BREEDING FOR COLD TOLERANCE IN RICE (*Oryza Sativa L.)* FOR THE HIGH ALTITUDE AREAS OF MADAGASCAR

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PLANT BREEDING AND BIOTECHNOLOGY

UNIVERSITY OF ELDORET, KENYA

NOVEMBER, 2016

DECLARATION

Declaration by the candidate

This thesis is my original work and has not been submitted for a degree in any other university; No part of this thesis may be reproduced without prior written permission from the author and/or University of Eldoret.

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DEDICATION

This thesis is dedicated to my parents; they sacrificed much to provide me with the quality of life and helped me to become the person I am today. Thank you for all you did throughout my life.

ABSTRACT

Low temperature or cold stress is one of the major abiotic stresses limiting rice (*Oryza sativa L.*) production and productivity in the high altitude of Madagascar; it is affecting an estimated 12.3% of the total rice cultivation. It causes poor seedling establishment and at the reproductive stage causes high sterility and decreases production. This study aimed to (*i*) introgress cold tolerance gene by crossing an *Indica* with a *japonica* rice, (*ii*) identify a BC_1F_2 population for cold tolerance at seedling and reproductive stage under control screening conditions and (*iii*) identify quantitative trait loci (QTLs) related to cold tolerance at seedling and reproductive stage using single nucleotide polymorphism (SNP) markers. Introgression was done by advance until BC_1F_2 from a cross between *Indica* rice (Vary botry, Soameva) used as the recurrent parent with *Japonica* rice (Chomrongdhan) used as a donor parent. Identification of BC_1F_2 plants as cold tolerance was done by phenotyping study of BC_1F_2 plants and their parents using a cold room at seedling and reproductive stages, cold room at temperature 12°C and 15°C for 10 days at seedling and reproductive stage respectively for cold treatment. At seedling stage after cold treatment, Chomrongdhan (Chom) showed higher percentage of survival plant (100%) than Vary botry (Var Bot) and Soameva (Soa) (50 and 15%) and BC_1F_2 plants 59 and 40% to Var bot//Chom and Soa//Chom respectively. The BC_1F_2 from the Var bot//Chom cross showed significant differences in seedling growth, seedling vigor while those from Soa// Chom showed significant differences for seedling growth, seedling vigor and seedling leaf growth at seedling stage. At the reproductive stage, BC_1F_2 from Var bot//Chom cross showed significant differences in plant height, panicle exsertion, number of full seeds, panicle weight, phenotypic acceptability, thousand seed weight and heading date while those from the Soa//Chom cross showed significant differences for tiller number, panicle weight, thousand seed weight, plant height, spikelet fertility and number of full seeds. Linkage map was constructed in 1566 (Var bot//Chom) and 1250 (Soa//Chom) polymorphic SNP markers and QTL declared at Logarithm of odds (LOD) greater than 2.5. Crosses between *Indica* and *Japonica* for this study produced fertile BC_1F_2 plants confirm that there are Introgress of cold tolerance gene from the *japonica* rice to the *indica*. A number of BC_1F_2 plants showed a good performance compared to the susceptible parent, and different QTLs for different traits related to cold tolerance at seedling and reproductive stage were identified in this study. The cold-tolerant breeding lines selected in this study had a QTL associated with cold tolerance. Four putative QTLs located on chromosome 2 and 10 conferred tolerances to cold at seedling stage and twelve putative QTLs located on chromosomes 2, 4, 5, 7, 8, 10 and 12 conferred tolerances to cold at reproductive stage from BC_1F_2 progeny of Var bot//Chom, while seven putative QTLs located on chromosome 1, 2, 4 and 9 were identified at seedling stage and six putative QTLs located on chromosome 2, 5,7, and 10 were identified tolerances to cold at reproductive stage from BC_1F_2 progeny of Soa//Chom. QTL associated with spikelet fertility of Var bot//Chom, and QTL associated with the panicle weight of Soa//Chom showed similar map position. Validation of the promising QTLs observed in this study could be useful to enhance the level of cold tolerance in Madagascar. BC_1F_2 populations should be tested under cold conditions in the field to validate the current results.

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LIST OF ACRONYMS

FAO:	Food and Agriculture Organization
INSTAT:	National Statistic Institute
GRiSP	Global Rice Science Partnership
QTL:	Quantitative trait loci
DNA:	Deoxyribonucleic acid
SNP:	Single-nucleotide polymorphism
IRRI:	International rice research institute
FOFIFA:	The National Center of Applied Research for Rural Development
IRGC:	International Rice Germplasm Center
AIEA:	International Atomic Energy Agency
STRASA	Stress-tolerant Rice for Africa and South Asia
INGER:	International Network for Genetic Evaluation of Rice
NERICA:	New Rice for Africa
SES:	Standard evaluation system for rice
KARLO:	Kenya Agricultural and Livestock Research Organization
GSL:	Genotyping Services Lab
PB Tools:	Plant breeding tools
SAS :	Statistical Analysis System

ACKNOWLEDGEMENT

- First, I would like to thank the Lord for the strength, courage and guidance He gave me to successfully complete this study.
- I extend my sincere gratitude to my supervisors: Prof. Miriam Kinyua, Dr. Alexis Ndayiragije and Dr Oliver Kiplagat for their time, effort, advice, constructive criticism, inspiration, friendship, supervision and encouragement during my study.
- I would like to thank Dr. R.K. Singh, from the International Rice Research Institute (IRRI) for his initial contribution in defining the scope of my thesis.
- I would like to express my thanks to the University of Eldoret, School of Agriculture and Biotechnology, Department of Biotechnology for the support during the period of this study. University of Eldoret not only recommended me for this scholarship but provided a suitable learning environment and for this reason I am very grateful.
- My special thanks go to IRRI for the full financial support of my scholarship; my sincere thanks go to the IRRI-ESA regional co-coordinator and his staff.
- Special gratitude goes to the Director of KARLO Njoro for allocating me greenhouse, cold room, and molecular lab for my research study. I appreciate the technician of KARLO Njoro and of biotechnology lab of the University of Eldoret for assisting me during my research work. I sincerely appreciate Dr. James Owuoche for his assistance in data analysis.

- I am grateful to my institution in Madagascar, The National Center of Applied Research for Rural Development (FOFIFA), particularly the Rice Research Department for granting me the study leave.

- I highly acknowledge my friends and colleagues from Kenya, Eldoret as well University of Eldoret for their support, motivation, understanding, assistance and friendship during my study; I am not feeling alone although my family is far from me. Finally, it's not easy to mention everyone who contributed to this study in one way or another; I therefore request them to accept my sincere appreciation.

- I sincerely thank my family for their great support during my study

Thank you all and God bless you.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Rice (*Oryza saliva L.*) cultivation or Asian rice was introduced in Madagascar from India, the Malay Peninsula and Indonesia approximately 800–1400 years ago (Mather *et al.,* 2010). Rice is both the main crop and the staple food of the majority of the population of Madagascar with average annual consumption of rice by an individual estimated at 154 kg/capita/year (paddy equivalent) (Raboin *et al.,* 2014; MinAgri, 2015). It is mainly grown by small-scale farmers as a food crop and as a primary source of household income for the majority of rural farmers (INSTAT, 2010; Rahaingo *et al.,* 2013). National rice production in 2015 was estimated at about 3.7 million tons (in paddy terms), representing a decline of 6 percent compared to the previous year (FAO, 2015), the country still imported about 200,000 tons of rice in the first three months of 2015 (Info rice, 2015) and the country's rice import requirement for the 2015/16 marketing year (April/March) is estimated at 331,000 tons (in milled terms) (FAO, 2015) to cover the needs of its population of around 24 million (Worldometers, 2016).

Importing rice to cover the deficient demand on rice was no longer a sustainable strategy in Madagascar. In recognition of this challenge, the Government of Madagascar through the Ministry of Agriculture, in the 2008-2018 national rice development strategy (NRDS), adopted a plan to double rice production by the end of 2018 (MinAgri, 2010). Hence, rice production is expected to increase from improving rural infrastructure, strengthening the provision of extension services and increasing access to input

(fertilizer, seeds) and credit (MinAgri, 2010; MinAgri, 2015). However, to achieve this goal, the country would require rice varieties with high yield potential and greater yield stability, especially against the adverse growing environments.

Yield instability is caused by several constraints including a range of both biotic and abiotic stresses, lack of access to agricultural equipment, good quality seed and mineral fertilizers (AfricaRice, 2015). Abiotic and biotic stresses frequently prevent the attainment of optimum growth and yield of rice (Das *et al.,* 2015). High salinity, drought, submergence and cold have a major negative effect and create risks to food security worldwide (Wani *et al.,* 2014). Among them, low temperature or cold stress is one of the major constraints that threaten the adaptability of rice and its production (Pan *et al.,* 2015).

Due to its origin in tropical and subtropical regions, rice is more sensitive to cold stress than other cereal crops such as wheat (*Triticum aestivum L.)* and barley (*Hordeum vulgare L.*) (Zhang *et al.,* 2014). Low temperature is a common problem in rice cultivation globally, (Zhou *et al.,* 2012) and still causes serious significant yield losses in worldwide rice production (Zhu *et al.,* 2015). Frequently occurring low temperature causes more than 50% yield loss (Basuchaudhuri, 2014). It is still one of the factors adding serious threats to global food security (Zulfqar *et al.,* 2016).

Cold stress affects rice cultivation not only in areas at high altitudes, but also in tropical and subtropical countries (da Cruz *et al.,* 2013), for example, low temperatures have resulted in a net loss of 75 and 44% of rice yield in Australia, and Japan (da Cruz *et al.,* 2013) respectively.

In Brazil, the largest rice producer outside Asia, the rice crop suffers damage from cold occurrence in Rio Grande doSul (RS), the southernmost state, where more than 60% of the Brazilian rice grain is produced. In this region, as well as in Uruguay and Argentina, *Indica* rice accounts for almost the totality of cultivated rice. Low temperature in this region can reduce up to 25% of the final yield (Clayton and Neves, 2011; Lima *et al.,* 2012).

In Africa, by seasonal temperature variation in Sahel regions of West Africa and because of the elevation in high-altitude regions of East Africa, low-temperature damage could occur, resulting in considerable yield losses (Wainaina *et al.,* 2015). Cold stress leads to high spikelet sterility, low tillering, stunting and delayed development and severe yield reduction in farmer's fields, particularly when they grow higher yielding, but susceptible rice varieties or a sensitive genotype (Manneh *et al.,* 2011; Eixarch, *et al.,* 2015); Around 15% of yield is lost (Manneh, 2009; Saito *et al.,* 2013).

In Madagascar, Rice is grown in six zones of the country; North, Northwest, and Central-Western regions; the East; the Central-Eastern part and the Central part of the Malagasy highlands at elevations up to 1500- 1900 meter above the sea level. In the highland region, where are located the densely populated area, with 102 inhabitants per km^2 (Gastineau *et al.,* 2010). Temperatures will normally drop with increasing altitude; this is a very important aspect of agriculture in Madagascar (Oldeman, 1990; GRiSP, 2013). In the highlands, smallholders traditionally grow irrigated or rain-fed lowland rice, mostly landraces (Raboin *et al.,* 2010; Raboin *et al.,* 2014). Cold stress reduces rice yields in this country and yield losses between 70 to 85% are experienced by farmers (GRiSP, 2013; DRR, 2014, 2015).

In order to cope with low temperature sensitivity, the development of tolerant cultivars is an important prerequisite in any rice breeding program. Therefore, this study was programmed to develop varieties that could withstand cold temperature during seedling and reproductive stage for the high altitude region of Madagascar.

Achieving tolerance to low temperature in rice is clearly a complex endeavor. Tolerance to low temperature at one stage may not correlate with tolerance at another stage. One other difficulty is that most of the sources of cold tolerance are from *Japonica* background, and breeding effort to incorporate cold tolerance from *Japonica* to *Indica* types have encountered some major difficulties. To make significant progress, molecular breeding methods are currently underway to introgress tolerant genes into cultivars that are high yielding, but susceptible to cold stress.

The complexity of plant tolerance to abiotic stress presents challenges for conventional breeding to make significant progress and has led to increased interest in molecular breeding methods. For multi-sample or multilocus genotyping, many researchers employed labor-intensive methods, such as cleaved amplified polymorphic sequences (CAPS), restriction fragment length polymorphisms (RFLP) and simple sequence repeats (SSR) (Lateef, 2015). SSR or microsatellites (SSRs) are widely used due to their high polymorphism and technically simple method of detection and low cost (McCouch *et al*., 2002; Gonzaga *et al.,* 2015). It has been used in crop breeding program such as genetic diversity analysis (Suh *et al.,* 2013); construction of a linkage map (Ranawake, *et al.,* 2014), molecular assisted selection and identification of genetic resources for cold tolerance rice (Shinada *et al.*, 2014).

Although SSRs have been useful in genetic studies, they have weaknesses. First there are limited numbers of SSR motifs in the genome which become a constraint when trying to saturate a region with markers or when trying to identify gene-based markers (Thomson, 2014). They may have low information content in the gene of interest (Macaulay *et al.*, 2001; Gonzaga *et al.,* 2015). In addition, the high information contents multiple alleles per locus from SSR also present difficulties when merging SSR data from different platforms and curating allele sizes in data bases. Also, gel-based SSRs are labor intensive and automated fragment sizing systems have limited scope for multiplexing, resolution of QTL mapping is frequently low because of limited molecular markers for a particular population and QTL are often mapped into intervals covering large DNA fragments (Thomson, 2014).Therefore, there is a need to use genotyping platforms that would map correctly the location of the tolerance QTL to accelerate their usage in molecular breeding.

To cover the SSR limitation, recent advances in rice genomics have led to the discovery of abundant single nucleotide polymorphism (SNPs) which have enormous potential for rice molecular breeding (Gonzaga *et al.,* 2015). Thus, SNP markers are ideal for high resolution genotyping for association studies, rapid genome-wide scans for genomic selection, as well as linkage mapping, and marker-assisted selection (MAS) (McCouch *et al*., 2010; Gonzaga *et al.,* 2015), SNPs are the most abundant and represent a robust class of molecular markers (Mammadov, *et al.,* 2012; Samarai *et al.,* 2015), feasible for automated high-throughput genotyping, and available for multiple assay options using different technology platforms to meet the demand for molecular breeding in crop plants (Singh *et al*., 2015).

SNPs are highly preferred in plant genetic and in plant breeding because of their excellent genetic attributes, such as wide genomic distribution, co-dominant inheritance, high reproducibility, and chromosome-specific location (Lateef, 2015; Singh, *et al.,* 2015), SNP is useful in detection of association mapping of genes controlling complex phenotypic traits (Thomson, 2014), it has greatly facilitated the production of much higher density maps than traditional marker systems (Kumar *et al.,* 2012). A favorable genetic map should have an adequate number of evenly-spaced polymorphic markers to accurately locate desired QTLs/genes (Singh, *et al.,* 2013; Jiang, 2013), and genetic maps are essential for the mapping of quantitative trait loci (QTL) (Ma *et al*., 2015). Some SNPs have been identified for cold tolerant genes in rice such as a single-nucleotide polymorphism in a gene called COLD1 that confers cold tolerance in *Japonica* rice (Ma *et al.,* 2015).

Identification of markers linked to genes and QTLs (quantitative trait loci) tolerance genes offer promising opportunities for rice improvement with regards not only for cold stress but also to others abiotic-stress tolerance (Thomson *et al*., 2010b; Dramé *et al.,* 2013). Precise high-throughput phenotyping protocols also need to be in place for effective screening of breeding lines. These major QTLs are being used for improved varieties that are widely popular among farmers in the target regions. (Dramé *et al.,* 2013).

1.2 Statement of the problem

In terms of cultivated area, irrigated rice is the most important, covering 82% of rice growing area in Madagascar, while the remaining is produced under rainfed lowland rice,

upland rainfed rice (called tanety), and rice as a first crop after slash and burn (called Tavy) (GRiSP, 2013). Similar to the high-altitude areas in the tropics and subtropical country because of the lower pressure in the high altitude areas, cold occurs as a result of altitude effect (Jacobson 2005; Ahrens, 2006). Rice production is affected very badly by cold weather and only one cropping a year is possible, making a double cropping difficult and low temperature affects both seedling and reproductive stages (Rasolofo *et al.,* 1986; Zenna *et al*, 2010).

The general complexity of cold problem with high altitude of Madagascar has often appeared by decreasing the optimum needed temperature at different stages for rice growing. Mean temperatures at 1,500 meters vary from 17°C in October, the rice-sowing period, to 20°C during the reproductive stage. Minimum temperatures can fall below 10°C during early vegetative stage and are below 14°C during reproductive stage and grain filling (Ahamadi 2004; Zenna *et al.*, 2010). Low-temperature damage is worse with a temperature drop during the seedling or reproductive stage as it slows rice growth at almost all stages: panicle initiation is delayed and grain-filling and maturation stages are lengthened. The cold and humid conditions increase the risk of panicle sterility and favor disease epidemics especially *Sarocladium* and blast (DRR, 2012). Cold during the reproductive stage may provoke high sterility rate (Raboin *et al.,* 2013).

Cold stress is one of the factors that adversely affect rice growth and productivity in this area, resulting in reduced yield losses which can range from 70 to 85% mostly due to low temperature during the seedling and reproductive stage (DRR, 2014, 2015), even though cold temperature can be harmful during the entire developmental stage of rice plants, from germination to grain filling (da Cruz *et al.,* 2013). In addition, traditional rice

cultivars have dominated in the rice area in this region (Radanielina, 2010) and Smallholders traditionally grow irrigated rice or rainfed lowland rice in inland valleys and on hillsides. Rice terraces also can be seen at 1,900 meters (Ahamadi 2004; Zenna *et al*., 2010). There are no traditional or elite irrigated rice varieties considered to have a cold tolerant gene, in addition, there is limited information concerning molecular work and biotechnology on rice in Madagascar.

1.3 Justification

Global food production will need to increase more than 50% before 2050 to satisfy the food demands of an increasing human population. To solve this food crisis, an increase in rice production will be necessary as rice is especially vital for the nutrition of much of the population in many countries in Asia, as well as in Latin America and Africa, and it is central to the food security of over half the world's population (Yamori *et al*., 2014) and a mainstay for the rural population and their food security (IRRI, 2013). Since rice cultivation is not sufficient for Madagascar and the country still has imported about 200,000 tons of rice in the first three months of 2015 (Info rice, 2015), strategies need to be put into place to increase production.

Rice is both the main crop and the staple food of the majority of the population of Madagascar, rice takes place on the quasi-totality of the Malagasy rural population, agricultural area is around 71.2% (World Bank, 2014) and 60% represents rice cultivated area (Rakotojaofeno, 2014). In the high altitude region of Madagascar (1500-1800m), because of the tropical altitude climate, this altitude has a large effect on the temperatures medium that decrease by0.6 °C per 100 m and affect cultivated plants (Sourisseau *et al.,*

2016), and an estimated around 122,886 ha (12.3%) of the total rice cultivation areas are affected by cold stress, in which, 12,889ha affected by cold occurring in seedling stage and 110,597ha affected by cold at the reproductive stage of rice plant, the last includes upland rice crop area from 1500m (Kumashiro, 2011; DRR, 2014,2015). Cold tolerant varieties would help to solve this problem.

About 70 to 85% of rice harvested was lost during seedling and reproductive stage in the 12.3% area affected by low temperature (DRR, 2014, 2015). Given these causes, these in turn result in lower rice quality, reduced returns to farmers, higher consumer prices, and farmers cannot produce enough rice to meet their household consumption requirements throughout the year, With colder temperatures at high elevations, rice yields are hampered (Rahaingo *et al.*, 2012). There is therefore need for cold tolerant varieties in Madagascar.

Due to the importance of rice in the high altitude region and cold stress effect, the Research Center in collaboration with International Institute activated an effort to advance cold tolerance rice research. In 1986, FOFIFA and IRRI started work together to identify donors and outstanding cold tolerant varieties by using Malagasy accessions from the International Rice Germplasm Center (IRGC) and from FOFIFA (Rasolofo *et al.*, 1986). In 2008, through collaboration between the University of Antananarivo and the International Atomic Energy Agency (AIEA), three cold tolerant induced mutants with high yield and seed set in the altitude 1300m were identified.

Since 2010 up to the present moment, FOFIFA through Rice Research Department (DRR) and the Stress-tolerant Rice for Africa and South Asia (STRASA) project are

trying to evaluate cold-tolerant rice germplasm materials from the International Network for Genetic Evaluation of Rice (INGER)-Africa and from AfricaRice (AfricaRice, 2015; MinAgri, 2016).

Although there have been a number of initiatives and investments for cold tolerant rice development, there are still problems of low rice yield for farmers in high altitude regions due to the effects of cold.

Farmers, as well as the Government of Madagascar don't have a concrete strategy or a method to manage the cold stress effects on crop cultivation including rice. One of the effective approaches to decrease the cold effect on rice production was by improving the most used varieties by farmers in this region, as the majority is traditional varieties. Hence, breeding varieties for cold tolerance could be the best economically viable and environmentally feasible solution for the problem, breeding work given priority as an entry point for increasing and stabilizing rice production in high altitude regions of Madagascar. As much as possible, this should be combined with improved management practices to realize the full yield potential of the new developed tolerant varieties.

This study will enable to improve elite varieties susceptible to cold at seedling and reproductive stages by applying traditional and molecular breeding to identify enhanced progeny, which is a key feature for developing better ones. Improvement in order to develop seedling growth and development, increase growth vigor and grain filling and can bring change to rice production and hence improve the farmer's livelihood in particular and food security in general.

1.4 Objectives of the Study

The overall objective of the study was to improve *indica* rice, traditional varieties susceptible to cold in irrigated rice ecosystem within the high altitude of Madagascar. The specific objectives of this research were to

1- Introgress of cold tolerance gene by crossing *Indica* with *japonica* rice

2- Identification of first backcross of second generation (BC_1F_2) population for cold tolerance at seedling and reproductive stage under controlled screening conditions;

3- Identification and mapping of QTLs related to cold tolerance at seedling and reproductive stage using SNP markers;

1.5 Research Hypotheses

1- There is no introgress cold tolerance gene by crossing *Indica* with *japonica* rice

2- There is no BC_1F_2 population for cold tolerance at seedling and reproductive stage that would be screened under controlled cold conditions

3- There are no QTLs related to cold tolerance at seedling and reproductive stage that could be used to improve tolerance of the cold susceptible rice cultivar using SNP markers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin, taxonomy and domestication of Rice

Consumption of Asian rice (*Oryza sativa L.*) was believed to have originated in Asia about 10,000 years ago (Chung *et al.,* 2015; Hirst, 2016), the oldest record dating its cultivation to have began as early as 5000 years BC, (Hirst, 2016).

Rice is a monocotyledonous angiosperm and belongs to the grass family Gramineae with a genome consisting of 430Mb across 12 (2n=24) chromosomes (Kirk, 1998; GRiSP, 2013). The genus, to which it belongs, *Oryza*, contains more than 20 species, only two of which are referred to as cultivated rice: *Oryza sativa* Asian cultivated rice, and *Oryza glaberrima* Africa cultivated rice.

The genus *Oryza,* to which grown rice belongs, originated at least 130 million years ago and dispersed as a wild grass in Gondwanaland, the super continent that eventually broke up and drifted apart to become Asia, Africa, Australia, and Antarctica (GRiSP, 2013). This shows the distribution of *Oryza* species all over the world. There are 22 wild species of genus *Oryza*, Nine of the wild species are tetraploid and the remaining wild species and the two cultivated species are diploid.

Oryza sativa, is cultivated worldwide and has two subspecies *indica* and *japonica* (Jena *et al.,* 2010; GRiSP, 2013) Subspecies *indica* widely cultivated in the hot and humid regions of Asia, Africa and Latin America, and accounts for 80% of world rice production. (Jena *et al*., 2010).

Subspecies *japonica* is cultivated in the temperate, sub-temperate and high-altitude regions of Asia, Europe, Latin America, North America and Oceania (Mackill and Lei, 1997). *Oryza glaberrima* originated from West Africa where it is an upland crop but is being replaced by *Oryza sativa*, it is planted on a limited scale in West Africa (Li *et al.*, 2011).

Morphologically there are two differences between these species mainly in ligule size and glumes pubescence but *O. glaberrima* always has a red pericarp. The common rice, *Oryza sativa*, and the African rice, *Oryza glaberrima*, are thought to be examples of directional evolution in crop plants (Li *et al.*, 2011; Dogara *et al*., 2014). The wild progenitor of *O. sativa* is the Asian universal wild rice, *O. rufipogon*, which shows a range of different from perennial to yearly types and given the specific name of *O. nivara* were tamed to become *O. sativa*. In a parallel origin path, *O. glaberrima* was tamed from annual *O. breviligulata*, which in turn arise from perennial *O. longistaminata* (Dogara *et al*., 2014). Domestication of wild rice's probably started about 9,000 years ago (Farooq, 2011). Development of annuals at different elevations in East India, northern Southeast Asia, and western China was enhanced by alternating periods of drought and variations in temperature during the Neothermal Age about 10,000 to 15,000 years ago (Dogara *et al*., 2014; Silva *et al.,* 2015).

Domestication in Asia could have occurred independently and concurrently at several sites within or bordering a broad belt that extends from the plains below the eastern foothills of the Himalayas in India through upper Myanmar, northern Thailand, Laos, and Vietnam to south western or southern China (Dogara *et al*., 2014).

The primary point of diversity for *O. glaberrima* is the swampy basin of the upper Niger River and two secondary points to the southwest near the Guinean coast (Li *et al.,* 2011; Dogara *et al*., 2014).

The importance of *glaberrima* has decreased due to the introduction of modern varieties of *O. sativa*. It has smooth hairless glumes, red grains, and short ligules with round tips, high seed dormancy and stiff upright panicles with few or no secondary branches. Because of the wide genetic gap between the two species (*O. sativa* and *O. glaberrima*) problems of sterility are experienced when crosses are made. However, making many crosses and selecting the few that are successful can breed out the sterility in few generations (Jones *et al*., 1997). The New Rice for Africa (NERICA) varieties were developed by crossing between *O glaberima* and *Oryza sativa* (Diagne *et al.,* 2010).

The island of Madagascar off the coast of East Africa, which was settled by humans only 2000 years ago (Burney *et al*., 2004), was one of the last major Old World areas of introduction after the domestication of rice and before extensive historical global trade in this crop. Asian rice was introduced into Madagascar from India, the Malay Peninsula and Indonesia (Ahmadi *et al,.* 1991; Mather *et al.,* 2010). Tropical *Japonica* varieties are believed to have been introduced first by these people from the Malay archipelago in the 5^{th} and 6^{th} century by the Malays migrating from Indonesia (Vaughan et *al.,* 2004; Dogara *et al.,* 2014).

2.2 The rice plant

2.2.1 Morphology

Cultivated rice is generally considered a semi aquatic annual grass; although in the tropics it can survive as perennial, producing new tillers from nodes after harvest (ratooning). At maturity, the rice plant has a main stem and several tillers. Each productive tiller bears a terminal flowering head or panicle. Plant height varies by variety and environmental conditions, ranging from approximately 0.4 meter (m) to more than 5 m in some floating rice. The morphology of rice is divided into the vegetative phase (including germination, seedling, and tillering stages) and the reproductive phase (including panicle initiation and heading stages) (GRiSP, 2013).

2.2. 2 Seeds

The rice grain, commonly called a seed, consists of the true fruit or brown rice (caryopsis) and the hull, which encloses the brown rice. Brown rice consists mainly of the embryo and endosperm. The surface contains several thin layers of differentiated tissues that enclose the embryo and endosperm (Figure 1).

The palea, lemmas, and rachilla constitute the hull of indica rice. In japonica rice, however, the hull usually includes rudimentary glumes and perhaps a portion of the pedicel. A single grain weighs 10–45 milligrams at 0% moisture content. Grain length, width, and thickness vary widely among varieties. Hull weight averages about 20% of total grain weight (GRiSP, 2013).

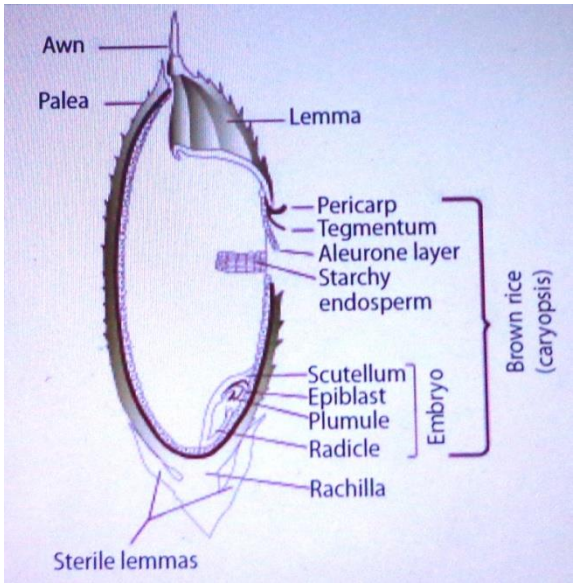


Figure 1: Cross-section of the rice grain **(Source: GRiSP, 2013)**

2. 2. 3 Seedlings

Germination and seedling development start when seed dormancy has been broken and the seed absorbs adequate water and is exposed to a temperature ranging from 10 to 40 °C. The physiological definition of germination is usually the time when the radicle or coleoptile (embryonic shoot) emerges from the ruptured seed coat. Under aerated conditions, the seminal root is the first to emerge through the coleorhizae from the embryo, and this is followed by the coleoptile. Under anaerobic conditions, however, the coleoptile is the first to emerge, with the roots developing when the coleoptile has reached the aerated regions of the environment. If the seed develops in the dark as when seeds are sown beneath the soil surface, a short stem (mesocotyl) develops, which lifts the crown of the plant to just below the soil surface (Figure 2). After the coleoptiles emerge, it splits and the primary leaf develops.

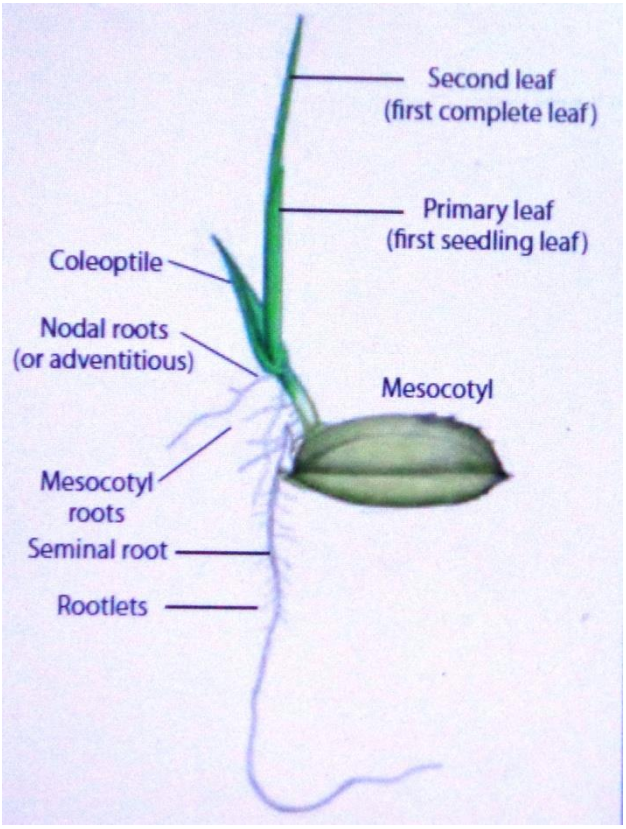


Figure 2: Parts of a young seedling **(Source: GRiSP, 2013)**

2.2.4 Tillering

Each stem of rice is made up of a series of nodes and internodes (Figure.3). The internodes vary in length depending on variety and environmental conditions, but generally increase from the lower to the upper part of the stem. Each upper node bears a leaf and a bud, which can grow into a tiller. The number of nodes varies from 13 to 16, with only the upper 4 or 5 separated by long internodes. Under rapid increases in water level, some deepwater rice varieties can also increase the lower internode lengths by more than 30 centimeters (cm) each (GRiSP, 2013).

The leaf blade is attached at the node by the leaf sheath, which encircles the stem. Where the leaf blade and the leaf sheath meet is a pair of claw like appendages, called the auricles. Coarse hairs cover the surface of the auricles. Immediately above the auricles is a thin, upright membrane called the ligule.

The tillering stage starts as soon as the seedling is self-supporting and generally finishes at panicle initiation. Tillering usually begins with the emergence of the first tiller when seedlings have five leaves. This first tiller develops between the main stem and the second leaf from the base of the plant. Subsequently, when the sixth leaf emerges, the second tiller develops between the main stem and the third leaf from the base.

Tillers growing from the main stem are called primary tillers. These may generate secondary tillers, which may in turn generate tertiary tillers. These are produced in a synchronous manner. Although the tillers remain attached to the plant, at later stages they are independent because they produce their own root cultural practices.

The rice root system consists of two major types: crown roots (including mat roots) and nodal roots (Figure 3). In fact, both these roots develop from nodes; but crown roots develop from nodes below the soil surface. Roots that develop from nodes above the soil surface usually are referred to as nodal roots. Nodal roots are often found in rice cultivars growing at water depths above 80 cm. Most rice varieties reach a maximum depth of 1 m or more in soft upland soils. In flooded soils, however, rice roots seldom exceed a depth of 40 cm. That is largely a consequence of limited oxygen diffusion through the gas spaces of roots (aerenchyma) to supply the growing root tips.

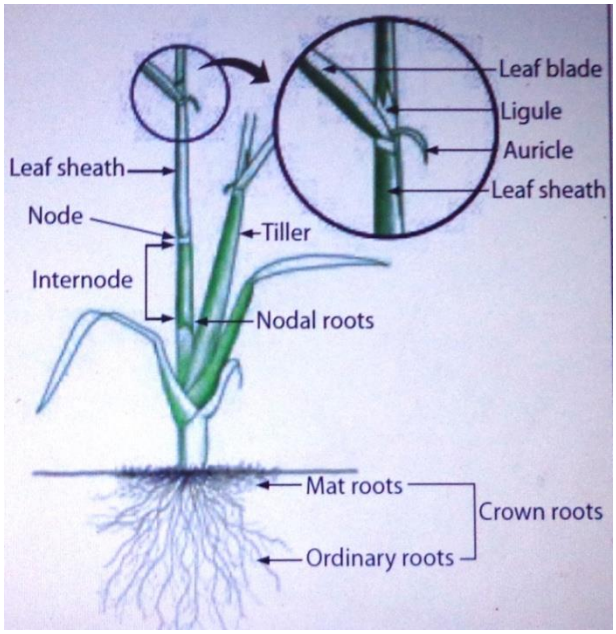


Figure 3: Parts of the rice stem and tillers (Source: GRiSP, 2013)

2.2.5 Panicle and spikelets

The major structures of the panicle are the base, axis, primary and secondary branches, pedicel, rudimentary glumes, and spikelets. The panicle axis extends from the panicle base to the apex; it has 8–10 nodes at 2- to 4-cm intervals, from which primary branches develop. Secondary branches develop from the primary branches. Pedicels develop from the nodes of the primary and secondary branches; the spikelets are positioned above them (Figure 4). The flower is enclosed in the lemma and palea, which may be either awned or awnless. The flower consists of the pistil and stamens, and the components of the pistil are the stigmas, styles, and ovary (GRiSP, 2013).

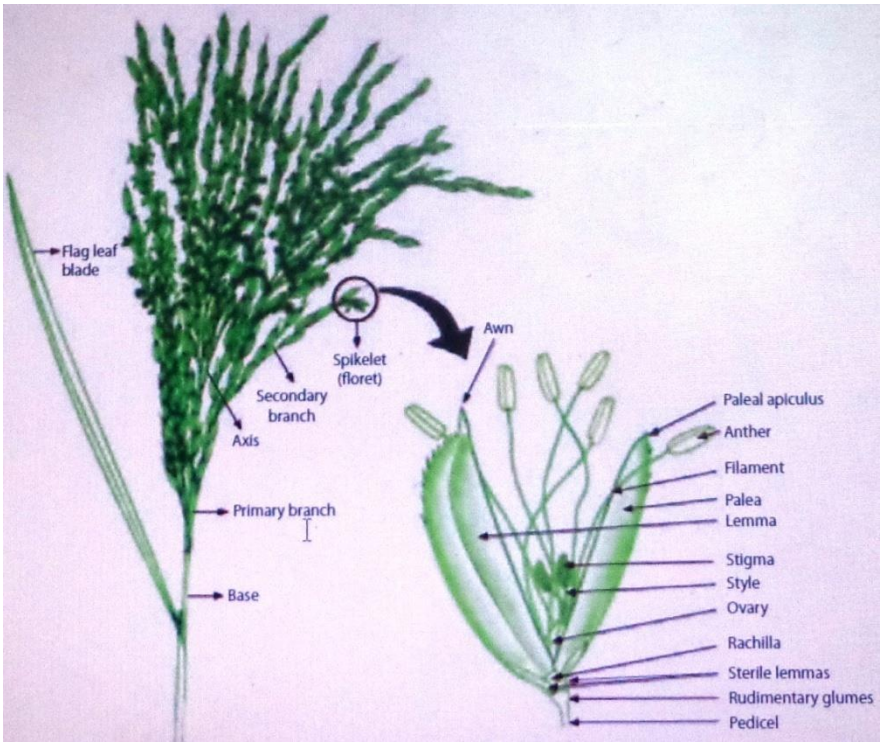


Figure 4: Rice panicle and spikelet **(Source: GRiSP, 2013)**

2.3 Importance of rice

Rice (*Oryza sativa L.)* is an important cereal staple food for a large part of the world population in terms of the area cultivated and amount consumed. Globally, more than 90 and 60 % of the global rice is grown and consumed in Asia and south East Asia, respectively. Rice is produced on about 10% of all crop land (144 million hectares) in over 110 countries ranking second to wheat in areas harvested (Bua *et al.,* 2013). Traditionally, rice has the potential to improve nutrition, boost food security, foster rural development and support sustainable land use in Africa. Accordingly, rice has grown to become an important food staple and source of cash income for the majority of the farming communities, Rice is also becoming increasingly popular in Africa, it is the

primary source of income and employment for more than 200 million households across countries in the developing world (FAO, 2004). In terms of health-and-its-nutritional-value, rice is considered a good source of vitamin B1 to prevent and cure Beriberi. Other major health benefits of rice include the provision of great source of instant energy; due to the high amount of carbohydrates in the rice, they are considered a good source of fuel to the body, rice also helps in normal functioning of the brain. Due to the absence of any harmful elements such as cholesterol and sodium, the eating of rice is highly beneficial for health. Rice is also considered a good source of different vitamins and other minerals including calcium fibber, iron and thiamine. The patients who are suffering from high blood pressure, the rice are the best diet for them as it does not contain sodium and cholesterol (Zahid, 2014).

2.4 Effect of cold stress on plants

The temperatures on the Earth's surface are very different, changing during the seasons as well as during the day and night (Zhang *et al*,. 2014). Low temperatures or cold stress is one of the main abiotic stress factor that has a strong impact on the survival, growth, reproduction and geographical distribution of crop plants (Zhu *et al.,* 2007) and it limits agricultural crop productivity and increase crop losses because plants are immobile (Kumar, 2013). Each plant is characterized by a certain genetically fixed level of resistance to low temperatures, which reduces its metabolic activity. This level of resistance can vary among individual plants and species. Cold stress can be classified as a chilling when the temperature is between 0 to15°C and freezing when the temperature is less than 0°C (Gulzar *et al*., 2011, Zhang *et al.,* 2014). Chilling damage can be observed

on many plant of tropical and subtropical origin when they are exposed to low positive temperatures (Aghae *et al*., 2011).

In general, plants from temperate climatic regions are considered as chilling tolerant with variable degree, and can increase their freezing tolerance by being exposed to chilling, non-freezing temperatures, a process known as cold acclimation (Miura *et al.,* 2013), which is associated with biochemical and physiological changes (Gilmour *et al*., 2000) and ultimately show marked changes in gene expression, biomembrane lipid composition, and small molecule accumulation (Sanghera *et al.,* 2011).

Many species of tropical or subtropical origins are injured or killed by non freezing low temperatures because of lack of mechanism of cold acclimation, and exhibit various symptoms of low-temperature injury such as chlorosis, necrosis, or growth retardation. In contrast, cold temperature tolerant species are able to grow at such cold temperatures (Gulzar *et al.,* 2011). Low temperature resistance in plants is a very complex trait, involving many different metabolic pathways and cell compartments (Sanghera *et al.,* 2011).

2.5 Symptom and effects of low temperature on rice growth and development

Rice, a kind of short day thermophilic crops requires certain temperature conditions at different stages of growth and reproduction. A low temperature is one of the main limiting factors for crop yield in the world (Sanghera et al., 2011).

Cold effect and damage depends on the function of the prevalent air, water temperature, photoperiod (day-length), cropping pattern, and variety (Zenna *et al.,* 2010; Hyun *et al.,* 2016). Crop failure can be observed when low temperature is manifested at different

growth stages, such as germination, seedling, vegetative, reproductive and grain maturity (Andaya and Mackill, 2003a, b).

At germination stage, temperatures below 20^{o}C can decrease both the speed and percentage of germination, resulting in poor seedling (da Cruz and Milach, 2000). Germination speed, in turn, relates to seedling vigor, and can be a significant determinant of good field performance (da Cruz and Milach, 2004). Under low temperatures, delayed germination, stunted seedling growth and leaf yellowing during early growth may result in non-uniform seedling growth and weak seedlings, which may affect final grain yield (da Cruz *et al.,* 2006). During the vegetative phase, cold during this phase slows down rice development and lengthens growth duration, Moreover, crop duration increases with low temperatures (Drame *et al.,* 2013).

During the reproductive phases of growth, including panicle initiation, booting, and flowering stages, the most sensitive stage for cold injury is the booting stage, especially the early pollen microspore stage, which occurs approximately 10–12 days prior to heading. Then, the flowering stage is the second most sensitive stage to the effects of low temperature (Matsuo *et al.,* 1995). Low temperature during the booting and flowering stages directly affects spikelet sterility which varies among varieties and increases with length of cold weather exposure (Andaya and Mackill, 2003a; Manneh *et al.,* 2007; Zenna *et al.,* 2010; da Cruz *et al.,* 2013). Spikelet sterility may result from pollen abortion due to cold during microsporogenesis when pollen grains are being formed at the booting stage (Mackill *et al.,* 1996; Sharifi, 2010). Sakata *et al.,* (2014) showed that microsporogenesis is disrupted by low temperature due to a reduction of bioactive gibberellins, GA4 and GA7. Sterility caused also by abnormalities in the reproductive

organs, including enlargement of anther cell walls and tapetal cells, inhibited anther dehiscence and pollination, reduction in the numbers of mature pollen, spikelet development and increased male sterility and reduces their fertility (Lee, 2001; Huang *et al*., 2012) those further influences the seed-setting rate of rice (Jing et al., 2012). During the grain filling stage, chilling temperature may cause delays and incomplete grain maturation and induce rapid leaf senescence, which can lead to a decrease in rice yields (Gunawardena *et al.,* 2003; Ye *et al.,* 2009; Shinada *et al.,* 2013).

Rice in the high-altitude areas has a lower efficiency of photosynthesis, while the rice in the low-altitude areas has a higher efficiency of photosynthesis, which reveals that the efficiency of photosynthesis tends to reduce with the increase of altitude (Jing *et al.,* 2012). Exposure to low temperatures causes physiological changes to the crop such as a decrease in total chlorophyll content (Aghaee *et al*., 2011), inhibition of photosynthetic activity and oxidative stress. Furthermore, there is an irreversible injury in leaves, such as necrosis, mottled chlorosis (Ye *et al*., 2009). Cold stress reduces photosynthesis, resulting in low dry matter accumulation (reduced growth), indirectly suppressing rice yields. The carbohydrates available for grain production become limited, leading to low yields (Wainaina *et al.,* 2015).

In the highlands of East Africa and the cold prone areas of the Sahel region, low temperature retards the rice plant's growth. This is a common problem among farmers who sow rice during cool seasons, and among those who grow rice at high altitudes and in areas that have a cold irrigation-water supply.

In Madagascar, in the high altitude region, cold stress, particularly during the winter months of May to October, is probably the most limiting factor for rice cultivation

additionally, the cultivation of local unimproved varieties, not adapted to the unfavorable climatic conditions, leads to high spikelet sterility (Rakotoarisoa, 2008).

Yoshida (1981) showed that sensitivity to cold varies between stages. According to his data, rice plants have a lower threshold temperature (10–13°C) for cold damage during the early stages of development (germination and vegetative), what makes them less sensitive to colder than during the reproductive stage, which has a higher threshold temperature for damage (18–20°C).

In order to cope with low temperature sensitivity, breeding is the most straightforward approach that has been used. Rice breeders have been making efforts to develop more cold-tolerant cultivars.

2.6 Variability for cold tolerance in rice

High variability for cold tolerance in rice has been reported in several studies. For instance, in a study on more than 700 japonica cultivars collected from Japan, Europe, China, Russia and other regions, Kotaka and Abe (1988) found a high genetic variability for seedling germinability. In addition, an evaluation of 20 Chilean rice genotypes and 192 Japanese accessions at 13ºC, led to the identification of cold tolerance genotypes, based on coleoptiles length reduction, coleoptiles length after cold treatment, coleoptiles length recovery and coleoptiles regrowth (Bosetti *et al* ., 2012; Ñanculao *et al.*, 2013). Similar studies were carried out in 477 landraces from five cropping regions in Yunnan, China, which is considered the center of genetic diversity and cold tolerance (Zeng *et al.*, 1999). This investigation showed that genetic variation for cold tolerance existed within accessions collected from this region and Northwest Yunnan housed the strongest cold tolerant landraces in China while South Yunnan had the most sensitive (Li *et al.*, 2004).

According to Dingkuhn and Miézan (1992), temperatures below 18 ^{o}C can induce up to 50% sterility and this can reach up 100% at 10^{o}C in very sensitive varieties.

In a study of reproductive stage cold tolerance conducted on 23 elite rice cultivars from eight countries, only accessions from Uzbekistan (Avangard and Mustaqillik) and Korea (Jinbu) showed high cold tolerance under cold-water and greenhouse conditions showing 71% to 79% spikelet fertility (Jiang *et al.,* 2010; Suh *et al.,* 2013).

2.7 Breeding for cold tolerance in Rice

Over the past 20 years, extensive efforts have been made to improve cold tolerance in rice, which is a very complex trait (Maruyama *et al.,* 2014). Introgression of cold tolerance into susceptible varieties has been a cost effective environmentally beneficial means of minimizing crop losses due to cold stress.

Breeding rice demands genetic variability. Fortunately, the rice species (*Oryza sativa L.*) have wide adaptability to cold, and cold-tolerant ecotypes are available for breeding.

The cultivated species *O. Sativa L* has two subspecies: *indica* and *japonica*. The *indica* subspecies includes cultivars better adapted to tropical environments such as India, China, and Indonesia, while *japonica* cultivars are more adapted to temperate climates such as the ones in Japan, Korea, and Java (Takahashi, 1984). Studies with large number of cultivars belonging to these two subspecies showed that *japonica* genotypes have a higher degree of cold tolerance at the germination stage (Mertz *et al.,* 2009) as well as at the vegetative and reproductive stages (Mackill and Lei, 1997), da Cruz and Milach (2004) also concluded that *japonica* genotypes presented higher cold tolerance at the germination stage than *indica* genotypes, although they found variability for this trait

within both subspecies. This agrees with previous reports of some *indica* genotypes from high-latitude regions that may present moderate level of chilling tolerance (Zhang *et al,* 2014).

Nevertheless, each country developed its own strategy for breeding for cold tolerance. However, it is clear that the main advancements have been obtained within the *japonica* cultivars. Therefore, the challenge still remains to develop *indica* type cultivars with adequate cold tolerance for the high-latitude regions, an apparent simple solution could be cross *indica* genotypes with *japonica* ones, in order to transfer genes for cold tolerance from *japonica*.

Continuous improvements in the tolerance of plants to unsuitable temperature in plant breeding programs have been performed using various strategies that have successfully produced many beneficial achievements (Sanghera *et al.*, 2011), as using combination of traditional breeding and molecular marker technology. Traditional breeding based on phenotypic selections and molecular marker technology through MAS based in the use of DNA markers that are tight-linked to target loci to follow regions of the genome that encode specific characteristics of a plant as a substitute or to assist phenotypic screening (Collard and Mackill 2008; Akhtar *et al.,* 2010) are a tool to improve cold tolerant on rice.

2. 8 Quantitative Trait Loci (QTL)

Many agriculturally important traits such as yield, quality and some forms of disease resistance, abiotic stress tolerant are controlled by many genes and are known as quantitative traits (also « polygenic », « multifactorial » or « complex » traits) (Collard *et al.*, 2005).

The regions within genomes that contain genes responsible for variation associated with a particular quantitative trait of interest are known as quantitative trait loci (QTLs) (Doerge, 2002; Collard *et al.,* 2005).

2. 8. 1 Principle of QTL mapping

QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. The markers are used to partition the mapping population in to different genotypic classes based on genotypes at the marker locus, and apply the correlative statistics to determine whether the individual of one genotype differ significantly with the individuals of other genotype with respect to the trait under study. A significant difference between phenotypic means of the two / more groups depending on the marker system and type of population indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait. A significant P value obtained for the differences between the marker and QTL is due to recombination. The closer a marker is from a QTL, the lower the chance of recombination occurring between marker and QTL. Therefore, the QTL and marker will be usually being inherited together in the progeny, and the mean of the group with the tightly-linked marker will be significantly different (P <0.05) to the mean of the group without the marker. When a marker is loosely-linked or unlinked to a QTL, there is independent segregation of the marker and QTL. In this situation, there will be no significant difference between means of the genotype groups based on the presence or absence of the loosely linked marker. Unlinked markers located far apart or on different chromosomes to the QTL are randomly inherited with the QTL; therefore, no significant differences between means of the genotype groups were detected (Veeresha *et al*., 2015).

Mapping means placing the markers in order, indicating the relative genetic distance between them and assaying them to their linkage groups on the basis of recombination values from all pair wise combination between the markers. Linkage map indicates the position and relative genetic distance between markers along chromosomes. We can analyze the segregation patterns for each of the markers by screening the mapping population using polymorphic molecular markers, which is referred as genotyping. A vanity of molecular markers viz., RFLPs, RAPD, SSRs, AFLP, and SNPs etc have been used to identify individual QTLs and to find out effects and position of these QTLs. The commonly used molecular markers along with important advantages and disadvantages are presented in Appendix 1.

2. 8. 2 Steps in QTL Mapping

The process of QTL mapping involves the four major steps Figure 5

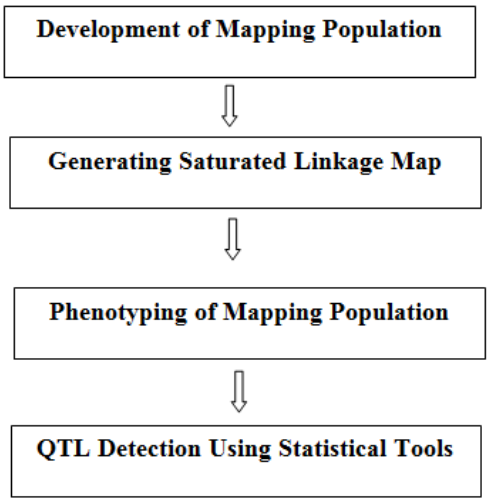


Figure 5: Various steps in the identification of quantitative trait loci (QTL) for use in marker assisted selection

2.9 Developing of Mapping Population

A suitable mapping population generated from phenotypically contrasting parents is prerequisite for QTL mapping (Example highly resistant and susceptible lines). The parental lines used in development of mapping population should be genetically diverse, which enhance the possibility of identifying a large set of polymorphic markers that are well distributed across the genome. Several different populations may be utilized for mapping within given plants species as shown in Figure 6. With each population type possessing advantages and disadvantages. The mapping population could vary based on the objective of study, the time frame line and resources available for undertaking QTL mapping. The ability to defect QTL in F_2 or F_2 derived populations and RILs are relatively higher than other mapping population. The $F_{2:3}$ families have the advantage that it is possible to measure the effects of additive and dominant gene actions at specific loci. The RILs are essentially homozygous and only additive gene action can be measured, the advantage with RILs is that the experiments can be performed at several locations in multiple years.

The size of the mapping population for QTL analysis depends on several factors viz., type of mapping population used for QTL analysis, genetic nature of the target trait, objective of the study, and resources available for handling a sizable mapping population in terms of phenotyping and genotyping. From the practical point of view the purpose of QTL mapping is to detect the QTL, with major effects. However, there is usually a high cost associated with genotyping (generation of molecular marker data) and phenotyping (field, greenhouse or screen house evaluation for the phenotypic trait) of large population size, particularly for traits requiring extensive field trials or complex analysis.

Consequently, the size of the mapping population and the number of replications and sites (environments) for phenotyping is often limited. Thus, most published experiments have used between 100 and 200 progenies (Somers *et al*., 2003).

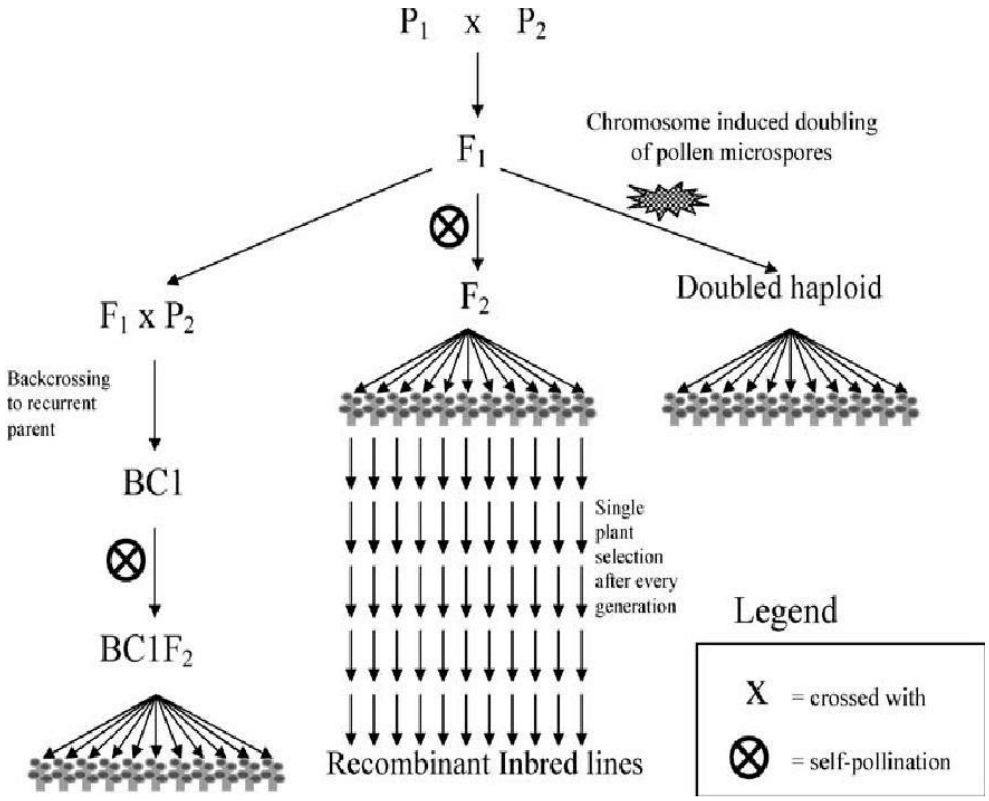


Figure 6: Diagram of the main types of mapping populations for self-pollinating species (Source: Collard *et al.*, 2005).

Overall, the QTL mapping literature has shown that if a breeder can develop a mapping population of 100-150 progenies derived from an F_2 or backcross population between two inbreds, obtain reasonably good phenotypic data for the traits of interest, and genotype the population with markers spaced about 10 to 15 cM apart, then an analysis of the phenotypic and marker data with an appropriate statistical method will almost always

lead to the identification of at least a few markers associated with each trait of interest (Bernardo, 2008).

Mapping means placing the markers in order, indicating the relative genetic distance between them and assaying them to their linkage groups on the basis of recombination values from all pair wise combination between the markers. Linkage map indicates the position and relative genetic distance between markers along chromosomes. We can analyze the segregation patterns for each of the markers by screening the mapping population using polymorphic molecular markers, which is referred as genotyping.

2.10 Markers and maps

One component of observed data in QTL mapping experiments is the markers. Various properties of different types of markers are important to consider in QTL mapping experiments.

2.10.1 DNA or Molecular markers

DNA markers or molecular markers are defined as a fragment of DNA revealing mutations/variations, which can be used to detect polymorphism between different genotypes or alleles of a gene for a particular sequence of DNA in a population or gene pool (Jiang, 2013). DNA markers, which reveal sites of variation in DNA are unlimited in number and are not affected by environmental factors and developmental stages of the plant (Winter and Kahl, 1995, Jones *et al*., 1997). Molecular markers are preferred for genotyping because these markers are unlikely to affect the trait of interest. Molecular marker is in a sequence of DNA, which are located with a known position on the

chromosome (Kumar, 1999), or a gene whose phenotypic expression is frequently easily discerned and used to detect an individual, or as a probe to mark a chromosome, nucleus, or locus (Schulmann, 2007). Markers show polymorphism, which may arise due to alteration of nucleotide or mutation in the genome loci (Hartl and Clark, 1997) and make it possible to identify genetic differences between individual organisms or species (Collard *et al.* 2005). Molecular markers can be used in, population studies (Hartl and Jones, 2005).

Various types of molecular markers are used to estimate DNA polymorphism and are classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers (Irshad, 2014).

Hybridization based markers

In hybridization based markers DNA profiles are visualized by hybridizing the restriction endonuclease digested DNA fragment, to a labeled probe, which is a DNA fragment of known sequence such as RFLP.

Polymerase chain reaction based markers

PCR based markers involve in vitro amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified DNA fragments are separated by electrophoresis and banding patterns are detected by different methods such as staining (using ethidium bromide dye) and autoradiography (Acquaah, 2012, Irshad, 2014).

2.10.2 Types of molecular markers

Several types of markers are used, including, restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs, or microsatellites), single nucleotide polymorphisms (SNPs) (Jonah *et al.*, 2011; Lateef; 2015).

2.10.2.1 Simple sequence repeats (SSRs, or microsatellites)

SSRs also called microsatellites; short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS) are PCR-based markers. They are randomly tandem repeats of short nucleotide motifs (2-6 bp/nucleotides long) found at high frequency in the nuclear genomes of most taxa (Idrees &Irshad, 2014). Di-, tri- and tetra-nucleotide repeats, e.g. (GT)n, (AAT)n and (GATA)n, are widely distributed throughout the genomes of plants and animals. The copy number of these repeats varies among individuals and is a source of polymorphism in plants. Because the DNA sequences flanking microsatellite regions are usually conserved, primers specific for these regions are designed for use in the PCR reaction. One of the most important attributes of microsatellite loci is their high- level of allelic variation, thus making them valuable genetic markers. The unique sequences bordering the SSR motifs provide templates for specific primers to amplify the SSR alleles via PCR, only small amounts of DNA need for amplification as thermocycling in this manner creates an exponential increase in the replicated segment (Irshad, 2014). SSR loci are individually amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence. The PCR-amplified products can be separated in high-resolution electrophoresis systems such as agarose gel electrophoresis (AGE) or polyacrylamide gel

electrophoresis (PAGE) and the bands can be visually recorded by fluorescent labeling or silver-staining. SSR markers are characterized by their hyper-variability, reproducibility, co-dominant nature, locus-specificity, and random genome-wide distribution in most cases. The advantages of SSR markers include that they can be readily analyzed by PCR and easily detected by PAGE or AGE. SSR markers can be multiplexed, have high throughput genotyping and can be automated. SSR assays require only very small DNA samples (~100 ng per individual) and low start-up costs for manual assay methods. However, SSR technique requires nucleotide information for primer design, labor-intensive marker development process and high start-up costs for automated detections. Since the 1990s SSR markers have been extensively used in constructing genetic linkage maps, QTL mapping, marker-assisted selection and germplasm analysis in plants. In many species, plenty of breeder-friendly SSR markers have been developed and are available for breeders (Jiang, 2013).

2. 10.2.2 Single nucleotide polymorphism (SNP)

A SNP is a single nucleotide base difference between two DNA sequences or individuals. SNPs can be categorized according to nucleotide substitutions either as transitions (C/T or G/A) or transversions (C/G, A/T, C/A or T/G). In practice, single base variants in cDNA (mRNA) are considered SNPs as being single base insertions and deletions (indels) in the genome. SNPs provide the ultimate/simplest form of molecular markers as a single nucleotide base is the smallest unit of inheritance, and thus they can provide maximum markers. SNPs occur very commonly in animals and plants. Typically, SNP frequencies are in a range of one SNP every 100-300 bp in plants (Xu, 2010). SNPs may present within coding sequences of genes, non-coding regions of genes or in the

intergenic regions between genes at different frequencies in different chromosome regions.

Based on various methods of allelic discrimination and detection platforms, many SNP genotyping methods have been developed. A convenient method for detecting SNPs is RFLP (SNPRFLP) or by using the CAPS marker technique. If one allele contains a recognition site for a restriction enzyme while the other does not, digestion of the two alleles will produce different fragments in length. A simple procedure is to analyze the sequence data stored in the major databases and identify SNPs. Four alleles can be identified when the complete base sequence of a segment of DNA is considered and these are represented by A, T, G and C at each SNP locus in that segment. There are several SNP genotyping assays, such as allele-specific hybridization, primer extension, oligonucleotide ligation and invasive cleavage based on the molecular mechanisms (Sobrino *et al.,* 2005), and different detection methods to analyze the products of each type of allelic discrimination reaction, such as gel electrophoresis, mass spectrophotometry, chromatography, fluorescence polarization, arrays or chips, etc. At the present, SNPs are also widely detected by sequencing (Xu, 2010).

SNPs are co-dominant markers, often linked to genes and present in the simplest/ultimate form for polymorphism, and thus they have become very attractive and potential genetic markers in genetic study and breeding. Moreover, SNPs can be very easily automated and quickly detected, with a high efficiency for the detection of polymorphism; it allows rapid and efficient genotyping of large numbers of samples (Davies *et al.,* 2016; Jiang *et al.,* 2016). Therefore, it can be expected that SNPs will be increasingly used for various

purposes, particularly as whole DNA sequences become available for more and more species (e.g., rice, maize, etc).

2.11 Phenotyping of Mapping Population

The target quantitative traits have to be measured as precisely as possible. Strictly speaking there should not be any missing data, but limited amounts of missing data can be tolerated. The missing data in the population causes the effective in the sample size and intern affect the power of QTL mapping. The data is pooled over location and replication to obtain a single quantitative value for the line. It is also necessary to measure the target traits in experiments conducted in multiple location to have better understanding of the QTL x Environment interaction.

2.12 Statistical methods for QTL detection

The basic purpose of QTL mapping is to detect QTL, while minimizing the occurrence of false positive (Type I Error) i.e. declaring an association between a marker and QTL when in fact it does not exists)

a) Single Marker Analysis (SMA)

It is referred as single point analysis. It is the simplest method for detecting QTL associate with single markers (Veeresha *et al.*, 2015). The statistical method used for the single point analyses includes T-test, analyses of variance (ANOVA) and linear regression. SMA is done for each marker locus independent of information for other loci. This method does not require complete linkage map and can be performed with basic statistical software programs. However the major disadvantage is that the further QTL is

from a marker, the less likely it will be detected. This is because recombination may occur between the marker and the QTL. The effect of QTLs is likely to be underestimated because these are confounded recombination frequencies. The use of large markers covering the entire genome may minimize these problems (Veeresha *et al.*, 2015).

b) Simple Interval Mapping (SIM)

Simple Interval Mapping was first proposed by Lander and Botstein in 1989. SIM method makes use of linkage maps and analysis intervals between adjacent pairs of linked markers along the chromosomes, simultaneously, instead of analyzing single markers. Presence of a putative QTL is estimated if the logarithm of odds ratios (LOD) exceeds a critical threshold which is more often fixed as ≥3. The use of linked markers for analysis compensates for recombination of the marker and the QTL, and is considered statistically more powerful than SMA. Many researchers have used Mapmaker/QTL, and QGene (Nelson, 1997) to construct SIM.

c) Composite Interval Mapping (CIM)

Composite Interval Mapping is one of the popular methods used to detect QTLs. CIM was developed by Zeng (1994) and MQM (Multiple QTL model or marker –QTL marker analysis) by Jansen and Stam (1994). This method combines interval mapping with linear regression. It considers a marker interval plus a few other well-chosen single markers in each analysis. The main advantage of CIM is that it is more precise and effective at mapping QTLs compared to SMA and SIM, especially when linked QTL are involved. Many researchers have used QTL Cartographer (Basten *et al.,* 1994) and Map manager QTL (Manly *et al.,* 2001) to perform CIM.

d) Inclusive composite interval mapping

Composite interval mapping (CIM) is one of the most commonly used methods for QTL mapping with populations derived from biparental crosses. However, the algorithm used in CIM cannot completely ensure that the effect of QTL at current testing interval is not absorbed by the background marker variables, and may result in biased estimation of QTL effect. Inclusive composite interval mapping (ICIM), developed by Meng *et al.,* (2015) has proved to be more efficient that CIM for background control via a two-step mapping strategy (Wang, 2009; Zhang *et al*., 2012). In the first step, stepwise regression was applied to identify the most significant regression variables. While in the second step; interval mapping is performed using phenotypes adjusted by the markers identified in the first step. ICIM provides intuitive statistics for testing additive, dominance and epistasis, and can be used for most experimental populations derived from two inbred parental lines. ICIM therefore, retains all advances of CIM over the simple interval mapping, and avoids the possible increase of sampling variance and the complicated background marker selection process in CIM (Li *et al*., 2012).

The ICIM Method has been used to map QTL in a number of studies including Map QTL for cold tolerance stress at seedling stage in rice (*Oryza sativa L*.) (Zhang *et al*., 2007). Since ICIM has the ability to identify QTL with increased precision then CIM, it was used to map QTL.

2.13 QTLs and Cold Tolerance in Rice

QTLs related to tolerance to low temperatures in rice have been identified by the use of restriction fragment length polymorphisms (RFLPs) (Takeuchi *et al.,* 2001) and

microsatellite/ simple sequence repeats (SSR) molecular markers Andaya and Mackill, 2003a,b; Lou *et al.*, 2007; Suh *et al.*, 2010).

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 (Andaya and Mackill 2003a, b; Fujino *et al.*, 2004), indicating that cold tolerance may be developmentally regulated and growth stage specific. About QTLs, at least 15% of the variability related to rice cold tolerance reported by different groups. These loci probably have large potential for successful application in rice genetic breeding aiming at cold tolerance in different developmental stages.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

Crosses between two different susceptible parents Vary botry and Soameva with a donor cold tolerant parent Chomrongdhan was carried out at the greenhouse, IRRI-ESA regional hub located in Burundi; and the F_{1s} were advanced up to the BC_1F_2.

Burundi is a landlocked country in the African great lakes region of East Africa, bordered by Rwanda to the north, Tanzania to the east and south, and the Democratic Republic of the Congo to the west. IRRI Burundi hub is situated in the capital Bujumbura within the campus of the University of Burundi, where laboratory and other facilities are shared with the Faculty of Agricultural Sciences. IRRI Burundi hub focused on developing and testing new rice varieties matched to the different rice production ecologies across Eastern and Southern Africa.

DNA extraction for molecular work and genotyping study was executed at the Molecular Laboratory of the Kenya Agricultural and Livestock Research Organization (KARLO, Njoro). Phenotyping study in a controlled condition experiment at seedling and reproductive stage was conducted also at KARLO, Njoro.

Genotyping analysis was accomplished at the Genotyping Services Lab (GSL, International Rice Research Institute IRRI, the Philippines); GSL is located at the IRRI headquarter, in the Philippines, GSL has been created to support the needs of rice breeding programs within the Global Rice Science Partnership. GSL has optimized the

sample preparation workflow to increase the efficiency of rice leaf tissue sampling, DNA extraction, and DNA quality control in preparation for Single nucleotide polymorphism (SNP) genotyping.

3.2 Plant materials

The recurrent parents used for this study were selected based on the importance of those varieties by farmers in the high altitude region of Madagascar. They belonged with an *Indica* group (Mather *et al.,* 2010). "Vary botry" is a traditional variety, while "Soameva" is an improved variety. They are the most common irrigated rice varieties used by the farmers in the high altitude region where cold stress affect more to rice cultivating.

Although Vary botry and Soameva have a moderately high yielding, and number desirable characters as submergence tolerant (because of having a taller plant), disease resistance and tolerant to phosphorus deficiency and iron toxicity particularly for Vary botry (Shahi *et al.,* 1988), limitation of Vary botry and Soameva was lack of cold tolerance like most of the *Indica* type.

The donor parent used for this study was Chomrongdhan, it belongs to the temperate *Japonica*, and it was sourced from Nepal. It was selected from a landrace well adapted to the cold conditions of high altitude in Nepal (Vales et Razafindrakoto, 1997). Chomrongdhan has an excellent panicle exsertion; it was also characterized by leaf blast and cold tolerant with short duration development. It is able to grow in the Irrigated and in the upland rice ecosystems at the high altitude between 1000 to 1850m of Madagascar.

3.3 Experimental design

3.3.1 Experimental design of cross between Vary Botry x Chomrongdhan

The experimental design used for the progeny BC_1F_2 from Vary botry//Chomrongdhan was augmented randomized complete block design (ARCBD); constructed the layout by using the Plant Breeding Tools software (IRRI, 2014). ARCBD is given for a pot culture experiment comprised ten blocks, within the donor and recurrent parents used as checks that appear exactly once in each block, and the 500 treatments that were not replicated in the trial. The 500 treatments were the seeds originated from randomized seeds of bulk harvested of BC_1F_2 plant from Vary botry//Chomrongdhan that are replicated once in the trial (Layout presented in Appendix 2).

3.3.2 Experimental design of cross between Soameva x Chomrongdhan

The experimental design used for the BC_1F_2 progeny from Soameva//Chomrongdhan was conducted in a completely randomized design (CRD), constructed the layout by using excels software. The layout of the CRD is given for a pot culture experiment with five seeds of 27 treatments and the parents, each replicated 4 times. 27 treatments were the seeds originated from $27BC_1F_2$ plants harvested from single plants from Soameva//Chomrongdhan (Layout shown in Appendix 3)

3.4 Experiment 1: Population development

3.4.1 F_1 Development

Two hundred fifty six (256) and sixty nine (69) seeds of F_1 progeny was obtained with initial cross between Vary botry (recurrent parent) with Chomrongdhan (donor parent), then Soameva (recurrent parent) with Chomrongdhan respectively.

The crosses were made at IRRI Burundi hub during the period of December 2013. The susceptible parents were used as female parent, whereas the tolerant parent was used as the male parent. The crossing achieved through staggered planting to synchronize the flowering times of parents for hybridization.

Emasculation was done to avoid self-fertilization and to produce a female parent. Rice is ready for emasculation when the panicles have emerged about 5 to 10 cm from the leaf sheath. The upper and lower part of the panicle was removed and only the middle part was used. Emasculation was carried out by removing the unopened spikelets and also cutting open the spikelets near its midpoint with small scissors so that the anthers can be easily seen. Anthers (6 per spikelet) were removed with fine-pointed forceps. The emasculated spikelets were covered with butter paper bag over each panicle to protect them from being naturally pollinated by unwanted foreign pollen.

Pollination was carried out by collecting the panicle with pollen of the male parent before anther dehiscence, the top of butter paper bag from the female parent was opened; at that time, the panicle of female parent was placed below the male panicle that have a pollen which was shaken gently to release the pollen. Rebagging was done with butter paper bag

after pollinated and the plant were tagged and labeled. The variety names of the male parent and date of pollination was added to the original identification tag on the female plant.

3.4.2 BC_1F_1 and BC_1F_2 population development

Through F_1 plants developed BC_1F_1 and BC_1F_2. BC_1F_2 was used as advanced progeny for phenotyping and genotyping for this study. The process to develop BC_1F_1 and BC_1F_2 was as follows, to have a backcross one for first generation (BC_1F_1), F_1 individual plant have been crossed to the recurrent parent, after that BC_1F_1 individual plant have been grown and seeds of BC_1F_2 were obtained through self pollination of the BC_1F_1 plant (Figure 7).

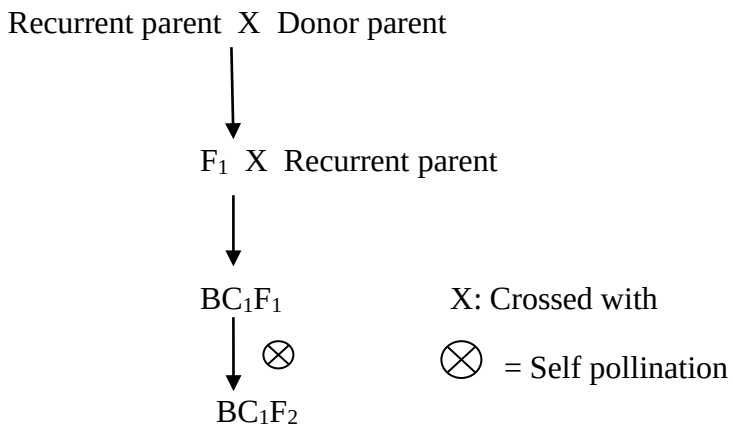


Figure 7: F_1, BC_1F_1 and BC_1F_2 population development

3.5 Experiment 2: Phenotyping and screening for cold tolerance in controlled condition

3.5.1 Cold treatment at seedling stage

For this study, plants from 500 BC_1F_2 seeds originated from BC_1F_2 plant harvested as a bulk from Vary botry//Chomrongdhan, and plants from 540 BC_1F_2 seeds originated from BC_1F_2 harvested as a single plant (27 plants) with 5 seeds for each plant and replicated for 4 times from Soameva//Chomrongdhan were used to evaluate cold tolerance at seedling and reproductive stage. The experiment was carried out in a greenhouse (have a normal condition) and the cold room (have a controlled condition) at KARLO Njoro, Kenya.

Pre germinated in a Petri dished with distilled water at 28-30^oC for 48 hours a dry and broken dormancy seeds of 500 BC_1F_2 seeds from BC_1F_1 plants (bulk harvested for Vary botry//Chomrongdhan) and 540 BC_1F_2 seeds from 27 BC_1F_1 plants (single plant harvested for Soameva//Chomrongdhan), and 10, 16 and 26 seeds of Vary botry, Soameva and Chomrongdhan respectively. Parents were used as cold-susceptible and cold-tolerant checks, respectively.

Pre germinated seeds were planted in a pot filled with soil around 5kg and animal manure and they were planted in the greenhouse under the natural conditions until seedling plant reached three and four leaf stage.

When seedling reached the three and four leaf stage, the 500 and 540 BC_1F_2 plants and check plants were moved to the cold room with a constant temperature of 12°C under a cycle of 12-h light (15000 LX) and 12-h dark (Yang *et al.,* 2013) for 10 days for cold treatment. The treated seedling plants were moved back to the greenhouse after 10 days to allow seedling to recover and resume normal growth.

3.5.2 Cold treatment at reproductive stage

When surviving plants of BC_1F_2 behind the cold treatment applied at seedling stage reached at reproductive stage as well at booting stage, the plants were taken to an artificially lighted room where the cold temperature treatment at reproductive stage was initiated. This room had a constant temperature of 15°C and a photoperiod of 9h light/15h dark and, after cold exposure for 10 days the pots were taken back to the greenhouse to allow the plants to recover and resume normal growth until the seed maturing stage. Light in the cold room was provided by fluorescent and incandescent lamps and corresponded to 15000 LX. Differences in the greenhouse were an uncontrolled variable, since in the latter the radiation source was the sun.

3.6 Scoring and phenotypic data collection

Cold tolerant related traits at seedling and reproductive stage were evaluated using standard evaluation system for rice (SES) (IRRI, 2002; IRRI 2014).

3.6.1 Cold stress evaluation at seedling stage

Cold tolerances related traits were evaluated at seedling as well plant survival rate, seedling growth, seedling vigor and seedling leaf growth.

The plant survival rate was evaluated by counting the number of surviving plant 7 days after recovering (7DAR).

Seedling growth, seedling vigor and seedling leaf growth was scored and measured at 0, 7 and 14 days after recovering

Seedling growth was evaluated by measuring the percentage number of leaf injury symptoms, then leaf injury symptoms were scored as follows: 1- dark green; 3- light green; 5- yellow color; 7- brown color and 9- seedlings dead. The rating of 1 to 3 was regarded as tolerant and moderately tolerant individuals while rating of 5 to 9 indicated a moderately susceptible to High susceptible to individuals

Seedling vigor was evaluated by computing the percentage of leaves number, tiller number, then seedling vigor was scored as follows: 1- Extra vigorous and plant has 5-6 leaves and 2 or more tillers; 3- Vigorous and plant has 4-5 leaves and 1-2 tillers; 5- Normal and plant at 4-leaf stage; 7- Weak and plants somewhat stunted, 3-4 leaves and no tiller formation; 9- Very weak, plant stunted growth and yellowing of leaves and no tiller formation. The rating of 1 to 3 was regarded as tolerant and moderately tolerant individuals while rating of 5 to 9 indicated a moderately susceptible to High susceptible to individuals.

Seedling leaf growth was evaluated by measuring the percentage of the degree of leaf wilting, and then the degree of leaf wilting was scored a scale of 1–3 with all leaves normal, no apparent visual injury to 4–9 with all leaves wilted, seedlings apparently dead. The rating 1 to 3 was regarded as tolerant plant while rating of 4-9 indicated susceptible plant.

3.6.2 Cold stress evaluation at reproductive stage

Scoring and data collection was done to the survival plant of each BC_1F_2 plants and the parents in the greenhouse after cold treatment. The numerical scores provided in the SES were used instead of the observed characters. In total ten traits were evaluated at reproductive stage. The morphological characters included, tiller number, plant height, panicle exsertion, plant fertility, number of full seeds, numbers of empty seeds, panicle weight, acceptability phenotypic, thousand seeds weight, and heading date. Visual observation, ruler for measurement and precision balance were used to observe clearly or score those traits.

Tiller number was computed with held the plant and the tiller was counted

Plant height was measured by holding the meter (ruler) from the soil surface to the top of the tallest plant

Panicle exsertion was evaluated by measuring the relative distance from the flag leaf ligule to the panicle node in centimeter, and then panicle exsertion was scored as follows: 1- Enclosed panicle; 3-partly exserted panicle; 5-just exserted panicle; 7- Moderately well exserted and 9- well exserted

Plant fertility was evaluated by assessing the spikelet fertility with counted the number of full seeds and the number of empty seeds, then calculated the percentage of spikelet fertility, and then spikelet fertility was scored as follows: 1- more than 90%; 3- 75 to 90%; 5- 50-75%; 7- less than 50% to trace and 9- 0%. The rating 1 to 3 was regarded as highly fertile to fertile while a rating of 5-9 indicated partly sterile to highly sterile.

Number of full seeds and Empty seeds were computed by counting the number of fertile and infertile grains respectively for each plant.

Phenotypic acceptability was evaluated with examined visually for overall acceptability of plant; then phenotypic acceptability was scored as follows: 1- excellent plant type and absence of disease; 3- very good appearance, 5- fair appearance, but has a few essential shortcomings; 7- poor appearance, but has a few important traits that make it suitable; 9- poor.

Thousand seed weight was evaluated by examining the weight of grains using precision balance

Heading date was evaluated with calculated the number of the days from sowing date to heading.

3.7 Correlation between Evaluated Traits

The correlation analysis was determined with measured the linear association between two phenotypic variables.

3.8 Experiment 3: Genotyping of BC_1F_2

3.8.1 SNP genotyping to cold tolerance

The aim of this experiment was to determine the genetic make-up (genotype) of each individual in the developed BC_1F_2 plants.

3.8.1.1 Extraction of genomic DNA

Young leaves of each individual BC_1F_2 plant and their parental were harvested and genomic DNA was extracted using CTAB (Cetyltrimethylammonium bromide) method with modifications based on the procedure described by (Murray and Thompson 1980).

Before grinding, the mortar and pestle were sterilized to avoid contamination by burning 95% ethanol solution in the mortar. Water baths was pre-heated to 65°C before beginning the extraction. CTAB extraction buffer were prepared and pre-heated in the 65°C water bath.

The tissues leaves (about 1 g of leaf tissue) were placed into the sterilized mortar and two times of 650µl CTAB buffer were added, then the leaves were ground and transferred into 2ml eppendorf tuber. The eppendorf tube with the sample was placed into the 65°C water bath and mixed by inversion every 10 min for 30 min to 1 h. After incubation, the sample tube was centrifuged for 10 min at 13500 rpm and the supernatant decanted into a new 1.5µl of eppendorf tube.

One volume of chloroform: isoamyl alcohol was added to the solution and mixed by shacking for 20min. The samples were centrifuged for 10 min at 13500 rpm and pipetted carefully the upper aqueous phase into a new eppendorf tube. Nucleic acids were precipitated by adding equal volume of cold-isopropanol and incubated at -20°C overnight.

The contents were centrifuged at 13500rpm for 10 minutes and then the isopropanol was discarded to obtain a nucleic acid. The pellet was washed with 70% ethanol

(500Microlitter), the ethanol was discarded and the pellet was dried, the tube was opened to evaporate residual, then the DNA palette was suspended in 50 µl of TE and was added of 5 µL of RNAse A (10 mg/mL) to the solution to digest RNA and it was incubated at 37°C for 30 minutes to enhance the activity of RNAse in the digestion of RNA.

3.8.1.2 DNA quality and quantity determination

The quality and quantity of the DNA were assessed by using agarose gel electrophoresis method as described by Sambrook and Russell (2001). 1% of agarose gel was prepared by dissolving 1g agarose in 100ml 0.5 x tris borate EDTA (TBE) electrophoresis buffer. the mixture was heated in a microwave for 2minutes to dissolve the agarose, and was added 3µl of Gel red stains when the gel was cooled, it was pour into a horizontal gel tray and put an appropriate comb to form a hole in the gel. After polymerization of gel the combs was removed carefully and the gel on the gel tray was immersed in electrophoresis tank containing electrophoresis buffer (0.5x TBE).

A five micro liter of suspended DNA mixed with 2 µl of loading dye was loaded into separate holes of submerged agarose gels. The sample was run at 80 volts for 30minutes then the gel was removed and photographed under UV gel documentation system.

The concentration and purity of rice genomic DNA was assessed by using NanoDrop spectrophotometer system. One microliter of each diluted DNA sample (50ng/ µl) was placed on spectrophotometer. The concentration was checked in $ng\mu l^{-1}$ and was read the ratio OD260/OD280 to conclude the DNA purity. A DNA ratio (OD260/OD280) of 1.8 to 2 was considered a good quality (Thermo Scientific, 2010), if the ratio is appreciably

lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm).

3.8.2 SNP Genotyping

3.8.2.1 Infinium SNP chip

The Infinium SNP chip rice 6K containing 4,606SNP markers evenly distributed on 12 rice chromosomes were used to genotype 144 and 128 BC_1F_2 from Vary botry//Chomrongdhan and Soameva//Chomrongdhan respectively the parents. Infinium SNP genotyping was carried out in the Genotyping Service Laboratory (GSL) at IRRI, the Philippines.

The major steps of illumina SNP genotyping: DNA amplification, chip hybridization, staining and scanning were performed according to the infinium assay standard protocol (Infinium HD Assay ultra, manual, (detail Appendix 4).

3.8.2.2 Linkage mapping

Polymorphic markers between two parents were tested for goodness of fit to establish the deviation of observed frequency from expected 1:2:5 Mendelian segregation ratio for BC_1F_2 population. The SNP markers that showed high segregation distortion were excluded from the analysis. The remaining markers were subjected to the BIN tool in the QTL IciMapping software to identify and remove redundant markers (i.e. markers that are completely correlated in a population and therefore, cannot provide additional information if more than one of them is considered in a data set (Wang *et al.*, 2014).

A logarithm of odds (LOD) criteria of 3.0 was used to construct genetic map (Wang *et al.,* 2014). Ordering of marker was done by input command since physical positions of the markers on the 12 chromosomes was known. After ordering, markers were rippled using sum of adjacent recombination frequencies (SARF) that gave a short linkage map. Conversion of recombination fractions between linked loci into centimorgan (cM) was obtained using Kosambi's mapping function (Kosambi, 1944). The final map was drawn by QTL IciMapping software version 4 (Wang *et al.,* 2014). Correspondence of linkage groups and order of the markers on chromosomes was inferred from the physical map of Nipponbare reference genome Michigan State University version 7 (Kawahara *et al.,* 2013). The process of linkage map is given in Appendix 5.

3.9 Data analysis

Phenotypic data

Analysis of variance (ANOVA) were performed to analyze phenotypic data related to cold stress using the SAS version 9.1.3 (SAS, 2000) with proc Mixed and using PBTools software (IRRI, 2014).

Correlation analysis between evaluated traits was performed using also the STAR software (IRRI, 2014).

The analysis of variance was fitted by the following statistical model:

Statistical model to "Vary botry" and Chomrongdhan

$Y_{ij} = \mu + ß_i + C_j + \tau_{k(i)} + \varepsilon_{ij}$ (mean + blocks + checks + new entries + error)

Where: Y_{ij}: = observed new genotype response; μ: overall population mean; ß: effect due to i^{th} block in the j^{th} check and $k(i)^{th}$ new genotype; C, is the effect due to j^{th} check in k(i) new genotypes; τ is the effect of $k(i)^{th}$ new genotypes by i^{th} block and j^{th} check, ε_{ij} represents the residual error for ij block.

Statistical model to "Soameva" and Chomrongdhan

$Y_{ij} = \mu+\lambda_i+\varepsilon_{ij}$

Where: Y_{ij}: = observed new genotype response; μ: overall population mean; λ: effect due to i^{th} treatment (new progeny and parents) of the jth rep; εij Are the error made in the j^{th} rep of the i^{th} treat

At seedling stage, plant survival rate was used to calculate the cold tolerance indices (CTI) as described by Li *et al (2006).* The CTI was derived as follows:

$$\text{Cold Tolerance Indice (CTI \%)} = \frac{\text{Surviving seedling}}{\text{Total seedling treated}} \text{X } 100$$

CTI less than 30% is indicate that seedling genotype presents a sensitive reaction, CTI between 30 and 70% is indicate that seedling genotype presents an intermediate reaction and CTI more than 70% is indicate that seedling genotype present tolerant reaction.

At reproductive stage spikelet fertility was calculated as follows:

$$\text{Spikelet fertility (\%)} = \frac{\text{Number of grains (Filled Spikelet)}}{\text{Total Number of spikelet (Filled, Partially filled, Empty)}} \text{ X } 100$$

3.9.2 Genotypic data

3.9.2.1 Mapping and QTL analysis

Data from all SNP markers that detected polymorphism between donor and recurrent parents were used to construct the linkage map. 4606 SNP were used for parental polymorphism survey. The polymorphic markers were selected for QTL analysis and construction of linkage map.

Mapping and QTL for Cold tolerance were detected using phenotypic data at seedling and at reproductive stage of the BC_1F_2 population. QTL analysis was conducted by the inclusive composite interval mapping (ICIM) using QTL IciMapping version 4 software (Wang *et al*, 2014).

A logarithm of Odds (LOD) threshold of 2.5 was initially used to declare major QTL in this study. After that, 1,000 permutations at a probability of 0.05 were used to declare definitive QTL. A chromosomal walk speed of 1.0 cM and default window size of 8cM was used for all QTL estimation. QTL effects were estimated as proportion of phenotypic variance explained (PVE) by the QTL within positive or negative additive effects that was used to identify the origin of the favorable alleles. Positive additive effect indicates that the allele from the donor parents contributed the phenotypic values, while a negative additive effect indicates provenience from the susceptible parent.

3.9.2.2 Nomenclature

Identified rice QTL was named following the nomenclature of McCouch *et al.,* (1997). QTL names was an italic and started with a lower case letter “q” to indicate that it is a

QTL, followed by a two to five letters standardized “trait name” (example PH for Plant height), Added a dash (-) and numbered to the end of the locus name to designate the rice chromosome (1- 12) on which it has been mapped. When more than one QTL for the same trait was identified on the same chromosome in the same study, a second numerical identified was added followed by another dash (-) and new number with the chromosomal designation to differentiate the individual QTLs.

For example in this system QTL name appears as qTN-6-1, it means the first QTL for tiller number reported on chromosome 6.

CHAPTER FOUR

RESULTS

4.1 Plant survival rate

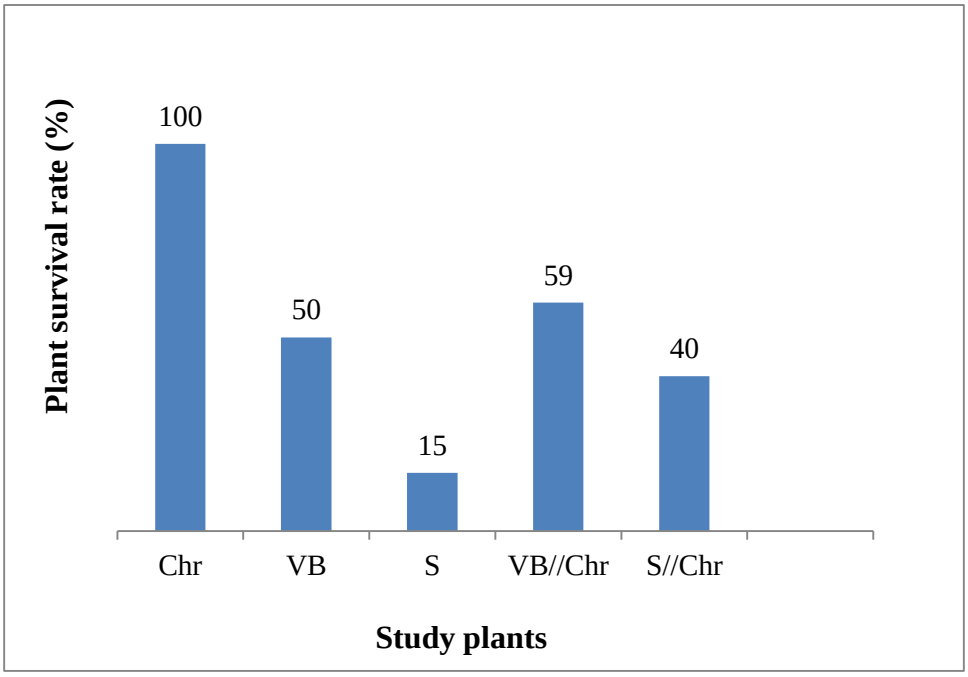
Vary botry// Chomrongdhan

The survival plant of Chomrongdhan (10 plants) as a donors parent was 100% classified as a tolerant reaction to cold, and Vary botry (10 plants) as the recurrent parent was 50% (5 plants) and classified as an intermediate reaction to cold (Figure 8). The BC_1F_2 plants (500 plants) showed varied tolerance to cold at seeding stage, with 59% (295 plants) of the plants showing survivals that classified as an intermediate reaction to cold at seedling stage (Figure 8).

Soameva//Chomrongdhan

Donor parent Chomrongdhan (16 plants), always showed higher percentage of survival plant by (100%) and Soameva (20 plants) the recurrent parent showed plant survival of 15% (3 plants) respectively, indicating a sensitive reaction to cold at seedling stage. The BC_1F_2 plants showed 40% (216 plants) survival at seedling stage that presented a reaction to cold classified as intermediate reaction (Figure 8).

In this study, regarding the BC_1F_2, plant survival was classified as an intermediate reaction to cold at seedling stage, indicating that a dominant gene action for cold tolerance at seedling stage was not expressed on plant survival.



Chr: Chomrongdhan; VB: Vary botry; S: Soameva; VB//Chr: BC_1F_2 plants from Vary botry// Chromrongdhan; S//Chr: BC_1F_2 plants from Soameva// Chromrongdhan

Figure 8: Frequency distribution of seedling survival percentage after cold treatment at seedling stage of Vary botry//Chomrongdhan and Soameva//Chomrongdhan BC_1F_2 progeny and the parents

4.2 Phenotypic screening of BC_1F_2 plants at seedling and reproductive stage

Cold tolerance was assessed by looking at the typical symptoms from BC_1F_2 plants from Vary botry//Chomrongdhan, Soameva//Chomrongdhan, and the parents.

Results showed that two hundred and five (205) and three hundred twenty four (324) plants from Vary botry//Chomrongdhan and Soameva//Chomrongdhan, respectively were killed during cold treatment at seedling stage and thus they were left out of from the analysis, 144 and 128 of BC_1F_2 plants from cross Vary botry//Chomrongdhan and Soameva//Chomrongdhan, respectively have been analyzed for this study.

4.2.1 Phenotypic screening at seedling stage of BC_1F_2 plants from Vary botry //Chomrongdhan

4.2.1.1 Assessment of seedling growth of BC_1F_2 plants from Vary botry //Chomrongdhan

Among the surviving plant, 144 BC_1F_2 have been analyzed, at the beginning of seedling growth, at 0 day after recovery (0 DAR) majority of BC_1F_2 plants showed a moderately susceptible reaction scored as 5 with 53.47% (77 plants), the seedling plants showed yellow and brown color on the leaves, the plants had an average of four leaves only and the plant just had 1 tiller. Only 35.42% (51 plants) of BC_1F_2 plants exhibited as moderately tolerant scored as 3 and 3.47 % (5 plants) like tolerant scored as 1 (Figure 9a). Seedling growth variation at 0 DAR behaved little skewed in favor of tolerant types (Figure 9a).

At 7 DAR the frequency distribution trend changed, a relative similarity between the percentage numbers of moderately tolerant with 38.19% (55 plants) and the moderately susceptible 33.33% (48 plants) of BC_1F_2 plants (Figure 9b) was observed. At 14 DAR, the result of seedling growth variation at 7 and 14 DAR behaved little skewed in favor of tolerant types (Figure 9b, c).

Analysis of variance following seedling growth in Vary botry//Chomrongdhan BC_1F_2 plants exhibited high significant differences between donor and recurrent parent and significant different between BC_1F_2 plants at 7 days after recovery (7 DAR) and at 14 days after recovery (14 DAR) (Table 1).

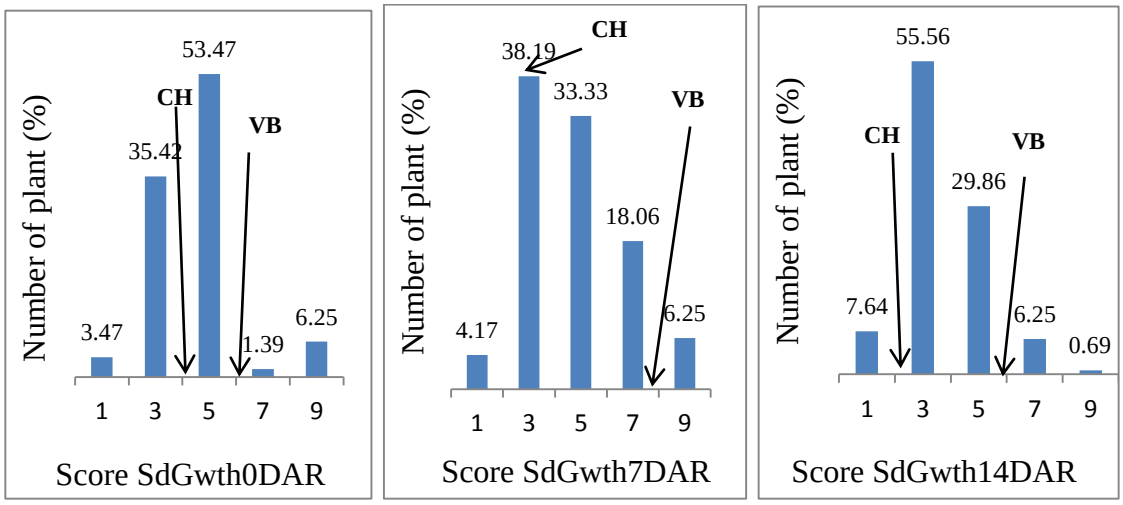


Figure 9: (a-b-c): Frequency distribution of seedling growth of BC_1F_2 plants from Vary botry//Chomrongdhan and the parent after cold treatment at seedling stage CH: Chomrongdhan, VB: Vary botry

4.2.1.2 Assessment of Seedling vigor of BC_1F_2 plants from Vary botry //Chomrongdhan

At 0 and 7 days after recovery (0 DAR, 7DAR) majority of BC_1F_2 plants showed as susceptible reaction scored as 7 with 59.03% (85 plants) and 47.22% (68 plants) respectively, the seedling plants showed as weak and plants somewhat stunted, 3-4 leaves and no tiller formation and 14DAR, a majority of seedling plant showed as moderately susceptible plants, scored as 5 with 35.42% (51 plants).

Variation of seedling vigor at 0, 7 and 14 DAR behaved little skewed in favor of susceptible types (Figure 10), and Analysis of variance of seedling vigor showed significant differences between BC_1F_2 plants at 7 DAR, and as well as the 14 DAR (Table 1).

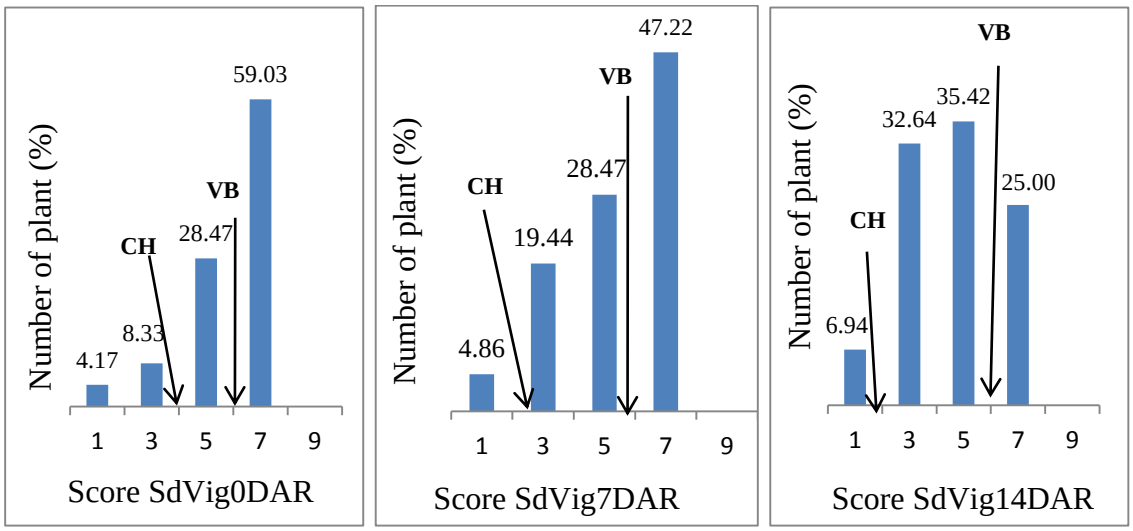


Figure 10: Frequency distribution of seedling vigor of BC_1F_2 plants from Vary botry//Chomrongdhan and the parent after cold treatment at seedling stage CH: Chomrongdhan, VB: Vary botry

4.2.1.3 Assessment of Seedling Leaf growth of BC_1F_2 plants from Vary botry //Chomrongdhan

Different BC_1F_2 plants showed variation in seedling leaf growth after cold treatment (Figure 11a). At 7 and 14 DAR, a majority of BC_1F_2 plant showed moderately susceptible plants scored as 5 with 37.50% (54 plants) and 53.47% (77 plants) respectively (Figure 11 (b-c)). At 7 DAR and 14DAR seedling leaf growth variation not fitted the normal distribution; variation behaved little skewed in favor of susceptible types.

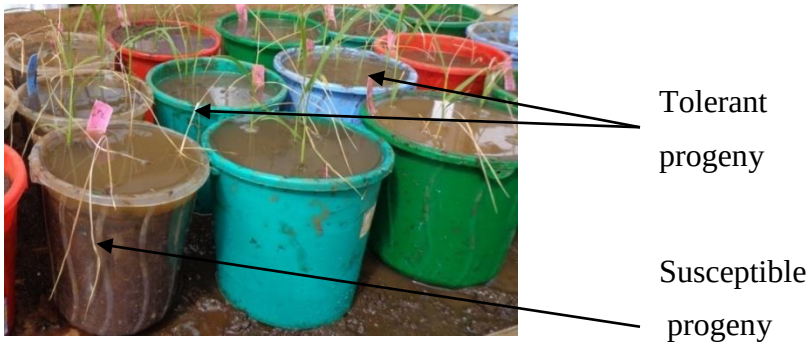
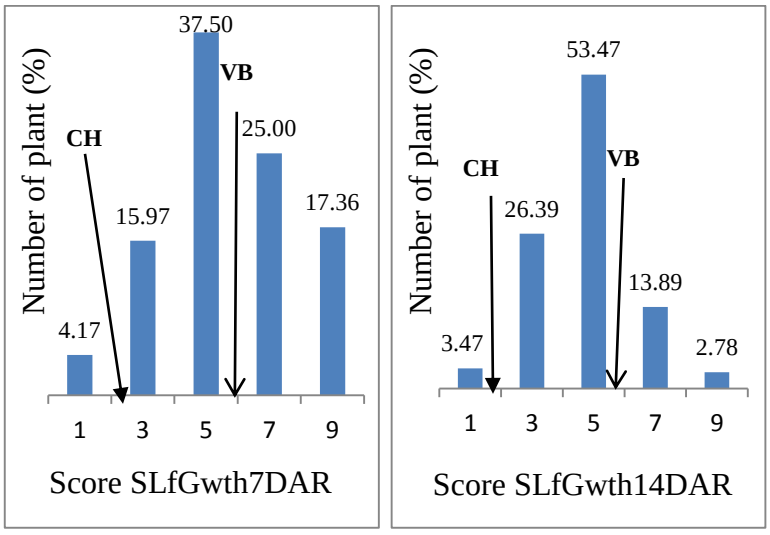


Figure 11(a): Variation of seedling leaf growth after cold treatment



b **c**

Figure 11 (b-c): Frequency distribution of seedling leaf growth of BC_1F_2 plants from Vary botry//Chomrongdhan and the parent after cold treatment at seedling stage CH: Chomrongdhan, VB: Vary botry

Table 1: Analysis of variance at seedling stage of BC_1F_2 plants from Vary botry//Chomrongdhan

Source of variation	Mean square							
	SdGwth0	**SdGwth7**	**SdGwth14**	**SdVig0**	**SdVig7**	**SdVig14**	**LfGwth7**	**LfGwth14**
Checks	21.19**	51.20*	36.22*	57.80*	57.80*	65.03*	80.00*	96.80*
Tested Plant	5.12	5.92**	6.02**	2.85	3.21***	4.31*	5.12	4.05*
CV (%)	29.83	23.15	26.86	18.32	17.196	17.19	28.05	28.12

***=Significant at p≤ 0.05;**=significant at p≤0.01, *= significant at p≤0.001; SdGrwt0DAR: seedling growth at 0 day after recovery; SdGwth7 DAR: seedling growth at 0 day after recovery; SdGwth14DAR: seedling growth at 14 day after recovery; SdVig7DAR: seedling vigor 7 day after recovery; SdVig14DAR: seedling vigor 14 day after recovery.

4.2.2 Phenotypic screening at reproductive stage of BC_1F_2 plants from Vary botry //Chomrongdhan

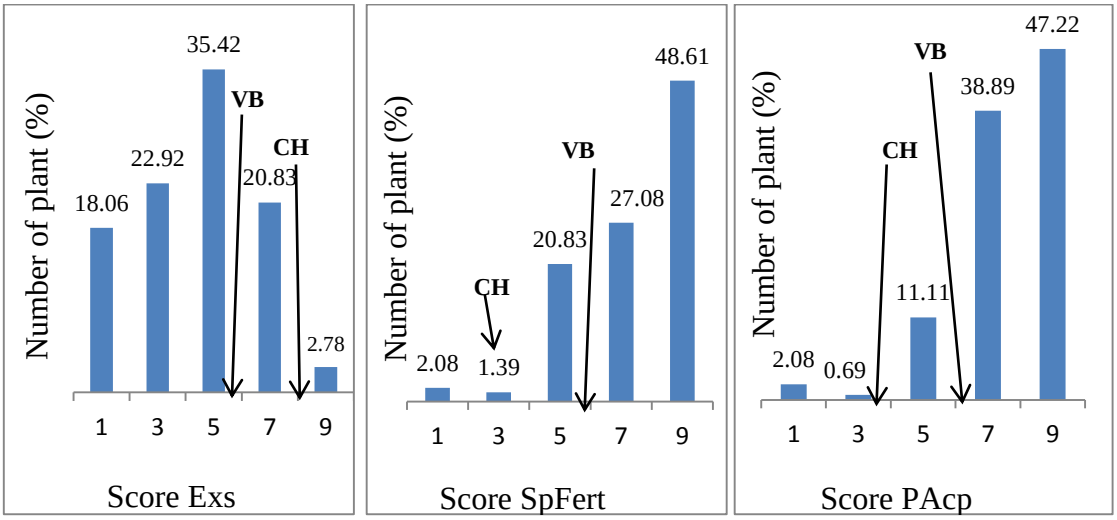
Cold stress injury at reproductive stage affected panicle exsertion (Exs), spikelet fertility (SpFert) and acceptability on phenotypic perception (PAcp). Figure 12a showed that majority of BC_1F_2 plant from Vary botry //Chomrongdhan exhibited a panicle exsertion as just exserted (score 5) with 35.42% (51 plants), 2.78% (4 plants) only was well exserted (score 9).

Similar to the panicle exsertion, the percentage number of BC_1F_2 plants that showed to be highly fertile (score 1) and fertile (score 3) were less than the plants that were partly sterile scored as 5 with 20.83 (30 plants), sterile scored as 7 with 27.08% (39 plants) and highly sterile scored as 9 with 48.61% (70 plants) (Figure 12b), then majority of BC_1F_2 plants showed as poor and unacceptable with score 7 and 9 respectively following the phenotypic acceptability, 38.89% (56 plants) and 47.22% (68 plants) (Figure 12c).

The results on the distribution frequency showed a normal distribution of the panicle exsertion, but the distribution of SpFert and PAcp not fit the normal distribution, BC_1F_2 plants were skewed towards the susceptible reaction. Analysis of variance (ANOVA) illustrated highly significant difference between BC_1F_2 plant progeny for Exs and PAcp (Table 2).

A part from panicle exsertion, spikelet fertility and acceptability phenotypic, other important traits related to yield included full seeds, the seeds fertility rate and the 1000 seeds weight, and were screened at BC_1F_2 for tolerance to cold. The results showed that 4.40 and 29.86% of BC_1F_2 plants were greater than the recurrent parent for the number of

full seed (>109), the seeds fertility rate (>43%) and 1000 seeds weight (>23g), respectively (List Appendix 6). Number of full seeds, the seeds fertility rate and the 1000 seeds weight were significantly different among the BC_1F_2 plants (Table 2).



a **b** **c**

Figure 12:(a-b-c): Frequency distribution of exsertion (Exs), spikelt fertility (SpFert) and phenotypic acceptability (PAcp) of BC_1F_2 plants from Vary botry//Chomrongdhan and the parent after cold treatment at reproductive stage ,CH: Chromrongdhan, VB: Vary botry

Table 2: Analysis of variance of BC_1F_2 plants from Vary botry//Chomrongdhan at reproductive stage

Source of variation	Mean square				
	TN	**PH (cm)**	**Exs**	**SpFert**	**FSN**
Checks	0.10	250.00*	14.4	19.60*	2402.5*
Tested plant	5.66	421.46**	7.73***	4.28	713.66***
CV (%)	56.19	6.44	20.34	18.83	20.06

Table 2 continued

Source of variation	Mean square			
	PWgt (g)	**PAcp**	**TSGW (g)**	**HD**
Checks	0.16***	19.60*	10.23*	23.82*
Tested plant	0.28***	3.86***	11.49***	12.56***
CV (%)	14.94	14.69	5.33	11.44

= Significant at P≤0.01; *=Significant at p≤ 0.05; TN: tiller number; PH: plant height; Exs: panicle exsertion; SpFert: spikelet fertility; FSN: number of full seeds; PWgt: panicle weight; PAcp: phenotypic acceptability; TSGW: 1000 grain weight; HD: heading date

4.2.3 Phenotypic screening at seedling stage of BC_1F_2 plants from Soameva//Chomrongdhan

4.2.3.1 Assessment of seedling growth of BC_1F_2 plants from Soameva//Chomrongdhan

Among the surviving plants, 128 BC_1F_2 from Soameva//Chomrongdhan were analyzed, at 0, 7 and the 14DAR majority of BC_1F_2 plants were highly susceptible reaction and scored as 9 with 30.47 (39 plants), 38.28 (49 plants) and 39.06 (50 plants). The percentage number of seedling that showed a dark green color, indicative of tolerance to cold was very low 1.56% (2 plants).

Frequency distribution of seedling growth did not fit the normal distribution with majority of BC_1F_2 plants skewed towards the susceptible types (Figure13). Analysis of variance following seedling growth in Soameva//Chomronghdan BC_1F_2 plants exhibited

high significant differences between donor and recurrent parent and significant different between BC_1F_2 plants (Table 3).

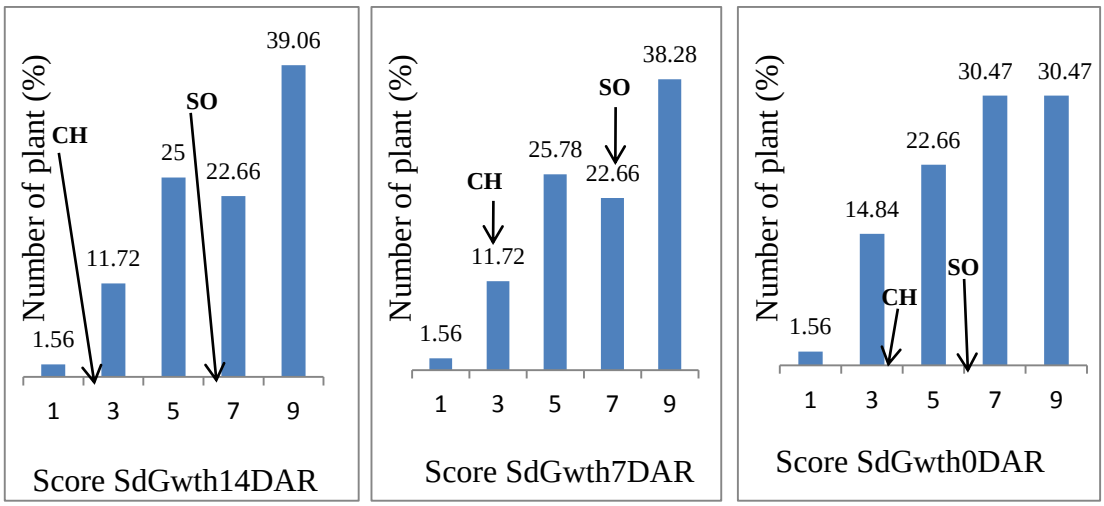


Figure 13: Frequency distribution of seedling growth of BC_1F_2 plants from Soameva//Chomrongdhan and the parent after cold treatment at seedling stage CH: Chromrongdhan, SO: Soameva

4.2.3. 2 Assessment of Seedling vigor of BC_1F_2 plants from Soameva//Chomrongdhan

Figure 14 shows the genotypic variations recorded for seedling vigor under cold stress. The majority of BC_1F_2 plants revealed highly susceptible plant types scored as 9 with 43.0 % (55 plants) and 37.5% (48 plants) at 0, 7 and 14 DAR respectively, the plants were very weak and stunted, yellowing of leaves and no tiller formation. It was only 1.6% (2 plants) showed like tolerant scored as 1 at 0 and 7 DAR, and 2.3% (3 plants) at 14DAR while 8.6% (11plants), 7.8% (10 plants) and 12.5% (16 plants) of BC_1F_2 plants were regarded as moderately tolerant scored as 3 at 0, 7 and 14DAR. Therefore, in relation to the plant seedling vigor, the percentage numbers of tolerant and moderately

tolerant of BC_1F_2 plants were less than the percentage number of BC_1F_2 plants that behaved as highly susceptible plant after cold treatment at seedling stage.

The seedling vigor did not follow the normal distribution and the BC_1F_2 plants skewed toward the susceptible type (Figure 14), and analysis of variance showed a significant difference within the parent and within the BC_1F_2 plant population in relation to seedling vigor (Table 3).

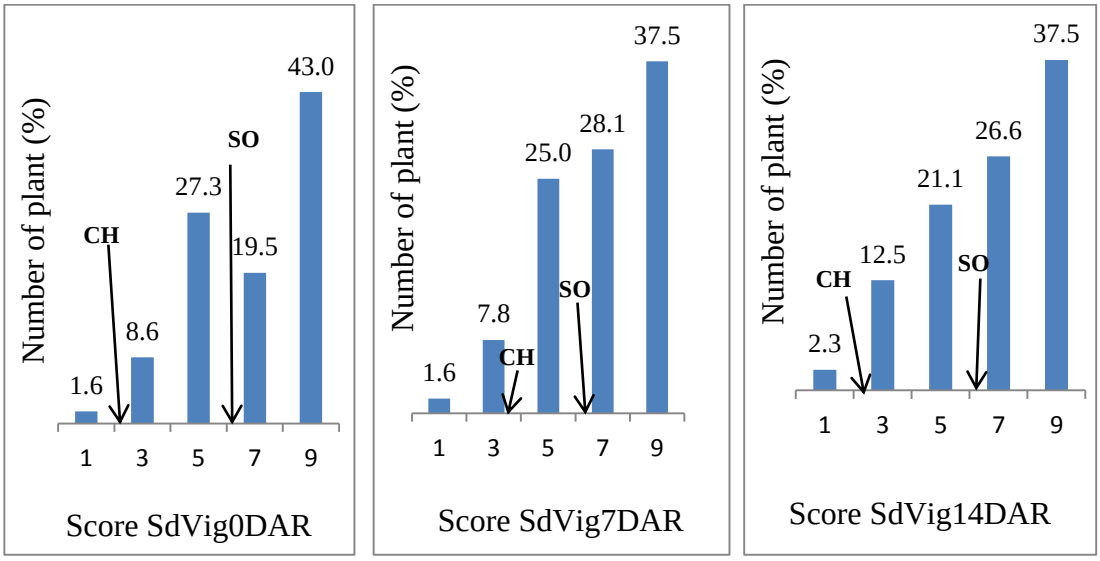


Figure 14: Frequency distribution of seedling vigor of BC_1F_2 plants from Soameva//Chomrongdhan and the parent after cold treatment at seedling stage, CH: Chomrongdhan, SO: Soameva

4.2.3.3 Assessment of Seedling Leaf growth of BC_1F_2 plants from Soameva//Chomrongdhan

At 7 DAR and 14 DAR, 38.845 (51 plants) and 38.28% (49 plants) respectively, of the BC_1F_2 plants showed a highly susceptible reaction scored as 9 with wilted leaves and seedlings apparently dead. At the end of the evaluation 14 DAR 14.84% (19 plants) of BC_1F_2 plants showed as moderately tolerant and only 1.56% (2 plants) of BC_1F_2 plants

were considered a tolerant in which all leaves were normal and in general they did not have any apparent visual injury. The frequency distribution (Figure15) shows an irregular distribution according to the seedling leaf growth; although in relation to seedling leaf growth, the analysis of variance showed that there was a significant difference within the parent and within the BC_1F_2 plant population (Table 3).

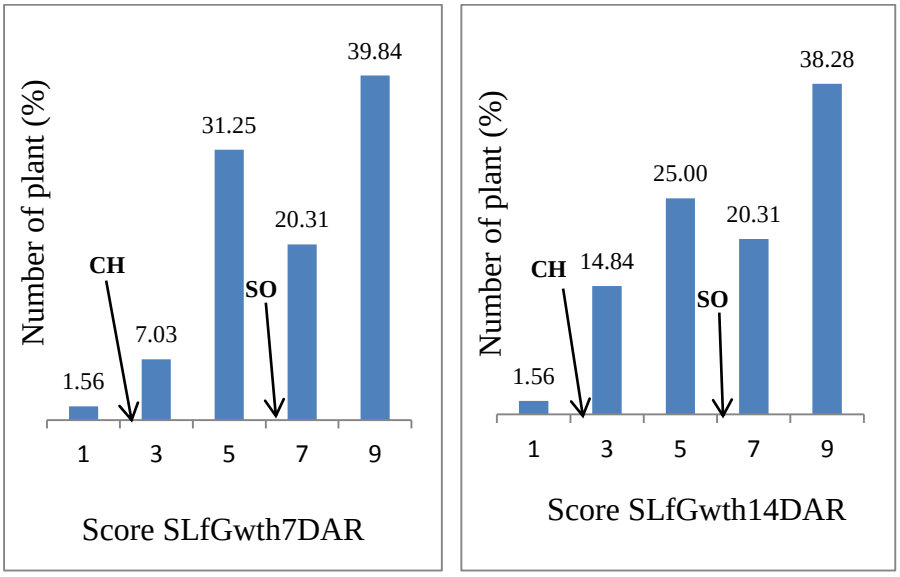


Figure 15: Frequency distribution of seedling leaf growth of BC_1F_2 plants from Soameva//Chomrongdhan and the parent after cold treatment at seedling stage, CH: Chromrongdhan, SO: Soameva

Table 3: Analysis of variance at seedling stage of BC_1F_2 plants from Soameva//Chromrongdhan

Source of variation	Mean square							
	SdGwth 0	SdGwth 7	SdGwth 14	SdVig 0	SdVig 7	SdVig 14	LfGwth 7	LfGwth 14
Tested Plant	7.13***	7.98***	7.71***	7.23***	7.98***	7.90***	7.87***	9.94***
CV (%)	21.29	18.07	19.86	18.17	18.07	20.42	17.93	21.26

***= Significant at p≤ 0.05; SdGrwt0: seedling growth at 0 day after recovery; SdGwth7: seedling growth at 0 day after recovery; SdGwth14: seedling growth at 14 day after recovery; SdVig0: seedling vigor 0 day after recovery; SdVig7: seedling vigor 7 day after

recovery; SdVig14: seedling vigor 14day after recovery; LfGwt7: leaf growth at 7 day after recovery; LfGwth14: leaf growth at 14 day after recovery.

4.2.4 Screening at reproductive stage of BC_1F_2 plants from Soameva//Chomrongdhan

Relating to the panicle exsertion, majority of BC_1F_2 plants showed panicle exsertion as partly exserted scored as 3 and enclosed scored as 1 with 36.72% (47 plants) and 31.25% (40 plants) respectively. Only 14.84% (19 plants) and 2.34% (3 plants) of BC_1F_2 plants were considered like moderately well exserted scored as 7 and well exserted scored as 9 (Figure 16a).

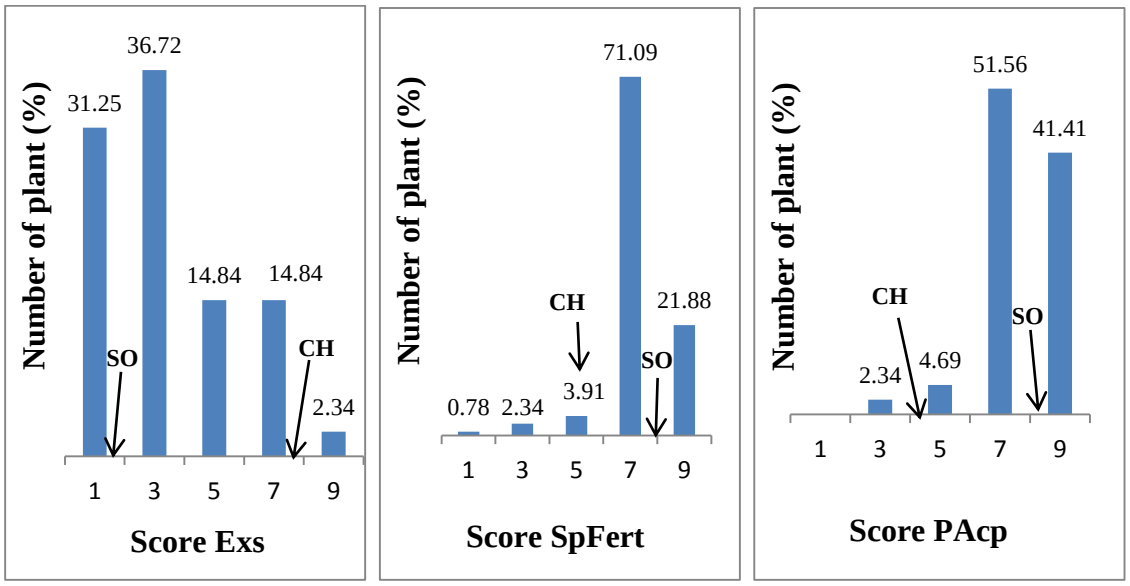
Regarding the Spikelet fertility (Figure 16b), common of BC_1F_2 plants were sterile with 71.09% (91 plants) ; it means that the percentage number of susceptible BC_1F_2 plants was very high compared to the percentage number of BC_1F_2 plants that showed less than 50% to trace of fertility scored as 7 to 9. Then majority of BC_1F_2 plants showed as poor and unacceptable with score 7 and 9 respectively, following the phenotypic acceptability, 38.89% (56 plants) and 47.22% (68 plants) (Figure 16c).

Concerning the phenotypic acceptability, no BC_1F_2 plant showed as an excellent plant (score 1), while the percentage number of BC_1F_2 plants that were considered a very good appearance scored as 3 and fair appearance, but has a few essential shortcoming scored as 5 were very low with 2.34% (3 plants) and 4.69% (6 plants) (Figure 16c).

The distribution frequency of panicle exsertion, spikelet fertility and phenotypic acceptability of BC_1F_2 plants were all not fitting the normal distribution, distribution frequency performed slightly skewed in favor of susceptible types. However, analyze of

variance showed that there was a significant difference between BC_1F_2 plants according to the spikelet fertility (Table 4).

Relating to some traits related to yield component, the result showed that 30.38 and 22.48% of BC_1F_2 plants were better than the recurrent parent based on the number of full seeds (>17.3), the seed fertility rate (> 47.32%) and 1000 seed weight (>17g) respectively (List appendix 7). According to the number of full seeds, the difference among BC_1F_2 plants was significant at $P \leq 0.001$ and 1000 seeds weight was significant at $p \leq 0.05$ (Table 4).



a **b** **c**

Figure 16(a-b-c): Frequency distribution of panicle exsertion (Exs), spikelt fertility (SpFert) and phenotypic acceptability (PAcp) of BC_1F_2 plants from Soameva//Chomrongdhan and the parent after cold treatment at reproductive stage

Table 4: Analysis of variance of Soameva//Chomrongdhan at reproductive stage

Source of variation	Mean square				
	TN	**PH (cm)**	**Exs**	**SpFert**	**FSN**
Tested plant	15.54**	3233.00*	13.43	19.26*	466.16*
CV (%)	59.48	51.76	59.27	55.74	76.98

Table 4 continued

Source of variation	Mean square			
	PWgt (g)	**PAcp**	**TSGW (g)**	**HD**
Tested plant	2092.94***	29.76	97.29***	4.66
CV (%)	78.32	84.66	65.86	18.74

*=significant at P≤0.001; **significant at P≤0.01;***= significant at p≤ 0.05;
TN: tiller number; PH: plant height; Exs: panicle exsertion;
SpFert: spikelet fertility; FSN: number of full seeds; PWgt: panicle weight;
PAcp: phenotypic acceptability; TSGW: 1000 grain weight; HD: heading date

4.3 Correlation

4.3.1 Correlation between evaluated traits of BC_1F_2 plants from Vary botry //Chomrongdhan at seedling stage (Table 5)

Seedling Growth at 0 day of recovery (SdGwth0) was highly significant and positively associated with Seedling Growth at 7 days of recovery (SdGwth7) (0.5163), Seedling Growth at 14 days of recovery (SdGwth14) (0.4681), Seedling vigor at 0 day of recovery (SdVig0) (0.3996), and leaf growth at 7 days of recovery (LfGwth7) (02813). On the

other hand, SdGwth0 was only significantly and positively correlated to SdVig14 (0.1726) and LfGwth14 (0.1998). SdGwth7 was positively and significantly correlated with SdGwth14 (0.5651), SdVig0 (0.2505), SdVig7 (0.3075), SdVig14 (0.224), LfGwth7 (0.4288) and LfGwth14 (0.3656). SdGwth14 had highly significant and positive correlation with SdVig0 (0.2341), SdVig7 (0.264), SdVig14 0.2268), LfGwth7 (0.3751) and LfGwth14 (0.4035). SdVig0 showed highly significant positive correlation with SdVig7 (0.5522), SdVig14 (0.4874), LfGwth7 (0.2575) and LfGwth14 (0.2825) while SdVig7 had highly significant positive correlation with SdVig14 (0.6261), LfGwth7 (0.3688) and LfGwth14 (0.3642). SdVig14 was highly significant and positively correlated with LfGwth7 (0.3339) and LfGwth14 (0.415).

4.3.2 Correlation between evaluated traits of BC_1F_2 plant from Vary botry //Chomrongdhan at reproductive stage

Table 6 shows the correlation of yield components for Vary botry// Chomrongdhan crosses. Plant height (PH) was highly significant and positively correlated with tiller number (TN) (0.2645). Spikelet fertility (SpFert) and panicle weight (PWgt) analysis revealed highly significant correlation with number of full seed (FSN) (0.775) and (0.4001) respectively. Panicle weight also had highly significant positive correlation with Spikelet fertility (0.3466) and heading date (HD) showed highly significant positive correlation with 1000 grain weight (TSGW) (0.4362).

Table 5: Phenotypic correlation coefficient among 8 observed traits of BC_1F_2 plants from Vary botry// Chomrongdhan at seedling stage

	SdGwth 0	SdGwth 7	SdGwth 14	SdVig 0	SdVig 7	SdVig 14	LfGwth 7	LfGwth 14
SdGwth0	1							
SdGwth7	0.51***	1						
SdGwth14	0.46***	0.56***	1					
SdVig0	0.39***	0.25***	0.23***	1				
SdVig7	0.26***	0.30***	0.2***	0.55***	1			
SdVig14	0.17**	0.2***	0.22***	0.48***	0.62***	1		
LfGwth7	0.28***	0.42***	0.37***	0.25***	0.36***	0.33***	1	
LfGwth14	0.19**	0.36***	0.40***	0.28***	0.36***	0.41***	0.58***	1

***= Highly significant at P≤0.01. **=significant at P≤ 0.05 level of probability **SdGwth0** Seedling growth after 0day recovery, **SdGwth7** Seedling growth after 7day recovery, **SdGwth14** Seedling growth after 14day recovery, **SdVig0** seedling vigor after 0day recovery, **SdVig7** seedling vigor after 7day recovery, **SdVig14** seedling vigor after 14day recovery, **LfGwth7** Leaf growth after 7day recovery, **LfGwth14** Leaf growth after 14day recovery

Table 6: Phenotypic correlation coefficient among 8 observed traits of BC_1F_2 plants from Vary botry//Chomrongdhan at reproductive stage

	TN	PH	FSN	SpFert	PWgt	TSGW	HD
TN	1						
PH	-0.26***	1					
FSN	0.00	0.05	1				
SpFert	0.02	-0.01	0.7***	1			
PWgt	-0.09	0.08	0.40***	0.34***	1		
TSGW	-0.03	-0.03	-0.06	-0.09	-0.08	1	
HD	0.09	-0.05	-0.07	0.04	-0.08	0.43***	1

***= Highly significant at P≤0.01. **=significant at P≤ 0.05 level of probability; TN: tiller number; PH: plant height; FSN: Number of full seeds; SpFert: spikelet fertility; PWgt: panicle weight; TSGW: 1000 seeds weight, HD: heading date.

4.3.3 Correlation between evaluated traits of BC_1F_2 plants from Soameva //Chomrongdhan at seedling stage

Each one of the trait was high significant and positively correlated with each other's traits in the cross between Soameva and Chomronghdan, as shown in Table 7.

4.3.4 Correlation between evaluated traits of BC_1F_2 plant from Soameva//Chomrongdhan at reproductive stage (Table 8)

Plant height (PH) was highly significant and positively correlated with tiller number (TN) (0.5668). Spikelet fertility (SpFert) was significantly and positively correlated with number of full seed (FSD) (0.8304), while Panicle weight (PWG) had significant positive correlation with PH (0.2082). FSD (0.5717) and SpFert (0.5654). 1000 seed weight (TSGW) was significantly and positively correlated with FSN (0.255) and SpFert (0.207). HD was significantly and negatively correlated with SpFert (-0.1818).

Table 7: Phenotypic correlation coefficient among observed traits of BC_1F_2 plants from Soameva//Chomrongdhan at seedling stage

	SdGwth 0	**SdGwth 7**	**SdGwth 14**	**SdVig 0**	**SdVig 7**	**SdVig 14**	**LfGwth 7**	**LfGwth 14**
SdGwth0	1							
SdGwth7	0.90***	1						
SdGwth14	0.85***	0.85***	1					
SdVig0	0.84***	0.80***	0.96***	1				
SdVig7	0.8***	0.84***	0.95***	0.95***	1			
SdVig14	0.86***	0.84***	0.93***	0.95***	0.92***	1		
LfGwth7	0.84***	0.83***	0.91***	0.94***	0.92***	073***	1	
LfGwth14	0.85***	0.84***	0.90***	0.92***	0.93***	0.90***	0.93***	1

***=Highly significant at P≤0.01 **SdGwth0** Seedling growth after 0day recovery, **SdGwth7** Seedling growth after 7day recovery, **SdGwth14** Seedling growth after 14day recovery, **SdVig0** seedling vigor after 0day recovery, **SdVig7** seedling vigor after 7day

recovery, **SdVig14** seedling vigor after 14day recovery, **LfGwth7** Leaf growth after 7day recovery, **LfGwth14** Leaf growth after 14day recovery

Table 8: Phenotypic correlation coefficient among 8 observed traits of BC_1F_2 plants from Soameva//Chomrongdhan at reproductive stage

	TN	PH	FSN	SpFert	PWgt	TSGW	HD
TN	1						
PH	0.5668***	1					
FSN	-0.0426	0.0731	1				
SpFert	-0.0585	0.0692	0.8304***	1			
PWgt	-0.0403	0.2082**	0.5717***	0.5654***	1		
TSGW	-0.0009	-0.0969	0.255**	0.207*	-0.0782	1	
HD	-0.0783	-0.0679	-0.0203	-0.1818*	0.0346	0.0036	1

***=Highly significant at P≤0.01, **=significant at P≤0.05, *=significant at P≤0.1; TN: tiller number; PH: plant height; FSN: Number of full seeds; SpFert: spikelet fertility; PWgt: panicle weight; TSGW: 1000 seeds weight, HD: heading date

4.4 Qualitative trait loci analysis

One hundred fifty one (151) of BC_1F_2 plants from Vary botry//Chomrongdhan and eighty eight (88) of BC_1F_2 plants from Soameva//Chomrongdhan were yielded poor DNA quality and thus couldn't be genotyped, the DNA sample was degraded during the transfer of DNA sample from KARLO/ Njoro, Kenya to GSL/IRRI the Philippines. One hundred forty four (144) and one hundred twenty eight (128) of BC_1F_2 plants were used to map QTL in the present study.

When a polymorphism parental survey was performed using 4606 SNP markers, 1566 and 1250 markers showed polymorphism between Vary botry//Chomrongdhan and Soameva//Chomrongdhan, respectively. No heterozygote alleles were found through the rice genome of all parents. The position of the polymorphic SNP is given in base pair (bp): A: Adenine, T: Thymine, G: Guanine, C: Cytosine. The markers were found to be evenly distributed across the 12 chromosomes of the rice parents studied.

4.4.1 Segregation of polymorphic markers in BC_1F_2

Markers showing polymorphism between Vary botry//Chomrongdhan and Soameva//Chomrongdhan were chosen for mapping and QTL analysis for the BC_1F_2 plant. These markers were tested for segregation distortion using Chi-square test (X^2) for goodness-of-fit to compare the observed and expected allelic frequency of 1:2:5 ratio at $P \leq 0.01$.

4.4.2 Distribution and density of polymorphic SNP Markers of BC_1F_2 plants from Vary botry//Chromrongdhan

For 282 markers, chromosomal length was 1073.45 (Table 9) with an average of chromosome length of 4.01cM. The highest marker distribution was found on chromosome 1 and 2 (38 markers each over a total number of 282, with an average chromosome length 3.24cM and 3.84cM, respectively. The largest average length was in chromosome 7 (6.25cM).

Table 9: Distribution and number of the polymorphic markers of BC_1F_2 plants from Vary botry//Chomrongdhan

Chromosome Number	Number of polymorphic SNPs markers	Total chromosomal length in cM	Average chromosomal length in cM
1	38	123.12	3.24
2	38	145.87	3.84
3	18	91.27	5.07
4	33	93.12	2.82
5	18	82.5	4.58
6	24	76.34	3.18
7	13	81.28	6.25
8	19	82.13	4.32
9	24	71.11	2.96
10	18	44.26	2.46
11	20	102.04	5.10
12	19	80.41	4.23
Total	**282**	**1073.45**	**4.01**

4.4.2.1 Quantitative Traits Loci Identified at seedling stage of BC_1F_2 plants from Vary botry//Chomrongdhan

Identified QTLs in BC_1F_2 plant populations from Vary botry//Chomrongdhan at seedling stage is given in Table 10 and Figure 17.

Four putative QTL were identified and mapped by Inclusive Composite Interval Mapping onto rice chromosomes 2 and 10, while no QTL was detected on chromosome 3, 4, 5, 6, 7, 8, 9, 11 and 12. The identified loci were associated with the log10- likelihood ratio (LOD) score threshold of 2.5 or above. Among four detected QTL, three QTL

(*qSdGwth14-10-1, qSdGwth14-10-2, qLfGwth14 -10-1*) detected on chromosome 10 were associated with cold tolerance for seedling growth and Leaf growth at 14 DAR.

qSdGwth14-10-1 and *qSdGwth14-10-2* were identified at the chromosome 10 at the position 10 and 20cM respectively, *qSdGwth14-10-1* were flanked by id10000391 and 10099158 with LOD scores of 3.66 with phenotypic variation expected by this QTL as 11.11% with negative (-0.4573) parental additive effect (-0.4573). While, *qSdGwth14-10*-2 were flanked with markers 10465477 and 10469362 with LOD Scores 2.65, expected 7.55% of phenotypic variation and positive of parental additive effect (0.2422).

One QTL, *qLfGwth14 -10-1* was identified for leaf growth at seedling stage. It was flanked by id10000391and 10099158 with LOD scores 2.89 and phenotypic expected variation of 8.87% and a negative parental additive effect (-0.4357). One QTL, *qSdVig0-2-1*, located on Chromosome 2 was identified to be related with seedling vigor at 0 day after recovery. This identified QTL was flanked to 2262412 and 2237404 markers with LOD scores 3.65 and phenotypic variation explained by the QTL as 12.80%, and negative parental additive effect (-0.1055).

Table 10: Quantitative Traits Loci Identified of BC_1F_2 plants from Vary botry//Chomrongdhan at seedling stage

[a]QTL	[b]Ch	[c]POS	[d]LM	[e]RM	[f]LOD	[g]PVE (%)	[h]Add
qSdGwth14-10-1	10	10	id10000391	10099158	3.6631	11.1104	-0.4573
qSdGwth14-10-2	10	20	10465477	10469362	2.6568	7.5502	0.2422
qSdVig0- 2-1	2	130	2262412	2237404	3.6576	12.8046	-0.1055
qLfGwth14-10-1	10	10	id10000391	10099158	2.8964	8.8726	-0.4357

[a]QTL identified at in the present study; [b]Chromosome on which QTL for cold tolerant is located; [c]POS Position of QTL on chromosome (cM); [d]LM Left marker; [e]RM Right

marker; [f]LOD scores (Log10-likelihood ratio) offer the strength of the data supporting the existence of a QTL in a defined interval at LOD≥2.5 at P≤0.0001; [g]PVE (%), Phenotypic variance explained by identified QTL; [h]Add Additive genetic effects of QTL, Positive and negative value indicates that alleles resulting in an increasing tolerance are from Vary botry and Chomrongdhan.

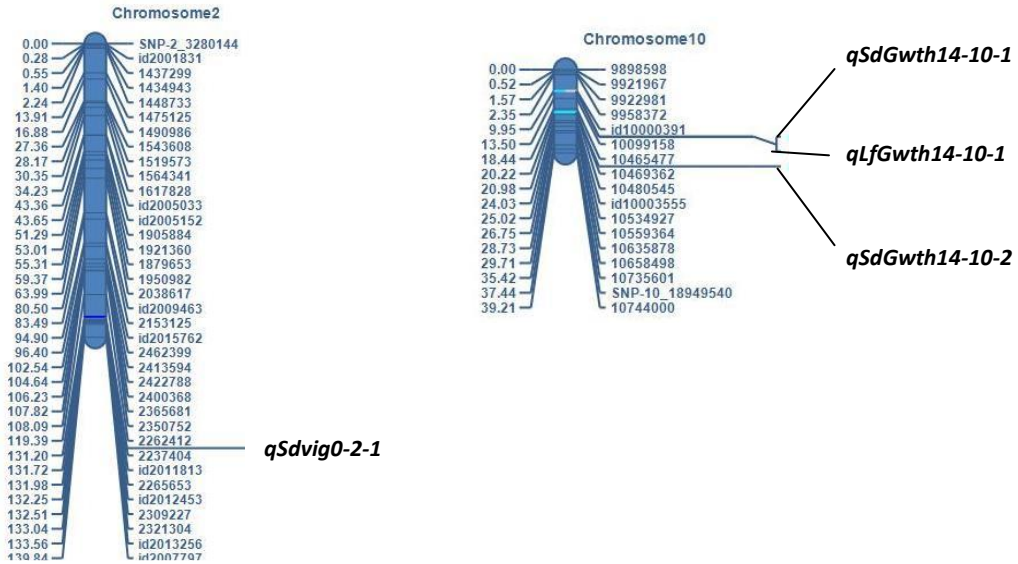


Figure 17: Genomic location of Quantitative trait loci affecting cold tolerance of BC_1F_2 plants from Vary botry//Chomrongdhan at seedling stage. Map units are expressed in centimorgan (cM)

4.4.2.2 Quantitative Traits Loci identified at reproductive stage of BC_1F_2 plants from Vary botry//Chomrongdhan

Twelve putative QTLs (Table 11, Figure 18) for plant height, number of full seed, spikelet fertility, and 1000 seeds weight were identified on chromosome 2, 4, 5, 7, 8, 10 and 12. Among the 12 Putative QTL, four QTL (*qSpFert-2-1, qSpFert-5-1, qSpFert-8-1* and *qSpFert-12-1*) for spikelet fertility were identified. *qSpFert-2-1* identified on chromosome 2 at position 43cM, and flanking by 1617828 and id2005033, with LOD scores 3.37 and phenotypic variation explained 9.48%, negative parental additive effect

is presented for this QTL(-0.92). *qSpFert-5-1* was positioned at 78cM on chromosome 5. It was flanking to 4904201 and 4928297 markers within LOD scores 2.93 and contributed to explaining 8.57% of the phenotypic variation with positive parental additive effect (7.69). *qSpFert-8-1*, identified on chromosome 8 at the position 16cM, was flanked by 8990744 and 8934988 with LOD scores 5.07 and phenotypic variation explained by this QTL 17.12% with positive parental additive effect (1.62) .

Three putative QTL (*qTSGW-4-1, qTSGW-10-1* and *qTSGW-10-2*) were identified for 1000 weight. *qTSGW-4-1* was identified on chromosome 4 at position 2cM. This QTL was flanked by 4733006 and 4729077 with LOD 3.47 and phenotypic variation explained by those QTL 11.94% and negative of parental additive effect (-2.26). *qTSGW-10-1* identified on chromosome 10 at 29cM were flanked by 10635878 and 10658498 with LOD 2.59, and contributed in explaining 8.59% of the phenotypic variation with negative of parental additive effect (-2.89). *qTSGW-10-2* was a QTL identified also on chromosome 10 but at position 33cM and flanked by 10658498 and 10735601 within LOD scores 3.64 and phenotypic variation explained by this QTL was 29.39% with negative parental additive effect (-4.99). One QTL (*qHD-4-1*) for heading date was identified on chromosome 4 at position 3cM; it was flanked by 4729077 and id4010985 with LOD scores 3.75 and explained 11.34% of phenotypic variation with negative of parental additive effect (-10.7) .

Three QTLs (*qFSN-5-1, qFSN-8-1* and *qFSN-8-2*) were identified for number of full seed. *qFSN-5-1*, QTL was identified on chromosome 5 at 26cM, it was flanked by 5684344 and 5649082 with LOD scores 2.56; phenotypic variation explained by this QTL was 7.37% and positive parental additive effect (5.95). *qFSN-8-1* and *qFSN-8-2*

were identified on chromosome 8 at position 17 and 37cM respectively, those QTL were flanked by 8990744 and 8934988 and by 8843060 and 8815450 respectively. LOD for those QTL were 2.56 and 3.57 respectively with explained 8.59 and 11.13% respectively of phenotypic variation. *qFSN-8-1* showed a positive of parental additive effect (5.11). Contrary *qFSN-8-2* showed a negative of parental additive effect (-9.99).

One QTL (*qPH-7-1*) relative to plant height was identified on chromosome 7 at 0cM position, it was flanked by id7000798 and 7110346 markers with LOD scores 2,57 and phenotypic variation explained by this QTL of 7.1% and negative of parental additive effect (-2.71) .

Table 11: Quantitative Traits Loci identified at reproductive stage of BC_1F_2 plants from Vary Botry//Chomrongdhan

[a]QTL	[b]Ch	[c]POS	dLM	[e]RM	[f]LOD	[g]PVE(%)	[h]Add
qPH-7-1	7	0	id7000798	7110346	2.55	7.18	-2.71
qFSN-5-1	5	26	5684344	5649082	2.56	7.37	5.95
qFSN-8-1	8	17	8990744	8934988	2.57	8.59	5.11
qFSN-8-2	8	37	8843060	8815450	3.57	11.14	-9.99
qSpFert-2-1	2	43	1617828	id2005033	3.37	9.48	-0.92
qSpFert-5-1	5	78	4904201	4928297	2.93	8.57	7.69
qSpFert-8-1	8	16	8990744	8934988	5.07	17.13	1.62
qSpFt-12-1	12	0	12023948	12108546	3.29	9.02	1.13
qTSGW-4-1	4	2	4733006	4729077	3.47	11.95	-2.27
qTSGW-10-1	10	29	10635878	10658498	2.60	8.59	-2.90
qTSGW-10-2	10	33	10658498	10735601	3.64	29.39	-4.99
qHD-4-1	4	3	4729077	id4010985	3.75	11.35	-10.71

[a]QTL identified at in the present study; [b]Chromosome on which QTL for cold tolerant is located; [c]POS Position of QTL on chromosome (cM),; [d]LM Left marker; [e]RM Right marker; [f]LOD scores (Log10-likelihood ratio) offer the strength of the data supporting the existence of a QTL in a defined interval at LOD≥2.5 at P≤0.0001; [g]PVE(%), Phenotypic

variance explained by identified QTL; [h]Add Additive genetic effects of QTL, Positive and negative value indicates that alleles resulting in an increasing tolerance are from Vary botry and Chomrongdhan.

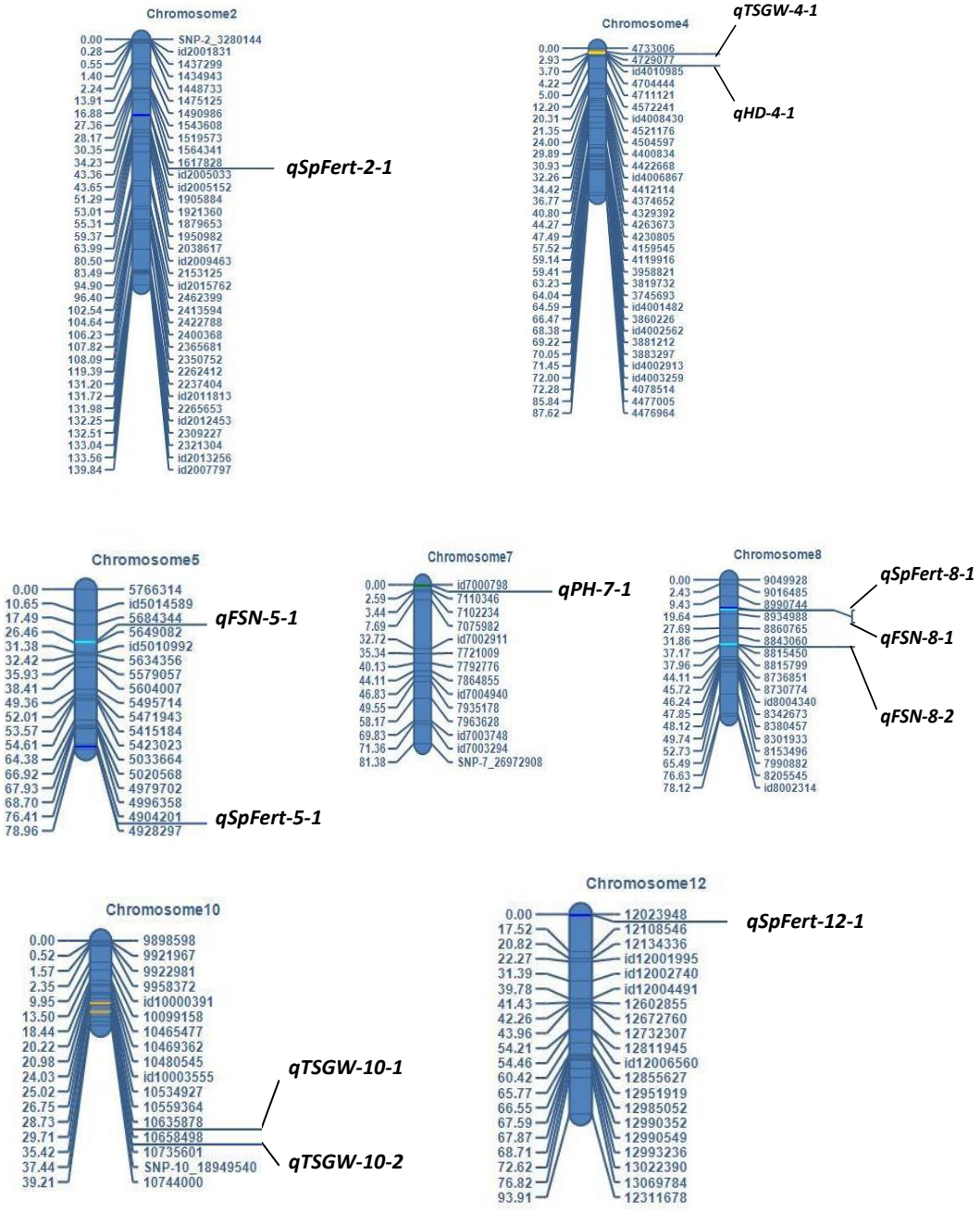


Figure 18: **Genomic location of Quantitative trait loci affecting cold tolerance at reproductive stage of BC_1F_2 plants from Vary botry//Chomrongdhan. Map units are expressed in centimorgan (cM)**

4.4. 3 Distribution and Density of Polymorphic SNP Markers of BC_1F_2 plants from Soameva//Chomrongdhan

The distribution of the 302 markers after binning 334 polymorphism markers on IciMapping software is given in Table 12, with a total chromosomal length of 1527.21cM (Table 12). The average of chromosome length was 5.62cM. High markers distribution was found on chromosome 3 (47 markers over a total number of 302), with an average chromosome length 4.45cM and the largest average length was in chromosome 10 (9.94cM).

Table 12: Distribution and number of the polymorphic markers of BC_1F_2 from Soameva//Chomrongdhan

Chromosome Number	Number of polymorphic SNPs markers	Total chromosomal length in cM	Average chromosomal length in cM
1	39	158.2	4.06
2	35	191.41	5.47
3	47	209.04	4.45
4	17	132.31	7.78
5	15	102.79	6.85
6	21	100.41	4.78
7	13	54.72	4.21
8	29	115.24	3.97
9	31	148.19	4.78
10	4	39.76	9.94
11	24	195.08	8.13
12	27	80.06	2.97
Total	**302**	**1527.21**	**5.62**

4.4.3.1 Quantitative Traits Loci Identified at seedling stage of BC_1F_2 from Soameva//Chomrongdhan

Identified QTL of BC_1F_2 plant from Soameva//Chomrongdhan at seedling stage is given in Table13 and Figure 19. Seven putative QTLs were identified and mapped by Inclusive Composite Interval Mapping onto rice chromosome 1, 2, 4 and 9.

The identified loci were associated with the log10- likelihood ratio (LOD) score threshold of 2.5 or above. Among seven identified QTL, four (*qSdgrwth14-2-1, qSdgwth7-4-1, qSdgwth7-9- 1* and *qSdgrwth14-9-1*) detected on chromosome 2, 4 and 9 respectively were linked to cold tolerance for seedling growth at 7 and 14 day after recovery. *qSdgwth7-4-1* and *qSdgwth7-9-1* were identified at the chromosome 4 and 9 at the position 85cM and 94cM respectively. *qSdgwth7-4-1* was flanked by 4355198 and 4422668 with LOD scores 2.56 and the phenotypic variation expected by this QTL was 9.94% with positive parental additive effect (0.6639). *qSdgwth7-9-1* was flanked by 9869869 and 9852552 with LOD scores 3.45 and expected 14.3% of phenotypic variation and negative of parental additive effect (-1.0317), while *qSdgrwth14-2-1* and *qSdgrwth14-9-1* were identified at the chromosome 2 and 9 at 68cM and 95cM respectively. *qSdgrwth14-2-1* was flanked by id2011509 and 2437583 with LOD scores 3.09 and the phenotypic variation expected by this QTL was 16.4% with negative parental additive effect (-0.5722). *qSdgrwth14-9-1* was flanked by 9869869 and 9852552 with LOD scores 2.86 and expected 10.7% of phenotypic variation and negative of parental additive effect (-0.9003).

Two QTLs, *qLfGwth7-1-1* and *qLfGwt14-2-1* were identified on chromosome 1 and 2. Both identified QTL conferred a QTL for leaf growth. *qLfGwth7-1-1* identified at the

position 83cM on the chromosome 1 was flanked by 745362 and 497200 with LOD scores 2.79. The phenotypic variation expected by this QTL was 12.35% within negative parental additive effect (-0.4912). *qLfGwt14-2-1* identified at the position 147cm on chromosome 2 was flanked to the markers id2005033 and id2005152 with LOD scores 2.56 and expected 8.55% of phenotypic variation against cold stress and negative of parental additive effect (-0.5076).

One QTL, *qSdVig7-2-1* was identified for seedling vigor. It was identified on chromosome 2 at position 148cm and flanked by id2005033 and id2005152 with LOD scores 2.52 and phenotypic expected variation of 8.24% and a negative parental additive effect (-0.5076).

Table 13: Quantitative Traits Loci identified at seedling stage of BC_1F_2 from Soameva//Chomrongdhan

[a]QTL	[b]Ch	[c]POS	LM	RM	LOD	PVE (%)	Add
qSdGwth7-4-1	4	85	4355198	4422668	2.5658	9.9461	0.6639
qSdGwth7-9-1	9	94	9869869	9852552	3.4538	14.3436	-1.0317
qSdGrwth14-2-1	2	68	id2011509	2437583	3.0941	16.4839	-0.5722
qSdGrwth14-9-1	9	95	9869869	9852552	2.8635	10.7174	-0.9003
qSdVig7-2-1	2	148	id2005033	id2005152	2.527	8.2458	-0.5256
qLfGwth7-1-1	1	83	745362	497200	2.7919	12.3524	-0.4912
qLfGwt14-2-1	2	147	id2005033	id2005152	2.5696	8.5544	-0.5076

[a]QTL identified at in the present study; [b]Chromosome on which QTL for cold tolerant is located; [c]POS Position of QTL on chromosome (cM); [d]LM Left marker; [e]RM Right marker; [f]LOD scores (Log10-likelihood ratio) offer the strength of the data supporting the existence of a QTL in a defined interval at LOD≥2.5 at P≤0.0001; [g]PVE(%), Phenotypic

variance explained by identified QTL; [h]Add Additive genetic effects of QTL, positive and negative value indicates that alleles resulting in an increasing tolerance are from Soameva and Chromrongdhan.

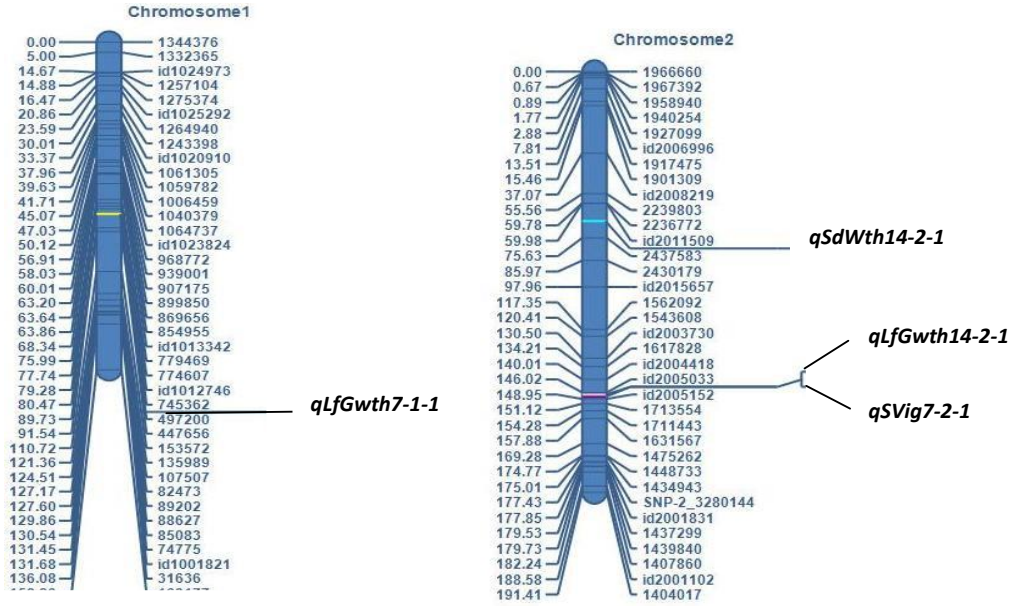


Figure 19: **Genomic location of quantitative trait loci affecting cold tolerance at seedling stage of BC_1F_2 plants from Soameva//Chomrongdhan. Map units are expressed in centimorgan (cM).**

4.4.3.2 Quantitative Traits Loci Identified at reproductive stage of BC_1F_2 plants from Soameva//Chomrongdhan

Six putative QTL (Table 14, Figure 20) for spikelet fertility, 1000 grain weight, tiller number and panicle weight were mapped onto rice chromosome and 2, 5, 7 and 10. Those identified loci were associated with the $\log_{10}$- likelihood ratio (LOD) score threshold more than or equal 2.5.

Among the six detected QTL, two QTLs *qSpFert-5-1* and *qSpFert-7-1* identified on chromosome 5 and 7 respectively were positioned at 19 and 51cM of the chromosome. *qSpFert-5-1* was flanked to id5008590 and id5013231 markers with LOD scores 2.77 and phenotypic variation expected against cold stress by this QTL was 10.49% within positive parental additive effect (4.79).

qSpFert-7-1 were flanked by 7930710 and 7947435 with LOD scores 2.71 and expected phenotypic variation of 9.01%, with negative parental additive effect (-5.82). One QTL, *qPWgt-2-1*, identified on chromosome 2 at 135cM conferred a QTL for panicle weight. This QTL was flanked by 1617828 and id2004418, with LOD scores 3.32 and the phenotypic variance expected by this QTL was 11.85% with negative parental additive effect (-0.03).

Two QTLs *qPH-2-1* and *qPH-2-2* were identified on chromosome 2 at different at position 149 and 184cM respectively. Both two identified QTL conferred a QTL for plant height. *qPH-2-1* was flanked by id2005152 and 1713554 with LOD scores 4.49. The phenotypic variation expected by this QTL was 13.73% within positive parental additive effect (8.94). *qPH-2-2* was flanked by 1407860 and id2001102 with LOD scores 3.66 and expected 12.01% of phenotypic variation against cold stress with positive of parental additive effect (1.28).

One QTL, *qTN-10-1*, was identified on chromosome 10 at 3cM. It was QTL for tiller number. This QTL was flanked by10480545 and SNP-10_18949540 with LOD scores 5.05 and the phenotypic variation expected by this QTL was 19.9% with positive parental additive effect (1.08).

Table 14: Identified putative QTL associated with cold tolerance rice at reproductive stage of BC_1F_2 plants from Soameva//Chomrongdhan

[a]QTL	[b]Ch	[c]POS	[d]LM	[e]RM	[f]LOD	[g]PVE(%)	[h]Add
qTN-10-1	10	3	10480545	SNP-10_18949540	5.05	19.90	1.09
qPH-2-1	2	149	id2005152	1713554	4.49	13.74	8.95
qPH-2-2	2	184	1407860	id2001102	3.66	12.01	1.29
qSpFert-5-1	5	19	id5008590	id5013231	2.78	10.49	4.79
qSpFert-7-1	7	51	7930710	7947435	2.71	9.02	-5.83
qPWgt-2-1	2	135	1617828	id2004418	3.32	11.86	-0.03

[a]QTL identified at in the present study; [b]Chromosome on which QTL for cold tolerant is located; [c]POS Position of QTL on chromosome (cM),; [d]LM Left marker; [e]RM Right marker; [f]LOD scores (Log10-likelihood ratio) offer the strength of the data supporting the existence of a QTL in a defined interval at LOD≥2.5 at P≤0.0001; [g]PVE(%), Phenotypic variance explained by identified QTL; [h]Add Additive genetic effects of QTL, Positive and negative value indicates that alleles resulting in an increasing tolerance are from Soameva and Chomrongdhan .

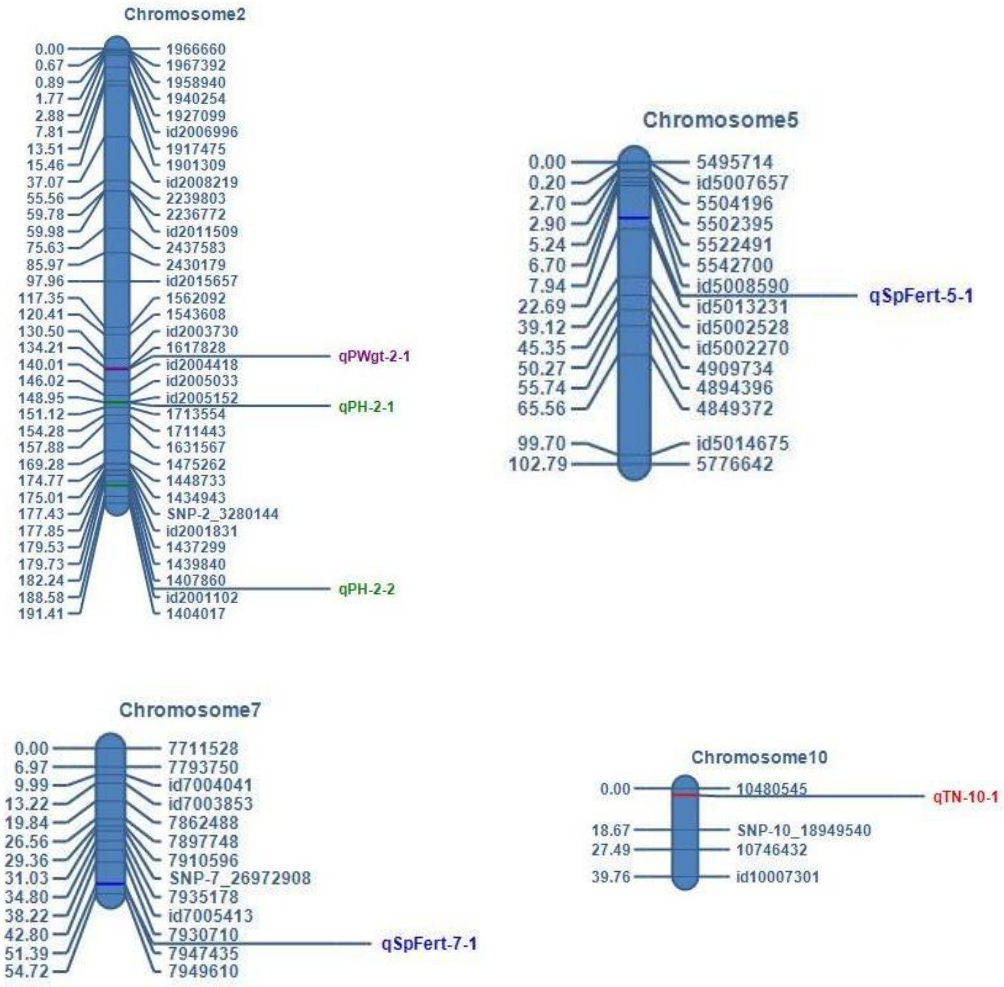


Figure 20: Genomic location of quantitative trait loci affecting cold tolerance at reproductive stage of BC_1F_2 plants of Soameva//Chomrongdhan. Map units are expressed in centimorgan (cM).

CHAPTER FIVE

DISCUSSION

5.1 Plant survival rate

BC_1F_2 plant of Vary botry//Chomrongdhan and Soameva//Chomrongdhan showed a clear difference in their rate of survival plant under the described cold stress condition at seedling stage.

The *indica* parent for both populations showed low survival rate ranging from 50 and 15% compared with the donor *japonica* parent which was Chomrongdhan. The *japonica* parent presented a relatively higher percentage of survival of the plants as also stated by Ma *et al.,* (2015). It confirms that Chomronghdan, the donor parent used in this study is a typical *japonica* rice and it exhibits more cold tolerance than *indica* varieties (da Cruz *et al.,* 2013; Ma *et al.,* 2015).

5.2 Phenotypic variation of BC_1F_2 progeny

Cold stress is one of the major environmental concerns in rice cultivation, especially in areas of high altitude and latitude including high-altitude regions in East Africa, and Madagascar (Jiang *et al.,* 2010, Wainaina *et al.,* 2015). Spikelet fertility (SpFert) was decreased when rice plants were exposed to low temperatures during the reproductive stage as well as the booting stages in this study. SpFert has been extensively used for evaluating cold tolerance during the reproductive stage (Suh *et al.,* 2010; Zhou *et al.,* 2010). Among the evaluated cold-related traits, SpFert was severely affected in recurrent parents (Vary Botry, Soameva) and the BC_1F_2 plants, and even in a cold-tolerant variety

(Chomrongdhan) that was moderately tolerant (Figure 13b and 17b). Importantly, this indicated that SpFert is a good indicator to use as a criterion for the routine selection of cold tolerance. The phenotypic Acceptability (PAcp) score showed highly significant differences between the parents and the BC_1F_2 population under cold treatment (Table 2). PAcp has been discussed as a complementary index of SpFert for cold tolerance in other studies (Lee, 2001, Jiang et al., 2011), that idea still confirmed on this study. The two parameters may therefore be used in the process of developing cold tolerant rice varieties. They are yield components that are related to tolerance to cold in rice. Additionally, heading was delayed, and the percentage number of BC_1F_2 plant considered as tolerance following panicle exsertion, full seeds number, panicle weight, spikelet fertility were less than the percentage number of BC_1F_2 plant considered as a susceptible and the recurrent parent, this given an indication that either minors genes or recessives genes were effective in causing the tolerant reaction confirm by additive effect by the identified QTLs.

Phenotypic variation was observed well in this study. These results indicated that a good selection method to evaluate cold tolerance in segregating populations is by the use of controlled air. Screening rice genotypes by imposing controls in air provides a conducive environment for selection, as it allows for correct measurement of traits associated with cold tolerance, and it is considered a reliable method of phenotyping for cold tolerance (Suh *et al.*, 2010). Screening for Cold tolerance using a greenhouse followed by controlled air temperature allows for evaluation of substantial differences in cold-related traits, and thus were suitable for the QTL study.

5.3 Quantitative trait loci analysis

5.3.1 Parental Diversity

The parents of mapping populations must have sufficient variation for the traits of interest at both the DNA sequence and the phenotypic level (Semagn *et al*., 2006c) for QTL analysis to be effectively carried out. The significant differences were found between donor parent Chomrongdhan and recurrent parent (Vary botry, Soameva) for the morphological studied traits. This allows for diversity studies using these parents. The recurrent parent relative number reduction was due to the cold stress and this confirmed the sensitivity of Vary botry and Soameva and tolerance of Chomrongdhan under cold stress.

The polymorphism level (34 and 27% between Vary botry//Chomrongdhan and Soameva//Chomrongdhan respectively) exhibited by the parents in this study was acceptable when compared with some studies. Therefore, it was good enough to be used for QTL analysis and linkage map as well. For example, Shinada *et al* (2013) when mapping QTLs for cold tolerance at fertilization stage, among a total of 949 SSR markers distributed throughout the genome only 87 markers (9.2%) exhibited polymorphisms. Xiao *et al* (2014), also mapping QTL for cold tolerance of rice roots at seedling and mature stages, only 113 markers showed the polymorphisms between the two used parents with a total of 653 SSR.

In this study, phenotyping data was relatively significant for any related study of the traits at seedling and reproductive stage after cold tolerance stress. Having confirmed acceptable Polymorphism for the genotyping data, the BC_1F_2 mapping population derived

from the cross between Vary botry//Chomrongdhan and Soameva//Chomrongdhan were suitable for mapping QTL for cold tolerance traits.

5.3.2 Identified Quantitative Traits Loci

This study allowed identification of QTLs linked to SdGwth, SdVig, and LfGwth at seedling stage (Vary botry//Chomrongdhan, Soameva// Chomrongdhan) and TN, PH, FSN, SpFert, HD (Vary botry// Chomronghdan), TN, PH, TSGW (Soameva//Chomrongdhan) at reproductive stage they were located on chromosomes number 1, 2, 4, 9 and 10 for seedling traits and on chromosome 2, 4, 5, 7, 8, 10 and 12 for the reproductive traits.

Both donor and recurrent parents were found to possess QTL alleles which increased phenotypic values.

Using one donor parent, in this study identified that, QTL associated with spikelet fertility *qSpFert-2-1* of Vary botry//Chomrongdhan, and QTL associated with panicle weight *qPWgt-2-1* of Soameva// Chomrongdhan showed a similar map positions; they were located in the chromosome 2 and close to 1617828 markers. The result suggests that this QTL is a common QTL for Vary botry//Chomrongdhan and Soameva// Chomrongdhan.

In this study, two different populations, one BC_1F_2 from Vary botry//Chomrongdhan and second BC_1F_2 from Soameva//Chomrongdhan identified a QTLs but not the same QTLs, this result confirm that QTLs for quantitative traits are influenced by genetic background effect. Used two different recurrent parents in this study confirmed that use of several

recurrent parents is essential to assess the effect of a QTL detected in one population when transferred to unrelated Genetic Backgrounds. When several recurrent parents were used, the consistency of the QTL effects in the different Genetic Backgrounds became less obvious

5.3.2.1 Identified Quantitative Traits Loci at seedling stage

The contribution of the parents to increase one trait was confirmed by the additive effect observed during QTL identification. At seedling stage, the result suggested that recurrent parent (Vary botry) was contributed 0.45, 0.10 and 0.43 by the alleles from the recurrent parent to increase *SdGwth14-10-1, SdVig0-2*-1, and *LfGwth14 -10-1* respectively. While, the donor parent (Chomrongdhan) contributed 0.2 by the alleles from the donor parent to increase *SdGwth14-10-2*. Then recurrent parent (Soameva) was contributed 1.0317, 0.5722, 0.2252, 0.8515 and 1.338 respectively by the alleles from the recurrent parent to increase, *qSdgwth7-9-1, qSdgrwth14-2-1, qSdgrwth14-9-1, qSdVig7-2-1, qLfGwth7-1-1* and *qLfGwt14-2-1* respectively. While, the donor parent (Chomrongdhan) contributed 0.6639 by the alleles from the donor parent to increase *qSdgwth7-4-1*.

This parental contribution values were relatively similar with the result of Liu *et al* (2015). They identified seven QTL and the susceptible parent contributed (-) 0.03 only to increase seedling cold tolerance when they were mapping QTL cold tolerance at the early seedling stage in landrace rice Xiang 743 using $F_{2:3}$ populations derived from a cross between cold tolerant landrace rice Xiang 743 and cold-sensitive variety Katy.

In addition, for this study at seedling stage, two cold tolerance loci *qSdGwth14-10-1* and *qLfGwth14 -10-1* with LOD 3.66 and 2.89 respectively coincided with the SNP markers

flanking between id10000391 and 10099158 on chromosome 10 in position 10 (Figure 21), moreover on chromosome 10 contained more than one QTL; it has up to three QTL for seedling growth and seedling leaf growth, hence *qSdGwth14-10-1* and *qSdGwth14-10-2*, those trait were considered as a major QTL (PVE >10%).

In this study at seedling stage, four putative QTL controlling seedling growth, seedling leaf growth and seedling vigor cold tolerance identified on chromosome 2 and 10 within donor and recurrent parent were contributed an additive effect for one trait also indicated that seedling under cold tolerance was controlled by multigenes (Shu *et al.*, 2011).

5.3.2.2 Identified Quantitative Traits Loci at reproductive stage

At reproductive stage, this study allowed identification of twelve QTLs linked to PH, FS, SpFert, TSW and HD. They were located on chromosome 2, 4, 5, 7, 8, 10 and 12 from Vary botry//Chomrongdhan. While six putative QTLs linked to TN, PH, SpFert and PWG from Soameva//Chomrongdhan. They were located on chromosome 2, 5, 7 and 10.

For the Vary botry//Chomrongdhan, 144 and for Soameva//Chomrongdhan 129 BC_1F_2 progeny were used for the mapping population to identify the QTL associated to cold tolerance at reproductive stage as well at booting stage. The results suggested that the Number of full seeds number, the spikelet fertility, 1000 seeds weight, heading date and plant height were contributed 9.99, 0.92, (2.27,2.90, 4.99), 10.71 and 2,71 by the alleles from susceptible parent Vary botry and contributed 5.83 and 0.03 to spikelet fertility and panicle weight respectively for Soameva.

This parental contribution values were high compared with the result of Kuroki *et al*., (2009). Five QTLs identified at booting stage with parental contribution was equal 0.08 to 0.09 when they were mapping QTLs for cold tolerance at booting stage using 114 recombinant inbred lines from cross between temperate japonicas, Kirara397 (cold-sensitive) and Hatsushizuku (cold-tolerant).

The increase of FSN, and SpFert (Vary botry//Chomrongdhan) and TN, PH, SpFert (Soameva//Chomrongdhan) under cold stress at booting stage were contributed by the alleles from the tolerant parent. This was confirmed by additive effect observed during identification of QTL affecting those traits. Donor and recurrent parent in this study were found to possess QTL alleles which increased phenotypic values, indicated that genes of cold tolerance are derived not only from the cold tolerant variety, but also from the cold susceptible variety (Jiang *et al.,* 2011).

5.3.3 Comparative analysis of identified QTLs

5.3.3.1 Comparative analysis of identified QTLs at seedling stage

In this present study at seedling stage, Vary botry//Chomrongdhan, seedling growth 14DAR (*qSdgwth14-10-1*) and leaf growth 14DAR (*qLfGwth14-10-1*) QTLs were identified on chromosome 10 at the same position at 10cM. The fact that those QTL were located at the same position, suggested that one or group of genes control the assessed trait. This means that the genes controlling both traits are pleitropic on the other hands, and this might contribute to significant positive correlation observed between seedling growth 14DAR and seedling leaf growth 14DAR (Table 10) in the BC_1F_2 progeny.

Two QTLs (*qSdGwth14-10-1* and *qSdGwth14-10-2*) which were located on chromosome10, one (*qSdGwth14-10-1*) was close to id10000391 at 10cM position while the other one (*qSdGwth14-10-2*) was at 20cM position from 10465477. These results suggest that these traits were controlled by multiples genes (polygenes) under cold stress.

While for Soameva//Chomrongdhan, seedling growth 14DAR (*qSdgwth14-2-1*) and seedling leaf growth 14DAR (*qLfGwth14-2-1*), QTLs were identified on chromosome 2 with *qSdgwth14-2-1* close to id2011509 at 68cM position and *qLfGwth14-2-1* closed to id2005033 at 147cM, suggesting that seedling growth 14DAR and seedling leaf growth 14DAR were controlled by multiples genes (polygenes) under cold stress.

Three QTL associated with seedling growth 7 DAR (*qSdGwth7-1-1,qSdGwth7-4-1* and *qSdGwth7-9-1* and) were identified on chromosome 2, 4 and 9, then two QTL associated with seedling growth 14 DAR (*qSdGwth14-2-1,qSdGwth14-9-1*) were identified on chromosome 2 and 9. Those results means that seedling growth 7 DAR were controlled by at least two different genes, and seedling growth 14 DAR were controlled by at least three different genes.

In this study QTL associated with seedling growth were identified on chromosome 2 and 10 (Vary botry//Chomrongdhan) and on Chromosome 2, 4 and 9 (Soameva//Chomrongdhan). In a previous similar study, Andaya and Tai (2007), Suh *et al.*, (2012), identified QTL associated with seedling growth at seedling stage on chromosome 4 (*qCTS4*, *qCTS4a*, and *qCTS4b*), and Lou *et al.*, (2007), identified QTL associated with seedling growth at seedling stage on chromosome 2 (*qCTS-2*). In contrast, Andaya and Mackill (2003a) identified QTL associated with seedling growth on

chromosome 12 (*qCTS12a*), Zhang *et al.,* (2005) on chromosome 3, 5, 8 (*qSV-3-1/2, −5, −8-1/20*), Koseki *et al.,* (2010) on chromosome 11 (*qCtss11*).

5.3.3.2 Comparative analysis of identified QTLs at reproductive stage

At reproductive stage into Vary botry//Chomrongdhan, three QTL associated with number of full seeds were identified, in which, one on chromosome 5 and two on chromosome 8. Four QTL associated with spikelet fertility were identified on chromosome 2, 5, 8 and 12, two QTLs associated with 1000 seeds weight were identified on chromosome 4 and 10. Those results indicate that number of full seeds, spikelet fertility and 1000 seeds weight were controlled by at least three, four and two different genes, respectively.

In the segregating population of Soameva//Chomrongdhan cross, two QTL associated with plant height were identified on chromosome 2 at different position (*qPH-2-1* at 149cM and *qPH-2-2* at 184cM), while two QTL associated with spikelet fertility were identified on chromosome 5 and 7. Those results indicate that plant height and spikelet fertility were controlled by at least two different genes for each one of the traits.

These results suggested that those traits were controlled by multiple genes (polygenes) under cold stress. This confirms the idea advanced by other authors, that rice cold tolerance at the booting stage is a quantitative trait controlled by multiple genes (da Cruz *et al.,* 2013). In other previous studies (Andaya and Mackill 2003b; Xu *et al.,* 2008; Saito *et al.,* 2010), there were reports that QTLs associated with Spikelet fertility on chromosome 4, 2, 3, 1,5,10, 11, 7, 9 (Ctb1*, Ctb2, qCTB2a, qCTB3, qCTB-1-1, −4-1/2, −5-1/2, −10-1/2, −11-1*) were identified.

Several studies related to identification of QTLs for cold tolerance rice at seedling and reproductive stage and using different mapping population and markers in different locations, have been undertaken it is difficult to compare the chromosomal locations of QTLs directly because different materials and molecular markers were used (Zhang *et al.*, 2014). However this present study has come up with molecular markers which have identified suggested positions of QTLs related to cold tolerance at seedling and reproductive stage.

5.4 Segregation distortion

Segregation distortion defined as deviation of the observed genotypic frequency from expected Mendelian ratio (Lu *et al.,* 2002; Li *et al.,* 2010). The segregation distortion from Mendelian ratio 1:2:5 of BC_1F_2 plants from Vary botry//Chomrongdhan and Soameva//Chomrongdhan, respectively was observed in 18.01% (282), and 24.16% (302) of the total polymorphic markers (1566) and (1250), respectively, reported in the present study. Since the genetic background of Vary botry and Soameva (*Indica*) and Chomrongdhan *(Japonica*) are different, a certain proportion of segregation distortion was expected in those crosses.

Segregation distortion is a common phenomenon that has been observed in many mapping studies (Alheit *et al.,* 2011) and it was generated with two parents that differed in genetic distance (Taylor and Ingvarsson, 2003).

In rice, 23.9 and 27.1% segregation distortion was reported from F_2 populations derived from japonica crossed with *indica* rice (Matsushita *et al.,* 2003; Wu *et al.,* 2010), Liu *et al* (2008) reported that there are segregation distortions in double haploid, RIL, F_2 and

BC_1F_2 populations. As an extreme example, a rice intraspecific population (CO39/Moroberekan) was reported to have distorted segregation ratio of up to 98% (Wang *et al.,* 1994). The percentage of distorted markers reported in this study is at relatively low level compare to other mapping populations. It is, therefore, expected that the polymorphic markers reported in this study are suitable for QTL mapping. Significant segregation distortion may affect linkage analysis in the estimation of linkage distance (Wu *et al.,* 2010, Reflinur *et al.,* 2012). The distorted markers reported in the present study were excluded from the data set for linkage mapping.

In the present study, the most distorted markers in the Vary botry//Chomrongdhan were found on chromosome 2, 4, and 11 while on chromosome 3, 1 and 2 in Soameva//Chomrongdhan and most of markers were distorted and skewed toward indica alleles, similar results as was observed from CO39 (*indica*) and Moroberekan (*japonica*) RI population, in which most of markers positioning was skewed toward the indica parent (Wang *et al.,* 2014). The presence of intespecific sterility genes could result in segregation distortion in rice (Gutierrez *et al.,* 2010). The theory suggests that the meiotic drive element such as gametophytic competition resulting in preferential fertilization or arbortion of the male or female gametes or zygotes is the main influence factors of segregation distortion in plants (Taylor and Ingvarsson, 2003). Since gametophyte genes and sterility genes have been reported on chromosomes 2, 4 and 11 to Vary botry//Chomrongdhan and on chromosome 3, 1 and 2 to Soameva//Chomrongdhan, segregation distortion reported in the present study may be due to gametophyte and sterility related genes, confirmed the idea that the presence of interspecific sterility genes

between *Indica* cross by *japonica* could also result in segregation distortion in rice (Gutierrez *et al*., 2010; Wu *et al.,* 2010).

5.5 Optimum conditions for evaluation of cold injury

For effective breeding variability in the germplasm is paramount. Although there are several reports to describe different methodologies to screen rice genotypes for cold reaction under controlled temperature conditions at different stages as well as temperature, time and evaluated traits, this fact has not been disputed.

At seedling stage, Bertin *et al.,* 1996, used 10^{o}C and 9 days for cold treatment and evaluate plant survival rate 10 DAR, Lee 2001 applied cool air treatment at 12^{o}C/10^{o}C (day/night) for 10 days at 3 leaf stage and evaluated growth and plant discoloration. Andaya and Mackill 2003b, Andaya and Tai 2006, used 9^{o}C for 4, 14, 16 and 18 days and evaluated visual scale (1-9). Qian *et al* (2000) applied 6 to 10^{o}C for 7 days and evaluated plant survival percentage.

At reproductive stage Koike *et al* (1990) used 12^{o}C at young microspore stage for 3-5days and evaluated the percentage of fertility, da Cruz *et al*., 2006b applied 17^{o}C for 7 days at the anthesis stage and to evaluate the percentage of fertility, Suh *et al* (2010); Jena *et al* (2012) used 17^{o}C at the booting stage for 10 days and assess the percentage of fertility. Besides the stage in which the plant is to be evaluated, duration of exposure to cold is also important, because it defines the degree of stress that will be imposed. In the present study exposing BC_1F_2 rice progeny to the temperature of 12°C for 10 days at seedling stage while 15^{o}C for 10 days at booting stage allowed for the distinction between cold tolerant and cold sensitive of new progeny following the different evaluated

traits at seedling and reproductive stage, those temperature presented the average of minimum temperature that observed during the 5 years ago (2009-2013) in the high altitude region of Madagascar (appendix 8), confirmed that optimum conditions are needed to evaluate stress tolerance (da Cruz *et al*, 2013).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

In this study, a cross between *Indica* rice used as a recurrent parent and *Japonica* rice used as a donor parent was produced a fertile BC_1F_2 plants, it confirms that there are introgress of cold tolerance gene from the *japonica* rice to the *indica*.

An experiment conducted in a greenhouse followed by cold treatment using a controlled air at seedling and reproductive stage to phenotype BC_1F_2 plants derived from crosses between *Indica* susceptible parent (Vary botry and Soameva) with tolerant *Japonica* (Chomrongdhan) allowed to distinguished cold tolerant and cold sensitive plant. A number of BC_1F_2 plants showed a good performance compared to the susceptible parent for this study, they were identified during phenotyping work.

The mapping population developed from the cross between Vary botry//Chomrongdhan and Soameva//Chomronghdan were suitable to genotype QTLs for cold tolerance, and within the development of molecular markers and linkage maps, different QTLs for different traits related to cold tolerance at seedling and reproductive stage were identified in this study. Therefore, Chomrongdhan, a tolerant check was found to be a novel source of cold tolerance;

At seedling stage, four putative QTL were identified on chromosome 2 and 10 of BC_1F_2 plants from Vary botry//Chomrongdhan crosses and seven putative QTL identified on chromosome 1, 2, 4 and 9 of BC_1F_2 plants from Soameva//Chomrongdhan crosses. While

at reproductive stage twelve putative QTLs were identified on chromosome 2, 4, 5, 7, 8, 10 and 12 of BC_1F_2 plants from Vary botry/Chomrongdhan crosses and Six putative QTL were identified onto rice chromosome 2, 5, 7 and 10 of BC_1F_2 plants from Soameva//Chomrongdhan crosses.

All of QTL occupied new positions in the rice genome compared with the results of previous study.

6.2 Recommendation

Cold is still one of the factors that decrease rice production in the high altitude region of Madagascar.

Introgression of tolerant QTLs/genes identified in this study could be useful to enhance the level of cold tolerance through markers assisted selection.

The gap between greenhouse research and field application is a major concern in cold tolerance research. Evaluating cold tolerance using open field criteria directly in the field should be continued to validate the result on this study;

Screening of segregating populations from this study should be continued until, hopefully, a variety or more will be released, that are cold tolerant;

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APPENDICES

Appendix i: Advantages and disadvantages of most commonly-used DNA markers for QTL analysis

Molecular marker	Codominant or Dominant	Advantages	Disadvantages	References
Restriction fragment length polymorphism (RFLP)	Codominant	• Robust • Reliable • Transferable across Populations	• Time-consuming, laborious and expensive • Large amounts of DNA required • Limited polymorphism (especially in related lines)	Beckmann and Soller (1986), Kochert (1994), Tanksley *et al*. (1989)
Random amplified polymorphic DNA (RAPD)	Dominant	• Quick and simple • Inexpensive • Multiple loci from a single primer possible • Small amounts of DNA Required	• Problems with reproducibility • Generally not transferable	Penner (1996), Welsh and McClelland (1990), Williams *et al*. (1990)
Simple sequence repeats (SSRs)∗ or 'microsatellites'	Codominant	• Technically simple • Robust and reliable • Transferable between Populations	• Large amounts of time and labour required for production of primers • Usually require polyacrylamide electrophoresis	McCouch *et al*. (1997), Powell *et al*. (1996), Taramino and Tingey (1996)
Amplified fragment Length Polymorphism (AFLP)	Dominant	• Multiple loci • High levels of polymorphism generated	• Large amounts of DNA required • Complicated methodology	Vos *et al*. (1995)

Appendix ii: Layout Vary botry//Chomrongdhan

Block	Treatment	Block	Treatment	Block	Treatment	Block	Treatment	Block	Treatment
1	new199	2	new476	3	new32	4	new285	5	new134
1	new109	2	new266	3	new4	4	new56	5	new217
1	new200	2	new30	3	new452	4	new233	5	new325
1	new445	2	new240	3	new437	4	new80	5	new98
1	new422	2	new499	3	new22	4	new159	5	new218
1	new20	2	new439	3	new166	4	new334	5	new466
1	new73	2	new490	3	new121	4	new414	5	new133
1	new124	2	new28	3	new284	4	new173	5	new373
1	new162	2	new302	3	new309	4	new55	5	new13
1	new129	2	new429	3	new177	4	new105	5	new37
1	new255	2	new262	3	check2	4	new348	5	new237
1	new258	2	new106	3	new484	4	new53	5	new442
1	check1	2	new473	3	new297	4	new180	5	new169
1	new482	2	new327	3	new491	4	new142	5	new319
1	new416	2	new397	3	new296	4	new234	5	new318
1	new481	2	new153	3	new443	4	new346	5	new244

1	new99	2	new423	3	new116	4	new214	5	new215
1	new289	2	new100	3	new436	4	new35	5	new340
1	new232	2	check2	3	new472	4	new379	5	new380
1	new317	2	new489	3	new350	4	new455	5	new197
1	new191	2	new206	3	check1	4	new279	5	new50
1	new222	2	new301	3	new353	4	check2	5	new67
1	new336	2	new49	3	new178	4	new150	5	new344
1	new122	2	new209	3	new163	4	new478	5	new251
1	new87	2	new59	3	new92	4	new179	5	new438
1	new111	2	new261	3	new342	4	new254	5	new91
1	new76	2	new375	3	new242	4	new228	5	new345
1	new259	2	new368	3	new399	4	new283	5	new25
1	new184	2	new140	3	new83	4	new60	5	new305
1	check2	2	new70	3	new420	4	new492	5	new181
1	new395	2	new430	3	new64	4	new146	5	new62
1	new84	2	new444	3	new440	4	new257	5	new276
1	new107	2	new495	3	new265	4	new74	5	new136
1	new448	2	new281	3	new194	4	new176	5	check2
1	new125	2	new365	3	new189	4	new268	5	new321

1	new398	2	new21	3	new45	4	new310	5	new43
1	new57	2	new384	3	new29	4	new483	5	new65
1	new263	2	new356	3	new89	4	new406	5	new202
1	new367	2	new113	3	new299	4	new322	5	new90
1	new174	2	new79	3	new388	4	new326	5	new171
1	new72	2	check1	3	new286	4	new290	5	new431
1	new479	2	new300	3	new131	4	new408	5	new407
1	new9	2	new26	3	new77	4	new467	5	check1
1	new248	2	new31	3	new464	4	new239	5	new97
1	new369	2	new94	3	new38	4	new391	5	new462
1	new378	2	new271	3	new69	4	new449	5	new216
1	new221	2	new205	3	new371	4	new363	5	new126
1	new385	2	new360	3	new338	4	new287	5	new354
1	new382	2	new123	3	new117	4	check1	5	new149
1	new323	2	new132	3	new156	4	new469	5	new372
1	new39	2	new145	3	new277	4	new104	5	new226
1	new320	2	new458	3	new461	4	new417	5	new332

Block	Treatment	Block	Treatment	Block	Treatment	Block	Treatment	Block	Treatment
6	check2	7	new500	8	new468	9	new253	10	new41
6	new24	7	new157	8	new270	9	new102	10	check2
6	new434	7	new130	8	new139	9	new193	10	new316
6	check1	7	new96	8	new394	9	new460	10	new364
6	new225	7	new249	8	new427	9	new288	10	new81
6	new402	7	new291	8	new8	9	new93	10	new267
6	new331	7	new390	8	new457	9	new137	10	new282
6	new183	7	new68	8	new58	9	new82	10	new152
6	new14	7	new447	8	new374	9	new496	10	new188
6	new424	7	new211	8	new386	9	check2	10	new108
6	new477	7	new293	8	new165	9	new196	10	new114
6	new229	7	new148	8	new15	9	new6	10	new250
6	new219	7	check2	8	check1	9	new403	10	new419
6	new198	7	new337	8	new12	9	new349	10	new19
6	new366	7	new359	8	new236	9	new203	10	new328

6	new46	7	new227	8	new33	9	new42	10	new292
6	new412	7	new78	8	new343	9	new167	10	new23
6	new411	7	new358	8	new269	9	new313	10	new10
6	new425	7	new312	8	new247	9	new207	10	new418
6	new120	7	new175	8	new154	9	new141	10	new454
6	new160	7	new377	8	new278	9	new48	10	new401
6	new118	7	new220	8	new387	9	new164	10	new295
6	new362	7	new155	8	new357	9	new383	10	new409
6	new231	7	new396	8	new474	9	new315	10	new471
6	new453	7	new182	8	new85	9	new86	10	new101
6	new404	7	new335	8	new158	9	new185	10	new238
6	new273	7	new351	8	new112	9	new223	10	new230
6	new303	7	new224	8	new17	9	new275	10	new204
6	new170	7	new456	8	new274	9	new361	10	new34
6	new16	7	new54	8	new497	9	new119	10	new405
6	new389	7	new432	8	new450	9	new381	10	new252
6	new410	7	new307	8	check2	9	new128	10	new18
6	new308	7	new2	8	new201	9	new190	10	new355
6	new187	7	new306	8	new36	9	new314	10	new298

6	new451	7	new241	8	new330	9	new352	10	new485
6	new465	7	new192	8	new347	9	new400	10	new272
6	new280	7	new63	8	new421	9	new151	10	new426
6	new143	7	check1	8	new415	9	new493	10	new115
6	new494	7	new138	8	new333	9	check1	10	new441
6	new341	7	new246	8	new5	9	new459	10	new3
6	new256	7	new47	8	new95	9	new392	10	new66
6	new339	7	new324	8	new498	9	new61	10	check1
6	new264	7	new40	8	new428	9	new433	10	new195
6	new235	7	new470	8	new435	9	new329	10	new260
6	new44	7	new475	8	new413	9	new186	10	new393
6	new88	7	new463	8	new488	9	new304	10	new7
6	new486	7	new487	8	new51	9	new27	10	new294
6	new213	7	new311	8	new168	9	new52	10	new376
6	new147	7	new208	8	new161	9	new243	10	new245
6	new446	7	new370	8	new71	9	new103	10	new11
6	new110	7	new1	8	new144	9	new212	10	new127
6	new210	7	new75	8	new480	9	new172	10	new135

Appendix iii: **Layout Soameva//Chomrongdhan**

1	23	2	3	4	28	6	7	8	9	10	29	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	1	5	9	13	17	21	25
10	1	5	3	1	9	13	2	12	8	16	7	18	4	20	14	5	26	15	2	19	10	24	11	21	13	6	25	22	2	6	10	14	18	22	26
2	22	6	17	15	9	22	19	1	21	25	12	23	9	18	26	6	20	8	23	29	17	4	27	13	3	21	18	16	3	7	11	15	19	23	27
29	27	24	10	25	14	7	20	4	15	19	7	28	16	11	3	27	11	17	5	14	8	24	12	28	11	5	26	29	4	8	12	16	20	24	28

1 to 27= Number of entry (BC_1F_2 plants), 28 to 29 Number of entry (parents)

Appendix iv: **Illumina SNP genotyping step according to the infinium assay standard protocol; Source: (Infinium HD Assay ultra, manual, from http://www.illumina.com)**

Sample preparation

Twenty microliter of DNA normalized to 50ng/ µl was dispersed into a deep-well, 96-sample plate using 8-channel pipette. The plates were labeled with a barcode sticker supplied by the kit and spin down in a centrifuge for 30 seconds, the plate were covered by a lid to keep out dust.

Amplification

The Illumina hybridization oven was preheated to 37^{o}C and allowed temperature to equilibrate. The MSA3 barcode label was applied to anew MIDI plate (infinium). The pack of tubes labelled MA1, MA2, and MSM (patented reagents) from Illumina were removed from the -20^{o}C freezer, set on bench at room temperature to thaw. These tubes were pulse centrifuged to 280xg. Using 8 channel pipettes, 20 µl of MAI was dispensed into each well of the MSA3 plate wells. Then, 4 µl of DNA sample from 96 well plates was transferred to the corresponding wells in the MSA3 plate. The original DNA sample identification for each well in MSA3 plate was recorded on the laboratory tracking form. Then, 4 µl of 0.1N NaOH was added into each well of the MSA3 plate containing MA1 and DNA sample. The MSA3 plate was sealed with the 96-well cap mat and vortexed for 1 min at 1,600 rpm on a microplate shaker and pulse centrifuged at 280 x g for 1 min.

The MSA3 plate was incubated at room temperature for 10 min. Then 24 µl of MA2 was dispensed into each of the MSA3 plate containing the DNA samples. This was followed by dispensing 38 µl of MSM into each well of MSA3 plate. The MSA3 plate was resealed with cap mat. The sealed MSA3 plate was vortexed at 1600 rpm for 1mn and pulse centrifuged at 280 x g for 1 min. The MSA3 plate was incubated in the Illumina hybridization oven for 24 hours at 37^{o}C.

Fragmentation

The FMS (patented reagent) tube was removed from the freezer and thawed on the bench and gently inverted to mix the contents. The FMS tube was pulse-centrifuged at 280 xg. MSA3 plate was removed from the illumina hybridization oven and centrifuged at 50 xg for 1 minute. Then, 25 µl of FMS was dispensed into each well of the MSA3 plate containing sample, covered with 96-well cap mat. The MSA3 plate was vortexed at 1,600 rpm for 1 min and centrifuged at 50 x g for 1 min at 22^{o}C. The sealed MSA3 plate was incubate on a preheated heat block set at 37^{o}C for 1 hour.

Precipitation

The PM1 (patented reagent) tube was thawed to room temperature and pulse-centrifuged at 50 x g for 1 min. The 96-well cap mat was removed from the MSA3 plate and 50 µl of PM1 dispensed into each well of the plate. The MSA3 plate was sealed with the cap mat and vortexed at 1,600 rpm for 1 min. The MSA3 plate was incubated on a preheated heat block set at 37^{o}C for 5 min. The MSA3 plate was centrifuged to 50 x g at 22^{o}C for 1 min. To the MSA3 plate containing the sample, 155 µl of 100% isopropanol was dispensed into each well, covered tightly with a new dry cap mat. The MSA3 plate was inverted at least 10 times to mix contents thoroughly. The contents in the MSA3 plate was incubated at 4oC for 30 min, centrifuged to 3,000 x g at 4^{o}C for 20 min. The cap mat was removed from the MAS3 plate and discarded. The supernatant was discarded by inverting the MAS3 plate and discarded. The supernatant was discarded by inverting the MSA3 plate and smacking it down onto an absorbed pad. The MSA3 plate was tapped firmly several times until all wells were devoid of liquid. The uncovered and inverted MSA3 plate was left on the tube rack for 1 hour at room temperature to air dry the pellet.

Resuspension

The RA1 (illumina) tube kept at -20^{o}C was removed from the freezer and thawed in a water bath set at room temperature. After thawing, 23µl of RA1 was dispensed into each well of the MAS3 plate containing DNA pellet. The remaining RA1 was maintained at 4^{o}C for later use during staining step. The MSA3 plate was sealed using a foil by firmly

holding the heat-sealer scaling block down for 5 seconds. The sealed MSA3 plate was placed in the Illumina hybridization oven preheated to 48ºC and incubated for 1 hour. The MSA3 plate was vortexed at 1,800 rpm for 1 min and pulse- centrifuged at 280 x g.

Hybridization

The hybridization (Hyb) chamber gaskets (Illumina) were placed into Hyb chambers. Then, 400 µl of PB2 (patented reagent, illumina) was dispensed into each of humidifying buffer reservoirs in each Hyb chamber (Illumina). The lid of each Hyb chamber was secured and kept on the bench at room temperature until ready to load beadchips. The re-suspended MSA3 plate was incubated on preheated heat block set at 95ºC for 20 minutes to denature the DNA samples. As the MSA3 plate was being denatured, a box of beadchips was removes from 4ºC and set on the bench. After 20 min incubation, MSA3 plate was removed from the heat block and placed on the benchtop at room temperature for 30 min. After, 30 min cool down, the MSA3 plate was pulse centrifuged at 280 x g. Just before loading the DNA samples, all the beadchips from their packages were removed making sure the beadstripe area and sample inlets are not touched.

The beadchip was placed in a Hyb chamber insert while orienting the barcode end so that it matches the barcode symbol on the insert. Using a multi–channel precision pipette, 12 µl of each DNA sample was dispensed onto the appropriate beadchip section, according to the chart on the laboratory tracking form. The samples were loaded by directly placing pipette tip to the array surface and the pipette held straight above the array surface making sure that all arrays contain the sample. DNA samples A1-F1 from the MSA3 plate were located into the left side beadchip inlet ports A1-F1 (every other inlet port on the left side of the beadchip). DNA samples in G1 and H1 from MSA3 plate were loaded into the left side beadchip inlet port G1 and H1. DNA samples A2-D2 from the MSA3 plate were loaded into the left side beadchip inlet port A2-D2. DNA samples E2-H2 from MSA3 plate were loaded into the right side beadchip inlet ports E2-H2. DNA smples A3 and B3 from the MSA3 plate was lode into the right side beadchip inlet ports A3 and B3. DNA samples C3-H3 from the MSA3 plate were loaded into the right side beadchip inlet ports C3-H3 (every other inlet port on the right side of the beadchip). The remaining

DNA samples were loaded following the colour-coded sections shown in the graphics supplied by Illumina. The beadchip barcode for each group of DNA samples was recorded on the laboratory tracking form. The sections of the beadchips were visually inspected ensuring that the DNA samples entirely covered each beadstripe.

The Hyb chamber insert (Illumina) containing beadchip were loaded into the Hyb chamber. The barcode end was positioned over the ridges indicated on the Hyb chamber. The back side of the lid was placed onto the Hyb chamber and the front end slowly brought down to avoid dislodging the hybridization chamber inserts. The clamps on both sides of the Hyb chamber were closed. The Hyb chamber was placed in a preheated Illumina Hyb oven set to 48^{o}C ensuring the clamps of the Hyb chamber face the left and right sides of the oven. The complete Hyb chamber containing the beadchips was incubated at 48^{o}C for 16 hours and the MSA3 plate discarded.

Washing of beadchip

The Hyb chambers were removed from Illumina hybridization oven and incubated at room temperature from 25 minutes prior to opening. Two wash dishes each were filled with 200 ml of PB1 (patented reagent, Illumina) and labelled as "PB1". Then, beadchip alignment fixture was filled with 150 ml of PB1 and clear plastic spacers were separated from the white glass backs. The glass back plates were cleaned as described in the Infinium assay Laboratory Set up and Procedure Guide. The wire handle was attached to the rack and submerged in the first wash dish, containing 200 ml PB1. Hyb chamber inserts one at time. IntelliHyb seal was removed from each beadchip using powder-free gloved hands. This was done by holding the beadchip in one hand with the thumb and forefinger on the long edges of the beadchip while avoiding contact with the sample inserts. The barcodes were ensured are facing up, close to the handler and the top of the beadchip angled slightly. The seal was removed in a single, slow, consistent motion by pulling it off in a diagonal direction. Beadchips were slide into the wash rack one at a time, making sure that the beachips are completely submerged in the PB1. After placing all the beadchips in the wash rack, it was moved up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation to remove bubbles. The wash rack was

then moved to the second wash dish containing PB1 making sure that the beadchips are completely submerged. Wash rack was moved up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation to remove air bubbles.

The flow-through chamber was assembly by filling the multi sample beadcheap alignment with 150 ml of PB1. For each beadchip, a black frame was placed into the multiple-sample beadchip alignment fixture (Illumina). Each beadchip was placed into a black frame, aligning its barcode with the ridges stamped onto the alignment fixture and immersed fully in PB1. A clear spacer was placed onto the top of each beadchip using the alignment fixture grooves to guide the spacers into proper position. Alignment bar was put onto the alignment fixture. Using a laboratory air gun, any accumulated dust from the glass plates were removed just before placing them onto the beadchip. A clean glass back plate was placed on top of the clear spacer covering each beadchip. The plate reservoir was placed at the barcode end of the beadchip facing inward to create a reservoir against the beadchip surface.

Metal clamps were attached onto each flow-through chamber by gently pushing the glass back plate up against the alignment bar with one finger. The first metal clamp was placed around the flow-through chamber so that the clamp was about 5mm from the top edge while the second metal clamp was placed around the flow-through chamber at the barcode end, 5mm from the reagent reservoir. Using the scissors, the spacers were trimmed at the non-barcode end of the assembly. Then, Hyb chamber reservoir was washed with distilled water and scrubbed with a cleaning brush ensuring that no PB1 remained. The assembled flow-through chambers were placed on the laboratory bench in a horizontal position while performing the preparation steps for beadchip staining.

Staining preparation

The water circulation was filled to the appropriate level. It was switched on and set to 44^{o}C to equilibrate. Bubbles trapped in the chamber rack were removed. The flow-through chambers was verified to ensure it reached 44^{o}C in multiple positions with an Illumina temperature probe. The reagent tube containing XC1, XC2, TEM, STM and ATM (patented reagents) were removed from the freezer, allowed to thaw at room

temperature, centrifuged to 3000 xg for 3 minutes and arranged in order in which they would be used. The XC4 re-suspended by adding 330ml of 100% ethanol. The XC4 bottle was

As the chamber rack reached 44^{o}C, each flow-through chamber assembly was placed into the chamber rack. In each reservoir of the flow-through chamber, 150µl of RAI was added and incubated for 30 minutes. This step was repeated five times. This was followed by adding 450 µl XC1 to each reservoir of the flow-through chamber and incubated for 10 minutes. Then, 450 µl of XC2 was added to the flow-through assemblies and incubated for 10 minutes. This was followed by addition of 200microliter TEM to the flow-through assemblies and incubated for 15 minutes. Then, 450 µl of 95% formamide/1mM EDTA was added to the flow-through assemblies and incubated for 1 minute. This step was repeated once and incubated for 5 minutes, the 95% formamide/1mM EDTA was prepared by mixing 95% formamide, 0.2% EDTA (0.5 M), 4.8% H_2O by volume and frozen in separate 15 ml increments. The temperature of the chamber rack was set to 37^{o}C and 450µl XC3 was added to the flow- through assemblies and incubated for 1 minutes. This step was repeated once more and chamber rack was left to attain desired temperature (37^{o}C).

The beadchip was stained by adding 250 µl STM and incubate for 10 min. Then, 450 µl of XC3 was dispensed into each of the flow-through assemblies and incubated for 1 min. This was repeated once more and set up left for 5 min. Then, 250µl of ATM was added to the flow- through reservoir and incubated for 10 min. This was followed by adding 450µl of XC3 to the flow-through assembly and incubated for 1 minute. This step was repeated once more and the set up left for 5 min before the next step. Then, 250 µl of STM was added and incubated for 10 min. This was followed by dispensing 450 µl of XC3 and incubates for 1 min. This step was repeated once more and the step up left for 5 minutes. Then, 250 µl of ATM was added and incubated for 10 min. This was followed by adding 450 µl of XC3 and incubated for 1 minute. This XC3 step was repeated once more and the set up left for 5 minutes before the next step. Then, 250µl of STM was added to the flow-through assemblies and incubated for 10 min. This was followed by adding 450 µl of XC3 and incubated for 1 minute. The XC3 step was repeated once more and left to set

for 5 min. The flow-through chambers were removed from the chamber rack and placed horizontally on a laboratory bench at room temperature.

About 310 µl of PB1 per 8 beadchip was poured into a wash dish and covered. The staining rack was placed inside the wash dish. For each beadchip, metal clamps were removed from flow-through chamber followed by the glass back plate, spacer and the beadchip. Beadchip were placed into the staining rack that was placed in the wash dish with barcodes facing away from the handler ensuring that all the chips were submerged.

Staining rack was moved up and down for 10 min, breaking the surface of reagent to remove bubbles and soaked for 5 minutes. Then, 310 µl of re-suspended XC4 was poured into a wash dish ensuring that it does stay unused for more than 10 min. The beadchip staining rack was moved into the XC4 dish ensuring that the barcodes are facing away from the handler. Staining rack was moved slowly up and down 10 times; breaking the surface of the reagent to remove air bubbles and soaked for 5 min. Staining rack was lifted out of the solution and placed on a tube rack with the staining rack and beadchips horizontal while barcodes facing up. The beadchips were removed from the staining rack with locking tweezers while working from top to bottom. Beadchip were dried in the vacuum desiccators for 50-55 min at 508 mmHg. The underside of each beadchip was cleaned with a ProStatEtOH wipe. The beadchip were stored overnight in the Illumina beadchip slide storage box inside vacuum desiccators at room temperature.

Scanning the beadchip

After the beadchip had dried, the vacuum was turned off by slowly returning the pressure to atmospheric. The iScan Reader software (Illumina) was activated and the beadchips moved into scanning tray. Beadchip's decoded filed were downloaded by activating the Decode File Client software and inputting the desires beadchip barcodes along with their corresponding box identification. Once the chips were properly seated in the tray and decode files recognized by the software, scanning was initiated. The iScan reader uses a laser to excite the fluorescence of the single-base extension product on the beads of the beadchip sections. Light emission from the fluorescence is then recorded in high resolution image of the beadchip sections.

Appendix v: The process of linkage map

1-Test of goodness of fit between two parents

MN	Ch	Position	Miss	A_Donor	Het	B_Recurrent	Ex.A	Ex.Het	Exp.B	Chisq	2	3	.	.
4824	1	194844	1	41	52	112	25.625	51.25	128.125	0.003579	H	B	.	.
7925	1	306611	1	41	53	111	25.625	51.25	128.125	0.003068	H	B	.	.
8144	1	315225	1	43	53	109	25.625	51.25	128.125	0.000644	H	B	.	.
9673	1	375814	1	41	53	111	25.625	51.25	128.125	0.003068	H	B	.	.
14005	1	530130	0	41	55	110	25.75	51.5	128.75	0.002479	H	B	.	.
20215	1	729666	1	41	52	112	25.625	51.25	128.125	0.003579	H	B	.	.
31684	1	1022215	1	40	52	113	25.625	51.25	128.125	0.007225	H	B	.	.
68081	1	2100471	0	17	2	187	25.75	51.5	128.75	2E-17	B	B	.	.
id1001821	1	2277961	0	18	1	187	25.75	51.5	128.75	1.04E-17	B	B	.	.
74775	1	2284907	0	46	55	105	25.75	51.5	128.75	3.46E-05	B	B	.	.
82473	1	2532252	1	15	0	190	25.625	51.25	128.125	2.67E-19	B	B	.	.
85083	1	2602798	1	44	55	106	25.625	51.25	128.125	0.000178	B	B	.	.
88627	1	2699862	0	45	56	105	25.75	51.5	128.75	6.89E-05	B	B	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12958034	12	23383250	0	15	0	191	25.75	51.5	128.75	2.03E-19	B	B	.	.
12958301	12	23387696	0	15	0	191	25.75	51.5	128.75	2.03E-19	B	B	.	.
12985052	12	24145303	0	35	35	136	25.75	51.5	128.75	0.011012	H	B	.	.
12990352	12	24317071	0	29	34	143	25.75	51.5	128.75	0.01893	B	B	.	.
12993236	12	24417433	0	29	36	141	25.75	51.5	128.75	0.04414	B	B	.	.

13009053	12	24913573	0	14	0	192	25.75	51.5	128.75	8.04E-20	B	B	.
13022382	12	25490919	0	11	0	195	25.75	51.5	128.75	3.8E-21	B	B	.
13022390	12	25491650	0	26	38	142	25.75	51.5	128.75	0.086084	B	B	.

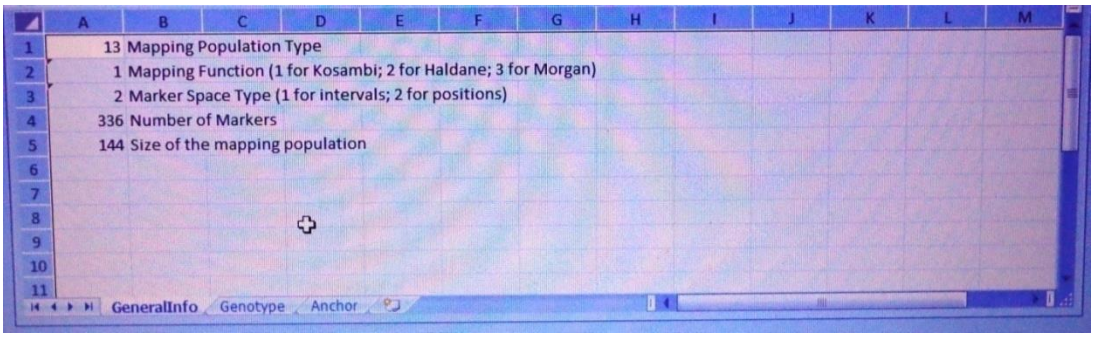
MN=Marker name; Chr=Chromosome

2- Binning of redundant markers

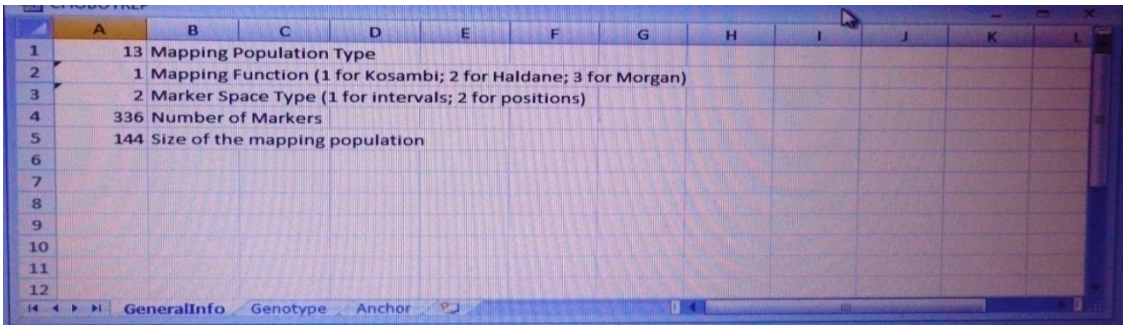
The remaining of markers after goodness of fit of Mendelian segregation ratio was binning to remove the redundant markers (Figure 2).

The input files for BIN have three formats: (1) general information, (2) marker types, and (3) anchor information (Figure 1).

1- General information



2- Marker types



3- Anchor information

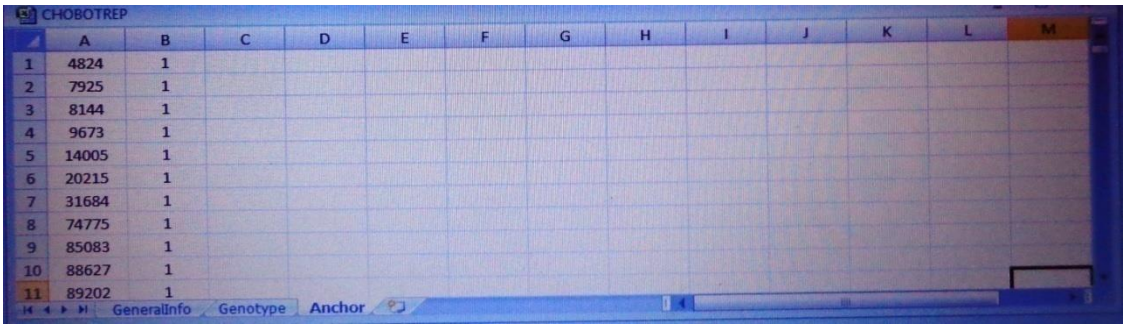


Figure 1: Input data for the BIN functionalities

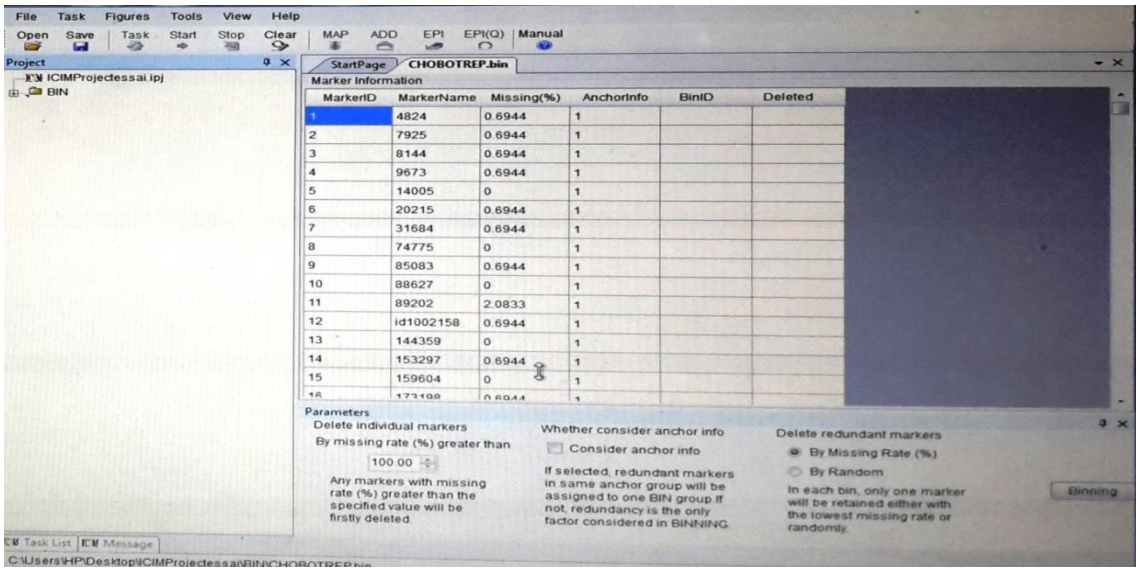
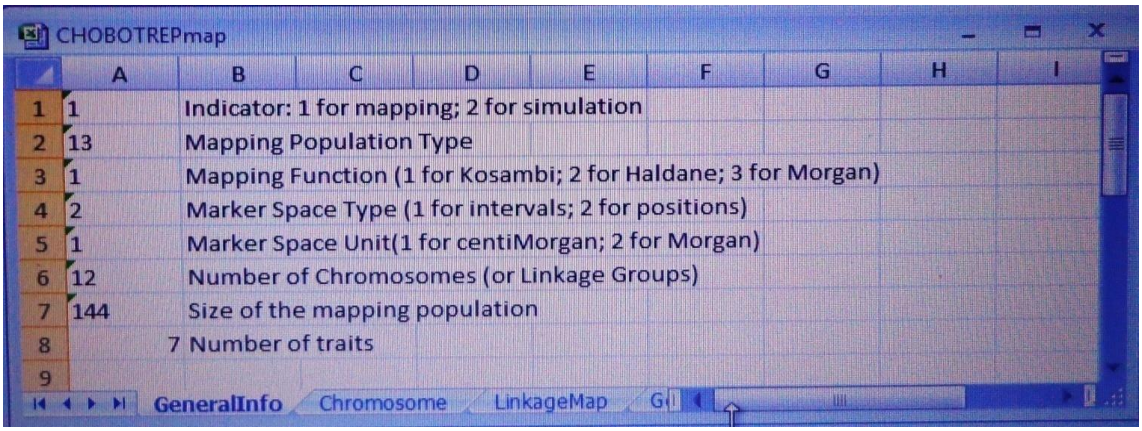


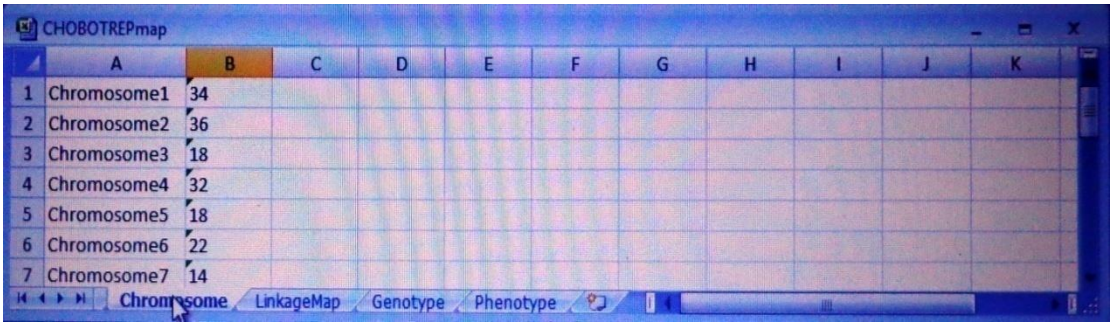
Figure 2: Interface of the BIN functionality. At the top of the interface are the menu and tool bars. At the left is the project window displaying the loaded input files and their output files under each functionality node. At the middle right is the marker summary display window. At the bottom right is the parameter setting window.

3- Mapping population

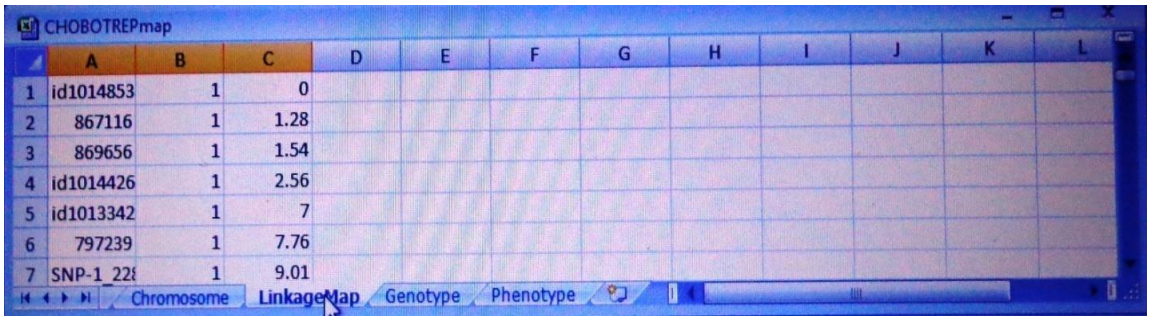
General information



'Anchor', Chromosomes

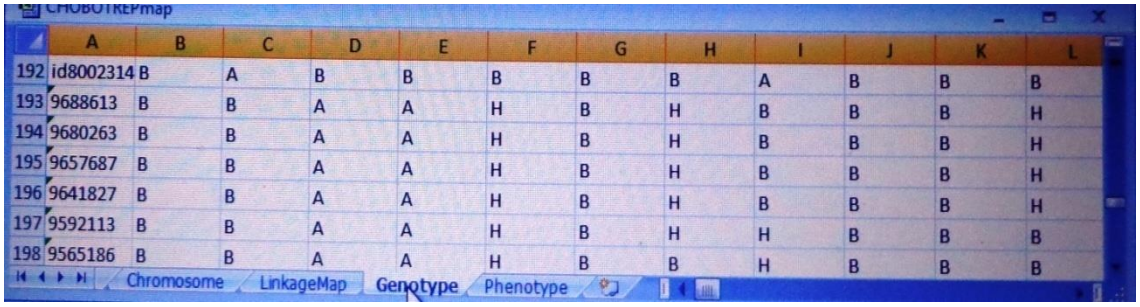


Linkage map



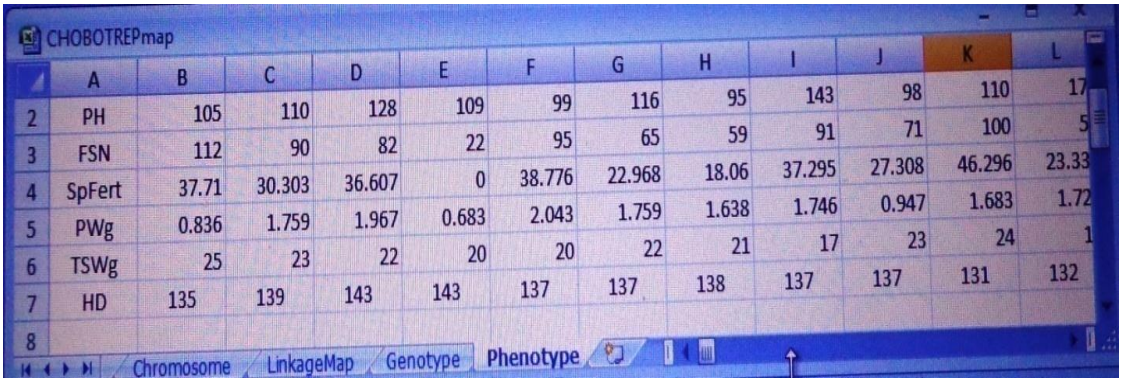
	A	B	C
1	id1014853	1	0
2	867116	1	1.28
3	869656	1	1.54
4	id1014426	1	2.56
5	id1013342	1	7
6	797239	1	7.76
7	SNP-1_228	1	9.01

Genotype



	A	B	C	D	E	F	G	H	I	J	K	L
192	id8002314	B	A	B	B	B	B	B	A	B	B	B
193	9688613	B	B	A	A	H	B	H	B	B	B	H
194	9680263	B	B	A	A	H	B	H	B	B	B	H
195	9657687	B	B	A	A	H	B	H	B	B	B	H
196	9641827	B	B	A	A	H	B	H	B	B	B	H
197	9592113	B	B	A	A	H	B	H	H	B	B	B
198	9565186	B	B	A	A	H	B	B	H	B	B	B

Phenotyp



	A	B	C	D	E	F	G	H	I	J	K	L
2	PH	105	110	128	109	99	116	95	143	98	110	17
3	FSN	112	90	82	22	95	65	59	91	71	100	5
4	SpFert	37.71	30.303	36.607	0	38.776	22.968	18.06	37.295	27.308	46.296	23.33
5	PWg	0.836	1.759	1.967	0.683	2.043	1.759	1.638	1.746	0.947	1.683	1.72
6	TSWg	25	23	22	20	20	22	21	17	23	24	1
7	HD	135	139	143	143	137	137	138	137	137	131	132
8												

Figure 3 Input data for the MAP functionalities

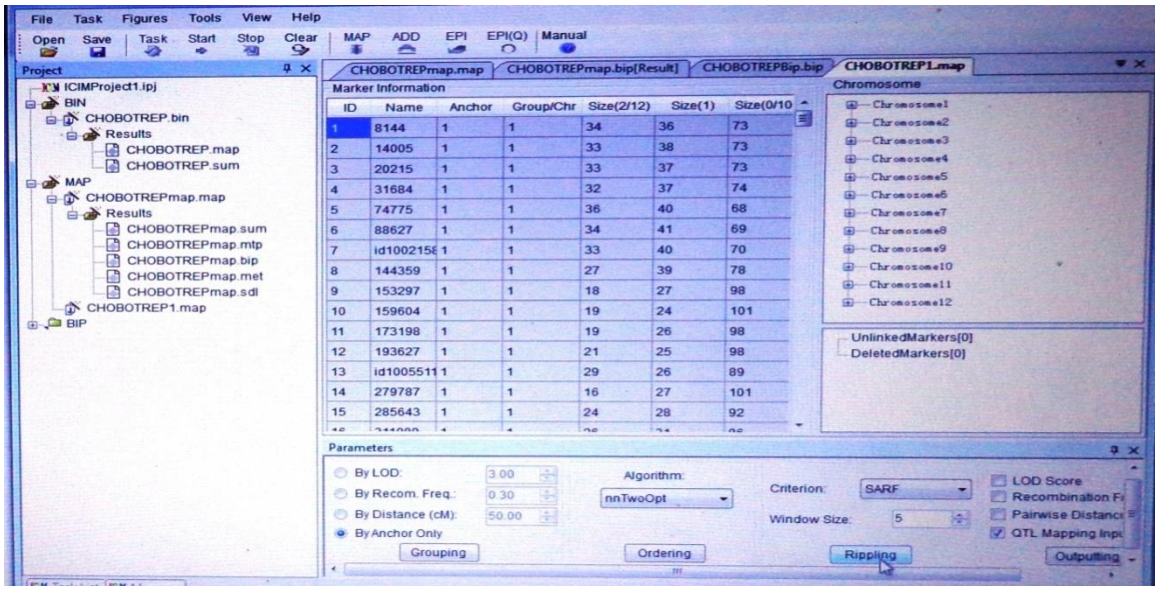


Figure 4: Interface of the MAP functionality. At the top of the interface are the menu and tool bars. At the left is the project window displaying the loaded input files and their output files under each functionality node. In the middle is the marker summary display window, and at the middle right is the linkage map display window. At the bottom right is the parameter setting window.

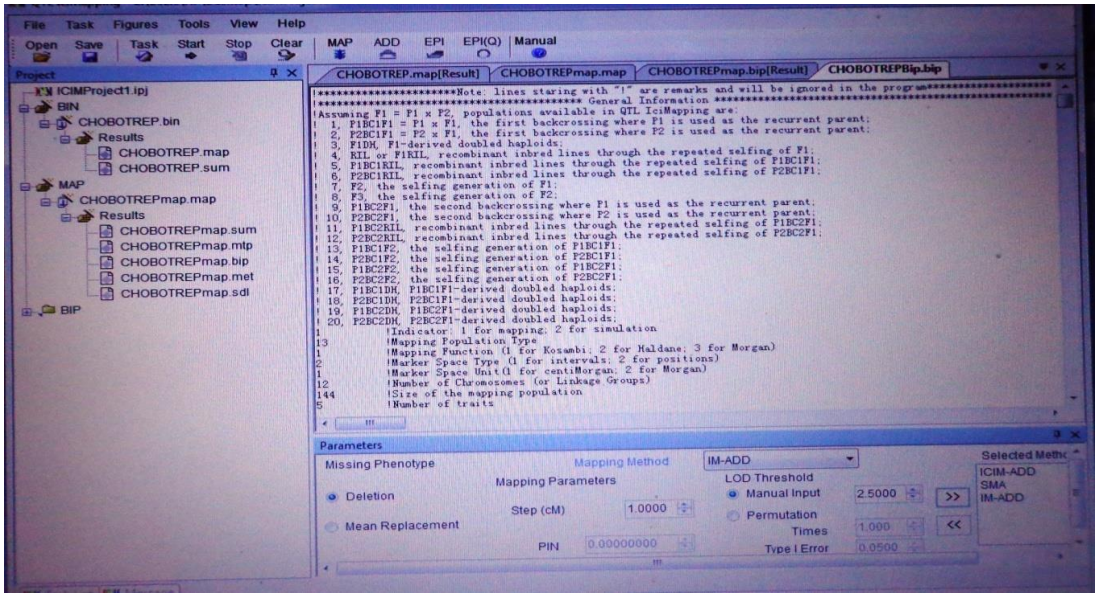


Figure 5: Interface of the QTL functionality. At the top of the interface are the menu and tool bars. At left is the project window displaying the loaded input files and their output files under each functionality node. At middle right is the input and output file display window. At bottom right is the parameter setting window.

Appendix vi

List of BC_1F_2 progeny from Vary botry//Chomrongdhan crosses that have number of full seeds more than recurrent parent

Order Number	New Progeny	Number of full seeds
1	New 2	112
2	New250	113
3	New49	110
4	New24	123
5	New33	132
6	New110	119

List of BC_1F_2 progeny from Vary botry //Chomrongdhan crosses that have higher Fertility rate more than recurrent parent

Order Number	New Progeny	Fertility rate
1	New32	46.30
2	New 52	45.76
3	New 55	45.99
4	New 72	63.36
5	New 77	75.37
6	New 80	79.37
7	New 105	47.34
8	New 109	57.35
9	New 121	65.58
10	New 126	56.79
11	New 133	46.26
12	New 158	63.70
13	New 163	63.64
14	New 208	52.35
15	New 250	68.49
16	New 257	52.66
17	New 263	47.83
18	New 273	65.65
19	New 29	54.84
20	New 49	77.47
21	New 89	66.17

22	New 205	54.02
23	New 118	50.76
24	New 18	46.15
25	New 24	58.02
26	New 33	49.81
27	New 213	75.78
28	New 110	58.91
29	New 180	72.46
30	New 14	48.00
31	New 178	62.88
32	New 245	49.02
33	New 147	65.67
34	New 206	45.58

List of BC_1F_2 progeny from Vary botry//Chomrongdhan crosses that have thousand seeds weight more than recurrent parent

Order Number	New Progeny	Thousand seed
1	New2	25
2	New 32	24
3	New 56	25
4	New 57	24
5	New 64	24
6	New 75	24
7	New 162	29
8	New 62	24
9	New 48	24
10	New 109	24
11	New 112	26
12	New 127	26
13	New 155	25
14	New 157	26
15	New 163	24
16	New 175	25
17	New 183	24
18	New 185	27
19	New 188	26
20	New 200	28

21	New 214	24
22	New 242	26
23	New 258	25
24	New 259	28
25	New 260	25
26	New 263	26
27	New 266	26
28	New 268	27
29	New 128	25
30	New 143	24
31	New 205	24
32	New 207	26
33	New 203	24
34	New 220	26
35	New 249	25
36	New 70	26
37	New 180	28
38	New 107	24
39	New 245	25
40	New 265	27
41	New 264	28
42	New 106	26
43	New 206	24

Appendix vii

List of BC_1F_2 progeny from Soameva //Chomrongdhan crosses that have number of full seeds more than recurrent parent

Order Number	New Progeny	Number of full seeds
1	New669	27
2	New 670	46
3	New 675	24
4	New 681	19
5	New 698	21
6	New 707	25
7	New 708	24
8	New 713	25
9	New 714	22
10	New 751	23
11	New 756	32
12	New 774	29
13	New 781	21
14	New 784	32
15	New 789	22
16	New 790	26
17	New 803	22
18	New 806	20
19	New 809	25
20	New 816	20
21	New 855	40
22	New 835	27
23	New 836	22
24	New 837	20
25	New 877	19
26	New 865	28
27	New 868	25
28	New 871	18
29	New 874	34
30	New 876	30
31	New 885	24
32	New 881	20
33	New 900	29
34	New 909	27
35	New 912	23
36	New 913	18

37	New 916	26
38	New 921	54
39	New 922	31
40	New 925	20
41	New 929	100
42	New 936	100
43	New 775	31
44	New 907	26
45	New 743	27
46	New 749	23
47	New 833	30
48	New 894	26
49	New 895	26
50	New 754	32
51	New 759	36
52	New 766	20
53	New 776	27
54	New 800	30
55	New 764	21
56	New 804	23

List of BC_1F_2 progeny from Soameva//Chomrongdhan crosses that have higher Fertility rate more than recurrent parent

Order Number	New Progeny	Fertility rate
1	New670	60.5
2	New674	53.6
3	New675	66.7
4	New682	50.0
5	New921	64.3

List of BC_1F_2 progeny from Soameva and Chomrongdhan crosses that have thousand seeds weight more than recurrent parent

Order Number	New Progeny	Thousand seed
1	New673	18
2	New 674	18
3	New 707	19
4	New 708	18
5	New 713	19
6	New 756	18
7	New 781	18
8	New 792	18
9	New 806	19
10	New 855	18
11	New 818	18
12	New 836	19
13	New 849	19
14	New 867	21
15	New 868	19
16	New 871	20
17	New 873	19.6
18	New 874	20
19	New 881	20
20	New 890	20
21	New 900	19
22	New 909	18
23	New 913	19
24	New 921	19
25	New 922	19
26	New 907	20
27	New 894	18
28	New 766	19
29	New 776	19

Appendix viii: Temperature 2009- 2013 in the part of high altitude of Madagascar; Source FOFIFA Andranomanelatra, Antsirabe

2013	Jan	Feb	Mar	Apr	Mai	June	July	Aug	Sept	Oct	Nov	Dec
Max Temp (°C)	25.3	23.5	24.5	24.1	22.3	21.2	21.2	22.9	26.5	25.4	26.8	25.6
Min Temp (°C)	15.3	15.9	14.6	12.1	9.9	4.4	4.6	5.8	8.2	13	14.5	15.3

2012	Jan	Feb	Mar	Apr	Mai	June	July	Aug	Sept	Oct	Nov	Dec
Max Temp (°C)									24.8	27.2	25.1	24.7
Min Temp (°C)									8.6	11.8	14.1	14.9

2011	Jan	Feb	Mar	Apr	Mai	June	July	Aug	Sept	Oct	Nov	Dec
Max Temp (°C)	24.5	26.5										
Min Temp (°C)	14.9	10.6										

2010	Jan	Feb	Mar	Apr	Mai	June	July	Aug	Sept	Oct	Nov	Dec
Max Temp (°C)	24.3	25.4	25.1	25.3	23.4	21.1	20.3	20.7	26.2	26.8	25.9	
Min Temp (°C)	16.1	15.2	16.1	11.8	10.4	8.2	6	7.6	4.9	10.8	12.2	

2009	Jan	Feb	Mar	Apr	Mai	June	July	Aug	Sept	Oct	Nov	Dec
Max Temp (°C)	26	25.4	25.4	23.4	23.2	22.2	20.4	20.8	23.8	25.3	28.2	25.5
Min Temp (°C)	15.6	14.1	16.1	13.2	9.5	7	6.7	9.1	9.6	11.9	13	15.2