comb was gently inserted between the two plates and allowed to polymerize for 30 min.

3.4.4.3. *Electrophoresis*

Two gel plates are assembled in the gasket such that the short plates of each plate is facing the inner side. 1X TBE buffer was added in to the plate assembly to fill it. The buffer was added to the electrophoresis tank and filled the tank up to the 4 gels mark. About 4 µl of the sample mixed with the 6 X loading dye was loaded in each well. DNA size marker like 100bp Ladder was loaded in one of the wells for size determination. The cover was placed on the tank corresponding to the leads (red

and black). Electrodes were connected to the power supply at 100 volts and ran the gel till the dye reaches the bottom of the gel.

3.4.4.4. *Visualization of PCR products by silver staining.*

3.4.4.4.1. *Reagents*

Table. 20. Fixer solution

Reagents	**Volume (per 500 ml)**
Absolute Alcohol	50 ml
Glacial Acetic Acid	2.5 ml

Make up the volume to 500 ml with Millipore water and reuse 3 times.

Table. 21. Silver Nitrate Solution

Reagents	**Volume (per 500 ml)**
Silver Nitrate	0.75 g
37% Formaldehyde	750 µl

Make up the volume to 500 ml with Millipore water, store in amber bottles and reuse 7 times.

Table. 22. Developer Solution

Reagents	**Volume (per 500 ml)**
Sodium hydroxide	7.5 g
37% Formaldehyde	750 µl

Make up the volume to 500 ml with Millipore water. This solution has to be freshly prepared.

3.4.4.4.2. *Silver staining protocol (*Benbouza *et al.*, 2006*)*

The gel electrophoresis unit was turned off and glass plates were removed from the tank. The glass plates were separated using a plastic wedge. The polyacrylamide gel was placed in a clean amber-coloured tray containing fixer solution for 5 min with continuous shaking using a shaker. After 5 min, the fixer was drained and the gel was rinsed thrice with Millipore water. The staining solution was added to the tray and incubated for 6-7 min with continuous shaking to ensure even staining. The amber-coloured tray was meant to maintain darkness and avoid interference of light affecting the staining procedure. After the stipulated time, the staining solution was drained quickly and the gel was rinsed twice with Millipore water. The band were developed by adding developer solution and incubating for 3-5 min with continuous shaking ensuring an even reaction between the stainer and developer. The developer was drained and gel was rinced. The gel was placed in the fixer solution once the bands had developed. The gel documentation BioRad Gel DOC XR+ system was used to document the images in jpeg format.

3.4.5. Parental polymorphism percentage

The parental polymorphism percentage was calculated between the recurrent and donor parent according to the markers selected which shows polymorphism (Yerva *et al*., 2018).

$$\text{Parental polymorphism \%} = \frac{\text{Markers which show polymorphism}}{\text{Total no.of markers screened}} \times 100$$

3.4.6. Analysis of molecular data

The molecular data were analysed using Graphical Geno Types (GGT 2.0) software (van Berloo, 2008). The recurrent parent genome recovery was obtained from GGT. The images depict the polymorphism, homozygous and heterozygous condition of donor and recurrent genome on chromosome basis.

Results

4. RESULTS

The present research titled "Introduction of *Saltol* gene into rice variety Sreyas" was conducted at Rice Research Station, Vyttila between 2019 and 2022 to introgress *Saltol* QTL for seedling stage salinity tolerance into rice variety Sreyas using the reliable salt-tolerant donor, FL478 through Marker Assisted Backcross Breeding using SSR markers. The project was divided into 4 experiments based on the stage of the breeding scheme and the generations being handled. The findings derived from this project are detailed hereby.

4.1. EXPERIMENT I

The first experiment comprised of hybridization of Sreyas and FL478 to generate the F_1 population and parental polymorphism assay between parents to identify foreground, recombinant and background markers to be used in MABB. Staggered planting was done to ensure the availability of pollen throughout the hybridization period.

The performance of the parents for agronomic characteristics and salinity tolerance were assessed as follows.

4.1.1. Agronomic performance

4.1.2. Salinity screening

4.1.3. Molecular screening

4.1.1. Agronomic performance

The mean values of each character was recorded and illustrated in Table. 23.The results showed that Sreyas (96.94 cm) recorded higher mean plant height than FL478 (90.04 cm). The comparison of the mean number of tillers revealed that Sreyas (8.3) had the higher number of productive tillers than FL478 (7.2). FL478 (73 days) bloomed earlier than Sreyas (81days). Days to maturity followed a similar pattern as days to flowering. FL478 completed its life cycle in 123 days. Sreyas

exhibited a longer duration of 128 days. Sreyas (20.75 cm) had a comparatively longer panicle than FL478 (20.07 cm).

Table. 23. Mean performance of parental lines

Characters	Sreyas	FL 478
Plant height (cm)	96.94	90.04
Number of productive tillers	8.30	7.20
Days to flowering	81.45	73.15
Days to maturity	127.05	123.30
Panicle Length (cm)	20.75	20.07
Grain L / B	2.25	3.00
Grain yield per plant (g)	26.08	23.01
Thousand Grain Weight (g)	29.64	23.26
Kernel Colour	Red	Red

Sreyas was a bold grain type with a grain length-to-breadth ratio of 2.25 whereas FL478 (3.00) exhibited slender grain type. Sreyas recorded better mean grain yield per plant (26.08 g) and mean thousand-grain weight (29.64 g) outweighing FL478 in mean grain yield per plant (23.01 g) and mean thousand-grain weight (23.26 g). The kernel colour was red for both genotypes.

4.1.2. Salinity screening:

The observations for each attribute were taken at varying electrical conductivities of 0, 3, 6, 9 and 12 dS m^{-1}. The results of the salinity screening are shown in Table. 24.

The results of each attribute are elaborated hereunder.

4.1.2.1. Germination percentage

The mean germination percentage was highest in FL478 (100%) at 3 dS m^{-1} while the lowest was recorded for Sreyas (83.33%) at 12 dS m^{-1}. At an EC of 0 dS m^{-1}, Sreyas showed a mean germination of 93.33% while FL478 showed 96.67%.

At 3 dS m^{-1}, FL478 recorded 100% mean germination while Sreyas recorded 96.67%.

FL478 exhibited a better mean germination percentage of 96.67%, 93.33% and 93.33% at 6 dS m^{-1}, 9 dS m^{-1} and 12 dS m^{-1} respectively. Sreyas, on the other hand recorded 93.33%, 86.67% and 83.33% at 6 dS m^{-1}, 9 dS m^{-1} and 12 dS m^{-1} respectively.

Table. 24. Mean salinity screening parameters of Sreyas and FL478

Electrical conductivity (dS m^{-1})	**0**	**3**	**6**	**9**	**12**
Germination percentage					
Sreyas	93.33	96.67	93.33	86.67	83.33
FL 478	96.67	100.00	96.67	93.33	93.33
Days to Germination (Initiation)					
Sreyas	1.25	1.10	1.36	1.23	1.32
FL 478	1.10	1.07	1.21	1.12	1.05
Days to Germination (Completion)					
Sreyas	6.25	6.14	6.46	6.27	6.36
FL 478	6.17	6.13	6.29	6.15	6.09
Survival percentage					
Sreyas	100.00	100.00	64.29	30.77	0.00
FL 478	100.00	100.00	96.55	92.86	78.57
SES Score					
Sreyas	1	1	4.86	5.89	7.96
FL 478	1	1	2.71	5.27	5.37
Leaf drying percentage					
Sreyas	0.00	0.00	61.90	74.07	98.67
FL 478	0.00	0.00	28.57	65.15	71.79

4.1.2.2. Days to germination

The mean days to germination were recorded at initiation and completion. The days to germination initiation and completion remained more or less the same for both genotypes across various stress levels. The shortest duration was recorded

for FL478 (1.05) at 12 dS m^{-1} while the longest duration was marked by Sreyas (1.36) at 6 dSm^{-1}.

The duration for germination initiation at 0 dS m^{-1}, ranged from 1.10 for FL478 to 1.25 for Sreyas. At 3 dS m^{-1}, Sreyas recorded 1.10 and FL478 1.07 mean days for germination initiation. When the electrical conductivity was 6 dS m^{-1}, Sreyas marked 1.36 and FL478 1.21. At 9 dS m^{-1}, germination initiated in Sreyas at a mean of 1.23 days while FL478 showed 1.12 mean days for germination initiation. At maximum stress (12 dS m^{-1}) germination initiated in Sreyas in 1.32 and FL478 at 1.05 days.

The duration for the completion of germination followed a similar fashion as initiation. At 0 dSm^{-1}, germination was completed at 6.25 days for Sreyas and 6.17 days for FL478. At 3 dS m^{-1}, Sreyas completed germination in 6.14 days and FL478 in 6.13 days. At 6 dS m^{-1}, Sreyas recorded 6.46 days while FL478 showed 6.29 days. When the electrical conductivity was 9 dS m^{-1}, germination was completed in Sreyas at a mean of 6.27 days while FL478 showed 6.15 days. At 12 dS m^{-1}, germination was completed in Sreyas in 6.36 and FL478 in 6.09 days.

4.1.2.3. Survival percentage

The mean survival percentage of germinated seedlings at 16 days of salinization for the parents at 0 and 3 dS m^{-1} was 100%. At 6 dS m^{-1}, FL478 (96.55%) survived better than Sreyas (64.29%). At 9 dS m^{-1} and 12 dS m^{-1}, FL478 prevailed in salinity stress with better percentages of 92.86% and 78.57% respectively. Sreyas on the other hand performed poorly at 9 dS m^{-1} with 30.77% and succumbed to stress at 12 dS m^{-1} where none of the plants survived.

4.1.2.4. SES Score

The mean SES score was the least (1) for both the genotypes at both 0 and 3 dS m^{-1}. However, Sreyas showed consistently high mean SES score of 4.86, 5.89 and 7.96 at 6, 9 and 12 dS m^{-1}. On the other hand, FL478 maintained a lower profile for SES score of 2.71, 5.27 and 5.37 at 6, 9 and 12 dS m^{-1}. Based on the SES score

recurrent parent shows salt injury at a susceptible to highly susceptible level while the donor parent showed moderate tolerance at 12 dS m^{-1}.

4.1.2.5. Percentage leaf drying

The mean percentage leaf drying showed a similar pattern to that of SES score. There was no evident leaf drying in both the genotypes (0%) at 0 and 3 dS m^{-1}. Similar to stress score Sreyas showed high mean percentage of leaf drying of 61.90%, 74.07% and 98.67% at 6, 9 and 12 dS m^{-1} respectively. FL478 exhibited lower leaf drying percentage of 28.57%, 65.15% and 71.79% at 6, 9 and 12 dS m^{-1} respectively.

4.1.3. Molecular screening

The genotypic screening included extraction of parental genomic DNA, measurement of quantity and quality of DNA and polymorphism assay of foreground, recombinant and background markers.

4.1.3.1. Quantity and Quality of DNA

Genomic DNA was isolated from the leaves of 21 days old rice plants using CTAB method . The quantity and quality of the DNA were measured using Thermo Scientific NanoDrop2000c. The observations are given in Table. 25.

Table. 25. Quantity and quality of parental genomic DNA

Sl. No.	Sample	DNA Quantity (ng μl^{-1})	A260	A280	DNA Quality 260/280
1	SREYAS	920.7	17.114	8.585	1.99
2	FL478	688.3	12.677	6.524	1.94

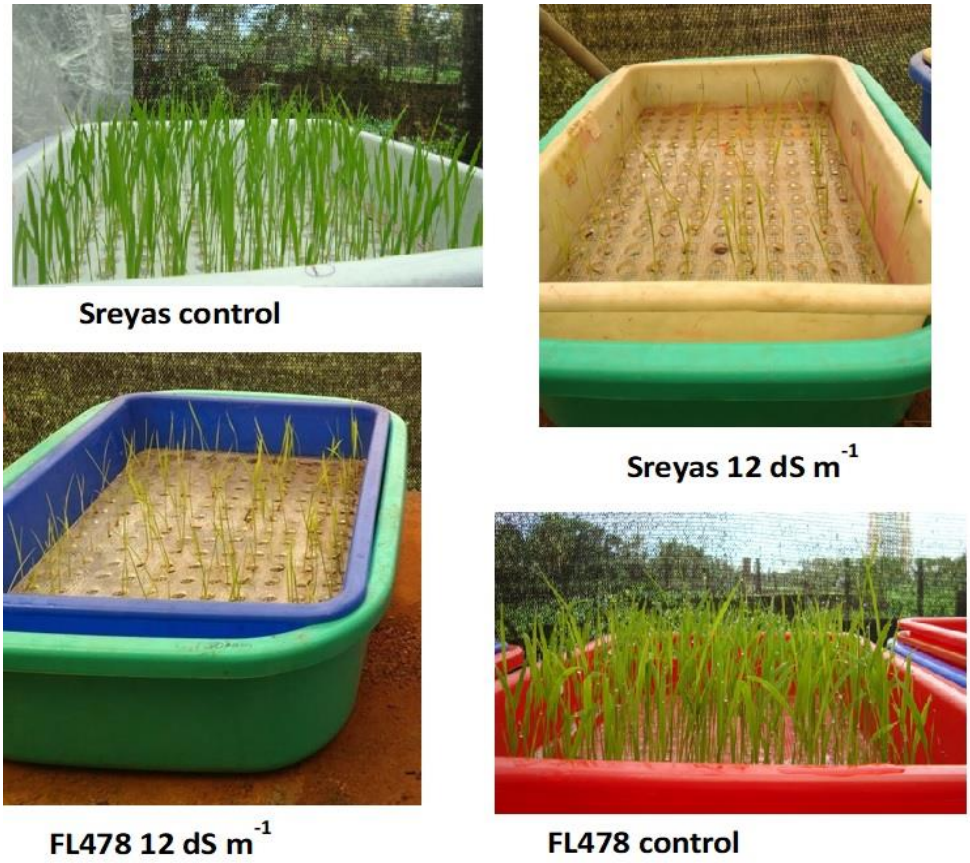


Plate. 3. Salinity Screening of parents at 12 dSm^{-1}

Plate. 4. Salt injury of parents at 12 dSm^{-1}

Plate. 5. Crossing block of parents

Plate. 6. Vacuum emasculation

A good quantity of quality (1.8-2.0) master DNA was isolated from Sreyas (920.7 ng μl^{-1}) and FL478 (688.3 ng μl^{-1}). A 20µl PCR reaction requires 25-50 ng of template DNA for a sufficient quantity of amplified product. The master DNA is diluted so that 15 ng μl^{-1} is the concentration of the diluted DNA 2 µl of which will be used per reaction.

4.1.3.2. Polymorphism assay

25 *Saltol* linked and 300 background SSR markers covering the 12 chromosomes were used for parental polymorphism assay. 9 among the QTL linked markers assayed were polymorphic which are compiled as Table. 26.

The polymorphism assay for *Saltol*-associated markers between Sreyas and FL478 revealed 9 polymorphic markers among which 8 were located within 10.8 – 12.3 Mb. The markers flanking the polymorphic regions are considered as recombinant markers while those located at the centre of the QTL are selected for foreground selection. The marker used for foreground selection were RM10711, RM8094, RM10713, RM10720 and RM3412. The markers RM1287 and RM10701 telomeric to the QTL and RM493 and RM10895 centromeric to the QTL were the flanking markers used for recombinant selection.

Table. 26. Polymorphic foreground and recombinant markers

Sl No.	Primer Name	Chromosome No:	Location (Mbp)
1	RM1287	1	10.83
2	RM10701	1	11.02
3	RM10711	1	11.16
4	RM8094	1	11.23
5	RM10713	1	11.23
6	RM10720	1	11.39
7	RM3412	1	11.58
8	RM493	1	12.28
9	RM10825	1	13.32

The software Graphical Genotypes 2.0 (GGT 2.0) was used to analyse the polymorphism assay and other molecular observations. The GGT image of

Chromosome 1(T) shows *Saltol* QTL (A) and locus to be introgressed (B) between Sreyas and FL478 as revealed in polymorphism assay (Fig. 2).

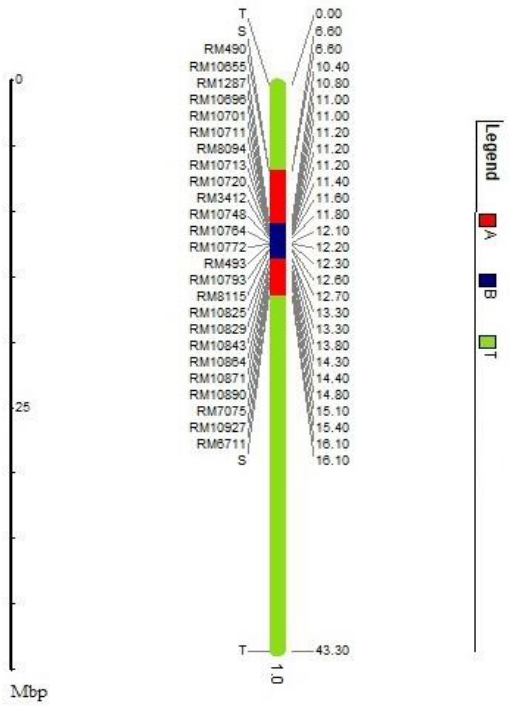


Fig 2. GGT image of Chromosome 1 showing *Saltol* QTL

The parental polymorphism percentage was 36% between the parents for the foreground markers. 9 out of 25 markers were found to be polymorphic.

Polymorphism assay for the background markers revealed that 83 markers were polymorphic between the parents with at least 5 per chromosome. The background markers exhibiting polymorphism are illustrated in Table. 27.

Table. 27. Polymorphic background markers between Sreyas and FL478

Sl No.	Primer Name	Chromosome No:	Location (Mbp)
1	RM10209	1	3.95
2	RM1196	1	21.90
3	RM237	1	26.82
4	RM246	1	27.34
5	RM472	1	37.89
6	RM5536	1	41.17

Sl No.	Primer Name	Chromosome No:	Location (Mbp)
7	RM3362	1	43.04
8	OSR 14	2	1.33
9	RM279	2	2.88
10	RM555	2	4.31
11	RM324	2	11.39
12	RM250	2	32.77
13	RM208	2	35.14
14	RM7485	2	35.74
15	RM546	3	6.16
16	OSR 13	3	7.13
17	RM7	3	9.83
18	RM5626	3	24.86
19	RM168	3	28.09
20	RM520	3	30.91
21	OSR 16	3	31.89
22	RM514	3	35.28
23	RM518	4	2.03
24	RM273	4	24.04
25	RM241	4	26.86
26	RM317	4	29.06
27	RM348	4	32.65
28	RM124	4	34.74
29	RM122	5	0.31
30	RM413	5	2.21
31	RM17960	5	3.85
32	RM249	5	10.78
33	RM6229	5	13.55
34	RM161	5	20.90
35	RM469	6	0.56
36	RM197	6	3.09
37	RM276	6	6.23
38	RM527	6	9.86
39	RM6818	6	16.58
40	RM162	6	24.04
41	RM494	6	31.09
42	RM3859	7	8.88
43	RM214	7	12.78
44	RM500	7	15.91
45	RM11	7	19.26
46	RM455	7	22.35
47	RM118	7	26.64
48	RM337	8	0.15
49	RM152	8	0.68

Sl No.	Primer Name	Chromosome No:	Location (Mbp)
50	RM1376	8	3.17
51	RM3481	8	9.14
52	RM6193	8	17.65
53	RM210	8	22.47
54	RM149	8	24.72
55	RM447	8	26.55
56	RM23654	9	0.15
57	RM8303	9	2.37
58	RM23805	9	4.60
59	RM5526	9	7.31
60	RM219	9	7.89
61	RM23958	9	8.00
62	RM296	9	10.79
63	RM3912	9	10.83
64	RM105	9	12.55
65	RM215	9	21.19
66	RM205	9	22.72
67	RM6364	10	0.07
68	RM474	10	1.82
69	RM3882	10	2.74
70	RM216	10	5.35
71	RM311	10	9.75
72	RM5689	10	13.48
73	RM536	11	8.99
74	RM7120	11	11.78
75	RM26652	11	15.07
76	RM287	11	16.77
77	RM144	11	28.28
78	RM247	12	3.19
79	RM27973	12	12.27
80	RM277	12	18.32
81	RM519	12	19.90
82	RM17	12	26.95
83	RM1227	12	27.31

The GGT image of the background polymorphism (Fig. 3) of 300 SSR markers is represented by M (monomorphic) and P (polymorphic) regions in the 12 chromosomes.

The parental polymorphism percentage was 27.67% between the genotypes for the markers under consideration. The highest parental polymorphism percentage

(44%) was observed in chromosome number 9 while lowest was observed in chromosome number 11(22.73%) (Table. 28).

Fig. 3. GGT image of background polymorphism assay

Table. 28. Parental polymorphism percentage between Sreyas and FL478

Chromosome no.	Total markers	Polymorphic markers	Parental polymorphism percentage
1	30	7	23.33
2	30	7	23.33
3	30	8	26.67
4	26	6	23.08
5	22	6	27.27

6	25	7	28.00
7	20	6	30.00
8	25	8	32.00
9	25	11	44.00
10	24	6	25.00
11	22	5	22.73
12	21	6	28.57
Total	**300**	**83**	**27.67**

4.2. EXPERIMENT II

The F_1 population was subjected to salinity screening and molecular screening. The population selected through *in-vitro* salinity screening at 12 dS m^{-1} was genotyped for foreground and recombinant markers to confirm the presence of the QTL. The selected plants were backcrossed with the recurrent parent Sreyas to develop the BC_1F_1 population.

4.2.1. Salinity screening

The F_1 population from 152 seeds was screened at 12 dS m^{-1} and observations were recorded at 16 days of salinization. A total of 132 seeds were germinated. Germination percentage and days to germination under salinity stress of the F_1 generation are tabulated in Table. 29.

4.2.1.1. Germination percentage

The mean germination percentage for the F_1 population under 12 dS m^{-1} was recorded and compared with both parents. The F_1 population showed a better germination percentage of 86.81% in comparison to the recurrent parent Sreyas (83.33%) but lesser than the donor parent FL478 (93.33%).

4.2.1.2. Days to germination

The number of days for initiation of germination under salinity stress for the F_1 population did not exhibit much variation for the three genotypes. On a comparative note, the least value was recorded for the donor parent (1.05), intermediate for the F_1 population (1.26) and highest for the recurrent parent (1.32).

Table. 29. Salinity screening observations of germination parameters

Salinity screening	Germination (%)	Days to Germination (Initiation)	Days to Germination (Completion)
Sreyas	83.33	1.32	6.36
F_1	86.81	1.26	6.25
FL 478	93.33	1.05	6.09

The average number of days taken for completion of germination followed a similar fashion. Sreyas recorded an average of 6.36 days and FL478 6.09 while the F_1 population showed an intermediate value of 6.25 days.

4.2.1.3. Survival percentage

The germinated seeds were transferred to salinity screening floats with nutrient solution. After a week the medium was replaced with saline nutrient solution at 6 dS m^{-1} which was gradually raised to 9 dS m^{-1} and 12 dS m^{-1} through a week's duration. On the 5^{th} day of salinization 101 from the 132 seedlings were surviving which reduced to 84 by the 10^{th} day of salinization. On the 16^{th} day of salinization, the saline nutrient medium was replaced by normal Yoshida solution and 69 seedlings were found to survive at a salt stress of 12 dS m^{-1}. The percentage of survival at 16^{th} day was noted. Comparative record of survival percentage, SS score and percentage leaf drying for Sreyas, FL478 and F_1 population is indicated in Table.30.

The mean values for survival percentage for Sreyas, the recurrent parent was 0% and donor parent FL478 was 78.57%. The F_1 population showed 52.27% survival.

Table. 30. Salinity screening observations of salt injury

Salinity screening	Survival percentage	SES Score	Leaf drying percentage
Sreyas	0.00	7.96	98.67
F_1	52.27	6.49	87.34
FL 478	78.57	5.37	71.79

4.2.1.4. SES Score

The average value of SES score was studied for Sreyas, FL478 and F_1 population under salinity stress. FL478 showed the best performance with a mean SES score of 5.37 whereas Sreyas accrued 7.96. F_1 population showed an intermediary score of 6.49 tending towards susceptibility.

4.2.1.5. Percentage leaf drying

The mean percentage leaf drying was observed for Sreyas, FL478 and F_1 population under salinity stress. The recurrent parent Sreyas exceeded both FL478 and F_1 population with an average percentage of 98.67% over 71.79% and 87.34% respectively.

At 21 days after salinization, 31 F_1 plants which survived were transferred to the crossing block. Among these only 16 F_1 plants recovered from stress injury. After 2-3 weeks DNA was extracted from young leaves of the F_1 plants.

4.2.2. Molecular screening

The molecular screening consisted of isolation of DNA, measurement of quantity and quality of DNA and marker assisted foreground and recombinant selection.

4.2.2.1. Quantity and Quality of DNA

Genomic DNA was isolated from young leaves of F_1 plants using CTAB method. The quantity and quality of the DNA were measured using Thermo Scientific NanoDrop2000c. The observations are given in Table.31.

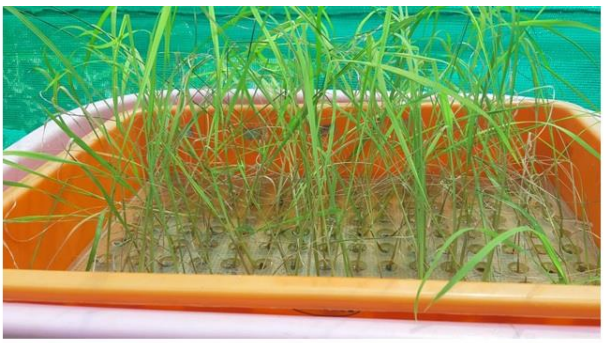
Most of the genotypes showed a good quantity of fine-quality DNA. The best quality was registered by SF1-9 with absorbance ratio 1.93 and SF1-8 recorded highest absorbance ratio of 2.04.

Table. 31. Quantity and quality of genomic DNA of F_1 population

Sl. No.	Sample	DNA Quantity (ng μl^{-1})	A260	A280	DNA Quality 260/280
1	SF1-1	271.2	5.425	2.737	1.98
2	SF1-2	353.8	7.076	3.551	1.99
3	SF1-3	357.5	7.151	3.612	1.98
4	SF1-4	257.6	5.151	2.572	2.00
5	SF1-5	355	7.101	3.559	2.00
6	SF1-6	86.6	2.032	1.034	1.97
7	SF1-7	310.2	6.103	3.005	2.03
8	SF1-8	240.5	4.810	2.354	2.04
9	SF1-9	199.2	3.985	2.065	1.93
10	SF1-10	213.4	4.269	2.117	2.02
11	SF1-11	297.2	5.944	2.987	1.99
12	SF1-12	278.4	5.569	2.827	1.97
13	SF1-13	200.7	4.014	1.997	2.01
14	SF1-14	317.3	6.346	3.173	2.00
15	SF1-15	249	4.980	2.515	1.98
16	SF1-16	230.8	4.615	2.319	1.99

Though few of them had an absorbance ratio of 2 and slightly above 2, it did not hinder the amplification process or PAGE. The quantity of DNA was found to range from 86.6 - 357.5 ng μl^{-1} with the highest recorded for the plant SF1-3 and lowest for SF1-6.

10 days of salinization



16 days of salinization

Plate. 7. Salinity screening F_1 population

Plate. 8. Crossing block of F_1 population

4.2.2.2. Foreground screening

Foreground selection using 5 markers tightly linked to the QTL namely RM10711, RM8094, RM10713, RM10720 and RM3412 was carried out for 16 F_1 plants. The loci in question were in heterozygous condition in all 16 plants. The GGT score of the plants is shown in Table. 32. The GGT image of the foreground and recombinant markers is illustrated in Fig. 4.

4.2.2.3. Recombinant screening

The markers RM1287 and RM10701 telomeric to the QTL and RM493 and RM10895 centromeric to the QTL were used for recombinant selection of the F_1 plants (Table. 33). All the F_1 plants showed heterozygosity for the recombinant markers also marking the presence of whole QTL segment under consideration in the selected progeny. These selected plants were backcrossed with the recurrent parent Sreyas to develop the BC_1F_1 population.

Table. 32. GGT score of foreground markers of F_1 plants

Markers	Chr. No.	Location (Mbp)	Genotype
RM10711	1	11.16	H
RM8094	1	11.23	H
RM10713	1	11.23	H
RM10720	1	11.39	H
RM3412	1	11.58	H

Table. 33. GGT score of recombinant markers of F_1 plants

Markers	Chr. No.	Location (Mbp)	Genotype
RM1287	1	10.83	H
RM10701	1	11.02	H
RM493	1	12.28	H
RM10825	1	13.32	H

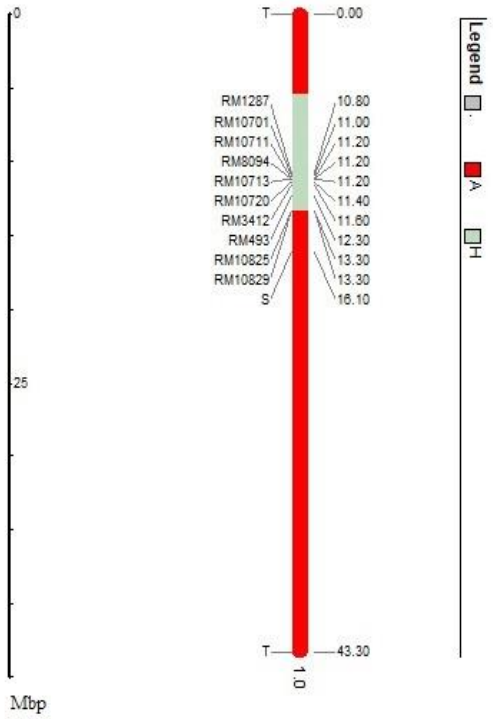


Fig. 4. GGT image of foreground and recombinant selection in F_1 plants

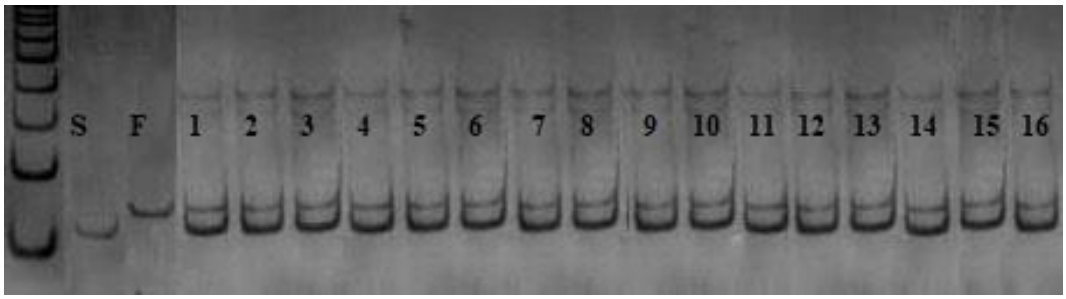


Plate. 9. Gel image of RM1287 of F_1 plants

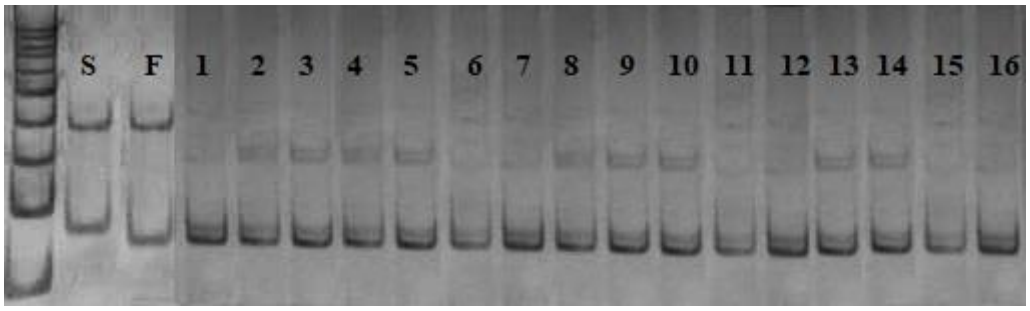


Plate. 10. Gel image of RM10701 of F_1 plants

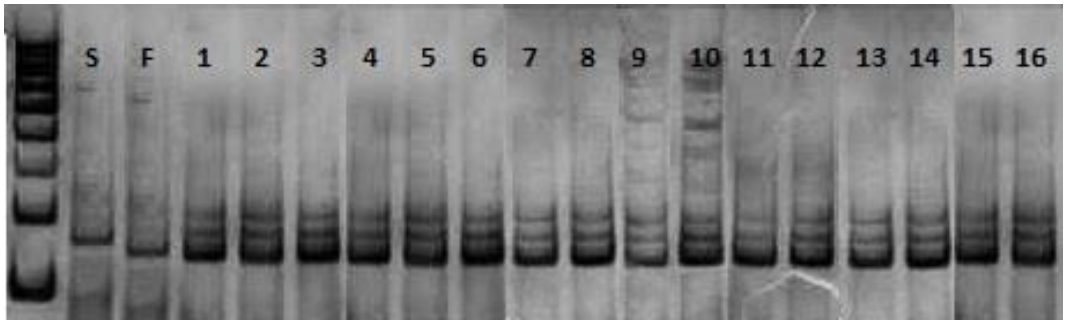


Plate. 11. Gel image of RM10711 of F_1 plants

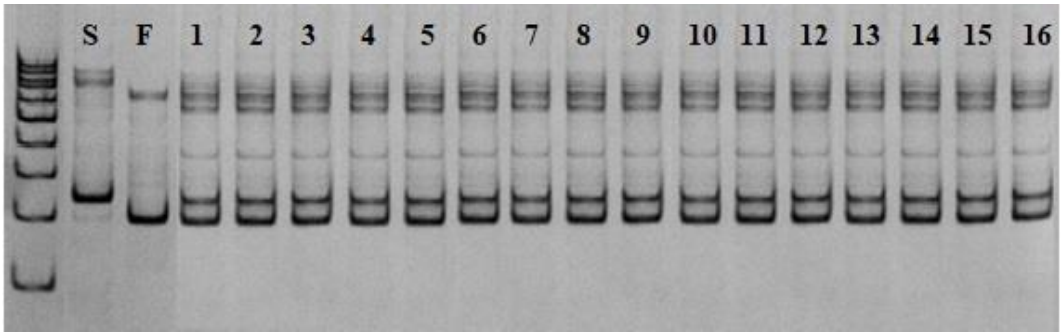


Plate. 12. Gel image of RM8094 of F_1 plants

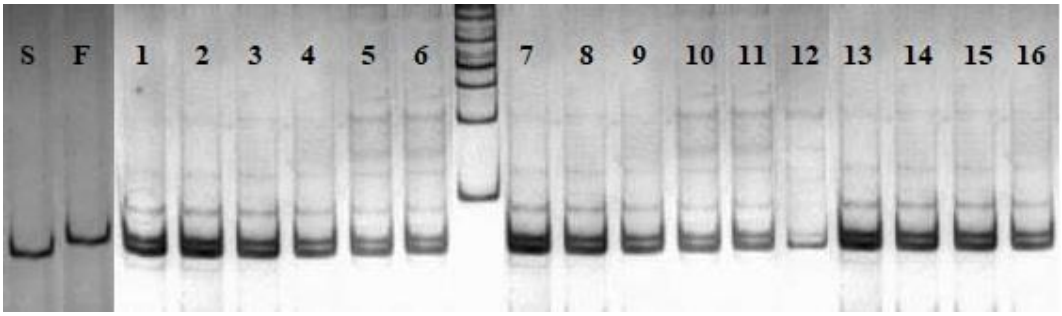


Plate. 13. Gel image of RM10713 of F_1 plants

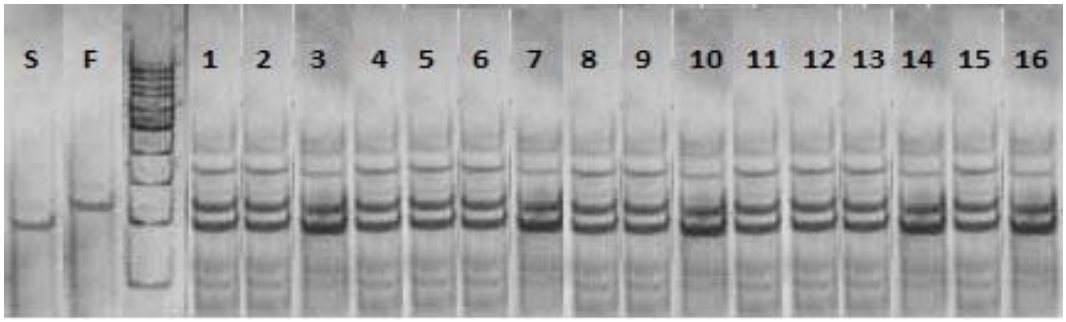


Plate. 14. Gel image of RM10720 of F_1 plants

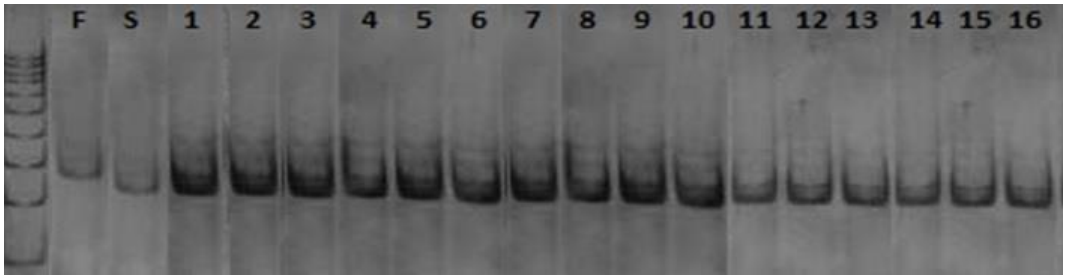


Plate. 15. Gel image of RM3412 of F_1 plants

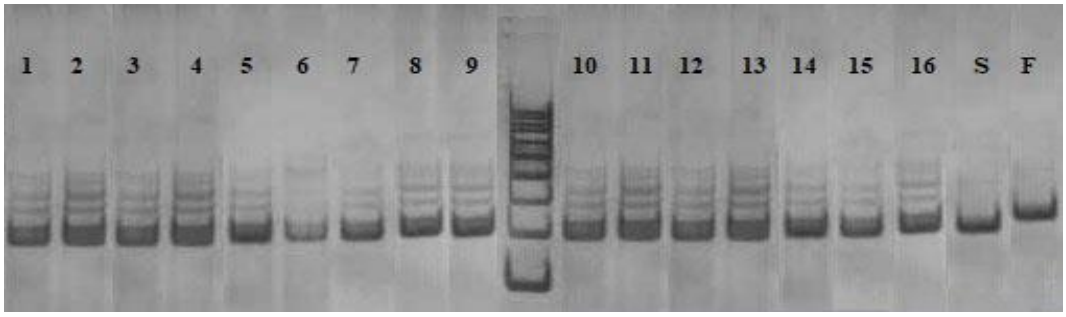


Plate. 16. Gel image of RM493 of F_1 plants

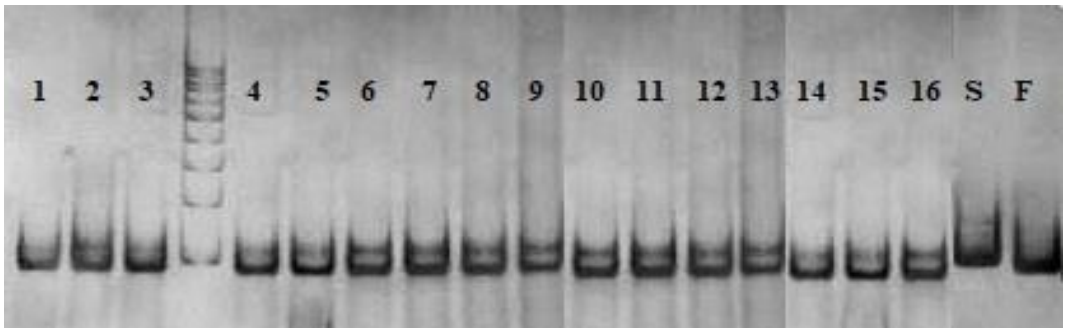


Plate. 17. Gel image of RM10825 of F_1 plants

4.3. EXPERIMENT III

Salinity screening and molecular screening of the BC_1F_1 population were performed to select the best genotypes to be forwarded to the next generation. The selected population under salinity screening at 12 dS m^{-1} was genotyped using foreground, recombinant and background markers. The selected plants were backcrossed with the recurrent parent Sreyas to develop the BC_2F_1 generation.

4.3.1. Salinity screening

The BC_1F_1 population was screened at 12 dS m^{-1} and observations were recorded at 16 days of salinization. A total of 288 BC_1F_1 seeds were sown for salinity screening. 249 seedlings emerged under saline environment. Germination parameters under salinity stress of the BC_1F_1 generation are tabulated in Table. 34.

Table. 34. Salinity screening for germination parameters of BC_1F_1 population

Salinity screening	Germination percentage	Days to Germination (Initiation)	Days to Germination (Completion)
Sreyas	83.33	1.32	6.36
F_1	86.81	1.26	6.25
BC_1F_1	86.45	1.28	6.28
FL 478	93.33	1.05	6.09

4.3.1.1. Germination percentage

The BC_1F_1 population showed better salinity tolerance than recurrent parent Sreyas. The mean germination percentage of BC_1F_1 (86.45%) plants improved compared to the recurrent parent Sreyas (83.33%). The mean germination percentage of BC_1F_1 generation under salinity stress was found to be on par with the F_1 generation (86.81%).

4.3.1.2. Days to germination

The mean days to germination initiation of BC_1F_1 (1.28) and F_1 (1.26) populations were closer to recurrent parent Sreyas (1.32). The donor parent, FL478 had a lesser number of days to germination (1.05).

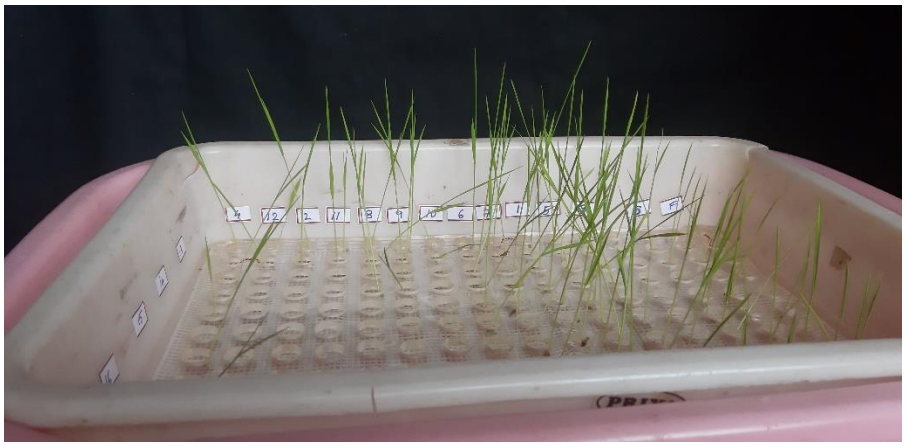


Plate. 18. Salinity screening of survived BC_1F_1 population at 12 dSm^{-1}

Plate. 19. Crossing block of BC_1F_1 population

The number of days to germination completion followed a similar pattern. The observation of BC1F1 (6.28) and F1 (6.25) populations were on par with Sreyas (6.36) while FL478 (6.09) was earlier.

4.3.1.3. Survival percentage

The germinated seedlings were transferred to salinity screening floats and the same salinity screening procedure as F_1 generation was followed. At the 10^{th} day of salinization 178 plants were found to survive which was reduced to 124 by 16^{th} day of salinization. On the 16^{th} day of salinization the saline nutrient medium was replaced by regular Yoshida solution. The survival percentage increased substantially in BC_1F_1 (49.79%) population compared to the recurrent parent Sreyas which did not survive at all. The F_1 (52.27%) population had better survival compared to the backcross generation. Salt injury observations of Sreyas, BC_1F_1, F_1 and FL478 are illustrated in Table. 35.

Table. 35. Salinity screening for salt injury aspects of BC_1F_1 population

Salinity screening	Survival percentage	SES Score	Leaf drying percentage
Sreyas	0.00	7.96	98.67
F_1	52.27	6.49	87.34
BC_1F_1	49.79	6.63	89.25
FL 478	78.57	5.37	71.79

4.3.1.4. SES Score

The mean SES scores which indicate salt injury in BC_1F_1 (6.63) population improved as against the susceptible parent, Sreyas (7.96). The BC_1F_1 population tends towards susceptibility when the mean value is considered.

4.3.1.5. Percentage leaf drying

The percentage leaf drying, another salt stress indicator was found to have improved in BC_1F_1 (89.25%) population compared to the recurrent parent Sreyas (98.67%) although higher than the F_1 (87.34%) population.

After 18 days of salinization, 58 BC_1F_1 plants were found to survive. At 21 days after salinization 26 plants which survived *in*-vitro screening were transferred to the crossing block. Among the survived plants 17 BC_1F_1 plants recovered from salt injury. After 2-3 weeks DNA was extracted from the young leaves of the recovered plants.

4.3.2. Molecular screening

The genotypic screening consisted of the isolation of DNA, measurement of quantity and quality of DNA and marker-assisted foreground, recombinant and background selection. The recurrent genome recovery was ascertained to aid in selecting plants to be advanced to the next generation.

4.3.2.1. Quantity and Quality of DNA

DNA required for genotypic screening was isolated from young leaves of BC_1F_1 plants using CTAB method. The quantity and quality of the DNA were measured using Thermo Scientific NanoDrop2000c. The observations are recorded in Table.36.

Table. 36. Quantity and quality of genomic DNA of BC_1F_1 population

Sl. No.	Sample	DNA Quantity (ng μl^{-1})	A260	A280	DNA Quality 260/280
1	2019-01-1	835.1	16.702	8.521	1.96
2	2019-01-2	770.8	15.416	7.376	2.09
3	2019-01-3	784.2	15.683	8.043	1.95
4	2019-01-4	789.7	15.794	7.937	1.99
5	2019-01-5	1033.2	20.665	10.437	1.98
6	2019-01-6	1346	26.919	13.460	2.00
7	2019-01-7	1348.8	26.977	13.694	1.97
8	2019-01-8	860.4	17.207	8.870	1.94
9	2019-01-9	1063	21.259	10.683	1.99
10	2019-01-10	679.4	13.588	7.040	1.93
11	2019-01-11	1304.4	26.088	13.044	2.00

12	2019-01-12	1107.9	22.158	11.248	1.97
13	2019-01-13	899.5	17.991	9.570	1.88
14	2019-01-14	730.3	14.606	7.452	1.96
15	2019-01-15	820.7	16.415	8.249	1.99
16	2019-01-16	805	16.101	8.386	1.92
17	2019-01-17	832.3	16.646	8.241	2.02

The absorbance ratios indicated that good quantity of template DNA with quality ranging from 1.88 - 2.09 were isolated. The DNA sample from plant 2019-01-13 was the purest with an absorbance ratio of 1.88. The highest absorbance ratio of 2.09 was registered for the plant 2019-01-2. The quantity of DNA ranged from 679.4-1348.8 ng μl^{-1} where 2019-01-10 recorded lowest and 2019-01-7 registered highest.

4.3.2.1. Foreground screening

Markers tightly linked to the QTL namely RM10711, RM8094, RM10713, RM10720 and RM3412 were used for foreground molecular screening of the 17 recovered BC_1F_1 plants. The loci in question were in heterozygous condition in all 17 plants. The GGT score of the markers is illustrated in Table. 37. GGT image of foreground and recombinant selection of BC_1F_1 plants is shown in Fig. 5. The region marked as A shows recurrent genome in homozygous condition and H shows heterozygosity.

4.3.2.2. Recombinant screening

The markers RM1287, RM10701, RM493 and RM10895 were used for the recombinant selection of the BC_1F_1 plants. The plants 2019-01-1, 2019-01-3, 2019-01-4, 2019-01-8, 2019-01-10, 2019-01-11, 2019-01-14 and 2019-01-16 among the 17 BC_1F_1 plants showed heterozygosity for all the recombinant markers. In the locus of RM1287, the plants 2019-01-6, 2019-01-9, 2019-01-13 and 2019-01-17 had recurrent parent genotype in homozygous condition. RM10701 was homozygous for Sreyas's genotype in 2019-01-17. The marker RM493 bore semblance to recurrent parent in 2019-01-5, 2019-01-7, 2019-01-12 and 2019-01-

15. RM10825 was homozygous for recurrent parent genotype in 2019-01-2, 2019-01-5, 2019-01-7 and 2019-01-15. The results obtained are as in Table. 38.

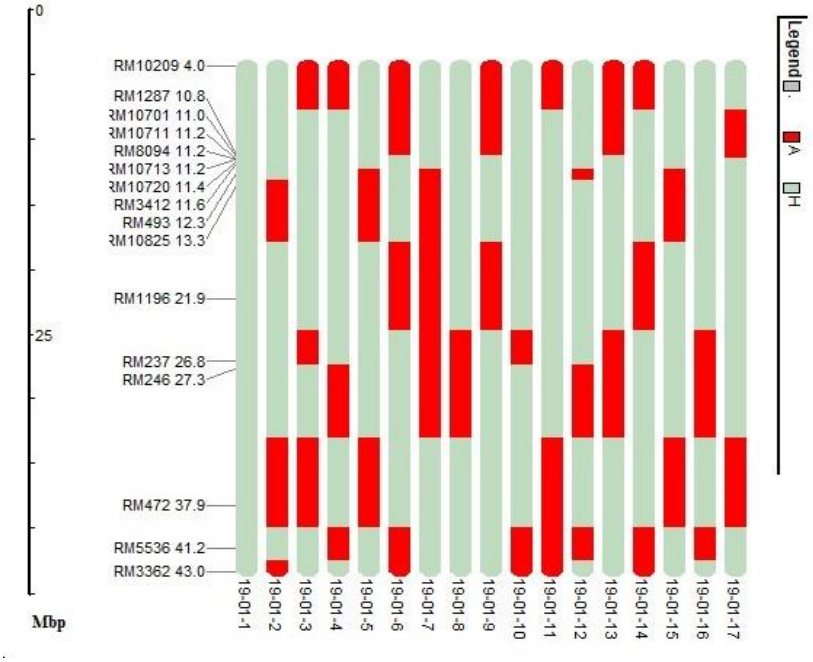


Fig. 5. GGT image of foreground and recombinant markers of BC_1F_1 population

4.3.2.3. Background screening

The background screening was conducted using 83 markers which were found to be polymorphic between the parents. The observation of genotyping using background markers in BC_1F_1 plants is compiled in Table. 39.

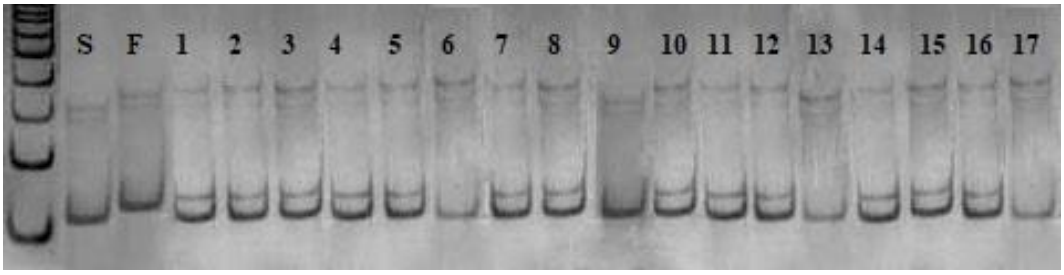


Plate. 20. Gel image of RM1287 of BC_1F_1 plants

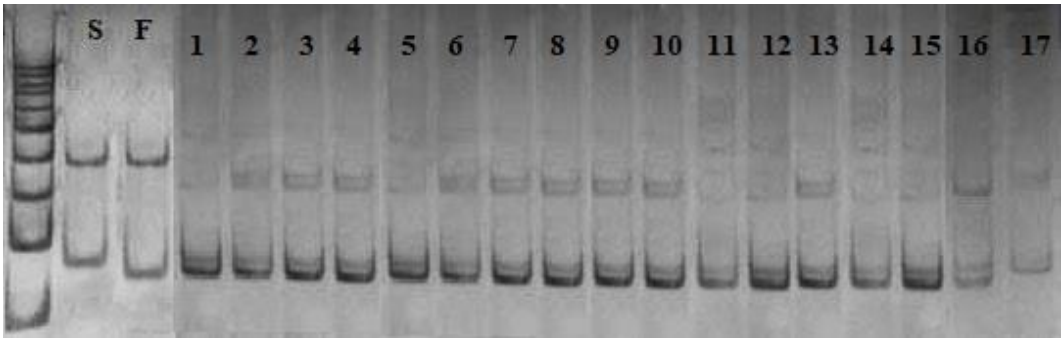


Plate. 21. Gel image of RM10701 of BC_1F_1 plants

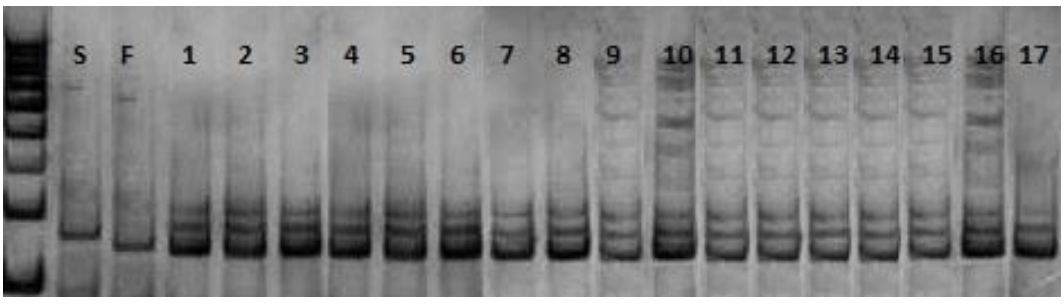


Plate. 22. Gel image of RM10711 of BC_1F_1 plants

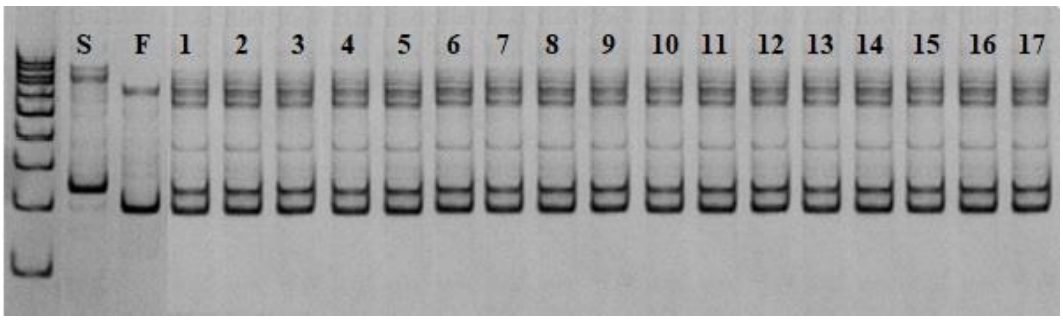


Plate. 23. Gel image of RM8094 of BC_1F_1 plants

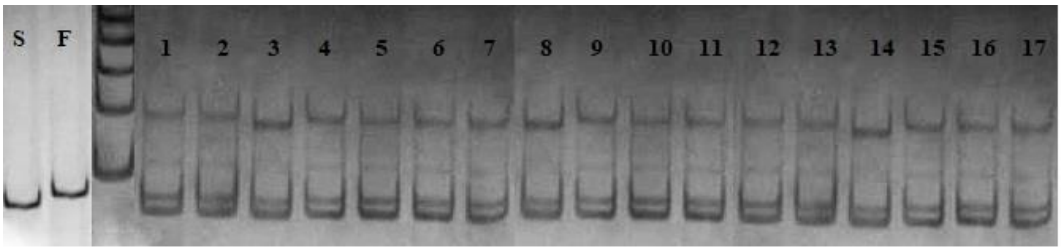


Plate. 24. Gel image of RM10713 of BC_1F_1 plants

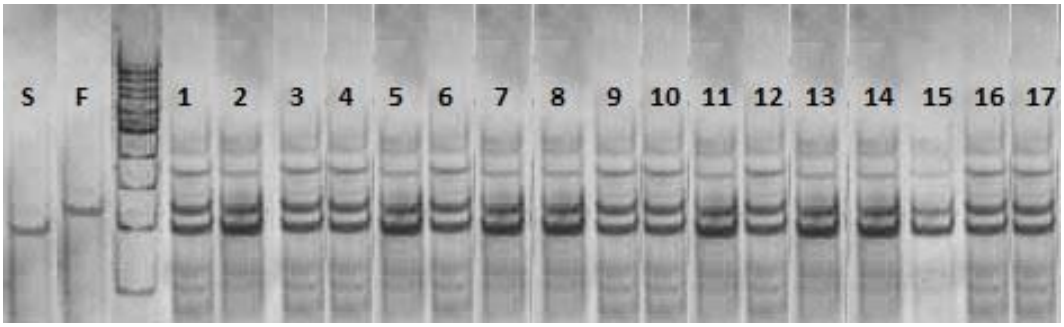


Plate. 25. Gel image of RM10720 of BC_1F_1 plants

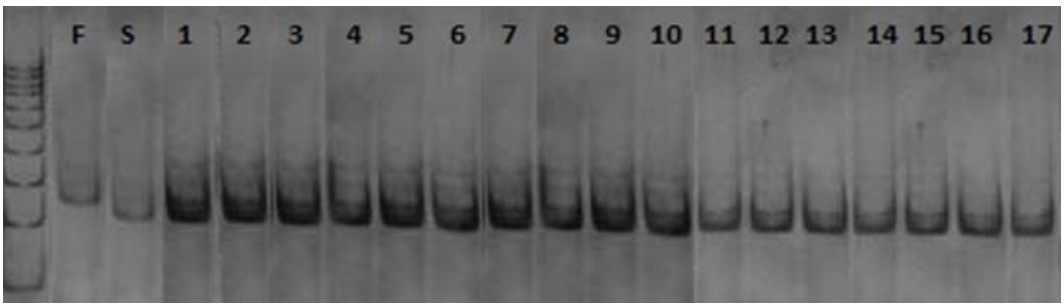


Plate. 26. Gel image of RM3412 of BC_1F_1 plants

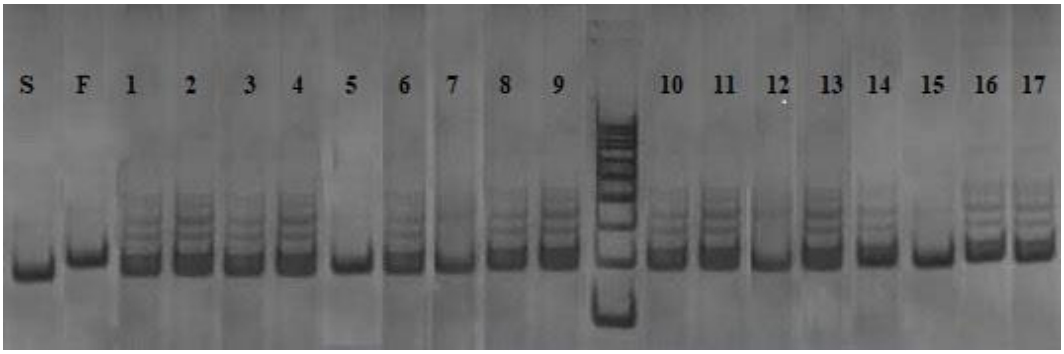


Plate. 27. Gel image of RM493 of BC_1F_1 plants

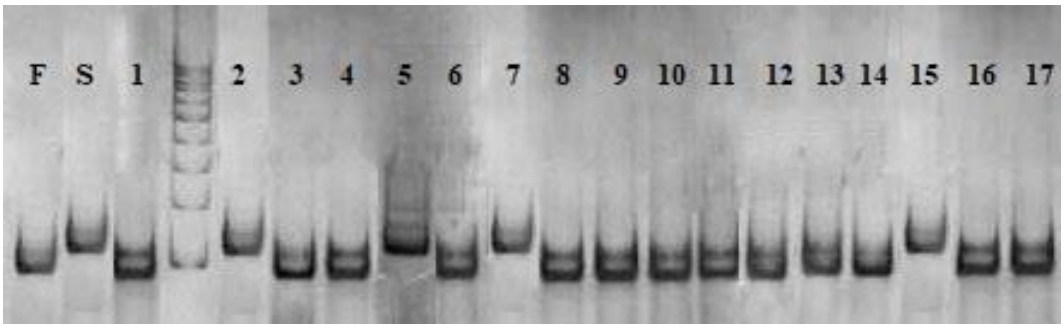


Plate. 28. Gel image of RM10825 of BC_1F_1 plants

Table. 37. GGT score of foreground markers of BC_1F_1 plants

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RM10711	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM8094	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM10713	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM10720	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM3412	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H

Table. 38. GGT score of recombinant markers of BC_1F_1 plants

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RM1287	H	H	H	H	H	A	H	H	A	H	H	H	A	H	H	H	A
RM10701	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	A
RM493	H	H	H	H	A	H	A	H	H	H	H	A	H	H	A	H	H
RM10825	H	A	H	H	A	H	A	H	H	H	H	H	H	H	A	H	H

Table. 39. GGT score of background markers of BC_1F_1 plants

Markers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Chromosome 1																	
RM10209	H	H	A	A	H	A	H	H	A	H	A	H	A	A	H	H	H
RM1196	H	H	H	H	H	A	A	H	A	H	H	H	H	A	H	H	H
RM237	H	H	A	H	H	H	A	A	H	A	H	H	A	H	H	A	H
RM246	H	H	H	A	H	H	A	A	H	H	H	A	A	H	H	A	H
RM472	H	A	A	H	A	H	H	H	H	H	A	H	H	H	A	H	A
RM5536	H	H	H	A	H	A	H	H	H	A	A	A	H	A	H	A	H
RM3362	H	A	H	H	H	A	H	H	H	A	A	H	H	A	H	H	H
Chromosome 2																	
OSR 14	H	H	H	H	H	H	H	A	H	H	H	H	H	H	H	H	A
RM279	H	A	H	H	A	H	H	A	A	A	H	A	H	H	H	A	A
RM555	H	H	H	A	H	A	H	H	H	A	H	H	H	H	A	A	H
RM324	H	H	A	H	H	H	H	H	A	H	H	H	A	H	H	H	H
RM250	H	H	H	H	A	H	H	H	H	H	H	H	H	A	H	H	H
RM208	H	H	H	H	H	A	H	H	H	H	A	A	A	H	H	H	H
RM7485	H	H	H	H	H	A	H	H	H	H	A	H	H	H	A	H	H
Chromosome 3																	
RM546	H	H	H	H	H	H	H	H	H	H	H	H	H	A	H	H	A
OSR 13	H	H	H	H	H	A	H	H	H	A	H	A	A	A	H	H	H
RM7	H	A	A	H	A	H	H	H	A	H	H	H	H	H	A	A	H
RM5626	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	A
RM168	H	H	H	H	H	H	H	H	H	H	A	H	H	H	H	H	H
RM520	H	A	A	H	H	H	H	A	H	H	A	A	H	H	H	A	H

Markers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
OSR 16	H	H	H	A	A	H	H	A	H	H	H	A	H	H	H	A	H
RM514	H	H	H	A	H	H	H	H	H	A	H	H	H	H	H	H	A
Chromosome 4																	
RM518	H	A	H	H	H	H	A	A	H	H	H	H	A	H	H	A	H
RM273	H	H	A	H	A	H	H	H	A	A	H	H	H	A	A	H	A
RM241	H	A	H	H	H	A	H	H	A	A	H	H	H	H	H	H	H
RM317	H	H	H	H	H	H	A	H	H	H	H	A	H	H	A	H	H
RM348	H	A	H	A	H	H	A	H	H	H	A	H	H	H	H	H	H
RM124	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H	H	A
Chromosome 5																	
RM122	A	H	H	H	H	H	A	H	H	A	H	H	H	H	A	A	H
RM413	H	A	H	H	H	H	H	A	H	H	A	H	A	A	A	A	A
RM17960	H	H	H	H	A	H	H	H	H	H	A	H	H	A	H	H	H
RM249	H	A	H	H	H	H	A	H	H	H	H	A	H	H	A	H	H
RM6229	H	H	H	H	H	H	H	H	A	H	H	H	A	H	H	H	H
RM161	A	H	A	H	H	H	A	H	H	H	H	A	H	H	H	H	H
Chromosome 6																	
RM469	H	H	H	H	H	H	H	A	H	H	H	H	H	A	H	H	H
RM197	H	H	H	H	A	H	A	H	A	H	H	H	H	H	H	H	H
RM276	H	H	A	A	H	H	H	H	H	A	H	H	A	H	H	A	A
RM527	H	H	H	H	H	H	A	H	H	H	H	A	H	H	H	H	H
RM6818	H	A	H	H	H	A	H	H	A	H	H	H	A	H	A	H	H
RM162	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	A	H
RM494	A	H	H	H	H	H	H	H	H	A	H	H	H	H	H	H	H
Chromosome 7																	

Markers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RM3859	H	A	H	H	A	H	A	H	H	H	H	H	H	H	H	H	H
RM214	H	H	A	H	H	H	A	H	H	A	H	A	A	H	H	H	A
RM500	H	H	H	H	H	A	H	H	A	H	H	H	H	A	H	H	H
RM11	A	H	H	A	A	H	H	A	H	H	H	H	H	H	H	A	H
RM455	H	H	H	H	H	H	H	A	H	H	A	H	A	H	H	A	H
RM118	A	A	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H
Chromosome 8																	
RM337	H	A	H	H	H	H	H	A	A	H	H	A	H	A	A	H	H
RM152	H	H	H	H	A	H	A	A	A	H	H	H	H	A	A	H	H
RM1376	H	H	A	H	H	H	A	H	H	A	H	H	A	H	A	A	H
RM3481	H	H	H	A	H	H	H	H	H	H	H	H	H	H	H	H	A
RM6193	H	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H	H
RM210	H	H	H	A	H	H	H	H	H	H	A	H	H	H	A	H	H
RM149	A	A	H	H	H	H	H	H	H	H	H	A	A	H	H	H	A
RM447	A	H	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H
Chromosome 9																	
RM23654	A	H	A	H	A	H	H	A	H	A	H	H	H	H	H	H	A
RM8303	H	H	H	H	H	A	A	H	H	H	A	H	H	H	H	H	A
RM23805	H	H	H	H	H	A	A	H	A	H	H	H	A	H	A	H	H
RM5526	H	H	H	H	H	A	H	H	A	H	H	A	H	A	A	H	H
RM219	H	H	H	H	H	H	A	H	A	H	H	A	H	A	A	H	H
RM23958	H	A	H	H	H	H	H	H	H	H	H	H	H	A	H	H	A
RM296	A	H	H	A	H	H	H	A	H	H	A	H	A	A	H	A	H
RM3912	H	H	A	H	A	H	A	A	H	H	A	H	H	A	H	A	H
RM105	H	A	H	A	H	H	H	H	H	H	H	H	H	H	A	A	H

Markers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RM215	A	H	A	H	H	A	H	H	H	A	H	A	H	H	H	H	A
RM205	A	H	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H
Chromosome 10																	
RM6364	A	H	H	H	A	H	A	H	A	H	H	H	H	H	H	H	H
RM474	A	A	A	H	H	A	A	H	A	A	H	H	A	H	H	H	A
RM3882	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM216	H	H	H	A	H	H	H	H	H	H	H	A	H	H	H	A	H
RM311	H	H	A	H	H	H	A	A	H	H	H	H	A	H	A	H	H
RM5689	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
Chromosome 11																	
RM536	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM7120	H	H	A	H	H	A	H	H	A	A	H	A	H	A	H	H	A
RM26652	H	A	H	H	H	H	A	H	H	H	H	H	A	H	H	H	H
RM287	H	H	H	A	H	H	H	H	H	H	H	H	H	H	A	A	H
RM144	A	H	H	H	A	H	A	A	H	H	A	A	A	H	H	H	H
Chromosome 12																	
RM247	A	A	H	A	H	H	A	H	H	H	H	H	H	H	H	H	H
RM27973	H	H	A	H	A	H	H	H	H	H	A	H	H	H	H	A	H
RM277	H	H	H	H	H	H	H	H	H	A	H	A	H	H	H	H	A
RM519	H	H	H	H	H	A	A	H	H	H	H	H	H	A	A	H	H
RM17	H	A	H	H	H	H	H	A	H	H	H	A	H	H	H	H	H
RM1227	H	H	H	H	H	H	H	A	H	H	H	H	H	H	H	H	H

The background screening data is well depicted in the GGT image of the 12 chromosomes of the 17 BC_1F_1 plants (Fig. 6.). The region marked as A shows recurrent genome in homozygous condition and H shows heterozygosity.

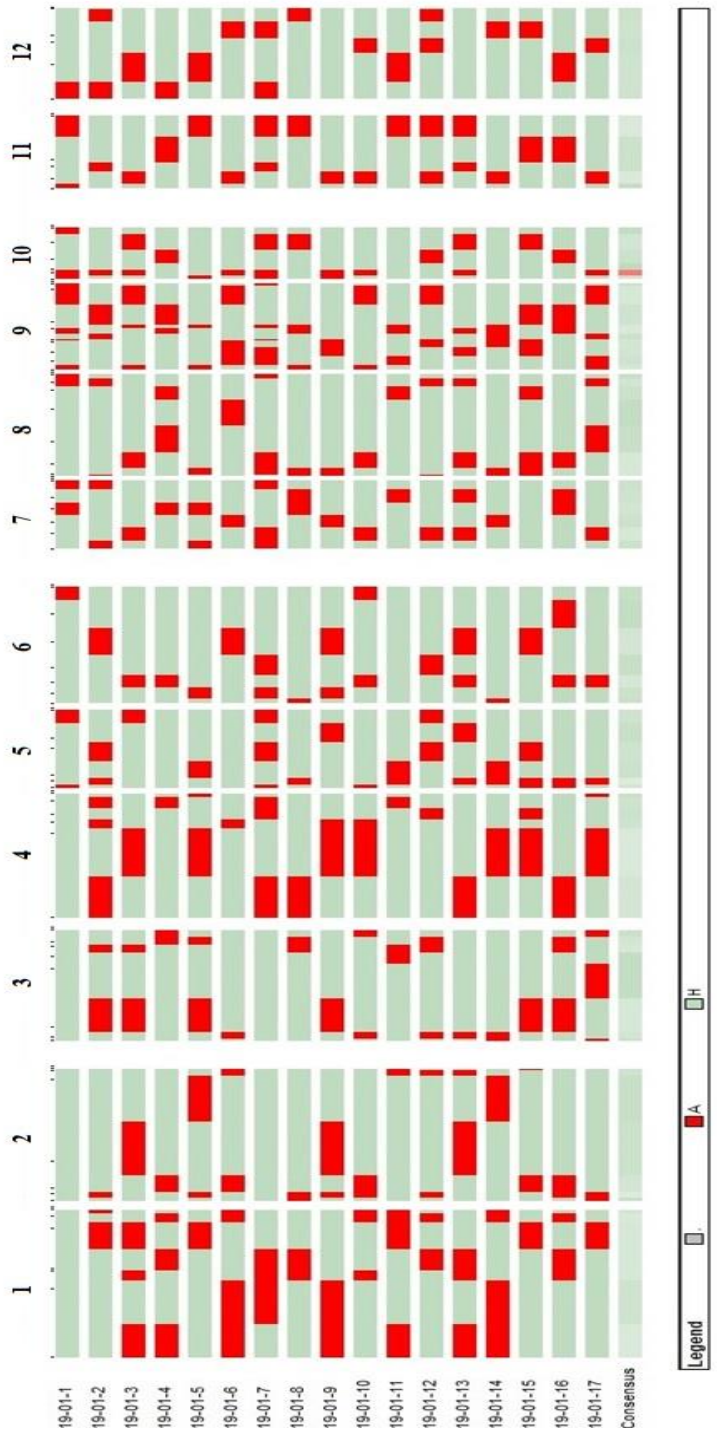


Fig. 6. GGT image of background markers of BC_1F_1 population

4.3.2.4. Recurrent genome recovery

The data from background screening were used to derive the recurrent genome recovery percentage which is a major criterion for the selection of plants for advanced generations (Table. 40).

Table. 40. Recurrent parent genome recovery of BC_1F_1 population

Sl. No.	Plant	A (%)	H (%)	RG Recovery%
1	19-01-1*	13.2	86.8	56.60
2	19-01-2	24.3	75.7	62.15
3	19-01-3*	30.3	69.7	65.15
4	19-01-4*	21.5	78.5	60.75
5	19-01-5	24.2	75.8	62.10
6	19-01-6	23	77	61.50
7	19-01-7	31.6	68.4	65.80
8	19-01-8*	17.6	82.4	58.80
9	19-01-9	28.9	71.1	64.45
10	19-01-10*	19.5	80.5	59.75
11	19-01-11*	19.3	80.7	59.65
12	19-01-12	21.4	78.6	60.70
13	19-01-13	30.3	69.7	65.15
14	19-01-14*	24.5	75.5	62.25
15	19-01-15	27.3	72.7	63.65
16	19-01-16*	29.1	70.9	64.55
17	19-01-17	22.6	77.4	61.30
	Mean			62.02

The mean recurrent genome recovery percentage ranged from 56.60% in plant 2019-01-1 to 65.80% in 2019-01-07. An average RG recovery of 62.02% was observed across the 17 BC_1F_1 plants. From the 17 plants survived in BC_1F_1 generation, plants 2019-01-1, 2019-01-3, 2019-01-4, 2019-01-8, 2019-01-10, 2019-01-11, 2019-01-14 and 2019-01-16 were observed to have the QTL intact and good RG recovery were advanced to the next generation by backcrossing.

4.4. EXPERIMENT IV

The BC_2F_1 population was subjected to salinity screening and molecular screening. *In-vitro* salinity screening at 12 dS m^{-1} was conducted and survived plants were genotyped using foreground, recombinant and background markers. The selected plants were self-pollinated to develop the BC_2F_2 generation.

4.4.1. Salinity screening

The salinity tolerance of BC_2F_1 population showed improvement with respect to the parent Sreyas. A total of 280 BC_2F_1 seeds were sown for salinity screening. 243 seedlings germinated under saline environment. Salinity screening observations of the progeny of the 8 selected BC_1F_1 plants *viz.*, 2019-01-1 (1), 2019-01-3 (3), 2019-01-4 (4), 2019-01-8 (8), 2019-01-10 (10), 2019-01-11 (11), 2019-01-14 (14) and 2019-01-16 (16) for germination parameters are tabulated in Table. 41.

4.4.1.1. Germination percentage

Mean germination percentage observed was highest for progeny of the plant 2019-01-16 (94.29%) followed by 2019-01-3 (91.43%) and lowest for 2019-01-11 (80%). The lines 2019-01-1 (85.71%), 2019-01-4 (88.57%), 2019-01-8 (82.86%), 2019-01-10 (88.57%), and 2019-01-14 (82.86%) showed intermediate values for germination percentage.

Table. 41. Germination parameters under salinity in progeny of 8 selected lines

Lines	Germination percentage	Days to germination (initiation)	Days to germination (completion)
2019-01-1	85.71	1.30	6.29
2019-01-3	91.43	1.31	6.32
2019-01-4	88.57	1.33	6.34
2019-01-8	82.86	1.29	6.30
2019-01-10	88.57	1.32	6.34
2019-01-11	80.00	1.33	6.32
2019-01-14	82.86	1.28	6.30
2019-01-16	94.29	1.29	6.31
Mean	**86.79**	**1.31**	**6.32**

The overall mean germination percentage of BC_2F_1 (86.79%) populations improved compared to the recurrent parent Sreyas (83.33%) and were on par with the F_1 and BC_1F_1 populations (Table. 42).

Table. 42. Germination parameters under salinity of BC_2F_1 population

Salinity screening	Germination percentage	Days to Germination (Initiation)	Days to Germination (Completion)
Sreyas	83.33	1.32	6.36
F_1	86.81	1.26	6.25
BC_1F_1	86.45	1.28	6.28
BC_2F_1	86.79	1.31	6.32
FL 478	93.33	1.05	6.09

4.4.1.2. Days to germination

The mean number of days taken for germination initiation was lowest for line 2019-01-14 (1.28) followed by 2019-01-8 (1.29) and 2019-01-16 (1.29). The lines 2019-01-1(1.30), 2019-01-3 (1.31), 2019-01-10 (1.32) showed higher days to germination initiation. The highest value was recorded by the lines 2019-01-4 (1.33) and 2019-01-11 (1.33) which were higher than the recurrent parent.

The mean days taken for completion of germination was lowest in the lines 2019-01-1 (6.29) followed by 2019-01-8 (6.30) and 2019-01-14 (6.30). The lines 2019-01-16 (6.31), 2019-01-3 (6.32) and 2019-01-11 (6.32) showed intermediary values. The longest duration for completion of germination was recorded by 2019-01-4 (6.34) and 2019-01-10 (6.34).

The overall days to germination initiation in BC_2F_1 (1.31) populations was on par with Sreyas (1.32) while completion of germination was earlier in BC_2F_1 (6.32) compared to the recurrent parent (6.36).

4.4.1.3. Survival percentage

The seedlings were germinated and transferred to salinity screening floats. On the 16th day of salinization, the saline nutrient medium was replaced by regular Yoshida solution. At the 16th day of salinization, 98 plants survived. Salinity screening observations for salt injury parameters of the progeny of the 8 selected BC_1F_1 plants *viz.,* 2019-01-1 (1), 2019-01-3 (3), 2019-01-4 (4), 2019-01-8 (8), 2019-01-10 (10), 2019-01-11 (11), 2019-01-14 (14) and 2019-01-16 (16) are compiled in Table. 43.

Table. 43. Salinity screening salt injury attributes in progeny of 8 selected lines

Lines	Survival percentage	SES score	Percentage leaf drying
2019-01-1	56.67	6.22	79.63
2019-01-3	40.63	7.31	89.74
2019-01-4	38.06	7.25	93.94
2019-01-8	35.17	7.31	93.33
2019-01-10	25.81	7.55	91.67
2019-01-11	31.03	7.89	92.59
2019-01-14	55.71	6.88	85.42
2019-01-16	39.39	7.60	92.31
Mean	**40.31**	**7.25**	**89.83**

The survival percentage was found to be highest in the line 2019-01-1 (56.67%) followed by 2019-01-14 (55.71%). The lines 2019-01-03 (40.63%), 2019-01-16 (39.39%), 2019-01-4 (38.06%) and 2019-01-8 (35.17%) recorded comparatively lower values. The least values were observed in 2019-01-11 (31.03%) and 2019-01-10 (25.81%). The mean survival percentage increased considerably in BC_2F_1 (40.31%) population compared to the recurrent parent Sreyas which did not survive at all (Table. 44.).

4.4.1.4. SES Score

The lowest SES score was registered by 2019-01-1 (6.22) followed by 2019-01-14 (6.88) which are closer to moderate tolerance. The lines 2019-01-4 (7.25), 2019-01-3 (7.31) and 2019-01-8 (7.31) showed higher visual injury score. The lines

2019-01-10 (7.55) and 2019-01-16 (7.60) suffered more damage. The highest injury level was observed in 2019-01-11 (7.89). These lines are in the susceptible category. The mean SES scores which indicate salt injury in BC_2F_1 (7.25) population improved as against Sreyas (7.96) yet shows susceptibility.

Table. 44. Salinity screening for salt injury aspects BC_2F_1 population

Salinity screening	Survival percentage	SES Score	Leaf drying percentage
Sreyas	0.00	7.96	98.67
F_1	52.27	6.49	87.34
BC_1F_1	49.79	6.63	89.25
BC_2F_1	40.31	7.25	89.83
FL 478	78.57	5.37	71.79

4.4.1.5. Percentage leaf drying

The leaf drying percentage, another salt stress indicator was found to be least in 2019-01-1 (79.63%) followed by 2019-01-14 (85.42%) and 2019-01-3 (89.74%). The highest was observed in 2019-01-4 (93.94%) followed by 2019-01-8 (93.33%), 2019-01-11 (92.59%), 2019-01-16 (92.31%) and 2019-01-10 (91.67%). The average percentage leaf drying was found to have improved in BC_2F_1 (89.83%) populations compared to the recurrent parent Sreyas (98.67%).

After 18 days of salinization, 56 BC_2F_1 plants were found to survive. At 21 days after salinization 31 plants which survived *in*-vitro screening were transferred to the crossing block. Among the transplanted plants 24 BC_2F_1 plants recovered from salt injury. After 2-3 weeks DNA was extracted from the young leaves of the recovered plants.

4.4.2. Molecular screening

The molecular screening consisted of the isolation of DNA, measurement of quantity and quality of DNA and marker-assisted foreground, recombinant and background selection of the BC_2F_1 plants. The recurrent genome recovery was ascertained to aid in selecting plants to be advanced in the breeding programme.

4.4.2.1 Quantity and quality of genomic DNA

DNA was extracted from young leaves of BC_2F_1 plants which recovered from salinity stress using CTAB method. Thermo Scientific NanoDrop2000c was used to measure the quantity and quality of the DNA. The readings are recorded in Table. 45.

The quantity of DNA obtained ranged from 343.1 to 2093.1 ng μl^{-1} with the highest value recorded in 2019-01-1-4 and the least in 2019-01-4-1. The absorbance ratio ranged from 1.89 to 2.01. The highest absorbance ratio was observed for 2019-01-1-1, 2019-01-10-1 and 2019-01-10-2 of 2.01. The best quality template DNA was obtained from 2019-01-8-1 (1.89).

Table. 45. Quantity and quality of genomic DNA of BC_2F_1 population

Sl. No.	Sample	DNA Quantity (ng μl^{-1})	A260	A280	DNA Quality 260/280
1	2019-01-1-1	1207	24.140	12.010	2.01
2	2019-01-1-2	806.7	16.134	8.108	1.99
3	2019-01-1-3	1900.9	38.018	19.298	1.97
4	2019-01-1-4	2093.1	41.863	21.143	1.98
5	2019-01-1-5	705.4	14.107	7.089	1.99
6	2019-01-1-6	1713.7	34.275	17.487	1.96
7	2019-01-1-7	1382.3	27.646	14.034	1.97
8	2019-01-1-8	822.9	16.457	8.270	1.99
9	2019-01-1-9	978.9	19.579	9.989	1.96
10	2019-01-3-1	1136.8	22.737	11.542	1.97
11	2019-01-3-2	1292.9	25.858	13.261	1.95
12	2019-01-4-1	343.1	6.861	3.448	1.99
13	2019-01-8-1	693	13.859	7.3328	1.89
14	2019-01-8-2	583.2	11.664	5.891	1.98
15	2019-01-10-1	730.6	14.612	7.270	2.01
16	2019-01-10-2	809.6	16.191	8.055	2.01
17	2019-01-11-1	1156.1	23.123	11.738	1.97
18	2019-01-14-1	1022.2	20.444	10.538	1.94
19	2019-01-14-2	889.6	17.793	8.897	2.00
20	2019-01-14-3	1568.9	31.378	16.009	1.96
21	2019-01-14-4	941.3	18.827	9.461	1.99

Sl. No.	Sample	DNA Quantity (ng μl^{-1})	A260	A280	DNA Quality 260/280
22	2019-01-14-5	1377.9	27.557	14.132	1.95
23	2019-01-14-6	565.4	11.309	5.741	1.97
24	2019-01-16-1	879.8	17.596	8.978	1.96

4.4.2.1. Foreground screening

Foreground selection was carried out using 5 markers tightly linked to the QTL namely RM10711, RM8094, RM10713, RM10720 and RM3412 in 24 BC_2F_1 plants. The loci in question were in the heterozygous condition in all plants except 2019-01-1-5 where the locus RM3412 showed homozygosity for recurrent genotype. The GGT score of the markers is illustrated in Table. 46.

4.4.2.2. Recombinant screening

The markers RM1287, RM10701, RM493 and RM10895 were used for recombinant selection of the BC_2F_1 plants (Table. 47).

The plants 2019-01-1-3, 2019-01-1-4, 2019-01-1-6, 2019-01-1-7, 2019-01-1-9, 2019-01-3-1, 2019-01-3-2, 2019-01-4-1, 2019-01-10-2, 2019-01-14-1, 2019-01-14-3, 2019-01-14-5 and 2019-01-14-6 showed heterozygosity for all the loci. The locus RM1287 showed recurrent genotype homozygosity in 2019-01-1-1, 2019-01-1-2, 2019-01-1-8, 2019-01-8-1, 2019-01-8-2, 2019-01-14-2 and 2019-01-16-1. In the case of the locus RM10701 was homozygous for Sreyas's genotype in 2019-01-1-2, 2019-01-1-8, 2019-01-8-2 and 2019-01-16-1. In the plants 2019-01-1-5, 2019-01-11-1 and 2019-01-14-4 the locus RM493 was in the homozygous recurrent parent genotype. The plants 2019-01-1-5, 2019-01-10-1, 2019-01-11-1 and 2019-01-14-4 resembled recurrent parent in the locus RM10825. The size of the introgressed fragments in each BC_2F_1 plant is shown in Table. 48.

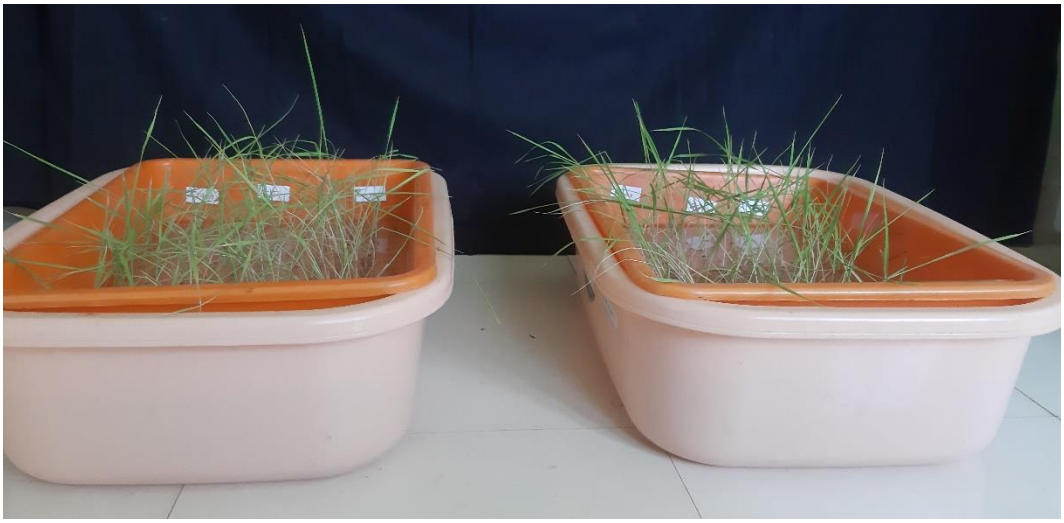


Plate. 29. Salinity screening of survived BC_2F_1 population at 12 dSm^{-1}

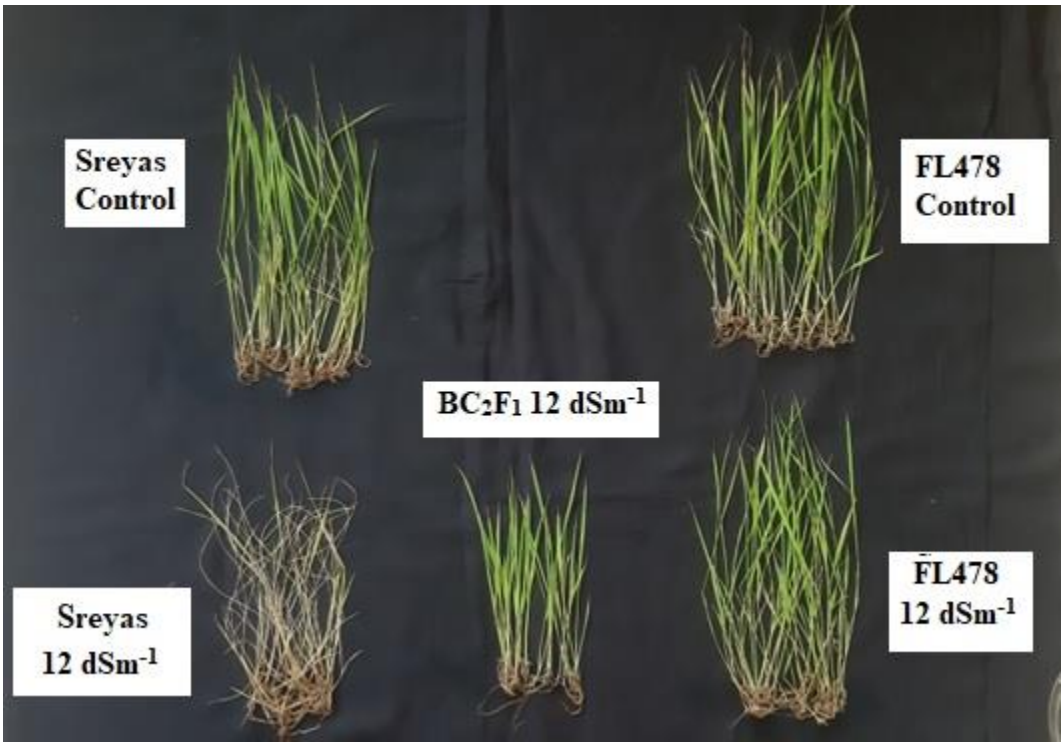


Plate. 30. Comparison of survived BC_2F_1 population at 12 dSm^{-1}

Plate. 31. Crossing block of BC_2F_1 population

Plate. 32. Survived plants of BC_2F_1 population

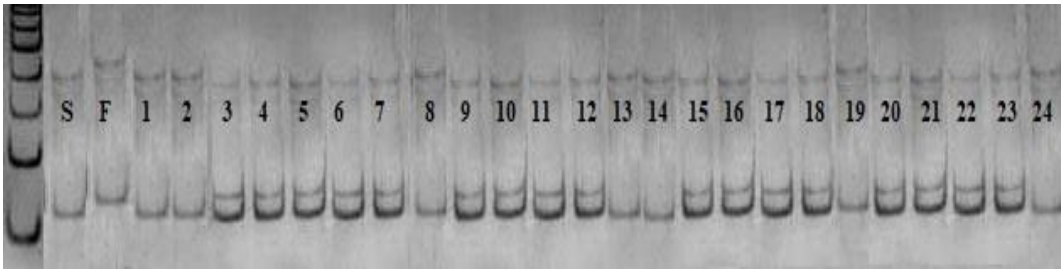


Plate. 33. Gel image of RM1287 of BC_2F_1 plants

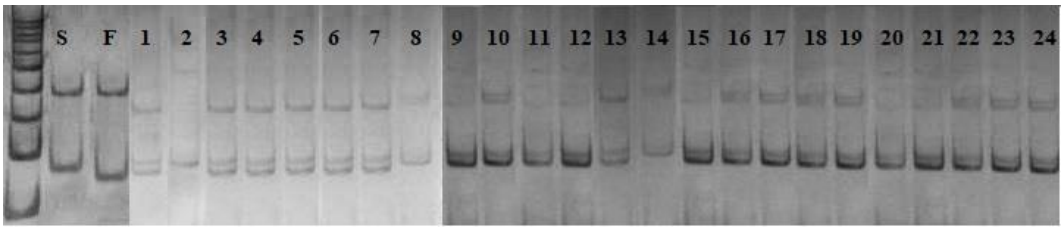


Plate. 34. Gel image of RM10701 of BC_2F_1 plants

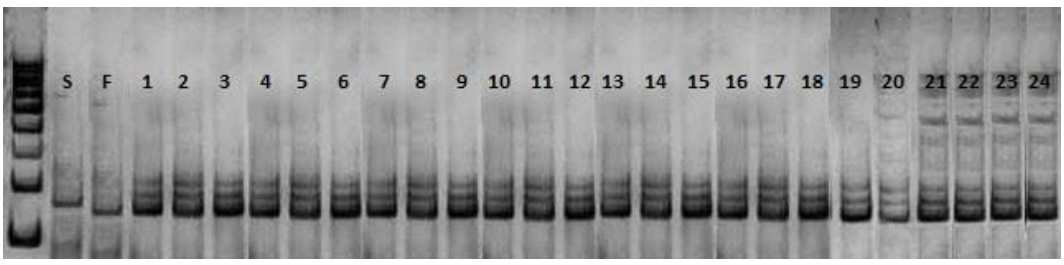


Plate. 35. Gel image of RM10711 of BC_2F_1 plants

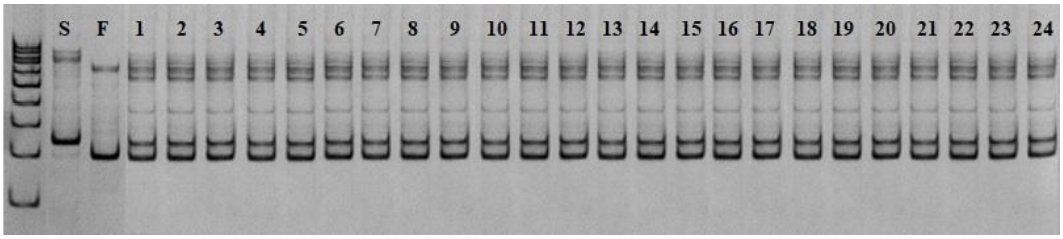


Plate. 36. Gel image of RM8094 of BC_2F_1 plants

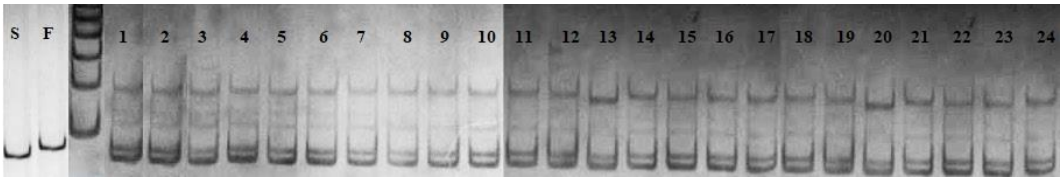


Plate. 37. Gel image of RM10713 of BC_2F_1 plants

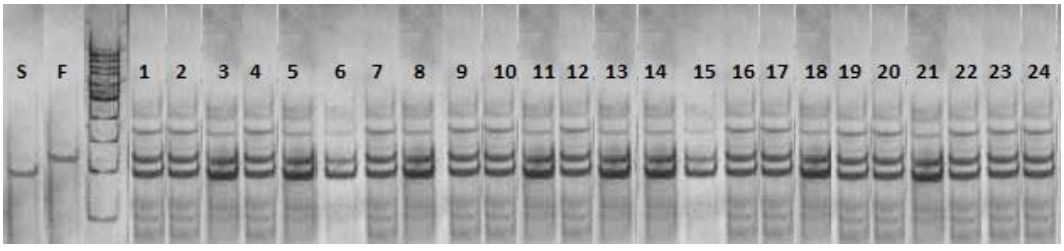


Plate. 38. Gel image of RM10720 of of BC_2F_1 plants

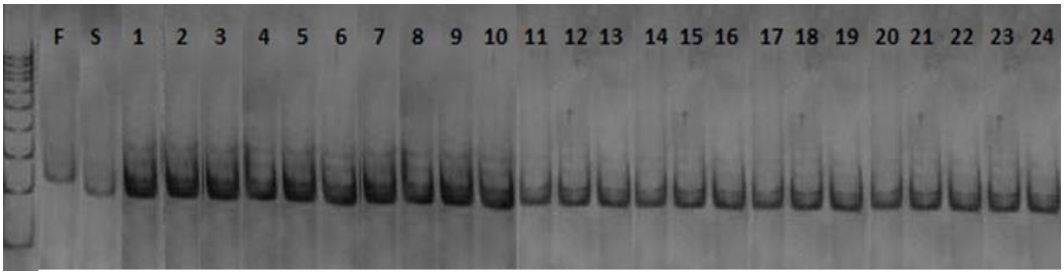


Plate. 39. Gel image of RM3412 of BC_2F_1 plants

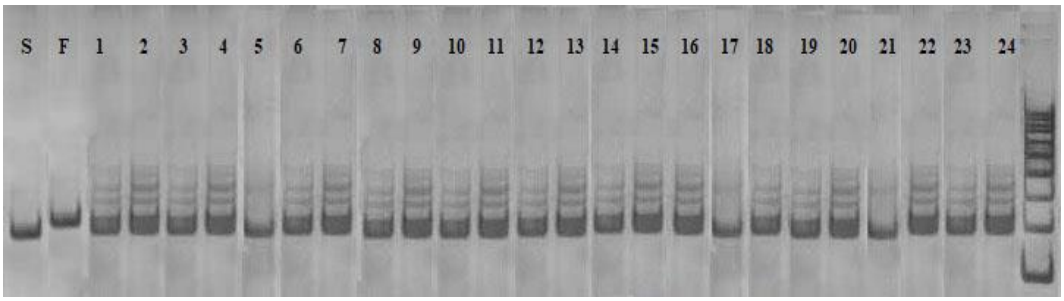


Plate. 40. Gel image of RM493 of BC_2F_1 plants

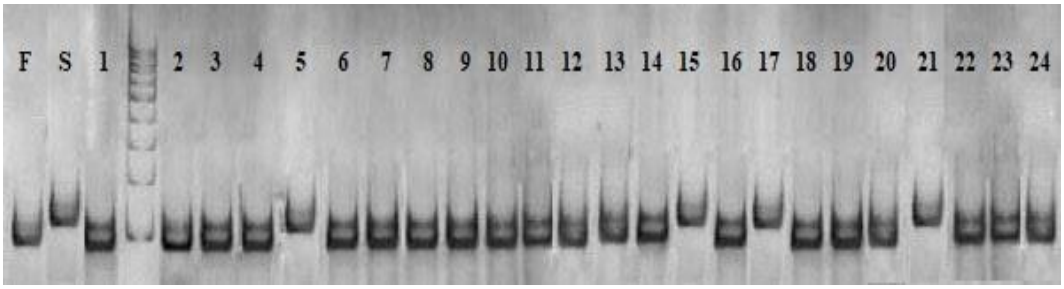


Plate. 41. Gel image of RM10825 of BC_2F_1 plants

Table. 46. GGT score of foreground markers of BC_2F_1 plants

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
RM10711	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM8094	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM10713	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM10720	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
RM3412	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H

Table. 47. GGT score of recombinant markers of BC_2F_1 plants

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
RM1287	A	A	H	H	H	H	H	A	H	H	H	H	A	A	H	H	H	H	A	H	H	H	H	A
RM10701	H	A	H	H	H	H	H	A	H	H	H	H	H	A	H	H	H	H	H	H	H	H	H	A
RM493	H	H	H	H	A	H	H	H	H	H	H	H	H	H	H	H	A	H	H	H	A	H	H	H
RM10825	H	H	H	H	A	H	H	H	H	H	H	H	H	H	A	H	A	H	H	H	A	H	H	H

Table. 48. Size of introgressed segment in BC_2F_1 population

Sl. No.	Plant	Size of introgressed segment (Mbp)
1	19-01-1-1	2.3
2	19-01-1-2	2.16
3	19-01-1-3*	2.49
4	19-01-1-4*	2.49
5	19-01-1-5	0.56
6	19-01-1-6*	2.49
7	19-01-1-7*	2.49
8	19-01-1-8	2.16
9	19-01-1-9*	2.49
10	19-01-3-1*	2.49
11	19-01-3-2*	2.49
12	19-01-4-1*	2.49
13	19-01-8-1	2.3
14	19-01-8-2	2.16
15	19-01-10-1	1.45
16	19-01-10-2*	2.49
17	19-01-11-1	0.75
18	19-01-14-1*	2.49
19	19-01-14-2	2.3
20	19-01-14-3*	2.49
21	19-01-14-4	0.75
22	19-01-14-5*	2.49
23	19-01-14-6*	2.49
24	19-01-16-1	2.16

In the BC_2F_1 population, the plants, 2019-01-1-3, 2019-01-1-4, 2019-01-1-6, 2019-01-1-7, 2019-01-1-9, 2019-01-3-1, 2019-01-3-2, 2019-01-4-1, 2019-01-10-2, 2019-01-14-1, 2019-01-14-3, 2019-01-14-5 and 2019-01-14-6 had an introgressed fragment of 2.49 Mbp within *Saltol* region (10.83 – 13.32 Mbp) in heterozygous condition. GGT image of the foreground and recombinant markers is illustrated in Fig. 7. The region marked as A shows recurrent genome in homozygous condition and H shows heterozygosity.

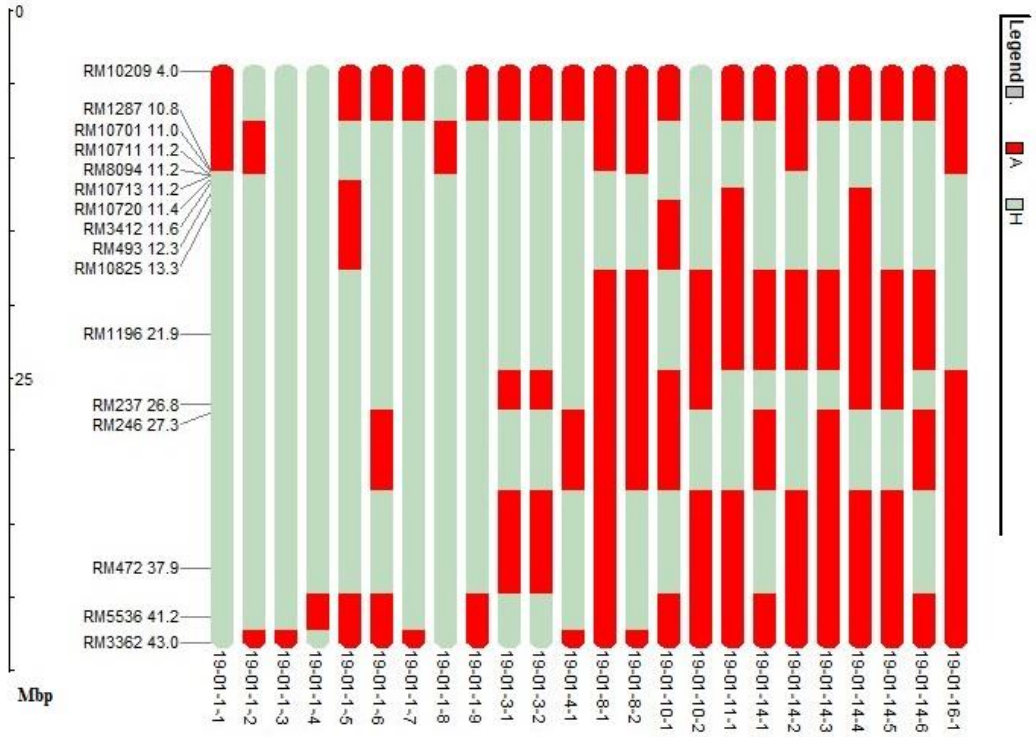


Fig. 7. GGT image of foreground and recombinant markers of BC_2F_1 population

4.4.2.3. Background screening

The background genotypic screening of BC_2F_1 plants was done using 83 polymorphic SSR markers. The observation of background screening is compiled in Table. 49.

Table. 49. GGT score of background markers of BC_2F_1 plants

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Chromosome 1																								
RM10209	A	H	H	H	A	A	A	H	A	A	A	A	A	A	A	H	A	A	A	A	A	A	A	A
RM1196	H	H	H	H	H	H	H	H	H	H	H	H	A	A	H	A	A	A	A	A	A	A	A	H
RM237	H	H	H	H	H	H	H	H	H	A	A	H	A	A	A	A	H	H	H	H	A	A	H	A
RM246	H	H	H	H	H	A	H	H	H	H	H	A	A	A	A	H	H	A	H	A	H	H	A	A
RM472	H	H	H	H	H	H	H	H	H	A	A	H	A	H	H	A	A	H	A	A	A	A	H	A
RM5536	H	H	H	A	A	A	H	H	A	H	H	H	A	H	A	A	A	A	A	A	A	A	A	A
RM3362	H	A	A	H	A	A	A	H	A	H	H	A	A	A	A	A	A	A	A	A	A	A	A	A
Chromosome 2																								
OSR 14	H	A	A	A	A	H	A	H	A	H	H	H	A	A	H	H	H	H	A	H	H	H	H	H
RM279	A	H	H	A	H	H	A	H	H	H	H	H	A	A	A	A	A	H	H	H	H	H	A	A
RM555	A	H	H	H	H	A	H	H	H	H	H	A	H	H	A	A	H	A	H	H	H	A	H	A
RM324	H	H	H	A	H	H	H	H	H	A	A	H	A	H	H	H	H	H	H	A	H	H	H	H
RM250	H	H	A	H	A	A	A	A	A	A	A	H	A	H	A	H	A	A	A	A	A	A	A	A
RM208	A	H	A	A	H	H	H	H	H	H	H	H	H	A	H	A	A	A	A	H	H	A	H	H
RM7485	A	H	A	A	H	H	A	A	A	H	H	H	H	H	H	H	A	H	A	H	A	H	H	H
Chromosome 3																								
RM546	H	H	H	H	H	H	H	A	A	A	H	A	H	H	A	H	H	A	A	A	A	A	A	A
OSR 13	H	H	H	A	A	H	A	A	H	H	H	H	H	H	A	A	H	A	A	A	A	A	A	H
RM7	A	A	A	A	A	A	H	H	H	A	A	A	H	A	H	A	H	H	H	H	A	H	A	A
RM5626	A	H	H	A	A	A	H	H	A	H	A	A	A	H	H	H	A	H	H	H	A	A	H	H
RM168	H	H	H	A	H	A	A	H	H	H	H	A	H	A	A	A	A	H	H	A	H	H	A	A
RM520	H	A	A	H	A	H	H	A	H	A	A	H	A	A	H	A	A	A	H	H	H	H	H	A

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
OSR 16	A	A	A	A	H	H	A	A	H	A	H	A	A	A	H	A	A	H	A	H	H	H	A	A
RM514	H	A	A	A	A	A	H	A	A	H	H	A	H	H	A	A	A	A	A	H	A	H	H	A
Chromosome 4																								
RM518	H	H	H	H	H	H	H	H	H	H	H	H	A	A	A	H	H	H	H	A	H	H	H	A
RM273	A	A	A	A	A	A	A	H	H	A	A	H	H	A	A	A	A	A	A	A	A	A	A	H
RM241	A	A	A	A	H	A	A	H	A	H	A	H	H	A	A	A	A	H	A	H	A	H	H	H
RM317	H	A	A	H	H	H	A	H	A	H	A	A	H	H	H	A	A	A	A	H	A	H	H	A
RM348	H	H	H	A	A	A	H	A	A	A	H	A	H	H	A	H	A	H	H	H	H	H	A	H
RM124	A	A	A	A	A	A	H	A	H	H	H	H	A	H	H	H	H	A	A	H	H	H	A	H
Chromosome 5																								
RM122	A	A	A	A	A	A	A	A	A	A	H	A	A	H	A	A	A	A	A	H	H	A	H	A
RM413	H	H	H	H	H	H	H	H	H	H	A	H	A	A	H	A	A	A	A	A	A	A	A	A
RM17960	H	A	A	A	A	H	H	A	H	H	H	H	H	A	H	A	A	A	A	A	A	A	A	A
RM249	H	H	H	H	A	A	A	H	A	A	H	A	H	H	A	H	H	H	H	A	H	A	H	H
RM6229	A	A	A	H	H	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	A	H	H	H
RM161	A	A	A	A	A	A	A	A	A	A	A	H	H	A	H	A	H	A	A	H	A	H	A	A
Chromosome 6																								
RM469	A	H	A	A	H	A	H	A	A	A	H	A	A	A	A	H	H	A	A	A	A	A	A	H
RM197	A	A	A	A	A	H	A	A	A	A	H	H	H	H	A	A	H	H	H	H	A	A	H	H
RM276	A	A	H	H	A	H	A	H	A	A	A	A	H	H	A	A	A	H	A	H	H	H	A	A
RM527	H	H	H	A	A	A	A	H	H	H	A	H	A	H	H	H	A	H	A	A	A	A	A	A
RM6818	H	A	H	H	H	H	H	H	H	H	H	A	H	A	H	H	H	H	H	H	H	A	A	H
RM162	A	A	A	A	A	H	A	H	H	H	H	H	A	A	H	A	H	H	A	H	A	H	H	A
RM494	A	A	A	A	A	A	A	A	A	A	H	H	A	H	A	A	H	H	H	A	A	A	A	A
Chromosome 7																								

Marker	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
RM3859	A	A	A	A	A	H	A	A	A	H	A	A	A	H	A	H	A	H	A	H	A	H	A	A
RM214	A	A	A	H	H	H	A	H	A	A	A	H	A	A	A	A	A	A	A	A	A	H	H	H
RM500	H	H	H	H	H	H	H	H	H	H	A	H	H	H	A	H	H	A	A	A	A	A	A	H
RM11	A	A	A	A	A	A	A	A	A	A	H	A	A	A	H	H	A	H	A	H	A	H	H	A
RM455	A	A	H	A	A	A	A	A	A	A	H	H	A	A	H	A	A	A	H	H	H	H	H	A
RM118	A	A	A	A	A	A	A	A	A	A	H	A	H	A	A	H	H	H	A	A	A	A	H	A
Chromosome 8																								
RM337	H	A	A	A	H	A	A	A	A	H	H	A	A	A	H	H	A	A	A	A	A	A	A	H
RM152	H	A	A	A	A	A	H	H	A	H	H	H	A	A	H	H	H	A	A	A	A	A	A	H
RM1376	A	H	A	H	A	H	H	A	H	A	A	A	H	A	A	A	A	H	H	A	H	H	A	A
RM3481	A	A	A	H	A	H	A	A	A	A	A	A	A	H	H	A	A	A	H	H	H	A	H	A
RM6193	H	H	A	A	A	H	A	H	A	H	A	H	H	A	A	A	H	H	H	A	H	H	H	H
RM210	A	A	H	H	A	H	A	A	H	A	H	A	A	A	H	A	A	H	H	H	H	A	A	A
RM149	A	A	A	A	A	A	A	A	A	H	A	H	H	A	A	H	A	A	H	A	A	H	A	H
RM447	A	A	A	A	A	A	A	A	A	A	A	A	H	H	H	A	H	A	A	A	H	H	A	A
Chromosome 9																								
RM23654	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	H	H	A	H	A	A	H	A
RM8303	H	A	H	H	A	A	H	H	A	A	H	A	A	A	A	H	A	H	A	H	A	A	H	H
RM23805	A	A	A	H	H	A	H	H	A	A	H	H	H	A	H	A	A	H	H	H	H	A	A	A
RM5526	H	H	H	H	H	H	H	H	H	H	H	A	A	H	A	H	H	A	A	A	A	A	A	H
RM219	H	H	H	H	H	H	H	H	H	H	H	H	A	H	H	H	H	A	A	A	A	A	A	H
RM23958	H	H	H	H	H	H	H	H	H	H	H	H	A	H	A	H	H	A	A	A	A	A	A	H
RM296	A	H	A	H	A	A	H	A	H	A	H	A	A	A	A	H	A	A	A	A	A	A	A	A
RM3912	A	H	H	A	H	H	H	A	H	A	A	H	A	A	A	A	A	A	A	A	A	A	A	A
RM105	H	H	A	H	A	H	H	H	A	H	A	A	A	H	H	A	A	A	H	H	H	A	H	A

Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
RM215	A	A	A	A	A	A	A	A	A	A	A	A	H	A	A	A	H	A	A	A	H	H	A	H
RM205	A	A	A	A	A	A	A	A	A	A	H	H	A	A	A	H	A	A	H	H	A	H	H	H
Chromosome 10																								
RM6364	A	A	A	A	A	A	A	A	A	H	H	A	H	H	H	A	H	H	A	H	A	H	H	A
RM474	A	A	A	A	A	A	A	A	A	A	A	A	H	H	A	A	H	H	H	H	H	A	A	A
RM3882	H	A	H	H	H	H	A	A	H	H	A	A	H	H	A	H	H	A	A	A	A	A	H	H
RM216	A	H	H	A	A	H	A	A	A	A	A	A	H	A	H	A	A	H	A	H	A	H	A	A
RM311	A	A	A	H	H	H	A	H	A	A	A	A	A	A	H	A	A	A	H	A	A	A	A	A
RM5689	A	A	A	A	A	A	A	A	A	H	A	A	A	H	H	A	A	H	H	A	H	H	H	H
Chromosome 11																								
RM536	A	A	A	A	H	A	A	A	A	A	H	A	H	H	H	A	A	A	H	H	A	H	A	A
RM7120	A	A	A	A	H	H	A	H	A	A	A	H	H	A	A	A	H	A	A	A	A	A	A	A
RM26652	A	H	A	A	A	H	A	A	A	H	H	A	H	A	H	A	H	A	A	A	A	A	H	H
RM287	A	A	A	A	A	A	A	A	H	A	H	A	A	H	A	H	H	A	A	H	A	A	H	A
RM144	A	A	A	A	A	A	A	A	A	A	H	A	A	A	H	A	A	A	H	A	A	A	H	A
Chromosome 12																								
RM247	A	A	A	A	A	A	A	A	A	H	A	A	A	A	A	H	A	A	A	H	H	A	H	H
RM27973	A	H	A	A	H	A	A	A	A	A	A	A	H	H	H	A	A	A	A	H	H	A	A	A
RM277	A	A	A	A	H	A	A	H	A	H	A	H	H	H	A	A	A	A	H	H	A	H	A	A
RM519	A	H	A	A	A	A	A	A	A	H	A	H	A	A	H	A	H	A	A	A	A	A	A	H
RM17	A	A	H	A	H	H	A	A	A	A	H	A	A	A	A	H	A	A	H	H	A	H	A	H
RM1227	A	A	A	A	H	H	A	A	A	A	H	A	A	A	H	H	A	H	H	A	H	A	H	A

The result of background screening is illustrated in the GGT image of the 12 chromosomes of the 24 BC_2F_1 plants (Fig. 8).

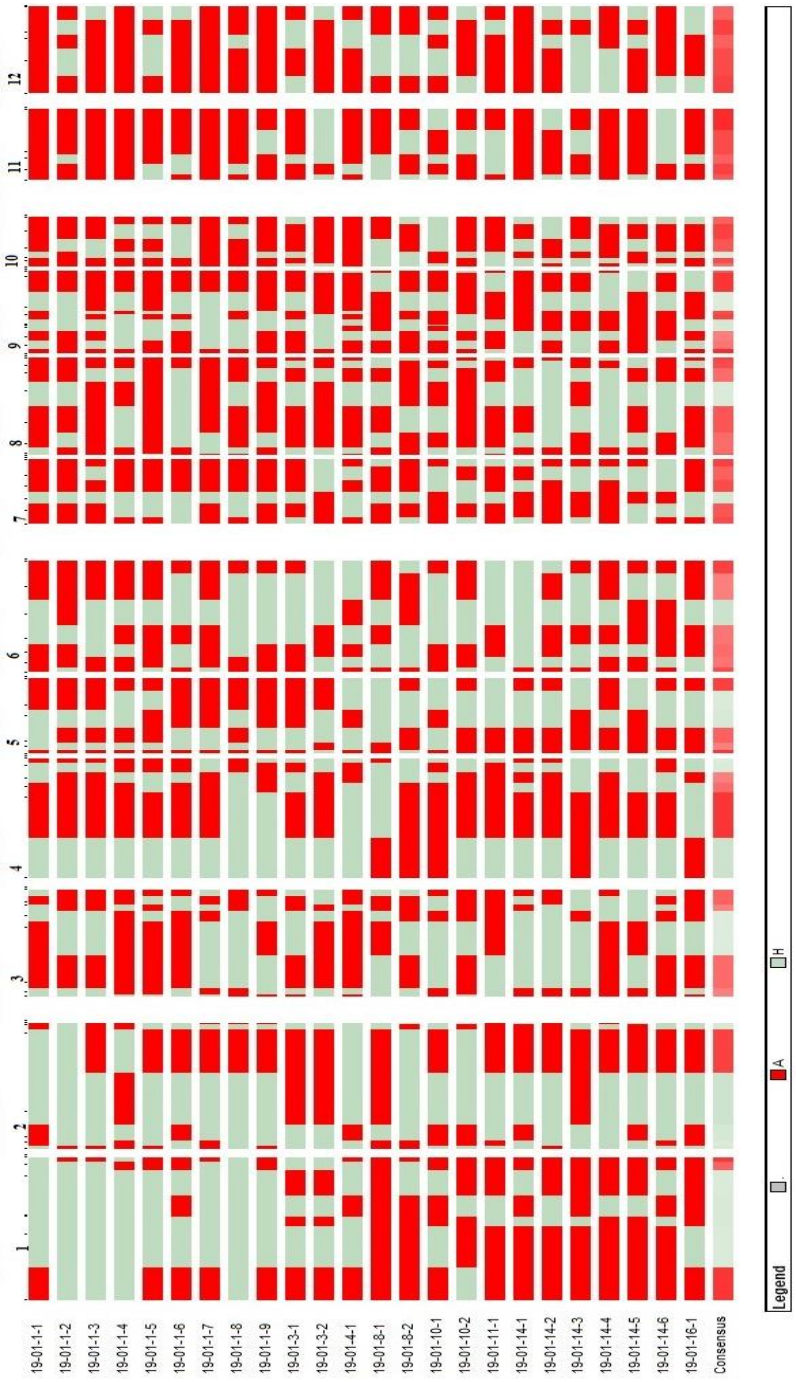


Fig. 8. GGT image of background markers of BC_2F_1 population

4.4.2.4. Recurrent genome recovery

The recurrent genome recovery percentage was assessed using observations from background screening. This was used to identify the plants to be advanced to next generation (Table. 50).

Table. 50. Recurrent parent genome recovery of BC_2F_1 population

Sl. No.	Plant	A (%)	H (%)	RG Recovery%
1	19-01-1-1	58	42	79
2	19-01-1-2	49.7	50.3	74.85
3	19-01-1-3*	56.6	43.4	78.3
4	19-01-1-4*	57	43	78.5
5	19-01-1-5	59.7	40.3	79.85
6	19-01-1-6*	53.2	46.8	76.6
7	19-01-1-7*	59.6	40.4	79.8
8	19-01-1-8	41.9	58.1	70.95
9	19-01-1-9*	54.5	45.5	77.25
10	19-01-3-1*	57.6	42.4	78.8
11	19-01-3-2*	57.9	42.1	78.95
12	19-01-4-1*	49.7	50.3	74.85
13	19-01-8-1	59.3	40.7	79.65
14	19-01-8-2	57.1	42.9	78.55
15	19-01-10-1	49.7	50.3	74.85
16	19-01-10-2*	58.5	41.5	79.25
17	19-01-11-1	57.8	42.2	78.9
18	19-01-14-1*	54.6	45.4	77.3
19	19-01-14-2	52.8	47.2	76.4
20	19-01-14-3*	55.2	44.8	77.6
21	19-01-14-4	61.1	38.9	80.55
22	19-01-14-5*	59.1	40.9	79.55
23	19-01-14-6*	54.6	45.4	77.3
24	19-01-16-1	59.2	40.8	79.6
	Mean			**77.8**

The mean recurrent genome recovery of BC_2F_1 population was 77.8%. The progeny of 19-01-1 and 19-01-14 BC_1F_1 lines were most promising based on salinity screening parameters. Based on genotypic selection and recurrent genome recovery of BC_2F_1 population 13 plants *viz.*, 2019-01-1-3, 2019-01-1-4, 2019-01-1-6, 2019-01-1-7, 2019-01-1-9, 2019-01-3-1, 2019-01-3-2, 2019-01-4-1, 2019-01-10-2, 2019-01-14-1, 2019-01-14-3, 2019-01-14-5 and 2019-01-14-6 were selected to be advanced in the breeding programme. Among these plants 19-01-1-7 (79.8%) and 19-01-14-5 (79.55%) were identified as most promising plants.

Discussion

5. DISCUSSION

Climate resilience and sustainability are reiterated notions among breeders owing to the fact that the evolution of adapted and stable genotypes is the most effective approach in imparting tolerance to abiotic stress. The differential manifestation in plants with respect to their morphological, physiological, biochemical or molecular parameters in response to stress needs to be tailor-made in order to achieve tolerance. Salinity is a major abiotic stress second only to drought in the global scenario. The present investigation titled "Introgression of *Saltol* gene into rice variety Sreyas" was drafted to introgress QTL *Saltol* into rice variety Sreyas using an identified donor FL478 through MABB.

Krishna (2016) used marker-assisted backcrossing to introgress *Saltol* QTL from donor parent FL478 to impart salt tolerance to the rice variety Karjat 6. The *Saltol* QTL was introgressed into two rice varieties of Kerala, Jyothi (Rohini and Shylaraj, 2017) and Aiswarya (Nair and Shylaraj, 2021) with FL478 as the donor using marker-assisted backcross breeding.

5.1. INTROGRESSION SCHEME

The breeding scheme involved the development of F_1, BC_1F_1 and BC_2F_1 generations. In each generation, phenotypic and genotypic screening was resorted to effectively select the best lines.

5.1.1. Development of F_1 population

The development of the F_1 population involved hybridization of the recurrent parent Sreyas with the donor parent FL478. Alongside hybridization, parental performance assay, salinity response study and polymorphism assay of molecular markers were done. To ensure availability of pollen grains throughout hybridization staggered planting was done. Vacuum or suction method was followed to emasculate the female parent. The female (Sreyas) parent was crossed with the male (FL478) in the crossing block and F_1 seeds were developed.

5.1.2 Development of BC_1F_1 population

The F_1 population from 152 seeds was sown and 132 seeds germinated. The germinated seeds were transferred to salinity screening floats with nutrient solution. After a week the medium was replaced with saline nutrient solution at 6 dS m^{-1} which was gradually raised to 9 dS m^{-1} and 12 dS m^{-1} through a week's duration. The number of plants that survived gradually reduced from 132 to 101 (5^{th} day), 84 (10^{th} day) and 69 seedlings by 16^{th} day of salinization. 31 F_1 plants which survived salinity screening were transferred to the crossing block after 21^{st} day of salinization. Among these only 16 F_1 plants survived the stress injury. These plants were subjected to foreground and recombinant selection. The selected plants were backcrossed with recurrent parent to obtain BC_1F_1 seeds.

5.1.3. Development of BC_2F_1 population

The BC_1F_1 population was constituted from 288 BC_1F_1 seeds. Under saline environment, 249 seedlings germinated. The seedlings were subjected to salinity screening in floats. On the 10^{th} day of salinization 178 plants were found to survive which was reduced to 124 by 16^{th} day of salinization. At this point, the saline nutrient medium was replaced by Yoshida solution. The number of surviving plants declined gradually from 58 (18 days) to 26 plants (21 days) which were transplanted to the crossing block 17 BC_1F_1 plants recovered from salt injury survived in the crossing block. After 2-3 weeks DNA was extracted from the young leaves of these plants. Genotypic screening of the plants was done and the selected plants were backcrossed to develop BC_2F_1 seeds.

5.1.4. Development of BC_2F_1 population

280 BC_2F_1 seeds were sown to constitute the BC_2F_1 generation. 243 seedlings germinated under salinity stress and were transferred to screening floats, of which 98 seedlings survived till 16^{th} day of salinization, . The saline nutrient medium was replaced with distilled water at 18^{th} day of salinization. Of the 31 seedlings transplanted to the crossing block 24 BC_2F_1 survived . DNA of these seedlings was extracted from the young leaves after 2-3 weeks for genotyping.

The observations recorded from the screening procedure are discussed here under the following heads

5.2. Agronomic performance

5.3. Salinity screening

5.4. Molecular screening

5.2. AGRONOMIC PERFORMANCE

The parents Sreyas and FL478 were raised in stress-free environment to confirm the agronomic performance. The morphological characters plant height (cm) and panicle length (cm) were found to higher for Sreyas compared to FL478 indicating the recurrent parent's ability to amass biomatter.

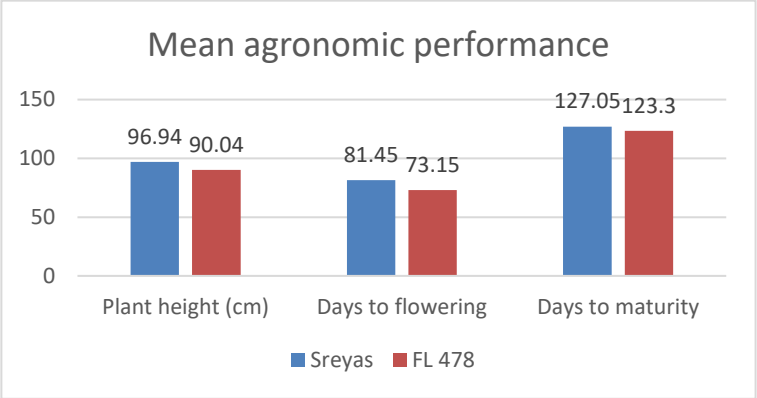


Fig. 9 (a). Mean agronomic performance under saline free environment

The yield attributes such as number of productive tillers, grain yield per plant (g), and thousand-grain weight (g) indicated that Sreyas had higher potential to convert assimilates into economic yield in a stress-free environment. The mean days to flowering and days to maturity were higher for the recurrent parent Sreyas though both parents are medium duration. The longer duration may be one of the contributing factors to higher biomatter and grain production.

The grain characteristics such as kernel colour and grain length-to-breadth ratio were assessed. Both the parents had red kernel colour. Sreyas with grain

length-to-breadth ratio of 2.25 whereas showed bold grain type against FL478 (3.00) which is slender.

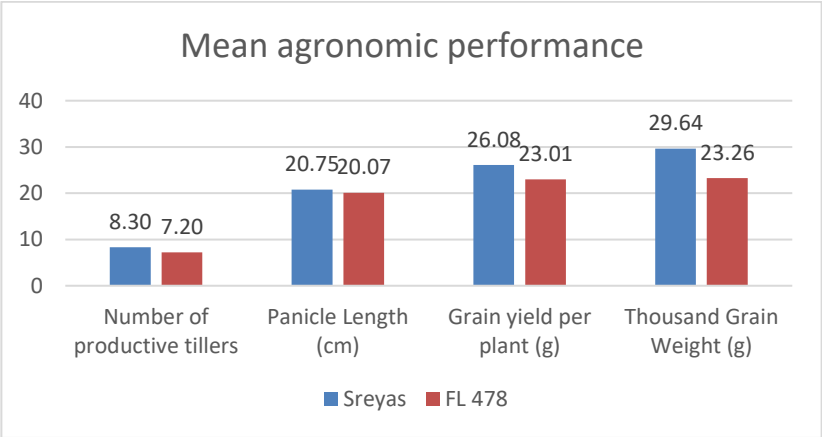


Fig. 9 (b). Mean agronomic performance under saline free environment

5.3. SALINITY SCREENING

The salinity screening included characters such as germination percentage, days to germination initiation and completion, survival percentage, SES score and percentage leaf drying of Sreyas and FL478 at different salinity levels in case of parents Sreyas and FL478. The observations for each character were taken at the electrical conductivities of 0, 3, 6, 9 and 12 dS m^{-1}. The progeny (F_1, BC_1F_1 and BC_2F_1) were screened at the highest level of salinity 12 dS m^{-1}.

5.3.1. Germination parameters

Seed germination can be divided into 3 phases (Weitbrecht *et al.,* 2011). The first phase is imbibition involving diffusion of water across the seed coat. The second phase is reactivation of metabolism including hormones and enzymes activation and active cell division. The third and the last phase is post-germination growth entailing seedling establishment and expression of genes for photosynthesis (Bewley *et al.*, 2013 and Nonogaki *et al.,* 2014).

5.3.1.1. Germination percentage

The mean germination percentage remained high for FL478 irrespective of the level of stress when compared to Sreyas (Fig. 10). However, the germination percentage was found to be above 80% in all levels of salinity stress.

The F_1, BC_1F_1 and BC_2F_1 populations were screened at 12 dS m^{-1} and the mean germination percentage was on par for the three populations and it was higher than the recurrent parent Sreyas but lower than FL478 (Fig. 11).

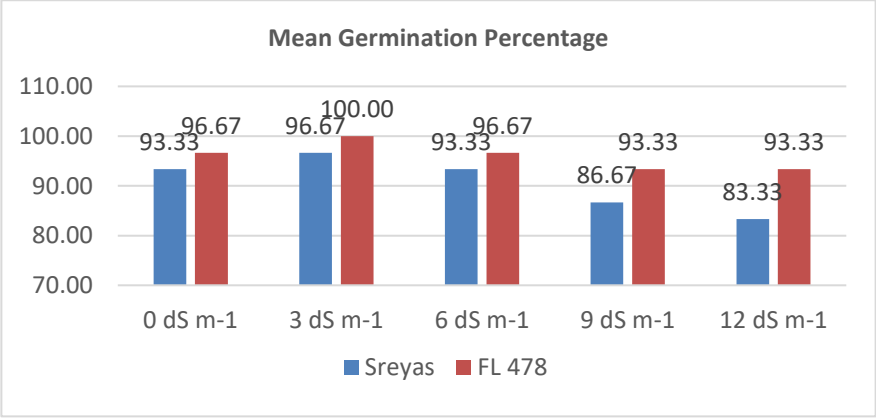


Fig. 10. Mean germination percentage of parents

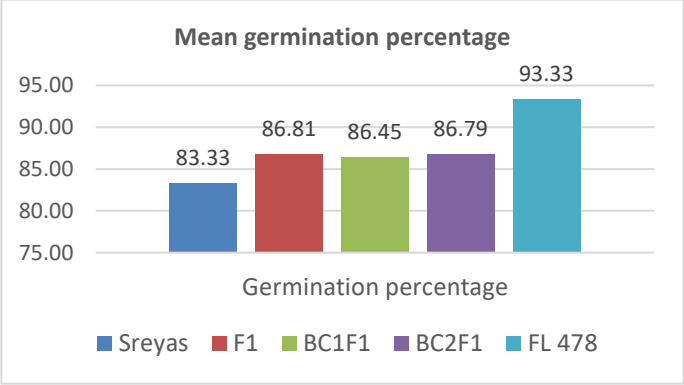


Fig. 11. Mean germination percentage of progeny at 12 dSm^{-1}

In the process of germination, it was observed that sprouting had happened in all genotypes at all levels of stress. This phenomenon signifies the imbibition of water by seeds for germination where only the water moves across the seed coat and not ions. Germination is considered to be a comparatively saline tolerant phase (Sajid *et al.*, 2019). The result obtained is in accordance with this record.

5.3.1.2. Days to germination

The mean days to germination initiation and completion were recorded at different stress levels for the parents (Fig. 12.a. and 12.b.). The duration of germination initiation in the parents was around 2 days with a gradual decline in mean values from 0 dSm^{-1} to 12 dSm^{-1}. This shows that days to initiation of germination is a seed's intrinsic characteristic rather than environment influenced. The observations are in agreement with Vibhuti *et al.* (2015) who reported that days taken to initiate and complete the germination processes varied in different rice varieties.

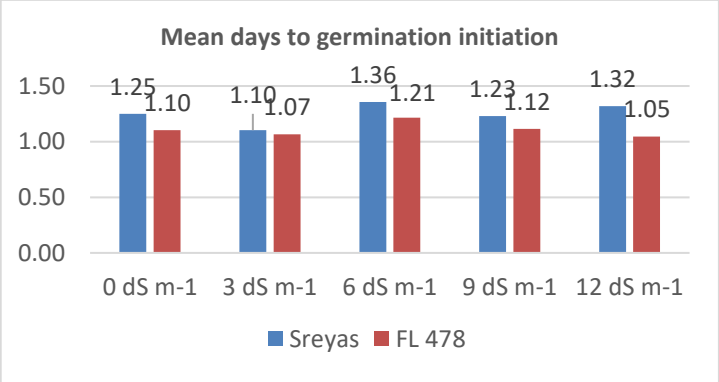


Fig. 12 (a). Mean days to germination initiation of parents

The mean days to completion of germination showed a similar pattern. The average duration taken by rice seedlings to complete germination is 3 – 5 days (ARS USDA, 2019). But here we observe that the germination completion takes 5-8 days with the average falling in the range of 6-7 days across different salinity levels.

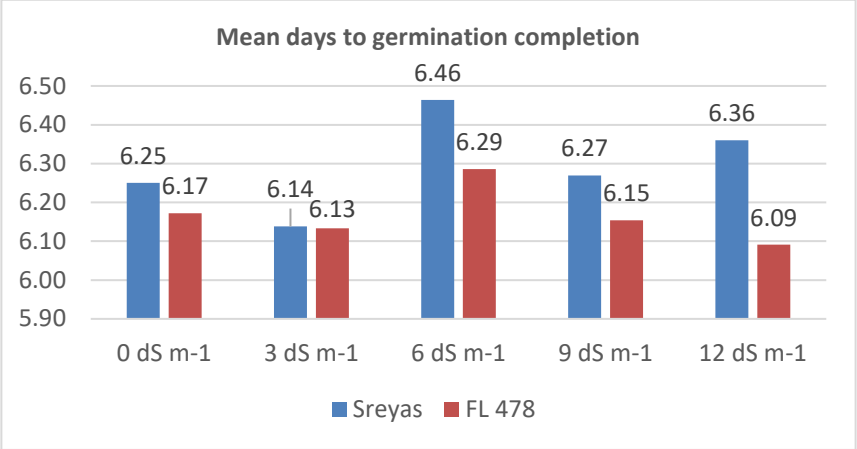


Fig. 12 (b). Mean days to germination completion of parents

The mean days to germination in the progeny (F_1, BC_1F_1 and BC_2F_1) also showed that the initiation of germination is less sensitive to salinity as the values are on par with the recurrent parent. Days to completion of germination of the progeny is also delayed and mean values were in the range of 6 - 7.

Salinity disrupts ionic and hormonal balances during germination which leads to delayed germination even inhibition of germination depending on tolerance of the seedling to salt stress (Mwando *et al.*, 2020).

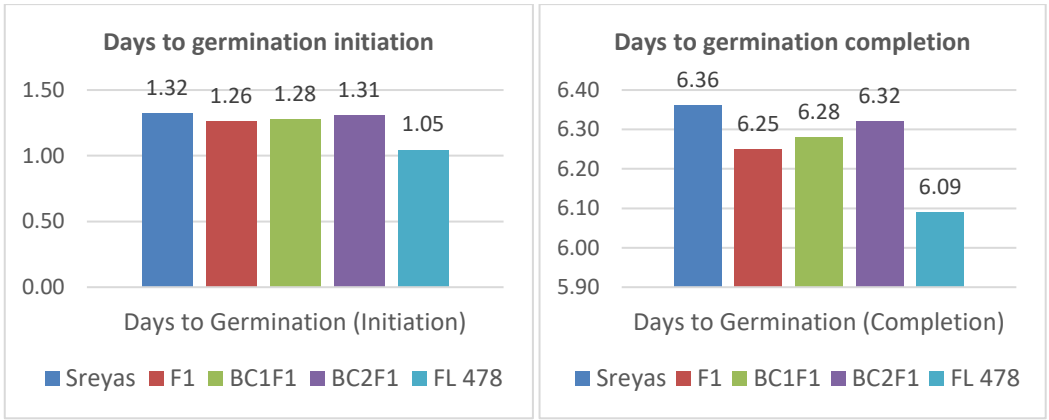


Fig. 13. Mean days to germination of progeny at 12 dSm^{-1}

The progeny of 8 BC_1F_1 plants were studied and it showed that the lines 19-01-16 (94.29%) showed highest germination percentage followed by 19-01-3 (91.43%). These values were greater than the mean value of the BC_2F_1 population. The days to germination initiation were on par for the lines but a comparative least

value was recorded by the lines 19-01-14 (1.28), 19-01-8 (1.29) and 19-01-16 (1.29). The days to completion of germination was comparatively lower for the lines 19-01-1 (6.29), 19-01-8 (6.30) and 19-01-14 (6.30) though the values were on par for all the lines. Hence, we can infer that the promising lines on the basis of germination parameters are the progeny of the plants 19-01-1, 19-01-8 and 19-01-14. Among the three stages, post-germination growth which decides days to completion of germination is sensitive to salt stress.

5.3.2. Salt injury

The morphological manifestations in response to salt stress can be used as a reliable screening criterion for salinity tolerance (IRRI, Rice Knowledge Bank). The physical expression of salt stress at the seedling stage includes white leaf tip followed by tip burning and leaf drying. Standard Evaluation Score (IRRI, 2002) is used for visual scoring of salt injury.

5.3.2.1. Survival percentage

Under high stress survival at seedling stage is a good criterion for selection of salt tolerant lines (IRRI, Rice Knowledge Bank). The observations were taken at 16 days after salinization.

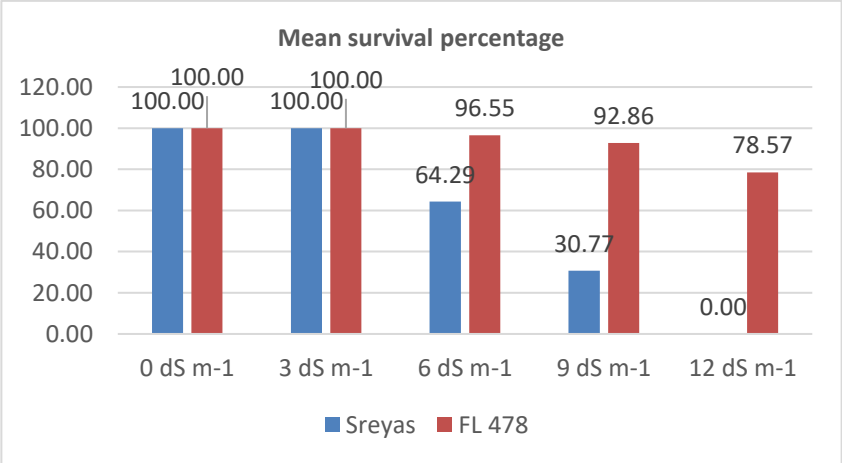


Fig. 14. Mean survival percentage of parents

The study of survival percentage of parents shows that upto 3 dSm^{-1} the effect of salt is nil on the crop irrespective of the variety. As the level of stress increases the differential response becomes obvious where Sreyas clearly shows higher plant mortality compared to FL478 (Fig. 14). At 12 dSm^{-1}, none of the recurrent plants survive while 78.57% of donor parent survived.

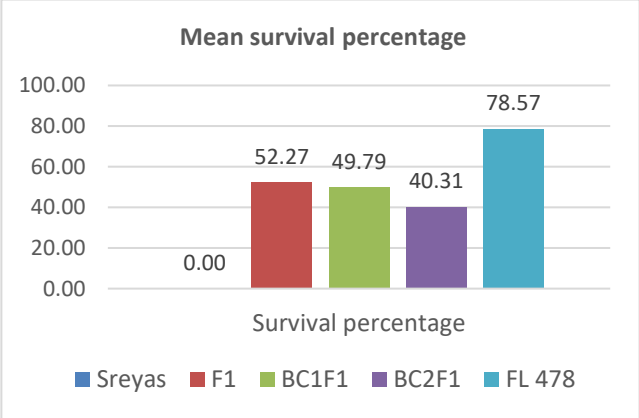


Fig. 15. Mean survival percentage of progeny at 12 dSm^{-1}

The F_1 (52.27%), BC_1F_1 (49.79%) and BC_2F_1 (40.31%) populations showed a better survival than the recurrent parent (Fig. 15). The percentage survival seems to be reducing in the backcross generation. This response may be attributed to the fact that the genotype is segregating in both these generations and more recurrent types emerge as a result.

In the BC_2F_1 population, the progeny of the plants 19-01-1 (56.67%) and 19-01-14 (55.71%) showed a survival rate better than the F_1 population (52.27%) which is much higher than the mean survival rate of the BC_2F_1 population (40.31%) itself.

5.3.2.4. SES Score

Visual scoring of salt injury at seedling stage has been devised by IRRI, Philippines as Modified Standard Evaluation Score (Gregorio *et al.*, 1997). According to Sexicon *et al.*, (2009), lower injury score can be used as a selection criterion in salinity tolerance breeding programmes.

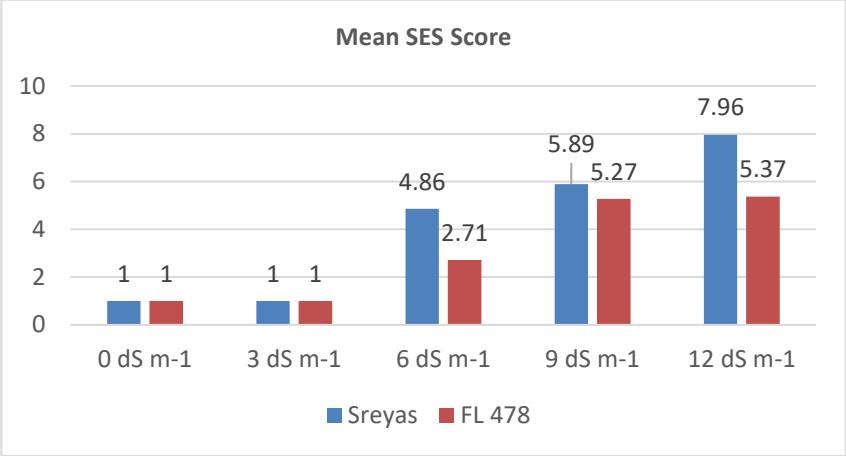


Fig. 16. Mean SES score of parents

The mean SES score implied no visual injury (1) for parental genotypes at both 0 and 3 dS m^{-1} (Fig. 16). At a salinity of 6 dSm^{-1}, Sreyas shows tolerance while FL478 shows highly tolerant response. At 9 dSm^{-1}, both parents show moderate tolerance. At 12 dSm^{-1}, FL478 shows moderate tolerance while Sreyas shows susceptibility.

The F_1, BC_1F_1 and BC_2F_1 populations showed mean SES score of 6.49, 6.63 and 7.25 respectively (Fig. 17). As in the case of survival percentage, the mean SES score also seems to be reducing in the backcross generation. This can be explained by the recurrent type plants that result from each generation of backcrossing.

In the study, BC_2F_1 population of the plants 19-01-1 (6.22) recorded mean SES score less than the F_1 population (6.49). The progeny of the plant 19-01-14 (6.88) also showed a visual injury score less than the average of the BC_2F_1 population (7.25).

In the case of the recurrent parent Sreyas, at 9 dSm^{-1} the salt stress injury was on par with the donor parent. This phenomenon led to tracing the parentage of Sreyas for presence of any salinity or any other abiotic stress-tolerant parent. Three generations down the breeding lane, the evidence of abiotic stress tolerance was attributed to the drought tolerance donor landrace, Kochuvithu.

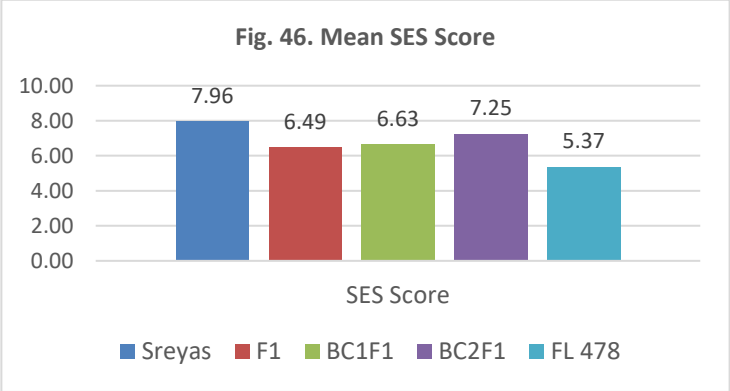


Fig. 17. Mean SES score of progeny at 12 dSm^{-1}

Salinity stress manifests in two phases (Munns, 2005). The osmotic stress induces drought stress-responsive pathway in Sreyas which leads to incipient salinity tolerance. The imminent ionic stress terminates plant growth due to ionic toxicity. Both stresses lead to cellular dehydration which results in the manifestation of signalling cross-talk between drought and salinity stress signalling and responsive pathways (Ji *et al.*, 2013). The phenomenon is also called "Common Adaptive Syndrome".

5.3.2.5. Percentage leaf drying

The plants have a survival mechanism of accumulating harmful ions in the old and senile parts of the plant. From the photosynthetic efficiency point of view the salt concentration in the old leaves causes leaf death, which is crucial for the survival of a plant (Munns *et al.*, 2005). Depending upon the concentration of salt and duration of exposure, increased mortality of leaves was observed in plants (Shereen *et al.*, 2005).

The mean percentage of leaf drying and SES score expressed a similar fashion. There was no evident leaf drying in Sreyas or FL478 at 0 and 3 dS m^{-1}. Sreyas showed high mean percentage of leaf drying which increased with an increase in salinity level. FL478 exhibited a lower leaf drying percentage against Sreyas though the stress injury increased at higher salinity levels (Fig. 18).

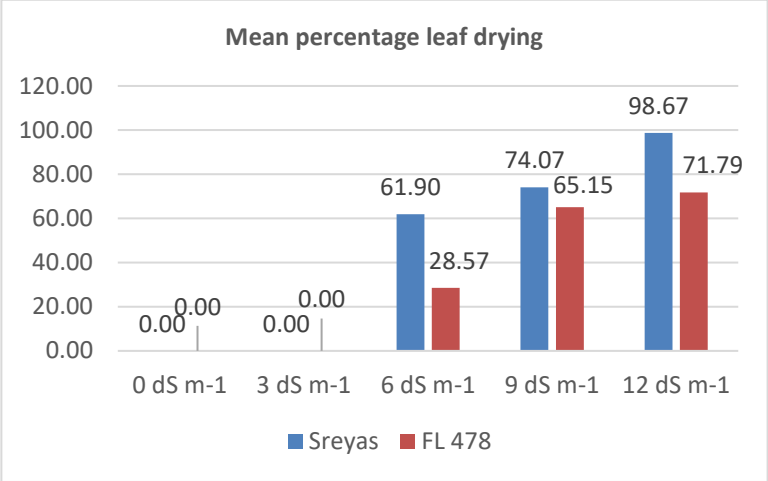


Fig. 18. Mean percentage leaf drying of parents

The leaf drying percentage of F_1 (87.34%), BC_1F_1 (89.25%) and BC_2F_1 (89.83%) populations showed lesser drying than Sreyas (98.67%). The observations seemingly reveal an increase in mean leaf drying with every generation of backcrossing.

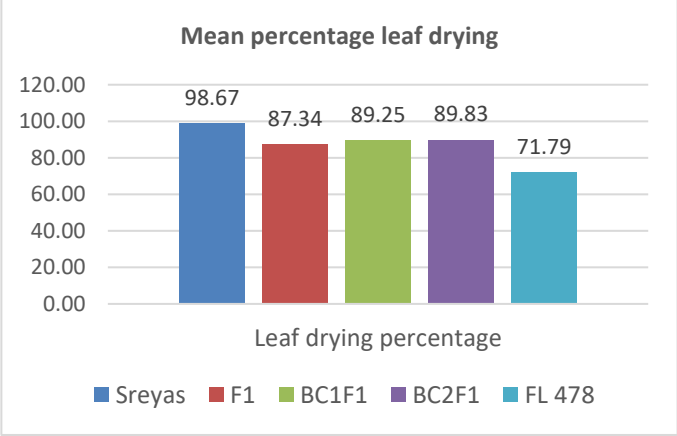


Fig. 19. Mean percentage leaf drying of progeny at 12 dSm^{-1}

The percentage leaf drying in BC_2F_1 population of the plants 19-01-1 (79.63%) registered a mean value closer to the donor parent. The progeny of the plant 19-01-14 (85.42%) showed lesser drying of leaves compared to the average values of F_1, BC_1F_1 and BC_2F_1 populations.

5.4. MOLECULAR SCREENING

In the present study, molecular screening involved isolation of genomic DNA, assessment of quantity and quality of DNA, parental polymorphism assay (recurrent parent Sreyas and donor parent FL478), foreground and recombinant screening in F_1, BC_1F_1 and BC_2F_1 generations, background screening in BC_1F_1 and BC_2F_1 and assessment of recurrent genome recovery percentage.

5.4.1. Isolation of Genomic DNA

In rice, the CTAB method is one of the most reliable DNA extraction methods which was used in the present study (Doyle and Doyle, 1987). CTAB acts as a detergent that solubilises the plant cell wall and lipid membranes of internal organelles. It also facilitates protein denaturation. Sodium chloride keeps the proteins dissolved in the solution and not precipitate with DNA. EDTA chelates divalent cations such as Mg^{2+} which act as co-factor of enzymes such as DNases inhibiting their activity. Tris acts as the buffer that facilitates the stability of the nucleic acids. The CTAB procedure is simple, rapid and cost-effective and ensures the isolation of high-quality DNA.

5.4.2. Quantity and Quality of isolated DNA

Molecular biology applications warrant accurate DNA concentration and purity measurements of the isolated DNA samples. The extracted DNA was quantified using Thermo Scientific NanoDrop2000c at 260nm. The ratio of Absorbance at 260 nm and 280 nm was used to check the purity of DNA and only pure DNA with A 260/ A280 ratio of 1.7 – 2.0. (Cawthorn *et al.,* 2011) was used in the present study. A low 260/280 ratio usually indicate that a sample is contaminated by residual phenol, guanidine, or other reagent used in the extraction protocol, However, a very high ratio can suggest a poor-quality blank eliminating too much signal near the 280 nm wavelength (Wilfinger *et al.,* 1997). In the present study, the quality of the DNA obtained from parents (1.94-1.99), F_1 (1.93-2.04), BC_1F_1 (1.88-2.02) and BC_2F_1 (1.89-2.01) were good quality. Though the sample

extracted from 2019-01-2 had a ratio of 2.09, it posed no adversity on the amplification process or PAGE.

5.4.3. Polymorphism assay

Simple sequence repeat (SSR)s are co-dominant markers distributed throughout the plant genome. SSR markers occur in high frequency and show a high level of polymorphism between the plant varieties. Parental SSR marker polymorphism study was performed between the salt-tolerant donor parent FL478 and susceptible recurrent parent Sreyas. The markers manifesting differential banding patterns between parents are used in screening process. The *Saltol* QTL is located between 10.8 – 12.3 Mb on chromosome 1 (Soda *et al.*, 2013). Assay for foreground, recombinant and background polymorphic markers was performed.

Amplification of the target locus was done using polymerase chain reaction (PCR). PAGE (polyacrylamide gel electrophoresis) is a technique for sorting DNA fragments shorter than 1000 base pairs, depending on the concentration. PAGE has higher DNA resolving ability than agarose owing to its finer pore size. Silver staining of double-stranded DNA is 100 times more sensitive than ethidium bromide staining (Beidler *et al.*, 1982). Silver is deposited directly on the nucleic acid within the transparent gel matrix (Kumar *et al*., 2015). Moreover, handling as well as disposing of ethidium bromide is a risk as it is a potent mutagen.

5.4.3.1. Foreground markers

Foreground selection refers to the use of markers that are tightly linked to the gene of interest in order to select the target allele or gene. The aim is to retain the target locus in a heterozygous state before the final backcross is completed. (Hospital and Charcosset, 1997).

25 markers in the vicinity of the *Saltol* region on chromosome 1 were used to identify polymorphic foreground markers. Five foreground markers (RM10711, RM8094, RM10713, RM10720 and RM3412) indicated clear polymorphism between Sreyas and FL478. The markers are located in the region between 11.16 –

11.58 Mbp. Thomson *et al.* (2010) suggested that the best markers for foreground selection within the *Saltol* QTL region were AP3206, RM8094, and RM3412. Singh *et al.*, 2016 used RM8094 for foreground selection to transfer Saltol QTL into seven common locally adapted rice varieties.

5.4.3.2. Recombinant markers

The objective of recombinant selection is to minimize the size of the donor chromosome segment containing the target locus and reduction of linkage drag (Hospital, 2005). The markers RM1287 (10.83 Mb) and RM10701 (11.02 Mb) telomeric to the QTL and RM493(12.28 Mb) and RM10895 (13.32Mb) centromeric to the QTL were identified as the flanking markers used for recombinant selection in the present study. According to Thomson *et al.* (2010), the most useful markers flanking the Saltol region were RM1287 and RM10694 (telomeric to *Saltol*) and RM493 and RM10793 (centromeric to *Saltol*).

5.4.3.3. Background markers

Background selection uses markers unlinked to the QTL of interest and are recurrent parent selection markers (Frisch *et al.*, 1999). 300 markers across the 12 chromosomes were screened to identify markers that can discriminate the donor against recurrent parent. Among these 83 were found to be polymorphic with at least 5 per chromosome. For a competitive background selection in a marker-assisted backcross breeding program, 40-50 SSR markers, at least 4 polymorphic markers per chromosome are ideal (Gopalakrishnan *et al*., 2008).

5.4.3.4. Parental Polymorphism percentage

The parental polymorphism percentage was 36% between the parents for the foreground markers. 9 out of 25 markers were found to be polymorphic. The parental polymorphism percentage for the background markers was 27.67% between the genotypes for the markers under consideration. Reddy *et al*. (2018) used the technique to assess the parental polymorphism percentage. The highest

parental polymorphism percentage (44%) was observed in chromosome number 9 while the lowest was observed in chromosome number 11 (22.73%).

5.4.4. Genotypic screening in introgression of *Saltol* QTL

The selected plants from *in-vitro* phenotyping were subjected to genotypic foreground, recombinant and background selection. This included genotypic screening of F_1 population, genotypic screening of BC_1F_1 population and genotypic screening of BC_2F_1 population.

5.4.4.1. Genotypic Screening of F_1 population

Foreground selection using the 5 markers tightly linked to the QTL namely RM10711, RM8094, RM10713, RM10720 and RM3412 was carried out for the F_1 plants. All the loci associated with foreground selection were in heterozygous condition in the F_1 plants. Regarding recombinant selection, all the F_1 plants showed heterozygosity for the recombinant markers These selected plants were backcrossed with the recurrent parent Sreyas to develop the BC_1F_1 population.

5.4.4.2. Genotypic Screening of BC_1F_1 population

The foreground markers RM10711, RM8094, RM10713, RM10720 and RM3412 were used for screening of the BC_1F_1 plants. All the plants expressed heterozygosity for these markers.

Recombinant selection of 17 BC_1F_1 plants revealed that 2019-01-1, 2019-01-3, 2019-01-4, 2019-01-8, 2019-01-10, 2019-01-11, 2019-01-14 and 2019-01-16 heterozygous condition for the markers in question.

The next stage of screening was background selection. The mean recurrent genome recovery percentage ranged from 56.60% to 65.80% in the BC_1F_1 generation. An average RG recovery was 62.02% in this generation.

From the 17 plants survived in BC_1F_1 generation, plants 2019-01-1, 2019-01-3, 2019-01-4, 2019-01-8, 2019-01-10, 2019-01-11, 2019-01-14 and 2019-01-16

were observed to have the QTL intact and good RG recovery. These 8 lines were backcrossed with Sreyas to develop the BC_2F_1 population.

5.4.4.3. Genotypic Screening of BC_2F_1 population

Foreground selection revealed that the 5 markers namely RM10711, RM8094, RM10713, RM10720 and RM3412 were in the heterozygous condition in all plants except 2019-01-1-5 where the locus RM3412 showed the recurrent parent genotype.

In recombinant selection, the plants 2019-01-1-3, 2019-01-1-4, 2019-01-1-6, 2019-01-1-7, 2019-01-1-9, 2019-01-3-1, 2019-01-3-2, 2019-01-4-1, 2019-01-10-2, 2019-01-14-1, 2019-01-14-3, 2019-01-14-5 and 2019-01-14-6 showed heterozygosity for the 4 loci. The plants 2019-01-1-1, 2019-01-1-2, 2019-01-1-8, 2019-01-8-1, 2019-01-8-2, 2019-01-14-2 and 2019-01-16-1 showed recurrent parent genotype for the marker RM1287. The marker RM10701 was homozygous recurrent genotype in 2019-01-1-2, 2019-01-1-8, 2019-01-8-2 and 2019-01-16-1. RM493 was in the homozygous recurrent parent genotype in the plants 2019-01-1-5, 2019-01-11-1 and 2019-01-14-4. The plants 2019-01-1-5, 2019-01-10-1, 2019-01-11-1 and 2019-01-14-4 showed recurrent parent type for the locus RM10825.

The plants, 2019-01-1-3, 2019-01-1-4, 2019-01-1-6, 2019-01-1-7, 2019-01-1-9, 2019-01-3-1, 2019-01-3-2, 2019-01-4-1, 2019-01-10-2, 2019-01-14-1, 2019-01-14-3, 2019-01-14-5 and 2019-01-14-6 were found to have an introgressed fragment of 2.49 Mb within *Saltol* region in heterozygous condition.

The mean recurrent genome recovery of BC_2F_1 population derived from background screening was 77.8%. Based on salinity screening and recurrent genome recovery of BC_2F_1 population 13 plants *viz.*, 2019-01-1-3 (78.3%), 2019-01-1-4 (78.5%), 2019-01-1-6 (76.6%), 2019-01-1-7(79.8%), 2019-01-1-9 (77.25%), 2019-01-3-1(78.8%), 2019-01-3-2 (78.95%), 2019-01-4-1 (74.85%), 2019-01-10-2 (79.25%), 2019-01-14-1 (77.3%), 2019-01-14-3 (77.6%), 2019-01-14-5 (79.55%) and 2019-01-14-6 (77.3%) were selected to be advanced in the

breeding programme. Among these plants 19-01-1-7 (79.8%) and 19-01-14-5 (79.55%) were identified as most promising plants.

Thus, in this study the *Saltol* QTL introgressed lines of Sreyas were developed and plants with a donor parent segment of 2.49 Mb in *Saltol* region and recurrent genome background of 74.85- 79.80% were successfully selected to be advanced to the next generation of the breeding programme.

Summary

6. SUMMARY

The present investigation titled “Introduction of *Saltol* gene into rice variety Sreyas” was intended to impart seedling stage salinity tolerance from FL478 into the genetic background of Sreyas. The study was performed at the Rice Research Station, Vyttila, Kerala Agricultural University during 2019-2022. Marker Assisted Backcross Breeding was employed for the introgression of the target locus. The research was divided into four experiments based on the generations involved.

In experiment I, the parents Sreyas and FL478 was hybridized. The agronomic characteristics were evaluated in a salinity-free environment. The salinity stress response of both the parents at gradient salinity levels of 0, 3, 6, 9 and at 12 dSm^{-1} was observed. Polymorphism assay of QTL-linked foreground and recombinant markers and genome-wide background markers was conducted. F_1 population was *invitro* screened at 12 dSm^{-1} for salinity in experiment II. The survived plants were genotyped for foreground and recombinant markers. The selected plants were backcrossed with recurrent parent Sreyas.

The BC_1F_1 population was subjected to seedling-stage salinity screening at 12 dSm^{-1} in experiment III. The survived plants were genotyped for the polymorphic foreground, recombinant and background markers. Plants with good salinity tolerance, heterozygosity for *Saltol* locus (foreground and recombinant) and high recurrent genome recovery were selected and backcrossed with the recurrent parent. Similarly, in experiment IV, the BC_2F_1 population was subjected to *in vitro* salinity screening at 12 dSm^{-1}. Molecular screening of the surviving plants was performed. The plants which performed well in both phenotypic and genotypic screening and showed the highest recurrent genome recovery percentage were selected and self-pollinated to develop the BC_2F_2 population.

The salient observations of the present study are summarized as follows

1. Morphological parameters plant height and panicle length were higher in Sreyas compared to FL478 in a salinity-free environment, evincing the recurrent parent's capacity to accumulate biomatter.
2. Yield attributes such as the number of productive tillers, grain yield per plant and thousand-grain weight indicated that Sreyas could better convert assimilates into economic yield in a stress-free environment.
3. The grain characteristics such as bold grain type (grain L/B ratio) of Sreyas commanded better market preference in Kerala compared to the slender grain type of FL478. The kernel colour was found to be red in both genotypes.
4. Sreyas is a native of Kerala while FL478 was introduced. The better performance may be attributed to the adaptive superiority of the recurrent parent.
5. The salinity gradient study of the parental population revealed no concurrence between the percentage of germination and the increase in salinity levels within genotypes. FL478 showed better germination at all salinity levels.
6. The mean germination percentage of the F_1, BC_1F_1 and BC_2F_1 populations at 12 dSm^{-1} were on par with each other, lower than FL478 and higher than Sreyas.
7. The mean germination percentage was above 80% in parents and progeny suggesting that germination is a comparatively salt tolerant phase in plant growth.
8. The days to germination initiation and completion were on par for parents and progeny. The average days for completion of germination was delayed which may have resulted from salinity stress.
9. The days to germination completion were found to be a salt-sensitive phase compared to germination initiation suggesting that early seedling stage is sensitive to salt stress.
10. The gradient study of the mean survival percentage showed that EC ranging from 0 - 3 dSm^{-1} does not affect the survival of rice plants at all.

However, the increase in stress level corresponds to the reduction in survival depending on the level of tolerance of the genotype.

11. The mean survival percentage though lesser than the donor parent increased considerably in the F_1, BC_1F_1 and BC_2F_1 generations compared to the recurrent parent where none of the plants survived at 12 dSm^{-1}.
12. The mean SES score showed that salt injury aggravates with the increase in salinity level within the genotype and depends on the inherent tolerance between genotypes. An exception was observed at 9 dSm^{-1} where the probability of signaling cross-talk between drought and salinity stress was observed due to presence of Kochuvithu in the parentage of Sreyas.
13. The visual salt injury score of the three progeny populations, F_1, BC_1F_1 and BC_2F_1 were intermediate to the parental populations with Sreyas showing highest salt injury.
14. The mean percentage leaf drying revealed that drying increases with increase in salinity within genotype and is influenced by the tolerance of the genotypes. The progeny were intermediate to the parental lines with FL478 showing least leaf drying percentage.
15. As the generations advanced the mean survival percentage, mean SES score and mean percentage leaf drying were found to tend towards the salt sensitive parent Sreyas which may have resulted from segregation and the emergence of recurrent parent genotypes in the backcross generations.
16. Polymorphism assay led to identification of 5 foreground, 4 recombinant and 83 background markers. The parental polymorphism percentage was 36% for the QTL linked markers and 27.67% for the genome wide background markers.
17. Genotyping of the F_1 population for foreground and recombinant markers revealed heterozygosity for all the 9 loci in the 16 plants which recovered from *in vitro* salinity screening.
18. Molecular assay of BC_1F_1 plants helped identify 8 superior lines from the 17 recovered plants from seedling-stage salinity screening based on the

heterozygous condition of the foreground and recombinant markers and recurrent parent genome recovery from background selection.

19. The performance of the progeny of BC_1F_1 plants indicated that the 2019-01-1 and 2019-01-14 were the most promising among the 8 superior lines.
20. Foreground, recombinant and background screening of the BC_2F_1 plants revealed that 13 out of 24 plants that survived salinity screening were superior genotypically and phenotypically.
21. The recurrent genome recovery percentage identified the BC_2F_1 plants 19-01-1-7 and 19-01-14-5 as the most promising among the 13 plants selected. The size of the introgressed segment in the selected lines was 2.49 Mb in the *Saltol* region and recurrent genome recovery ranged from 74.85- 79.80%.
22. The progeny of the selected plants may be advanced to BC_3 generation followed by 5-6 generations of selfing to obtain a uniform stable population which could be forwarded to evaluation and variety release.

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INTROGRESSION OF *Saltol* GENE INTO RICE VARIETY SREYAS

ANJU MARIAM JOSEPH

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ABSTRACT

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VELLAYANI, THIRUVANANTHAPURAM – 695 522

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ABSTRACT

Salinity is a major abiotic stress affecting rice productivity. The research work titled "Introgression of *Saltol* gene into rice variety Sreyas" was conducted to introgress QTL *Saltol* into rice variety Sreyas using identified donor FL478 through marker-assisted backcross breeding.

The research work included hybridization of the variety Sreyas (MO-22) with the donor FL478 to transfer the *Saltol* locus. Polymorphism assay of foreground, recombinant and background SSR markers was conducted to identify the markers polymorphic between both parents. *In vitro* phenotyping of F_1 plants under 12 dS m^{-1} (NaCl) and genotypic foreground selection of survived plants was done to select plants for the breeding programme. *In vitro* phenotyping of BC_1F_1 plants under 12 dS m^{-1} (NaCl) and genotypic foreground, recombinant and background selection of survived plants was carried out to identify the lines carrying *Saltol* QTL with adequate genome recovery. These plants were backcrossed with the recurrent parent Sreyas to advance to the next generation. *In vitro* phenotyping of BC_2F_1 plants under 12 dS m^{-1} (NaCl) and genotypic foreground, recombinant and background selection of survived plants were done to identify the most promising lines.

The performance of the parents was compared in stress-free conditions. Parent Sreyas recorded better performance for plant height (96.94 cm) number of productive tillers (8.3), longer panicles (20.75 cm), had bold grains (L/B ratio 2.25) and high yield (26.08 g) with a mean 1000-grain weight of 29.64g. FL478 (73.15 days) flowered earlier than Sreyas (81.45 days) and matured in 123 days with a panicle length of 20.07cm, grain L/B ratio of 3.00. The kernel colour of both genotypes was red.

The polymorphism assay for *Saltol*-associated markers between Sreyas and FL478 revealed 9 polymorphic markers which were located within 10.8 – 12.3 Mb. The foreground parental polymorphism percentage in 36%. The marker used for

foreground selection are RM10711, RM8094, RM10713, RM10720 and RM3412. The markers RM1287 and RM10701 telomeric to the QTL and RM493 and RM10895 centromeric to the QTL are the flanking markers used for recombinant selection. Polymorphism assay for the background markers revealed that 83 markers were polymorphic between the parents (at least 5 per chromosome) with a parental polymorphism percentage of 27.67%.

The F_1 population showed a better germination percentage of 86.81% at 12 dSm^{-1} in comparison to the recurrent parent Sreyas (83.33%) but lesser than the donor parent FL478 (93.33%). Days to germination initiation (1.26) and mean days for completion of germination (6.25) of F_1 was intermediate to both parents. The F_1 population showed 52.27% survival and an intermediary SES score of 6.49. Foreground genotypic selection resulted in the selection of 16 plants which were forwarded to the next generation by backcrossing with Sreyas.

The BC_1F_1 and BC_2F_1 populations showed better salinity tolerance than recurrent parent Sreyas. The mean germination percentage of BC_1F_1 (86.45%) and BC_2F_1 (86.79%) populations improved compared to the recurrent parent (83.33%). Days to germination initiation and completion in BC_1F_1 (1.28, 6.28) and BC_2F_1 (1.31, 6.32) populations was earlier than Sreyas (1.32,6.36). The mean survival percentage increased substantially in BC_1F_1 (49.79%) and BC_2F_1 (40.31%) populations. The mean SES scores which indicate salt injury in BC_1F_1 (6.63) and BC_2F_1 (7.25) populations improved as against Sreyas (7.96). The percentage leaf drying, was found to have improved in the backcrossing generations, BC_1F_1 (89.25%) and BC_2F_1 (89.83%)) Foreground, recombinant and background selection were used to identify the lines to be selected for the next generation. In the BC_1F_1 generation, 17 plants survived in the field from which 8 were forwarded to the next generation by backcrossing. In the BC_2F_1 generation, 24 plants survived in the field from which 13 were selected to be advanced to further generations.

Mean recurrent genome recovery was 62.02% in BC_1F_1 and 77.8% in BC_2F_1 populations. The progeny of 19-01-1 and 19-01-14 BC_1F_1 lines were most

promising based on salinity screening parameters. Based on genotypic selection and recurrent genome recovery of BC_2F_1 population 19-01-1-7 and 19-01-14-5 were identified as promising plants. In the BC_2F_1 population, the selected plants had an introgressed fragment of 2.49 Mb within *Saltol* region (10.83 – 13.32Mb) in heterozygous condition.

The selected plants may be backcrossed followed by screening for salinity attributes to identify superior lines.

APPENDIX I

List of genome-wide background primers

Sl No.	Primer Name	T	Forward sequence	Reverse Sequence	EPP	P
Chromosome 1						
1	RM495	55	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC	159	0.22
2	RM3252	55	GGTAACTTTGTTCCCATGCC	GGTCAATCATGCATGCAAGC	172	0.30
3	RM10209	55	AGCGACCAACCCTAATTATTGC	TCTCCCTGCTGCTACTACTCTTGG	186	3.95
4	RM323	55	CAACGAGCAAATCAGGTCAG	GTTTTGATCCTAAGGCTGCTG	244	4.21
5	RM1	55	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	113	4.64
6	RM283	55	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG	151	4.89
7	RM6289	55	GGGTTTTGCATTCTTTGGGG	CTTAGCTACAACCTTCGCCC	173	6.09
8	RM259	55	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT	162	7.45
9	RM600	55	AAACGTGTGTTAGCCTGTTAGG	CATATGCTAGTGGTGCTAGCG	220	9.46
10	RM140	55	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG	261	12.30
11	RM562	55	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC	243	14.63
12	RM595	55	CCTTGACCCTCCTCTTACTT	TCCTATCAAAATTTGGCAAC	189	15.12
13	RM113	55	CACCATTGCCCATCAGCACAAC	TCGCCCTCTGCTGCTTGATGGC	151	18.83
14	RM129	55	TCTCTCCGGAGCCAAGGCGAGG	CGAGCCACGACGCGATGTACCC	205	19.01
15	RM1196	55	AGCTGCCGTGAGCCTCAAG	TCCAAAACGCTCTCTTCGTC	187	21.90
16	RM5	55	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	113	23.97
17	RM237	55	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC	130	26.82
18	RM246	55	GAGCTCCATCAGCCATTCAG	CTGAGTGCTGCTGCGACT	116	27.34

19	RM443	55	GATGGTTTTCATCGGCTACG	AGTCCCAGAATGTCGTTTCG	124	28.34
20	RM128	55	AGCTTGGGTGATTTCTTGGAAGCG	ACGACGAGGAGTCGCCGTGCAG	157	30.74
21	RM212	55	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG	136	33.05
22	RM312	55	GTATGCATATTTGATAAGAG	AAGTCACCGAGTTTACCTTC	97	33.90
23	RM315	55	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	133	36.73
24	RM472	55	CCATGGCCTGAGAGAGAGAG	AGCTAAATGGCCATACGGTG	296	37.89
25	RM431	55	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC	251	38.89
26	OSR 23	55	TGATACGTGGTACGTGACGC	TAATCGCTTCCCTACCCCTG	151	40.11
27	RM8236	55	GGGATTATTTGAAATCTTTGC	ATATAGCATTGCCAGTTTGC	171	40.14
28	RM5536	55	GAATCCTGCAGGGATGAAAC	ATACTAATCCCGTCATCCGG	150	41.17
29	RM3362	55	AAGTTGAAGCAGTCGCCAAC	GAATTGCGTGGGATATGGAC	139	43.04
30	RM6840	55	TACCAAGACTCCGCTATGGC	GAAGAAGGGATCATGGATCG	191	43.17
Chromosome 2						
1	RM7382	55	GCTCCTCGAATCTGTCGATC	CACTCCGAACTCCTACGCTC	166	0.13
2	RM3340	55	GCATGCGGCCAACTGATT	TCCATCATCTCGATCTTGACGAA	117	0.39
3	OSR 17	55	GCTGGTTGATTCAGCTAGTC	GCCTCGTTGTCGTTCCACAC	163	0.83
4	RM154	61	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	183	1.08
5	OSR 14	55	AAATCCACGCACACTTTGCG	AGGTAAACGAGCTTGAGGTG	202	1.33
6	RM279	55	GCGGGAGAGGGATCTCCT	GGCTAGGAGTTAACCTCGCG	174	2.88
7	RM1075	52	CCAGTTCAGTAGTTCACACACC	GTTGGGTTGCTGTGTTGTTC	200	3.83
8	RM555	55	TTGGATCAGCCAAAGGAGAC	CAGCATTGTGGCATGGATAC	223	4.31
9	RM1347	55	AACAAATTAAACTGCCAAG	GTCTTATCATCAGAACTGGA	119	5.31
10	RM174	67	AGCGACGCCAAGACAAGTCGGG	TCCACGTCGATCGACACGACGG	208	7.01
11	RM71	55	CTAGAGGCGAAAACGAGATG	GGGTGGGCGAGGTAATAATG	149	8.76
12	RM438	57	CTTATCCCCCCGTCTCTCTC	CTCTCTGCCACCGATCCTAC	137	9.16
13	RM452	55	CTGATCGAGAGCGTTAAGGG	GGGATCAAACCACGTTTCTG	209	9.56

14	RM324	55	CTGATTCCACACACTTGTGC	GATTCCACGTCAGGATCTTC	175	11.39
15	RM550	55	CTGAGCTCTGGTCCGAAGTC	GGTGGTGGAAGAACAGGAAG	231	12.46
16	RM5812	55	CGCTGACATCTTGCCCTC	GTAGGACCCACGTGTCATCC	144	15.89
17	RM4499	55	AGCAACTTGCAAGCTTTAAT	GCTGAACCCTGAGAATATGT	134	18.53
18	RM341	55	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC	172	19.34
19	RM5430	55	TAAAAACTGAGCCGTGAGCC	ACCATGGGGAGCTGCTTC	181	22.80
20	RM106	67	CGTCTTCATCATCGTCGCCCCG	GGCCCATCCCGTCGTGGATCTC	297	25.14
21	RM526	55	CCCAAGCAATACGTCCCTAG	ACCTGGTCATGACAAGGAGG	240	26.66
22	RM221	55	ACATGTCAGCATGCCACATC	TGCAAGAATCTGACCCGG	192	27.61
23	RM6	55	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC	163	29.58
24	RM240	55	CCTTAATGGGTAGTGTGCAC	TGTAACCATTCCTTCCATCC	132	31.50
25	RM425	55	CCAACGAAGATTCGAAGCTC	CAGCACCATGAAGTCGCC	126	32.30
26	RM250	55	GGTTCAAACCAAGCTGATCA	GATGAAGGCCTTCCACGCAG	153	32.77
27	RM208	55	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC	173	35.14
28	RM406	55	GAGGGAGAAAGGTGGACATG	TGTGCTCCTTGGGAAGAAAG	146	35.24
29	RM498	55	AATCTGGGCCTGCTCTTTTC	TCCTAGGGTGAAGAAAGGGG	213	35.39
30	RM7485	55	GCCAGTTTCTCCAAAAGACG	AACTAGCCTCGACAGCGAAC	161	35.74
Chromosome 3						
1	RM60	55	AGTCCCATGTTCCACTTCCG	ATGGCTACTGCCTGTACTAC	165	0.11
2	RM523	55	AAGGCATTGCAGCTAGAAGC	GCACTTGGGAGGTTTGCTAG	148	1.32
3	RM22	55	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTCACTCGTC	194	1.52
4	RM569	55	GACATTCTCGCTTGCTCCTC	TGTCCCCTCTAAAACCCTCC	175	1.91
5	RM175	67	CTTCGGCGCCGTCATCAAGGTG	CGTTGAGCAGCGCGACGTTGAC	95	3.87
6	RM489	55	ACTTGAGACGATCGGACACC	TCACCCATGGATGTTGTCAG	271	4.33
7	RM545	55	CAATGGCAGAGACCCAAAAG	CTGGCATGTAACGACAGTGG	226	4.95

8	RM546	55	GAGATGTAGACGTAGACGGCG	GATCATCGTCCTTCCTCTGC	268	6.16
9	OSR 13	57	CATTTGTGCGTCACGGAGTA	AGCCACAGCGCCCATCTCTC	99	7.13
10	RM7	55	TTCGCCATGAAGTCTCTCG	CCTCCCATCATTTCGTTGTT	180	9.83
11	RM282	55	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG	136	12.41
12	RM338	55	CACAGGAGCAGGAGAAGAGC	GGCAAACCGATCACTCAGTC	183	13.22
13	RM3204	55	GCAACCCTTTCTTCCTCCTC	CCAAGGAGAGCGCACTAGC	93	14.99
14	RM7642	55	ACGAAATATCAGGGCACCTG	GTTGACTTTGGTCATGAGGG	194	18.63
15	RM5626	55	GATCAGTCGGTCATAAACG	CACCTTCCTCTTCTGCTG	188	24.86
16	RM168	55	TGCTGCTTGCCTGCTTCCTTT	GAAACGAATCAATCCACGGC	116	28.09
17	RM532	55	TCTATAATGTAGCCCCCCCC	TTTCAGGGGCTTCTACCAAC	180	28.13
18	RM55	55	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAGGCG	226	29.05
19	RM5172	55	ATATGCATGCGTTTATTACC	TGGCTGTTATGTGAAATACA	144	30.42
20	RM6213	52	TTGTTGGGGTCTCCGAGG	CTCATCGAGTACGGCGTCTC	119	30.69
21	RM520	55	AGGAGCAAGAAAAGTTCCCC	GCCAATGTGTGACGCAATAG	247	30.91
22	RM416	55	GGGAGTTAGGGTTTTGGAGC	TCCAGTTTCACACTGCTTCG	114	31.25
23	OSR 16	55	AAAACTAGCTTGCAAAGGGGA	TGCCGGCTGATCTTGTTCTC	125	31.89
24	RM130	55	TGTTGCTTGCCCTCACGCGAAG	GGTCGCGTGCTTGGTTTGGTTC	85	33.39
25	RM319	55	ATCAAGGTACCTAGACCACCAC	TCCTGGTGCAGCTATGTCTG	134	33.68
26	RM422	55	TTCAACCTGCATCCGCTC	CCATCCAAATCAGCAACAGC	385	33.71
27	RM227	55	ACCTTTCGTCATAAAGACGAG	GATTGGAGAGAAAAGAAGCC	106	34.93
28	RM514	55	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG	259	35.28
29	RM148	55	ATACAACATTAGGGATGAGGCTGG	TCCTTAAAGGTGGTGCAATGCGAG	129	35.84
30	RM85	55	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC	107	36.35
Chromosome 4						
1	RM551	55	AGCCCAGACTAGCATGATTG	GAAGGCGAGAAGGATCACAG	192	0.18

2	RM537	55	CCGTCCCTCTCTCTCCTTTC	ACAGGGAAACCATCCTCCTC	236	0.19
3	RM518	55	CTCTTCACTCACTCACCATGG	ATCCATCTGGAGCAAGCAAC	171	2.03
4	RM6770	55	ACCAATTCCACCTTCACTCG	GGAGGAAGAGTTGTTGCTGC	158	2.82
5	RM261	55	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	125	6.57
6	RM5953	55	AAACTTTCTGTGATGGTATC	ATCCTTGTCTAGAATTGACA	129	9.38
7	RM307	55	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC	174	13.15
8	RM401	55	TGGAACAGATAGGGTGTAAGGG	CCGTTCACAACACTATACAAGC	283	13.15
9	RM1100	50	GAAAGAGCGAAGGCGGTG	TCTCTGTCTCTCTCGCTCTCG	130	17.78
10	RM185	55	AGTTGTTGGGAGGGAGAAAGGCC	AGGAGGCGACGGCGATGTCCTC	197	18.58
11	RM564	55	CATGGCCTTGTGTATGCATC	ATGCAGAGGATTGGCTTGAG	228	18.59
12	RM471	55	ACGCACAAGCAGATGATGAG	GGGAGAAGACGAATGTTTGC	106	18.82
13	RM417	55	CGGATCCAAGAAACAGCAG	TTCGGTATCCTCCACACCTC	265	19.43
14	RM142	55	CTCGCTATCGCCATCGCCATCG	TCGAGCCATCGCTGGATGGAGG	240	20.52
15	RM177	61	CCCTCTTAGACAGAGGCCAGAGGG	GTAGCCGAAGATGAGGCCGCCG	195	22.41
16	RM273	55	GAAGCCGTCGTGAAGTTACC	GTTTCCTACCTGATCGCGAC	207	24.04
17	RM1018	55	ATCTTGTCCCACTGCACCAC	TGTGACTGCTTTTCTGTCGC	160	26.78
18	RM241	56	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	138	26.86
19	RM317	55	CATACTTACCAGTTCACCGCC	CTGGAGAGTGTCAGCTAGTTGA	155	29.06
20	RM1703	55	CATACCGTATGTATGATGTA	GTGTAAAGCTTAAAATCAAG	170	29.43
21	RM3217	55	GTTGCAAGGTTGCAACACAG	GTGGCAGCCAAGATGGAC	194	30.12
22	RM348	55	CCGCTACTAATAGCAGAGAG	GGAGCTTTGTTCTTGCGAAC	136	32.65
23	RM567	55	ATCAGGGAAATCCTGAAGGG	GGAAGGAGCAATCACCACTG	261	34.53
24	RM124	67	ATCGTCTGCGTTGCGGCTGCTG	CATGGATCACCGAGCTCCCCCC	271	34.74
25	RM5047	55	GGTATAGGTCACCAACTTTC	ATCAACTGTGTCCTCAACTA	135	34.91
26	RM559	55	ACGTACACTTGGCCCTATGC	ATGGGTGTCAGTTTGCTTCC	160	35.15

Chromosome 5						
1	RM507	55	CTTAAGCTCCAGCCGAAATG	CTCACCCTCATCATCGCC	258	0.10
2	RM153	55	GCCTCGAGCATCATCATCAG	ATCAACCTGCACTTGCCTGG	201	0.19
3	RM122	55	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	227	0.31
4	RM413	55	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	79	2.21
5	RM17960	55	CTAGGTTTGTGTCTCTTTGTGG	TCCATATGCATACTCCTACAGC	282	3.85
6	RM194	55	GCCCTGCTTCTTGCCCACCACC	TCCAGGGAGGGCAAGGCTGAGC	250	5.33
7	RM169	67	TGGCTGGCTCCGTGGGTAGCTG	TCCCGTTGCCGTTCATCCCTCC	167	7.50
8	RM249	55	GGCGTAAAGGTTTTGCATGT	ATGATGCCATGAAGGTCAGC	121	10.78
9	RM6229	56	CGAGGGGATTCGATCGAC	ATCCCTTCACACTGCTCCAC	101	13.55
10	RM509	55	TAGTGAGGGAGTGGAAACGG	ATCGTCCCCACAATCTCATC	141	16.32
11	RM598	55	GAATCGCACACGTGATGAAC	ATGCGACTGATCGGTACTCC	159	16.75
12	RM163	55	ATCCATGTGCGCCTTTATGAGGA	CGCTACCTCCTTCACTTACTAGT	124	19.19
13	RM161	57	TGCAGATGAGAAGCGGCGCCTC	TGTGTCATCAGACGGCGCTCCG	187	20.90
14	RM188	55	TCCGCCTCTCCTCTCGCTTCCC	GCAACGCACAACCGAACCGAGC	210	22.67
15	RM421	55	AGCTCAGGTGAAACATCCAC	ATCCAGAATCCATTGACCCC	234	23.98
16	RM534	55	ACAAAACCAAGGGCCTAACC	CTTCGTGCGAGCCATCTC	156	24.01
17	RM178	55	TCGCGTGAAAGATAAGCGGCGC	GATCACCGTTCCCTCCGCCTGC	117	25.10
18	RM538	55	TCCGCCTCTCCTCTCGCTTCCC	GCAACGCACAACCGAACCGAGC	274	26.03
19	RM480	55	GCTCAAGCATTCTGCAGTTG	GCGCTTCTGCTTATTGGAAG	225	27.31
20	RM334	55	GTTCAGTGTTCAGTGCCACC	GACTTTGATCTTTGGTGGACG	182	28.55
21	RM1054	55	TGCATATGTACCGCAACCTC	TTTCTGCATGATCCCCTCTG	155	29.16
22	RM5818	56	CTTGTCTTGGCTTGGCTAGG	ATCCACGCAGATAGACCTGC	153	29.69
Chromosome 6						
1	RM133	55	TTGGATTGTTTTGCTGGCTCGC	GGAACACGGGGTCGGAAGCGAC	230	0.23

2	RM508	55	GGATAGATCATGTGTGGGGG	ACCCGTGAACCACAAAGAAC	235	0.44
3	RM469	55	AGCTGAACAAGCCCTGAAAG	GACTTGGGCAGTGTGACATG	105	0.56
4	RM510	55	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	122	2.83
5	RM197	55	GATCCGTTTTTGCTGTGCCC	CCTCCTCTCCGCCGATCCTG	106	3.09
6	RM111	55	CACAACCTTTGAGCACCGGGTC	ACGCCTGCAGCTTGATCACCGG	124	5.10
7	RM276	55	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA	149	6.23
8	RM402	55	GAGCCATGGAAAGATGCATG	TCAGCTGGCCTATGACAATG	133	6.40
9	RM1925	55	AATTCATTCAAGCCTTGATA	ATTAGTTTCACCAAAGCAAC	113	9.17
10	RM527	55	GGCTCGATCTAGAAAATCCG	TTGCACAGGTTGCGATAGAG	233	9.86
11	RM5745	55	ATGCCAAGTGGACGATGTAC	ACATGTGGGTAGTGGGATGG	203	12.49
12	RM1161	56	AAACTGTTTTACCCCTGGCC	ATCCCCTTCTGCGGTAAAAC	80	13.75
13	RM6818	55	GTCGCATTCGTCTCCACC	ACCATTTCCAGATGACTCGG	130	16.58
14	RM1340	55	TCCAAACTAGTGGGAACGC	CTCAACGCCATGAACCTC	163	23.34
15	RM454	55	CTCAAGCTTAGCTGCTGCTG	GTGATCAGTGCACCATAGCG	268	23.38
16	RM162	61	GCCAGCAAAACCAGGGATCCGG	CAAGGTCTTGTGCGGCTTGCGG	229	24.04
17	RM6071	61	GAACGCCATTAGCATCATCC	TGGGAGAGTAGTGGTGGTGG	94	25.02
18	RM30	55	GGTTAGGCATCGTCACGG	TCACCTCACCACACGACACG	105	27.25
19	RM340	55	GGTAAATGGACAATCCTATGGC	GACAAATATAAGGGCAGTGTGC	163	28.60
20	RM439	55	TCATAACAGTCCACTCCCCC	TGGTACTCCATCATCCCATG	269	29.62
21	RM461	55	GAGACCGGAGAGACAACTGC	TGATGCGGTTTGACTGCTAC	195	30.11
22	RM1150	55	ACAGTGGCCACAGTGTGTTG	GGATTCGGGAGGTTGACG	147	30.38
23	RM103	55	CTTCCAATTCAGGCCGGCTGGC	CGCCACAGCTGACCATGCATGC	336	30.89
24	RM5753	55	AACATGCTCAACTTCTGGGC	GCTAGGTACGATCCAGCTGC	201	30.97
25	RM494	55	GGGAGGGGATCGAGATAGAC	TTTAACCTTCCTTCCGCTCC	203	31.09
Chromosome 7						

1	RM192	61	GCGGCGGATCATGAATTGCGAG	CTTGTTCCCCGGCGTCGAGTCC	267	0.26
2	RM295	55	CGAGACGAGCATCGGATAAG	GATCTGGTGGAGGGGAGG	180	0.41
3	RM436	55	ATTCCTGCAGTAAAGCACGG	CTTCGTGTACCTCCCCAAAC	81	2.55
4	RM427	55	TCACTAGCTCTGCCCTGACC	TGATGAGAGTTGGTTGCGAG	185	2.68
5	RM6111	52	GAGTCGTCGTCTTCGTCTCC	TCTAGGGCTAGCTCTTCCCC	94	4.09
6	RM3484	55	CGCAGGTGTCCAACTTCC	CTTGCTCCCACATCGTCG	195	4.42
7	RM125	57	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC	127	5.48
8	RM6776	52	AGCCCGGACATGCAAAAC	GAAGCAGGCGAAATCTCCTC	169	6.84
9	RM3859	55	TTGCAGATCGGTTTCCACTG	GGTCCTGGATTCATGGTGTC	191	8.88
10	RM214	55	CTGATGATAGAAACCTCTTCTC	AAGAACAGCTGACTTCACAA	112	12.78
11	RM500	55	GAGCTTGCCAGAGTGGAAAG	GTTACACCGAGAGCCAGCTC	259	15.91
12	RM1135	55	AGCCAACCAAGCAAGATAGC	ACACACATGTAAGCCTCCCC	148	16.93
13	RM445	55	CGTAACATGCATATCACGCC	ATATGCCGATATGCGTAGCC	251	17.46
14	RM11	55	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	140	19.26
15	RM560	55	GCAGGAGGAACAGAATCAGC	AGCCCGTGATACGGTGATAG	239	19.58
16	RM455	55	AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC	131	22.35
17	RM1132	55	ATCACCTGAGAAACATCCGG	CTCCTCCCACGTCAAGGTC	93	23.98
18	RM118	67	CCAATCGGAGCCACCGGAGAGC	CACATCCTCCAGCGACGCCGAG	156	26.64
19	RM420	55	GGACAGAATGTGAAGACAGTCG	ACTAATCCACCAACGCATCC	197	29.43
20	RM172	55	TGCAGCTGCGCCACAGCCATAG	CAACCACGACACCGCCGTGTTG	159	29.56
Chromosome 8						
1	RM408	55	CAACGAGCTAACTTCCGTCC	ACTGCTACTTGGGTAGCTGACC	128	0.13
2	RM337	55	GTAGGAAAGGAAGGGCAGAG	CGATAGATAGCTAGATGTGGCC	192	0.15
3	RM433	55	TGCGCTGAACTAAACACAGC	AGACAAACCTGGCCATTCAC	224	0.46
4	RM152	55	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG	151	0.68

5	RM1376	55	CATGTGTGATGACTGACAGG	GGTGCTGTGATGATTCTTTC	199	3.17
6	RM25	55	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC	146	4.37
7	RM310	55	CCAAAACATTTAAAATATCATG	GCTTGTTGGTCATTACCATTC	105	5.12
8	RM547	55	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG	235	5.59
9	RM3481	55	CTCGTCGCGTTCGTCAAC	CATCTCATCACCTCACGTCG	224	9.14
10	RM6032	55	ATCAAAACCCTCTTCGGCTC	GGAGGCAGACGAGCATAAAG	96	11.90
11	RM6471	56	TCTCCCATCTCCCATCTCAC	TGGTGATTGTGACAGATCGC	83	12.38
12	RM5999	55	ACCTTAAACCCCAGACACCC	AAGTCGAGGATGAGGACCAG	144	14.35
13	RM6193	61	CAAGAAGCTCTGGGCTAACG	GTTCTTGTGCCGTATCCTCC	133	17.65
14	RM515	55	TAGGACGACCAAAGGGTGAG	TGGCCTGCTCTCTCTCTCTC	211	20.28
15	RM1109	55	TCAAAATCACGTGTATGTAAGC	TTTACAAAGGACAGAGGGC	198	20.48
16	RM223	55	GAGTGAGCTTGGGCTGAAAC	GAAGGCAAGTCTTGGCACTG	165	20.65
17	RM284	55	ATCTCTGATACTCCATCCATCC	CCTGTACGTTGATCCGAAGC	141	21.14
18	RM531	55	GAAACATCCCATGTTCCCAC	TCGGTTTTTCAGACTCGGTC	128	22.47
19	RM210	55	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG	140	22.47
20	RM149	55	GCTGACCAACGAACCTAGGCCG	GTTGGAAGCCTTTCCTCGTAACACG	253	24.72
21	RM308	55	GGCTGCACACGCACACTATA	TTACGCATATGGTGAGTAGGC	132	24.79
22	RM1345	52	ACCACCACGCCATTAGAGAC	TGAGCATCCCGTGCTGTC	124	26.14
23	RM502	55	GCGATCGATGGCTACGAC	ACAACCCAACAAGAAGGACG	266	26.49
24	RM447	55	TCTCGCGGTATAGTTTGTGC	ACCACTACCAGCAGCCTCTG	111	26.55
25	RM264	55	GTTGCGTCCTACTGCTACTTC	GATCCGTGTCGATGATTAGC	178	27.93
Chromosome 9						
1	RM23654	55	CTCCGATGCCTTCTTCCTCTTGC	AAAGGGAGTAGCAAGCCGAGTGG	186	0.15
2	RM316	55	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC	192	1.07
3	RM8303	60	AGGGGAGAGGACACACACAC	GGATCCTCCTGCAAAATCAA	129	2.37

4	RM5799	55	ATCGAACCATCCAGGATGAC	TTGCACAAGAGGCAACACTC	199	3.80
5	RM23805	63	CACATAGTTTCCATGCTCGTTCAC	GGTAGAATCCATGACCGTCTCATC	225	4.60
6	RM464	55	AACGGGCACATTCTGTCTTC	TGGAAGACCTGATCGTTTCC	262	6.58
7	RM5526	55	TCAGCCTGGCCTCTCTTATC	ATGATCCTCCACCCACTAGC	171	7.31
8	RM219	60	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	202	7.89
9	RM23958	63	GAGACAGATGTGTACGGTTTGGTG	TTGACAAGGGAATTGAAGGAGAAG	88	8.00
10	RM1817	55	TAGTATTCTTTCCTTACAGA	ATTGAAAACTTAACAAATAG	129	10.62
11	RM296	55	CACATGGCACCAACCTCC	GCCAAGTCATTCACTACTCTGG	123	10.79
12	RM3912	55	TGTGTGCCCGATCTACCC	CCCCCATCCCCACTAAATAC	205	10.83
13	RM1896	55	GGACAGGGTAAAGTGTTAGA	CCTAAGACCTATCAACTCCA	108	11.77
14	RM105	55	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC	134	12.55
15	RM460	55	TGATCGACAGCGTTCTTGAC	GCCTGGCCCACATAATTAAG	265	13.93
16	RM409	55	CCGTCTCTTGCTAGGGATTC	GGGGTGTTTTGCTTTCTCTG	96	14.37
17	RM566	55	ACCCAACTACGATCAGCTCG	CTCCAGGAACACGCTCTTTC	239	14.70
18	RM434	55	GCCTCATCCCTCTAACCCTC	CAAGAAAGATCAGTGCGTGG	152	15.66
19	RM410	55	GCTCAACGTTTCGTTCCTG	GAAGATGCGTAAAGTGAACGG	183	17.64
20	RM242	55	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	225	18.81
21	RM553	55	AACTCCACATGATTCCACCC	GAGAAGGTGGTTGCAGAAGC	162	19.32
22	RM160	57	AGCTAGCAGCTATAGCTTAGCTGGAGATCG	TCTCATCGCCATGCGAGGCCTC	131	19.79
23	OSR 28	55	AGCAGCTATAGCTTAGCTGG	ACTGCACATGAGCAGAGACA	175	19.79
24	RM215	55	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	148	21.19
25	RM205	55	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	122	22.72
Chromosome 10						
1	RM6364	55	GTAGGTGAGGAGGATCTTGT	AATTTCTCGATTCTTCCTTC	163	0.07
2	RM474	55	AAGATGTACGGGTGGCATTC	TATGAGCTGGTGAGCAATGG	252	1.82

3	RM3882	52	GGTGCCCAATTTAGCAGAAC	CGGTGGGTTCCGAAATTTC	100	2.74
4	RM216	55	GCATGGCCGATGGTAAAG	TGTATAAAACCACACGGCCA	146	5.35
5	RM2504	57	TAACACAACAATAGCGTCAG	TAGGAAGAACTGAAGAAGCA	184	7.61
6	RM239	55	TACAAAATGCTGGGTACCCC	ACATATGGGACCCACCTGTC	144	9.69
7	RM311	55	TGGTAGTATAGGTACTAAACAT	TCCTATACACATACAAACATAC	179	9.75
8	RM294A	55	TTGGCCTAGTGCCTCCAATC	GAGGGTACAACTTAGGACGCA	173	10.08
9	RM5689	52	GCACATGGTGAGACGTCCTC	AAGTCCTGTAGTAGGTCACACCG	103	13.48
10	RM467	55	GGTCTCTCTCTCTCTCTCTCTC	CTCCTGACAATTCAACTGCG	221	13.49
11	RM596	55	ATCTACACGGACGAATTGCC	AGAAGCTTCAGCCTCTGCAG	188	15.21
12	RM184	55	ATCCCATTCGCCAAAACCGGCC	TGACACTTGGAGAGCGGTGTGG	219	16.36
13	RM271	55	TCAGATCTACAATTCCATCC	TCGGTGAGACCTAGAGAGCC	101	16.71
14	RM6100	55	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC	144	18.82
15	RM171	55	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG	328	19.05
16	RM1108	55	GCTCGCGAATCAATCCAC	CTGGATCCTGGACAGACGAG	124	19.16
17	RM6128	50	CTCTCTCTCCCCACCCAATC	GAGGGAGGAGGAGGTGTAGG	99	20.28
18	RM147	55	TACGGCTTCGGCGGCTGATTCC	CCCCCGAATCCCATCGAAACCC	97	20.95
19	RM484	55	TCTCCCTCCTCACCATTGTC	TGCTGCCCTCTCTCTCTCTC	299	21.07
20	RM1374	61	TAGATATGTTGGGCCGGAAG	AGATCGATGCCGTTTCAGAC	168	21.57
21	RM3123	50	ATTTCCCACACATCTCGCTG	GTGTCGCCGGTCAAGAAC	191	21.76
22	RM228	55	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	154	22.24
23	RM591	55	CTAGCTAGCTGGCACCAGTG	TGGAGTCCGTGTTGTAGTCG	258	22.90
24	RM590	55	CATCTCCGCTCTCCATGC	GGAGTTGGGGTCTTGTTCG	137	23.04
Chromosome 11						
1	RM7173	55	GAGCGTTTTTAGGATGCCAC	GTGATGTCGGATTCTTGGTG	120	0.44
2	RM1240	55	CCATGAGCTAGTAACTGCAGC	GGATCGCAAAATCTGGCATC	215	1.46

3	RM332	55	GCGAAGGCGAAGGTGAAG	CATGAGTGATCTCACTCACCC	183	2.84
4	RM1124	55	AAGCTATCCCCCTTTTTGGC	AGGGATCGGTAGACCCAATC	180	3.85
5	RM552	55	CGCAGTTGTGGATTTCAGTG	TGCTCAACGTTTGACTGTCC	195	4.84
6	RM5704	55	AAAAGTTTTGAATAAAACGAATG	ATGTGATTCTCCAAGCAGAG	210	5.48
7	RM116	55	TCACGCACAGCGTGCCGTTCTC	CAAGATCAAGCCATGAAAGGAGGG	258	5.74
8	RM479	55	CCCCTTGCTAGCTTTTGGTC	CCATACCTCTTCTCCTCCCC	253	7.69
9	RM536	55	TCTCTCCTCTTGTTTGGCTC	ACACACCAACACGACCACAC	243	8.99
10	RM7120	55	TGCCCAAAATATATGAAACC	TTTTCTTGTTGAATGGGAAC	153	11.78
11	RM26652	55	CAATCCATTGCTGGTTGATGC	CAAGATCTCCAAGGTGCTGAGG	169	15.07
12	RM7303	55	ACAGGAGGGGAATTGACCAG	CAGTGCTTAGCTGTAAGCTGC	131	16.52
13	RM287	55	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC	118	16.77
14	RM209	55	ATATGAGTTGCTGTCGTGCG	CAACTTGCATCCTCCCCTCC	134	17.81
15	RM229	55	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	116	18.41
16	RM457	55	CTCCAGCATGGCCTTTCTAC	ACCTGATGGTCAAAGATGGG	228	19.06
17	RM206	55	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	147	22.01
18	RM27172	57	GAAAGAAGGGATGTCTTGCATGAGG	GAACATCCTAACCACGTCGGAAGC	305	25.11
19	RM224	55	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	157	27.67
20	RM144	55	TGCCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG	237	28.28
21	RM6094	50	TGCTTGATCTGTGTTCGTCC	TAGCAGCACCAGCATGAAAG	182	28.41
22	RM2136	55	ATGTTTGAGAAAATGCAGAC	CACTAAGCTCGTTTTCAAAG	136	28.41
Chromosome 12						
1	RM1880	55	ACCACTAAATAAGCACATAC	GGCATCATACATTAAAATAC	128	0.75
2	RM19	55	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	226	2.43
3	RM453	55	CGCATCTCTCTCCCTTATCG	CTCTCCTCCTCGTTGTCGTC	178	2.69
4	RM247	57	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	131	3.19

5	RM491	55	ACATGATGCGTAGCGAGTTG	CTCTCCCTTCCCAATTCCTC	263	3.58
6	RM512	55	CTGCCTTTCTTACCCCCTTC	AACCCCTCGCTGGATTCTAG	214	5.10
7	RM1036	55	CTCATTTGTCGATTGCCGTC	ATGGGAGGAGTGATCAAACG	146	8.79
8	RM101	55	GTGAATGGTCAAGTGACTTAGGTGGC	ACACAACATGTTCCCTCCCATGC	324	8.83
9	RM27973	55	CCACACTGCCCAGGATTTAAGC	CTGTTCCCATCATCCAAATGACC	287	12.27
10	RM179	61	CCCCATTAGTCCACTCCACCACC	CCAATCAGCCTCATGCCTCCCC	190	14.45
11	RM465	55	GTGCCTCCATCATCATCATC	TAGGACAAGCGAAGAAACCG	212	16.75
12	RM277	55	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG	124	18.32
13	RM1246	55	CTCGATCCCCTAGCTCTC	TCACCTCGTTCTCGATCC	162	19.09
14	RM519	55	AGAGAGCCCCTAAATTTCCG	AGGTACGCTCACCTGTGGAC	122	19.90
15	RM463	55	TTCCCCTCCTTTTATGGTGC	TGTTCTCCTCAGTCACTGCG	192	22.09
16	RM1103	55	CAGCTGCTGCTACTACACCG	CTACTCCACGTCCATGCATG	216	23.54
17	RM6396	55	TAGGCGTATAAGGAGAGAGCG	TCGCTATCATCGTCGTCATC	169	24.97
18	RM235	55	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC	124	26.11
19	RM17	55	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	184	26.95
20	RM1296	55	CAGCTAGCACTGATCAAATAAG	GAAATTAACCAAGTTGGATTTG	192	27.06
21	RM1227	55	CATGGTAGCACACACCCTTG	CATCGACATGTGGACCACTC	176	27.31

T – Annealing temperature (^{0}C), P- Position (Mbp), EPP – Expected PCR Product (bp)