BULKED SEGREGANT ANALYSIS FOR HEAT TOLERANCE IN SEGREGATING GENERATION OF RICE (*Oryza sativa* L.) USING SSR MARKERS

By

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(2015-11-103)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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VELLANIKKARA, THRISSUR – 680 656
KERALA, INDIA
2017

DECLARATION

I, hereby declare that the thesis entitled **'Bulked segregant analysis for heat tolerance in segregating generation of rice (*Oryza sativa* L.) using SSR markers'** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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ACKNOWLEDGEMENT

*First of all I would like to thank and praise almighty '**God**' the most beneficent and merciful, for all the blessing conferred on mankind.*

*With immense pleasure, I wish to express my heartfelt respect and affectionate gratitude to my beloved father **Gorakh Ganpat Waghmare** and mother **Sangita Gorakh Waghmare** whose everlasting love, unfading faith, incessant inspiration, moral and financial support and blessings kept me enthusiastic throughout my life and moulded me to the present position, and whose constant encouragement brings out the best in every one of my endeavours and without which this work could not have been completed.*

*I humbly take this opportunity to place my profound debt of gratitude on record to my esteemed major advisor and Chairperson of the Advisory Committee, **Dr. P. Sindhumole,** Assistant professor, Department of Plant Breeding and Genetics, RARS Pattambi, for her valuable suggestions, encouragement, affection, personal guidance, keen interest, immeasurable help and constructive criticism given throughout my work and making it possible to bring out this thesis. It was an honour and privilege for me to be associated with her who stood by me with boundless patience in many difficult situations during my M. Sc. Degree Programme.*

*I consider it as my privilege to express my heartfelt gratitude to **Dr. M. R. Shylaja,** Professor and Head, Centre for Plant Biotechnology and Molecular Biology for her constant support, co-operation throughout the research programme and critical scrutiny of the manuscript.*

*I deem it my privilege in expressing my fidelity to **Dr. Deepu Mathew**, Assistant Professor, Centre for Plant Biotechnology and Molecular Biology, for his scholastic guidance on the work in all of its stages. I owe my sincere thanks to him for his transcendent suggestions and constant encouragement to embellish the study, inspite of his busy schedule.*

*With immense delight and deep respect, I express my profound etiquette sense of gratitude and thanks to **Dr. Rose Mary Francies**, Professor and Head, Dept. of Seed Science and Technology, for her most valuable and painstaking guidance, concrete suggestions, constructive criticism, constant inspiration and cordial encouragement during the course of investigation and in the successful completion of present study.*

I sincerely thank ***Dr. P. S. Abida,*** *Professor, Dept. of Plant Breeding and Genetics RARS Pattambi, for her expert advice, constant inspiration, precious suggestions and generous support during my entire study, which helped in successful completion of this work. I am delighted to place on record my profound sense of gratitude to* ***Dr. P. A. Valsala,*** *Professor (Retired), Centre for Plant Biotechnology and Molecular Biology for her valuable advices.*

I am highly grateful to ***Dr. M. C. Narayanan Kutty****, Associate Director of Research, RARS Pattambi, for permitting me to carry out this research work at RARS, Pattambi, and for providing all the facilities as well as accommodation in the campus. I express my gratitude to* ***Dr. R. Ilangovan,*** *Professor, Dept. of Agronomy, RARS Pattambi, for his valuable assistance, immense help and for providing the required instruments during the field study. I also express my thanks to* ***Dr. K.M. Sunil,*** *Assistant Professor, KVK Pattambi, Palakkad, for sharing his experience and providing instruments. My sincere thanks are due to* ***Dr. P. Raji,*** *Professor, Dept. Plant Pathology, RARS Pattambi, for her valuable suggestions during field study.*

Moreover, my special thanks to ***Dr. A.T. Francis*** *for providing excellent facilities in Library and easy access to journals for my thesis reference.*

I express my sincere thanks to my classmates ***Kishor, Pramod, Ashiwin, Basil, Debashish, Arunima, Geethu, Deepali, Priya, Marjan*** *and* ***Giridhari****. I am happy to place on record my sincere thanks to my seniors, especially* ***Mangalam, Naresh, Reddy, Manjesh, Darshan, Sandesh****,* ***Saurav****,* ***Rosmol, Kalavati, Vinusri, Anju*** *and* ***Brinda****, for their immense help and support during the course of this study. I take this opportunity to appreciate the help rendered by* ***Mr. Ajinkya Ambavane*** *during the writing of this thesis.*

I also wish to express my sincere and heartful thanks to all the Staff members and Research Assistants of RARS, Pattambi, especially ***Sajini****,* ***Krishna****,* ***Vini, Khaeeja, Vismaya****,* ***Ranjith****,* ***Ali****,* ***and Ammu chechi****.*

I am extremely thankful to ***Mr. M.V. Yusuf,*** *Farm Superintendent, and* ***labourers*** *of RARS Pattambi, for their co-operation and support during the conduct of field experiment. I am also grateful to* ***Dr. S.M. Purushothaman****, Associate Professor, RARS, Pattambi,* ***Mr. Tijesh*** *and* ***Mess workers*** *for providing me good accommodation facility and healthy food at Trainees' Hostel, RARS Pattambi, during course of research. I also thankful to* ***Mr. Baveesh*** *for his help at RARS, Pattambi.*

I would like to acknowledge the kind help and affection extended by ***Ms. Komal Pawar****, during the writing of my thesis.*

I am forever indebted to my loving ***sisters, brothers*** *and* ***my entire family****, without whose blessings and support I would not have completed this work.*

I once again appreciate and acknowledge the facilities provided at ***RARS, Pattambi*** *and* ***CPBMB*** *for the successful completion of my research work. I also acknowledge all the other facilities, research grant and Junior Research Fellowship provided by the* ***Department of Biotechnology***, ***Kerala Agricultural University*** *for financial and technical support for persuasion of my study and research work.*

It would be impossible to list out all those who have helped me in one way or another in the successful completion of this work. I once again express my heartfelt thanks to all those who helped me in completing this venture on time.

Swapnil Gorakh Waghmare

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ABBREVIATIONS

BSA	:	Bulked Segregant Analysis
°C	:	Degree Celsius
cM	:	Centimorgan
cm	:	Centimeter
m	:	Meter
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
CTAB	:	Cetyl Trimethyl Ammonium Bromide
dNTP	:	Di-Nucleotide Triphosphate
DNA	:	Deoxyribose Nucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic acid
Ha	:	Hectare
g	:	Gram
KAU	:	Kerala Agricultural University
kb	:	Kilo base
M	:	Molar
mg	:	Milligram
min.	:	Minute
mm	:	Milimeter
ml	:	Milliliter
nm	:	Nanometer
ng	:	Nano gram
µg	:	Microgram
µl	:	Microliter
µm	:	Micromole
MAS	:	Marker Assisted Selection
MAB	:	Marker Assisted Breeding
OD	:	Optical Density
pH	:	Hydrogen ion concentration
PCR	:	Polymerase Chain Reaction

rpm	:	Revolution per minute
%	:	Per cent
RIL	:	Recombinant Inbred Lines
RNA	:	Ribonucleic Acid
SMA	:	Single Marker Analysis
SSR	:	Simple Sequence Repeat
Sec.	:	Second
QTL	:	Quantitative Trait Locus
V	:	Volt
USDS	:	United States Department of Agriculture
MT	:	Million Tones
Mha	:	Million Hectare
IPCC	:	Intergovernmental Panel on Climate Change
FAPRI	:	Food and Agricultural Policy Research Institute
IMD	:	India Meteorological Department
IL	:	Introgression Lines
IRRI	:	Rice Research Institute
RARS	:	Regional Agricultural Research Station
RM	:	Rice Microsatellite
LOD	:	Logarithm of Odds
SNPs	:	Single Nucleotide Polymorphisms

1. INTRODUCTION

Rice (*Oryza sativa* L.) occupies 23 per cent of the total area under cereal production in the world. Rice is also the staple food for more than half of the world's population. The United States Department of Agriculture (2016) reported Asia is the largest producer and consumer of rice. India is the second largest producer of rice after China. In India, during 2015-16, rice occupied an area of about 43.86 Mha with a production of 10,600 MT. Rice is the staple food of Kerala also, and production in the state is decreasing owing to the declining trend in rice cultivation, due to various stresses. In the present situation of climate alterations, mainly in the form of global warming, the high temperature is directly affecting growth, development and yield of rice.

The global mean temperature may increase by 2 to 4°C by the termination of this century (IPCC, 2007). Increasing high temperature acts as an abiotic stress factor that shows a strong impact on the survival, growth and reproduction of rice plant resulting in the loss of yield. Reproductive and ripening stages are the very sensitive stages in rice and heat stress during this period may lead to spikelet sterility. Now, in the major rice growing tracts of Kerala *viz*., Palakkad, Kole and Kuttanad, the temperature tends to rise up to 39°C or more especially during the second/third crop. Hence, heat stress induced spikelet sterility has become a severe problem in these tracks.

Rice origin is from tropical and sub-tropical areas and due to that, it can survive under high temperature but the usual growth may diminish as temperature exceed the optimal temperature (Jarrod *et al*., 2010). The ideal temperature required for the growth of rice crop is 20 to 37.5°C, but it often comes across various adverse climates during its rising season. Temperature over 35°C during the reproductive stages reduces rice production. The yield of rice is estimated to reduce by 41 per cent due to climate change by the termination of the 21st century (Ceccarelli *et al*., 2010).

High temperature during the flowering period can harm anther dehiscence, pollen germination and pollen tube growth that lead to sterility (Jagadish *et al*., 2007). The high-

temperature stress particularly arising during flowering stage in rice leads to low seed setting rate and less grain yield (Peng *et al.*, 2004). High temperature during flowering also affects grain quality as it produces chalkiness and can hamper the transportation of photosynthates to grains, shorten the effective grain filling stage and decrease the endosperm cell separation (Thangapandian *et al.*, 2010). To tackle this, high yielding varieties coupled with heat stress must be developed urgently. Since most of the prevailing high yielding varieties in Kerala like Uma are highly susceptible to heat stress, the heat tolerance trait must be incorporated into them using potential donors. However, study on heat tolerance using Kerala rice varieties, especially on linked markers, is not available at present.

The conventional breeding takes many years to complete the breeding cycle and to reach the farmers. The major problem in conventional breeding is the difficulty in confirming the presence of tolerance/resistance gene. Marker assisted selection (MAS) has been identified as a dependable, reproducible, and time-saving strategy to confirm the presence of the desirable gene and to quicken the breeding cycle.

Currently, with the progress of molecular marker techniques and their extensive application, there is numerous information on genetic interpretation of high temperature stress tolerance in rice (Cao *et al.*, 2002; Zhang *et al.*, 2009; Barakat *et al.*, 2011; Liao *et al.*, 2011; Xiao *et al.*, 2011; Cheng *et al.*, 2012; Poli *et al.*, 2013). Simple sequence repeats (SSRs), also recognised as the microsatellite markers, is an active tool for detecting genetic variances of germplasm resources with benefits of convenience, simplicity, high polymorphism, codominance and stability (Ishii *et al.*, 2001; Liao *et al.*, 2011).

Bulked segregant analysis is a technique to identify molecular markers linked to the gene of interest by screening for variances between two pooled (bulked) DNA samples derived from a segregating population created from a single cross. The two bulks constitute the DNA from 5 to 10 tolerant/resistant progenies and sensitive/susceptible progenies. These bulks are then compared with two parents for the identification of linked marker with a specific trait, for which the bulk is constituted (Michelmore *et al.*, 1991).

The objective of the present research programme is the identification of SSR markers linked to gene/s for heat tolerance in rice through bulked segregant analysis approach using segregating generation.

2. REVIEW OF LITERATURE

High-temperature stress is one of the key factor controlling crop growth and yield especially when temperature excesses coincide with precarious stages of plant development (McWilliam, 1980). High-temperature stress induces many biochemical, molecular and physiological changes and responses that influence various cellular and entire plant metabolism that affects crop yield and quality. The increase in atmospheric temperature causes unfavourable effects on development, yield and quality of the rice by disturbing its phenology, physiology and yield components (Peng *et al.*, 2004).

Susceptibility to high temperature in rice differs with growth phase, the rise in day and night temperature and genotype (Yoshida *et al.*, 1981; Peng *et al.*, 2004). Ceccarelli *et al.* (2010) reported that yield of rice may be reduced by 41 per cent due to 2 to 4 °C increase in temperature by the termination of the 21st century. The expansion of more sustainable and strong agricultural systems could be achieved by detecting heat tolerant rice genes, QTLs and improvement of rice varieties for mitigating the yield losses under high-temperature stress. The relevant literature on different aspects of the present study has been reviewed under the following sub heads:

2.1 Climate change and crop yield

More than 40 per cent of the world's arable surface is utilised for agriculture (Easterling *et al.*, 2007). In developing countries, agriculture supports more than 70 per cent of the rural population along with providing approximately 20 per cent of Gross Domestic Product (Easterling *et al.*, 2007). The worldwide human population is expected to increase by 34 per cent by 2050 and this should be coordinated with a 70 per cent increase in crop yields (Powell *et al.*, 2012). These forecasts are alarming given that 24-39 per cent of global wheat, rice, soybean and maize growing areas have deteriorated (Ray *et al.*, 2013).

Using the population forecasts from the United Nations and income estimates from the FAPRI (Food and Agricultural Policy Research Institute), worldwide rice demand is projected to increase from 439 million tonnes (milled rice) in 2010 to 496

million tonnes in 2020 and an additional increase to 555 million tonnes in 2035. This is a general increase of 26 per cent in the next 25 years, but the degree of growth will drop from 13 per cent for the first 10 years to 12 per cent in the next 15 years as population growth drops and people diversify from rice to other foods (Ricepedia, 2017). Decreasing crop yield can be attributed to growing population and the subsequent loss of agricultural land, as well as abiotic events such as increased drought, tropospheric ozone, and high temperature resulting from global climate change (Easterling *et al.,* 2007; Battista and Naylor, 2009; Lobell *et al*., 2011).

During 2016, India was considerably warmer than normal. The yearly average land superficial air temperature averaged over +0.91°C during 2016 beyond the 1961-1990 average, thus making 2016 as the warmest year on record (IMD, 2017).

Thangapandian *et al.* (2010) opined that temperature increase of 2°C is sufficient to generate the chalky characteristic of rice grain, the trait which is more influenced by high temperature and will result in quality and yield losses.

Global warming is predicted to increase the number of extremely hot days as well as increasing night-time temperature (Mora *et al.,* 2013). The frequency and duration of extreme heat events that exceed maximum temperature on record are predicted to increase by 50 per cent by 2050, and 90 per cent by 2100 (Handmer *et al*., 2012). This increase will affect crop yield more than the average annual increase in surface temperature (Lobell *et al*., 2011).

Given the substantial impact of high temperature on crop yield, numerous models have been put forth predicting the influence of climate alteration on crop yield (Battista and Naylor, 2009; Lobell *et al*., 2011; Ray *et al*., 2013). For every 1-3 °C rise in temperature, grain yield of major cereal crops *viz*., rice, wheat and maize are projected to initially increase in high latitude areas whereas to decrease in low latitudes (Easterling *et al.,* 2007; Lobell *et al.,* 2011). However, above a 3 °C increase, crop yields are predicted to decline at high latitudes as well (Easterling *et al.,* 2007; Lobell *et al.,* 2011; Handmer *et al.,* 2012). By the end of this century, rice yield was predicted to decline by 20-40 per

cent in the tropics and 3.1 per cent (19 million metric tonnes) globally (Lobell *et al.,* 2011).

A 'greening effect' due to elevated CO_2 increases yield in crops like wheat only up to an increase of 1.5 °C in temperature and yield reduction will be observed above this range (Easterling *et al.,* 2007; Lobell *et al.,* 2011). Similarly, doubling CO_2 concentration at high temperature has no positive impact on rice yield (Ziska *et al.,* 1996).

The IPCC, 2007 (Inter-governmental Panel on Climate Change) stated that each of the past three decades had been consecutively warmer at the Earth's surface than any previous decade since 1850. Cruz *et al.* (2007) point out that India is one of the world's most vulnerable country to climate change. Emission of greenhouse gases from human activities is responsible for climate change (IPCC, 2007). Nearly half of India's population is reliant on agriculture (Bureau of Labor Statistics, India, 2010). Climate change leads to increased temperature, changing rainfall patterns and amount, and a higher occurrence and intensity of thrilling climate events such as flood, cyclone, drought, and heat wave (IPCC, 2007).

Temperature rise and irregular rainfall pattern affect agriculture most directly and unfavourably (Almaraz *et al.,* 2008). Fluctuating climate over time disturbs rice crop production harmfully (Behnassi *et al.*, 2011). Two major climate variables involved in climate change are rainfall and temperature. The rise in temperature affects the crop planting, flowering, harvesting, plant growth and development (Teixeira *et al.,* 2011). The fluctuations and occurrence of extreme climate events, particularly at critical crop growth stages, reduce rice yield significantly (Teixeira *et al.,* 2011).

Since ancient ages, India's agriculture is more reliant on monsoon. Any variation in monsoon trend severely affects agriculture. Increasing temperature is the disturbing onset of monsoon in the country. IPCC (2007) points out that soaring temperatures (>0.5 °C increase during 2010-2039) in the Indo-Gangetic Plain, is leading to pre-monsoon changes that will mainly affect the wheat crop. In the states of Odisha, Jharkhand and Chhattisgarh alone, rice production damage through severe drought averaged about 40 per cent of total production with projected price of $800 million (Pandey, 2007).

Climate change directly affects on food production throughout the world. The rise in the average seasonal temperature can decrease the duration of many crops thereby falling the ultimate yield. In areas where the temperature is close to the physiological extreme for crops, warming will affect the yield more instantly (IPCC, 2014).

2.2 Heat tolerance in rice

During 2003 and 2004, parents of mapping population of rice for high-temperature stress tolerance were screened at optimum (30 °C) and extreme (35 °C and 38 °C) air temperature to determine the differences in the daily flowering time which may be a potential heat avoidance mechanism (Jagadish *et al.*, 2008). Peak anthesis earlier in the morning (1.5 hr after dawn) was seen by cultivar CG14 (*O. glaberrima*) under both control (30 °C) and extreme (38 °C) temperature conditions than *O. sativa* genotypes (≥3 hr after dawn). More than 6 hr at high-temperature exposure abridged the spikelet fertility than the exposure for 2 hr. Similarly, fertility was lower with exposure to 38 °C temperature, than 30 °C. In this study, N22 identified as the most heat tolerant genotype (64-86 % spikelet fertility) whereas cultivars Azucena and Moroberekan (*Oryza sativa* ssp. *japonica*) identified as the most susceptible (<8 % spikelet fertility) genotypes.

Two rice cultivars *viz.*, 996 and 4628, their F_1 and F_2 generations were utilised in an experiment to detect QTL related to high-temperature stress tolerance (Zhang *et al.*, 2009). At the same growth stage during heading, 279 F_2 plants were selected from the 500 F_2 plants along with parents placed at optimal temperature (30°C day 25 °C night) and at high temperature (37°C day 30 °C night) of controlled environment chambers, respectively. After seven days of continuous high-temperature stress treatment, plants were placed under ambient field conditions. The heat tolerant variety, 996, showed 81.55 per cent and 66.31 per cent spikelet fertility under optimal and high temperature, respectively whereas 4628 showed 79.32 per cent and 30.11 per cent spikelet fertility under optimal and high temperature respectively and was identified as heat sensitive variety.

Huang *et al.* (2009) studied the effect of high-temperature stress on the yield of three rice cultivars and twenty new strains at the University of Arkansas, Pine Bluff, U.S.

and noticed a general reduction in the yield of most of them due to heat stress at reproductive stage. However, one cultivar and six new strains showed good tolerance to high temperature with a grain setting rate of 70.4-87 per cent.

Mohammed and Tarpley (2010) studied effects of night temperature and spikelet position in the panicle on the yield factors including number of productive tillers, spikelet sterility and the length, width and weight of grains. Ambient night temperature (27 °C) and high night temperature (32 °C) in the greenhouse were used to treat the plants. Yield reduction (90%) was noticed in plants exposed to high night temperatures as a result of disturbing spikelet sterility (61%) and grain traits. Rise in spikelet sterility under high night temperature was linked with reduced pollen germination (36%), augmented respiration rates (28%) and relative membrane injury (86%), but not with photosynthesis. In addition to high night temperature, the location of the spikelet in the panicle also played a significant role in defining its weight and nitrogen concentration (Mohammed and Tarpley, 2010).

Allah *et al.* (2011) reported that the physiological parameters are necessary and reliable for screening a heat tolerant variety. Due to high temperature, the sensitive physiological processes will be affected thereby inducing sterility. They observed considerable variation for heat tolerance traits among twenty genotypes under heat stress and non-stress environments. Under heat stress, average grain yield decreased by 22 per cent as compared to non-stress conditions. Higher spikelet sterility (14% and 80%) and pollen sterility (15% and 54%) were observed under heat stress whereas less spikelet sterility (10% and 18%) and pollen sterility (16% and 34%) were observed under non-stress conditions. Pollen and spikelet sterility were negatively correlated with grain yield under both heat stress and non-stress conditions. ARC11094, Pinulupotl, Tupa729, Shinriki, Kameji and Giza 178 were identified as potential donors for heat tolerance.

Frequent exposure of rice crop to water scarcity and high-temperature stress at flowering stage will lead to lesser spikelet fertility and yield loss (Jagadish *et al.*, 2011). Water scarcity alone and in combination with high-temperature stress considerably abridged peduncle elongation, tricking 32 per cent and 55 per cent of spikelets inside the

leaf sheath, respectively. Spikelet fertility was higher in those exerted normally (>93%) than trapped spikelet (66%) and mean weighted fertility of exerted spikelets was lowest with heat stress (35%) but highest with collective stress (44%), signifying acquired thermotolerance.

According to Shah *et al.* (2011), the influence of high night temperatures is more disturbing than daytime temperature. Booting and flowering are the sensitive stages to high-temperature stress, which may lead to whole spikelet infertility. Morphological markers for high-temperature stress tolerance comprises flowering at cooler times of the day, higher pollen viability, bigger anthers, extended basal dehiscence and presence of extended basal pores. Biochemical processes like protection of structural proteins, enzymes, and membranes, and expression of heat shock proteins was found to impart thermotolerance.

High spikelet fertility was observed for IR64 (94 %) and N22 (95 %) in together control and treated plants (Ye *et* al., 2011). However, in the treatment of high-temperature stress, spikelet fertility of IR64 (21 %) was considerably lower than that of N22 (81 %), whereas it ranged from 0 per cent to 89 per cent in 158 F_2 progenies.

From the North-Eastern hills of India, 600 rice germplasm accessions were evaluated by Pale *et al.* (2012) for their tolerance to high temperature. Seeds of each genotype (presoaked overnight) were germinated at three different temperatures *i.e.*, 40 °C, 45 °C and 50 °C. Seventy-eight genotypes showed >80 per cent germination at 40 °C, while at 45 °C, only 27 genotypes showed >60 per cent germination. Among the latter 27 genotypes, eighteen successfully recovered from 40 °C treatment. However, only nine recovered from 45 °C treatment and they were found to contain 30-60 per cent relative water content under 22 days of drought stress.

Forty rice genotypes including 23 introgression lines (IL) derived from BC_2F_6 of Swarna × *Oryza nivara* and KMR3 × *Oryza rufipogon* and three mutants of N22 were evaluated for high-temperature stress tolerance at germination, seedling and early vegetative stage, under different temperature treatments (Prasanth *et al.*, 2012). Higher germination (%), mean length of shoot and root, dry weight, chlorophyll, carotenoids and

leaf senescence index observed seen in three Swarna x *O. nivara* ILs (166-2, 175-2, 3-1K), four KMR3 × *O. rufipogon* ILs (377-13, 50, 117, 13-7) and three IET hybrids (21528, 20907 and 20114) as compared to N22 at high temperature. IL117 along with two deep-water rice varieties, Jalmagna and Madhukar, showed the highest values for the first four traits at 40 °C temperature, while NH686 and NH787 mutants of N22 showed the least shoot and root length. The highly significant effect was noticed for heat on carotenoid content also.

During the evaluation of 100 hybrid combinations of rice for heat resistance during flowering, heat treatment of 38 °C was given for 3 days as 50 per cent spikelets flowered (Bo *et al.*, 2012). Based on spikelet fertility percentage, the population was separated into heat tolerant, semi-heat tolerant, and heat susceptible groups. Y Liangyou 646, Guangzhan 63-4S × R558, Yixiang 5979, Y Liangyou 2, and Y Liangyou 896 were heat tolerant, whereas II you 838, Fengyou 199, and Jingchuyou 37 were heat susceptible groups.

Pot experiments were conducted by Xie *et al.* (2012) during 2008 with Yangdao 6 and Nanjing 44 by giving temperature stresses (35 °C for 3 days, 35 °C for 5 days, 39 °C for 3 days and 39 °C for 5 days) at heading in environment-controlled chambers. The traits *viz.* grain yield, chlorophyll content, net photosynthetic rate, superoxide dismutase content and leaf area index exhibited a sharp drop, while relative conductivity and malondialdehyde content improved gradually with the rise of high-temperature stress and extension of stress period. Under the same temperature stress, the proportion of some physiological and biochemical indices in Yangdao 6 were inferior to that in Nanjing 44. Response to high-temperature stress was sturdier in Nanjing 44 than Yangdao 6.

During 2008 dry season, the spikelet fertility of 455 tested accessions ranged from 7.3 - 92.7 per cent in the field (Tenorio *et al.*, 2013). When 200 selected accessions with high spikelet fertility (55.6 - 92.7%) were grown in the outdoor growth chamber, their spikelet fertility ranged from 0 - 44 per cent and only 28 varieties had relatively higher spikelet fertility. Further screening involving these 28 promising varieties along with 12 new breeding materials (from IRRI) resulted in 48.5-90.4 per cent spikelet fertility in the

field (2009 dry season) and 0.5- 60.1 per cent in controlled conditions (2009 wet season). Thus, 23 varieties with higher spikelet fertility were identified as potential donors of high-temperature stress tolerance.

Phenotypic and physiological characters like pollen viability, plant height, panicle length, yield/plant, tiller number, spikelet fertility, number of panicles, relative water content, chlorophyll a/b ratio, Fv/Fm and electron transport rate were studied in N22, NH219 and IR64 (Poli *et al.*, 2013). Experiments were conducted in the field with two sets, one at optimal environmental conditions and the other at high-temperature stress conditions. Except for the difference in chlorophyll a/b ratio and ETR, all the observed trait values at optimal conditions were higher in NH219 compared to N22 and IR64. All three genotypes showed an increase in plant height and reduction in all other observed traits. Under high-temperature stress, the rise in plant height was more in NH219 (12.82 %) than in N22 (4.59 %). A substantial decrease in yield was seen under high-temperature stress conditions in both N22 (33 %) and NH219 (23 %).

During Kharif 2012, under raised temperature of 2 °C and 4 °C, the days taken for development was less (96 days and 102 days) as compared to optimal temperature condition (108 days). The grain yield was also less (23 and 13.3 %, respectively) than yield at ambient condition. The maximum grain yield was observed from the treatment under ambient temperature with 6.20 t/ha, followed by 5.30 t/ha under 2 °C level and 4.70 t/ha at 4 °C level. Hence, the yield reduction under raised temperature was due to the high number of sterile spikelets and reduced crop duration (Rani and Maragatham, 2013).

Lowland genotypes (Swarna, Pooja and Ranjit) and upland genotypes (Satabdi, Annapurna and Khitish) were screened for high temperature stress tolerance at six diverse day and night temperature systems (50/40, 45/35, 40/30, 35/25, 30/20 and 25/15 °C with a constant diurnal temperature variation of 10 °C) during panicle initiation for three days. High-temperature stress above 35/25 °C harmfully affected panicle extrusion, flowering period and number of flowered spikelets. In addition, a significant decrease was observed for tube length and viability of pollens, number of pollens on stigma and anther

dehiscence. Qualitative and quantitative variations in pollen proteins of these genotypes were seen, which possibly led to the differential damage of viability (Das *et al.*, 2014).

Manigbas *et al.* (2014) used three heat-tolerant donors (N22, Dular and Nipponbare) for hybridization with widely grown high-yielding rice varieties from Korea, Vietnam, Cambodia, Thailand, and the Philippines. In different breeding institutions, F_1 seeds were developed and shared among the country partners. To select individuals with heat tolerance and high yield, screening was performed in the field and glasshouse condition. During this experiment, several advanced breeding lines from Gayabyeo × N22 were produced desirable individuals with heat tolerance, resistance to pests and diseases, and high yield potential.

Field screening 400 indigenous germplasm accessions during the hot season, helped identify ninety accessions with high spikelet fertility as potential donors for heat tolerance (Patel *et al.*, 2015). The high temperature of 39 °C was suitable for screening heat tolerance at booting stage, while 38°C was suitable for evaluation at flowering stage. K 1434 had the highest spikelet fertility percent followed by J 222 and N 646.

Of the 1217 lines from diverse warm rice growing areas screened by Masuduzzaman *et al.* (2016), only 2 per cent of them showed some degree of high-temperature stress tolerance. Though, most of the high temperature stress tolerant types were traditional varieties with detrimental agronomic traits, which make it problematic to use them in breeding programme. Based on spikelet fertility under high-temperature stress (average 32 °C at flowering stage), best heat tolerant materials were selected.

IWP, a medium-duration fine rice variety from the Tamil Nadu Rice Research Institute, Aduthurai, susceptible to high-temperature stress was evaluated along with N22 (an ideal donor of high-temperature tolerance). The spikelet fertilities of N22 under control and high-temperature treatments were 86.63 per cent and 72.85 per cent, respectively, while those of IWP were 68.82 per cent and 16.52 per cent, respectively. Some of the individuals of mapping population developed from the two genotypes showed 60 to 67 per cent spikelet fertility and used for finding the QTLs linked to high-temperature stress tolerance (Vivitha *et al.*, 2017).

Prasanth *et al.* (2017) tested six rice varieties (Vandana, N22, KMR3, IR64, Swarna and BPT5204) for their reaction to high-temperature stress enforced by delayed sowing at field conditions. Flag leaf temperature, Flag leaf chlorophyll content, stomatal conductance, net photosynthetic rate, spikelet fertility and grain yield were recorded to evaluate high-temperature stress. High-temperature stress diminished whole spikelet fertility by 16 per cent and grain yield by 29 per cent. KMR3 and N22 were recognised as greatly high-temperature stress tolerant and BPT5204 and Vandana were recognised as highly vulnerable to heat stress.

2.3 Mechanisms of heat tolerance in rice

The internal pressure generated by pollen swelling is essential for the breaking of the anther tissues *viz.,* stomium and septum, which play a major function in anther dehiscence. The breaking of these tissues apparently results in pollen release from the anther (Matsui *et al.*, 1999). High-temperature stress just before the days of flowering lowers the function of thecae (a lateral pair of anther sacs) to dehisce, whereas, high temperature at flowering decreases the capability of pollen grains to swell and result into reduced thecae dehiscence. The temperature of 32-38 °C inhibits anther dehiscence and prevents pollination, which is the first step in the fertilisation process. The primary cause of yield reduction during high-temperature stress is the improper anther dehiscence during anthesis (Matsui *et al.,* 2001).

Various cultivars were observed for the severity of anther indehiscence response to high-temperature stress (Matsui and Omasa, 2002; Jagadish *et al.,* 2007). Heat sensitive rice cultivars were found to have altered developmental patterns within the stomium and septum, and these included fewer cell numbers in the septum of the locule and larger substomial lacunae that operate to weaken the septum for dehiscence, following pollen swelling (Matsui and Omasa, 2002).

Heat tolerant cultivars were proposed to shed more pollen than less tolerant cultivars at both optimal and high temperature, due to a longer basal split in the theca (Matsui *et al.,* 2005; Tian *et al.,* 2010). Instead, Matsui and Omasa (2002) found no definitive role for pollen swelling in anther indehiscence under drought stress and opined

that the role of pollen swelling as the driving force in anther dehiscence under abiotic stress, remains to be clarified.

Rice cultivars that undergo anther dehiscence earlier during the daytime for avoiding anther indehiscence and cultivars with large basal dehiscence slits that allow for more pollen shedding and escaping the heat stress were identified as high-temperature stress tolerant cultivars (Shah *et al.,* 2011).

Jagadish *et al.* (2010) observed, even if tolerant cultivars manage to shed pollen at high temperature, pollen viability could be lost within as little as ten minutes, and pollen germination as well as pollen tube growth would be reduced, suggesting this feature as ineffective in the selection of cultivars for high-temperature tolerance.

Adversely affected anther dehiscence, pollen production and pollen viability are assumed to result in yield reductions at high-temperature stress (Jagadish *et al.,* 2010; Shah *et al.,* 2011). Impaired division of meiocytes (Prasad *et al.,* 2006) or microspore development (Satake and Yoshida, 1978) occurring due to high-temperature stress resulted in lesser pollen grain production. The number of meiocytes initiated was directly proportional to the number of pollen grains produced by an anther and reduction in pollen grain number indicated the direct influence of heat stress on cell division during anther development, particularly at meiosis (Pressman *et al.,* 2002).

Heat stress also had a negative effect on mitosis in rice. After the first mitotic division of microspores, the two pollen grains undergo starch deposition and the high temperature reduced the number of pollen grains with starch deposition (Raghavan, 1988; Shah *et al.,* 2011).

More than ten germinated pollen grains on the stigma are required for fertilisation in rice. Significant reductions in pollination, pollen viability, and pollen germination on the stigma, were linked with the decline in pollen production, and pollen grains with starch (Jagadish *et al.,* 2010).

2.4 Effect of high temperature on various growth stages of rice

The growth of a rice plant is generally divided into three phases *i.e.* vegetative, reproductive and grain filling and these stages can be affected by high-temperature stress. The developmental phase at which the plant is exposed to high-temperature stress defines the harshness of the possible injury to the crop (Wahid *et al.,* 2007). Rice with comparatively higher tolerance at the vegetative phase is extremely susceptible to high-temperature stress during the reproductive phase, typically during flowering (Prasad *et al.*, 2006; Jagadish *et al.*, 2007, 2008, 2010).

Floret sterility and abnormal pollination may be caused by high-temperature stress above 35 °C (Prasad *et al.*, 2006). Heat stress at the booting stage can lead to irregular pollen development and floret distortion, thus resulting in anomalous fertilisation (Shi *et al.*, 2008).

The period from booting to flowering is the most sensitive stage to high-temperature stress in rice. Regular temperatures higher than 30 °C or daily extreme temperatures higher than 35 °C through the flowering period will result in reduced anther dehiscence and inferior pollen production. This results in fewer number of germinating pollen grains and a low fertilisation percentage (Matsui and Omasa, 2002; Prasad *et al.*, 2006).

High night temperature during the precarious stages of development decreases rice yield and quality. A study was conducted by Mohammed and Tarpley (2009) to determine the effects of higher night temperature and defensive exogenous effectors on growth, development, physiology, and yield of rice plants. Plants were exposed to optimal night temperature (27.80 °C) and high night temperatures (32.80 °C) with constantly controlled infrared heaters, starting from 20.00 hours till 06.00 hours. The higher night temperature did not affected leaf photosynthetic rate but affected the chlorophyll content, leaf nitrogen content, percent pollen germination and spikelet fertility. Averagely, plants grown under higher night temperature exhibited 72 per cent and 90 per cent decrease in spikelet fertility and yield respectively, compared to the plants grown under optimal night temperature. Higher night temperature stress on spikelet fertility and pollen germination

affected the dry matter content of the grains. As compare to the plants grown under optimal night temperature, those under higher night temperature showed 10 per cent reduction in the number of viable leaves during the vegetative stage and also 7 per cent and 13 per cent reduction in overall chlorophyll content, and 13 per cent and 12 per cent decrease in chlorophyll a content, during the vegetative and booting stages, respectively (Mohammed and Tarpley, 2009).

2.4.1. Effect of high temperature at vegetative stage

Exposure to high temperature affected different characters of rice plant depending upon the stage of crop growth. The impact of increased temperature has an accumulative effect on the later phases of plant development. Changes that occur during the vegetative and ripening phases can alter the grain filling phase and grain quality of rice (Yoshida *et al.*, 1981).

Evaluation for heat tolerance at germination, seedling and early vegetative stage of development under different temperature treatments was carried out using introgression lines (ILs) derived from BC_2F_6 of Swarna × *O. nivara* and KMR3 × *O. rufipogon* and 3 mutants of N22 (Prasanth *et al.*, 2012). Swarna × *O. nivara* ILs (166-2, 175-2, 3-1K), KMR3 × *O. rufipogon* ILs (377-13, 50, 117, 13-7) and three IET hybrids (21528, 20907 and 20114) showed higher germination per cent, mean shoot and root length, dry weight, chlorophyll, carotenoids and leaf senescence index, compared to the tolerant check Nagina 22 at high temperature. Two deep water rice varieties, Jalmagna and Madhukar and IL117 showed the highest germination, mean shoot and root length, and dry weight whereas the two N22 mutants, NH686 and NH787 had the least shoot and root length at 40 °C.

2.4.2 Effect of high temperature at reproductive stage

Anthesis and microsporogenesis reproductive phases of rice are very sensitive to high (>35 °C) (Matsui and Omasa, 2002; Prasad *et al.,* 2006) in addition to low (15 °C) (Jacobs and Pearson, 1999) temperature. Spikelet sterility may be caused due to the effect

of extreme temperature on anther dehiscence, pollination, and pollen germination (Gunawardena *et al.*, 2003)

Many researchers (Yoshida, 1981; Matsui *et al.*, 2001; Matsui and Omasa, 2002; Prasad *et al.*, 2006; Jagadish *et al.*, 2007; Jagadish *et al.*, 2008) had reported significant genotypic and phenotypic variation due to high temperature stress tolerance during the flowering period in rice.

Jagadish *et al.* (2007) studied the influence of temperature on a flowering pattern by growing rice varieties at 30 °C day and 24 °C night temperature in a greenhouse and then relocated them to growth chamber for the temperature treatments. Spikelet fertility was abridged by about 7 per cent per °C above 29.60 °C in IR64 and by 2.40 per cent above a threshold of 33 °C in Azucena.

Cao *et al.* (2011) investigated the pollen development, yield components and physiological parameters of *indica* rice genotypes under high-temperature treatment during heading and early filling stages. Two heat-sensitive genotypes (Shuanggui 1 and T219) and two heat-tolerant genotypes (Huanghuazhan and T226) were grown in pots under high temperature and natural temperature. In the heat-sensitive genotypes, high-temperature treatment significantly reduced the rates of spikelet fertility and seed setting rate. The results suggested that the relatively high yield in heat tolerant genotypes under high-temperature stress was associated with low leaf temperature, high root activity, and the high levels of ATPase activity in grains, photosynthetic rate, and activities of antioxidant enzymes in leaves.

Model for predicting spikelet sterility under high temperature was constructed by Nguyen *et al.* (2013). Temperature rise at meiosis and flowering affected the spikelet fertility. The heating degree hour causing 50 per cent spikelet sterility at meiosis was higher in a *japonica* cultivar (Hwaseongbyeol) than in a Tongil type (Dasanbyeol), while the air temperature causing 50 per cent spikelet sterility at spikelet flowering time was higher in Tongil type cultivars than in *japonica* ones.

During the testing of nearly 25 different genotypes under severe conditions (39 °C temperature and 75% relative humidity), N22 sustained higher tolerance compared with the other selected entries at IRRI and also at other places, presenting that the tolerance trait of N22 was extremely stable (Jagadish *et al.,* 2016). Similarly, among a diverse set of genotypes, N22 was recognised as a perfect donor of the high-temperature stress tolerance gene at flowering phase (Prasad *et al.*, 2006; Jagadish *et al.*, 2008).

Opening flowers in the early morning is an advantageous response to escape spikelet sterility caused by high-temperature stress at anthesis, because the sensitivity of rice flowers to high temperatures stress drops during the 1 hour period after flower opening (Satake and Yoshida, 1978). Thus, flower opening time 1 hour previous than usual may decrease sterility, since it may lead to anthesis before the air temperature touches the critical level; air temperature can increase at a degree of >3 °C per hour starting around 10.00 (Nishiyama and Blanco, 1980).

Early flower opening in rice is a significant method for reducing high temperature induced spikelet sterility. For example, the flowers of *O. glaberrima* Steud. opens earlier than those of *O. sativa* L. (Nishiyama and Blanco, 1980; Jagadish *et al.*, 2008), and the flowers of interspecific hybrids among *O. glaberrima* and *O. sativa* opens prior than those of *O. sativa* (Nishiyama and Blanco, 1980).

Sterility caused by high-temperature stress is more expected to arise in warm humid than hot arid environments. When rice genotypes were grown under irrigated flooded conditions in four different environments *viz.*, the relatively humid dry season in the Philippines, the summer season in southern France, and the cold dry as well as hot dry seasons in northern Senegal. The spikelet sterility at grain maturity was observed up to 40 per cent sterility was noticed in the Philippines due to high temperature (Julia and Dingkuhn, 2013).

During 2008 and 2009, 59 rice varieties from seven countries, IRRI and WARDA were assessed in International Rice Heat Tolerant Nursery and N22 and IDSA 77 were found as high-temperature stress tolerant rice varieties. During the screening of 1669 F_4 breeding lines for high temperature stress tolerance, 154 breeding lines were promoted to

F_5 generation, based on pollen sterility and spikelet sterility (%), high-yielding potential with high grain quality, above 35 °C under optimal field condition and 38°C under controlled phytotron screening (Thangapandian *et al.*, 2010).

Jagadish *et al.* (2010) screened F_6 population of 181 RIL's (recombinant inbred lines) of tolerant variety Bala and susceptible Azucena for high temperature tolerance at anthesis by determining spikelet fertility at ambient (30°C) and high temperature stress (38 °C) in experiments conducted in the Philippines and the United Kingdom. The parents varied considerably for absolute spikelet fertility under ambient (79-87%) and at high-temperature stress (2.90 to 47.10%). Spikelet fertility under ambient conditions was averaged at 62.10%, while at high-temperature stress it was seriously tilted towards Azucena (2.90 to 6.90%), in the RILs.

The Institute of Agricultural Sciences (IAS) and Cuu Long Delta Rice Research Institute (CLRRI) made 22 crosses, and subsequently their F_4, F_5 and BC_2 were developed, to set up RIL and BC mapping populations (Buu *et al.*, 2013). Backcrossing was done with the donors as N22 and Dular and the recurrent parents used were OM5930, AS996. Heat treatment of 36-40°C was given at heading stage under field and phytotron conditions. Some promising varieties were identified OM6707, OM8108, TLR391, TLR392 and TLR397 based on spikelet fertility and high yield under heat stress.

Wang *et al.* (2004) revealed that during summer 2003, the Yangtze River rice growing region in China experienced high temperature above 39 °C which caused a great decrease in rice production due to spikelet sterility (30 to 70%). Matsui *et al.* (1997) reported that spikelet sterility can be critically increased at temperatures higher than 35°C.

Edilberto *et al.* (2006) conducted a series of trials with a set of 455 IRRI Gene bank accessions brought from hot countries (Iraq, Iran, India, Afghanistan and Pakistan). According to flowering duration, materials were grouped and staggered sowing of the entries was done. Twenty-eight best accessions with the minimum grain sterilities were selected and further tested during 2009 dry season along with 12 new promising germplasm lines. Entries *viz.* Darbari Roodbar, Larome and Mulai were some of the

superlative entries, which were equivalent or better than N22 for characters like spikelet sterility, pollen sterility, early time anthesis and grain yield per plant.

2.5 Varietal selection

N22 is the most heat tolerant variety of rice found so far. Jagadish *et al.* (2010) studied the proteomic and physiological approaches to know heat tolerance during anthesis. Some of the heat shock proteins in N22 were considerably upregulated and it may be the reason behind the high-temperature stress tolerance of N22. Experiments to compare the heat tolerance nature of N22 revealed N22 as one of the most heat tolerant variety. N22 can be used in breeding programs to transfer its high-temperature stress tolerance trait into other economically important high, yielding and heat susceptible rice varieties (Jagadish *et al.*, 2010; Thangapandian *et al.,* 2010; 2011; Buu *et al.*, 2013; Poli *et al.*, 2013; Tenorio *et al.*, 2013; Buu *et al.*, 2014; Manigbas *et al.*, 2014; Lang *et al.*, 2015; Ye *et al.*, 2011).

2.6 Bulked segregant analysis and marker assisted selection for heat tolerance in rice

Current progress in rice genomics research and completion of the rice genome sequence is convenient to identify genes and for gene mapping through linkage analysis and DNA markers (Jena and Mackill, 2008). Molecular markers are potent tools for finding the genetic variations and in the interpretation of genetic relationships within species (Thomson *et al.,* 2007).

Bulked segregant analysis (BSA) is a method to detect the molecular markers linked to the gene of interest by screening for alterations between two bulked (pooled) DNA samples derived from a segregating population made from a single cross. DNA bulks with fewer than ten individuals are normally bulked, rather than screening dozens of individuals, for linkage analysis with selected polymorphic primers saving time and resources. These pooled DNA samples vary genetically only in the certain region but heterozygous and monomorphic for all other regions. Segregating population, from which

the bulks were constituted, is used for validation of linkage between target loci and polymorphic marker. Markers for loci that are polymorphic in nature and totally associated with the region are used to discriminate the individuals constituting the bulks and this will detect clear variances between the bulks. If the marker is not associated, then loci will look heterozygous with nearly identical band intensities in each bulk. BSA is not a suitable method to find novel types of variances but rather allows the quick screening of many loci and hence the identification of segregating markers (Michelmore *et al.*, 1991). Many researchers (Zhang *et al.*, 2009; Prashant *et al.*, 2012; Yang *et al.*, 2013; Dixit *et al.*, 2014; Palanog *et al.*, 2014; Sandhu *et al.*, 2014; Tiwari *et al.*, 2016) conducted bulked segregant analysis for identification of molecular markers associated with several traits in rice.

Zhang *et al.* (2009) studied the genetic influence of QTLs conferring high-temperature stress tolerance during flowering stage in rice through BSA. The results of single marker analysis showed that SSR markers, RM3735 and RM3586 on chromosome 4 and chromosome 3 has significant linkage with high-temperature stress tolerance, indicating that high-temperature stress tolerance at flowering stage in rice was controlled by numerous genes.

Currently, with the progress of molecular markers and its wide application, there were reports on heat tolerance in rice by using molecular markers (Cao *et al.,* 2002; Zhu *et al.,* 2005; Zhao *et al.,* 2016). Genotypic variation in high-temperature stress tolerance at the flowering time (usually 5 to 6 days duration) in *japonica* and *indica* species had been documented (Yoshida *et al.,* 1981; Matsui *et al.,* 2001; Matsui and Omasa, 2002; Prasad *et al.,* 2006; Jagadish *et al.,* 2007; Jagadish *et al.,* 2008).

The genetic influence of QTLs conferring high-temperature stress tolerance during flowering in rice was studied by using 279 F_2 individuals from the cross between 996 (heat tolerant) and 4628 (sensitive) (Zhang *et al.*, 2009). Results showed that markers RM3735 (on chromosome 4) and RM3586 (on chromosome 3) indicated significant linkage with high-temperature stress tolerance, respectively.

Liao *et al.* (2011) used 791 BC_1F_8 backcross recombinant lines, resulted from the cross of Xieqingzao B / N22 // Xieqingzao B for evaluation of high-temperature stress tolerance at natural high temperature in fields. The rice lines with high temperature stress tolerance index greater than 90 per cent or lesser than 40 per cent were nominated to compare the morphological traits. Out of the 887 SSR markers, the genomic polymorphism between heat tolerant 703T line and heat sensitive 704S line was the minimum in the six pairs of rice lines, with only 16 polymorphic loci, with 22 diverse alleles.

Xiao *et al.* (2011) found two QTLs controlling high-temperature stress tolerance using the RILs developed from a cross among the high-temperature stress tolerant cultivar 996 and the heat susceptible cultivar 4628. Two QTLs that affected pollen fertility were detected in the interval between RM 5687 - RM 471 and RM 190 - RM 225 on chromosome number 4 and chromosome number 6, respectively.

In Saudi Arabia, heat tolerant (Debra) and heat susceptible (Yecora Rojo), two rice cultivars were crossed to develop F_1 and F_2 generations to assess high-temperature stress tolerance at the grain filling time during 2009 at high-temperature stress conditions (Barakat *et al.*, 2011). Bulked segregant analysis showed that three microsatellite markers *viz.*, Xgwm617, Xgwm577 and Xgwm132, were associated with spikelet fertility.

To find QTLs affecting heat tolerance at anthesis stage, 240 IL's (introgression lines) were developed from the advanced backcross generation of a cross between Xiushui 09 (*japonica* cultivar) and IR2061(*indica* breeding line) (Cheng *et al.,* 2012). *qSF4* and *qSF6* were identified as linked to high-temperature stress tolerance.

Nine polymorphism markers identified between IR64 (heat tolerant) and NH219 (heat susceptible) cultivars were primarily used for genotyping all the F_2 population. SSR marker RM1089 was found to be linked with yield per plant and number of tillers, RM 423 to leaf senescence, RM584 to leaf width, and RM 229 to yield per plant, all associated with high-temperature stress tolerance (Poli *et al.*, 2013).

Bharathkumar *et al.* (2014) used three SSR markers *viz.*, RM3735, RM3586 and RM6100 linked to high-temperature stress tolerance at flowering stage to screen seven rice landraces *viz.*, Rupsal, Nagalmutha, Ravana, Marishal, Polai, Talmugra and Raspanjar. Marker RM6100 marker was found to be linked with a major QTL on chromosome 10 for high-temperature stress tolerance at flowering stage.

Pradhan *et al.* (2016) studied a group of breeding lines and landraces including 240 germplasms. Tolerant and susceptible plants were selected by comparing spikelet fertility percentage under high-temperature stress. TASSEL analysis showed strong linkage of marker RM547 with spikelet fertility and the markers like INDEL3, RM314, RM205, RM242, RM247 and RM228, ultimately regulating the high-temperature stress tolerance.

Prasanth *et al.* (2012) used BSA, selective and whole population genotyping approaches for finding the QTLs for grain yield under drought stress in rice. Comparative analysis revealed that selective genotyping and bulked segregant analysis needs 63.50 per cent and 92.10 per cent fewer data points, respectively, than whole population genotyping in N22 × MTU1010 $F_{3:4}$ mapping population. *qDTY1.1* a QTL for grain yield under drought stress was identified using BSA and *qDTY8.1*, by whole population genotyping and selective genotyping. Results revealed BSA as the most effective and energy saving genotyping approach for detecting QTLs.

Next generation sequencing (NGS) assisted BSA was carried out by Yang *et al.* (2013) to identify the QTLs controlling cold tolerance during the seedling stage in rice. Two large DNA bulks derived from 10,800 F_3 individuals were used for sequencing and ~450,000 SNPs (single nucleotide polymorphisms) were obtained after screening. Six QTLs were identified on chromosomes 10, 8, 5, 2 and 1 and from that most significant three QTLs were present on chromosomes 8, 2 and 1.

BSA was carried out by Dixit *et al.* (2014) to identify the QTLs, that are *qDTY3.1* (RM168 to RM468), *qDTY6.1* (RM586 to RM217), and *qDTY6.2* (RM121 to RM541) linked to grain yield per plant in a $BC_1F_{3:4}$ populations derived from the cross IR5541904 × 2*TDK1 under drought stress. QTL class analysis was conducted to test the QTL effect

and it showed complementary nature of *qDTY3.1* and *qDTY6.1*. These QTLs presented particular patterns of effect across various maturity groups inside the mapping population and greater consistency for grain yield per plant was seen across stress levels for lines with both QTLs as compared to those with single or no QTL.

BSA followed by complete genotyping with nominated markers were carried out to detect important markers associated with high grain yield per plant under drought stress (Palanog *et al.*, 2014). BSA of Kali Aus/IR64 mapping population showed six significant polymorphic markers (RM450, RM232, RM250, RM246, RM518, and RM19) whereas, eight polymorphic markers (RM3, RM572, RM495, RM211, RM231, RM340, RM250 and RM246) were found significant in the mapping population. *qDTY2.3* and *qDTY2.2*, two major grain yield QTLs were identified under drought stress over two seasons in the Kali Aus/IR64 and Kali Aus/MTU1010 populations, respectively.

BSA was used to identify markers associated to grain yield QTLs *qDTY2.3, qDTY2.2, qDTY1.2*, and *qDTY1.3* in the Kali Aus/2*MTU1010 and Kali Aus/2*IR64 populations under drought stress (Sandhu *et al.*, 2014). The epistatic interaction was noticed for days to flowering between RM154-RM324 and RM263-RM573 (chromosome number 2) however major epistatic QTL for grain yield showing the interaction between the genomic location at RM488-RM315 (chromosome number1) and RM324-RM263 (chromosome number 2) in Kali Aus/2*IR64 mapping populations.

Tiwari *et al.* (2016) used bi-parental RIL's for BSA to described a technique for quick identification of QTLs for reproductive stage salt tolerance. Thirty each RILs were used for the constituting bulks with two extreme phenotypes. Genotyping of bulks and parents was done using a 50 thousand SNP chip. CSR11 × MI48 RILs segregating population was used for salt tolerance study. 6,068 polymorphic SNPs and 21 QTL loci were identified as associated with salt tolerance. CSR27 × MI48 RILs were used for further confirmation and mapping QTLs using low-density microsatellite markers. BSA with 50 thousand SNP chip discovered 5,021 polymorphic loci and 34 QTL loci.

3. MATERIALS AND METHODS

The study on 'Bulked segregant analysis for heat tolerance in segregating generation of rice (*Oryza sativa* L.) using SSR markers' was carried out at the Regional Agricultural Research Station, Pattambi, Palakkad and Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Thrissur during 2015 to 2017. The research work, including the materials used and the methodologies adopted, are discussed in this chapter.

3.1 Materials

3.1.1 Plant Materials

The F_3 progenies of the hybrid of two rice varieties *viz.*, a heat sensitive high yielding variety Uma and a heat tolerant variety N22 comprised the experimental population for the present study. The F_1 hybrid of Uma and N22 was developed at RARS, Pattambi during 2014. Subsequently, the F_2 population was developed and evaluated during 2015. From this population, the seeds produced from a selected heat tolerant F_2 plant formed the material for the present study. Hence in the present study, raising of F_3 progeny population of the cross Uma x N22 along with parent varieties (Uma and N22) (Plate 1.) was carried out at RARS, Pattambi.

3.1.2 Laboratory chemicals, glassware and plastic wares

The chemicals used in this study were of AR grade procured from Labcare, SRL, HIMEDIA and Merck India Ltd. The *Taq* DNA polymerase, *Taq* buffer and molecular weight marker were supplied by SRL and RNase were supplied by HIMEDIA. The plastic wares used for the study were purchased from Tarsons India Ltd.

3.1.3 Equipment and machinery

The present research work was carried out using the molecular biology facilities and equipments available at RARS, Pattambi and CPBMB, Vellanikkara. Centrifugation was done in a high speed refrigerated centrifuge (REMI C-24BL). NanoDrop®ND-1000

A) N22

B) Uma

Plate 1. Raising of parents for the study of parental polymorphism

spectrophotometer was used for the estimation of quality and quantity of DNA. Thermal cycling was done in the thermal cycler of Eppendorf. Horizontal gel electrophoresis system (BIO-RAD, Sub-Cell Model 192, USA) was used for agarose gel electrophoresis. Gel Doc (GELSTAN 4X Advanced – Medicare) was used for imaging and documenting the agarose gel.

3.2 Methods

3.2.1 Molecular analysis

Identification of SSR markers linked to the gene/s for heat tolerance in rice through Bulked segregant analysis (BSA) was carried out using the F_3 plants derived from the hybrid of two rice varieties, Uma (heat sensitive high yielding variety) and N22 (heat tolerant variety). 197 SSR markers were used for checking parental polymorphism (Table 1.). The detailed methodology of DNA isolation, PCR, gel electrophoresis, gel documentation is given below:

3.2.1.1 DNA isolation

Young leaves (one month old) were selected as the ideal part for extraction of the genomic DNA. Tender, green leaves yielded good quality DNA in sufficient quantity. From individual plants, leaves were collected early in the morning. The collected leaves were quickly covered in aluminium foil and brought to the laboratory. The CTAB method (Dellaporta *et al.* 1983) was used for the extraction of genomic DNA. The concentration of extraction buffer and reagents is mentioned below. Composition of reagents required for DNA isolation are given in Appendix - I.

3.2.1.1.1 Reagents

I. CTAB Buffer (2X)

a) 4.0 per cent CTAB (w/v)
b) 100 mM Tris HCl (pH-8.0)
c) 20 mM EDTA (pH-8.8)

Table 1. List of 197 SSR markers used for parental polymorphism study

Sr. No.	Marker Name	Chromo some number	Froward primer Sequence	Reverse primer sequence
1	RM212	1	AAGGTCAAGGAAACAGGGACTGG	AGCCACGAATTCCACTTTCAGC
2	RM9	1	GGCCCTCATCACCTTCGTAGC	CGTCCTCCCTCTCCCTATCTCC
3	RM1003	1	GATTCTTCCTCCCCTTCGTG	TTCCTGTCAGAACAGGGAGC
4	RM431	1	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC
5	RM302	1	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG
6	RM443	1	GATGGTTTTCATCGGCTACG	AGTCCCAGAATGTCGTTTCG
7	RM315	1	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG
8	RM10346	1	GCTTGATCTGCCCTTGTTTCTTGG	AACTCGAGCGGCCTTCTCAGC
9	RM10086	1	GTCCAATCCACAATCTCTTCTCC	AGTGTGTGTATTGTGGTGTGTGG
10	RM6703	1	GCTTTCCTCTCCTCTCCTCTCC	CAAATCAGTGTCGTATGCAGTGG
11	RM495	1	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC
12	RM237	1	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC
13	RM3825	1	CCACTAGCAGATGATCACAGACG	GAGCACCTCATAAGGGTTTCAG
14	RM5	1	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG
15	RM1201	1	GCTACGTACGAGCCCTAGTTACCG	TACCGCGCCACATATACACAACC
16	RM1	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
17	RM283	1	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG
18	RM151	1	TGCTGATCAGTTACACGAATCAGAGC	GCGTACGTGCACAAATTAAACAGACC
19	RG128	1	TGATTTCTTGGAAGCGAAGAGTGAGG	CCTCCTTGTGCTCAGCCATGC
20	RM246	1	GAGCTCCATCAGCCATTCAG	CTGAGTGCTGCTGCGACT
21	RM473A	1	AAAGGATGTGGCGGTAGAATGC	GACTCCAACCCTAAAGCCTCTCC
22	RM265	1	CGAGTTCGTCCAAGTGAGC	CATCCACCATTCCACCAATC
23	RM14	1	CCGAGGAGAGGAGTTCGAC	GTGCCAATTTCCTCGAAAAA
24	RM84	1	TAAGGGTCCATCCACAAGATG	TTGCAAATGCAGCTAGAGTAC
25	RM259	1	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT
26	RM490	1	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG
27	RM312	1	GTATGCATATTTGATAAGAG	AAGTCACCGAGTTTACCTTC
28	RM104	1	GGAAGAGGAGAGAAAGATGTGTGTCG	TCAACAGACACACCGCCACCGC
29	RM462	1	ACGGCCCATATAAAAGCCTC	AAGATGGCGGAGTAGCTCAG
30	RM406	2	GAGGGAGAAAGGTGGACATG	TGTGCTCCTTGGGAAGAAAG
31	RM106	2	CGTCTTCATCATCGTCGCCCCG	GGCCCATCCCGTCGTGGATCTC
32	RM452	2	CTGATCGAGAGCGTTAAGGG	GGGATCAAACCACGTTTCTG
33	RM154	2	GACGGTGACGCACTTTATGAACC	CGATCTGCGAGAAACCCTCTCC
34	RM525	2	GGCCCGTCCAAGAAATATTG	CGGTGAGACAGAATCCTTACG
35	RM166	2	GGTCCTGGGTCAATAATTGGGTTACC	TTGCTGCATGATCCTAAACCGG
36	RM208	2	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC
37	RM3692	2	TAGAGAGAGAGGGGAAGCGG	TGGTCTCCGTCTTCGTCTTC
38	RM3340	2	TCTTGGCAAGCTCTCCTCTC	CCATCATCTCGATCTTGACG
39	RM48	2	TGTCCCACTGCTTTCAAGC	CGAGAATGAGGGACAAATAACC
40	RM6	2	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC
41	RM240	2	CCTTAATGGGTAGTGTGCAC	TGTAACCATTCCTTCCATCC
42	RM485	2	CACACTTTCCAGTCCTCTCC	CATCTTCCTCTCTTCGGCAC
43	RM3515	2	CATGCTAGTAAGCAAAGGGCAACG	TTGCACGTCCAACTGTCCAAGC
44	RM236	2	GCGCTGGTGGAAAATGAG	GGCATCCCTCTTTGATTCCTC

45	RM183	2	GGAGCGGGAGAGAGAGCCACG	TGCCGATGAAGGACTGCGACGC
46	RM110	2	TCGAAGCCATCCACCAACGAAG	TCCGTACGCCGACGAGGTCGAG
47	RM489	3	ACTTGAGACGATCGGACACC	TCACCCATGGATGTTGTCAG
48	RM570	3	GTTCTTCAACTCCCAGTGCG	TGACGATGTGGAAGAGCAAG
49	RM157	3	CCTCCTCCTCACGAATCCCGCC	GGGCTTCTTCTCCGCCGGCTTC
50	RM3894	3	CGCACTTGCTTAGAAGTCAATCATCC	ATGCTCTCTCCTTCAGGCCATCC
51	RM7117	3	GTTGGCTGGTTGCTACCACTACACG	AGCTCCGACTCTTCGCCTTGC
52	RM3586	3	GAAGAGAGAGCCAGAGCCAG	ACACGATCGAGCTAGAAGACG
53	RM520	3	AGGAGCAAGAAAAGTTCCCC	GCCAATGTGTGACGCAATAG
54	RM282	3	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG
55	RM148	3	ATACAACATTAGGGATGAGGCTGG	TCCTTAAAGGTGGTGCAATGCGAG
56	RM49	3	TTCGGAAGTTGGTTACTGATCA	TTGGAGCGGATTCGGAGG
57	RM231	3	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG
58	RM251	3	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
59	RM514	3	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG
60	RM468	3	CCCTTCCTTGTTGTGGCTAC	TGATTTCTGAGAGCCAACCC
61	RM85	3	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC
62	RM203	3	CCTATCCCATTAGCCAAACATTGC	GATTTACCTCGACGCCAACCTG
63	RM554	3	GTTCGTCCGTCTCTCGTCTC	CCCAAAAATCTGTGCCTCTC
64	RM338	3	CACAGGAGCAGGAGAAGAGC	GGCAAACCGATCACTCAGTC
65	RM232	3	CCGGTATCCTTCGATATTGC	CCGACTTTTCCTCCTGACG
66	RM7076	3	ATCAACTCCGGCGTCAGAGACC	GAGCAGGGTCCATGAAATTCTCC
67	RM157	3	CCTCCTCCTCACGAATCCCGCC	GGGCTTCTTCTCCGCCGGCTTC
68	RM518	4	CTCTTCACTCACTCACCATGG	ATCCATCTGGAGCAAGCAAC
69	RM5687	4	GATCGCTGGCGATTGATC	GACTTGTGGGGTGGTTTTTG
70	RM5749	4	GTGACCACATCTATATCGCTCG	ATGGCAAGGTTGGATCAGTC
71	RM417	4	CGGATCCAAGAAACAGCAG	TTCGGTATCCTCCACACCTC
72	RM471	4	ACGCACAAGCAGATGATGAG	GGGAGAAGACGAATGTTTGC
73	RM3042	4	CAAAAAGGAATCAATGTGAA	GGCTGTTGAGAGGTAGAGAA
74	RM252	4	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG
75	RM349	4	TTGCCATTCGCGTGGAGGCG	GTCCATCATCCCTATGGTCG
76	RM6314	4	GATTCGTGTCGGTTGTCAAG	GGTTCAGGGACGAATTTCAG
77	RM317	4	CATACTTACCAGTTCACCGCC	CTGGAGAGTGTCAGCTAGTTGA
78	RM255	4	GAGGAGGAGGAGGAGAGATCAGG	AACGAAACCGCTCAGTTCAACC
79	RM280	4	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAGG
80	RM1018	4	CCTCACTGACACCAGTATCCTCTCC	GCTCTTGGCAGATGGTGTAGGG
81	RM3735	4	GCGACCGATCAGCTAGCTAG	ATAACTCCTCCCTTGCTGCC
82	RM241	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG
83	RM307	4	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC
84	RM124	4	ATCGTCTGCGTTGCGGCTGCTG	CATGGATCACCGAGCTCCCCCC
85	RM537	4	CCGTCCCTCTCTCTCCTTTC	ACAGGGAAACCATCCTCCTC
86	RM348	4	CCGCTACTAATAGCAGAGAG	GGAGCTTTGTTCTTGCGAAC
87	RM401	4	TGGAACAGATAGGGTGTAAGGG	CCGTTCACAACACTATACAAGC
88	RM163	5	ATCCATGTGCGCCTTTATGAGGA	CGCTACCTCCTTCACTTACTAGT
89	RM161	5	TGCAGATGAGAAGCGGCGCCTC	TGTGTCATCAGACGGCGCTCCG
90	RM274	5	CCTCGCTTATGAGAGCTTCG	CTTCTCCATCACTCCCATGG
91	RM249	5	CAACTCCACTCCAGACTCAACTCC	GGTATGATGCCATGAAGGTCAGC
92	RM440	5	CATGCAACAACGTCACCTTC	CTTCTCCATCACTCCCATGG

93	RM1054	5	TGCATATGTACCGCAACCTC	TTTCTGCATGATCCCCTCTG
94	RM122	5	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC
95	RM430	5	AAACAACGACGTCCCTGATC	GTGCCTCCGTGGTTATGAAC
96	RM1090	5	AATATTAATGGCCGGAACCGAAGG	GCAAACTTTGGGTGAGTGTTTGTGC
97	RM153	5	CCTCGAGCATCATCATCAGTAGG	TCCTCTTCTTGCTTGCTTCTTCC
98	RM164	5	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC
99	RM169	5	TGGCTGGCTCCGTGGGTAGCTG	TCCCGTTGCCGTTCATCCCTCC
100	RM334	5	GTTCAGTGTTCAGTGCCACC	GACTTTGATCTTTGGTGGACG
101	RM178	5	TCGCGTGAAAGATAAGCGGCGC	GATCACCGTTCCCTCCGCCTGC
102	RM13	5	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG
103	RM507	5	CTTAAGCTCCAGCCGAAATG	CTCACCCTCATCATCGCC
104	RM413	5	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC
105	RM405	5	TATGCTTTCTGTCAGCTTCC	CTGCTGTGAAAGAGTTGACG
106	RM588	6	GTTGCTCTGCCTCACTCTTG	AACGAGCCAACGAAGCAG
107	RM1130	6	GGGAGATCGGATTGGGATGG	CAGCACTTAACCCAACCAATTAGTGC
108	RM589	6	ATCATGGTCGGTGGCTTAAC	CAGGTTCCAACCAGACACTG
109	RM190	6	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG
110	RM136	6	GAGAGCTCAGCTGCTGCCTCTAGC	GAGGAGCGCCACGGTGTACGCC
111	RM111	6	CACAACCTTTGAGCACCGGGTC	ACGCCTGCAGCTTGATCACCGG
112	RM130	6	ATCAGCAGCATTCAGCATTTGG	CCGGACGATGTGTATATCTCTTGG
113	RM527	6	CGGTTTGTACGTAAGTAGCATCAGG	TCCAATGCCAACAGCTATACTCG
114	RM162	6	TTGTTCCAGTTCAGGTCTTGTGC	CCCTACAAACACCATAAGAAGCAACC
115	RM528	6	GGCATCCAATTTTACCCCTC	AAATGGAGCATGGAGGTCAC
116	RM454	6	CTCAAGCTTAGCTGCTGCTG	GTGATCAGTGCACCATAGCG
117	RM6836	6	GCGTGTTCAGAAATTAGGATACGG	GATCTCGCCACGTAATTGTTGC
118	RM7555	6	CAACTGACAGGTGTGCATTTCC	ATAACCGTCTGGTTTCACTG
119	RM103	6	ATCAGCAGCATTCAGCATTTGG	CCGGACGATGTGTATATCTCTTGG
120	RM225	6	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
121	RM461	6	GAGACCGGAGAGACAACTGC	TGATGCGGTTTGACTGCTAC
122	RM170	6	TCGCGCTTCTTCCTCGTCGACG	CCCGCTTGCAGAGGAAGCAGCC
123	RM510	6	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC
124	RM133	6	TTGGATTGTTTTGCTGGCTCGC	GGAACACGGGGTCGGAAGCGAC
125	RM314	6	CTAGCAGGAACTCCTTTCAGG	AACATTCCACACACACACGC
126	RM1209	7	AATGGAGCTCCTGACTCTAAAGC	TGCATCTCCTACAGAAACAAGG
127	RM234	7	TTCAGCCAAGAACAGAACAGTGG	CTTCTCTTCATCCTCCTCCTTGG
128	RM11	7	TGGTAGTATAGGTACTAAACAT	TCCTATACACATACAAACATAC
129	RM47	7	ACTCCACTCCACTCCCCAC	GTCAGCAGGTCGGACGTC
130	RM7121	7	TACCAGCTGCATGTTACCCGATACC	CGGAATCAAATTCCAGCAACAGC
131	RM118	7	CCAATCGGAGCCACCGGAGAGC	CACATCCTCCAGCGACGCCGAG
132	RM125	7	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC
133	RM455	7	AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC
134	RM336	7	GTATCTTACAGAGAAACGGCATCG	GGTTTGTTTCAGGTTCGTCTATCC
135	RM447	8	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC
136	RM5545	8	CAGCACTCCTCCCCTACCAG	GGCTAAGTCAGCGTGAGACC
137	RM210	8	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG
138	RM152	8	AAGGAGAAGTTCTTCGCCCAGTGC	GCCCATTAGTGACTGCTCCTAGTCG
139	RM72	8	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG
140	RM223	8	GCTGGGCCTATATGGACTTTCG	AGGCAAGTCTTGGCACTGTAAGC

141	RM515	8	CGAGATACGGTGAATGACACACG	ACCAATCAGAGAGCACACACAGG
142	RM342	8	GTTGCCGGTGAAGGTCCATCC	TGTCACCCTCATCAACATCAGTGG
143	RM408	8	CAACGAGCTAACTTCCGTCC	ACTGCTACTTGGGTAGCTGACC
144	RM25	8	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC
145	RM433	8	TGCGCTGAACTAAACACAGC	AGACAAACCTGGCCATTCAC
146	RM256	8	GACAGGGAGTGATTGAAGGC	GTTGATTTCGCCAAGGGC
147	RM149	8	GGAAGCCTTTCCTCGTAACACG	GAACCTAGGCCGTGTTCTTTGC
148	RM547	8	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG
149	RM284	8	ATCTCTGATACTCCATCCATCC	CCTGTACGTTGATCCGAAGC
150	RM80	8	CTCATCTCCGCCCTTGATTCC	GCCCATCAACCTCGTCTTCACC
151	RM502	8	CATCTCTGTTCCACTTGCTTTGC	CTACCAACAACCCAACAAGAAGG
152	RM108	9	TCTCTTGCGCGCACACTGGCAC	CGTGCACCACCACCACCACCAC
153	RM160	9	CCCAAATCAGGAAAGTTTCTCAGC	AGTCATCCTTGGCTACCAGATGC
154	RM7038	9	AGGTGGTGAGGGTGAACTTG	TGGGATTAGAGCTTTGGTGG
155	RM566	9	ACCCAACTACGATCAGCTCG	CTCCAGGAACACGCTCTTTC
156	RM7039	9	GCACATTTGCCATTCTACCG	GCCTTCCAGTGAGGTGACTC
157	RM316	9	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
158	RM201	9	GTACTCTCGCCGTTCACAACTCC	TTAGTGACCGGGATGACACAGC
159	RM205	9	CCTAAGAGGAGCCATCTAACAACTGG	CTTGGATATACTGGCCCTTCACG
160	RM105	9	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC
161	RM278	9	GTAGTGAGCCTAACAATAATC	TCAACTCAGCATCTCTGTCC
162	RM328	9	CATAGTGGAGTATGCAGCTGC	CCTTCTCCCAGTCGTATCTG
163	RM242	9	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG
164	RM219	9	CGTCGGATGATGTAAAGCCT	CGTCGGATGATGTAAAGCCT
165	RM258	10	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC
166	RM6100	10	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC
167	RM6132	10	CCGCCATCTCTCTTCAGTTC	CAGTGCATAGAGGAGAGGACG
168	RM147	10	TACGGCTTCGGCGGCTGATTCC	CCCCCGAATCCCATCGAAACCC
169	RM271	10	TCAGATCTACAATTCCATCC	TCGGTGAGACCTAGAGAGCC
170	RM474	10	AAGATGTACGGGTGGCATTC	TATGAGCTGGTGAGCAATGG
171	RM171	10	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG
172	RM222	10	CTTAAATGGGCCACATGCG	CAAAGCTTCCGGCCAAAAG
173	RM467	10	GGTCTCTCTCTCTCTCTCTCTC	CTCCTGACAATTCAACTGCG
174	RM484	10	TCTCCCTCCTCACCATTGTC	TGCTGCCCTCTCTCTCTCTC
175	RM228	10	TCTAACTCTGGCCATTAGTCCTTGG	AAGTAGACGAGGACGACGACAGG
176	RM26212	11	GTCGCTCCTCTCCTCCAATCC	GCTCGCTGCTTCTAATCTCTTGC
177	RM332	11	GCGAAGGCGAAGGTGAAG	CATGAGTGATCTCACTCACCC
178	RM3701	11	GAGCTAGAGGGAGGAGGTGC	TTGACTGATAGCCGATTGGG
179	RM144	11	CATGTTGTGCTTGTCCTACTGC	AGCTAGAGGAGATCAGATGGTAGTGC
180	RM167	11	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTTGTC
181	RM202	11	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA
182	RM206	11	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG
183	RM254	11	AGCCCCGAATAAATCCACCT	CTGGAGGAGCATTTGGTAGC
184	RM229	11	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT
185	RM287	11	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC
186	RM536	11	TACCAGGATCATGTTTCTCTCC	ACTGTGAGATTGACTGACAGTGG
187	RM552	11	CGCAGTTGTGGATTTCAGTG	TGCTCAACGTTTGACTGTCC
188	RM120	11	CACACAAGCCCTGTCTCACGACC	CGCTGCGTCATGAGTATGTA

189	RM209	11	ATATGAGTTGCTGTCGTGCG	CAACTTGCATCCTCCCCTCC
190	RM224	11	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
191	RM247	12	AAGGCGAACTGTCCTAGTGAAGC	CAGGATGTTCTTGCCAAGTTGC
192	RM235	12	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC
193	RM7119	12	AGGCTGAGGCTTATAGGCAG	GGATGATACAACTTGACCCC
194	RM17	12	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA
195	RM102	12	TGCTCCTCCACCGTCACGTACC	CTATCCGTGCGCCTCAACTTTCC
196	RM313	12	TGCTACAAGTGTTCTTCAGGAC	GCTCACCTTTTGTGTTCCAC
197	RM277	12	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG

d) 1.4 M Nacl

e) 2.0 per cent Polyvinyl pyrolidin (PVP)

f) 0.2 per cent β-mercaptoethanol

II. Chloroform: Isoamyl alcohol (24:1 v/v)

III. Chilled isopropanol

IV. 70 and 100 per cent ethanol

V. Sterile distilled water

Reagents I and III were autoclaved separately and stored at room temperature.

3.2.1.1.2 Procedure for DNA isolation

Total cellular DNA of two parents includes Uma and N22 was extracted by following protocol described by CTAB method (Dellaporta *et al.,* 1983).

1. 0.1g of tender leaves of rice was weighed and added to a pre-chilled mortar and pestle.

2. The leaves were grounded by adding β-mercapto ethanol (50 µl) and pinch of Poly vinyl pyrrolidine (PVP) along with liquid nitrogen and made it into a fine powder.

3. The grounded leaves were transferred to a sterile tube (2 ml) containing 1 ml of pre-warmed CTAB extraction buffer and mixed well.

4. This mixture incubated at 65° C for 30 min with occasional mixing by gentle inversion.

5. After incubation, 1ml of Chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion to the emulsion.

6. The tube was centrifuged at 15,000 rpm for 15 min at 4° C.

7. The aqueous phase was transferred to a clean tube.

8. 2 µl of RNase solution was added and kept for incubation at room temperature for 30 min.

9. An equal volume of Chloroform: Isoamyl alcohol was added to the tube.

10. The tube was mixed gently by inversion and centrifuged at 10,000 rpm for 5 min. at 4° C.

11. The aqueous phase was removed with a pipette to a clean tube, the 0.6^{th} volume of ice cold Isopropanol added and mixed well until the DNA was precipitated and kept at 4° C for 2 hours.

12. The tube was centrifuged at 15,000 rpm for 20 min. and gently poured the supernatant out by inverting tube.

13. The pellet was washed with 70 per cent ethanol and dried.

14. After drying, the DNA was dissolved in the sterile distilled water (100 ml) and stored at -20 °C.

3.2.2 Assessing the quality and quantity of DNA by spectrophotometer

The purity of DNA was further checked using NanoDrop ND-1000 spectrophotometer. Nucleic acids show absorption maxima at 260 nm whereas proteins show peak absorbance at 280 nm. Absorbance was recorded at both wavelengths and purity was indicated by the ratio OD ($A_{260/280}$). The values between 1.8 and 2.0 indicated that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was given as ng/µl.

Procedure for quantity detection using NanoDrop

1. NanoDrop spectrophotometer was connected to the System and the software ND-1000 was opened.
2. The option Nucleic acid was selected.
3. The Sampling arm was opened and 1 µl distilled water poured on measurement pedestal.

4. Spectral measurements were started after closing sampling arm.
5. The reading was set to zero with the blank sample.
6. 1 µl of the sample was poured onto the measurement pedestal and measure was selected.
7. After each reading, a soft laboratory wipe was used to clean the upper and lower pedestals of sampling.
8. OD260/OD280 ratio and DNA quantity were recorded by the above procedure.

3.2.3 Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through Agarose gel electrophoresis (Sambrook *et al*., 1989).

3.2.3.1 Reagents and equipments

1. Agarose
2. 50X TAE buffer (pH 8.0)
3. 6X loading /tracking dye
4. Ethidium bromide (10 mg/ml)
5. Electrophoresis unit, power pack (BIO-RAD, Sub-Cell Model 192), gel casting tray, comb
6. Gel documentation and analysis system (GELSTAN 4X Advanced – Medi ccare)

3.2.3.2 Procedure for Agarose gel electrophoresis of DNA

1. Electrophoresis of an aliquot of the DNA samples in 1 per cent Agarose gel was used to check the DNA degradation and contamination with other substances. Large molecular weight DNA appears as a band, while partially degraded DNA or RNA forms a smear of long to small fragments.
2. Both the sides of gel tray were closed with cello tape and comb was inserted.
3. 1 g Agarose was added to 100 ml of 1X TAE buffer to prepare 1 per cent Agarose gel.
4. The Agarose was boiled in borosil flask in a microwave oven and boiled Agarose then cooled to room temperature.

5. 3 µl of ethidium bromide (10mg/ml) was added to the cooled gel and mixed smoothly.
6. The gel was transferred into tray carefully without forming air bubbles. Solidified gel then transferred to electrophoresis tank after removing cello tape and comb.
7. To cover the gel to a depth of 5mm, nearly 3.5 litre of 1X TAE buffer was poured into the electrophoresis tank.
8. About $1/10^{th}$ volume of 6X loading dye (Bromophenol blue) was added to DNA samples and mixed by moderate tapping and spinning 2-3 sec.
9. Total 7 µl DNA samples along with loading dye were loaded into the wells.
10. The power supply of about 80 V was provided to run the gel until the dye reaches to 3/4th of the gel and the gel was removed from the gel apparatus.
11. After complete electrophoresis, the gel was examined using gel documentation system.

3.2.4 Gel documentation

The gel image was documented in gel documentation system (GELSTAN 4X Advanced – Medi ccare). The gel profile was examined for intactness, clarity of DNA band, the presence of contamination such as RNA and proteins. An image of a gel was captured using the controls in the imaging device window and displayed on computer screen.

3.2.5 Normalisation of DNA concentration for PCR

Normalisation of DNA was done to bring all DNA concentrations to a relatively equal level (25ng/µl) by appropriate dilutions. Dilutions were done with distilled water.

3.2.6 Parental screening for polymorphism

DNA samples isolated from the parents (Uma and N22) were genotyped using 197 SSR markers for identifying the polymorphic markers. These markers were selected randomly from Gramene database and also based on the available literature.

3.2.6.1. Polymerase chain reaction (PCR)

Amplification reaction mixture was prepared in 0.2 ml thin walled flat cap PCR tubes containing the following components. The total volume of each reaction mixture was 10µl.

Genomic DNA (50 ng)	:	2.0 µl
10X Taq assay buffer	:	1.0 µl
$MgCl_2$ (25 mM)	:	0.5 µl
dNTP mix (10 mM each)	:	0.5 µl
Taq DNA polymerase (3U)	:	0.4 µl
Forward primer (10 µM)	:	1.0 µl
Reverse primer (10 µM)	:	1.0 µl
Autoclaved distilled water	:	3.6 µl
Total volume	:	10.0 µl

Amplification was carried out on Master Cycler Gradient Eppendorf PCR. The amplification profile was as follows:

a) Initial denaturation at 95° C temperature for 3 min.

b) Denaturation at 94° C for 50 sec.

c) Primer annealing at ~57° C for 30 sec.

d) Primer extension at 72° C for 1 min.

Later, steps b) to d) were repeated 35 times.

e) Complete primer extension at 72°C for 10 min.

f) Hold at 4°C until removal.

3.2.6.2 Separation of amplified products by Agarose gel electrophoresis

2 per cent Agarose gel was prepared using electrophoresis grade Agarose (Sigma Alorich) of the volume of 200 ml 1X TAE (electrophoresis buffer). Ethidium bromide was added at a concentration of 0.5 µl per 10 ml (10 mg/ml) of gel. The gel was allowed to solidify fully before removing the comb. 2 µl of loading dye was added to 10 µl of PCR products and mixed well before loading into the wells. A voltage of 80 volt and 400 amp was given for 90 min for separation of PCR product. After 90 min of the run, the gel was examined under gel doc and the DNA banding pattern was photographed directly in documentation unit.

3.2.6.3 Analysis of bands for parental polymorphism

The banding pattern itself was noted from the digital image of the gels and analysed for identifying polymorphic primers between parents and these were used for further evaluation through bulked segregant analysis.

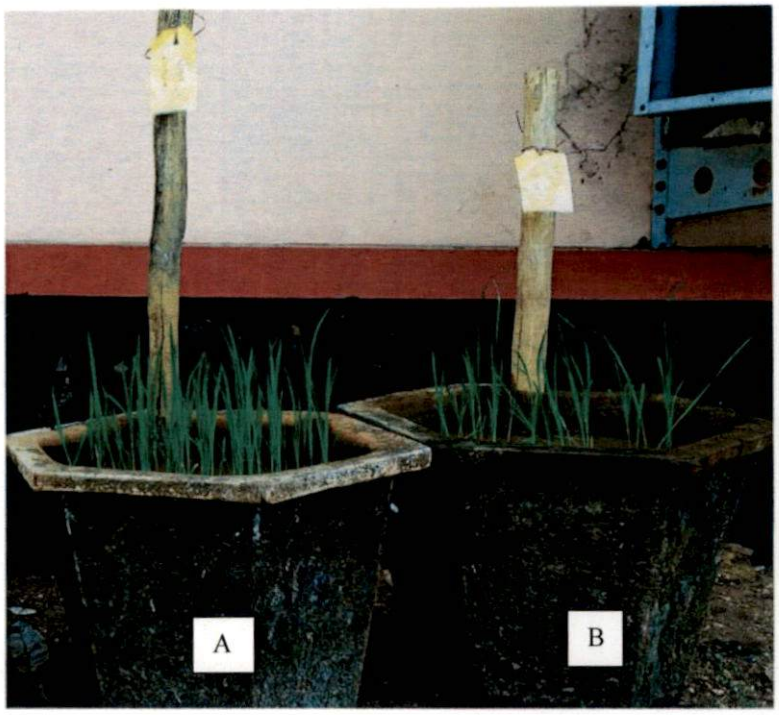
3.2.7 Bulked Segregant Analysis in progenies

3.2.7.1 Field analysis

Seedlings of F_3 progeny (>100 nos.), and parents, Uma and N22 (Plate 2.), were raised during the last week of December 2016 and individually transplanted into pots (Plate 3.) after three weeks *i.e.* during the last week of January 2017. The 49 F_3 plants that survived were labelled in serrattum (F_3-1 to F_3-49) and used for study. Ten seedlings (1 per pot) each of both the parents were also transplanted on the same day. All the plants were kept under ambient conditions.

At booting stage, all the F_3 plants and five plants each of both parental plants were transferred to polyhouse and exposed to high-temperature stress (Plate 4, 5 and 6.). Five plants each of both the parental genotypes retained in ambient conditions served as control. The F_3 progenies and N22 plants reached booting stage around 70 days of sowing while parent Uma reached booting stage at about 98 days

The average maximum temperature was 35.19° C at RARS, Pattambi during January to May 2017. Thermo Hygrometer was used to record the polyhouse temperature. Average temperature recorded during morning time (8.30 to 9.00 am) inside and outside the polyhouse was around 35° C and 32° C, respectively. Inside the polyhouse, the temperature ranged from 37.2 to 42° C during March 2017 to 37.1° C to 42.7° C in April



A) Uma and B) N22

C) F_3 plants

Plate 2. Raising of F_3 generation and parents (Uma and N22) for bulked segregant analysis

Plate 3. Transplanting of F_3 plants and parents (Uma and N22)

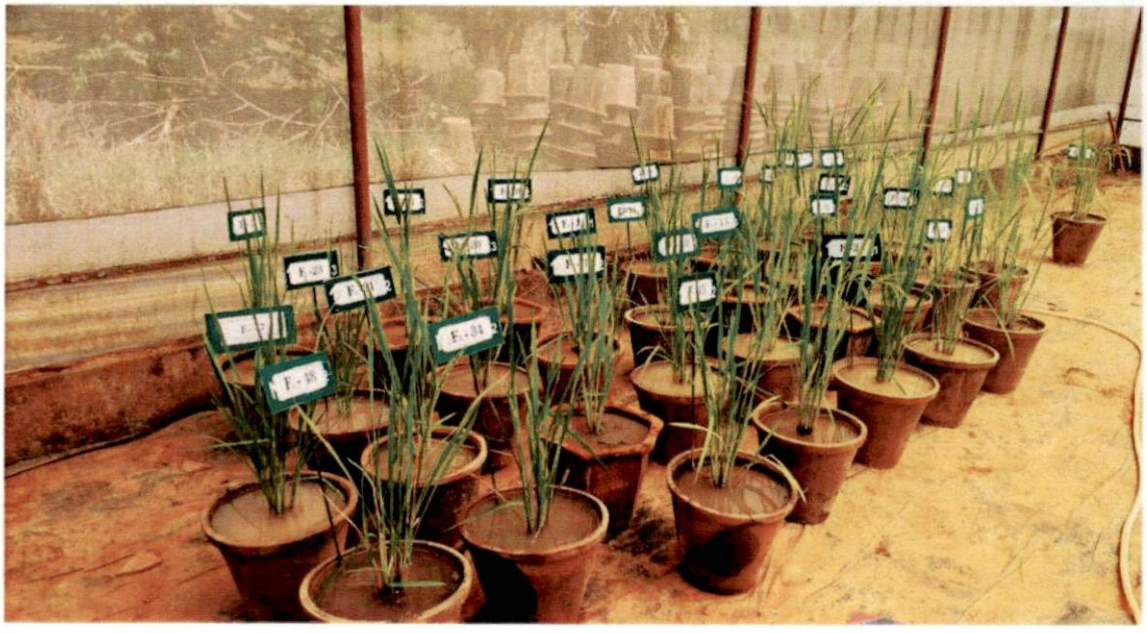


Plate 4. High temperature stress screening of F_3 plants in polyhouse

A) Heat treated

B) Control

Plate 5. High temperature stress screening of Uma

A) Heat treated

B) Control

Plate 6. High temperature stress screening of N22

Table 2. Ambient temperature and Poly house temperature

March 2017			April 2017		
Date	**Ambient temperature**	**Poly house temperature**	**Date**	**Ambient temperature**	**Poly house temperature**
	T max (^{0}C)	**T max (^{0}C)**		**T max (^{0}C)**	**T max (^{0}C)**
1/03/2017	37.8	41.2	1/04/2017	39.3	42.7
2/03/2017	37.5	40.3	2/04/2017	37.5	41.2
3/03/2017	35.4	39.4	3/04/2017	36.5	41.4
4/03/2017	37.5	40.5	4/04/2017	36.0	41.2
5/03/2017	36.4	39.7	5/04/2017	35.3	39.1
6/03/2017	34.4	39.2	6/04/2017	35.8	39.4
7/03/2017	32.7	37.2	7/04/2017	36.8	40.0
8/03/2017	35.3	38.8	8/04/2017	37.1	41.1
9/03/2017	35.8	37.7	9/04/2017	37.0	41.2
10/03/2017	35.5	39.4	10/04/2017	37.0	41.8
11/03/2017	34.9	39.2	11/04/2017	35.8	39.8
12/03/2017	35.0	39.4	12/04/2017	36.0	40.2
13/03/2017	34.8	39.7	13/04/2017	35.2	39.2
14/03/2017	35.5	39.1	14/04/2017	36.0	39.4
15/03/2017	36.5	39.3	15/04/2017	34.3	38.4
16/03/2017	36.2	40.1	16/04/2017	36.3	39.7
17/03/2017	32.4	37.2	17/04/2017	35.3	39.2
18/03/2017	34.8	38.2	18/04/2017	36.0	39.9
19/03/2017	34.4	39.4	19/04/2017	35.0	39.8
20/03/2017	34.3	37.4	20/04/2017	33.4	37.4
21/03/2017	34.5	38.7	21/04/2017	33.2	37.1
22/03/2017	35.2	39.1	22/04/2017	32.0	37.4
23/03/2017	36.3	39.5	23/04/2017	34.0	38.6
24/03/2017	36.3	39.2	24/04/2017	35.0	38.4
25/03/2017	38.0	42.0	25/04/2017	36.2	40.2
26/03/2017	37.8	41.2	26/04/2017	35.2	39.9
27/03/2017	37.5	41.3	27/04/2017	36.0	41.2
28/03/2017	38.0	41.7	28/04/2017	36.0	40.0
29/03/2017	35.8	38.9	29/04/2017	36.2	41.0
30/03/2017	35.8	39.0	30/04/2017	37.0	41.2
31/03/2017	36.8	39.4			
Average	**35.78**	**39.44**	**Average**	**35.75**	**39.91**

2017 (Table 2.). Generally, the temperature measured inside the polyhouse was 3 to 4° C higher than the normal outside temperature.

All the plants were removed from polyhouse to ambient conditions after fifteen days of heat treatment. Morphological parameters of all the plants were recorded during the first week of April 2017.

3.2.7.2 Study of morphological characters and statistical analysis

a) Study of morphological characters

1. **Plant height** (cm): Height of plant was measured from the base at ground level to the tip of the panicles (excluding the awns, if any) and expressed in centimetres.
2. **Number of tillers**: Number of tillers per plant was counted.
3. **Number of panicles**: Number of panicles per plant was counted.
4. **Panicle length** (cm): In each plant, the length of three panicles was measured from base to panicle tip (excluding the awns, if any) and the average was recorded in centimetres.
5. **Days for flowering:** Days taken by each plant for flowering from the date of sowing was counted.
6. **Number of filled grains per panicles:** In each plant, filled grains of five panicles were counted and the average was recorded.
7. **Number of sterile grains per panicle:** In each plant, sterile grains of five panicles were counted and the average was recorded.
8. **Spikelet fertility (%):** For each panicle, spikelet fertility (%) was calculated as below:

$$\frac{\text{No. of Filled grains per panicle}}{\text{Total No. of grains per panicle}} \times 100$$

9. **100 seed weight (g):** For each plant, the weight of 100 fully developed filled grains was recorded and expressed in grams.
10. **Grain weight per plant (g):** Weight of total grains harvested from each plant was recorded and expressed in grams.

b) Statistical analysis of parameters of variability

1. Mean: The mean value of each observation was worked out by dividing the totals by the corresponding number of observation:

$$\mathbf{X} = \frac{\sum X_i}{N}$$

Where,

X_i - any observation in i^{th} treatment

N - Total number of observations

2. Range: Range of each observation was recorded by showing the highest and lowest values present in the observations of a sample.

3.2.7.3 Genotyping of F_3 plants for heat tolerance

The bulked segregant analysis, proposed by Michelmore *et al.* (1991), is the technique to tag the genes of interest, using a structurally developed population, segregating for the gene of interest. In the present study, DNA was isolated from seven highly tolerant and seven highly susceptible F_3 plants. BSA was performed for each polymorphic primer using the DNA samples from a tolerant parent, susceptible parent, tolerant F_3 bulk and susceptible F_3 bulk. Then, the marker identified as linked to heat tolerance was again screened with individuals of bulks and parents for the confirmation of efficiency of the marker to differentiate between tolerant and susceptible lines.

3.2.7.4 Single marker analysis

The data on genotyping the F_3 individuals were analysed using the free software QTL IciMapping capable of building high-density linkage maps and mapping quantitative trait loci (QTL) in biparental populations. Out of the eight functionalities, MAP (construction of linkage maps in bi-parental populations) was used for the single marker analysis.

The analysis using this programme consisted of the preparation of an excel file containing five different sheets *viz*., (1) General Info, (2) Chromosome (3) Linkage Map (4) Genotype and (5) Phenotype (Meng *et al.*, 2015).

Single marker analysis fits the data to the simple linear regression model.

$y = b0 + b1\ x + e$

The logarithm of the odds ratio (LOD score):

$$\text{LOD} = \frac{\text{Probability of data occurring with a QTL}}{\text{Probability of data occurring with no QTL}}$$

LOD of 2 indicates that it is 100 times more likely that a QTL exists in the interval than that there is no QTL. Similarly, LOD of 3 indicates that it is 1000 times more likely that a QTL exists in the interval than that there is no QTL.

4. RESULTS

The results of the study on 'Bulked segregant analysis for heat tolerance in segregating generation of rice (*Oryza sativa* L.) using SSR markers' was undertaken during the period 2016 to 2017 at Regional Agricultural Research Station, Pattambi and Centre of Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, are described in this chapter.

4.1 Parental polymorphism study using molecular markers

4.1.2 DNA isolation and quantification

Tender leaves of N22 (P1) and Uma (P2) were collected during early morning for DNA isolation. The genomic DNA was isolated through CTAB method (Dellaporta *et al.*, 1983) with some modifications. RNase treatment was used during isolation procedure to remove all RNA contamination.

4.1.3 Quality of DNA by agarose gel electrophoresis

The quality of DNA was assessed using agarose gel electrophoresis. Clear and single intact band of high intensity appeared after gel documentation indicating the non-degraded good quality of isolated DNA, free from RNA and protein contamination (Plate 7).

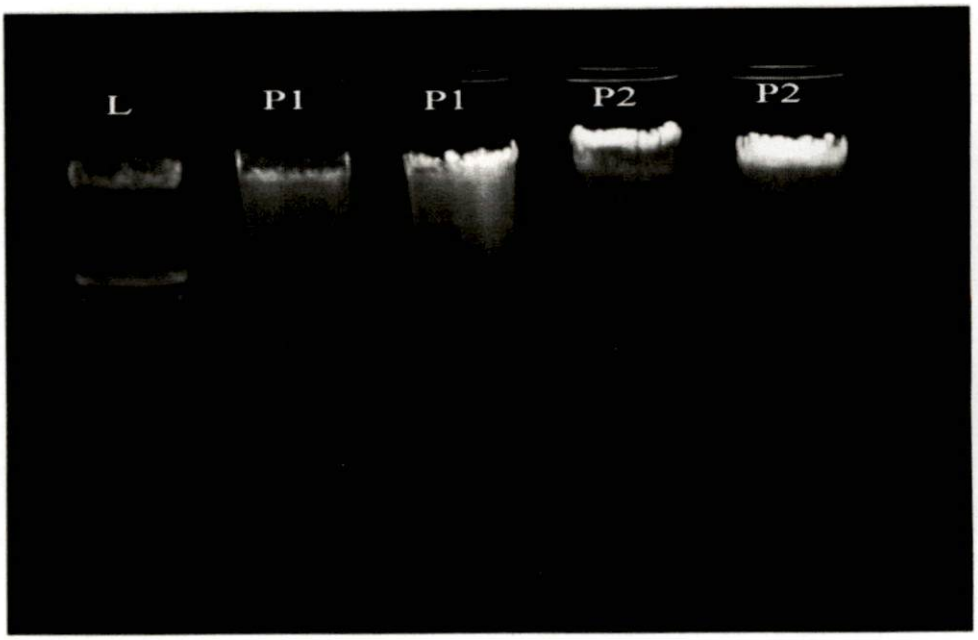
4.1.4 Quality and quantity of DNA using NanoDrop method

The result of the NanoDrop® ND-1000 spectrophotometer analysis is presented in Table 3.

Table 3. Quality and quantity of DNA isolated from N22 (P1) and Uma (P2) assessed by NanoDrop method

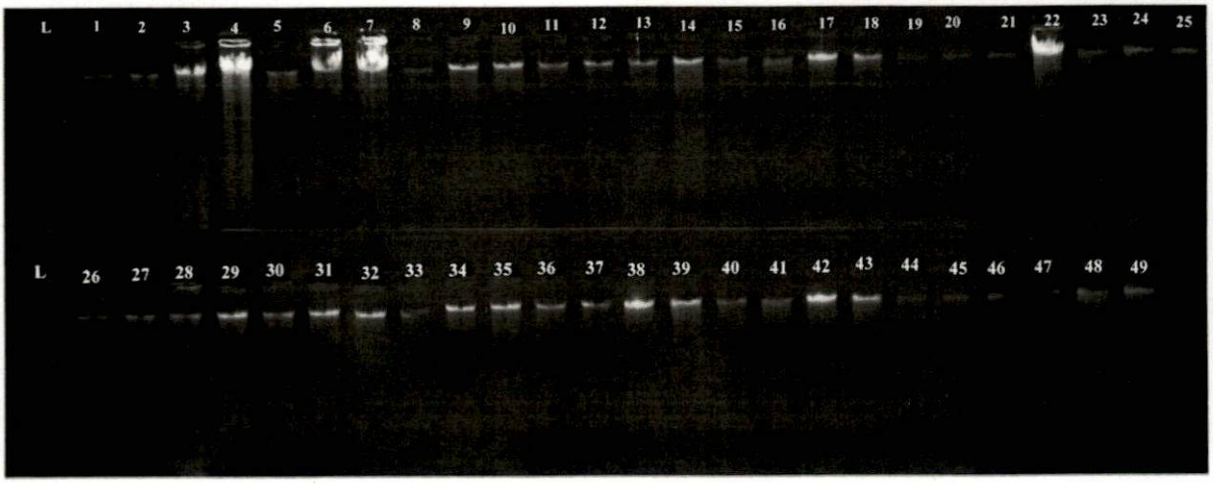
Genotypes	A $_{260/280}$	Quantity (ng/µl)	Quality
P1	1.87	1534.90	Good
P2	1.97	2144.82	Good

Plate 7. Genomic DNA isolation from parents N22 (P1) and Uma (P2)



L- Lambda double digest ladder, P1- N22, P2- Uma

Plate 8. Genomic DNA isolation from F_3 plants



L- Lambda double digest ladder, 1 - 49 F_3 plants

The ratio of UV absorbance at OD260/OD280 ranged between 1.87-1.97, and hence DNA samples were rated as good and standard. The quantity of DNA in the isolated samples ranged from 1534.90 to 2144.82 ng/µl.

4.1.5 SSR marker screening to assess parental polymorphism

Parental polymorphism at the molecular level was determined by genotyping them with SSR markers. The genomic DNA of the two parents N22 (P1) and Uma (P2) were initially screened using 197 Rice Microsatellites (RM) markers for parental polymorphism studies. These 197 Rice Microsatellites markers are distributed throughout the entire genomic area of all twelve chromosomes of the rice plant.

One or two amplicons were observed in the different RM markers of two parents in parental polymorphism study. The size of amplicons resolved among the RM markers ranged from 85bp (RM458) to 478bp (RM166). Data on nature of amplification, the number of amplicons and size of amplicons of these 197 rice microsatellites for genotyping parents presented in Table 4.

Out of the 197 RM markers, the 41 markers (Plate 9; Table 5) were observed to be polymorphic between N22 (heat tolerant parent) and Uma (heat susceptible parent).

Among these, seven RM markers were on chromosome number 1, three markers on chromosome number 2, four markers on chromosome number 3, four markers on chromosome number 4, five markers on chromosome number 5, three markers on chromosome number 6, single marker on chromosome number 7, three markers on chromosome number 8, three markers on chromosome number 9, single marker on chromosome number 10, five markers on chromosome number 11, and two markers on chromosome number 12.

Table 4. Parental polymorphism study

Sl. No.	Rice Microsatellite	Nature of amplification	No. of amplicon		Size of amplicon (bp)	
			P1	P2	P1	P2
1	RM212	Polymorphic	1	1	125	140
2	RM9	Polymorphic	1	1	177	138
3	RM1003	Monomorphic	1	1	119	119
4	RM431	Monomorphic	1	1	248	248
5	RM302	Polymorphic	1	1	164	173
6	RM443	Monomorphic	1	1	147	147
7	RM315	Monomorphic	1	1	210	210
8	RM10346	Polymorphic	1	1	302	321
9	RM10086	Monomorphic	1	1	390	390
10	RM6703	Monomorphic	1	1	198	198
11	RM495	Polymorphic	1	1	183	172
12	RM3825	Monomorphic	1	1	124	124
13	RM5	Polymorphic	1	1	107	114
14	RM1201	Monomorphic	1	1	178	178
15	RM1	Monomorphic	1	1	135	135
16	RM283	Monomorphic	1	1	102	102
17	RM151	Monomorphic	1	1	241	241
18	RG128	Monomorphic	1	1	227	227
19	RM473A	Polymorphic	1	2	132	109, 150
20	RM265	Monomorphic	1	1	118	118
21	RM259	Monomorphic	1	1	239	239
22	RM312	Monomorphic	1	1	125	125
23	RM462	Monomorphic	1	1	367	367
24	RM406	Monomorphic	1	1	126	126
25	RM106	Monomorphic	2	2	370, 290	370, 290
26	RM452	Monomorphic	1	1	210	210
27	RM154	Monomorphic	1	1	204	204
28	RM525	Monomorphic	1	1	103	103
29	RM208	Polymorphic	1	1	194	211
30	RM3692	Monomorphic	1	1	120	120
31	RM3340	Polymorphic	1	1	164	186
32	RM240	Monomorphic	1	1	120	120
33	RM485	Monomorphic	1	1	367	367
34	RM3515	Monomorphic	1	1	169	169
35	RM236	Monomorphic	1	1	217	217
36	RM110	Monomorphic	1	1	148	148
37	RM489	Monomorphic	1	1	234	234
38	RM570	Monomorphic	2	2	186, 154	186, 154
39	RM3894	Monomorphic	1	1	111	111
40	RM7117	Monomorphic	1	1	167	167
41	RM3586	Polymorphic	1	1	164	142
42	RM520	Monomorphic	1	1	388	388
43	RM282	Monomorphic	1	1	156	156
44	RM148	Monomorphic	1	1	123	123
45	RM514	Monomorphic	1	1	245	245
46	RM468	Monomorphic	1	1	310	310
47	RM338	Monomorphic	1	1	185	185

48	RM7076	Polymorphic	1	1	228	280
49	RM157	Monomorphic	1	1	120	120
50	RM518	Polymorphic	1	1	197	180
51	RM5687	Monomorphic	2	2	146, 121	146, 121
52	RM5749	Polymorphic	1	1	189	160
53	RM417	Monomorphic	1	1	235	235
54	RM471	Monomorphic	1	1	207	207
55	RM3042	Monomorphic	1	1	338	338
56	RM252	Polymorphic	1	1	210	267
57	RM349	Monomorphic	1	1	266	266
58	RM6314	Monomorphic	1	1	128	128
59	RM317	Monomorphic	1	1	165	165
60	RM255	Monomorphic	1	1	157	157
61	RM280	Polymorphic	1	1	180	153
62	RM1018	Monomorphic	1	1	149	149
63	RM3735	Monomorphic	1	1	149	149
64	RM241	Monomorphic	1	1	164	164
65	RM307	Monomorphic	1	1	146	146
66	RM124	Monomorphic	1	1	281	281
67	RM537	Monomorphic	1	1	311	311
68	RM348	Monomorphic	1	1	156	156
69	RM401	Monomorphic	1	1	160	160
70	RM163	Polymorphic	1	1	314	284
71	RM274	Monomorphic	1	1	128	128
72	RM249	Monomorphic	1	1	120	120
73	RM440	Monomorphic	1	1	191	191
74	RM1054	Monomorphic	1	1	169	169
75	RM122	Monomorphic	2	2	221, 200	221, 200
76	RM430	Monomorphic	1	1	154	154
77	RM1090	Monomorphic	1	1	191	191
78	RM153	Monomorphic	1	1	117	117
79	RM164	Polymorphic	1	1	283	314
80	RM169	Polymorphic	1	1	176	193
81	RM334	Polymorphic	1	1	190	184
82	RM178	Monomorphic	1	1	241	241
83	RM507	Monomorphic	1	1	279	279
84	RM413	Monomorphic	1	1	107	107
85	RM405	Monomorphic	1	1	85	85
86	RM588	Monomorphic	1	1	113	113
87	RM1130	Monomorphic	1	1	139	139
88	RM589	Monomorphic	1	1	188	188
89	RM190	Monomorphic	1	1	112	112
90	RM136	Monomorphic	1	1	160	160
91	RM111	Monomorphic	1	1	242	242
92	RM130	Monomorphic	1	1	135	135
93	RM527	Monomorphic	1	1	226	226
94	RM162	Monomorphic	1	1	167	167
95	RM528	Monomorphic	1	1	266	266
96	RM454	Monomorphic	1	1	280	280
97	RM6836	Polymorphic	1	1	254	224
98	RM103	Monomorphic	2	2	321,284	321,284

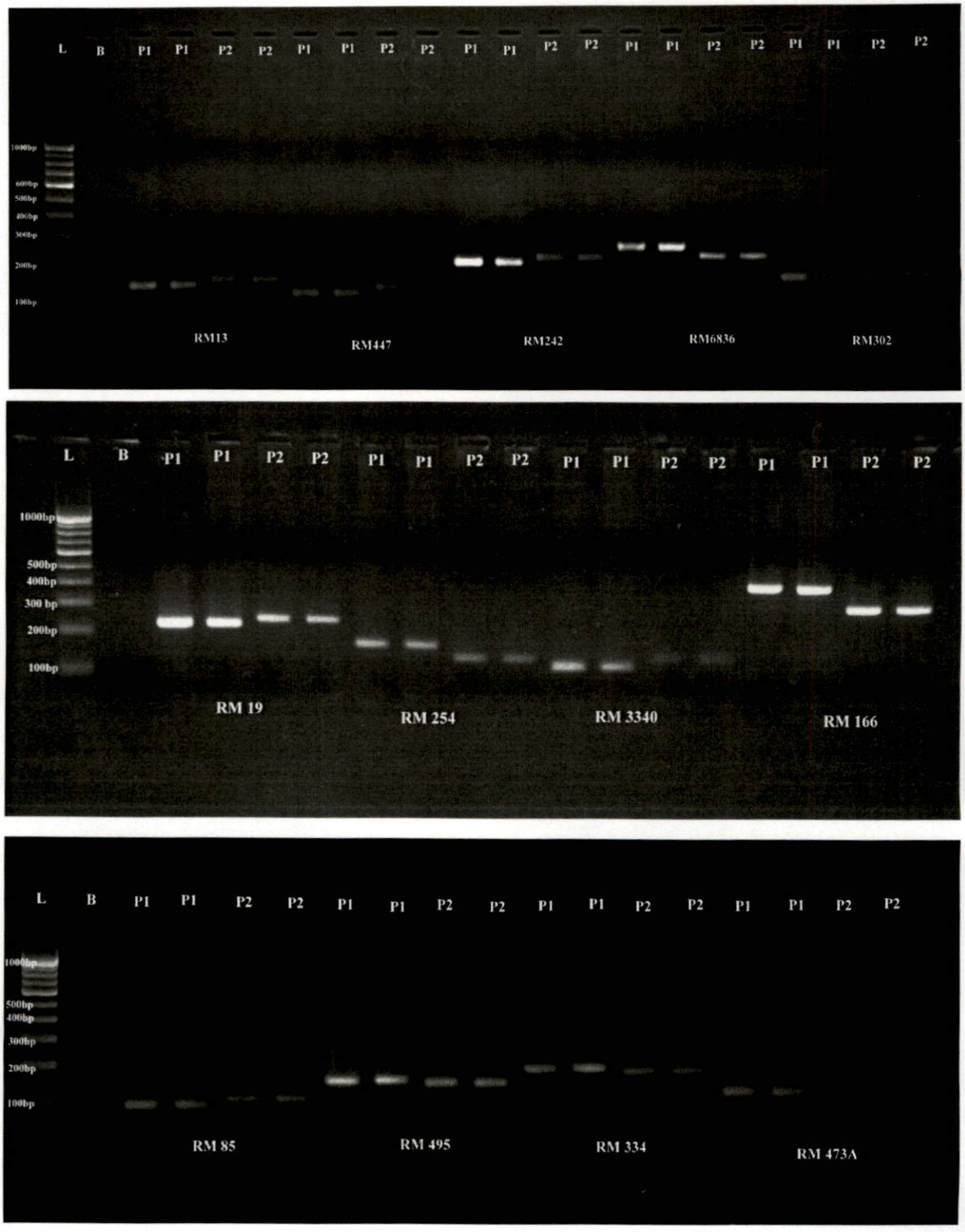
99	RM225	Polymorphic	1	1	158	180
100	RM461	Monomorphic	1	1	259	259
101	RM510	Monomorphic	1	1	139	139
102	RM314	Monomorphic	1	1	128	128
103	RM1209	Monomorphic	1	1	121	121
104	RM234	Monomorphic	1	1	192	192
105	RM11	Monomorphic	1	1	208	208
106	RM7121	Monomorphic	1	1	142	142
107	RM118	Monomorphic	1	1	201	201
108	RM125	Monomorphic	1	1	153	153
109	RM455	Monomorphic	1	1	141	141
110	RM336	Polymorphic	1	1	172	215
111	RM447	Polymorphic	1	1	130	142
112	RM5545	Polymorphic	2	1	179, 160	160
113	RM210	Monomorphic	2	2	167, 140	167, 140
114	RM152	Monomorphic	1	1	139	139
115	RM223	Monomorphic	1	1	243	243
116	RM515	Monomorphic	1	1	319	319
117	RM342	Monomorphic	1	1	263	263
118	RM408	Monomorphic	1	1	131	131
119	RM25	Monomorphic	1	1	161	161
120	RM256	Polymorphic	1	1	162	139
121	RM149	Monomorphic	1	1	231	231
122	RM547	Monomorphic	1	1	283	283
123	RM284	Monomorphic	1	1	154	154
124	RM80	Monomorphic	2	2	207, 154	207, 154
125	RM502	Monomorphic	1	1	248	248
126	RM108	Monomorphic	1	1	110	110
127	RM160	Monomorphic	1	1	145	145
128	RM7038	Monomorphic	1	1	207	207
129	RM566	Monomorphic	1	1	268	268
130	RM7039	Monomorphic	1	1	168	168
131	RM316	Polymorphic	1	2	201	213, 169
132	RM201	Polymorphic	1	1	340	165
133	RM205	Monomorphic	1	1	188	188
134	RM105	Monomorphic	1	1	156	156
135	RM242	Polymorphic	1	1	203	219
136	RM258	Monomorphic	1	1	158	158
137	RM6100	Polymorphic	1	1	169	160
138	RM6132	Monomorphic	1	1	124	124
139	RM147	Monomorphic	1	1	200	200
140	RM474	Monomorphic	1	1	236	236
141	RM171	Monomorphic	1	1	378	378
142	RM467	Monomorphic	1	1	299	299
143	RM484	Monomorphic	1	1	296	296
144	RM228	Monomorphic	1	1	114	114
145	RM26212	Polymorphic	1	1	170	137
146	RM332	Monomorphic	1	1	152	152
147	RM3701	Polymorphic	1	1	172	156
148	RM144	Monomorphic	1	1	331	331
149	RM167	Monomorphic	1	1	248	248

150	RM202	Monomorphic	1	1	319	319
151	RM206	Monomorphic	1	1	148	148
152	RM287	Monomorphic	1	1	299	299
153	RM536	Monomorphic	1	1	318	318
154	RM552	Polymorphic	1	1	304	252
155	RM209	Monomorphic	1	1	178	178
156	RM224	Polymorphic	1	1	151	165
157	RM247	Monomorphic	1	1	156	156
158	RM235	Monomorphic	1	1	306	306
159	RM7119	Monomorphic	1	1	121	121
160	RM17	Polymorphic	1	1	167	178
161	RM102	Monomorphic	1	1	433	433
162	RM219	Monomorphic	1	1	195	195
163	RM183	Monomorphic	1	1	127	127
164	RM229	Monomorphic	1	1	116	116
165	RM554	Monomorphic	1	1	266	266
166	RM313	Monomorphic	1	1	127	127
167	RM755	Polymorphic	1	1	118	147
168	RM254	Polymorphic	1	1	199	173
169	RM120	Monomorphic	1	1	307	307
170	RM166	Polymorphic	1	1	478	357
171	RM161	Monomorphic	1	1	126	126
172	RM133	Monomorphic	1	1	233	233
173	RM203	Monomorphic	1	1	256	256
174	RM170	Monomorphic	1	1	153	53
175	RM237	Monomorphic	1	1	189	189
176	RM277	Monomorphic	1	1	131	131
177	RM222	Monomorphic	1	1	209	209
178	RM6	Monomorphic	1	1	317	317
179	RM246	Monomorphic	1	1	133	133
180	RM278	Monomorphic	1	1	158	158
181	RM328	Monomorphic	1	1	154	154
182	RM72	Monomorphic	1	1	231	231
183	RM231	Monomorphic	1	1	274	274
184	RM19	Polymorphic	1	1	254	265
185	RM433	Monomorphic	1	1	199	199
186	RM14	Monomorphic	1	1	189	189
187	RM232	Monomorphic	1	1	171	171
188	RM271	Monomorphic	1	1	97	97
189	RM49	Monomorphic	1	1	113	113
190	RM84	Monomorphic	1	1	112	112
191	RM13	Polymorphic	1	1	144	160
192	RM251	Polymorphic	1	1	120	146
193	RM48	Monomorphic	1	1	312	312
194	RM490	Monomorphic	1	1	216	216
195	RM47	Monomorphic	1	1	371	371
196	RM85	Polymorphic	1	1	105	117
197	RM104	Monomorphic	1	1	195	195

Table 5. Selected SSR RM markers showing polymorphism between parents

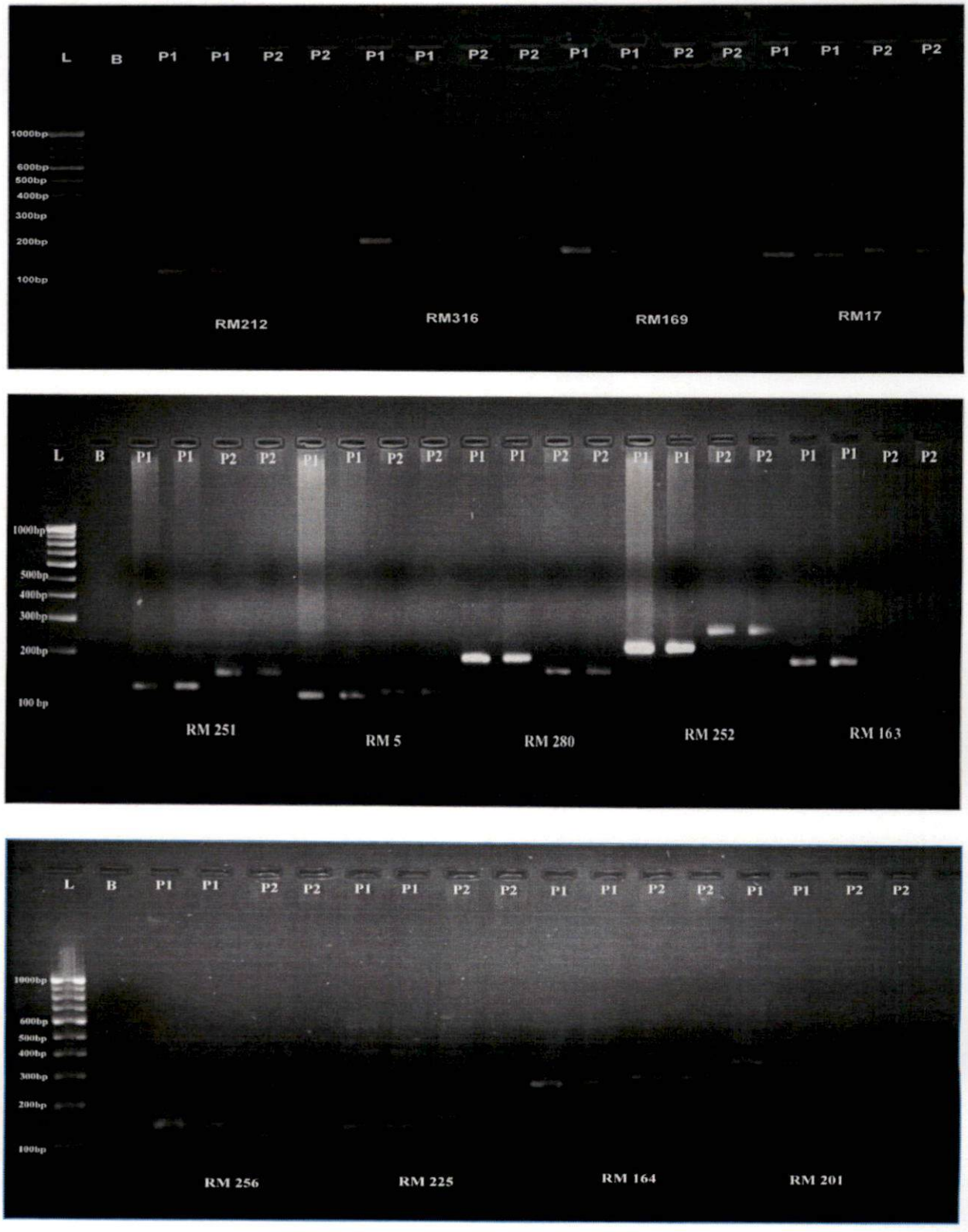
Sl. No.	Marker Name	Chromosome No.	Sl. No.	Marker Name	Chromosome No.
1	RM302	1	22	RM334	5
2	RM212	1	23	RM13	5
3	RM5	1	24	RM6836	6
4	RM495	1	25	RM225	6
5	RM473A	1	26	RM7555	6
6	RM10346	1	27	RM336	7
7	RM9	1	28	RM447	8
8	RM3340	2	29	RM256	8
9	RM166	2	30	RM5545	8
10	RM208	2	31	RM242	9
11	RM251	3	32	RM316	9
12	RM85	3	33	RM201	9
13	RM7076	3	34	RM6100	10
14	RM3586	3	35	RM254	11
15	RM280	4	36	RM552	11
16	RM252	4	37	RM26212	11
17	RM518	4	38	RM3701	11
18	RM5749	4	39	RM224	11
19	RM169	5	40	RM19	12
20	RM163	5	41	RM17	12
21	RM164	5			

Plate 9. Polymorphic SSR markers between parents N22 (P1) and Uma (P2)



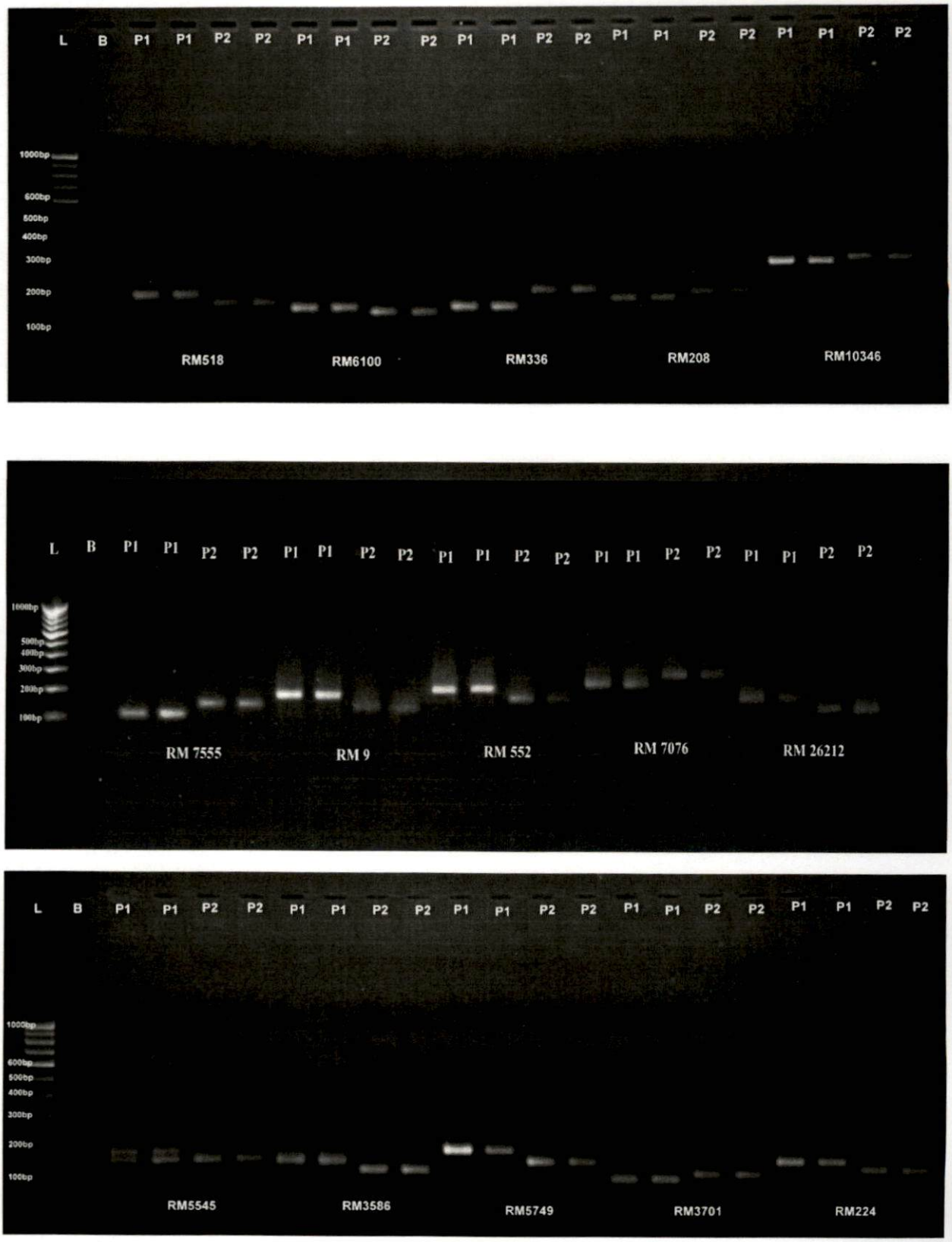
L- 100bp ladder, B- Blank, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent)

Plate 9. Polymorphic SSR markers between parents N22 (P1) and Uma (P2) (continued)



L- 100bp ladder, B- Blank, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent)

Plate 9. Polymorphic SSR markers between parents N22 (P1) and Uma (P2) (continued)



L- 100bp ladder, B- Blank, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent)

4.2 Bulked Segregant Analysis (BSA)

4.2.1 Phenotyping of F_3 plants for heat tolerance

Results of phenotyping of F_3 genotypes and parents for heat tolerance done at RARS, Pattambi during March and April 2017. F_3 plants were exposed to high temperature along with 5 plants of each parent (Uma and N22). As the control, 5 plants of each parent (Uma and N22) were kept outside the polyhouse at natural temperature. Morphological observations like plant height, number of tillers, number of panicles, panicle length, days for flowering, number of filled grains per panicles, number of sterile grains per panicle, spikelet fertility, 100 seed weight and grain weight per plant were measured for all the F_3 plants along with parents (Uma and N22). Morphological data of above mentioned 10 observations of F_3 plants are presented in Appendix - II. Estimates of range and mean for F_3 population are shown in Table 6. Morphological data of above mentioned 10 observations of parent plants are presented Table 10.

1. **Plant height (cm)**

Plant height in F_3 plants varied from 63 cm to 99 cm with a mean of 83.25 cm. Average plant height in heat treated and untreated control N22 was 101.80 cm and 103.80 cm respectively while it varied between 88.00 to 88.40 cm, respectively in treated and untreated control Uma.

2. **Number of tillers**

Number of tillers in F_3 plants varied from 3 to 14 tillers per plant with a mean of 8.64 tillers per plant. Mean number of tillers in heat treated and untreated control N22 was 10.2 and 11.80 tillers per plant respectively while it varied between 9.2 to 70.40 tillers per plant respectively in treated and untreated control Uma.

3. **Number of panicles**

Mean number of panicles in F_3 plants was 7.05 and varied from 3 to 11 panicles per plant. 8.80 and 11.80 panicles per plant were the mean numbers of panicles, respectively in heat treated and untreated control N22. Mean number of panicles in heat

treated Uma was 9.20 panicles and in untreated control Uma plants, mean number of panicles was 6.4 panicles per plant.

4. Panicle length (cm)

Mean panicle length in F_3 plants was 20.07 cm and it varied from 14.47 cm to 23.64 cm. In heat treated and untreated control N22 plants, mean panicle lengths were 18.82 cm and 19.12 cm, respectively. Mean panicle length of heat treated and untreated control Uma were 22 cm and 21.90 cm, respectively.

5. Days for flowering

Mean days for flowering in F_3 plants was 80.37 days and it varied from 73 to 104 days. In heat treated and untreated control N22 plants, mean days for flowering was 75.60 days. Mean days for flowering in heat treated and control Uma plants was 99 days with no variation.

6. Number of filled grains per panicle

Mean number of filled grains per panicle in F_3 plants was 64.89 filled grains and it varied from zero to 131.6 filled grains. 98.52 and 98.20 filled grains were the means of filled grains per panicle in heat treated and untreated control N22 plants respectively. 5.12 and 28.16 filled grains were the mean values of number of filled grains in heat treated and untreated control Uma plants respectively.

7. Number of sterile grains per panicle

Mean number of sterile grains per panicle in F_3 plants was 89.82 sterile grains and it varied 11.60 to 201.60 sterile grains. Mean number of sterile grains per panicle in heat treated and untreated control N22 were 10.12 and 8 sterile gains respectively. Mean number of sterile grains per panicle in heat treated Uma plant was 190.92 sterile grains and in untreated control Uma plant, it was172.60 sterile grains per panicle.

8. Spikelet fertility (%)

Mean spikelet fertility in F_3 plants was 44.78 per cent and in it varied from 0 per cent to 91.88 per cent Mean spikelet fertility in heat treated and control N22 plant were

90.92 per cent and 89.60 per cent respectively. Mean spikelet fertility in heat treated and untreated control Uma plant were 2.62 per cent and 14.10 per cent, respectively.

9. 100 seed weight (g)

Mean 100 seed weight in F_3 plants was 1.52 g and it varied from 0 g to 2.12 g. Mean 100 seed weight in heat treated N22 plant was 1.78 g and in the untreated control N22 plant was 1.82 g while in heat treated and control Uma it varied from 1.90 g to 2.17 g respectively.

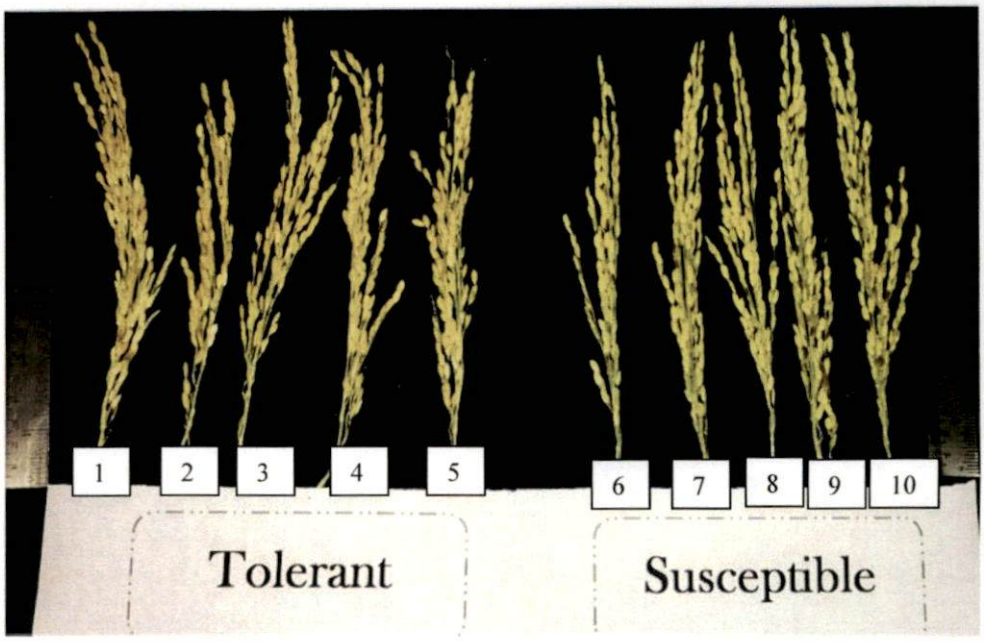
10. Grain weight per plant (g)

Mean grain weight per plant in F_3 plants was 5.37 g, it varied from 0 g to 12.1 g. Mean grain weight per plant in heat treated and untreated control N22 were 8.46 g and 9.29 g respectively. Mean grain weight per plant in heat treated and untreated control Uma plant were 0.89 g and 4.70 g, respectively.

4.2.2 Selection of tolerant and susceptible F_3 plants for BSA

F_3 plants for bulked segregant analysis were selected based on spikelet fertility percentage and grain weight per plant. Top seven F_3 plants having highest spikelet fertility were, F_3 - 31, F_3 - 15, F_3 - 41, F_3 - 13, F_3 - 16, F_3 - 12 and F_3 - 45 with spikelet fertility 91.88, 84.98, 82.85, 81.91, 81.18, 80.90, and 78.06 per cent respectively. Grain weight per plant of F_3 - 31, F_3 - 15, F_3 - 41, F_3 - 13, F_3 - 16, F_3 - 12 and F_3 - 45 were 12.10 g, 9.30 g, 8.10 g, 7.20 g, 7.70 g, 7.10 g and 8.73 g respectively. These seven plants, F_3 - 31, F_3 - 15, F_3 - 41, F_3 - 13, F_3 - 16, F_3 - 12 and F_3 - 45 were used to constitute the tolerant bulk. Top seven plants having lowest spikelet fertility were, F_3 - 4, F_3 - 5, F_3 - 11, F_3 - 29, F_3 - 34, F_3 - 37 and F_3 - 42 with zero per cent spikelet fertility and no grain weight per plant. These seven plants, F_3 - 4, F_3 - 5, F_3 - 11, F_3 - 29, F_3 - 34, F_3 - 37 and F_3 - 42 were used to constitute the susceptible bulk (Table 7 and Plate 10).

Plate 10. Spikelets of tolerant and susceptible F_3 plants



1 to 10 representative panicles of F_3 plants; 1 = F_3-31, 2 = F_3 - 15, 3 = F_3 - 41, 4 = F_3 - 13, 5 = F_3 - 16, 6 = F_3 - 4, 7 = F_3 - 5, 8 = F_3 - 11, 9 = F_3 - 29 and 10 = F_3 - 34.

Table 6. Variability in F_3 plants under high-temperature stress

Trait	Range		Mean
	Maximum	Minimum	
Plant Height (cm)	99.00	63.00	83.25
No. of tillers	14.00	3.00	8.64
No. of Panicles	11.00	3.00	7.05
Panicle length (cm)	23.64	14.47	20.07
Days for flowering	104.00	73.00	80.37
No. of filled grains/panicle	131.60	0.00	64.89
No. of sterile grains/panicle	201.60	11.60	89.82
Spikelet fertility (%)	91.88	0.00	44.78
100 seed weight (g)	2.12	0.00	1.52
Grain weight/plant (g)	12.10	0.00	5.37

Table 7. Phenotyping of seven tolerant and seven susceptible F_3 plants

Plant No.	Plant Height (cm)	No. of tillers	No. of Panicles	Panicle length (cm)	Days for flowering	No. of filled grains/ panicle	No. of sterile grains/ panicle	Spikelet fertility %	100 seed weight (g)	Grain weight/ plant (g)
F3 – 12	79	11	11	19.67	77	90.20	20.20	80.90	2.10	7.10
F3 – 13	91	10	5	19.00	77	121.40	27.00	81.91	1.97	7.20
F3 – 15	81	11	10	22.50	82	117.40	20.40	84.98	1.91	9.30
F3 – 16	82	7	5	19.17	75	100.60	23.40	81.18	1.63	7.70
F3 – 31	79	12	11	19.77	79	130.80	11.60	91.88	2.03	12.10
F3 – 41	76	8	7	17.57	76	105.20	21.40	82.85	1.73	8.10
F3 – 45	87	9	8	19.67	77	108.20	31.20	78.06	1.88	8.73
F3 – 4	81	8	8	18.67	80	0.00	200.00	0.00	0.00	0.00
F3 – 5	76	6	3	14.47	90	0.00	183.00	0.00	0.00	0.00
F3 – 11	89	8	7	16.80	81	0.00	175.20	0.00	0.00	0.00
F3 – 29	90	7	5	17.90	79	0.00	171.40	0.00	0.00	0.00
F3 – 34	82	8	8	17.67	86	0.00	183.00	0.00	0.00	0.00
F3 – 37	79	9	6	20.74	83	0.00	189.20	0.00	0.00	0.00
F3 – 42	90	7	6	20.84	81	0.00	166.20	0.00	0.00	0.00

4.2.2. DNA Isolation and quantification of F_3 plants

Tender leaves of F_3 plants (one month old) were collected early morning for DNA isolation. The genomic DNA isolated through the CTAB method reported by Dellaporta

et al. (1983) with some modifications, *i.e.* RNase treatment was used during isolation procedure to remove all RNA contamination. The quality and quantity of DNA was checked using agarose gel electrophoresis. Clear and single intact band of high intensity appeared upon gel documentation indicating, non-degraded good quality DNA free from RNA and protein contamination was isolated (Plates 8). The result of the NanoDrop® ND-1000 spectrophotometer analysis is presented in Table 8. The quantity of DNA in the isolated samples ranged from 1408.47 to 2042.72ng/µl.

Table 8. Quality and quantity of DNA isolated from F_3 plants assessed by NanoDrop method

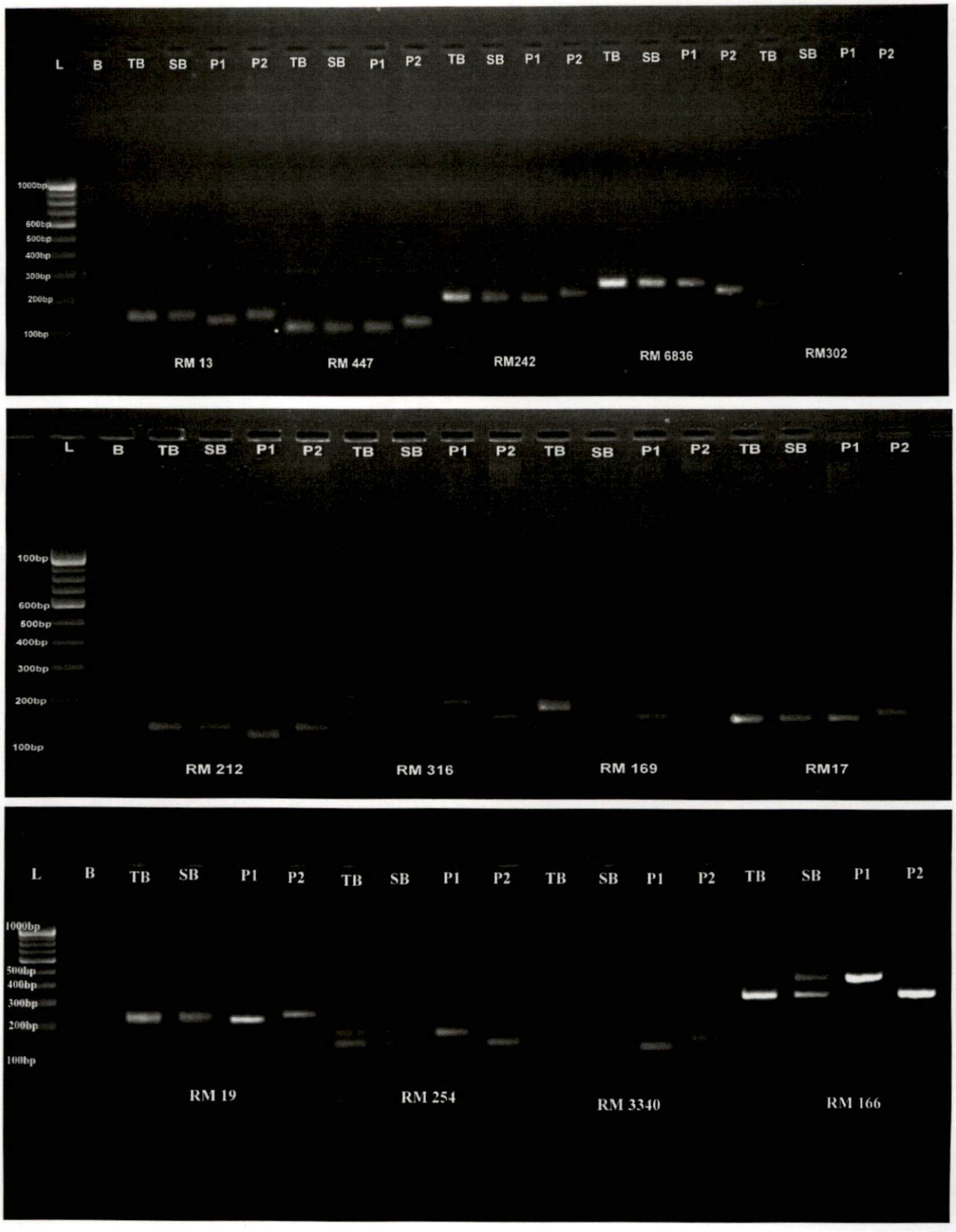
Genotypes	A 260/280	Quantity (ng/µl)	Genotypes	A 260/280	Quantity (ng/µl)
F3 – 1	1.87	1634.75	F3 – 26	1.88	1725.23
F3 – 2	1.92	2042.72	F3 – 27	1.89	1490.55
F3 – 3	1.83	1720.99	F3 – 28	1.91	1925.26
F3 – 4	1.88	1408.47	F3 – 29	1.90	1845.54
F3 – 5	1.87	1833.74	F3 – 30	1.82	1628.19
F3 – 6	1.81	1864.12	F3 – 31	1.84	1467.22
F3 – 7	1.83	1691.22	F3 - 32	1.84	1528.45
F3 – 8	1.88	1401.23	F3 – 33	1.87	1725.56
F3 – 9	1.91	1587.54	F3 – 34	1.90	1836.69
F3 – 10	1.80	1679.22	F3 – 35	1.82	1725.25
F3 – 11	1.91	1957.78	F3 – 36	1.87	1935.88
F3 – 12	1.93	1721.33	F3 – 37	1.91	1436.25
F3 – 13	1.86	2017.36	F3 – 38	1.88	1780.25
F3 – 14	1.85	1978.07	F3 – 39	1.87	1601.49
F3 – 15	1.87	1902.44	F3 – 40	1.90	1826.18
F3 – 16	1.92	1852.33	F3 – 41	1.92	1566.40
F3 – 17	1.82	1542.22	F3 – 42	1.90	1628.47
F3 – 18	1.88	1744.62	F3 – 43	1.84	1988.20
F3 – 19	1.91	1492.30	F3 – 44	1.87	2001.29
F3 – 20	1.82	1431.10	F3 – 45	1.88	1589.48
F3 – 21	1.90	1549.28	F3 – 46	1.93	1873.25
F3 – 22	1.84	1726.22	F3 – 47	1.87	1871.83
F3 – 23	1.89	1430.75	F3 – 48	1.83	1782.49
F3 – 24	1.89	1433.22	F3 – 49	1.85	1954.72
F3 – 25	1.92	1452.80			

4.2.3. Genotyping parents, tolerant and susceptible bulks

Total 41 polymorphic RM markers were selected from 197 rice microsatellite markers by parental polymorphism observed betweenN22 and Uma. These 41 polymorphic RM markers were used for genotyping parents (N22 and Uma), tolerant and susceptible bulks through bulk segregant analysis (Plate 11). Genotyping of parents, tolerant and susceptible bulks was done based on the position of the alleles (band of amplified products) of parents, tolerant and susceptible bulks for these 41 polymorphic RM markers. Genotyping of 41 polymorphic RM markers were given for allele position of the tolerant parent (N22) as 0 and 1 for the susceptible parent (Uma). Similarly, genotyping 41 polymorphic RM markers are given for allele position of susceptible bulks and tolerant bulks as either 0 or 1. Genotypic score '0' is given when allele position of susceptible bulks or resistant bulks similar to allele position of the susceptible parent (Uma) and '1' is given when allele position of susceptible bulks or resistant bulks similar to allele position of the tolerant parent (N22). Genotypic score '2' is given when two alleles present in tolerant bulk or susceptible bulk and similar position to both bands of resistant parent and susceptible parent.

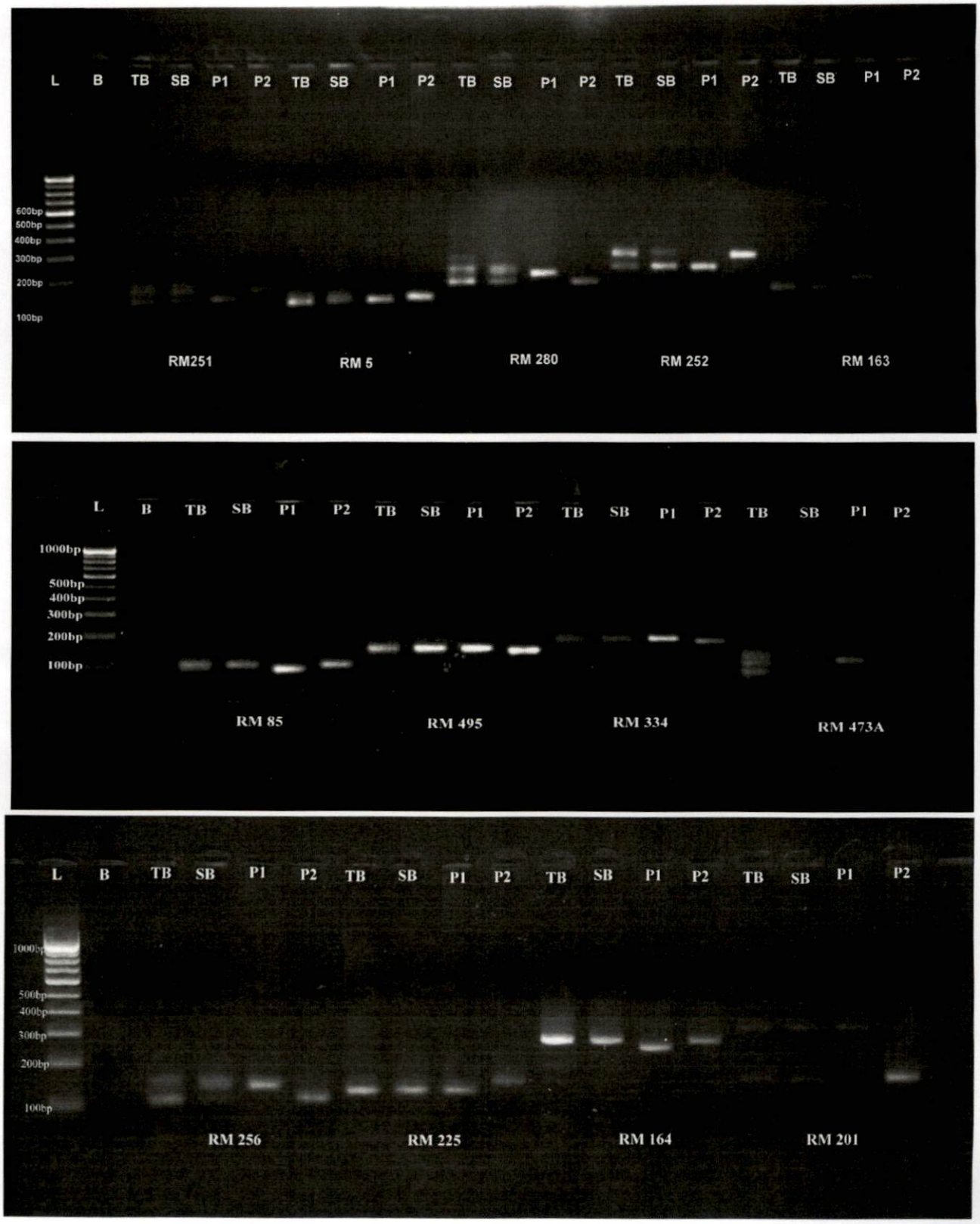
One RM marker out of 41 RM markers (polymorphic RM markers between two parents, N22 and Uma) showed genotypic score as 2 and 0 between tolerant bulks and susceptible bulks through bulk segregant analysis as between parents (N22 and Uma) and treated as polymorphic RM marker between tolerant bulks and susceptible bulks for heat tolerance. Remaining forty RM markers out of 41 RM markers (polymorphic RM markers between two parents, N22 and Uma) showed genotypic score as 0 or 1 or 2 in both cases of tolerant bulks and susceptible bulks through bulk segregant analysis. Genotyping score data of parents, susceptible and tolerant bulks through bulk segregant analysis for these 41 polymorphic RM markers is presented in Table 9. So, this one polymorphic RM marker (RM5749) was used for confirmation of putative marker by genotyping seven most tolerant plants and seven most susceptible plants along with parents.

Plate 11. Genotyping tolerant bulk (TB), susceptible bulk (SB) and parents with selected 41 polymorphic SSR markers.



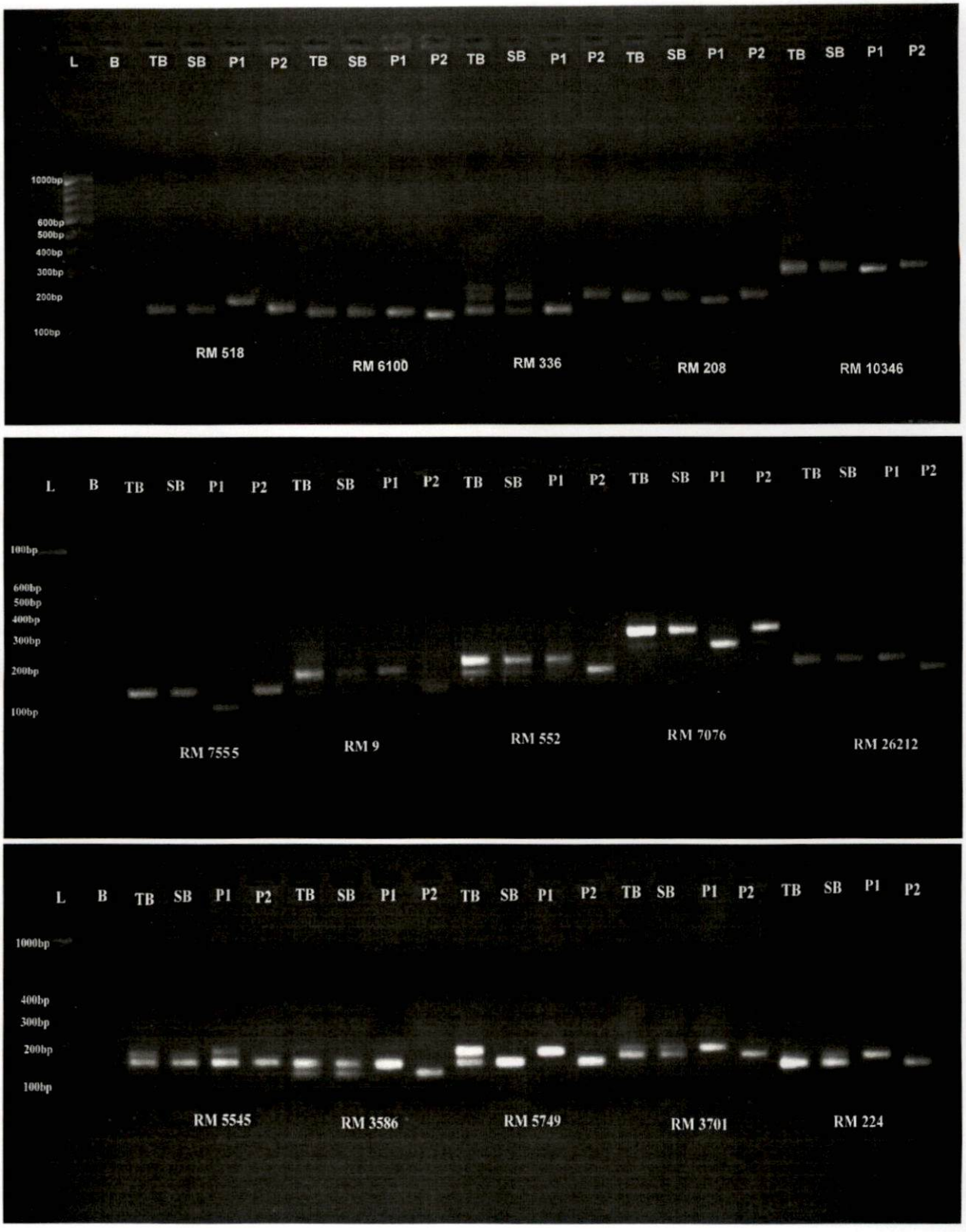
L- 100bp ladder, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent), TB- Tolerant bulk, SB- Susceptible Bulk

Plate 11. Genotyping tolerant bulk (TB), susceptible bulk (SB) and parents with selected 41 polymorphic SSR markers (continued)



L- 100bp ladder, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent), TB- Tolerant bulk, SB- Susceptible Bulk

Plate 11. Genotyping tolerant bulk (TB), susceptible bulk (SB) and parents with selected 41 polymorphic SSR markers (continued)



L- 100bp ladder, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent), TB- Tolerant bulk, SB- Susceptible Bulk

Table 9. Genotyping of tolerant bulk (TB), susceptible bulk (SB) and parents using polymorphic markers

Rice microsatellite	N22 (Tolerant parent)	Tolerant bulk	Uma (Susceptible parent)	Susceptible bulk
RM13	1	0	0	0
RM447	1	1	0	1
RM242	1	1	0	1
RM6836	1	1	0	1
RM302	1	0	0	0
RM19	1	2	0	2
RM254	1	2	0	2
RM3340	1	2	0	2
RM166	1	2	0	2
RM85	1	0	0	0
RM495	1	1	0	1
RM334	1	1	0	1
RM473A	1	2	0	2
RM212	1	0	0	0
RM316	1	2	0	2
RM169	1	0	0	0
RM17	1	1	0	1
RM251	1	2	0	2
RM5	1	1	0	1
RM280	1	2	0	2
RM252	1	2	0	2
RM163	1	0	0	0
RM256	1	2	0	2
RM225	1	1	0	1
RM164	1	0	0	0
RM201	1	2	0	2
RM518	1	0	0	0
RM6100	1	1	0	1
RM336	1	2	0	2
RM208	1	0	0	0
RM10346	1	2	0	2
RM7555	1	0	0	0
RM9	1	2	0	2
RM552	1	2	0	2
RM7076	1	0	0	0
RM26212	1	1	0	1
RM5545	1	1	0	1
RM3586	1	2	0	2
RM5749	1	2	0	0
RM3701	1	2	0	2
RM224	1	2	0	2

Tolerant allele - 1, Susceptible allele - 0, Heterozygous allele - 2

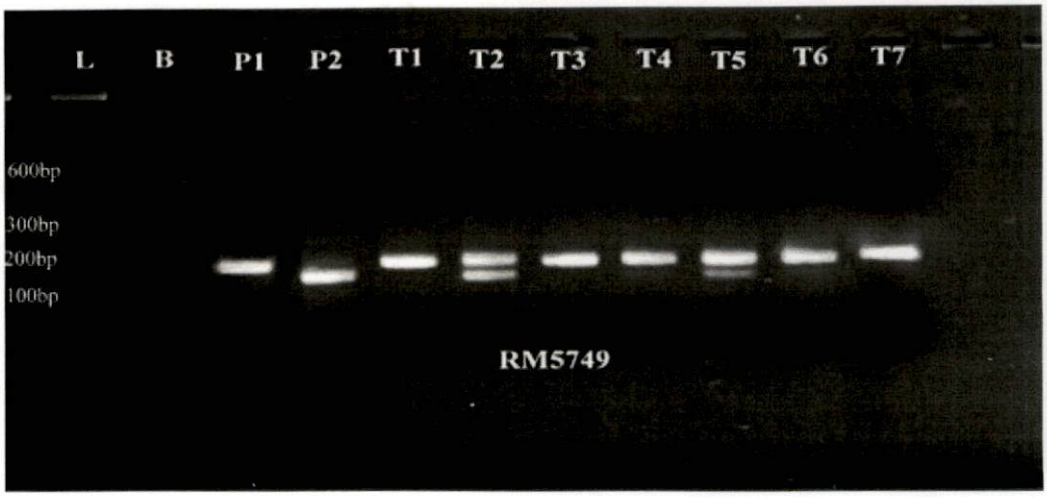
4.2.4 Confirmation of putative marker

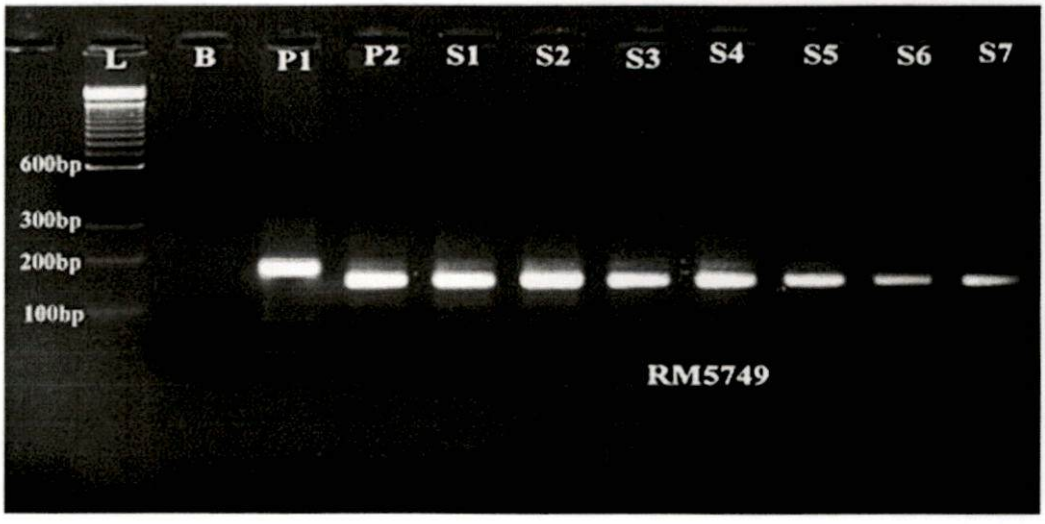
RM5749 showed polymorphism between parents as well as tolerant bulk and susceptible bulk. This polymorphic marker, RM5749 was validated by screening individuals of tolerant and susceptible bulks and their parents (Plate 12).

The seven individuals of tolerant bulk F_3 - 12 (T1), F_3 - 15 (T3), F_3 - 16 (T4), F_3 - 31 (T6) and F_3 - 45 (T7) showed monomorphic bands as in tolerant parent N22 and two individuals namely, F_3 - 13 (T2) and F_3 - 41 (T5) showed heterozygous bands of both parents. The seven individuals of susceptible bulk F_3 - 4 (S1), F_3 - 5 (S2), F_3 - 11 (S3), F_3 - 29 (S4), F_3 - 34 (S5), F_3 - 37(S6) and F_3 - 42 (S7) showed monomorphic bands like susceptible parent Uma (Plate 12).

Again, RM5749 was screened in all 49 F_3 individuals for the single marker analysis (Plate 13.). The genotyping and phenotyping data for the single marker analysis is given in Table 11. Single marker analysis showed, RM5749 was significantly ($P < 0.001$) associated with spikelet fertility on chromosome number 4 under high-temperature stress. The position of RM5749 was reported to be at 22.80 Mb on chromosome number 4 (Gramene Database). RM5749 was identified as tightly linked marker to spikelet fertility with 6.86 LOD value (Plate 14).

Plate 12. Selective genotyping of individual F3 plants of bulk and parents with RM5749



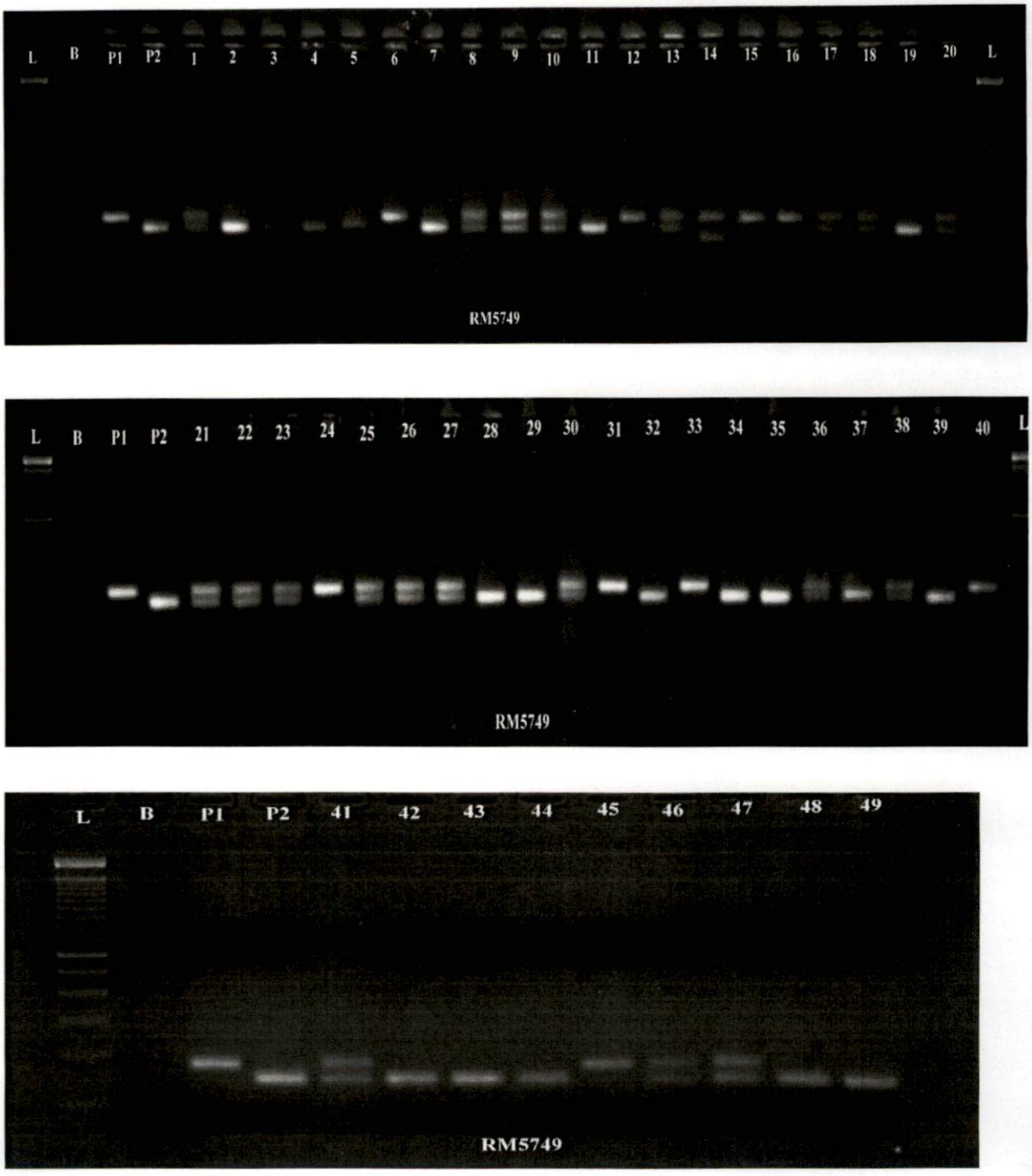


L- 100bp ladder, P1- N22 (Tolerant parent), P2- Uma (Susceptible parent)

T1, T2, T3…T7- Individuals of tolerant bulk,

S1, S2, S3…S7- Individuals of susceptible bulk.

Plate 13. Individual genotyping of F_3 plants with RM5749 for SMA



L- 100bp ladder (Geni), B- Blank,

P1- N22 (Tolerant parent), P2- Uma (Susceptible parent),

1, 2, 3…49- F_3 Plants

Fig. 1. Association of RM5749 with spikelet fertility

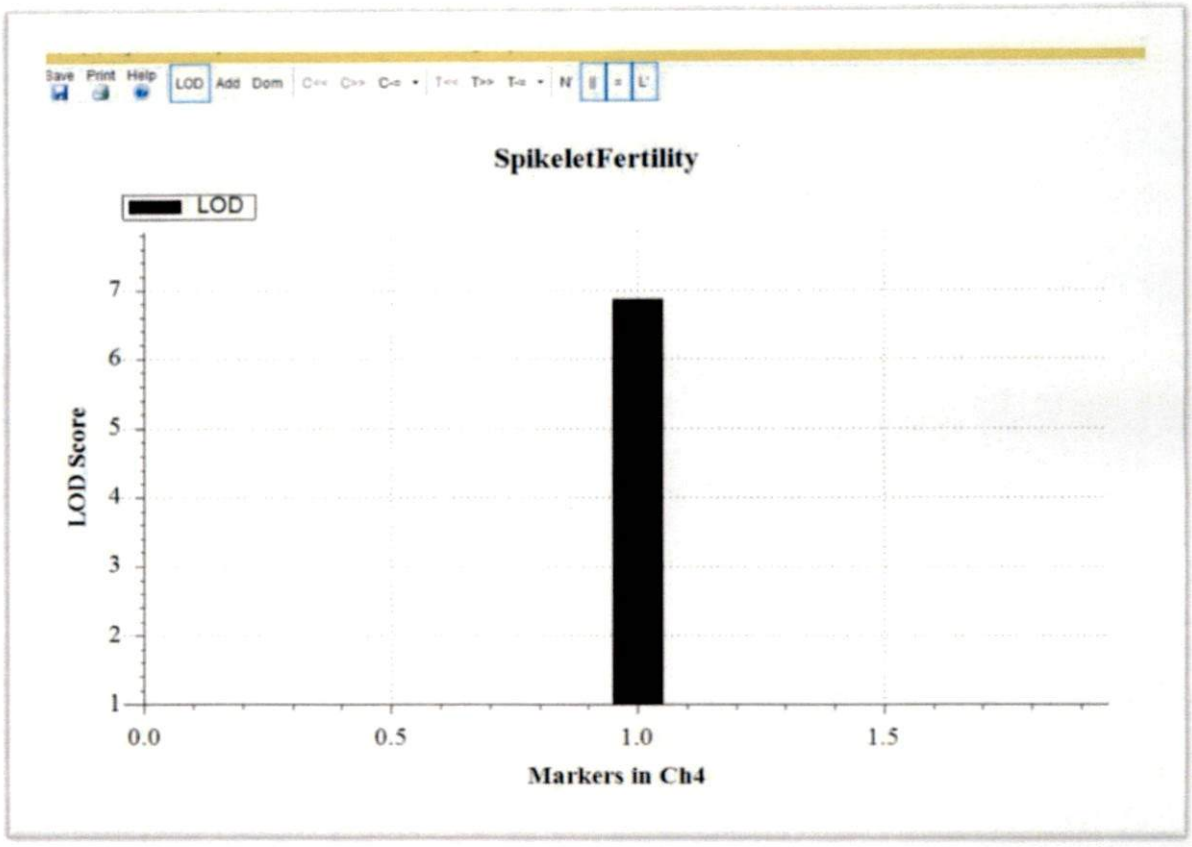


Fig. 2. Linkage map of RM5749 on chromosome number 4

Table 10. Phenotyping of parents (N22 and Uma)

Treatment	Plant Number	Plant Height (cm)	No. of tillers	No. of Panicles	Panicle length (cm)	Days for flowering	No. of filled grains/panicle	No of sterile grains/panicle	Spikelet fertility (%)	100 seed weight (g)	Grain weight/plant (g)
High temperature stress	N22 – 1	101	8	8	20.20	77	102.40	15.60	87.14	1.85	7.97
	N22 – 3	99	10	9	18.94	73	104.20	8.20	92.81	1.63	8.39
	N22 – 5	102	9	8	18.87	77	101.60	12.80	88.71	1.80	8.27
	N22 – 6	103	11	8	18.20	76	100.80	6.00	94.46	1.65	8.93
	N22 – 7	104	13	11	17.90	75	83.60	8.00	91.49	1.97	8.77
	Mean	**101.80**	**10.20**	**8.80**	**18.82**	**75.60**	**98.52**	**10.12**	**90.92**	**1.78**	**8.46**
	Uma-3	87	9	9	21.00	99	4.60	191.00	2.33	2.05	0.86
	Uma-5	89	9	9	22.00	99	6.80	194.80	3.31	1.83	1.12
	Uma-8	86	10	10	21.67	99	3.00	197.60	1.52	1.90	0.57
	Uma-9	88	7	7	23.00	99	4.60	192.40	2.32	1.97	0.63
	Uma10	90	11	11	22.34	99	6.60	178.80	3.58	1.71	1.23
	Mean	**88.00**	**9.20**	**9.20**	**22**	**99**	**5.12**	**190.92**	**2.62**	**1.90**	**0.89**
Non- heat treated control	N22 – 2	102	13	13	21.50	78	106.20	8.80	92.64	1.76	9.94
	N22 – 4	111	14	14	17.20	75	75.80	6.00	77.93	1.72	8.69
	N22 – 8	101	12	10	15.00	76	94.60	8.80	91.75	1.76	9.95
	N22 – 9	103	12	12	20.87	74	98.80	7.40	92.92	1.97	8.88
	N22 -10	102	8	10	21.04	75	115.60	9.00	92.75	1.85	9.03
	Mean	**103.80**	**11.80**	**11.80**	**19.12**	**75.60**	**98.20**	**8.00**	**89.60**	**1.82**	**9.29**
	Uma-1	84	7	4	22.34	99	37.80	170	18.15	2.20	5.06
	Uma-2	87	8	8	23.34	99	40.20	158.40	20.86	2.19	4.61
	Uma-4	89	6	4	22.34	99	16.60	179.60	8.44	2.18	4.44
	Uma-6	83	7	7	19.65	99	20.00	182.40	9.90	2.10	5.06
	Uma-7	99	9	9	21.84	99	26.20	172.60	13.14	2.18	4.33
	Mean	**88.40**	**7.40**	**6.40**	**21.29**	**99.00**	**28.16**	**172.60**	**14.10**	**2.17**	**4.70**

Table 11. Phenotyping and genotyping for Single marker analysis

Plant Number	Phenotyping		Genotypic score	Plant Number	Phenotyping		Genotypic score
	Spikelet fertility (%)	Class			Spikelet fertility (%)	Class	
F_3-1	75.30	4	1	F_3-26	66.48	3	1
F_3-2	57.07	3	0	F_3-27	58.82	3	1
F_3-3	50.03	3	0	F_3-28	11.09	2	0
F_3-4	0.00	1	0	F_3-29	0.00	1	0
F_3-5	0.00	1	0	F_3-30	66.22	3	1
F_3-6	74.70	3	2	F_3-31	91.88	5	2
F_3-7	64.61	3	0	F_3-32	0.00	1	0
F_3-8	64.67	3	1	F_3-33	76.70	4	2
F_3-9	58.04	3	1	F_3-34	0.00	1	0
F_3-10	44.91	2	1	F_3-35	60.20	3	0
F_3-11	0.00	1	0	F_3-36	37.04	2	1
F_3-12	80.90	4	2	F_3-37	0.00	1	0
F_3-13	81.91	4	1	F_3-38	10.36	2	1
F_3-14	53.27	3	1	F_3-39	45.17	2	0
F_3-15	84.98	4	2	F_3-40	77.03	4	2
F_3-16	81.18	4	2	F_3-41	82.85	4	1
F_3-17	68.75	3	1	F_3-42	0.00	1	0
F_3-18	76.38	4	1	F_3-43	0.00	1	0
F_3-19	35.74	2	0	F_3-44	16.05	2	0
F_3-20	45.65	2	1	F_3-45	78.06	4	2
F_3-21	17.37	2	1	F_3-46	44.10	2	1
F_3-22	70.64	3	1	F_3-47	33.17	2	1
F_3-23	48.31	2	1	F_3-48	0.00	1	0
F_3-24	20.94	2	2	F_3-49	46.87	2	0
F_3-25	36.78	2	1				

IRRI spikelet fertility classification		
Spikelet fertility (%)	Scoring	Class
0	Complete sterile	1
1 – 49	Highly sterile	2
50 - 74	Partial sterile	3
75 - 90	Fertile	4
90 - 100	Highly fertile	5

Genotypic classification	
Allele	Score
Tolerant allele	2
Susceptible allele	0
Heterozygous allele	1

5. DISCUSSION

Currently, rice production is considerably affected by climate change and seasonal climate variations. Global warming is predicted to rise the number of extremely hot days as well as increasing night-time temperature (Mora *et al.*, 2013). The frequency and duration of extreme heat events that exceed maximum temperature on record are predicted to increase by 50 per cent by 2050, and 90 per cent by 2100 (Handmer *et al.*, 2012).

The rise in atmospheric temperature causes unfavourable effects on growth, yield and quality of rice crop by affecting its phenology, physiology and yield components (Satake and Yoshida 1978; Yoshida *et al.*, 1981; Peng *et al.*, 2004). Heat stress induces numerous biochemical, molecular and physiological alterations and responses that stimulate numerous cellular as well as whole plant processes affecting crop yield and quality.

Reproductive stages like, microsporogenesis and anthesis are the most sensitive to heat stress (>35 °C). Heat stress at flowering has shown to be a major limitation for grain yield in rice. (Jagadish *et al.*, 2010). The temperature of 32-38 °C inhibits anther dehiscence and prevents pollination, which is the first step in the fertilisation process. The primary cause of yield reduction during high-temperature stress is the improper anther dehiscence during anthesis (Matsui *et al.*, 2001).

Uma is the popular high yielding variety of Kerala but it is extremely susceptible to high-temperature stress, particularly during the reproductive and grain filling stages. Now, in the major rice growing tracts of Kerala namely, Palakkad, Kole and Kuttanad, the temperature tends to rise up to 39 °C or more especially during the second/third crop. Hence heat stress induced spikelet sterility has become a severe problem in Uma variety. Hence it is the need of the hour to incorporate heat tolerance into this high yielding variety during the current scenario of climate change.

Molecular markers offer a great potential in breeding for biotic and abiotic stress tolerance as they can be used for selection of genotypes with specific trait or character. Microsatellite markers (SSR) are very useful as they are abundant and present throughout the genome. SSR markers can be used in various populations like F_2, F_3, back cross, NIL, RIL and double haploid for identification of linked marker to the specific trait.

Identification of molecular markers associated with specific genes will help to select the plant carrying these genes in various generations without subjecting them to any treatment, and at any stage of growth. Identified reliable markers, tagged with the specific gene of interest would prove to be powerful tools for screening and selection of plants having desirable qualitative and quantitative characters. This technique is very useful to reduce the breeding cycle time and the burden of plant breeders.

N22 is the most heat tolerant variety of rice found so far and used as a heat tolerance donor in various breeding programme. During the evaluation of physiological and proteomic approaches to study high-temperature stress tolerance at anthesis in rice, some of the heat shock proteins in N22 were considerably upregulated and it may be the reason behind the heat tolerance of N22 (Jagadish *et al.*, 2010).

In the present study, F_3 population developed from Uma x N22 was used for bulked segregant analysis to identify the SSR markers linked to heat tolerance. Two major aspects involved in the programme were:

1. Parental polymorphism study using molecular markers
2. Bulked Segregant Analysis

5.1 Parental polymorphism study using molecular markers

Two parents, Uma and N22 were planted during August 2016 for parental DNA isolation. Tender leaves were used for the DNA isolation. After PCR and gel electrophoresis, clear intact bands of DNA were observed. NanoDrop® ND-1000

spectrophotometer analysis also revealed the good quality of isolated DNA. Isolated DNA samples were used for studying the parental polymorphism using rice microsatellite markers.

One hundred and ninety-seven SSR markers distributed among the twelve chromosomes of rice were used to deduce the molecular level polymorphism between the high-temperature stress susceptible rice variety, Uma and high-temperature stress tolerant rice variety, N22. Forty-one markers were identified to differentiate these two parents. These 41 polymorphic rice SSR markers were found to be distributed over all 12 linkage groups of rice, among these, seven RM markers were on chromosome number 1, three markers on chromosome number 2, four markers on chromosome number 3, four markers on chromosome number 4, five markers on chromosome number 5, three markers on chromosome number 6, single marker on chromosome number 7, three markers on chromosome number 8, three markers on chromosome number 9, single marker on chromosome number 10, five markers on chromosome number 11, and two markers on chromosome number 12.

The parental survey revealed 20.82 per cent polymorphism between the two parents (N22 and Uma) used in the present study. Parental polymorphism per cent between any two parents depended on number of relevant primers selected for screening. Similar to the findings of the present study, Zhang *et al.* (2009) had identified 30 per cent polymorphism by using 200 SSR markers in heat tolerant 996 and heat susceptible 4628 rice cultivars.

Kanagaraj *et al.* (2010) screened 1206 SSR markers between IR20/Nootripathu and identified 134 polymorphic SSR markers between these two parents showing 11.12 per cent polymorphism. Vikram *et al.* (2011) also studied the polymorphism of N22 with IR64, Swarna and MTU1010 that was 42.50 per cent 43.50 per cent and 40.10 per cent respectively. Salunkhe *et al.* (2011) identified 96 SSR markers to be polymorphic among 343 SSR markers between two parents IR20 and Nootripathu indicating 27.99 per cent parental polymorphism. Wei *et al.* (2013) screened 2304 SSR markers and identified 322 polymorphic SSR markers between two breeding lines, HT54 (heat stress tolerant) and HT13 (heat stress

susceptible with 13.98 per cent polymorphism. Vikram *et al.* (2012) used 880 SSR markers and they identified 71 SSR markers between two rice varieties, Basmati334 and Swarna with 8.07 per cent polymorphism. Buu *et al.* (2014) used 501 SSR markers for the parental polymorphism, from that they found 264 polymorphic SSR markers between two parents, OM5930 (heat sensitive variety) and N22 (heat tolerant variety). Yadav *et al.* (2015) studied the parental polymorphisms between two land races of rice (BPT-5204 and ARC-10531) with 500 SSR markers and found 70 polymorphic markers between the parents.

The forty-one rice microsatellite (SSR) markers identified as polymorphic between the two parents (N22 and Uma) is a pointer to the existence of different alleles at each of the 41 marker loci. As the two parents differ from each other with respect to various traits (*e.*g., kernel colour, plant height, maturity duration etc.) other than their reaction to heat stress, the 41 polymorphic markers may or may not be linked to spikelet fertility, which is the reliable indicator of heat tolerance.

Out of these 41 polymorphic markers, fourteen were recognised by earlier researchers as associated with QTL for heat tolerance. RM 473A was a polymorphic marker between parents for heat tolerance, as identified by Liao *et al.* (2011). Buu *et al.* (2013) reported RM251 as a polymorphic marker between parents for heat tolerance. Poli *et al.* (2013) reported RM225 as a polymorphic marker between parents for heat tolerance. As per the reports of Bharathkumar *et al.* (2014), RM6100 was polymorphic marker between parents for heat tolerance. Buu *et al.* (2014) had identified RM7076, RM3586, RM26212 and RM5749 as polymorphic marker for heat tolerance between parents. Wei *et al.* (2013) identified RM242 as associated with heat tolerance. Zhao *et al.* (2016) reported RM3340, RM447, RM5545, RM3701 and RM336 as being polymorphic markers for heat tolerance. Various researchers like Zhang *et al.* (2009), Lang *et al.* (2015) and Buu *et al.* (2014) also reported RM3586 as a polymorphic marker for heat tolerance.

5.2 Bulked Segregant Analysis

5.2.1 Morphological screening of segregating population for heat tolerance

Spikelet fertility is the major trait for the identification and selection of F_3 plants for bulked segregant analysis. High-temperature stress directly affects spikelet fertility. During heat stress in this study, it is observed that N22 (heat tolerant parent) exhibited the highest mean spikelet fertility (90.92%) and Uma (heat susceptible parent) exhibited the lowest mean spikelet fertility (2.62%). In some panicles of Uma plants, fungal infection was noticed which was controlled by fungicide application. F_3 plants showed 44.78 per cent mean spikelet fertility and some of the individuals (F_3 - 31, F_3 - 15, F_3 - 41, F_3 - 13, F_3 - 16, F_3 - 12 and F_3 - 45) showed spikelet fertility similar to heat tolerant parent (N22) and some of the individuals (F_3 - 4, F_3 - 5, F_3 - 11, F_3 - 29, F_3 - 34, F_3 - 37 and F_3 - 42) showed spikelet fertility similar to heat susceptible parent (Uma).

Similar to the findings of the present study, Jagadish *et al.* (2008) found N22 as the highest temperature stress tolerant genotype (64 to 86% fertility at 38 °C) whereas cultivars *Oryza sativa ssp. japonica* Azucena and Moroberekan as the most susceptible (<8%) genotypes. Allah *et al.* (2011) also observed 20 genotypes and they found higher spikelet sterility (14% to 80%) and pollen sterility (15% to 54%) were observed under heat stress whereas lesser spikelet sterility (10% to 18%) and pollen sterility (16% to 34%) were observed under non-stress conditions. High spikelet fertility was observed for IR64 (94.20%) and N22 (95.10%) in both control and treatment (Ye *et al.*, 2011). Though, in the high-temperature stress treatment, spikelet fertility of IR64 (21.50%) was considerably lesser than that of N22 (81.10%) whereas it ranged from 0 to 89 per cent in 158 segregating (F_2) progenies as in the present study. Vivitha *et al.* (2017) also reported the spikelet fertility in N22 under control and high-temperature treatments as 86.63 per cent and 72.85 per cent respectively, while in IWP as 68.82 per cent and 16.52 per cent respectively. Some of the individuals of mapping population showed 60 to 67 per cent spikelet fertility and used for finding the QTLs related to heat tolerance.

Grain weight per plant is another major morphological observation to know the effect of high-temperature stress on the crop. Mean grain weight per plant was the lowest in heat treated Uma (0.89 g) and highest in control N22 (9.29 g). Uma is the high yielding variety, due to summer season mean grain weight per plant of the control Uma plants decreased. 39.30 to 32.00 °C was the range of temperature at control condition during the flowering period of Uma, due to that mean grain weight per plant of the control Uma was less as compared to N22 variety. Grain weight per plant of both heat treated and control N22 plants was higher with compare to Uma and F_3 plants. This justifies the fact that N22 is a highly heat tolerant variety and hence used in many breeding programs for development of new heat tolerant rice varieties.

There was much segregation in F_3 plants for the characters like spikelet fertility, 100 seed weight and grain weight per plant. Mean grain weight per plant of F_3 plants was 5.37 g, varying from 0 to 12.10 g per plant. Some of the F_3 plants did not produce any grains as like heat treated and control Uma plants. It is due to the heat susceptible nature of the Uma plants and 10 plants out of 49 F_3 plants represented this character of the parent Uma. At the same time, some of the F_3 plants showed grain weight per plant similar to N22 plants.

Researchers like Poli *et al.* (2013) carried out a similar study and found the substantial decrease in yield per plant under high-temperature stress conditions in the case of both NH219 (23%) and N22 (33%) rice varieties. Allah *et al.* (2011) also reported that the average grain yield was decreased by 22 per cent under heat stress condition as compared to the ambient environment.

F_3 - 31, F_3 - 15, F_3 - 41, F_3 - 13, F_3 - 16, F_3 - 12 and F_3 - 45 with spikelet fertility 91.88 per cent 84.98 per cent 82.85 per cent 81.91 per cent per cent 81.18 per cent 80.90 per cent and 78.06 per cent respectively and the grain weight per plant of these heat treated F_3 plants was 12.10 g, 9.30 g, 8.10 g, 7.2 g, 7.7 g 7.1 g and 8.7 g, respectively. These two traits were similar to the tolerant parent (N22) and based on these observations, F_3 plants were used for constituting the tolerant bulk. F_3 - 4, F_3 - 5, F_3 - 11, F_3 - 29, F_3 - 34, F_3 - 37 and F_3 - 42 showed zero per cent

spikelet fertility and no grain weight per plant, which was similar to the susceptible parent (Uma) and used to constitute the susceptible bulk.

5.2.2 Genotyping of parents, susceptible and tolerant bulks

Results of parental polymorphism survey with 197 microsatellite markers revealed that 41 were polymorphic between the parents (N22 and Uma). The procedure of associating putative markers by DNA pooling from selected segregants was proven by QTL mapping method (Michelmore *et al.,* 1991).

Genotyping the tolerant parent, tolerant bulk, susceptible parent and susceptible bulk with the 41 polymorphic markers indicated that one marker showed complete co-segregation among tolerant parent and tolerant bulk, susceptible parent and susceptible bulk (Plate 11). The marker that co-segregated, RM5749 marker was present on chromosome number 4. The co-segregation of the RM5749 marker between the tolerant parent and tolerant bulk, the susceptible parent and the susceptible bulk indicated this might be putatively linked to spikelet fertility, which is the strong indicator of heat tolerance. Buu *et al.* (2014) reported markers linked with high-temperature stress tolerance were located mostly on chromosomes 11, 10, 8, 6, 4, and 3. Buu *et al.* (2014) also identified RM5749 on chromosome 4, linked to heat tolerance in BC_2F_2 lines obtained from the cross of OM5930 × N22. In current study RM5749 was also identified linked to spikelet fertility under high-temperature stress So, RM5749 can be used efficiently for marker-assisted selection in the subsequent generations of the cross Uma x N22.

Similar to the findings of the study, Zhang *et al.* (2009) had also found markers RM3735 and RM3586 located on chromosome number 3 and 9, respectively and showed substantial linkage with high-temperature stress tolerance, indicating that high-temperature stress tolerance at flowering stage in rice was under control of multiple genes. However, in the present study, though RM3586 was a polymorphic marker between two parents (N22 and Uma), it was not polymorphic between the two bulks hence it cannot be considered as a linked marker to heat tolerance in the present population. As RM3735 was monomorphic

between the two parents (N22 and Uma) in this study, it was not included further for screening the bulks.

5.2.3 Confirmation of putative markers

Putative marker identified from genotyping of parents, susceptible bulk and tolerant bulks was used to genotype the F3 individuals. In our study, RM5749 marker was identified as putatively linked to spikelet fertility based on the analysis of bulked extremes (Plate 12). As RM5749 markers was associated with spikelet fertility which is considered as a reliable indicator of heat tolerance, the marker can be considered putatively linked the genomic region governing heat tolerance.

The probability of association of the putative marker RM5749 with spikelet fertility during high-temperature stress was highly significant ($p < 0.001$). Results indicated the strong association of this putative marker with the genomic region of spikelet fertility under high-temperature stress. The position of RM5749 was reported to be at 22.80 Mb on chromosome number 4 (Gramene Database). As this marker was linked to the quantitative trait loci for spikelet fertility which is considered as a reliable indicator of heat tolerance, the marker can be considered as putatively linked with the genomic region governing heat tolerance.

A quantitative trait loci (QTL) was identified based on the probability of association of the putative marker with spikelet fertility percentage under high-temperature stress through single marker analysis. A significant QTL was detected for the trait spikelet fertility percentage under high-temperature stress. LOD value of (6.86) indicates that the chance of the presence of QTL on chromosome 4 is more (106 times) than the absence of QTL for this character (Plate 14). Hence, it can be inferred that strong linkage exists for this QTL with spikelet fertility percentage under high-temperature stress.

Parallel to the findings of the present study, a QTL on chromosome number 4, at the locus RM5749 was identified as linked to grain yield under high-temperature stress by Buu *et al.* (2014). The same researchers also identified four

QTLs for filled grains per panicle on chromosome number 4 (RM241 - RM26212 and RM468 - RM7076), two QTLs regulating sterile grain percentage on chromosome number 3 (RM3686 and RM554) and one QTL for 1000-grain weight on chromosome 6 (RM103).

Similar to the findings of the present study, Zhu et al. (2005) identified three OTLs for heat tolerance on chromosome number 7, 4 and 1 with LOD scores of 12.86, 11.08 and 8.16, respectively. Whereas Zhang *et al.* (2009) found two microsatellite (SSR) markers, RM3586 (on chromosome number 3) and RM 3735 (on chromosome number 4) linked to high-temperature stress tolerance in rice through single marker analysis.

6. SUMMARY

The study on 'Bulked segregant analysis for heat tolerance in segregating generation of rice (*Oryza sativa* L.) using SSR markers' was carried out at the Regional Agricultural Research Station, Pattambi, Palakkad and Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Thrissur during 2015 to 2017. The main objective of the study was to identify the SSR markers linked to the gene/s for heat tolerance in rice through Bulked segregant analysis (BSA). The plant materials used in the study were two rice varieties *viz.*, high yielding heat susceptible Uma, and heat tolerant N22, and the F_3 progenies of the cross Uma × N22.

The results of the study are summarised below:

I. Parents, Uma and N22, were raised during August 2016 to assess the parental polymorphism. Tender leaves were used for DNA isolation and DNA samples were isolated by CTAB method with RNase treatment after first Chloroform:Isoamylalcohol wash. The ratio of UV absorbance ($A_{260/280}$) ranged between 1.87-1.97 and the quantity of DNA in the isolated samples ranged between 1534.90-2144.82 ng/µl.

II. Genomic DNA samples of the two parental lines (Uma and N22) were screened with 197 Rice Microsatellites (RM) markers and 41 RM markers were identified as polymorphic between these two parents. These polymorphic markers identified between the parents included the markers located on all the 12 chromosomes of rice.

III. More than 100 F_3 seeds were kept for soaking and 49 F_3 plants were germinated and screened for heat tolerance along with two parents, Uma and N22. Phenotypic observations of F_3 plants indicated he presence of high variability among F_3 plants.

IV. Seven most tolerant and seven most susceptible F_3 plants were selected for BSA, based on spikelet fertility. DNA samples from selected tolerant individuals were used to constitute the tolerant bulk and DNA from the selected susceptible individuals were used to constitute the susceptible bulk.

V. Polymorphic SSR markers identified between Uma and N22 were used to screen the tolerant bulk, susceptible bulk, tolerant parent (N22) and susceptible parent (Uma) and BSA was performed.

VI. RM5749 showed polymorphism between tolerant bulk and susceptible bulk as well as parents during BSA and this marker was considered as a putative marker for heat tolerance.

VII. This putative marker was confirmed by single marker analysis. The probability of putative marker was highly significant indicating the strong association of the marker with the genomic region of spikelet fertility percentage under high-temperature stress.

VIII. A quantitative trait loci (QTL) was identified based on significant LOD value (LOD= 6.86) of RM5749 marker associated with spikelet fertility percentage under high-temperature stress through single marker analysis. This QTL was present on Chromosome 4.

Future line of work includes linkage analysis of the identified 41 markers and QTL mapping in rice. RM5749 can be used for marker-assisted selection (MAS) for heat tolerance in rice and SSR markers near to RM5749 on chromosome number 4 can be screened for heat tolerance.

7. REFERENCES

Allah, A. A. A., Badawy, S. A., Elrewainy, I. M. O., and Elkhtyar, A. M. 2011. Identifying heat tolerant rice genotypes under field conditions. *Egyptian J. Agron*. 33(2): 167-178.

Almaraz, J. J., Mabood, F., Zhou, X., Gregorich, E. G., and Smith, D. L. 2008. Climate change, weather variability and corn yield at a higher latitude locale: Southwestern Quebec. *Climatic Change* 88(2): 187-97.

Barakat, M. N., Al-Doss, A. A., Elshafei, A. A., and Moustafa, K. A. 2011. Identification of new microsatellite marker linked to the grain filling rate as indicator for heat tolerance genes in F_2 wheat population. *Australian J. Crop Sci.* 5(2): 104-110.

Battista, D. S. and Naylor, R. L. 2009. Historical warnings of future food insecurity with unprecedented seasonal heat. *Science* 323: 240-244.

Behnassi, M., Shahid, S. A., and D'Silva, J. (eds.). 2011. *Sustainable agricultural development: recent approaches in resources management and environmentally-balanced production enhancement*. Springer Science & Business Media. 274p.

Bharathkumar, S., Pragnya, P. J., Jitendra, K., Archana, B., Singh, O. N., and Reddy, J. N. 2014. Identification of rice germplasms associated with microsatelite (SSR) markers for heat tolerance at reproductive stage and expression of heat stress related gene. *Indian Res. J. Genet. Biotech*. 6: 424-27.

BLS (Bureau of Labour Statistics, India). 2010. Report on Employment & Unemployment Survey (2009–10). Bureau of Labour Statistics, Government of India. Available at: http://labourbureau.nic.in/Final _Report_Emp_Unemp_2009_10.pdf [Accessed 25 April 2017].

Bo, H. S., Ping, Z. Y., Feng, Z. D., Qing, L. X., and Jing, X. 2012. Evaluation of heat resistance in hybrid rice. *Chinese J. Rice Sci.* 26(6): 751-756.

Buu, B. C., Anh, T. Q., Anh, B. P. N., Nguyen, N. N., Minh, L. T., Cuong, N. T., Bang, H. V., Hai, T. V., Quynh, L. V., Hieu, N. V., Tam, B. P., Ha, P. T. T., Nha, C. T., and Lang, N. T. 2013. Rice breeding for heat tolerance at initial stage. *Omonrice* 19: 1-10.

Buu, B. C., Ha, P. T. T., Tam, B. P., Nhien, T. T., Hieu, N. V., Phuoc, N. T., Minh, L. T., Giang, L. H., and Lang, N. T. 2014. Quantitative trait loci associated with heat tolerance in rice (Oryza sativa L.) *Plant Breed. Biotech.* 2: 14-24.

Cao, L. Y., Zhu, J., Zhao, S. T., He, L. B., and Yan, Q. C. 2002. Mapping QTLs for heat tolerance in a DH population from *indica japonica* cross of rice (*Oryza sativa*). *J. Agric. Biotech.* 10: 210-14.

Cao, Y. Y., Duan, H., Yang, L. N., Wang, Z. Q., Liu, L. J., and Yang, J. C. 2011. Effect of high temperature during heading and early filling on grain yield and physiological characteristics in *indica* rice. *Acta Agron. Sin.* 35: 512-21.

Ceccarelli, S., Grando, S., Maatougui, M., Michael, M., Slash, M., Haghparast, R., Rahmanian, M., Taheri, A., Alyassin, A., Benbelkacem, A., Labdi, M., Mimoun, H., and Nachit, M. 2010. Plant breeding and climate changes. *J. Agric. Sci.* 148: 627-37.

Cheng, L. R., Wang, J. M., Uzokwea, V., Meng, L. J., Wang, Y., Sun, Y., Zhu, L. H., Xu, J. L., and Li, Z. K. 2012. Genetic analysis of cold tolerance at seedling stage and heat tolerance at anthesis in rice. *J. Integrative Agric.* 11: 359-67.

Cruz, R. V., Harasawa, H., Lal, M., Wu, S., Anokhin, Y., Punsalmaa, B., Honda, Y., Jafari, M., Li, C., and Huu, N. N. 2007. Asia Climate Change 2007:

Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Available at: http://www.ipcc.ch/publications_and_data/ar4/wg2/en/ch10.html [Accessed 2 November 2016].

Das, S., Krishnan, P., Nayak, M., and Ramakrishnan, B. 2014. High temperature stress effects on pollens of rice (*Oryza sativa* L.) genotypes. *Environ. Exp. Bot.* 101: 36-46.

Dellaporta, S. L.; Wood, J., and Hicks, J. B. 1983. A plant DNA minipreparation: version II. *Plant Mol. Biol. Reporter* 1: 19-21.

Dixit, S., Singh, A., Sta Cruz, M. T., Maturan, P.T., Amante, M., and Kumar A. 2014. Multiple major QTL lead to stable yield performance of rice cultivars across varying drought intensities. *BMC Genet.* 15: 16.

Easterling, W. E., Aggarwal, P. K., Batima, P., Brander, K. M., Erda, L., Howden, S. M., Kirilenko, A., Morton, J., Soussana, J. F., Schmidhuber, J. and Tubiello, F. N. 2007. Food, fibre and forest products. In:*Climate Change 2007: Impacts, Adaptation and Vulnerability.* [Parry, M. L., Canziani, O. F., Palutikof, J. P., van der Linden, P. J. and Hanson, C. E. (eds).]. Cambridge University Press, Cambridge, UK, pp.273-313.

Edilberto, D. R., Marcelino, A. L., Sheryl, N. S., Manangkil, O. E., and Barroga, W. V. 2006. Identifying Heat-tolerant Rice Genebank Accessions (Workshop 2) Crop Production under Heat Stress. IRRI, Phillipines. p.2.

Gramene Database. Microsatellite markers. [On line]. Available: http://archive.gramene.org/markers /microsat/ [20 Aug 2016].

Gunawardena, T. A., Fukai, S., and Blamey, P. 2003. Low temperature induced spikelet sterility in rice - effects of panicle and root temperature. *Aust. J. Agric. Res.* 54: 937-46.

Handmer, J., Honda, Y., Kundzewicz, Z. W., Arnell, N., Benito, G., Hatfield, J., Mohamed, I. F., Peduzzi, P., Wu, S., Sherstyukov, B., Takahashi, K., and Yan, Z. 2012. Changes in impacts of climate extremes: human systems and ecosystems. In: *Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation* [Barros, C. B. V., Stocker, T. F., Qin, D., Dokken, D. J., Ebi, K. L., Mastrandrea, M. D., Mach, K. J., Plattner, G. K., Allen, S. K., Tignor, M., and Midgley, P. M. (eds.)]. Cambridge University Press, Cambridge, UK, and New York, NY, USA pp. 231-290.

Huang, B., Zong, Y. B., and Mei, J. Y. 2009. Effect of high temperature at rice reproductive stage to yield. *Guizhou Agric. Sci*. 7: 26-29.

IMD [India Metrological Department]. 2017. Statement on Climate of India during 2016 [On line]. Available: http://www.imd.gov.in/pages/press_release_view.php?ff=20170113_pr_89 [26 Feb 2017].

IPCC (Intergovernmental Panel on Climate Change). 2007. *Climate Change 2007*. Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Parry, M. L., Canziani, O. F., Palutikof, J. P., van der Linden, P. J. and Hanson, C. E. (eds)., Cambridge University Press, Cambridge, UK 976p.

IPCC (Intergovernmental Panel on Climate Change). 2014. *Climate Change 2014*. Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland pp-151.

Ishii, T. and McCouch, S. R. 2001. Nuclear and chloroplast microsatellite variation in genome species of rice. *Genome* 4: 658-666.

Jacobs, B. C. and Pearson, C. J. 1999. Growth, development and yield of rice in response to cold temperature. *J. Agron. Crop Sci.* 182: 79-88.

Jagadish, S. V. K., Craufurd, P. Q., and Wheeler, T. R. 2007. High temperature stress and spikelet fertility in rice. *J. Exp. Bot.* 58(7): 1627-1635.

Jagadish, S. V. K., Craufurd, P. Q., and Wheeler, T. R. 2008. Phenotyping parents of mapping populations of rice for heat tolerance during anthesis. *Crop Sci.* 48: 1140-1146.

Jagadish, S. V. K., Muthurajan, R., Oane, R., Wheeler, T. R., Heuer, S., Bennet, J., and Craufurd, P. Q. 2010. Physiological and proteomic approaches to address heat tolerance during anthesis in rice (*Oryza sativa* L.). *J. Exp. Bot.* 61(1): 143-156.

Jagadish, S. V. K., Muthurajan, R., Rang, Z. W., Malo, R., Heuer, S., Bennet, J., and Craufurd, P. Q. 2011. Spikelet proteomic response to combined water deficit and heat stress in rice (*Oryza sativa* cv. N22). *Rice* 4(1): 1-11.

Jagadish, S. K. V, Bahuguna, R. N., Djanaguiraman, M., Gamuyao, R., Prasad, P. V., and Craufurd, P. Q. 2016. Implications of high temperature and elevated CO2 on flowering time in plants. *Frontiers plant sci.* 7: 1-11.

Jarrod, R. W., Jeffrey, R. V., Maximilian, A., Piedad, F. M., Achim, D., and David, D. 2010. Rice yields in tropical/subtropical Asia exhibit large but opposing sensitivities to minimum and maximum temperatures. *Proc. Natl. Acad. Sci. USA* 107: 14562-14567.

Jena, K. K. and Mackill, D. J. 2008. Molecular markers and their use in marker-assisted selection in rice. *Crop Sci.* 48: 1266-1276.

Julia, C. and Dingkuhn, M. 2013. Predicting temperature induced sterility of rice spikelets requires simulation of crop generated microclimate. *Eur. J. Agron.* 49: 50-60.

Kanagaraj, P., Prince, K. S. J., Sheeba, J. A., Biji, K. R., Paul, S. B., Senthil, A., and Babu, R. C. 2010. Microsatellite markers linked to drought resistance in rice (*Oryza sativa* L.). *Curr. Sci*. 98: 836–839.

Lang, N.T., Ha, P. T. T., Tru, P. C., Toan, T.B., Buu, B. C., and Cho, Y. C. 2015. Breeding for heat tolerance rice based on marker-assisted backcrossing in Vietnam. Plant Breed. Biotech. 3: 274-281.

Liao, J., Zhang, H., Shao, X., Zhong, P., and Huang, Y. 2011. Identification for heat tolerance in backcross recombinant lines and screening of backcross introgression lines with heat tolerance at milky stage. *Rice Sci*. 18(4): 279-286.

Lobell, D. B., Schlenker, W., and Costa-Roberts, J. 2011. Climate trends and global crop production since. *Science* 333: 616-620.

Manigbas, N. L., Lambio, L. A. F., Madrid, L. B., and. Cardenas, C. C. 2014. Germplasm innovation of heat tolerance in rice for irrigated lowland conditions in the Philippines. *Rice Sci.* 21(3): 162-169.

Masuduzzaman, A. S. M., Ahmad, H. U., Haque, M. and Ahmed, M. M. E. 2016. Evaluation of rice lines tolerant to heat during flowering stage. *J. Rice Res.* 4: 170.

Matsui, T., Kobayasi, K., Kagata, H., and Horie, T. 2005. Correlation between viability of pollen and length of basal dehiscence of the theca in rice under a hot-and-humid condition. *Plant Prod. Sci.* 2: 109-114.

Matsui, T., Omasa, K., and Horie, T. 1999. Mechanism of anther dehiscence in rice (*Oryza sativa* L.). *Ann. Bot*. 84: 501-506.

Matsui, T., Omasa, K., and Horie, T. 2001. Comparison of two rice (*Oryza sativa* L.) cultivars with tolerance to high temperatures at flowering or susceptibility. *Plant Prod. Sci.* 4: 36-40.

Matsui, T. and Omasa, K. 2002. Rice (*Oryza sativa* L.) cultivars tolerant to high temperature at flowering: anther characteristics. *Ann. Bot.* 89: 683-687.

McWilliam, J. R. 1980. Summary and synthesis adaptation to high temperature stress. In: Turner, N. C., Krammer, P. J., (eds). *Adaptation of Plants to High Temperature Stress*. New York: John Wiley: 444-446.

Meng, L., Li, H., Zhang, L., and Wang, J. 2015. QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. *Crop J.* 3(3):269-283.

Michelmore, R. W., Pran, I., and Kessali, R. V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA.* 88: 9828-9832.

Mohammed, A. R., and Tarpley, L. 2009. High night time temperatures affect rice productivity through altered pollen germination and spikelet fertility. *Agricu. For. Meteorol.* 149(6): 999-1008.

Mohammed, A. R. and Tarpley, L. 2010. Effects of high night temperature and spikelet position on yield-related parameters of rice (*Oryza sativa* L.) plants. *Eur. J. Agron.* 33: 117-123.

Mora, C., Fraizer, A. G., Longman, R. J., Dacks, R. S., Walton, M. M., Tong, E. J., and Sanchez, J. J. 2013. The projected timing of climate departure from recent variability. *Nature* 502: 183-187.

Nguyen, D. N., Lee, K. J., Kim, D. I., Anh, N. T., and Lee, B. W. 2013. Modeling and validation of high temperature induced spikelet sterility in rice. *Fld. Crops Res.* 156: 293-302.

Nishiyama, I. and Blanco, L. 1980. Avoidance of high temperature sterility by flower opening in the early morning. *Japan Agric. Res.* 14: 116-17.

Palanog, A. D., Swamy, B. P. M., Shamsudin, N. A. A., Dixit, S., Hernandez, J. E., Boromeo, T. H., and Kumar, A. 2014. Grain yield QTLs with consistent-effect under reproductive-stage drought stress in rice. *Field Crops Res.* 161: 46-54.

Pale, G., Das, A., and Pattanayak, A. 2012. Screening of rice (*Oryza sativa* L.) germplasm of North-Eastern India for early vegetative stage heat tolerance. *Indian J. Genet. Plant Breed.* 72(2): 217-220.

Pandey, S. 2007. Economic costs of drought and rice farmers' coping mechanisms. *Int. Rice Res. News* 32: 5-11

Patel, T. S., Sharma, R. N., Mishra, H., and Verma, R. K. 2015. Screening of indigenous rice germplasm accessions for heat tolerance. *Ann. Plant Soil Res.* 17: 9-11.

Peng, S., Huang, J., Sheehg, J. E., Laza, R. C., Visperas, R. M., Zhong, X., Centeno, G. S., Khush, G. S., and Cassman, K. G. 2004. Rice yields decline with higher night temperature from global warming. *Proc. Natl. Acad. Sci.* 101: 9971-9975.

Poli, Y., Basava, R. K., Panigrahy, M., Vinukonda, V. P., Dokula, N. R., Voleti, S. R., Desiraju, S., and Neelamraju, S. 2013. Characterization of a Nagina22 rice mutant for heat tolerance and mapping of yield traits. *Rice* 6(36): 1-9.

Powell, N., Xuimei, J., Ravash, R., Edlington, J., and Dolferus, R. 2012. Yeild stability for cereals in a changing climate. *Funct. Plant Biol.* 39: 539 -552.

Pradhan, S. K., Barik, S. R., Sahoo, A., Mohapatra, S., Nayak, D. K., and Mahender A. 2016. Population structure, genetic diversity and molecular marker-trait association analysis for high temperature stress tolerance in rice. *PLoS ONE.* 11(8): e0160027.

Prasad, P. V. V., Boote, K. J., Allen, L. H., Sheehy, J. E., and Thomas, J. M. G. 2006. Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field Crops Res.* 95: 398-411.

Prasanth, V. V., Babu, M. S., Venkata, T., Kiran, T. V., Swamy, K. N., Babu, V. R., Mangrauthia, S. K., Subrahmanyam, D., Voleti, S. R., and Sarla, N. 2017. Response of popular rice varieties to late sown high temperature conditions in field. *Indian J. Plant Physiol.* DOI 10.1007/s40502-017-0287-y.

Prasanth, V. V., Chakravarthi, D. V. N., Kiran, T. V., Rao, Y. V., Panigrahy, M., Mangrauthia, S. K., Viraktamath, B. C., Subrahmanyam, D., Voleti, S. R., and Sarla, N. 2012. Evaluation of rice germplasm and introgression lines for heat tolerance. *Ann. Biol. Res.* 3(11): 5060-5068.

Pressman, E., Peet, M., and Pharr, D. M. 2002. The effect of heat stress on tomato pollen characteristics associated with changes in carbohydrate concentration in the developing anthers. *Ann. Bot.* 90: 631-636.

Raghavan, V. 1988. Anther and pollen development in rice (*Oryza sativa* L.). *Am. J. Bot.* 75: 183-196.

Rani, B. A., and Maragatham, N. 2013. Effect of elevated temperature on rice phenology and yield. *Indian J. Sci. Technol.* 6(8): 5095-5097.

Ray, D. K., Mueller, N. D., West, P. C., and Foley, J. A. 2013. Yield trends are insufficient to double global crop production by 2050. *PLoS ONE* 8(6): e66428.

Ricepedia. 2017. Ricepedia the online authority on rice [On line]. Available: http://ricepedia.org/rice-as-a-crop/rice-productivity [26 Feb. 2017].

Salunkhe, A., Poornima, R., Prince, K. S. J., Kanagaraj, P., Sheeba, J. A., Amudha, K., Suji, K. K., Senthil, A., and Babu, R. C. 2011. Fine mapping QTL for drought resistance traits in rice (*Oryza sativa* L.) using bulk segregant analysis. *Mol. Biotechnol.* 49: 90–95.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular cloning: a laboratory manual* (Ed. 2). Cold Spring Harbor Laboratory Press, New York 1546p.

Sandhu, N., Singh, A., Dixit, S., Cruz, M. T. S., Maturan, P. C., Jain, R. K., and Kumar, A. 2014. Identification and mapping of stable QTL with main and epistasis effect on rice grain yield under upland drought stress. *BMC Genet.* 15(1): 63.

Satake, T. and Yoshida, S. 1978. High temperature induced sterility in *indica* rice at flowering. *Japanese J. Crop Sci.* 47: 6-10.

Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C., and Wang, K. 2011. Impact of high-temperature stress on rice plant and its traits related to tolerance. *J. Agric. Sci.* 149: 545-556.

Shi, W., Ishimaru, T., Gannaban, R. B., Oane, W., and Jagadish, S. V. K. 2008. Popular rice (*Oryza sativa* L.) cultivars show contrasting responses to heat stress at gametogenesis and anthesis. *Crop Sci.* 55: 589-596.

Teixeira, E. I., Fischer, G., van Velthuizen, H., Walter, C., and Ewert, F. 2013. Global hot-spots of heat stress on agricultural crops due to climate change. *Agric. For. Meteorol.* 170: 206-215.

Tenorio, F.A., Ye, C., Redoña, E., Sierra, S., Laza, M., and Argayoso, M. A. 2013. Screening rice genetic resources for heat tolerance. *SABRAO J. Breed. Genet.* 45(3): 371-381.

Thangapandian, R., Jayaraj, T., Redona, E. D. and Sheryl, S. 2010. Development of heat tolerant breeding lines and varieties in rice. *Indian J. Plant Genet. Resour.* 23: 1-3.

Thomson, M. J., Septiningsihn, E. M., Suwardjo, F., Santoso, T. J., Silitonga, T. S., and McCouch, S. R. 2007. Genetic diversity analysis of traditional and improved Indonesian rice (*Oryza sativa* L.) germplasm using microsatellite markers. *Theor. Appl. Genet.* 114: 559-568.

Tian, X., Matsui, T., Li, S., Yoshimoto, M., Kobayasi, K., and Hasegawa, T. 2010. Heat-induced floret sterility of rice under humid and low wind conditions in the field of Jianghan basin, China. *Plant Prod. Sci.* 13(3): 243-251.

Tiwari, S., Kumar, V., Singh, B., Rao, A., and Mithra, S. V. A. 2016. Mapping QTLs for salt tolerance in rice (*Oryza sativa* L.) by bulked segregant analysis of recombinant inbred lines using 50k SNP chip. *PLoS ONE* 11(4): e0153610.

USDA [United States Department of Agriculture]. 2016. Economic Research Service. [On line]. Available: https://www.ers.usda.gov/topics/crops/rice/ [26 Feb 2017].

Vikram, P., Swamy, B. P. M., Dixit, S., Ahmed U. H., Cruz, M. T. S., Singh, A. K., and Kumar, A. 2011. qDTY1.1 a major QTL for rice grain yield under reproductive-stage drought stress with a consistent effect in multiple elite genetic backgrounds. *BMC Genet.* 12: 89.

Vikram, P., Swamy, B. M., Dixit, S., Ahmed, H., Cruz, M. S., Singh, A. K., and Kumar, A. 2012. Bulk segregant analysis: "An effective approach for mapping consistent-effect drought grain yield QTLs in rice". *Field Crops Res.* 134: 185-192.

Vivitha, P., Raveendran, M., and Vijayalakshmi, D. 2017. Introgression of QTLS controlling spikelet fertility maintains membrane integrity and grain yield

in improved White Ponni derived progenies exposed to heat stress. *Rice Sci*. 24(1): 32-40.

Wahid, A., Gelani, S., Ashraf, M., and Foolad, M. R. 2007. Heat tolerance in plants: an overview. *Environ. Exp. Bot.* 61: 199-223.

Wang, C., Yang, J., Wa, J., and Cai, Q. 2004. Influence of high and low temperature stress on fertility and yield of rice (*Oryza sativa* L.): Case study with the Yangtze River rice cropping region in China. Abstract, World Rice Research Conference 2004, Tsukuba, Japan. 97.

Wei, H., Liu, J., Wang, Y., Huang, N., Zhang, X., Wang, L., Zhang, J., Tu, J., and Zhong, X. 2013. A dominant major locus in chromosome 9 of rice (*Oryza sativa* L.) confers tolerance to 48 °C high temperature at seedling stage. *J. Heredity* 104(2): 287-294.

Xiao, Y. H., Pan, Y., Luo, L. H., Deng, H. B., Zhang, G. L., Tang, W. B., and Chen, L. Y. 2011. Quantitative trait loci associated with pollen fertility under high temperature stress at flowering stage in rice (*Oryza sativa*). *Rice Sci.* 18: 11-15.

Xie, X. J., Li, B. B., and Shen, S. H. 2012. Impact of high temperature stress on photosynthetic characteristic and yield of rice (*Oryza sativa* L.) at heading. *Indian J. Agric. Sci*. 82(6) 516-522.

Yadav, S., Anuradha, G., Kumar, R. R., Vemireddy, L. R., Sudhakar, R., and Donempudi, K. 2015. Identification of QTLs and possible candidate genes conferring sheath blight resistance in rice (Oryza sativa L.). Springer Plus 4: 175.

Yang, Z., Huang, D., Tang, W., Zheng, Y., and Liang, K. 2013. Mapping of Quantitative Trait Loci underlying cold tolerance in rice seedlings *via* High Throughput sequencing of pooled extremes. *PLoS ONE* 8(7): e68433.

Ye, C., Argayoso, M., Redoña, E., Sierra, S., Laza, M., Dilla, C., Mo, Y. J., Thomson, M. J., Chin, J. H., Delaviña, C. B., Diaz, G. Q., and Hernandez, J. 2011. Mapping QTL for heat tolerance at flowering stage in rice using SNP markers. *Plant Breed.* 131: 33-41.

Yoshida, S. 1978. Tropical climate and its influence on rice. *IRRI Research Paper Series* 20.

Yoshida, S., Satake, T., and Mackill, D. S. 1981. High temperature stress in rice. *IRRI Research Paper Series* 67: 1-15.

Zhang, G., Chen, L., Xiao, G., Xiao, Y., Chen, X., and Zhang, S. 2009. Bulked segregant analysis to detect QTL related to heat tolerance in rice using SSR markers. *Agric. Sci. China* 8(4): 482-487.

Ziska, L. H., Manalo, P. A., and Ordonez, R. A. 1996. Intraspecific variation in the response of rice (*O. sativa* L.) to increased CO_2 and temperature: growth and yield responses of 17 cultivars. *J. Exp. Bot.* 47(302): 1353-1359.

Zhao, Z. G., Jiang, L., Xiao, Y. H., Zhang, W. W., Zhai, H. Q., and Wan, J. M. 2006. Identification of QTLs for heat tolerance at the booting stage in rice (*Oryza sativa* L.). *Acta Agron. Sin.* 32: 640-644.

Zhu, C. L., Xiao, Y. H., Wang, C. M., Jiang, L., Zhai, H. Q., and Wan, J. M. 2005. Mapping QTLs for heat tolerance during grain filling in rice. *Chinese J. Rice Sci.* 19: 117-121.

APPENDIX-I

Composition of reagents used for DNA isolation and purification

I. 4X CTAB extraction buffer

a. CTAB (4% V/V)

b. 100 mM Tris buffer (p^H 8)

c. 20 mM EDTA (p^H 8)

d. 1.4 M NaCl

II. Washing solution

70% and 100% Ethanol

III. Chilled Isopropanol

IV. RNase stock

RNase A	-100 mg
Autoclaved distilled water1 ml	-1 ml

Stocks was prepared by dissolving 10 mg RNase A in 1 ml water and was stored under refrigerated conditions at -20°C. The RNase A was used to prepare RNase. Ten per cent RNA solution was prepared by dissolving the same in water at 1:10 ratio. The solution was stored at -20°C and use for RNase treatment.

V. TAE Buffer (1X)

40mM Tris

1mM EDTA

20mM Acetic acid

VI. Agarose gel composition

Agarose

TAE buffer – 2 ml per 100 ml of autoclaved distilled water

APPENDIX-II

Phenotyping of F_3 population under high-temperature stress

Plant Number	Plant Height (cm)	No. of tillers	No. of Panicles	Panicle length (cm)	Days for flowering	No. of filled grains/panicle	No of sterile grains/panicle	Spikelet fertility (%)	100 seed weight (g)	Grain weight/plant (g)
F3 - 1	73	9	7	20.94	83	104.40	34.20	75.30	2.01	7.30
F3 - 2	63	12	8	17.94	79	92.80	69.80	57.07	1.75	6.10
F3 - 3	85	10	9	20.10	75	64.40	69.60	50.03	1.93	5.70
F3 - 4	81	8	8	18.67	80	0.00	200.00	0.00	0.00	0.00
F3 - 5	76	6	3	14.47	90	0.00	183.00	0.00	0.00	0.00
F3 - 6	72	9	9	18.40	77	97.60	32.60	74.7	1.94	8.10
F3 - 7	78	3	3	21.90	77	97.20	53.80	64.61	1.97	7.00
F3 - 8	81	9	9	20.10	74	71.80	39.40	64.67	2.00	5.90
F3 - 9	66	5	3	17.50	77	78.20	57.00	58.04	1.50	4.00
F3 - 10	90	9	8	22.44	80	63.00	75.40	44.91	2.12	5.80
F3 - 11	89	8	7	16.80	81	0.00	175.20	0.00	0.00	0.00
F3 - 12	79	11	11	19.67	77	90.20	20.20	80.90	2.10	7.10
F3 - 13	91	10	5	19.00	77	121.40	27.00	81.91	1.97	7.20
F3 - 14	98	7	7	23.20	79	113.60	100.20	53.27	1.73	6.60
F3 - 15	81	11	10	22.50	82	117.40	20.40	84.98	1.91	9.30
F3 - 16	82	7	5	19.17	75	100.60	23.40	81.18	1.63	7.70
F3 - 17	91	11	10	22.34	73	118.40	52.80	68.75	2.10	7.50
F3 - 18	84	7	7	20.00	77	97.60	30.20	76.38	2.01	7.10
F3 - 19	89	8	7	23.64	80	56.20	94.80	35.74	1.91	6.90
F3 - 20	92	9	9	20.40	80	67.80	81.00	45.65	1.85	6.90
F3 - 21	84	7	7	19.57	82	23.40	111.00	17.37	1.80	5.00
F3 - 22	92	8	8	23.37	78	91.80	39.40	70.64	1.94	7.30
F3 - 23	81	9	5	19.54	87	48.20	56.20	48.31	1.79	5.20
F3 - 24	84	11	9	20.80	83	29.20	124.00	20.94	2.02	4.50

Phenotyping of F_3 population under high-temperature stress (Continued)

Plant Number	Plant Height (cm)	No. of tillers	No. of Panicles	Panicle length (cm)	Days for flowering	No. of filled grains/panicle	No. of sterile grains/panicle	Spikelet fertility (%)	100 seed weight (g)	Grain weight/plant (g)
F3 - 25	89	12	5	20.80	90	47.00	84.80	36.78	1.94	7.30
F3 - 26	79	7	7	19.20	75	80.80	41.00	66.48	1.84	6.20
F3 - 27	81	10	4	20.87	90	75.80	57.00	58.82	2.07	6.20
F3 - 28	79	9	4	16.20	79	16.60	131.60	11.09	1.90	4.00
F3 - 29	90	7	5	17.90	79	0.00	171.40	0.00	0.00	0.00
F3 - 30	89	10	10	22.24	77	90.60	44.20	66.22	2.10	7.80
F3 - 31	79	12	11	19.77	79	130.80	11.60	91.88	2.03	12.10
F3 - 32	69	7	8	15.67	104	0.00	166.40	0.00	0.00	0.00
F3 - 33	89	9	8	20.67	77	110.80	33.00	76.7	1.90	8.60
F3 - 34	82	8	8	17.67	86	0.00	183.00	0.00	0.00	0.00
F3 - 35	87	8	7	23.27	80	131.60	85.80	60.20	2.03	7.30
F3 - 36	69	8	8	22.04	82	68.40	120.40	37.04	2.02	5.20
F3 - 37	79	9	6	20.74	83	0.00	189.20	0.00	0.00	0.00
F3 - 38	99	8	8	22.80	80	15.60	145.40	10.36	1.70	4.30
F3 - 39	91	8	8	18.04	76	92.80	113.20	45.17	1.83	7.00
F3 - 40	88	8	7	20.24	73	118.80	37.60	77.03	1.74	7.80
F3 - 41	76	8	7	17.57	76	105.20	21.40	82.85	1.73	8.10
F3 - 42	90	7	6	20.84	81	0.00	166.20	0.00	0.00	0.00
F3 - 43	80	11	6	17.04	83	0.00	201.60	0.00	0.00	0.00
F3 - 44	82	4	3	19.84	77	27.20	141.60	16.05	1.89	4.50
F3 - 45	87	9	8	19.67	77	108.20	31.20	78.06	1.88	8.73
F3 - 46	82	8	8	22.94	83	75.20	82.60	44.1	1.79	7.30
F3 - 47	84	14	6	21.57	89	39.60	82.60	33.17	1.93	6.00
F3 - 48	93	8	3	22.44	79	0.00	176.20	0.00	0.00	0.00
F3 - 49	84	10	10	20.94	80	99.20	111.4	46.87	1.93	6.12

BULKED SEGREGANT ANALYSIS FOR HEAT TOLERANCE IN SEGREGATING GENERATION OF RICE (*Oryza sativa* L.) USING SSR MARKERS

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(2015-11-103)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE
(PLANT BIOTECHNOLOGY)

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ABSTRACT

Rice (*Oryza sativa* L.) is the staple food crop of Kerala. Rice production in the state is decreasing owing to the declining trend in rice cultivation, due to various stresses. In the present context of climate change, mainly in the form of global warming, the high temperature is one of the most important environmental factor influencing the growth, development and yield of rice. Rice is highly susceptible to heat stress, particularly during the reproductive and ripening stages. Hence high temperature induced sterility has become a serious problem. Since most of the prevailing high yielding varieties are highly susceptible to heat stress, the heat tolerance trait must be incorporated into them using potential donors. Marker-Assisted Selection (MAS) has been identified as a dependable, reproducible and time saving strategy to confirm the presence of the desirable gene and to quicken the breeding cycle. The study entitled 'Bulked segregant analysis for heat tolerance in segregating generation of rice (*Oryza sativa* L.) using SSR markers' was carried out with the objective to identify the SSR markers linked to the gene/s for heat tolerance in rice through bulked segregant analysis.

Two rice varieties, heat sensitive high yielding variety Uma and heat tolerant variety N22 and their hybrid (Uma × N22, developed at RARS, Pattambi during 2014) were used in this study. Initially, parents were grown to assess the parental polymorphism by SSR markers. DNA was isolated by CTAB method with some modification. Total 197 SSR markers were used to assess the parental polymorphism. SSR markers were obtained from Gramene database (*www.gramene.org*). The 41 SSR markers were polymorphic among 197 SSR markers between Uma and N22 indicating 20.81 per cent parental polymorphism.

After this, F_3 generation along with parents were kept for germination during last week of December 2016 and transplanted during last week of January 2017. Heat tolerance screening was done in polyhouse during March and April

2017. The temperature inside the polyhouse ranged from 37.1 to 42.7°C during these two months. High-temperature stress was given at booting stage of the plants for fifteen days. After 15 days of heat treatment, all the treated plants were taken outside of the polyhouse to ambient conditions. High-temperature stress directly affects spikelet fertility. Hence spikelet fertility was the major trait for identification and selection of F_3 plants for BSA. During heat stress in this study, N22 (heat tolerant parent) exhibited the highest mean spikelet fertility (90.92 %) and Uma (heat susceptible parent) exhibited the lowest mean spikelet fertility (2.62 %) whereas, F_3 plants showed 44.78 per cent mean spikelet fertility. DNA from seven most tolerant plants and seven most susceptible F_3 plants were used to constitute the tolerant and susceptible bulk, respectively.

The identified 41 parental polymorphic markers were used for the genotyping of tolerant bulk, susceptible bulk, tolerant parent (N22) and susceptible parent (Uma). Among these, one RM marker (RM5749 on chromosome number 4) showed polymorphism between tolerant bulk and susceptible bulk during BSA. This marker, which was found to co-segregate with the trait, was again used to screen with the individuals of tolerant and susceptible bulks. The LOD value obtained during Single marker analysis (SMA) for the linkage between marker and spikelet fertility was 6.86, indicating that RM5749 on chromosome number 4 is tightly linked to the spikelet fertility trait under heat stress.

The identified 41 polymorphic markers could be further used for the identification of QTLs and linkage analysis. RM5749 on chromosome number 4 could be used for the marker-assisted selection for heat tolerance in rice. SSR markers near to RM5749 on chromosome number 4 could be screened for heat tolerance. High-level heat tolerance in N22 could be transferred to high yielding heat susceptible variety Uma, through marker assisted breeding using RM5749.