MARKER ASSISTED SELECTION FOR COLD TOLERANCE AT SEEDLING STAGE IN RICE (*Oryza sativa* L.)

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DECEMBER, 2015

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REGISTRATION NO. 10-03807

A Thesis
submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
IN
GENETICS AND PLANT BREEDING
SEMESTER: JULY-DECEMBER, 2015

Approved by:

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*This is to certify that the thesis entitled, "**MARKER ASSISTED SELECTION FOR COLD TOLERANCE AT SEEDLING STAGE IN RICE (Oryza sativa L.)**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN GENETICS AND PLANT BREEDING,** embodies the result of a piece of bona fide research work carried out by **MD. MOZAHID-UL-ISLAM; Registration No. 10-03807** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that any help or sources of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: December, 2015

_____
Prof. Dr. Md. Sarowar Hossain
Supervisor

Dedicated to

My Beloved Parents

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ABSTRACT

This study was undertaken to introgress cold tolerance from IR90688-20-1-1-1-1-1 which showed strong cold tolerance under Bangladesh condition into BRRI dhan28 using marker assisted backcross (MABC) technique. The experiment was conducted in the Molecular Biology Laboratory and experimental field of Bangladesh Rice Research Institute (BRRI) during the period of April 2015 to October 2016. This technique was powered by phenotypic selection and screening of seedling stage cold tolerance. A total of 47 polymorphic markers distributed at an average distance of 18.8 cM over the genome and showing distinct difference in band position were used in background genotyping of 41 BC_1F_1 plants very close to BRRI dhan28 in phenotype. In total, nine plants with RPG value 60% or more from the BC_1 cycle were subjected to screening against cold stress at seedling stage and finally BC_2F_1 progenies of two plants (BC_1-239 and BC_1-265) having LD values ≤5, RPG (≥70%) and number of chromosomes with 75% to 100% R alleles were selected to proceed further for next backcross cycle.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my heartfelt gratitude and all out praises to the Supreme Ruler of the Universe, the Almighty Allah for His kind favor to me to complete this thesis work successfully. Many times I called on His name for help, comfort and guidance. I am the author deem it a great pleasure to express my profound gratefulness to my respected parents, who entitled much hardship inspiring for prosecuting my studies, receiving proper education.

I would like to express my deepest sense of gratitude to my respected supervisor Prof. Dr. Md. Sarowar Hossain, Chairman, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh for his scholastic guidance, support, encouragement, valuable suggestions and conducting and successfully completing the research work and in the preparation of the manuscript writing including data analysis.

I would like to expresses my gratefulness to my respected Co-supervisor and Rice Breeder, Dr. Partha Sarathi Biswas, Principal Scientific Officer, Plant Breeding Division, Bangladesh Rice Research Institute (BRRI), Gazipur for his continuous involvement, supervision, encouragements, patience, devotion, scholastic guidance, helpful comments and constant inspiration, inestimable help, valuable suggestions throughout the research work and in preparation of the thesis. His constructive criticism, unfailing patience and enthusiasm have made the completion of this venture possible.

I also express my deep sense of gratitude to Tamal Lata Aditya, Head, Plant Breeding Division, BRRI for her valuable and scholastic suggestions and comments during my work at BRRI.

I also want to express heartfelt thanks to all the teachers of the Department of Genetics and Plant Breeding, SAU, for their valuable suggestions, instructions, and cordial help.

Heartfelt appreciation is expressed to Md. Anisuzzaman(Senior Scientific Officer), Md. Abu Sayed(Scientific Officer), Hasina Khatun(Scientific Officer), Maksudul Haque (Scientific Officer-Golden Rice Project), Plant Breeding Division, Bangladesh Rice Research Institute, Gazipur for their inspiration, loving care in experimental and laboratory work to achieve the goal.

I am grateful to my friends specially to Mahathir Sarker for his cooperation, encouragement and cordial assistance. I would like to thank Nomita Das for providing valuable support in the Molecular Biology Laboratory, BRRI. I want to thank all the

staffs, especially Md. Shariful Islam and Md. Ajijul Islam and concerned laborers of Plant Breeding Division, BRRI for their co-operation in various ways during research work.

I would also like to thank my mother specially and other family members for their wise counsel, sympathetic ear, understanding, encouragement, sacrifice and inspiration throughout the whole period of this study.

In conclusion, I recognize that this research would not have been possible without the funding support, Molecular Biology Laboratory and experimental field facilities of Plant Breeding Division, Bangladesh Rice Research Institute, Gazipur.

December, 2015 *The Author*

SOME COMMONLY USED ABBREVIATIONS

Full word	Abbreviation
ABA	Abscisic acid
AFLP	Amplified Fragment Length Polymorphism
APX	Ascorbate Peroxidase
BAC	Bacterial Artificial Chromosome
BC	Backcross
BC	Before Christ
BILs	Backcross Inbred Lines
bp	Base Pair
BRRI	Bangladesh Rice Research Institute
CAMTA	Calmodulin-binding Transcription Activator
CAPS	Cleaved Amplified Polymorphic Sequences
CAT	Catalase
CBB	Conventional Backcross Breeding
CGMS	Cytoplasmic Genetic Male Sterility
cM	Centi Morgan
CO_2	Carbon dioxide
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAE	Department of Agricultural Extension
DNA	Deoxyribonucleic Acid
EB	Ethidium Bromide
ECV	Environmental Coefficient Of Variation
FAO	Food And Agriculture Organization
FAOSTAT	Food And Agriculture Organization Of The United Nations Statistics Division
GDP	Gross Domestic Product
GRiSP	Global Rice Science Partnership
GRP	Glycine Rich cell wall Protein
GS	Genomic Selection
GWS	Genomic-wide Selection
InDel	Insertion-Deletion
IRCTN	International Rice Cold Tolerance Nursery
IRGSP	International Rice Genome Sequencing Project
IRRI	International Rice Research Institute
LD	Leaf Discoloration
LPR	Laryngopharyngeal reflux
LRT	Likelihood Ratio Test

SOME COMMONLY USED ABBREVIATIONS (Cont'd)

Full word	Abbreviation
MAB	Marker Assisted Breeding
MABC	Marker Assisted Backcrossing
MARS	Marker Assisted Recurrent Selection
MAS	Marker Assisted Selection
MB	Molecular Breeding
MDAR	Mono DehydroascorbateReductase
MOAF	Ministry of Agriculture and Forests
MT	Million Tons
ng	Nanogram
NILs	Near Isogenic Lines
OMAP	*Oryza* Map Alignment Project
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
POD	Peroxidase
QTL	Quantitative Trait Loci
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction fragment length polymorphism
RILs	Recombinant Inbred Lines
RPG	Recurrent Parent Genome
SCAR	Sequence Characterized Amplified Region
SES	Standard Evaluation System
SNP	Single Nucleotide Polymorphisms
SOD	Superoxide
SPAD	Stability of Soil Plant Analytical Development
SRFA	Selective Restriction Fragment Amplification
SSR	Simple Sequence Repeat
STS	Sequence Tagged Sites

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CHAPTER I
INTRODUCTION

Rice, a monocot, self-pollinated cereal species of plant kingdom having chromosome number 2n = 24 (Hooker, 1979) provides foods for more than half of the world's human population, especially in Asia. It is the agricultural commodity with the third-highest worldwide production, after sugarcane and maize (FAOSTAT, 2012). It belongs to the genus *Oryza* which includes two cultivated species and 25 other wild species (GRiSP, 2013). The wild species are widely distributed in the humid tropics and subtropics of Asia, Africa, Central and South America, and Australia (Chang, 1985). Of the two cultivated species, African rice (*O. glaberrima* Steud.) is confined to West Africa, whereas common or Asian rice (*O. sativa* L.) is now commercially grown in over a hundred and twelve countries except Antarctica (Bertin *et al.*, 1971). Rice is generally grown as an annual plant, even though in tropical areas it can survive as a perennial and can generate a ratoon crop for up to thirty years(IRRI, 2009).

Rice is one of the three major cereals and it is produced annually more than 600 million tons worldwide and will be more than 800 million tons (MT) as early as 2025 (FAO, 2004). The Green Revolution technology developed at the International Rice Research Institute (IRRI) in the 1960s increased world rice production. It is consumed by about three billion people and is the most common staple food of a large number of people in the world; in fact, it feeds more people than any other crop. Rice contributes more than 80% to the total food supply. More than 95 percent population consumes rice and it alone provides 76 percent of calorie and 66 percent protein requirement of daily food intake (Bhuiyan*et al.*, 2002). In Asian countries, such as Bangladesh, India, Nepal, Myanmar and the Philippines' it is more or less the only food for many people and most of the energy intake comes from rice. Rice has been playing key role in human nutrition and culture for the past 10,000 years.

Bangladesh is the 4th largest rice growing country in the world in terms of area coverage and production. Cultivable land is shrinking day by day and demand for food is increasing with the rapid growth of the population of Bangladesh. During the last three decades, the average growth of rice production in Bangladesh was 2.60 percent and in the same period, the growth of population was 2.10 percent. Even though, during this period, rice production has been increased from 10.97 MT(1970) to 34.86 MT(2014) (Kabir *et al*., 2015). The country is now self-sufficiency in rice production with surplus of about 2.06 MT (Kabir *et al*., 2015). Nevertheless, there is no reason to be complacent. The population of Bangladesh is still growing by 2 M ever year and cultivable land is shrinking annually by 0.4% due to urbanization and infrastructure development. All these will entail Bangladesh to produce 44.6 MT of rice by the year 2050 (Kabir *et al*., 2015) simultaneously, during this period, total rice producing area will also shrink to 10.28 M hectares.

Particularly, variety development for the areas prone to different biotic and abiotic stresses is very important. Unfavorable environmental conditions pose a huge threat for agriculture and challenge the future food security (Kumari *et al*., 2009). Various abiotic stresses enormously affect rice yield, particularly low temperature, salinity, drought and submergence. Abiotic stresses have become increasingly important because more than 50% of crop yield is negatively affected by these stresses (Vij and Tyagi, 2007). Low temperature stress is one of the major abiotic stresses affecting plant growth and development and the rice growing areas in northern districts of Bangladesh are mostly affected by this stress, particularly at seedling stage. Germination and seedling establishment are sensitive growth stages for rice to cold stress. Although temperature does not prevent rice germination, it delays beginning and, consequently, plant emergence. Optimum temperature range for rice germination lies between 20°C and 35°C, and 10°C is cited as the minimum critical value below which rice does not germinate (Yoshida, 1980). Good

performance during germination is significant to guarantee fast establishment and uniform crop stand (Krishnasamy and Seshu, 1989).

Boro rice production is affected by cold stress at both seedling and reproductive stage. Seedling mortality occurs ranging from 10-90 percent in the northern parts of Bangladesh during severe cold spells in the late December to early January. Low temperature at the vegetative stage of rice increases the growth period, diminish the tiller number and thereby reduced biomass production (Alvarado and Hernaiz, 2007). Sometimes, farmers delay Boro rice production until the increment of ambient temperature. Thus, they delay the next crop also, which in turn comes into light as low total productivity of the farm. On the other hand, up to 100% sterility is observed in the short duration rice varieties when cold spells occurs during early to mid-February when Boro rice crop reaches to at reproductive stage. Cold stress at the reproductive stage promotes belated heading, incremented panicle sterility and reduced grain productivity and yield (Shimono *et al.,* 2007). In all cases, the level of damage largely relies on the developmental stage and intensity of the cold spell (Jacobs and Pearson, 1994). To overcome this problem, development of cold tolerant rice varieties for seedling and reproductive stage having high yield potential is necessary.

In the recent past, scientists used morphological data to identify the varieties which can withstand in the cold stresses. But this ultimately limits the development of adaptable variety tolerant to cold stress. Thus, very little progress has been achieved in the development of cold tolerant high yielding rice varieties. Bangladesh Rice Research Institute is working since long for the development of cold tolerant rice varieties in collaboration different international partners. Under this collaborative research BRRI imported IR90688-20-1-1-1-1-1 along with a series of RILs developed from a cross between cold susceptible BRRI dhan29 and cold tolerant Jinbubyeo in Korea (Kim *et al*., 2014) and evaluated under natural and artificial cold condition and found highly tolerant at seedling and reproductive stage (BRRI 2014). Thus

IR90688-20-1-1-1-1-1 was used as donor parent to introgress cold tolerance for seedling stage into cold susceptible BRRI dhan28, which is widely cultivated by the farmers across the country. The area under BRRI dhan28 is increasing day by day due to its better grain quality, higher clean rice yield and shorter duration than popular Boro rice variety.

The specific objectives of the study are as follows-

1. Identification of polymorphic SSR markers between to parents to fast-track recovery of recurrent parental genome
2. To introgress seedling stage cold tolerance from IR90688-20-1-1-1-1-1 into BRRI dhan28
3. To isolate backcross progenies that are tolerant to cold stress at seedling stage

CHAPTER II

REVIEW OF LITERATURE

Rice, a grass species of plant kingdom provides foods for more than half of the world's human population, especially in Asia. It is the agricultural commodity with the third-highest worldwide production, after sugarcane and maize (FAOSTAT, 2012). It influences the livelihoods and economies of several billion people. Cultivated rice is generally a semi aquatic annual grass; although in the tropics it can survive as perennial, producing new tillers from nodes after harvest (ratooning). Rice belongs to the genus *Oryza* which includes two cultivated species and 25 other wild species (GRiSP, 2013). Although the taxonomy of rice is still a matter of research and debated. The two cultivated species, *Oryza saliva* and, *Oryza glaberrima* are important cereals for human nutrition. *Oryza sativa* is grown all over the world, while *Oryza glaberrima* has been cultivated in parts of West Africa for the last 3500 years. Rice was first cultivated in tropical Asia, the oldest record dating 5000 years BC, and then extended to temperate regions (Watanable *et al*., 1997). It is cultivated in about 40 countries as a stable food, between latitudes 55° north and 36° south in different ecosystems., including irrigated, rainfed low land, rainfed upland, and flood-prone areas, and is subject to a variety of biotic and abiotic constrains (Brondani *et al*., 2006). Rice can be grown in different environments, depending upon water availability (IRRI, 2009). It is the only cereal crop that can grow for long periods of time in standing water. Around 57% of rice is grown in irrigated land,25% in rainfed lowland, 10% in the uplands, 6% in deep-water, and 2% in tidal wetlands (GRiSP, 2013).

Worldwide, more than 3.5 billion people depend on rice for more than 20% of their daily calories (FAO, 2014). It is the predominant dietary energy source for 17 countries in Asia and the Pacific, nine countries in North and South America and eight countries in Africa. Rice provides 20% of the world's dietary energy supply, while wheat supplies 19% and maize (corn) 5% (FAO, 2004). Rice is

the staple food of about 160 million people of Bangladesh. It provides nearly 48% of rural employment, about 69.6% calorie intake and 56.2% of the total protein intake of an average person in the country (GRiSP, 2013). Rice sector contributes one-half of the agricultural GDP and one-sixth of the national income in Bangladesh. Almost all of the 15 million farm families of the country grow rice. Rice is grown in about 10.5 million hectares. About 75% of the total cropped area and over 80% of the total irrigated area is planted with rice. Thus, rice plays a vital role in the livelihood of the people of Bangladesh. Total rice production in Bangladesh was about 10.79 million tons in the year 1970 when the country's population was only about 70.88 millions. However, the country is now producing about 34.7 million tons (in 2014) to feed her 160 million people (DAE, 2016). This indicates that the growth of rice production was much faster than the growth of population. This increased rice production has been possible largely due to the adoption of modern rice varieties on around 66% of the rice land which contributes to about 73% of the country's total rice production. However, there is no reason to be complacent. The population of Bangladesh is still growing by 2 million every year and may increase by another 45 million over the next 35 years. Thus, Bangladesh will require about 45.8 million tons of rice for the year 2050 (GRiSP, 2013). During this time total rice area will also shrink to 10.28 million hectares.

2.1 Different Abiotic Stress

Agriculture production is dwindled mainly due to biotic and abiotic stresses. Abiotic stress is one of the major factors which negatively affect the crop growth and productivity world-wide. These are the major constraints to achieve food security and alleviating poverty for millions of the rural people in the world and estimated as the major limitation for food production in Bangladesh as well as for the developing world (FAO, 2011). Abiotic stresses including high salinity, drought, flood, high and low temperatures are largely limiting productivity of rice crops in large areas of the world. According to Hossain (1996), abiotic stresses affect rice cultivation more than the biotic stresses.

Improving the resistance to abiotic stresses will increase agricultural productivity and extend cultivatable areas of rice.

Among the abiotic stresses cold stress is a common problem for rice cultivation, and is a significant factor affecting global food production since cold stress can cause poor germination, slow growth, withering, and anthers injury on rice plants (Andaya *et al.*,2007). Annually about 15 million hectares of rice in the world is suffered from cold damage (Zhang *et al*., 2005). In south Asia, about seven million hectares cannot be planted timely because of the low temperature stress (Sthapit *et al.,* 1998). Consequently, development of rice cultivars with cold tolerance is recognized as one of the important breeding objectives.

Low-temperature stress is an important factor affecting the growth and development of rice (*Oryza sativa* L.) in temperate and high-elevation areas. Cold stress may cause various seedling injuries, delayed heading and yield reduction due to spikelet sterility. Only one-third of the total land area on Earth is free of ice and 42% of land experiences temperatures below - 20°C (Ramankutty *et al.,* 2000). In such areas, plants require specialized mechanisms to survive at exposure to low temperature. Cold stress, which can be classified as chilling (0-15°C) and freezing (<0°C) stress, is a major environmental factor limiting the growth, productivity and geographical distribution of crops (Zhu *et al.,* 2007).

Rice (*Oryza sativa* L.) is a sensitive plant to low temperature stress, which under this condition germination and emergence can decrease and consequently, lose yield (Cruz and Milach, 2004; Jiang *et al.,* 2008; Sharifi, 2010). Cruz and Milach (2004) revealed that genotypes belonging to the Japonica subspecies showed higher cold tolerance than Indica, but there was variability within genotypes. Temperatures lower than 20°C decrease both the speed and percentage of germination (Yoshida, 1981) and result in lower crop stands and higher production costs. The reproductive stage of rice is the most susceptible to cool weather damage (Nishiyama *et al.,* 1995). Cold stress is also

very harmful for Boro rice crop in the northern part of Bangladesh. The effect of low temperature on rice plant is diverse and complex depending on different physiological properties and among different organs of rice plants.

2.2 Effect of Cold Stress on Plants

Abiotic stresses adversely affect growth, productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Cold stress is a major environmental factor that limits the agricultural productivity of plants in different areas. Plants respond and adapt to this stress to survive under stress conditions at the molecular and cellular levels as well as at the physiological and biochemical levels. However, expression of a variety of genes is induced by different stresses in diverse plants. Cold stress is a common problem in rice cultivation and a crucial factor in global production (Zhou *et al.,* 2012). Rice is a cold-sensitive plant that originated from tropical or subtropical zones. When low temperature occurs during the reproductive stages, it can cause serious yield and yield components losses (Farrell *et al.,* 2006). The optimum temperature for rice cultivation is between 25°C and 35°C, and in temperate regions, rice growth is impressed by limited period that favors its growth (De Los Reyes *et al.,* 2003). Exposure to cold temperature affects all phonological stages of rice and lower grain production and yield.

Low temperature in vegetative stage can cause slow growth and reduce seedling vigor (Ali *et al.,* 2006) low number of seedlings, reduce tillering (Shimono *et al.,* 2002) increase plant mortality (Farrell *et al.,* 2006; Baruah *et al.,* 2009; Fujino *et al.,* 2004) increase the growth period (Alvarado and Hernaiz, 2007) and in reproductive stage, it can cause to produce panicle sterility and lower grain production and yield tillering (Shimono *et al.,* 2002). Tahir *et al.* (2002); reported that the environmental coefficient of variation (ECV) was the highest for grain yield/plant and grains/panicle showing these characters was sensitive to environment. Low temperature often affects plant growth and crop productivity, which causes significant crop losses (Xin and Browse, 2001). Low-temperature stress is a major factor limiting rice

production in temperate and high altitude areas (IRRI, 1979; Sthapit and Witcombe, 1998). Cool temperature below 15-17°C can cause severe injuries leading to poor establishment, decrease in the plant competitive ability against weeds, delayed crop maturation and subsequently decrease in yields (Yoshida *et al.,* 1996; Andaya and Mackill, 2003a).

The low temperature at seedling and reproductive stages is the major problem resulting slow establishment and low seed set which leads to poor yield of the crop (Sanghera and Wani, 2008). The low temperature limits the crop productivity when temperatures remain above freezing that is > 0°C, it is called as chilling stress. Chilling sensitive cultivars are typically tropical genotypes. There is wide range of cold stress in temperate areas differing in both timing and intensity of low temperature. Yield losses are more severe when cold stress occurs during reproductive stage/anthesis in rice which lead to high spikelet sterility (Sanghera *et al.,* 2001).

2.3 Mechanism of Cold Tolerance in Plants

Ability of crop genotypes / lines to survive / perform better under low temperature than other genotypes is called as cold tolerance. Ordinarily, it is the consequence of cold hardening *i.e.* an earlier exposure to a low temperature for a specific period as a result of which chilling tolerance of the concerned plants increases. Cold tolerant rice plant develops different defense mechanisms at different vegetative and reproductive organs to be able to cope with low temperature as illustrated in Figure 1.

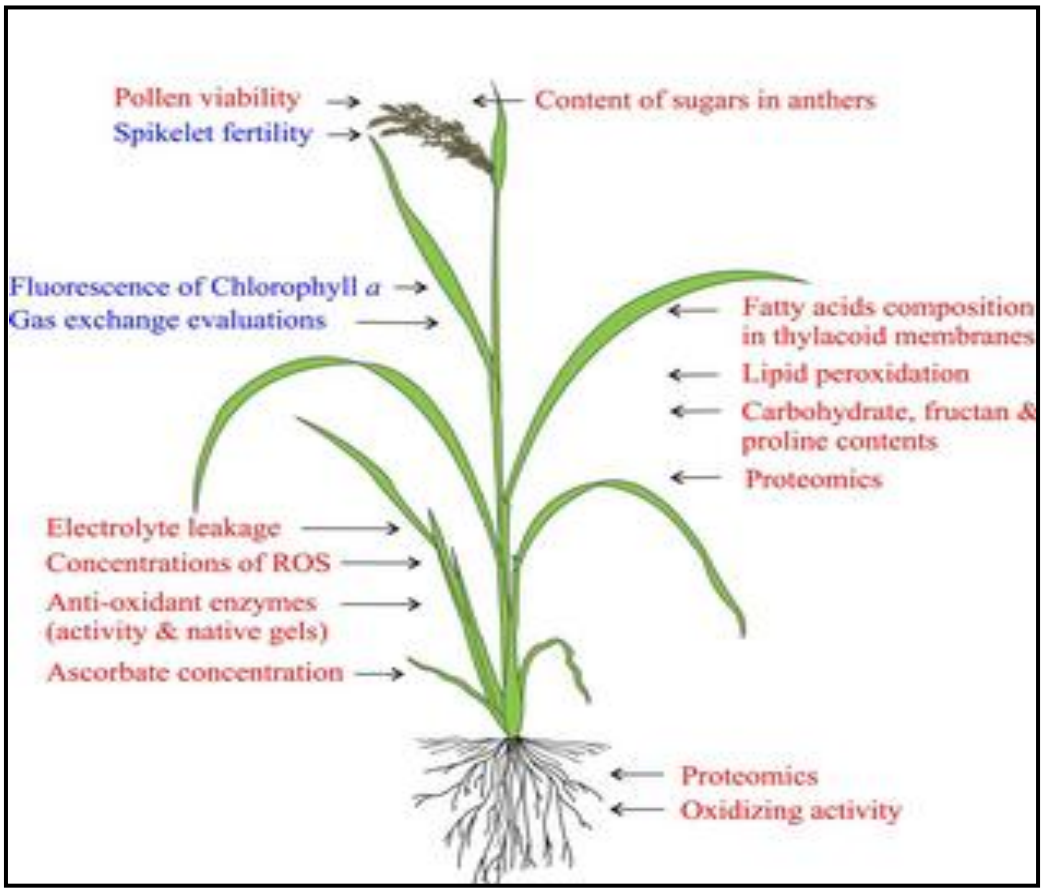

Figure 1. Schematic representation of defense mechanism in rice plant against cold stress. (Source: Cruz *et al.*, 2013)

Cold tolerance involves increased chlorophyll accumulation, reduced sensitivity of photosynthesis, improved germination, pollen fertility and seed set which are desirable as:

2.3.1 Reduced Sensitivity to Hydraulic Conductivity

Low temperature influences crop establishment, vegetative growth, spikelet fertility and thus the resulting low yield. It has been shown that low soil temperature inhibits water-uptake by roots (Kramer and Boyer, 1995). Since the root system is one of the major regulatory sites for water movement through whole plants, a decrease in hydraulic conductivity (LPR) affects plant water status, stomatal conductance, photosynthesis, growth and hence productivity. In rice, leaves roll or dry out by the low root temperature (LRT), because LRT causes dehydration stress even when the soil is abundant in water. Murai-Hatano *et al.* (2008); reported that root temperature of rice below 15°C reduces hydraulic conductivity and thereby water uptake by rice root is inhibited and plants suffer from dehydration.

2.3.2 Increased Chlorophyll Accumulation

Low temperature inhibits chlorophyll accumulations in actively growing leaves. In rice, cold tolerant lines, for example, japonica accumulation of chlorophyll is not affected even under cold stress condition than do cold sensitive line (Glaszmann *et al.,* 1990). Rasolofo (1986), evaluated 181 accessions to identify donor and outstanding cold tolerant lines using leaf discoloration score (1-3) and found 19 remained green (dark) after 10 days in the 12°C cold water tank. Sanghera *et al.* (2001); found 18 cold tolerance IRCTN rice genotypes based on dark green color and high spikelet fertility (>90%) under temperate conditions. Chlorophyll fluorescence parameters were strongly correlated with whole-plant mortality in response to environmental stresses (Pei-Guo and Ceccarelli, 2006). Koc *et al.* (2010); and Aghaee *et al.* (2011); reported that chlorophyll a and b content were decreased in plants when

plants were subjected to cold treatment. Reduced chlorophyll content can indicate the effect of low temperature on rice plants (Sharma *et al.,* 2005).

2.3.3 Reduced Sensitivity of Photosynthesis

Photosynthesis is one of the main physiological processes which is affected by low temperature (Mohabbati *et al.,* 2013). Low temperature is one of the most important factors that may limit photosynthetic activity (Larcher, 2000). Decrease of photosynthesis induced by low temperatures is a well-known response of chilling-sensitive plants. It is well documented that low temperature disturbs the growth and development of plants such as rice by affecting photosynthesis, membrane system, respiration, water uptake and metabolism of nucleic acids (Dai *el al.,* 1990; Liang *et al.,* 2007). In cold tolerant lines, photosynthetic activity is much less affected by low environment temperature. Jeoung *et al.* (2002); examined the mechanism of chilling on photosynthesis using two rice accessions. When the leaves were exposed to various low temperatures, the leaf photosynthesis of Milyang23 decreased faster than that of Stejaree45, indicating that Stejaree45 (temperate Japonica) was more resistant to light-chilling stress than Milyang23 (Indica). Because there is a positive relationship between leaf photosynthesis and leaf chlorophyll content (Kura-Hotta *et al.,* 1987; Kim *el al.,* 2002), it should be possible to use chlorophyll content as a manifestation of cold damage.

2.3.4 Improved Germination

Good cold tolerance at the seedling stage is an important character for stable rice production, especially in direct seeding fields. Temperature lower than 20°C decreases both the speed and percentage of germination of rice (Yoshida, 1981) and results in lower crop stands and higher production costs.*Oryza sativa* with the subspecies Japonica provides the main source of genetic variation in cold tolerance (Takahashi, 1984) at germination stage. Genetic variation in cold tolerance at germination and seedling stage has been documented by many researchers. Saini and Tandon (1985); found that L62G, Heng Jodo, Jodo,

Heugdo, IRAT 102, Khonorullo, K 78-28, Daegaldo, Mujudo and L62-2A genotypes were cold tolerant having more than 85% germination and good seedling vigor (score 3) at an average of 11° C field temperature. Massardo *et al.* (2000); reported that higher metabolic rates and less oxidative damage during germination greatly enhance germination rate in cold tolerant cultivar of Oat.

2.3.5 Improved Pollen Fertility and Seed Set

Low temperatures represent one of the environmental stresses that most adversely limit plant growth (Xiong *et al.,* 2002; Cui *et al.,* 2005) affecting mostly at the reproductive stage, followed by germination and emergence (Freitas, 2005; Amaral, 2010). When low temperature occurs during the reproductive stages, it can cause serious yield and yield components losses (Farrell *et al.,* 2006). Lia *et al.* (1998); reported plant cold tolerance in rice is associated with anther size, number of pollen grain, diameter of fertile pollen grains at booting stage. However, Sanghera *et al.* (2001); reported that cold tolerance is associated with high spikelet fertility (>90%) and well panicle exertion under temperate conditions.

Cold stresses cause a reaction in the plant that prevents sugar getting to the pollen. Without sugar there is no starch build-up which provides energy for pollen development and pollen tube development (pollen germination). And without pollen, pollination cannot occur, thereby, no grain is produced. A cell layer surrounding the pollen, called the `tapetum', is responsible for feeding the pollen with sugar. The tapetum is only active for 1-2 days — so if a cold snap occurs at this time, then there is no further chance for pollen growth. But the sugar cannot freely move into the tapetum and pass through it to the pollen. Instead the sugar has to be broken down then transported in bits to the pollen. Invertase is the catalyst that helps in breakdown of the sugar molecule to transport it into the tapetum before it is transported to the pollen (Oliver *et al.,* 2005). Quantities of invertase are decreased in conventional rice when it is

exposed to cold temperatures, but they remain at normal levels in a cold tolerant variety when it experiences cold. By comparing a cold tolerant strain of rice with conventional rice, CSIRO (2008); has found that the gene responsible for invertase looks exactly the same in the cold tolerant variety as it does in conventional rice. So the invertase gene itself does not make the rice plant cold tolerant — but instead a mechanism that regulates the invertase gene is different. Early research indicates that the invertase gene is regulated by the hormone abscisic acid (ABA). Oliver *et al.* (2007); has experimented injecting plants with ABA. The resulting rice plants are sterile, just like that they experienced with a cold snap. Also, ABA levels increase when conventional rice is exposed to cold, but they remain the same in the cold tolerant variety. Recent studies have indicated that the difference between cold-sensitive and tolerant rice is due to a different ability to control ABA levels (Zhao *et al.*, 2008). It has also been shown that this mechanism may require interactions with other plant hormones like auxins (Bhatnagar-Mathur *et al.*, 2008). Further, Zhao *et al.* (2008); also reported that low temperature turns off the genes responsible for sugar transport into the pollen grains and therefore starch cannot be produced in the pollen in cold conditions. Cold did not cause repression of sugar delivery in cold tolerant Chinese rice and fertile pollen was still produced following cold treatment. The sugar metabolism genes also continued to function normally during cold treatment of cold tolerant rice. Ample genetic variation for cold tolerance is available in well adapted breeding population. Germplasm collected from high altitude and low temperature areas, cold tolerant mutant, somaclonal variants and wild species can be exploited for breeding improved cold tolerant genotypes in hilly areas (Sanghera and Wani, 2008)

2.4 Effect of Low Temperature on Rice Production

Rice is a cold sensitive crop that has its origin in tropical or sub-tropical areas and temperatures dramatically reduce its production (Kuroki *et al.*, 2007). Low-temperature stress is common for rice (*Oryza saliva* L.) cultivation in

temperate zones and high-elevation environments. In parts of south and south-east Asia, an estimated 7000000 hectares cannot be planted with modern varieties because low-temperature stresses (Sthapit and Witcombe, 1998). In temperate-growing regions such as California (USA), cold temperature is an important stress that results in delayed heading or maturation and yield reduction due to spikelet sterility (Peterson *et al.*, 1974).

In the double-crop rice-growing regions in Asia, cold current outbreak in April often makes early-season rice seedlings rotten, causing heavy seed loss and a delayed growth period. In 1980, such seed loss reached 750000 kg in the Hangzhou region (Xiong *et al.,* 1984). In northern regions of Iran, rice is sown from early April to middle of May, when mean temperature is around 15°C which leads to rotten rice seedlings, causing heavy seed loss and a delayed growth period (Sharifi, 2010). Yield loss from rotten seedlings caused by low temperature is a serious and global problem in rice production (Han *et al.*, 2006). Low temperatures represent one of the environmental stresses that most adversely affect plant growth (Xiong *et al.*, 2002; Cui *et al.*, 2005) affecting mostly at the reproductive stage, followed by germination and emergence (Freitas, 2005; Amaral, 2010).

The growth of indica and japonica rice under temperature below 18°C and 15°C, respectively, is inhibited to different extents at early growth stage (Kwon *et al.*, 1979; Han *et al.*, 2002). Taking South China, for instance, low temperature stress in early spring often causes the seedlings of early indica varieties rot (Chung, 1979; Xiong *et al.*, 1990). Spikelet fertility decreases significantly by a low temperature at the booting stage (Satake and Hayase, 1970). Low temperature at reproductive stage has had adverse effects on the yield of rice in Australia, China and Korea since 2000 (Lee, 2001; Xu *et al.*, 2008). Analysis of mutants from the cultivar Taichung 65 treated with cold water at 19°C revealed that pollen development was inhibited, spikelet fertility was reduced due to malformed embryo sac (Nagasawa *et al.*, 1994). . In years of extreme low temperatures, all rice-growing areas are susceptible to cold at

the reproductive stage. For example, in 1980 and 1993, low temperatures seriously damaged the Korean rice crop, with grain yields dropping by 26% and 9.2%, respectively, compared with the national average yield on either side of these years (MOAF, 1994).

Cold stress is also a major problem for the Northern parts of Bangladesh. Particularly, rice crop becomes affected by cold stress in this part of the country in Boro season. Around 2 million ha of rice areas in the northern districts becomes affected by variable degrees of cold injury, which accounts for sometimes up to 90% seedling mortality incurring increased cost of cultivation depending on the strength and span of cold spell (Biswas *et al.*, 2012). Even sometime it affects reproductive stage of rice crop causing partial to total loss of crop. In the Northeaster haor areas, when short duration Boro rice is grown with an aim to harvest the crop prior to onset of flash flood in mid-April, severe cold shock usually occurs during booting stage of the crop causing total loss of crop due to cold induced failure of grain filling.

2.5 Tools to Evaluate Cold Stress in Rice

Most of the physiological analyses to study tolerance or sensibility of rice to low temperatures have been made in two stages of development: seedling and booting. In both of them cold temperature has harmful effects on crop productivity, as in the first one the number of established plants is affected and in the booting stage pollen sterility can be induced by cold, decreasing the final number of grains. A large array of methodologies, as different cold intensities and periods of exposure, has been applied to evaluate damage and tolerance in these developmental stages. Only a few of them are nondestructive.

2.5.1 Scoring Chilling Injury

At the seedling stage, visual characteristics, as wilting and yellowing of leaves are related to cold stress (Su *et al.*, 2010; Song *et al.*, 2011; Yang *et al.*, 2012). Tolerance to cold stress has been evaluated by scoring chilling injury and low-temperature chlorosis in seedlings by many researchers (Yoshida, 1981;

Andaya and Mackill, 2003; *Xu et al.,* 2008). The degree of leaf withering was used as a criterion for scoring chilling injury (Nagamine, 1991).

2.5.2 Survival Percentages

The survival rate (%) (the number of surviving plants divided by the total number of plants treated × 100) is determined after 4°C treatment, which is a severe condition for rice growth that explicitly distinguishes the cold tolerance of cultivars in short time periods ranging from 6 to 7 days (Zhang *et al.,* 2011b). As this method clearly and efficiently distinguishes degrees of cold tolerance among cultivars and individuals, it is recommended for gauging cold tolerance in the laboratory. Moreover, new leaf emergence can also be used to assess cold tolerance in transgenic rice in terms of maintained vigor and increased growth (Xie *et al.,* 2012). For some cold-tolerant wild type plants and their transgenic lines, cold stress (4°C treatment) only retards growth rather than causing lethal damage. So, new leaf emergence would be a better choice for distinguishing the cold tolerance of these lines.

2.5.3 Water Loss and Shoot-root Biomass

As water loss often occurs concomitantly with plant damage, changes in fresh weight can be used to indicate water loss and growth retardation of rice plants under cold stress (Bonnecarrère *et al.,* 2011). However, water loss is not always an accurate indicator of cold stress because it can also be affected by traits including plant variety and leaf size, and also by other stressors. Plant growth is often negatively influenced by cold stress (Sanghera *et al.,* 2011) and quantitative analyses, as shoot and root biomass, are also used in the evaluation of contrasting genotypes (Aghaee *et al.,* 2011) or transgenic plants (Tian *et al.,* 2011) at any developmental stage. However, these analyses have the disadvantage of being destructive and time consuming, and are not adequate to breeding programs where a large number of lines need to be evaluated.

2.5.4 Quantifications of Gas Exchange, CO_2 Assimilation, and Stomatal Conductance

Quantifications of gas exchange and, in particular, of photosynthetic rate (Wang and Guo, 2005), CO_2 assimilation (Saad *et al.*, 2012), and stomata conductance (Saad *et al.*, 2012) have also been used to understand the photosynthetic metabolism in different genotypes or transgenic plants of rice submitted to cold stress. The combination of gas exchange analyses and chlorophyll fluorescence determination allows the detailed understanding of the photosynthetic process (Saad *et al.*, 2012), because cold stress impacts on luminous and carboxylation reactions of photosynthesis.

2.5.5 Determination of Chlorophyll Content

Cold stress reduces significantly the concentration of chlorophyll in susceptible rice genotypes (Dai *et al.*, 1990; Aghaee *et al.*, 2011). Chlorophyll content was used as a tool to evaluate the degree of cold tolerance of transgenic plants (Tian *et al.*, 2011), to monitor plant recovery after stress (Kuk *et al.*, 2003), and to compare chilling tolerance between distinct hybrid lines during grain filling (Wang *et al.*, 2006).

2.5.6 Quantification of Antioxidant Activity

Activities of antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) can also be used to evaluate cold tolerance in rice. The higher activities of antioxidant enzymes often expressed in transgenic rice lines can indicate their relatively improved cold tolerance compared to wild-type plants (Bonnecarrère *et al.*, 2011; *Huang et al.*, 2009). Increased activities of antioxidant enzyme, including OsPOX1, APXa, and related kinase OsTrx23 (Kim *et al.*, 2011; Sato *et al.*, 2011; Xie *et al.*, 2012) may serve to maintain the functions of many other genes during cold stress. Therefore, rather than only physical evaluation of whole-plant cold tolerance, evaluation of antioxidant enzyme activities could both reveal cold-tolerant plants and reveal the mechanisms of cold tolerance.

Abscicic acid (ABA) content under cold stress may be used as a determinant of cold tolerant plants. Oliver *et al*. (2007); reported that the rice plants that were injected with ABA were found sterile, just like that they experienced with a cold snap. In fact, ABA levels increase when conventional rice is exposed to cold, but they remain the same in the cold tolerant variety.

2.5.7 Evaluation of Pollen Grain Viability, Determination of Sugars Content, and Invertases Activity at the Anthers

The methods described so far are mostly related to cold stress at the vegetative stage of rice plants. At the reproductive stage, mainly at anthesis, exposure of susceptible plants to cold leads to accumulation of sucrose and hexoses in anthers, starch exhaustion at the pollen grain and, consequently, flower sterility. This happens as a function of the reduction in activity of acid invertases bound to the cellular walls of the pollen grain, responsible for sugar hydrolysis. In tolerant plants, sugar accumulation at the anthers is not observed and, consequently, there is no sterility of the pollen grain (Oliver *et al.*, 2007). In this context, techniques of coloration for the evaluation of pollen grain viability, determination of sugars content, and invertases activity at the anthers may be used for evaluation of plant responses to cold during reproductive stage (Oliver *et al.*, 2005).

2.5.8 Spikelet Fertility

Cold tolerance may also be evaluated based on spikelet fertility (Sato *et al.*, 2011). Transgenic rice lines overexpressing OsAPXa (higher Ascorbate Peroxidase activity) were produced to obtain plants with increased ROS scavenger activity, and those plants also showed increased spikelet fertility under cold stress (Sato *et al.*, 2011). Therefore, analyses focused on physiological and biochemical responses to cold stress have contributed significantly with the understanding of rice plant responses to cold stress and with the identification of genotypes or transgenic rice plants tolerant to low temperature.

2.6 Tools for Crop Improvement

The conventional breeding techniques such as pedigree and bulk method are mainly used in the improvement of rice for non-stress condition. Although bulk method is sometimes practiced in handling breeding population for abiotic stress tolerance.Crop loss, low yield or increased cost of cultivation is common phenomena in rice production in low temperature environment. Indica rice cultivars are more sensitive to cold stress than the japonica cultivars. But still there are many indica germplasm that show considerable tolerance to cold stress. However, using different breeding techniques cold tolerance can be improved in the high yielding background. Hybridization with tolerant germplasm (indica or japonica) followed by selection is an well-established breeding technique for crop improvement, although this approach may take long time to attain success for the improvement of abiotic stress tolerance particularly cold tolerance. Mutation by irradiation is also practiced since 1960s for the development stress tolerant, but it did not produce any practical outcomes. The double haploid technique has opened up a new avenue in rice breeding utilizing the anther culture technique to introgress traits from japonica into indica rice in 1980s. The first variety 'Hwaseongbyeo' was developed with this technique in 1986. The rice breeding period was shortened to 5~ 6 years by this technique. A total of 15 rice cultivars were developed by anther-culture breeding system during 1980s~1990s. Although the hybrid rice breeding using the cytoplasmic genomic male sterility (CGMS) was started from the early 1970s, the real breeding work was actively conducted during 1980s and the first development of hybrid rice 'Suweonjapjong No.1 and No.2' was realized with a milled rice yield of 7.3~8.3t/ha in 1989. The establishment of testing system and effective selection of breeding kinds for cold tolerance was really carried out with the construction of cold-tolerance test nursery. The establishment of embryo rescue technique was realized to get the interspecific hybrid between the different genomic wild species and cultivars and to introduce resistant genes for disease and insect pests from wild rice species to

cultivars by recurrent backcrossing in 1990s. Also, the basic techniques to ensure the practical utilization of biotechnologies in rice breeding were developed, and studies on improvement of evaluation techniques and breeding systems were actively driven forward for rice breeding of direct seeding and high-quality or diversification of food-processing utility in recent years. However, all of these systems have also limitations in different aspects. Backcross breeding is the appropriate breeding technique for the development of high yielding stress tolerant variety.

2.6.1 Conventional Backcross Breeding (CBB)

This method was first proposed by Harlan and Pope (1922). Now-a-days this method is employed in improvement of both self and cross-pollinated crops where varieties are deficient in one or two aspects. This method is used particularly for transferring a single simply inherited character like disease, frost or drought resistance and earliness from an undesirable variety to a good commercial variety. The desirable variety is called as recurrent or recipient parent and it is crossed to an undesirable variety, called as donor or non-recurring parent (called donor because the desirable genes are transferred). F_1 plants instead of permitting to self-pollination as in pedigree or bulk method care crossed with the recurring parent and therefore, it is called as back cross method (A back cross may be defined as a crossing of F_1 hybrid with any of its parents). This method requires repetitive backcrossing with the recipient to incorporate a specific train into recipients' background without changing its other traits, which typically takes 6-8 backcrosses to fully recover the recurrent parent genome. Nevertheless, large amount of donor DNA along with target gene maybe retained even after many backcrosses due to linkage drag (Ribaut and Hoisington, 1998). A good example of surprising amount of the linkage drag was reported by Young and Tanksley (1989). They found that the cultivars that were developed even after 20 backcrosses contained as large as 4 cM introgressed donor segments flanking the target locus and another cultivar that was developed after 11 backcrosses still contained the entire arm of a

chromosome carrying the gene from the donor parent. This type of linkage drag is very common in CBB resulting poor outcome in the final product for example, spikelet sterility, susceptibility to biotic and abiotic stresses, etc.

2.6.2 Molecular Breeding

Molecular breeding (MB) may be defined in a broad-sense as the use of genetic manipulation performed at DNA molecular levels to improve characters of interest in plants and animals, including genetic engineering or gene manipulation, molecular marker-assisted selection, genomic selection, etc. More often, however, molecular breeding implies molecular marker-assisted breeding (MAB) and is defined as the application of molecular biotechnologies, specifically molecular markers, in combination with linkage maps and genomics, to alter and improve plant or animal traits on the basis of genotypic assays. This term is used to describe several modern breeding strategies, including marker-assisted selection (MAS), marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), and genome-wide selection (GWS) or genomic selection (GS) (Ribaut *et al.*, 2010).

Marker assisted backcrossing (MAB) approach represents a clear advantage over CBB, because this strategy results in the development of isogenic or near-isogenic lines (NILs) of the mega variety in a short period of time which cannot be derived through CBB. There are a set of basic advantages of MAB over phenotypic selection in CBB, such as, it is simpler, it can save time, resources, and effort needed for phenotypic screening of the traits particularly that are difficult and laborious to measure and quality traits, which generally require expensive screening procedures. In MAS scheme, selection can be performed at early vegetative stage for many traits specially the traits that are expressed at later developmental stage. This may have tremendous benefits in rice breeding because typical rice production practices involve raising of seedlings in the seedbed followed by transplanting of ¾-week old seedlings in the main field, making it easy to transplant only the selected seedlings to the main field.

Furthermore, single plant selection from conventional screening methods for many traits is unreliable due to environmental factors but with MAS, it can be done beyond doubt since there is no environmental influence on DNA markers. In addition, for most traits, homozygous and heterozygous plants cannot be distinguished by conventional phenotypic screening. "Gene pyramiding" or combining multiple genes simultaneously, avoidance of linkage drag of undesirable or deleterious genes during introgression of genes/QTLs from wild species, selecting individuals for traits with low heritability, traits controlled by recessive gene, testing for specific traits where phenotypic evolution is not feasible, can be handled easily through MAS. These advantages of MAS can be exploited by breeders to accelerate the breeding process (Ribaut and Hoisington, 1998; Morris *et al.*, 2003). Target genotypes can be more effectively selected, which may enable certain traits to be "fast-tracked", resulting in quicker line development and variety release. Markers can also be used as a replacement for phenotyping, which allows selection in of-season nurseries and permits more generations to be grown within a year (Ribaut and Hoisington, 1998). Another benefit of using MAS is that the total number of lines that need to be tested can be reduced. Since many lines can be discarded after MAS early in a breeding scheme, this permits more efficient use of glass house and/or field space because only important breeding material is maintained. Zheng *et al.* (1995); stated that MAS selects individuals carrying target genes in segregating population based on patterns of tightly linked markers rather than on their phenotypes. Therefore, the population can be screened at any growth stage and in various environments. In addition, MAS can eliminate interference from interactions between different alleles of locus or different loci. Thus MAS can increase the efficiency and accuracy of selection. Selecting plants from a segregating population those contain appropriate combination of genes is a critical component of plant breeding (Weeden *et al.*, 1994; Ribaut and Betran, 1999). Moreover, plant breeders typically work with hundreds or even thousands of populations, which often with large numbers of traits (Ribaut and Betran, 1999; Witcombe and Virk,

2001). MAS may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods. Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA markers alleles as a diagnostic to identify plants carrying the genes or QTLs (Mischelmore, 1995; Young, 1996; Ribaut *et al.*, 1997).

2.7 Genetic Markers

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as 'signs' or 'flags'. Genetic markers that are located in close proximity to genes (*i.e*. tightly linked) may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes (like genes) called 'loci' (singular locus'). There are three major types of genetic markers as stated by Jones *et al.* (1997); and Winter and Kahl, (1995); that are, (1) Morphological (also 'classical' or 'visible') markers, which are a phenotypic traits or characters; (2) Biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA. Morphological markers are usually visualized phenotypic characters such as flower colour, seed shape, growth habits or pigmentation. Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining. The major disadvantages of morphological and biochemical markers are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter and Kahl, 1995). However, despite these limitations, morphological and biochemical markers have been extremely useful to plant breeders (Eagles *et al.,* 2001). Due to the abundance of DNA markers are used the most widely. They are arising from different types of mutations such as substitution, insertion, deletion or errors in replication of tandemly repeated DNA (Paterson,

1996). These markers are selectively neutral because they are usually located in non-coding regions of DNA and also they are unlimited in number and are not affected by environment or developmental stage of plants (Winter and Kahl, 1995). Apart from the use of DNA markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity (Baird *et al.*, 1997; Henry, 1997).

There are three major type of DNA markers based on the methods of their detection: (1) hybridization-based; (2) polymerase chain reaction (PCR)-based and (3) DNA sequence-based (Gupta *et al.*, 1999; Jones *et al.*, 1997; Joshi *et al.*, 1999; Winter & Kahl, 1995). Essentially, DNA markers may reveal genetic differences that can be visualized by using a technique called gel electrophoresis and staining with chemicals (ethidium bromide or silver) or detection with radioactive or colorimetric probes. Restriction fragment length polymorphism (RFLP) is a hybridization based DNA markers. A number of PCR-based markers are used for linkage analysis such as Random amplified polymorphic DNA (RAPD), Sequence Tagged Sites (STS), Amplified fragment length polymorphism (AFLP) and Simple sequence repeats (SSR or microsatellite) markers. Greater advantage of PCR- based markers compared to RFLP because no southern blots, DNA hybridization, or autoradiography are necessary. PCR products can detect 1-2 kb efficiency (Kochert, 1994). Most recently the use of Single nucleotide polymorphism (SNPs) is becoming very much popular to the researchers.

2.7.1 Restriction Fragment Length Polymorphism (RFLP) Marker

RFLP markers are the first generation of DNA markers and one of the important tools for plant genome mapping. They are a type of Southern-Boltting based markers. In living organisms, mutation events (deletion and insertion) may occur at restriction sites or between adjacent restriction sites in the genome. Gain or loss of restriction sites resulting from base pair changes and insertions or deletions at restriction sites within the restriction fragments

may cause differences in size of restriction fragments. These variations may cause alternation or elimination of the recognition sites for restriction enzymes. As a consequence, when homologous chromosomes are subjected to restriction enzyme digestion, different restriction products are produced and can be detected by electrophoresis and DNA probing techniques.

RFLP markers are powerful tools for comparative and systematic mapping. Most RFLP markers are co-dominant and locus-specific. RFLP genotyping is highly reproducible, and the methodology is simple and no special equipment is required. By using an improved RFLP technique, i.e., cleaved amplified polymorphism sequence (CAPS), also known as PCRRFLP, high-throughput markers can be developed from RFLP probe sequences. Very few CAPS are developed from probe sequences, which are complex to interpret. Most CAPS are developed from SNPs found in other sequences followed by PCR and detection of restriction sites. CAPS technique consists of digesting a PCR-amplified fragment and detecting the polymorphism by the presence/absence of restriction sites (Konieczny and Ausubel, 1993). Another advantage of RFLP is that the sequence used as a probe need not be known. All that a researcher needs is a genomic clone that can be used to detect the polymorphism. Very few RFLPs have been sequenced to determine what sequence variation is responsible for the polymorphism. However, it may be problematic to interpret complex RFLP allelic systems in the absence of sequence information. RFLP analysis requires large amounts of high-quality DNA, has low genotyping throughput, and is very difficult to automate. Radioactive autography involving in genotyping and physical maintenance of RFLP probes limit its use and share between laboratories. RFLP markers were predominantly used in 1980s and 1990s, but since last decade fewer direct uses of RFLP markers in genetic research and plant breeding have been reported. Most plant breeders would think that RFLP is too laborious and demands too much pure DNA to be important for plant breeding.

2.7.2 Random Amplified Polymorphic DNA (RAPD) Marker

RAPD is a PCR-based marker system. In this system, the total genomic DNA of an individual is amplified by PCR using a single, short (usually about ten nucleotides/bases) and random primer. The primer which binds to many different loci is used to amplify random sequences from a complex DNA template that is complementary to it (maybe including a limited number of mismatches). Amplification can take place during the PCR, if two hybridization sites are similar to one another (at least 3000 bp) and in opposite directions. The amplified fragments generated by PCR depend on the length and size of both the primer and the target genome. The PCR products (up to 3 kb) are separated by agarose gel electrophoresis and imaged by ethidium bromide (EB) staining. Polymorphisms resulted from mutations or rearrangements either at or between the primer-binding sites are visible in the electrophoresis as the presence or absence of a particular RAPD band.

RAPD predominantly provides dominant markers. This system yields high levels of polymorphism and is simple and easy to be conducted. First, neither DNA probes nor sequence information is required for the design of specific primers. Second, the procedure does not involve blotting or hybridization steps, and thus it is a quick, simple and efficient technique. Third, relatively small amounts of DNA (about 10 ng per reaction) are required and the procedure can be automated, and higher levels of polymorphism also can be detected compared with RFLP. Fourth, no marker development is required, and the primers are non-species specific and can be universal. Fifth, the RAPD products of interest can be cloned, sequenced and then converted into or used to develop other types of PCR-based markers, such as sequence characterized amplified region (SCAR), single nucleotide polymorphism (SNP), etc. However, RAPD also has some limitations/disadvantages, such as low reproducibility and incapability to detect allelic differences in heterozygotes.

2.7.3 Sequence Tagged Site (STS) Marker

STS markers are another class of DNA markers, which are also extremely useful for MAS (Sharp *et al.,* 2001; Sanchez *et al.,* 2000; Shan *et al.,* 1999). STS is a short (200- 500bp) DNA sequence that has single occurrence in the genome, location and base sequence are known. These markers can be detected easily with specific primers by PCR amplification. STS markers are co-dominant, highly reproducible, suitable for high throughput and automation, and technically simple for use (Reamon-Butter and Jung, 2000). STS primers are designed from the sequence of cloned DNA fragments that are usually obtained by either RFLP or AFLP (Semagn *et al.,* 2006b). Sometimes the amplified fragment of this marker may be monomorphic and needs restriction digestion with different restriction enzymes to produce a different size of new fragments as polymorphic markers. STS are used for the detection of target sequence in the introgression lines.

2.7.4 Amplified Fragment Length Polymorphism (AFLP) Marker

AFLPs are PCR-based markers, simply RFLPs visualized by selective PCR amplification of DNA restriction fragments. Technically, AFLP is based on the selective PCR amplification of restriction fragments from a total double-digest of genomic DNA under high stringency conditions, i.e., the combination of polymorphism at restriction sites and hybridization of arbitrary primers. Because of this AFLP is also called selective restriction fragment amplification (SRFA). An AFLP primer (17-21 nucleotides in length) consists of a synthetic adaptor sequence, the restriction endonuclease recognition sequence and an arbitrary, non-degenerate ‘selective’ sequence (1-3 nucleotides). The primers used in this technique are capable of annealing perfectly to their target sequences (the adapter and restriction sites) as well as a small number of nucleotides adjacent to the restriction sites. The first step in AFLP involves restriction digestion of genomic DNA (about 500 ng) with two restriction enzymes, a rare cutter (6-bp recognition site, EcoRI, PtsI or HindIII) and a frequent cutter (4-bp recognition site, MseI or TaqI). The adaptors are then

ligated to both ends of the fragments to provide known sequences for PCR amplification. The double-stranded oligonucleotide adaptors are designed in such a way that the initial restriction site is not restored after ligation. Therefore, only the fragments which have been cut by the frequent cutter and rare cutter will be amplified. This property of AFLP makes it very reliable, robust and immune to small variations in PCR amplification parameters (e.g., thermal cycles, template concentration), and it also can produce a high marker density. The AFLP products can be separated in high-resolution electrophoresis systems. The fragments in gel-based or capillary DNA sequencers can be detected by dye-labeling primers radioactively or fluorescently. The number of bands produced can be manipulated by the number of selective nucleotides and the nucleotide motifs used.

A typical AFLP fingerprint (restriction fragment patterns generated by the technique) contains 50-100 amplified fragments, of which up to 80% may serve as genetic markers. In general, AFLP assays can be conducted using relatively small DNA samples (1-100 ng per individual). AFLP has a very high multiplex ratio and genotyping throughput, and is relatively reproducible across laboratories. Another advantage is that it does not require sequence information or probe collection prior to generating the fingerprints, and a set of primers can be used for different species. This is especially useful when DNA markers are rare. However, AFLP assays have some limitations also. For instance, polymorphic information content for bi-allelic markers is low (the maximum is 0.5). High quality DNA is required for complete restriction enzyme digestion. AFLP markers usually cluster densely in centromeric regions in some species with large genomes (e.g., barley and sunflower). In addition, marker development is complicated and not cost-efficient, especially for locus-specific markers. The applications of AFLP markers include biodiversity studies, analysis of germplasm collections, genotyping of individuals, identification of closely linked DNA markers and construction of genetic DNA marker maps, construction of physical maps, gene mapping, and transcript profiling.

2.7.5Microsatellites (SSR) Marker

SSR are tandem repeats of simple sequence that occur abundantly and at random throughout the most of eukaryotic genomes. They are relatively small in site and usually 2-10 by long and embedded in DNA with unique sequence. These DNA markers can be directly amplified by PCR using the unique sequences that flank either side of it (Tautz, 1989). SSR markers are ideal markers for constructing high-resolution genetic maps, assisting selection and studying the genetic diversity in germplasm. It has been extensively exploited for genome mapping and for wide range of population and evolutionary studies in Arabidopsis (Innan *et al.*, 1997), rice (Yang *et al.*, 1994), and other animal and plant species (Powell *et al.*, 1996). Condit and Hubbell (1991) first time reported that microsatellite occur abundantly in plant species, This marker has the advantage of reliability, reproducibility, discrimination, standardization, highly informative and cost efficiency over RFLPs, In many cases, microsatellite have been isolated from DNA libraries and published sequence data (Wu and Tanksley, 1993).

Microsatellites consist of tandem of one to six nucleotides, which are hyper variable. All SSRs with a repeat length of few base pairs could be considered as microsatellite and one extreme class of repeated sequence is satellite DNAs. Specific amplification indicates that microsatellite markers are more variable than RFLPs. Polymorphism of tandem repeat is thought to be generated by unequal crossing over or by slippage during DNA polymerization (Burr, 1994). Microsatellite arrays of tandemly repeated sequences have been found to be abundant and widely distributed throughout the genomes of eukaryotic organisms (Cornall *et al.*, 1991; Condit and Hubbell, 1991), It requires the small amount of DNA. The relatively low cost, high loci of allelic diversity, high mutation rate and high power of resolution arc important attributes of SSR markers (Panaud *et al.*, 1995; McCouch *et al.*, 1997; Karp *et at.*, 1997; Rivera *et al.*, 1999). The assay is PCR based; making them easily detectable on polyacrylamide gels. This considerably reduces the time required to obtain

results compared with RFLPs using southern blotting. Finally, use of radio isotopes can be avoided, polymorphism between alleles is large enough to be detected in agarose gels and generally, microsatellites behave as co-dominant markers. Panaud *et al.* (1995); reported that SSR occurred approximately every 80 kb of rice. Mackill *et al.* (1996); found microsatellites to have an average polymorphism at least 1.5 times than AFLP and RAPD markers in a comparison of 12 cultivars. Many studies have also reported significantly greater allelic diversity of microsatellites over RFLPs (McCouch *et al.,* 1997).

SSR markers have been extensively used as a powerful tool in variety protection (Rongwen *et al.,* 1995), molecular diversity studies (Sebastian *et al.,* 1998), QTL analysis, pedigree analysis and marker assisted breeding (Chen *et al.,* 1997). SSRs have been developed for many crop species, including wheat (Prasad *et al.,* 2000), maize (Senior *et al.,* 1998), sorghum (Dean *et al.,* 1999; Smith *et al.,* 2000), cotton (Liu *et al.,* 2000), tomato (Tanksley and Nelson, 1996), and soybean (Akkaya *et al.,* 1992). In rice, SSRs have been used to assess the genetic diversity of both wild and cultivated species (Brondani *et al.,* 2005; Siwach *et al.,* 2004; Ni *et al.,* 2002). SSR markers are particularly suitable for evaluating genetic diversity and relationships among closely related plant species, populations, or individuals (Kostova *et al.,* 2006; Tu *et al.,* 2007).

Many genes/QTL for biotic and abiotic stresses have been mapped and utilized in MAS using SSR markers. Ding *et al.* (2011); used SSR markers for mapping QTL of arsenic concentration in different tissues of maize. QTL was mapped for cadmium (Cd) accumulation and distribution (Yang *et al.,* 2013) and Cd tolerance in rice (Xue *et al.,* 2009) using SSR markers. The quantitative trait loci (QTLs) associated with arsenic (As) accumulation in rice was also mapped using SSR markers. Microsatellite marker analysis is attractive for developing marker-assisted selection programs.

2.7.6 Single Nucleotide Polymorphisms (SNPs)

The new generation of molecular markers, called single nucleotide polymorphisms (SNPs) may employ gel-based or non-gel-based assays (Gupta *et al.,* 2001), a more efficient and cost-effective marker systems with varying levels of multiplexing to suit a range of genetics and breeding applications. SNPs are bi-allelic (having two alleles per locus; Vignal *et al.,* 2002) and are the most abundant type of polymorphism in plant genomes, with recent studies identifying approximately five SNPs/kb across 20 Arabidopsis accessions and nine SNPs/kb across 517 resequenced rice varieties (Clark *et al.,* 2007; Huang *et al.,* 2010). A SNP is a genetic variation when a single nucleotide (*i. e*. A, T, C, G) is altered and kept through heredity. The discovery of single nucleotide polymorphisms and insertions/deletions (InDels) are the basis of most differences between alleles. Shen *et al.* (2004); constructed a genome-wide DNA polymorphism database between rice subspecies Nipponbare and 93-11 based on publicly available genomic sequence information, and the database include SNP and InDel frequencies and distribution, an InDel comparison between Nipponbare/93-11 and Nipponbare/GLA4, and the association of existing SSRs and InDel polymorphisms. This database contained 1,703,176 SNPs and 479,406 InDels, approximately one SNP at every 268 by and one InDel every 953 by in rice genome. InDel polymorphisms were experimentally validated and 90% (97 of 108) of InDels in the database could be used as molecular markers, which are comparatively cheap and easy to use, and their applicability for mapping *Indica/japonica* crosses analyzed. Comparison of the sequences of japonica rice with the indica rice showed that on average, one SNP occur in every 170 by and one InDel in every 540 by (Yu and Hu, 2002).

The Illumina Golden Gate assay is capable of multiplexing from 96 to 1,536 SNPs in a single reaction over a 3-day period (Fan *et al.,* 2003). A one 96-plex and six 384-plex SNP sets were designed and tested on the BeadXpress platform, targeting different germplasm groups in rice (Thomson *et al.,* 2012). Furthermore, a recent study designed a 384-plex SNP set in rice using data

from Minghui 63, Zhenshan 97 and Nipponbare, and identified 372 high quality SNPs (Chen *et al.,* 2011). A high-density Affymetrix SNP chip with 44,100 SNPs has also been developed using OryzaSNP loci in combination with SNPs from bacterial artificial chromosome (BAC)-end sequences from the Oryza Map Alignment Project (OMAP), of which over 90% passed quality criteria when genotyped on over 400 rice accessions (Zhao *et al.,* 2008). Thus, SNP markers can be used as ideal molecular markers for high-resolution genotyping for association studies, rapid genome-wide scans for genomic selection, as well as routine genetic diversity analysis, linkage mapping, and marker-assisted selection (MAS; Tung *et al.,* 2010; McCouch *et al.,* 2002; Ye *et al.,* 2010).

2.8 Genetic Markers and QTLs Linked to Cold Tolerance in Rice

Cold tolerance in rice is a quantitative trait controlled by multiple genes. Because it is often difficult to directly associate plant phenotypes with the genes responsible for cold tolerance, marker-assisted selection is an effective means of developing cold-tolerant cultivars (Shirasawa *et al.,* 2012; Foolad *et al.,* 1999). The development of molecular markers and linkage maps has made it possible to identify QTL that control cold tolerance in rice. QTL analyses have been carried out using rice populations with large levels of genetic variation for cold tolerance (Futsuhara and Toriyama, 1969).

QTLs related to tolerance to low temperatures in rice have been identified by the use of restriction fragment length polymorphisms (RFLPs; Li *et al.,* 1997; Harushima *et al.,* 1998; Takeuchi *et al.,* 2001) and microsatellite/ simple sequence repeats (SSR) molecular markers (McCouch *et al.,* 2002; Andaya and Mackill, 2003a,b; Fujino *et al.,* 2004; Kuroki *et al.,* 2007; Lou *et al.,* 2007; Suh *et al.,* 2010). The technical efficiency and multiplex potential of SSR makes them suitable to high-throughput mapping and marker-assisted breeding. SSR markers are codominant, multiallelic, and can be efficiently used in indica and japonica germplasm (McCouch *et at.,* 2002). Most recently, technical advances have allowed the identification of the genes (and their functions) responsible

for rice QTLs, such as a combination of PCR-based screening, development of near-isogenic lines and searches for hits in EST databases (Saito *et al.,* 2004), fine mapping based on microsatellite markers including markers identified from publicly available genomic sequences (Andaya and Tai, 2006) and map-based cloning (Fujino *et al.,* 2008). It has been suggested that QTLs related to cold tolerance in the germination stage are independent from QTLs conferring tolerance at the vegetative and reproductive phase (Saito *et al.,* 2001; Andaya and Mackill, 2003a,b; Fujino *et al.,* 2004), indicating that cold tolerance may be developmentally regulated and growth stage specific. Most recently, the complete and high-quality sequence of the rice genome has provided a genome-wide SNP resource (IRGSP, 2005) that leads to high-quality and reliable markers. Until the present moment, however, only few studies have been able to link cold tolerance to SNPs in rice (Koseki *et al.,* 2010; Shirasawa *et al.,* 2012), and only one could effectively locate a SNP which resulted in amino acid substitution leading to reduced enzyme activity (glutathione transferase isoenzyme (OsGSTZ2) (Kim *et al.,* 2011). According to the authors, this functional difference in the OsGSTZ2 isoform could explain the differential response observed between cold-tolerant and cold-sensitive rice cultivars.

2.8.1 QTLs Related to Cold Tolerance at the Germination Stage

Low temperature stress may affect rice seed germination, avoiding development to the seedling stage and eventually leading to the heterogeneous maturation of the culture (Andaya and Mackill, 2003b). A wide range of phenotypic variation of low-temperature germinability is found in rice cultivars. QTLs analyses for low-temperature germinability revealed that multiple genes control this trait. Using a genome-wide expression profiling analysis, Fujino and Matsuda, (2010); identified 29 qLTG3-1 dependent genes with diverse functions. Several genes involved in defense responses were upregulated by qLTG3-1, indicating that qLTG3-1 expression is required for the expression of defense response genes in low-temperature germinability in

rice (Fujino and Matsuda, 2010). On the other hand, Fujino *et al.* (2008); suggest that qLTG3-1 may not be involved in the response to low temperature, but rather in seed germination itself, under different kinds of stress.

Fujino *et al.* (2004); identified three QTLs controlling low-temperature germinability using backcross inbred lines (BILs) derived from a cross between a vigorous and a weak low-temperature germinability cultivars (Italica Livorno and Hayamasari, respectively). A major QTL for low-temperature germinability on chromosome 3, qLTG3- 1, explained 35% of the total phenotypic variation in the mapping population. High-resolution mapping placed qLTG3-1 in a 4.8-kb region. Only one gene, 0s03g0103300, was predicted to this region, and sequence analysis showed that an unknown function protein containing two known conserved domains, GRP (glycine-rich cell wall protein) and Tryp alpha amyl of the protease inhibitor/seed storage/LTP family, is encoded by this gene (Fujino *et al.*, 2008).

2.8.2 QTLs Related to Cold Tolerance at the Vegetative Stage

Cold tolerance at the seedling stage is an important trait affecting stable rice production. Different groups have located chromosome regions responsible for cold tolerance during the vegetative stage of rice growth. Kim *et al.* (2014); used an F_7 recombinant inbred line (RIL) population of 123 individuals derived from a cross of the cold-tolerant japonica cultivar Jinbubyeo and the cold-susceptible indica cultivar BRRI dhan29 for QTL mapping. Six QTLs including two on chromosome 1 and one each on chromosome 2, 4, 10, and 11 for seedling cold tolerance were identified with phenotypic variation (R2) ranging from 6.1 to 16.5 %. The QTL combinations (qSCT1 and qSCT11) were detected in all stable cold-tolerant RII, groups, which explained the critical threshold of 27.1 % for the le value determining cold tolerance at the seedling stage. Two QTLs (qSCT1 and qSCT11) on chromosomes 1 and 11, respectively, were fine mapped. The markers In 1-c3, derived from the open reading frame (ORF) LOC_Os01g69910 encoding calmodulin-binding transcription activator (CAMTA), and In11-dl, derived from ORF LOC_Osl 1

(Duf6 gene), co-segregated with seedling cold tolerance. Xiao et al. (2014); constructed 151 BC_2F_1, mapping plants using DX as a cold-tolerant donor and the indica variety Nanjing 11 (NJ) as a recurrent parent. Based on this, two seedling cold-tolerant QTLs, named qRC10-1 and qRC10-2, were detected on chromosome 10 by composite interval mapping. qRC10-1 (LOD = 3.1) was mapped at 148.3 cM between RM171 and RM1108, and qRC10-2 (LOD = 6.1) was mapped at 163.3 cM between RM25570 and RM304, which accounted for 9.4% and 32.1% of phenotypic variances, respectively. Xiao *et al.* (2014); also fined map the major locus qRC10-2, NJ was crossed with a BC_4F_2 plant (L188-3), which only carried the QTL qRC10-2, to construct a large BC_5F_2 fine-mapping population with 13,324 progenies. Forty-five molecular markers were designed to evenly cover gRC10-2, and 10 markers showed polymorphisms between DX and NJ. As a result, gRC10-2 was delimited to a 48.5-kb region between markers qc45 and qc48. In this region, Os10g0489500 and Os10g0490100 exhibited different expression patterns between DX and NJ. Our results provide a basis for identifying the gene(s) underlying qRC I 0- 2, and the markers developed here may be used to improve low-temperature tolerance of rice seedling and maturity stages via marker-assisted selection (MAS).

Park *et al.* (2013); evaluated leaf discoloration and SPAD value (chlorophyll content) of 80 recombinant inbred lines (RILs) from an inter-subspecific cross between Milyang23 (*0. sativa* ssp. Indica) and Hapcheonaengmi3, a japonica weedy rice. A total of four QTLs for SPAD were identified that explained phenotypic variance ranging from 5.4 to 16.0%. QTLs detected under the control condition were located on chromosome 2 and 5. Further, two QTLs inherited from Hapcheonawngmi3 that were found associated to an increased SPAD values were identified on chromosome 1 and 4. To delimit the position of qSPA-4, substitution mapping was performed which enabled its location in 810 kb region flanked by RM16333 and RM16368. The results indicated that Hapcheonaengmi3 contains QTL alleles that are likely to improve cold tolerance in indica rice.

Kim *et al.* (2011); suggested that cold sensitivity in rice is strongly correlated with a naturally occurring 11e99Val mutation in the multifunctional glutathione transferase isoenzyme GS7Z2. A mapping population of F_2 plants derived from a cold-tolerant wild rice, W 1943 (*Oryza rufipogon*), and a sensitive indica cultivar, Guang-lu-al 4, was used to identify QTLs associated with cold tolerance at the seedling stage. The work was based on phenotypic evaluation and development of SNP markers.

Koseki *et al.* (2010); detected three QTLs on chromosomes 3, 10, and 11. A major locus, qCtss11, was located on the long arm of chromosome 11, explaining about 40% of the phenotypic variation. Additional markers were developed to enable fine mapping of the qCtss11 region, where six putative open reading frames were identified. Two of those candidate genes (OslIg0615600 and Os 1 1g0615900) were considered the best candidates for qCtss11. The protein encoded by the 0s11 g0615900 gene was characterized as containing a NB-ARC domain (nucleotide-binding adaptor shared by APAF-1, R protein and CED-4). A comparison of the genetic location of gOssl I and qCTS11-2 (identified by Andaya and Mackill, 2003b) suggests that these loci are likely coincident and therefore possibly allelic.

Andaya and Tai, (2006); raised the possibility that these two QTLs on chromosome 12 are in fact the same, naming them as qCTSI2. Although the injuries from wilting and necrosis appeared to be distinct, there was strong correlation between the two traits. Moreover, both QTLs mapped to the same position on chromosome 12. Fine mapping of this locus was performed by saturating the short arm of chromosome 12 with microsatellite markers. Ten open reading frames were identified in this region, and two of them (OsGSTZ1 and OsGSTZ2 — two zeta class glutathione S-transferases) were considered the best candidates to correspond to qCTSI2 (Andaya and Tai, 2006).

Andaya and Mackill, (2003b); were evaluated recombinant inbred lines (RILs) derived from a cross between a cold-tolerant temperate japonica cultivar (M-202) and a cold-sensitive indica cultivar (1R50) using microsatellite markers.

Fifteen QTLs were identified, mostly with small effects. The QTL identified on chromosome 4, designated as qCTS4-1, accounted for about 21% of the phenotypic variation for general cold tolerance. In this same category, a QTL on chromosome 6, qCTS6-1, explained 15% of the variation. A major QTL was identified on chromosome 12, named qCTS12a, which accounted for 41 % of the phenotypic variation in tolerance to cold-induced wilting tolerance. Another QTL on chromosome 12, qCTS12b, explained 42% of the cold-induced necrosis tolerance.

2.8.3 QTLs Related to Cold Tolerance at the Reproductive Stage

Rice cold tolerance at the booting stage is a quantitative trait controlled by multiple genes. Identification of QTLs is based on phenotypical evaluation of distinct genotypes, an approach that is not prone to allow the simultaneous identification of multiple genes. To be able to combine a large number of genes in one genotype, it has been considered that the most effective approach is to identify single genes, based on phenotype analyses, and then combine the genes by marker-assisted selection.

Shirasawa *et al.* (2012); reported a single QTL for booting stage cold tolerance on the long arm of chromosome 3. This QTL was named qLTB3 and explained 24.4% of the phenotypic variance. Seven SNP markers were identified in five genes within the qLTB3 region, all of them causing amino acid substitutions. One of those SNPs (in the 0s03g0790700 gene) caused a mutation in a conserved amino acid and was considered the strongest candidate for conferring cold tolerance. The 0s03g0790700 gene encodes a protein similar to the Arabidopsis AAO2 aldehyde oxidase, which is believed to function in ABA biosynthesis (Koiwai *et al.*, 2004; Seo *et al.*, 2004).

Saito *et al.* (2010); indicated that an F-box protein gene confers the cold-tolerance trait and that cold tolerance is associated with larger anther length. Moreover, the F-box protein interacts with a subunit of the E3 ubiquitin ligase, Skp I, suggesting that an ubiquitin-proteasome pathway is involved in cold

tolerance at the booting stage (Saito *et al.*, 2010). The amount of pollen available for fertilization is directly related to anther length, and cold affects pollen grain maturation, reducing fertility as a consequence. Cold-tolerant varieties hold larger anthers and, consequently, they produce a larger number of pollen grains than susceptible varieties. Therefore, a strong correlation was suggested to exist between cold tolerance QTLs and anther length QTLs, being the pollen amount an important component of the tolerance mechanism (Saito *et al.*, 2001).

Zhou *et al.* (2010); reported a QTL for cold tolerance at the booting stage on chromosome 7, named riCTB7. The QTL explained 9 and 21% of the phenotypic variances in the F2 and F3 generations, respectively. Twelve putative cold tolerance genes from this QTL region were identified by fine mapping and candidate gene cloning. On the basis of genetical and physical mapping, the authors suggested that two other QTLs previously identified in the same location (qRCT7: Dai *et al.*, 2004; and qCT-7:Takeuchi *et al.*, 2001) may correspond to the same locus as qCTB7. Although identified in diverse genetic backgrounds and environments, the three QTIs explained similar percentages of the phenotypic variance, ranging from 20.6% to 22.1%.

Kuroki *et al.* (2007); identified a QTL for booting stage cold tolerance on chromosome 8 (qCTB8) through the analysis of F2, F3, and F7 populations, using SSR markers. This QTL explains 26.6% of the phenotypic variance. About 30 open reading frames were identified at the qC7T8 region. One of them encodes monodehydroascorbate reductase (MDAR), which was shown to be up regulated by cold treatment in rice anthers during the young microspore stage.

Saito *et al.* (2001); detected two closely linked QTLs (Ctb-1 and Clb-2) for cold tolerance and suggested their association with anther length. Later, Saito *et al*. (2004); reported the physical mapping of Ctb 1 and confirmed the association of Ctb 1 with anther length. According to the authors, seven open reading frames (ORFs) were found within the 56-kb region where Ctb 1 was

located: two receptor-like protein kinases, three ubiquitin-proteasome pathway-associated proteins (two of which encoded F-box proteins), a protein with an OTU domain and an unknown protein.

Takeuchi *et al.* (2001); had constructed a linkage map with RFLP and RAPD molecular markers for detection of QTLs controlling cold tolerance, and a total of eight QTLs were found. Among them, the ones with higher contributions were associated with general cold tolerance (qCT-7, on chromosome 7), and cold tolerance related to culm length (qCL-1, on chromosome 1) and heading date (qHD-3-2, on chromosome 3 and qHD-6, on chromosome 6), explaining 22.1%, 31.1%, 15.5%, and 50.5% of the respective phenotypic variation.

2.9 Benefits of Marker Assisted Backcrossing (MAB) Approach

Among the MAS schemes, MAB is the most appropriate method for incorporating a gene/QTL into the genetic background of a mega variety (Mackill, 2004). In backcross breeding the theoretical proportion of the recurrent parent genome after 'n' number of generations of backcrossing is given by: $(2^{n+1}-1)/2^{n+1}$ (where, n=number of backcrosses; assuming an infinite population size). However, there are some individuals that possess more of the recurrent parent genome than others (Collard *et al.*, 2005) although the average percentage of the recurrent parent genome is 75% for the entire BC1 population. Phenotypic selection of such plants from early backcross generation is not only difficult rather impossible. Advancement in molecular marker technology and availability of high resolution molecular map of rice genome have made it possible to track these type of plants, thus speeding up this process by reducing the number of backcross cycles required for full recovery of recurrent parental genome (RPG). Tightly-linked DNA markers flanking the gene/QTLs of interest and evenly spaced markers for other chromosomes (i. e. unlinked to genes/QTLs) of the recurrent parent are used for introgression of gene/QTLs and recovery of the RPG in this selection system. The use of additional markers to accelerate cultivar development is

sometimes referred to as ‘Full MAS’ or ‘Complete Line Conversion’ (Ribaut *et al.,* 2002; Morris *et al.,* 2003).

A simulation study based on PLABSIM, a computer program that simulates recombination during meiosis also indicated that efficiency of recurrent parent recovery using markers is far better compared to conventional backcrossing (Frisch *et al.,* 1999a, 2000). Therefore, considerable time savings can be made by using markers compared to conventional backcrossing (Collard *et al.,* 2005). Although the initial cost of MAB could be higher compared to conventional breeding in the short term, the time savings could lead to economic benefits. This is an important consideration for plant breeders because the accelerated release of an improved variety may translate into more rapid profits by the release of new cultivars in the medium to long-term (Morris *et al.,* 2003).

2.10 Strategies of Marker Assisted Backcrossing (MAB) Approach

In MAB, plant selection is performed in general at three levels as discussed by Holland (2004). At the first level, tightly linked or gene/QTL specific markers are used in combination with or without phenotypic screening for plant selection, which is referred to as ‘target gene selection’ or ‘foreground selection’ (Hospital and Charcosset, 1997). This selection is very much useful for the traits particularly those which are laborious or time-consuming to phenotype. It can also be used to select for reproductive-stage traits at the seedling stage allowing the best plant to be identified for backcrossing. Furthermore, recessive alleles can be selected, which is difficult to do using conventional methods (Babu *et al.,* 2004).

The second stage involves selecting backcross progenies with the target gene and tightly linked flanking markers, which is referred to as ‘recombinant selection’ (Collard and Mackill, 2007). The advantage of using flanking markers on both sides of a target gene is that the amount of donor chromosome segment (S) surrounding the target gene can be minimized. This is important

because the rate of decrease of this donor fragment if slower than from the unlinked regions and many undesirable genes that negatively affect crop performance may be linked to the target gene from the donor parent (Hospital, 2005). Using conventional breeding methods, the donor segment can remain very large even after many backcross generations (e. g., >10) (Ribaut and Hoisington, 1998; Salina *et al.,* 2003). By using markers flanking a target gene (e. g., <5 cM on either side), this type of linkage drag can be minimized. Since double recombination events occurring on both sides of a target locus are extremely rare events in the same generation, recombinant selection is usually performed using at least two backcross (BC) generations (Frisch *et al.*, 1999b). However, there are two options for obtaining an individual with recombination on both sides of the targeted gene. The first option is selection for simultaneous recombination on both sides, which will result in the desired recombinant in one generation. The alternative is a tandem selection, i. e., selection for recombination on one side in the first backcross generation and selection for recombination on the other side in the next backcross generation. Although the first option would save one generation of backcrossing, it is much more costly and laborious than the second option as it needs genotyping of a very large population. For example, assuming a distance of one cM on both sides, simultaneously recombination on both sides would occur at a frequency of 0.01%. Thus, one would have to screen 10,000 positive individuals containing the gene (representing only 50% of the plants in a backcross population) to obtain one double recombinant. This is obviously not realistic for the molecular marker assay as well as for the production of large number of backcross seeds. Additionally, there are possible complications of cross over interference in obtaining a double cross over. In contrast, also assuming a distance of 1 cM on both sides, a recombinant event between the targeted gene and flanking marker on either of the two sides would occur at a frequency of 2%. Thus, only 50 positive individuals would be needed in the first generation of backcrossing to expect a recombinant event between the targeted gene and one of the flanking markers, and 100 positive individuals would be needed to obtain a recombinant

on the other side of the gene in the second generation of backcrossing. Thus, the second option obviously costs much less in terms of labor and resources than the first one (Chen *et al.*, 2000). Young and Tanksley, (1989a); prescribed to perform recombinant selection on at least two successive generations allowing a single homozygote for recurrent parent on one side of the target QTL then on the other side. Individuals those are heterozygous at the target locus and homozygous for the recipient-type alleles for two markers flanking the target locus on each side have been termed as 'double homozygote' by Hospital and Decoux (2002).

The third stage of MAB involves selecting target plants with the greatest proportion of RPG, using markers which are unlinked to the target locus. This level of selection is referred to as 'background selection'. Background selection refers to the use of unlinked markers to select for the recurrent parent (Hospital and Charcosset, 1997; Frisch *et al.*, 1999b). This was also referred to as 'negative selection' by Takeuchi *et al.* (2006). Background selection has already been shown to be efficient by previous theoretical studies (e. g., Hillel *et al.*, 1990; Hospital *et al.*, 1992; Groen and Smith, 1995; Visscher *et al.*, 1996), and experimental studies (e. g., Ragot *et al.*, 1995). This level of selection is extremely useful as RPG recovery is greatly accelerated. With conventional backcrossing, plant breeders must select backcross progeny with visual observation of visible differences between the recurrent parent and the individual plant of backcross progenies in every backcross generation. This becomes increasingly difficult as the generation advances. In CBB, it takes a minimum of 6 successive backcross generations to recover RPG and there may still be several donor chromosome fragments unlinked to the target gene. Using markers, it can be achieved by BC_4, BC_3, or even BC_2 (Frisch *et al.*, 1999a, 1999b; Hospital and Charcosset, 1997; Visscher *et al.*, 1996), thus saving two to four backcross generations.

2.11 Limitations of Marker Assisted Backcrossing (MAB) Approach

The MAB scheme works best when a large number of backcross seeds are available in each cycle. Frisch *et al.* (1999a,b); suggested some guidelines for the population size needed for the MAB approach. The critical factors in a MAB scheme are the number of target genes, the distance between the flanking markers and the target gene (2-20 cM), and the number of genotypes selected in each backcross generation. Depending upon the objectives, the experimental design for line conversion through MAB needs to be modulated based on the available resources, nature of the germplasm (e. g., agronomic quality and number of lines to be converted) and technical options available at the marker level. Using a computer simulation technique on three different selection strategies, viz. i) two stage selection strategy – selection of individuals at the target locus followed by homozygous recurrent parent alleles over the genome; ii) three stage selection strategy – selection of individuals at the target locus followed by homozygous flanking recurrent parental alleles and homozygous recurrent parent alleles over the genome; iii) four stage selection strategy, which includes all of the steps of three stage selection with an additional step – selection of individuals homozygous for the recurrent parental alleles at all additional markers on the carrier chromosome immediately after target locus selection Frisch *et al.* (1999b); suggested that one can expect to find BC_3 progeny with at least 96% RPG with 90% probability using four stage selection strategy from reasonable size (50-100) backcross population. They also opined that in four-stage selection system, a reduction of marker data point can be attained by as much as 50-75% then three or two stage selection strategies. Although, two-stage selection seems to be appropriate for two generation backcross program with limited population size due to higher RPG recovery. In fact two-stage selection is suggested when no information of marker linkage map is available and hence, the only option of application of this strategy BC_1 generation. By comparing different selection approaches in MABC, Iftekharuddaula (2011); showed that selecting individuals at the target locus followed by homozygous flanking recurrent parental alleles and homozygous

recurrent parent alleles over the genome can improve the efficiency of backcross breeding to identify isogenic lines with target locus free from maximum possible linkage drag in only two generation of backcross. He, however, suggested that phenotypic selection has some effect after recombinant (i. e. homozygous flanking recurrent parent allele) selection over small population size in first backcross cycle.

The success of MABlargely depends on the number of the markers per chromosome and population size in each backcross generation. From the cytogenetic studies, it was observed that the number of chiasmata per chromosome per meiosis is 0, 1 or 2. The number of crossovers is roughly proportional to chromosome length. More than 5 or 6 crossovers per chromosome per meiosis are extremely rare (Kearsey and Pooni, 1996). So, for the short chromosomes of rice like 10, 12, 8, etc, at least five markers are needed while for long chromosome viz. 1, 3, 2, etc, 8-10 background markers are required to achieve recurrent parental recovery. As the range of crossing over interference is around 15 cM (Kersey and Pooni, 1996), the minimum distance between the two adjacent background markers should be at least 15 cM. However, Openshaw *et al.* (1994); and Visscher *et al.* (1996); suggested that and average marker density of approximately 20 cM is sufficient to warrant a good coverage of the genome for MAS programs.

2.12 Success Stories of Marker Assisted Backcrossing (MAB) in Crop Improvement

MAB has been widely used in rice varietal improvement. There are many success stories of this technique for the improvement of tolerance to different biotic and abiotic stresses, and grain quality improvement. Development of BB resistant pyramided lines (Chen *et al.,* 2000; Sanchez *et al.,* 2000; Singh *et al.,* 2001; Joseph *et al.,* 2004) is well documented in this regard. The cooking and eating quality of Zhenshan 97 was improved by introgressing Waxy gene region from Minghui 63 (wx-MH) through MAB. The improved versions of

Zhenshan 97 A and B have been widely used in hybrid rice production (Zhou *et al.,* 2003). The most recent success of MAB is the conversion of several popular rice varieties of South and Southeast Asia into submergence tolerant version (Xu *et al.,* 2006; Neeraja *et al.,* 2007 and Iftekharuddaula, 2011) with a sub1 QTL derived from a landrace, FR13A. this sub1 rice varieties are expected to have tremendous impact in alleviation of poverty and improvement of livelihoods of millions of farmers in the flood prone areas of South and Southeast Asia saving their crop from flooding. Salinity tolerance in rice is another avenue where MAB is being successfully practiced. 'Saltol', a salinity tolerant QTL from FL378 and FL478 are being introgressed and validated in different *indica* rice backgrounds (Ismail, A. M. Pers. Comm). A set of NILs conferring phosphorus (P) uptake efficiency of rice plants in phosphorus deficient soil have been developed transferring a QTL called PUP1 allele from Khasalath showed a 170% increase in P uptake and 250% increase in yield when grown under low-P conditions. A MAB scheme has been developed and is being used to transfer these QTL into three upland rice varieties that are sensitive to phosphorus deficiency, particularly in acid soils (Ismail, M. A., M. Wissuwa and S. Heurer, unpublished). Drought is the most widespread and damaging abiotic stress for rice cultivation in water limited conditions. Molecular approaches to drought tolerance have been widely applied to rice, beginning with QTL analysis. Numerous QTLs for secondary traits that are expected to be associated with drought response were identified (Lafitte *et al.,* 2006) but recently a major QTL (qtl12.1, Bernier *et al.,* 2007) for yield under drought has been mapped on chromosome12, which improves yield under drought by 47% and explained more than 50% of the genetic variance. A set of NILs have been developed for further fine mapping of this QTL. Once the marker information of this fine mapping is available, qtl12.1 would have great impact to enhance drought tolerance in rice through MAB. Kim *et al.* (2014); identified three main effect QTLs (*qSCT1, qSCT4,* and *qSCT11)* from a cross between BR29 and Jinbubyeo of which *qSCT1* and *qSCT11* were further fine maple and tightly linked insertion–deletion polymorphisms (InDel) markers

derived from the two candidate gene regions associated with seedling cold tolerance have been developed for use in rice improvement.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Site

The cold screening experiments were conducted at the cold screening facilities laboratory, backcross population and parental materials were raised in the net house and crossing blocks, and SSR marker analysis was carried out in the Molecular Biology Laboratory of Plant Breeding Division, BRRI, Gazipur from April 2015 to October 2016.

3.2 Geographical Location

The experimental site was situated at 23°59′21″N latitude and 90°24′23″E longitude at an altitude of 16 meter above the sea level in BRRI Head Quarter, Gazipur. It belongs to the Agro-ecological zone of "The Madhupur Tract", AEZ-28, which was a region of complex relief and soils developed over the Madhupur clay, where floodplain sediments buried the dissected edges of the Madhupur Tract leaving small hillocks of red soils as ‘islands' surrounded by floodplain. However, the soil in the BRRI farm was transformed to clay loam though land development. The experimental site was shown in the map of AEZ of Bangladesh in Figure 2.

3.3 Climate

Area has subtropical climate, characterized by high temperature, high relative humidity and heavy rainfall in Kharif season (April-September) and scanty rainfall associated with moderately low temperature during the Rabi season (October-March).

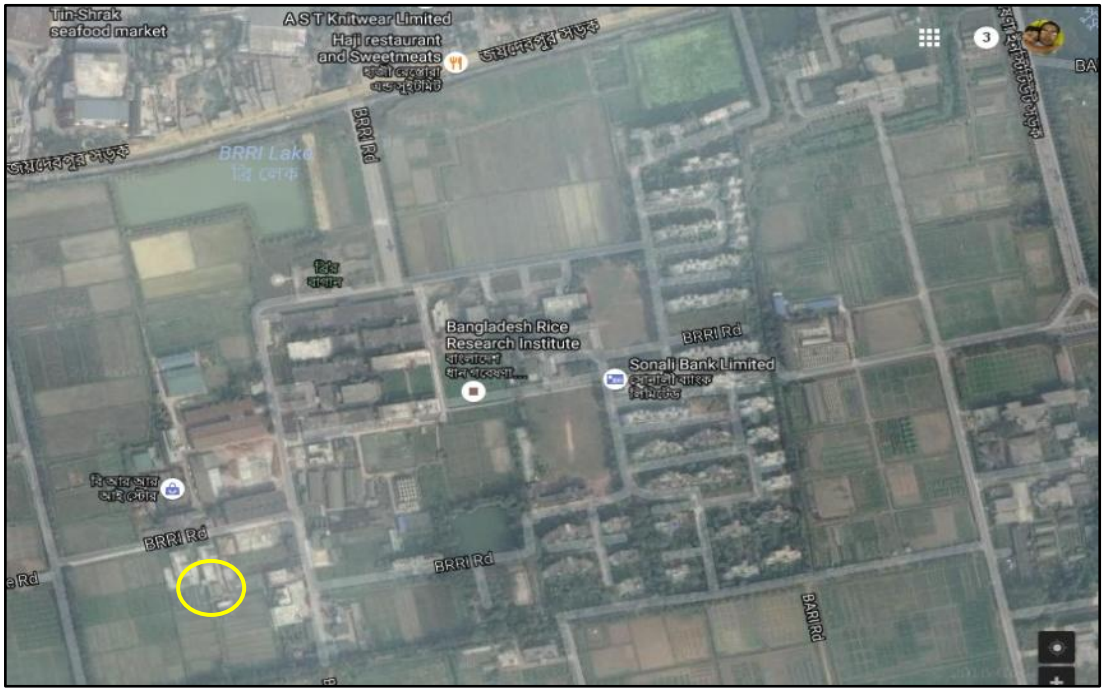

Figure 2. Google Map view of experimental site (yellow circled) in the BRRI Gazipur Campus under agro-ecological zone of "Madhupur Tract".

3.4 Characteristics of Soil

Soil of the experimental site belongs to the general soil type, Shallow Red Brown Terrace Soils. Top soils were clay loam in texture, olive-gray with common fine to medium distinct dark yellowish brown mottles. The pHand organic carbon of the experiment site were 6.5 and 1.18%, respectively. Experimental area was flat having available irrigation and drainage system and above flood level.

3.5 Experimental Materials

A total of 483 BC_1F_1 seeds and seeds of their parents, BRRI dhan28 and IR90688-20-1-1-1-1-1 were available at the Plant Breeding Division. A brief description of the parental lines is given below:

BRRI dhan28: It was a widely cultivated rice variety in Bangladesh occupying around 41 % area in Boro season. Short growth duration, long slender grain and higher milling yield are the main reason behind the popularity of this variety among the farmers. However, this variety is most susceptible to cold stress at both seedling and reproductive stage. The salient features of this variety are –

- long slender grain
- life cycle 140 days
- high yielder(6.0 t/ha)

IR90688-20-1-1-1-1-1: It is backcross introgression line derived from a cross between BRRI dhan29 and a tolerant Korean variety Jinbubyeo. This line showed strong cold tolerance at seedling stage under both artificial and natural cold temperature in Bangladesh. The salient features of this line are –

- long slender grain
- life cycle 157 -162 days
- high yielder (4.5 t/ha)

3.6 Methods

The whole study was conducted in different activities under a backcross approach involving phenotypic plant selection followed by genotyping and cold screening. A detailed description of methodology followed for different activities are described below.

3.6.1 Raising of Backcross Population and Parents

In total, 483 BC_1F_1 seeds were used in germination, of which 317 seedlings were survived and transplanted in the main field. Seeds of all the parents were sown at five staggers at an interval of 7 days. Backcross seeds were sown at the third staggers of parental seeds to synchronize flowering time with recipient parent BRRI dhan28. The seeds were pre-germinated by soaking with water in the petridishes for 8 hours. For avoiding contamination during soaking, seeds were soaked in the 0.1% solution of Provax. The treated seeds were rinsed with distilled water 4-5 times and were placed in petridish lined with wet blotting paper and covered with lid. Then the petridishes were incubated at 26^0C in an incubator for 48 hours (Plate 1a). The germinating seeds were transferred to the seeding trays filled with gravel free fertilized granular soil using forceps. After that, loose soil was spread over the seeds and sprinkled with some water. Water was applied to the trays as required.

Seedlings of backcross generation and parents were transplanted in puddle fertilized soil in the field at 18 days after seeding with a spacing of 25 X 25 cm (Plate 1b). Single seedling was transplanted at each hill. Fertilizer management was done as standard practice usually taken in Boro season. All fertilizers except urea was applied at final land preparation. Urea was applied in three equal splits at 15 days, 30 days and 45 days after transplanting. The experimental plots were kept weed free throughout the growing period of the crop. Disease and insect management was performed as and when necessary.

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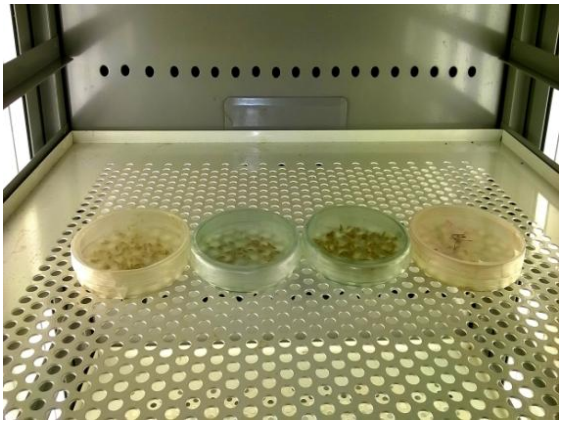

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Plate 1(a). (i) The treated seeds were placed in petridish lined with wet blotting paper and covered with lid, (ii) The petridishes were incubated at 26^0C in an incubator for 48hours, (iii) Field preparation, (iv) Field visit with supervisor.

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Plate 1 (b). (i & ii) Field preparation and Weeding, (iii) Seedlings, (iv) Transplanting of seedlings maintaining the spacing of 25cm X 25cm.

3.6.2 Phenotypic Selection Backcross Plants

Phenotypic selection of backcross plants was performed based on visual similarity of the backcross plant to the recurrent parent BRRI dhan28 in phenotype at maximum tillering stage. An arbitrary score, for example, 1 for very close phenotype, 2 for similar phenotype, and 3 for different phenotype to that of BRRI dhan28 was assigned.

3.6.3 Parental Polymorphism Survey of SSR Markers

A total of 236 simple sequence repeat (SSR) markers were analyzed between two parents. SSR analysis was performed following the protocol described below.

- **Collection of Leaf Samples**

Leaf samples were collected from young leaves 10-12 DAT. About 2 cm long leaf tips were collected from the plants and kept inside 1.5 ml microfuge/eppendorf tube. The ice buckets were carried to the transplanting field and the eppendorf tubes containing leaf samples were kept in sealed plastic bags and immediately preserved in ice. Sampling was performed in the early morning to avoid desiccation of the leaves in high temperature. Soon after bringing the samples to the laboratory, the eppendorf tubes containing leaf samples were stored in -80°C freezer for farther use. In most cases, the leaf samples were crushed(Chopping of leaf samples into small pieces, Plate 2) immediately for DNA extraction. Before collecting leaf samples, eppendorf tubes were labeled properly. It was very important to collect leaf samples from the backcross segregates keeping plat serial correctly.

- **Isolation of Genomic DNA**

Genomic DNA was extracted from the parents and the backcross progenies following modified Miniscale CTAB method as described in Virk*et al*., (1995).

Plate 2. Chopping of leaf samples into small pieces.

Approximately 20 mg leaf tissue was used for DNA extraction using modified CTAB method in 2.0 µl autoclaved eppendorf tube (Virk*et. al.*, 1995). Just before extraction mercaptoethanol was mixed with preheated (65°C) 4% CTAB extraction buffer (CTAB dissolved in 860ml sterile ddH2O+81.82 gm NaCl, 100ml 1M Tris pH 8.0 (autoclaved), and 40ml 0.5MEDTA pH8.0 (autoclaved) in 1L at rate of 10 µl per 10ml CTAB. Leaf samples were chopped into small pieces (Plate 2) and two stainless steel balls were put in each tube. The tubes containing the leaf samples were immersed in liquid nitrogen in a cryogenic tray and the frozen samples were immediately ground by shaking on a GENO grinder at 300strokes/min for 3 minute to make fine powder. 500 µl of 4% CTAB-mercaptoethanol extraction buffer was added to the leaf powder in tubes, mixed thoroughly and incubated in preheated water bath at 65°C with occasional shaking for 30min to 1 hour. The tubes were removed from water baths and allowed to cool briefly before adding 500 µl of choloroform: Isoamyle alcohol (24:1). Tubes were gently shaken at room temperature for 30 minutes and centrifuged at 12,000rpm for 10 minutes. The supernatant (aquous/top phase) was decanted into a fresh 1.5 ml eppendorf tube. 500 µl of ice-cold isopropanol (isopropyle alcohol) was added followed by gentle mixing, inverting the tubes 3-4 times and incubated at -20°C for 1 hour or overnight to precipitate DNA. The tubes were centrifuged at 13.000rpm for 10 minutes. After decanting the isopropanol, pellets were recovered. The pellets were washed with 70% ethanol and centrifuged again at 13.000rmp for 10 minutes. The ethanol was discarded by tilting the tubes gently and the pellets were air dried until no trace/smell of ethanol was detected. The pellets were then dissolved in 100 µl TE and stored at -20°C for farther use (Plate 3).

Plate 3. Equipments used in DNA Extraction: (i). Shaking Frozen Leaf Samples in Genogrinder, (ii). Shaking the 600 µl of Chloroform/isoamyl Alcohol Added Tubes in Orbital, (iii). Micro Centrifuge Tube with Dual Phase Aquas After centrifuge at 13,000 rpm for 10 Minutes, Shaker, (iv). Incubation of DNA Pellets with 1x TE buffer for 1 Hour at 37^{o}C in an Incubator, (v). Shaking water bath.

- **DNA Quantification and Dilution**

DNA samples required dilution for using them in PCR. The quality and quantity of the isolated concentrated DNA was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the concentration was determined using module Nucleic Acid, Software: 3.3.1. The NanoDrop is a cuvette free spectrophotometer (Plate 4). It uses just 2 ul DNA to measure from 5 ng/ulto 3,000 ng/ul. The ratio of the readings at 260 nm and 280 nm was used to evaluate the quality of the extracted DNA, with the ratio of 1.8 and 2.0 considered optimum for pure DNA. When the ratio is less than 1.8 the sample could be contaminated by proteins and a ratio of 2.0 or more indicates the presence of high proportion of RNA in the sample. Concentrated DNA Samples were diluted to working concentration of 25ng/µl using sterilized nanopure water before use in subsequent molecular assays. The diluted DNA samples were placed in a 96-well plate and stored at -20^0C.

- **Polymerase Chain Reaction for Simple Sequence Repeats (SSRs)**

The PCR reaction was prepared in a 96-well plate. The 10µl reaction mixture contains 50 ng of DNA template, 1 µl 10xTB buffer (containing 200mM Tris-HCl pH 8.3, 500mM KCl, 15mM $MgCl_2$), 0.2 µl of 1mM dNTP, 0.50 µl each of 5 µM forward and reverse primers and 0.2 µl of *Taq* DNA polymerase (5 U/µl). At the end, 10-15µl of mineral oil was added to each well and finally PCR plate was sealed with PCR sealing film/mat (Table 1).

- **Polyacrylamide Gel Electrophoresis (PAGE)**

Mini vertical system (C.B.S, Scientific, CA, USA) polyacrylamide gel electrophoresis (PAGE) was used to amplify the PCR products. Eight percent polyacrylamide gels (vertical) were used for better separation and visualization of PCR amplified microsatellite products. The PCR products of SSR markers having amplification size ranging from 100-350 bp were resoluted using Polyacrylamide gel electrophoresis. However, the details protocol of PAGE is given below:

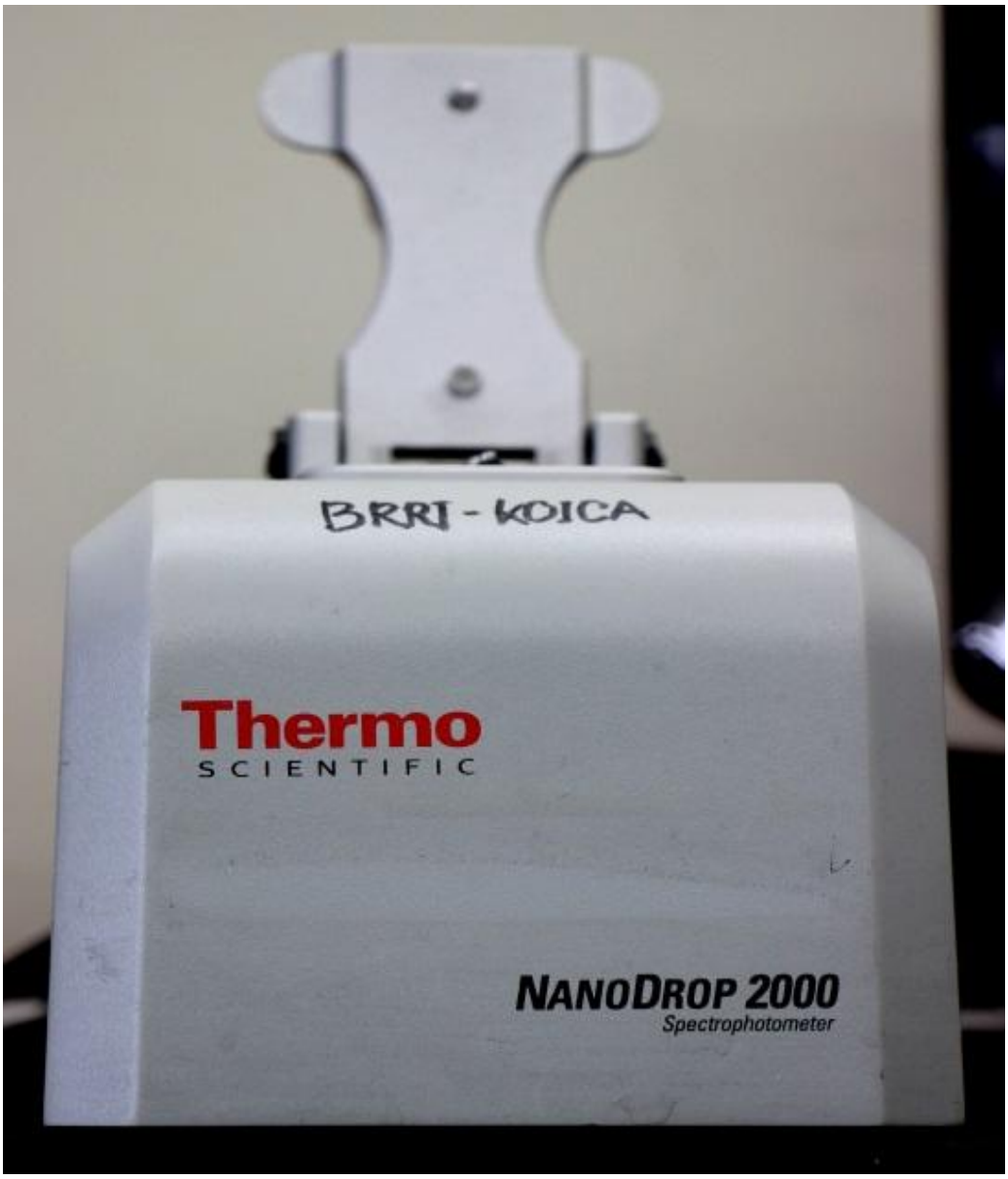


Plate 4. Nanodrop 2000 spectrophotometer.

Table 1. PCR components and thermal profile.

PCR component	**Stock concentration**	**Final concentration**	**Amount in one reaction**
ddH_2O			4.8 µl
PCR buffer	10X	1X	1 µl
dNTP mix	1mM	0.1mM	0.2µl
Primer Forward	5µM	0.25 µM	0.5 µl
Primer Reverse	5µM	0.25 µM	0.5 µl
Taq polymerase	5U	1U	0.2 µl
DNA template	25ng	5 ng	2 µl
Total volume			10 µl

PCR profile

1. 95 °C for 5 min (initial denaturation)
2. 32 cycles of the following steps
    - 95 °C for 45 sec (denaturation)
    - 55 °C for 45 sec (primer annealing)
    - 72 °C for 1 min 30 sec (primer extension)
3. 72 °C for 5 min (final extension)
4. 4 °C forever (storage)

- **Assembly of the Glass Plates**

a) Both glass plates (outer glass plate inner glass plate) were washed by hands thoroughly with high quality lab detergent then with deionized water. Glass plates were also washed by 0.5 M NaOH solution. There should not have any stickiness of previously used gel on the surface of glass. Glass plates were air dried or lint-free tissue papers were used to dry up the plates quickly. The chosen inner surfaces of the plate was sprayed with 100% ethanol and wiped with lint-free tissue.

b) The short plate (round-bottom) was hold and the rubber gasket was attached starting from one side of the plate. The notches on the gasket were aligned on the corners. The circular portion of the gasket was exposed to the inner side of the plate.

c) The short plate was lain on the table with the inner side up. Spacers of 1.5 mm thickness were placed along the inside edges of the gasket.

d) Large plate was put on small plate so that treated surfaces faced each other. It was taken care to see that spacers fit well against the edge of the plates, so that there were no gaps or leakage.

e) Clamps were put on both sides of the plates so that there was no leakage from bottom side and the plate assembly was laid flat on the table. The surface of the table was even. Plate 5 shows the assembly of glass plates with the comb inserted inside the two glass plates.

- **Preparation and Casting of the Polyacrylamide Gel**

For casting each gel, 60 ml of 8% polyacrylamide gel solution was prepared in a beaker with a magnetic stirring bar just prior to pouring (Table 2).

After adding TEMED, the solution was stirred using magnetic stirrer for few seconds on a stirrer machine at a speed to mix the chemicals properly. The speed of stirring was maintained such so that mixing was achieved but no

Table 2. Composition of gel component.

Reagents	**Final conc.**	**8% gel**
Sterile nanopure H_2O		41.35ml
10X TBE buffer	5X	6.0 ml
40% Acrylamide	8%	12 ml
10% APS	0.1%	600 µl
TEMED (N-N-N-N-Tetramethylethylenediamine)	1 µl/ml	50 µl
Total		60.0 ml

Plate 5. Assembly of glass plates with the comb inserted inside the two glass plates.

chemicals were spilt away. Immediately after stirring, the gel solution was poured into glass plate assembly. Gel solution was poured smoothly and continuously avoiding air bubbles starting from one corner until it reached top portion of the short plate. Shark tooth comb of 1.5 mm (102 wells) was inserted gently between the glass plates in the gel and the gel was allowed to polymerize for 25-30 minutes.

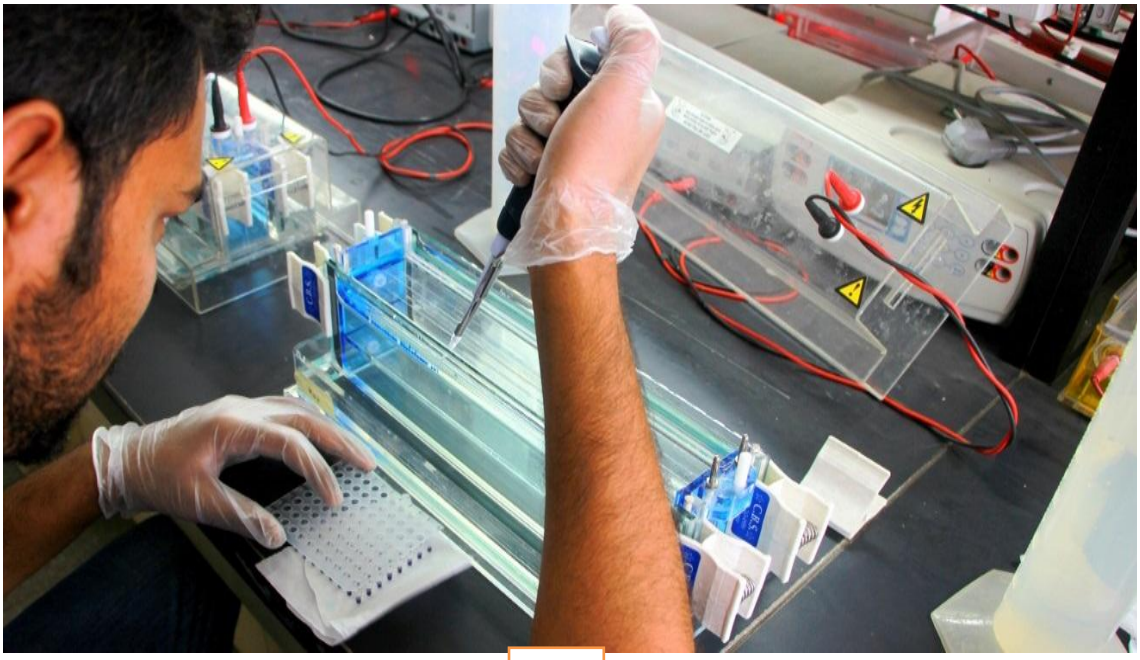
- **Setting Glass Plate in Vertical Rig**

    a) After the gel was polymerized, the rubber gasket was removed starting from one corner to another by removing the clamps. The base of the gel tank of the vertical rig was filled with 500 ml of 0.5X TBE buffer and then the casted plates were assembled in the tank with the shorter plate facing inner side. The plate was checked for presence of air bubbles at the bottom of the gel, adjusted to remove them if there was any to avoid interference with the electrical current. Around 300 ml of 0.5X TBE buffer was added to the top of the tank.

    b) Comb was removed and excess polyacrylamide gel was removed with a razor blade. Tissue paper was used to clean the glass plates with buffer.

    c) Two µl of 10X loading dye was added to the each well containing 20 µl PCR product and the plates were centrifuged at a speed of 3000 rpm for 30 sec in a high speed refrigerated centrifuge machine to mix the loading dye with PCR product. Around 2 µl of the mixer was loaded in the wells of PAGE gel with the help of 2-2.5 µl pipette. DNA size marker like 1 Kb^{+} DNA ladder was loaded in the first, middle and last wells for size determination.

    d) After loading, the cover of the tank was put and the electrodes were connected to the power supply and the gel was run for about 2.0-2.5 hours at 100 volts (Plate 6). It was noted that the running time varies depending on the size of PCR fragments.



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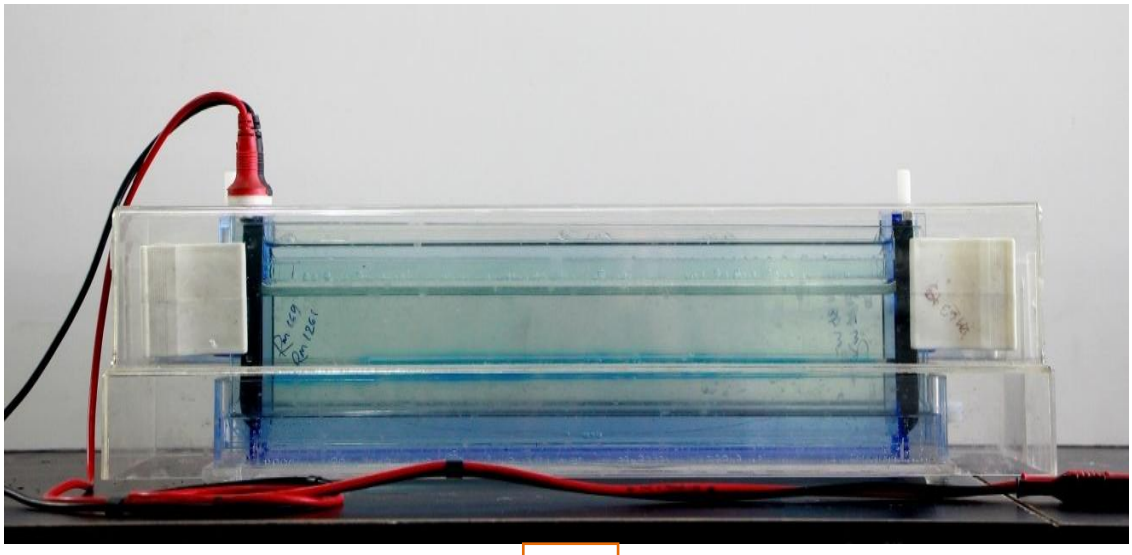

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Plate 6. (i & ii)Loading of PCR products into gel plate and Running polyacrylamide gel in vertical rig (CBS Scientific Co. Inc., CA, USA).

- **Staining and Visualization of the Gel**

    a. The power supply unit was turned off and the plates were removed from the tank. The glass plates were separated using a knife without damaging the gel. Ethidium bromide staining solution was carefully prepared by adding 40 µlethidium bromide in 500 ml of nanopure water. The acrylamide gel was removed carefully and transferred in the ethidium bromide staining solution (0.5 mg/ml) for around 25 minutes. All safety precautions were followed in handling ethidium bromide.

    b. The stained gels were put in the exposure cabinet of the gel documentation system. The gels were scanned under UV light with BIO-RAD scanner andsaved as a Jpeg file (Plate 7).

- **Determination Polymorphism**

Polymorphism of the SSR markers between the parents was determined through the difference in band position on a gel image of a specific SSR comparing the band position of known DNA ladder.

3.6.4 Genotyping of Backcross Progenies

A set of 47 polymorphic SSRs identified from the parental survey were used in genotyping of the selected backcross plants. For genotyping, DNA extraction and SSR analysis were performed following the protocol described in previous section (Sec. 3.3.3).

Allele Scoring

The size (in nucleotide base pairs) of the most intensely amplified band for each microsatellite marker was determined based on the migration, relative to a molecular-weight size marker (1 Kb DNA Ladder). The band having same level of BRRI dhan28 was scored as ‘A’ which indicated the homozygous allele of the recipient parent for the particular SSR marker. Again, the band having same level of IR90688-20-1-1-1-1-1 was scored as ‘B’ which indicated

Plate 7. Molecular Imager Gel Doc XR System, BIO-RAD, Korea.

the homozygous allele of the donor parent for particular SSR marker. However, heterozygous alleles were scored as 'H' having both the bands of two parents. Importantly, heterozygous allele always had the extra band in most of the cases with bigger size (Figure 3).

3.6.5 Making Backcrosses and Seed Production

The selected backcross plants were emasculated at the day before anthesis. The upper one-third of the flowering panicles of the backcross plants were clipped off with sharp scissors and anthers were removed using a vacuum emasculator. Then the emasculated panicles were bagged with glycine bags (Plate 8). Two panicles of each plant were kept un-emasculated to produce self-seeds. Pollen from BRRI dhan28 was dusted over the emasculated panicles on the following morning and bagged again to avoid contamination with foreign pollen (Plate 9). The mature naked backcross seeds were collected at 21 days after pollination. The seeds with main axis of the panicle were collected in glycine bags and were dried in the sun for 3-4 days. The seeds were then carefully removed from the spikelets and kept in the small brown paper envelops. The parentage, date of harvesting and number of seeds were written on the top of the small brown envelops. After that, the seeds were dried in the oven at 50°C temperature for 72 hours for breaking dormancy to use immediately to raise further backcross population.

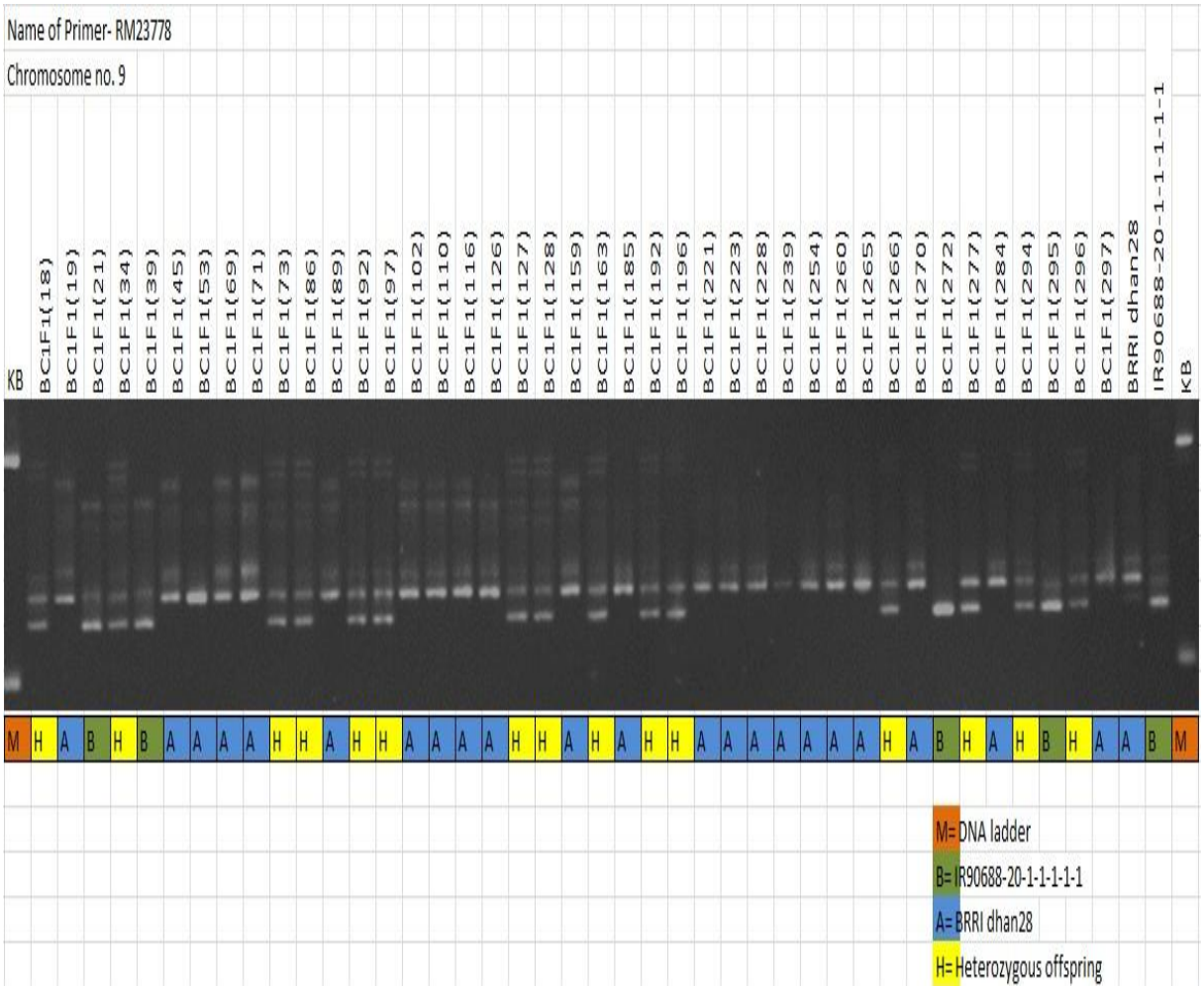

Figure 3. Allele scoring.

Plate 8. The first one showing the emasculated plant and another is panicle of BRRI dhan28.

Plate 9. Dusting of pollen into emasculated panicle.

3.6.6 Cold Screening of Backcross Derivatives

BC_1F_2 progenies of 41 plants including 9 selected plants through genotyping were evaluated against 13^0C cold stress in cold water tank at seedling stage. Seeds were grown in cold screening tray along with BRRI dhan28 as susceptible check and Hbj.BVI as tolerant check. The seedlings were allowed to grow at ambient temperature. At 3-leaf stage, the plastic trays were placed in cold water tank (Plate 10, 11). The depth of water in the tank was maintained at 5 cm. Temperature of the cold water tank was kept constant at 13°C for 6 days or until the susceptible check variety died. An imperial leaf discoloration (LD) scores (1 to 9) (Plate 12) was assigned following standard evaluation system (SES) of IRRI as described in Biswas *et al.* (2012); to each entry considering magnitude of discoloration of leaf due to cold temperature treatment.

3.6.7 Data Analysis

The marker data was analyzed using computer software called Graphical Genotyper (GGT 2.0) developed by Dr. Ralph van Berloo, Laboratory of Plant Breeding, Wageningen Agricultural University (Van Berloo R, 2008). This software was freely available from: http://www.dpw.wau.nl/PV/. The software provided the high quality graphical illustration at the percentage of recurrent parent chromosomal segments in the selected segregates of the backcross population.

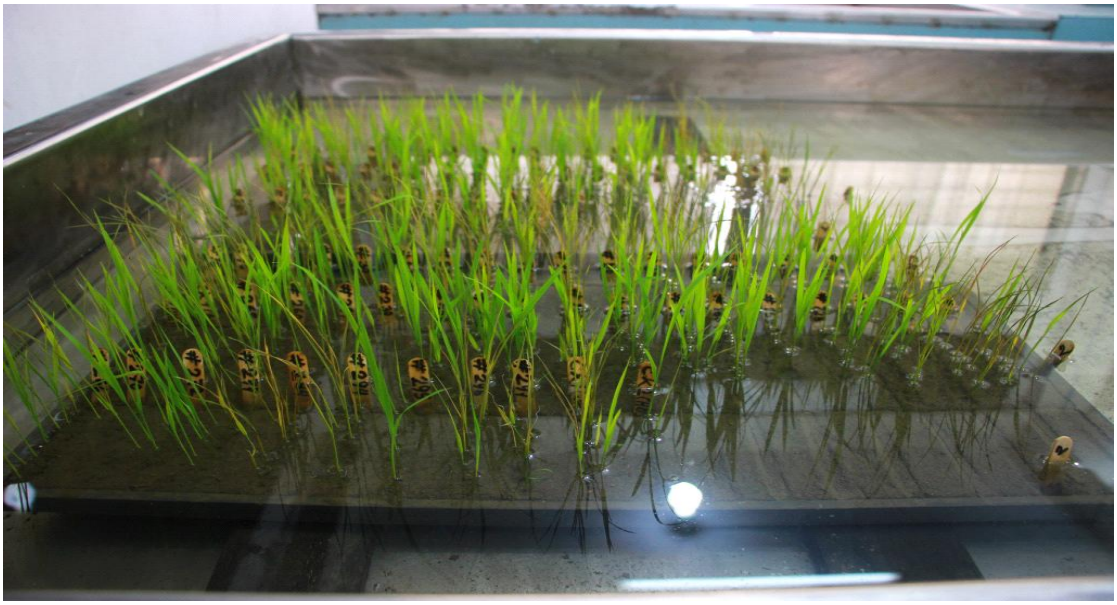

Plate 10. Preparation seedlings in seedling trays for cold screening.

Plate 11. Screening of backcross progenies of BRRI dhan28XHbj.BVI in cold water tank at 10.6^{0}C temperature.

Plate 12. Scale of Leaf Discoloration Scoring (1-9).

3.6.8 Selection Strategies

Phenotypic selection of backcross plants was performed based on the similarity of plant in phenotype with the recurrent parent BRRI dhan28 at maximum tillering stage. The plants having dissimilar phenotype (score 3) were discarded and the remaining plants were considered to select backcross progenies for background selection. The plants selected through background genotyping were subjected to cold stress. Finally, the plants having cold tolerance, maximum background recovery and very similar to BRRI dhan28 in phenotype were selected to proceed for next cycle of backcrossing. A schematic flow chart of the backcross approach followed for this study is given in Figure 4.

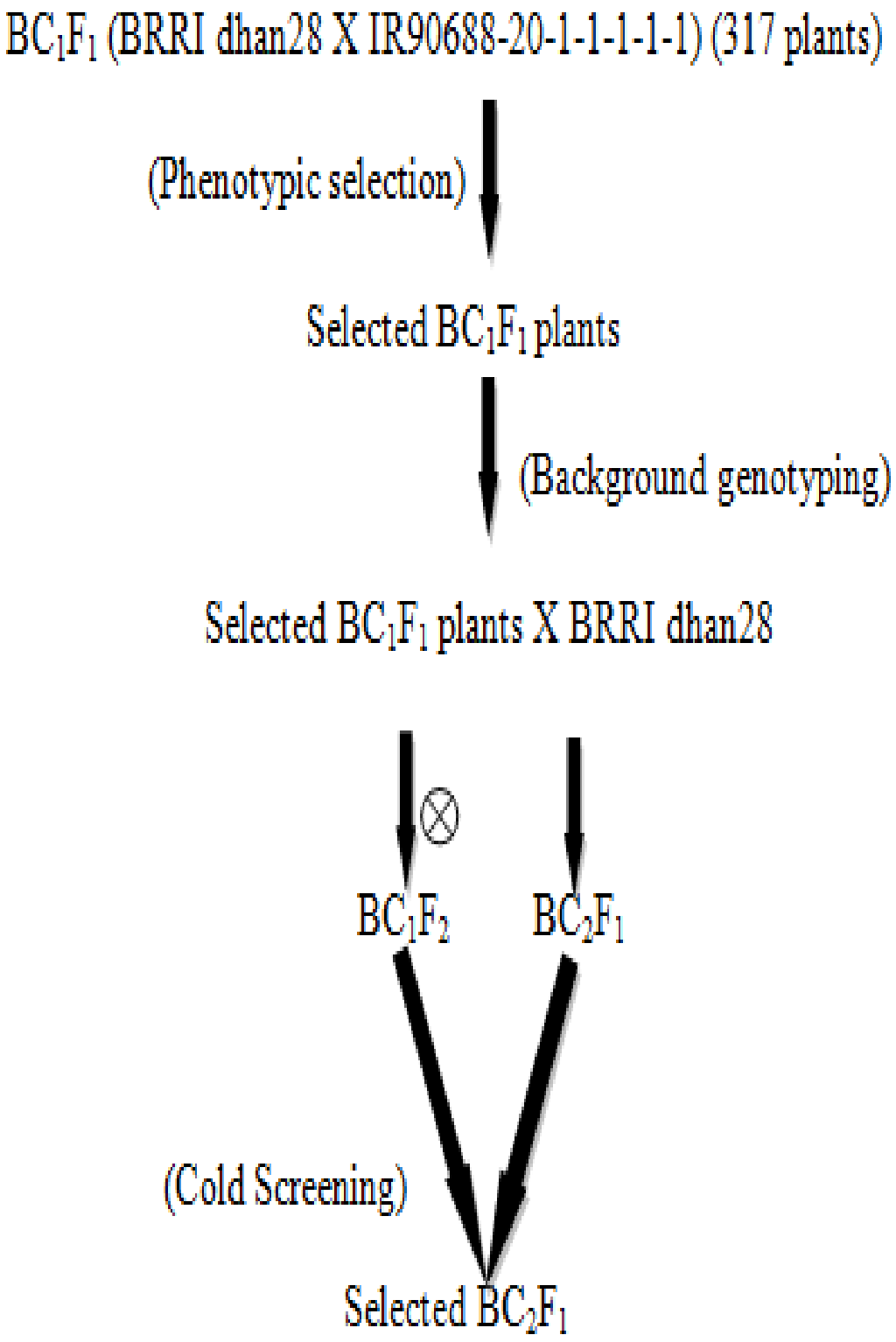

Figure 4. Schematic flow chart of the backcross approach.

CHAPTER IV

RESULTS AND DISCUSSION

Introgression of cold tolerance for seedling stage from IR90688-20-1-1-1-1-1 into cold susceptible BRRI dhan28 was performed through marker-assisted backcrossing approach. Results of different experiments conducted under this approach are described below.

4.1 Parental Polymorphism Survey and Marker Selection for Background Genotyping

A total of 236 SSRs were analyzed over the genome of BRRI dhan28 and IR90688-20-1-1-1-1-1 to find out polymorphic markers (Table 3 and Appendix I). Markers with distinct polymorphism were used for background genotyping of backcross progenies.

4.1.1 Parental Polymorphism Survey

A total of 236 SSRs were analyzed of them 103 markers were found as polymorphic which accounts for 44.55%, while 72 markers were monomorphic and 61 markers did not amplify at all (Figure 5 & 6). The possible reasons of non-amplification of those primers might be due to presence of null alleles between parents or may be due to experimental errors or problematic chemicals used in genotyping work.

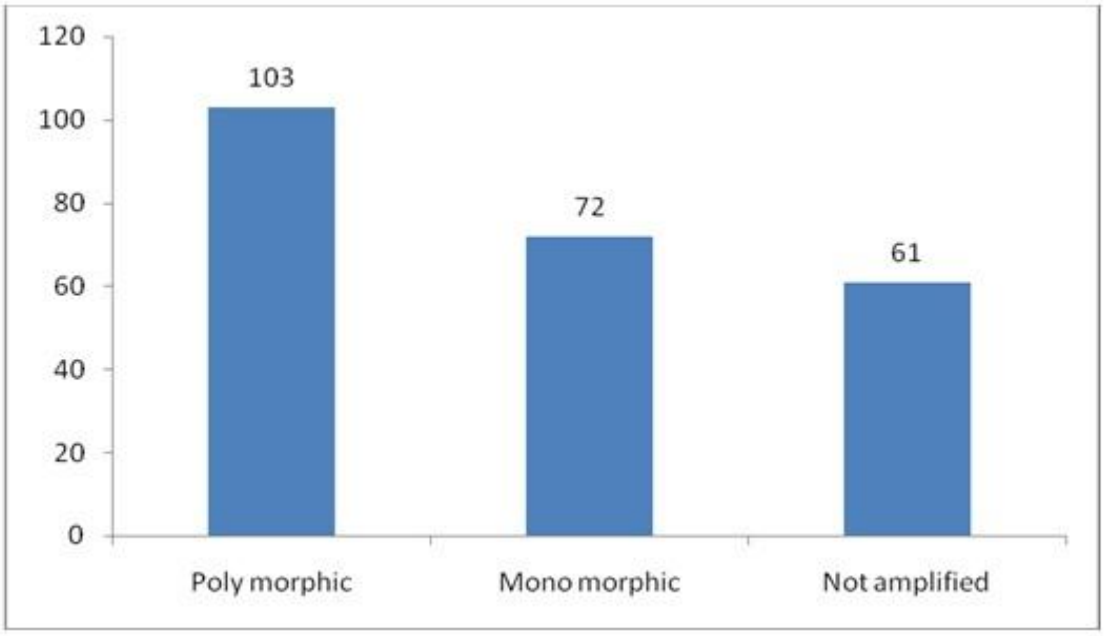

Figure 5. Distribution of SSR markers in different polymorphic classes.

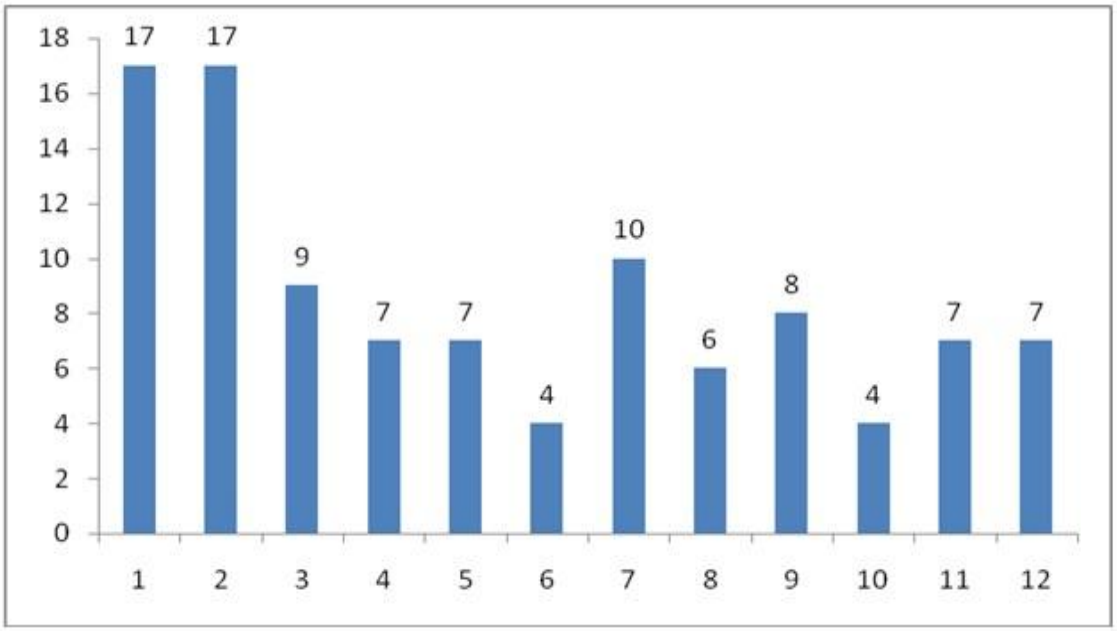

Figure 6. Chromosome-wise distribution of polymorphic SSR markers between BRRI dhan28 and IR90688-20-1-1-1-1-1.

Table 3. Summary results of parental polymorphism survey using 236 SSRs over two parents BRRI dhan28 and IR90688-20-1-1-1-1-1.

Chromosomes	Poly morphic	Mono morphic	Non-amplification	Total number	% Polymorphism
1	17	15	9	41	41.46
2	17	6	5	28	60.71
3	9	2	5	16	56.25
4	7	4	6	17	41.18
5	7	3	1	11	63.64
6	4	6	5	15	26.67
7	10	11	2	23	43.48
8	6	6	5	17	35.29
9	8	2	7	17	47.06
10	4	3	3	10	40.00
11	7	13	8	28	25.00
12	7	1	5	13	53.85
Total	103	72	61	236	Ave = 44.55

Table 3 shows the summary results of chromosome-wise SSR analysis and Figure 5 shows distribution of polymorphic SSR marker in 12 chromosomes of parental lines. In chromosome 1**,** a total of 41 primers were surveyed of which 17 primers were found as polymorphic, 15 primers were found as monomorphic, and 9 primers did not give any amplification. The marker polymorphism level of chromosome 1 was 41.46%. In chromosome 2**,** the second maximum percentage of polymorphism was obtained (60.71%). Out of 28 primers surveyed for chromosome, 17 primers were polymorphic, 6 primers were monomorphic, and 5 primers did not amplify. In chromosome 3, total 16 primers were surveyed. Out of them, 9 primers were found as polymorphic, 2 primers were found as monomorphic, and 5 primers did not give any amplification. The marker polymorphism level of chromosome 3 was 56.25%. In chromosome 4 the percentage of polymorphism was obtained 41.18%. A total of 17 primers were surveyed from chromosome 4. Out of them, only 7 primers were found as polymorphic, 4 primers were found as monomorphic, and 6 primers did not give any amplification. A total of 11 primers were surveyed from chromosome 5. Out of them, 7 primers were found as polymorphic, 3 primers were found as monomorphic, and 1 primers did not give any amplification. The polymorphism level of chromosome 5 was 63.64% showing the highest polymorphism among all the chromosomes. In chromosome 6, a total of 15 primers were surveyed for finding out suitable polymorphic markers. Out of them, 4 primers were found as polymorphic, 6 primers were found as monomorphic, and 5 primers did not give any amplification. The polymorphism level of chromosome 6 was 26.67%. : The second lowest percentage of polymorphism was obtained in this chromosome. In chromosome 7, a total of 23 primers were surveyed for finding out polymorphic markers. Out of them, 7 primers were found as polymorphic, 11 primers were found as monomorphic and 2 primers did not give any amplification. The polymorphism level of chromosome 7 was 43.48%. Chromosome 8 showed third lowest polymorphism among all the chromosomes. A total of 17 primers were surveyed from chromosome 8. Out of

them, 6 primers were found as polymorphic, 6 primers were found as monomorphic, and 5 primers did not give any amplification. The polymorphism level of chromosome 8 was 35.29%. A total of 17 primers were surveyed from chromosome 9. Out of them, 8 primers were found as polymorphic, 2 primers were found as monomorphic, and 7 primers did not give any amplification. The polymorphism level of chromosome 9 was 47.06%. Importantly, chromosome 9 showed third highest polymorphism among all the chromosomes. In chromosome 10, a total of 10 primers were surveyed for finding out suitable polymorphic markers. Out of them, 4 primers were found as polymorphic, 3 primers were found as monomorphic, and 3 primers did not give any amplification. The polymorphism level of chromosome 10 was 40.00%. The polymorphism level of chromosome 11 was 25.00% which is the lowest percentage among all of chromosomes. A total of 28 primers were surveyed from chromosome 11. Out of them, 7 primers were found as polymorphic, 13 primers were found as monomorphic, and 8 primers did not give any amplification. In chromosome 12, a total of 13 primers were surveyed. Out of them, 7 primers were found as polymorphic, 1 primers were found as monomorphic, and 5 primers did not give any amplification. The polymorphism level of chromosome 8 was 53.85%.

4.1.2 Selection of Marker for Background Genotyping

A total of 47 polymorphic SSR markers distributed over 12 chromosomes were used for background selection (Appendix II). Maximum number of markers (7) was on chromosome 2 and minimum number (1) on chromosome 11 with an average number of 4 markers per chromosome. Success of MAB largely depends on the number of the markers per chromosome and population size in each backcross generation. Since, the number of crossover is roughly proportional to the length of chromosome, more than 5 or 6 crossover per chromosome per meiosis are extremely rear (Kearsey and Pooni, 1996) which implies five or six markers are good enough for short length chromosomes of rice like chromosome 10, 12 and 8, while 8-10 background markers are needed

for long length chromosomes like 1 and 3 to achieve recurrent parental recovery in background selection. Although the background markers of this study were stretched at an average distance of 18.8 cM over the genome, but a significant proportion of chromosomes (chr. 3, 6, 10, 11 and 12) lacked the requirement of minimal number of markers for recovery of parental genome due to monomorphism and absence of adequate polymorphism between the parental lines(Figure 7). This might be due to close similarity in their genome. It should be noted here that IR90688-20-1-1-1-1-1 is a near isogenic lines developed from a cross between BRRI dhan29 and a Korean Cold tolerant variety Jinbubyeo. Marker diversity showed that BRRI dhan28 and BRRI dhan29 share more than 75% common alleles (BRRI, 2013). However, Frisch *et al.* (2001); suggested that a set of 100 markers evenly spaced with an average marker density of 20-30 cM is good enough to guarantee a good coverage of the genome to recover recurrent parental genome in a marker assisted selection scheme.

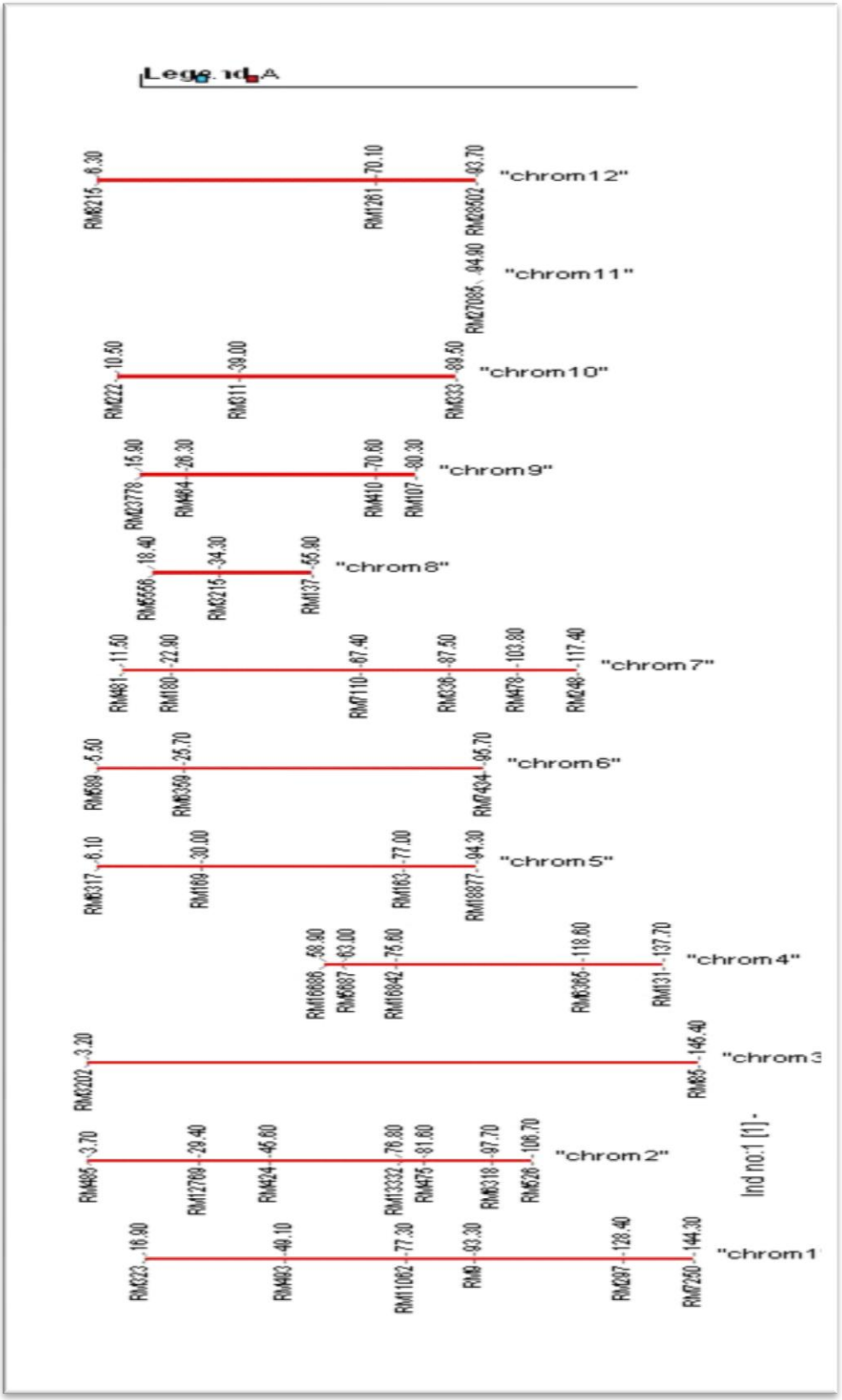

Figure 7.Graphical genotype of 47 polymorphic SSR markers over 12 chromosomes of BRRI dhan28

4.2 Phenotypic Selection and Background Genotyping of BC_1F_1 Progenies

A total 317 plants were survived in the main field after transplanting out 483 seeds used in this study. Phenotypic selection of plants was performed during maximum tillering stage based on phenotypes resemblance of the backcross derived plant with the recipient parent BRRI dhan28. Background selection was performed with the plants that were very close to BRRI dhan28 in phenotype.

4.2.1 Phenotypic Selection of BC_1F_1 Progenies

A total of 74 plants (Table 4) out of 317 were found to have phenotypic score 1 (very close to BRRI dhan28 in phenotype), 85 plants were scored as 2 (similar to BRRI dhan28), and 158 plants were scored as dissimilar to BRRI dhan28 (score 3).Although the selected plants were very close to BRRI dhan28 in phenotype during maximum tillering stage, many of them showed delayed heading and dissimilar grain type than BRRI dhan28. Therefore, finally 41 plants having almost closer heading dates and similar grain type (LS grain) to BRRI dhan28 were selected for background genotyping.

4.2.2 Background Genotyping of BC_1F_1 Progenies

Background genotyping was performed using 47 SSR markers selected from previous experiment described in section 4.1.2.The selected 41 plants showed wide range of recovery of recurrent parental genome (RPG) starting from 21% to 72% with an average of 46.8% (Appendix III). This finding supported the opinion of Collard *et al.*, (2005) who reported that although average percentage of recurrent parental genome is 75% for entire BC_1F_1 population, but there are individuals that possess more recurrent parent genome than others. Based on RPG value of 60% or above, a total of nine plants were selected for further backcrossing with BBRI dhan28 to make BC_2F_1 seeds. Table 5 shows the summary results of background genotyping of the selected nine plants. Plant number BC_1-265 showed the highest 72% recovery of RPG followed by plant number BC_1-239 (70%), BC_1-196 (68%) and BC_1-260 (68%). The remaining

seven BC_1-9, BC_1-69, BC_1-223, BC_1-266 and BC_1-297 showed bit higher RPG than 60% (Table 5).

BC_1-19

Plant number BC_1-19 showed wide range of recurrent parental allele ranging from 100% in chromosome 6 and 8 to 50% in chromosome 3, 11 and 12. A total of eight chromosomes of this plant had R allele at more than 75% loci (Table 6).

BC_1-69

Plant number BC_1-69 had full recovery (100%) of R (BRRI dhan28) allele in chromosome 1, 6, 9, 10 and 11. While, only 57% loci in chromosome 2 had R alleles at either homozygous or heterozygous state. In the chromosome 3 and 7, the occurrence of R alleles was 75% and 83%, respectively (Table 7).

BC_1-196

Plant number BC_1-196 showed 100% recovery of R alleles in chromosome 3, 4, 6 and 11. Minimum (63%) recovery of R allele was with chromosome 9. The other chromosomes had a range of R alleles starting from 67% to 88% (Table 8).

BC_1-223

Plant number BC_1-223 showed 100% recovery in R alleles in chromosome 2. Recovery of % R allele in the chromosome 1 was 92% and chromosome number 3, 4, 6, 7, 9 and 12 were partially recovered at 75% SSR loci or higher up to 90%. The remaining three chromosomes (11, 10 and 5) had the lowest recovery in R allele (0 – 67%) (Table 9). The null recovery in chromosome 11 was due to occurrence of homozygous donor alleles resulting from accidental selfing during backcrossing.

Table 4. Number of BC_1F_1 plants in different phenotypic similarity/dissimilarity classes at maximum tillering stage.

Phenotypic score	No. of plants
1	74
2	85
3	158
Total=	317

1= Very close to BRRI dhan28, 2= Similar to BRRI dhan28, 3=Dissimilar to BRRI dhan28

Table 5. Summary results of background genotyping of the selected nine BC_1F_1 progenies.

Item	Plant ID								
	19	69	196	223	239	260	265	266	297
No. of A	28	28	32	29	33	32	34	30	30
No. of B	0	0	1	1	2	1	1	0	1
No. of H	18	18	12	15	7	13	12	16	14
No. of MDP	1	1	2	2	5	1	0	1	2
% RPG	60	60	68	62	70	68	72	64	64

A=homozygous to recipient allele, B=homozygous to donor allele, H=heterozygous allele, MDP=missing data point & RPG=recipient parental genome

Table 6. Percentage of recipient alleles in different chromosomes in the plant BC_1-19.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	5	4	0	3	2	3	4	3	2	1	0	1
No. of H	1	3	2	2	2	0	2	0	2	2	1	1
% R allele	92	79	50	80	75	100	83	100	75	67	50	50

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage.

Table 7. Percentage of recipient alleles in different chromosomes in the plant BC_1-69.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	6	1	1	1	1	3	4	1	4	3	1	2
No. of H	0	6	1	4	3	0	2	2	0	0	0	0
% R allele	100	57	75	60	63	100	83	67	100	100	100	67

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage

Table 8. Percentage of recipient alleles in different chromosomes in the plant BC_1-196.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	4	4	2	5	3	3	4	2	2	2	1	2
No. of H	1	3	0	0	1	0	2	1	1	0	0	1
% R allele	75	79	100	100	88	100	83	83	63	67	100	83

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage

Table 9. Percentage of recipient alleles in different chromosomes in the plant BC_1-223.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	5	7	1	4	0	2	4	0	2	2	0	2
No. of H	1	0	1	1	3	1	2	3	2	0	0	1
% R allele	92	100	75	90	38	83	83	50	75	67	0	83

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & % R=recipient allele percentage

BC_1-239

Plant number BC_1-239 showed 100% recovery in R alleles in chromosome 5, 6, 10 and 11. Chromosome number 1, 2 and 7 were partially recovered at 75% SSR loci or higher up to 86%. The other chromosomes had a range of R alleles starting from 50% to 70% (Table 10, Figure 8).

BC_1-260

Plant number BC_1-260 showed 100% recovery of R alleles in chromosome 3, 5, 9 and 11, while chromosomes 1, 2, 4, 6 and 7 had R alleles in at least 75% SSR loci. The remaining chromosomes were partially recovered at 33 % to 50% loci (Table 11).

BC_1-265

Plant number BC_1-265 showed 100% recovery of R alleles in chromosome 1, 7, 9 and 11. Chromosome 2, 3, 4, 5, 6 and 12 were partially recovered at 75% SSR loci or higher up to 90%. The remaining two chromosomes were partially recovered at 50% loci (Table 12, Figure 9).

BC_1-266

Plant number BC_1-266 showed 100% recovery of R alleles in chromosome 3, 4, 5, 6 and 11. The lowest recovery of % R allele was observed in the chromosome 12. Other chromosomes (1, 2, 7, 8, 9 and 10) were partially recovered at 63% - 86% SSR loci (Table 13).

Table 10. Percentage of recipient alleles in different chromosomes in the best plant BC_1-239.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	4	6	0	3	4	3	5	1	2	3	1	1
No. of H	1	0	2	1	0	0	0	2	0	0	0	1
% R allele	75	86	50	70	100	100	83	67	50	100	100	50

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage

Table 11. Percentage of recipient alleles in different chromosomes in the plant BC_1-260.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	4	4	2	4	4	2	3	0	4	1	1	1
No. of H	2	3	0	1	0	1	3	3	0	2	0	0
% R allele	83	79	100	90	100	83	75	50	100	67	100	33

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage.

Table 12. Percentage of recipient alleles in different chromosomes in the best plant BC_1-265.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	6	5	1	4	3	2	6	0	4	0	1	2
No. of H	0	2	1	1	0	1	0	3	0	3	0	1
% R allele	100	86	75	90	75	83	100	50	100	50	100	83

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage.

Table 13. Percentage of recipient alleles in different chromosomes in the plant BC_1-266.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	2	5	2	5	4	3	3	2	1	2	1	0
No. of H	4	2	0	0	0	0	3	1	3	1	0	2
% R allele	67	86	100	100	100	100	75	83	63	83	100	33

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage

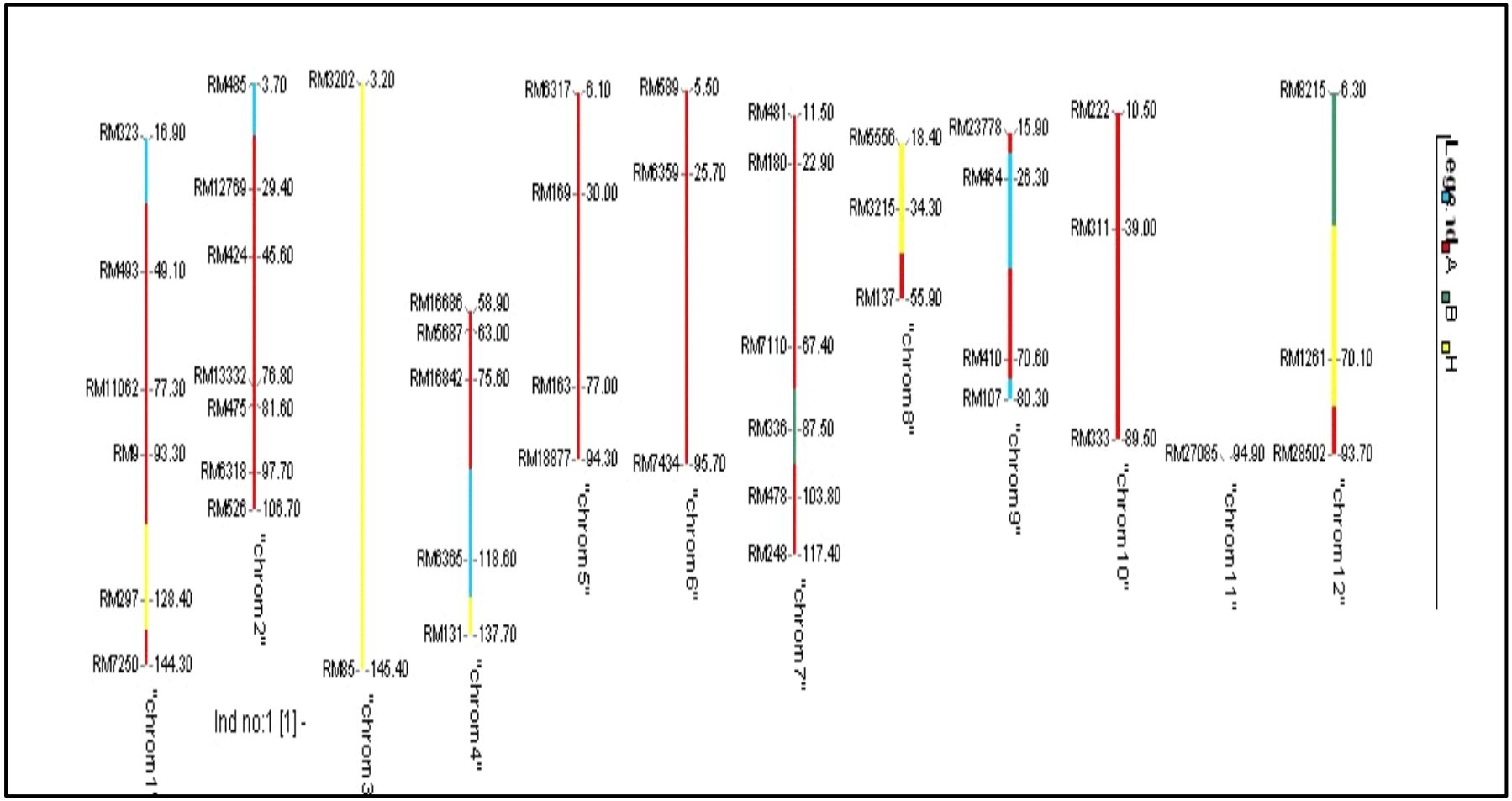

Figure 8. Graphical genotypes of plant number BC_1-239 showing recovery of homozygous recipient alleles (green color), heterozygous segments (yellow) and segments of unknown alleles (purple).

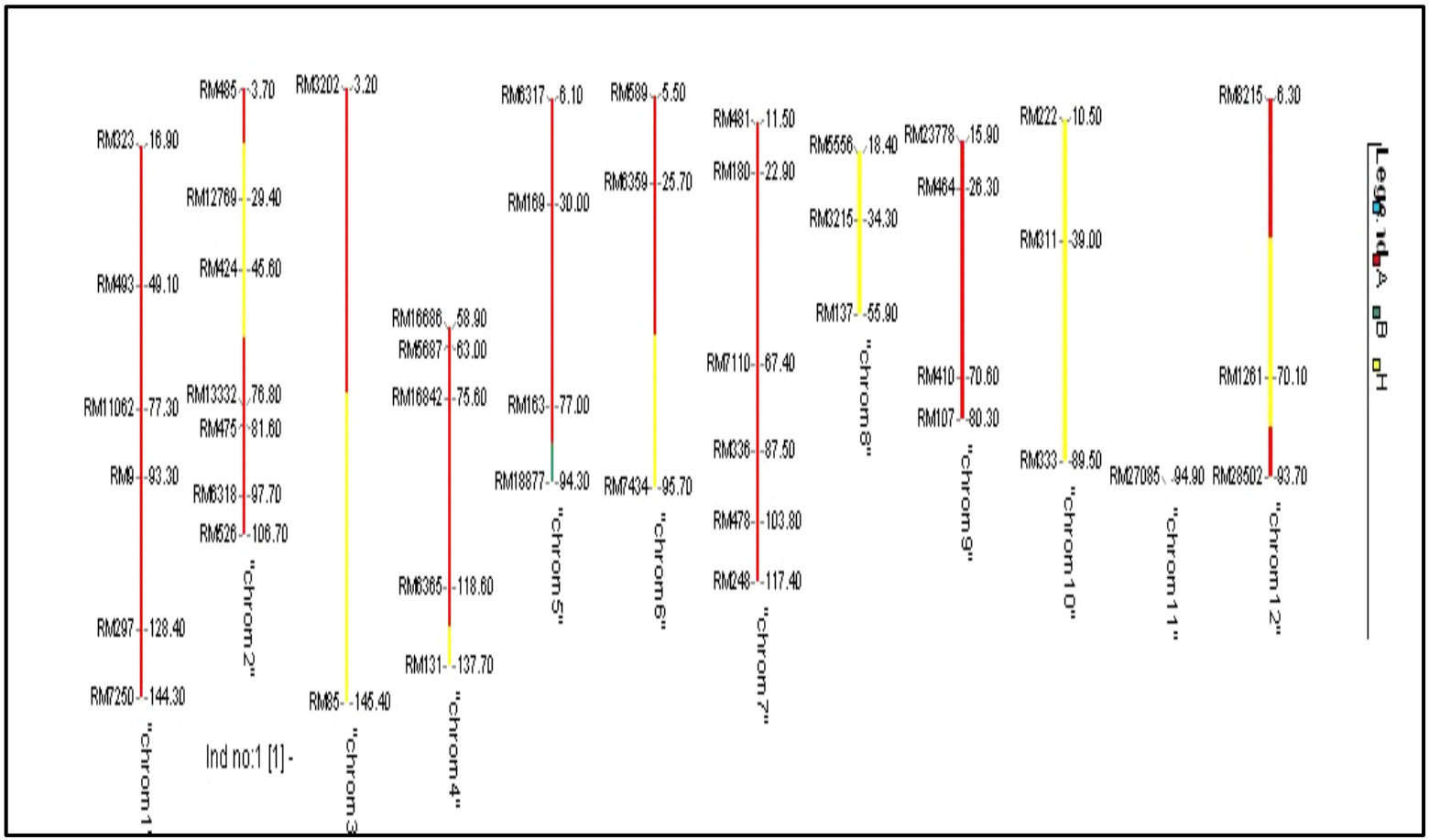

Figure 9. Graphical genotypes of plant number BC_1-265 showing recovery of homozygous recipient alleles (green colour), homozygous donor allele (red), heterozygous segments (yellow) and segments of unknown alleles (blue).

BC_1-297

Plant number BC_1-297 showed 100% recovery of R alleles in chromosome 3, 8 and 11. The chromosome 1, 2,7 and 9 had R alleles in 92%, 86%, 92% and 88% SSR loci, respectively. The remaining chromosomes had 50% or less number of loci with R alleles (Table 14).

Plant number BC_1-196 and BC_1-265 showed that a total of 10 chromosomes had 75% to 100% recovery of R alleles. 9 chromosomes had recovered (75%-100%)R allele in plant number BC_1-260 and BC_1-266 where 8 chromosomes in plant number BC_1-19, BC_1-223 and BC_1-297. The remaining two selected plant BC_1-69 and BC_1-239 had recovered 7 chromosomes (Table 15).

All the 9 BC_1F_1 plants were used in backcrossing to produce BC_2F_1 seeds. In addition, BC_1F_2 seeds were produced allowing self-pollination of two panicles of each plant (Table 16).

Table 14. Percentage of recipient alleles in different chromosomes in the plant BC_1-297.

Item	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of A	5	5	2	1	1	2	5	3	3	1	1	1
No. of H	1	2	0	4	1	1	1	0	1	2	0	1
% R allele	92	86	100	60	38	83	92	100	88	67	100	50

A=homozygous to donor allele, B=homozygous to recipient allele, H=heterozygous allele & %R=recipient allele percentage

Table 15. No of chromosome with recovery of R alleles starting from 75% to 100%.

Plant ID	No. of chromosome with R allele of 100%	No. of chromosome with R allele≥75% to 99%	Total
BC_1-19	2	6	8
BC_1-69	5	2	7
BC1-196	4	6	10
BC_1-223	1	7	8
BC_1-239	4	3	7
BC_1-260	4	5	9
BC_1-265	4	6	10
BC_1-266	5	4	9
BC_1-297	3	5	8

Table 16. No of BC_2F_1 and BC_1F_2 seeds/selected plant.

Plant ID	BC_2F_1 seeds	BC_1F_2 seeds
BC_1-19	20	85
BC_1-69	130	64
BC_1-196	102	22
BC_1-223	90	25
BC_1-239	208	14
BC_1-260	68	88
BC_1-265	152	29
BC_1-266	80	24
BC_1-297	64	55
Total=	**914**	**406**

4.3 Cold Screening of BC_1F_2 Progenies

Analysis of variance showed that the BC_1F_2 progenies of 41 plants including the selected nine plants described in section 4.2.2 varied significantly in leaf discoloration (LD) scores obtained from cold screening at 13^0C cold water treatment (Table 17).The LD values of the BC_1F_2 plants ranged from 3-9, while the susceptible check variety BRRI dhan28 obtained LD value of 8 and the tolerant check variety obtained LD value of 4 (Table 18, Appendix IV).

The average LD values of the BC_1F_2 progenies of selected nine plants are given in Table 18. The results showed that progenies of BC_1-223 showed the lowest LD value (3) indicating tolerance to cold stress at seedling stage, while progenies of BC_1-19, BC_1-297, BC_1-69 and BC_1-260 had LD values of 6 to 7 suggesting them to be susceptible to cold stress. However, the progenies of BC_1-196, BC_1-239, BC_1-265 and BC_1-266 scored LD values of 4 – 5 reflecting moderately tolerant reaction to cold stress.

Table 17. Analysis of variances and descriptive statistics of leaf discoloration score of BC_1F_2 progenies

Source of variation	DF	MS	F	P
Replication	1	3.2		
Genotypes	40	3.02	2.72	0.001
Error	40	1.11		
Range of LD values			3 - 9	
CV of LD values			17.6	
Av. LD value of BRRI dhan28 (Sus. Check)			8	
Av. LD value of Hbj.BVI (Tol. Check)			4	

Table 18. Recovery of RPG using 47 SSR markers, phenotypic resemblance and LD score of backcross derivatives of 9 BC_1F_1 plants.

Plant ID	LD score
BC_1-19	7
BC_1-69	6
BC_1-196	5
BC_1-223	3
BC_1-239	4
BC_1-260	6
BC_1-265	5
BC_1-266	5
BC_1-297	7
BRRI dhan28 (Sus. Ck)	8
Hbj.BVI (Tol. Ck)	4

Considering cold tolerance (LD ≤5) reaction, recovery of RPG (≥70%) and maximum numbers of chromosomes (≥4) with 100% R alleles over the genome (Table 18, 5 and 15) it was evident that the BC_2F_1 progenies of BC_1-239 and BC_1-265 were the best ones to initiate further backcrossing program to develop near isogenic lines in background of BRRI dhan28.

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CHAPTER V

SUMMARY AND CONCLUSION

Cold injury at seedling stage is one of the major abiotic stresses for rice production in the cold prone northern districts in Boro season causing some years up to 90 percent seedling mortality. This increases cost of cultivation. Farmers are compelled to procure green and fresh seedlings from other places for replanting. Also low temperature at the vegetative stage increases growth period, decrease tiller numbers and thereby reduces biomass production, which is translated into low yield of rice. On the other hand, farmers sometimes adjust planting time with the increment of ambient temperature by delaying Boro rice production that brings about the risk of low yield due to exposure of reproductive stage into high temperature. BRRI dhan28, a widely cultivated Boro rice variety, lacks cold tolerance. Thus this study was undertaken to introgress cold tolerance from IR90688-20-1-1-1-1-1 which showed strong cold tolerance under Bangladesh condition into BRRI dhan28.

A total of 236 SSR markers were used to identify polymorphic markers between BRRI dhan28 and IR90688-20-1-1-1-1-1. Of them, only 44.55% markers showed polymorphism. A set of 47 polymorphic markers distributed over 12 chromosomes and showing distinct difference in band position were used in background genotyping of BC_1F_1 backcross progenies that were very close to the recipient parent BRRI dhan28 in phenotype. Among the selected markers, maximum 7 SSRs were on chromosome 2 and only one SSR was on chromosome 11. Success of a MAB program largely depends on the number of the markers per chromosome and population size in each backcross generation. Five or six markers are good enough for short length chromosomes of rice like chromosome 10, 12 and 8, while 8-10 background markers are needed for long length chromosomes like 1 and 3 to achieve recurrent parental recovery in background selection. Although the background markers of this study were stretched at an average distance of 18.8 cM over the genome, but a significant

proportion of chromosomes (chr. 3, 6, 10, 11 and 12) lacked the requirement of minimal number of markers. This might be due to close similarity of the parents in genetic constituents.

A total 317 BC_1F_1 plants were subjected to phenotypic selection by visual resemblance with BRRI dhan28 at maximum tillering stage. Background genotyping of the selected 41 BC_1F_1 plants for 47 polymorphic SSRs showed wide range of recovery of RPG starting from 21% to 72% with an average of 46.8%. The plant number BC_1-265 showed the highest RPG value (72%) followed by plant number BC_1-239 (70%), BC_1-196 (68%) and BC_1-260 (68%). Nine plants (BC_1-19, BC_1-69, BC_1-196, BC_1-223, BC_1-239, BC_1-260, BC_1-265, BC_1-266 and BC_1-297) having RPG value more than 60% were selected for further backcrossing to produce BC_2F_1 seeds. Among these plants, BC_1-196 and BC_1-265 had 10 chromosomes with 75% to 100% R alleles followed by BC_1-260 and BC_1-266 (9 chromosomes), BC_1-19, BC_1-223 and BC_1-297 (8 chromosomes). The remaining two plants BC_1-69 and BC_1-239 had 75% to 100% R alleles in 7 chromosomes. A total of 914 BC_2F_1 seeds were produced from these nine plants.

Analysis of variance of LD scores obtained from cold screening with BC_1F_2 progenies of 41 plants including the selected nine plants used in backcrossing showed significant variation among them. The LD values of the BC_1F_2 plants ranged from 3-9, while the susceptible check variety BRRI dhan28 obtained LD value of 8 and the tolerant check variety obtained LD value of 4. The BC_1F_2 progenies of BC_1-223 had the lowest LD value (3) indicating tolerance to cold stress at seedling stage, while the progenies of BC_1-196, BC_1-239, BC_1-265 and BC_1-266 showed moderately tolerant reaction (LD: 4-5).

Considering compliance with at least two selection criteria out of three such as, cold tolerance (LD value 5 or less) reaction, recovery of RPG (70% or above) and numbers of chromosomes with 75% to100% R alleles over the

genomeBC_2F_1 progenies of BC_1-239 and BC_1-265 plants were finally selected to proceed further for backcrossing program to develop near isogenic lines in the background of BRRI dhan28.

Based on the findings of this study, the following conclusions and recommendations can be made:

Conclusion:

1. Out of 236 SSR markers used this study, 44.55% markers were found polymorphic between BRRI dhan28 and IR90688-20-1-1-1-1-1. A set of 47 polymorphic markers distributed at an average distance of 18.8 cM over the genome and showing distinct difference in band position were selected for background genotyping.

2. A total 41 BC_1F_1 plants very close to BRRI dhan28 in phenotype out of 317 plants grown for this study were used in background selection with 47 polymorphic SSR markers. Nine plants showing RPG value more than 60% were selected for further backcrossing to produce BC_2F_1 seeds.

3. The LD values obtained from cold screening of BC_1F_2 plants ranged from 3-9. The BC_1F_2 progenies of BC_1-223 showed tolerance to cold stress (LD: 3), while BC_1F_2 progenies of BC_1-196, BC_1-239, BC_1-265 and BC_1-266 showed moderately tolerant reaction (LD: 4-5).

4. Considering LD values ≤5, RPG (≥70%) and number of chromosomes with 75% to 100% R alleles BC_2F_1 progenies of BC_1-239 and BC_1-265 plants were finally selected.

Recommendation:

1. Additional SSR makers are required to saturate the gaps in majority of the chromosomes to realize reliable background recovery in backcross progenies avoiding linkage drag of undesired traits from the donor parent.

2. The number of backcross seeds from each selected plants need to be increased to have bigger population in next cycle of backcross for exploring better recombinants with desired levels of cold tolerance.

3. The backcross seeds produced in this study need to be used in further research to develop future cold tolerant rice variety.

CHAPTER VI
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APPENDICES

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions.

Sl. No.	GRHPC SL No.	Primer Name	Chromosome number	Starting at (bp)	Expected product size	BR28XIR90688
1	1	RM6464	1	171,992	146	NA
2	3	RM6672	1	281,868	150	M
3	5	RM3252	1	301,879	172	M
4	7	RM499	1	388,887	116	P
5	9	RM1282	1	546,313	157	P
6	19	RM323	1	4,213,951	244	P
7	21	RM220	1	4,424,392	127	P
8	23	RM562	1	14,626,324	243	VLP
9	43	RM200	1	8251397	122	VLP
10	53	RM10590	1	9,404,186	447	NA
11	55	RM600	1	9,463,544	220	NA
12	73	RM10671	1	29278026	384	M
13	75	RM10696	1	10,981,692	294	P
14	77	RM10701	1	11,026,953	69	VLP
15	79	RM493	1	12,280,117	211	P
16	85	RM10829	1	13,341,347	122	M
17	87	RM10843	1	13,793,636	162	M
18	91	RM10871	1	14,382,558	234	VLP
19	95	RM10890	1	14,759,384	240	VLP
20	99	RM595	1	15,305,529	189	NA
21	104	RM6711	1	16,111,715	118	NA
22	109	RM11062	1	19,321,343	219	P
23	121	RM9	1	23,325,018	136	VLP
24	125	RM5	1	23,971,321	113	VLP
25	139	RM11504	1	27,784,726	281	M
26	143	RM128	1	31,065,597	157	NA
27	145	RM297	1	32,099,566	148	VLP
28	149	RM212	1	33,053,493	136	M
29	151	RM11745	1	33,237,620	196	P
30	167	RM315	1	36,734,135	133	M
31	169	RM472	1	37,889,084	296	M
32	173	RM3468	1	40,141,956	201	M
33	175	RM104	1	40,166,840	222	NA
34	177	RM529	1	40,670,383	273	NA

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions (Cont'd).

35	179	RM414	1	40,756,026	223	M
36	181	RM5794	1	41,080,893	151	M
37	183	RM5362	1	41,087,022	101	M
38	185	RM5536	1	41,166,774	150	M
39	187	RM14	1	41362794	191	NA
40	189	RM6407	1	42,703,854	145	M
41	195	RM485	2	934,340	291	P
42	207	RM6151	2	1,949,258	159	M
43	209	RM211	2	2,020,515	161	P
44	211	RM3495	2	2,849,299	179	NA
45	213	RM279	2	2,882,052	174	P
46	215	RM5654	2	3,422,754	156	NA
47	217	RM423	2	3,836,813	273	P
48	219	RM3703	2	3,863,789	124	VLP
49	223	RM53	2	4414183	182	P
50	225	RM6654	2	4,816,997	102	M
51	231	RM12769	2	7,353,157	163	P
52	237	RM452	2	9,563,257	209	M
53	239	RM290	2	10,806,902	142	NA
54	241	RM324	2	11,389,704	175	VLP
55	243	RM424	2	11,389,704	142	P
56	247	RM13155	2	15,260,262	478	NA
57	251	RM5578	2	18,632,692	150	NA
58	253	RM561	2	18763965	190	VLP
59	255	RM3858	2	19,182,901	132	M
60	257	RM13332	2	19208210	354	VLP
61	263	RM475	2	20399300	235	P
62	269	RM5789	2	22,384,459	84	M
63	271	RM6318	2	24,420,594	199	VLP
64	273	RM13628	2	25,112,252	282	P
65	277	RM263	2	25,865,334	199	P
66	279	RM526	2	26,664,820	240	P
67	295	RM425	2	32,297,904	126	M
68	297	RM250	2	32,774,365	153	VLP
69	315	RM6147	3	796,986	151	NA
70	317	RM3202	3	809,952	189	P
71	323	RM569	3	1,908,094	175	P
72	325	RM231	3	2453245	182	VLP
73	335	RM546	3	6,163,117	268	NA
74	351	RM563	3	11,070,670	185	NA

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions (Cont'd).

75	367	RM487	3	22,019,474	176	VLP
76	373	RM5626	3	24,864,350	188	M
77	375	RM3513	3	25,112,673	144	NA
78	391	RM293	3	31649990	207	NA
79	395	RM1230	3	32,753,323	155	P
80	401	RM7076	3	33660308	266	P
81	407	RM565	3	35247925	172	P
82	409	RM570	3	35,534,495	208	P
83	413	RM442	3	35781324	257	M
84	417	RM85	3	36341072	107	P
85	425	RM537	4	185,131	236	NA
86	429	RM16327	4	1,526,461	320	NA
87	433	RM6487	4	4,670,293	129	VLP
88	439	RM417	4	9,426,246	265	M
89	443	RM16612	4	11,755,450	138	NA
90	445	RM307	4	13141577	174	NA
91	447	RM401	4	13,154,172	283	NA
92	449	RM16686	4	14,718,643	93	VLP
93	451	RM5687	4	15,742,285	158	P
94	457	RM471	4	18,824,746	106	VLP
95	459	RM16842	4	18,888,814	98	P
96	461	RM16852	4	19,011,780	96	M
97	475	RM3288	4	27,323,766	143	M
98	483	RM6365	4	29,647,666	191	P
99	491	RM349	4	32,499,412	136	NA
100	495	RM131	4	34,423,536	215	VLP
101	503	RM559	4	35,151,595	160	M
102	517	RM6317	5	1,522,561	168	P
103	527	RM574	5	3,450,870	155	M
104	529	RM17954	5	3,651,365	122	P
105	539	RM169	5	7,497,918	167	P
106	547	RM3838	5	16,496,039	132	NA
107	553	RM163	5	19251933	124	VLP
108	567	RM3870	5	22,900,326	193	M
109	569	RM18877	5	23,577,721	156	P
110	579	RM6972	5	25,325,414	158	P
111	583	RM538	5	26,033,392	274	VLP
112	589	RM480	5	27,313,250	225	M
113	603	RM589	6	1,380,866	186	VLP
114	609	RM4923	6	2,174,478	139	NA

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions (Cont'd).

115	611	RM587	6	2,291,804	217	M
116	617	RM585	6	3169313	174	NA
117	619	RM314	6	4844259	118	P
118	621	RM19545	6	4,999,931	127	NA
119	627	RM276	6	6,230,045	149	M
120	629	RM50	6	6375830	201	NA
121	633	RM6359	6	6,434,833	142	P
122	635	RM549	6	6,976,491	148	M
123	641	RM2523	6	8,918,950	146	NA
124	667	RM7434	6	23,934,663	109	P
125	671	RM275	6	24,324,733	114	M
126	681	RM400	6	28,431,560	321	M
127	697	RM141	6	31007437	136	M
128	701	RM51	7	238679	141	M
129	705	RM5211	7	501,190	197	M
130	711	RM6222	7	1,005,777	181	NA
131	713	RM7454	7	1,102,314	99	M
132	717	RM6965	7	2,809,213	187	M
133	719	RM481	7	2876165	169	P
134	723	RM1243	7	3,554,642	157	M
135	729	RM180	7	5,735,196	110	P
136	733	RM21333	7	9,010,772	281	M
137	743	RM8021	7	13356692	250	M
138	745	RM21438	7	13,409,205	250	M
139	753	RM21539	7	16443198	395	NA
140	755	RM7110	7	16,853,304	176	VLP
141	757	RM6427	7	17,449,481	162	VLP
142	765	RM418	7	18,132,231	142	M
143	767	RM320	7	18,693,223	121	VLP
144	777	RM336	7	21,871,205	154	P
145	779	RM455	7	22,350,593	131	VLP
146	785	RM5847	7	23,648,589	194	M
147	787	RM3753	7	23,660,194	145	VLP
148	795	RM478	7	25,949,521	205	VLP
149	801	RM248	7	29,339,144	102	P
150	803	RM172	7	29,561,293	159	M
151	811	RM506	8	124962	123	M
152	815	RM152	8	682,963	151	VLP
153	821	RM5432	8	4,371,975	216	M
154	825	RM5556	8	4,588,384	102	P

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions (Cont'd).

155	827	RM310	8	5,115,740	105	P
156	829	RM126	8	5221268	171	M
157	833	RM72	8	6762710	166	VLP
158	835	RM3215	8	8,566,867	197	VLP
159	841	RM331	8	12,288,777	176	NA
160	847	RM137	8	13984999	218	M
161	865	RM556	8	22,209,687	239	NA
162	869	RM256	8	24,270,635	127	M
163	871	RM80	8	24,478,642	126	NA
164	873	RM149	8	24,721,365	163	NA
165	877	RM433	8	25821467	234	M
166	883	RM3754	8	26,970,193	132	VLP
167	889	RM264	8	27923988	178	NA
168	893	RM23668	9	653,228	159	VLP
169	899	RM3609	9	1,156,791	158	NA
170	905	RM23778	9	3,965,989	327	P
171	911	RM444	9	5,925,016	162	NA
172	913	RM464	9	6,575,147	262	VLP
173	915	RM23902	9	6955618	533	NA
174	919	RM5515	9	7,146,625	123	VLP
175	937	RM3769	9	11,747,444	103	NA
176	939	RM321	9	12348284	200	M
177	941	RM105	9	12,549,315	134	NA
178	943	RM6051	9	12,830,382	136	M
179	957	RM410	9	17,642,699	183	P
180	965	RM5661	9	18,960,711	167	P
181	969	RM553	9	19,324,676	162	NA
182	971	RM107	9	20,068,688	189	P
183	975	RM1553	9	21,003,387	161	VLP
184	977	RM2144	9	22,209,322	132	NA
185	989	RM5271	10	1,935,557	184	NA
186	991	RM24953R	10	2136389	400	M
187	993	RM222	10	2619245	213	P
188	1001	RM311	10	9,747,442	179	P
189	1005	RM6142	10	12,802,044	122	M
190	1015	RM258	10	17,756,102	148	NA
191	1023	RM3773	10	19,895,046	150	VLP
192	1025	RM6691	10	20,232,723	152	NA
193	1031	RM5352	10	21,120,515	125	M
194	1041	RM333	10	22,372,009	191	P

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions (Cont'd).

195	1063	RM26038	11	1,644,853	121	M
196	1069	RM26063	11	2,256,991	163	M
197	1085	RM167	11	4,073,024	128	M
198	1093	RM26224	11	5,134,981	112	NA
199	1113	RM26362	11	8,023,301	134	VLP
200	1121	RM202	11	9,001,608	189	VLP
201	1141	RM206	11	22,014,679	147	P
202	1143	RM187	11	23224277	146	NA
203	1143	RM187	11	23224277	146	M
204	1145	RM27085	11	23,728,180	121	M
205	1145	RM27085	11	23,728,180	144	P
206	1147	RM254	11	23764303	165	NA
207	1147	RM254	11	23764303	165	P
208	1149	RM224	11	27201707	157	M
209	1151	RM144	11	28,281,693	237	NA
210	1151	RM144	11	28,281,693	237	NA
211	1155	RM457	11	19,064,76	238	M
212	1155	RM457	11	19,064,76	238	M
213	1159	RM1880	12	746,745	128	M
214	1171	RM8215	12	1,585,781	221	P
215	1199	RM511	12	17,395,485	130	NA
216	1201	RM1261	12	17,531,111	167	P
217	1209	RM28268	12	19,245,131	143	NA
218	1211	RM260	12	19520752	111	NA
219	1221	RM28502	12	23,414,322	109	VLP
220	1227	RM1194	12	25,864,560	128	NA
221	1229	RM235	12	26,107,904	124	P
222	1233	RM4585	12	26,132,676	165	P
223	1233	RM28746	12	26,331,864	120	VLP
224	1235	RM28748	12	26,369,378	122	VLP
225	1237	RM17	12	26,954,657	184	NA
226	1461	RM26881	11	19330394	144	M
227	1463	RM26902	11	19736505	114	M
228	1465	RM26931	11	20428445	127	M
229	1467	RM26945	11	20662146	185	NA
230	1469	RM26976	11	21277661	183	VLP
231	1471	RM26987	11	21456979	163	M
232	1509	RM26877	11	19222525	190	M
233	1511	RM26927	11	20253572	187	NA
234	1513	RM26975	11	21245079	195	NA

APPENDIX I: List of SSR primers used in parental polymorphism survey with their descriptions (Cont'd).

235	1525	In1-C3	1			VLP
236	1527	In11-d1	11			VLP

APPENDIX II: List of Polymorphic SSR markers used in background selection

Primer Name	Chromosome location	Physical distance (bp)	Converted Genetic distance (1 Mb =4 cM)
RM323	1	4213951	16.9
RM493	1	12280117	49.1
RM11062	1	19321343	77.3
RM9	1	23325018	93.3
RM297	1	32099566	128.4
RM7250	1	36085237	144.3
RM485	2	934340	3.7
RM12769	2	7353157	29.4
RM424	2	11389704	45.6
RM13332	2	19208210	76.8
RM475	2	20399300	81.6
RM6318	2	24420594	97.7
RM526	2	26664820	106.7
RM3202	3	809952	3.2
RM85	3	36341072	145.4
RM16686	4	14718643	58.9
RM5687	4	15742285	63.0
RM16842	4	18888814	75.6
RM6365	4	29647666	118.6
RM131	4	34423536	137.7
RM6317	5	1522561	6.1
RM169	5	7497918	30.0
RM163	5	19251933	77.0
RM18877	5	23577721	94.3
RM589	6	1380866	5.5
RM6359	6	6434833	25.7
RM7434	6	23934663	95.7
RM481	7	2876165	11.5
RM180	7	5735196	22.9
RM7110	7	16853304	67.4
RM336	7	21871205	87.5
RM478	7	25949521	103.8
RM248	7	29339144	117.4

APPENDIX II: List of Polymorphic SSR markers used in background selection (Cont'd).

RM5556	8	4588384	18.4
RM3215	8	8566867	34.3
RM137	8	13984999	55.9
RM23778	9	3965989	15.9
RM464	9	6575147	26.3
RM410	9	17642699	70.6
RM107	9	20068688	80.3
RM222	10	2619245	10.5
RM311	10	9747442	39.0
RM333	10	22372009	89.5
RM27085	11	23728180	94.9
RM8215	12	1585781	6.3
RM1261	12	17531111	70.1
RM28502	12	23414322	93.7

APPENDIX III: Percentage of R alleles in BC_1F_1 plants

Item	BC_1F_1(18)	BC_1F_1(19)	BC_1F_1(21)	BC_1F_1(34)	BC_1F_1(39)	BC_1F_1(45)	BC_1F_1(53)	BC_1F_1(69)	BC_1F_1(71)	BC_1F_1(73)	BC_1F_1(86)	BC_1F_1(89)	BC_1F_1(92)	BC_1F_1(97)	BC_1F_1(102)	BC_1F_1(110)	BC_1F_1(116)	BC_1F_1(126)	BC_1F_1(127)	BC_1F_1(128)	BC_1F_1(159)	BC_1F_1(163)	BC_1F_1(185)	BC_1F_1(192)	BC_1F_1(196)	BC_1F_1(221)	BC_1F_1(223)	BC_1F_1(228)	BC_1F_1(239)	BC_1F_1(254)	BC_1F_1(260)	BC_1F_1(265)	BC_1F_1(266)	BC_1F_1(270)	BC_1F_1(272)	BC_1F_1(277)	BC_1F_1(284)	BC_1F_1(294)	BC_1F_1(295)	BC_1F_1(296)	BC_1F_1(297)
No. of A	22	28	16	21	10	19	27	28	19	21	15	21	22	13	26	23	14	25	25	16	24	13	23	24	32	12	29	24	33	23	32	34	30	25	16	19	16	17	15	20	30
No. of B	1	0	10	0	13	0	0	0	0	0	0	0	2	12	0	0	1	0	0	1	0	7	0	1	1	13	1	1	2	1	1	1	0	1	16	6	13	1	10	1	1
No. of H	23	18	20	25	23	28	17	18	28	25	30	25	20	21	19	21	29	20	19	29	20	27	22	22	12	20	15	21	7	22	13	12	16	21	15	21	18	28	22	25	14
No. of MDP	1	1	1	1	1	0	3	1	0	1	2	1	3	1	2	3	3	2	3	1	3	0	2	0	2	2	2	1	5	1	1	0	1	0	0	1	0	1	0	1	2
%RPG	47	60	34	45	21	40	57	60	40	45	32	45	47	28	55	49	30	53	53	34	51	28	49	51	68	26	62	51	70	49	68	72	64	53	34	40	34	36	32	43	64

APPENDIX IV: LD score and SD value of BC_1F_1 plants

SL	PLANT ID	Mean	SD
1	18	8	2.3
2	19	7	2.1
3	21	8	1.0
4	34	7	1.4
5	39	6	0.1
6	45	7	0.2
7	53	8	1.1
8	69	6	2.4
9	71	7	1.1
10	73	7	0.3
11	86	7	1.1
12	89	7	0.5
13	92	7	0.6
14	97	6	0.0
15	102	7	1.7
16	110	7	0.8
17	116	7	0.3
18	126	8	1.3
19	127	8	0.4
20	128	6	0.5
21	159	7	0.1
22	163	5	0.9
23	185	5	0.3
24	192	4	0.1
25	196	5	1.0
26	221	4	0.7
27	223	3	0.3
28	228	5	0.4
29	239	4	0.2
30	254	6	1.9
31	260	6	0.7
32	265	5	1.5
33	266	5	1.1
34	270	6	0.2
35	272	5	0.7
36	277	6	0.9

APPENDIX IV: LD score and SD value of BC_1F_1 plants (Cont'd)

37	284	9	0.3
38	294	6	1.3
39	295	5	0.4
40	296	6	1.6
41	297	7	1.0
42	ck1(BR28)	8	0.7
43	ck2(HBJ vi)	4	0.7